Supplemental material

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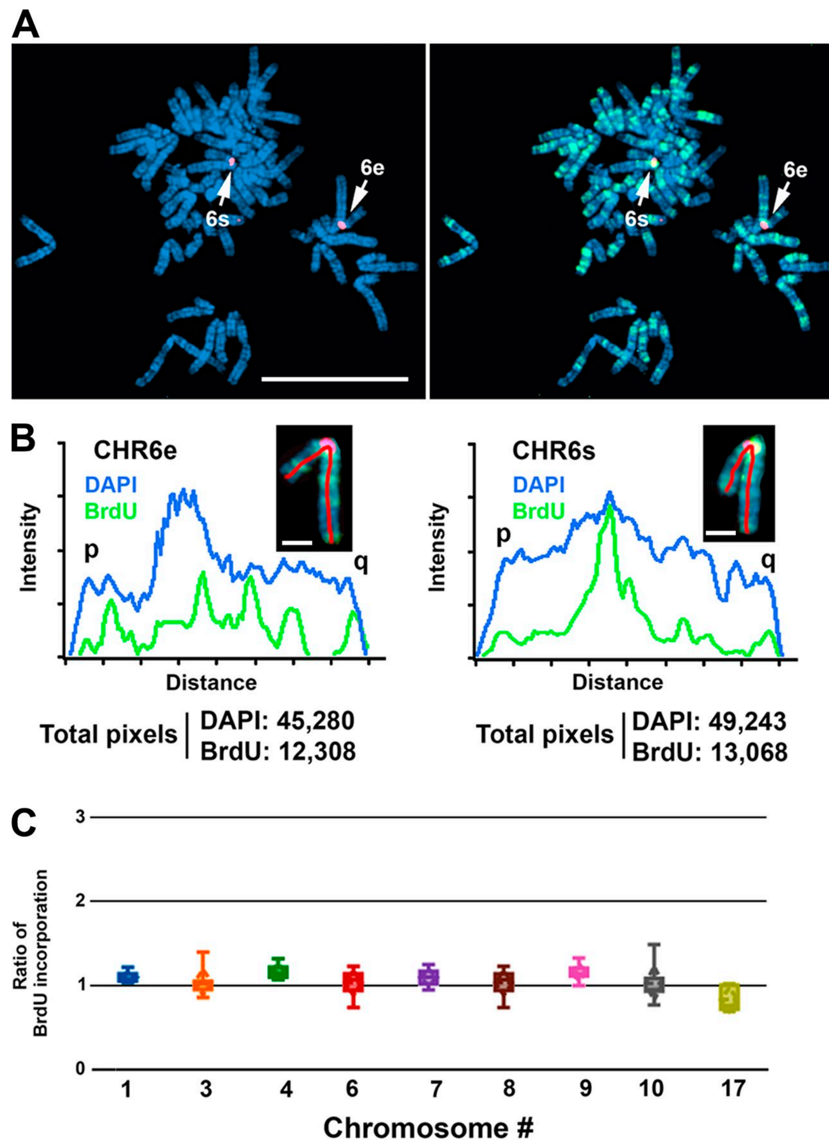
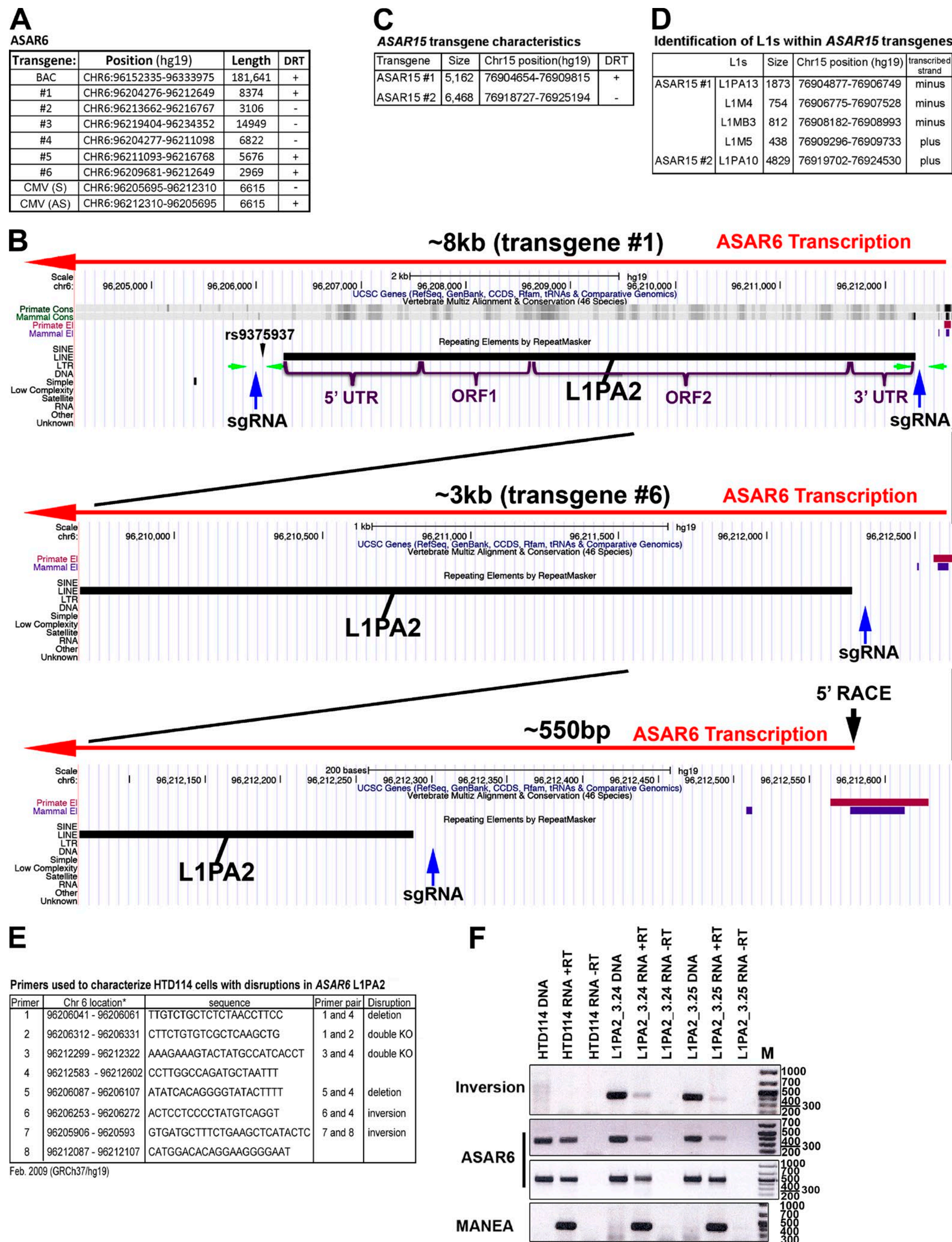


Figure S1. **Replication timing analysis of parental HTD114 cells.** HTD114 cells were exposed to BrdU and harvested, and mitotic cells were assessed for BrdU incorporation. **(A)** Left: Mitotic spread processed for DNA FISH with a chromosome 6 centromeric probe (red) and counterstained with DAPI (blue). Right: The same cell visualized for BrdU incorporation (green). Arrows mark the centromeres of the chromosome 6 homologues with either the silent *ASAR6* allele (6s, smaller signal) or expressed *ASAR6* allele (6e, larger signal). Bar, 10 µm. **(B)** Quantification of BrdU incorporation in chromosome 6. Left: Expressed chromosome 6e. Right: Silent chromosome 6s. BrdU incorporation and DAPI staining are shown for each homologue. The total number of pixels were quantified by multiplying the intensity by the area. Bars, 1 µm. **(C)** Replication timing analyses of homologous chromosome pairs in HTD114 cells. During the analysis of the chromosome 6 homologues, which were identified using a centromeric FISH probe, we were also able to measure replication timing of 8 other chromosome pairs (1, 3, 4, 7, 8, 9, 10, and 17) in the same mitotic cells. These additional chromosome pairs were identified using inverted DAPI staining (which essentially results in a G-banded pattern). We were not able to evaluate every chromosome in this set of mitotic cells because of chromosomes being crossed or our inability to unambiguously identify certain chromosomes with inverted DAPI. The ratio of BrdU incorporation in homologous chromosomes was visualized using a box plot and shown in C; the means are shown as horizontal dotted lines, medians as solid lines, and SDs as diagonal dotted lines. Values near 1 indicate that homologous chromosomes replicated synchronously.



A

ASAR6

Transgene:	Position (hg19)	Length	DRT
BAC	CHR6:96152335-96333975	181,641	+
#1	CHR6:96204276-96212649	8374	+
#2	CHR6:96213662-96216767	3106	-
#3	CHR6:96219404-96234352	14949	-
#4	CHR6:96204277-96211098	6822	-
#5	CHR6:96211093-96216768	5676	+
#6	CHR6:96209681-96212649	2969	+
CMV (S)	CHR6:96205695-96212310	6615	-
CMV (AS)	CHR6:96212310-96205695	6615	+

C

ASAR15 transgene characteristics

Transgene	Size	Chr15 position(hg19)	DRT
ASAR15 #1	5,162	76904654-76909815	+
ASAR15 #2	6,468	76918727-76925194	-

D

Identification of L1s within *ASAR15* transgenes

	L1s	Size	Chr15 position (hg19)	transcribed strand
ASAR15 #1	L1PA13	1873	76904877-76906749	minus
	L1M4	754	76906775-76907528	minus
	L1MB3	812	76908182-76908993	minus
	L1M5	438	76909296-76909733	plus
ASAR15 #2	L1PA10	4829	76919702-76924530	plus

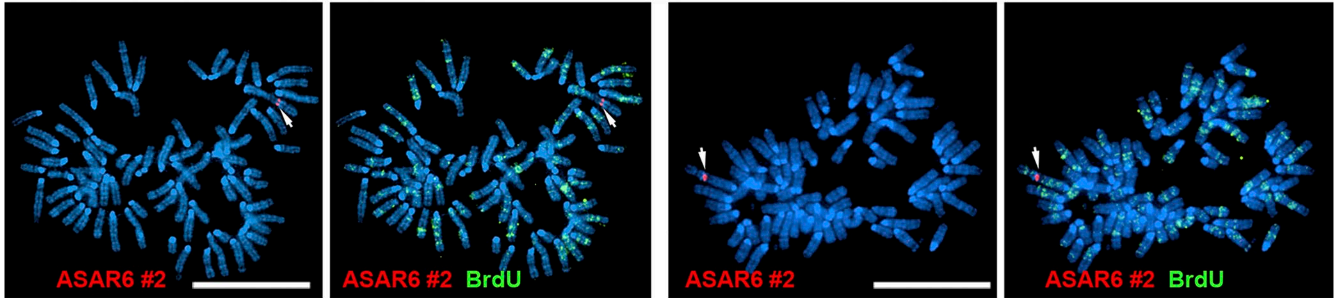
E

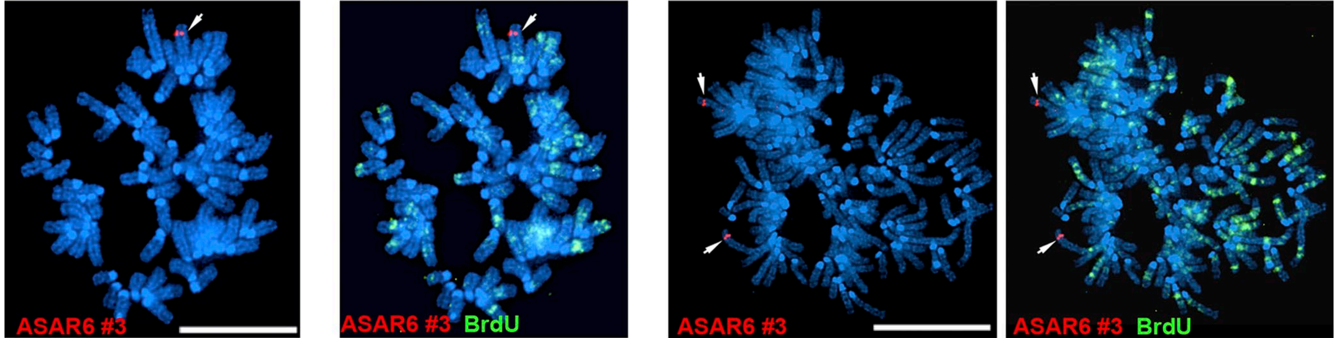
Primers used to characterize HTD114 cells with disruptions in *ASAR6* L1PA2

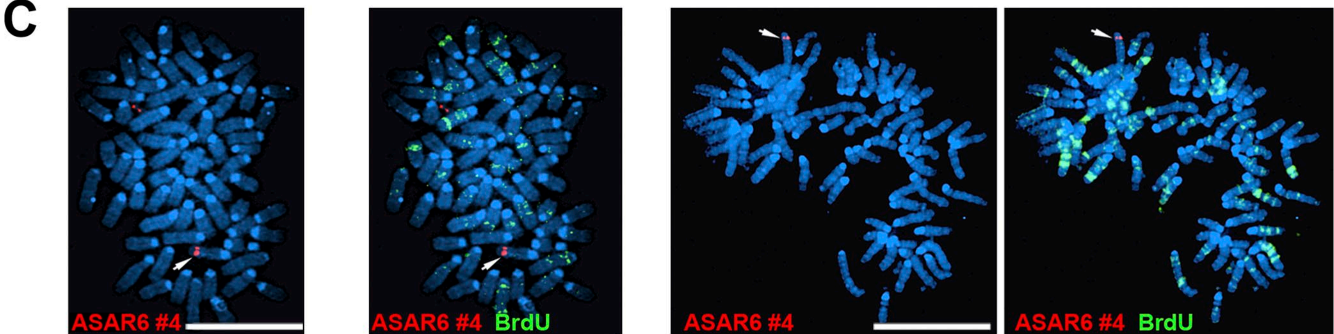
Primer	Chr 6 location*	sequence	Primer pair	Disruption
1	96206041 - 96206061	TTGTCTGCTCTCTAACCTTCC	1 and 4	deletion
2	96206312 - 96206331	CTTCTGTGTCGCTCAAGCTG	1 and 2	double KO
3	96212299 - 96212322	AAAGAAAGTACTATGCCATCACCT	3 and 4	double KO
4	96212583 - 96212602	CCTTGGCCAGATGCTAATTT		
5	96206087 - 96206107	ATATCACAGGGGTATACTTTT	5 and 4	deletion
6	96206253 - 96206272	ACTCCTCCCCTATGTCAGGT	6 and 4	inversion
7	96205906 - 9620593	GTGATGCTTTCTGAAGCTCATACTC	7 and 8	inversion
8	96212087 - 96212107	CATGGACACAGGAAGGGGAAT		

Feb. 2009 (GRCh37/hg19)

Figure S2. ***ASAR6* and *ASAR15* transgene characteristics and location of sgRNAs used to disrupt the *ASAR6* L1PA2. (A)** Location and chromosome replication timing results from mouse cells with integrated *ASAR6* BAC, transgenes 1–6, and CMV-driven L1PA2 in the sense (S) and antisense (AS) orientation. All transgenes in A were derived from the ~29-kb critical region of *ASAR6*. The presence or absence of the DRT phenotype are indicated with + or −, respectively. **(B)** The locations of *ASAR6* transgene 1 and 6 are visualized showing the Conservation and Repeat Masker Tracks on the UCSC Genome Browser. The location of the L1PA2, with the 5′ and 3′ UTRs, and ORF1 and ORF2 are shown. The L1PA2 is 6026 bp in length and lacks 125 bp at the 5′ end within the 5′ UTR and 4 bp at the 3′ end within the 3′ UTR. Both ORF1 and ORF2 contain multiple bp changes that disrupt the protein coding capacity. PCR primers used for screening CRISPR/Cas9-modified cells are indicated by green arrows. sgRNA binding sites are indicated by blue arrows, and the rs9375937 SNP location is indicated by a black arrowhead. The transcriptional start site within the 360 bp of unique sequence in *ASAR6* transgenes 1, 5, and 6 was determined by 5′ RACE (chromosome 6: 96,212,578) and is indicated by a black arrow. E1 conservation is indicated by magenta (primate) and purple (mammal) lines using the UCSC Genome Browser. The direction of *ASAR6* transcription is indicated by the red arrows. **(C)** Position on chromosome 15 of *ASAR15* transgenes 1 and 2. **(D)** Size, position, and transcribed strand of L1s within *ASAR15* transgenes 1 and 2. **(E)** Sequence and position on chromosome 6 of the primers used to characterize L1PA2 deletions and inversions. **(F)** Validation and expression analysis of inversions at the L1PA2. Total RNA isolated from parental cells (HTD114) and the inversion clones L1PA2_3.24 and L1PA2_3.25 was subjected to reverse transcription reactions in the presence (+RT) or absence (−RT) of reverse transcription followed by semiquantitative PCR using primers (7 and 8 in E) to detect the inversion (top). Genomic DNA was used as positive control. Note that parental HTD114 genomic DNA lacks the inversion PCR product. The PCR products were sequenced to confirm the inversions (Table 2). *ASAR6* expression outside the inverted region was also assayed using PCR primers that detected expression 5′ of the inversion (top ASAR6 panel), and 3′ of the inversion (bottom ASAR6 panel). PCR primers that detected MANEA cDNA were used as positive control for the reverse transcription reactions. The MANEA primers fail to detect human DNA because they span a large intron. DNA size ladder was used for size reference (M).







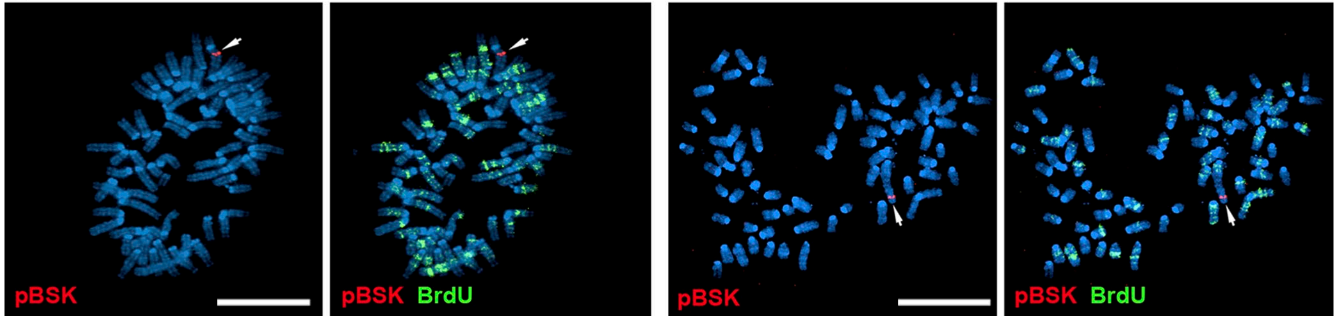


Figure S3. **Replication timing assays on *ASAR6* transgenes.** Cells from independent clones were exposed to BrdU, harvested, and subjected to DNA FISH using *ASAR6* transgene–derived probes. Mitotic cells were analyzed for BrdU incorporation (green) and the presence of the *ASAR6* transgene (red). For each transgene, we assayed replication timing in >100 mitotic cells from a minimum of three independent clones. For *ASAR6* transgenes 2, 3, and 4 and pBSK-containing cells, we failed to detect delayed chromosomes in any mitotic cells in any clones. Representative images of mouse cells containing integrated transgenes are shown. **(A)** Left two panels and right two panels: Representative cells with *ASAR6* transgene 2 integration. **(B)** *ASAR6* transgene 3. **(C)** *ASAR6* transgene 4. **(D)** Empty pBSK vector control. White arrows indicate integrated transgenes or empty pBSK. DNA is stained with DAPI (blue). Bars, 10 µm.

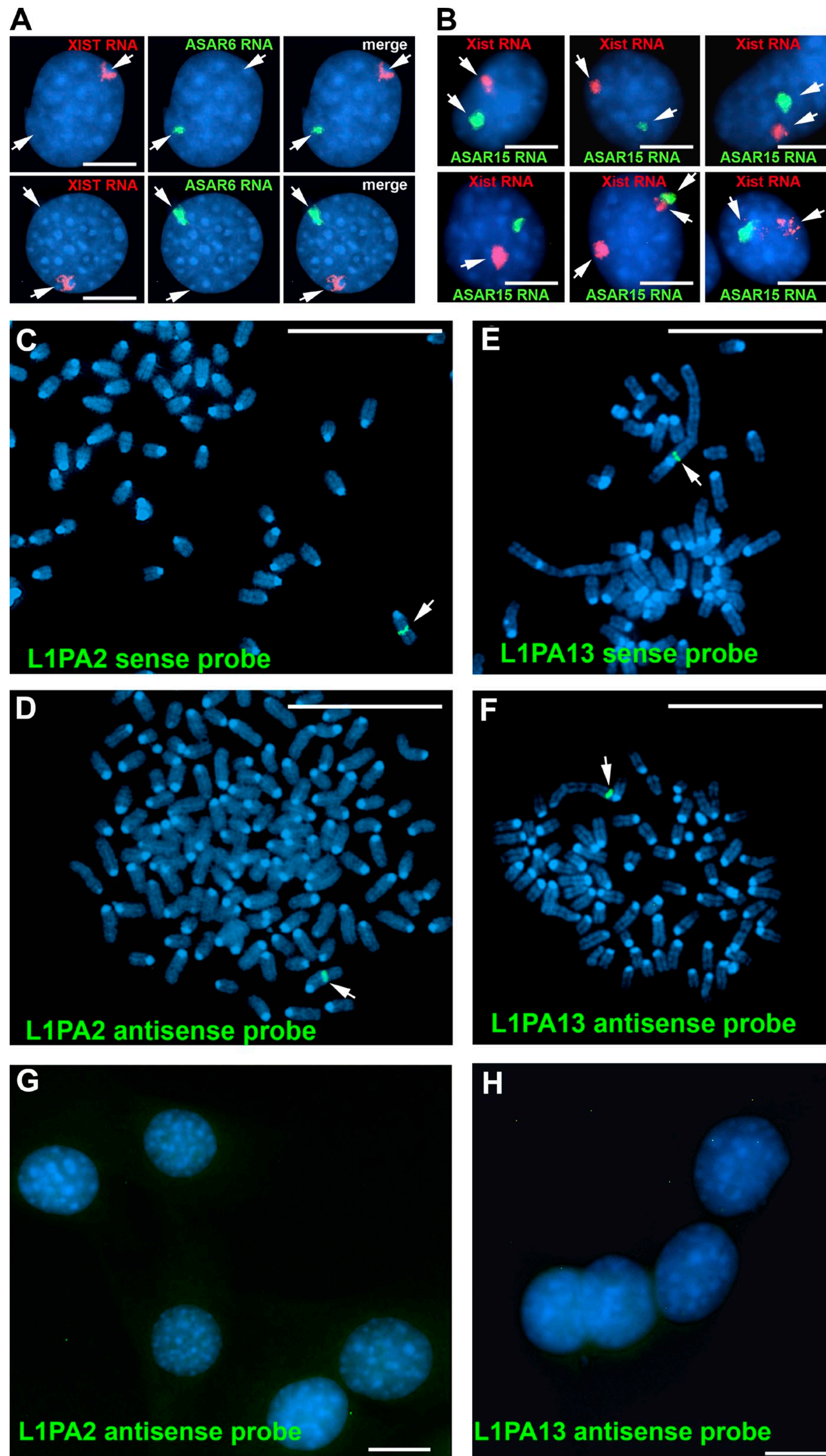


Figure S4. **Visualizing *ASAR* RNA by RNA-FISH. (A)** Comparison of RNA FISH signals expressed from *ASAR6* transgene 1 (middle, green) and Xist (left, red) in two cells (top and bottom rows); panels on the right are merged images. **(B)** Comparison of RNA FISH signals for Xist (red) and ASAR15 (green) in cells containing *ASAR15* transgene 1; each panel (top or bottom) shows independent cells. **(C and D)** Mitotic cells with the *ASAR6* BAC transgene insertion were incubated with the sense (C) or antisense (D) L1PA2 strand-specific probes. **(E and F)** Mitotic cells with an *ASAR15* transgene 1 insertion incubated with the sense (E) or antisense (F) L1PA13 strand-specific probes. Arrows indicate the transgene integration sites revealed by FISH signals (green dots). **(G)** RNA FISH using the antisense strand-specific probe to the L1PA2 on cells expressing the *ASAR6* BAC transgene. Note that G and D were hybridized with the same L1PA2 probe cocktail. **(H)** RNA FISH using the antisense strand-specific probe (green) to the L1PA13 on cells containing *ASAR15* transgene 1–expressing cells. Note that F and H were hybridized with the same L1PA13 probe cocktail. DNA was stained with DAPI (blue). Bars, 10 µm.

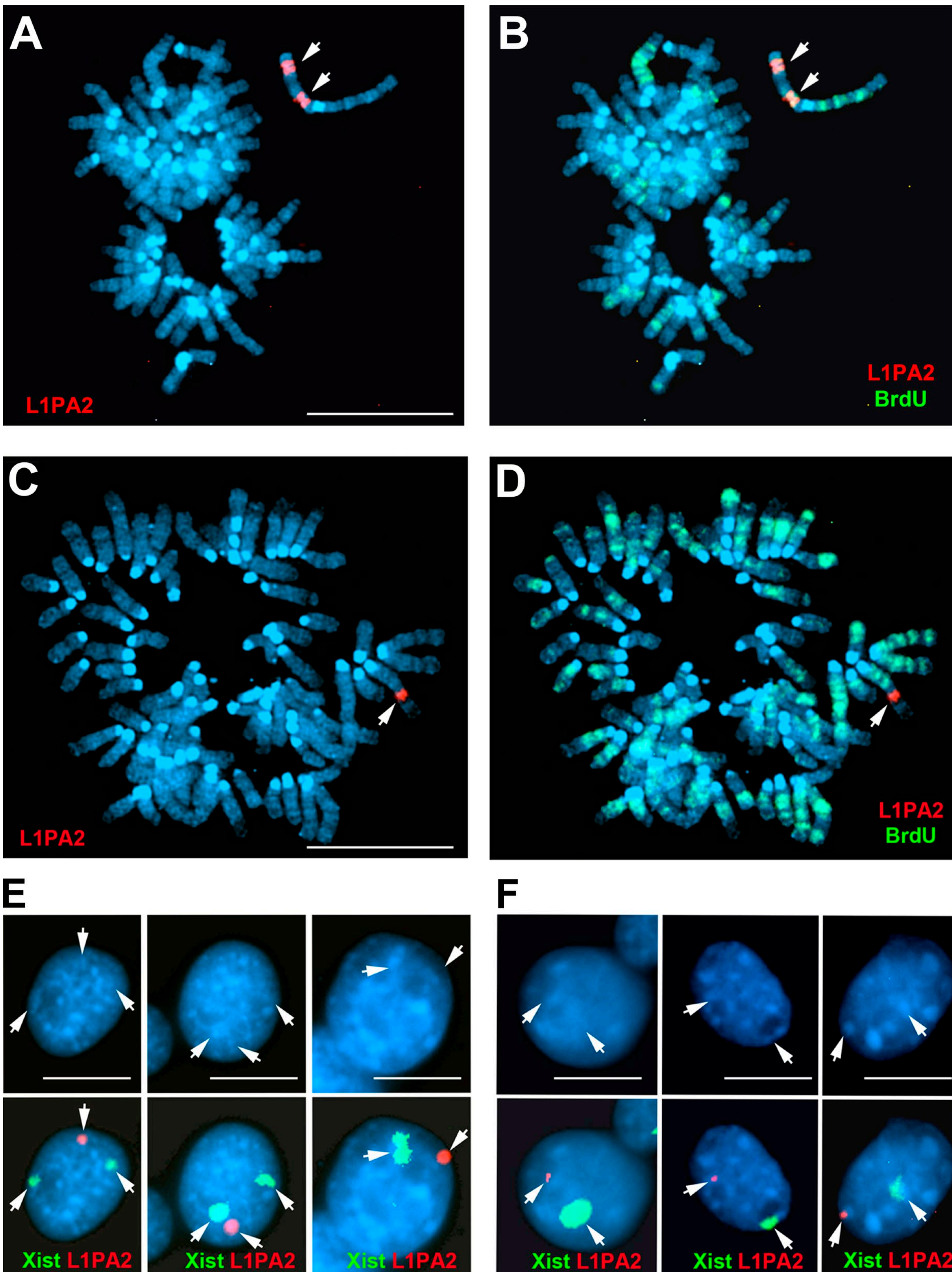


Figure S5. **Replication timing and RNA FISH assays on CMV-L1PA2 transgene–containing cells.** Mouse cells with integrated transgenes containing the critical L1PA2 in either orientation with respect to a CMV promoter were exposed to BrdU, harvested, and subjected to DNA FISH using *ASAR6* transgene–derived probes. Mitotic cells were analyzed for BrdU incorporation and the presence of the *ASAR6* transgene. **(A and B)** A representative cell containing a transgene expressing the L1PA2 expressed in the antisense orientation. **(A)** DNA FISH using the CMV-L1PA2 transgene as probe (red). The DNA was stained with DAPI. **(B)** The same cell as in A showing BrdU incorporation (green). **(C and D)** A representative cell containing a transgene expressing the L1PA2 expressed in the sense orientation. **(C)** DNA FISH using the CMV-L1PA2 transgene as probe (red). **(D)** The same cell as in C showing BrdU incorporation (green). **(A–D)** White arrows indicate integrated transgenes. DNA is stained with DAPI (blue). **(E and F)** Mouse cells containing CMV-L1PA2 transgene insertions were subjected to RNA FISH with probes that detected expression of the L1PA2 (red) and mouse Xist RNA (green). The DNA was stained with DAPI. The top and bottom panels show the same nuclei for two different cells. Arrows indicate the sites with RNA FISH signals. Bars, 10 µm.