Girds ‘n’ cleeks o’ cytokinesis: microtubule sticks and contractile hoops in cell division

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Abstract
Microtubules maintain an intimate relationship with the rings of anillin, septins and actomyosin filaments throughout cytokinesis. In Drosophila, peripheral microtubules emanating from the spindle poles contact the equatorial cell cortex to deliver the signal that initiates formation of the cytokinetic furrow. Mutations that affect microtubule stability lead to ectopic furrowing because peripheral microtubules contact inappropriate cortical sites. The PAV-KLP (Pavarotti-kinesin-like protein)/RacGAP50C (where GAP is GTPase-activating protein) centralspindlin complex moves towards the plus ends of microtubules to reach the cell equator. When RacGAP50C is tethered to the cell membrane, furrowing initiates at multiple non-equatorial sites, indicating that mis-localization of this single molecule is sufficient to promote furrowing. Furrow formation and ingression requires RhoA activation by the RhoGEF (guanine-nucleotide-exchange factor) Pebble, which interacts with RacGAP50C. RacGAP50C also binds anillin, which associates with actin, myosin and septins. Thus RacGAP50C plays a pivotal role during furrow formation by activating RhoA and linking the peripheral microtubules with the nascent rings through its interaction with anillin.

Spindle microtubules and cleavage rings
The title for this short paper reflects the Edinburgh venue of the meeting that catalysed its writing and the realization that the Scots tongue, a variant of Old English, has acquired status as a distinct language on the website of the Scottish Assembly. The Scots ‘girds ‘n’ cleeks’ corresponds to the English ‘hoops and sticks’, universal children’s toys that provide an analogy for the multiple hoops of anillin, actomyosin and septins and the microtubules that organize them for the process of cell division. We have previously reviewed how these interactions orchestrate the process of cytokinesis and refer the reader to that article for a more in-depth discussion [1]. The importance of spindle microtubules in directing the position of cytokinesis was clear from the early experiments of Rappaport [2]. Since that time, two apparently opposing models have emerged to account for their role: one proposing that astral microtubules inhibit furrow formation close to the spindle poles and the other suggesting that a sub-population of microtubules actually deliver a signal to the equatorial cortex. Most probably, elements of both models are relevant and these mechanisms are used to varying extents in different organisms or cell types. In this short article we focus on a series of experiments from our own laboratory that support the predominance of the second model in Drosophila cells, and we apologize in advance both for not giving a wider viewpoint and to scientists whose work we have not been able to cite because of space restraints.

Unashamedly, we will also focus upon one particular cell type in Drosophila, the primary spermatocyte. This is our favourite cell because of the advantages it offers for studying cytokinesis.

Cytokinesis in Drosophila spermatocytes
One difficulty in using genetic approaches to study cell division is the fact that many proteins that have a particular role in prometaphase are reutilized for different ends during cytokinesis. Thus mutants for such proteins tend to show metaphase arrest in mitosis due to the spindle assembly checkpoint. In male meiosis, however, the absence of a strong spindle assembly checkpoint enables such mutant cells to proceed through anaphase and telophase and to attempt cytokinesis. Mutations in asp, for example, cause somatic cells to arrest at metaphase with highly abnormal spindle poles, whereas asp mutant spermatocytes proceed beyond this stage and show pronounced defects in the organization of the central spindle microtubules that are necessary for cytokinesis [3,4]. Added to this peculiarity of cell cycle control is the fact that the primary spermatocyte is one of the largest cell types in Drosophila and is amenable to short-term culture and direct observation of meiosis by time-lapse microscopy.

Such observations on living spermatocytes expressing tubulin tagged with GFP (green fluorescent protein) show quite clearly that the initiation of furrow formation takes place when a set of peripheral microtubules extend from the centrosomes to ‘touch’ the cell cortex at the cell equator (Figure 1A) [5]. The equatorial rings that form at this time, first of anillin and subsequently of actin and septins, retain their contact with and progressively constrict the central spindle microtubules and will finally divide the cell.

Key words: anillin, central-spindle microtubule, centralspindlin, cytokinesis, Rac GTPase-activating protein (RacGAP), Rho guanine-nucleotide-exchange factor (RhoGEF).
Abbreviations used: GAP, GTPase-activating protein; GEF, guanine-nucleotide-exchange factor; GFP, green fluorescent protein; KLP, kinesin-like protein; PAV-KLP, Pavarotti-KLP; RNAi, RNA interference.
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Mechanics and Control of Cytokinesis

**Figure 1 | Furrowing is initiated in Drosophila spermatocytes when peripheral microtubules emanating from the spindle poles touch the cell cortex**

(A) Selected frames from a time-lapse sequence of a single cell shown at low (∼1 min) and higher (subsequent panels) magnification of the boxed region. After anaphase onset, peripheral microtubules (arrowheads) approach the cortex, which they probe (2 min) until cortical contact is made (4 min), at which time they become bundled (7 min). Shortly thereafter, the cleavage furrow initiates and ingresses, thereby coalescing peripheral and interior microtubules (12 and 17 min). The fluorescence signal for tubulin-GFP is inverted. Time is relative to anaphase onset. Scale bars, 10 μm. (B) Loss of orbit preferentially prevents formation of interior but not peripheral microtubules. Peripheral microtubules still form and probe the cytoplasm (8 min). A more robust set of peripheral microtubules form (arrowheads and arrows) and contact the cortex, immediately before furrow formation on the right and left sides of the cell (23 min). Furrow ingression arrests on the right but continues on the left side. This furrow advances before also then ultimately regressing (35 min). Times are relative to anaphase onset. Scale bar, 10 μm. (C) Abnormally long astral microtubules in klp67A mutants collect in the upper portion of the cell, where they initiate an abnormally positioned unilateral cleavage furrow that advances but fails to fully cleave the cell (12–23 min; arrows). Time is relative to anaphase onset. Scale bar, 10 μm.

Furrow initiation requires microtubules to contact the cell cortex

We have studied how the site of the initiation of furrowing depends upon the contact made by microtubules with the cortex in mutants in which microtubule stability is altered. One such mutant is in the gene orbit/mast that encodes the counterpart of the mammalian protein, CLASP. At metaphase, microtubule subunits flux along the kinetochore fibres; tubulin subunits are incorporated at the kinetochore while being simultaneously lost from microtubule minus ends at the centrosome. The Orbit protein is required for tubulin subunit addition, a function that is balanced by the microtubule-depolymerizing properties of the kinesin-type depolymerase KLP (kinesin-like protein) 10A [7]. Thus down-regulation of both Orbit and KLP10A rescues the monopolar spindle phenotype shown by down-regulation of either protein alone. During cytokinesis, Orbit relocates specifically to an interior population of central spindle microtubules. Consistently, this subset of microtubules are unstable in orbit mutants. However, the peripheral astral microtubules still grow out from the centrosomes to touch the cortex and initiate furrow formation, but in the absence of the interior central spindle, the furrow is unstable and cytokinesis fails (Figure 1B) [5]. Thus the peripheral microtubules are required to initiate the furrow and the interior central spindle microtubules for its maintenance. Another microtubule-associated protein whose function changes in late M-phase is the microtubule-destabilizing KLP67A protein. In prometaphase and anaphase, KLP67A is required to facilitate chromosome biorientation and anaphase movement, whereas in cytokinesis it acquires a microtubule stabilizing function [7,8]. In KLP67A mutant spermatocytes, anaphase B spindles elongate with normal kinetics but then bend towards the cortex. Both peripheral and interior spindle microtubules then form diminished bundles of abnormally positioned central spindle microtubules. Importantly, furrows initiate at the sites of central spindle bundles but can be unilateral or non-equatorially positioned (Figure 1C). Ectopic furrows can also be stimulated by the interior central spindle but in general are observed only to form after this structure buckles to contact the cortex. The central spindles become increasing unstable over time, actin and anillin fail to form homogenous bands and cytokinesis fails. Thus the
signal to furrow can be delivered wherever the peripheral microtubules contact the cortex but it can also be provided by the interior central spindle microtubules under these aberrant circumstances. Collectively this body of results from wild-type and mutant cells indicate that microtubules do deliver the signal that initiates furrowing in *Drosophila*.

**RacGAP50C provides the central platform of the furrow-initiation signalling machinery**

What then is this signal and can we see its association with microtubules? A likely candidate is the centralspindlin complex. At the core of this conserved complex are a motor component, known as PAV-KLP (Pavarotti-KLP) in *Drosophila*, ZEN-4 in *Caenorhabditis elegans* and MKLP-1 in mammals, and a GAP (GTPase activating protein), RacGAP50C or Tum in flies, CYK-4 in nematodes, and MgcRacGAP in mammals. The motor protein displays plus-end directed activity [9–12] and so is potentially able to deliver a signal to the cell equator. Furthermore, our own studies of GFP-tagged PAV-KLP in embryonic cells showed it to become concentrated at the equator of the cell prior to cytokinesis [13]. The GAP component had been reported to down-regulate the *in vitro* activity of Rac and Cdc42 GTPases more efficiently than Rho [14–16] and so did not seem like the ideal candidate for the signalling molecule. RhoA seemed more likely because its inactivation prevents cytokinesis in most systems [17] and its active form accumulates at the future cleavage site in a microtubule-dependent manner [18]. Studies in *Drosophila* and mammals indicated that RhoA activation at the cleavage furrow requires the RhoGEF (guanine-nucleotide-exchange factor) Pebble-ECT2 [19–21]. The finding by Saint and Somers [22] that *Drosophila* Pebble could complex to RacGAP50C led them to propose that the onset of cytokinesis could be triggered by the microtubule-mediated delivery of the centralspindlin complex to the cortex where its RacGAP component would activate the RhoGEF Pebble and, in turn, RhoA. The finding that MgcRacGAP interacts with ECT2 in mammals suggests that this proposed mechanism has been conserved during evolution [23–26].

Could the centralspindlin complex then indeed be a platform that permits Pebble/Ect2 to activate RhoA and initiate cytokinesis? To begin this question we were led to ask what happens to microtubules and the initiation of furrowing following the inactivation of either the PAV-KLP or the RacGAP50C components of centralspindlin. We found that down-regulation of either component by RNAi (RNA interference) in cultured cells prevented the onset of furrowing in spite of the continued growth of astral microtubules to touch the cell cortex at its equator [27]. Thus the centralspindlin complex seemed to be essential for the astral microtubules to transmit their signal. This interpretation was reinforced by our direct time-lapse observations of the dynamics of the centralspindlin complex in both mitotically dividing cells in culture and in primary spermatocytes. In both cell types, we were able to observe PAV-KLP tagged with GFP translocate to the plus ends of the astral microtubules as they touched the cell cortex to determine the cleavage plane (Figure 2). If PAV-KLP was just providing the motor function to take RacGAP50C to the cortex, we argued that a membrane-tethered version of RacGAP50C ought to provoke ectopic furrowing. To test this, we fused an integral plasma membrane component, the human T-cell receptor CD8, with the full coding region of RacGAP50C and a GFP tag was inserted between the two genes to follow its distribution (Figure 3A). When we induced the expression of this construct in cultured *Drosophila* cells, we observed the formation of multiple ectopic furrows all over the cell’s cortex (Figure 3B) [27]. These furrows were able to form in the absence of PAV-KLP and they recruited both anillin and septins. We strongly suspect that RacGAP50C induces these ectopic furrows because it serves as a means of recruiting and/or activating the RhoGEF Pebble. Consistently no equatorial or ectopic furrowing activity was observed in the CD8–GFP–RacGAP50C cells after RNAi depletion of Pebble.

We cannot rule out in the above experiments that the membrane-tethered version of RacGAP50C can also recruit other factors that co-operate with it to induce ectopic furrows. Indeed recent results from both our laboratory [28], and Robert Saint and co-workers [29], indicate that anillin, one of the first molecules to be recruited to the furrow in the form of a ring, also physically interacts directly with RacGAP50C. As anillin also interacts with actin, myosin and septins, this provides the basis for a physical
Figure 3 | Mislocalization of RacGAP50C causes ectopic furrowing
(A) Schematic diagram of the inducible CD8–GFP and CD8–GFP–RacGAP50C constructs that were stably expressed in Drosophila cell lines. Mt, metallothionein. (B) Following induction of the two constructs from the metallothionein promoter, cells were examined by immunostaining. The arrows indicate the ectopic furrows, whereas the arrowhead indicates the equatorial furrow. Scale bar, 10 μm.

Future challenges
The mechanics of the gird ‘n’ cleek are quite different from those of cytokinesis. Whereas the gird requires a simple stroke from the cleek to set it in motion, the contractile apparatus of the cell is mechanically far more complex. We are on the verge of understanding the events that initiate formation of the cleavage furrow and its associated contractile rings. Akin to the gird ‘n’ cleek, this requires that contact is first established between peripheral microtubules and the nascent contractile ring. There then begins a process likely to involve multiple cycles of incremental ring constriction in which the central spindle microtubules play an essential role. What are the roles of the active versus inactive forms of various GTPases might be in co-ordinating these events remains to be clarified. We also have much to learn about the mechanisms that maintain membrane association with the contractile ring and the relative roles of phosphatidylinositol lipids in regulating addition of new membrane and behaviour of the filamentous actin. Microtubules continue to be central to the whole process, not least by providing the transport network that cycles vesicles to and from the constricting furrow. We are beginning to have some insight into these processes through studies in a variety of model systems. The future challenge is to integrate this knowledge into an understanding of what is one the most fundamental processes in cell biology.

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References

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