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Review

Replication timing as an epigenetic mark

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Although early replication has long been associated with accessible chromatin, replication timing is not included in most discussions of epigenetic marks. This is partly due to a lack of understanding of the mechanisms behind this association but the issue has also been confounded by studies concluding that there are very few changes in replication timing during development. Recently, the first genome-wide study of replication timing during the course of differentiation revealed extensive changes that were strongly associated with changes in transcriptional activity and subnuclear organization. Domains of temporally coordinate replication delineate discrete units of chromosome structure and function that are characteristic of particular differentiation states. Hence, although we are still a long way from understanding the functional significance of replication timing, it is clear that replication timing is a distinct epigenetic signature of cell differentiation state.

Introduction

The present era is experiencing a burst of research activity directed toward understanding how the eukaryotic genome is packaged in the cell nucleus. Animal cloning and the ability to induce pluripotency have underscored the concept that different cell types share an identical and complete genome despite their functional non-equivalence. It is now a general belief that chromatin is packaged in characteristic ways that define how genes respond to developmental cues. However, it is clear that many modifications of chromatin structure, commonly referred to as "epigenetic marks," are short-lived and dynamically reversed during primary transcriptional responses.[1,2] This is not consistent with the concept of epigenetic "inheritance" originally invoked by Waddington[3,4] and Holliday[5-7] to explain the process by which cells become irreversibly committed to a particular lineage, even when transplanted into another region of a developing embryo. Clearly then, one of the important missions of the field of "epigenetics" should be to distinguish events associated with lineage determination from those that are intimately associated with the transcriptional mechanism itself.

In this sense we find it curious that replication timing has been left out of important discussions of epigenetics such as the NIH Epigenomics Roadmap Initiative (http://nihroadmap.nih.gov/epigenomics/) and the first textbook on Epigenetics.[8] Replication timing is a mitotically stable yet cell-type specific feature of chromosomes.[9,10] Chromatin is assembled at the replication fork and different types of chromatin are assembled at different times during S-phase.[11] Every multi-cellular organism studied to date exhibits a strong positive correlation between early replication and transcription.[12] At the same time, changes in replication timing are not directly influenced by nor do they have a direct influence on transcription but rather define a level of higher-order organization of the genome,[9,10,12] which is thought to affect transcriptional competence independent of transcription per se. Replication timing is therefore more in line with the concept of epigenetic inheritance than most histone modifications: indeed, replication defines mitotic inheritance. In fact, the time point of commitment for X chromosome inactivation in mammals is independent of transcriptional down-regulation but is coincident with a nearly chromosome-wide change in replication timing of the inactive X,[12,13] which is one of the best-conserved characteristics of mammalian X chromosome inactivation.[14] Recent work demonstrates convincingly that segments of all autosomes undergo similar changes in replication timing during cell fate determination.[10] Hence, changes in replication-timing profiles reveal chromosome segments that undergo large changes in organization during differentiation and may provide a handle into previously impenetrable levels of chromosome organization and their relationship to cellular identity. In this essay, we highlight features of replication timing that warrant its attention as an epigenetic mark.

Units of Coordinate Replication are Stably Inherited Through Multiple Cell Cycles

A great deal of cytogenetic evidence has established replication timing as a mitotically stable property of chromosomes.[9] Early studies of X chromosome inactivation in female mammals demonstrated that once one of the two X chromosomes becomes late replicating, the same chromosome remains inactive and late replicating throughout the remainder of somatic development.[14] More recently, replication of megabase-sized chromosome segments have been visualized as discrete 'replication foci' by pulse labeling with nucleotide analogs.[9,15] Early replication takes place within the interior euchromatic compartment of the nucleus, excluding nucleoli and blocks of heterochromatin, while late replication takes place at the nuclear periphery, the nucleolar periphery, and at internal blocks of heterochromatin.[15] Pulse-chase experiments have demonstrated that each chromosomal segment takes 45–60 minutes to complete

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replication,[16,17] and labeling foci in living cells demonstrates that these segments remain in their respective sub-nuclear locations throughout interphase.[18] Labeled foci, even adjacent chromosome segments that replicate less than two hours apart, remain distinct and retain their size and intensity for multiple generations.[16,17] Intriguingly, the entire cohort of foci replicated simultaneously in one short time interval have been observed to replicate in almost perfect synchrony at the same time in subsequent cell cycles.[16-19]

These cytogenetic data provide compelling evidence that the DNA that replicates together stays together as a stable structural and functional chromosomal unit for many generations.[9] However, they cannot evaluate the extent to which the molecular boundaries between these coordinately replicated units are stable within populations of cells. A recent genome-wide microarray analysis has demonstrated the existence of precise molecular boundaries between coordinately replicating units of chromosomes.[10] Strikingly, three different mouse embryonic stem cell (ESC) lines, two from independent mouse strains, as well as induced pluripotent stem (iPS) cells derived from mouse tail tip fibroblasts, revealed nearly identical replication timing profiles. The distant genetic and temporal histories of these cell lines demonstrate convincingly that coordinately replicated units of chromosomes or "replication timing domains," are a stable and characteristic property of a particular cell type.

Replication Timing is an Epigenetic Signature of Cellular Differentiation State

Developmental regulation is a key hallmark of an epigenetic mark.[7] While the aforementioned observations make a strong case for the mitotic inheritance of replication timing profiles in a given cell type, evidence for developmental regulation has been primarily anecdotal.[12] As described, the first and most well-established example of developmental regulation of replication timing is X chromosome inactivation in female mammals, which accompanies a shift from early- to late-replication of the inactive X in the epiblast of ~6.0 dpc mice.[20] The β-globin locus represents another classic example. In non-erythroid cells, β-globin is late replicating and shows features of silent chromatin, while in murine erythroleukemia cells that can be induced to express high levels of β-globin, the locus is DNaseI-accessible and early replicating.[12,21-23] Several other gene loci have been shown to exhibit replication-timing differences between different cell lines[12] and in the case of the mouse immunoglobulin heavy chain locus, earlier replication is also associated with a more DNaseI-accessible chromatin conformation.[24-26] A limitation to most of these studies is that a comparison of different established cell culture lines cannot rule out genetic differences or chromosomal aberrations that have occurred during long term cell culture. Moreover, most genes do not show difference in replication timing between different cell types,[27,28] and replication timing correlates quite strongly with static sequence features of chromosomes (e.g., isochore GC content and gene density),[29-32] raising legitimate questions as to the significance of those few differences that had been identified.[33,34] In fact, a microarray-based comparison of human chromosome 22 between fibroblast and lymphoblastoid cells revealed that only 1% of this chromosome differed in replication time.[31]

Dynamic changes in replication timing were first confirmed for a handful of gene loci during mouse ESC neural differentiation,[27,28] and it was proposed that changes in replication timing are limited to a specific class of genes that reside within AT-rich regions,[27] providing a potential explanation for why the GC-rich human chromosome 22 showed so few differences. Hence, conflicting data from limited comparisons of different cell types were unable to distinguish the extent to which replication-timing changes occur during development until in 2008, when a genome-wide study of replication timing during ESC differentiation to neural precursor cells (NPCs) revealed changes across ~20% of the mouse genome.[10] Importantly, the replication-timing changes that occurred were nearly identical using two different neural differentiation protocols and three cell lines. The ~20% changes seen are certain to be a fraction of the total changes that take place during development, since different chromosomal segments change replication timing in different cell lineages. Thus, it can now be stated confidently that a significant portion of the genome is subject to dynamic changes in replication timing during development, to create replication timing profiles that are cell-type specific. Addressing the extent of profile differences between cell lineages is an important future direction.

Early Replication Timing is Associated with "Active" Chromatin Structure

A close relationship has been observed between replication timing, chromatin structure and transcriptional activity. By the mid 1970's it was already known that late-replicating DNA coincided with AT-rich, Giemsa-dark, G-bands on metaphase chromosomes with low transcriptional activity, while early-replicating DNA coincided with GC-rich, R-bands with high transcriptional activity.[35] In the 1980's and 90's these cytogenetic observations were confirmed at a few dozen individual gene loci using molecular approaches, with the finding that early replicating genes could be either expressed or silent, while late replicating genes were almost always silent, leading to the hypothesis that early replication is necessary for transcriptional competence but is not sufficient for transcription per se.[12] This notion was supported by experiments deleting the Locus Control Region (LCR) of the human β-globin gene, which abolished β-globin gene transcription while early replication timing and DNaseI hypersensitivity remained unaffected.[21,22] In fact, reporter plasmids injected into early but not late S-phase mammalian nuclei assemble into a hyperacetylated chromatin structure permissive for transcription.[36] In the 2000's, the correlation between early replication timing and transcription was validated in higher eukaryotes using various types of microarray approaches. In Drosophila, mouse and human, a nearly identical strong and statistical positive correlation between early replication and transcription was found.[10,30,31,37-40] These same studies also confirmed that a small number (less than 5% of total genes) of late replicating genes were expressed. These genes were enriched for CpG-island rich, strong promoters that may render regulatory elements accessible even from within hypoacetylated chromatin.[10] Together, these results support a model in which different types of chromatin are assembled onto replication forks at different times during S-phase,[11] which can influence the expression of many but not all genes.

There is also evidence for the reverse type of model: that the structure of chromatin can influence replication time. Mutations, overexpression, or tethering to specific locus of chromatin modifying proteins in both yeast and mammalian systems have varying effects

on replication timing.[12,41-47] However, these studies have detected relatively modest changes in replication timing of certain genomic regions and no one mechanism has been found that regulates the global replication timing profile. Moreover, in fission yeast, the replication times of three different types of heterochromatin appear to be regulated by different mechanisms[46] and there are examples of heterochromatin that replicate early[48] and its early replication appears to depend upon the heterochromatic structure,[46] underscoring the complexity of these relationships. The most parsimonious explanation for these various results is that different chromosome segments have different chromatin components that contribute combinatorially to replication time.

In summary, the link between replication timing and chromatin structure is still unclear with indirect evidence supporting two rather intuitive genres of models in which early replication dictates open chromatin or open chromatin dictates early replication.[12] Combining these models creates an attractive scenario in which replication timing provides a means to inherit chromatin states that, in turn, regulate replication timing in the subsequent cell cycle, so that once a timing program is established, it forms a self-reinforcing auto-regulatory loop that is very stable for many generations. Moreover, since replication is coordinately regulated at the level of large megabase-sized domains, a change in replication timing would rapidly transmit a change in chromatin state to the entire chromosomal domain.

Replication Timing Reflects Global Nuclear Programming

Recent success in generating reprogrammed iPS cells highlight the importance of transcription factors as initiators of a change in cellular state.[49] However, cellular reprogramming is an inefficient (~1% of cells achieve pluripotency) and gradual process, requiring many cell generations and sequential apparently stochastic intermediate steps.[50] Reprogramming efficiency decreases with developmental progression, consistent with increased intrinsic restrictions in cellular competence as cells become canalized farther down specific lineages.[50] These restrictions are presumably due to changes in the epigenetic chromatin landscape that transcription factors must act upon during the reprogramming process. However, localized changes in chromatin structure, such as those mediated by histone modifications are for the most part easily erased enzymatically.[51] In contrast, higher-order organization of chromosomes at the megabase domain level and the 3-dimensional organization of these domains in the nucleus, such as is seen with X chromosome inactivation with the formation of a Barr body,[52] would seem to be much more difficult structures to reverse once established. Spatial organization of the genome in the nucleus is becoming increasingly recognized as a form of epigenetic regulation[53,54] and there is a close association of replication timing with sub-nuclear organization of chromatin.[9] As discussed earlier, early and late replicating sequences occupy different subnuclear compartments.[15] In addition, genes that changed their replication timing during differentiation of mouse ESCs also change their subnuclear position.[10,55] This suggests that changes in replication timing may provide a more tractable molecular handle into previously impenetrable higher-order levels of chromosomal organization in the nucleus. This is significant, given that there is currently no high-throughput methodology to assess changes in 3-dimensional genome organization, yet replication timing can be evaluated genome-wide in a relatively simple manner.

De-Regulation of Replication Timing is Associated with Disease Phenotypes

Temporal control of DNA replication is linked to many basic cellular processes that are regulated both during the cell cycle and during development. In several model systems, defects in replication timing are associated with defects in chromosome condensation, sister chromatid cohesion and genome stability.[56,57] Abnormal replication-timing control has become a clinical marker for predicting malignant cancers.[58-62] In particular, specific chromosome translocations result in a chromosome-wide delay in replication timing that triggers additional chromosome translocations at a high frequency.[63,64] Finally, cells from patients with several inherited human diseases show defects in replication timing that correlate with misregulation of genes during development.[65-72] These studies, at present still limited in scope, associate aberrations in replication timing with phenotypic consequences and strengthen the case for a role of replication timing in chromosome structure and function. Its role is still elusive and may extend beyond regulation of transcription: the locations and directions of replication forks, the organization of replication complexes that coordinate replication of large domains, and the locations of domain boundaries may constitute an epigenetic basis for tissue-specific or cancer-promoting differences in genome stability. Nonetheless, these studies demonstrate that replication timing is a property of chromosomes that is not dictated by DNA sequence but is somatically heritable and linked to phenotype, consistent with both old and new definitions of an epigenetic mark.

Conclusions

A large body of evidence has confirmed strong associations between replication timing and important properties of chromatin. While the contribution of replication timing to phenotype remains a mystery, a strong case can be made for replication timing as an epigenetic mark that is a stable, heritable property of different cell types. We anticipate that studies of replication timing in many cell types from varied differentiation pathways will provide novel insights into epigenetic regulation, particularly with regards to previously impenetrable higher-order multi-megabase levels of chromosome organization. The mere fact that different types of chromatin are assembled at different times during S-phase places replication timing at the intersection of physical, spatial and temporal organization of chromosomes.

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