Get round and stiff for mitosis

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Cell rounding is a common feature of cell division. The spherical shape that cells adopt during mitosis is apparently neither a simple detachment nor a global softening or stiffening that allows cells to adopt what seems to be a mechanical equilibrium. It is a highly complex mechanical transformation by which membrane folding and peripheral signals focusing can match spindle size in order to ensure a proper cell division. Recent new insight into the mechanism involved will prompt the scientific community to focus on the regulation of the physical links that exist between the lipid bilayer membrane and the underlying actin cytoskeleton since it now appears that these will strongly influence some crucial cellular events such as the spatial organization of cell division.

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In most tissues as in culture conditions cells round up when entering mitosis. The association between lipids of the plasma membrane and the underlying actin filaments network constitutes the so-called cell cortex. The profound reorganization of cell cortex structure and modifications of its mechanical properties induce large morphologic changes of cells entering mitosis. The cell architecture eventually reached is directly implicated in mitotic spindle formation and cleavage plane positioning (Théry and Bornens, 2006). But the mechanisms involved in this interaction are still obscure. Two recent publications in *Current Biology* and in the *Journal of Cell Biology* shed light on a new and major contribution of ezrin-radixin-moesin proteins in the regulation of the mechanical changes of the cell cortex in mitosis and in the shaping of the mitotic spindle (Carreno *et al.* 2008; Kunda *et al.* 2008).

MITOTIC CELL ROUNDING

Simple geometrical considerations highlight the complexity of mitotic cell rounding. A sphere corresponds to the smallest possible area for a given volume. Similarly it can be seen as the largest volume that can be contained in a given area. So the transforma-

tion of an object into a sphere can be performed either by a volume increase or an area reduction [Fig. 1(a)]. Recent experiments showed that during mammalian cell rounding the cell volume is even reduced (Boucrot and Kirchhausen, 2008). This is made possible by an important cell surface reduction. Large part of the surface is endocytosed and transformed in small internal vesicles (Boucrot and Kirchhausen, 2007). Another part of the surface is folded in many small membrane spikes, wrinkles and fibers that have been observed in electronic microscopy [Fig. 1(b)] (Porter *et al.*, 1973; Erickson and Trinkaus, 1976; Sanger *et al.*, 1984). These surface transformations require a large increase of membrane curvature and thus many internal structural changes to support membrane convolutions. The mechanical organization of cortical actin with respect to plasma membrane in interphase [Fig. 1(c)] has to be profoundly remodeled during mitotic cell rounding [Fig. 1(d)].

In interphase cells, actin cytoskeleton is organized in distinct structures having specific mechanical properties. The spatial arrangement of these structures defines a cortical polarity which reflects, and supports, the entire structural and functional polarity of the cell. Actin stress fibers step over specific areas

Figure 1. Cell cortex remodeling during mitotic cell rounding. Several changes of cell surface and cell volume can lead to cell rounding. To achieve an important degree of compaction, cells reduce both their volume and membrane area by folding their membrane in cortical bulges and internal vesicles (a). Electron microscopy images showed the appearance of cortical spikes and retraction fibers on mitotic cells (b, reproduced from *The Journal of Cell Biology*, 1973, **57**, 815–836. Copyright 1973 The Rockefeller University Press). In interphase the actin network form a branched network that push the membrane (c). In mitosis several structural changes can be imagined to participate for cell shape folding (d). A thin layer of actin filaments might follow the little membrane outgrowth while a thick, rigid and contractile network of filaments ensures entire surface compaction. The network is bound to the lipid bilayer by ERM proteins (d, blue dots). Myosin II molecules ensure filaments sliding and network contraction (d, yellow dots). RNA interference with the expression of ERM proteins strongly impaired cortex remodeling and cell rounding in mitosis [e, adapted from movie S2 in (Kunda *et al.* 2008)]. ERM protein is sufficient to induce cell rounding without Myosin II. The regulation of actin filaments length and cross-linking can modulate network compliance and cell shape (f). Cell rounding could be favored in an intermediate regime between short and flexible filaments and long and rigid bundles. ERM proteins recruit actin and actin associated proteins and anchor the network at the surface. Thereby they modulate cortical compliance and impinge on shape transformation.

where cells are not attached to their environment. They are rigid and contractile (Rotsch *et al.*, 1999; Rotsch and Radmacher, 2000; Peterson *et al.*, 2004). On adhesive regions, actin filaments assemble in a meshwork which is much softer (Laurent *et al.*, 2005; Park *et al.*, 2005). The growth of this meshwork pushes on the cell periphery and induces membrane protrusions. When entering mitosis this

complex actin network is completely deconstructed and reformed to ensure cell rounding (Théry and Bornens, 2006). This transformation is a complex and poorly described combination of local contractions and relaxations but also of modulation of the actin network density. It starts with a process called de-adhesion. Most adhesion protein complexes get dissociated and become cytosolic. Concomitantly stress

fibers disassemble and actin relocates to the cell periphery. This causes the previously contractile cell edges to retract inward. At the other edges, membrane protrusions cease and the cell margins start to move inward thereby forming retraction fibers from the small sites of attachment which remain after de-adhesion. Retraction fibers are thin actin-rich membrane tubes connected at their distal end to the adhesive substrate and to the mitotic cell body at their proximal end. During this movement cells start to round up upon the reorganization of the cortical acto-myosin network and reach an almost spherical and rigid shape. At this stage cell surface is not smooth but instead displays many microvilli and blebs (Erickson and Trinkaus, 1976; Sanger *et al.*, 1984). This and the presence of retraction fibers suggest that the interaction between the cortical and contractile acto-myosin network and the lipid bilayer is not simple. The activity of many enzymes is known to be modified when entering mitosis. But only a few of them have been shown to be implicated in the dramatic reorganization of cell architecture.

RhoA activity has been shown to be partially involved in cell rounding and cortical stiffening (Maddox and Burridge, 2003). But the downstream targets have not been identified. Acto-myosin gel mechanical properties or connection between this gel and the lipid membrane have been envisaged (Maddox and Burridge, 2003). However, the inactivation of myosin light chain kinases and Rho-associated kinases only partially interferes with cell rounding (Kanada *et al.*, 2005, and our own unpublished observations). It might thus be necessary to distinguish the effect of myosin binding activity from that of its motor property. Tail-to-tail myosin II filaments are still able to cross-link filaments after inhibition of Rho kinases. The variations of actin network density are directly correlated to the gel rigidity (Laurent *et al.*, 2005). A thick and dense gel is rigid. But is it sufficient to drive cell rounding? Clearly the driving force for cell rounding is still not known.

The stiffening of the cortex during cell rounding participates to the immobilization of specific proteins in the actin network. The biochemical heterogeneity of the cell cortex can play a determinant role in mitotic progression by controlling spindle orientation and cleavage plane positioning (Théry and Bornens, 2006). Recently LIMkinase-mediated phosphorylation of cofilin in mitosis has been shown to be required for the stabilization of the actin network and retention of cortical signals (Kaji *et al.*, 2008). Inactivation of LIMK leads to actin severing, rupture of the cortical network and loss of the defined localization of some specific cortical cues. Specific lipids in the plasma membrane could also be necessary to recruit and confine actin-related proteins, thus contributing to cortical heterogeneity (Toyoshima *et al.*, 2007).

Two recent studies provide new insight in the mechanical and biochemical connections that exist between the lipid bilayer and the underlying cytoskeleton. The ezrin-radixin-

moesin (ERM) protein family has been shown to be implicated in this interplay between plasma membrane and actin filaments (Bretscher *et al.*, 2002). They are usually localized in highly curved cortical regions. In these two studies, authors took advantage of the presence of a unique member of the ERM family in *Drosophila*, the moesin, to follow its activation state during mitosis and the consequences of its inactivation. It appeared that it plays a determinant role in cell shape regulation and spindle assembly.

Moesin was shown to be phosphorylated by Slik kinase at the onset of mitosis (Carreno *et al.*, 2008). Phosphorylation induces the opening of moesin conformation and allows it to bind actin (Simons *et al.*, 1998). The phosphorylated form of moesin accumulates in retraction fibers during cell rounding (Kunda *et al.*, 2008) and thus establishes a heterogeneous spatial pattern of ERM activation on the round mitotic cell body in early metaphase. Such patterns were also observed in mitotic cortex of mammalian cells (Théry *et al.*, 2005). Interestingly the formation of thin retraction fibers in which membrane is highly curved provides a geometrical configuration that could specifically recruit associated proteins and lipids and trigger defined signaling pathways (Roux *et al.*, 2005; Antonny, 2006; Meyers *et al.*, 2006). So the spatial distribution of retraction fibers is an indirect way to control the location of specific biochemical signals in the mitotic cell cortex.

The expression of constitutively active or inactive forms of moesin and of myosin II showed that moesin activation is necessary and sufficient to induce cortex stiffening. Surprisingly, the expression of the active form of moesin in a myosin II mutant was sufficient to induce not only cell cortex stiffening but also cell rounding, leading the authors to conclude that moesin plays a dominant effect on myosin II activity (Kunda *et al.*, 2008). Both studies showed that inactivation of moesin expression by siRNA had a dramatic effect on cell rounding. Cells initiate retraction but cannot achieve a complete rounding. Instead they stay flat with a floppy cortex adopting highly irregular shapes [Fig. 1(e)]. Atomic force microscopy measurements confirm that the irregular aspect of cell shape was correlated with the incapability of moesin defective cells to get stiffer when entering mitosis (Kunda *et al.*, 2008). The authors proposed that the ability of ERM protein to align actin fibers parallel to the cell surface accounts for cortex stiffening and cell rounding. Indeed the recruitment of actin and the densification of the actin network at the membrane could result in a global stiffening of the cortex (Laurent *et al.*, 2005). But how alignment of actin fibers along the cortex could induce cell rounding remains an interesting question to be investigated. The regulation of actin filaments length and cross-linking, by the control of severing and binding proteins (Kaji *et al.*, 2008), could modulate actin bundles compliance. In a defined regime, their alignment along the membrane could prevent high membrane curvature and promote cell rounding [Fig. 1(f)].

Cortical actin cross-linking is apparently necessary, for example, to keep human lymphoblasts with a round shape when tubulin is induced to fully polymerized by taxol (Bornens *et al.*, 1989). More direct evidence that actin cross-linking promotes a rounded morphology has been reported also: the addition of filamin in actin-containing vesicles caused the vesicles to become smooth and spherical upon actin polymerization, whereas an irregular morphology occurred in the absence of filamin (Cortese *et al.*, 1989). Myosin II could then induce actin filaments sliding along each other and cortex contraction. The shrinkage of the actin network under the lipid bilayer would normally induce local membrane detachment and the formation of cortical bulges. ERM protein would prevent bulge outgrowth by firmly binding the contractile actin network with the lipid bilayer.

Observations of F-actin localization and cell shape changes by video microscopy suggested that the cortex of moesin-depleted cells was not homogeneously softer but instead erratically and locally contractile leading to highly unstable cortical shapes. F-actin was seen to accumulate at the basis of membrane blebs. They were growing out but could not resorb (Carreno *et al.*, 2008; Kunda *et al.*, 2008). Blebs are membrane extrusions generated by a local increase of hydrostatic pressure due to a local contraction of the cell cortex (Charras *et al.*, 2005; Charras, 2007). The extrusion occurs in places where the binding energy between the lipid membrane and the actin cytoskeleton cannot sustain the pressure increase. When entering mitosis the actin cytoskeleton contracts itself. If moesin is inactivated, the link with the membrane is so weakened that the pressure generated outflow will push on the membrane and induce bleb formation. Actin will be transported by the flow and will reform a cortical network in the bleb. But the absence of moesin linking activity to the plasma membrane will preclude bleb resorption driven by actin network contraction (Charras *et al.*, 2006). Such a mechanism can account for large bleb formation in Moesin RNAi cells during mitosis. These deformations were also described to be highly unstable, leading to permanent cell shape changes. This dynamic phenomena could be the manifestation in multiple and randomly dispersed locations of a contractile oscillating behavior of the cell cortex described in nonadherent cells (Paluch *et al.*, 2005). A local increase in acto-myosin contraction or a locally reduced actin shell thickness can lead to actin network rupture. The hole in the contractile shell induces a wave of contraction that starts from the hole and propagates to the rest of the cell [Fig. 2(b)]. When the cell cortex is rather homogeneous the propagating contraction wave takes the appearance of a contractile ring oscillating from one end of the cell to the other (Paluch *et al.*, 2005). The propagation of the contraction wave from the newly formed hole can also induce large cell deformation particularly if the absence of ERM protein prevents actin reorganization and bleb retraction. This could account for the highly unstable cell cortex described by the authors. One

should also envisage the deregulation of mitotic shape in moesin-depleted cells to be partially due to deadhesion defects since ERM protein are known to regulate cell adhesion (Takeuchi *et al.*, 1994). Thus, instead of regularly oscillating rings, several distinct contractile domains could form and propagate irregularly due to the presence of remaining adhesion sites that have not been properly disassembled.

Interestingly, if the localization of contractile areas is not evenly distributed during cell rounding but instead spatially controlled by the cell adhesion pattern as hypothesized above, and if pressure is not equilibrated in the cell (Charras *et al.*, 2005), one should see a preferential localization of blebs in moesin-depleted cells where contractility was higher before entering mitosis. Bleb formation could thus be used to probe cortical heterogeneity during mitosis if one could grow *Drosophila* cells depleted for moesin on specific adhesive patterns.

SPINDLE FORMATION AND POSITIONING

Actin integrity and more precisely the specific segregation of lipid domains and actin related proteins in defined regions of the cell cortex are required for proper mitotic spindle positioning (Théry *et al.*, 2005; Kaji *et al.*, 2008; Toyoshima *et al.*, 2007). The two discussed studies showed how the activation of ERM proteins is also required. They describe a long metaphase delay and defective spindle morphology in response to moesin inactivation. Spindles were slightly shorter, and off centered. Time-lapse imaging of tubulin-GFP revealed spindle rocking movement and complete loss of orientation during metaphase (Carreno *et al.*, 2008; Kunda *et al.*, 2008). The authors suggest that these movements are not simply due to the global cortex relaxation or an increase in cytoplasmic space because they did not see such movement in myosin II depleted cells which present similar geometrical modifications (Carreno *et al.*, 2008). Remarkably the spindle defects could be rescued externally by artificially cross-linking and stiffening the cortex with concanavalinA in solution (Kunda *et al.*, 2008). This suggests that spindle abnormal morphology was not due to the biochemical inactivity of moesin but was a mechanical consequence of cortex instability. The investigation of the role of LIMkinase and cofilin on spindle positioning showed other examples of mutants in which mitotic cell shape was not drastically affected but spindle position was perturbed in response to the mechanical destabilization of the cortex (Kaji *et al.*, 2008). Together these results raise the possibility that spindle positioning is sensitive to the heterogeneity in the spatial distribution of cortical rigidity. Spindle displays abnormal movements when the cell cortex displays erratic contractions. Importantly, such a mechanism when accurately regulated could possibly be used to orient the spindle in normal conditions.

The study of the mechanics involved in bleb formation revealed that hydrostatic pressure is not always equilibrated

Figure 2. Mechanical changes of the cell cortex during mitosis. In Moesin RNAi S2 *Drosophila* cells the cortex displays large and chaotic bleb outgrowths as well as wide spindle displacements a, reproduced from *The Journal of Cell Biology*, 2008, **180**, 739–746. Copyright 2008 The Rockefeller University Press). The two phenotypes might be mechanically connected. If astral microtubules push on free membrane (black) and pull on rigid cortical areas (red) the distribution of forces acting on the spindle could induce highly disturbed spindle movements (a). Cortical rupture induces bleb formation and contraction waves [b, from (Paluch *et al.* 2005)]. The erratic apparition of cortex ruptures could explain the highly unstable patterns of cell contractility in moesin RNAi cells and the disorganized forces that position the spindle. In normal conditions, the localization of ERM proteins (green) and myosin II (red) change from prophase (c-1) to telophase (c-5). They first accumulate at the top of retraction fibers (1-2). They are associated with the production of forces that pull on spindle poles and orient the spindle. Under an unknown signal possibly coming from spindle poles they disappear locally and accumulate in the midzone which will become the cleavage furrow. The propagation of cortical contraction waves initiated at the poles could account for such a displacement (4).

in cells (Charras *et al.*, 2005). Blebs follow a local increase of pressure due to local actin contraction but this pressure increase is not transmitted in the entire cell. The porosity, elasticity and contractility of the cytoskeleton that span the cell volume are responsible for this unexpected cell mechanical property. A local increase of pressure, revealed by a membrane detachment, has only local consequences. This means that if the membrane of moesin-depleted cells undergoes irregular contractions, only the portion of the spindle close to the restricted areas of pressure increases will be locally deformed. The rest of the spindle will not be submitted

to the same pressure increase. This could participate in the abnormal spindle morphologies described in moesin-depleted cells (Carreno *et al.*, 2008). However, some caution should be taken here as we do not know if bleb formation takes place in mitotic cells as it does in interphase cells. Due to the considerable increase in cytoskeleton dynamics of assembly and the complete remodeling and fragmentation of most intracellular membrane compartments, which could be proficient to chromosome movements, the mitotic cytoplasm could demonstrate more fluidity and homogeneity than the interphase cell, with pressure equilibrated within the cell.

This would be compatible with the spherical shape of mitotic cells which in control conditions do not show evidence for domains having differential curvatures with line tension such as what can be observed in biomembrane models (Baumgart *et al.*, 2003; Subramaniam *et al.*, 2005). For example, the periodic surface contractions of the very large *Xenopus* egg, in phase with the mitotic state, propagate smoothly, suggesting cortical homogeneity (Hara *et al.*, 1980). Indeed eggs do not depend on adhesion for their shape which is spherical even in interphase thanks to the jelly layer. Therefore the mechanical heterogeneity of mitotic adherent cells could be entirely linked to adhesion. In the hypothesis of a fluid and homogeneous mitotic cytoplasm, spindle deformation in moesin mutant cells would only be due to heterogeneous astral microtubules behavior in response to uneven contraction of the cell cortex.

The fact that spindles are generally shorter in moesin RNAi cells suggests that astral microtubules were under tension and that spindle poles were pulled toward the cortex in normal conditions. These tensions have been shown to drive spindle positioning in yeasts, worms, insect and mammalian cells (Palmer *et al.*, 1992; Grill *et al.*, 2001; Cytrynbaum *et al.*, 2003; Théry *et al.*, 2007). Relaxation of these tensions induces spindle shrinkage (Cytrynbaum *et al.*, 2005; Goshima *et al.*, 2005). Tension in the microtubules can induce spindle movement or spindle elongation only if the cortex is sufficiently rigid to resist the tension at the other end of the microtubule. So tension forces on the spindle can only be exerted from rigid cortical areas. Blebs form where the cortex is less rigid and where the actin network breaks. Therefore blebs might certainly not be rigid enough for the tension to be developed on astral microtubules going into the bleb. So cell deformation by bleb expansion cannot be followed by spindle repositioning toward the bleb. In contrast, microtubules might push strongly (Brangwynne *et al.*, 2006) and deform the membrane which is too soft to resist. Thereby microtubules amplify membrane detachment and bleb growth while moving the spindle away from the bleb. When comparing spindle position and cell shape, the spindles look abnormally off centered because tension and pressure are irregularly distributed in astral microtubule network. The abnormal spindle positions might therefore be due to the chaotic spatial distribution of rigid areas in the cell cortex [Fig. 2(a)].

Finally, Kunda *et al.* observe moesin disappearance from cortical regions close to spindle pole and relocalization in the midzone in early anaphase to eventually accumulate in the cytokinetic furrow. Similarly, myosin II have been shown to accumulate at the top of retraction fibers (Cramer and Mitchison, 1995) and then to concentrate in the cytokinetic furrow. How all these proteins change their localization from retraction fibers to the future cleavage plane has not been specifically investigated. It is tempting to speculate that the mechanistic basis of the chaotic contractions of mitotic cell

shape in moesin-depleted cells is the same that governs cortical modification during anaphase of wild-type cells in a very controlled way. Paluch *et al.* proposed that cortical contraction waves could bring the future constituents of the future furrow in the midzone (Paluch *et al.*, 2006). The local inactivation of moesin at the poles could indeed trigger the weakening of the membrane-actin link and thereby define the location where membrane detachment would take place upon gel contraction. How this would result in two contractile waves propagating from the two opposite poles, merging in the middle zone and inducing the ingression of the future cytokinetic furrow (Paluch *et al.*, 2006) [Fig. 2(c)] would have to be worked out.

CONCLUSION

The two studies by Kunda *et al.* and Carreno *et al.* provide additional evidence of the critical role played by the cell cortex during mitosis not only for morphological changes but also for spindle positioning. They bring new key elements to the understanding of the mechanical integrity of the cell cortex during mitosis and insights into the molecular and mechanical interactions between the lipid bilayer and the actin network. They revealed the critical implication of ERM protein in cortical actin network stiffening and the maintenance of cell surface mechanical integrity during membrane folding. It would be interesting to understand how the amplitude of cell shape compaction during cell rounding is associated with the regulation of spindle size in order to bring them together. Their observations of the temporal regulation of ERM localization during mitosis should also help further understanding the dramatic cell shape transformations that occur all along mitosis. These studies should stimulate further investigations of the role of ERM proteins in other systems and particularly in mammalian cells where only few works have focused on it during mitosis. In this regard, the use of adhesive micropatterns to control the localization of ERM proteins during interphase and mitosis might be an interesting experimental tool. The possible heterogeneous mechanical properties of the cell cortex during mitosis and their effect on spindle positioning will be challenging investigations that could bring new key elements to the unraveling of cell division mechanism.

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