Cell-cycle checkpoints that ensure coordination between nuclear and cytoplasmic events in *Saccharomyces cerevisiae*

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Cytoskeletal organization is crucial for several aspects of cell-cycle progression but cytoskeletal elements are quite sensitive to environmental perturbations. Two novel checkpoint controls monitor the function of the actin and microtubule systems in budding yeast and operate to delay cell-cycle progression in response to cytoskeletal perturbations. In cells whose actin cytoskeleton has been perturbed, bud formation is frequently delayed and the morphogenesis checkpoint introduces a compensatory delay of nuclear division until a bud has been formed. In cells whose microtubule cytoskeleton has been perturbed, anaphase spindle elongation often occurs entirely within the mother cell, and the post-anaphase nuclear migration checkpoint introduces a compensatory delay of cytokinesis until one pole of the anaphase nucleus enters the bud. Recent studies indicate that regulators of entry into mitosis are localized to the daughter side of the mother-bud neck whereas regulators of exit from mitosis are localized to the spindle pole bodies. Thus, specific cell-cycle regulators are well-placed to monitor whether a cell has formed a bud and whether a daughter nucleus has been delivered accurately to the bud following mitosis.

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Abbreviations
hsl *histone synthetic lethal*
MAPK mitogen-activated protein kinase

Introduction

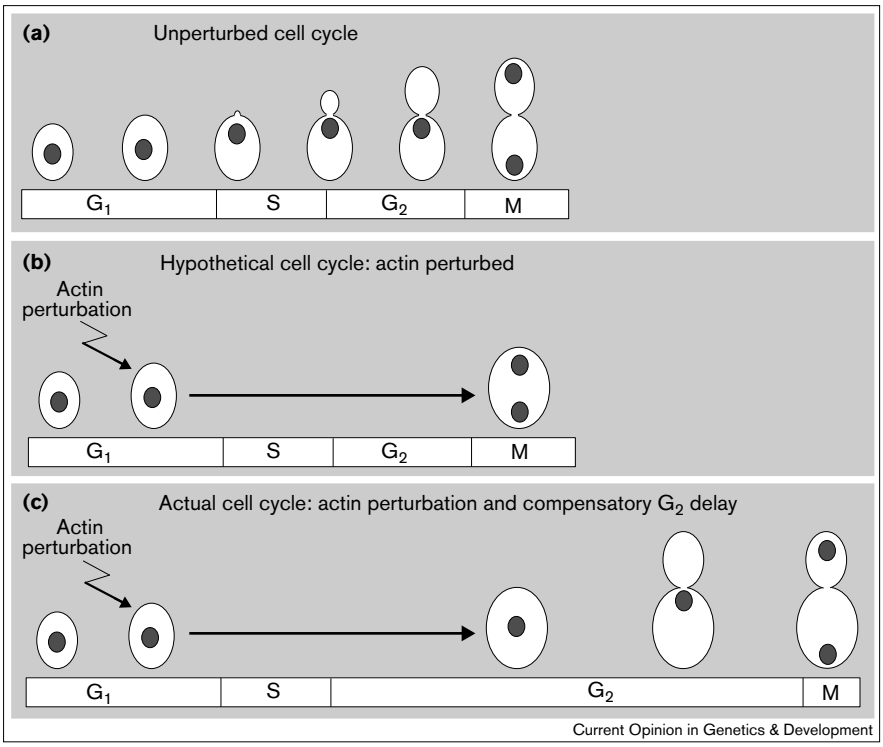
Errors in key cell-cycle processes can have catastrophic consequences for proliferating cells. Surveillance pathways termed checkpoint controls [1] have evolved to protect cells from such damage, by monitoring error-prone processes and providing extra time as needed for their completion. In addition to the well known checkpoint controls monitoring DNA replication and bipolar attachment of chromosomes to the mitotic spindle [2], recent studies with the budding yeast *Saccharomyces cerevisiae* have revealed the existence of checkpoint controls monitoring both actin organization (the morphogenesis checkpoint) and nuclear position in cells. Yeast cells must form a bud — a process that requires a polarized actin cytoskeleton — prior to nuclear division to avoid generating binucleate cells. Similarly, nuclear division must accurately partition the daughter nuclei between mother and bud prior to cytokinesis, to avoid generating binucleate and anucleate cells.

The morphogenesis checkpoint

In the laboratory, yeast cells are propagated under optimal conditions in which bud formation is rapid and continuous. However, many perturbations (e.g. shifts in temperature [3] or osmolarity [4]) that presumably occur quite frequently in more natural environments provoke a transient depolarization of the actin cytoskeleton, during which bud construction is delayed while cells adapt to the particular insult. Recent findings suggest that actin depolarization is an adaptive response triggered by a putative plasma-membrane sensor (Wsc1p), presumably helping to maintain cell integrity in times of stress [5••]. If the cell cycle were to continue unchecked in these cells, then nuclear division would often occur prior to bud formation, generating binucleate cells (Figure 1). This is not observed: rather, the cell cycle halts in G_2 until the actin can repolarize and complete bud construction [6] (Figure 1). A similar G_2 delay can be triggered by mutations or drugs that specifically impair actin organization in the absence of any environmental perturbation [7], suggesting that it is the actin disorganization rather than the specific insult that causes the cell-cycle delay. The sensor(s) that detect actin disorganization remain unknown but the resulting G_2 delay is enacted by Swe1p — the sole *S. cerevisiae* relative of the Wee1 kinase in the fission yeast *Schizosaccharomyces pombe* — which phosphorylates and inhibits the cyclin-dependent kinase Cdc28p [8,9]. This actin-monitoring Swe1p-regulatory pathway has been called the 'morphogenesis checkpoint' [6,7,9]. The past year has brought significant advances in our understanding of Swe1p regulation during the normal cell cycle, which can now be applied to elucidate how the checkpoint pathway affects Swe1p.

The abundance of Swe1p in unperturbed cells fluctuates during the cell cycle, accumulating during late G_1 and S phase and disappearing during G_2 and M phase [10]. In response to actin perturbations, however, Swe1p continues to accumulate during G_2. This regulation involves both increases in *SWE1* transcription [9] and suppression of Swe1p degradation [10]. Swe1p is normally targeted for rapid degradation in G_2 but its degradation is prevented by the morphogenesis checkpoint. Swe1p is a substrate of the ubiquitin ligase SCF^{Met30}, which catalyzes poly-ubiquitination of Swe1p leading to recognition and digestion by the proteasome [11]. Substrate recognition by SCF-family ubiquitin ligases often requires prior phosphorylation of the substrate [12] and Swe1p undergoes hyperphosphorylation during the cell cycle [10] — raising the possibility that Swe1p phosphorylation may control its degradation.

Figure 1



The morphogenesis checkpoint in budding yeast. **(a)** During the unperturbed cell cycle, a mature bud has formed by the time cells undergo nuclear division. Many environmental perturbations cause a transient depolarization of the actin cytoskeleton, causing delays in bud formation. **(b)** If such delays were not accompanied by a delay of nuclear division, then cells would frequently become binucleate. **(c)** The morphogenesis checkpoint prevents this fate by introducing a compensatory G_2 delay in the cell cycle.

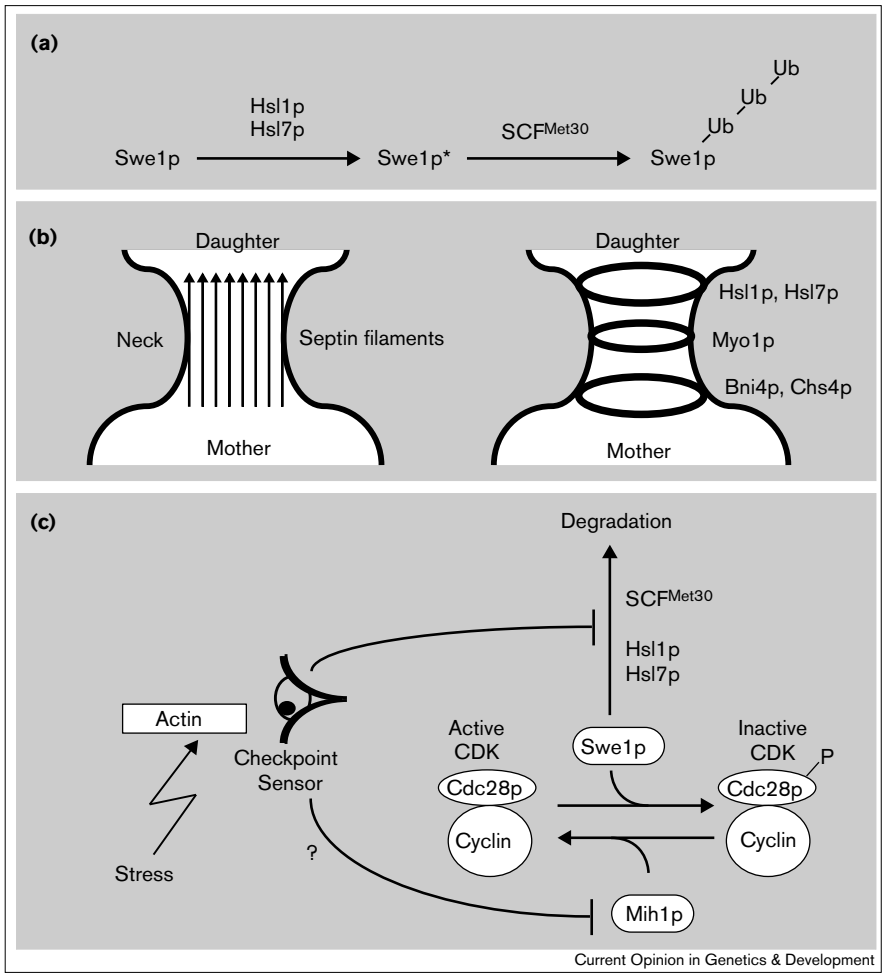
Role for Hsl1p and Hsl7p in targeting Swe1p for degradation

Mutation of a control element that targets Swe1p for degradation would be expected to provoke a Swe1p-dependent G_2 delay even in the absence of actin perturbation. Two mutants with just this phenotype (*hsl1* and *hsl7*) were discovered serendipitously in a screen for 'histone synthetic lethal' (*hsl*) mutants [13]; *HSL1* (also called *NIK1*) was discovered independently in a screen for *S. cerevisiae* genes that could serve as multicopy suppressors of a temperature-sensitive *cdc2* mutant in *S. pombe* [14]. *HSL1* encodes one of three *S. cerevisiae* protein kinases with amino-terminal catalytic domains related to that of *S. pombe* Nim1, which directly phosphorylates and inhibits Wee1 [15–17]. Hsl1p and Nim1 each contain a large carboxy-terminal 'regulatory domain' but show no homology in this region. Hsl7p is a phylogenetically conserved protein and a human homologue of Hsl7p (JBP1) was shown recently to possess protein methyltransferase activity [18••]. Overexpression of either Hsl1p or Hsl7p is sufficient to override most of the G_2 delay in cells exposed to actin perturbations [19••], suggesting that they act as negative regulators of Swe1p.

As Nim1 phosphorylates Wee1 directly, it is tempting to speculate that Hsl1p phosphorylates Swe1p directly. Consistent with this, Swe1p hyperphosphorylation is reduced in *hsl1* (and also in *hsl7*) strains [20••], although direct evidence that Hsl1p phosphorylates Swe1p is lacking. Interestingly, Hsl7p is also phosphorylated in an Hsl1p-dependent manner [19••], raising the possibility that Hsl7p is a direct substrate of Hsl1p. Two-hybrid and co-immunoprecipitation data indicate that Hsl7p associates with both Swe1p and Hsl1p, suggesting that Hsl7p might form a bridge connecting a Swe1p–Hsl7p–Hsl1p complex [19••,20••]. Examination of Swe1p stability in cells lacking or overexpressing either Hsl1p or Hsl7p revealed that both proteins are required for rapid Swe1p degradation and that Hsl1p (but not Hsl7p) is rate-limiting for degradation of excess Swe1p [19••]. In aggregate, these data suggest that Swe1p is first acted upon by Hsl1p and Hsl7p, which prepare Swe1p (perhaps via phosphorylation) for recognition by SCF^{Met30}, which then ubiquitinates Swe1p leading to its degradation by the proteasome (Figure 2a).

It is intriguing that while Nim1 appears to inhibit Wee1 kinase activity, Hsl1p targets Swe1p for degradation. It is not known whether Nim1 also targets Wee1 (or its relative Mik1) for degradation in *S. pombe*. Similarly, we do not know whether Hsl1p also inhibits Swe1p catalytic activity in *S. cerevisiae*. The expectation that Hsl1p and Nim1 might function via similar mechanisms stems from the homology between their catalytic domains. However, this domain is shared by two other kinases in *S. cerevisiae*, Gin4p and Kcc4p [21,22••,23,24], neither of which is able to target Swe1p for degradation in the absence of Hsl1p [19••]. Gin4p has a distinct role in promoting septin organization (see below) that is not shared with either Hsl1p or Kcc4p, whereas the role of Kcc4p remains unknown

Figure 2



Regulation of Swe1p. **(a)** Swe1p is targeted for rapid degradation during G_2/M through a pathway involving the Nim1-related kinase Hsl1p and the putative methyltransferase Hsl7p, which prepare Swe1p for recognition by the ubiquitin ligase SCF^{Met30}. The nature of Swe1p* – a hypothetical intermediate in this pathway – is not known but a reasonable hypothesis would be that it is a phosphorylated form of Swe1p. Both Swe1p and Hsl7p are likely substrates for the Hsl1p kinase. **(b)** Organization of the mother-bud neck. Left: one hypothesis for the organization of septin filaments underneath the plasma membrane at the neck. A polar filament array of this type could underlie the specific recruitment of various proteins to one or other side of the neck (right). Assembly of Hsl1p and Hsl7p at the daughter side of the neck is probably important for their ability to target Swe1p for degradation, in which case bud formation and the consequent neck organization could provide a direct trigger for Swe1p degradation. **(c)** Actin perturbations monitored by the morphogenesis checkpoint cause Swe1p stabilization but the detailed mechanism for this remains unknown. In addition, the checkpoint must utilize other pathways – here shown speculatively as an inhibition of the phosphatase Mih1p – in order to produce a robust G_2 delay.

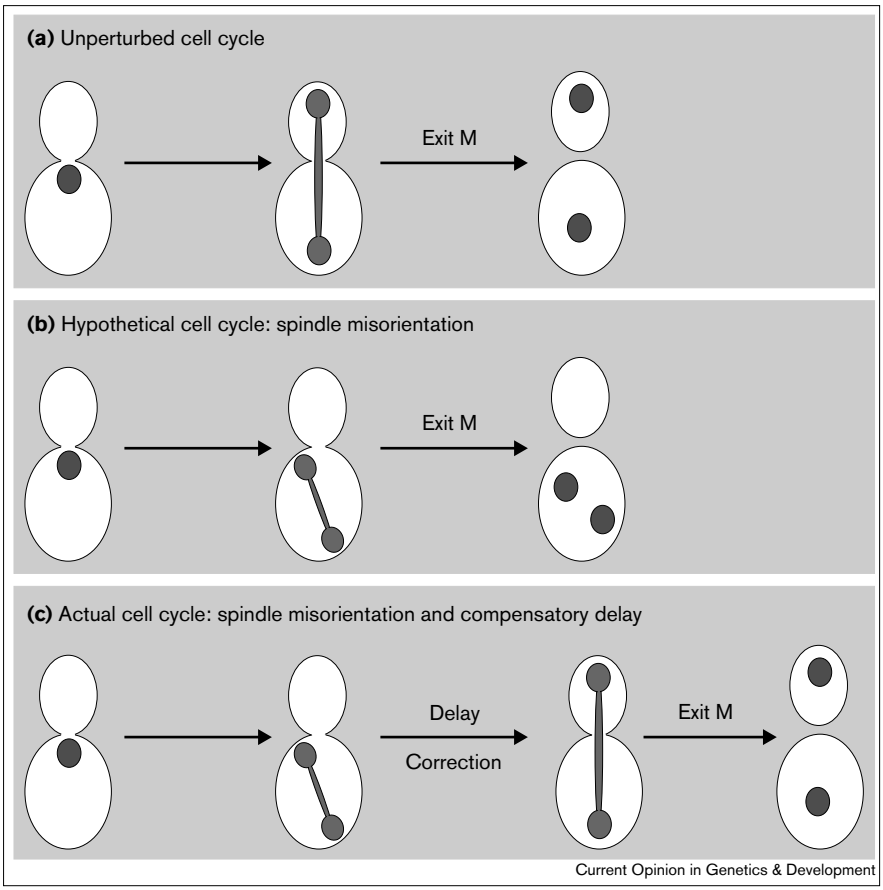
[21,25••]. Thus, kinases of this family do not act redundantly and the apparent similarity between Nim1 and Hsl1p may not reflect identical mechanistic roles in cell-cycle control.

Localization of Hsl1p and Hsl7p to the daughter side of the mother-bud neck

One of the most surprising findings to emerge from studies of Hsl1p and Hsl7p is that both proteins are concentrated on the daughter side of the mother-bud neck [20••,22••,25••]. Localization of most (if not all) proteins to the neck region in yeast depends on the septins, a family of evolutionarily conserved filament-forming proteins that serve as scaffolds for the assembly of functional complexes [26,27]. A prominent role for septins in many cells is the targeting of cytoskeletal and other elements to cleavage sites during cytokinesis, but they also play key roles in other processes including (in *S. cerevisiae*) bud-site selection and chitin deposition in the cell wall [26,27]. One model for the organization of septin filaments at the neck is that they line the neck in a parallel, polar array [28] (Figure 2b). The hypothesis that septin filaments are oriented along the mother-bud axis has received support from examination of *gin4* mutants, in which septins are frequently misorganized as a set of 5–8 bars traversing the neck [21]. The hypothesis that the septin filaments form a polar array is attractive in that this polarity could underlie the specific localization of septin-interacting proteins to one or other side of the neck.

Although some proteins — such as Bud3p and Bud4p, involved in axial bud site selection in haploid cells — are spread throughout the septin zone [29,30], a number of proteins targeted to the neck display more restricted distributions. For instance, Bni4p and Chs4p (which contribute to the deposition of the chitin-rich 'bud scar' on the mother cell) are found exclusively on the mother side of the neck [31]; Myo1p (the myosin that participates in actomyosin ring contraction during cytokinesis) is restricted to the middle of the neck [32,33]; and Hsl1p and Hsl7p are localized to the daughter side of the neck (Figure 2b). A ring of septins assembles at the presumptive bud site prior to bud formation [26,27], and recruits a number of proteins (though not Hsl1p and Hsl7p) to that site. The organization of the septin filaments within the ring in unbudded cells is not known but both geometrical considerations and

Figure 3



The post-anaphase nuclear migration checkpoint. **(a)** During the unperturbed cell cycle, anaphase spindle elongation segregates one spindle pole (with associated chromosomes) to the mother and one to the bud prior to exit from mitosis (M). Mutations (e.g. those affecting dynein function) and environmental perturbations (e.g. cold temperatures) can lead to a misoriented anaphase in which both spindle poles remain in the mother. **(b)** If such errors were not corrected prior to exit from mitosis, then cell division would produce binucleate and anucleate progeny. **(c)** The post-anaphase nuclear migration checkpoint prevents this fate by introducing a compensatory delay in the cell cycle, allowing correction of the error and delivery of one pole to the bud prior to cytokinesis.

electron-microscopy studies [34] indicate that this organization is distinct from that in the neck of budded cells.

In budded cells, Hsl1p and Hsl7p assemble onto the septin scaffold in a hierarchical manner that depends on proper septin organization. Co-immunoprecipitation data indicates that Hsl1p binds to the septin Cdc3p [22••] and Hsl1p is required to target Hsl7p to the neck [20••,25••]. In contrast, Hsl7p is not required to target Hsl1p to the neck [20••,25••]. Neck localization of both proteins is largely eliminated in mutants (e.g. *gin4*) in which septins are misorganized at the neck [25••], even though this misorganization does not prevent neck targeting of other proteins such as Bud4p and Bni4p [21], suggesting that localization of Hsl1p and Hsl7p is particularly sensitive to septin organization. Interestingly, mutants that perturb septin organization also display a Swe1p-dependent G_2 delay in the cell cycle [22••,25••], suggesting that delocalization of Hsl1p and Hsl7p from the neck may make them less effective in down-regulating Swe1p. A possible mechanism for this effect is suggested by the observation that Hsl1p isolated from septin-mutant cells undergoes significantly less autophosphorylation *in vitro* than Hsl1p isolated from wild-type cells [22••]. The simplest interpretation of these data is that Hsl1p catalytic activity is dependent upon (or at least stimulated by) Hsl1p recruitment to the neck which, in turn, depends upon the normal organization of septin filaments at the neck.

Why should Hsl1p/7p function be responsive to septin organization? An appealing hypothesis [25••] is that this is a way to link Swe1p degradation to bud formation. The septin organization that promotes Hsl1p/7p recruitment to the daughter side of the mother-bud neck is presumably unique to budded cells. By making Hsl1p/7p activity dependent on that septin organization, cells can ensure that Swe1p degradation will not begin until a bud has formed. In this context, it is interesting to note that whereas unbudded and small-budded cells undergo a Swe1p-dependent G_2 arrest upon depolymerization of F-actin, larger-budded cells fail to arrest [7]. Constitutive but modest Swe1p overexpression, which is insufficient to delay the normal cell cycle, allows larger-budded cells to arrest in G_2 in response to actin depolymerization [7]. This indicates that large-budded cells retain the ability to sense actin perturbation, but under normal circumstances such cells no longer contain sufficient Swe1p to enforce a G_2 arrest. Perhaps once a bud has been formed the cells no longer require the morphogenesis checkpoint and Swe1p is then degraded so that subsequent actin perturbations do not affect the cell cycle.

An alternative (not mutually exclusive) hypothesis is that Hsl1p/7p serve as sensors monitoring septin organization as part of a 'septin checkpoint' responsive to septin perturbations [20••,22••]. It is not known, however, whether yeast cells in their natural environment ever experience perturbations of septin organization, and it is unclear how introduction of a short G_2 delay might help cells to cope with such perturbations — as septin perturbations, unlike actin perturbations, do not impair bud formation. Moreover, even complete septin delocalization causes only a brief (30–50 minute) G_2 delay [22••,25••] as compared to the much longer delays (> 15 hours) that can occur upon actin perturbation [7].

These studies have led us to a much better understanding of Swe1p regulation in budding yeast, but many open questions remain. In particular, the basis for control of Swe1p degradation by the morphogenesis checkpoint remains unclear, as does the detailed mechanism whereby Hsl1p and Hsl7p target Swe1p for degradation. In addition, we now know [19••] that stabilization of Swe1p (as occurs in *hsl1* or *hsl7* mutants) is not sufficient to produce a long G_2 delay (such as that produced upon actin depolymerization). Thus, the checkpoint must employ other, parallel strategies to effect appropriate G_2 delays. One such strategy may be the inhibition of Mih1p, the Cdc25-related phosphatase that reverses the Cdc28p phosphorylation catalyzed by Swe1p [19••] (Figure 2c). Finally, the way in which actin perturbations are sensed by the morphogenesis checkpoint is still a complete mystery.

The post-anaphase nuclear migration checkpoint

Studies using time-lapse microscopy to examine the behavior of living cells lacking dynein function have suggested the existence of a novel checkpoint control monitoring nuclear position during anaphase [35]. The nuclear envelope does not break down during mitosis in yeast; an intranuclear spindle assembles between spindle pole bodies embedded within the nuclear envelope, and the position of the spindle is determined by astral microtubules that emanate from the cytoplasmic face of the spindle pole bodies and interact with the cell cortex [36]. In cells lacking cytoplasmic dynein, anaphase spindle elongation frequently occurs entirely within the mother cell, rather than between the mother and the bud [35]. The mutant cells are able to correct this defect, apparently by stochastically 'pushing' one pole of the late-anaphase spindle into the bud (Figure 3). The time taken for this correction varies widely from cell to cell and can take more than one hour [35]. The timing of cytokinesis is also highly variable in these cells, but the time between successful delivery of one spindle pole to the bud and subsequent cytokinesis is relatively constant, suggesting that initiation of cytokinesis is delayed until one spindle pole has entered the bud [35] (Figure 3). A search for putative checkpoint mutants has identified *bim1*/*yeb1* mutants, in which the delayed entry of one spindle pole into the bud is not always accompanied by a corresponding delay in cytokinesis [37]. The net result of abrogating dynein function and Bim1p function is the frequent generation of binucleate and anucleate cells. It seems likely that cells may require this checkpoint when they are exposed to cold temperatures, which impair microtubule function and may trigger defects similar to those observed in dynein mutants [37].

How do cells monitor whether one pole of the late-anaphase spindle has successfully penetrated into the bud? One possibility is that geometrically constrained interactions of astral microtubules with portions of the cell cortex (e.g. the neck) may occur in a manner dependent upon whether the spindle pole body is on the mother or bud side of the neck. Intriguingly, astral microtubules are shorter and less dynamic in *bim1* mutant cells [38••], which might prevent them from efficiently providing such positional information. Observations on cells harboring the cold-sensitive β-tubulin allele *tub2-401* grown at 18°C — a temperature at which the mutation causes the selective depolymerization of astral microtubules — are consistent with the hypothesis that astral microtubules are responsible for monitoring spindle-pole position. These cells frequently undergo cytokinesis despite a misoriented anaphase spindle, generating binucleate and anucleate cells [36]. In this context, the recent finding that Bim1p is concentrated at the plus ends of astral microtubules [38••] may indicate a more direct role for Bim1p in cortical interactions that provide positional information.

Another hypothesis to explain these observations would be that cells can somehow directly evaluate the position of the spindle pole(s) and that penetration of one pole into the bud provides a signal to exit mitosis and undergo cytokinesis. According to this hypothesis, the finding that mutants (*bim1*, *tub2-401*) with altered astral microtubule behavior can undergo cytokinesis with both spindle poles still in the mother could be explained by postulating that spindle poles in the mutant cells transiently, but ultimately unsuccessfully, penetrate into the bud prior to cytokinesis. Video microscopy of live cells containing GFP-marked spindles or spindle poles should reveal whether such frustrated penetrations occur and whether they can account for the checkpoint failure observed in mutant strains.

How do cells delay exit from mitosis until anaphase nuclear migration has delivered one spindle pole into the bud? Studies over the past several years have revealed the existence of a group of signaling proteins, sometimes called the 'mitotic exit network', required for spindle disassembly and cytokinesis following anaphase [39,40]. The reason for the existence of such a complex signaling system (involving four kinases, a ras-related GTPase, and a phosphatase) controlling exit from mitosis is unclear, but one role for this network may be to detect whether anaphase has successfully segregated one spindle pole into the bud. Homologues of several mitotic exit network proteins in

S. pombe have been shown to be concentrated at one or both spindle poles during mitosis [41–44] and recent results suggest that these proteins may also be localized to spindle poles in *S. cerevisiae* [45••,46••]. These proteins are, therefore, well placed to adjust the timing of cytokinesis in response to information detected at the spindle poles.

Conclusions

Yeast cells exposed to fluctuating weather conditions presumably experience frequent problems with the polarization of the actin cytoskeleton responsible for bud formation (e.g. at high temperature) and with the functioning of the microtubule cytoskeleton responsible for nuclear migration (e.g. at low temperature). This probably explains why checkpoint controls monitoring bud formation or nuclear position have evolved, but it is still a major challenge to elucidate how these monitoring processes operate. The unexpected discoveries that proteins controlling entry into mitosis are localized to the daughter side of the mother-bud neck, and that proteins controlling exit from mitosis are localized to the spindle pole bodies, provide tantalizing clues to the surveillance mechanisms monitoring bud formation and anaphase nuclear position, respectively.

Are similar surveillance mechanisms likely to exist in other cells? Although a mechanism to monitor bud formation is clearly inapplicable to cells that do not grow by budding, there is very good evidence for the existence of mechanisms that monitor some aspect of cytoskeletal tension and/or cell morphology in metazoan cells, generating important inputs into proliferative and differentiation decisions in many cell types [47]. In addition, spindle position in animal cells determines the subsequent cleavage plane and is therefore critical to ensure the equal, or in selected cases the unequal, size of the daughter cells. It is conceivable, therefore, that animal cells may use a monitoring system to ensure appropriate positioning of the mitotic spindle. Finally, cells may possess yet more self-surveillance mechanisms to coordinate the intricate process of cell division [48,49].

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In this paper, we document the septin-dependent, stepwise recruitment of a cell-cycle-regulatory module consisting of Hsl1p, Hsl7p, and Swe1p to the daughter side of the mother-bud neck. Various mutations that perturb septin organization at the neck prevent effective targeting of this module to the neck and cause Swe1p-dependent G_2 delays associated with the generation of elongated buds. Perturbations that affect actin organization, however, do not cause delocalization of this module from the neck. We suggest that the location of the module serves to couple Swe1p degradation to bud formation.

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