

## p38 and Chk1 kinases: different conductors for the G<sub>2</sub>/M checkpoint symphony

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The mechanism controlling G<sub>2</sub>/M checkpoint activation after DNA damage was thought to be mediated primarily by nuclear Chk1/Chk2 kinases. Recent evidence indicates that this checkpoint is more complex, involving at least two different biochemical systems that target the Cdc25B and Cdc25C phosphatases. Following genotoxic stress, different kinases integrate signaling from the damaged DNA and other damaged cellular components to regulate Cdc25 inactivation. Our current model for G<sub>2</sub>/M checkpoint activation after genotoxic stress is discussed emphasizing the roles for Chk1 and p38 kinases in checkpoint regulation.

### Addresses

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### Abbreviations

IR ionizing radiation

MAPK mitogen-activated protein kinase

### Introduction

For protection from a variety of different types of stress, eukaryotic cells have developed a system of checkpoints that delay progression to the next phase of the cell cycle. A critical evolutionarily-conserved checkpoint exists at the boundary between the G<sub>2</sub> phase of the cell cycle and mitosis. Considering the wide variety of agents that can trigger a G<sub>2</sub>/M checkpoint, its regulation is complex and many of its components are still being defined. In addition, there is significant redundancy in the control of this checkpoint allowing for responses to a broad spectrum of genotoxic and non-genotoxic stresses.

All checkpoint mechanisms can be classified into two general groups: transcription dependent and transcription independent. The p53 tumor-suppressor protein is a major transcription factor that is involved in the regulation of G<sub>2</sub>/M checkpoint activation after stress by suppressing (cyclin B1, Cdc2 or Cdc25C) or transactivating (Gadd45a, 14-3-3σ) specific genes (more details in review [1]). In addition, p53-independent transcriptional mechanisms also suppress expression of key cell-cycle genes such as cyclin B1 [2]. Transcription-dependent control generally requires several hours for full effectiveness and can contribute to the maintenance of a G<sub>2</sub> delay after stress. Activation of the checkpoint is a very rapid event, however, highlighting the dominant role of transcription-independent mechanisms in checkpoint regulation.

During normal cell-cycle progression, initiation of mitosis is triggered by a complex process of activation of the cyclin-dependent protein kinase Cdc2. Prior to mitosis, the Cdc2–cyclin B1 complex is held in the cytoplasm in an inactive state by Cdc2 phosphorylation at Thr14 and Tyr 15. Transcription-independent regulation of the G<sub>2</sub>/M checkpoint involves several stress-inducible signal-transduction pathways regulating Cdc2 phosphorylation at inhibitory sites after stress. Dephosphorylation of these sites in mammals is regulated by two Cdc25 phosphatases, Cdc25B and Cdc25C. Different kinases are required to regulate the activity of Cdc25B and Cdc25C phosphatases after DNA damage [3\*,4\*]. Along with the previously characterized functions of the Chk1 kinase, we discuss the role of the p38 kinase in regulation of G<sub>2</sub>/M checkpoint activation.

### Mechanism for G<sub>2</sub>/M checkpoint control after genotoxic stress

Cdc2 kinase is the major regulator or ‘engine’ that drives the G<sub>2</sub>→M transition. Cdc2 is phosphorylated at inhibitory sites during the G<sub>2</sub> phase of the cell cycle, and previous studies have shown that a G<sub>2</sub> arrest caused by DNA-damaging agents involves ‘stabilization’ of these phosphorylation sites in yeast and *Aspergillus* [5,6]. In mammals, conditional expression of the inhibitory sites mutant, Cdc2AF, in HeLa cells reduces but does not completely abolish a G<sub>2</sub> delay after DNA damage [7]. These data suggest that mechanisms in addition to Cdc2 phosphorylation can delay mitosis following stress. Some reports raised the possibility that DNA-damage signaling acts in part by stabilizing the cytoplasmic localization of cyclin B1, and constitutive nuclear targeting of cyclin B1 was indeed shown to cause a reduction in damage-induced G<sub>2</sub> delay [8,9]. However, it is still unclear whether the nuclear import of a cyclinB/Cdc2 complex in human cells follows and is either dependent or independent of Cdc2 activation. It is possible that nuclear translocation is simply a consequence of Cdc2 activation, and is not a separately regulated process. In the case of DNA damage, the prediction would be that if there is a mechanism to trap cyclin B in the cytoplasm, inclusion of the nuclear export inhibitor leptomycin B, would not cause a cyclin B re-distribution to the nucleus in the presence of DNA damage. However, leptomycin B was able to sequester cyclin B in the nucleus regardless of DNA damage (DV Bulavin, AJ Fornace Jr, unpublished data), arguing against the presence of a mechanism regulating cytoplasmic retention of cyclin B after stress.

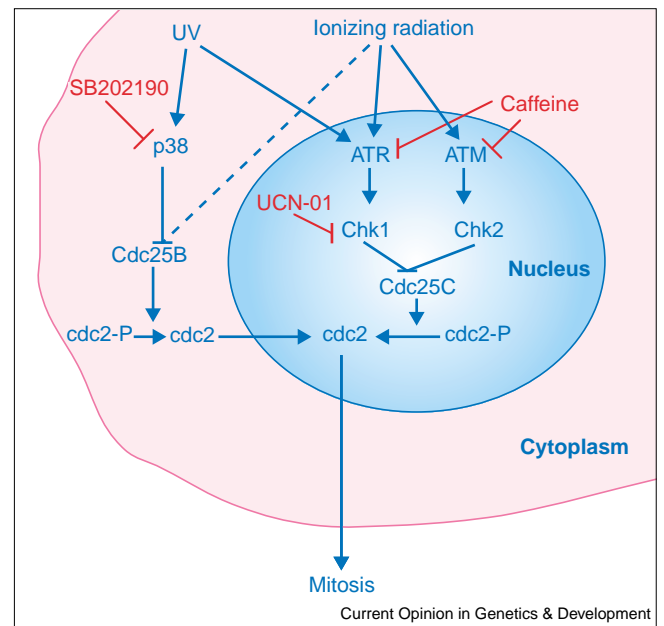
Although an accumulating body of evidence supports the idea of several stress-inducible pathways regulating G<sub>2</sub> delay after stress, the caffeine-sensitive cascade is a major

one operating through the nuclear ATM/ATR kinases [10,11] (Figure 1). Both enzymes can be activated by genotoxic stress transducing the signal(s) to the downstream kinases Chk1 and Cds1/Chk2. Analysis of 'knockout' cells — where either the Chk1 or the Chk2 gene was disrupted by gene targeting — further supports a central role for these genes in the regulation of G<sub>2</sub>/M checkpoint after DNA damage [12\*,13\*,14]. One of the most feasible targets in this cascade is Cdc25C phosphatase that can be phosphorylated at its 14-3-3 binding site, serine 216, by both the Chk1 and Chk2 kinases *in vitro* [15,16]. Although *in vivo* evidence for the role of Chk2 in phosphorylation of Cdc25C phosphatase is still lacking, recent analysis of Cdc25C serine 216 phosphorylation after ionizing radiation (IR) showed its complete ablation in the presence of the chemical compound UCN-01 [4\*]. UCN-01 inhibits several kinases *in vitro*, including Chk1, Chk2, cTak1 and p38  $\beta$  kinase [4\*,17] (DV Bulavin, AJ Fornace Jr, unpublished data) but the physiological effect of this drug can be seen at doses effective only for Chk1 inhibition, implicating Chk1 kinase as a major regulator for phosphorylation of serine 216 of Cdc25C after IR [4\*].

Recent evidence indicates the existence of another, caffeine-insensitive pathway regulating G<sub>2</sub> delay after stress that involves the p38 kinases [3\*,18\*]. The p38 portion of the mitogen-activated protein kinase (MAPK) system consists of four distinct kinases: the  $\alpha$  and  $\beta$  isoforms are expressed ubiquitously, whereas the  $\gamma$  and  $\delta$  show limited tissue-specific expression. In the case of p38 $\gamma$ , one report indicates its activation after IR could be dependent on ATM [19]. However, the p38 kinases show robust induction by stresses such as UV radiation, whereas their induction by IR is infrequent [20]. The commonly expressed forms of p38 are rapidly activated by UV radiation and can phosphorylate a variety of cellular targets including important regulatory sites in Cdc25B [3\*] as well as p53 [21,22\*]. The importance of Cdc25B in initiating G<sub>2</sub>/M transit is exemplified by the premature onset of mitosis that occurs with Cdc25B but not Cdc25C over-expression [23]. As with Cdc25B, p38 is localized to the cytoplasm which would facilitate rapid phosphorylation of Cdc25B after p38 activation. *In vivo*, the specific p38 kinase inhibitor, SB202190, significantly reduced serine 309 phosphorylation of Cdc25B, decreased its interaction with 14-3-3 proteins and abrogated the rapid initiation of G<sub>2</sub>/M checkpoint after UV irradiation [3\*].

Besides caffeine sensitivity, there is at least one more characteristic that can be used to classify different pathways: the role in initiation or in maintenance of a G<sub>2</sub> checkpoint. This distinction is supported by studies with inhibitors for p38, ATM/ATR, and Chk1 after UV radiation. As shown in Figure 2a, UV radiation triggered rapid checkpoint activation and prevented G<sub>2</sub> phase cells from progressing to mitosis in the first several hours after irradiation, whereas the p38 inhibitor blocked much of this checkpoint delay. In contrast, inhibitor of ATM/ATR (caffeine), and Chk1

Figure 1

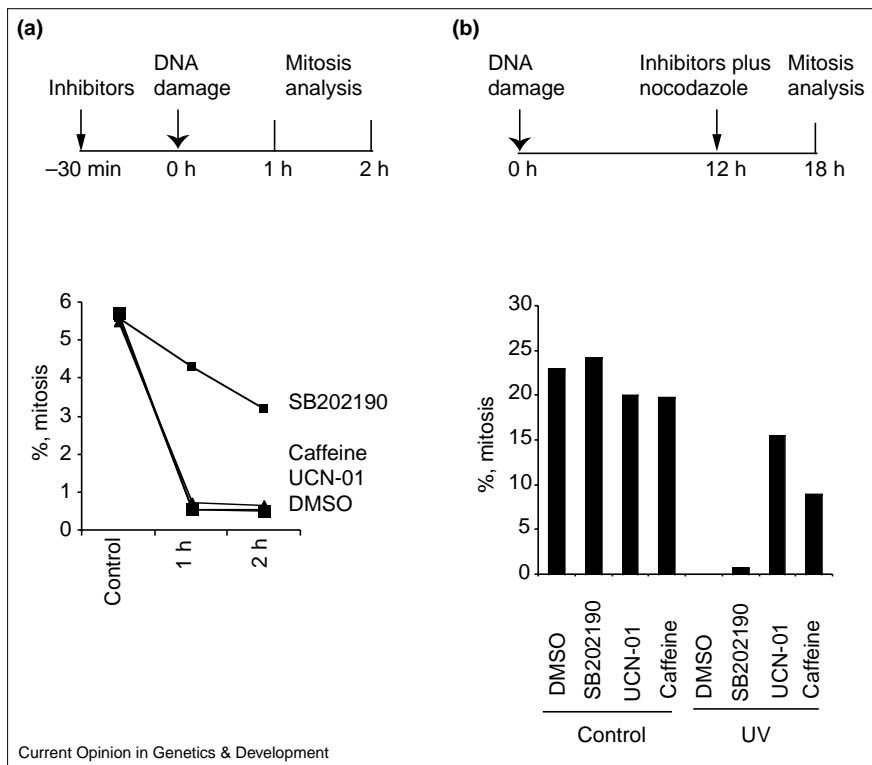


Overview of signaling pathways that target Cdc25 inactivation in G<sub>2</sub>. A simplified scheme is shown whereby Cdc25B removes inhibitory phosphates in cytoplasmic Cdc2; with G<sub>2</sub> progression, Cdc2 translocates to the nucleus where it is a target for Cdc25C, which maintains Cdc2 dephosphorylation at inhibitory sites. Cyclin B1 is not shown but is also required for Cdc2 activity. As described in the text, p38 can inhibit Cdc25B after UV irradiation and Cdc25C is a target for Chk1 and Chk2 kinases both after UV and IR.

kinase (UCN-01) had no effect on checkpoint initiation. These latter inhibitors did markedly attenuate the ability of the cell to maintain this checkpoint at later times after UV irradiation while inhibition of p38 had no appreciable effect on maintenance (Figure 2b). Although this example may vary with the stress agent employed and perhaps even the cell type, it highlights that p38 can have a prominent role early in this checkpoint.

The mechanism of phosphorylation-dependent inactivation of Cdc25 phosphatase is still not completely understood. Some reports have shown that in yeast and *Xenopus* phosphorylation of Cdc25 at 14-3-3 site induces binding with 14-3-3 proteins and subsequent redistribution of Cdc25 from the nucleus to cytoplasm [24,25]. This translocation disrupts Cdc25 association with and activation of nuclear Cdc2 kinase. However, this still has not been convincingly confirmed for Cdc25C phosphatase in mammals, and several reports have shown nuclear localization for endogenous Cdc25C during interphase when serine 216 is phosphorylated [26,27]. Some evidence from fission yeast showing that forced nuclear localization of Cdc25 fails to override the damage checkpoint also argues against a cytoplasmic retention of Cdc25 phosphatase as a major mechanism in G<sub>2</sub>/M checkpoint regulation [28\*]. Recently, several groups have proposed a reasonable model for inactivation of Cdc25 upon phosphorylation [29,30].

Figure 2



Evidence for differences in the mechanism for early G<sub>2</sub> checkpoint activation versus maintenance after UV irradiation. A. HeLa cells were treated with the p38 inhibitor SB202190, the Chk1 inhibitor UCN-01, or the ATM/ATR inhibitor caffeine and then UV-irradiated with 20 Jm<sup>-2</sup> as shown previously [3<sup>•</sup>]; progression into mitosis over the next 2 h was then determined. B. Cells were UV-irradiated with 10 Jm<sup>-2</sup> and then treated in a similar manner with these inhibitors 12 h later; 0.2 μM nocodazole was added at the same time to arrest any cells that had progressed to mitosis, and mitoses were scored 6 h later. As shown in this panel, none of these checkpoint inhibitors reduced progression to mitosis in the absence of UV radiation (designated 'control'), while UCN-01 and caffeine blocked UV-checkpoint maintenance; the effect with SB202190 was similar to solvent (DMSO) alone.

According to this model, phosphorylation of Cdc25 phosphatase causes rapid complexing with 14-3-3, masking residues required for interaction with cyclin B, thus preventing cyclinB1/Cdc2 interaction with the catalytic site of Cdc25. In this case, the inability of Cdc25 to dephosphorylate and activate Cdc2 kinase would be due to its interaction with 14-3-3 proteins rather than direct inactivation of Cdc25 phosphatase activity or its redistribution to cytoplasm after stress.

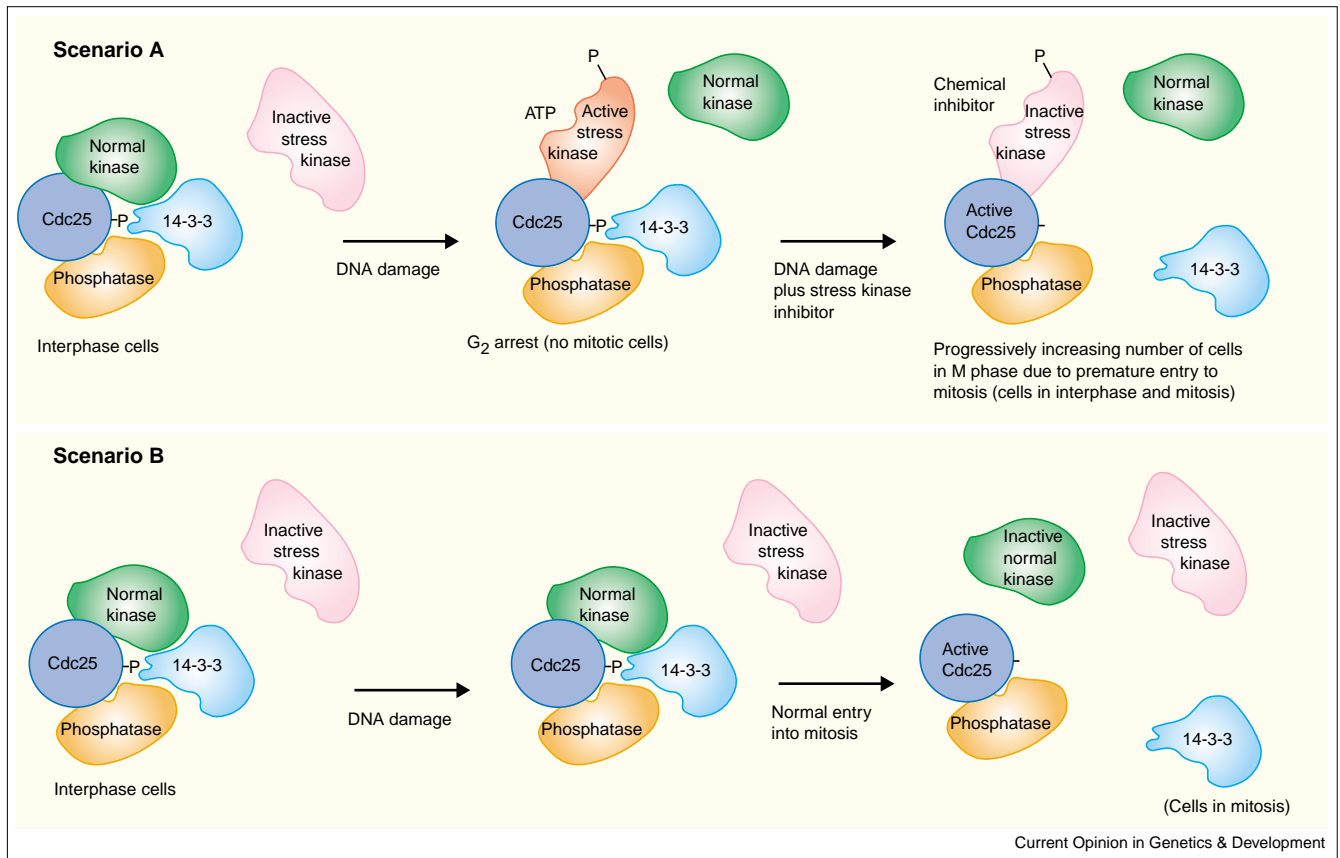
There is only one 14-3-3 binding site in human Cdc25C. This site contains serine 216, which is phosphorylated by Chk1 kinase both *in vitro* and *in vivo* [4<sup>•</sup>,15]. The 14-3-3 binding region for the mouse Cdc25C has not yet been defined, however, the mouse protein (National Center for Biotechnology Information accession number NM\_009860) does not have strong homology in the region where human serine 216 of Cdc25C is located. Recent evidence from *Cdc25C*<sup>-/-</sup> mouse embryo fibroblasts also argues against Cdc25C phosphatase being the only regulator for G<sub>2</sub>/M checkpoint activation following DNA damage [31<sup>•</sup>]. In contrast, human Cdc25B phosphatase contains four 14-3-3 binding sites (containing serines 137, 216, 309 and 361). At least two of the sites (homologues for serine 309 and 361 in human Cdc25B) are present in mouse Cdc25B (Cdc25M2) phosphatase (National Center for Biotechnology Information accession number NM\_023117). In the case of human Cdc25B, both these sites have been shown to be phosphorylated *in vitro* by p38 kinase, whereas serine 309

(serine 323 in Cdc25B2 isoform) is an *in vivo* site for p38 kinase after UV irradiation [3<sup>•</sup>].

During interphase, Cdc25C phosphatase is phosphorylated at the 14-3-3 binding site, serine 216, and becomes dephosphorylated in mitosis [15]. An analogous situation has been described for Cdc25B phosphatase which is also phosphorylated at its 14-3-3 binding site, serine 309, prior to mitosis [3<sup>•</sup>]. The 'normal' kinase(s) that phosphorylates these sites in Cdc25B and Cdc25C *in vivo* during interphase is still unknown. Some reports have shown that c-Tak1 kinase, purified from a crude cellular extract, has Cdc25C serine 216 phosphorylation activity [32]. cTak1 can also phosphorylate serine 309 of Cdc25B *in vitro* (DV Bulavin, AJ Fornace Jr, unpublished data), although the *in vivo* role of cTak1 in Cdc25s phosphorylation remains to be confirmed. As Cdc25C and Cdc25B are phosphorylated at 14-3-3 sites during interphase, it was reasonable to speculate that G<sub>2</sub>/M checkpoint activation through phosphorylation of Cdc25 phosphatases occurred in mitosis when these sites were dephosphorylated. However, it is still unclear if serine 309 in Cdc25B or serine 216 in Cdc25C can be phosphorylated after DNA damage while cells are in mitosis. Rather, evidence exists that the inhibitory phosphorylation of Cdc25 after genotoxic stress is regulated during interphase [3<sup>•</sup>,4<sup>•</sup>].

A major impediment to understanding the role of stress kinases in the regulation of Cdc25 phosphorylation is

Figure 3



Models for a switch mechanism regulating Cdc25 phosphorylation upon stress. **Scenario A:** regulation of Cdc25 after stress in the presence of the stress kinase inhibitors. **Scenario B:** regulation of Cdc25 after stress after knocking out a stress kinase or inactivation of upstream

components. Cdc25 phosphorylation at 14-3-3 sites in unstressed condition is regulated by the balance between a normal kinase (green) and a normal phosphatase (orange). Inactive stress kinase is shown in pink and activated in red. For more details see the text.

posed by the following question: how can stress kinases regulate Cdc25 phosphorylation in G<sub>2</sub> phase (interphase) when these inhibitory sites are already phosphorylated in non-stressed cells? A reasonable explanation for the role of stress-kinase-dependent regulation of G<sub>2</sub> delay came from phosphorylation analysis of the 14-3-3 sites on Cdc25 phosphatases in the presence of chemical inhibitors for either Chk1 or p38 kinases [3,4]. Inactivation of Chk1 kinase by UCN-01 completely abrogated serine 216 phosphorylation of Cdc25C after IR. A similar situation was found for p38-kinase-dependent phosphorylation of serine 309 in Cdc25B: this phosphorylation was affected by p38 inhibitor only after UV irradiation, but not under normal conditions. These data demonstrate that following DNA damage the phosphorylation of Cdc25 phosphatases at inhibitory sites becomes dependent on stress kinases, rather than on the normal kinases that phosphorylate these sites during interphase. However, the question remains as to what is the mechanism that causes Cdc25 phosphorylation following stress to switch from dependence on constitutive kinase(s) to stress-inducible kinases. As we discussed above, there is a 'normal' kinase(s) that is responsible for

keeping the 14-3-3 site phosphorylated during interphase, and, presumably, a 'normal' phosphatase that dephosphorylates the 14-3-3 binding site upon entry into mitosis (Figure 3). Neither the exact enzymes nor the precise mechanism of their regulation during the cell cycle is known, significantly complicating our understanding of the switch mechanism after stress; however, several lines of evidence do support this idea and merit more detailed discussion. Owing to different experimental protocols for inactivation of stress kinases, at least two possible scenarios can be proposed (Figure 3). Once activated by phosphorylation, stress kinases could adopt an active conformation and increased affinity for different substrates, including Cdc25 phosphatases. Displacement of the normal kinase from Cdc25 complex provides the simplest way to explain the switch mechanism (Figure 3, Scenario A). This could also explain why cells are then arrested in G<sub>2</sub> phase and do not enter mitosis: even if the normal kinase is inactivated through the normal mitotic mechanism, it does not affect Cdc25 dephosphorylation, because this phosphorylation is now under the control of the stress kinases. Subsequent inhibition of stress kinases by chemical inhibitors — such

as UCN-01 for Chk1 or SB202190 for p38 — may allow the stress kinase to remain in its active (phosphorylated) conformation [33] and to be complexed to the Cdc25 phosphatases (Figure 3, Scenario A). At the same time, the stress kinase would be unable to phosphorylate its substrate (14-3-3 sites in Cdc25B and C) because the chemical inhibitor has displaced ATP from the ATP-binding pocket as has been described for p38 kinase [34]. As a result, dephosphorylation of Cdc25 and a resultant decrease in the binding to 14-3-3 proteins occurs following DNA damage in the presence of the specific inhibitors, as has been published for both Chk1 and p38 kinases [3\*,4\*].

The consequences would be expected to be different in a situation where the stress kinases have been knocked out or the stress pathway has been deregulated upstream, such as at MKK6 for the p38 or ATM for the Chk1 kinases (Figure 3, Scenario B). According to this scenario, the level of Cdc25 phosphorylation at the 14-3-3 binding site probably would not be affected after DNA damage as the stress kinase due to its depletion or remaining in an inactive conformation would not be able to displace the 'normal' kinase. As a result, the Cdc25 phosphatase will remain in the complex with the normal kinase, which would still maintain phosphorylation of Cdc25 at the 14-3-3 inhibitory site in a cell-cycle-dependent manner. In this scenario, the cells would enter mitosis normally regardless of DNA damage. Continued phosphorylation of Cdc25 by the 'normal' kinase in this case could lead to efficient 14-3-3 binding in cells with a disrupted *Chk1* gene even after DNA damage, as has been shown in fission yeast [35]. In this scenario, cells would progress from interphase to mitosis regardless of damage — essentially the damage checkpoint signal would be ignored.

### A model for G<sub>2</sub>/M checkpoint activation after genotoxic stress

On the basis of the data discussed here, a simplified model can be proposed for the mechanism of G<sub>2</sub>/M checkpoint activation after stress. Different types of stress induce several independent pathways. The first group of pathways is responsible for checkpoint initiation and regulates mostly Cdc25B phosphorylation in the cytoplasm. One example of this group is the p38 MAPK pathway that induces G<sub>2</sub> delay at early time points after UV irradiation through the serine 309 phosphorylation of Cdc25B [3\*].

Another group of pathways is activated by specific types of damaged DNA, such as double-strand breaks induced by ionizing radiation or thymidine dimers produced by UV radiation. These pathways involve proteins participating in the detection of and signaling from the damage-modified DNA. Activation of nuclear Chk1 kinase in this case acts as a DNA-damage sensor and remains turned on until the damaged DNA is repaired. Thus, the Chk1 kinase continues to target Cdc25C phosphorylation at serine 216 and ensures the maintenance of the G<sub>2</sub>/M checkpoint while unrepaired DNA damage remains. An early response to

stress involves activation of a G<sub>2</sub>/M checkpoint by targeting Cdc25B in the cytoplasm. If the DNA is not damaged, cells enter mitosis and continue the cell cycle once the stress is eliminated. However, when the DNA is damaged, exit from G<sub>2</sub> comes under the control of the system regulating Cdc25C phosphorylation in the nucleus, such as the ATM/Chk1-dependent pathway. In this case, cells would enter mitosis only when damaged DNA was repaired.

### Conclusions

We are just beginning to realize the complexity of the stress-induced G<sub>2</sub>/M checkpoint and the exact role that different Cdc25 phosphatases play in controlling mitotic entry in mammalian cells. Recent findings showing the importance of p38 and Chk1 kinases in the regulation of Cdc25B and Cdc25C phosphorylation at inhibitory sites have revealed an additional layer of complexity in when and how the components that regulate these phosphorylations can interact with each other. Multiple pathways are involved after genotoxic stress, orchestrating a spatially organized system where both cytoplasmic and nuclear events coordinately conduct the regulation of a G<sub>2</sub>/M checkpoint after stress.

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