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Programming asynchronous replication in stem cells

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Many regions of the genome replicate asynchronously and are expressed monoallelically. It is thought that asynchronous replication may be involved in choosing one allele over the other, but little is known about how these patterns are established during development. We show that, unlike somatic cells, which replicate in a clonal manner, embryonic and adult stem cells are programmed to undergo switching, such that daughter cells with an early-replicating paternal allele are derived from mother cells that have a late-replicating paternal allele. Furthermore, using ground-state embryonic stem (ES) cells, we demonstrate that in the initial transition to asynchronous replication, it is always the paternal allele that is chosen to replicate early, suggesting that primary allelic choice is directed by preset gametic DNA markers. Taken together, these studies help define a basic general strategy for establishing allelic discrimination and generating allelic diversity throughout the organism.

The entire genome is divided into large zones that are replicated according to a fixed program whereby some regions undergo DNA synthesis early and others become copied at later times during S phase. In general, genes located in early zones are expressed in that particular cell type, and those located in late bands are silenced[1]. In contrast, imprinted genes are embedded within asynchronously replicating regions in which one allele, usually the paternal allele, replicates early and the other replicates late. This pattern is set up in gametes and is then maintained through every cell division, suggesting that replication timing represents a major chromosomal marker that serves to distinguish between the two alleles[2].

In addition to imprinted regions, there is another class of asynchronously replicating domains that are random in nature, with the paternal allele replicating early in some cells and the maternal allele replicating early in others[3], and this pattern appears to be present in all cells of the body. Using both *in situ* hybridization and biochemical techniques, it has been shown that in several different somatic cell types, the replication-timing profile of all of the loci is often preserved in a clonal manner so that the daughter cells always inherit the allele-specific profile present in the mother cell[4]. Many genes expressed monoallelically are located in domains that replicate asynchronously. In the case of the immunoglobulin κ (*Igk*) locus, early replication in pre-B-cell clones is even predictive for which allele will ultimately undergo rearrangement in the subsequent B-cell stage, strongly suggesting that the differential replication timing structure may serve as a mechanism for selecting one allele for activation[4,5]. This might represent a sophisticated built-in mechanism for allele selection.

How are random asynchronous replication timing patterns established during development? Early studies showed that these regions replicate synchronously in the early preimplantation embryo, becoming asynchronous around the time of implantation, and this can indeed be observed in ES cells, which mimic this stage[2,4]. Unlike somatic cells, however, these stem cells do not show a clonal pattern of asynchronous replication, so clones derived from single ES cells still contain some cells with an early replicating paternal allele and others with an early replicating maternal allele[5–7]. This finding implies the existence of a switching mechanism that could provide the plasticity to retain both possible options of allele specificity. Here, we provide evidence that both the initial assignment of replication timing and the switching process occur in a highly programmed manner and represent a built-in mechanism for controlling allele specificity in the genome.

RESULTS

Allelic plasticity in stem cells

Based on prior reports that both early embryos and ES cells in culture demonstrate allelic plasticity[5,8], we attempted to follow the progression of this state throughout development. In the mouse, naive ES cells are generally derived from the blastocyst, but at later stages, pluripotent stem cells can also be derived from the epiblast[9]. To assess whether these cells retain allelic plasticity, we produced epiblast stem cells (EpiSCs) by differentiating ES cells[10] carrying a defined deletion in the T-cell receptor β (*Tcrb*) region on one allele of chromosome 6 (ref. 5) (**Supplementary Fig. 1a–c**). We then carried out double-label fluorescence *in situ* hybridization (FISH) analysis for the *Igk* and an olfactory receptor (*Olfr*) locus[3] on single-cell subclones and used a *Tcrb* probe to distinguish between the two alleles (**Supplementary Fig. 1g**). In every individual clone, we found cells in which the maternal allele replicates early mixed with cells in which the paternal allele replicates early (**Fig. 1a**,**b**). We next asked what happens when these same ES cells are induced to undergo differentiation into neuronal progenitors (NPCs)[11,12] (**Supplementary Fig. 1d**). Here too, each subclone

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yielded a mixture of cells with either the maternal or paternal allele replicating early (**Fig. 1c**,**d**), indicating that these neural precursors also maintain allelic plasticity.

Previous results have also shown that hematopoietic stem cells (HSCs), as well as their multipotent precursor derivatives, retain allelic plasticity of replication timing, and this plasticity probably provides the hematopoietic system with a way to generate a variety of different somatic cell types with both allelic options[5,13]. To determine whether this plasticity is a general property of other adult stem cells, we took advantage of a mouse strain carrying a large deletion within the *Tcrb* on one copy of chromosome 6 to examine allele-specific asynchronous replication timing in any tissue[14]. As a first step, we isolated neural stem cells and grew them into individual neurosphere clones in culture[15]. Strikingly, we found equal numbers of cells with the paternal or maternal allele replicating early in each neurosphere, and similar results were obtained for an asynchronous-replicating *Olfr* region on chromosome 6 (**Fig. 1e**,**f**). We then isolated mammary stem cells from these mice and generated individual mammospheres in culture[16] (**Fig. 1g**). Once again, the FISH assay indicated that these cells retain their allelic plasticity and are able to generate daughter cells with either maternal-early or paternal-early replication patterns (**Fig. 1h**,**i**). Taken together, these results suggest that plasticity of asynchronous replication timing in multipotent stem cells is preserved from the embryonic stem cell stage.

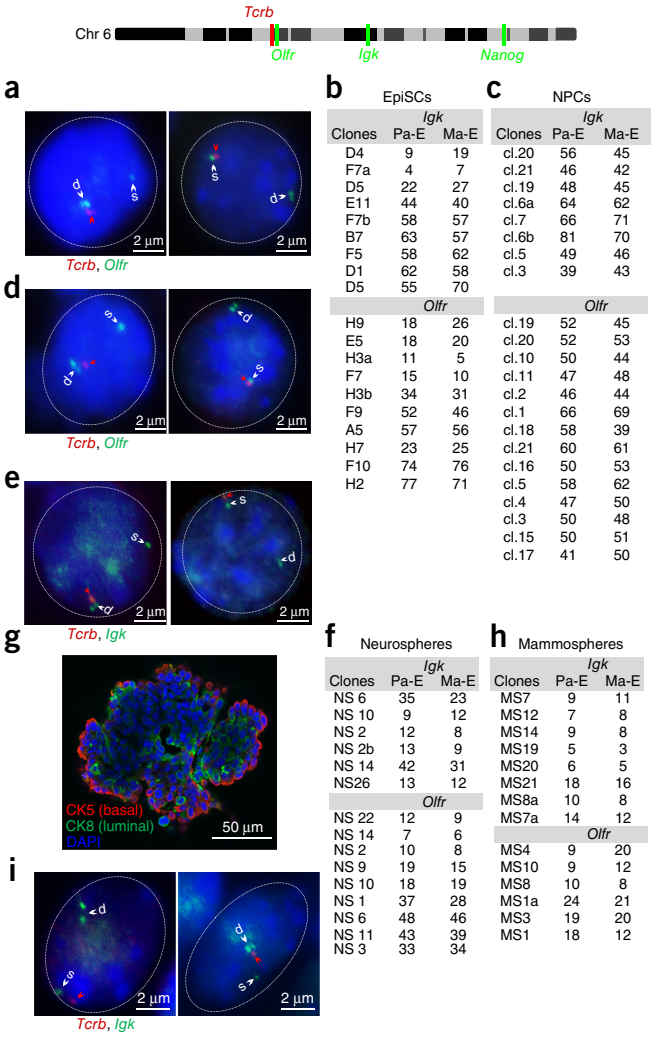
Allelic-switch programming

The concept of plasticity in single-cell-derived clones strongly suggests that there must be a mechanism for switching from the maternal-early state to the paternal-early state and vice versa. As a first step in understanding this process, we asked whether switching takes place in a stochastic manner or, alternatively, operates according to a preprogrammed schedule. To this end, we set up a system to examine allele-specific replication timing in pairs of daughter cells following cell division. We anticipated three potential outcomes: that both daughters replicate the same allele early, that each pair shows opposite allele specificity, or that there is no fixed pattern, which would suggest that switching occurs in a stochastic manner. ES cells carrying the T-cell-receptor deletion on chromosome 6 (ES (LN3) and ES (LN11/12)) were plated at low density, grown in culture to allow cell division, and then subjected to double-label FISH (**Fig. 2**).

Cell pairs with a single and a double *Igk*-locus signal (single–double) were scored for allelic replication. All pairs analyzed showed identical allele specificity, with half of the pairs being maternal early and the other half being paternal early. Strikingly, no pairs with opposite allele specificity were observed (**Fig. 2a**,**b**). Similar results were obtained for additional asynchronously replicating regions on this same chromosome (**Fig. 2a**). We repeated this experiment using iPS cells (BiPS 6.3 and 1.3) containing a deletion on one allele of the proximal *Igh* locus (chromosome 12)[17], using the distal V(H) region to detect asynchronous replication. Here too, all pairs showed identical allele specificity (**Fig. 2a**), indicating that this is a general phenomenon. Adult HSCs also appeared to undergo this same mode of switching (**Fig. 2a**).

Biochemical assay for switching

We next developed a biochemical assay to detect replication-time switching. Using standard methodology for BrdU labeling and S-phase fractionation, we demonstrated that, in contrast to synchronously replicating controls, the *Igk* locus has a broad replication timing pattern that covers both early and late S phase (**Supplementary Fig. 2**). On this basis, we devised a scheme to label specific early or late alleles



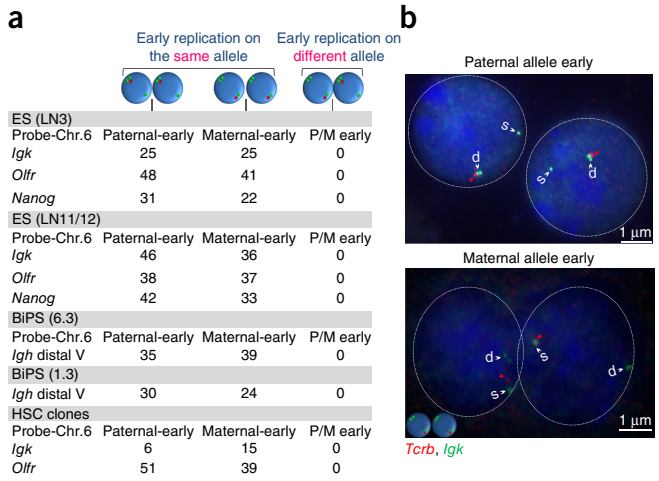
b EpiSCs	Igk	
Clones	Pa-E	Ma-E
D4	9	19
F7a	4	7
D5	22	27
E11	44	40
F7b	58	57
B7	63	57
F5	58	62
D1	62	58
D5	55	70
	Olfr	
H9	18	26
E5	18	20
H3a	11	5
F7	15	10
H3b	34	31
F9	52	46
A5	57	56
H7	23	25
F10	74	76
H2	77	71

c NPCs	Igk	
Clones	Pa-E	Ma-E
cl.20	56	45
cl.21	46	42
cl.19	48	45
cl.6a	64	62
cl.7	66	71
cl.6b	81	70
cl.5	49	46
cl.3	39	43
	Olfr	
cl.19	52	45
cl.20	52	53
cl.10	50	44
cl.11	47	48
cl.2	46	44
cl.1	66	69
cl.18	58	39
cl.21	60	61
cl.16	50	53
cl.5	58	62
cl.4	47	50
cl.3	50	48
cl.15	50	51
cl.17	41	50

f Neurospheres	Igk	
Clones	Pa-E	Ma-E
NS 6	35	23
NS 10	9	12
NS 2	12	8
NS 2b	13	9
NS 14	42	31
NS26	13	12
	Olfr	
NS 22	12	9
NS 14	7	6
NS 2	10	8
NS 9	19	15
NS 10	18	19
NS 1	37	28
NS 6	48	46
NS 11	43	39
NS 3	33	34

h Mammospheres	Igk	
Clones	Pa-E	Ma-E
MS7	9	11
MS12	7	8
MS14	9	8
MS19	5	3
MS20	6	5
MS21	18	16
MS8a	10	8
MS7a	14	12
	Olfr	
MS4	9	20
MS10	9	12
MS8	10	8
MS1a	24	21
MS3	19	20
MS1	18	12

Figure 1 Allelic plasticity in mouse stem cells. A schematic representation of chromosome 6 indicating the location of relevant probes is shown at top. EpiSCs, NPCs, neurospheres and mammospheres carrying a deletion of the *Tcrb* maternal allele gene were assayed by FISH for asynchronous DNA replication by counting the number of single and double dots hybridized to *Igk* or *Olfr* probes. The number of paternal-early or maternal-early nuclei was determined using a *Tcrb*-specific probe as a reference. (**a**) Representative FISH of EpiSC show one cell with the early-replicating *Olfr* (green, double dot, white "d") on the *Tcrb* WT allele (red arrowhead), whereas the other shows the unreplicated *Olfr* allele (green, single dot, white "s") associated with this allele. (**b**) EpiSCs single-cell clones assayed by FISH. Pa-E, paternal early; Ma-E, maternal early. *Igk*, Pa-E $n = 375$ cells, Ma-E $n = 397$; *Olfr*, Pa-E $n = 379$, Ma-E $n = 366$. (**c**,**d**) Representative FISH images and NPC single-cell clones were assayed by FISH as described for **a** and **b**. *Igk*, Pa-E $n = 449$ cells, Ma-E $n = 424$; *Olfr*, Pa-E $n = 727$, Ma-E $n = 717$. (**e**,**f**) Single-cell neurosphere colonies assayed by FISH as described in **a** and **b**. *Igk*, Pa-E $n = 124$ cells, Ma-E $n = 95$; *Olfr*, Pa-E $n = 227$, Ma-E $n = 204$. (**g**) Confocal microscopy image of single mammosphere colonies subjected to immunofluorescence using antibodies against basal (CK5, red) and luminal (CK8, green) keratin markers. DAPI was used to visualize the nuclei. (**h**,**i**) Single mammosphere colonies were assayed by FISH for asynchronous DNA as in **e** and **f**. *Igk*, Pa-E $n = 78$ cells, Ma-E $n = 71$; *Olfr*, Pa-E $n = 89$, Ma-E $n = 93$. In **b**, **c**, **f** and **h**, there was no statistically significant difference between maternal early and paternal early signals for both probes ($P > 0.3$ as determined by the exact two-tail binomial test). It should be noted that, as a control, both the *Igk* and *Olfr* regions were shown to replicate asynchronously by standard FISH analysis (**Supplementary Fig. 1g**).



ES (LN3)			
Probe-Chr.6	Paternal-early	Maternal-early	P/M early
Igk	25	25	0
Olfr	48	41	0
Nanog	31	22	0
ES (LN11/12)			
Probe-Chr.6	Paternal-early	Maternal-early	P/M early
Igk	46	36	0
Olfr	38	37	0
Nanog	42	33	0
BiPS (6.3)			
Probe-Chr.6	Paternal-early	Maternal-early	P/M early
Igh distal V	35	39	0
BiPS (1.3)			
Igh distal V	30	24	0
HSC clones			
Probe-Chr.6	Paternal-early	Maternal-early	P/M early
Igk	6	15	0
Olfr	51	39	0

Figure 2 Asynchronous gene replication in daughter cells. (**a**) Diagram at top illustrates three possible outcomes of examining allele-specific replication timing in pairs of daughter cells following mother-cell division. ES (LN3, deletion on the maternal allele, and LN11/12, deletion on the paternal allele), BiPS (deletion on the maternal allele) and HSC clones were sparsely plated and grown for 9 h until two-cell couplets were observed. The cells were fixed *in situ* and then subjected to FISH analysis using probes for *Igk*, *Olfr* and *Nanog* loci (on chromosome 6), as well as *Igh* distal V (on chromosome 12). The pattern of asynchronously replicated genes was compared in every two daughter cells showing a single–double dot pattern. The number of paternal-early or maternal-early nuclei was determined by reference to a *Tcrb* probe (chromosome 6) or by reference to the *Igh*-proximal probe (chromosome 12). There was no statistical difference between the number of maternal early and paternal early daughter cells in all cell lines and for all probes ($P > 0.3$ using the exact two-tail binomial test). Table summarizes replication timing patterns of each allele LN3: *Igk*, $n = 50$ cells, *Olfr*, $n = 89$, *Nanog*, $n = 53$; LN11/12: *Igk*, $n = 82$, *Olfr* $n = 75$, *Nanog*, $n = 75$. BiPS (6.3): *Igh*, $n = 74$. BiPS (1.3): *Igh*, $n = 54$. HSC: *Igk*, $n = 21$, *Olfr*, $n = 90$. (**b**) Representative FISH pictures from ES (LN3) daughter couples using *Igk* as a probe.

in S-phase and test whether the same alleles replicate at the alternate time point in the subsequent S phase[18,19]. We labeled ES cells with BrdU for 2 h (late S), waited an additional 2 h (G2) and then carried out shakeoff to isolate cells in mitosis[20] that were then replated and grown for 2 h (G0 and G1 phase) before being labeled a second time with BrdU (early S), as depicted in **Figure 3a**.

This approach enabled us to distinguish whether the original late allele switches to early replication or, alternatively, remains late replicating in the daughter cell. In the case of switching, the original late allele (labeled with BrdU on one strand) will replicate early in the presence of BrdU, yielding two copies, one of which is labeled with BrdU on one strand, and the other is labeled on both strands. In contrast, if switching does not occur, the original late-replicating allele will remain labeled with BrdU on one strand, and the original early-replicating unlabeled allele will undergo replication in the daughter cell, producing two copies, each labeled with BrdU on one strand.

In order to detect these differential labeling patterns, we used a variation of FISH that employs a single-strand nuclease to remove BrdU-labeled DNA, thereby exposing the opposite unlabeled strand to specific probe hybridization without denaturing the DNA[21]. This is accomplished by treating nuclei on slides with Hoechst 33258, irradiating with UV to cause nicks specifically in the BrdU-labeled strands and then digesting the nicked DNA with ExoIII nuclease, thereby exposing the opposite strand for probe hybridization. When switching occurs, the originally late allele appears as two copies: one is labeled with BrdU on a single strand, and the other is labeled on both DNA strands and will therefore be completely digested by replication-timing-specific hybridization (ReTiSH) (**Fig. 3a**). After hybridization, these nuclei will be marked by a single fluorescent dot. In contrast, if switching does not occur, the original late-replicating allele will have a single copy labeled with BrdU on one strand, and the original early allele will produce two copies that are newly labeled with BrdU on a single strand. Following ReTiSH, one should observe nuclei with one single dot and one double dot.

When hybridization was carried out using an *Igk*-locus probe, over 70% of the nuclei showed a single-dot pattern, indicating that this locus underwent replication-time switching. Similar results were obtained with probes for a variety of different asynchronously replicating regions within the genome (**Fig. 3b**).

To validate these findings, we carried out the converse experiment, whereby cells were labeled during early S-phase in the first round of replication and then labeled again in early S, following mitotic shakeoff. In this case, the resulting hybridization patterns were opposite of those seen in the previous strategy, with switching yielding a single–double hybridization pattern, whereas the lack of switching is characterized by nuclei with single-dot hybridization (**Fig. 4a**). For *Igk*, about 70% of the nuclei showed the switching pattern, and the same was observed for other asynchronously replicating regions as well (**Fig. 4b**). We also included two key controls, showing that known fixed-replication-time regions do not undergo switching in our assay (**Supplementary Fig. 3**) and that asynchronously replicating imprinted domains do not switch from one S phase to the next (**Supplementary Fig. 4**).

Allelic choice

These results strongly suggest that many DNA domains are marked in a manner that enables the cell to distinguish between the two alleles. Unlike imprinted gene regions, this differential epigenetic state is not inherited from the parents, but rather appears to be erased in the early embryo through a process that causes the two alleles to replicate synchronously[2,4,22]. Although naive mouse ES cells (grown in serum with LIF) appear to represent the preimplantation stage of development, it is possible to shift these cells to an earlier ground state that probably better mimics the inner cell mass of the blastula by growing them in the presence of GSK3 and MEK/ERK inhibitors (2i medium)[23]. It was previously reported that the *Nanog* locus reverts to synchronous replication under these conditions[8]. To test whether this is true of other randomly asynchronous loci, we treated parentally marked ES cells with 2i medium and used FISH to assess the replication-timing status of a number of different regions. Growth in this medium brings about erasure of random asynchronous replication timing at the *Igk* locus, causing it to replicate synchronously, but subsequent removal of 2i medium returned this region to its original asynchronous state (**Supplementary Fig. 5**). Similar results were obtained for an *Olfr* gene on chromosome 6, as well as for the *Igh* locus on chromosome 12. In contrast, imprinted domains remained asynchronous (**Fig. 5a**).

Although all cells are marked with differential replication timing patterns of alleles, it is not clear how this difference between alleles is first established. ES cells growing in 2i medium represent an excellent system for mimicking the initiation of differential replication timing patterns. We used ES cells carrying a *Tcrb* deletion on one allele that had been adapted to the synchronous state by multiple passages in 2i medium. We then removed the GSK3 and MEK/ERK inhibitors and used double-label FISH to examine the DNA replication timing pattern characteristic of the first replication cycle (15 h) under the new conditions. All daughter-cell pairs were analyzed for

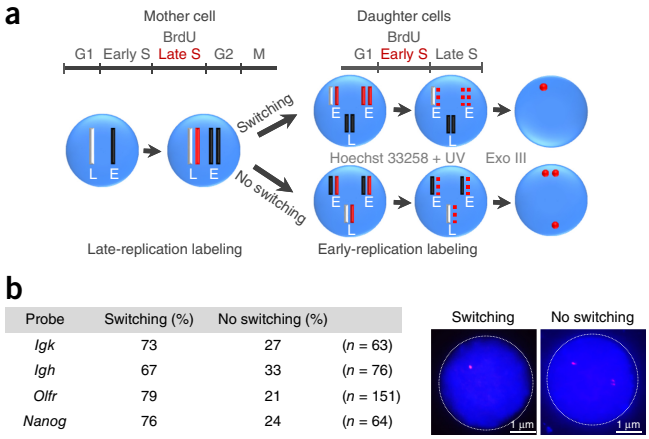


Figure 3 Replication time switching (late to early). (**a**) Illustration of the late-to-early ReTiSH assay. Diagram at top displays the experimental time course. Black and gray lines within blue circles represent early and late-replicating chromosomes, respectively. Cells were pulse labeled for 2 h with BrdU (red lines), grown another 2 h to enable them to pass through G2 and enter M-phase. Mitotic cells were collected by shakeoff and replated. After entering S phase (~2 h), cells were labeled with BrdU for 3.5 h (early replication), harvested and fixed for ReTiSH analysis. Daughter cells in which allelic switching occurred before S phase incorporate BrdU only into the early replicating chromosome. In daughter cells that do not switch between early and late replication, BrdU incorporates in both the early and late-replicating chromosomes. ReTiSH analysis is based on BrdU-specific digestion, leaving an unlabeled single strand that is free to hybridize. Switched cells show a single dot, whereas unswitched cells show a single dot for the late-replicating allele and two dots (double) for the early replicating allele. For simplicity, the diagram depicts two possible outcomes for one daughter cell only. (**b**) Late-to-early ReTiSH experiments using ES (LN3) cells. The percentage of cells showing 'switching' as opposed to 'no switching' was highly significant for all probes (*Igk*, $n = 63$ cells, $P < 0.0003$ using the exact 2-tail binomial test; *Nanog*, $n = 64$, $P < 10^{-4}$; *Igh*, $n = 76$, $P < 0.004$; *Olfr*, $n = 151$, $P < 10^{-9}$). As a control, we confirmed that >75% of the nuclei indeed have only one BrdU signal after the first round of labeling. It should be noted that we still detected many cells (~30%) with ReTiSH patterns consistent with a lack of switching. Although this probably represents a fixed background resulting from lack of total synchrony, it is also possible that switching does not take place systematically in every cell cycle.

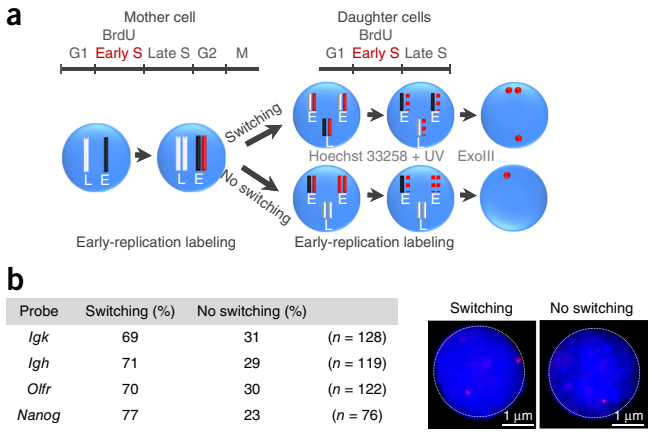


Figure 4 Replication time switching (early-to-late). (**a**) Illustration of early-to-early ReTiSH assay. Diagram at top displays the experimental time course. Early replication products were pulse labeled with BrdU for 2 h. After BrdU was washed off, cells were left to grow for 5.5 h so that all labeled cells went through G2 and entered M phase. Cells were then collected by mitotic shakeoff and replated. After entering S phase (~2 h), cells were labeled with BrdU for 3.5 h (early replication), harvested and fixed for ReTiSH analysis. In this experiment, which is the converse of that in **Figure 3**, switching yields a single–double hybridization pattern, whereas the lack of switching is characterized by nuclei with single-dot hybridization. (**b**) Early-early ReTiSH experiments using ES (LN3) cells. Nuclei (DAPI stained) were counted using different probes for asynchronous genes. The percentage of cells showing switching as opposed to no switching was highly significant (*Igk*, $n = 128$ cells, $P < 10^{-4}$; *Nanog*, $n = 76$, $P < 10^{-5}$; *Igh*, $n = 119$, $P < 10^{-5}$; *Olfr*, $n = 122$, $P < 10^{-5}$). *P* values were determined by the exact two-tail binomial test.

single–double signals, with allele specificity being determined by the *Tcrb* probe. In a truly striking manner, the paternal allele was found to be early replicating in every nucleus examined ($P < 10^{-68}$ by two-tailed binomial test), and this held true for both the *Igk* locus and an *Olfr* locus on the same marked chromosome (**Fig. 5b**). Similar results were obtained for the distal V (H) region on chromosome 12 using BiPS cells (**Fig. 5b**,**c**) and for an *Olfr* locus on chromosome 4 (data not shown). These findings suggest that allelic choice is built into the chromosomes themselves, probably by taking advantage of stable imprinting marks introduced in the gametes.

Role of DNA methylation

Previous studies have established that asynchronous replication takes place in ES cells carrying a triple knockout (TKO)[24] of all DNA methyltransferases and are therefore deficient in DNA methylation[8,25], suggesting that this modification is not necessary for distinguishing the two alleles. These prior experiments, however, did not examine whether allelic switching can occur in TKO as it does in wild-type (WT) ES cells. We therefore carried out an S-phase-specific labeling study on TKO ES cells[24] using ReTiSH to determine whether a BrdU-labeled late *Igk* allele in a mother cell becomes early replicating in the daughter. Strikingly, we found that this allele does not change its time of replication from one cell cycle to the next (**Table 1**). Similar results were obtained for both *Nanog* and an *Olfr* on chromosome 6, as well as *Igh* on chromosome 12. This suggests that asynchronous-replication switching does not take place in TKO cells. The synchronous *Adyc8* gene was assayed for percent single–single dots (S) (late replicating). Consistent with this observation, we found that transient treatment of normal ES cells with 5-azaC was also able to turn off switching (data not shown).

As further validation, we employed CRISPR technology to introduce a large deletion into one allele of chromosome 6 in TKO (IIAC2 Clone) and then used a probe derived from this region to distinguish between the two alleles. FISH analysis showed that the *Igk* locus replicates late on the deleted paternal allele in a large percentage of cells (**Table 2**), as opposed to WT ES in which the maternal and paternal alleles replicate late in an equal manner[5]. Similar results were also obtained for a number of different asynchronous loci on the same chromosome. Furthermore, this specificity appears to be fixed, because this same allele is also late in all single-cell-derived clones (IIAC2 subclones) (**Table 2**). This suggests that in the absence of methylation, allele switching does not occur and, as a result, TKO cells are locked into a single direction with the same allele always replicating early.

We next asked whether DNA methylation is also necessary for the process of allelic choice that takes place at the time of implantation, at which these loci normally undergo a change from synchronous to asynchronous replication timing. To this end, we first incubated TKO cells in 2i, thereby converting them to a synchronous replication profile and then transferred them back to normal medium lacking 2i. After several cell divisions, we examined asynchronous replication at a number of different loci by FISH analysis. As opposed to that

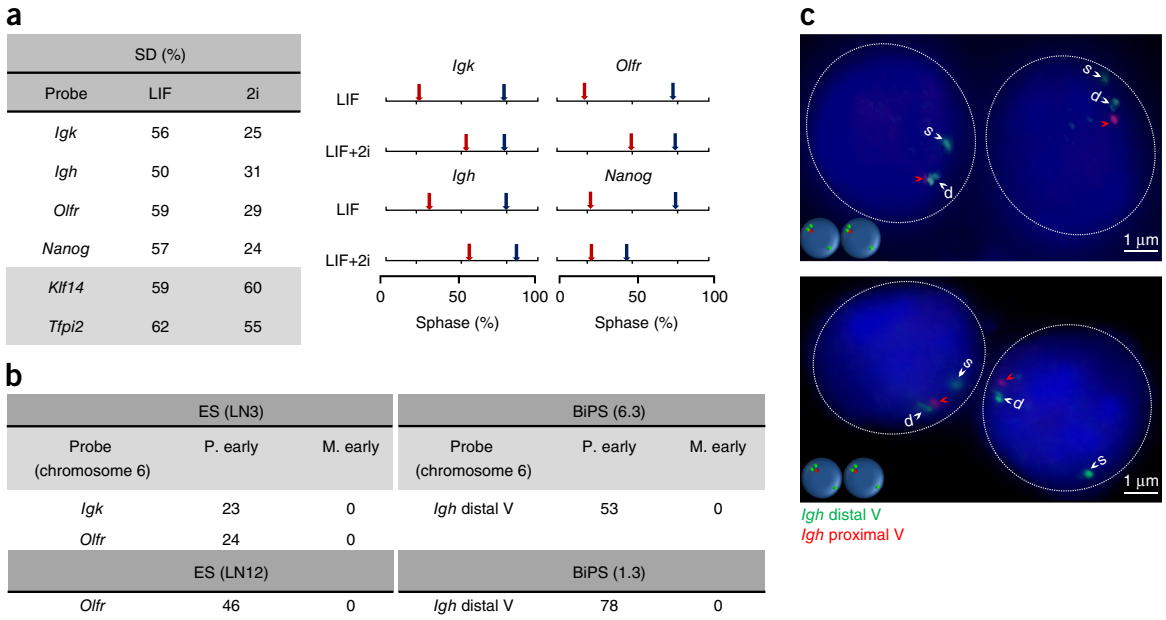


Figure 5 Initiation of asynchronous replication. (**a**) ES cells were grown in medium containing LIF or LIF supplemented with 3 µM of CHIR and 1 µM of PD (LiF + 2i), labeled with BrdU and assayed for replication timing using probes for *Nanog* (182 cells), *Igk* (100 cells), *Olfr* (300 cells) (chromosome 6) or *Igh* (198 cells) (chromosome 12). Left, table summarizes replication time as determined by the percentage of cells carrying a single–double (SD%) dot pattern. Right, graphic depiction of the approximate replication time of each allele; red and blue arrows represent the percentage of single–single (SS) (early) and SS + single–double (SD) (late) cells, respectively. (**b**) ES and BiPS clones were grown in 2i for several passages and then the inhibitors were removed. Double-label FISH was used to detect the DNA replication timing pattern characteristic of the first replication cycle (15 h) by examining two cell pairs (as described for **Fig. 2**), using probes for *Igk*, an *Olfr*, *Nanog* and for the *Igh* distal variable (V) region. The number of paternal-early (P. early) or maternal-early (M. early) nuclei was determined by reference to the *Tcrb* probe (chromosome 6) or the *Igh* proximal probe (chromosome 12). It should be noted that similar results were obtained regardless of whether the *Tcrb* deletion was on the maternal or paternal allele. (**c**) Representative FISH images from ES BiPS daughter pairs using *Igh*-proximal and distal V-region probes.

of WT cells, all of these sites showed a relatively low percentage of single–double signals, clearly indicating that they remained synchronously replicating (**Supplementary Fig. 5**) even after they exited the ground state, as shown by their pattern of embryonic markers (**Supplementary Fig. 5**). These observations clearly suggest that the entire process of allelic choice does not take place in the absence of DNA methylation.

DISCUSSION

It has previously been shown that asynchronous replication timing behaves in a clonal manner in differentiated somatic cells but not in stem cells, indicating that there must be a cellular mechanism for switching allelic timing in a reciprocal manner. Here, we demonstrate that this switching is not random, but rather occurs in an orderly fashion, with both daughter cells carrying the same epigenotype, opposite that present in the mother cell. This finding was confirmed by biochemical studies showing that replication itself, as detected by BrdU, actually changes its timing from one cell generation to the next, implying that ES cells have a molecular program responsible for carrying out the switching process. Because twin daughter cells always have the same allelic replication pattern, the switched state must already be marked on both chromosome copies of the alleles following their replication during S in the mother cell, and these signals are preserved through mitosis and cell division, during which they appear to instruct the replication-timing machinery of daughter cells.

On the basis of our present analyses, we speculate that the switching mechanism works like a toggle switch that alternates between two states, late and early, on each allele. Following replication of the early allele, the switch is reset so that the same allele will replicate late in the daughter cell. Conversely, the switch on the late-replicating allele in the mother cell will be reset to early replication timing in anticipation of the next cell-division cycle. According to this model, the two alleles are controlled independently and do not need to communicate with each other in order to remain asynchronously replicating.

Asynchronous replication itself is independent of DNA methylase activity[8,25], suggesting that the replication-timing identity of each

Table 1 Role of DNA methylation in replication time switching

Probe	Switching (%)	No Switching (%)	# cells	Switching (%)	No Switching (%)	# cells
		J1			TKO	
Igk	75	25	$n = 151$	25	75	$n = 133$
Igh	68	32	$n = 111$	22	78	$n = 136$
Olfr	62	38	$n = 132$	40	60	$n = 48$
Nanog	75	25	$n = 164$	26	74	$n = 66$
Adyc8	63		$n = 82$	67		$n = 94$

The percentage of cells showing switching or no switching was highly significant for all probes in J1 cells (*Igk*, $P < 10^{-9}$; *Nanog*, $P < 10^{-10}$; *Igh*, $P < 10^{-4}$; *Olfr*, $P < 10^{-12}$) and in TKO cells (*Igk*, $P < 10^{-8}$; *Nanog*, $P < 10^{-5}$; *Igh*, $P < 10^{-10}$; *Olfr*, $P < 0.005$), respectively, as determined using the exact two-tail binomial test.

Table 2 Role of DNA methylation in replication time choosing

IIAC2 clone Probe (chr6)	Paternal late (%)	Maternal late (%)	# cells
Igk	92	8	$n = 114$
Olfr	77	23	$n = 123$
Nanog	81	19	$n = 110$
IIAC2 subclones (chr6-*Olfr*)	**Paternal late (%)**	**Maternal late (%)**	**# cells**
IIAC2-a	85	15	$n = 107$
IIAC2-b	85	16	$n = 85$
IIAC2-c	85	15	$n = 91$
IIAC2-d	80	20	$n = 94$
IIAC2-e	88	11	$n = 99$

The difference in replication time between paternal late and maternal late is highly significant (IIAC2: *Igk*, $P < 10^{-13}$; *Olfr*, $P < 10^{-9}$; *Nanog*, $P < 10^{-11}$; IIAC2 subclones: *Olfr*, $P < 10^{-9}$) as determined using the exact two-tail binomial test.

allele does not involve methylation marks. DNA methylation does, however, appear to play a role in regulating the switching process, and in its absence, switching cannot occur, even though the asynchronous state can still be maintained. This same control mechanism may be used *in vivo* during normal development when tissues convert from the stem cell to the differentiated state[5]. Our experiments do not reveal how methylation influences this process. One possibility is that this modification regulates the switching phenomenon in *cis*. Alternatively, DNA methylation may work indirectly by controlling the transcription of genes involved in the switching process.

Asynchronous replication timing of designated loci appears to be present from the ES-cell stage throughout development in all cell-division cycles[4]. Here, we asked the key question of how this state is initially set up from early preimplantation embryonic cells, in which these loci replicate synchronously. Our studies demonstrate that during the first replication cycle, when ES cells are released from their ground state, they initiate asynchronous replication and always choose the paternal allele to replicate early. These findings are reminiscent of the X chromosome in early female embryos that always undergoes inactivation on the paternal early-replicating allele in extraembryonic tissues[26,27].

It should be noted that each individual chromosome may harbor multiple asynchronously replicating domains, but several studies indicate that these domains are regulated in a coordinated manner, so that in each cell, all of these regions replicate early on one chromosomal copy and replicate late on the other chromosomal copy[6,7,28,29]. Each chromosome evidently operates independently, so that some chromosome pairs are maternal early, whereas others are paternal early in any given cell. According to our model for allelic choice, probes on chromosomes 4, 6 and 12 are initially set to be paternal early and then alternate to maternal early during the next division cycle, but it is not yet known how other chromosomes initiate their first asynchronous replication cycle. It is possible that initially, some chromosomes activate the paternal allele early, and others activate the maternal allele early. Alternatively, initiation of asynchronous replication may not occur on all chromosomes during the first replication cycle.

In light of the observation that over 10% of the genes in higher diploid organisms may be expressed monoallelically[30,31], one of the dilemmas of molecular biology is to figure out how cells choose between two alleles in a binary manner. In the case of imprinting, this problem is solved by the fact that the two alleles can be chosen and marked separately in the haploid gametes and then maintained in this manner throughout development[2,22]. However, for random asynchronous replication, the choice of one allele in the early embryo must take place in cells that contain both chromosomal copies. Several models have been suggested for explaining allelic choice, and all are based on the idea that there must be some line of communication between the two alleles, either via a system of *trans*-acting factors that provides feedback from one allele to the other[32–34] or by physical juxtaposition of both alleles at the critical moment in the cell cycle when the decision must be made[35–39]. One problem with the *trans*-acting-factor concept is that it requires a precise range of factor concentrations in order to operate both efficiently and effectively[33,40,41].

We have uncovered evidence for a brand-new concept that could explain allelic choice and that has the potential to resolve this problem in a general manner. Our data suggest that the choice of one allele to replicate early and the other to replicate late is actually carried out by using preset imprinted markers that are already present on the two alleles. It is for this reason that when ES cells progress from their ground state, in which the alleles are synchronous, they always choose one parental paternal allele over the other. This mechanism does not require communication between alleles and can be carried out by recognition of markers *in cis*. This finding represents a breakthrough in our understanding of allelic choice.

To explain how asynchronous replication timing always designates a specific parental allele to be early, it must be assumed that all chromosomes in the early embryo carry a mark for 'remembering' their parental origin[42,43]. These marks certainly exist at many known imprinting centers, but it is also possible that additional signs are set up at other regions, perhaps specifically designated to this purpose[44–46]. In the case of imprinted genes, there is a sophisticated mechanism for preserving allele-specific methylation in the early embryo[42]. The fact that allelic choice is abolished in TKO cells might suggest that DNA methylation is indeed a key component that marks parental alleles, but additional work is required to identify the relevant molecular components that direct random allelic choice.

Asynchronous replication timing represents a basic aspect of genome structure that is programmed into the very fabric of early development and is intimately associated with allelic choice, particularly in the immune system[5]. We have shown that in HSCs, the *Igk* locus is in a switching mode and completely plastic in the choice of allele, but following differentiation to common lymphoid precursor cells (CLPs), replication timing becomes clonal and, in parallel, B-cell progeny are restricted to choosing a single specific allele. Thus, asynchronous replication plasticity in HSCs appears to be a critical component of adaptive immunity.

We propose the following model for the programming of allele selection throughout development. Many monoallelically expressed genes are embedded in asynchronously replicating domains[41]. These regions are originally set up in the embryo to be early replicating on one allele and late replicating on the other, and this process sets up a differential epigenetic mark to distinguish between the two alleles. Once established, differential replication timing itself will be maintained through each cell division. Initially, in ES cells, parent-specific timing undergoes switching following each cell generation, and this process continues in adult stem cells. During differentiation, however, the asynchronous replication pattern becomes clonal in a number of different cell types[5].

Allelic choice at other gene regions, such as those containing olfactory or NK receptors[41], may be based on this same principle, with asynchronous replication timing established very early in embryogenesis, serving as a stable marker throughout development that can then instruct monoallelic choice specifically in the cell type of expression. Thus, this system may represent a general fundamental mechanism governing allelic choice.

METHODS

Methods, including statements of data availability and any associated accession codes and references, are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

H.M. and M.F. designed and conducted the experiments, interpreted the results and assisted in manuscript preparation. M.H. validated the replication timing data by ReTiSH analysis. R.C. generated the mammospheres, K.M. and Y. Buganim generated ES lines 11 and 12 and assisted in generating the EpiSCs, T.B.-C. generated the neurospheres and assisted in designing methods for the other spheroids. H.C. and Y. Bergman directed the study and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Mice. LN3 ($V\beta1^{NT/NT}$) mice[14,47] containing the preassembled $V\beta1D\beta1J\beta1.4C\beta1$ gene were crossed with WT mice, yielding the LN3 ($V\beta1^{NT/+}$) mice. Cells isolated from these mice harbor a WT *Tcrb* allele as well as a preassembled allele that contains a large deletion resulting from the rearrangement process. Mice were housed and cared for under specific-pathogen-free conditions, and all animal procedures were approved by the Animal Care and Use Committee of the Hebrew University of Jerusalem.

Cell lines. ES cells (LN3, LN11, LN12 and J1 and its derived TKO)[24] were cultured without feeders on 0.2%-gelatin-coated plates in DMEM supplemented with 15% inactivated FCS (HyClone), 1 mM L-glutamine, 100 U/ml penicillin, 100 μg/mL streptomycin, 0.1 mM β-mercaptoethanol, 1× nonessential amino acids and homemade leukemia inhibiting factor (LIF). For 2i conditions, MEK inhibitor PD0325901 (1 mM) and GSK3 inhibitor CHIR99021 (3 mM)[23] were added. ES cells were also grown in N2B27 media (50% DMEM F12, 50% neurobasal medium, 1× N2 supplement (Invitrogen 17502048), 1× B27 supplement (Invitrogen 17504044), 2% FBS, 1 mM L-glutamine, 1× nonessential amino acids, 0.1 mM β-mercaptoethanol, 100 U/ml penicillin, 100 μg/mL streptomycin, 5 μg/ml BSA and homemade LIF with or without 2i. BiPS[17] were cultured on feeders using regular ES media containing leukemia LIF with or without 2i.

Generation of ES lines 11 and 12 (LN11, LN12). An LN3 ($V\beta1^{NT/NT}$) male mouse was crossed with a WT female to produce LN3 ($V\beta1^{NT/+}$) blastocysts from which ES cells were derived, as described in ref. 48. These ES lines were expanded on feeders using the N2B27 media supplemented with LIF.

All cell lines were tested and found to be negative for mycoplasma.

Neuronal stem cells. Neuronal stem cells isolation and neurosphere culturing were performed as previously described[15,49]. Briefly, LN3 ($V\beta1^{NT/NT}$) mice were mated with WT to generate E14.5 embryos. The telencephalic cortices were dissected from each embryo and kept on ice. CNS tissue was first diced using aggressive pipetting and digested with trypsin for 15 min at 37 °C. Trypsin inhibitor was then added for 2 min. The suspension was treated with DNaseI and then centrifuged. The pellet was resuspended in 1 ml media and filtered through a 70-mm mesh to get a single-cell suspension. Cells were then counted and seeded at a density of 150,000 cells/ml to 300,000 cells/ml in 10-cm tissue culture plates containing growth media (DMEM F12, B27 without vitamin A (Gibco 12587-010), 1 mM L-glutamine, 100 U/ml of penicillin, 100/μg ml streptomycin, 10 μg/ml Human-FGF). Every 24–30 h, the same amount of human-FGF was added to the media until nice floating neurospheres could be detected under the microscope. Single neurospheres were picked up separately under the microscope and dissociated into single cells using either 5 mM EDTA or NeuroCult Dissociation Solution supplied with NeuroCult Enzymatic Dissociation Kit (STEMCELL Technologies). Cells from each neurosphere were then fixed for FISH analysis.

Mammosphere formation. Primary mammospheres were isolated and cultured as previously described[50–52]. Briefly, mammary glands were dissected from 8-week-old female LN3 ($V\beta1^{NT/+}$) mice. After mechanical dissociation, the tissue was cut into small pieces and placed in culture medium (DMEM, 1 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin , 10% FBS (FBS) , 300 U/ml collagenase and 100 U/ml hyaluronidase , 0.1 mg/ml DNaseI) and digested for 1.5–2 h at 37 °C in a shaker at about 180 r.p.m. The resultant organoid suspension was sequentially resuspended in trypsin-EDTA for 3 min along with aggressive pipetting and brought to 0.64% NH_4Cl for 3 min at room temperature before filtration through a 40 mm mesh to obtain a single-cell suspension. Cells were counted and seeded in a flat-bottom low-affinity-binding 96-well plate. About 150–250 cells were seeded per well in Epicult-B medium (EB supplements, 5% FBS, 10 ng/ml hEGF, 5 μM Y-27632 (rho inhibitor), 20 ng/ml βFGF and 4 μl/ml heparin) containing 5% Matrigel. Single mammospheres were picked separately under a microscope. Each mammosphere was dissociated into single cells using 5 mM EDTA or with 1× TrypLE solution (Gibco). Cells from each mammosphere were then fixed for FISH analysis. Mammospheres were stained using immunofluorescent antibodies to distinguish between luminal and basal types[16,53].

Replication-timing FISH analysis. Cells were pulse labeled for 1 h with BrdU and isolated nuclei were then fixed with methanol/acetic acid (3:1). After denaturation and dehydration, slides were hybridized with labeled probes detecting the genes of interest. BrdU was detected by an anti-BrdU antibody (NeoMarkers) followed by rhodamine-labeled anti-mouse antibody (Jackson Laboratories). Replication timing profiles (percent single–single, single–double and double–double) were determined by counting 100–300 BrdU-positive nuclei for each sample[4,21]. FISH analysis was performed using rhodamine to label artificial chromosome BAC_RP24-297J5 that specifically recognizes a large region deleted on the prerearranged *Tcrb* allele on chromosome 6 (refs. 14,47) or BAC_RP23-262I5, which specifically recognizes a large region deleted on the pre-rearranged *Igh* allele on chromosome 12 (VHQ52-DJH (BiPS 6.3) / VHJ558-DJH (BiPS 1.3))[17] and FITC-labeled BACs containing *Igk* (RP24-387E13), the olfactory receptor cluster 453, 454 and 38 (RP23-62H2) or *Nanog* (RP23-117I23) located on chromosome 6 and FITC-labeled BACs containing *Igh* distal V genes (RP24-76I22) located on chromosome 12. For chromosome 4, we used the olfactory receptor probes (RP23-387N12, RP23-398A1). In some cases we used *Igh* Bac (chromosome 12 - RP23-375J21), which recognizes both alleles in our ES LN3$^{(NT/+)}$ cells as another asynchronous replicating marker.

ReTiSH. ReTiSH analysis was performed on interphase nuclei labeled as described with BrdU[21]. Cells were harvested and treated with hypotonic KCl solution (0.5 M) for 10 min at room temperature, washed with methanol-glacial acetic acid (3:1), dropped gently on superfrost-plus slides and air dried. Slides were then washed once with PBST (0.1% Triton X-100 in PBS), fixed again with 4% formaldehyde in PBS for 10 min, washed in PBST (three time, 5 min each), and treated with pepsin (1 mg/ml in 2N HCl) or with RNase A (2 mg/Ml in water) for 20 min at 37 °C. After an additional 5-min wash with PBST, slides were treated with Hoechst 33258 (0.5 μg/μl) for 15 min at room temperature, exposed to 365 nm UV light for 20 min using a UV Stratalinker 2400 transilluminator (Stratagene), washed again in 2× SSC and dried. Digestion by ExoIII at 3.2 units/μl (Promega) was carried out in the manufacturer-supplied buffer for 30 min at 37 °C, and the slides were then washed in PBST for 5 min, fixed for 2 min in 4% formaldehyde in PBS and washed three times for 5 min each in PBST. Slides were dehydrated through a series of gradual transition in 20% formamide/2× SSC, 40% formamide/2× SSC and 50% formamide/2× SSC. Samples were incubated sequentially for a minimum of 10 min each. The hybridization mixture containing 50% deiodinized formamide, 10% dextran sulfate in SCC X2 and 0.5 μg labeled probe was added to the samples, and hybridization was carried out at 37 °C overnight. Following hybridization, samples were washed once in 50% formamide/2× SSC, once in 25% formamide/2× SSC, and three times in 2× SSC. They were then mounted in VECTASHIELD (Vector) and mixed with DAPI, and images were recorded using confocal microscopy (Nikon).

In order to assay replication time switching, we measured the cell-cycle dynamics of ES cells and on this basis chose the time intervals for early- or late-specific BrdU labeling. Nuclei were isolated after two rounds of label and subjected to ReTiSH. Only those cells that showed single or single–double signals were counted. In parallel, for each experiment we confirmed that over 75% of the nuclei had a single signal when labeled for only one round of BrdU in either early or late S, thus insuring that the procedure is indeed cell-cycle specific.

Replication timing by S-phase fractionation. ES cells were analyzed for their replication timing by S-phase fractionation, as previously described[4]. Briefly, cells were pulse labeled in BrdU before collection, and nuclei were then FACS sorted according to DNA content into G1, S1-S5, G2 fractions. To each fraction, BrdU-labeled *Drosophila* DNA (S2 cells) was added as an internal IP control. BrdU DNA was isolated by immunoprecipitation (IP) from each fraction, and assayed by semiquantitative PCR using primers specific for the *Igk* locus (forward primer: 5′-ATAAAC TGG TCT GAA TCT CTG TCT G-3′ reverse primer: 5′-ACG CCATTTTGTCGT TCACTGCCA-3′). The *Igk* gel was quantified using Image-Studio-lite and normalized to IP internal control of the *Drosophila Gbe* gene: (forward primer: 5′-GCAAAGCCTAACGTGCACTCGTGGGAC-3′, reverse primer 5′-CGTGACAAGCTTTTAGAGCCACAGACGCACTTG-3′). α-globin and amylase were used as controls for early and late replication, respectively (α-globin forward primer: 5′-AAGGGGAGCAGAGGCATCA-3′, reverse primer: 5′-AGGGCTTGGGAGGGACTG-3′. Amylase forward primer: 5′-AGCACTGAGGATTCAGTCTATG-3′, reverse primer: 5′-CCCGTACA AGGAGAATTACAAC-3′). All others primers for qPCR analysis are listed in **Supplementary Table 1**.

CRISPR technology. We introduced a large deletion into the *Tcrb* gene on one allele of chromosome 6, using two gRNAs to target both sides of the region of interest: Right deletion (Forward: CACCGACACATTAGAAAAGTACTG; Reverse: AAACCAGTACTTTTCTAATGTGTC), Left deletion (Forward: CAC CGGGGGTAGTCACCTCCAACA; Reverse: AAACTGTTGGAGGTGACTACC CCC). We cloned the gRNAs into a pX330-based plasmid following the Addgene target-sequence cloning protocol. The plasmid was cotransfected with a zeocin-selectable plasmid into the cells using the Mirus TransIT-LT1 reagent (Invitrogen). Single-cell clones were picked following selection and were verified using specific primers; Left deletion, primer A- forward 5′-TCTCAGCCAGAGGGACCTTA-3′ and primer B reverse 5′-CATTTCCACTCTGCCTCCAT-3′ to detect the wt allele, or the same left deletion primer A with the left deletion primer 5′-TAAAATGGCCTGACCATCAGTT-3′ to detect the deleted allele.

Statistics. Statistical analysis was performed with either the exact two-tail binomial test or the exact Kruskal–Wallis test, as indicated. Differences with P values <0.05 were considered significant.

Data availability. All data supporting the findings of this study are available from the corresponding author upon reasonable request. A **Life Sciences Reporting Summary** for this article is available.

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Experimental design

1. Sample size

Describe how sample size was determined.	The exact sample size (n) for each experimental group was given as a number. All qPCR experiments were performed 3 times (3 independent experiments). All FISH and ReTISH experiments were done with different independent clones, and the exact number of counted cells per each experiment is noted in the text.

2. Data exclusions

Describe any data exclusions.	Data was excluded when probes were not appropriately labeled.

3. Replication

Describe whether the experimental findings were reliably reproduced.	The experimental findings were reliably reproduced. Moreover, each experiment (FISH and ReTISH) included several cell lines and numerous probes to ensure reproducibility.

4. Randomization

Describe how samples/organisms/participants were allocated into experimental groups.	NA

5. Blinding

Describe whether the investigators were blinded to group allocation during data collection and/or analysis.	All FISH and ReTISH experiments were documented by two investigators. Frequently, one of them was blinded to group allocation.

Note: all studies involving animals and/or human research participants must disclose whether blinding and randomization were used.

6. Statistical parameters

For all figures and tables that use statistical methods, confirm that the following items are present in relevant figure legends (or in the Methods section if additional space is needed).

n/a	Confirmed	
☐	☒	The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement (animals, litters, cultures, etc.)
☒	☐	A description of how samples were collected, noting whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
☒	☐	A statement indicating how many times each experiment was replicated
☐	☒	The statistical test(s) used and whether they are one- or two-sided (note: only common tests should be described solely by name; more complex techniques should be described in the Methods section)
☐	☒	A description of any assumptions or corrections, such as an adjustment for multiple comparisons
☐	☒	The test results (e.g. *P* values) given as exact values whenever possible and with confidence intervals noted
☐	☒	A clear description of statistics including central tendency (e.g. median, mean) and variation (e.g. standard deviation, interquartile range)
☐	☒	Clearly defined error bars

See the web collection on statistics for biologists for further resources and guidance.

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Software

Policy information about availability of computer code

7. Software

Describe the software used to analyze the data in this study.	Images were recorded with confocal microscopy (Nikon), using the NIS-Elements software. Real-time PCR was analyzed with CFX-manager software (Biorad).

For manuscripts utilizing custom algorithms or software that are central to the paper but not yet described in the published literature, software must be made available to editors and reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). *Nature Methods* guidance for providing algorithms and software for publication provides further information on this topic.

Materials and reagents

Policy information about availability of materials

8. Materials availability

Indicate whether there are restrictions on availability of unique materials or if these materials are only available for distribution by a for-profit company.	No unique materials were used.

9. Antibodies

Describe the antibodies used and how they were validated for use in the system under study (i.e. assay and species).	Anti-BrdU antibody (NeoMarkers) MS-1058-P. Rhodamin labeled anti-mouse antibody (Life Technologies) R6393. Anti CK-5 antibody (Abcam) ab52635. Anti CK-8 antibody (Progen) GP-K8.

10. Eukaryotic cell lines

a. State the source of each eukaryotic cell line used.	Murine cell lines.
b. Describe the method of cell line authentication used.	TKO cells were validated by RRBs, BiPS cells were validated by PCR for IgH rearrangement, LN3 cells were validated by PCR and by FISH for the expected deletions. LN11,12 and IIAC2 cells were validated by FISH for the expected deletion.
c. Report whether the cell lines were tested for mycoplasma contamination.	All lines were routinely tested for Mycoplasma (EZ-PCR, Biological industries cat #20-700-20).
d. If any of the cell lines used are listed in the database of commonly misidentified cell lines maintained by ICLAC, provide a scientific rationale for their use.	No commonly misidentified cells were used.

Animals and human research participants

Policy information about studies involving animals; when reporting animal research, follow the ARRIVE guidelines

11. Description of research animals

Provide details on animals and/or animal-derived materials used in the study.	NA

Policy information about studies involving human research participants

12. Description of human research participants

Describe the covariate-relevant population characteristics of the human research participants.	NA

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