The role and regulation of cell matrix adhesions during contact inhibition of locomotion

Alice Roycroft

A thesis submitted in partial fulfilment for the degree of Doctor of Philosophy

University College London

March 2017

I, Alice Roycroft confirm 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.

Abstract

Contact inhibition of locomotion (CIL) was first characterised over 60 years ago and defined as the behaviour of a cell to cease its continued migration in the same direction upon a collision with another cell. It has been implicated in multiple developmental processes including the precise dispersion of haemocytes and the directional migration of the cranial neural crest. In addition its absence has been linked to metastasis in cancer. Although many molecular mechanisms have recently been implicated in CIL, the role of cell-matrix adhesions (CMAs) during this process remains unknown. It has been hypothesised that cellular forces play an important role in CIL; however the role of traction forces generated via CMAs and intercellular tension during CIL is unclear. In this present study neural crest cells are used to elucidate the role and regulation of CMAs during CIL. The findings presented here demonstrate a rapid disassembly of CMAs near the cell-cell contact between colliding cells. This disassembly is shown to be dependent upon the formation of N-cadherin based cell-cell adhesions and driven by Src and FAK kinase activity. Furthermore this rapid disassembly of CMAs during CIL leads to a redistribution of intercellular forces from the substrate to the cell-cell contact and is essential to drive cell-cell separation after a collision.

Acknowledgments

I would like to start by thanking all those who have contributed to the work presented in this thesis. In particular I would like to thank my supervisor Prof. Roberto Mayor for the invaluable guidance and supervision I have received. I would also like to thank Prof. Maddy Parsons who performed the imaging and analysis for the FRET experiments, Dr. András Szabó who calculated the tension across the contact, Isabel Bahm who ran the Western Blots, and Iain Birkett who helped with the illustrations. In addition I would like to thank the entire Mayor lab for the camaraderie and the Patel lab for their friendship and support over the last 5 years.

I would also like to thank my friends and family who have helped and encouraged me along the way: my Mum for her unconditional support and nurturing the 'frogologist' in me, my Dad who taught me the concept of wave-particle duality from an early age, and my siblings who understand how science 'only adds'. In addition I would like to thank my partner Iain whose unwavering patience and understanding has made all the difference. I have also been very lucky to have been inspired and encouraged by some amazing teachers. In particular Mr. Clayton who ignited my passion for literature, Mr. Peck who introduced me to the world of science, and Dr. Byfield who I wanted to be when I grew up, they have each made an incredible impact on my life and I owe these teachers so much.

Bernard of Chartres famously mused that new findings are built on previous observations and discoveries like 'dwarfs standing on the shoulders of giants'. So finally I wish to thank all the giants.

Table of contents

List of figures and tables

List of supplementary movies

Movie 1 Cell-matrix adhesions near the contact during CIL

Zoom of the contact region of neural crest cells undergoing CIL. Cells expressing memraneRFP (red) and GFP-FAK (green). Frames every 30 seconds. Scale bar 20 μm.

Movie 2 Neural crest cells undergoing a collision

Live-imaging of neural crest cells colliding. Conditions either control, cells treated with 2 μM of PF-573228 or 5 μM of SU6656. Cells expressing nuclearRGFP (red), membraneGFP (green) and also imaged with brightfield. Cells collide at 0 min. Frames every 3 minutes. Scale bar 20 μm.

Movie 3 Cell-matrix adhesion ablation at edge of control cell

Neural crest cell expressing membraneRFP (red) and GFP-FAK (green). CMA ablated at the edge for 30 seconds from t = 0 min in region illustrated as yellow box on second repeat. Frames every 30 seconds. Scale bar 20 μm.

Movie 4 Cells with no ablation

Neural crest cells expressing membraneRFP. Either control or treated with 2 μM of PF-573228. Frames every 30 seconds. Scale bar 20 μm.

Movie 5 Cell-matrix adhesions ablated near the cell-cell contact

Neural crest cells expressing membraneRFP (Red) and GFP-FAK (green). Either control or treated with 2 μM of PF-573228. CMAs ablated near the contact for 30 seconds from t=0 in region illustrated as yellow box on second repeat. Frames every 30 seconds. Scale bar 20 μm.

Movie 6 Cell-cell adhesion ablated

Neural crest cells expressing membraneRFP. Either control or treated with 2 μM of PF-573228. CCA ablated for 30 seconds from t=0 in region illustrated as yellow box on second repeat. Frames every 30 seconds. Scale bar 20 μm.

List of abbreviations

ADAM	A disintegrin and metalloprotease
BA	Blocking antibody
BMP	Bone morphogenetic protein
CCA	Cell-cell adhesion
CIL	Contact inhibition of locomotion
CMA	Cell-matrix adhesions
CNC	Cranial neural crest
Cx43	Connexion 43
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
EMT	Epithelial-to-mesenchymal transition
F-actin	Filamentous actin
FAK	Focal adhesion kinase
FAT	Focal adhesion targeting
FERM	Four-point-one, ezrin, radixin, moesin
FGF	Fibroblast grown factor
FRET	Fluorescence/Förster resonance energy transfer
GAP	GTPases activating protein
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
Hif	Hypoxia-inducible factor
IRM	Interference reflection microscopy
MAPK	Mitogen-activated protein kinase
MDCK	Madin-Darby Canine Kidney
MET	Mesenchymal-to-epithelial transition
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MO	Morpholino oligomer
mRFP	Membrane-red fluorescent protein
NC	Neural crest

nRFP	Nuclear-red fluorescent protein
PAK	p21-activated kinase
PCP	Planar cell polarity
PDGF	Platelet-derived growth factor
PI3-kinase	Phosphoinositide-4,5-bisphosphate 3-kinase
ROCK	Rho kinase
SDF	Stromal cell-derived factor
TFM	Traction force microscopy
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metalloproteases
VASP	Vasodilator-stimulated phosphoprotein
VEGF	Vascular endothelial growth factor
YAP	Yes-associated protein

1. Introduction

1.1 Cell-matrix adhesions

Cell-matrix adhesions (CMAs) are large dynamic transmembrane multi-protein complexes that crosslink the extracellular matrix (ECM) to the intracellular cytoskeleton via integrin and adapter proteins. The ability of CMAs to couple the cytoskeleton to the ECM allows them to generate both a physical and a regulatory connection. This connection can result in force generation, cytoskeletal rearrangements and the activation of many signalling pathways leading to the regulation of cell migration, proliferation, gene expression and cell survival (Wolfenson et al., 2013).

The presence of CMAs was first speculated upon in the early decades of the 1900s (Harrison, 1911; Horwitz, 2012; Lewis, 1922), although it was not until the 1960s that they were first visualised thanks to the development of interference reflection microscopy (IRM) (Curtis, 1964; Izzard and Lochner, 1976). It was at this time that CMAs were shown to bind to the intracellular cytoskeleton (Heath and Dunn, 1978; Izzard and Lochner, 1976) as well as the ECM (Hynes and Destree, 1978). The ability of CMAs to regulate so many different processes relies on their capacity to bind to a vast range of adapter proteins, scaffolds and signalling proteins with hundreds of different proteins being identified as components of CMA complexes (Horton et al., 2015; Wolfenson et al., 2013; Zamir and Geiger, 2001).

1.1.1 Structure and Assembly of CMAs

Integrin-based CMAs are the most common and widely studied form of CMAs. There are other forms of CMAs, such as those composed of syndecans and selectins but these will not be discussed further here. Please refer to these reviews for a more in-depth discussion on these forms of CMAs (Barthel et al., 2007; Carey, 1997; Tedder et al., 1995; Woods and Couchman, 2001). Integrin heterodimers form the core basis of integrin-based CMAs and are transmembrane receptor proteins, so called ‘to denote [their] role as an integral membrane complex involved in the transmembrane association between the extracellular matrix and the cytoskeleton’ (Tamkun et al., 1986). They form a heterodimer consisting of an α and a β subunit. The many different

α and β subunits result in a wide range of possible combinations and give rise to the specificity for various ECM components, such as for different laminins, collagens and fibronectin. In humans 18 different α subunits and 8 different β subunits have been identified. Together they can form a minimum of 24 different combinations with specificity for various components of the ECM (Bökel and Brown, 2002). This specificity facilitates the migration of cells to the correct location during embryogenesis and tissue morphogenesis (Bökel and Brown, 2002).

Integrin activation

Integrin-mediated adhesion is a tightly regulated process. Integrins are found in the plasma membrane in an inactive bent conformation where they are neither bound to the ECM nor the cytoskeleton. To become fully activated integrins need to change their conformation to an extended open conformation via the intermediate low affinity extended but closed conformation (Fig. 1.1). This conformational change and subsequent activation can be driven by different mechanisms, namely inside-out activation or outside-in activation (Wehrle-Haller, 2012a). Outside-in activation requires the initial binding of the extracellular domain of integrin to its ligand in the ECM. This binding pulls on integrin and induces a conformational change in the integrin heterodimer that causes the separation of the α and β subunits and triggers the extension of the cytoplasmic tails (Kim et al., 2003; Yu et al., 2012). The unfurling of the cytoplasmic tail exposes a binding site for the adapter protein talin, which facilitates the binding of integrin to the actin cytoskeleton (Miyamoto et al., 1995). Inside-out activation denotes the reverse direction of activation. Intercellular signals induce the activation of talin or kindlin that allows them to bind to the cytoplasmic tail of β integrin subunit at sites of actin polymerisation (Watanabe et al., 2008). The binding of talin to integrin disrupts a salt bridge between the integrin α and β subunits inducing a conformation extension of integrin exposing a higher affinity binding site for its ligand in the ECM. In addition it induces a separation of the cytoplasmic domains exposing binding sites for adapter proteins (Anthis et al., 2009; Calderwood, 2004; Watanabe et al., 2008; Wehrle-Haller, 2012a). Kindlin acts as a co-activator of integrin and its binding also induces changes in the integrin heterodimer (Karaköse et al., 2010; Moser et al., 2008).

Crosslinking integrin to actin cytoskeleton

Once integrin dimers are in their active conformation and crosslinked to the ECM and intracellular cytoskeleton, integrin clustering occurs. Clustering is the process by which integrin heterodimers in the plasma membrane come together. Due to the relatively low binding affinity of integrin for the ECM, clustering of active integrins bound to the ECM is critical to form large multiprotein complexes that facilitate the strong anchoring of the actin cytoskeleton to the ECM via the CMA complexes. Integrin clustering is triggered by the binding of talin to the cytoplasmic tail of integrin following integrin binding to its ECM ligand (Cluzel et al., 2005). There is still some uncertainty as to whether integrin clustering occurs before or after integrin activation (Ginsberg et al., 2005). Integrins bind directly to their ECM ligand, however it is believed they do not directly bind actin, instead they can be coupled to actin by a vast number of different adapter proteins. These adapter proteins can either bind directly to both integrin and actin, such as talin (Horwitz et al., 1986) and α-actinin (Otey et al., 1990), directly to integrin and other adapter proteins, such as paxillin (Liu et al., 1999) or directly to actin and other adapter proteins, such as vinculin (Burridge and Mangeat, 1984; Johnson and Craig, 1995). All these adapter proteins help crosslink integrins to filamentous actin (F-actin) within the cell and are vital for the role of CMAs in facilitating cell migration. Actomysosin driven contractile forces lead to further clustering of integrin resulting in the recruitment of more proteins and enhancing the size and strength of the CMAs (Yu et al., 2011).

FAK and Src

In addition to their ability to crosslink the ECM to the cytoskeleton, CMAs also recruit a vast number of signalling proteins such as focal adhesion kinase (FAK), Src and p130Cas, a key scaffolding protein that can bind to many proteins and facilitate many of the downstream functions of CMAs (Fig. 1.2) (Mitra et al., 2005; Wozniak et al., 2004). Src and FAK are both tyrosine kinases. Src, so named due its discovery as a proto-oncogene in sarcomas, is 60kDa in size and consists of three domains, the SH2 and SH3 domains, which play a role in protein-protein interactions, and the kinase catalytic domain (Roskoski, 2015). It can regulate many cellular functions including cell

migration, growth and differentiation through its ability to control integrin signalling events by phosphorylating multiple downstream targets (Klinghoffer et al., 1999). For this reason Src activity and its localisation has to be tightly controlled and its dysregulation is linked to cancer (Mitra and Schlaepfer, 2006; Playford and Schaller, 2004). The vast majority of Src within a cell is in an inactive state due to inhibitory interactions between the SH2 and SH3 domains (Kaplan et al., 1995; Zheng et al., 2000). Phosphorylation on the residue Tyr527 in chicken (Tyr530 in human and Tyr526 in *Xenopus*) holds Src in its inactive conformation through its interaction with SH2 domain (Cooper et al., 1986; Kaplan et al., 1994; Zheng et al., 2000). The dephosphorylation of this residue leads to the partial activation of Src. In addition the Tyr416 residue in chicken (Tyr 419 in human and Tyr415 in *Xenopus*) must be phosphorylated to allow substrates access to the binding site for Src to phosphorylate them (Roskoski, 2004). Active Src localises to CMAs, in a process dependent on its SH3 domain (Kaplan et al., 1994; Machiyama et al., 2015), where it phosphorylates important proteins involved in signalling pathways downstream of integrin such as p130Cas, paxillin and FAK (Kanner et al., 1990; Mitra and Schlaepfer, 2006; Playford and Schaller, 2004). The targeting of Src to CMAs is crucial to their regulation as well as their function (Li et al., 2002). Src can be recruited to CMAs through various means. Interestingly Src does not require its catalytic activity in order to correctly localise to CMAs, however it is required in order for Src to modulate downstream functions of integrin signalling (Fincham and Frame, 1998). Src can bind directly to the cytoplasmic domain of β integrin and this interaction can lead to the activation of Src (Arias-Salgado et al., 2003). In addition Src can bind to other components of CMAs, in particular to FAK, which also leads to the activation of Src at CMAs (Carragher et al., 2003).

FAK is recruited to CMAs early on in their maturation (Zaidel-Bar et al., 2004) through its targeting to paxillin (Liu et al., 2002; Tachibana et al., 1995). Along with Src it is a key signalling component of CMAs and requires localisation to cell adhesion complexes in order to become activated. It is a large protein of 125kDa consisting of an N-terminal four-point-one, ezrin, radixin, moesin (FERM) domain, a central kinase domain and a C-terminal focal adhesion targeting (FAT) domain. Both the FERM domain and FAT

domain mediate protein-protein interactions and are involved in the recruitment of FAK to integrin and the CMA complex (Hildebrand et al., 1993; Schaller et al., 1995). In its inactive state the FERM domain is bound to the kinase domain to prevent autophosphorylation of the Tyr397 residue. Clustering of integrins triggers the autophosphorylation of FAK on this residue and in addition causes the FERM domain of FAK to interact with β integrin; both these events facilitate the activation of FAK (Cooper et al., 2003; Parsons, 2003). The phosphorylation of Tyr397 creates a binding site for Src via its SH2 domain, localising Src to the CMAs (Fig. 1.2) (Xing et al., 1994). Src phosphorylates further tyrosine residues on FAK resulting in a fully activate FAK-Src complex (Calalb et al., 1995; Owen et al., 1999). Further phosphorylation of FAK exposes additional sites for various proteins including Grb2 (Mitra et al., 2006; Mitra and Schlaepfer, 2006), p130Cas (Vuori et al., 1996) and PI3-kinase (Guinebault et al., 1995). In addition FAK also helps recruit GEF and GAP proteins for the regulation of small GTPases (Tomar and Schlaepfer, 2009). Interestingly kinase dead FAK still plays an important role in the function of CMAs suggesting that FAK plays a role as a scaffold protein in addition to its kinase function (Schaller et al., 1999). Due to the multitude of binding partners FAK has, it plays a vital role in coordinating signalling downstream of CMAs and can act as a switch determining what downstream pathways are turned on.

Assembly

CMAs are hugely diverse structures that vary in terms of size, strength, and composition. They can range from very small CMAs unable to withstand 2 pN of force across them without breaking (Jiang et al., 2003), to huge complexes that can support over 10 nN across them (Balaban et al., 2001) and the exact structure and composition of the CMAs varies according to its specific location and function. For example small punctate CMAs, known as focal complexes, are induced by Rac1 activity and form in the growing protrusions of migrating cells and are enriched in talin, paxillin and vinculin (Nobes and Hall, 1995; Zamir and Geiger, 2001). In addition they contain high levels of paxillin phosphorylated on the Tyr118 residue (Zaidel-Bar et al., 2007). Focal complexes are short lived adhesions; within minutes of their formation they either complete disassembly or mature into focal adhesions (Zaidel-Bar et al., 2003). The application of force to the focal complexes, such as that generated by Rho activation or

from the external environment, stimulates their maturation into focal adhesions (Riveline et al., 2001; Rottner et al., 1999). Focal adhesions are larger adhesions and are associated with myosin containing actin stress fibres (Zamir and Geiger, 2001). They contain zyxin as well as increased levels of paxillin and vinculin (Zaidel-Bar et al., 2004). Under increased actomyosin contractility focal adhesions increase in size and can develop further into fibrillar adhesions (Bershadsky et al., 2003). Fibrillar adhesions are stable adhesions that play a role in fibronectin fibrillogenesis and bind specifically to fibronectin via the integrin dimer α5 β1 (Pankov et al., 2000). They are also characterised by their association with actin cables, rather than actin stress fibres, the presence of tensin and low levels of tyrosine phosphorylated proteins (Zamir et al., 2000). Other forms of CMAs are podosomes and invadopodia which are involved in the degradation of the ECM. They form a distinctive ring structure consisting of actin-associated proteins, such as talin and vinculin, around an F-actin core (Linder and Kopp, 2005). The incorporation of the different proteins that together constitute the different CMAs is understood to be a hierarchical process, whereby certain proteins must be present in order for others to be recruited. Initially talin is recruited to integrin, closely followed by paxillin. Vinculin and α-actinin are subsequently recruited to CMAs and play a role in their maturation (Choi et al., 2008; Zaidel-Bar et al., 2004). FAK is also recruited early on to maturing CMAs where it can act as a scaffold for other proteins as well as coordinating downstream signalling events. Although the initial formation of CMAs does not require actomyosin contractility (Choi et al., 2008), myosin II driven actomyosin contractility and actin bundling is required for the maturation of small CMAs into large adhesions (Chrzanowska-Wodnicka and Burridge, 1996; Riveline et al., 2001). This is most likely due to tension induced conformational changes in tension-sensing molecules, such as p130Cas (Sawada et al., 2006) and vinculin (Grashoff et al., 2010) exposing additional binding sites allowing the binding of additional proteins to the adhesion complexes. In addition α-actinin has recently been shown to act as a mechanotransducer thanks to its ability to transmit cytoskeletal forces to CMAs triggering their maturation (Roca-Cusachs et al., 2013). The vast number of proteins involved in CMA complexes reflects their ability to perform multiple functions including binding the cell to the substrate, force transduction, intracellular signal transduction and actin polymerisation regulation. Due to their

complexity and multiple functions, CMAs can influence a vast number of cellular processes including cell migration, gene expression and cell survival. The role of CMAs in force transduction and cell migration is the focus here but for more information on its role in coordinating other cell processes please refer to these reviews (Geiger et al., 2009; Wehrle-Haller, 2012b; Wozniak et al., 2004).

1.1.2 Turnover and disassembly

Despite being such large protein complexes, CMAs are highly dynamic structures. They undergo constant protein turnover and integrins themselves are constantly binding and releasing the ECM. The rate of protein turnover differs for each protein and depends on the state of the CMA and the tension they are under (Digman et al., 2008; Horton et al., 2015; Wolfenson et al., 2011; Wolfenson et al., 2013). Furthermore CMAs are constantly growing and shrinking and their fates shift from assembly to disassembly depending on their location in the cell and the surrounding environment. Interestingly the disassembly of CMAs is not just the reverse of assembly; whereas assembly is a sequential and hierarchical process, it appears disassembly involves the detachment of many components at the same time (Laukaitis et al., 2001).

Microtubules

What causes the switch from growth and assembly to disassembly is not clear although it is known to be coordinated by tyrosine phosphorylation events and cytoskeletal tension (Crowley and Horwitz, 1995; Wolfenson et al., 2011). The presence of microtubules has been shown to reduce cell contractility and microtubule targeting to CMAs can enhance the disassembly of the CMAs, most likely through the relaxation of the actin bundles crosslinked to the CMAs (Kaverina et al., 1999) and the targeting of disassembly factors to CMAs (Ezratty et al., 2005; Nagano et al., 2012). When microtubule polymerisation is perturbed with the use of nocodazole, CMAs are inhibited from undergoing disassembly (Ezratty et al., 2005). This process is independent of assembly as nocodazole treatment does not appear to affect CMA assembly. There is also evidence that the microtubule motor protein kinesin-1 is important for CMA disassembly, potentially through the targeted delivery of proteins

involved in promoting the disassembly of CMAs, such as the kinase MLK2, via the microtubules (Kaverina et al., 2002; Krylyshkina et al., 2002; Nagata et al., 1998). Although there is much evidence on the role of microtubules in stimulating CMA disassembly, how microtubules are specifically targeted to CMAs is less clear. It is known that Rho GTPases regulate the targeting of microtubules to the cell cortex and it is possible they are playing a similar role here.

FAK-Src signalling

FAK-Src signalling plays a key role in mediating CMA disassembly (Fig. 1.3). It has been demonstrated that cells lacking either FAK or Src activity have impaired CMA disassembly and consequently reduced motility (Chen et al., 2002; Fincham and Frame, 1998; Ilić et al., 1995; Myers and Gomez, 2011; Webb et al., 2004). Furthermore increasing localised Ca^{2+} at CMAs has been shown to increase FAK and result in an increase in CMA disassembly (Giannone et al., 2004). In the same way increased Src activity, such as in transformed cells, can lead to a decrease in CMAs through an increase in the rate of CMA turnover (Frame et al., 2002). FAK-Src signalling is believed to influence the fate of CMAs through various mechanisms. One mechanism through which FAK-Src signalling can induce CMA disassembly is through the regulation of actomyosin contractility. FAK-Src signalling has been implicated in the localised suppression of Rho activity (Arthur et al., 2000; Ren et al., 2000; Schober et al., 2007). Transient suppression of Rho activity in the lamellipodia promotes CMA disassembly and is dependent on FAK localisation and activity (Ren et al., 2000). Conversely consistently elevated Rho activity leads to an increase in CMA stability, as is the case in cells lacking FAK (Ren et al., 2000). There is evidence that FAK-Src signalling leads to the localised suppression of Rho activity through the activation of p190RhoGAP, a deactivator of Rho (Arthur et al., 2000; Fincham et al., 1999; Schober et al., 2007). In addition FAK-Src signalling has also been shown to modulate actomyosin contractility through its ability to target and regulate extracellular signal-regulated kinase (ERK) which in turn phosphorylates myosin light chain kinase (MLCK) promoting actomyosin contractility (Webb et al., 2004). Interestingly there is evidence that microtubule induced CMA disassembly requires FAK-Src signalling (Ezratty et al., 2005). The GTPases dynamin is believed to localise to CMAs via its interaction with FAK (Fig. 1.3).

Microtubule extension to CMAs has been shown to activate dynamin at the CMAs and this event is required for the promotion of CMA disassembly (Ezratty et al., 2005). An alternative method through which FAK-Src may be stimulating CMA disassembly is through the activation of calpain, a calcium dependent proteolytic enzyme. Calpain enhances the disassembly of CMAs through the proteolysis of various CMAs components such as integrin (Flevaris et al., 2007), talin (Franco et al., 2004) and FAK itself (Chan et al., 2010). In the absence of calpain cell motility is reduced and CMAs are larger and more stable due to a reduction in CMA disassembly (Bhatt et al., 2002; Dourdin et al., 2001). There is evidence that Src-dependent phosphorylation of FAK stimulates the association of calpain to the FAK-Src complex thereby recruiting calpain to CMAs (Westhoff et al., 2004) where it is involved in driving CMA disassembly (Carragher et al., 2003). In addition Src has been implicated in the modulation of the Cas family protein Nedd9 at CMAs, a protein known to play a role in regulating the stability of CMAs; it is possibly Src could be promoting CMA disassembly through its effect on Nedd9 (Bradbury et al., 2014).

Above are just some of the mechanisms by which CMA disassembly is regulated and is by no means an extensive list. There are many other mechanisms involved in regulating CMA disassembly including the regulation of phosphatases and changes to the lipid membrane composition (Nagano et al., 2012; Webb et al., 2002; Wehrle-Haller, 2012a).

1.1.3 CMAs in cell migration

The ability of CMAs to crosslink the ECM to the intracellular cytoskeleton via integrin and the many adapter proteins is fundamental to their role in generating the force required for cell migration. Interestingly some cells, such as leukocytes, can migrate in the absence of CMAs (Lämmermann et al., 2008). In recent years this mode of migration has become increasingly well studied but it will not be discussed further here (Bergert et al., 2015; Renkawitz and Sixt, 2010). As a cell migrates it is constantly exposed to external and internal forces, such as the resistance from the surrounding environment and internal cytoskeletal rearrangements (Ananthakrishnan and Ehrlicher, 2007). The forward movement of a cell is driven by the polymerisation of F-

actin in the leading edge of a cell, a process coordinated by small GTPases. F-actin is filamentous structure of semiflexible polymers about 17 μm in length and 7 nm in diameter (Gittes et al., 1993). It is polar with a fast growing plus end and a slow growing minus end. The polymerisation of F-actin in the leading edge of migrating cells generates the driving force behind most cell migration (Ananthakrishnan and Ehrlicher, 2007; Hofman et al., 1999; Pollard and Borisy, 2003). In addition to driving the front of the cell forward by actin polymerisation, F-actin also binds myosin motors and together they generate contractile forces required for the retraction of the rear of the cell and the translocation of the cell body (Murrell et al., 2015). As well as constant polymerisation, F-actin also undergoes a process known as retrograde flow. This is the flow of actin away from the edge of the cell backwards in respect to the substrate. It is driven by actin polymerisation and myosin motors (Cramer, 1997; Lin et al., 1997; Watanabe and Mitchison, 2002). Overall the migration of the cell can be broken down into stages: initially actin polymerisation in the leading edge of the cell pushes the cell membrane forward generating cell protrusions, secondly cells form CMAs in these protrusions adhering the cell to the substrate, concurrently CMAs disassemble at the rear of the cell releasing the cell from the substrate and actomyosin driven contractile forces pull the rear of the cell and the cell body towards the front of the cell (Ananthakrishnan and Ehrlicher, 2007; Parsons et al., 2010; Sheetz et al., 1999).

CMA as a molecular clutch

The formation of CMAs in developing protrusions at the front of the cell is vital to help anchor the leading edge to the substrate by linking the actin cytoskeleton to the substrate. This connection causes a reduction in actin retrograde flow and so CMAs are said to behave as ‘molecular clutches’ through their ability to couple retrograde flow to the substrate below. This coupling has multiple implications including promoting the growth of protrusions, suppressing membrane contraction and transmitting contractile forces to the substrate (Alexandrova et al., 2008; Gardel et al., 2010; Parsons et al., 2010). When actin retrograde flow is reduced by CMA engagement, cell membrane protrusions increase due to the continuous actin polymerisation (Alexandrova et al., 2008). In addition reduced actin retrograde flow causes a build-up of contractile forces within the cell which are transmitted to the substrate as traction forces via the CMAs

consequently pulling the cell forward. Together protrusion formation and traction force generation help drive cell migration. The ability of CMAs to generate traction varies according to the strength of the coupling of the cytoskeleton with the substrate. When the coupling of the cytoskeleton with the substrate is weak actin retrograde flow continues at a faster rate and the transmission of force from the cytoskeleton to the substrate is less effective, consequently the speed of cell migration is reduced (Alexandrova et al., 2008; Gardel et al., 2010; Hu et al., 2007). Interestingly the relationship between the rate of actin retrograde flow and the traction force generated is biphasic (Gardel et al., 2008). When CMAs are large the relationship is inversely linear with migration velocity at its peak when both flow and traction are at intermediate levels. However when small CMAs are newly forming and retrograde flow is great the relationship is no longer linear (Alexandrova et al., 2008; Beningo et al., 2001; Han et al., 2012). The strength of a CMA corresponds to the traction force it can generate and is determined by its composition and the clustering of integrin dimers (Gardel et al., 2010). It can be modulated by both chemical and mechanical signals with mechanical force often increasing CMA maturation and size (Case and Waterman, 2015; Geiger et al., 2009).

In addition to their role as molecular clutches transmitting force from the cell to the substrate, CMAs also drive migration and other cell fates through the stimulation of various signalling pathways. One consequence of the downstream signalling of CMAs is the regulation of the small GTPases that drive migration (Vicente-Manzanares et al., 2009). CMAs can activate Rac1 and Cdc42 in protrusions (Price et al., 1998) and transiently inhibit RhoA activity through the activation of p190RhoGAP, a process mediated by the activation of Src (Arthur et al., 2000). Paxillin is a key mediator of small GTPase activity at CMAs (Vicente-Manzanares et al., 2009). The phosphorylation of paxillin by FAK or Src recruits Crk-II and p130cas to the CMAs which in turn activates Rac through the RacGEF DOCK180 (Reddien and Horvitz, 2000; Schaller and Parsons, 1995). In addition paxillin can also recruit the adaptor proteins GIT1-GIT2 and the Rac-GEF βPIX resulting in the activation of Rac and the recruitment of p21-activated kinase-1 (PAK1) to protrusions (Manser et al., 1998; Nayal et al., 2006). PAK1 phosphorylates MLCK resulting in the inactivation of myosin II (Sanders et al., 1999). The controlled

regulation of Rac, Rho and Cdc42 help coordinate actin polymerisation to the protrusions in migrating cells and inhibit premature stress fibre formation. However, RhoA, and consequently ROCK and myosin II activity, can be stimulated downstream of CMAs where their activation leads to actomyosin contraction and enlargement and stabilisation of the CMAs (Chrzanowska-Wodnicka and Burridge, 1996; Ren et al., 1999). In summary, CMA-dependent signalling can control the actin cytoskeleton through the mediation of the small GTPases which can lead to the activation, assembly or disassembly of the CMAs depending on the downstream effectors.

CMAs in mechanosensing

As well as facilitating migration on various substrates, CMAs also allow the cell to sense the external environment and respond to the mechanical forces applied to the cell from the substrate. The ability of a cell to sense substrate stiffness relies on the cells ability to exert force on the substrate, such as traction force through the CMAs, and, depending on the stiffness, composition and density of the substrate, respond to these cues converting them to biochemical signals via force induced conformation changes within the cell (Hytönen and Wehrle-Haller, 2016). Tension across CMAs can induce multiple downstream events including changes in gene expression, tyrosine phosphorylation cascades and further growth of the CMAs (Balaban et al., 2001; Hytönen and Wehrle-Haller, 2016; Riveline et al., 2001). A beautiful example demonstrating how substrate stiffness could lead to downstream signalling events was recently shown in mouse embryonic fibroblasts (Elosegui-Artola et al., 2016). Integrin has an intrinsic on/off rate of binding and releasing its ligand in the ECM. On a stiff substrate above 5 kPa, talin bound to integrin in the CMA complex, unfolded as force was applied across it from the contractile forces in the cytoskeleton to the substrate below. This unfolding of talin exposed a binding site for vinculin, another mechanosensing protein, and the CMA grew in size. The transcription factor YAP translocated to the nucleus allowing the cells to alter their gene expression in response to the stiffer substrate. Conversely when the cells were on a soft substrate the force across talin was weaker and therefore its rate of unfolding was slower. Integrin unbound from the ECM before talin had the chance to unfold meaning vinculin could not be recruited to the complex and YAP remained in the cytoplasm (Elosegui-Artola et

al., 2016). This is just one example of how cells can sense and respond to substrate stiffness but there are many other components of CMAs that can sense and respond to tension by undergoing conformational changes including other adapter proteins, such as paxillin, vinculin, the actin crosslinking protein filamin-A, which can couple the actin cytoskeleton to integrin as well as promoting actin branching, and integrin itself. It is still not fully understood how integrin can mediate rigidity sensing but its intrinsic rate of binding and unbinding the substrate, which varies depending on the heterodimer composition, has been shown to play a role (Elosegui-Artola et al., 2014; Novikova and Storm, 2013). As mentioned above, in order for integrin to become activated it must undergo a conformational change from a bent inactive conformation to an extended active conformation. As discussed, it has been demonstrated the binding of talin induces a conformational change in integrin to its active conformation (Anthis et al., 2009). However, in order for integrin to transform from a low-affinity conformation to a high-affinity conformation it must bind to its ligand in the ECM. This binding is believed to induce a conformational change in the extracellular domains of integrin that only occurs once integrin is under tension (Kong et al., 2009). In the absence of tension, such as when talin is bound to integrin but not to the actin cytoskeleton, integrin association with the ECM is weak and it undergoes rapid integrin exchange. However, when actin is bound to talin and tensile force is exerted on integrin, the binding of integrin to its ECM ligand is much stronger and integrin exchange is reduced (Cluzel et al., 2005). The dynamic behaviour of integrin can vary depending on the tensile force applied to it and this demonstrates a mechanism through which integrin can detect and respond to tension. In addition to integrin and adapter proteins that couple actin to integrin, FAK can also respond to tension across the CMA and the rigidity of the ECM (Plotnikov et al., 2012). FAK can bind to paxillin through its FAT domain and at the same time interact with the acidic lipids of the plasma membrane with its FERM domain (Hayashi et al., 2002; Liu et al., 2002), mechanically coupling FAK to both the membrane and the CMA. It is believed that this mechanical link under tension could accelerate FAK activation and expose its kinase domain to more targets (Goñi et al., 2014). This could lead to the accelerated recruitment of various proteins such as Src and enhance the phosphorylation of downstream targets. In addition FAK-paxillin can also work with vinculin to probe the rigidity of the substrate (Plotnikov et

al., 2012). In order for cells to carefully control their migration they need to be able to respond to mechanical cues. Above are just some of the mechanisms through which CMAs allow cells to do this.

CMAs are an essential part of cell migration in most cells. They are vital for the generation of traction forces that are required to drive cell migration. In addition their disassembly at the rear of the cell is required to allow the translocation of the cell body towards the direction of migration. Thus, in order for cells to migrate effectively their assembly and disassembly must be carefully coordinated.

1.1.4 CMAs *in vivo*

Historically much of the work investigating CMAs has mainly been carried out *in vitro* on 2D plastic or glass surfaces. This environment is very different to what cells would be experiencing *in vivo*. This led to speculation that the CMAs studied *in vitro* do not mirror what is happening *in vivo, a*nd it was suggested that large CMA complexes may be an artefact of culturing cells *in vitro* on hard substrates. The importance of integrin based adhesions and the components making up CMAs has been demonstrated *in vivo* during embryogenesis and development in various animal models. Integrin mutations in *Drosophila* and *C. elegans* have been shown to cause defects between tissues, loss of dorsal closure and defects in endoderm migration (Brown, 2000). In *Xenopus* FAK activity in mechano-receptive Rohon-Beard neurons was shown to be required for neurite extension *in vivo*; when FAK activity is absent there are fewer CMAs (Robles and Gomez, 2006). This is different from what was previously observed with other cell types *in vitro* although the requirement of FAK activity for migration still holds true. Different α and β integrin subunits were systematically knocked out in mice. Many knockouts were embryonic lethal due to an inability of embryos to develop their inner cell mass (Bouvard et al., 2001). Other mutants had defects in kidney and lung organogenesis (Kreidberg et al., 1996), problems with neural tube closure (De Arcangelis et al., 1999), limb abnormalities (De Arcangelis et al., 1999) and cardiac defects (Yang et al., 1995). Furthermore knockdown of other CMA associated proteins, such as kindlin 1, showed defects related to loss of adhesion such as skin atrophy and a detached colon (Ussar et al., 2008). However this evidence demonstrating the

importance of integrins does not address whether their behaviour *in vivo* is the same as what is occurring *in vitro*. In recent years two main methods have been used to try and address this problem; to develop new techniques in order to better mimic the 3D *in vivo* environment and to directly image CMAs *in vivo* (Doyle and Yamada, 2016; Worth and Parsons, 2010).

Mimicking in vivo conditions

In order to understand how CMAs respond to softer substrates more like those found *in vivo*, cells have been plated on acrylamide or collagen gels. As well as providing a more realistic environment, these gels can be used to quantify various properties of CMAs such as their ability to generate traction force on the substrate, a technique known as traction force microscopy (Wang and Lin, 2007). Interestingly the use of these softer substrates has shown that the size and dynamics of CMAs varies according to the stiffness of the substrate the cells are plated on; cells have smaller and more unstable CMAs when they are plated on softer substrates such as gels, compared to when they are plated on plastic or glass (Balaban et al., 2001; Pelham and Wang, 1998). Another technique developed to try and better mimic the *in vivo* environment is the generation of 3D matrices or collagen gels (Doyle and Yamada, 2016; Harunaga and Yamada, 2011). The existence of discrete CMAs have been identified in 3D matrices in many studies and in various cell types (Hakkinen et al., 2011; Harunaga and Yamada, 2011; Tamariz and Grinnell, 2002). CMAs have been observed even in cells deep in 3D matrices where the environment is most likely to mimic *in vivo* conditions (Kubow and Horwitz, 2011). However, some studies were unable to identify CMAs in cells deep within the 3D matrices, although the components of CMAs were still required in order for the cells to migrate (Fraley et al., 2010). Why some studies were able to detect discrete CMAs and others were not is unclear. It is possible that the imaging techniques used, the cell type or the preparation of the gels had a part to play in these contradictory observations. An alternative technique used to mirror the *in vivo* environment is the modulation of the substrate (Adutler-Lieber et al., 2014; Geiger et al., 2009). Substrates can vary in terms of the distance between the ligands to which integrin binds (Cavalcanti-Adam et al., 2007), the various substrate components in one area and the specific organisation of the substrate (Geiger et al., 2009; Wolfenson et

al., 2013). For example it is known that the ECM can form fibrillar structures *in vivo* (Provenzano et al., 2008) with cells aligning along these fibres (Sidani et al., 2006). Thanks to the use of micropatterning techniques these fibres can be mimicked *in vitro* (Théry et al., 2006). The use of 3D matrices and carefully modulating the substrate can be combined to address further questions as to how CMAs sense their environment and how they vary depending on the conditions they are exposed to (Doyle et al., 2015; Doyle and Yamada, 2016).

CMAs in vivo

CMAs have been visualised in some animal models, such as *Drosophila* and Zebrafish, where imaging CMAs *in vivo* is made easier thanks to the size and varying transparency of these organisms. *Drosophila* haemocytes provide an opportunity to image the effects of CMAs *in vivo* as they are a migratory population of cells that can be visualised beneath the epithelium at the ventral surface with high spatio-temporal resolution (Davis et al., 2015; Stramer et al., 2010). Interestingly many findings on the effects perturbing CMA components and regulation has on haemocyte migration mimics what has been described from previous work *in vitro*. For example the loss of Rho1 activity was shown to result in elongated haemocytes where the rear of the migrating cell was tethered to the ECM (Stramer et al., 2005), a result very similar to what is seen in Rho mutants *in vitro* (Cox and Huttenlocher, 1998). In addition knockdown of zyxin, an adapter protein only found in mature CMAs *in vitro* (Zaidel-Bar et al., 2004), increased the velocity of haemocyte migration as only smaller more dynamic CMAs could form (Parsons et al., 2010). This again mimics what has previously been shown from *in vitro* studies (Hoffman et al., 2006). Stress fibre associated CMAs have been visualised during the development of follicular epithelium in *Drosophila* and many CMA associated proteins were found to be localised to these structures (Delon and Brown, 2009). In addition the dynamics of CMAs have been investigated in *Drosophila* embryos and larvae in myotendinous junctions. CMA proteins such as integrin and talin were shown to have the same dynamic nature as has previously been described *in vitro* (Yuan et al., 2010). Furthermore the turnover rate varied depending on the tensile force applied (Pines et al., 2012), once again supporting previous observations made *in vitro* (Gardel et al., 2010). Zebrafish also provide an excellent

opportunity to image CMAs *in vivo* due to their transparency. There is evidence that active phosphorylated FAK is localised to CMAs in the cells of the notochord where it plays a role in somite morphogenesis (Henry et al., 2001). In addition proteins found in the CMA complex, such as vinculin, paxillin and talin, have all been shown to form CMAs at the intersomite septa in Zebrafish and the proteins accumulate in a similar manner to what has previously been described *in vitro* (Costa et al., 2008).

The imaging of CMAs in mammals has proved much harder. Immunoelectron microscopy has been used to demonstrate the presence of CMAs in mouse epithelium with the basal lamina in fixed tissue (Fuchs et al., 1997) . Now, thanks to vastly improved imaging techniques, such as multiphoton microscopy and second harmonic generation, CMAs can be imaged at much higher resolution *in vivo* in mammals (Worth and Parsons, 2010). Zyxin-labelled CMAs have now been visualised in metastasising cells in living-mice and the CMAs appear remarkably similar to how they appear *in vitro* (Sahai et al., 2005). Together these results suggest that CMAs *in vivo* have many of the same properties as what was originally described *in vitro* on stiff 2D surfaces. The improved mechanisms now available to probe CMAs *in vitro* in a more realistic environment and advanced imaging technique *in vivo* will together greatly improve the current understanding of the vital importance of CMAs and their regulation.

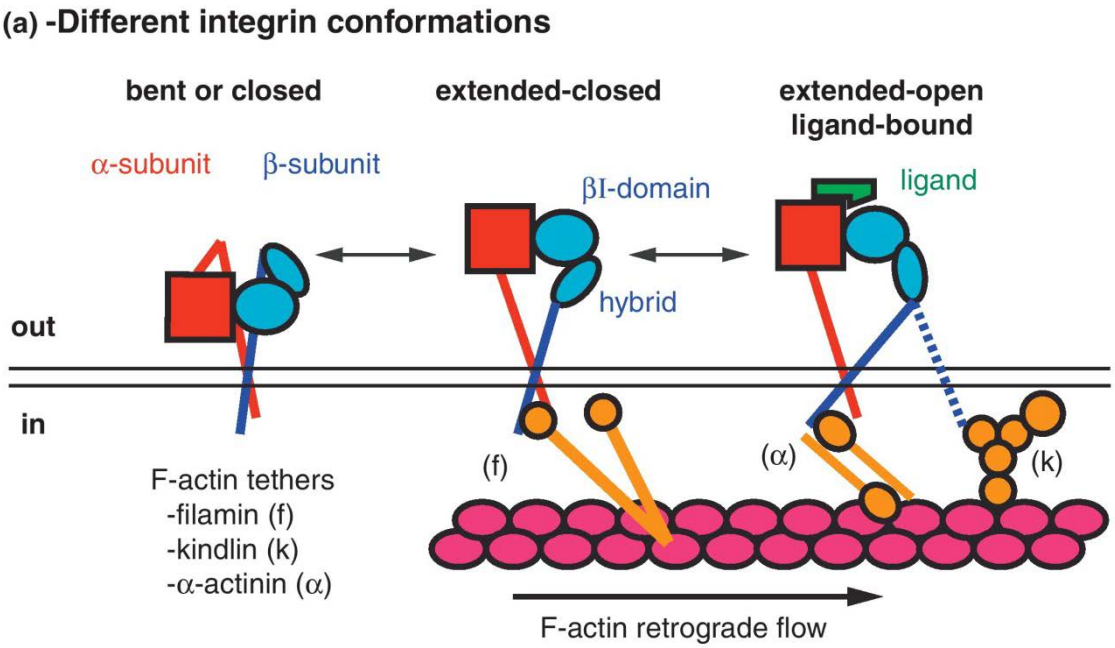

Figure 1.1 Integrin activation

Schematic of the different integrin conformations: the bent-closed conformation, the extended-closed conformation and the extended-open conformation. When integrin is neither bound to the ECM nor the intracellular cytoskeleton it is in an inactive bent-closed conformation in the plasma membrane. Upon crosslinking to the cytoskeleton via F-actin tethers, integrin is pulled into an extended-closed conformation with low affinity for its ligand in the ECM. The application of intracellular tension on integrin, such as that generated by actin retrograde flow, induces a conformational change in integrin to an extended-open conformation with high affinity for its ligand.

[Adapted from (Wehrle-Haller, B. 2012a)]

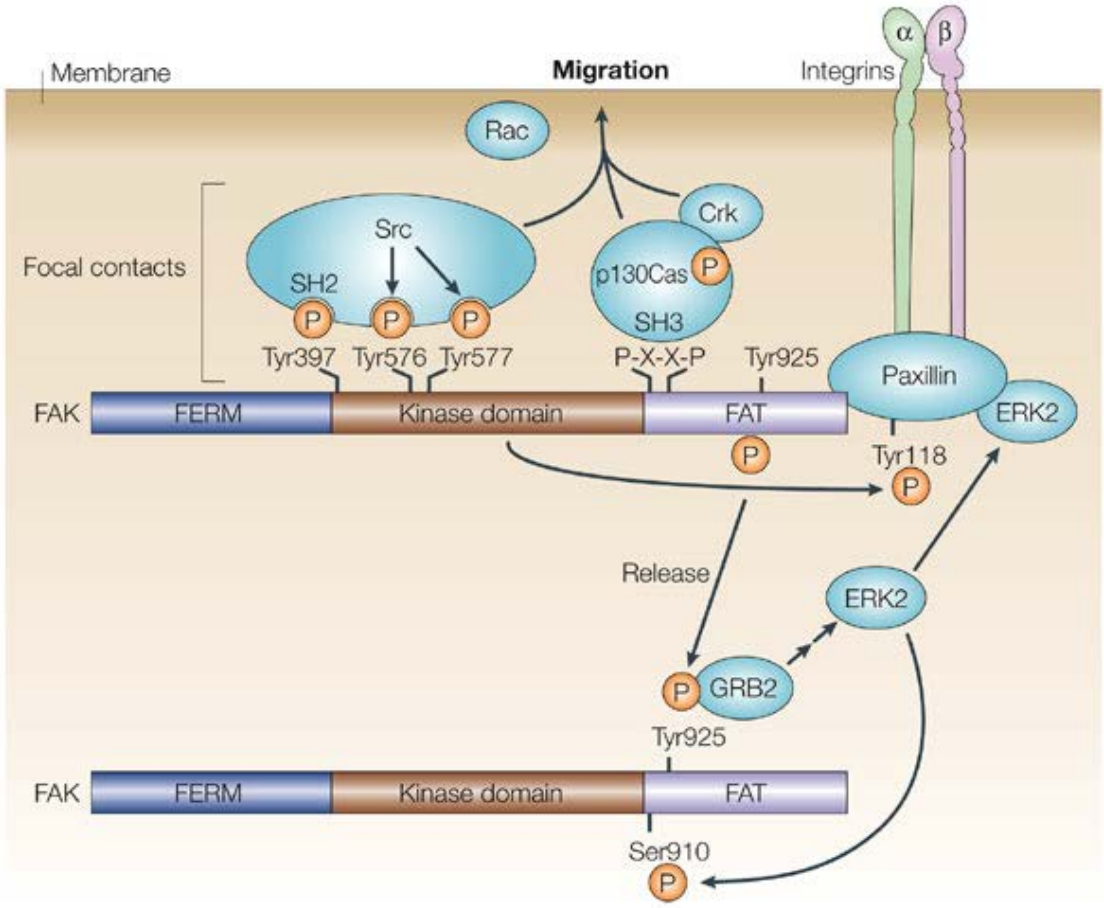

Figure 1.2 Src FAK interactions at CMAs

FAK is recruited to CMAs via its FAT domain. Integrin clustering promotes FAK autophosphorylation on its Tyr397 residue. This creates a binding site for the SH2 domain of Src. Src further phosphorylates FAK on other tyrosine residues including Tyr576, 577 and 925, resulting in a fully active FAK-Src complex able to recruit other proteins to the CMA. For example active FAK-Src recruits p130Cas which is subsequently phosphorylated by the complex. Phosphorylated p130Cas can recruit Crk and together they facilitate Rac activity driving actin driven protrusion formation and consequently cell migration. In addition phosphorylated Tyr925 can recruit Grb2 which in turn can activate the MAPK/ERK cascade.

[Adapted from (Mitra et al., 2005)]

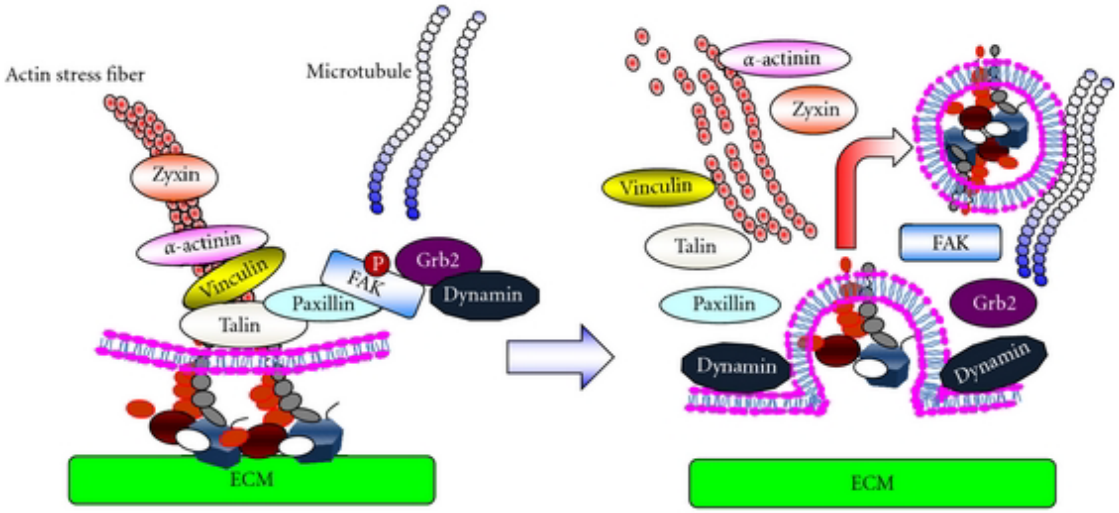

Figure 1.3 CMA disassembly

FAK plays an important role in regulating the disassembly of CMAs. Src phosphorylates FAK on Tyr925 residue. This creates a binding site for the adapter protein Grb2 which in turn can recruit the GTPase dynamin to the CMAs. Dynamin is a regulator of endocytosis. It is thought the extension of microtubules to the CMAs can initiate dynamin-dependent endocytosis of integrin which triggers the rapid disassembly of the CMA components. FAK becomes rapidly dephosphorylated and returns to an inactive conformation.

[Adapted from (Nagano et al., 2012)]

1.2 Cross-regulation between cell-cell adhesion and cell-matrix adhesions

The ability of cells to sense and respond to external cues and their surrounding environment is essential for their function. Not only do cells bind to the extracellular matrix (ECM) via cell-matrix adhesions (CMAs) where they can sense and respond to the substrate, as discussed in the previous chapter, they also bind to neighbouring cells via cell-cell adhesions (CCAs). CCAs are vital for the morphology and integrity of tissues and are involved in the communication of cells with their neighbours. Broadly there are three main classes of CCAs: tight junctions, gap junctions and cadherin-based junctions. The role of tight junctions and gap junctions will not be discussed here, for information on these junctions please refer to the following reviews (Evans, 2015; Goodenough and Paul, 2009; Hartsock and Nelson, 2008; Kotini and Mayor, 2015; Van Itallie and Anderson, 2014). Cadherin-based junctions (herein referred to as cell-cell adhesions or CCAs) are the sole focus here. CMAs and CCAs are closely related complexes; they both crosslink the intracellular actin cytoskeleton to the cell exterior and by doing so act as force transducers. In addition they can regulate the cytoskeleton, signal through similar pathways and have many common components. Despite their obvious similarities, they are found in distinct locations in the cell and mutually regulate each other, with the presence of one often leading to the suppression of the other (Burute and Thery, 2012). The idea that CCAs and CMAs can communicate has been speculated upon for a long time, particularly in regard to their roles in migrating cells (Abercrombie and Heaysman, 1953). Cross-regulation between the closely related CCAs and CMAs is an ever growing field and the focus of this chapter.

1.2.1 Cadherin-based adhesions

Cadherins are a large superfamily of Ca^{2+} dependent adhesion molecules, and are so named because of this. They are the basis behind junctions that couple neighbouring cells together generating strong adhesion between cells (Fig. 2.1). They are highly conserved among metazoan organisms and are found in almost all vertebrate tissues due to the vital role they play in maintaining tissue integrity during both homeostasis

and morphogenesis (Martin et al., 2010; Maître and Heisenberg, 2013; Wang et al., 2012). Cadherins were first identified in carcinoma cells as the glycoprotein responsible for homophillic Ca^{2+} dependent adhesions between cells (Yoshida and Takeichi, 1982). Over 100 cadherins have now been identified; the common feature between all cadherins is the existence of the cadherin domain or repeats in the extracellular domain. The cadherin superfamily is broadly subdivided into two main types: classical or non-classical. The non-classical cadherins are subdivided further into several subfamilies, including desmosomal, protocadherin, Flamingo and Dachsous and Fat cadherins and are classified according to the organisation of their extracellular cadherin domain and sequence similarities (Hirano et al., 2003; Nollet et al., 2000; Tanoue and Takeichi, 2005). Classical cadherins are the best characterised cadherins and the focus here; they are defined by their characteristic cytoplasmic sequence that binds to catenins (Fig. 2.1) (Leckband and de Rooij, 2014). Classical cadherins are class one transmembrane glycoproteins, meaning they only have a single transmembrane span. The extracellular N-terminal region consists of 5 cadherin repeats and mediates the binding of cadherins from neighbouring cells via the most distal domain, termed the EC1 domain (Chen et al., 2005; Hatta et al., 1988; Hirano et al., 2003; Niessen et al., 2011). The C-terminal cytoplasmic domains are highly conserved and interact with the cytoskeleton, cytoplasmic proteins, such as catenins, and modulate the activity of small GTPases. The binding of catenins is essential for the function of classical cadherins (Davis et al., 2003; Dufour et al., 2013; Hirano et al., 1992; Leckband and de Rooij, 2014). Out of the six identified catenin proteins that bind to cadherin the best characterised are β- α- and p120-catenin which each bind cadherin with a stoichiometry of 1 of each catenin per cadherin (Jou et al., 1995; Niessen et al., 2011). β-catenin associates with cadherins co-translationally (Chen et al., 1999) where it binds to the distal region of the cytoplasmic tail and facilitates the binding of various proteins to cadherin junctions, such as α-catenin (Aberle et al., 1994). In addition β-catenin also plays a role in Wnt signalling and its binding to cadherin can sequester it from this function. When β-catenin is released from cadherin it can translocate to the nucleus and promote various cell fates including the stimulation of an epithelial-to-mesenchymal transition (EMT) (Cadigan and Peifer, 2009). α-catenin can regulate actin dynamics through its role as an actin-binding protein whereby it links cadherin to the

actin cytoskeleton, a role essential for strong adhesion formation (Hirano et al., 1992). In addition it recruits other actin-binding proteins to developing CCAs, such as vinculin, formin-1, VASP and zyxin, which can lead to various consequences such as the nucleation of unbranched actin and mechanosensing (Kobielak and Fuchs, 2004; Leckband and de Rooij, 2014; Vasioukhin et al., 2000). p120-catenin was first identified as a target of the tyrosine kinase Src before it was shown to associate with cadherins (Kanner et al., 1991) where it binds to the juxtamembrane region of cadherin (Reynolds et al., 1994; Thoreson et al., 2000). p120-catenin plays an important role in coordinating the crosstalk between cadherin and the actin cytoskeleton as well as regulating the clustering of adhesions through its ability to control the activity of small GTPases (Anastasiadis et al., 2000; Anastasiadis and Reynolds, 2001; Yap et al., 1998). It can inhibit the activity of RhoA directly (Anastasiadis et al., 2000) or indirectly through the recruitment of p190RhoGAP (Wildenberg et al., 2006). Furthermore p120-catenin can also indirectly activate Rac1 and Cdc42 via the GEF-Vav2 (Noren et al., 2000). Furthermore p120-catenin binding to the cytoplasmic tail of cadherin is required for the stabilisation of CCAs (Davis et al., 2003; Xiao et al., 2005; Yap et al., 1998).

Classical cadherins are subdivided into two types: type I and type II. Type I cadherins, which include E-, N- and P-cadherin, are characterised by the existence of a His-Ala-Val tripeptide motif in their most distal EC1 domain which mediates strong adhesion between cells. Type II cadherins, such as VE-cadherin and cadherin-11, lack this motif and mediate weaker adhesions between cells (Chen et al., 2005; Halbleib and Nelson, 2006; Patel et al., 2006). E-cadherin, the first cadherin identified and so called due to its predominant expression in epithelial cells, is the best characterised cadherin. It is expressed in all mammalian epithelia where it plays an essential role in maintaining apico-basal polarity and regulating proliferation and cell survival (Baum and Georgiou, 2011; Lien et al., 2006). N-cadherin, so called due to its role in neural tissue, was first identified in embryonic chick neural retina cells (Grunwald et al., 1982). In addition to its expression in neural cells, N-cadherin is expressed in other tissues such as in the mesoderm during development (Hatta and Takeichi, 1986) and in cardiomyocytes (Hertig et al., 1996). Throughout development epithelial-to-mesenchymal transitions

(EMT) occur and this can be linked to a switch in cadherin expression from E-cadherin to the expression of N-cadherin. This switch from E- to N-cadherin can disrupt tissue integrity and lead to a more loosely associated collection of cells with enhanced individual motility and reorganised actin cytoskeleton (Wheelock et al., 2008). Although the exact cause of the variance in cell behaviour downstream of E- or N-cadherin is unclear, one possibility is due to their differential effects on FGF-signalling and the small GTPases (Wheelock et al., 2008). For example N-cadherin, but not E-cadherin, has been shown to physically interact with FGF receptors and initiate both FGF-receptor dependent and FGF-ligand dependent signalling (Suyama et al., 2002; Utton et al., 2001). In addition elevated E-cadherin levels in the neural crest have been shown to increase Rac1 activity at the cell-cell contact which is normally inhibited in the presence of N-cadherin (Scarpa et al., 2015). An E- to N- cadherin switch has been identified in multiple processes during development, such as during gastrulation and the onset of the migration of the neural crest, discussed in more detail in the following chapter (Dady et al., 2012; Hatta and Takeichi, 1986). Not only does this switch have implications in development, it has also been identified as a driving force behind invasion in cancer (Araki et al., 2011; Hazan et al., 2004).

1.2.2 Crosstalk between cell-matrix adhesions and cell-cell adhesions

Cadherin-based CCAs and integrin-based CMAs are closely related complexes due to their ability to crosslink the internal cytoskeleton to the extracellular environment, i.e. the substrate or neighbouring cells. Both CCAs and CMAs influence the behaviour of the cytoskeleton and play a role in mechanosensing. Despite their similarities, these structures have distinct functions with CCAs and CMAs cross-regulating each other in either a positive manner, by reinforcing the other, or negative manner, by suppressing the other. Evidence of this mutual cross-regulation and the mechanisms behind it are the focus of this discussion.

Spatial segregation of cell-cell and cell-matrix adhesions in tissue

During development there is an ever growing body of evidence demonstrating that CCAs and CMAs having an antagonistic effect on each other leading to their spatial

segregation in tissue (Fig. 2.2), a process required to drive tissue polarity, morphogenesis and the re-distribution of forces (McMillen and Holley, 2015; Wilson, 2011; Yeaman et al., 1999). The mutual exclusion and consequently spatial segregation between CCAs and CMAs appears to be driven by local negative feedback between these two adhesion types. For example during tubulogenesis, such as the formation of the pancreas and bile ducts, carefully coordinated cytoskeleton rearrangements are required within the tissue in order to establish cell polarity (Kesavan et al., 2009). It has been demonstrated that the formation of this polarity is coordinated by the formation of CCAs and laminin, an ECM to which CMAs can bind, to distinct poles (Fig. 2.2) (Antoniou et al., 2009; Burute and Thery, 2012; Kesavan et al., 2009). Furthermore the initiation of epithelial branching, such as that which occurs during lung, kidney and salivary gland development, requires the assembly of fibronectin and consequently CMAs in small cleft regions resulting in the localised loss of cadherin from these regions (Sakai et al., 2003). The spatial segregation of CMAs and CCAs is not just a property of epithelial tissues. It has been demonstrated during the formation of the paraxial mesoderm that the formation of fibronectin and consequently CMAs at the basal surface during somitogenesis in the chick embryo restricts cadherins to the apical surface (Fig. 2.2) (Rifes and Thorsteinsdóttir, 2012). A recent study in zebrafish paraxial mesoderm mesenchyme has revealed that the presence of N-cadherin based CCAs stabilise the inactive conformation of integrin and prevents it binding to fibronectin. In the regions where CCAs do not form, such as on the surface of the tissue, integrin is no longer held in its inactive conformation so can become activated and bind to fibronectin. It appears that the localised suppression of integrin by N-cadherin is required for the elongation and segmentation of the zebrafish body (Jülich et al., 2015). In addition during *Xenopus* gastrulation the mesendoderm expresses N-cadherin and tension is generated across these CCAs leading to the displacement of CMAs away from the CCAs (Davidson et al., 2002; Dzamba et al., 2009). Furthermore neural crest cells lacking β1 integrin show increased aggregation suggesting an increase in CCA strength in the absence of CMAs (Breau et al., 2006). Although the observation of the requirement of the spatial segregation between CMAs and CCAs *in vivo* highlights the presence and importance of crosstalk between the two, much of the work carried out to try and elucidate the mechanism behind it has been carried out

in vitro. A simple but effective experiment carried out *in vitro* to demonstrate the requirement of ECM for apico-basal polarity in epithelial cells was carried out on Madin-Darby Canine Kidney epithelial (MDCK) cells. MDCK cells plated in collagen gels formed doughnut like structures with the apical surface towards the central lumen and the basal surface forming the outside of the structure [Fig. 2.3], a structure that closely mirrors the formation of lumen containing structure during development such as during tubulogenesis events. In the absence of collagen MDCK instead formed an inverted cyst (Wang et al., 1990). This simple experiment is interesting for multiple reasons. As the cells in the absence of collagen form an inverted cyst with apico-basal polarity reversed in all the cells, it demonstrates that CCAs are sufficient to trigger the segregation of apico-basal polarity proteins. However, only when collagen is present do the MDCK cells reverse their polarity so the apical surface is internal to the structure and the basal surface external, demonstrating CMAs can orientate polarity and lead to the redistribution of CCAs. More recently this technique has helped identify the importance of the PI3-kinase pathway and consequently Rac1 activity in establishing this apico-basal polarity (Liu et al., 2007) and has helped demonstrate that integrin binding to the ECM at the basal surface disrupts E-cadherins in the vicinity (Ojakian et al., 2001).

CMAs and CCAs are mutually antagonistic

The cross-regulation between CCAs and CMAs can act in either direction; the presence of CCAs can supress CMAs and vice versa. In addition crosstalk between the two can also lead to a reinforced network where both adhesions are strengthened. The outcome appears to be very much context dependent. For example one study on S180 murine sarcoma cells demonstrated that integrin engagement to fibronectin increased the adhesion strength of E-cadherin through the activation of Src family kinases and a consequent increase in actomyosin contractility (Martinez-Rico et al., 2010). However, S180 murine sarcoma cells plated on micropatterned fibronectin stripes showed a significant reduction in the E-cadherin adhesion strength (Al-Kilani et al., 2011). The reason behind the discrepancy between these two studies is unknown, although it has been suggested it could be due to a difference in substrate rigidity or density leading to differential behaviour of the actin cytoskeleton (McMillen and Holley, 2015). These

conflicting results demonstrate that the consequence of the complex feedback loops involved is CCA/CMA crosstalk is dependent on many factors and the context the cells are in plays a role in the outcome of this crosstalk. Each of the different directions of cross-regulation between CCAs and CMAs will be discussed below.

CCAs antagonise CMAs

As discussed above, the plating of MDCK cells in collagen gels identified that the presence of CMAs can locally disrupt E-cadherin based CCAs (Ojakian et al., 2001). E-cadherin has also been shown to locally impair CMA formation, downstream signalling, protrusive activity and traction force in MDCK cells (Borghi et al., 2010; Yamada and Nelson, 2007). Together these results show a mutual antagonistic crosstalk is occurring between these two types of adhesion complexes leading to spatial segregation. *In vitro* cultures have highlighted many examples of the presence of CCAs locally supressing CMAs. For example the overexpression of E-cadherin in *Xenopus* XTC cell lines was shown to result in a reduction in CMAs (Finnemann et al., 1995). In vascular smooth muscle cells an increase in cell density corresponded to an increase in VE-cadherin based CCAs and led to a decrease in CMAs (Nelson et al., 2004). Interestingly the effect cell density had on the fate of the CMAs was dependent upon on the rigidity of the substrate suggesting mechanotransduction is important in the consequence of the cross-regulation between the CCAS and CMAs (Sazonova et al., 2011). Traction force measurements carried out on keratinocyte colonies demonstrated traction forces, a readout of CMA driven stress on the substrate, were solely generated at the periphery of the cluster (Mertz et al., 2013). However, when E-cadherin was blocked, traction force increased at the contact inferring an increase in CMAs in this region. This study suggests the presence of E-cadherin based adhesions at the cell-cell contact inhibit CMAs and consequently traction force near the contact and coordinate them solely to the periphery of the cluster (Mertz et al., 2013). There is also evidence of N-cadherin based CCAs locally disrupting CMAs in mesenchymal cells. For example astrocytes, which express N-cadherin, showed only very small punctate CMAs near the contact between cells (Camand et al., 2012). However, when N-cadherin expression was inhibited the CMAs increased near the contact, demonstrating the importance of N-cadherin based CCAs in their suppression. Furthermore mouse embryonic fibroblasts

show clear polarisation of CMAs away from N-cadherin based CCA where they mediate the activation of PI3-kinase and Rac away from the contact (Ouyang et al., 2013). In the absence of N-cadherin, or when N-cadherin that can no longer bind to p120-catenin was expressed, CMAs were observed near the cell-cell contact, demonstrating the requirement of N-cadherin:p120-catenin in the localised suppression of CMAs near to the contact. Furthermore an investigation on myocyte doublets, which are known to express N-cadherin (Soler and Knudsen, 1994), showed the formation and maturation of CCAs at the contact between two cells corresponded to the reduction of traction forces and CMAs in this region (McCain et al., 2012). Together these examples demonstrate that the suppression of CMAs by the formation of CCAs is not cell type specific as it has been observed in multiple cells types including epithelial and mesenchymal cells. Furthermore the suppression of CMAs can be driven by different cadherin based CCAs as E-, N- and VE-cadherin have all been shown to trigger the localised suppression of CMAs.

CMAs antagonise CCAs

Not only has the presence of CCAs been demonstrated to locally impair CMAs in *in vitro* cultures, the reverse has also been shown with the presence of CMAs causing an antagonistic effect on CCAs. For example S180 murine cells, a sarcoma cell line, plated on micropatterned fibronectin stripes showed a reduction in E-cadherin adhesion strength near the fibronectin (Al-Kilani et al., 2011) and a human hepatocellular carcinoma cell line showed reduced E-cadherin adhesion strength when plated on a substrate compared to when the cells were in suspension in a manner dependent upon β1 and β5 integrins (Genda et al., 2000). Furthermore MCF-7 breast cancer cells plated on micropatterned substrates of either E-cadherin or fibronectin showed the formation of CMAs inhibited E-cadherin adhesions forming in the local proximity (Tsai and Kam, 2009). Interestingly this local suppression of E-cadherin by CMAs was shown to be dependent on the rigidity of the substrate; on soft substrates CCAs and CMAs localised to the same regions (Tsai and Kam, 2009) suggesting high tension across the adhesion complexes and coupling to the actin network is required for their mutual suppression. MDCK cells plated on substrates with different rigidities have shown a similar result (de Rooij et al., 2005). Cells plated on rigid substrates showed a

disruption of CCAs. However, cells plated on the softer substrates, where the CMAs could not generate as much traction, demonstrated more stabilised CCAs. While actomyosin contractility can stimulate CCAs, it appears that increased actomyosin contractility driven by CMAs can lead to the disassembly of CCAs (de Rooij et al., 2005; Papusheva and Heisenberg, 2010). These results highlight a role for the actin network in the crosstalk between CMAs and CCAs.

Positive regulation between CCAs and CMAs

While there is much evidence of the mutual suppression of CCAs and CMAs, the opposite has also been shown where the presence of one can reinforce the other. For example blocking CMAs, which are required during *Xenopus* gastrulation for cell intercalation and convergent extension, reduced the binding of C-cadherin (Marsden and DeSimone, 2003) suggesting the presence of CMAs was required to increase C-cadherin adhesion. Furthermore, mouse kidney epithelial cells lacking α3β1 failed to form the subcortical actin and cadherin-junctions were no longer uniformly oriented and their association with catenins, α-actinin and consequently actin were reduced (Wang et al., 1999). This suggests integrin is required for cadherin function through mediating the subcortical actin and the association of cadherins with catenins. In HeLa cells the loss of the CMA proteins paxillin and FAK were shown to result in aberrant N-cadherin based CCAs due to increased Rac1 activity (Yano et al., 2004). This suggests that the presence of signalling downstream of CMAs in HeLa cells is required for normal N-cadherin adhesion formation in these cells.

1.2.3 Mechanisms for crosstalk between CCAs and CMAs

CCAs and CMAs both physically couple to the actin cytoskeleton and are mechanosensitive, responding to the force applied to them as well as generating and balancing forces across the cell (Collins and Nelson, 2015; Liu et al., 2010; Riveline et al., 2001). It has been demonstrated in MDCK cells that an increase in traction forces generated by CMAs result in an increase in tension across the cell-cell contact (Maruthamuthu et al., 2011). Furthermore in MCF epithelial cells the organisation of the ECM was shown to coordinate the positioning of the CCAs to areas lacking ECM

due to reduced intercellular tension in these regions (Tseng et al., 2012). It is unsurprising therefore that the actin network plays a central role in the crosstalk between CCAs and CMAs. The small GTPases, such as Rac1 and RhoA, drive the reorganisation of the cytoskeleton and are involved in both the formation and downstream signalling of CCAs and CMAs and in the cross-regulation that occurs between these two distinct adhesion complexes (Collins and Nelson, 2015; Weber et al., 2011). The specific activity of the small GTPases can lead to either the disruption or enhancement of the adhesions (Papusheva and Heisenberg, 2010). Cadherin engagement initially leads to an increase in Rac1 activity and inhibition of RhoA activity (Noren et al., 2001), potentially through p120-catenin which is known to increase the activity of Rac1 and Cdc42 whilst inhibiting the activity of RhoA (Braga and Yap, 2005). Low levels of RhoA activity are required for the initial formation of both CCAs and CMAs, however if levels become too great and actomyosin contractility increases beyond a certain limit, it then has a negative effect on the adhesions (Burute and Thery, 2012). RhoA activity downstream of VE-cadherin based CCAs in vascular endothelial cells can either stimulate or inhibit CMAs depending on the context (Nelson et al., 2004). The consequence of RhoA activity on the adhesions appears to be dependent on the downstream effectors through which it signals (Fig. 2.4) (Weber et al., 2011). For example if RhoA signals through the formin Dia it reorganises the actin cytoskeleton resulting in the stabilisation of CCAs (Sahai and Marshall, 2002). However, signalling through Rho-kinase (ROCK) results in actomyosin contractility leading to an excess of tension across the CCA promoting their disassembly (Sahai and Marshall, 2002). The activation of ROCK and consequently actomyosin contractility is generally regarded to occur downstream of CMAs (Bhadriraju et al., 2007; Ren et al., 1999) leading to increased tension which can enhance the growth of CMAs (Chrzanowska-Wodnicka and Burridge, 1996) resulting in a positive feedback loop. Furthermore ROCK activity, through myosin II, and consequently tension across the contact is required in order to stabilise newly forming CCAs (Shewan et al., 2005). Excessive tension however can disrupt both CCAs and CMAs. p120-catenin is a known regulator of RhoA activity and potential candidate in the crosstalk between CCAs and CMAs. p120-catenin has been shown to interact with p190RhoGAP resulting in the inhibition of RhoA (Wildenberg et al., 2006). Interestingly, as discussed in the previous chapter, CMAs

also activate p190RhoGAP through Src activity (Arthur et al., 2000; Fincham et al., 1999), and therefore p190RhoGAP could be a point of convergent signalling between CCAs and CMAs. In addition cytoplasmic p120-catenin can inhibit the maturation of CMAs due to inhibition of RhoA; the overexpression of a constitutively active form of RhoA can rescue the effect on p120-catenin (Grosheva et al., 2001). Furthermore the suppression of CMAs downstream of N-cadherin in fibroblasts was shown to require the association of p120-catenin with N-cadherin (Ouyang et al., 2013). Together these results demonstrate the role of p120-catenin in regulating actin dynamics and consequently the behaviour of CMAs and CCAs. In addition to their ability to regulate the cytoskeleton through p120-catenin, CCAs have also been shown to activate Rac1 activity through Src driven P13-kinase signalling (Fukuyama et al., 2006). Due to the fact that both CCAs and CMAs can lead to actin reorganisation and both are tension sensitive structures, the actin cytoskeleton is vital in the crosstalk between them leading to either positive or negative effects on the relative adhesions.

Further to their ability to communicate via the actin network, CCAs and CMAs are also linked through downstream signalling molecules such as various tyrosine kinases (Weber et al., 2011). The tyrosine kinase Src localises to both CMAs, as discussed in the previous chapter, and CCAs (McLachlan et al., 2007; Tsukita et al., 1991). Its recruitment to E-cadherin based CCAs drives the activation of the PI3-kinase pathway which promotes a positive feedback loop promoting the stability of the CCA complex (McLachlan et al., 2007). However, Src activity has also been shown to disrupt CCAs (Avizienyte et al., 2002; Behrens et al., 1993; Owens et al., 2000). The presence of CMAs were shown to destabilise CCAs in carcinoma cell lines through the dissociation of α-catenin with the cadherin complex (Genda et al., 2000). Interestingly active Src was shown to localise to the CCAs in a manner dependent on the presence of the CMAs, where its presence was believed to disrupt the CCAs by enhancing the dissociation of α-catenin from the cadherin complex. An alternative mechanism through which Src activity can disrupt CCAs is through FAK. Src phosphorylates and activates FAK (Calalb et al., 1996; Westhoff et al., 2004) and Src and FAK activity have been shown to result in the phosphorylation of β-catenin disrupting its association with the cadherin complex and promoting the disassembly of the CCA complex

(Behrens et al., 1993; Koenig et al., 2006; Wang et al., 2006). In addition phosphorylation of VE-cadherin by FAK stimulated by VEGF results in the disruption of the CCAs (Chen et al., 2012; Jean et al., 2014). Furthermore TGF-β induced Src/FAK activity leads to the internalisation and degradation of E-cadherin in hepatocytes undergoing EMT (Cicchini et al., 2008; Palacios et al., 2005). Both Src and FAK are activated at CMAs and their involvement in promoting the disassembly of CCAs downstream of CMAs has also been observed in squamous cell carcinoma *in vivo* (Canel et al., 2010). The inhibition of Src or FAK activity with small molecule inhibitors led to a stabilisation of E-cadherin and increased CCA strength (Canel et al., 2010). In colon cancer cells elevated Src and consequently FAK activity was shown to inhibit the assembly of CCAs and redistribute some shared components, such as vinculin, to the CMAs (Avizienyte et al., 2002). In addition Src activity resulted in an increase in myosin II phosphorylation and consequently actomyosin contractility leading to an enlargement of the CMAs and the disruption of the CCAs (Avizienyte et al., 2004). Interestingly both Src and FAK also play a role in the formation of CCAs through their ability to regulate Rho activity and actomyosin contractility (Martinez-Rico et al., 2010; Playford et al., 2008). This suggests that carefully controlled levels of Src and FAK activity are required to form, stabilise or disrupt the different adhesion complexes in different contexts.

Src and FAK are also key components in reinforcing both CCAs and CMA downstream of the other. Interestingly although the activation of Src downstream of CMAs can lead to CCA disruption, as discussed above, it is also required to enhance cell-cell adhesion strength in S180 murine sarcoma cells (Martinez-Rico et al., 2010). In S180 murine sarcoma cells, the force required to separate a doublet of cells was greater when the cells were adhered to the substrate compared to cells in suspension, suggesting the presence of CMAs reinforce the CCA in this context. This reinforcement was shown to be dependent on integrin-dependent activation of the Src family kinases leading to increased ROCK activity and consequently enhance actomyosin contractility (Martinez-Rico et al., 2010). More recently it has been demonstrated in the same cell line that E-cadherin based CCAs enhanced CMA driven traction forces through Src activity and activation of PI3-kinase activity (Jasaitis et al., 2012). Furthermore in some colon

cancer cells TGF-β elevated CMAs resulting in increased FAK activity which consequently promoted E-cadherin expression and enhanced cell-cell adhesion strength (Wang et al., 2004). Together these results once again highlight the critical role Src, FAK and the actin cytoskeleton play in the cross-regulation between CMAs and CCAs.

The mechanisms discussed above are by no means an extensive list of the mechanisms CMAs and CCAs can use to communicate and regulate each other. For example CCAs and CMAs share common components that link them both to the cytoskeleton such as the tension sensitive proteins vinculin and α-actinin, and the actin nucleator complex Arp2/3 (Epifano and Perez-Moreno, 2012). The differential regulation or recruitment of these proteins to CCAs and CMAs could result in either an antagonistic or positive effect. Furthermore CCAs and CMAs are believed to communicate through other means such as through phosphatases, the threonine/serine kinase ILK and the small GTPase Rap1 (Canel et al., 2013; Epifano and Perez-Moreno, 2012; Weber et al., 2011)

There is a vast body of evidence demonstrating the importance of crosstalk between CCAs and CMAS *in vivo*. Multiple candidates in this crosstalk, such as p120-catenin, the small GTPases and Src and FAK kinases have been identified *in vitro*. However, many of the cell types investigated in this crosstalk form stable CCAs. It is therefore uncertain how transient CCAs, such as those that have been identified during contact inhibition of locomotion discussed in the following chapter, regulate CMAs. This is one of the questions the work presented here wishes to address.

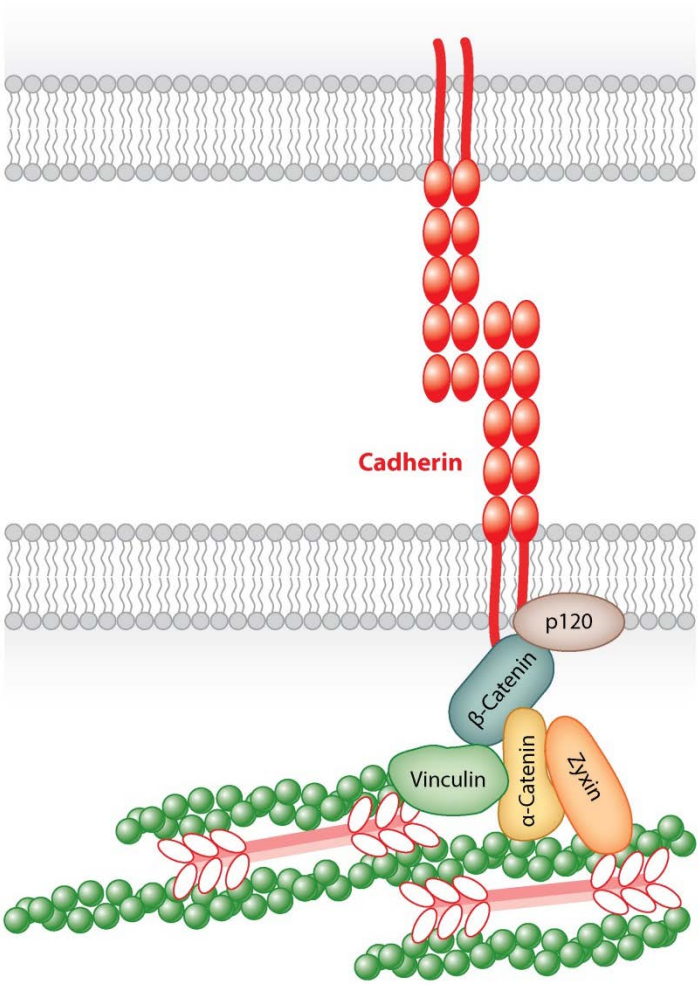

Figure 2.1 Classical cadherin cell-cell adhesion

Classical cadherins, such as E- and N-cadherin, consist of a single transmembrane span. They form an adhesion with cadherins from neighbouring cells via the most distal domain of the extracellular N-terminal region. The C-terminal cytoplasmic domain associates with p120-catenin and β-catenin, which in turn binds α-catenin. Together α- and β-catenin are involved in crosslinking cadherin to the F-actin cytoskeleton, either directly or via adapter proteins such as vinculin or zyxin. p120-catenin is involved in regulating the disassembly of cadherin junctions.

[From (Leckband and de Rooij, 2014)]

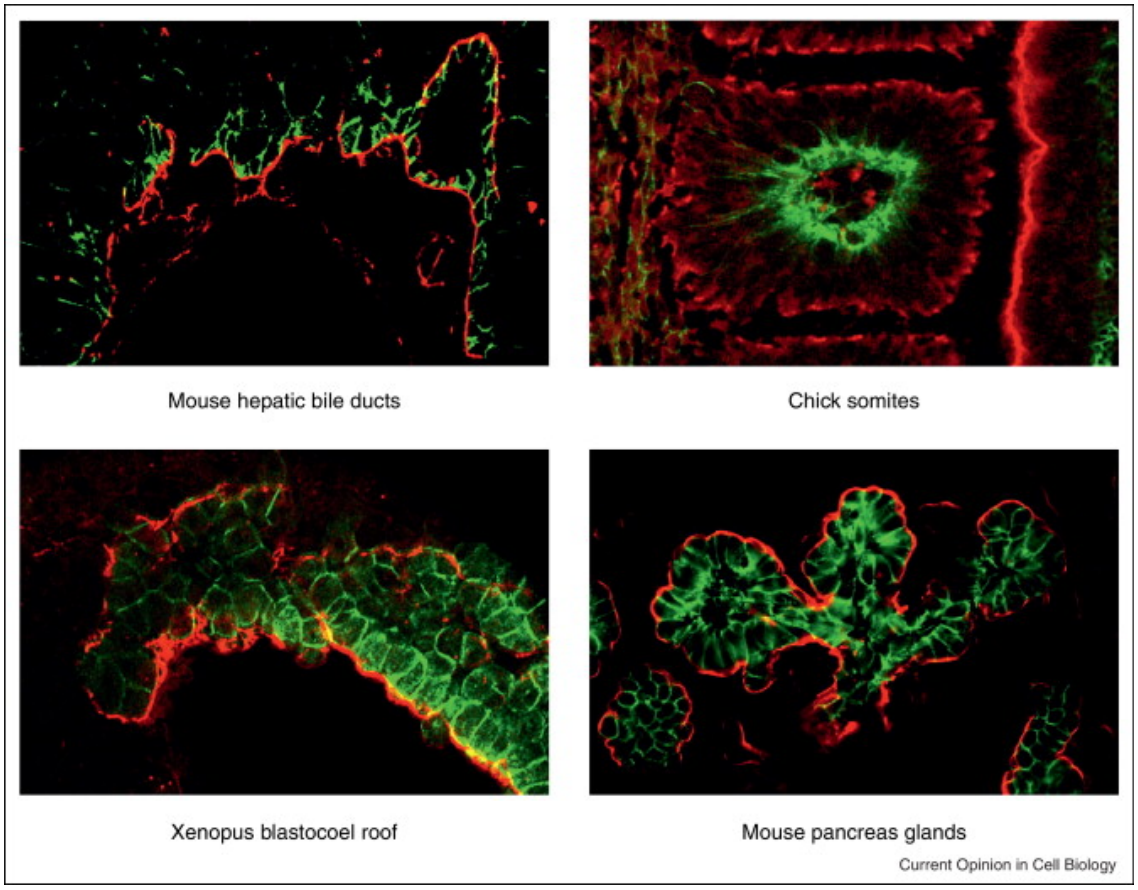

Figure 2.2 Spatial segregation of CCAs and CMAs

Examples of the spatial segregation of CCAs (green) and CMAs (red) in various tissues. In mouse hepatic bile duct, E-cadherin is green and laminin is red. In chick somites N-cadherin is green and laminin is red. In *Xenopus* blastocoel roof N-cadherin is green and fibronectin is red. In mouse pancreas glands E-cadherin in green and laminin is red.

[From (Burute and Thery, 2012)]

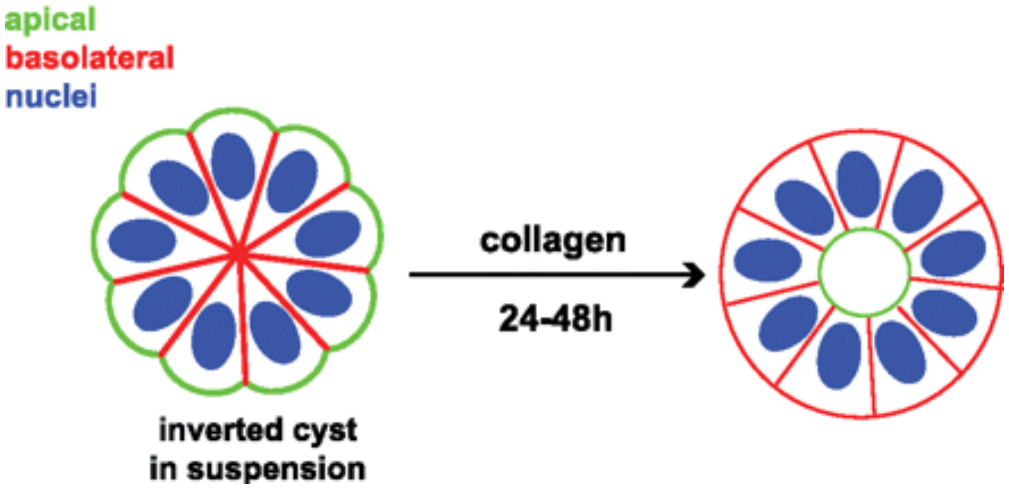

Figure 2.3 Effect of collagen on apico-basal polarity

Schematic of MDCK cells in the presence or absence of collagen. In the absence of collagen MDCK cells form an inverted cyst in suspension with their apical surface on the outside. In the presence of collagen MDCK cells reverse their polarity so the apical surface faces in creating a lumen. This lumen containing structure is similar to those that form during development and therefore conserved mechanisms are likely to occur in their formation. This system can be used to study the effect substrate and consequently cell-matrix adhesions have on cell-cell adhesions.

[Adapted from (Liu et al., 2007)]

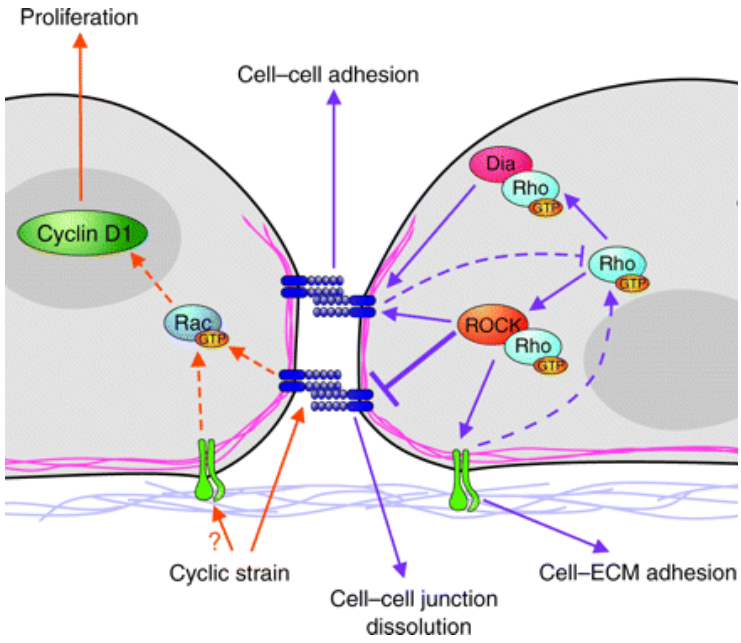

Figure 2.4 Molecular mechanism of crosstalk

Both CCAs and CMAs regulate the small GTPases and consequently the actin cytoskeleton. Both CCAs and CMAs can activate Rac which can result in the expression of cyclin D1 and proliferation. In addition CCAs and CMAs antagonistically influence Rho. CCAs can inhibit Rho activity, potentially through the activation of the GAP p190RhoGAP. CMAs however can activate Rho and, depending on the downstream effector through which it signals, can alter CCA behaviour. If Rho signals through Dia the actin cytoskeleton is reorganised in such a way that CCAs become stabilised. However, if Rho signals through ROCK actomyosin contractility is increased and this can result in an excess of tension across the CCA leading to their disassembly.

[Adapted from (Weber et al., 2011)]

1.3 Contact inhibition of locomotion

Contact inhibition of locomotion (CIL) is a multi-faceted process whereby cells that come into contact with each other cease their migration towards the site of contact before repolarising and migrating away from each other (Roycroft and Mayor, 2016). Leo Loeb initially observed this phenomenon in the 1920s among amoebocyte haemocytes in horseshoe crabs (*Limulus*) where he noted that the haemocytes 'move toward each other and meet and stick together. Subsequently the agglutinating cells send out pseudopods in such a way that the cells become again separated from each other' (Loeb, 1921). It was not until the 1950s however, that CIL was first characterised by the influential cell and developmental biologist, Michael Abercrombie (Abercrombie and Heaysman, 1954b). Abercrombie was interested in the social behaviour of cells, i.e. how a cell is influenced by other cells in its surrounding. His early observations of migrating chick heart embryonic fibroblasts revealed an interesting behaviour where the mean velocity of a single migrating cell was inversely proportional to the amount of collisions it underwent with other fibroblasts (Abercrombie and Heaysman, 1953). Abercrombie made more extensive observations of this behaviour and noted that not only was velocity restricted upon a collision with another cell, but the directionality was affected as well with cells migrating away from each other after colliding (Abercrombie and Heaysman, 1953). He coined the term 'contact inhibition' in order to describe this phenomenon.

1.3.1 Defining CIL

The term contact inhibition can also refer to a density dependent inhibition of cell growth first identified in the 1960s (Eagle et al., 1965; Todaro and Green, 1963). This is a process where the rate of proliferation is reduced in cells when they become confluent. Contact inhibition of cell growth and replication is distinct from CIL and they are driven by independent mechanisms (Stoker and Rubin, 1967).

The precise definition of CIL has evolved as the understanding of this phenomenon has increased. Abercrombie initially defined CIL as 'the prohibition, when contact between cells occurred, of continued movement such as would carry one cell over the surface of

another' (Abercrombie, 1970a). This description is still the defining characteristic of CIL; however, more detailed observations of CIL in a variety of cell types have allowed this definition to be expanded. CIL is often subdivided into two categories: type I and type II (Veselý and Weiss, 1973). Type I, as first observed in fibroblasts by Abercrombie, is characterised by paralysis of membrane ruffling and a contraction at the leading edge (Abercrombie and Ambrose, 1958). Type II, as described by Carter, does not involve contraction of the leading edge; the cessation of migration in the direction of contact is inhibited solely due to the difficulty of the cell to migrate across the surface of the other cell (Carter, 1967). Abercrombie himself questioned whether collisions without contraction at the leading edge, as observed in type II collisions, were in fact CIL, stating that type II collisions bear 'little resemblance to contact inhibition' (Abercrombie, 1970b) and many believe that contraction of the leading edge is a necessity for CIL (Heaysman and Pegrum, 1973a). The identification of the molecular mechanisms involved in type I CIL indicate that it is an active process and distinct from the more passive type II CIL. A key characteristic of type I CIL is that an unrestricted cell upon a collision not only ceases 'to continue moving in the same direction after contact with another cell' (Abercrombie, 1979), but also repolarises and migrates away from the contact if possible. A restricted cell i.e. one that is completely surrounded by cells, such as those in a cluster, would have their protrusions inhibited on all sides (Mayor and Carmona-Fontaine, 2010; Theveneau et al., 2010). The process of type I CIL, which is focused on here, can be broken down into four discrete stages (Fig. 3.1): (i) Initially a contact is formed between the cells, (ii) protrusive activity is inhibited at the site of contact, (iii) the cells repolarise and new protrusions form away from the contact, (iv) the cells separate and migrate away from each other.

CIL can occur between cells of the same type (homotypic CIL), or between cells of a different type (heterotypic CIL). In the 60 years following its initial characterisation in chick heart embryonic fibroblasts, homotypic CIL has been identified in a variety of other cells types including somitic cells (Newgreen et al., 1979) neural crest cells (Carmona-Fontaine et al., 2008; Newgreen et al., 1979), haemocytes (Stramer et al., 2010), and Cajal-Retzius neurons (Villar-Cerviño et al., 2013). Heterotypic CIL can occur

between cell types that independently show homotypic CIL, such as between fibroblasts and DU-145 prostate cancer cells (Batson et al., 2013) and neural crest and somitic cells (Gooday and Thorogood, 1985). Interestingly heterotypic CIL can also occur between a cell type that shows homotypic CIL and one that does not, such as between neural crest cells and placodes (Theveneau et al., 2013), where placodes, although able to repolarise away from the contact after a collision, do not separate from each other (Theveneau et al., 2013).

1.3.2 The role of CIL *in vivo*

Upon discovering CIL between chick heart embryonic fibroblasts, Abercrombie speculated on its possible role within living organisms. He suggested CIL may be one of the mechanism behind re-epithelialisation in wound healing, whereby the release of any cell-cell contacts induced by wounding would promote cells at the free edge to migrate into the free space (Abercrombie, 1979). Furthermore Abercrombie suggested CIL may be required in maintaining stationary cells within heathy tissue, the loss of which could lead to cells becoming invasive (Abercrombie, 1979; Abercrombie and Heaysman, 1954a; Abercrombie et al., 1957). He demonstrated that heterotypic CIL was perturbed between sarcoma cells and healthy fibroblasts; instead of repolarising after colliding with a healthy fibroblast the sarcoma cells continued migrating (Abercrombie and Heaysman, 1954a; Abercrombie et al., 1957). More recently CIL and its loss towards healthy cells has been demonstrated in prostate cancer cells (Astin et al., 2010; Batson et al., 2013; Batson et al., 2014) and adenocarcinoma cells (Lin et al., 2015), supporting the idea that CIL is important to maintain healthy tissue and its loss could be a prerequisite for metastasis (Parish et al., 1987; Tanaka et al., 2012).

CIL has also been identified to play a role in embryo development (Stramer et al., 2013). Neural crest cells, a population of pluripotent embryonic cells whose derivatives include craniofacial cartilage, melanocytes and ganglia of the peripheral nervous system (Bronner and LeDouarin, 2012), were first shown to exhibit CIL behaviour in the 1970s (Newgreen et al., 1979) and the cranial neural crest were the first cells visualised undergoing CIL *in vivo* when they were observed in *Xenopus* and Zebrafish (Carmona-Fontaine et al., 2008; Theveneau et al., 2010). During embryo development the cranial

neural crest migrates ventrally in distinct streams (Theveneau and Mayor, 2011). It has been demonstrated that CIL between neural crest cells is required for their directional collective migration (Carmona-Fontaine et al., 2008; Theveneau et al., 2010; Theveneau et al., 2013; Woods et al., 2014). How CIL can contribute to collective migration is not entirely understood. It has been proposed that CIL could be contributing to this phenomenon by inhibiting protrusions forming within the cluster and driving the polarisation of the cells at the leading edge into the space away from the cluster (Mayor and Carmona-Fontaine, 2010) (Fig. 3.2). Interestingly it has been observed that single cells that undergo CIL can form persistent polarised chains of cells coherently migrating in a given direction in a 1D environment (Desai et al., 2013). The more cells in the chain the more persistent the migration of the collective of cells is and the less likely the train is to repolarise upon a collision with a single cell. The formation of these chains is due to the fact that cells that display CIL do not do so in all collisions; the probability of CIL occurring in a given collision determines the likelihood of chains forming and is fundamental in determining the dynamics of the overall population (Desai et al., 2013; Scarpa et al., 2013). This suggests that CIL not only assists collective migration, it can in fact give rise to collective behaviour. Furthermore it was also observed that cells within these chains became coupled together through cell-cell adhesions (CCAs) (Desai et al., 2013), suggesting CIL could be functionally linked to collective migration through the coupling of CCAs. CIL is just one of the many factors that has to be carefully mediated for the collective migration of the neural crest (Theveneau and Mayor, 2012b), chemotaxis also plays a role in their collective migration (Olesnicky Killian et al., 2009; Theveneau et al., 2010; Theveneau et al., 2013). The interplay between CIL and chemotaxis is not fully understood. However, it has recently been shown that the outcome of CIL collisions changes in the presence of a chemoattractant gradient (Lin et al., 2015). When cells collide in the presence of a chemoattractant gradient they are more likely to repolarise in the direction of the chemoattractant, even if this means they are not polarising away from the contact. However, the outcome of a collision is dependent on the balance of CIL versus chemotactic response and can be shifted between one outcome or another depending on the signalling pathways activated (Lin et al., 2015). In neural crest explants it appears that while CIL polarises the cells at the edge of the cluster away from the

contact, the chemoattractant SDF1 stabilises these protrusions at the leading edge (Theveneau et al., 2010; Theveneau et al., 2013). One reason for this could be that the chemoattractant is biasing the outcome of collisions to repolarise cells towards the chemoattractant cue so the cells are the rear of the cluster, which would otherwise be polarised in the reverse direction to the leading edge, also transiently polarise in the direction of the chemoattractant. Overall these experiments demonstrate how directional migration and CIL could work together to polarise the cells and drive collective migration. Although the role of CIL in collective migration has predominantly been studied in the neural crest, it is likely to play a similar role in the collective migration of other cell types. It is also possible that CIL plays a role in the contact-dependent polarity that drives the tightly coordinated migration of border cells in the *Drosophila* ovary (Cai et al., 2014; Pocha and Montell, 2014).

CIL has also been observed *in vivo* between haemocytes in *Drosophila* where the behaviour observed between these cells is strikingly similar to what Abercrombie first observed in fibroblasts *in vitro* over 50 years earlier (Abercrombie and Heaysman, 1954b; Davis et al., 2012; Stramer et al., 2010) Haemocytes are large highly migratory macrophages involved in the immune response. They initially migrate out from the head mesoderm before dispersing throughout the embryo through defined migratory routes (Tepass et al., 1994). Haemocyte dispersion can be imaged within the haemocoel, an acellular cavity just beneath the epithelium along the ventral surface, a region superficial enough to allow high spatio-temporal resolution imaging (Stramer et al., 2010; Stramer et al., 2005). Imaging haemocytes in this region has revealed that they form a precise three-lined dispersion pattern where the cells have uniform cell spacing (Wood et al., 2006). This dispersion pattern has now been shown to be a consequence of CIL between the haemocytes (Davis et al., 2012). Interestingly a similar role for CIL has been shown in mouse embryos where CIL is required for the regular dispersion of Cajal-Retzius neurons throughout the cortex (Villar-Cerviño et al., 2013). It is possible that CIL plays a role in the precise dispersion patterning seen in other cell types in developing embryos (Grueber and Sagasti, 2010; Kay et al., 2012). For many decades following its initial characterisation by Abercrombie, the molecular mechanisms underlying CIL remained unknown. Its implication in invasion in cancer

(Abercrombie, 1979; Batson et al., 2013) and discovery in the developing embryo (Carmona-Fontaine et al., 2008; Stramer et al., 2013) has led to a resurgence in the field of CIL and the molecular components that drive CIL have finally begun to be elucidated.

1.3.3 Methods used to study CIL *in vitro*

In order to characterise CIL and better understand its role in cancer and development, several different assays have been developed over the years. Abercrombie first characterised CIL using a technique whereby two chick heart explant cultures were plated between 0.5 - 1 mm apart (Abercrombie and Heaysman, 1954b). The cells would grow out from these explants and their behaviour towards each other could be observed in the gap between them. He used this assay to characterise CIL and demonstrate that sarcoma cells lose CIL towards healthy fibroblasts (Abercrombie and Ambrose, 1958; Abercrombie and Heaysman, 1953; Abercrombie and Heaysman, 1954b; Abercrombie et al., 1957). Similar techniques are still used to address whether cells are invasive towards chick heart explants (Bracke et al., 2014) and 3D image reconstructions can give a more detailed view of the invasion taking place. A comparable confrontation assay was used to establish the role of CIL in the neural crest where the behaviour of explants towards each other could be observed (Carmona-Fontaine et al., 2008; Scarpa et al., 2015; Theveneau et al., 2010; Theveneau et al., 2013). In order to probe the distribution of traction forces during CIL, the explant confrontation assay has been carried out on polyacrylamide gels labelled with fluorescent beads in an assay known as traction force microscopy (Theveneau et al., 2013; Wang and Lin, 2007). CIL between single cells has predominantly been characterised on 2D glass or plastic substrates (Dunn and Paddock, 1982; Paddock and Dunn, 1986). Individual cells migrate randomly and stochastic collisions between cells are observed. This method has been used to investigate why cancerous cells lose CIL towards normal fibroblasts and has helped elucidate mechanisms controlling CIL (Astin et al., 2010; Batson et al., 2013; Batson et al., 2014; Carmona-Fontaine et al., 2008; Kadir et al., 2011; Moore et al., 2013; Theveneau et al., 2013). Cells on a 2D substrate can collide from any incoming angle. It has long been established that head-to-head

collisions show distinct CIL behaviour whereas other collisions, such as head-to-side where lamellae do no overlap, often do not display CIL (Abercrombie and Dunn, 1975; Davis et al., 2012). In order to restrict cell-cell interactions to more reproducible head-to-head collisions 1D collision assays have been generated (Desai et al., 2013; Scarpa et al., 2013). One method confines cells to micropatterned extracellular matrix lanes restricting the angle of collision to head-on only and forcing the cells to repolarise 180° (Scarpa et al., 2013). Forcing the cells to completely reverse their front-rear polarity makes it easier to establish the steps required for this repolarisation and the temporal regulation of these events. In addition restricting the cells to 1D lanes makes it easier to predict when cells are going to interact and allows for easier analysis (Desai et al., 2013; Scarpa et al., 2013). An additional assay has been generated that restricts cells to 1D migration through the use of microchannels (Lin et al., 2015). In this assay microfluidic chambers constrain cell migration to 1D channels whilst allowing chemoattractant gradients to be generated across the chamber (Li Jeon et al., 2002). These chambers are proving useful in understanding how CIL is affected by chemotactic cues found *in vivo*. This is of particular interest as cancer cells are known to migrate through tracks generated in the extracellular matrix (Desai et al., 2013) and respond to chemotactic cues (Desmarais et al., 2009; Roussos et al., 2011). Observations of cells undergoing CIL *in vitro* are similar to observations generated *in vivo*. For example the trajectories of Zebrafish cranial neural crest cells undergoing a collisions in *vivo* are similar to those of neural crests colliding *in vitro* (Carmona-Fontaine et al., 2008) and haemocytes imaged in the *Drosophila* mirror the behaviour and dynamics of fibroblasts in culture (Abercrombie and Heaysman, 1954b; Davis et al., 2015; Kadir et al., 2011; Stramer et al., 2010). These methods used to investigate CIL both *in vitro* and *in vivo* have helped elucidate some of the molecular mechanisms driving CIL in a variety of cell types.

1.3.4 Molecular machinery driving CIL

CIL is a complex multi-faceted process that involves many different molecular mechanisms. CIL can be broken down into four distinct steps, each of which requires changes to the cytoskeleton driven by a variety of molecular components (Fig. 3.1)

(Davis et al., 2015; Kadir et al., 2011; Roycroft and Mayor, 2015; Roycroft and Mayor, 2016). In the following section the stages of CIL are described in more detail and the key components identified in driving each of the steps are highlight.

(i) A contact is formed between the cells

The fact that an adhesive contact must be forming between colliding cell partners was first made evident by the early observation that tension was generated in the lamellae across a contact (Abercrombie and Ambrose, 1958; Harris, 1973; Heaysman and Pegrum, 1973a). After Abercrombie's discovery of CIL in fibroblasts, work was done to elucidate the nature of these adhesions using the microscopy techniques available at the time. Heaysman and Pegrum coupled the behaviour of the adhesions to the different stages of CIL in fibroblasts (Heaysman and Pegrum, 1973a). They noted that adhesion between the colliding cells formed soon after a collision and speculated that the abrupt separation of the cells was due to the loss of these adhesions. Interestingly sustained adhesion between the cells were not observed when fibroblasts collided with sarcoma cells (Heaysman and Pegrum, 1973b), where normal CIL behaviour is known to be lost (Abercrombie et al., 1957). Although the exact nature of these adhesions was speculated upon (Abercrombie, 1970a), the limitations of the microscopy and molecular biology techniques available prevented the identification of the molecular components involved. It was not until decades later that the nature of these adhesions could begin to be elucidated. One potentially surprising aspect of the adhesion molecules between cells identified in driving CIL is that they do not all belong to the same family of adhesion complexes. This suggests that CIL may be driven through a variety of different mechanisms.

Cadherins: The first family of CCA molecules to be identified in CIL were the cadherins (Chen and Obrink, 1991). Their importance in CIL was first identified in L-cell line fibroblasts where it was demonstrated that the presence of E-cadherin caused paralysis of the lamellae upon a collision (Chen and Obrink, 1991). Furthermore, E-cadherin has since been identified as the adhesion molecule required to inhibit the protrusive activity and migration of confluent epithelial cells (Bracke et al., 1997) and its disruption has been associated with the loss of this behaviour in carcinoma cells

(Ayollo et al., 2009). N-cadherin is required for CIL in a variety of cell types (Huttenlocher et al., 1998; Tanaka et al., 2012; Theveneau et al., 2010). In myoblasts and glial cells it is required for the cessation of migration and paralysis of lamellae upon a collision (Huttenlocher et al., 1998; Tanaka et al., 2012). In addition N-cadherin and cadherin-11 are essential for CIL between neural crest cells, their loss inhibits the migration of the neural crest *in vivo* and the neural crest no longer undergo CIL *in vitro*, instead they continue their direction of migration and do not repolarise away from the contact (Becker et al., 2013; Theveneau et al., 2010). In addition there is an increase in protrusive activity at the contact, indicating that the normal paralysis of lamellae is lost. Interestingly blocking N-cadherin junctions in Schwann cells seems to promote a CIL like process, where the cells pull away from each other after coming into contact (Letourneau et al., 1991). Why Schwann cells show this differential behaviour is unknown and could be due to the different downstream signals evoked in each case.

Eph-ephrin: Another group of proteins that are known to mediate cell-cell interactions during CIL are the Eph receptors. These are a group of tyrosine kinase receptors that bind transmembrane ephrin ligands from neighbouring cells. The binding of the ligand by the receptor triggers bidirectional signalling cascades in both the ligand-expressing and the receptor-expressing cells (Kullander and Klein, 2002). Eph/ephrins are expressed in all germ layers. They are essential for many aspects of development including vascular and skeleton morphogenesis, boundary formation and axon guidance (as reviewed in (Klein, 2012)) and their dysregulation is associated with disease (Pasquale, 2008). Interestingly Eph-ephrin mediated cell-cell interactions are often, but not always, associated with a repulsive response in the coupled cells causing the cells to retract upon contact in a process similar to CIL (Dudanova et al., 2010; Marston et al., 2003; Wang and Anderson, 1997). Like E- and N-cadherin, both EphA and EphB can both promote or suppress CIL response in a manner dependent on the cell-type (Batson et al., 2013; Lin et al., 2015; Tanaka et al., 2012). For example EphA signalling can facilitate CIL in prostate cancer cells by promoting a repulsive behaviour between cells (Batson et al., 2013; Batson et al., 2014); whereas EphB signalling suppresses CIL and increases membrane ruffling at the site of contact by promoting cell-cell attraction (Astin et al., 2010; Marston et al., 2003). Interestingly this difference

in behaviour controlled by a shift in the balance of activities of EphA to EphB, is strikingly similar to the cadherin switch from E- to N- that dictates whether neural crest cells undergo CIL or not (Scarpa et al., 2015). In agreement with these results the expression of ephrin-B in glioblastoma cells perturbs CIL (Tanaka et al., 2012). However, both EphA and EphB are required for CIL in Cajal-Retzius neurons in order to drive their proper dispersion (Villar-Cerviño et al., 2013). Furthermore EphB signalling gives rise to CIL in a carcinoma cell line and can induce high levels of CIL behaviour, which can override chemotactic cues (Lin et al., 2015). Neural crest cells, which undergo CIL, are known to express Ephs and ephrins and it is therefore possible that Eph signalling is contributing to CIL in the neural crest (Smith et al., 1997). Whether the full spectrum of cell-cell adhesion complexes that contribute to CIL have been identified is unknown. During CIL of haemocytes in *Drosophila* (Davis et al., 2012; Davis et al., 2015; Stramer et al., 2010) zyxin has been shown to localize at the cell-cell contact (Davis et al., 2015); however the molecular nature of the cell-cell adhesion molecule at the contact remains unknown. The engagement of this unidentified cell-cell adhesion is essential for CIL through its ability to couple the cytoskeletons in the colliding partners, allowing tension to be built up in their lamellae prior to separation (Davis et al., 2015).

(ii) Protrusive activity is inhibited at the site of contact

The distinct steps of CIL are each driven by cytoskeleton rearrangements and dynamics that are controlled by the activity of Rho family GTPases (Hall and Nobes, 2000; Roycroft and Mayor, 2016). Rac1 and RhoA are the best understood members of the RhoGTPases. The canonical understanding is that Rac1 drives the formation of lamellipodia (Ridley et al., 1992) through the mediation of actin polymerisation, while RhoA generates contraction through the regulation of actomyosin and activation of ROCK (Chrzanowska-Wodnicka and Burridge, 1996).

One distinct feature of CIL is the paralysis of membrane ruffling and inhibition of protrusive activity at the leading edge upon a collision (Abercrombie and Ambrose, 1958; Davis et al., 2015; Heaysman and Pegrum, 1973a; Theveneau et al., 2010; Trinkaus et al., 1971). In a free migrating cell Rac1 is active in the leading edge. This

drives actin polymerisation and subsequently protrusion formation at this site (Ridley et al., 1992). Upon a collision a switch in the activity of the RhoGTPases occurs at the contact site, whereby RhoA is activated and Rac1 is inhibited, driving paralysis of the membrane and loss of protrusions (Carmona-Fontaine et al., 2008; Matthews et al., 2008; Theveneau et al., 2010). In neural crest cells, this switch is dependent upon the activation of the non-canonical Wnt-planar cell polarity (PCP) pathway (Carmona-Fontaine et al., 2008; Matthews et al., 2008; Mayor and Theveneau, 2014). Upon a collision many PCP elements, including Dishevelled, Prickle1 and Strabismus, are recruited to the receptor Frizzled7 at the cell-cell contact where their presence is required to drive CIL (Carmona-Fontaine et al., 2008; Theveneau et al., 2013). The activation of the PCP pathway results in the activation of RhoA, which drives the contraction of the lamellae in a manner dependent on ROCK activity. If ROCK activity is blocked the protrusions fail to collapse at the contact and normal CIL behaviour is lost (Carmona-Fontaine et al., 2008; Matthews et al., 2008; Theveneau et al., 2013). In addition Rac1 activity is inhibited at the contact site, resulting in collapse of the protrusions (Mayor and Carmona-Fontaine, 2010; Mayor and Theveneau, 2014). This loss of Rac1 activity could in part be due to the antagonistic behaviour that is known to occur between RhoA and Rac1, where the activation of one results in the inhibition of the other (Shoval and Kalcheim, 2012). The requirement of RhoA/ROCK activity at the contact site in CIL has also been further established in chick embryonic heart fibroblast where their absence prevents the cells from undergoing CIL, instead they continue migrating in their given direction upon contact as there is no paralysis of membrane ruffles and protrusions (Kadir et al., 2011). Furthermore the perturbation of Rac1 in NIH-3T3 fibroblasts, either through the use of dominant active Rac1, dominant negative Rac1 or an increase in RhoA activity, results in the loss of CIL when they confront chick heart embryonic fibroblasts (Anear and Parish, 2012). As well as its inhibition downstream of PCP signalling, the inhibition of Rac1 is also driven by the formation of N-cadherin junctions at the contact in the neural crest. Blocking N-cadherin, either by antisense morpholinos or blocking antibodies, results in a loss of CIL due to an increase in Rac1 activity at the contact driving protrusions at this site (Theveneau et al., 2010). In addition the overexpression of E-cadherin in the neural crest also results in an increase in Rac1 activity at the contact (Scarpa et al., 2015).

Furthermore E-cadherin overexpressing cells no longer undergo CIL and instead demonstrate a large overlap of protrusions.

The precise mechanism through which the CCAs that drive CIL lead to the activation of RhoA and inhibition of Rac1 is not entirely clear and varies depending on the signalling pathway activated (Fig. 3.3) (Roycroft and Mayor, 2016). There is evidence that the RhoGTPase switch that occurs at the contact upon a collision could be mediated by the inhibition of the GEF-Trio at this site. Trio can activate Rac1 and modulate the activity of RhoA. It localises to the cell-cell contact in the neural crest *in vivo*, downstream of the polarity protein Par3, where its inhibition appears to be required for CIL (Moore et al., 2013). Furthermore there is evidence that Trio is recruited downstream of cadherin-11 and its inhibition could provide a mechanism for RhoA activation and Rac1 inhibition upon a collision (Kashef et al., 2009). It is possible the cadherin junctions recruit Par3 to the contact where they inhibit Trio activity, preventing Trio from activating Rac1. An additional mechanism that has been identified in driving the RhoGTPase switch is through the interaction between the nucleotide diphosphate kinase–nm23, and the GEF-Tiam1 that activates Rac1. Nm23 has been identified at the cell-cell contact site in glial cells undergoing CIL where it is localised to N-cadherin (Tanaka et al., 2012). At the cell-cell contact nm23 associates with Tiam1 and inactivates it resulting in the inhibition of Rac1 at this site. Another mechanism through which N-cadherin may be leading to the switch in RhoGTPase activity upon a collision is through its interaction with p120-catenin, which binds to N-cadherin and regulates its turnover (Davis et al., 2003). Cytosolic p120-catenin can enhance protrusion formation through the activation of Rac1 (Grosheva et al., 2001; Noren et al., 2000). Interestingly when it is sequestered to the CCA it can no longer promote the activation of Rac1 and protrusions are inhibited (Grosheva et al., 2001). During CIL N-cadherin could be sequestering p120-catenin preventing it from activating Rac1 at the contact. Furthermore, the elevation of Rac1 at the contact in neural crest cells overexpressing E-cadherin appears to be dependent on its interaction with p120-catenin and when this interaction is blocked Rac1 activity is once again reduced at the contact (Scarpa et al., 2015). This suggests the ability to prevent p120-catenin from activating Rac1 may be specific to the way it is sequestered by N-cadherin. p120-

catenin has also been implicated in modulating RhoGTPase activity downstream of Wnt signalling (Chacon-Heszele et al., 2012; Hong et al., 2010; Stocker and Chenn, 2015) . It is also possible that p120-catenin may be modulating the activity of RhoA and Rac1 at the contact after activation of the PCP pathway.

EphA/ephrinA signalling can activate RhoA/ROCK at the contact (Astin et al., 2010), via the GEF-Vav2, which is recruited to EphA when it is activated upon binding ephrinA (Batson et al., 2014). Furthermore it has recently been discovered that Rac1 activity in the overlapping protrusions of colliding fibroblasts is regulated by the GAP srGAP2 (Fritz et al., 2015). It appears that slit-robo signalling is activated in overlapping protrusions during a collision resulting in the activation of srGAP2 and the localised regulation of Rac1 activity (Fritz et al., 2015). This localised signalling event is required to prevent the cells continued migration and drive the repolarisation of the cells.

In addition to their role in regulating the actin cytoskeleton, RhoGTPases also play an essential role in the regulation of microtubules. Microtubules are stabilised in the leading edge where they are important for maintaining the polarity of a cell and driving directional migration (Bershadsky et al., 1991; Glasgow and Daniele, 1994). Stabilised microtubules promote membrane ruffling and the formation of lamellipodia (Bershadsky et al., 1991), whilst inhibiting contractility through the down regulation of stress fibre and focal adhesion formation (Bershadsky et al., 1996). Furthermore microtubules help maintain CCA complexes (Waterman-Storer et al., 2000). In haemocytes, microtubule bundles are observed in the leading edge where they stabilise the protrusions (Stramer et al., 2010). When two haemocytes collide the microtubule bundles align across the two colliding cells (Stramer et al., 2010), this coincides with a deceleration of the cells during CIL (Davis et al., 2015). It is likely the alignment of microtubule bundles in colliding haemocytes plays a role in the inhibition of the forward movement of the cells, potentially by generating a physical barrier that prevents the cells' continued migration. If the microtubules cannot be stabilised then polarity is lost in the haemocytes and they no longer undergo CIL (Stramer et al., 2010). It is possible that the initial coupling of microtubules in colliding cells promotes the formation of the CCA complex that is required to drive CIL.

(iii) The cells repolarise and new protrusions form away from the contact

Another key feature of CIL is the repolarisation of the cells away from the contact after a collision. The repolarisation of colliding cells requires a switch in front-rear polarity. In order for this switch to occur not only does RhoA have to be elevated and Rac1 inhibited at the contact, as discussed above, but a new leading edge must form away from the contact. The formation of a new leading edge is dependent on the interplay between adhesions, RhoGTPases and the cytoskeleton. Rac1 activity is elevated away from the contact driving the formation of new protrusions in this region (Scarpa et al., 2015; Theveneau and Mayor, 2010). During collisions between neural crest cells the switch in the localisation of Rac1 activity has been visualised (Scarpa et al., 2015). In a free migrating cell Rac1 is activated in the leading edge of the cell. Upon a collision Rac1 is inhibited at the contact and subsequently becomes active away from the contact (Fig. 3.1) (Scarpa et al., 2015).

In addition to a switch in Rac1 activity, a switch in the dynamics of microtubules is also required to drive the repolarisation of cells after a collision (Kadir et al., 2011; Moore et al., 2013; Nagasaki et al., 1992) Microtubules are stabilised in the leading edge of a cell where they are required to reinforce its polarity (Bershadsky et al., 1991; Glasgow and Daniele, 1994). Upon a collision there is a change in the dynamic behaviour of the microtubules at the site of contact, with an increase in the frequency of catastrophe events and rates of shrinkage and growth (Moore et al., 2013). This increase in microtubule dynamic behaviour at the contact is required for CIL (Batson et al., 2013; Kadir et al., 2011; Moore et al., 2013; Stramer et al., 2010). In the neural crest the dynamic behaviour of microtubules seems to be dependent upon the cell polarity protein - Par3 (Moore et al., 2013). Par3 localises to the cell-cell contact where it promotes microtubule catastrophe through the inhibition of the GEF-Trio and subsequent inhibition of Rac1. In haemocytes microtubule bundles align between colliding cells upon a collision and their subsequent collapse is required for a normal CIL response (Stramer et al., 2010). In addition to an increase in their dynamics at the contact site, microtubules also become stabilised away from the contact further driving the repolarisation of the cell (Nagasaki et al., 1992).

(iv) The cells separate and migrate away from each other

The events driving the separation of cells after a collision are still not clear and there may be many different mechanisms promoting separation in a cell type specific manner (Roycroft and Mayor, 2016). The disassembly or internalisation of the CCA would uncouple the cells and allow them to separate but what triggers the breakdown of the CCA complex is as yet unknown. The repolarisation of the cells away from the contact has been shown to be necessary to induce the separation of cells after a collision in both fibroblasts and neural crest cells (Nagasaki et al., 1992; Scarpa et al., 2015). In neural crest cells CCAs remain intact when protrusions are inhibited from forming away from the contact due to physical constraint (Scarpa et al., 2015). This suggests the cells need to pull apart from each other in order promote the disassembly of the CCAs. Furthermore, stimulating protrusion formation through the use of a photoactivatable Rac1 in the free edge of cells overexpressing E-cadherin, which do not separate upon a collision, is sufficient to drive the separation of these cells (Scarpa et al., 2015). This indicates that neural crest cells start migrating away from each other prior to the loss of CCAs and this pulling apart is necessary and sufficient to drive the breakdown of these adhesions. In fibroblasts, microtubule dynamics are required to drive the repolarisation of the cell and stabilise new protrusions away from the contact, an event required in order for the cells to separate (Kadir et al., 2011; Moore et al., 2013; Nagasaki et al., 1992). The repolarisation of cells away from the contact may promote separation by generating tension across the cell-cell contact pulling the cells apart and promoting the disassembly of CCAs.

The idea that tension may be required across the cell-cell contact in order to induce separation during CIL has long been discussed (Abercrombie and Ambrose, 1958; Abercrombie and Dunn, 1975; Davis et al., 2015; Harris, 1973; Heaysman and Pegrum, 1973a; Roycroft and Mayor, 2015; Roycroft and Mayor, 2016). Albert Harris observed a loss of cell-substrate adhesions near the contact upon a collision and speculated this led to a transfer of tension from the cell-substrate to the cell-cell contact and this transfer of tension was sufficient to break the cell-cell adhesion (Harris, 1973). Abercrombie however argued that cell-substrate adhesions persisted near the contact and that an alternative mechanism for tension build up across the cell-cell contact was

required (Abercrombie and Dunn, 1975). There is some evidence supporting the hypothesis that tension across the cell-cell contact is required to induce cell separation. In haemocytes a sudden retraction of lamellae is observed as the cell-cell adhesion complex is broken and the tension across the complex is released (Davis et al., 2015). Myosin II coated stress fibres align between colliding haemocytes and myosin II or formin mutants, where stress fibre formation was aberrant, showed a reduction in tension in the contacting lamellae and separation after a collision was perturbed (Davis et al., 2015). It has been hypothesised that the myosin driven contraction of these stress fibres could be sufficient to drive the separation of the cells by generating tension across the contact (Davis et al., 2015; Roycroft and Mayor, 2015).

The build-up of tension across the cell-cell contact could, however, be generated by other means. The activation of RhoA and subsequently ROCK at the contact upon a collision was believed to trigger actomyosin contractions (Astin et al., 2010; Carmona-Fontaine et al., 2008; Matthews et al., 2008). Interestingly, however, there is evidence that RhoA/ROCK activation at the contact site does not act through actomyosin contraction as normal CIL behaviour can still occur when myosin II is inhibited but not when Rho or ROCK activity is inhibited as this prevents cells from separating (Kadir et al., 2011). It appears instead that RhoA/ROCK activity acts through the mediation of microtubule dynamics (Kadir et al., 2011). As discussed above an increase in microtubule dynamics and catastrophe events upon a collision is required for CIL (Batson et al., 2013; Kadir et al., 2011; Moore et al., 2013; Stramer et al., 2010). Thus, a microtubule catastrophe at the contact could trigger the separation of the cells after a collision, potentially by causing an increase in tension across the cell-cell contact. Another mechanism that could lead to the build-up of tension across the cell-cell contact is the coupling of the actin cytoskeletons in colliding cells (Davis et al., 2015; Roycroft and Mayor, 2015). This could generate tension by linking the actin retrograde flow in the lamellae of both cells via cell-cell adhesions across the contact. In a mechanism similar to integrin, the cell-cell adhesions may act as a clutch by anchoring the cytoskeleton to a point of resistance (Davis et al., 2015; Gardel et al., 2008; Roycroft and Mayor, 2015). This causes a deceleration of the continuous actin

retrograde flow and results in a build-up of tension across the cell-cell contact and in the lamellae. Actin retrograde flow alone could generate enough tension across the cell-cell contact to induce separation.

It appears that a variety of mechanisms may be stimulating tension generation across the cell-cell contact and may be stimulating the disassembly of the cell-cell adhesions. Each event alone may not be sufficient to drive the separation of the cells but together they could generate enough force and possibly stimulate a signalling event that results in the disassembly of cell-cell adhesions and the subsequent separation of the cells. It is unclear how cell dependent the precise mechanism of separation is, or whether it is conserved across different cell types. A more thorough examination of this event is required to fully understand what drives the separation of cells after a collision during CIL.

1.3.5 Cell-matrix adhesions during CIL

As discussed in the opening chapter, cell-matrix adhesions (CMAs) play a key role in facilitating cell migration and are therefore likely to be a vital part of CIL. The behaviour of CMAs during CIL was first speculated upon by Abercrombie (Abercrombie, 1970a), although their behaviour and importance during this process is still not understood. Integrin signalling has been identified in myoblasts where ectopic expression of either α5 integrin, β1 integrin or downstream effectors of integrin – such as paxillin and FAK – result in a paralysis of membrane ruffling and lamellae activity upon a collision (Huttenlocher et al., 1998). There is further evidence of the importance of CMAs during CIL in the neural crest. Syndecan-4, a transmembrane heparan sulphate proteoglycan that can crosslink the extracellular matrix to actin via the adapter protein α-actinin (Greene et al., 2003) and stimulate the formation of CMAs (Alexopoulou et al., 2007), is essential for the directional migration of the neural crest *in vivo* (Matthews et al., 2008). In addition the loss of syndecan-4 results in a loss of CIL with protrusions no longer inhibited towards the contact, as is the case in control cell, due to a huge increase in Rac1 activity across the whole cell periphery. This suggests the presence of syndecan-4 inhibits Rac1 activity at the contact, although where syndecan-4 is localised in the neural crest or how it inhibits Rac1 activity has not

yet been identified. In fibroblasts however, there is evidence that syndecan-4 regulates Rac1 activity through the mediation of protein kinase C-α, which plays a role in localising Rac1 activity to the leading edge (Bass et al., 2007). Further to the evidence of the importance of syndecan-4 in the neural crest during CIL, Integrin based CMAs have also been visualised in the neural crest (Scarpa et al., 2015; Theveneau et al., 2013). The morphology of the CMAs in the free edge of cells are distinct from those adjacent to the site of contact. Large elongated adhesions are observed in the free edge whereas the adhesions near the contact are much smaller, punctate and rounded in shape. Interestingly these small adhesions near the contact become enlarged when E-cadherin is overexpressed in the neural crest (Scarpa et al., 2015). Whether this enlargement is a contributing factor or just a consequence of the loss of CIL in E-cadherin overexpressing cells is unknown. As discussed in the opening chapter, CMAs are important mediators of actin retrograde flow rates (Gardel et al., 2010). The engagement of these adhesions slow actin retrograde flow by generating friction between the actin network and the substrate, consequently generating traction on the substrate (Gardel et al., 2008). Changes in actin retrograde flow during CIL have recently been visualised in haemocytes *in vivo* (Davis et al., 2015). It is possible these changes are not solely due to the engagement of the cell-cell adhesion complex, as discussed above, but may also be driven by changes in the engagement of CMAs.

CIL is a complex process that requires careful coordination of adhesions, the activity of RhoGTPases and cytoskeleton dynamics. Perturbing any of these factors disrupts CIL. Although many molecular mechanisms and components of CIL have been identified the precise role and regulation of many others are still not fully understood. One outstanding question is the driving force behind the separation of the cells after a collision. Another is the precise role and regulation of CMAs during CIL. The neural crest cells of *Xenopus* provide an excellent model to address these outstanding questions.

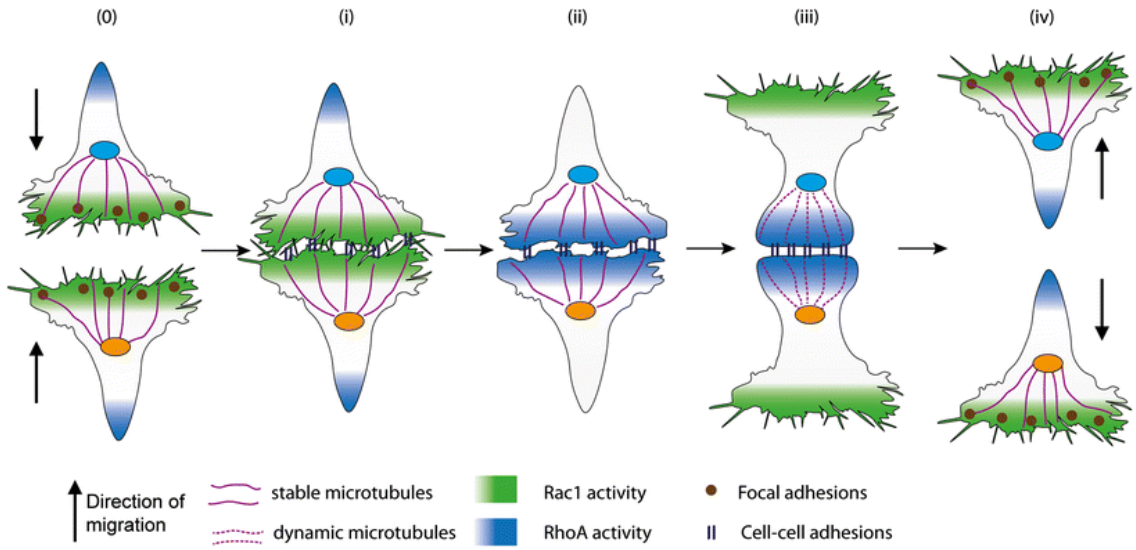

Figure 3.1 The multiple steps of contact inhibition of locomotion

Contact inhibition of locomotion is a multi-facetted process involving several stages. (0) Free migrating cells are polarised with elevated Rac1 activity at the leading edge stimulating protrusion formation. Microtubules are stabilised towards the leading edge and focal adhesions form in the growing protrusions. RhoA activity is elevated in the trailing edge of the cell. (i) Upon a collision a contact in formed between the cells: the protrusions of the colliding cells overlap and cell-cell contacts form between the cells coupling the cytoskeletons of the colliding cells. (ii) Protrusive activity is inhibited at the site of contact: Rac1 activity is lost at the contact site and RhoA activity is elevated in this region resulting in the subsequent collapse of protrusions. (iii) The cells repolarise and new protrusions form away from the contact: Rac1 activity increases away from the contact promoting the formation of new protrusions in this area. Focal adhesions begin to form in these new protrusions. Microtubule dynamics increase at the contact site with an increase in growth and shrinkage rates and catastrophe events. (iv) The cells separate and migrate away from each other: the cell-cell adhesions disassemble, cells continue migrating away from the contact and the cells finally separate.

[Adapted from (Roycroft and Mayor, 2016)]

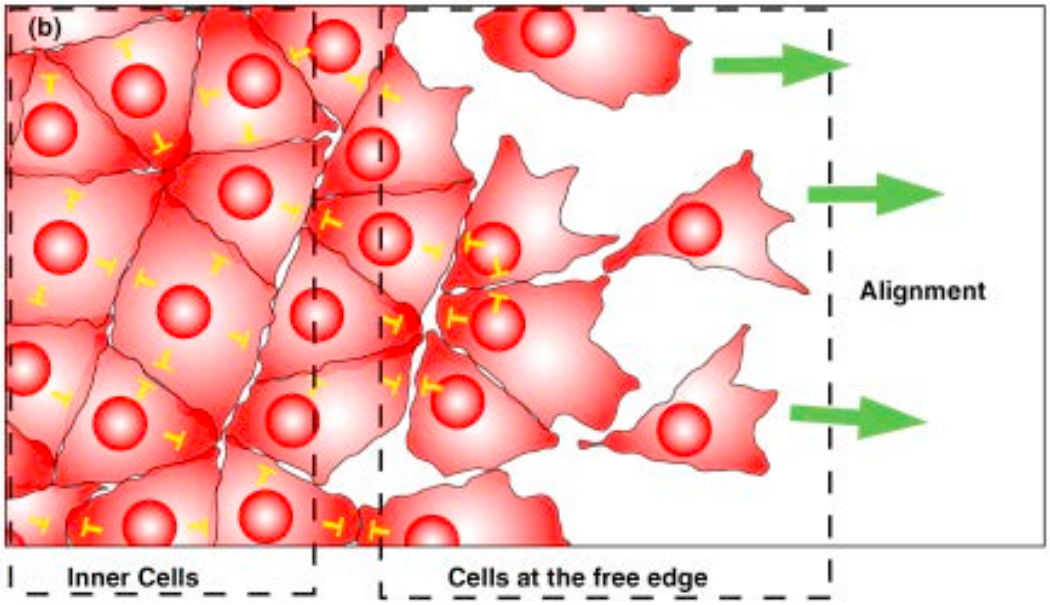

Figure 3.2 Contact inhibition of locomotion driving collective migration

Contact inhibition of locomotion is required for the collective directional migration of the cranial neural crest. Contact inhibition of locomotion is believed to contribute to directional collective migration by inhibiting the inner cells within the cluster from forming protrusions. Furthermore CIL between cells with a free edge drive the cells to become polarised and form protrusions into the free space thus creating the leading edge and driving the polarisation of the cluster.

[Adapted from (Mayor and Carmona-Fontaine, 2010)]

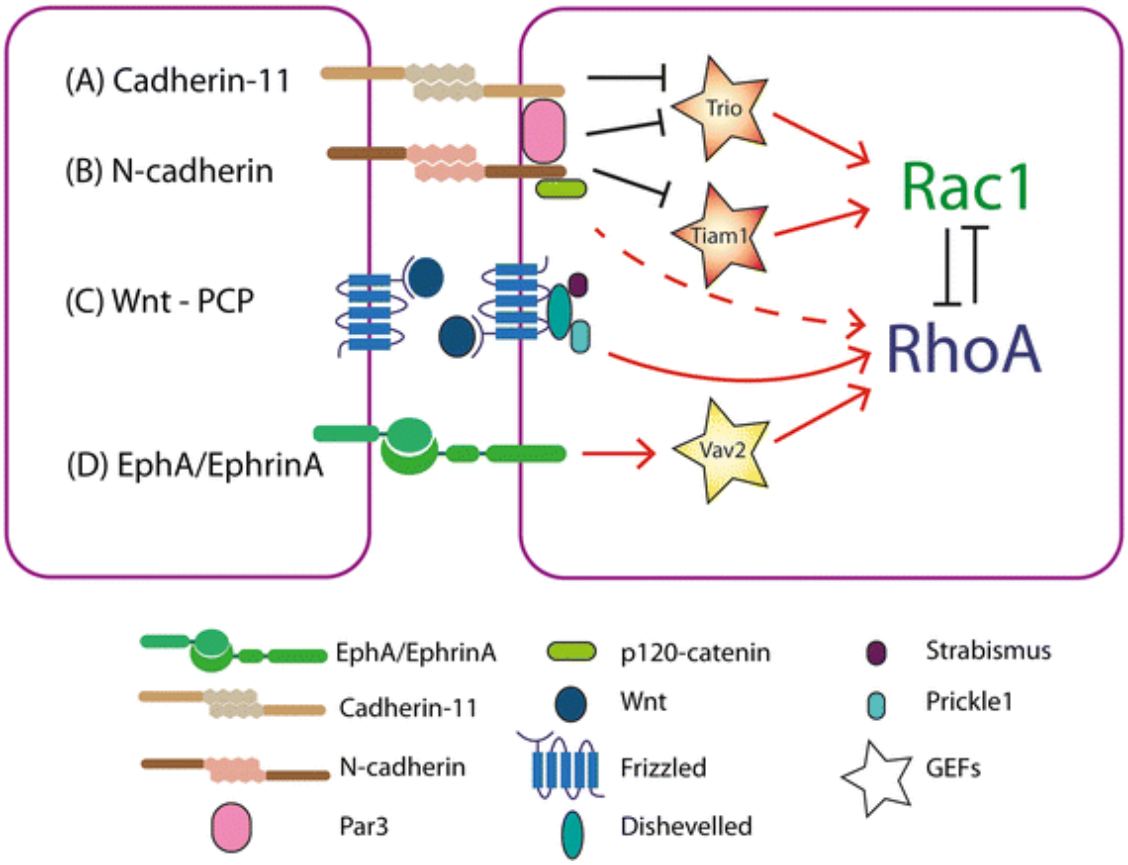

Figure 3.3 Regulation of Rac1 and RhoA activity at the cell-cell contact

During CIL a switch in RhoGTPase activity occurs at the cell-cell contact whereby Rac1 is inhibited and RhoA activity is elevated. Although some of the mechanisms leading to this switch are unclear some GEFs have been identified downstream of cell-cell adhesions involved in CIL that promote this switch. (A) cadherin-11 binds and sequesters Trio-GEF inhibiting it and preventing it from activating Rac1 and inhibiting RhoA as it otherwise would do. The inhibition of Trio-GEF downstream of cadherin-11 is possibly through its interaction with the polarity protein Par3 (B) N-cadherin may be inhibiting Rac1 activity through the same means. Furthermore N-cadherin leads to the inhibition of the GEF-Tiam1 via its association with nm23 which binds and inhibits Tiam1 at the contact site. Tiam1 is an activator of Rac1 and its inhibition prevents Rac1 activation at the contact site. There is also evidence that p120-catenin is involved in influencing the differential activity of the RhoGTPases downstream of E- and N-cadherin. (C) The Wnt-planar cell polarity pathway is activated by Wnt11 binding it is receptor Frizzled7. The PCP proteins Dishevelled, Prickle1 and Stabismus are all recruited to the receptor at the contact upon a collision. The activation of the PCP

pathway during CIL results in the activation of RhoA at the contact. (D) EphA binds EphrinA from a neighbouring cell. This stimulates bidirectional signalling that leads to the activation of the GEF-Vav2 which stimulates RhoA activity near the contact.

[From (Roycroft and Mayor, 2016)]

.

1.4 The neural crest

The neural crest (NC) is a population of multipotent embryonic cells unique to vertebrate embryogenesis and first described in 1868 by the embryologist Wilheim His (His, 1868). It has long been proposed that the emergence of the NC was central to the evolution of vertebrates (Gans and Northcutt, 1983; Northcutt and Gans, 1983), leading a professor of zoology to once described them as 'the only interesting thing about vertebrates' (Thorogood, 1989). NC cells are a transient population that are induced during neurulation in the ectoderm at the neural plate border, the interface between the neural plate and the prospective epidermis. They subsequently separate from the neural plate, a process known as delamination, and undergo an epithelial-to-mesenchymal transition (EMT), before migrating expansively throughout the developing embryo. The NC then differentiates into a vast number of cell types that can be broadly divided into ectomesenchymal (e.g. bone and cartilage) or non-ectomesenchymal cells (e.g. neurons, glia and melanocytes). The fate of the cells is dependent upon their destination which in turn is determined by the population of NC from which they are derived (Fig. 4.1). There are four populations of NC cells: cranial or cephalic neural crest (CNC), truncal NC, vagal and sacral NC and cardiac NC. The CNC migrates dorsolaterally through the embryo into the pharyngeal arches and gives rise to the craniofacial mesenchyme that further differentiates into cartilage, bone, cranial neurons, glia and connective tissue. The truncal NC migrates in one of two directions: those that migrate dorsolaterally over the somites become melanocytes, while those that migrate ventrolaterally between somites and the neural tube become either dorsal root ganglia, part of the sympathetic nervous system or the adrenal medulla depending on how far they migrate. The vagal and sacral NC migrates ventrally into the splanchnic mesenchyme of the gastrointestinal tract where they derive into parasympathetic ganglia of the gut. Cardiac NC cells migrate into the pharyngeal arches where they derive into melanocytes, neurons, cartilage and connective tissue. Some migrate beyond the pharyngeal arches to the cardiac outflow tract where they contribute to arteries and the cardiac septum. The work in this thesis was carried out on CNC cells and this is the population of the NC discussed here unless otherwise stated.

1.4.1 NC specification and maintenance

The formation of the NC is a two-step process consisting of its initial induction followed by its maintenance. The NC is initially induced in the ectoderm at the interface between the neural ectoderm and the non-neural ectoderm, at late blastula stage, and can be induced wherever a border between these two tissues forms (Bronner-Fraser, 1994; Mancilla and Mayor, 1996; Sauka-Spengler and Bronner-Fraser, 2008; Selleck and Bronner-Fraser, 1995). In *Xenopus* and Zebrafish induction is driven by a reduction of bone morphogenetic protein (BMP) signalling in the ectoderm (Marchant et al., 1998; Nguyen et al., 1998). In other vertebrates, such as chick and mouse, induction appears to be driven by the confrontation of BMP expressing ectoderm with non-BMP expressing ectoderm (Streit and Stern, 1999). In addition to the reduction of BMP signalling, Wnt activity, FGF signalling, Retonic acid and notch signalling all play an important role in the induction and maintenance of the NC. Detailed information behind the signalling and gene regulatory network coordinating NC induction can be found in these extensive reviews (Basch et al., 2004; Huang and Saint-Jeannet, 2004; Sauka-Spengler and Bronner-Fraser, 2008). Once induced the NC undergoes maintenance. This occurs during neurulation and is driven by Wnt and BMP activity from the intermediate mesoderm (Steventon et al., 2009). A gene regulatory network, consisting of multiple transcription factors, is activated in order to maintain the NC population. Snail1 and Snail2, members of the SNAI family of transcription factors, are expressed within the NC where they are required for the further development of the NC (del Barrio and Nieto, 2002; LaBonne and Bronner-Fraser, 2000; Shi et al., 2011). Snail promotes the expression of other maintenance genes, such as Twist, FoxD3 and Sox9 & 10 (Aybar et al., 2003). These maintenance genes are required to ensure a steady state in the population of the NC by inhibiting apoptosis and proliferation (Sauka-Spengler and Bronner-Fraser, 2008; Vega et al., 2004).

1.4.2 Delamination and epithelial-to-mesenchymal transition

Following their specification, the NC is a highly cohesive and non-migratory population of cells. In order to become migratory they need to undergo EMT and delamination, these processes are intimately linked and required to enable the NC to invade the ECM

and migrate large distances in the embryo. Although the terms delamination and EMT are sometimes used interchangeably when describing the neural crest they are in fact distinct processes. Delamination denotes the process by which the NC separates from the neural tube, whereas EMT refers to the process by which cells lose epithelial apical-basal polarity and gain front-rear polarity and a mesenchymal migratory phenotype. The process of delamination requires the NC to undergo either complete or partial EMT, depending on the species and the NC cannot separate from the neural tube if EMT does not occur (Duband, 2006). In order to successfully migrate *in vivo* the NC must successfully undergo both these processes. The onset of these two processes relative to each other varies for different populations of the NC and for different species (Theveneau and Mayor, 2012a). In the CNC of *Xenopus*, the model used in this thesis, NC all delaminate at once as a collective whilst the neural tube is still open (Sadaghiani and Thiébaud, 1987). However trunk NC cells delaminate gradually one at a time after the neural tube has closed (Clay and Halloran, 2010; Duband, 2010). Delamination of the NC is triggered by BMP signalling and many of the transcription factors involved in induction, such as Snail1 & 2, and Sox9 & 10, are also important in promoting this process (Cheung et al., 2005). Recently it has been shown that Wnt signalling has to be transiently inhibited in order for the NC to delaminate from the neural tube (Rabadán et al., 2016).

As mentioned above, EMT is the process by which cells lose their epithelial apical-basal polarity and gain the mesenchymal front rear polarity. It is characterised by the reduction in intercellular adhesions and increased motility converting highly cohesive epithelial cells to the loosely connected mesenchymal cells (Hay, 1995). EMT plays a key role in development; not only is it essential for the migration of the NC, it is also required for many other developmental processes such as during gastrulation to form the mesoderm, heart morphogenesis and the formation of the sclerotome (Larue and Bellacosa, 2005). In addition the invasiveness of cancer cells is associated with cells undergoing EMT (Nieto, 2013; Thiery and Morgan, 2004). The switch from epithelial to mesenchymal requires many changes to the cells: these include actin reorganisation, cell-cell adhesion disassembly and changes to cell matrix adhesions. EMT is a reversible

process and cells can revert to epithelial (MET) once they have reached their destination (Chaffer et al., 2007).

A hallmark of EMT is the downregulation of epithelial cell-cell junction proteins such as E-cadherin, gap junction components and tight junction components (Kirby and Hutson, 2010; Vandewalle et al., 2005). Many transcription factors that trigger EMT in the NC, including Slug, Snail and Twist, repress the expression of E-cadherin and other epithelial junction components (Carl et al., 1999; Kang and Massagué, 2004; Nieto et al., 1994; Taneyhill et al., 2007). In addition BMP signalling is required to induce EMT in the NC (Burstyn-Cohen et al., 2004). BMP activates Smad-interacting protein 1 (Sip1), a transcriptional repressor and known promoter of EMT, which specifically targets the genes involved in epithelial cell-cell junctions and inhibits the expression of E-cadherin. (Rogers et al., 2013; Vandewalle et al., 2005). Sip1 is expressed in the NC and its absence prevents their migration *in vivo* (Van de Putte et al., 2003). Recently the hypoxia factor Hif-1 has been shown to play a role in promoting EMT in the NC by promoting the expression of Twist (Barriga et al., 2013). One key feature of EMT is a cadherin switch where the expression of one type of cadherin is repressed and another promoted. During EMT the NC initially switches from expressing E-cadherin to N-cadherin (Dady et al., 2012; Rogers et al., 2013; Scarpa et al., 2015). This switch has been shown to be mediated by Sip1 in the CNC and required for their migration (Rogers et al., 2013; Scarpa et al., 2015). Interestingly in the trunk NC this switch is not sufficient to promote EMT and delamination from the neural tube, instead the cadherins switch again from N-cadherin to cadherin 6 to cadherin 7 (Clay and Halloran, 2014; Nakagawa and Takeichi, 1995; Park and Gumbiner, 2012).

In addition to the loss of epithelial cell-cell junctions, NC cells also acquire a migratory phenotype during EMT. This involves the expression of intermediate filament proteins, the flattening of the cells and the ability to generate lamellipodia and filopodia to facilitate migration. The onset of migration in the NC is characterised by blebbing in the membrane, a process mediated by myosin II and Rho-kinase (ROCK) activity (Berndt et al., 2008). Once the NC have separated from the neural tube the cells start to produce more robust lamellipodia and filopodia that generate the traction required for migration (Berndt et al., 2008).

It is interesting to note that EMT is also a hallmark of metastasis in cancer. Studying the NC undergoing EMT can help elucidate the molecular mechanism that drive this process and can therefore be used as an important model to investigate what may be driving metastasis in cancer cells (Kirby and Hutson, 2010).

1.4.3 Migration of the cranial neural crest

Further to the specific expression of cadherins, the expression of specific integrins is also an essential part of EMT. Avian NC cells require the expression of α4β1 integrins in order to migrate (Testaz and Duband, 2001). In addition expression of the glycoprotein tenascinC is critical for NC migration (Tucker, 2001). The ECM provides a permissive substrate for the NC to migrate on. The CNC migrates on fibronectin thanks to the expression of α5β1 integrin (Alfandari et al., 2003). Fibronectin has been shown to surround the migrating NC streams (Alfandari et al., 2003). In order for the NC to migrate it must break down the ECM through which it migrates. In order to do this the NC expresses a disintegrin and metalloprotease 13 (ADAM13) that cleaves matrix glycoproteins (Alfandari et al., 2001). The overexpression of this protein increases the invasiveness of the NC into surrounding tissue. Tissue inhibitor of metalloproteases-2 (TIMP2), a regulator of the activity of matrix metalloproteases, is also required for NC migration (Cantemir et al., 2004).

CNC cells migrate ventrally in three well defined distinct streams. These streams consist of clusters of loosely associated NC cells. There are multiple factors involved in NC migration including the physical restraints, chemotaxis, cell-cell interactions and interactions with the extracellular matrix (Perris, 1997; Theveneau and Mayor, 2012b; Woods et al., 2014). The role of each of these will briefly be discussed below.

Physical restraint of the neural crest streams

The fate of the CNC cells is determined by the stream they are in, for this reason it is important that the cells maintain their streams. There are a variety of factors promoting and maintaining the restriction of the streams. The death of some NC cells in specific areas is thought to contribute to the initial formation of the streams by creating the crest free regions between the streams (Kirby and Hutson, 2010). For

example it has been shown in Zebrafish that NC cells in rhombomeres 3 and 5 die and this may play a role in the separation seen between the first and second stream at these rhomobomeres (Kirby and Hutson, 2010). These streams are then maintained by the presence of semaphorins and versican in the crest free regions (Eickholt et al., 1999; Szabó et al., 2016; Yu and Moens, 2005). Semaphorins are a family of guidance molecular that can either attract or repels cells and named from the Greek meaning 'sign bearer'. Semaphorins 3A, 3F and 3G are expressed in the crest free area between the streams and their receptors, neurophilin 1 and 2, and the coreceptor plexin A1 are expressed by the NC (Koestner et al., 2008). NC cells expressing these receptors are repulsed by the presence of the semaphorins and therefore do not migrate on the regions where they are present (Chilton and Guthrie, 2003; Eickholt et al., 1999; McLennan and Kulesa, 2007). More recently the presence of the extracellular matrix proteoglycan versican has been shown to be expressed in the region between streams and its presence confines the migration of the NC to versican free regions (Szabó et al., 2016). In addition it has been shown that restricting the NC to streams can facilitate their migration *in silico* (Szabó et al., 2016; Woods et al., 2014).

Eph/Ephrins are another family of receptors and ligands that are believed to play a role in restricting the migration of the NC to distinct streams. Ephs are tyrosine kinase receptors and they bind to ephrins which are membrane bound ligands. A variety of Ephs and ephrins are expressed in and around the CNC and although the exact expression is not conserved across species, the role they play is (Davy and Soriano, 2007; Mellott and Burke, 2008; Smith et al., 1997; Wang and Anderson, 1997). It appears that Eph/Ephrin signalling is required to direct the CNC to their specific streams and maintain them there. If Eph/Ephrin signalling is perturbed non-distinct streams form with the NC either invading surrounding tissue or migrating across to the wrong stream (Smith et al., 1997).

Cell-cell interactions: chemotaxis, co-attraction and contact inhibition of locomotion

Different cell-cell interactions play an important role in NC migration (Fig. 4.2). CNC cells, even though they become mesenchymal, always maintain contact with each other via lamellipodia or filopodia (Teddy and Kulesa, 2004). Specific cadherins, such as

cadherin-11 and N-cadherin are kept at a tightly regulated level as their presence is required for migration although too high levels can prevent migration (Becker et al., 2013; Kashef et al., 2009; Kuriyama et al., 2014; Theveneau et al., 2010). It appears that cell-cell adhesions and the ability for cells to communicate with each other are both necessary for efficient NC migration. Connexion 43 (Cx43), a gap junction that facilitates cell-cell communication, is important in regulating the speed and directionality of the NC with a reduction in expression resulting in reduced migration (Huang et al., 1998). NC cells with reduced Cx43 expression demonstrate an increase in protrusive activity and a loss of polarised migration. In addition cells lacking Cx43 fail to retract in response to Semaphorin (Xu et al., 2006). Further to its role in cell-cell communication, Cx43 is also involved in regulating adhesion through interactions with N-cadherin (Xu et al., 2001).

Chemotaxis and co-attraction

The migration of the NC is coordinated by chemotactic cues, over both long and short distances (Shellard and Mayor, 2016). The NC has been shown to respond to several positive chemotactic cues including vascular endothelial growth factors (VEGFs), platelet-derived growth factors (PDGFs) and stromal cell-derived factor 1 (SDF-1). The exact role and localisation of these secreted growth factors in still not fully understood *in vivo*. The CNC migrates towards sources of VEGF and blocking VEGF signalling reduces the migration of the NC into the second branchial arch (McLennan et al., 2010). Furthermore VEGF is expressed in the ectoderm of the second branchial arch (McLennan et al., 2010). More recently it has been shown that VEGF particularly influences the expression pattern and behaviour of NC cells at the leading edge, suggesting that influencing the migration of the leading edge is sufficient to drive the directional migration of the NC as a whole (McLennan et al., 2015). PDGF belongs to the VEGF superfamily. PDGF is expressed in the optic stalk, branchial arches and oral and nasal cavities (Ding et al., 2000; Ho et al., 1994) while the receptors are expressed in the CNC (Ho et al., 1994; Schatteman et al., 1992; Smith and Tallquist, 2010). Knockout of the PDGF-receptors in mouse results in defects in the aortic arches and craniofacial cartilage (Richarte et al., 2007; Tallquist and Soriano, 2003). Live-imaging in Zebrafish has demonstrated the requirement of PDGF signalling in coordinating the

migration of the NC around the eye and into the oral ectoderm (Eberhart et al., 2008). The chemokine SDF-1 has also been identified as a chemoattractive cue for the CNC. Its expression has been identified in the pharyngeal arch endoderm and optic stalk in zebrafish (Olesnicky Killian et al., 2009). SDF-1 signalling via the Cxcr4 receptor, a G-protein coupled receptor expressed in the CNC, is required for the migration of the NC around the eye and to the pharyngeal arch (Olesnicky Killian et al., 2009; Theveneau et al., 2010). SDF-1 works through increasing the activity of Rac1 towards the chemoattractant and stabilising protrusions in this direction. Hif-1 is also required for chemotaxis towards SDF-1 through its ability to promote Cxcr4 expression in the NC (Barriga et al., 2013). Interestingly SDF-1 has been shown to be expressed in the epibranchial placodes in *Xenopus*, a tissue that lies adjacent to the premigratory NC (Theveneau et al., 2013). NC cells are attracted to placode explants *in vitro* and it is believed this could be providing a short range attractive cue promoting the directional migration of the NC *in vivo* (Theveneau et al., 2013).

As well as long range chemotaxis, NC cells are also mutually attracted to each other in a process termed co-attraction (Carmona-Fontaine et al., 2011). Co-attraction is important in NC migration in order to keep the cells together as a collective as their migration requires this collective nature (Theveneau et al., 2010; Woods et al., 2014). Interestingly the complement factors, more usually associated with the immune response, have been identified to play a role in co-attraction in the NC (Carmona-Fontaine et al., 2011). The complement factor C3a and its receptor C3aR are both expressed in the NC where their presence and function are required in order to maintain the cohesive nature of the NC. In the absence of co-attraction the NC disperses and can no longer efficiently migrate towards a chemoattractive source (Carmona-Fontaine et al., 2011; Woods et al., 2014).

Contact inhibition of locomotion

In addition to cues keeping the NC together, the NC also undergoes contact inhibition of locomotion (CIL). As discussed in more detail in the previous chapter, CIL is the phenomenon where cells in contact collapse their protrusions towards each other and polarise away from the contact (Roycroft and Mayor, 2016; Theveneau et al., 2010).

CIL between NC cells is required for their directional migration as demonstrated *in vitro*, *in vivo* and *in silico* (Carmona-Fontaine et al., 2008; Theveneau et al., 2010; Theveneau et al., 2013; Woods et al., 2014). CIL appears to play a two-fold role in NC migration. CIL between NC cells is believed to promote their collective directional migration by inhibiting the formation of protrusions in the cluster and promoting the formation of protrusions forming at the leading edge (Mayor and Carmona-Fontaine, 2010). A more detailed explanation of how CIL between NC cells may be driving the directional collective migration of the NC can be found in the previous chapter (Fig. 3.2). In addition to the homotypic CIL found between NC cells, heterotypic CIL also occurs between the NC and placodes (Theveneau et al., 2013). As mentioned above, the NC are attracted to the placodes by the chemokine SDF-1. When NC cells collide with placodes CIL occurs and both the protrusions of the NC and the placodes collapse. The placodes then repolarise and migrate away from the contact. This generates a 'chase and run' mechanism whereby the placodes move away from the NC when they collide and the NC chase after them, once their protrusions have recovered, due to the placodes providing a chemoattractive source (Theveneau et al., 2013). As mentioned in the previous chapter, several different components required for CIL have been identified in the NC. The N-cadherin based adherens junctions between cells are essential for CIL (Theveneau et al., 2010), in their absence protrusions are no longer inhibited at the contact. The presence of E-cadherin however suppresses CIL (Scarpa et al., 2015). In addition a variety of Wnt-PCP proteins also contribute to CIL in the NC through the activation of RhoA at the contact and subsequent inhibition of protrusions at the contact (Carmona-Fontaine et al., 2008; Matthews et al., 2008; Roycroft and Mayor, 2016; Theveneau et al., 2013). The tight junction complex protein Par3 has also been shown to play a role in CIL in the NC (Moore et al., 2013).

Many different cues are required to drive the carefully coordinated migration of the NC (Kirby and Hutson, 2010). It is difficult to dissect the exact requirements of the individual components due to the close interaction and crosstalk between them and it is only by looking at migration as a whole that we can fully appreciate this complex process. CIL, the focus of this thesis, is just one of many mechanisms contributing to NC migration and it is important to consider how it may be influencing other factors

driving the migration of the NC. An additional aspect that makes the understanding of NC migration so important is the similarities between these cells and cancer cells. The highly migratory, invasive and mesenchymal properties of the NC are also hallmarks of cancer cells. For this reason studying the NC can help elucidate common molecular mechanisms and components that drive metastasis and invasion in cancer.

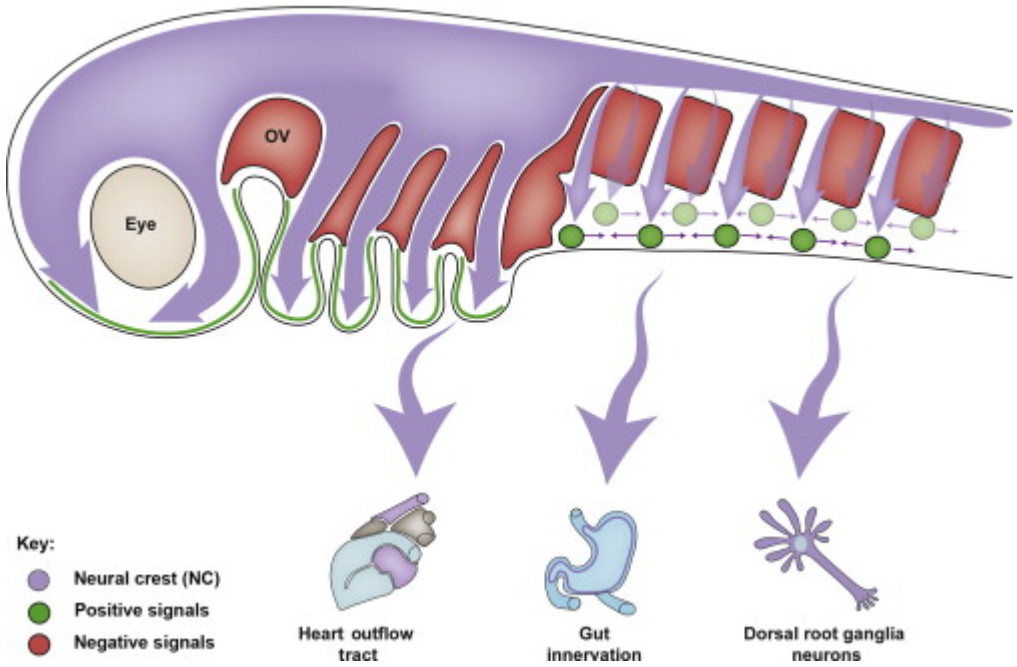

Figure 4.1 In vivo migration of the neural crest

The neural crest (purple) migrates in the developing embryo towards chemoattractive cues (green) in streams defined by repulsive signals (red). The thick purple arrows show the migratory pathway of the cranial neural crest cells which migrate in streams around the eye and otic vesicle (OV). Trunk neural crest cells are shown by the thinner purple arrows. Once the migratory neural crest cells reach their target tissue they differentiate into specific cell types depending on their destination. For example neural crest cells contribute to the heart outflow tract, the gut and dorsal root ganglia neurons.

[From ((Barriga and Mayor, 2015)]

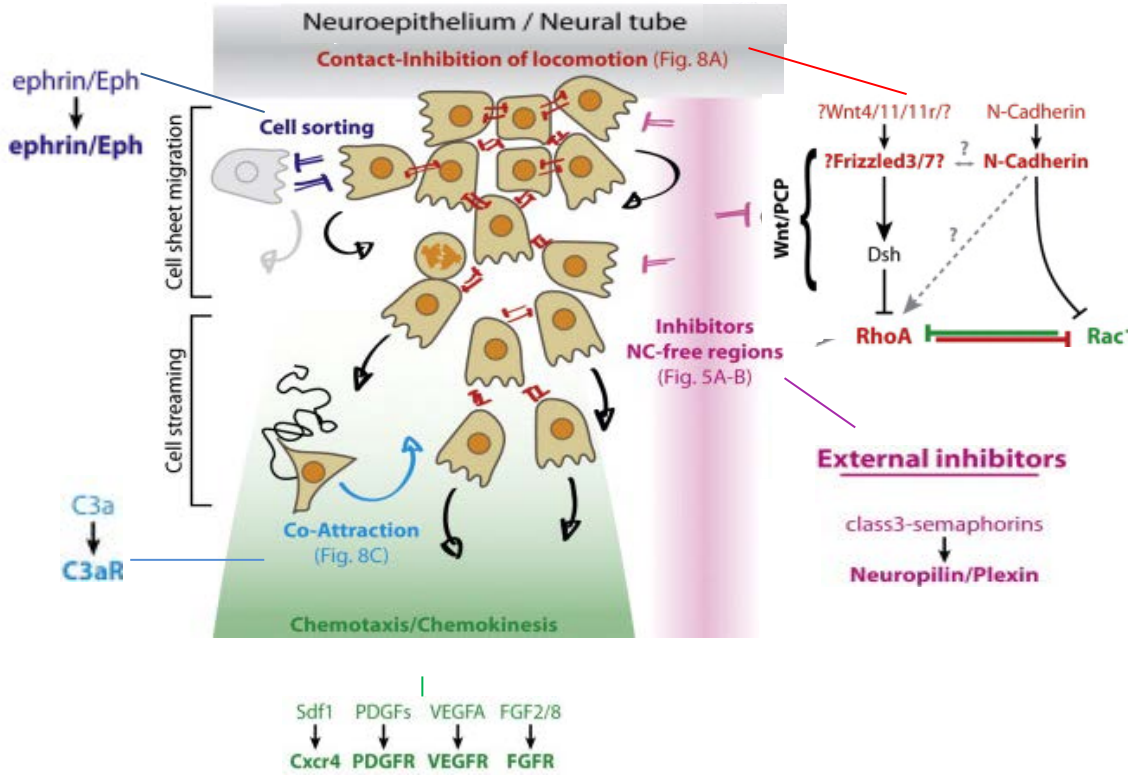

Figure 4.2 Cell-cell interactions and migratory cues

The migration of the cranial neural crest is coordinated by cell-cell interactions and external signals. A careful balance between contact inhibition of locomotion (red) and collective migration (light blue) orientates the polarity of the cells towards the free space whilst keeping them together as a collective. Contact inhibition of locomotion causes the collapse of protrusions at the cell-cell contact allowing only those at the free edge to form protrusions. Co-attraction, driven by C3 and its receptor, helps to attract stray cells back to the collective. This is important as individual neural crest cells respond poorly to chemoattractive cues. Chemotaxis (green) is driven by multiple different paracrine chemoattractants. Neural crest cells are kept in their respective streams by external inhibitor signals (pink), such as semaphorins and versican. In addition neural crest cells at the borders of streams can be sorted thanks to differential expression of ephrin/Eph molecules to prevent mixing (dark blue).

[Adapted from (Theveneau and Mayor, 2012a)]

1.5 Hypothesis

1.5.1 Hypothesis background

Contact inhibition of locomotion (CIL) is a multistep migratory phenomenon whereby colliding cells collapse their protrusions towards each other upon a collision before repolarising away from the contact and migrating away from each other. Although it was first identified in the 1920s (Loeb, 1921) and later characterised in the 1950s (Abercrombie and Heaysman, 1954b), it was not until its discovery as an important factor driving the directional migration of the neural crest (NC) in developing Zebrafish and *Xenopus* embryos that the molecular mechanisms driving CIL began to be revealed (Carmona-Fontaine et al., 2008). Now, thanks to the resurgence of interest in CIL, the role of cell-cell adhesions (CCAs), the small GTPases and the cytoskeleton have been elucidated. However, although initially discussed in the 1970s (Abercrombie, 1970a; Abercrombie and Dunn, 1975; Harris, 1973), the role and importance of cell-matrix adhesions (CMAs) during CIL remains unknown. It has been hypothesised that cellular forces play an important role in CIL; however the role of traction forces generated by CMAs and intercellular tension during CIL is unclear.

In the work presented here NC cells are used as a paradigm of CIL in order to try and address the unknown role of CMAs and traction forces during CIL. It is well established that NC cells form N-cadherin based CCAs required for CIL (Theveneau et al., 2010). CCAs and CMAs undergo a vast amount of cross-regulation, with the presence of CCAs often leading to the suppression of nearby CMAs (Borghi et al., 2010; Camand et al., 2012; Nelson et al., 2004).

1.5.2 Hypothesis

With this understanding the following hypotheses were proposed:

1) N-cadherin based CCAs that form between colliding cells during CIL negatively regulate the CMAs in the local vicinity upon a collision.
2) CMAs and the traction forces they generate play an important role in driving CIL. In order for cells to undergo CIL adhesion to the substrate and their associated forces need to be redistributed.

In order to test these hypotheses the following questions will be addressed:

1. What is the dynamic behaviour of CMAs during a collision?

2. Does the presence or absence of N-cadherin alter this behaviour?

3. If so how is N-cadherin regulating CMAs?

4. What is the requirement of CMA regulation during CIL?

2. Experimental and Analytical Procedures

2.1 Solutions

Solutions and buffers for embryology and neural crest cultures

Cysteine solution

2% L-cysteine

pH 7.8

Danilchick's Medium (DFA)

53mM NaCl

5mM Na_2CO_3

4.5mM K-Gluconate

32mM Na-Gluconate

1mM $MgSO_4$

1mM $CaCl_2$

0.1% BSA

pH 8.3 adjusted with 1M Bicine

50µg.ml^{-1} streptomycin

Calcium and Magnesium free made without $MgSO_4$ & $CaCl_2$

FICOLL

3% Polysucrose 400

in NAM 3/8

Modified Ringers Solution (10x MMR)

1M NaCl

20mM Kcl

10mM $MgSO_4$.7H20

20mM $CaCl_2$

50mM HEPES

1mM Disodium-EDTA

pH 7.8

Normal Amphibian Medium 1/10 (NAM 1/10)

11mM NaCl

0.2mM KCl

0.2mM Na_2HPO_4 pH 7.5

0.1mM $Ca(NO_3)_2$

0.1mM $MgSO_4$

0.1mM $NaHCO_3$

0.01mM Disodium EDTA

50µg.ml^{-1} Streptomycin

pH 7.2

Normal Amphibian Medium 3/8 (NAM 3/8)

41mM NaCl

0.75mM KCl

0.75mM Na_2HPO_4 pH 7.5

0.36mM $Ca(NO_3)_2$

0.36mM $MgSO_4$

0.1mM $NaHCO_3$

0.036mM Disodium EDTA

50µg.ml^{-1} Streptomycin

pH 7.2

Tricaine Solution

0.5% Ethyl 3-aminobenzoate methanesulfonate

Solutions and buffers for in situ hybridisation and immunofluorescence

Alkaline Phosphatase (AP) buffer

100mM Tris-HCl *pH 9.5*

100mM NaCl

50mM $MgCl_2$

0.1% Tween-20

Bleaching Solution

20% H_2O_2

5% Formamide

2.5% 20 x SSC

In DEPC Water

Diethylpyrocarbonate Water (DEPC)

0.1% diethylpyrocarbonate

Formamide/SSC post-hybridisation washes

Solution 1: 50% Formamide, 2x SSC, 0.1% Tween – 20

Solution 2: 25% Formamide, 2x SSC, 0.1% Tween – 20

Solution 3: 12.5% Formamide, 2x SSC, 0.1% Tween

Solution 4: 2x SSC, 0.1% Tween

Solution 5: 0.2x SSC, 0.1% Tween

Hybridisation Buffer

50% Formamide

5 x SSC

1 x Denhardt's Solution

1mg.ml^{-1} Ribonucleic acid

100µg.ml^{-1} heparin

0.1% CHAPS

10mM EDTA

0.1% Tween-20

pH 5.5

Phosphate-Buffered Saline (PBS)

137mM NaCl

2.7mM KCl

10mM Na_2HPO_4

1.8mM KH_2PO_4

pH 7 – 7.4

(add 0.1% Tween-20 for PTW)

Mowiol

25% Glycerol

10% Mowiol 4-88

50% 0.2M Tris HCl *pH 8.5*

SSC (20x)

3M NaCl

0.3M Tri-sodium citrate

pH 7.0

Maleic acid buffer (MAB)

100mM Maleic acid

150mM NaCl

0.1% Tween-20

pH 7.5

MEMFA

3.7% Formaldehyde

0.1 MOPS

1mM $MgSO_4$

2mM EGTA

2.2 Experimental procedures

Xenopus laevis oocyte collection and fertilisation

Mature male and female *Xenopus laevis* were procured from Portsmouth Animal Facility, UK and Nasco, USA. Frogs were pre-primed a few days before use by a subcutaneous injection of 100 units of the gonadotropic hormone PMSG (Intervet). The day before oocyte collection the frogs were injected subcutaneously with 500 units of Human Chorionic Gonadotrophin (Intervet) in order to stimulate ovulation. The induced frogs were left to lay overnight at 17°C in MMR solution in order to preserve oocytes for fertilisation. Testes were obtained from mature male frogs anaesthetised in 0.5% Tricaine solution for a minimum of 40 minutes. The removed testes were stored in Leibovitz L-15 medium (Invitrogen) with streptomycin added at 5 $\mu g.ml^{-1}$ (Sigma) at 4°C. The oocytes were collected and fertilised in a petri dish with a small piece of pulverised testis. NAM 1/10 solution was added 20 minutes after the addition of the sperm. After cortical rotation and the first division the jelly surrounding the embryos was removed in a 2% L-cysteine (Sigma) solution for 5 minutes. The embryos were raised in NAM 1/10 and staged according to the Nieuwkoop and Faber method, 1967.

Microinjection

Xenopus embryos were injected with mRNA, DNA or antisense morpholinos using a Narishige IM300 Microinjector. Needles were generated from borosilicate glass capillaries (Harvard Apparatus) with a 0.58mm inner diameter by pulling the capillaries on a Narishige PC-10 set to 2 step mode; both at 69.5% capacity. The needles were calibrated to inject the required volume, either 5 or 10 nl, using an eye piece graticule. The needles were filled with the solution to be injected, either mRNA, DNA or antisense morpholinos. The embryos were injected in Ficoll solution (Sigma), a highly branched hydrophilic polysaccharide used to preserve the integrity of the embryo during injection, at either 4 or 8-cell stage of development depending on the targeting and expression required. For mRNA expression the embryos were injected at 4- cell stage, once into the dorsal blastomere and once into the ventral blastomere on the right hand side of the embryo. For the expression of DNA the embryos were injected twice into the dorsal and ventral blastomeres at 4-cell stage. For injection of

morpholinos the neural crest was more precisely targeted by injecting the embryos at the 8-cell stage into the dorsal animal blastomere and the ventral animal blastomere. After injection the embryos were left in Ficoll solution overnight in 14.5°C before being transferred to NAM 1/10 once gastrulation has started. They were generally developed at 14.5°C although temperature could be varied from 14°C - 18°C to alter the speed of development.

Table 1 Constructs injected

Construct	Type	Amount injected	Stage
GFP-FAK	mRNA	200 pg	4-cell
nuclearRFP	mRNA	150 pg	4-cell
membraneRFP/GFP	mRNA	200 pg	4-cell
N-cadherin-RFP	mRNA	200 pg	4-cell
E-cadherin	mRNA	800 pg	8-cell
Src Y527F	mRNA	500 pg	8-cell
Src-FRET probe	DNA	600 pg	8-cell
N-cadherin MO (sequence 5'-GAAGGGCTCTTTCCGGCACATGGTG-3')	Morpholino oligomer	8 ng	8-cell

Neural crest dissection and culture

When the *Xenopus* embryos reached stage 18 of development, the cranial neural crest cells were isolated and explanted from the embryos. This was carried out at 18°C in NAM 3/8 to preserve the integrity of the embryo. Initially the vitelline membrane protecting the embryo was removed using a pair of forceps and the embryos were left to heal for a minimum of 15 minutes. The embryos were then embedded in plasticine and the superficial layer of the ectoderm was removed from the region of interest to expose the neural crest. The neural crest was then removed from the embryo and transferred to a plastic dish containing DFA before being further dissected into smaller clusters and plated on a fibronectin coated dish containing DFA. For single cell experiments the explants were fully dissociated in calcium and magnesium free DFA for 5 minutes prior to culturing on fibronectin in normal DFA. Cells and explants were

left to settle for a minimum of 30 minutes prior to imaging. Plastic dishes (Falcon 50 x 9 mm; used for dispersion, low resolution CIL and single cell motility assays) were incubated with fibronectin (Sigma) at 10 $\mu g.ml^{-1}$ for 1 hour at 37°C. Glass bottomed dishes (Greiner bio-one 35 mm 4 compartments or FluoroDish 35 mm; used for laser ablation experiments and high resolution live-cell imaging of CMAs) and coverslips (Academy Dia. 13, 0.13-0.16 mm thick; used for immunofluorescence) were incubated with fibronectin at 100 $\mu g.ml^{-1}$ for 1 hour at 37°C. Glass coverslips were sterilised in ethanol and air dried prior to fibronectin incubation in 4 well dishes (ThermoFischer multidish 4 wells). After coating with fibronectin, the dishes were washed with PBS before being blocked with 0.1% bovine serum albumin (BSA) in PBS for 20 minutes at 37°C. The dishes were then washed once again with PBS before DFA was added and the dishes were cooled to 18°C.

Immunofluorescence

Cells and explants were plated on glass coverslips and left to spread for a minimum of 2 hours. They were fixed in 3.7% formaldehyde for 30 minutes at room temperature (RT) before being washed multiple times with PBS. They were permeablised in 0.2% Triton X 100 in PBS for 8 minutes and blocked in 2% serum in PBS for a minimum of 1 hour. Primary antibodies were incubated in 2% serum in PBS at various conditions specific for the antibody as indicated below. Secondary antibodies were all incubated in 2% serum in PBS for 1 to 2 hours at room temperature along with any counterstains. Cells were mounted in Mowiol and imaged on a Leica TCS SPE upright confocal microscope using a 63x oil immersion lens and a 1.5x digital zoom.

Table 2 Primary antibodies

Antibody target	Supplier	Species	Dilution	Incubation
Phospho-paxillin pY118	Upstate	Rabbit	1:200	30 minutes at 37°C then overnight at 4°C
N-cadherin MNCD2	DSHB	Rat	1:100	2 hours at room temperature

E-cadherin	DSHB	Mouse	1:200	2 hours at room temperature
Src active (28)	Life Technologies	Mouse	1:300	1 hour at 37°C

Table 3 Secondary antibodies

Antibody	Supplier	Species	Dilution	Incubation
Rabbit - 488	Invitrogen	Goat	1:250	1.5 hours at room temperature
Rabbit - 555	Invitrogen	Goat	1:250	1 hour at 37°C
Mouse - 488	ThermoFisher	Goat	1:200	2 hours at room temperature
Rat - cys3	ThermoFisher	Goat	1:150	1 hour at 37°C
Hoescht 3342	ThermoFisher	-	1:1000	With secondary
Phalloidin Rhodamine	Sigma	-	1:500	With secondary

Fluorescence Resonance Energy Transfer (FRET)

Embryos were injected with the FRET probe and the neural crest was dissected as normal. Explants were plated on glass coverslips and left to spread for 1 hour prior to fixing in 4% paraformaldehyde for 30 minutes before being mounted in Mowiol. FRET imaging and analysis was carried out with the help of Prof. Maddy Parsons at King's College London. Confocal imaging was carried out on a Nikon A1R inverted laser scanning confocal microscope using a 63x oil-immersion objective. The intensity-based FRET method of acceptor photobleaching was used. The CFP and YFP channels were excited using the 440 nm diode laser and the 514 nm argon line respectively. The two emission channels were split using a 545 nm dichroic mirror, which was followed by a 475-525 nm bandpass filter for CFP and a 530 nm longpass filter for YFP (Chroma). One pre-bleach image for each channel was collected followed by bleaching with a minimum of 50 iterations of the 514 nm argon laser line at maximum power. A second post-bleach image was collected for each channel. Control non-bleached areas were

acquired for all samples in the same field of view as bleached cells to confirm specificity of FRET detection.

In situ hybridisation

The protocol for whole mount *in situ* hybridisation was adapted from (Harland, 1991). Solutions used up to and including the post hybridisation washes were made with DEPC water to remove the risk of RNAse contamination. Embryos were fixed in MEMFA for 1 hour at room temperature or overnight at 4°C before being washed in 100% methanol. The embryos could be stored in methanol overnight at -20°C. They were rehydrated by a series of washes containing decreasing amounts of methanol in PBS (75%, 50% and 25%). Embryos were then washed in PTW before being bleached with a hydrogen peroxide based bleaching solution for 30 minutes whilst being protected from light. After bleaching the embryos were washed in PTW before they were re-fixed in MEMFA for a further 30 minutes and washed once again with PTW. Embryos were then incubated in hybridisation buffer, either for 1 hour at room temperature before being stored at -20°C, or for 6 hours at 62°C. The digoxigenin labelled T*wist* probe was then added to the hybridisation buffer at a concentration of 1 $\mu g.ml^{-1}$ and incubated with the embryos at 62°C overnight. The probe was then removed and the embryos were washed in a series of Formamide/SSC post-hybridisation washes at 62°C for 10 minutes each for solutions 1 to 4 and 30 minutes for solution 5. The embryos were then washed twice in PTW and twice in MAB at room temperature before they were blocked in 2% BMBR blocking reagent (Roche) in MAB for 2 hours at room temperature. The embryos were then incubated with anti—digoxigenin-alkaline phosphatase conjugated antibody (Roche) diluted 1:4000 in 2% BMBR in MAB overnight at 4°C. The embryos were then washes 6 times in MAB and room temperature to remove any excess antibody before being transferred to AP buffer for two 15 minute washes. Finally the AP colour reaction was developed using 4-nitroblue tetrazolium chloride (NBT, Roche) & 5-bromo-4-chloro-3-indoylphosphate (BCIP, Roche) at 150 $\mu g.ml^{-1}$ and 75 $\mu g.ml^{-1}$ respectively. Once the colour was fully developed, the reaction was stopped by washing the embryos in PTW before they were fixed in 3.7% formaldehyde. The embryos were imaged on a Leica MZ FLIII stereomicroscope with a DCGC420 Leica camera and Plan objective at 2.4x zoom.

N-cadherin substrate

Plastic dishes were incubated in 10 µg.ml^{-1} of fibronectin with 3 µg.ml^{-1} of N-cadherin Fc chimera (R&D systems) for 1 hour at 37°C. Control coverslips were incubated in fibronectin only at the same concentration.

Inhibitors and blocking antibodies

The small molecule inhibitors SU6656 (Sigma) and PF-573228 (Sigma) were used to inhibit Src and FAK kinase activity respectively. For *in vivo* NC migration experiments embryos were incubated in the inhibitor with their membranes removed between stage 17, after NC induction, and stage 24 when embryos were fixed. SU6656 was used at 200 µM and PF-573228 was used at 100 µM. For *in vitro* experiments explants were plated in normal DFA and left to settle for 1 hour before the inhibitor was added. SU6656 was used at 5 µM and PF-573228 was used at 2 µM. The cells were left for a further hour before being imaged. Explants that were to be fixed for immunofluorescence experiments were left in the inhibitor for 4 hours before being fixed. The same volume of DMSO was added to the controls. To block N-cadherin, cells were incubated in 100 µg.ml^{-1} of the N-cadherin blocking antibody CDH2 (ThermoFisher) for 40 minutes prior to being plated.

Traction Force Microscopy

Polyacrylamide gels containing the fluorescent beads covalently crosslinked to the fibronectin with N-hydroxysuccinimide (Sigma) were prepared at a stiffness of 600 Pa by adjusting the Bis-acrylamide and acrylamide concentrations accordingly. A stiffness of 600 Pa was chosen as this was previously optimised in the lab to be soft enough to show distortion of the gel but not too soft the cells could not attach and migrate. Cells were injected with membrane-GFP so the outline of the cells could be identified. Only the uppermost layer of the gel was imaged in order to focus only on the beads closest to the substrate the cells were migrating on. This is essential in order to correctly calculate the traction forces generated on the gel. Images were taken at 3 minute intervals. After the movie the cells were removed by treatment with proteinase K for 5 minutes so an image of the gel in a relaxed state could be obtained. From the distortion of the gel the traction forces were extrapolated as described in (Theveneau

et al., 2013). Traction force microscopy movies were carried out on an inverted Zeiss compound microscope with a motorised stage with a 32x objective.

Laser ablation

Laser ablation experiments were carried out on an Olympus FV1000 microscope using a 60x lens. Both cell-matrix adhesions and cell-cell adhesions were ablated for 30 seconds with a PicoQuant picosecond pulsed diode laser turned to 405 nm with 40 MHz repetition rate . Images were taken every 30 seconds.

2.3 Analytical procedures

pY118 paxillin staining immunofluorescence

The area, number and size of pY118 stained CMAs were quantified by thresholding the confocal images. The same threshold was used for all images taken together. The 'Analyze Particle' tool on ImageJ was used to calculate the total area of staining, the number of individual CMAs and the average size of the stained CMAs. When cells were in contact the half of the cell adjacent to the cell free area was termed the free edge whilst the remaining portion of the cell, which was in contact with other cells, was termed the contact. In a single cell the front of the cell was determined by the presence and phenotype of protrusions. This half of the cell was termed the leading edge whilst the rear half of the cell was termed the trailing edge. If no clear leading edge could be established the cells were excluded from analysis.

Contact inhibition of locomotion and single cell migration assay

In order to analyse single cell behaviour, NC cells were incubated in Ca^{2+} and Mg2+ free DFA medium so the calcium dependent cadherin-based adhesions were lost. Individual cells were then plated in normal DFA out on a fibronectin coated plastic dish and imaged on an upright compound microscopes with motorised stages, either a DM550B Leica Microscope with a DFC 300FX Leica camera or a DMRXA2 Leica Microscope with a Hamamatsu Digital camera in a controlled environment of 18°C. Frames were taken every 3 minutes with a 20x objective. Movies were analysed for collisions on ImageJ. In order to determine single cell velocity and persistence cells were tracked using their nuclear label as a marker. The 'Manual Tracking' plugin and 'Chemotaxis Tool' were used on ImageJ in order to generate tracks of the migrating cells and covert these tracks into readouts of velocity and persistence. Single cells were tracked for a time period of no less than an hour. If a cell collided with another cell in this time they were excluded. The duration of collisions were determined by noting the number of frames cells were in contact for and multiplying that by 3 minutes, the frequency of the frames, to give the best estimate of the total duration of the collision.

Cell dispersion assay

NC explants were plated on a plastic dish for 30 minutes to settle and inhibitors were added for a further 30 minutes before the explants were imaged. Explants were imaged using the same microscopes as for single cells assays and imaged for 18 hours with frames taken every 10 minutes with a 10x objective. Cells within the explant were tracked using their nuclear marker. To analyse cell dispersion the average triangle area between the nuclei of cells was calculated using Delaunay triangulation algorithm as described in (Carmona-Fontaine et al., 2011). This algorithm connects each cell with its closest neighbours and calculates the triangle area between them. It does this for all the cells within the explant. The average triangle area was used as a readout of cell dispersion and calculated at every 6 hour timepoint, starting from time 0 hours, using a 'Dispersion tool' plugin available on ImageJ. Furthermore the triangles were colour-coded according to their area to produce a visual representation of the dispersion using the same 'Dispersion tool' plugin. In order to control for the spread of the explants at the start of the movie, the triangle areas were made relative to the mean area of controls at time 0 for each repeated experiment.

CMA dynamics

The disassembly rates of CMAs and the longevity of CMAs in live cells were analysed using the 'Focal Adhesion Analysis Server' developed by the Shawn Gomez Lab at the University of North Carolina at Chapel Hill (Berginski and Gomez, 2013). NC cells were injected with GFP-FAK to label the CMAs and membraneRFP to visualise the cell. Movies were generated on either a VoX spinning disk confocal microscope with a 60x objective or a Leica TCS SP8vis microscope with a 63x objective to give high resolution both spatially and temporally.

Src-FRET probe analysis

Pre- and post-bleached CFP and YFP images were imported into Image J for processing. Briefly, images were smoothed using a 3 x 3 box mean filter, background subtracted and post-bleach images fade compensated. A FRET efficiency ratio map over the whole cell was calculated using the following formula:

FRET efficiency = $(CFP_{postbleach}-CFP_{prebleach})/CFP_{postbleach}$.

Ratio values were then extracted from pixels falling inside the bleach region as well as an equally sized region outside of the bleach region and the mean ratio determined for each region and plotted on a histogram. The non-bleach ratio was then subtracted from the bleach region ratio to give a final value for the FRET efficiency ratio. Data from images were used only if YFP bleaching efficiency was greater than 70 %.

Laser ablation cell length

Laser ablation experiments were carried out on an Olympus FV1000 microscope. Cells were labelled with membraneRFP so the cells could be visualised. Cells were imaged for 2 frames prior to ablation and for 3 minutes after ablation with frames taken every 30 seconds. The length of the cell-cell contact was measured on ImageJ at each timepoint using the membraneRFP to visualise the contact. The values were made relative to the length of contact at the frame prior to the start of ablation.

Protrusion dynamics

Protrusion dynamics were determined from movies generated on either a VoX spinning disk confocal microscope with a 60x objective or a Leica TCS SP8vis microscope with a 63x objective using NC cells expressing membraneRFP. Images were imported to ImageJ and the images were thresholded to generate a binary image. In order to determine the area of growth a frame was subtracted from the following frame. The difference was the growth in the time between frames. This area was calculated for frames one minute apart.

Traction force microscopy

Traction force microscopy analysis was carried out with the help of András Szabó within the lab. In order to determine the traction forces generated by the cells on the polyacrylamide gels the fluorescent bead displacement was measured using Particle Intensity Velocimetry (PIV) analysis. This was carried out by importing the relaxed state reference frame and the frames from the movies as Tiff images into ImageJ. Images were optimised by subtracting the background and correcting for any xy shift using 'StackReg' and 'TurboReg' tool on ImageJ. To determine the displacement of the beads between each frame and the relaxed state a 3-step PIV was calculated using the

following parameters where piv = PIV grid, sw = search window and vs = vector spacing.

piv1 = 128, sw1=256, vs1 = 64

piv2 = 64, sw2 = 128, vs2 = 32

piv3 = 48, sw3 = 128, vs3 = 23

The PIV calculated from bead displacement was then used to determine the traction forces using the algorithm described in (Lin et al., 2010) with the following parameters: gel thickness 100 μm; Young's Modulus 600 Pa; Poisson's ratio 0.5.

The traction forces within the different regions of the cell were then determined using a plugin tool developed by András Szabó within the lab.

Statistical analysis

Graphs were constructed using GraphPad Prism which was also used for statistical analysis. All line graphs represent mean and error bars represent standard error mean (SEM). All bar charts represent median with error marks showing the interquartile range. For all graphs * = $p \leq 0.05$, ** = $p \leq 0.005$, *** = $p \leq 0.001$. The statistical analysis carried out was determined according to whether the data was normally distributed, the number of data sets to be compared and whether the data was paired or not. For non-normally distributed paired data, such as CMA size, area and number at different areas or time points within the same cell, a Wilcoxon signed rank test was used. For non-normally distributed unpaired data either a Mann Whitney test or Kruskal-Wallis test was performed depending on the number of data sets. For normally distributed data, such as the gray value of active Src and the contact, a one-way analysis of variance (ANOVA) test was used.

3. Results

3.1 Characterisation of CMAs during CIL

Neural crest (NC) cells exhibit CIL behaviour and can therefore be used to study this phenomenon (Carmona-Fontaine et al., 2008; Newgreen et al., 1979; Scarpa et al., 2013). When NC cells collide they form cell-cell adhesions (CCAs) between the cells, collapse their protrusions, repolarise and eventually separate and migrate away from each other (Mayor and Carmona-Fontaine, 2010; Roycroft and Mayor, 2016; Scarpa et al., 2015; Theveneau et al., 2010). It is well established that CIL is driven by multiple molecular components including CCAs, small GTPases and cytoskeletal rearrangements, as discussed in the introductory chapter 1.3 and described in (Roycroft and Mayor, 2016). Cell-matrix adhesions (CMAs) facilitate cell migration through the generation of traction forces and downstream signalling events, as discussed in the introductory chapter 1.1. It is well established that the migration of the CNC on fibronectin relies on the integrin heterodimer α5β1 (Alfandari et al., 2003; Kil et al., 1998; Kil et al., 1996). Despite the known importance of CMAs for NC migration and the likelihood that they are involved in CIL, the role of CMAs and the forces they generate during CIL in the NC is still not understood. The significance of CMAs during CIL was first speculated upon by Abercrombie when he discussed the possibility that separation upon a collision during CIL could be driven by the redistribution of forces between CMAs and CCAs (Abercrombie, 1970a). In the 1970s, Harris first investigated the dynamic behaviour of adhesion to the substrate during CIL using a micromanipulation technique whereby a capillary was moved under a cell to show where the cell was attached to the substrate (Harris, 1973). Harris observed a loss of cell-substrate adhesion near the contact during a collision (Harris, 1973). He suggested this loss of adhesion to the substrate could result in the transfer of tension from the cell-substrate to the cell-cell contact and that this transfer of tension was sufficient to break the adhesions holding the cells together (Harris, 1973). Abercrombie, however, used interference reflection microscopy (IRM) to visualise CMAs during CIL (Abercrombie and Dunn, 1975). Abercrombie concluded that adhesion to the substrate persisted near the contact during CIL and that an alternative mechanism for tension build-up across the cell-cell contact was required (Abercrombie and Dunn, 1975). IRM uses polarised light to visualise where a cell is attached to the

substrate. When the membrane of the cell is close to the glass the reflected light from the cell membrane cancels out the reflected light from the glass and therefore regions where the membrane is closest to the glass are visualised as dark patches (Curtis, 1964). Neither IRM nor micromanipulation techniques directly image CMAs; instead they infer their presence via indirect methods. Now, thanks to the Nobel Prize winning discovery of GFP (Chalfie et al., 1994) and vastly improved imaging techniques, CMAs can be directly visualised during live collisions and their dynamic behaviour during this process is now much easier to elucidate, allowing the contradictory observations of Harris and Abercrombie to be readdressed.

3.1.1 CMAs are reduced near the contact

In order to visualise the dynamic behaviour of CMAs during CIL, NC cells were made to express a GFP-tagged FAK construct to label CMAs (Myers and Gomez, 2011). FAK was chosen as a marker for CMAs as it is recruited early on to CMAs during their maturation and acts as a central mediator of integrin signalling (Miyamoto et al., 1995; Parsons, 2003). NC cells expressing GFP-FAK were imaged undergoing CIL. The CMAs near the region of contact were significantly reduced upon a collision compared to the CMAs in the same region prior to a collision (Fig. 5.1 A-D; Movie 1). This reduction was evident in terms of total area of the CMAs, their size and their number (Fig. 5.1 B-D). Furthermore the rate of disassembly of CMAs was increased near the contact upon a collision compared to CMAs in the leading edge of cells that were not colliding (Fig. 5.1 E). This suggests that the reduction of CMAs upon a collision is most likely due to an increase in the rate of their disassembly induced by cells coming into contact.

The endogenous levels of CMAs and the effect that a contact with another cell has on their localisation were investigated by immunofluorescence against the adapter protein paxillin phosphorylated on tyrosine 118 residue. Paxillin is an important scaffolding protein recruited early on to nascent adhesions. The phosphorylation of paxillin on the tyrosine 118 residue is required for the assembly of the CMA complex (Zaidel-Bar et al., 2007) and therefore staining against this residue can be used to label CMAs. NC cells that were in contact with another cell showed a significant reduction in the total area and size of CMAs near the contact compared to the area away from the

contact, termed the free edge (Fig. 5.2 A-C). This reduction in CMAs near the contact is similar to the decrease observed upon a collision (Fig. 5.1 A-C). This helps to confirm the previous observations that suggest the transient contact formed during CIL is equivalent to the contact present within clusters of NC cells already in contact (Scarpa et al., 2015; Theveneau et al., 2013).

Single NC cells that are not in contact with any other cells showed a reduction in CMAs in the trailing edge of the cell, the edge away from the presumed direction of migration, compared to the leading edge of the cell both in terms of total area and size (Fig. 5.2 D-F). This is unsurprising as it is known that CMAs form in the leading edge of a migrating cell where they can mature into large elongated adhesions that are required to facilitate cell migration (Zaidel-Bar et al., 2003). Interestingly, the magnitude of the reduction of CMAs in the trailing edge compared to the leading edge was less than the reduction observed near the contact of cells in contact. This was confirmed by calculating the polarised distribution of the CMAs by making a ratio of the area of CMAs in the front of the cell (i.e. the free/leading edge) over the area of CMAs in the rear of the cell (i.e. the contact/trailing edge). The higher the number the more polarised the CMA distribution is towards the front of the cell. Cells in contact showed a significant increase in the polarised distribution of CMAs towards the front of the cell compared to single cells (Fig. 5.2 G). This observation suggests it is not just the inherent polarisation of the cell that is leading to a reduction of CMAs near the contact, but the very presence of the cell-cell contact itself that is enhancing the polarised distribution of CMAs by increasing CMA disassembly in the region near the cell-cell contact.

CMAs form a physical link that couples the actin cytoskeleton to the ECM, as discussed in the introductory chapter 1.1. The cytoskeleton generates contractile forces within the cell and these forces are transmitted to the ECM via the CMAs, producing traction on the substrate (Ananthakrishnan and Ehrlicher, 2007). As CMAs generate traction on the substrate and larger CMAs can produce greater traction (Han et al., 2012), traction forces can be used as a functional readout of CMAs (Balaban et al., 2001; Trichet et al., 2012). The traction forces generated by NC cells were measured using traction force microscopy (TFM) on polyacrylamide hydrogels (Scarpa et al., 2015; Theveneau et al.,

2013). Cells that were in contact with other cells showed a significant reduction in the magnitude of traction near the contact compared to the traction generated at the free edge (Fig. 5.3 A, B). Single migrating cells however, showed equal traction at the front and rear of the cell, demonstrating that traction forces are usually balanced across the cell (Fig. 5.3 C, D). When a doublet of cells separated after a collision and the cell-cell contact was broken, a sudden increase in traction was observed at the rear of the cell (Fig. 5.3 E, F). Interestingly the reverse is true for the traction force generated at the free edge; as soon as the cells came apart the traction in the free edge was reduced (Fig. 5.3 E, G). Together these results illustrate that when a NC cell is in contact with another cell there is an asymmetric distribution of CMAs and consequently traction forces away from the contact that is not seen to the same extent in single cells. This suggests that the presence of the cell-cell contact may be driving this asymmetry.

3.1.2 Reduction of CMAs near the contact occurs after N-cadherin junction formation and before repolarisation

As discussed in the introductory CIL chapter 1.3, CIL is a multi-step process (Mayor and Carmona-Fontaine, 2010; Roycroft and Mayor, 2016). First the colliding cells form a cell-cell adhesion between each other and collapse their protrusions at the site of contact. Subsequently the cells repolarise, separate and migrate away from each other (Abercrombie, 1970a). From the above results it is evident that CMAs are reduced in the vicinity of the cell-cell contact upon a collision. However when this reduction of CMAs occurs during the process of CIL is not clear. The temporal sequence of events during CIL was investigated using GFP-tagged FAK to visualise CMA behaviour in relation to other aspects of CIL. CMAs near the contact were rapidly reduced upon a collision with the CMAs starting to decrease within the first minute after a collision (Fig. 5.4 A; Movie 1) as seen by the reduction in the total area of CMAs (Fig. 5.4 C) the average size of the CMAs (Fig. 5.4 D) and number of CMAs (Fig 5.4 E) near the contact from 30 seconds after a collision. To determine whether the repolarisation of the cells away from the contact, a hallmark of CIL, was leading to the reduction observed, repolarisation away from the contact was quantified in respect to CMA reduction near the region of contact. Repolarisation was quantified using two methods: first by

quantifying the formation of CMAs away from the cell contact during CIL (Fig. 5.4 A, B), and second by analysing protrusion formation away from the contact (Fig. 5.4 E, F). These methods can be used to quantify repolarisation as cells must form new protrusions away from the contact and these protrusions need to be stabilised by CMAs in order for cells to generate traction and migrate (Gardel et al., 2010). By correlating the loss of CMAs at the contact to the growth of CMAs away from the contact, it was evident that the reduction of CMAs at the contact starts prior to the growth of CMAs away from the contact (Fig. 5.4 A, B). Furthermore the growth of protrusions away from the contact, quantified by comparing the position of cells in frames one minute apart (Scarpa et al., 2015), also occurred after the loss of CMAs at the contact (Fig. 5.4 E, F). Together these results demonstrate that CMAs are disassembled near the contact during a collision prior to the cells repolarising away from each other. Due to this sequence of events it is therefore unlikely that the loss of CMAs is a consequence of repolarisation. An alternative mechanism therefore, must be stimulating the CMA disassembly observed near the cell-cell contact. One possible event that could trigger the disassembly of the CMAs is the formation of CCAs at the contact.

In NC cells N-cadherin based adhesions form between colliding cells and their formation is required for CIL (Scarpa et al., 2015; Theveneau et al., 2010). In order to establish whether CMAs were reduced at the contact before or after the formation of N-cadherin-based adhesions, the recruitment of N-cadherin to the contact was visualised using a RFP-tagged N-cadherin construct (Fig. 5.5 A). An instantaneous recruitment of N-cadherin to the contact was observed upon a collision (Fig. 5.5 A, B red line). This recruitment occurred prior to the start of reduction of the CMAs in the vicinity near the contact (Fig. 5.5 A, B blue line). The reduction of CMAs near the contact upon a collision could therefore be a consequence of the formation of the N-cadherin based CCA.

Discussion

The results presented in this chapter have helped establish the temporal sequence of events during CIL addressing the contradictory observations of Abercrombie and Harris

(Abercrombie and Dunn, 1975; Harris, 1973). Freely migrating NC cells have large elongated CMAs in their leading edge and smaller CMAs in their trailing edge. Traction forces driving migration are balanced across the cell (Fig. 5.6 i). When NC cells collide they immediately form an N-cadherin based CCA between the cells (Fig. 5.6 ii). The CMAs are then rapidly reduced near the site of contact, most likely due to an increase in the rate of their disassembly. Concurrently traction forces are also reduced in this region due to the reduction in CMAs (Fig. 5.6 iii). The cells then repolarise, forming protrusions and large CMAs away from the contact which generate large traction forces on the substrate (Fig. 5.6 iv). Finally the cells separate and migrate away from each other. Traction forces are reduced at the front of the cell and increase again at the rear of the cell so once again the traction forces are balanced across the cell (Fig. 5.6 v). The sequence of events established here support Harris' initial observation, that CMAs are rapidly lost near the contact prior to the cells pulling apart from each other (Harris, 1973). In addition they highlight that a redistribution of adhesive forces is occurring during CIL.

The reduction of CMAs near the contact occurs after N-cadherin is recruited to the cell-cell contact and prior to the repolarisation of protrusions away from the contact. In addition when cells are in contact the CMA polarity is enhanced towards the free edge of the cell. Together these results suggest it is not the inherent polarity of the cell that is driving the reduction of CMAs near the cell-cell contact, instead it is possible that the formation of the cell-cell contact itself is promoting the large asymmetric distribution of CMAs, and consequently traction forces, away from the cell-cell contact. It is therefore of interest to establish whether the presence of the CCA between the cells is promoting CMA disassembly near the contact upon a collision.

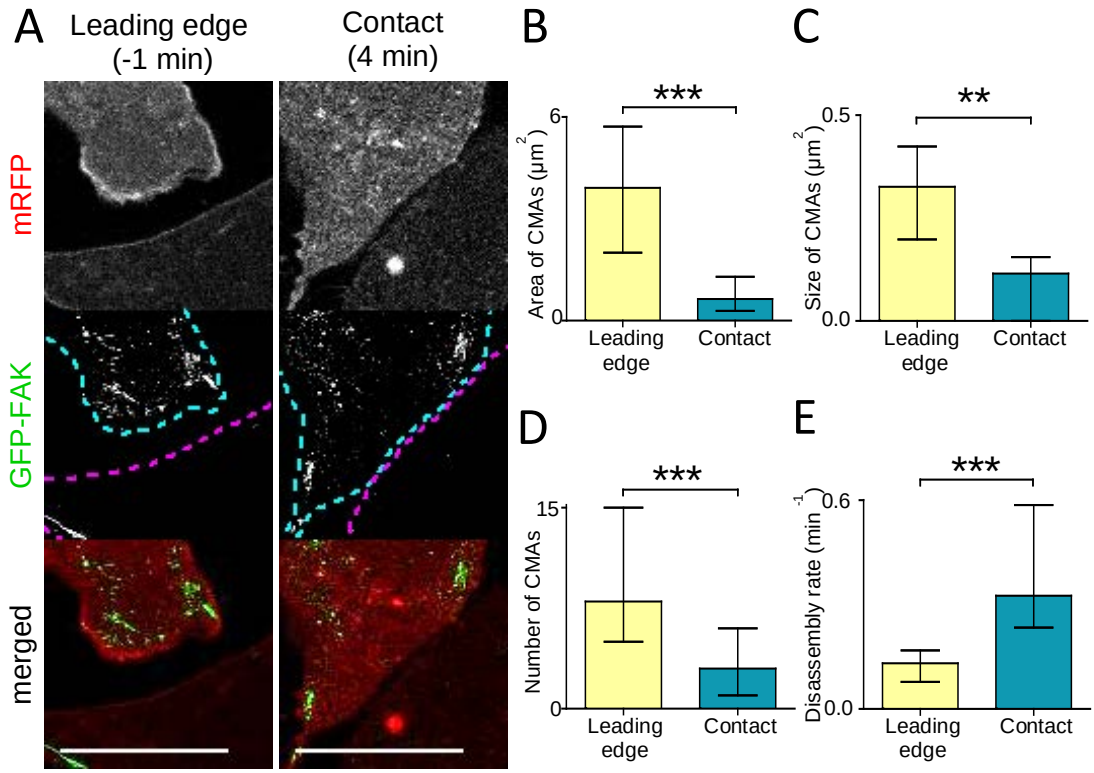

Figure 5.1 CMAs are reduced at the contact upon a collision

(A) Contact region between two colliding cells before collision (leading edge) and during collision (contact). Cells expressing membraneRFP (red) and GFP-FAK (green). Cells outlined in cyan and magenta. (B-D) Quantification of total area, average size and number of CMAs in the colliding portion of the cell before a collision (leading edge) or during a collision (contact). n = 15 cells. (E) Quantification of the rate of disassembly of CMAs in the leading edge of a cell and a cell during a collision. Leading edge: n = 24 cells, contact: n = 27 cells. Scale bars 20 µm. Bar on graphs: median, errors: 25-75 percentiles. Wilcoxon signed rank test for B-D, Mann Whitney test for E: *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

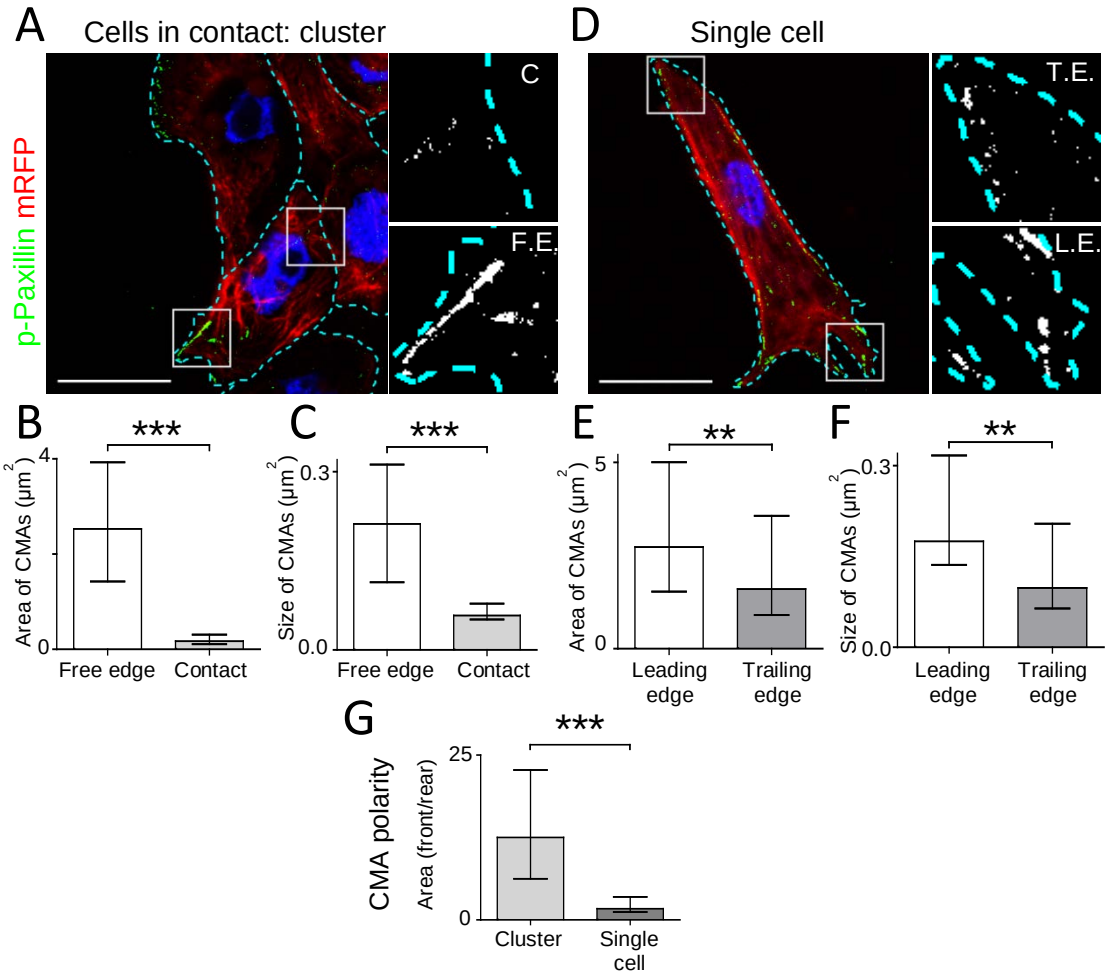

Figure 5.2 CMAs are reduced near the contact

(A, D) Endogenous CMAs in cells in contact and single cells respectively. Cells stained with pY118-paxillin (green), phalloidin (red), Hoescht (blue), outline of cells (cyan). Zoom shows contact/trailing edge and free edge/leading edge. (B, C, E, F) Quantification of area and size of CMAs related to (A) and (D) n = 80 for cells in contact and n = 23 for single cells. (G) CMA polarity is the ratio of the total area of CMAs at the front of the cell (either the free edge or leading edge) over the rear of the cell (either the contact or trailing edge). Scale bars 20 µm. Bar on graphs: median, errors: 25-75 percentiles. Wilcoxon signed rank test for B, C, E, F, Mann Whitney test for G: *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

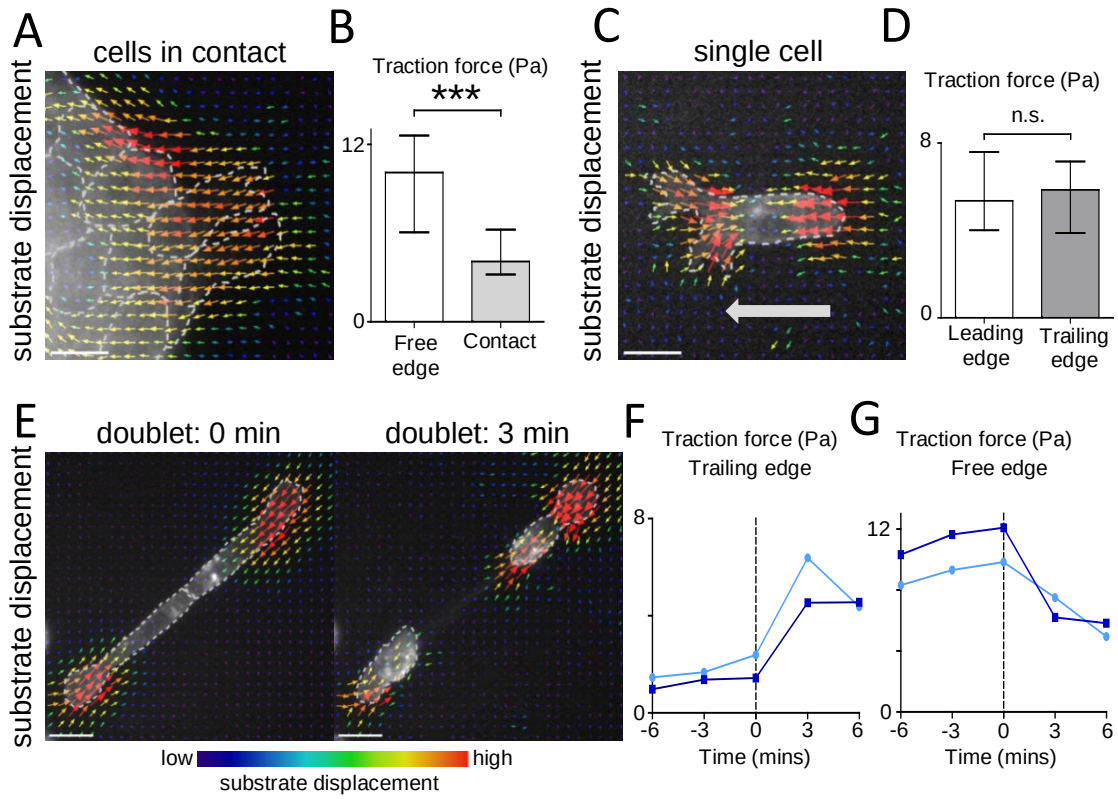

Figure 5.3 Traction forces in cells in contact and single cells

(A, C, E) TFM showing substrate displacement vectors. Cells labelled with membraneRFP and outlined in grey, vectors: substrate displacements. Grey arrow indicates direction of migration of single cell (B, D) Average traction at free edge (leading edge) and contact (trailing edge) areas. n = 27 for cells in contact and n = 17 for single cells. (E) Cell doublet one frame before separation and first frame after separation. (F, G) Time course of traction at contact/free edge of two cells as they separate. Different shade of blue indicates the two different cells. 0 mins = last frame together. Scale bars 20 µm. Bar on graphs: median, errors: 25-75 percentiles. Wilcoxon signed rank test for B and D. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

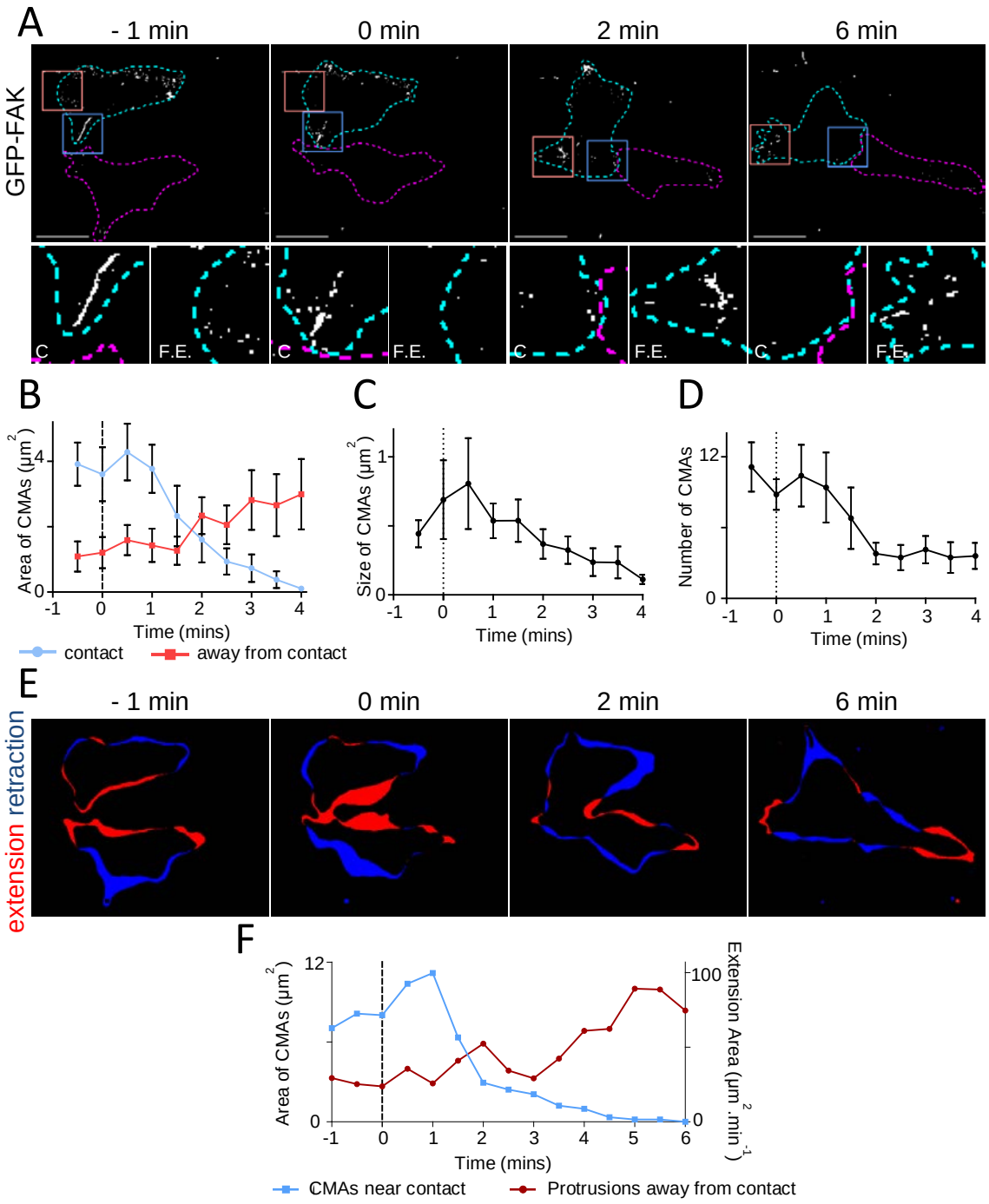

Figure 5.4 CMAs are downregulated prior to repolarisation

(A) Frames from a movie of two cells undergoing a collision labelled with GFP-FAK and outlines in cyan and magenta. Pink box marks zoom of free edge and blue box marks zoom of contact. (B) Total area of CMAs at contact (blue) or away from contact (red). Time relative to collision, n = 7 collisions. (C, D) Average size and total number of CMAs at contact during a collision. Time relative to collision, n = 15 collisions (E) Frames showing extension of membrane per minute in red and retraction per minute in blue.

(F) Area of CMAs at contact upon a single collision (blue) or extension of membrane away from the contact per minute (red). Scale bars 20 μm. Line graph shows mean , errors ± SEM.

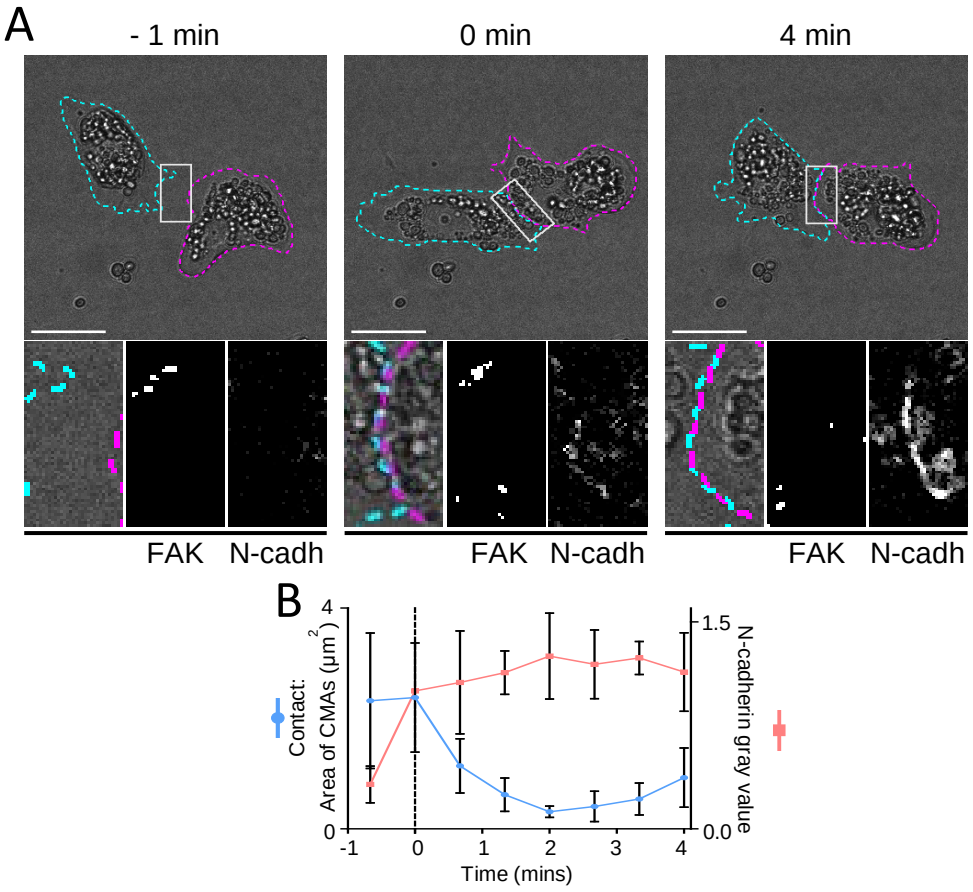

Figure 5.5 N-cadherin is rapidly recruited to the contact prior to the loss of CMAs

(A) Frames from a movie of two cells colliding labelled with N-cadherin-RFP and GFP-FAK and outlined in cyan and magenta. Box shows region of zoom. (B) CMA area at contact upon a collision (blue) and N-cadherin recruitment to the contact quantified as fluorescence intensity (red). Time relative to collision, n = 3. Scale bars: 20 µm. Line graphs show mean, errors ± SEM.

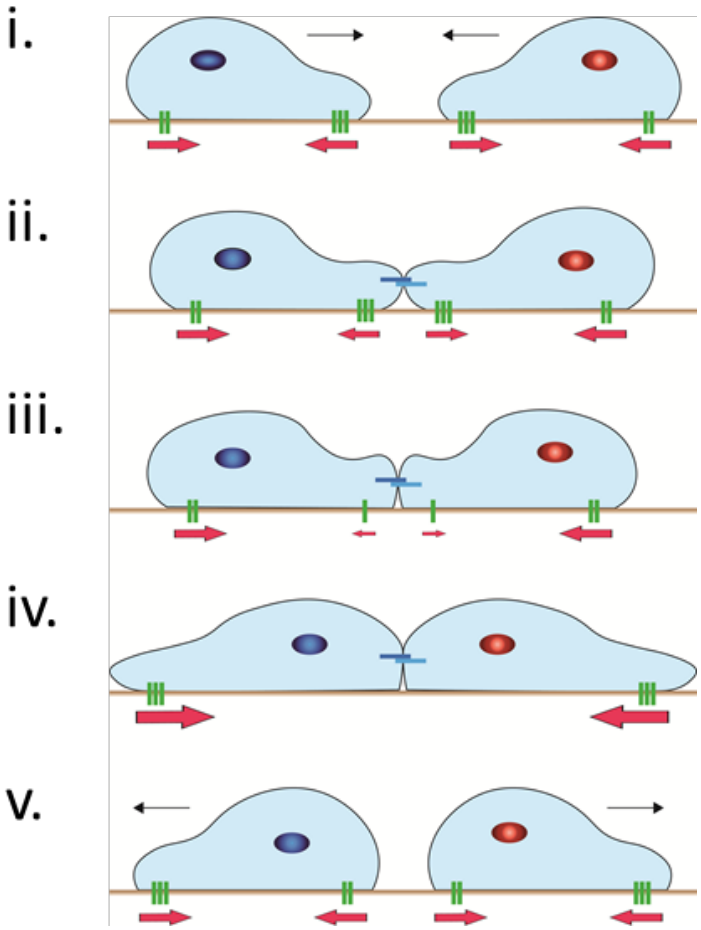

Figure 5.6 Sequence of events

Schematic of sequence of events during CIL. Traction forces: pink arrows; cell-cell adhesion, blue; cell-matrix adhesions, green; direction of migration, thin black arrows. (i) cells migrate towards each other, large CMAs at front, smaller CMAs at rear, traction forces are balanced across the cell. (ii) Cells collide and a N-cadherin based CCA forms between the cells. (iii) CMAs and consequently traction forces are reduced near the cell-cell contact. (iv) Cells repolarise away from the contact and form large CMAs away from the contact and generate increased traction. (v) Cells separate, traction is reduced at front and increased at rear of cell so it once again becomes balanced.

3.2 N-cadherin leads to the disassembly of CMAs at the contact through Src/FAK activity

In the previous chapter it was established that CMAs are rapidly reduced near the contact during CIL. This loss appears to be due to an increase in the rate of CMA disassembly upon a collision. Furthermore it seems that the presence of the cell-cell contact induces the reduction in the CMAs observed. How the presence of the cell-cell contact is leading to the reduction in CMAs remains unclear. It is well established that cell-cell adhesions (CCAs) and CMAs are closely related complexes that often have an antagonistic effect on each other, whereby the presence of one can lead to localised suppression of the other, as discussed in the chapter 1.2 and reviewed in (Burute and Thery, 2012). It has previously been established that N-cadherin based adherens junctions are required for CIL between NC cells (Theveneau et al., 2010). When N-cadherin junction formation was perturbed NC cells no longer underwent CIL upon a collision. Instead when two NC cells collided they continued migrating in their given direction and did not repolarise away from the contact (Theveneau et al., 2010). Furthermore protrusions were no longer inhibited at the contact between cells and the cells showed enhanced Rac1 activity at the cell-cell contact and reduced Rac1 activity at the free edge away from the contact compared to controls, demonstrating the vital importance of N-cadherin for CIL in the NC. However, the mechanism through which N-cadherin suppresses Rac1 activity at the contact in NC cells is still unclear. The work presented in the previous chapter established that N-cadherin is recruited to the cell-cell contact prior to the reduction of CMAs near the contact. As discussed in the introductory chapter 1.2, the presence of cadherin-based CCAs can either positively or negatively regulate CMAs and vice versa. Whilst there is evidence supporting a role for cadherins in the localised suppression of CMAs in various different systems (Borghi et al., 2010; Camand et al., 2012; Dzamba et al., 2009; Ouyang et al., 2013), the role of cadherin junctions in regulating CMAs during a dynamic process involving the separation of cells, such as CIL, where only transient N-cadherin junctions form (Scarpa et al., 2015) is unclear. It is therefore of interest to establish whether the formation of the N-cadherin junctions at the cell-cell contact upon a collision in the NC is involved in the disassembly of CMAs that occurs near the contact during CIL.

The regulation of CMA disassembly is a complex process involving many different molecular components and mechanisms, as discussed in chapter 1.1 and reviewed in (Wehrle-Haller, 2012a). The non-receptor tyrosine kinases Src and its downstream target FAK play multiple roles in the regulation of CMAs including the regulation of CMA disassembly (Myers et al., 2012; Webb et al., 2004; Woo et al., 2009). Furthermore the direct recruitment of Src to adherens junctions (Tsukita et al., 1991) and the activation of Src downstream of cadherins at the cell-cell contact (Camand et al., 2012; Leonard et al., 2013; McLachlan et al., 2007; Shao et al., 2016; Truffi et al., 2014) has previously been established. Src could therefore be involved in driving the disassembly of CMAs near the cell-cell contact during CIL and it is therefore of interest to investigate its role during this process in the NC.

3.2.1 N-cadherin is required for the reduction of CMAs near the contact

In order to investigate whether the formation of N-cadherin based adhesions at the cell-cell contact is required for the disassembly of CMAs near the contact during CIL, N-cadherin was perturbed and the levels of CMAs near the contact were investigated. N-cadherin was inhibited by using either an antisense Morpholino oligomer (MO) (Nandadasa et al., 2009; Theveneau et al., 2010) or an N-cadherin blocking antibody (BA) (Hatta and Takeichi, 1986; Theveneau et al., 2013). Endogenous levels of p-paxillin labelled CMAs near the cell-cell contact were significantly increased in cells treated with either the MO or the BA compared to controls (Fig 6.1 A, B). This was confirmed by the live cell imaging of cells expressing a GFP-FAK construct where cells incubated in the N-cadherin BA showed an increase in the CMAs near the contact compared to controls (Fig. 6.1 C, D). Furthermore the CMAs in cells where N-cadherin was blocked showed an increased longevity compared to controls (Fig 6.1 E, F), suggesting reduced N-cadherin junction formation results in more stable CMAs. These results suggest that N-cadherin is required to drive the reduction in CMAs observed near the contact during CIL. In order to investigate whether the presence of N-cadherin could stimulate a reduction in CMAs, NC cells were plated on fibronectin either with or without N-cadherin Fc in the substrate (Theveneau et al., 2013). NC cells plated on a substrate composed of both fibronectin and N-cadherin showed reduced CMAs both in terms of the total amount per cell and average size compared to those plated on fibronectin

alone (Fig. 6.2 A-C). Together these results suggest that N-cadherin is both able and required to stimulate the reduction in CMAs observed near the cell-cell contact during CIL.

3.2.2 FAK-Src signalling regulates CMA disassembly downstream of N-cadherin

The previous results demonstrate that the formation of N-cadherin based adhesions between cells play a role in the downregulation of CMAs observed in the region adjacent to the cell-cell contact. However, how the presence of N-cadherin is contributing to this downregulation is not clear. As the non-receptor tyrosine kinase Src and its target FAK are known to play a role in CMA disassembly as previously discussed, Src was investigated as a potential candidate involved in the crosstalk between N-cadherin and the CMAs. In order to investigate whether Src activity was affected downstream of N-cadherin several readouts of active Src were used. Src directly phosphorylates FAK on the tyrosine residue 861 and therefore phosphorylation of this residue can be used as functional readout of Src (Calalb et al., 1996). In order to investigate whether N-cadherin altered Src activity, N-cadherin expression was inhibited in *Xenopus* embryos by the use of an N-cadherin MO. Western blot analysis demonstrated a reduction in FAK pY861 in embryos in which N-cadherin was inhibited compared to controls, suggesting Src activity was reduced in the absence of N-cadherin (Fig 6.3 A, B). In order to verify this result NC cells were made to express a Src-FRET probe in order to visualise Src activity within the cell (Wang et al., 2005). In the absence of active Src the probe is not phosphorylated and remains in a closed conformation and high FRET efficiency is observed. In the presence of active Src the probe is directly phosphorylated by Src, it opens up and FRET efficiency is decreased. Therefore, FRET efficiency is inversely related to Src activity. Cells where N-cadherin junction formation was blocked with the use of an N-cadherin BA showed increased FRET and therefore decreased Src activity compared to controls (Fig. 6.3 C, D). Together these results show the loss of N-cadherin results in reduced Src activity in the cell. To further support the connection between N-cadherin and Src, and to probe the localisation of active Src in the cell, immunocytochemistry against an active form of Src was carried out (Kawakatsu et al., 1996). In its inactive conformation Src is phosphorylated on the tyrosine residue 527 (Cooper et al., 1986). In order for Src

to become activated this residue must first be dephosphorylated (Cooper et al., 1986). The active Src antibody used here recognises the region adjacent to Tyr527, which is only exposed when Tyr527 is dephosphorylated and consequently Src is in its active conformation (Kawakatsu et al., 1996). In control NC cells immunocytochemistry against active Src showed a clear recruitment of active Src to the cell-cell contact between cells (Fig. 6.4 A, B). However, when N-cadherin was inhibited, either with the MO or BA, active Src recruitment to the cell-cell contact was significantly reduced (Fig. 6.4 A, B). Together these results demonstrate that N-cadherin leads to increased Src activation at the cell-cell contact.

It is known that FAK-Src signalling can stimulate the disassembly of CMAs (Webb et al., 2004; Woo et al., 2009) (discussed in chapter 1.1). As it has been demonstrated that N-cadherin is required for CMA disassembly near the contact and that active Src is recruited to the cell-cell contact downstream of N-cadherin, it is of interest to investigate the effect FAK-Src signalling has on CMAs near the cell-cell contact in NC cells. In order to investigate the effect of FAK-Src signalling, Src and FAK kinase activity was inhibited with the use of the small molecule inhibitors SU6656 and PF-573228 which were used to target Src and FAK respectively (Blake et al., 2000; Slack-Davis et al., 2007). Blocking either Src or FAK kinase activity led to an increase in the CMAs near to the cell-cell contact (Fig. 6.5 A, B). As mentioned above, in order for Src to become activated it must be dephosphorylated on Tyr527 residue (Cooper et al., 1986). A Src mutant Y527F works as a constitutively active form of Src by preventing this inhibitory phosphorylation (Sandilands et al., 2004; Timpson et al., 2001) The effect of Src inhibition could be rescued by the ectopic of this constitutively active form of Src (Fig. 6.5 A, B). In addition CMAs were also stabilised when cells were incubated with the FAK inhibitor, demonstrated by an increase in longevity compared to controls (Fig. 6.5 C, D).

In order to confirm that Src activity lies downstream of N-cadherin in promoting the disassembly of CMAs near the cell-cell contact, cells treated with the N-cadherin BA, where CMAs were enhanced near the contact (Fig. 6.1 A, B ; Fig. 6.6 A, B), were made to express the constitutively active form of Src. NC cells expressing constitutively active Src were able to rescue the increase in CMAs near the cell-cell contact observed in cells

treated with the N-cadherin BA and return CMAs to control levels (Fig. 6.6 A, B). These results help to confirm that Src and FAK activity lie downstream of N-cadherin in their ability to lead to the disassembly of CMAs.

It has previously been demonstrated that the overexpression of E-cadherin in NC cells results in an increase in CMAs near the contact (Scarpa et al., 2015), similar to that observed in cells where N-cadherin is blocked (Fig. 6.1). To investigate whether this increase in CMAs could be due to a decrease in Src activity near the cell-cell contact, NC cells were made to overexpress E-cadherin. Interestingly active Src is reduced at the contact when E-cadherin is overexpressed compared to control cells (Fig. 6.7 A, B). This result could help explain the increase in CMAs observed near the contact in E-cadherin overexpressing cells.

Discussion

The results presented in this chapter demonstrate the presence of N-cadherin at the cell-cell contact is involved in driving the downregulation of CMAs in this region; when N-cadherin is perturbed CMAs near the contact are increased both in terms of total area and stability. In addition Src has been shown to be activated downstream of N-cadherin and the kinase activity of both Src and its downstream target FAK are required for the disassembly of CMAs near the cell-cell contact. The formation of transient N-cadherin adherens junctions during CIL and their requirement for CIL has previously been established in the NC (Scarpa et al., 2015; Theveneau et al., 2010). Control NC cells undergo normal CIL behaviour when two cells collide where they repolarise away from the contact and migrate away from each other. When N-cadherin is perturbed or when E-cadherin is overexpressed NC cells no longer display CIL behaviour, instead when two NC cells collide they continue their direction of migration and no longer repolarise (Scarpa et al., 2015; Theveneau et al., 2010). As discussed in the introductory chapter 1.2, crosstalk between CCAs and CMAs has previously been demonstrated in various systems. The presence of N-cadherin has been shown to suppress the formation of CMAs in astrocytes near the cell-cell contact (Camand et al., 2012) and the presence of N-cadherin in the paraxial mesoderm mesenchyme in Zebrafish has recently been shown to locally inhibit CMAs (Jülich et al.,

2015). Furthermore during *Xenopus* gastrulation tension across N-cadherin junctions results in the distribution of CMAs away from N-cadherin (Davidson et al., 2002; Dzamba et al., 2009). The results presented here demonstrate a similar crosstalk and further support the role of N-cadherin in the localised suppression of CMAs. Whilst it is tempting to speculate N-cadherin leading to a downregulation of CMAs near the cell-cell contact is a conserved phenomenon, it should be noted that cross-regulation can also occur in the opposite direction as CCAs can both negatively and positively regulate CMAs and vice versa with the overall outcome of the crosstalk appearing to be very much context specific (Collins and Nelson, 2015; McCain et al., 2012; McMillen and Holley, 2015; Mui et al., 2016; Tsai and Kam, 2009; Weber et al., 2011; Yano et al., 2004).

The downregulation of CMAs downstream of N-cadherin junction formation in the NC appears to be mediated by the tyrosine kinase Src. Recruitment and activation of Src downstream of CCAs has been described in other systems previously (Leonard et al., 2013; McLachlan et al., 2007; Shao et al., 2016; Truffi et al., 2014; Tsukita et al., 1991). It appears a similar mechanism is at work during CIL in the NC. One known role of Src is in the activation of the CMA protein FAK (Calalb et al., 1995), which consequently exposes a binding site for Src itself. This allows the formation of a FAK-Src complex (Xing et al., 1994) and leads to further phosphorylation of FAK in the kinase loop (Owen et al., 1999). This FAK-Src signalling activity has been implicated in the disassembly of CMAs (Myers and Gomez, 2011; Webb et al., 2004; Westhoff et al., 2004; Woo et al., 2009). The results presented here demonstrate that FAK-Src signalling is required for the normal disassembly of CMAs near the cell-cell contact as inhibition of FAK-Src activity leads to an increase in CMAs in term of total amount and their stability. This is consistent with previous findings that have shown that cells lacking FAK or Src activity have enlarged CMAs due to a reduction in disassembly (Chen et al., 2002; Ilić et al., 1995). Interestingly a decrease in Src recruitment to the cell-cell contact is observed in cells overexpressing E-cadherin. Previous findings have established that overexpressing E-cadherin in NC cells leads to an increase in CMAs near the contact (Scarpa et al., 2015). When N-cadherin is perturbed CMAs are also increased near the contact and Src activity is reduced. Taken together these results

demonstrate a clear correlation between Src activity, the downregulation of CMAs near the contact and CIL response.

Overall these results demonstrate that N-cadherin based adhesions at the cell-cell contact lead to the recruitment and activation of Src. Src phosphorylates and fully activates FAK and both Src and FAK activity are required for the disassembly of CMAs near the contact.

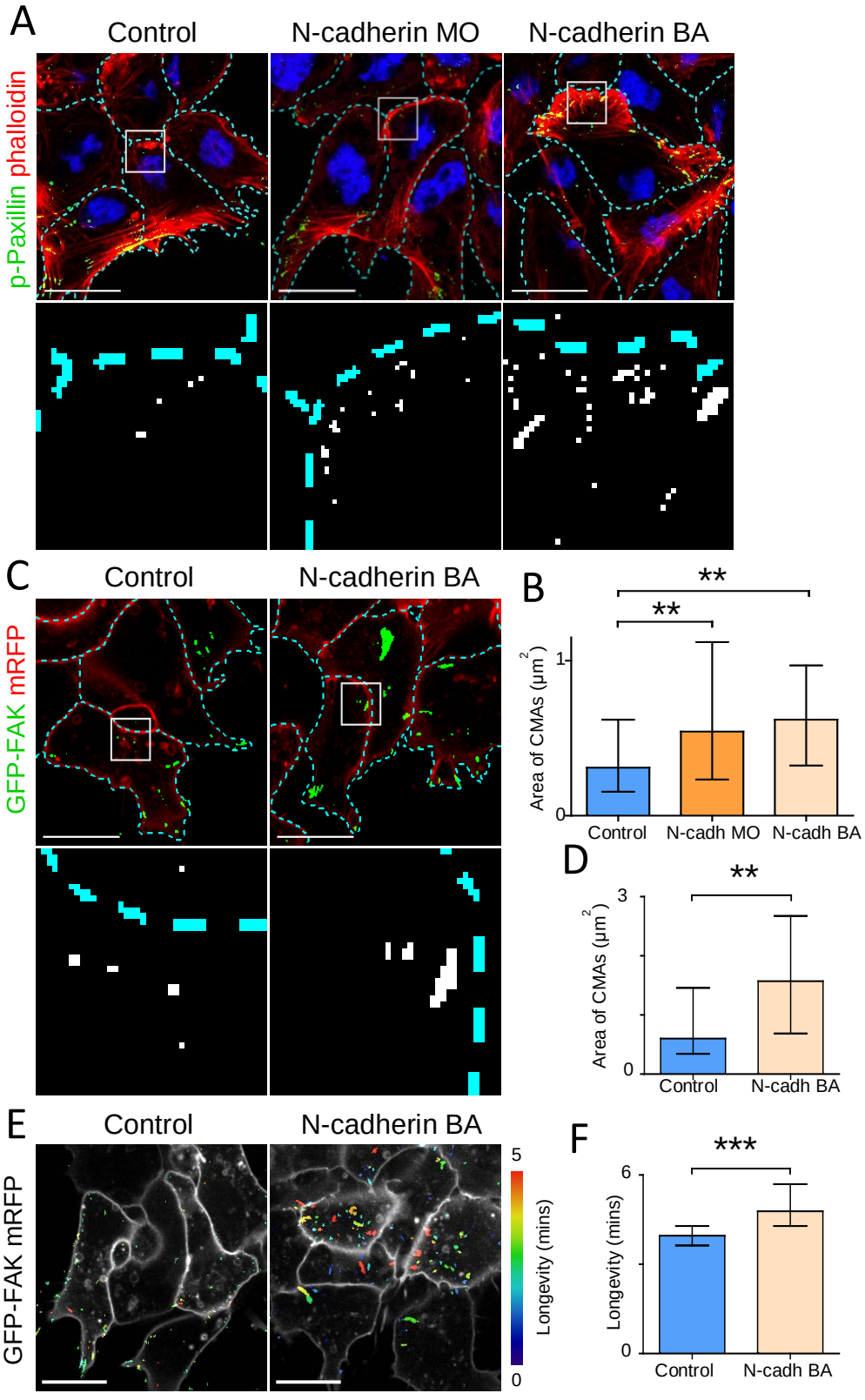

Figure 6.1 CMAs are increased near the contact when N-cadherin is perturbed

(A) Immunofluorescence against p-Paxillin (green), phalloidin (red) and Hoescht (blue) of control cells, cells injected with N-cadherin morpholino and cells pre-treated in N-

cadherin blocking antibody. Cells outlined in cyan and box illustrates zoom at contact. (B) Area of CMAs near the contact. Control n = 172, N-cadh MO n = 89, N-cadh BA n = 68. (C) Live-imaging of control cells and cells treated with N-cadherin BA, expressing membraneRFP (red) and GFP-FAK (green). Cells outlined in cyan. Box shows region of zoom. (D) Area of CMAs at contact in live cells shown in (C). n = 28 for both conditions. (E) Frame from a movie of control cells and N-cadherin BA treated cells, expressing membraneRFP (grey) and GFP-FAK (colour code shows CMAs longevity). (F) Longevity of CMAs at contact of cells expressing GFP-FAK. n = 30 for both conditions. Scale bars 20 μm. Bars: median, error bars: 25-75 percentiles. Kruskal-Wallis test for B, Mann-Whitney test for D and F. *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

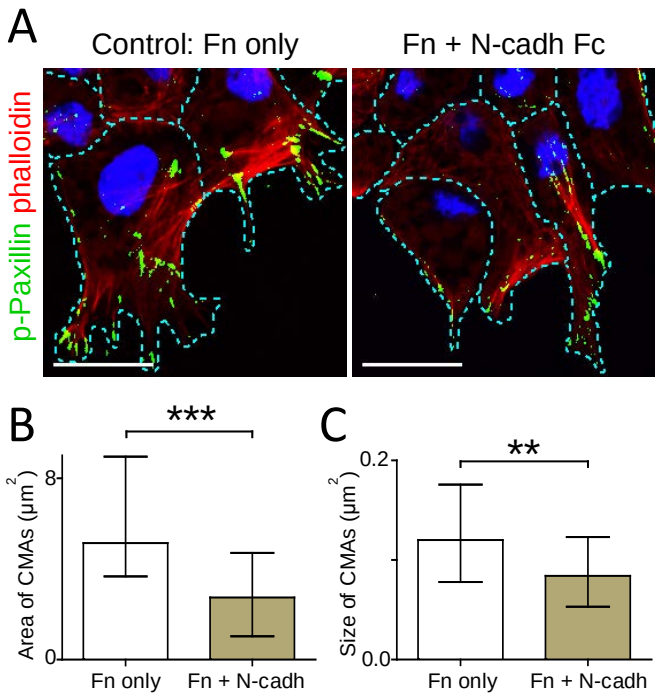

Figure 6.2 N-cadherin downregulates CMAs

(A) Immunofluorescence against p-Paxillin (green), phalloidin (red) and Hoescht (blue) of cells plated on a substrate of fibronectin only at 10 µg.ml^{-1} and cells plated on a substrate consisting of fibronectin and N-cadherin Fc at 10 µg.ml^{-1} and 3 µg.ml^{-1} respectively. Cells outlined in cyan. (B and C) Quantification of CMAs as total accumulative area of CMAs per cell and average size of CMAs per cell. Fn only n = 51, Fn + N-cadh Fc n = 43. Scale bars 20 µm. Bars: median, errors: 25-75 percentiles. Mann-Whitney for B & C. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

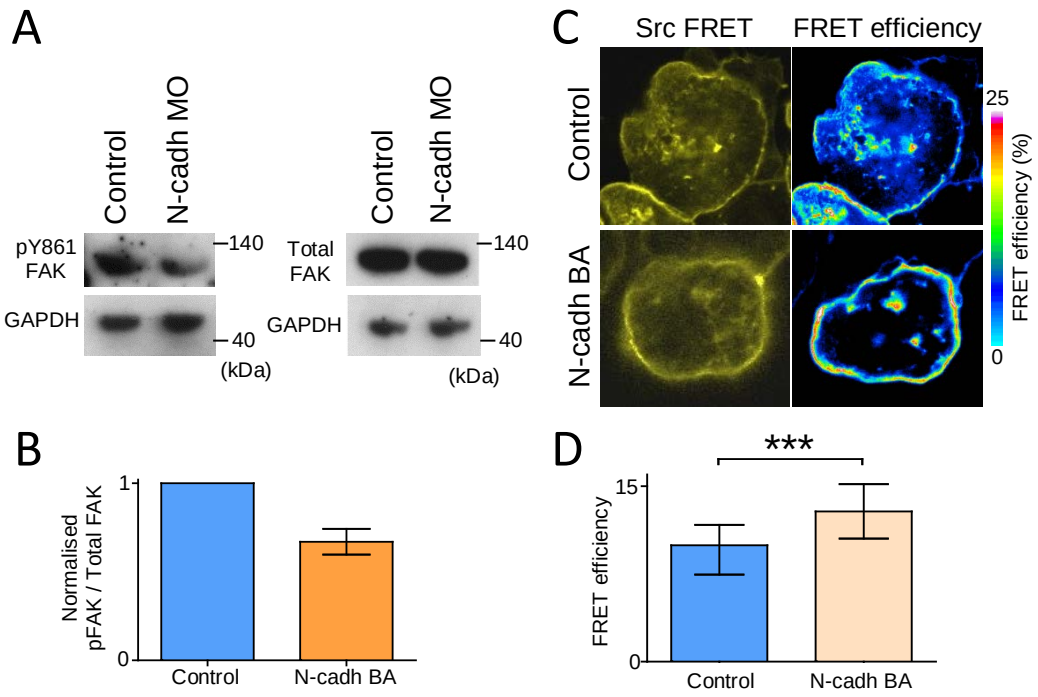

Figure 6.3 Loss of N-cadherin reduces Src activity

(A) Western blot against total FAK and pY861 FAK in control embryos or those injected with N-cadherin MO. (B) Quantification of Western blot normalized to control n = 3 repeats (C) Cells injected with Src FRET probe, either control or cells treated with N-cadherin blocking antibody. (D) Quantification of Src FRET efficiency n = 23 for each condition. Bar: median, error: 25-75 percentiles. Mann-Whitney test for B and D. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

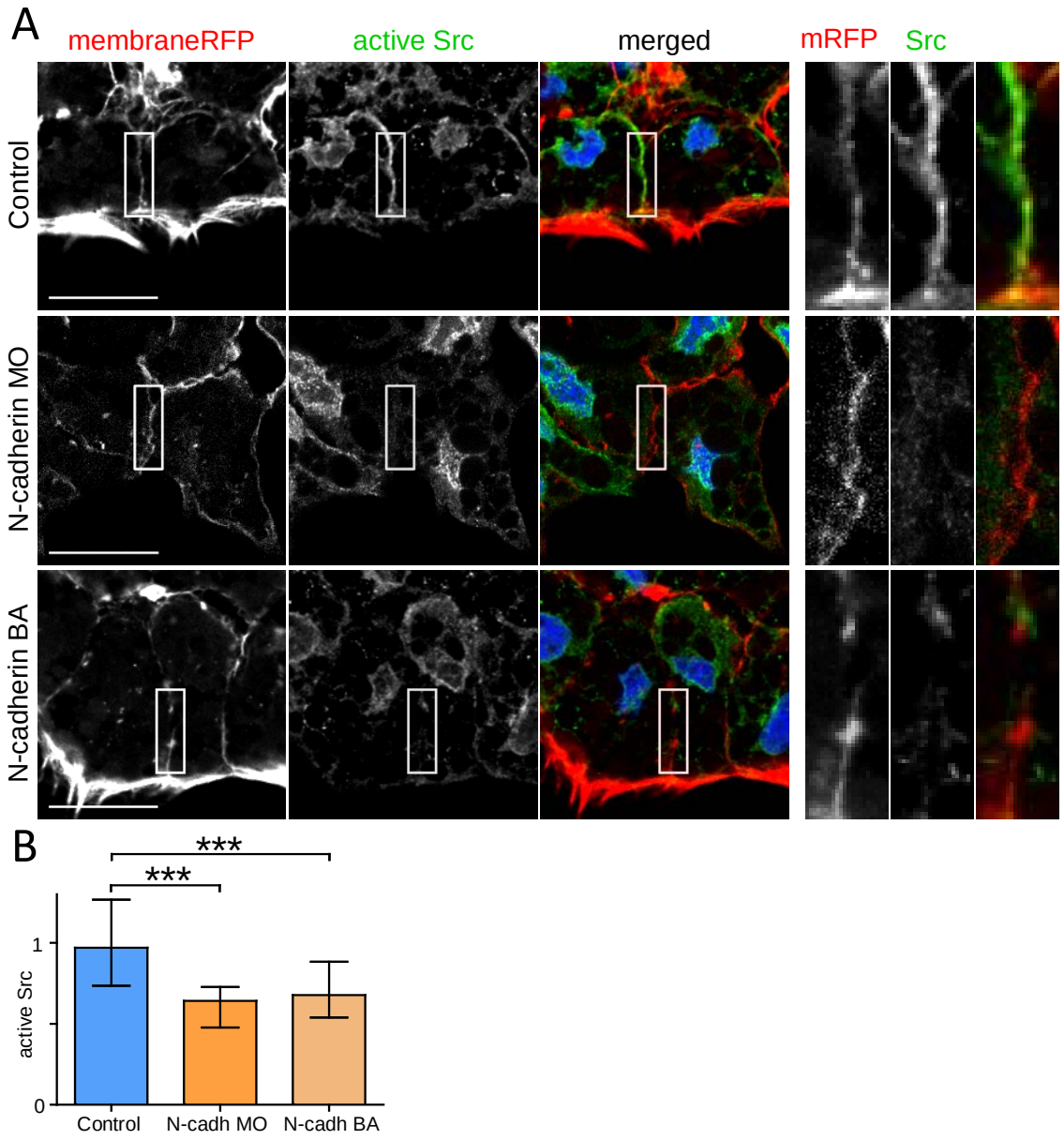

Figure 6.4 N-cadherin recruits and activates Src at the cell-cell contact

(A) Immunofluorescence against active Src (green) in cells injected with membraneRFP (red) and stained with Hoescht (blue) in control cells, cells with N-cadherin inhibited by either MO or blocking antibody. Box shows region of zoom over contact. Scale bar 20 µm. (B) Average gray value of active Src at the contact. Values are relative to average control for each experiment. Control n = 125, N-cadh MO n = 60, N-cadh BA n = 64. Bar: median, error: 25-75 percentiles. ANOVA test for B. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

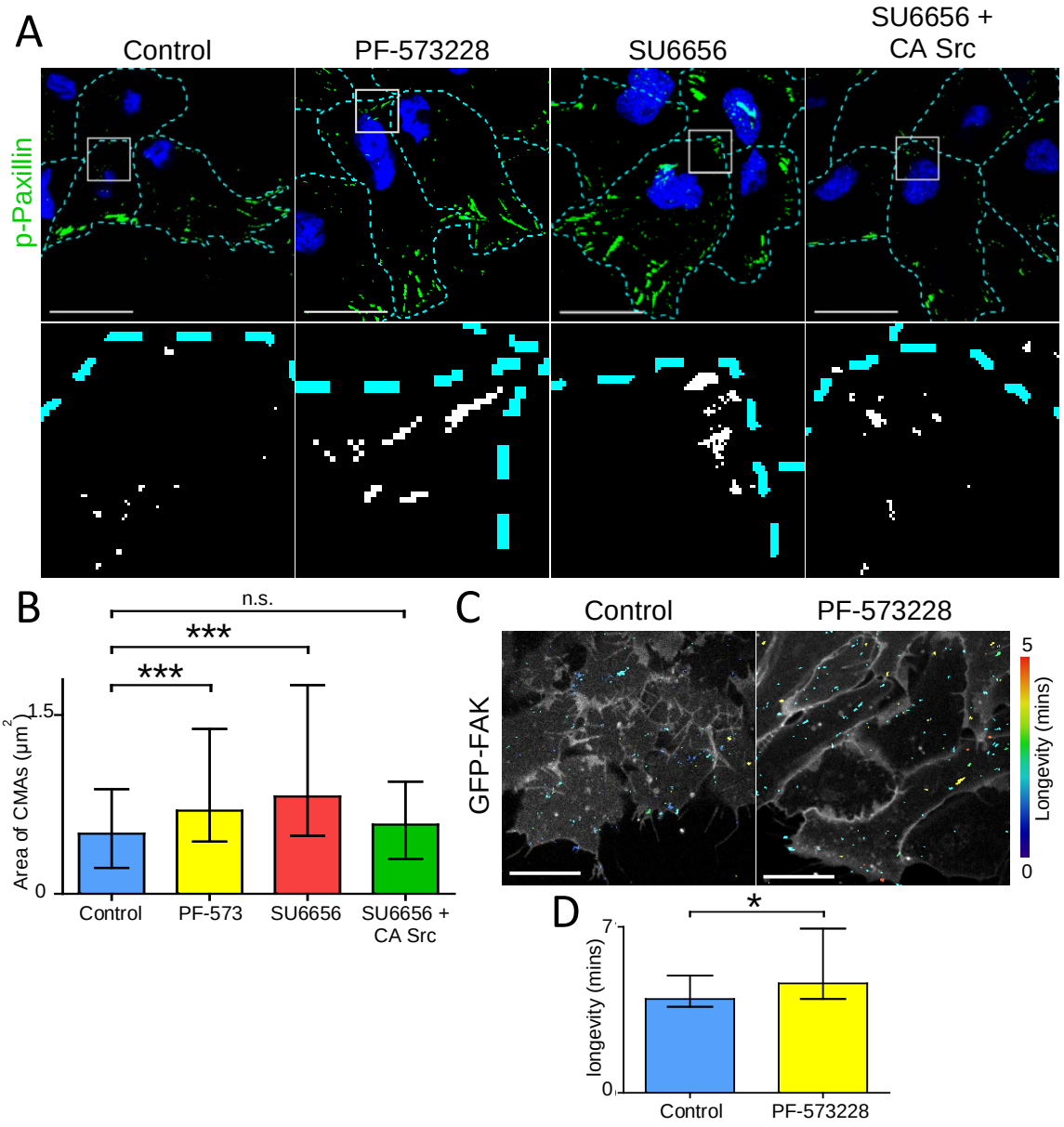

Figure 6.5 FAK-Src inhibition increases cell-matrix adhesions near the contact

(A) Immunofluorescence against p-Paxillin (green) and Hoescht (blue); outlines in cyan in control cells, cells incubated in 2µM of PF-573228 and cells incubated in 5µM of SU6656 or 5µM of SU6656 injected with Src Y527F. Box shows region of zoom. (B) Quantification of CMAs at contact. Control n = 182, PF-573228 n = 99, SU6656 n = 103, SU6656 + Src Y527F n = 84. (C) Frame from movie of control cells or cells treated with 2µM of PF-573228. Cells express membraneRFP (grey) and GFP-FAK (colour code indicates CMA longevity). (D) Quantification of longevity of CMAs near the contact. Control n = 29, PF-573228 n = 66. Scale bars: 20 µm. Bars: median, errors: 25-75

percentiles. Kruskal-Wallis test for B, Mann-Whitney test for D *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

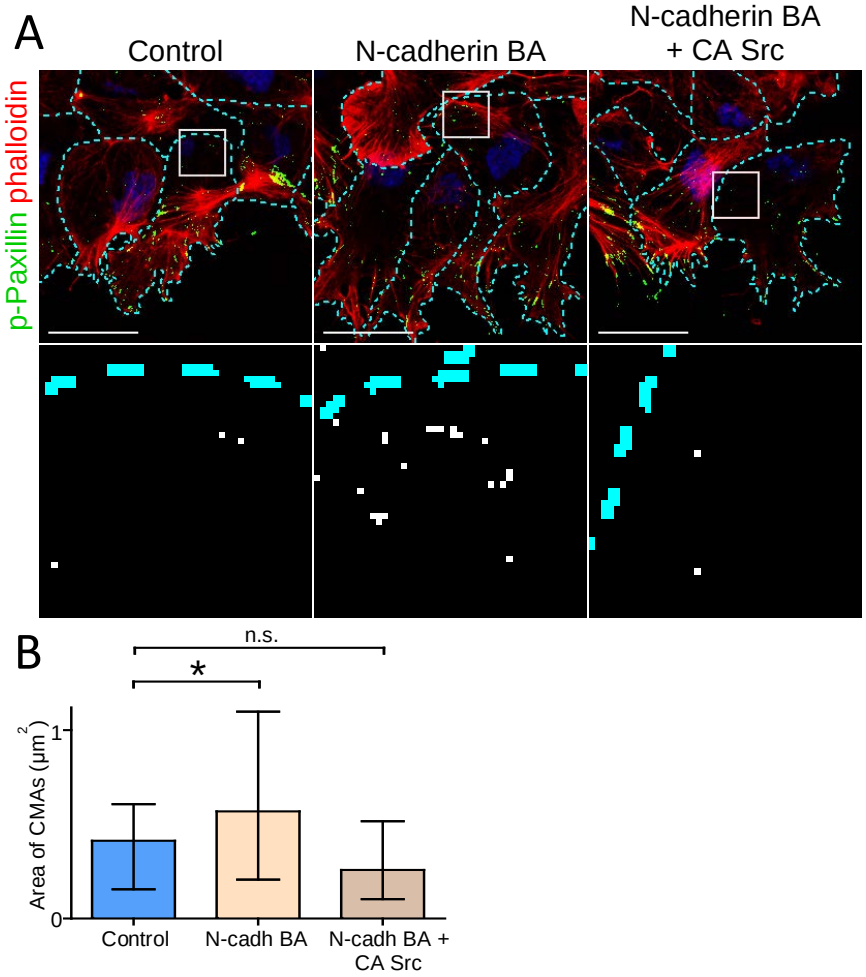

Figure 6.6 Src activity lies downstream of N-cadherin

(A) Immunofluorescence against P-paxillin (green), phalloidin (red) and Hoescht (blue) in control cells, cells pre-treated with N-cadherin blocking antibody or cells expressing Src Y527F and pre-treated with N-cadherin blocking antibody. Cells outlined in cyan. Box shows region of zoom. (B) Quantification of CMAs near the contact. Control n = 52, N-cadh BA n = 50, N-cadh BA + Src Y527F n = 49. Scale bars: 20 µm. Bars: median, errors: 25-75 percentiles. Kruskal-Wallis test for B. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

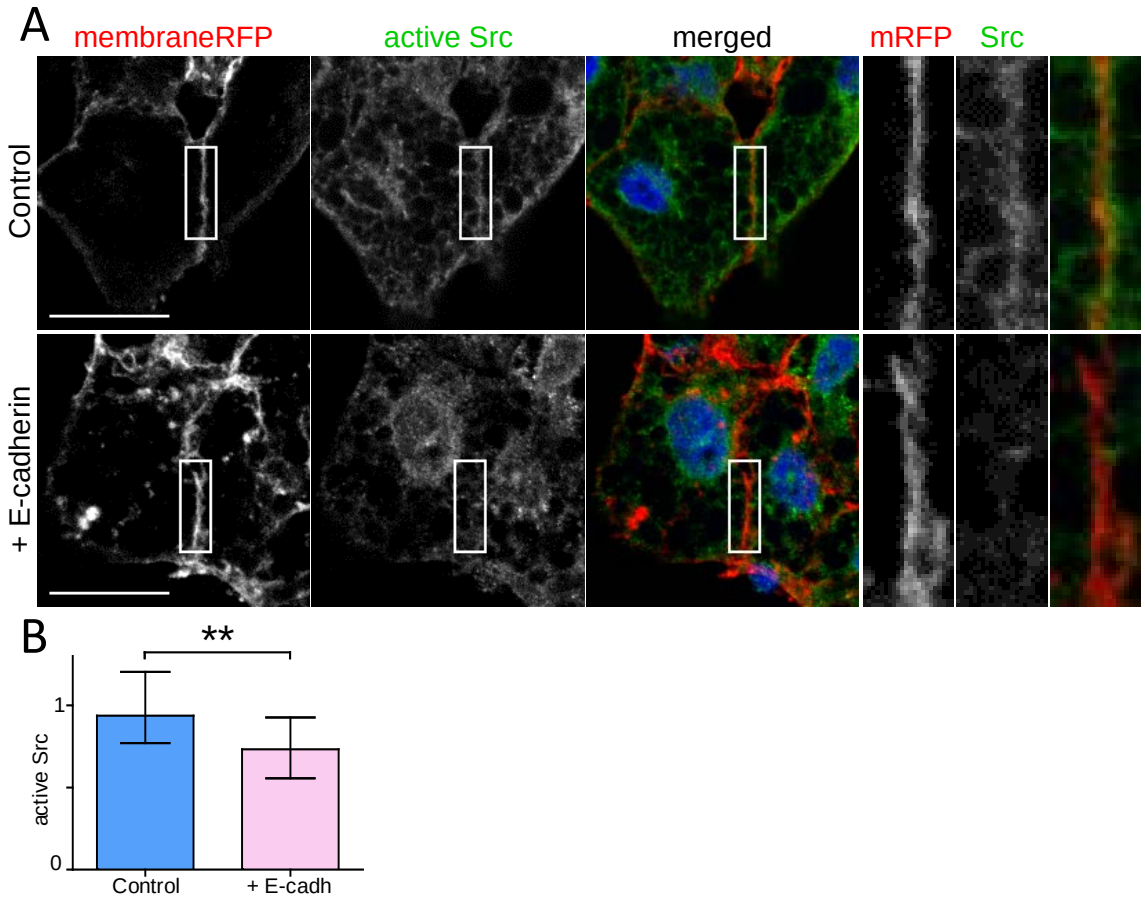

Figure 6.7 E-cadherin reduces active Src at the contact

(A) Immunofluorescence against active Src (green) in cells injected with membraneRFP (red) and stained with Hoescht (blue) in control cells and cells overexpressing E-cadherin. Box shows region of zoom over contact. Scale bar 20 µm. (B) Average gray value of active Src at the contact. Values are relative to average control for each experiment. Control n = 51, E-cadh n = 60. Bar: median, error: 25-75 percentiles. t-test for B. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

3.3 CMAs disassembly upon a collision is required for separation during CIL

The results presented in the previous chapters have helped establish that CMAs are rapidly disassembled near the cell-cell contact upon a collision during CIL. Furthermore this disassembly appears to be driven by Src and FAK activity downstream of N-cadherin junction formation. Although observed during CIL, the role this localised disassembly of CMAs plays during CIL is unclear. When Harris first inferred a loss of CMAs near the contact during CIL, he speculated that this loss leads to tension across the cell-cell contact (Harris, 1973). It is possible that tension is required across the cell-cell contact in order to drive the separation of cells after CIL. It is well established that there is a build-up in tension across the contacting lamellae upon a collision (Abercrombie and Ambrose, 1958; Davis et al., 2015; Harris, 1973; Heaysman and Pegrum, 1973a; Scarpa et al., 2015). How this tension builds up and whether this tension is sufficient to drive separation remains unknown. One potential consequence of the localised disassembly of CMAs near the cell-cell contact during CIL could be the redistribution of tension generated across the cell to the cell-cell contact to initiate cell separation after a collision. If the CMAs near the contact persisted during a collision it is possible their presence may alleviate the tension across the contact and prevent the generation of sufficient force across the contact required for cell separation. Consequently it is of interest to establish whether the stabilisation of CMAs near the cell-cell contact, through the inhibition of Src or FAK activity, has any effect on CIL and the transfer of tension.

3.3.1 Src/FAK inhibition prevents NC cells from separating after a collision

It is well established that CIL is required for the NC to undergo directional migration both *in vivo* and *in vitro* (Carmona-Fontaine et al., 2008; Theveneau et al., 2010). In order to investigate the effect that the inhibition of either Src or FAK has on NC migration, *Xenopus laevis* embryos were incubated in the Src or FAK inhibitor (SU6656 and PF-573228 respectively) from developmental stage 18 until stage 24. Stage 18 was chosen as the starting point for incubation as this is prior to when the NC start to migrate but after the NC are induced. It is possibly that Src and FAK could play a role in

NC induction so it was important to leave this process unperturbed. At stage 24, when the embryos were fixed, the cranial NC is migrating ventrally in its distinct streams. Embryos incubated with either the Src or FAK inhibitor showed reduced migration compared to the control embryos [Fig. 7.1 A, B]. Although it is well established that CIL is required for NC migration (Carmona-Fontaine et al., 2008; Theveneau et al., 2010; Theveneau et al., 2013; Woods et al., 2014), there are many other elements of NC migration that may be affected by the inhibition of Src and FAK that could result in reduced NC migration (Barriga and Mayor, 2015; Carmona-Fontaine et al., 2011; Kuriyama et al., 2014; Shellard and Mayor, 2016; Theveneau and Mayor, 2012b). In order to investigate whether NC cells with inhibited Src and FAK activity were still migratory, single NC cells where plated *in vitro* and their migratory properties were investigated. Cells lacking Src and FAK activity showed persistent migration similar to the controls [Fig. 7.1 D]. However, control cells migrated at a faster velocity than cells where Src or FAK were inhibited [Fig. 7.1 C]. This reduced velocity is not surprising as it has previously been demonstrated that cells lacking FAK activity, where CMAs are more stable, show reduced migration (Chen et al., 2002; Ilić et al., 1995; Webb et al., 2004; Westhoff et al., 2004).

To establish whether CIL is affected by Src and FAK inhibition, NC cells were cultured *in vitro* and both cluster behaviour and single cell collisions were analysed. When NC cells are plated on fibronectin they disperse and the cells migrate away from each other (Alfandari et al., 2003). CIL is the driving force behind this dispersion (Carmona-Fontaine et al., 2011; Davis et al., 2012; Stramer et al., 2010) and therefore dispersion can be used as a readout of CIL (Moore et al., 2013; Scarpa et al., 2015). Dispersion was quantified by Delaunay triangulation where the triangle areas between cells and their closest neighbours were measured (Carmona-Fontaine et al., 2011). After 18 hours the average triangle area between cells in control explants was over double that of the NC explants incubated with the SU6656 or PF-573228 inhibitors to inhibit Src or FAK kinase activity respectively [Fig. 7.2 A, B]. The reduction in dispersion caused by the Src inhibitor could be rescued by the ectopic expression of constitutively active Src.

There are three observed outcomes when two NC cells collide: they can undergo CIL where they cease migrating in their current direction, repolarise, separate and migrate

away from each other, they can remain adhered to each other, or they can continue their migration and move past each other without repolarising (Scarpa et al., 2013). Control NC cells undergo CIL in around 90% of collision (Fig. 7.3 A, B; Movie 2) (Scarpa et al., 2013). In contrast, cells treated with the Src or FAK inhibitor showed a dramatic decrease in CIL response (Fig. 7.3 A, B; Movie 2). This reduction in CIL could either be due to the cells migrating past each other or not separating within the 30 minutes of analysis. To address this the duration of the cell-cell contact after a collision was measured. Whilst the largest proportion of control NC cells separated in under 15 minutes, the largest proportion of treated cells still remained together after 60 minutes (Fig. 7.3 A, C). These results indicate that the inhibition of Src and FAK impairs CIL by preventing cells from separating.

3.3.2 Disassembly of CMAs at the contact is required for cell separation

It has been demonstrated that cells treated with either the FAK or Src inhibitor were unable to undergo CIL as they normally would. This reduction in CIL appears to be due to the inability of cells to separate after coming into contact. There are many possibilities as to why the cells could be unable to separate including increased cell-cell adhesion, inability to repolarise or the cells not generating sufficient tension across the contact to pull away from each other. Endogenous levels of E- and N-cadherin were investigated across the cell-cell contact when cells were treated with either the Src or FAK inhibitor. Interestingly the levels of N- and E-cadherin across the contact were unaffected by treated with either of the inhibitors (Fig. 7.4). It has recently been demonstrated that repolarisation away from the contact is required for separation after a collision during CIL in NC cells (Scarpa et al., 2015). It was therefore of interest to analyse whether cells treated with the inhibitors, where either Src or FAK activity was inhibited, were forming protrusions away from the contact in the same manner as control cells. In both Src and FAK inhibited cells the protrusions in the free edge mirrored the dynamic behaviour of protrusions in control cells (Fig. 7.5 A, B). In addition traction forces generated in the free edge of cells were not significantly different between FAK inhibited cells and control cells (Fig. 7.5 D, E). However, cells treated with the inhibitors showed an increase in the area of overlap between the

cells, demonstrating protrusions were not inhibited to the same extent at the contact as they were in control cells (Fig. 7.5 A, C). Whether this increase in overlap is due to the increase in CMAs or vice versa is unclear. Furthermore traction forces measured near the contact were significantly increased in cells treated with the FAK inhibitor compared to controls (Fig. 7.5 D, E). As protrusions at the free edge were unaffected by the inhibition of either Src or FAK and cell-cell adhesions appeared unaffected, it appears likely that the absence of cell separation in cells where Src or FAK is inhibited is due to the effect on CMAs, and possibly protrusions, at the contact. During the separation phase of CIL, tension across the cell-cell contact is built up and forces pulling the cells apart overcome those keeping the cells together (Davis et al., 2015; Scarpa et al., 2015). Tension across the cell-cell contact can be inferred from TFM (Maruthamuthu et al., 2011). Using this method to calculate the tension across the contact in control and FAK inhibited cells, tension was shown to be significantly reduced when FAK was inhibited compared to control cells (Fig. 7.5 F). Taken together, these results suggest that during CIL the disassembly of CMAs near the contact is required in order to redistribute tension generated across the cell to the cell-cell contact. However, when FAK is inhibited the CMAs that persist near the contact act to relieve part of the tension from the cell-cell contact and instead transmit it to the substrate in the form of traction. Indeed, tension has been shown to build up across the cell-cell contact as a consequence of repolarisation and the coupling of the cytoskeletons across the cell-cell junction during normal CIL (Davis et al., 2015; Scarpa et al., 2015). When the build-up of tension is perturbed, for example by preventing repolarisation or inhibiting stress fibres across the contact, the cells no longer separate during CIL indicating the requirement for tension across the cell-cell contact for driving separation (Davis et al., 2015; Scarpa et al., 2015). Therefore, the decreased tension across the cell-cell contact could explain the lack of separation seen in FAK inhibited cells.

If this conclusion is true the lack of separation phenotype observed in cells where CMAs are stabilised near the contact, such as those in FAK inhibited cells, could be rescued solely by removing the CMAs in this region. According to this idea, the removal of CMAs near the contact in FAK inhibited cells could induce the cells to behave the

same as controls and start to separate. This notion was tested directly by laser ablating the CMAs near the contact in FAK inhibited cells. It has previously been demonstrated that cell separation during CIL is preceded by a reduction in the length of the cell-cell contact (Scarpa et al., 2015). Therefore the thinning of the cell-cell contact was used as a read out of cell separation initiation; ablation could not be maintained for a sustained period of time to induce full separation without an adverse effect on the cells. In order to ablate cells at the same phase during CIL, cells were only ablated once they had formed protrusions away from the contact. Control NC cells that were not ablated showed a characteristic reduction in the length of the cell-cell contact over time (Fig. 7.6 A, D blue line; Movie 4) whereas the cells with inhibited FAK activity did not have a reduction in the length of the cell-cell contact (Fig. 7.6 A, D orange line; Movie 4). To ensure that laser ablation was sufficient to destroy CMAs, CMAs were ablated in the free edge of cells and recoil of protrusions were observed, demonstrating the loss of CMAs in this region and proving that laser ablation was sufficient to destroy the CMAs (Movie 3). Importantly, when the CMAs near to the contact of FAK inhibited cells were ablated, the length of the cell-cell contact decreased to levels similar to those seen in control cells (Fig. 7.6 B, D green line; Movie 5). Ablation of CMAs in control cells near the cell contact showed no significant difference compared to the non-ablated controls (Fig. 7.6 B, D red line; Movie 5), indicating that the ablation per se did not reduce the cell-cell contact independently of its effects on CMAs. Moreover, to verify that in FAK inhibited cells separation was not being prevented by stronger cell-cell adhesion, the cell-cell contact was ablated apical to the CMAs. Control cells ablated at the cell-cell contact showed a sudden reduction in the length of the cell contact and many cells separated completely after ablation (Fig. 7.6 C, E purple line; Movie 6). However, FAK inhibitor treated cells ablated at the cell-cell contact showed only a brief shortening of the contact immediately after ablation before the cell-cell contact length became constant once again (Fig. 7.6 C, E navy line; Movie 6). This result demonstrates that the loss of cell separation in FAK inhibited cells cannot be rescued by ablating the cell-cell adhesion. Overall these results indicate that the presence of increased numbers of CMAs at the contact in FAK inhibited cells prevents cell separation and further suggests that these CMAs have to be disassembled in order for the cells to separate during CIL.

Discussion

The results presented here demonstrate that normal Src and FAK kinase activities are required for NC migration *in vivo*. Furthermore it is apparent that Src and FAK are required for a normal CIL response between cells as their loss inhibits the separation of cells after a collision. As E-cadherin and N-cadherin levels at the contact and protrusions at the free edge remain unaffected by Src and FAK inhibition, it appears that loss of cell separation is a consequence of the increase in CMAs near the cell-cell contact resulting in increased traction in this region and decreased tension across the contact. Together these results suggest tension generated across the cell needs to be directed to the cell-cell contact in order to induce separation; stabilising CMAs near the contact dissipates some of the tension away from the contact and instead transmits it to the substrate as traction.

The idea that tension across the cell-cell contact could be inducing cell separation after a collision during CIL has been speculated upon since the initial characterisation of CIL and has been the subject of much discussion in the decades following (Abercrombie and Ambrose, 1958; Abercrombie and Dunn, 1975; Davis et al., 2015; Harris, 1973; Heaysman and Pegrum, 1973a; Roycroft and Mayor, 2015; Roycroft and Mayor, 2016; Scarpa et al., 2015). There is, however, minimal evidence demonstrating tension is required for separation. A recent study in haemocytes undergoing CIL demonstrated a build-up of tension in the overlapping haemocytes with the use of recoil analysis induced by laser ablation at the cell-cell contact (Davis et al., 2015). In addition the presence of a myosin II coated stress fibre that aligns between two colliding haemocytes was highlighted. When either myosin II or the formin diaphanous was mutated stress fibre formation was aberrant and there was a reduction in tension across the contact. In addition separation after a collision was perturbed (Davis et al., 2015). This result is suggestive of a role for tension across the contact in driving separation upon a collision and supports the results presented here. Further evidence for the requirement of tension across the cell-cell contact to stimulate cell separation was observed in the NC where NC cells made to overexpress E-cadherin showed a reduction in CIL response and failed to separate after a collision (Scarpa et al., 2015). These NC cells overexpressing E-cadherin had reduced traction in the free edge and

increased traction near the contact. Furthermore their polarity was aberrant with increased Rac1 near the contact and decreased Rac1 at the free edge, the reverse of control cells. However, when a photoactivatable Rac1 construct was induced in the free edge of NC cells overexpressing E-cadherin, the cells then formed protrusions away from the contact and were able to separate. One possible explanation for this result is that the increase in Rac1 activity in the free edge, and consequent the protrusions and traction forces generated in this area, leads to an increase in tension across the cell-cell contact with the increased traction generated in the free edge pulling the cells away from each other is transferred to the cell-cell contact inducing the cells to separate. Although it has not been demonstrated that this is the case, this result supports the results shown hear for the requirement of tension across the contact to induce cell separation during CIL.

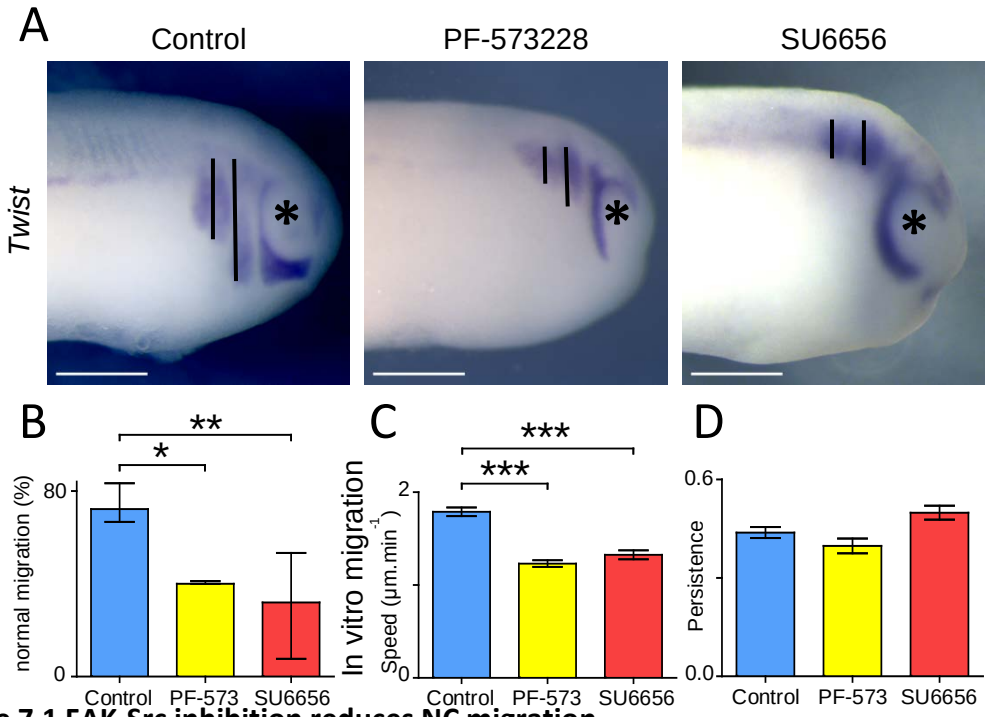

Figure 7.1 FAK-Src inhibition reduces NC migration

(A) In situ hybridisation against *Twist* to label the migrating NC in *Xenopus laevis* embryos at stage 24 in control embryos, embryos treated with 100 µM of PF-573228 and embryos treated with 100 µM of SU6656. Black lines mark distance of NC migration in second and third streams. Asterisks marks eye. Scale bar 500 µm (B) Quantification of percentage of embryos that show normal NC migration. n = 3 repeated experiments for PF-573228 and 4 repeats for SU6656. Total number of embryos analysed: Control = 108, PF-573228 = 46, SU6656 = 55. (C, D) Quantification of single cell migration in vitro in terms of speed and persistence in control cell, cells treated with PF-573228 at 2 µM and cells treated with SU6656 at 5 µM. Control n= 195, PF-573228 n= 95, SU6656 n = 100. Bar: median, errors: 25-75 percentiles. ANOVA for B, C, D *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

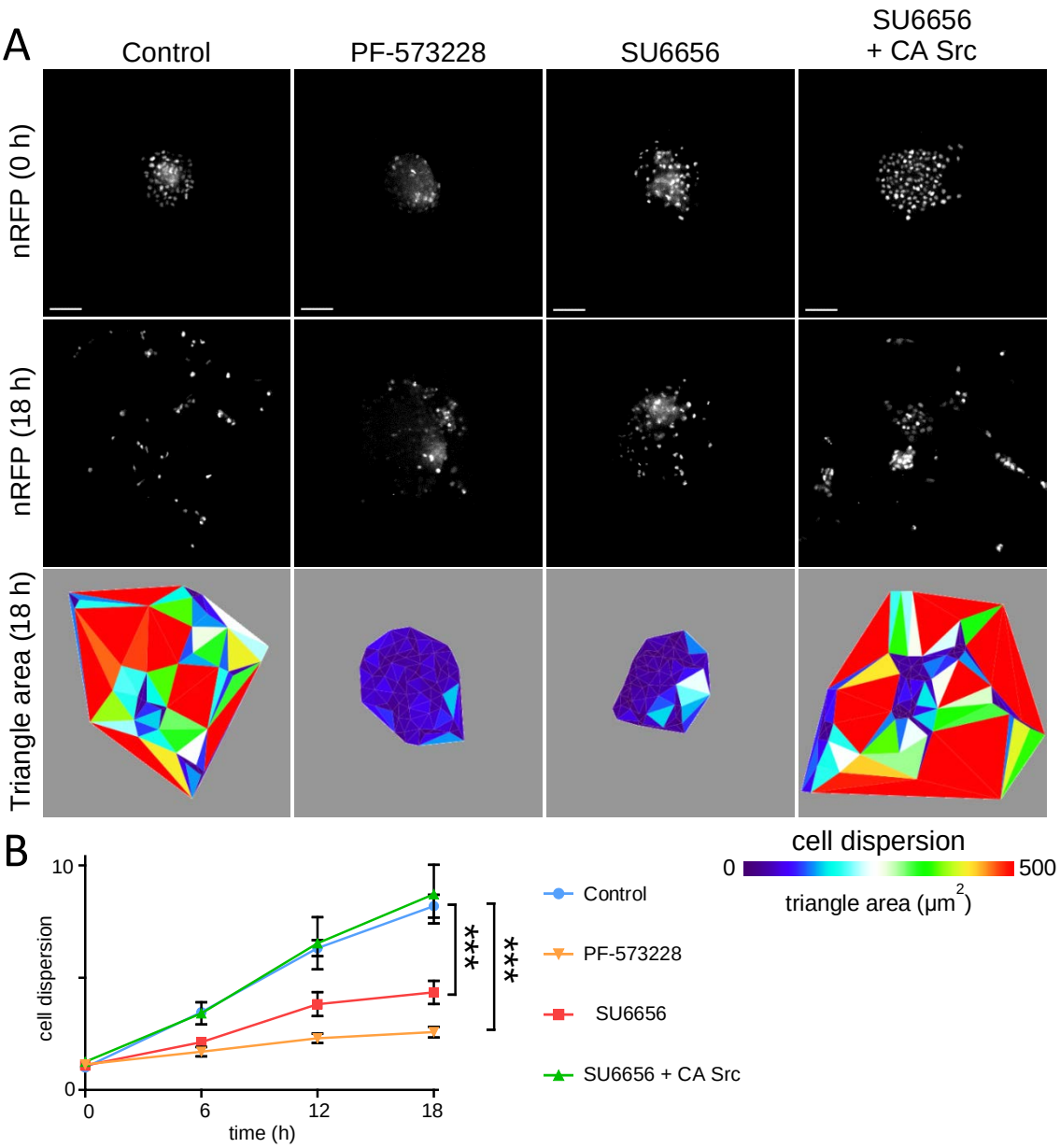

Figure 7.2 FAK-Src inhibition decreases NC dispersion

(A) Initial frame (0 hours) and last frame (18 hours) from movies of cell clusters expressing nuclearRFP from control explants, explants treated with 2 μM of PF-573228, explants treated with 5 μM of SU6656 and Src Y527F explants treated with 5 μM of SU6656. Scale bar 100 μM. Dispersion analysis with Delaunay triangles colour coded for area size. (B). Mean triangle areas over time relative to control at 0 h for each experiment. Control n = 52, PF-573228 n = 28, SU6656 n = 32, SU6656 + Src Y527F n = 32. Line graph: mean ± SEM. Analysis is ANOVA for B. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

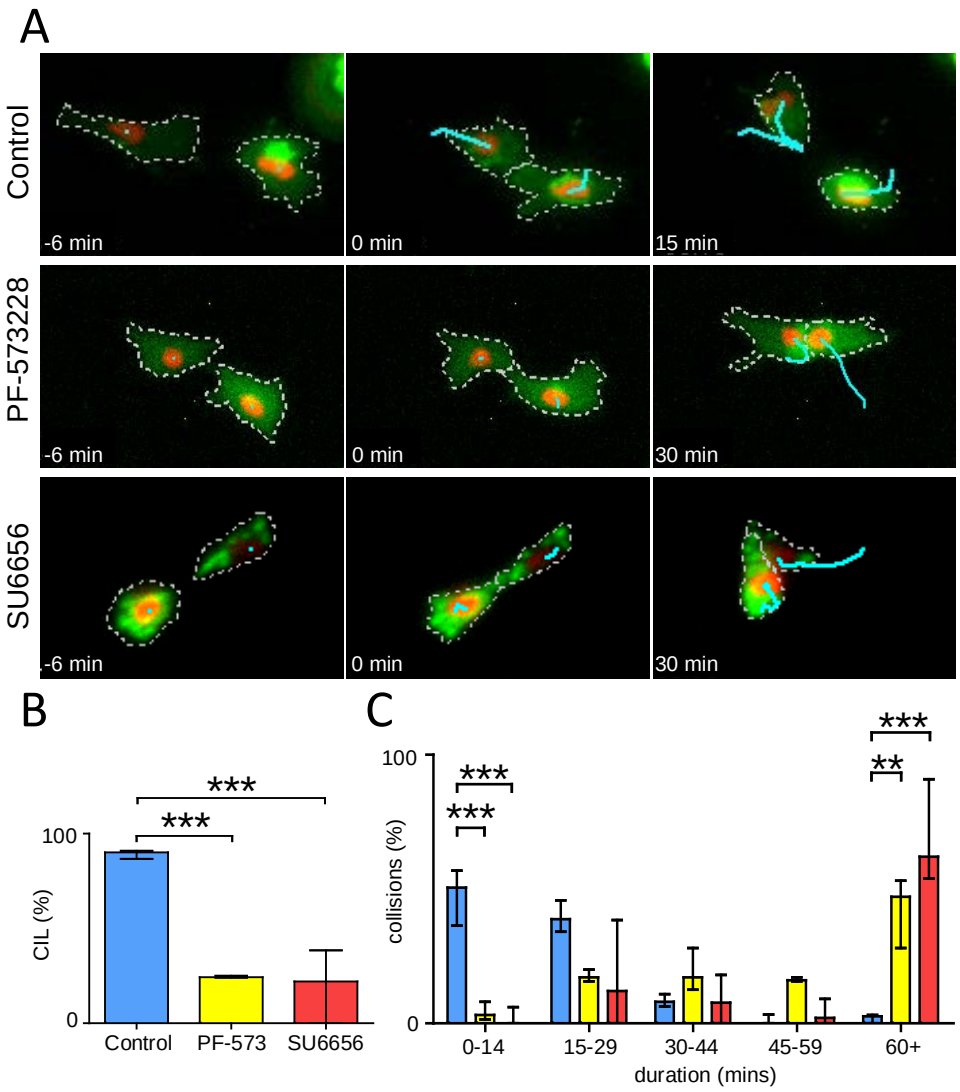

Figure 7.3 FAK-Src inhibition inhibits separation during CIL

(A) Frames from movies of colliding pairs of cells either control cells, cells treated with 2 µM of PF-573228 or cells treated with 5 µM of SU6656. Cells express membraneGFP (green) and nuclearRFP (red). Cells outlined in grey. Cell tracks in cyan. Scale bar 20 µM (B) Percentage of cells that undergo CIL within 30 minutes of colliding, n = 3 repeated experiments for PF-573228 and SU6656, number of collisions analysed: control = 250, PF-573228 = 127, SU6656 = 74. (C) Histogram showing duration of contact; 3 repeated experiments for PF-573228 and SU6656; number of collisions analysed: control = 250, PF-573228 = 127, SU6656 = 74. Bar graphs: median with 25-75 percentiles. Analysis ANOVA for B and C. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

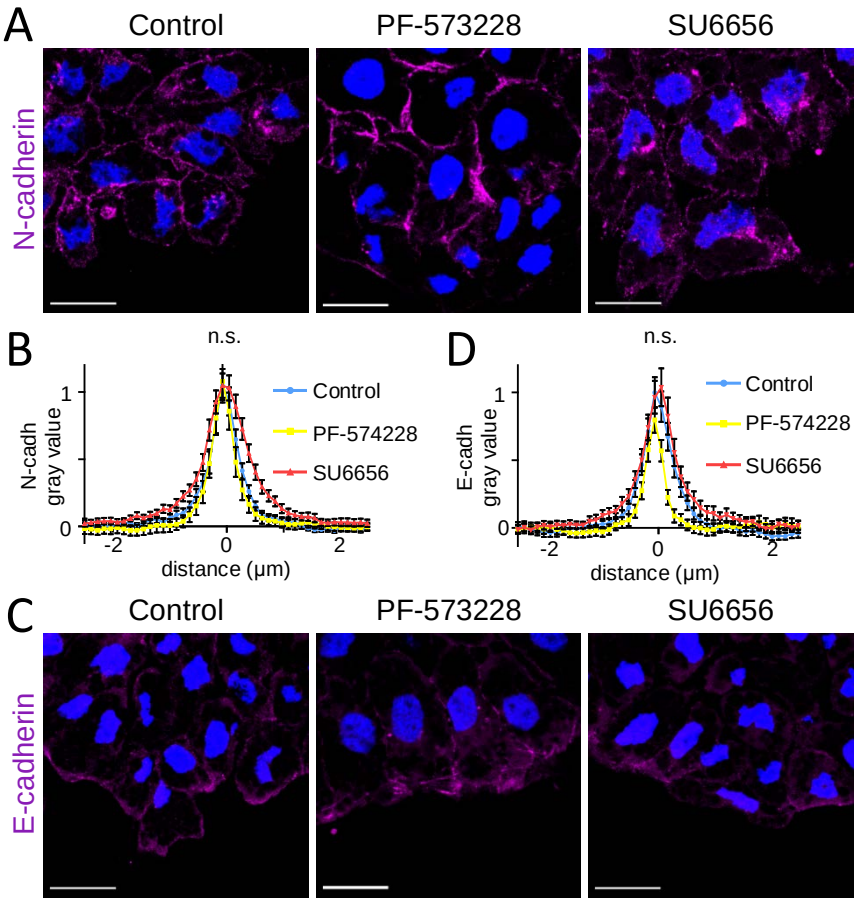

Figure 7.4 FAK-Src inhibition does not affect cadherin levels at the cell-cell contact

(A) Immunofluorescence against N-cadherin (magenta) and stained with Hoescht (blue) in control cells, cells treated with 2 µM of PF-573228 or cell treated with 5 µM of SU6656. (B) Quantification of fluorescence intensity of N-cadherin across contact with 0 µm being the centre of the contact. Values are normalized to average control at 0 µm for each repeat. Control n = 52, PF-573228 n = 52, SU6656 n = 50. (C) Immunofluorescence against E-cadherin (magenta) and stained with Hoescht (blue) in control cells, cells treated with 2 µM of PF-573228 or cell treated with 5 µM of SU6656. (D) Quantification of fluorescence intensity of E-cadherin across contact with 0 µm being the centre of the contact. Values are normalized to average control at 0 µm for each repeat. Control n = 52, PF-573228 n = 39, SU6656 n = 29. Scale bars: 20 µm. Line graph: mean ± SEM. Analysis is ANOVA for B and D. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

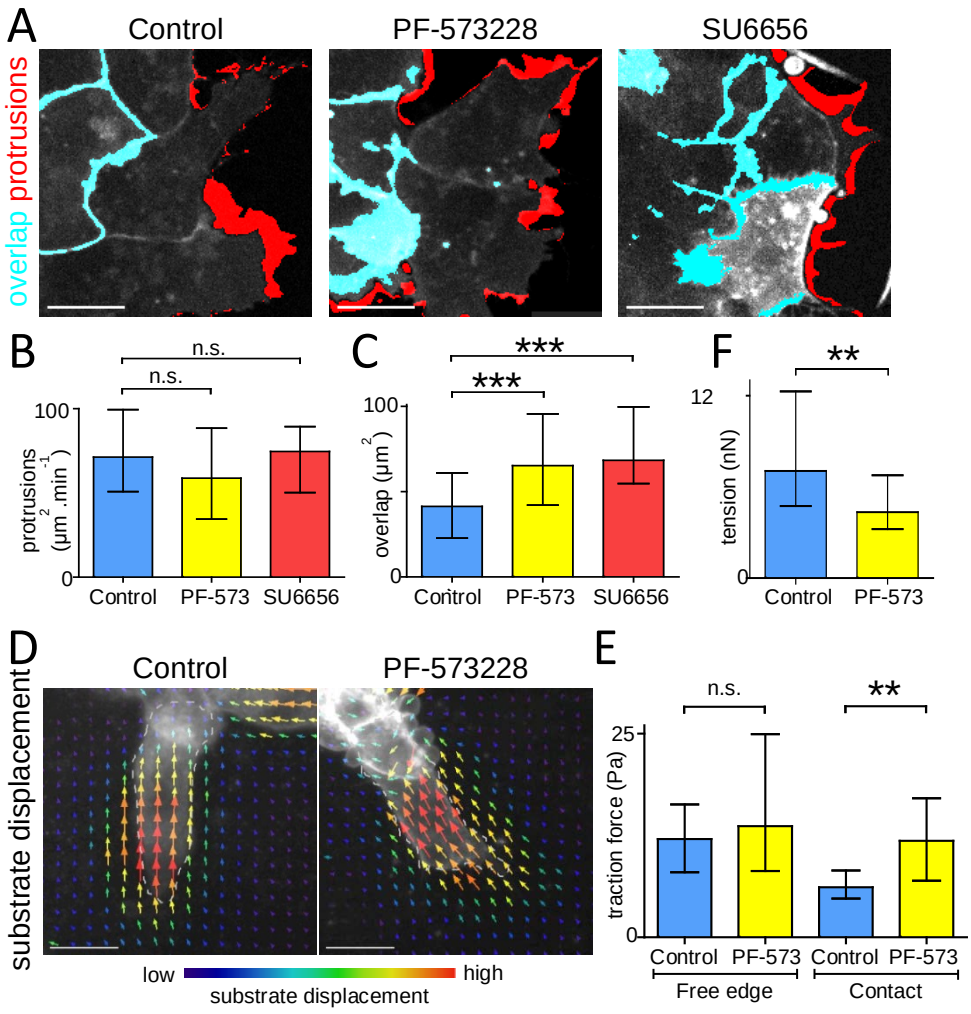

Figure 7.5 Tension is reduced across the contact in FAK inhibited cells

(A) Frames from movies of cells injected with membrane RFP (grey) overlap with extension area of protrusion per minutes (red) and overlap between cells (cyan) for control cells, cells treated with 2 µM of PF-573228 and, cells treated with 5 µM of SU6656 (B) Quantification of extension area of protrusions per minute. Control n = 62, PF-573228 n = 30, SU6656 n = 31. (C) Quantification of overlap area between cells. Control n = 111, PF-573228 n = 43, SU6656 n = 53 (D) Traction force microscopy of cells expressing membraneRFP (grey) and substrate displacement vectors colour coded to their magnitude for control and 2 µM PF-573228 treated cells (E) Quantification of traction force at the free edge and at the contact. n = 24 cells for both conditions. (F) Quantification of tension across the contact inferred from the balance of traction at free edge with the traction near the contact and tension across the contact. Control

n=39, PF-573228 n=18. Scale bars: 20 µm. Bar: median, Error: 25-75 percentiles. Analysis was Kruskal-Wallis test for B, C. Mann-Whitney test for E, F. *** = p≤0.001, ** = p≤0.01, * = p≤0.05.

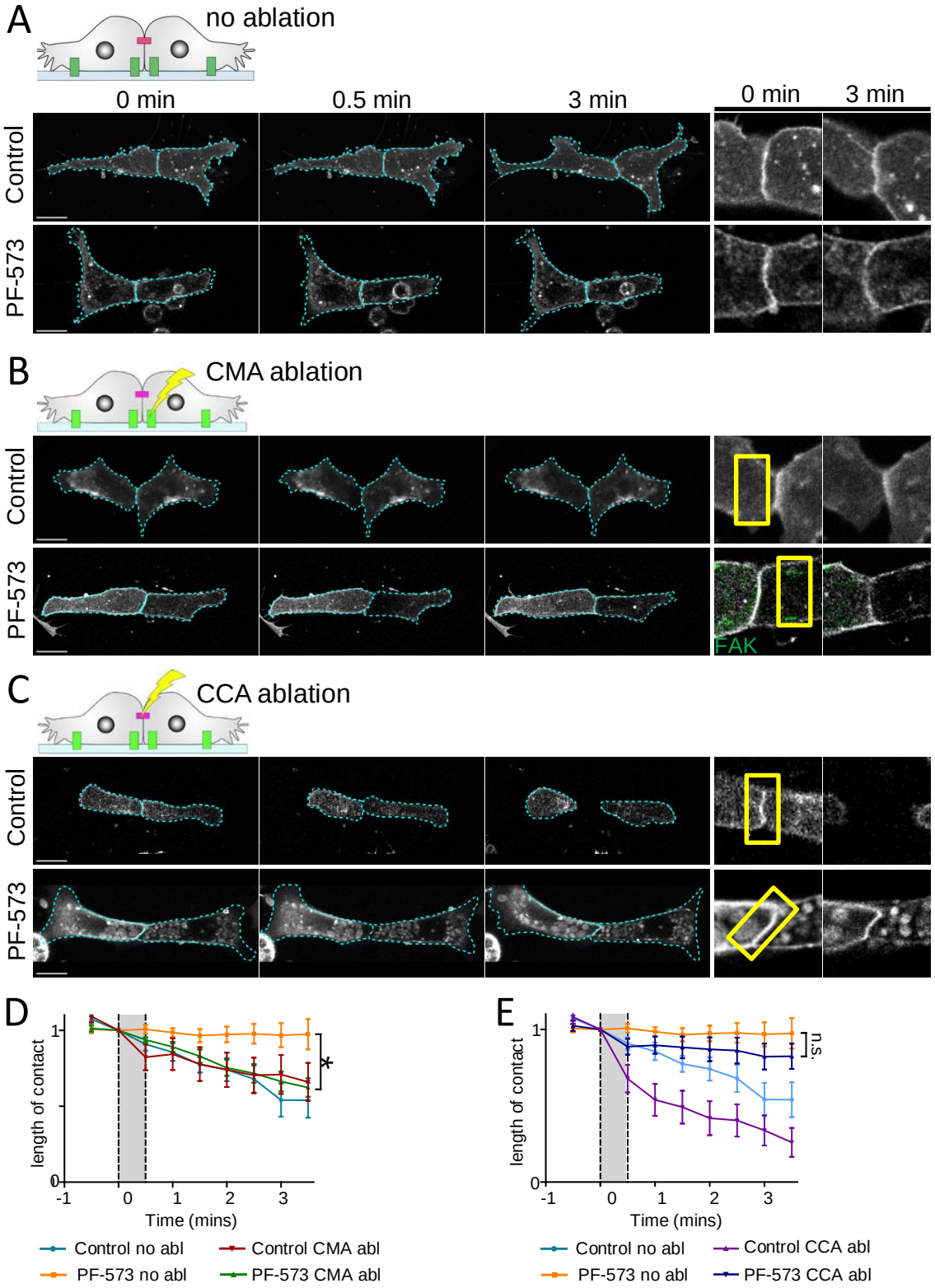
A
no ablation
0 min
0.5 min
3 min
0 min
3 min
Control
PF-573
B
CMA ablation
Control
PF-573
FAK
C
CCA ablation
Control
PF-573
D
length of contact
Time (mins)
Control no abl
Control CMA abl
PF-573 no abl
PF-573 CMA abl
*
E
length of contact
Time (mins)
Control no abl
Control CCA abl
PF-573 no abl
PF-573 CCA abl
n.s.

Figure 7.6 Disassembly of cell-matrix adhesions at the contact is required for separation during CIL

(A-C) Frames from movies after collisions, either control cells or 2 µM PF-573228 treated cells not ablated (A), ablated at the CMA near the contact (B) or ablated at the cell-cell contact (C). Cells expressing membraneRFP (grey) and GFP-FAK (green in zoom). Cells are outlined in cyan. Zoom of cell-cell contact. Ablation area marked with a yellow box on zoom. (D) Length of the cell-cell contact relative to the start of laser ablation (0 min). Grey area shows time of laser ablation. n = 15 for both no ablation conditions, Control CMA ablation n = 15, PF-573228 CMA ablation n = 18. (E) Length of the cell-cell contact relative to the initiation of laser ablation (0 min). Grey area corresponds to laser ablation. n = 15 for all conditions. Scale bars 20 µm. Line graphs show mean ± SEM. Kruskal-Wallis test. *** = $p \leq 0.001$, ** = $p \leq 0.01$, * = $p \leq 0.05$.

4. Discussion

4.1 Overview of results

CIL is a dynamic multi-faceted process that drives the inhibition of protrusions at the site of contact between cells and promotes the formation of protrusions away from the contact, eventually leading to the separation of the cells. CIL is a particularly important process to study as it is involved in multiple developmental events including the dispersion of haemocytes (Davis et al., 2012), the directed distribution of Cajal-Retzius neurons throughout the cortex (Villar-Cerviño et al., 2013) and the collective migration of the NC (Carmona-Fontaine et al., 2008), the system used here to study CIL. Furthermore CIL has been identified in cancer and its loss towards healthy tissue has been implicated in metastasis (Batson et al., 2013; Batson et al., 2014; Lin et al., 2015; Parish et al., 1987; Tanaka et al., 2012). CIL is a complex process due to the multiple steps involved and the many different molecular mechanisms and components that contribute to this migratory phenomenon (see chapter 1.3 and (Roycroft and Mayor, 2016). The work presented here was carried out in order to try and elucidate the sequence of events during CIL and establish the importance of CMAs during this process.

The dynamic sequence of the behaviour of CMAs during CIL has been identified as follows (Fig. 8.1):

1) Free migrating NC cells exhibit large elongated CMAs at their leading edge. The CMAs at the trailing edge of the cell appear smaller and are not as elongated as those at the leading edge. The traction forces generated at the front and rear of a single migrating cell are balanced.
2) During CIL, when two NC cells collide, functional N-cadherin based cell-cell adhesions rapidly form between the colliding cells. This results in increased Src activity at the contact.
3) Active Src phosphorylates FAK at the CMAs leading to a fully active FAK-Src complex enhancing the rate of disassembly of the CMAs at the contact. This leads to an overall downregulation of CMAs near the site of contact and reduced traction in this region, redirecting any tension generated across the cell to the cell-cell contact.

4) New protrusions stabilised by elongated CMAs form away from the contact generating traction in this region and lead to increased tension across the cell-cell contact.
5) The cells migrate away from each other, the cell-cell adhesion breaks down and the cells separate.

The results presented here have helped demonstrate a redistribution of the intracellular forces from the CMAs to the CCAs upon a collision. Furthermore this redistribution appears to be essential for cell separation during CIL.

4.1.1 Loss of CMAs near the contact result in tension across the cell-cell contact required for cell separation

The work presented here demonstrates the loss of CMAs at the contact leads to the redistribution of tension to the cell-cell contact and this redistribution is required to drive cell separation during CIL. Upon publishing his initial work characterising CIL, Abercrombie, the hugely influential cell and developmental biologist, speculated on the potential role and importance of the dynamic regulation of CMAs upon a collision during CIL (Abercrombie, 1970a; Abercrombie and Ambrose, 1958). He proposed that adhesion to the substrate may be lost in the contacting lamellae upon a collision and this loss may be leading to the tension observed across the cell-cell contact. Abercrombie investigated this hypothesis in chick heart embryonic fibroblasts using interference reflection microscopy (IRM) (Abercrombie and Dunn, 1975). Images generated using IRM show regions where the cell membrane is closest to the coverslip as dark patches and has historically has been used to infer the presence of CMAs at these regions (Curtis, 1964). Abercrombie concluded that CMAs in fact persist near the site of contact during CIL and therefore their dynamic behaviour cannot be responsible for the tension generated in the contacting lamellae (Abercrombie and Dunn, 1975). A conflicting observation however was demonstrated by Harris using a micromanipulation technique alongside live-imaging to reveal the points where cells were attached to the substrate (Harris, 1973). Harris concluded ‘the intercellular adhesion does not come under tension until the contacting lamella has been detached entirely from the substratum, so that the only remaining retraction fibres are between

the two cells. Thus it appears that the contacting cell gradually transfers its adhesion from the substratum to the contacted cell'. These conflicting results have not be re-examined in the 40 years since, hence the dynamic regulation and role of CMAs during CIL remains unknown. The work presented here was carried out to try and address these conflicting results and has demonstrated that CMAs are rapidly lost upon a collision, thus supporting Harris' conclusion. Furthermore a transfer of tension seems to be occurring from the substrate to the cell-cell contact as the CMAs near the contact are lost, further supporting Harris' observations. In addition it appears that this increase in tension across the cell-cell contact is required for cell separation as FAK inhibited cells, where CMAs are stabilised near the contact and tension is reduced across the cell-cell contact, cannot separate after a collision. As discussed in the introductory chapter 1.3, the idea that tension across the cell-cell contact could be involved in inducing cell separation during CIL has been discussed for a long time (Abercrombie and Ambrose, 1958; Abercrombie and Dunn, 1975; Davis et al., 2015; Harris, 1973; Heaysman and Pegrum, 1973a; Roycroft and Mayor, 2015). How tension is generated across the cell-cell contact during CIL in the NC remains unclear. Recent work investigating CIL in haemocytes in *Drosophila* demonstrated the presence of tension across the cell-cell contact and the role of stress fibre formation and actin retrograde flow in generating tension and promoting cell separation after a collision (Davis et al., 2015). It is possible a similar mechanism is at play in the NC. It is understood that large CMAs reduce the rate of actin retrograde flow (Alexandrova et al., 2008). One possible cause for the reduced tension observed across the cell-cell contact in NC cells treated with the FAK inhibitor, where CMAs persist near the contact, is due to large CMAs reducing actin retrograde flow in this region resulting in a decrease in actin pulling at the contact and consequently reduced tension generation across the cell-cell contact. Further to the idea that actin retrograde flow or stress fibre formation could be contributing to tension across the contact, previous work in the NC demonstrated the requirement for repolarisation away from the contact and generation of traction forces in the free edge in order to induce the disassembly of the CCA and cell separation (Scarpa et al., 2015). As cells have to balance any traction generated across the cell (Maruthamuthu et al., 2011), it can be speculated that the increased traction at the free edge may be counter-balanced by the cell-cell contact as

tension. If CMAs persist near the contact, as is the case with FAK inhibited cells, they could act to counteract the traction generated at the free edge and alleviate some on the tension across the cell-cell contact. In agreement with this idea, NC cells overexpressing E-cadherin, which do not separate after a collision, show reduced protrusion formation activity, and consequently reduced traction, at the free edge and increased CMAs and traction near the contact (Scarpa et al., 2015). However, activating Rac1 activity in the free edge, with the use of a photoactivatable Rac1 construct, generates protrusive activity in the free edge and induces cells separation (Scarpa et al., 2015). These results, taken together with the work presented here, demonstrate the requirement of tension across the cell-cell contact to induce cell separation. How tension is stimulating separation however is still unknown. Due to the rapid nature of separation during CIL in fibroblasts, Abercrombie speculated that the build-up of tension across the cell-cell contact was sufficient to break the adhesion between cells (Abercrombie and Ambrose, 1958). However, it is also possible that increased tension across the CCA triggers an event resulting in their disassembly through an induced signalling event. For example it is known that p120-catenin regulates the internalisation of cadherin by inhibiting clathrin-dependent endocytosis through its ability to bind to the cadherin cytoplasmic tail (Chiasson et al., 2009; Xiao et al., 2005). It is possible that tension across the contact could disrupt the interaction between p120-catenin and cadherin resulting in their endocytosis. The function of p120-catenin is heavily regulated by phosphorylation (Alemà and Salvatore, 2007). It is known applying force to CCAs can induce conformational changes in the proteins (Ladoux et al., 2015; Yao et al., 2014). It is therefore possible that increased tension across the cell-cell contact is needed to induce a conformational change in proteins in the CCA exposing a binding or phosphorylation site that consequently stimulates the disassembly or internalisation of the cadherin complex, such as the phosphorylation of p120-catenin. During CIL it has previously been demonstrated that the activation of RhoA and suppression of Rac1 at the contact is required for CIL (Carmona-Fontaine et al., 2008; Kadir et al., 2011; Matthews et al., 2008; Scarpa et al., 2015). When CMAs are stablished near the cell-cell contact and tension across the contact is low it appears that protrusions still form at the contact as there is an increase in membrane overlap between cells. It is possible tension may be required across the contact in order to

elevate the RhoA levels at the contact required to collapse cell protrusions and induce cell separation. When CMAs persist near the contact, their presence may be supressing RhoA, potentially through the activation of p190RhoGAP whose activity is known to be regulated by CMAs (Arthur et al., 2000), leading to elevated protrusion activity at the contact not seen in control cells where CMAs are disassembled.

4.1.2 Src activity and recruitment to N-cadherin

One interesting finding demonstrated by the work presented here is the recruitment and activation of Src downstream of N-cadherin in the neural crest. The recruitment and activation of Src downstream of cadherins has previously been demonstrated in other system (Leonard et al., 2013; McLachlan et al., 2007; Tsukita et al., 1991; Veracini et al., 2015). For example Src has been shown to localise to adherens junctions in hepatocytes and keratinocytes (Tsukita et al., 1991) and more recently in embryonic lens epithelial explants Src was shown to co-immunoprecipitate with N-cadherin where it is required to prevent N-cadherin junction maturation (Leonard et al., 2013). In addition Src has been shown to be recruited to E-cadherin junctions where its recruitment results in a downstream Src signalling event that appears to be involved in the regulation of the integrity of E-cadherin based adhesions (McLachlan et al., 2007; Veracini et al., 2015). How Src is recruited to cadherins and activated downstream of them is not entirely clear. It appears that the β-catenin binding region of the cadherin cytoplasmic tail plays a role in Src activation as mutants that lack the this region do not activate Src (McLachlan et al., 2007). Interestingly mutants that lack the ability to bind p120-catenin show similar levels of Src activation as controls (McLachlan et al., 2007). This suggests a possible role for β-catenin in the activation of Src downstream of cadherins. In addition it appears that upstream protein tyrosine phosphatase activity is required for the localisation and activation of Src to CCAs (Gomez et al., 2015; McLachlan and Yap, 2011; Truffi et al., 2014). A recent study in carcinoma cell lines suggests the recruitment of active Src to E-cadherin-based adhesions is regulated by the core fucosylation of E-cadherin (Shao et al., 2016). In addition the desmosomal cadherin Desmoglein 3 can form a complex with E-cadherin and recruit Src to the cell-cell junction and positively regulate its activity (Wan et al., 2016). It has recently been demonstrated that Desmoglein 3 and inactive Src

competitively bind to the scaffolding protein caveolin-1 (Wan et al., 2016). When Src is bound to caveolin-1 it is held in an inactive conformation (Li et al., 1996). Desmoglein 3 competes with inactive Src for binding to caveolin-1 thus releasing Src leading to its auto-activation (Wan et al., 2016). The work carried out in this thesis has demonstrated the recruitment and activation of Src is N-cadherin dependent in the NC; when N-cadherin was inhibited Src activity was reduced. However how Src is recruited to cell-cell contact by N-cadherin and activated by it is unclear; some of the mechanisms discussed above could be involved.

Interestingly, when E-cadherin is overexpressed in NC cells, active Src is reduced at the contact compared to controls. This observation raises two questions: first why levels of active Src at the contact differ between NC cells expressing endogenous levels of N-cadherin and those overexpressing E-cadherin, and second why this result appears contradictory to previously published results demonstrating Src is recruited and activated downstream of E-cadherin in epithelial cells (Gomez et al., 2015; McLachlan and Yap, 2011; Truffi et al., 2014). The fact that N- and E-cadherin can have a differential effect in the NC has previously been demonstrated (Scarpa et al., 2015). Whereas the presence of N-cadherin leads to a reduction in Rac1 activity at the contact, the overexpression of E-cadherin results in elevated Rac1 activity at the contact. Interestingly, elevated Rac1 activity is no longer observed when E-cadherin mutants unable to interact with p120-catenin are overexpressed instead (Ciesiolka et al., 2004; Scarpa et al., 2015). This suggests the differential Rac1 activation downstream of E- and N-cadherin is due to the way they interact with p120-catenin. Whether p120-catenin is playing a similar divergent role in the recruitment of active Src to the contact downstream of E- and N-cadherin in the NC is unknown. However, as p120-catenin is also a target for Src (Reynolds et al., 1994), it would be of interest to explore this further. Another possibility for the contrasting activation of Src downstream of E- and N-cadherin in the NC could be through their differential effects on FGF-signalling (Wheelock et al., 2008). It has been demonstrated that N-cadherin can initiate both FGF-receptor dependent signalling (Utton et al., 2001) and FGF-ligand dependent signalling (Suyama et al., 2002). By contrast, E-cadherin has been shown to suppress FGF-signalling (Bryant et al., 2005). As FGF-2 has been implicated in Src

activation and consequent CMA disassembly, (Kanda et al., 2006), it is possible that FGF signalling is playing an important role in the activation of Src downstream of N-cadherin, but not E-cadherin, in the NC. In order to investigate how active Src is recruited to N-cadherin, but not E-cadherin, in the NC, Fluorescence Covariance Analysis could be used to establish where and when Src binds to the adherens junction complex and could give a clear indication as to the proteins required in the complex to allow Src recruitment and activation (Vedula et al., 2016).

Why active Src is reduced downstream of E-cadherin in the NC when it has previously been demonstrated that Src can be activated downstream of E-cadherin in epithelial cells is unclear (Gomez et al., 2015; McLachlan and Yap, 2011; Truffi et al., 2014). One possibility is that the role of E-cadherin is cell type specific. The migratory NC are mesenchymal; as the NC undergo EMT they switch from expressing E-cadherin to predominantly expressing N-cadherin with very low endogenous levels of E-cadherin (Dady et al., 2012; Rogers et al., 2013; Scarpa et al., 2015). It is possible that due to the mesenchymal nature of the NC they lack the components involved in the activation of Src downstream of E-cadherin that epithelial cells possess. For example Src activation downstream of E-cadherin in epithelial cells relies on the transmembrane receptor protein tyrosine phosphatase RPTPα (Truffi et al., 2014). As cells undergo EMT a decrease in specific phosphatases, correlating to the decrease in E-cadherin, can occur (Fan et al., 2015). It is possibly migratory NC cells, which have already undergone EMT, lack the specific phosphatases required to activate Src downstream of E-cadherin. Whether this is the case however is unknown. Further investigation would need to be carried in order to understand the difference in Src activity observed downstream of E-cadherin in NC and epithelial cells.

4.1.3 FAK-Src activity results in CMA disassembly

The work presented here demonstrated that an increase in CMA disassembly occurred near the point of contact upon a collision in the NC. The requirement of both Src and FAK activity for CMA disassembly near the cell-cell contact has also been highlighted. When either Src of FAK kinase activity was perturbed the CMAs near the cell-cell contact increased and became more stable. The importance of Src and FAK in driving

CMA disassembly has been known for some time. The transformation of fibroblasts with v-Src leads to a decrease in CMAs in terms of size and number (Nakamura et al., 1993). Conversely, fibroblasts expressing kinase defective v-Src mutants have abnormally large CMAs (Fincham and Frame, 1998) and fibroblasts taken from FAK deficient mice show reduced motility and an increase in stress fibre associated CMAs (Ilić et al., 1995). Keratinocytes lacking FAK show reduced rates of CMA disassembly (Schober et al., 2007). Furthermore an increase in CMA turnover stimulated by brain-derived neurotrophic factor appears to be dependent upon FAK activity in growth cones (Myers and Gomez, 2011). In addition localised increases in Ca^{2+} at CMAs increases FAK and results in increased CMA disassembly (Giannone et al., 2004). Together these results demonstrate a role for Src and FAK in regulating CMA disassembly. In addition a large body of evidence suggests that FAK and Src work together to regulate CMA disassembly, hence the similarities in CMA behaviour when either is inhibited. The autophosphorylation of FAK on Tyr397 residue creates a binding site for Src at CMAs (Xing et al., 1994). Src further phosphorylates FAK resulting in a fully active FAK-Src complex at the CMAs (Calalb et al., 1995; Owen et al., 1999), thus FAK and Src act together in the regulation of CMA disassembly. How Src and FAK work together to promote CMA disassembly is not entirely clear although there is some evidence to suggest potential mechanisms through which they may be working. One mechanism for which there is evidence of FAK-Src signalling inducing CMA disassembly is through their ability to regulate the small GTPases, predominantly Rac1 and RhoA, and consequently control the actin network and actomyosin contractility. Fibroblasts from FAK deficient mice demonstrate a decreased rate of CMA disassembly and show elevated levels of Rho activity (Ren et al., 2000). Furthermore the overexpression of Rho mirrors the phenotype of loss of FAK with an increase in CMAs and a reduced rate of CMA disassembly. Reversely inhibition of Rho increases the rate of CMA disassembly (Ren et al., 2000). Therefore the rate of CMA disassembly and Rho activity appear to be inversely related. It appears that Src activity may be modulating Rho as cells that expressing Src show a transient decrease in Rho activity upon RGD stimulation to mimic integrin binding (Arthur et al., 2000). However, when Src activity is inhibited, Rho activity is no longer decreased upon RGD stimulation. Src can regulate Rho activity through the activation of p190RhoGAP, a deactivator of Rho (Fincham et

al., 1999). Upon RGD treatment p190RhoGAP is tyrosine phosphorylated and shows increased Rho-directed GAP activity in a manner dependent on Src (Arthur et al., 2000) and in cells lacking FAK p190RhoGAP is no longer activated at the CMAs (Schober et al., 2007). There is evidence that the effect Rho has on CMA disassembly may be through the activation of Rho-associated kinase (ROCK) and subsequent actomyosin contractility the myosin light chain (MLC) activation (Chen et al., 2002; Schober et al., 2007). Cells lacking FAK show elevated active MLC and the inhibition of ROCK and actomyosin contractility can rescue the impaired cell spreading and increased CMAs seen in FAK deficient cells (Chen et al., 2002; Schober et al., 2007).

As well as their role in regulating the actin cytoskeleton, FAK-Src signalling may be driving CMA disassembly though other means. The phosphorylation by Src on the Tyr925 residue of FAK drives CMA disassembly. Growth cones expressing Y925F FAK show a reduced rate of CMA turnover (Myers and Gomez, 2011), as do a colon cancer cell line where Src is inhibited preventing Tyr925 phosphorylation (Brunton et al., 2005). Phosphorylation of Tyr925 on FAK creates a binding site for the adapter protein, growth factor receptor-bound protein 2 (Grb2) (Mitra et al., 2006; Schlaepfer and Hunter, 1996). As mentioned in the introductory chapter, microtubules have been implicated in CMA disassembly (Ezratty et al., 2005; Kaverina et al., 1999; Krylyshkina et al., 2002). Grb2 binding to Tyr925 on FAK has been shown to recruit the GTPase dynamin to CMAs forming a Grb2-dynamin-FAK complex, required for microtubule driven CMA disassembly (Ezratty et al., 2005). Furthermore the direct interaction between FAK and dynamin results in it becoming phosphorylated by Src and activated, another step shown to be essential for CMA disassembly (Wang et al., 2011). Dynamin is responsible for driving endocytosis and it appears its ability to mediate the endocytosis of integrins is driving the disassembly of CMAs downstream of FAK-Src (Wang et al., 2011). In addition to its importance in driving dynamin recruitment to CMAs, Grb2 binding may also be an important activator of the MAPK/ERK pathway (Schlaepfer et al., 1994) and may be stimulating CMA disassembly through this pathway (Webb et al., 2004). Fibroblasts where MAP kinase kinase-1 activation is blocked, either by an inhibitor or by the use of a dominant negative, show a decreased rate of CMA disassembly (Webb et al., 2004). Furthermore the expression of

constitutively active MAP kinase kinase-1 can rescue the rate of CMA disassembly in cells lacking FAK activity (Webb et al., 2004), suggestive of a role for MAPK/ERK signalling downstream of FAK in CMA disassembly. Melanoma cells where MAPK/ERK signalling is impaired have defects in CMA disassembly (Coló et al., 2012). It is known that ERK can localise to CMAs and promote myosin light chain kinase (MLCK) activation and consequently actomyosin contractility (Fincham et al., 2000; Klemke et al., 1997). Interestingly cells treated with MLCK inhibitors show a decrease in the rate of CMA disassembly (Webb et al., 2004). Together these results suggest that the Src dependent phosphorylation of FAK on Tyr925 recruits Grb2 which in turn activates MAPK/ERK signalling potentially leading to the activation of MLCK and consequently actomyosin driven contractility resulting in the disassembly of CMAs. In addition to the activation of the MAPK/ERK pathway through Grb2, the FAK-Src complex formation at the CMAs also results in the phosphorylation of paxillin (Mitra et al., 2005; Scheswohl et al., 2008) which in turn leads to the localised activation of the MAPK/ERK pathway (Coló et al., 2012; Fincham et al., 2000; Ishibe et al., 2003). MAPK/ERK signalling can activate the protease Calpain 2, which has also been shown to promote the disassembly of CMA (Glading et al., 2000). In addition FAK has been shown to directly recruit Calpain 2 to CMAs, in a manner dependent of Src activity (Carragher et al., 2003). Calpain activation at the CMAs drives the proteolysis of CMA proteins such as FAK, talin and integrin itself, consequently leading to CMA disassembly (Bhatt et al., 2002; Chan et al., 2010; Flevaris et al., 2007; Franco et al., 2004).

The mechanisms that are responsible for driving the FAK-Src dependent CMA disassembly upon a collision in NC cells are unknown. It is likely to involve some of the pathways discussed above, although as it stands there is no evidence to support or negate any particular pathway at this time. It would be of interest to see whether MAPK/ERK signalling is playing a role downstream of FAK-Src at the CMAs and investigate further the behaviour of the small GTPases and their potential effect on CMA disassembly upon a collision during CIL between NC cells. In addition Src activity at E-cadherin based adhesions has been shown to activate myosin II and generate contractile forces (Gomez et al., 2015). Whether Src is playing a role in force

generation independent of its role in regulating CMA disassembly in the NC is of interest and would need to be investigated further.

As discussed in the introductory chapter 1.1., CMAs can be classified into various forms. The short lived focal complexes develop in the leading edge of cells and can be characterised by high levels of tyrosine phosphorylated paxillin and low levels of zyxin (Zaidel-Bar et al., 2004; Zaidel-Bar et al., 2007). Focal complexes only exist for a few minutes before either disassembling or developing into the more mature focal adhesions. Focal adhesions are associated with actin stress fibres and can be identified by high levels of tyrosine phosphorylated proteins, zyxin and low levels of tensin (Zaidel-Bar et al., 2004). Under increased actomyosin contractility focal adhesions can mature into the large and more stable fibrillar adhesions (Bershadsky et al., 2003). Fibrillar adhesions bind to fibronectin via integrin α5 β1 and are easily identified by the presence of tensin and only low levels of tyrosine phosphorylated protein (Pankov et al., 2000; Zamir et al., 2000). The exact composition and nature of the CMAs identified in the work presented here is unclear; further investigation is required to uncover this and establish how it may change over time.

4.1.4 Future perspectives

The work presented in this thesis has highlighted a functional link between N-cadherin at the cell-cell contact, the activation of Src and the localised FAK-Src driven disassembly of CMAs during CIL in the NC. However, the exact mechanism by which N-cadherin is activating Src and how FAK-Src activity is stimulating CMA disassembly remains unclear. Further investigation is needed to determine these mechanisms. It is well established that CIL plays vital roles *in vivo* (Stramer et al., 2013), including driving haemocyte dispersion in *Drosophila* (Davis et al., 2012) and contributing to the migration of the NC (Carmona-Fontaine et al., 2008). The haemocytes in the haemocoel of *Drosophila* provide an excellent opportunity to image cells undergoing CIL *in vivo* (Davis et al., 2012; Davis et al., 2015; Stramer et al., 2010). To determine whether the inhibition of Src or FAK stabilises CMAs *in vivo* and if so whether this perturbs cell separation during CIL in the embryo the same as *in vitro*, Src or FAK could be perturbed in haemocytes with the use of dominant negative mutants. Furthermore

NC cells can be imaged undergoing CIL *in vivo* in Zebrafish (Carmona-Fontaine et al., 2008; Scarpa et al., 2015). Dominant negative Src or FAK could be expressed in the NC of Zebrafish to see whether their loss perturbs CIL and the migration of the NC *in vivo*. It would be of interest to establish whether the *in vitro* results demonstrated in this thesis can be mirrored within the developing embryo.

4.2 Concluding remarks

In conclusion, the results presented here demonstrate that the separation of cells during CIL requires the disassembly of CMAs near the contact upon a collision in order to the build-up of tension across the cell-cell contact. The downregulation of CMAs is dependent upon N-cadherin at the contact and its ability to activate Src and subsequently FAK. Cells lacking either Src or FAK kinase activity do not separate during CIL due to the stabilisation of CMAs near the contact which in turn increase traction on the substrate at this site and reduces tension across the contact. Although this body of work has answered many questions regarding the nature, regulation and importance of CMAs during CIL, many questions remain unanswered such as the exact mechanism through which N-cadherin is activating Src, how FAK-Src signalling is driving the disassembly of the CMAs upon a collision and how tension across the contact is inducing the separation of the cells. CIL occurs between many different cell types and contributes to different morphological processes. The mechanism proposed here sheds a new light on the redistribution of forces during CIL, an important insight due to the role CIL plays in development and cancer metastasis.

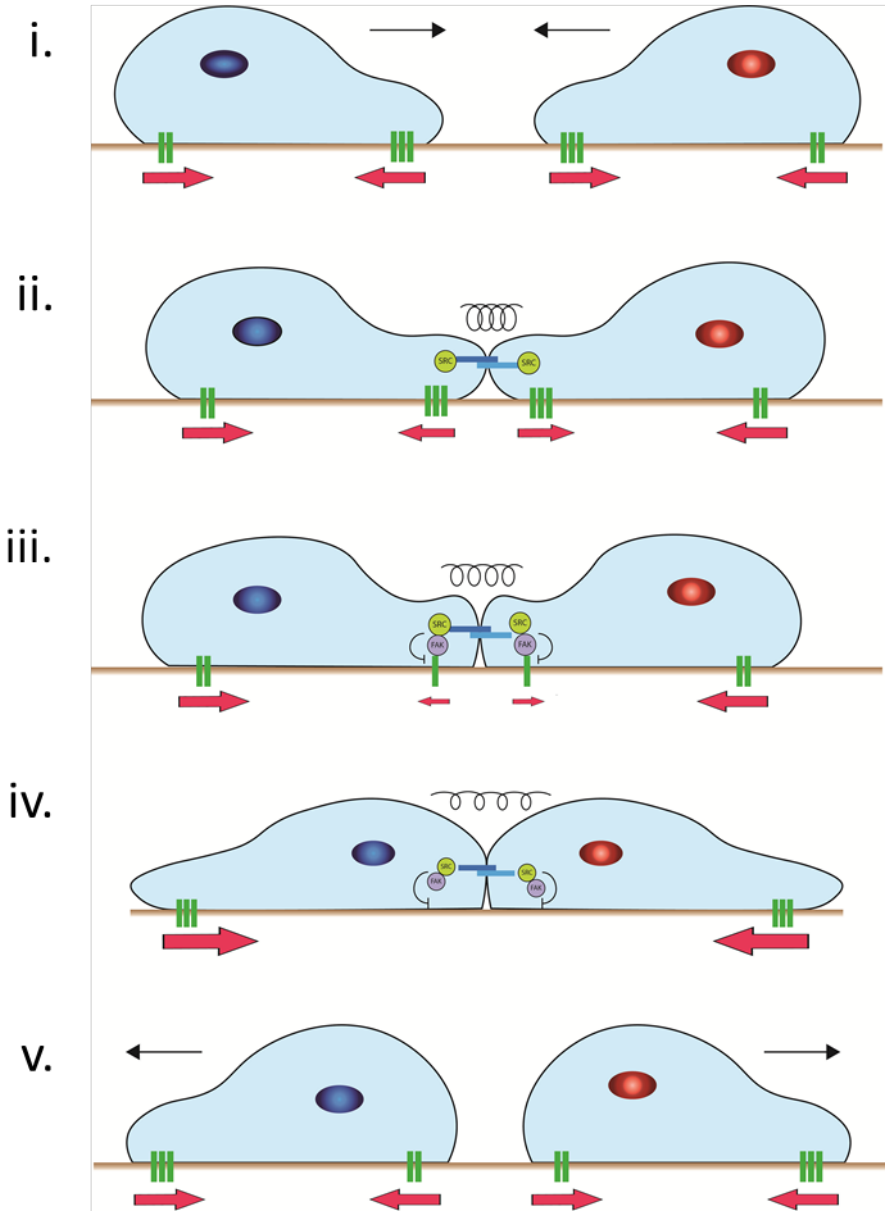

Figure 8.1 Model summarising results

Schematic of results. Traction forces: pink arrows; cell-cell adhesion, blue; cell-matrix adhesions, green; tension, coil; direction of migration, thin black arrows. (i) single cells migrate with large CMAs in their leading edge, smaller CMAs in the trailing edge, traction forces are balanced across the cell. (ii) Cells collide and a N-cadherin based cell-cell adhesion forms between the cells. The presence of N-cadherin results in the activation of Src at the cell-cell contact (iii) Src phosphorylates and fully activates FAK at the CMAs. Consequently CMAs are disassembly and the contact and traction forces are reduced at near the cell-cell contact. The loss of CMAs results in the transfer of tension to the cell-cell contact. (iv) Cells repolarise away from the contact forming

large CMAs and generating increased traction forces in this area, most likely leading to increased tension across the cell-cell contact. (v) Cells separate, traction is reduced at front and increased at rear of cell so it once again becomes balanced.

References

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

Abercrombie, M. 1970b. Control mechanisms in cancer. *Eur J Cancer*. 6:7-13.

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

Abercrombie, M., and E.J. Ambrose. 1958. Interference microscope studies of cell contacts in tissue culture. *Exp Cell Res*. 15:332-345.

Abercrombie, M., and G.A. Dunn. 1975. Adhesions of fibroblasts to substratum during contact inhibition observed by interference reflection microscopy. *Exp Cell Res*. 92:57-62.

Abercrombie, M., and J.E. Heaysman. 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., and J.E. Heaysman. 1954a. Invasiveness of sarcoma cells. *Nature*. 174:697-698.

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

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

Aberle, H., S. Butz, J. Stappert, H. Weissig, R. Kemler, and H. Hoschuetzky. 1994. Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J Cell Sci*. 107 (Pt 12):3655-3663.

Adutler-Lieber, S., I. Zaretsky, I. Platzman, J. Deeg, N. Friedman, J.P. Spatz, and B. Geiger. 2014. Engineering of synthetic cellular microenvironments: implications for immunity. *J Autoimmun*. 54:100-111.

Al-Kilani, A., O. de Freitas, S. Dufour, and F. Gallet. 2011. Negative feedback from integrins to cadherins: a micromechanical study. *Biophys J*. 101:336-344.

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

Alexandrova, A.Y., K. Arnold, S. Schaub, J.M. Vasiliev, J.J. Meister, A.D. Bershadsky, and A.B. Verkhovsky. 2008. Comparative dynamics of retrograde actin flow and focal adhesions: formation of nascent adhesions triggers transition from fast to slow flow. *PLoS One*. 3:e3234.

Alexopoulou, A.N., H.A. Multhaupt, and J.R. Couchman. 2007. Syndecans in wound healing, inflammation and vascular biology. *Int J Biochem Cell Biol*. 39:505-528.

Alfandari, D., H. Cousin, A. Gaultier, B.G. Hoffstrom, and D.W. DeSimone. 2003. Integrin alpha5beta1 supports the migration of Xenopus cranial neural crest on fibronectin. *Dev Biol*. 260:449-464.

Alfandari, D., H. Cousin, A. Gaultier, K. Smith, J.M. White, T. Darribère, and D.W. DeSimone. 2001. Xenopus ADAM 13 is a metalloprotease required for cranial neural crest-cell migration. *Curr Biol*. 11:918-930.

Ananthakrishnan, R., and A. Ehrlicher. 2007. The forces behind cell movement. *Int J Biol Sci*. 3:303-317.

Anastasiadis, P.Z., S.Y. Moon, M.A. Thoreson, D.J. Mariner, H.C. Crawford, Y. Zheng, and A.B. Reynolds. 2000. Inhibition of RhoA by p120 catenin. *Nat Cell Biol*. 2:637-644.

Anastasiadis, P.Z., and A.B. Reynolds. 2001. Regulation of Rho GTPases by p120-catenin. *Curr Opin Cell Biol*. 13:604-610.

Anear, E., and R.W. Parish. 2012. The effects of modifying RhoA and Rac1 activities on heterotypic contact inhibition of locomotion. *FEBS Lett*. 586:1330-1335.

Anthis, N.J., K.L. Wegener, F. Ye, C. Kim, B.T. Goult, E.D. Lowe, I. Vakonakis, N. Bate, D.R. Critchley, M.H. Ginsberg, and I.D. Campbell. 2009. The structure of an integrin/talin complex reveals the basis of inside-out signal transduction. *EMBO J*. 28:3623-3632.

Antoniou, A., P. Raynaud, S. Cordi, Y. Zong, F. Tronche, B.Z. Stanger, P. Jacquemin, C.E. Pierreux, F. Clotman, and F.P. Lemaigre. 2009. Intrahepatic bile ducts develop according to a new mode of tubulogenesis regulated by the transcription factor SOX9. *Gastroenterology*. 136:2325-2333.

Araki, K., T. Shimura, H. Suzuki, S. Tsutsumi, W. Wada, T. Yajima, T. Kobayahi, N. Kubo, and H. Kuwano. 2011. E/N-cadherin switch mediates cancer progression via TGF-β-induced epithelial-to-mesenchymal transition in extrahepatic cholangiocarcinoma. *Br J Cancer*. 105:1885-1893.

Arias-Salgado, E.G., S. Lizano, S. Sarkar, J.S. Brugge, M.H. Ginsberg, and S.J. Shattil. 2003. Src kinase activation by direct interaction with the integrin beta cytoplasmic domain. *Proc Natl Acad Sci U S A*. 100:13298-13302.

Arthur, W.T., L.A. Petch, and K. Burridge. 2000. Integrin engagement suppresses RhoA activity via a c-Src-dependent mechanism. *Curr Biol*. 10:719-722.

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

Avizienyte, E., V.J. Fincham, V.G. Brunton, and M.C. Frame. 2004. Src SH3/2 domain-mediated peripheral accumulation of Src and phospho-myosin is linked to deregulation of E-cadherin and the epithelial-mesenchymal transition. *Mol Biol Cell*. 15:2794-2803.

Avizienyte, E., A.W. Wyke, R.J. Jones, G.W. McLean, M.A. Westhoff, V.G. Brunton, and M.C. Frame. 2002. Src-induced de-regulation of E-cadherin in colon cancer cells requires integrin signalling. *Nat Cell Biol*. 4:632-638.

Aybar, M.J., M.A. Nieto, and R. Mayor. 2003. Snail precedes slug in the genetic cascade required for the specification and migration of the Xenopus neural crest. *Development*. 130:483-494.

Ayollo, D.V., I.Y. Zhitnyak, J.M. Vasiliev, and N.A. Gloushankova. 2009. Rearrangements of the actin cytoskeleton and E-cadherin-based adherens junctions caused by neoplasic transformation change cell-cell interactions. *PLoS One*. 4:e8027.

Balaban, N.Q., U.S. Schwarz, D. Riveline, P. Goichberg, G. Tzur, I. Sabanay, D. Mahalu, S. Safran, A. Bershadsky, L. Addadi, and B. Geiger. 2001. Force and focal adhesion assembly: a close relationship studied using elastic micropatterned substrates. *Nat Cell Biol*. 3:466-472.

Barriga, E.H., P.H. Maxwell, A.E. Reyes, and R. Mayor. 2013. The hypoxia factor Hif-1α controls neural crest chemotaxis and epithelial to mesenchymal transition. *J Cell Biol*. 201:759-776.

Barriga, E.H., and R. Mayor. 2015. Embryonic cell-cell adhesion: a key player in collective neural crest migration. *Curr Top Dev Biol*. 112:301-323.

Barthel, S.R., J.D. Gavino, L. Descheny, and C.J. Dimitroff. 2007. Targeting selectins and selectin ligands in inflammation and cancer. *Expert Opin Ther Targets*. 11:1473-1491.

Basch, M.L., M.I. García-Castro, and M. Bronner-Fraser. 2004. Molecular mechanisms of neural crest induction. *Birth Defects Res C Embryo Today*. 72:109-123.

Bass, M.D., K.A. Roach, M.R. Morgan, Z. Mostafavi-Pour, T. Schoen, T. Muramatsu, U. Mayer, C. Ballestrem, J.P. Spatz, and M.J. Humphries. 2007. Syndecan-4-dependent Rac1 regulation determines directional migration in response to the extracellular matrix. *J Cell Biol*. 177:527-538.

Batson, J., J.W. Astin, and C.D. Nobes. 2013. Regulation of contact inhibition of locomotion by Eph-ephrin signalling. *J Microsc*. 251:232-241.

Batson, J., L. Maccarthy-Morrogh, A. Archer, H. Tanton, and C.D. Nobes. 2014. EphA receptors regulate prostate cancer cell dissemination through Vav2-RhoA mediated cell-cell repulsion. *Biol Open*. 3:453-462.

Baum, B., and M. Georgiou. 2011. Dynamics of adherens junctions in epithelial establishment, maintenance, and remodeling. *J Cell Biol*. 192:907-917.

Becker, S.F., R. Mayor, and J. Kashef. 2013. Cadherin-11 mediates contact inhibition of locomotion during Xenopus neural crest cell migration. *PLoS One*. 8:e85717.

Behrens, J., L. Vakaet, R. Friis, E. Winterhager, F. Van Roy, M.M. Mareel, and W. Birchmeier. 1993. Loss of epithelial differentiation and gain of invasiveness correlates with tyrosine phosphorylation of the E-cadherin/beta-catenin complex in cells transformed with a temperature-sensitive v-SRC gene. *J Cell Biol*. 120:757-766.

Beningo, K.A., M. Dembo, I. Kaverina, J.V. Small, and Y.L. Wang. 2001. Nascent focal adhesions are responsible for the generation of strong propulsive forces in migrating fibroblasts. *J Cell Biol*. 153:881-888.

Bergert, M., A. Erzberger, R.A. Desai, I.M. Aspalter, A.C. Oates, G. Charras, G. Salbreux, and E.K. Paluch. 2015. Force transmission during adhesion-independent migration. *Nat Cell Biol*. 17:524-529.

Berginski, M.E., and S.M. Gomez. 2013. The Focal Adhesion Analysis Server: a web tool for analyzing focal adhesion dynamics. *F1000Res*. 2:68.

Berndt, J.D., M.R. Clay, T. Langenberg, and M.C. Halloran. 2008. Rho-kinase and myosin II affect dynamic neural crest cell behaviors during epithelial to mesenchymal transition in vivo. *Dev Biol*. 324:236-244.

Bershadsky, A., A. Chausovsky, E. Becker, A. Lyubimova, and B. Geiger. 1996. Involvement of microtubules in the control of adhesion-dependent signal transduction. *Curr Biol*. 6:1279-1289.

Bershadsky, A.D., N.Q. Balaban, and B. Geiger. 2003. Adhesion-dependent cell mechanosensitivity. *Annu Rev Cell Dev Biol*. 19:677-695.

Bershadsky, A.D., E.A. Vaisberg, and J.M. Vasiliev. 1991. Pseudopodial activity at the active edge of migrating fibroblast is decreased after drug-induced microtubule depolymerization. *Cell Motil Cytoskeleton*. 19:152-158.

Bhadriraju, K., M. Yang, S. Alom Ruiz, D. Pirone, J. Tan, and C.S. Chen. 2007. Activation of ROCK by RhoA is regulated by cell adhesion, shape, and cytoskeletal tension. *Exp Cell Res*. 313:3616-3623.

Bhatt, A., I. Kaverina, C. Otey, and A. Huttenlocher. 2002. Regulation of focal complex composition and disassembly by the calcium-dependent protease calpain. *J Cell Sci*. 115:3415-3425.

Blake, R.A., M.A. Broome, X. Liu, J. Wu, M. Gishizky, L. Sun, and S.A. Courtneidge. 2000. SU6656, a selective src family kinase inhibitor, used to probe growth factor signaling. *Mol Cell Biol*. 20:9018-9027.

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

Bouvard, D., C. Brakebusch, E. Gustafsson, A. Aszódi, T. Bengtsson, A. Berna, and R. Fässler. 2001. Functional consequences of integrin gene mutations in mice. *Circ Res*. 89:211-223.

Bracke, M.E., H. Depypere, C. Labit, V. Van Marck, K. Vennekens, S.J. Vermeulen, I. Maelfait, J. Philippé, R. Serreyn, and M.M. Mareel. 1997. Functional downregulation of the E-cadherin/catenin complex leads to loss of contact inhibition of motility and of mitochondrial activity, but not of growth in confluent epithelial cell cultures. *Eur J Cell Biol*. 74:342-349.

Bracke, M.E., V.S. Parmar, A.L. Depass, C.V. Stevens, B.W. Vanhoecke, and M.M. Mareel. 2014. Chick heart invasion assay. *Methods Mol Biol*. 1070:93-106.

Bradbury, P., C.T. Bach, A. Paul, and G.M. O'Neill. 2014. Src kinase determines the dynamic exchange of the docking protein NEDD9 (neural precursor cell expressed

developmentally down-regulated gene 9) at focal adhesions. *J Biol Chem*. 289:24792-24800.
Braga, V.M., and A.S. Yap. 2005. The challenges of abundance: epithelial junctions and small GTPase signalling. *Curr Opin Cell Biol*. 17:466-474.
Breau, M.A., T. Pietri, O. Eder, M. Blanche, C. Brakebusch, R. Fässler, J.P. Thiery, and S. Dufour. 2006. Lack of beta1 integrins in enteric neural crest cells leads to a Hirschsprung-like phenotype. *Development*. 133:1725-1734.
Bronner, M.E., and N.M. LeDouarin. 2012. Development and evolution of the neural crest: an overview. *Dev Biol*. 366:2-9.
Bronner-Fraser, M. 1994. Neural crest cell formation and migration in the developing embryo. *FASEB J*. 8:699-706.
Brown, N.H. 2000. Cell-cell adhesion via the ECM: integrin genetics in fly and worm. *Matrix Biol*. 19:191-201.
Brunton, V.G., E. Avizienyte, V.J. Fincham, B. Serrels, C.A. Metcalf, T.K. Sawyer, and M.C. Frame. 2005. Identification of Src-specific phosphorylation site on focal adhesion kinase: dissection of the role of Src SH2 and catalytic functions and their consequences for tumor cell behavior. *Cancer Res*. 65:1335-1342.
Bryant, D.M., F.G. Wylie, and J.L. Stow. 2005. Regulation of endocytosis, nuclear translocation, and signaling of fibroblast growth factor receptor 1 by E-cadherin. *Mol Biol Cell*. 16:14-23.
Burridge, K., and P. Mangeat. 1984. An interaction between vinculin and talin. *Nature*. 308:744-746.
Burstyn-Cohen, T., J. Stanleigh, D. Sela-Donenfeld, and C. Kalcheim. 2004. Canonical Wnt activity regulates trunk neural crest delamination linking BMP/noggin signaling with G1/S transition. *Development*. 131:5327-5339.
Burute, M., and M. Thery. 2012. Spatial segregation between cell-cell and cell-matrix adhesions. *Curr Opin Cell Biol*. 24:628-636.
Bökel, C., and N.H. Brown. 2002. Integrins in development: moving on, responding to, and sticking to the extracellular matrix. *Dev Cell*. 3:311-321.
Cadigan, K.M., and M. Peifer. 2009. Wnt signaling from development to disease: insights from model systems. *Cold Spring Harb Perspect Biol*. 1:a002881.
Cai, D., S.C. Chen, M. Prasad, L. He, X. Wang, V. Choesmel-Cadamuro, J.K. Sawyer, G. Danuser, and D.J. Montell. 2014. Mechanical feedback through E-cadherin promotes direction sensing during collective cell migration. *Cell*. 157:1146-1159.
Calalb, M.B., T.R. Polte, and S.K. Hanks. 1995. Tyrosine phosphorylation of focal adhesion kinase at sites in the catalytic domain regulates kinase activity: a role for Src family kinases. *Mol Cell Biol*. 15:954-963.
Calalb, M.B., X. Zhang, T.R. Polte, and S.K. Hanks. 1996. Focal adhesion kinase tyrosine-861 is a major site of phosphorylation by Src. *Biochem Biophys Res Commun*. 228:662-668.
Calderwood, D.A. 2004. Talin controls integrin activation. *Biochem Soc Trans*. 32:434-437.
Camand, E., F. Peglion, N. Osmani, M. Sanson, and S. Etienne-Manneville. 2012. N-cadherin expression level modulates integrin-mediated polarity and strongly impacts on the speed and directionality of glial cell migration. *J Cell Sci*. 125:844-857.
Canel, M., A. Serrels, M.C. Frame, and V.G. Brunton. 2013. E-cadherin-integrin crosstalk in cancer invasion and metastasis. *J Cell Sci*. 126:393-401.
Canel, M., A. Serrels, D. Miller, P. Timpson, B. Serrels, M.C. Frame, and V.G. Brunton. 2010. Quantitative in vivo imaging of the effects of inhibiting integrin signaling via Src and FAK on cancer cell movement: effects on E-cadherin dynamics. *Cancer Res*. 70:9413-9422.

Cantemir, V., D.H. Cai, M.V. Reedy, and P.R. Brauer. 2004. Tissue inhibitor of metalloproteinase-2 (TIMP-2) expression during cardiac neural crest cell migration and its role in proMMP-2 activation. *Dev Dyn*. 231:709-719.

Carey, D.J. 1997. Syndecans: multifunctional cell-surface co-receptors. *Biochem J*. 327 (Pt 1):1-16.

Carl, T.F., C. Dufton, J. Hanken, and M.W. Klymkowsky. 1999. Inhibition of neural crest migration in Xenopus using antisense slug RNA. *Dev Biol*. 213:101-115.

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

Carmona-Fontaine, C., E. Theveneau, A. Tzekou, M. Tada, M. Woods, K.M. Page, M. Parsons, J.D. Lambris, and R. Mayor. 2011. Complement fragment C3a controls mutual cell attraction during collective cell migration. *Dev Cell*. 21:1026-1037.

Carragher, N.O., M.A. Westhoff, V.J. Fincham, M.D. Schaller, and M.C. Frame. 2003. A novel role for FAK as a protease-targeting adaptor protein: regulation by p42 ERK and Src. *Curr Biol*. 13:1442-1450.

Carter, S.B. 1967. Haptotaxis and the mechanism of cell motility. *Nature*. 213:256-260.

Case, L.B., and C.M. Waterman. 2015. Integration of actin dynamics and cell adhesion by a three-dimensional, mechanosensitive molecular clutch. *Nat Cell Biol*. 17:955-963.

Cavalcanti-Adam, E.A., T. Volberg, A. Micoulet, H. Kessler, B. Geiger, and J.P. Spatz. 2007. Cell spreading and focal adhesion dynamics are regulated by spacing of integrin ligands. *Biophys J*. 92:2964-2974.

Chacon-Heszele, M.F., D. Ren, A.B. Reynolds, F. Chi, and P. Chen. 2012. Regulation of cochlear convergent extension by the vertebrate planar cell polarity pathway is dependent on p120-catenin. *Development*. 139:968-978.

Chaffer, C.L., E.W. Thompson, and E.D. Williams. 2007. Mesenchymal to epithelial transition in development and disease. *Cells Tissues Organs*. 185:7-19.

Chalfie, M., Y. Tu, G. Euskirchen, W.W. Ward, and D.C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science*. 263:802-805.

Chan, K.T., D.A. Bennin, and A. Huttenlocher. 2010. Regulation of adhesion dynamics by calpain-mediated proteolysis of focal adhesion kinase (FAK). *J Biol Chem*. 285:11418-11426.

Chen, B.H., J.T. Tzen, A.R. Bresnick, and H.C. Chen. 2002. Roles of Rho-associated kinase and myosin light chain kinase in morphological and migratory defects of focal adhesion kinase-null cells. *J Biol Chem*. 277:33857-33863.

Chen, C.P., S. Posy, A. Ben-Shaul, L. Shapiro, and B.H. Honig. 2005. Specificity of cell-cell adhesion by classical cadherins: Critical role for low-affinity dimerization through beta-strand swapping. *Proc Natl Acad Sci U S A*. 102:8531-8536.

Chen, W.C., and B. Obrink. 1991. Cell-cell contacts mediated by E-cadherin (uvomorulin) restrict invasive behavior of L-cells. *J Cell Biol*. 114:319-327.

Chen, X.L., J.O. Nam, C. Jean, C. Lawson, C.T. Walsh, E. Goka, S.T. Lim, A. Tomar, I. Tancioni, S. Uryu, J.L. Guan, L.M. Acevedo, S.M. Weis, D.A. Cheresh, and D.D. Schlaepfer. 2012. VEGF-induced vascular permeability is mediated by FAK. *Dev Cell*. 22:146-157.

Chen, Y.T., D.B. Stewart, and W.J. Nelson. 1999. Coupling assembly of the E-cadherin/beta-catenin complex to efficient endoplasmic reticulum exit and basal-lateral membrane targeting of E-cadherin in polarized MDCK cells. *J Cell Biol*. 144:687-699.

Cheung, M., M.C. Chaboissier, A. Mynett, E. Hirst, A. Schedl, and J. Briscoe. 2005. The transcriptional control of trunk neural crest induction, survival, and delamination. *Dev Cell*. 8:179-192.

Chiasson, C.M., K.B. Wittich, P.A. Vincent, V. Faundez, and A.P. Kowalczyk. 2009. p120-catenin inhibits VE-cadherin internalization through a Rho-independent mechanism. *Mol Biol Cell*. 20:1970-1980.

Chilton, J.K., and S. Guthrie. 2003. Cranial expression of class 3 secreted semaphorins and their neuropilin receptors. *Dev Dyn*. 228:726-733.

Choi, C.K., M. Vicente-Manzanares, J. Zareno, L.A. Whitmore, A. Mogilner, and A.R. Horwitz. 2008. Actin and alpha-actinin orchestrate the assembly and maturation of nascent adhesions in a myosin II motor-independent manner. *Nat Cell Biol*. 10:1039-1050.

Chrzanowska-Wodnicka, M., and K. Burridge. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. *J Cell Biol*. 133:1403-1415.

Cicchini, C., I. Laudadio, F. Citarella, M. Corazzari, C. Steindler, A. Conigliaro, A. Fantoni, L. Amicone, and M. Tripodi. 2008. TGFbeta-induced EMT requires focal adhesion kinase (FAK) signaling. *Exp Cell Res*. 314:143-152.

Ciesiolka, M., M. Delvaeye, G. Van Imschoot, V. Verschuere, P. McCrea, F. van Roy, and K. Vleminckx. 2004. p120 catenin is required for morphogenetic movements involved in the formation of the eyes and the craniofacial skeleton in Xenopus. *J Cell Sci*. 117:4325-4339.

Clay, M.R., and M.C. Halloran. 2010. Control of neural crest cell behavior and migration: Insights from live imaging. *Cell Adh Migr*. 4:586-594.

Clay, M.R., and M.C. Halloran. 2014. Cadherin 6 promotes neural crest cell detachment via F-actin regulation and influences active Rho distribution during epithelial-to-mesenchymal transition. *Development*. 141:2506-2515.

Cluzel, C., F. Saltel, J. Lussi, F. Paulhe, B.A. Imhof, and B. Wehrle-Haller. 2005. The mechanisms and dynamics of (alpha)v(beta)3 integrin clustering in living cells. *J Cell Biol*. 171:383-392.

Collins, C., and W.J. Nelson. 2015. Running with neighbors: coordinating cell migration and cell-cell adhesion. *Curr Opin Cell Biol*. 36:62-70.

Coló, G.P., P. Hernández-Varas, J. Lock, R.A. Bartolomé, N. Arellano-Sánchez, S. Strömblad, and J. Teixidó. 2012. Focal adhesion disassembly is regulated by a RIAM to MEK-1 pathway. *J Cell Sci*. 125:5338-5352.

Cooper, J.A., K.L. Gould, C.A. Cartwright, and T. Hunter. 1986. Tyr527 is phosphorylated in pp60c-src: implications for regulation. *Science*. 231:1431-1434.

Cooper, L.A., T.L. Shen, and J.L. Guan. 2003. Regulation of focal adhesion kinase by its amino-terminal domain through an autoinhibitory interaction. *Mol Cell Biol*. 23:8030-8041.

Costa, M.L., R.C. Escaleira, F. Jazenko, and C.S. Mermelstein. 2008. Cell adhesion in zebrafish myogenesis: distribution of intermediate filaments, microfilaments, intracellular adhesion structures and extracellular matrix. *Cell Motil Cytoskeleton*. 65:801-815.

Cox, E.A., and A. Huttenlocher. 1998. Regulation of integrin-mediated adhesion during cell migration. *Microsc Res Tech*. 43:412-419.

Cramer, L.P. 1997. Molecular mechanism of actin-dependent retrograde flow in lamellipodia of motile cells. *Front Biosci*. 2:d260-270.

Crowley, E., and A.F. Horwitz. 1995. Tyrosine phosphorylation and cytoskeletal tension regulate the release of fibroblast adhesions. *J Cell Biol*. 131:525-537.

Curtis, A.S. 1964. The mechanism of adhesion of cells to glass. A study by interference reflection microscopy. *J Cell Biol*. 20:199-215.

Dady, A., C. Blavet, and J.L. Duband. 2012. Timing and kinetics of E- to N-cadherin switch during neurulation in the avian embryo. *Dev Dyn*. 241:1333-1349.

Davidson, L.A., B.G. Hoffstrom, R. Keller, and D.W. DeSimone. 2002. Mesendoderm extension and mantle closure in Xenopus laevis gastrulation: combined roles for integrin alpha(5)beta(1), fibronectin, and tissue geometry. *Dev Biol*. 242:109-129.

Davis, J.R., C.Y. Huang, J. Zanet, S. Harrison, E. Rosten, S. Cox, D.Y. Soong, G.A. Dunn, and B.M. Stramer. 2012. Emergence of embryonic pattern through contact inhibition of locomotion. *Development*. 139:4555-4560.

Davis, J.R., A. Luchici, F. Mosis, J. Thackery, J.A. Salazar, Y. Mao, G.A. Dunn, T. Betz, M. Miodownik, and B.M. Stramer. 2015. Inter-cellular forces orchestrate contact inhibition of locomotion. *Cell*. 161:361-373.

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

Davy, A., and P. Soriano. 2007. Ephrin-B2 forward signaling regulates somite patterning and neural crest cell development. *Dev Biol*. 304:182-193.

De Arcangelis, A., M. Mark, J. Kreidberg, L. Sorokin, and E. Georges-Labouesse. 1999. Synergistic activities of alpha3 and alpha6 integrins are required during apical ectodermal ridge formation and organogenesis in the mouse. *Development*. 126:3957-3968.

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

del Barrio, M.G., and M.A. Nieto. 2002. Overexpression of Snail family members highlights their ability to promote chick neural crest formation. *Development*. 129:1583-1593.

Delon, I., and N.H. Brown. 2009. The integrin adhesion complex changes its composition and function during morphogenesis of an epithelium. *J Cell Sci*. 122:4363-4374.

Desai, R.A., S.B. Gopal, S. Chen, and C.S. Chen. 2013. Contact inhibition of locomotion probabilities drive solitary versus collective cell migration. *J R Soc Interface*. 10:20130717.

Desmarais, V., H. Yamaguchi, M. Oser, L. Soon, G. Mouneimne, C. Sarmiento, R. Eddy, and J. Condeelis. 2009. N-WASP and cortactin are involved in invadopodium-dependent chemotaxis to EGF in breast tumor cells. *Cell Motil Cytoskeleton*. 66:303-316.

Digman, M.A., C.M. Brown, A.R. Horwitz, W.W. Mantulin, and E. Gratton. 2008. Paxillin dynamics measured during adhesion assembly and disassembly by correlation spectroscopy. *Biophys J*. 94:2819-2831.

Ding, H., X. Wu, I. Kim, P.P. Tam, G.Y. Koh, and A. Nagy. 2000. The mouse Pdgfc gene: dynamic expression in embryonic tissues during organogenesis. *Mech Dev*. 96:209-213.

Dourdin, N., A.K. Bhatt, P. Dutt, P.A. Greer, J.S. Arthur, J.S. Elce, and A. Huttenlocher. 2001. Reduced cell migration and disruption of the actin cytoskeleton in calpain-deficient embryonic fibroblasts. *J Biol Chem*. 276:48382-48388.

Doyle, A.D., N. Carvajal, A. Jin, K. Matsumoto, and K.M. Yamada. 2015. Local 3D matrix microenvironment regulates cell migration through spatiotemporal dynamics of contractility-dependent adhesions. *Nat Commun*. 6:8720.

Doyle, A.D., and K.M. Yamada. 2016. Mechanosensing via cell-matrix adhesions in 3D microenvironments. *Exp Cell Res*. 343:60-66.

Duband, J.L. 2006. Neural crest delamination and migration: integrating regulations of cell interactions, locomotion, survival and fate. *Adv Exp Med Biol*. 589:45-77.

Duband, J.L. 2010. Diversity in the molecular and cellular strategies of epithelium-to-mesenchyme transitions: Insights from the neural crest. *Cell Adh Migr*. 4:458-482.

Dudanova, I., G. Gatto, and R. Klein. 2010. GDNF acts as a chemoattractant to support ephrinA-induced repulsion of limb motor axons. *Curr Biol*. 20:2150-2156.

Dufour, S., R.M. Mège, and J.P. Thiery. 2013. α-catenin, vinculin, and F-actin in strengthening E-cadherin cell-cell adhesions and mechanosensing. *Cell Adh Migr*. 7:345-350.

Dunn, G.A., and S.W. Paddock. 1982. Analysing the motile behaviour of cells: a general approach with special reference to pairs of cells in collision. *Philos Trans R Soc Lond B Biol Sci*. 299:147-157.

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

Eagle, H., E.M. Levine, and C.W. Boone. 1965. Cellular Growth, Contact Inhibition, and Macromolecular Synthesis. *Science*. 148:665.

Eberhart, J.K., X. He, M.E. Swartz, Y.L. Yan, H. Song, T.C. Boling, A.K. Kunerth, M.B. Walker, C.B. Kimmel, and J.H. Postlethwait. 2008. MicroRNA Mirn140 modulates Pdgf signaling during palatogenesis. *Nat Genet*. 40:290-298.

Eickholt, B.J., S.L. Mackenzie, A. Graham, F.S. Walsh, and P. Doherty. 1999. Evidence for collapsin-1 functioning in the control of neural crest migration in both trunk and hindbrain regions. *Development*. 126:2181-2189.

Elosegui-Artola, A., E. Bazellières, M.D. Allen, I. Andreu, R. Oria, R. Sunyer, J.J. Gomm, J.F. Marshall, J.L. Jones, X. Trepat, and P. Roca-Cusachs. 2014. Rigidity sensing and adaptation through regulation of integrin types. *Nat Mater*. 13:631-637.

Elosegui-Artola, A., R. Oria, Y. Chen, A. Kosmalska, C. Pérez-González, N. Castro, C. Zhu, X. Trepat, and P. Roca-Cusachs. 2016. Mechanical regulation of a molecular clutch defines force transmission and transduction in response to matrix rigidity. *Nat Cell Biol*. 18:540-548.

Epifano, C., and M. Perez-Moreno. 2012. Crossroads of integrins and cadherins in epithelia and stroma remodeling. *Cell Adh Migr*. 6:261-273.

Evans, W.H. 2015. Cell communication across gap junctions: a historical perspective and current developments. *Biochem Soc Trans*. 43:450-459.

Ezratty, E.J., M.A. Partridge, and G.G. Gundersen. 2005. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. *Nat Cell Biol*. 7:581-590.

Fan, L.C., C.W. Shiau, W.T. Tai, M.H. Hung, P.Y. Chu, F.S. Hsieh, H. Lin, H.C. Yu, and K.F. Chen. 2015. SHP-1 is a negative regulator of epithelial-mesenchymal transition in hepatocellular carcinoma. *Oncogene*. 34:5252-5263.

Fincham, V.J., A. Chudleigh, and M.C. Frame. 1999. Regulation of p190 Rho-GAP by v-Src is linked to cytoskeletal disruption during transformation. *J Cell Sci*. 112 (Pt 6):947-956.

Fincham, V.J., and M.C. Frame. 1998. The catalytic activity of Src is dispensable for translocation to focal adhesions but controls the turnover of these structures during cell motility. *EMBO J*. 17:81-92.

Fincham, V.J., M. James, M.C. Frame, and S.J. Winder. 2000. Active ERK/MAP kinase is targeted to newly forming cell-matrix adhesions by integrin engagement and v-Src. *EMBO J*. 19:2911-2923.

Finnemann, S., M. Kühl, G. Otto, and D. Wedlich. 1995. Cadherin transfection of Xenopus XTC cells downregulates expression of substrate adhesion molecules. *Mol Cell Biol*. 15:5082-5091.

Flevaris, P., A. Stojanovic, H. Gong, A. Chishti, E. Welch, and X. Du. 2007. A molecular switch that controls cell spreading and retraction. *J Cell Biol*. 179:553-565.

Fraley, S.I., Y. Feng, R. Krishnamurthy, D.H. Kim, A. Celedon, G.D. Longmore, and D. Wirtz. 2010. A distinctive role for focal adhesion proteins in three-dimensional cell motility. *Nat Cell Biol*. 12:598-604.

Frame, M.C., V.J. Fincham, N.O. Carragher, and J.A. Wyke. 2002. v-Src's hold over actin and cell adhesions. *Nat Rev Mol Cell Biol*. 3:233-245.

Franco, S.J., M.A. Rodgers, B.J. Perrin, J. Han, D.A. Bennin, D.R. Critchley, and A. Huttenlocher. 2004. Calpain-mediated proteolysis of talin regulates adhesion dynamics. *Nat Cell Biol*. 6:977-983.

Fritz, R.D., D. Menshykau, K. Martin, A. Reimann, V. Pontelli, and O. Pertz. 2015. SrGAP2-Dependent Integration of Membrane Geometry and Slit-Robo-Repulsive Cues Regulates Fibroblast Contact Inhibition of Locomotion. *Dev Cell*. 35:78-92.

Fuchs, E., J. Dowling, J. Segre, S.H. Lo, and Q.C. Yu. 1997. Integrators of epidermal growth and differentiation: distinct functions for beta 1 and beta 4 integrins. *Curr Opin Genet Dev*. 7:672-682.

Fukuyama, T., H. Ogita, T. Kawakatsu, M. Inagaki, and Y. Takai. 2006. Activation of Rac by cadherin through the c-Src-Rap1-phosphatidylinositol 3-kinase-Vav2 pathway. *Oncogene*. 25:8-19.

Gans, C., and R.G. Northcutt. 1983. Neural crest and the origin of vertebrates: a new head. *Science*. 220:268-273.

Gardel, M.L., B. Sabass, L. Ji, G. Danuser, U.S. Schwarz, and C.M. Waterman. 2008. Traction stress in focal adhesions correlates biphasically with actin retrograde flow speed. *J Cell Biol*. 183:999-1005.

Gardel, M.L., I.C. Schneider, Y. Aratyn-Schaus, and C.M. Waterman. 2010. Mechanical integration of actin and adhesion dynamics in cell migration. *Annu Rev Cell Dev Biol*. 26:315-333.

Geiger, B., J.P. Spatz, and A.D. Bershadsky. 2009. Environmental sensing through focal adhesions. *Nat Rev Mol Cell Biol*. 10:21-33.

Genda, T., M. Sakamoto, T. Ichida, H. Asakura, and S. Hirohashi. 2000. Loss of cell-cell contact is induced by integrin-mediated cell-substratum adhesion in highly-motile and highly-metastatic hepatocellular carcinoma cells. *Lab Invest*. 80:387-394.

Giannone, G., P. Rondé, M. Gaire, J. Beaudouin, J. Haiech, J. Ellenberg, and K. Takeda. 2004. Calcium rises locally trigger focal adhesion disassembly and enhance residency of focal adhesion kinase at focal adhesions. *J Biol Chem*. 279:28715-28723.

Ginsberg, M.H., A. Partridge, and S.J. Shattil. 2005. Integrin regulation. *Curr Opin Cell Biol*. 17:509-516.

Gittes, F., B. Mickey, J. Nettleton, and J. Howard. 1993. Flexural rigidity of microtubules and actin filaments measured from thermal fluctuations in shape. *J Cell Biol*. 120:923-934.

Glading, A., P. Chang, D.A. Lauffenburger, and A. Wells. 2000. Epidermal growth factor receptor activation of calpain is required for fibroblast motility and occurs via an ERK/MAP kinase signaling pathway. *J Biol Chem*. 275:2390-2398.

Glasgow, J.E., and R.P. Daniele. 1994. Role of microtubules in random cell migration: stabilization of cell polarity. *Cell Motil Cytoskeleton*. 27:88-96.

Gomez, G.A., R.W. McLachlan, S.K. Wu, B.J. Caldwell, E. Moussa, S. Verma, M. Bastiani, R. Priya, R.G. Parton, K. Gaus, J. Sap, and A.S. Yap. 2015. An RPTPα/Src family kinase/Rap1 signaling module recruits myosin IIB to support contractile tension at apical E-cadherin junctions. *Mol Biol Cell*. 26:1249-1262.

Gooday, D., and P. Thorogood. 1985. Contact behaviour exhibited by migrating neural crest cells in confrontation culture with somitic cells. *Cell Tissue Res*. 241:165-169.

Goodenough, D.A., and D.L. Paul. 2009. Gap junctions. *Cold Spring Harb Perspect Biol*. 1:a002576.

Goñi, G.M., C. Epifano, J. Boskovic, M. Camacho-Artacho, J. Zhou, A. Bronowska, M.T. Martín, M.J. Eck, L. Kremer, F. Gräter, F.L. Gervasio, M. Perez-Moreno, and D. Lietha. 2014. Phosphatidylinositol 4,5-bisphosphate triggers activation of focal adhesion kinase by inducing clustering and conformational changes. *Proc Natl Acad Sci U S A*. 111:E3177-3186.

Grashoff, C., B.D. Hoffman, M.D. Brenner, R. Zhou, M. Parsons, M.T. Yang, M.A. McLean, S.G. Sligar, C.S. Chen, T. Ha, and M.A. Schwartz. 2010. Measuring mechanical tension across vinculin reveals regulation of focal adhesion dynamics. *Nature*. 466:263-266.

Greene, D.K., S. Tumova, J.R. Couchman, and A. Woods. 2003. Syndecan-4 associates with alpha-actinin. *J Biol Chem*. 278:7617-7623.

Grosheva, I., M. Shtutman, M. Elbaum, and A.D. Bershadsky. 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.

Grueber, W.B., and A. Sagasti. 2010. Self-avoidance and tiling: Mechanisms of dendrite and axon spacing. *Cold Spring Harb Perspect Biol*. 2:a001750.

Grunwald, G.B., R.S. Pratt, and J. Lilien. 1982. Enzymic dissection of embryonic cell adhesive mechanisms. III. Immunological identification of a component of the calcium-dependent adhesive system of embryonic chick neural retina cells. *J Cell Sci*. 55:69-83.

Guinebault, C., B. Payrastre, C. Racaud-Sultan, H. Mazarguil, M. Breton, G. Mauco, M. Plantavid, and H. Chap. 1995. Integrin-dependent translocation of phosphoinositide 3-kinase to the cytoskeleton of thrombin-activated platelets involves specific interactions of p85 alpha with actin filaments and focal adhesion kinase. *J Cell Biol*. 129:831-842.

Hakkinen, K.M., J.S. Harunaga, A.D. Doyle, and K.M. Yamada. 2011. Direct comparisons of the morphology, migration, cell adhesions, and actin cytoskeleton of fibroblasts in four different three-dimensional extracellular matrices. *Tissue Eng Part A*. 17:713-724.

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

Hall, A., and C.D. Nobes. 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.

Han, S.J., K.S. Bielawski, L.H. Ting, M.L. Rodriguez, and N.J. Sniadecki. 2012. Decoupling substrate stiffness, spread area, and micropost density: a close spatial relationship between traction forces and focal adhesions. *Biophys J*. 103:640-648.

Harland, R.M. 1991. In situ hybridization: an improved whole-mount method for Xenopus embryos. *Methods Cell Biol*. 36:685-695.

Harris, A. 1973. Location of cellular adhesions to solid substrata. *Dev Biol*. 35:97-114.

Harrison, R.G. 1911. ON THE STEREOTROPISM OF EMBRYONIC CELLS. *Science*. 34:279-281.

Hartsock, A., and W.J. Nelson. 2008. Adherens and tight junctions: structure, function and connections to the actin cytoskeleton. *Biochim Biophys Acta*. 1778:660-669.

Harunaga, J.S., and K.M. Yamada. 2011. Cell-matrix adhesions in 3D. *Matrix Biol*. 30:363-368.

Hatta, K., A. Nose, A. Nagafuchi, and M. Takeichi. 1988. Cloning and expression of cDNA encoding a neural calcium-dependent cell adhesion molecule: its identity in the cadherin gene family. *J Cell Biol*. 106:873-881.

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

Hay, E.D. 1995. An overview of epithelio-mesenchymal transformation. *Acta Anat (Basel)*. 154:8-20.

Hayashi, I., K. Vuori, and R.C. Liddington. 2002. The focal adhesion targeting (FAT) region of focal adhesion kinase is a four-helix bundle that binds paxillin. *Nat Struct Biol*. 9:101-106.

Hazan, R.B., R. Qiao, R. Keren, I. Badano, and K. Suyama. 2004. Cadherin switch in tumor progression. *Ann N Y Acad Sci*. 1014:155-163.

Heath, J.P., and G.A. Dunn. 1978. Cell to substratum contacts of chick fibroblasts and their relation to the microfilament system. A correlated interference-reflexion and high-voltage electron-microscope study. *J Cell Sci*. 29:197-212.

Heaysman, J.E., and S.M. Pegrum. 1973a. Early contacts between fibroblasts. An ultrastructural study. *Exp Cell Res*. 78:71-78.

Heaysman, J.E., and S.M. Pegrum. 1973b. Early contacts between normal fibroblasts and mouse sarcoma cells. An ultrastructural study. *Exp Cell Res*. 78:479-481.

Henry, C.A., B.D. Crawford, Y.L. Yan, J. Postlethwait, M.S. Cooper, and M.B. Hille. 2001. Roles for zebrafish focal adhesion kinase in notochord and somite morphogenesis. *Dev Biol*. 240:474-487.

Hertig, C.M., M. Eppenberger-Eberhardt, S. Koch, and H.M. Eppenberger. 1996. N-cadherin in adult rat cardiomyocytes in culture. I. Functional role of N-cadherin and impairment of cell-cell contact by a truncated N-cadherin mutant. *J Cell Sci.* 109 (Pt 1):1-10.

Hildebrand, J.D., M.D. Schaller, and J.T. Parsons. 1993. Identification of sequences required for the efficient localization of the focal adhesion kinase, pp125FAK, to cellular focal adhesions. *J Cell Biol*. 123:993-1005.

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

Hirano, S., S.T. Suzuki, and C. Redies. 2003. The cadherin superfamily in neural development: diversity, function and interaction with other molecules. *Front Biosci*. 8:d306-355.

His, W. 1868. Untersuchungen über die erste Anlage des Wirbeltierleibes: die erste Entwickelung des Hühnchens im Ei. *Vogel FCW, Leipzig*:237 p.

Ho, L., K. Symes, C. Yordán, L.J. Gudas, and M. Mercola. 1994. Localization of PDGF A and PDGFR alpha mRNA in Xenopus embryos suggests signalling from neural ectoderm and pharyngeal endoderm to neural crest cells. *Mech Dev*. 48:165-174.

Hoffman, L.M., C.C. Jensen, S. Kloeker, C.L. Wang, M. Yoshigi, and M.C. Beckerle. 2006. Genetic ablation of zyxin causes Mena/VASP mislocalization, increased motility, and deficits in actin remodeling. *J Cell Biol*. 172:771-782.

Hofman, P., L. d'Andrea, E. Guzman, E. Selva, G. Le Negrate, D.F. Far, E. Lemichez, P. Boquet, and B. Rossi. 1999. Neutrophil F-actin and myosin but not microtubules functionally regulate transepithelial migration induced by interleukin 8 across a cultured intestinal epithelial monolayer. *Eur Cytokine Netw*. 10:227-236.

Hong, J.Y., J.I. Park, K. Cho, D. Gu, H. Ji, S.E. Artandi, and P.D. McCrea. 2010. Shared molecular mechanisms regulate multiple catenin proteins: canonical Wnt signals and components modulate p120-catenin isoform-1 and additional p120 subfamily members. *J Cell Sci*. 123:4351-4365.

Horton, E.R., A. Byron, J.A. Askari, D.H. Ng, A. Millon-Frémillon, J. Robertson, E.J. Koper, N.R. Paul, S. Warwood, D. Knight, J.D. Humphries, and M.J. Humphries. 2015. Definition of a consensus integrin adhesome and its dynamics during adhesion complex assembly and disassembly. *Nat Cell Biol*. 17:1577-1587.

Horwitz, A., K. Duggan, C. Buck, M.C. Beckerle, and K. Burridge. 1986. Interaction of plasma membrane fibronectin receptor with talin--a transmembrane linkage. *Nature*. 320:531-533.

Horwitz, A.R. 2012. The origins of the molecular era of adhesion research. *Nat Rev Mol Cell Biol*. 13:805-811.

Hu, K., L. Ji, K.T. Applegate, G. Danuser, and C.M. Waterman-Storer. 2007. Differential transmission of actin motion within focal adhesions. *Science*. 315:111-115.

Huang, G.Y., A. Wessels, B.R. Smith, K.K. Linask, J.L. Ewart, and C.W. Lo. 1998. Alteration in connexin 43 gap junction gene dosage impairs conotruncal heart development. *Dev Biol*. 198:32-44.

Huang, X., and J.P. Saint-Jeannet. 2004. Induction of the neural crest and the opportunities of life on the edge. *Dev Biol*. 275:1-11.

Huttenlocher, A., M. Lakonishok, M. Kinder, S. Wu, T. Truong, K.A. Knudsen, and A.F. Horwitz. 1998. Integrin and cadherin synergy regulates contact inhibition of migration and motile activity. *J Cell Biol*. 141:515-526.

Hynes, R.O., and A.T. Destree. 1978. Relationships between fibronectin (LETS protein) and actin. *Cell*. 15:875-886.

Hytönen, V.P., and B. Wehrle-Haller. 2016. Mechanosensing in cell-matrix adhesions - Converting tension into chemical signals. *Exp Cell Res*. 343:35-41.

Ilić, D., Y. Furuta, S. Kanazawa, N. Takeda, K. Sobue, N. Nakatsuji, S. Nomura, J. Fujimoto, M. Okada, and T. Yamamoto. 1995. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*. 377:539-544.

Ishibe, S., D. Joly, X. Zhu, and L.G. Cantley. 2003. Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. *Mol Cell*. 12:1275-1285.

Izzard, C.S., and L.R. Lochner. 1976. Cell-to-substrate contacts in living fibroblasts: an interference reflexion study with an evaluation of the technique. *J Cell Sci*. 21:129-159.

Jasaitis, A., M. Estevez, J. Heysch, B. Ladoux, and S. Dufour. 2012. E-cadherin-dependent stimulation of traction force at focal adhesions via the Src and PI3K signaling pathways. *Biophys J*. 103:175-184.

Jean, C., X.L. Chen, J.O. Nam, I. Tancioni, S. Uryu, C. Lawson, K.K. Ward, C.T. Walsh, N.L. Miller, M. Ghassemian, P. Turowski, E. Dejana, S. Weis, D.A. Cheresh, and D.D. Schlaepfer. 2014. Inhibition of endothelial FAK activity prevents tumor metastasis by enhancing barrier function. *J Cell Biol*. 204:247-263.

Jiang, G., G. Giannone, D.R. Critchley, E. Fukumoto, and M.P. Sheetz. 2003. Two-piconewton slip bond between fibronectin and the cytoskeleton depends on talin. *Nature*. 424:334-337.

Johnson, R.P., and S.W. Craig. 1995. F-actin binding site masked by the intramolecular association of vinculin head and tail domains. *Nature*. 373:261-264.

Jou, T.S., D.B. Stewart, J. Stappert, W.J. Nelson, and J.A. Marrs. 1995. Genetic and biochemical dissection of protein linkages in the cadherin-catenin complex. *Proc Natl Acad Sci U S A*. 92:5067-5071.

Jülich, D., G. Cobb, A.M. Melo, P. McMillen, A.K. Lawton, S.G. Mochrie, E. Rhoades, and S.A. Holley. 2015. Cross-Scale Integrin Regulation Organizes ECM and Tissue Topology. *Dev Cell*. 34:33-44.

Kadir, S., J.W. Astin, L. Tahtamouni, P. Martin, and C.D. Nobes. 2011. Microtubule remodelling is required for the front-rear polarity switch during contact inhibition of locomotion. *J Cell Sci*. 124:2642-2653.

Kanda, S., Y. Miyata, H. Kanetake, and T.E. Smithgall. 2006. Fibroblast growth factor-2 induces the activation of Src through Fes, which regulates focal adhesion disassembly. *Exp Cell Res*. 312:3015-3022.

Kang, Y., and J. Massagué. 2004. Epithelial-mesenchymal transitions: twist in development and metastasis. *Cell*. 118:277-279.

Kanner, S.B., A.B. Reynolds, R.R. Vines, and J.T. Parsons. 1990. Monoclonal antibodies to individual tyrosine-phosphorylated protein substrates of oncogene-encoded tyrosine kinases. *Proc Natl Acad Sci U S A*. 87:3328-3332.

Kaplan, K.B., K.B. Bibbins, J.R. Swedlow, M. Arnaud, D.O. Morgan, and H.E. Varmus. 1994. Association of the amino-terminal half of c-Src with focal adhesions alters their properties and is regulated by phosphorylation of tyrosine 527. *EMBO J*. 13:4745-4756.

Kaplan, K.B., J.R. Swedlow, D.O. Morgan, and H.E. Varmus. 1995. c-Src enhances the spreading of src-/- fibroblasts on fibronectin by a kinase-independent mechanism. *Genes Dev*. 9:1505-1517.

Karaköse, E., H.B. Schiller, and R. Fässler. 2010. The kindlins at a glance. *J Cell Sci*. 123:2353-2356.

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

Kaverina, I., O. Krylyshkina, and J.V. Small. 1999. Microtubule targeting of substrate contacts promotes their relaxation and dissociation. *J Cell Biol*. 146:1033-1044.

Kaverina, I., O. Krylyshkina, and J.V. Small. 2002. Regulation of substrate adhesion dynamics during cell motility. *Int J Biochem Cell Biol*. 34:746-761.

Kawakatsu, H., T. Sakai, Y. Takagaki, Y. Shinoda, M. Saito, M.K. Owada, and J. Yano. 1996. A new monoclonal antibody which selectively recognizes the active form of Src tyrosine kinase. *J Biol Chem*. 271:5680-5685.

Kay, J.N., M.W. Chu, and J.R. Sanes. 2012. MEGF10 and MEGF11 mediate homotypic interactions required for mosaic spacing of retinal neurons. *Nature*. 483:465-469.

Kesavan, G., F.W. Sand, T.U. Greiner, J.K. Johansson, S. Kobberup, X. Wu, C. Brakebusch, and H. Semb. 2009. Cdc42-mediated tubulogenesis controls cell specification. *Cell*. 139:791-801.

Kil, S.H., C.E. Krull, G. Cann, D. Clegg, and M. Bronner-Fraser. 1998. The alpha4 subunit of integrin is important for neural crest cell migration. *Dev Biol*. 202:29-42.

Kil, S.H., T. Lallier, and M. Bronner-Fraser. 1996. Inhibition of cranial neural crest adhesion in vitro and migration in vivo using integrin antisense oligonucleotides. *Dev Biol*. 179:91-101.

Kim, M., C.V. Carman, and T.A. Springer. 2003. Bidirectional transmembrane signaling by cytoplasmic domain separation in integrins. *Science*. 301:1720-1725.

Kirby, M.L., and M.R. Hutson. 2010. Factors controlling cardiac neural crest cell migration. *Cell Adh Migr*. 4:609-621.

Klein, R. 2012. Eph/ephrin signalling during development. *Development*. 139:4105-4109.

Klemke, R.L., S. Cai, A.L. Giannini, P.J. Gallagher, P. de Lanerolle, and D.A. Cheresh. 1997. Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol*. 137:481-492.

Klinghoffer, R.A., C. Sachsenmaier, J.A. Cooper, and P. Soriano. 1999. Src family kinases are required for integrin but not PDGFR signal transduction. *EMBO J*. 18:2459-2471.

Kobielak, A., and E. Fuchs. 2004. Alpha-catenin: at the junction of intercellular adhesion and actin dynamics. *Nat Rev Mol Cell Biol*. 5:614-625.

Koenig, A., C. Mueller, C. Hasel, G. Adler, and A. Menke. 2006. Collagen type I induces disruption of E-cadherin-mediated cell-cell contacts and promotes proliferation of pancreatic carcinoma cells. *Cancer Res*. 66:4662-4671.

Koestner, U., I. Shnitsar, K. Linnemannstöns, A.L. Hufton, and A. Borchers. 2008. Semaphorin and neuropilin expression during early morphogenesis of Xenopus laevis. *Dev Dyn*. 237:3853-3863.

Kong, F., A.J. García, A.P. Mould, M.J. Humphries, and C. Zhu. 2009. Demonstration of catch bonds between an integrin and its ligand. *J Cell Biol*. 185:1275-1284.

Kotini, M., and R. Mayor. 2015. Connexins in migration during development and cancer. *Dev Biol*. 401:143-151.

Kreidberg, J.A., M.J. Donovan, S.L. Goldstein, H. Rennke, K. Shepherd, R.C. Jones, and R. Jaenisch. 1996. Alpha 3 beta 1 integrin has a crucial role in kidney and lung organogenesis. *Development*. 122:3537-3547.

Krylyshkina, O., I. Kaverina, W. Kranewitter, W. Steffen, M.C. Alonso, R.A. Cross, and J.V. Small. 2002. Modulation of substrate adhesion dynamics via microtubule targeting requires kinesin-1. *J Cell Biol*. 156:349-359.

Kubow, K.E., and A.R. Horwitz. 2011. Reducing background fluorescence reveals adhesions in 3D matrices. *Nat Cell Biol*. 13:3-5; author reply 5-7.

Kullander, K., and R. Klein. 2002. Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol*. 3:475-486.

Kuriyama, S., E. Theveneau, A. Benedetto, M. Parsons, M. Tanaka, G. Charras, A. Kabla, and R. Mayor. 2014. In vivo collective cell migration requires an LPAR2-dependent increase in tissue fluidity. *J Cell Biol*. 206:113-127.

LaBonne, C., and M. Bronner-Fraser. 2000. Snail-related transcriptional repressors are required in Xenopus for both the induction of the neural crest and its subsequent migration. *Dev Biol*. 221:195-205.

Ladoux, B., W.J. Nelson, J. Yan, and R.M. Mège. 2015. The mechanotransduction machinery at work at adherens junctions. *Integr Biol (Camb)*. 7:1109-1119.

Larue, L., and A. Bellacosa. 2005. Epithelial-mesenchymal transition in development and cancer: role of phosphatidylinositol 3' kinase/AKT pathways. *Oncogene*. 24:7443-7454.

Laukaitis, C.M., D.J. Webb, K. Donais, and A.F. Horwitz. 2001. Differential dynamics of alpha 5 integrin, paxillin, and alpha-actinin during formation and disassembly of adhesions in migrating cells. *J Cell Biol*. 153:1427-1440.

Leckband, D.E., and J. de Rooij. 2014. Cadherin adhesion and mechanotransduction. *Annu Rev Cell Dev Biol*. 30:291-315.

Leonard, M., L. Zhang, B.M. Bleaken, and A.S. Menko. 2013. Distinct roles for N-Cadherin linked c-Src and fyn kinases in lens development. *Dev Dyn*. 242:469-484.

Letourneau, P.C., F.K. Roche, T.A. Shattuck, V. Lemmon, and M. Takeichi. 1991. Interactions of Schwann cells with neurites and with other Schwann cells involve the calcium-dependent adhesion molecule, N-cadherin. *J Neurobiol*. 22:707-720.

Lewis, W.H. 1922. The adhesive quality of

cells. *The Anatomical Record*. 23:387-389.

Li Jeon, N., H. Baskaran, S.K. Dertinger, G.M. Whitesides, L. Van de Water, and M. Toner. 2002. Neutrophil chemotaxis in linear and complex gradients of interleukin-8 formed in a microfabricated device. *Nat Biotechnol*. 20:826-830.

Li, L., M. Okura, and A. Imamoto. 2002. Focal adhesions require catalytic activity of Src family kinases to mediate integrin-matrix adhesion. *Mol Cell Biol*. 22:1203-1217.

Li, S., J. Couet, and M.P. Lisanti. 1996. Src tyrosine kinases, Galpha subunits, and H-Ras share a common membrane-anchored scaffolding protein, caveolin. Caveolin binding negatively regulates the auto-activation of Src tyrosine kinases. *J Biol Chem*. 271:29182-29190.

Lien, W.H., O. Klezovitch, and V. Vasioukhin. 2006. Cadherin-catenin proteins in vertebrate development. *Curr Opin Cell Biol*. 18:499-506.

Lin, B., T. Yin, Y.I. Wu, T. Inoue, and A. Levchenko. 2015. Interplay between chemotaxis and contact inhibition of locomotion determines exploratory cell migration. *Nat Commun*. 6:6619.

Lin, C.H., E.M. Espreafico, M.S. Mooseker, and P. Forscher. 1997. Myosin drives retrograde F-actin flow in neuronal growth cones. *Biol Bull*. 192:183-185.

Lin, Y.C., D.T. Tambe, C.Y. Park, M.R. Wasserman, X. Trepat, R. Krishnan, G. Lenormand, J.J. Fredberg, and J.P. Butler. 2010. Mechanosensing of substrate thickness. *Phys Rev E Stat Nonlin Soft Matter Phys*. 82:041918.

Linder, S., and P. Kopp. 2005. Podosomes at a glance. *J Cell Sci*. 118:2079-2082.

Liu, G., C.D. Guibao, and J. Zheng. 2002. Structural insight into the mechanisms of targeting and signaling of focal adhesion kinase. *Mol Cell Biol*. 22:2751-2760.

Liu, K.D., A. Datta, W. Yu, P.R. Brakeman, T.S. Jou, M.A. Matthay, and K.E. Mostov. 2007. Rac1 is required for reorientation of polarity and lumen formation through a PI 3-kinase-dependent pathway. *Am J Physiol Renal Physiol*. 293:F1633-1640.

Liu, S., S.M. Thomas, D.G. Woodside, D.M. Rose, W.B. Kiosses, M. Pfaff, and M.H. Ginsberg. 1999. Binding of paxillin to alpha4 integrins modifies integrin-dependent biological responses. *Nature*. 402:676-681.

Liu, Z., J.L. Tan, D.M. Cohen, M.T. Yang, N.J. Sniadecki, S.A. Ruiz, C.M. Nelson, and C.S. Chen. 2010. Mechanical tugging force regulates the size of cell-cell junctions. *Proc Natl Acad Sci U S A*. 107:9944-9949.

Loeb, L. 1921. Amoeligboid movement, tissue formation and consistency of protoplasm. *Science*. 53:261-262.

Lämmermann, T., B.L. Bader, S.J. Monkley, T. Worbs, R. Wedlich-Söldner, K. Hirsch, M. Keller, R. Förster, D.R. Critchley, R. Fässler, and M. Sixt. 2008. Rapid leukocyte migration by integrin-independent flowing and squeezing. *Nature*. 453:51-55.

Machiyama, H., T. Yamaguchi, Y. Sawada, T.M. Watanabe, and H. Fujita. 2015. SH3 domain of c-Src governs its dynamics at focal adhesions and the cell membrane. *FEBS J*. 282:4034-4055.

Mancilla, A., and R. Mayor. 1996. Neural crest formation in Xenopus laevis: mechanisms of Xslug induction. *Dev Biol*. 177:580-589.

Manser, E., T.H. Loo, C.G. Koh, Z.S. Zhao, X.Q. Chen, L. Tan, I. Tan, T. Leung, and L. Lim. 1998. PAK kinases are directly coupled to the PIX family of nucleotide exchange factors. *Mol Cell*. 1:183-192.

Marchant, L., C. Linker, P. Ruiz, N. Guerrero, and R. Mayor. 1998. The inductive properties of mesoderm suggest that the neural crest cells are specified by a BMP gradient. *Dev Biol*. 198:319-329.

Marsden, M., and D.W. DeSimone. 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., S. Dickinson, and C.D. Nobes. 2003. Rac-dependent trans-endocytosis of ephrinBs regulates Eph-ephrin contact repulsion. *Nat Cell Biol*. 5:879-888.

Martin, A.C., M. Gelbart, R. Fernandez-Gonzalez, M. Kaschube, and E.F. Wieschaus. 2010. Integration of contractile forces during tissue invagination. *J Cell Biol*. 188:735-749.

Martinez-Rico, C., F. Pincet, J.P. Thiery, and S. Dufour. 2010. Integrins stimulate E-cadherin-mediated intercellular adhesion by regulating Src-kinase activation and actomyosin contractility. *J Cell Sci*. 123:712-722.

Maruthamuthu, V., B. Sabass, U.S. Schwarz, and M.L. Gardel. 2011. Cell-ECM traction force modulates endogenous tension at cell-cell contacts. *Proc Natl Acad Sci U S A*. 108:4708-4713.

Matthews, H.K., L. Marchant, C. Carmona-Fontaine, S. Kuriyama, J. Larraín, M.R. Holt, M. Parsons, and R. Mayor. 2008. Directional migration of neural crest cells in vivo is regulated by Syndecan-4/Rac1 and non-canonical Wnt signaling/RhoA. *Development*. 135:1771-1780.

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

Mayor, R., and E. Theveneau. 2014. The role of the non-canonical Wnt-planar cell polarity pathway in neural crest migration. *Biochem J*. 457:19-26.

Maître, J.L., and C.P. Heisenberg. 2013. Three functions of cadherins in cell adhesion. *Curr Biol*. 23:R626-633.

McCain, M.L., H. Lee, Y. Aratyn-Schaus, A.G. Kléber, and K.K. Parker. 2012. Cooperative coupling of cell-matrix and cell-cell adhesions in cardiac muscle. *Proc Natl Acad Sci U S A*. 109:9881-9886.

McLachlan, R.W., A. Kraemer, F.M. Helwani, E.M. Kovacs, and A.S. Yap. 2007. E-cadherin adhesion activates c-Src signaling at cell-cell contacts. *Mol Biol Cell*. 18:3214-3223.

McLachlan, R.W., and A.S. Yap. 2011. Protein tyrosine phosphatase activity is necessary for E-cadherin-activated Src signaling. *Cytoskeleton (Hoboken)*. 68:32-43.

McLennan, R., and P.M. Kulesa. 2007. In vivo analysis reveals a critical role for neuropilin-1 in cranial neural crest cell migration in chick. *Dev Biol*. 301:227-239.

McLennan, R., L.J. Schumacher, J.A. Morrison, J.M. Teddy, D.A. Ridenour, A.C. Box, C.L. Semerad, H. Li, W. McDowell, D. Kay, P.K. Maini, R.E. Baker, and P.M. Kulesa. 2015. VEGF signals induce trailblazer cell identity that drives neural crest migration. *Dev Biol*. 407:12-25.

McLennan, R., J.M. Teddy, J.C. Kasemeier-Kulesa, M.H. Romine, and P.M. Kulesa. 2010. Vascular endothelial growth factor (VEGF) regulates cranial neural crest migration in vivo. *Dev Biol*. 339:114-125.

McMillen, P., and S.A. Holley. 2015. Integration of cell-cell and cell-ECM adhesion in vertebrate morphogenesis. *Curr Opin Cell Biol*. 36:48-53.

Mellott, D.O., and R.D. Burke. 2008. Divergent roles for Eph and ephrin in avian cranial neural crest. *BMC Dev Biol*. 8:56.

Mertz, A.F., Y. Che, S. Banerjee, J.M. Goldstein, K.A. Rosowski, S.F. Revilla, C.M. Niessen, M.C. Marchetti, E.R. Dufresne, and V. Horsley. 2013. Cadherin-based intercellular adhesions organize epithelial cell-matrix traction forces. *Proc Natl Acad Sci U S A*. 110:842-847.

Mitra, S.K., D.A. Hanson, and D.D. Schlaepfer. 2005. Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol*. 6:56-68.

Mitra, S.K., D. Mikolon, J.E. Molina, D.A. Hsia, D.A. Hanson, A. Chi, S.T. Lim, J.A. Bernard-Trifilo, D. Ilic, D.G. Stupack, D.A. Cheresh, and D.D. Schlaepfer. 2006. Intrinsic FAK activity and Y925 phosphorylation facilitate an angiogenic switch in tumors. *Oncogene*. 25:5969-5984.

Mitra, S.K., and D.D. Schlaepfer. 2006. Integrin-regulated FAK-Src signaling in normal and cancer cells. *Curr Opin Cell Biol*. 18:516-523.

Miyamoto, S., S.K. Akiyama, and K.M. Yamada. 1995. Synergistic roles for receptor occupancy and aggregation in integrin transmembrane function. *Science*. 267:883-885.

Moore, R., E. Theveneau, S. Pozzi, P. Alexandre, J. Richardson, A. Merks, M. Parsons, J. Kashef, C. Linker, and R. Mayor. 2013. Par3 controls neural crest migration by promoting microtubule catastrophe during contact inhibition of locomotion. *Development*. 140:4763-4775.

Moser, M., B. Nieswandt, S. Ussar, M. Pozgajova, and R. Fässler. 2008. Kindlin-3 is essential for integrin activation and platelet aggregation. *Nat Med*. 14:325-330.

Mui, K.L., C.S. Chen, and R.K. Assoian. 2016. The mechanical regulation of integrin-cadherin crosstalk organizes cells, signaling and forces. *J Cell Sci*.

Murrell, M., P.W. Oakes, M. Lenz, and M.L. Gardel. 2015. Forcing cells into shape: the mechanics of actomyosin contractility. *Nat Rev Mol Cell Biol*. 16:486-498.

Myers, J.P., and T.M. Gomez. 2011. Focal adhesion kinase promotes integrin adhesion dynamics necessary for chemotropic turning of nerve growth cones. *J Neurosci*. 31:13585-13595.

Myers, J.P., E. Robles, A. Ducharme-Smith, and T.M. Gomez. 2012. Focal adhesion kinase modulates Cdc42 activity downstream of positive and negative axon guidance cues. *J Cell Sci*. 125:2918-2929.

Nagano, M., D. Hoshino, N. Koshikawa, T. Akizawa, and M. Seiki. 2012. Turnover of focal adhesions and cancer cell migration. *Int J Cell Biol*. 2012:310616.

Nagasaki, T., C.J. Chapin, and G.G. Gundersen. 1992. Distribution of detyrosinated microtubules in motile NRK fibroblasts is rapidly altered upon cell-cell contact: implications for contact inhibition of locomotion. *Cell Motil Cytoskeleton*. 23:45-60.

Nagata, K., A. Puls, C. Futter, P. Aspenstrom, E. Schaefer, T. Nakata, N. Hirokawa, and A. Hall. 1998. The MAP kinase kinase kinase MLK2 co-localizes with activated JNK along microtubules and associates with kinesin superfamily motor KIF3. *EMBO J*. 17:149-158.

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

Nakamura, N., J. Tanaka, and K. Sobue. 1993. Rous sarcoma virus-transformed cells develop peculiar adhesive structures along the cell periphery. *J Cell Sci*. 106 (Pt 4):1057-1069.
Nandadasa, S., Q. Tao, N.R. Menon, J. Heasman, and C. Wylie. 2009. N- and E-cadherins in Xenopus are specifically required in the neural and non-neural ectoderm, respectively, for F-actin assembly and morphogenetic movements. *Development*. 136:1327-1338.
Nayal, A., D.J. Webb, C.M. Brown, E.M. Schaefer, M. Vicente-Manzanares, and A.R. Horwitz. 2006. Paxillin phosphorylation at Ser273 localizes a GIT1-PIX-PAK complex and regulates adhesion and protrusion dynamics. *J Cell Biol*. 173:587-589.
Nelson, C.M., D.M. Pirone, J.L. Tan, and C.S. Chen. 2004. Vascular endothelial-cadherin regulates cytoskeletal tension, cell spreading, and focal adhesions by stimulating RhoA. *Mol Biol Cell*. 15:2943-2953.
Newgreen, D.F., M. Ritterman, and E.A. Peters. 1979. Morphology and behaviour of neural crest cells of chick embryo in vitro. *Cell Tissue Res*. 203:115-140.
Nguyen, V.H., B. Schmid, J. Trout, S.A. Connors, M. Ekker, and M.C. Mullins. 1998. Ventral and lateral regions of the zebrafish gastrula, including the neural crest progenitors, are established by a bmp2b/swirl pathway of genes. *Dev Biol*. 199:93-110.
Niessen, C.M., D. Leckband, and A.S. Yap. 2011. Tissue organization by cadherin adhesion molecules: dynamic molecular and cellular mechanisms of morphogenetic regulation. *Physiol Rev*. 91:691-731.
Nieto, M.A. 2013. Epithelial plasticity: a common theme in embryonic and cancer cells. *Science*. 342:1234850.
Nieto, M.A., M.G. Sargent, D.G. Wilkinson, and J. Cooke. 1994. Control of cell behavior during vertebrate development by Slug, a zinc finger gene. *Science*. 264:835-839.
Nobes, C.D., and A. Hall. 1995. Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. *Cell*. 81:53-62.
Nollet, F., P. Kools, and F. van Roy. 2000. Phylogenetic analysis of the cadherin superfamily allows identification of six major subfamilies besides several solitary members. *J Mol Biol*. 299:551-572.
Noren, N.K., B.P. Liu, K. Burridge, and B. Kreft. 2000. p120 catenin regulates the actin cytoskeleton via Rho family GTPases. *J Cell Biol*. 150:567-580.
Noren, N.K., C.M. Niessen, B.M. Gumbiner, and K. Burridge. 2001. Cadherin engagement regulates Rho family GTPases. *J Biol Chem*. 276:33305-33308.
Northcutt, R.G., and C. Gans. 1983. The genesis of neural crest and epidermal placodes: a reinterpretation of vertebrate origins. *Q Rev Biol*. 58:1-28.
Novikova, E.A., and C. Storm. 2013. Contractile fibers and catch-bond clusters: a biological force sensor? *Biophys J*. 105:1336-1345.
Ojakian, G.K., D.R. Ratcliffe, and R. Schwimmer. 2001. Integrin regulation of cell-cell adhesion during epithelial tubule formation. *J Cell Sci*. 114:941-952.
Olesnicky Killian, E.C., D.A. Birkholz, and K.B. Artinger. 2009. A role for chemokine signaling in neural crest cell migration and craniofacial development. *Dev Biol*. 333:161-172.
Otey, C.A., F.M. Pavalko, and K. Burridge. 1990. An interaction between alpha-actinin and the beta 1 integrin subunit in vitro. *J Cell Biol*. 111:721-729.
Ouyang, M., S. Lu, T. Kim, C.E. Chen, J. Seong, D.E. Leckband, F. Wang, A.B. Reynolds, M.A. Schwartz, and Y. Wang. 2013. N-cadherin regulates spatially polarized signals through distinct p120ctn and β-catenin-dependent signalling pathways. *Nat Commun*. 4:1589.
Owen, J.D., P.J. Ruest, D.W. Fry, and S.K. Hanks. 1999. Induced focal adhesion kinase (FAK) expression in FAK-null cells enhances cell spreading and migration requiring both auto- and activation loop phosphorylation sites and inhibits adhesion-dependent tyrosine phosphorylation of Pyk2. *Mol Cell Biol*. 19:4806-4818.

Owens, D.W., G.W. McLean, A.W. Wyke, C. Paraskeva, E.K. Parkinson, M.C. Frame, and V.G. Brunton. 2000. The catalytic activity of the Src family kinases is required to disrupt cadherin-dependent cell-cell contacts. *Mol Biol Cell*. 11:51-64.

Paddock, S.W., and G.A. Dunn. 1986. Analysing collisions between fibroblasts and fibrosarcoma cells: fibrosarcoma cells show an active invasionary response. *J Cell Sci*. 81:163-187.

Palacios, F., J.S. Tushir, Y. Fujita, and C. D'Souza-Schorey. 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., E. Cukierman, B.Z. Katz, K. Matsumoto, D.C. Lin, S. Lin, C. Hahn, and K.M. Yamada. 2000. Integrin dynamics and matrix assembly: tensin-dependent translocation of alpha(5)beta(1) integrins promotes early fibronectin fibrillogenesis. *J Cell Biol*. 148:1075-1090.

Papusheva, E., and C.P. Heisenberg. 2010. Spatial organization of adhesion: force-dependent regulation and function in tissue morphogenesis. *EMBO J*. 29:2753-2768.

Parish, R.W., C. Schmidhauser, T. Schmidt, and R.K. Dudler. 1987. Mechanisms of tumour cell metastasis. *J Cell Sci Suppl*. 8:181-197.

Park, K.S., and B.M. Gumbiner. 2012. Cadherin-6B stimulates an epithelial mesenchymal transition and the delamination of cells from the neural ectoderm via LIMK/cofilin mediated non-canonical BMP receptor signaling. *Dev Biol*. 366:232-243.

Parsons, J.T. 2003. Focal adhesion kinase: the first ten years. *J Cell Sci*. 116:1409-1416.

Parsons, J.T., A.R. Horwitz, and M.A. Schwartz. 2010. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat Rev Mol Cell Biol*. 11:633-643.

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

Patel, S.D., C. Ciatto, C.P. Chen, F. Bahna, M. Rajebhosale, N. Arkus, I. Schieren, T.M. Jessell, B. Honig, S.R. Price, and L. Shapiro. 2006. Type II cadherin ectodomain structures: implications for classical cadherin specificity. *Cell*. 124:1255-1268.

Pelham, R.J., and Y.L. Wang. 1998. Cell locomotion and focal adhesions are regulated by the mechanical properties of the substrate. *Biol Bull*. 194:348-349; discussion 349-350.

Perris, R. 1997. The extracellular matrix in neural crest-cell migration. *Trends Neurosci*. 20:23-31.

Pines, M., R. Das, S.J. Ellis, A. Morin, S. Czerniecki, L. Yuan, M. Klose, D. Coombs, and G. Tanentzapf. 2012. Mechanical force regulates integrin turnover in Drosophila in vivo. *Nat Cell Biol*. 14:935-943.

Playford, M.P., and M.D. Schaller. 2004. The interplay between Src and integrins in normal and tumor biology. *Oncogene*. 23:7928-7946.

Playford, M.P., K. Vadali, X. Cai, K. Burridge, and M.D. Schaller. 2008. Focal adhesion kinase regulates cell-cell contact formation in epithelial cells via modulation of Rho. *Exp Cell Res*. 314:3187-3197.

Plotnikov, S.V., A.M. Pasapera, B. Sabass, and C.M. Waterman. 2012. Force fluctuations within focal adhesions mediate ECM-rigidity sensing to guide directed cell migration. *Cell*. 151:1513-1527.

Pocha, S.M., and D.J. Montell. 2014. Cellular and molecular mechanisms of single and collective cell migrations in Drosophila: themes and variations. *Annu Rev Genet*. 48:295-318.

Pollard, T.D., and G.G. Borisy. 2003. Cellular motility driven by assembly and disassembly of actin filaments. *Cell*. 112:453-465.

Price, L.S., J. Leng, M.A. Schwartz, and G.M. Bokoch. 1998. Activation of Rac and Cdc42 by integrins mediates cell spreading. *Mol Biol Cell*. 9:1863-1871.

Provenzano, P.P., D.R. Inman, K.W. Eliceiri, S.M. Trier, and P.J. Keely. 2008. Contact guidance mediated three-dimensional cell migration is regulated by Rho/ROCK-dependent matrix reorganization. *Biophys J*. 95:5374-5384.

Rabadán, M.A., A. Herrera, L. Fanlo, S. Usieto, C. Carmona-Fontaine, E.H. Barriga, R. Mayor, S. Pons, and E. Martí. 2016. Delamination of neural crest cells requires transient and reversible Wnt inhibition mediated by DACT1/2. *Development*.

Reddien, P.W., and H.R. Horvitz. 2000. CED-2/CrkII and CED-10/Rac control phagocytosis and cell migration in Caenorhabditis elegans. *Nat Cell Biol*. 2:131-136.

Ren, X.D., W.B. Kiosses, and M.A. Schwartz. 1999. Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *EMBO J*. 18:578-585.

Ren, X.D., W.B. Kiosses, D.J. Sieg, C.A. Otey, D.D. Schlaepfer, and M.A. Schwartz. 2000. Focal adhesion kinase suppresses Rho activity to promote focal adhesion turnover. *J Cell Sci*. 113 (Pt 20):3673-3678.

Renkawitz, J., and M. Sixt. 2010. Mechanisms of force generation and force transmission during interstitial leukocyte migration. *EMBO Rep*. 11:744-750.

Reynolds, A.B., J. Daniel, P.D. McCrea, M.J. Wheelock, J. Wu, and Z. Zhang. 1994. Identification of a new catenin: the tyrosine kinase substrate p120cas associates with E-cadherin complexes. *Mol Cell Biol*. 14:8333-8342.

Richarte, A.M., H.B. Mead, and M.D. Tallquist. 2007. Cooperation between the PDGF receptors in cardiac neural crest cell migration. *Dev Biol*. 306:785-796.

Ridley, A.J., H.F. Paterson, C.L. Johnston, D. Diekmann, and A. Hall. 1992. The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. *Cell*. 70:401-410.

Rifes, P., and S. Thorsteinsdóttir. 2012. Extracellular matrix assembly and 3D organization during paraxial mesoderm development in the chick embryo. *Dev Biol*. 368:370-381.

Riveline, D., E. Zamir, N.Q. Balaban, U.S. Schwarz, T. Ishizaki, S. Narumiya, Z. Kam, B. Geiger, and A.D. Bershadsky. 2001. Focal contacts as mechanosensors: externally applied local mechanical force induces growth of focal contacts by an mDia1-dependent and ROCK-independent mechanism. *J Cell Biol*. 153:1175-1186.

Robles, E., and T.M. Gomez. 2006. Focal adhesion kinase signaling at sites of integrin-mediated adhesion controls axon pathfinding. *Nat Neurosci*. 9:1274-1283.

Roca-Cusachs, P., A. del Rio, E. Puklin-Faucher, N.C. Gauthier, N. Biais, and M.P. Sheetz. 2013. Integrin-dependent force transmission to the extracellular matrix by α-actinin triggers adhesion maturation. *Proc Natl Acad Sci U S A*. 110:E1361-1370.

Rogers, C.D., A. Saxena, and M.E. Bronner. 2013. Sip1 mediates an E-cadherin-to-N-cadherin switch during cranial neural crest EMT. *J Cell Biol*. 203:835-847.

Roskoski, R. 2004. Src protein-tyrosine kinase structure and regulation. *Biochem Biophys Res Commun*. 324:1155-1164.

Roskoski, R. 2015. Src protein-tyrosine kinase structure, mechanism, and small molecule inhibitors. *Pharmacol Res*. 94:9-25.

Rottner, K., A. Hall, and J.V. Small. 1999. Interplay between Rac and Rho in the control of substrate contact dynamics. *Curr Biol*. 9:640-648.

Roussos, E.T., J.S. Condeelis, and A. Patsialou. 2011. Chemotaxis in cancer. *Nat Rev Cancer*. 11:573-587.

Roycroft, A., and R. Mayor. 2015. Forcing contact inhibition of locomotion. *Trends Cell Biol*. 25:373-375.

Roycroft, A., and R. Mayor. 2016. Molecular basis of contact inhibition of locomotion. *Cell Mol Life Sci*. 73:1119-1130.

Sadaghiani, B., and C.H. Thiébaud. 1987. Neural crest development in the Xenopus laevis embryo, studied by interspecific transplantation and scanning electron microscopy. *Dev Biol*. 124:91-110.

Sahai, E., and C.J. Marshall. 2002. ROCK and Dia have opposing effects on adherens junctions downstream of Rho. *Nat Cell Biol*. 4:408-415.

Sahai, E., J. Wyckoff, U. Philippar, J.E. Segall, F. Gertler, and J. Condeelis. 2005. Simultaneous imaging of GFP, CFP and collagen in tumors in vivo using multiphoton microscopy. *BMC Biotechnol*. 5:14.

Sakai, T., M. Larsen, and K.M. Yamada. 2003. Fibronectin requirement in branching morphogenesis. *Nature*. 423:876-881.

Sanders, L.C., F. Matsumura, G.M. Bokoch, and P. de Lanerolle. 1999. Inhibition of myosin light chain kinase by p21-activated kinase. *Science*. 283:2083-2085.

Sandilands, E., C. Cans, V.J. Fincham, V.G. Brunton, H. Mellor, G.C. Prendergast, J.C. Norman, G. Superti-Furga, and M.C. Frame. 2004. RhoB and actin polymerization coordinate Src activation with endosome-mediated delivery to the membrane. *Dev Cell*. 7:855-869.

Sauka-Spengler, T., and M. Bronner-Fraser. 2008. A gene regulatory network orchestrates neural crest formation. *Nat Rev Mol Cell Biol*. 9:557-568.

Sawada, Y., M. Tamada, B.J. Dubin-Thaler, O. Cherniavskaya, R. Sakai, S. Tanaka, and M.P. Sheetz. 2006. Force sensing by mechanical extension of the Src family kinase substrate p130Cas. *Cell*. 127:1015-1026.

Sazonova, O.V., K.L. Lee, B.C. Isenberg, C.B. Rich, M.A. Nugent, and J.Y. Wong. 2011. Cell-cell interactions mediate the response of vascular smooth muscle cells to substrate stiffness. *Biophys J*. 101:622-630.

Scarpa, E., A. Roycroft, E. Theveneau, E. Terriac, M. Piel, and R. Mayor. 2013. A novel method to study contact inhibition of locomotion using micropatterned substrates. *Biol Open*. 2:901-906.

Scarpa, E., A. Szabó, A. Bibonne, E. Theveneau, M. Parsons, and R. Mayor. 2015. Cadherin Switch during EMT in Neural Crest Cells Leads to Contact Inhibition of Locomotion via Repolarization of Forces. *Dev Cell*.

Schaller, M.D., J.D. Hildebrand, and J.T. Parsons. 1999. Complex formation with focal adhesion kinase: A mechanism to regulate activity and subcellular localization of Src kinases. *Mol Biol Cell*. 10:3489-3505.

Schaller, M.D., C.A. Otey, J.D. Hildebrand, and J.T. Parsons. 1995. Focal adhesion kinase and paxillin bind to peptides mimicking beta integrin cytoplasmic domains. *J Cell Biol*. 130:1181-1187.

Schaller, M.D., and J.T. Parsons. 1995. pp125FAK-dependent tyrosine phosphorylation of paxillin creates a high-affinity binding site for Crk. *Mol Cell Biol*. 15:2635-2645.

Schatteman, G.C., K. Morrison-Graham, A. van Koppen, J.A. Weston, and D.F. Bowen-Pope. 1992. Regulation and role of PDGF receptor alpha-subunit expression during embryogenesis. *Development*. 115:123-131.

Scheswohl, D.M., J.R. Harrell, Z. Rajfur, G. Gao, S.L. Campbell, and M.D. Schaller. 2008. Multiple paxillin binding sites regulate FAK function. *J Mol Signal*. 3:1.

Schlaepfer, D.D., S.K. Hanks, T. Hunter, and P. van der Geer. 1994. Integrin-mediated signal transduction linked to Ras pathway by GRB2 binding to focal adhesion kinase. *Nature*. 372:786-791.

Schlaepfer, D.D., and T. Hunter. 1996. Evidence for in vivo phosphorylation of the Grb2 SH2-domain binding site on focal adhesion kinase by Src-family protein-tyrosine kinases. *Mol Cell Biol*. 16:5623-5633.

Schober, M., S. Raghavan, M. Nikolova, L. Polak, H.A. Pasolli, H.E. Beggs, L.F. Reichardt, and E. Fuchs. 2007. Focal adhesion kinase modulates tension signaling to control actin and focal adhesion dynamics. *J Cell Biol*. 176:667-680.

Selleck, M.A., and M. Bronner-Fraser. 1995. Origins of the avian neural crest: the role of neural plate-epidermal interactions. *Development*. 121:525-538.

Shao, K., Z.Y. Chen, S. Gautam, N.H. Deng, Y. Zhou, and X.Z. Wu. 2016. Posttranslational modification of E-cadherin by core fucosylation regulates Src activation and induces epithelial-mesenchymal transition-like process in lung cancer cells. *Glycobiology*. 26:142-154.

Sheetz, M.P., D. Felsenfeld, C.G. Galbraith, and D. Choquet. 1999. Cell migration as a five-step cycle. *Biochem Soc Symp*. 65:233-243.

Shellard, A., and R. Mayor. 2016. Chemotaxis during neural crest migration. *Semin Cell Dev Biol*.

Shewan, A.M., M. Maddugoda, A. Kraemer, S.J. Stehbens, S. Verma, E.M. Kovacs, and A.S. Yap. 2005. Myosin 2 is a key Rho kinase target necessary for the local concentration of E-cadherin at cell-cell contacts. *Mol Biol Cell*. 16:4531-4542.

Shi, J., C. Severson, J. Yang, D. Wedlich, and M.W. Klymkowsky. 2011. Snail2 controls mesodermal BMP/Wnt induction of neural crest. *Development*. 138:3135-3145.

Shoval, I., and C. Kalcheim. 2012. Antagonistic activities of Rho and Rac GTPases underlie the transition from neural crest delamination to migration. *Dev Dyn*. 241:1155-1168.

Sidani, M., J. Wyckoff, C. Xue, J.E. Segall, and J. Condeelis. 2006. Probing the microenvironment of mammary tumors using multiphoton microscopy. *J Mammary Gland Biol Neoplasia*. 11:151-163.

Slack-Davis, J.K., K.H. Martin, R.W. Tilghman, M. Iwanicki, E.J. Ung, C. Autry, M.J. Luzzio, B. Cooper, J.C. Kath, W.G. Roberts, and J.T. Parsons. 2007. Cellular characterization of a novel focal adhesion kinase inhibitor. *J Biol Chem*. 282:14845-14852.

Smith, A., V. Robinson, K. Patel, and D.G. Wilkinson. 1997. The EphA4 and EphB1 receptor tyrosine kinases and ephrin-B2 ligand regulate targeted migration of branchial neural crest cells. *Curr Biol*. 7:561-570.

Smith, C.L., and M.D. Tallquist. 2010. PDGF function in diverse neural crest cell populations. *Cell Adh Migr*. 4:561-566.

Soler, A.P., and K.A. Knudsen. 1994. N-cadherin involvement in cardiac myocyte interaction and myofibrillogenesis. *Dev Biol*. 162:9-17.

Steventon, B., C. Araya, C. Linker, S. Kuriyama, and R. Mayor. 2009. Differential requirements of BMP and Wnt signalling during gastrulation and neurulation define two steps in neural crest induction. *Development*. 136:771-779.

Stocker, A.M., and A. Chenn. 2015. The role of adherens junctions in the developing neocortex. *Cell Adh Migr*. 9:167-174.

Stoker, M.G., and H. Rubin. 1967. Density dependent inhibition of cell growth in culture. *Nature*. 215:171-172.

Stramer, B., S. Moreira, T. Millard, I. Evans, C.Y. Huang, O. Sabet, M. Milner, G. Dunn, P. Martin, and W. Wood. 2010. Clasp-mediated microtubule bundling regulates persistent motility and contact repulsion in Drosophila macrophages in vivo. *J Cell Biol*. 189:681-689.

Stramer, B., W. Wood, M.J. Galko, M.J. Redd, A. Jacinto, S.M. Parkhurst, and P. Martin. 2005. Live imaging of wound inflammation in Drosophila embryos reveals key roles for small GTPases during in vivo cell migration. *J Cell Biol*. 168:567-573.

Stramer, B.M., G.A. Dunn, J.R. Davis, and R. Mayor. 2013. Rediscovering contact inhibition in the embryo. *J Microsc*. 251:206-211.

Streit, A., and C.D. Stern. 1999. Establishment and maintenance of the border of the neural plate in the chick: involvement of FGF and BMP activity. *Mech Dev*. 82:51-66.

Suyama, K., I. Shapiro, M. Guttman, and R.B. Hazan. 2002. A signaling pathway leading to metastasis is controlled by N-cadherin and the FGF receptor. *Cancer Cell*. 2:301-314.

Szabó, A., M. Melchionda, G. Nastasi, M.L. Woods, S. Campo, R. Perris, and R. Mayor. 2016. In vivo confinement promotes collective migration of neural crest cells. *J Cell Biol*. 213:543-555.

Tachibana, K., T. Sato, N. D'Avirro, and C. Morimoto. 1995. Direct association of pp125FAK with paxillin, the focal adhesion-targeting mechanism of pp125FAK. *J Exp Med*. 182:1089-1099.

Tallquist, M.D., and P. Soriano. 2003. Cell autonomous requirement for PDGFRalpha in populations of cranial and cardiac neural crest cells. *Development*. 130:507-518.

Tamariz, E., and F. Grinnell. 2002. Modulation of fibroblast morphology and adhesion during collagen matrix remodeling. *Mol Biol Cell*. 13:3915-3929.

Tamkun, J.W., D.W. DeSimone, D. Fonda, R.S. Patel, C. Buck, A.F. Horwitz, and R.O. Hynes. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell*. 46:271-282.

Tanaka, M., S. Kuriyama, and N. Aiba. 2012. Nm23-H1 regulates contact inhibition of locomotion, which is affected by ephrin-B1. *J Cell Sci*. 125:4343-4353.

Taneyhill, L.A., E.G. Coles, and M. Bronner-Fraser. 2007. Snail2 directly represses cadherin6B during epithelial-to-mesenchymal transitions of the neural crest. *Development*. 134:1481-1490.

Tanoue, T., and M. Takeichi. 2005. New insights into Fat cadherins. *J Cell Sci*. 118:2347-2353.

Tedder, T.F., D.A. Steeber, A. Chen, and P. Engel. 1995. The selectins: vascular adhesion molecules. *FASEB J*. 9:866-873.

Teddy, J.M., and P.M. Kulesa. 2004. In vivo evidence for short- and long-range cell communication in cranial neural crest cells. *Development*. 131:6141-6151.

Tepass, U., L.I. Fessler, A. Aziz, and V. Hartenstein. 1994. Embryonic origin of hemocytes and their relationship to cell death in Drosophila. *Development*. 120:1829-1837.

Testaz, S., and J.L. Duband. 2001. Central role of the alpha4beta1 integrin in the coordination of avian truncal neural crest cell adhesion, migration, and survival. *Dev Dyn*. 222:127-140.

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

Theveneau, E., and R. Mayor. 2010. Integrating chemotaxis and contact-inhibition during collective cell migration: Small GTPases at work. *Small GTPases*. 1:113-117.

Theveneau, E., and R. Mayor. 2011. Collective cell migration of the cephalic neural crest: the art of integrating information. *Genesis*. 49:164-176.

Theveneau, E., and R. Mayor. 2012a. Neural crest delamination and migration: from epithelium-to-mesenchyme transition to collective cell migration. *Dev Biol*. 366:34-54.

Theveneau, E., and R. Mayor. 2012b. Neural crest migration: interplay between chemorepellents, chemoattractants, contact inhibition, epithelial-mesenchymal transition, and collective cell migration. *Wiley Interdiscip Rev Dev Biol*. 1:435-445.

Theveneau, E., B. Steventon, E. Scarpa, S. Garcia, X. Trepat, A. Streit, and R. Mayor. 2013. Chase-and-run between adjacent cell populations promotes directional collective migration. *Nat Cell Biol*. 15:763-772.

Thiery, J.P., and M. Morgan. 2004. Breast cancer progression with a Twist. *Nat Med*. 10:777-778.

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

Thorogood, P. 1989. Developmental and Evolutionary Aspects of the Neural Crest. *Trends in neurosciences*. 12:38-39.

Théry, M., V. Racine, M. Piel, A. Pépin, A. Dimitrov, Y. Chen, J.B. Sibarita, and M. Bornens. 2006. Anisotropy of cell adhesive microenvironment governs cell internal organization and orientation of polarity. *Proc Natl Acad Sci U S A*. 103:19771-19776.

Timpson, P., G.E. Jones, M.C. Frame, and V.G. Brunton. 2001. Coordination of cell polarization and migration by the Rho family GTPases requires Src tyrosine kinase activity. *Curr Biol*. 11:1836-1846.

Todaro, G.J., and H. Green. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. *J Cell Biol*. 17:299-313.

Tomar, A., and D.D. Schlaepfer. 2009. Focal adhesion kinase: switching between GAPs and GEFs in the regulation of cell motility. *Curr Opin Cell Biol*. 21:676-683.

Trichet, L., J. Le Digabel, R.J. Hawkins, S.R. Vedula, M. Gupta, C. Ribrault, P. Hersen, R. Voituriez, and B. Ladoux. 2012. Evidence of a large-scale mechanosensing mechanism for cellular adaptation to substrate stiffness. *Proc Natl Acad Sci U S A*. 109:6933-6938.

Trinkaus, J.P., T. Betchaku, and L.S. Krulikowski. 1971. Local inhibition of ruffling during contact inhibition of cell movement. *Exp Cell Res*. 64:291-300.

Truffi, M., V. Dubreuil, X. Liang, N. Vacaresse, F. Nigon, S.P. Han, A.S. Yap, G.A. Gomez, and J. Sap. 2014. RPTPα controls epithelial adherens junctions, linking E-cadherin engagement to c-Src-mediated phosphorylation of cortactin. *J Cell Sci*. 127:2420-2432.

Tsai, J., and L. Kam. 2009. Rigidity-dependent cross talk between integrin and cadherin signaling. *Biophys J*. 96:L39-41.

Tseng, Q., E. Duchemin-Pelletier, A. Deshiere, M. Balland, H. Guillou, O. Filhol, and M. Théry. 2012. Spatial organization of the extracellular matrix regulates cell-cell junction positioning. *Proc Natl Acad Sci U S A*. 109:1506-1511.

Tsukita, S., K. Oishi, T. Akiyama, Y. Yamanashi, and T. Yamamoto. 1991. Specific proto-oncogenic tyrosine kinases of src family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. *J Cell Biol*. 113:867-879.

Tucker, R.P. 2001. Abnormal neural crest cell migration after the in vivo knockdown of tenascin-C expression with morpholino antisense oligonucleotides. *Dev Dyn*. 222:115-119.

Ussar, S., M. Moser, M. Widmaier, E. Rognoni, C. Harrer, O. Genzel-Boroviczeny, and R. Fässler. 2008. Loss of Kindlin-1 causes skin atrophy and lethal neonatal intestinal epithelial dysfunction. *PLoS Genet*. 4:e1000289.

Utton, M.A., B. Eickholt, F.V. Howell, J. Wallis, and P. Doherty. 2001. Soluble N-cadherin stimulates fibroblast growth factor receptor dependent neurite outgrowth and N-cadherin and the fibroblast growth factor receptor co-cluster in cells. *J Neurochem*. 76:1421-1430.

Van de Putte, T., M. Maruhashi, A. Francis, L. Nelles, H. Kondoh, D. Huylebroeck, and Y. Higashi. 2003. Mice lacking ZFHX1B, the gene that codes for Smad-interacting protein-1, reveal a role for multiple neural crest cell defects in the etiology of Hirschsprung disease-mental retardation syndrome. *Am J Hum Genet*. 72:465-470.

Van Itallie, C.M., and J.M. Anderson. 2014. Architecture of tight junctions and principles of molecular composition. *Semin Cell Dev Biol*. 36:157-165.

Vandewalle, C., J. Comijn, B. De Craene, P. Vermassen, E. Bruyneel, H. Andersen, E. Tulchinsky, F. Van Roy, and G. Berx. 2005. SIP1/ZEB2 induces EMT by repressing genes of different epithelial cell-cell junctions. *Nucleic Acids Res*. 33:6566-6578.

Vasioukhin, V., C. Bauer, M. Yin, and E. Fuchs. 2000. Directed actin polymerization is the driving force for epithelial cell-cell adhesion. *Cell*. 100:209-219.

Vedula, P., L.A. Cruz, N. Gutierrez, J. Davis, B. Ayee, R. Abramczyk, and A.J. Rodriguez. 2016. Quantifying cadherin mechanotransduction machinery assembly/disassembly dynamics using fluorescence covariance analysis. *Sci Rep*. 6:28822.

Vega, S., A.V. Morales, O.H. Ocaña, F. Valdés, I. Fabregat, and M.A. Nieto. 2004. Snail blocks the cell cycle and confers resistance to cell death. *Genes Dev*. 18:1131-1143.

Veracini, L., D. Grall, S. Schaub, S. Beghelli-de la Forest Divonne, M.C. Etienne-Grimaldi, G. Milano, A. Bozec, E. Babin, A. Sudaka, J. Thariat, and E. Van Obberghen-Schilling. 2015.

Elevated Src family kinase activity stabilizes E-cadherin-based junctions and collective movement of head and neck squamous cell carcinomas. *Oncotarget*. 6:7570-7583.
Veselý, P., and R.A. Weiss. 1973. Cell locomotion and contact inhibition of normal and neoplastic rat cells. *Int J Cancer*. 11:64-76.
Vicente-Manzanares, M., C.K. Choi, and A.R. Horwitz. 2009. Integrins in cell migration--the actin connection. *J Cell Sci*. 122:199-206.
Villar-Cerviño, V., M. Molano-Mazón, T. Catchpole, M. Valdeolmillos, M. Henkemeyer, L.M. Martínez, V. Borrell, and O. Marín. 2013. Contact repulsion controls the dispersion and final distribution of Cajal-Retzius cells. *Neuron*. 77:457-471.
Vuori, K., H. Hirai, S. Aizawa, and E. Ruoslahti. 1996. Introduction of p130cas signaling complex formation upon integrin-mediated cell adhesion: a role for Src family kinases. *Mol Cell Biol*. 16:2606-2613.
Wan, H., K. Lin, S.M. Tsang, and J. Uttagomol. 2016. Evidence for Dsg3 in regulating Src signaling by competing with it for binding to caveolin-1. *Data Brief*. 6:124-134.
Wang, A.Z., G.K. Ojakian, and W.J. Nelson. 1990. Steps in the morphogenesis of a polarized epithelium. I. Uncoupling the roles of cell-cell and cell-substratum contact in establishing plasma membrane polarity in multicellular epithelial (MDCK) cysts. *J Cell Sci*. 95 (Pt 1):137-151.
Wang, H., V. Radjendirane, K.K. Wary, and S. Chakrabarty. 2004. Transforming growth factor beta regulates cell-cell adhesion through extracellular matrix remodeling and activation of focal adhesion kinase in human colon carcinoma Moser cells. *Oncogene*. 23:5558-5561.
Wang, H.U., and D.J. Anderson. 1997. Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. *Neuron*. 18:383-396.
Wang, J.H., and J.S. Lin. 2007. Cell traction force and measurement methods. *Biomech Model Mechanobiol*. 6:361-371.
Wang, Y., E.L. Botvinick, Y. Zhao, M.W. Berns, S. Usami, R.Y. Tsien, and S. Chien. 2005. Visualizing the mechanical activation of Src. *Nature*. 434:1040-1045.
Wang, Y., H. Cao, J. Chen, and M.A. McNiven. 2011. A direct interaction between the large GTPase dynamin-2 and FAK regulates focal adhesion dynamics in response to active Src. *Mol Biol Cell*. 22:1529-1538.
Wang, Y., G. Jin, H. Miao, J.Y. Li, S. Usami, and S. Chien. 2006. Integrins regulate VE-cadherin and catenins: dependence of this regulation on Src, but not on Ras. *Proc Natl Acad Sci U S A*. 103:1774-1779.
Wang, Y.C., Z. Khan, M. Kaschube, and E.F. Wieschaus. 2012. Differential positioning of adherens junctions is associated with initiation of epithelial folding. *Nature*. 484:390-393.
Wang, Z., J.M. Symons, S.L. Goldstein, A. McDonald, J.H. Miner, and J.A. Kreidberg. 1999. (Alpha)3(beta)1 integrin regulates epithelial cytoskeletal organization. *J Cell Sci*. 112 (Pt 17):2925-2935.
Watanabe, N., L. Bodin, M. Pandey, M. Krause, S. Coughlin, V.A. Boussiotis, M.H. Ginsberg, and S.J. Shattil. 2008. Mechanisms and consequences of agonist-induced talin recruitment to platelet integrin alphaIIbbeta3. *J Cell Biol*. 181:1211-1222.
Watanabe, N., and T.J. Mitchison. 2002. Single-molecule speckle analysis of actin filament turnover in lamellipodia. *Science*. 295:1083-1086.
Waterman-Storer, C.M., W.C. Salmon, and E.D. Salmon. 2000. Feedback interactions between cell-cell adherens junctions and cytoskeletal dynamics in newt lung epithelial cells. *Mol Biol Cell*. 11:2471-2483.

Webb, D.J., K. Donais, L.A. Whitmore, S.M. Thomas, C.E. Turner, J.T. Parsons, and A.F. Horwitz. 2004. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol*. 6:154-161.

Webb, D.J., J.T. Parsons, and A.F. Horwitz. 2002. Adhesion assembly, disassembly and turnover in migrating cells -- over and over and over again. *Nat Cell Biol*. 4:E97-100.

Weber, G.F., M.A. Bjerke, and D.W. DeSimone. 2011. Integrins and cadherins join forces to form adhesive networks. *J Cell Sci*. 124:1183-1193.

Wehrle-Haller, B. 2012a. Assembly and disassembly of cell matrix adhesions. *Curr Opin Cell Biol*. 24:569-581.

Wehrle-Haller, B. 2012b. Structure and function of focal adhesions. *Curr Opin Cell Biol*. 24:116-124.

Westhoff, M.A., B. Serrels, V.J. Fincham, M.C. Frame, and N.O. Carragher. 2004. SRC-mediated phosphorylation of focal adhesion kinase couples actin and adhesion dynamics to survival signaling. *Mol Cell Biol*. 24:8113-8133.

Wheelock, M.J., Y. Shintani, M. Maeda, Y. Fukumoto, and K.R. Johnson. 2008. Cadherin switching. *J Cell Sci*. 121:727-735.

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

Wilson, P.D. 2011. Apico-basal polarity in polycystic kidney disease epithelia. *Biochim Biophys Acta*. 1812:1239-1248.

Wolfenson, H., A. Bershadsky, Y.I. Henis, and B. Geiger. 2011. Actomyosin-generated tension controls the molecular kinetics of focal adhesions. *J Cell Sci*. 124:1425-1432.

Wolfenson, H., I. Lavelin, and B. Geiger. 2013. Dynamic regulation of the structure and functions of integrin adhesions. *Dev Cell*. 24:447-458.

Woo, S., D.J. Rowan, and T.M. Gomez. 2009. Retinotopic mapping requires focal adhesion kinase-mediated regulation of growth cone adhesion. *J Neurosci*. 29:13981-13991.

Wood, W., C. Faria, and A. Jacinto. 2006. Distinct mechanisms regulate hemocyte chemotaxis during development and wound healing in Drosophila melanogaster. *J Cell Biol*. 173:405-416.

Woods, A., and J.R. Couchman. 2001. Syndecan-4 and focal adhesion function. *Curr Opin Cell Biol*. 13:578-583.

Woods, M.L., C. Carmona-Fontaine, C.P. Barnes, I.D. Couzin, R. Mayor, and K.M. Page. 2014. Directional collective cell migration emerges as a property of cell interactions. *PLoS One*. 9:e104969.

Worth, D.C., and M. Parsons. 2010. Advances in imaging cell-matrix adhesions. *J Cell Sci*. 123:3629-3638.

Wozniak, M.A., K. Modzelewska, L. Kwong, and P.J. Keely. 2004. Focal adhesion regulation of cell behavior. *Biochim Biophys Acta*. 1692:103-119.

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

Xing, Z., H.C. Chen, J.K. Nowlen, S.J. Taylor, D. Shalloway, and J.L. Guan. 1994. Direct interaction of v-Src with the focal adhesion kinase mediated by the Src SH2 domain. *Mol Biol Cell*. 5:413-421.

Xu, X., R. Francis, C.J. Wei, K.L. Linask, and C.W. Lo. 2006. Connexin 43-mediated modulation of polarized cell movement and the directional migration of cardiac neural crest cells. *Development*. 133:3629-3639.

Xu, X., W.E. Li, G.Y. Huang, R. Meyer, T. Chen, Y. Luo, M.P. Thomas, G.L. Radice, and C.W. Lo. 2001. N-cadherin and Cx43alpha1 gap junctions modulates mouse neural crest cell motility via distinct pathways. *Cell Commun Adhes*. 8:321-324.

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

Yang, J.T., H. Rayburn, and R.O. Hynes. 1995. Cell adhesion events mediated by alpha 4 integrins are essential in placental and cardiac development. *Development*. 121:549-560.

Yano, H., Y. Mazaki, K. Kurokawa, S.K. Hanks, M. Matsuda, and H. Sabe. 2004. Roles played by a subset of integrin signaling molecules in cadherin-based cell-cell adhesion. *J Cell Biol*. 166:283-295.

Yao, M., W. Qiu, R. Liu, A.K. Efremov, P. Cong, R. Seddiki, M. Payre, C.T. Lim, B. Ladoux, R.M. Mège, and J. Yan. 2014. Force-dependent conformational switch of α-catenin controls vinculin binding. *Nat Commun*. 5:4525.

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

Yeaman, C., K.K. Grindstaff, and W.J. Nelson. 1999. New perspectives on mechanisms involved in generating epithelial cell polarity. *Physiol Rev*. 79:73-98.

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

Yu, C.H., J.B. Law, M. Suryana, H.Y. Low, and M.P. Sheetz. 2011. Early integrin binding to Arg-Gly-Asp peptide activates actin polymerization and contractile movement that stimulates outward translocation. *Proc Natl Acad Sci U S A*. 108:20585-20590.

Yu, H.H., and C.B. Moens. 2005. Semaphorin signaling guides cranial neural crest cell migration in zebrafish. *Dev Biol*. 280:373-385.

Yu, Y., J. Zhu, L.Z. Mi, T. Walz, H. Sun, J. Chen, and T.A. Springer. 2012. Structural specializations of α(4)β(7), an integrin that mediates rolling adhesion. *J Cell Biol*. 196:131-146.

Yuan, L., M.J. Fairchild, A.D. Perkins, and G. Tanentzapf. 2010. Analysis of integrin turnover in fly myotendinous junctions. *J Cell Sci*. 123:939-946.

Zaidel-Bar, R., C. Ballestrem, Z. Kam, and B. Geiger. 2003. Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J Cell Sci*. 116:4605-4613.

Zaidel-Bar, R., M. Cohen, L. Addadi, and B. Geiger. 2004. Hierarchical assembly of cell-matrix adhesion complexes. *Biochem Soc Trans*. 32:416-420.

Zaidel-Bar, R., R. Milo, Z. Kam, and B. Geiger. 2007. A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. *J Cell Sci*. 120:137-148.

Zamir, E., and B. Geiger. 2001. Molecular complexity and dynamics of cell-matrix adhesions. *J Cell Sci*. 114:3583-3590.

Zamir, E., M. Katz, Y. Posen, N. Erez, K.M. Yamada, B.Z. Katz, S. Lin, D.C. Lin, A. Bershadsky, Z. Kam, and B. Geiger. 2000. Dynamics and segregation of cell-matrix adhesions in cultured fibroblasts. *Nat Cell Biol*. 2:191-196.

Zheng, X.M., R.J. Resnick, and D. Shalloway. 2000. A phosphotyrosine displacement mechanism for activation of Src by PTPalpha. *EMBO J*. 19:964-978.