CHAPTER

AUTOSOMAL LYONIZATION OF REPLICATION DOMAINS DURING EARLY MAMMALIAN DEVELOPMENT

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Abstract: It has been exactly 50 years since it was discovered that duplication of the eukaryotic genome follows a defined temporal order as cells progress through S-phase. While the mechanism of this replication-timing program still remains a mystery, various correlations of this program with both static and dynamic properties of chromatin render it an attractive forum to explore previously impenetrable higher-order organization of chromosomes. Indeed, studies of DNA replication have provided a simple and straightforward approach to address physical organization of the genome, both along the length of the chromosome as well as in the context of the 3-dimensional space in the cell nucleus. In this chapter, we summarize the 50-years history of the pursuit for understanding the replication-timing program and its developmental regulation, primarily in mammalian cells. We begin with the discovery of the replication-timing program, discuss developmental regulation of this program during X-inactivation in females as well as on autosomes and then describe the recent findings from genome-wide dissection of this program, with special reference to what takes place during mouse embryonic stem cell differentiation. We make an attempt to interpret what these findings might represent and discuss their potential relevance to embryonic development. In doing so, we revive an old concept of "autosomal Lyonization" to describe "facultative heterochromatinization" and irreversible silencing of individual replication domains on autosomes reminiscent of the stable silencing of the inactive X chromosome, which takes place at a stage equivalent to the post-implantation epiblast in mice.

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INTRODUCTION

With all phenomena in nature there are two major questions asked: the underlying mechanism (how?) and its biological significance (why?). All eukaryotic cells replicate their DNA in a specific temporal sequence but both the mechanism of this "replication timing program" and its biological significance remain a mystery. Understanding the mechanism might allow us to manipulate replication timing and query significance, but even with the power of molecular genetics in budding and fission yeasts, very little mechanistic insights have been gained.[1] Many studies point to regulation at the level of large chromosomal domains or subnuclear compartments, making replication timing an excellent gateway into the higher order structure and functional organization of chromosomes, albeit refractory to traditional molecular and biochemical approaches. By corollary, understanding significance might provide clues as to mechanism. Some studies have suggested evolutionary roles for replication timing in focusing mutation rates and/or suppressing recombination,[2,3] while others have suggested housekeeping roles such as the overall coordination of replicating large genomes in the presence of limited metabolic precursors.[4] In addition, a longstanding correlation between early replication timing and transcriptional competence has been substantiated by recent genome-wide studies,[1,5] but it is not clear whether transcription drives early replication or vice versa.

If replication timing were related to transcriptional competence, it should be developmentally regulated. In this chapter, we summarize the evidence for developmental regulation of replication timing in mammals historically from its original inception as a property associated with the process of X-inactivation in females[6] to very recent studies verifying an unanticipated degree of autosomal replication timing changes taking place at the level of megabase-sized chromosomal "replication domains."[7,8] In particular, we revive an old concept of "autosomal Lyonization" to describe "facultative heterochromatinization" and irreversible silencing of individual replication domains on autosomes reminiscent of the stable silencing of the inactive X chromosome (Xi),[9] which takes place at the epiblast stage in mice.[8] Moreover, comparative studies have revealed that replication timing programs and the changes that occur during development are evolutionarily conserved, to a greater extent than either the positions of replication origins or the overall GC content of chromosomal isochores.[10] These observations suggest positive selection for a replication program that is not arbitrarily dividing the genome into temporally separated segments for housekeeping purposes but is intimately related to chromosome structure and function.

REPLICATION TIMING PROGRAM: AN ELUSIVE MEASURE OF GENOME ORGANIZATION

Early Experiments

Early studies of DNA replication led to the discovery of key concepts in chromosomal organization. J. Herbert Taylor's synthesis of tritiated thymidine in 1953 allowed the first glimpses of DNA synthesis in living cells[11] at a time when the structure of DNA was just being resolved. Taylor's series of thymidine labeling experiments not only provided the first demonstration of the semi-conservative anti-parallel nature of DNA replication (more than one year prior to Messelson and Stahl[12]), [13]but also revealed a specific temporal

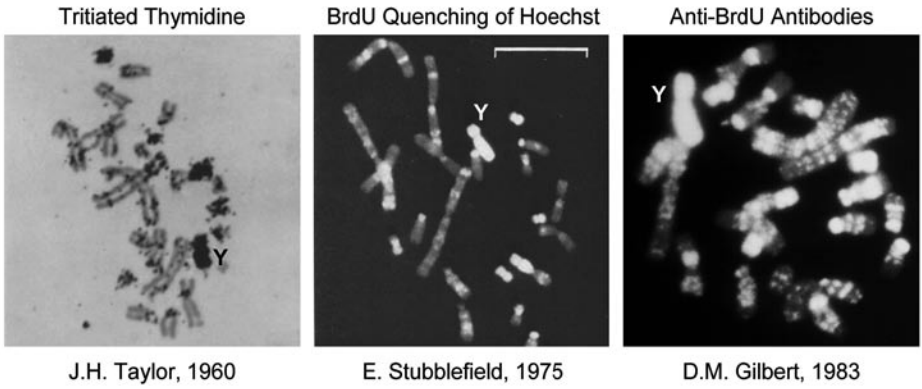

Figure 1. Brief Pulse-Labeling of DNA Synthesis Highlights Megabase-Sized "Replication Domains". Chinese hamster cells were pulse labeled for 10 minutes with either tritiated thymidine (left) or BrdU (right) during late S-phase or labeled continuously with BrdU except for 1 hour in late S-phase during which cells were labeled with thymidine (middle) and then chased with unlabeled medium into mitosis. Metaphase spreads were analyzed by autoradiography (left), BrdU quenching of Hoechst dye (middle) or indirect immunofluorescence using anti-BrdU antibodies (right). These cytogenetic methods demonstrated that megabase-sized segments of the genome are labeled in very short periods of time, producing banding patterns that were characteristic for each chromosome and varied during S-phase, with euchromatic R bands replicating early and heterochromatic G bands replicating late. Note that the heterochromatic Y chromosome is almost entirely labeled in a 10-minute period late in S-phase. With known rates of replication fork movement, this could only be achieved by the nearly synchronous firing of clusters of replication origins. Figure was adapted from J.H. Taylor[15] (left-© Taylor, 1960. Originally published in *The Journal of Biophysical and Biochemical Cytology*. 7: 455-463), E. Stubblefield[18] (middle) and D.M. Gilbert[102] (right) with permissions from *Journal of Biophysical and Biochemical Cytology*[15] *and Chromosoma*.[18]

program to the replication of DNA in the chromosomes of both plant[14] and animal[15] cells. In particular, by pulse-labeling Chinese hamster cells (whose chromosomes are easily distinguished by size) and then examining metaphase chromosomes at various times after the pulse, he found that different segments of chromosomes replicate within specific time intervals during S-phase (Fig. 1). From this study,[15] Taylor concluded that: "parts of chromosomes have a genetically controlled sequence in duplication, which may have some functional significance." Exactly 50 years later, that functional significance remains a total mystery.

By the 1970s, these coordinately labeled segments of chromosomes were found to be similar in appearance and size to chromomeric banding patterns of chromosomes seen using banding methods such as Giemsa staining. When it was discovered that incorporation of BrdU into DNA (instead of tritiated thymidine) could squelch the fluorescence of Hoechst dye,[16] a novel chromosome banding method ("replication banding") was developed that avoided the use of radioactivity and long autoradiography exposures (Fig. 1). In general, the transcriptionally active, GC-rich, R-bands were found to be early replicating, while the transcriptionally inactive, AT-rich, G-bands were late,[17] although the alignment was not absolute.[18,19] These results supported the hypothesis that heterochromatin is late replicating, which was originally proposed by Lima-de-Faria based on studies of grasshopper sex chromatin.[20] The finding that the replication time of individual chromosome segments is related to their transcriptional activity raised the possibility that coordinately replicated segments may represent not only structural but also functional units of chromosomes.

This was an attractive hypothesis, but if replication were related to transcription, one might expect to find different banding patterns in different cell types. Unfortunately, comparisons of replication banding patterns in different cell types failed to detect such differences.[21] Of course, the resolution of such studies could not rule out the existence of localized changes.[10] Moreover, studies in frog embryos[22] and studies of mammalian X-inactivation (discussed below) demonstrated that replication timing could change during development in a manner correlated with transcriptional activity.

The Lessons from X Chromosome Inactivation

In his 1960 study, Taylor noticed that, in female cells, the X chromosomes replicated asynchronously.[15] Coincidentally, in 1961, Mary Lyon proposed her famous hypothesis that the cytological manifestation of X-inactivation, the Barr body,[23] appears coincident with its genetic inactivation in early development and that both the structural and functional alterations of the homolog randomly chosen for inactivation are stably maintained in all subsequent somatic generations.[24] Taylor then went on to verify that the Barr body was late replicating in female human cells and that in cells with several X chromosomes all the Barr bodies replicated late.[6] These findings introduced a completely novel and mysterious notion: the fact that the two genetically identical X chromosomes behave differently meant that homologous chromosomes can be either heterochromatic or euchromatic in the same cell, leading to the unavoidable conclusion that replication timing is determined epigenetically—not by sequences alone.

Over the next two decades, the appearance of a late replicating Xi during early embryonic stages was used as a reliable cytological marker for X-inactivation, as it is one of the most conserved features of X-inactivation.[25] In fact, while late replication of the Xi seen in placental mammals (eutherians) is conserved in marsupials and at least partly in the egg-laying platypus (monotremes),[26,27] eutherian X-inactivation features such as the *Xist* gene, enrichment of "repressive" histone modifications and possibly promoter DNA methylation are either missing or not reported to date in marsupials and monotremes.[26,28] Upon random X-inactivation in the mouse embryo proper, a late-replicating Xi emerges at the post-implantation epiblast stage,[29] which precedes de novo DNA methylation of gene promoters on the Xi.[30] Later, the development of in vitro differentiation systems for embryonic stem cells (ESCs) allowed for the temporal order of events to be more precisely determined. ESC differentiation studies suggest *Xist* coating of the Xi to be the earliest event upon random X-inactivation, followed by an exclusion of RNA polymerase II from the Xi, then by the loss of "active" histone modifications and the acquisition of "repressive" histone marks.[31] Xi's switch to late replication either coincides with or occurs shortly after changes in histone modifications (based on metaphase spread analyses[32,33]), whereas de novo promoter DNA methylation occurs much later.[32] While it is tempting to speculate a causal role for earlier events in regulating the Xi's switch to late replication, this idea is difficult to reconcile with the fact that *Xist* and "repressive" histone modifications are missing from the Xi in marsupials[28] and yet a switch to late replication is observed.[26] Indeed no report to date has demonstrated a causal role for chromatin modifiers in regulating replication timing of the Xi. Interestingly, however, using a mouse ESC differentiation model with an inducible *Xist* transgene, Wutz et al demonstrated that the time point of commitment for X-inactivation is independent of transcriptional down-regulation but is temporally closely associated with a nearly chromosome-wide shift to late replication of the Xi.[32,34]

Compared to the late replicating state of the Xi in the embryo proper, the situation is somewhat different in the extra-embryonic lineages, in which the paternal X chromosome (Xp) is inactivated in an imprinted manner. Both in trophectoderm and primitive endoderm, the Xp temporarily becomes replicated precociously very early in S-phase, earlier than any autosomes.[35] The switch from synchronous to asynchronous replication timing of the X chromosomes takes place just after these extra-embryonic tissues are specified, at embryonic day (E) 3.5 in trophectoderm and E4.0–4.5 in primitive endoderm.[35] Then, around E6.0–6.4, the Xp in these two lineages (primitive endoderm and trophectoderm become visceral endoderm and extra-embryonic ectoderm, respectively, at this stage) switches to late replication and stably maintains its late replication state thereafter.[29] This abrupt nearly chromosome-wide switch may occur within a single cell cycle.[36] These observations indicate that the unusual precocious replication of the Xp in the extra-embryonic lineages is transient, whereas the switch to late replication of the Xi is conserved between embryonic and extra-embryonic lineages and is stably maintained during development. Interestingly, the emergence of both trophectoderm at E3.5 and primitive endoderm at E4.0 represent the first obvious manifestations of lineage commitment, segregating these extra-embryonic lineages from the embryo proper. Thus, the first two major cell fate transitions during early embryogenesis accompany alteration in replication-timing regulation of the Xi. It is not known, however, what the subsequent, abrupt switch to late replication at E6.0–6.4 in these extra-embryonic lineages represents.[29] Interactions between extra-embryonic and maternal tissues may be involved.

Replication Timing Landscape on Autosomes

Are replication timing changes unique to the X chromosome or are there equivalent events on autosomes during differentiation that escape cytological detection? Might there be a similar program to "Lyonize" (i.e., facultatively heterochromatinize) individual replicons or clusters of replicons[9] that are too small to visualize microscopically? In the 1970s, Carl Schildkraut and Walt Fangman pioneered the use of molecular methods to investigate the temporal order of replication in mammals[37] and budding yeast,[38] respectively. Together, these studies provided strong evidence that a precisely regulated replication-timing program is a conserved property of all eukaryotic cells. In the 1980s, the replication times of a few dozen genes became cataloged in different stable cell lines,[39-42] unambiguously identifying autosomal replication timing differences. The emerging rule of thumb was that if a gene were transcriptionally active, it would be early replicating, while late replicating genes were always inactive. In fact, a study comparing active and inactive clusters of *Xenopus* 5S rDNA genes that were known to compete for the same transcription factors revealed that the active gene clusters replicate substantially earlier, suggesting a model in which early replication could provide a competitive advantage for access to limiting quantities of activating factors at the replication fork ("first come, first served").[43] This model has yet to be refuted or substantiated.

The extent of developmental changes, however, had been elusive. Due to technical difficulties of working with differentiating cell cultures, replication timing studies through the early 2000s were limited to a few dozen gene loci in established, usually transformed, cell lines. Hence, it remained possible that many of the replication differences observed between cell lines resulted from genetic or epigenetic changes accumulated during long term culture. The advent of directed ESC differentiation

systems sufficiently homogeneous to perform molecular analyses permitted the first direct demonstration of differentiation-induced replication timing changes of autosomal loci in 2004.[44,45] However, replication timing of a fraction of ~100 genes analyzed was regulated and with only neural differentiation pathway analyzed, it was difficult to conclude whether developmental replication timing changes were frequent or rare. Various studies led to the conclusion that many genes replicate at the same time in all cell types, consistent with the cytogenetic studies.[46,47] Thus, these reports clearly provided evidence for differentiation-induced replication timing changes, but the small sampling demanded the use of genome-wide approaches to statistically determine the extent of changes.

The first genome-wide replication timing analysis was performed in budding yeast.[48] Unexpectedly, no correlation was found between replication timing and transcription, a finding that has since been corroborated in fission yeast.[49] Shortly thereafter, a series of microarray-based studies in *Drosophila* and mammalian cells provided evidence for a strong correlation between early replication and transcriptional activity in these higher eukaryotes,[7,50-58] suggesting that this relationship might be restricted to metazoa.[59] This correlation, along with the fact that gene expression programs change during differentiation, raised the possibility that a considerable degree of replication-timing changes might take place during development. However, the first report that examined more than one cell line found only 1% difference in replication timing across chromosome 22 in human fibroblasts vs lymphoblasts.[53] Moreover, several of these studies found that replication timing correlated strongly with static sequence features of mammalian chromosomes such as GC content and gene density.[7,52-56] Indeed, as recently as in 2008, many investigators had concluded that replication-timing changes are very rare and hence their significance to development came into question.[60-62] Still, the resolution and limited genomic coverage of existing studies and the paucity of data comparing cell lines left this fundamental question unanswered.

The advent of high-density oligonucleotide microarrays to query the genome at sufficient probe density, combined with ESC differentiation systems, offered an unprecedented opportunity to potentially induce and study changes in the replication-timing program during major cell fate transitions. In 2008, we were able to achieve such an analysis during differentiation of mouse ESCs to neural precursor cells (NPCs).[7] This study revealed that several polymorphic ESC lines showed virtually identical replication timing profiles, with clearly delineated patterns of coordinately replicated megabase-sized chromosome domains. Upon differentiation of ESCs to NPCs, changes took place across nearly 20% of the genome.[7] Combined with a follow-up study,[8] we have constructed replication profiles for 22 cell lines representing 10 different cell types that model differentiation of three germ layers during early mouse development (Fig. 2). Results revealed cell-type specific replication-timing profiles resulting from extensive developmental changes affecting nearly half the genome.[8] Replication timing changes occur coordinately across 400–800 kb segments of chromosomes within larger regions of constitutive replication, explaining why they escape cytological detection. Moreover, although the correlation between early replication and GC content or gene density is always positive and the most GC- or AT-rich genomic segments remain early or late replicating respectively in all cell types, it is the segments with intermediate GC content and gene density that change replication timing and these changes can substantially alter the overall degree to which replication timing correlates with static sequence features.[8]

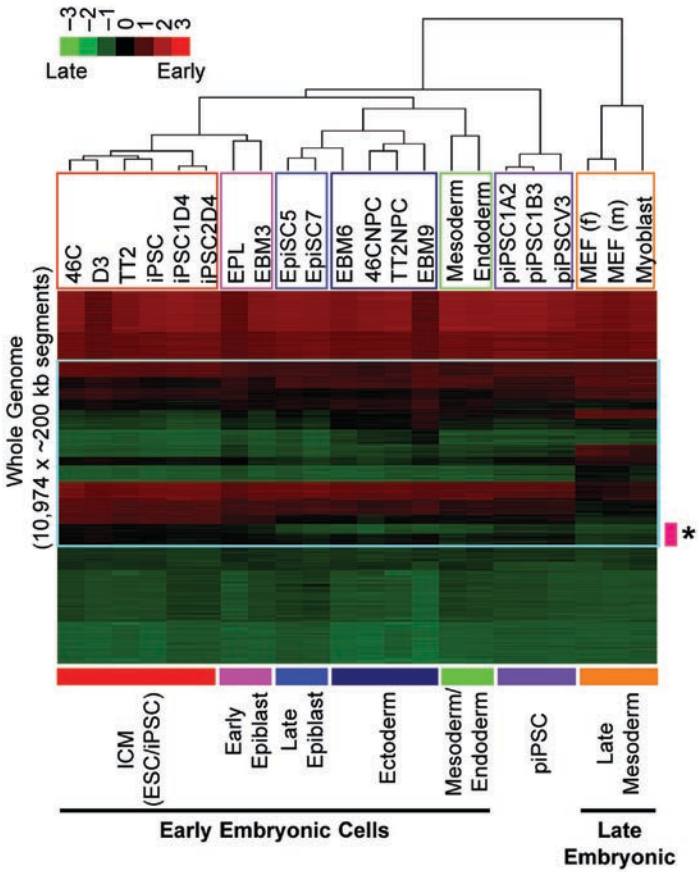

Figure 2. Relationship between cell culture models of mouse embryogenesis based on replication timing profiles. Hierarchical clustering of 22 mouse cell lines based on replication timing profiles obtained by microarrays.[8] The dendrogram reveals an epigenetic separation of cell types representing the late epiblast (EpiSCs) from the early epiblast (EPL and EBM3) as well as the ICM [ESCs (46C, D3, TT2) and fully reprogrammed iPSCs]. EpiSCs were more related to committed germ layer cell types of the early embryo [ectoderm (EBM6), neurectoderm (46CNPC, TT2NPC and EBM9), nascent mesoderm and endoderm]. Three partially reprogrammed iPSC (piPSC) lines were distinct from late embryonic cell types (MEFs and myoblasts), but were also distinct from ICM, epiblast or early germ layer cell types, forming an independent branch. The asterisk on the right indicates genomic segments that complete lineage-independent EtoL changes by the post-implantation epiblast stage (which roughly corresponds to 155 Mb total). Late replication of these segments was stably maintained in all downstream lineages and not reversed in piPSCs, which also exhibited difficulty in transcriptional reprogramming of genes within these segments. Figure was adapted from Hiratani et al with permission from *Genome Research*.[8]
Methods: Cells were pulse-labeled with BrdU, separated into early and late S-phase fractions by flow cytometry and BrdU-substituted DNA from each fraction was immunoprecipitated with an anti-BrdU antibody. The early and late replicating DNA samples were differentially labeled and cohybridized to whole-genome oligonucleotide microarrays. The ratio of the abundance of each probe in the early and late fraction ["replication timing ratio" = $\log_2$(Early/Late)] was then used to generate a replication timing profile for the entire genome at a density of one probe every 5.8 kb. Then, the whole genome was divided into 10,974 ~200-kb segments and their average replication timing ratios were compared between cell lines by hierarchical clustering. The heatmap shows the replication-timing ratios [= Log_2(Early/Late)] of 10,974 ~200-kb segments, with red and green representing early and late replication, respectively. Segments framed in blue shows those with significant differential between any cell types, which represent 45% of the genome.

AN EVOLUTIONARILY CONSERVED EPIGENETIC FINGERPRINT

It is now clear that replication-timing differences are extensive during early mammalian development. Moreover, comparison of two *Drosophila* cultured cell lines derived from embryonic or imaginal disc tissue also revealed approximately 20% differences in replication timing,[57] suggesting that extensive developmental changes are common in higher eukaryotes. But are these changes meaningful to the development of the animal or are they merely stochastic events with very little consequence to the fitness of the organism? Our current understanding of the mechanisms regulating replication does not permit a direct manipulative approach to this question. An indirect alternative is to evaluate whether the replication timing programs of individual cell types have been positively selected during evolution. To this end, we extended our analyses to differentiating human ESCs. Consistent with the mouse data, we found that multiple human ESC lines displayed nearly identical replication timing profiles that changed across approximately 20% of the genome during differentiation to NPCs (T. Ryba, I.H. and D.M.G, *unpublished*). As in mice, changes in replication timing generally occurred coordinately across 400–800 kb chromosome domains, suggesting a conserved unit size of replication timing changes that most likely involves a coordinated regulation of at least 2-3 replicons. However, human ESCs differed substantially in their replication timing profiles from mouse ESCs within regions of conserved synteny (T. Ryba, I.H. and D.M.G, *unpublished*). In fact, they aligned much more closely with stem cells derived from the post-implantation mouse epiblast, the EpiSCs (epiblast-derived stem cells),[63,64] providing a genome-wide support for the hypothesis that human ESCs represent an epiblast-like state that is downstream from the inner cell mass(ICM)-like state that mouse ESCs represent (T. Ryba, I.H. and D.M.G, *unpublished*). In addition, human cells showed a significantly lower correlation of early replication to GC content and GC content was significantly less well conserved than replication timing between human and mouse (T. Ryba, I.H. and D.M.G, *unpublished*).

Furthermore, recent unpublished comparisons of distantly related fission (N. Rhind, *personal communication*) and budding (K. Lindstrom and B. Brewer, *personal communication)* yeast species find a lack of conserved replication origin positions but a remarkable conservation of the replication-timing program. This is consistent with many observations suggesting that replication timing is independent of where replication initiates. For instance, the human beta-globin locus frequently replicates from one of two closely spaced origins while the mouse locus uses many widely dispersed origins and yet replication timing is conserved.[65] Moreover, the replication time of chromosomal domains is re-established in each cell cycle at a time point in G1 (TDP; timing decision point) prior to and independent of origin site specification, which takes place later in G1 at the ODP (origin decision point).[66-69] Altogether, these findings indicate that the replication-timing program is under considerably stronger positive selection during evolution than either overall GC content or replication origin positions.

The significance of this program and whether or not it is reflecting a mechanistic linkage to another chromosome property that is the direct object of the selective pressure remains to be determined. For example, early replication correlates positively with overall transcriptional activity domain-wide and with histone modifications associated with transcriptionally active chromatin (H3K4me3, H3K36me3) and inactive chromatin (H3K9me2) (albeit not with known inactive modifications H3K9me3 or H3K27me3).[7,70] However, ablation of several chromatin-modifying enzymes (Mll, Mbd3, Eed, Suv39h1/h2,

G9a, Dnmt1/3a/3b, Dicer) has surprisingly modest effects on replication timing.[1,70-72] Meanwhile, a recent study in fission yeast showed that Swi6, an HP1 (heterochromatin protein (1) ortholog enriched in heterochromatic domains, regulates replication timing through the loading of the replication initiation factor, Sld3.[73] Although no mammalian Sld3 orthologs have been reported and HP1 does not appear to regulate the late replication timing of pericentric heterochromatin in mice,[71] this study raises the intriguing possibility that domain-wide chromatin factors could regulate replication timing through initiation factor accessibility. For example, indirect observations suggest that a competition between histone H1 vs HMG-I/Y proteins across large chromosomal segments could regulate replication timing during differentiation.[74]

REPLICATION TIMING AS A QUANTITATIVE INDEX OF 3-DIMENSIONAL GENOME ORGANIZATION

During X-inactivation, the switch to late replication leads to an almost synchronous late replication of the entire X chromosome, which becomes highly condensed and localized to the periphery of the nucleus (Fig. 3A), forming what is known as a Barr body in a process that used to be called "Lyonization."[23] This relationship between replication timing and subnuclear position is not confined to the X chromosome, but is also reflected in the positions of individual autosomal replication domains. Domains that replicate at different times during S-phase are localized to different compartments within the nucleus that can be visualized by pulse labeling with nucleotide analogs and staining with antibodies against them (Fig. 3B), giving rise to the appearance of punctate labeled sites known as "replication foci."[1] These replication foci are clearly not artifacts of fixation as they can also be observed in living cells labeled with fluorescent nucleotides.[75] In virtually every animal cell examined, the first half of S-phase consists of hundreds of dispersed internally localized sites of replication that dramatically transition into more clustered peripherally localized foci near the middle of S-phase.[75,76] When these labeled foci are chased through subsequent cell cycles, the labeled segments do not mix, separate or change in shape, size or intensity, suggesting that the DNA that replicates together remains together as a stable structural and functional unit of interphase chromosome structure.[77] Quantitative microscopic methods in human cells estimate close to 1 Mb of DNA is replicated within each focus.[78] It is tempting to speculate that the 400–800 kb units of replication change observed during both mouse and human cell differentiation are the molecular equivalents of replication foci, although this remains a very difficult hypothesis to test.

If a mechanism resembling "Lyonization" were occurring on autosomes, then we would expect autosomal replication-timing changes to be accompanied by changes in subnuclear position. Indeed, this was found to be the case for all seven loci tested during neural differentiation.[7,8,79] Interestingly, whereas replication-timing changes occurred over the course of several cell cycles before stabilizing, subnuclear repositioning was relatively abrupt, occurring primarily during the time when replication-timing changes traversed the mid-late stages of S-phase (Fig. 3C).[8] For example, genes found to undergo significant shifts to later replication but confined to the first half of S-phase remained in the interior of the nucleus, whereas even smaller shifts to late replication that traverse the mid-late stages of S-phase were accompanied by movement toward the periphery.[8] Moreover, genes replicated throughout the first half of S-phase have an equal probability of being expressed, whereas the strongest correlation between transcription and replication

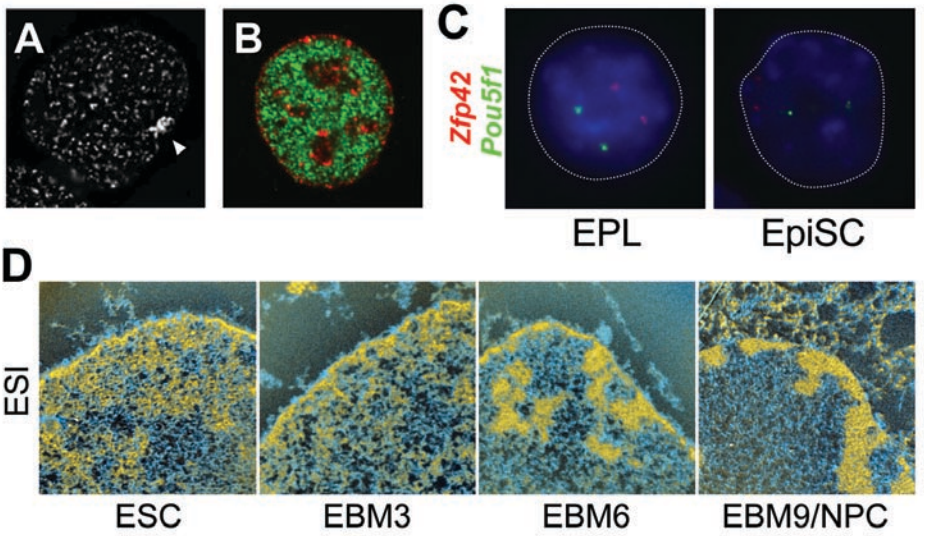

Figure 3. Subnuclear genome organization revealed by studies of DNA replication. A) Late-replicating, condensed inactive X chromosome (Xi, arrowhead) at the nuclear periphery in MEF cells revealed by a 10-minute BrdU pulse labeling during mid-late stages of S-phase. Figure was adapted from Wu et al with permission from Journal of Cell Biology.[71] B) Sites of DNA replication in early S (green, a 10-minute CldU pulse in early S) vs late S-phase (red, a 10-minute IdU pulse in late S of the same cell cycle) revealed by a "pulse-chase-pulse" experiment in mouse C127 fibroblast cells. Note that DNA replication during late S-phase takes place preferentially at the nuclear periphery and nucleolar periphery, whereas during early S-phase, it takes place in the interior of the nucleus excluding these two subnuclear compartments. C) Representative 2D DNA-FISH of Zfp42 (also known as Rex2) and Pou5f1 (also known as Oct4) loci in EPL cells and EpiSCs, which model early and late epiblast stages in mice, respectively.[8] Zfp42 locus is a representative lineage-independent EtoL locus that completes its EtoL change during the transition from early to late epiblast equivalent stage (see Figure 2, asterisk), during which its timing change traverses the mid-late stages of S-phase. By contrast, Pou5f1 locus is constitutively early replicating. Zfp42 locus (red signals) is repositioned from the interior toward the nuclear periphery in EpiSCs but not EPL cells, whereas Pou5f1 locus (green signals) maintains its internal positioning in both cell types. Figure was adapted from Hiratani et al with permission from Genome Research.[8] D) Electron spectroscopic imaging (ESI) analysis of nuclei during ESC differentiation.[8] Images from left to right are: ESC, EBM3 (day 3 differentiated cells), EBM6 (day 6 differentiated) and EBM9 (day 9 NPCs). Relative levels of phosphorus and nitrogen levels were used to delineate chromatin (yellow) vs protein and ribonucleoprotein (blue).[81] ESC nucleus is a relatively uniform meshwork of 10 nm chromatin fibers with a low degree of chromatin compaction along the nuclear envelope or throughout the nucleoplasm. EBM3 showed a landscape very similar to ESCs. However, note that in EBM6, a dramatic accumulation of compact chromatin was evident near the nuclear periphery, the boundaries of which became further sharpened in EBM9. The EBM3–EBM6 transition roughly corresponds to the early to late epiblast transition, based on gene expression, replication timing profiles and subnuclear position analysis of several gene loci.[8] Figure was adapted from Hiratani et al with permission from *Genome Research*.[8]

time is found for genes replicating in the mid-late stages of S-phase.[1] Together, these results predict that genes replicating in the second half of S-phase will be located near the periphery of the nucleus. Given that studies of gene position in the nucleus are currently very laborious, these results imply that replication-timing profiling can provide a genome-wide prediction of genes that change position during differentiation. In fact, we have recently discovered that spatial proximity of chromatin as measured by Hi-C

analysis (A novel form of chromosome conformation capture analysis, or 3C, applied genome-wide)[80] shows a strikingly high correlation to replication timing profiles in a cell-type specific manner (T. Ryba, I.H. and D.M.G., *unpublished*).

REPLICATION TIMING REVEALS AN EPIGENETIC TRANSITION: AUTOSOMAL LYONIZATION AT THE EPIBLAST STAGE

Perhaps most importantly for our appreciation of the significance of replication timing changes, these studies have unveiled a close association of replication timing changes with cell fate transitions during early mouse development (Fig. 2).[7,8] Replication timing changes were coordinated with changes in transcription, with expression of weak CpG-poor promoters showing the strongest relationship. The earliest events during mouse development include a distinct set of early-to-late (EtoL) replication timing changes completed during the post-implantation epiblast stage (Fig. 2, asterisk), coincident with repositioning of EtoL loci toward the nuclear periphery (Fig. 3C) and Xi's shift to late replication.[8] Moreover, by electron microscopy using an analytical technique called electron spectroscopic imaging (ESI),[81] a dramatic chromatin conformation change in the nucleus was revealed, with the nuclei showing an emergence of compact chromatin mass near the nuclear periphery during the transition from early to late epiblast equivalent stage (Fig. 3D),[8] coincident with radial subnuclear repositioning of EtoL loci (Fig. 3C). This reorganization was evident in EpiSCs, demonstrating that it is prior to down-regulation of Oct4/Nanog/Sox2 and germ layer commitment.[8] This suggests that the epigenetic landscape that these core pluripotency circuitry factors[82] must act upon is considerably different in late epiblast (EpiSCs) vs ICM/early epiblast [ESCs or early primitive ectoderm-like (EPL) cells[83]]. In contrast to EtoL changes, late-to-early (LtoE) changes occurred later during germ layer commitment in a lineage-dependent manner to generate cell-type specificity (Fig. 2). Taken together, there are extensive changes before and after the epiblast stage, corresponding to lineage-independent and lineage-dependent changes during development, respectively. Moreover, these results suggest that the replication timing program is specific to a given differentiation state, reflects global organization of chromatin within the nucleus and changes in this program represent or reflect epigenetic commitment of cells during key cell fate transitions. In such a way, replication profiling has revealed previously unappreciated epigenetic distinctions between closely related cell culture models that represent early vs late epiblast cells.

An epigenetic distinction between ICM/early epiblast vs late epiblast cell culture models is consistent with the fact that they exhibit major phenotypic differences[84] despite showing only small differences in gene expression.[85] First, unlike pre-implantation ICM cells, the post-implantation epiblast cells fail to colonize the blastocyst, despite the expression of many 'pluripotency' marker genes. Second, the success rate in isolating ESCs from epiblast in the permissive 129 mouse strain also seems to decline precipitously between E5.0 and E6.0.[84] Third, in vitro models of early and late epiblast cells demonstrate that the latter cell types have progressed beyond an as-yet unidentified epigenetic barrier that is difficult to overcome upon nuclear reprogramming. That is, an early epiblast model, EPL cells, have lost the ability to contribute to chimeric mice formation, but can readily revert back to the ESC state by culturing in ESC medium containing LIF, upon which they can contribute to chimeric mice.[83] In contrast, a late post-implantation epiblast model, EpiSCs, have lost the ability to easily revert back to the ESC state.[63,64] In fact, generating

ESC-like iPSCs (induced pluripotent stem cells) from EpiSCs is as inefficient (0.1-1%) as from other somatic cell types.[86] By these criteria, epiblast development appears to accompany a major cell fate transition that is not accompanied by major transcriptional differences but is reflected by significant changes in the replication timing profile and spatial genome organization (Figs. 2 and 3).

As discussed earlier, the first two major cell fate decisions during early mammalian embryogenesis accompany alterations in replication timing of the Xi, upon the emergence of trophectoderm at E3.5 and primitive endoderm at E4.0, both representing the divergence of extra-embryonic lineages from the embryo proper. In this regard, the post-implantation epiblast, in which the Xi's signature shift to late replication is first observed in the embryo proper, may represent the next major cell fate transition after the divergence of these two extra-embryonic lineages. We speculate that it is an important determination step for the late epiblast cells to first shut down their reversibility to the ICM state. Obviously, it is not only the female embryos but also male embryos that go through these series of cell fate decisions during early embryogenesis. Thus, it is reasonable to speculate that Xi's unique replication behavior is a part of a larger scale "Lyonization" event not limited to X-inactivation but that involves autosomes and may be related to the emergence of compact chromatin near the nuclear periphery (reminiscent of Barr body formation near the nuclear periphery[23]). In this sense, it is of interest that the lineage-independent autosomal EtoL changes at the epiblast stage (Fig. 2, asterisk) take place primarily in GC-poor/LINE-1-rich chromosomal segments, which is a hallmark sequence feature of the X chromosome.[8] Thus, Xi may be simply manifesting a putative "default" mode of behavior associated with cell fate changes that is somehow shared with chromosomal segments possessing GC-poor/LINE-1-rich sequence properties. If this were the case, it follows that the remaining active X is the one that bears an imprint to escape such regulation. Indeed, this is what Lyon and Rastan had proposed in 1984.[87] They argued that, in the case of imprinted X-inactivation in the extra-embryonic lineages, the experimental data fits better with a scenario in which the maternal X that stays active is the one that bears an imprint that preserves its activity, rather than the opposite scenario in which the paternal X that undergoes inactivation is the one that bears an inactivation imprint.

REPLICATION TIMING AND CELLULAR REPROGRAMMING: FURTHER SUPPORT FOR AUTOSOMAL LYONIZATION

iPSCs derived from adult somatic cells hold great promise for regenerative medicine in the 21st century, but they also provide an opportunity for understanding the nuclear reprogramming process. iPSCs share a replication-timing profile indistinguishable from that of ESCs in mice, consistent with the conclusion that replication-timing profiles reflect cell identity.[7,8] Thus, dissecting how replication-timing program is altered as somatic cells are reprogrammed back to pluripotent ESCs may reveal novel insight into the reprogramming process. In particular, partially reprogrammed iPSC lines (piPSCs), which are clonal cell lines that emerge from reprogramming experiments based on selection by morphology or reporter gene expression, provide a unique opportunity to view an intermediate state of the replication timing reprogramming process and to assess the reprogramming efficiency of different chromosomal regions. piPSCs fail to express many pluripotency genes and cannot contribute to chimeric mice formation, suggesting that they are blocked at an intermediate stage of the reprogramming process.[88,89]

Replication timing profiling of three independent piPSC lines derived from mouse embryonic fibroblasts (MEFs) revealed that their profiles were distinct from ESCs/iPSCs, early epiblast-like EPL cells, EpiSCs or nascent cells of the three germ layers (Fig. 2).[8] The three piPSCs were very similar to each other, suggesting that they were trapped at a common epigenetic state despite having independent retroviral integration site.[88] Interestingly, the majority of chromosomal segments that had experienced lineage-independent EtoL replication changes at the epiblast stage maintained their late replicating state in piPSCs (Fig. 2, asterisk),[8] which in females included the late-replicating Xi, underscoring the stability of these EtoL switches that were completed in the epiblast. In contrast, replication-timing changes that occurred later during development in a lineage-dependent (i.e., MEF-specific) manner were more readily reprogrammed. Furthermore, in these same piPSCs, expression of genes located within the lineage-independent EtoL switching segments showed the least similarity to ESCs when transcription profiles were compared.[8] Likewise, the Xi fails to become transcriptionally reactivated in female piPSCs.[88,89] Overall, these results suggest that many autosomal replication timing switches and in particular the EtoL replication timing switches at the epiblast stage coincident with X-inactivation, are stable epigenetic changes that are as difficult to reprogram as X-inactivation, supporting the notion of "autosomal Lyonization."

MAINTENANCE AND ALTERATION OF REPLICATION TIMING PROGRAM AND ITS POTENTIAL ROLES

As mentioned earlier, replication timing is re-established during early G1-phase at the TDP.[67] Intriguingly, this is coincident with the repositioning of chromosomal domains in the nucleus after mitosis.[67,90] The precise timing of TDP during G1 varies between cell types but is typically 1-3 hours into G1 in mammals[67] and at some point between mitosis and START in budding yeast.[91] In a parallel line of studies, chromatin mobility has been shown to be relatively high during the first 1-2 hours of G1-phase, after which it is locally constrained through the remainder of interphase.[92,93] Moreover, inducible targeting of loci to the nuclear lamina requires passage through mitosis and takes place during late telophase to early G1-phase.[94,95] Together, early G1 period seem to offer a temporal window for 3D organization of chromosomes to be re-established during each cell cycle, or alternatively, for a novel 3D chromosome organization pattern to be established, which in turn might dictate the replication timing program executed in the upcoming S-phase. Hence, this cell cycle regulation may offer a point of intervention for developmentally regulated changes in replication timing. It is also possible that the regulation of G1-phase length itself may influence the extent to which nuclei are reorganized before replication initiates and in turn affect the replication-timing program. Indeed, G1 length is highly variable between cell types and lengthens upon differentiation of mouse ESCs when a large degree of replication-timing changes is observed.[8]

Regarding the roles of the replication timing program, it should be emphasized that chromatin is assembled at the replication fork, providing a convenient window of opportunity to regulate this assembly process. Indeed, when reporter plasmids are injected into early or late S-phase mammalian nuclei, they assembled into hyper- or hypo-acetylated chromatin, respectively, providing evidence for different chromatin structure assembly at different times during S-phase.[96] Taking advantage of the fact that bovine papilloma virus plasmids replicate at different times in consecutive cell cycles,[97] the same authors recently

showed that tightly packaged, late replicating chromatin becomes loosely packaged when the mini-circle is replicated early in the subsequent cell cycle.[98] Hence, reports using an artificial experimental system support a positive feedback loop whereby replication timing dictates chromatin states that in turn regulate replication timing in the subsequent cell cycle.[46] While in vivo evidence for recruitment of different sets of chromatin modifiers to replication forks at different times during S-phase remains scarce, this represents an attractive scenario for stable epigenetic inheritance of a given chromatin state.

CONCLUSION

The precise role of a replication-timing program and why this program is developmentally regulated remains to be elucidated. However, temporal regulation of genome duplication and the existence of multi-replicon domains are conserved from humans to budding and fission yeasts.[49,99,100] DNA replication is centrally linked to many basic cellular processes that are regulated during the cell cycle and development and defects in replication timing have been observed in various disease models.[101] Recent studies allow us to conclude that widespread developmental replication timing switches occur in flies,[57] mice,[7] and humans (T. Ryba, I.H. and D.M.G., *unpublished*). Moreover, there is significant conservation of the replication-timing program when regions of conserved synteny from similar cell types are compared between human and mouse (T. Ryba, I.H. and D.M.G., *unpublished*). As discussed, the first two major cell fate decisions during early mouse development accompany changes in the replication timing program of the Xi.[35] We have proposed that the post-implantation epiblast may experience the next major cell fate transition through a process of "Lyonization," involving a change in the replication timing program of Xi as well as autosomes that is stably maintained thereafter.[8] Whatever their role, whether causal or reflective, replication-timing programs are cell-type specific and appear to be an integral part of cell identity. In such ways, studies of DNA replication, more than 50 years after the discovery of the double helical structure and successful visualization of replicating DNA in the nucleus, continue to provide new insights into the organization of chromosomes and its changes during differentiation.

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