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Cell Cycle News & Views

Targeting Chk1 in the replicative stress response

Comment on: McNeely S, et al. Cell Cycle 2010; 9:995–1004.

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In response to DNA damage or interference with DNA synthesis, transformed cells characteristically activate various checkpoints, which together comprise an important component of a complex signaling network that has been termed the DNA damage response (DDR). Checkpoints identified so far are classified in two ways: (1) cell cycle checkpoints: the G_1/S, intra-S or S, and G_2/M checkpoint; or (2) function-specific checkpoints: the DNA damage checkpoint, the replication checkpoint, the mitotic spindle checkpoint, the cytokinesis checkpoint, etc.[1] The observation that transformed cells are characterized by defects in the checkpoint apparatus[2] has prompted intense efforts to exploit this differential response (relative to normal cells) therapeutically.

Many of these efforts have focused upon the checkpoint kinase Chk1, which, along with Chk2, constitute "distal transducers" within the checkpoint signal transduction pathway. In response to DNA damage [specifically single strand DNA (ssDNA) lesions], Chk1 is activated by the "proximal transducers" ATR and to a certain extent ATM, large PI3 kinase-like proteins which are in turn activated by DNA damage "sensor" proteins in cooperation with "mediator" proteins.[1] Once activated by phosphorylation on Ser317 and Ser345, Chk1 phosphorylates and targets for degradation or cytoplasmic sequestration members of the Cdc25 phosphatase family (e.g., A, B and C), leading to inhibitory phosphorylations of cyclin-dependent kinases (CDKs), most notably cdk1 ($p34^{cdc2}$) and cdk2. Inhibition of such CDKs is critical for cell cycle arrest in the face of DNA damage or disruption of the DNA replicative machinery. Conversely, inhibition of Chk1 disables this checkpoint mechanism, allowing cells that have sustained DNA damage to continue their cell cycle traverse inappropriately. This leads to cell death, although the mechanisms by which this event occurs have not been fully elucidated. Notably, recent studies suggest that in addition to its critical role in checkpoint control, Chk1 serves multiple other functions, including direct involvement in cell survival and DNA repair, among others.[3]

In general, attempts to exploit disruption of Chk1 function have focused on two distinct strategies: potentiation of the lethality of agents that (1) induce DNA damage (e.g., topoisomerase inhibitors[4] or (2) interfere with DNA replication (e.g., nucleoside analogs).[5] Attempts to translate such strategies into the clinic have been limited by the toxicities and lack of specificity of available Chk1 inhibitors (e.g., UCN-01).[6] However, a new generation of more selective Chk1 inhibitors has recently emerged, raising the possibility that newer combination regimens will display enhanced efficacy.

Although the concept of enhancing nucleoside analog activity by Chk1 inhibition is not entirely new,[7] the mechanisms underlying such interactions have not yet been fully defined. However, the recent discovery that Chk1 plays a central role in the DNA replication checkpoint induced by replication stress absent exogenous insults[8] has focused attention on this therapeutic strategy. In the study by McNeely et al., the authors investigated factors contributing to synergism between AZD7762, a new selective and clinically relevant Chk1 inhibitor and the nucleoside analog gemcitabine in various epithelial malignancies. The major finding was that enhanced lethality for the combination very likely involved multiple mechanisms, including reversal of inhibition of replication origin firing and alterations replication fork dynamics, accompanied by DNA damage. In essence, AZD7762 converted gemcitabine-related stalled replication forks into double-strand breaks (DSBs). Significantly, cells with defects in the DNA repair machinery were particularly sensitive to this strategy. Collectively, these findings provide a theoretical foundation for rational attempts to enhance the activity of clinically useful nucleoside analogs by novel and selective Chk1 inhibitors.

The findings described in this study have potentially important implications for the development of second-generation Chk1 inhibitors such as AZD7762, particularly in combination with nucleoside analogs such as gemcitabine which are active against slowly proliferating epithelial tumors. For example, the observation that multiple mechanisms contribute to the lethality of such regimens could explain why a single pharmacodynamic determinant may not predict for activity. Conversely, distal events such as DNA damage induction, reflected by γH2A.X formation, may represent a final endpoint indicative of tumor cell responsiveness. A particularly interesting finding was that cells exhibiting defects in the DNA repair process may be uniquely susceptible to this strategy, analogous to the sensitivity of BRCA1 mutant cells to PARP[9] and Chk1 inhibitors.[10] Such cells may also display "synthetic lethality" when exposed to regimens combining Chk1 inhibitors with agents that induce DNA damage and disrupt DNA replication. Finally, it will be important to determine if the theoretical promise of this strategy can be realized with newer generation and more selective Chk1 inhibitors that have now entered the clinic.

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Targeting the MYCN effector, FAK, in neuroblastoma

Comment on: Beierle EA, et al. Cell Cycle 2010; 9:1005–15.

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Neuroblastoma is the fourth most common pediatric malignancy, comprising about 8% of all cancer diagnosed in children under 15 years of age.[1] Yet it is responsible for a disproportionate number of deaths in children from cancer, approximately 15% of all pediatric cancer deaths.[2] Neuroblastoma is a very heterogeneous disease: tumors can spontaneously regress or mature or display a very aggressive, malignant phenotype. And so, it has been of great interest to both clinicians and basic scientists. Progress in molecular and cellular biology has contributed greatly to a better understanding of neuroblastoma. In fact, neuroblastoma has served as a model for a molecular approach to treating patients with cancer, as increasing evidence indicates that the biologic and molecular features of neuroblastoma are highly predictive of clinical behavior. Therefore, current treatment of children with neuroblastoma is based not only on stage but also on risk stratification. This risk stratification takes into account both clinical and biologic variables predictive of disease relapse, with the status of the *MYCN* proto-oncogene (amplified or non-amplified) within the tumor cells currently being the most powerful biologic factor.[3,4] Overall, approximately 25% of primary neuroblastomas in children have *MYCN* amplification, with *MYCN* amplification being present in 40% with advanced disease.[3]

Unfortunately, this progress and understanding have not significantly altered the clinical outcome for children with advanced-stage, high-risk neuroblastoma. Although the prognosis for these patients has improved somewhat recently, the long-term outcome remains very poor. This is particularly true for patients whose tumors are *MYCN*-amplified. Clearly new treatment strategies are needed for these patients. One of the most exciting prospects for improving anti-tumor activity, as well as overcoming the problem of tumor resistance to therapy, involves targeted therapy. Information about the molecular profile of a given tumor type, specifically the mechanisms of tumorigenesis and proliferation control, can be translated into new drug development designed to induce differentiation of tumor cells, block their growth pathways and/or cause tumor cell death. These new agents can be used independently or in concert with traditional regimens. Elucidation of the complex molecular pathways involved in tumorigenesis hopefully will lead to the production of targeted anticancer agents with high specificity, efficacy and therapeutic index.

The study by Beierle et al. published in the last issue of *Cell Cycle*[5] presents exciting new data using just such an approach. This group has previously shown that MYCN regulates intratumoral expression of focal adhesion kinase (FAK) in neuroblastoma.[6] FAK is an intracellular kinase that regulates both cell adhesion and apoptosis,[7] and is overexpressed in a number of human cancers, including neuroblastoma,[8] where its overexpression is found in association with *MYCN*-amplification. Therefore, in this study, the authors have chosen to inhibit FAK activity as a downstream effector of MYCN, using the small molecule inhibitor of FAK phosphorylation, 1,2,4,5-benzenetetraamine tetrahydrochloride (Y15), in a targeted approach to treating neuroblastoma. The authors report that this drug successfully inhibited FAK phosphorylation at the Y397 site both in vitro and in vivo, increased neuroblastoma detachment and apoptosis and decreased cell viability in vitro and decreased heterotopic xenograft growth in nude mice in vivo. Interestingly and importantly, they found that the observed effects were much more profound in *MYCN*-amplified neuroblastoma cell lines, thereby supporting the rationale for the use of this FAK inhibitor.

A number of pre-clinical questions remain, however, including the efficacy of this approach in more relevant xenograft models of orthotopic and disseminated disease, as well as transgenic mouse models of *MYCN*-amplified neuroblastoma and the potential toxicity of systemic FAK inhibition. In addition, although "significant" in statistical terms, the results of FAK inhibition, when used as monotherapy to effect tumor growth inhibition in vivo, were fairly modest. Perhaps this approach might be better suited for use as a component of combination therapy. Or perhaps refinements in the timing of drug administration and/or dosing are needed. Nevertheless, these results are the latest in an increasing number of exciting proof-of-principle studies whereby a targeted anti-cancer approach has been used successfully in a pre-clinical model, providing hope that this general approach will provide new reagents for the successful treatment of neuroblastoma and other challenging cancers in the near future.

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NFκB and p53: A life and death affair

Comment on: O'Prey J, et al. Cell Cycle 2010; 9:947–952.

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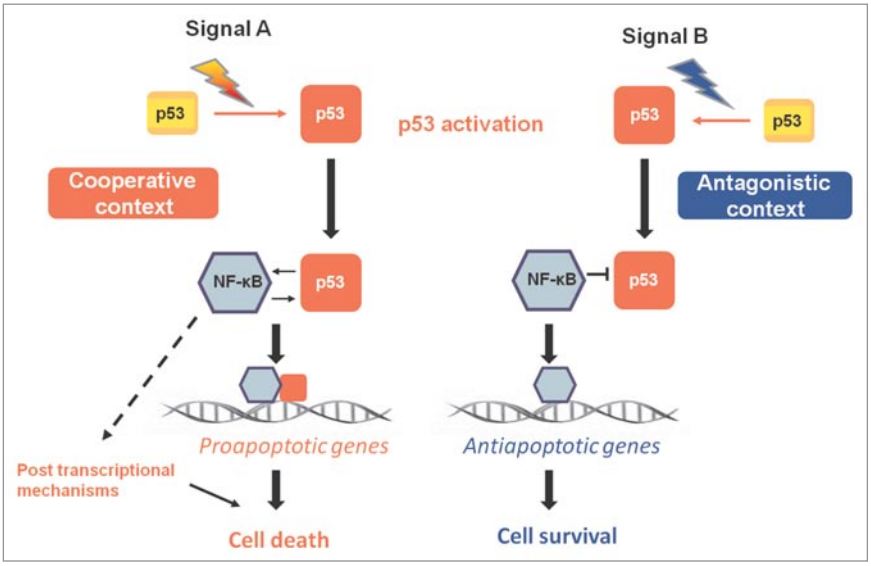
p53 and nuclear factor kappaB (NFκB) are two pivotal transcription factors that play crucial roles in human cancer. Common wisdom has it that p53 promotes cancer cell death while NFκB blocks it. This is consistent with the observation that p53 is very frequently inactivated in tumors, whereas NFκB is often hyperactive.[1] Yet, the picture is much more complex. In fact, p53 can also exert prosurvival effects,[2] whereas NFκB can actually sometimes augment cell death rather than prevent it. Not surprisingly, there is ample documentation of functional and molecular cross-talk between p53 and NFκB, a cross-talk that is highly context-dependent.[3] Thus, while these two transcription factors were initially shown to engage in mutually-inhibitory interactions, for instance by competing for limited amounts of coactivators such as p300, subsequent reports also demonstrated positive interactions. Notably, it was shown that the p65 subunit of NFκB is required for efficient induction of apoptosis by p53 in some settings.[4,5] The complexity of these two transcription factor pathways, their intimate association with cancer and the fact that they are coordinately activated by certain types of signals, most notably genotoxic stress, call for further intensive research into the molecular details and biological outcome of their interaction.



In a previous issue of *Cell Cycle*, O'Prey and collaborators[6] tackle this complicated cross-talk. By blocking NFκB function in cells with inducible p53, the authors could show that p53-driven cell death was strongly dependent on constitutive NFκB activity. Despite its surprising ability to augment p53-induced apoptosis, NFκB attenuated TNFα-induced death as expected, further reinforcing the notion that its impact on cell death is highly context dependent. NFκB blockade did not compromise nuclear p53 accumulation nor did it affect the general ability of p53 to transactivate a variety of target genes. Yet, the authors found a selective defect in the induction of two key proapoptotic proteins encoded by p53-regulated genes. Thus, the p53-induced increase in the levels of NOXA protein, a proapoptotic member of the Bcl-2 family, was considerably compromised when NFκB was constitutively inhibited. Surprisingly, NOXA mRNA levels were unaffected by NFκB blockade, implying a post-transcriptional mechanism. Further analysis of many p53-regulated transcripts revealed that, while most were not affected by NFκB blockade, the levels of p53aip1 mRNA were strongly reduced. Together, these observations revealed new molecular links between p53 and NFκB in the positive regulation of cell death.

The study of O'Prey et al. still leaves many unanswered questions. One intriguing finding is that NFκB maintains high NOXA protein levels via a post-transcriptional mechanism. It will be of interest to find out whether this involves regulation of NOXA protein stability, a largely uncharted territory. Alternatively, might it be that NFκB represses a particular microRNA, which normally restricts the translation of NOXA mRNA? Further exploration of this interesting preliminary finding is likely to be rewarding.

In sum, complementing earlier studies, the findings of O'Prey and coworkers suggest that the impact of NFκB on p53-mediated apoptosis is largely dependent on the cellular environment and the nature of the signal that activates p53 (Fig. 1), and that both transcriptional and post-transcriptional mechanisms are at play. Furthermore, these findings argue compellingly that the cross-talk between p53 and NFκB must be investigated in a context-dependent way; broad generalizations are likely to be misleading. Gaining further insights into the rules of this complicated game and the ways in which its outcome can be manipulated may pave the road towards better defined, more effective therapeutic approaches.[7,8]

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Novel findings on endoribonuclease activity of proteasomes

Comment on: Kulichkova VA, et al. Cell Cycle 2010; 9:840–9.

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26S proteasomes are large multisubunit enzymatic complexes that play the key role in degradation and processing of cellular proteins. They maintain the level of important regulatory proteins in the cell, which makes them crucial for the cell life in general and for regulation of cellular events in particular. The proteasome population is structurally and functionally heterogeneous. 26S proteasomes consist of a 20S core particle and its associated various regulatory particles. Apart from several endopeptidase activities, the eukaryotic 20S core displays a number of other activities, including protein chaperone-, DNA-helicase- and endoribonuclease activities. The proteasome activities are very well regulated, specifically due to post-translational modifications.[1,2]

The endoribonuclease activity of the 20S core was discovered and thoroughly studied in the 1990s. This activity is tightly associated with the 20S core and preserved after treatment with strong detergent (lauroylsarcosinate) or even with 6M urea. It showed high thermolability, unlike many other endoribonucleases. Structurally, the 20S core is a cylinder formed by four stacked heptameric rings. Two inner rings are formed by β-subunits and responsible for all endopeptidase activities, while two outer rings are composed of α-subunits. As shown, two of the latter, zeta (α5) and iota (α1), are responsible for the endoribonuclease activity. This activity was found to be RNA-specific, because the 20S core efficiently cleaved TMV RNA, 18S- and 28S rRNAs, and RNAs from adenovirus-infected HeLa cells, but failed to cleave 5S rRNA, yeast tRNAs and globin mRNA.[3,4]

20S cores isolated from Friend leukemia virus-infected mouse spleen cells were found to be associated with RNA fragments showing high homology to the 3′-untranslated region (UTR) of tumor necrosis factor-β that contains an AUUUA sequence known as the ARE motif, the instability element typical of many short-lived mRNAs. In vitro experiments using model substrates showed that the association of 20S cores with AUUUA-containing mRNA 3′UTR fragments resulted in the endoribonuclease-induced rapid degradation of these fragments at specific cleavage sites. Since not all of the 20S core-cleaved RNAs contained ARE, it was proposed that there may exist other proteasome-recognized elements, like a secondary structure common for all proteasome-sensitive RNAs. The selective mRNA degradation under the action of 20S cores correlates with the finding that the latter are capable of selective binding to some mRNAs and inhibiting their translation in a cell-free system. This suggests that proteasomes are involved in selective translational control through degradation of free mRNAs containing some specific sequences or secondary structures.[5]

The paper by Kulichkova et al., published in a recent issue of *Cell Cycle* describes the endoribonuclease activity of 26S proteasomes and the effects of extracellular signals on this activity. The authors are the first to show that similar to the 20S core, 26S proteasomes exhibit endoribonuclease activity and are capable of hydrolyzing various cellular RNAs, including AU-rich mRNAs of *c-mic* and *c-fos* in vitro. It is reported that the endoribonuclease activity of 26S proteasomes is conferred by the subunit α5 that also provides similar activity of 20S cores. The endoribonuclease activity of 26S proteasomes varies in response to different extracellular signals [hemin in case of human erythroleukemia cells K562 or epidermal growth factor (EGF) in case of human epidermoid carcinoma cells A431]. Besides, this activity is shown to contribute to degradation of *c-mic* mRNA at hemin-induced differentiation of the K562 cells.

As found by this team previously, the alkaline phosphatase-induced dephosphorylation of 26S proteasomes resulted in complete suppression of its endoribonuclease activity in vitro.[6] So, the current paper addresses the correlation between the endoribonuclease activity of proteasomes isolated from stimulated cells and the phosphorylation level of the proteasome subunits in vivo. It appears that the endoribonuclease subunit α5 of the K562 cells shows no notable phosphorylation. However, its adjacent subunit α6 undergoes phosphorylation at hemin-induced cell differentiation, and the authors propose that phosphorylation of α6 may affect endoribonuclease activity of α5, possibly by changing its conformation.

Previously, the authors showed that proteasomes penetrated into cells from the incubation mixture by an unknown mechanism and produced different effects on expression of different genes.[7] Here they report about similar experiments, except that the protease activity of the proteasome was inhibited by MG132 to avoid its effect on gene expression. Addition of such a proteasome with preserved endoribonuclease activity to the K562 cells resulted in a 20% decrease of *c-myc* mRNA, while in the same cells stimulated by hemin this decrease reached 40%. The authors believe that the endoribonuclease activity of 26S proteasomes contributes to *c-myc* mRNA degradation in vivo and thereby promotes cell differentiation.

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BubR1 highlights essential function of Cdh1 in mammalian oocytes

Comment on: Wei L, et al. Cell Cycle 2010; 9:1112–21.

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BubR1 is one member of the Spindle Assembly Checkpoint (SAC) family of proteins. Two recent papers[1,2] have taken an antisense approach to explore its role in female mammalian meiosis. They add to the body of evidence confirming a surveillance role of the SAC in female meiosis I, challenging the idea that high aneuploidy incidence in oocytes is due lack of SAC function. Interestingly they reveal some unexpected insights into a SAC-independent BubR1 function.

Both groups report on the ability of BubR1 knockdown to promote meiotic resumption from prophase I arrest, an effect mediated by its ability to stabilize levels of Cdh1. The importance of Cdh1 to prophase I arrest was first observed in 2006,[3] and has now been established by a number of groups.[4-7] Proteins familiar to the mitotic field, such as securin, Cdc14B and Emi1 have all been shown to modulate APC^{Cdh1} mediated cyclin B1 degradation, a process that appears to be essential to maintain arrest[4,6,7] (Fig 1A, i). What could not have been predicted from mitotic studies however is that BubR1 stabilizes Cdh1 levels,[2] a relationship made more intriguing by the observation that Cdh1 knockdown also leads to a loss in BubR1. One exciting idea based on these findings is that there exists a novel meiotic ubiquitin ligase, possibly an APC activator, recognizing as substrates both Cdh1 and BubR1. Loss of either Fzr1/Cdh1 or BubR1 would therefore lead to greater degradation of the other (Fig 1A, ii). Both substrates contain degradation signals that could be recognized by APC^{Cdh1}. However in oocytes it is unlikely to be APC^{Cdh1} degrading BubR1, given Cdh1 knockdown leads to BubR1 loss rather then stabilization.

Following exit from prophase arrest during prometaphase I both papers report on a SAC function for BubR1. Wei et al.[1] observed accelerated passage through meiosis associated with antisense BubR1, consistent with a compromised SAC. However, for Homer et al.[2] BubR1 depletion blocked oocytes from undergoing meiosis. The reason for the arrest was found to be excess securin, which was degraded by APC^{Cdh1}. High levels of securin would overwhelm APC^{Cdc20} at metaphase I,

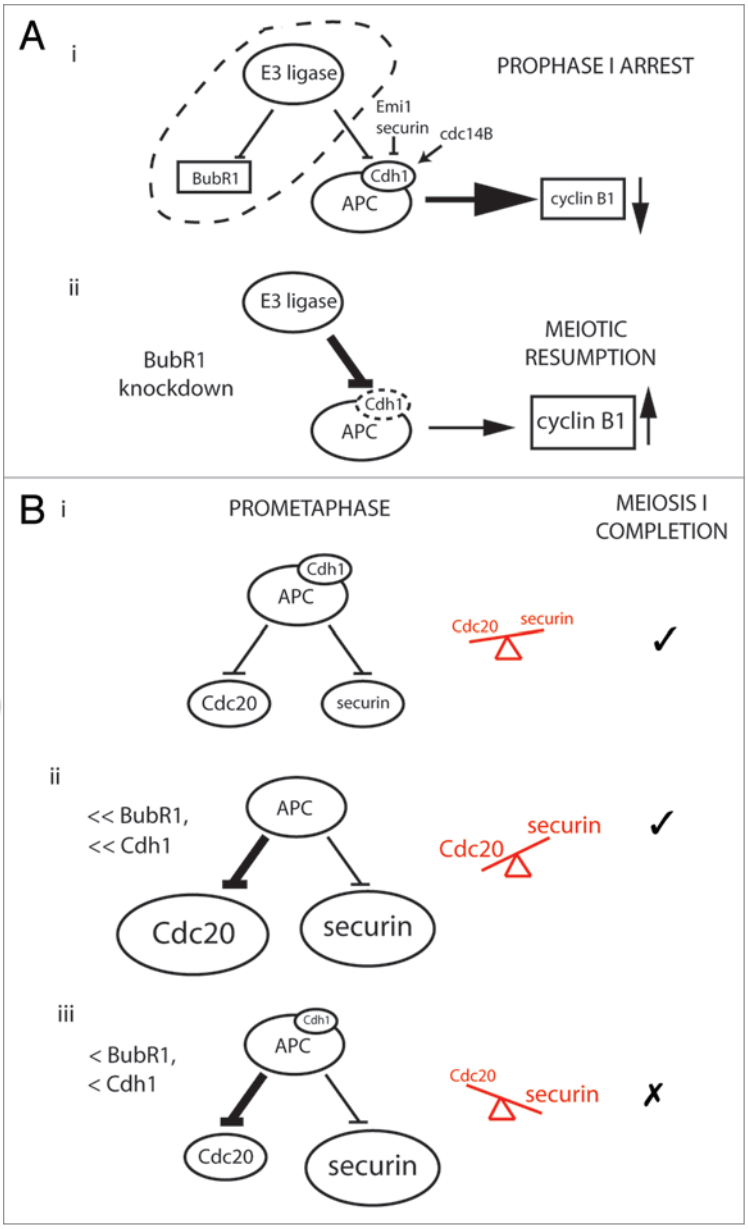

Figure 1. Prophase I meiotic arrest (A) and MI completion (B) in mammalian oocytes requires Cdh1 and BubR1. (A,i) Arrest is maintained via APC^{Cdh1} -mediated cyclin B1 degradation. Cdc14B positively regulates APC^{Cdh1} whilst Emi1 and securin are negative regulators. Recent knockdown of BubR1 in mouse oocytes[1,2] shows BubR1 also influences APC^{Cdh1} stability, possibly via another E3 ligase (dotted lines indicate proposed pathway). (A,ii) Loss of BubR1 causes oocytes to undergo premature meiotic resumption due to rising cyclin B1. (B,i) During prometaphase, APC^{Cdh1} activity maintains an appropriate balance of Cdc20 and securin to allow meiotic progression. (B,ii) Near-complete knockdown maintains Cdc20 and securin levels such that Cdc20 levels are high enough to degrade any excess securin, override the SAC and allow meiotic progression. (B,iii) In the case of incomplete BubR1/Cdh1 knockdown remaining APC^{Cdh1} favors Cdc20 degradation resulting in excess securin that prevents meiotic progression. Such a model relies on the premise that APC^{Cdh1} preferentially degrades Cdc20 in conditions of low APC^{Cdh1}.

preventing it from degrading enough cyclin B1 and securin to allow anaphase. Why would APC^{Cdh1} be active in prometaphase I, and why was arrest not observed by Wei and colleagues?

APC^{Cdh1} activity during prometaphase I has been observed previously where it was found to degrade Cdc20.[8] Assuming prometaphase APC^{Cdh1} degrades both securin and Cdc20 its physiological function may be explained in the following way. Prometaphase securin, present in excess, as a result of its cyclin B1 protective role during prophase I arrest,[6] needs to be degraded in order to ensure that it does not overwhelm APC^{Cdc20} activity at the end of meiosis I. This is achieved by APC^{Cdh1}, which at the same time also degrades Cdc20, so preventing the possibility of any premature APC^{Cdc20} activity, which can segregate homologous chromosomes too early and lead to aneuploid eggs[8] (Fig 1B, i).

Although the above ideas may help explain a physiological need for prometaphase I APC^{Cdh1} activity, do they help reconcile the phenotype observed between the two studies?[1,2] One consideration may be the extent of BubR1 (and so Cdh1) knockdown. Large amounts of BubR1/Cdh1 knockdown may lead to completion of meiosis I, as observed by both Wei et al. (for BubR1)[1] and Reis et al. (for Cdh1)[8] (Fig 1B, ii). The predominant factor here being the much larger amount of Cdc20 present in these eggs, and as such APC^{Cdc20} activity being high enough to degrade any amount of excess securin. If a smaller level of Cdh1 knockdown occurs then it may be possible to arrest oocytes (as in ref. 2) if there is too little Cdc20 and too much securin. Such a scenario would be possible if Cdc20 were degraded in preference to securin at lower levels of APC^{Cdh1} (Fig 1B, iii).

Unfortunately there is much supposition in the above. It would be useful now to have an oocyte-specific knockout of Cdh1 (an unconditional knockout is embryonic lethal, ref. 9) to examine its meiotic role in more detail and with no concerns regarding the level of knockout achieved through antisense approaches.

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A dexter exo within p53

Comment on: Bakhanashvili M, et al. Cell Cycle 2010; 9:In press.

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More than 14 years ago a 3´-5´ exonuclease activity has been found as intrinsic part of the p53 tumor suppressor protein.[1] Already then it was suggested that the 3´-exo might act as a proofreader that corrects errors made by DNA polymerases and thereby increases their fidelity. Thereafter, it was demonstrated that p53 indeed binds to the proofreading-deficient DNA polymerase (Pol) α[2] and, moreover, enhances the accuracy of this enzyme at least in vitro.[3,4] However, these findings were not well received, mainly because transgenic mice lacking functional p53 apparently did not display a mutator phenotype.[5] Despite this, several groups independently demonstrated subsequently that p53 exerts an exonuclease function that may be involved in error correction during DNA replication and various DNA repair processes. Very recently the group of Carol Prives demonstrated the importance of the L1 loop and particularly the histidine at position 115 for the exonuclease activity of p53. Mutating His115 to asparagine (H115N) increased DNA binding and caused a better expression of the p53-responsive genes p21, PIG3 and MDM2, but markedly reduced the exonuclease activity and the ability to induce apoptosis.[6]

A new twist came to the story, when it was demonstrated that p53 interacts physically with the catalytic subunit of the mitochondrial Polγ in response to mtDNA damage particularly induced by reactive oxygen species.[7] This came as a surprise as Polγ carries a proofreading activity on its own. In accord with this, Mary Bakhanashvili's lab showed a functional cooperation between p53 and Polγ that increased the fidelity of DNA replication in this cellular organelle[8] and Allan Fersht´s lab reported a physical and functional interaction between p53 and the mitochondrial single-strand DNA binding protein mtSSB[9] that was strongly reminiscent to the earlier observed interaction between human p53 and the nuclear single-strand binding protein and Polα interactor RPA. Like nuclear RPA, the mtSSB bound to the N-terminal part of p53. Moreover, mtSSB markedly enhanced the exonuclease activity, particularly in hydrolyzing 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxodG) present at the 3′-end of oxidized DNA.[9] In the forthcoming issue of *Cell Cycle* the Bakhanashvili group reports on the ability of p53 to recognize and bind to 3´ mismatched ends, a prerequisite for the excision of mismatched or damaged nucleotides. Their data demonstrate that p53 binds oligonucleotides possessing a 3´-mismatch with no apparent sequence preference. Therefore, p53 can be regarded as a genuine mismatched nucleotide binding protein. A very important caveat about the biological significance of the recent exciting findings still is the rather low specific activity of the exonuclease of p53. In comparison with nucleases involved in DNA repair and recombination and even proofreading nucleases, the reported activities of p53 from all labs falls about 10- to 100-fold lower. Could p53 therefore be a proofreader for damaged bases only? In the light of the existing recent results, it will become extremely important to thoroughly quantify to what extent damaged nucleotides, particularly oxidized ones, will be recognized and excised by p53. p53 has already been good for many surprises. Why not once again?

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