Regulating the onset of mitosis

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In eukaryotes, G_2/M progression is mediated by activation of mitosis promoting factor (MPF). To ensure faithful chromosome segregation, the activity of key mitotic inducers and inhibitors are coupled with chromosome replication, spindle pole duplication, morphogenesis, and DNA damage. Evidence gathered in the past two years has underscored the importance of positioning MPF and its regulators in the proper place at the proper time to ensure orderly progression through the G_2/M transition. Altering the spatial organization of G_2/M regulators also contributes to prevention of mitosis following DNA damage.

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Abbreviations

CAK	Cdk-activating kinase
CDK	cyclin-dependent kinase
CRS	cytoplasmic retention signal
MPF	mitosis promoting factor
NES	nuclear export signal
SPB	spindle pole body

Introduction

The initiation of mitosis in eukaryotic cells is governed by a spatially and temporally complex phosphorylation cascade which culminates in the activation of mitosis promoting factor (MPF). MPF consists minimally of the cyclin dependent kinase Cdc2 in yeast (Cdk1 in higher eukaryotes) and a B-type cyclin regulatory subunit. A primary challenge in revealing the mechanics of G_2/M regulation has been to identify the proteins that antagonize and promote MPF activation. During the past eight years, this field has been reviewed extensively owing to the significant progress that has been made [1–3].

As first demonstrated in the fission yeast *Schizosaccharomyces pombe*, MPF activation centers on the phosphorylation state of the Tyr15 residue of Cdc2. During interphase, MPF is kept inactive through Tyr15 phosphorylation of Cdc2 and, upon entry into mitosis, MPF is activated by dephosphorylation of this residue. The onset of mitosis is triggered by simultaneous activation of the Tyr15 phosphatase Cdc25 and inactivation of the Tyr15 kinase Wee1. As Cdc2–cyclin B is capable of phosphorylating and thereby activating and repressing the activity of Cdc25 and Wee1, respectively, it is thought that Cdc2 activation depends in part on a positive-feedback loop. Proteins other than MPF that regulate Cdc25 and Wee1 activity have also been identified. In *S. pombe*, the nim1p (cdr1p) kinase phosphorylates and inactivates wee1p; furthermore, *Xenopus* Plx, which is orthologous to *Drosophila* Polo kinase, has been identified as an activator of Cdc25C [4]. Cdc25C is one of three Cdc25 isoforms present in mammalian cells and in combination with Cdc25B promotes activation of MPF. In vertebrate cells, phosphorylation by Myt1 of the Cdc2 Thr14 residue, which is adjacent to the Tyr15 residue, also contributes to negative regulation of MPF. Phosphorylation of a conserved threonine residue that is localized in the activation-loop of Cdc2 molecules (Thr167 in *S. pombe* and Thr161 in higher eukaryotes) contributes positively to the regulation of Cdc2–cyclin B. This event is catalyzed by the Cdk-activating kinase (CAK) [5].

Eukaryotic cells tightly couple MPF activation with a number of cell-cycle-dependent events, such as chromosome replication and spindle pole body (SPB)/centrosome duplication. In addition, MPF activity is repressed by extracellular cues or insults that threaten faithful chromosome transmission, such as changes in osmolarity or agents that cause DNA damage. The mechanisms that lead to inactivation of Cdc2–cyclin B following DNA damage have been reviewed recently [6,7]. With the exception of the budding yeast *Saccharomyces cerevisiae*, G_2 delay in response to DNA damage is known to require repression of Cdc25 activity and thus maintenance of Cdc2 Tyr15 phosphorylation. This occurs through phosphorylation of Cdc25 on a conserved serine residue (Ser216 in human Cdc25C) by the protein kinases chk1p and possibly cds1p in fission yeast [8], and the orthologous Chk1 and Chk2(Cds1) proteins in vertebrate systems [9,10]. Subsequently, 14–3–3 proteins bind to and facilitate inactivation of Cdc25. 14-3-3 proteins comprise a family of phosphoserine binding proteins which plays roles in diverse cellular processes including cell cycle control. Whether Wee1 activity is positively regulated following DNA damage to ensure maintenance of Tyr15 phosphorylation has not been reported.

Although we now understand many of the biochemical interactions that influence MPF activation, how and when they occur within the space of the cell are ill defined. To understand fully the complexity of MPF regulation and specificity of signal transmission, it will be necessary to reveal the mechanisms that deliver MPF and its regulators to the proper place at the proper time. These mechanisms may in turn be regulated in response to cell cycle perturbations. In this review, we cover recent progress regarding G_2/M regulation with an emphasis (where possible) on how spatial and temporal organization of MPF and its regulators contribute to mitotic entry both under normal cellular conditions and under conditions of cellular insult.

B-type cyclins

Location of B-type cyclin isoforms

In vertebrate cells, the cellular location of Cdc2–cyclin B complexes is determined by the cyclin B variant to which

Table 1

Localization of mammalian Cdc2 regulators.

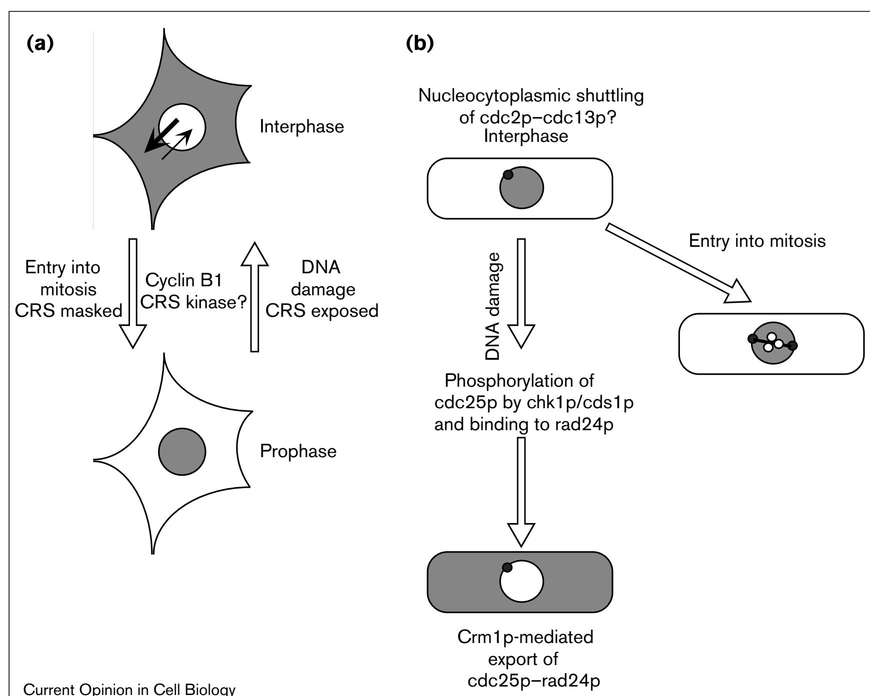
Name of Cdc2 regulator	Interphase location	Mitotic location	References
Cyclin B1	Cytoplasmic	Nuclear	[11–14]
Cyclin B2	Golgi	Golgi	[14]
Cyclin B3	Nuclear	Nuclear	[15]
Cdc25C	Cytoplasmic	Nuclear	[13]
Cdc25B	Cytoplasmic	Cytoplasmic	[21]
Wee1	Nuclear	Cytoplasmic localized to the midbody during cytokinesis	[13,64]
Myt1	ER/Golgi	ER/Golgi	[20•]
CAK	Nuclear	Throughout cell body	[65]

ER, endoplasmic reticulum.

Cdc2 is bound (Table 1). Cdc2–cyclin B1 complexes colocalize with cytoplasmic microtubules during interphase, and abruptly translocate to the nucleus upon entry into mitosis [11–14]. Cdc2–cyclin B2 complexes colocalize with the Golgi apparatus [14], and cyclin B3 — which shares properties with both A- and B-type cyclins — is constitutively nuclear throughout the cell cycle [15]. In *Drosophila*, the localization patterns of Cyclins B and B3 are similar to those of vertebrate cyclin B1 and B3, respectively [16,17]. The contribution of each class of MPF complex to mitotic progression is not understood at this point. Analyses of mice that are nullizygous for either cyclin B1 or B2 have shown that cyclin B1, but not cyclin B2, is essential for viability and fertility [18]. This indicates that Cdc2–cyclin B2 is dispensable for mitotic progression or that Cdc2–cyclin B1 is capable of compensating for the loss of Cdc2–cyclin B2. It will be interesting to determine the consequence of deleting cyclin B3 in mice and to investigate potential functional redundancies with cyclin B1 and B2. In *Drosophila*, genetic studies have demonstrated that, surprisingly, neither Cyclin B nor B3 are essential for cell division; however, removal of both cyclins results in delayed entry into mitosis and formation of aberrant and functionally compromised spindles [17].

A potential reason for targeting Cdc2–cyclin B2 to the Golgi was revealed by the demonstration that Cdc2 is required for mitotic fragmentation of the Golgi apparatus [19••]. Cdc2 complexed with either cyclins B1 or B2 — but not Cdk2–cyclin A or Cdk2–cyclin E — is able to phosphorylate Ser25 of the *cis*-Golgi matrix protein GM130. This phosphorylation inhibits the binding of GM130 to the vesicle-docking protein p115, an interaction which antagonizes Golgi fragmentation. Interestingly, the Cdc2-Thr14 kinase, Myt-1 also localizes to the Golgi apparatus [20•], and Cdc25B accumulates in the cytoplasm (Table 1) [21]. These distribution patterns contrast with those of Wee1 and Cdc25C, which are thought to regulate Cdc2–cyclin B1 activity (Table 1); Wee1 is nuclear until the onset of

Figure 1



Nucleocytoplasmic shuttling of mammalian Cdc2–cyclin B1 and *S. pombe* cdc25p during unperturbed cell cycle progression and following response to DNA damage. **(a)** The cytoplasmic steady state localization of Cdc2–cyclin B1 (shaded area) during interphase is mediated through a higher rate of nuclear export (thick arrow) *versus* nuclear import (thin arrow). Export of cyclin B1 is mediated through CRM1 (exportin 1) whereas nuclear import is mediated through importin-β. Nuclear export of cyclin B1 requires an unphosphorylated CRS. Following entry into mitosis, Cdc2–cyclin B1 is retained in the nucleus as a result of phosphorylation and masking of the cyclin B1 CRS. In response to DNA damage, G_2/M progression is inhibited by preventing Cdc2–cyclin B1 from accumulating in the nucleus. The CRS kinase remains to be identified. **(b)** cdc25p (shaded area) accumulates in the nucleus during G_2 and remains there until anaphase. It is unknown if cdc25p also localizes to SPBs. Following DNA damage, cdc25p is phosphorylated by chk1p (and possibly cds1p). This phosphorylation allows rad24p to bind cdc25p and escort cdc25p from the nucleus into the cytoplasm. Export of cdc25p–rad24p from the nucleus requires crm1p. CRS, cytoplasmic retention signal.

mitosis, and Cdc25C is imported into the nucleus at the G_2/M transition [13]. Perhaps Myt1 and Cdc25B function to coordinate cytoplasmic mitotic events which are regulated through Cdc2–cyclin B2 with nuclear mitotic events, which are mediated through Cdc2–cyclin B1 (and possibly Cdc2–cyclin B3). The division of labor between the various MPF complexes may not be this straightforward, however, as cytoplasmic accumulation of Cdc25B has recently been implicated in triggering centrosomal microtubule nucleation in HeLa cells [21].

Nucleocytoplasmic shuttling of Cdc2–cyclin B1

Recent investigations of cyclin B1 localization in *Xenopus* and HeLa cells have demonstrated that it is more dynamic than previously appreciated; cyclin B1 shuttles continuously between the cytoplasm and nucleus during interphase (Figure 1) [22••–24••]. Import of Cdc2–cyclin B1 into the nucleus occurs through binding of cyclin B1 to importin-β — Cdc2 is dispensable for cyclin B1 nuclear import [25]. Prior to the G_2/M transition, Cdc2–cyclin B1 is maintained in the cytoplasm by the activity of the nuclear export factor CRM1 (also known as exportin 1). Recognition of cyclin B1 by CRM1 occurs through the previously identified cytoplasmic retention signal (CRS), which is required for the cytoplasmic localization of cyclin B1 [26]. Injection of CRS–ovalbumin or CRS–GST (glutathione *S* transferase) conjugates into the nuclei of HeLa cells or *Xenopus* oocytes, respectively, result in rapid export of ovalbumin and GST [23••,24••]. Thus, the cyclin B1 CRS is a nuclear export signal (NES) rather than a CRS.

Phosphorylation of four serine residues located within the CRS has been implicated in nuclear retention of Cdc2–cyclin B1 during the onset of mitosis [27••]. Supporting this notion is the observation that mutations encoding serine to glutamic acid substitutions within the cyclin B1 CRS result in impaired nuclear export of cyclin B1 as a result of lowering the affinity of the CRS for CRM1 [24••]. The kinase which phosphorylates cyclin B1 and regulates its nuclear retention remains to be identified. In addition, whether cyclins B2 and B3 are subject to regulation by nucleoplasmic shuttling remains to be determined. Notably, cyclin B2 does possess a functional CRS that is required for its cytoplasmic localization [26].

In *Aspergillus nidulans* and *S. pombe*, MPF is nuclear during interphase indicating that in these organisms prevention of premature MPF substrate phosphorylation does not occur by nuclear exclusion of MPF [30,31]. A recent study in *A. nidulans*, however, has indicated that nuclear retention of $\mathrm{NIMX^{CDC2}}$–$\mathrm{NIME^{cyclin\ B}}$ may be a dynamic process that requires the NIMA protein kinase; *nimA* mutations result in the mislocalization of MPF to the cytoplasm. Consistent with this notion, a mutation in *sonA*, which encodes a protein related to the *S. cerevisiae* and *S. pombe* nucleocytoplasmic transporters Gle2p and rae1p, respectively, suppresses the $\mathrm{NIMX^{CDC2}}$–$\mathrm{NIME^{cyclin\ B}}$ nuclear localization and nuclear division defects of *nimA* [29,30]. Whether NIMA regulates nuclear retention of MPF through phosphorylation and masking of a $\mathrm{NIME^{cyclin\ B}}$ NES, and whether this process requires *A. nidulans* CRM1, remains to be determined.

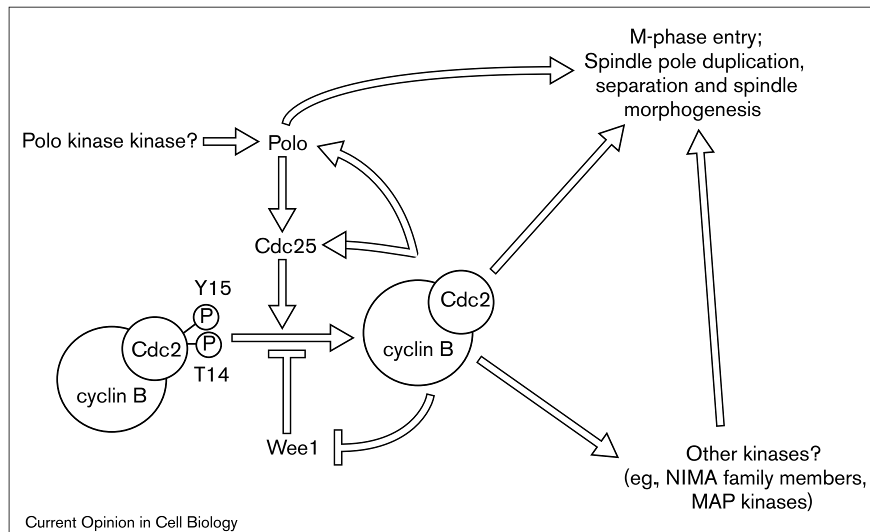
Polo kinases and Cdc2 at spindle poles

The *Drosophila* polo kinase and its orthologues in other eukaryotes are also important regulators of G_2/M transit, mitotic progression, cytokinesis, and exit from mitosis [4,33•]. Relevant to this discussion is the finding that *Xenopus* Plx is capable of binding, phosphorylating and thereby activating Cdc25C *in vitro*, suggesting a potential involvement in the Cdc2–cyclin B1 activation pathway (Figure 2) [32]. Such a role for Plx is supported by the observation that its depletion from *Xenopus* extracts abolishes or delays activation of Cdc25C. Interestingly, in *Xenopus* oocytes and extracts, Plx is activated by introduction of exogenous Cdc25C indicating the existence of a positive-feedback loop [34•,35]. Recently, a *Xenopus* kinase, xPlkk1, has been purified which phosphorylates and activates Plx indicating that a kinase cascade may be involved in regulating the mitotic activation of Plx [37].

At the G_2/M transition, Polo kinases have been localized to spindle pole bodies (SPBs) in *S. cerevisiae* and *S. pombe*, and centrosomes in *Drosophila* and vertebrate cells where they are thought to play an important role in spindle pole duplication and bipolar spindle formation [31]. A portion of Cdc2–cyclin B1 is also found at spindle poles [1]. An important function of MPF and Polo colocalization may be to facilitate their rapid co-activation in proximity to target proteins which participate in spindle pole duplication and separation. An exception to this notion is the *S. cerevisiae* Polo-like kinase Cdc5p. Cells with *cdc5* mutations do not show SPB duplication or spindle formation defects and are, instead, exclusively defective for promoting mitotic exit through degradation of the B-type cyclin Clb2 [36–38]. This difference is likely to be due to the fact that *S. cerevisiae* undergoes SPB duplication during S-phase.

A potential spindle pole target of cdc2p–cdc13p in *S. pombe* is the SPB component cut12p (stf1p) [39••,40]. The *cut12*$^+$(*stf1*$^+$) gene was identified in two independent screens. A loss-of-function allele was identified as a ‘cut’ (cell untimely torn) mutation, as cells with this mutation undergo septation and subsequent bisection of a single undivided nucleus as a result of failure to properly segregate chromosomal DNA. Although *cut12*$^-$ cells are capable of SPB duplication, they form monopolar spindles indicating that one SPB is defective for nucleating microtubules [39••]. A gain-of-function allele of the *cut12* gene was isolated as a suppressor of a *cdc25* mutation [41•]. The fact that an activating mutation in a spindle pole component suppresses a loss of cdc25p activity — and therefore probably bypasses the need for cdc2p activation during G_2/M — suggests that the major function of cdc2p–cdc13p during G_2/M transit is to promote bipolar spindle formation.

Figure 2



Involvement of Polo kinases in mitotic progression. Activation of Cdc2–cyclin B by Cdc25-mediated Thr14/Tyr15 dephosphorylation of Cdc2 is promoted by activation of Cdc25 by Polo. A recently identified Polo kinase kinase (xPlkk) in *Xenopus* suggests that a phosphorylation cascade may be involved in the activation of Polo. Cdc2–cyclin B and Polo kinases both localize to SPBs and centrosomes in all eukaryotic organisms examined where they are thought to participate in spindle pole duplication and bipolar spindle formation.

Coupling Tyr15 kinase activity to cell cycle progression

The morphogenesis checkpoint in *S. cerevisiae*

In *S. cerevisiae*, phosphorylation of Cdc28p Tyr19 (which is analogous to Cdc2 Tyr15) regulates the ability of cells to prevent mitosis when the actin cytoskeleton is perturbed [2,41•,42]. This prevents nuclear division from occurring until the daughter bud is large enough to accommodate one of the two separating nuclei. One mechanism that enforces a G_2/M arrest upon activation of this 'morphogenesis' checkpoint is stabilization of the Cdc28p Tyr19 kinase Swe1. In an unperturbed cell cycle, Swe1p abundance oscillates in a cell-cycle-dependent manner reaching maximal levels during G_2/M. Swe1p degradation appears to require phosphorylation by Cdc28p suggesting that a positive-feedback loop for Cdc28–Clb complex activation also exists in *S. cerevisiae* [43••]. The degradation of Swe1p occurs through ubiquitin-dependent proteolysis. Ubiquitination of Swe1p occurs through the concerted activities of the E2 Cdc34p and the E3 ligase complex termed SCF containing the F-box protein Met30p as the substrate-specificity determining subunit [44•].

Although changes in actin dynamics are likely to lie at the heart of the morphogenesis checkpoint [41•], the precise molecular signals that lead to activation of this checkpoint are not defined. Interestingly, an absence of septin function is also associated with Swe1p activation [45••]; the inability of cells to form a septin ring results in a failure to activate MPF due to Tyr19 phosphorylation of Cdc28p by Swe1p. Swe1p-dependent cell-cycle arrest in the absence of septin function is the result of inactivation of the redundant Swe1p inhibitory kinases Hsl1p, Kcc4p, and Gin4p. All three kinases are related to *S. pombe* cdr2p [46,47] and nim1p(cdr1p), which phosphorylates and inactivates wee1p [1–3]. Hsl1p, Kcc4p, and Gin4p all localize to the septin ring and require septin function for activity [45••,48•,49•]. These observations raise the possibility that the morphogenesis checkpoint may sense septin defects rather than disruption of the actin cytoskeleton. Of course, this model requires that septin function is dependent on that of actin. Alternatively, the morphogenesis and septin checkpoints may be distinct, and have only Swe1p in common as the factor which delays mitotic progression. Further studies are required to distinguish between these possibilities.

DNA replication

In *Xenopus* extracts, ubiquitin-mediated proteolysis of Wee1 was found to be required for timely cell cycle progression [50•]. Wee1 proteolysis requires the *Xenopus* orthologue of *S. cerevisiae* Cdc34p indicating that Wee1 degradation occurs in an evolutionarily conserved manner. Importantly, Wee1 proteolysis is prevented by treatment of extracts with the DNA replication inhibitor aphidicolin suggesting that regulation of Wee1 proteolysis is one mechanism that ensures completion of S-phase (and DNA synthesis) prior to triggering the onset of mitosis.

In *S. pombe*, inhibition of DNA replication results in activation of the chk1p and cds1p kinases [51,52], which are also required for the DNA damage checkpoint (see above). The inhibitory phosphorylation of cdc25p by cds1p and chk1p is a conserved feature of the DNA-damage-checkpoint and replication-checkpoint pathways. Two novel mechanisms utilized in the DNA replication checkpoint pathway to maintain Tyr15 phosphorylation of cdc2p appear to be phosphorylation of wee1p and accumulation of the redundant Tyr15 kinase mik1p — both mechanisms are mediated through cds1p [52]. As this study used GST fusions with amino-terminal fragments of wee1p lacking the catalytic domain to assay wee1p phosphorylation it remains to be seen whether the observed phosphorylation does modulate

wee1p activity [52]. Implicit in the observation that mik1p accumulates following activation of the DNA replication checkpoint is that mik1p has an important role in this checkpoint pathway. Consistent with this notion, cells lacking *mik1+* are hypersensitive to the DNA replication inhibitor hydroxyurea [52]. These findings are significant as mik1p was previously thought to play a relatively minor role in the regulation of Tyr15 phosphorylation of cdc2p.

Nucleocytoplasmic shuttling of mitosis promoting factor and its regulators in response to DNA damage

Regulating Cdc25 localization

Analysis of the DNA damage response in *S. pombe*, mammalian cells, *Xenopus* and *Aspergillus* have demonstrated that the G_2 delay in response to DNA damage involves repression of MPF activity through maintenance of Cdc2 Tyr15 phosphorylation. This requires phosphorylation of Cdc25C on Ser216 by the Chk1 protein kinase and subsequent binding of 14–3–3 proteins [6,7]. Studies in the past year have indicated that the related human Chk2(Cds1) kinase [9,10] cooperates with Chk1 to regulate Cdc25 phosphorylation. Human Chk1 and Chk2(Cds1) are nuclear proteins that display a punctate staining pattern as determined by indirect immunofluorescence of endogenous proteins [10,53].

The binding of 14–3–3 proteins to Cdc25 does not affect its catalytic activity indicating that prevention of Tyr15 dephosphorylation of Cdc2 occurs through another mechanism [54•,55•]. In *S. pombe*, this mechanism involves physical separation of cdc25p from cdc2p–cdc13p (Figure 1). In wild-type cells, cdc25p is synthesized during G_2 [56] and a subpopulation of the protein accumulates in the nucleus [57••]. Upon treatment with ionizing radiation, cdc25p is exported from the nucleus in a rad24p- (a 14–3–3 protein in *S. pombe* [58]) and crm1p-dependent manner. Although *S. pombe* cdc25p lacks an obvious NES, rad24p does have one which is essential for it to escort cdc25p to the cytoplasm following DNA damage [57••].

Regulating MPF localization

Although it is not yet known whether DNA damage regulates nucleocytoplasmic shuttling of Cdc25C in mammalian cells, nuclear export of Cdc2–cyclin B1 has recently been shown to play an important role in achieving G_2 arrest following DNA damage (Figure 1) [23••,59••]. Previous reports have shown that phosphorylation of Thr14 and Tyr15 of Cdc2 plays an important role in the G_2 delay observed following DNA damage: HeLa cells expressing Cdc2AF (Thr14→Ala, Tyr15→Phe) display a partial bypass of DNA damage-induced G_2 delay [60,61]. HeLa cells expressing a constitutively nuclear variant of cyclin B1 also partially override DNA damage-induced G_2 delay indicating that nuclear exclusion of Cdc2–cyclin B1 is a second mechanism which prevents entry into mitosis in cells carrying damaged DNA. Strikingly, expression of both Cdc2AF and constitutively nuclear cyclin B1 in HeLa cells completely abolishes radiation-induced G_2 arrest [59••]. These results suggest that maintenance of Thr14 and Tyr15 phosphorylation of Cdc2 and nuclear export of Cdc2–cyclin B1 are the two primary mechanisms through which eukaryotic cells achieve a G_2 arrest following DNA damage. Consistent with this notion, cells treated with both etoposide and caffeine override a G_2 arrest either in the presence of the CRM1 inhibitor leptomycin B or a cyclin B1 mutant which contains valine to alanine substitutions within the CRS [23••].

Conclusions

It is an exciting time to be studying the mechanisms that govern the G_2/M transition in eukaryotic cells. Although it is clear that G_2/M transit centers on Cdc2–cyclin B activation, there is evidence that entry into mitosis can occur in the absence of detectable MPF activation [62,63]. In these exceptional cases, it is possible that Cdc2–cyclin B is localized properly during the G_2/M transition, and that it has sufficient kinase activity to phosphorylate and activate downstream effectors (e.g. Polo- or NIMA-like kinases). Indeed, if downstream mitotic kinases utilize a positive- feedback loop similar to that of Cdc2, very little Cdc2 kinase activity may be required to promote the onset of mitosis. These observations underscore the importance of determining the mechanisms that regulate localization of MPF and its regulators during the cell cycle. Without accomplishing this, it will be difficult to accurately predict how mitotic progression occurs and how it is coupled to other cell-cycle-dependent events. Thus, as we learn the identity of more molecules comprising the G_2/M regulatory circuit and reveal their localization patterns throughout the cell cycle, we will see even more progress towards understanding how entry into mitosis is controlled.

Acknowledgement

We apologize to those colleagues whose work we have not cited due to space constraints.

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