Stabilizers and Destabilizers Controlling Cell Cycle Oscillators

Minireview

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Various destabilizing factors of the ubiquitin system contribute to the synchrony and unidirectionality of the cell cycle clock by finely tuning the activity of various CDKs. The recent findings of hierarchical and connected waves of cyclin stabilizers highlight the complexity of this network.

How to Keep CDK Activity Low in G0 and High in "C Phase"

The cell relies on three major systems to maintain the quiescent state (or G0 phase): the pocket proteins (pRb, p107, and p130), Cdh1, and certain CDK inhibitors (CKIs) (Figure 1A). Critical downstream targets of the three G0 guardians are Cdk1 and Cdk2, two kinases that lie at the very core of the cell cycle machinery and constitute its major driving force (Murray, 2004). Pocket proteins repress the activity of the members of the E2F transcription factor family (particularly E2F1-3), which induce the transcription of many positive cell cycle regulators, including CDK activators such as cyclins (type A, B, and E) and Cdc25 protein phosphatases (Yamasaki and Pagano, 2004). Cdh1 activates the anaphase promoting complex/cyclosome (APC/C) ubiquitin ligase to promote the degradation of a number of positive regulators of S phase and mitosis (Ang and Harper, 2004; Peters, 2002). These include cyclins (type A and B) and Cdc25A. A further activator of Cdk1 and Cdk2, Skp2, is also a target of APC/C^{Cdh1} (Kurland and Tansey, 2004). Skp2 is an F box protein and constitutes the substrate recognition subunit of an SCF (Skp1/Cul1/F box protein) ubiquitin ligase complex (Cardozo and Pagano, 2004) that targets the CKIs p21 and p27 for degradation. Degradation of Skp2 by APC/C^{Cdh1} contributes to increase the levels of these CKIs in quiescent cells.

Quiescence is self-sustaining; however, mitogenic stimuli are able to induce G0 cells to enter the cell cycle. To do so, mitogens need to inhibit each of the G0 regulators: pocket proteins, Cdh1, and CKIs. This inhibition is the product of complex signaling pathways that start at the membrane and eventually lead to the activation of Cdk1 and Cdk2 (Figure 1B). These two kinases will then remain active from late G1 until anaphase. We propose to define this interval of time "C phase," a phase with active Cdk1/Cdk2, as opposed to G0, early G1, and telophase that are characterized by no Cdk1/Cdk2 activity.

Significantly, the start of the cell cycle requires CDKs to act upstream of pocket proteins, Cdh1, and CKIs, reversing the inhibition of CDKs exerted by these factors in G0 (Figure 1B). The gradual phosphorylation of each of

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these inhibitors by Cdk1 and Cdk2 (and other CDKs, at least in the case of pocket proteins) induces their inactivation. Phosphorylation of pocket proteins induces their dissociation from E2F transcription factors, thereby allowing their activation. Phosphorylation of Cdh1 leads to its dissociation from APC/C, resulting in the inactivation of this ubiquitin ligase. Finally, phosphorylation of p21 and p27 promotes their degradation through SCF^{Skp2}. All this creates autoamplification loops that render Cdk1 and Cdk2 increasingly more active.

An important consequence of E2F activation is the transcription of *Emi1*, a central inhibitor of APC/C (Hsu et al., 2002). In addition, APC/C contributes to its own inactivation by mediating the degradation of both its activator Cdh1 and its ubiquitin conjugating enzyme, Ubc10 (Lukas and Bartek, 2004). Thus, at least three signals cooperate to keep APC/C^{Cdh1} inactive from G1/S until late mitosis: CDKs, Emi1, and APC/C^{Cdh1} itself. This intricate mechanism clearly highlights the need for the cell to inhibit APC/C^{Cdh1} during a time in which CDKs need to be active.

How to Keep Cdk1 Activity Low in S-G2 and High at G2/M

Cdk1 activity accumulates slowly during S and G2 and reaches maximal activation only in mitosis, whereas Cdk2 reaches its peak of activity earlier in S phase. The sum of Cdk1 and Cdk2 activities during S and G2 is much lower than the activity of Cdk1 in early mitosis alone (Bashir and Pagano, 2005). Low CDK activity is permissive for DNA replication, whereas the inhibition of DNA rereplication and entry into mitosis require higher thresholds. Thus, the attenuation of Cdk1 activity prevents entry into mitosis prior to the completion of DNA synthesis and the verification of its fidelity. The inhibitory phosphorylation of Cdk1 on its ATP binding site by the kinase Wee1 is responsible for restraining Cdk1 activity during S and G2 phase. To avoid the action of Wee1 being neutralized, the phosphatase Cdc25A needs to be kept at bay. This is achieved by βTrcp-mediated degradation of Cdc25A triggered through Cdc25A phosphorylation by the DNA replication checkpoint kinase Chk1. At G2/M, full activation of Cdk1 is allowed by turning the checkpoint off, leading to the consequent inactivation of Chk1 and accumulation of Cdc25A. At the same time, Cdk1 (in conjunction with Plk1) furthermore phosphorylates Wee1, creating a phospho-degron for the recognition by βTrcp. Thus, SCF^{βTrcp} attenuates Cdk1 activity during S and G2 (by inducing Cdc25A degradation) but contributes to its full activation at G2/M (by promoting the proteolysis of Wee1).

How the Circle Closes

CDKs not only phosphorylate downstream substrates to generate autoamplification loops (as is the case for pocket proteins, Cdh1, p21, p27, and Wee1), but they also generate negative feedback signals that will induce their own inactivation in late M. For example, in G2, Cdk1 and Cdk2—in association with cyclin A—phosphorylate
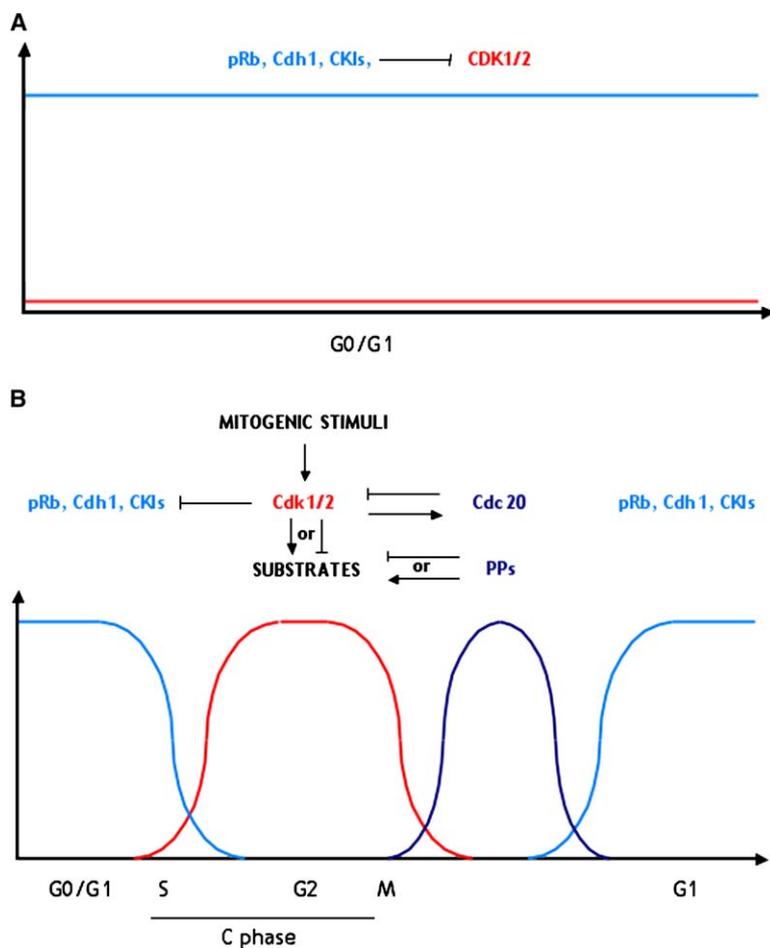
A

B

Figure 1. The G0 State Is Self-Sustaining, whereas the C Phase Contains the Program for Its Own Shutdown

(A) In the absence of mitogenic stimuli, pRb and other pocket proteins, Cdh1, and certain CKIs are responsible for maintaining the quiescent state or G0. This is achieved, at least in part, by preventing the activation of CDKs. (B) In response to mitogenic signals, CDKs become active and subsequently phosphorylate and inactivate pocket proteins, Cdh1, and CKIs. This generates positive autoamplification loops that render CDKs increasingly more active as the cell progresses from G1/S to mitosis. However, the C phase (or phase of CDK activity) is programmed to end in late mitosis. For example, Cdk1 promotes the activation of APC/C^{Cdc20}, both by phosphorylating certain subunits of APC/C and by inducing the degradation of Emi1 (see Figure 2). In turn, active APC/C^{Cdc20} causes the degradation of cyclins with the consequent inactivation of Cdk1. At the end of the cell cycle, Cdc14 and other phosphatases (PPs) dephosphorylate many of the substrates phosphorylated by CDKs during the C phase. Please note that the amplitude of the curves representing each phase is not in scale with the actual time length. For example, the C phase is much longer than the interval between late mitosis and the next G1.

E2F1 and E2F3, resulting in the inactivation of these two transcription factors (Yamasaki and Pagano, 2004). In prometaphase, phosphorylation of Emi1 by Cdk1 and subsequently by Plk1 induces the βTrcp-dependent degradation of this APC/C inhibitor (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003). This event triggers the activation of a different form of APC/C, namely APC/C^{Cdc20} (Figure 1B). Cdk1 further contributes to the activation of APC/C^{Cdc20} by phosphorylating some of its subunits. However, only when all chromosomes have completed bipolar attachment to the mitotic spindle does the APC/C^{Cdc20} ubiquitin ligase reach full activity and allow the metaphase-anaphase transition to occur by inducing the degradation of securins and thus promoting the separation of the sister chromatids (Reed, 2003). Significantly, the same ubiquitin ligase responsible for the segregation of the chromosomes destined to the two daughter cells also induces the degradation of A and B type cyclins, thereby inhibiting Cdk1 activity and resetting the cell cycle to low CDK activity for the "newborn/G1" cells.

What occurs at the exit from mitosis is better understood in yeast than in higher eukaryotes, although the general mechanisms seem to be conserved. It appears that at the end of the long-lasting reign of CDKs there is a short time in which phosphatases (Cdc14 and likely others such as PP2A and PP1) rule to revert the effects of CDKs by dephosphorylating substrates previously targeted by CDKs (Figure 1B), among them pocket pro-

teins (resulting in the inhibition of E2F1-3) and Cdh1 (Bosl and Li, 2005). Cdh1 in its unphosphorylated state is free to bind to APC/C and keep it active from late mitosis to late G1. In turn, APC/C^{Cdh1} induces the degradation of Skp2, giving rise to upregulation of p21 and p27. The circle is finally closed, and the cell cycle clock is reset: the two daughter cells generated by one cell division cycle are back in G1 and will proceed into S only if favorable conditions are still present. If not, pocket proteins, Cdh1, and CKIs will again cooperate in maintaining the G1 state and eventually in promoting entry into quiescence. Significantly, the keepers of the G0 state have also been implicated in cell differentiation, and most of them are also expressed in postmitotic cells. Thus, they also appear to be inducers and guardians of the differentiated state.

In summary, the cell cycle is characterized by a buildup of CDK activity, and many negative feedbacks emanating from CDKs themselves ensure that at the end of one cycle CDK activity is downregulated to allow exit from mitosis. In fact, unless cyclins are ubiquitylated by APC/C, an intramitotic checkpoint prevents departure from anaphase (Murray, 2004). Clearly, the ubiquitin system strongly contributes to the synchrony and unidirectionality of the cell cycle by ensuring that various cell cycle gates are opened at the right time. A variety of CDKs and cyclins (combined with their differential transcription, translation, degradation, subcellular localization, and posttranslational modifications) have evolved to
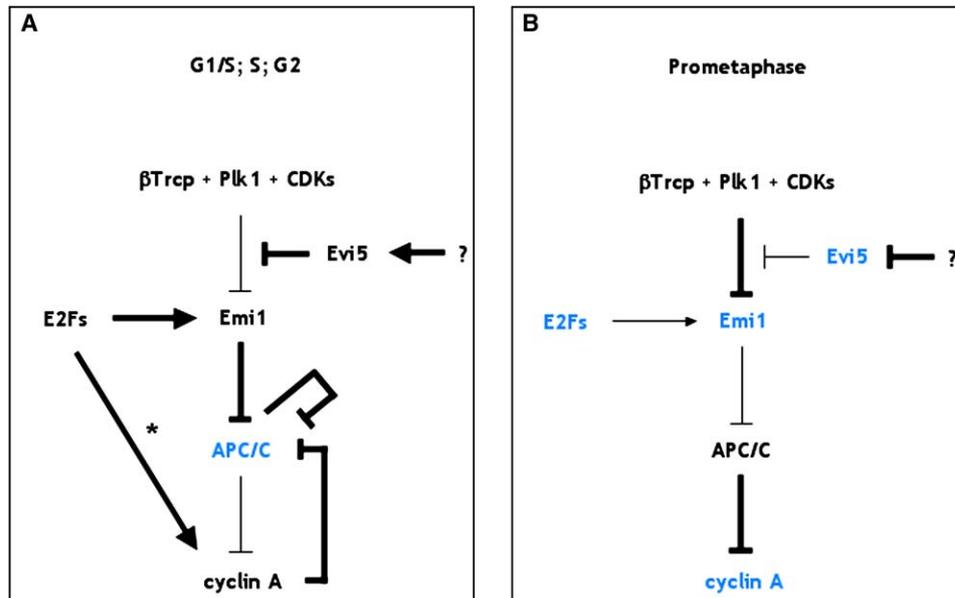
Figure 2. The Evi5-Emi1-APC/C Axis as an Example of Stabilizers and Destabilizers Controlling Both Positively and Negatively CDK Activity

Black color signifies activated forms of the respective proteins, and turquoise indicates inactive forms or degraded proteins.

(A) New results suggest that during most of the C phase, Evi5 prevents Emi1 phosphorylation by Plk1 and its consequent recruitment by SCF$^{\beta Trcp}$. Emi1 is thus protected from degradation and, by blocking the activity of APC/C, drives the accumulation of cyclin A, which contributes to the activation of Cdk1 and Cdk2. The asterisk indicates that the E2F-mediated transcriptional activation of cyclin A is not present during G2. In fact, during this phase, cyclin A induces the phosphorylation and inactivation of certain E2F family members.

(B) In prometaphase, Plk1 phosphorylates Evi5, triggering its destruction. Unbound Emi1 is in turn phosphorylated by Cdk1 and Plk1, ubiquitylated by SCF$^{\beta Trcp}$, and degraded by the proteasome. APC/C becomes active and induces both the entry into anaphase and the degradation of mitotic cyclins, thereby inactivating Cdk1.

provide a complex control system for the cell cycle machinery. Focusing on the proteolytic mechanisms only, the following scenario appears: in early G1, CDKs are kept in check by APC/C^{Cdh1}, during the C phase, CDK activity is stimulated by SCF^{Skp2}, and in late mitosis, it is inhibited by APC/C^{Cdc20}. This system appears to induce a binary response: OFF, ON, and back to OFF. However, another ubiquitin ligase, SCF$^{\beta Trcp}$, provides exquisitely fine regulation of Cdk1 activity thresholds by controlling Cdc25A, Wee1, and Emi1 during different phases of the cell cycle.

How to Stabilize Cyclin A: Emi1 and Evi5
A recent study (Eldridge et al., 2006) has introduced a new player controlling the oscillation of cell cycle regulatory proteins. This protein is called Evi5 and its activity is to inhibit the degradation of Emi1 (Figure 2A). Earlier work demonstrated that, in addition to destabilizing factors (i.e., most ubiquitin conjugating enzymes and ubiquitin ligases), the ubiquitin system also includes stabilizers. These consist of certain deubiquitylating enzymes (e.g., Usp7), inhibitors of ubiquitin ligases (e.g., Emi1 and Mad proteins), certain kinases (e.g., CDKs phosphorylating Cdh1), inhibitors of kinases (e.g., inhibitors of GSK3β in the WNT pathway), etc. Evi5, however, works by a novel mechanism. It associates with Emi1 and protects it from SCF$^{\beta Trcp}$ ubiquitylating activity.

Specifically, Evi5 binds directly to a region of Emi1 surrounding the degron that is phosphorylated by Plk1. As a result, it prevents the phosphorylation and the subsequent recruitment by SCF$^{\beta Trcp}$. It is believed that the timing of association between substrates and

SCF ligases is dictated by either the availability of the F box protein (e.g., inverse correlation of Skp2 with p27 levels) or the kinase that phosphorylates the substrate (e.g., inverse correlation of Chk1 activity with Cdc25A levels) (Petroski and Deshaies, 2005). Thus, the new finding has profound implication, as Evi5 provides additional means of control by blocking the phosphorylation of the SCF substrate.

Importantly, Evi5 levels themselves oscillate during the cell cycle, being high from early G1 to the beginning of mitosis. The mitotic decrease in Evi5 levels is once again due to the activity of Plk1, which phosphorylates a C-terminal degron and thus triggers Evi5 destruction by an as yet unknown ubiquitin ligase. In late mitosis, the destruction of Plk1 allows the restabilization and reaccumulation of Evi5.

Thus, the C phase is characterized by an ensemble of three hierarchical and largely concomitant waves of stability: Evi5 stabilization leads to Emi1 accumulation, which in turn causes the stabilization of cyclin A (Figure 2A). Later, in early mitosis, Plk1 phosphorylates Evi5, promoting its degradation. As a result, Emi1 also becomes accessible to Plk1-mediated phosphorylation and is consequently eliminated via SCF$^{\beta Trcp}$. This event, together with the phosphorylation of specific APC/C subunits by Cdk1, leads to the full activation of APC/C^{Cdc20} and the consequent degradation of mitotic cyclins (Figure 2B). At the end of the C phase, Plk1 is degraded via APC/C^{Cdh1}, permitting the accumulation of Evi5 and the rebuilding of a protective shield for the newly synthesized Emi1 during the next G1/S. However, because Plk1 is only active in G2 and mitosis, the

protective function of Evi5 during S could be crucial to shield Emi1 from phosphorylation by other members of the polo-like kinase family. Alternatively, Evi5 would protect Emi1 from low Plk1 activity and from premature Plk1 activation.

Although the function of Evi5 in establishing a defined period of Emi1 stability during the C phase is mostly understood, it is still unclear where in the cell all this takes place. Evi5 is a protein localized mostly to the centrosome (Eldridge et al., 2006; Faitar et al., 2005), and potentially important subpopulations of most of the other players (βTrcp, Skp1, Cul1, cyclins, Plk1, Plk2, and Emi1) localize to the centrosome as well. One possibility is that Evi5 promotes the stabilization of the centrosomal pool of Emi1 only, causing cyclin A accumulation on the centrosome. This might explain how Evi5 stabilizes a centrosomal pool of cyclin A, a protein that is required for centrosomal duplication during S phase. However, this scenario does not clarify why the downregulation of Evi5 leads to the disappearance of the total cellular pools of Emi1 and cyclin A. It is possible that Emi1 is only ubiquitylated and degraded on the centrosome and thus generates a "draining force," which siphons off the entire pool of Emi1 and cyclin A. According to this model, Evi5 would act as a plug to stop the degradation of cyclin A and therefore provide a direct link between the centrosome cycle and the cell division cycle.

There are additional unclear issues. How is Evi5 expression induced in G1? And what is the nature of the ubiquitin ligase that triggers Evi5 destruction in early mitosis? Evi5 appears in G1 a few hours before Emi1. Do other proteins exist that are stabilized by Evi5 during this window of time? All these unanswered questions are of particular importance given the role of Evi5 in the regulation of APC/C activity.

The Evi5-Emi1-APC/C Axis and Cancer

It is worth mentioning that the G0 keepers are either tumor suppressors or at least display some cancer suppressor characteristics (Sherr, 2004). In contrast, many positive regulators of cell cycle progression are prooncogenic (e.g., A and E type cyclins, Cdc25A, E2F3, Emi1, and Skp2) (Sherr, 1996; Yamasaki and Pagano, 2004). Evi5 may belong to the latter group. Indeed, Evi5 was originally isolated as a product of a gene frequently targeted by retroviral integration in murine T cell lymphomas (Liao et al., 1997). In another study, it was found truncated as the result of a chromosomal translocation in a patient with stage 4S neuroblastoma (Roberts et al., 1998). It is still uncertain if these genomic alterations suppress Evi5 expression or generate a truncated version with modified biological activity. In spite of this, the fact that *Evi5* is a target of genomic rearrangements suggests a role for Evi5 in oncogenesis. Evi5 may favor tumorigenesis by its ability to stabilize Emi1. Experiments in cultured cells have shown that deregulation of Emi1 proteolysis during prometaphase causes mitotic aberrations such as misalignment of the chromosomes and abnormal spindle formation (Guardavaccaro et al., 2003; Margottin-Goguet et al., 2003), which may potentially be a cause of aneuploidy in daughter cells able to escape mitotic catastrophe. In addition to aneuploidy, these daughter cells would have a G1 phase characterized by high levels of Emi1

with consequent inhibition of APC/C^{Cdh1} and accumulation of positive regulators of the cell cycle. This, in turn, would lead to premature entry into S phase, a further cause of genomic instability (Murray, 2004). Theoretically, this scenario could be mimicked by deregulated expression of Evi5. Additional studies will be needed to fully understand the importance of the Evi5-Emi1-APC/C axis in oncogenesis.

Outlook

Despite the large body of work on the cell cycle and the ubiquitin system, we are only scraping at the surface of a more complex network. The findings on Emi1 and Evi5 may potentially implicate that additional proteins with similar stabilizing functions control the cell cycle and other cellular processes. Moreover, hundreds of different ubiquitin ligases and isopeptidases exist in mammalian cells, which begs the question of how many of them play a role in cell cycle control. How are all these enzymes regulated and how do they functionally interact with each other? Is phosphorylation the only language understood by cell cycle ubiquitin ligases or can additional posttranslational modifications impinge on their activity and/or recognition of substrates? Finally, what are the variations on the theme in different cell types in the various tissues? These are only some of the areas that will likely experience intense research activities in the near future.

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