PRC1 controls spindle polarization and recruitment of cytokinetic factors during monopolar cytokinesis

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ABSTRACT **The central spindle is a postanaphase array of microtubules that plays an essential role in organizing the signaling machinery for cytokinesis. The model by which the central spindle organizes the cytokinetic apparatus is premised on an antiparallel arrangement of microtubules, yet cells lacking spindle bipolarity are capable of generating a distal domain of ectopic furrowing when forced into mitotic exit. Because protein regulator of cytokinesis (PRC1) and kinesin family member 4A (KIF4A) are believed to play a principal role in organizing the antiparallel midzone array, we sought to clarify their roles in monopolar cytokinesis. Although both factors localized to the distal ends of microtubules during monopolar cytokinesis, depletion of PRC1 and KIF4A displayed different phenotypes. Cells depleted of PRC1 failed to form a polarized microtubule array or ectopic furrows following mitotic exit, and recruitment of Aurora B kinase, male germ cell Rac GTPase-activating protein, and RhoA to the cortex was impaired. In contrast, KIF4A depletion impaired neither polarization nor ectopic furrowing, but it did result in elongated spindles with a diffuse distribution of cytokinetic factors. Thus, even in the absence of spindle bipolarity, PRC1 appears to be essential for polarizing parallel microtubules and concentrating the factors responsible for contractile ring assembly, whereas KIF4A is required for limiting the length of anaphase microtubules.**

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INTRODUCTION

Cytokinesis is the terminal stage of cell division by which a dividing cell partitions the cytoplasm following chromosome segregation, and these temporally and spatially distinct events are coordinated in such a manner that dividing cells are protected from the negative consequences of aneuploidy or tetraploidy (Glotzer, 2001; Barr and Gruneberg, 2007). Unlike budding yeast and plants, in which the cleavage plane is determined by nuclear or cortical landmarks prior to the onset of mitosis, the cleavage plane in animal cells is specified by microtubules of the mitotic apparatus following anaphase onset (Field *et al.*, 1999; Burgess and Chang, 2005; Oliferenko *et al.*, 2009; Almonacid and Paoletti, 2010). Remarkable progress has been made in understanding how microtubules provide positional information to define the cleavage plane (Glotzer, 2009), and studies of variations among different cell types and the regulation of postanaphase microtubule organization and dynamics remain areas of active investigation.

Following anaphase onset, an antiparallel microtubule array known as the central spindle assembles with the aid of a diverse set of microtubule-associated proteins and motors that is also critical for the placement and assembly of the contractile ring (Glotzer, 2009; Douglas and Mishima, 2010; Hornick *et al.*, 2010). Principal among these is centralspindlin, a heterotetrameric complex comprising mitotic kinesin-like protein (MKLP1), and male germ cell Rac GTPase-activating protein (MgcRacGAP), a Rho-family GTPase-activating protein (Mishima *et al.*, 2002). Together these two components display robust microtubule-bundling activity (Mishima *et al.*, 2002; Hizlan *et al.*, 2006; Pavicic-Kaltenbrunner *et al.*, 2007) and are subject to regulation by Aurora B kinase, whose phosphorylation of MKLP1 is required for formation of the central spindle (Guse *et al.*, 2005; Neef *et al.*, 2006). Aurora B also phosphorylates MgcRacGAP, increasing its GAP activity toward RhoA in vitro (Minoshima *et al.*,

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Abbreviations used: Cdk1, cyclin-dependent kinase 1; CPC, chromosomal passenger complex; Ect2, epithelial cell–transforming protein 2; INCENP, inner centromeric protein; KIF4A, kinesin family member 4A; MgcRacGAP, male germ cell Rac GTPase-activating protein; MKLP1, mitotic kinesin-like protein; Plk1, Polo-like kinase-1; PRC1, protein regulator of cytokinesis.

2003). MgcRacGAP serves as critical regulator of RhoA activation and contractile ring assembly by recruiting the guanine nucleotide exchange factor epithelial cell transforming protein 2 (Ect2) to the central spindle (Somers and Saint, 2003; Yuce *et al.*, 2005; Zhao and Fang, 2005; Chalamalasetty *et al.*, 2006; Nishimura and Yonemura, 2006; Petronczki *et al.*, 2007; Burkard *et al.*, 2009; Wolfe *et al.*, 2009). Concomitantly, its role as a RhoGAP is critical to limiting the zone of RhoA activation to a narrow band at the cell equator (Miller and Bement, 2009). Finally, studies in *Caenorhabditis elegans* have shown that in addition to regulating RhoA, MgcRacGAP also antagonizes Rac1, and this activity is essential for the cleavage furrow ingression (Canman *et al.*, 2008). Thus centralspindlin organization of the midzone and recruitment of RhoA regulatory machinery provides a critical link between the mitotic spindle and reorganization of the actomyosin cytoskeleton during cytokinesis (Piekny *et al.*, 2005).

In addition to the centralspindlin signaling cascade that provides structural and regulatory inputs for cleavage plane determination, the Map65 family of microtubule-associated proteins also plays a critical role in the formation and assembly of the central spindle and cytokinesis in all organisms examined (Jiang *et al.*, 1998; Mollinari *et al.*, 2002, 2005; Schuyler *et al.*, 2003; Muller *et al.*, 2004; Verbrugghe and White, 2004; Verni *et al.*, 2004; Loiodice *et al.*, 2005; Yamashita *et al.*, 2005). Originally identified in humans as a cyclin-dependent kinase 1 (Cdk1) substrate, antibody inactivation of protein regulator of cytokinesis (PRC1) results in cytokinesis failure (Jiang *et al.*, 1998). Depletion PRC1 by RNA interference resulted in two spindle halves (Mollinari *et al.*, 2002), and whereas time-lapse microscopy revealed that PRC1-depleted cells were able to produce a furrow and ingress, they were unable to undergo abscission, ultimately giving rise to binucleated cells (Jiang *et al.*, 1998; Mollinari *et al.*, 2005; Zhu and Jiang, 2005; Zhu *et al.*, 2006). Domain analyses of PRC1 reveal that the N-terminal domain of PRC1 is important for its localization at the central spindle, dimerization, and interactions with MKLP1, CENP-E, and kinesin family member 4A (KIF4A; Jiang *et al.*, 1998; Zhu and Jiang, 2005; Zhu *et al.*, 2006; Mollinari *et al.*, 2002; Kurasawa *et al.*, 2004), whereas the C-terminus contains a docking site for Polo-like kinase 1 (Plk1; Neef *et al.*, 2007). KIF4A colocalizes with PRC1 and is required for concentrating PRC1 at the central spindle (Zhu and Jiang, 2005). In vitro studies demonstrated that Ase1/PRC1 can autonomously bundle antiparallel microtubules (Janson *et al.*, 2007; Bieling *et al.*, 2010; Subramanian *et al.*, 2010), and it has been proposed that through PRC1 bundling and the microtubule-modulating activities of KIF4A, these two factors regulate the width of microtubule overlap at the central spindle (Bieling *et al.*, 2010).

Although the bipolar arrangement of the mitotic spindle has long been believed to be a requirement for the specification of cleavage furrow, recent studies indicate that this is not an absolute requirement for furrow positioning. Indeed, when cultured cells containing monopolar spindles are forced into anaphase and mitotic exit, cells form an array of polarized microtubule bundles that orient toward the cortex and produce ectopic furrows (Canman *et al.*, 2003; Hu *et al.*, 2008). Although it is well established that PRC1 and KIF4A are key elements of the central spindle, it is not known how these factors contribute to the formation of parallel microtubule bundles such as those observed during monopolar cytokinesis. In an effort to understand how these factors function during monopolar cytokinesis, we depleted PRC1 and KIF4A individually or in combination and examined ectopic furrowing, spindle morphology, and the recruitment of known cytokinetic factors. Our results show that PRC1 is required for microtubule polarization and the recruitment of cytokinetic factors during monopolar cytokinesis, suggesting that PRC1 contributes to microtubule organization regardless of orientation. In contrast, KIF4A played no role in microtubule polarization but was required for maintenance of microtubule length and restricted localization of PRC1 and cytokinetic factors to the distal ends of microtubules. Together these observations suggest that by altering the normal geometric arrangement of spindle microtubules, independent roles may be revealed for PRC1 and KIF4A in the formation and maintenance of the central spindle.

RESULTS

Cdk1 inhibition induces multiple ectopic furrows in cells containing monopolar spindles

The assembly of a bipolar spindle requires class 5 kinesins (Eg5) in all eukaryotic cells (Ferenz *et al.*, 2010), and genetic or pharmacological inactivation of Eg5 results in the formation of a monopolar spindle and mitotic arrest (Saunders and Hoyt, 1992; Mayer *et al.*, 1999; Kapoor *et al.*, 2000). In light of reports demonstrating that cytokinesis can be induced in cells containing monopolar spindles (Canman *et al.*, 2003; Hu *et al.*, 2008), we sought to examine the recruitment dynamics of cytokinetic regulators in cells lacking spindle bipolarity. Treatment of HeLa cells with 5 µM *S*-trityl-L-cysteine (STLC; Skoufias *et al.*, 2006) resulted in the generation of monopolar spindles with microtubules radiating outward and the chromosomes arranged in a rosette manner as previously described (Sawin *et al.*, 1992; Blangy *et al.*, 1995; Gaglio *et al.*, 1996; Walczak *et al.*, 1998; Figure 1A, a). These checkpoint-arrested cells were then treated with 10 µM flavopiridol, an inhibitor of Cdk1, to bypass the spindle assembly checkpoint and force the cells into mitotic exit (Potapova *et al.*, 2006). In cells treated in this manner, the radial symmetry of the spindle was broken as early as 10 min following flavopiridol treatment, with microtubules oriented or polarized in a particular direction (Figure 1A, c). By 25–30 min, ectopic furrows formed at the cortical domain most proximal to the polarized microtubule array (Figure 1A, d). Localization of γ-tubulin revealed that both prior to and following flavopiridol treatment, γ-tubulin remained associated with the monopole, with no detectable signal at the distal microtubule ends (Supplemental Figure S1), suggesting that the polarized array was composed of parallel microtubules. We also observed that in HeLa cells, monopolar spindles did not consistently polarize in a single direction but instead displayed multiple sites of polarization and furrowing (Figure 1B), in contrast to earlier studies using Ptk1 cells, which are more adherent to the substratum and display a more uniform directionality of polarization (Canman *et al.*, 2003). Analysis of cells fixed 25 min following flavopiridol treatment (n = 218) revealed that 39% of furrowing cells had more than one axis of polarization. To investigate the dynamics of ectopic furrowing in living cells, we examined monopolar cells expressing green fluorescent protein (GFP)–tubulin and forced into mitotic exit with flavopiridol. Consistent with immunofluorescence data, we were able to detect polarized microtubule bundles and multiple furrows when monopolar cells were treated with flavopiridol (Figure 1C, i–l, and Supplemental Movie S1). Robust membrane blebbing was observed both at the site of polarization and along the periphery in nearly all cells (97%), with a recognizable microtubule bundle and ectopic furrow observed in 84% of cells (n = 61).

Recruitment of key cytokinetic factors to sites of ectopic furrowing in cells lacking bipolarity

We next examined the recruitment dynamics of key cytokinetic factors during monopolar cytokinesis. Aurora B, the catalytic subunit of the chromosomal passenger complex (CPC), relocalized to the

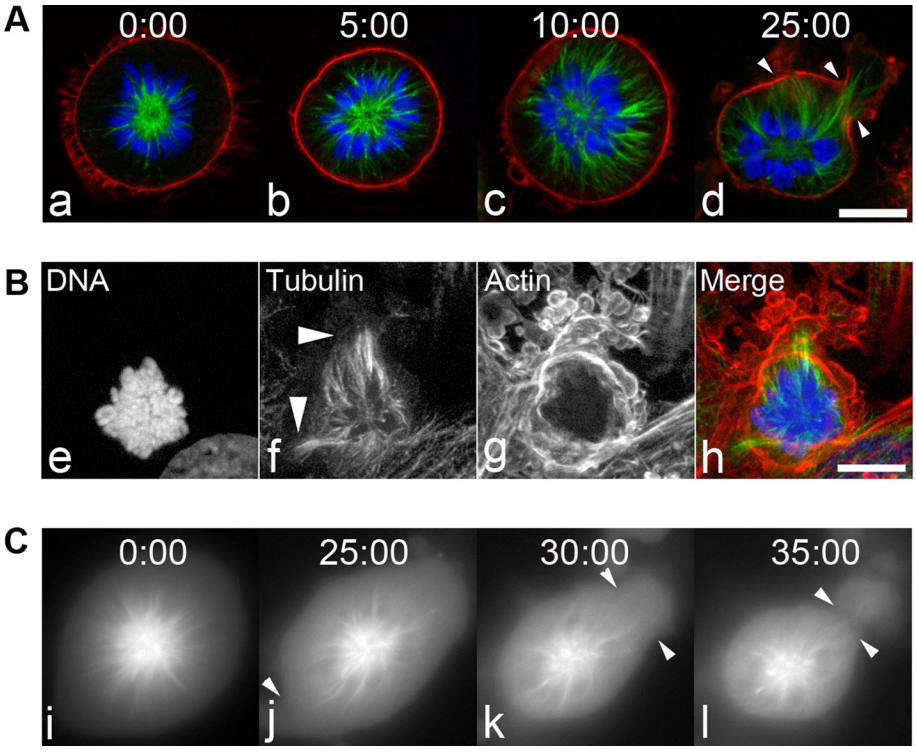

FIGURE 1: Characterization of ectopic furrow formation during forced cytokinesis. (A) HeLa cells were treated with 5 μM STLC for 6 h to generate monopolar spindles. Cells were subsequently treated with 10 μM flavopiridol in the presence of STLC to induce mitotic exit. Cells were fixed at different time points and processed for actin (red), tubulin (green), and DNA (blue) localization. Numbers denote time in minutes following flavopiridol treatment. (B) Three-dimensional reconstruction of a monopolar HeLa cell undergoing forced cytokinesis to highlight multiple sites of ectopic furrowing, with arrowheads denoting the major axes of polarization. (C) Selected frames from a time-lapse sequence showing the dynamics of ectopic furrow formation in an enhanced GFP (EGFP)-tubulin–expressing cell following flavopiridol treatment. Arrows denote the position of ectopic furrow formation (also see Supplemental Movie S1). Bar, 10 μm.

distal ends of microtubules, as well as to the cell cortex, as previously reported (Hu *et al.*, 2008; Figure 2, a–d). Identical patterns were also observed with inner centromeric protein (INCENP; unpublished data). We also examined the localization dynamics of Aurora B kinase during forced cytokinesis in cells containing bipolar spindles, in which Aurora B was preferentially recruited to the equatorial region of the cell where the central spindle would normally form following anaphase onset. Moreover, although a trace amount of Aurora B could be seen at the cortex at the vicinity of the central region, we did not observe Aurora B signal at the astral microtubules pointing toward the polar regions or at the cortex or the space between the tips of astral microtubules and the cortex (Supplemental Figure S2, a–c).

One of the principal targets of Aurora B during anaphase is centralspindlin, a complex comprising MKLP1 and MgcRacGAP that is required for central spindle formation and completion of cytokinesis (Mishima *et al.*, 2002). When we examined the recruitment of centralspindlin components during forced monopolar cytokinesis, we found these regulators at the tips of polarized microtubules, as previously reported (Figure 2, e–h; Hu *et al.*, 2008), and in bipolar cells, and they displayed a localization pattern similar to that of Aurora B (Supplemental Figure S2, d–f). Similarly, both KIF4A and PRC1 were found at the tips of the polarized microtubules in monopolar spindles (Figure 2, i–p), whereas in bipolar spindles undergoing forced mitotic exit, PRC1 displayed a slightly broader localization pattern (Supplemental Figure S2, g–i).

In contrast to the other cytokinetic factors, Plk1 did not exhibit a robust recruitment to sites of ectopic furrowing (Supplemental Figure S3A). Plk1 could be observed at sites of ectopic furrowing in only 5% of monopolar cells, whereas in the vast majority of the cells (92%), Plk1 was weakly associated with the polarized microtubules as analyzed by immunofluorescence (n = 673). To dissect whether this poor recruitment is unique to the monopolar cytokinesis or a phenomenon occurring during forced cytokinesis in general, we forced cells with bipolar spindles and arrested in metaphase to exit mitosis with flavopiridol. Immunofluorescence analysis of these cells revealed that the cortical enrichment of RhoA in bipolar cells undergoing forced mitotic exit was not always accompanied by robust presence of Plk1 at the ectopic furrow site (Supplemental Figure S3B), suggesting that although Plk1 is required for Ect2-mediated RhoA activation (Petronczki *et al.*, 2007; Burkard *et al.*, 2009; Wolfe *et al.*, 2009), it may be able to accomplish this modulation over a distance.

PRC1 is required for microtubule polarization during monopolar cytokinesis

The Ase1/PRC1/MAP65 family preferentially bundles antiparallel microtubules and is required for the organization of the spindle midzone and completion of cytokinesis (Jiang *et al.*, 1998; Schuyler *et al.*, 2003; Muller *et al.*, 2004; Verbrugghe and White, 2004; Verni *et al.*, 2004; Loiodice *et al.*, 2005; Yamashita *et al.*, 2005). Given that antiparallel organization of microtubules appears to be dispensable for cytokinesis (Canman *et al.*, 2003; Hu *et al.*, 2008), we sought to determine how PRC1 participates in monopolar cytokinesis. Toward these ends, PRC1 was depleted from HeLa cells with control or targeting small interfering RNA (siRNA) for 42 h followed by STLC arrest and analysis by Western blotting (Figure 3A) and immunostaining (unpublished data) to confirm the efficiency of siRNA depletion. When control or PRC1-depleted cells were treated with STLC and then forced into mitotic exit, PRC1-depleted cells failed to polarize and produce ectopic furrows. Examination of fixed cells stained for actin and microtubule localization revealed that >70% of the PRC1-depleted cells failed to polarize and furrow (n = 1366), whereas >90% of the control cells polarized or were furrowing by 25 min posttreatment (n = 1128; Figure 3B). Microtubule organization in PRC1-depleted cells was characterized by a radial array of microtubules, frequently tracking circumferentially around the cortex (Figure 3C, f and n). Examination of RhoA recruitment in PRC1-depleted cells revealed that RhoA was found throughout the cortex (Figure 3C, e–h), whereas in controls, RhoA was enriched at the cortical region where the polarized microtubules were oriented (Figure 3C, a–d). In contrast to control cells in which actin was enriched at the site of microtubule polarization and ectopic furrowing (Figure 3C, i–l), the organization of F-actin in PRC1-depleted cells resembled actin

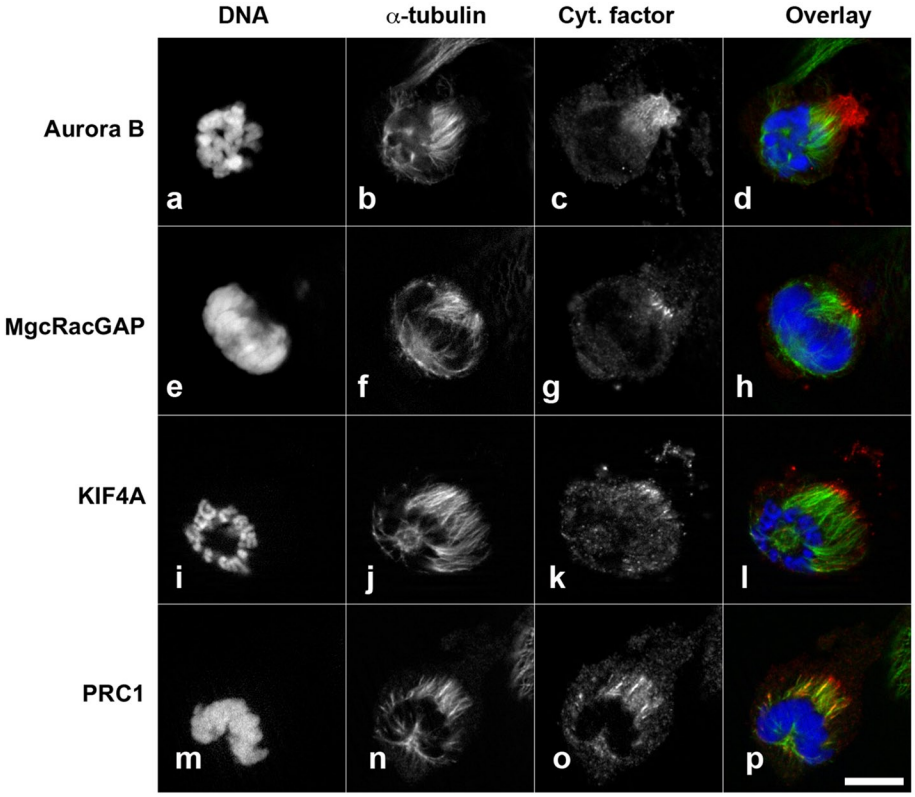

FIGURE 2: Recruitment of cytokinetic factors during forced mitotic exit. Monopolar HeLa cells were generated by incubation in the presence of STLC for 6 h, followed by addition of flavopiridol for 25 min. Following fixation, cells were probed for the recruitment of different cytokinetic markers. Bar, 10 μm.

organization prior to flavopiridol treatment (Figure 1A, a) and also displayed partial colocalization with microtubules (Figure 3C, m–p). This lack of actin organization appears to be consistent with studies in *Drosophila*, in which the depletion of Feo, the *Drosophila* homologue of PRC1, resulted in failure in actin organization and formation of contractile ring (Verni *et al.*, 2004).

To further explore the effect of PRC1 depletion on microtubule organization and monopolar cytokinesis, we performed live-cell imaging on GFP-tubulin–expressing cells transfected with either control or PRC1 siRNA (Figure 4). Whereas 94% (n = 35) of control cells produced ectopic furrows within 20–40 min following flavopiridol treatment (Figure 4A; Supplemental Movie S2), 91% of PRC1-depleted cells (n = 33) failed to display a polarized array of microtubules, furrow, or undergo membrane blebbing (Figure 4B and Supplemental Movie S3). Of interest, extended observation of cells revealed that one-third of PRC1-depleted monopoles underwent spindle rotation, with 68% rotating clockwise and 32% counterclockwise (Figure 5, bottom, and Supplemental Movie S5). This "swirling" effect was observed as early as 20 min following flavopiridol treatment, with the period of

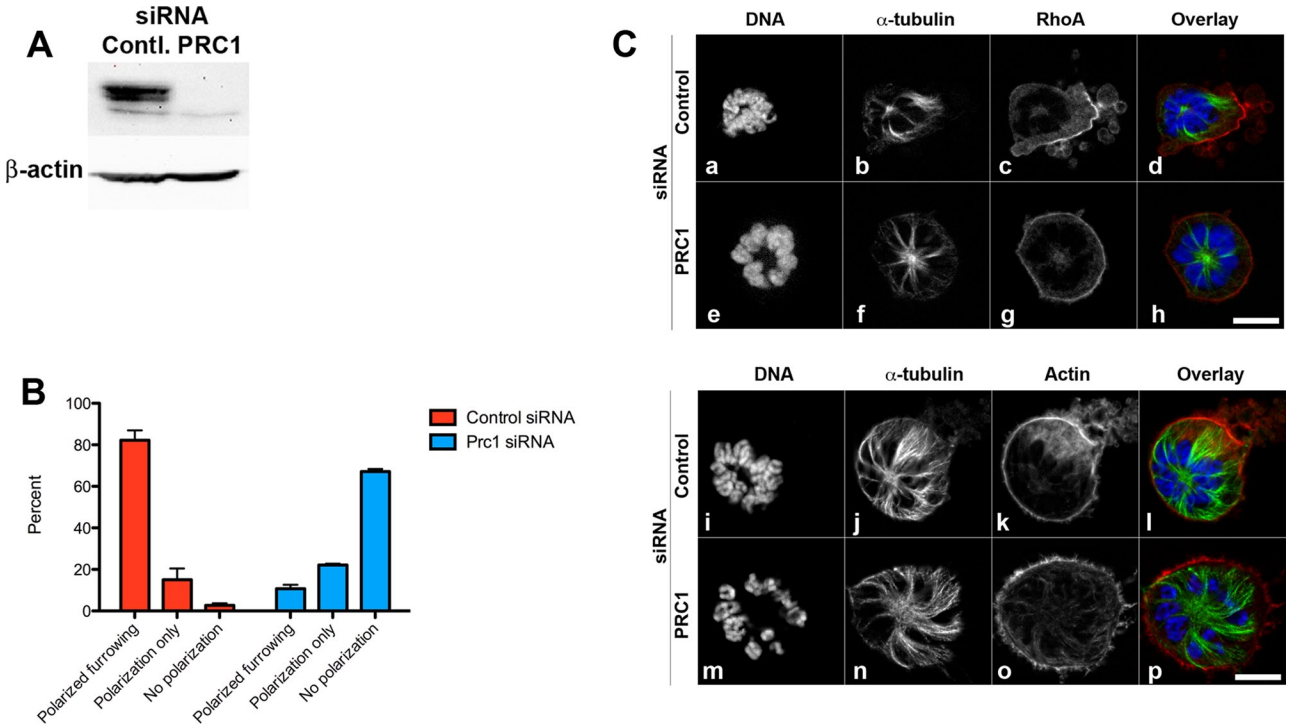

FIGURE 3: Microtubule polarization and ectopic furrowing is inhibited in PRC1-depleted cells. (A) Western blot showing the efficiency of depletion of PRC1. Control siRNA-transfected cell lysates are shown in the left lane, and the right lane contains PRC1 siRNA-transfected cell lysates. β-Actin was used as the loading control. (B) The effect of PRC1 depletion on microtubule polarization and ectopic furrowing during forced monopolar cytokinesis (n = 1128 for control siRNA and n = 1366 for PRC1 siRNA–treated cells), with bars representing SE. (C) Control and PRC1-depleted cells were treated with STLC and flavopiridol, fixed, and probed for the presence of microtubules and RhoA (a–h) or F-actin (i–p). Bar, 10 μm.

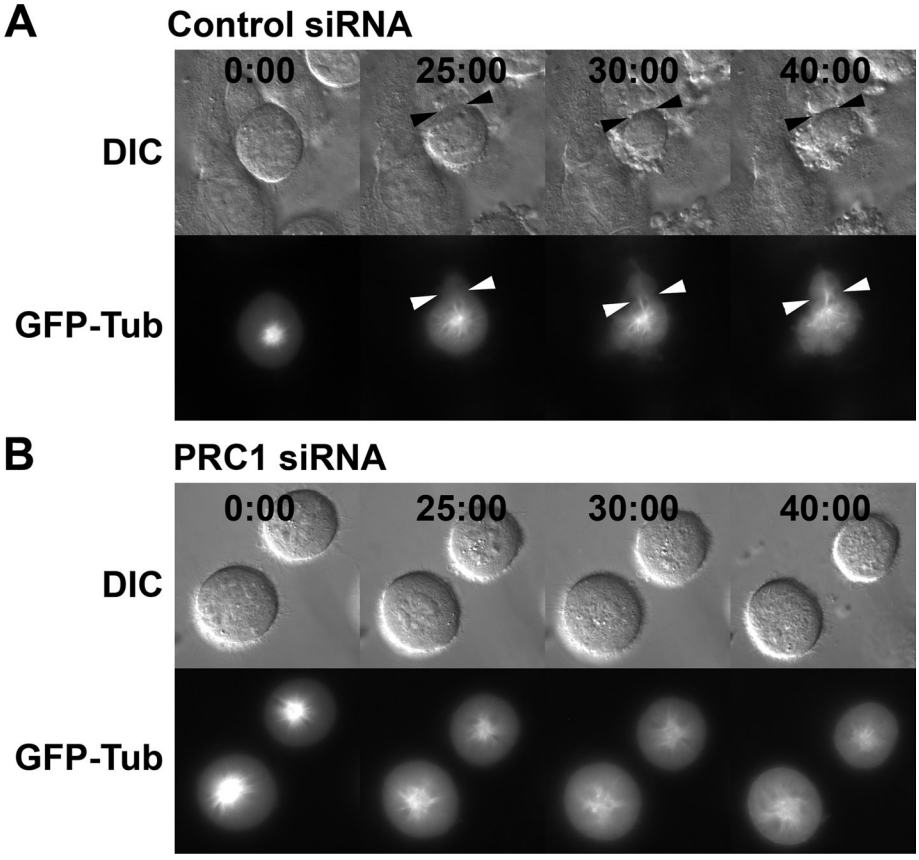

FIGURE 4: Depletion of PRC1 prevents microtubule polarization and ectopic furrow formation in monopolar cells. EGFP-tubulin–expressing HeLa cells were transfected with either control (A) or PRC1 (B) siRNA for 42 h. Cells were then incubated with 5 μM STLC for 6 h. Subsequently, coverslips were mounted on a temperature-controlled microscope stage, and prewarmed medium containing 10 μM flavopiridol was perfused through the chamber to induce mitotic exit and forced cytokinesis. (A) Selected frames of live-cell imaging of control siRNA undergoing forced cytokinesis. Arrows denote the position of the ectopic furrow. (B) Frames of PRC1-depleted monopolar cells treated in identical manner as in A. The numbers indicate time in minutes following flavopiridol addition.

rotation lasting between 20 and 83 min, with a mean duration of 40.64 ± 4.9 min. Moreover, the microtubules in the rotating monopoles appeared to be elongated compared with controls. In contrast, control cells furrowed, and they eventually flattened as they reentered interphase (Supplemental Movie S4). Only two control-transfected cells (n = 35) were observed to undergo spindle rotations (lasting 10 and 18 min, respectively), and in the second case, the cell failed to form an ectopic furrow (unpublished data).

PRC1 is required for the recruitment of key cytokinetic factors to ectopic sites during monopolar cytokinesis

Depletion of PRC1 resulted in a loss of spindle polarization and ectopic furrowing (Figures 3 and 4 and Supplemental Movies S3 and S5). We next examined how PRC1 depletion affected the recruitment of other key regulators of central spindle assembly during monopolar cytokinesis. Whereas in control cells, Aurora B localized both at the plus end of polarized microtubules and at the cortical cytoskeleton (Figure 6A, a–c), in PRC1-depleted cells Aurora B was found in punctate regions at the cortex but not enriched in any particular region (Figure 6A, d–f). MgcRacGAP recruitment was similarly impaired in PRC1-depleted cells (Figure 6B, j–l) relative to controls, in which MgcRacGAP was observed at the tips of polarized microtubules (Figure 6B, g–i). Finally, consistent with previous reports in bipolar spindles (Kurasawa *et al.*, 2004), the recruitment of KIF4A to microtubule ends observed in controls (Figure 6C, m–o) was not observed in PRC1-depleted cells (Figure 6C, p–r).

KIF4A limits spindle length during monopolar cytokinesis

We next investigated the role of KIF4A because this motor interacts with PRC1 and regulates the width of the spindle midzone by length-dependent microtubule depolymerization (Bieling *et al.*, 2010; Walczak and Shaw, 2010). To determine whether the cellular morphology and cytokinesis defects observed in the PRC1 knockdown could be phenocopied by KIF4A knockdown, we depleted cells of PRC1 or KIF4A alone or in combination (Figures 7–9). Western blotting indicated that depletion of one factor had no effect on the abundance of the other (Figure 7A). In contrast to PRC1 depletion (Figure 3C), KIF4A-depleted cells were capable of furrowing, and the localization of RhoA and actin was indistinguishable between control and KIF4A-depleted cells (Figure 7B). Furthermore, examination of living cells undergoing forced mitotic exit revealed that, on average, KIF4A-depleted cells initiated ectopic furrow formation ~14 min earlier than their control counterparts (n = 33 for each treatment; $p < 0.0001$; Figure 7C, Supplemental Figure S4 and

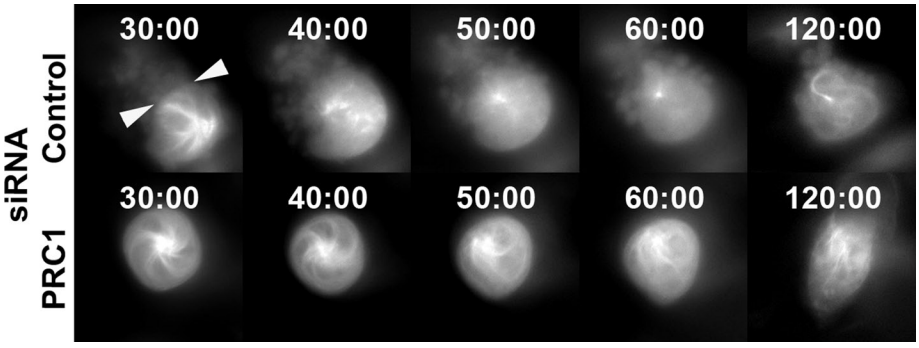

FIGURE 5: Induction of spindle rotation in PRC1-depleted monopolar cells undergoing forced cytokinesis. Following siRNA transfection for 42 h, EGFP-tubulin–expressing HeLa cells were treated with STLC for 6 h. Coverslips were mounted on a temperature-controlled stage and subsequently forced into mitotic exit with flavopiridol and followed by time-lapse microscopy. Shown are the selected frames of control (top) and PRC1-depleted monopolar cells (bottom) undergoing forced mitotic exit The numbers indicate time in minutes following flavopiridol addition.

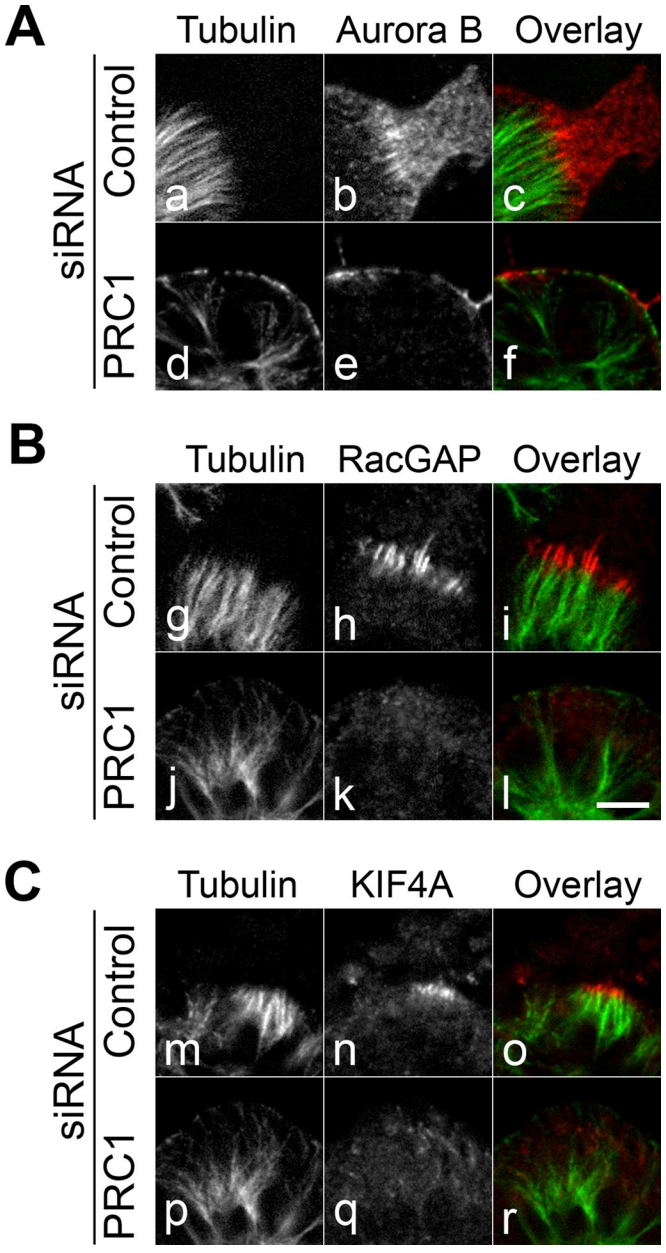

FIGURE 6: PRC1 depletion impairs recruitment of cytokinetic factors during monopolar cytokinesis. HeLa cells were transfected with control or PRC1 siRNA 42 h before treatment with STLC and flavopiridol. At 25 min postflavopiridol treatment, cells were fixed and probed for the localization of microtubules, DNA, and Aurora B (A), MgcRacGAP (B), or KIF4A (C). Bar, 5 μm.

Supplemental Videos S6 and S7). Given that KIF4A-depleted cells furrowed earlier than control cells, we examined the kinetics of microtubule polarization, reasoning that KIF4A-depleted monopoles should polarize microtubules earlier than controls. Analysis of fixed cells (n = 600 cells per condition) revealed that 10 min following flavopiridol treatment, 52% of KIF4A-depleted cells displayed a polarized spindle (Figure 8, A and B), whereas only 13% of controls had polarized by this time point. However, by 25 min posttreatment, there was no difference in the percentage of cells that were polarized or furrowing (unpublished results). In addition, measurements of the long axis of monopolar spindles in control and KIF4A-depleted cells (n = 36 from three independent experiments) revealed that, whereas control monopoles had an average length of 14 μm, KIF4A-depleted cells had an average spindle length of 20 μm (Figure 8C), corresponding to a 42% increase over controls.

Depletion of KIF4A in bipolar cells revealed that key cytokinetic factors, such as PRC1, centralspindlin complex, and chromosomal passenger proteins, failed to properly localize at the central spindle midzone during anaphase; instead these regulators were found broadly dispersed throughout the central spindle (Kurasawa *et al.*, 2004). To examine how the KIF4A depletion affected the recruitment of these factors during monopolar cytokinesis, we examined the localization dynamics of key cytokinetic regulators in control or KIF4A-depleted cells (Figure 9). Whereas in control cells Aurora B was recruited to the cortex with no detectable localization in main cell body (Figure 9A, a–d), in KIF4A-depleted cells Aurora B was also found along the entire length of the polarized microtubule array (Figure 9A, e–h). Similarly, the centralspindlin component MKLP1 could be seen both at the tips of polarized microtubules and along polarized microtubules (unpublished data). Finally, PRC1 was found along the entire length of the polarized microtubule array in KIF4A-depleted cells, whereas in controls PRC1 was found mostly at the distal regions of the polarized spindles (Figure 9B). This suggests that, whereas binding of PRC1 to polarized microtubules is KIF4A independent, its overall enrichment at the distal ends of microtubules does depend on KIF4A.

Analysis of PRC1-depleted monopoles forced into mitotic exit revealed that one-third of these cells exhibited a period of spindle rotation that appeared to be accompanied by the elongated microtubules (Figure 5). Because KIF4A negatively regulates microtubule length (Bringmann *et al.*, 2004; Bieling *et al.*, 2010), we surmised that simultaneous depletion of both PRC1 and KIF4A would increase the percentage of cells undergoing spindle rotation, possibly facilitated by hyperelongated microtubules. To test this idea, we simultaneously depleted cells of KIF4A and PRC1 (confirmed by Western blotting as shown in Figure 7A) and examined live and fixed cells. Live–cell imaging of doubly depleted monopoles revealed that 38% of GFP-tubulin–expressing cells underwent spindle rotation (n = 26), with all cells observed failing to bleb or furrow. Moreover, the pattern of F-actin and tubulin localization in KIF4A/PRC1-depleted cells was essentially indistinguishable from that in cells depleted of PRC1 alone (Supplemental Figure S5). Together these data suggest that although KIF4A may be indirectly responsible for the focused localization of cytokinetic regulators, PRC1 was the primary factor controlling the polarization of parallel microtubules and the recruitment of those factors required for localized RhoA activation.

DISCUSSION

Although the bipolar arrangement of microtubules is conventionally believed to be an absolute requirement for specifying the cleavage plane, studies using PtK1 cells demonstrated that the cleavage plane can be specified in the absence of spindle bipolarity (Canman *et al.*, 2003). Subsequent reports using HeLa cells, whose spherical shape and spindle morphology during mitosis do not impose a directional bias for placement of the cleavage furrow, confirmed and extended this original report and demonstrated that the principal factors believed to direct contractile ring assembly are recruited to sites of ectopic furrowing (Hu *et al.*, 2008, 2011). Monopolar spindles offer an excellent system with which to gain novel insights into microtubule organization, as well as into the recruitment dynamics and function of key cytokinetic markers during cytokinesis. In this report, we examined the behavior of PRC1 and KIF4A, which together are believed to organize the antiparallel microtubules of the central spindle. By disrupting the normal geometrical arrangement

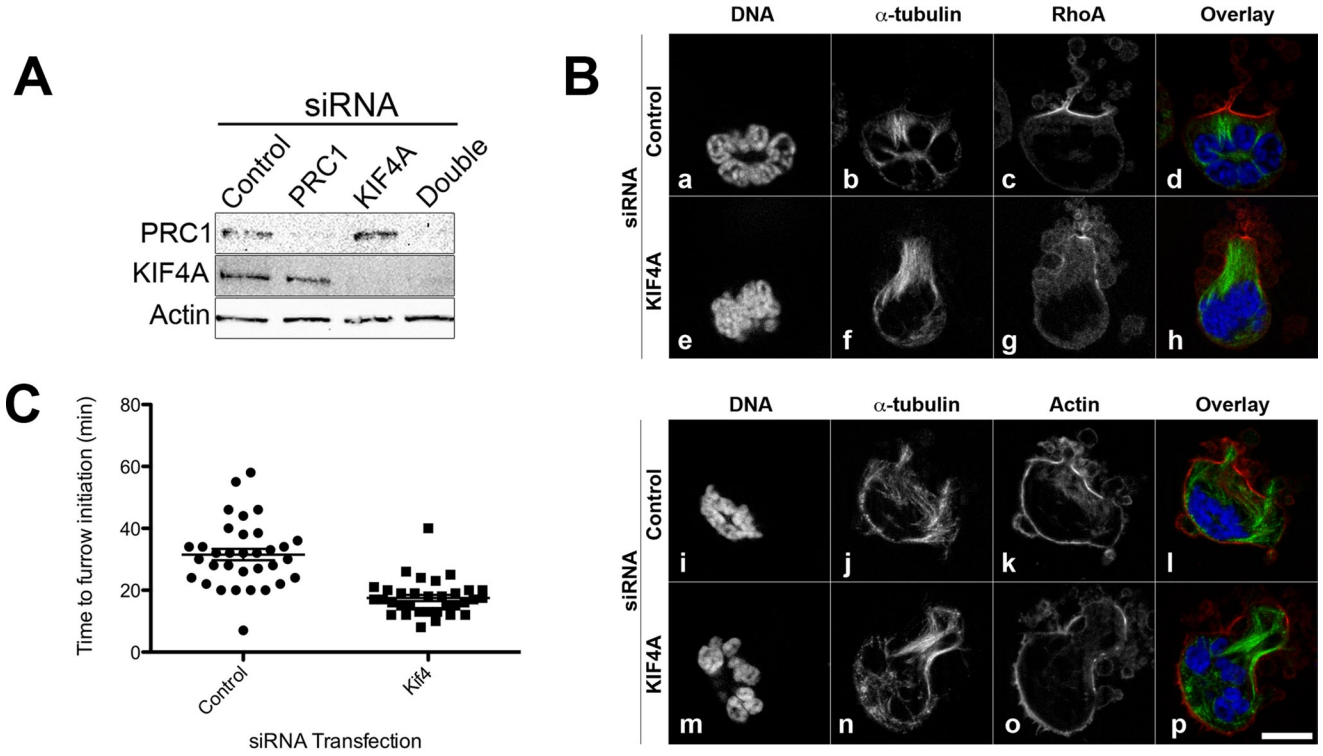

FIGURE 7: KIF4A depletion induces early onset of ectopic furrow formation during monopolar cytokinesis. (A) Western blot to confirm the efficiency of KIF4A and PRC1 depletion. (B) HeLa cells were transfected with either control or KIF4A siRNA for 42 h. Transfected cells treated with STLC and then with flavopiridol were probed for RhoA (top) and actin (bottom) localization. (C) The timing of furrow initiation obtained from live-cell data for control and KIF4A-depleted monopoles (n = 33 for each condition). On average, KIF4A-depleted cells initiated ectopic furrow formation 14 min earlier than the control siRNA, $p < 0.0001$. Bar, 10 μm.

of spindle fibers, we determined that although PRC1 and KIF4A depend on each other for correct localization in the absence of bipolarity, these two factors play distinct roles in organizing the parallel microtubule array and recruiting factors necessary for furrow induction.

PRC1 is required for microtubule polarization during monopolar cytokinesis

Studies in yeast, plants, and animal cells firmly established that the Ase1/MAP65/PRC1 family of microtubule cross-linking proteins preferentially bundles antiparallel microtubule arrays both in vivo and in vitro (Loiodice *et al.*, 2005; Janson *et al.*, 2007; Gaillard *et al.*, 2008; Kapitein *et al.*, 2008). In addition, studies with purified components identified the structural basis for how PRC1 (in conjunction with KIF4A) may act as a compliant cross-linker, and the accumulation of which at regions of antiparallel overlap may ultimately act as a break on microtubule sliding and define the length of spindle midzone (Kapitein *et al.*, 2008; Bieling *et al.*, 2010; Subramanian *et al.*, 2010; Braun *et al.*, 2011). Given that the depth of our understanding of this protein family extends to the structural level, we found it quite surprising that monopolar cytokinesis (where furrowing is induced by parallel microtubules) was dependent on PRC1 (Figures 3–6 and Supplemental Movies S3 and S5). Indeed, there was a striking difference in microtubule organization between control- and PRC1-depleted monopolar spindles, where PRC1-depleted spindles lacked the defined polarization visible in controls, instead displaying a characteristic "pinwheel" morphology (Figures 3–5). How might PRC1 contribute to the polarization and bundling of parallel arrays? Although the PRC1 family member Ase1 displays a 9.4-fold preference for antiparallel microtubules (Janson *et al.*, 2007), it is not exclusive, leading to the possibility that weak parallel bundling activity may be sufficient to polarize the microtubule array. However, in vitro studies of purified PRC1 on microtubules failed to detect parallel bundling activity (Bieling *et al.*, 2010), raising the alternative hypothesis that PRC1 may indirectly assist in microtubule polarization through interactions with other motors or microtubule-associated proteins. Indeed, PRC1 has been reported to interact with a number of factors, including MKLP1, MKLP2, KIF4A, CENP-E, CLASP, and KIF14 (Kurasawa *et al.*, 2004; Gruneberg *et al.*, 2006; Liu *et al.*, 2009). In addition, whereas depletion of KIF4A had no effect on monopole polarization (Figures 7–9 and Supplemental Figure S4), the role of MKLP1 in central spindle assembly is well documented (Glotzer, 2009; Douglas and Mishima, 2010), and others reported a polarization defect in MKLP1-depleted cells (Hu *et al.*, 2011). Alternatively, KIF14 has been implicated in regulating minus end dynamics and maintenance of the central spindle during cytokinesis (Carleton *et al.*, 2006; Cai *et al.*, 2010) and conceivably could also participate in spindle polarization. Thus, whereas individual PRC1 molecules may interact with motors in an mutually exclusive manner (Kurasawa *et al.*, 2004), interactions between PRC1 and multiple motors at the population level may ultimately contribute to the organization of the postanaphase central spindle.

In addition to having a pronounced polarization defect, cells with monopolar spindles and forced into mitotic exit also failed to recruit critical cytokinetic factors in the absence of PRC1 (Figure 6). Whereas the CPC was found to be enriched at the ectopic furrow sites in control cells (Figure 6A, a–c), little or no CPC was could be detected at the cortex in monopolar cells that failed to polarize following

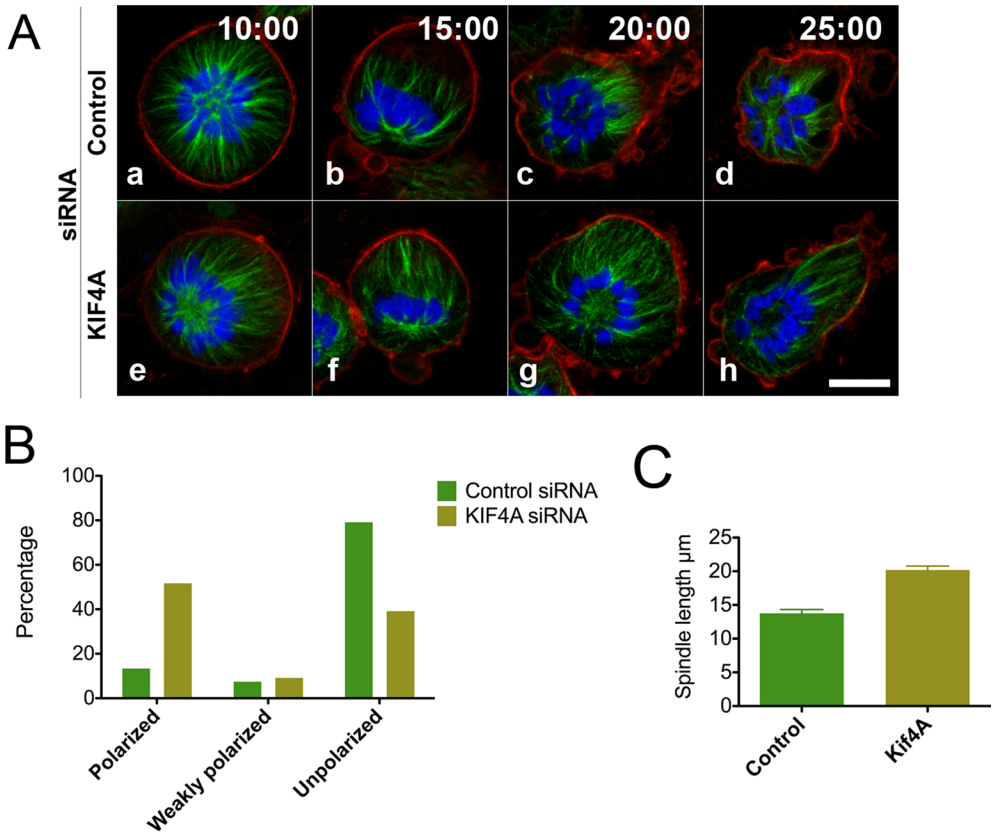

FIGURE 8: KIF4A depletion results in early spindle polarization and hyperelongation during monopolar cytokinesis. (A) HeLa cells were transfected with control or KIF4A siRNA, and 42 h following transfection, cells were treated with STLC to generate monopolar spindles and flavopiridol for 25 min to induce mitotic exit. Cells were fixed at different time points and processed for microtubule (green), F-actin (red), and DNA (blue) localization. (B) Cells at the 10 -min postflavopiridol condition were then scored for microtubule polarization and furrowing (n ≈ 600 for each condition). (C) The long axes of spindles 25 min postflavopiridol treatment were measured in triplicate for each cell (n = 36 for each condition). Bar, 10 µm.

flavopiridol treatment (Figure 6A, d–f). Similarly, the centralspindlin component MgcRacGAP (Figure 6B) and the chromokinesin KIF4A (Figure 6C) were also mislocalized. Although the loss of these factors could be due to the lack of a polarized microtubule array, biochemical interactions between Aurora B, centralspindlin, and KIF4A have been reported (Kurasawa *et al.*, 2004; Zhu and Jiang, 2005; Ozlu *et al.*, 2010). Thus the lack of recruitment of these factors could very well be a function of a lost interaction between binding partners.

Similar analyses of the centralspindlin and chromosomal passenger complexes have been performed on cells with bipolar spindles depleted of PRC1, with slightly different results. In two reports, components of the CPC and centralspindlin complexes localized to microtubule distal ends of the two spindle halves in the absence of PRC1 but failed to form a focused band (Kurasawa *et al.*, 2004; Neef *et al.*, 2007). However, in another report, centralspindlin was completely lost in PRC1-depleted cells, whereas TD60 and the CPC localized to the equator presumably via the tips of astral microtubules (Mollinari *et al.*, 2005). The loss of PRC1 had a more pronounced effect on microtubule organization and CPC/centralspindlin recruitment in monopolar spindles (Figures 3–6) than what has been reported for bipolar spindles (Kurasawa *et al.*, 2004; Mollinari *et al.*, 2005; Neef *et al.*, 2007) raising the possibility that the furrowing observed in PRC1-depleted cells with bipolar spindles may arise from the additive effect of the two spindle halves. Thus the midzone created by two spindle halves may afford the recruitment of factors that would not be possible in the absence of spindle bipolarity.

PRC1-depleted monopoles underwent a period of spindle rotation during reentry into interphase (Figure 5 and Supplemental Movie S5). This was not observed in KIF4A-depleted cells, suggesting that spindle rotation was not due to a compromise in KIF4A regulation of microtubule length. Previous studies of monopolar cytokinesis described a cooperative interaction between microtubules, Rho signaling, and the actomyosin cytoskeleton (Hu *et al.*, 2008), and it is possible that in the absence of a polarized spindle (and thus recruitment of the Rho signaling apparatus), those cooperative interactions were lost in the absence of PRC1. Untethered by an active zone of furrowing, the monopole may then rotate in response to either dynein-driven forces, recently described flows of subcortical actin (Fink *et al.*, 2011; Yi *et al.*, 2011), or both. Subcortical actin represents a poorly understood component of the cytoskeleton (Field and Lenart, 2011), and during monopolar cytokinesis, cytoplasmic actin was concentrated at the zone of polarization (Figure 3C, k), whereas in PRC1-depleted cells, actin was found throughout the cytoplasm (Figure 3C, o). Thus it is entirely possible that the observed spindle rotation in PRC1-depleted cells was a function of actin dynamics within the cytoplasm.

KIF4A regulates the length of polarized microtubules during forced monopolar cytokinesis

KIF4A and PRC1 physically interact and are mutually dependent on each other for localization to the central spindle (Kurasawa *et al.*, 2004; Zhu and Jiang, 2005), and consistent with these studies, KIF4A

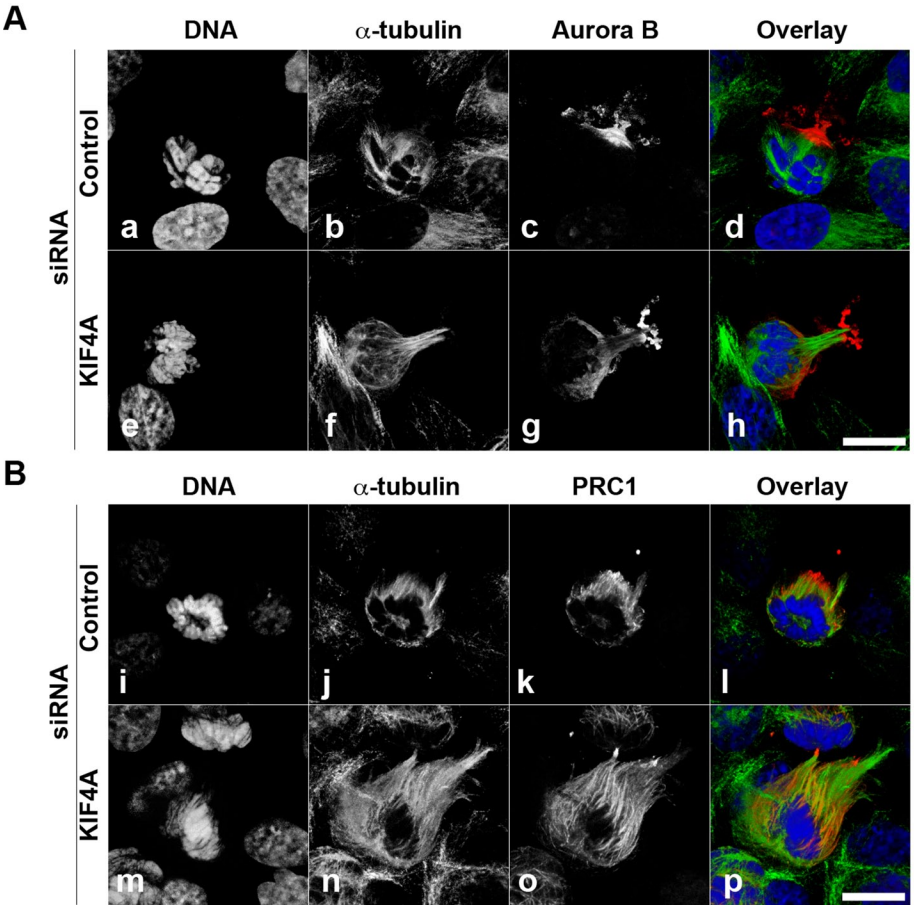

FIGURE 9: KIF4A is required for the focused recruitment of cytokinetic factors at ectopic sites during monopolar cytokinesis. HeLa cells transfected with control or KIF4A siRNA were treated with STLC, and monopoles were treated with flavopiridol for 25 min prior to fixation and processing for immunofluorescence. Three-dimensional reconstructions of cells imaged at 0.25-μm intervals. Bar, 10 μm.

was not recruited to microtubule ends in PRC1-depleted monopoles (Figure 6C). Depletion of KIF4A leads to disorganization of the central spindle, mislocalization of key cytokinetic factors, and cytokinesis failure (Kurasawa *et al.*, 2004; Zhu and Jiang, 2005), and since PRC1 and KIF4A are involved in central spindle assembly, we predicted that depletion of KIF4A would phenocopy the PRC1 knockdown. However, KIF4A depletion inhibited neither microtubule polarization nor furrow formation during monopolar cytokinesis (Figures 7–9, Supplemental Figures S4 and S5, and Supplemental Movie S7). Instead, we observed that spindle polarization and furrowing initiated earlier in KIF4A-depleted cells than in controls (Figures 7 and 8 and Supplemental Movies S6 and S7) and that the long axis of KIF4A-depleted monopolar spindles was 42% longer than in controls (Figure 8, A and C). The elongated-microtubule phenotype is consistent with the notion that one of the primary roles for KIF4A at the spindle midzone is to control microtubule length, a notion supported by recent in vitro reconstitution experiments (Bieling *et al.*, 2010) and live-cell imaging studies using both monopolar and bipolar spindles (Hu *et al.*, 2011). This mode of regulation of microtubule length is also found in yeast, in which Kip3p depolymerizes plus end of the microtubule (Varga *et al.*, 2006). The rate of depolymerization for the longer microtubules is faster than for the shorter polymer (Varga *et al.*, 2006). Thus the elongated microtubules observed in PRC1-depeleted cells may stem from the loss of KIF4A recruitment, thus relieving microtubules from length-limiting regulation. Finally, KIF4A depletion resulted in the Aurora B and PRC1 distribution throughout the long axis of the cells (Figure 9), but given the total loss of microtubule polarization and furrowing observed in double-knockdown cells (Supplemental Figure S5), it seems likely that the altered distribution observed in KIF4A knockdowns is a function of altered PRC1 distribution. One possibility that cannot be completely dismissed is that KIF4B may compensate for KIF4A depletion in these experiments. Indeed, KIF4A and B share 90% identity, and siRNA depletion of KIF4B results in a disrupted anaphase spindle and binucleate cells (Zhu *et al.*, 2005). However, the PRC1-binding domain lies within the C-terminal 200 residues of KIF4A (Zhu and Jiang, 2005), and this domain is truncated in KIF4B by 49 residues. It is unknown whether this truncation affects KIF4B binding to PRC1.

How might studying monopolar spindles inform our understanding of cytokinesis in cells with bipolar spindles?

Since the initial observation in PtK1 cells, the notion that the factors directing cytokinesis may be positioned in the absence of spindle bipolarity has raised questions regarding how these factors are recruited to ectopic furrows and how this may inform the process of cleavage plane specification in normal cells. We chose to study PRC1 since it has been firmly establish that it acts as an antiparallel microtubule cross-linker in a wide variety of cell types (Walczak and Shaw, 2010). However, despite the parallel organization of microtubules in monopolar spindles, PRC1 was essential for spindle polarization, recruitment of cytokinetic factors, and furrow formation. Although the loss of midzone factors has been reported in studies of cells with intact spindles, the organizing activity of PRC1 (i.e., polarization of the monopolar spindle; Figure 3) could not have been predicted based on studies in bipolar spindles. Similarly, whereas depletion of KIF4A results in longer-anaphase spindles in normal cells (Kurasawa *et al.*, 2004; Hu *et al.*, 2011), it was studies in monopolar cells that provided direct evidence that KIF4A limited microtubule elongation at the equator (Hu *et al.*, 2011, and the present results). Thus, by altering the normal geometric relationship between spindle microtubules and the cell cortex, we gained novel insights into the process of cleavage plane determination in normal bipolar cells.

MATERIALS AND METHODS

Cell culture

HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in Earle's balanced salt solution (Lonza, Walkersville, MD) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Norcross, GA), 2.0 mM L-glutamine, 0.6 mM sodium pyruvate, and 0.075% sodium bicarbonate, with or without penicillin–streptomycin solution (PSF; 50.0 IU/ml), at 37°C in the presence 5% CO_2. Cells were seeded onto 18- or 25-mm^2 coverslips at a density of (3–5) × 10^5 cells/ml for all fixed- or live-cell analyses.

Transfection and drug treatments

HeLa cells were transfected with either nontargeting control siRNA or pooled targeting PRC1, Eg5, or KIF4A (Dharmacon; Thermo Fisher Scientific, Lafayette, CO; Supplemental Table S1) at the final concentration of 100 nM for 24 h, using Dharmacon DhermaFECT-1 transfection reagent according to manufacturer's specifications. Following transfection, cells were released in the media without antibiotics for 18 h. Subsequently, the cells were treated with 5.0 μM Eg5 inhibitor STLC (provided by Jeffrey Arterburn, Department of Chemistry and Biochemistry, New Mexico State University) for 6.0 h to generate monopolar cells. To induce forced mitotic exit and cytokinesis, monopolar cells were treated with 10.0 μM Cdk1 inhibitor flavopiridol (Sigma-Aldrich, St. Louis, MO; Potapova *et al.*, 2006).

For live-cell imaging, cells were transfected with pEGFP-tubulin (Clontech, Mountain View, CA) for 6 h using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and were allowed to grow overnight before being further transfected with 100 nM control, PRC1, or KIF4A siRNA as described. Eighteen hours following release from siRNA transfections, cells were treated with 5.0 μM STLC for 6 h before mounting onto a temperature-controlled perfusion chamber for live-cell imaging.

Lysate preparation and Western blot analyses

To confirm the efficiency of siRNA depletion of target proteins, transfected cells were washed three times with sterile phosphate-buffered saline (PBS), and lysates were prepared using RIPA buffer (50 mM Tris-HCl, pH 7.0, 120 mM NaCl, 5 mM ethylene glycol tetraacetic acid, 5 mM $MgCl_2$, 0.5% SDS, and 0.5% NP40) supplemented with 220 μM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, and protease inhibitors (Calbiochem, Gibbstown, NJ). SDS–PAGE was performed using 4–15% precast gradient gels (Bio-Rad, Hercules CA) and blotted onto Immobilon membranes (Millipore, Billerica, MA). Blots were blocked in 5% milk or bovine serum albumin and probed using rabbit anti-PRC1 (Santa Cruz Biotechnology, Santa Cruz, CA, and Abcam, Cambridge, MA) or rabbit anti-KIF4A (Bethyl, Montgomery, TX). Mouse anti–β-actin (Sigma-Aldrich) was used as a loading control. Bound primary antibodies were detected using peroxidase-conjugated anti–rabbit/anti–mouse immunoglobulin G (GE Healthcare, Pittsburgh, PA) and Immun-Star HRP Chemiluminescent Kit (Bio-Rad). Images were acquired using a ChemiDoc XRS molecular imaging system (Bio-Rad). Eight-bit images were exported, and figures were prepared using Photoshop CS software (Adobe Systems, Mountain View, CA).

Immunofluoresence and image acquisition

Immunofluorescence analyses of different cytokinetic factors were performed using different fixatives. For the visualization of MgcRacGAP, MKLP1, MKLP2, and KIF4A cells grown in coverslips were fixed in cold methanol at −20°C for 30 min, followed by rehydration in PBS for 10–15 min. Subsequently, fixed cells were blocked with 3% bovine serum albumin (BSA)/PBS for 1 h. For the visualization of F-actin, PRC1, Aurora B, INCENP, and Plk1, cells were fixed in 3.7% formaldehyde/PBS, followed by permeabilization with 0.5% Triton X-100/PBS (PBST) for 20 min and blocking in 3% BSA/PBS for 1 h. Fixation for RhoA localization was carried out by incubating cells in ice-cold 10% trichloroacetic acid for 15 min as described (Hayashi *et al.*, 1999), followed by permeabilization (PBST) for 5–10 min. The following primary antibodies were used in the dilutions as recommended by manufacturers: rabbit anti–γ tubulin (Sigma-Aldrich), rabbit anti–Aurora B kinase (Abcam), rabbit anti-INCENP (Cell Signaling, Danvers, MA), mouse anti-Plk1 (Abcam), rabbit anti-MKLP1 (Cytoskeleton, Denver, CO), goat anti-MgcRacGAP (Abcam), rabbit anti-PRC1 (Santa Cruz Biotechnology), goat anti-KIF4A (Abcam), rabbit anti-RhoA (Cell Signaling), mouse anti-RhoA (Santa Cruz Biotechnology), and mouse anti–α-tubulin (Sigma-Aldrich). Primary antibodies were detected with Alexa Fluor–labeled secondary antibodies (Invitrogen, Carlsbad, CA), and F-actin was detected using rhodamine–phalloidin (Sigma-Aldrich). DNA was visualized using Hoechst 33342 (Invitrogen) at a concentration of 1 μg/ml. Images were acquired using a 63× Plan Apochromat, 1.4 numerical aperture, oil immersion objective mounted on an Axiovert 200M inverted microscope (Carl Zeiss, Thornwood, NY) outfitted for standard transmitted light and epifluorescence microscopy, as well as for optical sectioning with an Apotome structured illumination module. Images were acquired with a 12-bit AxioCam MrM charge-coupled device camera driven by AxioVision 4.5 software (Carl Zeiss). The acquired 8-bit images were exported, and figures were generated using Photoshop CS5 software.

Live-cell imaging

Live-cell imaging was performed on cells cultured on coverslips and mounted into a 1.0-ml chamber within a temperature-controlled, microscope stage insert containing ports for media perfusion. Cells were cultured in media containing phenol-free L-15 medium supplemented with 20% FBS, 20 mM glutamine, sodium bicarbonate, and PSF (Frigault *et al.*, 2009). The chamber was maintained at 37°C on the inverted Zeiss Axiovert 200M microscope. To induce mitotic exit, medium containing 10 mM flavopiridol was perfused through the chamber, and recording was initiated at the moment that perfusion was initiated. Differential interference contrast and/or fluorescence images were captured using a 63×, 1.3 numerical aperture, Plan Apochromat water objective. Time-lapse sequences were exported into 8-bit image sequences and then cropped and converted into QuickTime movies (Apple, Cupertino, CA) using ImageJ (National Institutes of Health), and figures were prepared using Photoshop.

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