The degradation of two mitotic cyclins contributes to the timing of cytokinesis

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SUMMARY

BACKGROUND: Cytokinesis occurs just as chromosomes complete segregation and reform nuclei. It has been proposed that cyclin/Cdk kinase inhibits cytokinesis until exit from mitosis, however the timer of cytokinesis has not been experimentally defined. While expression of a stable version of Drosophila cyclin B blocks cytokinesis along with numerous events of mitotic exit, stable cyclin B3 allows cytokinesis even though it blocks late events of mitotic exit [1]. We examined the interface between mitotic cyclin destruction and the timing of cytokinesis.

RESULTS: In embryonic mitosis 14, the cytokinesis furrow appeared 60 sec after the metaphase/anaphase transition and closed 90 sec later during telophase. In cyclin B or cyclin B3 mutant cells, cytokinesis furrow appeared at an earlier stage of mitosis. Expression of stable cyclin B3 delayed and prolonged furrow invagination; nonetheless cytokinesis completed during the extended mitosis. Reduction of Pebble function, a Rho GEF required for cytokinesis, also delayed and slowed furrow invagination, but incomplete furrows were aborted at the time of mitotic exit. In functional and genetic tests, cyclin B and cyclin B3 inhibited Pebble contributions to cytokinesis.

CONCLUSIONS: Temporal coordination of mitotic events involves inhibition of cytokinesis by cyclin B and cyclin B3 and punctual relief of the inhibition by destruction of these cyclins. Both cyclins inhibit Pebble-dependent activation of cytokinesis, while cyclin B can inhibit cytokinesis by additional modes. Stable cyclin B3 also blocks the later return to interphase which otherwise appears to impose a deadline for completion of cytokinesis.

INTRODUCTION

Cells don’t simply exit mitosis: the chromosomes are faithfully segregated, the mitotic spindle is transformed and ultimately disassembled, nuclei and other organelles are rebuilt, and the cell pinches into two in the process of cytokinesis. Little is known about the regulation of these events. In particular, cytokinesis must be properly coordinated with spindle function for accurate chromosome partitioning into daughter cells.

Activation of protein degradation by the Anaphase Promoting Complex (APC) is essential for mitotic exit, and one important pathway of its action has been identified [2-5]. APC directs metaphase destruction of securin, a protein that inhibits separase. Separase, a specific endoprotease, then cleaves cohesin, a protein that holds sister chromatids together. The discovery of this pathway for release of sister chromatids superseded an earlier supposition that destruction of cyclins would reverse cyclin/Cdk activation to initiate exit from mitosis. Nonetheless, destruction of mitotic cyclins does contribute to mitotic exit.

Stable versions of cyclin B in metazoans or Clb2 in S. cerevisiae failed to block transition to anaphase, but blocked final mitotic exit [1, 2, 6-17]. Thus, cyclin destruction controls steps such as spindle disassembly and cytokinesis, and recent results suggest contributions beyond those seen in early work, which tested only one member of the family of mitotic cyclins. Three evolutionarily conserved classes of cyclin – a cyclin A, cyclin B and cyclin B3 – contribute to mitosis in Drosophila, while S. cerevisiae has six mitotic cyclins (CLB1-6). In systems from yeast to frog, different cyclin types have overlapping but non-identical functions [8, 12, 18-21]. The mitotic cyclins of Drosophila are degraded in succession: cyclin A prior to metaphase/anaphase; cyclin B at

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the transition to anaphase and cyclin B3 early during anaphase [12, 20, 22-24]. Additionally, stabilization of each of these cyclins blocks diminishing subsets of mitotic exit events as if the destruction schedule orders and times these events [1, 12]. In this regard, stabilization of cyclin B blocked cytokinesis, while stabilization of cyclin B3 did not [1].

The onset of cytokinesis is marked by assembly of a cortical actin ring, a step thought to be regulated the small GTP-binding protein, RhoA [25-27]. Drosophila pebble (pbl), which encodes a RhoA specific exchange factor, is required for cytokinesis [25]. pbl mutant embryos show no obvious defect during the first 13 mitotic cycles, which occur in a syncytial cytoplasm without cytokinesis, but show failures of cytokinesis 14 [28, 29]. Immunostaining reveals intriguing changes in Pebble abundance and localization: Pebble declines in early mitosis, and then intensifies cortically, focusing to the equatorial cortex as cytokinesis furrowing occurs [25]. Pebble staining persists along the furrow as it deepens and then migrates into the newly-formed telophase nuclei. The requirement for Pebble in cytokinesis and cortical presence of the protein exclusively when it might be needed, suggests that regulation of its abundance and location might define a window of opportunity for cytokinesis.

We describe the roles that cyclin B and cyclin B3 play in timing cytokinesis and in inhibiting Pebble-dependent contributions to cytokinesis.

RESULTS

Cytokinesis initiates during anaphase B and is completed in 90 seconds

As a foundation for our analysis, we examined the normal progress to cytokinesis in live and fixed Drosophila embryos during cell cycle 14 (Figure 1A-A”). Metaphase cells exhibited fan shaped arrays of microtubules organized into a broad spindle, short astral microtubules, and a cortex that stained brightly with phalloidin, but they showed no sign of cytokinesis (cell #1).

During anaphase, microtubule bundles became parallel as the spindle narrowed, astral microtubules reached the equatorial cell cortex, and the cytokinesis furrow appeared at this position (cells #2-3) just as the elongating spindle reached 11 μm (Figure 1 B). The furrow deepened during anaphase and telophase, and cytokinesis was completed before the midbody depolymerized (cells #4-5). Finally, cortical actin staining declined to interphase levels.

Real-time detection of a plasma membrane protein fused to GFP (supplementary data: movie 1) or a HistoneH2-GFP fusion (data not shown) revealed membrane and chromosome dynamics, respectively. The cytokinesis furrow appeared about 60 seconds after the metaphase/anaphase transition and 30 seconds after the beginning of cell elongation (anaphase B). Moreover, cytokinesis was completed about 90 seconds after its initiation (Figure 1C). Since the furrow was not initiated until after cyclin degradation [12, 20, 22-24], we asked if cyclin B and/or cyclin B3 activities prevent earlier onset of cytokinesis initiation.

Cytokinesis furrows appear at an earlier stage of mitosis in cyclin B and cyclin B3 mutant cells

Mitotic cyclins B and B3 are partially redundant and each is individually dispensable for many mitoses in Drosophila [19, 20]. Nonetheless, it was suggested that coordination of mitotic exit events was disturbed in cells deficient in cyclin B [19]. We tested whether loss of cyclin B or cyclin B3 affected cytokinesis timing during mitosis of cell cycle 16, a stage of embryogenesis with a negligible residue of maternal cyclin products [19, 20]. Wild-type cells that have not yet separated their daughter complements of DNA beyond a threshold degree can be reliably predicted to lack a cytokinesis furrow (Figure 2A and 2D). In contrast, cells below this threshold show well established cytokinesis furrows in cyclin B and cyclin B3 mutant embryos (Figure 2B, C, E, F and supplementary data: Figure 1). Furthermore, as expected for precocious cytokinesis in the cyclin B and cyclin B3 mutants, the proportion of anaphase cells that had not yet begun cytokinesis was reduced (Figure 2D-F). It can also be noted that an
increased proportion of anaphase cells are in the process of cytokinesis in the mutants (Figure 2E, F) suggesting that furrow ingression is slowed, perhaps as a secondary consequence of early initiation and interference by the as yet immature spindle. In any case, cyclin B and cyclin B3 are required to delay cytokinesis until chromosomes separation is well on its way. Using real-time analysis of histone-GFP in wild-type embryos during cycle 16, we have developed a conversion table relating chromosome separation to time. The degree of chromosome separation when cytokinesis furrows are first detected in cyclin mutants versus controls (Figure 2D-F) corresponds to an advance of roughly 25 and 15 sec in cyclin B and cyclin B3 mutants, respectively. If it is mitotic destruction that ordinarily relieves cyclin inhibition of cytokinesis in wild-type, stabilization of the cyclins ought to extend the period of inhibition and thus further delay cytokinesis.

**Destruction of cyclin B and cyclin B3 promotes cytokinesis furrow initiation and ingression**

Studies in numerous organisms have shown that stabilization of cyclin B blocks mitotic exit events, including cytokinesis [1, 6, 7, 9, 10, 12-16]. In *Drosophila* the arrest occurs with robust cortical phallloidin staining indicating F-actin accumulation (Figure 3A, A’), and incomplete chromosome separation [1, 12]. In marked contrast, stabilization of *Drosophila* cyclin B3 blocked exit from mitosis without blocking cytokinesis [1], as if destruction of cyclin B, but not cyclin B3, governed onset of cytokinesis. However, intermediates in cytokinesis were frequently seen in embryos expressing cyclin B3 (Figure 3B, B’ versus Figure 1A, A’), suggesting that cytokinesis progressed slowly. This was confirmed in live records following induction of stable cyclin B3 in embryos marked with histone H2-GFP (supplementary data: movie 2) or a membrane-GFP marker (supplementary data: movie 3). The time between metaphase/anaphase and visible furrowing increased from one min in control to three min (movie 2) and furrow ingression was slower (360 versus 90 sec). The delay was observed consistently, although its duration ranged from 30 (see movie 3) to 300 sec (not shown). Similarly, the rate of furrow ingression varied but often took more than five min (e.g. movies 2 and 3).

Though retarded by stable cyclin B3, cytokinesis continues apparently to completion. In fixed preparations, some cells with completed furrows appeared to be escaping the arrest as they have reduced phospho-histone staining and a midbody-like structure (Figure 3B-B’, cell #7), but other cells retained robust phospho-histone staining, condensed chromosomes, obvious spindles, and lacked a nuclear envelope (Figure 3B-B’,E-E’,F-F’, and data not shown). Thus, cyclin B3/Cdk1 levels sufficient to block many features of the exit from mitosis slowed but did not block cytokinesis. Consequently, whereas the previous work suggested an absolute distinction between the ability of cyclin B and cyclin B3 to inhibit cytokinesis, we now conclude that they both have this ability, but that only cyclin B gives a complete block.

To explore the regulatory interface between cyclin destruction and initiation of cytokinesis, we examined the influence of cyclin B and cyclin B3 on Pebble, an upstream activator of cytokinesis.

**Reduction of Pebble function delays and slows cytokinesis**

Since Pebble activation of RhoA is likely to be a key upstream step in reorganizing cortical actin to initiate cytokinesis, we expected *pbl* mutants to lack cytokinesis furrows. Surprisingly, in cells of *pbl* mutant embryos, we frequently detected furrow initiation and ingression during mitosis 14 (data not shown), and, to a lesser extent, in mitosis 15 (Figure 4A-C’). When cells in the final steps of mitosis were ordered according to the degree of spindle disassembly, chromosome decondensation and reformation of the nuclear envelope, it appeared that ingressed but incomplete furrows were in the process of regression (Figure 4B-C’). Furthermore, abortion of incomplete furrows appears to be the source of the binucleate cells that typify interphase 15 *pbl* mutant embryos. These findings indicate that either Pebble is not absolutely essential for furrow initiation or the mutation does not fully remove Pebble function.
Staining for Pebble in \( pbl^2 \) mutant embryos reveals substantial staining in cycle 15 nuclei (supplementary data: Figure 2). Since the \( pbl^2 \) allele drastically truncates the protein and should eliminate Pebble epitopes [25], we infer that there is persistence of maternally-encoded Pebble into cycle 15. Consequently, the phenotype at cytokinesis 14 appears to be due to reduction in function, rather than absence of function.

We used RNA interference (RNAi) to further probe the requirement for Pebble. Analysis of fixed embryos showed that \( pbl \) RNAi mimicked the \( pbl \) mutant phenotype (producing binucleate cells) while only reducing, not eliminating, Pebble staining (supplementary data: Figure 2). In real-time images of uninjected and control injected cycle-14 embryos, cytokinesis furrows appeared 30 sec after cells began to elongate (taken as initiation of anaphase B), while cytokinesis furrows appeared more than 100 sec after onset of cell elongation in embryos injected with \( pbl \) dsRNAs (supplementary data: movies 1, 4 and 5). Moreover, cytokinesis furrows aborted either soon after cytokinesis initiation (supplementary data: movie 4), or when furrows had deeply ingressed (Figure 4D-I and supplementary data: movie 5). Thus, \( pbl \) RNAi delays appearance of the cytokinesis furrow, and slows furrow ingress. As cells exit mitosis, nuclear envelopes reform, and the still incomplete furrows abort to produce binucleate cells. The abortion of furrows delayed by reduction of Pebble function contrasts with the successful completion of the dramatically delayed furrows seen in cyclin B3 expressing cells.

**Synergetic inhibition of cytokinesis by stable cyclin B3 and \( pbl \) mutation**

Hypothesizing that extending mitosis might allow completion of the slow cytokinesis events in \( pbl^2 \) mutants, we expressed stable cyclin B3 in the mutant embryos. Rather than permitting completion, the combination gave an arrest where cytokinesis furrows were absent (or exceptional) (Figure 4J-J'). Moreover, late spindles were broad and short (Figure 4J-J' and data not shown). We conclude that stable cyclin B3 and the \( pbl^2 \) mutant act in synergy to inhibit cytokinesis initiation and mitotic progression.

These results suggested that cyclin B3 (and possibly cyclin B) inhibits a residual activity of the Pebble pathway or suppresses a parallel pathway that acts in conjunction with Pebble. To further test interactions with Pebble, we developed a genetic approach to assay whether cyclin B or cyclin B3 mutations modify the cytokinesis defects induced by expression of a dominant-negative Pebble construct.

**Pbl\(^{DH} \) expression in the wing leads to cytokinesis defects, a reduction in cell density and a multiple wing hair phenotype**

A deletion of pebble sequences encoding a conserved DH (Dbl Homology) domain created a dominant-negative construct (Pbl\(^{DH} \)), which induced rough eyes when expressed in the eye under the control of GAL4 [25]. For our studies, we used a similarly sensitized background, but did this in the context of wing development where we could show that the phenotype was associated with a cytokinesis defect. The wing-specific driver MS1096-Gal4 [30] in conjunction with UAS-Pbl\(^{DH} \) produced a “multiple wing hair” phenotype (Figure 5A-E). The severity of the phenotype, which was scored according to cell density, frequency of multiple-wing-hair cells and the multiplicity of hairs, increased with temperature consistent with temperature influences on GAL4 (Figure 5B-D), and with the effective dose of the X-chromosome driver, which is higher in hemizygous males than in heterozygous females (data not shown).

To test whether wing expression of UAS-Pbl\(^{DH} \) produced a defect in cell division, we stained pupal wings with Hoechst just before eclosion of the adults. In control wings, each hair was associated with a single nucleus, consistent with studies showing that each cell initiates one, and only one, hair (Figure 5F) [31]. In contrast to the orderly array of nuclei in the control, Pbl\(^{DH} \)-expressing wings showed clusters of nuclei (Figure 5G). These nuclei were heterogeneous in size, consistent with descriptions of nuclear fusion following cytokinesis failures in \( pbl \) mutants. The wing territories in which we found clusters of nuclei corresponded to the territories showing multiple wing hairs, and tufts composed of many
hairs were associated with clusters having more or bigger nuclei. We assessed the time of action of Pbl\textsubscript{DH} using temperature shift experiments (from 18˚C to 30˚C). No enhancement of the phenotype was observed when the shift was done after the last pupal wing cell divisions (data not shown). We conclude that cytokinesis failure occurs in wings expressing Pbl\textsubscript{DH} and we used the reduced cell density and the multiple wing hair phenotype as a readout of cytokinesis failures.

We next tested whether the severity of the wing phenotype is sensitive to the dose of endogenous genes known or thought to function in the Pebble pathway. Removal of one endogenous copy of either \textit{pbl}, \textit{RhoA} or \textit{zipper} (gene for myosin II heavy chain) strongly enhanced the Pbl\textsubscript{DH} phenotype (Figure 5H-J, compared to B). We used an overproduction strategy to test for modification by the \textit{Drosophila} Rho-associated kinase (Drok), a likely of RhoA action [32]. Expression of a catalytically-activated, truncated form of Drok (Drok-CAT, [32]), strongly suppressed the multiple wing hair and the cell density Pbl\textsubscript{DH} phenotype in one third of the flies (Figure 5K, compared to B). In the other flies, the Drok-CAT transgene appeared to have hyperactivated the Pebble pathway, as it mimicked overexpression of a wild-type \textit{pbl} transgene (reduced and folded wings, data not shown) – as expected for a positive factor in the Pebble pathway. Altogether, these genetic interactions are consistent with the notion that Pebble acts in a cascade involving RhoA, Drok and myosin II to promote cytokinesis and to prevent the development of multiple wing hairs (see Figure 7 and discussion).

Cyclin B and cyclin B3 inhibit the contributions of the Pebble pathway to cytokinesis

While pleiotropic regulators such as cyclin B and cyclin B3 are likely to have multiple inputs into cytokinesis, we can probe their involvement in regulating a specific pathway by partially crippling that pathway and thereby making the system uniquely sensitive to further changes in the activity of the pathway. Because our studies of cytokinesis in the embryo suggested a synergy in the inhibition of cytokinesis by reduction in \textit{pbl} function and expression of stable cyclin B3, we posited that cyclin B3 inhibits \textit{pbl} function. To test this further, we asked whether reduction of cyclin B3 function would modify the wing phenotype due to Pbl\textsubscript{DH} expression. Since the cyclin B3 mutant is zygotically viable, we were able to test \textit{cycB3} homozygotes as well as heterozygotes. The removal of one or two copies of cyclin B3, a proposed inhibitor of \textit{pbl}, ought to improve \textit{pbl} function and hence suppress the actions of the dominant-negative construct. We compared the severity of the Pbl\textsubscript{DH} phenotype in flies having two, one or zero functional copies of cyclin B3 by counting the frequency of cells with single hairs and multiple hairs. Both criteria showed a dose-dependent suppression of the Pbl\textsubscript{DH} by reduction of cyclin B3 (Figure 6A-C). Similar analysis using a cyclin B mutation showed that it also acts as a dose-dependent suppressor of Pbl\textsubscript{DH}, and that the strength of suppression is similar to that of cyclin B3 (Figure 6D-F). Mutation in cyclin A did not modify this phenotype, suggesting that suppression of Pbl\textsubscript{DH} is a specific feature of cyclin B and B3 mutations (data not shown). We conclude from these data that both cyclin B and cyclin B3 genetically inhibit the contribution of the Pebble pathway to cytokinesis.

DISCUSSION

Successful segregation of chromosomes to the spindle poles is futile unless cytokinesis acts at the right instant and position to reliably trap full genetic complements in each daughter cell. The onset of anaphase is triggered by targeted protein degradation, which is initiated upon activation of the APC, but it is not known to what extent the subsequent events run free according to their own internal schedule, progress under constraints that couple different events, or are timed by independent inputs from central regulatory circuits. While several post-anaphase events are blocked by a non-degradable form of cyclin B [1, 2, 6, 7, 9-17, 33], it was unclear whether cyclin B destruction, which occurs at the transition to anaphase, has a timing input into the later event of cytokinesis or whether it is simply a necessary precondition. Here, we probe the regulatory coupling between
cyclin destruction and cytokinesis in *Drosophila*. We detail the timing of the processes, demonstrate that destruction of cyclin B and cyclin B3 influences the timing of cytokinesis, and provide evidence that one pathway of cyclin action involves inhibition of Pebble contributions to the initiation of cytokinesis.

**The timing and context of cytokinesis**

Signals from the spindle contribute to positioning of the cytokinesis plane [27, 34]. In order to guide the process, these signals must precede cytokinesis and ought to interface with signals timing the initiation of cytokinesis. Whether the spindle signals emanate from the astral microtubules, the central spindle, or both, is still a matter of debate [34, 35]. *Drosophila* embryonic spindles develop peripheral bundles of microtubules that extend during early anaphase to reach the cortex at the future furrow position (Figure 1A′, cell #2). The proximity to the site of furrowing might make these fibers particularly efficient in delivering a spindle signal to initiate cytokinesis [35]. Other spindle changes, such as the maturation of the midbody, occurs in concert with the onset and progression of cytokinesis.

Observations showing that cyclin stabilization can block cytokinesis drew our attention to a possible role of cyclin destruction in timing cytokinesis. Importantly, cyclin destruction ordinarily precedes cytokinesis. The onset of cyclin B destruction precedes the onset of furrowing by about 60 sec and immunohistochemical staining of cyclin B disappears prior to cytokinesis initiation [11, 12, 22, 24]. Cyclin B3 destruction occurs slightly after cyclin B, but it is also over prior to the onset of cytokinesis [20].

**Cyclin destruction provides a timing signal for cytokinesis**

We used three complementary approaches to test the influence of cyclin B and cyclin B3 destruction on the timing of cytokinesis. In the most direct of these, we demonstrated that genetic elimination of cyclin B or cyclin B3 led to cytokinesis furrow initiation at an earlier stage of mitosis (Figure 2). In a second genetic approach, we partially crippled a pathway activating cytokinesis, and showed that removing doses of cyclin B or cyclin B3 alleviated the phenotype as if each encoded cytokinesis inhibitors of similar potency (Figure 6). In the third approach, we examined the consequence of persistence of function. As previously reported, stabilized cyclin B but not stabilized cyclin B3 blocked cytokinesis [1]. However, we document that stable cyclin B3 retards the onset and slows the progression of cytokinesis, agreeing with the mutant analysis in showing that both cyclin B and cyclin B3 suppress precocious cytokinesis (Figure 3).

Inhibition of cytokinesis by cyclin B has been observed in numerous systems. In contrast, there are few studies of cyclin B3 other than those in *Drosophila*, and the continued, albeit slowed, cytokinesis during a mitotic arrest has not been reported in other systems. Even though the detailed isotype specificities of the *Drosophila* cyclin B3 might differ in other phyla, the conservation of the A, B and B3 classes of cyclin and their relative timing of degradation [33] leads us to suspect that our findings will, at least in part, be generalizable.

Together, our results show that *Drosophila* cyclin B and cyclin B3 can inhibit cytokinesis, that their normal function defers cytokinesis, and that their prompt destruction is required for timely advance to cytokinesis. However, more detailed considerations are required to address whether this regulatory input from the cyclins reflects a direct timing control of cytokinesis, or whether the actions of cyclins on cytokinesis might be mediated indirectly via inhibition of a distinct mitotic event that is a necessary prerequisite for cytokinesis.

**The cyclin/cytokinesis interface**

Stabilization of cyclin B inhibits events that precede the onset of cytokinesis. For example, stable cyclin B inhibits the transition to anaphase B [1] and blocks the dissociation of passenger proteins from centromeres ([14, 36], Parry et al. in prep.), events thought to be required for cytokinesis. Consequently, indirect routes of inhibition of cytokinesis are likely to contribute to the very effective block induced by stable cyclin B. We propose that stable cyclin B has both direct and indirect actions that inhibit cytokinesis (Figure 7).
Mitosis progresses normally in the presence of stable cyclin B3 up to the time at which cytokinesis would usually initiate in mid anaphase B. Stable cyclin B3 alters or slows some steps of spindle maturation that are coincident with cytokinesis. The peripheral microtubules that contact the cortex at the midzone do not develop fully and the midbody does not develop the highly-compacted structure that characterizes the late spindle in Drosophila. While these effects of stable cyclin B3 might influence progression of already initiated cytokinesis, one of the earliest and perhaps most direct effect of cyclin B3 stabilization is to delay onset of cytokinesis (Figure 7).

Inhibition of the Pebble pathway is one way by which cyclins B and B3 inhibit cytokinesis

We were attracted to the pbl gene product as a candidate target for the regulation of cytokinesis because it is specifically required for cytokinesis [28, 29], and its action as a RhoA GEF implied early action in the pathways promoting actin assembly into the ring that preasges the furrow [27, 37]. Thus, onset of cytokinesis might be triggered by activating Pebble. We showed, using two different approaches, that mitotic cyclins influence Pebble action.

A cytokinesis furrow is still evident in embryos homozygous for pbl, but it is delayed, slowed and eventually aborted (Figure 4, supplementary data: movies 4 and 5). Persistence of maternal Pebble suggested that residual furrowing was due to persisting Pebble function. Furrowing was also delayed and slowed upon expression of stable cyclin B3 (Figure 3 and supplementary data: movies 2 and 3). Importantly, furrows were suppressed when stable cyclin B3 was expressed in pbl (Figure 4). This indicates that both Pebble and cyclin impinge on early steps in cytokinesis.

We developed a genetic test to assess the activity of Pebble promotion of cytokinesis. Expression of a dominant-negative version of Pebble (Pbl(DH)) in wing discs partially suppressed Pebble function and led to cytokinesis failures, a reduction in cell density, and a multiple wing hair phenotype (Figure 5). In this background, inactivation of just one of two doses pbl or other genes in the Pebble pathway modified the phenotype indicating that small changes in the residual activity of the Pebble pathway were detected. Our findings support a pathway of sequential activation of RhoA, Rho-associated kinase (Drok) and myosin II leading to cytokinesis (Figure 7) [27, 37]. Like mutations in Pebble pathway functions, cyclin B and cyclin B3 mutations dominantly modified the Pbl(DH) induced phenotypes (Figure 6) – suppressing, as expected for reduction of inhibitors. A similar study showed that a deficiency encompassing the cyclin B gene suppressed an eye phenotype induced by Pbl(DH) (R. Saint, personal communication). These findings show that cyclin B and cyclin B3 inhibit a step or pathway with regulatory connections to Pebble action on cytokinesis.

The genetic interaction experiments, while implying a regulatory connection between the cyclins and Pebble function, do not define how direct this connection might be. It had previously been suggested that cyclin/Cdk1 directly inhibit a downstream component, myosin II [38, 39]. However, tests of this idea have been negative [16, 40-42]. This led us to consider inhibition of the pathway at more upstream levels, perhaps at the level of Pebble itself.

Consistent with a limiting and regulatory role, Pebble appears to be absent or at low levels early during mitosis, and to accumulate cortically and then concentrate in the region of the furrow at the time of cytokinesis [37]. Furthermore, the level of Pebble early in mitosis is likely to be inadequate to promote cytokinesis given that RNAi treatment of embryos or mutation of pbl provoked cytokinesis defects while leaving detectable amounts of protein (supplementary data: Figure 2, movies 4 and 5). From this we suggest that the apparent rise in Pebble levels as anaphase progresses is significant and contributes to initiation of cytokinesis. We found that expression of stable cyclin B or stable cyclin B3 suppressed the rise in Pebble immunostaining (data not shown). We propose that cyclin/Cdk regulation of Pebble accumulation controls onset of cytokinesis, but note that the pathway includes numerous additional opportunities for regulation that might be used to create a robust program.
Limiting cytokinesis to mitosis

In addition to supporting the model that cyclin B3 degradation times the onset of cytokinesis, our results with stable cyclin B3 suggest that termination of the window of opportunity for cytokinesis is also influenced by cyclin B3. We found that both reduction of Pebble function and expression of stable cyclin B3 delayed and slowed cytokinesis, but the outcome differed. When Pebble function is reduced, furrow ingression terminates and the furrow recedes as the cell exits mitosis with two nuclei. In contrast, cytokinesis is completed in the presence of stable cyclin B3 after a long slow course. Apparently, stable cyclin B3, while inhibiting the progression of cytokinesis, also promotes successful cytokinesis, by maintaining the cell in a state conducive to cytokinesis. Perhaps this opposing positive effect of stable B3 is due to its ability to prolong the mitotic state and block nuclear membrane formation which is usually followed by nuclear recruitment of Pebble away for its site of action at the furrow.

CONCLUSIONS

Our results show that inhibition of cytokinesis by cyclin B and cyclin B3 normally prevents precocious cytokinesis, and that mitotic destruction of these cyclins contributes to timely cytokinesis. Although stable cyclin B inhibits cytokinesis by additional modes, both cyclins B and B3 inhibit activation of cytokinesis by the Pebble pathway.

We propose that an as yet undefined regulatory interface between cyclin activity and Pebble function connects the central cell cycle machinery to cytokinesis to provide temporal regulation. In addition, we suggest that cyclin B3 can delay a deadline for completion of cytokinesis that might ordinarily be imposed by nuclear envelope reformation and nuclear recruitment of Pebble away from its site of action.

EXPERIMENTAL PROCEDURES:

Fly stocks and stable cyclin expression

We used the wild-type strain Sevelen, and the following mutant alleles for cyclin B: cycB2 [20], cyclin B3: cycB32 [20], pebble: pbl2 [43]; RhoA: Rhoa720 [44] and zipper: zip1 [45]. The G289 plasma membrane GFP fusion strain was described in [46]. Stable cyclin B3 or Cdc2 with stable cyclins B were expressed under a heat shock promoter induced during G2 of embryonic cycle 14, as described in [1] together (or not) with histone H2-GFP (2AvD-GFP [47]). The plasma membrane GFP fusion and the stable cyclin B3 expressed under a heat shock promoter strains were combined and used in supplementary data: movie 3. We used the driver MS1096 [30] at the indicated temperature for wing expression of a truncated dominant-negative version of Pebble (UAS-Pbl_DH497-549B, UAS-Pbl_DH497-549A) [25] and a catalytically-active form of the Drosophila Rho-Associated kinase Drok (UAS-Drok-CAT) [32].

Immunohistochemistry and imaging

Embryos were fixed as in [48], devitellinized by hand and stained as described in [1]. The following primary antibodies/stains were used: anti-phospho-histone H3 (Upstate Biotechnologies, 1:800), anti-α-tubulin (Sigma, 1:400), anti-Pebble ([25], 1:800), phalloidin-Rhodamine (Molecular Probes, 1:1000), Hoechst 33258 (Molecular Probes, 1_g/ml). Secondary antibodies (except for the Donkey anti-Rat IgG (H+L) Cy3 conjugates (Jackson ImmunoResearch Laboratories, 1:600)), imaging and videomicroscopy procedures were described in [1]. For nuclear staining of wing cells, adult flies were removed from their pupal cases 1-2 hours before eclosion, wings were fixed in 37% formaldehyde for 10 minutes, incubated with Hoechst 33258 (1_g/ml) for 1 hour, then quickly washed and mounted for imaging.

Genetic interactions

Right wings from adult flies of the appropriate genotype were removed and dehydrated in 100% Ethanol for 1 hour and mounted in Ethanol:Glycerol 1:1. Pictures were taken from the dorsal surface between wing veins III and IV, in a 166_m x 140_m rectangle centered above the posterior crossvein. For quantification, at least 15 independent right wings of each genotype were analyzed.

RNA interference experiments

dsRNAs covering the first 714 base pairs of the Pebble coding sequence [25] were polymerized by the T7 RNA polymerase using the Ribomax kit (Promega). We used a PCR product flanked with two T7 promoter sequences (written in uppercase, see below) as a template for in vitro transcription. This PCR product was amplified from pBlueScriptSKII-Pblwt1a [25] with the following primers: TAATACGACTCACTATAGGGAGAatggaaatggagaccattgaa and TAATACGACTCACTATAGGGAGAagcaaacgcctccgttttctaaaag. Pebble dsRNAs were annealed during cooling at room
temperature after denaturation for 1 minute at 94°C, and adjusted to 2 mg/ml in injection buffer (5mM KCl, 0.1mM NaPhosphate buffer pH 7.8). Control dsRNAs were made for part of the coding sequence of the bacterial LacI, from a PCR product amplified with the following primers: TAATACGACTCATACTAGGGAGAtctgaccagacacccatcaac and TAATACGACTCATACTAGGGAGAgtttccagtcgggaaacctg . Twenty min Sevelen or G289 embryo collections were injected with control or Pebble dsRNAs immediately after egg deposition, at the posterior or the anterior pole, using standard injection protocols, and processed for videomicroscopy as described in [1].

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FIGURE LEGENDS

FIGURE 1: Wild-type cytokinesis initiates during anaphase B of mitosis 14 and is completed in 90 seconds.
Fluorescence micrographs of wild-type cells fixed during mitosis 14. (A) Phospho-histone H3 staining (blue), F-actin/phalloidin staining (red and A’) and _-tubulin staining (green and A’’) label mitotic chromosomes, mitotic spindles and the cortical actin cytoskeleton that underlines the plasma membrane, respectively. The numbers in panel A’ indicate cells or cell pairs at progressively later stages of cytokinesis. Note the decondensation of the DNA and reorganization of the spindle as cells near completion of cytokinesis (cell pair 4). Scale bar, 5_m. (B) The distances between the microtubule organizing centers (spindle length) were measured in 128 cells at mitosis 14 (metaphase or later) and individually plotted as a narrow vertical bar. The bars were colored red if there were no signs of cytokinesis, green if initiated or clearly in progress, blue if almost completed (actin ring being almost closed) or black if completed. Within each category, the bars are ordered with spindle length increasing from left to right. Note that because the spindle elongates progressively through anaphase B but shortens abruptly at the end of mitosis, the ordering according to length should correspond to an order in time except for the last cells represented in black, which are presumably in the reverse temporal order. (C) Schematic of the stereotyped transformations of actin and microtubule-based cytoskeletons during mitotic exit with the timing indicated for mitosis 14. Cytokinesis is initiated during anaphase B, when the spindle length is greater than 11 _m (see B). Thirty-four sec (SD = 8.5 sec) elapsed from anaphase A/B transition to initiation of cytokinesis and 93 sec (SD = 13 sec) from cytokinesis initiation to completion (n = 20), as measured by recording lived embryos (see supplementary data: movie 1, showing real-time imaging of cytokinesis progression in embryos that express a plasma membrane protein fused to GFP). Measurement of the time elapsed between metaphase/anaphase to anaphase A/B transitions were obtained from a combination from fixed and live data (D. Parry and P.O.F., unpublished data).

FIGURE 2: Endogenous cyclins B and B3 influence the time of cytokinesis furrow formation within mitosis.
(A-C) Fluorescence images of ventral ectoderm cells fixed at mitosis 16 from wild-type embryos (A), homozygous cyclin B mutant embryos (B) or homozygous cyclin B3 mutant embryos (C). Red, green and blue colors represent F-actin/phalloidin, _-tubulin and DNA staining, respectively. The dashed white lines provide a reference for the degree of DNA separation in a wild-type cell prior to initiation of cytokinesis. Scale bar, 2.5_m. (See also (A-C) displayed in separated colors in supplementary data: Figure 1)
(D-F) Plots in which bars representing the degree of chromosome separation (measured from the leading/outer edge) of individual cells (118-162) have been grouped according to whether they have a cytokinesis furrow (green) or not (red) and then displayed along the abscissa according to degree of separation within their group. Bar thickness was normalized according to the number of cells to allow comparison between the different genotypes. It can be seen that the presence of a cytokinesis furrow is correlated with separation. The threshold separation distance at which we observed cytokinesis furrows is shorter in cyclin B (5-6_m, E) and cyclin B3 mutant cells (7-7.2_m, F) than in controls (~8_m, D), and the proportion of anaphase cells (those having chromosome separation greater than 1-1.5_m) lacking cytokinesis furrows is much reduced in mutants (E, F).
We conclude that cyclin B and cyclin B3 are required to prevent precious cytokinesis during the early stages of anaphase.

FIGURE 3: Mitotic destruction of cyclins B and B3 is required for normal initiation and progression of cytokinesis.
(A-F’) Fluorescence images of cells fixed at mitosis 14 from embryos in which a stable (non-degradable) version of cyclin B (A-A’) or a stable
(non degradable) version of cyclin B3 (B-F’) were induced during the G2 phase of cycle 14. In the colored panels, red, green and blue represent F-actin/phalloidin, _-tubulin, and phospho-histone H3 stainings, respectively. In prime-letter panels, the phalloidin stainings from the corresponding letters are separately shown in gray scale. Scale bars, 5_m.

In stable cyclin B expressing cells, cytokinesis is not initiated (A-A’), whereas in stable cyclin B3 expressing cells, cytokinesis progresses (B-F’). Staining for actin (B’ and C’-F’) shows the degree of cell elongation and furrow invagination, revealing a progression as indicated by the numbers in (B’). Visualization of _-tubulin and DNA in these fields shows that exit from mitosis does not accompany this progression of cytokinesis (B, C-F) and that completion of cytokinesis appears to occur without disassembly of the spindle or decondensation of the DNA (E,F). Although cytokinesis occurs in cells expressing stable cyclin B3, the cytokinesis initiation is delayed and progresses slowly to completion (see supplementary data: movie 2).

**FIGURE 4:** Reduction of Pebble function delays the onset and slows the progression of cytokinesis, and acts in synergy with stable cyclin B3 to inhibit cytokinesis.

(A-C’) Fluorescence images of cells fixed at mitosis 15 from embryos homozygous for pbl mutant allele of the RhoA activator Pebble. (A-C) Red, green and blue colors represent F-actin/phalloidin, _-tubulin, and DNA staining, respectively. (A’-C’) show separately the F-actin/phalloidin staining in gray scale from (A-C), respectively. The arrowheads in B’ indicate the partially regressed furrow in a cell displaying signs of return to interphase (see text). Scale bar, 5_m.

(D-I) Selected frames from supplementary data: movie 5 of cells in mitosis 14 in an embryo expressing a plasma membrane protein fused to GFP, after reduction of Pebble function by RNA interference. Time is given in min:sec, with time 0:00 representing the beginning of anaphase B marked by the onset of cellular elongation (red arrow) that accompanies spindle elongation. White arrowheads indicate the initial site of cytokinesis constriction (E) and the maximal ingression just prior to rapid regression (G). Scale bar, 5_m. (See also supplementary data: movies 3 and 4).

(J-J’) Fluorescence images of cells fixed at mitosis 14 from embryos homozygous for pbl mutation in which a stable version of cyclin B3 was induced during the G2 phase of cycle 14. (J) Red, green and blue colors represent, F-actin/phalloidin___-_tubulin, and phospho-histone H3 staining, respectively. (J’) shows separately the F-actin staining in gray scale from (J). Scale bar, 5_m.

Reduction of Pebble function either by mutation or RNA interference leads to cytokinesis failure after abortion of the cytokinesis furrows that initiate (albeit after a delay) and progress (albeit slowly and to variable degrees). Although frequent at mitosis 14 in pbl mutant and in stable cyclin B3 expressing cells, cytokinesis initiation figures are absent in stable cyclin B3 expressing cells that are also mutant for Pebble (J’), showing that stable cyclin B3 and the pbl mutation cooperate to inhibit cytokinesis.

**FIGURE 5:** Wing expression of a dominant-negative form of Pebble leads to cytokinesis failures and results in a reduction of the cell density and a multiple wing hair phenotype.

The truncated dominant-negative Pebble version UAS-Pbl_DH was expressed in the wing disc during late larval and pupariation stages, using the X-linked MS1096-Gal4 driver. Female flies (of the specified genotype) were raised at the indicated temperatures. In all micrographs, a field of the dorsal surface between wing veins III and IV is shown with the following orientation: anterior is up, distal is to the right.

(A) Control flies (X/X; UAS-Pbl_DH/+) at 24˚C. (B, C, D) Pbl_DH-expressing flies (MS1096/+; UAS-Pbl_DH/+) at 24˚C, 18˚C and 29˚C, respectively. (E) Left panel: Quantification of the number of cells with 1, 2 or more than 2 hairs per field, in control flies (black bars) as in (A) or in the Pbl_DH-expressing flies (gray bars) as in (B). (E) Right panel: Quantification of the total number of cell per field (cell density) in control (black bar) or Pbl_DH expressing flies (gray bar). (F-G) Hoechst
staining of pupal wings several hours before eclosion at 29°C: control flies (F), or Pbl\textsuperscript{DH} expressing flies (G). (H-J) Pbl\textsuperscript{DH} expressing flies at 24°C. heterozygous mutant for Pebble (H) (MS1096/+; UAS-Pbl\textsuperscript{DH}/+; pbl\textsuperscript{2}/+), for RhoA (I) (MS1096/+; UAS-Pbl\textsuperscript{DH}/Rho\textsuperscript{2O}), or for the myosin II heavy chain Zipper (J) (MS1096/+; UAS-Pbl\textsuperscript{DH}/zip\textsuperscript{1}). (K) Pbl\textsuperscript{DH} expressing flies at 24°C that co-expressed a catalytically-activated version of the Rho-associated kinase Drok (MS1096/+; UAS-Pbl\textsuperscript{DH}/+; UAS-DrokCAT/+). Wing expression of the dominant-negative Pbl\textsuperscript{DH} transgene resulted in cytokinesis defects (G), a reduction of the wing cell density and appearance of cells with multiple hairs (E). The severity of this phenotype depends on the expression levels of the Pbl\textsuperscript{DH} transgene, and the number of nuclei resulting from cytokinesis defects is increased in cells that have a high number of multiple hairs. The genetic modifications of the Pbl\textsuperscript{DH} phenotype by other genes known to act in cytokinesis suggests that quantification of the cell density and the number of cells with multiple wing hairs provides a read-out of the activity of a Pebble-dependent pathway contributing to cytokinesis (H-K).

FIGURE 6: Mitotic cyclin B and cyclin B3 inhibit the Pebble pathway.
The UAS-Pbl\textsuperscript{DH} transgene expressed in the wing disc of females raised at 24°C, in which 0, 1 or both endogenous copies of the cyclin B3 (A-C) or cyclin B (D-F) genes were removed by mutation. (A) Pbl\textsuperscript{DH}-expressing flies with wild-type levels of cyclin B3 (MS1096/+; UAS-Pbl\textsuperscript{DH}/+). (B) Pbl\textsuperscript{DH}-expressing flies homozygous mutant for cyclin B3 (MS1096/+; UAS-Pbl\textsuperscript{DH}/+; cycB3/cycB3). (C) Quantification of the number of cells with 1 or more than 2 hairs per cell from at least 15 independent wings of Pbl\textsuperscript{DH}-expressing flies that were wild-type (white bars), heterozygous mutant (gray bars) or homozygous mutant (black bars) for cyclin B3. (D) Pbl\textsuperscript{DH}-expressing flies with wild-type levels of cyclin B (MS1096/+; UAS-Pbl\textsuperscript{DH}/+). (E) Pbl\textsuperscript{DH}-expressing flies homozygous mutant for cyclin B (MS1096/+; cycB, UAS-Pbl\textsuperscript{DH}/cycB). (F) is the same as indicated in (C), with cyclin B instead of cyclin B3. The wing phenotype resulting from the expression of the dominant-negative Pbl\textsuperscript{DH} transgene is suppressed in a dose-dependent manner by mutations in either cyclin B or cyclin B3. Thus the presence of cyclin B and cyclin B3 normally inhibit the Pebble pathway.

FIGURE 7: Model for cytokinesis inhibition by cyclin B and cyclin B3.
Our work shows that inhibition of cytokinesis by cyclin B and cyclin B3 and relief of inhibition by cyclin destruction controls the timing of cytokinesis, but the circuitry of the regulation is likely to include indirect as well as more direct actions of the cyclins. There are many factors with inputs into cytokinesis that might be targets for action of the cyclins. We propose that the cyclins act by inhibiting (red lines) one or more of the factors/events (grouped in boxes) that usually promote cytokinesis (green arrows).

Whereas cyclin B and cyclin B3 appear to be similarly effective in inhibition of Pebble-dependent contributions to cytokinesis, stabilization of cyclin B can completely block cytokinesis, while stable cyclin B3 only delays and slows it. We thus suggest that cyclin B inhibits cytokinesis by additional modes, such as blocking mitotic exit events thought to be prerequisites for cytokinesis. Importantly, stable cyclin B but not stable cyclin B3 blocks these events. For instance,
cyclin B, but not cyclin B3, inhibits the release of passenger proteins from chromosomes, a step believed to promote cytokinesis [14, 36, Parry et al. in prep). Moreover, persistence of cyclin B inhibits astral microtubule (MT) growth, spindle elongation (anaphase B) and maturation into a compact midbody structure -events all suggested to convey a cytokinesis stimulus to the cortex.

SUPPLEMENTARY DATA: FIGURE 1: Endogenous cyclin B and cyclin B3 influence the onset of cytokinesis.
(A-C) As in panels A-C of Figure 2 these panels show mitosis 16 in wild-type, cyclin B mutant and cyclin B3 mutant embryos, respectively. Phalloidin staining is shown in red, tubulin in green and DNA in blue and these are repeated in isolated gray scale images in the prime, double-prime and triple-prime panels, respectively. Scale bar, 2.5_m.

SUPPLEMENTARY DATA: FIGURE 2: Pebble levels in cycle 15 cells from wild-type and pbl2 mutant embryos or wild-type embryos injected with Pebble dsRNAs.
(A-C) Pebble levels detected by indirect immunofluorescence on cells fixed at cycle 15 from wild-type embryos (A), homozygous pbl2 mutant embryos (B) or wild-type embryos injected with Pebble dsRNAs after egg deposition (C). (D) Background immunofluorescence was assayed by treating wild-type embryos as in (A) but without Pebble primary antibodies. Images of A-D embryos were taken with the same settings and processed in parallel. Scale bar, 5_m.
Both RNA interference and the pbl2 mutation reduced, but did not completely abolish the Pebble staining in cycle 15 embryonic cells. As the allele truncates the protein before the epitopes recognized by the Pebble antibodies, we conclude that only partial reduction of Pebble function is sufficient to induce cytokinesis defects.

SUPPLEMENTARY DATA: MOVIE 1: Cytokinesis in a live control embryo expressing a plasma membrane protein fused to GFP.

Real-time videomicroscopy of cells at mitosis 14 from embryos expressing a plasma membrane protein fused to GFP allowing visualization of the membrane dynamics during mitosis. Images were acquired every 7 sec at room temperature. Time is given in min:sec. Scale bar, 5_m.
Mitotic cells first rounded up and increased their volume before elongating along one axis (anaphase B). During the elongation process, cytokinesis was initiated and completed, before the cell shrunk to an interphase size. The first cell to divide in this movie started to elongate (beginning of anaphase B) at frame #5, initiated cytokinesis 35 sec later at frame #10 and completed cytokinesis 91 sec later at frame #23. See also Figure 1C for a detailed analysis of the time elapsed between different transitions during mitotic exit.

SUPPLEMENTARY DATA: MOVIES 2 AND 3: The presence of stable cyclin B3 delays the onset and slows the progression of cytokinesis.
Real-time videomicroscopy of cells at mitosis 14 that had been induced to express of a stable (non-degradable) version of cyclin B3 during G2 of cycle 14. Chromosome behavior was revealed by the histone H2 fused to GFP (movie 2) and cytokinesis initiation and progression could be followed either thanks to the cytoplasmic background fluorescence (movie 2), or directly in cells expressing a plasma membrane protein fused to GFP (movie 3). Images were acquired every 7 sec at room temperature, and time is given in min:sec. Scale bar, 5_m.
After the metaphase/anaphase transition (movie 2, frame #10), chromosomes were segregated to the poles with normal kinetics, but arrested in this anaphase B configuration without any signs of decondensation. The cytokinesis furrow was first evident at frame #38, 196 sec after the metaphase/anaphase transition, and is thus delayed by more than 2 min compared to the normal schedule (the metaphase/anaphase transition and the onset of cytokinesis are separated by 1 min in control embryos, as described in Figure 1). Moreover, cytokinesis progressed very slowly and lasted more than 360 sec (completion not reached before frame #94), compared to 90 sec in control embryos.
In the cell presented in movie 3, cytokinesis furrow was initiated at frame #10 (63 sec after the anaphase A/B transition, thus delayed by about 30 sec compared to the normal schedule) and cytokinesis lasted 7 minutes (completion reached at frame #70).

SUPPLEMENTARY DATA: MOVIE 4: Reduction of Pebble function by RNA interference delayed the onset and slowed the progression of cytokinesis (I).
Real-time videomicroscopy of cells at mitosis 14 from embryos that expressed a plasma membrane protein fused to GFP, and that had been injected with Pebble dsRNAs after egg deposition. Images were acquired every 7 sec at room temperature, revealing the membrane dynamics during mitosis. Time is given in min:sec. Scale bar, 5 μm. Pebble RNAi resulted in reduction of Pebble protein levels (see supplementary data: Figure 2) and a failure of cytokinesis. The first cell to divide in this movie started anaphase B at frame #1, and initiated cytokinesis 1 min and 49 sec later at frame #16, i.e. more than 1 min 15 sec behind the normal schedule (the time elapsed between the beginning of anaphase B and the cytokinesis onset is about 30 sec in control embryos, see Figure 1C and supplementary data: movie 1). The furrow progressed midway to completion, but eventually aborted (it started to regress at frame #34, about 2 min after the beginning of ingestion), resulting in a failure of cytokinesis. A similar real-time analysis of cytokinesis in pbl2 mutant embryos revealed a similar delay and slowing of furrowing suggesting that both approaches reduced Pebble function to a comparable degree and with similar consequences (data not shown).

SUPPLEMENTARY DATA: MOVIE 5: Reduction of Pebble function by RNA interference delayed the onset and slowed the progression of cytokinesis (II).
This movie shows another example of cytokinesis failure from embryos injected with Pebble dsRNAs, as described in supplementary data: movie 4. In this case, the cytokinesis furrow appeared to reach completion but abruptly regressed within 7 sec, between frames #34 and #35.
cyclin B / Cdk1

passenger protein release:
- AuroraB
- INCENP
- TD60

astral MT growth
- spindle elongation
- midbody formation

Pebble
- RhoA
- Drok
- Zipper

cytokinesis