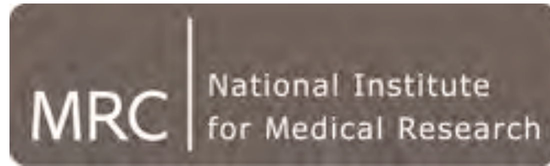

Investigation into the cellular mechanisms underlying cell sorting by Eph receptors and ephrins

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Declaration

This PhD project has been completed in the laboratory of Dr. David Wilkinson in the Division of Developmental Neurobiology at the MRC National Institute for Medical Research, London.

I, Rosalind Morley, hereby declare that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. I acknowledge that the *in vitro* assays used in this work were established in collaboration with Lauren Gregory in the Wilkinson laboratory.

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Abstract

The mechanisms that control the segregation of cells in the developing embryo are essential for normal development. Eph receptors and ephrins are responsible for cell segregation and the maintenance of sharp boundaries between regions of cells, such as those in the adult intestine or the compartments of the developing vertebrate hindbrain. The mechanisms through which they achieve this are not well understood.

One widely discussed theory, based on the differential adhesion hypothesis, is that Eph receptors and ephrins influence the relative adhesion between cells in adjacent compartments. The other is that active migratory or repulsive mechanisms are responsible for segregation.

Using *in vitro* assays that were established as part of this project, I have shown that N-cadherin is required for EphB2-ephrinB1 mediated cell sorting, consistent with an important role of cell-cell adhesion in this process. p120 and p0071, which are downstream targets of signalling through EphB2 and have established roles in regulating cadherin stability, are also required for cell segregation by EphB2 and ephrinB1.

However, comparison with the segregation of cells expressing different cadherins suggests that differential adhesion is not the main mechanism driving sorting downstream of Eph-ephrins. Instead, I propose that repulsion is the main mechanism driving segregation mediated by EphB2 and ephrinB1 and that N-cadherin is required for general adhesion between all cells, which stabilises the formation of EphB2 cell clusters.

Cell behaviour analyses indicate that N-cadherin is not required for the repulsion response of EphB2 cells after interactions with ephrinB1 cells, although it does play a contact-dependent role in cell migration. However, there is a cadherin-independent role of p120 in repulsion downstream of Eph-ephrins, which could contribute to cell sorting.

These results support a model where Eph-ephrin mediated repulsion acts in combination with a basal level of cell-cell adhesion to drive cell segregation.

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Abbreviations

AP	:	Anterior-Posterior
ADAM	:	A-Disintegrin-And-Metalloprotease
DV	:	Dorsal-Ventral
Dvl	:	Dishevelled (also Dsh)
ECM	:	Extracellular matrix
Eph	:	Erythropoietin-producing hepatocellular receptor
GAP	:	GTPase activating protein
GDI	:	GDP dissociation inhibitor
GEF	:	Guanine nucleotide exchange factor
HCI	:	Heywood Circularity Index
MO	:	Morpholino Oligonucleotide
MSD	:	Mean Squared Displacement
NN	:	Nearest Neighbour
p120	:	p120 catenin
p0071	:	Member of the p120 catenin family
Par	:	Partitioning defective protein
PCP	:	Planar Cell Polarity
PDF	:	Probability Distribution Function
PRI	:	Perimeter Regularity Index
r	:	Rhombomere
RTKs	:	Receptor tyrosine kinases

1. Introduction

A major aim of developmental biology is to understand how cells within an embryo cooperate to form the patterned set of tissues that make up an animal. A developing embryo undergoes extensive cell proliferation and re-arrangement, yet cells are able maintain their correct positions relative to one another. As an embryo grows, it becomes subdivided into regions which will later develop into specific tissue types. From an early stage, it is important that the cells in adjacent regions remain distinct from one another in order for tissues to develop in the correct pattern later on. Understanding how different groups of cells are able to maintain their positional identities, despite constant pressure from cell division and motility, is therefore crucial to our understanding of development.

Compartments and boundaries

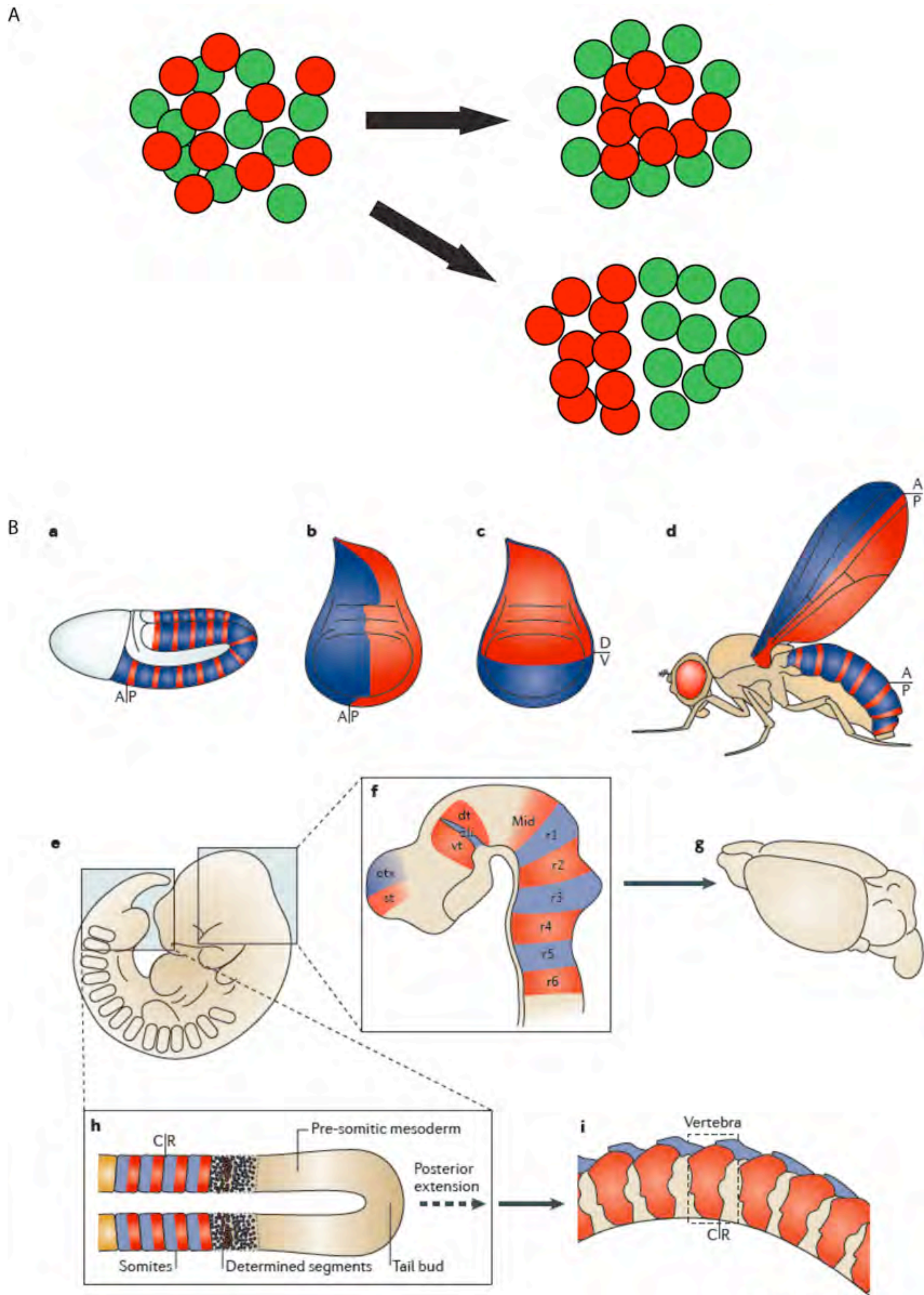
What are developmental compartments?

Within a developing tissue, newborn cells are often confined to a particular region and do not mix with cells of the adjacent region. Such groups of cells, called compartments are lineage-restricted, since cells from the same parent will always stay in the same compartment (Dahmann & Basler, 1999). The boundaries that these lineage-restricted cells cannot cross are not always identifiable based on morphology. However, they can be visualised by clonal analysis or by looking at the expression of selector genes, which mark the fate of the cells in the compartment in which they are expressed. For example, one of the first compartments discovered, the posterior compartment of the *Drosophila* wing imaginal disc, is marked by the expression of *engrailed* (Figure 1.1; Morata & Lawrence, 1975). In this case, engrailed activity is also crucial for the maintenance of a sharp boundary between the anterior and posterior compartments of the wing disc. In vertebrates, segments of the developing hindbrain are compartments which can be distinguished by differential expression of *hox* genes (Pasini & Wilkinson, 2002).

Figure 1.1: Cell segregation and boundary sharpening

(A) Cell sorting is the separation of two initially intermingled populations of cells. Separation can be by sorting of one population to the middle, surrounded by the other, or side-by-side segregation. (B) There are many examples of sharp boundaries in development across which cells do not migrate, the most commonly studied of which are shown here. Anterior-posterior (AP) boundaries of gene expression between parasegments (a) of the Drosophila embryo precede the formation of morphological segments in the adult fly (d). Similarly, the AP and dorso-ventral (DV) boundaries in the wing imaginal discs generate a plan of cell identities which pattern the adult wing. (e-g) Sharp boundaries maintain the segments of the embryonic vertebrate brain, which underlie the patterning of the adult brain. ctx, cortex; st, striatum; vt, ventral thalamus; zli, zona limitans intrathalamica; dt, dorsal thalamus; Mid, midbrain; r1-6, rhombomeres 1-6. Boundaries in the somites also establish a pattern that is maintained in the adult spinal column (h,i). The boundary between each somite, between the caudal (posterior) domain of one somite and the rostral (anterior) domain of the next is marked C/R (Dahmann *et al.*, 2011).

Figure 1.1



Why are compartments important?

The establishment of compartments is important for two reasons. Firstly, to make a functional tissue, cells need to be grouped together and unable to mix with the surrounding tissue cell types. Secondly, cells at compartment boundaries often take on a new identity and become organisers, regions that produce signals to pattern the surrounding cells. For example, antero-posterior boundary cells in the *Drosophila* wing imaginal disc secrete the morphogen DPP, which forms a gradient across the disc to induce different cell types in a concentration dependent manner (Dahmann *et al.*, 2011). The restriction of an embryo into compartments helps to establish a blueprint for development.

The vertebrate hindbrain

The hindbrain is made of compartments

One well-studied example of compartments in vertebrates is found in the developing neuroepithelium. The vertebrate hindbrain is made up of a series of metameric compartments called rhombomeres. Lineage tracing studies in chick, carried out by injecting intracellular dye to mark clones of cells, demonstrated that labelled cells were able to mix freely with other cells in the same rhombomere, but were restricted from entering adjacent rhombomeres (Fraser *et al.*, 1990). The compartmentalisation of the hindbrain later gives rise to a segmented pattern of neuronal development.

Each rhombomere has some similarity with the next, but also has an individual identity, based on the expression of specific genes and the types of neuron which derive from each segment. For instance, every rhombomere will give rise to a set of eight types of projection interneuron (Clarke & Lumsden, 1993). There is also a two-segment repeat pattern of branchio-motor neurons, which arise only from even-numbered rhombomeres (Lumsden & Keynes, 1989). Rhombomeres also have some individual identity. For example, in chick, rhombomere 4 gives rise to a group of migrating neurons which go on to innervate hair cells of the inner ear (Simon & Lumsden, 1993). The specification of the different rhombomeres is required to generate neurons in the right pattern later in development.

The compartments in the hindbrain appear morphologically as "bumps" in the neural tube, which correspond with the boundaries of expression of several genes including the hox genes. For example, hoxb2 is expressed in rhombomeres 3-8 (r3-8) and has a sharp boundary between r2 and r3 (Pasini & Wilkinson, 2002). Similarly, the transcription factor krox-20 is expressed in r3 and r5 and is important in establishing their genetic identity (Schneider-Maunoury *et al.*, 1993). These boundaries are initially fuzzy and sharpen quickly, as can be seen by the expression pattern of krox-20 by whole-mount in situ hybridisation (Cooke *et al.*, 2005; Irving *et al.*, 1996). Subsequent segmental expression of Eph receptors and ephrins is important in maintaining the boundaries between compartments (discussed in more detail later). Disruption of this early segmentation pattern disturbs the pattern of neuronal projections from the hindbrain. This illustrates the importance of compartment maintenance for tissue patterning (Cooke *et al.*, 2005; Moens *et al.*, 1996).

Introduction to cell sorting and tissue segregation

The mechanisms which underlie the segregation of distinct cell populations have long been studied. Key to our understanding of the segregation between tissues are classic experiments such as those by Townes & Holtfreter. These researchers observed that when embryos were dissociated into single cells and mixed together, these intermingled cells were capable of "sorting-out" into their original tissue types (Moscona & Moscona, 1952; Townes, 1955; Trinkaus & Groves, 1955). Following these experiments came over 60 years of investigation into the mechanisms cells employ to recognise one another as "like" or "unlike", to segregate from one another and to maintain this segregation over time.

It is important to note here the distinction between cell sorting and the maintenance of sharp interfaces between regions of cells *in vivo*. Cell sorting is the process by which random mixtures of cells segregate from one another. *In vivo*, cells are already largely segregated and there is sharpening and maintenance of this segregation at boundaries. It is considered that the principles which govern cell sorting also underlie the local segregation and maintenance of sharp boundaries. However, there may be mechanisms contributing to cell sorting that

are not applicable to boundary sharpening *in vivo*. Equally, there may be additional mechanisms involved in boundary maintenance which would not explain sorting.

Three main principles have been proposed to explain cell sorting or the restriction of intermingling between cells. *Differential adhesion* between groups of cells can drive them to sort *in vitro* (Duguay *et al.*, 2003; Foty & Steinberg, 2005; Steinberg, 1970) and is capable of maintaining segregation *in vivo*, for example in compartments of the developing mouse brain (Inoue *et al.*, 2001). *Repulsion* between cells of adjacent compartments is also thought to be capable of maintaining segregation and driving cell sorting *in vitro* (Abercrombie, 1962; Pasquale, 2005; Poliakov *et al.*, 2008). A further mechanism involves *tension* in cell sorting (Krieg *et al.*, 2008; Schotz *et al.*, 2008) and in the restriction of cell intermingling, for example in the form of myosin cables which become physical barriers between tissue compartments (Harris, 1976; Landsberg *et al.*, 2009; Major & Irvine, 2005; Monier *et al.*, 2010). In addition, *Eph receptors and ephrins* are known to be important in segregation at boundaries, although the cellular mechanisms they employ to achieve this are still debated (Cooke *et al.*, 2005; Mellitzer *et al.*, 1999; Xu *et al.*, 1999). The ideas which seeded these hypotheses and experimental evidence that supports them are discussed in the following sections.

History of cell sorting

Early experiments showed that dispersed cells of an embryo could re-aggregate and sort into their original tissue types, (Moscona & Moscona, 1952; Townes, 1955; Trinkaus & Groves, 1955). Several concepts about cell behaviour in embryonic development emerged from experiments such as these. For example, Moscona and Moscona described the two main processes that occur in dissociation and sorting experiments: firstly the cells re-aggregate, suggesting that cohesion between the cells must occur; and secondly they reorganise, indicating that there is cell movement (Moscona & Moscona, 1952). How such processes are coordinated to cause cells to segregate has been the subject of much debate.

Moscona & Moscona suggested that “active amoeboid movement was mostly responsible for the movement of the cells” and proposed that either selective

differences in adhesiveness or chemotactic migration were responsible for sorting (Moscona & Moscona, 1952).

The most widely accepted explanation for cell sorting has been the "differential adhesion hypothesis" (Steinberg, 1963; Steinberg, 1970). This theory suggests that different populations of cells, which have intrinsic, non-directional motility, will sort out from one another due to differences in the attractive forces between them. It derives from the observation that the sorting of cells is similar to sorting of immiscible liquids. Steinberg suggested that the thermodynamic principles explaining the segregation of liquids, which are well understood, also apply to cells. In this theory, attraction between like-cells tends to minimise their surface area in contact with cells of different adhesive strength. The differential adhesion hypothesis was supported by experiments demonstrating a hierarchy of cell sorting, whereby six embryonic cell types would either sort to the centre of a cluster or the outside depending on their relative position in the hierarchy (Steinberg, 1970). This position also corresponded to the extent of deformation of a cluster of cells subjected to centrifugal force, suggesting that an increase in the relative strength of interactions between cells could predict their pattern of sorting (Steinberg, 1970). More recent evidence (discussed later) confirms the predictions made by the differential adhesion hypothesis, demonstrating that cells can sort based on differences in adhesion (Duguay *et al.*; Foty & Steinberg, 2005).

Whilst the differential adhesion hypothesis was prevalent in the field, alternative theories were also proposed. Harris argued that cells have various properties which are not exhibited by molecules and which might undermine the assumption that they behave according to normal thermodynamic principles (Harris, 1976). For example, the differential adhesion hypothesis does not take into account the ability of cells to introduce new energy into the system, which they do in the form of intrinsic motility. It also assumes that cells are equally adhesive across their surface, whereas it was known that there were adhesive puncta that are more adhesive than other regions of the membranes between apposing cells (Harris, 1976). Instead, Harris favoured a model of differential contractility, where cells on the outside of an aggregate would contract, generating a force which could explain the tendency for aggregates to minimise their surface area.

Abercrombie advocated the idea of migratory differences between cell types. Analysis of cells in culture led to the observation of contact inhibition of locomotion, where collisions between cells restricts their migration such that they are prevented from overlapping one-another (Abercrombie & Heaysman, 1954). By extension of this idea, differences in cells' abilities to restrict the migration of different cell types could result in cell segregation or the prevention of cell mixing (Abercrombie, 1962; Weston & Abercrombie, 1967).

In 1978, Curtis advocated the idea of a "morphogen or interaction modulation factor theory", whereby a diffusible factor from one cell type is capable of "diminishing the adhesion of some unlike cell types so that they tend to allow unlike cell types to escape from their own environment" (Curtis, 1978). He proposed that the gradient of diffusible signal would be able to contribute to the relative positioning of cells.

Recently, much work has emerged in which specific molecular mechanisms have been identified that contribute to cell segregation. Modern views of how these mechanisms could fit with the hypotheses outlined above to underlie sorting and the restriction of intermingling between groups of cells are discussed below.

Molecular mechanisms of cell sorting and boundary maintenance

The Differential Adhesion Hypothesis

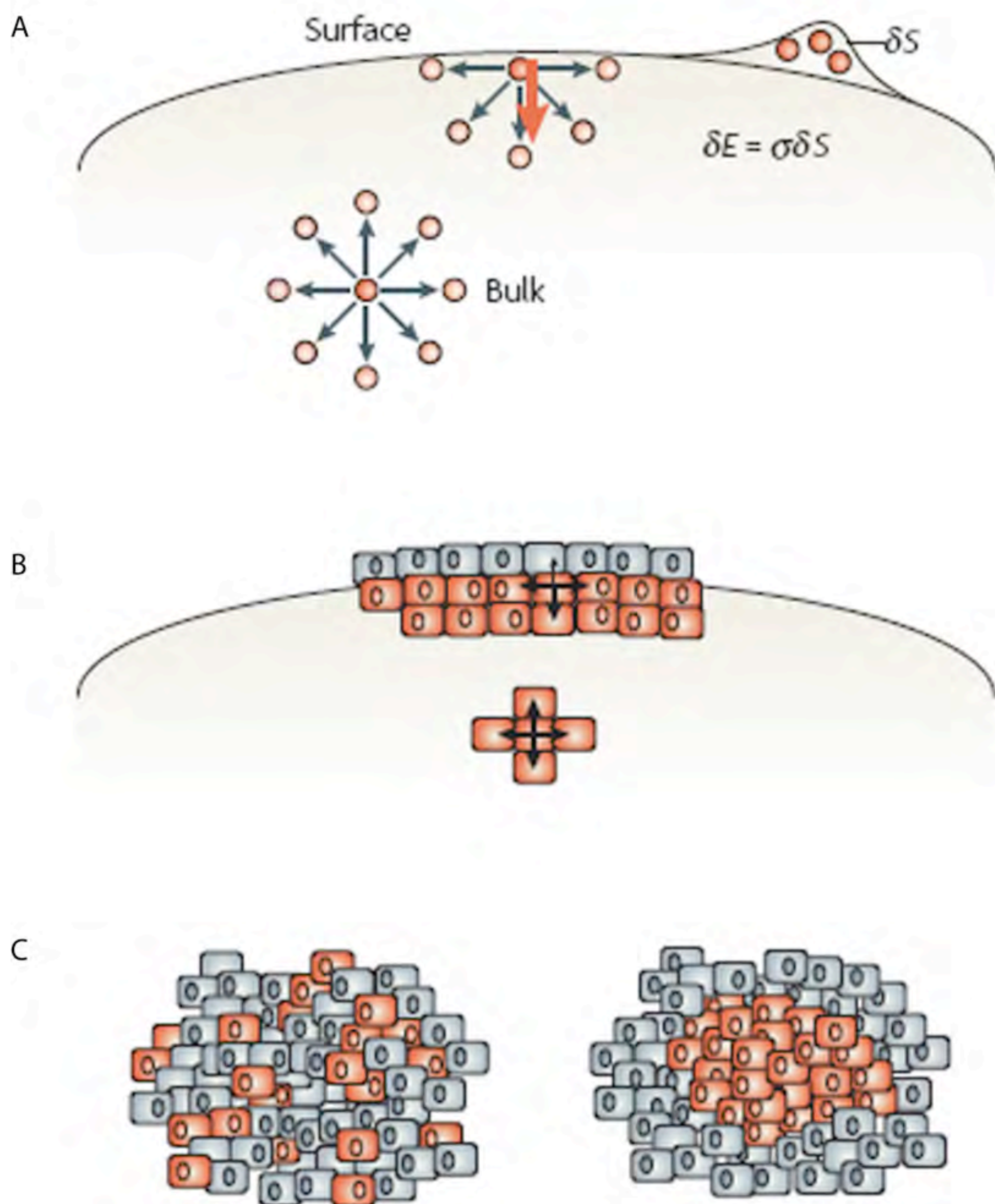
Until recently, the differential adhesion hypothesis was the most prevalent theory to explain cell segregation. The theory says that a population of cells with similar affinities for one another will sort from cells with different affinities, in a process similar to the segregation of oil and water molecules.

Steinberg first proposed his hypothesis of differential adhesion based on the observation that mixtures of cells share three key characteristics with mixtures of immiscible liquids: they are composed of discrete units of two major types; these units are motile; and the units appear to adhere and cohere with different strengths (Steinberg, 1962). Following this logic, cells are modelled as balls with certain adhesiveness, which sort based on thermodynamic principles into two phases with high interfacial surface tension between them.

Figure 1.2: The differential adhesion hypothesis

(A) Surface tension in a fluid. Molecules experience adhesive forces from other molecules surrounding them. Within the bulk of the liquid, these forces are distributed evenly on all sides of the molecule. At the surface, however, molecules experience more attraction towards the bulk of the liquid, resulting in contraction of the surface of the liquid. The energy (δE) required to change the liquid's surface area (δS) is directly proportional to the surface tension (σ). (B) Parallels can be drawn between this molecular model of surface tension and cell sorting. Red cells express higher levels of cadherins, so are more adhesive than the grey cells. At the interface between these two populations, the group of red cells minimises its surface area by adopting a spherical shape and forming a sharp interface with the grey cells. Grey cells will also tend to minimise their surface area in contact with the media because it is energetically favourable. (C) This behaviour of cells results in them sorting out from one another when they are intermingled, with all cells forming a ball and the red, more adhesive cells, sorting to the interior (Lecuit & Lenne, 2007).

Figure 1.2



Interfacial tension occurs between two immiscible liquids, much like the surface tension at the interface between liquid and air. Within a liquid, all molecules are experiencing similar attractive forces from all sides (Figure 1.2). However, near the edge there is an imbalance, since molecules are only experiencing attractive forces from molecules in the bulk of the liquid. This results in a net attraction of the molecule towards the liquid, increasing surface tension and minimising surface area.

When it was first proposed, the differential adhesion hypothesis relied on the prediction that there would be some factor on the surface of cells which could confer them with adhesive properties (Moscona & Moscona, 1952; Steinberg, 1970). This proved to be true with the discovery of the cadherin cell-cell adhesion molecules (Yoshida & Takeichi, 1982). Members of this large family of trans-membrane proteins are able to interact with one-another homophilically between cells in a calcium-dependent manner (Hatta & Takeichi, 1986). In addition, some members of the cadherin family are able to interact with one another heterophilically to different extents. For example, cells expressing E-cadherin will mix with cells expressing P-cadherin (Duguay *et al.*, 2003) but will segregate from those expressing N-cadherin (Niessen & Gumbiner, 2002; Shan *et al.*, 2000).

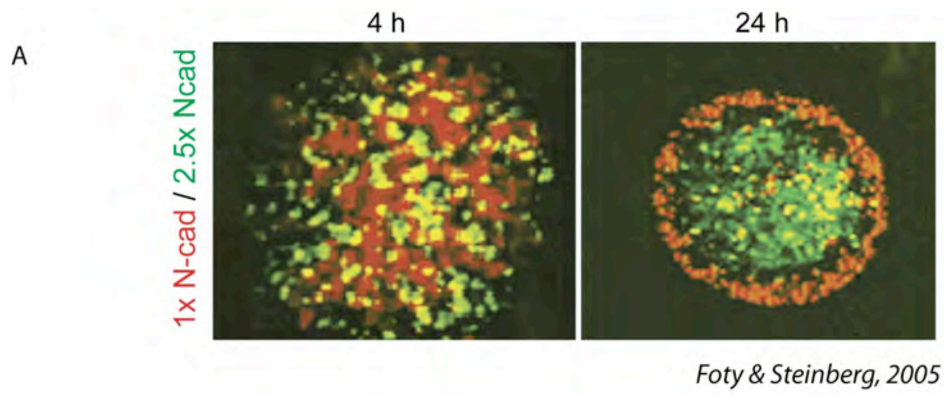
Subsequent experiments exploiting these properties of cadherins allowed the various predictions of the differential adhesion hypothesis to be tested. It has been shown that the size of re-aggregates of cells correlates with their aggregate surface tension, which in turn correlates with the number of cadherins expressed on the cell surface (Foty & Steinberg, 2005). This supports the prediction from the differential adhesion hypothesis that differences in affinity alone could specify differences in tissue surface tension. Another verification of the hypothesis was that more adhesive cells sort to the centre of an aggregate and less adhesive cells to the outside. This was demonstrated by mixing cells which express different levels (either 1x or 2.5x) of N-cadherin. The cells expressing more N-cadherin sorted to the centre of the aggregates (Figure 1.3 A). In this configuration, cells maximise their adhesive bonds and minimise the energy in the system. These data demonstrate that differences in levels or types of cadherin expression, and therefore differences in tissue tension, can drive cell sorting, consistent with the

Figure 1.3: Segregation models

(A) Cell sorting through differential adhesion. L cells transfected with 1x N-cadherin (red) sort from L cells transfected with 2.5x N-cadherin (green), with the more adhesive, green, cells sorting to the middle of the cluster (Foty & Steinberg, 2005). (B) Cell segregation by Eph-ephrins. Zebrafish embryos were labelled with rhodamine dextran or fluorescin dextran and injected with EphB2, ephrinB2 or a truncated EphB2 incapable of signalling. Animal caps were dissected from these embryos and differently labelled caps were juxtaposed. Cells expressing ephrinB2 (green) form a sharp boundary with cells expressing EphB2 (red), but not from cells expressing a truncated, signalling incompetent EphB2 (ΔEphB2) (Mellitzer *et al.*, 1999). (C) Eph-ephrins are important for the segregation of adjacent hindbrain rhombomeres *in vivo*. Morpholinos (MO) to EphA4, ephrinB2 or both disrupt the sharp boundaries created between rhombomeres 2-5 (r3, r5, marked by in situ hybridisation for krox-20) in the zebrafish hindbrain (Cooke *et al.*, 2005).

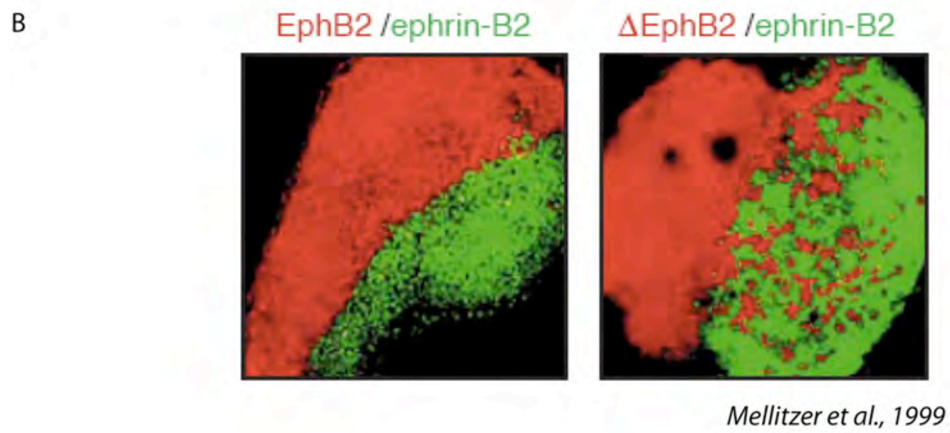
Figure 1.3

A



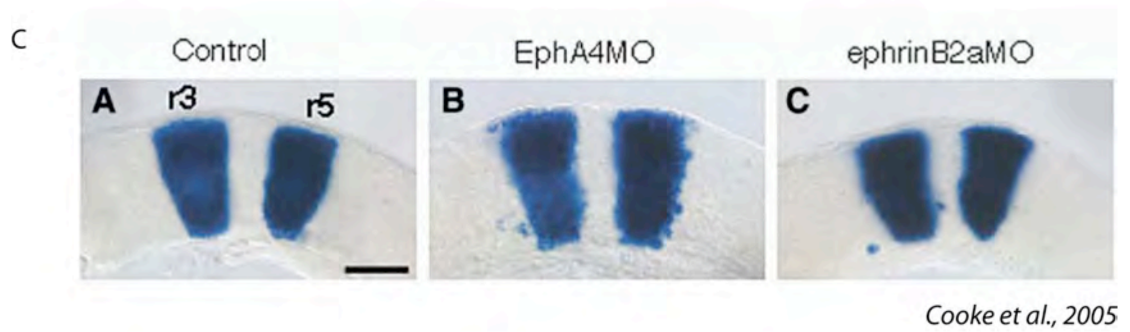
Foty & Steinberg, 2005

B



Mellitzer et al., 1999

C



Cooke et al., 2005

differential adhesion hypothesis. The next question is whether this is relevant to cell segregation *in vivo*.

In vivo – differential adhesion in boundary sharpening

Since its conception, differential adhesion has been thought to help maintain segregation at boundaries. There is good evidence for this. Firstly, the boundaries of expression of different types of cadherin often coincide with morphological boundaries early in development (Redies & Takeichi, 1996). In *Drosophila* development and in the vertebrate brain, differential cadherin expression has been shown to correlate with a functional role. DE-cadherin in the *Drosophila* oocyte is required for its correct positioning in the ovary (Godt & Tepass, 1998; Gonzalez-Reyes & St Johnston, 1998). Its expression is required in the oocyte itself and in the surrounding posterior follicle cells in order to position the oocyte at the posterior of the ovary.

In chick brain development, R-cadherin and cadherin-6 are expressed in the future cerebral cortex and the lateral ganglionic eminence respectively, with their expression marking the lineage restriction between the compartments. Overexpression of R-cadherin in the LGE resulted in sorting to the cortex and the opposite is true for cadherin-6, suggesting that they are required to maintain a clear boundary between these two regions (Inoue *et al.*, 2001).

Expression of different cadherins is also observed between different pools of motor neurons in the developing chick spinal cord. In this case, mis-expression of MN-cadherin was found to disrupt the segregation of two motor pools which are normally distinguished by expression of this protein (Price *et al.*). The above results demonstrate that differential cadherin expression is found between adjacent regions of a developing embryo and in some cases is involved in their segregation.

The differential adhesion hypothesis is not the only mechanism responsible for sorting and boundary maintenance.

There are still several observations, however, which do not fit with the differential adhesion hypothesis being the only mechanism driving sorting. Two of the best-studied boundaries in development are the dorso-ventral and the anterior-

posterior boundaries in the fly wing imaginal disc. Much progress has been made in understanding that the maintenance of the AP boundary requires *engrailed* expression in posterior cells which activates hedgehog signalling, to which anterior cells adjacent to the boundary can respond. At the DV boundary, local Notch signalling is important in preventing cell mixing (Tepass *et al.*, 2002). However, the mechanistic link between these signalling pathways and boundary maintenance remains unknown. Interestingly, forced overexpression of DE-cadherin in the wing disc leads to these cells sorting from the endogenous tissue (Dahmann & Basler, 2000). However, there has been little progress in identifying cell adhesion molecules which are differentially expressed between the compartments.

It has been suggested that the transmembrane proteins Capricious and Tartan confer differences in affinity to cells in the dorsal compartment of the *Drosophila* wing disc (Blair, 2001; Milan *et al.*, 2001). Their expression is able to rescue boundary formation in discs with reduced *apterous* expression, a selector gene for dorsal compartment identity. Similarly, Lrrm1, a vertebrate orthologue of Cap/Tar is required for the formation of the midbrain/hindbrain boundary in chick (Tossell *et al.*, 2011). However, the intracellular domains of these proteins are required for their function in segregation and it has been shown that their expression in cells does not induce aggregation, indicating that they may function more by intracellular signalling than cell-cell adhesion (Milan *et al.*, 2005; Tossell *et al.*, 2011). Such evidence suggests that there are other mechanisms which are important in the maintenance of this boundary.

In addition, there is little loss of function data to support a requirement for differential adhesion in the maintenance of boundaries *in vivo* (Batlle & Wilkinson, 2012). For example, the protocadherin, PAPC, is required for the separation of mesoderm and ectoderm in Xenopus gastrulation (Winklbauer, 2009). PAPC is also capable of reducing the strength of C-cadherin mediated adhesion. However, expression of a truncated version of PAPC, which cannot interact with C-cadherin, does not affect segregation behaviour (Winklbauer, 2009). This result is consistent with the idea that differential adhesion does not drive separation of these tissues.

Also mechanistically, the differential adhesion hypothesis does not always hold true. The proposition is that different cadherin subtypes sort from one another due to differences in relative affinities. However, Niessen and Gumbiner show that the relative adhesion strength, based on adhesion flow assays, of C, E and N-cadherins does not predict the pattern in which they sort (Niessen & Gumbiner, 2002). A similar observation was made in studying the sorting of embryonic zebrafish cells into the three germ layers, where the expression of adhesion molecules and relative adhesive strengths of the three cell types does not predict the order in which they sort (Krieg *et al.*, 2008; Schotz *et al.*, 2008). These data suggest that there are other cell properties than adhesiveness which can influence the order of cell sorting.

Taken together, this indicates that differential adhesion is able to drive cell sorting. However, there are circumstances where it is insufficient to do so, in certain *in vitro* cell lines and at some developmental boundaries *in vivo*.

Tension: Cortical tension and acto-myosin fences

Another mechanism which appears to be important in sorting and the maintenance of some boundaries is cortical acto-myosin tension (Landsberg *et al.*, 2009; Major & Irvine, 2005; Major & Irvine, 2006; Monier *et al.*, 2010). Evidence for the importance of cortical tension comes from studies in fly epithelia and zebrafish germ layers.

One place where tension is involved in boundary maintenance is in the *Drosophila* wing imaginal disc. Notch signalling is responsible for the maintenance of the dorsal-ventral (DV) boundary in the *Drosophila* wing disc, and it has been suggested that Notch activity creates a “fence” between dorsal and ventral cells (Major & Irvine, 2005). In support of this “fence model”, F-actin and myosin II are enriched at this boundary and their activity is required for boundary maintenance downstream of Notch (Major & Irvine, 2005; Major & Irvine, 2006).

A similar enrichment of actin and myosin II is seen at the anterior-posterior (AP) compartment boundary of the *Drosophila* wing disc. There is an alignment of cell bonds, and an enrichment of F-actin and myosin II occurs at the level of adherens junctions, without any increase in cadherin accumulation (Landsberg *et al.*, 2009).

The cell bonds aligned at the boundary were revealed, by specific laser ablation of individual cell bonds, to be under 2.5-fold more tension than surrounding cell bonds. Computer modelling demonstrated that an increase in tension is able to maintain a straight boundary, although a 2.5-fold difference between boundary and non-boundary bonds was not sufficient to form a completely sharp boundary.

There is also an enrichment of actin and myosin at the AP parasegment boundaries (Monier *et al.*, 2010). These "myosin cables" were shown to maintain the straightness of the boundary after it was challenged by cell division. Local chromophore-assisted light inactivation (CALI) of myosin II disrupted the straight compartment boundaries indicating the importance of myosin cables in boundary maintenance.

Actin accumulation is also seen at the edges of DE-cadherin and Echinoid (Ed) mutant clones in fly epithelia (Chang *et al.*, 2011). Ed is the *Drosophila* homologue of nectin, a transmembrane protein which mediates cell-cell adhesion primarily at adherens junctions. Chang *et al*. propose that proper sorting of these clones consists of two steps: sorting is controlled by differential Ed expression; but maintenance of a smooth boundary requires the production of an actin cable. The development of this cable appears to require the intracellular domain of Ed and is only required in the Ed positive cells, in contrast to the situation at the parasegment boundaries where acto-myosin accumulation is seen on both sides of the boundary (Chang *et al.*, 2011; Monier *et al.*, 2010).

Cortical tension generated by actin and myosin is also important for cell sorting in zebrafish. Atomic force microscopy was used to measure the relative cell-cortex tensions and the adhesive strength between cells from the three germ layers (Krieg *et al.*, 2008; Manning *et al.*, 2010; Schotz *et al.*, 2008). When the different combinations of cells were mixed, their pattern of sorting correlated with increased cortical tension as opposed to adhesive strength. Disruption of the cortex using the myosin II inhibitor blebbistatin was able to reduce sorting, and differential expression of a dominant-negative Rho kinase was sufficient to induce sorting. Combined with computer modelling, these data suggest that differential acto-myosin-mediated cortical tension is required to explain cell sorting of the zebrafish germ layers (Krieg *et al.*, 2008). Further *in vivo* and computational work

has demonstrated that this is because cortical tension is a key component in generating tissue surface tension in embryos. The authors conclude that, consistent with the differential adhesion hypothesis, differential surface tension between the germ layers specifies their positioning. However, they argue that surface tension consists of a combination of the cortical tension of a cell as well as its adhesive properties (Manning *et al.*, 2010; Schotz *et al.*, 2008).

In summary, acto-myosin tension is important for boundary sharpening and maintenance in two respects. In one case, it is required to generate differences in cortical tension between the different cell populations, which contributes to differences in their overall tissue tension, which underlies cell sorting. In the other, a local alignment of acto-myosin-rich cell bonds contributes to the sharpening and maintenance of epithelial cell compartments.

Repulsion

It has been suggested that one mechanism of driving cell sorting might be repulsion between cells of different types (Mellitzer *et al.*, 1999; Xu *et al.*, 1999). Abercrombie advocated the idea of contact inhibition of migration, the process by which a cell ceases to migrate on contact with another cell (Abercrombie & Heaysman, 1954). Collision with a different cell type would prevent the forward migration of a cell and thus prevent its invasion into the adjacent territory (Astin *et al.*, 2010). In this context, repulsion is an active response of the cell after contact with another cell, involving cytoskeletal collapse and migration away from the point of contact. In contrast, the term repulsion is sometimes used to refer to de-adhesion between cells, which could fit with Steinberg's hypothesis where local adhesion differences could result in sorting (Cooke *et al.*, 2005; Steinberg, 2007).

Contact inhibition can be seen between both like and unlike cells in culture by a collapse of cell protrusions after contact and subsequent migration away from the point of contact (Abercrombie, 1962; Theveneau *et al.*, 2010). A similar effect is seen in culture between cells expressing Eph receptors, a family of receptor tyrosine kinases, and cells expressing their ligands, ephrins (Astin *et al.*, 2010; Monschau *et al.*, 1997; Poliakov *et al.*, 2008).

Eph receptors and ephrins are expressed in apposed regions of cells throughout developing vertebrate embryos. For example, they are expressed in a largely alternating pattern in the rhombomeres of the hindbrain (Gale *et al.*, 1996; Pasini & Wilkinson, 2002). They are vital for the maintenance of sharp boundaries between these compartments (Mellitzer *et al.*, 1999; Xu *et al.*, 1999). By analogy with their known roles in growth cone guidance, it has been suggested that signalling through Eph receptors and ephrins could mediate repulsion between compartments and that this could maintain the boundaries between the compartments (Mellitzer *et al.*, 1999; Xu *et al.*, 1999).

Eph receptors and ephrins

Eph receptors are receptor tyrosine kinases which mediate signalling upon binding to membrane-bound ephrins on an adjacent cell (Poliakov *et al.*, 2004). Eph-ephrin signalling can affect a range of cell processes including repulsion and adhesion, proliferation and survival. It has been implicated in a variety of diseases and physiological processes including development and regeneration of the nervous system, oncogenic transformation, stem cell maintenance and immune function (Pasquale, 2008; Pasquale, 2010; Poliakov *et al.*, 2004). In addition, Eph receptors and ephrins are expressed throughout developing and adult tissues and their mis-regulation often correlates with poor prognosis in cancer patients. There is thus increasing interest in understanding the activity of these molecules and their therapeutic potential (Pasquale, 2008).

Eph receptors and ephrins are also unusual as signalling molecules in that signal transduction has been shown to occur in both the Eph-expressing cell (forward signalling) and the ephrin-expressing cell (reverse signalling). They are split into two classes, with the EphB receptors mostly binding transmembrane ephrinBs, and the EphA receptors binding glycosyl phosphatidyl inositol (GPI) linked ephrinAs. In general, binding is promiscuous within groups such that EphAs interact with all ephrin-As and all EphBs with ephrinBs. The exceptions to this rule are EphA4, which can interact with ephrinB2 and ephrinB3 as well as ephrinAs (Gale *et al.*, 1996), and EphB2, which interacts not only with ephrinBs but also ephrinA5 (Himanen *et al.*, 2004).

Since both Eph receptors and ephrins are membrane-tethered, their activation requires direct contact between cells, a characteristic not exhibited by other receptor tyrosine kinases. In common with other receptor tyrosine kinases, clustering of the receptor is necessary for downstream signalling. Eph receptors cannot be stimulated by soluble monomeric ligands (Davis *et al.*, 1994), but only by ephrins that are membrane-tethered or artificially clustered. Experiments using ephrinA5-coated beads demonstrate that the actively phosphorylated area covered by EphA3 clusters exceeds that in contact with the bead (Wimmer-Kleikamp *et al.*, 2004), suggesting a non-linear correlation between ephrin contact and Eph activation.

Roles of Eph receptors and ephrins

Collectively, Eph receptors and ephrins are expressed in every tissue in the developing vertebrate embryo (Gale *et al.*, 1996; Murai & Pasquale, 2003; Poliakov *et al.*, 2004). They have been implicated in a variety of cellular processes, including proliferation, survival, adhesion and repulsion and are important for embryonic patterning as well as tissue maintenance in adult life (Pasquale, 2005; Poliakov *et al.*, 2004; Solanas *et al.*, 2011). Their role is well characterised in angiogenesis where they are important for the guidance and development of new blood vessels, with Eph receptors and ephrins expressed in veins and arteries respectively (Gerety *et al.*, 1999). Of clinical relevance, Eph-ephrins are frequently found to be mis-regulated in tumours (Pasquale, 2010). Their roles are perhaps best understood in the development of the nervous system.

Eph receptors and ephrins have a particularly well-studied role in the guidance and patterning of neurons. For example, EphB2 is involved in the guidance of commissural axons across the midbrain (Henkemeyer *et al.*, 1996) and gradients of expression of ephrinA2 and ephrinA5 direct motor axons expressing different levels of EphA4 to the correct position in the limb (Helmbacher *et al.*, 2000).

One established role of Eph receptors and ephrins in neuronal guidance is the topographic mapping of retinal neurons to the optic tectum (Suetterlin *et al.*, 2011; Triplett & Feldheim, 2011). Neurons positioned at the nasal side of the retina project axons to more anterior positions within the optic tectum and increasingly

temporal neurons project axons more posteriorly within the tectum. It was postulated that graded molecular cues within the retina and tectum could guide each retinal axon to a unique tectal position (Sperry, 1963). EphA3 and EphA4 receptors and ephrinA2 were later found to be expressed in opposing gradients in these regions, consistent with the idea that they could be the molecular cues (Cheng *et al.*, 1995). Drescher *et al*. provided the first functional evidence of this using an *in vitro* stripe assay to demonstrate that ephrinA2 (RAGS) was capable of repelling retinal axons (Drescher *et al.*, 1995). Thus, the graded expression of EphAs and ephrinAs underlies neuronal positioning.

The situation has become more complicated since the discovery of opposing gradients of ephrinAs and EphAs within the retina and the tectum, suggesting that interactions between ephrinAs and EphAs *in cis* may have modulatory effects on the repulsive behaviour of these axons (Hornberger *et al.*, 1999; Suetterlin *et al.*, 2011). Co-expression of different Eph receptors and ephrins in the same cell has been shown to alter their behaviour in other contexts as well. For example, EphB2-ephrinB2 interactions usually result in cell repulsion, but when both are expressed together in cells in the urethra, they can promote adhesion. These different responses can be attributed either to interactions *in cis* between the Eph and ephrin, or to the combined effect of signalling responses through both the receptor and ligand in the same cell.

Binding, activation and disengagement

Eph receptors and ephrins bind one-another with high affinity and yet one of the major cell responses they induce is disengagement and subsequent retraction of cell membranes. Two mechanisms have been described which explain the disengagement of the proteins: proteolytic cleavage and endocytosis.

Proteolytic cleavage of ephrin by the Adam10 metalloproteinase has been demonstrated to be important for the disengagement of EphA3 and ephrinA2 (Hattori *et al.*, 2000) or ephrinA5 (Janes *et al.*, 2005). A similar mechanism also exists for EphB-ephrinB detachment, but through secreted metalloproteinases rather than Adam10 (Lin *et al.*, 2008; Litterst *et al.*, 2007).

The second mechanism for disengagement involves endocytosis of the Eph-ephrin signalling complex (Marston *et al.*, 2003; Zimmer *et al.*, 2003). The complex can be endocytosed into either the Eph receptor or the ephrin-expressing cell. For example, EphB4 activation by ephrinB2 was found to cause actin assembly and endocytosis, both of which are Rac-dependent (Marston *et al.*, 2003). This endocytosis could directly cause cell disengagement by physically severing the membranes of apposing cells. Since the complex remains intact after endocytosis, there is the possibility that it can continue to signal once it has been internalised (Marston *et al.*, 2003).

This evidence suggests that either proteolytic cleavage or endocytosis mechanisms or both could function to break contacts between Eph receptors and ephrins. This is important for terminating signalling and could also underlie the repulsive or de-adhesive responses of cells to Eph-ephrin signalling discussed below.

Eph-ephrins in boundary maintenance and cell sorting

Eph receptors and ephrins are expressed from early in development and are commonly, but not always, found in a complementary pattern between different regions of an embryo (Gale *et al.*, 1996; Wilkinson, 2001). Segregation by Eph receptors and ephrins is also found to be important in adult life; for example EphBs and ephrinBs are important in compartmentalising cells in the adult intestine (Batlle *et al.*, 2002; Solanas *et al.*, 2011).

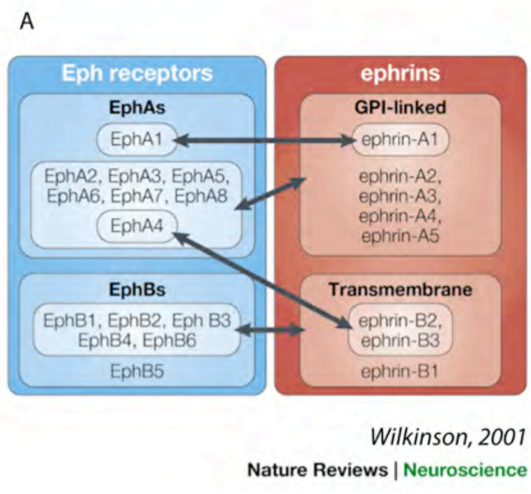
In the vertebrate hindbrain, Eph receptor expression largely alternates with that of ephrins (Pasini & Wilkinson, 2002). For example, EphA4 is expressed in r3 and r5, whereas its ephrinB ligands are highly expressed in r2, r4 and r6 (Figure 1.4). The boundaries between these rhombomeres in wild type embryos are sharp and straight. However, when EphA4 or ephrinB2 are knocked down using anti-sense morpholino oligonucleotides (MOs), these boundaries become fuzzy, and the effect is exaggerated when both MOs are used in combination (Figure 1.3 C; Cooke *et al.*, 2005), indicating the importance of Eph-ephrin signalling in the sharpening of these boundaries. Studies with juxtaposed animal caps demonstrate that overexpression of EphA4 or EphB2 receptor in one cap and ephrinB2 in another can restrict intermingling between the two populations (Mellitzer *et al.*, 1999).

Figure 1.4: Eph receptors and ephrins

(A) Eph receptors are divided into two families, EphAs and EphBs, which generally interact with GPI-linked ephrinAs or transmembrane ephrinBs respectively. Binding is quite promiscuous within the groups with most EphAs binding most ephrinAs and most EphBs binding most ephrinBs. The main exception is EphA4, which can also interact with specific ephrinBs (Wilkinson, 2001). (B) Eph-ephrins are bi-directional signalling molecules. Both receptor and ligand are membrane-bound and both can transduce signals. A response in the Eph-expressing cell is referred to as forward signalling, whereas a response in the ephrin-expressing cell is called reverse signalling (Klein, 2009). (C) Alternating expression in the hindbrain. Several Eph receptors and ephrins are expressed in the developing vertebrate neuroepithelium. The transcription factor Krox20 governs the expression of certain Eph receptors and ephrins in r3 and r5, such that EphA4 expression for example, is enriched in these rhombomeres. The expression of Eph and ephrins is largely complementary between rhombomeres such that, for example EphA4 is expressed in r3 and r5 whilst ephrins B2 and B3 are expressed in adjacent rhombomeres. Blue bars indicate expression. Dark blue indicates higher levels of expression than light blue (Pasini & Wilkinson, 2002).

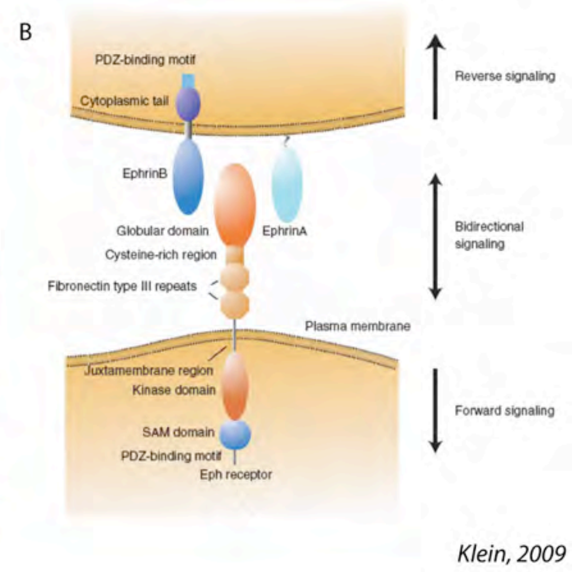
Figure 1.4

A



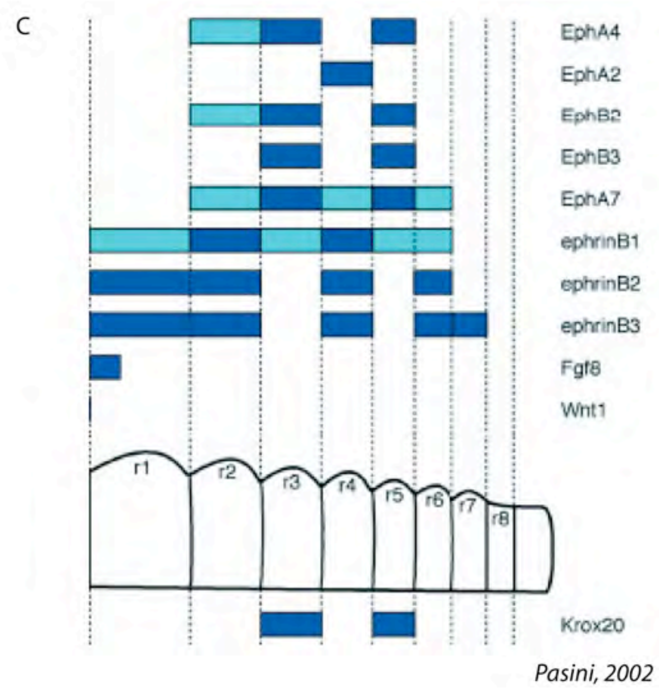
Wilkinson, 2001

B



Klein, 2009

C



Pasini, 2002

Furthermore, they show that bi-directional Eph-ephrin signalling is necessary for this process, as overexpression of a truncated form of either the Eph-receptor or the ephrin disrupts this effect so that a sharp boundary is not maintained. This role of Eph receptors and ephrins in segregating cells has been confirmed by an *in vitro* assay recently developed in our lab in which EphB2-expressing HEK293 cells sort out from ephrinB1-expressing cells into clearly defined clusters (Jorgensen *et al.*, 2009; Poliakov *et al.*, 2008).

Overlapping expression of Eph receptors and ephrins also frequently occurs *in vivo*, with several Eph receptors or ephrins expressed within any given region of the embryo (Sobieszczuk & Wilkinson, 1999). Their combinatorial function can be important for their role in segregation. For example, both ephrinBs and EphB receptors are important in the adjacent ectoderm and mesoderm for boundary maintenance between these tissues in Xenopus embryos (Rohani *et al.*, 2011).

Whilst it is well established that Eph receptors and ephrins are involved in the maintenance of specific developmental boundaries, the mechanisms through which they achieve this are not well understood. One school of thought is that they segregate cells by contact-dependent repulsion. The other suggests that they could be responsible for regulating adhesion.

Eph receptors, ephrins and repulsion

One possible mechanism through which Eph-ephrins could mediate cell sorting is by generating repulsion between adjacent groups of cells. Eph-ephrins are known to bring about a repulsive response in many contexts in development and this has been particularly well studied in the nervous system.

One example is in the guidance of axonal growth cones. EphBs are required to guide commissural axons down the ventral midline of the mouse spinal cord. In mice lacking ephrinB3 or EphBs, these axons migrate either side of the midline, suggesting that EphB forward signalling is restricting the migration of these axons on interaction with an ephrinB3 barrier at the midline (Kadison *et al.*, 2006). ephrinB3 has also been shown to mediate midline repulsion of EphA4-expressing neurons in postnatal mice (Yokoyama *et al.*, 2001). In addition, ephrinB2 has been

demonstrated to act as a repellent in the projection of neurons across the optic chiasm in both Xenopus and mouse (Nakagawa *et al.*, 2000; Williams *et al.*, 2003).

Repulsion mediated by Eph-ephrin signalling can be seen directly *in vitro*, characterised by a collapse response of axonal growth cones (Jurney *et al.*, 2002; Monschau *et al.*, 1997) and also of EphB2-expressing HEK293 cells after contact with ephrinB1-expressing cells (Poliakov *et al.*, 2008). EphA receptors have been shown to be vital for the contact inhibition response between prostate cancer cells and are antagonised by EphB over-expression in these cells, which has been implicated in the malignancy of prostate cancer (Astin *et al.*, 2010). Thus, Eph receptors and ephrins can generate repulsion by initiating a local collapse of cell protrusions.

Eph-ephrin regulation of repulsion

One of the main mechanisms through which Eph receptors and ephrins can mediate repulsion is by regulation of the Rho family of GTPases (Poliakov *et al.*, 2004). Rho GTPases play an important role in the regulation of the actin cytoskeleton and in the control of cell migration. They are modulated by cycling from a GDP-bound to an active GTP-bound form via the activity of guanine-nucleotide exchange factors (GEFs) and the intrinsic GTPase activities of the Rho proteins.

Eph-ephrin regulation of Rho-GEFs is the main mechanism through which they can modulate cell migration and repulsion (Noren & Pasquale, 2004; Poliakov *et al.*, 2004). GEFs have been found which bind with Eph receptors and can interact with different RhoGTPases including Rac1, RhoA and Cdc42. These RhoGTPases regulate the dynamics of the cytoskeleton. In general, Rac1 is considered to be involved in actin polymerisation and promoting lamellipodial protrusions, RhoA with actin dynamics, stress fibre formation and cell contractility and Cdc42 in the formation of filopodia, although there is some overlap in their function (Hall & Nobes, 2000; Spiering & Hodgson, 2011). In addition, Eph receptor binding to adaptor proteins Dishevelled (Tanaka *et al.*, 2003), Crk (Lawrenson *et al.*, 2002) and Ras-GAP (Holland *et al.*, 1997) leads to activation of RhoGTPases.

Altering the balance of activity of the different RhoGTPases Rac, Rho and Cdc42 can be responsible for a change in cell behaviour. Eph-ephrin signalling often acts to shift this balance. For example, in neuronal cells, a shift towards RhoA results in growth cone retraction (Shamah *et al.*, 2001; Wahl *et al.*, 2000). The GEF ephexin is important in this process. Binding of ephexin to Eph activates RhoA and inhibits Cdc42 and Rac1 in the growth cone, promoting outgrowth (Shamah *et al.*, 2001). Stimulation by ephrin results in tyrosine phosphorylation of ephexin which shifts the RhoGTPase balance to a repulsive response (Knoll & Drescher, 2004).

It has recently been proposed that the repulsion exhibited by Eph-ephrin signalling is analogous to the process of contact inhibition of locomotion (CIL) (Astin *et al.*, 2010; Mayor & Carmona-Fontaine, 2010). This process was defined by Abercrombie as “the stopping of the continued locomotion of a cell in the direction which has produced a collision with another cell” (Abercrombie, 1970). In the case of prostate cancer cells, activation of Cdc42 by EphB-ephrinB signalling results in cell migration and metastatic invasion of fibroblasts (Astin *et al.*, 2010). Conversely, contact inhibition between cancer cells is facilitated by EphA-induced ROCK signalling (Astin *et al.*, 2010). These data reinforce the idea that Eph-ephrin signalling regulates the balance between different RhoGTPases, which can control cell repulsion and invasion.

Eph receptors, ephrins and adhesion

It has been suggested that Eph-receptors and ephrins could mediate cell sorting by effecting differential adhesion (Cooke *et al.*, 2005; Steinberg, 2007). Steinberg suggested that repulsion between Eph and ephrin expressing cells could result in de-adhesion, which would lead to an effective difference in adhesiveness between the two populations (Steinberg, 2007). Other studies suggest mechanistic links between Eph-ephrins and the adhesive machinery (Cortina *et al.*, 2007).

In zebrafish transplantation experiments, cells in which EphA4 has been knocked down by morpholino sort to the boundaries of rhombomeres which express EphA4. Meanwhile, wild-type cells sort into discrete clumps within the EphA4 morphant rhombomeres, rather than to the boundaries. This result suggests that

Eph receptors must be active within the segments and is consistent with an adhesive function of Eph signalling (Cooke *et al.*, 2005; Kemp *et al.*, 2009)

In some contexts, Eph-ephrin signalling seems to promote attraction. Activation of EphB1 by ephrinB1 is capable of upregulating adhesion to the ECM via α_v β_3 and $\alpha_5\beta_1$ Integrins (Huynh-Do *et al.*, 1999). In contrast to its role in repulsive guidance of growth cones to the limbs, EphA4 attractively guides motor axons to the axial muscles via interaction with ephrinA5 in the rostral sclerotome (Eberhart *et al.*, 2002). An attractive role is also seen in topographic mapping of vomeronasal neurons to the accessory olfactory bulb (Knoll & Drescher, 2002). Here, vomeronasal axons expressing high levels of ephrinA5 project to regions of the accessory olfactory bulb that express high concentrations of EphA6, and this pattern is disrupted in the ephrinA5 mutant. Interestingly, *in vitro*, these axons grow preferentially on substrates containing EphAs, suggesting an adhesive or attractive guidance mechanism. This modulation of EphA and ephrinA activity by different levels of co-expression has also been seen in the topographic mapping of retinotectal neurons. In this case, however, it has been suggested that this activity could also be explained by a *cis*-inhibition of repulsion (Hornberger *et al.*, 1999; Suetterlin *et al.*, 2011). Attraction between Eph receptors and ephrins is context dependent and the difference between an attractive and repulsive response often depends on the co-expression of other Eph receptor or ephrin molecules.

In urethra development, EphB2 and ephrinB2 expression is required for epithelial fusion, which separates the urethra endoderm from the urinary and alimentary tracts (Dravis *et al.*, 2004). At the fusion site, both EphB2 and ephrinB2 are co-expressed, and signalling through both molecules is required for efficient fusion. This could indicate that combinatorial forward and reverse signalling within the same cells could impact on the cell response. Another possibility is that *cis*-inhibition of the Eph receptor by interaction with ephrin could cause a low level of signal activity.

Although the examples above demonstrate that Eph-ephrin signalling can switch between repulsive and adhesive roles in development, little is known about the regulation of cell-cell adhesion molecules through Eph-ephrins. Recent work has

suggested that Eph-ephrin signalling may directly affect the cadherin-mediated adhesion between cells.

Eph-ephrin regulation of cell-cell adhesion

Cell-cell adhesion is mediated by a variety of membrane-bound proteins. The first discovered and most prevalent of these are the cadherins, which mediate adhesion by binding to cadherins of the same type on adjacent cells.

Consistent with the idea that Eph receptors and ephrins could function by influencing cell-cell adhesion are *in vitro* experiments demonstrating that EphB-ephrinB-mediated cell sorting is disrupted when E-cadherin is knocked down (Cortina *et al.*, 2007). Colorectal cancer cell lines expressing EphB3 or EphB2 sorted into clusters when mixed with similar lines expressing ephrinB1-RFP. However, the expression of E-cadherin shRNA in either or both cell lines led to a reduction in sorting, measured by a reduction in the size of EphB clusters. The authors also report a translocation of E-cadherin to the membranes of EphB2-expressing cells stimulated with a soluble ephrinB1-Fc chimera (Cortina *et al.*, 2007). The authors proposed that E-cadherin regulation by EphB-ephrinB signalling could be driving sorting by establishing differential adhesion between cells.

It has been described that, in Schwann cells, EphB2 activation can lead to upregulation of N-cadherin (Parrinello *et al.*, 2010). This correlates with increased clustering of EphB-expressing Schwann cells and sorting from ephrinB-expressing fibroblasts. This upregulation of N-cadherin appears to be a long-term mechanism mediated by Sox2 expression downstream of EphB-ephrinB signalling. It has been suggested that this pathway could help to maintain migrating Schwann cells in groups, facilitating axonal outgrowth in the wound healing process.

More recently, a mechanism has been described in which EphB-ephrinB signalling could induce differential adhesion at the post-transcriptional level (Solanas *et al.*, 2011). Solanas *et al*. propose a model in which the metalloproteinase Adam10 binds EphBs, and signalling through the EphB receptor activates Adam10 to cleave the extracellular domain of E-cadherin. As adhesion remains normal between the other sides of the cell, which are in contact with other EphB cells, this localised

cleavage results in an effective differential adhesion between EphB and ephrinB cells. Treatment of EphB and ephrinB expressing cells with a metalloproteinase inhibitor, or the expression of a dominant negative version of Adam10 result in a disruption to sorting in colorectal cancer cells. There is a decrease in the co-localisation of E-cadherin between EphB and ephrinB cells which have interacted. In HEK293 cells over-expressing EphB2, there is an apparent increase in the level of cleaved extracellular E-cadherin after treatment with ephrinB1-Fc chimera. The authors also demonstrate that expression of a dominant-negative Adam10 in mouse phenocopies the disruption of intestinal crypt cell sorting seen in the EphB2 mutant mouse, suggesting that this cadherin cleavage mechanism is also relevant *in vivo*.

Further evidence for a link between Eph-ephrin signalling and the regulation of adhesion comes from two screens for phosphorylation targets of EphB2-ephrinB1 signalling, which identified a number of proteins involved in the regulation of cell-cell adhesion. These include p120 and the related protein p0071, and plakophilins, as well as various other molecules involved in the assembly of adherens junctions and polarity (Jorgensen *et al.*, 2009; Zhang *et al.*, 2008). The modulation of these proteins by Eph-ephrin signalling provides the potential for a direct mechanistic link between Eph receptors and ephrins and the regulation of cell-cell adhesion.

A requirement for adhesion for Eph-ephrin signalling?

Some reports suggest that cadherin-mediated adhesion is required upstream of Eph-ephrin signalling. Mouse ES cells lacking E-cadherin express decreased levels of EphB3 and EphB4 as well as ephrinB1 and ephrinB2, suggesting that E-cadherin is required for their correct expression (Orsulic & Kemler, 2000). In addition, cell lines in which E-cadherin was not present showed a decrease in the membrane localisation of EphA2. Low levels of phosphorylated EphA2 were found in a breast cancer cell line with low E-cadherin, and this could be rescued by increased E-cadherin expression (Zantek *et al.*, 1999). Together, these results suggest that E-cadherin may be required for the normal function of EphA2. No such relationship has yet been reported for other members of the cadherin or Eph receptor families.

Regulation of cell-cell adhesion

In vertebrates, cells stick to one another mainly through three specialised structures: tight junctions (TJs), adherens junctions (AJs) and desmosomes (Figure 1.6 A; Hofmann *et al.*, 2009; Takeichi, 2011). Of these, tight junctions are important for creating the permeability barrier across epithelial sheets. Desmosomes are also found only in a subset of cells and are thought to be important in withstanding mechanical stress (Hatzfeld, 2007). Adherens junctions are detected between most cells *in vivo* (Takeichi, 2011).

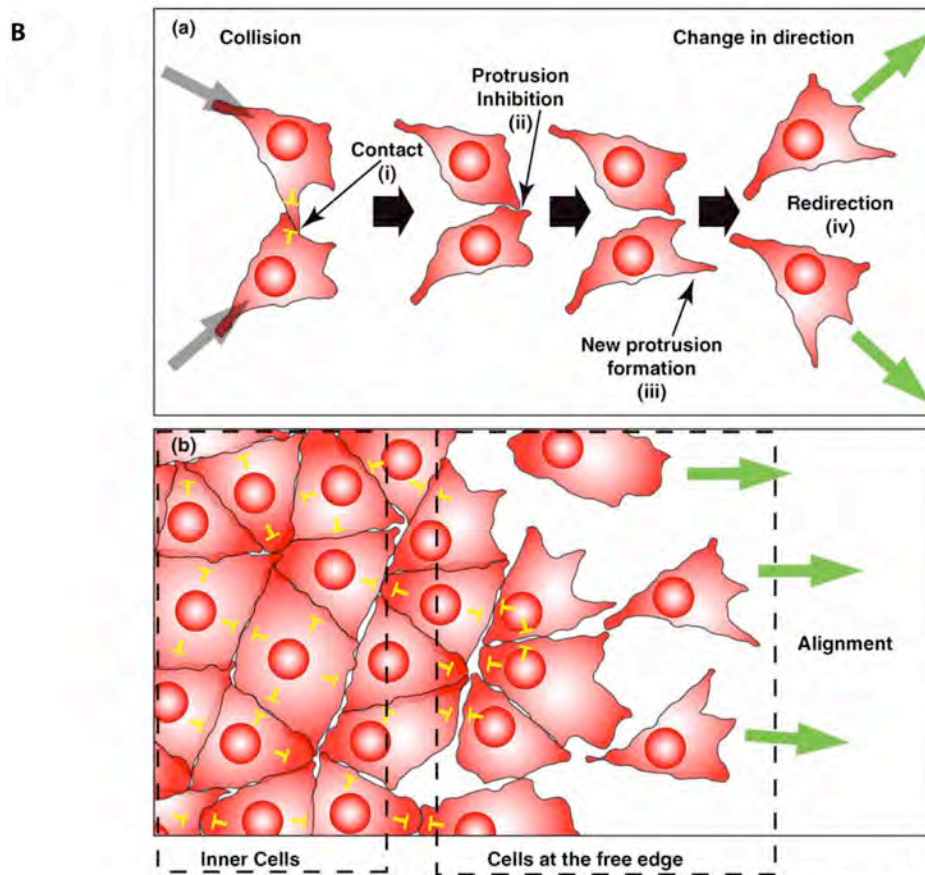
Cells adhere to one another through a variety of proteins expressed on the cell surface. The most widely studied are the classical cadherins, which interact in *trans* between cells, and are a key component of adherens junctions. They consist of one-pass transmembrane cadherins, such as E-cadherin, N-cadherin and P-cadherin (named after their discovery in epithelia, neuronal tissues and placenta, respectively) (Figure 1.6; Stemmler 2008). They were discovered as calcium-dependent adhesion molecules that are essential for tissue integrity, but have since been implicated in a range of cell behaviours such as synapse formation and cell motility (Takeichi, 2011).

In addition to classical cadherins, the cadherin subfamily also consists of non-classical cadherins, including desmosomal cadherins, FAT cadherins and protocadherins, which differ in their structure and function. Desmosomal cadherins are similar in structure to classical cadherins although desmosomes appear to play a different junctional role to adherens junctions. The other members of the cadherin superfamily are seven-pass transmembrane proteins and are not localised to specific cell-cell junctions. These include protocadherins, which have been implicated in regulation of adhesion through classical cadherins (Chen & Gumbiner, 2006; Meng & Takeichi, 2009), and FAT and Flamingo proteins which are involved in setting up planar cell polarity (Meng & Takeichi, 2009; Takeichi & Abe, 2005). Hereafter, the term cadherin refers to classical cadherins unless otherwise specified.

Figure 1.5: Cadherin functions: adhesion and contact inhibition

(A) The formation of cadherin adhesions in migrating cells. Cell protrusive activity, through lamellipodia or filopodia, will create random contacts between cells. These contacts form cadherin puncta, connected to the circumferential actin cable. Some cells form an intermediate stage called an adhesion zipper, created by myosin tension. As the contacts expand and mature, more cadherins build up and actin begins to accumulate at the adhesion belt, strengthening the adhesion between cells (Cavey & Lecuit, 2009). (B) Cadherins are also important for contact inhibition between migrating cells. After contact, if the cells do not form strong adhesions, they migrate away from one another and this directionality is dependent on the activity of cadherins at the point of contact (a). In a group of migrating cells, constant cadherin-dependent contact inhibition causes random orientation of cells within the group, but allows cells at the free edge to migrate directionally away from the group (b). Subsequently, cells which were previously part of the group are exposed to the free edge and they then move forward. This process can drive the collective migration of a whole group of cells (Mayor & Carmona-Fontaine, 2010).

Figure 1.5



Mayor & Carmona-Fontaine, 20

Cadherin-mediated adhesion

Classical cadherins are transmembrane proteins consisting of tandemly repeated extracellular cadherin (EC) domains and a highly conserved intracellular domain containing the juxtamembrane and the catenin-binding domains (Figure 1.6; Suzuki, 2008). In vertebrates, classical cadherins have 5 EC repeats, and are classified into two classes, type I and type II, depending on the presence or absence of a conserved His-Ala-Vav motif in EC1 (Stemmler, 2008). Interactions between the EC1 domain in *trans* between cadherins on apposing cells initiate cadherin binding (Meng *et al.*, 2007; Zhang *et al.*, 2009).

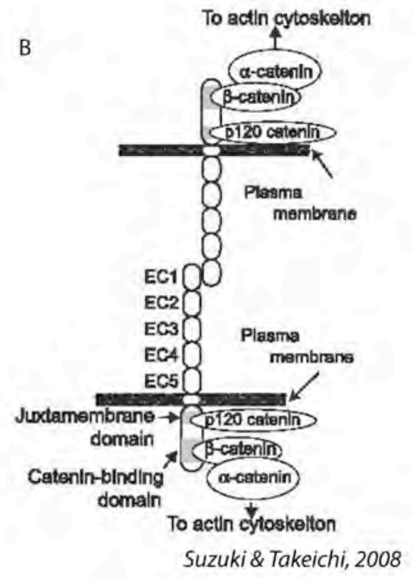
The juxtamembrane domain contains a defined sequence of amino acids which is responsible for binding the p120 family of catenins. There is still some debate about the precise functions of these molecules (see below), but they are important for stabilising the cadherins at the cell membrane (Chen *et al.*, 2003; Davis *et al.*, 2003). The catenin-binding domain binds β–catenin, which in turn binds α–catenin and the resulting complex is responsible for anchoring cadherins to the actin cytoskeleton. α–catenin can bind *in vitro* to cadherin and to F-actin but recent studies have demonstrated that it cannot bind both at once, suggesting that another protein must be required to make the link (Yamada *et al.*, 2005). One candidate is EPLIN, which can bind both α–catenin and actin, and whose activity is required for stabilising adherens junctions, although punctate junctions do still form in its absence (Abe & Takeichi, 2008; Suzuki & Takeichi, 2008). The linkage of cadherins to the cytoskeleton is essential for cadherin-mediated cell-cell adhesion (Hirano *et al.*, 1992; Suzuki & Takeichi, 2008).

Cadherins bind one another *in trans* either homophilically (binding to an identical protein) or they can bind heterophilically to other cadherins. For example, *in vitro* co-cultures demonstrate heterophilic binding between L cells transfected with P-cadherin and E-cadherin (Foty & Steinberg, 2005) and between N-cadherin and E-cadherin (Niessen & Gumbiner, 2002). However, the strength of binding is generally greater between molecules of the same type than in a heterophilic situation (Duguay *et al.*, 2003).

Figure 1.6: Cadherin structure and function

(A) Typical junctions in an epithelial cell. Interactions with other cells occur largely through the adherens junctions at the adhesion belt. Adhesion through classical cadherins is concentrated here. Tight junctions, desmosomes and gap junctions also mediate cell-cell adhesion. Cells interact with the extracellular matrix through integrins at focal adhesions and through hemi-desmosomes (php.med.unsw.edu.au). (B) Cadherin interactions. Classical cadherins consist of an extracellular domain containing 5 extracellular (EC) repeat regions. EC1 interacts with cadherin on the adjacent cell to mediate adhesion. The intracellular region consists of the catenin-binding domain, which anchors cadherins to the actin cytoskeleton, and a juxtamembrane domain which binds p120catenin, regulating its expression at the membrane (Suzuki & Takeichi, 2008). (C) Different families of cadherins, their common features and common examples (Stemmler, 2008).

Figure 1.6



When two cells come into contact, the EC domains of cadherins on apposing membranes interact. More cadherins are subsequently recruited to the site of contact and form a complex which links to the actin cytoskeleton (Ehrlich *et al.*, 2002; Yamada & Nelson, 2007). Cadherins can interact homotypically *in cis*, which could help regulate the formation of these complexes (Stemmler, 2008). Another model suggests that they interact *in trans* in a zipper-like manner to recruit more cadherins to the site of contact (Boggon *et al.*, 2002).

Adhesive junctions form when actin-driven cell surface projections, filopodia or lamellipodia, make contact with the membrane of another cell (Figure 1.5; (Green *et al.*, 2010). Initially, punctate adherens junctions form at this site of contact which develop into actively expanded cell contacts. This process requires organised control of the actin cytoskeleton including myosin II-mediated contractility (Krendel & Bonder, 1999). This process involves precise spatiotemporal regulation of RhoGTPases (Green *et al.*, 2010; Watanabe *et al.*, 2009). In MDCKII cells, RhoA and acto-myosin contractility as well as Rac1 and lamellipodia formation are localised to different regions of the membranes of contacting cells and are required for the establishment of stable contacts (Yamada & Nelson, 2007). The regulation of cytoskeletal activity is thus vital for the establishment and maintenance of stable cell adhesions.

In epithelial cells, the adherens junction is present between cells near their apical surface, characterised by the adhesion complexes and actin belt which form there. Assembly of the adherens junction requires the activation of RhoGTPases Rac1 and Cdc42 (Noren *et al.*, 2000), which contribute to stabilising cadherins at the cell surface. Actin branching via Arp2/3 is inhibited by homodimeric α-catenin, favouring actin bundling at sites of mature cell contact (Weis & Nelson, 2006). The requirement of catenins to form stable junctions highlights the importance of regulation of the actin cytoskeleton in this process. The high turnover and constant fluctuations of RhoGTPases and F-actin at the junctions contribute to their highly dynamic state.

Cadherin-mediated cell contacts are highly dynamic and cadherin complexes are being constantly trafficked away from cell junctions even in apparently stable situations (Yap *et al.*, 2007). Thus, the control of cadherin endocytosis is a key

mechanism for regulating cell adhesiveness. Certain signalling pathways result in increased endocytosis. For example, VEGF stimulation of sub-confluent endothelial cells increases VE-cadherin internalisation (Gavard & Gutkind, 2006; Yap *et al.*, 2007). Conversely, NMDA stimulation of neurons stabilises N-cadherin at the synapse (Tai & Truong, 2007; Yap *et al.*, 2007). One known regulator of cadherin endocytosis is p120, discussed below, which inhibits VE-cadherin endocytosis to stabilise the cadherin at the cell surface (Xiao *et al.*, 2005; Yap *et al.*, 2007).

In summary, cadherins mediate adhesion, but they function in consort with a range of other proteins, which regulate their stability and their linkage to the actin cytoskeleton. In addition, the actin cytoskeleton is tightly regulated to facilitate initial contacts, by the extension of cell protrusions, but also to maintain the structural integrity of the junctions. The tight coordination between these processes is crucial for the regulation of cell-cell adhesion.

p120 catenin in the regulation of adhesion

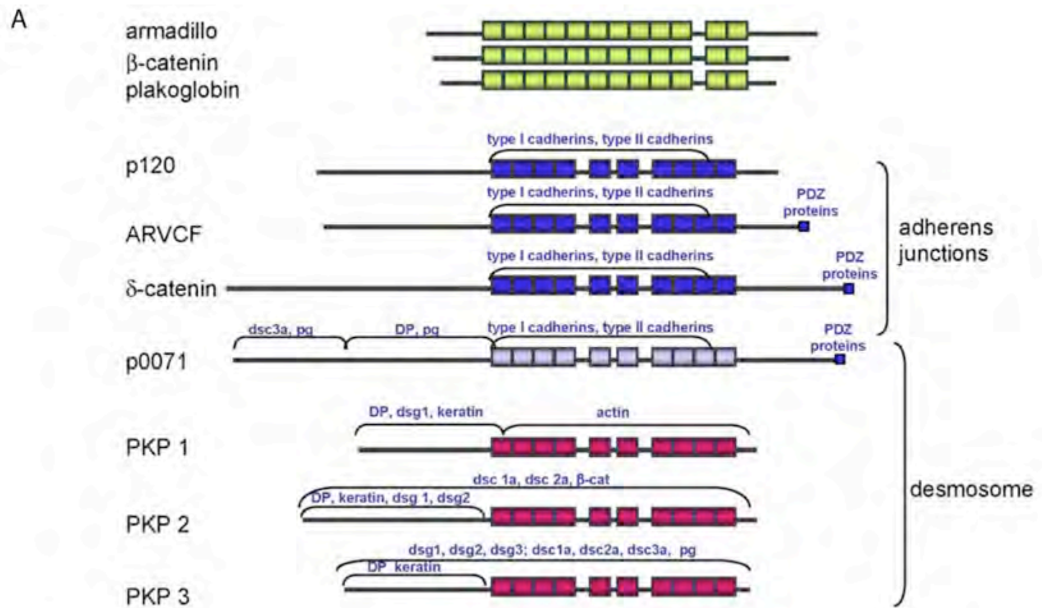
p120 was originally identified as a substrate of Src (Reynolds *et al.*, 1989). Its homology with β-catenin led to the idea that it could be involved with cadherins, and it was subsequently found to co-localise with (Reynolds *et al.*, 1992) and bind to E-cadherin (Ishiyama *et al.*, 2010). Further investigation has demonstrated that it binds to a specific motif in the juxtamembrane cytoplasmic domain of classical cadherins (Yap *et al.*, 1998) and that interaction with cadherins is necessary and sufficient for its recruitment to the cell membrane (Figure 1.7; Thoreson *et al.*, 2000).

The interaction of p120 with cadherin is also required to form stable cell-cell adhesions. p120 deficiency is embryonic lethal in mice but a conditional knockout of the gene in the mouse salivary gland shows decreased levels of E-cadherin by antibody staining (Davis & Reynolds, 2006). p120 knockouts in *Drosophila* and *C. elegans* are viable, likely through redundancy with the other close family members, p0071, ARVCF and δ–catenin (Davis & Reynolds, 2006). Decreased levels of p120 also cause rapid degradation of cadherins on delivery to the cell surface (Davis *et al.*, 2003; Xiao *et al.*, 2003). In mammalian cell lines, a p120-uncoupled form of E-cadherin has been shown to be incapable of promoting cell compaction, suggesting

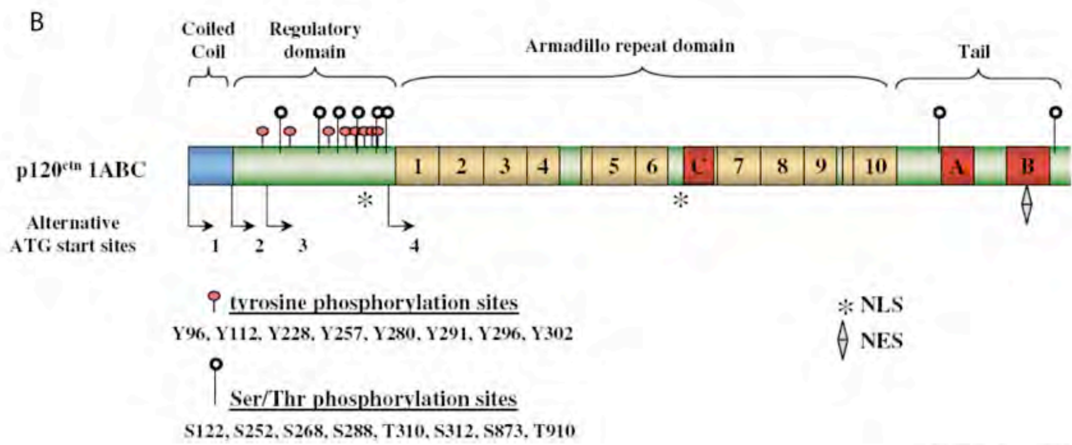
Figure 1.7: The p120ctn family

(A) Comparison of structures and binding partners of members of the p120-family proteins and their relatives. All of the proteins contain an armadillo repeat domain, similar to the armadillo/ β-catenin. p120, ARVCF, δ-catenin and p0071 are p120-like proteins which bind type I and II cadherins and are localised to adherens junctions. PKP1, 2 and 3 are plakophilins which bind cadherins localised at desmosomes. p0071 has also been found localised at desmosomes. DP, desmoplakin; pg, plakoglobin; dsg, desmoglein; dsc, desmocolin; PKP, plakophilin (Hatzfeld, 2005). (B) More detailed structure of p120 showing: a central armadillo repeat domain which is important for cadherin binding; a regulatory domain consisting of tyrosine (red dots) and serine/threonine (white dots) phosphorylation sites which vary with the dynamic regulation of p120 function. Alternative splicing in this region gives rise to 4 different isoforms; exons A, B and C are also alternatively spliced. NLS, nuclear localisation signal; NES, nuclear export signal (Reynolds *et al.*, 2004).

Figure 1.7



Hatzfeld, 2005



Reynolds, 2004

an impact on the ability of E-cadherin to form stable adhesions (Thoreson *et al.*, 2000). p120 overexpression leads to increased aggregation of cells (Aono *et al.*, 1999). Where E-cadherin is overexpressed in cell lines it can compensate for the effect of p120 knockdown, suggesting that p120 may not be absolutely required for junction formation (Ireton *et al.*, 2002). Nevertheless, p120 is an important component of adherens junctions and a key player in cadherin-mediated adhesion.

Whilst it is clear that there is a requirement for p120 in stabilising cadherins at the cell surface, there is some debate as to how it achieves this. Binding of p120 to cadherin could stabilise it at the membrane by protecting it from degradation or by decreasing the rate of turnover by endocytosis (Reynolds, 2007; Yap *et al.*, 2007). Another possibility stems from the fact that p120 has some RhoGTPase activity. The binding of p120 to cadherin localises this activity to the point of cell contact which could be important for cytoskeletal re-modelling at the junctions (Wildenberg *et al.*, 2006).

The phosphorylation state of p120 appears to be important, perhaps in modifying its ability to bind cadherins. There is a transient increase of tyrosine-phosphorylated p120 at nascent adhesive contacts (Calautti *et al.*, 1998; Calautti *et al.*), suggesting that phosphorylation is important at the establishment of contacts, but that phospho-p120 is not required to maintain cadherins in stable contacts. In support of the idea that phospho-p120 is required in stabilising E-cadherin, it has been demonstrated that tyrosine-phosphorylated p120 binds cadherin preferentially to the unphosphorylated version (Reynolds, 2007). However, it has also been shown that phospho-tyrosine defective mutants are also able to effectively bind and stabilise E-cadherin at the cell surface (Mariner *et al.*, 2004; Xiao *et al.*, 2003). p120 lacking the N-terminal domain (which contains regulatory phosphorylation sites) is capable of restoring E-cadherin stability more efficiently than full length p120 in Colo205 cells which normally grow dispersed (Aono *et al.*, 1999). The discrepancy between these results could be due to different roles of p120 in the different models discussed. It could also be the case that small variations in the levels of expression of the different p120 constructs could have significant effects on the phenotypes of the cells in which they are expressed.

p120 catenin regulation of cell motility and migration

Cell motility and migration are controlled by complex interactions with the ECM and surrounding cells as well as by regulation of the cytoskeleton within cells. Small GTPases including RhoGTPases are key players underlying these dynamic processes.

When not bound to cadherins, p120 family members can regulate RhoGTPases via GEFs or GAPs (McCrea & Gu, 2010). For example, p0071 is involved in the regulation of Rho necessary for cytokinesis (Wolf *et al.*, 2006). The p120 family generally activates Rac and inhibits Rho (Anastasiadis, 2007; Hatzfeld, 2007). Reduction of p120 levels leads to an increase in activated Rho in many cell lines (Reynolds, 2007). Forced expression of p120 in fibroblasts results in an abnormal branching morphology, consistent with altered cytoskeletal regulation by RhoGTPases in these cells (Reynolds *et al.*, 1996). It has been shown that in SV80, NH3T3 and CHO cells, expression of a full-length p120 induces protrusive activity. This correlates with an increase in migration of these cells and occurs via activation of Rac and Cdc42 without inhibiting Rho activity (Grosheva *et al.*, 2001). Therefore p120 can act through RhoGTPases to regulate cell morphology and motility.

The RhoGTPase regulating activity of p120 family members may be, in part, dependent on their association with cadherins. For example, it has been suggested that cadherin binding to p120 may facilitate the activation of Rac by cadherins (Goodwin *et al.*, 2003). Similarly, p120 has been shown to recruit p190RhoGAP to repress Rho near cadherins (Wildenberg *et al.*, 2006). Conversely, p0071 interacts directly with the RhoGEF, Ect2, to activate Rho (Wolf *et al.*, 2006). p120 is also able to interact with Rho and keep it in the GDP-bound/inactive form (Yanagisawa *et al.*, 2008). The fibroblast branching phenotype caused by overexpression of full-length p120 can be rescued by co-expression of the cadherin cytoplasmic domain. This suggests that Rho and cadherin bind to the same site on p120, precluding a cooperative role for p120 in recruiting Rho-inhibition from the site of cell contact (Reynolds, 2007). The differences in the regulatory activity of p120 family molecules may reflect a difference between the cell lines used in terms of: their

ability to activate Rac/Rho; expression of different cadherins; and interaction with different receptor tyrosine kinases.

Alternatively, it has been postulated that p120 family members act as a rheostat of adhesion and motility and that the release of p120 from cadherins could underlie EMT in some contexts (Grosheva *et al.*, 2001; McCrea & Gu, 2010). Therefore, variations in the levels of p120, or relative levels of phosphorylated p120, could underlie significant changes in cell responses.

Signalling and non-adhesive roles of cadherins

Aside from their role in mediating cell adhesion, cadherins also have a signalling role and have been implicated in the control of morphology, motility and migration. One widely found example is that of cadherin switching, a change in the expression of one cadherin for another. In development, a cadherin switch occurs during neurulation where E-cadherin expressing cells switch to N-cadherin expression as they invaginate to form the neural tube (Halbleib & Nelson, 2006; Hatta & Takeichi, 1986).

Cadherin switching frequently accompanies the increased motility of cells after epithelial to mesenchymal transition (EMT). The delamination of neural crest from the vertebrate neuroepithelium is one example of EMT. Neural crest cells (NCCs) derive from the N-cadherin expressing neural tube. During EMT, N-cadherin downregulation is accompanied by increased expression of cadherin-6B, followed later by a switch to cadherin-11 when cells become more migratory (Clay & Halloran, 2011; Nakagawa & Takeichi, 1995). Aside from the resulting change in adhesion, downregulation of N-cadherin also has a direct signalling role. Its regulation involves a proteolytic cleavage, resulting in release of a cytoplasmic domain, which signals to the nucleus to stimulate expression of other EMT-promoting genes (Clay & Halloran, 2011; Nakagawa & Takeichi, 1998; Shoval *et al.*, 2007). Knockdown experiments in chick indicate that cadherin-6B also has a role in increasing migration, in part by stimulating BMP signalling (Park & Gumbiner, 2010). Furthermore, studies of Xenopus cranial neural crest demonstrate that cadherin-11 is essential for NCC migration, and can induce filopodia and lamellipodia formation (Kashef *et al.*, 2009). In these cases, the cadherin switch

underlies the transition of cells to a more migratory cell type and is an important part of the developmental programme.

In other cases, cadherin switching represents a deviation of cells from their normal identity. EMT is a common feature of cancer and cells which have undergone the epithelial to mesenchymal switch become increasingly invasive. For example, E-cadherin is commonly downregulated and replaced with N-cadherin, R-cadherin, cadherin-6 or cadherin-11 (Halbleib & Nelson, 2006). N-cadherin can bind and activate FGFR resulting in MAPK signalling and extracellular metalloproteinase secretion, both of which stimulate increased invasiveness (Halbleib & Nelson, 2006). *In vitro*, N-cadherin expression in myoblasts has been shown to activate RhoA and inhibit Rac1 and Cdc42 (Charrasse *et al.*, 2002; Mayor & Carmona-Fontaine, 2010). Collectively, their roles in EMT in both normal development and in disease indicate that cadherins have a signalling function in the promotion of cell migration as well as their role in adhesion.

Experiments carried out culturing cells on alternating stripes of collagen (ECM) or E-cadherin-Fc demonstrate a cross-talk between E-cadherin mediated adhesion and lamellipodia activity and directionality (Borghi *et al.*, 2010). Adhesion of cells to E-cadherin decreased migratory activity and influenced the direction of migration, although the ECM was required to facilitate migration. Such a result demonstrates that there is cross-talk between cadherin and integrin mediated adhesion.

Collectively, these data demonstrate a variety of roles for cadherins. In addition to their requirement in cell-cell adhesion, they also contribute to cell morphology and migration via the activation of downstream signalling pathways, including the regulation of RhoGTPases.

Contact Inhibition of Locomotion

Two migratory cells in a dish which come into contact will generally migrate away from one another. This phenomenon was first recognised by Abercrombie and Heaysman in 1953 and was called contact inhibition of locomotion (CIL) (Abercrombie & Heaysman, 1953). It was later defined as: “the phenomenon of a cell ceasing to continue moving in the same direction after contact with another

cell" (Abercrombie, 1979). The behaviour consists generally of four phases: cell-cell contact; inhibition of protrusions, such as lamellipodia and filopodia, at the point of contact; formation of new protrusions away from the point of contact; and migration in the direction of the new protrusions (Mayor & Carmona-Fontaine, 2010).

CIL is a general property of most cells. For example, most cells when cultured on a dish will spread out to form a monolayer because they are prevented from migrating on top of one-another (Abercrombie *et al.*, 1957). However, in some cases contact inhibition is not seen, for example in some invasive cancers where cancer cells are freely able to ignore contact cues from the surrounding cells (Astin *et al.*, 2010). Neural crest cells are able to migrate through surrounding tissue as part of their normal development but retain the ability to contact inhibit one another's migration (Mayor & Carmona-Fontaine, 2010). Study of this common cell behaviour will help to increase our understanding of collective cell dynamics in development and later life.

Whilst the concept has existed for nearly 60 years, little progress has been made in elucidating the molecular mechanisms underlying CIL. Only very recently did analysis of neural crest migration in Xenopus provide the first evidence that CIL occurs *in vivo* (Carmona-Fontaine *et al.*, 2008a). These cells have a clear polarity, with a large lamellipodium at the leading edge of migration, which collapses on contact with another cell and changes the direction of migration. FRET analysis revealed an increase in RhoA activity at the point of contact between the cells, which is required to maintain CIL. Planar cell polarity pathway components Dsh and DEP are also required for this process and present one mechanism through which cells can recognise one another (Carmona-Fontaine *et al.*, 2008b). A more recent paper demonstrates that N-cadherin is also required for this process, and inhibits Rac1 activity, thus inhibiting protrusive activity at the site of cell-cell contact (Theveneau *et al.*, 2010). Theveneau *et al.* propose a model where N-cadherin is required for two processes to allow the neural crest cells to migrate as a coherent group. The first is to regulate adhesion between cells. The second is that in a cell that is not completely surrounded by N-cadherin contacts, the free edge

will be relieved from Rac1 inhibition and protrusions will form, driving directed migration.

Cadherins have also been implicated in directing polarity in relation to cell-cell contact in other cell types. For example, in rat astrocytes, N-cadherin is required to control positioning of the centrosome relative to the nucleus (Dupin *et al.*, 2009). The position of the centrosome is generally in front of the nucleus in migrating cells (Etienne-Manneville & Hall, 2001). In rat kidney cells (NRK-52E), E-cadherin was found to be required for the establishment of cell polarity, organising organelles towards the leading edge of the cell (Desai *et al.*, 2009). This cadherin-dependent polarisation of the cells was found to require the actin cytoskeleton and the activity of Cdc42. As with other cell processes involving cadherins, the function of cadherins in re-polarisation during CIL is dependent on small GTPases RhoA, Rac1 and Cdc42, whose polarised activity within the cell is required to direct migration away from the point of cell-cell contact.

It is notable that CIL displays many of the characteristics associated with repulsion mediated by Eph-ephrin signalling. Cell surface contact between Eph receptor and ephrin expressing cells results in the retraction of cell processes and repulsion, for example in axon retraction (Jurney *et al.*, 2002; Poliakov *et al.*, 2008). Eph-ephrins also regulate the activities of RhoA, Rac1 or Cdc42 after cell contact (Noren & Pasquale, 2004). Furthermore, EphB-ephrinBs have been shown to activate RhoA via Dsh/PCP signalling (Tanaka *et al.*, 2003). One intriguing possibility is that Eph-ephrins are heterotypic mediators of CIL, allowing recognition and repulsion between unlike populations of cells, which could facilitate their correct positioning in the embryo.

Aims of this study

My aim was to analyse whether cell-cell adhesion is involved in cell sorting mediated by EphB2-ephrinB1 signalling. Differential adhesion is a prominent theory in the field to explain cell sorting in development and disease. Little evidence existed for a role of differential adhesion downstream of Eph-ephrin signalling, but a recent paper suggested that EphB-ephrinB signalling required E-cadherin to mediate cell sorting (Cortina *et al.*, 2007). Previous work from the

Wilkinson lab had suggested that directional migration plays a role in the segregation of EphB2 and ephrinB1 expressing cells (Alexei Poliakov, unpublished) but a role of cell-cell adhesion in this process had not been addressed. I was interested to further investigate the idea that Eph-ephrins could drive cell sorting by implementing differential adhesion. I also wanted to investigate the role that repulsion downstream of Eph receptors could play in this model.

I used cell segregation and cell migration assays developed in the Wilkinson lab to investigate the role of cadherin-mediated adhesion in cell segregation between EphB2 and ephrinB1 expressing cells, with a particular focus on the potential for Eph-ephrin signalling to set up differential adhesion. The roles of p120 family catenins, p120 and p0071, were then analysed for their potential role downstream of Eph-ephrin signalling in the regulation of cell sorting. The effect of these molecules on migration downstream of Eph activation was also assessed, to investigate the relationship between cell-cell adhesion and migration in cell segregation.

2. Materials and Methods

Reagents

Antibodies

For Western blot analysis, primary antibodies were used at the indicated dilutions against: T-cadherin 1:1000 (3583, rabbit, ProSci); Cleaved-caspase 3, 1:1000 (9661, rabbit, Cell Signalling Technology); E-cadherin, 1:1000 (610181, mouse, BD); N-cadherin (C-ter) 1:1000 (610920, mouse, BD); N-cadherin (N-ter) 1:1000 (clone GC-4, C3865, SIGMA);Pan-cadherin, 1:500 (C1821, mouse SIGMA); p120 catenin, 1:1000 (610133, BD); p0071, 1:10 (651166, Progen); γ-tubulin, 1:1000 (T3559, rabbit, SIGMA); EphB2, 1:1000 (A467 , R&D Systems); ephrinB1, 1:1000 (AF473, R&D systems); α-Tubulin, 1:5000 (T9026, Sigma); β-tubulin, 1:1000 (T8328, mouse, SIGMA); phospho-Eph receptor antiserum, 1:500 (from C. Nobes, University of Bristol, England, UK); Adam10, 1:1000 (AB19026, Millipore); phosphotyrosine, clone 4G10, 1:1000 (05-321, Millipore); FLAG clone M2, 1:1000 (F3165, SIGMA);. Secondary antibodies used were compatible with the Odyssey scanner system: IRDye800CW, IRDye700DX, 1:5000 (raised in donkey against rabbit, mouse or goat, Rockland/Tebu-bio).

For immunocytochemistry, the same primary antibodies were used that are listed above, at the following dilutions: N-cadherin, 1:250; pan-cadherin, 1:500; EphB2, 1:100; ephrinB1, 1:100; γ-tubulin, 1:1000.

Recombinant ephrinB1-Fc chimera (473-EB-200, R&D Systems, 200ug/ml stock) was used at a final concentration of 5 μg/ml to stimulate EphB2 activation.

Plasmids

N-cadherin-eGFP (mouse, Addgene ,plasmid 18870); mAdam10 deltaMP in pQCXIP cFLAG (mouse, Addgene, plasmid 19138); E-cadherin (Alexei Poliakov); pCDNA3.1; pCS2+.

siRNA

Silencer select pre-designed siRNAs were purchased from Applied Biosystems, Ambion: Negative Control #1 siRNA: 4390844; N-cadherin (Cdh2): s2771, s2772, s2773; Adam10: s1004, s1005, s1006; p120 (Ctnnd1): s3725, s3726, 3727

p0071 (Pkp4): s16148, s16149, s16150.

Cell lines

HEK293 cells stably transfected with EphB2, EphB2 and a myristylated-GFP (EphB2 cells) or ephrinB1 (ephrinB1 cells) were generated using G418 and/or hygromycin (Poliakov *et al.*, 2008). HEK293 and melanoma lines stably expressing E-cadherin, T-cadherin or a control pCDNA3.1 plasmid were obtained from K. Rubina. Untransfected L-cells and an L-cell line expressing N-cadherin were generously donated by N. Itasaki (MRC National Institute for Medical Research, London, UK) and an L-cell line expressing E-cadherin from Y. Fujita (MRC Laboratory for Molecular Cell Biology, London, UK). HEK293 cell lines stably transfected with E-cadherin-GFP, Adam10ΔMP-FLAG and EphB2 or E-cadherin-mCherry and ephrinB1 were generously provided by Guiomar Solanas (Institut de Recerca en Biomedicina, Barcelona, Spain).

Cell maintenance

Cells were cultured at $37^{o}C$ with 5% CO_2 in DMEM (high glucose media 4.5g/l without glutamine) supplemented with 1% L-Glutamine (200mM), 1% Sodium Pyruvate (100mM) and 10% Fetal Bovine Serum Mycoplex (all PAA). Where the number of cells plated was important, they were counted using a Cellometer T4 Cell Counter (Nexcelom Bioscience).

Microscopy

For the majority of images, fixed and live, including the cell segregation assay, boundary assay, and hanging drop assay, the RT live-imaging workstation (Deltavision; Applied Precision, LLC) on a microscope (IX-70; Olympus) was used with a charge-coupled device camera (MicroMax 1300 YHS; Roper Scientific) and a heated environmental chamber ($37^{o}C$; 5% CO_2). Images were acquired using SOFTWORX acquisition software (Applied Precision, LLC).

Confocal images were captured using a Leica TCS SP2 laser scanning confocal microscope. Images were processed with Fiji, Image J, Photoshop, GMimPro (Mashanov & Molloy, 2007) or LabView (National Instruments) as discussed in the relevant sections.

siRNA knockdown

Knockdown of human genes in HEK293 cells and stable lines was achieved using pre-designed Silencer Select siRNAs (Applied Biosystems, Ambion). GFP-EphB2, ephrinB1, E-cadherin or control HEK293 stable cell lines were seeded 24 h before siRNA transfection at 400,000 cells/ml, 1 ml per well of a 6-well dish. 60 pmol/ml total siRNA and 5 μl Lipofectamine RNAiMAX (Invitrogen) transfection reagent were used in a total of 500 μl Opti-MEM I Reduced Serum Medium-with GlutaMAX (Invitrogen) according to manufacturer's instructions. In most cases, 3 siRNAs to the same gene were pooled to minimise off-target effects. For N-cadherin, siRNAs were tested individually and s2772 was found to be toxic to the cells. s2771 and s2773 were therefore pooled, which gave a similar phenotype and level of knockdown as when each was used alone. Where 2 genes were knocked down in combination, the total concentration of siRNA was kept the same (60pmol/ml). Cells were incubated in siRNA for 6h then 1.5ml pre-warmed medium was added to each well. They were incubated for 48 h before re-plating for sorting, boundary or hanging drop assays. For Western blot analysis, they were incubated for 24 h, 48 h or 72 h. Other transfection reagents, XtremeGene (Roche) and Lipofectamine 2000 (Invitrogen), were used initially in experiments comparing transfection efficiency and non-specific effects of these reagents compared with Lipofectamine RNAiMAX.

Cell labelling

Cells were fluorescently labelled 48h after siRNA transfection by addition of 2ml of 5mM Cell Tracker CMRA or CMFDA (Molecular Probes, Invitrogen) in pre-warmed DPBS containing 1000mg/l D-glucose and 36mg/l sodium pyruvate, calcium and magnesium (GIBCO, Invitrogen). They were incubated at 37°C for 30 min

according to manufacturer's instructions. After 30 min, labelling solution was replaced with 2ml pre-warmed medium for at least 1 h.

Cell sorting assay

Protocol

Glass slides (2 well chambered slide system (1.0 borosilicate; Lab-Tek II, Nunc)) were coated with fibronectin. 700µl 50µg/ml fibronectin (from bovine plasma; Sigma) was incubated in each well for 30 min at room temperature. Slides were then washed 3 times in PBS and left in PBS.

Cells were plated for the segregation assay 48 h after siRNA transfection. They were labelled as described above, or used without labelling. They were dissociated in Accutase (PAA laboratories), pelleted, resuspended in fresh medium and filtered through 0.45 µm filters to create a single cell suspension. They were counted using an automatic cell counter. Two cell lines were mixed in equal proportion and plated into each well of the chamber slide at a concentration of 200,000 total cells/cm^2 (2ml/well). Where chemical inhibitors were used (i.e. TAPI-1, 20µM), they were added at the same time as cells were plated. This culture was then incubated for 48 h, until confluent, before fixation.

Analysis

Cells were visualised using a Deltavision microscope and images of segregated cells were acquired with a 4x /0.13 NA objective (Olympus). These were subjected to the following analyses:

Nearest Neighbour analysis

To quantify the pattern of cell sorting seen in this assay, I initially carried out Nearest Neighbour analysis (Mochizuki *et al.*, 1998). Grayscale images of one cell population (usually GFP-Eph cells) were binarised and resized so that each pixel represented roughly the area of one cell and the clusters appeared black. These images were then analysed using custom-written software which assessed the proportion of cells surrounded by like cells. The result of this analysis is represented as a single number – the Nearest Neighbour value – for each image. A

value of 0.5 would represent a totally random pattern, where a cell is surrounded by 2 black and 2 white cells, whereas values approaching 1 represent increasingly segregated populations. 3 or more images were analysed per condition and experiments were repeated at least once.

Perimeter Regularity Index

Another method of quantifying cell segregation is calculating the perimeter regularity index (PRI) (Hueck *et al.*, 2000) of clusters. Grayscale images of one cell population (usually GFP-Eph cells) were inputted (three per condition) into an custom-designed Labview program (Chen Qian, Confocal Imaging and Analysis Laboratory, NIMR). This identifies cell clusters based on a threshold pixel intensity, and calculates the mean PRI value across all clusters within each image. This programme was also used to calculate the mean area and Heywood Circularity Index of clusters. Statistical significance between a control and experimental condition was calculated using the Student's T-Test ($p<0.05$) within each experiment. Experiments where the PRI value for the control siRNA condition with Eph-ephrin signalling was not significantly different to the control siRNA condition without Eph-ephrin signalling were discarded from further analysis. The mean PRI value for each condition was calculated from the PRI values of the condition for each experiment (minimum of three repeats). The significance of the mean PRI value for each condition compared to the control condition was calculated using the Student's T-Test ($p<0.001$).

Boundary assay

Protocol

For the boundary assay, siRNA-transfected cells were fluorescently labelled 48 h after transfection as described above. A 4 well chambered slide system (1.0 borosilicate; Lab-Tek II, Nunc) was pre-coated with fibronectin, and a silicone barrier (Culture insert, Ibidi) was placed in each culture slide well using sterilised forceps after PBS was removed but before the fibronectin dried out. Each of two cell lines was plated in equal proportion into either side of the silicone barrier, at a concentration of 1.26 million total cells/ml (70µl each side of the barrier). Cells

were incubated at 37°C for 6 h before the barrier was removed (with sterile forceps) and 1ml fresh medium was added to each well. Cells were incubated at 37°C for 48 h before being fixed and mounted in ProLong Gold.

Analysis

Boundary sharpness was quantified by measuring the length of the boundary. Grayscale images of one cell population (usually GFP-Eph cells) were inputted (three per condition) into an custom-designed program (Chen Qian, Confocal Imaging and Analysis Laboratory, NIMR) which outlined and calculated the boundary length for each image based on a pixel intensity threshold. Experiments were excluded from further analysis according to the same criteria described for the perimeter regularity index calculations. The boundary length shown represents the mean of the boundary length of the condition calculated from at least 3 experiments. The significance of the overall boundary length value for each condition compared a control condition was calculated using the Student's T-Test ($p<0.01$) on the average values for each experiment.

All error bars on all bar charts represent standard error of the mean (SEM).

Hanging drop assay

Cells were dissociated and filtered as for the sorting assay. Two cell lines were mixed in equal proportions at a final concentration of 100,000 cells/ml and plated in 10 μl drops (1000 cells/drop) onto a clean coverslip. The inverted drops were then grown at 37°C for 48 h suspended across a well of a 6 well plate containing 1 ml medium to prevent them from drying out. For imaging, the coverslips were turned over, so the drops were no longer hanging, and imaged immediately on a Deltavision microscope. For confocal analysis, the drops were fixed by flooding the drop with 4% formaldehyde (4% Formaldehyde Ultrapure (Thermo Scientific), MEM (GIBCO), sterile water and sodium hydroxide to pH 7.5 (filtered and pre-warmed at 37°C)) for 15 min. The fixative was removed and replaced with PBS or 70% glycerol, and the coverslip was placed on a glass slide, slightly raised using silicone gel.

Immunocytochemistry

Cells were fixed for immunocytochemistry at 37°C in 4% formaldehyde (4% Formaldehyde Ultrapure (Thermo Scientific), MEM (GIBCO), sterile water and sodium hydroxide to pH 7.5 (filtered and pre-warmed at 37°C)). They were rinsed 3 times in PBS, once for 5 min in PBS/0.1% Triton to permeablise the cell membranes, rinsed in PBS/0.1% Triton, and rinsed in PBS. They were then shaken for 1 h in blocking buffer (4% donkey serum (Jackson Immuno Research), 2%BSA (Jackson Immuno Research), PBS). This was replaced with blocking buffer containing primary antibody, and shaken for 1h. The following primary antibodies were used against: EphB2, 1:100 (A467, goat, R&D Systems); ephrinB1, 1:100 (AF473, goat, R&D Systems); N-cadherin 1:1000 (610920, mouse, BD); Pan-cadherin 1:500 (C1821, mouse SIGMA); T-cadherin (3583, rabbit, ProSci); Cleaved-caspase 3 (9661, Cell Signalling Technology).

Cells were washed 3 times for 5 min in PBS, before incubation in blocking buffer containing 1:250 secondary antibody. Secondary antibodies were used from Jackson Immunoresearch: Cy3 or Cy5 conjugated, raised in donkey. Cells were rinsed three times in PBS, before removal of chambers from the slide and rinsing in dH_2O. Slides were mounted in Prolong Gold or Prolong Gold with DAPI (Molecular Probes, Invitrogen). Images were taken on a Deltavision microscope using a 4x objective. Higher magnification confocal images were taken on a Leica SP2 confocal microscope using a 63x oil objective.

Western blot analysis

The extent of siRNA knockdown was analysed by Western blot 72 h post-transfection. For other conditions, cells were grown for at least 24 h before harvesting for Western blot. Western blots were carried out according to a previously published protocol (Poliakov *et al.*, 2008). Cells were chilled on ice for 10 min, collected in ice-cold PBS and pelleted by centrifugation. They were lysed in 30µl cell lysis buffer (1% NP-40, 20mM Hepes, pH7.4, 100mM NaCl, 10mM $Na_4P_2O_7$, 1mM $CaCl_2$, 1mM $MgCl_2$, Halt protease inhibitor cocktail (Thermo Fisher Scientific, 1:100) for 10 min at 4°C. Cell lysate was obtained by centrifugation at 13,000rpm for 10 min at 4°C, and the supernatant collected. Protein concentration

in cell lysates was measured using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. NuPAGE LDS sample buffer (4x) and Reducing Agent (10x) (both Invitrogen), were mixed 2.5: 1 respectively and added to each lysate condition in a ratio 3.5:6.5 respectively, before denaturing for 10 min at 65°C. Samples were loaded (>30μg protein per well) on NuPAGE 10% Bis-Tris Gels (Invitrogen) with 5μl Novex Sharp Pre-stained protein standard, and run in Novex Mini-Cell tanks (Invitrogen) in NuPAGE MOPS SDS Running Buffer (Invitrogen) for 45 min at 200V. Transfer of proteins from gels to rehydrated Immobilon-FL membranes (Millipore) was performed using XCell II Blot modules (Invitrogen) in NuPAGE Transfer Buffer (Invitrogen; containing 10% methanol, 0.1% NuPAGE Antioxidant (Invitrogen)) for >1 h at 30V.

Membranes were blocked in blocking solution (50% Odyssey Blocking Buffer (LI-COR Biosciences), 50% PBS) for 1 h at room temperature or 4°C overnight. Blocking solution was replaced with primary antibodies in blocking solution/0.1% Tween for 1 h at room temperature or overnight at 4°C, and washed 4x 5 min in PBS/0.1% Tween. Membranes were then incubated with secondary antibodies in blocking solution/0.1% Tween/0.01% SDS (Bio-Rad laboratories) for 1 h at room temperature or overnight at 4°C. After staining, the membranes were washed 3x 5 min in PBS/0.1% Tween, rinsed in PBS and scanned using 700- and 800-nm channels on an imager (Odyssey; Li-cor Biosciences). The intensity of staining was determined using the median background method in the Li-cor software. At least 3 blots were run for each condition, but in some cases only two were quantified due to noisy staining on the membrane.

EphrinB1-Fc stimulation

For analysis of cadherin expression after Eph activation, cells were plated on fibronectin-coated chamber slides and grown for 24 h before stimulation with 5 μg/ml ephrinB1-Fc chimera (R&D Systems) for 0, 5, 15 or 30 min before fixing and staining as described elsewhere.

To assess the extent of Eph activation after knockdown of siRNA, cells were stimulated with 1ug/ml ephrinB1-Fc chimera for 30min at 37°C, 72 h after siRNA transfection. They were then harvested and cell lysate prepared in lysis buffer

containing 1:100 phosphatase inhibitor cocktail set II (EMD) before Western blot as described above using anti-phospho-Eph and anti-EphB2 antibodies.

Immuno-precipitation

Cells were plated at 200,000 cells/cm^2 in 6 well plates (2ml/well) with or without 20µM TAPI-1, and incubated at 37°C for 48 h. They were harvested by incubation on ice for 10 min then collection in ice cold PBS, before being lysed in 500µl lysis buffer. 1-2mg antibody per reaction were incubated with Protein G (GE Healthcare) for 1h in lysis buffer on a rotor. Cell lysate was incubated with pre-bound antibodies for at least 1 h at 4°C on a rotor, before several washes in lysis buffer, lysis buffer with 1M NaCl, and lysis buffer containing 10mM HEPES. The beads were then boiled at 65°C for 10 mins in 2x sample buffer. All the supernatant was loaded on a 10% Bis-Tris SDS-Page gel in MOPS buffer as described for Western blotting.

Quantitative Real Time PCR

RNA extraction

RNA was extracted from approximately 200,000 cells using a PicoPure RNA Isolation kit (MDS Analytical Technologies), according to the manufacturer's instructions.

cDNA preparation

Approximately 1µg RNA was used for cDNA preparation using a Superscript kit (Superscipt^{TM} First Strand, Invitrogen), according to the manufacturer's instructions.

Quantitative Real Time PCR (RT-PCR)

RT-PCR was performed with an ABI 7500 Real-Time PCR System (Applied Biosystems) using SYBR Green (Platinum SYBR Green qPCR Supermix-UDG, Invitrogen) in a 20µl reaction volume, according to the manufacturer's instructions (based on (Lekanne Deprez *et al.*, 2002)). Relative levels of gene expression were calculated compared to β-actin controls. Complementary DNA PCR primers were designed using Primer Express software (version 2.0, Applied Biosystems).

Live Imaging

High density sorting

Cells were fluorescently labelled and plated for the segregation assay as described above but at a concentration of 400,000 total cells/ cm^2 in a 2 well chambered coverglass (chambered 1.0 borosilicate; Lab-Tek, Nunc). Cells were set up in a heated environmental chamber (37°C; 5% CO_2) and images taken with a 10x /0.4 NA objective (Olympus) on a Deltavision microscope every two minutes for 24-48 h.

Boundary assay

For time-lapse imaging of boundary cell behaviour, a 2 well chambered coverglass (1.0 borosilicate; Lab-Tek, Nunc) was used and cells were visualised using a Deltavision microscope, in a heated environmental chamber (37°C; 5% CO_2),and images were acquired with a 10x /0.4 NA objective (Olympus) every 2-5 min for 48 h. Videos of cell behaviour were created using ImageJ software (National Institute of Health).

Motility analysis

For cell motility analyses, cells were fluorescently labelled 48 h after siRNA transfection as described above, re-suspended in medium containing 2% HEPES (PAA), and then filtered through 40µm filters to obtain a single cell suspension before counting. For whole population analysis, two populations of cells were plated in equal proportions at a total concentration of 30,000 cells/cm^2 (200µl/well) into an 8 well chambered coverglass (1.0 borosilicate; Lab-Tek, Nunc) which had been coated with fibronectin. They were incubated for 1 h at 37°C to settle before time-lapse imaging was set up. Images were acquired every minute for 2 h using SOFTWORX acquisition software (Applied Precision, LLC). For analysis of cells in the absence of interactions, cells were plated at 5,000 cells/cm^2 and images were recorded every minute for 2 hours. For analysis of individual cell interactions, cells were plated at 20,000 cells/cm^2 and tracked for 8 h (these movies were taken by Alexei Poliakov).

Mean Squared Displacement analysis

Whole population analysis was carried out on the EphB2 cell movies. Movies were tracked using a single particle tracking algorithm in GMimPro software (Mashanov & Molloy, 2007), which tracks the XY coordinates of cell centroids based on their pixel intensity. This data is then used to calculate the mean squared displacement (MSD, μm^2) using overlapping time intervals (DiMilla *et al.*, 1993; Martens *et al.*, 2006) which is plotted over a change in time (ΔT) up to 57min (half the total time for which the cells were tracked). In each experiment, 4 different cell combinations were filmed in parallel, and two different fields of view were combined for each condition. The results shown are an example of one such experiment.

The ratio of MSD endpoints between EphB2 cells mixed with EphB2 cells and EphB2 mixed with ephrinB1 cells was calculated using the MSD of each track at 57min intervals. The data presented shows the mean of three experimental repeats. Statistical differences in the average MSD between conditions in an experiment were calculated using the Student's T-Test ($p<0.05$) for all 57 min intervals of tracks of each condition compared to another. The significance of the overall MSD ratio increase with Eph-ephrin signalling between control and siRNA conditions was calculated by combining the p-values calculated for each individual experiment using Fisher's method ($P<0.005$).

Directionality analysis

Turning angles of the tracks were calculated and endpoints and tracks plotted using a custom-written Python script (TrackParser, Robert Gilchrist). This programme used the XY coordinates of tracks extracted from GMimPro and analysed only full-length (2 h) tracks. The turning angle is the angle between 2 consecutive 5 min intervals relative to movement in a straight line. These were calculated for overlapping intervals and are plotted as a probability distribution function histogram. TrackParser was also used to plot a random selection of tracks on a common origin. 50 full-length tracks were picked at random and their XY coordinates were plotted. For endpoint analysis, the endpoint positions of all full-length tracks for a condition are plotted as individual points around a central origin and the root mean square (RMS) displacement of these is shown as a solid line around the origin.

3. Are the sorting activities of Eph-ephrin signalling and differential adhesion similar?

Cell segregation mechanisms are frequently investigated using assays in which two populations of cells are randomly mixed together then allowed to sort out from one another (Moscona & Moscona, 1952; Townes, 1955; Trinkaus & Groves, 1955). The use of such assays led to several theories of cell sorting. The differential adhesion hypothesis has been the most widely accepted of these (Steinberg, 2007). This suggests that cells sort if there is a difference in cohesion between two populations of cells which move randomly. It has been demonstrated to work *in vitro* (Duguay *et al.*, 2003; Foty & Steinberg, 2005) and to play a role in certain *in vivo* systems, including the separation between the cerebral cortex and the lateral ganglionic eminence in the chick brain (Inoue *et al.*, 2001).

When Eph receptor or ephrin expressing cells are mixed *in vitro*, they sort from one another (Cortina *et al.*, 2007; Jorgensen *et al.*, 2009; Poliakov *et al.*, 2008). Eph receptors and ephrins are also important in the segregation of adjacent regions of cells *in vivo*, for example rhombomeres of the vertebrate hindbrain, and are involved in the formation of sharp boundaries between these regions (Cooke *et al.*, 2005; Mellitzer *et al.*, 1999; Poliakov *et al.*; Xu *et al.*, 1999).

Signalling between Eph receptor and ephrin expressing cells often results in cell repulsion. For example, in stripe assays, cultured neurons expressing EphAs are excluded from stripes of ephrinA2 (Drescher *et al.*, 1995). EphBs and ephrinBs are important in the repulsive guidance of commissural axons cells along the midline (Kadison *et al.*, 2006; Yokoyama *et al.*, 2001). In addition, ephrin stimulation results in cytoskeletal collapse and growth-cone or lamellipodial retraction of Eph-expressing cells *in vitro* (Groeger & Nobes, 2007; Jurney *et al.*, 2002; Poliakov *et al.*, 2008). Repulsion between Eph and ephrin expressing cells could result in decreased adhesion between them. This would create differential adhesion between the two cell populations, since like cells would still stick to one another. It has been proposed that this difference in adhesion could also sharpen boundaries, consistent with the differential adhesion hypothesis (Steinberg, 2007).

There is also some evidence to suggest that Eph-ephrin signalling could be linked to processes directly affecting cell-cell adhesion. Cortina *et al.* showed that siRNA knockdown of E-cadherin in colorectal cancer cell lines disrupted sorting between EphB and ephrinB expressing cells (Cortina *et al.*, 2007). They also demonstrated that there is a redistribution of E-cadherin from the cytoplasm to the membrane in EphB cells stimulated with ephrinB1, suggesting that E-cadherin distribution is correlated with EphB-ephrinB signalling. More direct evidence for the involvement of Eph-ephrins in regulating cell adhesion comes from recent screens which have identified several proteins involved in cell-cell adhesion as phosphorylation targets of EphB2-ephrinB1 signalling (Jorgensen *et al.*, 2009; Zhang *et al.*, 2008). These include members of the p120 and plakophilin families as well as several proteins involved in the regulation of adherens junctions and polarity.

These results suggest that EphB-ephrinB signalling can affect cell-cell adhesion. However, it is still not clear whether differential adhesion is sufficient to explain sorting through Eph-ephrins or whether the generation of differential adhesion is the only role cell-cell adhesion molecules play in this system. I thus set out to investigate the role of cell-cell adhesion in Eph-ephrin mediated cell sorting.

The process of cell segregation mediated by Eph receptors and ephrins is challenging to study *in vivo* for two reasons. Firstly, because cells generally express more than one Eph receptor or ephrin, and *cis*-interactions between them can affect their response to external signals (Astin *et al.*, 2010; Hornberger *et al.*, 1999). Secondly, because cells *in vivo* are generally in contact with several cells at once, which makes it difficult to interpret how they are responding to each individual cell or interaction.

To better understand the response of individual cells to Eph-ephrin signalling, one can take advantage of *in vitro* systems where cells can be plated in the absence of confounding factors (Astin *et al.*, 2010; Drescher *et al.*, 1995; Marston *et al.*, 2003; Poliakov *et al.*, 2008). In addition to this, in this study I used HEK293 cell lines which express naturally very low levels of EphB receptor and ephrinBs and over-express only EphB2 or ephrinB1, to ensure that the responses are due to Eph-ephrin signalling.

In this chapter, I will describe three assays that were used to investigate the segregation of EphB2 receptor and ephrinB1 expressing cells. I then present a side-by-side comparison of cell segregation driven by EphB2 and ephrinB1 and cell sorting driven by differential adhesion. This comparison suggests that differential adhesion is unlikely to be the main mechanism through which EphB2-ephrinB1 interactions are driving cell segregation.

EphB2 receptors and ephrinB1 mediate cell segregation and boundary sharpening

EphB2 receptor and ephrinB1 mediated cell segregation

An *in vitro* cell sorting assay has been developed in the Wilkinson lab to assess the role of Eph-ephrin signalling in cell segregation (Jorgensen *et al.*, 2009; Poliakov *et al.*, 2008). HEK293 cells, which express relatively low levels of EphB receptors and ephrinBs, have been stably transfected with either EphB2 or ephrinB1. Cells stably expressing EphB2 alone or co-expressing EphB2 and GFP (EphB2 cells) are mixed with cells stably expressing ephrinB1 (ephrinB1 cells) and grown in a monolayer. The EphB2 cells are observed to sort into clusters surrounded by ephrinB1 cells. This sorting phenotype is disrupted when specific targets of EphB2-ephrinB1 signalling are knocked down by siRNA (Jorgensen *et al.*, 2009) or on ectopic activation of FGFR in EphB2 cells (Poliakov *et al.*, 2008). I used three variants of this assay to assess segregation in different contexts.

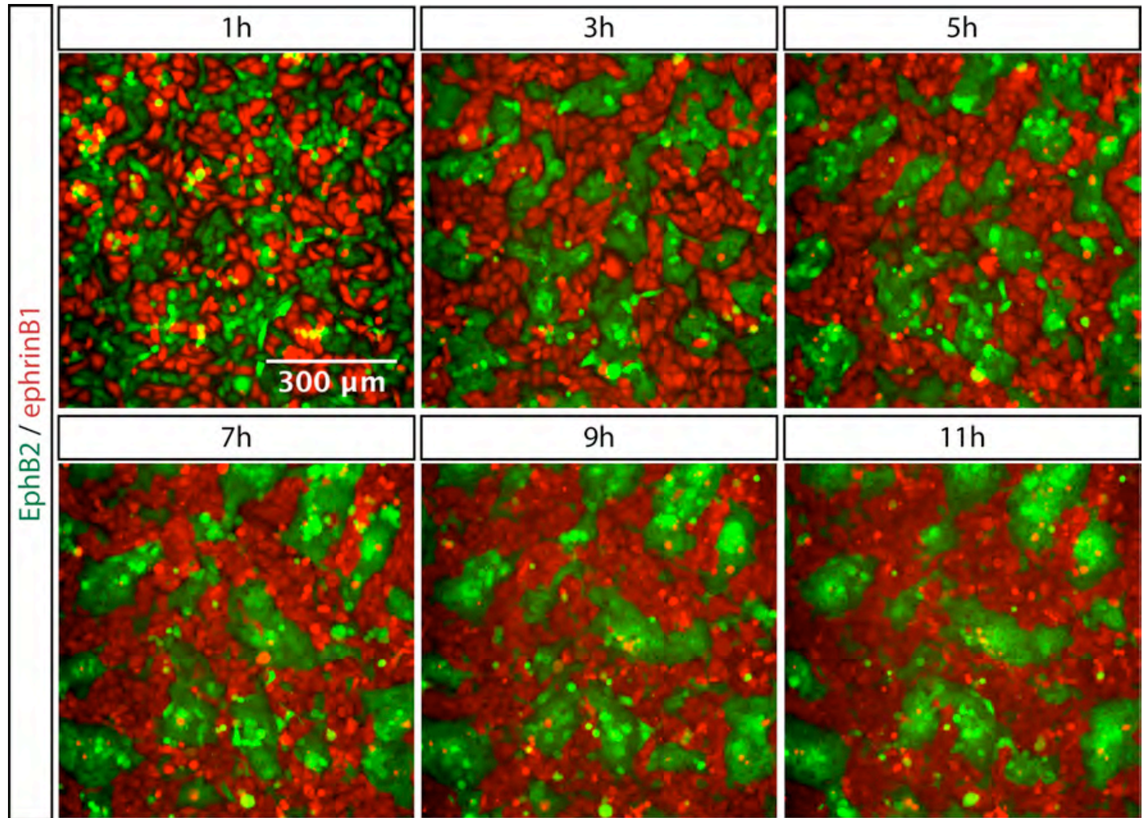
Segregation assay

The cell segregation assay has been previously published by the Wilkinson lab (Jorgensen *et al.*, 2009; Poliakov *et al.*, 2008). In this assay, EphB2 cells and ephrinB1 cells are mixed in equal proportions in a single cell suspension and are plated onto fibronectin-coated dishes. EphB2 cells sort out over time to form clusters surrounded by ephrinB1 cells. Figure 3.1 shows a 10 h time course of this process. At this relatively high cell density, the cells have begun to sort at the first time point, 1 h after the cells are mixed (Figure 3.1, 1 h). With time, the clusters increase in size and their edges become increasingly smooth. At the end of the

Figure 3.1: Time-lapse movie of EphB2-ephrinB1 cell sorting

(A) EphB2 cells (green) sort from ephrinB1 cells (red). Images represent 2 h intervals of a time-lapse movie showing sorting over the course of 10 h. The movie was started 1 h after the cells were plated to allow them to settle on the dish (1 h). Even at this point, cells are beginning to sort. Clusters refine over time.

Figure 3.1



time-course, large clusters of EphB2 cells have formed, which maintain relatively sharp borders with the ephrinB1 cells.

The segregation assay shows that EphB2 cells sort from ephrinB1 cells. It is a good broad readout for sorting and is quick to quantify crudely (discussed in more detail below). However, the EphB2 cell clusters are irregular in shape and it is often difficult to distinguish two closely associated clusters, making them difficult to quantify using conventional algorithms.

In light of this, Lauren Gregory in the Wilkinson lab devised the boundary assay.

Boundary assay

In the boundary assay, cells are plated either side of a removable silicone barrier (Ibidi). After allowing the cells to settle on the dish, the barrier is removed and the two populations of cells migrate towards one another. Figure 3.2 shows a time course of this process. It takes approximately 6 hours for the two populations of cells to come into contact. EphB2 cells immediately begin to form a smooth boundary with ephrinB1 cells, which sharpens over time. The boundary also shifts to the left over the course of the movie (discussed in Chapter 4). In contrast, when EphB2 cells meet other EphB2 cells, the boundary does not sharpen and clear intermingling between the two cell populations remains after 18h. The sharpness of the boundary is easily quantified using an automated programme to measure the length of the boundary between EphB2 and ephrinB1 cells. Variation in the size and shape of the EphB2 cell territory is no longer a problem, since the EphB2 and ephrinB1 cells are segregated into two regions from the start. This also makes it a better model than the segregation assay for boundary sharpening *in vivo* since this most frequently occurs between regions of cells which are already largely segregated.

Hanging drop assay

Both the segregation and boundary assays are 2-dimensional, whereas cell segregation *in vivo* is usually occurring in a 3-dimensional environment. In addition, the majority of previous studies into cell sorting have been carried out in a 3D assays. In order to check that the main experimental observations in 2D and 3D systems are similar, I established a 3D assay, where there are a high number of

Figure 3.2: Time-lapse movie of EphB2-ephrinB1 boundary sharpening

Cells were plated either side of a barrier, which is removed at 0 h, and time-lapse images taken. Frames shown are at 2 h intervals over a total of 18 h. EphB2 cells (green) form a sharp boundary with red ephrinB1 cells (A) but not with EphB2 cells (B). In addition, the EphB2-ephrinB1 boundary shifts to the left over the course of the movie.

Figure 3.2

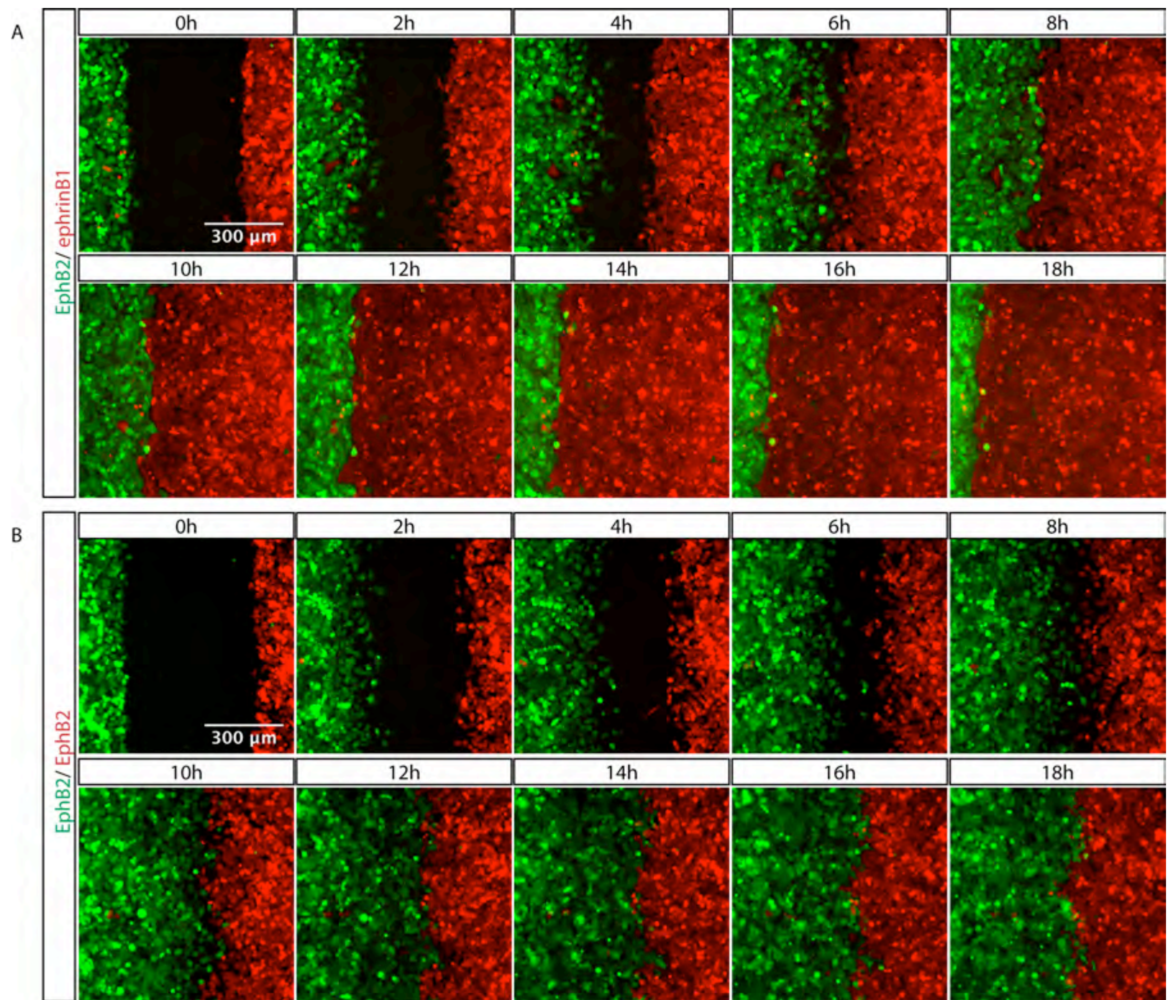
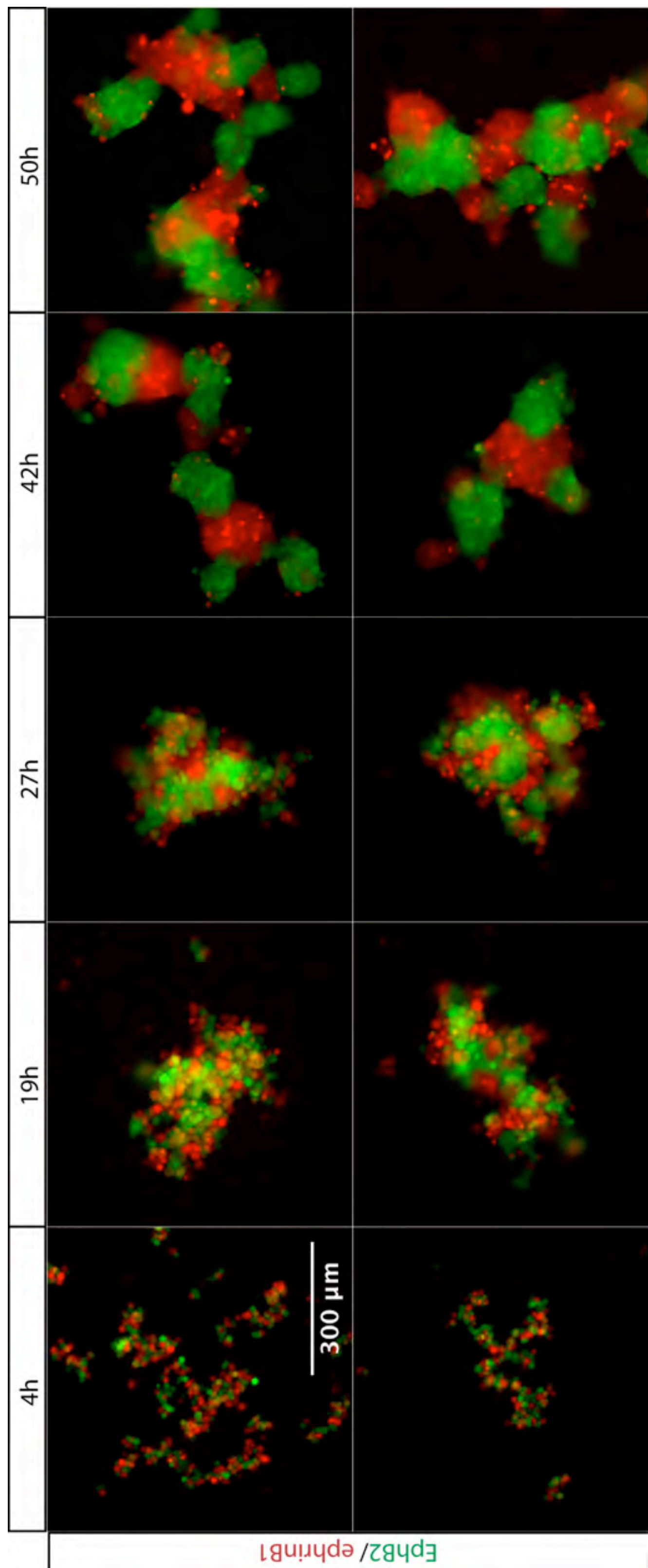


Figure 3.3: A time course of EphB2-ephrinB1 sorting in hanging drops

EphB2 cells (green) sort from ephrinB1 cells (red) when cultured in hanging drops. 2 images are shown of representative cell aggregates at the time points indicated: 4, 19, 27, 42 and 50 h after plating. Cells begin to appear sorted at 19 h but have segregated more completely by 50 h.

Figure 3.3



interactions between cells. This assay is based on previous studies of cell sorting, most of which use cell aggregates in suspension, either on shaking platforms or in hanging drops (Duguay *et al.*, 2003; Foty & Steinberg, 2005; Shi *et al.*, 2008).

Two differently labelled cell populations were mixed in a single cell suspension as in the segregation assay. 10ul drops containing 1000 cells were then placed on coverslips and suspended in humid conditions. Figure 3.3 shows a time course of the hanging drop assay. Unlike the segregation and boundary assays, hanging drops were a more challenging subject for live time-lapse microscopy, so the images show a time-course of still images of different aggregates. Interestingly, segregation of EphB2 cells from ephrinB1 cells was much slower in hanging drops. No segregation is seen at 4 hours, and a high level of segregation is not seen until 42 hours after plating.

The hanging drop assay is a good system for studying cell sorting driven by cell-cell interactions. EphB2 cells and ephrinB1 cells segregate well in this assay, indicating that cell-cell contact mechanisms are capable of driving sorting downstream of EphB2-ephrinB1 signalling, although this process is slower than sorting in the 2-dimensional assays.

Effects of cell numbers on the degree of sorting

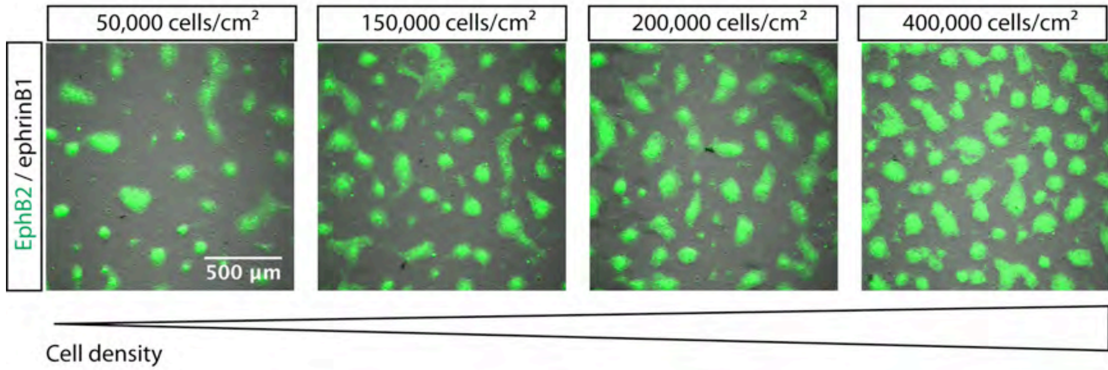
Initially, I wanted to better characterise the pattern of cell sorting in the segregation assay, where the size and shape of cell clusters is variable. One possible cause of this variability is the initial density of cells when they were plated. Cells plated at lower densities would have more opportunity to migrate so may be able to sort more completely than cells which are crowded to begin with.

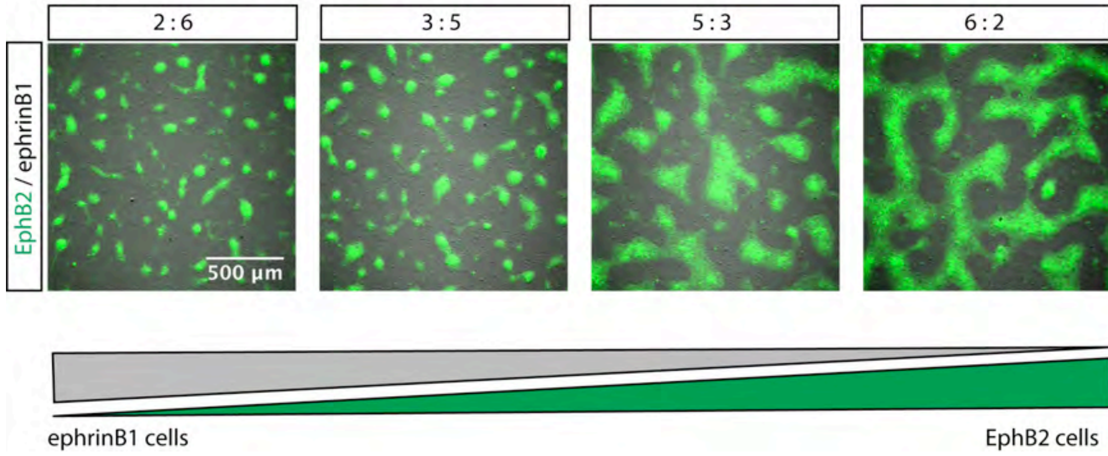
To assess the contribution of cell numbers on the size and distribution of EphB2 cell clusters, I varied both the total number of cells plated and the relative ratios of EphB2 to ephrinB1 cells (Figure 3.4). Increasing the total cell number does not dramatically affect the sizes of EphB2 cell clusters. At very high cell densities (400,000 cells/cm^2) clusters are more frequent than at lower cell densities. There are fewer, more spaced out, clusters at 50,000 cells/cm^2 yet the surrounding ephrinB1 cells appear still to cover the dish. This effect is likely due to the compaction of EphB2 cells when they cluster. EphB2 cells in clusters appear less

Figure 3.4: Varying total cell number and ratio of different cell populations affects the EphB2-ephrinB1 sorting pattern

Analysis of the effect that changing the total cell number or the relative cell numbers has on the cell sorting pattern of EphB2 and ephrinB1 cells. (A) Increasing the total number of cells has little affect on cluster size although there is an increase in the frequency of clusters between 50,000 cells/cm^2 and 400,000 cells/cm^2. (B) Increasing the ratio of EphB2 to ephrinB1 cells does increase cluster size. EphB2 cells always form clusters surrounded by ephrinB1 cells regardless of the total cell density or relative numbers.

Figure 3.4





spread than ephrinB1 cells, so at low densities the ephrinB1 cells are able to spread out and fill the available space (Figure 4.2). When plated at higher densities, there are more Eph clusters, so ephrinB1 cells do not have room to spread out. Intermediate changes in cell density, between 150,000 and 200,000 cells/cm^2, a 33% increase in cell number, have very little effect on the sorting phenotype.

A change in the relative ratios of the different cell types has a more profound effect on cluster size and distribution. Increasing the proportion of EphB2 cells to ephrinB1 cells increases cluster size. However, the order of sorting remains the same, with EphB2 cells always forming clusters surrounded by ephrinB1 cells. As described above, the compaction of cells within the EphB2 clusters leads to them covering less surface area than the surrounding ephrinB1 cells (Figure 3.4).

These results demonstrate that a change in the overall density of cells has little effect on the sorting phenotype. However, a change in the ratio of EphB2 cells to ephrinB1 cells has a dramatic effect on the resulting size and distribution of clusters. It was therefore important for all subsequent experiments using the segregation assay that EphB2 and ephrinB1 cells were treated the same. This would ensure that any changes in proliferation or growth that a manipulation (siRNA, drug or plasmid expression) might induce, would affect all cells equally and prevent variation in the ratio between the cell types.

Optimising an siRNA-based cell segregation assay

My initial goal was to use the cell segregation assay to screen potential targets of Eph-ephrin signalling to look for their effect on cell segregation. I planned to use small interfering RNAs (siRNAs) to knock down the genes of interest in EphB2 cells and ephrinB1 cells and use the segregation assays as a read-out for their possible role in Eph-ephrin mediated segregation. The optimisation of this assay was done in collaboration with Lauren Gregory.

I performed preliminary experiments to optimise the conditions to achieve a maximal level of gene knockdown as well as a good degree of sorting after siRNA transfection. I tested 3 different lipid-based transfection reagents (XtremeGene, Lipofectamine 2000 and Lipofectamine RNAiMax), several nonsense (siCtrl)

siRNAs and a range of different concentrations of siRNA. To begin with, I used siRNAs to Rac1, a well-characterised target of Eph signalling that is vital for cell motility, which should act as a positive control. I also used siRNAs to GFP, whose knockdown is easy to assess in GFP-positive cells, and one to N-cadherin. Initially, I used two different siRNAs to each gene to control for non-specific effects.

Table 3.1 summarises a subset of these preliminary experiments, where I compared the Lipofectamine transfection reagents, Lipofectamine 2000 and Lipofectamine RNAiMax, with various amounts of siRNA and plating conditions. From these experiments I decided that Lipofectamine RNAiMax used to transfect 60pmoles siRNA in 1ml medium containing serum gave both good knockdown of the genes tested and also sorting results consistent with expectations. This is in contrast to several other conditions tested, where control siRNA treatment gave a significantly different sorting phenotype to that seen in untransfected cells.

Once transfection conditions had been established, I began to test the efficiency of siRNA knockdown of the genes of interest. I tested three different pre-designed siRNAs (Applied Biosystems, Ambion), which target different regions of the N-cadherin mRNA. Western blot analysis to assess gene knockdown was carried out at different time points. All three siRNAs showed a high level of knockdown at 48 hours post-transfection (Figure 3.5, H). I also tested the effects of each of these siRNAs in the cell segregation assay. All three siRNAs disrupted cell segregation in this assay (Figure 3.5, A-G). However, N-cadherin siRNA s2772 resulted in high cell death (cleaved-caspase-3 staining, data not shown). The other two, s2771 and s2773, did not result in cell death and had similar phenotypes individually and in combination. Further experiments were therefore conducted using these two siRNAs in combination, to minimise any off-target effects. For all subsequent gene knockdown experiments, the siRNAs were not tested individually, but 3 different siRNAs to the same gene were pooled to minimise off-target effects.

It was also noticed that cells transfected with any of the siRNAs studied grow more slowly, i.e. reach confluence later, than cells transfected with siCtrl. We had shown already that total number of cells has little effect on the size of sorted clusters but that changing the ratio between the two cell types does (see previous section). Therefore, siRNA was always transfected to both cell populations (EphB2 and

Table 3.1: Summary of preliminary experiments comparing siRNA transfection conditions

Cell segregation experiments and Western blot analysis of Rac1 and N-cadherin siRNA knockdown were used to determine optimum siRNA transfection conditions. Two different transfection reagents, Lipofectamine RNAiMax and Lipofectamine2000 (Invitrogen) were tested. Variables altered were: concentration of siRNA (based on the range recommended on reagent protocol); plating cells in serum-free medium (OPTIMEM) or medium containing serum (DMEM). In all experiments, conditions were considered to be good where the controls were consistent i.e. cells which had not been transfected, transfected with only transfection reagent, or with a control siRNA as well as transcfection reagent all gave the same sorting phenotype. In addition, the efficiency of protein knockdown was assessed by Western blot of Rac1 and N-cadherin after siRNA knockdown of these proteins. Cells plated in DMEM and transfected with 60pmoles siRNA using Lipofectamine RNAiMAX gave the most consistent controls in sorting experiments and the best knockdown as analysed by Western blot.

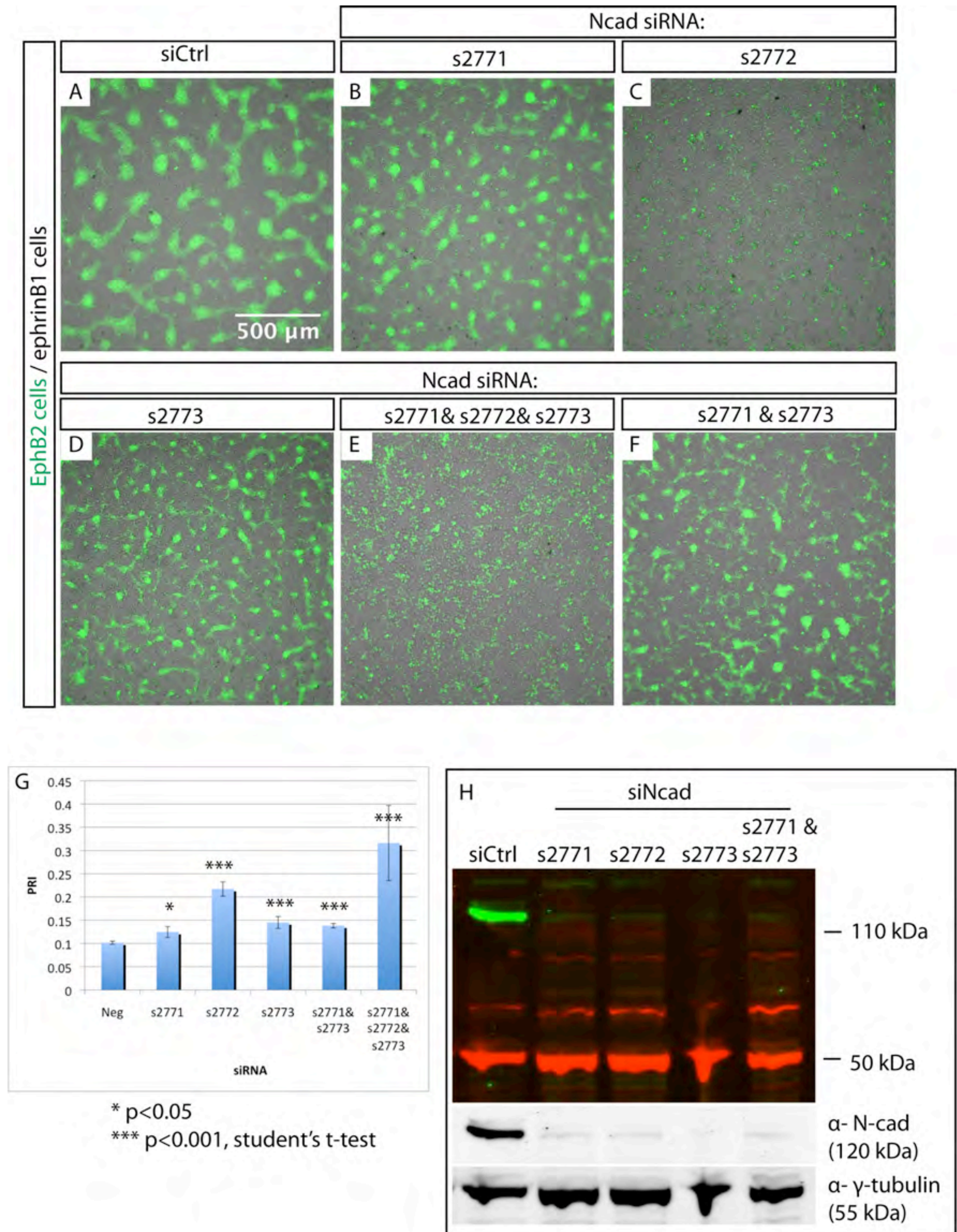
Table 3.1

TRANSFECTION REAGENT	SORTING EXPERIMENT						WESTERN ANALYSIS OF KNOCKDOWN						
	siRNAs	Concentration (pmoles)		Controls consistent?	Phenotype with siRNA knockdown? Rac	Cad1	siRNAs	Concentration (pmoles)		Controls consistent?	Best knockdown (%) Rac	Cad1	Cad2
Lipofectamine RNAiMAX	Ctrl 1,Ctrl 2	30		×									
	Ctrl1,Ctrl 2, Rac, Ncad1	30, 60	DMEM	✓	✓	✓	Ctrl1,Ctrl 2, Rac, Cad1	60	DMEM	✓	24hrs 21%	72hrs 90%	
			OPTIMEM	×	×	×			OPTIMEM	~	72hrs 57%	48hrs 69%	
	Ctrl1,Ctrl 2, Rac, Cad1, 2	100, 200	DMEM	~ Smooth	~	✓	Ctrl1,Ctrl 2, Rac, Cad1, 2	100 200	DMEM	✓	42% 26%	82% 69%	
			OPTIMEM	~ Messy	×	✓	(at 72hrs only)	100 200	OPTIMEM	✓	40%	54%	40%
Lipofectamine 2000	Ctrl2 (+pCDNA3)	10, 15, 20, 40		✓ Messy			Ctrl1,Ctrl 2, Rac, Cad1	20 40		×	24hrs 29% 48hrs 49%	48hrs 55% 24hrs 54%	
	Ctrl1,Ctrl2 (+pCDNA3) pCDNA3, pCS2, mCherry	10, 15, 20, 40		~									
	Ctrl1,Ctrl 2, Rac, Cad1 (+pCDNA3)	100, 200		✓	×	✓	Ctrl1,Ctrl 2, Rac, Cad1, 2	100 200		~	72hrs 31% 48hrs 46%	48hrs 79% 48hrs 83%	72hrs 85% 72hrs 82%

Figure 3.5: Validation of N-cadherin knockdown phenotypes with different siRNAs

Three different siRNAs were used to knock down the expression of N-cadherin. Their effects on EphB2-ephrinB1 segregation were assessed as well as the level of gene knockdown. siRNAs s2771 and s2773 have similar phenotypes in the segregation assay both individually and in combination (B, D, F). However, s2772 appears to have a toxic effect on the cells. They are much more sparse than the other conditions both when the siRNA has been used alone and in combination with s2771 and s2773 (C, E). Quantification of EphB2 cell clusters under these conditions shows an increase in the Perimeter Regularity Index (PRI) after knockdown of all the siRNAs which is statistically significant (G; Student's t-test, $n \geq 3$). Each of the siRNAs causes a considerable reduction of N-cadherin protein detected by Western blot analysis of EphB2 cell lysates, 48 h after transfection (H). Error bars represent standard error of the mean.

Figure 3.5



ephrinB1 cells) so that any off-target effect due to proliferation would affect all cells and have only a minor and predictable impact on the cell sorting phenotype.

Quantification of the assays

An important consideration in the development of these assays was to find effective ways to quantify them. Segregation was initially quantified using the nearest neighbour method. In this analysis, images were made binary and adjusted such that one pixel represented the size of an average cell. The nearest neighbour score was calculated by considering the average number of "like" pixels surrounding each pixel. A score of 0.5 represents an even distribution of cells and scores approaching 1 represent increasingly segregated populations. This approach gave a significant but not very large difference between the sorted and unsorted controls. This is likely to be largely due to the inaccuracy in thresholding and re-sizing the images (Figure 3.6, G).

Another approach I took was to quantify the images using an automated program to identify and quantify clusters of green (EphB2) cells (designed by Chen Qian, Imaging lab, NIMR, in Labview). Three images per condition were entered into the programme, which uses automated thresholding to recognise individual clusters before calculating specific parameters: area; Heywood Circularity Index; and perimeter regularity index (PRI). The average area of clusters was significantly higher in EphB2/EphB2 than EphB2/ephrinB1 cell mixtures, indicating that EphB2 cells are largely connected in unsorted populations (Figure 3.6, H, F). Heywood Circularity Index is a measure of roundness, which compares the perimeter of the cluster to the perimeter of a circle with the same area (Christensen *et al.*, 2010). The closer the index is to 1, the more round a cluster is. The Heywood circularity index is significantly higher in EphB2/EphB2 than EphB2/ephrinB1 cell mixtures indicating that sorted clusters are more rounded (Figure 3.6, I).

Another measure of the roughness of boundaries is the perimeter regularity index (PRI). This gives a ratio of the real length of the boundary of a cluster relative to a smooth boundary (Hueck *et al.*, 2000). The PRI proved to correlate well with the observed cell sorting patterns, and was significantly lower for sorted EphB2/ephrinB1 cells than unsorted EphB2/EphB2 cell mixtures (Figure 3.6, J).

Figure 3.6: Quantification of the segregation assay

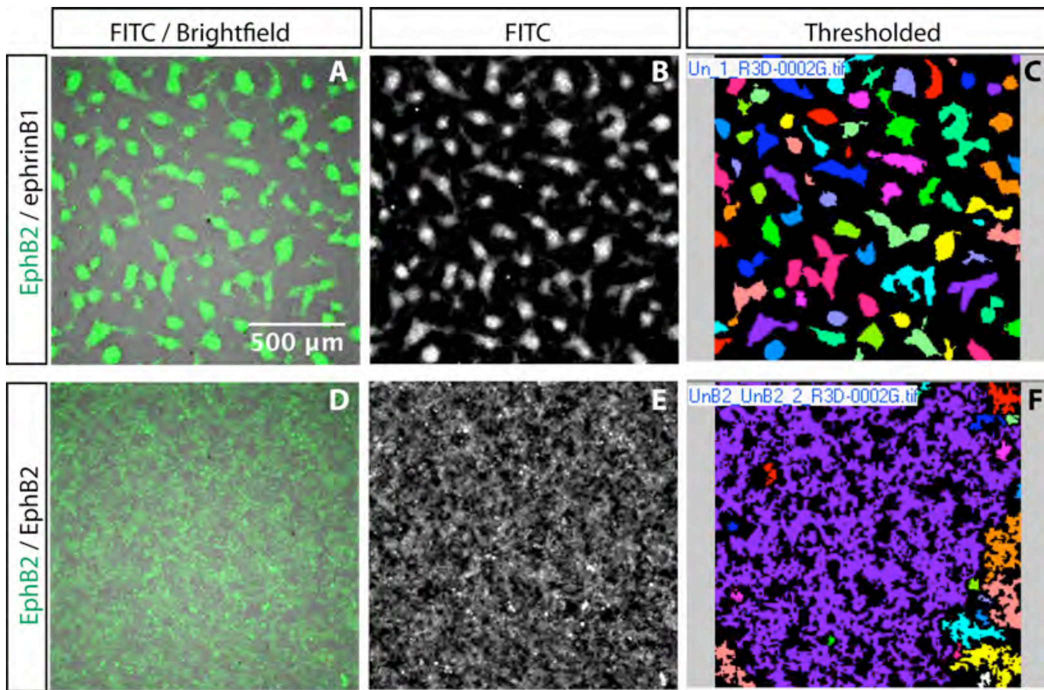
EphB2 cell segregation from ephrinB1 cells was quantified by nearest neighbour analysis, Perimeter Regularity Index (PRI), Heywood's Circularity Index (HCI) and cluster area.

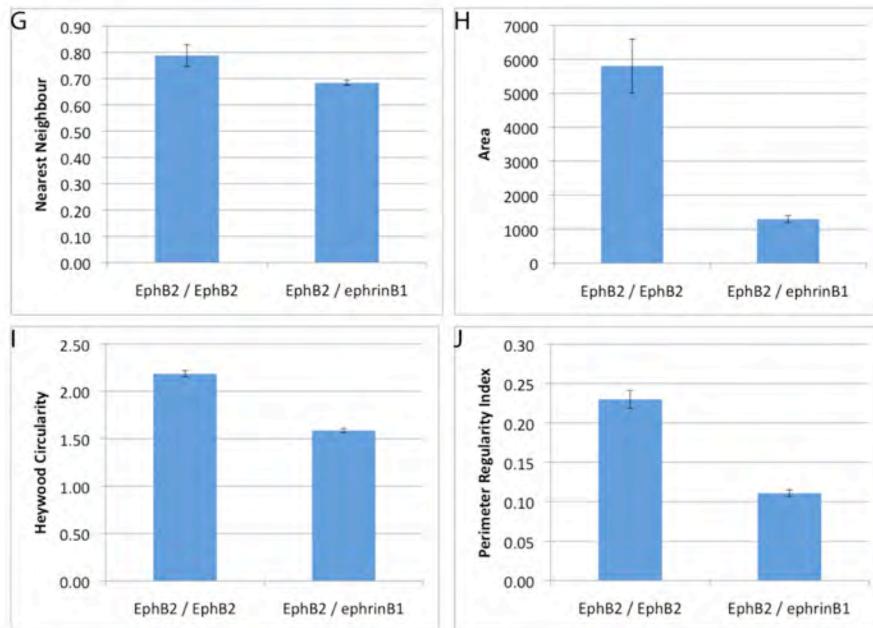
Segregation was initially quantified using nearest neighbour analysis (G). Images were made binary and adjusted such that one pixel represented the size of a cell. The clustering of cells was calculated by considering the average number of like pixels surrounding each pixel. A score of 0.5 represents random dispersal and scores approaching 1 tend towards total segregation.

The images were also quantified using an automated program to identify clusters of green (EphB2) cells by thresholding the green channel image (C,F). These clusters were then quantified in one of three ways: Area (H); HCI (I); and PRI (J).

The nearest neighbour score, cluster area, HCI and PRI are significantly reduced for EphB2-ephrinB1 cell mixtures compared to EphB2-EphB2 cell mixtures ($p<0.01$, Student's t-test). Error bars represent standard error of the mean.

Figure 3.6





Since it is a measurement of the roughness of the boundary rather than comparing it to a perfect circle, PRI was a more appropriate index for measuring the extent of segregation in these cells than the Heywood Circularity Index. It is important to note here, that the PRI is not a perfect solution as it will be influenced by cluster size to some extent, but it represents the best available method for quantifying cluster smoothness.

Differential adhesion leads to mild segregation phenotypes

It has been suggested that the segregation between Eph cells and ephrin cells could be due to differential adhesion between the two populations (Steinberg, 2007). To establish whether this could be true, it would be useful to have a direct comparison of the pattern of cells after sorting mediated by differential adhesion compared to that mediated EphB2-ephrinB1 signalling. Differential adhesion can be generated by the expression of different levels or of different types of cadherin cell-cell adhesion molecules (Duguay *et al.*, 2003; Foty & Steinberg, 2005). Although many previous studies have analysed cell sorting in hanging drops or in shaking suspensions, I was unable to identify studies in which cells expressing different levels or types of cadherins were plated on a dish and allowed to sort in a 2-dimensional assay, as in my experiments. I thus performed a direct comparison between differential adhesion-driven and EphB2-ephrinB1 driven sorting by mixing cells expressing different combinations of cadherins in the assays I have previously described.

Differential adhesion in the sorting of HEK293 cells

Initially, I made use of HEK293 stable cell lines expressing E-cadherin at high levels (from K. Rubina in Moscow) mixed with control HEK293 cells in which N-cadherin, but not E-cadherin, is endogenously expressed. It was expected that the difference in cadherin types as well as the altered levels of cadherins expressed between these two cell types, would drive cell sorting, consistent with the differential adhesion hypothesis. However, these cells do not segregate in the sorting assay, and boundaries are not sharpened in the boundary assay, although they do show a small degree of sorting in the hanging drop assay (Figure 3.7, B,F,J). Since the E-cadherin expressing cells are also expressing N-cadherin, this could be

Figure 3.7: Segregation of HEK293 cells by differential cadherin expression

Cell sorting via differential adhesion in the 3 different assays: (A-D) segregation; (E-H) boundary; (I-J) hanging drops. (A, E & I) Un-sorted control HEK293 cells, which express endogenous N-cadherin. (B, F & J) E-cadherin cells, which are stably overexpressing E-cadherin as well as endogenous N-cadherin, sort to a small extent from control cells. (C, G &K) E-cadherin cells in which N-cadherin is knocked down by siRNA sort from control cells which only express N-cadherin. Boundary length is significantly decreased compared to apposed control cells (N). (D, H & L) E-cadherin cells in which N-cadherin is knocked down by siRNA do not sort from E-cadherin cells. Error bars represent standard error of the mean.

Figure 3.7

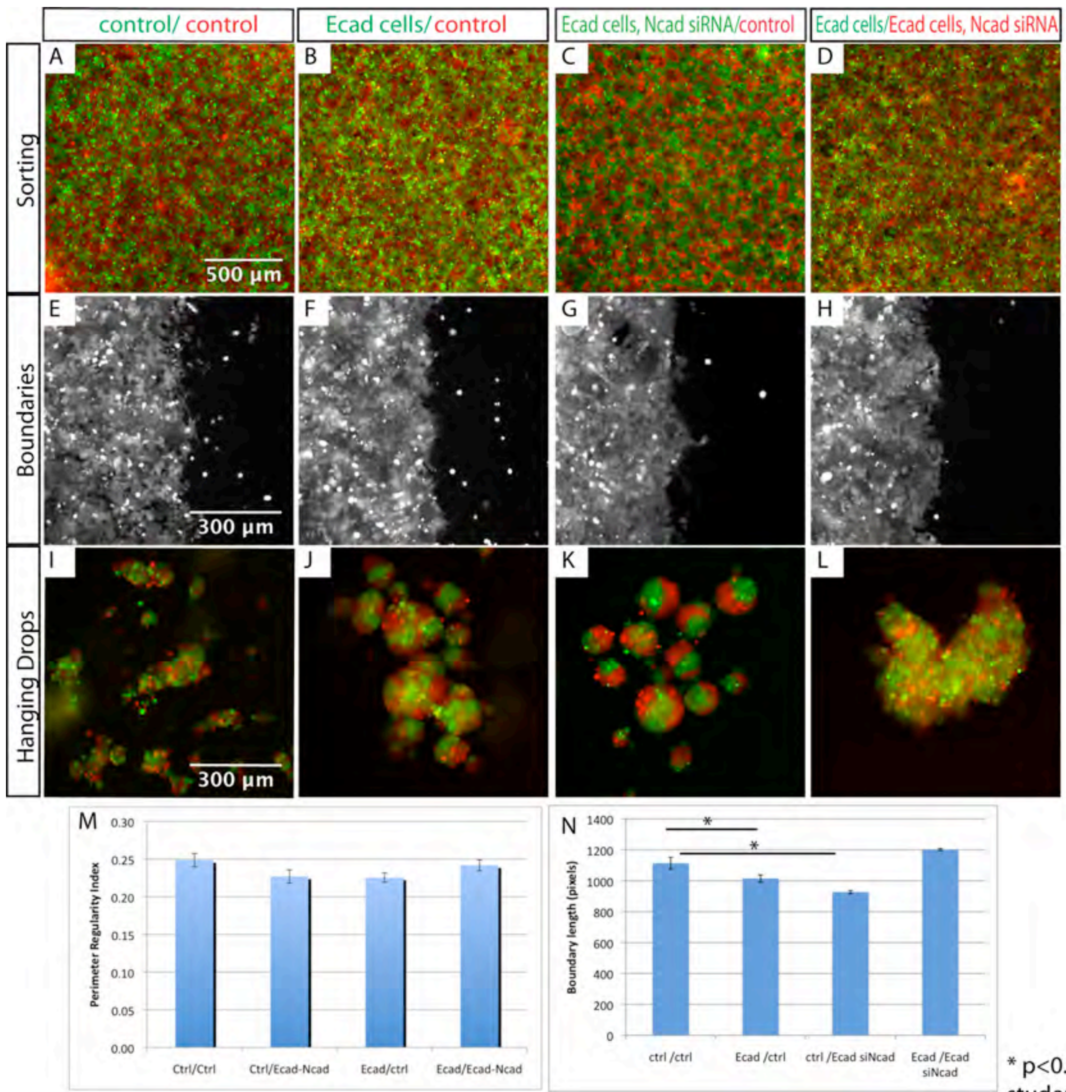
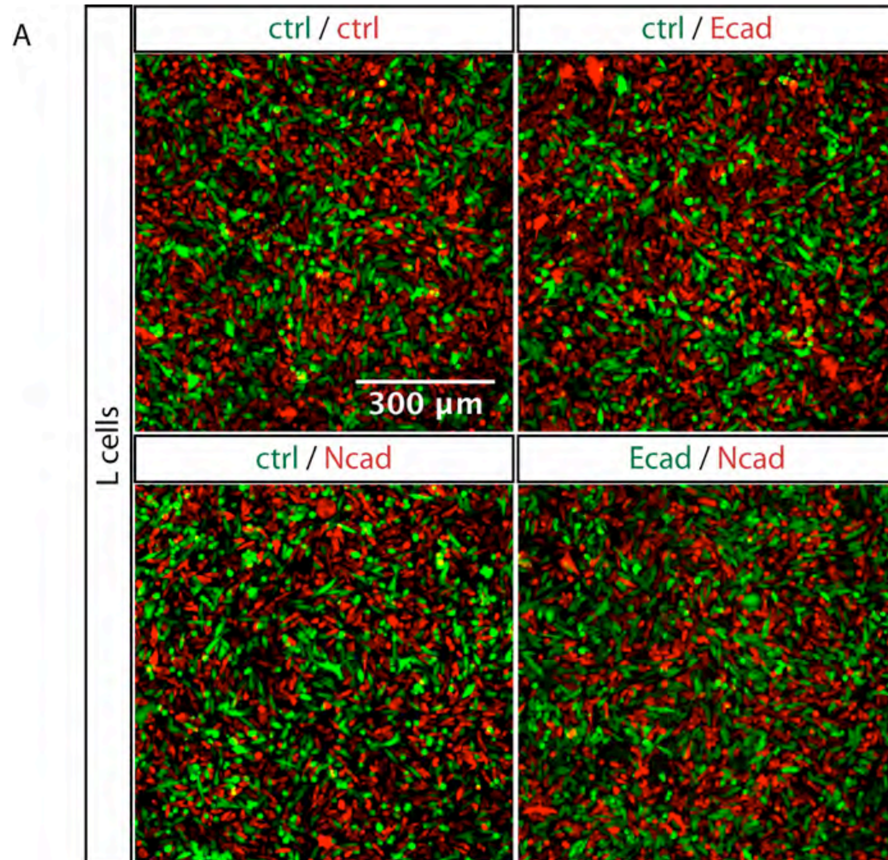


Figure 3.8: Segregation of L cells by differential cadherin expression

(A) L cells, which do not express endogenous cadherins, were mixed with L cells stably expressing E-cadherin or N-cadherin. No sorting is seen in any of the possible combinations.

Figure 3.8



interfering with their ability to mediate differential adhesion. To address this, I used siRNA to knock down N-cadherin in these cells, creating a more substantial difference in cadherin expression between the cell populations.

First, $Ecad^+$ cells were mixed with these $Ecad^+/Ncad^-$ cells. This mixture does not segregate significantly in 2D segregation or boundary assays or in the 3D hanging drop assay, but the aggregates are generally larger and more compact, consistent with an increase in adhesion between all cells (Figure 3.7, D,H,L). When $Ecad^+/Ncad^-$ cells are mixed with control cells ($Ncad^+$) in hanging drops, they clearly sort into distinct regions of the aggregate (Figure 3.7, K). In the segregation assay, the control cells form rough clusters, although the PRI value is not significantly different from unsorted control cells (Figure 3.7, C,M). In the boundary assay, there is a small but significant decrease in boundary length, indicating that there is some sorting between these cells (Figure 3.7, G, N). Taken together, these results show that segregation occurs between E-cadherin expressing and N-cadherin expressing HEK293 cells in hanging drops and, to only a small extent, in the 2D segregation and boundary assays.

Differential adhesion in the sorting of L cells

As well as testing sorting by differential adhesion in HEK293 cells, I also examined its effect in a different cell line. L cells have been used in previous studies of differential adhesion and are good control cells for studying cadherin-mediated processes since they do not endogenously express cadherins. L cells expressing either N-cadherin or E-cadherin were kindly donated by Nobue Itasaki (NIMR, UK). It was anticipated that, since the only difference between these cell lines was the expression of N-cadherin or E-cadherin, this difference would drive sorting, as has been seen previously in aggregated L cells (Shan *et al.*, 2000) as well as in Chinese Hamster Ovary lines (Niessen & Gumbiner, 2002). When I mixed these L cells in the segregation assay in 2D, however, I did not see any sorting between the cells (Figure 3.8).

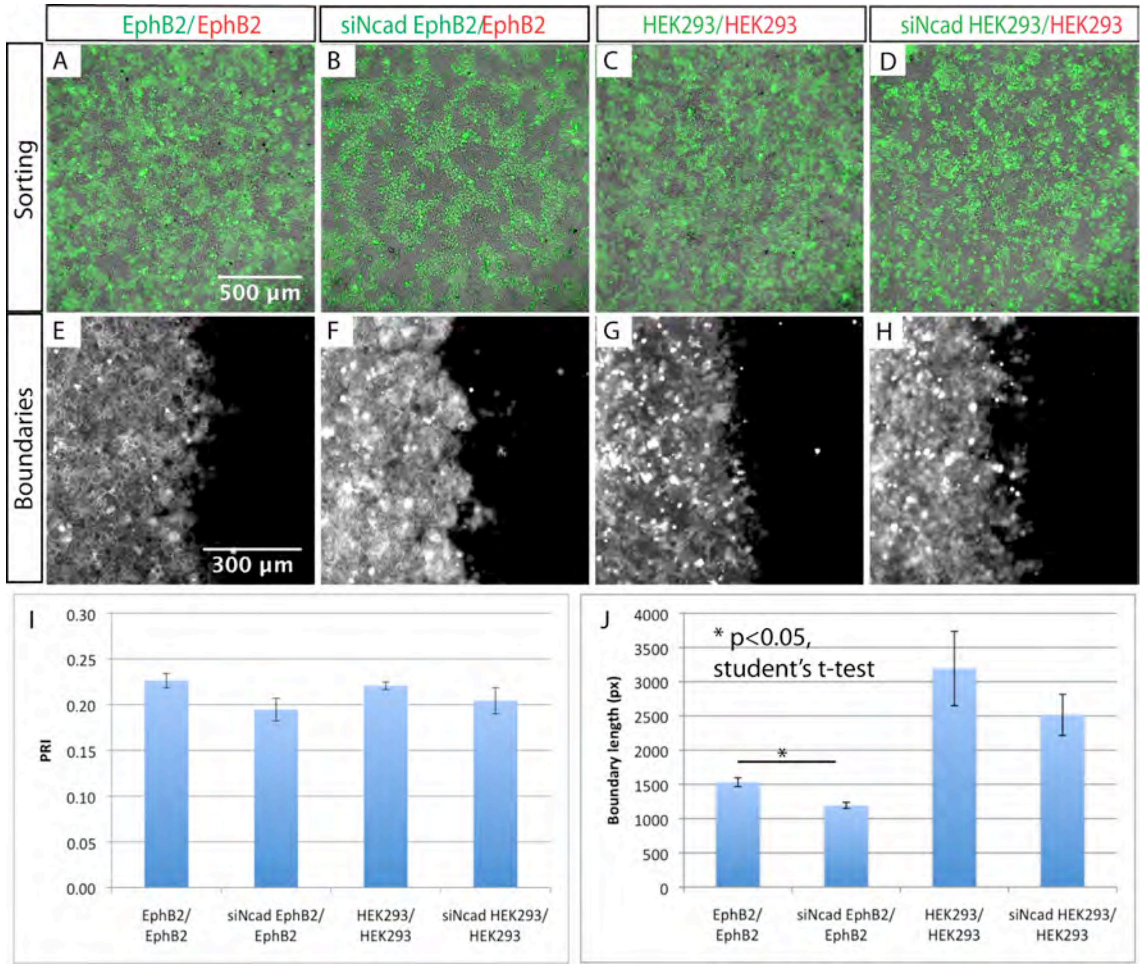
The presence of Eph receptor increases sorting by differential adhesion

Differential adhesion can be driven both by the expression of different types of adhesion molecule and by different levels of the same adhesion molecule. To test

Figure 3.9: Segregation by differential adhesion in EphB2 and control HEK293 cells

Differential adhesion is set up using siRNA knockdown of N-cadherin in one cell population and mixing them with cells where N-cadherin is still endogenously expressed. In EphB2 cells, this leads to sorting of the two cell populations (B) and the formation of a significantly shorter boundary (F) than where two EphB2 cell populations are mixed (A, E, J). In HEK293 cells without EphB2 expression, sorting and boundary sharpening between cells with or without N-cadherin are less pronounced and not significantly different to controls (C,D,G,H,I,J).

Figure 3.9



whether different levels of N-cadherin were capable of driving HEK293 cells to sort, siRNA was used to knock down N-cadherin in one of two mixed populations of cells (Figure 3.9 C,D,G,H). In this case, cells expressing less N-cadherin sort slightly from cells with more N-cadherin to form small clusters with rough edges.

To test whether the presence of EphB2 receptor in cells would affect sorting via differential adhesion, I used EphB2 cells in which N-cadherin has been knocked down. These were mixed with untransfected control or EphB2 cells which express endogenous N-cadherin. In contrast to the situation with cells which do not express EphB2, when differential adhesion is set up between cells which do express EphB2, segregation is increased: clusters in the segregation assay appear larger and the boundary is significantly shortened (Figure 3.9, B, F). These results indicate that EphB2 expression improves segregation between cells with differential adhesion.

Discussion

Eph–ephrin signalling drives cell sorting

When EphB2 and ephrinB1 expressing cell lines are mixed together and plated on a cell culture dish, they consistently sort from one another, with EphB2 cells forming clusters surrounded by ephrinB1 cells, as previously reported (Jorgensen *et al.*, 2009; Poliakov *et al.*, 2008). Maintenance of segregation between EphB2 and ephrinB1 cells has also been shown to occur in 3D aggregates in suspension, using zebrafish animal caps expressing EphB2 or EphA4 juxtaposed with animal caps expressing ephrinB2. In these assays, the two cell types began separated, and segregation is maintained since cells from one aggregate fail to invade the other (Mellitzer *et al.*, 1999; Xu *et al.*, 1999). The current study shows that Eph-ephrin signalling can also drive sorting from initially intermingled populations in 3D culture. When HEK293 cell lines expressing EphB2 or ephrinB1 are mixed and allowed to aggregate in hanging drops, they sort out. ephrinB1 cells will often sort side-by-side with EphB2 cells but occasionally EphB2 cells sort to the outside of the aggregate (Figure 3.3).

In the differential adhesion hypothesis, cells which are more adhesive will sort to the middle of an aggregate, surrounded by less adhesive cells. This minimises the surface area between each of the three phases – more adhesive cells, less adhesive cells and the cell culture medium – and provides the most energetically favourable configuration. If the two cell types express cadherins which do not interact heterophilically with one another but have similar cohesive properties, these cells will sort side by side (Duguay *et al.*, 2003; Foty & Steinberg, 2005; Steinberg, 2007). Comparison with the differential adhesion hypothesis would predict that EphB2 cells are less adhesive or a similar adhesiveness to ephrinB1 cells, since they sort side-by-side or take up external positions in the cluster. However, the sorting pattern may reflect other mechanisms activated by Eph-ephrin signalling such as cell repulsion.

The opposite pattern is seen in 2D cultures, however. Here, clusters of EphB2 cells are surrounded by ephrinB1 cells. According to the differential adhesion hypothesis, clustering would be predicted to occur between cells that are more adhesive. This pattern has been described in the context of differential adhesion. When chick limb bud cells and liver cells are mixed in reaggregate assays, they sort from one another, with heart cells sorting to the inside of the cluster (Garrod & Steinberg, 1973; Garrod & Steinberg, 1975). However, it is the limb bud cells which form clusters when plated in 2D. The authors propose that this is because the limb bud cells are taller, so have a greater surface area in contact with other cells, allowing them to stick together more strongly than heart cells in a 2D situation. It is worth noting, however, that these cell types likely differ not only in their adhesive properties. Limb bud cells and heart cells *in vivo* express different Eph receptors and ephrins too (Gale *et al.*, 1996; Oshima *et al.*, 2008; Wada *et al.*, 2003), which may be the cause of that pattern of sorting independent of their adhesive properties.

Differential adhesion is less powerful than EphB2-ephrinB1 at mediating sorting and boundary sharpening

Despite the fact that differential adhesion is the most widely accepted explanation for cell sorting, I could find no examples where it has been studied in two dimensional cell culture assays. Since Eph receptors and ephrins can drive very

clear cell sorting in 2D, I was interested to know how this compared to cell sorting driven by differential adhesion alone. I found that the extent of sorting driven by differential expression of cadherins was low (Figure 3.7).

One potential explanation for this is the choice of cadherins used in these assays. It has been reported that E-cadherin and N-cadherin do not sort out in a shaking suspension assay (Duguay *et al.*, 2003), and that they have similar affinities which might preclude them from sorting (Shi *et al.*, 2008). However, in some circumstances cells expressing these cadherins do sort. Indeed, in the hanging drop assay, clear sorting can be seen between the E-cadherin and N-cadherin expressing HEK293 cells (Figure 3.7 K). In addition, overexpression of E-cadherin should mean that the total levels of cadherins are significantly higher in these cells than in control HEK293 cells, which should alone cause sorting.

Segregation between E-cadherin and N-cadherin expressing cells has also been reported in the literature. For example, Chinese Hamster Ovary cells expressing human E-cadherin sort from human N-cadherin expressing cells (Niessen & Gumbiner, 2002) and L cells expressing E-cadherin do not co-aggregate with L cells expressing N-cadherin (Shan *et al.*, 2000) in shaking suspensions. Also, there are frequent occasions where N-cadherin and E-cadherin expressing regions of cells are found adjacent to one another in development. The delamination of the neural crest from the neuroepithelium coincides with a switch from N-cadherin to E-cadherin consistent with the cells expressing these cadherins being immiscible with one another (Clay & Halloran, 2011; Nakagawa & Takeichi, 1995). For the avoidance of any doubt, it will be important to repeat these experiments using cadherins that are more unanimously considered to sort from one another in other contexts, for example P-cadherin or R-cadherin and E-cadherin (Duguay *et al.*, 2003). However, the results presented here strongly indicate that differential adhesion is not able to drive efficient cell segregation in 2D assays.

Another way of varying cell adhesion is to express different levels of a cadherin. I hoped to achieve this by knocking down N-cadherin in one population of cells and mixing them with cells in which N-cadherin levels had not been reduced. It has been shown that a 2.5-fold difference in N-cadherin levels is sufficient for cells to sort in hanging drops (Foty & Steinberg, 2005). However, despite nearly complete

knockdown of N-cadherin in one cell population, resulting in at least a 10-fold difference in cadherin levels between the cells, very little sorting occurred. One possibility is that the cells were unable to sort because the knockdown was so efficient that the cells could not stick to one another at all, resulting in a lower rate of sorting. Another is that the knockdown is not uniform, so cells in which N-cadherin is knocked down only a little, could intermingle with cells expressing normal levels of N-cadherin. However, the average knockdown efficiency across the whole population is very high – around 98% - a figure which could not be obtained if many knockdown cells expressed high levels of N-cadherin.

Thus, it seems that differential adhesion is not capable of driving the same degree of sorting in a 2D assay as it can in 3D aggregates. The nature of 3D assays is such that cells are able to form contacts with more neighbours, being surrounded on all sides by other cells. By contrast, in 2D assays, cells are only in contact with other cells at their sides, and interactions with the cell culture medium on one side and the dish on the other will influence their behaviour. I suggest that the 3D assay is more suited to detecting sorting driven by cell-cell adhesion, since the majority of cell behaviour in this assay is governed by cell-cell interactions. On the other hand, cells in a 2D assay are able to migrate around the culture dish, and are not confined to cell-cell interactions, suggesting that this assay is detecting more migration-driven mechanisms.

Unlike cells expressing different levels or types of cadherins, EphB2 cells segregate well from ephrinB1 cells in both 2D and 3D assays. I suggest therefore, that regulation of cell-cell adhesion is not the main mechanism through which Eph-ephrins can mediate sorting. This argues that there must be another mechanism through which sorting can occur.

4. Is N-cadherin downstream of Eph-ephrin signalling?

Is cell-cell adhesion regulated by Eph-ephrin signalling?

In the previous chapter, I described how differential adhesion cannot fully explain segregation driven by Eph-ephrin signalling. However, there is published evidence that adhesion is affected by Eph activation. In colorectal cancer cells, for example, activation of EphB2 by a soluble ephrinB1-Fc chimera causes an accumulation of cadherins at the cell junctions (Cortina *et al.*, 2007). Furthermore, in these same cells, knockdown of E-cadherin results in a disruption to cell sorting mediated by EphB3 and ephrinB1. In addition, two biochemical screens have identified several signalling targets of Eph-ephrins which are involved in the regulation of cell-cell adhesion (Jorgensen *et al.*, 2009; Zhang *et al.*, 2008). Such results indicate that there is a link between adhesion and Eph-ephrin signalling, and I aimed to investigate this link further.

One possibility is that adhesion is decreased between Eph and ephrin cells and this generates differential adhesion (Solanas *et al.*, 2011). As discussed in the previous chapter, this is unlikely to be the only mechanism mediating cell sorting, but it may still be a contributing factor. A second possibility is that adhesion is required for Eph-ephrin signalling to occur. There is evidence, for example, that E-cadherin is required for EphA2 activity in breast cancer cell lines (Zantek *et al.*, 1999). Thirdly, Eph-ephrin signalling could have no impact on cell adhesion, but it could be a basal requirement that cells to stick to one another in order to sort by other mechanisms. A fourth scenario is that cadherins are required independently of their role in cell adhesion, for example in contact inhibition of locomotion. How an adhesion-independent role of cadherins could contribute to sorting through Eph-ephrin signalling will be discussed in more detail in Chapter 5.

To address these questions, I have used the assays described in Chapter 3 to assess segregation and boundary sharpness driven by EphB2 and ephrinB1 after knockdown of genes involved in cell-cell adhesion.

Initially, I tested whether there is a requirement for cadherins in EphB2-ephrinB1 sorting in HEK293 cells, using siRNA to abrogate N-cadherin expression. I then

explored possible biochemical links between Eph signalling and cadherins by knocking down genes which were identified as targets of Eph-ephrin signalling: p120, which plays a key role in cadherin-mediated adhesion; the related gene p0071; and the cadherin-cleaving metalloproteinase Adam10.

N-cadherin knockdown decreases the efficiency of EphB2-ephrinB1 sorting

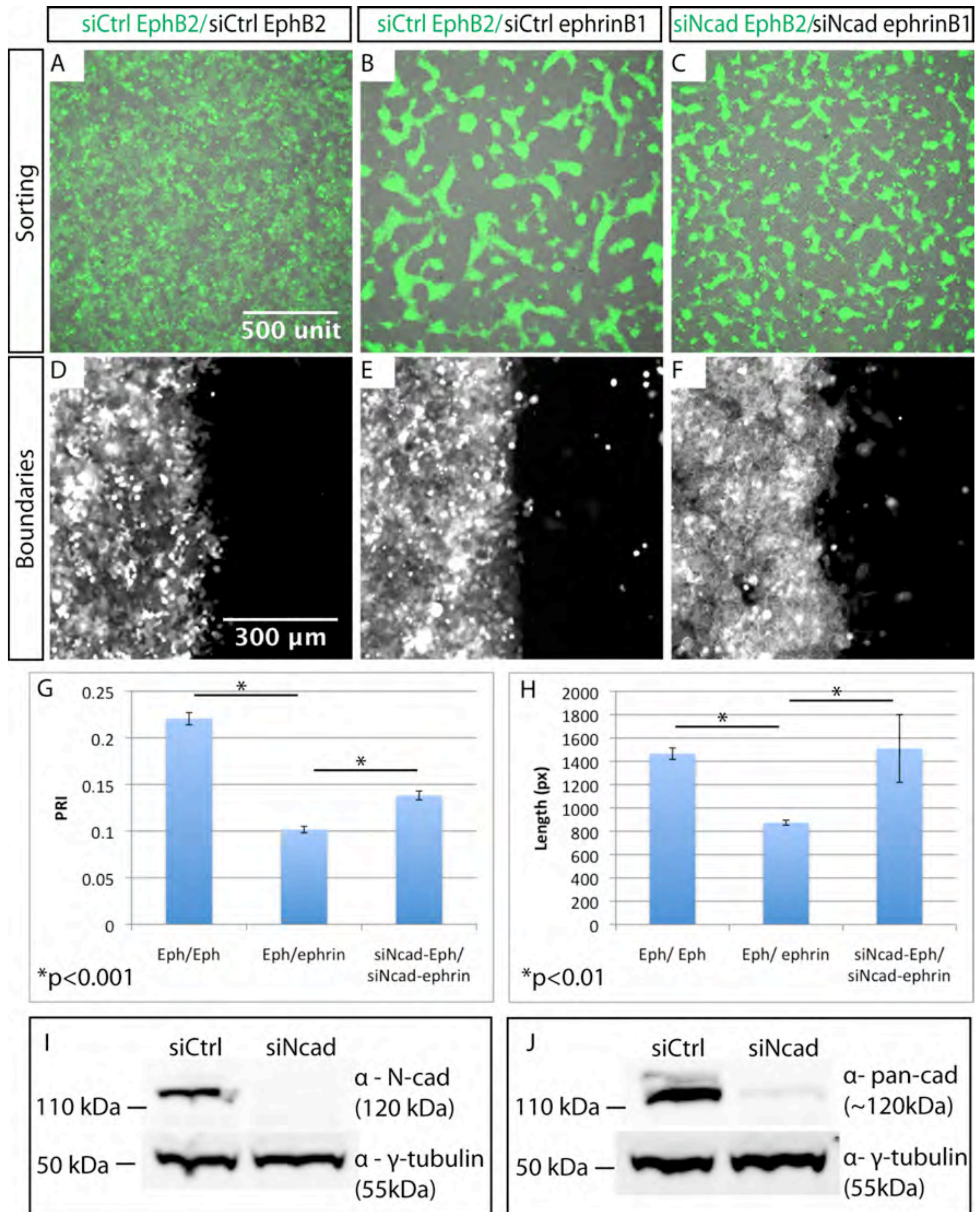
It has been reported that the knockdown of E-cadherin by shRNA disrupts EphB-ephrinB mediated cell sorting in epithelial colorectal cancer cell lines (Cortina *et al.*, 2007). HEK293 cells predominantly express N-cadherin, rather than E-cadherin, so I first wanted to test whether N-cadherin is similarly required for EphB2-ephrinB1 cell sorting. I used siRNA to knock down N-cadherin in both EphB2 and ephrinB1 cells before mixing them and plating them in equal proportions in the segregation assay; or plating them either side of a removable barrier in the boundary assay (Figure 4.1). Whereas EphB2-ephrinB1 mixtures transfected with a control siRNA form large, smooth-edged clusters (Fig 4.1 B) and sharp boundaries (Fig 4.1 E), cells in which N-cadherin has been knocked down form smaller, rougher clusters and disordered boundaries (Fig 4.1 C,F). This is confirmed by a significant increase in the roughness of the clusters, assessed with the PRI, and in boundary length (Fig 4.1 G, H). Interestingly, although sorting was disrupted in these conditions, some degree of segregation still occurred since clusters of EphB2 cells do form. This confirms that EphB2 and ephrinB1 can drive a degree of sorting independently of N-cadherin expression, but that N-cadherin is required for complete segregation. Alternatively, the residual sorting could be due to incomplete knockdown of the N-cadherin protein. The latter idea was tested by Western blot analysis.

The efficiency of the siRNA at knocking down N-cadherin was tested by Western blot analysis of cell lysates 72 h after transfection. This time point corresponds to the mid-point of a segregation assay. Lysates from cells transfected with siNcad were compared to siCtrl. Staining of the Western blot with an antibody to N-cadherin shows a 98±1% knockdown, after normalising to a γ-tubulin loading control (Fig 4.1 I). There is a high efficiency of N-cadherin knockdown in these cells, suggesting that the remaining sorting that is seen in these conditions is due

Figure 4.1: N-cadherin is required for EphB2-ephrinB1 cell segregation

EphB2 cells sort out from ephrinB1 cells to form clusters with defined boundaries. Knockdown of N-cadherin by siRNA significantly disrupts this segregation (A-C, G; Student's t-test, $p<0.001$). (D-F, H) siRNA knockdown of N-cadherin also significantly increases the length, therefore roughness, of the boundary between EphB2 and ephrinB1 cells in the boundary assay (Student's T-test, $p<0.01$). px: pixels. (I,J) The siRNA is effective at knocking down N-cadherin as tested by Western blot using antibodies for N-cadherin (98 ±1% knockdown) and pan-cadherin (93±6% knockdown), compared to a nonsense siRNA. γ-tubulin was used as a loading control. Error bars represent standard error of the mean.

Figure 4.1



to N-cadherin independent mechanisms. However, other cadherins could still be expressed in the cells, compensating for N-cadherin loss.

The presence of other cadherins was assessed by staining equivalent Western blots with an anti-pan-cadherin antibody, which recognises classical cadherins including E-cadherin and N-cadherin. Staining with this antibody showed a knockdown of 93±6% in cells treated with siNcad compared to siCtrl (Figure 4.1 J). This suggests that there may be some other cadherins present in the N-cadherin knockdown condition. However, since less than 10% cadherin staining remains after siNcad treatment, N-cadherin constitutes the major cadherin expressed by these cells. The remaining levels of total classical cadherin are very low, suggesting that the residual sorting in the cells is likely to be through a cadherin-independent mechanism. This is supported by studies revealing a role of cell migration in Eph-ephrin mediated cell segregation (Chapter 5).

Nevertheless, there is a clear disruption to segregation when N-cadherin is knocked down, indicating that N-cadherin is required for Eph-ephrin mediated sorting in HEK293 cells. The next step was to determine how N-cadherin is involved in dynamic responses during the sorting of these cells. One possibility is that EphB2 activation results in the relocalisation of N-cadherin.

The localisation of cadherins is affected in the segregated EphB2 cells and after EphB2 activation.

It has previously been shown that activation of EphB receptors leads to an accumulation of E-cadherin at the cell membrane in colorectal cancer cells (Cortina *et al.*, 2007). I used immunochemistry to determine whether Eph activation also affects the expression of N-cadherin in HEK293 cells. Initially, EphB2 cells were stimulated with soluble ephrinB1-Fc chimera (Figure 4.2 A-D). After 30 min of stimulation, the cells have begun to round up and retract their processes consistent with previous observations (Poliakov *et al.*, 2008). There is also an increase in the intensity of pan-cadherin antibody staining at the cell surface as predicted from the colorectal cancer cell experiments (Cortina *et al.*, 2007). However, the intensity of staining of EphB2 and of membrane-bound GFP (not shown) is similarly increased between these cells. This could suggest that the

increase in staining of membrane proteins could be a secondary effect to the change in cell shape.

I was interested to see if a similar effect could be seen in mixtures of EphB2 and ephrinB1 cells in the segregation assay and the boundary assay (Figure 4.2 E-N). There is an increase in cadherin staining within the EphB2 cell clusters. This also co-localises with the membrane GFP expressed by these cells. The increase in cadherin intensity is most clear between cells in the centre of the clusters. There are several examples of EphB2 cells in contact with ephrinB1 cells, which are indistinguishable from their neighbours in terms of cadherin staining (Figure 4.2 E-H, *). Similar to what is seen after ephrinB1-Fc stimulation, the increase in cadherin staining appears to be concomitant with a change in cell shape. The same is true in the boundary assay. There is no clear difference in cadherin localisation between EphB2 cells in direct contact with ephrinB1 cells and adjacent cells which are only in contact with other EphB2 cells.

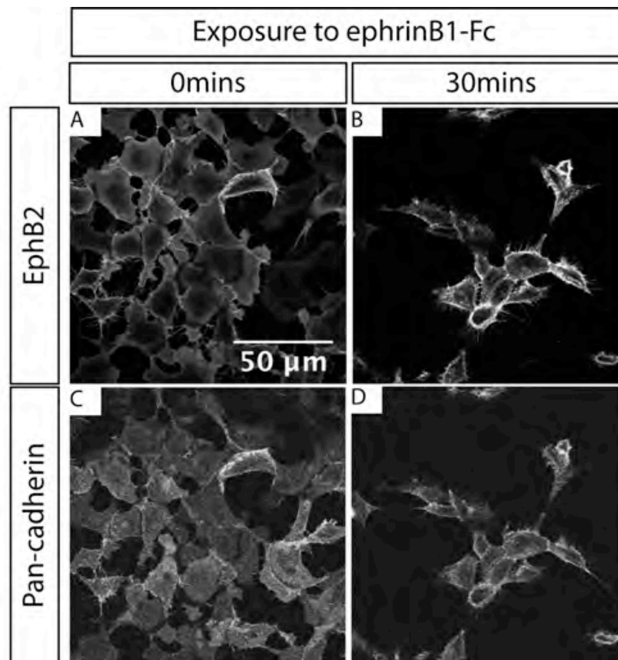
These results show that cells within sorted EphB2 cell clusters have enriched cadherin staining at their membranes. This could be due to an upregulation or stabilisation of cadherins at cell membranes. However, since this increase in cadherin staining is not detected in EphB2 cells at the edges of clusters, it is unlikely to be a direct consequence of Eph-ephrin signalling. Alternatively, this increased staining could be a secondary effect of the crowding and change in cell shape that occurs after EphB2 stimulation or clustering (Ehrlich *et al.*, 2002; Yamada & Nelson, 2007). This is supported by the fact that membrane bound GFP, whose distribution is not likely to be affected by Eph-ephrin signalling, is also enriched between cells in clusters and colocalises with the cadherin staining.

To assess this more directly, I looked at earlier stages of EphB2-ephrinB1 sorting, before large clusters have formed and where EphB2 cells and ephrinB1 cells have similar shapes (Figure 4.3). After 5h, the cultures are still sparse but the EphB2 cells have clearly sorted from ephrinB1 cells. Pan-cadherin staining is enriched at the membranes between any two cells which are juxtaposed. Cadherin staining is enriched between cells in some large clusters where EphB2 cells are tightly packed. However, cadherin staining is not enriched between EphB2 cells in

Figure 4.2: Cadherin localisation in EphB2 and ephrinB1 cells

Cadherin localisation after EphB2 activation. Confocal images showing the localisation of EphB2 (A,B) and pan-cadherin (C,D) in EphB2-cells before (A,C) and after (B,D) stimulation with ephrinB1-Fc. (E-H) Confocal images of pan-cadherin staining (white/red) of EphB2-ephrinB1 cell mixtures 48hrs after plating. EphB2 cells are marked by co-expression of membrane-GFP. Intersections between EphB2 and ephrinB1 cells which show no difference in cadherin localisation compared to surrounding cells (*) and downregulation of cadherin at the membrane (>) are indicated as shown. (I-K) Wide-field images of pan-cadherin staining (white) of EphB2-EphB2 (I,K) and EphB2-ephrinB1 (J,L) boundaries at the end point of the boundary assay. There is more pan-cadherin staining in the bulk of the EphB2 cells than ephrinB1 cells, but there is no clear difference in staining between the two populations at the interface. Cells are more tightly packed within the clusters, as can be seen by densely packed nuclei shown by DAPI staining (M,N).

Figure 4.2



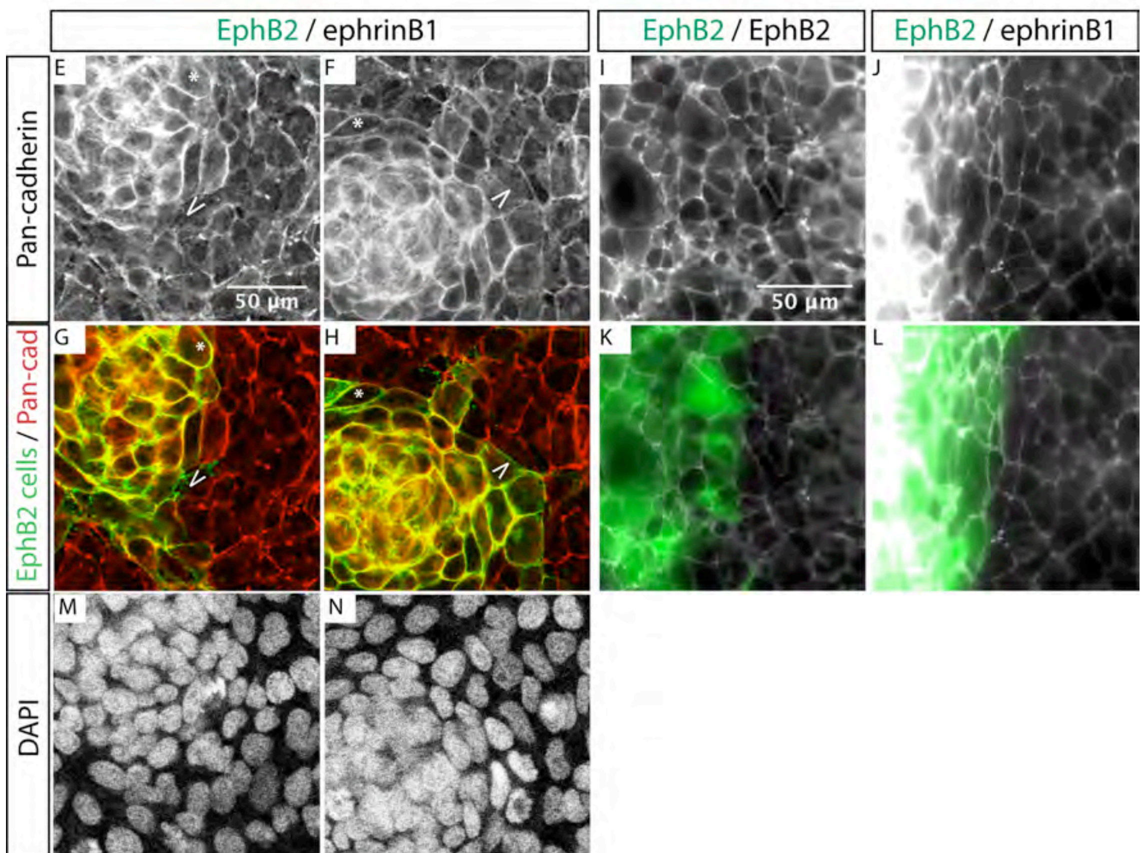
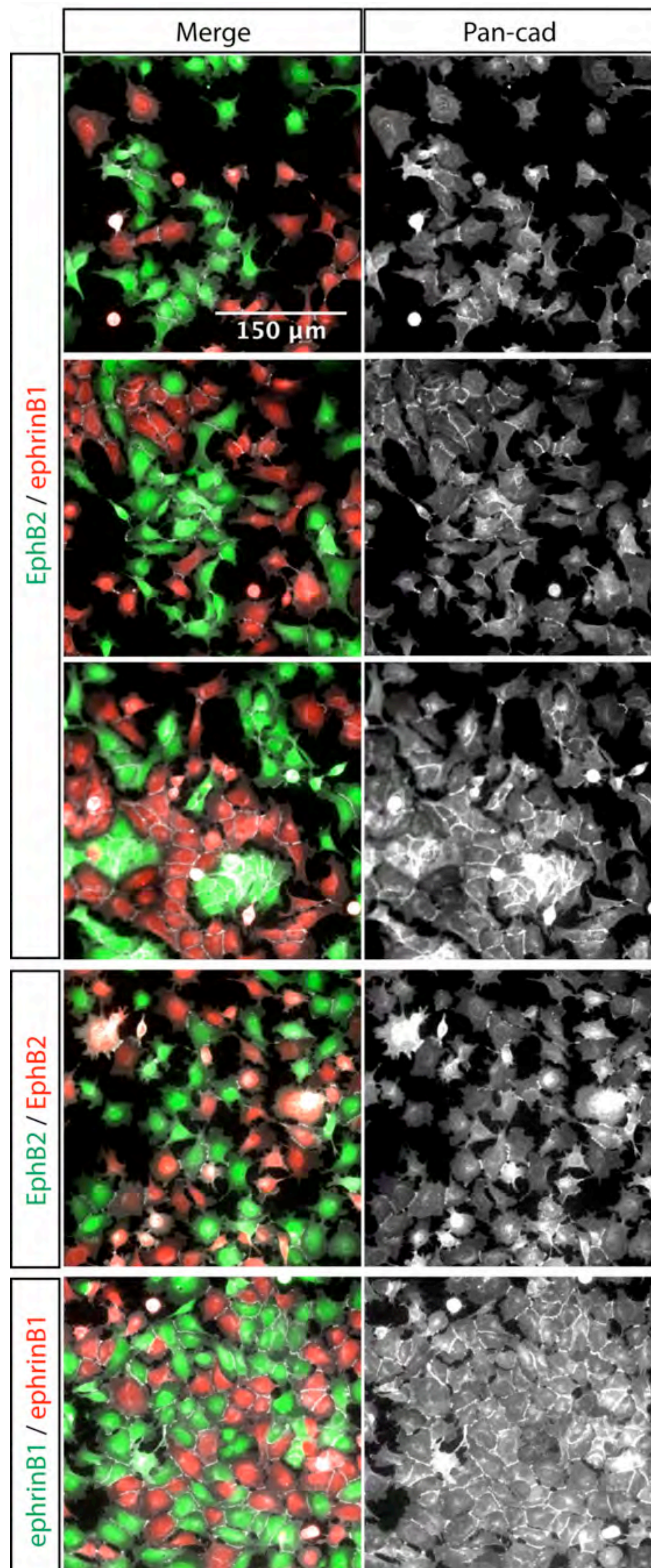


Figure 4.3: Cadherin expression in EphB2-ephrinB1 sorting cells at low density

Pan-cadherin staining of EphB2 and ephrinB1 cells 5 hours after the cells were mixed. Increased staining (white) can be seen at the junctions between cells, and is higher between cells within EphB2 clusters. However, in less dense regions of cells, where sorting can still be clearly seen, clusters cannot be distinguished based on the intensity of staining. Slides prepared by Alexei Poliakov.

Figure 4.3



clusters which are less tightly packed. This suggests that the initial stages of sorting are independent of cadherin enrichment between EphB2 cells.

EphB2 or ephrinB1 expression or EphB2 activation do not affect the expression of cadherins.

One explanation for increased cadherin staining within sorted EphB2 cell clusters could be regulation of the levels of N-cadherin, for example by an increase in protein synthesis or degradation. To test this, I carried out Western blot analysis of cells expressing EphB2 or ephrinB1 compared with control cells, as well as cells after EphB2 activation by ephrinB1-Fc stimulation (Figure 4.4). The expression of EphB2 (with or without co-expression of mGFP) or ephrinB1 alone does not affect the level of protein detected by N-cadherin or pan-cadherin antibodies. Equally, activation of the EphB2 receptor by stimulating the cells with a clustered, soluble form of the ligand, ephrinB1-Fc chimera, does not affect the levels of expression detected by these two antibodies. This indicates that there is no effect of EphB2 expression, ephrinB1 expression or EphB2 activation by ephrinB1 on the cellular levels of N-cadherin. Therefore, if there is an enrichment of cadherin staining between cells within EphB2 cell clusters, it must be due to a change in localisation, rather than total cell levels, of the protein.

N-cadherin is required in both EphB2 and ephrinB1 cells for efficient sorting

When N-cadherin is knocked down in both EphB2 and ephrinB1 cells, sorting is disrupted. One question that arises from this experiment is whether N-cadherin is required in both EphB2 and ephrinB1 cells or predominantly in one type or the other. Reduction of N-cadherin levels in either cell type by siRNA knockdown could help to answer this question. This will reduce cadherin-mediated adhesion between cells of that type (cohesion) and will also set up differential adhesion between the EphB2 cells and ephrinB1 cells which may contribute to the sorting phenotype (Figure 4.5 M). Experiments in the previous chapter have shown that EphB2 cells in which N-cadherin has been knocked down do not sort out well from control EphB2 cells (Figure 3.9). Therefore, the effect of differential adhesion in this experiment may be negligible compared to the effect of cohesion in Eph-ephrin mediated sorting.

Figure 4.4: Cadherin expression levels in EphB2 and ephrinB1 cells

Western blots showing levels of cadherins detected in cell lysates of ephrinB1, EphB2 and GFP-EphB2 cells detected using an antibody to N-cadherin (A) and pan-cadherin (B). N-cadherin and pan-cadherin antibodies detect the same protein levels in all the cell types studied, including GFP-EphB2 cells stimulated with ephrinB1-Fc chimera which activates signalling through EphB2. γ-tubulin is used as a loading control.

Figure 4.4

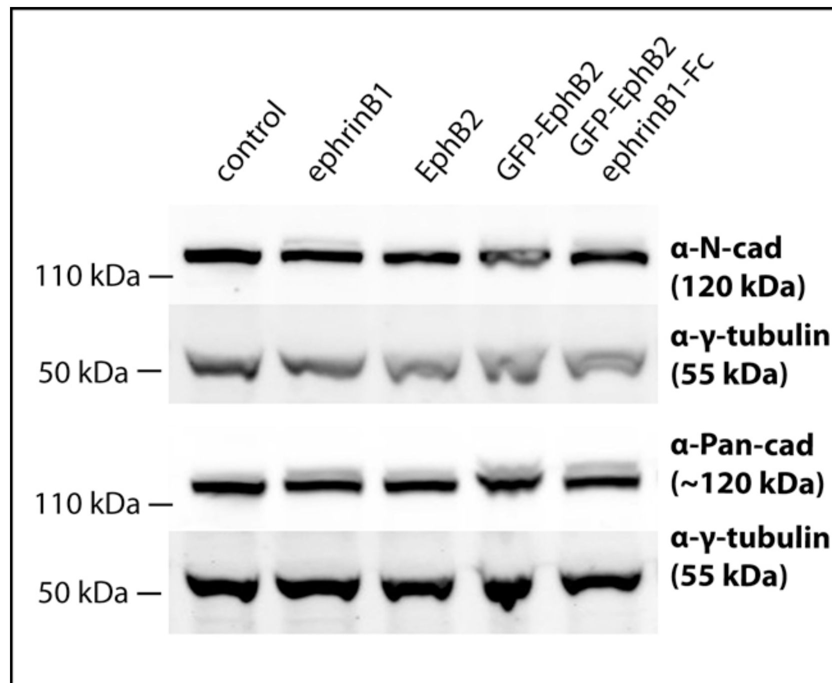
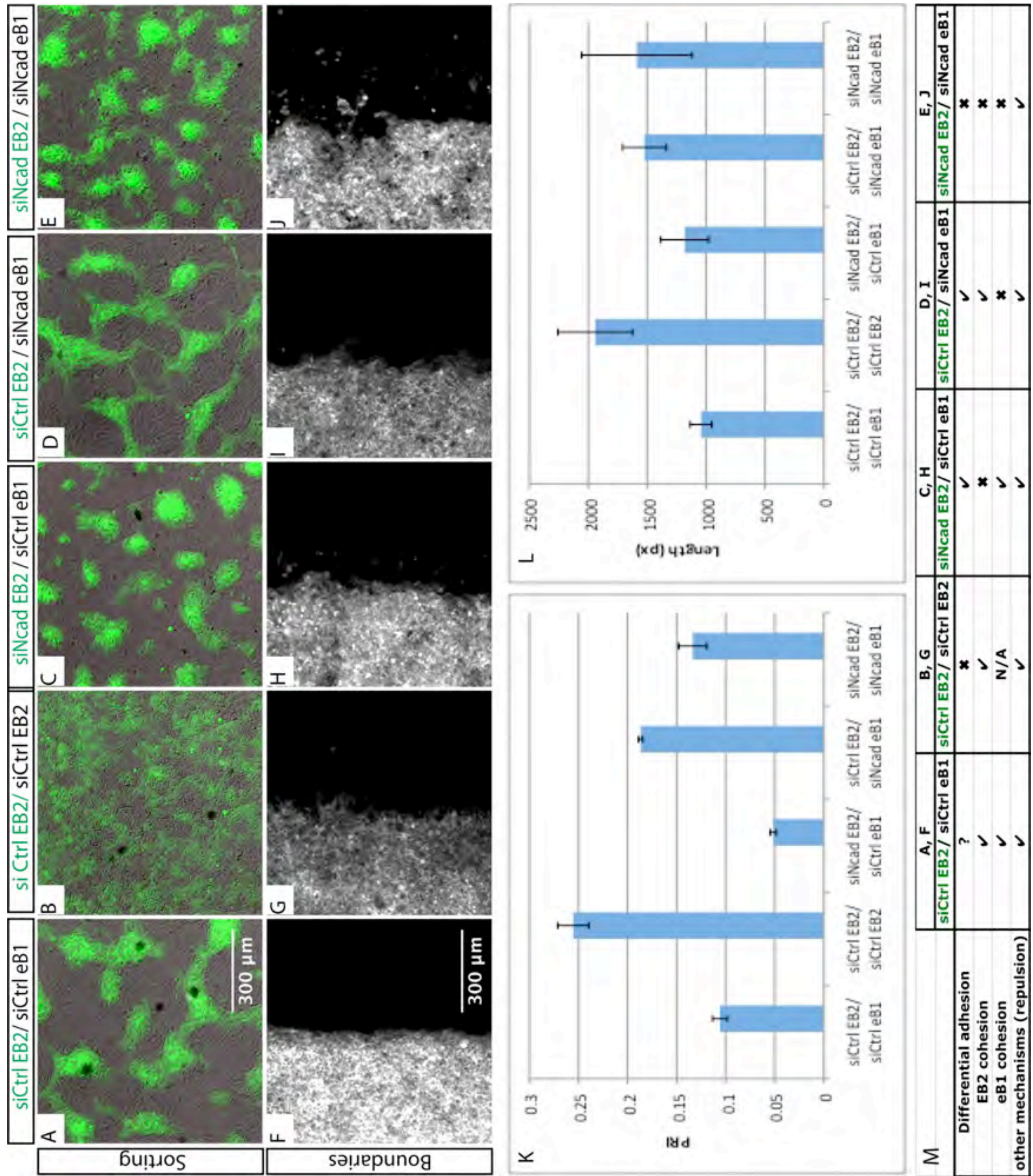


Figure 4.5: N-cadherin is required for basal cell-cell adhesion, to stabilise segregation

(A-E) Segregation assay where N-cadherin has been knocked down by siRNA in EphB2 cells (EB2), ephrinB1 cells (eB1) or both. Clusters are smaller and rounder compared to controls when N-cadherin is knocked down in only EB2 cells, with more isolated EB2 cells in the eB1 territory (C). When N-cadherin is knocked down in only eB1 cells, EB2 clusters are larger and more interconnected (D). Knockdown of N-cadherin in both cell populations gives a phenotype which appears like a combination between these two (E). A similar pattern is seen in the boundary assay, with knockdown of N-cadherin in only eB1 resulting in a fuzzy boundary, knockdown in only EB2 a slightly less fuzzy boundary but with more isolated cells, and knockdown in both an even fuzzier boundary (F-J). Images taken at 10x magnification on a Deltavision microscope. EB2 cells detected by membrane-GFP co-expression. Quantification based on 3 repeat experiments of the sorting (K) and boundary assays (L) confirms these phenotypes. px: pixels. (M) Table outlining the presence of cell cohesion, differential adhesion and other mechanisms in the different cell combinations shown in images A-J. Error bars represent standard error of the mean.

Figure 4.5



	A, F siCtrl EB2/ siCtrl eB1	B, G siCtrl EB2/ siCtrl EB2	C, H siNcad EB2/ siCtrl eB1	D, I siCtrl EB2/ siNcad eB1	E, J siNcad EB2/ siNcad eB1
Differential adhesion	?	✖	✔	✔	✖
EB2 cohesion	✔	✔	✖	✔	✖
eB1 cohesion	✔	N/A	✔	✖	✖
other mechanisms (repulsion)	✔	✔	✔	✔	✔

When N-cadherin was reduced in the EphB2 cells alone, cells were able to sort into rounded clusters (Figure 4.5 C,H). These varied in size, with more small clusters than in cells treated with siCtrl. By contrast, when N-cadherin is knocked down in ephrinB1 cells, EphB2 cells form more spread clusters with long branches interlinking them (Figure 4.5 D, I).

In the boundary assay where N-cadherin is knocked down in EphB2 cells only, boundary length is comparable to control EphB2-ephrinB1 cell mixtures, although more isolated EphB2 cells can be seen in the ephrinB1 cell territory (Figure 4.5 J,L). The boundary length in co-cultures where N-cadherin has been knocked down in ephrinB1 cells only is longer than with siCtrl-treated cells (Figure 4.5 I,L).

These results support the idea that there are different requirements for N-cadherin in EphB2 and ephrinB1 cells. These results can be explained in terms of cohesion between cells. When cohesion is reduced in EphB2 cells only, there is an apparent increase in the number of smaller clusters, which would be expected if they were unable to stick together to stabilise larger clusters. Conversely, when cohesion is reduced in ephrinB1 cells only, they do not form a continuous sheet but are broken up by branches of EphB2 cells.

These results support a key role for N-cadherin in mediating cohesion between cells and stabilising clusters. Nevertheless, molecules involved in cell-cell adhesion are regulated downstream of EphB2-ephrinB1 signalling so the regulation of adhesion may still play a role in segregation. To investigate this further, I studied signalling targets of EphB2 with roles in the regulation of cell-cell adhesion.

Regulatory molecules downstream of cadherin-mediated adhesion are also required for EphB2-ephrinB1 sorting and boundary sharpening

Two screens have been published which searched for biochemical targets of EphB2-ephrinB1 signalling. These screens identified proteins with altered tyrosine phosphorylation states in EphB2-expressing cells after stimulation with ephrinB1-Fc or ephrinB1-expressing cells (Jorgensen *et al.*, 2009; Zhang *et al.*, 2008). Amongst the proteins identified as EphB2 targets were several involved in mediating cell-cell adhesion. These suggest a link between Eph-ephrin signalling and the regulation of cell-cell adhesion.

I shortlisted these proteins by considering their relevance to cell-cell adhesion, based on a literature search. I was particularly interested in proteins with a key role in cell-cell adhesion. The most interesting candidates are listed in Figure 4.6 A. I carried out qPCR of these genes in HEK293 cells to see which were expressed (Figure 4.6 B). Of these, I found p120 and p0071 to be expressed at similar levels to N-cadherin. Af-6 was not detectably expressed so was not investigated further. Although Rab7 is expressed at high levels and has been implicated in E-cadherin endocytosis (Palacios *et al.*, 2005), it is also involved in a wide range of other endocytic processes. On the other hand, many studies have linked p120 to the regulation of cell adhesion and p120 activity has been shown to be regulated by phosphorylation (Alema & Salvatore, 2007; Castano *et al.*, 2007; Fukumoto *et al.*, 2008). I therefore decided to focus on the role of p120 and its related family member p0071 downstream of Eph-ephrin signalling.

siRNA knockdown of N-cadherin, p120 or p0071 does not prevent Eph activation.

Any effect that knockdown of molecules has on Eph-ephrin sorting could be due to a specific role they play downstream of Eph-ephrin signalling. However, these proteins could also be acting upstream of Eph-ephrins, affecting the ability of the receptor to be phosphorylated and therefore to activate downstream pathways. To test this possibility, I carried out Western blot analysis of EphB2 cells which had been stimulated with ephrinB1-Fc, compared to cells which had been mock-stimulated with PBS, after knockdown of N-cadherin, p120 and p0071. The Western blots were then stained using an antibody against the phosphorylated form of Eph receptor (phospho-Eph) and compared to staining for total EphB2 (Figure 4.7).

There is a low background level of phospho-Eph staining without ephrinB1-Fc stimulation, which may be due to high levels of expression of EphB2 in these cells leading to auto-activation of the receptor (Poliakov *et al.*, 2004). However, there is a substantial increase in phospho-Eph staining after stimulation with ephrinB1-Fc in all conditions. This demonstrates that siRNA knockdown of Ncad, p120 or p0071 has no effect on the ability of EphB2 to be phosphorylated. Interestingly,

Figure 4.6: Targets of ephrinB1-EphB2 signalling

Several of the proteins identified as downstream targets of ephrinB1-EphB2 signalling are involved in the regulation of cadherin-mediated adhesion. Table (A) lists four of these that stood out as having a clear role in this process. (B) The expression levels of these four genes as well as N-cadherin in HEK293 cells as determined by qPCR. Results are shown relative to the expression of β-actin (n=2). With the exception of Af-6, they are all expressed in these cells. Error bars represent standard error of the mean.

Figure 4.6

A

Gene	Alternative gene names	Protein Function
p120	CTNND1; CAS; p120; CTNND; P120CAS; P120CTN; KIAA0384; p120(CAS); p120(CTN); catenin (cadherin-associated protein), delta 1	An armadillo protein, which functions in adhesion between cells and signal transduction. Phosphorylation of ctnnd1 causes defects in cadherin-mediated stability. (Ireton *et al.*, 2002; Ozawa & Ohkubo, 2001; Thoreson *et al.*, 2000)
p0071	PKP4 (plakophilin4); FLJ31261; FLJ42243	PKP4 is an armadillo protein which may be a component of desmosomal plaque and other adhesion plaques and is thought to be involved in regulating junctional plaque organization and cadherin function. (Hatzfeld *et al.*, 2003)
Rab7	RAB7A; PRO2706; FLJ20819	Involved in vesicle trafficking and has been implicated in the lysosymal targeting of E-cadherin. (Palacios *et al.*, 2005)
Af6/ afadin	MLLT4; FLJ34371; RP3-431P23.3; myeloid/lymphoid or mixed-lineage leukemia (trithorax homolog, Drosophila); translocated to, 4	Af6 is a Ras target that regulates cell-cell adhesions downstream of Ras activation. Af6 knockout mice have severe adherens junction defects. (Ikeda *et al.*, 1999; Takai & Nakanishi, 2003)

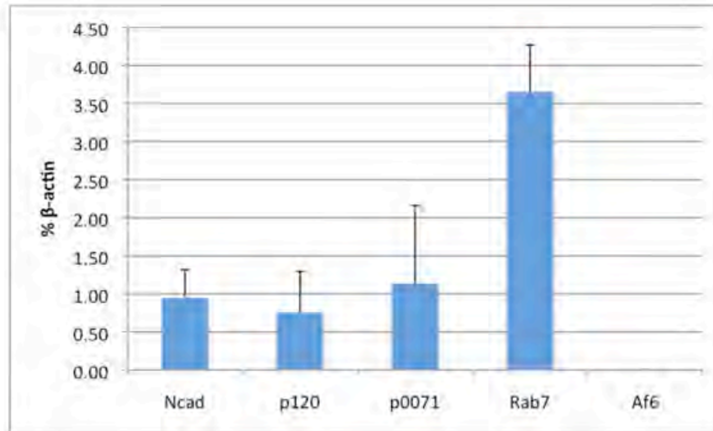
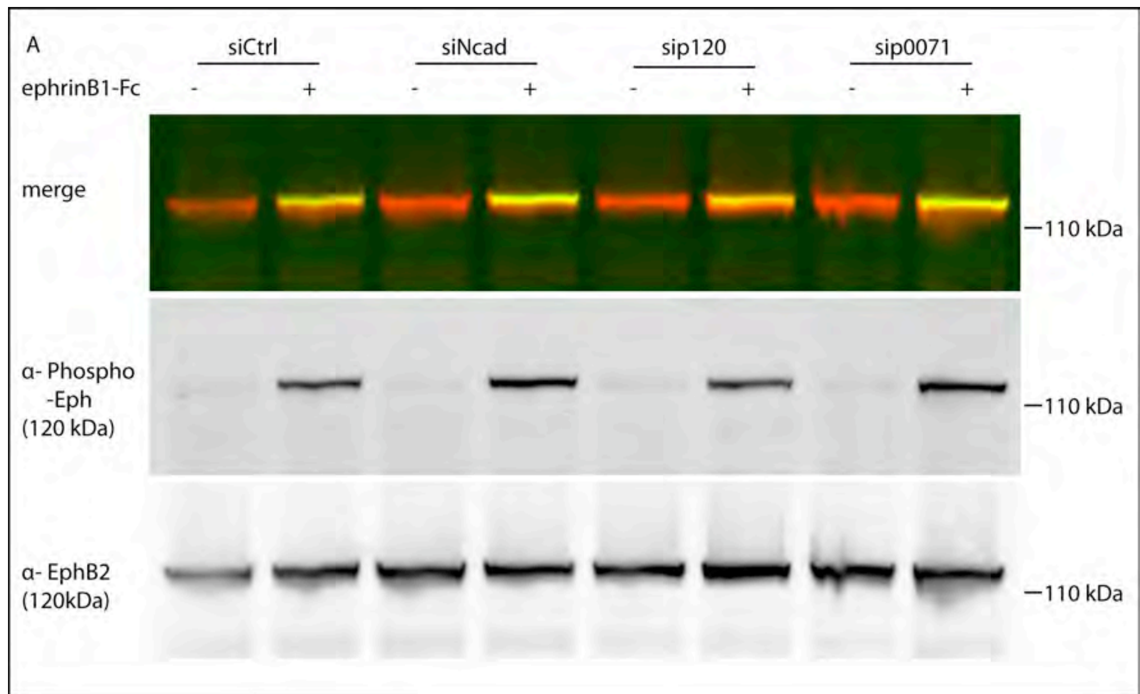
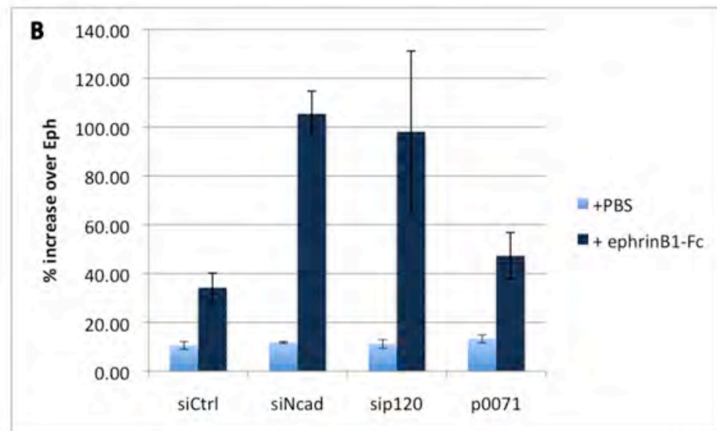


Figure 4.7: Phospho-Eph staining as a readout for EphB2 activity in siRNA knockdowns of N-cadherin, p120 and p0071

siRNA knockdown of N-cadherin, p120 and p0071 does not decrease the activity of EphB2 receptor. Western blot analysis of EphB2 cells for a phosphorylated form of Eph receptor, before or after stimulation with soluble ephrinB1-Fc. Phosphorylation of Eph is not affected when N-cadherin, p120 or p0071 are knocked down by siRNA (A), but increases relative to a control siRNA when cells are stimulated by ephrinB1-Fc. Total EphB2 is used as a loading control. Quantification of these Western blots (n=3) confirms this increase. Error bars represent standard error of the mean.

Figure 4.7





there is a significant increase in the phosphorylation of EphB2 after N-cadherin and, less consistently, p120 are knocked down. This could reflect a direct effect of these proteins inhibiting Eph signalling. Alternatively, it could be a secondary effect due to an increase in the surface of the cell exposed to the ligand after cell-cell contacts are disrupted. Either way, treatment of EphB2 cells with siRNAs to N-cadherin, p120 or p0071 does not decrease ephrinB1-EphB2 forward signalling.

p120, but not p0071, knockdown reduces levels of N-cadherin

Previous studies have shown that reduction in p120 expression results in degradation and decreased expression of cadherins (Davis *et al.*, 2003; Davis & Reynolds, 2006). It was unclear whether a comparable relationship might exist for p0071. To evaluate how N-cadherin is affected by p120 and p0071 in HEK293 cells, I carried out Western blot analysis of EphB2 cells treated with sip120, sip0071, siNcad, and siCtrl (Figure 4.8 A). This analysis shows that cadherin levels, detected by N-cadherin or pan-cadherin antibodies, are significantly reduced when p120 is knocked down. In contrast, p0071 has no effect on the total cell levels of N-cadherin. Neither siNcad nor sip0071 disrupts p120 levels in these cells. These results support previous evidence that p120 is required for cadherin stability.

To confirm this result, I carried out immunochemistry on these cells. Under control conditions, N-cadherin is highly enriched at the cell-cell contacts (Figure 4.8 B). As expected, this enrichment is abolished in the presence of siNcad. sip120 treatment results in reduction of total protein levels detected, consistent with the Western blot analysis, and no enrichment of the remaining protein is seen at cell contacts. sip0071 treatment does not appear to reduce N-cadherin levels, although there is a reduction in the number of cell contacts at which N-cadherin is enriched. This could be due to reduced stability of N-cadherin protein at contacts, without subsequent change in protein levels. Alternatively, p0071 knockdown could decrease the stability of contacts through another mechanism, for example by increasing the activity of cell protrusions.

p120 and p0071 knockdown disrupt EphB2-ephrinB1 sorting

The requirements for p120 and p0071 in EphB2-ephrinB1 cell sorting were assessed using the segregation and boundary assays.

Firstly, I tested the efficiency of knockdown of each of these genes using a pool of 3 siRNAs targeted to different regions of the gene. Pooling siRNAs minimises off-target effects as described in Chapter 3. Western blot analysis was carried out on lysates of cells, which were harvested 72 h after transfection with siCtrl, sip120 or sip0071. I tested several commercially available antibodies to the proteins of interest before finding ones which worked. The antibodies to either p120 or p0071 detect several bands which may correspond to different isoforms of these proteins. siRNA that targets p120 gives an average protein knockdown of 85±12% relative to a γ-tubulin control (n=2, Figure 4.9 I). sip0071 reduces protein levels by 74±12% on average (n=2; Figure 4.9 J).

siRNA knockdown of either p120 or p0071 disrupts the pattern of cell sorting of EphB2 and ephrinB1 cells (Figure 4.9 A-C, G). EphB2 clusters appear smaller and less dense and the PRI is significantly increased. In the boundary assay, however, only sip120 and not sip0071 has a significant effect on the sharpness of the boundary (Figure 4.9 D-F, H). This points to a possible difference in the sensitivities of the two assays to detecting sorting, where cells migrate freely at first, versus boundary sharpness. The migration of cells in the boundary assay may be more restricted since they are at a higher density from the moment the EphB2 and ephrinB1 cells interact.

The similarities between p120 and p0071 suggest that they may play redundant roles in the stabilisation of cadherins at the membrane (Hatzfeld, 2005) . Alternatively, they could act together or independently of N-cadherin, to regulate RhoGTPase activity and cell migration. To assess whether Ncad, p120 and p0071 work together or through separate pathways, I knocked them down in combination (Figure 4.10).

So that the total concentration of siRNA transfected into the cells was kept the same across all conditions, siRNAs to N-cadherin, p120 and p0071 were mixed either with one another or with siCtrl. Since this halves the amount of siRNA to each gene, I first checked the knockdown efficiency of p120 and N-cadherin in these situations. p120 protein was knocked down by 78±7% (n=2) similar to before, and N-cadherin by 76±14% (n=3), less than the 98% when it was

Figure 4.8: Cadherin expression and localisation in cells treated with sip120 or sip0071

Western blot analysis and immunochemistry were used to determine the levels and localisation of N-cadherin, p120 and p0071 under conditions where each was knocked down. (A) Western blot analysis of EphB2 cells treated with siNcad, sip120, sip0071 or siCtrl. Cadherin protein levels, detected using an anti-N-cadherin and a pan-cadherin antibody, are high in siCtrl and sip0071 treated cells, but are reduced in cells treated with siNcad and sip120. p120 levels are unaffected by either siNcad or sip0071. Anti-γ–tubulin antibody was used as a loading control.

(B) Immunochemistry of EphB2 cells treated with siNcad, sip120, sip0071 or siCtrl. N-cadherin is enriched at the membranes of cells in contact with one another in siCtrl conditions. N-cadherin expression is abolished in siNcad-treated EphB2 cells and there is greatly reduced expression in sip120-treated cells, with no enrichment seen at cell junctions. In cells treated with sip0071, N-cadherin levels remain high and there is some enrichment at cell-cell contacts, although this appears reduced compared to siCtrl treated cells.

Figure 4.8

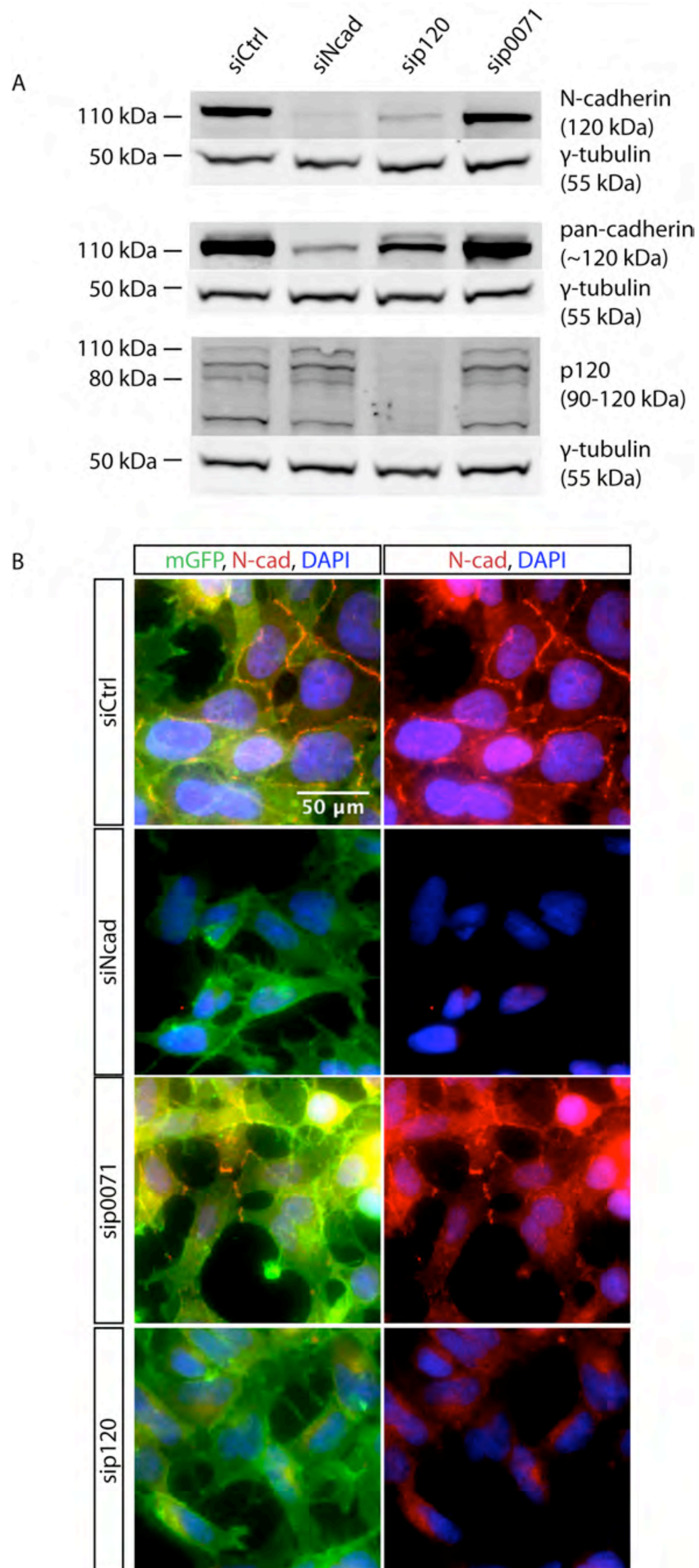
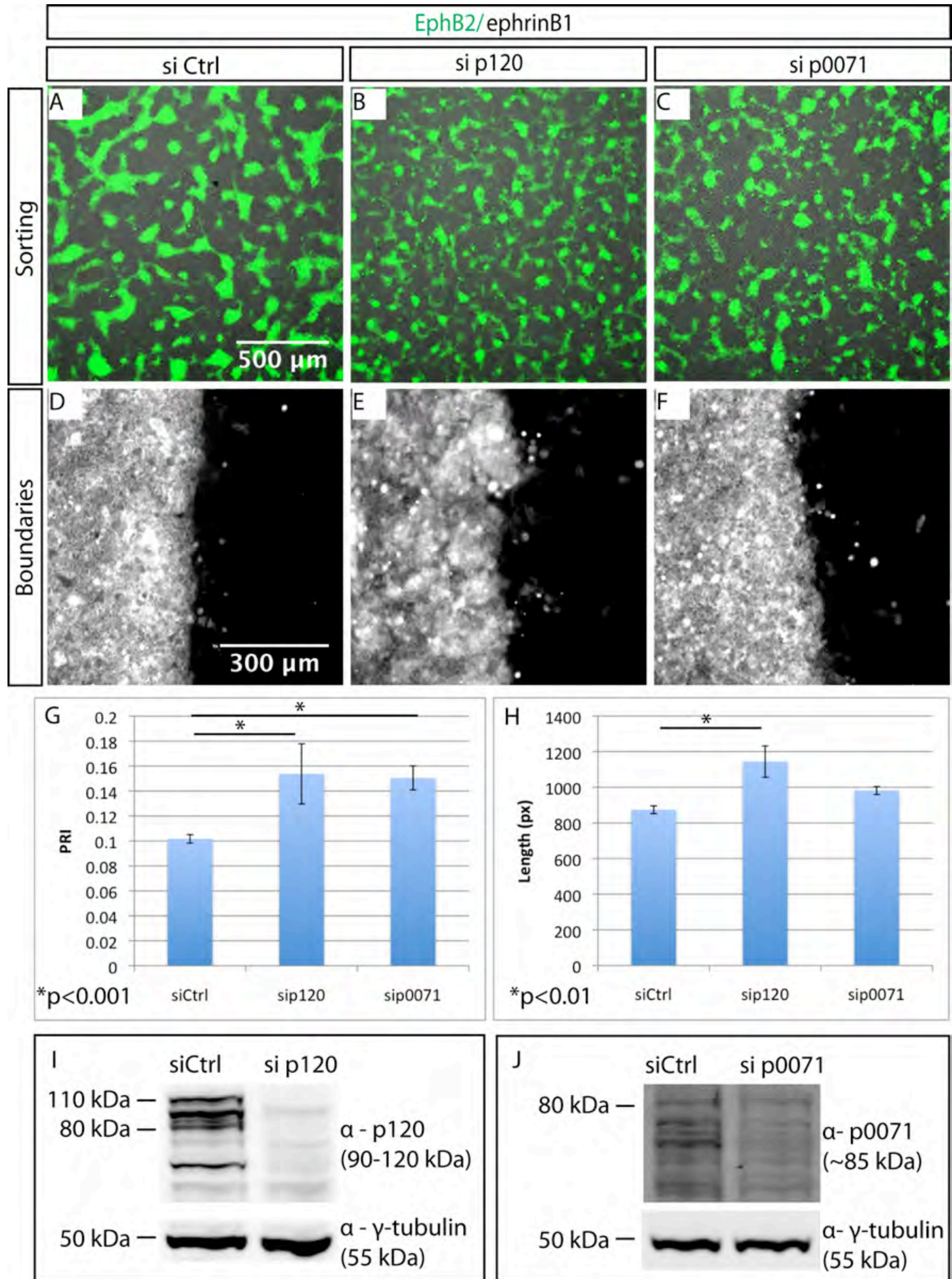


Figure 4.9: p120 and p0071 effects on sorting and boundary sharpness

(A-C) EphB2-ephrinB1 sorting is disrupted by siRNA knockdown of p120 or p0071. The perimeter regularity index (PRI) of these conditions is significantly higher than when a nonsense control siRNA is used (G; Student's t-test, $p<0.001$). (D-E, H) siRNA knockdown of p120 also disrupts boundary sharpness, significantly increasing boundary length above the control. p0071 does not have this effect and boundaries are not significantly longer than the control. px: pixels. (I,J) Western blot analysis showing effectiveness of siRNA knockdown of p120 ($84\pm12\%$ knockdown) and p0071 ($74\pm12\%$ knockdown). Knockdown is measured compared to a control siRNA and relative to γ-tubulin, which is used as a loading control. Error bars represent standard error of the mean.

Figure 4.9



knocked down alone. p0071 levels were not checked in these experiments due to a lack of available antibody.

When siNcad & siCtrl or p120 & siCtrl were transfected into EphB2 and ephrinB1 cells, they disrupted sorting and boundary sharpness to similar extents (Figure 4.10 C, D, K, L). A similar phenotype is seen when siNcad & sip120 are transfected together in the same cells (Figure 4.10 F, N, Q, R). This supports the idea that N-cadherin and p120 are working in the same pathway to contribute to sorting consistent with p120 being required for N-cadherin stability (see Figure 4.8).

Treating EphB2 and ephrinB1 cells with sip0071 & siCtrl has very little effect on sorting or boundary sharpness (Figure 4.10 E, M, Q, R). This discrepancy with previous results is probably due to a lower level of knockdown when sip0071 is used in combination with siCtrl, since less of each siRNA is used in this situation. In contrast to this result, transfection of sip0071 in combination with either siNcad or sip120 greatly disrupts sorting and boundary sharpness (Figure 4.10 G, H, O-R). This indicates that p0071 is acting in a different pathway to both p120 and N-cadherin and that both pathways are necessary for segregation between EphB2 and ephrinB1 cells.

Adam10 is not necessary for sorting or boundary sharpening

A recent study from the Batlle lab has proposed a differential adhesion mechanism for EphB-ephrinB sorting by the regulation of E-cadherin via Adam10 (Solanas *et al.*, 2011). Adam10 is a trans-membrane metalloproteinase which, they demonstrate, cleaves E-cadherin on EphB activation. They propose that this sets up decreased adhesion at the site of EphB-ephrinB cell contact and that this difference in adhesion can drive sorting. It was important to test whether such a mechanism could be involved in our cell system as well.

I attempted to abrogate the expression of Adam10 using three different approaches. Firstly, I used siRNAs to Adam10 to knock down its expression, and carried out the segregation and boundary assays (Figure 4.11 A-H). Using this approach, knockdown of Adam10 did not disrupt either sorting or boundary sharpness. I quantified the level of knockdown of the protein by Western blot, as described for previous siRNAs. However, the antibody detected many non-specific

Figure 4.10: Knockdown of combinations of N-cadherin, p120 and p0071 enhance cell sorting abrogation phenotypes

EphB2-ephrinB1 segregation (EphB2 cells and brightfield; A-H, R) and boundary (EphB2 cells; I-P, Q) assays in which N-cadherin, p120 or p0071 are knocked down by siRNA. siRNA knockdown of N-cadherin, p120 or p0071 combined with siCtrl disrupts cell sorting by EphB2 receptor and ephrinB1 (A-E, I-N). Combining siNcad and sip120 does not disrupt sorting further than either of these siRNAs used alone (F, N). p0071 knocked down with N-cadherin or with p120 increases disruption of sorting and boundary sharpness (G,H, O, P). Error bars represent standard error of the mean.

Figure 4.10

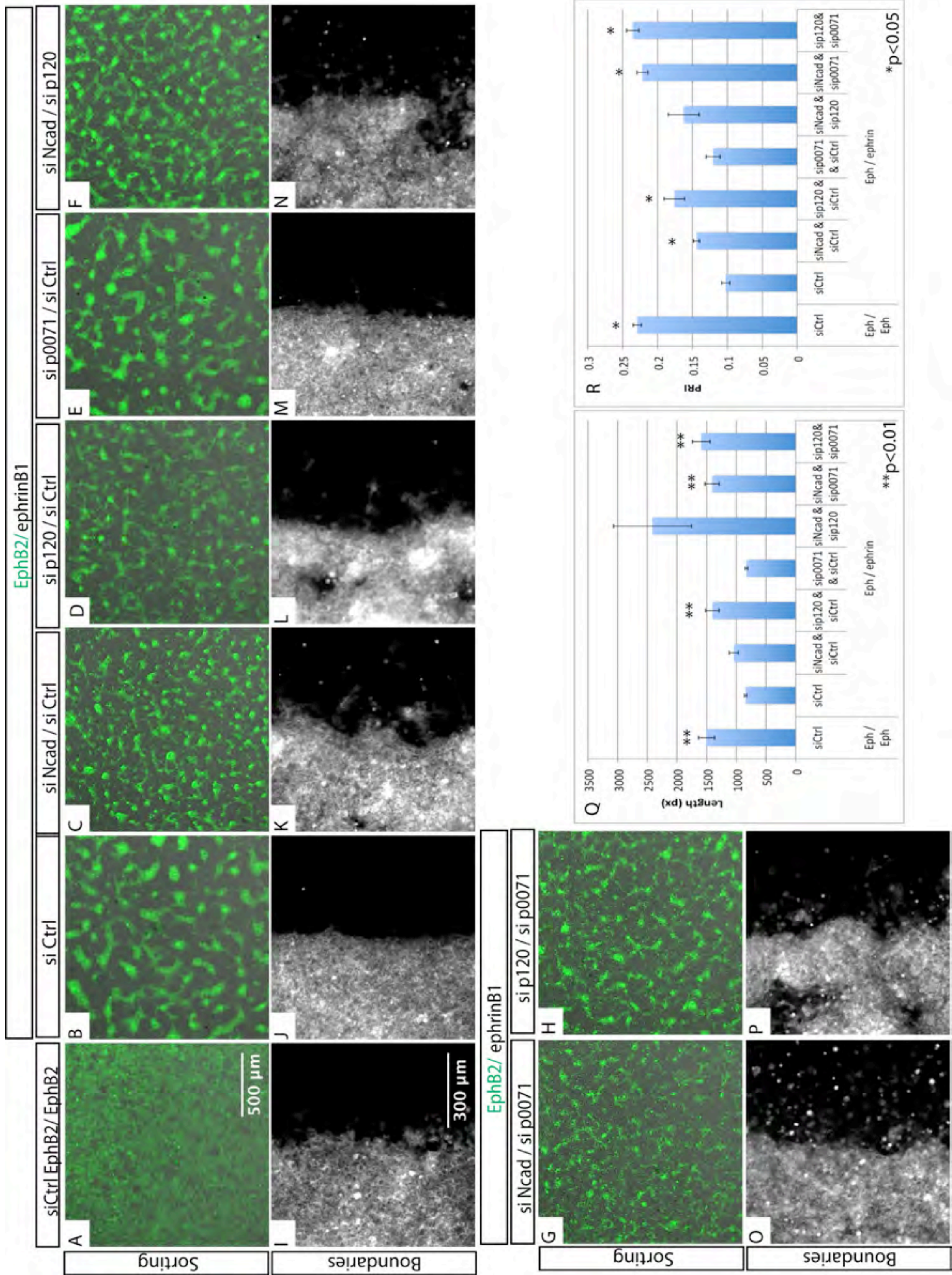


Figure 4.11: Adam10 does not affect EphB2-ephrinB1 cell sorting in HEK293 cells

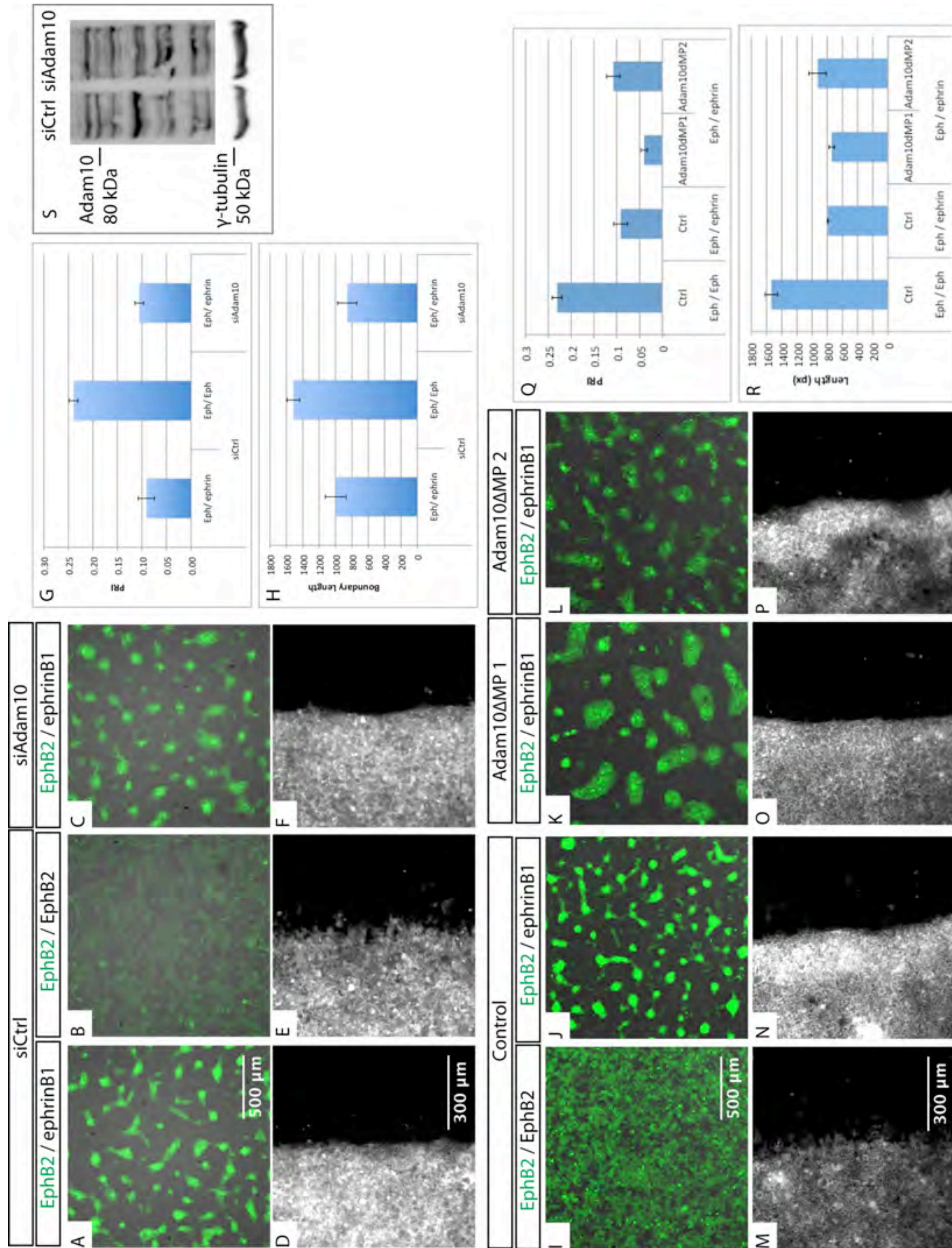
(A-H) Cell segregation (green EphB2 cells and brightfield) and boundary (green EphB2 cells) assays. Treatment of cells with Adam10 siRNA does not perturb sorting or boundary sharpening between EphB2 and ephrinB1 cells (C,F).

(I-R) Expression of a dominant negative Adam10 (Adam10ΔMP) does not disrupt signalling. Several stable cell lines were generated which expressed Adam10ΔMP to similar levels. Examples where two different Adam10ΔMP-expressing EphB2 lines were mixed with two different ephrinB1 lines are shown (K,L,O,P).

(S) Western blot analysis showing knockdown of Adam10 by siRNA relative to a γ-tubulin loading control. The knockdown of the 85kDa band, corresponding to the correct size of Adam10 protein is 80±27% (n=3) by mean intensity measurement.

Error bars represent standard error of the mean.

Figure 4.11



bands, and varying levels of knockdown were measured (average knockdown 80±27%, n=3; Figure 4.11 S). Since the knockdown of Adam10 was not 100% efficient, it was possible that any remaining protein was able to compensate for incomplete siRNA knockdown.

Another approach was to express a dominant negative version of Adam10, which lacks the metalloproteinase domain, rendering it catalytically inactive (Adam10ΔMP; Lemieux *et al.*, 2007). No effect could be seen on segregation or boundary sharpening when the construct was expressed in transient transfections (data not shown). To create a uniform population of cells expressing Adam10ΔMP, I generated stable cell lines expressing this dominant negative Adam10 and tested several which expressed the protein to similar levels (Figure 4.11 K-P). There is some variability in the size and shape of clusters, likely due to selection of different levels of EphB2 or ephrinB1 in the different stable lines. However, the PRI and boundary length are similar to those for cells which do not express the dominant negative Adam10 protein (Figure 4.11 Q,R). The expression of dominant negative Adam10 did not have any effect on sorting in hanging drop assays either. Importantly, reduced sorting was not seen with any of the Adam10ΔMP expressing cell lines in any of the segregation assays.

The third strategy was to use a broad-spectrum metalloproteinase inhibitor, TAPI-1, to see if any disruption to sorting could be seen. Even when used at 20μM (twice the concentration used by Solanas *et al.*), there is no disruption to segregation or boundary sharpness (data not shown).

Since none of these methods of abrogating Adam10 function showed a significant effect on Eph-ephrin sorting, I decided to investigate whether cadherin is cleaved downstream of EphB2-ephrinB1 signalling. Using an antibody to the extracellular domain of N-cadherin, I performed immuno-precipitation on the media in which EphB2-ephrinB1 cell mixtures had been cultured. This should concentrate any extracellular cleaved N-cadherin. When run on an SDS-PAGE gel and subjected to Western blot analysis, no band could be detected which was different between EphB2-EphB2 and EphB2-ephrinB1 cell mixtures (data not shown). This was possibly due to the EC-N-cadherin band running close to the antibodies used for IP. Another method of detecting cleaved cadherin is to look at the intracellular

cleavage products of cadherin. When N-cadherin is cleaved extra-cellularly, it will subsequently be cleaved intra-celluarly, separating it from the membrane and giving rise to a 35kDa fragment. This could be detected using an anti-C-terminal N-cadherin antibody. When treated with the Adam metalloproteinase inhibitor TAPI, there was an increase in the intensity of this 35kDa band in EphB2 cells, suggesting that the inhibitor is effective at preventing cadherin cleavage through Adam10. However, this band could not be detected in EphB2-ephrinB1 cell mixtures. These results suggest that there is no detectable increase in cadherin cleavage in mixtures of HEK293 cells expressing EphB2 receptor or ephrinB1.

Taken together, these experiments show that Adam10 cleavage of cadherin does not play an important role in EphB2-ephrinB1 sorting in this system.

Discussion

Cadherin mediated adhesion is required for EphB2 receptor and ephrinB1 cell sorting and boundary sharpening

The results described in this chapter show that siRNA knockdown of N-cadherin in EphB2 and ephrinB1 cells disrupts their normal pattern of sorting in segregation, boundary and hanging drop assays. This indicates that cadherin mediated adhesion is required for EphB2-ephrinB1 signalling to drive efficient cell sorting. Moreover, known downstream targets of EphB2 include regulators of cadherin-mediated adhesion, p120 and p0071. Despite being reported to have an important role in assays with other cell types, Adam10 cadherin cleavage does not seem to be necessary in this sorting system.

N-cadherin could be required in EphB2-ephrinB1 mediated cell sorting for one or more of three reasons. Firstly, it could simply be required for cohesion between cells, which is required to allow EphB2 and ephrinB1 cells to sort. Secondly, it could be regulated by EphB2–ephrinB1 signalling to set up differential adhesion between cells, which could assist in sorting. Thirdly, it could be important upstream of EphB2 to facilitate signalling. Each of these predicted roles is discussed below.

Model 1: Cadherin is required for cell-cell adhesion. Eph-ephrins drive sorting through another mechanism.

Knockdown experiments demonstrate that cadherins are required for Eph-ephrin driven sorting (Figure 4.1; Cortina *et al.*, 2007). The experiments presented here suggest that a major function of cadherins is to mediate cohesion between cells, which re-enforces segregation, but that sorting is driven by cadherin-independent mechanisms.

When N-cadherin is knocked down, segregation is disrupted. However, under these conditions in the segregation assay, clusters of EphB2 cells are still formed, indicating that sorting is taking place in the absence of N-cadherin. The reduction of N-cadherin is more than 95% efficient so any effect of the residual protein is likely to be negligible. A likely explanation is that another, cadherin-independent mechanism drives segregation of cells downstream of EphB2-ephrinB1 signalling (see Chapter 5).

Segregation and boundary assays where N-cadherin was knocked down in either EphB2 cells or ephrinB1 cells support the idea that the cadherin is mostly required for cohesion between cells. When N-cadherin was reduced in EphB2 cells only, more, smaller clusters of EphB2 cells formed, consistent with a requirement for cohesion in stabilising these clusters. Similarly, when N-cadherin was reduced in ephrinB1 cells only, these cells were more frequently interrupted by connections between clusters of EphB2 cells. This is consistent with a cadherin-independent role of EphB2-ephrinB1 signalling in segregating cells, but a requirement for cadherins in stabilising contacts between segregated cells.

The idea that the regulation of cadherins is not a principal mechanism for cell sorting through Eph-ephrins is supported by immunochemical staining of EphB2 cells and ephrinB1 cells at early stages of segregation. After 5h in co-culture, EphB2 cells have begun to segregate from ephrinB1 cells, yet cadherin staining is the same between the two cell types and there is no variation in cadherin localisation within any given cell. Cadherins may be upregulated within clusters at a later stage of sorting, since cells in the centres of large clusters have enriched cadherin staining. This could be a specific mechanism downstream of Eph-ephrin signalling to aid in the maintenance of segregation. It could also be a secondary

effect due to cluster formation. EphB2 cells in the middle of clusters appear more densely packed together and their shapes change in accordance with that. The increase in staining between them could be a result of this shape change. In accordance with this, when a change in cell shape occurs after stimulation of EphB2 cells with ephrinB1-Fc, there is an increase in cadherin staining. However, this is accompanied by an increase in the staining of other membrane-bound proteins such as EphB2 (Figure 4.2).

The strength of cadherin mediated adhesion increases with increased time of cell contact (Ehrlich *et al.*, 2002; Yamada & Nelson, 2007), so another possibility is that EphB2 cells which have formed clusters could have increased cadherin at their surfaces through this mechanism. This would also account for the fact that cadherin increases throughout the cluster even if the cluster is many cells in diameter and the EphB2 cells in the centre are not in direct contact with ephrinB1 cells or directly responding to ephrin signalling. I therefore propose that cadherins are upregulated between cells in EphB2 clusters, but the initial stages of EphB2-ephrinB1 sorting are independent of any detectable cadherin re-localisation.

One way that Eph-ephrins are likely to be influencing cell sorting without regulating cell-cell adhesion is through cell repulsion. Repulsion of Eph-expressing cells has been shown to occur on contact with ephrin-expressing cells in cell culture assays (Poliakov *et al.*, 2008). Unpublished modelling data from the Wilkinson lab has suggested that repulsion, in the form of persistent migration after cell collisions, can drive cells to sort. Computer simulations based on parameters taken from the HEK293 cell system (e.g. speed of cell migration, length of time in contact with one another) demonstrate that repulsion of one cell type from another is sufficient for sorting. Interestingly, sorting by repulsion in this model is reliant on a baseline ability for cells to stick to one another, consistent with a requirement for cadherin to facilitate cohesion in Eph-ephrin segregation (see Chapter 6).

This data supports the idea that N-cadherin is required to provide basal cell-cell cohesion, which is needed for EphB2 and ephrinB1 cells to sort.

Model 2: Regulation of N-cadherin generates differential adhesion

A main role of cadherins in Eph-ephrin sorting may be to facilitate cohesion, which can stabilise the sorting pattern. However, differential adhesion may still represent an important mechanism through which Eph-ephrins mediate segregation. In support of this, p120, which has a well-described role in regulating adhesion, and its close family member p0071, are regulated downstream of EphB2 activation. This suggests that p120 or p0071 could mediate mechanisms through which adhesion is differentially regulated in EphB2 cells.

Model 2a: Regulation of N-cadherin via p120 generates differential adhesion between EphB2 and ephrinB1 cells

Knockdown of p120 and N-cadherin together disrupts sorting to the same extent as knockdown of each on its own, suggesting that they function in the same pathway in EphB2 and ephrinB1 cell sorting. Furthermore, p120 expression is required for normal protein expression of N-cadherin. Could p120 be downstream of Eph-ephrin signalling to modulate N-cadherin expression on the cell surface, resulting in differential adhesion?

p120 is generally considered to be required for cadherin-mediated adhesion. Its binding to the juxtamembrane domain of E-cadherin is required and sufficient to recruit E-cadherin to the membrane (Thoreson *et al.*, 2000; Yap *et al.*, 1998). Furthermore, decreased levels of p120 result in rapid degradation of cadherins on delivery to the cell surface (Davis *et al.*, 2003; Xiao *et al.*, 2003). Interestingly from the point of view of its control by Eph receptor activation, p120 activity is regulated by phosphorylation of serine/threonines and tyrosines in the regulatory domain of the protein (Reynolds, 2007); Chapter 1, Figure 1.7). Tyrosine-phosphorylated p120 binds cadherin preferentially than the unphosphorylated version, suggesting a role in the stabilisation of E-cadherin at the cell surface (Mariner *et al.*, 2004). This is consistent with the observation that there is a transient increase of tyrosine-phosphorylated p120 at nascent adhesive contacts (Calautti *et al.*, 1998; Calautti *et al.*). However, phosphotyrosine defective mutants are also able to efficiently bind and stabilise E-cadherin at the cell surface (Mariner *et al.*, 2004; Xiao *et al.*, 2003), which suggests that tyrosine phosphorylation is not universally required for p120 to stabilise E-cadherin.

The large-scale screens identifying phosphorylation targets of EphB2-ephrinB1 signalling demonstrate that Y217, Y228, Y257 and Y904 have consistently *decreased* phosphorylation in EphB2 cells mixed with ephrinB1 cells but *increased* phosphorylation in EphB2 cells stimulated with *soluble* ephrinB1-Fc (Jorgensen *et al.*, 2009). This difference in response to different activating ligands could be due to differences in the level of clustering between Eph receptors when stimulated with soluble versus membrane-bound ligands. The degree of clustering of receptor-ligand complexes has been shown to affect the signalling response of the Eph receptor (Huynh-Do, 1999). The response to membrane-bound ligand represents a more physiological scenario, suggesting that p120 phosphorylation decreases in EphB2 cells on contact with ephrinB1 cells.

Since EphB2 is a kinase, it must depend on an intermediate target to dephosphorylate p120. Direct targets of EphB2 which would be capable of regulating p120 phosphorylation include Shp1 (Keilhack *et al.*, 2000) and Src (Mariner *et al.*, 2001; Reynolds *et al.*, 1989). Shp1 and Src have both been shown to directly interact with p120. Specifically, Src has been shown to phosphorylate Y217 and Y228 of p120 (Castano *et al.*, 2007). Shp1 and Src are therefore likely intermediates in the regulation of p120 downstream of EphB2 activation.

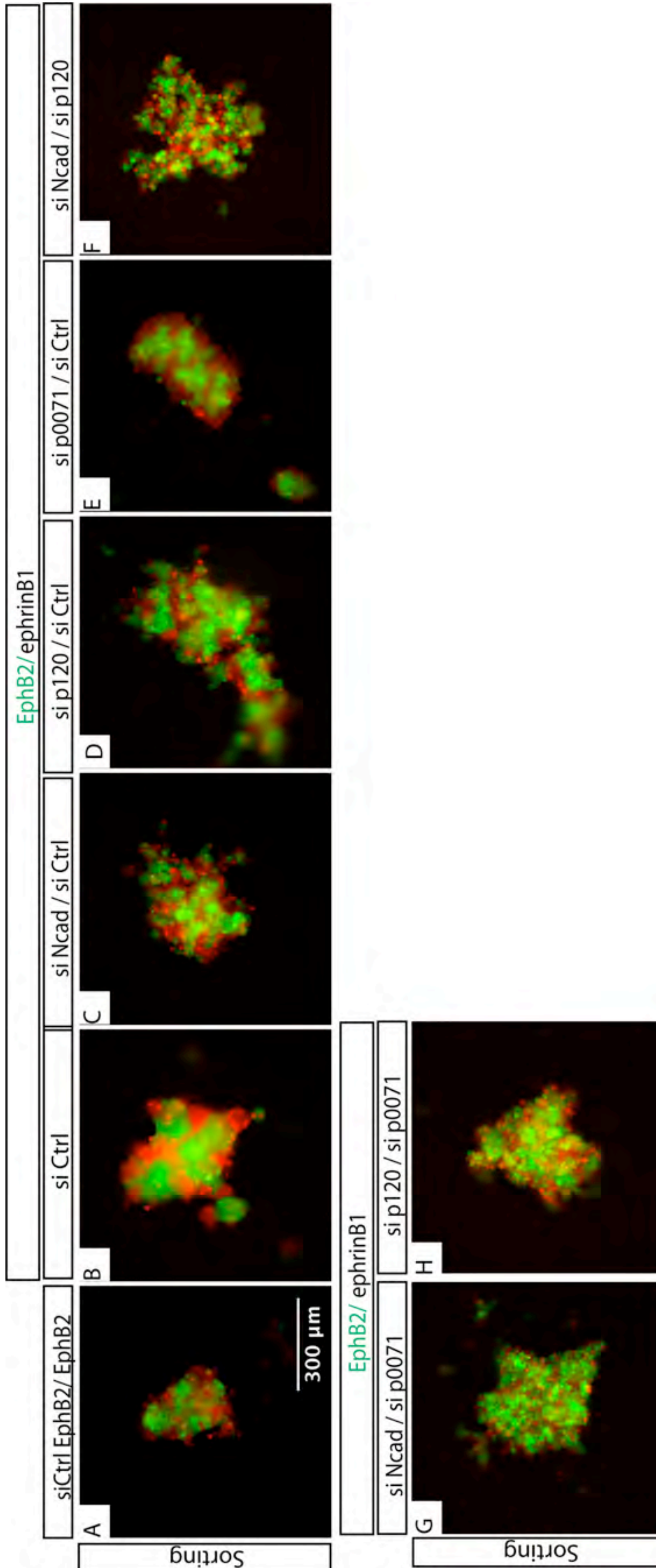
Sorting is not more disrupted when siNcad and sip120 are mixed in the segregation or boundary assays, compared to either siNcad or sip120 alone. However, there is an additive phenotype of siNcad and sip120 in hanging drops (Figure 4.12). This highlights the different sensitivities of the hanging drop and sorting assays. Cell rearrangement in the hanging drop assay is dependent only on interactions of cells with the medium or with other cells. By contrast, the segregation and boundary assays rely on cell interactions with the cell culture dish as well as between cells and the medium. This may render the segregation and boundary assays more suitable to detecting changes in migration-dependent segregation, which are not detected in the hanging drop assay, and less sensitive to differences in adhesion between cells (see Chapter 3).

The strong phenotypes in the segregation assay of each of N-cadherin and p120 knockdown are consistent with their key roles in cell-cell adhesion. The increased strength of phenotype when siRNAs to the two genes are combined in the hanging

Figure 4.12: Knockdown of combinations of N-cadherin, p120 and p0071 disrupt cell sorting in hanging drops

EphB2-ephrinB1 segregation in hanging drops (green EphB2 cells and red ephrinB1 cells) in which N-cadherin, p120 or p0071 are knocked down by siRNA. siRNA knockdown of N-cadherin, p120 or p0071 combined with siCtrl disrupts cell sorting by EphB2 and ephrinB1 (A-E, I-N). Combining siNcad and sip120 or p0071 in any combination of two greatly increases disruption of sorting and boundary sharpness (F-H).

Figure 4.12



drop assay is most easily explained by the fact that each gene is knocked down incompletely in this experiment. When N-cadherin is knocked down incompletely, the remaining protein could still function in combination with normal levels of p120. If the pool of available p120 is also reduced, this could reduce the formation of stable N-cadherin-p120 complexes.

In summary, N-cadherin and p120 act in the same pathway to regulate cell-cell adhesion, which is required for EphB2-ephrinB1 cell sorting. p120 is regulated by Eph receptor activation. However, it is still not clear that the requirement for N-cadherin and p120 in segregation by Eph-ephrins is due to a general need for the adhesive properties of these molecules or because they are regulated by Eph-ephrin signalling.

Model 2b: Eph-ephrin signalling activates Adam10 to generate differential adhesion between cells

Evidence for this model comes from experiments on colorectal cancer cells in which E-cadherin is cleaved at the interface of EphB-ephrinB cells by activation of Adam10 metalloproteinase (Cortina *et al.*, 2007; Solanas *et al.*, 2011). Cortina *et al.* also show an increase in E-cadherin staining at the membranes of EphB2 cells after stimulation with ephrinB1-Fc (Cortina *et al.*, 2007), suggesting that EphB2 acts to increase adhesion between cells (Figure 4.13). Collectively, this would set up a difference in adhesion between EphB2 cells, which stick more strongly together, and ephrinB1 cells, which cannot stick to EphB2 cells.

In HEK293 cells, there is also an increase in cadherin intensity at the membrane of EphB2 cells after ephrinB1-Fc stimulation (Figure 4.2). There is also an accumulation of cadherin at the membranes between EphB2 cells after they have formed clusters, although not at the early stages of clustering (Figures 4.2, 4.3), as discussed above.

Despite extensive efforts to abrogate the function of Adam10, there seems to be no effect of this on the level of sorting of HEK293 cells by EphB2-ephrinB1 signalling. This could be for two reasons: either the reagents are not efficiently blocking Adam10 function; or Adam10 does not have a role in EphB2-ephrinB1 sorting in HEK293 cells.

Figure 4.13: Model for differential adhesion through EphB-ephrinB signalling

Model suggesting a mechanism through which differential adhesion is generated by EphB-ephrinB activity in epithelial cells (Solanas *et al.*, 2011). EphB-expressing cells respond to ephrinB signalling by: a) activating ADAM10 at the point of contact, which cleaves E-cadherin, decreasing adhesion; b) a relative increase in adhesion between EphB-expressing cells, resulting in stable associations between these cells.

Figure 4.13

Initially, Adam10 was knocked down by siRNA. The detected level of Adam10 protein knockdown by siRNA is much less than 100%, either because the siRNA is inefficient, or because the antibody binds non-specifically to other proteins. If there is not complete knockdown of the protein, the remaining Adam10 could be sufficient to carry out its normal function.

A second approach used to abrogate Adam10 function was to express high levels of a metalloproteinase-deficient Adam10 (Adam10ΔMP) which has been shown to act as a dominant negative (Lemieux *et al.*, 2007), but expression of this did not disrupt sorting either. One possibility is that the construct was expressed at too low a level, which was insufficient to completely abrogate endogenous Adam10 activity. However, Adam10ΔMP is strongly detected in the cells by both immunocytochemistry and Western blotting suggesting this is not the case.

Thirdly, a more general approach was to treat the sorting cells with the Adam inhibitor TAPI-1. This had no effect on Eph-ephrin segregation, when it was added once at the beginning of the experiment, from the point the cells are mixed. However, TAPI-1 can lose activity after 12h (Guiomar Solanas, personal communication). To address this, I added TAPI-1 to the segregating cells every 12h throughout their incubation. This still had no effect on cell segregation (data not shown).

These three different methods were used to abrogate Adam10 function, and still segregation of EphB2 and ephrinB1 cells was not disrupted. Therefore, it is likely that Adam10 is not playing a role downstream of EphB2-ephrinB1 sorting in these cells.

The cells in which the role of Adam10 was discovered are epithelial colorectal cancer cells, where Adam10 is able to cleave E-cadherin downstream of EphB2 or EphB3. By contrast, HEK293 cells express N-cadherin rather than E-cadherin. This difference in the type of cadherin cannot be the sole reason for the difference in results, since Adam10 has been shown to cleave both proteins (Uemura *et al.*, 2006). Another difference between the cells could be their motility. Cells in epithelia tend not to change position a lot, relative to one another (Lecuit, 2010),

but HEK293 cells are very motile and are frequently making and breaking contacts with one another. Consistent with this is the fact that HEK293 cells express N-cadherin which is commonly associated with increased cell motility. The Adam10 mechanism was proposed based on experiments using E-cadherin expressing, epithelial cells. It is likely to be more important in epithelial tissues in which adhesions between cells are strong and can prevent them from moving, and less important in cell types which are constantly able to break contact with one another.

Model 3: Cadherins potentiate Eph-ephrin signalling

There is some evidence that E-cadherin is required upstream of EphA2 activation (Zantek *et al.*, 1999). The fact that EphB2 is still tyrosine phosphorylated in response to ephrinB1 ligand, even in the absence of N-cadherin, demonstrates that there is not a cell autonomous requirement for N-cadherin to facilitate EphB2-ephrinB1 signalling (Figure 4.7). From this experiment, however we cannot tell whether N-cadherin is required to assist interactions between cells. It is possible that N-cadherin could be required to help cells come into contact and allow signalling to occur. However, Eph receptors and ephrins have high affinities for one another, so it is very likely that they could allow cells to interact even in the absence of cadherin-mediated adhesion. Furthermore, N-cadherin knockdown actually results in an increase in Eph receptor phosphorylation. One possibility is that N-cadherin presence at the membrane may exclude Eph-ephrin interactions. Another possibility is that N-cadherin knockdown decreases contacts between cells, increasing the surface area of the cell on which EphB2 is exposed to the ephrinB1-Fc ligand. It would be interesting to repeat the staining for phospho-tyrosine using mixtures of EphB2 and ephrinB1 cells, rather than a soluble ligand, to address these possibilities. From these data, N-cadherin is not required upstream of EphB2-ephrinB1 signalling to mediate cell sorting.

The potential role of p0071

I chose to investigate the function of p0071 in Eph-ephrin segregation as it is regulated downstream of EphB2 activation, and because of its similarity to p120 in both structure and known function. p0071 binds classical cadherins and co-

localises with β-catenin at adherens junctions (Hatzfeld *et al.*, 2003; Hofmann *et al.*, 2008). It has been suggested that it has similar roles in linking cadherins to the cytoskeleton (Hatzfeld, 2007) as well as in the regulation of RhoGTPase activity (Wolf *et al.*, 2006). It was originally thought to localise to desmosomes, so it might play an analogous role to that of p120 at adherens junctions by regulating desmosomal cadherins at desmosomes, although this is under some dispute (Hatzfeld *et al.*, 2003; Hofmann *et al.*, 2008).

Unexpectedly, knocking down p0071 has a very different effect than knockdown of p120 on EphB2-ephrinB1 segregation. When knocked down on its own (Figure 4.9 C,F), p0071 has only a small effect on sorting and no effect on boundary sharpening, suggesting that it is either unimportant for sorting or that its activity is compensated by similar proteins such as p120. However, when knocked down in combination with p120 or N-cadherin, there is a striking additive effect and sorting is almost completely prohibited (Figure 4.10 Q,R). p0071 co-knockdown with N-cadherin or p120 decreases sorting in hanging drops, consistent with the 2D sorting results (Figures 4.12, 4.9). This could suggest that p0071 is acting independently of N-cadherin and that both together are vital for efficient sorting through EphB2 and ephrinB1.

Cadherin staining of EphB2 cells in which p0071 has been knocked down demonstrates that fewer cadherin-enriched junctions form between these cells. These results are consistent with the possibility that p0071 is required for normal cell-cell adhesion. One possibility is that p0071 could be regulating adhesion by influencing the protrusive activity of the cell membrane. A more dynamic membrane would be less able to form stable interactions, so would render the cell less adhesive and vice versa. This would fit with the observed role of p0071 in cytoskeletal dynamics through RhoGTPase regulation (Wolf *et al.*, 2006).

In summary, p0071 is required for normal sorting in the absence of N-cadherin or p120, though knockdown of p0071 alone has a mild segregation phenotype. Since p0071 is required for sorting in hanging drop assays, it is likely to be involved in a cell contact-dependent process, possibly by contributing to N-cadherin regulation and possibly by the dynamic regulation of the cytoskeleton.

Conclusions

In this chapter, I have shown that cadherins are necessary for cell sorting by Eph-ephrins. They are important in mediating cohesion between cells, which facilitates sorting. p120, which is found downstream of EphB2 signalling, is also required for EphB2-ephrinB1 sorting as a regulator of cadherin stability. p0071, a p120 family protein, is also found downstream of EphB2 and is also important in EphB2-ephrinB1 cell segregation possibly through a mechanism independent of p120 or N-cadherin. However, it is still unclear if the requirement for these proteins in EphB2-ephrinB1 cell segregation extends beyond their ability to mediate cohesion. This could be cell-type dependent and the requirement for regulation of differential adhesion could be more important in E-cadherin expressing cells. Another approach to this question is to investigate alternative mechanisms which could drive segregation through EphB2-ephrinB1 signalling. How N-cadherin, p120 and p0071 affect cell migration downstream of Eph-ephrin signalling will be discussed in the following chapter.

5. <u>Links between motility and adhesion</u>

Cell movement is key to segregation by Eph receptors and ephrins

Contact inhibition of locomotion is a common characteristic of cells where, upon contact, they cease to migrate forwards and re-orient to migrate away from the collision. It was first identified through the observation that cells plated in a dish tend to form a monolayer (Abercrombie, 1970) and has also been demonstrated to be required *in vivo* for neural crest cells to migrate coherently (Theveneau *et al.*, 2010). Recent evidence has shown that N-cadherin is required for contact inhibition of locomotion (Theveneau *et al.*, 2010). Therefore, in addition to their primary role in cell-cell adhesion, there is also evidence suggesting a role for cadherins in the regulation of cell motility.

It has been suggested that p120 could be involved in the process of contact inhibition of locomotion (Mayor & Carmona-Fontaine, 2010). p120 family molecules are generally thought to activate Rac and inhibit RhoA, supporting a role for p120 in promoting cell migration (Anastasiadis, 2007; Hatzfeld, 2007). One possibility is that cadherin binding prevents this activity at the point of cell-cell contact, so p120 is only active at the other side of the cell, facilitating Rac activation and protrusion formation and re-directing migration. It has been postulated that p120 family members act as the rheostat of adhesion and motility and that the release of p120 from cadherins could underlie EMT (Grosheva *et al.*, 2001; McCrea & Gu, 2010).

Eph receptors and ephrins can also regulate cell motility. Eph-ephrins have a well-characterised role in axon guidance, and their complementary expression in neurons and the underlying tissue generates repulsive cues which direct the axon to the correct path. For example, they play key roles in directing commissural axons across the midbrain (Henkemeyer *et al.*, 1996), and motor axons to the limb (Helmbacher *et al.*, 2000). Repulsion mediated by Eph-ephrin signalling can be seen directly *in vitro*, characterised by a collapse response of axonal growth cones (Jurney *et al.*, 2002; Monschau *et al.*, 1997). Such repulsion has been proposed to underlie cell segregation by Eph receptors and ephrins (Cooke *et al.*, 2005;

Poliakov *et al.*, 2008). Recent work by Alexei Poliakov has shown that EphB2 cells display not only a collapse response, but also they migrate with more directional persistence after contact with ephrinB1 cells. Computer modelling has suggested that this change in cell migration could be sufficient to explain cell sorting (manuscript in preparation).

In the previous chapter, I demonstrated how N-cadherin, p120 and p0071 are required for EphB2-ephrinB1 mediated cell sorting to occur, and discussed the effects they might have in relation to cell-cell adhesion. Given the potential for these proteins to regulate cell migration, and the suggestion that cell repulsion could be the driving force for sorting, I investigated whether any of these three molecules were involved in the regulation of migration in EphB2 cells.

EphB2-ephrinB1 signalling affects directional persistence

The behaviour of EphB2 cells after collision with ephrinB1 cells was assessed using time-lapse imaging and cell tracking. Alexei Poliakov obtained data from time-lapse movies of EphB2 cells after interaction with ephrinB1 cells. The cells were labelled with green (EphB2) and red (ephrinB1) fluorescent dyes, plated at low density on fibronectin-coated glass plates and imaged every 2 mins for 5 h. The cells were tracked using single particle tracking software GMimPro (Mashanov & Molloy, 2007) (Figure 5.1 A). Tracks of green cells were selected which lasted 1 h from the point of interaction with a red cell, without colliding with any further cells. These tracks were then pooled and used to calculate various parameters (Figure 5.1 B-H).

Initially, Alexei used GMimPro to calculate the mean squared displacement (MSD) of the tracks. MSD is the square of the straight-line distance between a cell's position at a given time interval (Figure 5.1 B). The MSD curve for EphB2 cells after collision with ephrinB1 cells (red line) is steeper than the MSD curve for EphB2 cells after interaction with EphB2 cells (blue line), resulting in a 200 μm^2 difference in MSD between the conditions at 25 min intervals. The MSD curve for pooled tracks of all EphB2 cells regardless of collisions is shown in green as a control. MSD is proportional to the squared speed of cells and the persistence with which they move, where persistence is defined as the average time period between

Figure 5.1: Increased directional persistence in EphB2 cells after contact with ephrinB1 cells

EphB2 cells and ephrinB1 cells were labelled green and red respectively and mixed at low density. (A) Time-lapse movies of these cells were tracked using automated tracking software, GMimPro. White lines show tracks of EphB2 cells (green) and yellow lines show the tracks of ephrinB1 cells (red) over the 7 h time-lapse movie. (B) Mean squared displacement (MSD) analysis of EphB2 cell tracks after interaction with EphB2 cells (blue line) or ephrinB1 cells (red). The green line shows the MSD of all tracks, regardless of interaction. MSD is higher after EphB2-ephrinB1 interactions than after EphB2 cells collide with EphB2 cells. The MSD of full-length tracks is similar to EphB2-EphB2 cell collisions. (C-E) EphB2 cells move further and more directionally after interaction with EphB2 cells than with ephrinB1 cells. XY coordinates of EphB2 cells after interaction with an EphB2 cell (C) or an ephrinB1 cell (D). 50 tracks picked at random are plotted on a common origin. (E) The endpoints, the XY coordinates of cells 25 min after interaction, are plotted on a common origin. The solid line represents the root-mean-square of these coordinates (red, after ephrinB1 interaction; blue after EphB2 interaction). (F) The probability distribution function (PDF) of turning angles of the tracks. The deviation from a straight line (turning angle) between two consecutive 5 min intervals was calculated for every possible combination of consecutive 5 min intervals in all tracks. There is a narrower distribution of turning angles in tracks of EphB2 cells after an ephrinB1 cell collision than after an EphB2 cell collision. (G,H) Values of speed (G) and persistence (H) derived from MSD curves for cells after interactions. EphB2 cells have higher persistence after contact with ephrinB1 cells (red) than EphB2-EphB2 or ephrinB1-ephrinB1 collisions (grey), but move at a similar speed. Raw data from Alexei Poliakov. Error bars represent standard error of the mean.

Figure 5.1

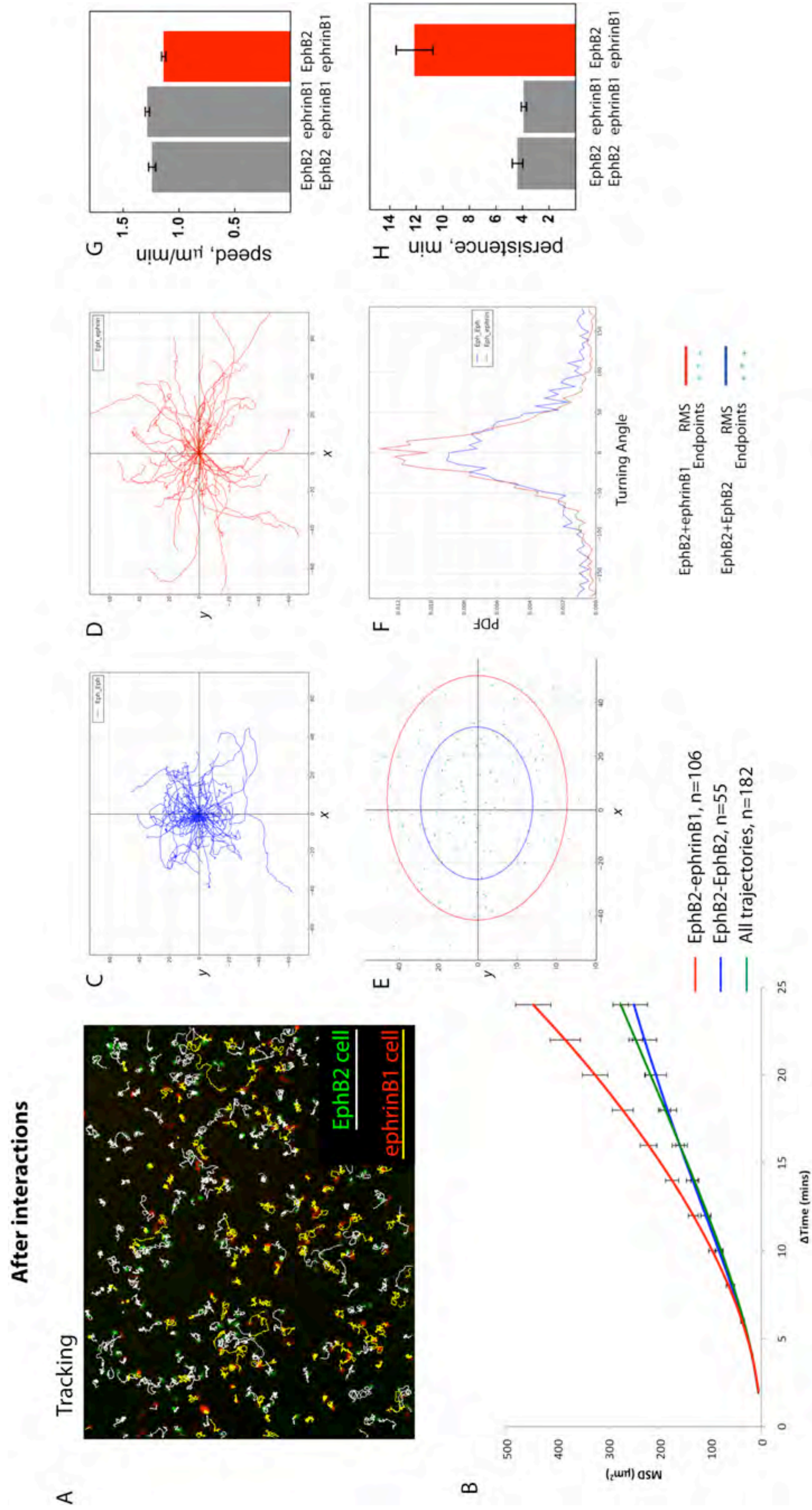
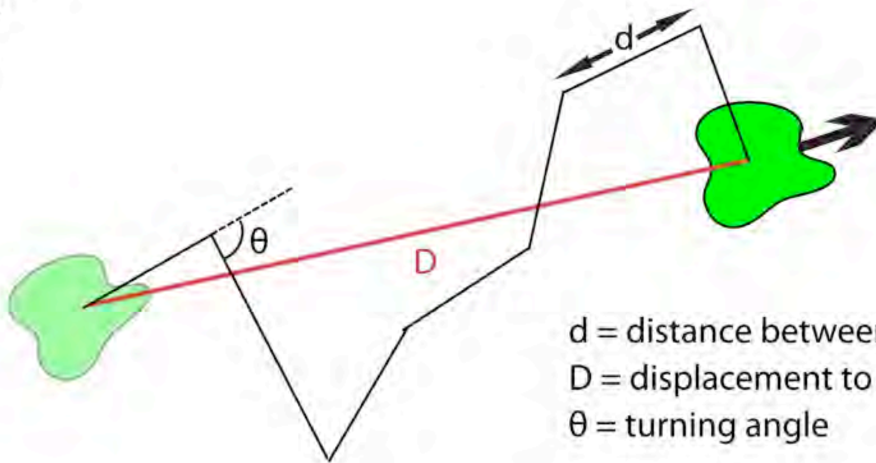


Figure 5.2: Tracking Analysis

(A) Analysis of a cell track. d is the distance moved by a cell between two frames of a movie. It is the smallest distance measurable from a time-lapse dataset. The sum of all ds of a track will give the total distance it moves in the course of the movie. D is the final displacement of the cell – the distance from the start to the end of the track. The more persistently or quickly the cell moves, the greater D will be. θ, the turning angle, is the angle of deviation from a straight line between time points. (B) Mean squared displacement equation for a cell moving with persistent random walk (Martens *et al.*, 2006).

Figure 5.2



B

$$\langle(\Delta x^2)\rangle = 2S^2P[(\Delta t) - P(1 - e^{-(\Delta t)/P})]$$

$\langle(\Delta x^2)\rangle$ = mean squared displacement

Δt = time interval

S = speed at Δt

P = persistence time

changes in the direction of movement greater than 67° (DiMilla *et al.*, 1993). Cells are assumed to travel with a persistent random walk, i.e. they do not travel directly backwards between two time intervals. Fitting of a persistent random walk equation (Figure 5.2 B) to these curves indicated that there was no difference in speed between the conditions, but that EphB2 cells were more persistent after ephrinB1 collisions than after collisions with other EphB2 cells (Figure 5.1, G, H).

Using this data and a custom-written programme (TrackParser, Robert Gilchrist) I plotted other parameters of these tracks. Rose plots of 50 randomly selected 1 h tracks, centred on a common origin, are shown for EphB2 cells after EphB2 collisions (Figure 5.1 C) and ephrinB1 collisions (Figure 5.1 D). EphB2 cell tracks appear straighter after EphB2 cell collisions with ephrinB1 cells. This is also represented by plotting the endpoint, the XY coordinate at 1 h, of each of the tracks analysed (Figure 5.1 E). The root mean square of these coordinates is represented by a solid line.

The final way in which these tracks were analysed was by measuring their turning angle distributions, also using TrackParser. Each turning angle represents the deviation from a straight line between two consecutive 4 min intervals (Figure 5.2A). The results of all cells were pooled and plotted as the probability distribution function over turning angle. The broader the PDF curve, the more randomly cells move. The distribution of turning angles after EphB2-ephrinB1 collisions (red) is narrower than after EphB2-EphB2 collisions (blue), suggesting that they move more directionally (Figure 5.1 F).

Whole population analysis of EphB2 cell motility

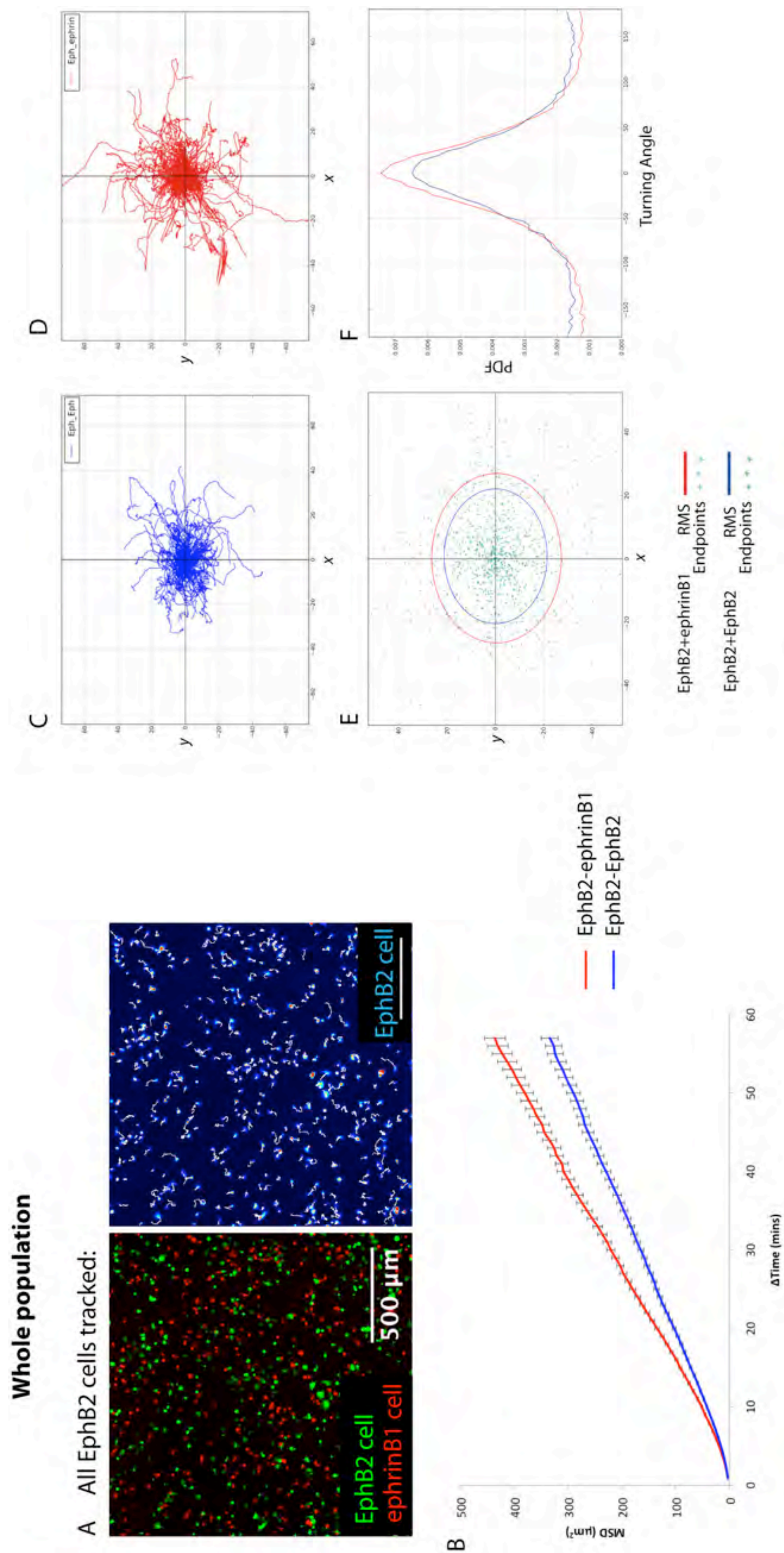
Whilst the data above provide evidence for the increased directionality of cells downstream of signalling through EphB2, such data is extremely time-consuming to generate. To be able to analyse several different conditions, it was necessary to establish a high-throughput method of analysis. To do this, we used an approach to track a whole population of EphB2 cells mixed with ephrinB1 cells.

Cells were differentially labelled green or red and mixed together on a fibronectin-coated glass slide. Green-labelled EphB2 cells were mixed with red-labelled

Figure 5.3: Whole population analysis of directional persistence in EphB2 cells after contact with ephrinB1 cells

EphB2 cells move further and more directionally after interaction with EphB2 cells than with ephrinB1 cells. EphB2 cells were labelled green and red respectively and mixed with red-labelled EphB2 cells or ephrinB1 cells at low density. Time-lapse movies of green cells were tracked using automated tracking software, GMimPro (A). White lines show tracks of the cells (blue) after a 2 h time lapse. (B) Mean squared displacement (MSD) analysis of EphB2 cell tracks mixed with populations of EphB2 cells (blue line) or ephrinB1 cells (red). MSD is higher for EphB2-ephrinB1 mixtures than for EphB2 cells mixed with EphB2 cells. XY coordinates of EphB2 cells mixed with EphB2 cells (C) or ephrinB1 cells (D). 50 full-length tracks (2 h) picked at random are plotted on a common origin from the start of the movie. (E) The endpoints, the XY coordinates of cells 2 h after the start of the movie, are plotted on a common origin. The solid line represents the root mean square of these coordinates (red, after ephrinB1 interaction; blue after EphB2 interaction). (F) The probability distribution function (PDF) of turning angles of the tracks. The deviation from a straight line (turning angle) between two 5 min intervals was calculated for every possible combination of 5 min intervals in all tracks. There is a narrower distribution of turning angles in tracks of EphB2 cells after an ephrinB1 cell collision than after an EphB2 cell collision. Error bars represent standard error of the mean.

Figure 5.3



ephrinB1 cells or red-labelled EphB2 cells. Cells were then imaged every minute for 2 h. They were plated at a density at which, on average, every cell would interact with one other cell, once in the movie. The green cells were then tracked using GMimPro single particle tracking software (Mashanov & Molloy, 2007; Figure 5.3 A) and MSD, turning angles and cell tracks were calculated, as described above. Around 700 cells were tracked per condition. It was found that the MSD curve for a population of EphB2 cells mixed with ephrinB1 cells was steeper than for EphB2 cells mixed with red-labelled EphB2 cells (red and blue lines respectively in Figure 5.3 B), consistent with previous data for individual collisions. The difference between the MSD after 57 min is 101 μm^2, about half that measured after individual interactions (197 μm^2 after 25 min), as expected because the signal is diluted using this method. The data presented is from one experiment but is representative of at least 3 experimental repeats.

Using TrackParser, 100 tracks from each condition were picked at random and plotted on a common origin. Tracks of EphB2 cells mixed with ephrinB1 cells are often longer and more directional than those of EphB2 cells mixed with EphB2 cells (Figure 5.3 C, D). This is also reflected in a plot of the endpoints of these tracks, showing their displacement after 2 h; the root mean square of these end points for EphB2 cells mixed with ephrinB1 cells is greater than for EphB2 cells mixed with EphB2 cells (Figure 5.3 E). Turning angle analysis of these tracks shows that there is a higher chance of cells in the EphB2-ephrinB1 mixture heading straight on than for cells in the EphB2-EphB2 mixture (Figure 5.3 F).

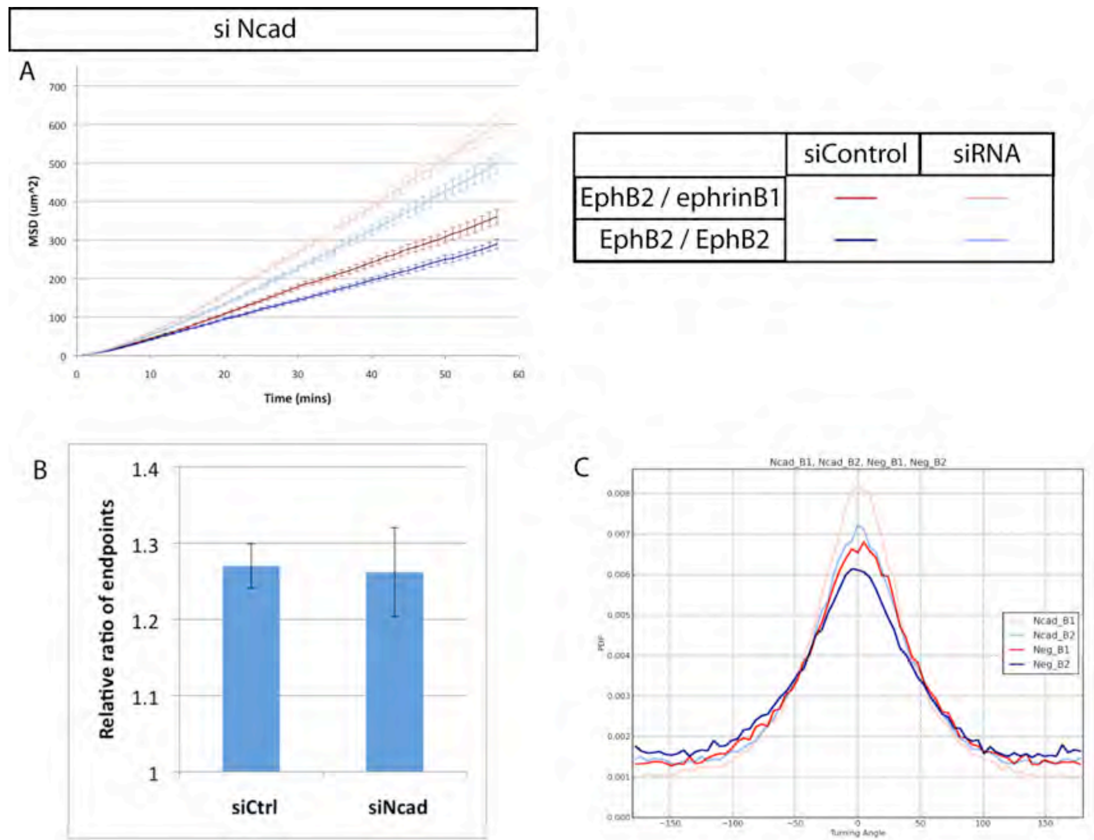
Due to a high variability between the results of experiments recorded on different days, the cell conditions being compared were imaged in parallel, to provide internal controls.

In summary, these results from the high-throughput, whole population analysis accurately reflect the increase in persistence of EphB2 cells after contact with ephrinB1 cells.

Figure 5.4: N-cadherin does not affect ephrinB1-EphB2 increase in cell motility

N-cadherin knockdown does not affect the ability of EphB2 cells to increase their motility in response to ephrinB1 signalling, however it does increase overall motility. (A) Mean-squared displacement (MSD) plot calculated from the 2 h tracks of whole populations of EphB2 cells mixed with EphB2 cells (blue lines) or ephrinB1 cells (red lines). EphB2 cells mixed with ephrinB1 cells have steeper MSD plots, so are more motile than EphB2 cells mixed with EphB2 cells. This is true even when N-cadherin is knocked down (pale blue and pink lines), although there is an increase in the motility of both conditions. (B) The ratio between the endpoints, MSD at 57mins, of EphB2 cells mixed with ephrinB1 cells compared to EphB2 cells mixed with EphB2 cells. The ratio between these points is the same whether the cells are treated with a control siRNA (siCtrl) or siRNA to N-cadherin (siNcad). (C) Comparison of the distribution of turning angles between conditions. The probability distribution function (PDF) is calculated across all possible turning angles for each condition. Where the turning angle is 0, a cell will move in a straight line, hence the narrower the distribution of turning angles, the more directionally the cells move. EphB2 cells move more directionally when mixed with ephrinB1 cells than with EphB2 cells, either with siNcad or siCtrl. siNcad increases the directionality of movement of the cells in both EphB2/EphB2 and EphB2/ephrinB1 mixtures. Error bars represent standard error of the mean.

Figure 5.4



N-cadherin does not affect the increase in EphB2 cell motility after contact with ephrinB1 cells

When N-cadherin is knocked down in mixtures of EphB2 and ephrinB1 cells, their segregation is reduced. Since N-cadherin has been implicated in the motility of cells, it was important to assess whether siNcad was affecting the migratory behaviour of cells required for segregation. To do this I used the whole population assay described above to compare the motility of EphB2 cells mixed with EphB2 cells or with ephrinB1 cells, in the presence of siNcad or siCtrl. All four conditions were imaged in parallel and the experiment repeated 3 times. The results shown are from one experiment but are representative of the three repeats.

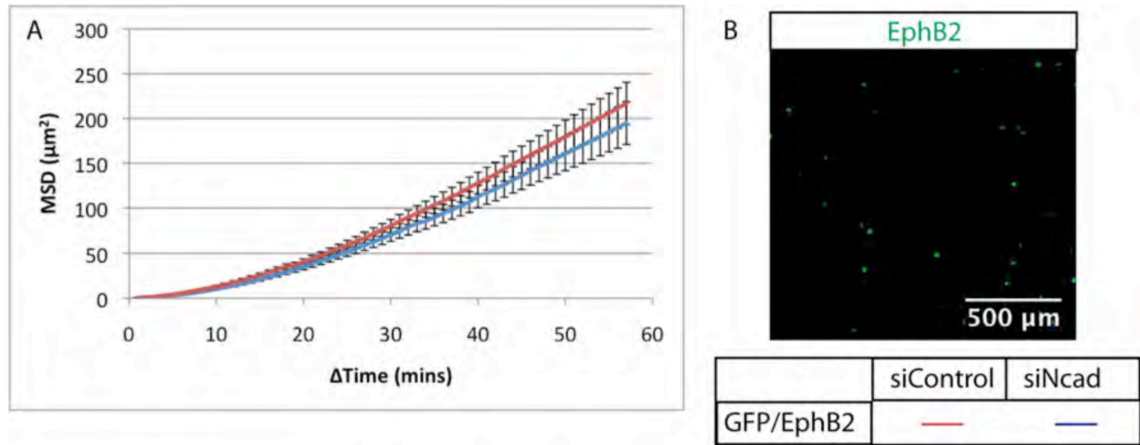
When cells are transfected with siCtrl, EphB2 cells mixed with ephrinB1 cells have an MSD curve which is steeper than for EphB2 cells mixed with EphB2 cells (red and dark blue respectively, (Figure 5.4 A). The average ratio between the MSD at 57 min (the endpoint) of these conditions is 1.27±0.03 (Figure 5.4 B). When transfected with siNcad, this ratio is the same, 1.26±0.06. A similar pattern is seen for the distribution of turning angles. There is a narrower distribution of turning angles, consistent with an increase in directional persistence, for EphB2 cells mixed with ephrinB1 cells compared with EphB2 cells mixed with EphB2 cells, in the presence of either siCtrl or siNcad (Figure 5.4 C). This indicates that when N-cadherin is knocked down, EphB2 cells still increase their directional persistence in response to collisions with ephrinB1 cells. This suggests that N-cadherin is not required downstream of EphB2-ephrinB1 interactions to drive directional persistence.

However, there is a large increase in the MSD gradient of siNcad-transfected EphB2 cells compared to siCtrl-transfected EphB2 cells, which is higher than the increase in MSD seen after ephrinB1-EphB2 signalling. This could be explained by an impairment of contact inhibition of locomotion after N-cadherin knockdown. If cells no longer changed direction on contact, their persistence would be increased. Alternatively, N-cadherin could be affecting cell migration independently of cell-cell contacts.

Figure 5.5: siNcad treatment does not affect the basal motility of cells

Knockdown of N-cadherin by siRNA does not increase the free migration of EphB2 cells. (B) EphB2 cells were plated at very low density to minimise interactions throughout the course of the time-lapse. Any cells which could be seen to be interacting were excluded from further analysis. (A) The mean squared displacement (MSD) of tracks is plotted against increasing time intervals. The MSD of cells transfected with siCtrl is the same as for cells transfected with siNcad. Error bars show standard error of the mean.

Figure 5.5



N-cadherin affects general cell motility in the presence of cell-cell contacts

To assess whether loss of N-cadherin affects migration cell-autonomously or through contacts with other cells, tracks of cells were analysed which did not contact any other cells. If the increase in MSD of cells after knockdown of N-cadherin was due to a disruption of contact inhibition of locomotion, rather than a cell autonomous effect on cell migration, then in the absence of contacts, there would be no difference between the MSD of siCtrl and siNcad transfected cells.

EphB2 cells transfected with siCtrl or siNcad were plated at a very low density to minimise cell-cell contacts (Figure 5.5). Time-lapse images were taken every minute for 2 h and cells were tracked using GMimPro as described above. Tracks of any cells which contacted another cell within the course of the movie were removed from further analysis. Under these conditions, there was no significant difference between the MSD of cells in which N-cadherin had been knocked down and those in which it had not. This suggests that the increased motility seen when N-cadherin is knocked down in EphB2 cells in the whole population analysis is due to a change in contact-dependent migratory responses of cells.

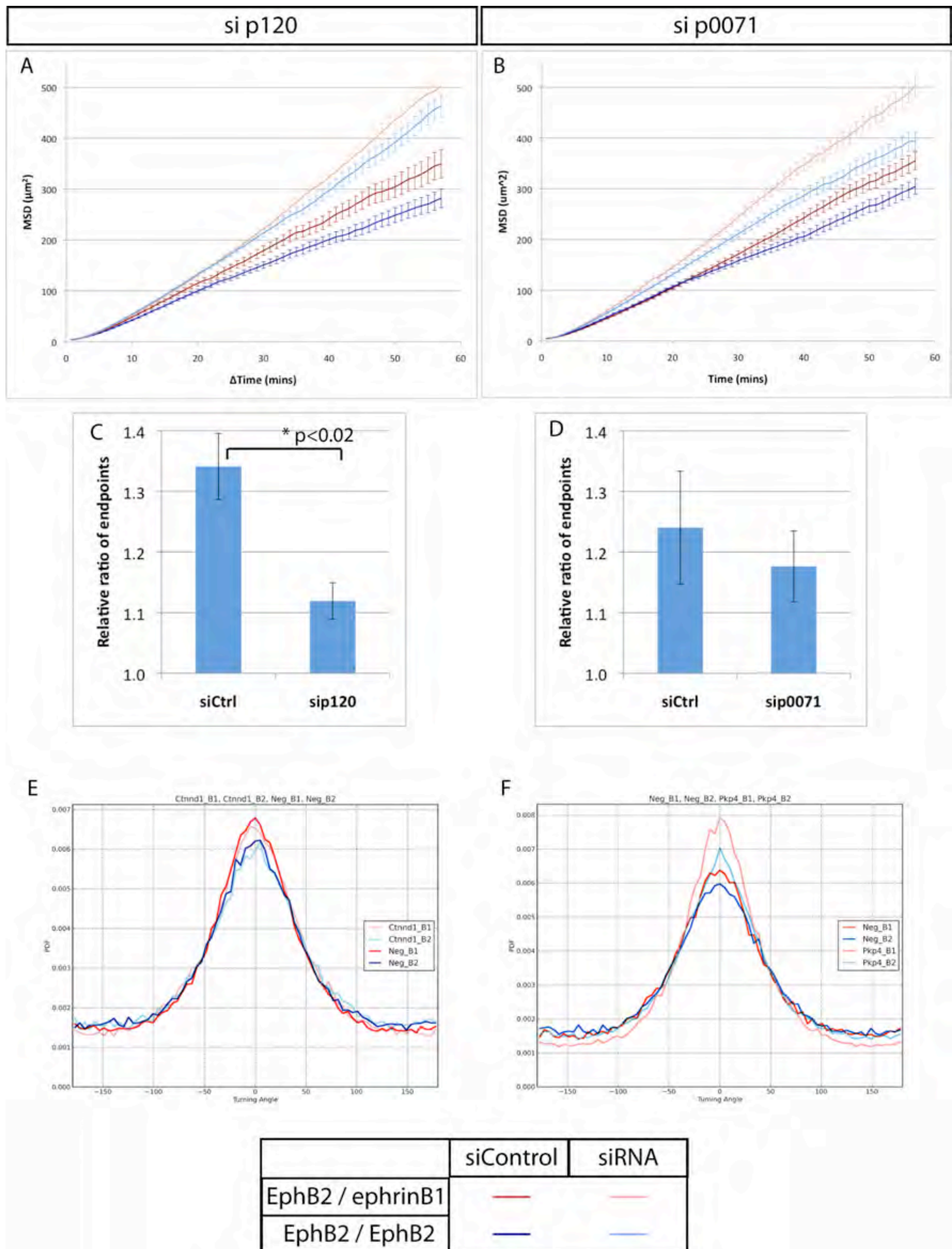
p0071 knockdown recapitulates the N-cadherin knockdown phenotype

I next tested the effect of knocking down p0071 on the motility of EphB2 cells. p0071 is capable of regulating Rho activity to remodel the actin cytoskeleton at the cleavage furrow of dividing cells (Hatzfeld, 2007; Reynolds, 2007). This RhoA-associated actin-regulating activity and the similarity of p0071 to p120, made it likely that p0071, could affect cell migration. If p0071 were downstream of EphB2-ephrinB1 signalling in the control of directional persistence, one would expect a decrease in the MSD ratio between EphB2-ephrinB1 and EphB2-EphB2 mixtures, and a similar shift in turning angle distribution. However, when p0071 is knocked down, there is still a substantial increase in the MSD of EphB2 cells mixed with ephrinB1 cells (red) compared to EphB2 cells mixed with EphB2 cells (blue; Fig 5.6 B). The relative ratio of MSD between these conditions at 57 min is 1.18±0.06 in the sip0071 knockdown compared to 1.24±0.09 when treated with siCtrl (Figure 5.6 D). Similarly, there is a narrower distribution of turning angles for EphB2 cells mixed with ephrinB1 cells than mixed with EphB2 cells, both in the control and the

Figure 5.6: sip120 but not sip0071 inhibits an increase in cell migration after EphB2-ephrinB1 interactions

sip120 and sip0071 increase the overall motility of cells. sip120 but not sip0071 specifically inhibits the EphB2-ephrinB1 induced increase in motility. (A, B) Mean-squared displacement (MSD) plot calculated from the 2 h tracks of whole populations of EphB2 cells mixed with EphB2 cells (blue lines) or with ephrinB1 cells (red lines). EphB2 cells mixed with ephrinB1 cells have steeper MSD plots, so are more motile than EphB2 cells mixed with EphB2 cells. This is true also when p0071 is knocked down (pale blue and pink lines), although there is an increase in the motility of both conditions. Knockdown of p120 also increases the motility of both conditions but there is no difference between the MSD plots of EphB2/EphB2 and EphB2/ephrinB1 cell mixtures, suggesting a specific effect on ephrinB1-EphB2 induced motility. (C, D) The ratio between the endpoints, MSD at 57 mins, of EphB2 cells mixed with ephrinB1 cells compared to EphB2 cells mixed with EphB2 cells. There is a significant difference between the ratio of endpoints between siCtrl and sip120 ($p<0.02$, Students t-test) but not between siCtrl and sip0071. (E, F) Comparison of the distribution of turning angles between conditions. The probability distribution function (PDF) is calculated across all possible turning angles for each condition. Where the turning angle is 0, a cell will move in a straight line, so the narrower the distribution of turning angles, the more directionally the cells move. sip0071 but not sip120 increases the directionality of the cells. In both cases, there seems to be little difference in the increase in directionality between EphB2 and ephrinB1 cells between siCtrl-transfected cells and cells transfected with sip120 or sip0071. Error bars represent standard error of the mean.

Figure 5.6



p0071-transfected conditions (Figure 5.6 F). This indicates that, as with N-cadherin, p0071 is not involved in the increase in directional motility induced by ephrinB1-EphB2 signalling.

However, as with the N-cadherin knockdown, there is an increase in the MSD of EphB2 cells after p0071 has been knocked down. This could reflect a similar role for p0071 and N-cadherin in the control of contact inhibition of locomotion.

p120 is involved in altering motility downstream of EphB2-ephrinB1 signalling

Unlike N-cadherin and p0071, which are not required for the ephrinB1-EphB2-induced increase in directional persistence, p120 does appear to be involved in changes in cell motility mediated by EphB2 activation.

When cells are treated with siRNA to p120, EphB2 cells mixed with ephrinB1 cells (pink) have a comparable MSD curve to EphB2 cells mixed with EphB2 cells (pale blue; Fig 5.6 A). The relative ratio between these conditions is 1.12±0.03, lower than the ratio of 1.34±0.05 for the siCtrl treated cells (Figure 5.6 C). This indicates that p120 knock down specifically disrupts the EphB2-activation-induced increase in cell motility.

As is the case for both N-cadherin and p0071, sip120 treatment also increases the MSD of the cells even in the absence of EphB2-ephrinB1 cell contacts, indicating a role in migration that is independent of EphB2-ephrinB1 contacts. This is not seen, however, in the analysis of turning angles (Figure 5.6 E). The turning angle distribution is narrower for EphB2-ephrinB1 mixtures (red) than EphB2-EphB2 mixtures (blue), but the sip120-treated cells (pale lines) have similar distributions to the siCtrl-treated cells (dark lines). This indicates that the effects seen in the MSD analysis are due to an alteration in the speed rather than the directional persistence of the cells. Nevertheless, the data support a model where p120 is downstream of EphB2-ephrinB1 interactions affecting cell migration.

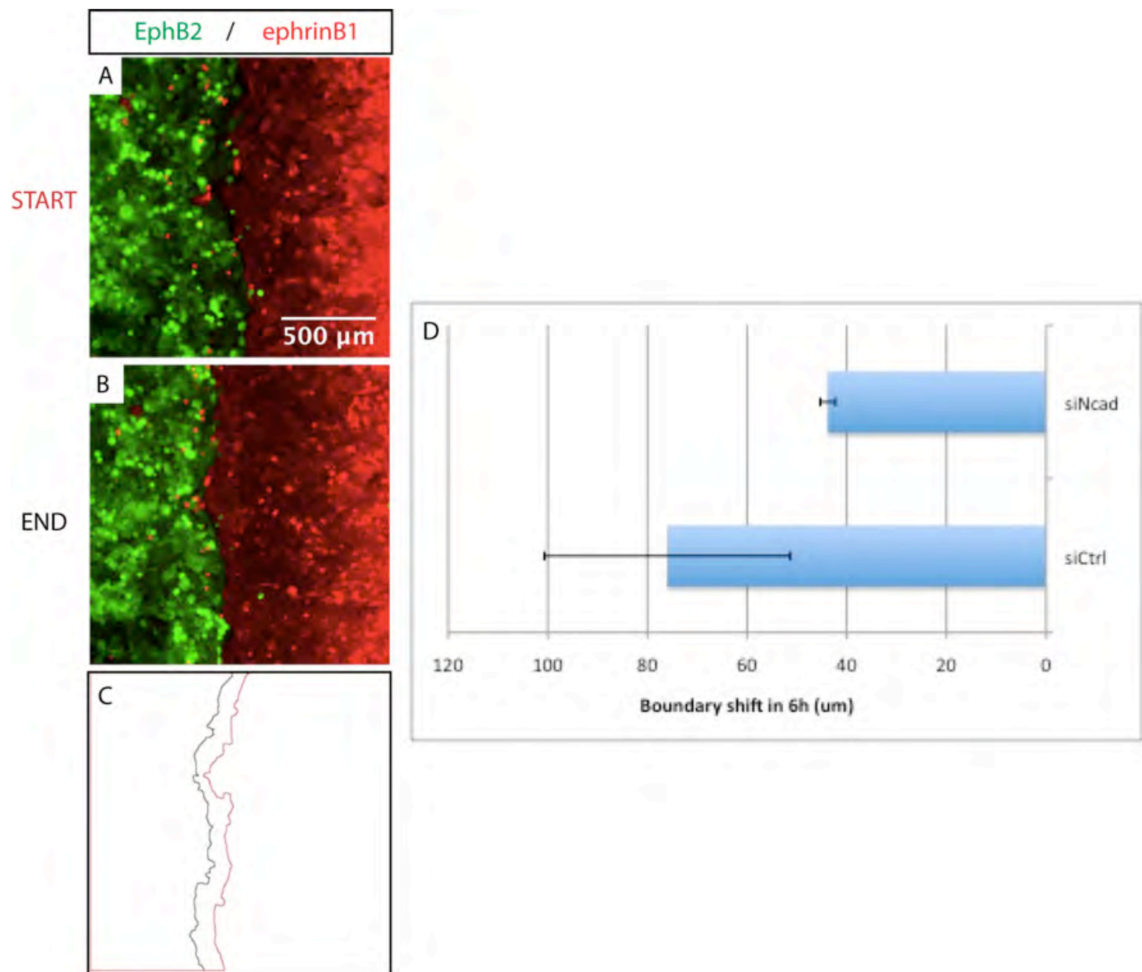
Analysis of EphB2-ephrinB1 boundary movement

In an attempt to visualise how N-cadherin knockdown was affecting boundary sharpening by EphB2 receptors and ephrinB1, I took time-lapse movies of the

Figure 5.7: Shifting of the EphB2-ephrinB1 cell boundary

Quantification of the movement over time of the boundary between EphB2 and ephrinB1 cells in time-lapse movies. (A) The start is the point where the average x-coordinate of the green boundary is equal to that of the red boundary, i.e. the point where the EphB2 and ephrinB1 cells meet (C, red line). (B) The end point is the position of the boundary after 6 h (C, black line). (D) The difference between these boundary positions was calculated for siCtrl and siNcad treated cells. Where cells are transfected with siCtrl, the boundary moves 76μm after 6 h. siRNA to N-cadherin reduces the extent of boundary movement to 44μm. Error bars represent standard error of the mean.

Figure 5.7



boundary assay with EphB2 and ephrinB1 cells transfected with either siCtrl or siNcad.

A striking feature in the movies of the boundary assay with EphB2 and ephrinB1 cells is that the boundary moves over time. When EphB2 cells are plated on the left and ephrinB1 on the right, the boundary moves to the left, away from the ephrinB1-expressing territory (Figure 3.2). This boundary shift is reduced after N-cadherin is knocked down. To quantify this effect, the average positions of the boundary were calculated at the point where EphB2 and ephrinB1 cells meet and 6 hours later. In control cells, the boundary between EphB2 and ephrinB1 cells shifts by 76 µm over this time. When N-cadherin is knocked down, there is still some movement, but the shift is less than in the control situation (44 µm; Figure 5.7). That N-cadherin knockdown affects both the shift of the boundary and the segregation assays could suggest that the assays are readouts of the underlying mechanisms of sorting. For example, it is likely that similar cell processes such as adhesion or contact inhibition of locomotion could be important for this shift of the boundary as well as being important in cell segregation. This assay could therefore provide useful insights into the process of segregation.

Discussion

ephrinB1-EphB2 cell collisions cause cell repulsion

Whilst repulsion is described in the literature as a process through which Eph-ephrins drive sorting, until recently there was no direct evidence that repulsion alone can cause cells to sort.

The definition of repulsion in this context is important. It can be used to refer to: de-adhesion between cells (Cooke *et al.*, 2005); the collapse response, which involves the retraction of lamellipodia from the point of cell-cell contact (Poliakov *et al.*, 2008); or an active movement of cells away from the point of contact. All three could be active together and the mechanisms interlinked, but it is important to think about them as different definitions of "repulsion".

All three of these definitions have been used to describe the behaviour of Eph and ephrin cells after contact. Collisions between EphB2 and ephrinB1 cells bring

about a collapse response between HEK293 cells (Poliakov *et al.*, 2008). This response has been suggested to bring about de-adhesion between the different populations of cells, which could cause them to segregate by generating differential adhesion (Cooke *et al.*, 2005; Steinberg, 2007). Additionally, EphB2 cells increase their directionality of migration after contact with ephrinB1 cells (Figure 5.1). By fitting parameters to the mean-squared displacement (MSD) curves, it was possible to derive the speed and persistence of the different cell types and show that after contact with ephrinB1 cells, there is an increase in the persistence of EphB2 cells (Figure 5.1 H). So, EphB2-ephrinB1 mediated repulsion refers to the collapse of protrusions and subsequent movement away of an EphB2 cell from the point of contact with an ephrinB1 cell.

The contribution of N-cadherin to cell migration

As well as its role in adhesion, N-cadherin also plays a signalling role in the recognition of other cells and in cell migration. Cadherins have been demonstrated to be important for the orientation of cells such that they migrate away from one another after contact (Desai *et al.*, 2009; Dupin *et al.*, 2009). This contact-dependent re-orientation of cells is similar to the function of Eph-ephrins in causing cell repulsion. The results presented here indicate that N-cadherin does not affect segregation downstream of EphB2 signalling, since even in the absence of N-cadherin, EphB2 cells respond to ephrinB1 cells with an increase in directional migration (Figure 5.4). This suggests that N-cadherin and EphB2-ephrinB1 are working in parallel pathways to affect cell migration.

One mechanism which has been proposed through which N-cadherin could affect cell migration is via the RhoGTPase-promoting activity of p120 (Mayor & Carmona-Fontaine, 2010). p120 family molecules are generally thought to activate Rac and inhibit RhoA, supporting a role for p120 in promoting cell migration (Anastasiadis, 2007; Hatzfeld, 2007). If the interaction with cadherin prevented this activity at the point of contact between cells, this could polarise the cell’s Rac activity, such that it would promote protrusions away from the point of contact. If this model were true, then p120 knockdown would phenocopy N-cadherin knockdown in the migration assay. This would be expected anyway, since p120 is required to stabilise N-cadherin expression at the cell surface.

In one respect, there is a similarity between the behaviour of siNcad and of sip120 treated cells. The MSD curve of EphB2 cells is much steeper when they are treated with sip120 than when treated with siCtrl. This could be indicative of their collective role in contact inhibition of locomotion. However, in contrast to N-cadherin knockdown, p120 does specifically affect the migration of cells downstream of ephrinB1-EphB2 interactions (Figure 5.6). When p120 is knocked down, EphB2 cells do not increase their migration in response to ephrinB1 cells. This suggests that p120 has a role in cell migration downstream of ephrinB1-EphB2 interactions which is independent of its association with N-cadherin (discussed in more detail in chapter 6).

p0071 is also helping to control the migration of cells, since its knockdown also increases EphB2 cell motility. In common with N-cadherin, however, it does not seem to be directly affecting the increase in directionality downstream of EphB2-ephrinB1 signalling. This result suggests that p0071 could be acting in consort with N-cadherin in contact inhibition of locomotion. This is in contrast to the results seen in sorting and boundary assays. There, sip0071 has a mild phenotype compared to siNcad, but both together create a dramatic disruption to sorting. As discussed in Chapter 4, this could suggest that there is an additive effect of the two siRNAs because there is incomplete knockdown of p0071 on its own. Altogether, the results suggest that p0071 could act in the same pathway as N-cadherin in contact inhibition of locomotion.

Therefore, it seems that p0071, p120 and N-cadherin could be acting together to effect contact inhibition responses of the EphB2 cells, and in addition p120 also has a specific role downstream of ephrinB1-EphB2 interactions. Knockdown of N-cadherin in cells at low density supports the idea that contact inhibition decreases the general migration of these cells. In this case, there is no difference between the migration of EphB2 cells in which N-cadherin has been knocked down and the migration of EphB2 cells transfected with a control siRNA. p120 is also likely to be involved in contact inhibition of locomotion given its role in the stabilisation N-cadherin, and p0071 could also be important given its relationship to p120. The involvement of p120 and p0071 in contact inhibition of locomotion could be tested by measuring the effects of sip120 or sip0071 on the migration of cells at low

densities, as described for N-cadherin knockdown. If the same result were seen with p120 and p0071, this would further reinforce the idea that they act through the same pathway as N-cadherin to increase migration. It would also be useful to test this idea more directly by looking at cells at medium density, to assess how often cells change direction on contact, with or without N-cadherin, p120 or p0071 expression.

Further evidence in support of the effect of N-cadherin on migration comes from studies linking cell-cell adhesion through cadherins and cell-matrix adhesion through integrins. Cadherin expression affects the traction exerted by cells on a substrate, indicating that there is cross-talk between cell-cell and cell-substrate adhesion (Dzamba *et al.*, 2009). The relationship seems to work both ways, since alterations in integrin-dependent traction forces affect cell-cell adhesion in MDCK cells (de Rooij *et al.*, 2005) and in Xenopus animal caps (Marsden & DeSimone, 2003). Integrin-mediated adhesion is indispensible for cell migration (Moissoglu & Schwartz, 2006), so these studies indicating direct links between cell-cell adhesion and matrix-adhesion provide another mechanism for cadherins to be influencing directed migration.

Technical aspects of cell tracking in dense mixtures

Investigation into cell migration poses a number of problems. Analysis of tracks of a whole population of 500 cells or more only gives an average idea of how cells are behaving. This is particularly problematic when we are only interested in a small proportion of each track – the time after it has interacted with a different cell type (red with green, e.g. EphB2 with ephrinB1). This means there will be a lot of non-specific signal in each dataset contributed by free migration of cells or migration of cells during contact or after contact with like-cells (red with red, e.g. EphB2 with EphB2). Thus, any change in migration seen will be an underestimate of the actual change of migration after interaction between different cell types. In addition, since the data does not represent a "pure" population of cells, it was not possible to fit any standard equation which represents cell movement, so it was not possible to derive values of speed or persistence. The data just give an average overview of the change in cell behaviour, which is represented by the MSD curve.

One way of "cleaning up" the dataset is to select the tracks of interest. Alexei Poliakov performed this analysis by manually selecting tracks of cells which have just interacted with unlike cells. To some extent, this method is prone to bias since the experimenter can choose which tracks to include in the analysis. An ideal method would be to automate this process computationally. This could be done by measuring proximity of cell centroids and only choosing tracks after cell centres have come within a certain range of one another. This sort of analysis would also be prone to error, however, since cells vary in their shape and size and two cells could be close to one another and not touching, or far away and contacting through fine protrusions. Selecting by hand ensures that the maximum number of interactions is detected. However, it is extremely time consuming and was not suitable for analysis of the several conditions that are described here.

For all of this analysis it is important to note the difficulty in distinguishing between speed and persistence. The distance moved by a cell between any time interval will be proportional to both speed and persistence, and we can not be sure that the speed we measure, even at the shortest time interval, is not contributed to by the directionality of cell movement. Another contributing factor is the small distance over which the cells move in this assay – over 1 h, a cell will move an average distance of 15μm, around the diameter of a cell. This means that small fluctuations in the cell centroid could account for some of the movement detected. A more reasonable estimate of cell speed can be gained by fitting an equation for the migration of cells to the MSD curve. However, this can only be done for tracks of cells after cell-cell interactions, since analysis of whole populations of cells proved too noisy to fit reliably to the equation (see above). An alternative approach was to try to directly quantify the directionality of cells. Measuring the turning angle distribution of a collection of tracks gives an idea of how persistently the cells are moving. We assume that the wider the turning angle distribution, the less persistent is the migration. This approach should be less affected by the speed of cells, although their speed may have an intrinsic effect on their ability to change direction.

Possible explanations of boundary movement

One of the striking phenotypes which appears in time-lapse movies of the boundary assay is the shift of the boundary back into the EphB2 domain (Figure 3.2), which is reduced in the absence of N-cadherin. What could be responsible for this movement?

The two most obvious explanations are the same as the mechanisms underlying cell segregation. Firstly, an increase in adhesion between EphB2 cells could cause their compaction and apparent retraction from ephrinB1 cells, shifting the boundary. Alternatively, the boundary movement could be a consequence of the repulsion of EphB2 cells from ephrinB1 cells. In this case, as in the segregation assays, N-cadherin could be required for basal cell-cell adhesion, which would allow the cells to migrate cohesively (Theveneau *et al.*, 2010). In these movies, siNcad cells appear to move more erratically than siCtrl treated cells, which is in agreement with the idea that the coherence of cells is important. This is consistent with the reported requirement for N-cadherin in the collective migration of neural crest cells by a combination of contact inhibition and coherent movement (Theveneau *et al.*, 2010). These boundaries are also less sharp, suggesting a relationship between boundary sharpness and coherent cell movement.

In EphB2 and ephrinB1 cell segregation assays, when N-cadherin is knocked down only in EphB2 cells, this has less effect on boundary sharpness than when it is knocked down in both populations (Figure 4.5). However, N-cadherin knockdown in only ephrinB1 cells has a dramatic effect on boundary sharpness. It would be interesting to observe the extent of boundary movement in each of these conditions to see whether movement is permissible where only ephrinB1 cells express N-cadherin, or if it is required more between EphB2 cells in facilitating cell cohesion. The movement of the boundaries could provide a new method for revealing mechanisms that underlie the collective behaviour of cells expressing Eph or ephrin, which may be important *in vivo*.

6. Discussion

Mechanisms of sorting through Eph-ephrins

It had previously been suggested that two cellular mechanisms could be regulated by Eph receptors and ephrins to achieve cell segregation. One was repulsion, based on the ability of Eph-ephrin signalling to mediate repulsion in other situations, such as axon guidance (Poliakov *et al.*, 2004 2010). Other evidence suggested that they could act by regulating cell adhesion (Cortina *et al.*, 2007; Parrinello *et al.*, 2010; Solanas *et al.*, 2011), which could generate differential adhesiveness between sorting cells (Steinberg, 2007). I have explored the relationship between these two mechanisms using *in vitro* assays to investigate the sorting and behaviour of EphB2 and ephrinB1 expressing cells.

Initially, I compared cell sorting driven by EphB2 and ephrinB1, with sorting driven by differential adhesion in the segregation, boundary and hanging drop assays. I found that, whilst differential adhesion can drive segregation in 3D assays, it is inefficient in two-dimensional assays (Figure 3.7), whereas EphB2 and ephrinB1 are capable of driving cell sorting in both three-dimensional and two-dimensional assays. This suggested that the mechanisms underlying sorting downstream of EphB2-ephrinB1 interactions are different from differential adhesion. Interestingly, siRNA knockdown of N-cadherin, a key cell-cell adhesion molecule, disrupted EphB2-ephrinB1 cell segregation, indicating that cadherin-mediated adhesion was required for cell sorting (Figure 4.1). To investigate this further, I took advantage of studies that have identified targets of Eph-ephrin signalling (Jorgensen *et al.*, 2009; Zhang *et al.*, 2008). These targets included members of the p120ctn family, p120 and p0071, which were strong candidates to regulate adhesion downstream of EphB2-ephrinB1 signalling. Knockdown of p120 or p0071 disrupts cell segregation mediated by EphB2 and ephrinB1 (Figure 4.9).

It has been previously reported that Eph-ephrin signalling mediates repulsion and directional migration on contact between cells (Figure 5.1; Astin *et al.*, 2010; Poliakov *et al.*, 2008). This is a behaviour also exhibited by cadherins which, as well as being important for cell-cell adhesion, are also required for contact

inhibition of locomotion (Mayor & Carmona-Fontaine, 2010; Theveneau *et al.*, 2010). An intriguing possibility was that cadherin-mediated contact inhibition of locomotion and Eph-ephrin mediated repulsion could be related. However, EphB2 cells were able to respond to ephrinB1 signalling with an increase in directional migration even in the absence of N-cadherin, suggesting that these two mechanisms are not directly linked. Interestingly, tracking analysis suggested that p120 has an N-cadherin independent function in promoting directional migration downstream of ephrinB1-EphB2 interactions.

How these observations could fit into a model for Eph-ephrin activity in the cell sorting process will be discussed (Figure 6.1).

Eph-ephrins drive sorting through repulsion

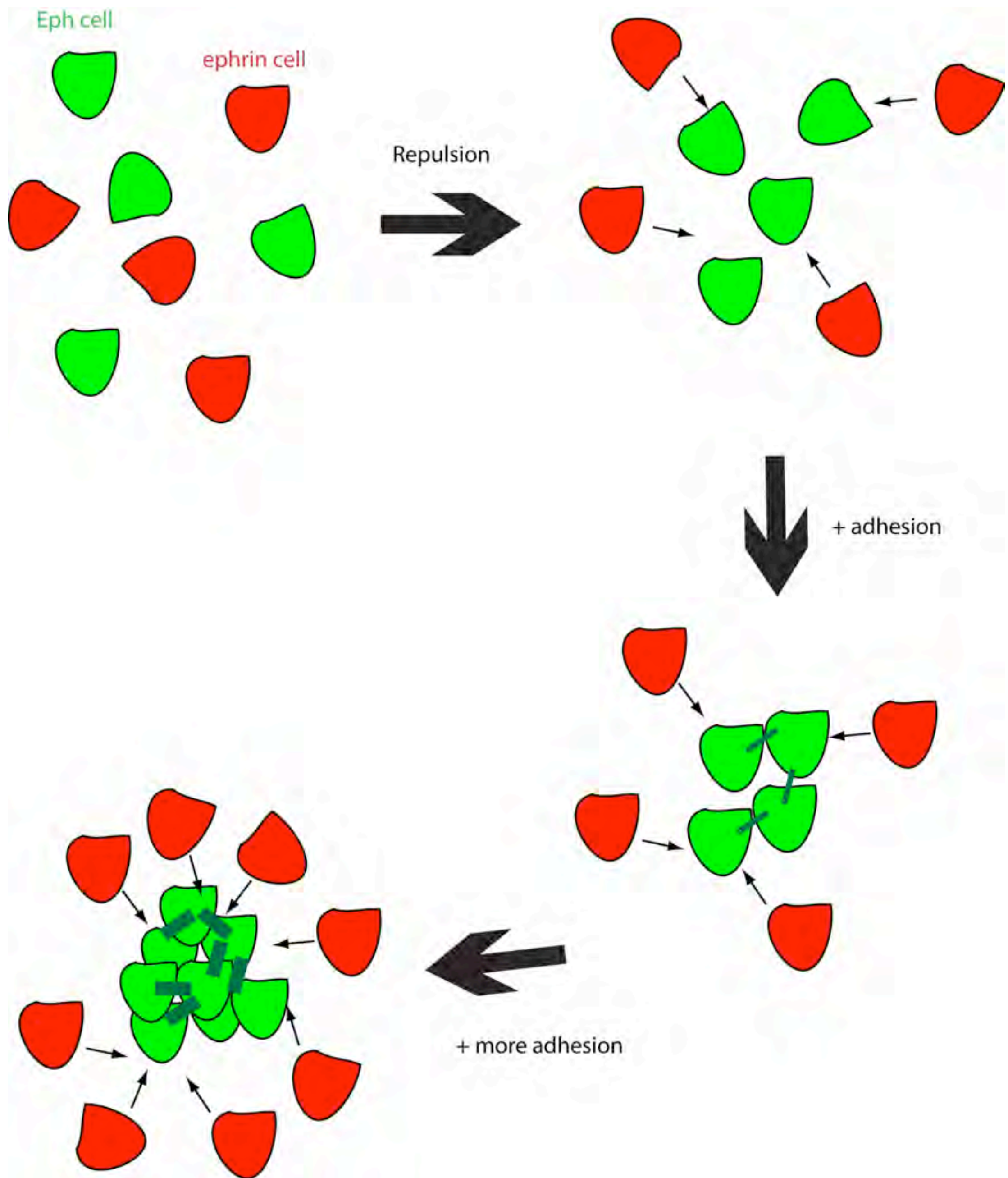
Repulsion is a cellular response downstream of signalling through Eph receptors and ephrins which consists of at least two phases (Astin *et al.*, 2010; Jurney *et al.*, 2002; Monschau *et al.*, 1997). It initially takes the form of a collapse response, characterised in EphB2 cells by the retraction of protrusions away from the point of contact with an ephrinB1 cell (Poliakov *et al.*, 2008). Secondly, the EphB2 cell moves with increased directionality after contact with an ephrinB1 cell (Figure 5.1). One possibility is that the mechanisms which cause the collapse response may persist and are the same as the mechanisms which allow the cell to migrate with increased directionality.

Such repulsive behaviour can be seen in neurons to guide growing axons, for example EphA4 is required for the correct localisation of developing motor neurons in the avian limb (Eberhart *et al.*, 2002). Experiments in Xenopus suggest that there is a continued cycle of repulsion and adhesion which is responsible for allowing migration of ectoderm over mesoderm during gastrulation, while maintaining a distinct boundary between the germ layers (Rohani *et al.*, 2011). Time-lapse imaging of membrane-labelled explants of the cell types allows visualisation of this dynamic process and demonstrates a requirement for EphB-ephrinB signalling in cell repulsion.

Figure 6.1: Eph-ephrin driven sorting

Model for Eph-ephrin driven cell sorting. Initially intermixed Eph and ephrin cells begin to sort by a repulsion response in the Eph cells on contact with ephrin cells. Clusters are stabilised by adhesive interactions between Eph cells. As cluster size increases over time, due to sorting and proliferation of cells, the strength of adhesions between cells increases by increased recruitment of cadherins.

Figure 6.1



RhoGTPase activity and cell migration

The change in cell migration downstream of Eph-ephrin signalling is likely to involve the activity of Rho GTPases, especially Rac1 and RhoA, which control cell protrusions via regulation of the actin cytoskeleton. RhoGTPases have a well-studied role in directing cell migration but there is extensive cross-talk between these proteins and their activity is complex. The traditional view is that active Rac1 promotes the formation of lamellae all around the cell, promoting random walk migration (Pankov *et al.*, 2005). Reduced levels of Rac1 increase the directional persistence of the cell concomitant with formation of a stable lamellipodium at the leading edge, to which active Rac1 is largely localised. Thus total levels of Rac and localisation of active Rac1 at the leading edge are important for directional migration. RhoA is also important for cell migration, but is active at the trailing edge of cells. There is mutual antagonism between Rac1 and RhoA, which helps to stabilise this polarised localisation of the proteins, and therefore directional migration (Mayor, 2010).

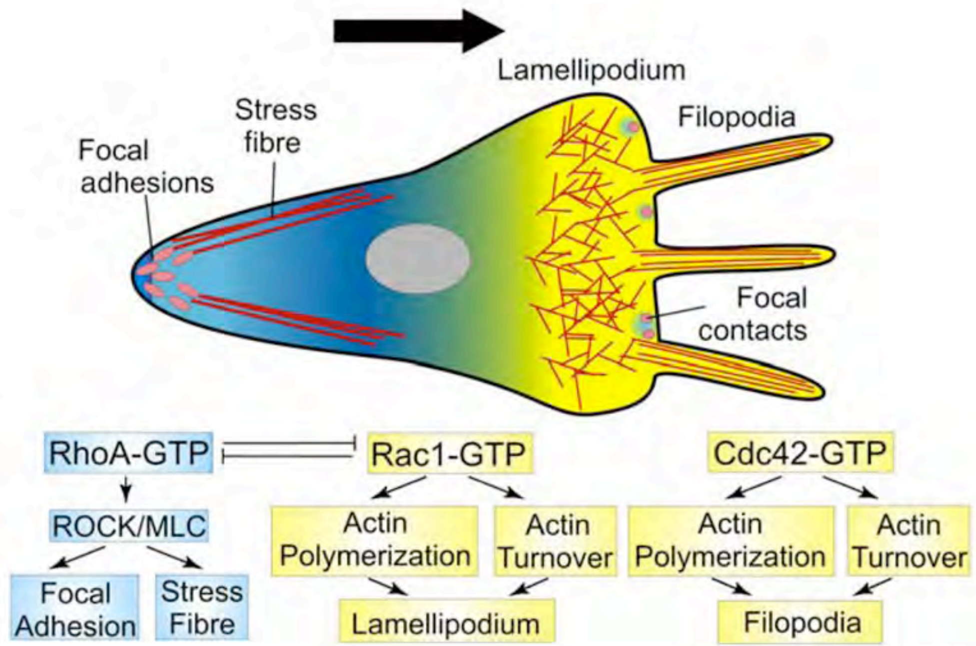
More recent work using high temporal resolution time-lapse and FRET (fluorescent resonance energy transfer) microscopy suggests a more complex relationship between the RhoGTPases (Spiering & Hodgson, 2011). Spiering *et al.* demonstrate that there is activity of Rho, Rac and Cdc42 at the forming lamellipodia of a cell, though they are excluded from each others' domains both spatially and temporally (Machacek *et al.*, 2009). Several effectors of RhoGTPases have been identified which explain their role in modulating the dynamics of the actin cytoskeleton (Figure 6.2). For example, RhoA can directly interact with ROCK which regulates actin contractility through myosin light chain phosphorylation, and Cdc42 and Rac1 can indirectly activate Arp2/3 to promote actin polymerisation (Spiering & Hodgson, 2011). The contribution of each Rho GTPase to motility is thus highly dynamic and its regulation equally so. More sophisticated methods of analysis will need to be used to fully understand the relative contributions of each component to migration.

Figure 6.2: RhoGTPase activity in a migrating cell

Localised activity of different RhoGTPases is important for directed migration of cells (A). Cdc42 is important in the formation of filopodial protrusions at the leading edge of the cell. Rac1 promotes activity of the lamellipodium at the leading edge and mutually inhibits the opposing activity of RhoA in this region. RhoA is active at the trailing edge of the cell where it regulates the activity of focal adhesions and the contraction of stress fibres which pull the back of the cell forwards. (B) Summary of the known pathways through which the RhoGTPases mediate their effects. Rac1, Cdc42 and RhoA all interact with the actin cytoskeleton, being responsible for its polymerisation or contractility.

Figure 6.2

A



Mayor, 2010

PCP and polarity components are important in the migratory responses downstream of Eph-ephrin interactions

Previous work has suggested that the PCP (planar cell polarity) and Par proteins play a role in regulating the balance of Rac and Rho to drive directional migration downstream of EphB-ephrinB signalling. Cell sorting assays demonstrated a requirement for PCP component Dishevelled (Dvl) and RhoA in sorting mediated by EphB-ephrinBs in Xenopus blastomeres (Tanaka *et al.*, 2003). Dvl was also required for migration in the Xenopus eye field, downstream of ephrinB1 (Lee *et al.*, 2006).

Several PCP and Par proteins were identified downstream of EphB2 in proteomics screens, including Dvl, Daam1, Par3 and Par6. It was found that siRNA knockdown of either Dvl2 or Par6B disrupted the increase in directional migration downstream of ephrinB1-EphB2 signalling (Lauren Gregory, Thesis, 2011). This led to a model where, in control cells, Dvl2 and Par6B form a complex with aPKC which is bound to EphB2 and is capable of activating Rac1 and inhibiting RhoA. When ephrinB1-binding induces phosphorylation of the EphB2 receptor, the complex dissociates, relieving RhoA inhibition and allowing its increased activity at the point of cell-cell contact. This results in polarisation of the cell, which allows migration away from the point of contact as well as sustained persistent migration.

p120 affects migration downstream of Eph-ephrins

p120 also acts downstream of Eph-ephrin interactions to mediate increased cell motility, since siRNA knockdown of p120 specifically inhibits the ability of EphB2 cells to migrate further after contact with ephrinB1 cells (Figure 5.6). The activity of p120 in directing cell migration can also be explained by its regulation of RhoGTPases. The p120 family generally activates Rac and inhibit Rho (Anastasiadis, 2007; Hatzfeld, 2007). Consistent with this, reduction of p120 levels leads to increased levels of activated Rho in many cell lines (Reynolds, 2007), and forced expression of p120 in fibroblasts leads to a branching morphology indicative of increased Rac activity (Reynolds *et al.*, 1996). It has been shown in SV80, NH3T3 and CHO cells, that expression of full-length p120 induces protrusive activity. This correlates with an increase in migration of these cells and occurs via activation of Rac and Cdc42 without inhibiting Rho (Grosheva *et al.*, 2001). The

behaviour of p120 has been attributed to its acting as a Rho guanine dissociation inhibitor (RhoGDI), whereby Rho activity is inhibited by preventing normal exchange of guanine nucleotides (Castano *et al.*, 2007).

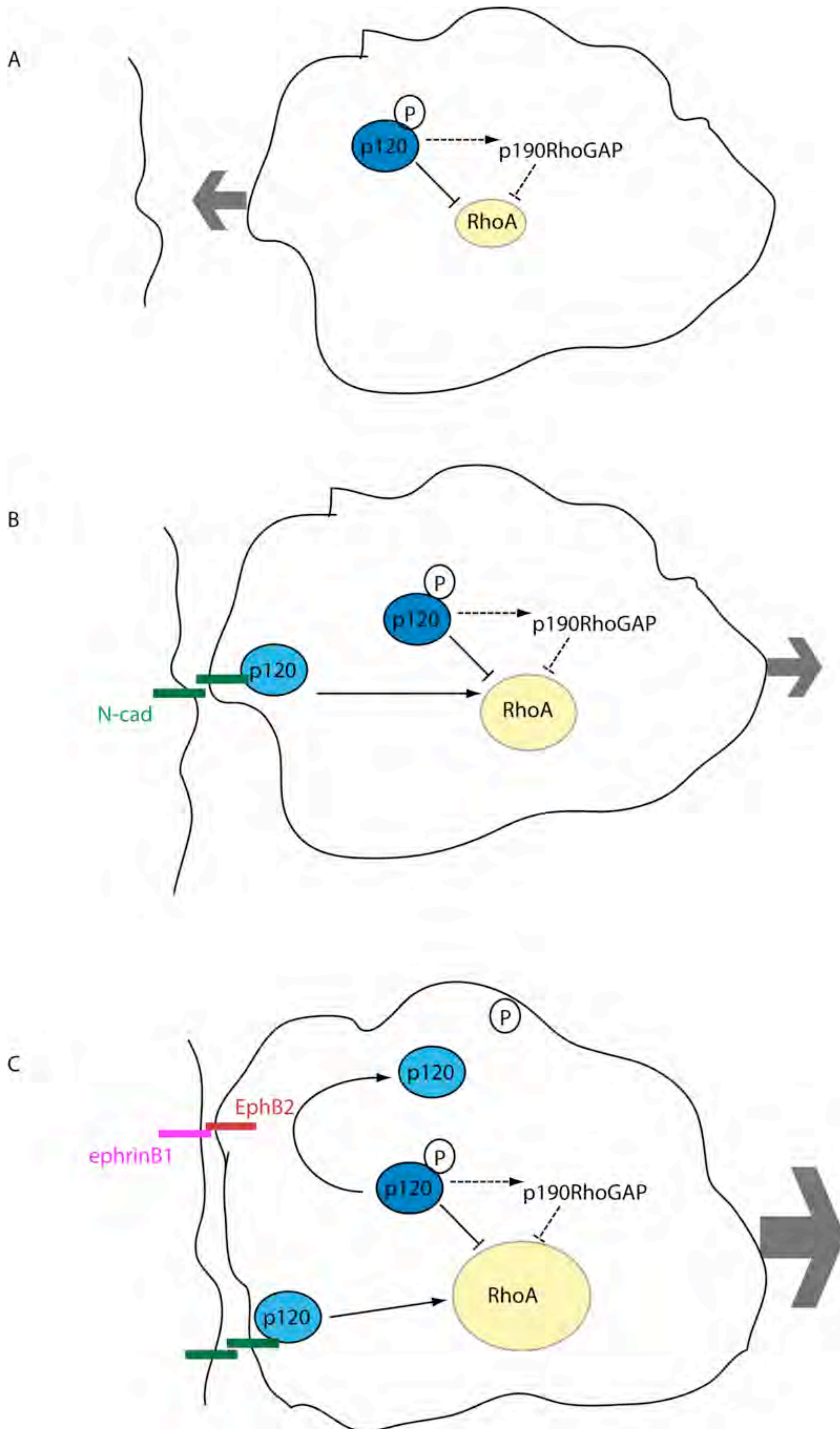
ephrinB1-EphB2 signalling impacts on the phosphorylation state of p120, which affects its activity. The large-scale screens identifying phosphorylation targets of ephrinB1-EphB2 signalling demonstrate that Y217, Y228, Y257 and Y904 or p120 have consistently decreased phosphorylation in EphB2 cells mixed with ephrinB1 cells but increased phosphorylation in EphB2 cells stimulated with soluble ephrinB1-Fc (Jorgensen *et al.*, 2009). So ephrinB1-EphB2 signalling could either upregulate or downregulate phosphorylation of p120. Use of soluble ligands, although they are artificially clustered, is thought not to be representative of normal signalling, since it does not induce the same level of multimerisation of the Eph-ephrin complex as occurs at cell-cell contacts (Jorgensen *et al.*, 2009). Thus, the mixture of EphB2- and ephrinB1- expressing cells is more representative of *in vivo* signalling. However, the difference in response between these two cases could also hints at a response to signalling that is variable depending on the timing of contacts and context in which the cells find themselves.

How do changes in the phosphorylation state of p120 affect its activity? Some reports suggest that none of the phosphorylation sites are necessary for cell-cell adhesion (Mariner *et al.*, 2004; Xiao *et al.*, 2003), since phospho-tyrosine defective mutants can efficiently bind and stabilise E-cadherin at the cell surface. This is consistent with the idea that changes in phosphorylation could instead modulate directional migration. Different isoforms of p120 have been implicated in the differential regulation of migration through their varying effects on Rho activity, although they do not affect Rac1 activity (Yanagisawa *et al.*, 2008). One hypothesis is that longer isoforms of p120 have increased affinity for RhoA, leading to reduced RhoA activity and increased migration and tumour invasiveness. p120 was shown to be able to bind RhoA *in vivo* (Magie *et al.*, 2002), consistent with its proposed role as a RhoGDI. Other evidence suggests that p120 may inhibit Rho via the recruitment of p190RhoGAP (Wildenberg *et al.*, 2006). Phosphorylation could also affect this binding to RhoA. For example, it has been reported that phosphorylation of Tyr217 and Tyr228 by Src promotes a higher affinity of p120 towards RhoA

Figure 6.3: p120 directs migration downstream of ephrinB1-EphB2 signalling and cadherins

In a freely migrating cell, p120 inhibits RhoA either by direct binding or by interaction of p190RhoGAP (A). On contact with a like cell, p120 association with N-cadherin changes its conformation and facilitates activation of RhoA (B). This causes a local activation of RhoA, which stimulates increased migration away from the point of contact. (C) When an EphB2 cell comes into contact with an ephrinB1 cell, signalling changes the phosphorylation state of p120. De-phosphorylation of tyr217 and 228 prevents binding to RhoA, resulting in higher levels of active RhoA and a stronger "push" away from the point of contact.

Figure 6.3



(Castano *et al.*, 2007), which would inhibit RhoA activity. Since both of these sites are affected by ephrinB1-EphB2 signalling, this could provide a mechanism through which ephrinB1-EphB2 signalling affects RhoA activity and migration. The phosphorylation state of p190RhoGAP is also influenced by ephrinB1-EphB2 signalling, providing another mechanism through which this could work (Jorgensen *et al.*, 2009). I propose a model where p120 is found at the leading edge of a freely migrating cell, inhibiting RhoA either by direct binding or through p190RhoGAP, and promoting lamella formation (Figure 6.3). On cell-cell contact, p120 binds to the cadherin which accumulates there, and this causes a conformational change in p120 which allows RhoA activation, resulting in a change of cell polarity and increased migration away from the point of contact. When an EphB2 cell contacts an ephrinB1 cell, the resultant signalling induces de-phosphorylation of tyr217 and tyr228 in cytosolic p120 near the contact, which will prevent binding to RhoA, allowing the latter to become active and strengthening the repulsion response.

This mechanism could be working in combination with the PCP and Par polarity components previously proposed. In addition, a direct link has been identified between the PCP pathway and p120 through the Xenopus transcription factor Kaiso (Kim *et al.*, 2004). Association of p120 with xKaiso relieves its repression of xWnt11, interactions which are vital for gastrulation movements in the developing embryo. It would be interesting to explore this connection further.

Basal requirement for adhesion for cell sorting driven by repulsion

Knockdown of N-cadherin or p120 disrupts EphB2-ephrinB1 cell sorting indicating that adhesion is required for this process. However, the data presented here also reveal that differential adhesion cannot explain EphB2-ephrinB1 mediated sorting and indicate that directional migration is a likely process driving the segregation of cells. This raises the question: what is the role of cell-cell adhesion in this process?

If cell repulsion can mediate sorting, there is no need for differential adhesion to do so, although the two processes could be working in parallel (see Chapter 4). Instead, the requirement for cadherins is likely to be in establishing a basal level of adhesion, so that cells can stick to one another. On this background, repulsion

would be able to drive sorting with adhesion helping to stabilise clusters, whose stability will be constantly disrupted by motile cells. This conclusion fits well with the data from this study and with the results of computer simulations described below.

However, Eph-ephrin interactions could set up differential adhesion which could contribute to cell sorting in addition to these migratory mechanisms. For one thing, repulsion between cells can contribute to the decrease in adhesion between Eph and ephrin expressing cells by breaking contacts between them. This sets up a difference in adhesiveness between the cell types that could contribute to their segregation.

There may also be a context-dependent requirement for differential adhesion to be set up downstream of Eph-ephrin signalling. In the intestinal epithelium, E-cadherin is required for cell compartmentalisation, in combination with EphB-ephrinB signalling (Solanas *et al.*, 2011). It has been suggested that specific cleavage of E-cadherin by Adam10 at the site of cell-cell contact, in response to EphB-ephrinB signalling, establishes an area of decreased adhesion and differential adhesion between EphB and ephrinB cells. These cells are epithelial, so potentially less motile than some other cell types, with better-established cell-cell adhesion through mature adherens junctions. Differential adhesion may be a more important mechanism in the sorting of strongly adhesive cells such as these.

The extensive separation between cells that occurs in a cell culture dish is something which would not occur *in vivo*, since adhesion is required to maintain the integrity of the embryo from very early stages. N-cadherin mutants (*parachute* and *glass onion* in zebrafish), for example, have strong defects in neural tissues and somites and are only viable if N-cad or E-cad expression is used to rescue defects in heart tissues (Lele *et al.*, 2002; Luo *et al.*, 2001). In this context, where cells adhere to one another on all sides, additional mechanisms may be required to downregulate adhesion in segregating cells. An alternative interpretation of the Solanas *et al*. results could be that cadherin is cleaved to facilitate repulsion between EphB and ephrinB cells. De-adhesion through an active cleavage process at the point of EphB cell and ephrinB cell contact could be important in allowing the cells to move away from one another.

I propose that whilst adhesion is required between cells in order for EphB2-ephrinB1 interactions to drive sorting, differential adhesion is not the main mechanism underlying the segregation of HEK293 cells. However, the regulation of cadherins by Eph-ephrins may be an important additional mechanism in some circumstances, such as between highly adhesive epithelial cells, to facilitate cell segregation.

Cadherin enrichment could help to maintain EphB2-ephrinB1 sorting

In HEK293 cells, there is an increase of N-cadherin staining at the membranes between EphB2 cells within sorted EphB2 clusters but not at earlier stages of sorting. This suggests a model in which changes in cadherin enrichment at cell-cell interfaces are not required to drive EphB2-ephrinB1 sorting. In support of this is the observation that N-cadherin is not required for EphB2 cells to cluster when mixed with ephrinB1 cells which do express N-cadherin (Figure 4.5). However, an increase in cadherin-mediated adhesion could be important for maintaining clusters of EphB2 cells once they have formed.

It has previously been reported that Eph receptor activation results in an upregulation of cadherins at the cell membrane. EphA2 cell stimulation by ephrinA1-Fc induces cell compaction and maturation of E-cadherin cell-cell junctions in MDCK cells (Miura *et al.*, 2009). This represents the start of a feedback loop, since E-cadherin is also able to enhance signalling through EphA2, indicating complex interactions between the pathways. In colorectal cancer cells, EphB2 activation by ephrinB1-Fc seems to induce a re-localisation of E-cadherin from the cytoplasm to the cell membrane (Cortina *et al.*, 2007). In Schwann cells, there is an upregulation of N-cadherin at the membrane, which correlates with an increase in total protein in the cell and is concomitant with increased cell clustering (Parrinello *et al.*, 2010). This upregulation is a longer-term result of EphB2 signalling, since it requires increased transcriptional activity of the transcription factor Sox2 and is seen only after several hours. This implies that the activity of N-cadherin is not required for early stages of sorting but rather for the maintenance of segregation.

Adherens junctions mature with time after a nascent contact, with increasing E-cadherin localised to them (Yamada & Nelson, 2007). It is possible that the increase in E-cadherin localisation in EphB2 cell clusters is a result of an increased duration of cell-cell contact, allowing adhesive complexes to increase in strength.

Whilst cadherin expression does affect the adhesive strength between cells (Duguay *et al.*, 2003; Foty & Steinberg, 2005), other factors, such as cortical tension, can also affect it (Krieg *et al.*, 2008; Schotz *et al.*, 2008). It would be useful to determine whether the expression or activation of EphB2 and ephrinB1 affects the cohesiveness of cells. Re-aggregation assays of gastrula stage Xenopus embryos demonstrate that Eph and ephrin expression affect the adhesiveness between the different germ layers. However, they also demonstrate that these differences in the adhesiveness of the ectoderm and mesoderm do not correlate with their segregation. Instead, they show that the repulsive activity of EphB-ephrinB signalling correlates better with the pattern of segregation and is likely to be important in maintaining a boundary between these compartments (Rohani *et al.*, 2011).

Atomic force microscopy has been used to measure the relative adhesiveness of different germ layers in zebrafish and could be used to determine differences in adhesion between Eph and ephrin cells (Krieg *et al.*, 2008). This sort of assay could provide insights into the intrinsic differences in adhesion between EphB2 and ephrinB1 expressing cells.

Modelling cell sorting

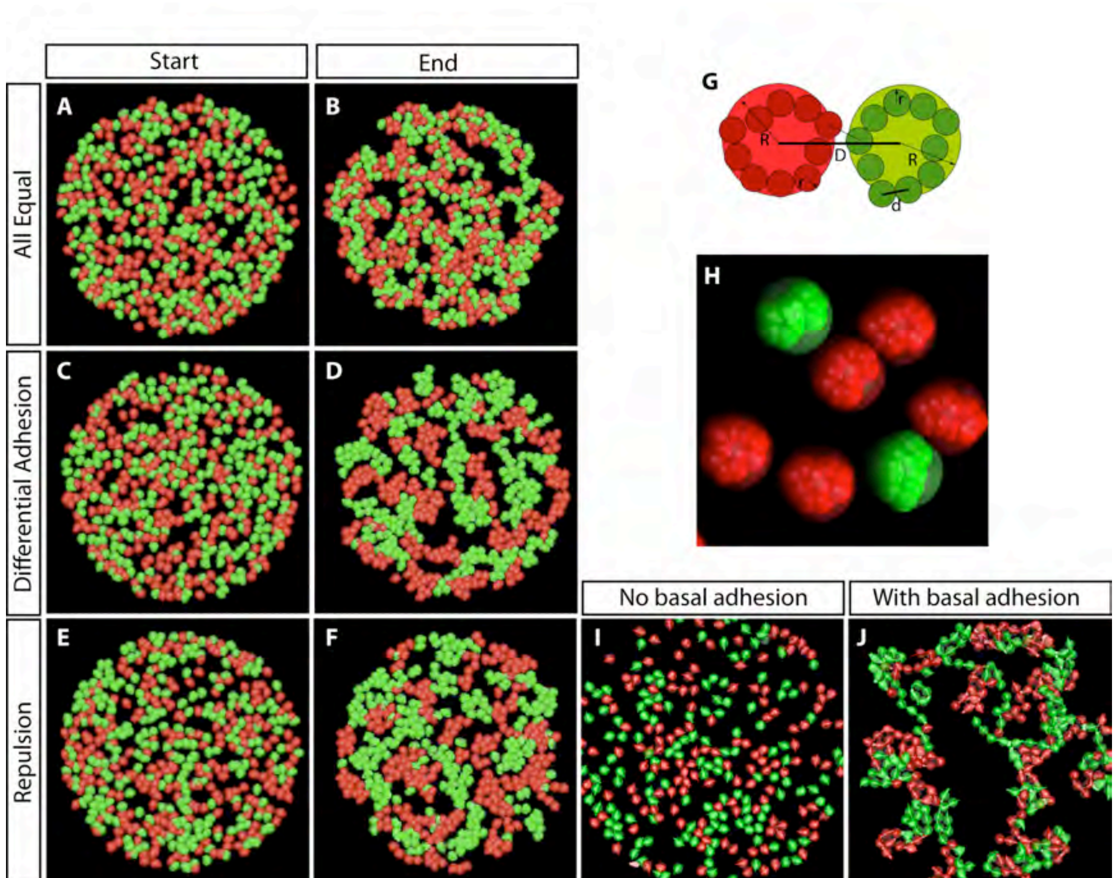
Whilst it is clear that Eph-ephrin signalling can lead to an increase in the directional persistence of cells, it was not known whether this was sufficient to explain cell sorting. To address this question, a collaboration between Alexei Poliakov and Willie Taylor led to the development of a computer model based on the EphB2 and ephrinB1 cell segregation assay (Taylor *et al.*, 2011).

This model was used to simulate cell sorting *in silico* (Figure 6.4). Briefly, cells were modelled based on parameters extracted from time-lapse imaging of HEK293 cells, similar to that described in previous sections. The model incorporates two basic parameters which control the of amount and direction of displacement of

Figure 6.4: Repulsion can generate sorting in a computer model

Computer simulations based on the parameters of cell behaviour of HEK293 cells demonstrate that repulsion can mediate cell sorting. (A, B) Start and end points of a control simulation where parameters of movement and stickiness are the same for each cell type and no sorting is seen. (C, D) Start and end points of a simulation of differential adhesion, in which cells of each type stick preferentially to other cells of the same type, demonstrate that there is segregation between red and green cells. (E, F) Start and end point of a simulation of repulsion, in which green cells move with increased directional migration after contact with red cells, demonstrate that repulsion is sufficient to segregate cells. (G, H) Cartoon and representative image of individual cells within the model. Cells are modelled as a string of 10 balls, each with radius, r, which are always within a fixed distance, d, of their neighbours and within a distance, R, from the centre of mass of the cell. Cells are described as coming into contact when D (the distance between their centres of mass) is equal to 2R. (I,J) Basal adhesion is required for repulsion driven cell sorting. Cells segregate by repulsion when a basal level of adhesion is applied to all cells. However, in the absence of this basal adhesion, cells do not sort by repulsion alone.

Figure 6.4



virtual cells and which can be manipulated to simulate different types of individual cell movement and cell-cell interactions. The real cells move with persistent random walk which can be modelled and scaled to fit real life. They have a "hook" parameter, such that when two cells collide, the probability of them staying together in the next step of the simulation is higher or lower. Secondly, the "kick" parameter defines the increase in distance cells move after a collision, which decays in time as cells move away from one another. Two populations of cells (red and green) can be assigned varying values of hook and kick.

Differential adhesion can be simulated by assigning different values of hook to the red or green population of cells (Figure 6.4 D). To assess the real rate of sorting of these *in silico* cells, the parameters of the model were scaled according to real life observations. It was found that the model could account for the extent of segregation by differential adhesion seen using L cells expressing different cadherins in 2D culture (Figure 3.8). However, the model indicates that differential adhesion could not account for the extent of segregation seen between EphB2 receptor and ephrinB1 expressing cells within the observed time interval. To achieve this amount of segregation, using only a difference in adhesion, would take 10 times longer *in silico*.

To simulate directional persistence, kick is applied to green cells after interaction with red cells. When a simulation is run with this condition, cells sort (Figure 6.4 F) more quickly than using differential adhesion alone (manuscript in preparation). Interestingly, this sorting is only seen when a uniform value of hook is applied to all cells, indicating that a basal level of adhesion is required for cell sorting to occur via differential migration (Figure 6.4 I,J). This is consistent with the findings in this study, which suggest that general cohesion between all cells in combination with cell repulsion is sufficient to explain sorting by Eph-ephrins.

Sharpening boundaries as part of the cell sorting process

By understanding cell sorting, we hope to gain some insight into the mechanisms which underlie boundary sharpening *in vivo*. However, there could be different mechanisms underlying these two stages of sorting. For example, sorting can result in a pattern with separate clusters which have rough boundaries (Figure 1.3

A; Foty, 2005) but would nevertheless be classified as sorted. Meanwhile, studies of clones in Drosophila epithelia, for example, are only considered fully sorted when they have smooth boundaries to separate them from the surrounding cells (Chang *et al.*, 2011). The key difference between these situations is the resolution at which they are observed. In the first, hundreds of cells are often considered, whereas a clone can be seen to sort even if it only consists of tens of cells. Thus, in the latter case, sorting will only be apparent based on local changes in the alignment and shape of cells, as opposed to their large-scale rearrangement.

Several mechanisms could be responsible for the long-term maintenance of boundaries in addition to the mechanisms that drive sorting. One is an accumulation of ECM at the boundary between adjacent compartments, which could provide a physical barrier to sorting. This has been demonstrated at boundaries between the zebrafish somites. Here Eph-ephrin signalling activates integrin clustering and fibronectin accumulation which is required for boundary maintenance (Koshida *et al.*, 2005; Julich *et al.*, 2009). Accumulation of fibronectin is also seen at the boundaries between rhombomeres in the chick hindbrain, which may also have a barrier function (Heyman *et al.*, 1993; Lumsden & Keynes, 1989).

Another method of creating a physical barrier between compartments is by the assembly of actin and myosin to create a contractile cable which prevents cell mixing (Landsberg *et al.*, 2009; Major & Irvine, 2005; Monier *et al.*, 2010). As has been discussed previously, this mechanism is involved in the sorting of cells at the dorso-ventral and anterior-posterior compartment boundaries of the Drosophila wing disc as well as at parasegment boundaries, where an enrichment of actin and phospho-myosin II correlates with alignment of cell bonds (Landsberg *et al.*, 2009; Major & Irvine, 2005; Monier *et al.*, 2010). Tension generated by actin and myosin could be an important mechanism for the maintenance of these compartments as well as the original establishment of their boundaries. A recent paper has demonstrated that the generation of acto-myosin tension is required to maintain a sharp boundary after the establishment of clusters by differential adhesion (Chang *et al.*, 2011).

These mechanisms work effectively in an epithelium whose main challenge to cell positioning is cell division. However, in other tissues, there is continuous

movement between cells and more dynamic mechanisms may be required to maintain boundaries. In support of this, acto-myosin cables are not seen at boundaries between somites in zebrafish (Fagotto, ISDB conference talk, 2009) or between hindbrain rhombomeres, although F-actin is enriched in an Eph-ephrin dependent manner at the boundary between ectoderm and mesoderm (Rohani *et al.*, 2011).

Another possibility is that fine-tuning of the mechanisms used for sorting is responsible for maintaining sharp boundaries. Eph-ephrin signalling is capable of driving both sorting and sharpening of the boundary. Sharpening is visible even at high magnification where a change in the shape of cells is particularly visible at the boundary (Figure 4.2 L). In this case, it has been suggested that continued repulsion of the membrane from the boundary at the cellular level is capable of maintaining separation between different cell types (Rohani *et al.*, 2011). This fits with the observations at high magnification that Eph-ephrin signalling drives a collapse response in cells in addition to a sustained change in their direction of migration (Poliakov *et al.*, 2008). Thus, the repulsive mechanisms that underlie directional migration downstream of ephrinB1-EphB2 signalling could also be responsible for the continued repulsion of the membrane required for sharpening of the boundary. As suggested above, in some circumstances Eph-ephrin signalling may result in clustering and increased adhesion between like-cells (Cortina *et al.*, 2007; Parrinello *et al.*, 2010; Solanas *et al.*, 2011). This is another mechanism for long term maintenance of separate regions of cells which could act in addition to continued repulsion to sharpen boundaries.

Cross-talk between adhesion and migration

The data described here are derived from experiments looking at a mixture of single cell responses or responses in a whole organism. It is likely that the different contexts in which cells are observed will influence their behaviour. This is particularly important when considering pathways which link cell-cell adhesion to cell migration since, for example, the dynamics of RhoGTPase activity are likely to vary greatly between cells surrounded with contacts and cells which are totally free of contacts with other cells. In light of this, it would be interesting to study the

dynamics of such molecules *in vivo*. Emerging techniques should allow us to look at high spatial and temporal resolution at the dynamics of Rho and Rac in response to Eph-ephrin signalling and how they are affected in the absence of p120 and N-cad.

It would also be interesting to observe the larger-scale movement of cells in an *in vivo* context. *In vitro*, EphB2 cells move more directionally after contact with ephrinB1 cells even at a high density (Poliakov, unpublished data). Whether this same directed movement away from a boundary is seen *in vivo* is not known, although there is dynamic membrane ruffling at some boundaries (Rohani *et al.*, 2011), and cells do shuffle around in some developing tissues (Marie Breau, unpublished data). Such data suggest that repulsion between Eph-ephrin cells at boundaries could be a common sharpening mechanism, but it would be interesting to track cells to establish how this occurs in a living embryo. This sort of behaviour would require dynamic interactions between cell-cell adhesion and cell-matrix interacting pathways.

Another way of testing these assumptions is to use computer models. Now that the framework exists for modelling cells which have properties based on the cell culture system used in the lab, it will be interesting to exploit this further. For example, it could be extended to considering more dense or three-dimensional mixtures of cells. It would be particularly interesting to adjust the parameters of adhesion and repulsion to determine the extent to which basal cell adhesion is required compared to repulsion in this model. Even more interesting would be to connect the two processes, as the mechanisms are connected through regulation of similar molecules *in vivo*, and to test whether extensive cross-talk between pathways is a requirement for sorting.

Finally, it would be interesting to know more about how Eph-ephrin signalling persists within the cell. For instance, adhesion generally appears to increase in EphB2 cell clusters after EphB2-ephrinB1 signalling. If this is a direct consequence of signalling, does it require that all EphB2 cells come into contact with ephrinB1 cells at one point, or is there a transduction of signals between EphB2 cells at the boundary and EphB2 cells within the cluster? It would be relatively straightforward in the sorting assay to track cells within the clusters to see

whether they do all contact an ephrinB1 cell during the course of sorting, and it would be interesting to find out how this translates to the embryo as well.

Final Comments

We have developed a series of *in vitro* assays which demonstrate that EphB2-ephrinB1 signalling is capable of driving cell sorting and boundary sharpening, and which have allowed us to begin to dissect the mechanisms behind this process.

Using cell sorting and boundary sharpening assays, I have been able to demonstrate that Eph-ephrin mediated cell sorting is unlikely to be driven by the generation of differential adhesion. This is in contrast to some previously published hypotheses (Solanas *et al.*, 2011; Steinberg, 2007), but is supported by the idea that cell repulsion could be the main driving force for sorting downstream of Eph-ephrins (Poliakov *et al.*, 2008; Rohani *et al.*, 2011; Xu *et al.*, 1999). Nevertheless, there is still a requirement for cadherins in basal cell-cell adhesion, which is important for stabilising segregation.

We have shown that EphB2 cells respond to ephrinB1 signalling by increasing their directional migration. N-cadherin also has a role in migration as described in neural crest cells (Theveneau *et al.*, 2010), but this requirement is basal and N-cadherin directed migration is not responsible for the directional migration downstream of ephrinB1-EphB2 interactions.

p120ctn family proteins p120 and p0071 are also required for cell sorting and boundary sharpening through Eph-ephrins, but are differently regulated. It is likely that both are required to assist in cadherin-mediated adhesion and contact inhibition of locomotion. In addition, p120 is specifically required in directional cell migration downstream of Eph-ephrin signalling, suggesting a novel, cadherin-independent role for this protein.

Taken together, these results support a model where EphB2-ephrinB1 interactions cause cell repulsion which drives segregation in the presence of a basal level of cell-cell adhesion. It will be interesting to see the extent to which this model applies to other systems and particularly how it relates to cell segregation and boundary sharpening *in vivo*.

Bibliography

Abe, K. & Takeichi, M. (2008). EPLIN mediates linkage of the cadherin catenin complex to F-actin and stabilizes the circumferential actin belt. *Proc Natl Acad Sci U S A* **105**, 13-19.

Abercrombie, M. & Heaysman, J. E. (1953). Observations on the social behaviour of cells in tissue culture. I. Speed of movement of chick heart fibroblasts in relation to their mutual contacts. *Exp Cell Res* **5**, 111-131.

Abercrombie, M. & Heaysman, J. E. (1954). Observations on the social behaviour of cells in tissue culture. II. Monolayering of fibroblasts. *Exp Cell Res* **6**, 293-306.

Abercrombie, M., Heaysman, J. E. & Karthauser, H. M. (1957). Social behaviour of cells in tissue culture. III. Mutual influence of sarcoma cells and fibroblasts. *Exp Cell Res* **13**, 276-291.

Abercrombie, M. (1962). Contact-dependent behavior of normal cells and the possible significance of surface changes in virus-induced transformation. *Cold Spring Harb Symp Quant Biol* **27**, 427-431.

Abercrombie, M. (1970). Contact inhibition in tissue culture. *In Vitro* **6**, 128-142.

Abercrombie, M. (1979). Contact inhibition and malignancy. *Nature* **281**, 259-262.

Alema, S. & Salvatore, A. M. (2007). p120 catenin and phosphorylation: Mechanisms and traits of an unresolved issue. *Biochim Biophys Acta* **1773**, 47-58.

Anastasiadis, P. Z. (2007). p120-ctn: A nexus for contextual signaling via Rho GTPases. *Biochim Biophys Acta* **1773**, 34-46.

Aono, S., Nakagawa, S., Reynolds, A. B. & Takeichi, M. (1999). p120(ctn) acts as an inhibitory regulator of cadherin function in colon carcinoma cells. *J Cell Biol* **145**, 551-562.

Astin, J. W., Batson, J., Kadir, S., Charlet, J., Persad, R. A., Gillatt, D., Oxley, J. D. & Nobes, C. D. (2010). Competition amongst Eph receptors regulates contact inhibition of locomotion and invasiveness in prostate cancer cells. *Nat Cell Biol* **12**, 1194-1204.

Batlle, E., Henderson, J. T., Beghtel, H. & other authors (2002). Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* **111**, 251-263.

Batlle, E. & Wilkinson, D. G. (2012). Molecular mechanisms of cell segregation and boundary formation in development and tumorigenesis. *Cold Spring Harb Perspect Biol* **4**, a008227.

Blair, S. S. (2001). Cell lineage: compartments and Capricious. *Curr Biol* **11**, R1017-1021.

Boggon, T. J., Murray, J., Chappuis-Flament, S., Wong, E., Gumbiner, B. M. & Shapiro, L. (2002). C-cadherin ectodomain structure and implications for cell adhesion mechanisms. *Science* **296**, 1308-1313.

Borghi, N., Lowndes, M., Maruthamuthu, V., Gardel, M. L. & Nelson, W. J. (2010). Regulation of cell motile behavior by crosstalk between cadherin- and integrin-mediated adhesions. *Proc Natl Acad Sci U S A* **107**, 13324-13329.

Calautti, E., Cabodi, S., Stein, P. L., Hatzfeld, M., Kedersha, N. & Paolo Dotto, G. (1998). Tyrosine phosphorylation and src family kinases control keratinocyte cell-cell adhesion. *J Cell Biol* **141**, 1449-1465.

Calautti, E., Grossi, M., Mammucari, C., Aoyama, Y., Pirro, M., Ono, Y., Li, J. & Dotto, G. P. (2002). Fyn tyrosine kinase is a downstream mediator of Rho/PRK2 function in keratinocyte cell-cell adhesion. *J Cell Biol* **156**, 137-148.

Carmona-Fontaine, C., Matthews, H. & Mayor, R. (2008a). Directional cell migration in vivo: Wnt at the crest. *Cell Adh Migr* **2**, 240-242.

Carmona-Fontaine, C., Matthews, H. K., Kuriyama, S., Moreno, M., Dunn, G. A., Parsons, M., Stern, C. D. & Mayor, R. (2008b). Contact inhibition of locomotion in vivo controls neural crest directional migration. *Nature* **456**, 957-961.

Castano, J., Solanas, G., Casagolda, D., Raurell, I., Villagrasa, P., Bustelo, X. R., Garcia de Herreros, A. & Dunach, M. (2007). Specific phosphorylation of p120-catenin regulatory domain differently modulates its binding to RhoA. *Mol Cell Biol* **27**, 1745-1757.

Cavey, M. & Lecuit, T. (2009). Molecular bases of cell-cell junctions stability and dynamics. *Cold Spring Harb Perspect Biol* **1**, a002998.

Chang, L. H., Chen, P., Lien, M. T., Ho, Y. H., Lin, C. M., Pan, Y. T., Wei, S. Y. & Hsu, J. C. (2011). Differential adhesion and actomyosin cable collaborate to drive Echinoid-mediated cell sorting. *Development* **138**, 3803-3812.

Charrasse, S., Meriane, M., Comunale, F., Blangy, A. & Gauthier-Rouviere, C. (2002). N-cadherin-dependent cell-cell contact regulates Rho GTPases and beta-catenin localization in mouse C2C12 myoblasts. *J Cell Biol* **158**, 953-965.

Chen, X., Kojima, S., Borisy, G. G. & Green, K. J. (2003). p120 catenin associates with kinesin and facilitates the transport of cadherin-catenin complexes to intercellular junctions. *J Cell Biol* **163**, 547-557.

Chen, X. & Gumbiner, B. M. (2006). Paraxial protocadherin mediates cell sorting and tissue morphogenesis by regulating C-cadherin adhesion activity. *J Cell Biol* **174**, 301-313.

Cheng, H. J., Nakamoto, M., Bergemann, A. D. & Flanagan, J. G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. *Cell* **82**, 371-381.

Christensen, J. H., Soerensen, M. B., Linghui, Z., Chen, S. & Jensen, M. O. (2010). Pre-diagnostic digital imaging prediction model to discriminate between malignant melanoma and benign pigmented skin lesion. *Skin Res Technol* **16**, 98-108.

Clarke, J. D. & Lumsden, A. (1993). Segmental repetition of neuronal phenotype sets in the chick embryo hindbrain. *Development* **118**, 151-162.

Clay, M. R. & Halloran, M. C. (2011). Regulation of cell adhesions and motility during initiation of neural crest migration. *Curr Opin Neurobiol* **21**, 17-22.

Cooke, J. E., Kemp, H. A. & Moens, C. B. (2005). EphA4 is required for cell adhesion and rhombomere-boundary formation in the zebrafish. *Curr Biol* **15**, 536-542.

Cortina, C., Palomo-Ponce, S., Iglesias, M. & other authors (2007). EphB-ephrin-B interactions suppress colorectal cancer progression by compartmentalizing tumor cells. *Nat Genet* **39**, 1376-1383.

Curtis, A. S. (1978). Cell-cell recognition: positioning and patterning systems. *Symp Soc Exp Biol* **32**, 51-82.

Dahmann, C. & Basler, K. (1999). Compartment boundaries: at the edge of development. *Trends Genet* **15**, 320-326.

Dahmann, C. & Basler, K. (2000). Opposing transcriptional outputs of Hedgehog signaling and engrailed control compartmental cell sorting at the Drosophila A/P boundary. *Cell* **100**, 411-422.

Dahmann, C., Oates, A. C. & Brand, M. (2011). Boundary formation and maintenance in tissue development. *Nat Rev Genet* **12**, 43-55.

Davis, M. A., Ireton, R. C. & Reynolds, A. B. (2003). A core function for p120-catenin in cadherin turnover. *J Cell Biol* **163**, 525-534.

Davis, M. A. & Reynolds, A. B. (2006). Blocked acinar development, E-cadherin reduction, and intraepithelial neoplasia upon ablation of p120-catenin in the mouse salivary gland. *Dev Cell* **10**, 21-31.

Davis, S., Gale, N. W., Aldrich, T. H., Maisonpierre, P. C., Lhotak, V., Pawson, T., Goldfarb, M. & Yancopoulos, G. D. (1994). Ligands for EPH-related receptor

tyrosine kinases that require membrane attachment or clustering for activity. *Science* **266**, 816-819.

de Rooij, J., Kerstens, A., Danuser, G., Schwartz, M. A. & Waterman-Storer, C. M. (2005). Integrin-dependent actomyosin contraction regulates epithelial cell scattering. *J Cell Biol* **171**, 153-164.

Desai, R. A., Gao, L., Raghavan, S., Liu, W. F. & Chen, C. S. (2009). Cell polarity triggered by cell-cell adhesion via E-cadherin. *J Cell Sci* **122**, 905-911.

DiMilla, P. A., Stone, J. A., Quinn, J. A., Albelda, S. M. & Lauffenburger, D. A. (1993). Maximal migration of human smooth muscle cells on fibronectin and type IV collagen occurs at an intermediate attachment strength. *J Cell Biol* **122**, 729-737.

Dravis, C., Yokoyama, N., Chumley, M. J., Cowan, C. A., Silvany, R. E., Shay, J., Baker, L. A. & Henkemeyer, M. (2004). Bidirectional signaling mediated by ephrin-B2 and EphB2 controls urorectal development. *Dev Biol* **271**, 272-290.

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M. & Bonhoeffer, F. (1995). In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. *Cell* **82**, 359-370.

Duguay, D., Foty, R. A. & Steinberg, M. S. (2003). Cadherin-mediated cell adhesion and tissue segregation: qualitative and quantitative determinants. *Dev Biol* **253**, 309-323.

Dupin, I., Camand, E. & Etienne-Manneville, S. (2009). Classical cadherins control nucleus and centrosome position and cell polarity. *J Cell Biol* **185**, 779-786.

Dzamba, B. J., Jakab, K. R., Marsden, M., Schwartz, M. A. & DeSimone, D. W. (2009). Cadherin adhesion, tissue tension, and noncanonical Wnt signaling regulate fibronectin matrix organization. *Dev Cell* **16**, 421-432.

Eberhart, J., Swartz, M. E., Koblar, S. A., Pasquale, E. B. & Krull, C. E. (2002). EphA4 constitutes a population-specific guidance cue for motor neurons. *Dev Biol* **247**, 89-101.

Ehrlich, J. S., Hansen, M. D. & Nelson, W. J. (2002). Spatio-temporal regulation of Rac1 localization and lamellipodia dynamics during epithelial cell-cell adhesion. *Dev Cell* **3**, 259-270.

Etienne-Manneville, S. & Hall, A. (2001). Integrin-mediated activation of Cdc42 controls cell polarity in migrating astrocytes through PKCzeta. *Cell* **106**, 489-498.

Foty, R. A. & Steinberg, M. S. (2005). The differential adhesion hypothesis: a direct evaluation. *Dev Biol* **278**, 255-263.

Fraser, S., Keynes, R. & Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-435.

Fukumoto, Y., Shintani, Y., Reynolds, A. B., Johnson, K. R. & Wheelock, M. J. (2008). The regulatory or phosphorylation domain of p120 catenin controls E-cadherin dynamics at the plasma membrane. *Exp Cell Res* **314**, 52-67.

Gale, N. W., Holland, S. J., Valenzuela, D. M. & other authors (1996). Eph receptors and ligands comprise two major specificity subclasses and are reciprocally compartmentalized during embryogenesis. *Neuron* **17**, 9-19.

Garrod, D. R. & Steinberg, M. S. (1973). Tissue-specific sorting-out in two dimensions in relation to contact inhibition of cell movement. *Nature* **244**, 568-569.

Garrod, D. R. & Steinberg, M. S. (1975). Cell locomotion within a contact-inhibited monolayer of chick embryonic liver parenchyma cells. *J Cell Sci* **18**, 405-425.

Gavard, J. & Gutkind, J. S. (2006). VEGF controls endothelial-cell permeability by promoting the beta-arrestin-dependent endocytosis of VE-cadherin. *Nat Cell Biol* **8**, 1223-1234.

Gerety, S. S., Wang, H. U., Chen, Z. F. & Anderson, D. J. (1999). Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol Cell* **4**, 403-414.

Godt, D. & Tepass, U. (1998). Drosophila oocyte localization is mediated by differential cadherin-based adhesion. *Nature* **395**, 387-391.

Gonzalez-Reyes, A. & St Johnston, D. (1998). The Drosophila AP axis is polarised by the cadherin-mediated positioning of the oocyte. *Development* **125**, 3635-3644.

Goodwin, M., Kovacs, E. M., Thoreson, M. A., Reynolds, A. B. & Yap, A. S. (2003). Minimal mutation of the cytoplasmic tail inhibits the ability of E-cadherin to activate Rac but not phosphatidylinositol 3-kinase: direct evidence of a role for cadherin-activated Rac signaling in adhesion and contact formation. *J Biol Chem* **278**, 20533-20539.

Green, K. J., Getsios, S., Troyanovsky, S. & Godsel, L. M. (2010). Intercellular junction assembly, dynamics, and homeostasis. *Cold Spring Harb Perspect Biol* **2**, a000125.

Groeger, G. & Nobes, C. D. (2007). Co-operative Cdc42 and Rho signalling mediates ephrinB-triggered endothelial cell retraction. *Biochem J* **404**, 23-29.

Grosheva, I., Shtutman, M., Elbaum, M. & Bershadsky, A. D. (2001). p120 catenin affects cell motility via modulation of activity of Rho-family GTPases: a link

between cell-cell contact formation and regulation of cell locomotion. *J Cell Sci* **114**, 695-707.

Halbleib, J. M. & Nelson, W. J. (2006). Cadherins in development: cell adhesion, sorting, and tissue morphogenesis. *Genes Dev* **20**, 3199-3214.

Hall, A. & Nobes, C. D. (2000). Rho GTPases: molecular switches that control the organization and dynamics of the actin cytoskeleton. *Philos Trans R Soc Lond B Biol Sci* **355**, 965-970.

Harris, A. K. (1976). Is Cell sorting caused by differences in the work of intercellular adhesion? A critique of the Steinberg hypothesis. *J Theor Biol* **61**, 267-285.

Hatta, K. & Takeichi, M. (1986). Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. *Nature* **320**, 447-449.

Hattori, M., Osterfield, M. & Flanagan, J. G. (2000). Regulated cleavage of a contact-mediated axon repellent. *Science* **289**, 1360-1365.

Hatzfeld, M., Green, K. J. & Sauter, H. (2003). Targeting of p0071 to desmosomes and adherens junctions is mediated by different protein domains. *J Cell Sci* **116**, 1219-1233.

Hatzfeld, M. (2007). Plakophilins: Multifunctional proteins or just regulators of desmosomal adhesion? *Biochim Biophys Acta* **1773**, 69-77.

Helmbacher, F., Schneider-Maunoury, S., Topilko, P., Tiret, L. & Charnay, P. (2000). Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons. *Development* **127**, 3313-3324.

Henkemeyer, M., Orioli, D., Henderson, J. T., Saxton, T. M., Roder, J., Pawson, T. & Klein, R. (1996). Nuk controls pathfinding of commissural axons in the mammalian central nervous system. *Cell* **86**, 35-46.

Heyman, I., Kent, A. & Lumsden, A. (1993). Cellular morphology and extracellular space at rhombomere boundaries in the chick embryo hindbrain. *Dev Dyn* **198**, 241-253.

Himanen, J. P., Chumley, M. J., Lackmann, M. & other authors (2004). Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. *Nat Neurosci* **7**, 501-509.

Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S. & Takeichi, M. (1992). Identification of a neural alpha-catenin as a key regulator of cadherin function and multicellular organization. *Cell* **70**, 293-301.

Hofmann, I., Kuhn, C. & Franke, W. W. (2008). Protein p0071, a major plaque protein of non-desmosomal adhering junctions, is a selective cell-type marker. *Cell Tissue Res* **334**, 381-399.

Hofmann, I., Schlechter, T., Kuhn, C., Hergt, M. & Franke, W. W. (2009). Protein p0071 - an armadillo plaque protein that characterizes a specific subtype of adherens junctions. *J Cell Sci* **122**, 21-24.

Holland, S. J., Gale, N. W., Gish, G. D., Roth, R. A., Songyang, Z., Cantley, L. C., Henkemeyer, M., Yancopoulos, G. D. & Pawson, T. (1997). Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. *EMBO J* **16**, 3877-3888.

Hornberger, M. R., Dutting, D., Ciossek, T. & other authors (1999). Modulation of EphA receptor function by coexpressed ephrinA ligands on retinal ganglion cell axons. *Neuron* **22**, 731-742.

Hueck, I. S., Hollweg, H. G., Schmid-Schonbein, G. W. & Artmann, G. M. (2000). Chlorpromazine modulates the morphological macro- and microstructure of endothelial cells. *Am J Physiol Cell Physiol* **278**, C873-878.

Huynh-Do, U., Stein, E., Lane, A. A., Liu, H., Cerretti, D. P. & Daniel, T. O. (1999). Surface densities of ephrin-B1 determine EphB1-coupled activation of cell attachment through alphavbeta3 and alpha5beta1 integrins. *EMBO J* **18**, 2165-2173.

Ikeda, W., Nakanishi, H., Miyoshi, J. & other authors (1999). Afadin: A key molecule essential for structural organization of cell-cell junctions of polarized epithelia during embryogenesis. *J Cell Biol* **146**, 1117-1132.

Inoue, T., Tanaka, T., Takeichi, M., Chisaka, O., Nakamura, S. & Osumi, N. (2001). Role of cadherins in maintaining the compartment boundary between the cortex and striatum during development. *Development* **128**, 561-569.

Ireton, R. C., Davis, M. A., van Hengel, J. & other authors (2002). A novel role for p120 catenin in E-cadherin function. *J Cell Biol* **159**, 465-476.

Irving, C., Nieto, M. A., DasGupta, R., Charnay, P. & Wilkinson, D. G. (1996). Progressive spatial restriction of Sek-1 and Krox-20 gene expression during hindbrain segmentation. *Dev Biol* **173**, 26-38.

Ishiyama, N., Lee, S. H., Liu, S., Li, G. Y., Smith, M. J., Reichardt, L. F. & Ikura, M. (2010). Dynamic and static interactions between p120 catenin and E-cadherin regulate the stability of cell-cell adhesion. *Cell* **141**, 117-128.

Janes, P. W., Saha, N., Barton, W. A. & other authors (2005). Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. *Cell* **123**, 291-304.

Jorgensen, C., Sherman, A., Chen, G. I. & other authors (2009). Cell-specific information processing in segregating populations of Eph receptor ephrin-expressing cells. *Science* **326**, 1502-1509.

Jurney, W. M., Gallo, G., Letourneau, P. C. & McLoon, S. C. (2002). Rac1-mediated endocytosis during ephrin-A2- and semaphorin 3A-induced growth cone collapse. *J Neurosci* **22**, 6019-6028.

Kadison, S. R., Makinen, T., Klein, R., Henkemeyer, M. & Kaprielian, Z. (2006). EphB receptors and ephrin-B3 regulate axon guidance at the ventral midline of the embryonic mouse spinal cord. *J Neurosci* **26**, 8909-8914.

Kashef, J., Kohler, A., Kuriyama, S., Alfandari, D., Mayor, R. & Wedlich, D. (2009). Cadherin-11 regulates protrusive activity in Xenopus cranial neural crest cells upstream of Trio and the small GTPases. *Genes Dev* **23**, 1393-1398.

Keilhack, H., Hellman, U., van Hengel, J., van Roy, F., Godovac-Zimmermann, J. & Bohmer, F. D. (2000). The protein-tyrosine phosphatase SHP-1 binds to and dephosphorylates p120 catenin. *J Biol Chem* **275**, 26376-26384.

Kemp, H. A., Cooke, J. E. & Moens, C. B. (2009). EphA4 and EfnB2a maintain rhombomere coherence by independently regulating intercalation of progenitor cells in the zebrafish neural keel. *Dev Biol* **327**, 313-326.

Kim, S. W., Park, J. I., Spring, C. M., Sater, A. K., Ji, H., Otchere, A. A., Daniel, J. M. & McCrea, P. D. (2004). Non-canonical Wnt signals are modulated by the Kaiso transcriptional repressor and p120-catenin. *Nat Cell Biol* **6**, 1212-1220.

Klein, R. (2009). Bidirectional modulation of synaptic functions by Eph/ephrin signaling. *Nat Neurosci* **12**, 15-20.

Knoll, B. & Drescher, U. (2002). Ephrin-As as receptors in topographic projections. *Trends Neurosci* **25**, 145-149.

Knoll, B. & Drescher, U. (2004). Src family kinases are involved in EphA receptor-mediated retinal axon guidance. *J Neurosci* **24**, 6248-6257.

Koshida, S., Kishimoto, Y., Ustumi, H., Shimizu, T., Furutani-Seiki, M., Kondoh, H. & Takada, S. (2005). Integrinalpha5-dependent fibronectin accumulation for maintenance of somite boundaries in zebrafish embryos. *Dev Cell* **8**, 587-598.

Krendel, M. F. & Bonder, E. M. (1999). Analysis of actin filament bundle dynamics during contact formation in live epithelial cells. *Cell Motil Cytoskeleton* **43**, 296-309.

Krieg, M., Arboleda-Estudillo, Y., Puech, P. H., Kafer, J., Graner, F., Muller, D. J. & Heisenberg, C. P. (2008). Tensile forces govern germ-layer organization in zebrafish. *Nat Cell Biol* **10**, 429-436.

Landsberg, K. P., Farhadifar, R., Ranft, J., Umetsu, D., Widmann, T. J., Bittig, T., Said, A., Julicher, F. & Dahmann, C. (2009). Increased cell bond tension governs cell sorting at the Drosophila anteroposterior compartment boundary. *Curr Biol* **19**, 1950-1955.

Lawrenson, I. D., Wimmer-Kleikamp, S. H., Lock, P., Schoenwaelder, S. M., Down, M., Boyd, A. W., Alewood, P. F. & Lackmann, M. (2002). Ephrin-A5 induces rounding, blebbing and de-adhesion of EphA3-expressing 293T and melanoma cells by CrkII and Rho-mediated signalling. *J Cell Sci* **115**, 1059-1072.

Lecuit, T. & Lenne, P. F. (2007). Cell surface mechanics and the control of cell shape, tissue patterns and morphogenesis. *Nat Rev Mol Cell Biol* **8**, 633-644.

Lecuit, T. (2010). alpha-catenin mechanosensing for adherens junctions. *Nat Cell Biol* **12**, 522-524.

Lee, H. S., Bong, Y. S., Moore, K. B., Soria, K., Moody, S. A. & Daar, I. O. (2006). Dishevelled mediates ephrinB1 signalling in the eye field through the planar cell polarity pathway. *Nat Cell Biol* **8**, 55-63.

Lekanne Deprez, R. H., Fijnvandraat, A. C., Ruijter, J. M. & Moorman, A. F. (2002). Sensitivity and accuracy of quantitative real-time polymerase chain reaction using SYBR green I depends on cDNA synthesis conditions. *Anal Biochem* **307**, 63-69.

Lele, Z., Folchert, A., Concha, M., Rauch, G. J., Geisler, R., Rosa, F., Wilson, S. W., Hammerschmidt, M. & Bally-Cuif, L. (2002). parachute/n-cadherin is required for morphogenesis and maintained integrity of the zebrafish neural tube. *Development* **129**, 3281-3294.

Lemieux, G. A., Blumenkron, F., Yeung, N. & other authors (2007). The low affinity IgE receptor (CD23) is cleaved by the metalloproteinase ADAM10. *J Biol Chem* **282**, 14836-14844.

Lin, J., Luo, J. & Redies, C. (2008). Differential expression of five members of the ADAM family in the developing chicken brain. *Neuroscience* **157**, 360-375.

Litterst, C., Georgakopoulos, A., Shioi, J., Ghersi, E., Wisniewski, T., Wang, R., Ludwig, A. & Robakis, N. K. (2007). Ligand binding and calcium influx induce distinct ectodomain/gamma-secretase-processing pathways of EphB2 receptor. *J Biol Chem* **282**, 16155-16163.

Lumsden, A. & Keynes, R. (1989). Segmental patterns of neuronal development in the chick hindbrain. *Nature* **337**, 424-428.

Luo, Y., Ferreira-Cornwell, M., Baldwin, H., Kostetskii, I., Lenox, J., Lieberman, M. & Radice, G. (2001). Rescuing the N-cadherin knockout by cardiac-specific expression of N- or E-cadherin. *Development* **128**, 459-469.

Machacek, M., Hodgson, L., Welch, C. & other authors (2009). Coordination of Rho GTPase activities during cell protrusion. *Nature* **461**, 99-103.

Magie, C. R., Pinto-Santini, D. & Parkhurst, S. M. (2002). Rho1 interacts with p120ctn and alpha-catenin, and regulates cadherin-based adherens junction components in Drosophila. *Development* **129**, 3771-3782.

Major, R. J. & Irvine, K. D. (2005). Influence of Notch on dorsoventral compartmentalization and actin organization in the Drosophila wing. *Development* **132**, 3823-3833.

Major, R. J. & Irvine, K. D. (2006). Localization and requirement for Myosin II at the dorsal-ventral compartment boundary of the Drosophila wing. *Dev Dyn* **235**, 3051-3058.

Manning, M. L., Foty, R. A., Steinberg, M. S. & Schoetz, E. M. (2010). Coaction of intercellular adhesion and cortical tension specifies tissue surface tension. *Proc Natl Acad Sci U S A* **107**, 12517-12522.

Mariner, D. J., Anastasiadis, P., Keilhack, H., Bohmer, F. D., Wang, J. & Reynolds, A. B. (2001). Identification of Src phosphorylation sites in the catenin p120ctn. *J Biol Chem* **276**, 28006-28013.

Mariner, D. J., Davis, M. A. & Reynolds, A. B. (2004). EGFR signaling to p120-catenin through phosphorylation at Y228. *J Cell Sci* **117**, 1339-1350.

Marsden, M. & DeSimone, D. W. (2003). Integrin-ECM interactions regulate cadherin-dependent cell adhesion and are required for convergent extension in Xenopus. *Curr Biol* **13**, 1182-1191.

Marston, D. J., Dickinson, S. & Nobes, C. D. (2003). Rac-dependent trans-endocytosis of ephrinBs regulates Eph-ephrin contact repulsion. *Nat Cell Biol* **5**, 879-888.

Martens, L., Monsieur, G., Ampe, C., Gevaert, K. & Vandekerckhove, J. (2006). Cell_motility: a cross-platform, open source application for the study of cell motion paths. *BMC Bioinformatics* **7**, 289.

Mashanov, G. I. & Molloy, J. E. (2007). Automatic detection of single fluorophores in live cells. *Biophys J* **92**, 2199-2211.

Mayor, R. & Carmona-Fontaine, C. (2010). Keeping in touch with contact inhibition of locomotion. *Trends Cell Biol* **20**, 319-328.

McCrea, P. D. & Gu, D. (2010). The catenin family at a glance. *J Cell Sci* **123**, 637-642.

Mellitzer, G., Xu, Q. & Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.

Meng, W. & Takeichi, M. (2009). Adherens junction: molecular architecture and regulation. *Cold Spring Harb Perspect Biol* **1**, a002899.

Meng, Y. G., Han, W. D., Zhao, Y. L., Huang, K., Si, Y. L., Wu, Z. Q. & Mu, Y. M. (2007). Induction of the LRP16 gene by estrogen promotes the invasive growth of Ishikawa human endometrial cancer cells through the downregulation of E-cadherin. *Cell Res* **17**, 869-880.

Milan, M., Weihe, U., Perez, L. & Cohen, S. M. (2001). The LRR proteins capricious and Tartan mediate cell interactions during DV boundary formation in the Drosophila wing. *Cell* **106**, 785-794.

Milan, M., Perez, L. & Cohen, S. M. (2005). Boundary formation in the Drosophila wing: functional dissection of Capricious and Tartan. *Dev Dyn* **233**, 804-810.

Miura, K., Nam, J. M., Kojima, C., Mochizuki, N. & Sabe, H. (2009). EphA2 engages Git1 to suppress Arf6 activity modulating epithelial cell-cell contacts. *Mol Biol Cell* **20**, 1949-1959.

Mochizuki, A., Wada, N., Ide, H. & Iwasa, Y. (1998). Cell-cell adhesion in limb-formation, estimated from photographs of cell sorting experiments based on a spatial stochastic model. *Dev Dyn* **211**, 204-214.

Moens, C. B., Yan, Y. L., Appel, B., Force, A. G. & Kimmel, C. B. (1996). valentino: a zebrafish gene required for normal hindbrain segmentation. *Development* **122**, 3981-3990.

Moissoglu, K. & Schwartz, M. A. (2006). Integrin signalling in directed cell migration. *Biol Cell* **98**, 547-555.

Monier, B., Pelissier-Monier, A., Brand, A. H. & Sanson, B. (2010). An actomyosin-based barrier inhibits cell mixing at compartmental boundaries in Drosophila embryos. *Nat Cell Biol* **12**, 60-65; sup pp 61-69.

Monschau, B., Kremoser, C., Ohta, K. & other authors (1997). Shared and distinct functions of RAGS and ELF-1 in guiding retinal axons. *EMBO J* **16**, 1258-1267.

Moscona, A. & Moscona, H. (1952). The dissociation and aggregation of cells from organ rudiments of the early chick embryo. *J Anat* **86**, 287-301.

Murai, K. K. & Pasquale, E. B. (2003). 'Eph'ective signaling: forward, reverse and crosstalk. *J Cell Sci* **116**, 2823-2832.

Nakagawa, S. & Takeichi, M. (1995). Neural crest cell-cell adhesion controlled by sequential and subpopulation-specific expression of novel cadherins. *Development* **121**, 1321-1332.

Nakagawa, S. & Takeichi, M. (1998). Neural crest emigration from the neural tube depends on regulated cadherin expression. *Development* **125**, 2963-2971.

Nakagawa, S., Brennan, C., Johnson, K. G., Shewan, D., Harris, W. A. & Holt, C. E. (2000). Ephrin-B regulates the Ipsilateral routing of retinal axons at the optic chiasm. *Neuron* **25**, 599-610.

Niessen, C. M. & Gumbiner, B. M. (2002). Cadherin-mediated cell sorting not determined by binding or adhesion specificity. *J Cell Biol* **156**, 389-399.

Noren, N. K., Liu, B. P., Burridge, K. & Kreft, B. (2000). p120 catenin regulates the actin cytoskeleton via Rho family GTPases. *J Cell Biol* **150**, 567-580.

Noren, N. K. & Pasquale, E. B. (2004). Eph receptor-ephrin bidirectional signals that target Ras and Rho proteins. *Cell Signal* **16**, 655-666.

Orsulic, S. & Kemler, R. (2000). Expression of Eph receptors and ephrins is differentially regulated by E-cadherin. *J Cell Sci* **113 (Pt 10)**, 1793-1802.

Oshima, T., Akaike, M., Yoshihara, K. & other authors (2008). Overexpression of EphA4 gene and reduced expression of EphB2 gene correlates with liver metastasis in colorectal cancer. *Int J Oncol* **33**, 573-577.

Ozawa, M. & Ohkubo, T. (2001). Tyrosine phosphorylation of p120(ctn) in v-Src transfected L cells depends on its association with E-cadherin and reduces adhesion activity. *J Cell Sci* **114**, 503-512.

Palacios, F., Tushir, J. S., Fujita, Y. & D'Souza-Schorey, C. (2005). Lysosomal targeting of E-cadherin: a unique mechanism for the down-regulation of cell-cell adhesion during epithelial to mesenchymal transitions. *Mol Cell Biol* **25**, 389-402.

Pankov, R., Endo, Y., Even-Ram, S., Araki, M., Clark, K., Cukierman, E., Matsumoto, K. & Yamada, K. M. (2005). A Rac switch regulates random versus directionally persistent cell migration. *J Cell Biol* **170**, 793-802.

Park, K. S. & Gumbiner, B. M. (2010). Cadherin 6B induces BMP signaling and de-epithelialization during the epithelial mesenchymal transition of the neural crest. *Development* **137**, 2691-2701.

Parrinello, S., Napoli, I., Ribeiro, S. & other authors (2010). EphB signaling directs peripheral nerve regeneration through Sox2-dependent Schwann cell sorting. *Cell* **143**, 145-155.

Pasini, A. & Wilkinson, D. G. (2002). Stabilizing the regionalisation of the developing vertebrate central nervous system. *Bioessays* **24**, 427-438.

Pasquale, E. B. (2005). Eph receptor signalling casts a wide net on cell behaviour. *Nat Rev Mol Cell Biol* **6**, 462-475.

Pasquale, E. B. (2008). Eph-ephrin bidirectional signaling in physiology and disease. *Cell* **133**, 38-52.

Pasquale, E. B. (2010). Eph receptors and ephrins in cancer: bidirectional signalling and beyond. *Nat Rev Cancer* **10**, 165-180.

Poliakov, A., Cotrina, M. & Wilkinson, D. G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Dev Cell* **7**, 465-480.

Poliakov, A., Cotrina, M. L., Pasini, A. & Wilkinson, D. G. (2008). Regulation of EphB2 activation and cell repulsion by feedback control of the MAPK pathway. *J Cell Biol* **183**, 933-947.

Price, S. R., De Marco Garcia, N. V., Ranscht, B. & Jessell, T. M. (2002). Regulation of motor neuron pool sorting by differential expression of type II cadherins. *Cell* **109**, 205-216.

Redies, C. & Takeichi, M. (1996). Cadherins in the developing central nervous system: an adhesive code for segmental and functional subdivisions. *Dev Biol* **180**, 413-423.

Reynolds, A. B., Roesel, D. J., Kanner, S. B. & Parsons, J. T. (1989). Transformation-specific tyrosine phosphorylation of a novel cellular protein in chicken cells expressing oncogenic variants of the avian cellular src gene. *Mol Cell Biol* **9**, 629-638.

Reynolds, A. B., Herbert, L., Cleveland, J. L., Berg, S. T. & Gaut, J. R. (1992). p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo. *Oncogene* **7**, 2439-2445.

Reynolds, A. B., Daniel, J. M., Mo, Y. Y., Wu, J. & Zhang, Z. (1996). The novel catenin p120cas binds classical cadherins and induces an unusual morphological phenotype in NIH3T3 fibroblasts. *Exp Cell Res* **225**, 328-337.

Reynolds, A. B. & Roczniak-Ferguson, A. (2004). Emerging roles for p120-catenin in cell adhesion and cancer. *Oncogene* **23**, 7947-7956.

Reynolds, A. B. (2007). p120-catenin: Past and present. *Biochim Biophys Acta* **1773**, 2-7.

Rohani, N., Canty, L., Luu, O., Fagotto, F. & Winklbauer, R. (2011). EphrinB/EphB signaling controls embryonic germ layer separation by contact-induced cell detachment. *PLoS Biol* **9**, e1000597.

Schneider-Maunoury, S., Topilko, P., Seitandou, T., Levi, G., Cohen-Tannoudji, M., Pournin, S., Babinet, C. & Charnay, P. (1993). Disruption of Krox-20 results

in alteration of rhombomeres 3 and 5 in the developing hindbrain. *Cell* **75**, 1199-1214.

Schotz, E. M., Burdine, R. D., Julicher, F., Steinberg, M. S., Heisenberg, C. P. & Foty, R. A. (2008). Quantitative differences in tissue surface tension influence zebrafish germ layer positioning. *HFSP J* **2**, 42-56.

Shamah, S. M., Lin, M. Z., Goldberg, J. L. & other authors (2001). EphA receptors regulate growth cone dynamics through the novel guanine nucleotide exchange factor ephexin. *Cell* **105**, 233-244.

Shan, W. S., Tanaka, H., Phillips, G. R., Arndt, K., Yoshida, M., Colman, D. R. & Shapiro, L. (2000). Functional cis-heterodimers of N- and R-cadherins. *J Cell Biol* **148**, 579-590.

Shi, B., Laudon, V., Yu, S., Dong, D., Zhu, Y. & Xu, Z. (2008). E-cadherin tissue expression and urinary soluble forms of E-cadherin in patients with bladder transitional cell carcinoma. *Urol Int* **81**, 320-324.

Shoval, I., Ludwig, A. & Kalcheim, C. (2007). Antagonistic roles of full-length N-cadherin and its soluble BMP cleavage product in neural crest delamination. *Development* **134**, 491-501.

Simon, H. & Lumsden, A. (1993). Rhombomere-specific origin of the contralateral vestibulo-acoustic efferent neurons and their migration across the embryonic midline. *Neuron* **11**, 209-220.

Sobieszczuk, D. F. & Wilkinson, D. G. (1999). Masking of Eph receptors and ephrins. *Curr Biol* **9**, R469-470.

Solanas, G., Cortina, C., Sevillano, M. & Batlle, E. (2011). Cleavage of E-cadherin by ADAM10 mediates epithelial cell sorting downstream of EphB signalling. *Nat Cell Biol* **13**, 1100-1107.

Sperry, R. W. (1963). Chemoaffinity in the Orderly Growth of Nerve Fiber Patterns and Connections. *Proc Natl Acad Sci U S A* **50**, 703-710.

Spiering, D. & Hodgson, L. (2011). Dynamics of the Rho-family small GTPases in actin regulation and motility. *Cell Adh Migr* **5**, 170-180.

Steinberg, M. S. (1962). On the mechanism of tissue reconstruction by dissociated cells. I. Population kinetics, differential adhesiveness. and the absence of directed migration. *Proc Natl Acad Sci U S A* **48**, 1577-1582.

Steinberg, M. S. (1963). "ECM": its nature, origin and function in cell aggregation. *Exp Cell Res* **30**, 257-279.

Steinberg, M. S. (1970). Does differential adhesion govern self-assembly processes in histogenesis? Equilibrium configurations and the emergence of a hierarchy among populations of embryonic cells. *J Exp Zool* **173**, 395-433.

Steinberg, M. S. (2007). Differential adhesion in morphogenesis: a modern view. *Curr Opin Genet Dev* **17**, 281-286.

Stemmler, M. P. (2008). Cadherins in development and cancer. *Mol Biosyst* **4**, 835-850.

Suetterlin, P., Marler, K. M. & Drescher, U. (2011). Axonal ephrinA/EphA interactions, and the emergence of order in topographic projections. *Semin Cell Dev Biol* **23**, 1-6.

Suzuki, S. C. & Takeichi, M. (2008). Cadherins in neuronal morphogenesis and function. *Dev Growth Differ* **50 Suppl 1**, S119-130.

Tai, K. K. & Truong, D. D. (2007). NMDA receptor-mediated excitotoxicity contributes to the cerebral hypoxic injury of a rat model of posthypoxic myoclonus. *Brain Res* **1133**, 209-215.

Takai, Y. & Nakanishi, H. (2003). Nectin and afadin: novel organizers of intercellular junctions. *J Cell Sci* **116**, 17-27.

Takeichi, M. & Abe, K. (2005). Synaptic contact dynamics controlled by cadherin and catenins. *Trends Cell Biol* **15**, 216-221.

Takeichi, M. (2011). Self-organization of animal tissues: cadherin-mediated processes. *Dev Cell* **21**, 24-26.

Tanaka, M., Kamo, T., Ota, S. & Sugimura, H. (2003). Association of Dishevelled with Eph tyrosine kinase receptor and ephrin mediates cell repulsion. *EMBO J* **22**, 847-858.

Taylor, W., Katsimitsoulia, Z. & Poliakov, A. (2011). Simulation of cell movement and interaction. *J Bioinform Comput Biol* **9**, 91-110.

Tepass, U., Godt, D. & Winklbauer, R. (2002). Cell sorting in animal development: signalling and adhesive mechanisms in the formation of tissue boundaries. *Curr Opin Genet Dev* **12**, 572-582.

Theveneau, E., Marchant, L., Kuriyama, S., Gull, M., Moepps, B., Parsons, M. & Mayor, R. (2010). Collective chemotaxis requires contact-dependent cell polarity. *Dev Cell* **19**, 39-53.

Thoreson, M. A., Anastasiadis, P. Z., Daniel, J. M., Ireton, R. C., Wheelock, M. J., Johnson, K. R., Hummingbird, D. K. & Reynolds, A. B. (2000). Selective uncoupling of p120(ctn) from E-cadherin disrupts strong adhesion. *J Cell Biol* **148**, 189-202.

Tossell, K., Andreae, L. C., Cudmore, C., Lang, E., Muthukrishnan, U., Lumsden, A., Gilthorpe, J. D. & Irving, C. (2011). Lrrn1 is required for formation of the midbrain-hindbrain boundary and organiser through regulation of affinity differences between midbrain and hindbrain cells in chick. *Dev Biol* **352**, 341-352.

Townes, P. L., Holtfreter, J. (1955). Directed movements and selective adhesion of embryonic amphibian cells. *Journal of Experimental Zoology* **128**, 53-120.

Trinkaus, J. P. & Groves, P. W. (1955). Differentiation in Culture of Mixed Aggregates of Dissociated Tissue Cells. *Proc Natl Acad Sci U S A* **41**, 787-795.

Triplett, J. W. & Feldheim, D. A. (2011). Eph and ephrin signaling in the formation of topographic maps. *Semin Cell Dev Biol* **23**, 7-15.

Uemura, K., Kihara, T., Kuzuya, A., Okawa, K., Nishimoto, T., Ninomiya, H., Sugimoto, H., Kinoshita, A. & Shimohama, S. (2006). Characterization of sequential N-cadherin cleavage by ADAM10 and PS1. *Neurosci Lett* **402**, 278-283.

Wada, N., Tanaka, H., Ide, H. & Nohno, T. (2003). Ephrin-A2 regulates position-specific cell affinity and is involved in cartilage morphogenesis in the chick limb bud. *Dev Biol* **264**, 550-563.

Wahl, S., Barth, H., Ciossek, T., Aktories, K. & Mueller, B. K. (2000). Ephrin-A5 induces collapse of growth cones by activating Rho and Rho kinase. *J Cell Biol* **149**, 263-270.

Watanabe, T., Sato, K. & Kaibuchi, K. (2009). Cadherin-mediated intercellular adhesion and signaling cascades involving small GTPases. *Cold Spring Harb Perspect Biol* **1**, a003020.

Weis, W. I. & Nelson, W. J. (2006). Re-solving the cadherin-catenin-actin conundrum. *J Biol Chem* **281**, 35593-35597.

Weston, J. A. & Abercrombie, M. (1967). Cell mobility in fused homo- and heteronomic tissue fragments. *J Exp Zool* **164**, 317-323.

Wildenberg, G. A., Dohn, M. R., Carnahan, R. H., Davis, M. A., Lobdell, N. A., Settleman, J. & Reynolds, A. B. (2006). p120-catenin and p190RhoGAP regulate cell-cell adhesion by coordinating antagonism between Rac and Rho. *Cell* **127**, 1027-1039.

Wilkinson, D. G. (2001). Multiple roles of EPH receptors and ephrins in neural development. *Nat Rev Neurosci* **2**, 155-164.

Williams, S. E., Mann, F., Erskine, L. & other authors (2003). Ephrin-B2 and EphB1 mediate retinal axon divergence at the optic chiasm. *Neuron* **39**, 919-935.

Wimmer-Kleikamp, S. H., Janes, P. W., Squire, A., Bastiaens, P. I. & Lackmann, M. (2004). Recruitment of Eph receptors into signaling clusters does not require ephrin contact. *J Cell Biol* **164**, 661-666.

Winklbauer, R. (2009). Cell adhesion in amphibian gastrulation. *Int Rev Cell Mol Biol* **278**, 215-275.

Wolf, A., Keil, R., Gotzl, O., Mun, A., Schwarze, K., Lederer, M., Huttelmaier, S. & Hatzfeld, M. (2006). The armadillo protein p0071 regulates Rho signalling during cytokinesis. *Nat Cell Biol* **8**, 1432-1440.

Xiao, K., Allison, D. F., Kottke, M. D., Summers, S., Sorescu, G. P., Faundez, V. & Kowalczyk, A. P. (2003). Mechanisms of VE-cadherin processing and degradation in microvascular endothelial cells. *J Biol Chem* **278**, 19199-19208.

Xiao, K., Garner, J., Buckley, K. M., Vincent, P. A., Chiasson, C. M., Dejana, E., Faundez, V. & Kowalczyk, A. P. (2005). p120-Catenin regulates clathrin-dependent endocytosis of VE-cadherin. *Mol Biol Cell* **16**, 5141-5151.

Xu, Q., Mellitzer, G., Robinson, V. & Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-271.

Yamada, S., Pokutta, S., Drees, F., Weis, W. I. & Nelson, W. J. (2005). Deconstructing the cadherin-catenin-actin complex. *Cell* **123**, 889-901.

Yamada, S. & Nelson, W. J. (2007). Localized zones of Rho and Rac activities drive initiation and expansion of epithelial cell-cell adhesion. *J Cell Biol* **178**, 517-527.

Yanagisawa, M., Huveldt, D., Kreinest, P., Lohse, C. M., Cheville, J. C., Parker, A. S., Copland, J. A. & Anastasiadis, P. Z. (2008). A p120 catenin isoform switch affects Rho activity, induces tumor cell invasion, and predicts metastatic disease. *J Biol Chem* **283**, 18344-18354.

Yap, A. S., Niessen, C. M. & Gumbiner, B. M. (1998). The juxtamembrane region of the cadherin cytoplasmic tail supports lateral clustering, adhesive strengthening, and interaction with p120ctn. *J Cell Biol* **141**, 779-789.

Yap, A. S., Crampton, M. S. & Hardin, J. (2007). Making and breaking contacts: the cellular biology of cadherin regulation. *Curr Opin Cell Biol* **19**, 508-514.

Yokoyama, N., Romero, M. I., Cowan, C. A., Galvan, P., Helmbacher, F., Charnay, P., Parada, L. F. & Henkemeyer, M. (2001). Forward signaling mediated by ephrin-B3 prevents contralateral corticospinal axons from recrossing the spinal cord midline. *Neuron* **29**, 85-97.

Yoshida, C. & Takeichi, M. (1982). Teratocarcinoma cell adhesion: identification of a cell-surface protein involved in calcium-dependent cell aggregation. *Cell* **28**, 217-224.

Zantek, N. D., Azimi, M., Fedor-Chaiken, M., Wang, B., Brackenbury, R. & Kinch, M. S. (1999). E-cadherin regulates the function of the EphA2 receptor tyrosine kinase. *Cell Growth Differ* **10**, 629-638.

Zhang, G., Fenyo, D. & Neubert, T. A. (2008). Screening for EphB signaling effectors using SILAC with a linear ion trap-orbitrap mass spectrometer. *J Proteome Res* **7**, 4715-4726.

Zhang, Y., Sivasankar, S., Nelson, W. J. & Chu, S. (2009). Resolving cadherin interactions and binding cooperativity at the single-molecule level. *Proc Natl Acad Sci U S A* **106**, 109-114.

Zimmer, M., Palmer, A., Kohler, J. & Klein, R. (2003). EphB-ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion. *Nat Cell Biol* **5**, 869-878.