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Replication timing and transcriptional control: beyond cause and effect—part III

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Abstract

DNA replication is essential for faithful transmission of genetic information and is intimately tied to chromosome structure and function. Genome duplication occurs in a defined temporal order known as the replication-timing (RT) program, which is regulated during the cell cycle and development in discrete units corresponding to topologically-associating domains (TADs) that are spatially compartmentalized in the nucleus. Correlations of RT to chromatin organization and gene regulation have been known for decades but causal and mechanistic links remain unknown. The complete elucidation of these intriguing liaisons is critical to understand the connection between the three-dimensional organization of the nucleus and cellular function. Here, we discuss emerging evidence providing new insights into regulation of chromosome architecture, transcription and its connections with RT.

Introduction

Two decades ago studies of DNA replication discovered the presence of stable submegabase units of chromosome structure and their spatio-temporal compartmentalization in the nucleus. Pulse-labeling followed by *in situ* detection revealed punctate sites of DNA synthesis ("replication foci") that segregated into distinct spatial compartments depending upon the time of S phase labeling (Figure 1A) [1–5]. When chased for multiple generations, replication foci persisted as stable chromosome units [6–8], each estimated to contain 0.5–1Mb of DNA replicated by clusters of several synchronously firing replicons [9]. Early replicated foci located at the nuclear interior while late replicating foci were more tightly clustered and associated with the periphery of the nucleus and nucleolus (Figure 1A), as well as other sites of heterochromatin [4,5,10*]. The development of genome-wide methods to map RT found that during cell fate commitment half of the genome changes RT, coinciding with changes in gene expression and select examples of re-localization within the nucleus (Figure 2) [11,12]. These RT changes occurred in units of 400–800 kilobases termed replication domains (RDs) that are likely to be molecular equivalents of replication foci.

Each 7 years, Current Opinions has asked us to review the enigmatic relationships between replication timing, sub-nuclear chromatin organization and transcriptional regulation

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[13,14]. 14 years ago, we had little more than anecdotal observations from cytogenetics and comparisons of individual gene behavior in several cell lines to suggest a mutually reinforcing relationship between RT and chromatin structure for which teasing out causality resembled the chicken and egg conundrum (Figure 1 of [13]). 7 years ago genomics had emerged to fill in gaps from anecdotal studies into complete descriptions of the complexity of RT and transcription, suggesting strong correlative links to 3D genome organization (Figure 1 of [14]). In the past 7 years, developments in stem cell differentiation have facilitated extensive mapping of regions of the genome subject to developmental control of RT and 3D organization, and identified unique properties of constitutive vs. developmental RDs that challenge longstanding correlations previously presumed to be clues to causal linkages between these complex phenomena. Mapping interactions with the nuclear lamina revealed chromosome domains physically associated with the nuclear periphery (lamina-associated domains; LADs) [15–18]. Intriguingly, live single-cell tracking of LADs from one cell cycle to the next revealed that a significant proportion of peripheral chromatin redistributed to locations throughout the nucleus that resemble the spatial distribution of late replicating foci (Figure 1A) [19], and *in situ* hybridization suggests that late replicating segments can associate with any of these locations [20*] suggesting that these varied locations that replicate simultaneously constitute a single functionally equivalent sub-nuclear compartment. The explosion of chromatin conformation capture methods (Hi-C) has permitted genome-wide mapping of early and late replicating spatial compartments, and has identified stable structural units of topologically self-associating chromatin (TADs) that correspond to RDs we discussed 7 years ago and likely to foci we discussed 14 years ago. Here we summarize the most recent findings, emphasizing structure-function relationships in the nucleus, and we predict that the next 7-year cycle will be dominated by genome editing methods that may finally get us “beyond cause and effect”.

Replication timing reflects the spatial organization of the genome

The biggest conceptual leap for RT in the last 7 years is the molecular elucidation of 3D genome conformation by Hi-C, providing genome map coordinates that indisputably align the spatio-temporal relationships revealed through cytogenetics to those mapped by RT functional genomics. RT profiles are generally displayed as the ratio of early/late replication along the length of each chromosome [21–24*] and reveal chromosome segments with uniform replication timing, known as constant timing regions or CTRs (Figure 1B), which often consist of several adjacent RDs that replicate within 1–2 hours [25–27]. Intriguingly, chromatin conformation methods (Hi-C) detect not only chromatin partitioning into TADs but also folding of TADs into multi-megabase compartments of active/open (A-compartments) or inactive/closed (B-compartments) chromatin [28]. Consistent with the cytogenetic studies, comparison of RT profiles and Hi-C compartments shows remarkable alignment of RT to physical compartmentalization of the genome (Figure 1B, [29–32]). Moreover, by mapping RT transitions in multiple cell types the majority of RD boundaries could be mapped revealing a near one-to-one alignment to TAD boundaries [26**] (Figure 1C). Hence, the “replication domain model” [26**,27,33] proposes that RD/TADs are stable chromosome units segregated into spatially distinct and coordinately replicated nuclear compartments (Figure 1D). Indeed, tracking the formation of TADs and 3D compartments

through the cell cycle revealed that 3D structure is dismantled during mitosis [34,35**], but both TAD structure and interaction compartments were re-established coincident with the re-establishment of RT in a very early window during G1 referred as the timing decision point (TDP) [35**], demonstrating a convincing intimate co-appearance of structure and function. However, TADs and compartments persist into G2 phase, when RT function is lost [36], suggesting that 3D structure is not sufficient for RT, but may provide a necessary scaffold on which cell cycle regulated factors operate to maintain the RT program [35**].

In fact, several factors have recently been implicated in regulating replication timing in the context of chromosome 3D organization, all of which act by antagonizing or recruiting the essential replication initiation cell division cycle 7 kinase (Cdc7). In budding yeast, forkhead proteins (Fkh1 and Fkh2) are thought to organize early replication origins into clusters that permit the concentration of Cdc7 activity [37]. In yeast and mammals, the large multi-functional rap1 interacting factor (Rif1) has been shown to localize to late replicating regions of the genome [38], and in yeast Rif1 recruits phosphatase PP1 to antagonize Cdc7 [38–41]. Rif1 depletion appears to disrupt 3D chromatin organization in mammals [42,43**], leading to speculation that Rif1 regulates RT through establishing 3D structure. However, there is still contradictory data as to whether Rif1 is removed from chromatin during replication [42,44], so whether Rif1 is directing 3D structure necessary but not sufficient for RT, or whether Rif1 interacts with the 3D scaffold in a cell cycle regulated fashion to influence RT is still a matter of debate.

Intrinsic and extrinsic variation in RT and large-scale chromosome architecture

An important gap in our understanding of RT is the degree of stochastic variation and cell-to-cell heterogeneity. Recent studies of budding and fission yeast replicon structure using isolated DNA fibers demonstrate a great deal of heterogeneity in the sites and timing of initiation for replication [45,46*]. However, current DNA fiber methods do not retain cell of origin information to distinguish extrinsic (cell to cell) and intrinsic (homologue to homologue) variation. Moreover, compelling cytogenetic evidence in mammalian cells suggests that the same cohort of replication foci labeled in one cell cycle are also labeled coordinately in the subsequent cell cycle [47], implying a great degree of coordination. It is now imperative to develop methods to probe how structure and function co-vary within single cells. Although single-cell RT has not been developed, great progress in single-cell isolation and whole genome amplification techniques have paved the way for transcriptome and epigenome analyses of individual cells [48,49]. The first glimpses of single-cell Hi-C suggest stability in the structure of TADs but considerable variability in long range interactions that nonetheless remained within their respective interaction compartment [50] while single-cell LAD mapping has confirmed cell to cell heterogeneity in chromatin interaction with the nuclear periphery [51*] predicted from the earlier single cell studies [19]. However, both studies examined haploid chromosomes, precluding measurements of intrinsic variation. In mammals, RT profiles may soon emerge from single-cell copy number measurements [52*], and high-throughput methods to analyze individual DNA fibers are

becoming state-of-the-art [53,54*], suggesting measurements of origin firing and RT variability are on the horizon.

While we do not yet have good assessments of the degree of stochastic intrinsic variation, there is evidence for deterministic influences on intrinsic RT variation. Genomic imprinting in mammals is associated with silencing and delayed replication of the imprinted allele [55,56] and mono-allelically expressed genes are also generally replicated earlier when active [57,58]. Moreover, during chromosome X inactivation in female mammals, the inactive chromosome X (Xi) replicates later than the active X (Xa) [59,60]. Intriguingly, the Xi is depleted of TADs [61–63] and replicated by a rapid and synchronous firing of origins throughout the whole chromosome [64,65*]. Analysis of RT by deep sequencing of phased genomes (i.e. with all allelic variants mapped to distinguish the parental origin of each haplotype) permits correlations between allelic DNA sequence variation and RT. In general, homologues replicate highly synchronously with very few regions showing allelic differences in RT or in origin usage related to DNA sequence variation, although some reach statistical or disease-associated significance worth further investigation [24*,66*,67,68*,69*]. An elegant series of recent papers has revealed a new class of *cis*-acting elements involved in the regulation of RT, mitotic condensation and chromosome stability [70–72**]. These elements consist of monoallelically expressed long non-coding RNAs that appear to be present on each mammalian chromosome, and interact in *cis* to regulate RT, monoallelic gene expression and structural stability of the entire chromosome [72**]. Overall, these results uncover specific mechanisms that control intrinsic allelic variation in RT that are certain to be the subject of much investigation in the coming years.

Clinging onto Proteus's neck

Strong correlations between genome organization, RT and gene regulation are found in all multicellular organisms studied [11,12,22,73–80]. Half of the genome changes RT during development in units of 400–800 kilobases (entire RD/TADs) closely coordinated with transcriptional competence (Figure 2) [11,12,77,81–83]. Just as holding the mythical Proteus throughout all his transformations was necessary to learn his secrets, understanding these intriguing liaisons requires careful observation of their order of events during cell fate transitions. However, until recently, systems capable of eliciting change found that the changes in RT, gene activity and nuclear positioning were too synchronous to temporally separate them [12]. By taking advantage of new human embryonic stem cell (hESC) differentiation systems that allow highly synchronous derivation of distinct cell types [84–86**], we were able to track changes in RT and gene expression through multiple intermediate steps in lineage specification [86**]. Surprisingly, our study revealed that the strong correlation between early replication and active transcription is restricted to RT-constitutive genes (i.e. genes that do not change their RT program), while the RT-switching genes have a much weaker correlation that is further diminished during differentiation. Moreover, some RT-switching genes are expressed only when replicated early (E-class), while the majority (C-class) can be strongly expressed in one or more cell types while late replicating, demonstrating that transcription is not sufficient for early replication (Figure 3). Additionally, a rare category of genes (L-class) were expressed exclusively when replicated late. Tracking C-class gene activity through cell fate transitions showed that this class of

genes commonly reach high expression levels in the same lineage where an RT switch occurs, but preceding the change to early RT by one or two intermediate stages of differentiation, while down-regulation often followed changes to late RT (Figure 3) [86**]. These results suggest that some aspects of transcriptional control could indirectly influence RT. One possibility is that RT responds to aspects of transcriptional regulatory circuitry, but is not directly related to transcription itself.

Studies of RT changes during differentiation also revealed that many other longstanding correlations to RT, in addition to transcription, apply only to the RT-constitutive domains and that RT-switching or "developmental domains" have independent organizational principles that challenge many of the assumptions that guided our hypotheses over the last few decades. In addition to their distinct sequence composition (see [86**] and Figure 1 of [14]), they are highly nuclease insensitive, depleted of replication origins [87,88], less confined to A/B Hi-C compartments and chromatin states correlated to RT in constitutive domains are much less correlated to RT in developmental domains [35**]. Hence, chromosome domains fall into categories that reflect their developmental control of DNA RT. Importantly, it is not known whether the lack of correlations in the RT-switching half of the genome is due to intermediate properties of these domains, increased cell-to-cell heterogeneity, or structural instability within a single cell cycle. It is now imperative to tease out what properties actually do correlate with RT within the half of the genome that changes RT and 3D organization during differentiation.

Twisting the lion's tail

Understanding causal relationships between RT, chromosome architecture and gene regulation requires experimental manipulation or *'twisting the lion's tail'* [89]. Several studies expressing artificial proteins consisting of sequence-specific DNA binding domains fused to domains that can target to sub-nuclear domains or strongly remodel chromatin have revealed some important principles. Rapid re-localization (1–2 hrs) of a chromosome site has been observed from the periphery to the nuclear interior upon robust transcriptional induction [90] or towards RNA processing sites following heat shock [91*]. Conversely, artificial anchoring of distinct genes to the nuclear lamina leads to their reversible repression [92–94], but this re-positioning occurs only after a passage through mitosis [93]. Tethering to the periphery is sufficient to suppress the expression of some but not all genes [92,95,96], implying the existence of different sensitivity classes of genes, as was found with the RT studies discussed above. Although RT was not measured in these studies, since the periphery is a late replicating compartment such analogies are reasonable to expect. Additionally, mechanisms tethering LADs to the nuclear periphery remain unknown and some evidence is contradictory: mES cells depleted of nuclear lamins preserved normal chromatin interactions with the nuclear periphery [97], while other studies suggest that lamin A/C is required to anchor chromatin to the nuclear periphery [98,99].

Several earlier studies suggested that targeting strong artificial transcriptional activators is sufficient to induce a change from late to early replication of some specific loci [100–103]. More recently, targeting an exceptionally strong transactivator induced changes in both nuclear repositioning and RT [104*], while targeting a mutated transactivator with chromatin

unfolding but not transcription activity [105] elicited repositioning but not a change in RT, prompting the authors to conclude the transcription is sufficient for the RT switch [104*]. This is clearly not a generalized conclusion, since genome-wide studies reveal numerous examples of transcriptional upregulation without alterations in RT and approximately 20% of late replicating genes are expressed [11,12,73,86**]. Moreover, RT changes in response to the artificial transactivator were less robust than changes observed at this same locus during differentiation [104*] and some evidence suggests that remodeling of chromatin during DNA replication precedes and is necessary for gene activation during differentiation at other loci [106]. Hence, we are beginning to witness a transition in the field from correlative to more manipulative studies that are providing glimpses of causality, but also complexity. It is possible that transcription, RT, chromatin structure and chromosome organization all influence each other in a contextually or quantitatively dependent manner. Clearly there is much exciting work ahead to establish the governing principles relating large-scale structure and function in the nucleus.

Conclusions and future directions

There is an intimate connection between, on the one hand, fundamental chromosome functions of RT and transcription and, on the other hand, the structural organization of chromosomes into TADs and their 3D organization in the nucleus. A critical issue to be resolved is the extent to which genome structure is cause or consequence of genome function. From the intriguing equivalence between RDs and TADs it is tempting to speculate that RT reflects the organization of the genome, but to date it is not clear how RT is regulated while TAD organization is not sufficient to dictate RT [35**]. Moreover, studies of RT have revealed that domains with developmentally plastic RT lack or have measurably weaker associations of RT to chromatin structure and transcriptional control as compared to the RT-constitutive domains. Transcriptional activity appears to be capable of influencing both gene position and RT, but only in as yet poorly defined contexts. Moreover, developmental domains can be just as early or late replicating as constitutive domains, yet they do not follow the correlations between RT and chromatin properties identified over the last 30 years. Figure 4 presents a hypothetical model in which chromosomes are partitioned into distinct RD/TADs that agglomerate to form early and late compartments. Dynamic changes in nuclear organization, initiated by the transcriptional induction of C-class genes, elicit a compartment switch in the following mitosis that changes RT, creating a new stable state reinforced by chromatin assembly at a different time and location in the nucleus. Newly emerging approaches may finally be capable of teasing out cause and effect in these relationships. For example, combining chromosome “domain engineering” with evaluating effects during cell fate transitions [107,108] will identify necessary and sufficient cis-acting elements of chromosome structure and function, while recently developed methodologies for analysis of thousands of randomly integrated reporters [109] will provide much needed insight into influence of large scale architecture on functional outputs.

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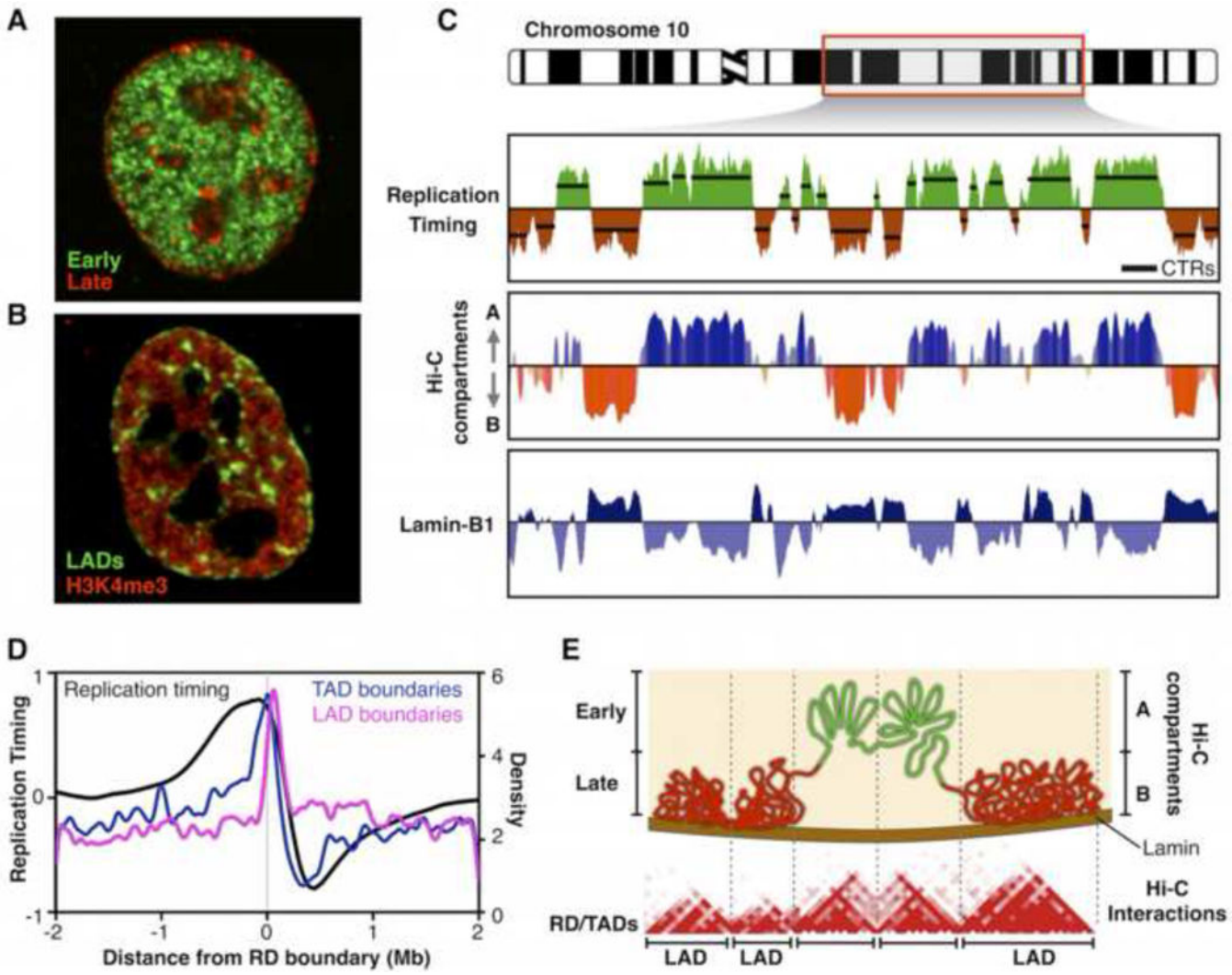

Figure 1.

Replication timing reflects genome organization. A) Cells were pulse-labeled with CldU, chased for 3 hours, pulse-labeled with IdU, and fixed and immunofluorescently stained for CldU (green) and IdU (red) [29]. Shown is a nucleus from a cell labeled with the early replicating compartments labeled in green and the late replicating compartment in red. B) LADs (green) labeled at the nuclear periphery during one cell cycle re-distribute in the next cell cycle to locations that resemble the late replicating foci. H3K4me3 labels active chromatin [19]. C) Exemplary RT profile of 50 Mb of human Chr10 from IMR90 fibroblasts, segments of chromosomes with uniform RT (CTRs) aligned with Hi-C interaction compartments (Eigenvector display) and LaminB1 contact maps (DamID). D) RD boundaries align with the boundaries of both topologically associating domains (TADs) and lamin-associated domains (LADs). E) Replication domain model. Early and late replicating regions correspond to the Hi-C A/B compartments and RDs are equivalent to TADs, while late RDs and RDs in the passively replicated transitional regions between early and late RDs are associated with the nuclear periphery (LADs). The graphs were plotted

using public data: RT from [29], HiC from [28], lamin-B1 from [16] and boundaries alignment from [26**].

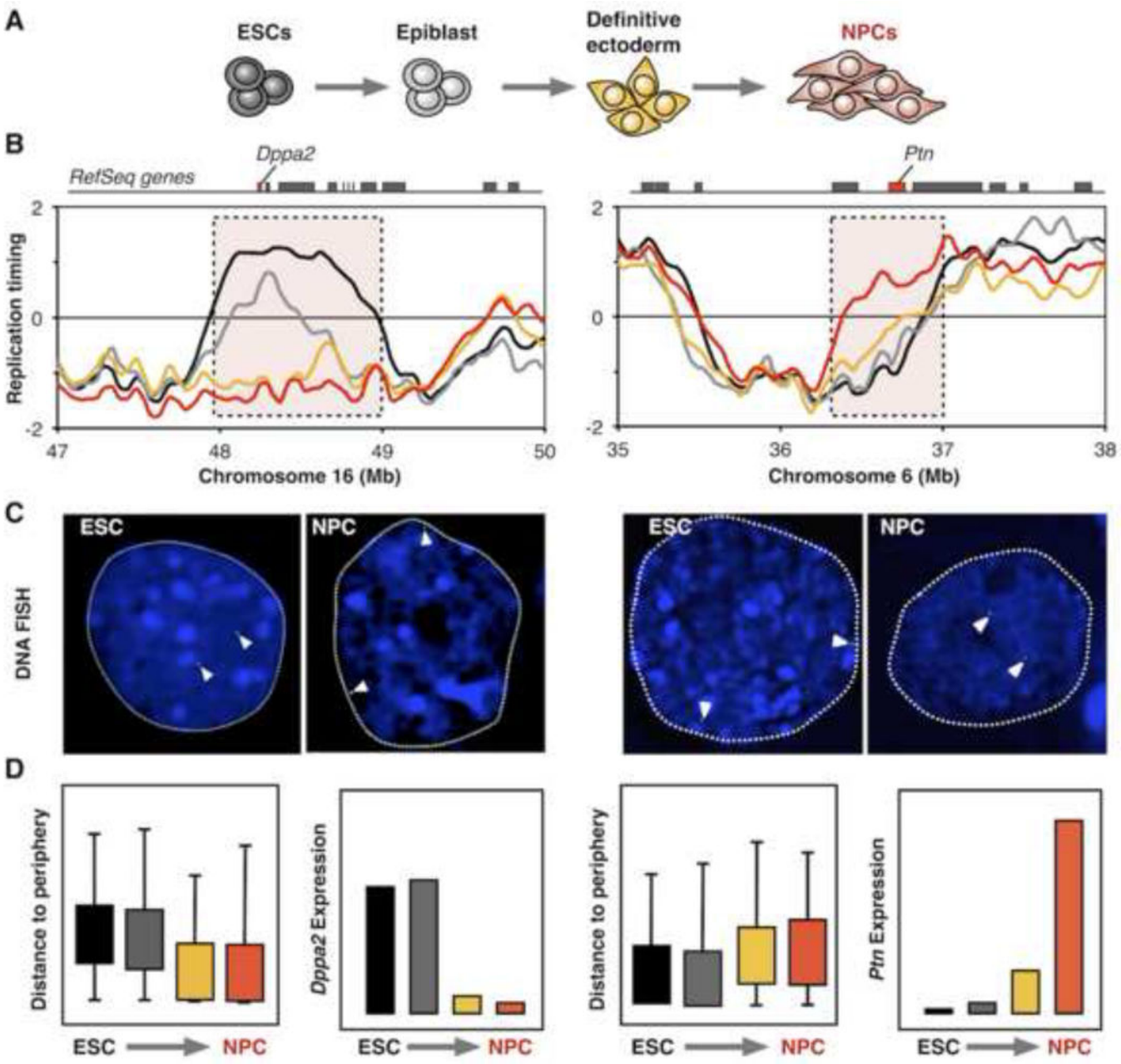

Figure 2.

RT changes are associated with nuclear re-localization and gene expression. A) Schematic depiction of neural differentiation (NPCs) from mouse embryonic stem cells (ESCs). B) RT changes during neural differentiation. Location of *Dppa2* and *Ptn* genes are shown at the top. C) Nuclear re-localization of *Dppa2* and *Ptn* domains during neural differentiation visualized by DNA FISH. D) Measurements of radial nuclear positioning (distance to the nuclear periphery) and gene expression of *Dppa2* and *Ptn* genes. Colors in the graphs represent the distinct differentiation stages as shown in (A). Data and FISH images were obtained from [12].

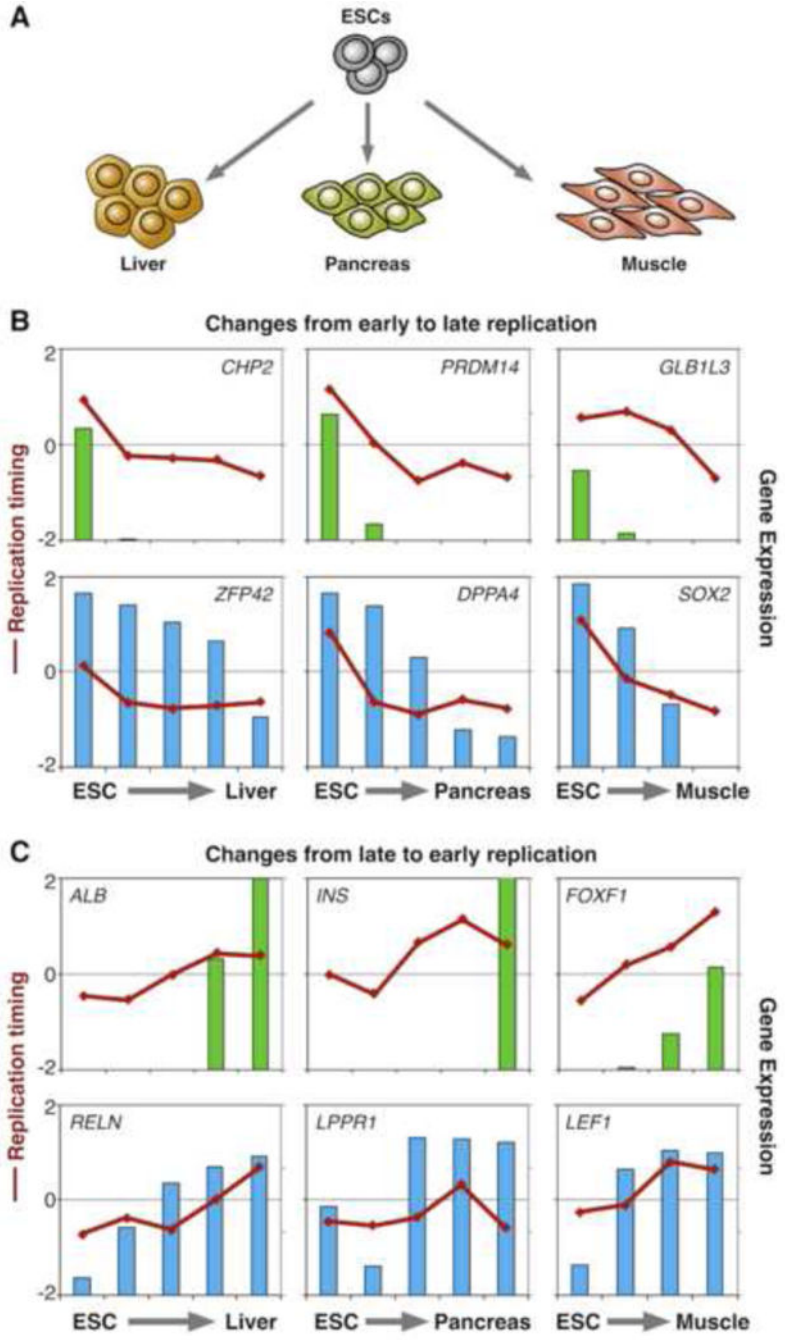

Figure 3.

Transcription changes precede or follow RT changes for different classes of genes. RT changes and gene regulation during differentiation of human ESCs to liver, pancreas and muscle (Schematized in A). Exemplary genes switching from early to late (B) and from late to early (C) replication are shown. Lines represent the RT and bars the expression values at distinct cell fate transitions. E-class genes (top panels in B and C) are transcribed only when replicate early. C-class genes (bottom panels in B and C) down-regulation follows changes

to late replication while their induction precedes changes to early replication. Specific details of differentiation stages and original data from [86**].

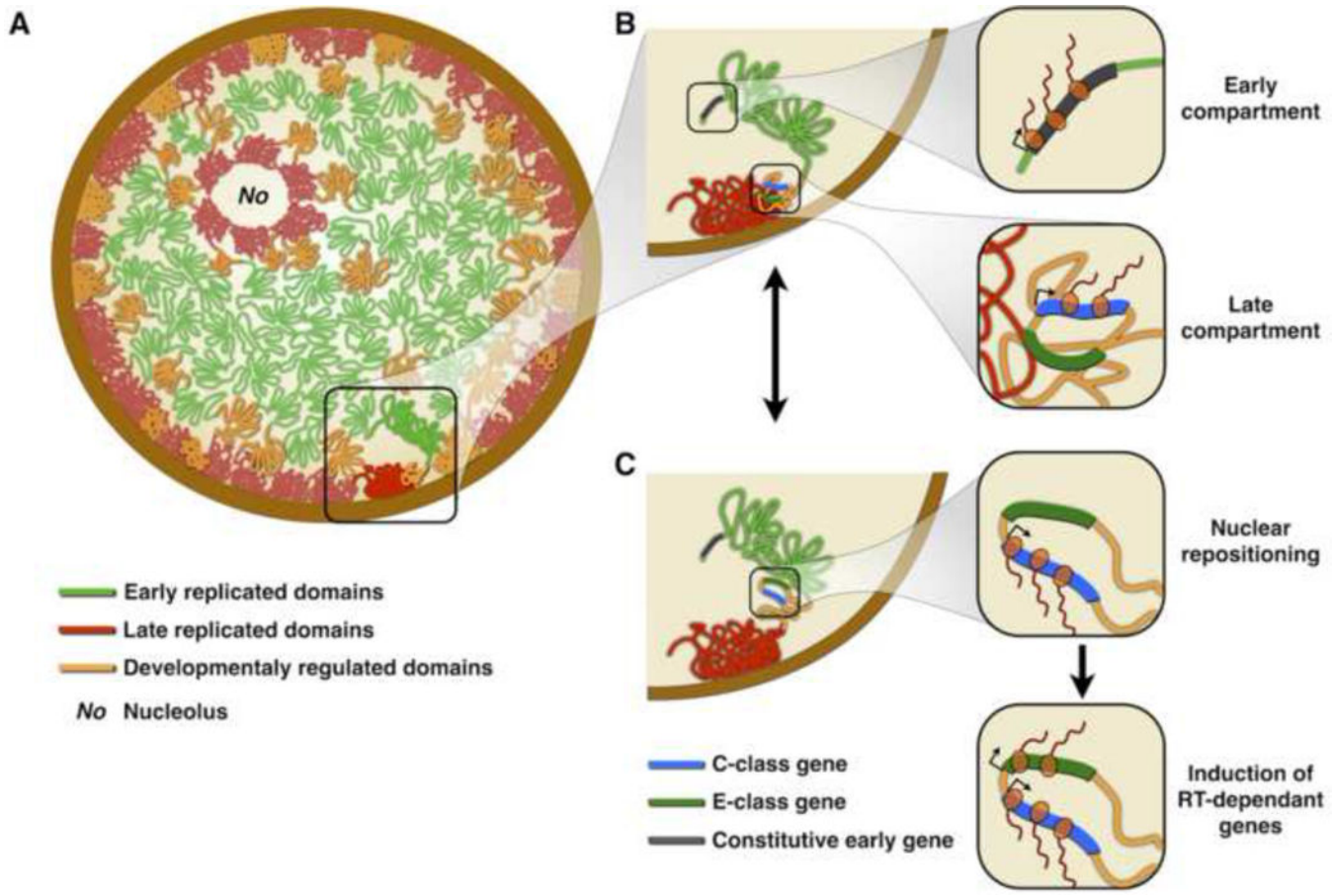

Figure 4.

Genome organization, RT and gene regulation in the nucleus. A) Organization of RDs at the nuclear interior. Late replicating domains are located at the nuclear and nucleolar periphery (and other regions as shown in Figure 1A), early replicating domains are located at the nuclear interior and developmental regulated domains are less well compartmentalized [35**]. B) Early replicating compartments contain open/active chromatin at the nuclear interior where genes can be highly expressed. Developmentally regulated RDs contain distinct classes of genes: E-class genes are silenced while C-class genes can be expressed despite being late replicating and close to the periphery [86**]. C) RT changes and nuclear repositioning are commonly accompanied by an increase in expression of C-class genes, while E-class genes are induced only after the RT change.