

Cell Cycle News & Views

Protecting normal cells from the cytotoxicity of chemotherapy

Comment on: van Leeuwen IMM, et al. *Cell Cycle* 2012; 11:1851–61;
PMID:22517433; <http://dx.doi.org/10.4161/cc.20254>

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The occurrence of intolerable side effects in patients undergoing chemotherapy is still a major clinical hurdle. Finding tumor-specific therapy and exploiting the differences between normal cells and tumor cells has led to strategies targeting oncogenic mutations or the deficiency of tumor suppressor pathways in cancers. Alternatively, focusing efforts to protect normal cells from the side effects of chemotherapy has gained much interest, and harnessing cell cycle checkpoints to target tumor cells while sparing normal cells is an attractive strategy.

Contrary to achieving drug synergism when targeting tumor cells, one hopes to achieve drug “antagonism” on normal cells in a proposed strategy, termed “cyclotherapy.”¹ The concept requires the use of two drugs, the first to arrest the normal cells and the second, to kill only cycling (tumor) cells (Fig. 1). The high frequency of p53 mutations in human cancers presents an opportunity for selectively targeting the p53-deficient tumors using S- or M-phase poisons, by activating a checkpoint arrest only in the normal cells. Indeed, several recent studies demonstrate that p53 activation

protects normal cells from the toxicity of drugs acting in the S-phase or M-phase, including Ara-C,² taxol,³ Aurora kinase inhibitors⁴ and Polo-like kinase inhibitors.⁵ The therapeutic window, in this case, is defined by (1) the toxicity of p53 activation in normal tissues, (2) the specificity of the “first” drug for p53 activity and (3) the effectiveness of the “second” drug in inducing p53-independent apoptosis. Exploring various combinations of p53-activating drugs and cell cycle-specific poisons may help to clarify the importance of each criteria. Ingeborg et al.⁶ recently performed a systematic screening of 16 drug combinations of p53 activators and S- or M-phase poisons for the protection of wild-type p53 cells while rendering selective toxicity against p53-deficient cells. Remarkably, all of the tested p53-activating drugs (Nutlin-3, a small-molecule antagonist of MDM2, Actinomycin D, Tenovin-6, Leptomycin B) antagonize the activity of microtubule-targeting compounds (vinblastine, vinorelbine) and antimetabolites (Ara-C and gemcitabine) in normal primary human fibroblasts, thus protecting them. They further explored the specificity of the observed

drug antagonism for wild-type p53 cells by testing the same combinations on cell lines deficient for p53 functions. Interestingly, different classes of p53-activating drugs have varied outcomes in mutant p53 cells. While Nutlin and Actinomycin D, as expected, do not induce any arrest in mutant p53 cells, Tenovin-6 and Leptomycin B activated a cell cycle arrest that protected the mutant p53 cells against vinca alkaloids. Curiously, the cell cycle arrest only partially protected the mutant p53 cells from Gemcitabine and Ara-C. The study revealed that the specificity of the p53-activating drug for wild-type p53 is critical for cyclotherapy against p53-mutant tumors. Understanding what invokes a p53-independent cell cycle arrest in response to Tenovin-6 and Leptomycin B may help to prescribe the use of these drugs as chemoprotectants in another context.

Before p53-based cyclotherapy can be used in the clinic, one would like to know (1) the consequences of p53 activation in various normal tissues, (2) the outcome of p53 activation by different drugs, which may differentially effect p53-dependent gene transcription, either favoring cell cycle arrest, apoptosis or

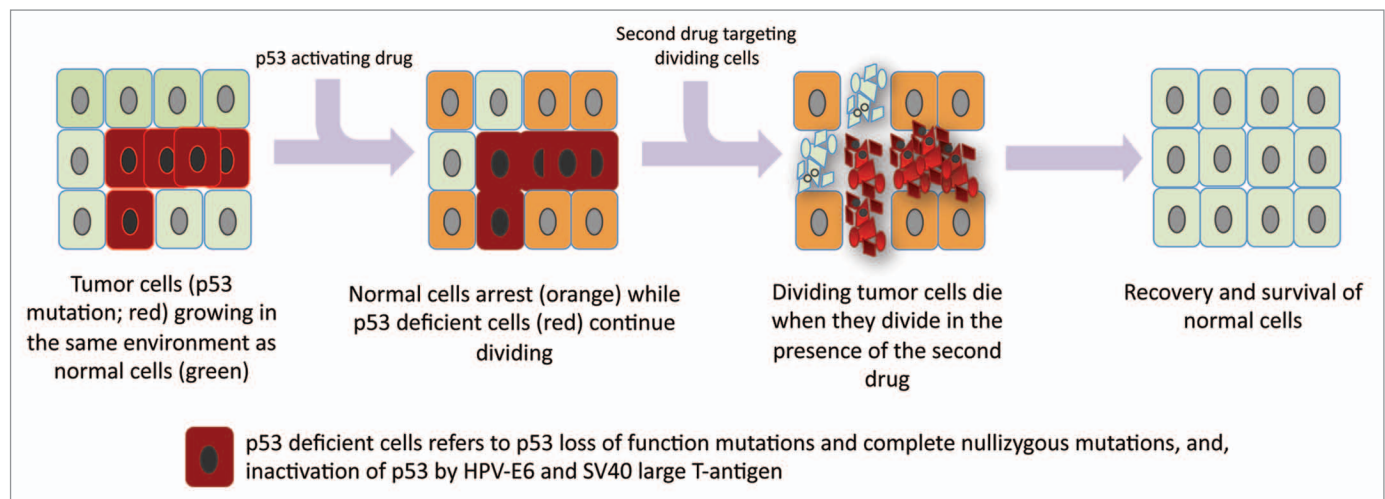


Figure 1. Harnessing p53-dependent cell cycle arrest to protect normal cells and render selective drug toxicity to p53 mutant cells. Sequential drug therapy using a p53-activating drug that results in cell cycle arrest in normal cells and a second drug that targets only proliferating cells (any S- or M-phase specific poisons), results in selective killing of p53-deficient tumor cells while sparing normal cells.

senescence, and (3) the right dosage of drugs to use for chemoprotection. High drug doses may induce other p53-independent cytotoxic effects that trigger DNA damage, synergizing with the activity of p53 to induce apoptosis instead. The observation that Nutlin can suppress neutropenia,⁵ a common side effect in chemotherapy, in mice treated with Polo-like kinase inhibitors provides great optimism.

Should the selection of new small molecules activating p53 for chemoprotection, be based on their selectivity for cell cycle arrest (over apoptosis)? Nutlin, for example, has low dose potency; however, because of its

specificity for p53 activity and its low toxicity on normal tissues, it seems like a good candidate as a chemoprotectant. Apart from the p53 pathway, perhaps other oncogenic pathways may be considered for the cyclotherapy approach? For example, inhibition of the EGF, MEK or PI3-K prevents proliferation in normal cells but not in some tumor cells. UCN-01, a CHK kinase inhibitor, also prevents cell cycle progression and suppresses the toxicity of Topotecan in bone marrow.⁷ Finally, establishing *in vivo* models to test for potential drug combinations in cyclotherapy would be exciting in the future.

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Setting the stage for cohesion establishment by the replication fork

Comment on: Rudra S, et al. *Cell Cycle* 2012; 11:2114–21
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The complex process of semi-conservative DNA replication involves a mechanism whereby the leading and lagging strands with opposite polarity serve as templates for concerted synthesis of complementary base pairs.¹ Lagging-strand synthesis creates discontinuous Okazaki fragments that require timely processing of the 5' flaps, so that adjacent nascent DNA strands are ligated together to insure genomic stability. While the genetic and

molecular requirements of Okazaki fragment maturation have been studied in much detail, the precise temporal and spatial relationship of lagging-strand processing to sister chromatid cohesion remains unclear.² The newly replicated daughter duplex DNA molecules (i.e., the sister chromatids) become tethered during DNA replication and remain paired in order to permit proper segregation of the chromosomes to respective poles during

mitosis and nuclear division. Elegant genetic studies in yeast have implicated posttranslational modification of cohesins (specialized protein complexes responsible for tethering sister pairs) by Ctf7/Eco1 acetylase as a key regulatory step in the process, enabling cohesins to perform their function in capturing the newly synthesized sister chromatids. Previous work suggested that genetic and physical interactions among the yeast acetyltransferase Ctf7/Eco1, helicase Chl1, Flap Endonuclease (Fen1) and accessory replication factors [e.g., RFC (clamp loader) and PCNA (clamp)] play an integral role in cohesion establishment. Based on these pieces of evidence, several models to explain the relationship between replication fork dynamics and sister chromatid cohesion have been proposed; however, our understanding of the precise timing of cohesin acetylation and the passage of the replication fork machinery has remained murky at best. Given the importance of proper chromosome segregation for chromosomal stability and the suppression of developmental disorders and tumorigenesis, a comprehensive understanding of the molecular acrobatics involved in sister chromatid cohesion is highly important.

In a recent study, the temporal relationship between sister chromatid establishment and lagging-strand synthesis was illuminated.³ The authors have elucidated the link between the catalytic functions of DNA unwinding, flap

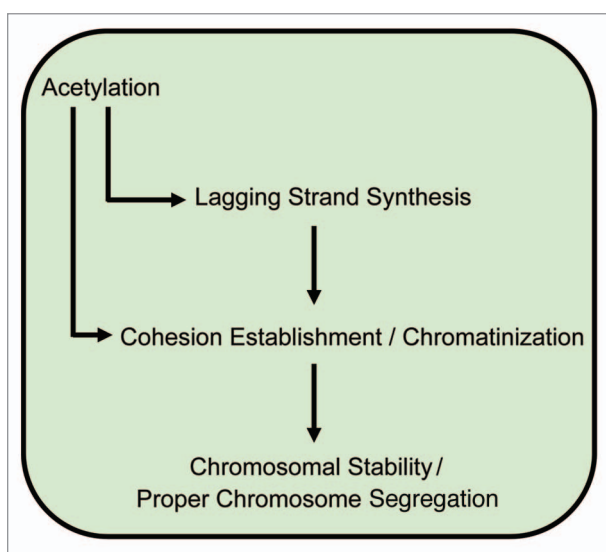


Figure 1. Interplay between acetylation, replication fork dynamics and cohesion establishment important for chromosomal integrity.

processing and acetylation, which supports a model of cohesion deposition and establishment that occurs after the passage of the replication fork, similar to how genomic DNA becomes chromatinized. This is a significant advance from an earlier and very popular model of sister chromatid cohesion predicted that Ctf7/Eco1 acetylated cohesin proteins before the encounter by the DNA replication fork, which was thought to permit fork progression and the proper cohesion state for sister chromatid tethering (for review, see ref. 2). Instead, the genetic evidence presented by the Skibbens lab supports a model whereby cohesion establishment is temporally coupled to lagging-strand processing.³ In support of the genetic proof, Rudra and Skibbens went on to show that both Ctf7/Eco1 and Chl1 are associated with the lagging-strand processing nuclease Fen1. Altogether, the experimental results implicate a post-fork establishment model that is analogous to how histone protein complexes are deposited onto newly synthesized sister chromatids and become posttranslationally modified to confer epigenetic status.

The discovery from the Skibbens lab that cohesion establishment is closely orchestrated

with Okazaki fragment processing prompts a new line of inquiry about the control of flap processing by acetylation and its dual purpose for proper sister chromatid cohesion and replication fidelity in eukaryotes (Fig. 1). The catalytic activity of human FEN-1^{4,5} and a functionally related endonuclease known as Dna2⁴ have been shown to be modulated by p300 acetylation, which suggested a model for creating long flap intermediates to promote genomic stability and suppress mutagenesis. Given evidence that ChlR1 is implicated in the genetic disorder Warsaw Breakage syndrome and that the human homolog of yeast Chl1⁶ interacts with the RFC complex and Fen1,⁷ it will be informative to determine if acetyltransferases such as the human orthologs Escp1 and Escp2, the latter mutated in the cohesinopathy Roberts syndrome,⁸ and perhaps other acetyltransferases (e.g., p300) are master regulators of lagging-strand synthesis that not only affect replication fidelity and genomic stability, but also sister chromatid cohesion. Coordination of sister chromatid cohesion establishment with lagging strand synthesis may also involve replication fork stabilization by the Timeless-Tipin protein

complex implicated in replication checkpoint.⁹ Defects in the efficient coupling of lagging-strand synthesis to sister chromatid cohesion may contribute to the chromosomal instability characteristic of age-related diseases and cancer.

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A role for CDK9 in UV damage response

Comment on: Shchebet A, et al. *Cell Cycle* 2012; 11:2122-7; PMID:22592529; <http://dx.doi.org/10.4161/cc.20548>

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Preserving genome stability is critically important for cell survival. DNA is under continuous attacks from damaging agents that threaten proper replication and gene expression. The DNA damage response (DDR) coordinates repair throughout the cell cycle.

Cyclin-dependent kinase-9 (CDK9) has been implicated in double-strand break (DSB) repair and suppression of DNA:RNA hybrid (R-loop) formation. Moreover, recently it has been shown that it is important in the response to replication stress induced by HU and aphidicolin.^{1,2}

CDK9, together with cyclins T1/T2, forms the positive transcription elongation factor-b (P-TEFb). CDK9 acts mainly through phosphorylation of Ser2 within the C-terminal repeat domain (CTD-S2ph) on RNA polymerase II and subsequent co-transcriptional histone modifications and mRNA processing.

CTD-S2 phosphorylation leads to histone H2B monoubiquitination (H2Bub1) and histone H3 trimethylations.¹

UBE2A (yeast Rad6 homolog) serves as the E2 and complexes with E3 ubiquitin ligase formed by RNF20/40 to monoubiquitinate H2B. CDK9-induced CTD-S2 phosphorylation recruits the RNF20/40 through interaction with WAC protein.³ Interestingly, in the absence of CTD-S2ph, some H2Bub1 still persists compared with CDK9 knockdown, suggesting additional modes of regulation.⁴

This report investigates the interaction between CDK9 and UBE2A. The authors show that CDK9 interacts with UBE2A and phosphorylates it *in vitro* and *in vivo*, similar to what has been found in yeast. Moreover, they explore the mechanisms for modulation of UBE2A activity and demonstrate that UBE2A phosphorylation affects the enzymatic activity but not its

interaction with binding partners. Knockdown of CDK9 affects not only H2B but also PCNA monoubiquitination in the UV damage response, potentially affecting the DNA damage tolerance (DDT) and/or the Fanconi anemia (FA) pathways. Interestingly, cyclin T1 seems to contribute to this activity. Collectively, this report shows that the role of P-TEFb-mediated phosphorylation is more complex than initially realized and contributes to understanding of CDK9 in the response to the DNA damage.⁵

Yeast cells have two CTD-S2 kinases, Bur1 and Ctk1.⁶ CDK9 was considered the only mammalian homolog fulfilling both roles. Cyclin K has been implied to be the important player for the different pathways. However, recent studies have characterized a new kinase complex, CDK12/CyclinK.⁷ More and more evidence points at CDK12 as the functional homolog of Ctk1. Both play a role in DDR;

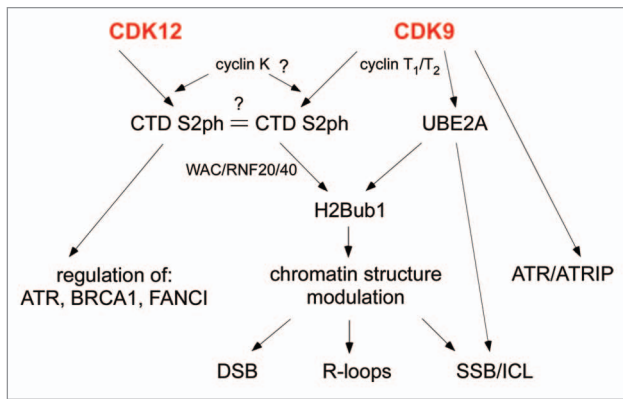


Figure 1. Schematic representation of CDK9/CDK12 influence on DNA damage response. CDK9 interacts with cyclins T1/T2 and possibly also cyclin K. CDK12 binds cyclin K. Both kinase/cyclin complexes can phosphorylate RNA polymerase II C-terminal on Serine 2 (CTD S2ph). There seem to be at least 2 forms of Ser2 marks, possibly distinct in response to the action of the two kinases. CDK12 affects genomic stability through the regulation of gene expression of DNA damage responsive genes (ATR, BRCA1, FANCI). CDK9 can have direct and indirect effects on genomic stability. CDK9 can directly form a complex with ATR/ATRIP. Moreover, CDK9 phosphorylates CTD (which recruits E3 ligases -RNF20/40) and E2 (UBE2A), both required for H2B monoubiquitination (H2Bub1). This modification plays a critical role in chromatin structure modulation, potentially affecting the response to different damaging stresses: double-strand breaks (DSB), DNA-RNA hybrid structures (R-loops), single-strand breaks (SSB) and interstrand DNA crosslinks (ICLs). UBE2A phosphorylation can also directly affect SSB and ICLs.

however, their contributions are quite distinct (Fig. 1). CDK12 maintains genomic stability by direct regulation of the expression of *ATR*, *BRCA1* and *FANCI*.⁷ However, CDK9 can act through both direct and indirect mechanisms. First it co-immunoprecipitates with the ATR-ATRIP-claspin complex, acting in a common pathway in response to HU.² On the other hand, it is a potential global chromatin modulator due to its induction of H2Bub1 modification.¹ H2Bub1 could orchestrate chromatin changes to allow repair mechanisms onto the DNA in response to specific DNA changes.

It is still not clear whether both CDK9 and CDK12 act in parallel or are activated in response to different stress signals. Interestingly, it seems that there are at least two forms of Ser2 marks, and they respond distinctly to depletion of the two kinases.⁷ Moreover, whether cyclin K is bound exclusively to CDK12 or if it also interacts with CDK9 under specific conditions remains to be verified. This study provides the first evidence that cyclin T1 contributes to DDR. It will be interesting to test whether the other cyclins (K and T2) can also play a role in the UV damage response.

This is particularly interesting in the light of the recent study showing importance of cyclin K in response to camptothecin and ionizing radiation but not MMS (a UV mimetic).⁸

Both CDK9 and CDK12 can potentially contribute to cancer. This could be especially important in the response to inter-strand DNA cross-links in the FA pathway, where CDK9 can contribute by PCNA modification and CDK12 can directly affect the FANCI and FANCD2 expression. Encouragingly, studies in gastric and breast cancers have shown that the CDK9 inhibitor flavopiridol can increase the efficiency of Mitomycin C in the induction of apoptosis.⁹ Combining the CTD-S2ph inhibitors with radiotherapy or chemotherapy could refine the present strategies to fight cancer.

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Cancers co-opt cohabitants' catabolism: Autophagy and senescence in the tumor stroma

Comment on: Capparelli C, et al. *Cell Cycle* 2012; 11:2272-84; PMID:22684333 and Capparelli C, et al. *Cell Cycle* 2012; 11:2285-302; PMID:22684298

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Autophagy is a fundamental cellular process in which surplus or modified cellular components, from individual proteins to whole organelles, are degraded in the lysosomes. Autophagy prevents the accumulation of random molecular damage in long-lived structures, particularly mitochondria, and more generally provides a means to reallocate cellular resources from

one biochemical pathway to another. Consequently, it is commonly upregulated in conditions where a cell is responding to stress signals, such as starvation, oxidative stress and exercise-induced adaptation.

While autophagy is a process of damage repair, senescence might be viewed as a state of damage limitation, occurring when the level

or nature of damage detected is likely to be irreparable. Triggered either by genotoxicity registered via p53 or via a secondary pathway involving p16^{INK4a}, the senescent state is characterized by cell cycle arrest, hypertrophy and flattening along with changes in a panel of genetic and proteomic biomarkers, especially the lysosomal protein β -galactosidase.

Although the two processes have distinct functions, recent work from Cambridge has determined that activation of autophagy is both typical of and, in some cases, sufficient to induce senescent transformation, and that inhibition of autophagy delays the acquisition of senescence.¹

Senescent cells are resistant to apoptotic signaling and are known to accumulate in many tissues during aging. Interestingly, significant numbers are frequently found in close proximity to benign tumors. This colocalization has traditionally been seen as a result of senescence programs acting successfully to terminate early-stage tumorigenesis. However, two studies^{2,3} published in this issue of *Cell Cycle* propose a different explanation, presenting compelling evidence for a direct metabolic link between neoplastic cells and the senescent fibroblasts surrounding the tumor. Analysis of clinical data indicates that a high level of autophagy and senescence in the stroma induced in vivo, particularly through hydrogen peroxide secretion by the tumor, correlates with poor prognosis in multiple cancers; however, the mechanism underlying this connection is not well understood.

To dissect the relationship between the autophagic state and tumor growth, hTERT-immortalized fibroblast cultures were transformed with BNIP3, cathepsin B or ATG16L1. Each of the three genes employed was independently able to support a state of constitutively upregulated autophagy, as determined by a panel of biomarkers including downregulation of the membrane protein caveolin-1.

Loss of caveolin-1—which alone is sufficient to induce autophagy in some cell types,⁴ suggesting a feedforward cycle—has been shown to result in ligand-independent activation of the TGFβ pathway, with a particularly prominent upregulation of connective tissue growth factor (CTGF). The authors demonstrate a key intracellular role for CTGF in inducing and supporting the chronic autophagic state, independent of its well-established role in enhancing extracellular matrix deposition. Specifically, CTGF overexpression is shown to promote HIF-1α signaling, resulting in increased transcription of glycolytic enzymes and components of the autophagic pathway.

The authors hypothesize that chronic autophagy, and particularly mitophagy, eventually results in a failure of mitochondrial respiration and a switch to predominantly aerobic glycolysis. As a result, the fibroblast produces a surplus of high-energy intermediates, including L-lactate and 3-hydroxybutyrate, which escape into the microenvironment and are taken up by nearby tumor cells as a supplemental energy source. The magnitude of this nutrient transfer is shown to be significant, promoting experimental tumor growth by around 50–100%; surprisingly, metastasis rates are increased even more profoundly (up to 11-fold for ATG16L1-induced fibroblasts).

The accumulation of permanently senescent cells over time is a well-established mechanism of systemic aging, contributing to the increased incidence of a number of conditions—osteoarthritis, atherosclerosis, prostatic hyperplasia, metabolic syndrome and

cancer among them.⁵ The secretion of a panel of inflammatory mediators, the “senescence-associated secretory phenotype,” or SASP, is the best-characterized mediator of the pathological effects observed, although it is not a universal feature of p16^{INK4a}-senescent cell populations.⁶

Although senescence is not always an irreversible phenotype, its undeniably important role in tumor suppression makes attempts to therapeutically reverse it, such as telomerase-activating drugs, particularly fraught. We have for some time advocated the more straightforward approach of selectively destroying (and, where necessary, replacing) senescent cells,⁷ and we are delighted to confirm that this approach has recently been shown to have significant rejuvenating effects in mice.⁸

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Dissecting the role of mTOR complexes in cellular senescence

Comment on: Kolesnichenko M, et al. *Cell Cycle* 2012; 11:2391-401; PMID:22627671 and Pospelova TV, et al. *Cell Cycle* 2012; 11:2402-407; PMID:22672902

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The current papers by Peiqing Sun and collaborators¹ and by Mikhail Blagosklonny and collaborators² add new important pieces to the role of the mTOR pathway in senescence and longevity.

Filling the Gaps

The Ser/Thr-kinase mTOR is a main metabolic sensor activated by the presence of nutrients, and whose activity orchestrates cell growth mainly through the inhibition of autophagy

and the activation of protein synthesis.³ A large body of evidence indicates that reduced levels of mTOR activity favor organismal and cellular longevity, and the small drug compound rapamycin has been instrumental for the consolidation of this concept thanks to its ability to inhibit mTOR. In particular, chronic administration of rapamycin extends the lifespan of mice⁴ and, indeed, rapamycin is the only known compound able to increase

longevity in mammals. Cellular senescence is a stress response that disables the proliferative capacity of cells while maintaining their viability,⁵ and there is evidence that cellular senescence and organismal aging share some mechanistic principles. Notably, contemporaneous to the description of the anti-aging activity of rapamycin in mice, it was also reported by Blagosklonny and collaborators that rapamycin delays cellular

senescence induced by chemical stresses.⁶ Chemically induced senescence is a well-accepted model to study cellular senescence, but the question still remained whether replicative and oncogene-induced senescence are also delayed by rapamycin. Replicative senescence results after extensive proliferation of cells in vitro, and it is considered the most relevant type of cellular senescence in relation to organismal aging.⁵ On the other hand, oncogene-induced senescence is relevant for tumor suppression.⁵ These pending questions are now answered in the accompanying reports, where it is demonstrated that rapamycin impairs replicative senescence in human¹ and rodent² cells, as well as oncogene-induced senescence.¹ This satisfactorily extends the longevity activity of rapamycin to in vitro cultured cells and validates cellular senescence as a model to study organismal aging in relation to the mTOR pathway.

Dissecting the mTOR Complexes

An important consideration regarding mTOR kinase derives from the fact that it assembles into two distinct multiprotein complexes named mTORC1 and mTORC2.³ To add further complexity, the inhibitory effect of rapamycin on these two complexes depends on the dose and duration of the treatment. In general, short treatments with rapamycin at low doses produce partial inhibition of mTORC1 (and activation of mTORC2 as a result of the attenuation of the negative feedback loop that connects mTORC1 with mTORC2), whereas chronic treatment or high doses can inhibit both mTORC1 and mTORC2.³ In this complex scenario, it is not obvious whether the effects of rapamycin on organismal and cellular longevity are mediated by mTORC1, mTORC2 or

both. An initial clue came from the observation that female mice lacking S6K1, a key substrate of mTORC1 (but not of mTORC2), have an increased longevity.⁷ This has been further supported by the report this year that female mice doubly heterozygous in mTOR and mLST8 are partially deficient in mTORC1 activity (but not in mTORC2 activity) and also present an extended lifespan.⁸ The molecular basis for the selective deficit in mTORC1 activity is not fully understood, because mLST8 is common to both mTORC1 and mTORC2 complexes, although a simple explanation could be their differential affinity for mLST8. The emerging central role of mTORC1 inhibition in longevity is now complete thanks to the accompanying report by Sun and collaborators.¹ In this paper, the authors demonstrate that selective inhibition of mTORC1, but not of mTORC2, impairs replicative and oncogene-induced senescence (Fig. 1).¹

Implications

These two new papers extend and solidify the anti-aging activity of rapamycin through inhibition of mTORC1.^{1,2} A point of worry is the inhibition of oncogene-induced senescence by rapamycin, which could suggest a pro-tumorigenic effect of rapamycin. This concern, however, is mitigated by the fact that chronic treatment of mice with rapamycin does not produce an increase in spontaneous tumors.⁴ Nonetheless, rapamycin is not without undesirable effects, due to its pro-diabetic activity.⁹ The pro-diabetic activity of rapamycin is now known to be due to the inhibition of mTORC2 (and not to the inhibition of mTORC1).⁸ In this state of affairs, the identification of rapamycin analogs highly selective for mTORC1 inhibition holds the promise of a more potent anti-aging activity.

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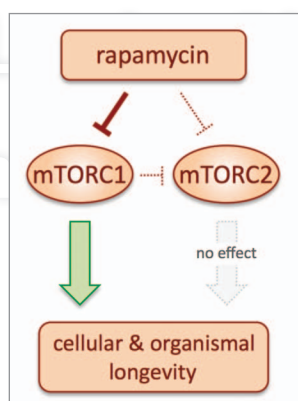


Figure 1. Inhibition of mTORC1, but not of mTORC2, promotes cellular and organismal longevity.