Local actin nucleation tunes centrosomal microtubule nucleation during passage through mitosis

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Abstract

Cells going through mitosis undergo precisely timed changes in cell shape and organisation, which serve to ensure the fair partitioning of cellular components into the two daughter cells. These structural changes are driven by changes in actin filament and microtubule dynamics and organisation. While most evidence suggests that the two cytoskeletal systems are remodelled in parallel during mitosis, recent work in interphase cells has implicated the centrosome in both microtubule and actin nucleation, suggesting the potential for regulatory crosstalk between the two systems. Here, by using both in vitro and in vivo assays to study centrosomal actin nucleation as cells pass through mitosis, we show that mitotic exit is accompanied by a burst in cytoplasmic actin filament formation that depends on WASH and the Arp2/3 complex. This leads to the accumulation of actin around centrosomes as cells enter anaphase and to a corresponding reduction in the density of centrosomal microtubules. Taken together, these data suggest that the mitotic regulation of centrosomal WASH and the Arp2/3 complex controls local actin nucleation, which may function to tune the levels of centrosomal microtubules during passage through mitosis.

Keywords Arp2/3 complex; centrosomal actin; mitosis; WASH complex

Subject Categories Cell Adhesion, Polarity & Cytoskeleton; Cell Cycle

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Introduction

The microtubule (Zhai et al, 1996; Meraldi & Nigg, 2002) and actin cytoskeletons (Rakmukar & Baum, 2016) undergo profound parallel changes in dynamics and organisation as cells go through mitosis. These changes play a vital role in the control of animal cell division and begin as cells enter prophase. At this time, the interphase microtubule cytoskeleton is disassembled (Cenotzone & Borisy, 1990; Niethammer et al, 2007; Mchedlishvili et al, 2018), allowing microtubule nucleation to become focused at centrosomes (Zhai et al, 1996; Piehl et al, 2004; Mchedlishvili et al, 2018), where gamma-tubulin accumulates (Khodjakov & Rieder, 1999; Bettencourt-Dias & Glover, 2007; Sulimenko et al, 2017). With the loss of the nuclear/cytoplasmic compartment barrier at the onset of prometaphase, this is followed by a sudden change in microtubule organisation (Mchedlishvili et al, 2018) and dynamics (Zhai et al, 1996). During prometaphase, the short, dynamic centrosomal microtubules that remain capture chromosomes (Mitchison & Kirschner, 1985) drive bipolar spindle formation (Magidson et al, 2011) and interact with the cortex to guide positioning of the mitotic spindle (McNally, 2013).

The actin cytoskeleton also undergoes changes over the same period. These begin in prophase when the interphase actin cytoskeleton is disassembled (Matthews et al, 2012). This likely frees up a pool of actin monomers (Kaur et al, 2014), which is then used to assemble a thin (Clark et al, 2013), mechanically rigid (Fischer-Friedrich et al, 2015), cortical actomyosin network that drives mitotic rounding (Reinsch & Karsenti, 1994; Ragkousi & Gibson, 2014; Sorce et al, 2015). While the mechanisms underlying this mitotic switch in actin organisation are not well

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understood, the process likely involves the following: (i) the loss of interphase focal adhesions (Dix et al., 2018; Lock et al., 2018), (ii) the loss of Arp2/3-dependent lamellipodia (Ibarra et al., 2005; Bovellan et al., 2014; Rosa et al., 2015) and (iii) the activation of formins downstream of Ect2/Pbl and the GTPase Rho (Maddox & Burridge, 2003; Matthews et al., 2012; Rosa et al., 2015; Chugh et al., 2017).

Interestingly, these parallel changes in actin and microtubule organisation appear to be largely independent of one another (Mchedlishvili et al., 2018). Thus, mitotic rounding is not much altered in cells entering mitosis without microtubules. Conversely, mitotic spindle assembly occurs with relatively normal kinetics in spherical cells that have been treated with latrunculin to remove their actomyosin cortex (Lancaster et al., 2013). Thus, during mitotic entry, the two systems appear to be independently regulated. However, this changes at anaphase, where the behaviour of the two filament systems is tightly coordinated for proper cell division. The signals emanating from the anaphase spindle polarises the overlying actomyosin cortex (Rappaport, 1996). This is achieved mainly through the activity of the centralspinlindin complex (White & Glotzer, 2012), which binds overlapping microtubules at the midzone. This complex, in turn, recruits Ect2 (Yuice et al., 2005; Su et al., 2011), leading to Rho activation and assembly of a contractile actomyosin ring (Rappaport, 1996; Fededa & Gerlich, 2012), which drives cytokinesis (Wagner & Glotzer, 2016; Basant & Glotzer, 2018). At the same time, as the anaphase spindle elongates, signals associated with the anaphase chromatin appear to aid relaxation of the polar cortical actomyosin network (Salmon & Wolniak, 1990; Motegi et al., 2006; von Dassow, 2009; Ramkumar & Baum, 2016). This leads to the de-phosphorylation of ERM proteins, which cross-link actin to the plasma membrane (Rodrigues et al., 2015), to the loss of anillin (Kiyomitsu & Cheeseman, 2013) and to activation of SCAR/WAVE and the Arp2/3 complex at opposing cell poles (Zhang & Robinson, 2005; King et al., 2010; Nezis et al., 2010; Bastos et al., 2012; Luo et al., 2014). In some instances, the process of polar relaxation and cell re-spreading is sufficient to drive division in cells that lack an actomyosin ring (Neujahr et al., 1997; Dix et al., 2018).

In studies looking at the role of the actin cytoskeleton in division, the mitotic cortical actomyosin network has been subject to most scrutiny. This is because cortical cytoskeleton controls animal cell shape and is by far the brightest actin-based structure visible under the microscope. However, two groups have reported the existence of dynamic cytoplasmic actin-based structures in dividing HeLa cells (Mitsushima et al., 2010; Field & Lénárt, 2011; Fink et al., 2011). While the precise function of this pool of cytoplasmic actin remains unclear, it has been reported to play a role in spindle assembly and positioning in various systems (Woolner et al., 2008; Sabino et al., 2015). In addition, cytoplasmic actin appears to work together with an unconventional Myosin, Myo19, to aid the partitioning of mitochondria at anaphase (Rohn et al., 2014).

Here, building on a previous study that identified the WASH/Arp2/3-dependent nucleation of actin at centrosomes in interphase cells (Farina et al., 2016), we have re-examined the dynamics and potential function of non-cortical actin at mitotic exit. Using a combination of cell biology and biochemistry, we report the identification of a pool of WASH/Arp2/3-dependent cytoplasmic actin that is nucleated around centrosomes in early anaphase, which appears to limit the nucleation of centrosomal microtubules.

Results

In order to explore the possibility that actin is nucleated at centrosomes during mitotic exit, as it is in interphase cells (Farina et al., 2016), we fixed a population of HeLa cells and examined the amount of F-actin (phalloidin) and microtubules in a region close to centrosomes at different cell cycle stages. This revealed an increase in the density of F-actin in a small region around the centrosomes during the passage from metaphase to early anaphase (Fig 1A and B). During this period, we observed no significant changes in the levels of non-centrosomal cytoplasmic actin (Fig EV1A and C). This increase in centrosomally associated actin was accompanied by a decrease in microtubules intensity in the same region (Figs 1A and EV1B). A similar increase in actin accumulation and a corresponding decrease in tubulin intensity were also observed around the centrosomes of early anaphase Jurkat cells, a T-lymphocyte cell line (Fig EV1E and F). To investigate the dynamics of actin during this period, we used a spinning disc confocal to image HeLa cells expressing Lifeact-GFP. We observed a dynamic pool of cytoplasmic actin in metaphase, as described previously (Mitsushima et al., 2010; Fink et al., 2011). At anaphase, this pool became concentrated sub-cortically around the opposing poles (Figs 1C and EV2, Movies EV1–EV3). In order to define the dynamics of actin accumulation around the centrosomes, we generated a stable HeLa cell line expressing RFP-Lifeact and GFP-tubulin and performed relatively high temporal resolution acquisition (Fig 1D). Shortly after anaphase onset, we observed an increase in the levels of actin around centrosomes (Fig 1D–F), while non-centrosomal cytoplasmic actin levels remained unchanged (Fig EV1A and D). This burst of actin filament formation was extremely transient, occurring within minutes of anaphase onset, and was over by the time the cytokinetic furrow became clearly visible (Figs 1C–F and EV2, Movies EV1–EV3). Further, we observed this transient accumulation of actin around centrosomes during anaphase using diverse actin reporters (si-R-actin, Lifeact-GFP and RFP-Lifeact), albeit with different accumulation dynamics that we attribute to the nature of the probes and their differing actin-binding kinetics (Figs 1 and EV2). During the same period, the density of microtubules, measured as an integrated intensity in a small region around spindle poles, dropped (Fig 1D and G). Thus, this transient appearance of cytoplasmic actin close to centrosomes at mitotic exit is associated with a reduction in the density of centrosomal microtubules.

In order to better visualise centrosomal actin in vivo, and as a method by which to isolate centrosomes for the in vitro experiments (see below), we also carried out similar analysis during monopolar cytokinesis (Hu et al., 2008). For these experiments, we arrested HeLa cells in prometaphase using STLC (Mayer et al., 1999; DeBonis et al., 2004)—a treatment that inhibits the Eg5 motor to prevent the assembly of a bipolar spindle (Fig 2). Actin nucleation was then followed as these prometaphase-arrested cells were forced to exit mitosis through the addition of a Cdk1 inhibitor (RO-3306) (Hu et al., 2008). Importantly, under these conditions, the analysis of centrosomal actin is facilitated by the fact that the monopole contains both centrosomes and remains far from the cell cortex (Fig 2A)—even though many other aspects of cytokinesis appear similar (Karayel et al., 2018). In this experiment, we observed little centrosomal-associated actin in the cells arrested in prometaphase with STLC (Figs 2A and EV3, Movie EV4). However, within ~6 min
Figure 1.

A) Metaphase and Early Anaphase images of Hela Kyoto cells stained with Actin, Tubulin, DAPI, and Pericentrin.

B) Hela centrosomal actin intensity comparison between metaphase and early anaphase.

C) Lifeact-GFP time-lapse images showing actin dynamics from 0 min to 12 min.

D) Metaphase and anaphase timeline with images of Hela cells stained with alpha-tubulin-GFP and RFP-Lifeact.

E) Normalized intensity plot showing actin and tubulin dynamics over time.

F) Hela centrosomal actin intensity comparison with p-values.

G) Hela centrosomal tubulin intensity comparison with p-values.
of Cdk1 inhibitor addition we observed a burst of actin filament formation close to centrosomes (Figs 2A and EV3, Movie EV4). Strikingly, these actin filaments formed parallel bundles that lay in between the astral microtubules emanating from the large monopole present in these cells (Fig 2A and B). To confirm this finding, cells arrested in prometaphase and cells after forced exit were fixed and stained to visualise actin filaments (phalloidin). We observed a similar increase in actin around centrosomes during forced exit in multiple cell lines: in HeLa cells (Fig 2C–E), the Jurkat T-cell line (Fig EV4A and B) and the MAVER1 B-cell line (Fig EV4C and D). Further, this actin accumulation was accompanied by a reduction in the density of centrosomal microtubules (Fig 2E). Thus, cytoplasmic actin appears to transiently accumulate around centrosomes during mitotic exit in both monopolar and bipolar divisions, and this is accompanied by a local decrease in microtubule density.

Previous work demonstrated that the actin formed at interphase centrosomes is nucleated by a local pool of Arp2/3 (Farina et al., 2016). To investigate the role of Arp2/3 complex in actin accumulation during mitotic exit, we treated cells with either DMSO or the Arp2/3 complex inhibitor, CK666, and determined the amount of centrosomal actin in fixed (Fig 3) and live cells (Fig EV5). While the DMSO control behaved as described above, the CK666 Arp2/3 inhibitor was effective in eliminating the formation of cytoplasmic actin, including the actin emanating from the centrosome (Fig 3A–D). This was the case in both regular bipolar mitosis (Fig 3A and B), forced monopolar exit (Fig 3C and D) and live monopolar exit (Fig EV5A and B). While the actin accumulation decreased in cells treated with the Arp2/3 inhibitor, interestingly we found that Arp2/3 inhibition prevented the reduction in the density of microtubules associated with centrosomes during normal mitotic exit (Figs 3E and EV6A) and in monopolar exit (fixed cells—Figs 3F and 6B; live cells—EV5C). This suggests that the pool of actin associated with the centrosomes may influence centrosomal microtubules in early anaphase. This could aid division, as we observed a small percentage of cells with spindle oscillations (data not shown).

These results indicate the role of Arp2/3 in generating the burst of cytoplasmic actin associated with centrosomes at mitotic exit. To determine the localisation dynamics of Arp2/3 during mitotic progression, we fixed and stained cells using an antibody against a component of the complex, p34 (Fig 4A). This revealed a pool of Arp2/3 at the centrosome, marked by centrin 1 (Fig 4A and B) that was very low to undetectable in metaphase, but increased significantly in intensity as cells were forced to leave mitosis (Fig 4A and B). A similar increase in the level of centrosomal Arp2/3 was also observed when we analysed the association of p34 with centrosomes purified from cells before and shortly after forced mitotic exit (Fig 4C and D). Additionally, we observed a pool of Arp2 colocalised with centrin at the centre of centrosomes, which was significantly higher during anaphase (Fig EV7A and B’). These data identify a pool of Arp2/3 that is recruited to centrosomes soon after the onset of anaphase, where it functions to nucleate the formation of local actin filaments.

To validate these findings and to determine whether centrosomes might be a potential source of the cytoplasmic pool of actin formed at anaphase, we performed biochemical experiments on centrosomes isolated from cells arrested in prometaphase and those
Figure 2.

A. Lifeact and α-tubulin imaging of Hela cells over time.

B. Graph showing intensity change over time (min).

C. Images showing Prometaphase arrest and Forced mitotic exit.

D. Hela centrosomal actin normalized intensity.

E. Hela centrosomal tubulin normalized intensity.

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Figure 3.

A. DMSO vs. 200μM CK666

B. Bipolar centrosomal actin

C. DMSO vs. CK666

D. Monopolar centrosomal actin

E. Bipolar centrosomal tubulin

F. Monopolar centrosomal tubulin

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Figure 3. Arp2/3-dependent actin accumulation at the centrosome.

A Maximum projection (2 z-slices) view of HeLa cells pre-treated with DMSO and 0.2 mM CK666 for 15 min during their mitotic exit showing that treatment with CK666 leads to reduced accumulation of actin around the centrosomes during anaphase. Scale bar = 10 μm.

B Quantification of actin around centrosomes for cells treated with DMSO or 0.2 mM CK666, showing the reduction in actin accumulation around the centrosomes following CK666 treatment. DMSO-metaphase = 1 ± 0.4325, n = 54; CK666-metaphase = 0.936 ± 0.4604, n = 43; DMSO-anaphase = 1.8 ± 0.9736, n = 76; CK666-anaphase = 1.087 ± 0.4597, n = 73, one-way ANOVA, P < 0.0001. Data pooled from three independent experiments.

C Z-projection of HeLa cells expressing GFP-centrin 1 pre-treated with DMSO or 0.2 mM CK666 during prometaphase arrest and forced mitotic exit and stained with phalloidin for F-actin.

D Quantification of the level of actin around the centrosome from (C), which shows the reduction in actin accumulation around centrosomes following CK666 pre-treatment. DMSO-STLC = 1 ± 0.02762, n = 99; CK666-STLC = 0.776 ± 0.02186, n = 87; DMSO-RO-3306 = 1.339 ± 0.30348, n = 127; CK666-RO-3306 = 0.7699 ± 0.02246, n = 70, one-way ANOVA, P < 0.0001.

E Quantification of tubulin around centrosomes for cells treated with DMSO or 0.2 mM CK666, showing the failure to reduce tubulin density around centrosomes following CK666 treatment during bipolar exit. DMSO-metaphase = 1 ± 0.1968, n = 54; DMSO-anaphase = 0.867 ± 0.2345, n = 78; CK666-anaphase = 0.7955 ± 0.1275, n = 43; one-way ANOVA, P < 0.0001. Data pooled from three independent experiments. Error bars indicated standard deviation.

F Quantification of tubulin around centrosomes for cells treated with DMSO or 0.2 mM CK666, during monopolar exit, showing the failure to reduce tubulin density around centrosomes following CK666 treatment. Data from three independent experiments. Prometaphase arrest (p.a.) 1 ± 0.34, n = 27, forced mitotic exit (f.m.e) 0.78 ± 0.30, n = 26, p.a. plus CK666 0.49 ± 0.34, n = 30, f.m.e plus CK666 0.92 ± 0.37, n = 30. One-way ANOVA with Sidak’s multiple comparisons test.

Figure 4. Arp2/3-mediated actin nucleation around centrosomes in vitro.

A Z-projection of HeLa cells expressing GFP-centrin 1 immunostained for p34-Arc (sub-domain Arp2/3) following prometaphase arrest and forced mitotic exit for 5 min. N = 3 independent experiments. Scale bar = 10 μm and for zoom = 2 μm.

B Levels of p34-Arc around the centrosome [μm area] were quantified relative to metaphase and increase during forced mitotic exit. STLC arrested = 1 ± 0.01576, n = 153; STLC+RO-3306 = 1.268 ± 0.01748, n = 191; ***P < 0.0001, unpaired t-test with Welch correction. Error bars represent standard deviation.

C Z-projection of centrosomes isolated from cells expressing GFP-centrin 1 during prometaphase arrest and forced exit, which were immunostained with p34-Arc. N = 2 independent experiments. Scale bar = 2 μm.

D Quantification of p34-Arc levels around the centrosome in (C), relative to metaphase, showing its increase during forced exit. STLC arrested = 1 ± 0.00779, n = 93; STLC+RO-3306 = 1.249 ± 0.02446, n = 134; ***P < 0.0001, unpaired t-test with Welch correction. Error bars represent standard deviation.

E Time lapse of in vitro assay on centrosomes isolated from prometaphase-arrested cell and cells forced to exit mitosis showing centrosomal actin nucleation over time. Scale bar = 10 μm.

F Quantification of actin nucleation from isolated centrosomes as in (E) over an area of 2 μm (white circle), showing an increase in actin nucleation around centrosomes isolated from cells undergoing forced mitotic exit. N = 2 isolations, five independent experiments, n = 16 STLC and n = 19 STLC-RO.

G Colour switch experiment using green- and red-labelled actin. Red actin was used first, followed by capping protein, and then green-labelled actin. The accumulation of green in the centre indicates that actin is nucleated at the centrosome. Scale bar = 10 μm.

H Quantification of (G) over the time. N = 2 isolations, two independent experiments, n = 19 STLC and n = 14 STLC-RO. Error bars represent standard deviation.

I Time lapse showing cytoplasmic actin filament formation at centrosomes isolated from cells undergoing forced exit when pre-treated with DMSO or CK666. Scale bar = 10 μm.

J Quantification of (I), showing that centrosomal actin nucleation fails when centrosomes isolated from cells undergoing exit are pre-treated with CK666. N = 2 isolations, two independent experiments, n = 17 RO+DMSO, 26 RO+CK666 24 STLC+DMSO, 23 STLC+CK666. Error bars represent standard deviation.

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WASH RNAi appears to have the same effect as a treatment with the Arp2/3 inhibitor, CK666.

**Discussion**

This paper identifies a role for Arp2/3 and its upstream activator, WASH complex, in the nucleation of actin filaments from centrosomes at mitotic exit. While it has long been clear that the spindle directs the assembly of a contractile actomyosin ring at anaphase, and that actin and microtubules work together to control cell shape and organisation (Huber et al., 2015), there has been little evidence of crosstalk between the two filament systems occurring in the opposite direction during mitosis. The data presented here, along with data in studies carried out in lymphocytes in interphase (Inoue et al., 2019) and in Xenopus egg extracts in vitro (Collin et al., 2018), suggest that actin may also play a role in tuning microtubule nucleation at the centrosome.
There are many ways in which the microtubule network can be influenced by actin filaments (Rodriguez et al., 2015; Coles & Bradke, 2015; Huber et al., 2015; Colin et al., 2018). Actin can direct the growth and alignment of microtubules (Kaverina et al., 1998; Therè et al., 2006; Lopez et al., 2014; Elie et al., 2015), can change MT dynamics (Zhou et al., 2002; Hutchins & Wray, 2014) and can subject MTs to mechanical forces and physical constraints (Gupton et al., 2002; Brangwynne et al., 2006; Fakhri et al., 2014; Huber et al., 2015; Robison et al., 2016; Katrucka et al., 2017; Colin et al., 2018). These interactions occur along the length of microtubules and at their growing plus ends (Ahkmanova & Steinmetz, 2015; Mohan & John, 2015), and are especially prevalent at the cell periphery (Waterman-Storer & Salmon, 1997; Wittmann et al., 2003), where the two filament systems converge in a crowded space.

The presence of actin at the centrosome (Farina et al., 2016) now provides an additional spatial region where the two filaments systems can interact to regulate one another’s behaviour. Here, we show that centrosomal actin is generated through the local recruitment and utilisation of the Arp2/3 complex during anaphase. This is likely to be regulated by the change in Cdk1 activity at mitotic exit. The anaphase burst of centrosomal actin is also dependent on the activity of the WASH complex. The timing of centrosomal actin nucleation correlates with the shift in the WASH1 band (Fig 5A and B), suggesting that this process might be regulated by the change in mitotic kinases and phosphatases activity at mitotic exit. This could be augmented by the mitotic regulation of Arp2/3 complex (Figs 4A–D, EV7A and B). The precise nature of the activation of these complexes and their crosstalk remains to be determined.

Like Arp2/3 on a bead, bacterium or patch (Pollard et al., 2000; Pollard & Borisy, 2003; Reymann et al., 2011), the local activation of Arp2/3 is expected to generate actin filaments that have their minus ends fixed in place, while their plus ends grow out into the cytoplasm (Figs 1C and D, EV2, Movies EV1–EV3). This is in keeping with the fact that the extension of these asters can be capped by capping protein (Fig 4G). However, in cells exiting mitosis normally (Figs 1C and D, EV2, Movies EV1–EV3), centrosomes also appear to nucleate a much more diffuse cytoplasmic pool of actin. This may result from Arp2/3-dependent branching of centrosomally nucleated filaments in the cytoplasm of anaphase cells. Together, this pool of cytoplasmic WASH-Arp2/3-dependent actin may have an impact on the viscosity of the cytoplasm (Moulding et al., 2012), as has been shown in meiotic cells (Chaigne et al., 2016). Cytoplasmic actin could act in this manner, to limit the movement of separated organelles after the disassembly of the anaphase spindle—as has been shown for mitochondria (Rohn et al., 2014).

Previous studies have suggested mitotic roles for cytoplasmic actin in centrosome separation (Rosenblatt et al., 2004; Cao et al., 2010), pole splitting (Sabino et al., 2015) and spindle assembly (Woolner et al., 2008; P’uha & Kavallaris, 2015; Vilmos et al., 2016).
Figure 6.
2016). Our data suggest that the generation of centrosomal actin in metaphase is limited. Moreover, in HeLa cells, where this cytoplasmic actin was first observed (Mitsushima et al., 2010; Fink et al., 2011), Arp2/3 complex activity has little influence on the timing of mitotic progression (Lancaster et al., 2013) and contributes little to metaphase cortical mechanics (Chugh et al., 2017). However, our data support the idea that cytoplasmic actin has a significant function at the onset of anaphase (including in the control of mitochondrial movement (Rohn et al., 2014)), when CDK1-mediated phosphorylation of WASH is relieved and active Arp2/3 returns to the centrosome to induce a burst of actin.

This burst in centrosomal actin is accompanied by a reduction in the microtubule density around the centrosomes at early anaphase. In the absence of centrosomal actin nucleation, with Arp2/3 inhibitor, this reduction in microtubule density is inhibited. This suggests an antagonistic crosstalk between the two filament systems at mitotic exit. The precise nature of this antagonism between local actin and microtubule formation at the centrosomes remains to be determined (Piel et al., 2001; Obino et al., 2016; Inoue et al., 2019). We speculate that this burst of anaphase actin nucleation at centrosomes may cause steric problems, as the two systems compete for physical space around the centrosome. However, in live cells, we cannot rule out the possibility of an indirect crosstalk mediated by changes in localisation or activity of proteins affecting post-translational modifications of tubulin during mitotic exit (Shi et al., 2019). Further, it is possible that the centrosomal actin pool could have a more profound effect depending on the nature of microtubules, i.e. astral vs spindle microtubules at mitotic exit.

The actin dependent reduction in microtubule density around centrosomes may help cells leaving mitosis to re-establish a telophase array of sparse, long centrosomal microtubules. These microtubules can then span the entire space of the cells, assisting in polar relaxation, adjusting spindle orientation (Kiyomitsu & Cheeseman, 2013) and/or promoting re-spreading (Ferreira et al., 2013). In short, this reduction in microtubule density at early anaphase could assist cells exiting mitosis to return to their interphase organisation and morphology.

Materials and Methods

Cell culture

HeLa Kyoto cells were cultured in DMEM, and Jurkat cells (immortalised human T lymphocytes) as well as MAVER1 CML B cells in RPMI 1640 (Gibco) at 37°C and 5% CO₂. All media were supplemented with 10% foetal bovine serum and penicillin/streptomycin (Gibco). Cells were synchronised in prometaphase using S-trityl-L-cysteine (STLC; Sigma) at 5 μM treatment for 18 h (Skoufias et al., 2006). Forced mitotic exit was performed by the addition of 20 μM RO-3306 (Enzo Life Sciences), an inhibitor of Cdk1/cyclin B1 and Cdk1/cyclin A to STLC-treated cells. For the Arp2/3 complex inhibition experiments, we used 0.2 mM CK666 (Sigma-Aldrich). Control experiments were performed using DMSO. For CK666 treatment (Fig 3), cells were plated with 2 mM thymidine for 22 h, were released from thymidine block for 9 h, following which they were treated with DMSO or 0.2 mM CK666 for 15 min. They were then processed for staining. Actin network disruption was performed by adding 10 μg/ml cytochalasin D (Sigma-Aldrich). Microtubule depolymerisation was performed by adding 1 μM nocodazole (Sigma-Aldrich) for 1 h at 37°C and 5% CO₂ and for 30 min at 4°C.

Stable and transient cell transfection

Stable HeLa cell lines expressing GFP-α-tubulin and RFP-Lifeact were established starting from HeLa stably expressing GFP-α-tubulin by lentiviral transduction with rLV-Lifeact-RFP (Ibidi) using 3 MOI (multiplicity of infection). After 24 h, cells were incubated with fresh medium for 48 h. After 72 h post-transduction, stable cells were selected using 1 μg/ml puromycin. Medium with puromycin was replaced every 2–3 days until resistant colonies were identified. ON-TARGETplus Human SMARTpool WASH1-targeting (siWASH1) siRNAs (Dharmacon, GE Healthcare) were transfected into HeLa cells at a final concentration of 20 nM using Lipofectamine RNAiMax (Life Technologies) according to supplier’s protocol. Negative control siRNA was performed using AllStars Negative Control siRNA (Qiagen).

Isolation of centrosomes

Centrosomes were isolated from cells arrested in prometaphase (STLC) or shortly after their forced mitotic exit (RO-3306 5′) using a previously published protocol (Farina et al., 2016). Cells were incubated for 18 h with STLC and then treated with nocodazole (0.2 μM) and cytochalasin D (1 μg/ml). For the mitotic exit, we added to STLC-treated cells 20 μM RO-3306 for 5 min, and cells were kept on ice for 30 min. Centrosomes were then harvested by centrifugation onto a 60% sucrose cushion and further purified by centrifugation through a discontinuous (70, 50 and 40%) sucrose gradient. Composition of sucrose solutions was based on TicTac buffer (10 mM Hepes, 16 mM Pipes (pH 6.8), 50 mM KCl, 5 mM MgCl₂, 1 mM EGTA). The TicTac buffer was supplemented with 0.1% Triton X-100 and 0.1% β-mercaptoethanol.

Protein expression and purification

Tubulin was purified from fresh bovine brain by three cycles of temperature-dependent assembly/disassembly in Brinkley Buffer 80 (BRB80 buffer: 80 mM Pipes pH 6.8, 1 mM EGTA and 1 mM MgCl₂) according to Shelanski (Shelanski, 1973). Fluorescent tubulin (ATTO-565-labelled tubulin) was prepared according to Hyman et al (1991). Actin was purified from rabbit skeletal-muscle acetone powder (Spudich & Watt, 1971). Monomeric Ca-ATP-actin was purified by gel-filtration chromatography on Sephacryl S-300 (MacLean-Fletcher & Pollard, 1980) at 4°C in G buffer (2 mM Tris–HCl, pH 8.0, 0.2 mM ATP, 0.1 mM CaCl₂, 1 mM NaN₃ and 0.5 mM dithiothreitol (DTT)). Actin was labelled on lysines with Alexa-488, Alexa-568 and Alexa-647 as described previously (Isambert et al., 1995; Egle et al., 1999). Recombinant human profilin, mouse capping protein, the Arp2/3 complex, GST-pWA and mDia1 were purified according to previous work (Almo et al., 1994; Egle et al., 1999; Machesky et al., 1999; Falck et al., 2004; Michelot et al., 2007).

In vitro assay

This was done essentially as in Farina et al (2016). Briefly, experiments were performed in polydimethylsiloxane (PDMS) open chambers in order to sequentially add experimental solutions when
needed. PDMS (Sylgard 184 Kit, Dow Corning) was mixed with the curing agent (10:1 ratio), degassed, poured into a Petri dish to a thickness of 5 mm and cured for 30 min at 100°C on a hot plate. PDMS layer was cut to 15 × 15 mm and punched using a hole puncher (Ted Pella) with an outer diameter of 8 mm. The PDMS chamber and clean coverslip (20 × 20 mm) were oxidised in an oxygen plasma cleaner for 20 s at 80 W (Femto, Diener Electronic) and brought into contact. Isolated centrosomes were diluted in TicTac buffer (10 mM Heps, 16 mM Pipes (pH 6.8), 50 mM KCl, 5 mM MgCl₂, 1 mM EGTA) and incubated for 20 min. Excess centrosomes were removed by rinsing the open chamber with large volume of TicTac buffer supplemented with 1% BSA to prevent the non-specific interactions (TicTac-BSA buffer). Microtubules and actin assembly at the centrosome were induced by diluting tubulin dimers (labelled with Alexa-488, or Alexa-568, or Alexa-647, 1 µM final) in TicTac buffer supplemented with 1 mM GTP and 2.7 mM MgCl₂, 1 mM EGTA) and incubated for 20 min. Excess centrosomes were removed by rinsing the open chamber with large volume of TicTac buffer supplemented with 1% BSA to prevent the non-specific interactions (TicTac-BSA buffer). Microtubules and actin assembly at the centrosome were induced by diluting tubulin dimers (labelled with Alexa-488, or Alexa-568, or Alexa-647, 1 µM final) in TicTac buffer supplemented with 1 mM GTP and 2.7 mM ATP, 10 mM DTT, 20 µg/ml catalase, 3 mg/ml glucose, 100 µg/ml glucose oxidase and 0.25% w/v methylcellulose. In addition, a threefold molar equivalent of profilin to actin was added in the reaction mixture. Antibody inhibition experiments were performed by incubating isolated centrosomes with primary antibodies (diluted in TicTac-BSA buffer) for 1 h. The control experiment without antibodies was performed incubating isolated centrosomes for 1 h with TicTac-BSA buffer. Arp2/3 complex inhibition experiments were performed by adding 0.2 mM CK666 in the reaction mixture.

**Immunofluorescence staining (in cell)**

This was done essentially as in Farina et al (2016). Briefly, cells were incubated for 18 h with STLC. Forced mitotic exit was performed incubating STLC cells with RO-3306 for 5 min. Cells were then fixed and stained. For actin filament staining, cells were fixed with 4% paraformaldehyde (PFA) for 20 min, blocked with antibody blocking buffer (PBS supplemented with 1% BSA, PBS-BSA) for 30 min. Permeabilisation was performed with 0.2% Triton X-100 for 1 min. Alexa-647-phalloidin (200 nM) was incubated for 20 min. DNA was labelled with a 0.2 µg/ml solution of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma). The coverslips were air-dried and mounted onto glass slides using Mowiol mounting medium. Arp2/3 complex was performed by fixing cells with methanol at −20°C for 3 min and blocking with PBS-BSA for 30 min. Primary and secondary antibodies, diluted in PBS-BSA, were incubated for 1 h and 30 min, respectively. DNA labelling and coverslip mounting were performed as previously described.

**Immunofluorescence staining (isolated centrosomes)**

This was done essentially as in Farina et al (2016). Briefly, staining of F-actin on centrosomes was performed without prior fixation. Isolated centrosomes were incubated with primary antibodies for 1 h and with secondary antibodies for 30 min at room temperature. The antibodies were diluted in TicTac-BSA buffer.

**Imaging, processing and analysis**

This was done essentially as in Farina et al (2016). Briefly, fixed cell images were captured on a confocal microscope (Leica SP5) using a 40× 1.25 N.A. objective lens, 63× 1.4 N.A. or Zeiss LSM800 with a 63× 1.4NA lens. Live cell imaging was performed on a UltraView Vox (Perkin Elmer) spinning disc confocal microscope with 60× NA 1.4 oil objective and 100× 1.4 N.A. and 3× spinning disc confocal with 63× 1.4 N.A and 100× 1.4 N.A objectives equipped with a temperature-controlled environment chamber. Image processing was performed using ImageJ software. All the images show the centrosome plane. Measurement of the actin amount around the centrosome was performed by measuring the integrated intensity of fluorescence in a 4-µm-diameter circle centred around the centrosome. p34-Arc measurements were performed measuring the integrated fluorescence intensity in a 3-µm-diameter circle centred around the centrosome. Data from separate experiments were normalised so that the average intensity in control cells was 1. Imaging of isolated centrosomes was performed with a total internal reflection fluorescence (TIRF) microscope (Roper Scientific) equipped by an iLasPulsed system and an Evolve camera (EMCCD 512 × 512, pixel = 16 µm) using a 60× 1.49 N.A objective lens. Actin nucleation activity was quantified measuring the actin fluorescence intensity integrated over a 2 µm diameter at the centre of the actin aster and normalised with respect to initial intensity over the time. Representative data for several experiments are shown.

**Actin staining and measurement bipolar divisions (Figs 1 and 3)**

Fixed cells—Cells were cultured in 96-well cell carrier plates. For staining, cells were fixed with 4% paraformaldehyde (PFA) for 20 min and permeabilised with 0.1% Triton X-100 for 10 min. They were blocked in antibody blocking buffer (PBS supplemented with 5% BSA, PBS-BSA) for 30 min and incubated with primary antibodies—anti-tubulin (1:400) and anti-pericentrin (1:1,000) overnight at 4°C. Cells were incubated with secondary antibodies—goat anti-mouse Alexa-647, goat anti-rabbit Alexa-568, phalloidin-FITC (1:500) and DAPI (4′,6-diamidino-2-phenylindole dihydrochloride-1:1,000) for 1 h at room temperature. Jurkat cells were fixed with 4% paraformaldehyde (PFA) for 20 min in suspension and were adhered to 96-well plates coated with Poly-L-lysine.

For analysis, a 4-µm-diameter (for HeLa) and 3-µm-diameter (for Jurkat) circle was centred around the centrosome using the pericentrin channel and the corresponding actin- and tubulin-integrated density around the same region was measured using Fiji. The images used were 2-z-projection around the centrosome plane. In order to combine data from multiple experiments, the data were normalised to metaphase average for each cell line. For live cells, a 4-µm-diameter circle was centred around centrosomal region using alpha-tubulin RFP as reference. A 4-z-projection around centrosome region was used for measurements. The intensity data for each cell was normalised to the intensity at t = 0, which is one frame before anaphase onset.

**Actin measurement forced exit (Figs 2E and F and EV1)**

A circular, 3 µm² ROI was centred on the centrosomes as determined by pericentrin staining. A series of slices equal to approximately 3 µm in height (with z interval of 0.39 µm, the number of slices was 9, including the central slice) were then analysed using the defined ROI to give a mean intensity per slice, which were then averaged to give the mean intensity within the 3 × 3 × 3 cylindrical
area. Individual cell averages were normalised to the experimental average for the STLC condition.

Statistics

Statistical analysis was performed with GraphPad Prism 7 (GraphPad Software). All graphs show mean and error bars are standard deviation. The test used is mentioned for each graph in figure legend. In all the graphs, prism convention: ns ($P > 0.05$), *($P < 0.05$), **($P < 0.01$), ***($P < 0.001$) and ****($P < 0.0001$).

Western blotting

Western blots were performed fractioning proteins on SDS polyacrylamide gels. Membrane blocking was carried out using 3% BSA in PBS. Primary and secondary antibodies were diluted in PBS supplemented with 1% BSA and 0.1% Tween-20 while washing steps were performed with PBS supplemented with 1% BSA and 1% Tween-20.

Blue NativePAGE

This was done essentially as in Tyrrell et al (2016). Briefly, cells were lysed using the NativePAGE™ Sample Prep Kit (Thermo Scientific—BN2008). Each sample was lysed in 400 μl of NP-lysis buffer [100 μl of NP buffer, 260 μl of ddH2O, 40 μl 5% digitonin, halt protease inhibitor (Pearce) and halt phosphatase inhibitor (Pearce)] for 10 min on ice and scraped with a cell scraper. Samples were centrifuged at 20,000 g for 30 min at 4°C, and pellets were discarded. Equal amounts of protein (determined using DC™ Protein Assay Kit (Bio-Rad—5000111)) were separated by BN-PAGE NativePAGE™ Novex™ 3–12% Bis-Tris Protein Gels as per the manufacturer’s instructions (NativePAGE™ Bis-Tris Gel protocol from Thermo Scientific). NativeMark™ Unstained Protein Standard (Thermo Fisher Scientific) was used as molecular weight standard. Protein was blotted onto PVDF membranes and fixed for 15 min in 8% acetic acid. Membranes were blocked in 5% milk for 1 h at room temperature and incubated overnight rocking at 4°C in 5% milk PBS-T. Membranes were washed in PBS-T and incubated for 1 h with peroxidase-conjugated secondary antibody (Cell Signalling, Hitchin, UK). Clarity Western blotting ECL substrate (Bio-Rad) was used to generate a signal that was detected using a Chemidoc imaging system (Bio-Rad).

Phos-tag band shifts

Cells were lysed using hot lysis buffer (2% SDS, 1 mM EDTA, 50mM HCl, preheated to 97°C). Samples were allowed to cool at RT before performing protein assay using DC™ Protein Assay Kit (Bio-Rad—5000111). Sample buffer was added, and equal protein was loaded into 8% polyacrylamide gels supplemented with 5 μM Phos-tag and 10 μM MnCl2. Phos-tag gels were resolved at 30 mA under constant current until the dye front migrated to the bottom of the gel. Gels were transferred onto nitrocellulose membrane with a 0.45 pore size (GE Healthcare) at 250 mA for 120 min under constant current.

Antibodies and chemicals

For immunofluorescence staining, we used the following antibodies: mouse anti-p34-Arc (Dubois et al, 2005) (undiluted), mouse anti-tubulin (Sigma T9026), rabbit anti-pericentrin (ab4448), phalloidin-FITC (P5282) and Alexa-647 phalloidin (Fluka 65906). For inhibition experiments on isolated centrosomes, we used rabbit anti-WASH antibodies (Derivery et al, 2009) (2 μg/ml) (gift Alexis).

For Western blot analysis, we used rabbit anti-WASH1 (1: 1,000, ab157592, Abcam), rabbit anti-WASH (1:1,000; Atlas), strumpellin (1: 1,000, ab101222, Abcam) and anti-beta-tubulin (sc-5274, Santa Cruz). Labelled anti-mouse and anti-rabbit secondary antibodies (1: 1,000) and HRP-conjugated goat IgG anti-mouse and anti-rabbit (1: 10,000) for Western blot were obtained from Jackson ImmunoResearch.

Cytochalasin D and nocodazole were purchased from Sigma-Aldrich. PFA was purchased from Delta Microscopies. CK666 was purchased from Sigma-Aldrich. Alexa-647-phalloidin was purchased from Life Technologies.

Expanded View for this article is available online.

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Author contributions

NR carried out the cell culture experiments for normal bipolar cytokinesis as well as measuring this. GB and ARHGEF10, MT and LB (Louise) carried out the experiments for monopolar and bipolar cytokinesis, respectively. FF and BB conceived the initial idea. NR and BB oversaw the development of the project and wrote the manuscript with assistance of TZ. LB (Louise) and TZ did WASH biochemistry. TW, JB and TZ did the monopolar cytokinesis in Jurkat and MAVE1 cell lines. MT and LB (Laurent) oversaw in vivo work and advised on in vivo work, together with help from GS.

Conflict of interest

The authors declare that they have no conflict of interest.

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