2-18-2009

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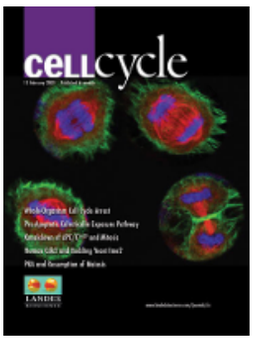
Steven R. Grossman
University of Massachusetts Medical School



Cell Cycle

ISSN: 1538-4101 (Print) 1551-4005 (Online) Journal homepage: http://www.tandfonline.com/loi/kccy20

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To cite this article: Steven R. Grossman (2009) Occam's dull razor: MDM2 and P/CAF keep Chk2 in check the hard way, Cell Cycle, 8:4, 515-517, DOI: 10.4161/cc.8.4.8056

To link to this article: https://doi.org/10.4161/cc.8.4.8056

Published online: 15 Feb 2009.

[Cell Cycle 8:4, 515-517; 15 February 2009]; ©2009 Landes Bioscience

Occam's dull razor

MDM2 and P/CAF keep Chk2 in check the hard way

As is the case for most stress response factors, the activity of Chk2 kinase, a central effector of the DNA damage response, is homeostatically regulated by a fine balance of positive and negative regulation. Prives and colleagues investigate the negative regulation of Chk2 by ubiquitin/proteasome degradation in the February 1, 2009 issue of *Cell Cycle*,[1] and in so doing, expose a significant gap in our somewhat dogmatic knowledge of MDM2 biology and function. Kass et al. show that both MDM2, a well-studied E3 that targets p53, and the recently identified E3, P/CAF[2] (better known for its HAT function and participation in transcription regulation), target Chk2 for ubiquitination and degradation. Targeting of Chk2 by MDM2 was blocked by DNA damage stimulated phosphorylation of Chk2 S456, allowing for stress induced stabilization of Chk2 during DNA damage responses. Unexpectedly, the ability of MDM2 to ubiquitinate Chk2 did not require its RING E3 domain, which is required for ubiquitination of all of its other known targets—most notably, p53. Given that P/CAF binds and ubiquitinates MDM2, the authors also looked for an impact of P/CAF on Chk2, and found that it independently ubiquitinated and destabilized Chk2. MDM2 and P/CAF together synergistically ubiquitinated Chk2, and all three molecules could be found in one complex. As seen with MDM2, mutation of the putative P/CAF E3 domain had no effect on its Chk2 destabilizing activity. The authors propose that P/CAF and MDM2 may act within a larger multi-subunit E3 (a la SCF or APC) to target Chk2, and serve mainly as recognition factors/scaffolds rather than actual E3 enzymes. In support of this idea, MDM2 interacted more strongly with Chk2-S456A, a mutant which is preferentially hyperubiquitinated in the presence of MDM2.

Presuming the effects of P/CAF and MDM2 on Chk2 ubiquitination are direct—this work leaves the obvious mystery of the identity of the Chk2 E3. What known MDM2-P/CAF binding partners might be viable candidates? Most obvious is MDM-X, the heterodimerization partner of MDM2.[3] MDM-X, though structurally similar to MDM2, with a conserved C-terminal RING E3 domain, does not target p53 for degradation by itself. It can participate with MDM2 in targeting p53 when heterodimerized (or perhaps in larger oligomeric complexes) with MDM2. Thus, if the MDM2 E3 defective mutant and the E3-defective P/CAF mutant can both interact with MDM-X, perhaps its RING contributes the missing E3. Another viable candidate would be CUL4 and its associated SCF complex, as two publications have identified CUL4 as a direct binding partner of MDM2, and also a participant with MDM2 in p53 ubiquitination.[4,5]

Lastly, p300 and/or CBP may provide the missing E3 activity. p300 interacts with both MDM2 and P/CAF through separate domains (C/H1-TAZ1 and C/H3-TAZ2, respectively), while p300 and P/CAF both interact with MDM2 through its central domain.[2,6,7] Thus, there is a distinct possibility given all the possible contact surfaces, that p300 (and/or CBP), P/CAF and MDM2 participate in a tripartite complex that could then interact with Chk2. p300/CBP are better known as HATs (as is the distantly related P/CAF) that globally regulate histone acetylation, but also act as non-histone acetylases that regulate dozens (at least) of transcription factors, most notably, p53. The ubiquitin ligase activity of p300 was recently described as specifically targeting p53 for polyubiquitination, but only when p53 had been oligoubiquitinated by MDM2 first.[8] CBP shares a similar "E4" activity for p53.[9] This activity is centered within the N-terminal 595 amino acids of p300 where a substrate independent generic E3 activity is also located. Initially perplexing was the lack of a canonical E3 domain in p300 (RING, Hect, U-box, etc.), but since 2003 there has been a growing list of such non-canonical E3's, which includes A20,[10] P/CAF and Rabex-5.[11] Though no evolutionarily conserved domains are shared by these proteins, at least p300 and A20 share non-RING (and non-homologous) Zn^{2+}-binding domains, which are required for E3 activity, or are at least present within the putative E3 domain. P/CAF has a potential Zn^{2+} binding sequence within its E3 domain, and this sequence aligns with a portion of p300 TAZ1 sequence.[12] Given that p300 and P/CAF do share evolutionary origins, their E3 domains may very well share structural similarity, perhaps centering on the known (for p300) and putative (for P/CAF) Zn^{2+}-binding domains of each protein.

Setting aside the details of exactly how MDM2 and P/CAF accomplish the ubiquitination of Chk2 without their own E3 domains, one wonders—why this way? Given evolutionary pressures, the regulation of Chk2 may have evolved in this manner due to the need for Chk2 ubiquitination in contexts where the MDM2 RING or P/CAF E3 domains are not available. Two possibilities arise. Perhaps the Chk2 regulatory function was encoded in a more evolutionarily ancient form of MDM2 or P/CAF, prior to E3 activities being joined to the molecules. More likely, especially for MDM2, there are prevalent forms of the molecule that lack the RING domain, yet should still bind to Chk2 and P/CAF. A substantial fraction of MDM2 protein exists in a caspase-cleaved form, where cleavage after residue 361 by distinct caspases in resting[13] and apoptotic[14] cells, leaves intact a C-terminally truncated RING-less form that maintains an intact central domain, and presumably, p300 and P/CAF binding activities. As the exact functional relevance of caspase-cleaved MDM2 has remained mysterious, perhaps examination of its effects on Chk2 in resting, DNA damaged and apoptotic cells would be worthwhile, and also shed light on why nature seems to have chosen an arcane mechanism more reminiscent of Rube Goldberg than Occam, when it comes to MDM2-P/CAF regulation of Chk2.

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Steven R. Grossman; Departments of Cancer Biology and Medicine; University of Massachusetts Medical School; Worcester, Massachusetts USA; Email: Steven.Grossman@umassmed.edu

Strong inducible knockdown of Cdc20 does not cause mitotic arrest in human somatic cells

Implications for cancer therapy?

Eukaryotes have evolved to initiate the separation of their sister chromatids in a highly synchronous manner at the onset of mitotic anaphase. This promotes the fidelity of chromosome segregation. Anaphase is triggered as a result of the degradation of proteins that become ubiquitinated by the Anaphase Promoting Complex/Cyclosome (APC/C). The APC/C is a multi-subunit complex[1] which requires a specificity factor, Cdc20, to facilitate the ubiquitination of the known anaphase targets, securin and cyclin B.[2] The spindle assembly checkpoint (SAC) is a mechanism that inhibits Cdc20 until anaphase initiation is appropriate. This mode of anaphase regulation has become widely accepted as the mechanism that controls chromosome segregation in eukaryotes. However, recent studies indicate that anaphase can occur in the absence of Cdc20 and also that the SAC can restrain anaphase independently of inhibiting APC/C activity. These

studies suggest that redundant mechanisms control anaphase progression.

In the article published in this issue of *Cell Cycle* by Baumgarten et al., the authors ask what are the consequences of Cdc20 depletion from human cultured cells. This question has been addressed in other published articles but the published data are somewhat contradictory, perhaps due to different depletion approaches and/or efficiencies. In this study the authors used probably the best method to achieve depletion (lentiviral vector-mediated RNA interference) and the efficiency seems exceptionally strong. Moreover, the authors derived clonal cell lines in which Cdc20 depletion is inducible. The findings of this work are quite striking as the authors provide evidence that Cdc20 depletion does not arrest the cell cycle or greatly stabilize the substrates of the APC/C, cyclin B and securin.[3] Their conclusion is that a redundant mechanism allows mitotic progression in the absence of APC/C-Cdc20 activity. The authors go on to show that securin and cyclin B become stabilized in the absence of Cdc20 only when the SAC is activated by nocodazole treatment. In other words, a part of the SAC pathway must be able to act independently of Cdc20 inhibition and this activity is capable of blocking anaphase onset.

This work adds to a growing literature which has revealed inconsistencies in the current model of anaphase control. Depletion of Apc2 (an essential catalytic subunit of the APC/C) from human somatic cells[4] delays but does not prevent sister chromatid separation in anaphase but these cells do arrest in telophase. This result indicates that the APC/C is not the sole pathway capable of promoting anaphase progression. Similar to the above study, in these experiments anaphase was initiated in the absence of APC activity but upon the addition of nocodazole, anaphase was blocked. Thus, an alternate SAC pathway can control anaphase onset independently of the APC/C. It is becoming clear that the APC/C promotes mitotic progression in collaboration with underlying redundant safety features that together provide fidelity.

Yeast cells that lack APC/C catalytic subunits and also lack Cdc20, securin and Clb5 (a B-type cyclin) are able to perform anaphase with sufficient fidelity to remain viable. In these cells, there remains a SAC response to nocodazole which results in metaphase arrest.[5] Deletion of the MAD2 SAC gene does not overcome the checkpoint arrest. These yeast genetic experiments are consistent with the results presented by Baumgarten et al. In both the yeast and human cells lacking Cdc20, mitotic arrest was induced upon activation of the SAC. The simplest interpretation of these data is that mitosis can be completed in the absence of Cdc20 and that the SAC does more that just inhibit Cdc20.

These studies may have implications for cancer therapy. An important discovery was that some aggressive tumors have reduced APC/C activity due to constitutive high levels of the Mad2 SAC protein.[6] It is plausible that these cells are able to undergo mitosis due to the APC/C-independent pathway. Anaphase is abnormal in these cells providing the opportunity for chromosome loss which could be an advantage to the cancer cells. However, the APC-independent mechanism that promotes anaphase might be crucial for tumor survival. The identification of this pathway could reveal novel therapeutic targets.

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Duncan J. Clarke; Department of Genetics, Cell Biology and Development, University of Minnesota; Minneapolis, Minnesota USA; Email; Duncan.J.Clarke-2@umn.edu

PKA and CDC25B

At last connected

Mouse oocytes grow while arrested in the first meiotic prophase. Following completion of growth a surge in luteinizing hormone initiates resumption of meiosis and ovulation. It is well established that activation of the cyclin-dependent protein kinase CDC2A-CCNB (M-phase promoting factor) triggers resumption of meiosis. There is also a large body of evidence that PKA is essential to maintain meiotic arrest. For example, a decrease in cAMP concentration occurs during the time when oocytes become committed to resume meiosis; cyclic nucleotide phosphodiesterase inhibitors and membrane-permeable cAMP analogs prevent maturation; and injecting oocytes with the catalytic subunit of PKA inhibits maturation, whereas injecting oocytes with the PKA inhibitor PKI overcomes inhibition of maturation in response to cyclic nucleotide phosphodiesterase inhibitors and membrane-permeable cAMP analogs. What is unresolved to date is how PKA prevents activation of CDC2A-CCNB and hence initiation of maturation.

The activity of CDC2A-CCNB is regulated by phosphorylation (as well as by proteolysis of cyclin). Phosphorylation of T14 and Y15 in CDC2A is catalyzed by members of the WEE1 family of protein kinases and inhibits the kinase activity of CDC2A. Reciprocally, dephosphorylation of these residues by a dual specificity protein phosphatase CDC25 activates CDC2A.

Much evidence indicates a role for PKA in activating WEE1.[1,2] Mouse oocytes contain an oocyte-specific form of WEE1, WEE1B. RNAi-mediated degradation of Wee1b mRNA results in oocytes maturing in the presence of high levels of cAMP and hence PKA activity. Furthermore, PKA-mediated phosphorylation of S15 on WEE1B increases its kinase activity and expressing in Xenopus oocytes a putative constitutively active form of WEE1B in which S15 is mutated to an aspartate reside inhibits progesterone-induced maturation; in Xenopus, in response to luteinizing hormone, follicle cells secrete a steroid, e.g., progesterone, that acts on a membrane receptor in the oocyte to induce maturation.

In a paper published in this issue of *Cell Cycle*, Pirino and colleagues provide compelling evidence that PKA-mediated phosphorylation of CDC25B is essential to maintain meiotic arrest in mouse oocytes.[3] Mouse oocytes express three forms of CDC25, namely, CDC25A, -B and -C. Cdc25c null mice are fully fertile, excluding a role for this isoform in maintaining meiotic arrest. Cdc25a null mice are embryonic lethal, but results of recent experiments suggest that although CDC25A is involved in maturation, it is not the primary family member responsible for maintenance of meiotic arrest.[4] In contrast, Cdc25b is clearly implicated in maintaining meiotic arrest because Cdc25b null female mice are infertile and oocytes obtained from such mice do not initiate maturation, i.e., breakdown of the nuclear membrane.[5] Moreover, expressing an active form of CDC25B in Cdc25b null oocytes results in resumption of meiosis and activation of CDC2A, whereas expressing a catalytically inactive form of CDC25B in these oocytes fails to initiate meiotic maturation.

Pirino and colleagues report that CDC25B is directly phosphorylated by PKA on S321, consistent with results of immunoprecipitation experiments using a FLAG-HA-mCherry-CDC25B that document an interaction with PKA. In addition, expressing a mutated form that cannot be phosphorylated (S321A) results in oocytes undergoing maturation in the presence of a phosphodisterase inhibitor. In Xenopus, CDC25C appears to be the target of PKA, and phosphorylation of CDC25C generates a binding site for a 14-3-3 protein that sequesters CDC25C within the cytoplasm, thereby preventing translocation to the nucleus where it functions. A similar situation appears in mouse in which CDC25B phosphorylation generates a 14-3-3 binding site that results in CDC25B localizing to the cytoplasm. For example, FLAG-HA-mCherry-CDC25BS321A quickly localizes to the nucleus whereas FLAG-HA-mCherry-CDC25B remains in the cytoplasm.

With CDC25B serving as a target for PKA in which CDC25B function is inhibited by its association with 14-3-3 and PKA mediating an activating phosphorylation of WEE1B, what emerges is a simple model for the role of PKA in maintenance of meiotic arrest and resumption of meiosis. In the presence of elevated levels of cAMP, the ability of PKA both to inhibit CDC25B and activate WEE1B maximally suppresses CDC2A-CCNB activity and thereby maintains meiotic arrest. The maturation-associated decrease in oocyte cAMP triggers resumption of meiosis by decreasing PKA activity with the concomitant activation of CDC25B and inhibition of WEE1B, the outcome being activation of CDC2A. After about 35 years since first ascribing a role for cAMP in oocyte maturation, the circle is finally closed.[6]

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Richard Schultz; Department of Biology; University of Pennsylvania; Philadelphia, Pennsylvania; Email: rschultz@sas.upenn.edu

Crossing borders in search of functions

The role of Cdk2 in meiosis

The eukaryotic cell cycle is tightly orchestrated to achieve optimal proliferation as dictated by external conditions or the surrounding cellular milieu. This finely tuned process can also be modified when necessary to allow cellular differentiation and the achievement of developmental goals. Meiosis and gamete formation is one example of a differentiation process in which the cell cycle is modified to produce cells that can perform a specific function. The molecular details of mammalian gametogenesis have been difficult to decipher due to our inability to effectively recapitulate the process in vitro. In contrast, the budding yeast *Saccharomyces cerevisiae* has proven to be an exceptional model for gametogenesis in that it is genetically tractable and can be induced to synchronously initiate and proceed through the process.

Cyclin dependent kinases (Cdks) provide the major impetus for progression through cell cycle transitions during both mitotic proliferation and meiotic differentiation. Mammalian cells express several Cdks that participate in cell cycle regulation: Cdk1, Cdk2, Cdk4 and Cdk6. Although deletion of any of the Cdks is deleterious, it has come as a surprise that only Cdk1 is essential.[1] Cdk2 appears to have a critical role in gamete formation. $Cdk2^{-/-}$ mice are viable but display infertility with germ-line cells arresting prior to the first meiotic division.[2]

In this issue of *Cell Cycle*, Szwarcwort-Cohen et al.,[3] have postulated that Cdk2 is a functional homolog of the budding yeast meiosis-specific protein kinase Ime2. The yeast Ime2 displays sequence similarity to Cdks but unlike Cdks does not require a cyclin partner for activity. Ime2 performs multiple functions in meiosis ranging from the regulation of meiosis-specific gene expression to initiating DNA replication and meiotic chromosome divisions. Although Ime2 is Cdk-like and performs many essential functions in meiosis it cannot replace the yeast Cdk1 homolog Cdc28 which is required to promote DNA replication and the meiotic chromosome divisions both during meiosis and during mitotic growth.

Szwarcwort-Cohen et al. tested their hypothesis by expressing Cdk1, Cdk2 or Cdk4 in yeast cells harboring an ime2 deletion and inducing the cells to initiate meiotic differentiation. While none of the Cdks tested could fully rescue meiosis and gamete formation, data are presented suggesting that Cdk2 may have the ability to partially suppress the defects in DNA replication and the expression of a subset of meiosis-specific genes. Although it may be an over statement to call Cdk2 a true functional homolog of Ime2, it does appear to perform a subset of the same functions. Given the array of meiosis-specific tasks performed by Ime2 it is perhaps not surprising that Cdk2 could not provide a full replacement of its functions. The consensus sites for phosphorylation by Cdk2 or the yeast Cdc28 (S/T*-P-x-K/R) and Ime2 (R-P-x-S/T*-A/G) are different however many of the same proteins phosphorylated by yeast Cdc28 can also be phosphorylated by Ime2. In some cases, the same sites are phosphorylated but in others different sites are targeted but the same end is achieved. Only a fraction of all Ime2 substrates have been identified and it is likely that at least a subset will be targeted by Ime2 but not Cdk activity. The difference in consensus sites likely ensures that some protein substrates that are specific for Cdk and some for Ime2.

Interestingly mammalian cells express a testis specific protein kinase Mak1 (male germ-line associated kinase) and a close homolog MRK1 (Mak1 Related Kinase) that are similar to Ime2 and display a similar phosphorylation site consensus (R-P-x-S/T-P).[4] At least in mice Mak1 is not essential for meiosis.[5] Perhaps the overlapping consensus phosphorylation sites between Mak1 and Cdk2 allow Cdk2 to perform all of the necessary meiotic functions in the absence of Mak1 but further investigation of the roles of both enzymes in meiosis are needed before such a conclusion can be drawn.

A particularly interesting question raised by this work is: what yeast cyclins are binding to and activating Cdk2 in these experiments? Cyclins typically provide substrate specificity to the Cdk and in yeast Clb5 and Clb6 bind yeast Cdk1 to promote meiotic DNA replication, however, they cannot replace Ime2. In mammalian meiosis Cdk2 pairs with cyclin A1 to perform its functions. Perhaps the heterologous pairing of human Cdk1 and yeast cyclins provides Cdk2 with a substrate specificity that allows it to perform Ime2 functions. Or perhaps Cdk2 is activated by a yeast non-cyclin protein providing it with a new substrate specificity. Whichever is the case, the authors suggest that Cdk2 can fulfill some of the same functions as Ime2, although the ability of Cdk2 to perform those functions is limited. A comprehensive understanding of the role Cdk2 plays in meiosis will require extensive investigations in an animal model. However, since the basic meiotic processes are similar between yeast and mammals, the genetically tractable yeast model may be a tool to reveal the essential functions and substrates of Cdk2 in mammalian gametogenesis.

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David T. Stuart; Department of Biochemistry; University of Alberta; Edmonton, Alberta, Canada; Email: dtstuart@gpu.srv.ualberta.ca