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Title: Topologically-associating domains and their long-range contacts are established during early G1 coincident with the establishment of the replication timing program

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Running title: Chromatin folding and cell cycle

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ABSTRACT

Mammalian genomes are partitioned into domains that replicate in a defined temporal order. These domains can replicate at similar times in all cell types (constitutive) or at cell type specific times (developmental). Genome-wide chromatin conformation capture (Hi-C) has revealed sub-megabase topologically-associating domains (TADs), which are the structural counterparts of replication domains. Hi-C also segregates inter-TAD contacts into defined 3D spatial compartments that align precisely to genome-wide replication timing profiles. Determinants of replication timing program are re-established during early G1 phase of each cell cycle and lost in G2 phase, but it is not known when TAD structure and inter-TAD contacts are re-established after their elimination during mitosis. Here we use multiplexed 4C-seq to study dynamic changes in chromatin organization during early G1. We find that both establishment of TADs and their compartmentalization occur during early G1, within the same time frame as the establishment of the replication-timing program. Once established, this 3D organization is preserved either after withdrawal into quiescence or for the remainder of interphase including G2 phase, implying 3D structure is not sufficient to maintain replication timing. Finally, we find that developmental domains are less well compartmentalized than constitutive domains and display chromatin properties that distinguish them from early and late constitutive domains. Overall, this study uncovers a strong connection between chromatin re-organization during G1, establishment of replication timing and its developmental control.

INTRODUCTION

Mammalian cells replicate their genomes in a defined temporal order (“replication-timing program”) that is cell type-specific and altered in many diseases (Rhind and Gilbert 2013). Almost half of the genome changes replication timing during development in units of 400-800kb, designated replication domains (RDs) (Ryba et al. 2010). As a result, RDs can be categorized as replicating either at the same time in all cell types (constitutive RDs) or at different times in different cell types (developmental RDs)(Hiratani et al. 2008). The boundaries of both constitutive and developmental RDs are stable across cell types but the sub-nuclear position, chromatin structure and transcriptional state changes coordinately with replication timing changes for developmental RDs. In general, early replication is correlated with transcriptional activity (Hiratani et al. 2010; Ryba et al. 2010; Hatton et al. 1988). Although RDs are regulated independently, tandem RDs often replicate at similar times resulting in larger regions of similar replication timing that can fractionate or consolidate during differentiation depending on the distribution of constitutive and developmental domains (Hiratani et al. 2008).

The replication-timing program is re-established in each cell cycle at a point during early G1 phase termed the timing decision point (TDP). The TDP is coincident with the cytogenetically observed 3D re-positioning and anchorage of chromatin domains to their final interphase positions in the newly formed nucleus (Dimitrova and Gilbert 1999; Thomson et al. 2004; Walter et al. 2003). The 3D organization of interphase chromatin in mammals has been linked to several genome-wide processes such as DNA replication, long-range gene regulation and chromosomal translocations (Ryba et al. 2010; Moindrot et al. 2012; Denholtz et al. 2013; Ay et al. 2014; Zhang et al. 2012; Sima

and Gilbert 2014). Applications of genome-wide chromatin conformation capture (Hi-C) have revealed organization of chromatin into sub-megabase topologically-associating domains (TADs) that are preserved across cell types and compartmentalization of chromatin contacts between TADs (inter-TAD interactions) into larger scale folding states that are cell type specific (Lieberman-Aiden et al. 2009; Dixon et al. 2012; Nora et al. 2012). Strong correlations between replication timing and chromatin contacts in the interphase nucleus have revealed two important structure-function relationships. First, boundaries of TADs align to the boundaries of RDs, demonstrating that TADs are the units of replication timing control (Pope et al. 2014). Second, inter-TAD interactions segregate the genome into spatial compartments that precisely reflect the replication-timing program (Ryba et al. 2010; Yaffe et al. 2010). This interphase organization of chromatin contacts is dismantled during mitosis (Naumova et al. 2013), suggesting the hypothesis that TADs and inter-TAD contacts are re-established after each mitosis at the TDP. Here, we test this hypothesis using 4C-seq (4C) to examine the re-establishment of interphase chromatin organization and its relation to replication timing in a mouse mammary epithelial cell line (C127) in which the cell cycle regulation of replication timing has been extensively studied (Lu et al. 2010; Wu et al. 2006; Lu and Gilbert 2007; Splinter et al. 2012).

RESULTS

Nuclear compartments are established during early G1

Employing replication timing profiles from 28 different mouse cell lines (replicationdomain.org) we identified 4C viewpoints (“baits”) across three mouse chromosomes (Chr 8, Chr 16, Chr 17) whose replication times are either constitutively early, constitutively late or developmentally regulated (**Supplementary Fig. 1,**

Supplementary Table 1). Cells were synchronized by mitotic shake-off after a brief nocodazole treatment and released into G1 phase for 0.5, 1, 1.5, 2, 3 and 4 hr (Methods). Using this protocol, cells are synchronized precisely and homogeneously in metaphase and retain features of mitotic cells that are lost upon longer nocodazole blocks, including retention of Cyclin A and activation of APC ubiquitin ligase activity within minutes of release (Wu et al. 1997; Okuno et al. 2001). Within one hour of release the mitotic index dropped from 98% to 2% demonstrating rapid and efficient reversibility of the block (**Supplementary Fig. 2A**). 4C profiles from each time point were highly reproducible across both biological and technical replicates (**Supplementary Fig. 3, Supplementary Table 2**). 4C profiles at 3 hr and 4 hr were consistent with virtual 4C profiles generated using previously published (Dixon et al. 2012) Hi-C data from asynchronous mouse embryonic stem cells (ESCs) (**Supplementary Fig. 3 A, B**). 4C contact frequencies at 3 hr and 4 hr were also correlated with distances between locus pairs measured by 3D FISH, demonstrating correspondence to proximity in nuclei (**Fig 1A, Supplementary Fig 4**).

4C data collected from cells in mitosis or at 0.5 hr after mitosis showed a gradual drop off of contact frequency with distance from the bait, which resembled the pattern seen in Hi-C data collected from human cells blocked in mitosis (**Supplementary Fig 5**) (Naumova et al. 2013). In contrast, by 4 hr the 4C profiles had drastically changed, with contact frequencies dropping precipitously at sites aligned with the RD boundaries flanking the bait location (**Fig 1C, D**). For both early and late replicating baits, the strongest contacts (top 5% highest z-scores) changed dramatically from being random with respect to replication timing to being clearly compartmentalized. Contacts with regions that replicated at times similar to the bait became strongly enriched, while

regions that replicated at different times from the bait became strongly depleted (**Fig 1C, D, E**).

TADs and inter-domain contacts are established coincident with the TDP.

The TDP in C127 cells occurs 2-3 hr after mitosis (**Fig. 1B**) (Lu et al. 2010; Wu et al. 2006). To find the precise time-point at which chromatin acquires inter-phase conformation, we compared 4C profiles between 12 baits positioned within 3 chromosomes across several G1-phase time points (**Fig. 1B**). The results revealed that the inter-phase organization of the chosen RDs is established coincident with the TDP (**Fig. 2**). Four lines of evidence support this folding behavior of the representative regions, independent of their replication timing. First, the temporal progression of spatial compartmentalization for domains harboring each bait was visually obvious without any computational analysis as an increase in the contact frequency with regions of similar replication timing as the bait. Compartmentalization of both early and late replicating RDs was detectable as early as 1 hr and reached completion by 3 hr after mitosis (**Fig. 2A, Supplementary Fig 6A**). This folding behavior is further revealed by quantification of spatial compartmentalization, determined by plotting the replication timing of the strongest contacts (top 5% highest z-scores) at each time point after mitosis (**Fig. 2B, Supplementary Fig 6B**). Alternatively, applying a more rigorous test for quantifying significant contacts (Methods), which also corrects for multiple testing, revealed that significant contacts did not appear until the TDP (**Supplementary Fig 7A**). As expected, inter-chromosomal contacts were rare and difficult to quantify. However, for those baits that had sufficient inter-chromosomal contact signal strength, significant contacts (Benjamin and Hochberg test) also became compartmentalized after the TDP (**Supplementary Fig 7B**). Second, the decay in contact frequency with increasing

genomic distance from the bait for each time point showed significant differences between pre- and post-TDP profiles (**Fig. 2C**), reminiscent of Hi-C data collected during mitosis vs. interphase (Naumova et al. 2013). The differences in decay of contact frequency showed a transition from a mitosis-like organization observed at 30min and 1Hr to interphase-like configuration observed by 3 and 4Hr (**Fig. 2C**). Third, we measured the 3D distance between two loci with similar replication timing on Chr 8 using Fluorescent In-Situ Hybridization (FISH) at different time-points after release from mitosis. Since FISH doesn't require a large number of cells, these experiments were performed in the complete absence of nocodazole. The results show that the relative 3D distance between the two loci decreases with time after release from mitosis and stabilizes by 2Hr post-mitosis consistent with the 4C data (**Fig. 2D**). Fourth, we examined the formation of TAD structures as measured by the appearance of periodic transitions in upstream or downstream contact bias (directionality bias) that disappear during mitosis (Dixon et al. 2012; Naumova et al. 2013). The kinetics of TAD formation during early G1 phase could be visualized by the appearance of a sharp drop in contact frequency at Replication Domain/TAD boundaries and also by the appearance of an asymmetrical distribution of contact frequency to either side of the bait (directionality bias) (**Fig. 3A**). We chose six bait regions with visually identifiable directionality bias and quantified their directionality biases at each G1-phase time point (Methods), revealing a switch from negligible to strong directionality bias that suggests formation of TADs (**Fig. 3B**).

Taken together, these data demonstrate that Replication Domains/TADs have distinct contact partners, establish both domain boundaries and inter-domain contacts within the

same brief early G1 phase time frame as the establishment of a replication-timing program.

Post-TDP chromatin organization is preserved in G0 and G2

155 To further probe the relationship between chromatin contacts and replication timing we examined quiescent and G2-phase cells, whose chromatin retains or has lost replication-timing determinants, respectively (Bridger et al. 2000; Lu et al. 2010). Cytogenetic studies in fibroblasts have demonstrated that chromatin undergoes dramatic organizational changes during entry into quiescence, including repositioning of
160 chromosomes, distortion of heterochromatin organization and changes in distribution of replication foci, while replication timing is retained (Bridger et al. 2000; Lu et al. 2010). By contrast, recent 4C study found that chromatin contacts in resting human lymphoblasts were identical to those during interphase (Moindrot et al. 2012), suggesting that either lymphocytes do not undergo the cytogenetically visible
165 organizational changes seen in fibroblasts during quiescence or that these dramatic organizational changes can occur while retaining interphase contacts. To distinguish between these two possibilities, we performed 4C in serum-starved C127 cells (**Supplementary Fig 2**) using a previously published protocol where quiescence-associated chromosome re-organization has been observed (Lu et al. 2010). 4C profiles
170 in quiescence were very similar to post-TDP cells and exhibited TAD organization and similar compartmentalization patterns predicted by the replication time of the bait region (**Fig. 4A, B, Supplementary Fig 8**). These results demonstrate that interphase chromatin contacts and the replication-timing program are established together and preserved together even under conditions that lead to widespread cytogenetically-
175 detectable chromatin spatial re-organization.

In contrast to quiescence, G2 phase is a period where RT is lost but the interphase spatial organization of chromatin as measured cytogenetically remains unchanged (Lu et al. 2010). The complete lack of temporal specificity to replication when induced to initiate within G2 phase nuclei, similar to what is seen in the pre-TDP stage of G1 phase, led us to hypothesize that chromatin organization within G2 nuclei may have returned to the pre-TDP configuration. We prepared G2 phase C127 cells by releasing from mitosis for 15 hr and including nocodazole during the last 3 hr to block fast cycling cells from entering the next G1, followed by eliminating mitotic cells by shake-off prior to collection (**Supplementary Fig 2**). Unexpectedly, the G2 4C profiles exhibited TAD organization and compartmentalization patterns similar to post-TDP (**Fig. 4A, B, Supplementary Fig 8**). Hence, while establishing of interphase chromatin contacts in early G1 may be necessary to establish replication timing, those chromatin contacts are not sufficient to dictate the program within G2 phase nuclei.

Developmental domains exhibit decreased compartmentalization and distinct chromatin properties.

We observed that, even after achieving their interphase configuration, certain baits were less spatially compartmentalized than others (**Supplementary Fig 6A**). Since only 1 out of 7 constitutive baits vs. 2 out of 5 developmental baits were poorly compartmentalized (**Supplementary Fig 6A**), we investigated whether structural plasticity (i.e., poor compartmentalization) might be related to developmental plasticity (tendency to change replication timing during development). Indeed, quantifying compartmentalization as the log ratio between contacts to the same compartment vs. contacts to the opposite compartment for each of our baits (Degree of compartmentalization, Methods) (**Fig. 5A**)

suggested that developmental domains were less compartmentalized. Applying this same definition to normalized Hi-C contact counts from 3 mouse cell lines and 4 human cell lines at 50 kb resolution (Dixon et al. 2012; Imakaev et al. 2012; Rao et al. 2014) validated genome-wide that developmental domains were indeed significantly less compartmentalized than constitutive domains (**Fig. 5B**). Although developmental domains can replicate as early or late as constitutive domains, they generally switch replication timing to or from mid-S phase (Hiratani, 2008; Hiratani 2010). However, their reduced compartmentalization was not a consequence of frequent middle S phase replication time, as the degree of compartmentalization of developmental domains (brown line) was always lower than constitutive domains (blue line) for any given replication timing value (**Fig. 5C**). Finally, we compared the contact strength and the difference in replication timing value (ΔRT) for pairs of interacting loci using Hi-C data, revealing that constitutive domains have significantly stronger contacts to domains with similar replication timing ($\Delta RT=0$) than developmental domains (**Fig. 5D**).

These results suggest that developmental RDs have fundamentally different chromatin organization principles. Indeed, we recently reported that developmental RDs display low nuclease sensitivity when either early or late replicating (**Supplementary Fig 9B**), despite a global correlation of nuclease sensitivity to early replication (Takebayashi et al. 2012). To systematically analyze the differences between constitutive domains and developmental domains, constitutive RDs were further sub-classified as Constitutive Early (CE) or Constitutive Late (CL) and similarly, for each given cell type, developmental RDs were sub-classified as Developmental Early (DE) or Developmental Late (DL). We next compared several properties of chromatin, exploiting data from this report as well as other published datasets (Dixon et al. 2012; Guelen et al. 2008; Peric-

Hupkes et al. 2010; Takebayashi et al. 2012; Besnard et al. 2012; Bernstein et al. 2012) (Fig. 6A, Supplementary Fig 9, Supplementary Table 3). Enrichment of various chromatin features was correlated to replication timing for constitutive domains, while developmental domains showed moderate enrichment that was less correlated to replication timing. For example, association with the nuclear lamina, known to play an important role in sequestering heterochromatin to the periphery of the nucleus (Burke and Stewart 2013), was less constrained by replication timing for developmental vs. constitutive RDs (Fig. 6B). Reduced compartmentalization of developmental RDs may be related to an intermediate affinity of these domains for the lamina.

Recently, high resolution Hi-C from human GM12878 was used to classify the genome into 6 sub-compartments (2 active (A1, A2) and 4 inactive (B1 to B4)) based on long-range chromatin interactions. Our classification of the genome into 4 categories by developmental regulation of replication parsed out predictably into these 6 sub-compartments (Rao et al. 2014) (Fig. 6C). Specifically A1, which replicates the earliest, was mostly comprised of constitutive early RDs, whereas A2 that has both active and repressive marks (H3K9me3) was mostly comprised of developmental early RDs. Sub-compartment B1, which correlated with facultative heterochromatin, was almost exclusively composed of developmental early and late domains, whereas B2 and B3, comprised mainly of heterochromatin and devoid of analyzed chromatin marks, was enriched for constitutive late and developmental late RDs (Fig. 6C). B4, which consists of a small percentage of the genome and heavily enriched for KRAB-ZNF genes, was comprised almost exclusively of developmental domains.

We next investigated a set of seven chromatin labels defined in human lymphoblasts and HeLa cells, via a combination of Segway and ChromHMM (Hoffman et al. 2013) (Methods). All but one of these labels was more correlated to replication timing for constitutive vs. developmental domains (**Fig. 6D**). The one label that did not correlate was repressive marks, which have individually been shown to have a poor correlation to replication timing (Ryba et. al., 2010). Furthermore, a recent segmentation method identified domains of cell-type specific vs. constitutive gene expression that were enriched for developmental vs. constitutive RDs, respectively (Libbrecht et al. 2015). Finally, we individually examined 44 trans-acting factor binding sites and 11 chromatin marks. Consistent with the chromatin segmentation analysis, we observed intermediate enrichments and depletions for developmental RDs in 42 trans-acting factors and 6 chromatin marks (**Fig. 6A, Supplementary Figure 8**). The remaining trans-acting factors and chromatin marks displayed either no enrichment (notably, the heterochromatin marks H3K9me3 and H3K27me3) or other patterns of enrichment between the domains (**Supplementary Figure 8**). Together, these results reveal that developmental RDs are distinct from constitutive RDs both in the level of enrichment of various chromatin properties and in the correlation of those properties with replication timing, which may underlie their compartmentalization plasticity.

DISCUSSION

In this study, we used 4C-seq at different time-points after mitosis to study the re-organization of interphase chromatin, categorized with respect to temporal and developmental regulation of DNA replication. Our results demonstrate that TADs, as well as the inter-TAD contacts that create distinct sub-nuclear chromatin compartments, are formed coincident with the establishment of a replication-timing program. This

275 organization is preserved when cells withdraw from the cell cycle into quiescence, a
state in which replication timing determinants are preserved despite cytogenetically-
visible re-organization of chromatin. These results provide compelling evidence
supporting the replication domain model for replication timing regulation, and suggest
that the 3D organization of chromatin is necessary to establish and maintain a temporal
280 order of replication. However, we found that TADs and inter-TAD contacts are
maintained during G2 phase when the determinants of replication timing are lost (Lu et.
al., 2010), demonstrating that 3D organization is not sufficient to maintain replication
timing. Additional factors essential for replication timing, such as RIF1 (Cornacchia et al.
2012; Hayano et al. 2012), may become absent or re-localized during G2 phase. The
285 formation of TADs and their segregation into distinct nuclear compartments may seed
the assembly of compartments that are enriched or depleted for such factors (Gilbert
2001).

We also show that developmental and constitutive replication domains have distinct
290 organization principles. In contrast to constitutive regions that interact almost exclusively
with chromatin of similar replication time, the contacts of developmental regions tend to
be less tightly constrained to chromatin of similar replication timing, suggesting a link
between developmental and structural plasticity. Our results cannot distinguish whether
developmental regions are more mobile, transiently visiting different compartments in
295 the same cell, or are equally anchored as constitutive domains but reside in different
compartments in different cells. Moreover, the enrichment of various chromatin features
was considerably more correlated to replication time for constitutive vs. developmental
regions. The lower degree of compartmentalization and intermediate enrichment of
transcription factors and histone marks in developmental domains may underlie their

ability to switch between active and inactive epigenetic states more easily during the course of development. Indeed, a comparison of constitutive and developmental domains between 2 cell types in *Drosophila* revealed similar findings (Lubelsky et al. 2014) and other studies have reported a similarity in nuclease sensitivity and origin density between developmental domains and constitutively late domains, regardless of their replication time (Takebayashi et al. 2012; Besnard et al. 2012).

In summary, our results show that 3D chromatin organization is established during early G1 phase coincident with the establishment of a replication-timing program at the TDP. 3D organization is not sufficient to maintain the replication-timing program in G2 phase, suggesting that it may create a scaffold to seed the assembly of essential, cell-cycle regulated, factors. We also show that domains whose replication timing is regulated during development, which represent approximately 50% of the genome (Hiratani et al. 2010; Hansen et al. 2010), comprise a novel sub-nuclear compartment defined by promiscuous nuclear compartmentalization and a chromatin composition that is poorly correlated to replication time. The evolutionary conservation of replication timing and its developmental regulation in all studied metazoans (Yue et al. 2014; Ryba et al. 2010) indicates that the novel properties of developmentally regulated RDs described here are a fundamental aspect of genome organization.

METHODS

Cell Culture and Synchronization

C127 cells (ATCC) were cultured and synchronized in mitosis as previously described (Wu et al. 1997; Lu et al. 2010). Quiescent cells were prepared by serum starvation for 5 days as previously described (Lu et al. 2010). Cells were synchronized in G2 by

releasing from mitosis for 15 hr and including nocodazole during the last 3 hr to block fast cycling cells from entering the next G1, followed by eliminating mitotic cells by shake-off prior to collection (Lu et al. 2010).

3D FISH

FISH experiments were performed as previously described (Takebayashi et al. 2012) with modifications described in supplementary methods. **For time course experiments, a significant increase in the radius of the nuclei was observed during the first few hours of G1, therefore the distance between probes was normalized to the radius of the nucleus, to give “relative distance”.**

Multiplex 4C-seq

4C-seq was performed and 4C reads were processed for statistical significance assignment as previously described (Splinter et. al., 2012), with modifications described in supplementary methods. Directionality Index (DI) was calculated as previously described (Dixon et al. 2012), with modifications described in supplementary methods.

Classification of human and mouse genomes into constitutive/developmental early and late regions

28 mouse replication timing datasets and 21 human published replication timing datasets (replicationdomian.org) were used for the annotation of the human and mouse genome into constitutive/developmental early and late regions. The datasets were windowed into 40 or 50 kb bins. Then the following criteria were used for the annotation of both human and mouse datasets. Windows with replication timing values greater than 0.5 in all datasets were labeled constitutive early (CE) and below -0.2 in all datasets

were labeled constitutive late (CL). Certain large late domains had replication timing values higher than -0.5 in few datasets. To prevent the removal of these large late domains from CL category just because of these outliers, we set the threshold of CL lower than CE. Windows where the magnitude of replication timing value is more than 0.5 in all datasets but with a sign change (positive to negative or vice-versa) in at least one dataset were labeled developmental. Developmental segments were further classified into developmental early (DE) or developmental late (DL) for a given cell line depending on whether the segment replicated early ($RT > 0$) or late ($RT < 0$) in that cell line.

DATA ACCESS

The 4C-seq data generated for this study have been submitted to the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE66579. Replication timing labels (Constitutive Early (CE), Constitutive Late (CL) or Developmental (D)) for both human (hg19) and mouse (mm9) genome are available as supplementary files (Supp-RT-human-50kb-bins.xlsx, Supp-RT-mouse-40kb-bins.xlsx).

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Author contributions: VD and DMG devised experiments; VD and JS performed the experiments; VD, FA, JS and DLV analyzed data; VD, FA, JS, WSN and DMG wrote the manuscript.

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FIGURE LEGENDS

Fig 1. Establishment of interphase chromatin compartments during early G1. A) Validation of 4C by 3D FISH. Cumulative frequency curves and boxplots of distances (in microns) between FISH signals from a pair of regions with low 4C contact count (red, n=51) or high 4C contact count (green, n=57). **B)** Schematic diagram of spatial distribution of early (red) and late (green) replicating RDs within the nucleus before and after the establishment of a replication timing program at the TDP (TDP) in mouse C127 cells. Time-points chosen for 4C after release from mitosis are indicated with black arrows. Nucleolus (No). **C and D)** “Contact count”: Smoothed 4C data (dark grey) overlaid with the replication timing (RT) profile (red/green is early/late replicating) for a late replicating bait Ch8.B53 (C) and an early replicating bait Ch8.B87 (D). Both baits were analyzed at 0.5 (top) and 4 (bottom) hours after mitosis. “-log(p-value):” Negative log10 of contact p-values (Methods) colored in red or green for early or late replicating regions, respectively. Between 0.5 to 4 hrs after mitosis, contacts between bait and

other intra-chromosomal regions become more significant and focused to those with similar RT as the bait. **E)** Distribution of Replication Timing (RT) values for the strongest (top 5% highest z-scores) contacts with either the early replicating (Ch8.B87, red) or the late replicating (Ch8.B53, green) bait. Positive values of RT indicate early replication, negative values of RT indicate late replication and zero indicates middle replication. The higher the magnitude the earlier or later the region replicates. For reference, the RT distribution of the whole Chr 8 is shown in grey. Contacts at 0.5 hr did not have statistical significance where as 4hr had significant contacts to regions with same RT as the bait (**Supplementary Fig 7A**).

Fig 2. Interphase chromatin compartments are established coincident with TDP.

A) Representative 4C contact count plots for baits positioned in an early replicating (red) and late replicating (green) replication domains for the entire chromosome. 4C profiles are overlaid with C127 replication timing (RT) data (black). **B)** Distribution of Replication Timing (RT) values for the strongest (top 5% highest z-scores) contacts with early replicating (red), and late replicating (green) baits across Chr 8 and Chr 16. For reference, the RT distributions of the whole chromosomes 8 and 16 are shown in grey. Contacts before the TDP did not have statistical significance where as significant contacts begin to appear coincident with the TDP and become more compartmentalized after the TDP (**Supplementary Fig 7A**). **C)** Loess (local polynomial regression fitting) fitted decay of 4C contact frequency with distance from the bait (decay curve) averaged for all baits from Chr 8 and Chr 16. **D)** FISH based measurement of 3D proximity normalized to the size of the nucleus (Relative distance, Methods) of two late loci (Supplementary Fig 4) at different points after release from mitosis (Nocodazole free synchrony). There is a significant increase in 3D proximity ($p < 0.001792$) between before

1hr and after 2 hr, which is coincident with TDP. N=91, 82, 57, 184, 67 for 30 min, 1 hr, 2 hr, 3 hr, 4 hr respectively. Mitosis is not included in the time course due to the inability to normalize to the state of condensation of mitotic chromatin (see Supplementary methods).

Fig 3. TADs are formed coincident with TDP. A) 4C contact counts shown for 1 to 3 Mb to either side of early/late late baits. Below the contact count plots is the directionality index (DI, Dixon et al. 2012) calculated from Hi-C in mouse ESC and cortex cell lines, shown to indicate the downstream (dark red) or upstream (dark green) bias. The vertical solid black line indicates TAD boundaries in mouse ESCs and the dotted black line indicates the position of the bait. The kinetics of TAD formation can be visualized by the appearance of a sharp drop in contact frequency at RD/TAD boundaries. **B)** Formation of TADs measured as an increase in directionality bias of the contacts with time, for baits positioned at constitutive early (red) and late (green) replicating RDs (Ch8.B26, Ch8.B44, Ch8.B118, Ch16.B30, Ch16.B46, Ch16.B48). Y-axis shows the percentage of DI relative to maximum DI for each bait.

Fig 4. Chromatin organization in G0 (Quiescence) and G2 are similar to post-TDP. A) 4C contact counts for an early (red) and late (green) replicating region, displayed as in Fig. 2A, comparing 4 hr interphase time point to G0 and G2. **B)** 4C contact counts shown for 1 to 3 Mb to either side of early/late late baits shows conserved TADs and directionality bias (as in Fig 3A) during G0 and G2 phase, similar to 4hr time point.

Fig 5. Developmental domains are less compartmentalized than constitutive domains. A) Degree of compartmentalization (Methods) measured for constitutive baits

(C baits) in blue vs developmental baits (D bait) in brown. The difference in degree of compartmentalization between C baits and D baits did not have statistical significance (p=0.7374, KS test), therefore we performed genome wide comparison using Hi-C. **B)** Degree of compartmentalization for constitutive (C) regions vs developmental domains (D) (50kb windows) across 3 mouse cells types and 4 human cell types using Hi-C data. The difference in degree of compartmentalization between constitutive domains and developmental domains showed high statistical significance ($p < 2.2e^{-16}$, KS test) **C)** Density contour plot (lighter colors indicate less density) with local regression fitting (loess) for degree of compartmentalization vs absolute replication timing value for developmental (brown) vs constitutive (blue) regions. **D)** Plot comparing frequency of Hi-C contacts vs difference in replication timing value for pairs of 50kb windows using Hi-C data. The top 50 percentile of contacts from the Hi-C data was used for the analysis. Blue and brown lines show local regression fitting of the data for constitutive and developmental regions.

Fig 6. Developmentally regulated regions have different organizational principles.

A) Table comparing chromatin features between constitutive early and late (CE, CL) and developmental RDs that are either early (DE) or late (DL) in the particular cell type analyzed for each respective chromatin feature (Supplementary Table 3). **B)** LaminB association for constitutive early/late and developmental early/late RDs. **C)** Distribution of constitutive early/late and developmental early/late RDs within the 6 sub-compartments defined by Hi-C data in GM12878 (Rao et al. 2014). **D)** Segway/ChromHMM analysis of human lymphoblast (GM12878) and HeLa cells showing the enrichment of 7 chromatin labels within constitutive early/late and developmental early/late RDs. WE: Weak enhancer, E: Predicted enhancer, R:

Predicted repressed regions, PF: Promoter flanking region, T: Predicted transcribed regions, TSS: Predicted promoter region including TSS, CTCF: CTCF enriched
 470 element. Note that in all cases, the properties of developmental domains are less distinct and less dependent on their replication time than constitutive domains.

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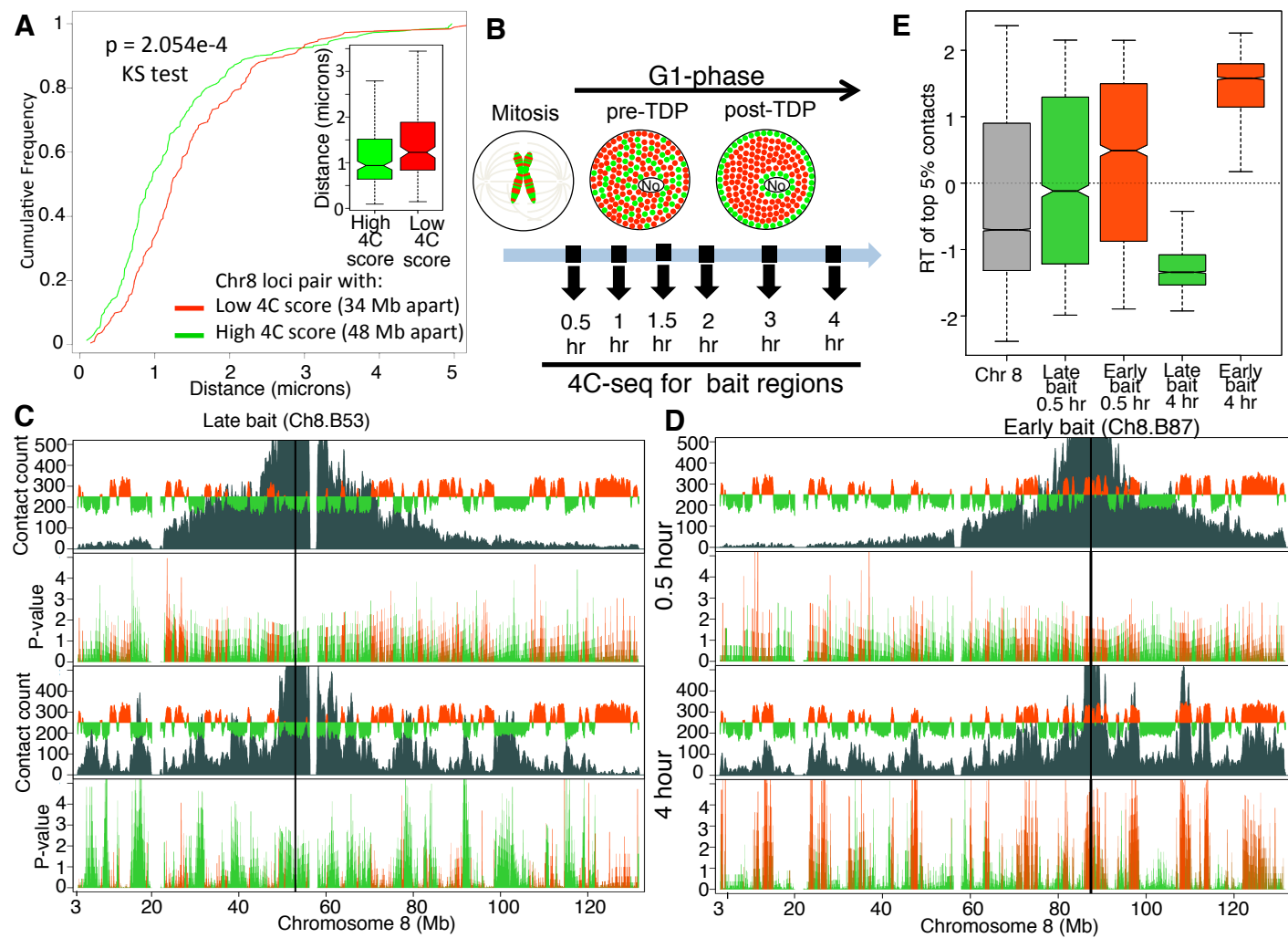
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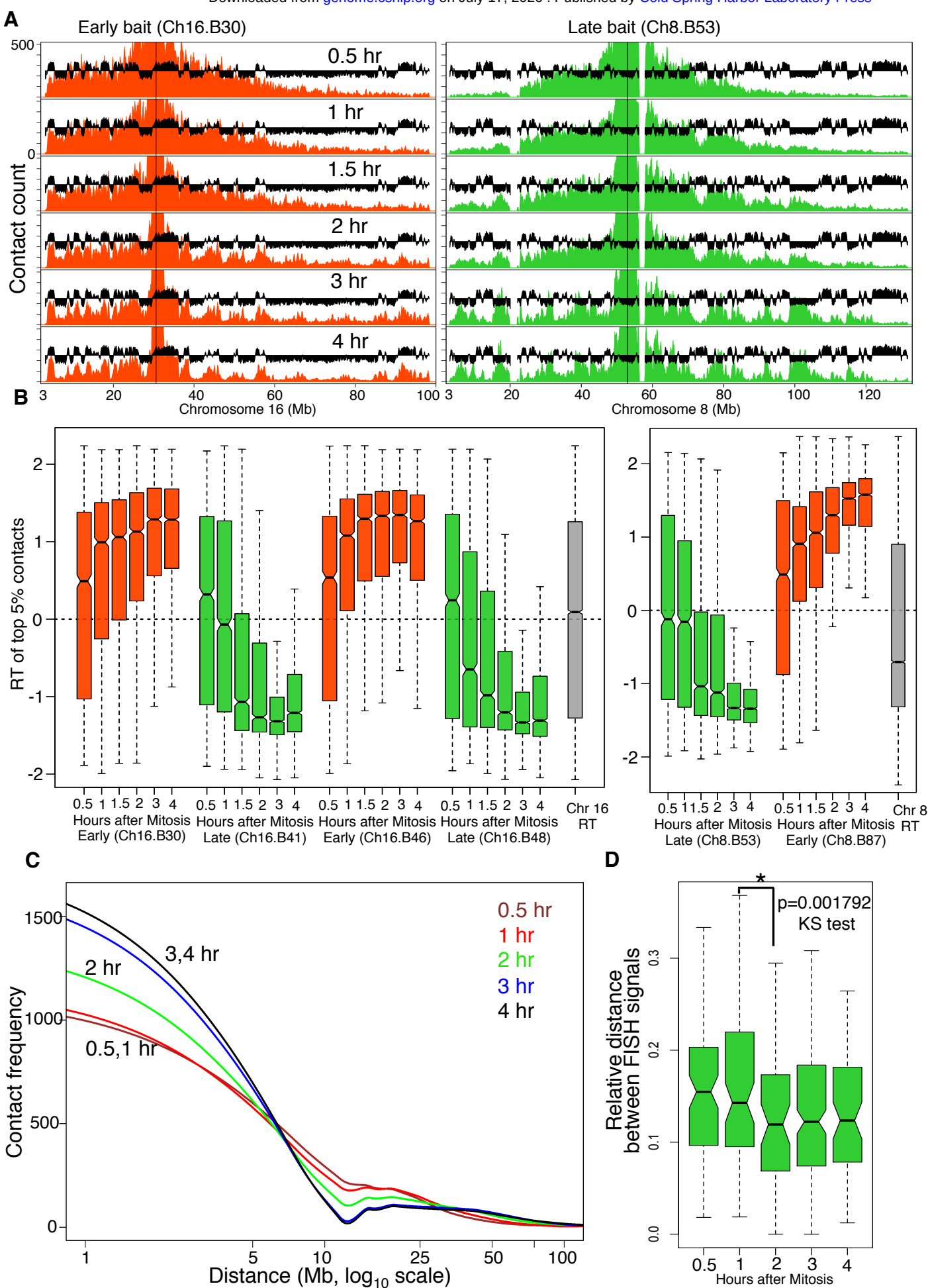
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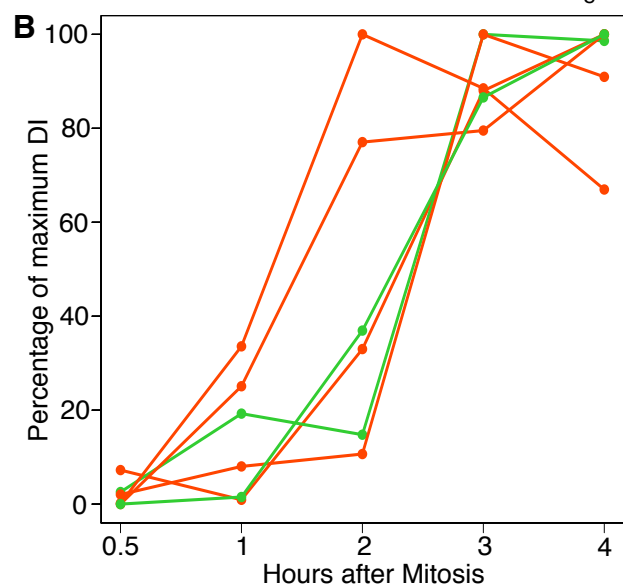
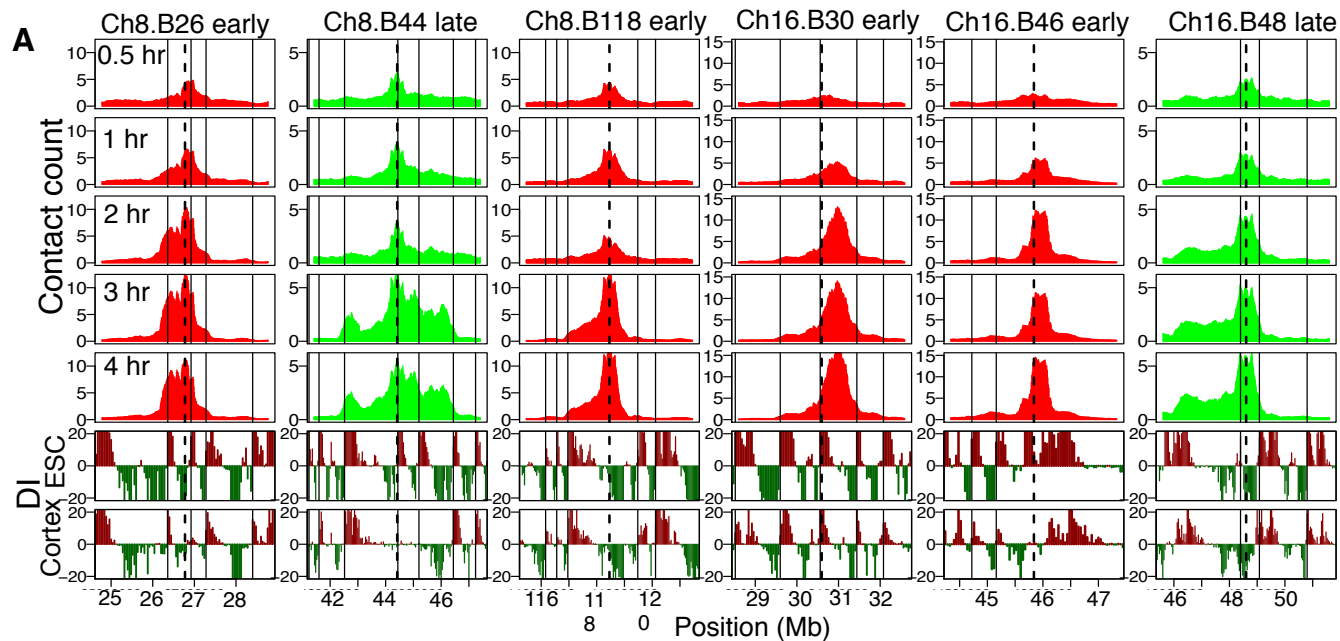
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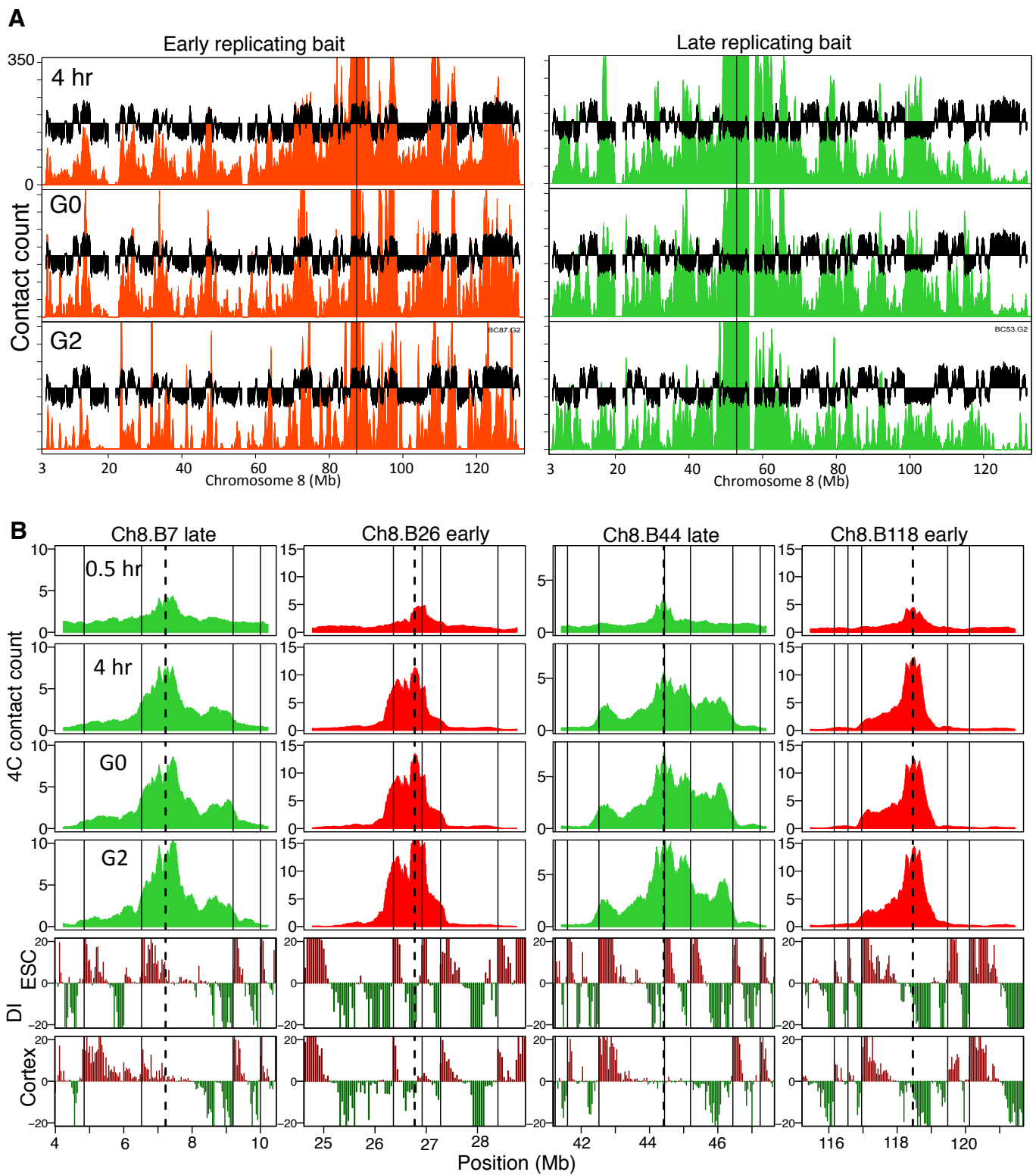
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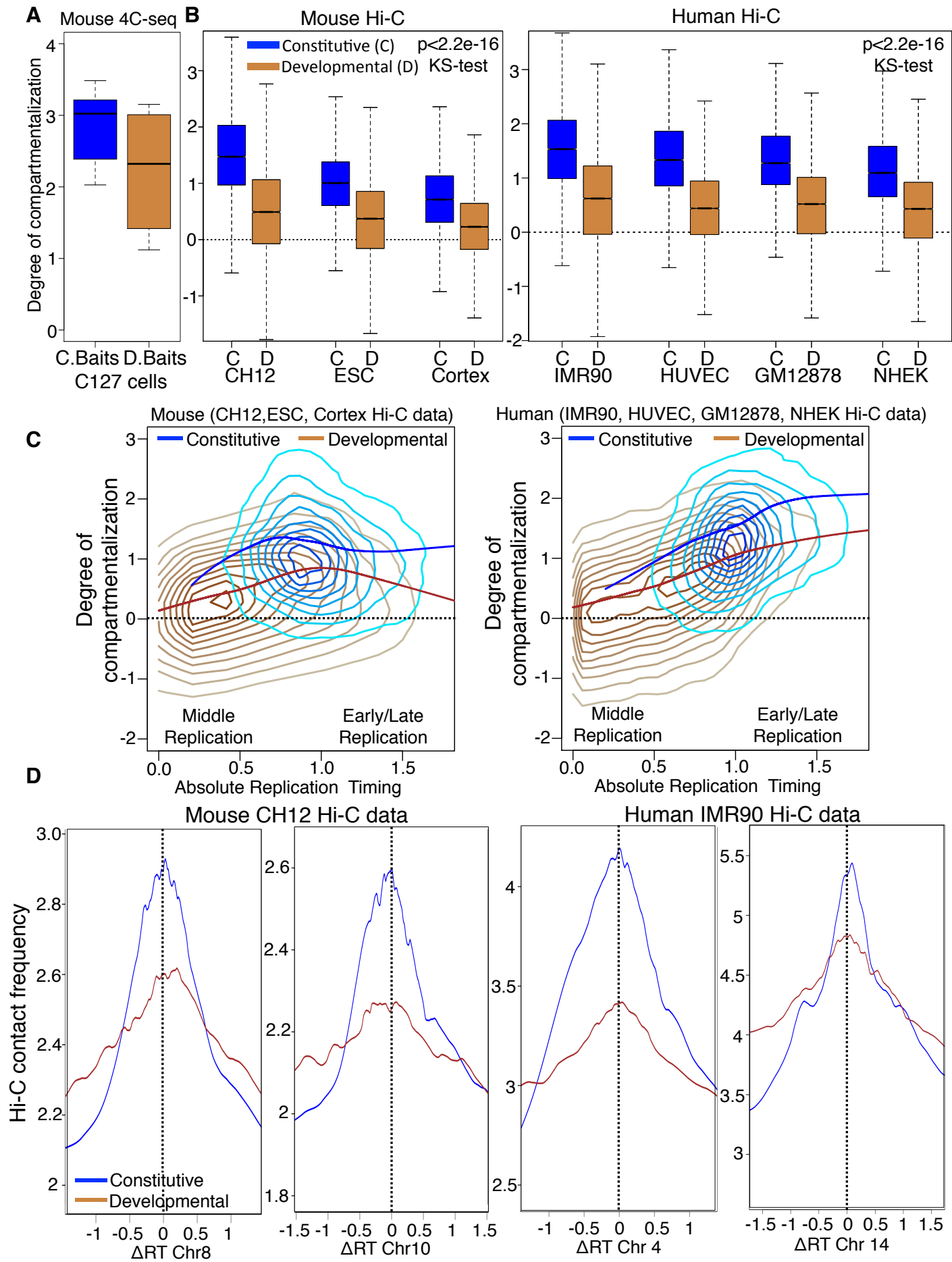
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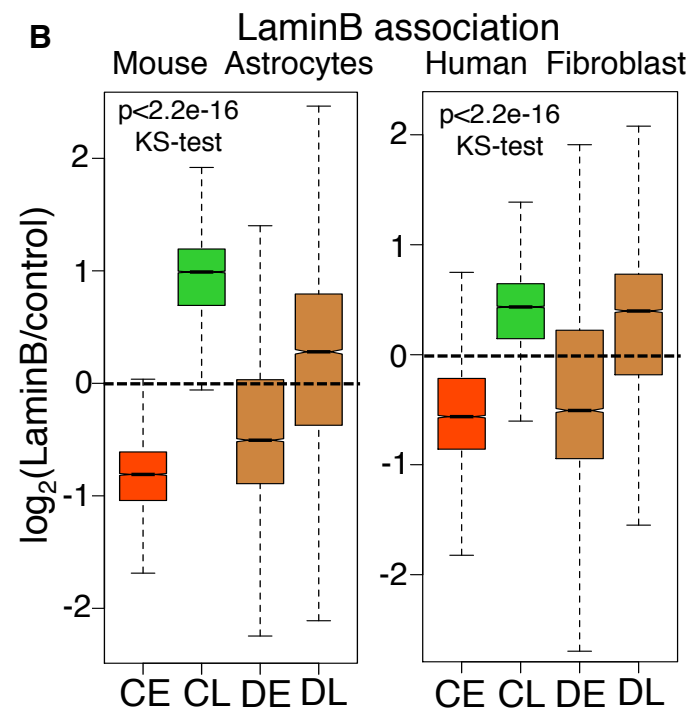




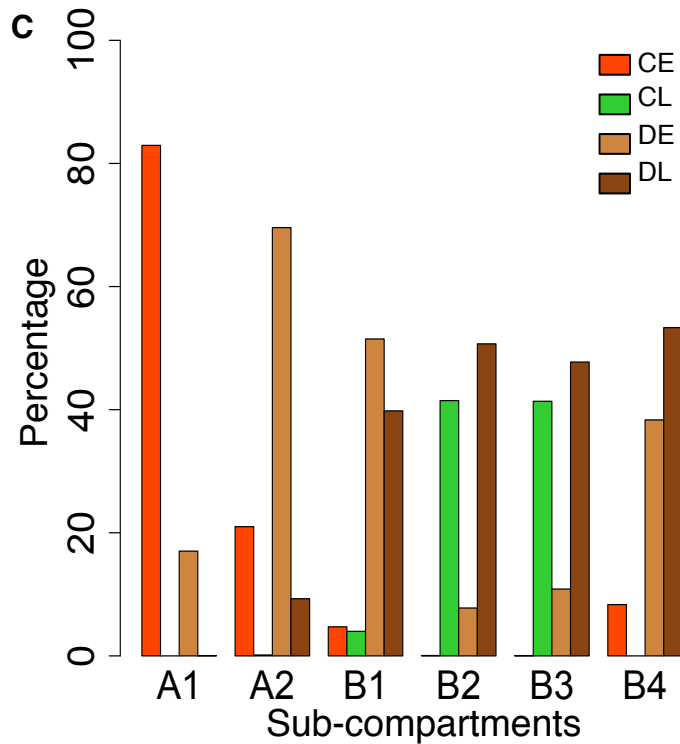


A

Property	CE	CL	DE	DL
Degree of compartmentalization	High	High	Low	Low
MNase sensitivity, Origin Density	High	Low	Low	Low
LaminB association	Low	High	Moderately Low	Moderately High
42 trans-acting factors (eg: CTCF, EZH2) Active chromatin marks: H3K27ac, H3K4me1, H3K4me2, H3K9ac, H4K20me1, H2az	Enriched	Depleted	Moderately Enriched	Moderately Depleted

B

C



D

