

The regulation of cell-cell adhesion by GTPase activating proteins

by

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**This thesis is submitted to Imperial College London for the Degree of Doctor
of Philosophy**

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Declaration of Originality

I declare that all data presented within this thesis is my own and any contribution from others is clearly acknowledged

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Acknowledgements

Firstly, I would like to thank my supervisor, Vania Braga, for allowing me to carry out this work, and all her enthusiasm, help and guidance throughout this project. I also want to thank Tony Magee for his support.

I am very grateful to everyone in the Braga lab for the help and advice (and cake) over the last few years. I have made many friends here who have made my time in the lab so enjoyable, and I feel very lucky to have been able to work in an environment where people have always been willing to go out of their way to offer help. Particularly, I wish to thank Seb for all the invaluable advice during the beginning of this project, Mat for kindly helping me prepare for my Viva, Susann for being exceptionally helpful (especially regarding cloning) and Natalie, for (amongst so many other things) listening to my moaning about experiments and then always trying to fix them, this thesis would probably not be finished without her help. I am also grateful to Irina for help with mutagenesis and Reiko for her supernatural protein production skills.

I wish to thank all my friends and family for always being so kind and supportive throughout my studies, and reminding me that there are other things worth talking about that are not related to cell-cell adhesion. I am especially grateful to Jen and Abdullah for tea breaks, Becky for her unwavering support, Charles, for technical advice, Lauren for always being so kind and supportive and Louis, for being so incredibly encouraging and looking after me during the write-up. I particularly wish to thank my Father, for always being interested in my work and offering me so much encouragement. To all these people I am exceptionally grateful.

Finally, this work was made possible thanks to funding from the BBSRC.

Abstract

Adherens junctions (AJs) are responsible for the adhesion of cells to their neighbours and influence a range of cellular processes. The formation and maintenance of AJs is governed by members of the Rho subfamily of small GTPases. Activation of the GTPases Rac1 and RhoA is necessary for the formation of cell-cell contacts in keratinocytes. However, how the GTPases themselves are regulated is not well understood. Rho GTPase activating proteins (GAPs) inactivate GTPases by promoting the hydrolysis of GTP to GDP. Whilst a large number of proteins containing Rho GAP domains have been identified, knowledge of this large family of proteins remains limited. It is predicted that multiple Rho GAPs are required in order to coordinate precise processes that are necessary for the formation and maintenance of cell-cell contacts to occur. However, relatively few Rho GAPs have been identified as regulators of cell-cell contacts. The aims of this project are to 1) identify GAPs involved in the regulation of cell-cell contact formation in keratinocytes and 2) to specifically investigate the role of CdGAP in junction formation and maintenance.

To this end, 20 GAPs have been identified from a siRNA screen as potential regulators of junction formation, the vast majority of which have not previously been linked to this process. Subsequently, two Rho-specific GAPs have been investigated to probe their roles in the regulation of Rho in both junction formation and maintenance. The second part of this thesis focuses on the Rac and Cdc42-specific GAP CdGAP, which I demonstrate is an important regulator of AJs in keratinocytes. I show that CdGAP is able to interact with the scaffold protein Ajuba. Ajuba is known to localise to cell-cell adhesions, where it is necessary for maintaining Rac activation at junctions. However, Ajuba itself has no catalytic activity. My data suggests cooperation between CdGAP and Ajuba allows Rac levels to be fine-tuned at AJs. Overall, this data identifies numerous Rho GAPs as potential regulators of junction formation, and confirms for the first time the importance of three Rho GAPs, ARAP1, ARHGAP6 and CdGAP, in the regulation of cell-cell contact formation and maintenance. Ultimately, this work provides further insight into the regulation of AJs by GAPs and enhances our understanding of how scaffold proteins, such as Ajuba, are able to influence this tightly controlled process.

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Abbreviations

%THA – Percentage threshold area

ABR – Active BCR-related gene

AJ – Adherens junction

ANOVA – Analysis of variance

AOS – Adams-Oliver syndrome

APC – adenomatous polyposis coli

APS – Ammonium Persulphate

ARAP – Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein

Arf – ADP-Ribosylation Factor

Arp2/3 – Actin-related protein 2/3

ASAP - Arf-GAP with SH3 domain, ANK repeat and PH domain-containing protein

Asef – APC stimulated exchange factor 1

BAR - Bin, amphiphysin, RSV161/167

BCIP - bromochloroindolylphosphate

BCR – Breakpoint cluster region

BSA - Bovine serum albumin

C2 – cysteine-rich phorbol ester binding

CBS – Citrate-buffered saline

CdGAP – Cdc42 GTPase activating protein

CDH1 – Cadherin-1

CFP – Cyan fluorescent protein

CIP4 – Cdc42-interacting protein 4

CKI/II – Casein kinase I/II

COS – CV-1 in origin and carrying SV40 genetic material

CRIB - Cdc42/Rac interacting binding

DAG – Diacylglycerol

DCS – Donor calf serum

DEPC - diethylpyrocarbonate

DH – Dbl homology

Dia - Diaphanous

DLC – Deleted in liver cancer

Dlg – Discs large homolog
DMEM - Dulbecco's modified Eagle's medium
DMSO - Dimethyl sulphoxide
DNA - Deoxyribonucleic Acid
dNTP - di-Nucleotide tri-phosphate
DOCK - Dedicator of cytokinesis
DRF – Diaphanous-related formins
DTT - Dithiothreitol
EC1-5 – Extracellular domain 1-5
E-cadherin - Epithelial cadherin
ECL - Enhanced chemiluminescence
ECM – Extracellular matrix
ECT2 - epithelial cell-transforming sequence 2 oncogene
EDTA - Ethylenediaminetetraacetic acid
EGF – Epidermal Growth Factor
EGFR – Epidermal Growth Factor Receptor
EPAC – Exchange protein directly activated by cAMP 1
EPLIN – Epithelial protein lost in neoplasm
ER - Endoplasmic Reticulum
ERK – Extracellular signal-regulated kinase
ERM - ezrin, radixin, moesin
FAK – Focal adhesion kinase
FCH – Fes/CIP4 homology
FCS – Foetal calf serum
FLIM - Fluorescence lifetime imaging
FRAP – Fluorescence recovery after photobleaching
FRET - Forster-resonance energy transfer
GAP – GTPase-Activating Protein
GBD – GTPase binding domain
GBF1 - Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1
GDI – Guanine-nucleotide Dissociation Inhibitor
GDP - Guanosine di-phosphate
GEF – Guanine-nucleotide Exchange Factor
GFP – Green Fluorescence Protein

GMIP – Gem interacting protein
 GRAF – GTPase regulator associated with focal adhesion kinase
 GRF1 – Ras-specific guanine nucleotide-releasing factor 1
 GSK3 β - Glycogen synthase kinase 3
 GST – Glutathione-s-Transferase
 GTP - Guanosine tri-phosphate
 HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
 HGF - Hepatocyte growth factor
 HRP – Horseradish Peroxidase
 INPP5B - inositol polyphosphate 5-phosphatase type II
 IP – Immunoprecipitation
 IPTG – Isopropyl β -D-1-thiogalactopyranoside
 IQ – IQ calmodulin-binding motif
 IQGAP - IQ motif containing GTPase activating protein
 IVT - in vitro translation
 JAM – Junctional adhesion molecule
 JNK – c-Jun N-terminal kinase
 LARG - Leukemia associated Rho GEF
 LB – Luria broth
 LEF-1 – Lymphoid enhancer factor 1
 LIM – Lin11, Isl-1 and Mec-3
 LIMK - LIM domain kinase
 LPP – Leupeptin, pepstatin, pefabloc
 MAPK – Mitogen-activated protein kinase
 MCF-7 – Michigan Cancer Foundation-7
 MDCK - Madin-Darby canine kidney
 MEF – Mouse embryonic fibroblast
 MHC – Myosin heavy chain
 miR – micro RNA
 MLC – Myosin light chain
 MLCK – Myosin light chain kinase
 MLCP – Myosin light chain phosphatase
 MLS - microphthalmia with linear skin defects syndrome
 MMP – Matrix metalloproteinase

mRNA - messenger RNA
 MTT - thiazolyl blue tetrazolium bromide
 Myo9A – Myosin IXA
 Myo9B – Myosin IXB
 MyoII – Myosin II
 NET – Neuroepithelial transforming protein
 NF- κ B – Nuclear factor-kappa beta
 NS – Not significant
 OCRL1 – Oculocerebrorenal Lowe syndrome phosphatase
 OV – Sodium orthovanadate
 Pan - Pangolin
 PAK - p21 activated kinase
 PAR - Protease-activated Receptor
 PARG1 - PTPL1 associated RhoGAP
 PBS – Phosphate Buffered Saline
 PBS-T – PBS-Tween
 PCR – Polymerase Chain Reaction
 PDGF – Platelet derived growth factor
 PDZ - post synaptic density protein, *Drosophila* disc large tumor suppressor, and zonula occludens-1 protein
 PFA – Paraformaldehyde
 PH - Pleckstrin homology
 PI3K – Phosphatidylinositol-3-Kinase
 PIP - Phosphatidylinositol phosphate
 PIP₃ - Phosphatidylinositol (3,4,5)-trisphosphate
 PIPES – piperazine-N,N'-bis(2-ethanesulfonic acid)
 PKC - Protein Kinase C
 PKN - PKC-related kinase
 PLC - Phospholipase C
 PLD - Phospholipase D
 PM - Plasma membrane
 PMSF - phenylmethanesulfonylfluoride
 PPI – Protein-protein interaction
 PVDF – Polyvinylidene difluoride

PX – Phox homology
RA – Ras associating
Rab – Ras-like in the brain
Ran – Ras-related nuclear protein
Rap – Ras-related protein
Ras - Rat Sarcoma
RBD – Rho binding domain
RFP - Red Fluorescence Protein
Rho – Ras homologous
RICH - RhoGAP interacting with CIP4 homologs protein
RICS – RhoGAP involved in the beta-catenin-N-cadherin and NMDA receptor signaling
RNA - Ribonucleic acid
RNAi - RNA interference
Robo – Roundabout
ROCK – Rho associated kinase
ROS – Reactive oxygen species
RSK – Ribosomal protein S6 kinase
RTK – Receptor tyrosine kinase
SAM – sterile α -motif
SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SH2/3 - Src homology 2/3
SH3BP1 – SH3 domain-binding protein 1
siRNA - small interfering RNA
Slp1 – synaptotagmin-like protein
Slug – Zinc finger binding protein SNAI2
SNAIL – Zinc-finger binding protein SNAI1
SRGAP - slit-robo GAP
START – StAR-related lipid transfer
TBS - Tris Buffered Saline
TCF – T-cell factor
TEMED - N,N,N,N'-Tetramethylethylenediamine
TGF- β – Transforming growth factor beta
Tiam1- T-cell lymphoma invasion and metastasis 1
TJ – Tight junction

TRIO – Triple functioning domain

Twist – Twist basic helix-loop-helix transcription factor 1

WASP - Wiskott–Aldrich syndrome protein

WAVE - WASP family verprolin-homologous protein

WT - Wild Type

YFP – Yellow fluorescent protein

ZEB1/2 - Zinc finger E-box-binding homeobox 1/2

ZO – Zonula adherens

Chapter 1.

Introduction

1.1. Epithelial tissue

Epithelial tissues, formed of tightly connected epithelial cells, are found lining internal organs and the external surface of the body. The epithelium provides an essential barrier protecting the underlying tissues from pathogenic, physical or chemical assault as well as serving a structural function by maintaining tissue architecture.

The epidermis is formed of multiple layers of squamous epithelium, largely consisting of keratinocytes, that lies on a complex meshwork of connective tissue called the basement membrane (**Figure 1.1**). Due to the continual barrage of environmental stressors, the uppermost layer of the skin is constantly being renewed. This is facilitated by stem cells in the basal layer that possess the proliferative capacity to maintain the homeostasis of this tissue. These give rise to transit amplifying cells, which are destined to undergo a finite number of divisions before committing to terminal differentiation and migrating upwards. The constant renewal of cells of the epidermis creates a highly dynamic and protective structure (Blanpain and Fuchs, 2009; Watt, 1998).

A common feature of epithelial cells is the presence of multiple adhesive structures both between neighbouring cells and between cells of the basal layer and the underlying basement membrane (Blanpain and Fuchs, 2009). The ability of cells of the epidermis to adhere strongly to one another is absolutely vital for their correct function. However, these dynamic intercellular adhesions are constantly changed, as cells move upwards upon terminal differentiation, and are susceptible to transient downregulation in response to certain extracellular cues, for example during wound healing (Fuchs and Raghavan, 2002).

1.1.1. Cell-cell adhesive complexes

Specialised cell-cell adhesive complexes are essential for maintaining the epidermal barrier by physically adjoining neighbouring cells, facilitating communication, and forming robust links to the cytoskeleton. These complexes also act as signalling centres in their own right, able to direct transcriptional changes that influence proliferation and differentiation

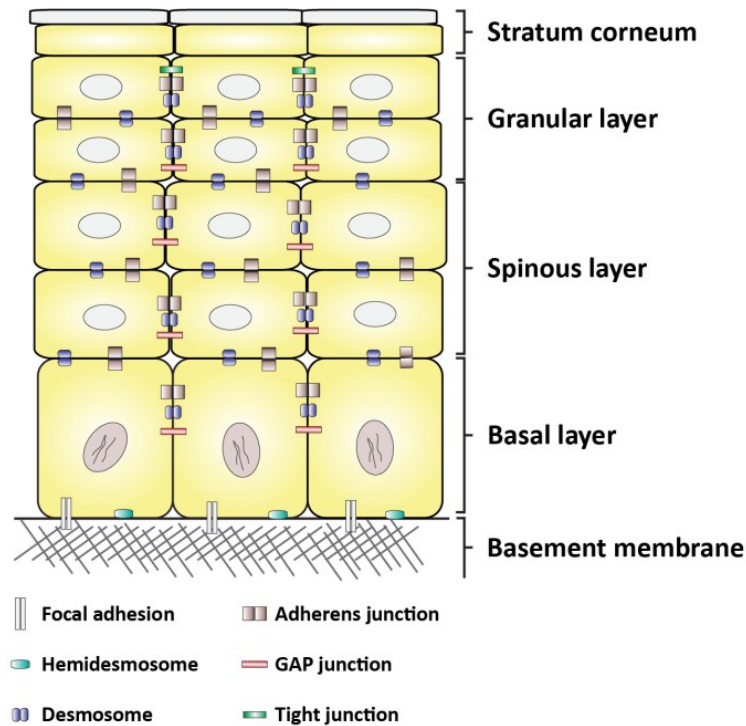


Figure 1.1 The structure of the epidermis The multiple layers of epithelium that make up the epidermis are anchored, via cells of the basal layer, to the basement membrane via specialised adhesive structures called focal adhesions and hemidesmosomes. Stem cells of the basal layer maintain homeostasis by replacing keratinocytes that are continually lost through differentiation or injury. This results in a stratified epithelium composed of terminally differentiating cells transiting from the spinous layer to the granular layer and finally the stratum corneum. These layers can be distinguished by the presence of different adhesive complexes and intermediate filaments that serve to rigidify the uppermost layers, culminating in the formation of a highly resistant barrier. Intercellular adhesion is mediated by adherens junctions and desmosomes, which are anchored to the actin cytoskeleton and intermediate filaments (keratin) respectively. Gap junctions allow the passage of small molecules and ions between cells. Finally, tight junctions are only formed in cells of the granular layer where they provide a barrier function and separate the apical and basolateral portions of the cell membrane

The main structures responsible for cell-cell adhesion and thus epithelial architecture are the adherens junctions (AJs) and desmosomes. Both of these junctions are formed via interactions between calcium-dependent cadherin transmembrane proteins that are coupled to the cytoskeleton (AJs to actin and desmosomes to intermediate filaments) via association with various intermediaries, including armadillo family proteins (Green et al., 2010).

AJs (discussed in detail in section 1.2) are found in large numbers in cells of the basal layer and their assembly precedes desmosome formation (Green et al., 2010). Loss of major components of AJs or desmosomes impairs barrier function (Tinkle et al., 2008; Vasioukhin et al., 2001b). However, these structures also serve as landmarks around which additional cellular structures orient themselves. For example, loss of E-cadherin can severely compromise tight junction (TJ) barrier function (Michels et al., 2009) whilst desmosomes can regulate microtubule organisation at cell-cell contacts (Lechler and Fuchs, 2007). Furthermore, the expression pattern of desmosomal cadherins alters during epidermal differentiation, implying specific roles for different desmosomal cadherins in the regulation of homeostasis (Green and Simpson, 2007). Indeed, misexpression of different desmosomal cadherins can cause a range of phenotypes (eg. hyperproliferation, abnormal differentiation, reduction in barrier function and increased susceptibility to carcinogens) (Delva et al., 2009; Green and Simpson, 2007).

TJs are composed of junctional adhesion molecules (JAMs), occludins and claudins which are supported by a selection of scaffold proteins, including those from the zonula adherens family (ZO-1, ZO-2 and ZO-3) (Niessen, 2007). Study of claudin-1-deficient mice reveals the importance of TJs in providing an efficient barrier, as mice die within one week of birth due to dehydration (Furuse et al., 2002). Interestingly, protein synthesis of TJ components precedes formation of the stratum corneum during epidermal regeneration, suggesting these junctions represent part of a rescue system (Brandner et al., 2002). TJs may also have a role in regulating polarity, as polarity proteins are associated with TJs in keratinocytes (Michels et al., 2009). In the epidermis TJ assembly is restricted to the granular layer. However, expression of some TJ components (Claudin-1, JAM-1) can be found in all layers implying TJ-independent functions for these proteins (Kirschner and Brandner, 2012; Niessen, 2007; Proksch et al., 2008).

Gap junctions are composed of hexamers of connexins (connexons) that are trafficked to the membrane where they fuse with connexons on an adjacent cell. This results in the formation of a channel that spans the two membranes and permits the passage of small molecules and ions (Goodenough and Paul, 2009; Proksch et al., 2008). As well as permitting cell-cell communication,

Gap junctions are also necessary for barrier function (Proksch et al., 2008). The epidermis of mice lacking the C-terminal of connexin 43 is highly permeable compared to wild-type mice, and the majority of these animals die within a week of birth (Maass et al., 2004). Further, numerous skin disorders have been characterised that arise from mutations in connexins, for example mutations of connexin 26 are associated with keratitis-ichthyosis-deafness, Vohwinkel syndrome and palmoplantar keratoderma, amongst others (Scott et al., 2012).

1.1.2. Cell-matrix adhesive complexes

Keratinocytes are anchored to the basement membrane via focal adhesions and hemidesmosomes. The interaction between the cell and the basement membrane can have profound effects on tissue morphology as these structures transmit mechanical signals from the extracellular environment to regulate differentiation, proliferation and migration. Importantly, cell-matrix adhesions must coordinate signals with cell-cell adhesions in order to regulate homeostasis (Burute and Thery, 2012; Watt, 2002).

Hemidesmosomes are formed from the transmembrane protein 180kDa-bullous pemphigoid antigen, $\alpha 6\beta 4$ integrin and CD151 which are linked to keratin filaments, thus providing robust mechanical support and also anchoring the cell to the basement membrane (Tsuruta et al., 2011). Loss of hemidesmosomes leads to uncoupling of keratinocytes from the basement membrane which manifests as severe blistering in affected individuals (notably in epidermolysis bullosa) (Sawamura et al., 2010). In cultured keratinocytes hemidesmosomes do not form, instead hemidesmosome proteins are aggregated at the cell-substrate interface. However, these lack some ultra-structural features of hemidesmosomes (Tsuruta et al., 2011).

Focal adhesions are formed from different combinations of integrins and are linked to the actin cytoskeleton via a complex mix of adaptor proteins (Burute and Thery, 2012; Watt, 2002). Focal adhesions contribute towards anchorage to the basement membrane, evidenced by findings that mutation in fermitin family homolog 1, a component of focal adhesions, causes the blistering disease Kindler syndrome (Jobard et al., 2003). However, the large variety in integrin configurations that can be adopted means focal adhesions can mediate the transmission of myriad extracellular signals. For example, signals transmitted via integrins are important in regulating cell migration during wound healing (Tsuruta et al., 2011).

1.2. Molecular overview of AJs

This project focuses on the regulation of AJs. The basic core of AJs is the cadherin-catenin complex, some form of which is conserved in all animals, and also found in other organisms, including arthropods (Hiroki, 2012). In epithelial cells the classical epithelial (E-)cadherin is predominant (Furukawa et al., 1997). Cell-cell adhesion is mediated by homophilic interactions between E-cadherin monomers on neighbouring cells (in *trans*) which are later stabilised via lateral clustering (in *cis*) (**Figure 1.2**) (Ishiyama and Ikura, 2012; Meng and Takeichi, 2009; Zhang et al., 2009).

E-cadherin possesses a large extracellular domain that can be subdivided into five subunits (EC1-5), through which the EC1 subunit mediates homophilic interactions (Nose et al., 1990). Association of the extracellular domains with calcium serves to rigidify this region, priming it for interactions and also preventing proteolytic cleavage (Ishiyama and Ikura, 2012; Nagar et al., 1996). Notably, the calcium dependence of E-cadherin can be exploited by researchers to specifically induce cell-cell contact formation in cells maintained in a low calcium environment, via the addition of calcium ions (O'Keefe et al., 1987).

1.2.1. The cadherin-catenin complex

The cytoplasmic portion of E-cadherin can be split into two major domains, the juxtamembrane domain, which binds p120-catenin (Reynolds et al., 1994), and the C-terminal catenin-binding domain, which binds β -catenin. This complex is then transiently or indirectly linked to the actin cytoskeleton via α -catenin (Hiroki, 2012; Meng and Takeichi, 2009).

Interaction with p120 catenin stabilises E-cadherin; downregulation of p120 catenin results in E-cadherin internalisation (Davis et al., 2003), whilst expression of E-cadherin binding-deficient p120 catenin reduces the amount of surface E-cadherin in MCF-7 and MDCK cells (Ishiyama et al., 2010). Binding of p120 catenin occupies a large portion of the E-cadherin juxtamembrane domain (Ishiyama et al., 2010). One mechanism by which p120 catenin could stabilise E-cadherin may be by blocking the binding site on E-cadherin for Hakai, interaction with which can promote E-cadherin ubiquitination and degradation (Fujita et al., 2002).

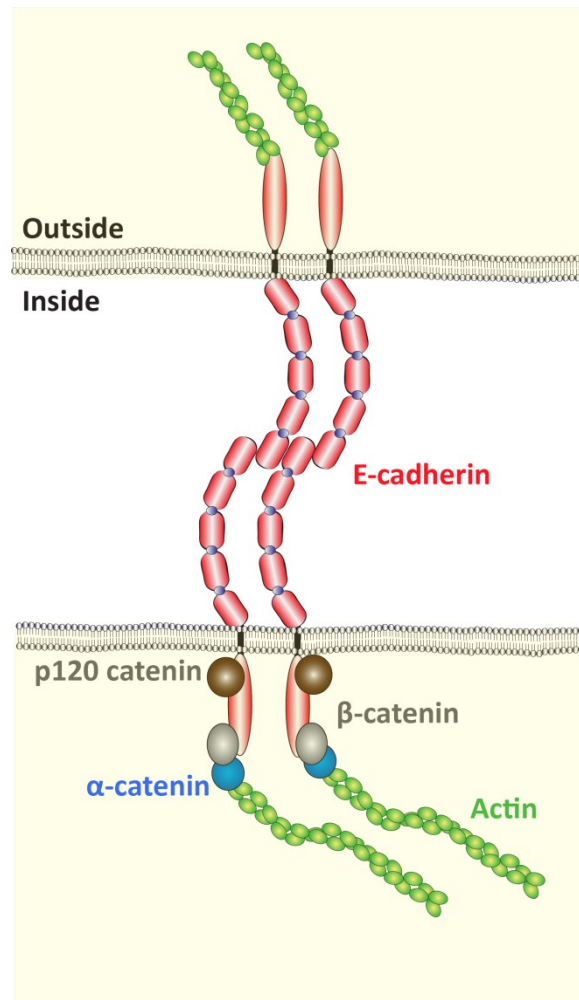


Figure 1.2 The organisation of AJs. Calcium ions (navy blue circles) bind to the E-cadherin extracellular domain and rigidify this region. This facilitates homophilic binding between E-cadherin monomers on adjacent cells via the last subunit of the extracellular domain (EC1). The E-cadherin cytoplasmic tail interacts with p120-catenin and β -catenin, which is in turn bound to α -catenin. The complex is linked to the actin cytoskeleton either via conformational changes in α -catenin that allow it to bind simultaneously actin and β -catenin, or via additional interactions mediated between α -catenin and actin binding proteins (not shown).

1.2.2. Links to the actin cytoskeleton

E-cadherin binds to β -catenin via the latter's central armadillo repeats (Huber and Weis, 2001). Simultaneously, β -catenin can interact with α -catenin via its N-terminal region providing a link to the cytoskeleton (Aberle et al., 1996). However, the exact nature of this association is not clear. The N-terminal of α -catenin facilitates homodimer assembly, which is essential for α -catenin's binding and bundling of F-actin through its C-terminus. Thus, α -catenin cannot simultaneously bind β -catenin and F-actin (Drees et al., 2005; Yamada et al., 2005a). Potentially α -catenin may need to be 'activated' when bound to β -catenin, for example in a similar way in which vinculin binding sites on α -catenin are revealed upon the application of mechanical force to α -catenin (Yonemura et al., 2010). Alternatively, α -catenin may provide an indirect link to actin via interaction with another actin binding protein. Indeed several proteins able to bind both α -catenin and actin are present at cell-cell contacts including vinculin (Ie Duc et al., 2010), formin (Kobielak et al., 2004), EPLIN (Abe and Takeichi, 2008), α -actinin (Yamada et al., 2005a) and Ajuba (Marie et al., 2003).

The presence of an array of actin binding proteins at AJs allows for precise manipulation of the actin cytoskeleton. Due to the nature in which these proteins interact with actin, different structures are formed. For example, the Actin-related protein 2/3 (Arp2/3) complex induces the formation of branched actin cables, whereas formin promotes the assembly of linear actin filaments (Han and Yap, 2012). Vitrally, the actions of actin binding proteins must be spatially constrained as they have the potential to influence the organisation of the entire cytoskeleton.

1.3. The formation and maintenance of AJs during homeostasis

AJs are not static, but highly dynamic structures, constantly being remodeled in order for developmental processes to proceed and in the adult organism to maintain tissue architecture. The formation and maintenance of cell-cell contacts can be separated into distinct steps (**Figure 1.3**). However, it is important to note that whilst, broadly speaking, similar morphological events may take place during the processes of cell-cell contact formation, stabilisation and disassembly, there can be vast differences in terms of the regulatory machinery that governs these changes when comparing different cell types, or in response to different stimuli (Cavey and Lecuit, 2009).

1.3.1. Cell-cell contact formation

In many stages of development as well as in the adult organism during, for example, wound healing, migrating cells undergo mesenchymal-to-epithelial transition and form new cell-cell contacts (Cavey and Lecuit, 2009). This can be recapitulated in cells in culture in calcium switch assays or by plating cells on cadherin-coated plates.

During development, initial contact formation is likely the result of collisions between exploratory actin-based protrusions (lamellipodia, filopodia or membrane ruffles depending on cell type and context) (Ehrlich et al., 2002; Vasioukhin et al., 2000; Yamada and Nelson, 2007). Upon membrane contact E-cadherin puncta (areas enriched in E-cadherin) are established (Adams et al., 1998; Adams et al., 1996), this leads to activation of myosin II (MyoII) which subsequently concentrates E-cadherin at puncta (Miyake et al., 2006; Shewan et al., 2005). E-cadherin that was previously 'free' in the membrane quickly becomes immobilised as it is anchored to actin (Adams et al., 1998; Sako et al., 1998). Actin polymerisation is required for junctions to form (Ivanov et al., 2005; Vasioukhin et al., 2000) and changes in the actin cytoskeleton are observed as puncta are stabilised by newly recruited actin. In subconfluent cells radial bundles of actin can be seen extending from the circumferential actin ring to cadherin puncta (Adams et al., 1998; Vasioukhin et al., 2000; Yonemura et al., 1995), although in confluent keratinocytes radial bundles are not present, actin bundles are still rapidly recruited at sites of contact (Zhang et al., 2005). The reorganisation of actin serves to strengthen cell-cell adhesion (Chu et al., 2004).

1.3.2. Contact expansion

Contact expansion is characterised by the recruitment of E-cadherin into portions of the membrane adjacent to the initial site of contact (Adams et al., 1998). Ultimately this leads to actin polymerisation-dependent strengthening of the contact and the appearance of a continuous line of E-cadherin staining delineating the membranes of neighbouring cells (Adams et al., 1998; Chu et al., 2004). Multiple populations of actin, with different dynamics and morphologies, are coordinated in this process (Cavey et al., 2008; Yamada and Nelson, 2007; Zhang et al., 2005). Data from *Drosophila melanogaster* suggests that E-cadherin incorporated into puncta is stabilised by a stable patch of actin over the cluster and a highly dynamic population which constrains lateral movement (Cavey et al., 2008). Similarly, in MDCK cells actin at the site of contact becomes less dynamic, whilst a more dynamic actin population exists at the edges of contacts, which are also enriched in E-cadherin puncta (Adams et al., 1998; Yamada and Nelson, 2007). In keratinocytes in culture, a dynamic pool of

junctional actin and less dynamic pool of loosely packed bundles coalesce as contacts are stabilised, until a single population is visible (Zhang et al., 2005).

How are these actin populations regulated? MyoII, which is localised to cell-cell contacts in numerous cell types, has been implicated in contact expansion (Krendel and Bonder, 1999; Yamada and Nelson, 2007; Zhang et al., 2005). Potentially MyoII is required to generate contractile forces at the cell membrane that pull away from the site of contact (Krendel and Bonder, 1999). Indeed, MyoII activity is increased at the contact edges (Krendel and Bonder, 1999; Yamada and Nelson, 2007). Interestingly a specific role for MyoII activity during contact expansion is demonstrated by the finding that inhibition of MyoII (via inhibition of its upstream regulator, Rho kinase (ROCK)) prevents new E-cadherin contacts forming at the edge of the contact but does not result in disassembly of the contact (Yamada and Nelson, 2007).

Alternatively, the formation of branched actin structures adjacent to sites of contact can stimulate formation of new adhesions. Imaging the formation of contacts between two MDCK cells reveals contact formation is followed by a wave of lamellipodia adjacent to the site of contact that further expands the contact area (Ehrlich et al., 2002). Furthermore, actin binding proteins able to promote branched polymerisation, such as Arp2/3, are found at site of contacts (Kovacs et al., 2002b; Verma et al., 2004).

1.3.3. Junction maturation and remodelling

Membrane protrusions are not conducive to stable adhesion and are limited in mature junctions (Ehrlich et al., 2002). This is achieved by depletion from contacts of actin binding proteins that induce branching (eg Arp2/3) (Yamada and Nelson, 2007), and recruitment (and/or activation) of proteins that promote the polymerisation of linear filaments (eg. formin, Dia or Ena/Vasp) (Kobielak et al., 2004; Sahai and Marshall, 2002b; Vasioukhin et al., 2000). The result is the generation of thick actin bundles underlying the plasma membrane and an increase in actomyosin tension (Gomez et al., 2011; Liu et al., 2010; Smutny et al., 2010; Zhang et al., 2005).

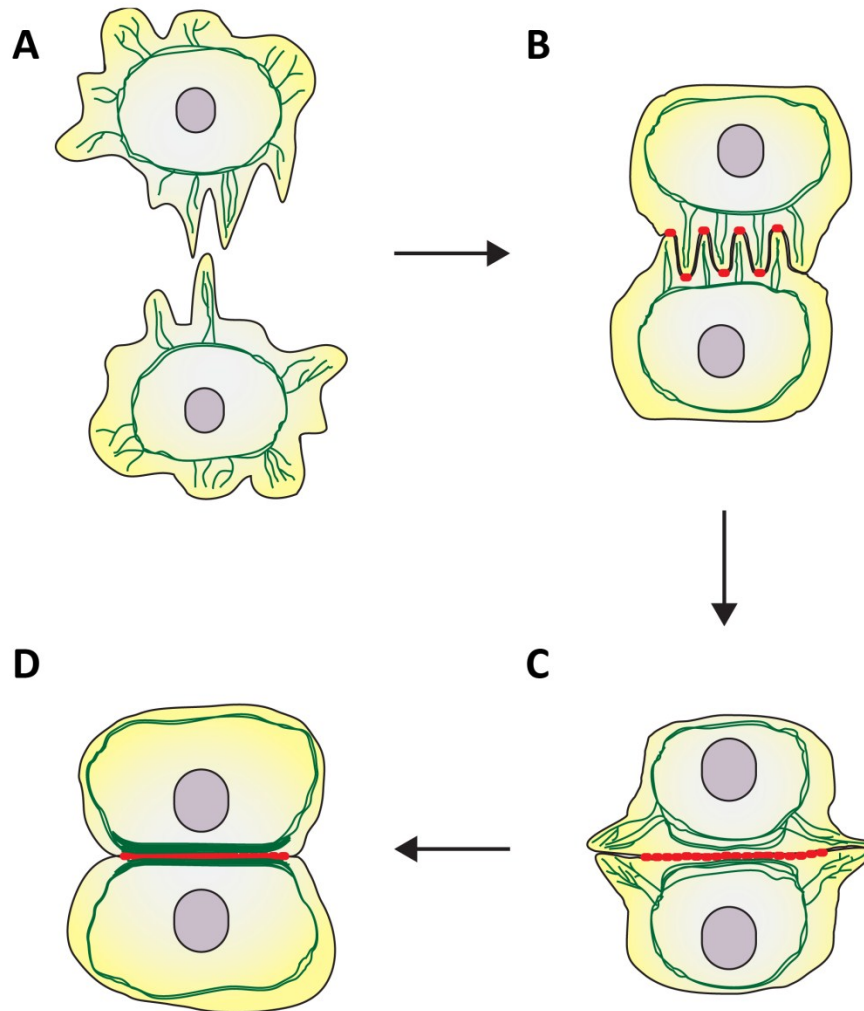


Figure 1.3 Actin reorganisation during junction formation, stabilisation and maintenance A) Prior to contact formation cells form actin-based protrusions (ruffles, lamellipodia and filopodia) (green). B) Junction formation is initialised by contact between actin protrusions on adjacent cells which leads the formation of cadherin puncta (red), areas highly enriched in E-cadherin. The neighbouring cells form a zipper-like structure, whereby actin protrusions embed into the opposite cell. Early events in junction stabilisation involve MyoII-generated force which serves to bring two membranes together. C) Contact expansion relies on the formation of branched actin filaments at the edges of cell-cell contacts whilst a more stable actin population restricts E-cadherin movement in the centre of the contact. MyoII is found at the edge of contacts and generates the force to expand the contact area outwards. D) Mature junctions display a single, continuous line of E-cadherin and thick actin bundles adjacent to contact sites.

Mature AJs are considerably dynamic structures and E-cadherin is constantly being turned-over. Adhesion strength can be adjusted by modulating the amount of E-cadherin at the membrane. Due to the long half-life of E-cadherin, transcriptional changes are not suitable for enacting rapid changes in adhesion (Shore and Nelson, 1991). Instead this is achieved by balancing endocytosis and degradation with synthesis and recycling of E-cadherin (eg. (Classen et al., 2005), reviewed in: (Kowalczyk and Nanes, 2012).

E-cadherin can be endocytosed via clathrin-dependent or independent pathways (Bryant et al., 2007; Le et al., 1999; Lu et al., 2003). In both cases, evidence exists that E-cadherin engaged in *trans*-interactions is less susceptible to endocytosis (Izumi et al., 2004; Paterson et al., 2003). Interestingly, AJ components can regulate these processes. For example loss of p120 catenin results in clathrin-mediated internalisation of E-cadherin (Xiao et al., 2005). Upon internalisation, E-cadherin is either degraded or recycled back to the membrane. Shifting this balance alters the amount of E-cadherin at the surface.

1.3.4. Disassembly of AJs

AJs must also be disassembled both in development, for example during gastrulation and neural crest development (Kalluri and Weinberg, 2009; Lim and Thiery, 2012), and in the adult organism during wound healing or apoptosis (Kessler and Muller, 2009; Leopold et al., 2012). Disassembly of AJs is often associated with epithelial-to-mesenchymal transition and can be triggered by growth factors (Kalluri and Weinberg, 2009; Lim and Thiery, 2012).

Gastrulation occurs early on in embryonic development and leads to the generation of the ectoderm, mesoderm and endoderm, from which all tissues arise. The first stage in gastrulation is the formation of a furrow in the blastula, called the primitive streak, whereby cells separate from the epithelial layer (delaminate) by either folding inwards (invagination) or collectively migrating into an adjacent area (involution) (Lim and Thiery, 2012). Cells in the primitive streak undergo successive epithelial-to-mesenchymal transitions whereby, through a process called ingression, single cells disassemble contact with the basal lamina, dismantle cell-cell adhesions and migrate. The cells that will become the mesoderm and the endoderm ingress at specific sites after which they revert back to an epithelial phenotype by undergoing mesenchymal-to-epithelial transition (Lim and Thiery, 2012). Importantly, epithelial-to-mesenchymal transition occurs in predefined areas and only in specific cells. The integrity of the basal membrane is maintained and cells ultimately revert back to an epithelial phenotype.

1.4. The modulation of cell-cell contacts in cancer

The metastatic process typically involves the breakdown of cell-cell contacts, detachment from adjacent cells at the primary site, invasion into surrounding tissues, entry and dissemination via the blood or lymphatic systems to a distinct secondary site and finally outgrowth and extravasion at the secondary site (Yilmaz and Christofori, 2009). Thus, numerous features associated with epithelial-to-mesenchymal transitions, from the morphological alterations to the molecular mechanisms in place to downregulate key adhesive proteins, are closely mirrored in pathogenic scenarios. This suggests that these vital developmental processes can be subverted by tumour cells to promote metastasis (Kalluri and Weinberg, 2009; Lim and Thiery, 2012; Yilmaz and Christofori, 2009). As such, valuable insights into tumour progression can be gained from understanding epithelial-to-mesenchymal transition during developmental processes and *vice versa* (Lim and Thiery, 2012).

Tumour progression and metastasis requires that cell-cell and cell-matrix contacts are extensively remodeled as cancer cells detach from their neighbours, migrate and re-adhere at secondary sites. In cells migrating individually this can involve complete disassembly of AJs (van Zijl et al., 2011). However, in other situations, for example when cancer cells migrate collectively, AJs are maintained (Friedl and Gilmour, 2009). As tumour progression relies on loss (or extensive modulation) of cell-cell contacts a central role for E-cadherin during tumour progression and metastasis is unsurprising (Berx and van Roy, 2009; Vasioukhin, 2012). Decreased expression of E-cadherin is found in many human epithelial tumours and is usually associated with poor prognosis (Berx and van Roy, 2009). Most likely, this is derived from E-cadherin's ability to coordinate multiple signaling pathways, as well as the structural role played in mediating cell-cell contact.

1.4.1. Downregulation of cadherins

Mutations in CDH1, the E-cadherin gene, resulting in deletion or truncations are found in gastric, prostate, hepatocellular and breast cancers (Becker et al., 1994; Berx et al., 1995). However, this does not seem to be the predominant form of downregulation of E-cadherin, as whilst there is a high incidence of CDH1 mutations associated with lobular breast and diffuse gastric cancer, it is a relatively rare occurrence in other cancers (Berx et al., 1998; Strathdee, 2002).

The downregulation of E-cadherin can be achieved via a number of mechanisms including epigenetic alterations, such as DNA methylation of the CDH1 promoter region, post-translational modification of AJ components or via the secretion of proteases from neighbouring cells (Strathdee, 2002;

Yoshiura et al., 1995). Unlike genetic mutations though, these are reversible which would allow for low levels of E-cadherin expression if necessary, and may account for why these are far more prevalent than genetic mutations.

Transcriptional downregulation of cadherin can be achieved via upregulation of transcription factors capable of inhibiting E-cadherin expression by binding to the E-cadherin promoter. The transcription factors Zinc-finger binding protein SNAI1 (SNAIL), Zinc finger E-box-binding homeobox 1/2 (ZEB1/2), twist basic helix-loop-helix transcription factor 1 (Twist) and Zinc finger binding protein SNAI2 (Slug) are able to repress E-cadherin expression (Batlle et al., 2000; Cano et al., 2000; Comijn et al., 2001; Grooteclaes and Frisch, 2000; Hajra et al., 2002; Yang et al., 2004) and elevated expression levels of these transcription factors have been reported in a range of cancers (Blanco et al., 2002; Elloul et al., 2005; Rosivatz et al., 2002; Spoelstra et al., 2006; Yang et al., 2004).

Interestingly, there is a degree of cross-talk between microRNAs (miRs) and transcriptional factors. For example, miR-141 and miR-200c suppress ZEB1 and transforming growth factor-beta (TGF- β) transcription and ZEB1 directly suppresses miR-141 and miR-200c in breast cancer and colorectal cancer cell lines (Burk et al., 2008). Thus, inhibition of these miRs may contribute to the ability of ZEB1 to promote invasion. MicroRNAs can also negatively regulate E-cadherin by binding directly to the E-cadherin 3' UTR (eg. mir-9 (Ma et al., 2003), mir-92a (Chen et al., 2011) and miR-495 (Hwang-Verslues et al., 2011)).

Destabilisation of the cadherin-catenin complex by post-translational modification also reduces cell-cell adhesion by indirectly promoting removal of E-cadherin from contact sites. Phosphorylation of E-cadherin in the cytoplasmic tail by casein kinase II (CKII) on serine residues is necessary for β -catenin binding (Huber and Weis, 2001; Lickert et al., 2000). Conversely, phosphorylation within the juxtamembrane domain by receptor tyrosine kinases (RTKs) and/or Src kinases creates a binding site for Hakai, which leads to E-cadherin ubiquitination and degradation (Fujita et al., 2002). RTKs can also phosphorylate β -catenin which decreases its binding affinity for E-cadherin (Roura et al., 1999).

Finally, tumour progression activates proteases (matrix metalloproteinases (MMPs), disintegrin and metalloproteinases and γ -secretases) in order to expedite passage through the ECM (eg. MMP2, MMP9 (Remacle et al., 1998), MMP7 (Li et al., 2006) and stromelysin-3 (Basset et al., 1990) amongst others (reviewed in: (Nelson et al., 2000)). These also serve to directly cleave E-cadherin molecules of nearby cells. The cleaved cadherin extracellular domain may act negatively on neighbouring cells by binding to free cadherin on adjacent cell surfaces and impairing function (Noe et al., 2001), whilst

the cytoplasmic tail of N-cadherin can translocate to the nucleus where it can regulate transcription (Ferber et al., 2008; Shoval et al., 2007).

1.4.2. Catenins and cancer

Although loss of E-cadherin is correlated with increased invasion and metastasis, studies of mice with deletion of E-cadherin in skin and mammary glands reveal that loss of E-cadherin alone is not sufficient to induce tumourigenesis (Boussadia et al., 2002; Tinkle et al., 2008; Tunggal et al., 2005). However, the concomitant loss of E-cadherin with p120-catenin or α -catenin is highly tumourigenic. Furthermore, the loss of either p120 catenin or α -catenin is sufficient to induce invasion; transplantation of α -catenin^{-/-} null keratinocytes onto nude mice cause skin lesions reminiscent of squamous cell carcinoma (Kobielak et al., 2004), whilst p120^{-/-} keratinocytes cause inflammatory lesions and tumour-like growths (Perez-Moreno et al., 2008). These are consistent with findings that loss of E-cadherin also correlates with loss of α -catenin and increased invasion (Aaltomaa et al., 1999).

As such, modulation of the levels and functionality of catenins presents an additional mechanism to promote tumourigenesis which may relate to both adhesive and non-adhesive roles for these proteins. For example, ablation of α -catenin in mouse skin does not prevent formation of cell-cell contacts, and E-cadherin and β -catenin localise to cell-cell contacts. However, hyperproliferative cells are present in the epidermis, similar to those seen in squamous cellular carcinoma (Vasioukhin et al., 2001a). Interestingly, loss of α -catenin is associated with activation of nuclear factor (NF)- κ B signaling pathways which enhances the inflammatory response (Kobielak and Fuchs, 2006). Furthermore, functions related to the regulation of transcription have been described for p120 catenin (Daniel and Reynolds, 1999; Ferber et al., 2008; Park et al., 2005).

As opposed to the tumour suppressive roles of α - and p120 catenin, β -catenin is found upregulated in many cancers (Clevers, 2006). In addition to its function in AJs, β -catenin is a central component of the Wnt signaling pathway that controls several aspects of cell fate. Wnt signalling induces the cytoplasmic pool of β -catenin to translocate to the nucleus, where it directs gene transcription in association with T-cell factor (TCF), lymphoid enhancer factor (LEF-1) and Pangolin (Pan) (Behrens et al., 1996; Funayama et al., 1995; McEwen et al., 2012). This is balanced by the actions of a destruction complex for β -catenin composed of adenomatous polyposis coli (APC), axin, Glycogen synthase kinase 3- β (GSK3- β) and casein kinase (CKI) (Behrens et al., 1998; Kikuchi, 1999). Mutations are often found that stabilise β -catenin. Commonly these affect the ability of β -catenin to become

phosphorylated (which regulates its degradation) resulting in constitutive activation and Wnt signaling (Vasioukhin, 2012). Interaction with E-cadherin effectively sequesters β -catenin at cell-cell contacts, preventing it from activating Wnt genes. Thus, loss of E-cadherin, or disruption of the E-cadherin- β -catenin interaction has the secondary effect of increasing the pool of cytoplasmic β -catenin (McEwen et al., 2012).

1.5. Small GTPases

Small GTPases regulate diverse cellular function including regulation of trafficking, migration, proliferation and adhesion (Wennerberg et al., 2005). These proteins function as biological switches, and through a range of post-translational modifications, and the actions of a cohort of regulatory proteins, their activity can be directed in order to bring about very specific cellular responses (Vetter and Wittinghofer, 2001; Wennerberg et al., 2005)

1.5.1. Diverse functions regulated by Small GTPases

Members of the Ras superfamily of small GTPases can be subdivided into five subgroups, Ras, Rho, Rab, Ran and Arf, based on sequence similarity and function and are important regulators of a wide variety of cellular processes (**Figure 1.4**) (Wennerberg et al., 2005). The focus of this work is on Rho GTPases, and these will be discussed in detail in a section 1.7.

Ras sarcoma (Ras) proteins are the founding members of this family and are involved in the coordination of many signaling pathways. Ras proteins typically regulate the transcription of genes that affect cell growth and survival and numerous Ras targets have been described (Schaefer and Sers, 2011; Wennerberg et al., 2005). Close relations of Ras are the Ras-like nuclear (Ran) proteins. Ran GTPases regulate nuclear transport of proteins and Ran is particularly important in regulating mitosis. Ran also regulates neurite outgrowth and microtubule organisation (Yudin and Fainzilber, 2009).

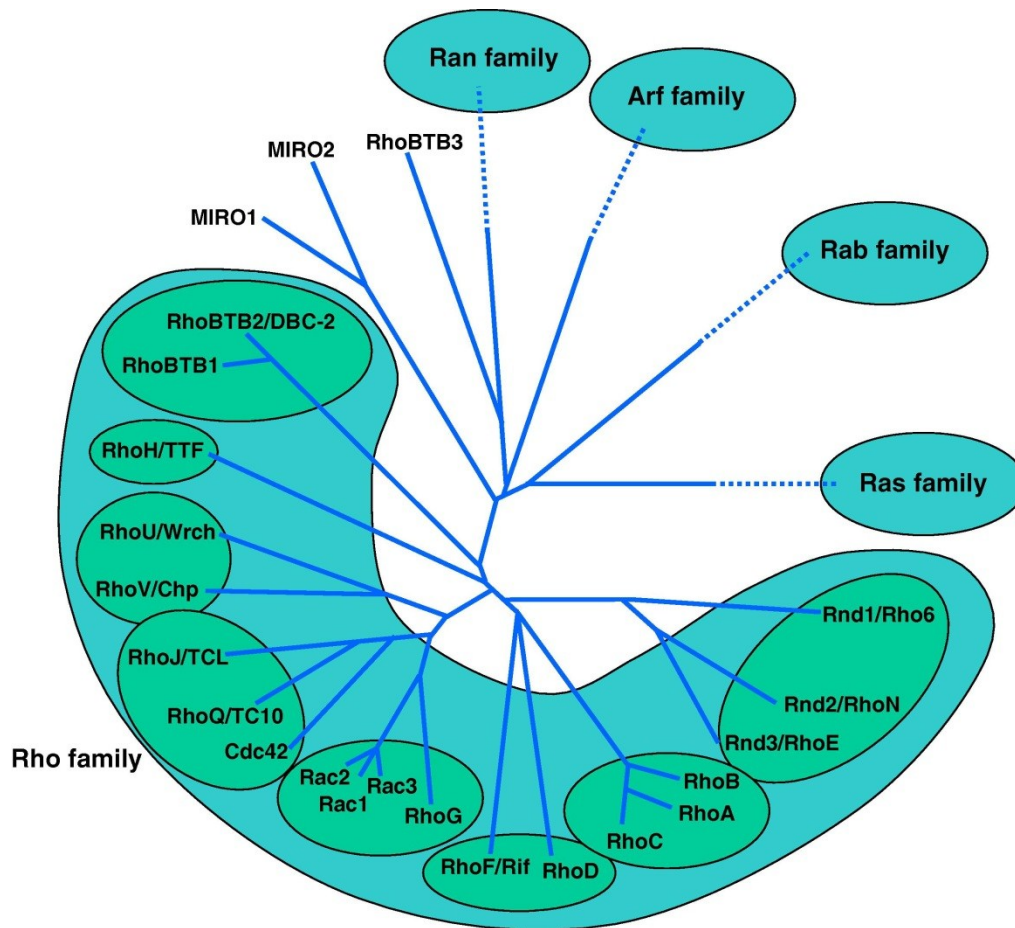


Figure 1.4 Phylogenetic tree of small GTPases The Ras superfamily is grouped into five main branches; Ras, Rab, Arf, Ran and Rho. Within the Rho family eight subfamilies exist. The GTPases Miro1, Miro2 and RhoBTB3 are separated due to differences in sequence and form their own branches. Figure reproduced from Vega and Ridley, 2005.

Ras homologous (Rho) family GTPases are found in all eukaryotes and are highly conserved (Hall, 2012). Eighteen Rho GTPases exist in mammals and these can be further subdivided into 8 groups (**Figure 1.4**) (Hall, 2012; Maria Rojas et al., 2012; Wennerberg et al., 2005). Rho GTPases regulate actin cytoskeleton dynamics by interacting with a range of proteins that can influence the assembly, disassembly and reorganisation of actin (Hall, 2012; Jaffe and Hall, 2005). As coordination of the actin cytoskeleton is required to drive so many fundamental cellular processes (eg. migration, cell division, endocytosis) Rho GTPases, by regulating actin organisation, are key players in these processes (Hall, 2012).

Members of the ADP-ribosylation (Arf) and Ras-like proteins in brain (Rab) families are regulators of membrane trafficking. At the membrane (cell membranes or organelles) Arf proteins control the recruitment of lipid-modifying enzymes, effector proteins and coat proteins to vesicles which subsequently influences the sorting of cargo into vesicles (Donaldson and Jackson, 2011). Rabs coordinate the transport of vesicles from one area of the cell to another via the cytoskeleton. Different Rab proteins regulate distinct steps throughout the journey of a cargo protein from the endoplasmic reticulum (ER) to the target membrane. For example, Rab5 regulates fusion of vesicles to the ER, recycling of the vesicle to the plasma membrane requires Rab4, transport to the Golgi requires Rab9 but fusion to the Golgi requires Rab1 (Mizuno-Yamasaki et al., 2012).

1.5.2. Small GTPases are molecular switches

Small GTPases are molecular switches that cycle between an active and inactive state depending on the status of nucleotide binding (McCormick and Wittinghofer, 1996) (**Figure 1.5 A**). When bound to GTP the GTPases are active and able to interact with their effectors, whilst in the GDP-bound form they are inactive (McCormick and Wittinghofer, 1996). Their activation status is influenced by three classes of regulatory proteins (Cherfils and Zeghouf, 2013). The intrinsic hydrolysis rate of GTPases is highly variable, but generally slow (Vetter and Wittinghofer, 2001). This can be catalysed by the actions of GTPase activating proteins (GAPs) that serve as negative regulators, effectively 'switching off' the GTPase (Vetter and Wittinghofer, 2001). Guanine nucleotide exchange factors (GEFs) are positive regulators of GTPases. These stabilise the nucleotide-free form of the GTPase, causing them to release bound GDP, which is replaced by the more prevalent GTP (Vetter and Wittinghofer, 2001). Guanine-nucleotide dissociation inhibitors (GDIs) are the final class of regulators, these serve to sequester GTPases in the cytoplasm that prevents them from signalling (Vetter and Wittinghofer, 2001).

Small GTPases all share a broadly conserved structure for the binding and hydrolysis of GTP, called the G domain (Hakoshima et al., 2003). This consists of a six-stranded β sheet and five α helices (Bourne et al., 1991). Binding of GTP changes the conformation of two switch regions (switch I and switch II), which facilitates the binding of effector proteins and subsequent downstream signalling (Bourne et al., 1991; Vetter and Wittinghofer, 2001). Magnesium ions are vital for binding of GTP and consequently activity of the GTPase as these stabilise the conformation of the GTPase (Hakoshima et al., 2003). GTPases all hydrolyse GTP at very different rates, and some GTPases lack any intrinsic hydrolysis at all (Bos et al., 2007; Scheffzek and Ahmadian, 2005). The intrinsic hydrolysis of GTP involves the transfer of the γ -phosphate of GTP to a water molecule present at the active site. The Mg^{2+} ion is required to polarise GTP, which then abstracts a proton from the water molecule. The water molecule then acts as an attacking nucleophile (Scheffzek and Ahmadian, 2005).

Knowledge of the structure of GTPases has aided development of constitutively active or inactive mutants of GTPases, as specific mutation of critical residues within the G domain can affect interactions with GTP, effectors or regulators. These have proven useful experimental tools (Feig, 1999). Dominant-negative mutants (eg. Rac and Ras mutants whereby serine 17 is replaced by arginine) bind GEFs with high affinity, effectively sequestering Rho GEFs from binding endogenous GTPases (Feig, 1999). This is thought to be due to important interactions mediated by serine 17 with the Mg^{2+} ion. Conversely, constitutively active mutants were created by mutation of leucine (in the switch II region) to glutamine (L61 in Ras, Rac and Cdc42, L63 in Rho) or mutations of glycine to valine (V14 in RhoA, V12 in Rac and Cdc42) which render GTPases unable to hydrolyse GTP.

Post-translational modifications serve to target GTPases to distinct membranes, where they exert their effects. Rho family proteins typically contain a CAAX motif at their C-terminus (C= cysteine, A= aliphatic, X= any amino acid), whilst Rabs contain similar, cysteine-containing motifs which are recognised by enzymes able to catalyse the addition of farnesyl or geranylgeranyl groups to these proteins (Magee et al., 1992; Takai et al., 2001). Conversely, Arfs are targeted via myristoylation of their N-terminal domains (Wennerberg et al., 2005). Mutation of amino acids within these regions can perturb function due to mislocalisation.

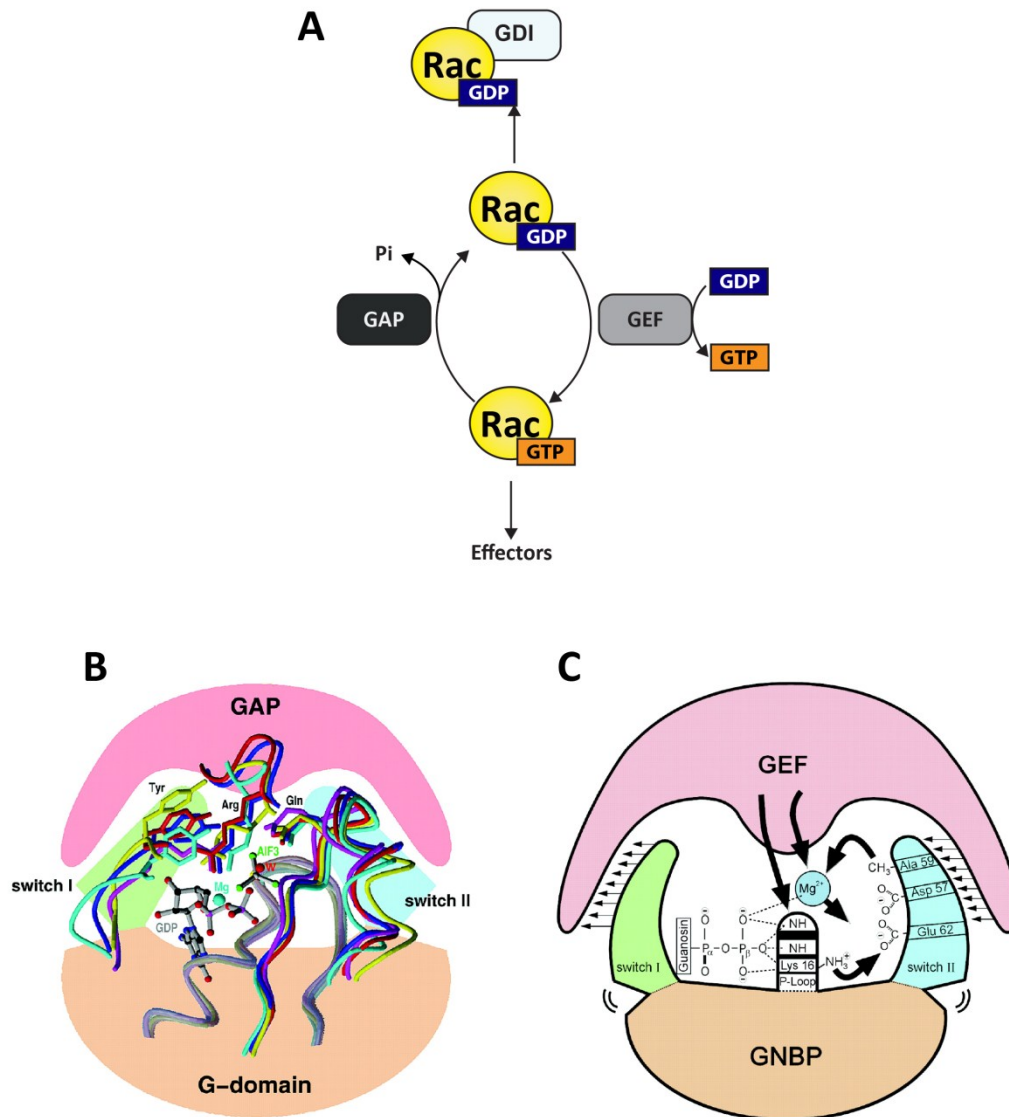


Figure 1.5 GTPases are molecular switches A) GTPases cycle between an active (GTP-bound) and inactive (GDP-bound) form. In the active form, they interact with their effectors and influence various cellular processes. The activity of GTPases is governed by the actions of three classes of regulators. Positive regulators, the guanine nucleotide exchange factors (GEFs) catalyse the release of tightly-bound GDP which would otherwise be released slowly. Two classes of negative regulators exist; GTPase activating proteins (GAPs) enhance the inherent catalytic activity of the GTPase, thereby inactivating it. Rho and Rab family GTPases can be regulated by GTPase dissociation inhibitors (GDIs), which sequester GDP-bound GTPases in the cytoplasm and bind GTP-bound GTPases to prevent their association with effectors proteins. B) GAPs (pink) bind to the G-domain of their target GTPase (orange) and insert a conserved arginine residue into the active site between the two switch regions. The interactions between different GTPases and GAPs are shown and coloured as follows; Ras-RasGAP (yellow), Rho-RhoGAP (red), Rac-ExoS (cyan) and G_iα-RGS (magenta). Catalytic water is shown as a red "W". C) GEF (pink) bound to GTPase (orange). Guanine nucleotides bind strongly to the P loop and magnesium ion. GEF binding disrupts this interaction, pulling switch I and pushing switch II out of position. B and C are adapted from Vetter and Wittinghofer, 2001.

1.5.3. Small GTPase regulators

GAPs bind to GTPases and enhance, by up to five orders of magnitude, the hydrolysis of GTP to GDP with the effect of 'turning off' the GTPase and ending downstream signaling (Scheffzek and Ahmadian, 2005). GAPs work by stabilising the catalytic complex, and aligning the water molecule to optimise nucleophilic attack. A conserved arginine in the GAP (the arginine finger) is critical in this reaction, as this protrudes into the active site and stabilises negative charges which emerge in the transition state (**Figure 1.5 B**) (Scheffzek and Ahmadian, 2005). Mutation of the arginine finger is commonly used to inactivate GAPs in order to investigate GAP-domain dependent functions of a protein.

GTPases bind GDP/GTP with high affinity, meaning their dissociation is slow (Bos et al., 2007). GEF binding reduces the affinity of the GTPase for the nucleotide so that the nucleotide is rapidly released and then replaced. The catalytic domains of GEFs from different families is not well conserved, but all use similar mechanisms to displace GDP (Bos et al., 2007; Cherfils and Zeghouf, 2013). GEFs bind to the switch I and II regions on the GTPase and residues from the GEF are placed in the vicinity of the Mg^{2+} ion, which pushes the switch regions out of position (**Figure 1.5 C**) (Cherfils and Zeghouf, 2013; Vetter and Wittinghofer, 2001). In some GEFs, acidic residues create repulsive electrostatic forces within this region, whilst others induce changes in the switch II region which results in destabilisation of the Mg^{2+} ion. The result is either partial or complete obstruction of the active site which makes it impossible for both the phosphates and the metal ion to bind (Cherfils and Zeghouf, 2013; Vetter and Wittinghofer, 2001). The nucleotide is then displaced and upon binding of the next nucleotide the GEF becomes dissociated.

Rho and Rab family GTPases can also be regulated by GDIs. Only three Rho GDIs and three Rab GDIs exist, and these are fairly well-conserved (Cherfils and Zeghouf, 2013). GDIs form two separate interfaces with their targets, one at the switch region and one at the prenylated C-terminus. The binding of GDI prevents the release of nucleotide, and by binding to the prenylated COOH-terminus prevents localisation of the GTPase to the membrane by shielding the hydrophobic tail. This effectively results in sequestration of the GTPase in the cytoplasm (Bos et al., 2007).

As GTPases control numerous facets of cellular signalling, GTPase regulators are of vital importance in directing the actions of their targets. GTPase regulators control the localisation and duration of signalling as well as influencing the interaction with GTPase effector proteins (Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007). Consequently, GAPs and GEFs can greatly influence the

outcome of GTPase activation (Kather and Kroll, 2013; McCormack et al., 2013; Tcherkezian and Lamarche-Vane, 2007; Tolia et al., 2011). For these reasons, the localisation and activity of the regulators must also be tightly controlled.

1.6. Regulating the activity of GTPase regulators

The activity of GTPase regulators is achieved via various mechanisms which may alter the conformation of the GEF/GAP domain, such that this is revealed or obscured, or promote the translocation of the regulator to a region of the cell where it is activated or sequestered (Bernards and Settleman, 2004; Garcia-Mata et al., 2011; Rossman et al., 2005).

1.6.1. Auto-inhibition

Many GEFs and GAPs can adopt autoinhibited states (Bernards and Settleman, 2004; Cherfils and Zeghouf, 2013; Rossman et al., 2005). For example, the Rho GAPs Oligophrenin, Cdc42 GTPase activating protein (CdGAP), p190 RhoGAP and β -chimearin are able to adopt auto-inhibitory configurations (Bustos et al., 2008; Canagarajah et al., 2004; Fauchereau et al., 2003; Southgate et al., 2011); expression of Oligophrenin N-terminal inhibits GAP activity when co-expressed with the GAP domain (Fauchereau et al., 2003) and truncation mutants of CdGAP lacking the C-terminal are more active on Cdc42 (Southgate et al., 2011). Similarly, several GEFs are constitutively activated following deletion of their N- or C-terminal regions, suggesting that these regions negatively regulate GEF activity (Cherfils and Zeghouf, 2013; Rossman et al., 2005). These include Rho-family GEFs APC stimulated exchange factor 1 (Asef1), T-cell lymphoma invasion and metastasis 1 (Tiam1), Vav1 and epithelial cell-transforming sequence 2 oncogene (ECT2) (Aghazadeh et al., 2000; Kawasaki et al., 2000; Miki et al., 1993; van Leeuwen et al., 1995), Ras GEF Ras-specific guanine nucleotide-releasing factor 1 (GRF1) (Baouz et al., 1997) and RapGEF Exchange protein directly activated by cAMP 1 (EPAC) (de Rooij et al., 1998).

The highly complex domain structures exhibited in the regulators mean these proteins can form a range of interactions with other cellular components, and so release from an inhibited state can be disrupted by lipids, protein-protein interaction and phosphorylation. These are discussed below.

1.6.2. Regulation by phosphorylation

Phosphorylation is an important mechanism used to activate, or inhibit regulators. The Rho GAP p190 can be activated via both phosphorylation (Chang et al., 1995; Haskell et al., 2001; Hu and Settleman, 1997) or dephosphorylation (Chiarugi et al., 2000; Shiota et al., 2003; Sordella et al., 2003). The kinase Src phosphorylates p190 which leads to its interaction with p120RasGAP, translocation of this complex to the cell membrane, and activation of p190 (Chang et al., 1995; Haskell et al., 2001; Hu and Settleman, 1997). Subsequently, other kinases such as breast tumour kinase and Fyn have been found to phosphorylate p190 and promote its interaction with p120RasGAP, thus this appears an important mechanism by which to coordinate Rho and Ras signaling (Shen et al., 2008; Wolf et al., 2001). Insulin receptor can also phosphorylate p190 which results in its rapid translocation to membranes (Sordella et al., 2003). The dephosphorylation of p190 by Low molecular weight protein tyrosine phosphatase, protein tyrosine phosphatase 20 and protein tyrosine phosphatase 11 can all inhibit p190 (Chiarugi et al., 2000; Shiota et al., 2003; Sordella et al., 2003).

The Rho-family GEF Vav1 is activated via phosphorylation (Aghazadeh 2000). Vav1 activation has been studied extensively (Cherfils and Zeghouf, 2013; Yu et al., 2010) and crystal structures reveal a tightly packed structure that requires the completion of several phosphorylation events to induce activation (Yu et al., 2010). This allows Vav1 to be efficiently inhibited but rapidly activated (Cherfils and Zeghouf, 2013). Multiple phosphorylation events are also required to activate GRF1, which is retained in an autoinhibitory state (Baouz et al., 2001; Baouz et al., 1997).

Phosphorylation of RhoGDIs (for example by p21 activated kinase 1 (PAK1), protein kinase C (PKC) α or ζ , or Src) can reduce their affinity for GTPases, which leads to their release (DerMardirossian et al., 2004; DerMardirossian and Bokoch, 2006; Garcia-Mata et al., 2011; Mehta et al., 2001). Interestingly, phosphorylation can specifically alter binding affinity for different GTPases. For example PAK1 can phosphorylate RhoGDI1 which promotes the release of the Rho GTPase Rac1, but not RhoA (DerMardirossian et al., 2004).

1.6.3. Regulation by lipids

Phosphatidylinositols are used as second messengers in signalling pathways. Diacylglycerol (DAG) is generated at cell membranes downstream of phospholipase C (PLC) activation. Rho GAPs of the chimaerin family contain DAG receptor sites (Hall 1990, Leung 1993, Hall 1993). In its inactive state,

β 2-chimaerin is inhibited by residues in the N-terminal region that obstruct the active site, binding of DAG to the C1 region dissociates several interactions within this area that reveals the GAP domain (Canagarajah et al., 2004). Binding of DAG also induces the translocation of chimaerins to the membrane (Caloca et al., 1997; Caloca et al., 2001) where they inactivate Rac (Wang et al., 2006). Interestingly, α -chimaerin is stabilised via interaction with DAG, which prevents its ubiquitination and degradation (Marland et al., 2011).

Furthermore, as integral components of cellular membranes, lipids may direct Rho GAP or GEF activation at specific subcellular sites. For example, several regulators contain domains that facilitate their recruitment to lipid membranes (eg. Bin, amphiphysin, RSV161/167 (BAR) domains) which allows them to interact with membranes and cause membrane curvature. The specialised nature of this interaction may be important to spatially confine Rho GTPase activation. For example the recruitment of ArfGAP1 to highly curved membranes activates its GAP activity (Bigay et al., 2003). Similarly, the Rho-family GAP, CdGAP binds several phospholipids via a basic N-terminal region and incorporation of phosphatidylinositol 3,4,5-triphosphate (PIP₃) into *in vitro* membranes enhances GAP activity of CdGAP (Karimzadeh et al., 2012).

1.6.4. Regulation by protein-protein interaction

Protein-protein interactions mediated by a range of molecules may serve to activate or inhibit regulators via conformational changes or by regulating localisation. Examples of protein-protein interactions that result in activation of regulators include the binding of Rac to p190B which releases p190B from its autoinhibited state and promote its association with its target, RhoA, at the membrane (Bustos et al., 2008). In addition, EPAC, is activated by cyclic AMP binding (de Rooij et al., 1998) which induces large conformational changes in EPAC to reveal the GEF domain (Cherfils and Zeghouf, 2013).

Protein-protein interactions can also induce the translocation of Rho GAPs to distinct cellular compartments which mediates their activation. An example is CdGAP which interacts with actopaxin (also called α -parvin), a protein found at focal adhesions in some cells. Interaction with actopaxin induces the localisation of CdGAP to focal adhesions where it inhibits spreading of osteosarcoma cells (LaLonde et al., 2006). Interaction with actopaxin has no effect on GAP activity of CdGAP *in vitro*, but loss of actopaxin increases spreading as CdGAP is not localised to focal adhesions (LaLonde et al., 2006).

CdGAP and another Rho-family GAP, RhoGAP interacting with CIP4 homologs protein 1 (Rich1, ARHGAP17), are also negatively regulated via interaction with the scaffold proteins intersectin (itself a GEF) (Hussain et al., 2001; Jenna et al., 2002; Primeau et al., 2011) and Amot (Wells et al., 2006) respectively. Rich1 is required for the maintenance of TJs via restriction of Cdc42 activity. However, expression of Amot prevents Rich1 from inactivating Cdc42 leading to loss of TJ integrity (Wells et al., 2006). CdGAP interacts directly with intersectin via a basic region adjacent to the GAP domain, which likely induces a conformational change in the protein (Primeau et al., 2011). The Rho GEFs Asef and Intersectin both contain Src homology 3 (SH3) domains that bind to their active sites to inhibit these proteins (Ahmad and Lim, 2010; Mitin et al., 2007; Murayama et al., 2007). Asef and Intersectin are activated via interaction with APC and the Cdc42 effector neural Wiskott–Aldrich syndrome protein (N-WASP) binding, respectively (Hussain et al., 2001; Zhang et al., 2012) which likely mediates conformational changes in these proteins.

RabGDIs can be regulated by GDI-displacement factors which catalyse the release of Rabs from GDIs (Sivars et al., 2003). No such proteins have thus far been identified for RhoGDIs. The release of GTPase can also be achieved via formation of protein-protein interactions. For example, both the kinase Ephrin type-A receptor 3, and ezrin, radixin, moesin (ERM) proteins can compete with GTPase for the binding of RhoGDI (Kim et al., 2002; Takahashi et al., 1997).

1.7. Cellular functions of Rho GTPases

The most studied GTPases within this family are RhoA, Rac1 and Cdc42 and their activation has specific effects on the organisation of actin; RhoA stimulates the formation of highly contractile stress fibres, Rac1 promotes the formation of lamellipodial and Cdc42 promotes the formation of filopodia (Nobes and Hall, 1995; Ridley and Hall, 1992; Ridley et al., 1992).

1.7.1. Rho GTPases

Three highly homologous Rho proteins exist; RhoA, RhoB and RhoC (Wheeler and Ridley, 2004). Despite this high homology, these proteins have distinct functions. RhoB has been implicated in endosomal trafficking (Ellis and Mellor, 2000) and is growth-suppressive (Huang and Prendergast, 2006), whereas RhoA and C are growth-promoting. The C-terminal regions of RhoA, B and C are where most differences in sequence are found, thus distinct functions may be due to the discrete localisation of these proteins (Wheeler and Ridley, 2004). Rho activity is necessary in cytokinesis for

the formation of the contractile ring (Jaffe and Hall, 2005) as well as for phagocytosis mediated by the complement receptor (Caron and Hall, 1998) and can inhibit neurite extension (Luo, 2000).

The Rac subfamily consists of Rac1, Rac2, Rac3 and RhoG as well as a Rac1 splice variant, Rac1b. Expression of the different Rac proteins varies in different tissues, for example Rac2 is highly expressed in hematopoietic cells, Rac3 is highly expressed in brain and Rac1 and RhoG are ubiquitously expressed (Haataja et al., 1997; Heasman et al., 2010; Shirsat et al., 1990). Only Rac1 null mice are embryonic-lethal (Sugihara et al., 1998), although tissue-specific defects are reported in Rac2, Rac3 and RhoG knockout mice (Heasman et al., 2010). Rac regulates neurite extension (Luo, 2000), the passage of G₂/M phase and can accumulate in the nucleus, where it promotes mitosis (Mack et al., 2011)

Cdc42 has frequently been associated with the regulation of polarity in numerous species and cell types (Etienne-Manneville and Hall, 2002; Heasman et al., 2010). Cells are required to polarise, that is to separate proteins and organelles into defined regions of the cell, during migration, cell division, and the formation of epithelial monolayers (Etienne-Manneville and Hall, 2002). Par proteins are well-conserved proteins that play a fundamental role in the establishment and maintenance of polarity (McCaffrey and Macara, 2009). Cdc42 regulates interactions between Par proteins to form a polarity complex (formed of Par-3, Par-6 and PKC) (McCaffrey and Macara, 2009). Similar polarity complexes are assembled in different contexts and across the animal kingdom. Thus, regulation of polarity by Cdc42 is critical for development and morphogenesis of many cell types and tissues (Etienne-Manneville and Hall, 2002; Jaffe and Hall, 2005).

Rho GTPases also coordinate their activity to regulate many processes, including cell migration and phagocytosis (Caron and Hall, 1998; Ridley, 2001; Sanz-Moreno et al., 2008). Cells can use a variety of mechanisms to control migration. For example, mesenchymal migration is characterised by an elongated morphology and protrusions at the leading edge (Ridley, 2001). In these cells, Rac promotes the turnover of focal adhesions and formation of lamellipodia at the leading edge of the cell whereas Rho generates contractile forces at the rear of the cell (Ridley, 2001; Sanz-Moreno et al., 2008). Also, Rac and Cdc42 activities are coordinated to bring about phagocytosis via Fc receptors as well as pinocytosis (Caron and Hall, 1998; Etienne-Manneville and Hall, 2002)

1.7.2. Rho effectors

Rho GTPases signal through numerous effectors to bring about downstream responses. Over 70 Rho effectors have been described, thus only some of the best characterised are discussed below (**Figure 1.6**).

Rho activation is typically characterised by the formation of stress fibres and the generation of contractile forces (Ridley and Hall, 1992). Rho activation promotes actomyosin contractility via regulation of its effector ROCK (Ishizaki et al., 1996; Leung et al., 1995). ROCK has many targets that regulate the actin cytoskeleton (Amano et al., 2010). Contractility relies on the actions of myosins, which are molecular motor proteins able to transduce force along actin cables. Myosins are formed of heavy (MHC) and light (MLC) chains and their activity is generally governed by phosphorylation of their light chains. ROCK promotes phosphorylation of the MLC of MyoII directly by phosphorylating MLC, and indirectly by inhibiting myosin light chain phosphatase (MLCP) (Kaibuchi et al., 1999; Kimura et al., 1996) thus controlling contractility.

Other important ROCK targets are the LIM kinases (LIMK) (Maekawa et al., 1999), these are serine kinases that target and inhibit actin-binding protein cofilin (Stanyon and Bernard, 1999). Cofilin depolymerises and severs F-actin, thus activation of LIMK by ROCK (or PAKs, discussed below) inhibits the effects of cofilin (Maekawa et al., 1999).

Rho also signals through several other kinases including Citron kinase (Madaule et al., 1998) and PKC-related kinase (PKN) (Amano et al., 1996). Citron kinase acts downstream of Rho to regulate cytokinesis by regulating contraction (Madaule et al., 1998). PKN interacts with RhoA, B and C (Zhao and Manser, 2005) and PKN activation downstream of RhoB has been implicated in endosomal trafficking (Mellor et al., 1998).

Rho can stimulate the formation of linear actin fibres and this is achieved via activation of formins (Goode and Eck, 2007). Rho interacts directly with the diaphanous-related formins (DRFs, including Dia) (Aspenstrom, 2010; Goode and Eck, 2007; Watanabe et al., 1997). Dia induces the rapid elongation of actin filaments which is critical for the formation of stress fibres, filopodia and the cytokinetic ring (Goode and Eck, 2007). DRFs are maintained in an inactive state via interaction of a Diaphanous autoinhibitory domain at the C-terminus with a Diaphanous inhibitory domain (Alberts, 2001; Li and Higgs, 2003). The binding of Rho to the GTPase binding domain (GBD) at the N-terminus relieves autoinhibition and activates the formin (Li and Higgs, 2003). Signaling downstream of Dia

can also activate the transcription factor, serum response factor leading to changes in transcription (Tominaga et al., 2000).

1.7.3. Rac and Cdc42 effectors

Rac and Cdc42 share a number of effectors, although these can initiate differing cellular responses. As such these will be discussed together.

PAKs are serine/threonine protein kinases and important Rac and Cdc42 effectors (Manser et al., 1994). Six PAK genes exist and Rac and Cdc42 interact with all members via a conserved Cdc42 and Rac interactive binding (CRIB) domain (Bokoch, 2003). PAKs regulate contractility, actin remodeling and microtubule organisation via interaction with various targets that have been described as signalling downstream of Rac/Cdc42 activation (Bokoch, 2003; Szczepanowska, 2009).

PAKs regulate contractility by influencing myosin activity either directly, via phosphorylation of the MLC of MyoII (Chew et al., 1998; Ramos et al., 1997; Zeng et al., 2000) therefore promoting contractility, and indirectly, via phosphorylation of MLCK, which inhibits MLCK and therefore indirectly inhibits MLC phosphorylation and contractility (Bokoch, 2003; Sanders et al., 1999). PAK3 is also able to phosphorylate the unconventional myosin VI (Yoshimura et al., 2001).

PAKs can regulate actin remodeling via phosphorylation and activation of LIMK (Dan et al., 2001; Edwards et al., 1999) which then inhibits cofilin. In addition, cortactin interacts with Arp2/3 and stabilises actin branching (Urano et al., 2001). Phosphorylation of cortactin by PAK3 decreases cortactin binding to F-actin (Webb et al., 2006).

Microtubule dynamics are regulated via phosphorylation of Stathmin, a microtubule-destabilising protein (Wittmann et al., 2004). Another PAK target is the scaffold protein Ajuba (Nola et al., 2011) and phosphorylation of Ajuba enhances its affinity for active Rac, promoting Rac localisation to cell-cell contacts (Nola et al., 2011). Numerous other PAK targets have been described and are reviewed elsewhere (Bokoch, 2003; Szczepanowska, 2009).

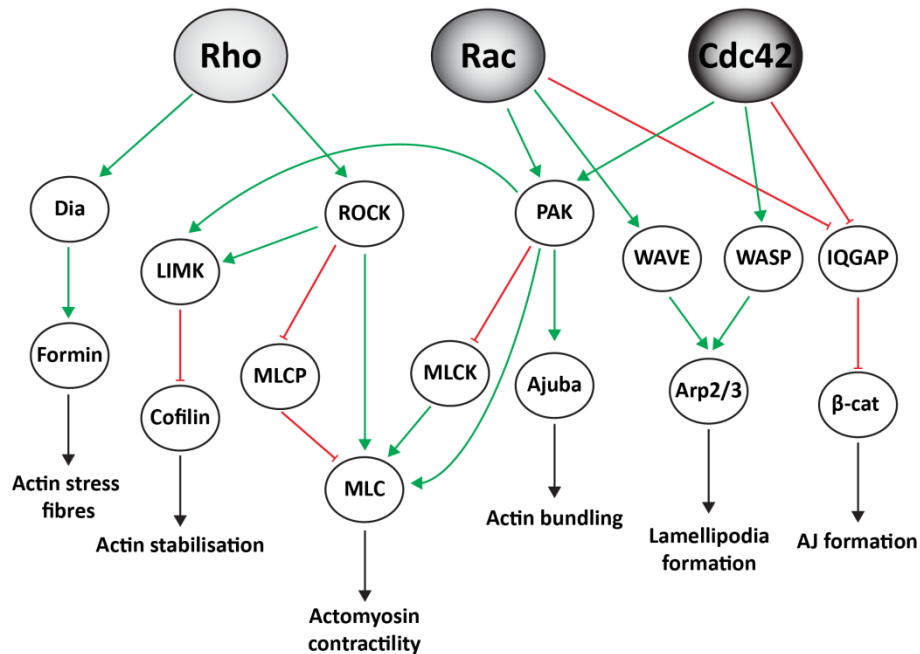


Figure 1.6 Rho GTPase effectors and cross talk A schematic illustrating some of the downstream effectors and signaling pathways activated downstream of RhoA, Rac1 and Cdc42. The subsequent effects these proteins have on the cytoskeleton, AJs and cellular processes are described. Green arrows represent activating interaction, red lines with bars represent inhibitory interaction and the black arrows describe the cellular outcome resulting from GTPase signaling. Additional details are provided in the text. Dia, Diaphanous; LIMK, LIM kinase; ROCK, Rho-associated kinase; MLCP, Myosin light chain phosphatase; MLC, Myosin light chain; MLCK, Myosin light chain kinase; PAK, p21 activated kinase; WAVE, WASP family verprolin homologous protein; WASP, Wiskott-Aldrich syndrome protein; Arp, actin-related protein; IQGAP, IQ motifs and GAP-related-domain containing protein; β -cat, β -catenin. Figure adapted from Citi *et al.*, 2011.

Both Rac and Cdc42 promote the formation of branched actin structures via the activation of the Arp2/3 complex. However, Rac and Cdc42 signal through different effectors, Rac via WASP family verprolin-homologous protein (WAVE) and Cdc42 through WASP or N-WASP (Eden et al., 2002; Stradal and Scita, 2006). The Arp2/3 complex promotes the formation of branched actin structures at a 70° angle from an existing (mother) actin filament (Pollard, 2007). Arp2/3 must be activated by nucleating promoting factors (of which WAVE and WASP/N-WASP are) (Welch and Mullins, 2002). However, WAVE and WASP/N-WASP are also maintained in inactive conformations (Pollard, 2007). WASP/N-WASP is autoinhibited via interactions between its GBD and the VCA domain (the region important for actin and Arp2/3 binding) and binding of Cdc42 to the GBD releases this inhibition to activate WASP (Kim et al., 2000a; Rotty et al., 2013). WAVE is maintained in an inactive multimeric complex (Eden et al., 2002) and Rac activates WAVE, possibly by freeing the VCA, resulting in Arp2/3 activation (Eden et al., 2002; Pollard, 2007; Rotty et al., 2013; Stradal and Scita, 2006).

IQ motif containing GTPase activating proteins (IQGAPs) (there are three in humans, but IQGAP1 has been most extensively studied) (White, Brown and Sacks 2009) contain diverse domain structures and are able to interact with a range of proteins including F-actin, β -catenin and MLC amongst others (Briggs and Sacks 2003). Rac and Cdc42 bind to IQGAP via a GAP-related domain which inhibits IQGAP interaction with E-cadherin and β -catenin (Kuroda 1996, 1999). However, Cdc42 can also promote actin cross-linking mediated by IQGAP *in vitro* (Fukata 1997).

1.7.4. Rho GTPases and cancer

The prominence of Rho GTPases in the regulation of so many fundamental processes means it is unsurprising these have also frequently been associated with cancers. The misregulation of Rho proteins can allow cancer cells to proliferate by promoting transformation, avoid signals that stimulate apoptosis and promote angiogenesis, that is necessary if cancer cells are to grow and survive (Sahai and Marshall, 2002a; Vega and Ridley, 2008). Tumour cells may then detach from their original location and invade secondary sites. This requires that cell-cell and cell-matrix contacts are remodeled and underlying basement membrane disrupted so that the tumour can enter the blood or lymphatic systems and travel to a distant site (Sahai and Marshall, 2002a; Vega and Ridley, 2008).

Unlike Ras proteins, which are frequently mutated in tumours, mutations in Rho GTPases are relatively rare (although an increasing number of activating mutations have recently been identified in a range of cancers eg. (Krauthammer et al., 2012)) (Vega and Ridley, 2008). Rather, several examples exist where expression levels or activity of Rho GTPases are altered in tumours. For

example, RhoA (Fritz et al., 2002; Fritz et al., 1999; Kamaï et al., 2001), RhoC (Clark et al., 2000; Fritz et al., 2002; Suwa et al., 1998; van Golen et al., 2000), Rac1 (Fritz et al., 2002; Fritz et al., 1999), Rac2 (Roberts et al., 1999), Rac3 (Mira et al., 2000), Cdc42 (Fritz et al., 2002; Fritz et al., 1999), and RhoF (Gouw et al., 2005) are all upregulated in a range of cancers. In contrast, RhoB and RhoE are frequently downregulated (Baldwin et al., 2008; Bektic et al., 2005; Huang and Prendergast, 2006; Luo et al., 2012; Zhao et al., 2012) (Reviewed in (Gomez del Pulgar et al., 2005; Sahai and Marshall, 2002a; Vega and Ridley, 2008). Interestingly, in some cases upregulation of a GTPase may promote one aspect of cancer progression but inhibit another, and cell-type specific functions are likely. For example, Cdc42 is upregulated in some breast cancers (Fritz et al., 1999), but its depletion in liver promotes tumourigenesis (Van Hengel et al., 2008).

Rho GTPases are vital regulators of AJs (discussed below) and TJs. As such the misregulation of their activity can have severe consequences for cell-cell adhesion. Loss of epithelial polarity may be the first stage in tumourigenesis as this subsequently leads to disassembly of cell-cell contacts. TJs are important regulators of epithelial polarity, thus disruption of Cdc42 may promote cancer progression by perturbing TJ organisation. In addition, RhoE expression can antagonise RhoA and lead to loss of epithelial polarity (Guasch et al., 1998).

Cancer cells typically invade either collectively or individually, but both require the generation of mechanical force facilitated by the actin cytoskeleton and modulation of cell-cell and cell-matrix contacts. Two modes of (interchangeable) individual cell migration have been described, mesenchymal and ameboid (Sahai and Marshall, 2003; Sanz-Moreno et al., 2008; Wolf et al., 2003). Mesenchymal migration is characterised by the formation of lamellipodia at the leading edge, focal adhesions that provide traction to enable the cell to progress, and the secretion of proteases to digest the surrounding ECM. This mode of migration is driven primarily by Rac activation but RhoA contributes to tail retraction (Sanz-Moreno et al., 2008). Cells migrating using the ameboid mode are characteristically round and do not excrete proteases, instead relying on actomyosin-based contractility to drive the cell forward (Sahai and Marshall, 2003; Wolf et al., 2003; Wyckoff et al., 2006). This mode is driven by high Rho activity coupled with Rac inhibition (Sanz-Moreno et al., 2008).

In collective cell migration the leading cell of the group generates the necessary force for movement, whilst the trailing cells are dragged behind (Vega and Ridley, 2008). In cultures of squamous cell carcinomas, the leading cell relies on RhoA activity for the secretion of MMPs to facilitate passage through the ECM and also regulation of MLC phosphorylation to generate necessary force to carry

the collection forward (Gaggioli et al., 2007). In the trailing cells, depletion of Cdc42 via siRNA reduces the ability of squamous cell carcinoma cells to invade (Gaggioli et al., 2007).

In order to survive, tumour cells must resist apoptotic signals and Rho GTPases have been implicated in this process. Several studies have found activation of Rac1 and its splice variant, Rac1b, protects cells from apoptosis (Friedland et al., 2007; Joneson and Bar-Sagi, 1999; Matos and Jordan, 2005). However, Rac1 can also promote apoptosis in PC-12 cells via p38 and c-Jun N-terminal kinase (JNK) signaling (Xia et al., 1995).

1.7.5. Rho regulators in cancer

The misregulation of Rho GTPases in cancer is often associated with perturbation in their activation or their inhibition (Vega and Ridley, 2008). Consequently, perturbations in the activity, localisation or expression of their upstream regulators, the Rho GEFs and GAPs, have been described in different cancers.

Due to their ability to activate GTPases, the overexpression of Rho GEFs has generally been considered oncogenic. Accordingly, several Rho GEFs are upregulated in cancers (Lazer and Katzav, 2011) including PIP₃-dependent Rac exchanger 1 protein which is upregulated in prostate adenocarcinoma and lymph node metastases (Qin et al., 2009), ECT2 which is upregulated in lung and esophageal cancers (Hirata et al., 2009), and Tiam1 which is overexpressed in hepatocellular carcinoma, colon cancer cell lines, breast carcinomas and giant-cell lung carcinomas and is associated with malignancy in these cases (Adam et al., 2001; Hou et al., 2004; Lazer and Katzav, 2011; Minard et al., 2005; Yang et al., 2010). In many cases, aberrant activation of the target GTPase is also observed upon Rho GEF overexpression. Leukemia associated Rho GEF (LARG) fuses to the mixed lineage leukemia gene in acute myeloid leukemia, and results in aberrant RhoA activation (Reuther et al., 2001). Vav family GEFs have repeatedly been associated with cancer progression; Vav1, 2 and 3 are overexpressed in numerous cancers and Vav1 expression is associated with poor prognosis (Lazer and Katzav, 2011). The GEF activity of Vav1 seems to contribute to malignancy (Lazer and Katzav, 2011).

Historically, GAPs have been considered as more likely to be tumour suppressors, due to their ability to 'switch off' GTPases. Indeed a number of these have been identified including Deleted in liver cancer 1 (DLC1), DLC2, p190, β 1-chimaerin and slit-robo GAP 3 (SRGAP3) (El-Sitt et al., 2012; Lahoz and Hall, 2012; Liao and Lo, 2008; Wang et al., 1997; Yuan et al., 1995). DLC family members are best

studied in terms of the tumour suppressive capabilities (Liao and Lo, 2008). Re-expression of DLC1 in breast, prostate and liver cancer cell lines can inhibit growth (Goodison et al., 2005; Guan et al., 2008; Wong et al., 2005).

Conversely, relatively few GAPs have been found upregulated in tumours, including ARHGAP8, ARHGAP21 and CdGAP (Bigarella et al., 2009; He et al., 2011; Johnstone et al., 2004; Lazarini et al., 2013). Expression of these GAPs often promotes migration in cancers, but the mechanisms behind this are not well understood. CdGAP protein and mRNA levels are increased in mammary tumour explants expressing an activated form of the ErbB2 receptor (He et al., 2011). In these cells CdGAP appears to be required for migration and invasion induced by TGF- β , suggesting CdGAP can promote cancer progression downstream of TGF- β (He et al., 2011). ARHGAP21 is overexpressed in head and neck squamous cell carcinoma and its depletion impairs migration of glioblastoma cells (Bigarella et al., 2009) and reduces proliferation and migration in some prostate cancer cell lines (Lazarini et al., 2013). Both ARHGAP24 and ARHGAP22 are activated downstream of Rho and inhibit Rac activity resulting in promotion of amoeboid-like migration in melanoma (ARHGAP22), breast, lung, prostate and colorectal carcinomas (ARHGAP24) (Saito et al., 2012; Sanz-Moreno et al., 2008).

1.8. Rho GAPs

This project focuses on the role of Rho GAPs of which more than 60 are predicted (Bernards, 2003). Rho GAPs out-number their GTPase targets by around 3 to 1 meaning each is likely to regulate GTPases in a specific signaling pathway. Indeed, Rho GAPs contain a mixture of structural domains, allowing them to perform a wide range of functions and mediate cross talk between other small GTPase families (Bernards, 2003; Tcherkezian and Lamarche-Vane, 2007) (**Figure 1.7**). Rho GAPs can be divided into several subfamilies based on sequence similarities (Bernards, 2003) (**Figure 1.7**). The general functions of these are discussed briefly below but discussion regarding the roles of Rho GAPs at AJs will be discussed later (section 1.10).

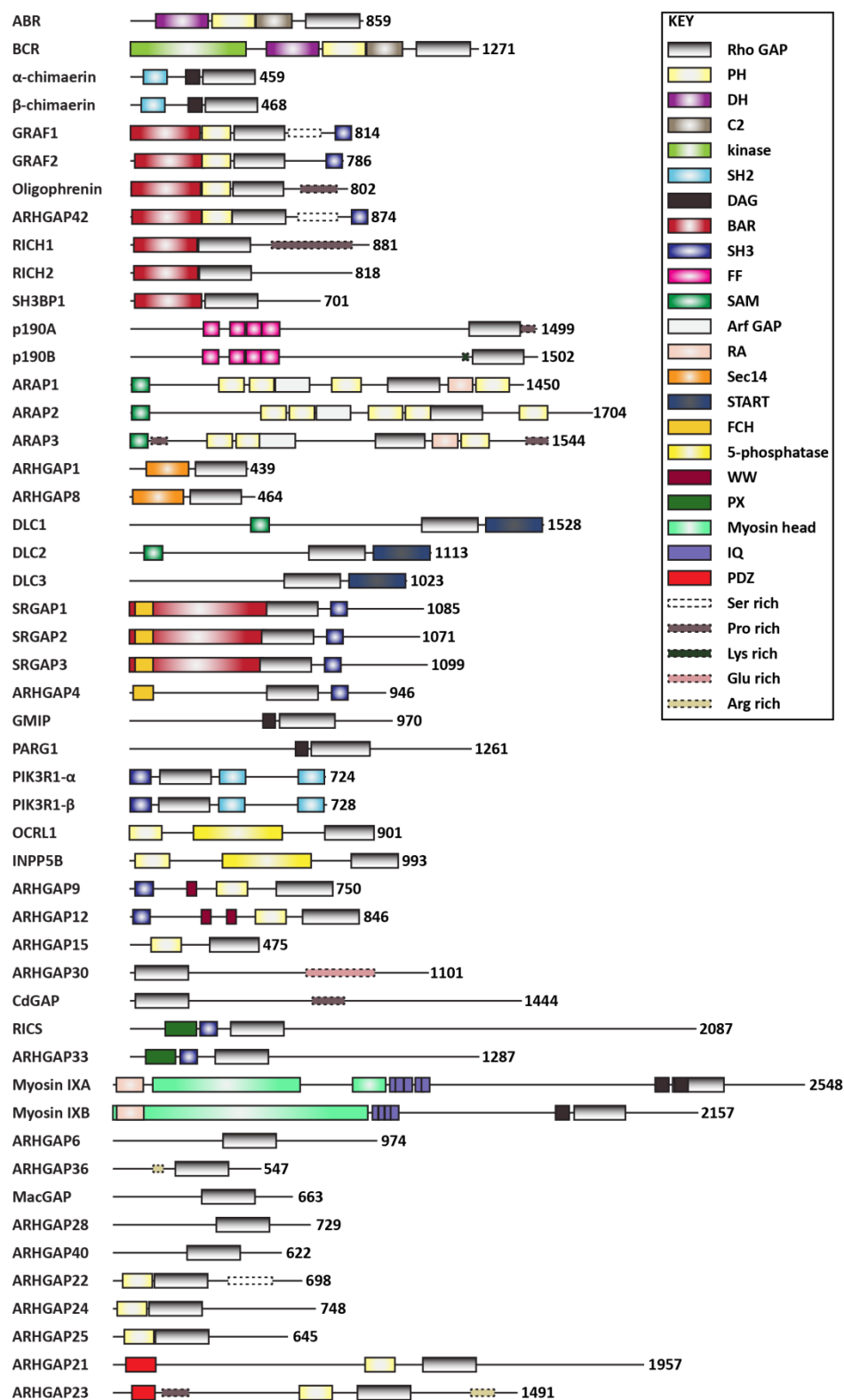


Figure 1.7 The domain structures of Rho GAP proteins Rho GAPs contain a wide variety of functional domains and can be grouped based on structural similarities (described (Bernards, 2003)). PH, pleckstrin homology; DH, Dbl homology; C2, cysteine-rich phorbol ester binding; SH2, Src homology 2, DAG, Phorbol-ester/DAG-type zinc finger; BAR, Bin, amphiphysin, RSV161/167; SH3, Src homology 3; FF, two conserved Phe residues; SAM, sterile alpha motif; RA, Ras-associating; Sec14, Sec14-like; START, StAR (steroidogenic acute regulatory)-related lipid transfer; FCH, Fes/CIP4 homology; WW, two conserved Trp residues; PX, Phox homology; IQ, calmodulin-binding motif; PDZ, post synaptic density protein, *Drosophila* disc large tumor suppressor, and zonula occludens-1 protein. Amino acid number is shown at protein C-terminals.

1.8.1. ABR and BCR

The breakpoint cluster region (BCR) gene was first identified as a cause myelogenous leukemia due to the fusion of BCR to ABL kinase which results in aberrant activation of the kinase (Gianfelici et al., 2012; Groffen et al., 1984). Active BCR-related gene (ABR) is highly similar to BCR, but lacks the N-terminal region (Heisterkamp et al., 1993). Both ABR and BCR contain GAP and GEF domains. The GAP domain is active against Rac and Cdc42, whilst the GEF domain targets Rho, Rac and Cdc42 (Chuang et al., 1995). The GEF and GAP domains of ABR and BCR can bind GTPases non-competitively, suggesting these proteins could act as dual regulators of GTPase activity (Chuang et al., 1995). Subsequently, ABR has been shown to control the spatial separation of Rho during single wound repair (Vaughan et al., 2011). In this case, ABR is targeted to areas of active Rho and potentiates Rho activity whilst simultaneously inhibiting Cdc42 activity in this area (Vaughan et al., 2011).

Both ABR and BCR have described roles in the regulation of innate immune cells (Cho et al., 2007; Cunnick et al., 2009). There is likely some redundancy in function between ABR and BCR, as ABR-null mice have no obvious phenotype. However, distinct functions have also been described, for example macrophages from BCR-null and double ABR/BCR-null mice produce more reactive oxygen species (ROS) than wild-type controls whereas ABR-null mice do not (Cunnick et al., 2009; Voncken et al., 1995).

1.8.2. The chimaerins

The α -chimaerin and β -chimaerin gene products can be alternatively spliced to yield $\alpha 1$ -, $\alpha 2$ -, $\beta 1$ - and $\beta 2$ -chimaerin (Tcherkezian and Lamarche-Vane, 2007). All can act as Rac GAPs (Diekmann et al., 1991; Leung et al., 1993). At their N-terminus chimaerins harbor cysteine-rich regions, which mediate interactions with phorbol esters and contribute to regulation of their activity (Ahmed et al., 1993). Functions related to the regulation of axonal growth in the nervous system have been described for α -chimaerin, including regulation of axon guidance and dendritic spine development (Buttery et al., 2006; Diaz et al., 2002). By interacting with ephrins, α -chimaerin is required to limit Rac activity in order to inhibit outgrowth which is essential for the correct development of neural networks (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007). Chimaerins are also able to regulate T-cell receptor signaling and localise to the immune synapse in T-cells (Caloca et al., 2008). Expression of either α - or β -chimaerin can inhibit nuclear factor of activated T-cells signaling (a

family of transcription factors that mediate activation and differentiation of T-cells) in a GAP-dependent manner (Caloca et al., 2008).

1.8.3. GRAF1, GRAF2, Oligophrenin and ARHGAP42

As well as a Rho GAP domain, members of this group all contain PH, BAR and (with the exception of Oligophrenin 1) SH3 domains (Bernards, 2003). GTPase regulator associated with focal adhesion kinase (GRAF1, also known as ARHGAP26) and GRAF2 (ARHGAP10) are Rho and Cdc42 GAPs (Shibata et al., 2001) (although *in vivo* GRAF1 is more active on Rho (Doherty et al., 2011b)) and both interact with the Rho target PKN β (Shibata et al., 2001). GRAF localises to focal adhesions where it interacts with focal adhesion kinase (FAK) (Hildebrand et al., 1996). GRAF2 also interacts with the FAK-related kinase and Protein-tyrosine kinase 2 (Ren et al., 2001) suggesting these proteins may regulate cell-matrix adhesions. Interestingly, GRAF1 can regulate clathrin-independent endocytosis (Doherty et al., 2011a; Lundmark et al., 2008) and by coordinating endocytosis at adhesion sites regulate cell spreading and migration (Doherty et al., 2011a). GRAF is also able to promote muscle differentiation in C2C12 cells and its regulation of both endocytosis and muscle differentiation are dependent on GRAF GAP activity and the BAR domain (Doherty et al., 2011b).

Oligophrenin 1 is predominantly expressed in the brain and is a GAP for Rac, Rho and Cdc42 (Fauchereau et al., 2003). Loss of Oligophrenin 1 is associated with X-linked mental retardation (Barresi et al., 2010; Billuart et al., 1998), and results in perturbations in dendritic spine morphology. In primary neurons this defect can be rescued by inhibition of ROCK, suggesting Oligophrenin 1 acts to inhibit RhoA signaling (Govek et al., 2004). Interestingly, Oligophrenin 1 is localised at pre- and post-synaptic neurons (Govek et al., 2004) and loss of Oligophrenin 1 impairs uptake of synaptic vesicles (Nakano-Kobayashi et al., 2009) implying a role for Oligophrenin 1 in endocytosis. So far, there have been no studies on ARHGAP42.

1.8.4. RICH1, RICH2 and SH3BP1

Members of this group all contain BAR domains at their N-terminus (Tcherkezian and Lamarche-Vane, 2007). All can act as Rac and Cdc42 GAPs *in vitro* (Cicchetti et al., 1995; Harada et al., 2000; Richnau and Aspenstrom, 2001) whilst Rich1 and SH3 domain-binding protein 1 (SH3BP1) can act as Rac and Cdc42 GAPs *in vivo* (Parrini et al., 2011; Wells et al., 2006; Yi et al., 2011).

Rich1 regulates TJ organisation and epithelial polarity via Cdc42 (Wells et al., 2006). ZO-1 and polarity complex proteins Par3 and Pals1 are mislocalised upon depletion of Rich1, whilst expression of a GAP-dead Rich1, or Amot impairs barrier function. Thus, Rich1 is required to confine Cdc42 activity in order to promote TJ maintenance and uphold barrier integrity (Wells et al., 2006). The inhibition of Rich1 by Amot may also have clinical significance as Amot signals downstream of the tumour suppressor protein Merlin. Upon formation of cell-cell contacts, Merlin localises to junctions, where it inhibits Extracellular signal-regulated kinase (ERK)/ Mitogen-activated protein kinase (MAPK) signaling to promote cell-cell contact (Jin et al., 2006; Lallemand et al., 2003; Morrison et al., 2007; Stokowski and Cox, 2000). At mature junctions Merlin binds Amot, which releases Rich1 and allows it to inactivate Rac leading to a decrease in MAPK signaling (Yi et al., 2011). Rich2 (ARHGAP44) also regulates epithelial polarity but in this case by coupling the lipid raft-associated membrane protein tetherin to the actin cytoskeleton via interaction with ezrin (Rollason et al., 2009). Loss of Rich2 disrupts this link and leads to the accumulation of actin basally and loss of cell height (Rollason et al., 2009).

SH3BP1 regulates cell migration via inactivation of Rac1, required to control actin dynamics at the leading edge (Parrini et al., 2011).

1.8.5. p190A and p190B

The GAPs p190A and p190B (ARHGAP35 and ARHGAP5 respectively) are structurally similar RhoA GAPs (Burbelo et al., 1995; Bustos et al., 2008; Su et al., 2009). These proteins contain four FF (two phenylalanine residues) and act predominantly on RhoA (Burbelo et al., 1995; Settleman et al., 1992; Tcherkezian and Lamarche-Vane, 2007). Diverse processes are regulated by p190A and p190B. The expression of p190A is regulated by the cell cycle (Su et al., 2003) and p190 has a role in cytokinesis. p190A can interact with general transcription factor II-I and control expression of serum-activated genes (Jiang et al., 2005). Both p190A and p190B are highly expressed in the nervous system and regulate the organisation of actin in the developing neuroepithelium (Brouns et al., 2000; Matheson et al., 2006). Mammary gland morphogenesis can be regulated by p190B which may be linked to tumour formation (Chakravarty et al., 2000).

1.8.6. ARAP1, ARAP2 and ARAP3

Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing proteins (ARAP) 1-3 contain a sterile α -motif (SAM), five PH domains, an ArfGAP domain, ankyrin repeats, a Rho GAP domain and a

Ras-association (RA) domain (Krugmann et al., 2002; Miura et al., 2002). This domain structure means that members of this group of proteins are well placed to coordinate cross-talk between different GTPase families. Furthermore, as well as acting as Arf and Rho GAPs, ARAP3 interacts with Rap1 (Krugmann et al., 2004).

The ArfGAP activities of these proteins are regulated by PIP₃ binding (Campa et al., 2009; Krugmann et al., 2004). ARAP1 acts on Arf1 and Arf5, ARAP2 acts on Arf6 and ARAP3 acts on Arf5 and Arf6 (Krugmann et al., 2002; Miura et al., 2002; Yoon et al., 2006). ARAP1 and ARAP3 are Rho specific GAPs, whilst ARAP2 can bind RhoA, it is unable to inhibit Rho as it lacks the catalytic arginine (Krugmann et al., 2004; Miura et al., 2002; Yoon et al., 2006). However, ARAP2 can indirectly regulate Rac levels through inactivation of Arf6 (Chen et al., 2013; Yoon et al., 2006). The loss of ARAP2 results in an increase of Arf6 and Rac (which can be activated downstream of Arf6) (Chen et al., 2013; Radhakrishna et al., 1999).

ARAP1 regulates actin cytoskeleton dynamics and endocytosis of epidermal growth factor receptor (EGFR) (Daniele et al., 2008; Hasegawa et al., 2012; Miura et al., 2002; Yoon et al., 2008). ARAP2 promotes the stabilisation of focal adhesions (Chen et al., 2013) and loss of ARAP2 reduces the appearance of focal adhesions and stress fibres (Chen et al., 2013; Yoon et al., 2006). ARAP3 is a phosphatidylinositol 3-kinase (PI3K) effector (Krugmann et al., 2002) and functions in a number of important cellular processes including promoting cell adhesion to the ECM, consequently inhibiting invasion in scirrhous gastric carcinoma cells (Yagi et al., 2011). ARAP3-null mice die due to defects in angiogenesis (Gambardella et al., 2010). Inactivation of RhoA by ARAP3 can promote neurite outgrowth downstream of Rap1 (Jeon et al., 2010a; Jeon et al., 2010b).

1.8.7. ARHGAP1 and ARHGAP8

ARHGAP1 (also known as p50RhoGAP or Cdc42GAP) was the first RhoGAP discovered (Garrett et al., 1989). ARHGAP1 and ARHGAP8 both contain N-terminal Sec14 domains (Tcherkezian and Lamarche-Vane, 2007). ARHGAP1 is active against RhoA, Rac1 and Cdc42 *in vitro* (Ridley et al., 1993). A knockout ARHGAP1 mouse shows diverse roles for this protein. ARHGAP1 null mice are considerably smaller than their littermates due to overactivation of the JNK signaling pathway, which leads to an increase in apoptosis (Wang et al., 2005). Defects in Rho inactivation by ARHGAP1 *in vivo* is demonstrated in neuroepithelial cells undergoing epithelial-to-mesenchymal transition (Clay and Halloran, 2013). In this case, ARHGAP1 is required to restrict Rho activity to the apical portion of

neural crest cells and this facilitates detachment of these cells prior to epithelial-to-mesenchymal transition (Clay and Halloran, 2013).

ARHGAP8 interacts with RhoA, Rac1 and Cdc42, but appears to inactivate only RhoA (Shang et al., 2003). Interestingly, ARHGAP8 enhances cell migration by coordinating RhoA, Rac1 and Cdc42 at the leading edge (Lua and Low, 2004a, b; Shang et al., 2003). ARHGAP8 can also promote EGFR endocytosis via binding to endophilin2 (Lua and Low, 2005) and activate ERK to promote differentiation of PC12 cells (Lua and Low, 2005; Ravichandran and Low, 2013).

1.8.8. DLC1, DLC2 and DLC3

DLCs have N-terminal SAM domains which mediate protein-protein interactions, and C-terminal StAR-related lipid transfer (START) domains which can bind lipids. DLC proteins act primarily on Rho (A-C) and to a lesser extent Cdc42 (Ching et al., 2003; Kawai et al., 2007; Wong et al., 2003). DLC proteins inhibit proliferation and migration of tumour cells (Liao and Lo, 2008). DLC proteins are often localised to focal adhesions and can be targeted via protein-protein interactions mediated by the N-terminus (El-Sitt et al., 2012; Kawai et al., 2009; Liao and Lo, 2008).

1.8.9. SRGAP1, SRGAP2, SRGAP3 and ARHGAP4

Slit-Robo GAPs (SRGAP1, SRGAP2 and SRGAP3) as well as closely related ARHGAP4 (sometimes called SRGAP4) all contain N-terminal Fes/CIP4 homology (FCH) domains (Bernards, 2003). Additionally, the SRGAPs contain F-BAR domains that can induce membrane curvature. SRGAP1, SRGAP2 and SRGAP3 were initially identified in a yeast two-hybrid screen to identify roundabout (robo)-binding proteins (Robo proteins are receptors for the axon guidance cue Slit) (Wong et al., 2001). Subsequently, Slit binding to robo was found to activate SRGAP1 in order to inactivate Cdc42 and regulate neuronal migration (Wong et al., 2001). The F-BAR domain of SRGAP2 contributes to neuronal morphogenesis via the formation of filopodia (Guerrier et al., 2009). Interestingly the F-BAR domains of SRGAP1, 2 and 3 all induce different effects on membranes of both neuronal and non-neuronal cells (Coutinho-Budd et al., 2012).

1.8.10. GMIP and PARG1

Members of this group all contain N-terminal FCH domains and C1 lipid binding domains. Gem-interacting protein (GMIP) and PTPL1-associated RhoGAP (PARG1) both preferentially activate Rho

(Aresta et al., 2002; Saras et al., 1997). PARG1 was identified in a screen for protein tyrosine phosphatase 1 binding partners (Saras et al., 1997). Subsequently, PARG1 was found to interact specifically with Rap2 (but not Rap1 or Ras) (Myagmar et al., 2005), which appears to inhibit PARG1 activity on Rho (Myagmar et al., 2005). GMIP interacts with the atypical Ras-related protein Gem (Aresta et al., 2002) and acts downstream of Gem and its binding partner ezrin to reorganize the actin cytoskeleton (Hatzoglou et al., 2007). GMIP also interacts with Slp1, a Rab27a effector, to regulate exocytosis (Johnson et al., 2012). By inactivating RhoA, GMIP can induce actin depolymerisation that is directed around Slp1-containing vesicles and facilitates their transport in phagocytes (Johnson et al., 2012).

1.8.11. PI3-kinase regulatory subunits p85 α and p85 β

The p85 α and p85 β subunits form part of the regulatory subunit of PI3K. PI3K regulates a variety of signaling pathways via the generation of phosphatidylinositol 3-phosphate. Structurally, both proteins are similarly composed of a Rho GAP domain, two SH2 domains and one SH3 domain (Bernards, 2003; Tcherkezian and Lamarche-Vane, 2007). Despite binding to Cdc42 and Rac (Zheng et al., 1994) p85 α has no GAP activity, even though the protein has the critical arginine finger (Zheng et al., 1993).

1.8.12. OCRL and INPP5B

Oculocerebrorenal Lowe syndrome phosphatase (ORCL1) and inositol polyphosphate 5-phosphatase type II (INPP5B) are phosphoinositide phosphatases, meaning they dephosphorylate the 5 position of the inositol ring of phosphoinositides (Liu and Bankaitis, 2010). Loss of OCRL causes Oculocerebrorenal Lowe syndrome, an X-linked disorder which causes ocular and renal defects and mental retardation (Attree et al., 1992; Pirruccello and De Camilli, 2012) and Dent disease (Hoopes et al., 2005). OCRL1 and INPP5B are the only inositol polyphosphate 5-phosphatases to contain Rho GAP domains. Both OCRL1 and INPP5B GAP domains lack the catalytic arginine required for GAP activity (Jefferson and Majerus, 1995), although OCRL does have low Rac GAP activity *in vitro* (Faucherre et al., 2003), and can interact with Rac and Cdc42 via its GAP domain (Erdmann et al., 2007; Faucherre et al., 2003). OCRL1 also interacts with Arf GTPases 1 and 6 (Krauss et al., 2003) and several Rab GTPases. The latter of which is thought to result in OCRL1 membrane localisation (Dambournet et al., 2011; Fukuda et al., 2008; Hyvola et al., 2006; Rodriguez-Gabin et al., 2010).

1.8.13. ARHGAP9, ARHGAP12 and ARHGAP15

These proteins all contain N-terminal PH domains and C-terminal GAP domains. ARHGAP9 and ARHGAP12 also contain WW domains (two conserved tryptophan residues) (Tcherkezian and Lamarche-Vane, 2007). ARHGAP15 and ARHGAP12 are Rac GAPs (Gentile et al., 2008; Seoh et al., 2003), whereas ARHGAP9 is active against Rac and Cdc42 (Furukawa et al., 2001). ARHGAP12 is localised to AJs (Matsuda et al., 2008) and its overexpression suppresses cell scattering in response to hepatocyte growth factor (HGF, a growth factor used to promote epithelial-to-mesenchymal transition), which also transcriptionally downregulates ARHGAP12 expression (Gentile et al., 2008).

ARHGAP15-null mice are viable and fertile. Analysis of macrophages and neutrophils taken from these mice reveal roles in limiting chemotactic responses, ROS production and phagocytosis (Costa et al., 2011). Interestingly, ARHGAP15 can interact with PAK1 and 2 and is directly phosphorylated by PAK2 downstream of Rac activation which results in inhibition of ARHGAP15 GAP activity (Radu et al., 2013). Interestingly, ARHGAP15 inhibits PAK activity (Radu et al., 2013) and an increase in PAK signaling increases ERK activation in ARHGAP15-depleted cells. ARHGAP9 also inhibits ERK signaling, although this is through direct interaction between ARHGAP9 WW domain and the MAP kinases ERK2 and p38 α (Ang et al., 2007).

1.8.14. ARHGAP30, CdGAP, RICS and ARHGAP33

ARHGAP30 and CdGAP (ARHGAP31) are highly similar proteins and share the same binding partners in some cases, including intersectin and the atypical Rho GTPase Wrch1 (Naji et al., 2011; Primeau et al., 2011). The Rho GAP domains of RhoGAP involved in the beta-catenin-N-cadherin and NMDA receptor signaling (RICS, ARHGAP32) and ARHGAP33 share approximately 70% homology with CdGAP (Tcherkezian and Lamarche-Vane, 2007). RICS and ARHGAP33 also contain phox homology (PX) domains at their N-terminals (Moon et al., 2003; Tcherkezian and Lamarche-Vane, 2007). ARHGAP30 may regulate cell-matrix adhesions (Naji et al., 2011). Diverse functions of CdGAP have been described including the regulation of cell-matrix adhesions (Jenna et al., 2002; Wormer et al., 2012), the maintenance of TJ (Togawa et al., 2010), cell spreading and migration (LaLonde et al., 2006; Wormer et al., 2012).

RICS-null mice appear normal and are viable. RICS is highly expressed in the brain and knockout mice have longer neurites with enhanced Cdc42 activity (Nasu-Nishimura et al., 2006), implicating RICS in

the inhibition of neurite outgrowth. ARHGAP33 is also highly expressed in the brain (Liu et al., 2006) and also negatively regulates neurite outgrowth (Liu et al., 2006; Shen et al., 2012).

1.8.15. Myosin IXA and myosin IXB

Myosin IXA (Myo9A) and Myosin IXB (Myo9B) are unconventional myosins with complex domain structures composed of calmodulin-binding IQ domains, myosin head-like domains, phorbol ester/DAG-type zinc fingers, an RA domain and Rho GAP domain. Myo9A and Myo9B are both Rho-specific GAPs (Chieriegatti et al., 1998; Post et al., 1998). Loss of Myo9A results in severe hydrocephalus due to abnormal epithelial organisation (Abouhamed et al., 2009). Myo9B-null mice are viable and fertile (Hanley et al., 2010). Myo9B is highly expressed in immune cells (Hanley et al., 2010; Wirth et al., 1996). Macrophages derived from knockout mice do not respond to chemotactic cues as normal, exhibiting fewer membrane protrusions and migrating more slowly suggesting Myo9B regulates migration (Hanley et al., 2010). These defects can be partially restored by inhibition of Rho via treatment with C3 (Hanley et al., 2010). Defects in cell migration are also observed in epithelial cells depleted of Myo9B during wound healing assays and assembly of TJs is also perturbed (Chandhoke and Mooseker, 2012).

1.8.16. ARHGAP6 and ARHGAP36

ARHGAP6 shares approximately 41% sequence similarity with ARHGAP36. No studies on ARHGAP36 have been published. ARHGAP6 was originally discovered as part of this gene is deleted in microphthalmia with linear skin defects syndrome (MLS) (Prakash et al., 2000; Schaefer et al., 1997). Mice where the ARHGAP6 Rho GAP domain has been deleted appear normal, suggesting GAP-independent functions of ARHGAP6 in MLS (Prakash et al., 2000). ARHGAP6 is also able to regulate actin remodeling and PLC δ activation via GAP-independent mechanisms (Ochocka et al., 2008; Prakash et al., 2000).

1.8.17. MacGAP, ARHGAP28 and ARHGAP40

Neither ARHGAP28, nor ARHGAP40 have been functionally characterised, and little is known about the function of MacGAP (ARHGAP18). MacGAP was initially identified from a library of epididymis-specific cDNA library (Li et al., 2008; Liu et al., 2001). MacGAP is localised to the leading edge in migrating cells and acts as a RhoA GAP to promote migration (Maeda et al., 2011).

1.8.18. ARHGAP22, ARHGAP24 and ARHGAP25

These proteins contain an N-terminal PH domain, central Rho GAP domain and C-terminal coiled-coil (Nakamura, 2013). All members of this group target Rac *in vivo*, although ARHGAP24 (FilGAP) may also target Cdc42 (Itoh et al., 2002; Ohta et al., 2006; Sanz-Moreno et al., 2008). Both ARHGAP24 and ARHGAP22 are activated downstream of Rho and inactivate Rac to regulate cell polarity and cancer cell migration (Itoh et al., 2002; Saito et al., 2012; Sanz-Moreno et al., 2008). ARHGAP25 is less well studied but is highly expressed in spleen and leukocytes and its expression in primary macrophages inhibits phagocytosis (Csepanyi-Komi et al., 2012).

ARHGAP24 is the best characterised of this group and binds the actin cross-linker Filamin-A, which regulates the activity and localisation of ARHGAP24 (Ohta et al., 2006). ARHGAP24 is localised to actin-rich protrusions such as lamellipodia and podocytes (Akilesh et al., 2011; Ohta et al., 2006). By targeting ARHGAP24 to lamellipodia, Filamin-A increases ARHGAP24 GAP activity against Rac (Ohta et al., 2006). Interestingly, this interaction is regulated by mechanical stress; strain disrupts the shape of Filamin-A, and consequently alters protein-binding sites of this protein leading to dissociation of ARHGAP24 from Filamin-A in response to mechanical forces (Ehrlicher et al., 2011).

1.8.19. ARHGAP21 and ARHGAP23

ARHGAP21 (sometimes referred to as ARHGAP10) and ARHGAP23 share approximately 45% sequence similarity (Bernards, 2003). No studies have been published on ARHGAP23. ARHGAP21 may play a role in cancer progression by modulating migration. ARHGAP21 regulates cellular trafficking via inactivation of Cdc42 and binding to Arf1 (Dubois et al., 2005; Hehny et al., 2009; Wang et al., 2012) and regulates actin dynamics via Cdc42 and Arp2/3 at the Golgi (Dubois et al., 2005).

1.8.20. Miscellaneous GAPs

Several Rho GAPs lack similarity with other Rho GAP proteins. These include RacGAP1, ARHGAP20 (RA-RhoGAP), RalBP1, ARHGAP11A, ARHGAP39, TAGAP and DEPDC1B. For some of these proteins little has been described regarding their functions (ARHGAP11A, ARHGAP39, TAGAP and DEPDC1B). However, for others diverse roles have been explored including the regulation of cytokinesis by RacGAP1 (Hirose et al., 2001; Lee et al., 2004; Minoshima et al., 2003). Furthermore, ARHGAP20 and

RalBP1 also mediate cross talk between Rho GTPases and Rap1 and Ral, respectively (Cantor et al., 1995; Yamada et al., 2005b).

1.9. Rho GTPases at AJs

The formation, stabilisation, maintenance and disassembly of AJs rely on the correct coordination of the actin cytoskeleton. The construction of different actin structures is vital for the initiation of contacts through the formation of exploratory protrusions, for driving contact expansion and for stabilising junctions by creating strong underlying actin cables. Rho family GTPases interact with a host of effectors that are able to modulate the actin cytoskeleton and drive cell-cell adhesion (**Figure 1.6**). The most well characterised members of this family are discussed below as well as GTPases from other families that can regulate AJs.

1.9.1. RhoA

The requirement for Rho activity during contact formation is somewhat contradictory, and may represent differences between cell type, stimulus and interaction with downstream effectors. In keratinocytes, RhoA is activated downstream of E-cadherin adhesion (Calautti et al., 2002) and may be required for contact stabilisation, as Rho activity remains high even 24 hours after junction formation (Calautti et al., 2002). Blocking Rho activity (via C3 transferase) results in removal of cadherins from junctions (Braga et al., 1999; Braga et al., 1997; Calautti et al., 2002; Takaishi et al., 1997), which is partially attributed to signaling through PRK2 (a PKN-related kinase), which promotes phosphorylation of p120 catenin and β -catenin (Calautti et al., 2002). However, Rho activity must be tightly constrained. RhoA is downregulated in some systems, and constitutive activation can disassemble junctions (Noren et al., 2003; Noren et al., 2001; Sahai and Marshall, 2002b). Furthermore, RhoA is required for disassembly of cell-cell contacts under certain stimuli (Samarin et al., 2007).

In MDCK cells, RhoA activity is confined to the edges of nascent contacts, and appears to drive contact expansion via activation of ROCK and MyoII (Yamada and Nelson, 2007). This increases actomyosin contractility necessary for driving expansion of the contacting membranes (Yamada and Nelson, 2007). However, ROCK-mediated actomyosin contractility can be disruptive in other systems. Activation of ROCK by RhoC in colon carcinoma cells perturbs α -catenin localisation and disrupts junctions (Sahai and Marshall, 2002b). Conversely, RhoA-mediated activation of the actin-

binding protein Dia promotes localisation of E-cadherin and α -catenin to the cell periphery in colon carcinoma cells (Sahai and Marshall, 2002b).

1.9.2. Rac1

The requirement for Rac activity at AJs is fairly well-established in various cell types. Rac activity is necessary for the formation, stabilisation and maintenance of AJs (Braga and Yap, 2005; Citi et al., 2011). As Rac drives lamellipodia formation, Rac activity is important in the early stages of cell-cell contact formation (Ehrlich et al., 2002; Yamada and Nelson, 2007). As contacts expand, Rac activity is restricted to the periphery of the contacting membranes (Ehrlich et al., 2002; Yamada and Nelson, 2007), where it overlaps with Arp2/3 and drives lamellipodia formation (Yamada and Nelson, 2007).

Cell-cell contact and cadherin engagement results in rapid activation and localisation of Rac at newly-forming AJs (Betson et al., 2002; Kovacs et al., 2002b; Nakagawa et al., 2001; Noren et al., 2001). Rac seems to be required for actin recruitment at newly forming contacts, as expression of dominant negative Rac blocks the recruitment of actin to clustered cadherins (Braga et al., 1997; Lambert et al., 2002).

Rac is also necessary for the maintenance of stable AJs as expression of both dominant negative or constitutively active Rac in keratinocytes or MDCK cells perturb AJs (Braga et al., 1997; Jou and Nelson, 1998; Takaishi et al., 1997). PAK activation is required for Rac-mediated junction disassembly, indicating that aberrant expression of PAK may promote contact disassembly in the context of tumour progression (Lozano et al., 2008). However, PAK activation also contributes to AJ stabilisation via phosphorylation of Ajuba (Nola et al., 2011). PAK targets are diverse, thus activation of PAK downstream of Rac (or Cdc42) may regulate AJs via multiple pathways (Bokoch, 2003; Szczepanowska, 2009) (**Figure 1.6**).

A second way in which Rac may stabilise cell-cell contacts is via binding IQGAP (Fukata et al., 1999; Kuroda et al., 1998; Noritake et al., 2005). IQGAP binds and cross-links actin filaments (Noritake et al., 2005) and interacts with β -catenin, inducing its dissociation from α -catenin (Kuroda et al., 1998). Active Rac binds to IQGAP, thereby preventing its interaction with β -catenin and strengthening contacts by stabilising actin (Fukata et al., 1999; Noritake et al., 2005). Finally, Rac can also regulate E-cadherin endocytosis, which may contribute to its ability to stabilise junctions (Akhtar and Hotchin, 2001; Izumi et al., 2004).

1.9.3. Cdc42

The role of Cdc42 at AJs is controversial. Expression of constitutively active or dominant negative forms of Cdc42 in various cell types does not consistently perturb AJs, as is the case with Rac (Chu et al., 2004; Jou and Nelson, 1998; Takaishi et al., 1997). Accordingly, cell-cell contact formation in MCF-7 cells activates Cdc42 (Kim et al., 2000b), whereas in keratinocytes Cdc42 is not activated upon junction formation (Erasmus et al., 2010). Thus Cdc42 may be required for junction formation in some, but not all cell types.

Cdc42 directs the formation of filopodia and these may promote initial cell-cell contacts forming in some cell types (Vasioukhin et al., 2000). As Cdc42 shares several of the same effectors with Rac, it may regulate AJs using similar mechanisms. For example, Cdc42 can also bind IQGAP to prevent its association with β -catenin (Kuroda et al., 1998) and inhibit E-cadherin endocytosis in MDCK cells (Izumi et al., 2004) (**Figure 1.6**).

1.9.4. Regulation of AJs by other small GTPases

Arf6 is localised to the plasma membrane and its participation in the regulation of cell-cell adhesion has been extensively characterised (D'Souza-Schorey, 2005). Arf6 activation promotes E-cadherin internalisation leading to AJ disassembly (Palacios et al., 2001). Treatment of cells with HGF induces breakdown of AJs and cell scattering. HGF promotes the activation of Arf6 which in turn activates Rac1 and perturbs junctions via endocytosis of E-cadherin. Interestingly, Arf6 is also activated downstream of the Par3/Par6 polarity complex upon epithelial polarisation in Eph4 cells. Depletion of Arf6 in these cells impairs the formation of AJs (Ikenouchi and Umeda, 2010). Thus, Arf6 is able to regulate both the assembly and disassembly of AJs.

Rabs can regulate cell-cell adhesion by controlling the destination of endocytosed E-cadherin and regulating the trafficking of junctional proteins. E-cadherin is recycled back to the plasma membrane in a Rab11-dependent manner (Desclozeaux et al., 2008). However, by directing E-cadherin to lysosomes for degradation via Rab5 and Rab7, cell-cell adhesion is reduced (Frasa et al., 2010; Kamei et al., 1999; Palacios et al., 2005). Interestingly, upon expression of v-Src (which induces epithelial-to-mesenchymal transition), Rab5 can direct E-cadherin-containing vesicles to the lysosome for degradation (Palacios et al., 2005). Rab8 and Rab35 have also been described to regulate E-cadherin trafficking and thus AJ stability (Charrasse et al., 2013; Yamamura et al., 2008).

Ras-related proteins (Raps) are closely related in sequence to Ras proteins. Rap1 and Rap1-specific GEFs are localised to cell-cell contacts in many epithelial cell types (Hogan et al., 2004; Yajnik et al., 2003). Loss of Rap1 in *Drosophila* wing disrupts the localisation of DE-cadherin (Knox and Brown, 2002). In mammalian cells, the expression of activated Rap1 rescues disruption of junctions by distinct stimuli: downstream of oncogenic Ras or following HGF treatment (Price et al., 2004). The expression of the Rap GEF Dedicator of cytokinesis (DOCK) 4 in osteosarcoma cells can also prevent junction disruption (Yajnik et al., 2003). Rap1 may regulate the endocytosis of E-cadherin, as interaction between Rap1 and the adaptor protein afadin/AF6 inhibits the endocytosis of E-cadherin (Boettner et al., 2000; Hoshino et al., 2005).

1.10. Regulation of Rho GTPases at AJs

As Rho GTPases are vital regulators of AJs, it is likely that a range of GEFs and GAPs are required to correctly coordinate their actions here. Due to the diversity of cellular processes Rho GAPs and GEFs are involved in there are many ways in which they may regulate AJs.

No GEFs have been described to specifically regulate cell-cell contact formation (McCormack et al., 2013). However, Rho GEFs that regulate the stabilisation, maintenance and disassembly of AJs have been reported. Similarly, several Rho GAPs have been identified to regulate various steps during cell-cell contact formation, maintenance and disassembly (**Figure 1.8**). There are also examples of Rho GEFs and GAPs localised to AJs. However, localisation does not necessarily imply activation, as some GEFs and GAPs are inactivated at AJs. Discussed below are only those GEFs and GAPs whose functions at AJs have been described.

1.10.1. GEFs regulating cell-cell contact stabilisation and maintenance

Tuba is a Cdc42-specific GEF that is found at cell-cell contacts in simple epithelial cells, where it localises with components of AJs and TJs (Otani et al., 2006). Localisation of Tuba to cell-cell contacts depends on interaction with ZO-1 and depletion of Tuba perturbs the organisation of both AJs and TJs. The depletion of Tuba does not prevent formation of junctions, but does alter the organisation of E-cadherin and F-actin in the apical portion of the cell which also correlates with reduced localisation of Cdc42. Thus, Tuba appears to recruit Cdc42 to nascent junctions and direct Cdc42 activity here (Otani et al., 2006).

ECT2 is localised at sites of cell-cell contact in MDCK cells (Liu et al., 2004), MCF-7 and Caco-2 cells (Ratheesh et al., 2012). Depletion of ECT2 has no effect on TJs, but E-cadherin is not concentrated as a single line as in control cells. Fluorescence recovery after photobleaching (FRAP) data reveals that E-cadherin is more mobile at junctions in the absence of ECT2. ECT2 appears to stabilise E-cadherin at junctions via RhoA and MyoIIA, as junctional levels of both of these proteins are also reduced upon depletion of ECT2 (Ratheesh et al., 2012).

Asef is a Rac and Cdc42 GEF that is localised to cell-cell contacts and has been shown to both increase and decrease the levels of E-cadherin at cell-cell contacts (Kawasaki et al., 2003; Muroya et al., 2007). The reason for this discrepancy is not yet known, thus the role of Asef at cell-cell contacts remains unclear.

In contrast to previous GEFs that regulate AJs via direct modulation of the cytoskeleton, Triple functioning domain protein (Trio) regulates E-cadherin transcription (Yano et al., 2011). Tara is a Trio-interacting protein and both proteins localise to cell-cell adhesions via interaction with E-cadherin (Seipel et al., 2001; Yano et al., 2011). Depletion of Tara reduces E-cadherin levels (both total protein and mRNA) and increases Rac1 activation at cell-cell contacts (Yano et al., 2011). The increase in Rac1 is dependent on Trio signaling, and results in activation of p38 MAP kinase. A target of p38 is the transcriptional repressor Tbx3, and this is also increased in response to Tara depletion (Yano et al., 2011). Thus, the inhibition of Trio by Tara is likely necessary for the stabilisation of AJs. Loss of Tara leads to release of Trio and activation of the E-cadherin transcriptional repressor Tbx3 via Rac and p38 with the outcome being a reduction of E-cadherin at contacts and disruption to the underlying actin cytoskeleton (Yano et al., 2011).

Tiam1 regulates both AJs and TJs. Expression of Tiam1 can block HGF- and H-Ras-induced transformation (Hordijk et al., 1997; Sander et al., 1998). Furthermore, Src-mediated junction disruption induces phosphorylation and degradation of Tiam1, suggesting that Tiam1 is required to maintain cell-cell adhesions (Woodcock et al., 2009). Depletion of Tiam1 in transformed MDCK cells leads to disassembly of AJs (Malliri et al., 2004). Indeed, keratinocytes derived from Tiam1 knockout mice form AJs and recruit AJ and TJ components normally. However, in the Tiam1 knockout cells, junctions fail to mature and actin is disorganised (Mertens et al., 2005). Interestingly, Tiam1 is also required for junction formation induced by expression of E1A in Ras-transformed MDCK cells, suggesting Tiam1 could also be required for AJ formation (Malliri et al., 2004).

1.10.2. GEFs regulating cell-cell contact disassembly

Invading pathogens subvert cellular machinery in order to gain access to the cell. *Listeria monocytogenes* entry into cells involves binding to the E-cadherin extracellular domain and leads to dynamic rearrangement of AJs and the actin cytoskeleton (Mengaud et al., 1996). The virulence protein InlC is essential for invasion of *L. monocytogenes* and infection induces the formation of curved junctions (similar to those seen in cells depleted of Tuba) (Otani et al., 2006; Rajabian et al., 2009). InlC can interact with Tuba at Tuba's N-terminus, which in turn prevents the interaction between Tuba and N-WASP (Rajabian et al., 2009). This leads to loss of tension, perturbation of junctions and promotes the spread of *L. monocytogenes* (Rajabian et al., 2009).

Neuroepithelial transforming protein isoform 1 (Net1) and 2 (Net1A) are RhoA GEFs. Net1A depletion disrupts AJs and leads to epithelial-to-mesenchymal transition. However, Net1 expression can also be transforming (Qin et al., 2005). Both Net1 and Net1A are predominantly localised in the nucleus, and their presence in the cytoplasm is facilitated via interaction with discs large homolog 1 (Dlg), which prevents Net1 from degradation (Carr et al., 2009; Garcia-Mata et al., 2007). Cell-cell contacts promote the Net1-Dlg interaction resulting in increased RhoA activation (Carr et al., 2009). Furthermore, treatment of HaCaTs with TGF- β leads to transcriptional upregulation of Net1A, RhoA activation and stabilises junctions (Papadimitriou et al., 2012). However, after 24-48 hours of TGF- β treatment Net1A and Net1 are degraded and epithelial-to-mesenchymal transition is initiated (Papadimitriou et al., 2012). Thus, Net1-mediated activation of Rho can stabilise junctions in some scenarios, but may be used to disassemble junctions during epithelial-to-mesenchymal transition.

1.10.3. GAPs regulating cell-cell contact formation

Only one GAP, the Cdc42 GAP SH3BP1, has so far been identified to specifically regulate the formation of cell-cell contacts. Depletion of SH3BP1 in Caco-2 and A431 cells disrupts the localisation of TJ and AJ markers ZO-1 and β -catenin and perturbs actin organisation (Elbediwy et al., 2012). Interestingly, these cells are able to form filopodia but unable to progress to form AJs. SH3BP1 is recruited to cell-cell contacts as part of a multiprotein complex with paracingulin and CD2AP in the early stages of junction formation. Forster-resonance energy transfer (FRET) analysis in A431 cells reveals Cdc42 is reduced at junctions in the absence of SH3BP1 suggesting SH3BP1 spatially regulates Cdc42 activity (Elbediwy et al., 2012). Interestingly, although a GAP for Rac and Cdc42, only Cdc42 levels are increased upon depletion of SH3BP1 (Elbediwy et al., 2012).

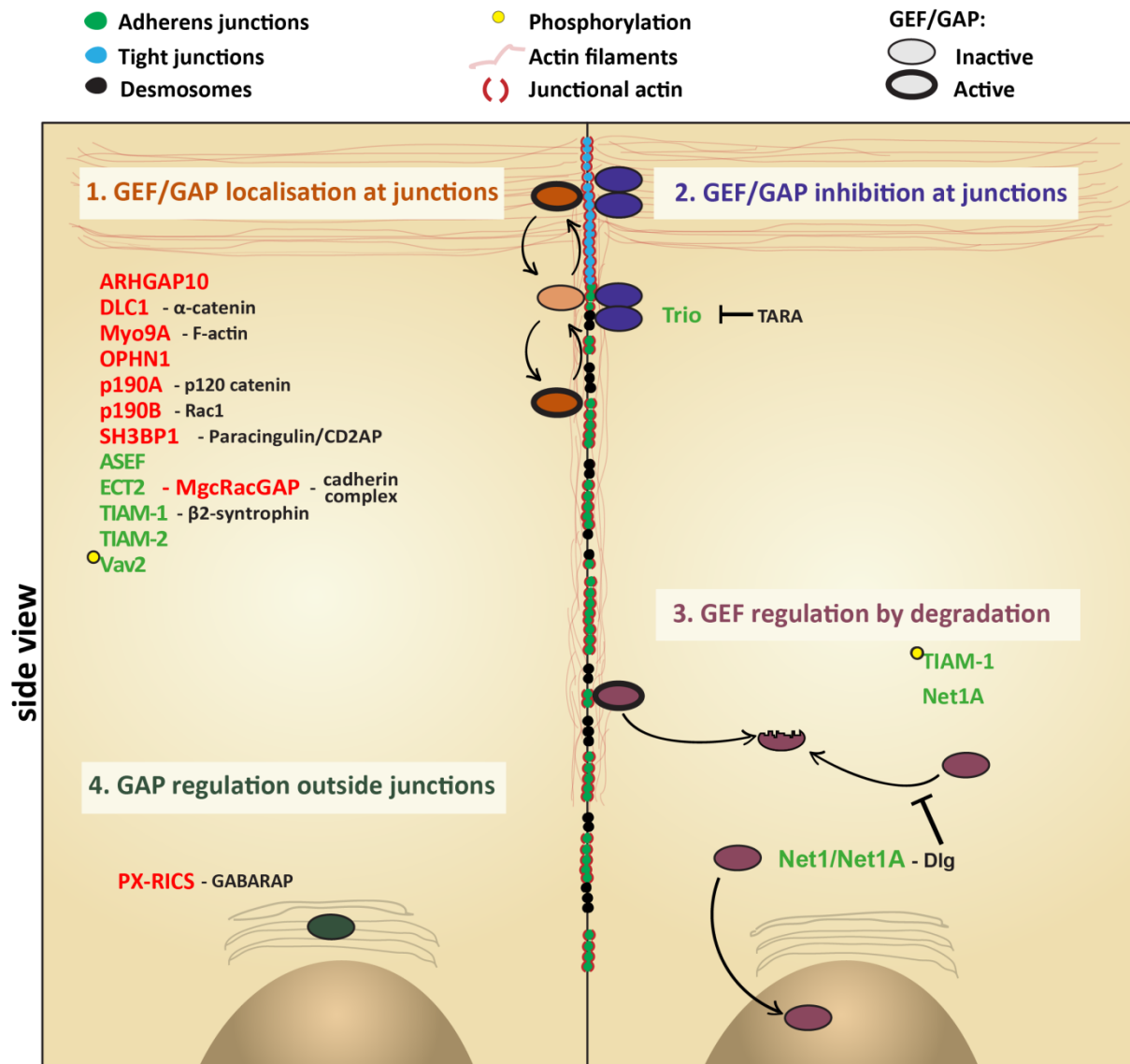


Figure 1.8 Diverse mechanisms are used by Rho GEFs and Rho GAPs to regulate AJs. The localisation of GEFs (green) and GAPs (red) to AJs is shown in the top left. Some regulators are localised to AJs via interaction with specific proteins (black), but for some proteins how junctional targeting occurs is not known. Inactivation of GEFs at junctions is shown in the top right. Inactivation can occur via interaction with specific proteins. Some GEFs are regulated via degradation, these are shown in the bottom right. Post-translational modification, such as ubiquitination, can target Rho GEFs for degradation via the proteasome. In some cases junctional regulation can occur at sites distinct from a location away from the junction (bottom left). An example of this is PX-RICS, which regulates transport of N-cadherin and β -catenin. Figure adapted from McCormack, Welsh and Braga, 2013.

1.10.4. GAPs regulating cell-cell contact stabilisation and maintenance

Myo9A localises to sites of cell-cell contact in Caco-2 (Elbediwy et al., 2012) and bronchial epithelial cells (Omelchenko and Hall, 2012). In Myo9A null mice AJ and TJ components are mislocalised and mice die from hydrocephalus due to defects in cell-cell contacts in the ependymal cells (Abouhamed et al., 2009), thus Myo9A is an important regulator of cell-cell contacts. Depletion of Myo9A in bronchial epithelial cells undergoing collisions reveals Myo9A regulates the early stages of cell-cell contact stabilisation (Omelchenko and Hall, 2012). In these cells, initial contacts are formed, but are not stabilised and cells ultimately retract from one another (Omelchenko and Hall, 2012). Myo9A appears to regulate stabilisation in two ways. Firstly, via its myosin head-like domains Myo9A regulates the formation of actin structures that are necessary for contact expansion (as depletion of Myo9A abolishes formation of these structures). Secondly, Myo9A is required to constrain RhoA activity; treatment of Myo9A-depleted cells with ROCK inhibitor partially rescues the junctional phenotype. FRET reveals that RhoA activity is enhanced specifically at cell-cell contacts in cells depleted of Myo9A (Omelchenko and Hall, 2012).

DLC1 positively regulates the stabilisation of AJs using two mechanisms 1) DLC1 interacts with α -catenin and may increase its presence at AJs (Tripathi et al., 2012) and 2) Expression of DLC1 raises protein levels of E-cadherin independently of α -catenin (although α -catenin is required for E-cadherin localisation) (Tripathi et al., 2013). DLC1 GAP-activity is vital for these functions, as expression of GAP dead constructs cannot localise α -catenin or increase E-cadherin levels (Tripathi et al., 2012; Tripathi et al., 2013). Interestingly, inhibition of RhoC, more so than RhoA, appears to be critical for upregulation of E-cadherin induced by DLC1 (Tripathi et al., 2013).

Both p190A and p190B can be activated downstream of C-, N- and E-cadherin to inactivate RhoA at contact sites (Noren et al., 2003; Ratheesh et al., 2012; Wildenberg et al., 2006). Depletion of p190 perturbs p120 catenin and N-cadherin localisation in mouse embryonic fibroblasts (MEFs) (Wildenberg et al., 2006). Interestingly, the recruitment of p190 proteins to junctions is driven by Rac signalling, and p190B can bind directly to Rac (Bustos et al., 2008; Wildenberg et al., 2006). RhoA activity is not always downregulated at sites of contact, rather it is confined to localised regions. In MCF-7 cells the Rac GAP, RacGAP1 (which forms part of the centralspindlin complex) is required to limit Rac signalling at nascent AJs (Ratheesh et al., 2012). This prevents the localisation of p190B to AJs and allows high RhoA activity to be maintained. The loss of the centralspindlin complex (via siRNA) leads to recruitment of p190B to junctions and inhibition of RhoA (Ratheesh et al., 2012).

Finally, PX-RICS (a splice variant of RICS) can regulate the trafficking of E-cadherin, thus controlling the amount of E-cadherin present on the surface that can be engaged in adhesions (Nakamura et al., 2010; Nakamura et al., 2008). Upon synthesis, cadherins are trafficked from the ER to the membrane with β -catenin (Chen et al., 1999). PX-RICS is a β -catenin binding partner necessary for trafficking of the N-cadherin/ β -catenin complex (Nakamura et al., 2008). Depletion of PX-RICS weakens cell-cell contacts due to a loss of N-cadherin and β -catenin from cell-cell contacts and a build-up of cadherin in the perinuclear region of HeLa and A431 cells. The GAP domain of PX-RICS is necessary for this role as a catalytically inactive PX-RICS GAP domain is unable to rescue the localisation of N-cadherin and β -catenin in PX-RICS null MEFs (Nakamura et al., 2008).

1.10.5. GAPs regulating cell-cell contact disassembly

As well as interacting with Tuba (see above) *L. monocytogenes* may also gain entry into the cell via ARHGAP21. ARHGAP21 interacts with α -catenin and localises to junctions in Caco-2 and JEG-3 cells (Sousa et al., 2005). Depletion of ARHGAP21 reduces α -catenin levels at the junctions of JEG-3 cells (Sousa et al., 2005) and leads to weaker cell-cell adhesions (Barcellos et al., 2013). Though E-cadherin is present, depletion of ARHGAP21 impedes entry of *L. monocytogenes*. Overexpression of the GAP domain reduces entry of *L. monocytogenes* compared to expression of a GAP-dead mutant or the N-terminal region (which lacks the GAP domain), indicating that GAP-dependent rearrangement of the actin cytoskeleton supports *L. monocytogenes* invasion (Sousa et al., 2005).

1.11. Identification of Rho GAPs regulating cell-cell adhesion

Whilst several GAPs regulating cell-cell adhesion have been identified, this highly complex process likely requires the coordination of multiple GAPs at each stage of assembly, stabilisation and maintenance. As such, there is still much to be understood regarding the identity of Rho GAPs participating in these processes. Screening approaches have been utilised to identify many Rho GAPs. For example, by screening for binding partners of proteins known to be enriched, or to play critical roles in certain cellular processes, important interactors can be identified (eg. (Aresta et al., 2002; Pulimeno et al., 2011; Saras et al., 1997)). Alternatively, the use of small inhibitory RNAs (siRNAs) has allowed researchers to effectively isolate proteins regulating specific cellular processes by assessing what effect their loss has in different cell types during various assays (Echeverri and Perrimon, 2006).

1.11.1. The use of siRNA screens to identify novel regulators of cellular processes

RNA interference was first described in 1998 when Fire *et al.* demonstrated that the injection of double stranded RNAs into *Caenorhabditis elegans* resulted in the specific degradation of messenger RNAs in the cytoplasm (Fire *et al.*, 1998). Since then the use of RNA interference as a tool for the study of gene function has increased substantially. In mammalian cells mRNAs can be specifically targeted for degradation using siRNA. These siRNAs present a robust method for gene silencing, enabling the function of a specific protein to be elucidated (Hammond *et al.*, 2001; Hannon, 2002).

The implementation of high-throughput screens using siRNAs has allowed whole genomes to be surveyed to identify novel components in signalling pathways, furthering our knowledge of, for example, development, cell division and migration and facilitated discovery of potential therapeutic targets (Berns *et al.*, 2004; Boutros *et al.*, 2004; Nybakken *et al.*, 2005; Paddison *et al.*, 2004). The quantitative assessment of particular cellular features is highly advantageous, as this can facilitate the identification of more subtle phenotypes, may be implemented more quickly than visual analyses, therefore allowing more targets to be tested and can remove any experimenter bias. The inclusion of a validation step also strengthens confidence in the data; genes are targeted using more than one distinct reagent and only if the phenotype is reproduced in two or more scenarios is the target protein classified a hit (Echeverri and Perrimon, 2006).

The motility of migrating cells during wound healing was assessed using a high-throughput siRNA screening approach whereby kinases, phosphatases and migration- and adhesion-related genes were targeted (Simpson *et al.*, 2008). In this screen, motility was inferred based on the objective quantification of the area of the wound that had been closed (Simpson *et al.*, 2008). Many genes were identified that had not previously been linked to migration, including several genes known to regulate cell-cell adhesions, and many genes found to enhance migration negatively regulated cell-cell adhesion (Simpson *et al.*, 2008). The organisation of focal adhesions were also assessed using the same library and the morphology and distribution of focal adhesions assessed, as well as cell spreading and elongation (Winograd-Katz *et al.*, 2009). This facilitated clustering of target proteins based on their effects on different parameters (Winograd-Katz *et al.*, 2009). In both of these screens the experimental parameters investigated were such that objective quantification was possible. This meant that the application of computational biological techniques complemented data derived from wet lab experiments in order to survey the effects of a vast number of genes on particular cellular processes.

The success of many screens has relied on the measurement of a single experimental parameter, and allowed whole genomes to be analysed. However, more complex phenotypes are increasingly being examined (Bakal et al., 2007; Fuchs et al., 2010). Such approaches have relied on the use of sophisticated computer software to classify specific phenotypes (Bakal et al., 2007). The assessment of more complex or multiple parameters gives a greater overview of the cellular process being studied and has led to the identification of known and unexpected proteins (D'Ambrosio and Vale, 2010). For example, in a screen for regulators of cell spreading in *Drosophila* S2 cells, several computational strategies were used to classify images according to specific phenotypes (eg. stellate cell morphology, ability to spread and presence of membrane ruffles) (D'Ambrosio and Vale, 2010). This led to the identification of actin-related proteins (eg. Arp2/3 complex proteins), as well as several uncharacterised genes, and proteins involved in the synthesis of lipids and regulating membrane synthesis (D'Ambrosio and Vale, 2010).

1.11.2. Screens for regulators of cell-cell adhesion

Several screens have been conducted with the aim of identifying novel regulators of cell-cell adhesion in different species. These range from whole genome screens to the study of a targeted subset of genes and have led to the successful identification of *bona fide* regulators of cell-cell contact formation (Elbediwy et al., 2012; Lynch et al., 2012; Omelchenko and Hall, 2012). However, several of these screens relied on the time-consuming task of visually assessing the phenotypes induced following the knockdown of target proteins (Elbediwy et al., 2012; Omelchenko and Hall, 2012). It would be far more effective to develop an automated, semi-quantitative approach in order to screen for regulators of cell-cell contact. However, disruption of proteins regulating the formation and maintenance of cell-cell adhesions may not induce readily measurable changes, rather alterations may be more subjective, and thus harder to assess.

Many high-throughput systems have been in place for a number of years that are capable of quantifying various cellular effects, such as tube formation, proliferation and migration with software often coupled to the microscope used to acquire images (Krausz, 2007). In order to make quantitative measurements related to the processes of cell-cell contact formation or maintenance it is first necessary to identify sites of cell-cell contact. However, the identification of the boundary between cells present in a confluent monolayer has proven challenging (Dima et al., 2011).

Although nuclei can be used to identify individual cells, many available algorithms segment isolated cells, which is obviously not appropriate for the identification of cells in confluent monolayers (Dima

et al., 2011; Krausz, 2007). The automated identification of the cell boundary is not trivial, and made more complicated by the fact that a hit protein may significantly perturb this boundary. Other factors, such as the large variation in size of keratinocytes, that cells are often found above the monolayer (that have died and been extruded), or that cell boundaries can overlap, complicate analysis meaning it is often inaccurate.

Current methods for segmenting confluent cells are still being developed (Karakaya et al., 2012) but one way of circumventing the problem of defining the cell edge is to apply thresholding-based methods. These work on the assumption that the intensity of E-cadherin (or other junctional proteins) staining at the junction is higher than in the rest of the cell. Pixels of intensity below the threshold are removed, leaving only the intense, junctional region. Although this means the junction area is only approximate, it is relatively efficient to apply to a large number of images simultaneously and allows the comparison of multiple siRNA treatments based on the relative amount of the image that is thresholded.

Overall, siRNA screening provides an excellent means of investigating the functions of multiple genes that may regulate cell-cell contact organisation and further use of systems biology may allow novel components of AJs to be identified.

1.12. Hypothesis and aims

The processes of cell-cell contact formation and maintenance are highly complex and regulated via the coordinated efforts of different GTPases. Clearly the actions of Rho GTPases must be tightly constrained in order for AJs to function correctly as these structures can influence so many cellular processes. The evidence so far suggests that multiple Rho GAPs are likely required to regulate very precise processes that must occur in order for cell-cell contact formation, stabilisation, maintenance and disassembly to proceed correctly.

The broad aims of this thesis are:

1. Identify Rho GAPs that can regulate specific steps in cell-cell contact formation in keratinocytes using a siRNA screening approach
2. Investigate the function of CdGAP at cell-cell contacts

Chapter 2.

Materials and Methods

2.1. Cell culture

Human keratinocytes isolated from neonatal foreskin (strain SF, passages 3-6) were maintained on mitomycin-C (Sigma, Dorset, UK) treated J2 mouse fibroblasts in standard medium (Dulbecco's Modified Eagle Medium (DMEM) DMEM:F12 BioWittaker, Lonza, Germany) supplemented with 1.8mM CaCl_2 , 10% foetal calf serum (FCS), 5mM glutamine, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 5 $\mu\text{g}/\text{ml}$ insulin, 10ng/ml epidermal growth factor (EGF), 0.5 $\mu\text{g}/\text{ml}$ hydrocortisone (all Sigma) and 0.1nM cholera toxin (Quadrantech Diagnostics Ltd, Surrey, UK) as described (Watt, 1994). Low calcium medium (approximately 0.1mM CaCl_2) had the same formulation as standard medium but contained FCS pre-treated with Chelex-100 resin (BioRad, Hemel Hempstead, UK) to deplete divalent calcium ions (Hodivala and Watt 1994). Cells to be grown in low calcium were seeded in standard medium and switched to low calcium when approximately 20 cells per colony were seen. Cells were seeded on 9cm dishes at a density of 2×10^5 , on 2cm² coverslips at a density of 3×10^4 or on 96 well plates (Corning Incorporated, Corning, NY) at a density of 2.2×10^3 cells per well. Cells were maintained at 37°C, 5% CO₂. J2 mouse fibroblasts were maintained in medium consisting of Dulbecco's modified medium (DMEM, Sigma-Aldrich), 10% DCS (Sera Laboratories International Ltd, West Sussex, UK) and 5mM L-glutamine (Sigma-Aldrich).

COS-7 cells were cultured in DMEM supplemented with 10% FCS, glutamine and the antibiotics penicillin and streptomycin. For pull-down experiments, cells were seeded in six-well plates (3cm area per well) and transfected when 70% confluent.

For RNAi experiments, keratinocytes were seeded at 3×10^4 on glass coverslips in standard medium for 3-5 days. Cells were then switched to low calcium medium. Cells were cultured until confluent. For all other experiments, keratinocytes were seeded at $1-3 \times 10^4$ on glass coverslips or 2×10^5 in 9cm dishes.

2.2. DNA constructs and cloning

2.2.1. DNA constructs

Mammalian expression plasmids used are displayed in **Table 2.1**. All constructs used were verified by sequencing carried out by Beckman-Coulter.

Protein	Plasmid	Product size (kDa)	Source
Ajuba	pCS2myc	58	G. Longmore, Washington University
Ajuba	mRFP	66	G. Longmore, Washington University
Ajuba	pGEX-2T	66	G. Longmore, Washington University
Ajuba LIM	pCS2myc	25	G. Longmore, Washington University
Ajuba LIM	pGEX-2T	15	G. Longmore, Washington University
Ajuba Pre-LIM	pCS2myc	35	G. Longmore, Washington University
ARAP1	pEGFP	162	S. Vermeren, University of Edinburgh
ARAP1 GAP-dead	pEGFP	162	S. Vermeren, University of Edinburgh
ARHGAP6	Flag	106	J-P Vincent, National Institute of Medical Research
ARHGAP6 RC	V5	122	J-P Vincent, National Institute of Medical Research
ARHGAP6 RD	V5	143	J-P Vincent, National Institute of Medical Research
ARHGAP6 RE	V5	129	J-P Vincent, National Institute of Medical Research
CdGAP ₁₋₂₂₁	pGEX-4T3	15	N. Lamarche, McGill University
CdGAP ₁₁₆₀₋₁₄₂₅	pGEX-4T3	25	N. Lamarche, McGill University
CdGAP ₁₁₆₀₋₁₄₂₅	pRK5 myc	40	N. Lamarche, McGill University
CdGAP ₁₁₆₀₋₁₄₂₅ ^{R1172A}	pGEX-4T3	25	J. McCormack, Imperial College London

CdGAP ₁₁₆₀₋₁₄₂₅ ^{R1172A}	pRK5 myc	40	J. McCormack, Imperial College
CdGAP ₁₁₆₀₋₁₄₂₅ ^{R1412A}	pRK5 myc	40	J. McCormack, Imperial College
CdGAP ₁₂₅₃₋₁₄₂₅	pGEX-4T3	15	N. Lamarche, McGill University
CdGAP-I	eGFP	250	N. Lamarche, McGill University
CdGAP-I	pRK5 myc	250	N. Lamarche, McGill University
CdGAP-I GAP-Dead	eGFP	250	N. Lamarche, McGill University
CdGAP-s	eGFP	125	N. Lamarche, McGill University
CdGAP-s	pRK5 myc	125	N. Lamarche, McGill University
Empty vector	eGFP	30	
Empty vector	pRK5 myc	30	
GST	pGEX-4T1	25	
mRFP-PAK1-Rac-GFP Rac tail	Raichu	45	T. Ng, King's College London
PAK-CRIB	pGEX2T	15	J. Collard, Netherlands Cancer Institute, Netherlands

Table 2.1 DNA constructs used in this study The vector, size of the protein in kilodaltons (excluding tag) and who the construct was obtained from is shown.

2.2.2. Site-directed mutagenesis

Primers were designed to mutate Arginine (R) residues in the CdGAP C-terminus (identified as being important for CdGAP-CdGAP and CdGAP-Ajuba binding) to Alanine (A) (**Table 2.2**).

Polymerase chain reaction (PCR) was carried out using pRK5myc-CdGAP-I, pRK5myc-CdGAP₁₁₆₀₋₁₄₂₅ or GST-CdGAP₁₁₆₀₋₁₄₂₅ as the template as follows; 50ng template, 1x polymerase buffer (New England Biolabs (NEB), Hitchin, UK), 1-2µl Phusion DNA polymerase (NEB), 3µl forward primer (5µM stock), 3µl reverse primer (5µM stock) (Invitrogen), 1µl dNTPs (20µM stock), 1µl Dimethyl sulphoxide (DMSO), made up to 50µl with dH₂O. Samples were subjected to PCR as follows: 95°C for 30 seconds, followed by 30 cycles of 95°C for 30 seconds, 72-75°C for 1 minute and 68°C for 9 minutes.

A 5µl sample was taken following PCR for future analysis. The remainder of the reaction was subjected to digestion by the DpnI enzyme (NEB) for 1 hour at 37°C in order to remove bacterially

derived template DNA and leave only the product of the PCR reaction. A 5µl sample was removed following this reaction and run alongside its undigested counterpart on an agarose gel. Where necessary, the DpnI digested product was gel-purified using the StrataPrep® DNA Gel Extraction kit (Stratagene, Cheshire, UK)) and the entirety of the purified product used to transform library efficient DH5-α (NEB) using a standard protocol; DpnI digested product was added to competent bacteria on ice and incubated for 20 minutes followed by heat shock at 37°C for 1 minute and a further 2 minutes incubation on ice. Sample were shaken in 160µl LB for 40 minutes at 37°C before being spread onto ampicillin plates (0.1mg/ml) and grown overnight at 37°C. The following day, several colonies were picked from each plate and grown overnight in 5ml of luria broth (LB) containing ampicillin in order to derive DNA. Samples were verified by sequencing to confirm the presence of the desired mutation.

Name	Sequence
CdGAP R1172A For	5'-CCTGTAAGTGTGTCAGCAGTGGCTACCTCCTTCATGGTCAAAATG-3'
CdGAP R1172A Rev	3'-GTAAACTGGTACTTCCTCCATCGGTGACGACTGTGTGAATGTCC-5'
CdGAP R1412A For	5- CTGTTTTTACCAGCCCCAGCGTGCCTCCGTGATTCTG – 3'
CdGAP R1412A Rev	3'-GTCTTAGTGCCTCCGTGCGACCCCGACCATTTTTGTC-5'

Table 2.2 Site-directed mutagenesis primers

2.2.3. Reverse-transcription PCR

mRNA was extracted from cells grown in standard calcium medium using the GTC method (Chomczynski and Sacchi, 1987) and reverse transcription performed using 1µg mRNA. Specific primers were designed and used to amplify human proteins (**Table 2.3**). PCR products were resolved on 1% (w/v) agarose gels containing GelRed™ (Biotium, Hayward, CA) (0.1µl/ml). Products were visualised under UV using Gel Doc XR system (BioRad).

2.3. Transfection techniques

2.3.1. cDNA transfections

For keratinocyte transfections, plasmid DNA was incubated with JetPrime buffer and JetPrime transfection reagent (Polyplus transfection, Illkirch, France) in a total volume of 50µl per coverslip.

The transfection reagent was used at a ratio of 2:1 DNA. Tubes were vortexed, centrifuged briefly and incubated at room temperature for 10 minutes. Cells were washed once and 350µl fresh medium was added to the cells. The DNA:transfection reagent mixture was incubated with the cells for 4 hours. Cells were washed once and medium was replaced with fresh medium. Cells were allowed to express DNA for a further 4-20 hours. Alternatively, plasmid DNA was diluted in Effectene buffer (Qiagen) and Enhancer added at a ratio of 1:8 Enhancer. Samples were vortexed and incubated for 5 minutes at room temperature. Effectene transfection reagent was added at a ratio of 1:25 Effectene and tubes vortexed prior to 5 minutes' incubation at room temperature. Standard medium was used to dilute the final plasmid DNA mixture and this added to cells. Medium was changed after 4 hours. COS-7 cells were transfected using 2.5-3.0µl of lipofectamine (Invitrogen) according to the manufacturers' instructions with between 0.25µg and 8µg of CdGAP-l, CdGAP-s or CdGAP₁₁₆₀₋₁₄₂₅ or 4µg Ajuba full-length, 3µg Ajuba Pre-LIM or 6µg Ajuba LIM domains. Media was changed after three hours and cells grown for approximately 24 hours.

Target	Sequence Forward	Sequence Reverse	Product size (bp)
ARAP1	5'-CTGGCGTCTCCAAGGTGCGG-3'	3'-GCTCTGCAAGAGCCAGGGGC-5'	372/189
ARHGAP20	5'-ACAGACCGAAAAGCCCGCGGT-3'	3'-CCCGGCTGTCCCCCTAGAGT-5'	252
ARHGAP26	5'-ACCCGGCCCAACTCACTCCCC-3'	3'-AGCAGTTCTGCTGGGGCCCA-5'	497/332
ARHGAP27	5'-ACACCAACCACTTCACTCAGGAGC-3'	3'-GTGCTTCTCCGGAGCCGCTT-5'	335/254
DEPDC1B	5'-TACGCGCCATGGAGCATCGC-3'	3'-GCTCCACGGTCTCATTCCACAGC-5'	75
GMIP	5'-TCGCCGCCAAACGGACTGAG-3'	3'-TGCCTGCACCGCCTCATTCATC-5'	95
RacGAP1	5'-TCAAACCGGAGCTGTGGGCG-3'	3'-GAGAATCTCCACCCGGCGCA-5'	342/353 /233

Table 2.3 RT-PCR primers used and sizes of products yielded in base pairs More than one different product shown is representative of multiple variants of the protein.

2.3.2. siRNA transfections

Oligonucleotides were dissolved in 1x siRNA buffer (containing 100mM KCl, 30mM HEPES-pH 7.5, 1.0mM MgCl₂) in RNase-free diethylpyrocarbonate (DEPC)-treated water (Dharmacon, Thermo Scientific, Colorado, USA). Keratinocytes were grown to approximately 80% confluence and transfected with oligonucleotides designed to target CdGAP, ARAP1 or a control, non-targeting oligonucleotide (**Table 2.4**, Dharmacon or Eurogentecs, Southampton, UK). Oligonucleotides (20-

75nM) were incubated with 2-3µl of INTERFERin (Polyplus transfection) in a total volume of 100µl of serum-free medium for 10 minutes at room temperature. Cells were prepared by replacing medium with 300µl fresh complete medium and the transfection mixture added to the cells for 4 hours. Cells were fixed or lysed after 48 hours. To determine the optimal time of RNAi treatment, SMART pools™ consisting of four oligonucleotides targetting CdGAP or ARAP1 were transfected for between 24 and 72 hours prior to lysis. Transfection efficiency was assessed relative to the levels of the target protein in the scrambled oligonucleotide control. Once the most effective time point was established, cells were transfected with individual oligonucleotides from the SMART pool™ (5-75nM) to ascertain the most effective oligonucleotide and the best concentration.

Oligonucleotide	Sequence
Non-targeting control	5'-AUGAACGUAAUUGCUCUA-3'
CdGAP oligo 1	5'-GCTGTGACCTGACGGAGTA-3'
CdGAP oligo 2	5'-GGATGTAACCCATTCAGTA-3'
ARAP1 oligo 1	5'-GAGAAGGAGUGGCCUAUUA-3'
ARAP1 oligo 2	5'-GCAGGGAUCUUAUCAUCUAU-3'
ARAP1 oligo 3	5'-GGAGAUACUGCCAUUGUG-3'
ARAP1 oligo 4	5'-ACAAGAATCTAGAGGAGTA-3'
ARAP1 oligo 5	5'-GTTGAGACCTCTTGGCCCA-3'
ARAP1 oligo 6	5'-GCATCAGGGAGAAGGACTA-3'

Table 2.4 siRNA oligonucleotides used

2.4. Electrophoresis and Western Blotting

Sample buffer (5x stock: 0.2mM Tris pH6.8, 50% glycerol, 5% SDS, 0.25M Dithiothreitol (DTT), 250mg bromo-phenyl Blue dye) was added to protein samples to 1x and these boiled for 5 minutes at 95°C. Protein samples were separated by electrophoresis through Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and transferred to a Polyvinylidene difluoride (PVDF) membrane (Millipore) using a standard wet transfer method (buffer: 50mM Tris, 380mM glycine and 20% methanol). For pull down assays, GST and GST-fusion proteins were visualised by amido black staining (Sigma-Aldrich).

For immunoblotting, membranes were blocked with 5% non-fat dry milk (Marvel) in Tris Buffered Saline (TBS) pH 7.5 containing 0.1% Tween-20 (Sigma-Aldrich) (TBS-T) or with Western Blocking Solution (GE Healthcare) for 30-60 minutes with agitation. Membranes were incubated with the indicated primary antibodies for 1 hour at room temperature or overnight at 4°C. Membranes were washed three times for 5 minutes in TBS-T followed by incubation with relevant conjugate diluted in 5% milk/TBS-T or TBS-T for 1 hour with agitation. Blots were subject to three washes in TBS-T and developed with ECL or ECL plus detection kit (GE Healthcare) and exposed to Hyperfilm ECL (Amersham Biosciences). Alternatively, blots incubated with fluorescent secondary antibodies were scanned using the Ettan DIGE Imager (GE Healthcare).

Antibody	Clone name	Supplier	Source	Dilution
α -catenin	VB1		Rabbit	1:2000
Actin	C4	Sigma-Aldrich	Mouse mAb	1:100,000
Ajuba	9104	Cell signalling	Rabbit	1:300
ARAP1		S. Vermeren, University of Edinburgh	Rabbit	1:2000
β -catenin	VB2		Rabbit	1:1000
β -tubulin	TUB 2.1	Sigma	Mouse mAb	1:10000
Cdc42	44/CDC42	BD Transduction Laboratories	Mouse	1:1000
CdGAP	CT	N. Lamarche	rabbit	1:250
E-cadherin	HECD-1	CRUK	Mouse mAb	1:1000
E-cadherin	ECCD2	Invitrogen	Rat	1:500
Flag-tag	M2	Sigma-Aldrich	Mouse mAb	1:4000
Flag-tag		Sigma-Aldrich	Rabbit	1:1000
GST	GST-2	Sigma	Mouse mAb	1:5000
GFP	3-E1	CRUK	Mouse mAb	1:5000
Myc-tag	9E10	CRUK	Mouse mAb	1:500
Myc-tag	A14	Santa Cruz	Rabbit	1:500
Rac	23A8	Upstate	Mouse mAb	1:1000
Rho	26C4	Santa Cruz	Mouse mAb	1:1000
V5-tag	2F11F7	Invitrogen	Mouse mAb	1:200

Table 2.5 Primary antibodies used for Western blotting and staining Details of all primary antibodies used in this study with information about clone name, supplier, source and dilution

Antibody	Supplier	Source	Dilution
Cyanine (Cy2)- conjugated anti-mouse IgG	Jackson Immuno Research laboratories	Goat	1:1000
Indocarbocyanine (Cy3)-conjugated anti-mouse IgG	Jackson Immuno Research laboratories	Donkey	1:5000
Indodicarbocyanine(Cy5)-conjugated anti-mouse IgG	Jackson Immuno Research Laboratories	Donkey	1:1000
Indocarbocyanine (Cy3)-conjugated anti-rabbit IgG	Jackson Immuno Research laboratories	Donkey	1:3000
Indodicarbocyanine (Cy5)-conjugated anti-rat IgG	Jackson Immuno Research laboratories	Donkey	1:4000
HRP-conjugated anti-mouse IgG	Pierce	Goat	1:5000
HRP-conjugated anti-rabbit IgG	Pierce	Goat	1:5000
HRP-conjugated anti-rat IgG	Pierce	Goat	1:5000
Alkaline-phosphatase-conjugated anti mouse IgG	Sigma-Aldrich	Goat	1:10000
DAPI	Sigma-Aldrich		1:3000
Phalloidin AF488	Molecular Probes		1:1000

Table 2.6 Secondary antibodies used for Western blotting and staining Details of secondary antibodies used in this study with information about clone name, supplier, source and dilution.

2.5. Immunofluorescence and microscopy

Cells were fixed with 3% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 10 minutes at room temperature followed by permeabilisation in 0.1% Triton X-100 and 10% FCS (Sigma-Aldrich) in PBS for 10 minutes at room temperature. Alternatively, cells were permeabilised (10mM PIPES pH6.8, 50mM NaCl, 3mM MgCl₂, 300mM Sucrose and 0.5% Triton X-100) for 10 minutes at room temperature prior to fixation as above, followed by blocking in 10% FCS for 10 minutes at room temperature. Coverslips or 96-well plates were incubated with primary and secondary antibodies (**Table 2.5** and **Table 2.6**) diluted in 10% FCS (final concentration) in PBS sequentially for 30-60 minutes. Following incubations, coverslips or 96-well plates were subjected to nine washes in PBS and three washes in water. Coverslips were mounted on glass slides (Fisher Scientific) using Mowiol (Calbiochem, Ca, USA). Each well of the 96-well plates was covered with 150µl Mowiol. Images were

acquired using an Olympus Provis AX70 microscope coupled to a SPOT RT monochrome camera using SimplePCI 6 software (Hamamatsu, Japan) or with a Leica SP5 inverted confocal microscope using Leica LAS AF Lite software. Pictures were processed using Adobe Photoshop CS6 and WCIF ImageJ software.

2.6. Microinjection

Microinjections were performed as described (Braga et al., 1997) using a phase contrast microscope (Axiovert 135m Zeiss Inc., Thornwood, New York) attached to an Eppendorf Microinjection Unit (Microinjector model 5242; micromanipulator model 5170; CO₂ Controller model 3700; Heat Controller model 3700). Cells were injected with DNA diluted in sterile PBS and allowed to express for 1-24 hours.

2.7. Determination of Rac and Cdc42 levels *in vivo*

2.7.1. PAK-CRIB pull-down assays

Keratinocytes grown in 9cm dishes in standard calcium medium were transfected with pEGFP-CdGAP-I, pEGFP-CdGAP-I GAP-Dead or empty vector control for 24 hours and lysed (50mM Tris-HCl pH7.5, 1% Triton X-100, 0.1% SDS, 150mM NaCl, 10mM MgCl₂, 1mM PMSF and 1mM leupeptin, pepstatin and pefabloc cocktail (LPP)). Lysates were cleared by centrifugation for 2 minutes, 14268.8 *g* at 4°C. Lysates were incubated with GST-PAK-CRIB immobilised on beads for 60 minutes. Beads were washed three times in wash buffer (50mM Tris pH7.5, 1% Triton X-100, 150mM NaCl, 10mM MgCl₂, 1mM of each protease inhibitor phenylmethanesulfonylfluoride (PMSF) and LPP). Samples were then subjected to electrophoresis and Western blotting.

2.7.2. Fluorescence lifetime imaging-Forster-resonance energy transfer

Keratinocytes grown in 96-well plates in standard calcium were transfected with pRK5-myc-CdGAP-I or pRK5-myc together with mRFP-PAK1-Rac1-GFP for 24 hours. Cells were fixed with 3% PFA and immediately stained for E-cadherin and myc-tagged exogenous protein. FRET in the mRFP-PAK1-Rac1-GFP probe was measured via fluorescence lifetime imaging (FLIM) of GFP (collaboration with D. Kelly and S. Kumar, Imperial College London). FLIM-FRET was performed using a multiwell plate reader built around a widefield microscope (Olympus IX81-ZDC) (described previously (Alibhai et al., 2013)). GFP was excited with a picosecond-pulsed supercontinuum source (Fianium UK Ltd, SC 400-

6) to 457-480nm and fluorescence was detected in the band 503-537nm. Cy5 was excited at 590-650nm and detected at 663-738nm; Cy5 images were integrated for 0.5s. Images were acquired using a long working distance Olympus 40x objective with N.A 0.6 (LUCPLFLN). Lifetime data was analysed using in-house software “FLIMFit” (Warren et al., 2013). Manual segmentation was performed using in-house software “CellSegmenter” (developed by D. Kelly). Lifetime data was fit to a single exponential model with background and instrument response taken into account with data taken from wells containing PBS and 20µM Erythrosin B, respectively (performed by D. Kelly).

2.8. Aggregation assays

Confluent coverslips of keratinocytes were trypsinised in 500µl Aggregation Assay buffer (60% versene (v/v), 0.1% trypsin, 0.1mM CaCl₂) and allowed to detach (the presence of calcium in this buffer prevents degradation of cadherin receptors by trypsin). Cells were then gently pipetted to disrupt clusters, counted and spun down at 900rpm for 5 minutes. The supernatant was removed and cells were resuspended in standard calcium medium to a density of 5 X 10⁴cells/ml. PBS was then added to the bottom of an 8cm² dish to create a humid chamber and six drops of 20µl of the resuspended cells pipetted onto the lid of the dish. The lid was placed back onto the top of the dish and photographs of the aggregates forming were taken 0, 60 and 120 minutes after standard calcium medium addition. After 120 minutes cells were pipetted gently 6 times to disaggregate and images of the remaining cell aggregates were taken. All images were taken using a phase contrast on an Axiovert 135m Zeiss or an Olympus CKX41 microscope linked to Colorview IIIu camera using cell^D v2.4 software (Olympus). The size of the aggregates and disaggregates was determined using ImageJ as described in (Nola et al., 2012).

2.9. Protein production and purification

2.9.1. Protein production from bacterial expression plasmids

Bacterial expression plasmids used are displayed in **Table 2.1**. All constructs used were verified by sequencing carried out by Beckman-Coulter. GST-Ajuba full-length and GST-Ajuba LIM were kindly produced by Reiko Daigakhu (Imperial College London).

Rosetta DE3 *Escherichia coli* (Novagen) cells were used for the production of GST-fusion proteins. Cells were freshly transformed with the desired DNA and grown on ampicillin plates. Overnight cultures (100ml) were set up in LB medium containing ampicillin and grown at 37°C with 220rpm

agitation. Cultures were then diluted to 1 litre and grown at 37°C until an optical density of between 0.6 and 0.8 was reached (600nm) (Aquarius, Cecil Instruments, Cambridge, UK). Protein production was induced via the addition of Isopropyl β -D-1-thiogalactopyranoside (IPTG) according to **Table 2.7**. To assess the efficiency of induction, 1ml samples of culture were taken before and after IPTG addition and these pelleted at 14268.8 *g*. Pellets were resuspended in water and sample buffer and a fraction analysed by SDS-PAGE. After the time indicated in **Table 2.7** bacteria were pelleted at 2620 *g* for 25 minutes and resuspended in 5ml ice cold lysis buffer (**Table 2.7**). The resuspended bacterial pellet was lysed by sonication (four times of 20 seconds on ice). Bacteria were centrifuged at 11000 *g* for 30 minutes at 4°C and the supernatant incubated with glutathione sepharose 4B beads (Pharmacia), previously equilibrated in lysis buffer for 30 minutes at 4°C. Samples were taken from the supernatant prior to the addition of the beads and of the pellet for analysis. Beads were washed four times by centrifugation at 352.8 *g* for 1 minute in 10ml of buffer (**Table 2.7**) and resuspended in 60% glycerol in wash buffer.

To elute purified proteins from beads, fusion proteins were resuspended in resuspension buffer (50mM Tris.HCl pH 7.5, 150mM NaCl, 5mM MgCl₂, 0.1mM DTT) containing reduced glutathione (20mM) and incubated for 2 minutes. Beads were centrifuged at 352.8 *g* and the supernatant was collected in a separate tube. This was repeated and the two supernatant samples combined. A sample of the beads post-elution was collected for SDS-PAGE analysis to check elution efficiency.

All eluted proteins were subject to dialysis. Dialysis tubing (Scientific Laboratory Supplies) with samples was incubated twice in 1 litre dialysis buffer (15mM Tris.HCl pH 7.5, 150mM NaCl, 5mM MgCl₂, 0.1mM DTT), initially for 2 hours at 4°C. The protein was removed from the dialysis tubing and centrifuged at 12303 *g* to remove any remaining beads and particulates. The supernatant was transferred to a fresh tube. Samples from each step of protein production and purification were analysed by SDS-PAGE and visualised by Coomassie Blue stain (0.1% Brilliant Blue dye, 45% methanol, 10% acetic acid). Protein concentration was calculated relative to bovine serum albumin (BSA) (Fluka) standards of known concentration.

Protein	IPTG (mM)	Induction Temp (°C)	Induction time (hrs)	Lysis buffer	Wash buffer
CdGAP ₁₋₂₂₁	1	30	3	A	A ₁
CdGAP ₁₁₆₀₋₁₄₂₅	1	4	O/N	B	2x B + 2x B ₁
CdGAP ₁₂₅₃₋₁₄₂₅	0.5	25	4	C	2x C + 2x C ₁
CdGAP ₁₁₆₀₋₁₄₂₅ R1172A	1	4	O/N	B	2x B + 2x B ₁
PAK-CRIB	0.5	30	4	D	D ₁
GST	0.3	30	3.5	E	E

Table 2.7 Protein production conditions

Buffer A: 50mM Tris HCl pH7.5, 50mM NaCl, 5mM MgCl₂, 1mM DTT, 1mM LPP, 1mM PMSF

Buffer A₁: 50mM Tris HCl pH7.5, 150mM NaCl, 5mM MgCl₂, 1mM DTT, 1mM LPP, 1mM PMSF

Buffer B: 50mM Tris HCl pH7.5, 100mM NaCl, 1% Triton, 1mM DTT, 1mM LPP, 2mM PMSF

Buffer B₁: 50mM Tris HCl pH7.5, 100mM NaCl, 1mM DTT, 1mM LPP, 2mM PMSF

Buffer C: 50mM Tris HCl pH7.5, 250mM NaCl, 1% Triton, 5mM DTT, 1mM LPP, 2mM PMSF

Buffer C₁: 50mM Tris HCl pH7.5, 250mM NaCl, 1mM DTT, 1mM LPP, 2mM PMSF

Buffer D: 50mM EDTA pH8, 0.1% Triton, 10% glycerol in PBS, 1mM DTT, 1mM LPP, 1mM PMSF

Buffer D₁: 50mM EDTA pH8, 1mM DTT in PBS, 1mM DTT, 1mM LPP, 1mM PMSF

Buffer E: 50mM Tris HCl pH7.5, 100mM NaCl, 1mM DTT, 1mM LPP, 1mM PMSF

O/N; Overnight incubation

2.9.2. Protein production from mammalian expression plasmids

CdGAP₁₁₆₀₋₁₄₂₅, CdGAP₁₁₆₀₋₁₄₂₅^{R1172A} and CdGAP₁₁₆₀₋₁₄₂₅^{R1412A} in the mammalian expression plasmid pRK5-myc were *in vitro* translated via the SP6 promoter using the TNT® Coupled Reticulocyte Lysate system (Promega) according to the manufacturers' instructions. Ajuba and Ajuba Pre-LIM in pCS2-6myc vectors were linearised via digestion with MfeI for 1 hour at 37°C. DNA was checked on an agarose gel and purified using the Stratagene PCR clean up kit. The linearised DNA was *in vitro* transcribed using the RiboMax kit (Promega) as per the manufacturers' instructions. The derived RNA was translated using the Retic lysate IVT™ kit (Ambion) according to the manufacturers' instructions. Protein synthesis was checked by subjecting 1µl of the reaction mixtures to SDS-PAGE followed by Western blotting.

2.10. Pull-down assays

2.10.1. Pull-down assays *in vivo*

For pull-down assays, COS-7 cells were washed once in PBS then lysed in 250µl of lysis buffer (10mM TrisHCl pH 7.5, 150mM NaCl, 1mM MgCl₂, 1% NP40 containing DTT, LPP and PMSF (1mM each) and sodium orthovanadate (OV) and sodium fluoride (NaF) (10mM each)) on ice, followed by centrifugation for 2 minutes at 4°C at 14268.8 *g*. The contents of two wells were pooled and the supernatant divided: half was incubated with 1.5µl GST and half with 10µg GST-fusion proteins in the presence of an additional 250µl lysis buffer. Samples were rotated at 4°C for one hour followed by three washes in lysis buffer. Sample buffer (0.0625M Tris-HCL pH 6.8, 0.5% SDS, 10% glycerol, bromophenol blue and 5% β-mercaptoethanol) was added and samples resolved using either 7.5% or 12% SDS-PAGE.

2.10.2. Pull-down assays *in vitro*

GST-tagged proteins, or GST alone immobilised on beads (2-5µg) were incubated with myc-tagged proteins produced by *in-vitro* translation in a total volume of 100µl (10mM Tris pH 7.5, 150mM NaCl, 2mM MgCl₂, 1% NP40, 1mM DTT, 1mM LPP, 1mM PMSF and 1mM NaF). Samples were rotated for between 1 and 2 hours at 4°C. Beads were washed three times in 500µl buffer (as above), and subjected to electrophoresis and western blotting. Alternatively, GST-tagged CdGAP₁₋₂₂₁ or GST alone was incubated with myc-tagged CdGAP₁₁₆₀₋₁₄₂₅ produced by *in-vitro* translation, in a total volume of 100µl (25mM HEPES, 100mM NaCl, 10mM MgCl₂, 5% glycerol, 1% NP40, 1mM OV, 10mM NaF, 1mM PMSF, 1mM DTT and 1mM LPP).

2.11. SPOT-peptide array experiments

CdGAP peptides, 25 amino acids in length, (25-mers), covering amino acids 1083 to 1425 (frame shifted by five residues) or covering amino acids 1147-1197, 1302-1327, 1337-1362 and 1387-1412 where each residue was sequentially mutated to an Alanine, were spot-synthesised on a nitrocellulose membrane using the MultiPep SPOT synthesizer (Intavis AG) (Synthesised at the Departamento de Bioquímica e Imunologia, Instituto de Ciências Biológicas, Universidade Federal de Minas Gerais, Belo Horizonte, Brazil). Membranes were blocked using 10% Western Blocking Reagent solution (10% purified casein protein in maleic acid) (Roche) dissolved in TBS (50mM Tris-HCl, pH 7.0) with 5% sucrose overnight. Membranes were overlaid for 2 hours at 4°C with *in vitro*

translated Ajuba or Ajuba Pre-LIM in 10mM Tris HCl pH 7.5, 150mM NaCl, 1mM MgCl₂, 1% NP40, 1mM DTT, 1mM LPP, 1mM PMSF, OV 10mM and NaF 10mM each. Alternatively, membranes were overlaid for 2 hours at 4°C with eluted GST-CdGAP₁₋₂₂₁ (200-400 nM) or GST in 25mM HEPES, 100mM NaCl, 10mM MgCl₂, 5% glycerol, 1% NP40, 1mM OV, 10mM NaF, 1mM PMSF, 1mM DTT and 1mM LPP. Bound protein was detected using rabbit or mouse anti-myc or anti-GST antibodies followed by either alkaline phosphatase-coupled anti-mouse Ig (Sigma) or Indocarbocyanine (Cy3)-conjugated anti-rabbit IgG or Indocarbocyanine (Cy5)-conjugated anti-mouse IgG. Between incubations, membranes were washed three times for 5 minutes with TBS-T. Membranes were then incubated with 0.6 mM bromochloroindolylphosphate (BCIP), 0.7 mM thiazolyl blue tetrazolium bromide (MTT) (both Sigma) and 5 mM MgCl₂ in citrate-buffered saline (CBS, 10mM sodium citrate, NaCl 137 mM, KCl 3 mM, pH 7.0) for up to 60 min and colour development stopped by washing membranes in Milli-Q Water. Alternatively, bound protein detected via fluorescently conjugated secondary antibodies was visualised using the Ettan DIGE Imager (GE Healthcare).

2.12. Quantifications and statistical analyses

All experiments, unless stated in figure legends, were performed independently at least three times. Significance was tested using Student's T-test or Analysis of Variance (ANOVA) depending on sample type and is specified in figure legends. Statistical testing was performed using Microsoft Excel or GraphPad Prism software. Western blots and SPOT membranes were scanned and pixel intensity quantified using Image J software.

Quantification of junction disruption in CdGAP-expressing cells was performed using Image J. The line selection tool was used to measure the junctional length in number of pixels between two cells. This was then overlaid onto the area covered by E-cadherin and a second measurement taken. The 'E-cadherin coverage' measurement was determined by dividing the area covered by E-cadherin by the total area of the junction.

Qualitative analysis of junction disruption in cells expressing CdGAP alone or in the presence of Ajuba was based upon the following criteria. A highly retractive phenotype was ascribed if the cell possessed at least one highly retractive junction, characterised by having at least two holes between the cell and its neighbour. All images for the quantification were acquired on a confocal microscope. For the qualitative analysis of CdGAP enrichment at cell-cell contacts, a junction was classified as being enriched if CdGAP was present in a continuous line covering more than 30% of the junction (as

determined by area where E-cadherin is present) and this line was of greater intensity than the adjacent area.

2.13. siRNA screen techniques

2.13.1. siRNA screen workflow

A primary siRNA screen had previously been conducted in the lab (A. Wheeler and J. Erasmus). A library of SMART poolTM oligonucleotides (Dharmacon) was used to target 70 GEFs, 46 GAPs, 3 GDIs, 23 GTPases and 64 GTPase interactors. The primary screen was analysed in collaboration with Natalie Welsh. Candidate proteins identified in the primary screen were analysed in validation screens. The Rho GEFs were validated by N. Welsh and the GTPases and Interactors validated by S. Bruche. For the GAP validation screen, 27 GAPs were followed up using four single oligonucleotides targeting different regions of the mRNA transcript (Dharmacon) (**Figure 2.1**). Oligonucleotides were resuspended in 1 x siRNA buffer to a final concentration of 2 μ M. Each oligonucleotide was tested in three independent experiments.

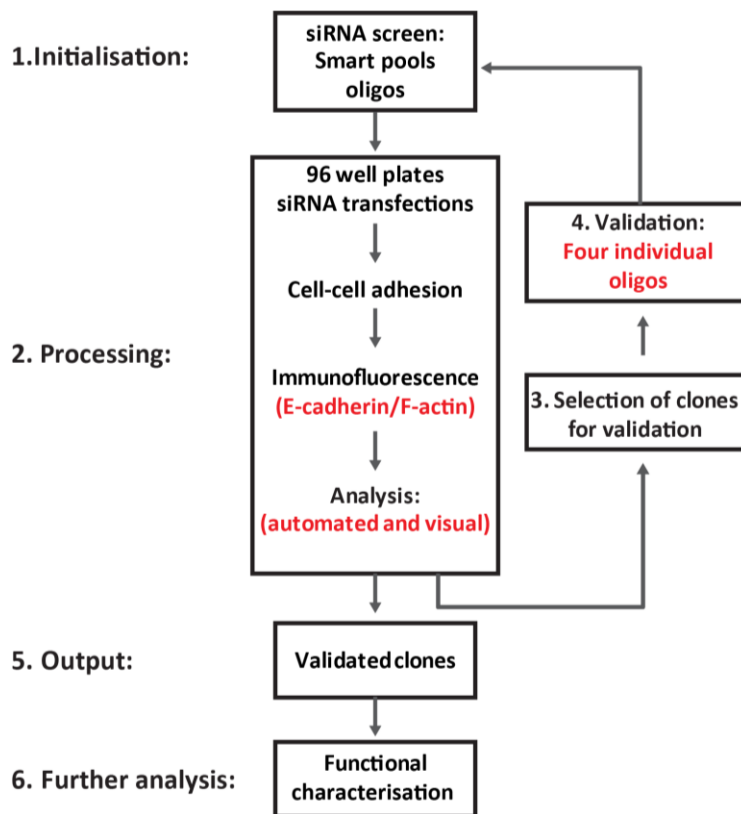


Figure 2.1 Workflow diagram showing the siRNA screening process For the primary screen SMART pools™ were used containing four oligos targeting a particular gene (step 1). Transfections were performed on 96-well plates and after 72 hours cells induced to form contacts via the addition of calcium ions for 30 minutes. Cells were fixed and stained for E-cadherin, F-actin and the cell nucleus. Images were analysed visually (J. Erasmus) and using an automated custom-made program (N. Welsh and J. McCormack) (step 2). Following this analysis 'candidate GAP proteins' were selected and tested in the Validation screen (step 3) (J. McCormack). At this point four individual oligos were used to target separate regions on the mRNA transcript. After this round of screening, a further stage of analysis lead to the generation of 'final hits' (step 5) which were then subjected to further characterisation (step 6).

2.13.2. siRNA transfections

Prior to the start of the screen siRNA transfections were optimised (A. Wheeler and V. Braga, unpublished). Seventy-two hours was demonstrated to be efficient for depletion of RhoA. Furthermore, the transfection of kif11 (Dharmacon) was used to demonstrate effective transfection (kif11 depletion leads to mitotic arrest thus transfection efficiency can be visually assessed). Cells maintained in low calcium were transfected when they reached approximately 70% confluence. For the validation screen, oligonucleotide (final concentration 100nM) was incubated with 0.4µl RNAifect (Qiagen) per well. Cells were washed once in low calcium medium. Following 15 minutes incubation, the transfection mixture in fresh low calcium medium was added to cells (final volume 50µl). Only the central 60 wells of the 96 well plates were used to avoid edge-effects. Any unused central wells were mock transfected with 1 x siRNA buffer. Four hours post-transfection medium was replaced with fresh low calcium medium. After 72 hours, cell-cell contacts were induced by the addition of 1.8mM CaCl₂. To prevent the influence of other signalling pathways old medium was used in which growth factors were depleted. Thirty minutes post-calcium stimulation cells were fixed using 3% PFA for 10 minutes at room temperature.

2.13.3. Image acquisition

For the primary screen images were acquired using GE Healthcare InCell Analyser 3000 (collaboration with Simon Stockwell, Cell Imaging Facility, ICR, London, UK). Using an infra-red autofocus (offset 1µM) images were taken at three different wavelengths (E-Cadherin, 647nm, actin, 488nm, DAPI, 364nm) at four different Z-planes 1µM apart. Exposure was 1.7ms and binning set at 1. Images were subsequently converted from 16 bit to 8 bit using InCell Analyser 3000 file exporter. For the validation screen images were acquired using a Cellomics ArrayScan IV (collaboration with Mike Howell, High Throughput Screening Laboratory, Cancer Research UK London Research Institute). Images were collected at the same wavelengths as above. At least two fields of view per well were acquired using a 10x objective. Autofocus was used to focus at the level of actin staining.

2.13.4. Visual analysis

A visual analysis was performed for the E-cadherin images in order to remove any non-confluent or unsuitable images. Each image was scored from 0 to 2. Zero values represented wells equal to controls. Values of 1 indicated wells above 90% confluence and values of 2 indicated wells of less

than 90% confluence or poor acquisitions and were removed from future analysis. Each plate was scored independently by two different observers and the mean value used

2.13.5. Computational analysis

All images were analysed using a custom-made program developed by Chris Tomlinson (Bioinformatics Support Service, Biochemistry, Imperial College London). For each plate, a set of representative control images were selected. These were either treated with scrambled oligonucleotides or were mock-transfected wells. The control E-cadherin and F-actin images were thresholded so that any pixel of intensity lower than the threshold was removed (**Figure 2.2**). The entire plate was then globally thresholded using the same values. As the intensity of the DAPI staining was variable, DAPI images were thresholded locally to facilitate the effective identification of all stained nuclei. The three experimental parameters E-cadherin, junctional actin and cytoplasmic actin were obtained from further processing of the thresholded images (**Figure 2.3**). All the processed images were globally thresholded to the control set and the percentage area remaining following thresholding measured (percentage of the total area). These values were normalised to the mean of the controls (**Box 1, equation 1**). To compare values between plates, Z-scores were calculated from the normalised values (**Box 1, equation 2**). The Z-score was used as a measure of how similar an experimental image is to the control group, where a control Z-score is 0.

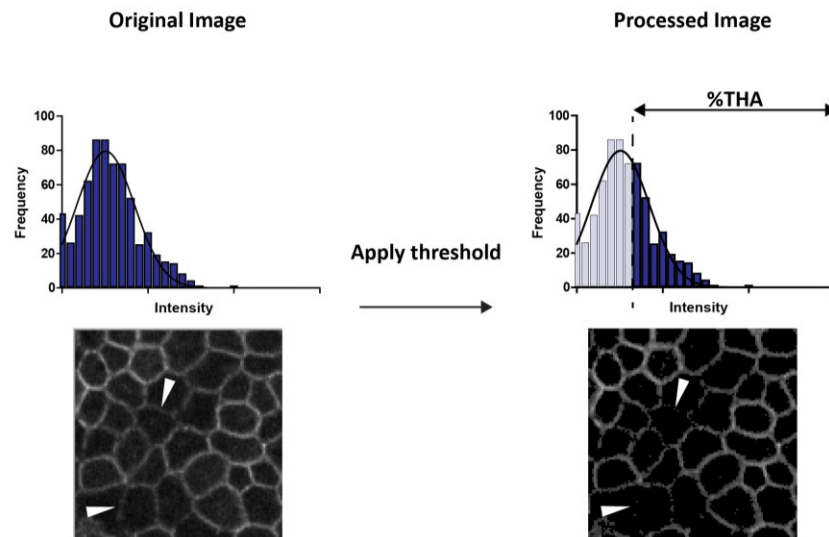


Figure 2.2 Image processing to return the percentage thresholded area value All images were analysed based on the percentage thresholded area (%THA). E-cadherin images are shown with example histograms above showing the distribution of the intensity of pixels within that image. Thresholds, set based on sets of control images unique to each plate, were applied to the image (dashed line right histogram). Any pixel above the threshold was kept (right hand side of the dashed line, dark blue, %THA) whilst any pixel below the threshold was removed (light purple) to give the processed image. The arrows on the images show areas where pixels have been removed from the processed image because these fell below the threshold.

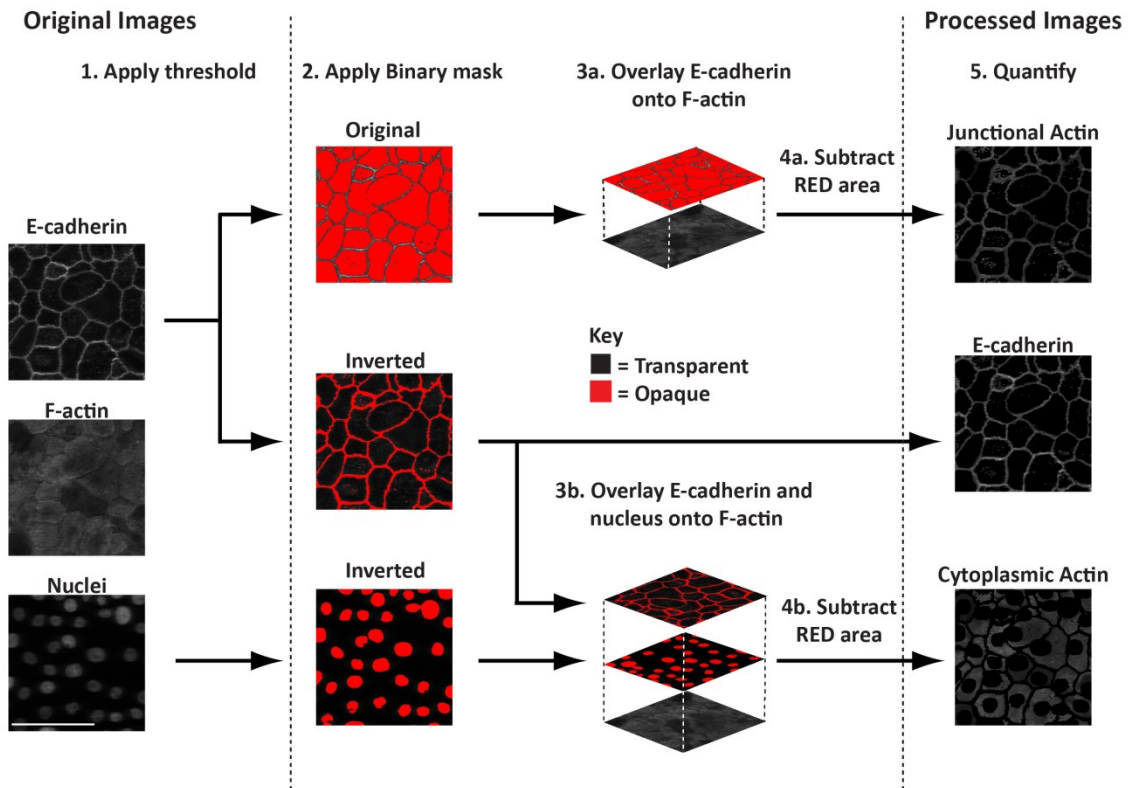


Figure 2.3 Flow chart demonstrating the steps taken to generate the three experimental parameters From the original E-cadherin and DAPI images (step 1) binary masks were created. Two masks were created from the E-cadherin image, an original mask, whereby the area covered by E-cadherin is transparent, and an inverted mask whereby the area covered by E-cadherin is opaque (step 2). To obtain the Junctional Actin image the E-cadherin binary mask was overlaid onto the F-actin image (step 3a). The area covered by the transparent portion of the mask gives the junctional actin image, ie the pool of actin that localises at junctions. In order to generate the cytoplasmic actin image, the inverted E-cadherin binary mask and the nuclei binary mask were overlaid onto the F-actin image (step 3b). Corresponding pixels to nuclei and cell-cell contacts were subtracted from the F-actin image (step 4a), leaving the F-actin pool that is present in the cytoplasm. Scale bar represents 50µm.

Equation 1: Normalisation to the controls. Where x_i is the percentage area remaining after thresholding of the i^{th} well and $\bar{c}$ is the mean percentage area remaining after thresholding of the controls

$$Norm = \frac{x_i}{\bar{c}}$$

Equation 2: Z-score. Where $Norm_i$ is the value normalised to the controls of the i^{th} well and S_c is the standard deviation of the normalised controls

$$Z\ Score = \frac{(Norm_i - 1)}{S_c}$$

Box 1: Equations used to analyse raw data Equation 1 was applied to all values of percentage thresholded area in order to normalise these to the control images for that plate. Following normalisation Equation 2 was used to calculate the Z-score. One was subtracted from the normalised value calculated in Equation 1 and this value divided by the standard deviation of the controls of that plate. This allowed values to be compared between plates.

2.13.6. Selection of hit proteins

For each of the target proteins, the Z-scores from each replicate, and for each of the experimental parameters were collected. The median Z-score value of the three replicates was calculated (the mean was used if only two replicates were found suitable following quality control of the images). To select proteins for further analyses, cut-off values were set based on the standard deviation of the mean of the controls. In the primary screen, samples with a median value outside of this range for any of the experimental parameters were classified as candidate proteins and brought forward to the validation screen. Four oligonucleotides targeting a single protein were tested individually in the validation screen. A hit protein was identified when at least two of the four tested oligonucleotides yielded images with Z-scores outside the cut-off. Further inspection of the individual repeats for each target protein was carried out to remove any unreliable z-scores. For example, if a mean z-score was used and the standard deviation was large this protein was discarded.

Chapter 3.

**The identification of Rho GAPs regulating cell-cell
contact formation via a medium-throughout siRNA
screen**

3.1. Introduction

The use of siRNA as a tool to screen libraries of genes in order to investigate particular cellular processes has led to the characterisation of countless proteins and helped uncover many novel signalling pathways (Berns et al., 2004; Boutros et al., 2004; Nybakken et al., 2005; Paddison et al., 2004; Simpson et al., 2008). Cell-cell contact formation is an ideal process to be studied using a high to medium-throughput screening approach as very clear morphological changes are observed over a relatively short amount of time (30 minute calcium switch) (Adams et al., 1998; Zhang et al., 2005). E-cadherin staining at cell-cell contacts becomes considerably more intense over the course of junction formation, whilst extensive transformations occur in the actin cytoskeleton (Adams et al., 1998; Vasioukhin et al., 2000; Zhang et al., 2005). Prior to the addition of calcium, actin appears in loose bundles throughout the cell. However, junction assembly triggers the appearance of thick, distinctive actin bundles at the site of contact. At early time points, two clear actin populations can be discerned (Zhang et al., 2005). Thus, defects in junction formation can be manifested as changes in E-cadherin staining, or in the compaction of actin bundles at the sites of contact.

Thus far very few screens have addressed the processes of cell-cell contact formation and maintenance. Furthermore, these have often involved visual analysis of a relatively small library of genes (Elbediwy et al., 2012; Omelchenko and Hall, 2012). The GAPs SH3BP1 and Oligophrenin-1 were identified as AJ regulators using a screening approach that assessed phenotypes based on a visual analysis of junctional markers (including ZO-1, E-cadherin and α -catenin) (Elbediwy et al., 2012). Subsequently, the function of SH3BP1 was verified and this protein found to control the spatial confinement of Cdc42 activity and regulate actin dynamics during cell-cell contact formation in a number of cell types (Elbediwy et al., 2012). However, these screens relied on the time-consuming task of visually assessing the phenotype produced by every siRNA. Such a qualitative analysis may allow more complex phenotypes to be identified, but this comes at the expense of throughput. Ideally, as many targets as possible should be examined and a robust and reliable automated analysis step employed to semi-quantitate the effects of the siRNA treatment. Such an approach also offers the possibility of identifying proteins whose depletion may have a more subtle effect on morphology (Jones et al., 2009).

Quantitative image analysis has been utilised successfully to identify, for example, regulators of cell shape and cell spreading (D'Ambrosio and Vale, 2010; Fuchs et al., 2010). Furthermore, the generation of multiple quantitative outputs allows further analysis of data in order to cluster targets

according to their phenotypes, possibly aiding the characterisation of signalling pathways (Bakal et al., 2007; D'Ambrosio and Vale, 2010; Fuchs et al., 2010).

To identify novel regulators of cell-cell contact formation a siRNA screen targeting a library of GTPases, GEFs, GAPs, GDIs and Rho GTPase interacting proteins (Interactors) was performed (Jenni Erasmus). Perturbations in junction formation or stabilisation caused by depletion of target proteins were assessed by staining for E-cadherin and actin. In collaboration with Natalie Welsh, the effects of depletion of each of the target proteins was assessed by subjecting images to thresholding (**Figure 2.2**) in order to generate three experimental parameters; E-cadherin, junctional actin and cytoplasmic actin. Images were then assigned Z-scores that reflected how similar these were to control images. Subsequently, validation screens were performed with candidate proteins identified in the primary screen (the GAP screen discussed in this chapter was performed by J. McCormack, whilst validation of the GEFs and GTPases and Interactors were performed by N. Welsh and S. Bruche, respectively). Prior to commencing this screen numerous rounds of optimisation ensured that this thresholding-based method was capable of detecting perturbations in junction organisation induced by proteins known to be disruptive to cell-cell contacts (A. Wheeler and V. Braga, unpublished).

The thresholding-based approach meant the effects of depletion of a target protein could be assessed in terms of its effect on E-cadherin and two separate populations of actin. This should allow for the identification of proteins that can regulate diverse processes, for example the depletion of a protein regulating the trafficking of E-cadherin to the membrane may not show any affect on either actin population at early time points, but perturb E-cadherin staining. Conversely, the depletion of a protein regulating the organisation of actin at the contact edges during contact expansion may affect junctional but not cytoplasmic actin. Thus, it is hoped that by processing images in this way we may be able to dissect mechanistically the early stages of junction formation and stabilisation.

3.2. Aims

1. Analyse the results from a primary siRNA screen that had previously been performed in the lab (J. Erasmus) using a customised quantitative program (collaboration with N. Welsh)
2. Perform a validation screen of the candidate GAP proteins identified in the primary screen (J.McCormack)

3.3. Results

3.3.1. Primary siRNA screen

In total 206 different proteins were targeted in the primary screen, covering GTPases, GAPs, GEFs, GDIs and interactors. To assess differences between siRNA treatments a thresholding-based method was employed (see **Figure 2.2**). This enabled three experimental parameters, E-cadherin, junctional actin and cytoplasmic actin, to be derived and quantified for each image (**Figure 2.3**). Every image was ultimately assigned a Z-score corresponding to each of these parameters which represented the degree of difference between that image and controls. Z-scores from three repeats were averaged and these compared to control images of untransfected cells (in collaboration with Natalie Welsh).

Before analysis of the Z-scores, we used the raw values of the percentage thresholded area (%THA) (**Figure 2.2**) to analyse the spread of the data for each of the parameters, E-cadherin, junctional actin, cytoplasmic actin and total actin from all screening plates, as this would dictate the statistical tests subsequently used on this data. The %THA represented the percentage of the image that was remaining after the thresholding step was performed for a particular well, and before any normalisation had taken place (**Equation box 2.1**). The %THA was used to generate histograms illustrating the distributions of these parameters (**Figure 3.1**). To test for normality D'Agostino-Pearson omnibus tests were performed. Despite large sample sizes, none of the parameters were normally distributed (**Figure 3.1**). This is consistent with the nature of proteins selected in this targeted screen, as they were particularly likely to have an influence on junction morphology when depleted.

3.3.2. Well confluence does not impact upon the %THA

In order to avoid false positives being included due to artefacts of the analysis, a visual analysis of every well was performed to remove sub-confluent wells. A scale of 0-2 was used to classify confluence with 0 representing fully confluent wells, 1 representing wells 90% confluent and 2 representing wells less than 90% confluent (**Figure 3.2 A**). All wells classified as 2 were removed from the analysis (as were any images that were out of focus or otherwise unsuitable).

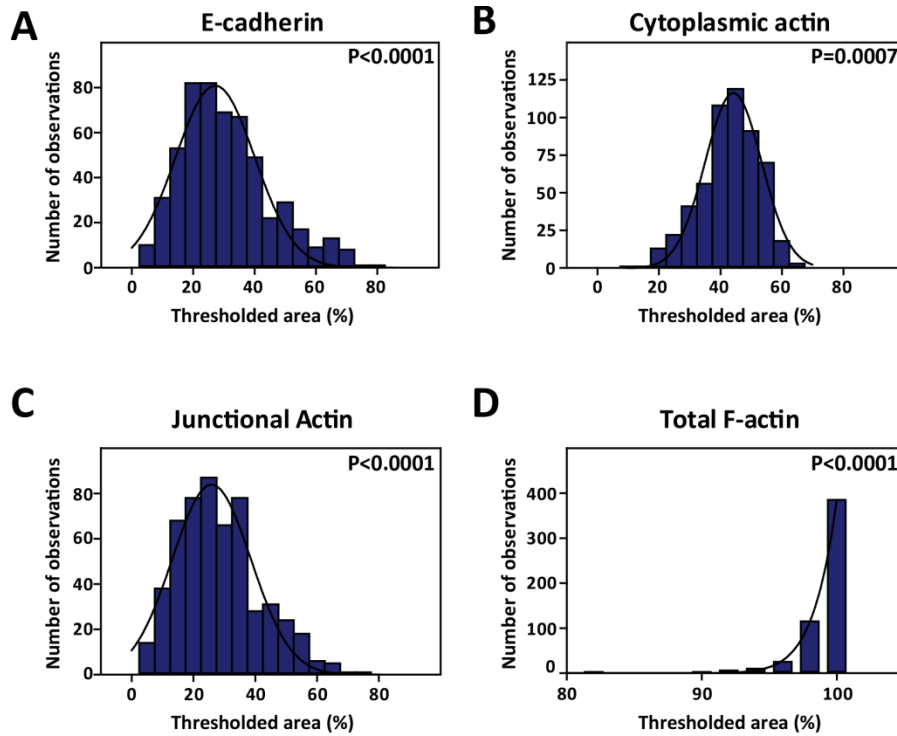


Figure 3.1 The experimental parameters are not normally distributed The percentage thresholded area for each of the parameters, E-cadherin (A), Cytoplasmic actin (B), Junctional actin (C) and Total F-actin (D) were plotted on histograms. Normality was tested using the D'Agostino-Pearson Omnibus test. P values, shown in the top right corners of the graphs, indicate that if the null hypothesis were true (that data is from a normally distributed population) the likelihood that this distribution would be obtained from random sampling of this population. Thus a significant P value indicates the null hypothesis should be rejected.

This is a time-consuming process, thus its automation would be desirable. We sought to determine if either cell count or %THA F-actin were useful predictors of confluence that may be used to remove automatically sub-confluent wells in future screens. Box plots were created with each confluence group plotted against cell count (**Figure 3.2 B**). The mean cell count for the wells classified as 0 (530.8) is higher than the mean cell count for wells classified as 1 or 2 (453.7 and 296.2 respectively). However, a considerable overlap in the spread of this data indicates that a confluent well can contain a large range of cell counts (from 285 to 928). As such, cell count is not a good predictor of confluence and the use of cell count to remove sub-confluent wells automatically is not possible due to the wide range of cell counts present amongst confluent wells.

As number of nuclei did not correlate with confluence, one possibility was to use the %THA of F-actin, which should represent the cell cytoplasm spreading on the well. The prediction is that subconfluent wells visually classified as 2 should have lower values for %THA. Box plots of the %THA F-actin against confluence were generated and show wells classified as 0 or 1 had similar amounts of F-actin (mean 99.33 and 98.03) whereas wells classified 2 had a lower mean (83.58) (**Figure 3.2 C**). Thus, confluent wells (0 or 1) included in the analysis all contained a similar overall %THA for F-actin, but a wide range of cell numbers. The %THA F-actin is therefore a better predictor of a confluent well than cell count, and could potentially be used in future analyses for the automated removal of sub-confluent wells. However, in order to ensure the removal of any sub-confluent well a high limit would have to be set, meaning that many confluent wells would also be excluded. This approach would not be suitable for a screen of this size, as too much data would be lost.

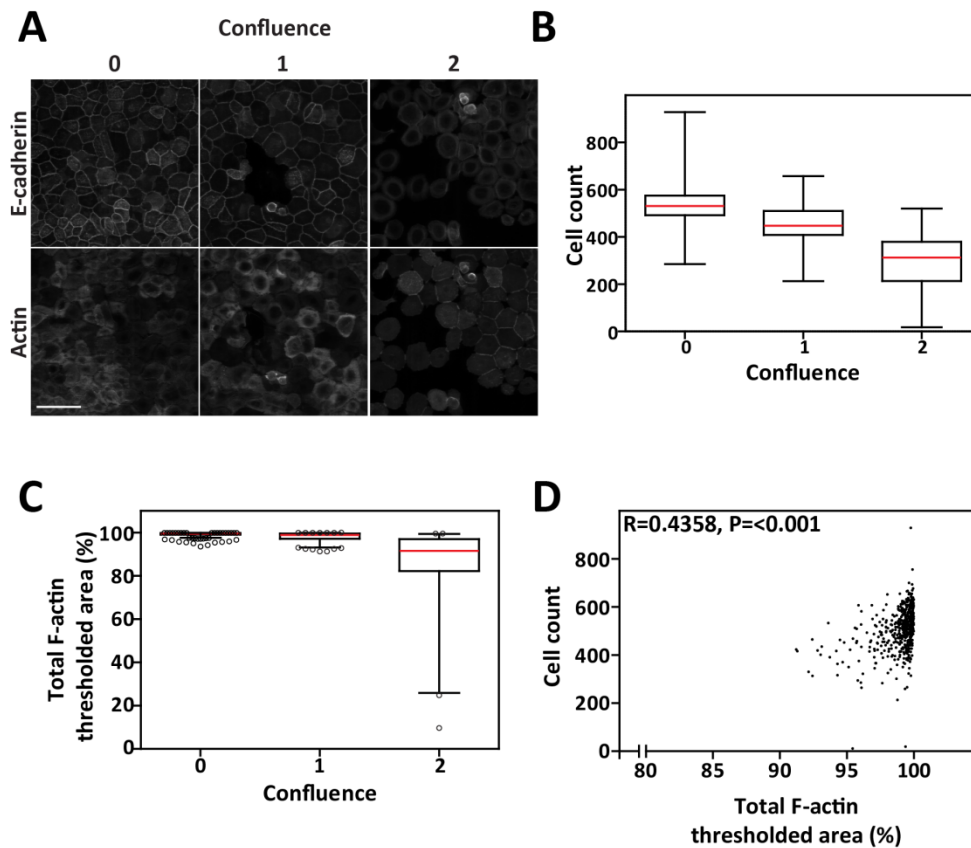


Figure 3.2 Confluence does not correlate with percentage thresholded area of F-actin A) Example images of E-cadherin and actin staining from wells classified 0, 1 or 2 based on confluence. Wells classified '0' were completely confluent, wells classified '1' were above 90% confluent and wells classified '2' were below 90% confluent and were removed from analysis. Scale bar represents 50 μ m. B) The number of cells in each well was quantified with ImageJ using the 'cell counter' plugin and cell count plotted against confluence classification on box plots. Red bars indicate means. Boxes show 25th and 75th percentiles, whiskers show minimum and maximum values. C) The total F-actin %THA was plotted against confluence classification on box plots. Red bars indicate means. Boxes show 25th and 75th percentiles, whiskers show 5th to 95th percentiles, circles show outliers. D) Cell count was plotted on a scatter graph against total F-actin %THA. Correlation between these parameters was measured using Spearman's ranked correlation coefficient and R value is shown in the top left corner of the graph. N=3

To confirm that cell count would not influence results independently of siRNA treatment, it was important to determine if any correlation existed between cell count and any of the experimental parameters (**Figure 3.3 A-C**). In the case of cell-count versus E-cadherin and junctional actin the correlations give R values approaching zero, suggesting no correlation exists. For cytoplasmic actin R is higher, at 0.1452, and P is significant (<0.05) indicating that this association although very low may not be random. As such, it is possible a very low association exists between these parameters.

The relationship between each of the experimental parameters to total F-actin was examined to exclude the possibility that (particularly for the junctional and cytoplasmic actin parameters) the total amount of F-actin could influence the %THA of any other parameter. All parameters gave very low R values which are significant ($p<0.05$) suggesting a very weak association between these parameters and F-actin (**Figure 3.3 D-F**). Such mild associations suggest total F-actin levels do not influence the amount of E-cadherin, or of either actin population, the latter indicating our actin segmentation is sound. The lack of correlation between both the cell count data and F-actin %THA and each of the experimental parameters indicates that neither of these variables can influence the %THA of the experimental parameters. Thus any deviation from the controls can be attributed to siRNA treatment, rather than artefacts of the analysis program.

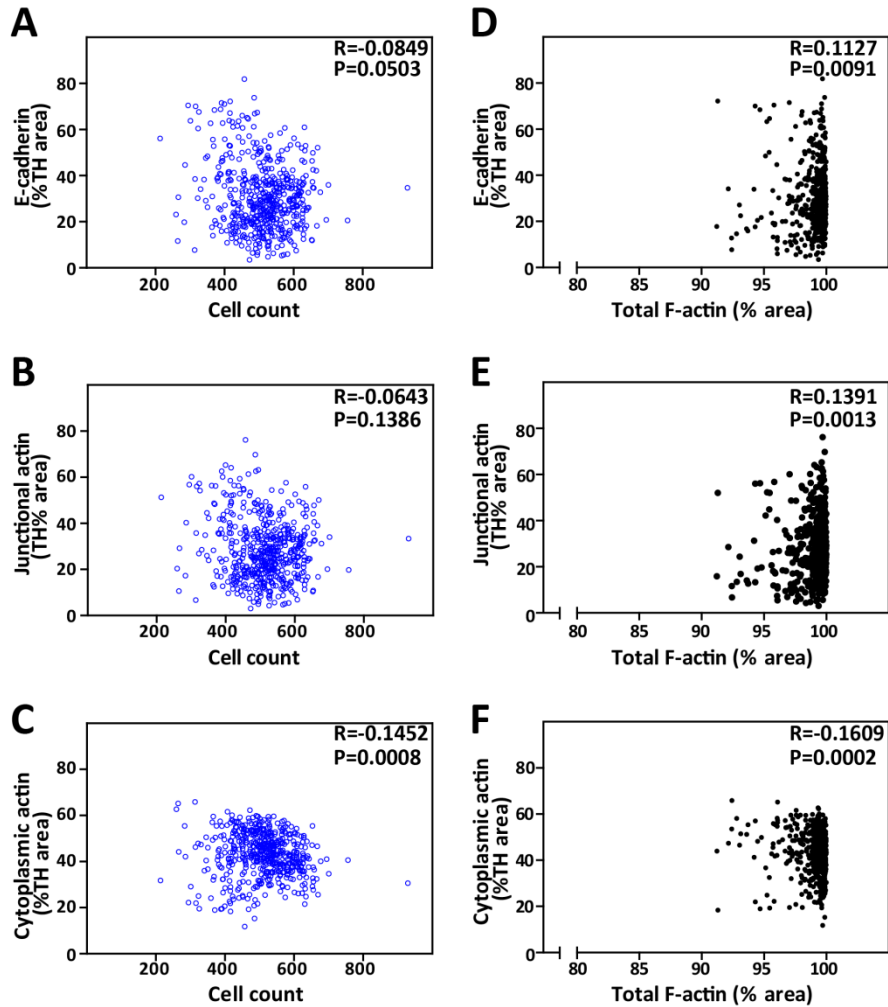


Figure 3.3 Experimental parameters do not correlate with cell count or Total F-actin Cell count was correlated with E-cadherin (A), Junctional actin (B) and Cytoplasmic actin (C). Total F-actin %THA was correlated with E-cadherin %THA (D), Junctional actin %THA (E) and Cytoplasmic actin %THA (F). The correlation between these variables was assessed using Spearman's rank correlation coefficient and R values are shown in the top right corner of graphs.

3.3.3. E-cadherin, Junctional actin and cytoplasmic actin Z-scores all correlate with each other

The relationship between the Z-scores from each parameter was examined for the primary screen of 206 proteins (**Figure 3.4 A**) and the candidate proteins (according to functional group) (**Figure 3.4 B**). E-cadherin and junctional actin are positively correlated ($R^2=0.9833$ and $R^2=0.9818$ for primary and candidate proteins respectively) likely due to the functional relatedness of these two parameters. Cells with more E-cadherin are likely to have a greater amount of actin at their junctions, whereas junctions containing less E-cadherin may be that way due to a lack of underlying junctional actin (although no causality can be assigned to this relationship from the correlation analysis). Interestingly, cytoplasmic actin correlates negatively with both E-cadherin and junctional actin in the total screen ($R^2=-0.8019$, E-cadherin and $R^2=-0.8190$, junctional actin) and amongst the candidate proteins ($R^2=-0.7450$, E-cadherin and $R^2=-0.7806$, junctional actin). This indicates that a low score for cytoplasmic actin is predictive of high scores for E-cadherin and junctional actin. This is not unexpected as with actin compaction towards the junctions the adjacent cytoplasmic loose bundles appear less intense compared to the junctional pool. In all cases correlations were significant ($P<0.0001$) indicating these associations are not due to chance.

For these correlations the candidate proteins were colour-coded according to functional group (**Figure 3.4 B**). Potentially more GAPs and GTPases are found to positively affect E-cadherin and junctional actin whilst negatively affecting cytoplasmic actin than other functional groups. However, for firm conclusions to be drawn a more thorough evaluation, for example using principal component analysis, would be required.

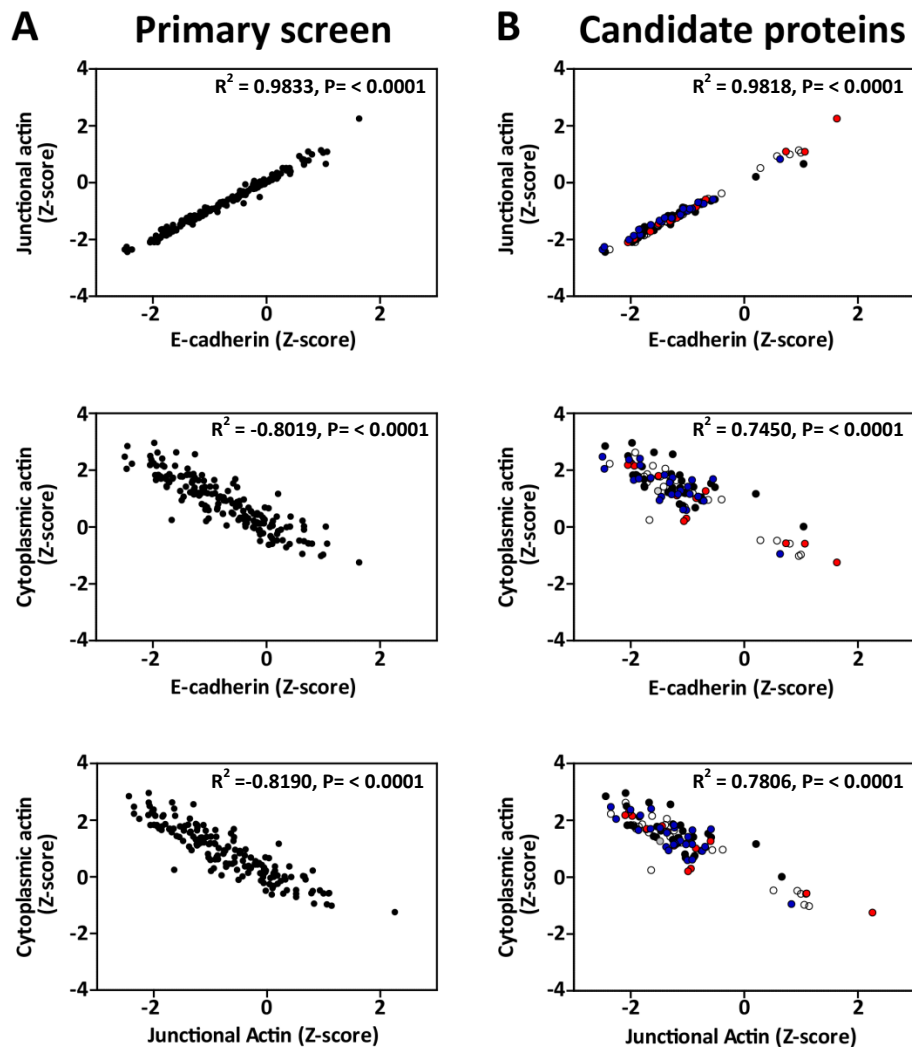


Figure 3.4 The experimental parameters correlate strongly with one another Z-scores for each of the experimental parameters E-cadherin, junctional actin and cytoplasmic actin were correlated with one another for all proteins in the primary screen (A) and just the candidates (B). The candidates were coloured according to the functional group they belonged to. White=GAPs, Black=GEFs, Grey= GDIs, Red= GTPases and Blue= Interactors. The degree of correlation was determined using Pearson's correlation coefficient (R^2).

3.3.4. Selection of candidate proteins

To select candidate proteins to be taken forward to the validation screen, Z-scores of each parameter were ranked (**Figure 3.5 A-C**) and cut-off values were defined as plus or minus one standard deviation of the mean of the controls (the mean Z-score of the controls was 0). Any protein with a Z-score above or below the cut-off for any parameter was designated a candidate. Candidate proteins were classified as 'positive' or 'negative', reflecting whether the Z-score was higher or lower than the control means (**Figure 3.5 A-C**). Fundamentally, a positive Z-score indicates an image with a higher proportion of pixels above the threshold compared to the controls. For both E-cadherin and junctional actin, a large skew in the Z-scores is seen, with the majority of candidates having a negative Z-score compared to controls. Conversely, cytoplasmic actin Z-scores are skewed in the opposite direction, meaning that most candidates have higher Z-scores than controls. Hence the depletion of the majority of proteins in the screen reduced the amount of E-cadherin and/or junctional actin and increased cytoplasmic actin, suggesting most proteins targeted may be positive regulators of cell-cell contact formation.

When candidate proteins (above and below cut off) and control Z-scores were plotted for each parameter there was no overlap between the inter-quartile ranges of the candidates and the controls (**Figure 3.5 A-C**). However, the range of Z-scores generated in the controls was large, due to the inherent variability of keratinocytes. As a relatively low cut-off value was set there was some overlap between the total spread of the controls and the candidate proteins. Whilst this approach increases the possibility of detecting false positives in the primary screen, it maximises the likelihood of identifying proteins causing more subtle phenotypes on junction morphology.

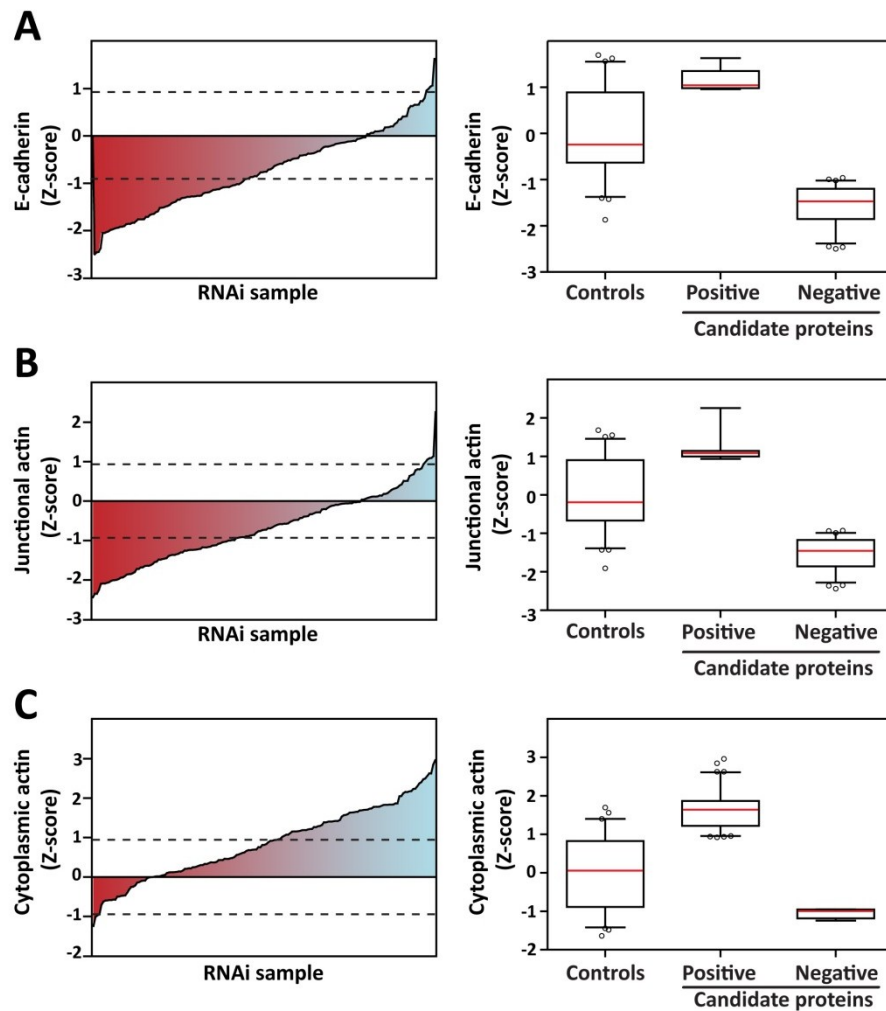


Figure 3.5 Selection of candidates from the primary screen E-cadherin (A), Junctional actin (B) and Cytoplasmic actin (C) Z-scores were ranked (RNAi sample) from negative (red) to positive (blue). Candidate proteins were selected based on cut-offs, set as one standard deviation above and below the mean of the controls (dashed lines). Shown alongside are box plots showing the spread of the Z-scores from controls and positive and negative candidate proteins for E-cadherin (A), Junctional actin (B) and Cytoplasmic actin (C). Red lines show mean values, boxes show 25th and 75th percentiles, whiskers show 5th to 95th percentiles, dots show outliers.

3.3.5. Summary of the candidate proteins

From the original 206 proteins tested, 101 were classified as candidates (49%). To see how each parameter was affected by the depletion, candidates were grouped according to whether they affected one, two or all three of the parameters (**Figure 3.6 A and B**). The majority of the candidates (69%) influenced all three experimental parameters when depleted (triple). Relatively few candidates (8%) had an effect on two parameters (double). Of which the vast majority (75%) influenced E-cadherin and junctional actin. These results are consistent with the fact that these two parameters are highly correlated. The remaining 23% of candidates caused a change in a single parameter, the majority affecting cytoplasmic actin (65%).

The original library contained a mixture of Rho GTPases, their regulators, and interacting partners. Interestingly, the Rho GTPases and the Rho GAPs both made up a larger percentage of the candidate proteins than they did the original library (from 11% and 22% of the library to 14% and 26% of the candidates respectively) (**Figure 3.6 C**). Their enrichment amongst the candidate proteins comes at the expense of the Rho interacting proteins, for which relative proportions dropped from 31% to 25% and suggests the importance of these groups in cell-cell contact formation.

A literature review was conducted on all the candidate Rho GAP proteins and GTPase specificity *in vitro* and *in vivo* recorded (**Table 3.1**). GAPs can have a very narrow specificity, acting on a single GTPase or multiple GTPases from different families (eg. ARAP1 (Miura et al., 2002)). Furthermore, activity *in vitro* does not necessarily translate to activity *in vivo*, as post-translational modification or protein-protein interactions can enhance or inhibit GAP activation in a cellular context (Minoshima et al., 2003). I collated the GTPase specificity of all candidate GAPs against the three best studied Rho GTPases, Rho, Rac and Cdc42 (**Figure 3.6 D**). None of the candidate GAPs possessed Cdc42 activity alone either *in vitro* or *in vivo* (although several could act on Cdc42 in conjunction with Rac and or Rho). This is interesting as Cdc42 is not required for cell-cell contact formation in keratinocytes (Erasmus et al., 2010). Rho GAPs narrowly made up the largest single group both in terms of *in vitro* and *in vivo* activity.

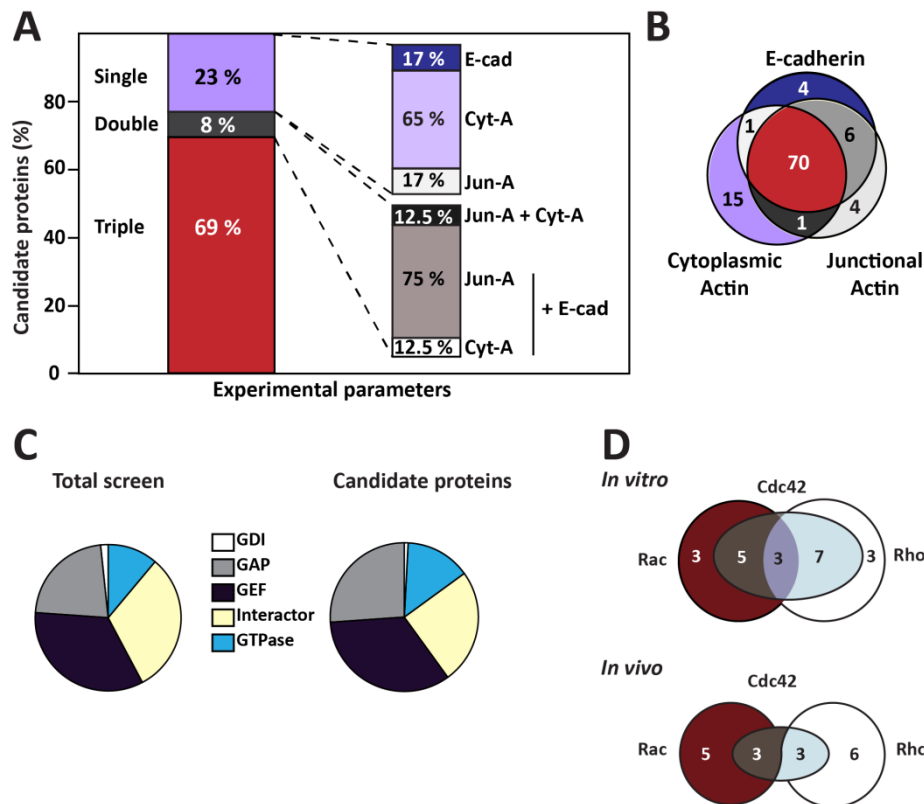


Figure 3.6 Summary of results from the primary screen A) Candidate proteins were grouped based on the number of parameters in which they had Z-scores outside of the cut-offs. For example, a candidate that produced changes in E-cadherin, Junctional actin and Cytoplasmic actin would be called 'triple' and a candidate affecting any combination of two parameters called 'double'. The candidates influencing two parameters (double) or one parameter (single) were broken down further to show which parameters were most affected within these groups. B) The breakdown of the candidates is shown in the venn diagram displaying absolute numbers of proteins. C) All the screened proteins were grouped by function as either GTPase, GEF, GAP, GDI or Interactor (proteins predicted or verified as interacting with a Rho GTPase) and this data represented as the proportions of each group within the total RNAi screen (left pie chart) or the candidate proteins subset (right pie chart). D) The GTPase activity of candidate GAPs is summarised in venn diagrams showing the *in vitro* GTPase activity (top, GTPase activation assays) and *in vivo* GAP activity of each candidate GAP (effector pull-down assays or FRET).

Gene	Protein name	GTPase specificity <i>in vitro</i>				GTPase specificity <i>in vivo</i>				Cell type	Species	Refs	
		Rac	Rho	Cdc42	Other	Rac	Rho	Cdc42	Other			In vitro	In vivo
ABR	Active breakpoint cluster region-related protein	X		X	Rac2	X			Rac2 Rac3	CHO	M	[1, 2]	[3]
ARHGAP4	Rho GTPase-activating protein 4 Rho-GAP hematopoietic protein C1	X	X	X								[4]	
ARHGAP6	Rho GTPase-activating protein 6		X									[5]	
ARHGAP9	Rho GTPase-activating protein 9	X		X		X			Rac2	COS-7	H	[6]	[7]
ARHGAP10	Rho GTPase-activating protein 10		X	X			X	X		HEK293	M	[8]	[9]
ARHGAP11A	Rho GTPase-activating protein 11A		X	X			X			U87	?	[10]	[10]
ARHGAP15	Rho GTPase-activating protein 15	X				X				BMDMs	M	[11]	[12]
ARHGAP20	Rho GTPase-activating protein 20		X				X			NG108	H	[13]	[13]
ARHGAP21	Rho GTPase-activating protein 21 Rho GTPase-activating protein 10		X*	X		X	X			PC3, T98G	H	[14]	[15, 16]
ARHGAP22	Rho GTPase-activating protein 22					X				A375M2	H		[17]
ARHGAP25	Rho GTPase-activating protein 25	X										[18]	
ARHGAP26	Rho GTPase-activating protein 26 Oligophrenin-1-like protein		X	X						C2C12	M	[19]	[20]
ARHGAP27	Rho GTPase-activating protein 27	X		X								[21]	
ARHGAP28	Rho GTPase-activating protein 28												
CENTD2	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1		X	X	Arf1 Arf5 [‡]							[22]	
CENTD3	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 3	X*	X	X*	Arf6 [‡]		X			COS-7, Sf9	H	[23]	[24]
DEPDC1B	DEP domain-containing protein 1B												
DLC1	Deleted in liver cancer 1 protein START domain-containing protein 12 Rho GTPase-activating protein 7		X	X	RhoB, RhoC		X			HEK293	H	[25, 26]	[27]
GMIP	GEM-interacting protein		X				X			HEK293	H	[28]	[28]
INPP5B	Type II inositol-1,4,5-trisphosphate 5- phosphatase Phosphoinositide 5-phosphatase	N	N	N								[29]	
RACGAP1	Rac GTPase-activating protein 1 Male germ cell RacGAP	X	X [†]	X		X		X		HeLa	H	[30, 31]	[32]
SH3BP1	SH3 domain-binding protein 1	X		X	Rac2 RhoG	X		X		HEK293, COS-7, A431	H	[33]	[34, 35]
SRGAP1	SLIT-ROBO Rho GTPase-activating protein 1 Rho GTPase-activating protein 13						X	X		HEK293	H		[36]
SRGAP2	SLIT-ROBO Rho GTPase-activating protein 2 Rho GTPase-activating protein 34	X										[37]	
SRGAP3	SLIT-ROBO Rho GTPase-activating protein 3 Mental disorder-associated GAP Rho GTPase-activating protein 14	X		X		X		X		SHSY-5Y	H	[38]	[39]
STARD8	START domain-containing protein 8 Deleted in liver cancer 3 protein		X	X								[40]	
TAGAP	T-cell activation Rho GTPase-activating protein Rho GTPase-activating protein 47												

Table 3.1 Summary of a literature survey of the candidate GAP proteins *In vitro* and *in vivo* GAP activity shown for the most studied GTPases, RhoA (grey), Rac1 (blue) and Cdc42 (maroon). Activity (if known) against other GTPases is shown in the 'other' column. *In vitro* activity was confirmed by GAP activity assays. *In vivo* activity was confirmed with either effector pull-downs or using FRET. For the *in vivo* data, the cell type in which activity was tested is noted and the species column shows the species the GAP construct used was (H; human, M; mouse). N, no GAP activity detected; *very low GAP activity; †GAP activity only when phosphorylated by Aurora B or ‡ dependent on phospholipids. References are listed in the appendix 1.

3.3.6. Analysis of the validation screen

The validation screen involved the use of four individual oligonucleotides targeting each of the candidate proteins. In total 27 GAP proteins were targeted. Thresholding was used as before to yield a Z-score for each well and mean or median Z-scores used to compare experimental wells to controls. The relationship between the Z-scores from each parameter was investigated (**Figure 3.7**). Strong correlations were found between all variables, although these were less than were seen in the primary screen. Again, the most correlated parameters were E-cadherin and junctional actin, whilst cytoplasmic actin correlated negatively with both E-cadherin and junctional actin. This may indicate that GAPs are more likely to affect a single parameter, as opposed to influencing multiple signalling pathways.

To pick the final hits, cut-offs were set as ± 1 standard deviation from the controls. A lower borderline cut-off was also set to detect more subtle phenotypes (Z-scores ± 0.8 standard deviations of the controls). In order to be classified a 'hit protein' at least two of the oligonucleotides had to produce Z-scores above or below the borderline cut-off for at least one parameter (**Figure 3.8 A-C**). Based on these criteria 20 GAPs (74%) were classified hit proteins (**Table 3.2**).

The screen has allowed the identification of both positive and negative regulators of cell-cell contact formation (**Figure 3.9 A**). ARAP1 depletion led to an overall increase in E-cadherin (with both E-cadherin present at the junctions and the cytoplasm increased) and junctional actin levels and a decrease in cytoplasmic actin. Conversely, SRGAP3 depletion resulted in a large decrease in E-cadherin and junctional actin levels compared to controls. In these images E-cadherin is lost in some sections of junctions or is much less intense than in control images. The same is true for junctional actin. Lower levels of E-cadherin or actin populations in images are clearly reflected in the Z-scores these images obtained. Thus, the segmentation and quantification strategy used yielded different Z-scores that are effective in distinguishing different phenotypes.

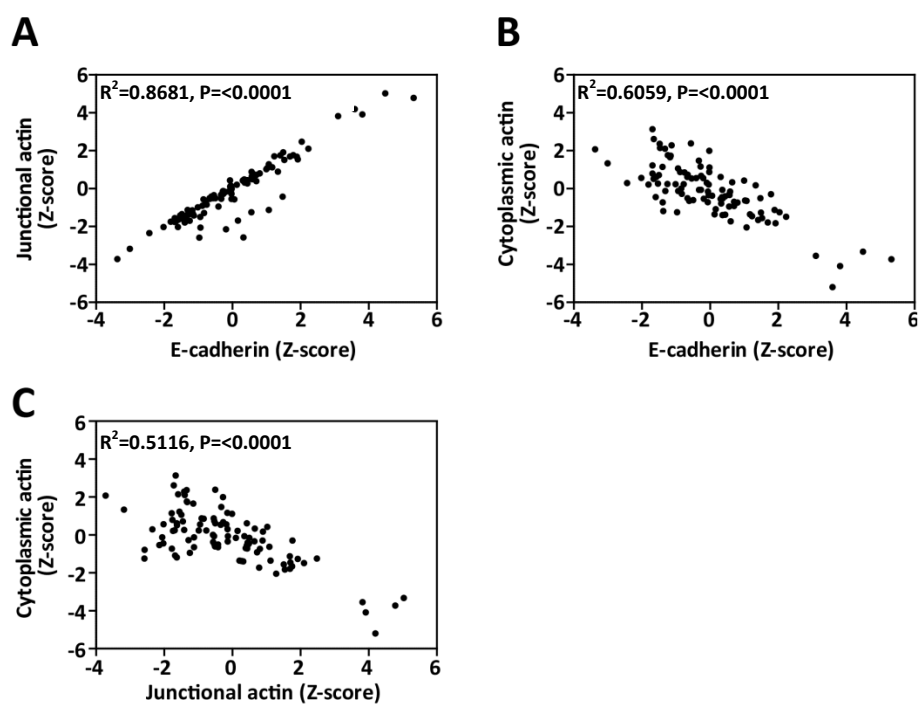


Figure 3.7 Correlations between the experimental parameters of candidate proteins Z-scores for each of the experimental parameters E-cadherin, junctional actin and cytoplasmic actin were correlated with one another for all oligonucleotides targeting proteins in the validation screen. The degree of correlation was determined using Pearson's correlation coefficient (R^2).

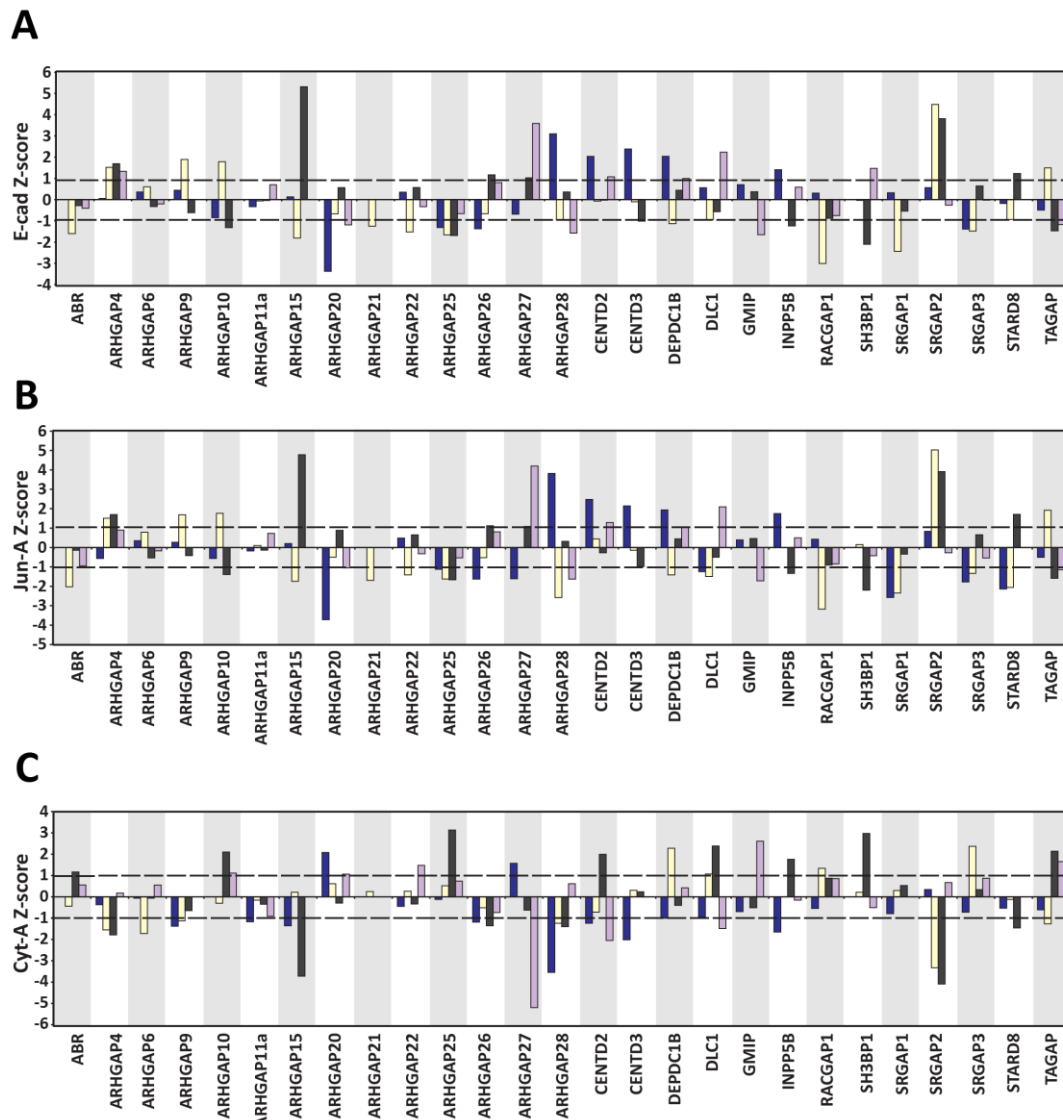


Figure 3.8 Bar charts showing Z-scores from each oligonucleotide tested in the validation screen Z-scores from four individual oligonucleotides used to target each protein, E-cadherin (A), Junctional actin (B) and Cytoplasmic actin (C) plotted on bar charts. Blue, cream, grey and purple are used to represent oligonucleotides 1 to 4 respectively. Dotted lines represent borderline cut-offs.

Gene name	Protein name	E-Cadh	Junc-A	Cyto-A
ABR	Active breakpoint cluster region-related protein		—	
ARHGAP4	Rho-type GTPase-activating protein 4 Rho-GAP hematopoietic protein C1	+	+	—
ARHGAP9	Rho-type GTPase-activating protein 9			—
ARHGAP10	Rho- GTPase-activating protein 10 GTPase regulator associated with focal adhesion kinase 2	—		+
ARHGAP11A	Rho GTPase-activating protein 11a			—
ARHGAP15	Rho GTPase-activating protein 15			—
ARHGAP20	Rho GTPase-activating protein 20	—	—	+
ARHGAP25	Rho GTPase-activating protein 25	—	—	
ARHGAP26	Rho-type GTPase-activating protein 26 Oligophrenin-1-like protein GTPase regulator associated with focal adhesion kinase	+	+	—
ARHGAP27	Rho-type GTPase-activating protein 27 CIN85-associated multi-domain-containing Rho GTPase-activating protein 1	+	+	
ARHGAP28	Rho-type GTPase-activating protein 28	—	—	—
CENTD2	Arf-GAP with Rho-GAP domain, ANK repeat and PH domain-containing protein 1	+	+	—
DEPDC1B	DEP domain-containing protein 1B	+	+	
DLC1	Deleted in liver cancer 1 protein START-domain containing protein 12 Rho-type GTPase activating protein 7		—	
RACGAP1	Rac GTPase-activating protein 1 Male germ cell RacGap	—	—	+
SRGAP1	SLIT-ROBO Rho GTPase-activating protein 1 Rho GTPase-activating protein 13		—	
SRGAP2	SLIT-ROBO Rho GTPase-activating protein 2 Formin-binding protein 2 Rho GTPase-activating protein 34	+	+	—
SRGAP3	SLIT-ROBO Rho GTPase-activating protein 3 Mental disorder-associated GAP Rho GTPase-activating protein 14	—	—	+
STARD8	StAR-related lipid transfer protein 8 START domain-containing protein 8 Deleted in liver cancer 3 protein		—	
TAGAP	T-cell activation GTPase-activating protein Rho GTPase-activating protein 47	—	—	+

Table 3.2 Summary of the hit proteins from the validation screen Parameter(s) affected by each hit protein shown. ‘+’ denotes a Z-score above the controls and ‘-’ denotes a Z-score below the controls.

All hits were grouped based on the number of experimental parameters their depletion affected (**Figure 3.9 B**). Interestingly, only 45% of GAPs affected three parameters (compared to 65% of proteins in the primary screen), possibly indicating a tendency of Rho GAPs to affect single, specific processes, rather than governing many cellular events. Consequently, 20% of hits influenced two parameters (up from 8% in the primary screen), of which 75% affected E-cadherin and junctional actin. Thirty-five percent of hits affected one parameter. Of these, none were found to affect E-cadherin alone, suggesting that perturbations in E-cadherin will also disrupt actin populations.

Finally, I sought to confirm the expression of a sub-set of GAPs in keratinocytes (**Figure 3.10**). Primers were designed to target ARAP1, DEPDC1B, ARHGAP20, ARHGAP26, ARHGAP27 and RacGAP1 and RT-PCR performed using keratinocyte RNA. All the targeted GAPs were expressed in keratinocytes. The primers were designed such that (if possible) if separate isoforms of the protein existed (based on sequences deposited on NCBI gene database) these could be distinguished. For ARHGAP20 only one form exists and this seen at 252bp. A larger band is also present above this which may be due to non-specific amplification of a different product. Two isoforms exist of ARHGAP26 (A, 497bp and B, 332bp products). Isoform B is the predominant form expressed in keratinocytes. This isoform is the shorter of the two, but maintains all of the recognised functional domains that are present in the longer isoform. ARHGAP27 also has two isoforms (A and B) and I found both expressed in keratinocytes (335bp and 254bp products). Two isoforms of DEPDC1B exist, but the sequences differ only in that a small section is missing in the middle of the second isoform. As such primers were unable to distinguish between these. DEPDC1B is expressed in keratinocytes, but which isoform predominates is unknown. Three variants of RacGAP1 exist, but variants 1 and 2 differ by only 11bp and could not be distinguished. The main band observed corresponds to either variant 1 or 2 (342 and 353bp products). However, whether or not the third variant is also expressed is not clear. A band around 233bp is present on the gel, but as there seems to be several unexplained bands here this may be non-specific. Three isoforms of ARAP1 exist. Keratinocytes predominantly express isoform C (372bp product) which is the longest of the isoforms.

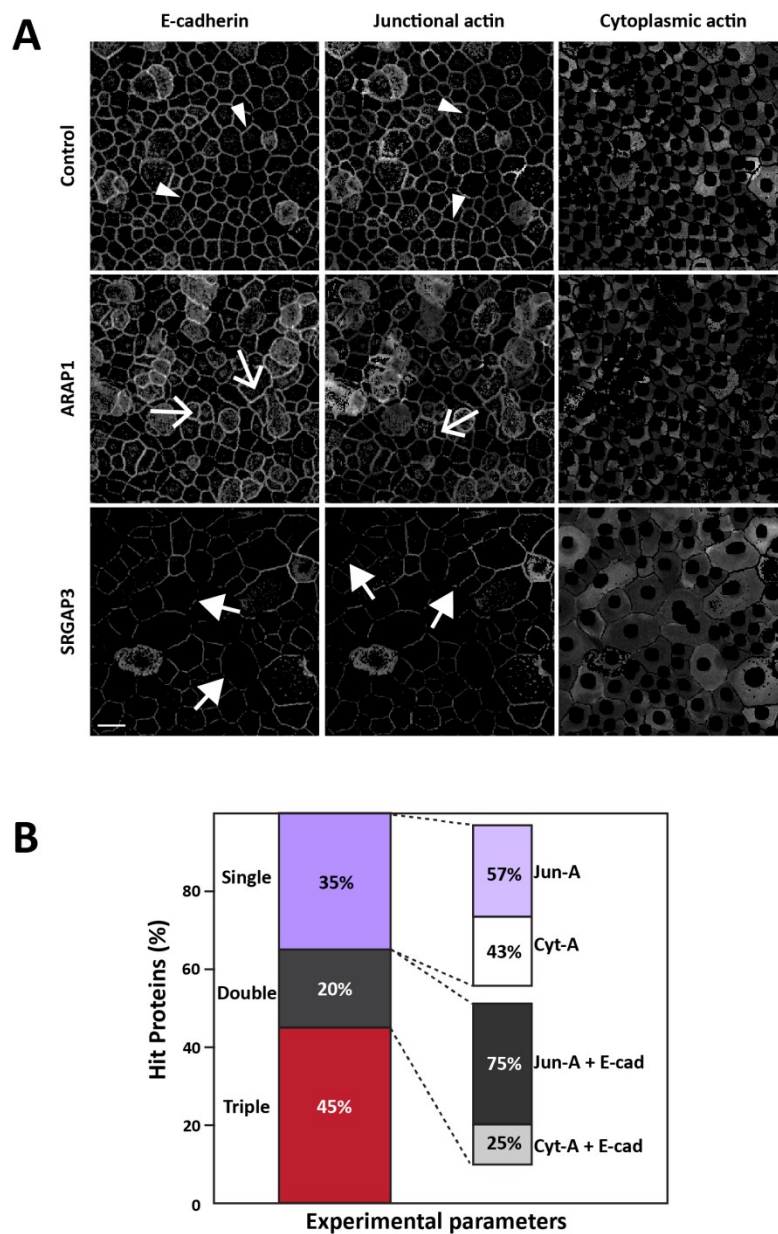


Figure 3.9 Positive and negative regulators of cell-cell contact formation were identified in the screen A) Candidate proteins were grouped based on the number of parameters in which they had Z-scores outside of the cut-offs. For example, a candidate affecting E-cadherin, Junctional actin and Cytoplasmic actin would be called 'triple' and a candidate affecting any combination of two parameters called 'double'. The candidates affecting two parameters (double) or one parameter (single) were broken down further to show which parameters were most affected within the group. B) Montage of example images from hit proteins that when depleted were found to positively (ARAP1) or negatively (SRGAP3) regulate E-cadherin levels. Arrow heads indicate normal junctions, open arrows indicate junctions enriched in E-cadherin or actin, arrows indicate gaps in E-cadherin or actin staining at junctions. Scale bar represents 50µm.

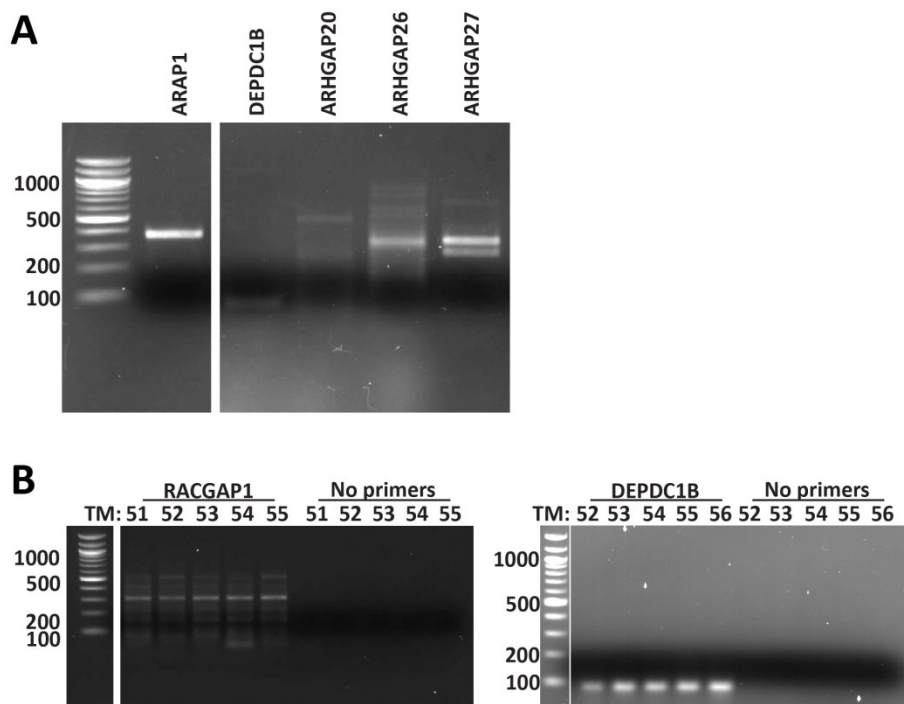


Figure 3.10 A subset of hit proteins are expressed in keratinocytes RT-PCR on keratinocyte samples amplified several of the hit proteins. PCR products were run on agarose gels to detect products A) Primers specific for ARAP1, DEPDC1B, ARHGAP20, ARHGAP26 and ARHGAP27 were used to amplify these proteins from keratinocyte RNA. B) PCR reactions were optimised using a temperature gradient between 51 and 55°C to confirm the presence of GMIP and RacGAP1 isoforms. Control reactions were run in the absence of primers. Ladders indicating size in basepairs of the product is shown on the left.

3.4. Conclusions

In this chapter, the use of an automated analysis program for the segmentation and quantification of junctional proteins during cell-cell contact formation was optimised (in collaboration with N. Welsh). We show that this program can accurately identify differences in the amount of E-cadherin, junctional actin and cytoplasmic actin, and that this is independent of cell count or the total amount of F-actin present. Importantly, the finding that several of the Rho GAPs identified have reported roles in the regulation of cell-cell contact supports the efficacy of this approach. As such, this program may be used to assess the effects of depletion of a range of targets on cell-cell contact formation and the early stabilisation of junctions. With the improvement of automatic analysis software to detect cell boundaries, and the increasing ability of these to distinguish complex morphological traits, even more proteins can be analysed in similar screens.

Multiple GAPs have been identified in this screen that can regulate the formation and early stabilisation of cell-cell contacts, many of which have not previously been linked to this process. However beyond this, these findings allude to a system that is very precisely governed; Rho and Rac activity is necessary for cell-cell contact formation, but the loss of several Rho and Rac-specific GAPs can also perturb cell-cell contacts, demonstrating that GTPase activity must be spatially constrained also. Possibly, the very fact that Rho/Rac activity is critical for this process necessitates the presence of numerous Rho-specific GAPs in order to ensure dynamic regulation of Rho GTPase activity. This suggests multiple Rho GAPs specific for the same GTPase are required for junction assembly to proceed correctly. Thus, these are likely required to govern very specific stages of complex cellular processes and to direct localised GTPase signalling appropriately. The diversity in terms of reported GTPase targets, the presence of multiple functional domains and the multitude of recorded functions associated with the hit proteins supports this conclusion and suggests the existence of a highly complex system underlying cell-cell contact formation where the inactivation of GTPase is just as important as its activation.

3.5. Discussion

3.5.1. The use of an automated analysis program to quantify cellular features upon cell-cell contact formation

We have used an automated analysis program to segment E-cadherin, junctional actin and cytoplasmic actin and quantified these regions in relation to a selection of controls. The quantification of E-cadherin appeared to compare favourably to visual analysis of wells, with images containing more E-cadherin (assessed visually) receiving a correspondingly higher Z-score. Using this approach a number of Rho GTPases have been identified as potential regulators of cell-cell contact formation.

This approach is useful to segment E-cadherin and quantify its levels at junctions. As such this could be used to segment other junctional proteins also. However, this type of analysis does have limitations in terms of the conclusions that should be drawn based on a Z-score. The main caveat associated with thresholding-based image analysis is that this relies on the assumption that E-cadherin will be most intense in the region of the junction. Consequently, interpretation of the Z-score is compromised in images with high levels of cytoplasmic E-cadherin, as any increase at cell-cell contacts may not be picked up. Furthermore, a large population of cytoplasmic E-cadherin may interfere with the junctional and cytoplasmic actin images due to the methods used to generate these. Indeed, whilst the E-cadherin segmentation seemed overly accurate, the generation of junctional actin, and particularly the cytoplasmic actin parameters was variably successful. Alternatively, the accurate segmentation of actin may require images of a higher quality than those used, as actin is more widely distributed within cells and generally, images had higher background. With continually improving software, it is likely that issues with segmentation can be resolved and methods could be employed to selectively detect E-cadherin at the junctions, thus improving the detection of actin populations. As such, the fact that segmentation was less successful with our actin images does not mean a similar thresholding-based approach would not be suited for similar screens in the future.

Overall, the method of thresholding used is well suited to identify changes in levels of a protein in a particular area, but unsuitable for identification of certain morphological traits, such as wavy junctions (unless these also have less/more of the examined protein). Potentially then, thresholding can be used to narrow-down from a large library a smaller subset of proteins that may subsequently be analysed using more time-consuming, but accurate methods.

3.5.2. The composition of proteins in the candidate screen

From the primary screen, we found that the E-cadherin and junctional actin Z-scores correlate. This is certainly consistent with the important roles both play in junction formation and maintenance. Indeed, clustering of E-cadherin triggers actin recruitment to contacts, which in turn strengthens junctions (Adams et al., 1998; Chu et al., 2004). Conversely, cytoplasmic actin was negatively correlated with both junctional actin and E-cadherin. The formation and maturation of contacts sees numerous changes in actin dynamics in order to stabilise cadherin molecules (Adams et al., 1998; Yamada and Nelson, 2007; Zhang et al., 2005). Over time, cytoplasmic actin changes from being loosely packed in the cytoplasm to concentrating at AJs (Yamada and Nelson, 2007; Zhang et al., 2005). Therefore, it is unsurprising that a junction with more E-cadherin and/or junctional actin has less cytoplasmic actin, as this has likely been redistributed to the vicinity of the junction (Yamada and Nelson, 2007; Zhang et al., 2005).

The distribution of the Z-scores in the primary screen showed that both E-cadherin and junctional actin distributions were heavily negatively skewed, whilst the cytoplasmic actin distribution was positively skewed. This may suggest that most proteins targeted were positive regulators of cell-cell contact formation, without them less E-cadherin and junctional actin was present at sites of contact.

Proteins positively regulating AJ formation may do so by controlling several cellular processes, for example by regulating the delivery of E-cadherin to sites of contact or by promoting engagement of E-cadherin with the cytoskeleton (Baum and Georgiou, 2011). However, loss of a protein regulating the coupling of E-cadherin to the cytoskeleton may not result in such a reduction of E-cadherin from cell-cell contacts that would yield a sufficiently different Z-score from controls (this would therefore be a false negative). For example, ARHGAP21 is required for the localisation of α -catenin to cell-cell contacts in some epithelial cell lines (Sousa et al., 2005) and loss of ARHGAP21 renders cells more susceptible to invasion by *L. Monocytogenes* (Sousa et al., 2005). However, despite apparent weakening of junctions and presumably less E-cadherin being coupled to α -catenin, in this case, the loss of ARHGAP21 does not affect the levels of E-cadherin at cell-cell contacts (Sousa et al., 2005). As such, it will be interesting to ascertain the nature of the positive regulators we have detected in this screen.

Negative regulators of junction formation, when depleted, will result in increased levels of E-cadherin and/or junctional actin. If these proteins control the engagement of E-cadherin rather than promoting its internalisation they may be more difficult to detect as surface E-cadherin levels may

remain unchanged. This may account for the small proportion of negative E-cadherin and junctional actin Z-scores obtained in this screen.

Comparison of the composition of candidate proteins and total screen in terms of functional group revealed an enrichment of GAPs and GTPases amongst the candidates. The importance of GTPases in multiple processes based around the remodelling of the actin cytoskeleton is well established (Hall, 2012; Watanabe et al., 2009). The enrichment of GAPs in our candidate proteins suggests this class of regulatory proteins play many varied roles in a large number of processes and are vital for the appropriate spatial and temporal regulation of GTPases (Bernards, 2003; Moon et al., 2003). This is substantiated by the occurrence of a varied cohort of candidate GAPs. However, there was no enrichment in GEFs in the screen, which is curious given the notion that the regulation of a GTPase in a particular process requires coordination between GEF and GAP (Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007).

Rho and Rac-specific GAPs were the most prevalent identified in the primary screen, even though both Rho and Rac are known to be necessary for cell-cell contact formation to occur (Braga et al., 1997; Calautti et al., 2002; Ehrlich et al., 2002; Kovacs et al., 2002a; Yamada and Nelson, 2007). However, expression of constitutively active forms of both Rho and Rac can disrupt contacts (Braga et al., 1997; Chu et al., 2004; Jou and Nelson, 1998; Noren et al., 2003; Noren et al., 2001) indicating the importance of tightly regulating their activation. As such, it is not surprising that the loss of several Rho and Rac GAPs can disrupt AJ organisation.

Conversely, Cdc42 is not activated upon cell-cell contact formation in keratinocytes (Erasmus et al., 2010) (but is activated by cell-cell contact in other cell types (Chu et al., 2004; Kim et al., 2000b)). This implies that a Cdc42-specific GAP must be present at cell-cell contacts. However, no Rho GAPs specific for Cdc42 alone were identified in the primary screen, although several able to act on Cdc42 in conjunction with another GTPase were present. As Rho GAPs can display cell-type specific activities it is possible that any of these identified Rac/Rho and Cdc42 GAPs act predominantly on Cdc42 in keratinocytes (this would have to be tested). GTPase activation occurs in precisely controlled zones during contact formation (Yamada and Nelson, 2007). Interestingly, the dual GEF and GAP ABR was a hit protein and can act simultaneously as a Cdc42 GAP and Rho GEF (Chuang et al., 1995; Vaughan et al., 2011). During wound healing ABR maintains the existence of localised zones of GTPase activity (Vaughan et al., 2011). Thus, this protein may perform a similar role in cell-cell adhesion and inactivate Cdc42 whilst also promoting activation of Rho.

3.5.3. The validated hits

The validation screen was conducted using four individual oligonucleotides targeting a total of 27 Rho GAPs. A total of 20 candidate GAPs were validated as hits (74%). As proteins in the validation screen are selected based on the high likelihood (based on the primary screen) that they would perturb junction formation, this hit rate is expected.

Since the completion of this screen several Rho GAPs have been implicated in the regulation of cell-cell contact formation and maintenance (McCormack et al., 2013). Some of these GAPs were either not included in our screen (RICH1, PX-RICS, MyoIXB (Chandhoke and Mooseker, 2012; Nakamura et al., 2008; Wells et al., 2006)) or were lost in the primary screen as not enough replicates were gathered (MyoIXA, Oligophrenin-1, PI3KR1 (Elbediwy et al., 2012; Gurney et al., 2011; Omelchenko and Hall, 2012)). Whilst SH3BP1, which specifically regulates cell-cell contact formation in A431 and Caco-2 cells by spatially confining Cdc42 signalling yielded highly variable Z-scores in the validation screen making interpretation of its effects on junction formation in keratinocytes difficult (Elbediwy et al., 2012).

Similarly, ARHGAP21 was classified as a candidate protein but not enough replicates were gathered in the validation screen, meaning the effect of depletion of this protein remains ambiguous. We may speculate that ARHGAP21 plays a role in stabilising cell-cell contacts as its depletion in Caco-2 and JEG-3 cells inhibits recruitment of α -catenin to cell-cell contacts (measured 72 hours after transfection in subconfluent cells) (Sousa et al., 2005). This is supported by the phenotype seen upon ARHGAP21 depletion in the primary screen (decrease in E-cadherin and junctional actin and an increase in cytoplasmic actin). In neither case are contacts prevented from forming, rather levels of junctional proteins are reduced, implying ARHGAP21 is not a critical for cell-cell contact formation to proceed, but may contribute to early stabilisation of junctional proteins. Indeed in Caco-2 cells E-cadherin staining is not disrupted by loss of ARHGAP21, possibly by this point E-cadherin had been stabilised via additional mechanisms (Sousa et al., 2005).

The related GAPs p190a and p190b have been implicated in cell-cell contact maintenance in several cell types and down regulate RhoA at AJs (Noren et al., 2003; Ratheesh et al., 2012; Wildenberg et al., 2006). Interestingly, depletion of either of these two GAPs did not produce a strong phenotype in keratinocytes in the primary screen, and were thus not forwarded to the validation. Assuming the depletion was efficient, this may reflect cell-type specific roles for these proteins. Indeed, p190a is necessary for cell-cell contact in NIH3T3 cells (Wildenberg et al., 2006), but in MCF-7 cells it is absent

from junctions, whilst p190b is prevented from localising at junctions by the centralsplindlin complex (Ratheesh et al., 2012). In MCF-7 cells the absence of Rac GTPase-activating protein 1 (RacGAP1) decreases levels of E-cadherin and active RhoA at the junctions, whilst p190b localisation at junctions is enhanced (Ratheesh et al., 2012). Thus, RacGAP1 plays a role in limiting the localisation of p190b to cell-cell contacts. Depletion of Rac GTPase-activating protein 1 (RacGAP1) (a component of the centralsplindlin complex (Mishima et al., 2002)) decreased E-cadherin and junctional actin in the validation screen. As keratinocytes require active RhoA to form junctions (Braga et al., 1997; Calautti et al., 2002) they may employ a similar mechanism as MCF-7 cells and limit p190b localisation to the junctions in order to promote Rho activation. Depletion of RacGAP1 may reflect an increase in p190b at the junctions, and also explain why the depletion of p190b failed to induce any phenotype in keratinocytes (if normally this protein is inhibited).

Other GAPs identified in the screen that have also been implicated in cell-cell contact regulation are the related proteins DLC1 and DLC3 (STARD8). Both are Rho-specific GAPs often found downregulated in different cancers and have been implicated in the positive regulation of cell-cell contacts (Holeiter et al., 2012; Tripathi et al., 2012; Tripathi et al., 2013). In the screen both DLC1 and DLC3 depletion reduced the amount of junctional actin without affecting either E-cadherin or cytoplasmic actin. Possibly the depletion of these GAPs lead to a weakening of junctions associated with a migratory phenotype.

Amongst the hit proteins were all members of the slit-robo GAPs (SRGAP1, SRGAP2, SRGAP3 and ARHGAP4). In *c. elegans* a single SRGAP homolog exists, SRGP-1, which regulates cell-cell contact stability via its F-BAR domain (Zaidel-Bar et al., 2010). F-BAR domains bind to and modulate membrane curvature, and SRGP-1 is required to increase dynamic membrane ruffling which promotes the establishment of cell-cell contacts during gastrulation (Zaidel-Bar et al., 2010). In the screen, the phenotypes seen upon depletion of each member of the SRGAP family were varied, possibly reflecting divergent roles for these proteins that have arisen in mammals (**Table 3.2**).

3.6. Future work

3.6.1. A bioinformatics survey of the hit proteins

The screen has yielded a wealth of data regarding the effects of depletion of a range of GTPases, their regulators and their effectors on junction morphology. This data can be used to create protein-protein interaction (PPI) networks of the candidate GAPs in order to identify novel pathways

regulating cell-cell contact formation, and identify underlying patterns associated with specific phenotypes (Wheeler *et al* manuscript in preparation)(Paris and Bazzoni, 2008). The analysis of large data sets to elucidate PPI networks facilitates the identification of novel participants in signalling pathways (Simpson *et al.*, 2008; Winograd-Katz *et al.*, 2009). Further analysis, including annotation of proteins with biological function and use of novel predictive tools then allows complex PPIs to be deconvoluted to identify, with a high degree of confidence, proteins involved in specific processes (Schaefer *et al.*, 2013; Zhang *et al.*, 2007). PPI analysis has been used successfully to uncover a range of candidate proteins involved in many diseases including atherosclerosis and cancer (Diez *et al.*, 2010; Hood *et al.*, 2004).

3.6.2. Re-testing the RNAi library under different conditions

Although our screen identified numerous hit proteins, false negatives can arise if the half-life of the target gene is not compatible with the screening conditions. In this screen cells were fixed 72 hours post transfection. However, by this point levels of some proteins may already have recovered. Repeating the screen using shorter time points, such as 24 and 48 hours would be beneficial.

Furthermore, the continual advances in automated image analysis mean that more sophisticated methods for the segmentation of E-cadherin have become available since the screen was analysed (Karakaya *et al.*, 2012). Whilst these may still be too laborious to implement in a medium-throughput screen, they could be used on a subset of hit proteins to verify the screen phenotypes. These may also allow more complex classification of hit proteins by grouping these into categories based on the morphological alterations their loss instigates (eg. wavy junctions).

3.6.3. Validation of the hit proteins

Although the screen identified several Rho GAPs with described functions regarding regulation of cell-cell junctions, the only way of truly validating the success of this method is to validate some of the hit proteins in keratinocytes. Initially siRNA must be optimised, as 72 hours will not yield optimal results for all proteins. Next, whether or not the screen phenotype can be recapitulated should be determined.

Finally, the functions of hit proteins during cell-cell contact formation and maintenance will be addressed. This will include ascertaining which GTPase the hit protein acts upon by performing effector pull down assays in cells either depleted or overexpressing the hit protein. Additionally use

of unimolecular FRET probes of Rac, Rho or Cdc42 will facilitate spatiotemporal information regarding the target GTPase to be gathered during cell-cell contact formation (Itoh et al., 2002; Makrogianneli et al., 2009; Yoshizaki et al., 2003).

Investigating potential interacting partners of the hit protein, which will be complemented by the bioinformatics networks generated, will offer clues as to the cellular processes affected by the hit protein.

Chapter 4.

The characterisation of Rho GAPs regulating cell-cell contact formation and maintenance

4.1. Introduction

Cell-cell contacts are governed by multiple GTPases whose actions must in turn be coordinated by a variety of regulators. As such, it is likely that multiple GAPs, with different specificities, are present at sites of contact in order to facilitate the inactivation of different GTPases.

In the previous chapter I identified putative GAPs that regulate cell-cell contact formation via a siRNA screen. To decide on GAPs to further characterise several factors were considered including; i) that the GAP had not previously been identified as a regulator of cell-cell contacts, ii) some literature on the GAP was available iii) that evidence existed that the GAP may be present at the cell membrane iv) that some reagents were available (eg. effective antibodies) and v) that the GAP was expressed in keratinocytes. Based on this analysis, ARAP1 (CENTD2) was selected to further characterise. ARAP1 is a dual Arf and Rho-specific GAP also containing an ankyrin repeat, five PH domains, a SAM and a RA domain (Miura et al., 2002). ARAP1 can localise to the plasma membrane in several cell types (Guo et al., 2003; Simova et al., 2008; Yoon et al., 2008) where it is able to regulate the endocytosis of EGFR (Daniele et al., 2008; Yoon et al., 2008). EGF promotes the recruitment of ARAP1 to the cell periphery where it associates with EGFR in a Rab5-dependent manner. The depletion of ARAP1 via siRNA accelerates the association of EGF and EGFR with early endosomal markers and hastens the degradation of EGFR (Daniele et al., 2008; Yoon et al., 2008). The regulation of EGFR is particularly interesting as in the laboratory it has previously been demonstrated that Rac is activated by EGFR signalling downstream of cadherin adhesion (Betson et al., 2002). Furthermore, the ability of ARAP1 to regulate both Arf and Rho GTPases means it is well placed to coordinate crosstalk between multiple signalling pathways, making it an interesting candidate for study.

The second GAP investigated in this chapter is ARHGAP6. ARHGAP6 is a RhoA-specific GAP associated with MLS (Prakash et al., 2000) and can regulate actin remodelling (Prakash et al., 2000) and PLC δ activity (Ochocka et al., 2008). ARHGAP6 orthologs are found in a range of species including *D. melanogaster*, *C. elegans* and *Saccharomyces cerevisiae*. To investigate a role for ARHGAP6 in cell-cell adhesion I have collaborated with the group of J-P Vincent (NIMR, London).

The final protein examined in this chapter is CdGAP. CdGAP is a Rac and Cdc42 specific GAP known to regulate cell migration, spreading and invasion (He et al., 2011; Jenna et al., 2002; LaLonde et al., 2006; Lamarche-Vane and Hall, 1998; Wormer et al., 2012). Mutations in the CdGAP gene were recently identified as being one cause of the developmental disorder Adams-Oliver Syndrome (AOS)

(Southgate et al., 2011). AOS is characterised by congenital absence of skin (often affecting the head this can vary from small hairless patches to complete loss of epidermis and underlying tissue of the skull) and transverse limb defects, typically affecting distal phalanges or entire digits but in extreme cases whole limbs can be missing (Whitley and Gorlin, 1991). A range of associated abnormalities have also been reported including cardiac and pulmonary complications (Snape et al., 2009). Mutations in the CdGAP gene were found to produce prematurely truncated proteins with enhanced activity for Cdc42 (Southgate et al., 2011). Although expressed at high levels in the developing heart, no cardiac defects were seen in subjects with the CdGAP mutations (Southgate et al., 2011) indicating that mutations in CdGAP do not account for the full range of AOS-associated abnormalities. Further, mutations in CdGAP were only found in a small proportion of subjects presenting with AOS. Subsequently, another Rac and Cdc42 regulator, DOCK6 (a Rho GEF), was also implicated in AOS. Mutations in two patients were found to result in DOCK6 truncation mutants lacking key catalytic domains, rendering this protein inactive (Shaheen et al., 2011). These findings demonstrate the importance of Rac and Cdc42 regulation in early organogenesis.

CdGAP is also an example of a potentially oncogenic GAP as its expression can promote TGF- β -mediated invasion in mammary explants expressing an activated ErbB2 receptor (He et al., 2011). Interestingly, the expression of CdGAP in these cells was found to correspond with a strong reduction in E-cadherin protein levels (He et al., 2011). These findings suggest that CdGAP promotes migration and invasion in response to TGF- β in epithelial breast cancer cells. CdGAP's regulation of cell-matrix adhesions and potentially E-cadherin levels prompted us to investigate CdGAP function in keratinocytes at cell-cell contacts.

4.2. Aims

1. Validate the efficacy of the siRNA screen by investigating the function of a hit protein, ARAP1, in cell-cell contact formation
2. Characterise the roles of the Rho GAPs ARHGAP6 and CdGAP in formation and maintenance of cell-cell adhesion.

4.3. Results

4.3.1. ARAP1 is a positive regulator of cell-cell contact formation

In the screen, the depletion of ARAP1 led to an increase in levels of E-cadherin and junctional actin and a decrease in cytoplasmic actin (**Table 3.1**). I sought to confirm the function of ARAP1 in cell-cell contact assembly. A pool of oligonucleotides targeting ARAP1 was used to determine the optimal time-point for the depletion of ARAP1 in keratinocytes to be 48 hours (**Figure 4.1 A**). Subsequently, this time point was used for further optimisation with individual oligonucleotides (**Figure 4.1 B**). ARAP1 was depleted from keratinocytes with about 50% efficiency. To measure the effect of ARAP1 depletion on E-cadherin at cell-cell contacts, monolayers of cells maintained in low calcium medium were transfected with ARAP1 oligonucleotides 4 and 6 for 48 hours before junction formation was induced. Depletion of ARAP1 by both oligonucleotides considerably reduced the amount of E-cadherin present at cell-cell contacts, as measured by a thresholding-based method (**Figure 4.1 C**). Unlike the screen phenotype, ARAP1 depletion decreased E-cadherin levels at junctions by between 60 and 80% compared to controls transfected with non-targeting scrambled siRNA (**Figure 4.1 C and D**).

As ARAP1 depletion reduced E-cadherin levels, I investigated if overexpression of ARAP1 could have the opposite effect and increase E-cadherin levels. Unfortunately, ARAP1 could not be efficiently expressed in cells maintained in low calcium, so expression of ARAP1 was investigated in cells with mature junctions. Neither expression of the wild-type protein or a PH-domain mutant that was unable to act as an Arf-GAP ((Campa et al., 2009) and personal communication) caused any changes to E-cadherin contacts in terms of the uniformity or the intensity of E-cadherin staining (based on a visual analysis) (**Figure 4.2**), indicating that ARAP1 may be a positive regulator for the earlier stages of contact stabilisation.

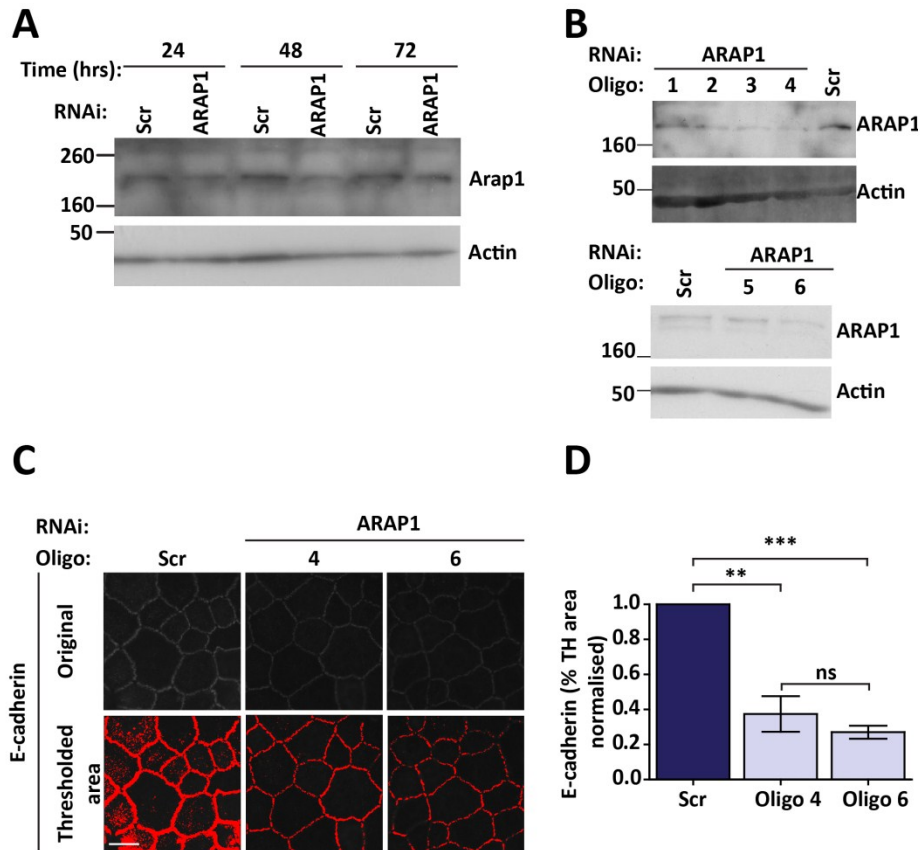


Figure 4.1 ARAP1 regulates cell-cell contact formation ARAP1 was depleted via RNAi using either a SMART pool™ consisting of four oligonucleotides (30nM) (A) or individual oligonucleotides 1-6 (20-50nM) (B). Cells grown in low calcium medium were lysed after the indicated times for the pools and after 48 hours for the single oligonucleotides. ARAP1 and actin were detected in lysates via Western blot. Depletion efficiency was calculated relative to the amount of ARAP1 in cells treated with a control scrambled oligonucleotide. C) ARAP1 was depleted using oligonucleotides 4 and 6 from cells grown in low calcium medium. Confluent monolayers were induced to form junctions for 30 minutes and fixed and stained for E-cadherin (Original). Images were processed for E-cadherin levels (Thresholded area). Scale bar = 50µm. D) Quantification of E-cadherin levels following ARAP1 depletion. The mean percentage of thresholded area relative to control is shown. Standard error of the mean is shown. Statistical significance was assessed using one-way ANOVA with Bonferroni's multiple comparison test. **, $P \leq 0.01$; ***, $P \leq 0.001$. Number of repeats (N) =3.

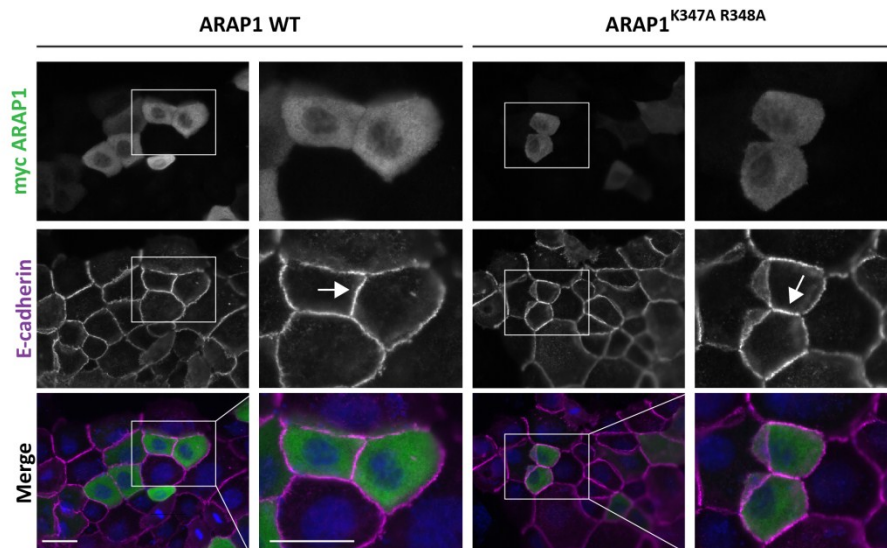


Figure 4.2 Exogenous expression of ARAP1 does not destabilise mature cadherin contacts myc-ARAP1 wild-type or myc-ARAP1^{K347A R348A}, a mutant deficient in PIP₃ binding and so unable to act as an Arf-GAP, were expressed in keratinocytes with mature junctions for 8 hours. Cells were fixed and stained for E-cadherin, myc-tagged exogenous protein and the cell nucleus with DAPI. Images were collected on a widefield microscope. Colour images from each channel were overlaid to create the merged image. The white box delineates zoomed area in adjacent image. Arrows indicate junctions with similar levels of E-cadherin as control surrounding, non-expressing cells. Scale bars = 50µm. N=3 for wild-type and N=2 for ARAP1^{K347A R348A}

4.3.2. ARHGAP6 expression disrupts mature cell-cell contacts

ARHGAP6 was depleted as part of the primary siRNA screen and found to decrease E-cadherin and junctional actin whilst increasing cytoplasmic actin (**Figure 4.3 A**). However, no consistent effect was seen in the validation screen using individual oligonucleotides on the levels of E-cadherin or either population of actin (**Figure 4.3 B and C**). ARHGAP6 may have been a false positive in the primary screen. However, it is also possible that the depletion of ARHGAP6 obtained by the single oligonucleotides were either less effective (Parsons et al., 2009), or only one or two oligonucleotides were efficient in depleting ARHGAP6 and so their effect was masked by the other oligonucleotides. Therefore, the function of ARHGAP6 during early stages of contact formation needs to be further tested.

Alternatively, ARHGAP6 is not required during the early stages of junction formation, but instead regulates their maintenance. ARHGAP6 was expressed in keratinocytes with mature junctions (**Figure 4.4**) and an unusual phenotype was observed. Several cells had extensive protrusions, which appear to grow above and below neighbouring cells. In addition, expressing cells in many cases lost E-cadherin from their junctions. This is in contrast to a control Rho-specific GAP that caused no such alterations in cellular morphology (**Figure 4.4**). To further characterise the nature of these phenotypes I looked into the expression of the *Drosophila* homolog of ARHGAP6 (collaboration with J-P Vincent, NIMR)

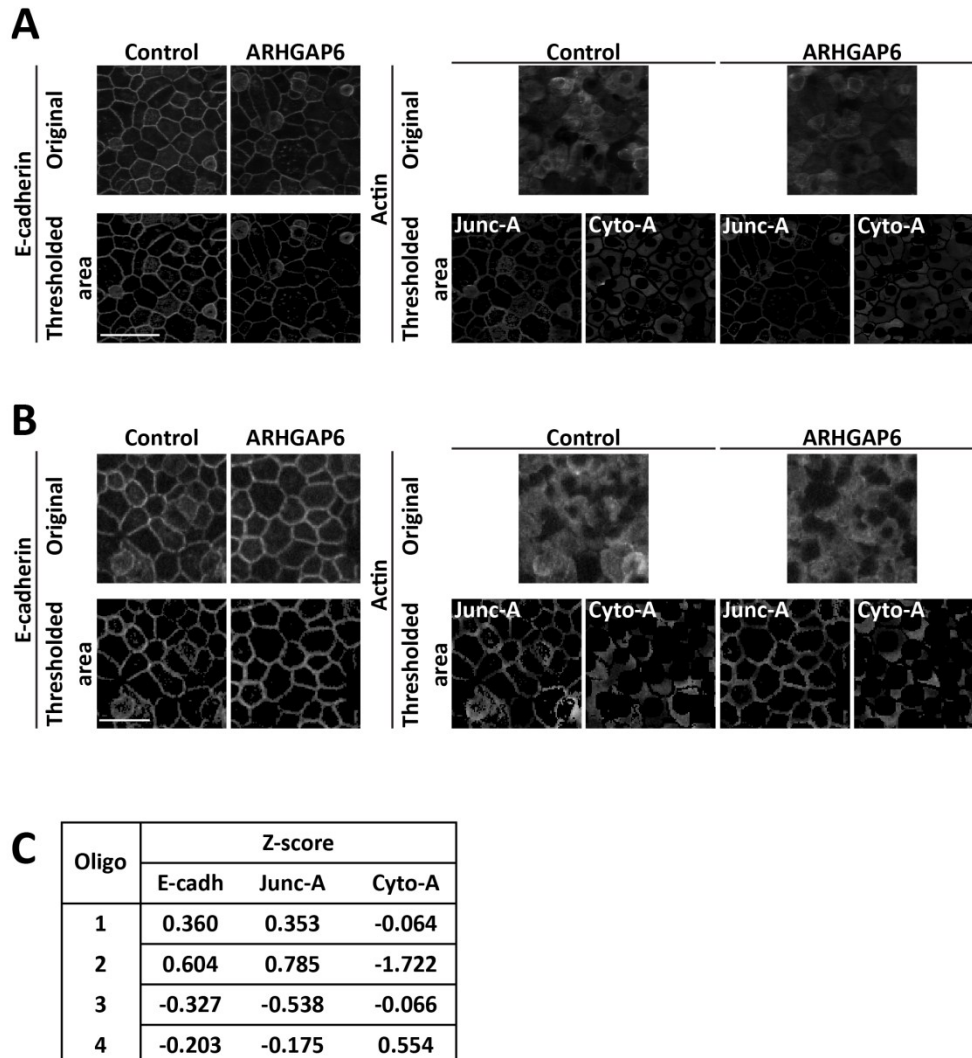


Figure 4.3 ARHGAP6 depletion does not affect cell-cell contact formation Depletion of ARHGAP6 with a pool of four oligonucleotides targeting separate regions of the ARHGAP6 transcript (A) or with individual oligonucleotides (set of representative images from oligonucleotide 3) (B) for 72 hours as part of a siRNA screen. Control images show wells of untransfected cells. Keratinocytes were grown in low calcium medium and confluent monolayers allowed to form junctions for 30 minutes prior to fixation and staining for E-cadherin, F-actin (Original) and the nucleus with DAPI (image not shown). E-cadherin images were processed (described Figure 2.3) for E-cadherin present at the junctions (Thresholded area). Actin images were processed to isolate actin present at the junctions (Junc-A) or in the cytoplasm (Cyto-A). Scale bars represent 50 μ m. C) Table showing the Z-scores for a pool and each ARHGAP6 oligonucleotide for the parameters E-cadherin, junctional actin and cytoplasmic actin (control images Z=0). N=3

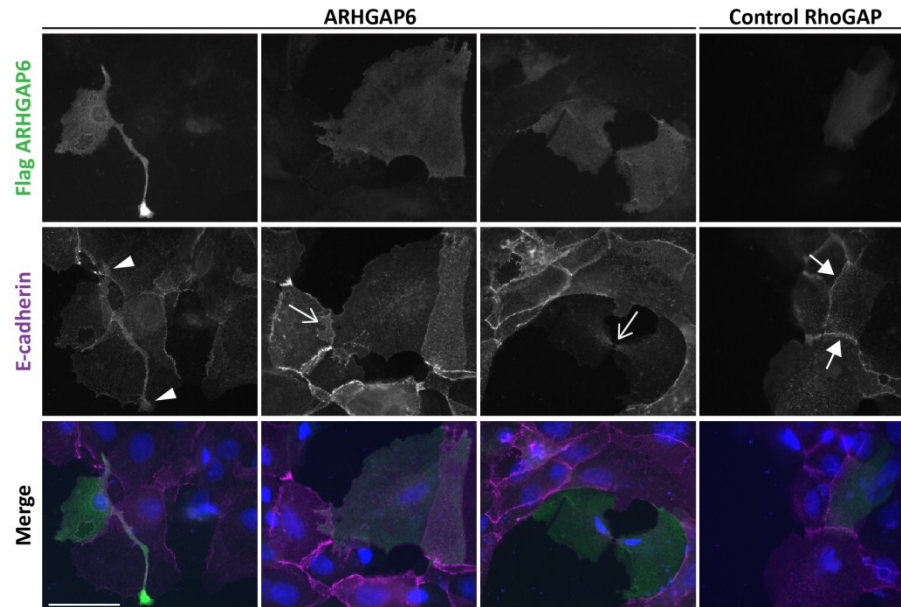


Figure 4.4 Expression of human ARHGAP6 perturbs mature cell-cell contacts Flag-ARHGAP6 or eGFP-ARAP1 (as a control Rho-specific GAP) were expressed in keratinocytes with mature junctions for 8 hours. Cells were fixed and stained for E-cadherin, Flag-tagged exogenous protein and the cell nucleus with DAPI. Images were collected on a widefield microscope. Colour images from each channel were overlaid to create the merged image. Filled arrows indicate junctions with uniform E-cadherin staining similar to E-cadherin in neighbouring, non-expressing control cells. Arrowheads show protrusions and open arrows show discontinuous junctions. Scale bar = 25 μ m. N=3

The *Drosophila* ARHGAP6 homolog is RhoGAP102a of which there exist three isoforms. Each of the isoforms is highly conserved, but only one, RD, retains the entirety of the Rho GAP domain (**Figure 4.5 A**). The Rho GAP domain begins at amino acid 858 in all isoforms, but the last 10 amino acids of the GAP domain are missing from isoforms RC and RE. All isoforms then have a distinct N-terminal region. The expression of these isoforms in *Drosophila* wing indicates that the RD isoform is responsible for enhancing migration and apical extrusion (L A. Baena-Lopez and J-P. Vincent). Furthermore, the RD isoform also alters the localisation of RhoA and results in loss of E-cadherin at the tips of migrating cells (L A. Baena-Lopez and J-P. Vincent). The effects on E-cadherin being consistent with my findings following expression of human ARHGAP6, I tested the expression of the *Drosophila* isoforms in keratinocytes. The RE isoform was not expressed efficiently, but preliminary data suggests that expression of this isoform had no effect on keratinocytes (data not shown). Similar to the data gained from expression in *Drosophila*, expression of the RC isoform had little effect; junctions appeared largely normal (uniform E-cadherin staining) and were disrupted infrequently (**Figure 4.5 B**). However, the RD isoform resulted in about 60% of junctions being disrupted in expressing cells (compared to 20% with the RC) (**Figure 4.5 C**). Qualitatively it appeared that junction disruption seen in the RD expressing cells was quite distinct to that seen upon expression of the RC isoform. Disrupted junctions were classified as being either discontinuous, wavy, or with protrusions (thin, finger-like protrusions extended from the expressing cell). Upon expression of the RD isoform disrupted junctions often had protrusions, whereas this was very uncommon in the RC expressing cells (**Figure 4.5 D**). Thus, a complete Rho GAP domain is necessary to induce the distinct morphological changes associated with ARHGAP6 expression.

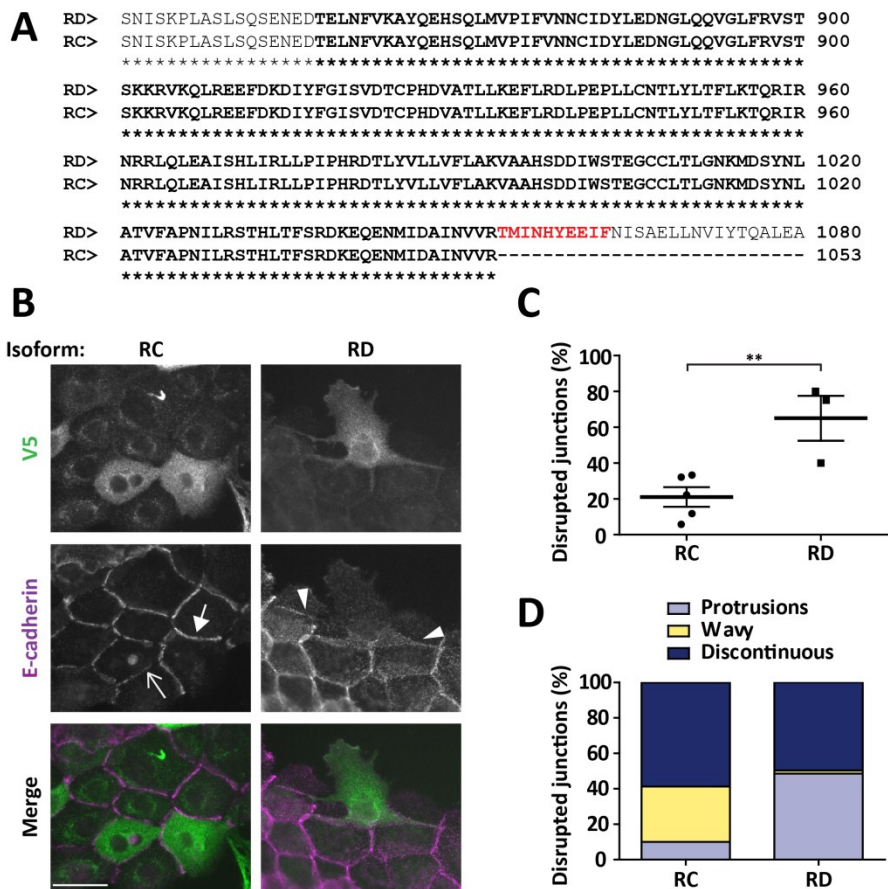


Figure 4.5 The complete Rho GAP domain is required for the *Drosophila* ARHGAP6 homolog to disrupt cell-cell contacts A) A Clustal W alignment was generated of the two isoforms, (RC and RD), of the *Drosophila* ARHGAP6 homolog, RhoGAP102a. Amino acid numbers are displayed on the far right hand side. The predicted GAP domain is highlighted in bold with the last amino acids of the GAP domain (only present in the RD isoform) coloured red. Asterisks (*) represent conserved residues. B) V5-tagged RhoGAP102a isoforms RC or RD were expressed in keratinocytes with mature junctions for 8 hours. A V5 empty vector control should have been expressed simultaneously but this construct was not available at this time, thus no negative control was performed. Expressing cells were only considered if non-expressing neighbours appeared normal. Cells were fixed and stained for E-cadherin and V5-tagged exogenous protein. Colour images from each channel were overlaid to create the merged image. Arrows indicate junctions with uniform E-cadherin staining. Arrowheads indicate protrusions and open arrows indicate discontinuous junctions. Scale bar = 25µm C) The number of junctions disrupted in cells expressing the RC and RD isoforms was quantified and the mean per experiment expressed as a percentage. The line represents the mean. Standard error of the mean is shown. Statistical significance was assessed using the Student's T-test. **, $P \leq 0.001$. D) The disruption induced upon expression of the RC and RD isoforms was scored qualitatively according to morphology (panel C) as either 'with protrusions', 'wavy' or 'discontinuous'. The percentage of disrupted junctions displaying each of these morphologies is shown. N=3 for expression of the RD isoform and N=5 for expression of the RC isoform.

4.3.3. CdGAP regulates AJ maintenance, not formation

CdGAP is a Rac and Cdc42 specific GAP known to regulate cell-matrix adhesion and cell migration (LaLonde et al., 2006; Lamarche-Vane and Hall, 1998; Wormer et al., 2012). I sought to determine if CdGAP was a regulator of AJ formation or maintenance. Depletion of CdGAP via siRNA did not affect the total levels of proteins from the junctional complex or the GTPases Rac1, RhoA or Cdc42 (**Figure 4.6 A**). However, although total cellular levels are unaffected, CdGAP depletion may potentially alter E-cadherin localisation at specific cellular compartments. To address this, CdGAP was depleted in cells maintained in low calcium where cadherins were not engaged in adhesion and junction formation induced for 30 minutes. I then measured the levels of E-cadherin at the membrane using a thresholding-based method (**Figure 4.6 B and C**). All data was normalised to the scrambled oligonucleotide control, which was set arbitrarily at 1. Depletion of CdGAP by two separate oligonucleotides resulted in a slight, but not significant, reduction in E-cadherin levels (0.89 and 0.91 respectively). This indicates that CdGAP does not play a major role in the regulation of junction formation in keratinocytes.

To test if CdGAP could instead regulate the maintenance of cell-cell junctions, I performed aggregation assays (Thoreson and Reynolds, 2002) (**Figure 4.7 A**). Cells are dissociated into a single cell suspension and allowed to form aggregates over two hours. Aggregates are then disrupted mechanically a fixed number of times and the size of the resulting disaggregates measured. The suspension of the aggregates removes any influence of cell-matrix adhesive structures and allows for the measurement of cell-cell contact formation (mediated by AJs or desmosomes) (ability of the cells to aggregate) and for the measurement of the ability of the cells to withstand the mechanical disruption. The depletion of CdGAP (**Figure 4.7 B**) had no effect on the ability of the cells to form aggregates after two hours (**Figure 4.7 C**). However, when disaggregates were measured, depletion of CdGAP resulted in larger sized disaggregates when compared to cells transfected with scrambled oligonucleotides (**Figure 4.7 D**). This finding was significant for one of the oligonucleotides tested. Whilst the other did not show a significant increase, the efficiency of this knockdown was comparatively lower. Thus, the depletion of CdGAP strengthens preformed cell-cell contacts and has no effect on the ability of cells to form junctions.

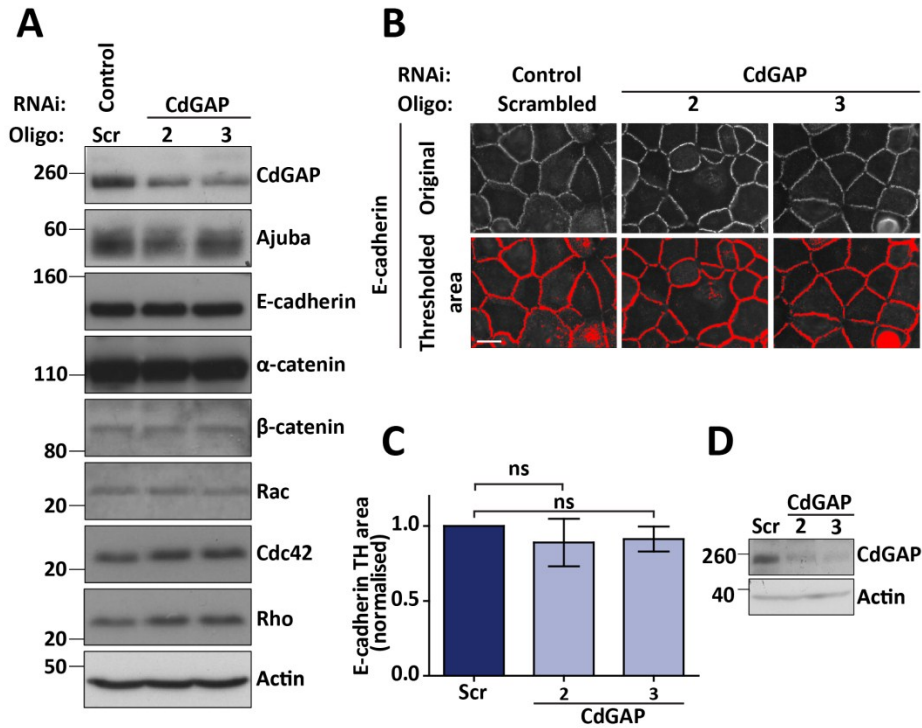


Figure 4.6 CdGAP does not regulate cell-cell contact formation A) Keratinocytes were depleted of CdGAP using two specific oligonucleotides for 48 hours. Cells grown in low calcium medium were lysed and the lysate probed for proteins shown on the right hand side of the panels (molecular weight markers are shown on the left) via Western blot analysis. B) E-cadherin levels at junctions in cells depleted of CdGAP. Confluent monolayers were induced to form junctions for 30 minutes and fixed and stained for E-cadherin (Original). Images were collected from a widefield microscope and processed for E-cadherin levels at the junction (Thresholded area). Scale bar = 25µm. C) The mean percentage of thresholded area E-cadherin for each condition was quantified relative to the control non-targeting oligonucleotide. CdGAP levels analysed by Western blot to confirm depletion (D). Standard error of the mean is shown. Statistical significance was assessed using one-way ANOVA with Bonferroni's multiple comparison test. ns= Not significant, N=3

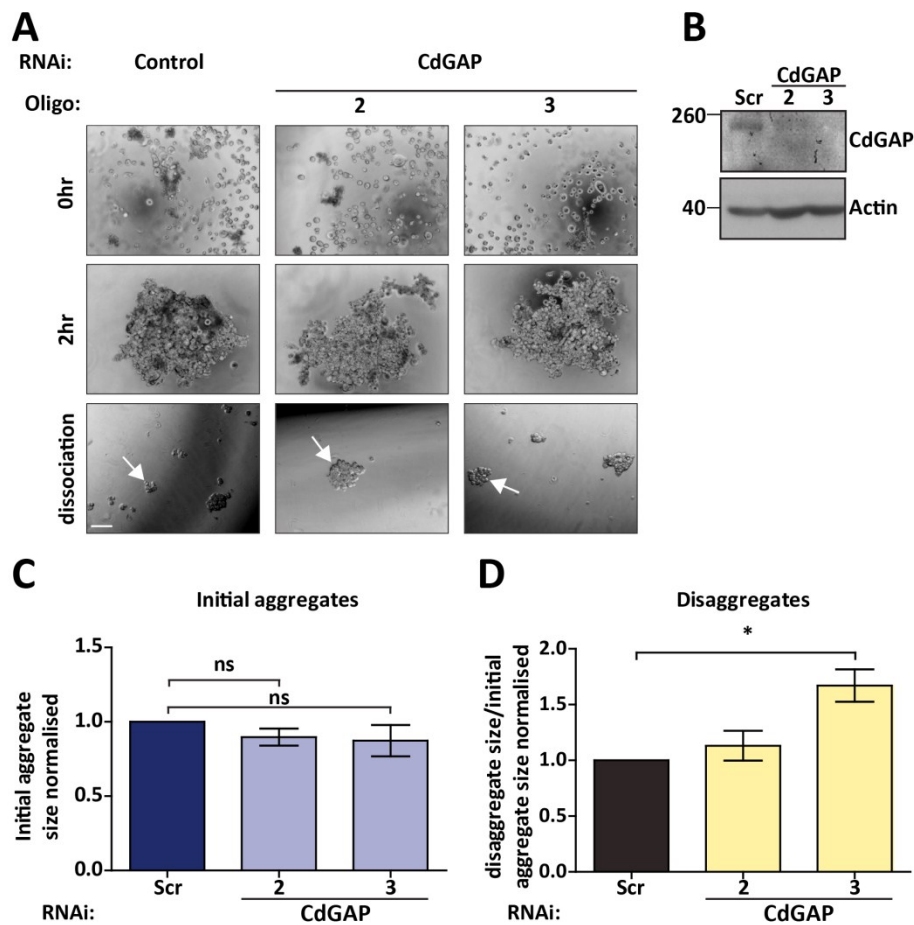


Figure 4.7 CdGAP regulates AJ maintenance CdGAP was depleted using two specific oligonucleotides and aggregation assays performed. A) Confluent monolayers of keratinocytes maintained in low calcium medium were depleted of CdGAP for 48 hours. Cells were detached from tissue culture dishes, dissociated into a single cell suspension and cell suspensions placed into at least six hanging drops in a humid chamber per condition (0hr). Cells were allowed to aggregate for 2 hours in the presence of calcium ions (2hr), dissociated by pipetting and all the resulting disaggregates imaged (dissociation). Images shown are representative of three independent experiments. Scale bar = 100 μ m. B) The depletion of CdGAP was verified by Western blot for every aggregation assay. Actin is used as a loading control. C, D) The area of aggregates formed after two hours (2hr) and disaggregates (dissociation) were measured using ImageJ (Nola et al., 2012). C) Average size of aggregates was expressed relative to control samples. D) The area of all disaggregates (dissociation) was corrected for the size of the initial aggregate (2hr). The average corrected size of disaggregates was expressed relative to control samples (Scr., set as 1). Standard error is shown. Statistical significance was assessed using one-way ANOVA with Bonferroni's multiple comparison test. ns= not significant, *, $P \leq 0.05$. N=3

4.3.4. CdGAP expression disrupts E-cadherin contacts in a GAP-dependent manner

Thus, the above result suggests that CdGAP could be a negative regulator of mature junctions. If this is the case, we would expect that expression of CdGAP in keratinocytes may reduce the amount of E-cadherin at mature junctions, or destabilise the junctions in some way. Two CdGAP isoforms exist. CdGAP-l is the longest at 1425 amino acids whilst CdGAP-s is 820 amino acids long (**Figure 4.8 A**). I microinjected the longest isoform of CdGAP, CdGAP-l, into keratinocytes for different amounts of time and then stained cells for E-cadherin and CdGAP-l (**Figure 4.8 B**). I observed that non-expressing cells maintained continuous E-cadherin along the length of their junctions, while, in contrast, junctions in CdGAP-l-expressing cells lost E-cadherin staining from the corners between neighbouring cells after 4 hours. This effect was similar between two expressing cells, but also between expressing and non-expressing cells. Preliminary data (N=1) suggests that this effect was more evident after longer times of expression, as 24 hours post injection CdGAP-expressing cells developed much larger holes between cells (**Figure 4.8 B**).

To compare the destabilising effect of different isoforms of CdGAP (**Figure 4.8 A**) I transfected CdGAP-l, the shorter CdGAP isoform, CdGAP-s, and the C-terminal fragment, CdGAP₁₁₆₀₋₁₄₂₅ into keratinocytes with mature junctions and allowed them to express for 8 hours (**Figure 4.8 C**). Both CdGAP-l and CdGAP-s produced striking phenotypes; cells appeared with large gaps at junctions between both two expressing cells as well as between expressing cells and their non-expressing neighbours. Expression of the C-terminal alone, which harbours no recognised structural domain, had no effect on morphology. Qualitatively, the CdGAP-s isoform appeared to be more effective at disrupting junctions.

Both CdGAP-l and CdGAP-s are Rac and Cdc42 GAPs, but CdGAP-s is predicted to be more active (Lamarche-Vane and Hall, 1998; Southgate et al., 2011; Tcherkezian et al., 2006). I hypothesised that junction disruption could be a GAP-dependent process, as this would explain the apparent higher level of disruption seen with CdGAP-s and why the C-terminal construct failed to disrupt junctions. GFP-tagged CdGAP-l wild-type and GAP-dead (LaLonde et al., 2006) were transfected into keratinocytes with mature junctions and E-cadherin stained for (**Figure 4.9 A**). CdGAP-l expression resulted in junction disruption as before, but expression of the GAP-dead construct appeared to have little effect on cells and E-cadherin distribution and levels appeared normal.

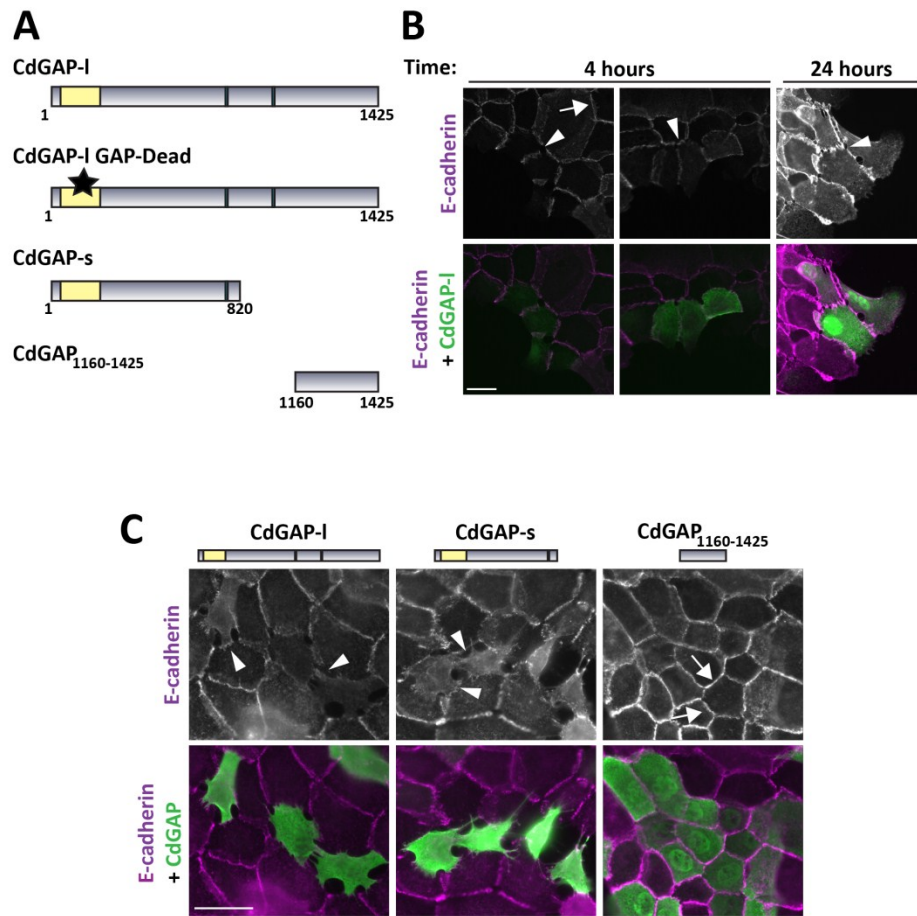


Figure 4.8 CdGAP expression disrupts mature junctions A) cDNA constructs used in this study. All constructs used have either a myc or GFP tag (not shown). The GAP domain is coloured light yellow, proline rich regions are represented as blue boxes. The GAP-dead construct has two mutations in the GAP domain, the catalytic arginine (R56A) and asparagine (N163V) that mean that this protein cannot catalyse the hydrolysis of GTP. Mutations are represented by a star. Amino acid numbers are listed below the protein. B, C). Keratinocytes maintained in standard calcium medium were microinjected with myc-CdGAP-I and allowed to express the exogenous protein for the indicated amounts of time (B) or transfected with myc-CdGAP-I, myc-CdGAP-s or myc-CdGAP₁₁₆₀₋₁₄₂₅ constructs as indicated for 8 hours (C). Cells were fixed and stained for E-cadherin (Cy5, purple) and myc-tagged exogenous protein (Cy2, green) and images collected with a widefield microscope. Colour images from each channel were overlaid to create the merged image. Arrows show junctions with continuous E-cadherin staining, arrowheads show junctions with interrupted E-cadherin staining. Scale bars = 50μm. N=1 for microinjection with expression for 24 hours, N=2 for microinjection with 4 hours expression, N=3 for transfections.

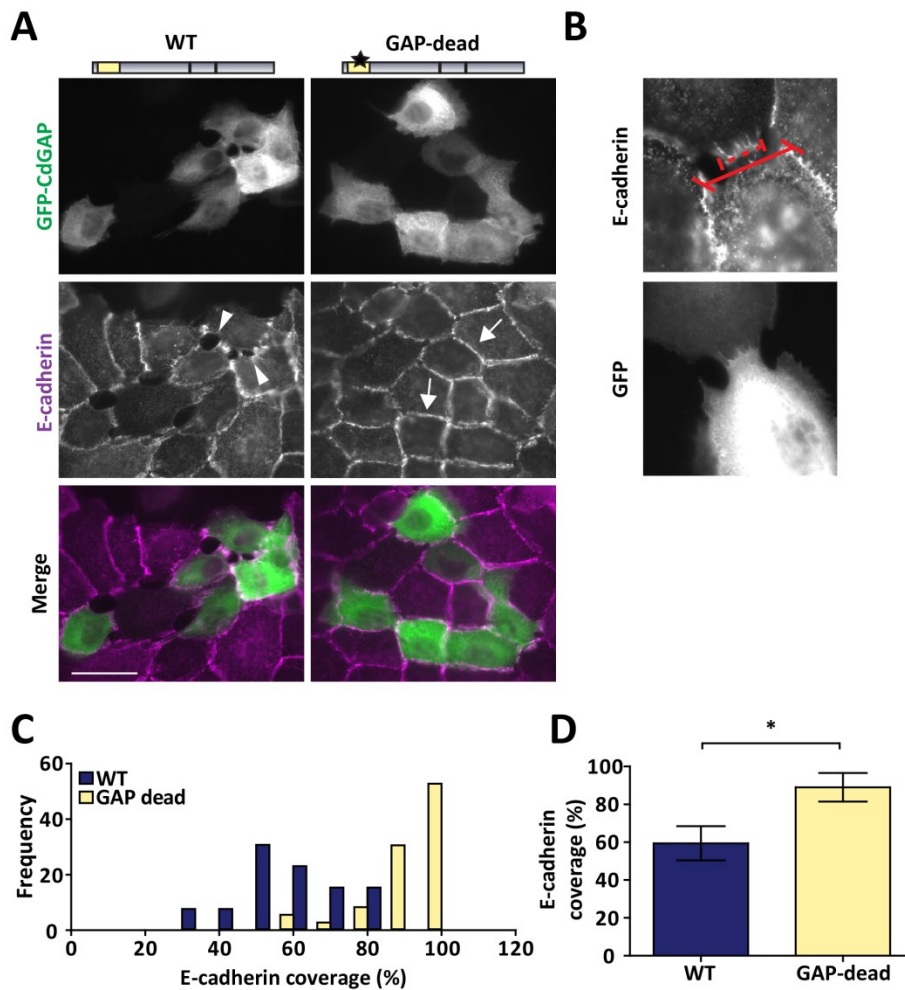


Figure 4.9 CdGAP-mediated junction disruption is dependent on the GAP domain GFP-CdGAP-I wild-type or a GAP-dead CdGAP-I (containing mutations in the GAP domain rendering it inactive) were expressed in keratinocytes with mature junctions. Cells were fixed after 8 hours and stained for E-cadherin. Images were collected from a widefield microscope and colour images from each channel overlaid to create the merged image. Arrows show junctions with continuous E-cadherin staining, arrowheads show junctions with interrupted E-cadherin staining. Scale bar = 50 μ m. B) The amount of E-cadherin covering junctions in CdGAP-expressing cells was quantified by measuring the total length in pixels of a junction (measured corner-corner) (solid line) and taking a second measurement over this area that is covered by E-cadherin (dashed line). 'E-cadherin coverage' was then calculated as E-cadherin (dashed line) divided by total junction length (solid line). C) E-cadherin coverage at junctions from CdGAP wild-type (blue) and GAP-dead (cream)-expressing cells was plotted on histograms (representative histogram from one experiment shown). D) The average E-cadherin coverage at junctions in CdGAP wild-type and GAP-dead-expressing cells. Standard error is shown. Statistical significance was assessed using Student's T-test. *, $P \leq 0.05$. N=3

The amount of E-cadherin present at junctions of CdGAP-expressing cells was quantified by measuring the total length of the junction and then the percentage of this area covered by E-cadherin staining (E-cadherin coverage) (**Figure 4.9 B**). These results were plotted on histograms for each experiment to compare the spread of the data between the conditions (**Figure 4.9 C**). The mean percentage E-cadherin coverage for junctions from CdGAP-I and CdGAP GAP-dead-expressing cells was also plotted (**Figure 4.9 D**). Overall, expression of wild-type CdGAP-I resulted in more junctions with a lower percentage coverage of E-cadherin (average covering 59%), whereas the junctions from GAP-dead CdGAP-I expressing cells were almost completely covered with E-cadherin (89%). Thus, junction disruption induced by CdGAP expression requires a functional GAP domain.

4.3.5. CdGAP is a Rac and Cdc42 GAP in keratinocytes and may regulate Rac at the membrane

CdGAP is a GAP for both Rac and Cdc42 *in vitro* and *in vivo* (Aoki et al., 2004; Lamarche-Vane and Hall, 1998). However, GAP activity will depend on cell type, pathway in which the GAP is activated and the cellular process regulated. Thus, it is necessary to test CdGAP GAP activity in keratinocytes. Effector pull-down assays in cells transfected with CdGAP-I and CdGAP-I GAP-dead were performed to assess global levels of active Rac and Cdc42 (**Figure 4.10 A and B**). Using an empty GFP-vector as a control I found CdGAP-I was able to inactivate both Rac and Cdc42 in keratinocytes, as expression of the wild-type construct reduced total levels of both of these GTPases (**Figure 4.10 A and B**). CdGAP significantly reduced the amount of active Rac. However, although levels of active Cdc42 were reduced, this was not significant, likely due to the degree of variability of this data set. The GAP-dead construct had no effect on the levels of active Rac, but inconclusive effects on active Cdc42. Therefore the phenotype seen following expression of CdGAP could be down to misregulation of either Rac or Cdc42 or both.

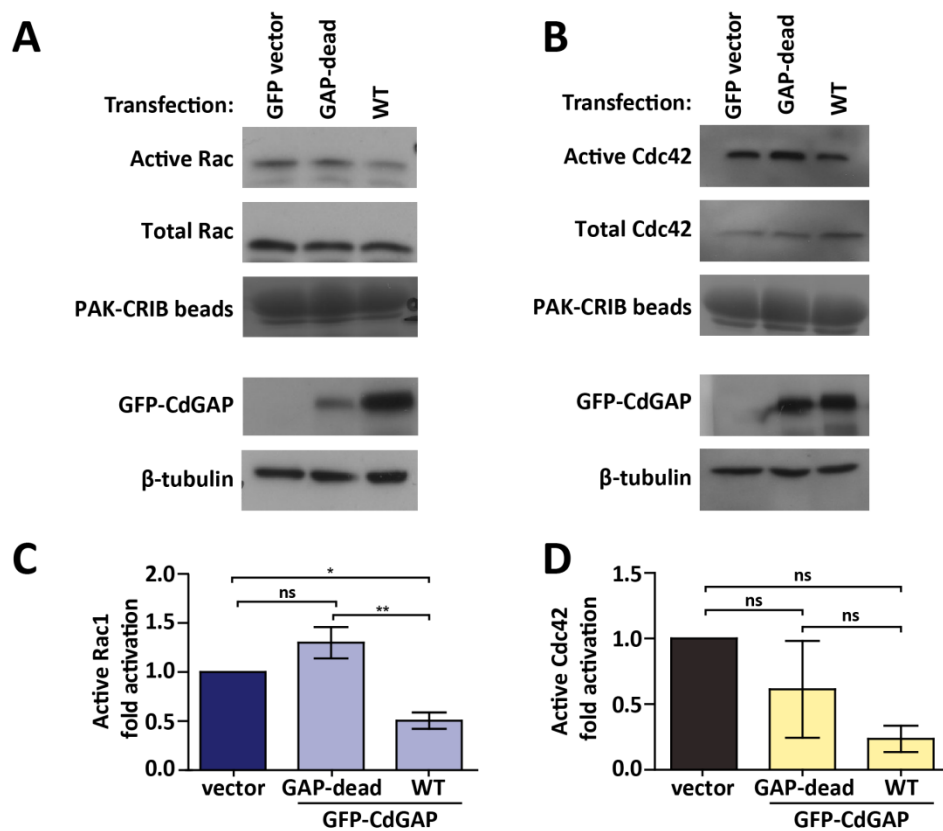


Figure 4.10 CdGAP is a Rac and Cdc42-specific GAP in keratinocytes Keratinocytes were transfected with CdGAP-I wild-type, CdGAP GAP-dead or an empty GFP vector for 24 hours. Cells were lysed and lysates incubated with GST-PAK-CRIB immobilised on glutathione sepharose beads for one hour. Western blot analysis was used to detect active, GTP-bound Rac (A) or Cdc42 (B) bound to beads (active), total Rac or Cdc42 (2% total lysate volume)(total), GFP-tagged CdGAP and β-tubulin. Fold activation of Rac (C) and Cdc42 (D) in the presence of the CdGAP constructs was quantified by dividing the active GTPase by the total GTPase and normalising this value to the GFP-vector control. Statistical significance was assessed using one-way ANOVA with Bonferroni's multiple comparison test. ns= Not significant. *, $P \leq 0.05$, **, $P \leq 0.01$, $N=3$

Whilst effector pull-down assays are useful to assess the ability of a protein to regulate a particular GTPase *in vivo*, no information about the local regulation of GTPase is attained. I optimised the expression of a unimolecular probe in keratinocytes that allowed for the measure of localised activation of Rac through FRET (Makrogianneli et al., 2009). This probe contains the CRIB domain of PAK1 together with Rac1 and is fused to GFP at the C-terminal and RFP at the N-terminal. We took advantage of FLIM to measure the change in the lifetime of this probe in the presence of CdGAP (**Figure 4.11**) (this work was done in collaboration with Doug Kelly and Sunil Kumar, Imperial College London). In FLIM, only the lifetime of the donor probe (GFP) is measured, which means there is no issue with spectral bleed-through, fluorophore concentration, or photobleaching. In its inactive state Rac remains unbound to PAK and there is no FRET between GFP and RFP. Consequently, the lifetime of the GFP signal is longer. Upon Rac activation Rac1 binds PAK, bringing the GFP and RFP-tagged terminals of the probe into close proximity and facilitating FRET. This can be detected as a reduction in the lifetime of GFP, as energy is transferred to the close-by RFP.

The expression of myc-tagged CdGAP-I and the Raichu probe was optimised on 96-well plates. Cells expressing both the Raichu probe and CdGAP-I (or vector control) were then manually segmented (**Figure 4.11 A**) and FLIM calculated in the whole cell or exclusively at the membranes (**Figure 4.11 B and C**) (lifetime measurements were computed by D. Kelly). The expression of CdGAP resulted in an overall increase in the lifetime of GFP, reflecting a decrease in active Rac both in whole cells and specifically at the membrane (**Figure 4.11 B and C**). This data corroborates biochemical data gathered from PAK-CRIB pull-downs that also show a global decrease in Rac activity in CdGAP-expressing cells. However, it also demonstrates that CdGAP expression causes perturbation in Rac activation at the membrane, strengthening the hypothesis that CdGAP-induced junction disruption is Rac-dependent.

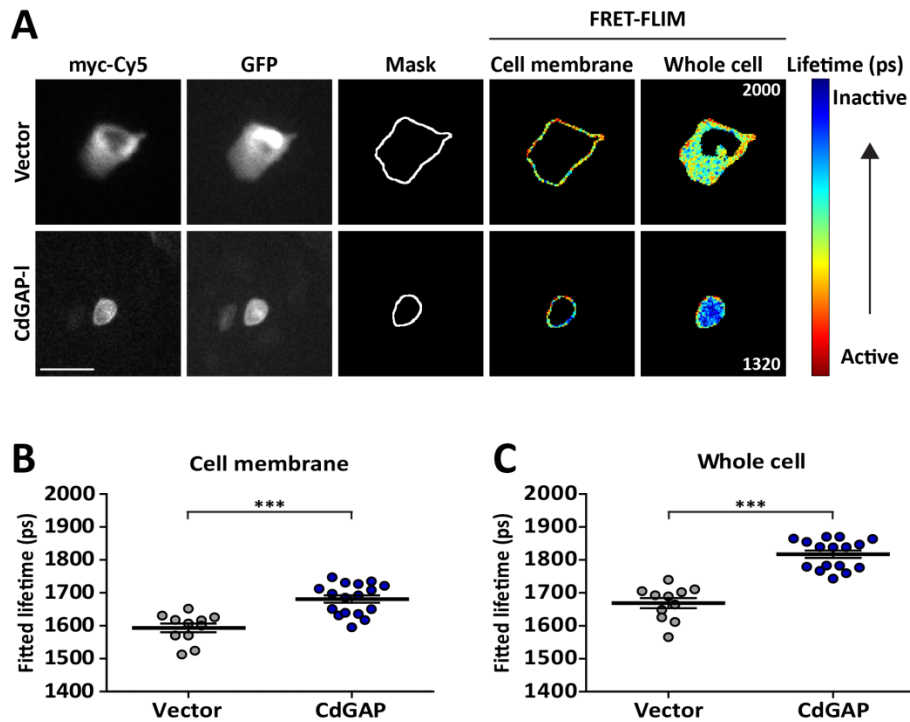


Figure 4.11 CdGAP expression inactivates Rac specifically at the membrane A) Myc- CdGAP-I or empty vector containing a myc tag were expressed in keratinocytes together with a dual mRFP-PAK-Rac1-GFP probe for 24 hours. Cells were fixed and stained for exogenous myc-tagged protein with a Cy5-tagged secondary antibody. Cells were imaged for Cy5 and GFP. Fluorescence lifetime of GFP was measured and lifetime data analysed using in-house software 'FLIMFit' (Lifetime analysis performed by D. Kelly, Imperial College London). Cells were segmented manually using a custom-designed program (CellSegmenter) based on the GFP channel. The membrane was then defined based on this outline. Fluorescence lifetimes of GFP are shown in false colours; red represents low lifetime indicating active Rac and blue represents high lifetime indicating inactive Rac. Scale bar represents 50µm. FLIM was calculated at segmented membranes (B) and in the whole of the cell (C). Standard error of the mean is shown. Significance was assessed using Student's T-test. ***, $P \leq 0.001$. N=16 for CdGAP-expressing cells and N=11 for Vector-expressing cells in one experiment.

4.4. Conclusions

In this chapter, I investigated the roles of different Rho GAPs in the related processes of cell-cell contact formation and junction maintenance. I have identified three different GAPs responsible for regulating the maintenance of cell-cell contacts in keratinocytes.

ARAP1 is the only positive regulator I have identified, seemingly acting at the very early stages of contact stabilisation. I have shown that its depletion reduces levels of E-cadherin at newly formed cell-cell junctions. How ARAP1 is mediating this effect is unclear, but may depend on its regulation of Rho or Arf GTPases.

In contrast, the Rho GAP ARHGAP6 is a negative regulator of junction maintenance. Depletion of ARHGAP6 had no effect on E-cadherin or F-actin during cell-cell contact formation (siRNA screen data). However, the expression of human ARHGAP6 as well as the *Drosophila* homolog RhoGAP102a containing the full GAP domain, had profound effects on cellular morphology, causing cells to generate long protrusions. The purpose of these structures is not yet clear, but evidence from *Drosophila* suggests they may be required for migration.

Finally, I have shown that CdGAP is a regulator of cell-cell contact maintenance, and not assembly. I have shown that CdGAP expression specifically removes E-cadherin from the junctions and leads to significant morphological changes in expressing cells, most likely associated with actin remodelling. Conversely, the depletion of CdGAP strengthens junctions, allowing them to more readily withstand mechanical disruption. I have also shown that CdGAP-induced junction disruption is mediated via its GAP domain, and expression perturbs Cdc42 and Rac signalling both globally and (in the case of Rac) locally at the cell membrane. CdGAP has previously been implicated in the development of the ectoderm and limb bud and my findings compliment earlier studies on the function of CdGAP, suggesting that regulation of cell-cell, as well as cell-matrix adhesions could be important for development and may be hijacked in the case of epithelial cancers.

Overall, I have identified three different Rho GAPs that all regulate the maintenance of cell-cell contacts. Interestingly, these all appear to operate at different times and use different mechanisms to exert their effects on junctions. Whether or not any of these proteins can work together or not to regulate the stabilisation of cell-cell contacts remains to be seen. But, it is interesting to speculate that the activity of ARAP1 could be balanced by CdGAP or ARHGAP6 in order to sustain a highly dynamic adhesive system, able to rapidly respond to external cues. Each of these GAPs harbours

structurally diverse domains, important for mediating protein-protein or protein-lipid interactions and allowing them to interact with a unique subset of proteins. Indeed, the fact that two Rho-specific GAPs, ARAP1 and ARHGAP6 can have such diverse effects on cellular architecture illustrates that Rho GAPs are not simply required to inactivate GTPases, but also provide a vital scaffold for the direction of specific cellular processes. Together, this data highlights the vastly complex systems underlying cell-cell contact homeostasis that exist.

4.5. Discussion

4.5.1. ARAP1 as a positive regulator of the early stages of cell-cell contact stabilisation

The depletion of ARAP1 in keratinocytes results in a significant decrease in the amount of E-cadherin present at cell-cell contacts following a 30 minute calcium switch (**Figure 4.1 D**). This is in contrast to the phenotype seen in the siRNA screen, in which ARAP1 depletion increased the amount of E-cadherin at the membrane. This finding was unexpected but may be due to off target effects of the original oligonucleotides. Indeed, three of the four original oligonucleotides were not effective in consistently depleting ARAP1 levels (**Figure 4.1 B**).

The depletion of ARAP1 does not prevent the formation of cell-cell contacts; rather, E-cadherin levels at the junctions are significantly reduced compared to control cells. Potentially ARAP1 is required to regulate early stabilising processes, such as contact expansion, the recruitment of cadherin, or actin polymerisation. As a RhoA, Arf1 and Arf5 GAP (Miura et al., 2002), ARAP1 may be required to influence the activity of any of these GTPases during such stabilisation processes.

We know that Rho activity is necessary for junction formation to occur in keratinocytes; inhibition of RhoA, B or C via treatment with C3 transferase inhibits formation (Braga et al., 1997). Furthermore, in MDCK cells Rho seems to be required to drive actomyosin contractility via ROCK during cell-cell contact formation, which promotes contact expansion (Yamada and Nelson, 2007). However, Rho activity is carefully localised during this process as MDCK cells exhibit high RhoA activity at the edges of contacts but low RhoA activity at the actual sites of cell-cell contact (Yamada and Nelson, 2007). Moreover, Rho activity can also drive contact disassembly through ROCK in HTC116 epithelial tumour cells with mature junctions (Sahai and Marshall, 2002b). Thus Rho activity must be spatially constrained in order for junction formation to proceed.

Indeed the depletion of another Rho-specific GAP, Myo9A, inhibits contact expansion in bronchial epithelial cells (Omelchenko and Hall, 2012). In this case, initial contacts can form, but these are never stabilised and eventually disperse. Although this phenotype is partially attributed to Myo9A's myosin-binding head domain, FRET analysis confirms that Myo9A depletion specifically increases Rho activation at sites of contact (Omelchenko and Hall, 2012). Furthermore, treatment of Myo9A-depleted cells with Y-27632 to inhibit ROCK partially rescues the defective contact expansion phenotype, and cells are able to maintain some contact, no longer pulling away from one another (Omelchenko and Hall, 2012).

Data from the lab has previously shown that EGFR contributes to activate Rac downstream of cadherin adhesion (Betson et al., 2002). Although Rac activation is reduced when EGFR is inhibited, junctions can still form. ARAP1 siRNA hastens the degradation of EGFR via interaction with Cbl-interacting protein of 85 kDa (Yoon et al., 2011; Yoon et al., 2008). As such, the depletion of ARAP1 may indirectly result in a reduction in Rac activation downstream of cadherin adhesion. As Rac activation is required for cadherin recruitment and stabilisation at contacts this may explain the reduction in E-cadherin we see, as without full activation of Rac, E-cadherin is not stabilised at the membrane.

Alternatively, ARAP1 may regulate contact stabilisation via its regulation of Arf1 or Arf5. The reduction of E-cadherin at the junctions seen upon ARAP1 depletion may reflect a net reduction in the amount of cadherin delivered to, or an increase in the internalisation of cadherin from the plasma membrane, processes regulated by Arf GTPases. Interestingly, depletion of the *Drosophila* homolog of Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1 (GBF1), an Arf1 GEF, inhibits transport of DE-cadherin to the plasma membrane in *Drosophila* salivary gland (Szul et al., 2011). This finding indicates disruption of Arf1 activity can perturb cadherin-mediated adhesion. The presence of Arf1-specific GAPs is vital in regulating trafficking, as without GTP hydrolysis of Arf1, cargo packaging and coat disassembly are perturbed (Lanoix et al., 1999). Indeed, both the depletion and overexpression of Arf1-GAP ASAP1 reduces the localisation of the cell-matrix adhesion protein paxillin to focal adhesions (Liu et al., 2002; Liu et al., 2005). Thus any disruption in dynamic Arf1 cycling can impact upon cell-matrix adhesive structures. Though less well studied, Arf5 can also regulate adhesive complexes as depletion inhibits internalisation of β 1-integrin in HeLa cells, leading to increased cell attachment and spreading on fibronectin (Moravec et al., 2012). Potentially similar mechanisms may be deployed by ARAP1 to regulate E-cadherin at cell-cell adhesions in keratinocytes.

4.5.2. ARHGAP6 negatively regulates cell-cell adhesion

Expression of both human and the longest *Drosophila* isoform of ARHGAP6 in keratinocytes disrupt mature E-cadherin contacts and induce the formation of protrusions (**Figures 4.4 and 4.5**). This is in agreement with data gathered from expression of ARHGAP6 and RhoGAP102a in *Drosophila* wing, which leads to alterations in cellular morphology, enhanced migration and disrupted Rho signalling (A. Baena-Lopez and J-P. Vincent).

Previous studies of ARHGAP6 have identified GAP-independent functions for this protein, including regulation of actin polymerisation (Prakash et al., 2000), PLC δ activation (Ochocka et al., 2008) and modulation of human ether-a-go-go gene (a potassium channel) current (Potet et al., 2009). Furthermore, part of the ARHGAP6 gene is deleted in patients with MLS (Schaefer et al., 1997). However, mice with deletion of the Rho GAP domain displayed normal phenotypes, suggesting ARHGAP6 GAP activity does not contribute to the MLS phenotype (Prakash et al., 2000). Thus, the role of the Rho GAP domain of ARHGAP6 *in vivo* is not clear.

Interestingly, the complete GAP domain is required for the formation of protrusions in keratinocytes and to enhance migration in *Drosophila* wing. The expression of human ARHGAP6 and the *Drosophila* RD isoform in keratinocytes induced the formation of protrusions; potentially these lamellipodial-like structures represent a necessary step in migration. Indeed a mesenchymal mode of migration is associated with upregulation of Rac and inhibition of Rho in melanoma cells (Sanz-Moreno et al., 2008) and expression of the RD isoform causes mislocalisation of active Rho in *Drosophila* wing. The finding that both human and *Drosophila* forms of the protein behave similarly in both keratinocytes (**Figure 4.4 and 4.5**) and *Drosophila* tissues (A. Baena-Lopez and J-P. Vincent) suggests an important evolutionarily conserved function.

4.5.3. CdGAP can act as a positive regulator of epithelial-to-mesenchymal transition (potentially in a developmental process)?

The expression of CdGAP in keratinocytes induces the loss of E-cadherin contacts and potentially extrusion from the monolayer without seeming to induce apoptosis (nuclear staining shows no abnormalities). This is accompanied by significant morphological changes, most likely a result of actin remodelling, that leads to the expressing cell appearing to retract from its neighbours. Potentially this could represent an early stage in epithelial-to-mesenchymal transition, as cells

expressing CdGAP are reminiscent of epithelial monolayers stimulated to scatter with HGF (de Rooij et al., 2005).

Epithelial-to-mesenchymal transition requires close coordination between cell-cell and cell-matrix adhesions and CdGAP has previously been implicated in regulating cell-matrix adhesions (LaLonde et al., 2006; Wormer et al., 2012). However, my data suggests a specific role for CdGAP at AJs because 1) depletion of CdGAP results in strengthening of cell-cell adhesion in aggregation assays (although an effect on desmosomes here cannot be excluded) (**Figure 4.7**), and 2) expression of CdGAP results in the rapid loss of E-cadherin from cell-cell contacts (**Figure 4.8**). I propose that the expression of CdGAP can promote loss of cell-cell adhesion and potentially lead to migration, whilst downregulation of CdGAP strengthens contacts.

Following loss of cell-cell contacts cells undergoing epithelial-to-mesenchymal transition are stimulated to migrate to distant sites. Interestingly, CdGAP has previously been linked to migration, but seems to have cell-type specific roles in this process (Southgate et al., 2011; Wormer et al., 2012). For example, in U2OS cells CdGAP depletion promotes migration in wound healing assays (Wormer et al., 2012), but in primary dermal fibroblasts expression of a truncated form of CdGAP enhances speed of migration in wound healing assays. The morphological changes I see upon CdGAP expression include retraction from neighbouring cells and preliminary data (not shown) suggests changes in actin reorganisation. These may reflect a switch to a more migratory phenotype, as the generation of actin-based structures such as lamellipodia and filopodia can be vital in promoting migration in some cell types.

The ability of CdGAP to regulate both cell-cell adhesion and migration may contribute to phenotypes observed in other CdGAP-regulated processes such as in development, cell scattering and metastasis (He et al., 2011; Southgate et al., 2011; Togawa et al., 2010). Mutations in CdGAP identified as a causative factor in the developmental disorder AOS indicate that CdGAP is an important regulator of the development of the ectoderm and limb buds (Southgate et al., 2011). Studies using conditional knock-outs have revealed Rac1 and Cdc42 to play an important role in the normal development of the limb bud and in interdigital programmed cell death (Aizawa et al., 2012; Suzuki et al., 2009; Wu et al., 2008). Interestingly, truncating mutants (1-683 and 1-1087) of CdGAP causing AOS have enhanced activity on Cdc42 (although Rac was not tested) compared to the wild-type protein. Furthermore, one mutant (1-683) migrates more quickly than wild-type controls, suggesting that the AOS phenotype can be caused by disrupted Cdc42 (or Rac) cycling. However, CdGAP expression, as well as regulating migration, may be required for the disassembly of E-cadherin cell-cell contacts

prior to migration. This is supported by the fact that expression of either CdGAP-I or CdGAP-s (which should behave in a similar way to the truncating mutants) results in the dismantling of E-cadherin mediated contacts in keratinocytes. Furthermore, CdGAP-I expression in keratinocytes is accompanied by a global and membrane-specific decrease in the levels of active Rac, indicating that Rac inactivation may be necessary for cell-cell contact disassembly to occur. Possibly, CdGAP regulates the development of the ectoderm and limb buds by regulating both cell-cell contact disassembly and migration via regulation of Rac/Cdc42. Its early expression may be required to confine Rac/Cdc42 signalling necessary for the development of the growing limb. However, the constitutive activation or overexpression of CdGAP leads to unregulated Rac/Cdc42 signalling and disrupts this finely balanced process.

CdGAP regulates several processes where coordination between cell-cell and cell-matrix adhesions is vital. CdGAP function on cell-cell contacts may be either cell-type specific, or regulated by stimulus. That a particular feature in AOS patients is loss of patches of skin, particularly the scalp, and my data from keratinocytes shows CdGAP-induced disassembly of contacts suggests that at least in skin cells hyperactivation of CdGAP is likely to have a negative effect on cell-cell adhesion. However, CdGAP can inhibit HGF-induced cell scattering in MDCK cells by inactivating Cdc42. Stimulation of cells with HGF results in inactivation of CdGAP via phosphorylation on Threonine 776 by ERK, causing cell-cell contacts to be dismantled and cells to scatter (Tcherkezian et al., 2005; Togawa et al., 2010).

An early stage in metastasis involves the downregulation of E-cadherin (Berx and van Roy, 2009). As the expression of CdGAP provokes loss of E-cadherin from contacts without apparent induction of apoptosis, this may reflect a role for CdGAP in cancer progression. Whilst several examples exist of Rho GAPs acting as tumour suppressors, there are very few examples of Rho GAPs upregulated in tumours (Johnstone et al., 2004; Lahoz and Hall, 2012; Liao and Lo, 2008; Wang et al., 1997; Yuan et al., 1995). However, CdGAP is required for TGF- β -induced migration and invasion in mammary tumour explants expressing an activated form of ErbB-2 receptor (although in these cells the GAP domain of CdGAP is not required) revealing that Rho GAPs can also act as positive regulators of cancer cell migration and invasion (He et al., 2011). Intriguingly, the depletion of CdGAP in these cells leads to an increase in E-cadherin levels, indicating that CdGAP could regulate E-cadherin levels. My data is supportive of this link as expression of CdGAP in keratinocytes reduces E-cadherin at junctions. However, CdGAP-mediated junction disruption in keratinocytes does require the GAP domain, suggesting that, at least in keratinocytes, CdGAP expression could act as a positive regulator of migration and/or invasion via the regulation of Rac or Cdc42. One interesting possibility is that following the loss of E-cadherin, CdGAP promotes migration via GAP-independent mechanisms (as

the GAP domain is not required for TGF- β -induced migration in breast epithelial cells). A number of proteins have been identified to interact with CdGAP, including the atypical GTPase Wrch1 (Naji et al., 2011) and these may offer insight into GAP-independent, CdGAP-mediated processes involved in cell migration.

4.5.4. CdGAP regulation of Rac, Rho (?) and Cdc42

The expression of CdGAP in keratinocytes correlates with a global decrease in Rac and Cdc42 activity and a specific reduction in active Rac at the membrane (**Figures 4.10 and 4.11**). The activation of GTPases is regulated both spatially and temporally meaning that a global decrease in the activity of a GTPase does not necessarily mean this is decreased at all cellular locations. For example, depletion of the Cdc42 GAP SH3BP1 leads to an increase in total Cdc42, but a decrease in Cdc42 at cell-cell junctions, which is where SH3BP1 exerts its effects (Elbediwy et al., 2012). Whilst we see a decrease in Rac activation at the membrane in CdGAP-expressing cells, there may be a localised increase in Rac or Cdc42 activity associated with the lamellipodial-like structures seen (as lamellipodia formation is driven by Rac).

Furthermore, the complex nature of the regulatory networks governing GTPase activation means that inactivation of one GTPase can result in the activation of another (Nimnual et al., 2003; Ratheesh et al., 2012; Warner and Longmore, 2009). By reducing the levels of active Rac at AJs CdGAP may be indirectly releasing suppression of RhoA activity. RhoA activity can be regulated at AJs downstream of Rac, which promotes the localisation of the Rho GAP p190A to junctions (Ratheesh et al., 2012; Wildenberg et al., 2006). Cdc42 has also been described as being able to antagonise Rho signalling at cell-cell contacts (Warner and Longmore, 2009). In *Drosophila* eye, Cdc42 inhibits RhoA in order to reduce apical tension. Loss of Cdc42 increases phosphorylation of MLC at AJs and this increases cellular tension (Warner and Longmore, 2009). The activation of RhoA is interesting as TGF- β -induced epithelial-to-mesenchymal transition has been described to signal through Rho and ROCK and brings about loss of E-cadherin and actin remodelling (Bhowmick et al., 2001). Thus, a more thorough analysis of localised GTPase activation in CdGAP-expressing cells is necessary.

4.6. Future work

4.6.1. Regulation of cell-cell contacts by ARAP1

ARAP1 depletion resulted in a significant reduction in the levels of E-cadherin at cell-cell contacts. However, expression of ARAP1 had no effect on mature AJs. Together these findings indicate ARAP1 regulates the early stages of cell-cell contact stabilisation. Immediate future work should address two broad questions. Firstly, as ARAP1 inactivates both Rho and Arf GTPases, through which GTPase(s) (if any) is ARAP1 regulating cell-cell contacts? Secondly, how does depletion of ARAP1 lead to reduction in E-cadherin at cell-cell contacts? For example, this may be through an increase in the rate of removal of E-cadherin from contacts, or a decrease in the rate of delivery.

Does ARAP1 influence the levels of E-cadherin at the junctions via regulation of Arf or Rho GTPases?

ARAP1 can regulate both Rho and Arf signalling. As important regulators of the cytoskeleton and intracellular trafficking, perturbation of Rho or Arf activation could disrupt AJs. Thus, to investigate the nature of E-cadherin loss in ARAP1-depleted cells it would first be informative to determine the involvement (if any) of these GTPases in this process. To determine Rho activity at the junctions FLIM-FRET can be used, taking advantage of a dual probe harbouring the binding domain of the Rho target, Rhotekin, together with RhoA and fused at either terminal to RFP and GFP (Heasman et al., 2010). Arf1 activation can also be tracked using FRET by measuring FRET between activated Arf1 (bound to CFP) and the N-terminus of its target Golgi-localised, gamma ear-containing, ARF-binding protein 1 fused to YFP (Beemiller et al., 2006). Such data will provide vital information on the localised regulation of Arf and Rho in ARAP1-depleted cells. A complementary approach could involve the expression of the dominant-negative forms of RhoA, Arf1 and Arf5 in cells depleted of ARAP1 to see if either could rescue the decrease in E-cadherin levels at cell-cell contacts. However, the levels of expression of these proteins would have to be carefully titrated to avoid toxic, off-target effects. By determining which GTPase(s) ARAP1 regulates during cell-cell contact stabilisation, we will be better able to investigate the mechanism behind the reduction of E-cadherin at cell-cell contacts upon ARAP1 depletion.

What is the mechanism behind the reduction of E-cadherin induced by ARAP1 depletion?

The reduction in E-cadherin at junctions in the absence of ARAP1 could reflect changes in the amount of E-cadherin delivered to the membrane after synthesis, the rate of recycling back to the membrane, or the rate of removal from the membrane (Bryant and Stow, 2004). To uncouple these

processes will be vital in uncovering ARAP1 function at AJs. Levels of E-cadherin on the surface and the rate of internalisation can be determined by performing recycling assays using cell surface biotinylation (Le et al., 1999). Following biotinylation of the cell surface, any remaining, unbound biotin is removed and cells either lysed to measure surface levels or incubated for a further time at 37°C before lysis. Biotinylated protein detected in lysates from cells lysed immediately after incubation tells us of the amount of E-cadherin present on the surface, whilst biotinylated protein derived from lysates after incubation tell us of the amount of E-cadherin that has been internalised. By lysing cells at different time points the rate of internalisation and recycling can be inferred. These can be compared in ARAP1-depleted and control cells.

Alternatively, FRAP can be used to determine the dynamics of E-cadherin at the membrane. In this method GFP-tagged E-cadherin (Adams et al., 1998) is transfected into cells and these cells imaged live. A small subset of fluorescent E-cadherin is photobleached and by monitoring the fluorescence recovery in this area two things may be inferred. Firstly, the percentage recovery is calculated, that is how much fluorescence returns to the site after bleaching. This can indicate how large the proportion of the mobile fraction of GFP-E-cadherin is. Secondly, the rate of recovery, how quickly the fluorescence returns to the original intensity, tells us how mobile the fraction of GFP-E-cadherin is. Comparison of FRAP data from cells depleted of ARAP1 with control cells can tell us if ARAP1 depletion perturbs the recovery of E-cadherin. It would also be possible to couple this analysis with investigation of ARAP1 GTPase targets, for example, it would be interesting to see if expression of activated Arf1, Arf5 or RhoA rescued any defects seen in FRAP caused by depletion of ARAP1.

4.6.2. Regulation of cell-cell adhesion by ARHGAP6

ARHGAP6 expression in keratinocytes led to distinct morphological changes in expressing cells that meant cell-cell contacts were disrupted. The effects were conditional on the presence of a complete GAP domain and were seen upon expression of both human and *Drosophila* ARHGAP6. ARHGAP6 also induced morphological changes upon expression in *Drosophila* wing, and affected migration and localised Rho activation. The investigation into ARHGAP6 and cell-cell adhesion should firstly focus on confirming in keratinocytes the phenotypes seen in *Drosophila* wing and should address whether ARHGAP6 expression can increase migration in keratinocytes and if this is due to ARHGAP6 regulation of RhoA.

Does ARHGAP6 expression increase migration?

Data from *Drosophila* wing and keratinocytes suggests that expression of ARHGAP6 can promote migration. In *Drosophila*, ARHGAP6-expressing cells are more migratory and in keratinocytes, E-cadherin is lost and cells often display protrusions, which may be consistent with a migratory phenotype. It would be interesting to determine if ARHGAP6 could enhance cell migration by performing migration assays with ARHGAP6-expressing cells. For example, wound healing assays could be performed with ARHGAP6-expressing cells to see if these are able to close wounds more rapidly than control cells. Another method to investigate the effect of ARHGAP6 expression on migration involves the tracking of single cells over time. Advanced algorithms are now available that facilitate the automation of this process. By tracking individual cells it will allow us to measure the rate of migration as well as providing morphological data on the studied cells, for example if these appear mesenchymal or amoeboid (Jaqaman et al., 2008; Sanz-Moreno et al., 2008; Wilkinson et al., 2005).

Is localised Rho activation disrupted in ARHGAP6 expressing cells?

Modes of migration are influenced by the activity of RhoA and Rac1. In *Drosophila* wing ARHGAP6 results in Rho activity being lost apically and building up basally. This is correlated with overall morphological changes and enhanced migration. As ARHGAP6 is a Rho GAP it would be important to determine if Rho activity is perturbed in ARHGAP6-expressing keratinocytes. To do this, pull-down assays can be performed taking advantage of the binding between active RhoA and Rhotekin. Immobilisation of Rhotekin Rho binding domain (RBD) on glutathione beads allows active RhoA to be isolated from cells transfected with or depleted of ARHGAP6 in order to assess the global changes in RhoA activity. To look more specifically at RhoA signalling in keratinocytes expressing ARHGAP6 FLIM-FRET can be performed utilising the dual RhoA-RBD probe fused to GFP and RFP at each terminal which allows information regarding the localised activation of RhoA to be determined based on the lifetime of GFP.

4.6.3 Regulation of cell-cell adhesion by CdGAP

CdGAP expression resulted in loss of E-cadherin from cell-cell contacts and significant morphological changes. This was associated with global changes in the activity of Cdc42 and Rac, and local changes in Rac activity at the membrane. Depletion had no effect on the levels of E-cadherin at junctions during cell-cell contact formation indicating CdGAP primarily regulates the maintenance of cell-cell contacts, and may be important in the regulation of developmental processes, particularly epithelial-to-mesenchymal transitions. Future work should firstly address how CdGAP expression results in AJ

disassembly. This should be achieved by further investigating the activation of different GTPases at cell-cell contacts in CdGAP-expressing cells and ascertaining what effect this has on the actin cytoskeleton. Secondly, whether or not CdGAP is inducing epithelial-to-mesenchymal transition in keratinocytes should be confirmed.

How does CdGAP expression disrupt cell-cell contacts?

FLIM-FRET data finds CdGAP-expression significantly decreases Rac activity at the membrane. CdGAP-expressing cells exhibited a striking morphology, and it is unlikely that there is a uniform inactivation of Rac across the whole of the cell membrane in CdGAP-expressing cells. As such, it would be informative to look very specifically at the levels of active Rac at the tips lamellipodial-like protrusions seen in CdGAP-expressing cells, where only a small fraction of the junction remains. This should involve FLIM-FRET but using a higher magnification than used previously, followed by manual segmentation of areas of interest.

As CdGAP is a Rac and Cdc42 GAP, and Cdc42 activity is globally reduced in CdGAP-expressing cells it will be important to determine the localised activity of Cdc42 in CdGAP-expressing cells. It is possible that CdGAP is localising to other sub-cellular structures, such as the Golgi, which is a site of Cdc42 activity and it will be interesting to look at the activation of Rac and Cdc42 at such structures as well as sites of contact. Due to the reduction of Cdc42 and Rac seen in CdGAP expressing cells it would also be informative to see if Rho levels are increased as a result of CdGAP expression. This can be done biochemically using Rhotekin beads or using FRET to gather spatial information.

The activation of different GTPases has different effects on the actin cytoskeleton. Cells expressing CdGAP are highly retractive and preliminary data (not shown) indicates that this is mediated by changes in the actin cytoskeleton. A comprehensive analysis of actin organisation in CdGAP-expressing cells would be important and can be complemented by analysis of Rho GTPase activity. Use of EGFP-Actin would facilitate study of actin organisation in CdGAP-expressing cells, and also facilitate live-cell imaging. Because the morphological changes associated with CdGAP expression occur by 8 hours expression, live cell imaging of the entire process is potentially feasible. Mature cell-cell contacts have tightly packed cortical bundles of F-actin present at adhesions (Cavey and Lecuit, 2009). However, formation of stress fibres can often be seen in cells undergoing epithelial-to-mesenchymal transition (Savagner, 2001). High resolution microscopy of EGFP-Actin and CdGAP-expressing cells would allow the number, length and dynamics of any stress fibres formed to be quantified. It would also be possible to observe the localisation of CdGAP and if there is a direct association with actin filaments. Finally, through which proteins CdGAP regulates actin

reorganisation is unknown. The identification of novel CdGAP-binding proteins will be informative in this sense and may be achieved through typical protein-protein interaction screens (such as yeast two-hybrid) or using a more modern bioinformatics approach to identify novel proteins that may be expressed in similar tissues at the same time as CdGAP, or possess a similar binding region as previously characterised CdGAP-binding proteins (Schaefer et al., 2013).

Is CdGAP expression inducing cells to undergo epithelial-to-mesenchymal transition?

The expression of CdGAP causes such considerable morphological changes that it is likely multiple signalling pathways are affected. If CdGAP expression is able to induce epithelial-to-mesenchymal transition these cells should express mesenchymal proteins, such as vimentin and MMPs, as well as modulate E-cadherin via upregulation of transcriptional repressors (for example via Snail) (Yilmaz and Christofori, 2009). A full analysis of gene expression, for example via q-PCR, would ascertain if these cells are undergoing epithelial-to-mesenchymal transition, and if so, if other interesting proteins are significantly up or downregulated. This may offer clues as to which proteins CdGAP can regulate, or is itself regulated by, that ultimately results in junction disruption. This approach may be complemented by analysis of tumour databases to identify if CdGAP is upregulated in cancers or cells lines. These methods will confirm if CdGAP is indeed a rare example of a GAP that can also promote invasion.

Cells that have undergone epithelial-to-mesenchymal transition are more motile than epithelial cells. Furthermore, as discussed above, CdGAP expression can have mixed effects on cell migration, with studies finding both increases and decreases in rates of migration upon expression of CdGAP. To investigate the ability of CdGAP to induce epithelial-to-mesenchymal transition by promoting migration in keratinocytes invasion assays can be performed. Transwell invasion assays measure the ability of cells to migrate through various types of matrigel. The ability of CdGAP to promote tumour cell invasion in breast epithelial cells does not require the GAP domain (He et al., 2011). However, I have shown that CdGAP-mediated junction disruption is GAP-dependent and so it will be interesting to determine if GAP activity is also necessary for (potentially) promoting migration and/or invasion in keratinocytes.

Chapter 5.

CdGAP is regulated by the scaffold protein Ajuba

5.1. Introduction

The maintenance of cell-cell contacts is governed by a variety of proteins. The GTPases Rac, Rho and Cdc42 have been implicated in cell-cell contact maintenance in various cells types and understanding of their regulation, directed by a host of GEFs and GAPs, has for some cases been elucidated (Citi et al., 2011; McCormack et al., 2013). However, the GTPases and their regulators rely on a cohort of additional proteins to coordinate not only their localisation, but also their temporal activation. These proteins can commonly be referred to as 'scaffold' proteins. Often lacking any catalytic activity themselves, they typically possess a variety of protein-protein interaction domains allowing them to integrate signalling between multiple pathways. Unravelling the mode of action of such scaffold proteins informs us as to how spatial and temporal separation of signalling pathways can be achieved.

Ajuba is a scaffold protein localised to AJs via interaction with α -catenin (Marie et al., 2003). Ajuba plays an important role in the maintenance of AJs by ensuring Rac activation is sustained at AJs and by regulating actin reorganisation (Nola et al., 2011). Depletion of Ajuba in keratinocytes reduces the amount of active Rac at cell-cell contacts and consequently weakens cell-cell adhesions. However, Ajuba does not regulate Rac recruitment (Nola et al., 2011). Ajuba also regulates Rac activation in wound healing; Ajuba null MEFs cannot activate Rac as efficiently at the leading edge of migrating cells (Pratt et al., 2005). The regulation of Rac activity by Ajuba suggests that Ajuba can either promote the activation/localisation of a Rac GEF, or inhibit the localisation/activation of a Rac GAP. In order to identify candidate proteins that may act downstream of Ajuba to regulate Rac, a yeast two-hybrid screening approach was taken (performed by A. Ferrand and D. Birnbaum). In this screen, CdGAP was identified as a potential Ajuba binding partner (A. Ferrand and D. Birnbaum).

In the previous chapter, I have shown that CdGAP regulates AJ maintenance. The expression of CdGAP causes severe junction disruption and a progressive loss of E-cadherin from cell-cell contacts. I also show that this is a GAP-dependent process, as the GAP-dead form of CdGAP fails to disrupt junctions. In this chapter I have sought to examine the mechanisms behind CdGAP-mediated junction disruption by investigating the possibility that Ajuba regulates Rac activity at cell-cell contacts via interaction with CdGAP.

I have also investigated how the regulation of CdGAP at cell-cell contacts could occur by probing the mechanism of CdGAP auto-regulation. The two truncated CdGAP mutants associated with AOS have augmented activity against Cdc42, implying that the C-terminal region of CdGAP may regulate

the GAP domain (Southgate et al., 2011). Indeed a CdGAP construct covering the N-terminal GAP domain (GFP-CdGAP₁₋₂₂₁) was detected in immunoprecipitates of myc-tagged CdGAP C-terminal (CdGAP₁₀₈₃₋₁₄₂₅) (Southgate et al., 2011). I sought to confirm this interaction was direct and further examine the binding between different regions of CdGAP.

5.2. Aims

1. Determine if Ajuba can influence the ability of CdGAP to perturb cell-cell contacts. I aim to ascertain if Ajuba can regulate the activity or localisation of CdGAP.
2. Determine if CdGAP and Ajuba can interact directly and map the regions on CdGAP and Ajuba responsible for the interaction.
3. Investigate the potential auto-inhibitory mechanism of CdGAP binding. If CdGAP is able to interact with itself and inhibit its activity, I wish to map in detail the binding site.

5.3. Results

5.3.1. Ajuba regulates the localisation of CdGAP and its ability to disrupt cell-cell contacts in keratinocytes

The long and short isoforms of CdGAP were expressed in keratinocytes with and without Ajuba to see if Ajuba could influence the localisation of CdGAP, or modulate in any way the effects of CdGAP expression on E-cadherin junctions (**Figure 5.1, 5.2**). In the presence of Ajuba, CdGAP-l localised at cell-cell contacts in a considerable number of cells, whereas this was relatively rare in cells expressing CdGAP-l alone. Representative line scans highlight the co-localisation of CdGAP and E-cadherin in an Ajuba expressing cell. Conversely, cells expressing CdGAP-l alone show fairly uniform CdGAP-l localisation, with no areas being specifically enriched. Interestingly, the presence of Ajuba seemed to have no effect on the localisation of CdGAP-s, which was enriched at junctions in a similar number of cells irrespective of Ajuba expression.

To quantify these effects I first counted the number of cells with an enrichment of CdGAP at junctions (**Figure 5.2 A**). In the case of cells expressing CdGAP-l and Ajuba, CdGAP-s alone, and CdGAP-s and Ajuba, CdGAP was enriched at one or more junction in approximately 40% of cells.

However, in cells expressing CdGAP-I alone the junctional enrichment of CdGAP was much less frequent (17% of cells). These findings indicate Ajuba contributes to the localisation of CdGAP-I, but not CdGAP-s at cell-cell contacts.

In the previous chapter, I observed that the expression of CdGAP induced a loss of E-cadherin from cell-cell contacts in quite a characteristic fashion; cells appeared highly retractive, with large holes appearing between neighbouring cells whilst remaining E-cadherin was concentrated in small patches at contacts. Co-expression of CdGAP and Ajuba resulted in a reduction in the number of cells exhibiting this highly retracted morphology compared to cells expressing CdGAP-I alone (**Figure 5.1**). Whilst in some cases small holes were seen between cells, the prevalence of very large holes was much lower in the Ajuba -CdGAP-I expressing cells. Again, no such effect was seen in CdGAP-s expressing cells as both in the presence and absence of Ajuba the highly retractive morphology was observed.

Junction disruption was quantified by counting the number of cells with a highly retractive morphology (defined as more than two holes between cells) (**Figure 5.2 B**). Levels of disruption in cells co-expressing Ajuba and a CdGAP isoform were normalised relative to the disruption observed upon expression of each isoform by itself. This enabled assessment of the effect of Ajuba expression on the respective CdGAP isoforms independently of the ability of each isoform to disrupt junctions. The levels of junction disruption between CdGAP-s and CdGAP-s and Ajuba were very similar. However, the presence of Ajuba in CdGAP-I expressing cells significantly reduced the amount of disruption caused by CdGAP-I expression (**Figure 5.2 B**).

CdGAP-mediated junction disruption is dependent on the GAP domain (**Figure 4.10**). The apparent ability of Ajuba to inhibit the capacity of CdGAP to disrupt junctions may be due to a direct regulation of CdGAP GAP activity. Preliminary experiments have been conducted to investigate this (these experiments were performed as part of a collaboration with Nathalie Lamarche's group at McGill University, Montreal by F. Karimzadeh) which suggest that in the presence of Ajuba, CdGAP GAP activity is inhibited in *in vitro* GAP assays. However, further experiments are required to verify this

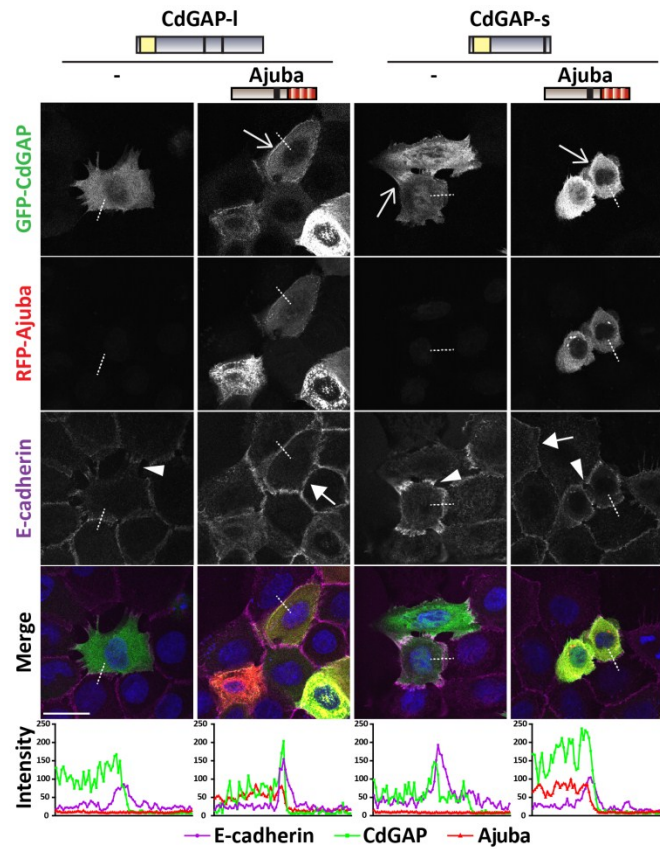


Figure 5.1 Ajuba regulates CdGAP localisation Keratinocytes with mature junctions were transfected with GFP-CdGAP-I or GFP-CdGAP-s in the presence or absence of RFP-Ajuba for 8 hours. Cells were fixed and stained for E-cadherin (Cy5, purple) and the nucleus with DAPI (blue). Images were collected on a confocal microscope. Merged images were created by overlaying the colour channels. Fluorescence line scans (dashed lines) were made over junctional areas between CdGAP-expressing and non-expressing cells. Graphs below the images represent fluorescence intensity for E-cadherin (purple), CdGAP (green) and Ajuba (red) over these areas. Arrows indicate junctions either in CdGAP-expressing cells or in neighbouring, non-expressing control cells with continuous E-cadherin staining. Arrowheads indicate highly disrupted junctions where E-cadherin has been partially lost from the junction area. Open arrows indicate regions enriched with CdGAP relative to the rest of the cell. Scale bar = 25µm. N=3

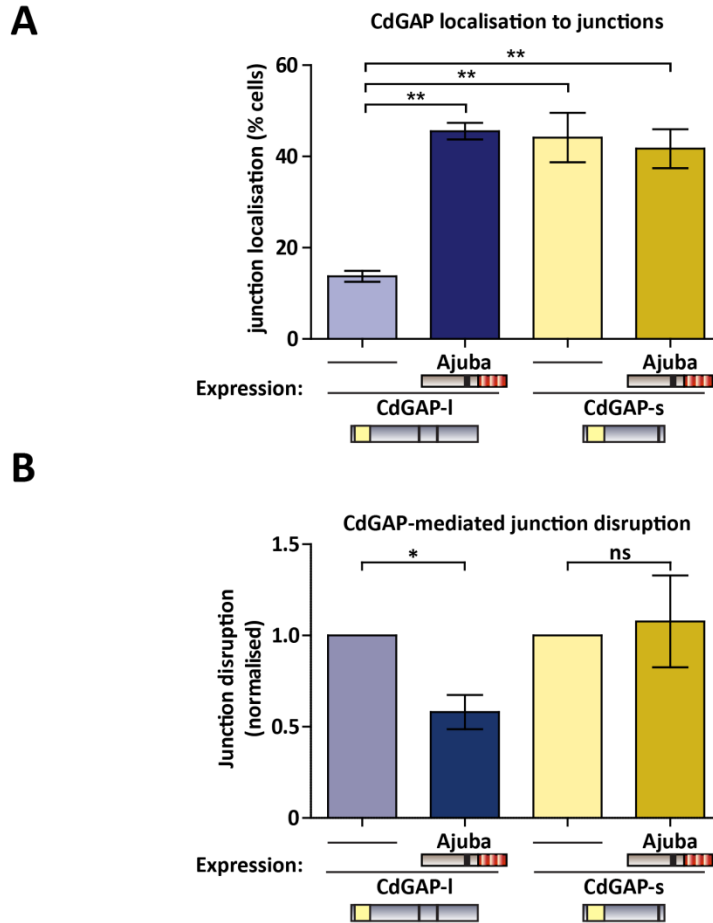


Figure 5.2 Ajuba regulates CdGAP ability to disrupt junctions and localisation in keratinocytes Keratinocytes expressing either CdGAP-I or CdGAP-s in the presence or absence of Ajuba (Figure 5.1) were examined in order to quantify the degree of junctional localisation of CdGAP (A) or number of cells with disrupted junctions (B) for each condition. A) The number of cells with CdGAP enriched at junctions compared to the rest of the cell was counted for each condition. The mean number of cells with junctional CdGAP localisation was expressed as a percentage of total number of cells and standard error bars shown. Statistical significance was assessed using one-way ANOVA with Bonferroni's multiple comparison test. **, $P \leq 0.01$. B) The number of cells with highly disrupted junctions (defined as more than two holes between neighbouring cells) was counted for each condition. The mean number of cells with disrupted junctions in the CdGAP-I + Ajuba and the CdGAP-s + Ajuba conditions were normalised relative to the disruption observed upon expression of each isoform by itself (arbitrarily set at 1). Statistical significance was assessed using Student's T-test. *, $P \leq 0.05$; ns= Not significant. N=3

5.3.2. Ajuba interacts directly via its LIM domain with CdGAP-I C-terminal

These results indicate that Ajuba is able to modulate the activity and localisation of CdGAP-I, but not CdGAP-s. Thus Ajuba likely interacts with CdGAP-I, but not CdGAP-s. To map the binding site between CdGAP and Ajuba, different CdGAP constructs were transfected into COS-7 cells and GST-tagged Ajuba (full-length) used to pull-down associated protein (**Figure 5.3 A**). CdGAP-I was found to bind Ajuba, whilst CdGAP-s did not. Furthermore, the C-terminal fragment, CdGAP₁₁₆₀₋₁₄₂₅ also bound Ajuba, indicating that the Ajuba binding site is within this region. This is in good agreement with the yeast two-hybrid data, which identified amino acids 1064 to 1183 of CdGAP binding to Ajuba (A. Ferrand and D. Birnbaum). To test if the Ajuba-CdGAP interaction is direct, CdGAP₁₁₆₀₋₁₄₂₅ was *in vitro* translated and incubated with GST-Ajuba (**Figure 5.3 B**). GST-Ajuba was able to pull-down CdGAP₁₁₆₀₋₁₄₂₅ indicating that these proteins bind directly.

I next located the CdGAP binding site on Ajuba. Cells were transfected with myc-tagged Ajuba constructs (full-length, Pre-LIM and LIM) and GST-tagged CdGAP₁₁₆₀₋₁₄₂₅ used to pull down Ajuba from cell lysates (**Figure 5.4 A**). Full-length Ajuba was bound to GST-CdGAP₁₁₆₀₋₁₄₂₅ as was Ajuba LIM. However, the Pre-LIM domain of Ajuba was unable to bind CdGAP₁₁₆₀₋₁₄₂₅, confirming the LIM domain is sufficient for this interaction.

To further narrow-down the Ajuba binding site on CdGAP, a second C-terminal CdGAP construct containing amino acids 1253 to 1425 (GST-CdGAP₁₂₅₃₋₁₄₂₅) was used to establish if this region was sufficient to mediate the interaction (**Figure 5.4 B**). Cells were transfected with the different myc-tagged Ajuba constructs and lysates incubated with GST-CdGAP₁₂₅₃₋₁₄₂₅ to pull-down bound Ajuba. Interestingly, GST-CdGAP₁₂₅₃₋₁₄₂₅ was unable to interact with Ajuba full-length and only a very small amount of Ajuba LIM was seen bound to CdGAP₁₂₅₃₋₁₄₂₅ and only when blots were over-exposed. These findings were verified by *in vitro* translating myc-tagged Ajuba full-length and using the two GST-CdGAP constructs (GST-CdGAP₁₁₆₀₋₁₄₂₅ and GST-CdGAP₁₂₅₃₋₁₄₂₅) to pull down Ajuba (**Figure 5.4 C**). Again, GST-CdGAP₁₁₆₀₋₁₄₂₅ was able to interact with Ajuba, but GST-CdGAP₁₂₅₃₋₁₄₂₅ was not. These findings illustrate that the Ajuba binding site lies between amino acids 1160 and 1253. Further considering the yeast two-hybrid result, the minimal Ajuba binding site apparently lies between amino acids 1160 and 1183 (**Figure 5.4 D**).

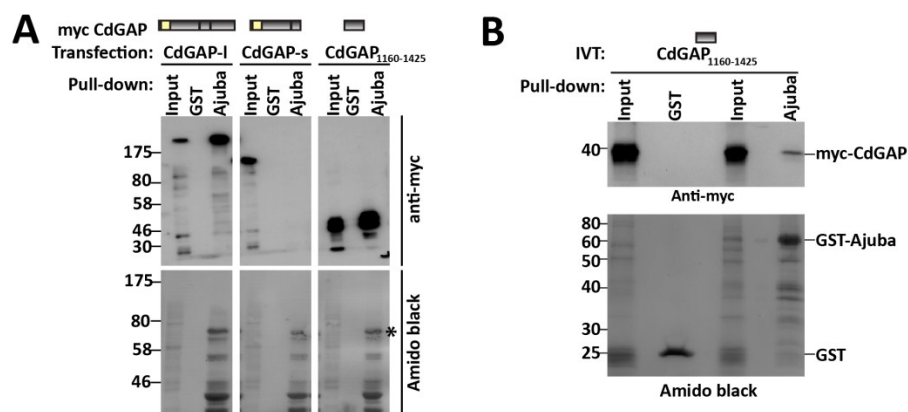


Figure 5.3 CdGAP and Ajuba interact directly via CdGAP C-terminus A) COS-7 cells were transfected with either myc-tagged CdGAP-I, CdGAP-s or CdGAP₁₁₆₀₋₁₄₂₅ for 24 hours. Cells were lysed and lysates incubated with GST or GST-Ajuba immobilised on glutathione sepharose beads for one hour. Western blot was used to detect myc-tagged CdGAP protein in input samples (5% total lysate) and bound to beads. N=3 B) myc-CdGAP₁₁₆₀₋₁₄₂₅ was produced via *in vitro* translation and incubated with GST or GST-Ajuba immobilised on glutathione sepharose beads for 2 hours. Western blot was used to detect myc-tagged CdGAP in input samples (20% total) and bound to beads. Molecular weight markers are shown on the left of each panel. N=3

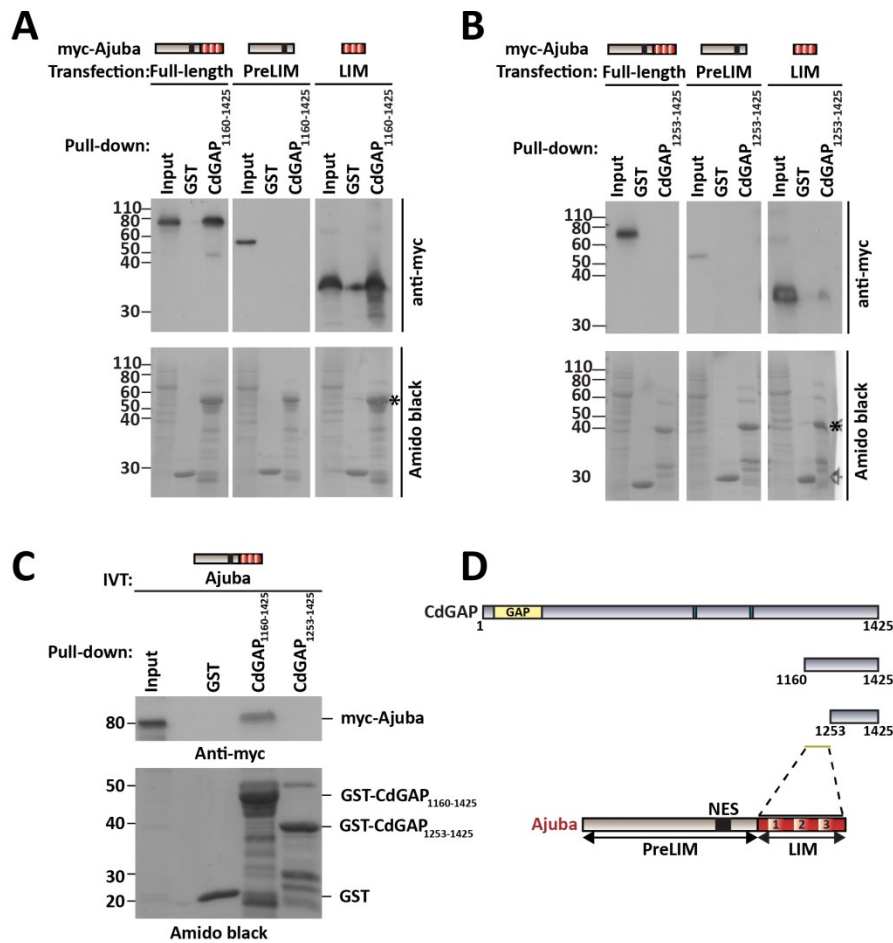


Figure 5.4 CdGAP interacts with Ajuba LIM domain COS-7 cells transfected with myc tagged Ajuba full-length, preLIM or LIM domain for 24 hours were lysed and lysates incubated with GST (A and B), GST-CdGAP₁₁₆₀₋₁₄₂₅ (A) or GST-CdGAP₁₂₅₃₋₁₄₂₅ (B) immobilised to glutathione sepharose beads for one hour. Western blot was used to detect myc-tagged Ajuba in input samples (5% total lysate) and bound to beads. In all cases, GST-tagged proteins (indicated by the asterisk) were visualised by amido black staining of the membrane (shown below respective Western blots). C) myc-Ajuba was produced via *in vitro* translation and incubated with GST, GST-CdGAP₁₁₆₀₋₁₄₂₅ or GST-CdGAP₁₂₅₃₋₁₄₂₅ immobilised on glutathione sepharose beads for 2 hours. Western blot was used to detect myc-tagged Ajuba in input samples (20% total) and bound to beads. COS-7 cells were transfected with either myc-tagged Ajuba full-length, Ajuba Pre-LIM or Ajuba LIM for 24 hours. IVT= *in vitro* translated. Molecular weight markers are shown on the left of each panel. N=3. D) Summary of the binding between CdGAP and Ajuba based on pull-down experiments. CdGAP-I (amino acids 1 to 1425) shown with GAP domain in pale yellow and proline-rich regions in blue. GST-CdGAP₁₁₆₀₋₁₄₂₅ and GST-CdGAP₁₂₅₃₋₁₄₂₅ shown below. Below is Ajuba with the PreLIM and LIM regions highlighted. The three LIM domains are numbered accordingly. The nuclear export signal (NES) is represented by the black box. The minimal binding region, based on pull-down experiments lies between 1160 and 1253 on CdGAP (yellow line) and maps to the LIM domain of Ajuba.

5.3.3. Ajuba interacts with CdGAP at several regions within CdGAP's C-terminal

The C-terminal of CdGAP does not share any similarity with other known proteins. Furthermore, there is no published information regarding the 3D structure of CdGAP. In order to identify specific residues of CdGAP mediating the CdGAP-Ajuba interaction, a library of overlapping peptides was prepared covering amino acids 1083 to 1425 of CdGAP. Peptides were either 15- or 25-mers each shifted by 2 or 5 amino acids, respectively and were SPOT-synthesised onto cellulose membranes. By incubating the SPOT membrane with purified Ajuba positive interactions with specific peptides can be observed by using a secondary antibody labelled with either a fluorescent tag or with alkaline phosphatase which produces coloration of the membrane. Initially a SPOT membrane comprising 15-mers of CdGAP was used (data not shown). However, no interaction was seen between any of the immobilised peptides and Ajuba. As no other known binding partner for the C-terminal of CdGAP is known, a positive control could not be tested. However, it is possible that 15-mers are not sufficient to support the interaction and a larger region is needed (Bass et al., 1999).

Thus, a second peptide array comprising a library of 25-mers was tested (**Figure 5.5**). In this case, at least four separate regions appear to be involved in mediating the CdGAP-Ajuba interaction (Ajuba Pre-LIM was used as a negative control and only weak coloration was observed (not shown)). Amino acids 1148 to 1202 (spots 13 to 20), that overlap with the region identified by both the pull-downs and yeast two-hybrid were identified. Within this region an obvious reduction in intensity at spots 16 and 17 potentially indicates the existence of two discontinuous binding sites. Interestingly, other regions were also able to interact with Ajuba. There is a small section between amino acids 1298 to 1337 (spots 44 to 47). Spots 46 and 47 showed a weaker interaction, suggesting amino acids located exclusively in spots 44 and 45 (amino acids 1298-1327) contribute to the interaction but are not vital for it. Additionally, several spots at the very C-terminus of CdGAP were highly reactive. Due to signal interruptions these could be considered as separate regions but may be brought into close proximity in the folded protein. I next sought to identify specific amino acids responsible for mediating this interaction using alanine scanning arrays.

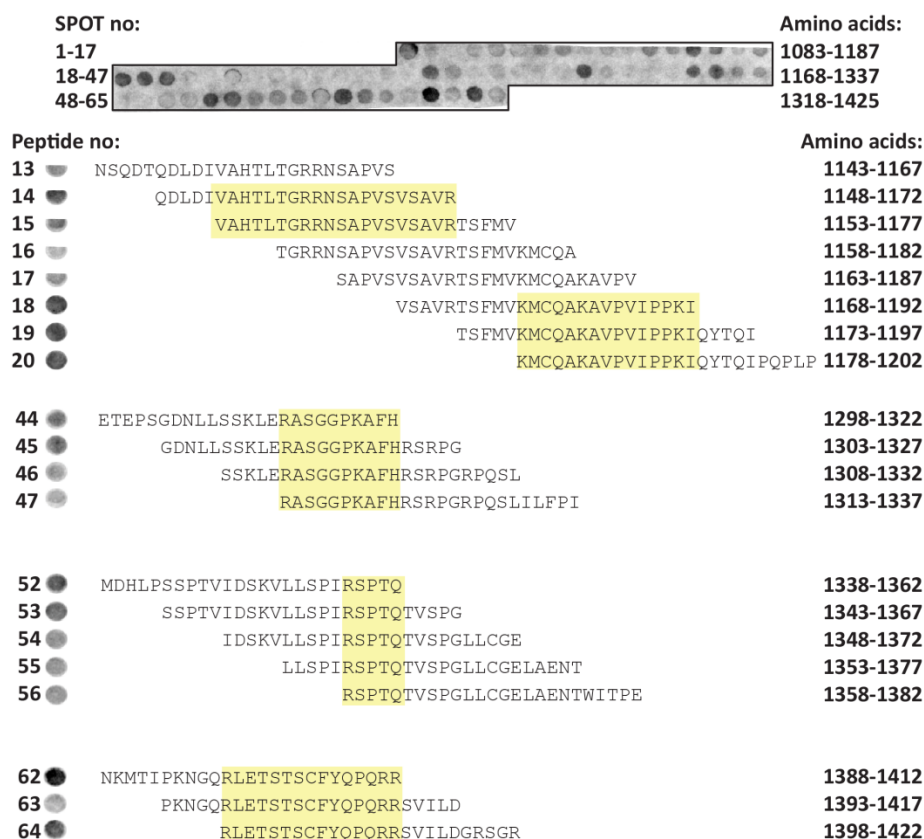


Figure 5.5 Ajuba interacts with several distinct regions on CdGAP C-terminus Peptides (25-mer) covering the C-terminus of CdGAP (amino acids 1083-1425) with 5 amino acid overlaps were spot synthesised on a cellulose membrane. The membrane was overlaid with *in vitro* translated myc-Ajuba for 2 hours then washed and incubated with rabbit anti-myc antibody followed by Indocarbocyanine (Cy3)-conjugated anti-rabbit IgG. Positive interactions are indicated by dark colouration on the membrane, visualised using the Ettan DIGE Imager. SPOT number refers to the position of the 25-mer in the CdGAP C-terminal sequence. For example, SPOT number 1 encompasses amino acids 1083-1107, and SPOT number 2 encompasses 1088-1112 etc. The SPOTs present on each row of the membrane are shown on the left of the membrane. The corresponding amino acids covered on each row are shown on the right hand side of the membrane. The sequences of highly reactive SPOTs are shown below the membrane alongside their respective SPOT number and the amino acids they cover. Amino acids predicted to be important in the interaction are shaded in yellow. N=3

5.3.4. Arginine residues 1172 and 1412 of CdGAP are important for mediating the CdGAP-Ajuba interaction

Several of the strongest interacting peptides from different areas of the CdGAP C-terminal were selected and each amino acid sequentially mutated to Alanine (**Figure 5.6**). Overall, the reactivity of the alanine scan membrane was much weaker than the previous peptide array, making quantitation of binding less reliable. In fact, some peptide sequences (not shown) that were highly reactive on the original membrane failed to bind Ajuba at all, potentially indicating a problem with the synthesis. However, Ajuba binding was semi-quantified relative to the wild-type peptide sequence and the results plotted on bar charts.

Semi-quantitation of SPOT membranes is not trivial and intensity can be influenced by many factors including antibody concentration, binding time and competition between peptides for binding to the partner protein (Weiser et al., 2005). As such it is important to compare only relative intensity, to an internal control for each experiment, rather than comparing between membranes (Weiser et al., 2005).

For peptide 14, covering amino acids 1148 to 1172 of CdGAP, the strongest reduction in the interaction with Ajuba came with the mutation of arginine 1172, which reduced binding by approximately 50% of the wild-type (**Figure 5.6 A**). Other amino acids, particularly arginines 1160 and 1161, asparagine 1162 as well as serine and valine residues 1167 and 1168 also notably decreased binding to Ajuba (**Figure 5.6 A**). This data is in good agreement with that gained from pull-downs, the yeast two-hybrid and the previous SPOT membrane showing that the region 1148-1172 is important for mediating binding between CdGAP and Ajuba. Peptide 62 (amino acids 1388-1412) was the most reactive in both the alanine scan and the original 25-mer membrane. From the alanine scan array, mutation of one amino acid (arginine 1412) reduced profoundly the binding of Ajuba by almost 90% compared to the wild-type. Several other residues within this region when mutated to alanine also reduced Ajuba binding by more than 50% indicating its importance (**Figure 5.6 B**).

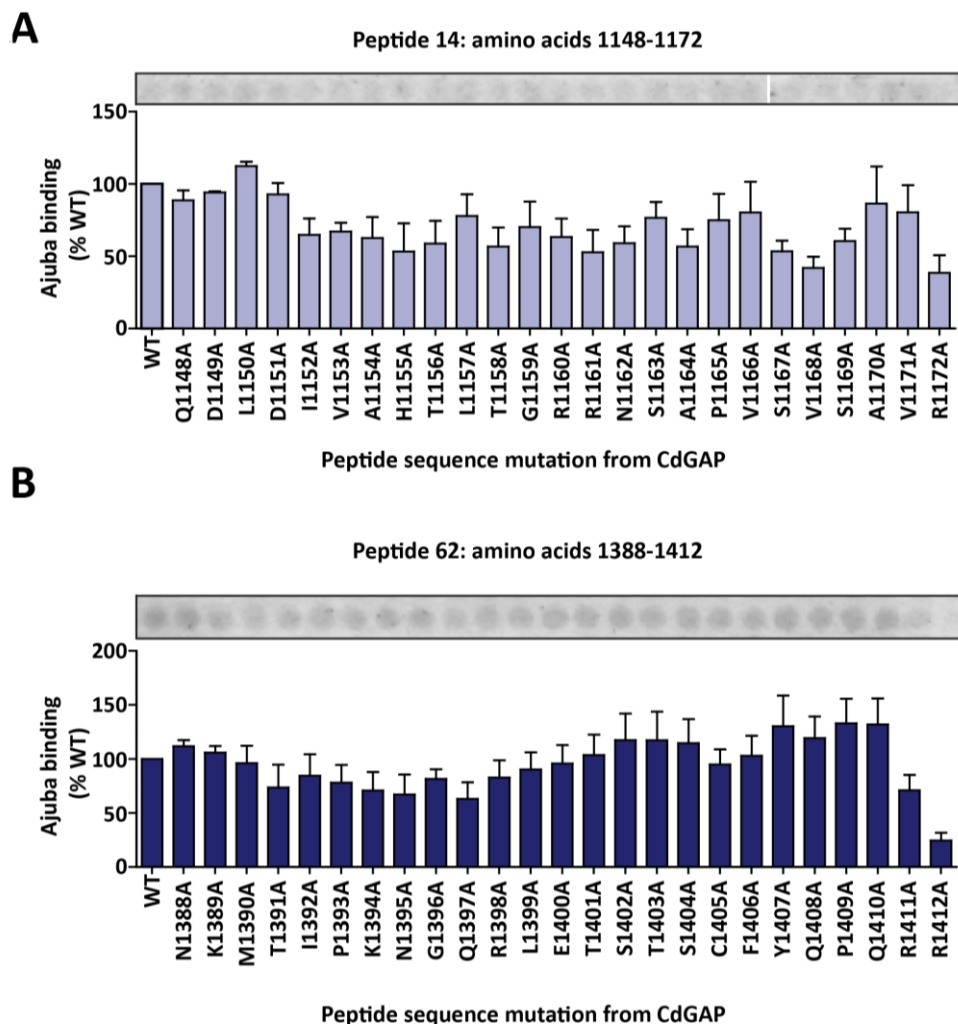


Figure 5.6 Arginine residues 1172 and 1412 are critical for the CdGAP-Ajuba interaction Peptides (25-mer) of highly reactive SPOTs identified in the peptide array (Figure 5.5) were synthesised whereby every amino acid was sequentially mutated to alanine. Peptide 14, covering amino acids 1148-1172 of CdGAP (A) and peptide 62, covering amino acids 1388-1412 of CdGAP (B) were overlaid with *in vitro* translated myc-Ajuba for 2 hours. Membranes were then washed and incubated with mouse anti-myc antibody followed by anti-mouse secondary antibody coupled to alkaline phosphatase. Bound protein was detected via the addition of alkaline phosphatase substrate ((bromochloroindolylphosphate (BCIP), thiazolyl blue tetrazolium bromide (MTT) MgCl₂ in citrate-buffered saline (CBS)) for up to 60 min. Positive interactions are indicated by dark colouration on the membrane. The reactivity of individual SPOTs was quantified by measuring intensity using ImageJ and subtracting background intensity. Reactivities are plotted on graphs and expressed relative to the wild-type (WT) sequence. The mutation present in the CdGAP sequence in each peptide is displayed underneath its reactivity value on the graphs. N=3

5.3.5. CdGAP C-terminal is able to bind directly its GAP domain and C-terminal

The C-terminal of CdGAP is predicted to interact with its GAP domain, as myc-CdGAP₁₀₈₃₋₁₄₂₅ co-immunoprecipitates with GFP-CdGAP₁₋₂₂₁ (Southgate et al., 2011). This interaction could provide an auto-inhibitory mechanism as the GAP activity of CdGAP constructs lacking the C-terminal (1-1087 and 1-683) have increased GAP activity on Cdc42 in *in vitro* assays (Southgate et al., 2011). Ajuba interaction with CdGAP C-terminus may somehow promote this auto-inhibition in order to regulate CdGAP activity. To test for direct binding between CdGAP GAP domain (1-221) and C-terminal (1160-1425), *in vitro* translated CdGAP₁₁₆₀₋₁₄₂₅, was used to test binding to GST-CdGAP₁₋₂₂₁ (**Figure 5.7 A**). Indeed, GST-CdGAP₁₋₂₂₁ interacted directly with CdGAP₁₁₆₀₋₁₄₂₅.

As such, CdGAP could be folding in on itself or could form antiparallel dimers. I also tested binding of CdGAP C-terminal to itself, to see if CdGAP could potentially form parallel dimers. Purified myc-CdGAP₁₁₆₀₋₁₄₂₅, produced via *in vitro* translation, was tested for binding to GST-CdGAP₁₁₆₀₋₁₄₂₅ (**Figure 5.7 B**). Interestingly, these two fragments are also able to interact with one another conceivably presenting another regulatory mechanism.

5.3.6. The CdGAP-CdGAP interaction is mediated by the same regions of CdGAP as the CdGAP-Ajuba interaction and these are well conserved across different species

The interaction between CdGAP₁₋₂₂₁ and CdGAP₁₁₆₀₋₁₄₂₅ may present an auto-inhibitory mechanism that Ajuba binding may exploit. To investigate this I utilised the SPOT-synthesised peptide array of CdGAP 25-mers covering amino acids 1083 to 1425 to explore the CdGAP-CdGAP binding region on CdGAP's C-terminal (**Figure 5.7 C**). GST-CdGAP₁₋₂₂₁ was incubated with the membrane and binding observed. Several distinct binding regions within the C-terminal were detected (GST-tag alone produced only weak binding so this is a specific interaction). Remarkably, these were highly similar to those found to be important for the CdGAP-Ajuba interaction. Specifically, amino acids 1138 and 1202, amino acids 1368 to 1202 and amino acids 1388 to 1422 were all highly reactive. Interestingly, whilst the regions themselves seem well conserved between Ajuba and CdGAP, the contribution of specific peptides was different. For example in the first common region (1138 to 1202) Ajuba reacted most strongly with the latter amino acids 1168 to 1202, whilst CdGAP reactivity in this area, whilst high, was less than with spots harbouring amino acids 1138 to 1172.

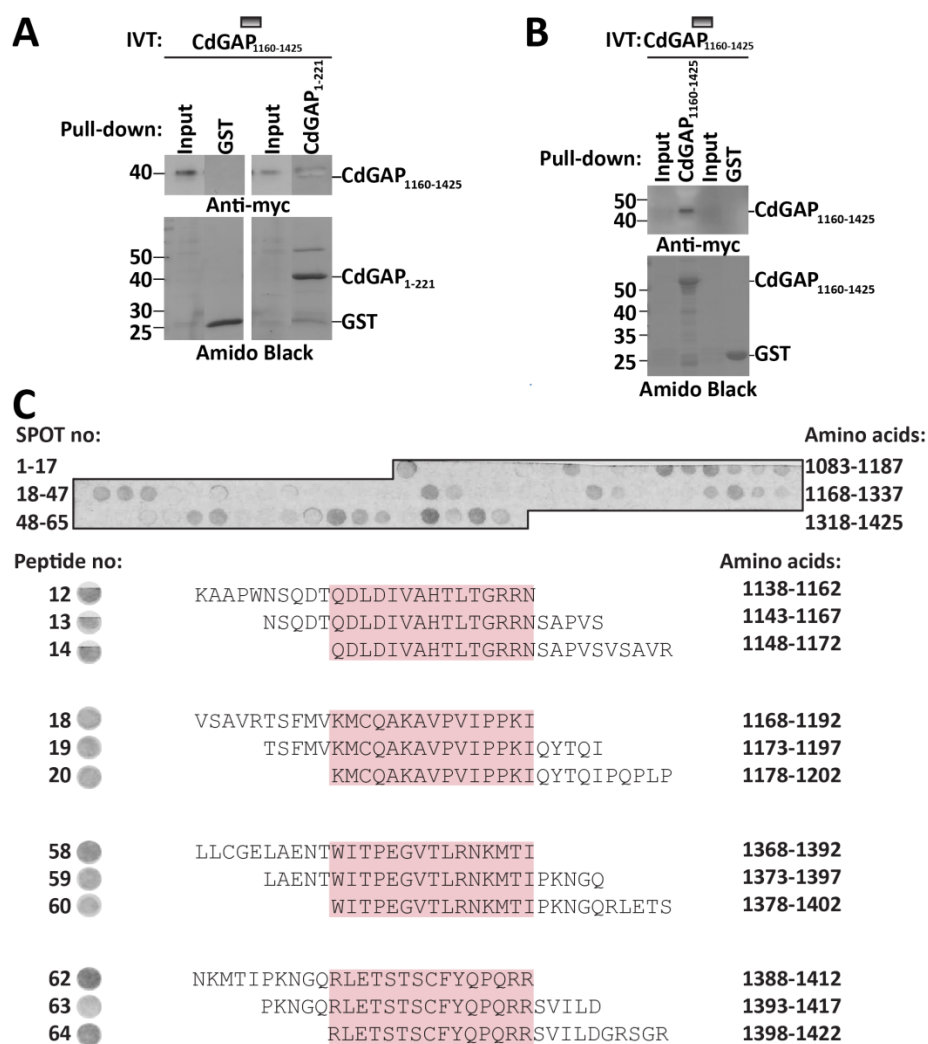


Figure 5.7 CdGAP interacts with itself in multiple regions myc-CdGAP₁₁₆₀₋₁₄₂₅ was produced via *in vitro* translation and incubated with GST (A and B), GST-CdGAP₁₋₂₂₁ (A) or GST-CdGAP₁₁₆₀₋₁₄₂₅ (B) immobilised on glutathione sepharose beads for 2 hours. Western blot was used to detect myc-tagged CdGAP in input samples (20% total) and bound to beads. GST-tagged proteins were visualised by amido black staining of the membrane. IVT= *in vitro* translated. Molecular weight markers are shown on the left of each panel. N=3 C) 25-mer peptides covering the C-terminus of CdGAP (amino acids 1083-1425) with 5 amino acid overlap were spot synthesised on a cellulose membrane. The membrane was overlaid with GST-CdGAP₁₋₂₂₁ for 2 hours then washed and incubated with mouse anti-GST antibody followed by Indocarbocyanine (Cy5)-conjugated anti-mouse IgG. Positive interactions are indicated by dark colouration on the membrane, visualised using the Ettan DIGE Imager. SPOT no. refers to the position of the 25-mer in the CdGAP C-terminal sequence. For example, SPOT no. 1 encompasses amino acids 1083-1107, SPOT no. 2 encompasses 1088-1112 etc. The SPOTs present on each row of the membrane are shown left of the membrane. The corresponding amino acids are shown right of the membrane. The sequences of highly reactive SPOTs are shown below the membrane alongside their respective SPOT number and the amino acids they cover. Amino acids predicted to be important in the interaction are shaded in pink. N=3

To assess the contribution of the individual amino acids I again made use of the alanine scanning peptide arrays, this time incubating them with CdGAP₁₋₂₂₁ (**Figure 5.8**). For peptide 14 (amino acids 1148-1172, **Figure 5.8 A**) the most effective mutation in disrupting binding was arginine 1172 to alanine, which also perturbed the CdGAP-Ajuba interaction. Interestingly, mutation of any amino acid between 1160 and 1172 severely compromise binding of CdGAP₁₋₂₂₁ indicating this is probably an important regulatory region. Comparison of the CdGAP and Ajuba binding profiles was facilitated by tabulation of the relative reactivities of each spot relative to wild-type (**Table 5.1**). Two amino acids, proline 1165 and valine 1166, when mutated to alanine had very little effect on the ability of Ajuba to bind CdGAP (95% and 105% respectively relative to wild-type) but reduced CdGAP binding considerably (both bound 13% relative to wild-type). Thus these may be important in CdGAP, but not Ajuba binding.

However, the alanine scanning arrays contained several peptides of wild-type sequence (as wild-type alanines were not mutated to any other amino acid) which gave varying results in terms of reactivity. One SPOT (A1164) had one of the lowest reactivities compared to the reference wild-type used in the CdGAP-CdGAP interaction. This may be a false negative, but could also indicate problems with the peptide synthesis, which may also explain the lower reactivity seen with the alanine scans compared to the original membrane.

For peptide 62 (amino acids 1388 to 1412 (**Figure 5.8 B**)), a pattern similar to that seen with Ajuba binding was observed. The most effective mutation was arginine 1412 to alanine which completely abolished binding of CdGAP₁₋₂₂₁ to CdGAP. Furthermore, mutation of any amino acid between positions 1392 and 1397 reduced binding by approximately 50%. The considerable overlap in the binding sites for CdGAP GAP domain and Ajuba on the C-terminus (**Table 5.1**) may prevent a simultaneous interaction with the GAP domain and Ajuba occurring. It will be interesting to determine if Ajuba and CdGAP₁₋₂₂₁ may compete for binding to CdGAP's C-terminal region.

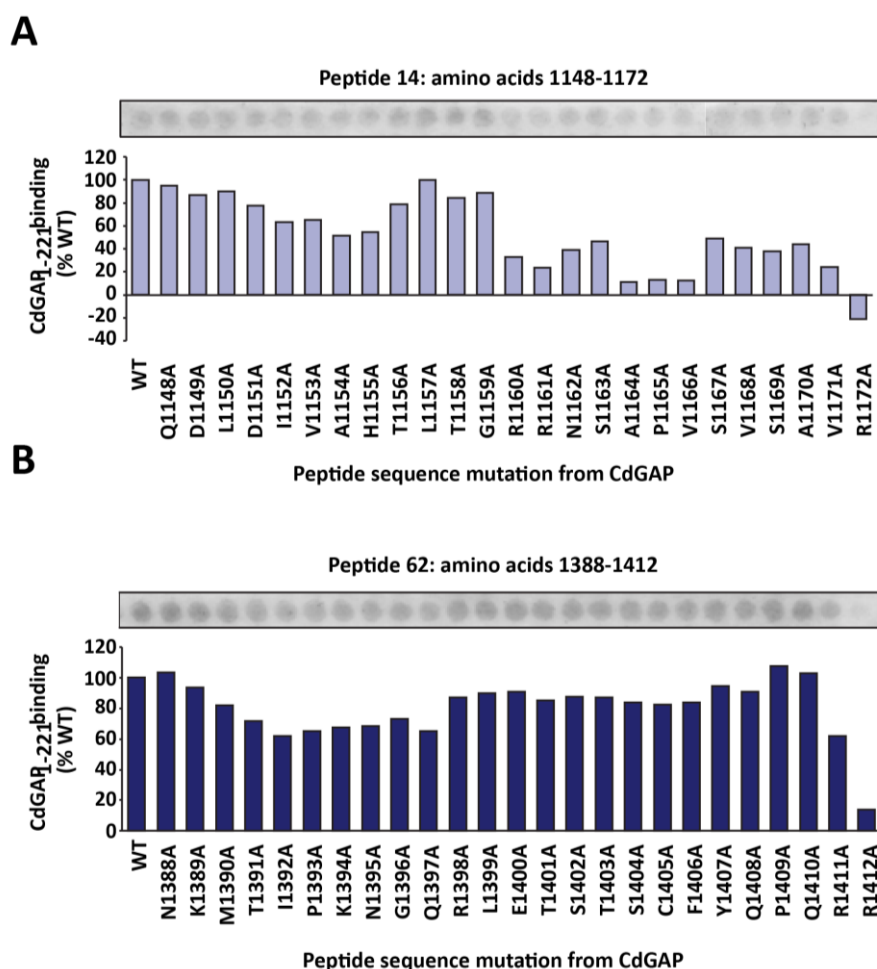


Figure 5.8 Residues crucial for the CdGAP-Ajuba interaction also regulate the CdGAP-CdGAP interaction 25-mer peptides of highly reactive SPOTs identified in the peptide array (Figure 5.7) were synthesised whereby every amino acid was sequentially mutated to alanine. Peptide 14, covering amino acids 1148-1172 of CdGAP (A) and peptide 62, covering amino acids 1388-1412 of CdGAP (B) were overlaid with GST-CdGAP₁₋₂₂₁ GST-CdGAP₁₋₂₂₁ expressed in and purified from *E.coli* for 2 hours. Membranes were then washed and incubated with mouse anti-GST antibody followed by anti-mouse secondary antibody coupled to alkaline phosphatase. Bound protein was detected via the addition of alkaline phosphatase substrate (BCIP, MTT and MgCl₂ in CBS) for up to 60 min. Positive interactions are indicated by dark colouration on the membrane. The reactivity of individual SPOTs was quantified by measuring intensity using ImageJ and subtracting background intensity. Reactivities are plotted on graphs and expressed relative to the wild-type (WT) sequence. The mutation present in the CdGAP sequence in each peptide is displayed underneath its reactivity value on the graphs. N=1

Peptide 14			Peptide 62		
Mutation	CdGAP binding (% reactivity WT)	Ajuba binding (% reactivity WT)	Mutation	CdGAP binding (% reactivity WT)	Ajuba binding (% reactivity WT)
Q1148	95	89	N1388	103	112
D1149	87	94	K1389	93	106
L1150	90	112	M1390	82	96
D1151	78	93	T1391	72	74
I1152	63	65	I1392	62	84
V1153	65	67	P1393	65	78
A1154	52	63	K1394	68	71
H1155	55	53	N1395	68	67
T1156	79	59	G1396	73	82
L1157	100	78	Q1397	65	63
T1158	85	57	R1398	87	83
G1159	89	70	L1399	90	90
R1160	33	63	E1400	91	96
R1161	24	53	T1401	85	104
N1162	39	59	S1402	87	117
S1163	47	77	T1403	87	117
A1164	11	57	S1404	84	115
P1165	13	75	C1405	83	95
V1166	13	80	F1406	84	103
S1167	49	53	Y1407	95	130
V1168	41	42	Q1408	91	119
S1169	38	61	P1409	108	133
A1170	44	86	Q1410	103	132
V1171	24	80	R1411	62	71
R1172	-21	89	R1412	14	25

Table 5.1 Comparison of residues important for mediating the CdGAP-CdGAP and CdGAP-Ajuba interactions

The alanine scanning arrays containing peptide 14, covering amino acids 1148-1172 of CdGAP and peptide 62, covering amino acids 1388-1412 of CdGAP where each amino acids was sequentially mutated to alanine were overlaid with *in vitro* translated myc-Ajuba (Figure 5.6) or GST-CdGAP₁₋₂₂₁ expressed in and purified from *E.coli* (Figure 5.8). The reactivity of individual SPOTs was quantified by measuring intensity using ImageJ and subtracting background intensity. Reactivities are expressed as a percentage of the wild-type SPOT and summarised for each peptide in reaction to either myc-Ajuba or GST-CdGAP₁₋₂₂₁.

Amino acid sequences important for protein regulation are often conserved both between species and in related proteins. The CdGAP sequences from several species were aligned and amino acids important for binding between CdGAP C-terminal region and Ajuba or GAP domain highlighted (**Figure 5.9**). Overall the C-terminus of several mammalian CdGAP is quite conserved. However, the highlighted regions (shaded in blue) show particular conservation across all species, implying that these are important, evolutionarily conserved regions.

The sequences of CdGAP (human and mouse) and closely related proteins (ARHGAP30, ARHGAP32 (RICS) and ARHGAP33) were also aligned. Interestingly, arginine 1412 is found within a sequence of four conserved amino acids present in all family members (**Figure 5.10 A**). This region may represent a conserved motif of Q(T/R/S)R(S/T). The presence of either a serine or threonine will confer relatively similar properties to a protein, structurally (the only difference being serine contains a hydrogen in place of the methyl group on threonine), and both may be phosphorylated. Mutation in arginine 1412 abolishes binding of CdGAP C-terminus to the GAP domain and Ajuba, this highly conserved sequence may represent an important regulatory region.

5.3.7. Mutations of arginines 1172 and 1412 on CdGAP severely compromise binding to Ajuba

To verify the results from the peptide arrays, mutations were made in the wild-type CdGAP protein. Without any knowledge of 3D structure other tools were used to predict with as high a confidence as possible the likelihood that these regions would be surface exposed. CdGAP is heavily phosphorylated on serine and threonine residues (Danek et al., 2007; Tcherkezian et al., 2005) so phosphorylation sites within this region were searched for based on the assumption that these would be on the surface. Serine 1163 and serine 1413 have both been identified via mass spectrometry in several screens (Huttlin et al., 2010; Villen et al., 2007; Zanivan et al., 2008) searching for phosphorylated proteins, suggesting these sites may be exposed.

Arginines 1172 and 1412 were mutated to alanines as these mutations had the most profound effect on binding in the alanine scans. Furthermore, arginines are large, charged amino acids that can be important in mediating protein-protein interactions via electrostatic interactions (Bogan and Thorn, 1998).

In vitro translated Ajuba full-length was tested for binding in *in vitro* pull-down assays using purified GST-CdGAP₁₁₆₀₋₁₄₂₅ wild-type or GST-CdGAP₁₁₆₀₋₁₄₂₅^{R1172A} (**Figure 5.10 B**). Although the R1172A mutation did not abolish binding of Ajuba to CdGAP, far less Ajuba was precipitated with this GST-CdGAP construct than the wild-type. Indeed, quantification (**Figure 5.10 C**) shows that this mutation significantly reduces the amount of Ajuba bound to CdGAP, indicating that this point mutation is sufficient to reduce binding of Ajuba by approximately 75%.

To confirm this result the reverse experiment was performed using GST-Ajuba LIM domain and *in vitro* translated CdGAP₁₁₆₀₋₁₄₂₅ wild-type, R1172A and R1412A (**Figure 5.10 D**). Although preliminary (N=1), it confirmed the previous result as GST-Ajuba LIM pulls down less of CdGAP₁₁₆₀₋₁₄₂₅^{R1172A} when compared with CdGAP wild-type. Interestingly, the effect was even more profound with the CdGAP₁₁₆₀₋₁₄₂₅^{R1412A} mutant, of which very little was bound to Ajuba. Thus, both arginines 1172 and 1412 appear to be important for the Ajuba-CdGAP interaction. A summary of the binding regions is shown in **Figure 5.11**.

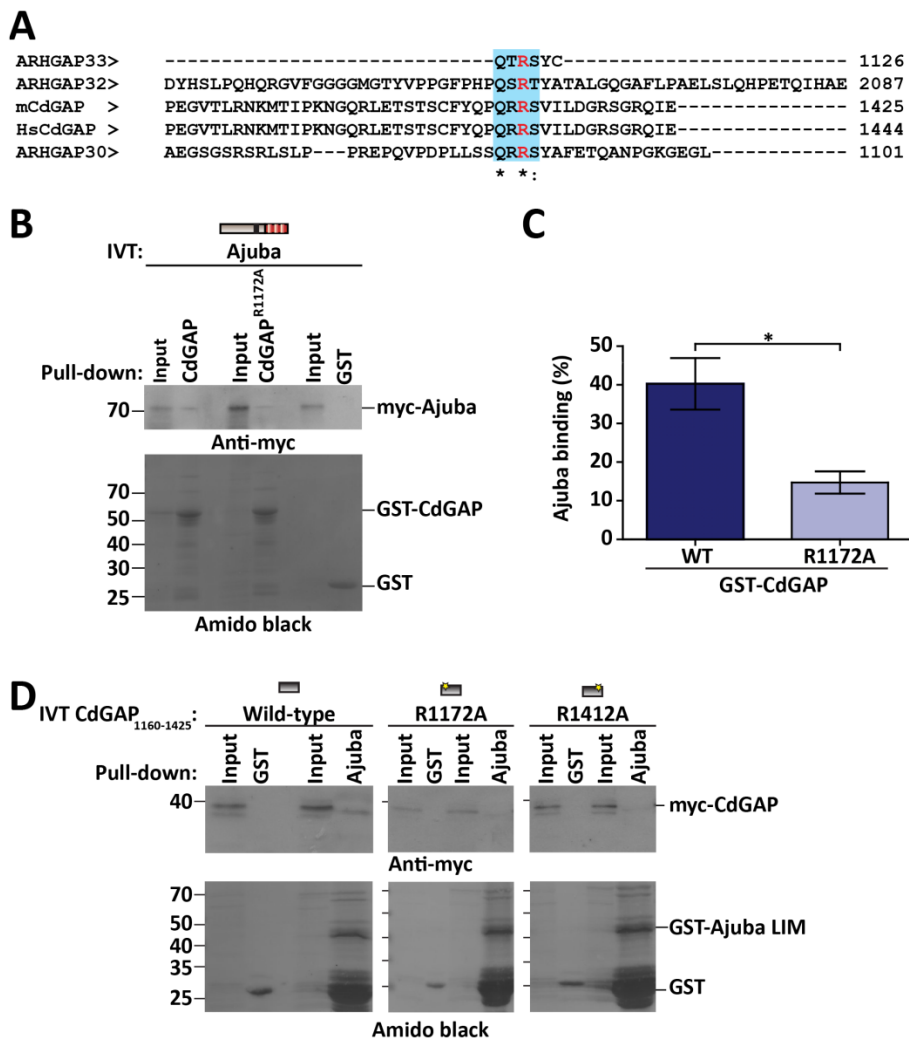


Figure 5.10 CdGAP arginine residues 1172 and 1412 are important for the Ajuba-CdGAP interaction A) A ClustalW alignment of the human and mouse CdGAP sequences, ARHGAP30, ARHGAP32 and ARHGAP33 (human). Conserved arginine (1412 in mouse CdGAP) highlighted in red and surrounding conserved amino acids shaded in blue. (*), conserved residues; (:) conserved substitutions. Amino acid number is shown on the right. B-C) *In vitro* translated myc-Ajuba full-length was incubated with GST, GST-CdGAP¹¹⁶⁰⁻¹⁴²⁵ wild-type (CdGAP, WT) or mutated on arginine 1172 (CdGAP R1172A) immobilised on glutathione sepharose beads. Precipitated associated proteins were detected by probing with anti-myc antibody. Amido black shows GST-tagged proteins. C) The amount of Ajuba precipitated with different CdGAP proteins in B was calculated by densitometry using ImageJ. Bound Ajuba was expressed as a percentage of the total Ajuba. Error bars show standard error of the mean. Statistical significance was assessed using Student's T-test. *, $P \leq 0.05$. N=3 D) myc-CdGAP¹¹⁶⁰⁻¹⁴²⁵ wild-type, myc-CdGAP¹¹⁶⁰⁻¹⁴²⁵^{R1172A} or myc-CdGAP¹¹⁶⁰⁻¹⁴²⁵^{R1412A} were produced via *in vitro* translation and incubated with GST or GST-Ajuba LIM domain. Input sample represents 20% of total. Amido black shows GST-tagged proteins. Preliminary data N=1. Molecular weight markers are shown on the left of blots.

5.4. Conclusions

Rho GAPs are vital regulators of GTPases, and as such coordinate multiple cellular processes. This means that understanding how GAP proteins themselves are regulated is crucial if we are to fully unravel the complex pathways governing cellular processes regulated by GTPases. In this chapter I have attempted to discern the mechanism underlying the ability of CdGAP to disrupt E-cadherin cell-cell contacts by probing CdGAP interaction with the scaffold protein Ajuba. Ajuba modulates the localisation of CdGAP-I at junctions and also partially rescues junction disruption caused by CdGAP-I expression. Furthermore, preliminary data suggests that Ajuba is able to directly regulate the GAP activity of CdGAP against Rac.

The LIM domain of Ajuba and the C-terminal of CdGAP mediated the interaction. Fascinatingly, the Ajuba binding site is highly similar to the site bound by CdGAP's GAP domain and the highly conserved nature of these regions could mean they represent important regulatory sites.

Overall these findings suggest that Ajuba coordinates CdGAP activity in order to maintain Rac activation at the membrane for the maintenance of cell-cell contacts. By directly binding to CdGAP, Ajuba is not only able to regulate its localisation, by sequestering CdGAP at the junctions, but also control its activity. The collection of Rac and a Rac GAP at the region of the junction is an elegant means of finely-tuning Rac activity in a confined area allowing for dynamic regulation of Rac at cell-cell contacts.

5.5. Discussion

5.5.1. Probing the binding sites on CdGAP C-terminal of Ajuba and CdGAP GAP domain

Several techniques have been used to firstly confirm and then to investigate the CdGAP-CdGAP and CdGAP-Ajuba binding sites. Pull-down assays both *in vitro* and from cell lysates confirmed the existence of these interactions. Subsequent use of peptide arrays then allowed specific regions responsible for these interactions to be identified. Peptide arrays have been used extensively to identify and characterise protein-protein interactions (Baillie et al., 2007; Gingras et al., 2005; Primeau et al., 2011). However, this technique does have its limitations. Interactions are detected between the chosen protein and a linearised portion of the target protein, devoid of tertiary structure. Thus more favourable, stable binding will be conferred in the properly assembled protein meaning false negatives are possible. False positive binding, as a result of non-specific electrostatic

interactions are also a possibility. Thus, drawing conclusions regarding the reactivity of specific SPOTs must be approached with caution (Katz et al., 2011; Weiser et al., 2005).

For this reason, any conclusions regarding the contributions of individual amino acids should be made based on the relative binding compared to wild-type, should be taken only as a guide, and must be confirmed using other methods (Katz et al., 2011). Despite these drawbacks, several regions were identified that mediate binding between CdGAP-CdGAP and CdGAP-Ajuba. Crucially, these findings were verified using pull-down assays with mutated proteins, thus validating the use of this method.

Both the Ajuba-CdGAP and the CdGAP-CdGAP binding sites appear to involve regions between amino acids 1138 and 1202 and between amino acids 1338 and 1422. However, disruptions in the signal were observed within both of these areas, indicating these may be composed of shorter, discontinuous sites that are brought together when the protein is folded. Without knowledge of the 3D structure and whether the regions identified are surface exposed it is impossible to ascertain this. The first region identified (1138-1202) is in good agreement with data from pull-down assays which predicted the minimal binding site for Ajuba to lie between amino acids 1160-1253 and the yeast two-hybrid which identified amino acids 1064-1183 (**Figure 5.11**). The second region, in the C-terminal may not be essential for binding between CdGAP and Ajuba, as CdGAP₁₂₅₃₋₁₄₂₅ does not bind Ajuba. However identification of this region in the peptide arrays cannot be a false positive as mutation of arginine 1412 to alanine severely compromises binding. Rather, the more C-terminal region may be necessary, but not sufficient for interaction with Ajuba (**Figure 5.11**).

Individual subsets of amino acids, often composed of arginine, tryptophan or tyrosine residues are termed hotspots, as they frequently contribute the majority of the energy necessary for binding within a protein-protein interaction interface (Bogan and Thorn, 1998; Castro and Anderson, 1996). Mutation of arginine 1172 or arginine 1412 profoundly reduced binding of both Ajuba and CdGAP GAP domain to CdGAP C-terminus, indicating that these sites may form part of a hotspot. Both residues are well conserved in CdGAP homologs, but interestingly, arginine 1412 is also found in related GAP proteins ARHGAP30, ARHGAP32 and ARHGAP33. Thus this region, may also facilitate auto-inhibition of CdGAP-related proteins, or alternatively constitute a novel Ajuba binding site. Indeed, ARHGAP30 and CdGAP are known to share binding partners; both can interact with intersectin (via a basic region adjacent to the GAP domain) (Primeau et al., 2011) and Wrch-1, and can signal downstream of this GTPase (Naji et al., 2011).

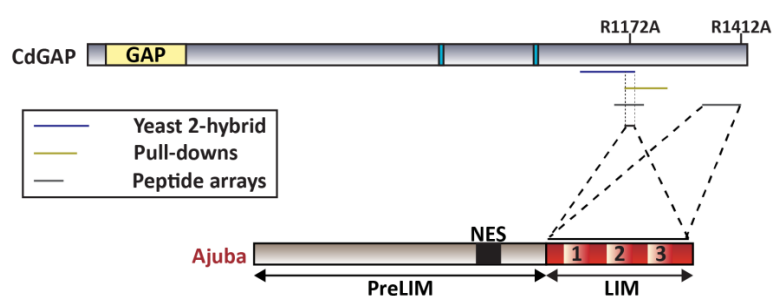


Figure 5.11 Summary of the CdGAP-Ajuba binding site The domain structures of CdGAP and Ajuba. CdGAP is shown on top with the GAP domain in pale yellow and proline-rich regions in light blue. Ajuba is shown below CdGAP with the PreLIM and LIM regions highlighted. The three LIM domains are numbered accordingly. The nuclear export signal (NES) is represented by the black box. Several methods were used to identify the minimal Ajuba binding site on CdGAP. Yeast two-hybrid identified binding of a fragment between amino acids 1064 and 1183 (navy blue line). The pull-down assays revealed Ajuba binding required amino acids from 1160 to 1253 (yellow line). Peptide arrays identified multiple, shorter sites, within the C-terminal (grey lines). Shown are the two longest and most reactive regions identified; the first between amino acids 1138 and 1202 and the second between 1338 and 1422. The importance of these regions was verified using pull-downs with CdGAP mutants with mutations R1172A and R1412A. The minimal binding site on CdGAP is delineated by the dashed black lines, highlighting amino acids 1160 to 1183. The locations of the two arginine mutations are shown above CdGAP. The Ajuba binding site was mapped to the LIM domain.

There is a clear overlap between the binding sites on CdGAP C-terminus for its GAP domain and to Ajuba. However, these sites may rely on slightly different amino acids to facilitate binding. For example, when values are normalised to internal controls for each binding protein, within the first binding region (peptides 12-20, amino acids 1138-1202) peptides 18-20 were much more reactive for Ajuba binding, whilst peptides 12-14 reacted most strongly with CdGAP. However, the close proximity of the bind sites makes it highly likely that binding of either Ajuba or CdGAP-GAP domain would preclude binding of the other.

5.5.2. The regulation of CdGAP localisation to AJs by Ajuba

The co-expression of CdGAP-I and Ajuba partially rescues junction disruption mediated by CdGAP-I. By binding CdGAP, Ajuba could regulate the recruitment of CdGAP to a specific cellular area or control its retention at AJs. The finding that CdGAP-s (which cannot bind Ajuba) is also found at cell-cell contacts and its localisation here is unaffected by Ajuba expression means Ajuba is unlikely to be necessary for CdGAP recruitment, which may be regulated by as yet unidentified proteins. Probably CdGAP and Ajuba are recruited separately to the junction and only associate once there.

Potentially junctional localisation is dictated by activation status. CdGAP-I can interact with itself not only via its C-terminus and GAP domains, but also via binding between two C-terminal regions. Therefore, CdGAP may exist in a folded conformation, as an anti-parallel dimer or as a parallel dimer. The prevailing conformation *in vivo* remains to be determined, but it seems likely that CdGAP-I is predominantly retained in an inactive state. Conversely, although both CdGAP isoforms can be regulated by other mechanisms (eg via interaction with Intersectin, or phosphorylation (Jenna et al., 2002; Tcherkezian et al., 2005), the absence of the C-terminal region on CdGAP-s indicates reduced ability for auto-inhibition with this isoform. The activation of CdGAP may somehow trigger its localisation to junctions, and would explain why a larger proportion of CdGAP-s is present here whilst CdGAP-I localisation is more diffuse.

Once localised to cell-cell contacts, the role of Ajuba may be to retain CdGAP-I here. However, the fact the co-expression of Ajuba and CdGAP-I reduces junction disruption implies that the enrichment of CdGAP-I seen at junctions when co-expressed with Ajuba represents an inactive pool of CdGAP. Ajuba binds CdGAP through Ajuba LIM domain, whereas Rac binding occurs via the Pre-LIM (Nola et al., 2011), meaning Ajuba may be able to simultaneously bind both CdGAP and Rac. The gathering of Rac and a Rac GAP would allow Ajuba to fine-tune Rac signalling and mean Ajuba could promote Rac activation at sites of contact but may also be induced to release CdGAP to quickly and locally reduce

Rac activity if required (for example targeting Ajuba for degradation could be a means of releasing CdGAP). A similar mechanism may be observed for the RhoA GEF GEF-H1 binding to cingulin, which results in inactivation and sequestration of GEF-H1 at TJs (Aijaz et al., 2005). Interestingly, GEF-H1 is required for disassembly of AJ and TJ in SK-CO15 cells in response to calcium removal (Samarin et al., 2007). Thus maintaining this Rho GEF in an inactive state at cell-cell contacts may facilitate rapid modulation of GTPase activation in response to external stimuli (in this case the removal of calcium) (Samarin et al., 2007).

Alternatively, that CdGAP-I is able to efficiently disrupt junctions despite relatively little localising here may indicate that junctional localisation is not necessary for CdGAP to perturb cell-cell contacts. PX-RICS can modulate cell-cell contacts in HeLa cells via regulation of endoplasmic reticulum to Golgi transport of β -catenin and N-cadherin, thereby influencing the amount of N-cadherin present at the cell surface (Nakamura et al., 2008). Whilst we cannot exclude the possibility of CdGAP disruption being mediated from the cytoplasm, there is no obvious enrichment of CdGAP at any other regions of the cell, implying this is not the case. Rather, it is likely that low concentrations of CdGAP are sufficient to disrupt junctions, and/or that CdGAP is only retained at junctions for a short amount of time.

5.5.3. The regulation of CdGAP activity by Ajuba

CdGAP activity can be regulated directly via protein-protein interaction (Jenna et al., 2002; Primeau et al., 2011), phosphorylation (Danek et al., 2007; Tcherkezian et al., 2005) and via binding PIP_3 (Karimzadeh et al., 2012). Thus, Ajuba binding may utilise any of these mechanisms to inhibit CdGAP.

Simultaneous binding of Ajuba and the GAP domain to CdGAP C-terminus would suggest that Ajuba could maintain CdGAP in its closed conformation, whereas if Ajuba binding out-competes the GAP domain, a conformational change in CdGAP upon Ajuba binding is more likely responsible for the reduction in activity. The close proximity of the Ajuba and GAP domain binding sites on the C-terminus of CdGAP mean it is unlikely both Ajuba and the GAP domain can bind simultaneously. Thus, if Ajuba is able to directly inhibit CdGAP activity it is more likely it does so by inducing distinct conformational changes within CdGAP that result in shielding of the GAP domain.

A possible mechanism via which Ajuba may facilitate CdGAP inactivation is via phosphorylation (Danek et al., 2007; Tcherkezian et al., 2005; Tcherkezian et al., 2006). CdGAP is heavily phosphorylated and some regulatory phosphorylation sites have been characterised, including

phosphorylation of threonine 776 by GSK3 β , ERK1/2 and Ribosomal protein S6 kinase (RSK1) (Danek et al., 2007; Tcherkezian et al., 2005). Interestingly, both Ajuba and CdGAP are able to interact with GSK3 β (Danek et al., 2007; Haraguchi et al., 2008). GSK3 β can act downstream of Ajuba in order to negatively regulate Wnt signalling (Haraguchi et al., 2008). Interestingly, GSK3 β phosphorylation of β -catenin requires the presence of Ajuba (Haraguchi et al., 2008). Further, as well as being phosphorylated by GSK3 β , CdGAP protein levels are also regulated in a GSK3 β -dependent manner in response to serum (Danek et al., 2007). Thus, CdGAP can be regulated in two ways by the actions of GSK3 β . Alternatively, CdGAP may be a novel PAK1 substrate, a kinase we know is present at the junctions in keratinocytes and phosphorylates Ajuba (Nola et al., 2011), although no PAK1 sites have yet been identified on CdGAP.

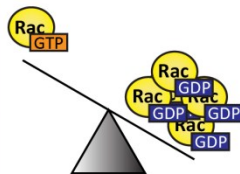
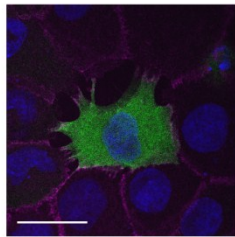
Together, a picture emerges whereby upon activation, CdGAP is localised to cell-cell contacts where it inactivates Rac (and potentially Cdc42) leading to the removal of E-cadherin from the junctions. In the presence of Ajuba, CdGAP is sequestered in an inactive form at junctions which prevents junction disassembly and also explains how Ajuba depletion can decrease Rac activation at cell-cell contacts (Nola et al., 2011) (**Figure 5.12**).

5.5.4. Other processes CdGAP and Ajuba might regulate

Ajuba has been implicated in a range of cellular processes regulating development and homeostasis (Das Thakur et al., 2010; Hou et al., 2008; Kanungo et al., 2000; Langer et al., 2008; Nola et al., 2011; Pratt et al., 2005). Conceivably, Ajuba may be required to regulate CdGAP activity in other cellular processes controlled by Rac (or Cdc42), where it is necessary to spatially confine signalling. In U2OS cells the depletion of CdGAP promotes migration in wound healing assays and cells display lamellipodia that rapidly extend and retract (LaLonde et al., 2006; Wormer et al., 2012). Conversely, Ajuba null MEFs, when stimulated with platelet-derived growth factor (PDGF) to migrate in wound healing assays, exhibit reduced activation of Rac at the leading edge and display defective lamellipodia resulting in retarded migration (Pratt et al., 2005). In these cells Ajuba signals upstream of p130Cas and is required to promote its localisation at focal adhesions (Pratt et al., 2005). A second role of Ajuba may be to inhibit CdGAP activity. Indeed, CdGAP is phosphorylated (and inhibited) *in vivo* in response to PDGF treatment in Swiss 3T3 fibroblasts (Tcherkezian et al., 2005). Perhaps Ajuba is required to facilitate phosphorylation of CdGAP in migrating cells downstream of PDGF, leading to inactivation of CdGAP at the leading edge and promoting Rac activation required for migration.

Expression of CdGAP-I

Loss of E-cadherin
Balance in favour of Rac.GDP



Co-expression of CdGAP and Ajuba

Uniform E-cadherin junctions
Balance in favour of Rac.GTP

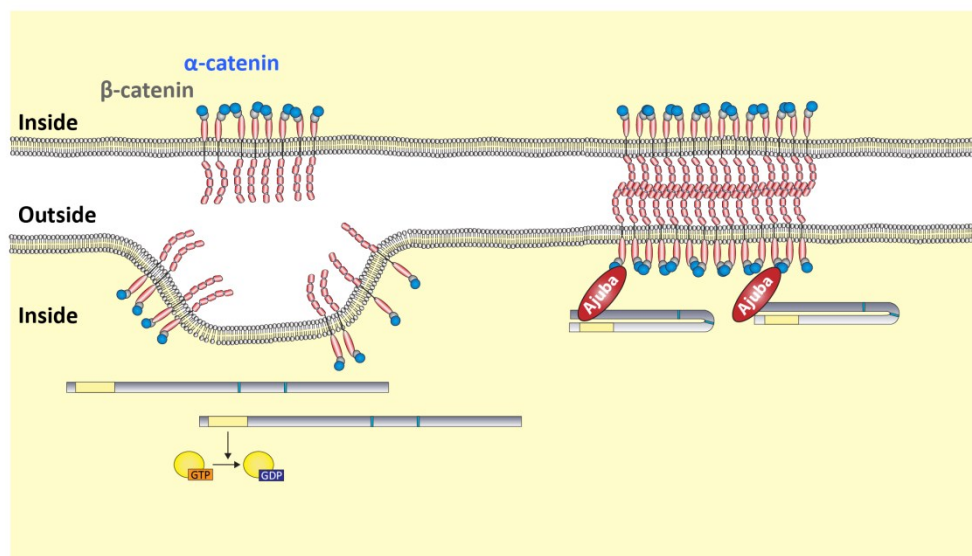
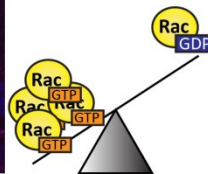
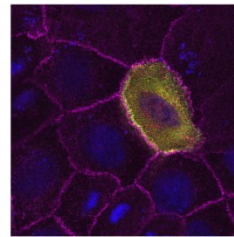


Figure 5.12 Summary of the potential mechanism employed by Ajuba to regulate CdGAP at junctions

Fluorescent images shown above the diagram are representative of cells expressing CdGAP (left) or co-expressing CdGAP and Ajuba (right) (scale bar is 25um). The expression of CdGAP results in inactivation of Rac at the membrane, shifting the balance in favour of Rac.GDP. E-cadherin is removed from the junctions and cells retract from their neighbours (left side). In cells expressing CdGAP and Ajuba junctions remain intact. CdGAP is accumulated at cell-cell contacts but is recruited separately from Ajuba. Ajuba binds to CdGAP at the C-terminus and may inhibit CdGAP activity either directly or indirectly via co-localising CdGAP with a kinase or another protein that can shield its GAP domain.

Ajuba is the first protein described able to interact with the C-terminal of CdGAP and thus the first able to exclusively regulate CdGAP-I and not CdGAP-s activity. Beyond knowledge of their tissue-specific expression patterns little is known about the isoform-specific roles of CdGAP (Tcherkezian et al., 2005; Tcherkezian et al., 2006) but potentially tissue-specific roles may be partially explained by an interaction with Ajuba.

5.6. Future work

I have shown that Ajuba is able to inhibit CdGAP's GAP activity and prevent CdGAP-mediated junction disruption. Future work should initially aim to better characterise the mechanism behind Ajuba's regulation of CdGAP. The Ajuba-binding-deficient mutants, as well as CdGAP peptides can be utilised to achieve this. Specifically, whether Ajuba induces conformational changes in CdGAP or regulates CdGAP activity via phosphorylation should be confirmed. Following this, a more thorough analysis of the dynamic CdGAP-Ajuba interaction, including how CdGAP may be released from Ajuba to inactivate Rac should be investigated. Finally, a role for Ajuba in AOS should be examined.

5.6.1. Characterisation of the Ajuba-binding deficient mutants

A useful tool to investigate the role of Ajuba in CdGAP regulation will be the CdGAP mutants that cannot bind Ajuba as efficiently as wild-type. Firstly, this result needs to be confirmed for the R1412A mutant, as data is only preliminary. Secondly, it may be necessary to create further mutants that have further reduced capacity for Ajuba binding (as the current mutants can still bind weakly to Ajuba). This may be achieved by mutation of two or more amino acids within the critical binding regions.

Given the similarity in the CdGAP-Ajuba and CdGAP-CdGAP binding sites these mutations are also likely to prevent or inhibit binding to CdGAP GAP domain, and thus could represent hyperactivated constructs. It will be important to determine this before any analysis is done *in vivo* as this will greatly influence the interpretation of results.

If CdGAP mutants do bind to the GAP domain normally, but are unable to bind Ajuba, these should be co-expressed with Ajuba in cells and their effects on cell-cell contacts observed. If Ajuba is required to inhibit CdGAP, these mutants should disrupt E-cadherin contacts with the same efficiency as cells expressing CdGAP-I alone and also not be retained at the junctions. If this is not

the case it would be interesting to investigate the possibility of other CdGAP-binding partners that may control localisation and/or activity.

Also, to confirm that the GAP activity of the non-binding mutants is not affected by the presence of Ajuba, *in vitro* GAP assays can be performed. If mutants can neither bind Ajuba or the GAP domain, we may expect these to induce even more junction disruption than the wild-type, but it will be difficult to interpret results from such mutants.

5.6.2. How does Ajuba regulate the localisation of CdGAP – recruitment vs retention?

Expression of CdGAP mutants that are unable to bind Ajuba can confirm whether Ajuba regulates the recruitment of CdGAP-I to cell-cell contacts. If Ajuba has no role in recruitment, the localisation of the mutant CdGAP should mirror that of wild-type CdGAP-I in the absence of Ajuba.

My data suggests that Ajuba does not recruit CdGAP to cell-cell contacts, but may instead contribute to the retention of CdGAP in this region. FRAP experiments can be used to measure the mobility of GFP-CdGAP in the presence and absence of Ajuba. If Ajuba is holding CdGAP-I at contacts then we can expect the mobility of GFP-CdGAP to be reduced in the presence of Ajuba. The use of a GFP-tagged CdGAP mutant that is deficient in Ajuba binding will also corroborate this result as this construct should be more mobile than the wild-type CdGAP if Ajuba is required to maintain CdGAP at contacts.

5.6.3. How does Ajuba regulate the activity of CdGAP – directly or indirectly?

Ajuba inhibits CdGAP's GAP activity and prevents CdGAP-mediated junction disruption. I envisage this regulation could occur either by a direct interaction that prevents CdGAP opening or changes the conformation of CdGAP, or indirectly by localising CdGAP with a kinase that phosphorylates and inhibits CdGAP.

To formally demonstrate if direct interaction with Ajuba inhibits CdGAP, GAP assays using purified proteins could be performed. This would rule out the influence of other mechanisms of regulation, such as post-translational modification, which may occur in cells.

To understand how Ajuba is able to inhibit CdGAP it will be important to learn if Ajuba and CdGAP GAP domain can bind simultaneously to the C-terminus. To address this question, plates coated with

CdGAP C-terminus can be incubated with equal amounts of GAP domain and Ajuba to determine 1) whether both are able to bind and 2) which binds preferentially by observing the proportions of each protein bound. By varying the amounts of GAP domain and Ajuba, we can test for competition between these proteins for interaction with the C-terminus of CdGAP. By maintaining the concentration of GAP domain, and adding increasing amounts of Ajuba it will be possible to see if Ajuba can displace the GAP domain for binding to C-terminus.

Peptides have been synthesised covering the strongest binding regions of the C-terminal. It would be most informative though, to characterise a range of peptides with different affinities for Ajuba and CdGAP GAP domain in order to fully separate signalling downstream of CdGAP activation dependent on Ajuba. These can be used to test for competition between CdGAP and Ajuba for binding of the C-terminus. It would then be useful to determine if these peptides could out-compete the C-terminal protein for binding to either Ajuba or the GAP domain. With the necessary modifications these peptides could then be used *in vivo* to disrupt these interactions.

As CdGAP activity is regulated in part by its C-terminal domain, visualisation of the activation of CdGAP may be ideally suited for FRET (Che et al., 2005; Itoh et al., 2002). This could be an important tool for monitoring how and where CdGAP is activated and how Ajuba can regulate this. By creating a unimolecular CdGAP construct harbouring GFP and RFP tags on the C- and N-terminals it may be possible to visualise activation using FLIM-FRET. The placing of the tags is critical as these must be sufficiently close to the protein that FRET is possible, but far away enough that their presence does not influence binding of the C- to the N-terminal. As amino acids in the very C-terminal of CdGAP are important for the interaction, this may be problematic. However, if these criteria can be met several things could be inferred. Firstly, *in vitro* experiments can be performed whereby the purified CdGAP probe is incubated with purified Ajuba and FRET is measured would complement *in vitro* GAP assays; if Ajuba inhibits CdGAP via changes in the conformation of CdGAP this will be reflected by a decrease in FRET, as the C- and N-terminal domains come closer together. Secondly, expression of the CdGAP probe in keratinocytes and measurement of FLIM-FRET at different regions of cell will reveal where CdGAP activity is highest. Finally, co-expression of the CdGAP probe and Ajuba and measurement of FLIM-FRET will uncover whether Ajuba expression reduces CdGAP activity *in vivo*.

Ajuba may also or additionally regulate CdGAP via directing its phosphorylation. CdGAP has the potential to be heavily phosphorylated (Tcherkezian et al., 2005), and phosphorylation by RSK1, ERK1/2 and GSK3 β can all result in inactivation of CdGAP (Danek et al., 2007; Tcherkezian et al., 2005; Tcherkezian et al., 2006). It would be interesting to determine if and on which residues CdGAP

is phosphorylated on at cell-cell contacts and if this is altered in the presence of Ajuba. Keratinocytes transfected with CdGAP and Ajuba or CdGAP alone could be labelled with [P^{32}]orthophosphate before lysis and immunoprecipitation of CdGAP. By detecting radiolabelled proteins by autoradiography (using SDS-PAGE), it would be possible to see if Ajuba enhances the degree of phosphorylation of CdGAP. *In vitro* kinase assays could be performed to determine if CdGAP is phosphorylated by GSK3 β or PAK1 in keratinocytes, and if this is influenced by the presence of Ajuba.

5.6.4. How is Rac and Cdc42 activity influenced by Ajuba and CdGAP?

As Ajuba seems to inhibit CdGAP GAP activity on Rac, it would be useful to determine if this translates to an increase in Rac activity at the membrane of cells co-expressing CdGAP and Ajuba. Co-expression of wild-type or mutant CdGAP unable to bind Ajuba with the unimolecular Rac1-PAK1 probe tagged with GFP and RFP would allow spatial data to be gathered to answer this question. If Ajuba binding is required to modulate Rac signalling through CdGAP, the CdGAP mutant unable to bind Ajuba will have a different lifetime to the wild-type construct. We would predict that this mutant is more active, and thus have a decreased lifetime compared to wild-type.

5.6.5. Does the regulation of CdGAP by Ajuba have clinical relevance in AOS or cancer?

Mutations in Rac and Cdc42 regulators DOCK6 and CdGAP have been found to cause AOS (Shaheen et al., 2011; Southgate et al., 2011). However, CdGAP mutations only account for a fraction of AOS cases (Southgate et al., 2011). Therefore, mutations in proteins necessary for regulating CdGAP may yet be identified in cases of AOS. It would therefore be interesting to see if any mutations in the Ajuba gene are associated with this disorder, using a similar approach to that used to identify CdGAP mutations (Southgate et al., 2011).

As CdGAP expression promotes invasion in breast epithelial cells (He et al., 2011) and my data shows CdGAP expression perturbs cell-cell contacts, CdGAP may play a role in cancer progression in some cells types. In this case it would be interesting to determine if CdGAP expression could be negatively correlated with Ajuba expression in tumours.

Chapter 6.

Final Discussion

6.1. Summary

Using both traditional (yeast two-hybrid) and contemporary (RNAi) screening methods, numerous Rho GAPs have been identified that can regulate distinct stages in the formation, maintenance and disassembly of AJs in keratinocytes (**Figure 6.1**). The siRNA screen utilised novel thresholding-based image analysis software to assess the effects of depletion of 206 proteins (46 GAPs) on junction formation. The efficacy of this screen is verified via the presence in the hit proteins of several Rho GAPs known to regulate cell-cell contacts and through characterisation of the function of one hit protein, ARAP1, in keratinocytes.

Additionally, I have shown that CdGAP regulates the maintenance of AJs and may participate in the disassembly of cell-cell contacts during development or in a pathological setting. The expression of CdGAP in keratinocytes severely disrupts AJs due to the misregulation of either (or both) of Rac and Cdc42. CdGAP binding to the scaffold protein Ajuba was first demonstrated in a yeast two-hybrid screen (A. Ferrand and D. Birnbaum) and I have confirmed that this interaction is direct and mediated by a seemingly well-conserved region on the CdGAP C-terminus. Ajuba is known to regulate Rac activity both at cell-cell contacts and during migration (Nola et al., 2011; Pratt et al., 2005). At AJs, Ajuba functions to maintain Rac activation at cell-cell contacts, but does not regulate the recruitment of Rac (Nola et al., 2011). I demonstrate that Ajuba may be required to sequester CdGAP at AJs in order to inhibit its activity against Rac, therefore promoting Rac activation and maintaining cell-cell adhesion.

6.2. Too many GAPs

Previous screens conducted to identify regulators of cell-cell contacts have involved the visual analysis of target genes, or used embryo lethality as a single readout (Elbediwy et al., 2012; Lynch et al., 2012; Omelchenko and Hall, 2012). However, a far more comprehensive understanding of cellular systems is gained from gathering multiparametric data (Fuchs et al., 2010; Winograd-Katz et al., 2009). We have optimised the use of an automated analysis program that is able to effectively segment junctional E-cadherin and used this to measure three experimental parameters associated with junction formation. This approach can therefore be used in the future to identify additional regulators of cell-cell contacts, with the possibility of segmenting different junctional proteins (eg. α -catenin). Using this method several Rho GAPs were identified that have been described subsequently as regulators of cell-cell contacts (eg. (Ratheesh et al., 2012; Tripathi et al., 2012)).

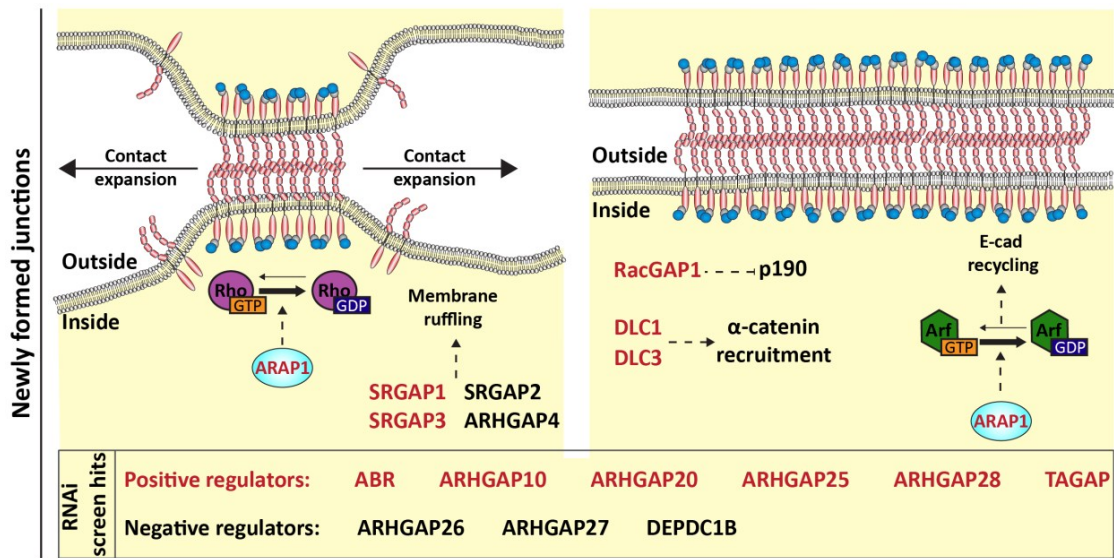
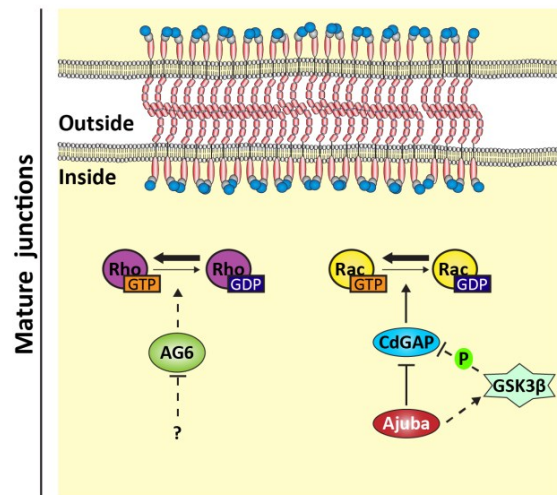
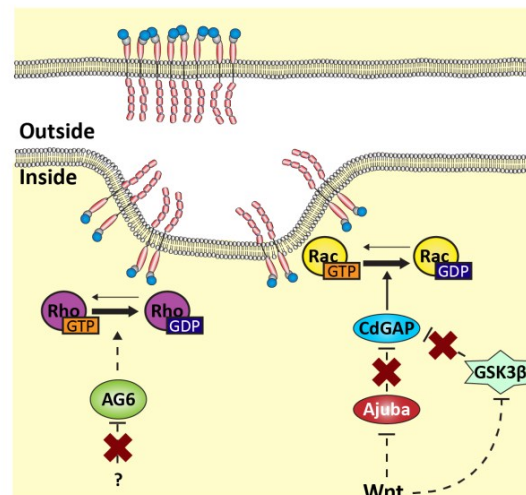
A**Junction formation (30' Ca²⁺ switch)****Junction stabilisation (30' adhesion)****B****Junction maintenance****C****Junction disassembly**

Figure 6.1 Summary of potential Rho GAP signalling pathways regulating junction formation, stabilisation, maintenance and disassembly

The Rho GAPs studied in this thesis, ARAP1, ARHGAP6 (AG6) and CdGAP are shown in ovals. A) GAPs identified as regulators of junction formation or stabilisation via the siRNA screen (hits) are shown in red (positive regulators; depletion reduced E-cadherin and/or junctional actin) or black (negative regulators; depletion increased E-cadherin and/or junctional actin). Hits for which a role in cell-cell contacts has not previously been described are shown in the box. Hits previously demonstrated to regulate cell-cell contacts are SRGP-1 (Zaidel-Bar et al., 2010), of which the mammalian homologs SRGAP1-3 and ARHGAP4 are shown, RacGAP1 (Ratheesh et al., 2012), DLC1 (Tripathi et al., 2012; Tripathi et al., 2013) and DLC3 (Holeiter et al., 2012). ARAP1 regulates the formation or early stabilisation of cell-cell contacts. Possible functions of ARAP1 may be to inhibit Rho activity at the centre of the nascent contact (arrows indicate the direction of contact expansion) or to regulate Arf1 or Arf5 to control E-cadherin trafficking. B) At mature cell-cell contacts AG6 and CdGAP are likely inhibited to prevent local inactivation of Rho and Rac/Cdc42 respectively. It is unknown how AG6 is inhibited but CdGAP may be inhibited by Ajuba either directly, or via a kinase, possibly GSK3β. C) Junctions may be disassembled as a result of overactivation of AG6 or CdGAP to imbalance levels of active Rho and Rac/Cdc42. Possibly extracellular signals (eg. Wnt) trigger Ajuba is degradation (Haraguchi et al., 2008) leading to the release of CdGAP, or the loss of phosphorylation signals (eg. from GSK3β, which is regulated by Wnt and Ajuba (Haraguchi et al., 2008)). Free CdGAP may then inactivate Rac at the membrane and promote junction disassembly.

Rho regulators (the GAPs and GEFs) considerably outnumber their GTPase targets and this is likely due to these proteins performing specific functions in specialised processes (Bernards, 2003; Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007). Rho GAPs and GEFs possess wide-ranging characteristics that allow them to control Rho GTPase signaling specificity by influencing GTPase localisation, receiving upstream signals and recruiting downstream effectors (Rossman et al., 2005; Tcherkezian and Lamarche-Vane, 2007). Consequently, multiple Rho regulators are required to correctly regulate complex cellular processes that rely on GTPase signaling, such as cell-cell contact formation, neuronal morphogenesis and migration (McCormack et al., 2013; Tolia et al., 2011; Tomar and Schlaepfer, 2009). Thus, the identification of 20 Rho GAPs potentially regulating cell-cell contact formation and stabilisation is not necessarily surprising. Examination of this group revealed a diverse set of proteins with disparate functions in other cellular processes which likely reflects the requirement of cells to coordinate a multitude of processes in order to correctly assemble cell-cell contacts.

Although a certain level of redundancy exists between related GAPs, even highly similar proteins fulfill niche functions that mean their loss cannot be compensated for in certain scenarios. For example, ABR and BCR are highly similar proteins involved in regulation of the immune response (Cho et al., 2007; Cunnick et al., 2009; Voncken et al., 1995) and comparison of macrophages derived from ABR-, BCR- and double ABR/BCR-null mice reveal that to an extent, loss of one protein can compensate for the other. ABR or BCR-null single knockouts show no difference in the release of granules from neutrophils compared to wild-type, whilst this is significantly enhanced in double knockout mice (Cunnick et al., 2009). However, macrophages from BCR-null and double knockout mice produce more ROS than wild-type controls whereas ABR-null mice do not (Cunnick et al., 2009; Voncken et al., 1995). This suggests ABR and BCR have some overlapping functions, but also regulate distinct processes (Cunnick et al., 2009).

The identification of several related GAPs (for example SRGAPs 1-3 and ARHGAP4) in our screen suggests that these proteins may all regulate similar underlying processes, indeed SRGAPs1-3 are known to share binding partners (Wong et al., 2001). However, the fact that loss of any one of these proteins can alter E-cadherin and/or actin populations at cell-cell contacts illustrates that they cannot compensate for each other. Interestingly, these proteins are active on different GTPases (Endris et al., 2002; Foletta et al., 2002; Guerrier et al., 2009; Wong et al., 2001; Yang et al., 2006) and the BAR domains of SRGAPs 1-3 can all induce different effects on membrane curvature (Coutinho-Budd et al., 2012), implying these may perform novel roles in the regulation of cell-cell contact formation and stabilisation.

It is likely that a range of Rho GAPs, specific for different GTPases, with different interacting domains, binding partners and enzymatic capabilities are necessary for cell-cell contact formation. The subsequent characterisation of ARAP1 and ARHGAP6 at cell-cell contacts illustrates that the shared ability to regulate a GTPase, in this case Rho, is not always a good predictor of functional outcome downstream of regulator and/or GTPase activation/inactivation.

6.3. Rho GAPs with opposing functions at AJs: ARHGAP6 and ARAP1

The requirement for Rho at cell-cell contacts is somewhat controversial. In keratinocytes Rho is required for contact formation (Braga et al., 1997; Calautti et al., 2002) whilst Rho-mediated actomyosin contractility can drive contact expansion in MDCK cells (Yamada and Nelson, 2007). However, Rho is downregulated in some systems downstream of adhesion, and activation can promote contact disassembly following certain stimuli (Noren et al., 2003; Noren et al., 2001; Sahai and Marshall, 2002b; Samarin et al., 2007). What is clear is that spatial constraint of Rho signaling is vital. For example, inhibition of ROCK prevents contact expansion, but does not result in disassembly of the contact (Yamada and Nelson, 2007). This implies that following initial contact formation Rho signaling is required at the edges of expanding contact but either not at the initial site, or is required to signal through a different subset of effectors in this region.

It is interesting that two Rho-specific GAPs, ARAP1 and ARHGAP6, are found to regulate cell-cell adhesions in keratinocytes but seem to have opposite effects (**Figure 6.1**). Loss of ARAP1 does not prevent junction formation, but reduces the amount of E-cadherin present at junctions, whilst the overexpression of ARAP1 has no effect on junction morphology. These findings suggest that ARAP1 is not required for formation, but more likely supports the stabilisation of nascent AJs. In contrast, the expression of ARHGAP6 severely disrupts E-cadherin and results in the generation of protrusions. Studies of ARHGAP6 expression in *Drosophila* compliment these findings, and show expression of ARHGAP6 promotes migration and disrupts Rho activation (L A. Baena-Lopez and J-P. Vincent). In both cases the GAP domain is required. ARHGAP6 therefore, negatively regulates AJs.

The interaction of GTPases with their downstream effectors determines the cellular outcome of their activation, and Rho activation has been shown to both promote and disrupt cell-cell junctions depending on which effector it binds (Sahai and Marshall, 2002b). Rho regulators can influence downstream signaling by recruiting effector proteins, thus it will be interesting to determine if Rho interacts with a different subset of effector proteins in ARAP1-depleted cells compared to in wild-type cells.

Currently, far more work is needed in order to characterise the functions of these proteins at cell-cell contacts. Whilst ARHGAP6 appears to act via inactivation of Rho (expression of ARHGAP6 isoforms lacking the complete Rho GAP domain does not perturb junctions), the function of ARAP1 may be to inactivate Arf1 or Arf5 (Miura et al., 2002). For example, depletion of the Arf1 GEF GBF1 in *Drosophila* perturbs DE-cadherin trafficking (Szul 2011) and loss of both Arf1 and Arf5-specific GAPs can disrupt cell-matrix adhesive complexes (Liu 2002, 2005, Moravec 2012). Overall, these findings hint at the existence of a highly dynamic system that would allow cells to respond rapidly to extracellular cues to inactivate Rho and bring about very specific downstream effects depending on the GAP protein activated. If two Rho-specific GAPs are able to elicit such different responses, then regulation of the localisation and activation of GAP proteins must clearly be tightly controlled.

6.4. CdGAP and Ajuba regulation of AJs

In chapters 4 and 5 I have presented evidence for the involvement of CdGAP in the stabilisation of AJs. Depletion of CdGAP results in cells better able to withstand trituration in aggregation assays (although at this point we cannot exclude the possibility that loss of CdGAP leads to an increase in desmosome-mediated adhesion also), whilst expression of CdGAP severely perturbs morphology and leads to the rapid loss of E-cadherin from junctions. Subsequently, I have shown that CdGAP interacts directly with Ajuba, a scaffold protein known to regulate Rac activity at cell-cell contacts (Marie et al., 2003; Nola et al., 2011). Taken together, these data compliment previous studies of CdGAP and points towards an important role for CdGAP in the development of the ectoderm via regulation of both cell-cell and cell-matrix adhesions (LaLonde et al., 2006; Southgate et al., 2011; Wormer et al., 2012). Furthermore, the observation that CdGAP expression rapidly induces loss of cell-cell contacts supports the notion that CdGAP activity maybe hijacked in epithelial cancers to promote migration and invasion (He et al., 2011).

Expression of CdGAP in keratinocytes leads to the removal of E-cadherin from AJs and causes cells to display a severely retracted morphology. Interestingly, cells expressing CdGAP do not seem to undergo apoptosis and appear reminiscent of cells stimulated with HGF that are undergoing epithelial-to-mesenchymal transition (de Rooij et al., 2005). The co-expression of CdGAP with Ajuba limits disruption and also results in increased levels of CdGAP at junctions. Several questions remain regarding how Ajuba may regulate CdGAP in order to promote maintenance of cell-cell contacts. As CdGAP-s (which cannot interact with Ajuba) is still able to localise to AJs, Ajuba is unlikely to regulate the recruitment of CdGAP, which must be mediated by an as yet unidentified mechanism. Presumably then, Ajuba regulates CdGAP activity and maintains CdGAP in an inactive state at

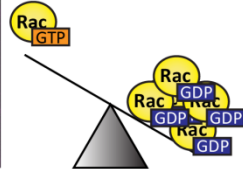
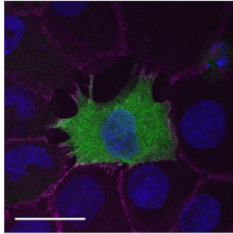
junctions. The inactivation of CdGAP may be mediated by 1) a direct interaction with Ajuba that induces conformational changes in CdGAP such that the GAP domain is obscured, 2) Ajuba may colocalise CdGAP with a kinase that phosphorylates CdGAP (**Figures 6.1 and 6.2**) (eg. (Jenna et al., 2002; Okabe et al., 2003; Tcherkezian et al., 2005; Wells et al., 2006).

I have shown Ajuba and CdGAP interact directly, and this interaction depends on several regions within the C-terminus of CdGAP that also appear to be conserved across species, and possibly in the related proteins, ARHGAP30, ARHGAP32 and ARHGAP33. This region is important for CdGAP auto-regulation (Southgate et al., 2011). However, the close proximity of the binding sites for CdGAP N-terminus and Ajuba on the C-terminus does imply that simultaneous binding of both Ajuba and the GAP domain to the C-terminal region of CdGAP would be unlikely. As such, it is unlikely that Ajuba simply maintains CdGAP in its inactive conformation (although its binding may still induce conformational changes in CdGAP that renders it inactive). Ultimately, in order to better understand both the Ajuba-CdGAP and the potential auto-inhibited CdGAP conformation more information regarding the 3D structures of these proteins is required.

A second possibility is that Ajuba facilitates the action of a kinase on CdGAP, as CdGAP is heavily phosphorylated and this negatively regulates its activity (Danek et al., 2007; Tcherkezian et al., 2005; Tcherkezian et al., 2006). The kinases GSK3 α , GSK3 β , ERK1/2 and RSK1 have all been identified as targeting CdGAP (Danek et al., 2007; Tcherkezian et al., 2005; Tcherkezian et al., 2006) and further kinases may also be identified able to phosphorylate CdGAP. As well as negatively regulating CdGAP, GSK3 β also interacts with Ajuba (Danek et al., 2007; Haraguchi et al., 2008). Ajuba binds to β -catenin and promotes its interaction with GSK3 β , resulting in phosphorylation and degradation of β -catenin, which ultimately prevents β -catenin from translocating to the nucleus to activate Wnt-dependent genes (Haraguchi et al., 2008). Thus, Ajuba may utilise a similar approach to inactivate CdGAP and maintain cell-cell contacts. Sequestering CdGAP in an inactive state at AJs may mean cells are able to rapidly respond to cues to locally inactivate Rac. Potentially, under certain stimuli Ajuba releases CdGAP which would result in the rapid and specific inactivation of Rac at these sites (**Figure 6.2**).

Expression of CdGAP-I

Loss of E-cadherin
Balance in favour of Rac.GDP



Co-expression of CdGAP and Ajuba

Uniform E-cadherin junctions
Balance in favour of Rac.GTP

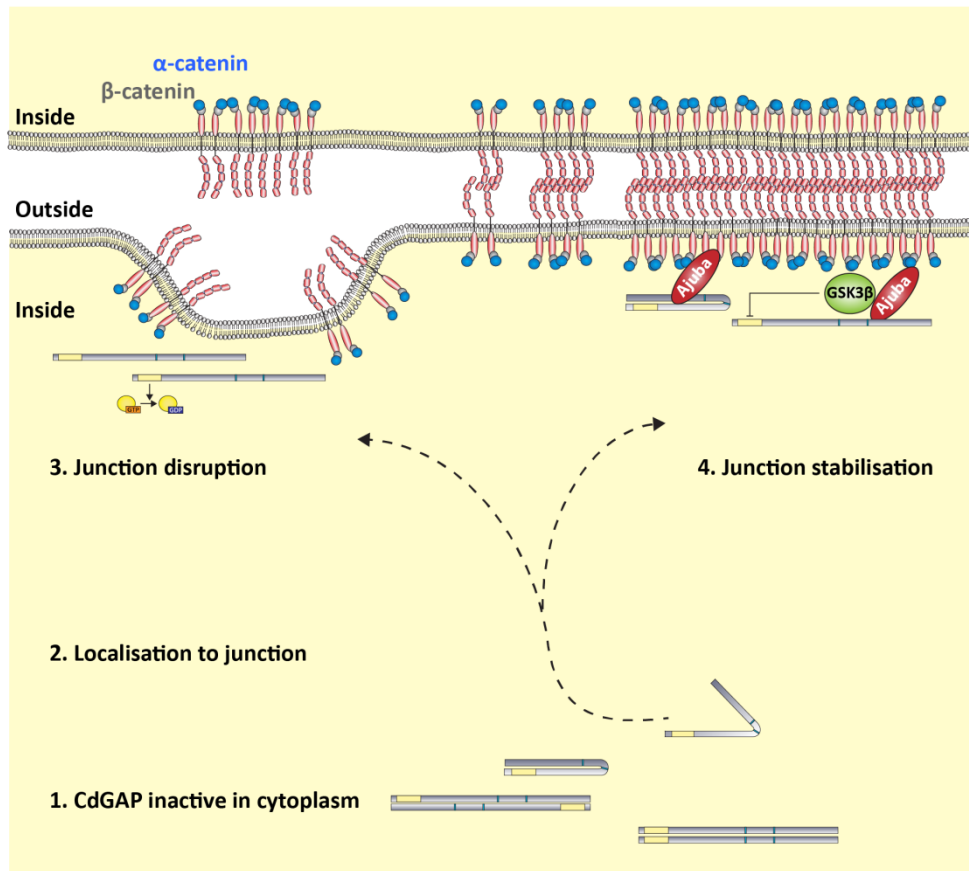
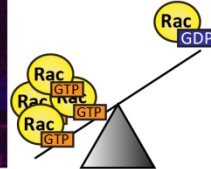
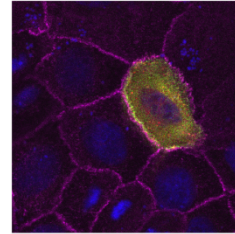


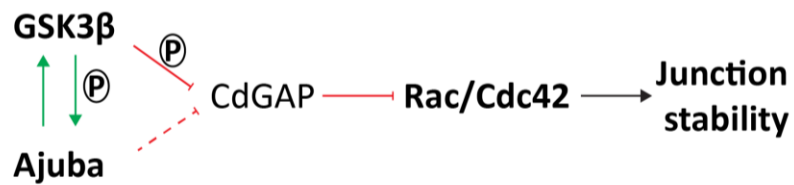
Figure 6.2 Possible mechanism for the regulation of CdGAP by Ajuba at cell-cell junctions The cellular outcomes on AJs are compared in CdGAP or CdGAP and Ajuba expressing cells. Representative images are shown at the top; scale bar 25μm. Expression of CdGAP alone induces loss of E-cadherin at junctions and inactivation of Rac at the membrane, thus the balance of active to inactive Rac is shifted in the favour of inactive, GDP-bound Rac. Co-expression of CdGAP and Ajuba shifts the balance in favour of active Rac, via either direct or indirect inactivation of CdGAP. 1. CdGAP is largely found in the cytoplasm where it is likely inactive. I speculate that CdGAP may exist in several conformations that could potentially shield the GAP domain; a folded monomer, a parallel dimer or an anti-parallel dimer. 2. How CdGAP is localised to junctions is not clear but does not require Ajuba. Junctional localisation may be triggered by activation, as more CdGAP-s (which may be hyperactivated) is found at junctions than CdGAP-I. 3. In the absence of Ajuba, CdGAP causes junction disassembly via inactivation of Rac at the membrane. 4. In the presence of Ajuba CdGAP is sequestered at junctions and presumably inactivated. Ajuba may inactivate CdGAP directly, via conformational changes, or indirectly, by colocalising CdGAP with a kinase (such as GSK3β).

The finding that mutations in CdGAP that result in truncated, hyperactive protein are a cause of AOS reveals a role for CdGAP in the developing limb bud (patients present with loss of skin from the scalp, defects in limb and digit formation and sometimes heart or pulmonary complications (Snape et al., 2009; Southgate et al., 2011). The formation of the limb bud requires precise control of Rac and Cdc42 activities (Aizawa et al., 2012; Suzuki et al., 2009; Wu et al., 2008) and CdGAP may be important in spatial constraint of Rac and Cdc42 signalling to control migration (Southgate et al., 2011) and AJ disassembly (this work). Interestingly, Wnt signalling is vital for the formation of the apical ectodermal ridge, an important signalling centre that controls growth and patterning of the limbs (Barrow et al., 2003; Hill et al., 2006) and a lack of Wnt, or conditional loss of β -catenin also result in the formation of truncated limbs (Barrow et al., 2003; Hill et al., 2006). In order to enhance the stabilisation of β -catenin (necessary so that it may localise to the nucleus and regulate transcription), Wnt signalling promotes the degradation of Ajuba (Haraguchi et al., 2008).

It is interesting therefore to speculate that Wnt signalling may also regulate CdGAP activity indirectly by modulating the levels of Ajuba and GSK3 β , and could be the signal required to release CdGAP from Ajuba, leading to junction disassembly. This would result in inactivation of Rac/Cdc42 at junctions and may promote epithelial-to-mesenchymal transition and early detachment from neighbouring cells prior to migration. The activation of CdGAP must be carefully localised as hyperactivation of CdGAP is detrimental to limb bud formation (Southgate et al., 2011) and loss of Rac inhibits the localisation of β -catenin to the nucleus (Wu et al., 2008). However, the sequestration of CdGAP at AJs by Ajuba may ensure that CdGAP activity is appropriately restricted and further (as yet unidentified) mechanisms are likely also to be in place (**Figure 6.3**).

Negative feedback loops must exist that later inhibit CdGAP, potentially by stabilising GSK3 β which in turn stabilises Ajuba (Haraguchi et al., 2008). Alternatively, as GSK3 β can also upregulate CdGAP mRNA (Danek et al., 2007), loss of GSK3 β may result in decreased levels of CdGAP, meaning only a limited amount of CdGAP is available to initially inactivate Rac/Cdc42. Such a regulatory network may also be exploited in epithelial cancers, as CdGAP is upregulated in mammary tumour explants and promotes migration and invasion of these cells (He et al., 2011). Possibly, upregulation of CdGAP leads to junction disassembly, loss of cell-cell contacts and epithelial-to-mesenchymal transition. It would therefore be of interest to determine if Ajuba or GSK3 β are downregulated in mammary epithelial cancers that show increased levels of CdGAP, or if expression of Ajuba or GSK3 β can inhibit TGF- β -induced migration and invasion (He et al., 2011).

A



B

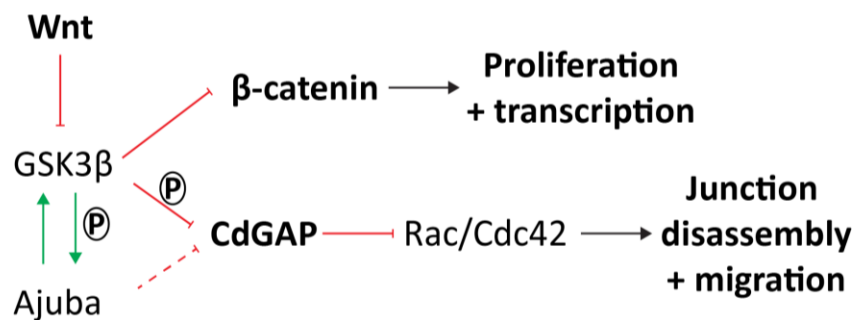


Figure 6.3 Possible signalling pathways regulating CdGAP activity A) In the presence of Ajuba, CdGAP activity may be inhibited either directly, or through facilitating the activity of GSK3β on CdGAP, which phosphorylates and inactivates CdGAP (Danek et al., 2007). Ajuba is also phosphorylated by GSK3β, which stabilises Ajuba and prevents its degradation (Haraguchi et al., 2008) (possibly to maintain CdGAP inactivation). The inhibition of CdGAP by either means may prevent CdGAP inactivating Rac/Cdc42 thus supporting the maintenance of AJs. B) Wnt signaling inhibits GSK3β and promotes the degradation of Ajuba. As well as promoting β-catenin translocation to the nucleus and the upregulation of Wnt genes, I speculate that this may lead to an increase in CdGAP activity. CdGAP may then inactivate Rac and/or Cdc42 resulting in junction disassembly. Black arrows indicate cellular outcome, red arrows indicate inhibition and green arrows indicate activation. Dotted lines represent unconfirmed inhibition. P indicates phosphorylation events. Proteins in bold typeface represent an increase in either their levels or their activity status.

6.5. Conclusions

What emerges from this thesis and other studies is that Rho GAPs are not only required to control the inactivation of their target GTPases (including where, when and for how long activation persists), but also strongly influence the outcome of GTPase activation. Time and time again activation of the classical GTPases Rac, Rho and Cdc42 is shown to result in downstream signalling through the same few effectors (ROCK, PAK, LIMK etc) but with vastly different consequences. Indeed, my findings demonstrate that two Rho GAPs, ARAP1 and ARHGAP6 can have completely different effects on cell-cell junctions. As such, the regulation of the activity and localisation of Rho GAPs may be just as important as the regulation of their GTPase targets.

I have shown that CdGAP potentially induces cell-cell contact disassembly. Misregulation of CdGAP can have devastating consequences, as it spatially constrains Rac and Cdc42 activity in different cellular processes, developmental and pathological events (He et al., 2011; Jenna et al., 2002; LaLonde et al., 2006; Southgate et al., 2011; Wormer et al., 2012). The identification of Ajuba as a regulator of CdGAP activity provides new insight into how Rac activity is regulated at cell-cell contacts, and may also aid our understanding of how CdGAP activity can be regulated in developmental and pathological settings. Much remains to be understood regarding the regulation of GAP proteins in different cellular contexts, including which GAPs are activated at cell-cell adhesions. What is clear is that this class of regulator is not simply around to terminate signaling from GTPases. Rather, GAPs control many diverse signaling processes via inactivation (and indirectly activation) of their GTPase substrates.

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Appendix I

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