Adhesion-dependent cell division

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I, Christina Lyn Dix, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Animal cells undergo a dramatic series of cell shape changes as they pass through mitosis and divide which depend both on remodelling of the contractile actomyosin cortex and on the release of cell-substrate adhesions. Here, I use the adherent, non-transformed, human RPE1 cell line as a model system in which to explore the dynamics of these shape changes, and the function of mitotic adhesion remodelling. Although these cells are highly motile, and therefore polarised in interphase, many pause migration and elongate to become bipolar prior to mitosis. Interestingly, and in contrast to most reported cell types, these cells do not round fully, and many leave long adhesive tails connected to the underlying substrate. These are typically bipolar, persist throughout mitosis, and guide cell respreading following mitotic exit. Further analysis shows that while many proteins are lost from focal adhesion complexes during mitotic rounding, integrin-rich contacts remain in place along these tails as well as defining the tips of retraction fibres. These adhesions are functionally important in RPE1 cells, since these cells fail to divide when removed from the substrate prior to entry into mitosis. The restoration of cell-substrate adhesions at anaphase are sufficient to rescue division in control cells. However, adhesions must persist into mitotic exit for division in cells compromised in their ability to construct an actomyosin ring. Division in these cells depends on respreading, since Ect2 RNAi cells fail to divide on small adhesive islands, but successfully divide on larger patterns with the cytoplasmic bridge connecting daughter cells narrowing as they migrate away from one another. Together these results reveal the importance of coupling adhesion remodeling to mitotic progression.

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List of Abbreviations

Arp 2/3 - Actin related proteins 2 and 3

Cdc42 - Cell division cycle protein 42

CDK - Cyclin dependent kinase

Cep55 - Centrosome protein 55

CHMP - Charged multivesicular body protein

CPC - Chromosomal passenger complex

ECM - Extracellular matrix

ERM - Ezrin, Radixin, and Moesin

ESCRT - Endosomal sorting complex required for transport

FAK - Focal adhesion kinase

G1 - Gap 1 phase

G2 - Gap 2 phase

GAP - GTPase activating proteins

GDP - Guanosine diphosphate

GEF - Guanine nucleotide exchange factors

GTP - Guanosine triphosphate

MRLCs - Myosin regulatory light chains

MTOC - Microtubule organising centres

NETO - New end take off

NEP - Nuclear envelope permeabilisation

RIAM - Rap1-interacting adhesion molecule

ROCK - Rho-Kinase

SAC - Spindle assembly checkpoint

VASP - Vasodilator-stimulated phosphoprotein

WASp - Wiskott-Aldrich Syndrome protein family

1. Acknowledgements

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2. Introduction

As animal cells progress through the cell cycle they undergo a series of shape changes. The most drastic of these occurs during mitosis, as cells round up and subsequently divide in two. These shape changes rely on a number of factors, primarily changes in the actomyosin cortex and the cell-substrate adhesions. In this study I shall explore the dynamics of these shape changes, and the function of mitotic adhesion remodelling.

2.1. Cell cycle overview

The cell cycle can be broken up into interphase and mitosis. During interphase the cell goes through 3 phases; Gap 1 (G1) phase when it duplicates it's cellular components (other than chromosomes), Synthesis (S) phase when it duplicates it's chromosomes, and Gap 2 (G2) phase when it checks and corrects replication errors, and undergoes addition growth, depending on the system. During mitosis, a cell goes through; prophase when it begins to condense it's chromosomes, prometaphase when nuclear envelope permeabilisation (NEP) occurs, metaphase when it aligns chromosomes on a spindle, anaphase when it separates the sister chromatids, and telophase when daughter cells decondense their chromosomes and reform the nuclear envelope (Figure 2.1 top half, on page 16). In addition, a cell in culture undergoes a series of drastic shape changes during mitosis; from a spread interphase morphology, to rounded during metaphase, elongated during anaphase, dumbbell shaped during telophase, and finally back to it's spread interphase morphology (Figure 2.1 top half, on page 16).

Mitosis is the mechanism by which a cell separates all of it's components into

two new daughter cells. This can occur either symmetrically or asymmetrically. Some cellular components segregate passively with cell volume, e.g. Golgi, mitochondria, and some components must be actively segregated so that each daughter cell gets the correct amount e.g. DNA, centrosomes. Mitosis is a highly complicated process which must occur correctly or give rise to severe defects in the cell such as aneuploidy. The key regulators driving most aspects of mitosis are cyclin-cyclin dependent kinase (CDK) pairs (reviewed by Nigg (1995); Satyanarayana and Kaldis (2009)).

In G1 CyclinD-CDK4/6 are activated and initiate phosphorylation of the retinoblastoma protein (Rb) family (Rb, p107 and p130) which, through E2F transcription factors and E2F responsive genes, allows progression of the cell cycle (reviewed by Dyson (1998)). CyclinE-CDK2 activity controls the transition from G1 into S-phase (Knoblich et al., 1994; Ohtsubo and Roberts, 1993). In S-phase CDK2 then binds CyclinA and triggers DNA replication (Girard et al., 1991; Pagano et al., 1992). At the end of G2, phophorylation of CyclinB1 causes the CyclinB1-CDK1 complex to be translocated into the nucleus (Li et al., 1997), where it is maintained and activated by positive feedback loops (Gavet and Pines, 2010b; Santos et al., 2012) (Figure 2.1 bottom half on page 16). There it triggers NEP (Lüscher et al., 1991; Peter et al., 1990), and at that point, mitosis is ireversible. The CyclinB1-CDK1 complex then initiates many other mitotic events such as golgi fragmentation (Lowe et al., 1998), chromosome condensation and centrosome separation (Gavet and Pines, 2010a). Levels of CyclinB-CDK1 drop in anaphase as the cell exits mitosis. Although these are the primary roles of these complexes, there have been a lot of compensatory functions and redundancy reported for many of the Cyclin-CDK pairs (reviewed by Malumbres (2014)).

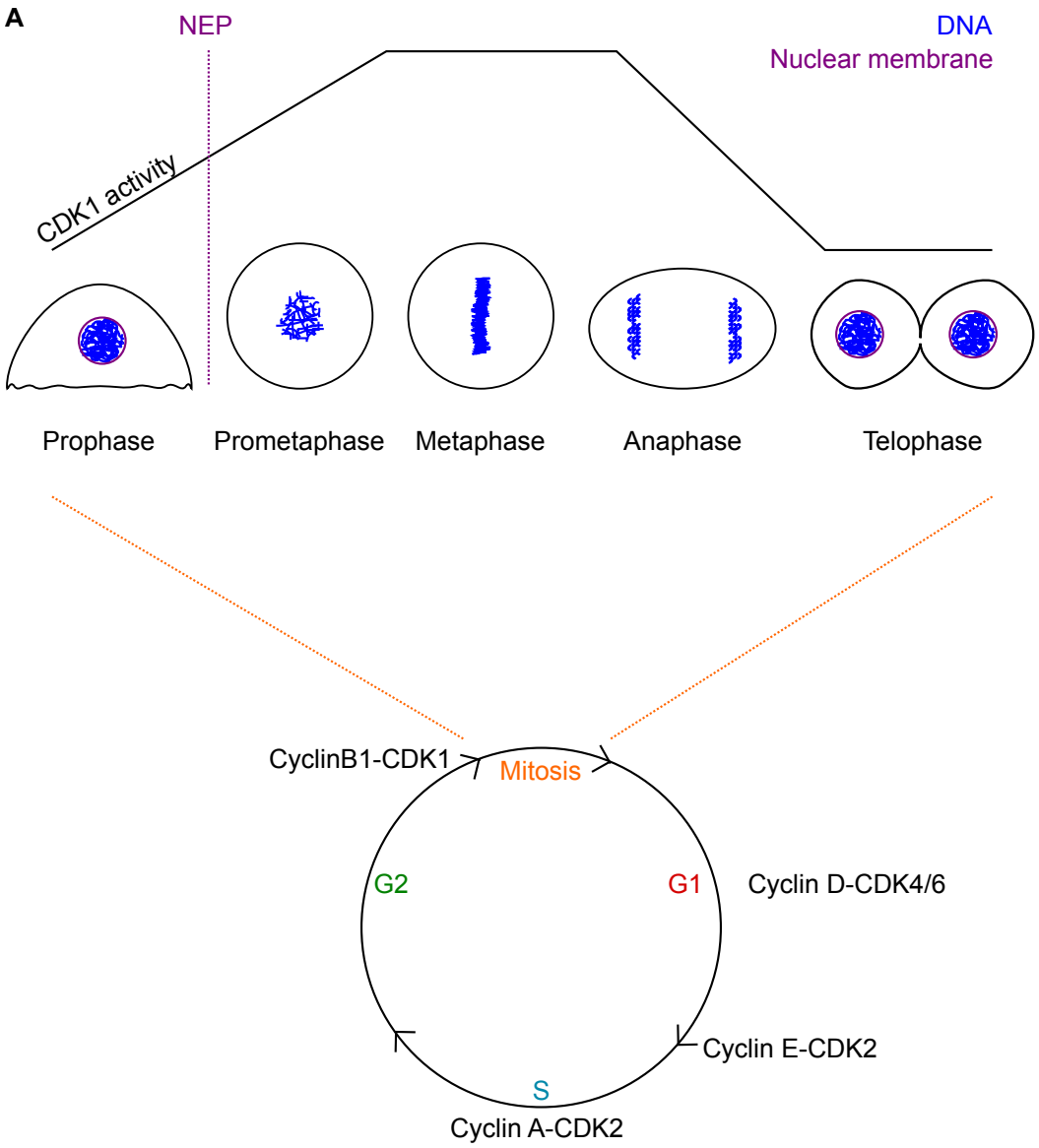

Figure 2.1: Cell cycle overview. (A) Schematic showing the phases of the cell cycle, and then more detail on the changes in DNA, cell shape and CDK1 activity during the phases of mitosis.

2.2. Key regulators of actomyosin activity

In animal cells, it is the actin cytoskeleton that regulates and drives changes in cell shape, like those that accompany cell cycle progression. Actin monomers polymerize to form actin filaments approximately 7nm in diameter, with the monomers oriented in the same direction. These filaments have a distinct polarity, with a fast-growing barbed (a.k.a plus) end where ATP-actin monomers are added, and a slow-growing pointed (a.k.a minus) end where ADP-actin monomers are lost. The main actin nucleators are formins and the Actin related proteins 2 and 3 (Arp 2/3) complex (Eisenmann et al., 2007; Tominaga et al., 2000).

Formins are activated by GTP-bound-Rho-GTPase. GTPases are activated when bound to guanosine triphosphate (GTP) and inactive when bound to guanosine diphosphate (GDP) (reviewed by Bourne et al. (1990)). GTP binding is induced by their interaction with guanine nucleotide exchange factors (GEFs), and the rate of hydrolysis is accelerated by GTPase activating proteins (GAPs). GTP-bound-Rho-GTPase binding disrupts the interaction of a formin's N-terminal GTPase binding domain (GBD) with it's C-terminal DAD domain and releases it from auto-inhibition. Formins promote linear actin filament nucleation by competing with capping proteins for binding to barbed ends. The FH1 domains of formins recruit profilin-bound or monomeric actin, facilitating nucleation and subsequent filament elongation. Formin nucleated actin filaments are implicated in many cellular processes such as in the formation of cytokinetic cleavage furrows (Chang et al., 1997), filopodia, actin cables and adherens junctions (Feierbach and Chang, 2001; Kobielak et al., 2004; Pellegrin and Mellor, 2005; Schirenbeck et al., 2005). The activity of the Arp 2/3 complex is promoted by SCAR/Wave and Wiskott-Aldrich Syndrome protein family (WASp) (Machesky et al., 1999; Yarar et al., 1999). It induces barbed

end elongation and actin branching by nucleating new filament formation at 70° angles to existing actin filaments (Mullins et al., 1998). This branching actin network allows the cytoskeleton to push against the plasma membrane and form lamellipodia in migrating cells (reviewed by Pollard (2007)).

Intracellular mechanical force is generated by the motor protein non-muscle Myosin II. This is a hexamer composed of two heavy chains, two light chains and myosin regulatory light chains (MRLCs). Phosphorylation on Thr18 and/or Ser19 of MRLC results in an increase of actin-activated Mg2+-ATPase activity (Ikebe and Hartshorne, 1985). Myosin II motors act within large bipolar ensembles known as myosin filaments, which in the case of mini-filaments of non-muscle Myosin II, contain a few dozen myosin hexamers. Myosin mini-filaments generate mechanical movement by binding to actin filaments and using the hydrolysis of ATP to drive a force-generating powerstroke and walk along them (reviewed by Mermall et al. (1998)). Several kinases, including myosin light chain kinase (MLCK; also known as MYLK), Rho-associated, coiled coil-containing kinase (ROCK), myotonic dystrophy kinase-related CDC42-binding kinase (MRCK), citron kinase, and leucine zipper interacting kinase (ZIPK) can phosphorylate MRLC to activate it (Reviewed by Somlyo and Somlyo (2003)).

2.3. Cell-substrate adhesion

During my PhD I focused on cell-substrate adhesions and their dynamics and function during mitosis. These focal adhesions play vital roles in a number of cell processes such as cell survival (reviewed by Frisch and Screaton (2001)), proliferation (reviewed by Schwartz and Assoian (2001)), migration (reviewed by Mitra et al. (2005)) and mechanotransduction (reviewed by Geiger et al. (2009)). Cells in epithelial monolayers also form cell-cell adhesions called adherens junctions, however, these are outside the scope of this study and shall not be discussed further.

Focal adhesions are complex macromolecular structures which can contain hundreds of different components (reviewed by Zaidel-Bar et al. (2007)). However, all focal adhesions have the same basic structure; integrins bind elements of the extracellular matrix (ECM) on the cell exterior, then span the plasma membrane to connect to the actin cytoskeleton via a complex of various adaptor proteins (Kanchanawong et al., 2010) (Figure 2.2 on page 20). Many kinases, GTPases and other adaptor proteins get recruited or activated at focal adhesions, triggering signalling cascades which allow the cell to respond to a variety of mechanical and chemical cues (reviewed by Geiger et al. (2009)).

Heterodimeric $\alpha - \beta$ integrins, contain a binding domain for ECM proteins, a transmembrane domain, and a binding domain for Talin (reviewed by Hynes (2002)). They can exist in a inactive conformation when they have low affinity for ligands and do not signal. Integrins can be activated to adopt a high affinity conformation so that they can bind ligands and signal, either through ”inside-out” activation as a result of cytoplasmic events, or through ”outside-in” activation as a result of extracellular factors (reviewed by Margadant et al. (2011)). Talin and Kindlin binding of integrin β tails triggers several signalling cascades and results in integrin activation and the initiation of focal adhesions (Tadokoro et al., 2003). Tyrosine kinase focal adhesion kinase (FAK) is recruited by the integrin-binding proteins Paxillin and Talin and phosphorylates α-actinin (amongst other substrates). This then interacts with Vinculin and Zyxin, which through it’s binding to vasodilator-stimulated phosphoprotein (VASP), crosslinks actomyosin stress fibres, connecting them to the focal adhesion complex (Fradelizi et al., 2001). The stress fibres then exert pulling forces (via the activity of Rho GTPases) on the focal adhesions, which leads to maturation of the complex.

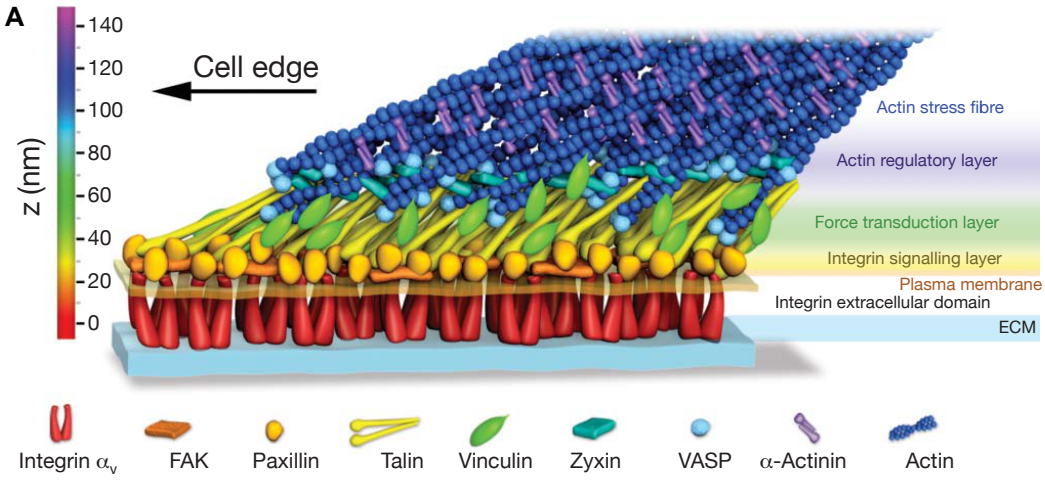

Figure 2.2: Composition of focal adhesions. (A) Schematic model of focal adhesion molecular architecture, depicting experimentally determined protein positions, from Kanchanawong et al. (2010), reproduced with permission.

2.4. Cell Migration

Although many cells in culture are migratory, relatively little is known about how they transition from this dynamic migratory state to a rounded mitotic state. It is intuitive that cells must pause their migration at the onset of mitosis, as the actin cytoskeleton gets remodelled into a mitotic cortex and is therefore unavailable for lamellipodia formation. Indeed Cramer and Mitchison (1997) found that PtK2 cells begin mitosis by stopping lamellipodia formation and retracting the cell margin. On the other hand, at mitotic exit, cells must re-spread and, in many cell types, the nascent daughter cells will specifically migrate away from each other in a polarised fashion.

Current models for how polarity is established and maintained in migrating cells in culture integrate several different aspects of cell biology (reviewed by Woodham and Machesky (2014)): The cell can become polarised spontaneously or in response to a variety of cues such as chemokines, growth factors, or ECM molecules. These set up spatial segregation of GTPases with cell division cycle protein 42 (Cdc42) and Rac1 at the front and RhoA at the rear (Nobes and

Hall, 1999; Waterman-storer et al., 1999), which are maintained and strengthened by positive feedback loops (Waterman-Storer and Salmon, 1999). This induces the formation of actin-based, polarised protrusions such as filopodia and lamellipodia due to the activation of WASp/Scar and the Arp2/3 complex at the leading edge of the cell (relative to migration) (Pollard and Borisy, 2003). These are stabilized by anchorage to the ECM via adhesions (Tadokoro et al., 2003). At the same time ROCK and Myosin II activation at the cell rear cause rear retraction and forward translocation of the cell body (Yam et al., 2007). Vesicle trafficking is polarised to deliver cargo (such as proteins and lipids) to specific membrane domains (Slaughter et al., 2013). All of the above are supported by intrinsically polarised cytoskeletal polymers and associated machinery (reviewed by Mitchison (1992)).

Focal adhesions have been shown to be polarised during migration (Ballestrem et al., 2001). They showed that at the leading edge, adhesions are formed and remain stable with respect to the substrate as the cell body moves over them. These adhesions have low density αVβ3-integrin induced by low tension and Rac1 activity. However adhesions at the retracting rear slide along the substrate behind the cell before being disassembled. These adhesions have high density αVβ3-integrin due to high tension caused by RhoA activity. The sliding of these adhesions has been shown to be due to a treadmilling effect, with monomeric paxillin being added to one side of the adhesion complex, and polymers being removed from the other (Digman et al., 2008).

2.5. Mitotic rounding

One of the first visible changes at the entry to mitosis is that of cell rounding. This is a near universal process that was first observed long ago $in\ vivo$ (Sauer, 1935). It has since been studied both in the context of an epithelia (Reinsch and Karsenti, 1994; Rosa et al., 2015) and in single cells (Cramer

and Mitchison, 1997; Matthews et al., 2012). Mitotic rounding is essential to make enough space for the cell to efficiently undergo spindle formation in cells in culture (Lancaster et al., 2013). A number of co-ordinated events must occur for the cell to undergo efficient rounding. Cells must remodel their cell membrane to change their surface-volume ratio. An increase in osmotic pressure has also been reported to aid mitotic rounding (Stewart et al., 2011). However, the key driver of mitotic rounding is that of actomyosin remodelling. In addition, in order to retract it's margins during mitotic rounding, a cell must remodel the focal adhesions anchoring it to the substrate (Dao et al., 2009).

Actomyosin Remodelling during mitotic rounding

During interphase, the actin cytoskeleton of animal cells in culture consists of an Arp 2/3 mediated branched actin network at the leading edge and formin nucleated actin stress fibres. However, during mitotic entry, the cell switches its cytoskeleton to instead form a stiff actomyosin cortex (Rosa et al., 2015). This cortex is a thin layer of dynamic actin filaments just inside the plasma membrane (Bray and White, 1988; Zieve et al., 1980). Myosin motors are integrated into this actin network and exert the contractile forces neccesary for cell rounding to occur.

Mitotic rounding is primarily regulated through the activity of the RhoGEF Ect2 (Matthews et al., 2012; Miki et al., 1993; Tatsumoto et al., 1999). During interphase, Ect2 is primarily localised to the nucelus of cells in culture, where it has no known function. During prophase, it is actively exported from the nucleus. This may occur through CDK1 activity as Ect2 is a known CDK1 substrate. Once in the cytoplasm, Ect2 activates the small GTPase RhoA (Maddox and Burridge, 2003; Tatsumoto et al., 1999) at the plasma membrane. At the same time, ERM (Ezrin, Radixin, and Moesin) proteins are activated and crosslink actin to the plasma membrane (Carreno et al., 2008; Kunda et al., 2008). To-

gether this causes the construction of a relatively isotropic (Rosa et al., 2015), and mechanically rigid (Kunda et al., 2008; Stewart et al., 2011) actomyosin cortex. Combined with adhesion remodelling and osmotic swelling, this leads to mitotic rounding.

It has been shown in culture that many cell types do not completely round up when they enter mitosis, but leave thin, actin rich tubes called retraction fibres connecting the rounded cell to the substrate. These were first described by Cramer and Mitchison (1997) in Potoroo tridactylis kidney (PtK2) cells. These attachments allow rounded cells to remain anchored to the substrate and to read cues from the environment (Théry et al., 2006).

Focal adhesion remodelling during mitotic rounding

At mitotic entry a cell in culture must remodel it's cell-substrate adhesions to release it's anchorage in order to round up. This has been shown to require the downregulation of Rap1 activity (Dao et al., 2009). Rap1 is a small GTPase which acts as a potent activator of many integrins by promoting the recruitment of talin to integrins through Rap1-interacting adhesion molecule (RIAM) (Han et al., 2006). Dao et al. (2009) showed that Rap1 is transiently inactivated during mitosis in HeLa cells, leading to a loss of Vinculin-positive puncta. Further, expression of constitutively active Rap1 inhibits cell-retraction and impairs mitotic rounding (Dao et al., 2009). It is not clear what regulates Rap1 activity during mitosis. However CDK1 seems a likely candidate, as CDK1 activity regulates many cellular processes during mitosis and it has been shown that CDK1 phosphorylates Rap1GAP (Janoueix-Lerosy et al., 1994). The activity of DEDPC1B, acting upstream of CDK1, has also been suggested to play a role in adhesion remodelling at the G2/M transition (Marchesi et al., 2014).

Despite the fact that adhesions must be remodelled during mitotic rounding

to release the cell's anchorage to the substrate, some adhesion must remain as retraction fibres are left connecting the cell to the substrate. In addition to this, it has been reported that some cell types require adhesion during mitosis, and will fail cytokinesis if forced to divide in suspension. This was decribed for mouse fibroblasts (3T3 and 3T6), epithelial monkey kidney cells (BSC-1) and four B16 melanoma cell variants based on a increase in multi-nucleate cells (Ben-Ze'ev and Raz, 1981). Interestingly, although primary human fibroblasts fail cytokinesis in suspension (and thus are deemed adhesion-dependent), V12 H-Ras-transformed fibroblasts and two cancer cell lines progressed through the entire cell cycle and succeded cytokinesis in suspension (Thullberg et al., 2007). HeLa are also capable of dividing in suspension without significant defects, and a suspension viable line of HeLa has been generated (Puck et al., 1955).

2.6. Mitotic spindle formation and orientation

The bipolar mitotic spindle is the mechanism by which a cell faithfully segregates it's DNA into two new daughter cells. Correct formation of a mitotic spindle is essential for proper cell division. It is formed primarily of microtubules, which extend from the centrosomes, acting as microtubule organising centres (MTOC), at opposite ends of the cell. These connect to the kinetochores of sister chromatids. Once chromosomes have been captured by this complex they are brought to the spindle midzone to form a metaphase plate (Magidson et al., 2011). Astral microtubules extend from the other side of the centrosomes to connect to the cortex in a bipolar manner.

Failure to properly assemble the spindle can activate the spindle assembly checkpoint (SAC) and arrest the cell in mitosis (Musacchio and Salmon, 2007), or lead to improper segregation of sister chromatids resulting in aneuploidy. Mitotic cell rounding has been shown to be important for efficient spindle for-

mation in HeLa cells. Cells in culture which have been prevented from rounding (either through Rap1 overexpression, or mechanically by being restrained by a stiff gel), are delayed in mitotic progression due to problems with spindle formation (Lancaster et al., 2013).

Correct positioning and alignment of the spindle is also important. Spindles mark the position that the furrow will form and daughter cells will be cleaved later in mitosis (Rappaport, 1985), therefore a mis-positioned or mis-aligned spindle will affect the symmetry of division. The spindle is positioned through the actions of astral-microtubules and Dynein motors at the cortex . The conserved cortical complex (Gαi/LGN/NuMA) captures astral microtubules to discrete regions on the cell cortex. It also recruits the dynein/dynactin motor proteins which exert pulling forces on these microtubules to position the spindle between the two capture sites (reviewed by McNally (2013)). Most cells in culture orient their spindle parallel to the substrate, this ensures that after anaphase and furrow formation, each daughter cell inherits contact with the substrate. In addition to this, many cell types will orient their spindle in XY perpendicular to the long axis of the interphase cell shape. This is known as Hertwigs rule and was first described in fertilised frog eggs, where the mitotic spindle aligned with the long axis when the eggs were geometrically constrained by lateral compression (Hertwig, 1884). Since then this work has been built on extensively. Through the use of microcontact printing to control the pattern of ECM on the substrate, (Théry et al., 2005) found that spindle orientation is biased by the spatial distribution of the ECM to which retraction fibres remain attached, which are remnants of interphase cell shape. Fink et al. (2011) went on to show that these retraction fibres are under tension through mitosis and this provides the mitotic spindle with a cue that directs it's orientation during mitosis. They went on to show that these external cues induce polarisation of dynamic subcortical actin structures that interact with microtubules and correlate with spindle

movements.

Finally, once all kinetochores are properly bioriented and the SAC is satisfied, the protease Separase is released from inhibition and cleaves the cohesin complex that holds sister chromatids together (Magidson et al., 2011), allowing cells to enter anaphase, moving the sister chromatids towards opposite poles through the shortening of kinetochore fibres (reviewed by Inoué and Salmon (1995)).

2.7. Furrow formation

It has long been known that the position of the mitotic spindle apparatus at anaphase dictates the position of the cleavage furrow (Rappaport, 1985). When the spindle assembly checkpoint has been satisfied and anaphase occurs, the chromosomal passenger complex (CPC), consisting of Aurora B and the three regulatory and targeting components INCENP, Survivin and Borealin, is relocated from the histones at the inner-centromere to the bundled anti-parallel microtubules at the spindle midzone between the separating chromosomes (Earnshaw and Cooke, 1991; Euteneuer and McIntosh, 1980). Aurora B then phosphorylates the MKLP1 subunit of the centralspindlin complex to promote its recruitment to the midzone (Kaitna et al., 2000). The centralspindlin complex in turn recruits the Rho-GEF Ect2, to the region of anti-parallel microtubule overlap, and loads it onto the plasma membrane (Lekomtsev et al., 2012; Somers and Saint, 2003; Su et al., 2011). Ect2 then activates Rho(Bement et al., 2005; Kimura et al., 2000; Nishimura and Yonemura, 2006; Tatsumoto et al., 1999; Yüce et al., 2005), leading to local formin-nucleation of actin filaments and Myosin II activation at the membrane (reviewed by Bohnert et al. (2013)), forming the contractile actomyosin ring.

At the same time, signals from anaphase chromatin inactivate the mitotic cor-

tex at the cell poles causing polar relaxation (Rodrigues et al., 2015). Cells then undergo cytokinesis as they relax at their poles and constrict at the centre (reviewed by Green et al. (2012)). The furrow thus constricts fully to separate the mitotic cell into two new daughter cells.

Although this RhoA dependent method of furrow formation utilising a contractile ring is the one most commonly seen in most animal cell types, an alternative, contractility free method has also been described. This has been most thoroughly characterised in $Dictyostelium\ discoideum$. These cells can utilise either cytokinesis method. They can divide both in suspension or on an adhesive substrate using the contractile ring method (Neujahr et al., 1997; Zang et al., 1997). However, if the formation of the ring is impaired in strains lacking $mhcA$, which codes for Myosin II, they can still succefully divide on a substrate (but not in suspension) (Neujahr et al., 1997; Zang et al., 1997), through the action of daughter cells respreading and moving away from each other in a SCAR dependent, polarised manner (King et al., 2010), which narrows the neck between them until they are fully separated (Nagasaki et al., 2001).

2.8. Respreading

After furrow formation, as the daughter cells are begining to exit mitosis, they must reverse the processes involved in mitotic cell rounding. This involves polarising their cytoskeleton to form lamellipodia and respread away from each other as decribed in section 2.4 on page 20 .

Rap1 activity (which is downregulated at the onset of mitosis) has been shown to be required for post-mitotic spreading, although the increase in Rap1 activity at the end of mitosis seems to happen slowly (Dao et al., 2009). It has also been reported that End-binding (EB) protein 3 (one of a conserved family of MT plus-end tracking proteins (+TIPs)) regulates the stabilisation of focal ad-

hesions during mitotic exit. This activity relies on Aurora B–mediated dephosphorylation of S176 (Ferreira et al., 2013). Therefore, through the formation of focal adhesion complexes and the activity of cytoskeleton remodelling, daughter cells are able to respread along the substrate and generate traction forces in a polarised manner (Burton and Taylor, 1997b). Strikingly, daughter cells tend to respread into the former interphase shape of the mother (Théry and Bornens, 2006), suggesting that during mitosis the cell somehow maintains a memory of its adhesive pattern. This may occur through retraction fibres left during mitotic rounding, as these have been shown to act as tracks to guide this daughter cell respreading (Cramer and Mitchison, 1993).

2.9. Abscission

After ingression of the cleavage furrow and after daughter cells have respread, abscsission occurs to split the plasma membrane and fully separate the two daughter cells (reviewed by McCollum (2005)). Contraction of the actomyosin contractile ring results in an intercellular bridge with a diameter of 1–2 µm between the two daughter cells. The overlapping region of antiparallel arrays of microtubules from the central spindle forms a dense structure, known as the Flemming body (Flemming, 1981), midbody matrix, or midbody dark zone, in the middle of the intercellular bridge. Abscission occurs on either side of the Flemming body and requires the removal of microtubule and actin cytoskeleton (Connell et al., 2009), the secretion of vesicles, and the assembly of the abscission machinery, which is primarily composed of endosomal sorting complex required for transport (ESCRT) proteins.

During telophase, endosomal vesicles are delivered in the intercellular bridge toward the Flemming body. At the same time, tumour susceptibility gene 101 (Tsg101), an ESCRT-I component, and Alix, an ESCRT-associated protein, move to the Flemming body through binding to centrosome protein 55 (Cep55),

which is recruited onto the Flemming body through interacting with the MKLP2 subunit of the centralspindlin complex (Carlton and Martin-Serrano, 2007; Morita et al., 2007). This targets ESCRT-III subunits to the midbody (Elia et al., 2011). In addition, microtubule-severing enzyme spastin removes microtubules from the midbody (Connell et al., 2009). This allows constriction of the cortex adjacent to the midbody and to the formation of 17nm filaments, which encircle the intercellular bridge as large helices (Guizetti et al., 2011; Mullins and Biesele, 1977).

Finally, ESCRT-III depolymerization factor vacuolar protein sorting 4 (VPS4) accumulates at the intercellular bridge by binding to ESCRT-III subunits and the related increased sodium tolerance (IST)1 protein (Agromayor et al., 2009; Bajorek et al., 2009; Elia et al., 2011). This triggers scission of the membrane (Adell et al., 2014; Babst et al., 1998). How VPS4 contributes to ESCRT-III remodeling at the abscission site is not well understood, but one model suggests that VPS4-mediated disassembly of ESCRT-III polymers may constrict membrane necks in a ”purse-string” mechanism to promote scission (Saksena et al., 2009). It has also recently been suggested that ESCRT-III is rapidly turned-over at the midbody via VPS4 activity, and this aids constriction of the ring (Mierzwa et al., 2017).

Abscission timing must be carefully regulated to protect against errors in dividing the genome. This occurs through the Aurora B dependent abscission checkpoint, which delays abscission in response to chromosome mis-segregation (Steigemann et al., 2009). This was first described as the ”no-cut” pathway in yeast (Norden et al., 2006). They found that Aurora B phosphorylates the two anillin-related proteins, Boi1 and Boi2, which in turn inhibit septin function. In addition, lagging chromosomes in the intercellular bridge are sensed by the Aurora B kinase, which then phosphorylates ESCRT-III subunit charged

multivesicular body protein (CHMP) 4C to delay assembly of ESCRT-III at the midbody (Capalbo et al., 2012; Carlton et al., 2012).

2.10. PhD Aims

As detailed above, it is known that animal cells undergo a dramatic series of cell shape changes as they pass through mitosis and divide, which depend both on remodelling of the contractile actomyosin cortex and on the release of cell-substrate adhesions.

The aim of my PhD was to use the adherent, non-transformed human RPE1 cell line as a model system in which to explore the dynamics of these shape changes, and the function of mitotic adhesion remodelling. In particular I was interested by how these processes were affected by a polarised migratory interphase cell shape. Later, I extended my research to include a study of adhesion-dependent division, and how cells can use adhesion based traction forces to divide in the absense of a contractile ring.

In the following chapters I will layout the methods I used and my major findings.

3. Materials and Methods

3.1. Cell culture and plasmid transfection

hTERT-RPE1 cells were cultured in DMEM F-12 Glutamax (Gibco 31331-028), with 10% fetal bovine serum (FBS), 3.4% sodium bicarbonate (Gibco 25080-060), 1% Penstrep (Gibco 15070-063) at 37°C under 5% CO_2. HeLa LifeAct Ruby cells (Steigemann et al., 2009) were cultured in DMEM (Gibco 41965-039) with 10% FBS and 1% PenStrep at 37°C under 5% CO_2. Leibovitz's L-15 CO_2-independent media (Gibco 21083-027) with 10% FBS was also used during imaging

Plasmid transfection of empty cells with the pArek1-EGFP-Zyxin plasmid (Gift from A. Welman (Welman et al., 2010)) was carried out in antibiotic-free media using Fugene HD (Promega E2311) diluted in Optimem (Gibco 51985-026), according to the manufacturer's instructions. Positive cells were selected and maintained with 500 µg/ml G418 (Calbiochem 345812) to generate a stable cell line. Transient transfection of HeLa with pRK5-Rap1[Q63E] (Dao et al., 2009) was carried out with the same protocol.

rLVUbi-LifeAct-TagRFP lentiviral vector (Ibidi 60142) was used to infect RPE1 Zyxin-GFP cells. Positive cells were then selected and maintained with 1 µg/ml puromycin to generate a stable cell line.

3.2. Ect2 siRNA

siRNA treatment was carried out in antibiotic-free media using AllStars negative control siRNA (Qiagen 1027280), Hs_ECT2_6 Flexitube siRNA (Qiagen SI03049249) and Lipofectamine RNAimax (Invitrogen 13778-075) diluted in

Optimem (Gibco 51985-026) according to the manufacterer's instructions. Cells were used approximately 20 hours post-transfection.

Where RNAi was caried out in conjunction with Rap1[Q63E] transfection, cells were first transfected with Rap1[Q63E], and then Ect2 siRNA the following day. Imaging was started 20 hours later.

3.3. Drug treatments

Cells were incubated with 300 µmol Arp2/3 inhibitor a.k.a. CK-666 (Sigma SML0006) and imaged immediately.

Cells were incubated with 500 nM Wee1 inhibitor a.k.a. MK 1775 (Selleckchem S1525) and imaged immediately.

Cells were incubated with 10 µmol PP2 (Calbiochem 529576) for 1.5 hours. This drug was then washed out and cells were imaged immediately.

To synchronise cells prior to mitosis they were incubated with 9 µmol CDK1 inhibitor a.k.a. Ro-3306 (enzolife sciences ALX-270-463) for 15-20 hours. This was replaced with drug free media immediately before imaging.

To synchronise cells in metaphase they were incubated with 10 µmol STLC (Sigma 164739) for 4 hours.

Where indicated, control treatments were performed with an equivalent volume of the solvent DMSO.

3.4. Micropatterning

Pre-patterned coverslips containing fibronectin lines of various widths were purchased from Cytoo. Versene 1X (Gibco 15040-066) was used to dissociate

cells before plating on these coverslips to ensure fast re-spreading on patterns 4 hours prior to imaging.

To pattern standard 25mm coverslips with discs, ovals or rings, the following protocol was used (optimised in collaboration wit L. Wolf and S. McLaren): HCL cleaned coverslips were passivated by plasma cleaning for 30 sec before incubating for 30 min at room temperatue with 0.1mg/ml PLL-g-PEG-633 (SuSoS PLL(20)-g[3.5]- PEG(2)/Atto633). A drop of MilliQ water was used to attach the coverslips to a quartz mask custom designed with the desired patterns. The coverslip was exposed to deep UV through the mask for 4 min. The illuminated coverslip surface was then incubated for 1 hour at room temperature with 25 µg/ml of fibronectin (Sigma F1141) solution in 100 mM NaHCO3 pH 8.5 (Gibco 25080-060). Versene 1X (Gibco 15040-066) was used to dissociate cells before plating on coverslips to ensure fast re-spreading on patterns and imaging started within 4 hours.

3.5. Cell fixation and immunostaining

16% warmed PFA was added to cells seeded in LabTek dishes for fixed imaging (Thermo Scientific 154526), to a final concentration of 4% and incubated at room temperature for 20 min. Alternatively 10% cold TCA was added and incubated at room temperature for 20 min. They were then washed 3 times and 0.2% Triton was added for 5 min. 5% bovine serum albumin (BSA)/PBS was used to block for 30 min at room temperature before the following primary antibodies were added in 1% BSA/PBS: Active β1 Integrin (Abcam ab30394), Paxillin (Abcam AB32084), Ect2 (Santa Cruz sc-1005), Anillin (Gift from Chris Field), α-tubulin (Sigma T9026), p-Myosin LC2 Ser19 (Cell Signaling Technology 3671L) and Aurora B (Abcam ab2254). Phalloidin Tritc (Sigma p1951) and Dapi (Invitrogen D3571) were added with secondary antibodies (Invitrogen AlexaFluor 647 anti-mouse or AlexaFluor 448 anti-rabbit). The chamber

from the LabTeks were removed and slides were mounted in Fluorsave (Merck Millipore 345789). Fixed samples were imaged on a Leica TCS SPE 2 microscope except for the online fixation experiment.

3.6. Online fixation

For online fixation RPE1 Zyxin-GFP CDK1-inhibited cells were imaged on a Nikon Eclipse Ti microscope with Andor Neo-Zyla camera. Using the pump system established by Almada and Henriques (paper in preparation), the media was exchanged at the microscope to remove inhibition and allow the cells to enter mitosis while imaging continued. 30 min after media exchange when many cells were in metaphase, the PFA fixation protocol as above was triggered to PFA fix and stain the cells at the microscope. The same cells were then imaged post fixation.

3.7. Live cell imaging

Wide field imaging at 37°C was carried out on Nikon Ti inverted microscope or a Zeiss Axiovert 200M microscope at 3 or 5 min timepoints using a 20x or 40x objective. Live confocal imaging at 37°C was carried out on a Nikon TiE inverted stand attached to a Yokogawa CSU-X1 spinning disc scan head, using the 40X air objective or the 60X oil objective, 1 µm z-steps and 3 min timepoints.

3.8. Western blot

Control and Ect2 siRNA cells were lysed using chilled RIPA buffer on ice. The protein concentration of the supernatant was determined using Bradford reagent. Samples of equal concentration were then prepared and run on 4-12% Tris Bis gel (Invitrogen NW04122). Gels were then blotted, blocked and probed with Ect2 (Santa Cruz sc-1005) and α-tubulin (Sigma T9026) primary anitbodies, and anti-mouse and anti-rabbit HRP-conjugated secondary antibodies (Dako).

Membranes were visualized using an ECL detection kit (GE Healthcare) and an ImageQuant LAS4000 system (GE Healthcare).

3.9. Image analysis and statistical analysis

Images were analysed using Fiji to generate qualitative and quantitative data as described in the results sections.

Graphs were produced in Microscoft Excel and Graphpad Prism. Statistical tests were carried out in Graphpad Prism. Normal data sets comparing distribution of values were analysed using the unpaired t test, two tailed. Non-normal data sets were analysed using Mann-Whitney two-tailed test. Binary data sets were analysed using the Chi-square test. *p<0.01 **p<0.001 ***p<0.0001 ****p<0.00001.

3.10. Cell respreading from suspension

RPE1 cells were plated in a 6 well plate and treated with control or Ect2 siRNA as previously described. 16 hours after siRNA transfection a mitotic shake-off was carried out to dislodge mitotic, dead or dying cells. To synchronise cells in metaphase they were incubated with 10 µmol STLC (Sigma 164739) for 4 hours. Then a mitotic shake off was carried out and the collected cells washed twice before being re-suspended in imaging media. They were then re-plated into fibronectin coated glass-bottomed LabTek dishes (Thermo Scientific 1555383). The dish was centrifuged at-200 x g, with zero braking for 1 minute, and imaged immediately.

3.11. Measuring mitotic failure in suspension

1X Versene was used to dissociate cells from the substrate so that they could either be plated directly into fibronectin coated LabTek dishes for fixed imaging (Thermo Scientific 154526), or into a 96-well Ultra-low attachment plate

(Corning 3474). 4 replicate wells per sample were used in the 96 well plate as pilot experiments found that many cells were lost in later washing steps. After 3.5 hours the cells in fibronectin-coated wells underwent the standard PFA-fixation as described above. The 4 wells for each sample in the non-adherent wells were combined and 16% PFA was added to make a final concentration of 4% and incubated for 20 min. Cells were then washed and added to LabTek dishes which had been pre-coated with Cell-Tak (Corning 354240, 354241) (3.5 µg Cell-Tak/cm^2 of surface area in 0.1 M sodium bicarbonate for 30 minutes, then washed with sterile MilliQ water) to make the cells adhere to the bottom of the dish. The dish was then centrifuged at 200 x g, with zero braking for 1 minute. All LabTek dishes were then permeabilised with 0.2% Triton, blocked with 5% BSA and incubated with a primary antibody against α-tubulin (Sigma T9026) for 1 hour at room temperature. This was then washed off and Dapi and Phalloidin TRITC was added with the secondary Alexa Flour anti-mouse 488 antibody. The chamber from the LabTeks were removed and slides were mounted in Floursave (Merck Millipore 345789).

4. Cells undergo drastic shape changes at mitotic entry and exit

4.1. Introduction

I began my PhD by carrying out a detailed characterisation of mitosis in RPE1 cells, a hTERT immortalised cell line (Bodnar et al., 1998). Although the process of cell division has been extensively studied, much is still unknown, and what is known has primarily been studied in transformed cell lines such as HeLa.

At the entry to mitosis almost all animal cell types, both single cells and those in tissues, will undergo a significant amount of mitotic rounding (Reviewed in Kunda and Baum (2009)). This drastic shape change was shown to be essential for efficient spindle orientation (Lancaster et al., 2013). It is a complex process, requiring the cell to stop all it's interphase activities, release it's anchorage to the substrate and remodel it's actomyosin cytoskeleton to form a stiff cortex.

At mitotic exit, after separation, the daughter cells must undergo the reverse process. They must re-spread, form new attachments to the substrate and reorganise their cytoskeleton to return to it's standard interphase morphology. In migratory RPE1 cells this morphology primarily consists of a clear Rac1-regulated lamellipodia at the leading edge and a clear RhoA-regulated retracting rear (Ballestrem et al., 2000; Nobes and Hall, 1999; Waterman-Storer and Salmon, 1999; Waterman-storer et al., 1999).

To understand how these human cells deal with the numerous tasks and prob-

lems a mitotic cell must overcome, I shall begin in this chapter by characterising how RPE1 cells progress through mitosis, and each of the stages they go through.

4.2. Cells pause migration prior to mitotic entry

The first thing that must be considered as regards an RPE1 cell entering mitosis, is that of motility. These are highly migratory cells, and are almost constantly in motion during interphase. How then does a cell enter mitosis while migrating?

To examine this I carried out long timelapse videos of an unsynchronised population of RPE1 cells stably expressing Zyxin-GFP, a component of the focal adhesion complex. As well as bright Zyxin-GFP positive puncta allowing me to study adhesion remodelling in these cells (studied extensively in Chapter 4), the cytoplasmic GFP signal allowed me to study the changes in cell shape as cells progress through mitosis. These cells were plated on a standard fibronectin substrate, and their behaviour observed as they entered mitosis.

I found that a large proportion of cells will stop lamellipodia extension and migration at some point before entering mitosis, which I termed ”pausing”. As the interphase shape of these cells can be quite variable and change stochastically as cells are moving, a cessation of lamellipodia extension was not always easy to define. Instead, I used a qualitative examination of the position of the nucleus from one timepoint to the next to determine when a cell stopped moving relative to the substrate (this could be improved upon in the future using automated or manual tracking). I then compared this to when it underwent NEP (Figure 4.1A on page 39). This analysis showed a wide distribution of the amount of time paused prior to mitotic entry (Figure 4.1B on page 39). I used the 25th percentile of this distribution (12 minutes) to establish a cut off time to separate

cells into ”Pause” and ”Don’t Pause” categories for analysis later in this chapter.

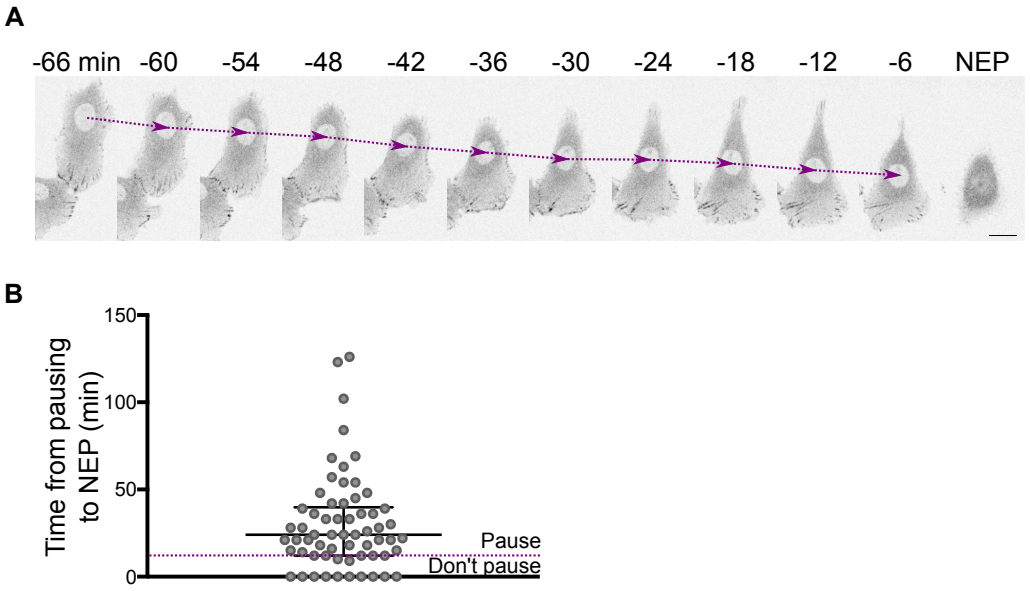

Figure 4.1: Migratory RPE1 cells pause prior their movement prior to mitotic entry. (A) Montage shows an RPE1 cell stably expressing Zyxin-GFP slowing down it’s migration prior to mitosis. Time is shown relative to NEP. Magenta arrow notes the centre of the nucleus at each timepoint. 1 basal z-slice. Scale-bar 20 µm. **(B)** Graph shows the time from when cells pause their migration (assessed qualitatively) to when they undergo NEP. Median $\pm$ interquartile range. For future analysis, any cells in the 25th percentile (below 12 min) were deemed not to have paused, any above 12 min were deemed to have paused. N=11 experiments.

This data shows that cells often stop migrating prior to mitosis, and that this can occur long in advance of mitotic entry. Pausing could either be a stochastic event that occurs throughout interphase, or it could be an event that occurs specifically as cells are preparing to enter mitosis, perhaps as a consequence of preparing to reogranise their cytoskeleton for events such as cell rounding. To examine this I tracked the nuclear position of 8 cells from 4 hours prior to the onset of rounding, and plotted the change in their velocity over time (Figure 4.2 on page 41). From this it could be seen that cells pause often during interphase and there is no clear pattern or trend towards an increase in pausing prior to mitosis. This suggests that pausing is a stochastic event which occurs independently of mitosis. However more sophisticated tracking at a higher time resolution and for longer periods would be required to prove this conclusively.

4.3. A subset of cells undergo a polarity switch prior to mitotic entry

As a migratory cell type RPE1 cells have a clear polarity, a lamellipodia at the front and a retracting rear. How does a cell account for this asymmetric interphase shape when preparing to undergo a symmetric division?

It had previously been observed in the lab that prior to mitosis some RPE1 cells will extend a second lamellipodia behind them and so become more symmetric in shape (A. Roycroft and R. Picone, unpublished data). Using the Zyxin-GFP puncta visible in this RPE1 cell line, I qualitatively assesed cells and defined any which extended new Zyxin-positive puncta backwards while maintaining the lamellipodia at the front, as having undergone this polarity switch and deemed them ”bipolar”. Those which didn’t undergo the polarity switch were termed ”monopolar” (Figure 4.3A on page 42). 48% of cells were seen to switch to bipolar prior to mitosis (N=70 cells from 12 experiments). As with pausing, the

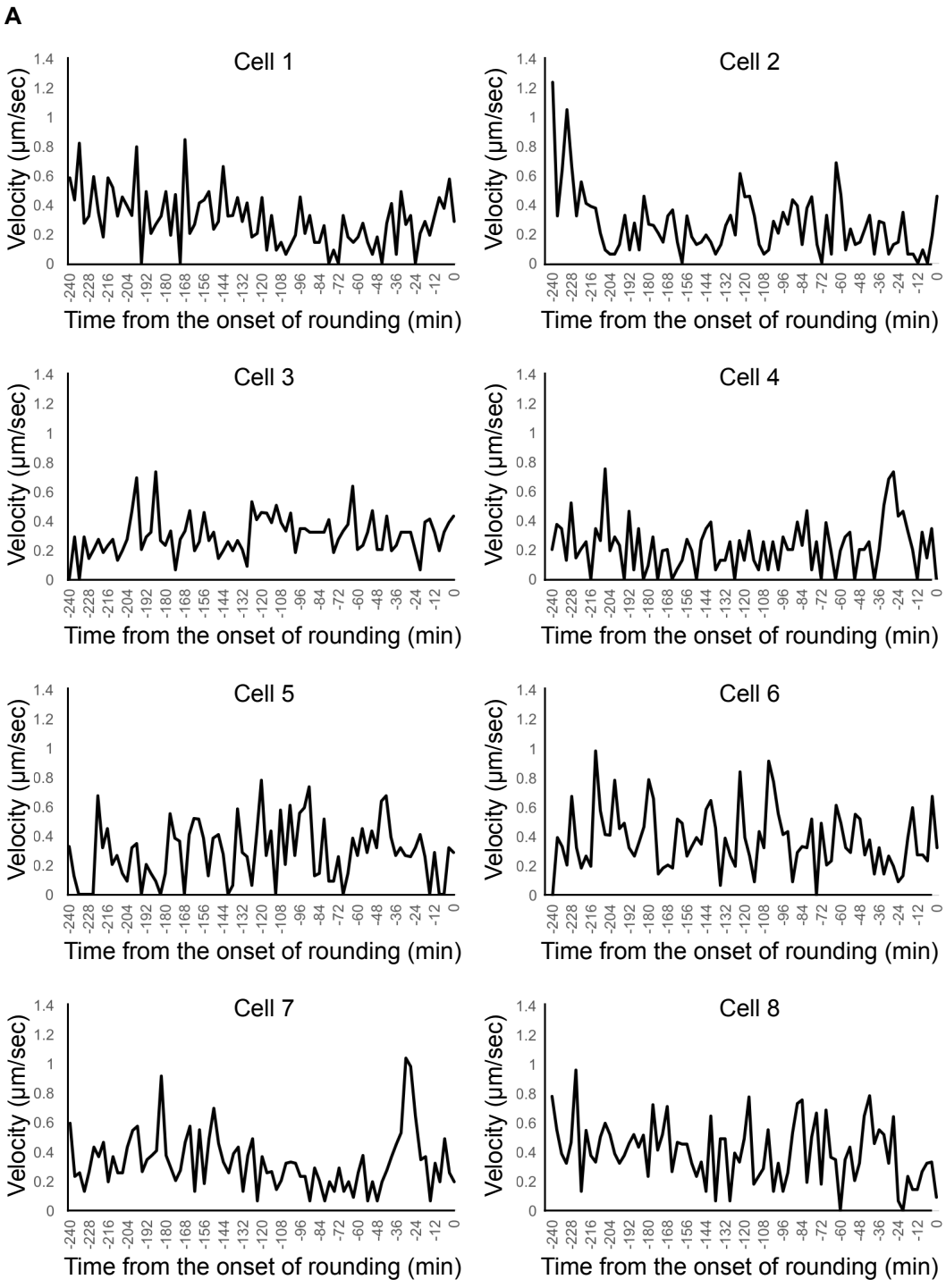

Figure 4.2: Cell pausing is a stochastic event. (A) Graphs from 8 sample cells showing the velocity of the nucleus as a proxy for cell migration. Time is shown relative to the onset of rounding.

time this switch would occur prior to mitosis was very variable betwen cells (Figure 4.3B on page 42).

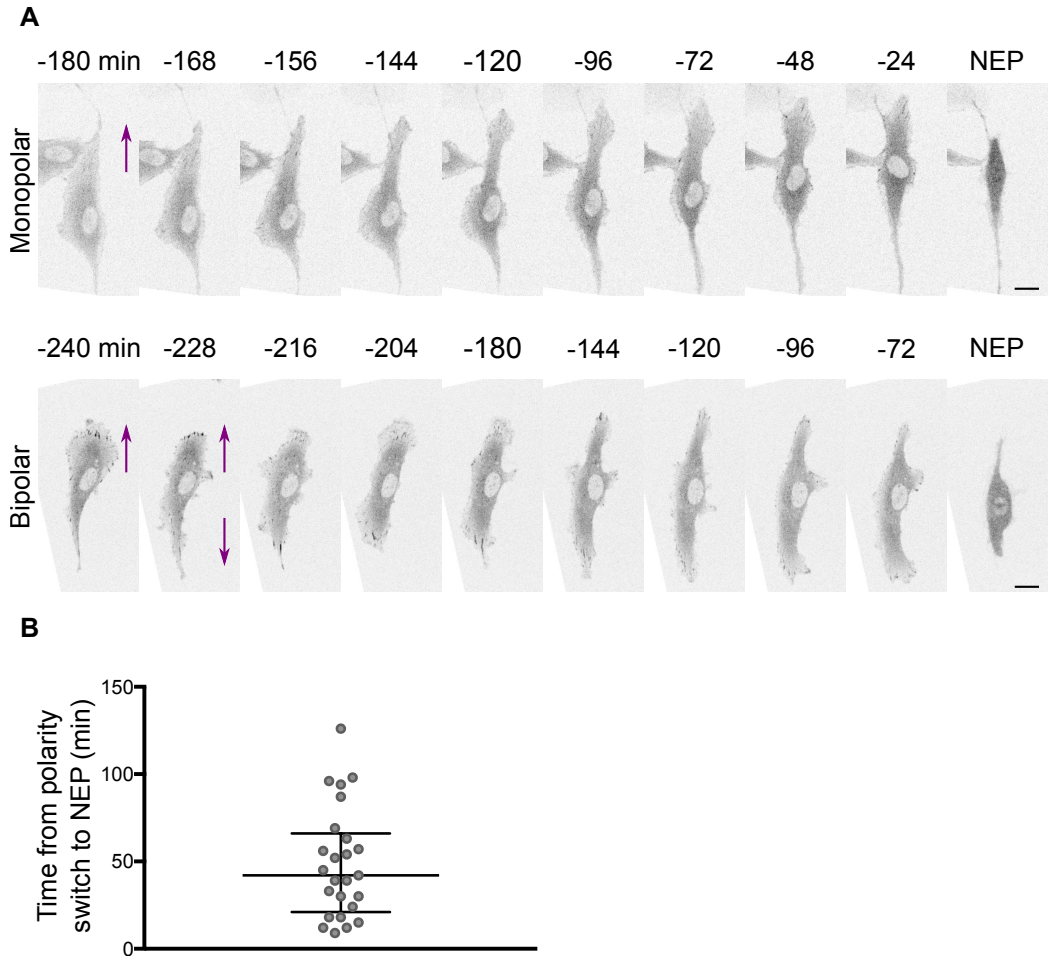

Figure 4.3: A subset of cells undergo a polarity switch prior to mitotic entry. **(A)** Montages show a monopolar and a bipolar cell stably expressing Zyxin-GFP. The bipolar cell undergoes a polarity switch prior to mitotic entry (arrows). Time is shown relative to NEP. 1 basal z-stack. Scale-bar 20 µm. **(B)** Graph shows the time from when bipolar cells underwent the polarity switch to when they underwent NEP. Median ± interquartile range. N=9 experiments.

As pausing seems to be a stochastic event, is the polarity switch also? To examine this I compared the relative number of monopolar and bipolar cells in cells at the onset of rounding, and cells 4 hours prior to that. From this I found a slight, but not significant increase in the amount of bipolar cells at the onset of rounding (Figure 4.4 on page 43). It may be that 4 hours prior to mitosis is too close and that by going further back a clearer difference would be seen.

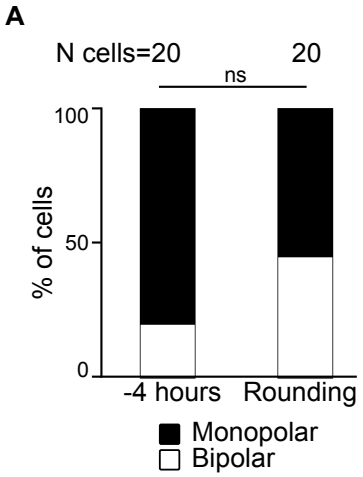

Figure 4.4: There is no increase in the polarity switch prior to mitosis. (A) Graph shows the percentage of cells which were monopolar or bipolar either at the onset of rounding or 4 hours prior to it. N=7 experiments. Statistics used two-sided Chi-Square test.

To see if there was a correlation between pausing prior to mitosis and undergoing the polarity switch I examined how many of either bipolar or monopolar cells had previously paused and found that bipolar cells were more likely to have done so (Figure 4.3A on page 42). In addition to this, bipolar cells tended to have paused for slightly longer than monopolar cells (Figure 4.3B on page 42). This suggests that either paused cells are more likely to undergo the polarity switch, or that cells undergoing the polarity switch are more likely to pause. To distinguish between these two possibilities would require more complete data on cell velocity to accurately define pausing, and markers for the polarity switch e.g. Rac1/CDC42 at the newly forming leading edge.

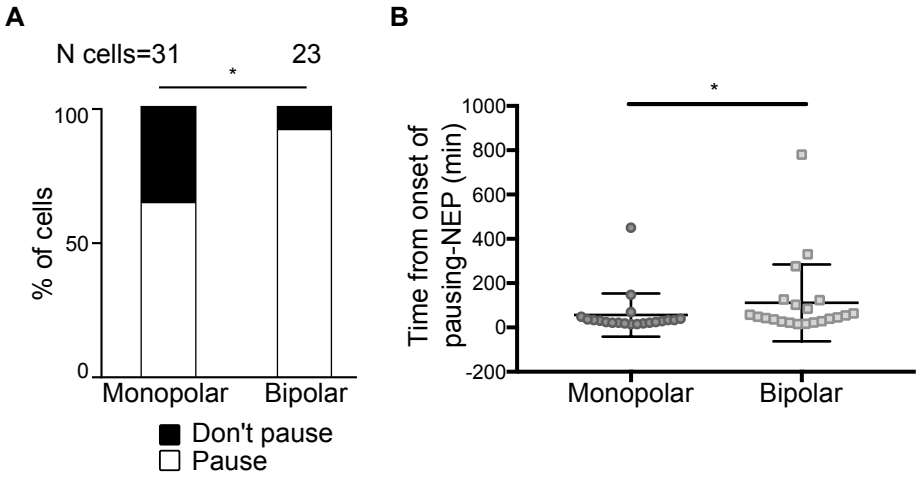

Figure 4.5: Bipolar cells tend to have paused more than monopolar cells. (A) Graph shows the percent of monopolar and bipolar cells which Pause or Don't pause just prior to mitotic entry. N= 14 experiments. Statistics used two-sided Chi-Square test. **(B)** Graph shows the time from the onset of pausing to NEP in monopolar and bipolar cells. Mean ± SD. N=10 experiments. Statistics used two-tailed Mann-Whitney test.

One difficulty in analysing RPE1 cells was that the cell shape could be quite variable. To combat this and further examine how cells pause and undergo the polarity switch, I decided to plate cells on micro-patterned fibronectin lines available from Cytoo. Preliminary experiments were carried out with lines of 2.5 µm, 10 µm and 20 µm width, and all constrained the cell to migrate in only 1 axis. I tracked the nuclear movement of cells for 4 hours prior to the onset of rounding, and noted when they were either monopolar or bipolar. From this analysis there seemed to be slight correlation between increased velocity when cells were in a monopolar conformation, but it was not completely clear (Figure 4.3A, arrows, on page 44). However when I compared the velocity in time-points where the cell was monopolar, to the velocity when cells were bipolar, a significant difference could be seen (Figure 4.3B on page 44).

Although largely stochastic, this polarity switch could be one way that cells such as RPE1, which are polarised and thus highly asymmetric during interphase, are able to organize their components so as to evenly and efficiently distribute

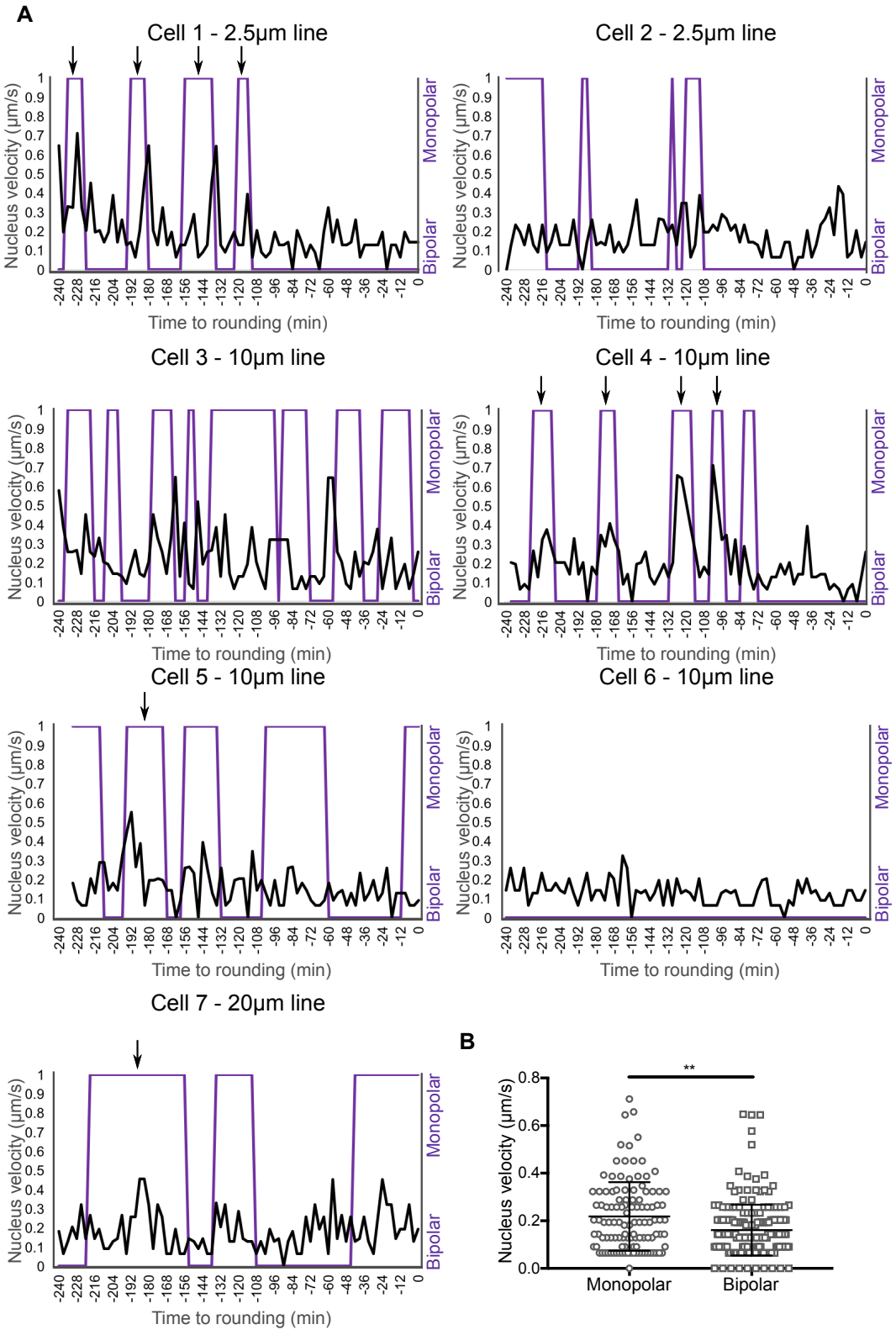

Figure 4.6: Cells move faster when in a monopolar conformation than in a bipolar conformation. (A) Graphs show the nuclear velocity (black), and switch between monopolar and bipolar conformation (magenta) for 7 individiual cells plated on fibronectin lines of 2.5 µm, 10 µm and 20 µm width. Time is shown relative to the onset of rounding. N=5 experiments. **(B)** Graph shows the nuclear velocity for each timepoint when cells were either in a monopolar or bipolar conformation. Each data point

(continued) represents one timepoint. Pooled from 4 cells on 10 µm lines. N= 4 experiments.Mean ± SD. Statistics used two-tailed Mann-Whitney test.

them to two new daughter cells during symmetric division. For example, the differences between the actomyosin distribution in the leading edge and rear of a monopolar cell are likely to be asymmetric in a similar fashion to that seen in an interphase, migratory cell (Ballestrem et al., 1998). However, undergoing the polarity switch and becoming bipolar likely leads to a more symmetric actomyosin distribution between the two ends of the cell. This may have downstream effects on later processes in mitosis such as mitotic rounding.

4.4. RPE1 cells undergo inefficient mitotic rounding which reflects the symmetry of interphase cell shape

After pausing, and potentialy undergoing a polarity switch, the next process a cell must go through at mitotic entry is mitotic rounding. Cramer and Mitchison (1997) carried out a detailed analysis of this process in PtK2 cells. They described how cells entering mitosis retract their cell margin to expose thin, actin rich fibres, known as retraction fibres, which remain attached to the substrate throughout mitosis. This drastic shape change requires a number of processes to occur in concert; primarily, adhesion to the substrate must be remodelled to release the cell so it can round up (explored extensively in Chapter 4)(Dao et al., 2009), and the actomyosin cytoskeleton must be significantly rearranged to form a stiff actomyosin cortex just inside the cell membrane (Matthews et al., 2012),

As I showed in the previous section that cells will enter mitosis and undergo mitotic rounding from two distinctly different shapes, monopolar and bipolar, I first wished to examine what effect this may have on the rounding process. Intriguingly, I found that a large number of RPE1 cells did not round fully and become

completely spherical. In fact, 79% (N=77cells from 7 experiments), would leave cytoplasmic tails along the long axis of the cell, which persisted throughout mitosis (Figure 4.7A, arrows, on page 48). Further, these tails tended to follow the symmetry of the interphase cell prior to mitosis i.e. monopolar cells tended to leave only 1 tail, bipolar cells tended to leave 2 (Figure 4.7B on page 48). This shows that the symmetry of the interphase cell shape prior to mitosis has an impact on the symmetry of the rounding process.

To analyse tails, the most extreme pattern of 2.5 µm was chosen as cells on these lines adopted the longest shape. I then focused on bipolar cells which left two tails. Even within this category there was great variability in mitotic rounding between cells. If a cell left tails they tended to gradually thin as most of the cell mass moved to the main cell body (Figure 4.8A on page 49). However, this could occur symmetrically or asymmetrically. Some cells left tails which remained throughout mitosis, some cells gradually retracted these tails to some extent, and some cells left tails at the begining of rounding which were retracted and fully incorporated into the main cell body prior to anaphase. Interestingly, the retraction of tails did not happen symmetrically. Two tails on opposite sides of the same cell could be retracted at different rates, and to different extents (Figure 4.8A on page 49).

The finding that tails get pulled in, along with the fact that not all cells leave them in the first place, suggests that they may be a simple consequence of inefficient mitotic rounding by RPE1 cells.

4.5. Cells round to the nucleus centre

A key interest of this study was about how well adhered to the substrate cells are during mitosis. It is known that mitotic cells are certainly less well adhered than interphase cells, hence they can be removed by mitotic shake off. How-

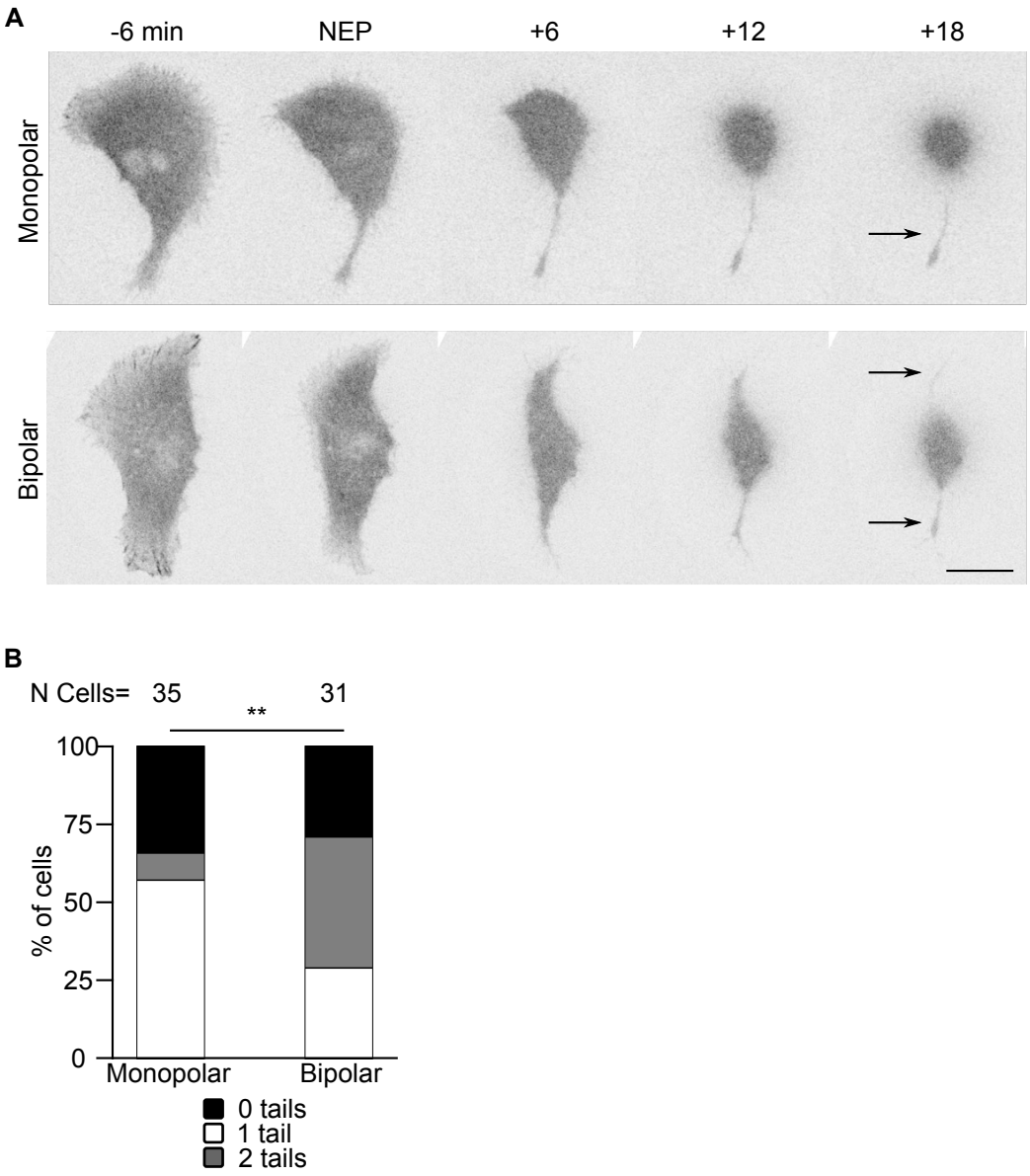

Figure 4.7: Undergoing a polarity switch effects cell rounding. (A) Montages show monopolar and bipolar cells stably expressing Zyxin-GFP and leaving tails (arrows). Time is shown relative to NEP. 1 basal z-slice. Scale-bar 20 µm. **(B)** Graph shows the distibution of monopolar or bipolar cells which leave 0, 1 or 2 tails. N=14 experiments. Statistics used two-sided Chi-squared test.

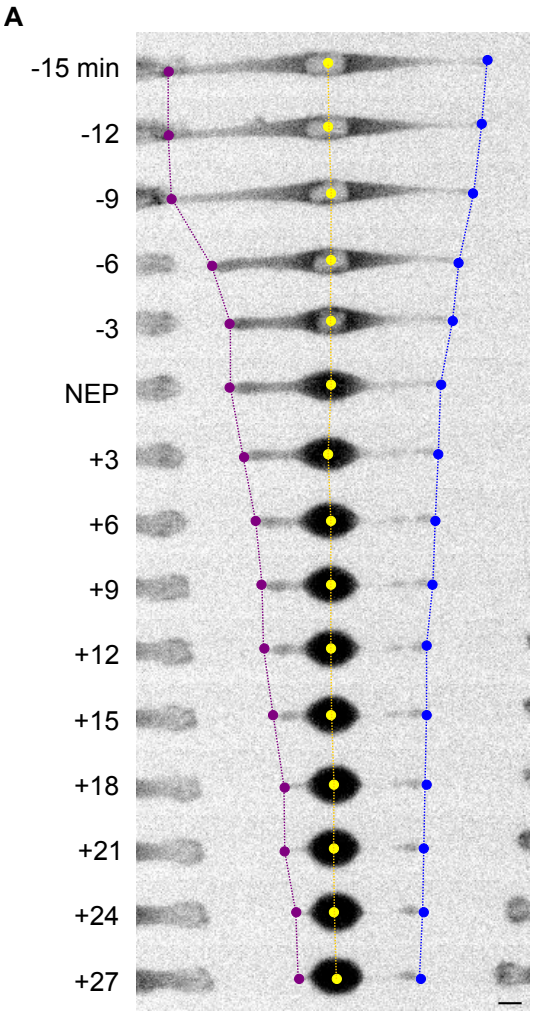

Figure 4.8: Tails are retracted asymmetrically. (A) Montage shows a bipolar RPE cell stably expressing LifeAct GFP, plated on a 2.5 µm line, entering mitosis and gradually retracting it's cytomplasmic tails. Time is shown relative to NEP. Wide-field image. Scale-bar 20 µm.

ever, they do still maintain some adhesion, as if left undisturbed they will remain attached to the substrate throughout division, without becoming dislodged and floating off. In my system there is a further argument for cells retaining attachements, as they also leave cytoplasmic tails as described earlier in this chapter (Section 4.4 on page 46). These tails must be anchored to the substrate somehow. The final rounded cell shape and position must be a result of both the cell substrate attachment being remodelled and the forces generated through the formation of an actomyosin cortex along with osmotic swelling. As I found that cells do not neccesarily round symmetrically to resolve these issues I wished

to examine the effect of this on the final rounded position.

I noted anecdotally that in cells which had their nucleus positioned offset to the cell centre, the final rounded cell body would usually be closer to the nucleus position, than the centre of the cell body. To study this quantitatively I measured the position of the centre of the nucleus, the position of the centre of the interphase cell shape (based on the longest axis of the cell through the nucleus), and the position of the final rounded cell, and compared these three positions (Figure 4.9A on page 51). Although in most cases, the nucleus centre and the cell centre would be in approximately the same place, by plotting the distribution of the distance between these points I could use the 75th percentile to generate a cut off of 20 µm to define ”offset nuclei” (Figure 4.9B on page 51). By comparing the distance from the cell centre to the rounded centre, and the distance from the nucleus centre to the rounded centre, in cells with offset nuclei, I found that cells round to their nuclei, i.e. the final rounding position tended to be closer to the nucleus centre than the interphase cell centre (Figure 4.9C on page 51).

The cell may round to the nucelus because there is more adhesion and greater anchorage underneath. I screened for small molecules that might perturb this behaviour, focusing on Src family kinase inhibitors as they are known to regulate cell-substrate adhesion dynamics (Di Florio et al., 2007). In this way, I showed that cells treated with PP2, a Src Tyrosine kinase inhibitor, fail to round to the nucleus efficiently (Figure 4.9D-F on page 51)). However, no significant difference over control could be seen between cells treated with the other Src family kinase inhibitors, SU6656 and Src inhibitor 1. Although this has to be repeated this suggests that rounding to the nucleus may be Src-dependent.

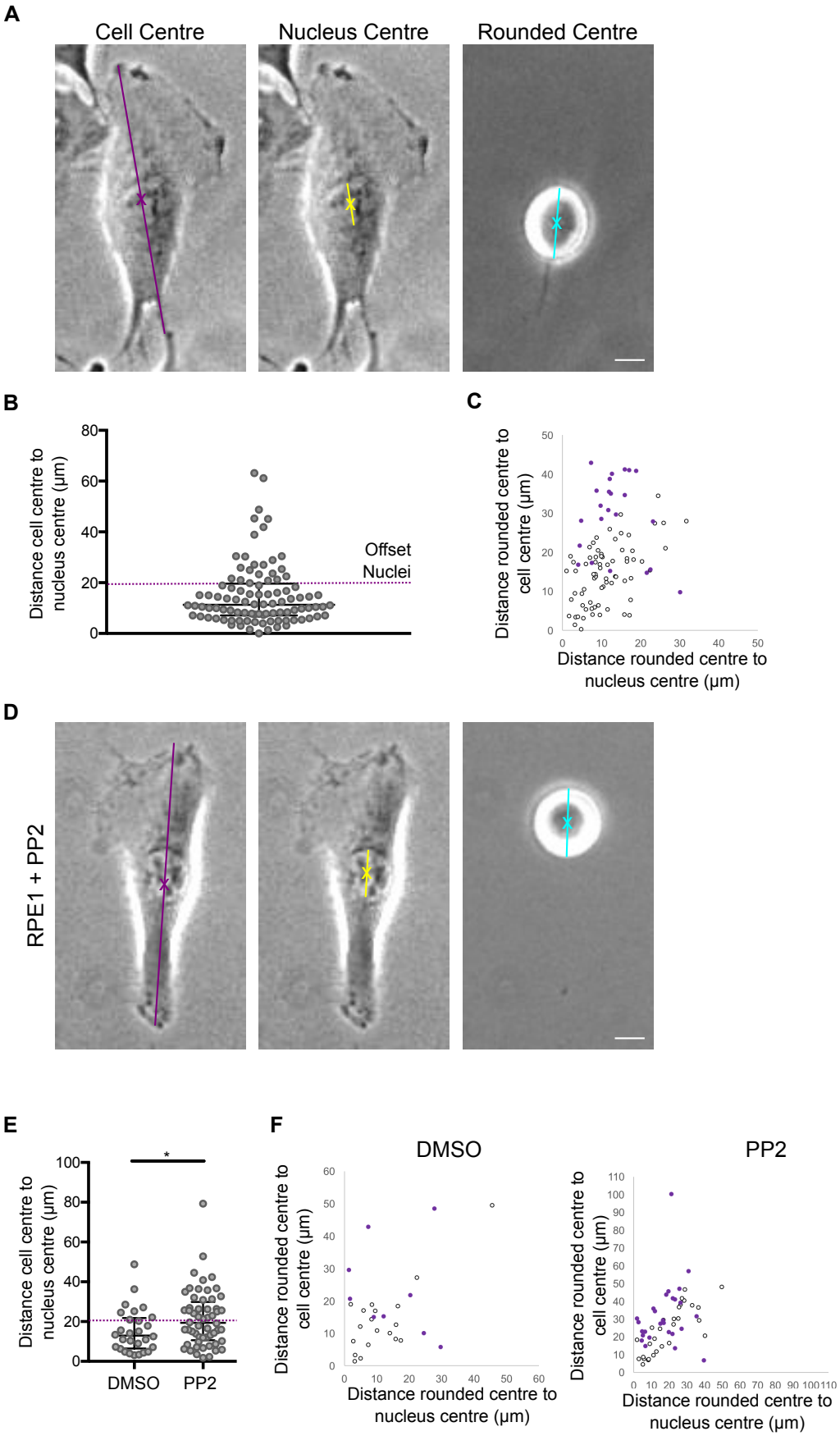

Figure 4.9: Cells round to the position of the nucleus. (A) Images show the positions taken for the cell centre (Magenta X, basal slice, timepoint prior to rounding), the

(continued) nucleus centre (Yellow X, basal slice, timepoint prior to rounding), and the rounded centre (Cyan X, middle slice, timepoint prior to anaphase) to generate the data in graphs (B-C). Wide-field images. Scale-bar 20 µm. **(B)** Graph shows the distance between cell centre and the nucleus centre. Median ± interquartile range. For future analysis, any cells above the 75th percentile (20 µm) (magenta line) were deemed to have an offset nucleus. N=5 experiments. **(C)** Scatterplot compares the distance between the rounded centre and the nucleus centre, and the distance between the rounded centre and the cell centre. Cells which were deemed to have offset nuclei are labelled in magenta. N=5 experiments.**(D)** Images show RPE1 cells treated with a PP2 inhibitor and the positions taken for the cell centre, the nucleus centre, and the rounded centre, as for control cells in (A), to generate the data in graphs (E-F). Wide-field images. Scale-bar 20 µm. **(E)** Graph shows the distance between the cell centre and the nucleus centre for cells treated with DMSO or 10 µmol PP2 for 1.5 hours before imaging. Median ± interquartile range. N=2 experiments. Statistics used two-tailed Mann-Whitney test. **(F)** Scatterplots compare the distance between the rounded centre and the nucleus centre, and the distance between the rounded centre and the cell centre, for cells treated with DMSO or 10 µmol PP2. Cells which were deemed to have offset nuclei are labelled in magenta. N=2 experiments.

4.6. Spindle position and orientation is not affected by the presence of tails

The metaphase plate and spindle are assembled as cells transition from prophase to prometaphase. The spindle must then be correctly positioned in the centre of the cell as it will later dictate the cleavage plane (Rappaport, 1985), and an offset spindle can lead to an unwanted assymetric division (Bringmann and Hyman, 2005). It must also align the spindle in Z so it is parallel to the susbstrate, enabling both daughter cells to inherit adhesive contacts. In addition, most animal cells orient to the interphase long axis of the cell. This was first described and defined as Hertwig's rule, and has been thoroughly studied since (Reviewed by Minc and Piel (2012)). RPE1 cells are very good at orienting their spindle parallel to the substrate and very rarely misorient it in Z (when assessed qualitatively 10% of 69 cells had a misorientation in Z of greater than 20°, N=11 experiments). However, as RPE1 cells enter mitosis from either an asymmetric (monopolar), or symmetric (bipolar) shape, and leave asymmetric or symmetric tails accordingly, I wished to study the impact adhesion to the substrate via tails

may have on the position and XY orientation of the spindle. These cytoplasmic tails likely reflect large retraction fibres, actin rich protrusions which have been reported to guide orientation of the metaphase spindle relative to the pattern of adhesions along the long cell axis (Fink et al., 2011; Petridou and Skourides, 2016; Théry et al., 2005). I found no difference in the ability of cells which had 0, 1 or 2 tails to position the spindle at the geometric centre (Figure 4.10A on page 54). A slight trend could be seen towards cells with tails orienting better relative to the long cell axis (Figure 4.10B on page 54). It is possible that increased numbers would reveal a clear difference which may suggest that the presence of tails helps cells to read cues from the substrate to orient their spindle to the former interphase long axis..

4.7. Daughter cells respread symmetrically along cytoplasmic tails

Having correctly positioned and aligned it's spindle, a cell will then undergo anaphase, separating sister chromatids to opposite sides of the cell. The furrow, position defined by the spindle (Bringmann and Hyman, 2005), then forms between the emerging daughter cells, separating them evenly. I wished to understand how leaving a large unretracted cytoplasmic tail might impact division symmetry. Can the cell somehow detect and compensate for the mass left in these tails?

The ideal way to judge this would be to compare the dry mass of each daughter cell just after abscission. However, since abscission can be difficult to detect, other timepoints had to be chosen. I carried out pilot experiments using a phasics camera to measure dry mass. However, for these measurements to be accurate a perfectly blank background must be subtracted, and this, along with other technical difficulties, made this experiment impossible at this time.

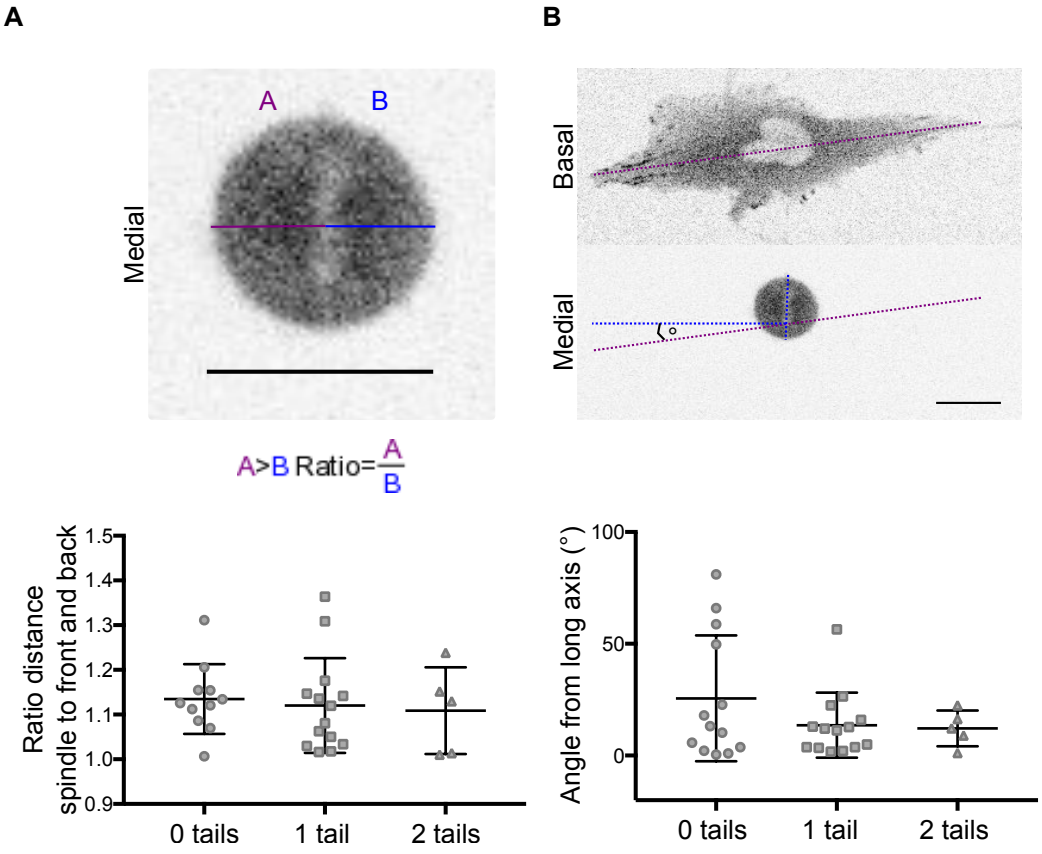

Figure 4.10: The presence of mitotic tails has no impact on spindle position or orientation. (A) Image shows the measurements taken for the graph. 1 middle z-slice. Measurements were taken in the timepoint prior to anaphase. Scale-bar 20 µm. Graph shows the ratio of the distance of the spindle from one edge of the rounded cell to the other, as a measure for how central it was, in cells which left 0, 1 or 2 tails. The ratio was always taken against the larger mesaurement. Mean ± SD. N=10 experiments. **(B)** Images show the measurements taken for the graph. 1 z-slice. Measurements were taken at the timepoint prior to the onset of rounding (magenta), and at the timepoint prior to anaphase (blue). Graph shows the angle off the long axis a cell was, in cells which left 0, 1 or 2 tails. Mean ± SD. N=10 experiments.

Instead, as a proxy for cell size, I measured both middle cell area just after furrow closure, and basal cell area 45 minutes after anaphase when the daughter cells had respread. By getting the ratio of the larger cell to the smaller cell, I could see no difference in division symmetry between cells which left 0, 1 or 2 tails (Figure 4.11 on page 55).

Although more accurate measurements need to be carried out before this can be concluded with certainty, it seems from these measurements that the pres-

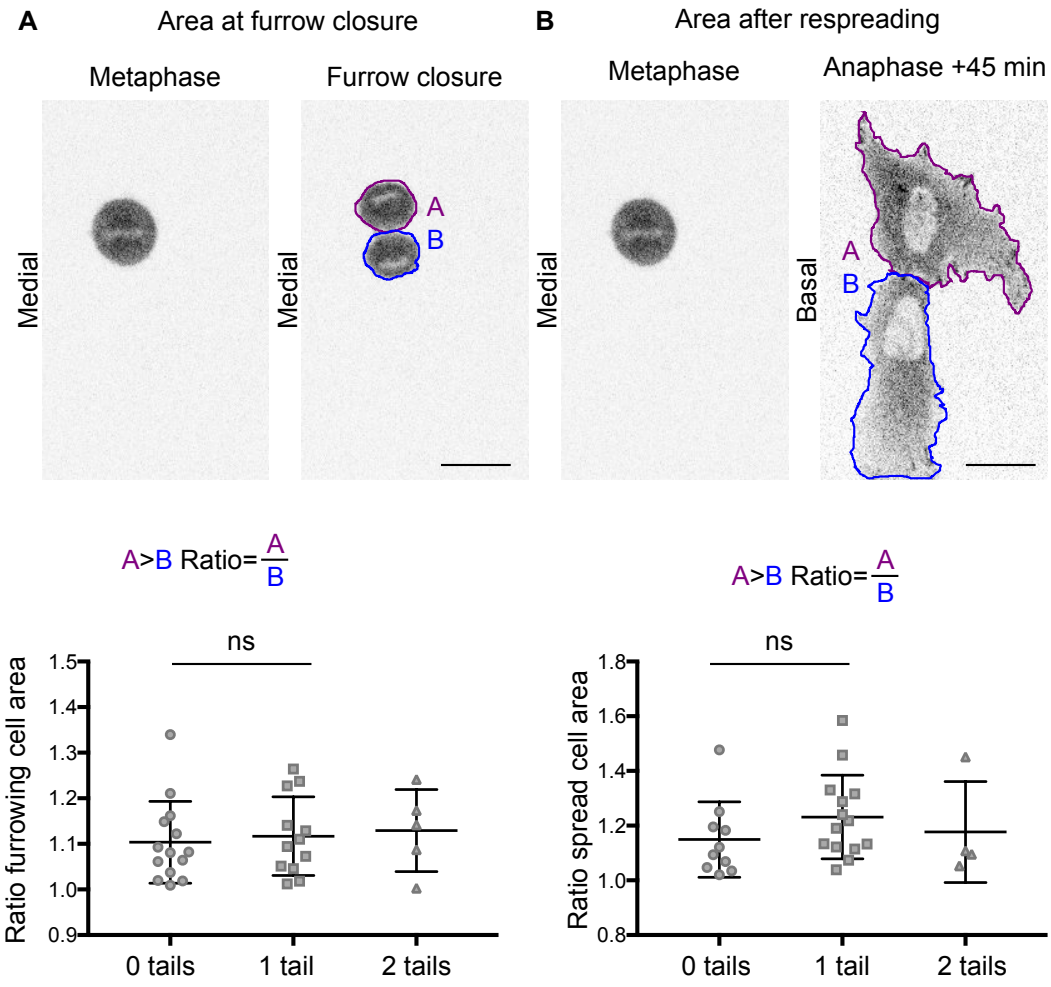

Figure 4.11: The presence of tails does not impact on daughter cell symmetry. **(A)** Images show the measurements taken for the graph. 1 z-stack. Scale-bar 20 µm. Graph shows the ratio of the middle area of daughter cells just after furrow closure, in cells which left 0, 1 or 2 tails. The ratio was always taken against the larger mesaurement. Mean ± SD. N=9 exp experiments. Statistics used two-tailed Mann-Whitney test. **(B)** Images show the measurements taken for the graph. 1 z-stack. Scale-bar 20 µm. Graph shows the ratio of the basal spread area of daughter cells 45 min after anaphase, in cells which left 0, 1 or 2 tails. The ratio was always taken against the larger mesaurement. Mean ± SD. N=7 experiments. Statistics used two-tailed Mann-Whitney test.

ence of a tail does not impact on the symmetry of daughter cell size.

However, as tails bear some similarities to retraction fibres, which have been previously described to act as tracks to guide daughter cell respreading (Cramer and Mitchison, 1993), I decided to look at whether the presence of tails effects daughter cell respreading. Indeed, I found that cells without tails have slower respreading of daughter cells than cells with tails, and in cases where only one daughter cell inherits a tail, that daughter cell will respread faster than its sister (Figure 4.12A on page 57). Similarly, when comparing the time from anaphase to the onset of respreading in any daughter cell which inherits a tail, to those which don't, it can be seen that daughter cells with tails start respreading faster (Figure 4.12B on page 57).

This confirms that the adhesive tails left by RPE1 cells during mitotic rounding, do not seem to have any deleterious effects on daughter cell symmetry. In fact they aid daughter cell respreading.

4.8. Dicussion

In this chapter I have carried out a detailed characterisation of the progress of human RPE1 cells through mitosis.

I show that cells can round incompletely at mitotic entry and leave large cytoplasmic tails. These seem to bear some similarities to the previously described retraction fibres Cramer and Mitchison (1995), as they too can guide daughter cell respreading. They also may play a minor role in spindle orientation, as has been previously described for retraction fibres (Fink et al., 2011). However, they do seem to be distinct from retraction fibres as retraction fibres have always been described as thin actin-rich tubes, and these are generally relatively wide. They are also highly variable; in shape, in the way they are formed,

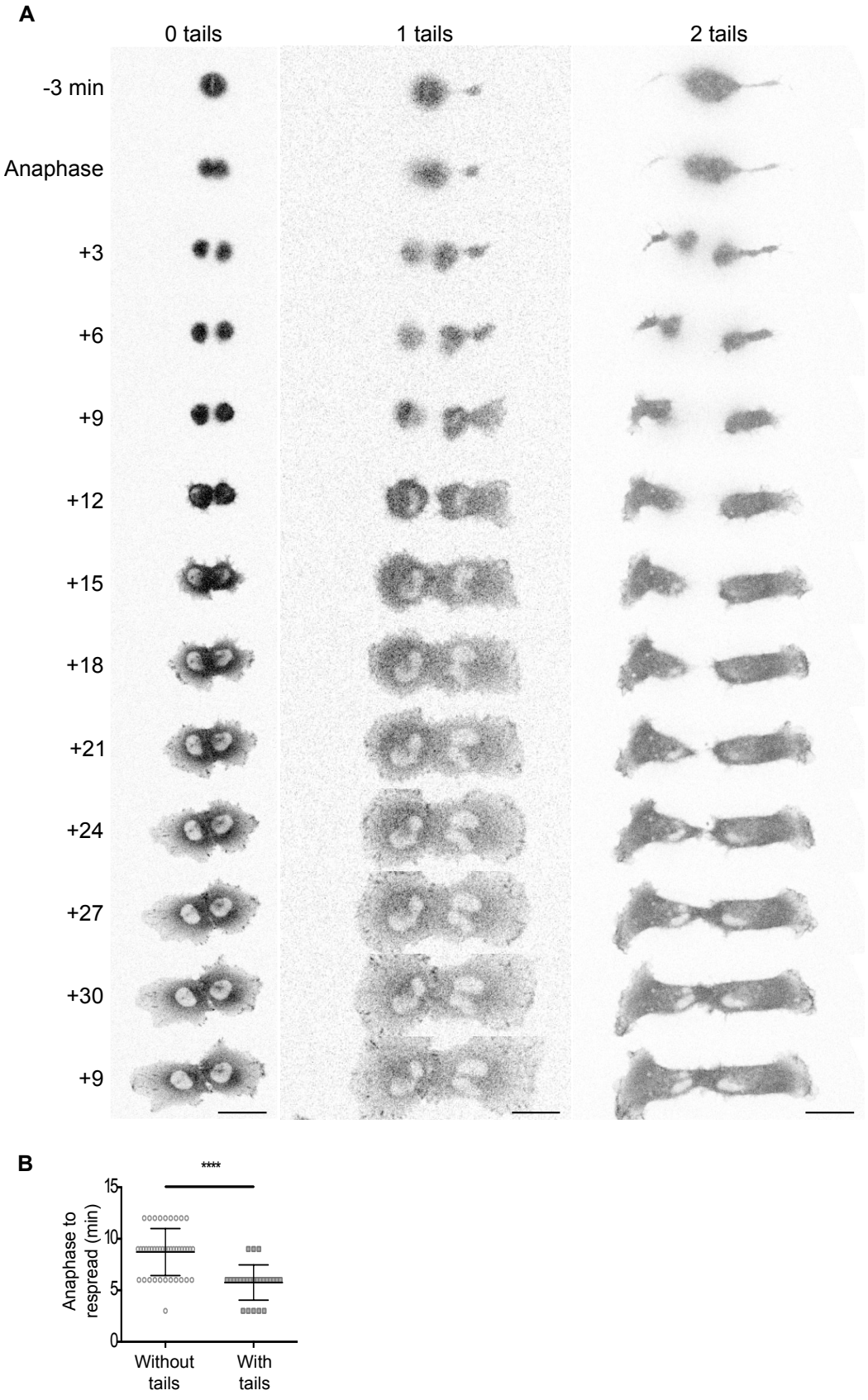

Figure 4.12: The presence of tails causes daughter cells to respread faster. (A) Montages show RPE1 cells stably expressing Zyxin GFP, exiting mitosis with 0, 1 or 2 tails. Time is shown relative to anaphase. 1 basal z-slice. Scale-bar 20 µm. Statistics used two-tailed t test.

(continued) (B) Graph shows the time from anaphase to the onset of respreading in daughter cells which either do or don't inherit a tail. Mean $\pm$ SD. N=6 exp.

and how they are sometimes retracted into the main cell body as it progresses through mitosis.

From this study, I also found that most of these highly motile cells will pause their migration prior to mitosis, and a subset will extend a second leading edge at the rear to become more symmetric. This switch seems to be a consequence of stochastic cell pausing, however it may have the indirect consequence of aiding the cell in equally segregating its components into two daughter cells. It would be easier to do this in a symmetric (bipolar), than an asymmetric (monopolar) cell.

It is not yet clear what causes this difference between these two populations of cells. It may be due to differences in tension between them as Ballestrem et al. (2001) showed that there is low tension in the leading edge and high tension in the tail. This sets up differences in actomyosin and adhesion dynamics, which in turn may effect cell rounding. This could partially explain the differences we see between monopolar and bipolar cells. The dynamics of adhesion remodelling in these two populations of cells will be more extensively studied in the next chapter.

5. Adhesion Remodelling at Mitotic Entry

5.1. Introduction

In the previous chapter I examined the dynamics of cell shape changes during mitotic entry. I found that during the process of mitotic rounding, although cells undergo drastic changes to become more spherical than in interphase, Human RPE1 cells rarely become fully round. Instead, as they undergo mitotic rounding, they tend to leave unretracted tails which lie along the interphase long cell axis. These tails then act as tracks to guide daughter cell re-spreading at mitotic exit.

As mitotic rounding requires the simultaneous formation of a stiff actomyosin cortex (Matthews et al., 2012) and changes in cell-substrate adhesion (Dao et al., 2009), in this chapter I explore how these processes are linked.

Focal adhesions are formed of a layered complex of numerous proteins connecting the ECM to the actin cytoskeleton (Kanchanawong et al., 2010). A previous study from the lab utilised a combination of immunostaining and live imaging in HeLa cells and found that Talin, Vinculin, Paxillin, FAK and pTyrosine (which mark interphase adhesions) puncta present in interphase cells are lost as cells enter mitosis. While previous studies have suggested that Paxillin is degraded in mitosis (Yamaguchi et al., 1997), western blotting against Talin, Vinculin, Paxillin and FAK in HeLa cells which were either synchronised in interphase (S-phase) using a thymidine block, or in mitosis using a thymidine and nocodazole block followed by a mitotic shake-off, suggested that levels of these proteins do not change (N. Heatley, MRes Thesis). This is not surprising as focal adhesions are only lost from the cell-substrate interface for a short

period of time and are rapidly reformed during daughter cell re-spreading. It is difficult to imagine cells being able to re-synthesise the adhesion complex components required for re-spreading as they exit mitosis. These data imply that the changes in adhesion that accompany mitotic entry are regulated downstream of the mitotic kinases. While it is not known how this works, a key fact is that it requires the inactivation of Rap1 (Dao et al., 2009). Thus cells expressing a constitutively active Rap1 cannot release their adhesions in order to round up.

To study the dynamics of adhesion remodelling in this chapter I utilise data generated by imaging an RPE1 cell line I developed early in my PhD which stably expresses Zyxin-GFP. Zyxin is a regulator of actin filament assembly and a component of mature cell-matrix adhesions. By imaging the basal-most part of these cells it is possible to follow the dynamics of the bright Zyxin-GFP puncta, representing focal adhesions. By studying Zyxin-GFP dynamics together with immunostaining for other components of the focal adhesion complex it is possible to build up a general picture of focal adhesion behaviour during mitosis.

5.2. Adhesion puncta are lost gradually

Previous work by Dao et al. (2009) has shown that the inactivation of Rap1 leads to the loss of focal adhesions (visualised using immunostaining for Vinculin) at mitotic entry. To confirm the loss of focal adhesions in my system I used the RPE1 Zyxin-GFP cell line to examine what happens to focal adhesions as cells enter mitosis. Zyxin-GFP puncta were brightest at the cell periphery, where cells are thin, while puncta under the main cell body were partially obscured by the high cytoplasmic GFP signal. By qualitatively assesing when Zyxin-GFP puncta were formed I found that new adhesion formation stopped 11.45 ± 6.1 min before NEP. This is followed by a loss of puncta upon entry into mitosis (Figure 5.1A on page 61). This does not occur all at once, rather, Zyxin-

GFP positive puncta are gradually lost over a period of several minutes as cells begin to round up. The timing of this process relative to NEP, proved to be variable between cells (Figure 5.1B on page 61). This is surprising and is different from many other mitotic events (e.g. microtubule remodelling (Mchedlishvili et al., 2017)). It indicates that NEP does not play a major role in driving adhesion loss during mitotic rounding. Therefore, instead of assesing adhesion loss relative to NEP, I qualitatively assesed when the last Zyxin-GFP puncta was formed and then examined how many puncta dissapeared after this. From this analysis it was clear that all cells eventually lost all their Zyxin-GFP puncta as they entered mitosis (Figure 5.1C and D on page 61).

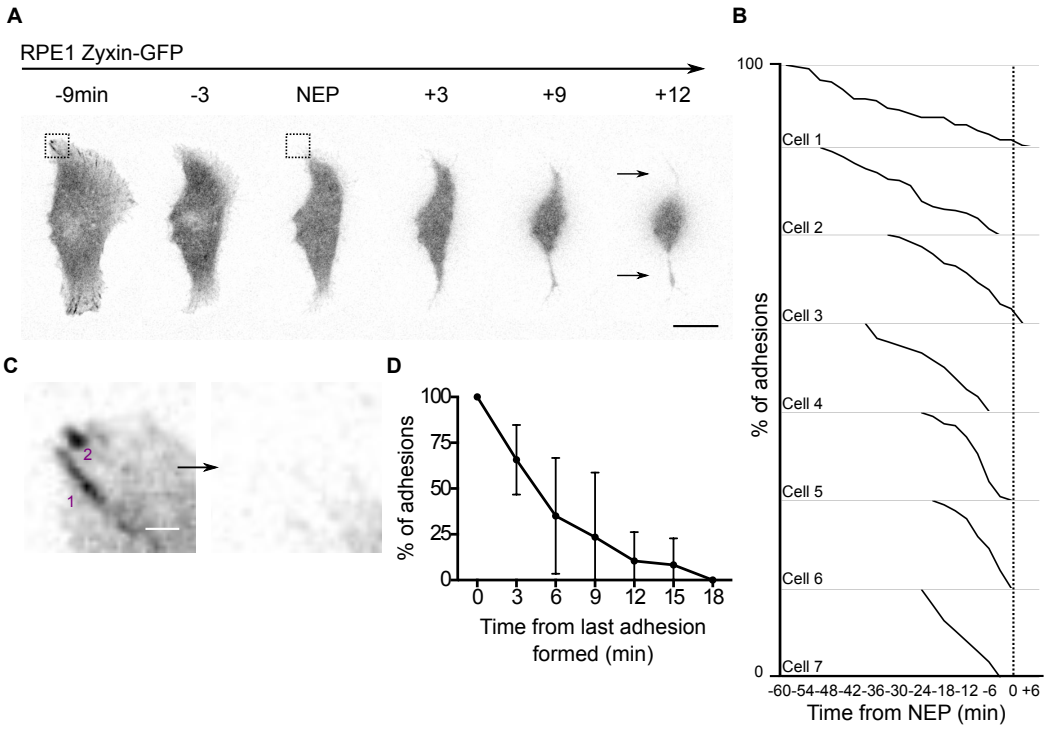

Figure 5.1: All Zyxin-GFP puncta are lost as cells enter mitosis. (A) Image depicts adhesion remodelling in a representative RPE1 cell stably expressing Zyxin-GFP rounding up as it enters mitosis. 1 basal z-slice. Time is shown relative to NEP. Scalebar 20 µm. **(B)** Graphs show adhesion loss relative to NEP in 7 sample cells at mitotic entry. **(C)** Images show representative puncta from boxed regions in (A) which were measured and tracked until lost to generate data for the graph in (D). **(D)** Graph showing loss of Zyxin-positive adhesion sites as cells progress through mitosis. N=10 cells from 7 experiments. Mean ± SD.

Having found that adhesions are gradually lost from cells entering mitosis, I

next examined the behaviour of neighbouring adhesions. By examining three neighbouring adhesions which were formed at approximately the same time, I found that the lifetime and dynamics of neighbouring adhesions was variable, with one adhesion being lost long after it's neighbours (Figure 5.2A and B on page 63). The intensity profiles of these 3 adhesions had a similar shape over time, becoming brighter, reaching a maximum intensity, and then fading before they are lost (Figure 5.2C on page 63). However the maximum intensity was quite variable, even between neighbouring adhesions. These results are slightly surprising, as we would assume that neighbouring adhesions would be subject to similar levels of local biochemical signaling. However, forces are likely to be different. If one adhesion bears little force, its neighbours have to bear more. This may drive asynchrony.

Moreover, there was no clear correlation between puncta intensity and lifetime (Figure 5.3A on page 64). However, the lifetime of adhesion complexes were shorter in cells entering mitosis than those in interphase (Figure 5.3B on page 64).

Thus it is not neccesarily the case that brighter adhesions last longer. Nor is it the case that adhesions formed in the same area at the same time have the same dynamics. Instead our findings suggest that the downregulation of cell-matrix adhesions is the result of global regulation (through the down-regulation of Rap1), and local control, as neighbouring adhesions act independently of one another, perhaps as a result of their being subject to different mechanical inputs. However, in the end all cells lost their Zyxin-GFP puncta.

5.3. Active integrin remains in mitosis, other markers are lost

To determine whether other adhesion proteins are regulated in the same way as Zyxin-GFP, I used immunostaining to examine Paxillin and Integrin. While

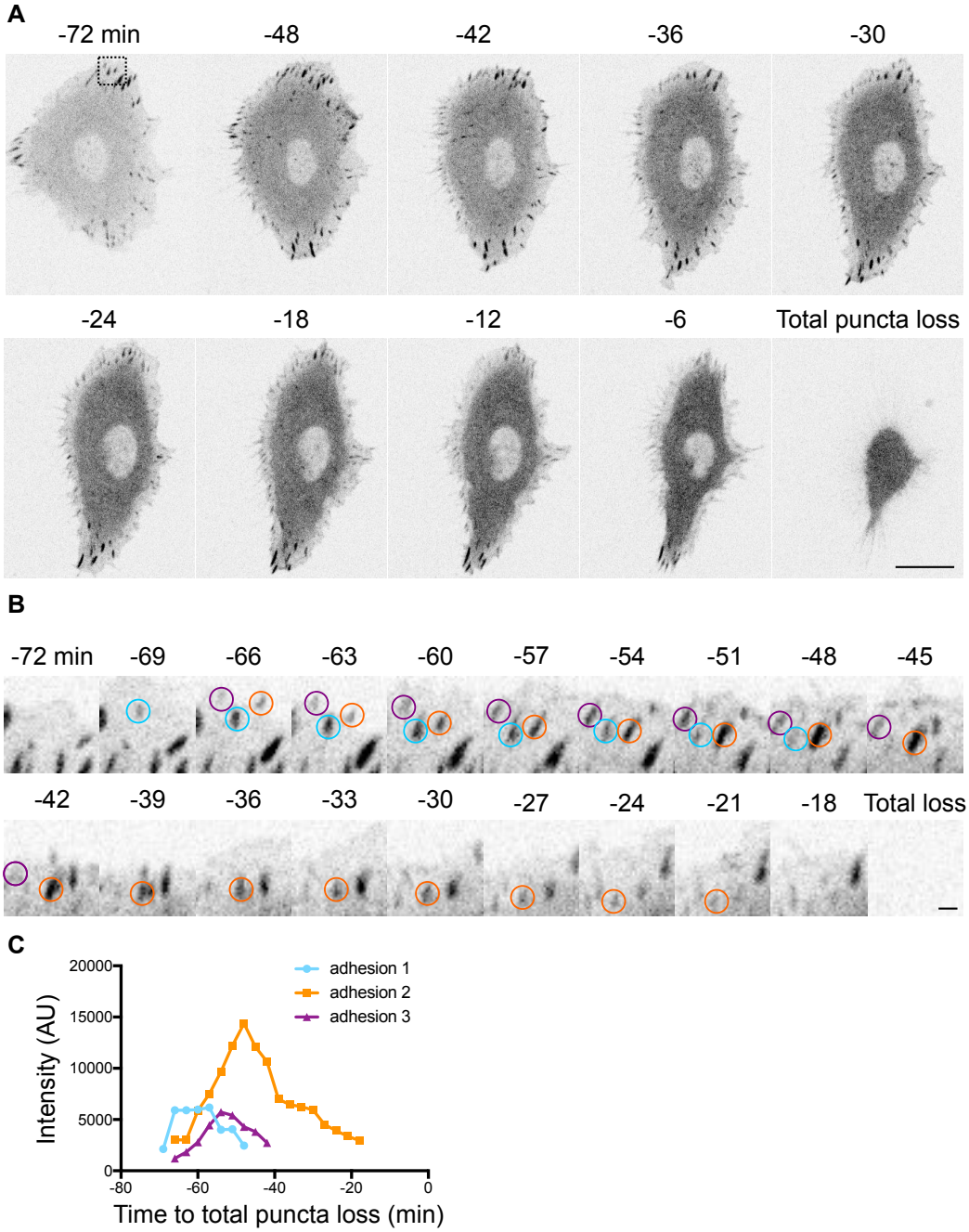

Figure 5.2: Adhesions are lost gradually. (A) Image depicts adhesion remodelling in a representative RPE1 cell stably expressing Zyxin-GFP rounding up as it enters mitosis. 1 basal z-slice. Time is shown relative to when all visible Zyxin-GFP puncta are lost. Scale-bar 20 µm. **(B)** Zoom of boxed region in (A) with 3 neighbouring adhesions higlighted as they are formed and eventually lost. Time is shown relative to when all visible Zyxin-GFP puncta are lost. Scale-bar 2 µm. **(C)** For the 3 neighbouring adhesions depicted in (B), a 2 pixel (0.476x 0.476 µm) width line was drawn along the length of the adhesion for each timepoint from it's birth to it's death, and the mean intensity was measured. The data for each adhesion had the background of a neighouring cytoplasmic region of the same size subtracted. Graph depicts this data.

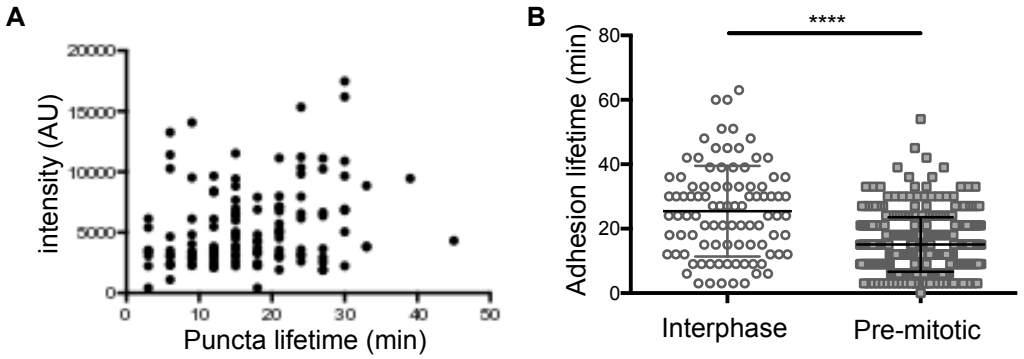

Figure 5.3: Adhesion lifetime decreases in cells about to enter mitosis. (A) Graph plots the maximum intensity of individual adhesions versus their lifetime. N= 3 pre-mitotic cells from 3 experiments. **(B)** Graph depicts the lifetime of adhesions of cells in interphase or just about to enter mitosis. N Interphase= 3 cells from 3 experiments, N Premitotic = 11 cells from 7 experiments. Mean $\pm$ SD. Statistics used two-tailed Mann-Whitney test.

Paxillin puncta were lost from metaphase RPE1 cells. Active β1 Integrin puncta were retained (Figure 5.4 on page 65). This is in line with data from the lab that identified integrin at the tips of retraction fibres in HeLa cells (H.K. Matthews, unpublished data).

To examine how Zyxin-GFP puncta are remodelled to leave integrin-positive adhesions that lack Zyxin and Paxillin upon entry into mitosis, I collaborated with P. Almada. Together we used an automated microfluidics system he designed to carry out online fixation in my Zyxin-GFP cells. We imaged the cells as they entered mitosis and lost all visible Zyxin-GFP puncta, then used this system to carry out PFA fixation and immunostaining at the microscope. As seen in fixed cells, integrin puncta were retained throughout mitosis, forming the outline of the interphase cell shape, connected to the main cell body via actin-rich retraction fibres and the unretracted cytoplasmic tails described in Chapter 3 (Figure 5.5A on page 66). More importantly, active β1 Integrin was found at sites where the Zyxin-GFP puncta had previously been (Figure 5.5B on page 66).

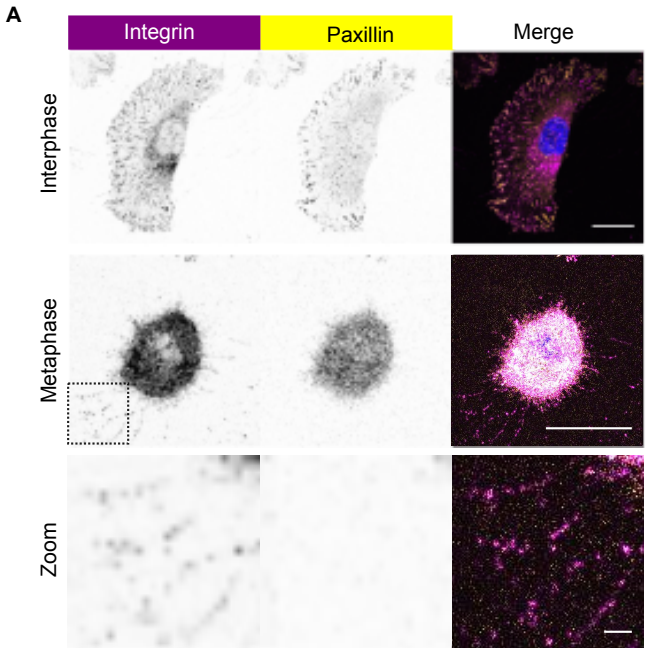

Figure 5.4: Adhesions are remodelled at mitotic entry. (A) Images depict fixed RPE1 cells in interphase and metaphase stained for active β1 Integrin (magenta) and Paxillin (yellow). Merge also shows DAPI (blue). 1 basal z-slice. Scale-bar 20 µm. Zoom of boxed region shows Paxillin loss. Scale-bar 2 µm.

Thus, although substantial adhesion remodelling occurs at mitotic entry, and many focal adhesion proteins such as Zyxin and Paxillin are lost, active integrin puncta remain place. This is surprising, as integrin is usually only thought to function in the context of a complex (Zaidel-Bar et al., 2007). In the future, it will be important to survey the full set of proteins, including Talin. However, it may be that this process is important to prevent cells losing contact with the substrate entirely. Moreover, these mitotic adhesions have been reported to aid spindle orientation to the long axis, as previously described (Petridou and Skourides, 2016; Théry et al., 2005), and to guide daughter cell re-spreading at mitotic exit: a process I examine later in this chapter.

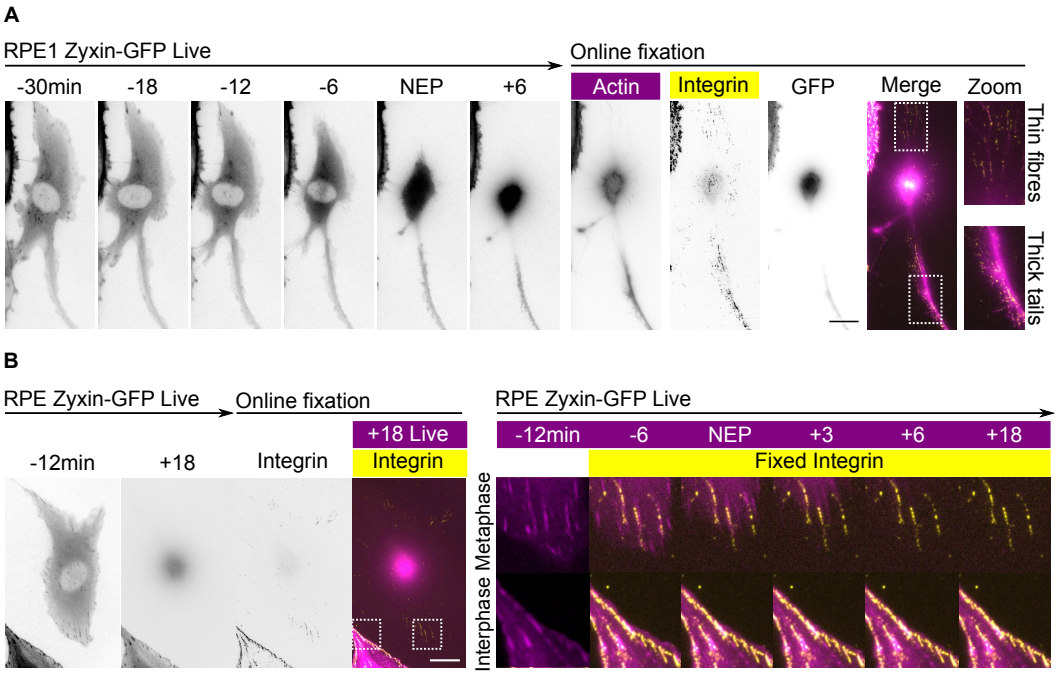

Figure 5.5: Integrin puncta persist throughout mitosis. (A) RPE1 cells stably expressing Zyxin-GFP were released from CDK1 inhibition to synchronise entry into mitosis (see Materials and Methods). Picture shows a representative cell (from a sample of 15 cells from 3 experiments), imaged during rounding, which was fixed in metaphase and stained on the microscope to visualize DNA (cyan), actin (magenta) and integrin (yellow) using DAPI, phalloidin TRITC and active β1 Integrin antibody. Zyxin-GFP remains visible after fixation. 1 basal z-slice. Scale-bar 20 µm. Zooms of boxed regions show thin retraction fibres and an adhesive tail. **(B)** Metaphase and interphase cells which underwent online fixation (as in A). Image shows montage of the boxed region of each cell, overlaying the fixed integrin staining (yellow), with live Zyxin-GFP puncta loss (magenta). 1 basal z-slice. 20 µm.

5.4. Adhesion remodelling is independent of acto-myosin cortex formation

At mitotic entry, adhesions are remodelling as cells are assembling a stiff acto-myosin cortex. Together these events allow efficient mitotic rounding to occur. To determine whether these two events are coupled mechanistically we used Ect2 RNAi (a key regulator for building the cortex (Matthews et al., 2012)) to reduce cortical contractility in cells entering mitosis. RPE1 cells stably expressing LifeAct-RFP were transfected with an Ect2 siRNA for 18 hours prior to imaging, as done previously in the lab (Matthews et al., 2012)). Cells were then

replated and adhesion remodelling was examined as cells progressed through mitosis. We could see efficient knockdown of the protein using this siRNA by both western blot and immunostaining (Figure 5.6A and B on page 68). Using live cell imaging we could see that the actomyosin cortex is disrupted in the Ect2 siRNA cells compared to control siRNA cells, as expected. In control cells actin becomes recruited to the cortex as they round, with very little remaining in the cytoplasm. In contrast, Ect2 siRNA cells have much higher levels of the LifeAct-RFP signal in the cytoplasm, with patchy localisation of actin around the cortex (Figure 5.6C-E on page 68). Without a proper actomyosin cortex to help the cell to round up away from the substrate, Ect2 siRNA cells have a larger spread area than Control siRNA cells when viewed in the basal-most layer (Figure 5.6F on page 68). This confirms previous findings that Ect2 is required for the assembly of a stiff actomyosin cortex (Matthews et al., 2012).

Despite these rounding defects, adhesion remodelling appeared unchanged in Ect2 siRNA treated cells relative to the control. Zyxin GFP puncta were gradually lost as cells rounded up at mitotic entry (Figure 5.7A-C on page 69). In addition to this, immunostaining showed that Paxillin puncta were absent in metaphase Ect2 RNAi cells, while active integrin puncta were retained, as was seen in control cells (Figure 5.7D on page 69). However, a closer inspection should be carried out in case there are more subtle defects.

Together these results show that efficient knockdown of Ect2 compromises cell rounding, but not adhesion remodelling, suggesting that these two processes can be uncoupled during mitotic entry.

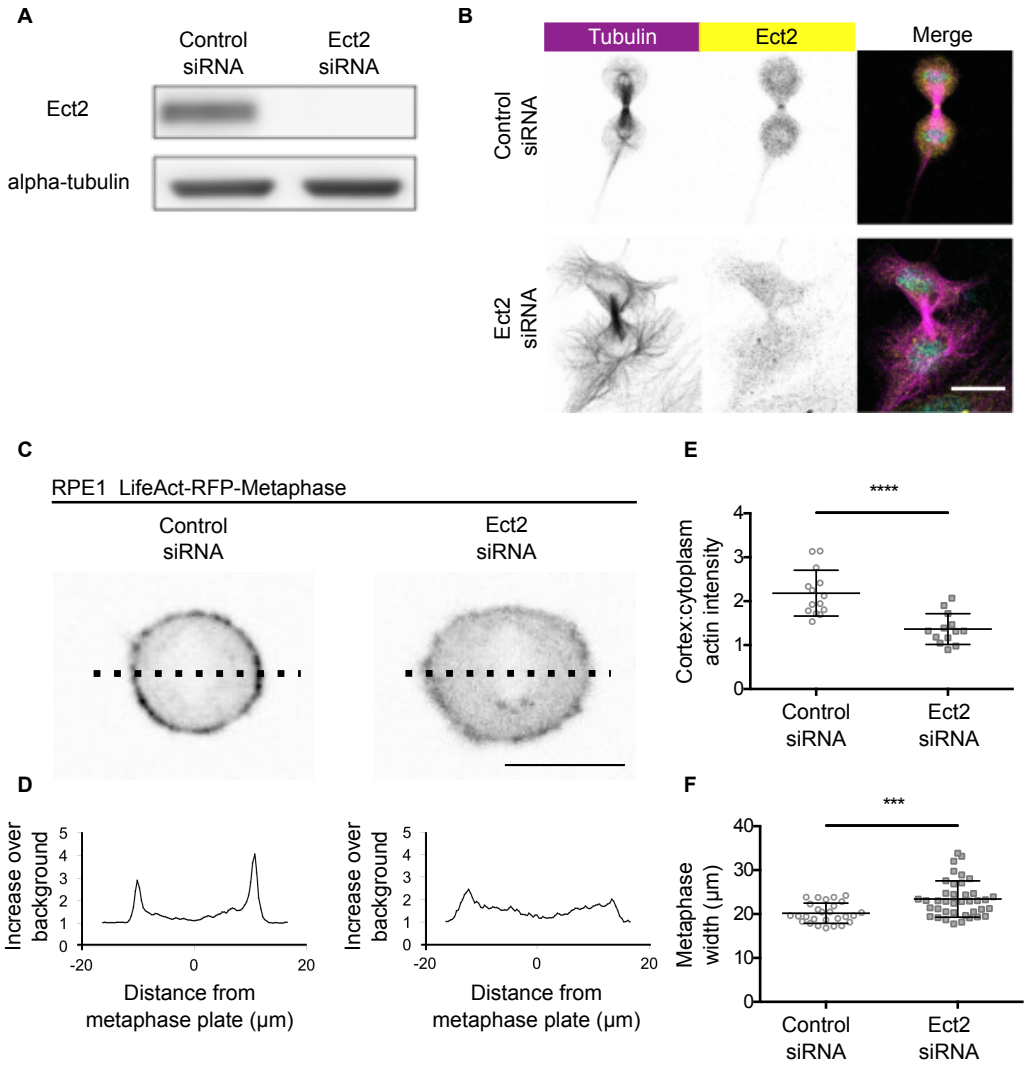

Figure 5.6: Ect2 RNAi gives efficient knockdown of the protein which results in rounding defects. **(A)** Western blot showing the extent of Ect2 silencing in RPE1 cells. α-Tubulin antibody was used as a loading control. Representative of 2 experiments. **(B)** Images depict fixed Control siRNA and Ect2 siRNA RPE1 cells stained for α-Tubulin (magenta) and Ect2 (yellow). The merge also shows DAPI (cyan). 1 middle z-slice. Scale-bar 20 µm. **(C)** Images show Control siRNA and Ect2 siRNA treated RPE1 metaphase cells stably expressing LifeAct-RFP. 1 middle z-slice. Scale-bar 20 µm. Dotted line shows measurement taken for (D). **(D)** Line-graph showing the actin distribution in the middle plane of sample cells. Data have been normalised to background. **(E)** Graph showing the ratio of actin intensity at the cortex compared to the cytoplasm in Control siRNA and Ect2 siRNA cells. For each cell, four square regions around the cortex were averaged and compared to the average of four square regions around the cytoplasm to create one data-point. N=2 experiments. Mean $\pm$ SD. Statistics used two-tailed t test. **(F)** Graph showing metaphase width of Control siRNA and Ect2 siRNA RPE1 cells. N=3 experiments. Mean $\pm$ SD. Statistics used two-tailed Mann-Whitney test.

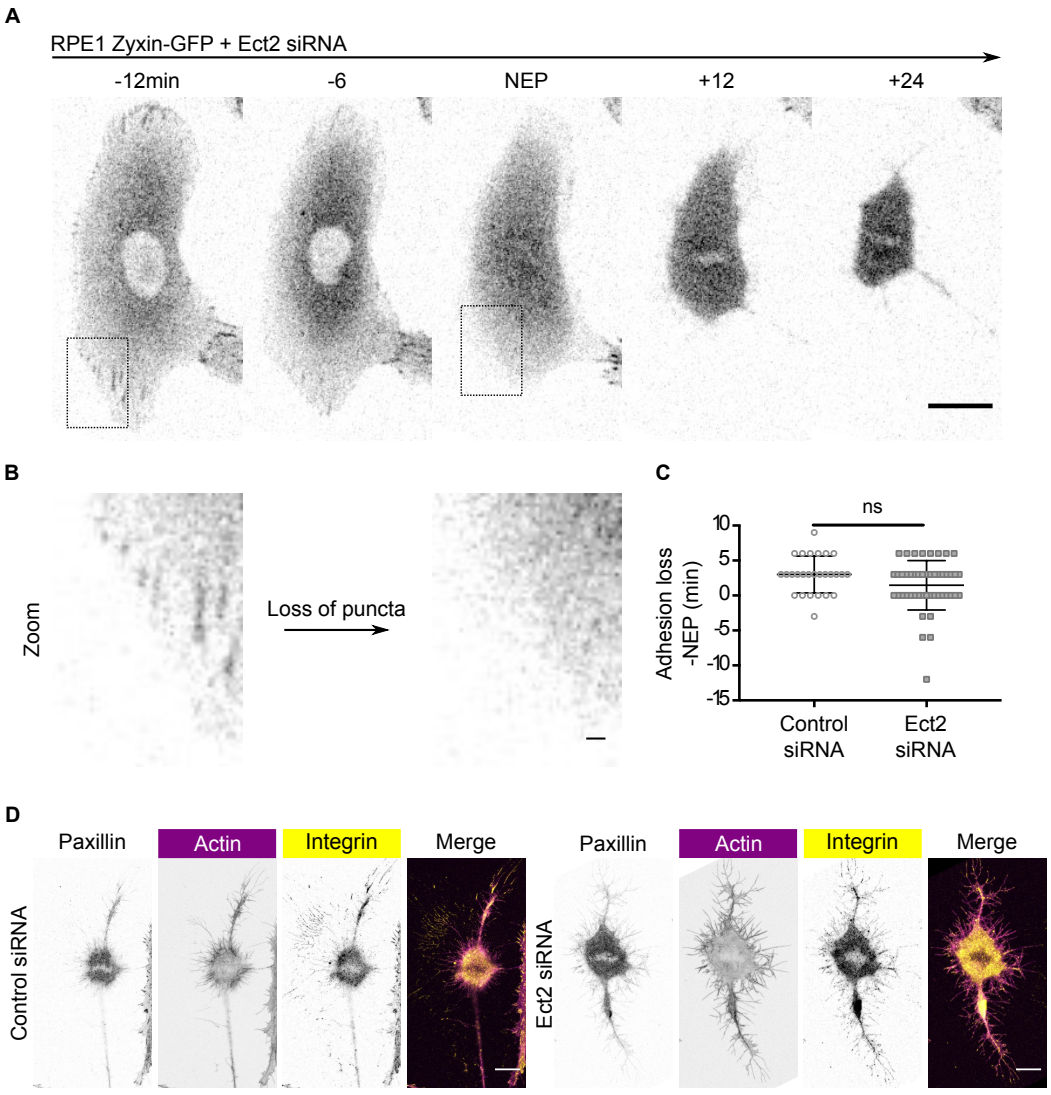

Figure 5.7: Adhesion remodelling occurs independently of cortical contraction during mitotic rounding. **(A)** Montage showing a representative RPE1 Zyxin-GFP cell treated with Ect2 siRNA entering mitosis. Scale-bar 20 µm. **(B)** Zoom of boxed regions at -12min and NEP in (A) shows loss of puncta. Scale-bar 2 µm. **(C)** Graph showing the time from when the last Zyxin-positive puncta disappears to NEP in Control and Ect2 siRNA cells. N=3 experiments. Mean ± SD. Statistics used two-tailed Mann-Whitney test.**(D)** Images show fixed Control siRNA and Ect2 siRNA cells stained with phalloidin TRITC (magenta) and Integrin β1 antibody (yellow). 1 basal z-slice. Scale-bar 20 µm.

5.5. Adhesion at the front versus the rear of migrating cells entering mitosis

In the previous chapter (Section 4.3 on page 40), I established that RPE1 cells can enter mitosis in one of two ways. Being migratory, in interphase they have a clear front with a lamellipodia, and a clear retracting rear that trails behind. However, prior to mitosis many of these cells stop migrating. A subset (48%) then extend a second lamellipodia at the rear, causing them to assume symmetric and bipolar geometry. I wanted to compare the behaviour of adhesions in cells which undergo this polarity switch and compare them to those which don't, in order to see how this might influence other events in mitosis.

It has previously been shown that adhesions at the front of a migrating cell behave differently to those at the rear. At the front of the cell, new adhesions are formed, and remain in place as the leading edge of the cell moves past them, until they eventually disappear. At the rear of the cell, adhesions are pulled along behind the cell as it moves (Ballestrem et al., 2001). This type of adhesion slippage can seen by tracking the position of Zyxin-GFP puncta over time to calculate their average displacement.

In interphase, migratory RPE1 cells plated on a standard fibronectin coating, adhesions formed at the leading edge tended to remained in place with respect to the substrate as cells moved. They were then either disassembled (seen as a gradual fading of the GFP signal), or became obscured by the cytoplasmic signal. In contrast to this, the adhesions observed at the rear of the cell were found to slide along the substrate as the cell pulls away from them (Figure 5.8A, top panel, and C on page 72). Interestingly, many of these adhesions tended to be formed in the middle of the cell either at the periphery or under the cell body, before becoming incorporated into the trailing edge as the cell moved

over them. A slight difference in adhesion lifetime could also be seen between the front and rear of migrating RPE (Figure 5.8B on page 72).

We could also see a similar difference in the displacement of Zyxin-GFP puncta at the front and rear when we looked at monopolar cells about to enter mitosis. The adhesions at the front moved less than the adhesions at the back, although there was no difference in adhesion lifetime (Figure 5.8A, middle panel, B and C, on page 72). However, when we looked at bipolar cells about to enter mitosis, there was no difference in the puncta displacement or puncta lifetime between the adhesions at the former front and former rear of the cell (Figure 5.8A, bottom panel, B and C, on page 72). These results show that cells which undergo a polarity switch prior to entry into mitosis become symmetric as far as lamellipodia extension and adhesion behaviour is concerned.

5.6. Zyxin-GFP puncta return

Having exmined the dynamics of adhesion remodelling at mitotic entry, I next wished to look at mitotic exit. By following RPE1 stably expressing Zyxin-GFP as they underwent anaphase and began to re-spread, Zyxin-positive puncta can be seen to return to the daughter cells along the lamellipodia first and then around the cell body as they re-spread (Figure 5.9A, top panel, on page 73). This occurs regardless of whether a cell has left cytoplasmic tails or not (Figure 5.9B, top panel, on page 73). It can also be seen that daughter cells tend to re-spread into the pattern of the cell just before it entered mitosis (Reviewed in Théry and Bornens (2006)). This is particularly noticable in bipolar cells which have left adhesive tails (Figure 5.9A and B, bottom panels, on page 73).

In addition to this, by using the online fixation technique as previously described, in a cell that had just started to re-spread and form new Zyxin-positive puncta, we could see that these puncta were occuring in the same positions

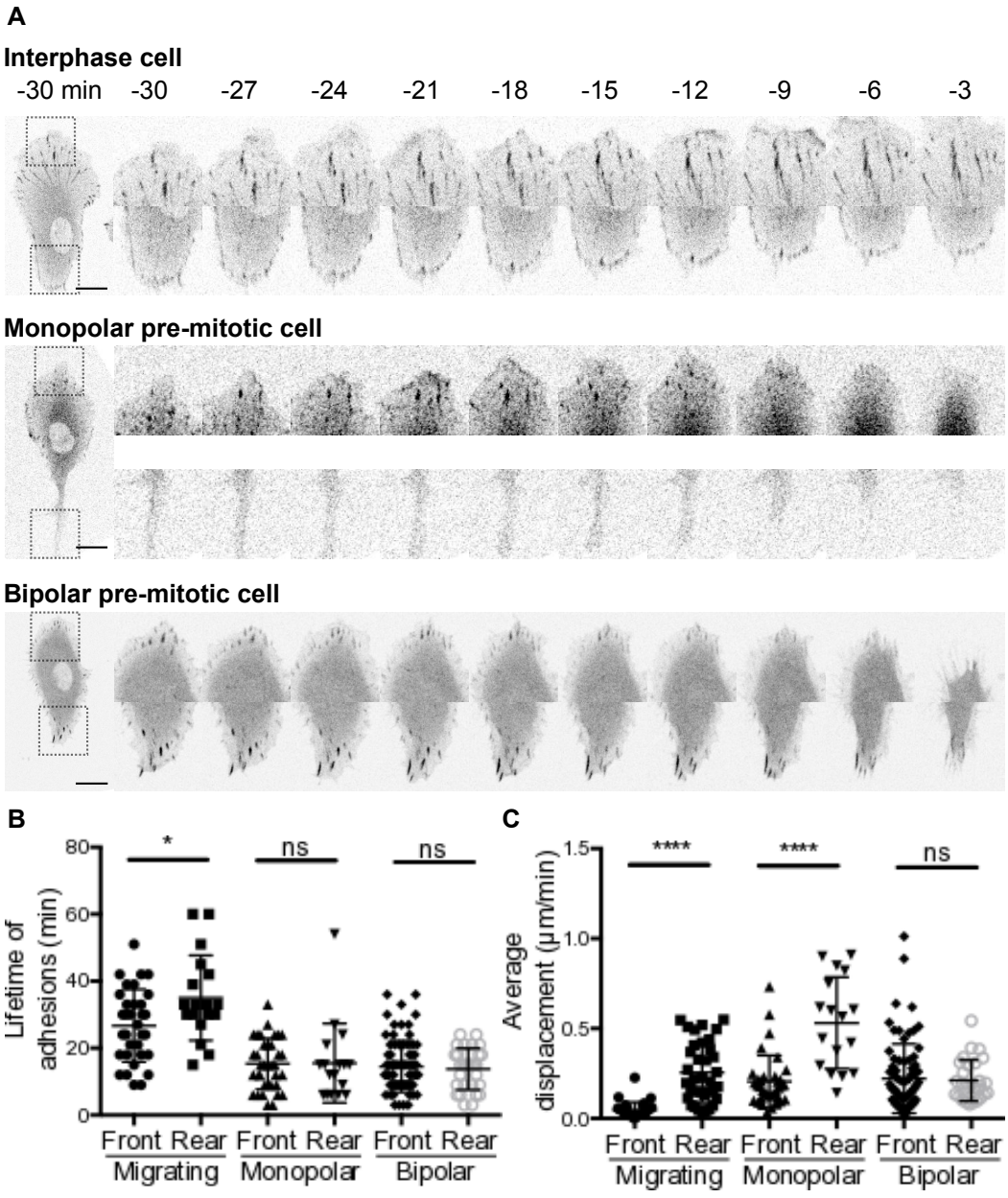

Figure 5.8: Differences in the symmetry of adhesion dynamics between monopolar and bipolar cells. (A) Montages show zooms of the boxed regions at the front and rear of representative migratory interphase, monopolar pre-mitotic and bipolar pre-mitotic cells stably expressing Zyxin GFP. Time is shown relative to NEP for pre-mitotic cells. 1 basal z-slice. Scale-bar 20 µm. **(B)** Graph shows the lifetime of adhesions at the front and rear of the cell, for migratory interphase cells, monopolar pre-mitotic and bipolar pre-mitotic cells. Mean $\pm$ SD. N Migrating= 3 cells from 3 experiments , N monopolar = 5 cells from 2 experiments, N bipolar = 6 cells from 5 experiments. Statistics used two-tailed Mann-Whitney test. **(C)** Graph shows the average displacement of adhesions at the front and rear of the cell, for migratory interphase cells, monopolar pre-mitotic and bipolar pre-mitotic cells. Mean $\pm$ SD. N Migrating= 3 cells from 3 experiments , N monopolar = 5 cells from 2 experiments, N bipolar = 6 cells from 5 experiments. Statistics used two-tailed Mann-Whitney test.

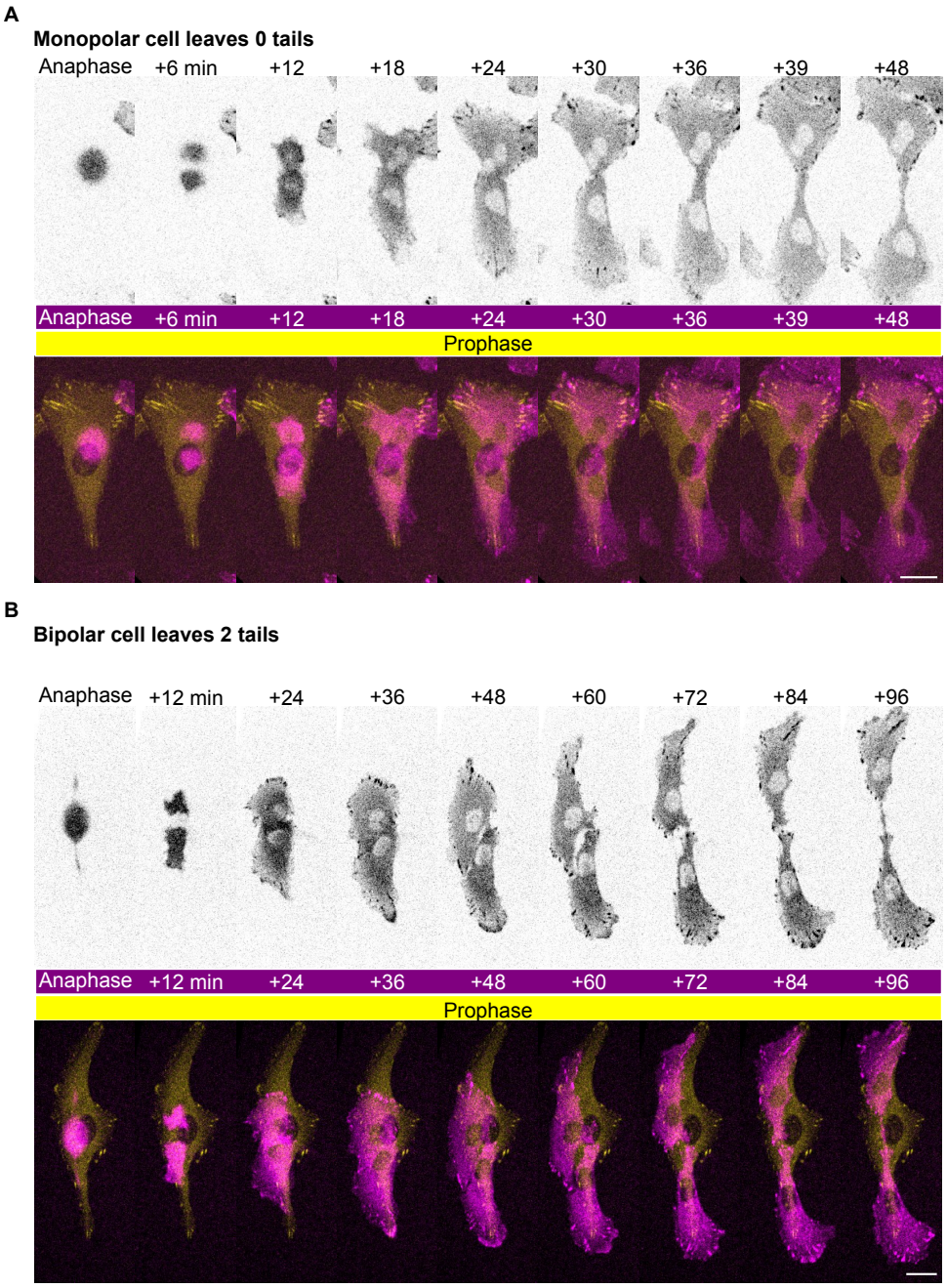

Figure 5.9: Zyxin-GFP positive puncta return to the cell at mitotic exit as daughter cells re-spread. (A) Montage shows a representative monopolar cell which left no tails re-spreading during mitotic exit. The prophase timepoint (yellow) overlays each timepoint as the cell exits mitosis (magenta) shown relative to anaphase. 1 basal z-stack. Scale-bar 20 µm. **(B)** Montage shows a representative bipolar cell which left two tails re-spreading during mitotic exit. The prophase timepoint (yellow) overlays each timepoint as the cell exits mitosis (magenta) shown relative to anaphase. 1 basal z-stack. Scale-bar 20 µm.

as integrin-positive puncta (Figure 5.10 on page 74); implying that Zyxin is re-cruited to the same sites it left during mitotic entry. However, as there are so many integrin puncta and so few Zyxin puncta in this cell, it would be better investigated in a cell which was slightly more respread, to better examine the co-localisation of integrin and Zyxin. Unfortunately, I did not manage to capture any later cells.

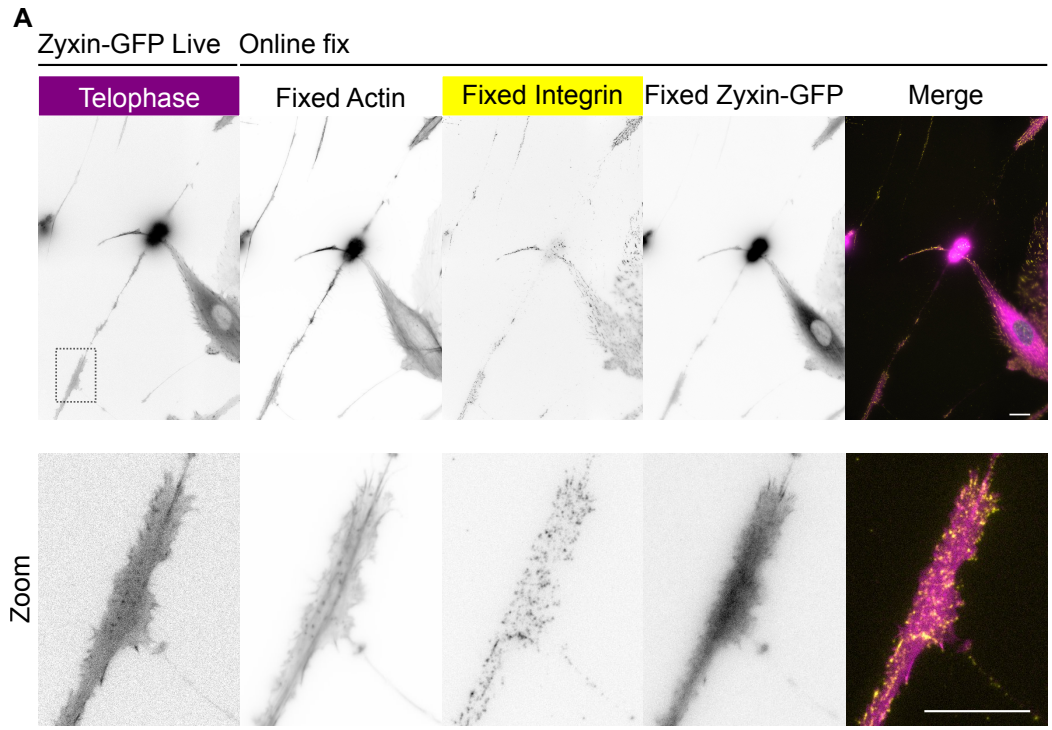

Figure 5.10: Integrin puncta are present at sites where Zyxin-GFP returns at telophase. (A) Images show an RPE1 cell stably expressing Zyxin-GFP which underwent the online fixation protocol (as in Figure 5.5 on page 66) as it was exiting mitosis and just begining to re-spread (magenta in merge). It was stained with Dapi (blue), phalloidin TRITC and Integrin β1 (yellow) antibody. The plasmid expressed Zyxin-GFP was also still visible after fixation. Zoom shows a region of the cell where Zyxin-GFP positive puncta have begun to return as it re-spreads. 1 basal z-slice. Scale-bar 20 µm

This suggests that the integrin positive puncta which are retained during mitosis, act as a memory of the former adhesive pattern of the cell which guides reformation of focal adhesions and daughter cell re-spreading.

5.7. Discussion

In this chapter I examined the interplay between the drastic shape changes and the adhesion remodelling that occur during mitotic entry and mitotic exit.

I confirmed previous findings by ours and other labs that many adhesion proteins are lost at mitotic entry and built on this further with the finding that Zyxin-GFP puncta were lost gradually as the cell rounds, and this could be uncoupled from the formation of a robust actomyosin cortex. The asymmetric nature of rounding and adhesion loss may imply that the dismantling of cell-matrix adhesions is not simply governed by a global switch that turns them all off at the same time. In addition to this, feedback in the form of tension on the adhesions may play a role in regulating their gradual dissasembly. Riveline et al. (2001) found that individual puncta in interphase cells could act as mechanosensors, and applying external force using a micropipette could mature them into stronger focal contacts. This may go some way towards explaining the finding that neighbouring adhesions can be lost at different times. It is possible that when an adhesion is lost, there is an increase in local tension leading to more pulling force on the remaining adhesions, causing an increase in adhesion proteins so the neighbouring adhesion lives slightly longer.

Although I could see this loss of Zyxin-GFP and Paxillin, I found that integrin puncta remain. Moreover, these puncta were retained at sites that appear to attach cells to the substrate through either actin-rich retraction fibres, or through larger, long tail-like structures. The retention of these structures, suggests a need for the cell to remain attached during the crucial process of mitosis. I shall examine this requirement further in Results Chapter 3 (Sections 6.2 and 6.3).

Interestingly, the polarity switch described in Results Chapter 1, could be reiter-

ated by the symmetry of adhesion behaviour at the front and rear of cells which switch compared to those which don't. Differences in adhesion displacement have been studied by Ballestrem et al. (2001) in motile B16 F1 melanoma cells and non-motile 3T3 fibro- blasts. They found that low tension, Rac1 and Cdc42 signaling at the leading edge led to the formation of small stationary adhesions with low αVβ3-integrin density, whereas high tension and RhoA signaling in the rear led to the formation of large, sliding adhesions with high αVβ3-integrin intensity. Considering the importance of adhesion dynamics, specifically the downregulation of adhesions, on the rounding process, this stark difference between monopolar and bipolar cells could have implications on the overall mitotic process.

Finally, I examined the nature of adhesion remodelling at mitotic exit. Having seen active integrin-positive puncta at sites where Zyxin-GFP puncta had dissapeared at mitotic entry, I could also see Zyxin-GFP puncta returning to integrin-positive puncta at mitotic exit. This provides further support for the hypothesis that the integrin puncta act as a memory of interphase cell shape and adhesive pattern, which may help guide re-spreading of the daughter cells. The impact of this shall be further examined in Chapter 3.

6. Adhesion is a requirement for division

6.1. Introduction

In the previous 2 chapters I have established that as RPE1 cells enter mitosis they leave substantial adhesive contacts to the substrate. Although extensive remodelling of focal adhesions occurs, active integrin-positive puncta remain, acting both as a memory of interphase shape, and as tracks to guide daughter cell re-spreading. In this chapter I shall further explore mitotic exit, and how adhesion to the substrate influences it.

Preliminary findings from a previous study in the lab by H. K. Matthews suggest that adhesion is an essential requirement for succesful cytokinesis in RPE1 cells. She removed cells from the substrate using trypsin-EDTA and plated individual cells into wells which were either coated with fibronectin, so the cells could form cell-substrate adhesions, or coated with PLL-Peg, forcing the cells to remain in suspension. She found that interphase RPE1 cells underwent cell cycle arrest in suspension. However, a proportion of these cells, presumably those at the G2/M boundary, entered mitosis. Strikingly, the cells which entered mitosis in suspension underwent mitotic exit and furrow formation, but failed to undergo abscission; leading to division failure. This compares to a division failure rate of 11.5% for RPE1 cells plated in fibronectin coated wells. By contrast, the majority of HeLa were able to divide in suspension under the same conditions (19/25 suspension HeLa cells completed a succesful division). In line with this, there are HeLa cells (S3) that can be propagated in suspension culture (Puck et al., 1955). These data suggest that adhesion is a vital requirement for successful division in non-transformed cells, but may not be essential for division in transformed cells.

To further study the influence of cell-substrate adhesion during mitotic exit, in this chapter we use microfabrication techiniques to manipulate the cell environment, and RNAi and small molecules to manipulate cell behaviour.

6.2. Cells require adhesion at mitotic exit for succesful division

Previous studies found a requirement for adhesion during division in several mammalian cell types (Ben-Ze'ev and Raz, 1981; Thullberg et al., 2007). I wished to confirm H.K. Matthews preliminary experiments that this was also the case in RPE1 cells. Due to technical difficulties with imaging mitotic cells in suspension, I instead decided to fix cells which had been kept in suspension and count bi-nucleate cells as a readout of division failure. For this analysis, cells were plated into 96-well Ultra-low attachment plates for 3.5 hours before being PFA-fixed and spun down onto CellTak coated coverslips. As the cell cycle time for RPE1 cells is approximately 22 hours, it would have been preferable to leave them in suspension for longer before fixing to allow more cells to go through mitosis. However, after 3.5 hours cells began to clump together into aggregates which could not be broken up and these made it impossible to get an accurate count of the proportion of bi-nucleate cells in a population. Moreover, as stated above, H.K. Matthews work suggested that RPE1 cells won't enter mitosis unless they are already in late G2. These fixed and permeabilised cells were then stained with Dapi and an anti-α-tubulin antibody in order to identify bi-nucleate cells (Figure 6.1A on page 79). Although many cells likely arrested in G1 and G2 in the suspension condition, I observed a significant increase in the number of bi-nucleate cells in the suspension culture compared to cells plated in control fibronectin wells (Figure 6.1B on page 79). This confirms the finding by H.K. Matthews that adhesion is essential for division in RPE1 cells.

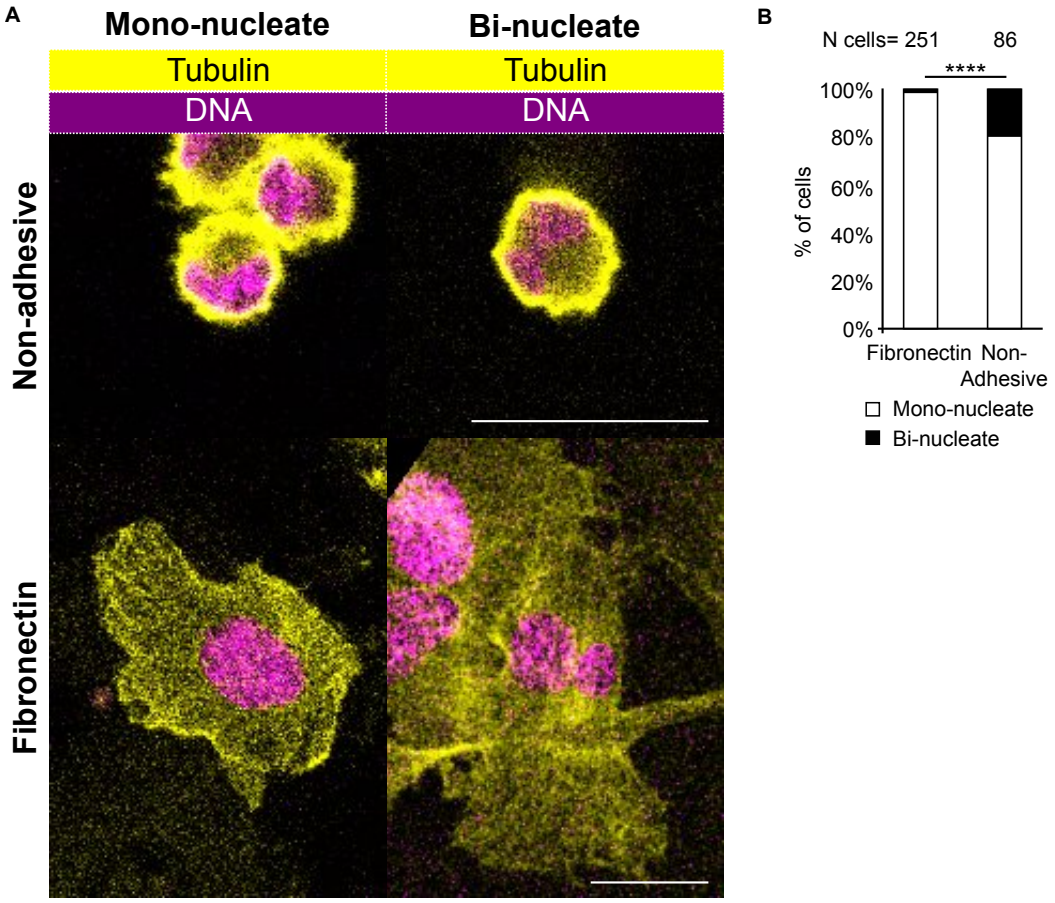

Figure 6.1: RPE1 cells in suspension tend to become bi-nucleate. (A) Images show DNA (magenta) and tubulin (yellow) staining in representative mono-nucleate and bi-nucleate cells which were either kept in fibronectin coated dishes, or in a 96 well non-adhesive plate where they were forced to remain in suspension for 3.5 hours before fixation. 1 z-stack. Scale-bar 20 µm. **(B)** Graph depicts the percentage of cells which were mono-nucleate or bi-nucleate when kept on fibronectin or in suspension. N=1 experiment. Statistics used two-sided Chi-squared test.

Having confirmed that adhesion is vital for division in RPE1 cells, I next wished to examine how much adhesion was required. To test this, micro-patterns were used to limit the adhesive area available to cells. Due to technical difficulties leading to a low number of mitotic cells imaged per experiment, this required a high number of repeats to get sufficient cell numbers to draw conclusions. Therefore I got help with this experiment from two visiting summer students, S. McLaren and L. Wolf.

The sizes of the patterns used were derived from pilot experiments and from

measuremements of the spread area of cells just prior to mitotic entry. The larger patterns (2800 μm^2) were designed to be slightly larger than the basal area of most interphase cells to enable cells to spread without being constrained. The smaller patterns confined cells to an area 4 times smaller than this (700 μm^2). As a result, cells entering mitosis were unable to leave long adhesive tails like those described in Section 4.4 (on page 46). Even smaller patterns of 300 μm^2 were also designed, however cells failed to even adhere to these patterns so they were abandoned. Two different pattern geometries were designed for each area; a circular shape that lacks external polarity, and an elongated elliptical shape (1:2.5 minor:major axis) that provides cells with a clear long axis.

Interestingly, although cells were able to divide on all the patterns, there was a slight, but significant increase in the total time in mitosis in cells plated on small circular micro-patterns, as compared to cells plated on the larger or more elongated patterns (Figure 6.2 on page 81). This delay in mitosis seems unlikely to be due to a delay in rounding time, as the cells on small patterns have a smaller interphase area and therefore should round faster. Instead the delay may be due to problems forming the spindle correctly and therefore activating the Spindle Assembly Checkpoint (SAC) (Minshull et al., 1994), although the mechanism by which this happens is not yet clear.

To determine whether adhesion is required for division as well as for timely mitotic progression, I carried out a different experiment. In this case, I replated a suspension cutlure of mitotic STLC (S-Trityl-L-cysteine)-treated cells (prevents cell cycle progression by inhibiting both separation of the duplicated centrosomes and bipolar spindle formation (Skoufias et al., 2006)) onto an adherent fibronectin coated substrate and tested them for their ability to divide as they exited mitosis after STLC washout. 86.5% of the cells that exited mitosis under

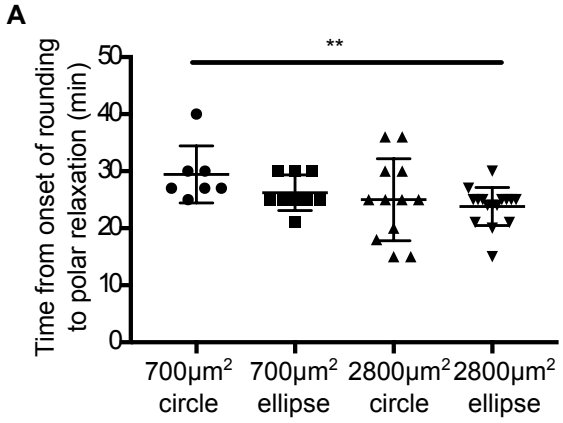

Figure 6.2: Minimal adhesion is sufficient for division but leads to an increase in total mitotic time. (A) Graph showing the time from the onset of rounding to when daughter cells undergo polar relaxation at mitotic exit. Mean ± SD. N=6 experiments. Statistics used two-tailed Mann-Whitney test.

these conditions succeeded in division (N=163 cells from 1 experiment) (Figure 6.3 on page 81). This shows that adhesion at mitotic exit is sufficient for successful division in control RPE1 cells (see Figure 6.14 on page 97 below for the same analysis of cells with a compromised cortex).

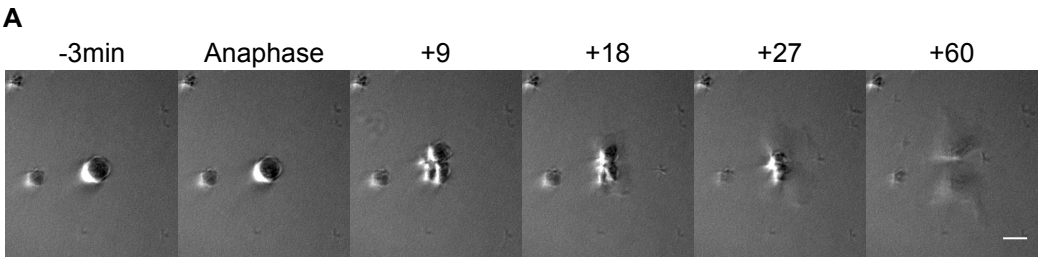

Figure 6.3: Cells require adhesion at mitotic exit for succesful division. (A) Bright-field montage of a cell undergoing mitotic exit after replating from suspension. Time is shown relative to anaphase. Wide-field image. Scale-bar 20 μm

6.3. Adhesion is not required under the furrow for successful division

Having shown that RPE1 cells require adhesion at mitotic exit to divide, we wished to know $where$ this adhesion is required. This was important as a previous study in NCI-H460 lung cancer cells found that adhesion was required

under the furrow at mitotic exit (Pellinen et al., 2008). For this analysis I worked with a student L. Wolf. Cells were plated on micro-patterns with a non-adhesive hole in the centre. On these patterns many cells divided along the edge of the pattern so that they fully remained on the adherent part of the pattern. However, I focused my analysis on the few cells which divided accross the centre of the pattern so that they developed a central furrow above the non-adherent part of the pattern. 11/11 cells that formed a furrow over the non-adhesive hole successfully completed division (Figure 6.4A on page 83). In addition to this, I noted that in cells on normal fibronectin substrate, the central part of the cell appeared to lift up from the basal plane as the cytokinetic ring constricted (Figure 6.4B on page 83), implying that it is not tightly connected to the substrate. Together these results confirm that adhesion is not required under the furrow for successful division in RPE1 cells. Therefore, in order to divide, it is probably sufficient for RPE1 cells to adhere to the substrate at their poles as they exit mitosis.

6.4. Tails rescue Ect2-independent division

Most work on cell division thus far has focused on the role of the actomyosin ring. Therefore, having shown that adhesion (at the cell periphery) is a vital requirement for efficient division in RPE1 cells, I wished to examine whether adhesion is sufficient to enable division in the absense of an actomyosin ring.

For this experiment I used Ect2 RNAi. Ect2 is a Rho GEF required for the formation of a stiff actomyosin cortex at mitotic entry (Matthews et al., 2012), as described in Results Chapter 2 (Figure 5.6 on page 68), and for the formation of the contractile actomyosin ring at mitotic exit (Kimura et al., 2000; Tatsumoto et al., 1999; Yüce et al., 2005). First I confirmed that Ect2 siRNA was sufficient to induce a significant reduction in the levels of Ect2 proteins via western blotting, leading to a defect in mitotic rounding in RPE1 (Figure 5.6 on page 68),

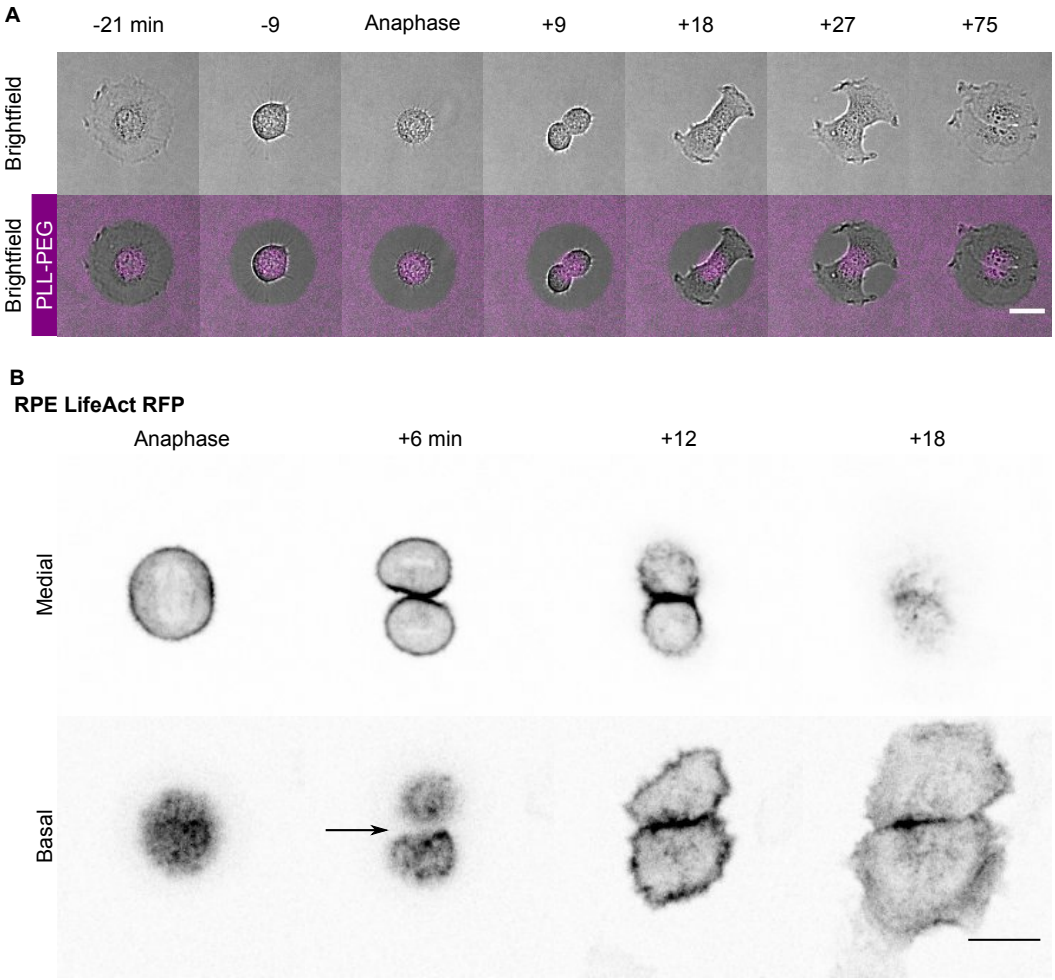

Figure 6.4: Adhesion is not required under the furrow for successful division. **(A)** Montage showing a representative RPE1 cell imaged by L. Wolf on fibronectin patterns with a non-adhesive hole in the centre. Time is shown relative to anaphase. Wide-field image. Merge shows the cell overlaid on PLL-Peg which is excluded from the fibronectin pattern. 11/11 cells from 3 experiments succeeded in dividing across a non-adhesive hole. Scale-bar 20 µm. **(B)** Montage shows a representative RPE1 cell expressing LifeAct RFP undergoing mitotic exit and lifting its furrow (arrow) off the substrate. Time is shown relative to anaphase. 1 middle and 1 basal z-slice. Scale-bar 20 µm. Representative of 45 cells from 3 experiments.

and as previously described (Matthews et al., 2012).

Despite the efficient protein knockdown, 58% of RPE1 cells treated with Ect2 siRNA successfully completed division on a fibronectin coated substrate (N=98 cells from 8 experiments). Interestingly, the chance of an individual Ect2 RNAi cell successfully dividing was positively correlated with the extent of it's contact with the substrate. Specifically, the presence of tails in metaphase was correlated with an increased chance of succesful division (Figure 6.5A and B on page 85). Similarly, the chance of an Ect2 RNAi cell undergoing a succesful division was positively correlated with the extent of it's basal cell length and, to a lesser extent, it's basal cell area (Figure 6.5C and D on page 85). This was surprising, since the better the knockdown, the flatter cells are in metaphase.

Therefore, in RPE1 cells, which adhere well to the substrate during metaphase, a robust contractile ring is not required for cell division. Is this the case for other, less adherent cell lines? To test this, I turned to HeLa, a transformed cell line that has been widely used in the field to study mitosis and division. Importantly for this analysis, in contrast to RPE1 cells, HeLa cells round up efficiently and do not leave adhesive tails when they enter mitosis. 70% of relatively non-adherent HeLa cells silenced for Ect2 failed to divide under these conditions (Matthews et al., 2012). To determine if this dependency on Ect2 was due to the loss of mitotic adhesion in these cells, I tested the impact of expressing an activated form of Rap1 in these cells. This is a small GTPase which must be downregulated at mitotic entry for normal adhesion remodelling (Dao et al., 2009). Ect2 RNAi HeLa cells transiently transfected with RapQ63E were able to divide at a rate similar to that seen for RPE1 cells (Figure 6.6 on page 86). Therefore, increased adhesion to the substrate during metaphase can rescue a cell's ability to divide without the need for a contractile ring.

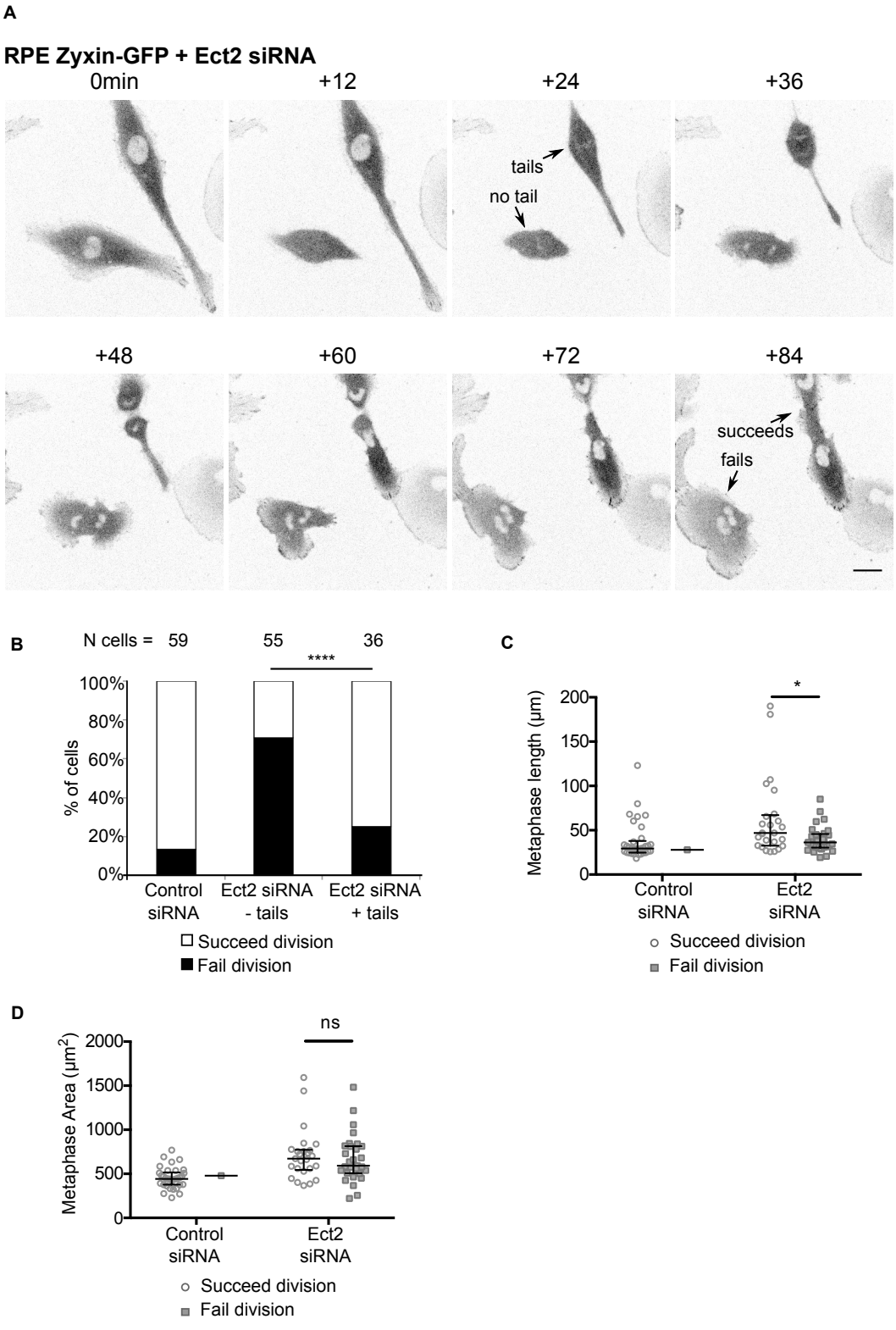

Figure 6.5: Tails rescue Ect2-independent division. (A) Montage showing Zyxin-GFP in Ect2 siRNA treated cells. One cell without a tail fails to divide, and one with tails succeeds. 1 basal z-stack. Scale-bar 20 µm. **(B)** Graph shows the percent of cells which succeed or fail division in Control siRNA cells, Ect2 siRNA cells without tails and Ect2 siRNA cells with tails. N=8 experiments. Statistics used two-sided Chi-squared test. **(C)** Graph depicting metaphase basal length in Control siRNA and Ect2 siRNA cells which succeed or fail division. Statistics used two-tailed Mann-Whitney test.

(continued) Median ± interquartile range. N=4 experiments. **(D)** Graph depicting metaphase basal area in Control siRNA and Ect2 siRNA cells which succeed or fail division. Median ± interquartile range. N=4 experiments. Statistics used two-tailed Mann-Whitney test.

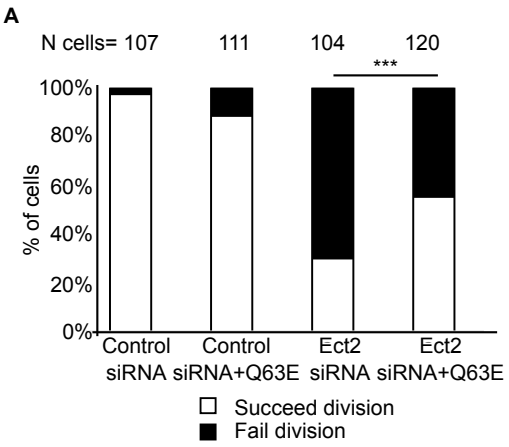

Figure 6.6: Increased mitotic adhesion rescues Ect2-independent division in HeLa. (A) Graph showing the percent of HeLa cells which succeed or fail division with or without Ect2 siRNA in combination with Rap1[Q63E]. N=3 experiments. Statistics used two-sided Chi-squared test.

6.5. Cells undergoing Ect2-independent division narrow the neck between cells using a different mechanism than in Ect2-dependent division

Having shown that RPE1 cells can utilise metaphase adhesion to the substrate to succesfully divide in the absense of Ect2, I wished to further examine the nature of this actomyosin-ring independent division. To study this I first used immunostaining to examine the localisation of key proteins that usually drive furrow formation downstream of Ect2 in these cells. In Control siRNA cells, actin, phosphorylated non-muscle Myosin II and Anillin were all clearly localised to the neck between post-anaphase daughter cells (Figure 6.7A top row, and B, on page 87). However following Ect2 RNAi, the levels of actin filaments at the furrow were significantly reduced relative to the control. Live imaging suggests that the actin filaments that remain in the neck of Ect2 RNAi cells appear to

be the remnants of the metaphase cortex (Figure 6.8A, bottom panel on page 89). More strikingly, phospho-Myosin II and Anillin were entirely absent from the neck forming between daughter cells (Figure 6.7A bottom row, and B, on page 87).

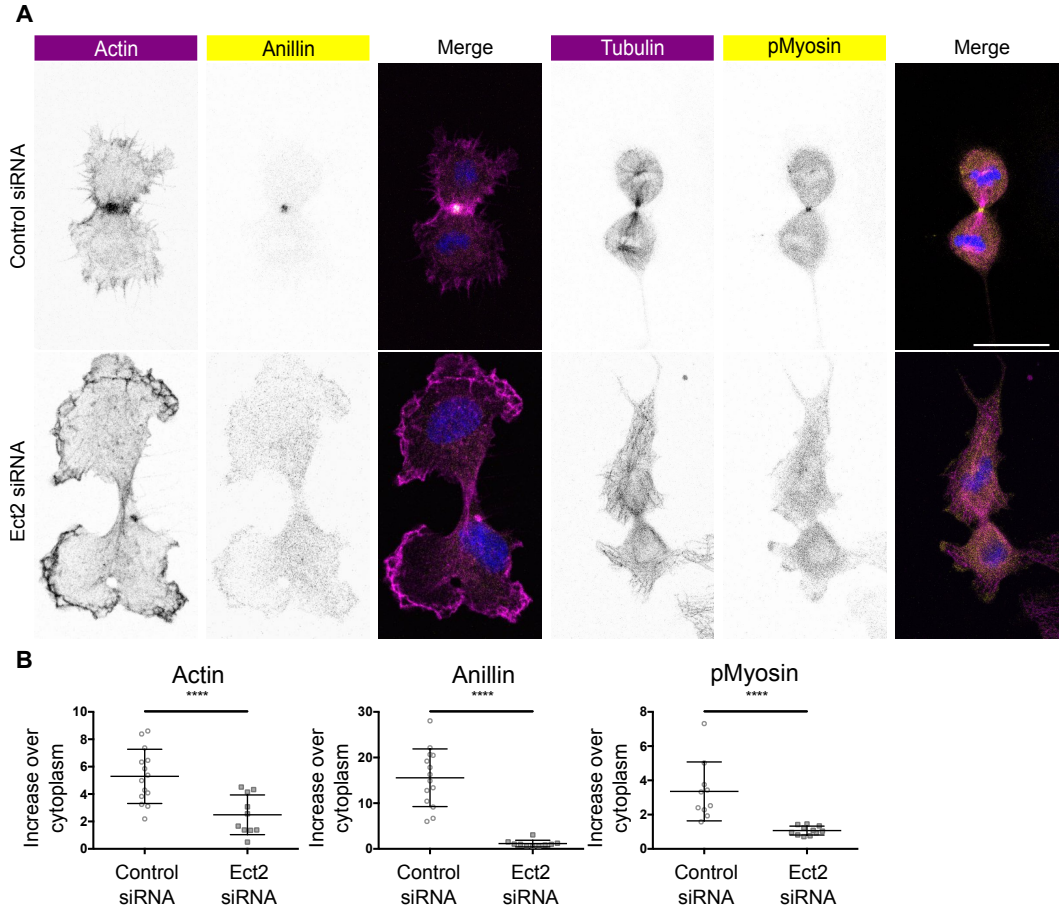

Figure 6.7: Key furrowing proteins are lost from the midzone with Ect2 siRNA. **(A)** Images show RPE1 Control siRNA and Ect2 siRNA cells fixed and stained with phalloidin TRITC (magenta), Anillin (yellow), Tubulin (magenta) and p-Myosin (yellow) antibodies. DAPI is also shown in blue in the merges. 1 middle z-stack. Scale-bar 20 µm. **(B)** Graphs quantifying of the loss of Actin (Statistics used two-tailed t test), Anillin (Statistics used two-tailed Mann-Whitney test) and pMyosin (Statistics used two-tailed Mann-Whitney test) proteins from the bridge connecting daughter cells, where the neck measures less than 5 µm. For each cell a 10x10 px (1.1 x 1.1 µm) square in the neck was normalised to the average intensity of two identical sized boxes in each daughter cell cytoplasm. Mean ± SD. N=1 experiment.

In other systems in which cells divide in the absense of an actomyosin ring, it has been suggested that division results from daughter cell migration (Kanada et al., 2005; Nagasaki et al., 2009). As I had previously found that cells which

leave tails are more likely to succeed Ect2-independent division (Section 6.4 on page 82), and that daughter cells which inherit tails re-spread faster (Section 4.7 on page 53), it seemed likely that polar migration of daughter cells might aid Ect2-independent division in RPE1 cells. To better examine how cells that lack Ect2, Anillin, and an actomyosin ring undergo cytokinesis I followed cells stably expressing LifeAct RFP as they exited mitosis. It was clear from this analysis that Ect2 RNAi cells divide in a very different manner from the control (Figure 6.8A on page 89). Following mitotic exit, control siRNA cells quickly accumulated a narrow actomyosin band at their centre, this then constricted to form a tight v-shaped furrow in the middle plane separating the daughter cells. Only when this had occured did cells begin to re-spread and migrate away from each other (Figure 6.8A, B and C top row, on page 89). Abscission then occured some time later (75-150 min after anaphase) to one side of the midbody. In sharp contrast to this, Ect2 siRNA cells did not accumulate actomyosin or build a furrow. Instead, after anaphase, the daughter cells underwent polar relaxation and re-spread away from each other (aided by the presence of tails). Only after re-spreading was well underway did the U-shaped connection linking them begin to slowly narrow (Figure 6.8A, B and C bottom row, on page 89), culminating in abscission.

As a normal cell undergoes cytokinesis microtubules are brought into alignment. As they come together, they form a discrete midbody, which electron microscopy shows as an electron dense structure into which microtubules are embedded. Microtubules then extend out on both sides of the midbody. Scission to fully separate daughter cells occurs when microtubules are cleaved by Spastin to one side of this midbody (Reviewed by Green et al. (2012)).

This was seen in control siRNA cells by the formation of a mid-body that was visible as a strucutre with two dense microtubule-based structures flanking a

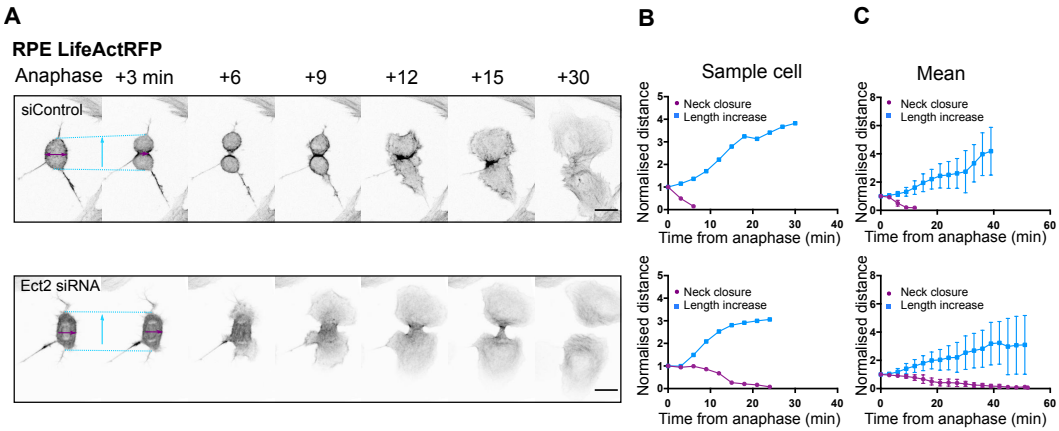

Figure 6.8: Cells undergoing Ect2-independent division narrow the neck between cells using a different mechanism than in Ect2-dependent division. (A) Montage of Control siRNA and Ect2 siRNA RPE1 cells stably expressing LifeAct RFP as they exit mitosis. The magenta arrow shows the measurement taken over time of neck closure. The blue arrow shows the measurement taken over time of the increase in length between the leading edge of the two daughter cells as they migrate away from each other. Time is shown relative to anaphase. 5 µm maximum projection of the basal-middle cell. Scale-bar 20 µm. **(B)** Graphs show neck closure and length increase of sample cells. Normalised to the first timepoint. **(C)** Graphs show mean neck closure and length increase. Normalised to the first timepoint. N=12 cells from 2 experiments. Error bars show SD.

gap. A discrete concentration of Aurora B staining could also be seen on either side of the midbody (Figure 6.9A top half, arrows, on page 90). Aurora B is a mitotic kinase which forms part of the Chromosomal Passenger Complex (CPC) (Kaitna et al., 2000). This complex localises to the central region of the mitotic spindle at anaphase and is involved in cytokinesis (Reviewed in Adams et al. (2001)). While tubulin was also localised to the midzone of dividing Ect2 siRNA cells, the appearance of the localisation pattern was much more disordered than in Control siRNA cells, even when I controlled for neck width (Figure 6.9A bottom half, arrows, on page 90). The same was true of Aurora B staining in Ect2 siRNA cells (Figure 6.9A bottom half, and B, on page 90). This suggests that in the absence of an Ect2-dependent actomyosin ring, cells can't assemble a proper midbody. This finding has implications for the mechanism of abscission in actomyosin ring-independent divisions.

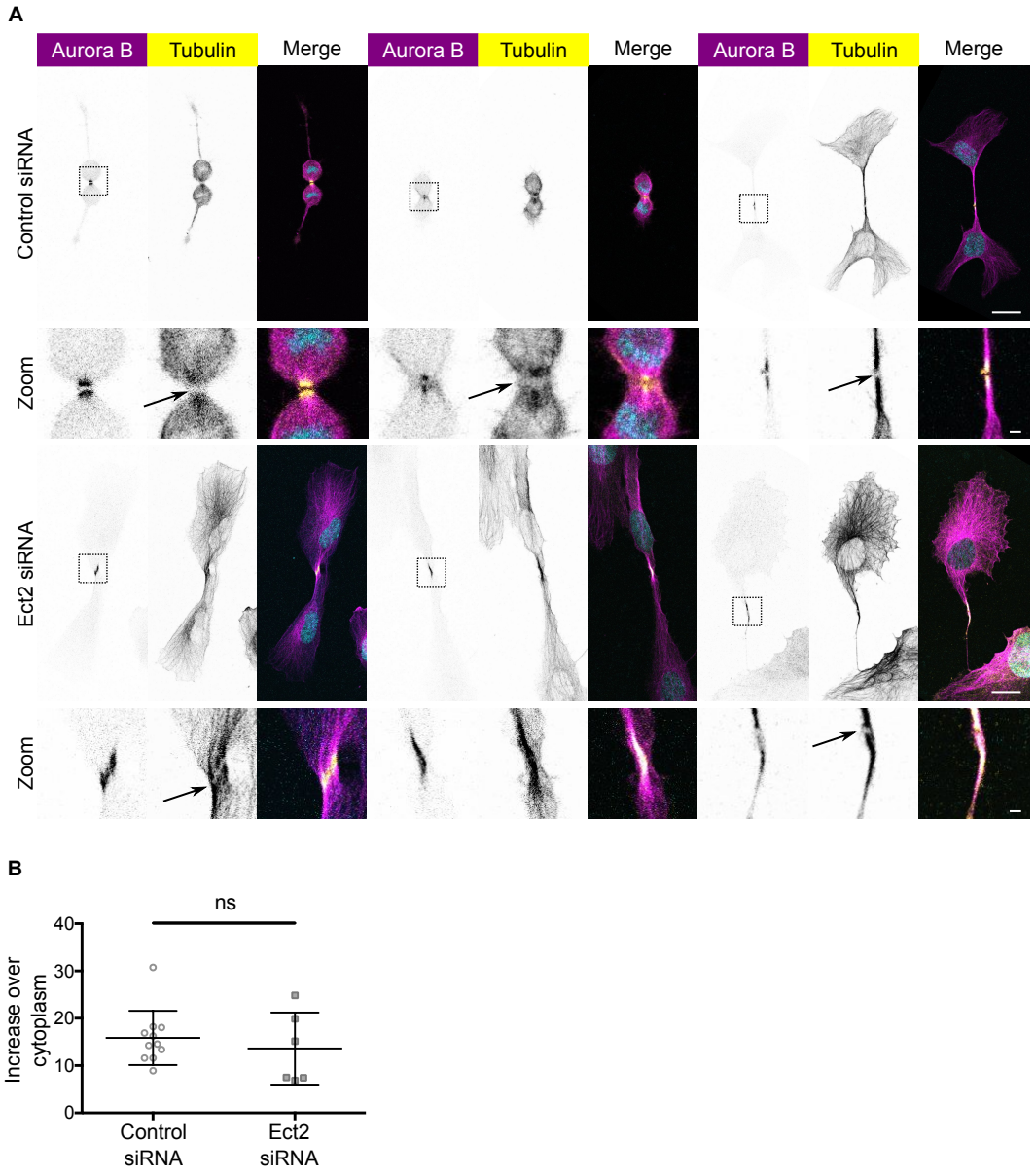

Figure 6.9: The midbody is disordered with Ect2 siRNA. (A) Images depict three sample cells each, treated with either Control or Ect2 siRNA, stained for AuroraB (yellow) and αTubulin (magenta). 1 middle z-slice. The merge also shows Dapi (cyan). Scale-bar 20 µm. Zoom of the boxed regions shows the midbody. Scale-bar 2 µm. Arrows show breaks in tubulin staining. **(B)** Graph quantifying of the intensity of Aurora B in the bridge connecting daughter cells. For each cell a 10x10 px (1.1 x 1.1 µm) square in the neck is normalised to the average intensity of two identical sized boxes in each daughter cell cytoplasm. Mean $\pm$ SD. N=1 experiment.

Having seen that neck closure occurs much more slowly in Ect2 RNAi cells, and that tubulin and Aurora B localisation in the midbody are disordered in these cells, I wished to examine what effect this may have on abscission timing. Surprisingly, abscission seems unimpaired by the loss of a well-ordered mid-body. (Figure 6.10A on page 92). Relative to anaphase, the timing of abscssion was found to be the same as in Control siRNA cells. Furthermore, relative to furow closure, it was slightly faster (Figure 6.10B and C on page 92). These data suggest that the downstream effectors of abscission (the ESCRT complexes), can be recuited even in the absense of a proper midbody.

Together these results confirm that the mechanism for Ect2-independent division does not rely on a typical actomyosin ring and instead, following polar relaxation, the neck linking daughter cells narrows as they re-spread and migrate away from each other, to provide a thin bridge that provides a substrate for the abscission machinery.

6.6. Polarised migration of daughter cells is required for Ect2-independent division

Having seen that cells undergoing Ect2-independent division narrow the neck between daughter cells only as they re-spread and migrate away from each other it seemed likely that respreading itself was aiding succesful division. Indeed, I found that cells which fail had re-spread less than those which succeed at 21 min post-anaphase (Figure 6.11 on page 93).

These findings suggest that it is the adhesion-dependent migration of daughter cells away from each other (Burton and Taylor, 1997a) that generates the traction forces required for Ect2-independent cell division.

To test this hypothesis we impaired the migration of daughter cells by plating

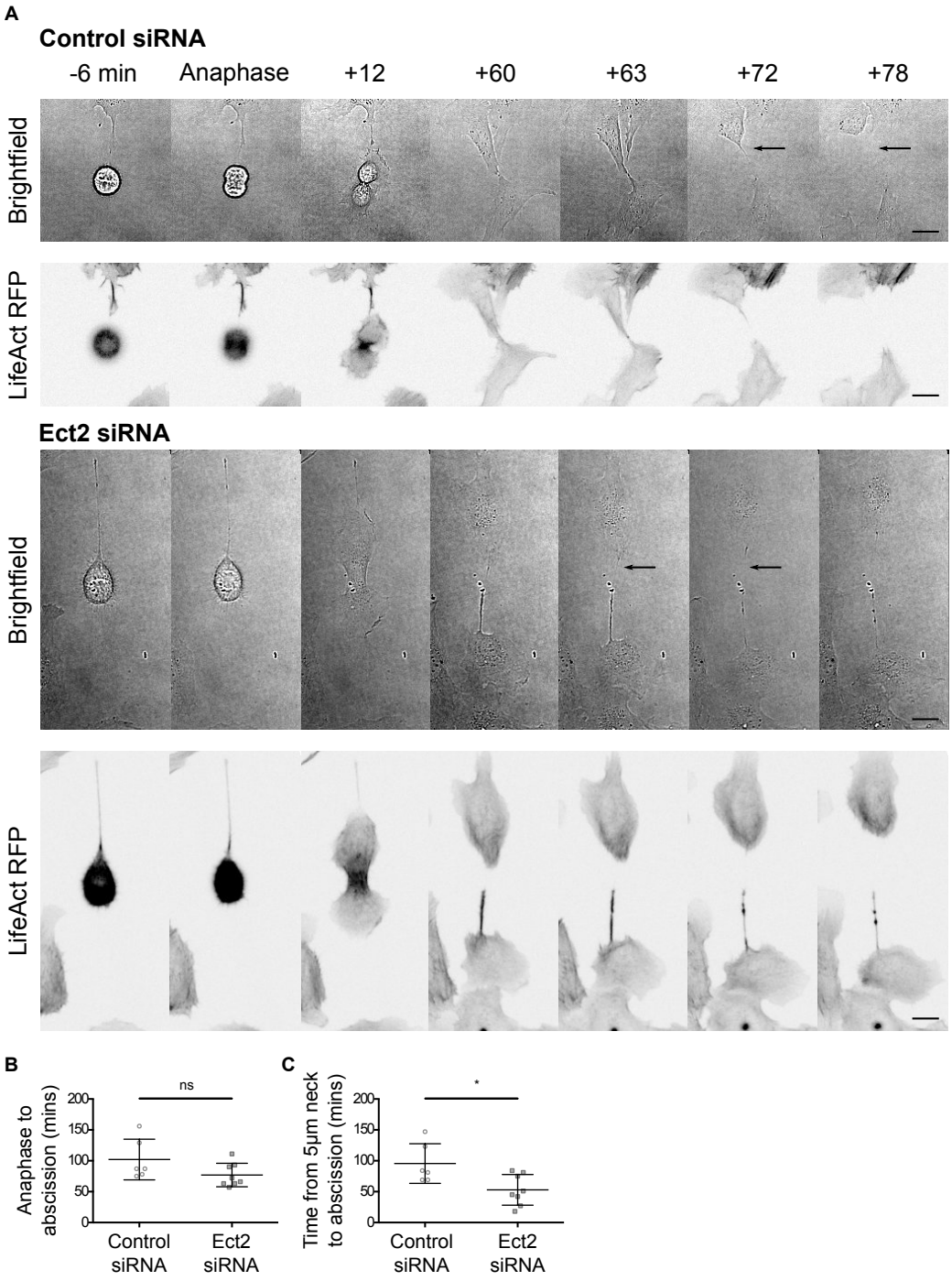

Figure 6.10: Abscission timing is not affected by Ect2 siRNA. (A) Montage showing brightfield and LifeAct RFP in RPE1 cells treated with Control or Ect2 siRNA exiting mitosis and undergoing abscission (arrows). Time is shown relative to anaphase. Wide-field image. Scale-bar 20 µm. **(B)** Graph showing the time from anaphase to abscission. N=1 experiment. Mean ± SD. Statistics used two-tailed Mann-Whitney test. **(C)** Graph showing the time from when the neck reaches 5 µm to abscission. N=1 experiment. Mean ± SD. Statistics used two-tailed t test.

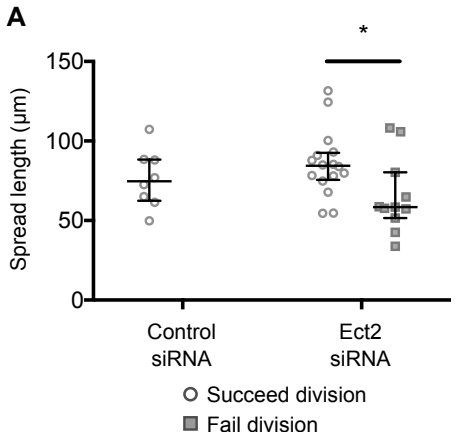

Figure 6.11: Cells which succeed in Ect2-independent division re-spread faster. (A) Graph showing the length daughter cells had re-spread to by 21 min after anaphase in Control siRNA and Ect2 siRNA cells which succeed or fail division. N=3 experiments. Median ± interquartile range. Statistics used two-tailed t test.

Control siRNA and Ect2 siRNA treated cells on micro-patterns of various sizes and geometries (as described in Section 6.2 on page 80). Control siRNA cells could successfully divide on all pattern sizes and shapes (Figure 6.12A and B top row, on page 94), however Ect2 siRNA treated cells all failed on small circle patterns (Figure 6.12A middle row, and C, on page 94). In these cases of division failure, the neck separating daughter cells appeared to narrow as they exited mitosis, before widening again when opposing poles of the re-spreading daughter cells reached the pattern edge (Figure 6.12A and B middle row, on page 94).

Patterns with an increased area, or with a more elongated shape (1:2.5 minor:major axis ellipse), give a slight increase in the rate of division success (Figure 6.12C on page 94). However, only by plating on large ellipses where daughter cells had plenty of space to migrate away in a polarised manner could the level of successful division compare to that of unpatterned Ect2 RNAi cells (Figure 6.12A and B bottom row, and C, on page 94).

This suggests that in the absense of an actomyosin ring to constrict the mid-

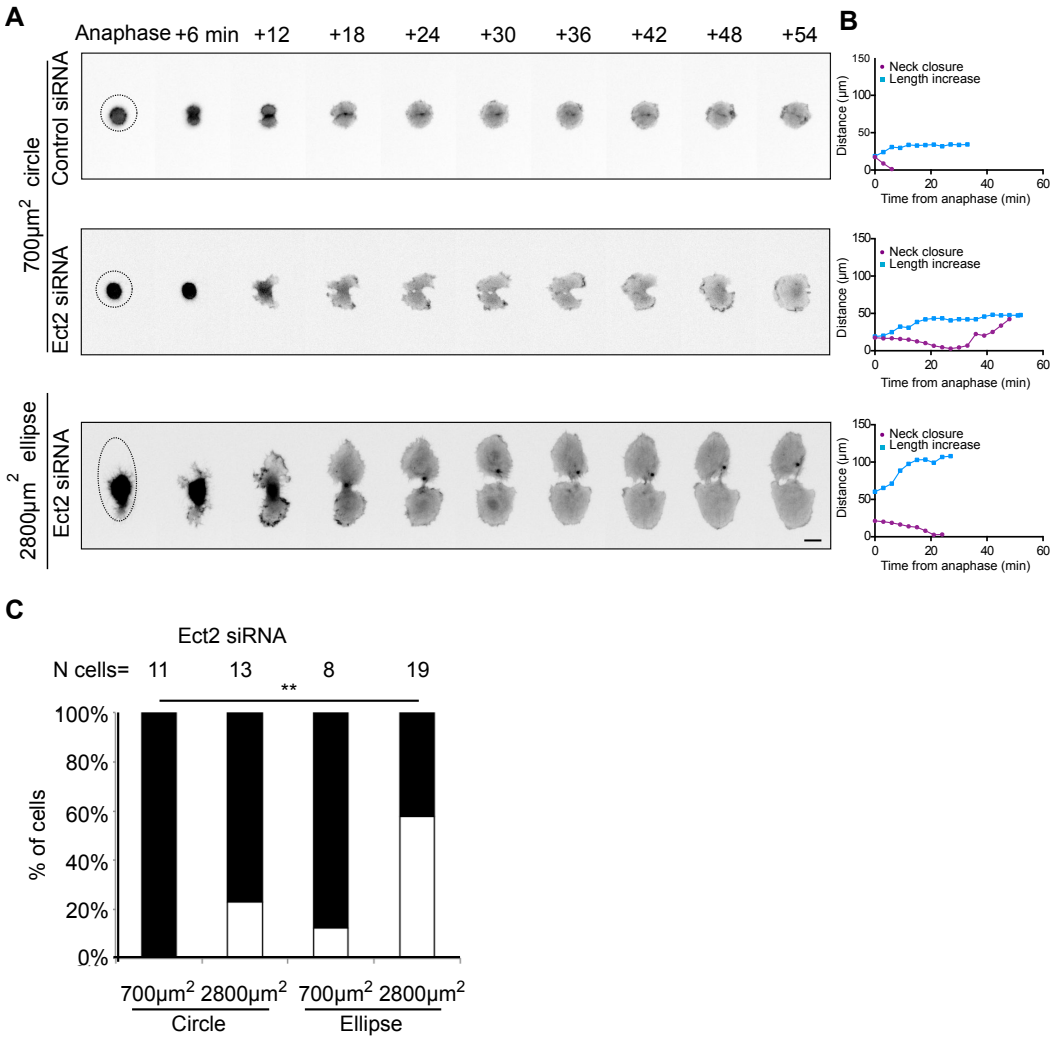

Figure 6.12: Daughter cells need space to migrate post-anaphase for Ect2-independent division. **(A)** Montages of cells expressing LifeAct RFP treated with Control siRNA or Ect2 siRNA plated on micro-patterns. Dotted line in first panel outlines the shape of the pattern. Time is shown relative to anaphase. Wide-field image. Scale-bar 20 µm. **(B)** Graphs shows neck closure and length increase of sample cells. Normalised to the first timepoint. **(C)** Graph shows percent of Ect2 siRNA treated cells which succeed or fail division on each pattern type. N=7 experiments. Statistics used two-sided Chi-squared test.

zone, generating a substrate for abscission, continuous and directed lamellipodial extension is required to narrow the bridge linking daughter cells. To confirm this I treated the cells with a small molecule targeting Arp2/3 to impair lamellipodia formation (CK-666) (Henson et al., 2015; Nolen et al., 2009). In most cases the presence of 300 µmol Arp2/3 inhibitor prevented cells entering mitosis. Therefore I only looked at cells immediately after drug addition. Interphase cells could be seen to rapidly stop extending lamellipodia and migrating, confirming the efficacy of the drug (data not shown). Of the 7 Ect2 RNAi cells treated with CK-666 that entered and progressed through mitosis immediately after drug addition, all re-spread as large bi-nucleate cells. They did so without any polar movement of daughter cells away from each other and barely any narrowing of the neck (Figure 6.13 on page 96). I repeated this experiment with a lower dose of 200 µmol of CK-666 and saw similar results. However, in this experiment I observed abnormally high levels of cell failure in the DMSO control. Therefore, while the result appears robust, the experiment needs repeating.

My previous results suggest that RPE1 cells treated with Ect2 siRNA can undergo succesful division through the action of daughter cells migrating away from each other. I showed in Section 4.7 (on page 53) that the presence of adhesive tails which presist throughout mitosis aid daughter cell re-spreading at mitotic exit, and the presence of these tails correlates with succesful division in the presence of Ect2 RNAi (Figure 6.5A on page 85). This implies that the tails left during mitotic rounding may aid actomyosin-ring independent division. To test this I replated a suspension culture of metaphase Ect2 RNAi cells (as I previously did with the control in Figure 6.3 on page 81), so that they could form new contacts as they exited mitosis. While control cells divide succesfully under these conditions, then Ect2 RNAi cells almost all fail to divide (Figure 6.14 on page 97).

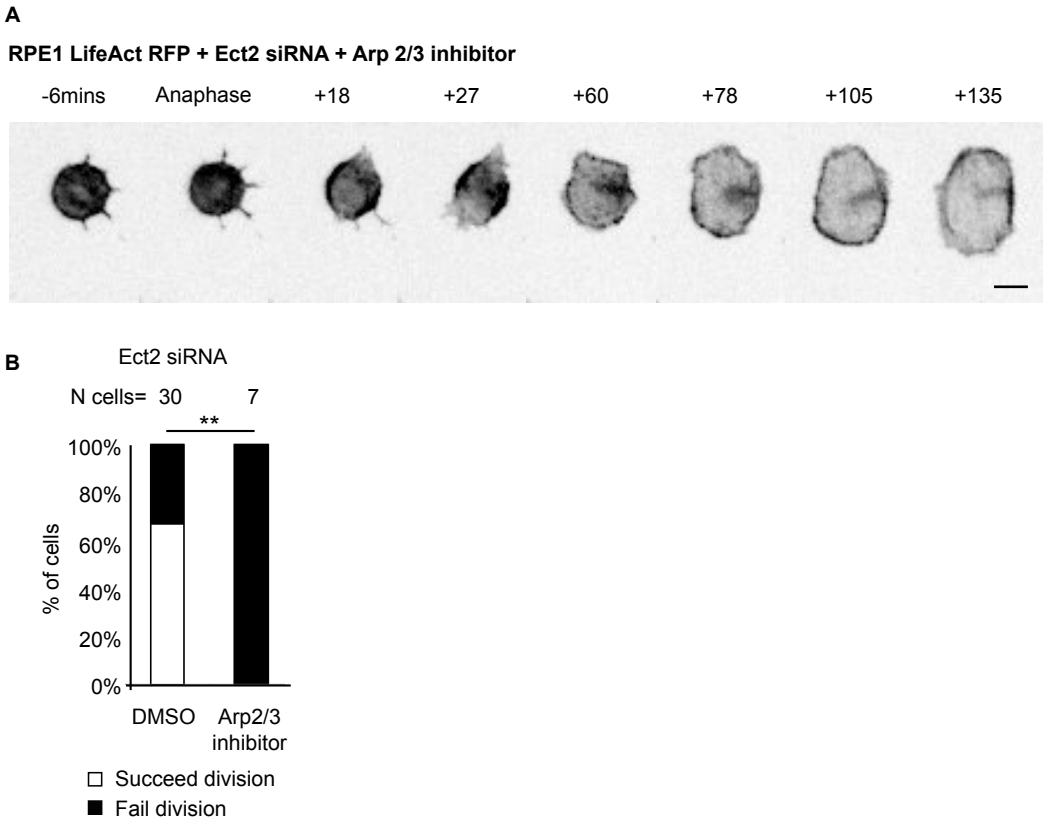

Figure 6.13: Lamellipodia formation of daughter cells is required for Ect2-independent division. (A) Montage showing a representative Ect2 siRNA treated cell treated with 300 µmol Arp 2/3 inhibitor re-spreading as it exits mitosis as one large bi-nucleate cell. Time is shown relative to anaphase. Wide-field image. Scale-bar 20 µm. **(B)** Graph showing the percent of cells which succeed or fail division in Ect2 siRNA treated cells treated with either DMSO or 300 µmol Arp 2/3 inhibitor. N=1 experiment. Statistics used two-sided Chi-squared test.

Together these results confirm that the active integrin-positive tails which aid re-spreading and generate traction force aid adhesion-mediated division in cells compromised in their ability to construct an actomyosin ring.

6.7. Discussion

In this chapter I expanded on previous work showing that many human cell types require adhesion for succesful cell division (Ben-Ze'ev and Raz, 1981). We found that adhesion is dispensible in metaphase and is only required at mitotic exit. Further to this, furrow formation does not require adhesion as cells in suspension can succesfully form a furrow which is only regresses later

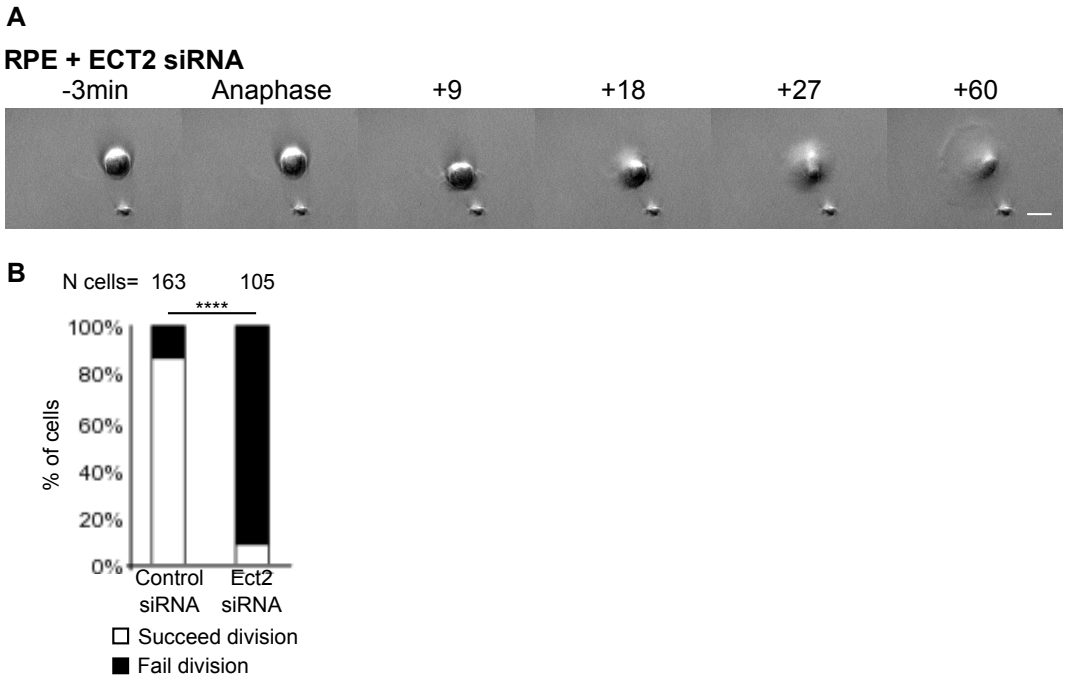

Figure 6.14: Adhesive contacts left during mitotic rounding are essential for Ect2-independent division. (A) Brightfield montage of an Ect2 siRNA treated cell undergoing mitotic exit after replating from suspension. Time is shown relative to anaphase. Wide-field image. Scale-bar 20 µm. **(B)** Graph showing the percentage to Control siRNA and Ect2 siRNA cells which succeed or fail division when replated from suspension at mitotic exit. N=1 experiment. Statistics used two-sided Chi-squared test.

(H.K Matthews, unpublished data). Also, as RPE1 cells divide on fibronectin-substrate, the furrow is raised from the substrate. These data lead me to conclude that control RPE1 cells require a low level of substrate adhesion at the cell poles for abcission, the final cut separating daughter cells as they complete division.

Taking this further, I found that adhesion is also sufficient to drive division in the absense of a robust actomyosin ring. Again, this is in line with previous work which suggested that some adherent eukaryotic cells, e.g. *Dictyostelium*, NRK and HT1080 fibrosarcoma cells, can utilise cell migration to divide succesfully despite compromised actomyosin ring formation (Kanada et al., 2005, 2008; Nagasaki et al., 2009).

Cells are able to utilise adhesion based migration, aided by the unretracted tails left during mitotic rounding, to separate daughter cells in the absense of a contractile ring. This relies on the ability of daughter cells to continuously extend Arp2/3 based lamellipodia away from each other and presumably use the traction force and tension this generates (Houk et al., 2012) to narrow the bridge between them until it is a good substrate for abscission to occur. This contrasts with myosin-dependent division where cells form an actomyosin ring which contracts to form a tight v-shaped furrow before cells begin to re-spread. These findings could be greatly strengthened through using traction force microscopy to examine the changes in forces as cells enter and exit mitosis.

In line with a role for mitotic adhesions in Ect2-dependent division, HeLa Kyoto cells, which fail to generate long adherent tail-like structures when they round up and enter mitosis and which attach poorly to the substrate (Matthews et al., 2012), fail to divide following Ect2 siRNA. However, as I show here, they can be induced to undergo adhesion-dependent division through the expression of an activated form of Rap1.

Interestingly, a previous study in HeLa cells found that abscission was actually delayed by tension in the bridge caused by daughter cells moving apart, and that they must relax back together for abscission to occur efficiently (Lafaurie-Janvore et al., 2013). We do not see this delay in our Ect2 treated cells which require migration-mediated tension to narrow the neck between daughter cells. This suggests that RPE1 cells that lack an actomyosin-ring undergo abscission soon after the bridge has thinned. This might be as soon as ESCRT III is recruited (Carlton and Martin-Serrano, 2007), since the midbody is not as well organised and actomyosin does not have to be removed. I shall explore the possible explanations for this difference between studies in more detail in the general discussion.

7. Discussion

Through this analysis of cell shape and adhesion dynamics in RPE1 cells (a migratory cell line) as they enter and exit mitosis, I have made several findings. While some of these support previously published work in the field, especially from $D.\ discoideum$. I have also endeavoured to reconcile these findings with our existing knowledge of normal and cancer cell division. In the following discussion, I will focus on three broad topics; the nature of mitotic cell rounding and variations between cell types, changes in cell polarity and the impact of this on cell division, and the robustness of cell division and the relative contribution of adhesion and the actomyosin ring to this division in different environments. I will then finish with a brief discussion on the future perspectives of this work.

7.1. Inefficient rounding in cells in culture

Cell rounding is a key event during mitosis and is the first visible sign of mitotic entry (Gavet and Pines, 2010a). It has been described in single cells in culture, epithelial monolayers in culture and in epithelia in vivo in a number of systems (some such examples include (Carreno et al., 2008; Cramer and Mitchison, 1997; Reinsch and Karsenti, 1994; Rosa et al., 2015; Sanger et al., 1984)).

However, in this study I have shown that not all cells round to a perfectly spherical shape as had been previously described. RPE1 cells are a non-transformed, migratory human cell line. They rarely round fully and instead tend to leave cytoplasmic tails attached to the substrate. This contrasts sharply with HeLa, a transformed human cell line, which round very well during mitosis. Other non-transformed cell lines (MCF10A- examined by H. Matthews), and transformed cell lines (RPMI, MDA-MB-231 and A375- examined by a L.

Wolf) also round very efficiently (unpublished data). There are several possible reasons for these difference in cell types. To round up efficiently a cell must remodel the focal adhesions anchoring it to the substrate while it retracts the cell margin and builds a stiff actomyosin cortex. If any of these process are impaired, the cell cannot round as efficiently (Figure 7.1 on page 102).

In section 5.3 on page 62 I studied the changes in focal adhesion composition as RPE1 cells enter mitosis. All of these cells lose Zyxin and Paxillin puncta, however they retain active β1-Integrin puncta that decorate the cytoplasmic tails and thin retraction fibres spread over the area of the former interphase cell shape which persist throughout cell division (it needs to be confirmed that these structures are still functionally attached to the cell body and not just remnants left behind during rounding). This is in line with previous findings from the lab in HeLa. N. Heatley used a combination of fixed and live imaging and found that interphase HeLa cells contain discrete puncta of Talin, Vinculin, Paxillin, Zyxin, FAK and phospho-Tyrosine, all of which are lost from metaphase cells. H. Matthews also found that active β1-Integrin puncta are present in both interphase and metaphase cells (unpublished data), as I found with RPE. Therefore, there is not a clear difference in the composition of mitotic adhesions between cells which round efficiently and those which don't. However, it is possible that the loss of these proteins from the cell-substrate adhesions happens with different dynamics between cell lines, which may lead to differences in the extent of rounding. As RPE1 cells have a considerably larger spread interphase area than HeLa, if it loses the proteins from the focal adhesion complex more slowly, it is possible that the remaining integrin puncta are more likely to retain anchorage to the substrate and prevent efficient rounding.

If there did prove to be differences in the dynamics of adhesion loss between cell lines it may be regulated through the activity of FAK. This was found to be

one of the first of the proteins lost from HeLa cells entering mitosis in HeLa (N. Heatley, MRes Thesis). FAK is a tyrosine kinase and regulates a lot of the tyrosine phosphorylation in focal adhesions (Mitra et al., 2005), which is required to create binding domains and enable recruitment of many focal adhesion components. Therefore if FAK is lost at differing rates between cell lines it may effect the stability of the rest of the complex and lead to differing rates of complex dissasembly. It must also be considered that differences in residual levels of focal adhesion proteins not detectable using the imaging techniques I employed may explain cell type specific differences in the efficiency of cell rounding (Figure 7.1 Left side, on page 102).

An alternative possibility is that the difference in rounding when compared to HeLa cells is due to inefficient or inconsistent formation of the actomyosin cortex in RPE1 cells (Figure 7.1 Right side, on page 102). It is not immediately clear whether this may be the case, as RPE1 cells do lose stress fibres and build a cortex. A direct comparison of the robustness of actomyosin cortex formation between cell lines is difficult if not impossible. An endogenous tag would be needed to avoid discrepancies due to overexpression of a plasmid and even then there are likely differences between cell types. This could potentially be controlled for by measuring the difference between cytoplasmic signal and cortex signal (as in Figure 5.6E on page 68) and comparing this to the middle area/width of the cell. Another read-out of the robustness of cell cortex formation is that of stiffness. Preliminary findings by K. Plak have found that mitotic HeLa cells are significantly stiffer than mitotic RPE1 cells. Assuming these findings to be accurate, less stiff cells suggest a less robust actomyosin cortex which may have had problems building the tension required to retract the cell margin. Coupled with the fact that RPE1 cells also have a significantly larger spread area during interphase, this may explain why HeLa can round up fully at mitotic entry, but RPE1 cannot.

Finally, it seems that adhesion remodelling and formation of the actomyosin cortex can be uncoupled from each other. When RPE1 were treated with Ect2 siRNA and therefore unable to build a proper actomyosin cortex, they still remodelled their adhesions in a similar fashion as control. Further investigation is needed to establish whether the dynamics of adhesion loss may be different with this treatment, however, ultimately Paxillin and Zyxin puncta were lost and active β1-Integrin puncta remained.

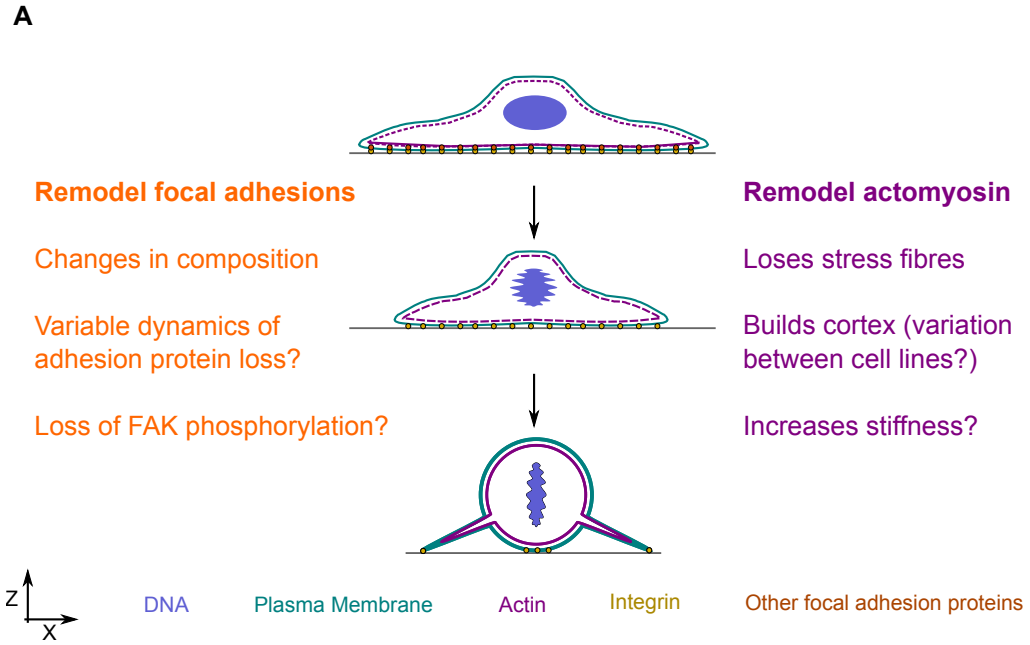

Figure 7.1: Inefficient rounding. (A) Model showing how a mitotic cell might remodel it's focal adhesions and it's actomyosin cytoskeleton.

7.2. Robustness of division

A key interest underlying much of this study was that of the robustness of division. In general, cells are very good at dividing succesfully and it takes extreme circumstances to cause cells to fail. It should be noted that a caveat for all work in tissue culture is that by definition, stable cell lines are those that can survive and divide in those conditions, and they are under constant unintentional selection in the lab through passaging. However, these are the tools currently at our disposal and through careful consideration of this limitation and ensuring

robust controls are included, they can be used to draw meaningful conclusions.

Although previous work has found that HeLa need to round robustly for efficient spindle formation, even HeLa cells which have been artificially flattened either through being physically constrained under a gel or through expression of constitutively active Rap1 will ultimately divide succesfully in the majority of cases (Lancaster et al., 2013). RPE1 cells round inefficiently in general and yet can still divide the vast majority of the time.

RPE1 cells can even divide in the absence of a proper cytokinetic ring (Figure 7.2A, on page 106), in a mechanism termed Myosin II independent division, which was first described in *D. discoideum* (Neujahr et al., 1997; Zang et al., 1997). This has since been most extensively studied by the Uyeda lab, primarily in *D. discoideum* , but also in some mammalian cell types. They suggested that this mechanism of division requires the traction forces generated by continuous polar extension to indirectly cause furrow ingression. (Nagasaki et al., 2001). They then confirmed that this requires focal adhesions as more sever cytokinetic defects were seen with double knockout strains lacking mhcA, which codes for Myosin II , and paxB or vinA (Nagasaki et al., 2009). In *D. discoideum* the forces driving adhesion-based cell division have been shown to depend on the polarized activity of the Arp2/3 activator, SCAR/WAVE (King et al., 2010).

The Uyeda lab then examined Myosin II independent division in mammalian cell lines. As they could not generate cell lines completely lacking Myosin II as they had for *D. discoideum* , they had to rely on other means to impair actomyosin ring formation. They did this using blebbistatin, a specific inhibitor of nonmuscle Myosin II (Straight et al., 2003). At intermediate concentrations of 30 µmol blebbistatin (which reduced Myosin II ATPase activity by >90% in vitro (Kovács et al., 2004; Straight et al., 2003)) they found that 7/16 NRK cells furrowed and divided in a manner strikingly similar to mchA-null *D. discoideum* cells and re-

quired polarised daughter cell migration. The result was less clear in human HT1080 fibrosarcoma cells as only 1/11 cells completed scission with 30 µmol blebbistatin. However, Myosin II has also been reported to be involved in post-mitotic spreading (Cramer and Mitchison, 1995), spindle formation (Rosenblatt et al., 2004), and maintenance of cell polarity during interphase (Pierini et al., 2003; Straight et al., 2003). Therefore, I feel that using a drug such as blebbistatin which will deplete Myosin II activity in the entire cell throughout the whole cell cycle is not the best way to examine the role of the contractile actomyosin ring. This is why in this study I chose to use Ect2 depletion to impair actomyosin ring formation, as it is spatially and temporally regulated and should only effect the cell at the time and place we are studying (cell rounding and furrow formation during mitosis).

The Uyeda lab did study the effect of Ect2 depletion on cytokinesis by using shRNA to generate HT1080 cells with a stable Ect2 Knock down (Kanada et al., 2008). However, the results they got with this are quite different to the results I got in RPE1 cells with a transient siRNA knock-down. Although slightly slower than controls, in their Ect2-depleted cells furrow formation happens relatively quickly and without obvious respreading of lamellipodia. In addition to this, RhoA is still present at furrow in these cells. They therefore concluded that Ect2 is not the primary GEF mediating activation of RhoA at the equatorial cortex of HT1080 cells.

HeLa cells are not normally able to divide in the absense of a proper cytokinetic ring (Figure 7.2A, on page 106), however if mitotic adhesion is increased, and therefore efficient rounding decreased through expression of constitutively active Rap1, more cells can divide succesfully. Further examination on the mechanism of this division in both HeLa and RPE1 needs to be carried out. One line of investigation would be examining the localisation of adhesion proteins. Are

they focused at the lamellipodia to help generate the required traction forces as has been previously suggested for $D.\ discoideum$ and NRK cells (Nagasaki et al., 2009)? It would also be good to confirm the assumption that cell migration generates traction forces that are required to narrow the neck. This could be done using RNAi or small molecule inhibitors to impair PLD2/mTORC2 and its downstream signalling. It has been shown that increased membrane tension at the leading edge acts through this pathway as a long range inhibitor of actin network assembly to maintain lamellipodia formation at the leading edge and not elsewhere in the cell (Diz-Muñoz et al., 2016).

Therefore RPE1 cells can use traction generated through adhesion to the substrate to divide in the absense of a proper cytokinetic ring. However, we found that some adhesion to the substrate is required even with a properly formed cytokinetic ring as they cannot divide sucesfully in suspension (Figure 7.2B on page 106). This is in line with a previous study which found that mouse 3T3 fibroblasts and monkey kidney epithelial BSC-1 cells becamse binucleate when adhesion was prevented both by seeding in methyl cellulose medium or on non-adhesive poly(2-hydroxyethylmethacrylate)(poly[HEMA]) (Ben-Ze'ev and Raz, 1981). This contrasts to HeLa, which complete division in suspension with relatively little difficulty (Figure 7.2B on page 106). In addition, a previous study suggested that oncogenic signaling itself may help override the requirement for adhesion, as they found that V12 H-Ras-transformed fibroblasts succeeded to divide in suspension whereas control cells did not (Thullberg et al., 2007). However, some cells do not fit this mould as four B16 melanoma cell variants were also found become binucleate in suspension (Ben-Ze'ev and Raz, 1981).

Another explanation for the differences between cell types could relate back to the robustness of mitotic rounding and cortical stiffness discussed in the previous section. It may be that cells which are better at building an actomyosin

cortex, will also be better at building a contractile ring and subsequently recruit the regulators of abscission, which helps overcome the lack of cell-substrate attachment so the cell can succesfully divide in suspension. Further investigation on the robustness of division could be carried out through examination of a wider panel of cell lines and their cortical stiffness, and by increasing stiffness in cells such as RPE to see if it rescues mitotis in suspension.

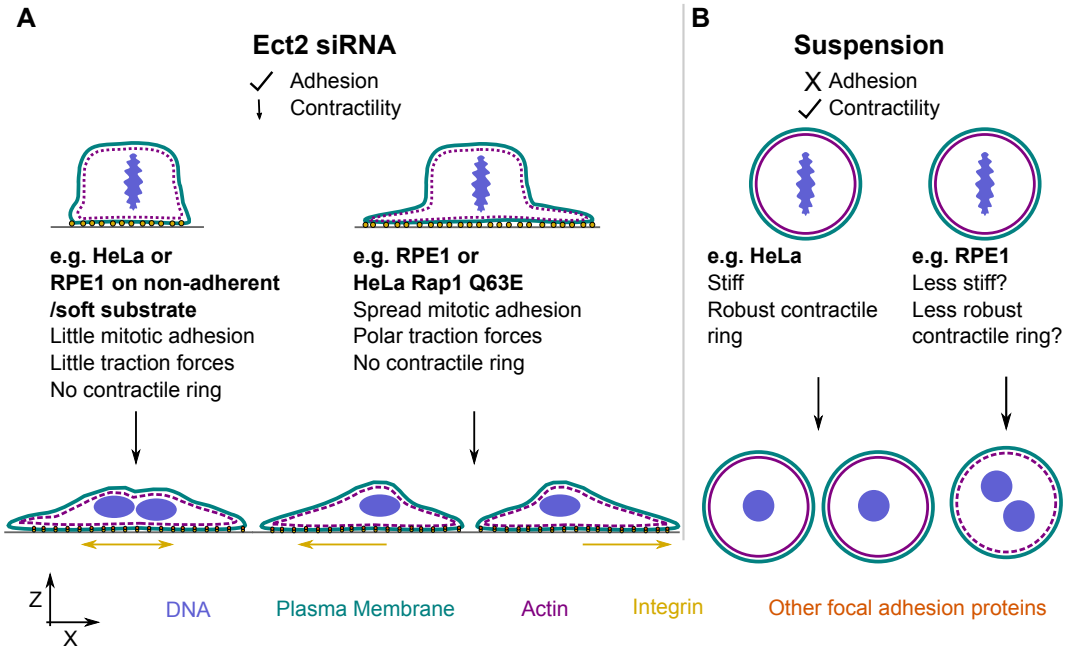

Figure 7.2: Robustness of mitosis. (A) Model showing mitotic HeLa and RPE1 cells with impaired contractile ring formation through Ect2 siRNA. **(B)** Model showing mitotic HeLa and RPE1 cells in suspension.

7.3. Polarity switching in migratory cells

Given that a migratory cell has a polar interphase shape, how does it deal with this asymmetry to allow it to undergo a symmetric division and give rise to two daughter cells which polarise and migrate away from each other? In this study I reported two stochasitc events which may help with this. Many RPE1 cells pause their migration prior to the onset of mitosis. A subset then extend a second leading edge behind them and become more symmetric in shape and in the distribution of cytoskeletal components. I also showed that the dynamics

of focal adhesions become more symmetric with the polarity switch. However, both pausing and the polarity switch seem to be stochastic events, with no obvious increase in the incidence prior to mitosis.

Why then do cells pause their migration and undergo a polarity switch? As these cells are not following a chemokine trail, the event to set up polarity to migrate in the first place was a stochastic symmetry breaking event. Changes in the substrate effecting the movement of the cell or intracellular changes in polarity signalling could all have the effect of disrupting the signal maintaining this polarity and causing cells to stop.

The elongation of the RPE1 cells as they pause and become bipolar is curious, as it goes against the accepted signalling pathway of Rac/Cdc42 regulated lamellipodia formation in one place inhibiting it in another through competition for GEFs (Osmani et al., 2010; Vicente-Manzanares et al., 2011). It is however reminiscent of the phenomenon of new end take off (NETO), which occurs as $Schizosaccharomyces\ pombe$ (Mitchison and Nurse, 1985) cells prepare for entry into mitosis. When one of these yeast cells undergoes division, the daughter cells each have an old end (furthest from the point of cytokinesis) and a new end (created by cytokinesis). Initially cells grow in a monopolar fashion from the old end. When they reach a minimum length and have appropriate levels of CDK1/CyclinB activity, NETO occurs and cells initiate growth from the new end as well, while maintaining growth at the old end (Grallert et al., 2013). Das et al. (2012) showed that NETO in $S.\ pombe$ is regulated by oscillations in Cdc42 levels between opposite cell poles. This is due to competition between poles for active Cdc42. When the cell reaches a certain length, the old end is too far away to compete with the new end for the pool of active Cdc42, so the new end accumulates Cdc42 and can begin to grow.

With this in mind, if a migratory cell becomes too large, then signals such as

Cdc42 or tension induced inhibition of lamellipodia formation (**?**) may dissipate before reaching the rear so it can break free from inhibition and form the second lamellipodia seen in RPE1 cells undergoing the polarity switch (Figure 7.3 on page 109). Although no obvious correlation between cell length and the polarity switch could be seen, this does bear further investigation. Plating on fibronectin lines would allow standardisation of cell width so that cell length and area could be more accurately measured in cells undergoing the polarity switch, and if possible, compared to flourescently tagged Cdc42.

Although seemingly stochastic, there are some interesting consequences of the polarity switch. This increased symmetry in interphase cell shape corelates with increased symmetry in mitotic cell shape, and through the presence of tails, increased symmetry in the dynamics of division and daughter cell respreading. This is of particular importance in cases where cells are dividing via contractile ring independent cytokinesis as was discussed in the previous section.

7.4. Future perspectives

Abscission

It was interesting to find abscission unimpaired in cells which divide via migration-mediated cytokinesis. The recruitment of the abscission machinery to the bridge connecting daughter cells may be purely physical, once it's thin enough they will be recruited. Alternatively, they may be recruited due to membrane tension-dependent signaling from the lamellipodium to the cell rear as previously described for migrating cells (Diz-Muñoz et al., 2016). In HeLa cells, the migration of daughter cells away from each other was found to delay ESCRT III -mediated abscission (Lafaurie-Janvore et al., 2013). This is not neccesarily incompatible with my findings. In that study the abscission delay may be due in part to the effect of tension on the actomyosin left in the bridge, as if actin is not cleared

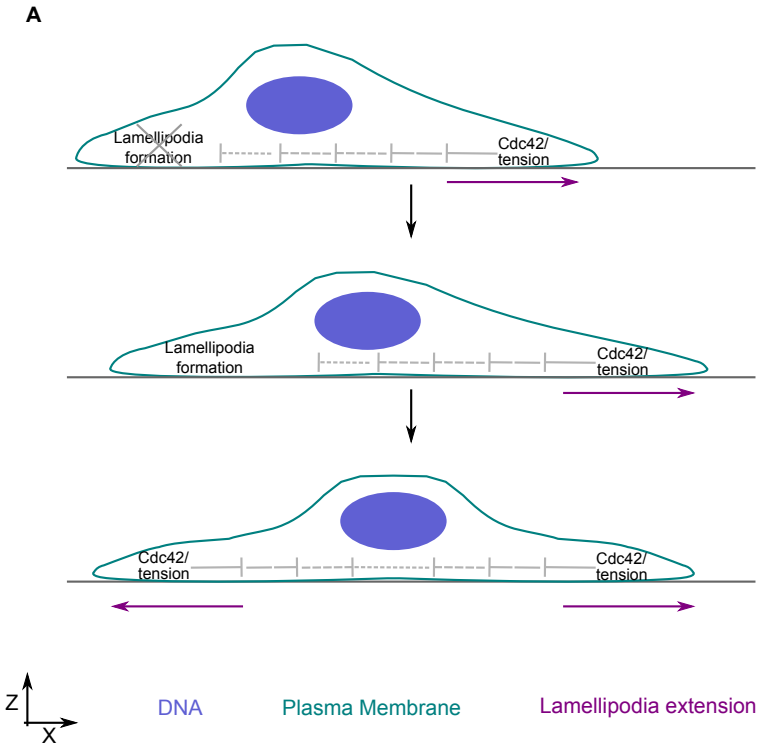

Figure 7.3: Polarity switching in migratory cells. (A) Hypothesis as to how loss of long range inhibition as a cell increases in length may contribute to the polarity switch seen in RPE1 cells.

from the mid-body region in a timely manner it can delay abscission (Echard, 2012). Therefore, it could be that under normal control conditions, the process of ring contraction leads to the formation of a mid-body rich in actin, which inhibits abscission until it is cleared and spastin and ESCRT III machinery are recruited to induce abscission (Connell et al., 2009; Guizetti et al., 2011). However, here I have shown that a cortical ring is not required for narrowing the neck between daughter cells, and migration (likely actin through traction force mediated tension) is neccesary for this in cells undergoing Ect2-independent division. This leads to a narrow neck, which contains Aurora B and seems to be a sufficient substrate for abscission to occur in a timely manner (Guizetti et al., 2011; Mierzwa and Gerlich, 2014). The exact mechanism of abscission in this system needs to be further investigated through examination of the ESCRT machinery and other proteins involved in abscission.

Endogenous integrin

will be going through this with Jigna when I'm back and fill out this section
One thing that would greatly add to this study would be to use live imaging to examine the dynamics of integrin puncta as they progress through division, both in the presence and absense of a robust contractile ring. I attempted to do this using Integrin-GFP and Integrin-RFP plasmids, however RPE1 are difficult to transfect, and integrin plasmids are also difficult to transfect and tend to cause overexpression effects, so this line of examination became too difficult with the given time restraints. However, with the recent boom in studies using CRISPR to endogenously tag genes, this may be possible. With the help of J. Patel in the lab I intend to... C or N terminal tag? single or double strand break? FACS sort and select to make a cell line.

In vivo parallels

As a final point, this work, and most similar work on the comparative roles of adhesion and cortical contractility in division, have all been done in single cells in culture. It would be good to study this either in vivo, or in conditions which more closely resemble in vivo. A possible candidate for the latter lies with MCF10A cells, or other similar cell lines which make cell-cell junctions and form epithelial monolayers in certain culture conditions. Preliminary work by H. Matthews has found that these cells round less efficiently when surrounded by neighbours than when on their own. They may also maintain adhesion to their neighbours during mitosis. Would this anchorage serve a similar role as tails do in RPE1 and rescue contractile ring independent division?

More difficult, but also possible, would be to examine this in vivo in $D. melanogaster$. Our lab has extensive experience in live imaging the developing fly notum to study various mitotic events in the context of an epithelia. Flies containing ge-

netic knockdowns for Pebble (the fly homologue of Ect2) could be crossed with those expressing markers for actin or adhesion proteins as has been used to examine the role of Pebble in mitotic rounding (Rosa et al., 2015).

A final thought is that of the basal process many epithelial cells leave conected to the basal substrate as they round up to undergo mitosis (Das et al., 2003). There has been speculation that these processes may impact on fate determination in asymmetric division (Fishell and Kriegstein, 2003; Huttner and Kosodo, 2005; Miyata et al., 2001). In some cases during symmetric division in neuroepithelial cells the basal process has been reported to split, and either each daughter cell will inherit one protrusion or one will inherit both and the other none (Kosodo et al., 2008). As I found that a daughter cell that inherits a tail respreads faster than one which doesn't, how would this affect asymmetric or symmetric divisions and daughter cell positioning in epithelia if only one daughter cell inherits basal processes.

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