Circular dorsal ruffles : a mode of steering cells

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CIRCULAR DORSAL RUFFLES: A MODE OF STEERING CELLS

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SCHOOL OF BIOLOGICAL SCIENCES

2018

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SCHOOL OF BIOLOGICAL SCIENCES

**A thesis submitted to the Nanyang Technological University in
partial fulfilment of the requirement for the degree of Doctor of
Philosophy**

2018

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CONTENTS

ACKNOWLEDGEMENTS	1
CONTENTS	2
LIST OF FIGURES	5
ABBREVIATIONS	6
ABSTRACT	8
THESIS OUTLINE	9
1. INTRODUCTION	11
1.1 Biological background of proteins involved in CDRs	14
1.1.1 Actin	15
1.1.2 Actin Nucleating Proteins	17
1.1.2.1 Arp2/3 (Actin related protein 2/ actin related protein 3)	17
1.1.2.2 Formins	20
1.1.3 Rho GTPases, their effectors and signalling	22
1.1.3.1 Rac1	24
1.1.3.2 RhoA and the cooperation between mDia1 and ROCK	25
1.1.4 Myosine Light Chain Kinase (MLCK)	27
1.1.5 Stress fibers and Focal adhesions	27
1.1.6 POPX2	30
1.2 Summary of molecular players that regulate CDRs	32
1.3 Objectives of this study	40
2. MATERIALS AND METHODS	41
2.1 Materials	41
2.1.1 General reagents	41
2.1.2 Commercial kits	41
2.1.3 Media	41
2.1.4 Buffers	42
2.1.5 Bacterial strains and mammalian cell lines	43

2.1.6 Primary antibodies	43
2.1.7 Secondary antibodies	44
2.1.8 Fluorescence Probes	44
2.1.9 Plasmids	44
2.1.10 Pharmacological Inhibitors and Stimulants	45
2.1.11 ECM Proteins/reagents used for coating and cell constraining	46
2.1.12 Microscopes	46
2.2 Methods	47
2.2.1 Plasmid purification	47
2.2.2 Cell culture	47
2.2.3 Transfection	47
2.2.4 Western blot	48
2.2.5 Preparation and characterization of patterned fibronectin strips	48
2.2.6 Usage of Reagents	50
2.2.7 G-actin incorporation assay	50
2.2.8 Immunofluorescence staining	50
2.2.9 Microscopy	51
2.2.10 Quantifying the fraction of cells exhibiting CDRs	52
2.2.11 Quantifying the fold changes/differences in cells exhibiting CDRs	52
2.2.12 Obtaining z-fluorescence intensity plots in CDRs	53
2.2.13 Extraction of focal adhesion properties	54
2.2.14 Time lapse imaging and reconstruction of SIM images	54
2.2.15 Visualization and photoactivation of mEos3.2-actin	54
2.2.16 Quantification of cell migration persistence (γ)	55
2.2.17 Statistical Analysis	55
3. RESULTS	56

3.1 Circular Dorsal Ruffles: co-operation between GTPases	56
3.1.1 RhoA inhibition attenuates CDR formation and dynamics	56
3.1.2 RhoA and Rac1 have clear and distinct zones of activation in a CDR	60
3.1.3 Formins are potential actin nucleating candidates in CDRs	63
3.1.4 Cooperation between mDia1 and Arp2 to form the actin architecture at different zones in a CDR	67
3.1.5 Filaments need not originate from the base of the CDRs	76
3.2 Localised Actomyosin Contractility pulls on focal adhesions to accommodate Circular Dorsal Ruffles	82
3.2.1 Focal adhesions are a mechanical component of CDR formation	82
3.2.2 Focal adhesions and MLCK cooperate to regulate CDRs	87
3.2.3 Focal adhesions act as cell anchors to support CDRs	92
3.2.4 POPX2 activity promotes CDR formation	96
3.2.5 POPX2 activity enhances localised actomyosin contractility around CDRs	99
3.3 Circular Dorsal Ruffles increase Directional Persistence of Cell Migration	103
3.3.1 CDRs contribute to directional persistence but not speed	103
3.3.2 Actin relocates from CDRs to the lamellipodia	108
3.3.3 MLC II localises around CDRs and lamellipodia	111
4. DISCUSSION	120
5. CONCLUSION	128
6. FUTURE WORK	133
7. REFERENCES	134
8. MANUSCRIPTS	144
9. CONFERENCES	145

LIST OF FIGURES

Figure 1. Dynamics of CDRs	14
Figure 2. Structure of actin monomer and filament.....	16
Figure 3. Structure and actin nucleation pattern due to Arp2/3 complex.....	19
Figure 4. Structure and role of formin	22
Figure 5. Regulation Rho GTPases.....	24
Figure 6. Rac1 regulates actin polymerization	25
Figure 7. Presumed Rho regulated steps in assembly of actomyosin bundles	27
Figure 8. Different types of stress fibers are found in migrating cells.....	30
Figure 9. The processes and pathways leading up to the formation of CDRs.....	39
Figure 10. Cells seeded on fibronectin strips prepared by micropatterning.....	49
Figure 11. RhoA inhibition attenuates CDR dynamics while CDRs could form on localised activated Rac1 regions	60
Figure 12. Spatially distinct active zones for RhoA and Rac1 in CDRs.	63
Figure 13. mDia1 is important to the formation dynamics and localises to CDRs	66
Figure 14. Arp2 localises more to the bottom while mDia1 localises to the top of a CDR.....	70
Figure 15. Arp2 localises more to the bottom while DNA Nuclease1 localises to the top of a CDR.....	72
Figure 16. DNA Nuclease 1 and mDia1 localise more to the top of a CDR	73
Figure 17. Arp2 and G-actin is localised throughout the height of the CDR	75
Figure 18. G-actin is localised throughout the height while mDia is more localised to the top of a CDR	76
Figure 19. Actin polymerisation in CDRs could initiate at both the top and bottom of CDRs	79
Figure 20. Proposed cooperation between Arp2/3 and formins in CDRs.....	81
Figure 21. Cell adhesion alone is insufficient for CDR formation	83
Figure 22. Focal adhesion signalling is required for CDR formation.....	86
Figure 23. MLCK can regulate CDR dynamics.....	88
Figure 24. Focal adhesions and MLCK cooperate to regulate CDRs.....	91
Figure 25. Focal adhesions act as cell anchors to support CDRs	95
Figure 26. POPX2 activity promotes CDR formation.....	98
Figure 27. POPX2 activity enhances localised actomyosin contractility around CDRs.....	102
Figure 28. Cells exhibiting CDRs show an increase in the persistence of directional migration with no change in migration speed	107
Figure 29. Cell protrusion and actin relocalisation in control and blebbistatin treated NIH 3T3 cells.....	110
Figure 30. Actomyosin activity is observed in the region between CDR and leading edge of cell.....	113
Figure 31. Microtubules do not contribute to CDR formation	115
Figure 32. MLC II current moves towards the leading edge in a CDR exhibiting cell	119
Figure 33. X2 cells display elevated actomyosin contractility	130
Figure 34. CDRs improve directional migrational persistence	131

ABBREVIATIONS

Arp2/3	Actin related protein 2/3
BSA	Bovine Serum Albumin
CDR	Circular Dorsal Ruffles
CDC42	Cell division cycle 42
Ctrl	Control
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
FAK	Focal adhesion kinase
FBS	Fetal Bovine Serum
FRET	Fluorescence resonance energy transfer
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
JMY	Junction mediating regulatory protein
LPA	Lysophosphatidic acid
MLC II	Myosin Light Chain II
MLCK	Myosin Light Chain Kinase
MLCP	Myosin Light Chain Phosphatase
NPF	Nucleation promoting factor
PAK	p21-activated kinase
PDGF	Platelet Derived Growth Factor
PDMS	Polydimethylsiloxane
PIX	PAK-interacting exchange protein
PI3K	Phosphoinositide 3-kinase
PLL	Poly L Lysine
POPX	Partner of PIX
PTM	Post translational modification
RFP	Red fluorescent protein
SIM	Structured Illumination Microscopy
SMIFH2	Small molecule inhibitor of formin homology 2 domains
TIRF	Total Internal Reflection Fluorescence

WASP	Wiskott-Aldrich Syndrome protein
WAVE	WASP and verprolin homologs
WHAMM	WASP homolog associated with actin, membranes and microtubules
WH2	Wiskott-Aldrich homology 2 domain
WCA	WH2 domain and Arp2/3 binding central acidic sequences domain

ABSTRACT

Circular Dorsal Ruffles (CDRs) are actin rich, ring-shaped structures that form across the dorsal surface of certain cell types upon stimulation by growth factors. Although previous studies have implicated the formation of CDRs as a Rac1 dependent process, substrate stiffness experiments have hinted a role for RhoA and its effectors. Herein, how RhoA and its effectors could affect CDR formation and its dynamics were investigated. It was found that RhoA and Rac1 have clear distinct zones of activation in CDRs. As a result, their effectors namely, mDia1 and Arp2/3 can co-operate to produce actin filaments to support the CDR structure. Interestingly, actomyosin contractility was found to be responsible for sustaining CDRs and that POPX2 phosphatase could harness enhanced localised actomyosin contractility to promote CDR formation. In addition, focal adhesions serve as anchors to accommodate CDRs when they traverse on the dorsal surface. In this study, I also found that CDRs can act as actin reservoirs from which actin can be re-directed to the leading edges after CDR disassembly. Surprisingly, the actomyosin network is also observed to move towards the leading edge after CDR disassembly. Consequently, cells that exhibit CDRs display enhanced directional migration persistence. This opens avenues to study CDRs in myriad cell migration contexts to better understand their biological function.

THESIS OUTLINE

This thesis aims to achieve answers and clarity to questions related to the structural features of CDRs, factors responsible in sustaining CDRs and the fate of actin after the disassembly of CDRs. The following Introduction chapter is dedicated to the current state of understanding on CDR itself, the molecular players crucial to CDRs and a summary of how they regulate CDRs. Chapter two contains detailed description of the materials and methods used.

Experimental results are presented in three sub-chapters as they attempt to uncover and explain separate features of CDRs. The first sub-chapter is dedicated to shed light on how CDRs are formed and the structural features one would expect to see. In this, the structural components of CDRs have been probed via z-stack time lapse confocal microscopy and structural illumination imaging. The possibility of cooperation between Rho GTPases and actin nucleators has been proposed for the formation of CDRs.

The second Results sub-chapter is dedicated to explore the factors required to sustain fully formed CDRs. In this study, factors associated with CDR formation such as the focal adhesions and the associated actomyosin contractility have been examined via total internal reflection fluorescence microscopy (TIRF) microscopy. The need to have actomyosin contractility in order to use focal adhesions as anchors has been highlighted.

Lastly, the third Results sub-chapter is dedicated to tracking the fate of actin after the disassembly of CDRs. In this study, photoactivatable actin constructs were used to determine the path/fate of actin after CDR disassembles. The possibility that CDRs can be a source of actin pool for lamellipodial protrusions to increase persistence for directional migration is suggested.

Next, a chapter is dedicated for the discussion of the results described in the previous three sub-chapters. Herein, the relevant literature that has indicated similar results and how this work has furthered these ideas is also described. This is followed by a final conclusion which aims to tie up the findings and their implications.

1. INTRODUCTION

The cells are dynamic machines that can sense their local environment, integrate biochemical and mechanical information they received, respond and adapt constantly by changing their metabolism, cell morphology, gene expression and behaviour. Understanding cellular dynamics can help to uncover mechanisms in disease progressions. Cellular dynamics can be altered by many different ways including external mechanical cues. These external mechanical cues are sensed by mechanosensors and the signals are transduced into biochemical cascades within the cell, triggering dynamic events. However, these dynamics can be either too fast or slow for human perception.

Alterations in cellular dynamics can be contributed by remodelling of the cytoskeleton. The cytoskeleton in mammalian cells is composed of actin, microtubules and intermediate filaments. In this study, I focused on the actin cytoskeleton. In particular, I was interested in the actin rich structures called circular dorsal ruffle (CDR). CDRs form on the dorsal surface of the cell upon stimulation by growth factors such as platelet derived growth factors (PDGF), human growth factors (HGF) and epidermal growth factors (EGF) (Mellstrom et al., 1988; Mellstrom et al., 1983; Tamura & Iwamoto, 1989). They take the form of ring shapes but the rings can sometimes be incomplete. These are membraneous projections in the z-axis and are highly dynamic such that they can erupt and traverse across the dorsal surface of the cell (Krueger et al., 2003). The membraneous projection is believed to be driven by polymerization of branched actin filament network (Dharmawardhane et al., 1997). CDRs erupt, constrict and finally disappear after about 2-3 minutes of PDGF stimulation (Fig. 1 & Movie 1). Interestingly, repeated stimulation by PDGF does not trigger more CDRs indicating that it is solely a ‘one-time’ event. Ironically, CDRs behave just like waves that propagate in an excitable medium. For

instance, two CDRs in a single cell have been observed to merge as one. However, they cannot be excited for a second time (Buccione et al., 2004).

CDRs have been known for decades. Initially, peripheral ruffling was discovered (Abercrombie et al., 1970). Much later, similar structures that were induced by growth factor stimulation and/or viral infection were observed (Ambros et al., 1975; Chinkers et al., 1979). A few years later in 1983, Mellstrom and colleagues spotted CDRs while stimulating human glial cells with PDGF and confirmed that these were actin rich structures. Since, then CDRs have been observed in various cell lines in response to a myriad of growth factor stimulation (Dowrick et al., 1993; Edgar et al., 1997). Recent reports have suggested that similar machinery involving lamellipodial formation could be involved in the formation of CDRs (Legg et al., 2007; Payne et al., 2014). Reviews on CDRs have focused on different aspects of this actin rich structure. CDRs were compared to other similarly actin-rich ring-like structures such as podosomes and invadopodia (Buccione et al., 2004). In addition, endocytosis was championed as one of the primary functions of CDRs (Orth & McNiven, 2006). This mechanism has been shown to be involved in integrin turnover (Gu et al., 2011). A compiled list of the localisation of different proteins to the CDR was made available (Hoon et al., 2012). In the same year, a list of possible pathways that could be involved in the formation of CDRs was also explained in detail (Hasegawa et al., 2011; Hasegawa et al., 2012).

CDRs do not occur as frequently in cancer cell lines compared to fibroblasts given the same stimulation conditions. Therefore, it was hypothesized that uncontrolled proliferation of the cancer cells could be facilitated by their inability to form CDRs (Orth et al., 2006). In contrast, the co-localisation of metalloproteases (MMP2) to CDRs has suggested that these actin structures can also contribute to the invasiveness of the cells. Nevertheless, little is known about how they assemble, traverse across the cell, what cellular function they contribute towards

and what happened to the cell after their disassembly. It is of interest to study these aspects because CDRs have been implicated in macrophage's functions. Macrophages patrol our tissues to constantly scan for pathogens, cell debris or even dead cells (Steinman et al., 1983). CDRs could be a mechanism for the uptake of extracellular materials by the macrophages.

Macrophages have unique and dynamic cell surface features that allow them to reorganise and sample the extracellular environment. Cellular extensions such as CDRs have been postulated to be the basis for macropinosomes for non-selective extracellular fluid sampling. Interestingly, exposing macrophages to lipopolysaccharide of Gram-negative bacteria increases the fraction of them exhibiting CDRs suggesting that CDR formation is also a pathogen-inducible cell response. In addition, CDRs can also generate phagocytic cups for receptor mediated uptake of particles and pathogens. After the collapse of CDRs, the receptors are sent to macropinosomes or phagosomes (Husebye et al., 2010) where different signalling adaptors assess the threat and modulate downstream responses (O'Neill & Bowie, 2007). In addition, newly synthesised tumour necrosis factor (TNF) can also be exported at sites of CDRs to neutralise the pathogenic threats (Barton & Kagan, 2009). Although the dynamic features of the cell surface are apt for counteracting pathogens, many viruses, bacteria and other pathogens have evolved to hijack macropinosomes for entry and survival. Therefore, studying CDR dynamics and function can help to uncover novel protein targets and strategies that can be employed for developing preventive measures to help fight infections.

Although it has been long established that CDRs are actin rich, the actual architecture that gives CDRs their shape still remains unclear (Hoon et al., 2012). Therefore, answering the question of how CDRs assemble will be the first objective of the thesis. After the CDR forms, it propagates across the dorsal surface before the ring starts to constrict and disassemble. Review articles have

highlighted that CDRs are excitable and propagating waves but these studies so far concentrated on ring contraction rather than the propagation. As a result, little is known about how fully assembled CDRs are propagated across the dorsal surface of a cell. Exploring how fully assembled CDRs are sustained will be the second objective of this thesis. After sustaining for over a few minutes, CDRs tend to constrict and disassemble. Earlier reports have proposed endocytosis and macropinocytosis as the main functions of CDRs and that downstream pathways are tuned according to what pathogen or debris was internalised. However, little is known of the fate of the actin that was used to make up the CDR and what other purpose CDR serves the cell. For the third objective of this thesis, I propose to study the fate of the actin after CDR disassembly and the outcome of exhibiting CDRs for the cell other than endocytosis.

Figure 1. Dynamics of CDRs

Upon stimulation by PDGF, CDRs form on the dorsal surface of cells and can typically last for 2-3 min. See Movie 1 at <https://drive.google.com/open?id=18WHui1tRHIIG9z0NLlQeVctok9z1i9dM>.

1.1 Biological background of proteins involved in CDRs

In this section, I introduce the proteins involved in forming CDR. Since the CDRs are actin rich structures, most of the proteins implicated in CDR formation are regulators of actin polymerization.

1.1.1 Actin

Actin is abundant in most eukaryotic cells such that they can form up to 20% of the total protein composition. It assembles reversibly to form one of the three major filaments that made up the cytoskeletal network. The actin protein is about 42 kDa and exists in a monomeric globular form (G-actin) or filamentous (F-actin) states. The actin filaments interact with a variety of associated proteins (e.g. myosin) to form a network of actin cytoskeleton. In particular, there is a network of actin cytoskeleton near the plasma membrane of eukaryotic cells and it is commonly called the actin or cell cortex. Myosin motor proteins interact with actin filaments (Blanchoin et al., 2014), thus enabling the sliding of actin filaments to facilitate processes such as intracellular transport, maintenance of cellular architecture and cytokinesis. Due to its propensity to form filaments, it was difficult to crystallise the protein. Eventually in the 1990s, G-actin was crystallised along with DNase I (Kabsch et al., 1990) and profilin (Schutt et al., 1993). This paved the way to obtain high resolution structures for eukaryotic and prokaryotic actin (Rao et al., 2014). The structures revealed four subdomains within which ATP binds in a deep cleft such that it interacts with all the subdomains with differing affinities. Actin filaments consist of two strands of subunits that are intertwined to form right handed helices that are staggered by about 1.35nm (Huxley, 1963). Actin filament has structural polarity since the monomers orient their nucleotide-binding sites toward the same end of the filament which is known to be the pointed end while the opposite end is known to be the barbed end (Fig. 2). The (+) or barbed ends usually have a higher concentration of actin-ATP while the (-) or pointed-ends have higher concentration of actin-ADP. Due to differences in affinity for actin-ATP, the barbed end polymerises faster than the pointed end (Fujiwara et al., 2007).

These filaments are highly dynamic as they are constantly remodelled to suit the cell's needs. Actin binding proteins regulate every aspect of actin assembly. Collectively, these proteins help

to maintain a reservoir of G-actin for polymerisation, nucleation, elongation, capping of pointed and barbed ends from polymerisation and depolymerisation, severing and cross-linking filaments. Amongst these, actin nucleation proteins will be a focus in this thesis as the first objective in this work is to study how initial CDRs are formed.

Figure 2. Structure of actin monomer and filament

Actin folds into 4 domains leaving a cleft for nucleotide binding and the monomers are stacked such that the nucleotide binding cleft is always positioned at the pointed end (Pollard and Earnshaw, 2007).

1.1.2 Actin Nucleating Proteins

1.1.2.1 Arp2/3 (Actin related protein 2/ actin related protein 3)

Actin filament nucleation involves two unfavourable steps namely, dimer formation and the addition of a third monomer to form a trimer. Although the association reactions are fast, dimers and trimers dissociate at 10^{-6} sec^{-1} and 100 sec^{-1} and therefore are deemed unstable. Thus, spontaneous polymerisation begins with a lag phase that is hugely dependant on the concentration of G-actin. In the 1960s, this lag phase was accurately identified as a slow nucleation that forms viable oligomers for elongation (Johnson, 1976). In addition, actin binding proteins such as, profilin and thymosin- β 4 sequester G-actin and thus suppress actin nucleation. As a result, cells rely on nucleating proteins to initiate actin polymerisation in a controlled manner. The Arp2/3 complex which consists of seven subunits and is mainly involved in the generation of branched actin filaments from an existing mother filament (Fig. 3). In contrast, formins are used to generate unbranched filaments. Other types of filaments can be formed via interaction with proteins containing tandem Wiskott-Aldrich Homology 2 (WH2) domains.

Of the seven subunits of the Arp2/3 complex, Arp2 and Arp3 display a similar homology to actin. However, the Arp2/3 complex is intrinsically inactive since the Arp 2 and Arp3 subunits are held apart by the others. Arp2/3 complex mediated actin nucleation requires the activity of nucleation promoting factors (NPFs) such as, the Wiskott-Aldrich Syndrome protein (WASP). WASP, WASP and verprolin homologs (WAVEs), WASP homolog associated with actin, membranes and microtubules (WHAMM) and the junction mediating regulatory protein (JMY). These NPFs are also regulated thereby controlling actin nucleation through the Arp2/3 complex. NPFs are usually regulated through allosteric modulation of accessibility to the G-actin binding

WH2 domain and Arp2/3 binding central acidic sequences (WCA) domain by auto inhibition as is the case for WASP (Derivery & Gautreau, 2010). WASP exists in a closed conformation due to the auto inhibition of the WCA domain by the CDC42/RAC interactive binding (CRIB) domain until it is activated by CDC42. This exposes the WCA domain so that it can interact with Arp2 and Arp3 (Fig. 3A). This binding induces conformational changes to each of the seven subunits in the Arp2/3 complex (Fig. 3B). WASP binding to Arp2/3 complex leads to a weak association with existing mother actin filaments. WASP also binds G-actin and this strengthens the association between Arp2/3 complex and the existing mother actin filaments. Further conformational changes anchor the Arp2/3 complex to the mother filament. This allows binding of G-actin to the Arp2/3 complex (Fig. 3C). Similarly, WAVE which is kept inactive by binding to a Nap-PIR-Abi2 subcomplex, is activated by Rac1, binds to the Arp2/3 complex to stimulate actin polymerisation (Fig. 3D). This starts the nucleation of the branched daughter filament and elongation ensues. This process leads to the formation of branched actin filaments. This model was initially obtained by docking the atomic resolution crystal structure of an inactive conformation of the Arp2/3 complex into a 3D reconstruction obtained via electron tomography (Rouiller et al., 2008). It was claimed that Arp2 and Arp3 interact with the pointed end of the daughter filament. On the other hand, ARPC2 and ARPC4 make significant contact with the mother filament. Mutagenesis studies involving ARPC2 and ARPC4 revealed that they are required for efficient nucleation. The branched actin filament nucleating ability of Arp2/3 complex can be inhibited pharmacologically by the treatment of CK666 (Nolen et al., 2009). This is achieved by allosteric inhibition that promotes the stability of the inactive state of the complex.

A

B

C

D

Figure 3. Structure and actin nucleation pattern due to Arp2/3 complex

(A) WASP exists in a closed conformation due to the auto inhibition of the WCA domain by the CDC42/RAC interactive binding (CRIB) domain until it is activated by CDC42. This exposes the WCA domain so that it can interact with Arp2 and Arp3. (B) Arp2/3 is made of seven subunits of which Arp2 and Arp3 have to be in close proximity to nucleate branched actin from mother filaments, from Pollard

and Earnshaw 2007. (C) The activation of the Arp2/3 complex is mediated by NPFs such as WASP from Smith et al., 2013. WASP also binds G-actin and this strengthens the association between Arp2/3 complex and the existing mother actin filaments. Further conformational changes anchor the Arp2/3 complex to the mother filament. This allows binding of G-actin to the Arp2/3 complex. (D) WAVE which is kept inactive by binding to a Nap-PIR-Abi2 subcomplex, is activated by Rac1, binds to the Arp2/3 complex to stimulate actin polymerisation.

1.1.2.2 Formins

Formins are a family of proteins involved in actin polymerization. They are characterized by the formin homology domain 2 (FH2) which is responsible for the interaction with the barbed end of an elongating actin filament (Goode & Eck, 2007; Paul & Pollard, 2009). Formins can form homodimers through their FH2 domains (Xu et al., 2004). In the absence of G-actin, the FH2 dimer can wrap around an actin filament (Otomo et al., 2005). Beside the FH2 domain, formins contain a proline rich FH1 domain that binds profilin. Near the C-terminus of the protein, a diaphanous auto-regulatory domain (DAD) interacts with a DAD interacting domain (DID) near the N-terminus resulting in a closed conformation which renders the formins inactive (Fig. 4A). Most formins are effectors of small GTPases. Binding of Rho GTPases at the GTPase binding domain which is located closer to the N-terminus leads to conformational changes that relieve the auto-inhibition.

The formins nucleate and catalyses polymerization of unbranched actin by initially stabilising actin dimers (Pring et al., 2003). There seems to be no requirement for a mother filament. Only G-actin appears to be involved in the nucleation process. These resulting unbranched filaments have been implicated in the formation of filopodia, contractile rings and stress fibers. Apart from nucleating unbranched filaments, formins can promote the elongation of the actin filament by interacting with the barbed end processively (Fig. 4B). The open and closed conformations of the FH2 homodimer control the addition of G-actin to the barbed end (Vavylonis et al., 2006). Depending on the formins, the barbed ends can be open from 5% and 90% of the time.

This control can be overcome by the rapid delivery of G-actin to the barbed end. The FH1 domain, acts like tentacles, traps profilin bound actin and delivers G-actin to the barbed end allowing rapid elongation. In the mouse diaphanous homolog 1 (mDia1) formin, whose FH2 homodimer is open 90% of the time and has an FH1 domain to trap profilin bound actin, elongation can be about five times faster than for a free barbed end (700 subunits/second) (Higashida et al., 2004). The processive nature allows formins to constantly “step over” the newly added G-actin for many cycles. This activity overcomes capping and allows the filaments to elongate rapidly. The activity of formin can be inhibited pharmacologically by the treatment of SMIFH2, an inhibitor targeting the FH2 domain (Rizvi et al., 2009). This inhibitor decreases formin’s affinity for the actin barbed end thus, preventing formin nucleation and processive barbed end.

Figure 4. Structure and role of formin

(A) Domain organisation of a diaphanous type formin. (B) Model of actin filament elongation, an incoming actin binds one of the dimers and this creates a binding site for the trailing FH2 domain to “step” toward the barbed end. The model is based on the crystal structure (Otomo et al., 2005).

1.1.3 Rho GTPases, their effectors and signalling

The Rho GTPases belong to a subfamily of the Ras superfamily. Rho GTPases can regulate the organisation of the actin cytoskeleton through their downstream effector proteins in response to various stimuli. Rho GTPases can be classified as molecular switches as they can cycle between active and inactive states (Etienne-Manneville & Hall, 2002). In its GTP bound state, Rho GTPases are activated and can spur a cascade of reactions to effect changes in cellular behaviour. There are three classes of proteins that regulate the activity of the Rho GTPases. GTPase activating proteins (GAPs) accelerate the hydrolysis of GTP in the Rho GTPases and thus switch them off (Fig. 5). In contrast, guanine nucleotide exchange factors (GEFs) catalyses the dissociation of GDP from Rho GTPases and replacement to GTP. Guanine nucleotide dissociation inhibitors (GDIs) bind to the carboxy terminal prenyl groups on Rho GTPases and prevent the exchange to a fresh GTP. In addition, GDIs also sequester the GTPases in the cytosol, keeping them away from their regulators and targets.

In addition, mechanisms other than cycling can also influence GTPase activity. This is especially the case in non-traditional GTPases that possess a high capacity for intrinsic nucleotide exchange activity or have substitutions in their GTPase domains that attenuates GTPase activity. Thus these proteins are better regulated via a wide range of post translational modifications (PTMs). PTMs such as lipid modification, phosphorylation, prenylation, palmitoylation, ubiquitylation and sumoylation ensure that these proteins are activated and attenuated in a coordinated spatiotemporal fashion.

In human, there are more than 20 Rho GTPase family members of which RhoA, Rac1 and CDC42 have been best characterized. These three GTPases are central to actin dynamics as their effectors influence the actin cytoskeleton organisation and thus, in cell migration mechanisms as well. RhoA activity is linked to the formation of stress fibers and integrin based focal adhesions in adherent cell types. In the cell migration context, RhoA is active at the front and rear of migrating cells and drives bleb based migration (Paluch & Raz, 2013). It promotes actomyosin contractility through its effector ROCK and actin polymerisation through the formin family of actin nucleators (Ridley, 2015). Another GTPase that belongs to the Rho-like subfamily is RhoC. RhoC is also implicated in the formation of invadopodia in ovarian cancer cells (Semprucci, et al., 2016). However, they can serve different functions. For example, RhoA and RhoC can localise to the plasma membrane however, RhoA often inhibits cancer cell invasion in vitro while RhoC enhances this (Simpson, et. al., 2004). Despite coming from the same subfamily, the regulation exerted by different GEFs in different cell types also contributes towards the different modes of cell migration. From the Rac GTPases, there exists Rac1, Rac2 and Rac3 which are members of Rho GTPases. Rac1 and Rac3 are ubiquitously expressed while Rac2 is hematopoietic lineage specific (Etienne-Manneville & Hall, 2002). Rac1, on the other hand, is linked with a range of functions such as, cytoskeletal rearrangements to the formation of lamellipodia and cell migration via a GIT- β -PIX-PAK complex (Frank & Hansen, 2008). This also results in focal adhesion turnover. From the CDC42-like subfamily, CDC42 activity is also linked to a range of functions such as invasion, actin polymerisation maintaining cell-cell contacts, cell-ECM contacts and the formation of filopodia (Gupton & Gertler, 2007). There is considerable overlap in the functions that GTPases due to the resultant downstream cascades that arise from the cross talks and various feedback loops. Thus, the differences in spatial-temporal signalling ensure that one constantly keeps check of the other while processes in cells take place in a coordinated fashion.

Figure 5. Regulation Rho GTPases

Guanine nucleotide-exchange factors (GEFs) activate Rho GTPases by promoting the release of GDP and the binding of GTP while, GAPs promote the hydrolysis of GTP to GDP and thus serve as negative regulators of GTPases. GDIs sequester GTPases in the cytosol thereby preventing their localisation to the membrane and effectors (Heasman & Ridley, 2008).

1.1.3.1 Rac1

Rac1 can be activated by signalling pathways initiated by extracellular ligands such as growth factors but its activation is restricted in a spatial-temporal manner. Rac1 activation results in lamellipodial extensions, membrane ruffles and migration in multiple cell types (Ridley, 2001). During lamellipodial extensions, Rac1 regulates actin polymerisation through a few effectors. Firstly, they do so by activating actin nucleating proteins such as, Arp2/3 complex via Scar/WAVE (Fig. 6) (Chhabra & Higgs, 2007; Goley & Welch, 2006). Secondly, they lead to the removal of barbed ends capping proteins and promote the severing of actin filaments through cofilin or gelsolin (Jaffe & Hall, 2005). Surprisingly, Rac1 null macrophages can form membrane ruffles and migrate at a similar speed compared to wildtype macrophages (Wells et al., 2004). However, they exhibit impaired lamellipodial extensions characterised by elongated morphologies and poor spreading (Wells et al., 2004). Another effector of Rac1 is the p21-activated kinase (PAK) which phosphorylates LIM kinase (LIMK) that in turn phosphorylates

cofilin to inhibit its actin severing activity (Edwards et al., 1999). In addition, Rac1 can also interact with and activate mDia2 to regulate actin polymerization (Lammers et al., 2008).

Figure 6. Rac1 regulates actin polymerization

Rac promotes actin polymerisation through the WASP and WAVE complex which activates Arp2/3. Rac is also involved in promoting actin cytoskeletal rearrangements via mDia2 in erythrocytes. Rac- or CDC42-mediated activation of the Ser/Thr kinase PAK (p21-activated kinase) phosphorylates LIM kinase (LIMK), which in turn phosphorylates and inhibits cofilin, thereby regulating actin-filament turnover (Heasman & Ridley, 2008).

1.1.3.2 RhoA and the cooperation between mDia1 and ROCK

RhoA can be activated by extracellular ligands such as lysophosphatidic acid (LPA) but its activation is restricted in a spatial-temporal manner. Its activation results in the assembly of stress fibers and focal adhesion complexes. Recent studies have also indicated the importance of RhoA in membrane protrusions (Pertz et al., 2006). RhoA activity can be inhibited by the treatment of C3 exoenzyme resulting in loss of stress fibers and poor focal adhesion complex formation. Two well-studied effectors of RhoA are Rho-associated coiled coil forming kinase (ROCK) and the formin mDia1 (mammalian homolog of the *Drosophila Diaphanous* protein) (Watanabe et al., 1999). ROCK is a serine threonine kinase that has many substrates. One major substrate of ROCK is the myosin binding subunit of myosin light chain phosphatase (MLCP). Phosphorylation of the myosin binding subunit of MLCP inactivates the phosphatase leading to

increased amounts of phosphorylated myosin light chain (MLC). ROCK can also directly phosphorylate MLC (Brozovich, 2002). Consequently, myosin light chain phosphorylation promotes crosslinking of actin filaments by myosin and enhances actomyosin contractility. In addition, ROCK activates LIMK via phosphorylation. Activated LIMK1 then phosphorylates and inactivates cofilin. On the other hand, binding of RhoA at the GTPase binding domain of mDia1 which is located closer to the N-terminus leads to conformational changes. These changes relieve the auto-inhibition thereby, activating mDia1's actin nucleating ability, which results in the formation of unbranched actin filaments. Taken together, the actions of ROCK and mDia1 on actin and myosin are thought to induce formation of actin structures and actomyosin contractility (Fig. 7). The involvement of ROCK and mDia1 in contractile ring formation was also reported (Ishizaki et al., 2000). However, expression of constitutive active ROCK is insufficient to promote the formation of organised actomyosin bundles and contractile rings (Ishizaki et al., 1997). Co-expression of active mDia1 can rescue this insufficiency (Watanabe et al., 1999). Although ROCK and mDia1 cooperate in forming stress fibers and focal adhesions in the cells, their downstream signalling pathways can also antagonise each other (Tsuji et al., 2002). ROCK activity attenuation can be achieved by the treatment of Y-27632 which competitively inhibits the ATP binding site of the catalytic domain (Ishizaki et al., 2000).

Figure 7. Presumed Rho regulated steps in assembly of actomyosin bundles

RhoA catalyzes actin nucleation and polymerization to form actin filaments and activate myosin to cross-link them, from (Narumiya et al., 2009).

1.1.4 Myosine Light Chain Kinase (MLCK)

MLCK is activated by an increase in Ca^{2+} concentration in smooth and skeletal muscle cells. MLC is the major substrate of MLCK. MLCK also binds actin with high affinity via its amino terminal actin binding sequence. This stretch of sequence has three repeat motifs whereby each one can bind an actin monomer along a filament. With its amino terminal bound to the actin filament, its catalytic site can reach for myosin for MLC phosphorylation to promote the actomyosin contractility. MLCK activity can also be regulated in a Ca^{2+} concentration independent manner. cAMP dependent protein kinase induces phosphorylation of MLCK, which inactivates MLCK. Similarly, PAK which is one of the effectors in the network of Rho GTPase signalling induces a negative effect on MLCK activity and MLC phosphorylation (Sanders et al., 1999). MLCK activity attenuation can be achieved by the competitive inhibition due to ML-7 treatment (Shi et al., 2007). Although other inhibitors like ML-9 have been used, ML-7 is still more general and potent as compared to ML-9.

1.1.5 Stress fibers and Focal adhesions

Stress fibers are prominent bundles of F-actin associated with myosin II, α -actinin and other cytoskeletal proteins. They are usually associated at one or both ends with focal adhesions (Fig. 8). Stress fibers can be further classified into ventral stress fibers, dorsal stress fibers and traverse arcs (Small et al., 1998). Ventral stress fibers are anchored at each end by a focal adhesion while, dorsal stress fibers are anchored at only one end by a focal adhesion near the

leading edge of a cell. Dorsal stress fibers may extend toward the nucleus or upwards toward the dorsal surface of the cell through formin and VASP based actin polymerisation which end serving as tracks for traverse arcs to slide on centripetally (Tojkander et al., 2015). (Fig. 8). Traverse arcs are found just behind the leading edge of the cell. They are long curved actin structures on which recurring bands of myosin and α -actinin can be observed. They do not directly bind to focal adhesions and slide centripetally along the dorsal stress fibers. Traverse arcs are also implicated in actin retrograde flow which plays an important role in the maturation of focal adhesions.

Many studies examining stress fibers and focal adhesions have identified RhoA as a key regulator in controlling the assembly and maturation of these structures (Ridley & Hall, 1992). The assembly of these structures are also blocked by the inhibitors of myosin activity and actomyosin contractility. The dynamic nature of stress fibers and focal adhesions can be observed when cells spread and migrate on extra cellular matrix (ECM) such as fibronectin, and collagen, just to name a few. The formation of adhesions is initiated by the binding of integrins to ECM substrates and the associated clustering. This clustering triggers the assembly of a fresh focal adhesion complex on their cytoplasmic domains. Proteomic approaches have revealed that integrins could cooperate to activate RhoA during adhesion to the ECM (Schiller et al., 2013). It was shown that the $\beta 1$ integrins were coupled to ROCK and myosin II activation. On the other hand, αv integrin was shown to be recruited along with mDia1 to the adhesions. This led to the conclusion that the cooperation between fibronectin binding integrins namely, $\beta 1$ and αv , promoted the activation of RhoA signalling thus, contributing to focal adhesion maturation via actin polymerisation and enhancing actomyosin contractility.

In the cell migration context, integrins engage ECM to form nascent adhesions at the leading edge where actin filaments orient with their growing ends facing the plasma membrane. Actin

assembly pushes against the plasma membrane thus driving actin towards the cell centre in a process termed as retrograde flow. Maturation of these nascent adhesions occurs during the transition where actin retrograde flow is mediated by myosin-based contractility and thus, creating tension. The maturing adhesions elongate in the direction of the retrograde actin flow and slow down its flow. Adhesion elongation was blocked by inhibiting RhoA activity but this could be rescued by expressing constitutively active mDia1 (Riveline et al., 2001). Numerous studies have found a correlation between adhesion size and tension (Riveline et al., 2001). Under tension, the affinity of F-actin for myosin II increases thus, driving bundling of bipolar actin filaments. This force transmitted on the focal adhesion leads to the recruitment of additional components. It has been shown that applying tension to specific proteins in the focal adhesion complex such as, talin, could expose previously buried binding sites for other adaptor focal adhesion proteins thus, facilitating newer interactions and undergoing reinforcements (Zhong, et al., 1998).

Although myosin II regulates adhesion maturation, the extent to which this process occurs depends on the efficiency of the linkage between stress fibers and the ECM. This linkage is often affected by extracellular mechanical cues such as the surface rigidity and the concentration of ECM proteins. Cells seeded on rigid surfaces coated with high concentration of ECM proteins exhibit thick stress fiber bundles along with large, myosin II dependent focal adhesions. On the contrary, cells seeded on pliable surfaces coated with low concentration of ECM proteins tend to exhibit thin stress fibers and small focal adhesions. Moreover, cells, which carry a net negative charge on the extracellular side of the cell membrane due to the dense negatively charged network of glycolipids and glycoproteins, when seeded on poly-L-lysine coated surfaces exhibit poor focal adhesion formation since the adhesion is mainly based on electrostatics. Thus, cells can sense extracellular mechanical cues and respond by modulating

integrin clustering, myosin II activity, stress fiber formation and density, focal adhesion composition, maturation and its downstream signalling.

Figure 8. Different types of stress fibers are found in migrating cells

Schematic representation of SFs in motile cells, (A) top and (B) side views. Four categories of SFs are observed: dorsal SFs, transverse arcs, ventral SFs and the perinuclear actin cap, from (Burrige & Guilluy, 2016)

1.1.6 POPX2

POPX2 is a serine threonine phosphatase that belongs to the PP2C family. It was isolated using the PAK interacting guanine exchange factor, PIX, as bait in a yeast two-hybrid screen (Koh et al., 2002). Substrates of POPX2 include PAK, calmodulin-dependent protein kinase II and kinesin family member 3A (KIF3A). POPX2 has been found to regulate numerous processes including apoptosis (Weng & Koh, 2017), serum response factor mediated transcription (Xie et al., 2008), cell polarity (Hoon et al., 2014), and vesicle transport along the microtubules (Phang et al., 2014). The level of POPX2 has been found to be much higher in the more invasive MDA-MB-231 than in the non-invasive MCF7 breast cancer cells. Knocking down POPX2 decreases cell motility and invasiveness of breast cancer cells suggesting a role for POPX2 in cell invasiveness (Susila et al., 2014). Overexpression of the phosphatase leads to an increase in the speed of cell migration and invasiveness (Singh et al., 2011; Susila et al., 2014). POPX2 is also associated with angiogenesis and cancer metastasis at later stages of tumorigenesis (Zhang et al., 2017).

More importantly and relevant to this study on CDRs, POPX2 activity is able to prevent stress fiber loss induced by expressing dominant negative mDia1 (Xie et al., 2008). In addition, POPX2 dephosphorylates PAK and thus downregulates its activity. As a result, it prevents the loss of stress fiber and changes in cell morphology induced by active PAK. Furthermore, silencing of POPX2 inhibits $\beta 1$ integrin expression (Susila et al., 2014). Correspondingly, POPX2-knockdown cells show poor attachment to collagen and fibronectin indicating that POPX2 may have a role in forming stronger focal adhesions in these cell lines. High expression level of POPX2 impairs nucleus-centrosome-leading edge alignment. High POPX2 expressing cells move faster but the persistence in migration and directionality are affected in scratch wound assays indicating that the phosphatase activity of POPX2 is involved in the regulation of migratory direction (Hoon et al., 2014).

1.2 Summary of molecular players that regulate CDRs

The small GTPase Rac1 and the phosphatidylinositol-3-kinase (PI3K) are the two main proteins often implicated in signalling events downstream of PDGF receptor activation that drives CDR formation. Proteins associated with the actin cytoskeleton such as cortactin, dynamin 2, N-WASP and Arp2/3 have been found to be co-localized to the actin ring of the CDR (Krueger et al., 2003; Orth et al., 2006; Orth & McNiven, 2006). Interestingly, clathrin, clathrin-adaptors and caveolin which are normally associated with endocytosis, are not required for CDR formation (Orth et al., 2006).

The formation of CDRs involves complex interplay of regulators and cross-talks between several major pathways. As such, one better understood pathway involves the Ras superfamily of small GTPases (Ras, Rac1 and Rab5) and the SH2-SH3 adaptor proteins (Nck) (Rivera et al., 2006; Rohatgi et al., 2001) that transduce extracellular signals to downstream actin dynamics (Buday et al., 2002; Innocenti et al., 2003; Rohatgi et al., 2001). The actin dynamics in CDRs is generally thought to be induced by Arp2/3 activation which is in turn activated by the Wiskot-Aldrich syndrome (WAS) family of proteins (WASP and WAVE) (Chhabra & Higgs, 2007; Goley & Welch, 2006) and several other regulatory proteins, such as PI3K, cortactin and dynamin.

Rac1 is one of the most implicated effectors in CDR formation. A lack of CDRs seen in Rac1 dominant negative expressing and Rac1^{-/-} fibroblasts supports the requirement for the GTPase (Ladwein & Rottner, 2008). Early studies have proposed a model whereby Rac1 activates type 1 PIP-5-kinase, leading to the formation of PIP₂. PIP₂ sequesters gelsolin and allows filament elongation. The WAVE-profilin complex together with Arp2/3 goes on to add fresh actin

monomers that contribute to CDR formation (Miki et al., 1998). Another known Rac1-dependent dorsal ruffling pathway describes the recruitment of WAVE1 by activated Rac1 to membrane ruffles (Eden et al., 2002) followed by the release of the active WAVE complex which mediates Arp2/3-dependent actin cytoskeleton reorganization.

However, sole activation of Rac1 is insufficient for CDR formation. It has been shown that constitutively active Rac1 induces peripheral ruffles (PRs) (Suetsugu et al., 2003) but not CDRs (Bar-Sagi & Feramisco, 1986; Lanzetti et al., 2004; Ridley et al., 1992) through WAVE2, while dominant negative Rac1 abolishes both types of ruffles. Therefore, Rac1 alone is sufficient for PRs (Rosario & Birchmeier, 2003) whereas supplementary signals parallel to or downstream of Rac1 are required for CDR formation.

Rab5, another small GTPase, participates in both Rac1 dependent and independent pathways leading to CDR formation. Firstly, Rab5 is activated by ligand bound receptor tyrosine kinases (RTKs) leading to the trafficking of Rac1 and the guanine exchange factor (GEF) T-cell lymphoma invasion and metastasis 1 (TIAM1) to early endosomal compartments (Lanzetti et al., 2004). Rac1 becomes activated there and is subsequently rerouted to the plasma membrane where it is involved in CDR formation. Rab5 associates with actinin 4 (ACTN4) via the Rab5 GTPase activating protein (GAP), RN-tre. ACTN4 binds and cross links F-actin and modulates actin dynamics favourable for CDR formation (Lanzetti et al., 2004).

Another kinase, PAK, has also been reported to play a role in CDR formation (Dharmawardhane et al., 2000). Activated PAK1 is found to co-localise with F-actin in immature CDRs (Dharmawardhane et al., 1997; Dharmawardhane et al., 2000; Lanzetti et al., 2004). Many more CDRs are observed in cells expressing active PAK suggesting an early regulatory role for PAK1.

Further downstream, cortactin, a cytoplasmic regulator of several cortical actin structures, mediates CDR formation. It forms a complex with the endocytotic GTPase dynamin 2 (Dyn2) (Krueger et al., 2003) in mediating actin remodelling. WASP exists in this complex in most instances (Legg et al., 2007) and it is thought that it regulates the ability of cortactin to stimulate Arp2/3-dependent actin polymerization (Buccione et al., 2004; Cortesio et al., 2010; Schafer et al., 2002). Within the complex, cortactin binds and cross-links F-actin while stabilizing the filament branch points. Arp2/3 serves by stimulating actin nucleation and polymerization. This WASP-Arp2/3-cortactin-Dyn2 complex is often implicated as the minimal essential machinery for a number of actin structures.

Interestingly, focal adhesion proteins also play an important role in CDR formation. β 1-integrin crosstalk with growth factor receptors mediates an array of downstream signalling events, including the formation of CDRs (King et al., 2011; Schwartz, 2010; Sturge et al., 2002). It was shown that cells lacking β 1-integrins exhibited almost no CDR while endogenous β 1-integrin localizes to CDRs along with PDGFRs and WASP (King et al., 2011). β 1-integrin could promote CDR formation by regulating the WASP-WIP association and preventing ubiquitin mediated degradation of WASP. On the other hand, β 1-integrin could function in an independent pathway since the engagement of integrin receptors can trigger focal adhesion kinase (FAK) mediated phosphorylation of p130^{cas} and its activation leading to Nck-/Crk-induced CDR formation. β 1-integrin is also responsible for localised activation of CDC42, which can in turn activate WASP (King et al., 2011). Integrin clustering sites also serve as points of enrichment for phospholipids that potentiate the synergistic activation of WASP in CDRs (J. Hasegawa et al., 2011; King et al., 2011). In addition, β 3-integrins have also been found to translocate from the ventral surfaces to CDRs after PDGF stimulation (Gu et al., 2011). These integrins are then internalized and recycled back to the focal adhesions after CDR

closure. Contrary to the previous study, Azimifar and co-workers have found that CDR formation is independent of $\beta 3$ -integrin signalling and is co-regulated by EGFR signalling and events downstream of $\beta 1$ -integrin and integrin-linked kinase (Azimifar et al., 2012). Similarly, many molecules involved in both ECM-cell adhesions (paxillin and Pyk2) and invasion (matrix metalloproteinase-2 (MMP-2)) are localized to CDRs and are also important components of ECM invasion (Buccione et al., 2004; Suetsugu et al., 2003). Interestingly, paxillin-knockout fibroblast (pax^{-/-}) exhibited more CDRs when stimulated with PDGF than pax^{+/+} cells (Sero et al., 2011). This finding suggests that CDRs might be closely related to invasive protrusions that form when the cells lose their focal adhesions.

CDRs have also been implicated in cell migration (Krueger et al., 2003; Sero et al., 2011). Cell migration is important in many physiological processes such as gastrulation (Keller, 2005), leukocyte extravasation (Carman, 2009) and wound healing (Martin, 1997), as well as pathological processes such as metastasis of cancer cells (Liotta et al., 1991; Yang et al., 2004). Migration for cells adherent to the substrates involves continuous formation and dissociation of cell-substrate anchorage sites (Ilić et al., 1995), where forces are transmitted from the cell to the substrate through traction (Munevar et al., 2001) as the cell protrudes and retracts its membrane to propel itself forward. As such, the compliance of the substrate plays a role in regulating cell migration speed (Lo et al., 2000).

To initiate cell migration, the stress fibers and cell-ECM attachments mediated by focal adhesions must first be disassembled (Buccione et al., 2004). There should be simultaneous lamellipodial protrusion in response to a stimulus and retraction of the cell's trailing edge to propel the cell forward. During this process, the cell undergoes massive actin cytoskeleton reorganization and membrane extension occurs at the leading edge. Stress fibers in PDGF stimulated NIH 3T3 fibroblasts undergo disassembly with the resulting G-actin relocating to

peripheral ruffles (PRs) and CDRs (Cortesio et al., 2010; Legg et al., 2007; Suetsugu et al., 2003). It has been shown that these ruffles are proximal to the leading edge and could be supporting the cortical meshwork in protruding lamellipodia (Buccione et al., 2004; Krueger et al., 2003; Orth et al., 2006).

The persistence in cell migration direction affects how quickly a moving cell reaches its target location. In wound healing assays, cells at the wound edge migrate directly into the wound (Martin, 1997; Rodriguez, 2005), exhibiting migrational persistence towards the direction of the wound. Cellular migrational persistence is evident in many other cellular processes such as chemotaxis (Corallino et al., 2018; Zigmond, 1973), durotaxis (Lo et al., 2000) and haptotaxis (Carter, 1967), where cells migrate along chemical, stiffness and substrate-bound chemoattractant gradients respectively. One important chemoattractant in which cells respond to via chemotaxis is PDGF, which induces the formation of CDRs. In addition, Corallino and co-workers have described how a PDGF based chemotactic environment induces CDR formation and promotes directed migration in a PI3K mediated fashion (Corallino et al., 2018). Since CDRs tend to form near the edge of cells, there may be a possibility that CDR themselves can solely contribute towards directional persistence of cell migration. However, till date, this remains unclear.

Besides priming the stationary cell for movement, CDRs have been implicated in mesenchymal migration, the acquired ability of cells to traverse in three-dimensions within the ECM. It has been found that mesenchymal migration encompasses both PR-dependent cell motility and CDR dependent matrix invasion (Suetsugu et al., 2003). Mesenchymal cells must have the ability to disrupt and remodel the ECM around them. It was found that MMP-2 colocalizes with WAVE1

at CDRs (Buccione et al., 2004; Suetsugu et al., 2003), suggesting a potential role for CDRs in invasion, indicating a possible correlation between CDRs and cancer.

In summary, CDRs have mainly been characterised as a feature of growth factor stimulated, and Rac1 mediated structures, there has been growing evidence that there may be a role for RhoA and its downstream effectors. Increasing substrate stiffness has been shown to increase the number of cells exhibiting CDRs and regulate their lifetimes and maximum area (Huynh et al., 2013; Zeng et al., 2011). In addition, it was found that the fraction of cells exhibiting CDRs could be controlled via the inhibition of myosin light chain kinase (MLCK) even when they were seeded on substrates with increasing stiffness (Huynh et al., 2013). Another effector of RhoA is the formin family protein, mDia1, which is an actin nucleator. mDia1 has a GTPase binding domain (GBD) at the N terminal and the diaphanous auto-regulatory domain (DAD) at the C terminal enclosing the FH3, FH1 and the FH2 domains. This closed conformation can be relieved by binding of active RhoA to the GBD. mDia1 has been found in the phagocytosis cup and has also been speculated to be part of membrane ruffles. However, the role of RhoA and its effectors remains unclear in the CDR context. Thus, probing into the contribution of RhoA and its effectors is of interest in this work.

It has also been postulated that CDRs are formed from disassembled stress fibers which are reorganised into dynamic actin rich structures. Bernitt and colleagues found that CDR formation was not substantial in cells grown on circular fibronectin islands (Bernitt et al., 2015). Zeng and colleagues found that cells seeded on increasing substrate stiffness led to more of them forming visible stress fibers and exhibiting more CDRs when stimulated by PDGF (Zeng et al., 2011). A substrate stiffness coupled MLCK activity dependant mechanism for CDR formation has also been proposed (Huynh et al., 2013). In addition, integrin signalling at the focal adhesions has been shown to be crucial to CDR formation in a Src dependent manner (Azimifar et al., 2012).

Considering the interplay between the stress fiber maintenance functions of POPX2 (Xie et al., 2008), MLCK, focal adhesion signalling, it was still unclear how CDRs are sustained after their formation. Thus, probing into the contribution of stress fibers, focal adhesions and POPX2 is of interest in this work.

Another important question that has not been addressed is the fate of disassembled actin from CDRs. Both CDRs and lamellipodia are actin-rich structures that have been constantly linked to cell migration (Gu et al., 2011; Ponti et al., 2004; Zicha et al., 2003). One cannot negate the possibility that CDRs can solely contribute towards directional persistence of cell migration. In addition, retrograde actin flow has been well described to be essential for cell migration. However, little is known if CDRs that are F-actin rich, serve as actin pools and thus can enhance actin flow towards the leading edge to support retrograde actin flow and thus directed cell migration. Thus, probing into the contribution of CDRs in actin flow towards the leading edge for directed cell migration is of interest in this work.

The figure below summarises the signalling molecules implicated in CDR formation and our current understanding of CDR regulation (Fig. 9). I have also highlighted the topics (in red boxes) that are not well studied and of interest to this thesis work.

Figure 9. The processes and pathways leading up to the formation of CDRs

There is a growing body of evidence that supports the contribution of substrate stiffness and focal adhesion signalling in CDR formation. The need for stress fibers to be disassembled to form a pool of actin monomers to be assembled into CDRs has also been postulated. Growth factor stimulation starts a cascade of reactions involving several protein regulators of CDRs and they mainly converge at Rac1 activation suggesting branched actin filament formation at CDRs via Arp2/3. The role of RhoA in CDRs, especially since substrate stiffness is implicated, is less well studied. The architecture of CDRs and nucleators involved as well as the final fate of actin after CDR disassembly still remain unexplored. In this figure, the boxes highlighted in red are topics of interest for this study.

1.3 Objectives of this study

The physiological roles of CDR have become more evident. There is growing evidence that suggest that CDR formation is a pathogen-inducible cell response and they form the basis for macropinocytosis in macrophages. Although many proteins and signalling pathways involved in CDR formation have been reported, there are still many unknowns related to CDR. These earlier findings and remaining unanswered questions give us further motivation to study CDR dynamics and the structural features one would expect to see, the factors required to sustain fully formed CDRs and the fate of actin after the disassembly of CDRs. The overall objectives for this study are (1) to resolve the contribution of RhoA and Rac1 and its effectors in the formation of CDRs to reveal the architecture of CDRs, (2) to uncover the contribution from stress fibers and focal adhesions to sustain CDRs, (3) to uncover how actin is reorganised after CDR disassembly and thus understand the advantages that CDRs confer to a migrating cell.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 General reagents

All chemicals and reagents used were purchased from the following companies:

Buffers and salts:

Sigma-Aldrich (St. Louis, MO, USA)
Merck (Darmstadt, Germany)
USB (Sampscott, MA, USA)
Bio-Rad (Hercules, CA, USA)

Cell culture and transfection reagents:

Sigma-Aldrich (St. Louis, MO, USA)
Gibco (Grand Island, NY, USA)
Invitrogen (Carlsbad, CA, USA)

SDS-PAGE, Western Blot reagents and Protein markers:

Sigma-Aldrich (St. Louis, MO, USA)
Bio-Rad (Hercules, CA, USA)

2.1.2 Commercial kits

QIAquick® Spin Maxiprep kit
Qiagen Plasmid Mini kit (25)
Alexa Fluor 633 Protein Labelling kit

2.1.3 Media

LB medium 1% (w/v) Bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl
LB agar LB medium plus 1.5% (w/v) bacto-agar
Ampicillin broth LB medium containing 60 µg/mL ampicillin
Ampicillin plate LB agar plate containing 60 µg/mL ampicillin
Kanamycin broth LB medium containing 30 µg/mL kanamycin
Kanamycin plate LB agar plate containing 30 µg/mL kanamycin
Cell culture media Dulbecco's Modified Eagle's Medium with 4500mg/L glucose
Cell freezing medium 10% (v/v) DMSO in FBS

2.1.4 Buffers

50× TAE buffer:

24.2g Tris base
57.1ml acetic acid
100ml 0.5M EDTA, pH8
Top up to 1L with water

Mammalian cell lysis buffer:

10ml 1M Hepes, pH7.5
24ml 5M NaCl
400μl 1M MgCl₂
0.8ml 0.5M EGTA
8ml 1M β-glycerol phosphate
4ml 0.1M sodium orthovanadate
8ml 0.5M sodium fluoride
2ml glycerol (neat)
10ml 20% Triton-X
Top up to 400ml with water
Add 1× protease inhibitor and 1× DTT before use

10× SDS-PAGE running buffer:

30.2g Tris base
144g glycine
10g SDS
1L water

10× PBS (phosphate buffered saline):

80g NaCl
2g KCl
14.4g Na₂HPO₄
2.4 g KH₂PO₄
1L water, pH 7.4

10× TBS:

24.2g Tris base
80g NaCl
1L water, pH 7.6

10× Nitrocellulose transfer buffer:

30.3g Tris base
144g glycine
1L water

1× Nitrocellulose transfer buffer (with 20% Methanol):

1× 50ml 10× nitrocellulose transfer buffer
50ml methanol
400ml water

2.1.5 Bacterial strains and mammalian cell lines

DH5α Chemically competent bacterial strain Invitrogen, Thermo Scientific, #18265017

NIH3T3 Mouse embryonic fibroblast ATCC-CRL-1658

NIH3T3-GFP Stable line overexpressing GFP, Puromycin resistant, (In house)

NIH3T3-POPX2 Stable line overexpressing GFP-POPX2, Puromycin resistant, (In house)

NIH3T3-POPX2m Stable line overexpressing GFP-POPX2m, Puromycin resistant, (In house)

2.1.6 Primary antibodies

Antibody, Species, Clonal, Source Catalog #

Active Rac1-GTP (Payne, et. al., 2014) Mouse, Monoclonal, NewEast Biosciences (Malvern, PA, USA), #26903

Active Rho-GTP (Gangli, et. al., 2013) Rabbit, Polyclonal, NewEast Biosciences (Malvern, PA, USA), #26904

p140mDia (Watanabe et. al., 1997) Mouse, Monoclonal, BD Transduction Laboratories, #610848

Arp2 (Schlau et. al., 2018) Rabbit, Polyclonal, Cell Signalling Technology, #3218S

MLC II (Ser 19) (Keil et. al., 2009) Rabbit, Polyclonal, Cell Singnalling Technology, #3671

Anti-FAK (Zeng, et. al., 2003) Mouse, Monoclonal, BD Transduction Laboratories, #610087

Anti-phospho-FAK (Y397) (Barcus, et. al., 2013) Rabbit, Polyclonal, Invitrogen, #44-624G

Paxillin (Kawada, et. al., 2013) Mouse, Monoclonal, Invitrogen, AH00492

α-tubulin (Chen, et. al., 2007) Mouse, Monoclonal, Sigma-aldrich, #T9026

2.1.7 Secondary antibodies

Antibody, Company, Catalog number

Goat anti-mouse IgG HRP conjugated, Thermo Scientific	#62-6520
Goat anti-rabbit IgG HRP conjugated, Thermo Scientific	#65-6120
Goat anti-mouse IgG, Alexa Fluor 488 Molecular Probes	#A-10680
Goat anti-mouse IgG, Alexa Fluor 546 Molecular Probes	#A-11060
Goat anti-rabbit IgG, Alexa Fluor 488 Molecular Probes	#A-11034
Goat anti-rabbit IgG, Alexa Fluor 546 Molecular Probes	#A-11010

2.1.8 Fluorescence Probes

NucBlue Fixed Cell Stain Ready Probes reagent	Molecular Probes by Life Technologies, #R37606
Phalloidin	Alexa Fluor 647 phalloidin, CST, #8940
Phalloidin	Alexa Fluor 633 Molecular Probes #1275739
Cy5 dye	Alexa Fluor 633 Molecular Probes, #A20170

2.1.9 Plasmids

pXJ-GFP-Flag	pXJ40 vector, GFP tag
pXJ-GFP-Flag-RhoG14V	Human RhoA full length cDNA, amino acid residue 1-193, mutation at G14V
pXJ-GFP-Flag-RhoT19N	Human RhoA full length, amino acid residue 1-193, mutation at T19N
pXJ-GFP- Flag-RacG12V	Human Rac1 full length, amino acid residue 1-191, mutation at G12V

pXJ-GFP- Flag-RacT17N	Human Rac1 full length, amino acid residue 1-191, mutation at T17N
pXJ-GFP-Lifeact	Full length Lifeact (MBI)
pXJ-GFP- Flag-Paxillin	Full length Paxillin (MBI)
pEGFP-mDia1	Full length mDia1, amino acid residue 1-1256 in pEGFP backbone
pEGFP-mDia1-ΔN3	Constitutive active mDia1 (543-1192aa) containing only FH1 and FH2 domain in pEGFP backbone
pXJ-RFP-Lifeact	Full length Lifeact in pXJ backbone (MBI)
pXJ-mCherry-MLC II	Full length Myosin Light Chain II in pXJ backbone (MBI)
mEosin3.2-actin	mEosin3.2 fused with full length actin in mEos3.2-C1 backbone (MBI)
pTriEx4-Rac1-2G-CFP/YFP FRET	Full length second generation Rac1 FRET biosensor in pTriEX4 backbone (Rac1-2G) (MBI)

2.1.10 Pharmacological Inhibitors and Stimulants

PDGF (Johansen, et. al., 1994)	Sigma-Aldrich, #P4306
RhoA activator II, (Valtcheva, et. al., 2013)	Cytoskeleton, #CN03-A
RhoA inhibitor I, (Balzer et. al., 2012)	Cytoskeleton, #CT04-A
SMIFH2, (Rizvi et. al., 2009)	ChemBridge Corporation #5992446
CK666, (Nolen, 2009)	Calbiochem, CAS 442633-00-3
Y-27632, (Ishizaki, et. al., 2000)	Calbiochem, CAS 146986-50-7
ML-7, (Saitoh, et. al., 1987)	Calbiochem, CAS 110448-33-4
Blebbistatin (Coffman, et. al., 2009)	Sigma-Aldrich, B0560
Nocodazole (Glottfelty, et. al., 2014)	Sigma-Aldrich, M1404

2.1.11 ECM Proteins/reagents used for coating and cell constraining

Fibronectin	Roche, #06475736103
Collagen, Type 1	Advanced BioMatirx, #5005
Poly-L-Lysine	Sigma-Aldrich, #P8920
Pluronic F-127	Sigma-Aldrich, #P2443
SYLGARD™ 184 Silicone Elastomer Curing Agent	Dow Corning Corporation, #0009387783
SYLGARD™ 184 Silicone Elastomer Base	Dow Corning Corporation, #0009387783

2.1.12 Microscopes

Perkin Elmer Spinning Disk	Olympus IX81 Inverted Microscope, Confocal EMCCD camera (C9100-13, Hamamatsu Photonics)
Nikon A1R si Polarizing Module	Nikon TI2-E Motorised Inverted Microscope, Andor Neo sCMOS camera (Andor Technology)
Olympus iLAS TIRF	Olympus IX81 Inverted Microscope, DualCam: Photometrics DC2; Dichroic (T585LP); Emitter (525/45; 609/54)
Olympus Live TIRF	Olympus IX81 Inverted Microscope Photometrics CoolSNAP K4 2048 x 2048
Nikon N-SIM	Nikon Ti-E Motorised Inverted Microscope 3D-SIM(100 EX V-R) DU-897 3 directions*5phases

2.2 Methods

2.2.1 Plasmid purification

Plasmids were amplified in DH5 α cells and purified from an overnight culture in LB medium supplemented with Ampicillin or Kanamycin using Qiagen Maxiprep Kit according to the manufacturer's protocol.

2.2.2 Cell culture

NIH3T3 cells were cultured in DMEM containing 4500mg/L glucose, supplemented with 3.7g/L sodium bicarbonate and 10% fetal bovine serum (FBS) at 37°C in 5% CO₂. 80000-100000 cells were seeded on surfaces (27mm glass bottom dishes, 35mm plastic bottom dishes and 6 well plates) and allowed to grow to 60%-80% confluency overnight for experiments. The cell culture media was replaced with media supplemented with 1% FBS to serum-starve the cells for 4hrs. Just before live cell imaging, the cell culture media was replaced with fresh cell culture media supplemented with 10% FBS and 30 ng/mL of recombinant PDGF was added to each sample to stimulate CDRs. Typically, about 30% of the cells exhibit CDRs upon PDGF stimulation.

2.2.3 Transfection

NIH3T3 cells were grown to 70-80% confluence and transiently transfected using jetPRIME reagent according to manufacturer's instructions. 1 μ g plasmid DNA was diluted in 100 μ l of

jetPRIME buffer. This mixture was vortexed for 10s and spun down. 3 μ l of jetPRIME reagent was added to the mixture and this was vortexed for 10s and spun down. The resulting transfection mixture was incubated at room temperature for 10min. The transfection mixture was then added to 100000 adherent cells in a 6-well/35mm dish already in serum containing media. The media was then changed after 4hr and the cells were incubated overnight. Cells were then re-plated on fresh extracellular matrix coated dishes so as to improve dynamics.

2.2.4 Western blot

Protein lysates were harvested from NIH3T3 cells and the concentration determined with the Bio-Rad protein assay kit. 50 μ g of sample was boiled in 1x SDS loading buffer and loaded into each well on an 8-12% polyacrylamide gel. The proteins were then transferred to nitrocellulose membranes. Blocking was carried out for 1h at room temperature in 5% BSA. Immunoblotting with primary antibody was carried out at 4°C overnight and secondary antibody for 1h at room temperature. The chemiluminescent signals were detected on film with BioRad® ECL reagent.

2.2.5 Preparation and characterization of patterned fibronectin strips

Micro-fabricated moulds were designed and made in collaboration with the Nano and Microfabrication Core at the Mechanobiology Institute at NUS, such that they had indentations that can be used to print 2 μ m, 5 μ m and 10 μ m wide long tracks (Fig. 10). SYLGARD™ 184 Silicone Elastomer Base was mixed with SYLGARD™ 184 Silicone Elastomer Curing Agent in a 9:1 ratio and this was degassed for about 1 hr to remove bubbles in the mixture. The degassed gel was then poured into the micro-fabricated mould until it covered the surface. The mould was

then placed at 80 °C for 3 hrs to cure to form a cross-linked semi-solid layer of PDMS. This layer was peeled from the micro-fabricated mould and was cut into small stamps (2 cm by 2 cm). These PDMS stamps were plasma treated at 8.5 kV for 5 min to decrease hydrophobicity of the stamps. 100× dilution of Alexa-633 cy5 dye was carried out in 10 µg/ml of fibronectin in PBS. 10 µl of this solution was added the side of the stamp that had the indentations for the shape of interest. The solution was allowed to spread throughout the stamp and dry for 10-15 min. Once the stamp was sufficiently dry, it was inverted and stamped down onto the 27 mm Iwaki glass bottom dish and held for about 30 sec. The stamp was then removed and discarded. The dish was further treated with 0.2% pluronic acid for 1 hr. After pluronic acid treatment, the dish was washed with PBS thrice and stored in 4 °C, to be used for seeding cells the next day (Fig. 10).

Figure 10. Cells seeded on fibronectin strips prepared by micropatterning

Fibronectin strips restrict cells to move in either a rightward or leftward direction along the strips. This restriction aids in determining if CDRs, which particularly form near the lamellipodia, help cells commit towards a certain direction in a persistent manner.

2.2.6 Usage of Reagents

RhoA activator II and inhibitor were purchased from Cytoskeleton. Cells were treated with RhoA activator II (Valtcheva, et. al., 2013) for 2 hrs at 1 $\mu\text{g/ml}$ and were constantly checked for cell health and increased stress fiber density. Cells were treated with RhoA inhibitor I (Balzer et. al., 2012) for 1.5-2 hrs at 2 $\mu\text{g/ml}$ and were constantly checked for cell health and decreased stress fiber density. Cells were treated with 20 μM and 100 μM of SMIFH2 (formin inhibitor) (Rizvi, et. al., 2009) and CK666 (Arp2/3 inhibitor) (Nolen, 2009) respectively for at most 20 min before proceeding to live imaging. Cells were treated with 20 μM Y-27632 (ROCK inhibitor) (Ishizaki, et. al., 2000) for 30 min. Cells were treated with 50 μM ML-7 (MLCK inhibitor) (Saitoh, et. al., 1987) for 30 min. Cells were treated with 5 μM PF-228 (FAK inhibitor) (Slack-Davis, et. al., 2007) for 30 min.

2.2.7 G-actin incorporation assay

For labelling of actin incorporation sites (barbed ends), cells were serum starved for 2-4 hrs and stimulated with PDGF for 2 min. On observing the first CDRs, the cells were permeabilized in the presence of 0.45 μM rhodamine-labelled G-actin in cytoskeletal buffer with 0.02% digitonin and 1% BSA for 2 min. After a brief rinse in cytoskeletal buffer, cells were fixed in 3.7% formaldehyde for 15 min. The cells were then processed for immunostaining, with the Triton permeabilization step omitted.

2.2.8 Immunofluorescence staining

After 3 min of PDGF induction, NIH 3T3 fibroblasts were fixed with 3.7% formaldehyde for 15 min. Fixed cells were permeabilised with 0.2% Triton-X/PHEM and blocked in 2% BSA. Primary antibodies were diluted in 4% BSA and added to cells for incubation overnight at 4°C. The cells were then incubated with secondary antibodies (diluted in 2% BSA) for 1 hr at room temperature. Immunostained samples were then counter-stained with NucBlue Fixed Cell Stain Ready Probes reagent. All primary and secondary antibodies were diluted at the ratio 1:200 and 1:1000 respectively.

2.2.9 Microscopy

Time lapse, live-imaging, phase contrast microscopy to visualise CDRs was carried out using the Widefield TIRF Olympus Live microscope with a 20x objective at 30 sec intervals, with a sample heater (37°C) and a CO₂ incubation chamber. Images of 6 field positions were taken to be representative of the entire sample. This was helpful in determining the fraction of cells exhibiting CDRs in a given sample. Fixed images were reconstructed by the Nikon A1 software and were further processed using ImageJ. Some immunostained samples were examined using confocal PerkinElmer Spinning Disk microscope with a 100x /1.3 oil immersion objective. Images were then analysed using ImageJ and Imaris. Images were acquired with z-step sizes of 0.1-0.2µm. Time lapse and fixed structured illumination microscopy was carried out using the super resolution NSIM microscope with a 100x /1.3 oil immersion objective. A value of 0.5 for Structured Illumination Contrast was used for image reconstruction. A default value of 0.05 was used for the width of the 3D-SIM filter.

2.2.10 Quantifying the fraction of cells exhibiting CDRs

Time lapse, live-imaging, phase contrast microscopy to visualise CDRs was carried out using the Widefield TIRF Olympus Live microscope with a 20x objective at 30 sec intervals for 15 min, with a sample heater (37°C) and a CO₂ incubation chamber. Images of 6 field positions were taken to be representative of the entire sample. This was helpful in determining the fraction of cells exhibiting CDRs in a given sample. The number of cells exhibiting CDRs was counted in a given field of view. The corresponding fraction of cells exhibiting CDRs was computed in a given field of view. This was repeated with images from 6-7 other field positions. Eventually, an average fraction of cells exhibiting CDRs was obtained for a given sample.

2.2.11 Quantifying the fold changes/differences in cells exhibiting CDRs

In experiments that required comparisons between samples, the average fraction of cells exhibiting CDRs was normalised against the average fraction of cells exhibiting CDRs in control samples to derive the fold changes/differences between samples. Since experiments were performed in triplicates; there is a spread in the data within the control sample. Hence, a box plot was used to represent this data to reflect the spread within each condition and the fold changes between the different conditions.

In experiments that involved the transfection of GFP tagged constructs, the fraction of cells exhibiting CDRs in GFP expressing cells (control) was initially obtained by observing the 488nm channel. Precautions were taken to analyse cells exhibiting similar intensities of GFP. Next, the fraction of cells exhibiting CDRs in GFP-construct expressing cells was also obtained

by the similar manner. Following this, the average fraction of GFP-construct expressing cells exhibiting CDRs was normalised against the mean of the GFP only expressing (control) samples to derive the fold changes/differences between samples. In addition, since experiments were performed in triplicates, there is a spread in the data within the control sample. Hence, a box plot was used to represent this data to reflect the spread within each condition and the fold changes between the different conditions.

2.2.12 Obtaining z-fluorescence intensity plots in CDRs

I had performed some immunofluorescence experiments to observe for the differences in fluorescence intensities due to different proteins along the height of the CDR. Z-stacks of the samples were obtained via imaging on the Perkin Elmer Spinning Disk and the Nikon N-SIM microscopes. Within the z-stack, the top and bottom positions of a CDR was obtained recording last slices in the stack that show clear focussed images of the CDR. In an upward scan, the first slice to show clear indication of a CDR is taken to be the base of a CDR. The last slice to show any remnant signal of CDR was taken to be the top of a CDR (the base of a CDR is usually out of focus near the top of the CDR and vice versa). The N-SIM microscope was preferred due to the need for a higher ~800nm resolution. However, bleaching issues had forced imaging via the Perkin Elmer Spinning Disk confocal microscope where samples seemed more protected. 5-10 points along the perimeter of the CDRs were used to generate the z fluorescence intensity profile. A mean profile from all of these profiles was obtained from the 5-10 points. This same procedure was repeated for the non-CDR regions in the cell. Subsequently, the mean profile obtained from the points in the CDR was normalized against the mean profile obtained from the points in the non-CDR region to yield the normalized z-fluorescence intensity plot.

2.2.13 Extraction of focal adhesion properties

TIRF images of the ventral portion of fixed cells stained for focal adhesion proteins were obtained by the Olympus iLAS TIRF microscope at a 100x oil objective. These images were saved as tiff files and uploaded on the Focal Adhesion Analysis Server (Berginski & Gomez, 2013). The received results were packaged with MS Excel files. Typical results from the server include, focal adhesion areas, focal adhesion length, etc.

2.2.14 Time lapse imaging and reconstruction of SIM images

Time lapse, live-imaging to visualise CDRs was carried out using the Nikon N-SIM super resolution microscope while selecting the 3D SIM mode at 30 sec intervals for 15 min, with a sample heater (37°C) and a CO₂ incubation chamber. Only cells transfected with different coloured tags that exhibited a high contrast against the background were chosen for imaging. Eventually, the time lapse images were reconstructed using the internal N-SIM software protocol and rendering was performed on Imaris from which orthogonal views were also generated.

2.2.15 Visualization and photoactivation of mEos3.2-actin

NIH 3T3 fibroblasts were imaged to obtain CDR and lamellipodial sizes, cell migration speed, and directional persistence using an inverted microscope (Axiovert 200; Carl Zeiss) with a 20x (0.3 NA) objective fitted with a temperature control incubator under phase contrast. Images were captured 1 hr before and after PDGF stimulation of cells, at an interval of 30 sec. To visualize and track the movement of actin in lamellipodia and CDRs, the cells were transfected with mEos3.2-actin cDNA construct encoding a green to red photoactivatable fluorescence protein conjugated to actin. Photoactivation experiments involving mEos3.2-actin were carried

out using confocal PerkinElmer Spinning Disk microscope under a 100x /1.3 oil immersion objective. After PDGF stimulation, the actin in the CDRs within the cells were photoactivated using a 405 nm laser and the red fluorescence intensity of actin at the lamellipodia at the cell edge close to the CDR was monitored. The kymograph function in ImageJ was eventually used to track the relocation of actin from CDRs to the leading edge of a cell.

2.2.16 Quantification of cell migration persistence (γ)

The cell migration speed and persistence were quantified by tracking the movement of the centroid of the cell in time by TrackMate and DiPer (Tinevez et al., 2017; Gorelik & Gautreau, 2014). The mean squared displacement r^2 , where $r = \sqrt{\Delta x^2 + \Delta y^2}$, of a tracked cell with coordinates (x, y) at any point in time is characterized as having a diffusivity D . I have described the movement of the cell as $r^2 = Dt^\gamma$, where t is the time elapsed since the start of cell tracking and γ is denoted as a cell migration persistence factor. When $\gamma = 1$, then $r^2 = Dt$, where the displacement is proportional to the square root of the elapsed time and the cell is said to exhibit a random migrational behaviour. When $\gamma = 2$, then $r^2 = Dt^2$, where the displacement is proportional to the elapsed time and the cell exhibits migratory persistence. The value of γ is obtained for each cell by plotting the log-log graph of r^2 against t and obtaining a linear fit, where γ is the slope of the line. Hence, cell migration persistence can be quantified using γ .

2.2.17 Statistical Analysis

The Student's two-tailed t test was used to test the statistical significance of the data. Data were derived from at least triplicates and the error bars reflect the standard errors that arise from them.

3. RESULTS

3.1 Circular Dorsal Ruffles: co-operation between GTPases

3.1.1 RhoA inhibition attenuates CDR formation and dynamics

Due to the growing evidence for the role of RhoA's effectors in the formation of CDRs, I had sought to confirm the influence of RhoA on CDRs. Control NIH 3T3 cells were compared against cells that were treated with RhoA activator or inhibitor. I consistently found that RhoA activated cells exhibited strong stress fiber formation and a greater fraction of them exhibited CDRs when compared to control and RhoA inhibited cells (Fig. 11A, 11B & Movie 2). I next proceeded to determine how RhoA activity mediated stress fiber formation could affect the dynamics of CDRs. The time taken to initiate CDRs was first examined to see if there was any enhancement in propensity of the cells to exhibit CDRs. I found that cells treated with the RhoA activator exhibited CDRs earlier than the control cells and those treated with RhoA inhibitor (Fig. 11C & 11D).

The maximum area of the CDR was also examined. It was found that all cells exhibited CDRs of similar maximum areas regardless of treatment with RhoA activator or inhibitor (Fig. 11E). In addition, the lifetimes of CDRs were compared for RhoA-activator or inhibitor treated cells. It was observed that all cells exhibited CDRs of similar lifetimes (Fig. 11F). These observations suggested that RhoA inhibition mediate stress fiber loss could attenuate CDR formation and RhoA activation mediated stress fiber formation could enhance CDR formation. However, I found that overexpression of wildtype RhoA in cells resulted robust stress fiber formation similar to overexpression of RhoG14V (Fig. 11G). To confirm that RhoA activity was crucial for CDR formation at the single cell level, I transfected NIH3T3 cells with GFP-only construct,

GFP tagged constitutively active and dominant negative constructs of RhoA and Rac1 to determine their effects on CDR formation. The transfection efficiency was around 20%. These cells were stimulated by PDGF and live imaging was performed over 15 mins at 30 sec intervals. I obtained a fraction of cells that also expressed GFP and exhibited CDRs against the total population of cells that expressed GFP. This was repeated with cells that expressed the GFP tagged constitutively active and dominant negative constructs of RhoA and Rac1. These fractions were then normalised against the fraction of GFP only expressing cells that exhibited CDRs. I found that relative to control cells (GFP-only expressing cells), overexpression of RhoAT19N and RacG12V attenuated the fraction of cells exhibiting CDRs. Overexpression of RacT17N also affected CDR formation but to a lesser extent.

On the other hand, overexpression of RhoAG14V did not affect CDR formation. I found similar fractions of cells exhibiting CDRs compared to control cells which were transfected with GFP vector plasmid (Fig. 11H and Movie 3). It was puzzling that expressing the dominant active Rac1 construct (RacG12V) resulted in attenuation in the fraction of cells exhibiting CDRs. I therefore used a FRET biosensor to investigate if there was localised activation of Rac1 at the region where CDRs form. Interestingly, localised Rac1 activation was observed at the CDR (Fig. 11I). Taken together, the observations suggest that both RhoA and Rac1 are involved in CDR formation.

A

B **C** **D** **E** **F**

G **H** **I**

Figure 11. RhoA inhibition attenuates CDR dynamics while CDRs could form on localised activated Rac1 regions

NIH 3T3 cells were treated with RhoA activator and RhoA inhibitor (Cytoskeleton Inc) prior to PDGF stimulation for CDR formation. Phase contrast live imaging was performed. Immunostaining with phalloidin was done to check for stress fiber formation. The fraction of cells exhibiting CDRs and CDR dynamics characterised by time taken to initiate CDRs, max area and lifetime were quantified. (B) A plot of fold differences in fraction of cells exhibiting CDR with respect to control cells and (C) the time taken to initiate CDRs in a given population of cells in the presence of RhoA activator and inhibitor. (D) A montage of time-lapse images to represent the time taken to initiate CDRs in a given population of cells in the presence of RhoA activator and inhibitor. CDRs were traced by dotted lines in the figure. Higher magnification images of the CDRs can be seen in the boxed areas. (E) max CDR area and (F) lifetime from cells exhibiting CDRs that were treated with RhoA activator and inhibitor. Derived from analysing at least 150-200 cells from each group over at least 3 independent experiments. CDR dynamics was extracted from at least 10 CDRs per group. See Movie 2 at https://drive.google.com/open?id=1l8xzIHPIZsP4Xo1uZh_1UaV8spcPJXAo. (G) Cells expressing RhoWT, RhoG14V and RhoT19N were compared with control cells to check for similarity in stress fiber formation in RhoWT and RhoG14V transfected cells. (H) A plot of fold differences in fraction of cells exhibiting CDR. Cells expressing GFP-tagged RacG12V, RacT17N, RhoG14V and RhoT19N were compared with control cells (expressing GFP). All cells were transfected with the respective plasmids, and stimulated with PDGF to induce CDR. At least 30 cells with similar GFP intensity per group were observed over 4 independent experiments. See Movie 3 at <https://drive.google.com/open?id=1omW7fVqT3WnMNPPJTWlWarErgmT37r72>. (I) NIH 3T3 cells were transfected with Rac1 single chain FRET biosensor construct which is encoded with Rac1 and a PAK binding domain (PBD) on the same single chain. Binding between Rac1 and PBD increases proximity between the fluorophores to promote FRET efficiency thus revealing the active zones of Rac1. A representation of Rac1 activity via FRET efficiency with respect to CDRs. For control NIH 3T3 cells, about 20% to 30% of the population will exhibit CDR upon PDGF stimulation. Scale bar=10 μ m.

3.1.2 RhoA and Rac1 have clear and distinct zones of activation in a CDR

Having established that both RhoA and Rac1 are important for CDR formation, I proceeded to examine the CDR structure for the localisation of active RhoA and Rac1. Through immunofluorescence experiments, I examined z fluorescence intensity plots of at least 5 to 10 points along the perimeter of the CDR ring and regions other than the CDR ring. I found that active Rac1 was enriched throughout the height of the CDR ring, which is similar to the profile of phalloidin marked F-actin at the CDR (Fig. 12A). In addition, active Rac1 enrichment was also observed at the base of the CDR where phalloidin (which stains for F-actin) intensity started to decrease. Outside the ring however, active Rac1 and phalloidin were not enriched. In addition, active RhoA was found to be enriched at the top of the CDR while phalloidin was enriched throughout the CDR height (Fig. 12B). Similarly, outside the CDR ring, intensity

levels of active RhoA and phalloidin were not enriched. These observations indicate that RhoA and Rac1 have clear and distinct zones of activation within a CDR; active RhoA is enriched at the top of CDR while active Rac1 is found along the CDR and at the base of the CDR (Fig. 12C).

A

B

C

Figure 12. Spatially distinct active zones for RhoA and Rac1 in CDRs.

NIH 3T3 cells were treated with PDGF to induce CDR. Cells were fixed and immunostained with antibodies against active Rac1 and active RhoA. Alexa-647-phalloidin and DAPI was used to visualize F-actin and DNA, respectively. (A) Top panel: Immunofluorescence staining for active Rac1 and phalloidin to indicate the localisation of active Rac1 and phalloidin at a single CDR and regions without CDRs. Bottom left: z axis profile plot of fluorescence intensities along the height of CDRs. Bottom right: z axis profile plot of fluorescence intensities at regions other than the CDR. (B) Top panel: Immunofluorescence staining for active RhoA and phalloidin to indicate the localisation of active RhoA and phalloidin at a single CDR and regions without CDRs. Bottom left: z axis profile plot of fluorescence intensities along the height of CDRs. Bottom right: z axis profile plot of fluorescence intensities at regions other than the CDR. Z-stack step size of 0.2 μm and the final z axis profile plot of fluorescence intensities are derived from at least 10 different locations on the CDR and outside the CDR. Scale bar=20 μm for (A) and 10 μm for (B). (C) Schematic diagram of the distinct active zones for RhoA and Rac1 in a CDR.

3.1.3 Formins are potential actin nucleating candidates in CDRs

I hypothesize that downstream effectors of RhoA and Rac1 are important for the nucleation of actin involved in CDRs. Therefore, I first determined if inhibition of formins and Arp2/3 affected CDR formation in cells stimulated by PDGF. I found that inhibition of Arp2/3 led to a significantly lower fraction of cells exhibiting CDRs when compared to control cells (Fig 13A & Movie 4). This was expected as earlier studies have described the involvement of Arp2/3 in the formation of CDRs. In addition, I found that formin inhibition was deleterious to the formation of CDRs (Fig. 13A). To further confirm that formin activity was important for CDR formation, cells were transfected with the full length mDia1 and its constitutively active form and scored for the fraction of cells exhibiting CDRs. It was found that a significantly greater fraction of cells expressing constitutive active mDia1 (mDia1- ΔN3) exhibited CDRs as compared to control and cell expressing full length mDia1 (Fig. 13B & Movie 5). In addition, CDRs formed in cells expressing constitutive active mDia1 were observed to appear significantly sooner compared to CDRs in control cells and cells overexpressing full length mDia1 (Fig. 13C & 13D). These observations suggest that the actin nucleating activity of mDia1 might be involved in the formation of CDRs. I further checked for any significant

differences in CDR area and lifetime. I found no significant differences in terms of maximum CDR area and lifetime (Fig. 13E & F), suggesting that constitutively active mDia1 might not be involved in sustaining CDR structures for a considerable time. I suspected that mDia1 activity could be spatially correlated with the dynamics of CDR formation. To validate this hypothesis, time lapse imaging was performed to determine if there are any differences in spatial dynamics in cells expressing the full length mDia1 or the constitutively active form of mDia1 with reference to a CDR. I found that full length mDia1 showed diffused localisation at the CDR and throughout the cell whereas, constitutively active mDia1 was enriched at the CDR (Fig. 13G & Movie 6). The observations suggest that mDia1 might serve as an actin nucleator in CDRs.

A

B

C **D** **E** **F**

G

Figure 13. mDia1 is important to the formation dynamics and localises to CDRs

Phase contrast and fluorescence live imaging was performed. The fraction of cells exhibiting CDRs and CDR dynamics characterised by time taken to initiate, max area and lifetime were quantified. CDRs were visualised under fluorescence and phase contrast microscopy. (A) NIH 3T3 cells were treated with Arp2/3 inhibitors and formin inhibitors prior to PDGF stimulation for CDR formation. A plot of fold differences in fraction of cells exhibiting CDRs in a given population of cells in the presence of Arp2/3 or formin inhibitors compared with control cells was obtained. See Movie 4 at <https://drive.google.com/open?id=17Hj7sb7-IGFviGCyICnpB1qlChzWFL4o>. (B) NIH 3T3 cells were transfected with full length (mDia-FL) and constitutively active (mDia-ΔN3) forms of mDia1. The cells were then treated with PDGF to induce CDRs. A plot of fold differences in fraction of cells exhibiting CDRs was obtained. (C) A plot of time taken to initiate CDRs in a given population of cells expressing the full length and constitutively active forms of mDia1. See Movie 5 at <https://drive.google.com/open?id=1OzsEuvkbUIUpD78IMuppKk51S1NH9ERW>. (D) A montage of time-lapse images to represent the time taken to initiate CDRs in a given population of cells expressing the full length and constitutively active forms of mDia1. White arrows indicate the initiation of CDR. NIH 3T3 cells were transfected with full-length (mDia1-FL) and dominant active mDia1 (mDia1-ΔN3). The transfected cells were stimulated with PDGF to induce CDR. The area (E) and lifetimes (F) of CDRs were measured. Plots of max CDR area and lifetime from cells exhibiting CDRs are presented. All plots were obtained from at least 3 independent experiments analysing > 80 cells per group and at least 10 CDRs per group for the dynamics (G) Time lapse structural illumination microscopy (SIM) performed at 30 sec intervals, representing the localisation of full length and active mDia1 in a cell exhibiting a CDR. See Movie 6 at <https://drive.google.com/open?id=1xp8KnTTBA2pWA3iUorCCa9eQ8Gjo73W5>. White arrows indicate the locations of CDR. Scale bar=10 μm.

3.1.4 Cooperation between mDia1 and Arp2 to form the actin architecture at different zones in a CDR

Next, I hypothesized that mDia1 could be involved directly with the formation of actin filaments in CDRs and wanted to investigate how mDia1 and Arp2 could cooperate in the incorporation of actin into the dynamic CDR ring. Immunofluorescence staining with specific antibodies for mDia1 and Arp2 was carried out. Fluorescence intensity plots along the z-axis at least 5-10 positions on the CDR ring and other regions were examined. Finally, z-axis fluorescence intensity profile was obtained by normalising against fluorescence intensities from other regions of the cell. I observed that mDia1 localisation was mostly confined to the top portion of a CDR and remained noticeable at z slices along the CDR region but was decreased at z slices corresponding to the base of the CDR (Fig. 14A). Arp2 was localised throughout the height of a CDR and remained stable at the base of the CDR but was decreased at z slices at the top of the CDR. The raw fluorescence z intensity profile showed a similar trend with Arp2 localisation mostly confined to the bottom of CDRs (Fig. 14B). In addition, the profiles of phalloidin, mDia1 and Arp2 staining appeared to be similar in regions with no CDR (Fig. 14C). Furthermore, orthogonal views of the CDR have revealed mDia1 localisation to be near the top of a CDR and also along its height (Fig. 14D). On the other hand, Arp2 localisation was found to be near the base of the CDR and also along its height. The observations suggest that mDia1 and Arp2 could operate at different zones of a CDR to regulate its actin filament formation.

Next, I was interested to find out how free actin (G-actin) could be incorporated into the actin rich CDR. Since mDia1 and Arp2 could operate at different zones of a CDR, I hypothesized that incorporation of G-actin could be aided by mDia1 at the top and Arp2/3 from the bottom of a CDR. I visualized G-actin with fluorescence-labelled deoxyribonuclease 1 (DNase1) due to their high affinity interaction. Visualization of F-actin was done using fluorescence-labelled

phalloidin. Therefore, I immunostained CDRs for DNase 1 (G-actin), mDia1, Arp2 and phalloidin (F-actin) to reveal the localisation of G-actin in relation to the actin nucleating proteins. Fluorescence intensity plots along the z-axis at least 5-10 positions on the CDR ring and other regions outside CDR were examined. The z-axis fluorescence intensity profile of CDR was obtained by normalising against fluorescence intensities from other regions of the cell. DNase1 was localised at z slices within and at the top of the CDR (Fig. 15A). Arp2 was mostly confined to the z slices corresponding to the base of the CDR (Fig. 15B). The raw fluorescence z intensity profile showed that G-actin localisation was similar to F-actin localisation however the Arp2 intensity profile did not fluctuate too much in the CDR ring (Fig. 15C). Raw fluorescence intensity z profiles at other regions revealed similar trends between G-actin and F-actin localisation (Fig. 15D). I then repeated the experiment with immunostaining for mDia1 instead of Arp2. In addition I found that DNase1 was localised at z slices at the top of the CDR. mDia1 was found to localise throughout the height of the CDR. Its normalised fluorescence intensity was found to be decreased in the region corresponding to the base of the CDR (Fig. 16A). The raw fluorescence z intensity profile showed that DNase1 and mDia1 intensities was localised close to the top region of a CDR (Fig. 16B). Raw fluorescence z intensity profile of regions other than the CDR revealed no such trend at the same z slices (Fig. 16C). These observations suggest that endogenous G-actin could be incorporated into actin filaments in CDRs throughout its height via Arp2 and also at the top perhaps, via mDia1.

A **B** **C**

D

Figure 14. Arp2 localises more to the bottom while mDia1 localises to the top of a CDR.

NIH 3T3 cells were treated with PDGF to induce CDRs. The cells were fixed and immunostained with antibodies against Arp2, mDia1, Alexa-647-phalloidin and DAPI to visualize the localisation of Arp2, mDia1, F-actin and DNA, respectively. (A) Top panel: Immunofluorescence staining for Arp2, mDia1 and phalloidin to indicate the localisation of Arp2, mDia1 and F-actin at a single CDR and regions without CDRs. Bottom left: A z stack of the immunofluorescence staining used to derive a normalised z axis profile plot of fluorescence intensities. Right: A normalised z axis profile plot of fluorescence intensities due to Arp2, mDia1 and phalloidin along the height of a CDR and regions other than the CDR. Raw fluorescent intensities measured at CDR normalised against (B) raw fluorescent intensities measured at a CDR and (C) regions other than the CDR. Z-stack step size of 0.2 μm and intensity plots are derived from at least 10 different locations on the CDR and outside the CDR. Scale bar=5 μm . (D) Orthogonal views of Arp2 and mDia1 signals are presented to show relative localisation of Arp2 and mDia1 along a CDR height. Scale bar=3 μm .

A

Normalised Fluorescent signals

B

C

Figure 15. Arp2 localises more to the bottom while DNA Nuclease1 localises to the top of a CDR

NIH 3T3 cells were treated with PDGF to induce CDRs. The cells were fixed and immunostained with antibodies against Arp2, DNA nuclease 1, Alexa-647-phalloidin and DAPI to visualize the localisation of Arp2, G-actin, F-actin and DNA at a CDR, respectively. (A) Top panel: Immunofluorescence staining for DNA nuclease 1, Arp2 and phalloidin to indicate the localisation of G-actin, Arp2 and F-actin at a CDR and regions without CDR. Left: A z stack of the immunofluorescence staining used to derive a normalised z axis profile plot of fluorescence intensities due to Arp2, DNA Nuclease 1 and phalloidin along the height of a CDR. (B) A z axis profile plot of fluorescence intensities solely due to Arp2 normalizing (C) raw fluorescent intensities measured at CDR against (D) raw fluorescent intensities measured at regions other than the CDR. Z-stack step size of 0.2 μm and intensity plots are derived from at least 10 different locations on the CDR and outside the CDR. Scale bar=20 μm .

Figure 16. DNA Nuclease 1 and mDia1 localise more to the top of a CDR

NIH 3T3 cells were treated with PDGF to induce CDRs. The cells were fixed and immunostained with antibodies against mDia1, DNA nuclease 1, Alexa-647-phalloidin and DAPI to visualize the localisation of mDia1, G-actin, F-actin and DNA, at a CDR, respectively. (A) Immunofluorescence staining for mDia1, DNA nuclease 1 and phalloidin to indicate the localisation of mDia1, DNA nuclease 1 and F-actin at CDR and regions without the CDR. A z stack of the immunofluorescence staining was used to derive a normalised z axis profile plot of fluorescence intensities due to mDia1, DNA nuclease 1 and phalloidin along the height of a CDR. (B) Raw fluorescent intensities measured at the CDR was normalised against (C) raw fluorescent intensities measured at regions other than the CDR. Z-stack step size of 0.2 μm and intensity plots are derived from at least 10 different locations on the CDR and outside a CDR. Scale bar=20 μm .

To further confirm how G-actin could be added to the CDR ring, I performed a G-actin incorporation assay to look at how exogenous fluorescence labelled G-actin could be incorporated into a CDR. I prepared an actin polymerisation master mix which contained fluorescence labelled G-actin. I then stimulated cells to exhibit CDRs and permeabilised them. Fluorescence labelled G-actin was added so that it can be taken up by the cells. The cells were then fixed within 2 minutes to minimise background signal and probed for either mDia1 or Arp2 and F-actin. Then, I examined the normalised fluorescence intensity plots along the z-axis at least 5-10 positions on the CDR ring and other regions. I observed exogenously added G-actin was localised throughout the height of the CDR (Fig. 17A). Similarly, Arp2 was localised throughout the height of a CDR but not at the top of a CDR. A similar trend was observed in the raw fluorescence intensity z profiles of the CDR ring (Fig. 17B). However, no similar trend was

observed in the raw fluorescence intensity z profiles of regions outside the CDR (Fig. 17C). The experiment was next repeated with probing for mDia1 instead of Arp2. I observed that exogenously added G-actin was localised throughout the height of CDR. Similarly, mDia1 was localised throughout the height of CDR and at the top of a CDR (Fig. 18A). A similar trend was observed in the raw fluorescence intensity z profiles of the CDR ring (Fig. 18B). However, no similar trend was observed in the raw fluorescence intensity z profiles of regions other than the CDR (Fig. 18C). These observations further lent evidence that G-actin could be incorporated in CDRs not just from the bottom and also via the top suggesting that mDia1 could be a potential actin nucleating protein involved in the formation of actin filaments in a CDR.

A

Figure 17. Arp2 and G-actin is localised throughout the height of the CDR

NIH 3T3 cells were treated with PDGF to induce CDRs. The cells were permeabilised to allow rhodamine labelled G-actin incorporation and was fixed and immunostained with antibodies against Arp2, phalloidin and DAPI. Alexa-647-phalloidin and DAPI was used to visualize F-actin and DNA, at a CDR, respectively. (A) Immunofluorescence staining Arp2, phalloidin and DAPI to indicate the localisation of Arp2, F-actin and DNA from which a normalized z intensity fluorescence plot is obtained. This is obtained by normalizing the (B) raw z axis profile plot of fluorescence intensities due to Arp2, incorporated G-actin and phalloidin along the height of the CDR against (C) regions other than the CDR. Z-stack step size of 0.2 μm and intensity plots are derived from at least 10 different locations on and outside a CDR. Scale bar=10 μm .

Figure 18. G-actin is localised throughout the height while mDia1 is more localised to the top of a CDR

NIH 3T3 cells were treated with PDGF to induce CDRs. The cells were permeabilised to allow rhodamine labelled G-actin incorporation and was fixed and immunostained with antibodies against mDia1, phalloidin and DAPI. Alexa-647-phalloidin and DAPI was used to visualize F-actin and DNA, at a CDR respectively. (A) Immunofluorescence staining mDia1, phalloidin and DAPI to indicate the localisation of mDia1, F-actin and DNA from which a normalized z intensity fluorescence plot is obtained. This is obtained by normalizing the (B) raw z axis profile plot of fluorescence intensities due to mDia1, incorporated G-actin and phalloidin along the height of a CDR against (C) regions other than the CDR. z-stack step size of 0.2 μm and intensity plots are derived from at least 10 different locations on and outside a CDR. Scale bar=10 μm.

3.1.5 Filaments need not originate from the base of the CDRs

Since actin nucleation facilitated by mDia1 results in thin and straight filaments without branching, I went further to investigate the actin architecture of CDR by taking short interval time lapse images of actin filament in CDRs. I hypothesized that straight vertical actin filaments could be nucleated by mDia1 near the membrane without an existing filament underneath to cause the vertical projections in CDRs. I observed time lapse images of CDRs in cells transfected with RFP-Lifeact construct to visualize F-actin. The different z-sections could reveal much about the formation dynamics of CDRs. I observed the formation of vertical filaments which would later go on to be branched (Fig. 19A & Movie 7) suggesting that vertical mother filaments formed initially and perhaps new filaments branched out from them.

Whether these vertical projections branched out from exiting filaments underneath was not clear. In addition, since I found that CDRs could grow to a height of around $1.6\mu\text{m}$ (Fig. 19B), I performed time lapse imaging to collect z-stack images (Fig. 19C). The z stack was obtained by a downward scan of the sample. Thus, in a given time period the bottom most slices were imaged the last while the top most slices were imaged the earliest with a time interval of 1 second between frames/slices. After 20 z-steps, I returned to the top z-stack position and start imaging downwards again. Although there is a time interval of 20 seconds between frames of the same z position, I was now able to see if there was any fresh actin nucleation from existing horizontal filaments at lower z slices closer to the base of the CDR. Due to the earlier results which suggest that G-actin could be incorporated via mDia1 at the top and via Arp2/3 throughout the height of the CDR, I hypothesised that the vertical filaments could be nucleated from both the top without any existing filaments and also from the base of the CDR from existing horizontal filaments. On closer inspection of the CDR region, I found vertical filaments at the dorsal surface. However, they were found to be diminished at the lower z slices (Fig. 19D & Movie 8). This indicates that there was no filament formed from an existing horizontal filament at the base portion of a CDR. On the other hand some horizontal filaments were spotted at lower z slices from which vertical filaments were formed. Some of these filaments were found to have branched out to form more filaments at higher z slices. These observations further confirmed earlier reports that branched actin filaments were found in CDRs (Buccione et al., 2004). In addition, I had also observed that actin filaments could originate from the top of the CDRs (Fig. 20A). Taken together these results suggest that a vertical actin assembly could push the membrane cortex upward. In addition, an upward actin assembly via branching from existing horizontal filaments underneath could also push the membrane cortex upward at regions where a CDR forms (Fig. 20B).

A

B

C

D

Figure 19. Actin polymerisation in CDRs could initiate at both the top and bottom of CDRs

NIH 3T3 cells were transfected with RFP-Lifeact. At 24 hrs post transfection, the cells were stimulated with PDGF to induce CDRs. (A) A montage of 2 sec interval time lapse images at a fixed z height of a cell transfected with RFP-Lifeact and exhibiting a CDR. Scale bar=5 μm . See Movie 7 at https://drive.google.com/open?id=1a68rTzTSi3IQJdPaiSXVev4t8Pp06H_b. Fixed z stack fluorescence imaging of CDRs revealed the height of a typical CDR (B) Box plot of height of CDRs from at least 10 CDRs. (C) The cells were then subjected to live z-stack confocal fluorescence imaging using a strategy that allowed obtaining (D) montages of time lapse and z-stack images (0.1 μm step size) of 20 sec period at 1 sec interval between step sizes depicting how actin filaments form in a CDR. Scale bar=10 μm (upper panels) and 2 μm (lower panels). The z-distance away from the base of the CDR was marked in each frame. See Movie 8 at <https://drive.google.com/open?id=1RkxNkBo7oPsFdrUffJgvpY0WOarIelpx>.

A

B

Figure 20. Proposed cooperation between Arp2/3 and formins in CDRs

(A) Time lapse and z stack imaging presents two scenarios of the sequence of events that could happen during the genesis of CDR. In scenario 1, actin filament formation is observed at the base of the forming CDR while no filament formation is observed at the top of the CDR. In scenario 2, actin formation is observed at the top of the CDR while no filament formation is observed from a mother filament at the base of the CDR. (B) Cooperation between actin nucleators is responsible for the formation of CDRs. The Arp2/3 and formins are important to remodel the actin cytoskeleton and promote the growth of branched and unbranched actin filaments to support CDRs. Branched filaments can arise from mother filaments from the dorsal stress fiber network using Arp2/3 as a nucleator while unbranched filaments can arise from elongation using formins as a nucleator. The disassembly of CDRs could finally lead to fragmented filaments and monomeric actin, potentially serving as a pool for further functions.

3.2 Localised Actomyosin Contractility pulls on focal adhesions to accommodate Circular Dorsal Ruffles

3.2.1 Focal adhesions are a mechanical component of CDR formation

Azimifar and colleagues had earlier established that CDR formation was mediated by focal adhesions through integrin-linked kinase (ILK) and Src kinase-dependent activation (Azimifar et al., 2012). However, little has been explored in relation to the mechanical properties of focal adhesions and herein I hypothesized that the mechanical properties of focal adhesions are important to CDR formation. I first seeded the cells on substrates with increasing fibronectin concentration, followed by stimulation with PDGF. I found that the fraction of cells exhibiting CDRs increased with increasing fibronectin concentration (Fig. 21A & Movie 9). I repeated the same experiment with cells seeded on increasing concentration of collagen. I found that the fraction of cells exhibiting CDRs increased as the collagen concentration increased (Fig. 21B). To confirm that focal adhesion signalling was vital I repeated the same experiment with cells seeded on increasing concentration of poly-L-lysine. As expected, I found that there was a significantly smaller fraction of cells exhibiting CDRs with increasing concentration of poly-L-lysine (Fig. 21C). I also found that poly-L-lysine surfaces interfered with CDR formation as there was a smaller fraction of cells exhibiting CDRs when plated on poly-L-lysine coated surfaces compared with control cell culture dishes. A higher fraction of cells that were seeded on fibronectin or collagen exhibited CDRs than those seeded on uncoated culture dishes. The observations suggest that FA and/or FA signalling was a key requirement of CDR formation.

Figure 21. Cell adhesion alone is insufficient for CDR formation

NIH 3T3 cells were seeded on increasing concentration of fibronectin, collagen and PLL and stimulated with PDGF to induce CDRs. (A) A plot of fold differences in fraction of cells exhibiting CDRs in a given population of cells seeded on increasing fibronectin concentration. See Movie 9 at <https://drive.google.com/open?id=1JhhvOpQEuoOwtkjnw5Fxkep3RSBIN2PQ>, (B) increasing collagen concentration and (C) increasing PLL concentration. All plots were derived from at least 3 independent experiments; having analysed at least 200 cells per condition.

I was interested to find out if increasing fibronectin concentration could affect CDR dynamics. I found that the time taken to initiate CDRs did not significantly shorten with increasing fibronectin concentration (Fig. 22A). Interestingly, the maximum CDR area increased with increasing fibronectin concentration (Fig. 22B). In addition, no significant differences in lifetime of CDRs were observed (Fig. 22C & D). Next, I went further to examine why more cells would exhibit CDRs with a larger maximum area on increasing concentration of fibronectin. I suspected that it might be correlated to the physical properties of focal adhesions. I then performed TIRF imaging to compare the focal adhesion lengths and areas of cells seeded on uncoated and fibronectin-coated dishes. I found that the focal adhesions were significantly longer in cells seeded on 0.5 μM fibronectin concentration compared to cells seeded on 0.02 μM fibronectin concentration (Fig. 22E). In addition, cells seeded on 0.02 μM fibronectin formed longer and larger focal adhesions than cells seeded on uncoated glass bottom dishes. These observations suggest that focal adhesion length/size might play a role in the mechanics of CDR formation. It has been reported that focal adhesions increase in sizes and become elongated during the maturation process (Choquet, et al., 1997; Galbraith et al., 2002) and under tension (Riveline et al., 2001). Taken together, these results might suggest that focal adhesion signalling, tension experienced by the focal adhesion and its length/size can influence CDR formation.

A **B** **C**

Figure 22. Focal adhesion signalling is required for CDR formation

NIH 3T3 cells were seeded on increasing concentration of fibronectin and stimulated for CDRs. A plot of raw scores of (A) the time taken to initiate CDRs, (B) CDR maximum area and (C) CDR lifetime. CDR dynamics were derived from at least 20 CDRs for each group, $p < 0.01$, from at least 3 independent experiments. (D) A montage to represent the time taken to initiate CDRs in a given population of cells seeded on increasing fibronectin concentration. (E) focal adhesion lengths in a given population of cells

seeded on increasing fibronectin concentration derived from at least 10 cells per group which involved at least 50 adhesions per cell per group. Scale bar=10 μ m. $p<0.01$, from at least 3 independent experiments.

3.2.2 Focal adhesions and MLCK cooperate to regulate CDRs

Previous studies have shown that more cells exhibited CDRs when plated on stiffer substrates (Zeng et al., 2011; Huynh et al., 2013). Although there was a link between the number of cells exhibiting CDRs and MLCK activity, how MLCK participate in CDR formation is not clear. Since I also observed that focal adhesion elongation was a feature of cells exhibiting more CDRs, I suspected that a higher extent of actomyosin contractility was a requirement for CDR formation. Therefore, I examined the dynamics of CDRs in control, Y-27632 and ML-7 treated cells. Y-27632 and ML-7 are inhibitors of ROCK and MLCK, respectively. I found that Y-27632 and ML-7 treatment did not significantly attenuate CDR formation (Fig. 23A & Movie 10). However, ML-7 treatment appeared to result in shorter time taken to initiate CDRs compared to the control and Y-27632 treated cells (Fig. 23B). In addition, I observed that Y-27632 treated cells exhibited CDRs that were of similar maximum CDR area compared to those of control cells (Fig. 23C). In contrast, ML-7 treated cells exhibited CDRs that were significantly smaller compared to the CDRs in control and Y-27632 treated cells. ML-7 treatment appeared to also result in CDRs having a shorter lifetime compared to the CDRs in the control and Y-27632 treated cells (Fig. 23D & E). These observations suggest that although MLCK inhibition does not entirely abrogate CDR formation, it could be attenuating the ability of the cell to support and sustain the life time and maximum size of CDRs.

Figure 23. MLCK can regulate CDR dynamics

NIH 3T3 cells were seeded and were subjected to Y-27632 and ML-7 treatment and then stimulated to induce CDRs. The fraction of cells exhibiting CDRs and CDR dynamics characterised by time taken to initiate, max area and lifetime were quantified. (A) A plot of fold differences of the fraction of cells exhibiting CDR, derived from analysing at 150 cells per group over 3 independent experiments, (B) time taken to initiate CDRs, (C) CDR max area and (D) CDR lifetime in a given population of control, Y-27632 and ML-7 treated cells derived from analysing at least 20 CDRs in each group, $p < 0.01$, from at least 3 independent experiments. (E) A montage to represent the time taken to initiate CDRs in a given population of control, Y-27632 and ML-7 treated cells. See Movie 10 at <https://drive.google.com/open?id=1KTn8VPOKeWbjwcsiR04MGDKgTPtiFuta>. Scale bar=10 μm .

Having observed that ML-7 treatment reduced the lifetime and maximum area of CDRs while Y-27632 treatment did not, I wanted to examine if Y-27632 and ML-7 had any significant effects on focal adhesions properties which would in turn affect the CDRs. Since CDRs mostly assemble and disassemble near the leading edge I hypothesized that the focal adhesions in that region would be bigger and longer to support the formation of CDRs. I also speculated that the actomyosin contractility in this region would also be elevated as a result of larger and elongated focal adhesions. I then carried out TIRF imaging to compare the focal adhesion properties of control, Y-27632, ML-7 and PF-228 (FAK inhibitor) treated cells. PF-228 treatment was used as a negative control for focal adhesion size. As expected, I found that PF-228 treated cells displayed the shortest focal adhesion length amongst the cells with different inhibitor treatment (Fig. 24A & B). Interestingly, ML-7 treated cells displayed the most elongated while Y-27632 treated cells displayed significantly less elongated focal adhesions.

From the same samples, I imaged the z-stacks by scanning from the bottom to the top with a step size of 0.2 μm . I then obtained a maximum intensity projection of these images and looked for the localisation of phosphorylated MLC II with reference to the focal adhesions. Firstly, I observed that the intensity of phosphorylated MLC II was significantly the highest in the DMSO control cells (Fig. 24A). On the other hand, Y-27632, ML-7 and PF-228 treated cells displayed significantly low phosphorylated MLC II signals. Fluorescence intensity line plots starting from the leading edge to the central portion of cells were obtained. Fold changes in fluorescence

intensity was derived from normalizing the raw fluorescence intensity signals against the mean fluorescence signal due to that particular protein. It revealed that, despite the significantly reduced phosphorylated MLC II intensity levels, its localisation in Y-27632 and ML-7 cells was spatially distinct (Fig. 24C-F). In Y-27632 treated cells, phosphorylated myosin light chain was mainly localised to the leading edge and the central portion. In ML-7 treated cells however, phosphorylated MLC II was mainly localised to the lamella region where CDRs would normally assemble and disassemble. However, no significant difference in phosphorylated MLC II localisation was observed in PF-228 treated cells (Fig. 24F). This suggests that the presence of phosphorylated MLC II at the cell edge and lamella could be important to the formation of CDRs.

Figure 24. Focal adhesions and MLCK cooperate to regulate CDRs.

NIH 3T3 cells were seeded and were subjected to Y-27632, ML-7 and PF-228 treatment and fixed. The cells were immunostained for phosphorylated myosin light chain II, phosphorylated FAK and F-actin to check for their localisation. (A) Immunolocalization of phalloidin, phosphorylated MLC II at serine 19 and phosphorylated FAK at tyrosine 397. A (B) plot of raw focal adhesion lengths in DMSO control, Y-27632, ML-7 and PF-228 treated cells derived from at least 10 cells per group involving at least 40 adhesions per cell. XY plane fluorescence intensity plot, measuring the fold changes in fluorescence intensities between phalloidin, phosphorylated MLC II at serine 19 and phosphorylated FAK at tyrosine

397 from the leading edge to the intra cell region in (C) DMSO control (D) Y-27632, (E) ML-7 and (F) PF-228 treated cells. Scale bar=10 μ m.

3.2.3 Focal adhesions act as cell anchors to support CDRs

CDRs behave like excitable waves (Bernitt et al., 2017; Bernitt et al., 2015) and since MLCK activity was linked to substrate stiffness and the propensity of cells to exhibit CDRs (Huynh et al., 2013), I sought to examine how MLCK activity could help in CDR formation. I hypothesized that the excitable waves require actomyosin contractility to pull on focal adhesions, which act like anchors when these waves traverse across the dorsal surface of the cell. Therefore, I was curious to determine if focal contacts in CDR regions would become elongated and if there was actomyosin contractile activity in that region. Firstly, I performed TIRF imaging to examine the focal adhesions (immunostained for phosphorylated-FAK) in the region proximal to a CDR. I found that the focal adhesions in the area at the leading edge and proximal to a CDR were larger and more elongated as compared to a region without CDR at another leading edge of the same cell (Fig. 25A). However, no physical connection was observed between CDRs and the focal adhesions. CDRs and the focal adhesions were clearly located at different z planes (Fig. 25B). In addition, the focal adhesions at leading edges proximal to CDR were observed to be more elongated than those in other leading edges without the CDR (Fig. 25C). An earlier report by Azimifar et al., (2012) suggests that integrin/integrin-linked kinase (ILK) and epidermal growth factor (EGF) receptor signalling are both required for CDR formation. They have implicated the tumour suppressor Cyld as a possible link to the eventual cytoskeletal rearrangement for the formation of the dorsal ruffles. Thus, I wanted to explore further to determine if there is any mechano-contribution besides the phosphorylation and signalling requirement of ILK and EGF receptor.

Cells transfected with RFP-Lifeact and GFP-paxilin were stimulated to exhibit CDR so that the focal adhesion properties could be tracked (Fig. 25D, E). I observed that CDRs generally formed at the lamellae region and traversed around this region. I observed that they also formed in regions that already had large and mature focal adhesions on the ventral surface. I then examined the lengths of focal adhesions at the leading edge where the CDR wave front had not reached. Live imaging was performed at 30 sec intervals to track the growth of focal adhesions. Focal adhesions that only appeared over a single time point were not included in analysing the changes in focal adhesion length. Interestingly I found that the focal adhesions elongated as the CDR approached the leading edge (Fig. 25D & Movie 11). More interestingly, these focal adhesions shortened as the CDR moved away from the leading edge (Fig. 25E & F). These observations suggest that tension on focal contacts was required to sustain the approach of CDRs and the tension was relieved when the CDR moved to another region. These observations suggest that focal adhesions could serve as anchors for CDRs when they traverse across the dorsal surface while depending on actomyosin contractility to pull on these anchors.

Figure 25. Focal adhesions act as cell anchors to support CDRs

NIH 3T3 cells were stimulated to exhibit CDRs and were immunostained for phosphorylated FAK and F-actin to check for their localisation. A TIRF z-stack was also performed to check for any physical connection between focal adhesions and CDRs. NIH 3T3 cells were transfected with RFP-Lifeact and GFP-paxillin to visualise the dynamic changes focal adhesions go through when CDRs are formed. (A) Immunolocalization of phalloidin and phosphorylated FAK at tyrosine 397. Scale bar=20 μm (B) z-stack representation of CDRs as visualised from the focal adhesions (imaged with a 0.2 μm , step size but presented as 0.4 μm increment per frame). (C) A plot of focal adhesion length of the focal adhesions located the leading edge proximal to CDR versus the focal adhesions at other leading edges, at least 500 focal adhesions were analysed from 10-20 cells. (D) Time lapse structural illumination microscopy (SIM) performed at 30sec intervals, representing the dynamic changes in the localisation of RFP-Lifeact and elongation of GFP-paxillin in a cell exhibiting a CDR heading towards the leading edge (left side of image) and back inward (right side of image). See Movie 11 at

https://drive.google.com/open?id=1AwqfkSN6_C9e4T2s9IGmNmogvc_QzNfq. Scale bar=5 μm and 1 μm (lower panels). (E) A plot of fold changes in distance of 32 focal adhesions in (D) to CDR wavefront and the 32 focal adhesion lengths against time. This is derived by normalizing the raw corresponding distances against the corresponding mean lengths of 32 focal adhesions at the leading edge and their distance to the CDR wavefront based on this cell. (F) A plot of the raw distance between an elongating focal adhesion in (D) to the CDR wavefront and the focal adhesion length against time based on this cell. Error bars represent standard error from the mean.

3.2.4 POPX2 activity promotes CDR formation

Since POPX2 overexpressing cells show robust stress fibers (Koh et al., 2002), and there is positive correlation between stress fibers and CDR, cells overexpressing POPX2 might exhibit more CDR. Indeed, I found that a greater fraction of POPX2-overexpressing (X2) cells exhibited CDRs as compared to control and cells overexpressing phosphatase-dead mutant (X2m) (Fig. 26A, E & Movie 12), suggesting that the phosphatase activity of POPX2 is important to promote CDRs. I then further probed at which point was the phosphatase activity critical to CDRs. I observed that X2 cells exhibited CDRs that were initiated earlier than those from control and X2m cells (Fig. 26B). I also examined the max CDR area but found that CDRs from control, X2 and X2m were of similar sizes (Fig. 26C). I then examined the lifetimes of the CDRs and found that CDRs exhibited by X2 cells had a significantly longer lifetime than those exhibited by control and X2m cells (Fig. 26D). These results suggested that POPX2 activity was important for the initiation and sustenance of CDRs.

Since POPX2 activity regulated the formative dynamics of CDRs in a similar manner as cells seeded on higher fibronectin concentration, I suspected that the regulation by POPX2 occurred via actomyosin contractility required to pull on focal adhesions as anchors or POPX2 participate in signalling downstream of the integrins. I proceeded to investigate if signalling from focal adhesions was elevated in X2 cells. For this, I performed TIRF imaging on control, X2 and X2m cells immunostained against phosphorylated FAK (Y397) to look for differences in focal adhesion length and area (Fig. 26F). I found that X2 cells had focal adhesion lengths that were

slightly longer than those of X2m and control cells (Fig. 26G). I also compared the focal adhesion areas of control, X2 and X2m cells. Interestingly, I found that X2 cells had significantly larger focal adhesions than those found in control and X2m cells (Fig. 26H). X2 cells might inherently have elevated signalling from the larger focal adhesions compared to control and X2m cells. Taken together, these results hinted that the focal adhesion area might be relevant to CDR formation, CDR sustenance and time taken to initiate CDR and POPX2 may play a role in regulating these events.

Figure 26. POPX2 activity promotes CDR formation

Control NIH 3T3, X2 and X2m cells were seeded and stimulated to exhibit CDRs. The fraction of cells exhibiting CDRs and CDR dynamics characterised by time taken to initiate, max area and lifetime were quantified. The cells were immunostained for phosphorylated FAK and analysed for differences in focal

adhesion properties in these cells. A plot of fold differences from normalising the raw scores against the mean of the control was obtained. See Movie 12 at <https://drive.google.com/open?id=1PvAl6nIEiFUPmFbCxhp5NykCcRUi10rM>. (A) Fold differences in fraction of cells exhibiting CDRs, (B) time taken to initiate CDRs, (C) CDR max area and (D) CDR lifetime in a given population of control, X2 and X2m cells, derived from at least 90 cells per group over at least 3 independent experiments. CDR dynamics were extracted from at least 10 CDRs per group, $p < 0.01$, from at least 3 independent experiments. (E) A montage to represent the time taken to initiate CDRs in a given population of control, X2 and X2m cells. Scale bar = 10 μm . (F) Representative TIRF images of control, X2 and X2m cells immunostained with anti-phospho-FAK (Y397). Scale bar = 10 μm . A plot comparing (G) focal adhesion lengths and (H) focal adhesion areas from control, X2 and X2m cells. $p < 0.01$, from at least 20 cells from which at least 40 adhesions per cell were analysed.

3.2.5 POPX2 activity enhances localised actomyosin contractility around CDRs

I was curious to find out if actomyosin contractility was important to sustain CDRs. I examined CDR exhibiting control, X2 and X2m cells immunostained for phosphorylated MLC II at serine 19 (pS19MLC II) to check for the extent of localised actomyosin contractility needed for CDR sustenance. I found that phosphorylated myosin light chain was enhanced at the exterior region of CDRs and the leading edge of control and X2m cells (Fig. 27A). However, phosphorylated MLC II was not localised to CDRs in control and X2m cells. In contrast, phosphorylated MLC II was found to be localised to CDRs in addition to the exterior region of CDRs and the leading edge of X2 cells. This suggests that POPX2 activity is essential to regulating actomyosin contractility at CDRs. Next I wanted to check if POPX2 activity was essential for MLC II phosphorylation globally. I performed western blot for cell lysates from control, X2 and X2m cells to probe against pS19MLC II and pan MLC II. I found that there were no significant differences in the pS19MLC II/MLC II ratio in control, X2 and X2m cells (Fig. 27B). The observations suggest that X2 activity did not affect global MLC II phosphorylation but might be required for localised elevated actomyosin contractility around and at CDRs.

Since POPX2 contributed to localised, elevated MLC II phosphorylation around and at the CDRs, I hypothesized that POPX2 could be affecting CDR formation and its dynamics through ROCK and MLCK. I scored for the fraction of cells exhibiting CDRs in Y-27632 and ML-7 treated control, X2 and X2m cells (Fig. 27C & Movie 13). Expectedly, a significantly higher fraction of X2 cells exhibited CDRs compared to control and X2m cells. Y-27632 treatment did not significantly attenuate CDRs in general. However, there was a decrease in the number of X2 cells exhibiting CDRs. Interestingly I found that ML-7 treatment significantly attenuated CDR formation in X2 cells compared to control and X2m cells. I further found that Y-27632 and ML-7 treatment significantly reduced the maximum CDR area in X2 cells (Fig. 27D & E). These results suggest that POPX2 activity could be regulating localised myosin light chain phosphorylation at the CDRs and thus increasing the ability of the cells to exhibit CDRs.

B

Figure 27. POPX2 activity enhances localised actomyosin contractility around CDRs

Control NIH 3T3, X2 and X2m cells were stimulated by PDGF to exhibit CDRs and were immunostained for phosphorylated MLC II at serine 19, F-actin (phalloidin) and DNA (DAPI) to view their localisation with respect to GFP labelled POPX2 and POPX2m. Cell lysates of control NIH 3T3, X2 and X2m cells were subjected to western blot analysis and were probed for pan MLC II and phosphorylated MLC II at serine 19. These cells were also subjected to Y-27632 and ML-7 treatment and stimulated to exhibit CDRs. The fraction of cells exhibiting CDRs was scored. (A) Immunolocalization of phalloidin and phosphorylated MLC II at serine 19. Scale bar=20 μm. (B) Western blot analysis to determine the ratio of phosphorylated MLC II at serine 19 against myosin light chain II (n=1). (C) A plot of fraction of cells exhibiting CDRs in a given population of control, Y-27632 and ML-7 treated control, X2 and X2m cells. See Movie 13 at <https://drive.google.com/open?id=1iWJSW0e1eKRwphTNFw3g4RiJZaWUtNB9>. (D) A plot of CDR max area in control, Y-27632 and ML-7 treated X2 cells. All plots were obtained from at least 3 independent experiments observing at least 80 cells per group and at least 20 CDRs per group analysed

for their dynamics. (E) A montage to represent the maximum CDR area in a given population of control, Y-27632 and ML-7 treated X2 cells. Scale bar=10 μ m.

3.3 Circular Dorsal Ruffles increase Directional Persistence of Cell Migration

3.3.1 CDRs contribute to directional persistence but not speed

When control NIH 3T3, X2 and X2m cells seeded on fibronectin strips were stimulated with PDGF and imaged (Fig. 28A), I found that cells that exhibited CDRs (Fig. 28B, *white arrow* & Movie 14) showed an increase in persistence in directional cell migration compared to cells which did not exhibit CDRs. Migration persistence and speed were quantified by observing the cell migration kymographs for each cell (Fig. 28B) as it migrated either to the left or right on the fibronectin strips. This persistence in cell migration direction can be quantified using a defined cell migration persistence factor γ where a value of 2 denotes directed motion and a value of 1 denotes random walking. The persistence factor, γ , for all three cell populations are quantified in (Fig. 28C). In each of the three cell populations of control, X2 and X2m groups, cells exhibiting CDRs show a significantly higher average γ of 1.83 ± 0.22 , 1.92 ± 0.16 and 1.24 ± 0.32 , respectively, compared to cell populations which did not exhibit CDRs where γ values are 1.42 ± 0.37 , 1.34 ± 0.19 and 0.81 ± 0.44 , respectively. In X2 cells which are known to have impairment in directional migration (Hoon et al., 2014), stimulation with PDGF and subsequent expression of CDRs significantly increased the migration persistence on patterned fibronectin strips. These results suggest that cells with CDRs exhibit an increase in migration persistence, and this suggests that CDRs might play a role in maintaining persistence in directional cell migration. However, no significant difference in migration speed was found between cells with and without CDRs (Fig. 28D). The same experiment was also repeated on unpatterned surfaces. I hypothesized that CDRs could increase migration persistence in X2 cells without a

need for fibronectin strips for linear motion. I found that X2 cells that exhibited CDRs could maintain a longer linear path of migration (Fig. 28E). In addition, migration persistence increased in X2 cells that exhibited CDRs and consistent with earlier result, there was no significant change in speed. (Fig. 28F & G).

C **D**

E**Plot from origin of cells on unpatterned surfaces**

F

Directional Persistence on unpatterned surfaces

G

Speed on unpatterned surfaces

Figure 28. Cells exhibiting CDRs show an increase in the persistence of directional migration with no change in migration speed

NIH 3T3 control, X2 and X2m cells were seeded on fibronectin tracks and stimulated to exhibit CDRs. They were imaged to see if cells exhibiting CDRs tended to have greater migration persistence. (A) Fibronectin-coated patterned strips of width 2, 5 and 10 μm were used to study the role of CDRs in migrating cells. NIH 3T3 control (Ctrl), POPX2 overexpressing (X2) and POPX2m overexpressing cells (X2m) cells were grown on patterned tracks and stimulated by PDGF and imaged live over 65 min at 30 sec intervals. See Movie 14 at https://drive.google.com/open?id=11_M-mAlJnQ5OKODhPdAmzkrKpEkzcUSx. Control NIH 3T3, X2 and X2m cells were also seeded on unpatterned surfaces and stimulated to exhibit CDRs and imaged live over 65 min at 30 sec intervals. Scale bar=50 μm . (B) Representative kymographs of time lapse imaging of 3 populations of migrating cells on fibronectin strips. (C) A plot of γ , which quantifies cell migration persistence for cells with

CDRs, for all three cell populations. γ for cells exhibiting CDRs is significantly higher than the other cell populations. (D) A plot of the cell migration speed of all three cell populations. Cells with CDRs did not show significant differences in migration speed compared to the other cell populations. (E) A plot of the trajectories of all the three cell populations was obtained to reveal the increase in migration persistence in X2 cells. (F) A plot of persistence factor which quantifies cell migration persistency for NIH 3T3, X2 and X2m cells with and without CDRs and a (G) plot of the cell migration speed of NIH 3T3, X2 and X2m cells with and without CDRs. The persistence factor of NIH 3T3 control and X2m cells do not significantly change whether or not they exhibit CDRs. An improvement in persistence factor is seen in X2 cells exhibiting CDRs as compared to X2 cells that do not exhibit CDRs. The migration speed is not affected by CDRs in NIH 3T3, X2 and X2m cells. All plots were derived from 3-30 cells per group over 3 independent experiments.

3.3.2 Actin relocates from CDRs to the lamellipodia

The observations indicated that the cells tend to move towards the direction where CDRs are located. To understand more about possible relationship between CDR and the direction of cell migration, I examined the differences in dynamics of CDRs in relation to cell protrusions in both control and blebbistatin (10 μ M for 10 min) treated NIH 3T3 cells. Blebbistatin is an inhibitor of myosin II. Blebbistatin is used in this experiment to determine if myosin II is involved in the regulation of migration direction in relation to CDR. From time lapse images, I consistently observed that CDR disassembly mostly preceded lamellipodial protrusions in control NIH 3T3 cells (Fig. 29A & Movie 15). However, the time elapsed between complete CDR disassembly and lamellipodial protrusions were longer in blebbistatin treated NIH 3T3 cells. In addition, control NIH 3T3 cells exhibited prominent cell protrusions whereas blebbistatin treated NIH 3T3 cells showed less prominent cell protrusions (Fig. 29B & Movie 16). I then hypothesized that myosin inhibition could retard the re-localisation of actin from the CDR to lamellipodia. Kymographs obtained from experiments using photoactivated fluorescence tagged actin, revealed that there was poor actin relocalisation to the lamellipodia in the absence of CDR (Fig. 29C & Movie 17). On the contrary, there was significant relocalisation of actin in cells which exhibited CDRs (Fig. 29D). Blebbistatin treatment revealed poor actin relocalisation to the lamellipodia in the absence of a CDR, just as in the control (Fig. 29E). Interestingly, poor actin relocalisation was also observed in the presence of a CDR under

blebbistatin treatment (Fig. 29F). Taken together, the results indicated that CDRs could function like a free actin pool that can be recycled and relocalised to form cell protrusions. However, this relocalisation could be blocked by myosin inhibition. These results hinted at a possible role for myosin II in the formation of cell protrusions from disassembly of CDRs.

Figure 29. Cell protrusion and actin relocalisation in control and blebbistatin treated NIH 3T3 cells

Control NIH 3T3 cells were seeded on unpatterned surfaces, subjected to blebbistatin treatment and stimulated to exhibit CDRs. They were tracked based on live phase contrast movies. The time lag between CDR closing and lamellipodia formation was measured to see if there is a delay in lamellipodia formation. Complete CDR closure was taken to be at $t=0$. When protrusions formed after complete CDR closure then the t value (time taken between CDR closure and lamellipodial formation) was recorded as a positive value. When protrusions formed earlier than complete CDR closure then the t value (time taken

between CDR closure and lamellipodial formation) was recorded as a negative value. After computing the average of these timings (presented as box plots) a significant delay in lamellipodia formation was found due to blebbistatin treatment. (A) Quantification of the time elapsed at the point of cell protrusion after CDR disassembly, derived from 29-30 cells per group. See Movie 15 at <https://drive.google.com/open?id=1BC8t33V5beJCzYICJsp1kz4AdWTSpJR7>. (B) Representative montages and kymographs from live time lapse imaging revealing the presence of sustained protrusions in control cells and cells subjected to blebbistatin treatment. See Movie 16 at <https://drive.google.com/open?id=1ZRG-igaPJsWUCjnVA8UNkZdiG1E5sDw7>. Scale bar=10 μ m. NIH 3T3 cells were transfected with the mEos3.2-actin cDNA construct and stimulated to exhibit CDRs. Photoactivation was induced by exposing regions of interest to strong light (405 nm). The photoactivated actin was then tracked around the cell. (C) Actin relocalisation within a cell that did not exhibit CDR as seen from photoactivated actin time lapse imaging. See Movie 17 at <https://drive.google.com/open?id=1ZqLLReWtpevv8JeVf0vub99RdGY2Ic0H>. Scale bar=10 μ m. Kymograph indicates no relocalisation to the lamellipodia over the course of the live imaging. (D) Actin relocalisation within a cell that exhibited CDR. Scale bar=5 μ m. Kymograph indicates relocalisation of actin from the CDR towards the lamellipodia. (E) Actin relocalisation within a blebbistatin treated cell that did not exhibit CDR. Scale bar=10 μ m. Kymograph indicates no relocalisation to the lamellipodia over the course of the live imaging. (F) Actin relocalisation within a blebbistatin treated cell that exhibited CDR. Scale bar=5 μ m. Kymograph indicates no relocalisation to the lamellipodia over the course of the live imaging.

3.3.3 MLC II localises around CDRs and lamellipodia

I was curious to study the link between MLC II and lamellipodial protrusions when CDRs disassemble. Firstly, through immunofluorescence experiments, I found that phosphorylated MLC II was mainly localised to the exterior region of CDRs (Fig. 30A). On closer inspection of fluorescence intensity plots, I found that phosphorylated MLC II had higher intensity levels than phalloidin just outside the CDR (Fig. 30B). At the CDR ring however, phosphorylated MLC II fluorescence intensity levels dropped and phalloidin intensity levels peaked. In addition, at the ring, residual signals due to phosphorylated MLC II mainly localized to the bottom of the ring and not along its z height (Fig. 30C). As expected, signals from both phosphorylated MLC II and phalloidin was markedly reduced within the CDR ring.

A

B

C

Figure 30. Actomyosin activity is observed in the region between CDR and leading edge of cell

NIH 3T3 cells were seeded and stimulated for CDRs. The localisation of phosphorylated MLC II and F-actin (phalloidin) and DNA (DAPI) was determined by immunostaining. (A) Immunolocalization of phalloidin and phosphorylated MLC II at serine 19 Scale bar=20 μm . (B) Left panel: XY plane fluorescence intensity plot based on line drawn on right panel, measuring relative intensities between phalloidin and phosphorylated MLC II at serine 19 from the leading edge to the intra CDR region Scale bar=5 μm . (C) Localisation of phosphorylated MLCII at a CDR was observed by examining the z fluorescence intensity plot. Right panel: A spot on the CDR is observed to derive the localization of phalloidin and phosphorylated MLC II at serine 19 along the height of a single CDR as visualized by a z fluorescence intensity plot and orthogonal views at the peak of a CDR. Top left panel: pMLCII S19 enrichment is observed at the base radially around a CDR. Bottom panel: pMLCII S19 enrichment occurs along the same z height as phalloidin enrichment in non CDR regions, derived from right panel. Scale bar=10 μm .

Next, I wanted to rule out the involvement of microtubules in the formation of cell protrusions. I immunostained for β -tubulin and F-actin in cells exhibiting CDRs and found that β -tubulin was not localized to CDRs (Fig. 31A). On closer inspection of fluorescence intensity plots, I found that β -tubulin had higher intensity levels than phalloidin just inside the CDR (Fig. 31B). At the CDR ring however, β -tubulin fluorescence intensity levels dropped and phalloidin intensity levels peaked. Outside the CDR ring and at the leading edge of a cell, β -tubulin fluorescence intensity levels were found to be low. As expected, phalloidin fluorescence intensity levels were also found to be low outside CDR. This indicates that CDRs are not composed of tubulin and microtubules are not involved in the relocalisation of actin from CDRs to the leading edge. Next, I wanted to see if nocodazole treatment delayed the time elapsed between complete CDR disassembly and lamellipodial protrusions just as blebbistatin treatment did. I found that there were no significant differences in the time elapsed between complete CDR disassembly and lamellipodial protrusions in control and nocodazole treated cells (Fig. 31C & Movie 18). These observations indicate that the microtubule network is not affecting CDR assembly, disassembly and cell protrusions. The results further suggest that the actin relocalisation from CDRs to the

leading edge is facilitated by myosin and not by other motor proteins that would require microtubules.

C

Figure 31. Microtubules do not contribute to CDR formation

NIH 3T3 cells were seeded and stimulated to exhibit CDRs. Cells were fixed and probed for F-actin and microtubules to reveal their localisation with respect to CDRs. The time lag between CDR closing phase and lamellipodial formation was measured to see if there is a delay in lamellipodial formation for cells treated with nocodazole. (A) Immunolocalization of phalloidin and β tubulin at CDR showing that microtubules are not found at CDR. Scale bar=20 μm . (B) Left panel: XY plane fluorescence intensity plot was used to measure relative intensities of phalloidin and β tubulin from the leading edge to the intra CDR region, as depicted by line in Right panel. The results show that microtubules do not localize to CDRs. Scale bar=5 μm . (C) Control NIH 3T3 were seeded on unpatterned surfaces, subjected to nocodazole treatment and stimulated to exhibit CDRs. They were tracked based on live phase contrast movies. See movie 18 at https://drive.google.com/open?id=1Vt4Xuy6homhflveAHb2_kpI72BNJoef. The time lag/time taken between CDR closure and lamellipodial formation was measured to see if there is a delay in lamellipodial formation. Complete CDR closure was taken to be $t=0$. When protrusions formed after complete CDR closure then the t value (time taken between CDR closure and lamellipodial formation) was recorded as a positive value. When protrusions formed earlier than complete CDR closure then the t value (time taken between CDR closure and lamellipodial formation) was recorded as a negative value. After computing the average of these timings (presented as box plots) no significant delay in the formation of lamellipodial protrusions was found due to nocodazole treatment. Quantification of the time elapsed at the point of cell protrusion after CDR disassembly, derived from 29-31 cells over 3 independent experiments, Scale bar=20 μm .

To determine the fate of MLC II during and after CDR disassembly, I used structured illumination microscopy live imaging to observe the movement of MLC II fluorescent signals around CDR in NIH 3T3 cells co-transfected with GFP-Lifeact and mCherry-MLC II. I found a distinct MLC II band around CDRs, consistent with earlier immunofluorescence experiments. In cells that exhibited CDRs, I found that there was a significant shift in the MLC II band from the around the CDR to the leading edge after the CDR was disassembled. On the contrary, when there was no CDR exhibited, no significant shift in the MLC II band to the leading edge was observed (Fig. 32A-C & Movie 19).

Next, I tracked the MLC II fluorescence signals in both CDR exhibiting and non CDR exhibiting cells to see if there were any differences in speed and diffusion constant due to the exhibition of CDRs. I found that MLC II fluorescence signals from around a CDR were committed to moving towards the lamellipodia without switching to the opposite direction. Therefore, these MLC II fluorescence signals exhibited increased directionality (d/D) which is obtained by the ratio of the displacement, d , to the distance, D , covered by the MLC II fluorescence signals. On the other hand, MLC II fluorescence signals in the absence of CDR were not committed to moving towards a particular direction. In addition, MLC II fluorescence signals around a CDR moved faster towards the lamellipodia when compared to MLC II fluorescence signals in the absence of a CDR. This observation suggested that there was a drive in the actomyosin network towards the leading edge from the disassembling CDRs.

I went further to compute the diffusion coefficients of MLC II to check if the presence of CDRs could affect the diffusion of MLC II from CDR site towards the leading edge (Fig. 32D). I applied the 2D diffusion equation, $\langle x^2 \rangle = 2Dt$; whereby x is the displacement of the fluorescent signals, D is diffusion coefficient and t is the time elapsed. I found the diffusion coefficient of MLC II fluorescence signals tracked from non-CDR regions to the leading edge to be $0.60 \pm$

0.42 $\mu\text{m}^2/\text{sec}$. This value was found to be of the same magnitude as the known range of diffusion coefficient values of myosin (0.200-0.800 $\mu\text{m}^2/\text{sec}$) (Uehara et al., 2010). On the other hand, I found the average diffusion coefficient of MLC II fluorescent signals tracked from around CDRs to the leading edge to be $4.59 \pm 2.21 \mu\text{m}^2/\text{sec}$. This value was not found to be of the same magnitude as the known range of diffusion coefficient values of myosin but it is of the same order of magnitude as the known range of diffusion coefficient values of cytosolic G-actin monomers, which are from 3 to 30 $\mu\text{m}^2/\text{sec}$. These results demonstrated that the actomyosin network translocated to the leading edge at a time scale that is close to the known diffusive time scale of actin monomers.

A

B

C

D

Figure 32. MLC II current moves towards the leading edge in a CDR exhibiting cell

NIH 3T3 cells were transfected with GFP-Lifeact and mCherry-MLC II and stimulated to exhibit CDRs. The positions of MLC II signals were tracked using time lapse structured illumination microscopy. See Movie 19 at <https://drive.google.com/open?id=1BdOleHIN6DaIGc6tERVynZ5Ts1sGrJ59>. (A) MLC II signals in a cell that does not exhibit a CDR move without committing towards the lamellipodia. Scale bar=5 μm . Bottom panel: Zoom of boxed regions revealing montages from the time lapse imaging. Scale bar=2 μm . (B) MLC II signals in a cell that exhibits a CDR commits its movement towards the lamellipodia. Scale bar=5 μm . Bottom panel: Zoom of boxed regions revealing montages from the time lapse imaging. Scale bar=1 μm . (C) Time lapse structured illumination microscopy of cells was performed to obtain representative kymograph of time lapse imaging over 2 min of mCherry MLCII moving either towards or away from the leading edge. Top panel: mCherry MLC II signals move together with GFP Lifeact toward the leading edge. Bottom panel: mCherry MLC II signals move together with GFP Lifeact but not towards the leading edge. (D) Quantitative analysis of MLC II signals on Lifeact signals with respect to directionality ratio, speed and diffusion coefficient. Number of cells and MLC II signals analysed for CDR+ve: 2 cells containing 58 signals, CDR-ve: 2 cells containing 28 signals.

4. DISCUSSION

Earlier studies have suggested that CDRs are induced by signalling pathways downstream of growth factor receptors and Rac1 GTPases (Ladwein & Rottner, 2008; Eden et. al, 2002). RhoA which is often found to antagonise Rac1 signalling has not been implicated in CDR formation. However, I have now found that RhoA plays an important role in the formation of CDRs, more specifically, in the initiation of CDR. This raises the possibility that an activated RhoA cellular environment might be a prerequisite for cells to exhibit CDRs (Fig. 11). Zeng et al. have shown earlier that having high stress fiber density favours the formation of CDRs and they postulated that the stress fibers disassemble upon PDGF stimulation to release the G-actin required for building the CDR (Zeng et al., 2011). In this study, I have found that localised but not global Rac1 activation is important for CDR formation. This is also supported by experiments done by Orth and his colleagues, whereby CDRs are found to form near the location of localized extracellular growth factor stimulation administered via a microneedle (Orth et al., 2006). Since both RhoA and Rac1 are found to be important for CDR formation, this opens the possibility of cooperation between the two GTPases (Fig. 12). This study demonstrated that RhoA contributed to CDR formation beyond creating a source of F-actin to be tapped upon after local activation of Rac1.

Many have reported the importance of cooperation between different GTPases and their different spatiotemporal dynamics (Pertz et al., 2006). Cell migration is a complex process that requires precise cooperation between GTPases to allow for timely lamellipodia formation, cell protrusions and retraction. As such, a clear antagonistic relationship between RhoA and Rac1 has been established (Pertz et al., 2006). In the CDR context, I have discovered cooperation between RhoA and Rac1. I found that active RhoA localises to the top of CDRs and could

facilitate unbranched actin filament nucleation from there. On the other hand, active Rac1 localises to the midsection and bottom of the CDRs and could facilitate branched actin filament formation from there (Fig. 19 & 20). This raises the possibility that RhoA serves a deeper role than just providing a high F-actin environment to be tapped upon for CDR formation.

Some of the actin filaments I have observed in CDRs appear to be unbranched filaments that nucleate from the top of the CDR. mDia1 could be involved in contributing such filaments in the CDRs. Furthermore, my data showed that inhibition of formins was deleterious to CDRs (Fig. 13A). Previous work has reported co-localisation of Arp2/3 along with its interacting partner, WAVE and cortactin at the CDRs, suggesting that the CDR contains branched actin (Krueger et al., 2003; Orth et al., 2006). Some of the filaments I have observed in CDRs indeed exhibited branching. Furthermore, I also found that the inhibition of Arp2/3 attenuated CDR formation which is in agreement with previous work (Fig.13A).

I propose that RhoA and Rac1 GTPases are required for CDR formation but they appear to localize to different parts of the CDR. I also suggest that there is cooperation between mDia1 and Arp2/3 to form the actin structure of the CDR. Isogai and colleagues had shown previously that cooperation between mDia1 and Arp2/3 in a sequential manner in the formation of lamellipodia and membrane ruffles (Isogai et al., 2012). This current study also supports the formation of a mother vertical filament that could be used for branching of other filaments as I was able to observe a concurrent formation of a mother vertical filament from the dorsal membrane cortex and daughter actin filament branching from horizontal actin filaments near the base of a CDR. Previous studies discovered that pre-existing mother actin filaments and WAVE are required for activation of the Arp2/3 complex, subsequently leading to branched actin filaments and formation of lamellipodia and ruffles (Krueger et al., 2003; Orth et al., 2006). As F-actin is the template which Arp2/3-complex uses for actin nucleation, it also supports the

possibility that Arp2/3 mediated actin filament assembly can be concurrent and independent of the filament formation arising from mDia1 (Chhabra & Higgs, 2007; Goley & Welch, 2006; Innocenti et al., 2003). My findings also show a clear spatial distinction such that Arp2/3 is localised more to the base and mDia1 is localised more to the top of a CDR. I found that endogenous and exogenous G-actin is also localised at the top of the CDR. This further provides evidence that apart from Arp2/3 mediated actin structures, another actin nucleating mechanism mediated by active RhoA and mDia1 is present in CDRs (Fig. 14-18). This raises the possibility of two spatially distinct signalling mechanisms acting in a synchronised temporal fashion to facilitate CDR formation.

In addition to the type of vertical protrusions seen in CDRs, little is known about why CDRs form a ring-like structure. Zeng and colleagues proposed a model for the ring-like structure of CDRs (Zeng et al., 2011). Rac1 activity is elevated at the cell membrane, where the PDGF receptor is activated. As a consequence, the WAVE-Arp2/3 protein complex is recruited for actin rearrangement into the CDR. Mathematical modelling by Zeng and colleagues, revealed that a RacGAP that is linked to WAVE should be required for negative feedback that downregulates Rac1 and causes a dip in Arp2/3 mediated actin filament assembly in CDRs (Zeng et al., 2011). Their simulations assumed that activated PDGF receptors are localized to a small area at the cell membrane. This is possible since experiments done by Orth and colleagues revealed that CDRs tend to form near the location of localized extracellular growth factor stimulation administered via a microneedle (Orth et al., 2006).

Activation of the PDGF receptor leads to the activation of Rac1 via PIP3 and it has been proposed that activated Rac1 then forms two peaks that travel away from the origin and maintain a stable spatial location. At the end, the two peaks eventually move toward the origin when Rac1-GTP is hydrolyzed to Rac1-GDP by a proposed RacGAP, which is linked to the

WAVE complex. My current data show that actin filaments can be generated from the top, mediated by RhoA and mDia1. The data also show that there could be initial actin filament generation from the bottom, mediated by Rac1 and Arp2/3 (Fig. 19-20) as speculated by Zeng and colleagues (Zeng et al., 2011). At further time points, I also propose that the disassembled actin from the top could accumulate at the base of a CDR causing the membrane cortex to collapse. This actin accumulation also opens possibilities of it being a pool of G-actin to be redistributed elsewhere in the cell for other functions.

Studies have shown and predicted that the formation of stress fibers might be a prerequisite for cells to exhibit CDRs (Boscher & Nabi, 2013; Huynh et al., 2013; Zeng et al., 2011). Activation of RhoA leads to enhanced stress fiber formation. It has been demonstrated that effectors of RhoA, ROCK and mDia1 are required for the regulation of stress fibers (Ishizaki, et al., 2000; Tsuji et al., 2002). ROCK phosphorylates myosin light chain to promote the bundling of actin filaments leading to actomyosin contractility whereas, mDia1 nucleates and elongates thin actin filaments. Earlier work has suggested that actomyosin contractility helps the transmission of mechanical signals from the focal adhesions via the actomyosin bundles (BurrIDGE & Guilluy, 2016). Actomyosin bundling is further enhanced with better engagement with the ECM and elevated focal adhesion signalling. Given the functional links between focal adhesions and stress fiber formation, I found that increased focal adhesion signalling, sizes, and the ability to elongate the adhesion complexes are important for CDR formation and sustenance (Fig. 21-22). Boscher and Nabi had even found that enhancement of outside-in integrin signalling by Mn^{2+} treatment could lead to actin reorganization via CDRs in response to EGF stimulation (Boscher & Nabi, 2013). This piece of work further lends evidence that activation of signalling from the focal adhesions regulates the formation of CDRs.

Studies done on traction forces found that MLCK activation is crucial to the formation of CDRs and have suggested the possibility of the connection between the focal adhesions and actomyosin contractility (Huynh et al., 2013). Similarly, I found that regulators of actomyosin contractility such as, MLCK is important to the sustenance of CDRs (Fig. 23). More specifically at the regions surrounding the CDR as I found increased phosphorylated MLC II at this region (Fig. 30). In contrast, Peleg and colleagues found that actomyosin contractility does not contribute to the formation of CDRs (Peleg et al., 2011). It was further predicted that the interplay between certain activators of actin and membrane bound curved proteins would be essential to sustain the propagation of the CDR excitable waves. However in this study, it is shown that the sustenance of CDRs requires the elongation of focal adhesions and thus I propose that actomyosin contractility contributes to the tension sensed by the focal adhesions (Fig. 25). Earlier work has shown that dorsal stress fibers initiate from focal adhesions mediated by VASP and mDia1 (Gateva et al., 2014; Hotulainen & Lappalainen, 2006; Watanabe et al., 1999). In addition, actin-nucleation promoting factors aid in the elongation of dorsal stress fibers. These elongating dorsal stress fibers have been found to associate with multiple myosin II containing arcs which arise from lamellipodial actin structures (Tojkander et al., 2015). The centripetal flow of this network has been found to generate a more contractile bundle. It has also been shown that the focal adhesions connected to the dorsal stress fibers sense a significant myosin II generated tension. In agreement, I observed elevated phosphorylated MLC II at the exterior regions of CDRs that could be responsible for generating the tension to elongate focal adhesions as the CDR draws closer (Fig. 30). Hence, this raises the possibility that actomyosin contractility within the dorsal stress fibers is required to support and sustain CDRs when they traverse across the dorsal surface.

Furthermore, POPX2, a serine threonine phosphatase was also shown to maintain stress fibers even in cells overexpressing dominant negative mDia1 (Xie et al., 2008). Interestingly, I

observed that POPX2 activity promotes CDR formation. I also found that POPX2 activity enhances focal adhesion area and phosphorylation of MLC II around and at CDRs. I have further confirmed that the CDR promoting effect due to POPX2 is significantly attenuated by ML-7 treatment (Fig. 26 & 27). This suggests that POPX2 could promote CDR formation through the regulation of signalling downstream of the focal adhesions leading to enhanced MLCK activity and localised MLC II phosphorylation around and at CDRs. As POPX2 is a negative regulator of PAK (Koh et al., 2002), the inhibition on MLCK by PAK (Sanders et al., 1999; Bokoch, 2003) could be relieved and thus preserve its activity. POPX2 has also been shown to co-localise with stress fibers (Xie et al., 2008) and CDRs (unpublished data, Koh lab). The findings so far have raised the possibility that there is a mechanical relation between CDRs, actomyosin contractility and the focal adhesion signalling. It also further shows how an activated RhoA environment is a requirement for cells to exhibit CDRs.

Till date, it is still not clear why cells exhibit CDRs. In addition, what benefit do CDRs provide the cells is also not well understood. Actin re-localisation from CDRs to the proximal leading edge sheds some light on the function of CDRs in cell migration context. The CDR appears to act as a G-actin pool for forming the lamellipodial protrusions at cell edges near the CDR. My hypothesis is supported by previous studies which have shown that CDRs almost always form near the leading edge of the cells after PDGF stimulation (Krueger et al., 2003). This allows cells to protrude lamellipodia in a localized manner near the leading edge. It has also been suggested that the stabilization of localized lamellipodia could result in directional cell migration (Harms et al., 2005).

The process of directed cell migration is not governed by lamellipodial protrusion alone; other factors such as cell trailing edge retraction and cell-substrate traction forces play important roles in the overall process (Discher et al., 2005). As such, I found that cell migration persistence

increases with CDR formation but the migration speeds were not affected by the presence of CDRs (Fig. 28). Although the cells have been shown to protrude more lamellipodia after CDR formation, this does not necessarily translate to higher migration speeds (Krueger et al., 2003). The employment of biophysical methods has also helped to suggest a strong coupling between cell speed and persistence (Maiuri et al., 2015). Maiuri et al., showed that faster moving cells turn less. This is due to cell polarity and faster retrograde actin flow which increases persistence time and cell speed. In addition, blebbistatin treatment was shown to markedly reduce cell polarization lifetime and prevent the increase in speed of retrograde actin flow. One could speculate that limited actin flow may impact on CDR formation and after the disassembly of CDR poor translocation of disassembled actin from CDRs to the proximal leading edge. Consequently, this further opens up a new avenue to pursue in the relationship between CDRs and actin flow. Based on my current observations, one could speculate that CDR formation could benefit from retrograde actin flow and thus promote increased directional persistence of cell migration. However, further experiments have to be designed to address this question specifically.

Although my current data suggest that actin from the CDRs moves to the protruding lamellipodia at the cell edge nearest to the site of CDR formation, how actin is transported between these two structures is not clear. Diffusion of actin from CDR to the lamellipodia could be one possibility since actin diffusion at the lamellipodia has been reported (Koestler et al., 2009). As the CDR breaks down, the actin components diffuse in the cytosol, in all directions away from the CDR ring, and actin that diffuses towards the site of lamellipodial protrusion eventually becomes part of the lamellipodia. This explanation is supported by the experiments presented through the photoactivation of photoconvertible fluorescence-tagged actin in the CDRs and the subsequent relocalization of the red fluorescent actin from the CDR to the lamellipodial protrusions (Fig. 29). Another possible mechanism that could explain the transport

of actin from the CDRs to the lamellipodia is via actin-binding proteins like Myo1c (Fan et al., 2012), which have been implicated in transporting G-actin components to the lamellipodia at the leading edge of endothelial cells.

The involvement of non-muscle myosin heavy chain IIA had been proposed in the assembly of CDRs (Casalou et al., 2014). However, its involvement in the disassembly of CDRs was left unexplored. Others have reported mechanisms involving myosin II for the disassembly of the actomyosin ring during cytokinesis. (Huang et al., 2016; Palani et al., 2017). It has been shown that addition of exogenous actin cross linking proteins could block ring contraction and this process was myosin II dependent. In the cytokinesis context, the diffusion coefficient of myosin II using GFP-tagged myosin heavy chain was estimated to be $0.8 \mu\text{m}^2/\text{sec}$ (Uehara et al., 2010). In parallel, the observations show that there is actin flow from a disassembling CDR and MLC II found around and underneath the CDR ring can promote the movement of the actomyosin network towards the leading edge. However, the mechanism of the disassembly of the CDRs still remains relatively unknown. It is possible that MLC II could help to limit the size and lifetimes of CDRs by exerting a counter force to close the ring. On the other hand, it is possible that MLC II could help sustain CDRs by absorbing the pressures of vertical cortical protrusions in the surrounding region. Taken together, the observations suggest that disassembly of CDRs is actomyosin-dependent and could lead to increased persistence in directional migration of the cells (Fig. 32).

The observations that CDR disassembly results in increase directed cell motility suggests that CDR formation could potentially be linked to the response of cells to extracellular chemical stimuli. Since CDR formation is triggered by the stimulation of cells with PDGF, the observed increase in persistence in the direction of migration for cells exhibiting CDRs could be in response to available growth factors in the extracellular environment. This is again supported by

experiments done by Orth et al. whereby CDRs are found to form near the location of localized extracellular growth factor stimulation administered via a microneedle (Orth et al., 2006).

5. CONCLUSION

In this study, the contribution of RhoA in the formation of CDRs was investigated. I found that RhoA was crucial to the time taken to initiate CDRs but not for spreading and sustaining CDRs. I also observed that localised Rac1 activity is required for CDR formation. Overexpression of a constitutively active or dominant negative Rac1 in the cells would result in attenuation of CDR formation. Interestingly, I found distinct activity zones for RhoA and Rac1 suggesting of a possible cooperation between these GTPases and their effectors in the formation of CDRs. I have shown that mDia1 and Arp2/3 have distinct localisation in a CDR. Although the involvement of Rac1 and Arp2/3 in CDR formation was already well characterised, the involvement RhoA and mDia1 in this process was relatively unknown. How these components could work in temporal synchrony despite being spatially distinct was also not clear. Herein, I have shown that there is a possibility of this cooperation between GTPases such as RhoA and Rac1 and their respective effectors mDia1 and Arp2/3 in actin filament formation in CDRs.

I also speculate that CDRs could serve as a pool of actin filaments after they have disassembled. The fate of the pool of actin filaments during or after CDR disassembly was still not well characterized previously, although I speculate that they might be recycled for lamellipodial protrusions since there are studies which suggest that CDRs prepare the cells for motility (Corallino et al., 2018; Krueger et al., 2003; Sero et al., 2011).

With the finding that RhoA could be more actively involved in CDR formation, I have also found that factors such as stress fiber formation and focal adhesion maturation can play a higher role in sustaining CDRs when they form. I found that certain focal adhesion physical properties positively contribute to CDR formation. I propose that focal adhesions can cooperate with MLCK to control the lifetime and maximum CDR area. I also further propose that focal adhesions serve as anchors for CDRs when they traverse across the dorsal surface while depending on actomyosin contractility to pull on these anchors. Furthermore, I have shown that POPX2 activity is important for forming larger focal adhesions and more CDRs.

Although substrate stiffness, outside-in integrin activation experiments and integrin trafficking involved in CDR formation are already well characterised I have shown that the localised dynamic changes to focal adhesion properties and actomyosin contractility occurs when CDRs traverse across the dorsal membrane. Specifically, I have shown that CDRs are accommodated when there is localised enhanced actomyosin contractility to pull on focal adhesions and this effect could be possibly tuned by POPX2 activity (Fig. 33).

This accommodation however is lost sooner or later and hence could be the driving force behind the disassembly of CDRs. Since CDRs assemble and disassemble near the leading edges of the cells, I speculate that the tension associated with accommodating CDRs could be relieved thus resulting in the drift of the under tense actomyosin network towards the proximal leading edge. This could unexpectedly favour the movement of the centroid of the cell to the leading edge thus further committing the cell to migrate in that particular direction.

Control

X2

Figure 33. X2 cells display elevated actomyosin contractility

X2 cells display larger focal adhesions, elevated stress fiber formation and localised myosin II phosphorylation at CDRs as compared to control NIH 3T3 cells.

Cell migration towards a particular direction involves a tightly coordinated process and needs constant actin retrograde flow. I found that cells exhibiting CDRs show an increase in directional persistence during cell migration but not in migration speed when compared to cells which did not exhibit CDRs. In addition, reorganization of actin to the edge closest to where CDR formation takes place, allows the cell to protrude lamellipodia locally at the leading edge. I have shown that actin from CDRs are being localized to lamellipodial protrusions through the use of green to red photoactivatable mEos3.2-actin. Hence, I propose that CDRs act as actin reservoirs that can be tapped on when they disassemble. This disassembled actin could possibly diffuse to the leading edge to power the retro grade actin flow or immediately participate in the formation of the lamellipodia. Consequently, an increased directional persistence of cell

migration could be established due to CDRs (Fig. 34). This opens new avenues as CDRs can serve as a signalling hub that link so many mechanical occurrences to confer cells a function in the migration context.

Figure 34. CDRs improve directional migrational persistence

It is observed that CDRs tend to disassemble near the leading edge of the cell. This disassembly of CDRs provides a pool of actin that is transported towards the leading edge. MLC II also move from CDR vicinity towards the leading edge and actomyosin contractility might be involved in this translocation. Actin from the disassembling CDRs is redistributed to the lamellipodia to enhance directional migrational persistence.

In this thesis, I have explored the possible F-actin structures in CDRs. I have established a role for mDia1 to function at the top of the CDR to nucleate and polymerize actin. I also found that co-operation between Rho GTPases and downstream actin nucleators were necessary for the formation of CDRs. In addition, different active zones for Rac1 and RhoA at CDRs were identified. The observations suggest that RhoA and Rac1 have different roles in the regulation of CDRs. The coupling or interplay between these two GTPases and their downstream effectors is important to modulate CDR dynamics.

I also explored the factors required to sustain fully formed CDRs. I discovered that POPX2 activity could promote CDR formation. Focal adhesions and actomyosin contractility were found to promote and in turn modulate CDR dynamics. The need to have actomyosin contractility in order to use focal adhesions as anchors in sustaining CDRs was also investigated.

Lastly, I unveiled the fate of actin after the disassembly of CDRs. I found that CDRs can act as an actin pool for lamellipodial protrusions. The involvement of actomyosin contractility was further probed and the flow of G-actin from CDRs and the surrounding actomyosin network to the leading edge of the cells was detected. This observation further suggests that CDRs serve as actin reorganisation centres, which use the actomyosin contractility around them to redistribute G-actin to the leading edge thus promoting lamellipodial formation and enhancing directional persistence. The contribution of actomyosin contractility further highlights the importance of RhoA in CDRs.

In summary, novel roles for RhoA and its effectors as well as POPX2 in CDR formation have been uncovered in this thesis. I believe that my current findings can be applicable to other cell types that exhibit CDRs such as macrophages. Since CDRs have been implicated in macropinocytosis, which in turn could be a process for the internalization of viruses, bacteria or pathogens, targeting CDR formation can be a possible strategy to limit infection.

6. FUTURE WORK

CDRs display a very highly dynamic and complicated scenario where the excitable waves can combine as and when needed and thus, it can be a hub for many more spatial-temporally intricate reaction mechanisms. Despite uncovering novel roles for RhoA and its effectors, further work is required to image and elucidate the turnover rates and mechanisms of actin dynamics in the CDR ring. Based on the location of mDia and Arp2/3 in the CDR, I have proposed that linear actin structure can be found at the top of the CDR and more branching actin network is found at the walls and base of the CDR. Higher resolution actin structure could be obtained from cryo-EM. Further studies into how microtubules and intermediate filaments support this structure should also be pursued. In vivo imaging of CDRs should also be pursued to get a better understanding of their features in 3D and physiological setting. However, there is a lack of tools that can be used to extract and identify many more proteins and their functions within CDRs. Hence, a method to isolate CDRs has to be developed so that proteomic studies can be done to validate and possibly open up a new understanding of actin structures and their dynamics.

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8. MANUSCRIPTS

1. Kamaladasan, K., Low, B.C, Koh, C.G. “Circular Dorsal Ruffles, a site for GTPases and actin nucleators to cooperate”, (2019) *Manuscript in preparation*.
2. Kamaladasan, K., Zeng, Y. K., Low, B.C, Chaim, K.H., Koh, C.G. “Circular Dorsal Ruffles Formation Reorganizes Actin and increases directional persistence of cell migration”, (2019) *Manuscript in preparation*.

9. CONFERENCES

1. *Circular Dorsal Ruffles: A collaboration between Rho GTPases*. Oral communication at Small G proteins in cellular signalling and disease, Cambridge, UK. Organised by the Biochemical Society. (July, 2018).
2. *Circular Dorsal Ruffles: A mode of steering cells*. Poster presentation at Science Improving Lives Symposium, NTU, Singapore. Organised by the NTU Graduate Student Council. (Feb, 2017).
3. *Circular Dorsal Ruffles: A mode of steering cells*. Poster presentation at Mechanobiology of Disease Thematic meeting, NUS, Singapore. Organised by the Biophysical Society. (Sept, 2016)