Molecular networks linked by Moesin drive remodeling of the cell cortex during mitosis

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The cortical mechanisms that drive the series of mitotic cell shape transformations remain elusive. In this paper, we identify two novel networks that collectively control the dynamic reorganization of the mitotic cortex. We demonstrate that Moesin, an actin/membrane linker, integrates these two networks to synergize the cortical forces that drive mitotic cell shape transformations. We find that the Pp1-87B phosphatase restricts high Moesin activity to early mitosis and down-regulates Moesin at the polar cortex after anaphase onset. Overactivation of Moesin at the polar cortex impairs cell elongation and thus cytokinesis, whereas a transient recruitment of Moesin is required to retract polar blebs that allow cortical relaxation and dissipation of intracellular pressure. This fine balance of Moesin activity is further adjusted by Skittles and Pten, two enzymes that locally produce phosphoinositol 4,5-bisphosphate and thereby, regulate Moesin cortical association. These complementary pathways provide a spatiotemporal framework to explain how the cell cortex is remodeled throughout cell division.

Introduction

A universal feature of animal cells undergoing mitosis is the series of transformations in their shape necessary to generate two identical daughter cells. Mitotic cell shape remodeling relies on a precise coupling of cortical actomyosin forces with the plasma membrane. At mitosis entry, increased hydrostatic pressure and isotropic cortical contractility drive the characteristic rounding of prometaphase cells (Matzke et al., 2001; Maddox and Burridge, 2003; Carreno et al., 2008; Kunda et al., 2008; Stewart et al., 2011). Subsequently, asymmetry in cortical tensions leads to polar relaxation and equatorial contraction, which contribute to anaphase cell elongation and to cytokinesis (Hickson et al., 2006; Surcel et al., 2010; Sedzinski et al., 2011). Although mitotic stages were originally described more than one century ago (Flemming, 1882), the molecular networks that modify the cortex to drive transformations in the shape of dividing cells remain to be identified.

We and others have shown that Moesin (Moe) plays essential roles in the regulation of cell shape during mitosis in Drosophila melanogaster (Carreno et al., 2008; Kunda et al., 2008). Moe is the sole Drosophila member of the ERM (Ezrin, Radixin, and Moesin) family of cytoskeletal regulators, which allow, in a signal-dependent manner, bridging of the actin cytoskeleton to the plasma membrane (Fehon et al., 2010). A flexible α-helical linker separates an N-terminal (FERM [4.1 and ERM]) domain from a C-terminal domain (C-ERMAD), which interact with the plasma membrane and with F-actin, respectively. ERM proteins are regulated by a conformational change: in their dormant cytoplasmic state, interaction between the FERM and the C-ERMAD domains masks the two binding surfaces. In response to various signals, ERM proteins open and provide a bridge between actin filaments and the plasma membrane. Activation of ERM proteins involves both the binding of the FERM domain to phosphoinositol 4,5-bisphosphate (PI(4,5)P2) and the phosphorylation of a conserved threonine residue (T559 in Drosophila Moe) located in the C-ERMAD moiety. Although phosphorylation is a hallmark of ERM activation,
interaction with PI(4,5)P_2 has emerged as playing important roles in their regulation (Coscosy et al., 2002; Hao et al., 2009; Roch et al., 2010). Current models state that PI(4,5)P_2 favors conformational opening and that phosphorylation further stabilizes this open active form at the cell cortex (Fehon et al., 2010).

ERM function and proper regulation is required during cell division in both flies (Carreno et al., 2008; Kunda et al., 2008; Cheng et al., 2011) and mammals (Luxenburg et al., 2011). In *Drosophila*, depletion of Moe or Slik, the kinase necessary to phosphorylate Moe on T559, leads to similar mitotic defects: mutant cells are unable to round at mitosis entry, and their cortex is continuously deformed throughout cell division. Importantly, these cortical defects impinge on additional aspects of mitosis, including spindle morphogenesis and positioning, as well as chromosome segregation (Carreno et al., 2008; Kunda et al., 2008). Therefore, unraveling ERM function and regulation at the cortex represents a key step to better understanding how cell shape transformations are coordinated during division.

Using functional approaches and high-resolution live imaging in *Drosophila* cultured cells, we show here that the regulated activity of Moe orchestrates changes in tension applied at the cortex and thereby, controls cell shape transformations at the successive steps of cell division. Through systematic screenings of candidate regulators, we identify two networks that collectively provide a spatiotemporal control of Moe activity. The first one relies on Pp1-87B, a phosphatase that counteracts activity of the Slik kinase to restrict high Moe function to early mitosis. Then, the PI(4)P 5-kinase Skittles and PI(3,4,5)P_3 phosphatase Pten further refine the pattern of activated Moe through the local production of PI(4,5)P_2, which is required for both Moe cortical recruitment and phosphorylation. Integration of these two regulatory networks provides a cell cycle–regulated burst of isotropic Moe activation at the cortex, which is required for cell rounding at G2/M transition. Subsequently, the concomitant equatorial enrichment and polar diminution of Moe activity after the anaphase onset synchronizes equatorial contractions with polar relaxation to allow cell elongation and cytokinesis.

**Results**

**Control of Moe activation participates in cell elongation and cytokinesis**

As deduced from the pattern of phosphorylated Moe (P-Moe) in fixed samples (Carreno et al., 2008), the location of activated Moe parallels the sites of cortical contractions during mitosis. To gain insight into the role and the regulation of Moe activity at the cell cortex throughout the cell cycle, we examined dynamics of a functional GFP-tagged Moe (Roch et al., 2010) stably expressed in *Drosophila* S2 cells. Time-lapse microscopy confirmed that Moe localization is tightly regulated during the cell cycle. Although mostly cytoplasmic in interphase, Moe-GFP was recruited to the cell cortex upon mitosis entry (Fig. 1 A). Then, it was redistributed into the cytoplasm after furrow ingress, before complete abscission. Throughout mitosis, the spatial pattern of Moe association with the cortex directly correlated with cell morphology. In prophase, metaphase, and early anaphase, when cells are spherical, Moe-GFP was isotropically associated with the cortex. When cells started to elongate in anaphase B, Moe-GFP was progressively lost from the poles and accumulated at the equator (Fig. 1, B and C; and Video 1).

This dynamic Moe localization likely requires precise regulation of its activity. We tested this hypothesis by substituting endogenous Moe with a constitutively active phosphomimetic mutant, Moe-TD-GFP. To this aim, cells stably expressing Moe-TD-GFP were treated by a double-strand RNA (dsRNA) specifically targeting the 3’ untranslated region (UTR) of endogenous Moe RNA. Under these conditions, hereafter referred to as Moe-TD cells, Moe-TD-GFP accumulated abnormally at the cortex during cell elongation (Fig. 1, D, F, and G). Moreover, Moe depletion triggered a low rate of cytokinesis failure (Carreno et al., 2008), substituting endogenous Moe by Moe-TD-GFP impaired cytokinesis in 50% of the cells (Fig. 1 E). Because in many cell types completion of cytokinesis relies on proper elongation in anaphase B (Rappaport, 1971), we evaluated the geometry of Moe-TD cells when progressing through anaphase. Indeed, most Moe-TD cells displayed defective elongation, a phenotype associated to cytokinesis failure (Fig. 1 E). It has been shown that anaphase cell elongation requires concomitant equatorial actomyosin contractions and polar relaxation (Hickson et al., 2006). Because activated Moe increases cortical rigidity (Kunda et al., 2008), we hypothesized that Moe overactivation at the polar cortex triggers excess stiffness by increasing F-actin association and, consequently, blocks cell elongation. Consistently, we measured an increase in F-actin associated with the polar cortex versus the equatorial region in Moe-TD cells (Fig. 1 H). These results indicate that reduction of Moe activity at the polar cortex contributes to cell elongation during anaphase B and, thus, to cytokinesis.

**Moe controls polar relaxation by regulating polar cortical blebbing**

To further investigate the role of Moe in anaphase cell elongation, we imaged cortical dynamics in dividing S2 cells. Since pioneering works (Prothero and Spencer, 1968), several studies have shown that short-lived blebs form at each pole during anaphase elongation (Porter et al., 1973; Fishkind et al., 1991; Burton and Taylor, 1997; Boucrot and Kirchhausen, 2007). These polar blebs have been recently shown to be necessary to release the cortical tension and cytoplasmic pressure created by cytokinetic ring furrowing (Sedzinski et al., 2011). High-resolution analyses of living Moe-GFP cells indicated that Moe was not initially associated with the bleb cortex during its expansion and was recruited at the bleb rim just before retraction. In normal conditions, blebs were rapidly retracted, within a couple of minutes (Figs. 2 A and S1 and Video 2), and thus, displayed a small size (Fig. 2 B). The absence of Moe activity leads to abnormally large cytoplasmic bulges deforming the cortex of dividing cells as observed in fixed samples (Carreno et al., 2008), suggesting that it disrupts cortical organization. We thus analyzed Moe-GFP dynamics in living cells during polar blebbing.
not associated with bleb membranes when blebs expand, they were recruited to the bleb cortex when they retract in a manner that parallels the recruitment of Moe (Figs. 2 C and S1 and Video 4). In cells depleted of Moe or Slik, actin and myosin were no longer recruited uniformly to the bleb cortex (Figs. 2 C and S1 and Video 5). Secondary blebs then formed, ultimately leading to multiple blebs on blebs and triggering the abnormal mitotic cytoplasmic bulges observed in fixed samples (Carreno et al., 2008).

Together, these results show that cell division involves a precise regulation of Moe activity to control polar relaxation via mitotic blebbing. Defective Moe cortical recruitment promotes abnormal, unregulated polar blebs and thus, excessive polar relaxation. On the contrary, overactivation of Moe at the polar cortex impairs anaphase relaxation and cell elongation, leading to cytokinesis failure.
associated with the polar cortex during anaphase elongation in Pp1-87B–depleted cells, similar to the distribution of the phosphomimetic Moe-TD mutant form (Fig. 1 D). As observed in Moe-TD cells, Pp1-87B depletion triggered frequent cytokinesis failure (21%, n = 554), further supporting that Moe down-regulation at the polar cortex is required to achieve proper cell division.

To evaluate how Pp1-87B controls Moe activity during the cell cycle, we examined Pp1-87B distribution using a functional GFP fusion (Fig. S2 C). Although mostly cytoplasmic in interphase, GFP–Pp1-87B was redistributed to the mitotic spindle upon mitosis entry (Figs. 3 D and S2 D). Similarly to Pp1-87B, its human orthologue (Trinkle-Mulcahy et al., 2006), GFP–Pp1-87B accumulated on chromosomes, close to the polar cortex in anaphase (Figs. 3 D and S2 D); then, it concentrated to the intercellular bridge where Moe is normally inactivated before abscission (Fig. 1 A). Therefore, dynamics of Pp1-87B distribution inversely correlates with sites of Moe activity. In contrast, Slik associated with the whole-cell cortex in interphase and metaphase and accumulated at the cleavage furrow after the anaphase onset (Fig. 3 D). These data show that a balance of Slik kinase and Pp1-87B phosphatase activities coordinates Moe activation with the cell cycle, restricting high levels of active cortical Moe to early mitosis.
As a first test of this hypothesis, we examined the distribution of the Moe FERM domain fused to GFP (FERM Moe-GFP) in live cells. Although FERM Moe-GFP isotropically associated with the cortex from interphase to metaphase, it was then enriched at the equatorial cortex in anaphase B and telophase (Fig. 4 A). Reciprocally, a full-length Moe mutant (Moe-KN-GFP) unable to bind to PI(4,5)P2 (Roch et al., 2010) was not associated with the cortex throughout mitosis (Fig. 4 A). Therefore, PI(4,5)P2 binding is required for Moe enrichment at the anaphase equator.

We then examined the localization of PI(4,5)P2 in a S2 cell line that stably expresses GFP-Tubby, a specific probe for this phosphoinositide (Szentpetery et al., 2009; Ben El Kadhi et al., 2011). As observed for Moe-GFP (and FERM Moe-GFP), GFP-Tubby cleared from polar regions and concentrated at the PI(4,5)P2 regulates Moe distribution during mitosis

Despite alteration of Moe cortical levels, neither Slik nor Pp1-87B depletion completely abolished the equatorial enrichment of Moe-GFP when cells progress through anaphase (Figs. 2 A and 3 C). Furthermore, the reciprocal phospho mutant forms of Moe, Moe-TD-GFP (Fig. 1 D) and Moe-TA-GFP (Fig. S3), remained enriched at the equatorial cortex in anaphase. These findings suggest that a phosphorylation-independent mechanism regulates the cortical distribution of Moe after the anaphase onset. Binding of the FERM domain to PI(4,5)P2 plays a key role in Moe function in vivo (Roch et al., 2010), opening the possibility that PI(4,5)P2 could contribute to the regulation of Moe activity/distribution during cell division.

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Figure 4. PI(4,5)P2 regulates Moe distribution during mitosis. [A] Time-lapse frames of cells stably expressing the N-terminal FERM domain of Moe fused to GFP (top) or a full-length mutant form of Moe (MoeKN-GFP; bottom) at which point mutations in the FERM domain abolish binding to PI(4,5)P2 (Roch et al., 2010). (B) Dynamics of GFP-Tubby, a PI(4,5)P2 probe in a living S2 cell undergoing mitosis. Max Proj., maximum projection. (C) Quantification of the equatorial enrichment of GFP-Tubby (green) and of anaphase cell elongation (blue). L, length along the spindle; l, length along the equator. (D) Schematic representation of rapamycin-induced dephosphorylation of PI(4,5)P2. Protein domains expressed from RC constructs heterodimerize upon rapamycin addition causing dephosphorylation of PI(4,5)P2 at the plasma membrane (Varnai et al., 2006). (E) Cell lines stably expressing GFP-Tubby, Moe-GFP, or Moe-TD-GFP were transfected (+RC) or not transfected (−RC) by RCs. Living interphase or metaphase cells were imaged just before and after rapamycin addition. (F) Rapamycin-treated Moe-GFP cells in telophase expressing (+RC) or not expressing (−RC) RC constructs. Bars, 10 µm.
equator when cells started to elongate in anaphase (Fig. 4, B and C). These results support the idea that PI(4,5)P_2 contributes to the local control of Moe activation during cell division.

To directly test this conclusion, we used a system that rapidly reduces PI(4,5)P_2 cortical levels (Varnai et al., 2006), through a rapamycin-inducible recruitment of a type IV phosphoinositide 5-phosphatase domain to the plasma membrane (Fig. 4 D). In this assay, rapamycin induces the heterodimerization between a membrane-targeted FKBP-rapamycin–binding domain fragment of mammalian target of rapamycin and a 5-phosphatase domain fused to FKBP12 (Varnai et al., 2006), individually expressed from two constructs, hereafter referred to as rapamycin constructs (RCs). Although addition of rapamycin to control cells did not modify the localization of GFP-Tubby (Figs. 4 E and S4 A), rapamycin promoted its rapid translocation between the membrane to the cytosol in RC cells (Figs. 4 E and S4 B [for quantification] and Video 6). These results validate the efficiency of the RC system in S2 cells and the specificity of the GFP-Tubby probe for PI(4,5)P_2. We then explored whether reduction of PI(4,5)P_2 affects the distribution of wild-type or TD Moe-GFP. Moe-TD displayed a strong cortical association in interphase, which was lost after rapamycin treatment in RC cells (Fig. 4 E). Furthermore, when rapamycin was added on RC cells in metaphase, Moe-GFP rapidly delocalized from the cortex to the cytoplasm (Figs. 4 E and S4 C [for quantification] and Video 7). Similarly to Moe dsRNA depletion, this rapamycin-inducible release of Moe from cortical membranes triggered excessive polar relaxation, with unregulated blebbing during cell elongation (Fig. 4 F and Video 8). Together, these results support that PI(4,5)P_2 is necessary to ensure mitotic cortical stability, at least in part, through regulation of Moe cortical distribution and activation.

Localized activity of Skittles and Pten controls sites of PI(4,5)P_2 production and of Moe activation

To further investigate how PI(4,5)P_2 regulates mitotic cortical stability and Moe activation, we individually depleted each of the *Drosophila* kinases (10), phosphatases (16), and phospholipases (5) predicted to directly or indirectly control PI(4,5)P_2 metabolism (Figs. 5 A and S5), and we assessed the consequences of these depletions on the mitotic cortex by time-lapse microscopy. Among the 31 enzymes we have tested, depletion of three proteins induced high cortical defects, including excessive polar relaxation and unregulated blebs during anaphase (Figs. 5 B and S5). Two of them, Skittles and Pten directly produce PI(4,5)P_2 by phosphorylating PI(4)P and dephosphorylating PI(3,4,5)P_3, respectively. The third enzyme, encoded by CG10260 and predicted to produce PI(4)P from phosphatidylinositol, was required to a lesser extent for mitotic cortical stability (Fig. 5 B). To quantify the respective contribution of these enzymes to PI(4,5)P_2 production, we measured cellular phosphoinositides using radiolabeling assays (Payrastre, 2004). PI(4,5)P_2 pools were decreased by 15, 31, and 48% after depletion of CG10260, Pten, or Skittles, respectively (Fig. 5 C). Consistently, although the cortical association of GFP-Tubby was only slightly reduced by depletion of CG10260 (not depicted), the absence of Pten or Skittles further prevented the cortical recruitment of this biosensor, showing that these enzymes generate PI(4,5)P_2 at the cortex (Fig. 5 D).

Having shown that Skittles and Pten play major roles in PI(4,5)P_2 production in S2 cells, we first examined whether their localization could account for PI(4,5)P_2 enrichment at the equator in anaphase. Comparable with PI(4,5)P_2 dynamics, Pten-GFP and Skittles-GFP uniformly associated with the cortex in prometaphase. Both enzymes then progressively accumulated at the equator, as cells underwent anaphase elongation (Fig. 6, A and B). Because Pten metabolizes PI(3,4,5)P_3, we analyzed the distribution of this PI(4,5)P_2 precursor during cell division using the Grp1-pleckstrin homology (PH)-GFP–specific PI(3,4,5)P_3 probe (Gray et al., 1999). In control cells, Grp1-PH-GFP was weakly associated with the cortex of mitotic cells, albeit enriched at the cleavage furrow in anaphase (Fig. 6 A). Pten depletion increased the cortical levels of Grp1-PH-GFP, with accumulation at the anaphase equator, where Pten normally localizes. This indicates that Pten locally dephosphorylates PI(3,4,5)P_3 to control PI(4,5)P_2 cortical levels during mitosis. Accordingly, depletion of Pten provoked the formation of abnormal long-lived polar blebs, a phenotype reminiscent of that seen after rapamycin-induced reduction of PI(4,5)P_2. The absence of Skittles further decreased cortical PI(4,5)P_2 levels during mitosis, as deduced from its impact on GFP-Tubby distribution (Fig. 6 B). The depletion of Skittles also enhanced unregulated polar blebbing in anaphase (Fig. 6 B), mimicking the defects observed in cells with reduced PI(4,5)P_2 levels by the RC experiment (Fig. 4 F) or those lacking Moe activity (Fig. 2, A and C).

We then tested whether Skittles or Pten was required for Moe phosphorylation during mitosis, using FACS analysis of cells costained for P-Moe and the mitotic marker phospho–histone H3. When compared with control conditions, Pten depletion caused a slight decrease in P-Moe levels (27.7% of mitotic cells negative for P-Moe staining vs. 17% in controls). These defects were enhanced when Skittles was depleted, with 81.7% of mitotic cells displaying reduced levels of Moe phosphorylation (Fig. 6 C).

Accordingly, Skittles depletion impaired the association of Moe-GFP with the mitotic cortex (compare Fig. 6 D and Video 9 with Fig. 1 A). This lack of Moe recruitment at the cortex of polar blebs correlated with defects in actin organization (Fig. 6 E and Video 10) and triggered the “blebs on blebs” phenotype and unregulated bleb growth (5.57 ± 1.58–µm length, n = 26 vs. 3.25 ± 0.44–µm length, n = 40 in controls; P = 1.5 x 10^{-5}) observed after Moe or Slik depletion (Fig. 2, A and C).

These results show that Skittles and Pten contribute to enrich PI(4,5)P_2 at the equator, when dividing cells progress through anaphase. Thus, Skittles and Pten spatially mediate Moe cortical activation to promote efficient bleb dynamics necessary to control anaphase cell elongation.

**Discussion**

These findings unravel how, by integrating two regulatory networks, Moe activity provides a spatiotemporal framework to control cell shape transformations during division (Fig. 7).
At mitosis exit, both cortical contractions and ERM activity must be downregulated to allow cells to go back to their interphase shape. In Drosophila cells, the Slik kinase was shown to activate Moe at mitosis entry (Carreno et al., 2008; Kunda et al., 2008). Here, we identify the Pp1-87B phosphatase as essential for Moe inactivation after cytokinesis and in interphase.

Although Slik homogenously associates with the cell cortex in both interphase and early mitosis, Pp1-87B is cytoplasmic in interphase and relocalizes to the spindle in pro/metaphase (Fig. 7 B). An attractive model would be that together with a "constitutive" cortical association of the Slik activator in interphase and pro/metaphase, intracellular redistribution of the Pp1-87B inhibitor represents an efficient way to restrict high levels of Moe phosphorylation to mitosis entry. During anaphase, Pp1-87B concentrates near the chromosomes migrating toward the polar cortex, whereas Slik accumulates at the cleavage furrow. In this model, redistribution of both Pp1-87B

We find that the increase in cortical rigidity that drives cell shape remodeling at the interphase/mitosis transition involves a Pp1-87B/Slik molecular switch that timely regulates Moe phosphorylation. We further identify PI(4,5)P₂ as a spatial cue that controls Moe distribution at the cortex. This latter aspect coordinates the spatial balance in cortical stiffness/contractility that is required for anaphase cell elongation and cytokinesis. We propose that the concerted action of these two regulatory networks ensures the proper series of mitotic cell shape transformations required for the fidelity of cell division (Fig. 7).

**A Pp1-87B/Slik molecular switch controls temporal activation of Moe**

A global increase in cortical actomyosin forces generate cell rounding at mitosis entry (Maddox and Burridge, 2003; Stewart et al., 2011). These forces are transmitted to the plasma membrane through the activation of ERM proteins (Carreno et al., 2008; Kunda et al., 2008; Luxenburg et al., 2011). At mitosis exit, both cortical contractions and ERM activity must be downregulated to allow cells to go back to their interphase shape. In Drosophila cells, the Slik kinase was shown to activate Moe at mitosis entry (Carreno et al., 2008; Kunda et al., 2008). Here, we identify the Pp1-87B phosphatase as essential for Moe inactivation after cytokinesis and in interphase.

Although Slik homogenously associates with the cell cortex in both interphase and early mitosis, Pp1-87B is cytoplasmic in interphase and relocalizes to the spindle in pro/metaphase (Fig. 7 B). An attractive model would be that together with a "constitutive" cortical association of the Slik activator in interphase and pro/metaphase, intracellular redistribution of the Pp1-87B inhibitor represents an efficient way to restrict high levels of Moe phosphorylation to mitosis entry. During anaphase, Pp1-87B concentrates near the chromosomes migrating toward the polar cortex, whereas Slik accumulates at the cleavage furrow. In this model, redistribution of both Pp1-87B
Figure 6. **Localized activity of Skittles and Pten controls sites of PI(4,5)P_2 production and Moe activation.** (A) Dynamic of Pten-GFP distribution during mitosis (top). A stable cell line that expresses Grp1-PH-GFP was used to probe PI(3,4,5)P_3 distribution during mitosis in control conditions or after Pten depletion. (B, top) Time-lapse frames of a Skittles-GFP cell progressing through mitosis. Bottom images show a living cell stably expressing GFP-Tubby after Skittles depletion. (C) FACS analysis showing the distribution of P-Moe intensity in mitosis (assessed by histone H3 phosphorylation) of control and Pten- or Skittles-depleted cells. a.u., arbitrary unit. (D) Distribution of Moe-GFP after Skittles depletion. (E) Rapid time-lapse imaging of α-GFP-UtrCH (GFP-UtrCH) and α-Tubulin-mCherry after depletion of Skittles showing maximum projection. Close-ups (focal planes) correspond to the framed region and show GFP signal in black. Arrowheads show cortical rupture and defects of F-actin recruitment at the bleb rim; arrows show unregulated mitotic blebs. Bars, 10 µm.
PI(4,5)P₂ controls stability of the mitotic cortex and spatial activation of Moe

Our results show that local levels of PI(4,5)P₂ provide an additional mechanism to regulate Moe function at the cortex of dividing cells. Several studies have established a role of PI(4,5)P₂ in the localization of ERM proteins in polarized processes of differentiated cells (Fievet et al., 2004; Hao et al., 2009; Roch et al., 2010). Here, we provide evidence that during mitosis, PI(4,5)P₂-rich membrane domains act as a spatial cue that regulates both Moe distribution and activation at the cortex. The distribution of PI(4,5)P₂ at the plasma membrane is tightly regulated during mitosis. As in mammalian cells (Emoto et al., 2005; Field et al., 2005), we find that PI(4,5)P₂ is actively enriched at the equator at anaphase in Drosophila S2 cells, suggesting that equatorial accumulation of PI(4,5)P₂ is a feature shared by most animal cells. Although a previous study did not detect...
Pl(4,5)P₂ enrichment at the cleavage furrow of Drosophila spermatocytes (Wong et al., 2005), whether this is caused by an intrinsic difference between mitosis and meiosis or by experimental limitations in vivo remains to be established. However, how this dynamic localization is regulated remains unknown. Here, we show that the equatorial enrichment of Pl(4,5)P₂ relies, at least in part, on the enzymatic activity of Skittles and Pten. During cytokinesis, the equatorial accumulation of Pl(4,5)P₂ plays a role in cleavage furrow formation and ingestion, through controlling the activity and/or recruitment of several components of the contractile ring (Janetopoulos and Devreotes, 2006; Ben El Kadhi et al., 2011). Pl(4,5)P₂ hydrolysis is also necessary for maintaining cleavage furrow stability and efficient cytokinesis (Emoto et al., 2005; Field et al., 2005; Wong et al., 2005). Our findings extend the functional repertoire of Pl(4,5)P₂ during mitosis to the control of local properties of the mitotic cortex, which are required for polar relaxation and cell elongation. Through functional screenings, we identify novel regulators of cell division among the entire set of enzymes implicated in phosphoinositide biosynthesis. Two main pathways regulate Pl(4,5)P₂ levels in mitotic cells, and their alterations provoke similar cortical disorganization. The first pathway involves the Pten tumor suppressor, a Pl(3,4,5)P₃-3-phosphatase. Pten was shown to accumulate at the septum of dividing yeast cells (Mitra et al., 2004), as well as at the cleavage furrow in Dictyostelium discoideum (Janetopoulos et al., 2005). Our results of living Drosophila cells show a progressive delocalization of Pten from the polar cortex to the equator after anaphase onset (Fig. 7B), suggesting that Pten dynamics rely on mechanisms conserved throughout evolution. Furthermore, depletion of Pten leads to a significant enrichment of Pl(3,4,5)P₃ at the cortex, especially at the cleavage furrow. These results show that Pten uses Pl(3,4,5)P₃ to spatially control Pl(4,5)P₂ levels at the mitotic cortex.

The second pathway relies on Skittles, a Pl(4)P 5-kinase that plays a major role in regulating the levels and localization of Pl(4,5)P₂ during mitosis. Skittles switches from an isotropic cortical distribution in pro/metaphase to equatorial enrichment after the anaphase onset (Fig. 7B). Depletion of Skittles results in a phenotype similar to the mitotic cortical defects observed after inducible Pl(4,5)P₂ hydrolysis. We also find that CG10260, a phosphoinositide 4-kinase, contributes to the organization of the mitotic cortex. Genetics screens have identified a role for phosphoinositide 4-kinases in the division of budding and fission yeast (Audhya et al., 2000; Hama et al., 2000; Desautels et al., 2001) as well as for cytokinesis of male spermatocytes in flies (Brill et al., 2000). CG10260 is involved in Pl(4)P synthesis, the major substrate of Skittles to produce Pl(4,5)P₂. Together, these data show that Skittles acts as a key regulator of Pl(4,5)P₂ levels and Moe activation at the mitotic cortex. Interestingly, Skittles is required for Moe activation in Drosophila oocytes (Gervais et al., 2008), suggesting that this enzyme plays a broad role in the regulation of ERM proteins.

An important question is how Skittles and Pten are enriched at the equator in anaphase. It has been reported that activated RhoA stimulates a Pl(4)P 5-kinase activity and promotes Pl(4,5)P₂ synthesis in mammalian cells (Chong et al., 1994). During anaphase, activated RhoA localizes at the equatorial cortex (Yoshida et al., 2009), where it could recruit and/or activates Skittles to promote Pl(4,5)P₂ production. This anisotropy in Pl(4,5)P₂ distribution might be in turn reinforced by the localized activity of Pten, whose membrane association is itself dependent on Pl(4,5)P₂ (unpublished data; Campbell et al., 2003; Rahdar et al., 2009). Together, the activity of Skittles and Pten could therefore provide a feed-forward regulatory loop of local Pl(4,5)P₂ levels at the cortex of dividing cells.

**Spatiotemporal regulation of Moe contributes to mitotic cell elongation**

The metaphase/anaphase transition is characterized by a break in cortical symmetry, with concomitant relaxation of the polar cortex and contraction of the equator. We find that the anisotropic distribution of Moe participates in coordinating this differential in cortical tension. Overactivation of Moe impairs cell elongation and causes cytokinesis failure, suggesting that the polar cortex is too rigid for cell division. Accumulation of F-actin at the cleavage furrow can be attributed, at least in part, to a cortical flow of F-actin filaments from polar regions to the equator (Chen et al., 2008). Overactivation of Moe at the poles could block this actin cortical flow, through an excessive bridging of the actin cytoskeleton with the plasma membrane, leading to an abnormal stiffness of the polar cortex. Therefore, redistribution of activated Moe from the polar cortex to the equator participates in polar relaxation, anaphase cell elongation, and cytokinesis fidelity.

Contraction of the equatorial actomyosin ring increases the cytoplasmic pressure exerted on the plasma membrane. Relaxation of the polar cortex is thus required to dissipate this extra pressure by increasing the cellular volume, a process that was proposed to involve short-lived polar blebs (Prothero and Spencer, 1968; Brugués et al., 2010; Sedzinski et al., 2011). These polar blebs were recently found to play important roles during cell division. Perturbation of their dynamics triggers anaphase spindle rocking (Rankin and Wordeman, 2010) and destabilization of cleavage furrow positioning (Sedzinski et al., 2011). Although recent studies have addressed how cortical blebs are regulated in interphase (Charras et al., 2005, 2006), our understanding of the signalization that controls dynamics of cortical blebs in mitosis has poorly progressed since pioneering studies. Our results show that a transient recruitment of Moe at the mitotic bleb membrane is required for efficient polar bleb retraction, as are the functions of the Moe positive regulators Slik, Skittles, and Pten. Active Moe contributes to cortical bleb organization because alteration of Moe function (or regulation) disrupts actin organization and efficient bleb retraction. This leads to disorganization of the mitotic cortex, characterized by giant blebs that continue growing in an unregulated manner. Therefore, although a global decrease in Moe activity at the polar cortex contributes to cell elongation and cytokinesis, transient and local association of Moe at the rim of polar blebs is important for their retraction. If the binding of Moe to Pl(4,5)P₂ is required at both the equator and bleb membrane, the influence of the Slik kinase on Moe activation appears different between these two regions of the anaphase cortex. Although Slik depletion abolishes
Moe recruitment to polar blebs, remnants of cortical Moe are still visible at the equator, likely as a result of high PI(4,5)P2 levels at the furrow.

Although these mechanisms synergistically contribute to the cortical contractility at the equator, they also allow cortical relaxation at the polar cortex through control of transient anaphase blebs. We propose that this dual mechanism of Moe regulation relaxes levels of the different GFP fusions. The distribution of Pp1-87B–GFP, Skit-tiles–GFP, and reverse, 5’-TAATAGCCTCATAATTGAGGAGAACATTTTGCAGGG-3’; Pten_2 forward, 5’- TAATACGACTTGCTCTATACTG GGGTAAGGC-3’; Pten forward, 5’- TAATACGACTTGCTCTATACTG GGGTAAGGC-3’; Pten forward, 5’- TAATACGACCTCATAGTGGGAGAACATTTTGCAGGG-3’; CG10260_1 forward, 5’- TAATACGACTTGCTCTATACTG GGGTAAGGC-3’; CG10260 forward, 5’- TAATACGACTTGCTCTATACTG GGGTAAGGC-3’. For live imaging, cell lines were cultivated in glass-bottom plates and multichannel images were acquired at 27°C with a microscope (DeltaVision; Applied Precision) equipped with a camera (Coolsnap HQ2) at 2 × 2 binning and 60× Plan Apochromat (1.42 NA) objective.

Dosage of phosphoinositides Phosphatase subunits and phosphoinositide enzymes were identified by phosphoinositol phosphate and PI(4,5)P2 levels. We used FACS sorting (FACS Aria; BD) to select cell lines expressing moderate levels of the different GFP fusions. The distribution of Pp1-87B–GFP, Skit-tiles–GFP, and reverse, 5’-TAATACGACCTCATAGTGGGAGAACATTTTGCAGGG-3’; CG10260_1 forward, 5’- TAATACGACTTGCTCTATACTG GGGTAAGGC-3’; CG10260 forward, 5’- TAATACGACTTGCTCTATACTG GGGTAAGGC-3’; Pten_2 forward, 5’- TAATACGACTTGCTCTATACTG GGGTAAGGC-3’; Pten forward, 5’- TAATACGACTTGCTCTATACTG GGGTAAGGC-3’. For live imaging, cell lines were cultivated in glass-bottom plates and multichannel images were acquired at 27°C with a microscope (DeltaVision; Applied Precision) equipped with a camera (Coolsnap HQ2) at 2 × 2 binning and 60× Plan Apochromat (1.42 NA) objective.

Flow cytometry Cells were fixed for 4 h using ethanol 75% and permeabilized by 0.02% saponin. Cells were labeled using anti–P-Moe (1:100), anti–phospho–histe H3 (Ser10) 647 conjugate (1:100; Cell Signaling Technology), and Alexa Fluor 488 goat anti–rabbit (1:1,000; Invitrogen) antibodies. Cells were then analyzed by FACS (LSR II; BD).

Online supplemental material Fig. S1 shows localization of sqh-GFP during mitotic blebbing and quantification of mitotic bleb dynamics in control and Slik dsRNA conditions. Fig. S2 shows characterization of Pp1-87B function during cell division. Fig. S3 shows localization of Moe-TA-GFP during cell division. Fig. S4 shows quantification

Materials and methods DNA constructs Pen cDNA obtained from V. Archambault (Institute for Research in Immunology and Cancer, Montréal, Québec, Canada; IP16020; Drosophila Genomics Resource Center) was amplified by PCR (Phusion; New England Biolabs, Inc.) and fused to GFP in the pAc5.1 vector (Invitrogen). RCS (PM-FRB-monomeric RFP [mRFP] and mRFP-FKBPs phosphatase domain) obtained from T. Balla (National Institutes of Health, Bethesda, MD; Yarnai et al., 2006) and GFP-Urophin-Ch obtained from W.B. Menter (University of Würzburg, Würzburg, Germany; Drath et al., 2007) were subcloned into the pAc5.1 vector (Invitrogen). pUAS-Skittles-GFP was obtained from A. Guichet (Institut Jacques Monod, Unité Mixte de Recherche 7592, Paris, France; Gervais et al., 2008), pUAS-Slik-GFP was obtained from D. Hipfner (Institut de Recherches Cliniques de Montréal, Montréal, Québec, Canada; Hipfner et al., 2004), and pUAS-GFP-Tubby and pU6b-p11-GFP were obtained from T. Balla (National Institutes of Heath, Bethesda, MD; Varnai et al., 2009). pAc5.1 vector (Invitrogen). pUAS-Skittles-GFP was obtained from A. Guichet (Institut Jacques Monod, Unité Mixte de Recherche 7592, Paris, France; Gervais et al., 2008), pUAS-Slik-GFP was obtained from D. Hipfner (Institut de Recherches Cliniques de Montréal, Montréal, Québec, Canada; Hipfner et al., 2004), and pUAS-GFP-Tubby and pU6b-p11-GFP were obtained from T. Balla (National Institutes of Heath, Bethesda, MD; Varnai et al., 2009). DNA constructs were previously described in Carreno et al. (2008). dsRNA primers dsRNA primers used in this study were as follows: Skittles_1 forward, 5’-TAATAGCCTCATAATTGAGGAGAACATTTTGCAGGG-3’; and reverse, 5’-TAATAGCCTCATAATTGAGGAGAACATTTTGCAGGG-3’. For live imaging, cell lines were cultivated in glass-bottom plates and multichannel images were acquired at 27°C with a microscope (DeltaVision; Applied Precision) using softWoRx software [Applied Precision] equipped with a camera (Coolsnap HQ2; Photometrics) at 1 × 1 binning and 60× Plan Apochromat (1.42 NA) objective. For live imaging, cell lines were cultivated in glass-bottom plates and multichannel images were acquired at 27°C with a microscope (DeltaVision; Applied Precision) using softWoRx software [Applied Precision] equipped with a camera (Coolsnap HQ2; Photometrics) at 1 × 1 binning and 60× Plan Apochromat (1.42 NA) objective.

Immunofluorescence and video microscopy Cells were cultured for 4 h on glass coverslips, fixed in 4% formaldehyde (or 10% TCA for P-Moe), and processed for immunostaining [Carreno et al., 2008]. We used anti–α Tubulin (Thueringer et al., 2008) at 1:100, anti–α-tubulin coupled to FITC (F2168; Sigma–Aldrich) at 1:50, and Texas red goat anti–rabbit (Invitrogen) at 1:200. Texas red phalloidin (Invitrogen) was used at 1:100 for F-actin staining. Fixed imaging was performed at room temperature using mounting medium (Vectashield; Vector Laboratories) on a microscope (DeltaVision; Applied Precision) using softWoRx software [Applied Precision] equipped with a camera (Coolsnap HQ2; Photometrics) at 1 × 1 binning and 60× Plan Apochromat (1.42 NA) objective.
of the RC experiments shown in Fig. 4. Fig. S5 shows the results of the functional screen for enzymes implicated in phosphoinositide metabolism shown in Fig. 5. Video 1 shows the dynamic localization of Moe-GFP during division of a control cell over a 25-min period with a 1-min interval (as shown in Fig. 4 B). Video 2 shows the dynamic localization of Moe-GFP during division of a Skittles-depleted cell over a 250-s period with a 20-s interval (as shown in Fig. 2 A). Video 3 shows the dynamic localization of Utoprin-CH-GFP (as an F-actin probe) during division of a control cell over a 337.5-s period with a 2.5-s interval (as shown in Fig. 2 C). Video 4 shows the dynamic localization of Utrophin-CH-GFP (as an F-actin probe) in interphase RC cells before and after addition of rapamycin over a 450-s period with a 15-s interval (as shown in Fig. 4 E). Video 5 shows the dynamic localization of Moe-GFP of a metaphase RC cell before and after addition of rapamycin over a 300-s period with a 10-s interval (as shown in Fig. 4 E). Video 6 shows the dynamic localization of Utrophin-CH-GFP (as an F-actin probe) during division of a Skittles-depleted cell over a 217.5-s period with a 7.5-s interval (as shown in Fig. 6 D). Video 10 shows the dynamic localization of Utrophin-CH-GFP (as an F-actin probe) during division of a Skittles-depleted cell over a 450-s period with a 15-s interval (as shown in Fig. 2 C). Video 6 shows the dynamic localization of GFP-Tubb3 (as a PI(4,5)P2 probe) in interphase RC cells before and after addition of rapamycin over a 450-s period with a 15-s interval (as shown in Fig. 4 E). Video 7 shows the dynamic localization of Moe-GFP of a metaphase RC cell before and after addition of rapamycin over a 300-s period with a 10-s interval (as shown in Fig. 4 E). Video 8 shows the dynamic localization of Moe-GFP of an ana/telophase RC cell 435 s after addition of rapamycin over a 603-s period with a 9-s interval (as shown in Fig. 4 F). Video 9 shows the dynamic localization of Moe-GFP during division of a Skittles-depleted cell over a 90-min period with a 10-min interval (as shown in Fig. 6 D). Video 10 shows the dynamic localization of Utoprin-CH-GFP (as an F-actin probe) during division of a Skittles-depleted cell over a 217.5-s period with a 7.5-s interval (as shown in Fig. 6 E). Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201106048/DC1.

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