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Cell Cycle News & Views

Running m(o)TOR with the brakes on leads to catastrophe at mitosis

Comment on: Leontieva OV, et al. Cell Cycle 2012; 11:3926–31;
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Two parallel and concordant activities characterize cell cycle progression. One is associated with preparation for and execution of DNA replication followed by mitosis ("cell cycle"). Cyclins are the motors of the cell cycle, as they activate the respective CDKs and thereby drive the cell through the sequential phases of the cycle. Their antagonists are CKIs, which inhibit the CDKs stalling the cycle progression. The second type of activities involves anabolic processes that contribute to growth in cell size and mass ("cellular growth"). Constitutive signaling along the mTOR/S6K pathways is the key factor mediating these anabolic processes.[1] During unperturbed and balanced growth these two activities are flawlessly coordinated. This synchronization ensures that the cell size, as well the ratio of protein or RNA content to DNA, remains invariable for cells in particular phases of the cycle or for particular cell type. However, during arrest in cell cycle progression, for example, when induced by inhibitors of DNA replication, these activities become uncoupled. The cell growth continues, resulting in an "unbalanced growth" phenotype when the ratio of cell protein/mass to DNA content is greatly augmented. While this phenomenon was initially observed nearly five decades ago,[2] recent evidence underscores its importance and links it mechanistically with senescence and aging.[3-5] Specifically, it has been postulated that cell cycle arrest when is concurrent with the ongoing or intensified mTOR/S6K signaling (growth cycle) results in induction of the unbalanced growth phenotype (cell hypertrophy), which is a characteristic feature of cell senescence as well as considered to be the primary cause of organismal aging.[3-5]

In the currently published article, Leontieva et al.[6] describe that constitutive mTOR signaling during the cell cycle arrest, induced by upregulation of p21, contributed to cell senescence (geroconversion); these cells were characterized by greatly increased levels of cyclin D1 and cyclin E as well as being under replication stress, manifested by markers of DNA damage signaling. When the cycle progression was restored by downregulation of p21, the cells were able to pass through S and G_2 and decrease the level of cyclins D1 and E, but then they underwent either mitotic catastrophe or entered higher DNA ploidy by endoreduplication. Suppression of mTOR signaling, either by rapamycin or by nutlin 3a in the cells arrested by p21, prevented geroconversion, lowered the level of cyclin D1 expression and, after removal of p21, restored ability to proliferate. In another model cell system, in which nutlin 3a was unable to suppress mTOR signaling but was inducing arrest in G_1 and the senescent phenotype, removal of nutlin 3a led to initiation of DNA replication but could not restore capability to proliferate.

The findings presented by Leontieva et al.[6] underscore the role of mTOR signaling during cell cycle arrest in the induction of either cell senescence or quiescence and restoration of replicative potential. Of particular interest is the observation of massive upregulation of cyclins D1 and E, which, upon restoration of the cycle progression, appeared to initially enhance DNA replication rate (providing the "hyper-mitogenic drive"), but then likely to contribute to the mitotic catastrophe. Clearly, progression of hypertrophic cells additionally accelerated during S appears to be catastrophic when passing later through mitosis.

Highly unbalanced ("unscheduled") expression of not only cyclins D and E but also cyclins A and B1 was previously observed in cells arrested and synchronized at the G1/S boundary by the inhibitors of DNA replication aphidicolin, mimosine or excess of thymidine.[7] These cells, in which the chromosome cycle and growth cycle were also dissociated, resulting in their hypertrophy, when released from the arrest and progressing through S phase, had several-fold higher levels of all the cyclins (D, E, A and B1) compared with S-phase cells in untreated cultures. The elevated level of cyclin A was likely reporting the replication stress, while the elevated level of cyclin B1 was considered to be due to the increased half-life of this protein stabilized by overexpression of cyclin E.[7] Interestingly, following successful mitosis and cytokinesis, the immediate G_1 progeny of these synchronized cells, while they had normal levels of the respective cyclins, still showed some degree of imbalance, characterized by > 30% higher protein to DNA ratio compared with G_1 cells from exponentially growing cultures, and had proliferative capacity.[7] These findings collectively with observations of Leontieva et al.[6] suggest that there is a threshold level of the growth imbalance (cell hypertrophy) defining the "point of no return." The cells that pass the threshold are losing the reproductive potential either through mitotic catastrophe or endoreduplication. The cells below this threshold attempt to return to balanced growth either through accelerated rate of cell cycle progression vis-à-vis growth cycle,[8] by autophagy[9] or by both mechanisms and preserve their replicative potential. The intensity of mTOR signaling combined with duration of the cell cycle arrest plays a critical role as to whether the cells do pass this point or not.

The possibility of decoupling "cell cycle" from the "growth cycle" to induce cell hypertrophy and irreversible senescence suggests an interesting anticancer strategy. The activation of many oncogenes and/or dysfunction of tumor suppressor genes, either one leading downstream to an increased mTOR signaling, is a hallmark of most cancers. Cells of such tumors, therefore, would be more predisposed to undergo senescence under conditions of persistent chromosome cycle arrest that induces DNA replication stress compared with normal cells. We have recently reported that prolonged treatment of the non-small cell lung carcinoma cells, characterized by strong mTOR signaling, with very low doses of DNA alkylating drug mitomycin C, induced cell cycle arrest, replication stress and, similarly, as in the case of p21-induced arrest,[6] endoreduplication.[10]

This led to their senescence and irreversible loss of reproductive capability.[10] The induction of cell senescence by the low doses of DNA damaging drugs or other means of arresting the cell cycle rather than induction of apoptosis by high drug doses, typical of standard chemotherapy, may be therefore more effective in treatment of cancers characterized by high level of mTOR signaling, with less side effects for the patient. Screening tumors for the presence of activation of such oncogenes may select patients sensitive for this type of chemotherapy, providing a personalized cancer treatment approach.

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The many roads to checkpoint activation

Comment on: Wang G, et al. Cell Cycle 2012; 11:3792–800; PMID:23070520; http://dx.doi.org/10.4161/cc.21987

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The genome of eukaryotic organisms is under constant surveillance by the DNA damage checkpoint (DDC) signaling network. In budding yeast, the sensor kinase Mec1^{hATR} and the downstream kinase Rad53^{hCHK2} are the main checkpoint kinases in the DDC network. Mec1 and Rad53 play crucial roles in the preservation of genomic integrity and cell viability, as they regulate key effector proteins involved in processes such as DNA replication, repair, transcription and cell cycle control. Over the past 15 y, significant progress has been made in understanding how checkpoint kinases are activated, but are we close to a full mechanistic understanding of DDC activation and regulation? According to a recent paper in the October 15, 2012 edition of *Cell Cycle* by Wang et al.,[1] the answer is: not really.

The work by Wang et al. addresses the mechanism of signal transduction from Mec1 to Rad53, which is a critical step in the activation of the downstream portion of the DDC network. In budding yeast, the Rad9 adaptor protein is a key player in this step, functioning to recruit Rad53 molecules to sites of DNA damage where Mec1 is located.[2] How Rad9 recognizes sites of DNA lesions is a central question that has attracted the attention of several laboratories studying DDC signaling. It is now clear that Rad9 can be recruited to sites of DNA damage through redundant mechanisms that rely on histone modifications. The TUDOR domain of Rad9 recognizes histone H3K79 methylated by the Dot1 methylase, and the BRCT domain of Rad9 binds to histone H2A phosphorylated by Mec1 at S129 (H2A^{pS129}).[3,4] Additionally, Rad9 can be recruited by the BRCT domain-containing protein Dpb11^{hTopBP1}, which directly interacts with the 9-1-1 clamp and Mec1 at sites of DNA damage.[5] The Rad9-Dpb11 interaction is mediated by Cdc28 (yeast CDK)-dependent phosphorylation of Rad9, which is subsequently recognized by the BRCT domains of Dpb11. How CDK-dependent phosphorylation of Rad9 is recognized by Dpb11 has been the focus of recent investigations.[6,7] An initial report by Granata et al. revealed that CDK-dependent phosphorylation of Rad9 on serine 11 (S11) is important for the interaction.[6] In contrast, a more recent report from Pfander and Diffley indicated that the CDK-dependent phosphorylation of Rad9 on S462 and T474 plays a preponderant role in mediating the Rad9-Dpb11 interaction.[7] In the October 15, 2012 edition of *Cell Cycle*, Wang et al. provide new insights that may reconcile these two previous reports. Using a series of point mutations aimed at disrupting different CDK consensus phosphorylation sites (over 17 phosphosites were mutated in different combinations), the authors show that multiple CDK phosphosites on Rad9 can function in a redundant manner to promote Rad53 activation. While the authors confirm that S462 and T474 are indeed important, their data show that other CDK phosphosites also contribute to the Rad9-Dpb11 interaction, revealing additional layers of redundancy in how Rad9 can be recruited.

Work by several laboratories has unveiled multiple modes of Rad9 recruitment, prompting the question of why there is so much redundancy toward Rad53 activation. It is clear that these different mechanisms of Rad9 recruitment are under distinct spatial regulation. While H3K79 methylation is widespread on chromatin and does not appear to be responsive to DNA damage, H2A^{pS129} is mostly induced by DNA damage at regions of a few kilobases surrounding the lesions.[3,8] On the other hand, recruitment of Dpb11 is mostly dependent on the 9-1-1 clamp that is likely loaded specifically at sites of lesions.[5] These observations suggest that each mode enables recruitment at distinct proximities to the site of lesion. It is tempting to speculate that in addition to providing "back-up" mechanisms, these different modes of recruitment cooperatively help cells fine-tune the levels and dynamics of Rad53 activation. To test this hypothesis, it would be important to use genotoxic conditions that more closely mimic physiological levels of stress. While relatively high doses of genotoxins are useful in identifying key players in the DNA damage response, they may mask many of the regulatory aspects of the response. Based on the work by Wang et al., it is possible to envision that different types or levels of DNA damage may lead to distinct patterns of Rad9 phosphorylation, creating distinct levels of interaction with Dpb11 and adding even more layers of regulation toward Rad53 activation.

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Alteration/deficiency in activation 3 (ADA3): Regulator of DNA repair and genome stability

Comment on: Mirza S, et al. Cell Cycle 2012; 11:4266–74; PMID:23095635; http://dx.doi.org/10.4161/cc.22613

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Faithful transmission and maintenance of the integrity of genetic information in the cell is controlled by a set of coordinated processes that involve chromatin remodeling, cell cycle checkpoint control, DNA replication, recombination and repair. A defect in any of these tightly regulated processes would result in gross chromosomal rearrangements, such as chromosome deletion, insertion, duplication, translocation and loss and lead to tumorigenesis or cell death.[1] In eukaryotic cells, DNA is packaged into a highly compacted inaccessible structure called the chromatin by both histones and nonhistone proteins.[1,2] Cells rely on post-translational histone modifications and ATP-dependent chromatin remodeling machines to gain access to, and perform various functions on, DNA. Histones can be reversibly modified in several ways, including methylation, ubiquitylation, acetylation and phosphorylation. Histone acetylation is regulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs are a diverse set of enzymes that are evolutionarily conserved from yeast to humans and are usually subunits of large co-activator complexes,[2] namely the human TBP-free TAF complex (TFTC), SPT3/TAF9/GCN5 complex (STAGA) (human homolog of yeast SAGA complex) and the activation 2a (Ada2a)-containing (ATAC) complex. These co-activator complexes function in many cellular processes, including transcription, cell cycle progression and DNA replication and repair.[2,3]

Human Ada3 is an essential component of the ATAC complex, which is composed of ADA2, ADA3 and GCN5.[2] Ada3 protein associates with and regulates transcriptional activity of estrogen receptor[4] and retinoic acid receptor.[5] Ada3 also binds and stabilizes the tumor suppressor p53 and is required for p53 acetylation by p300.[6] Although several cellular functions of Ada3 as part of multiple HAT complexes have been identified, the in vivo physiological roles of mammalian Ada3 were unclear, until a recent study by the Band Laboratory that demonstrated an essential role of mammalian Ada3 in embryonic development as well as cell cycle progression.[7] In a recent issue of *Cell Cycle*, Mirza et al.[8] discovered another interesting function of Ada3: it participates in DNA repair and in the maintenance of genomic stability. Using adenovirus-Cre-mediated Ada3 deletion in $Ada3^{fl/fl}$ mouse embryonic fibroblasts, Mirza et al. showed that Ada3 depletion is accompanied by increased levels of pATM, γH2AX, p53BP1 and pRAD51, even before ionizing radiation, indicating that Ada3 deficiency results in increased DNA damage in the cell.[8] Ada3 does not appear to be required for DNA damage sensing or checkpoint activation; however, a delay in DNA repair was observed in Ada3−/− cells.[8]

The authors further asked whether Ada3 deletion would affect chromosomal stability. They found that chromosome condensation was normal in $Ada3^{-/-}$ cells, whereas increased chromosome aberrations, ranging from chromosome breaks, fragments, deletions and translocations, were observed in these cells,[8] suggesting that Ada3 plays an important role in maintaining genome integrity. Interestingly, the frequency of chromosomal aberrations in Ada3-deleted S-phase cells upon DNA damage was significantly higher than that in control or Ada3-deleted G_1- or G_2-phase cells.[8] The authors also observed delayed disappearance of CtIP foci after DNA damage in $Ada3^{-/-}$ cells.[8] Since CtIP is known to act in both cell cycle checkpoint and DNA repair,[8,9] this delay in the disappearance of CtIP foci may explain both the checkpoint defects[7] and DNA repair deficiency[8] observed in $Ada3^{-/-}$ cells.

The findings of Mirza et al. not only establish a novel function of Ada3 in DNA repair and genome maintenance, but also open up a new direction for the studies of HAT complexes in DNA damage response. It would be interesting to further decipher the precise molecular mechanism underlying the functions of Ada3 and its associated HAT complexes in cell cycle checkpoint control, DNA repair and the maintenance of genome stability.

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Micromanaging a large tumor suppressor

Comment on: Fang L, et al. Cell Cycle 2012; 11:4352–65;
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Studies of microRNA (miRNA) in cancer are relatively recent but have exciting possibilities to improve our understanding and treatment of this disease.[1] Over 1,000 miRNAs (1–4% of the human genome) exist in non-coding genes or gene clusters or in introns of coding genes. Primary miRNA transcripts are cleaved into stem-loop structures in the nucleus and trimmed into 19–24 nucleotide duplexes in the cytoplasm. One strand of a duplex is incorporated into an RNA-induced silencing complex (RISC), where it binds complementary sequences in 3′-untranslated regions (UTRs) and decreases protein expression by inhibiting translation or causing mRNA degradation. Each miRNA can potentially bind 200 mRNAs and regulate multiple genetic pathways.

Aberrant miRNA expression has been documented in a number of cancers. For example, the miR-17-92a cluster consisting of miR-17, -18a, -19a, -19b-1, -20a and -92a-1 is overexpressed in lymphomas and lung cancers and may act like an oncogene.[2] In contrast, let-7 family members, miR-15 and miR-16 are downregulated in a number of cancers and may act as tumor suppressors by decreasing the expression of oncogenes or anti-apoptotic proteins such as Ras or Bcl-2.[1,3] While members of an miRNA cluster are often expressed coordinately, they may not have identical functions. For example, miR-17 acts like a brake on miR-92a, which appears to be the most potent oncogene of the miR-17-92 cluster.[2]

In this issue of *Cell Cycle*, Burton Yang's laboratory identifies a new role for miR-93 in breast cancer.[4] MiR-93 is part of the miR-106b-25 cluster on chromosome 7 and was found to be overexpressed in human primary breast cancer cells compared with normal breast tissue. Overexpression of miR-93 in MT-1 human breast cancer cells caused them to survive better in serum-free conditions and increased their invasive properties and interactions with endothelial cell lines in vitro. These properties were reflected in increased metastatic capability and angiogenesis in immunodeficient mice. To explain these observations, in silico studies implicated the tumor suppressor large tumor suppressor 2 (LATS2) as a miR-93 target. LATS2 is a serine-threonine protein kinase that is activated by mitotic stress, DNA damage and oncogenes and regulates a number of cell processes, including apoptosis and motility.[5] LATS2 is downregulated in a number of cancers, including breast cancer, through epigenetic mechanisms involving promoter methylation. However, LATS2 has also been shown to be downregulated by miR-372, miR-373, miR-31 and miR-195 in a number of cancer cell lines.[5,6]

Fang, et al. found a reciprocal relationship between LATS2 protein expression and miR-93 in breast cancer lines and primary cells. Expression of a luciferase construct harboring the target site in the LATS2 3′-UTR was decreased by miR-93, but not when this region was mutated to prevent binding of miR-93. Treatment of MT-1 cells with siRNA to LATS2 conferred the same phenotype as miR-93, while ectopic expression of a LATS2 gene lacking the 3'-UTR reversed the effects of miR-93. Taken together, these results establish miR-93 as an oncogene that downregulates LATS2 and promotes malignant behavior of breast cancer cells.

This study is an important contribution to our understanding of breast cancer. However, as with any good research, the findings raise additional questions. Why is miR-93 upregulated? Is there a cytogenetic basis for this finding? Interestingly, previous reports documented loss of heterozygosity at this locus rather than amplification.[7] Alternatively, is there a constitutively activated signaling pathway that increases miR-93 expression? Is miR-93 associated with specific breast cancer types?[8]

It is interesting that so many different miRNAs appear to regulate LATS2. This observation probably attests to the biological importance of this molecule, but is LATS2 the only pathogenic protein that is regulated by miR-93 in breast cancer? Certainly, increased interactions with endothelial cells suggest that cytokines and adhesion molecules are likely also increased by miR-93 and may be independent of LATS2.

What is the role of the other members of the miR-106b-25 cluster? Are miR-106b and miR-25 also oncogenes, or do they function to balance the oncogenic properties of miR-93, as described for the paralogous miR-17–92 cluster?[2]

Intriguing therapeutic possibilities arise from the finding that miR-93 is overexpressed in breast cancer. In the future, the results of Fang and coworkers may lead to more effective breast cancer treatments that use agents such as anti-sense oligonucleotides[1] to block miR-93.

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Mesenchymal traits and cancer stem cells in mammospheres: Chicken or egg?

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The cancer stem cell (CSC) hypothesis states that tumors are initiated and maintained by a subset of cancer cells with the ability to self-renew and to differentiate into cells of various lineages. Putative CSCs have been identified in a variety of human malignancies including breast cancer.[1] Breast CSCs expressing the cell surface markers CD44high/CD24low were able to generate tumors when transplanted in small numbers in immunocompromised NOD/SCID mice.[1] In addition to the cell sorting method, breast CSCs have been identified in vitro by their ability to grow as mammospheres (MS) in non-adherent, serum-free conditions.[2] MS obtained in these pro-stem culture conditions have been shown to be enriched in cells expressing CD44high/CD24low markers.[3]

Beside stem-like features, the epithelial-mesenchymal transition (EMT) appears to play a critical role in breast cancer progression. EMT is a process wherein epithelial cells undergo multiple changes that enable them to adopt mesenchymal features, including enhanced capacity for migration, invasiveness and elevated resistance to apoptosis.[4] EMT programs are orchestrated by a set of pleiotropic transcription factors, including Slug, Snail, Twist and Zeb1, which can directly repress levels of E-cadherin, the hallmark of the epithelial state.[4] N-cadherin, the mesenchymal intermediary filament vimentin and extracellular matrix (ECM) components such as fibronectin are mesenchymal markers. Mani et al.[5] and Morel et al.[6] independently found that the activation of the EMT program in both normal and transformed mammary epithelial cells is associated with the acquisition of stem cell properties, including the ability to form spheres in non-adherent cultures. In addition, recent studies have shown that the subtypes of breast carcinomas enriched in stem features, such as claudin-low tumors, also express EMT markers.[7]

Borgna et al.[8] have studied distinct subtypes of breast cancer cell lines (luminal, HER2-positive, basal-like and claudin-low) under MS-proficient conditions (suspension). Their results suggest that the growth in suspension as MS favors the expansion of cells with mesenchymal traits (**Fig. 1**). For instance, an increase in the expression of vimentin has been observed in seven out of 10 cell lines, along with a significant decrease in the expression of the E-cadherin epithelial marker CDH1. Moreover, Borgna et al.[8] have also observed a global increment in the expression of EMT transcription factors Zeb1, Zeb2, Snail2 and Twist 1. Overall, their data provide evidence for a shift toward a mesenchymal phenotype, along with stemness features, under proficient culture conditions. This supports the use of MS as an in vitro model for the study of therapeutic approaches targeting mesenchymal phenotypes.

The enrichment in mesenchymal features under MS-proficient conditions may be due to an increase in the proportion of a pre-existing subpopulation of cells with mesenchymal phenotype, or by de novo induction of EMT. Interestingly, Borgna et al.[8] have observed the gain of mesenchymal features in short-term MS in MCF7 cells (a luminal cell line) lacking enrichment in the CD44high/CD24low fraction, suggesting that the gain of mesenchymal phenotype may precede the acquisition of stem-like features. This is an important observation that can be further validated by a functional assessment of MS under different culture conditions, as cells acquire an EMT and then a stem cell profile. We have recently developed a quantitative approach[9] to validate different possible scenarios that can lead to the enrichment of stem cell activity following induction of EMT.[5,6] Additionally, we have suggested the utility of comparing mammosphere data to computational mammosphere simulations in elucidating the growth characteristics of mammary CSCs. I anticipate that this "modus operandi" of using quantitative modeling (grounded in experimental data) to gain new insights may well provide a rational means for predicting the timeline of the acquisition of mesenchymal and stem features. The process of EMT and its relationship to CSCs are novel and rapidly converging directions of research, and many related questions may be considered. For instance, it would be interesting to use the suggested MS culture conditions to develop and validate novel and effective therapeutic strategies, followed by in vivo validations. Moreover, in vitro MS studies under tumor microenvironmental conditions, such as hypoxia, may provide further information, which can be also validated by in vivo experiments.

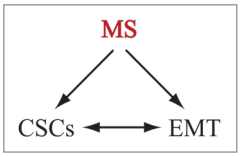

Figure 1. Borgna et al.[8] have shown that breast cancer cells grown as MS are enriched in both SC features and EMT markers.

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