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Review

The Renaissance or the cuckoo clock

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'. . .in Italy, for thirty years under the Borgias, they had warfare, terror, murder and bloodshed, but they produced Michelangelo, Leonardo da Vinci and the Renaissance. In Switzerland, they had brotherly love, they had five hundred years of democracy and peace—and what did that produce? The cuckoo clock'.
Orson Welles as Harry Lime: *The Third Man*

Orson Welles might have been a little unfair on the Swiss, after all cuckoo clocks were developed in the Schwartzwald, but, more importantly, Swiss democracy gives remarkably stable government with considerable decision-making at the local level. The alternative is the battling city-states of Renaissance Italy: culturally rich but chaotic at a higher level of organization. As our understanding of the cell cycle improves, it appears that the cell is organized more along the lines of Switzerland than Renaissance Italy, and one major challenge is to determine how local decisions are made and coordinated to produce the robust cell cycle mechanisms that we observe in the cell as a whole.

Keywords: mitosis; kinase; phosphatase; checkpoint

1. INTRODUCTION

To proliferate successfully, the eukaryotic cell must coordinate multiple signalling pathways (both extrinsic and intrinsic) to produce robust, ordered decisions: DNA replication should normally be followed by cell division, and division by another round of replication. This cell cycle is controlled by the cyclin-dependent kinases (Cdks), whose activities regulate each of the major events: the assembly of origins of replication, the firing of these origins to synthesize DNA and the rearrangement of the whole cell architecture to assemble the mitotic apparatus that segregates sister chromatids [1]. These events require coordination with multiple other protein kinases and some of the mechanisms by which this is achieved are becoming apparent. Moreover, as low Cdk activity is required for cells to exit mitosis and for origins of replication to assemble, whereas high Cdk activity prevents origin assembly and promotes mitosis [2], it is not simply the kinase that is important, the antagonistic phosphatases have an equally important role.

The balance between an important cell cycle kinase and its antagonistic phosphatase has assumed increasing prominence in cell cycle research as the specificity of distinct phosphatase holoenzyme complexes has been realized. The PP1 and PP2A families of phosphatases are most clearly implicated in the control of the cell cycle and although the catalytic subunit of these families has little specificity, the regulatory subunit imparts considerable specificity through both substrate binding and, very importantly, localization [3]. Similarly, a number of protein kinases are activated by partner subunits that have distinct subcellular localizations. This aspect is omitted from many descriptions of cell cycle regulation, which is mostly described in global terms that treat the cell as a homogeneous milieu, where the balance between a protein kinase and its antagonistic phosphatase is altered uniformly throughout the cell. Therefore, one of the major challenges for the cell cycle field is to refine these ideas to take into account cell structure and protein dynamics because it is becoming clear that the local balance of enzyme activities is crucial for many processes. Here, we cite examples, mostly from the control of mitosis, of how local decision-making controls specific cell cycle events and we discuss the mechanisms by which the balance between enzymes can be altered at specific locations, and how these local decisions can be coordinated to control the cell cycle as a whole.

2. COORDINATING PROTEIN KINASES

For several important cell cycle protein kinases, it has become clear that their activity is fundamentally regulated at the local level. This is exemplified by two families of mitotic kinases: the Aurora and Polo kinases. The Aurora kinases are activated by binding to a partner protein and these are often localized to specific structures in the cell. For example, Aurora A in vertebrate cells is activated by binding to TPX2, which binds to microtubules [4], whereas Aurora B is activated by binding to an inner centromere protein ([5]; see below). By comparison, the Polo-like kinases (Plks) are usually recruited to their substrates through a conserved polo-box domain in their carboxyl

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One contribution of 16 to a Theme Issue 'The cell cycle'.

terminus that is a phospho-binding domain [6]. Many of the substrates of the Plks are important regulators of different aspects of mitosis and their phosphorylation is often coordinated with the actions of the Cdks through recruitment of the Plk to a particular substrate after it has been phosphorylated by a Cdk to create a polo-box-binding domain [6]. The importance of recruitment to a phospho-primed substrate may help to explain how Plk1 and the Mps1 kinases have very different roles in mitosis and yet phosphorylate a similar consensus sequence at the primary amino acid level [7]. In some circumstances, notably recovery from DNA damage, Aurora A is required to activate Polo [8,9], thereby creating the potential to generate higher levels of spatial control by demanding coordination between two independent recruitment events.

3. FEEDBACK LOOPS

The key to generating a localized change in enzyme activity is the feedback loop. Turing [10] first suggested that symmetry can be broken if stochastic fluctuations can be amplified auto-catalytically, and one of the most elegant demonstrations of this is symmetry breaking in budding yeast [11,12]. Here, even in the absence of polarity cues, a positive feedback loop at the cell cortex is set up between the Rho family GTPase, Cdc42, its guanine-nucleotide exchange factor (GEF) and its effector kinase (p21-activated kinase, PAK) by the scaffold protein Bem1. When bound to guanosine triphosphate (GTP), cortical Cdc42 can bind PAK, and thereby recruit further GEF activity through Bem1 [11]. As a result, small fluctuations in the concentration of GTP–Cdc42 will be rapidly amplified and competition for limiting amounts of the Bem1–GEF–PAK complex subsequently results in only one focus of active Cdc42 that polarizes the actin cytoskeleton [13]. Speed is of the essence in establishing this single, dominant, Cdc42 focus, as retarding the amplification process can lead to two foci that drive two independent budding events [13].

Feedback loops, both positive and negative, are integral parts of the cell cycle control machinery where they can confer a switch-like response or bi-stable state. The clearest examples are the feedback loops between the major mitotic Cdk, Cyclin B–Cdk1 and its inactivator (Wee1) and activator (Cdc25) that have been delineated in *Xenopus* extracts [14,15]. Cyclin B–Cdk1 and Wee1 form a double-negative feedback loop: cyclin B–Cdk1 is kept inactive by the Wee1 kinase that phosphorylates Cdk1 on tyrosine 15 in its adenosine triphosphate (ATP)-binding site, but once activated Cyclin B–Cdk1 can inhibit Wee1 by phosphorylating it. In contrast, Cyclin B–Cdk1 and Cdc25 form a positive feedback loop: phosphotyrosine 15 is dephosphorylated by the Cdc25 phosphatase, and the newly activated Cyclin B–Cdk1 further promotes its activation by phosphorylating and activating Cdc25. Thus, once sufficient Cyclin B–Cdk1 is activated, the whole pool of the kinase is rapidly activated, such that in a uniform environment Cyclin B–Cdk1 exhibits switch-like behaviour: it is either on or off. But, we have seen that there is an inherent danger in feedback loops because they can be inappropriately triggered by stochastic fluctuations. Militating against this is that both Wee1 and Cdc25 are ultrasensitive substrates of Cyclin B–Cdk1 [16]. A small amount of Cyclin B–Cdk1 activity is only able to phosphorylate sites on Wee1 that have no effect on Wee1 activity. Higher Cyclin B–Cdk1 activity levels are required to phosphorylate the sites on Wee1 that inhibit it [16]. Simultaneously, Cdc25 is rapidly activated with only a marginal increase in Cyclin B–Cdk1 levels, once a relatively low threshold is met [17]. Thus, Cyclin B–Cdk1 activity must reach a critical threshold before it triggers the amplification loop, whereupon the bi-stable switch from inactive to fully active is rapid and complete. The lock that sets this switch is provided by PP2A–B55δ, because fully activated Cyclin B–Cdk1 is not sufficient to drive *Xenopus* extracts into mitosis unless the PP2A–B55δ form of the PP2A phosphatase is inhibited by phosphorylated endosulphine [18] or phosphorylated Arpp19 [19].

These studies inform the prevailing model for how mitosis is triggered: a threshold level of Cyclin B–Cdk1 triggers its auto-amplification, and the downstream activation of the greatwall kinase subsequently inactivates PP2A–B55δ by phosphorylating endosulphine or Arpp19. Thus, the problem of understanding the control of mitotic commitment becomes one of understanding the thresholds that trigger the feedback loops. Moreover, as well as triggering feedback loops, different thresholds of kinase activity can themselves promote different processes in the cell cycle. Seminal work by Coudreuse & Nurse [20] highlighted the importance of thresholds with the elegant demonstration that in fission yeast DNA replication and mitosis can be driven by different threshold levels of a single Cyclin B–Cdk1 complex.

4. DIFFERENT CYCLIN–CDK THRESHOLDS DRIVE DNA REPLICATION AND MITOSIS

There are multiple types of Cdk in most organisms and a division of labour among them would seem an obvious mechanism by which each event in the cell cycle could be triggered at the right time and in the right order. Some substrate specificity is indeed observed between Cyclin and Cdk complexes [21–23]. However, through the analysis of cyclin-deletion strains, Fisher & Nurse [24] established that fission yeast could effectively control the cell cycle with a single Cyclin B–Cdk1 complex (Cdc2 in partnership with the B type cyclin Cdc13). By explanation, Stern & Nurse [25] proposed that a lower threshold level of Cdc13–Cdc2 is required to drive S phase than is required to drive M phase. Technological developments recently made possible a rigorous test of this model. Coudreuse & Nurse [20] exploited the ability to fine-tune the activity of a Cdc2–Cdc13 fusion protein in which the Cdc2 catalytic component had been mutated to render it exclusively sensitive to non-hydrolysable ATP analogues. This enabled them to dictate the level of Cdc2/CyclinB activity and ask which cell cycle events could be triggered by this level of activity. The result was an overwhelming endorsement of the Stern–Nurse model proposed some 14 years earlier [20].

This work establishes a critical principle: a single Cyclin–Cdk can drive different control loops that force the cell into a fully committed state depending

upon the absolute level of the activity. How then, are these thresholds set and met for each discrete event? The simplest view would be that the critical 'entry point' substrates for each phase have distinct thresholds, through intrinsic affinity for the protein kinase or through association with an opposing phosphatase. Differing abilities to engage phosphatase 'locks' could work equally well. But another intriguing option, also to emerge from studies in fission yeast, is that of spatial control over where the threshold is reached. Rather than arising through the inevitable accumulation of a critical level within a homogeneous milieu, the threshold activity of Cyclin B–Cdk1 required for mitotic commitment in this system maybe met at the fission yeast spindle pole body (SPB; equivalent to the centrosome in animal cells).

5. THE SPINDLE POLE BODY AS A SIGNAL INTEGRATION PLATFORM FOR ENTRY INTO MITOSIS

Fission yeast Cdc25 and Plk both require Cyclin B–Cdk1 (Cdc13–Cdc2) to be activated, indicating that this yeast is no exception to the rule of feedback control of mitotic commitment [26,27]. Gain of function mutations in the SPB component Cut12 (*cut12.s11*) enables cells to overcome an otherwise lethal loss of Cdc25 [28]. They appear to do so by a change in feedback controls because this mutation increases Plk activity, and relies upon this acquired function to suppress the Cdc25 deficiency [29]. Downregulation of Wee1 mirrors the *cut12.s11* mutation in suppressing Cdc25 [30,31]; therefore, a favourable interpretation of these data is that the SPB component triggers feedback loop amplification of Cyclin B–Cdk1 independently of the normal reliance upon the priming activation by Cyclin B–Cdk1. The implication is that the threshold for feedback loop amplification is normally first met on the SPB before being propagated throughout the cell to drive mitotic commitment.

Alongside the important role of the SPB in mitotic commitment, the control of Wee1—by the protein kinases Cdr1, Cdr2 and Pom1—couples the commitment to mitosis with the geometry of these rod-shaped cells [32,33]. Wee1 is inhibited by Cdr1 and Cdr2, which, in turn, are inhibited by the DRYK kinase, Pom1. Wee1, Cdr1 and Cdr2 are localized to numerous foci that form an equatorial belt around the middle of the cell. In contrast, Pom1 is distributed as a gradient that diminishes from peak levels at cell tips to a minimum at the cell equator. The strength of the Pom1 gradient is proposed to be sufficient for Pom1 to be able to inhibit Cdr1/Cdr2/Wee1 at the equatorial nodes in short cells, but as cells lengthen, the Pom1 gradient migrates away from the centre until it is no longer able to inhibit Cdr1/Cdr2. This enables Cdr1/2 to inactivate Wee1 and promote entry to mitosis [32,33]. Thus, feedback loop activation from the SPB is coupled with the attainment of a critical cell size because once cells reach this size the amplification loop emanating from the centrally located SPB no longer encounters inhibitory Wee1 activity and can propagate, unimpeded, throughout the entire cell.

The control of mitotic progression in budding yeast graphically illustrates how the simple imposition of spatial regulation into the conserved Wee1/Cdc25 feedback loop can impart a powerful control. In this case, the spatial control is exerted through localized proteolysis and ensures that genome segregation cannot occur until it is guaranteed that there are two daughter cells to receive the genomes.

Saccharomyces cerevisiae cell cycles differ in several key respects from those of most other eukaryotes. The most immediately apparent is growth by budding in which a daughter cell emerges from a discrete point on the mother cell cortex and is ultimately pinched off by the contraction of the neck that forms at the time of bud emergence. It has been argued that budding represents a specialized form of cytokinesis [34]. Consequently, because cytokinesis must not normally be uncoupled from mitosis, a second unusual feature of the budding yeast cycle arises; the mitotic spindle forms at the start of the cell cycle. Forming a spindle so early in the cell cycle places a significant challenge upon using Wee1/Cdc25 to control mitotic commitment because the B type cyclin–Cdk complexes regulating S phase and those regulating mitosis have to be independently controlled. To ensure that Wee1 (Swe1) regulates genomic segregation and not replication in this system, the B type cyclins that dock Wee1 have different sensitivities to Wee1: both the cyclins that promote S phase (Clb5/6) and those that promote spindle assembly (Clb3/4) are active in the presence of high Wee1 activity and only the 'mitotic' B type cyclins (Clb1/Clb2) are inhibited by Swe1 [35,36]. Thus, Swe1 mirrors the Wee1 kinases in other eukaryotes in governing the timing of chromosome segregation.

The spatial control of Swe1 degradation is an exquisite exploitation of the unique architecture of the budding yeast cell to ensure that the genomes cannot be segregated until there are two daughter cells to receive each copy. Swe1 is destroyed only when it is recruited to a complex of Hsl1 kinase and its partner protein Hsl7 [37]. Importantly, Hsl1 associates with the proteins that form a ring between the mother and daughter cells [38,39] (it associates with the budward side of the ring). As a consequence, cells are unable to degrade Swe1 when there is no bud. If Swe1 cannot be degraded, then neither Clb1–Cdk1 nor Clb2–Cdk1 complexes can be activated, making it impossible to segregate the chromosomes until a daughter cell has been generated. Thus, the simple imposition of a spatial constraint on Swe1 degradation couples mitotic progression with bud emergence in a 'morphogenesis checkpoint' [40]. This principle of restricting degradation to one defined locale to generate local activity gradients is emerging as a common theme in a number of other processes, including signal transduction, the DNA damage response and potentially mitosis, as explained later.

6. MITOTIC COMMITMENT AND THE CENTROSOME

The decision to enter mitosis in animal cells has parallels with the decision in fission yeast. The animal cell equivalent of the yeast SPB is the centrosome, and, as in

fission yeast, there is evidence that Cyclin B–Cdk1 is first activated here in human cells: an antibody that recognizes the phosphorylated form of the auto-phosphorylation site of Cyclin B1 first stains centrosomes in prophase cells [41]. Although centrosomes themselves are not required for some animal cells to enter mitosis [42,43], it is likely that they could facilitate the initial activation of Cyclin B–Cdk by raising the local concentration of Cyclin B–Cdk1 and its activator, Cdc25. Immunofluorescence and live-cell [44,45] analyses show that Cyclin B1 binds to microtubules and centrosomes before mitosis, but fluorescence recovery after photobleaching (FRAP) analysis shows that a Cyclin B1–green fluorescent protein (GFP) marker rapidly diffuses throughout the cell: the Cyclin B1 signal recovers to 100 per cent within 1 s of bleaching the centrosome (J. Richardson & J. Pines 2004, unpublished data). Some Cdc25 can also be observed at the centrosome [46–48]; therefore, in a manner similar to Turing-type symmetry breaking, it is likely that the auto-amplification of Cyclin B–Cdk1 and Cdc25 will first occur on centrosomes.

Centrosomes appear to have a similar role in facilitating mitosis in the *Caenorhabditis elegans* embryo where the pronucleus to which centrosomes are attached (normally the male pronucleus) is the first to accumulate active Cdk1 and to break down its nuclear envelope [49]. In this case, the Aurora A kinase has been implicated as the centrosome-associated factor that facilitates mitosis, which may relate to the role of the Aurora family kinase Ark1 in modulating the feedback loop at the fission yeast SPB [50].

Although centrosomes may act as nucleation sites for the activation of Cyclin B–Cdk1, simply activating Cyclin B–Cdk1 is not sufficient for mitosis: the PP2A–B55δ phosphatase must also be inhibited, at least in *Xenopus* [18,51]. This is achieved by the greatwall kinase that generates a specific inhibitor for PP2A–B55δ by phosphorylating either of two small molecules: α-endosulphine or Arpp19 [18,19]. Once phosphorylated, α-endosulphine and Arpp19 specifically bind to the B55δ regulatory subunit, but not to any of the other members of the family [18]. This is crucial because some of these other regulatory subunits are components of phosphatase holoenzymes that have other critical roles in mitosis. For example, the PP2A–B55α subunit is recruited to centromeres by the Shugoshin family of proteins [52,53], where in mitotic cells it is required to maintain sister chromatid cohesion at centromeres until anaphase by antagonising the Plk1 kinase [54,55].

7. LOCAL DECISIONS AT KINETOCHORES: ERROR CORRECTION

Once cells have entered mitosis, the key event is the capture of microtubules by specialized structures on the chromosomes called kinetochores to enable the sister chromatids to be segregated at anaphase. This is a stochastic process because individual kinetochores will capture microtubules at different times and any improper attachments must subsequently be corrected; therefore, even one unattached kinetochore (and there are 92 kinetochores in a normal human cell) must be able to prevent the cell from initiating anaphase [56]. Consequently, a number of different processes are integrated at the kinetochore: microtubule attachment, error correction and the spindle assembly checkpoint (SAC) that prevents the proteolysis of anaphase inhibitors. Some of the clearest evidence for local decision-making is provided by signalling at the kinetochore where the key regulators are the Aurora B kinase and its antagonistic PP1 phosphatase.

Regulation by Aurora B and PP1 at kinetochores requires spatial control of their activity to set up a local gradient of phosphorylation activity, and a feedback loop between them to sharpen the gradient. Aurora B is one component of the chromosomal passenger complex (CPC) whose other members are survivin, borealin and inner centromere protein (INCENP) [57,58]. Borealin is required to load the CPC onto chromatin [59] and Aurora B has to bind to INCENP to be active [60,61]. In turn, INCENP is recruited to centromeres by binding to survivin, which docks via its BIR (*b*aculovirus *i*nhibitor of apoptosis protein *r*epeat) domain to threonine 3 phosphorylated histone H3, mediated by the haspin kinase that is restricted to the centromere [62–64]. Aurora B activity is further spatially restricted because INCENP has to be phosphorylated on its C-terminus in *trans* by other Aurora B kinases [59]; therefore, with another nod to Turing, Aurora B only gains full activity when CPCs are clustered. Opposing Aurora B is the PP1γ phosphatase that is recruited to the outer kinetochore by the kinetochore-null 1 (KNL1) protein [65]. This sets up a gradient of phosphorylation activity between the centromere and the kinetochore. This is sharpened by a feedback loop between Aurora B and PP1 because Aurora B phosphorylates PP1 and prevents its binding to KNL1 [65,66]. As a result, the closer the kinetochore is to the centromere, the higher the effective activity of Aurora B. This has led to the following model for microtubule capture and error correction, although it should be noted that the recruitment of PP1 to KNL1 may have an equally, or more, important role in the SAC [66] (see below). When a microtubule is first captured by the kinetochore, it enters a region of high Aurora B activity. Improper attachments will be unable to push the kinetochore away from the centromere [67,68] and consequently be destabilized by Aurora B activity. In contrast, correct, end-on attachments will push the kinetochore away from the centromere, increasing the recruitment of PP1γ and generating a low Aurora B activity region, which stabilizes the attachment.

Although a number of elements of this model remain untested, elegant studies using a Förster resonance energy transfer (FRET) biosensor for Aurora B activity support its validity [69]. The biosensor consists of an Aurora B phosphorylation site and a phospho-binding forkhead-associated (FHA) domain that link together two fluorescent proteins (the FRET pair). When the FHA domain binds to the phosphorylated linker site, this promotes a conformational change and alters the FRET signal from the FRET pair [70]. Thus, the biosensor reads out the balance between Aurora B activity and its opposing phosphatase. These studies showed

that Aurora B activity at the centromere remains high after a microtubule attaches because there was no change in biosensor activity if it was targeted to the centromere [69]. In contrast, the signal from a biosensor targeted to the kinetochores decreased on kinetochores to which microtubules had correctly attached [69]. Thus, microtubule attachment does not change Aurora B activity *per se*; it alters the balance between Aurora B and PP1γ at the kinetochore.

8. LOCAL AND GLOBAL DECISIONS AT KINETOCHORES: THE SPINDLE ASSEMBLY CHECKPOINT

To prevent chromosome loss or mis-segregation, any unattached kinetochores must prevent both the separation of all the sister chromatids and exit from mitosis. This is achieved by the SAC. The target of the SAC is the Cdc20 protein that binds to the anaphase promoting complex/cyclosome (APC/C) and allows it to recognize the securin and Cyclin B proteins to target them for ubiquitin-mediated proteolysis. Recent structural [71,72] and biochemical [73,74] studies support the idea that Cdc20 binds to the APC/C to form—with the APC10 subunit—a bi-partite receptor for securin and Cyclin B. The SAC is mediated by several proteins (the Mad and Bub proteins and the Mps1 kinase) that accumulate on unattached kinetochores [75,76], as does Cdc20 [77] and the APC/C [78]. It is unclear exactly how the SAC proteins target Cdc20 but the most persuasive theory is the Mad2 template model [79]. There are two conformations of Mad2: open and closed [80,81]. It is thought that the closed conformation can catalyse the binding of the open form to Cdc20, and once bound the open form is converted to the closed form. Thus, a population of closed Mad2 stably bound to unattached kinetochores through the Mad1 protein continually promotes the binding of open Mad2 to Cdc20 by a mechanism that requires the Mps1 kinase [82]. The Mad2–Cdc20 complex subsequently binds to the Mad3/BubR1–Bub3 complex [83,84], which prevents Cdc20 forming the substrate receptor on the APC/C. Once a kinetochore binds microtubules, the Mad1–Mad2 complex is removed by dynein-mediated transport along the microtubules [85], and the Mps1 kinase also leaves, both of which are required to silence the SAC. If Mps1 is artificially targeted to kinetochores the Mad1–Mad2 complex is continually recruited to the kinetochore, or if Mad1 is artificially tethered to the kinetochore, the SAC continually inactivates Cdc20 regardless of microtubule attachment [86,87].

The SAC is not simply coupled to microtubule attachment by dynein-mediated removal of the Mad1–Mad2 complex. Evidence from studies on budding and fission yeast [66,88–91], and recently on human cells [92], indicates that Aurora B has an important role in the SAC, and that this role again depends on the antagonism between Aurora B and PP1. More specifically, the recruitment of PP1γ to KNL1 (or Spc7 in fission yeast) at kinetochores is required to silence the SAC [66,90,93]. Moreover, as Aurora B prevents PP1γ binding to kinetochores [65], this means that the SAC can only be silenced when kinetochores are pushed away from the centromeres by microtubules, thereby coupling the SAC to error correction. It is unclear, however, how many microtubules must bind to animal cell kinetochores to satisfy the SAC. For example, one vertebrate kinetochore binds between approximately 10 and 50 microtubules depending on the species and stage of mitosis [94,95] but whether each microtubule binds to a site that acts as a SAC signalling centre, and how SAC signalling is coordinated over the kinetochore as a whole remain obscure. The recently identified Ska complex that has been implicated in coordinating microtubule attachment may provide insights into this question [96–99].

9. HOW CAN A LOCAL SIGNAL COORDINATE THE CELL AS A WHOLE?

Each kinetochore acts as a signalling centre for the SAC and responds to microtubule attachment and error correction, but we are left with the question of how this local signal can coordinate anaphase in the cell as a whole. One unattached kinetochore must be able to prevent any APC/C complex recognizing securin or Cyclin B. One theory is that the Mad2–Cdc20 complex can catalyse the binding of further open Mad2 molecules to Cdc20 in the cytoplasm [79], thereby amplifying the SAC signal to sequester all the Cdc20. But unless the Mad2–Cdc20 complex is very short-lived, this putative positive feedback loop is not easily reconciled with the rapid silencing of the SAC: live-cell observations show that the APC/C is activated within a couple of minutes of the last kinetochore attaching to a microtubule [100]. Moreover, size exclusion chromatography shows that there is a large fraction of Cdc20 that is not bound to Mad2 or any other checkpoint proteins even in nocodazole-treated cells where all the kinetochores are unattached [83].

As yet, the amplification of the SAC is an important, unresolved question but there are some straws in the wind that implicate some form of spatial control. The first of these is the intriguing observation that after fusing two cells to generate a cell with two spindles, the unattached kinetochores in one spindle are unable to prevent anaphase in the adjacent spindles if it is separated by more than 20 μm [101]. This tends to preclude the idea that there is cytoplasmic amplification of the SAC signal, and may indicate that the region of the spindle represents a specialized zone for both the SAC and the APC/C. Potential support for this hypothesis is that live-cell assays of Cyclin B–GFP proteolysis show that its fluorescence first declines on the centrosomes and the chromosomes, although the rapid diffusion of Cyclin B–GFP does not allow one to conclude that these are the only sites where it is degraded [100,102].

Should this specialized zone exist, there are a number of mechanisms by which it could be created. For the SAC, one obvious candidate is the gradient set up by Aurora B, KNL1 and PP1γ. Similarly, there could be a gradient of Mps1 activity generated by the targeting of Mps1 to kinetochores and an opposing phosphatase. Gradients of the other two checkpoint kinases, Bub1 and BubR1, are also possible, although

whether the kinase domain of BubR1 is required for the SAC is currently controversial [103]. A phosphorylation gradient could also regulate APC/C activity; a number of protein kinases can phosphorylate the APC/C, of which Cyclin B–Cdk1 is the most well characterized [104,105]. Although Cyclin B–Cdk diffuses rapidly around the mitotic cell, it does concentrate on specific structures, including the kinetochores, the centrosomes and the spindle microtubules [45,106]. Indeed, our unpublished data (M. Jackman & J. Pines 2000) support the idea that the kinetochore population of Cyclin B–Cdk activity is crucial to maintaining the SAC [107]. To generate a gradient of activity would require an opposing phosphatase, preferably one that is itself negatively regulated by Cyclin B–Cdk activity. This makes PP1 an obvious candidate because the catalytic subunit is repressed when phosphorylated by Cdk [108].

Alternatively, it has already been demonstrated that the Ran GTPase can create a specialized zone around the chromatin in *Xenopus* extracts. As with Cdc42-symmetry breaking, the GEF for Ran GTPase (RCC1) clusters on chromatin to create a Ran gradient that peaks on the chromosomes [109,110]. The GTP-bound form of Ran promotes the dissociation of the importin proteins from their cargoes; therefore, these complexes dissociate around chromosomes [111]. Some of these complexes contain proteins that are important for microtubule nucleation, whose activity is shielded by binding to an importin, and are, therefore, most active nearest to chromosomes [112]. Should any of the key SAC or APC/C components also be shielded by binding to importins, this mechanism could also account for their spatially limited activity. There are, however, caveats to extending this model to other systems; for example, the Ran GAP that antagonizes the GEF is bound to kinetochores in human cells [113], which would tend to disrupt the gradient. Thus, it is possible that the Ran gradient is primarily important in very large cells, such as oocytes.

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Finally, the local recruitment of protein phosphatases is essential to return the cell to the interphase state. In budding yeast, the Cdc14 phosphatase is particularly important, and its localization is controlled by the Cdc14 early anaphase release (FEAR) and mitotic exit network (MEN) pathways that monitor spindle position in the bud [114]. In animal cells, the PP1 phosphatase is recruited to a number of different sites at different times by distinct targeting subunits. For example, the RepoMan protein recruits PP1 to chromosomes in anaphase and this is important for chromosome decondensation [115]. Many more examples are likely to emerge in the near future as the role of phosphatases in cell cycle control continues its recent Renaissance.

11. FUTURE CHALLENGES

The local control of cellular events poses a number of significant challenges for future research. In order to understand how decisions are made, we must be able to assay the local balance of the principal post-translational regulation pathways: kinases and phosphatases, ubiquitin ligases and deubiquitylases, GTP-exchange factors and GTPase-activating proteins, to name but three. This means that the increasing power and sophistication of biochemical analyses, exemplified by the advances in protein analysis by mass spectrometry, cannot be taken in isolation. These analyses tend to average out the changes in the protein levels and modifications within the cell and within the population of cells; therefore, we must develop tools and methods that allow us to assay pathways in specific cells, and in specific locations within the cells.

Until now, subcellular analyses have been largely qualitative, relying on intrinsically nonlinear assays such as immunofluorescence. Therefore, we suggest that progress in cell biology requires quantitative assays: it is important to measure both molecule numbers and activity *in situ*, which is becoming possible through tagging at the genomic locus with fluorescent proteins and the development of biosensors. These measures will have to be combined with the measurement of protein dynamics by FRAP and related techniques to measure the dwell times and the flux of proteins at specific locations. The advent of super-resolution microscopy will be crucial to define the gradients of protein levels and activity with sufficient precision to generate meaningful data for mathematical models. These imaging techniques will in turn need to be combined with quantitative mass spectroscopy, first to obtain absolute numbers of molecules of specific proteins per cells, and then to determine the stoichiometries with which they are post-translationally modified. These data can then reveal the number of molecules in specific places at specific times and be used to generate predictive models.

Ultimately, *in vitro* reconstitution of critical interactions with recombinant proteins will be required to understand molecules composed of functionally antagonistic domains. Determining the specific post-translational modifications that regulate a particular association *in vitro* should provide crucial clues to decipher otherwise confusing or contradictory observations *in vivo*. Such correlative assessments may prove particularly powerful in understanding how structural molecules such as centrosome components act as scaffolds to coordinate localized activities in response to multiple convergent signalling pathways, or the means by which the structural components of the kinetochore respond to microtuble association to trigger signalling pathways.

More challenging will be to measure protein–protein interactions in living cells to obtain dissociation constants and to assay the composition and half-life of specific complexes, which are necessary to determine the kinetics of specific reactions and pathways *in vivo*. Some progress has already been made using techniques such as FRET and fluorescence cross correlation spectroscopy, but these remain specialized techniques and limited in their practical applications. Crucially, we will need to be able to measure the flux of molecules through pathways. This will require pulse labelling a subset of molecules, for example by photo-activation/photo-conversion or SNAP-tagging [116],

and determining their half-lives, post-translational modifications and protein interactions over very short periods of time.

Meeting these challenges will require advances on many different fronts but an increased emphasis on quantitative assays is, we believe, essential to understand the democratic decision-making in the cell.

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