

Cell Cycle News & Views

Replication timing: Findings from fibers

Comment on: Frum RA, et al. Cell Cycle 2009; In this issue

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DNA synthesis in eukaryotes must be performed with absolute precision as defects compromise genetic integrity. Recent progress in understanding the mammalian S phase have focussed on 'post-genomic' style experiments in which nascent DNA is hybridized to microarrays to define active sites. This is epitomized by three recent studies,¹⁻³ which together define the reliability of the replication timing program and how features such as RNA synthesis, genome architecture and epigenetic modifications provide landmarks to effect orderly replication.

While many studies⁴ confirm the general timing of synthesis during S phase the temporal resolution of these protocols is insufficient to reveal subtleties in replication timing. Significantly, genome-wide approaches, which inevitably provide an average view of cell behavior, cannot reveal heterogeneity in cell populations. Hence, mechanistic understanding of replication control demands that genome-wide information is complimented by experiments performed on single cells.

Using DNA fibers to analyze replication

Analysis of replication using DNA fibers presents the best opportunity to understand events that define stability or heterogeneity of replication within individual cells. Historically, DNA fibers from cells labelled with 3H-thymidine were used to measure rates of synthesis and show that replicons were activated in small clusters during synthesis. Subsequently, versatile labelling protocols using modified thymidine analogues⁵⁻⁷ have been widely used to analyze DNA synthesis. Key experiments have confirmed the structure of replicon clusters within DNA foci,⁵ measured the rate of synthesis throughout S phase^{6,7} and explored how rates compensate to accommodate replicon clusters with variable inter-origin spacing.⁶ In addition, fibers have allowed analysis of the distribution of potential origins at specific loci and confirmed the redundancy of potential origins and stochastic activation of a minority of origins during each synthetic cycle.⁸

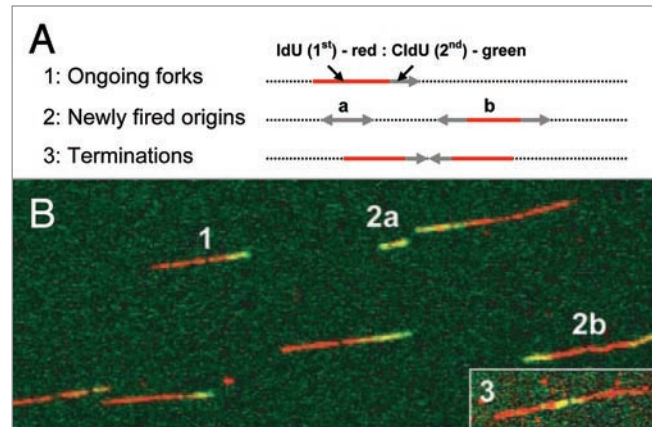


Figure 1. Replication track forms visualized by fiber labelling. (A) A schematic of double-labelled replication tracks as they would appear after synthesis with short, consecutive pulses of thymidine analogues IdU (1st label, red) and CldU (2nd label, green); the dotted black line represents the unlabelled DNA fiber. (B) An example of the labelled tracks seen on isolated DNA fibers after indirect immuno-labelling. Image reproduced from Merrick et al. J Biol Chem 279:20067-75 with permission from the American Society for Biochemistry and Molecular Biology.

Notably, fiber analysis has shown that checkpoint proteins contribute to the suppression or squelching of redundant potential origins⁹ and regulate the transition from early to mid/late S phase.¹⁰

Comparing normal and cancer cells

DNA fibers support high-resolution quantitative measurements and so allow direct comparison of replication in different cell types. An excellent example of this is shown in a recent study from David Kaufman and colleagues.¹¹ The study compared the structure of active replicons as human fibroblasts (transformed by telomerase expression) and glioblastoma (T98G) cells progressed through S phase. Cells were synchronized using a combination of release from serum deprivation and replication and pulse labelled with short consecutive pulse of IdU and CldU. After spreading and indirect immunolabelling (Fig. 1), fibers were scored into four categories based on the structure of replication tracks:

(1) Bi-directional forks—contain a track of 1st label with two flanking tracks of the 2nd,

consistent with origin activation during the 1st pulse period;

(2) Uni-directional forks—contain a track of 1st label with an adjacent track of 2nd label, consistent with individual growing forks;

(3) Clustered replicons—contain intermingled and contiguous tracks of 1st and 2nd label, which must represent clustered replicons that engaged and completed synthesis during labelling. Because labelling was performed for 30 min, origins in clustered replicons must be <50kbp apart;

(4) Terminated forks—contain a track of 2nd label with two flanking tracks of the 1st, consistent with opposing forks that meet during the 2nd pulse period.

Fiber analysis was performed at 1 h intervals during transit through S phase. As expected, replication at the onset of S phase correlated with a high frequency of bi-direction forks from origins that were activated during labelling; most of the remainder were growing forks. Interestingly, in the following period of synthesis—between 1st and 2nd h of S phase—origin activation in normal cells fell to the level seen throughout the remainder of S phase whereas

two-fold more bi-directional forks were seen in the cancer cells. This is consistent with the regulation of initiation being compromised in cancer cells so that synchronous origin activation seen in somatic cells is lost. This may relate to previous observations, which showed that normal cells display a brief period of reduced elongation soon after initiation whereas cancer cells continue to replicate at a constant rate.¹² This implies that normal cells, unlike transformed cells, engaged a form of regulatory checkpoint to assess the efficacy of assembly of the replication machinery.

Interesting cell-type specific differences in replication were seen throughout S phase.¹¹ In human cells, S phase takes 9–10 h and synthesis of euchromatin in chromosomal R-bands completes before replication of heterochromatin in G bands is attempted;⁴ in normal cells, the switch from R to G band synthesis occurs at 4–5 h. In normal diploid cells, Kaufman and colleagues see that this transition zone correlates with a notable increase in initiation

and subsequent dramatic increase in fork termination—both within clusters (class iii) and remote forks (class iv). This implies that as cells approach the R/G transition normal cells engage a mechanism for efficient selection of any remaining origins within euchromatin and ensure that euchromatin and heterochromatin are replicated at appropriate times. Notably, in some cell types, the R/G transition can be accentuated to reveal a so called 3C-pause,¹³ which emphasizes the imperative of preserving the timing with which euchromatin and heterochromatin are replicated in somatic cells. Excitingly, detailed analysis of the normal and cancer cell lines shows that key features seen in normal cells are deregulated in glioblastoma.¹¹

There is no doubt that DNA fibers will continue to provide fundamental insights into the way replication is regulated in human cells. More detailed studies might even begin to reveal how S phase regulation is compromised as cells acquire pathogenic properties and contribute to our understanding of genome stability while

providing tools to monitor changes in DNA synthesis during the development of disease.

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Selectively killing transformed cells through proteasome inhibition

Comment on: Kazi A, et al. *Cell Cycle* 2009; 8:1940–51.

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The ubiquitin-proteasome system (UPS) is responsible for degrading a broad array of proteins involved in diverse cellular processes, including signal transduction, cell cycle regulation and cell survival, among numerous others. This system involves two principal processes: protein ubiquitination, mediated by ubiquitin activating, conjugating and ligating proteins, and protein degradation by the 20S component of the 26S proteasome mediated through the activity of peptidase, trypsin-like and chymotryptic-like enzymes.¹ Because the disposition of proteasome target proteins represents such an essential function for so many cellular regulatory events, it seems counter-intuitive that inhibition of the proteasome could be a viable anticancer strategy. Nevertheless, preclinical studies suggested that proteasome inhibitors might preferentially target transformed cells and display relatively little toxicity toward their normal counterparts.² These and similar findings supported the development of proteasome inhibitors as antineoplastic compounds, exemplified by bortezomib (Velcade), a reversible inhibitor of the 20S proteasome that has been approved for the treatment of patients with refractory

multiple myeloma,³ and more recently, mantle cell lymphoma.⁴ The success of bortezomib, as well as the identification of several dose-limiting toxicities (e.g., neurotoxicity and thrombocytopenia), has prompted the search for even more effective proteasome inhibitors, and several second generation compounds of this class, including NPI-0052⁵ and PR-171 (carfilzomib)⁶ are currently undergoing clinical evaluation.

Despite the success of bortezomib in multiple myeloma and mantle cell lymphoma, the therapeutic range of activity of this and similar compounds remains rather narrow. For example, to date, the activity of proteasome inhibitors against epithelial tumors appears limited for reasons that are not entirely clear. In addition, the mechanism(s) by which proteasome inhibitors kill transformed cells remain(s) to be fully elucidated. For example, it has long been assumed that proteasome inhibitors kill myeloma cells so effectively because these cells are highly dependent upon NFκB activation for survival, and inhibition of the proteasome leads to accumulation of the NFκB-inhibitory protein IκBα, which inactivates NFκB.⁷ However, results of a very recent study raise certain questions

about this assumption based on evidence that in multiple myeloma cells, bortezomib can actually increase rather than decrease NFκB activation.⁸ Moreover, results of studies in both epithelial⁹ and hematopoietic cells¹⁰ suggest that induction of oxidative injury (e.g., reactive oxygen species/ROS generation) may underlie proteasome inhibitor toxicity. Finally, the UPS is involved in DNA repair processes,¹¹ raising the possibility that proteasome inhibitors may act, at least in part, by promoting DNA damage.

Despite their limited therapeutic range, and uncertainties about their mechanism of action, the search for more effective as well as more selective proteasome inhibitors continues unabated. In a recent report in *Cell Cycle*, Kazi et al. described the activity of a new proteasome inhibitor, designated PI-083, identified by in silico and experimental screening of the NCI's chemical library to target compounds active against the chymotrypsin-like activity of the proteasome.¹² This agent exhibited several noteworthy characteristics, including rapid onset of activity against diverse epithelial neoplasms, including those of breast, ovarian, lung, prostate and myeloma cells. Notably, PI-083

was relatively non-toxic toward the normal counterparts of these transformed cells. In contrast, bortezomib displayed limited activity against epithelial tumors, and did not exhibit anti-tumor selectivity. Consistent with its in vitro actions, PI-083 was active in nude mouse xenograft lung and breast cancer model systems, whereas bortezomib was significantly less effective. Finally, PI-083 inhibited tumor but not normal liver chymotrypsin-like activity whereas bortezomib inhibited activity in both normal and transformed tissues, raising the possibility that this capacity might account for or contribute to PI-083 selectivity. The authors concluded that PI-083 warrants further attention as an antineoplastic agent, particularly in the setting of epithelial tumors.

Given the established activity of bortezomib in hematopoietic malignancies, the identification of a compound that, at least in preclinical studies, appears to have a more rapid onset of action, exhibit greater activity against epithelial tumors in vitro and in vivo, and which shows evidence of enhanced antitumor selectivity, is certainly noteworthy. Whether these desirable preclinical characteristics will translate into

improved activity in patients remains to be established. One natural question is what is the basis for the enhanced preclinical selectivity of PI-083 compared to bortezomib? This issue will be hard to resolve until the mechanism of action of PI-083, and of proteasome inhibitors, is clearly identified. For example, selective toxicity toward transformed cells may reflect their enhanced dependence on an intact ubiquitin-proteasome system, or, alternatively, failure of PI-083 to inhibit proteasome activity in neoplastic cells. The results of the Kazio report suggest that the latter possibility may be particularly relevant in the case of PI-083.

Clearly, additional studies will be necessary to answer this and related questions. For example, to what extent does PI-083 inhibit NF κ B activity, which is often critical to the survival of transformed cells?¹³ Do the selective actions of PI-083 reflect preferential induction of oxidative injury or DNA damage in neoplastic cells? Does the selective lethality of PI-083 toward tumor cells stem from enhanced proteasome inhibition, greater dependence upon an intact UPS, or a combination of these factors? Why

does PI-083 display superior activity toward epithelial tumor cells than bortezomib, and will this capacity translate into improved activity against solid tumor malignancies in the clinical arena? Although much work will be needed to resolve these questions, the preclinical evidence presented here makes a strong case for further exploration of the therapeutic potential of PI-083 and analogous compounds, particularly in solid tumor malignancies.

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c-Myc and telomerase activation

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There is an overwhelming consensus that telomerase activation (a.k.a. de-repression) is required during cancer development; however, the mechanism of its activation in cancer cells is far from clear. At present, the rate-limiting step in telomerase activation appears to be the transcription of the hTERT gene, which encodes the catalytic subunit of the telomerase complex. After the cloning and characterization of hTERT, several groups embarked upon characterization of 5' regulatory sequences of hTERT.^{1,2} From these studies, it became clear that one of the positive factors regulating hTERT expression is c-Myc, which was shown to bind an E-box present in the promoter region of hTERT. Soon after it was established that c-Myc is a direct transcriptional activator of hTERT, many studies also showed a positive correlation between c-Myc overexpression and telomerase activation in tumor cells. Along these lines, Wang et al. showed that c-Myc overexpression leads to telomerase activation and immortalization of human mammary epithelial cells (HMECs).³

Similar results were reported in additional strains of HMECs, human prostate epithelial cells and human fibroblasts.^{4,6}

c-Myc is a potent oncogene. Besides regulating a vast number of genes, it also causes DNA damage and induces genomic instability.⁷ Immortalization of cells is a slow process, occurring over several passages. A continuous expression of c-Myc over several passages is likely to result in genomic changes in cells. Therefore, it is possible that c-Myc induced immortalization of HMECs entails additional genomic changes. In the forthcoming issue of *Cell Cycle*, Bazarov et al. performed careful studies examining the kinetics of telomerase activation and accumulation of additional genomic changes during immortalization of HMECs by c-Myc.⁸ While confirming the earlier results that c-Myc overexpression resulted in immortalization of two different strains of HMECs, the authors reported some intriguing results pointing towards the role of additional genomic changes that may facilitate c-Myc

induced upregulation of endogenous hTERT and immortalization of HMECs.

First, authors report that c-Myc overexpression led to increased activity of a transiently transfected luciferase reporter driven by hTERT promoter. However, as suggested by real time RT-PCR analysis, the levels of hTERT transcripts in c-Myc overexpressing cells did not rise until passage 18, and the mean telomere length continued to shorten until passage 18 plus few more additional passages. From around passage 18, control cultures (vector-infected cells) start entering senescence. The authors suggest that c-Myc starts recognizing endogenous hTERT promoter only after the mean telomere length has reached a limit at which without c-Myc, cells would have entered senescence. If cells are encouraged to proliferate using exogenous factors beyond the senescence point, critically short telomeres can promote genomic instability, the authors speculate that continued c-Myc expression not only promoted proliferation but also possibly resulted in accumulation of

genomic changes. To identify additional genomic changes, the authors carried out array comparative genomic hybridization (aCGH) assays, which identified copy number gains in part or all of chromosome 5 in c-Myc overexpressing cells after passage 18 or so.

So what are the potential targets for copy gain in chromosome 5 and what is the relevance of these copy gains with respect to immortalization? One of the targets in one cell line appears to be hTERT itself. In another cell line, the targets remain to be identified. Although, the functional significance of targets on chromosome 5 remains to be determined, authors speculate that they may participate in chromatin remodeling allowing c-Myc to access the hTERT locus. The chromatin remodeling via DNA methylation or histone modifications including methylation and acetylation is thought to play a role in telomerase repression in somatic cells and its de-repression in tumor cells.⁹⁻¹¹ Moreover,

histone H3-K4 trimethylation by a chromatin remodeling factor SMYD3 was recently shown to be required for hTERT expression and occupancy of its promoter by c-Myc and Sp1 factors in malignant cells.¹²

Myc is expressed reasonably well in most somatic cells, as do most if not all of c-Myc targets, yet telomerase is undetectable in somatic cells under normal circumstances. This argument favors the hypothesis put forward by the authors that chromatin accessibility of the hTERT locus by c-Myc is important for its activation. This accessibility is lost in somatic cells and regained during chromatin remodeling of HMECs post senescence phase (or agonescence¹³). It is unclear what dictates this chromatin remodeling. It may involve genomic instability caused by dysfunctional telomeres or c-Myc overexpression itself. Because it takes many passages, during which telomeres continue to shorten, the authors favors a model

that involves telomere dysfunction. Most breast cancer cells have stable but critically short telomeres; therefore, it is likely that telomere dysfunction coupled with overexpression of c-Myc or another positive regulator of hTERT imparts immortality to breast cancer cells.

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Aurora-C: The youngest of "The Three (Aurora kinase) Tenors" of mitotic symphony

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Faithful equal segregation of duplicated chromosome into the two daughter cells during mitosis is pivotal to maintaining genomic integrity. This exquisitely regulated process of mitotic chromosome segregation by sequential activation and silencing of a host of genes cross communicating in consonance is almost the cellular equivalent of a melodious symphony being played by a group of tenors in perfect harmony. Details of this "mitotic symphony" has been emerging in recent years with the discovery of multiple critical genes and their pathways involved in the process aided by increasing technical capabilities achieved to resolve their phenotypic effects at both the cellular and biochemical levels. Chromosomal passenger protein complex (CPC), consisting of Aurora-B protein kinase, the inner centromere protein INCENP, survivin and borealin, has been mentioned as an essential "conductor" of this symphony due to their well documented roles in orchestrating proper execution of multiple critical steps in mitosis ranging from chromosome-microtubule interactions to sister chromatid cohesion to cytokinesis.¹ In a recent paper, Slattery et al.² now provide compelling evidence that another member of the Aurora kinase family, Aurora-C

is capable of performing virtually all the essential functions ascribed to Aurora-B kinase of the CPC in human cells. The authors have utilized a high throughput automated imaging microscopy technique, developed by them, in conjunction with live cell imaging of cells to demonstrate that the wild type but not the inactive form of Aurora-C, in absence of Aurora-B, can regulate chromosome congression, spindle assembly checkpoint (SAC) and cytokinesis appropriately. In an interesting set of experiments, designed on the basis of the observation that elevated expression of Aurora-C causes depletion of Aurora-B, the authors followed the mitotic phenotypes in human HeLa cells expressing either the wild type or the kinase inactive form of Aurora-C under doxycycline induction in what they referred to as a "single step Aurora-B loss of function and rescue system." The cells expressing the wild type or the mutant Aurora-C were allowed to enter mitosis in a synchronized manner in absence or presence of specific mitotic targeting drugs and analyzed with their custom developed high throughput automated image analysis algorithms coupled with qualitative fluorescence analytical techniques. It was reported that cells expressing the kinase inactive mutant

but not the wild type kinase revealed unaligned and lagging chromosomes in addition to cytokinetic defects. Following release of the cells into mitosis in the presence of proteasome inhibitor MG132 to prevent anaphase entry, unaligned chromosomes with monotelic and syntelic kinetochore-microtubule attachments were observed only in the mutant expressing cells and not in the cells expressing the wild type kinase thus proving that wild type Aurora-C can correct kinetochore-microtubule misattachments. These observations were also corroborated with live cell imaging experiments. Furthermore, expression of the kinase inactive Aurora-C unlike the wild type protein mimicked the mitotic progression defects seen in presence of an inhibitor of Aurora-B kinase. Finally, with their Aurora-C expression/Aurora-B loss of function cells authors demonstrated that Aurora-C, like Aurora-B, can mediate SAC in response to lack of tension across the centromeres in presence of the microtubule stabilizing drug paclitaxel.

The Slattery et al. paper,² while convincingly demonstrating overlapping functional capabilities of Aurora-C and Aurora-B kinases in a human cancer cell line raises interesting possibilities

about the physiological role of Aurora-C in mammalian somatic cells that has remained debatable so far. The findings of this and other published reports on Aurora-C structure-function characterizations in human cells,^{3,4} makes it reasonable to suggest that Aurora-C despite its more recent evolutionary origin retains the conserved functional characteristics of its ancestral family member while acquiring specialized functions of its own in mammalian cells. Aurora kinase family consists of three members, Aurora-A, -B and -C kinases. Having arisen from Aurora-B through gene duplication during mammalian evolution,⁵ it is not surprising that Aurora-C shares functional roles with Aurora-B in mammalian somatic cells and phosphorylate some of the same substrates including those, which form the CPC.^{3,4,6} The debate about the physiological relevance of Aurora-C in somatic cells, however, primarily stems from the facts that Aurora-C knockout mice appear nearly normal with reduced male fertility and that among humans, mutations in the gene is associated with male infertility and no overt somatic phenotypes.^{7,8} While, these are strong evidence of a non-redundant functional role of Aurora-C in male meiosis, they do not necessarily rule out possible redundant yet important functions with non-lethal loss of function phenotypic effects for the kinase in mammalian somatic cells. In fact, reports of Aurora-C expressing at levels similar to Aurora-B in the prostate and spleen⁹ together with the findings that Aurora-C is involved in controlling circadian rhythm in the pineal gland by mediating chromatin remodeling through histone H3 phosphorylation¹⁰ justify such a conclusion. On the other hand, Aurora-A, the well characterized member of this kinase family, essential for embryonic development and

somatic cell proliferation and survival, has been shown to have a role in female meiosis.¹¹ These observations suggest that although the three members of this kinase family have important roles in both somatic and germ cell proliferation and development, the relative significance of each may be physiologically more critical in either of the two developmental compartments. With this understanding, it now becomes imperative to precisely elucidate the molecular and biochemical details of the functional interactions of Aurora-C in mammalian somatic cells that appear both similar as well as distinct from those of Aurora-B and -A kinases. In this respect, it is curious that Aurora-C has been reported to be localized at the centromeres and the midbody in a CPC complex in mitosis like Aurora-B^{3,4} and also at the centrosomes in the interphase cells like Aurora-A.^{12,13} These observations also acquire significance in view of the recent reports that a single amino acid change of Gly-198 in Aurora-A to the equivalent residue of Asn-142 of Aurora-B (Asn-105 of Aurora-C) in the catalytic domain transforms Aurora-A into Aurora-B like kinase in terms of cellular localization and function.¹⁴ Do these observations suggest that besides their organ and tissue specific functions at different developmental stages, all the three Aurora kinase family members have relevant regulatory roles, albeit redundant to some extent in certain instances, through different stages of the mitotic cell cycle? The findings of the Slattery et al. paper² together with those cited above certainly indicate that to be the case. In light of this scenario, the three members of the Aurora kinase family, with Aurora-C as the youngest member in mammalian cells, remind us of the three legendary singing tenors orchestrating the mitotic symphony.

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