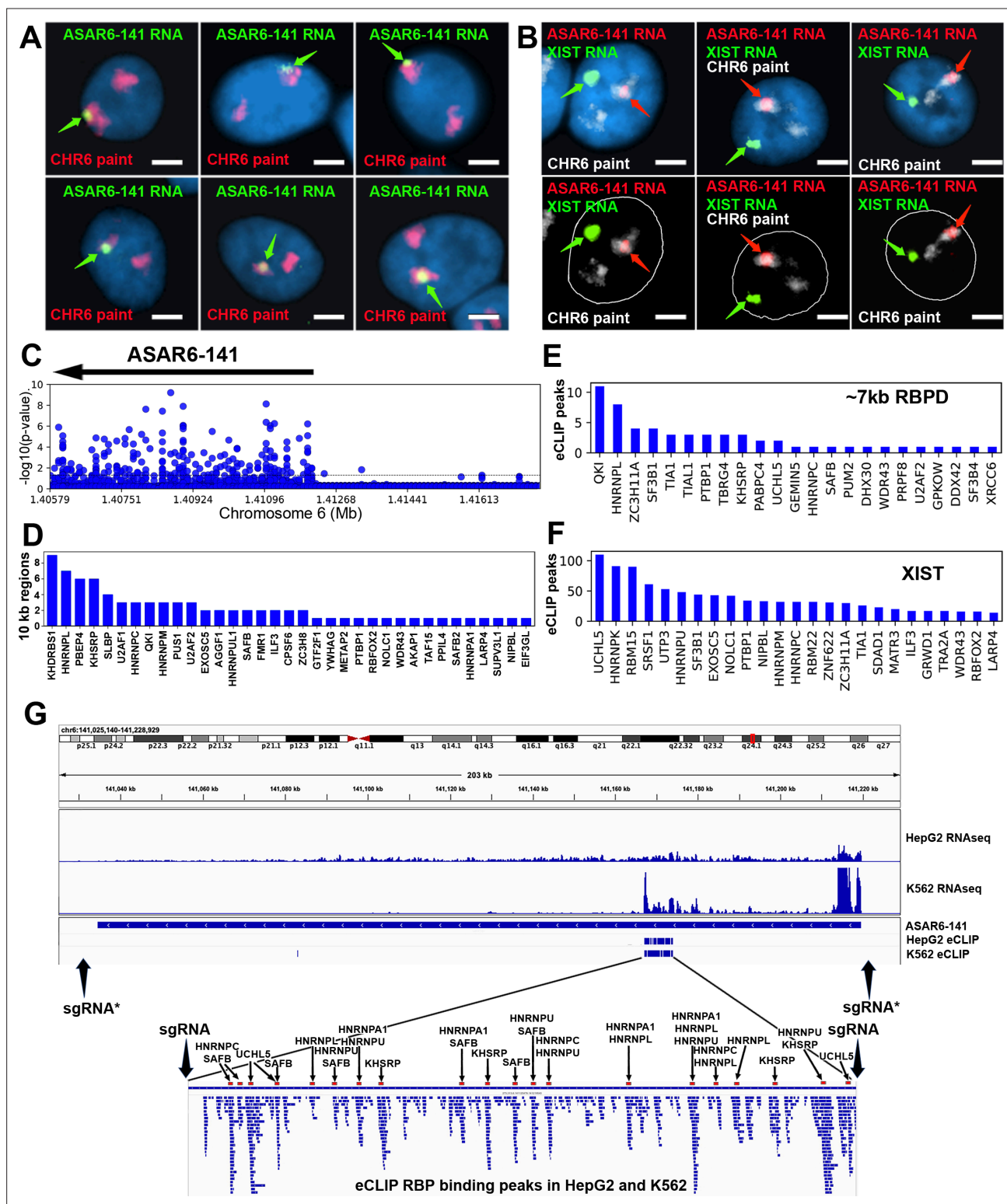


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## Figures and figure supplements

ASAR lncRNAs control DNA replication timing through interactions with multiple hnRNP/RNA binding proteins

**Mathew Thayer and Michael B Heskett *et al.***

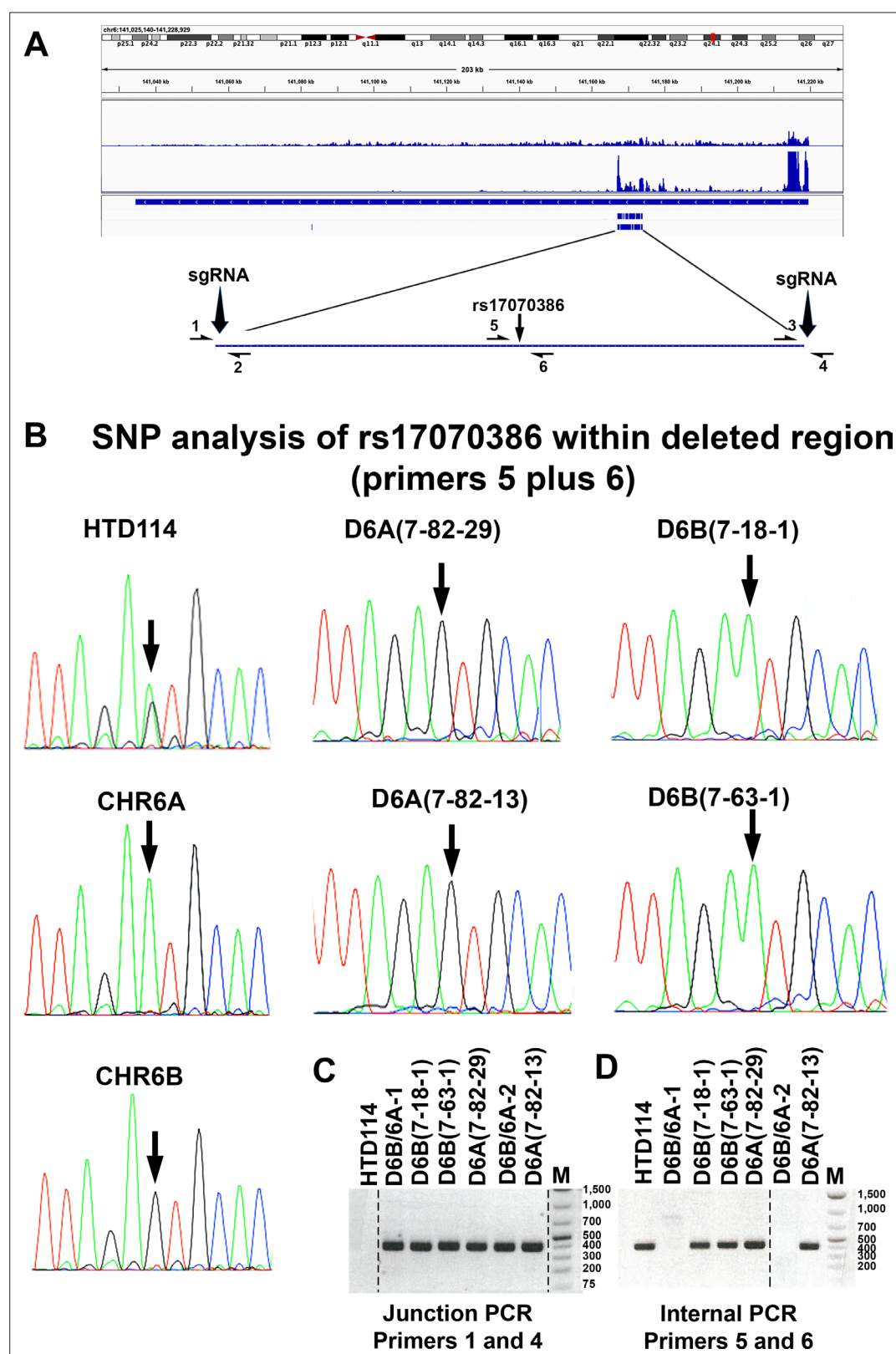


**Figure 1.** RNA binding proteins interact with an ~7 kb domain within ASAR6-141 RNA. **(A)** RNA-DNA FISH images of ASAR6-141 expression in six individual HTD114 cells. ASAR6-141 RNA (green; green arrows) and chromosome 6 DNA (chromosome paint, red), and DNA was stained with DAPI. **(B)** RNA-DNA FISH of ASAR6-141 RNA (red, red arrows), CHR6 DNA (chromosome paint, white), and XIST RNA (green, green arrows) visualized within individual GM12878 cells, top and bottom panels represent the same three cells with the nuclear outline drawn in white. **(C)** RNA-protein interaction data (eCLIP from ENCODE) for 120 RBPs expressed in K562 cells for the genomic region that contains ASAR6-141, and ~200 kb of upstream non-transcribed DNA (chromosome 6 140.8 mb-141.6 mb). Each point represents the FDR-corrected p-value of the z-score of log<sub>2</sub>-ratio between eCLIP vs

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*Figure 1 continued*

control for each RBP within a 10 kb sliding window. **(D)** Number of 10 kb regions with significant enrichments of eCLiP reads vs control within ASAR6-141. **(E)** Histogram of eCLiP peaks count per RBP within ASAR6-141 7 kb RBP. **(F)** Histogram of eCLiP peaks count per RBP within lncRNA XIST. **(G)** Genome browser view of ASAR6-141 with RNA-seq expression and eCLiP peaks shown in K562 and HepG2. The zoomed in view shows the ~7 kb RBP with the location of the peaks of eCLIP reads that map within the region (see **Figure 1—source data 1**). The eCLIP peaks for the RBPs used in the shRNA knockdown experiments are indicated and highlighted with arrows and red bars. The location of sgRNAs are shown with arrows (see **Figure 1—source data 3**), and the asterisks mark the sgRNAs from **Heskett et al., 2020**.



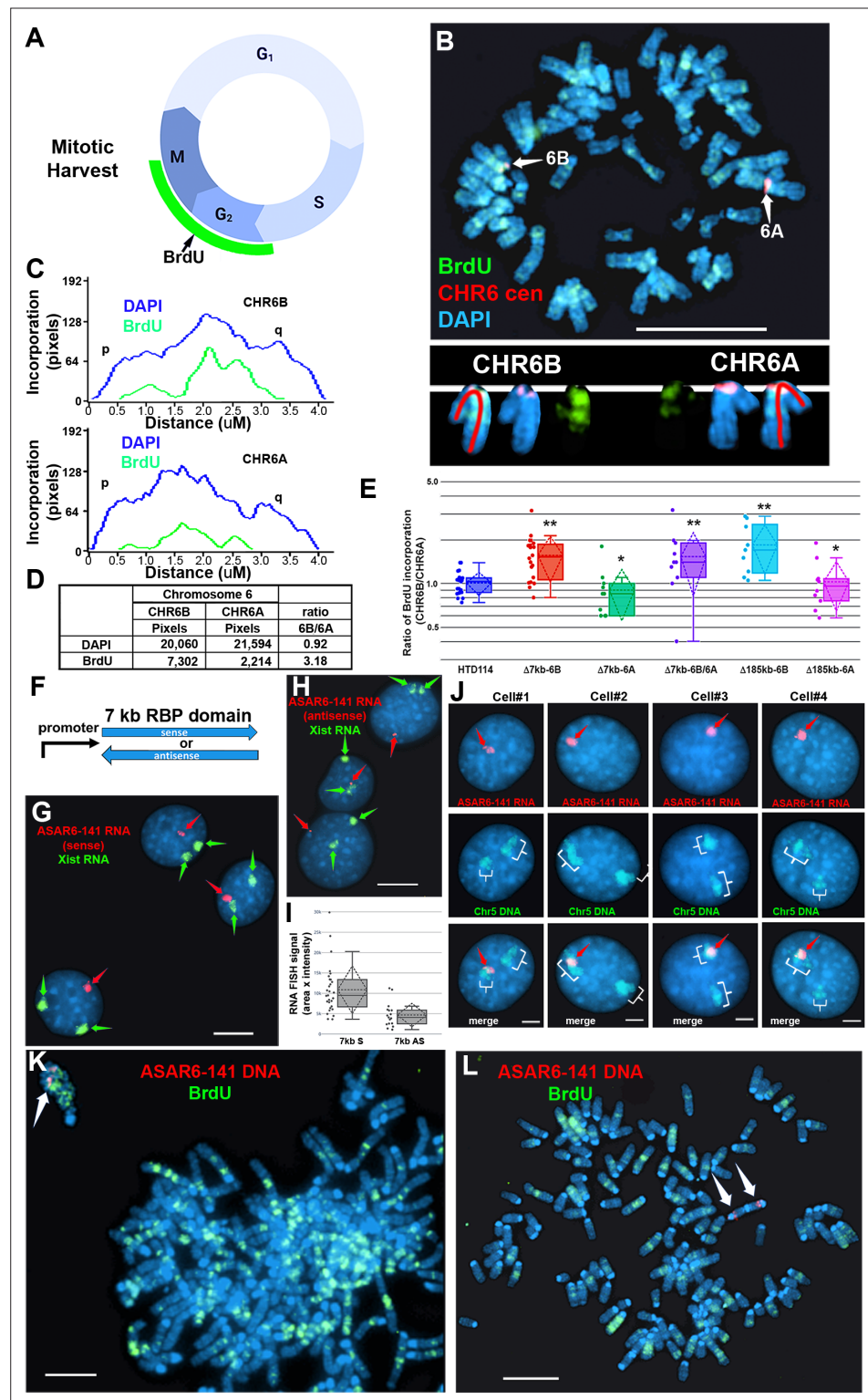
**Figure 1—figure supplement 1.** CRISPR/Cas9 deletion of the ~7 kb RBDP. (A) Integrative Genomics Viewer view showing the genomic location of ASAR6-141. The genomic locations of RNAseq and eCLIP reads from HepG2 and K562 (from ENCODE) are shown. The zoomed in view shows the ~7 kb RBDP with the location where all of the peaks of eCLIP reads map within the region (see **Figure 1—source data 1**). The location of sgRNAs

Figure 1—figure supplement 1 continued on next page



*Figure 1—figure supplement 1 continued*

that flank the ~7 kb RBDP are shown. The location of PCR primers (1-6) and the heterozygous SNP rs17070386 are also shown (see **Figure 1—source data 3**). **(B)** Sanger sequencing traces on PCR products generated from genomic DNA using primers 5 and 6 (see **Figure 1—source data 3**). In previous studies we generated mouse monochromosomal hybrid cells containing the two chromosome 6 homologs from HTD114 (**Stoffregen et al., 2011**), and sequencing traces from these two hybrids, contain either chromosome 6 A (CHR6A) or chromosome 6B (CHR6B) are shown below the HTD114 panel. Sequencing traces from PCR products generated from 4 heterozygous deletions, two with CHR6A deletions {D6A(7-82-29) and D6A(7-82-13)} and two with CHR6B deletions {D6B(7-18-1) and D6B(7-63-1)} are shown. **(C)** Junction PCR (primers 1 and 4) on genomic DNA isolated from heterozygous {D6A(7-82-29), D6A(7-82-13), D6B(7-18-1) and D6B(7-63-1)} or homozygous deletions {D6B/6 A-1 and D6B/6 A-2} are shown. Parental HTD114 DNA was used as control. Size markers are in base pairs. **(D)** Internal PCR products (primers 5 and 6) on genomic DNA isolated from heterozygous {D6A(7-82-29), D6A(7-82-13), D6B(7-18-1) and D6B(7-63-1)} or homozygous deletions {D6B/6 A-1 and D6B/6 A-2} are shown. Parental HTD114 DNA was used as control. Size markers are in base pairs.

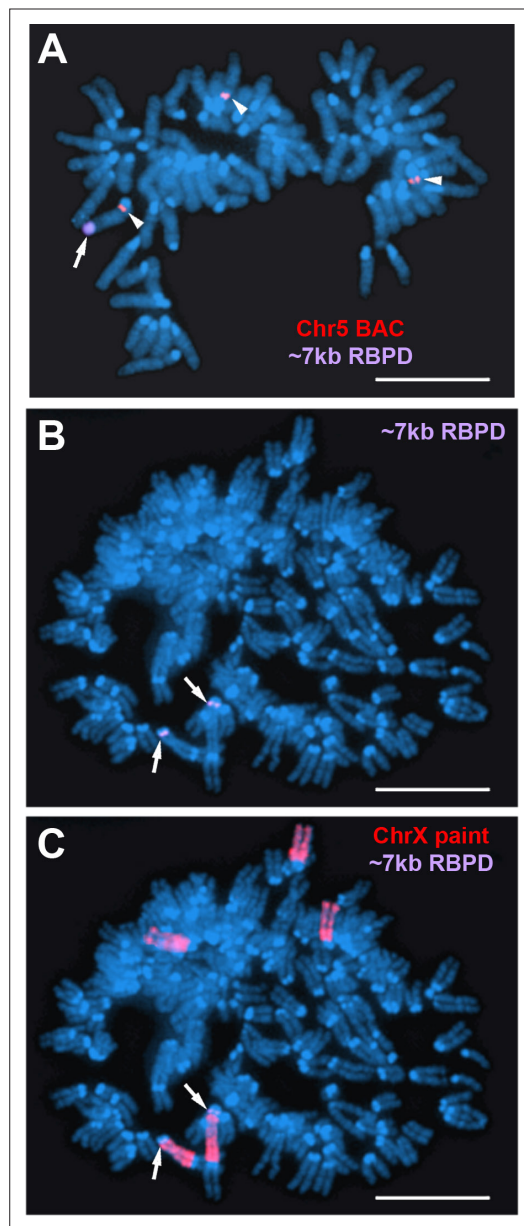


**Figure 2.** Replication timing in cells with ASAR6-141~7 kb RBP deletion or ectopic integration. **(A)** Schematic illustration of the BrdU terminal label protocol (*Smith and Thayer, 2012*). Cells were treated with BrdU (green) for 5 hr and then harvested for mitotic cells. **(B)** BrdU incorporation in HTD114 cells containing a heterozygous deletion of the ~7 kb RBP. Cells containing a deletion of the ~7 kb RBP from the expressed allele of ASAR6-141 were exposed to BrdU, harvested for mitotic cells, and subjected to DNA FISH using a chromosome 6 centromeric probe (red; **B**). The larger centromere resides on the chromosome 6 with the silent ASAR6-141 allele (CHR6A),

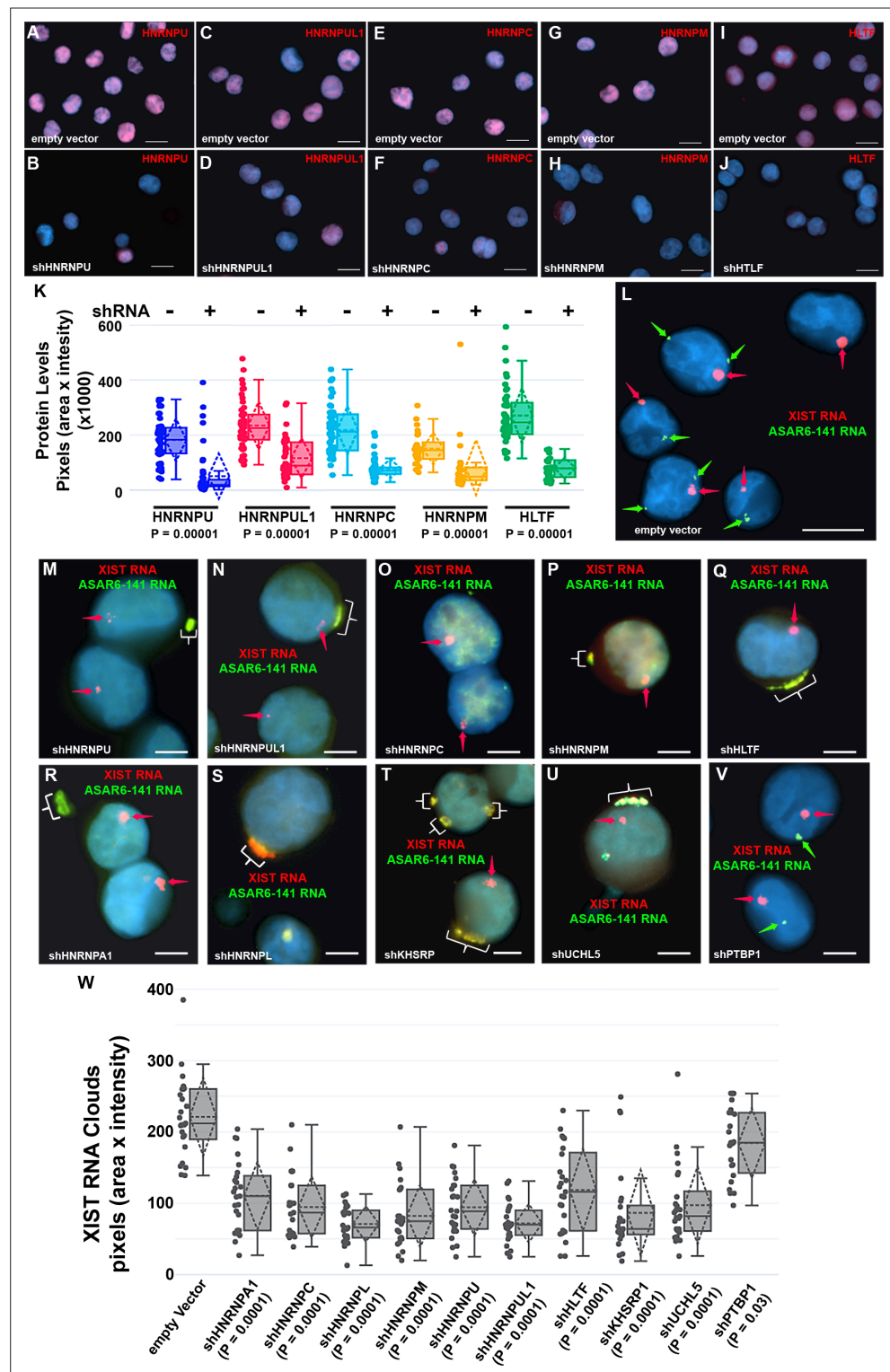
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# Figure 2 continued

and the smaller centromere resides on the chromosome 6 with the expressed ASAR6-141 allele (CHR6B). DNA was stained with DAPI (blue). **(C and D)** DAPI staining and BrdU incorporation were quantified by calculating the number of pixels (area x intensity) and displayed as a ratio of BrdU incorporation in CHR6B divided by the BrdU incorporation in CHR6A. **(E)** Quantification of BrdU incorporation in multiple cells with heterozygous ( $\Delta 7\text{kb-6B}$ , expressed allele; or  $\Delta 7\text{kb-6A}$ , silent allele) or homozygous ( $\Delta 7\text{kb-6B/6A}$ ) deletions of the  $\sim 7$  kb RBP. Also shown is the quantification of BrdU incorporation in heterozygous deletions of the entire  $\sim 185$  kb ASAR6-141 gene from chromosome 6B ( $\Delta 185-6B$ ) or 6A ( $\Delta 185-6A$ ) (Heskett et al., 2020). Box plots indicate mean (solid line), standard deviation (dotted line), 25th, 75th percentile (box) and 5th and 95th percentile (whiskers) and individual cells (single points). p Values were calculated using the Kruskal-Wallis test (Kruskal, 1964). Values for a minimum of 10 individual cells are shown as dots.  $**p=0.00027$  and  $*p=0.03$ . **(F)** Schematic view of the  $\sim 7$  kb RBP in both the sense and antisense orientation. The promoter used to drive expression is from ASAR6<sup>9</sup>. **(G and H)** Two color RNA FISH assay for expression of the  $\sim 7$  kb RBP transgenes. Individual clones were screened for RNA expressed from the ASAR6-141 ( $\sim 7$  kb RBP) transgenes (red, arrows), and RNA hybridization using an Xist probe (green, arrows) was used as positive control. **(G and H)** represent examples of cells containing the sense and antisense transgenes, respectively. **(I)** Quantitation of the size (pixels = area x intensity) of the RNA FISH signals expressed from the 7 kb sense (7 kb S) or antisense (7 kb AS) transgenes are shown. Box plots indicate mean (solid line), standard deviation (dotted line), 25th, 75th percentile (box) and 5th and 95th percentile (whiskers) and a minimum of 10 individual cells (single points) are shown. p Value of 0.006 was calculated using the Kruskal-Wallis test (Kruskal, 1964). Values for individual cells are shown as dots. **(J)** RNA from the  $\sim 7$  kb RBP remains localized to mouse chromosomes. RNA-DNA FISH using the  $\sim 7$  kb RBP as RNA FISH probe (red, arrows), plus a mouse chromosome 5 paint to detect chromosome 5 DNA (green, brackets). **(K)** Cells containing the sense  $\sim 7$  kb RBP transgene integrated into mouse chromosome 5 were exposed to BrdU (green), harvested for mitotic cells, and subjected to DNA FISH using the  $\sim 7$  kb RBP (red, arrows). Note that the chromosome that contains the transgene shows delayed mitotic condensation and more BrdU incorporation than any other chromosome within the same cell. **(L)** Cells containing the antisense  $\sim 7$  kb RBP transgene integrated into a mouse chromosome were exposed to BrdU (green), harvested for mitotic cells, and subjected to DNA FISH using the  $\sim 7$  kb RBP (red). Note that the chromosome that contains the transgene shows normal mitotic condensation and only a small amount of BrdU incorporation. Scale bars are 10  $\mu\text{M}$  (**B, G, H**) 5  $\mu\text{M}$  (**K, L**) and 2  $\mu\text{M}$  (**J**).



**Figure 2—figure supplement 1.** Identification of mouse chromosomes containing the ~7 kb RBPB transgenes. **(A)** DNA FISH on mouse chromosomes from clone ~7 kb(+)A5. Mitotic spread showing DNA FISH using the ~7 kb RBPB (magenta; arrow) and a mouse chromosome 5 BAC (RP24-107D17; red; arrowheads). **(B and C)** DNA FISH on mouse chromosomes from clone ~7 kb(+)X1. Mitotic spread showing DNA FISH using the ~7 kb RBPB (magenta; arrows) and a mouse X chromosome paint (red). Scale bars are 10uM.

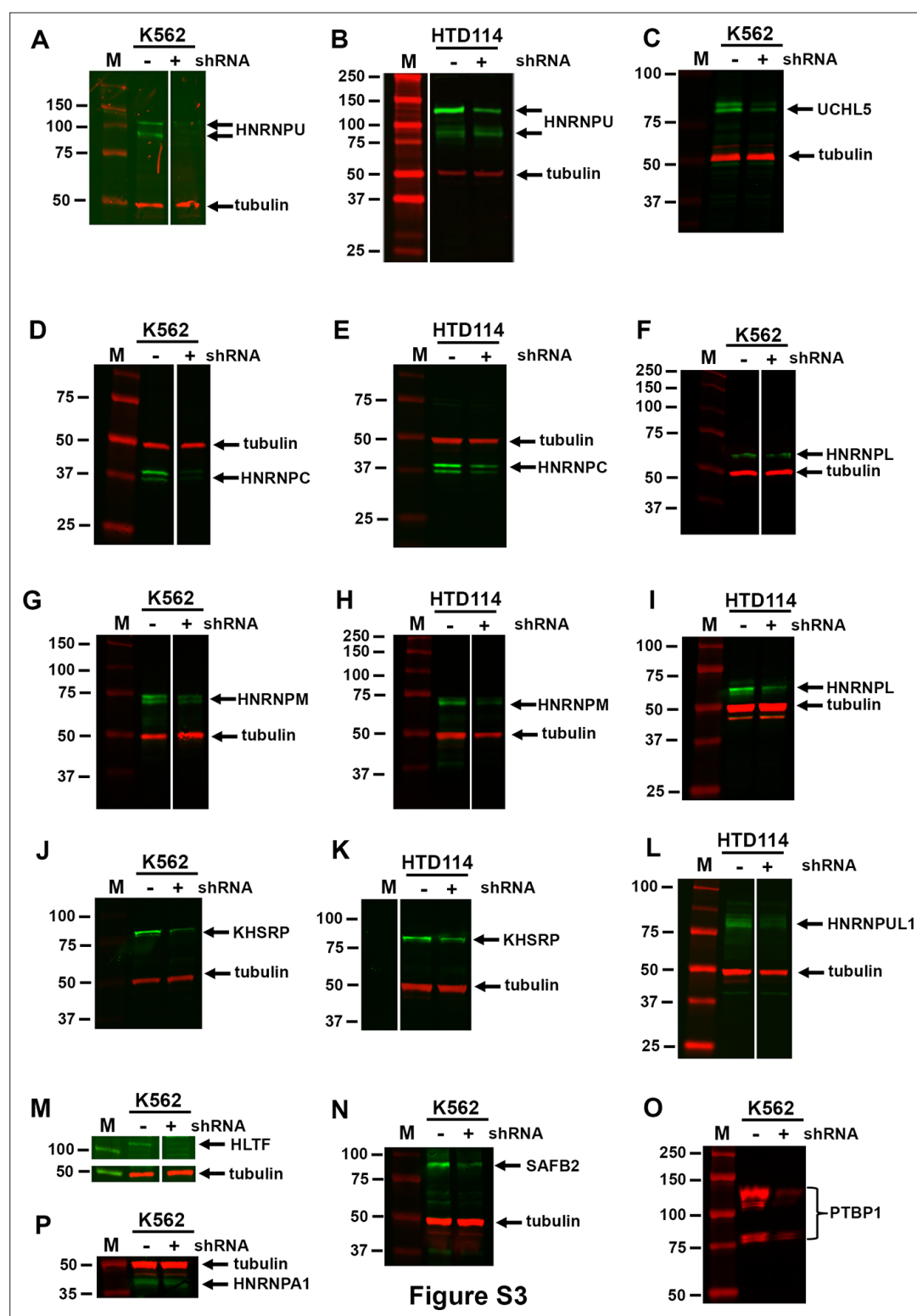


**Figure 3.** Depletion of RBPs results in disruption of the chromosome territory localization of ASAR6-141 RNA. (A–J) shRNA mediated depletion of RBPs. K562 cells were transfected with empty vector (A, C, E, G, and I) or vectors expressing shRNAs directed against *HNRNPU* (B), *HNRNPUL1* (D), *HNRNPC* (F), *HNRNPM* (H), or *HLTF* (J). (K) Cells were stained with the appropriate antibodies and quantitation of each RBP was determined in >25 individual cells. Box plots indicate mean (solid line), standard deviation (dotted line), 25th, 75th percentile (box), 5th and 95th percentile (whiskers), and individual cells (single points) are indicated. p Values were calculated using the Kruskal-Wallis test (Kruskal, 1964). (L–V) RNA-DNA FISH for ASAR6-141 (green) and XIST (red) RNA. K562 cells were

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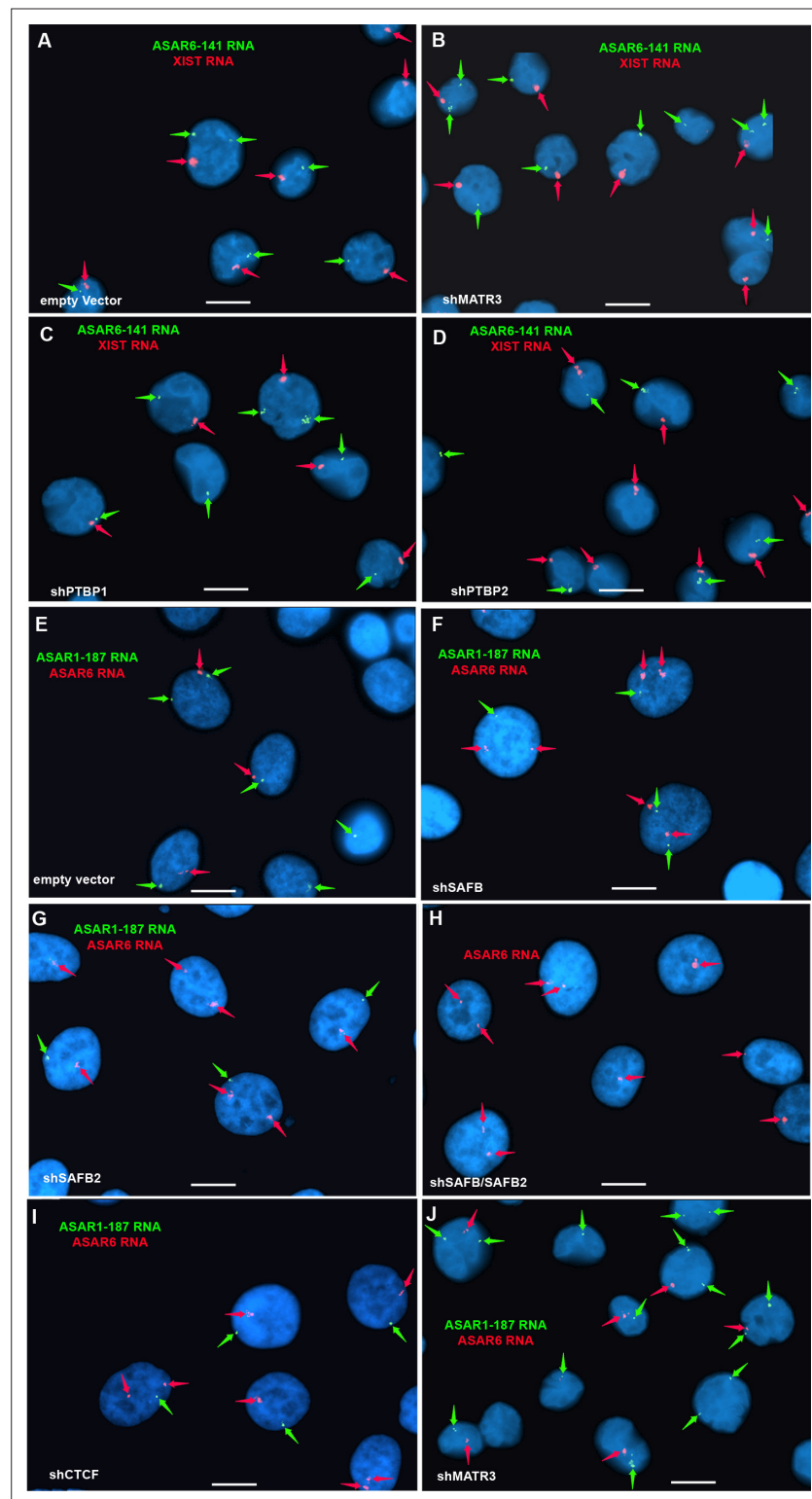
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transfected with empty vector (**L**) or vectors expressing shRNAs against *HNRNPU* (**M**), *HNRNPUL1* (**N**), *HNRNPC* (**O**), *HNRNPM* (**P**), *HLTF* (**Q**), *HNRNPA1* (**R**), *HNRNPL* (**S**), *KHSRP* (**T**), *UCL5* (**U**), or *PTBP1* (**V**). The red arrows mark the RNA FISH signals for XIST. The brackets mark cytoplasmic regions that hybridized to both RNA FISH probes. DNA was stained with DAPI, and Bars are 10  $\mu$ M (**A–J and L**) or 5  $\mu$ M (**M–V**). (**W**) Quantitation of the XIST RNA FISH signals. K562 cells transfected and processed for RNA FISH as in L–V were analyzed for quantitation of XIST RNA cloud size (pixels: area  $\times$  intensity) in  $>25$  individual cells. Box plots indicate mean (solid line), standard deviation (dotted line), 25th, 75th percentile (box), 5th and 95th percentile (whiskers) and individual cells (single points). p Values were calculated using the Kruskal-Wallis test (**Kruskal, 1964**).



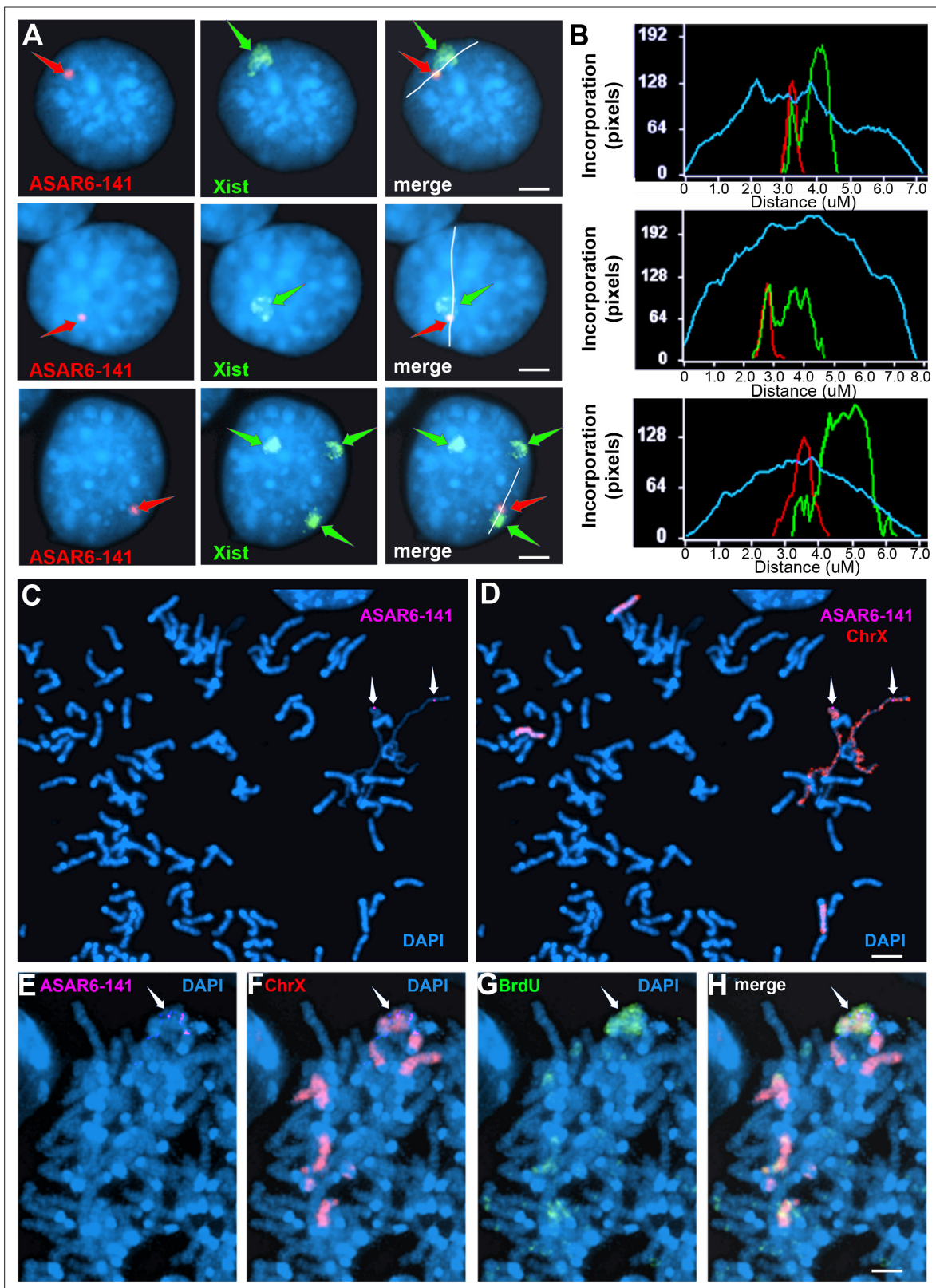
**Figure 3—figure supplement 1.** RBP protein levels in cells expressing shRNAs. K562 or HTD114 cells were transfected with empty vector (-) or shRNA against RBPs and cell extracts were processed for western blots using antibodies (Key Resource Table) as indicated: (A and B) HNRNPU (green); (C) UCHL5 (green); (D and E) HNRNPC (green); (F and I) HNRNPL (green); (G and H) HNRNPM (green); (J and K) KHSRP (green); (L) HNRNPUL1 (green); (M) HLTF (green); (N) SAFB2 (green); (O) PTBP1 (red); or (P) HNRNPA1 (green). Tubulin (red) was used as loading control, and size markers are indicated.





**Figure 3—figure supplement 2.** RNA FISH on cells transfected with shRNAs that did not disrupt ASAR localization. (A–D) K562 cells were transfected with empty vector (A) or shRNA expression vectors for *MATR3* (B), *PTBP1* (C), or *PTBP2* (D). Cells were processed for RNA FISH using probes for ASAR6-141 (green; arrows) or *XIST* (red; arrows). (E–J) HTD114 cells were transfected with empty vector (E) or shRNA expression vectors for *SAFB* (F), *SAFB2* (G), *SAFB* plus *SAFB2* (H), *CTCF* (assayed for only ASAR6 RNA) (I), or *MATR3* (J). DNA was stained with DAPI, and scale bars are 10 μm.



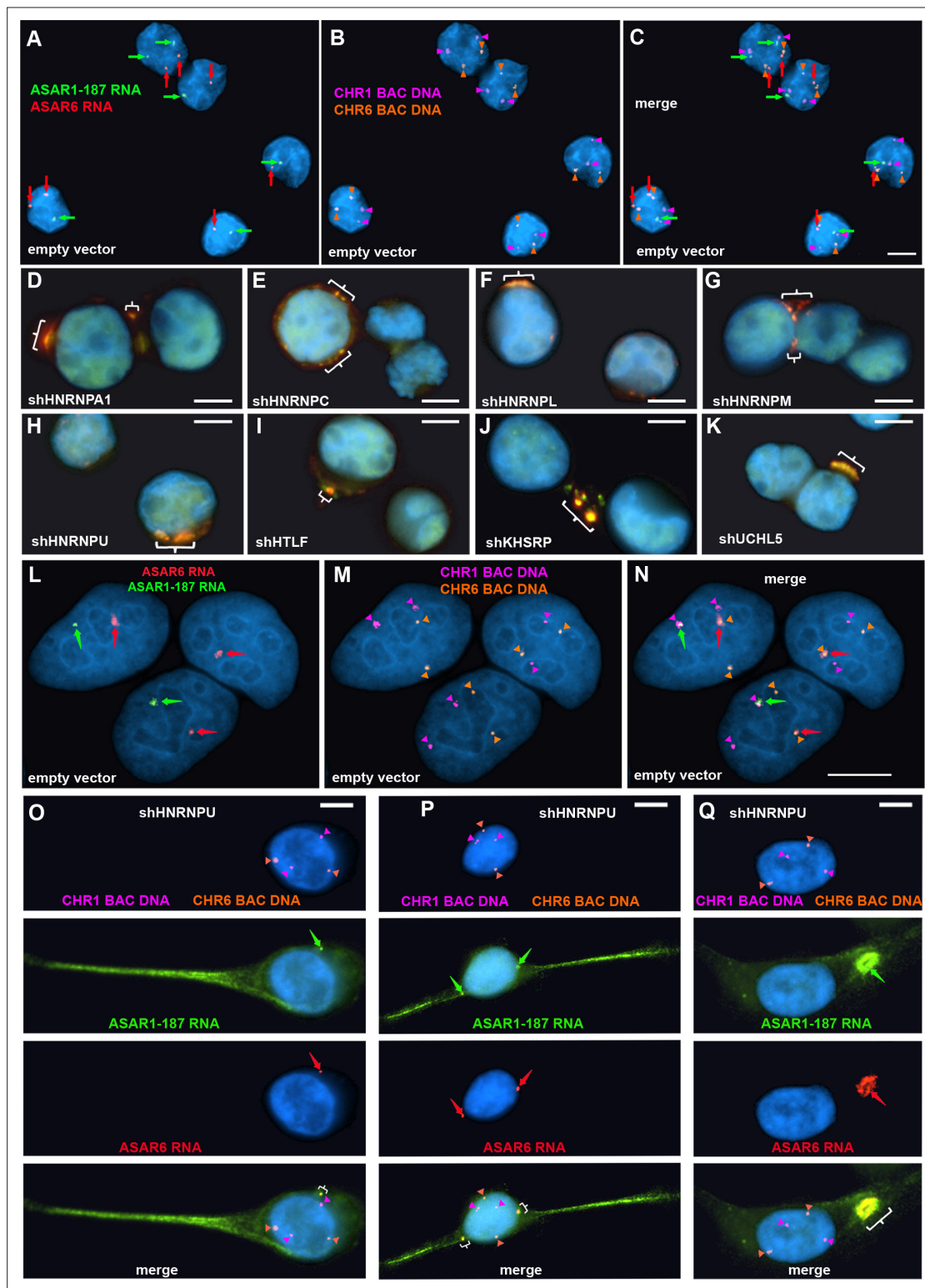


**Figure 4.** Ectopic integration of the ASAR6–141~7 kb RBPD transgene into an inactive X chromosome. **(A)** Two color RNA FISH assay for expression of the sense ~7 kb RBPD transgene plus Xist RNA. Examples of three different cells showing colocalization of the ASAR RNA and Xist RNA. Individual cells were processed for expression of the sense strand ~7 kb RBPD transgene (left panels, red, arrow) in combination with RNA FISH for Xist (middle panels, green, arrow). The right panels show the merged images. The white lines show the path for the pixel intensity profiles used for panel B. **(B)** Pixel intensity profiles for the three cells. The y-axis is 'Incorporation (pixels)' and the x-axis is 'Distance (uM)'. The profiles show peaks corresponding to the ASAR6-141 (red) and Xist (green) signals. **(C)** ASAR6-141 (magenta) and DAPI (blue) FISH images. White arrows point to the ASAR6-141 signal. **(D)** ASAR6-141 (magenta), ChrX (red), and DAPI (blue) FISH images. White arrows point to the ASAR6-141 signal. **(E)** ASAR6-141 (magenta) and DAPI (blue) FISH images. White arrows point to the ASAR6-141 signal. **(F)** ChrX (red) and DAPI (blue) FISH images. White arrows point to the ChrX signal. **(G)** BrdU (green) and DAPI (blue) FISH images. White arrows point to the BrdU signal. **(H)** Merge of ASAR6-141 (magenta), ChrX (red), BrdU (green), and DAPI (blue) FISH images. White arrows point to the ASAR6-141 signal.

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*Figure 4 continued*

intensity profiles across the *Xist* RNA hybridization domain showing enhanced signal intensity over the ~7 kb RBP RNA hybridization domain. **(C and D)** Delayed mitotic chromosome condensation of the X chromosome that contains the ~7 kb RBP transgene. An example of a mitotic spread processed for DNA FISH using the ~7 kb RBP as probe (magenta, panel C) plus a mouse X chromosome paint (red, panel D). The arrows mark the location of the transgene hybridization signal. **(E–H)** Cells containing the ~7 kb RBP transgene integrated into the mouse X chromosome were exposed to BrdU (G, green), harvested for mitotic cells, and subjected to DNA FISH using the ~7 kb RBP (E, magenta) plus a mouse X chromosome paint (F, red). The merged images are shown in panel H. Scale bars are 2  $\mu$ M.

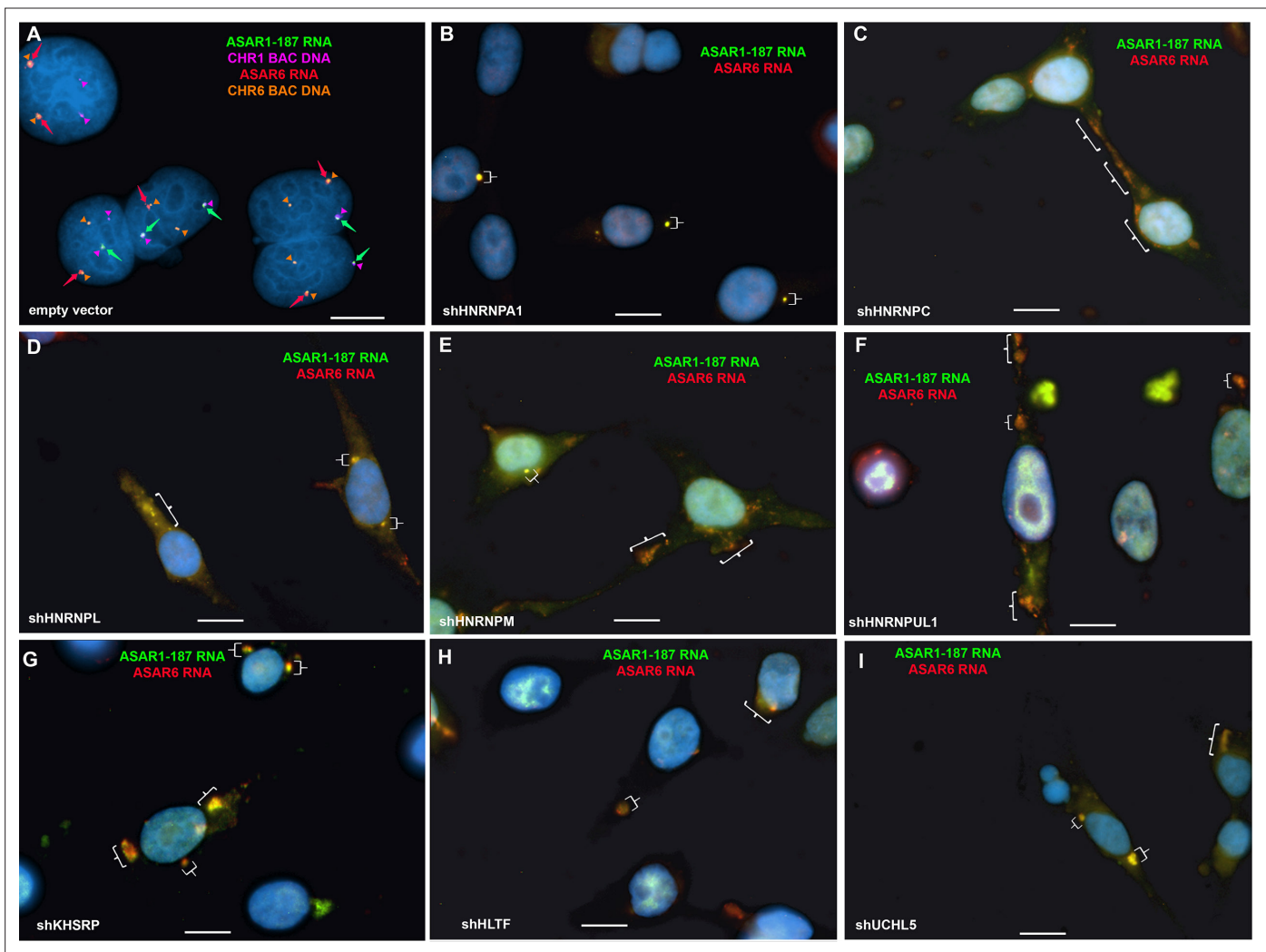


**Figure 5.** Depletion of RBPs results in disruption of the chromosome territory localization of ASAR RNAs. shRNA-mediated depletion of RBPs in K562 cells (A–K) or HTD114 cells (L–Q). Cells were transfected with empty vector (A–C and L–N) or vectors expressing shRNAs directed against *HNRNPA1* (D), *HNRNPC* (E), *HNRNPL* (F), *HNRNPM* (G), *HNRNPU* (H, and O–Q), *HLTF* (I), *KHSRP* (J), or *UCHL5* (K). Cells were processed for RNA FISH with probes for ASAR6 (red, arrows) and ASAR1-187 (green, arrows). Cells were also processed for DNA FISH using BAC DNA to detect chromosome 1 (CHR1 BAC DNA, magenta) and chromosome 6 (CHR6 BAC DNA, orange; A–C and L–Q). Arrows mark the sites of RNA FISH hybridization and arrowheads mark the sites of DNA hybridization. RNA-DNA FISH for ASAR1-187 (RNA in green, arrows; DNA in magenta and arrowheads), ASAR6 (RNA in red, arrows; DNA in orange and arrowheads), ASAR1-187 (RNA in green, arrows; DNA in magenta and arrowheads), ASAR6 (RNA in red, arrows; DNA in orange and arrowheads).

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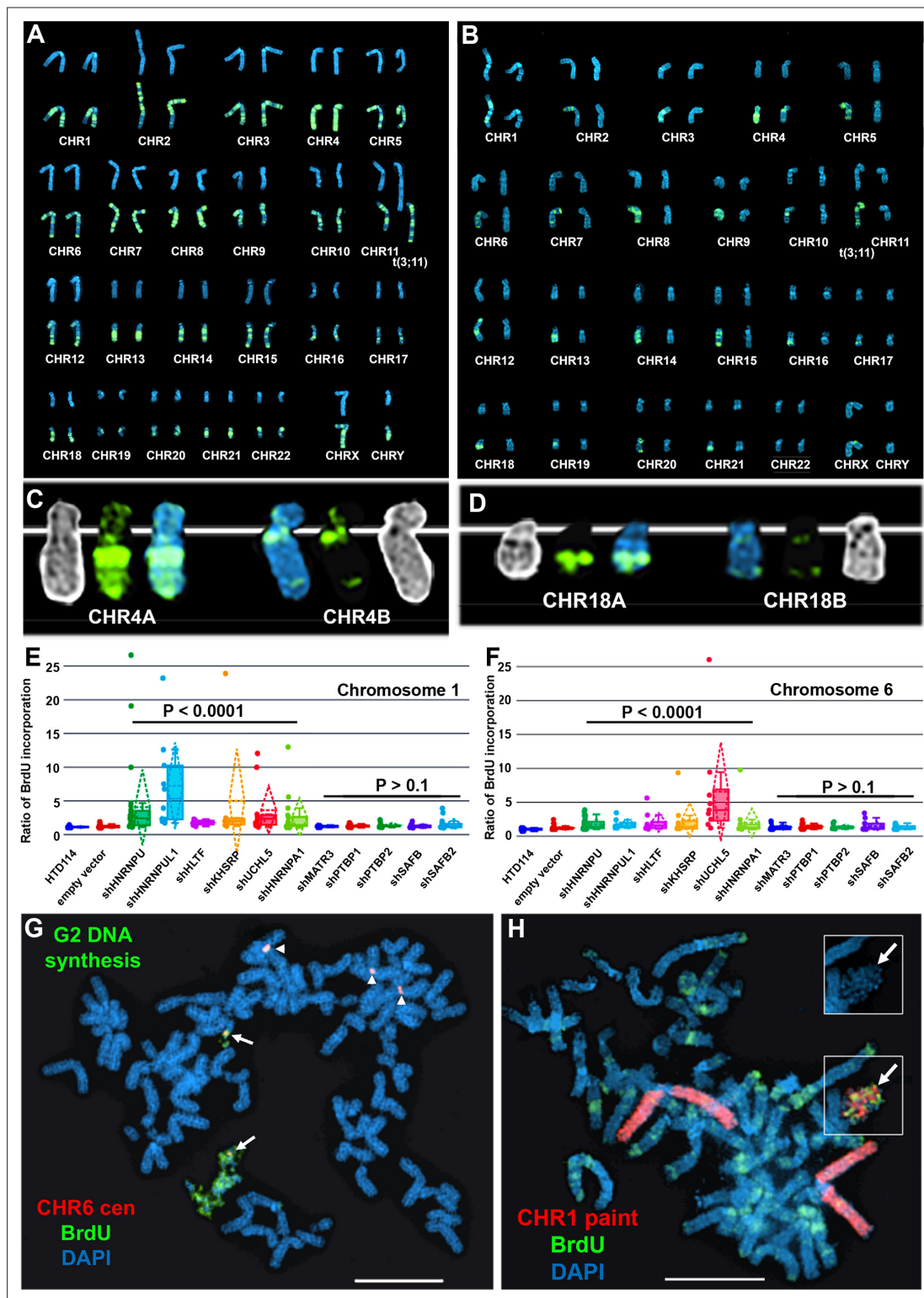
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DNA in orange and arrowheads). The brackets mark the cytoplasmic regions that hybridized to both RNA FISH probes. DNA was stained with DAPI, and scale bars are 10  $\mu$ M.



**Figure 5—figure supplement 1.** Disruption of the chromosome territory localization of ASAR1-187 and ASAR6 RNAs following shRNA depletion of RBPs. HTD114 cells were transfected with empty vector (A) or shRNA expression vectors for *HNRNPA1* (B), *HNRNPC* (C), *HNRNPL* (D), *HNRNPM* (E), *HNRNPUL1* (F), *KHSRP* (G), *HLTF* (H), or *UCL5* (I). Cells were processed for RNA FISH using Fosmid probes (see Figure 1—source data 3) for ASAR1-187 (green, arrow) and ASAR6 (red, arrow). (A) DNA FISH hybridization signals for chromosome 1 (CHR1 BAC DNA; magenta) and chromosome 6 (CHR6 BAC DNA; orange) are indicated by arrowheads. The brackets mark cytoplasmic regions that hybridized to both ASAR RNA FISH probes. DNA was stained with DAPI, and scale bars are 10  $\mu$ M.



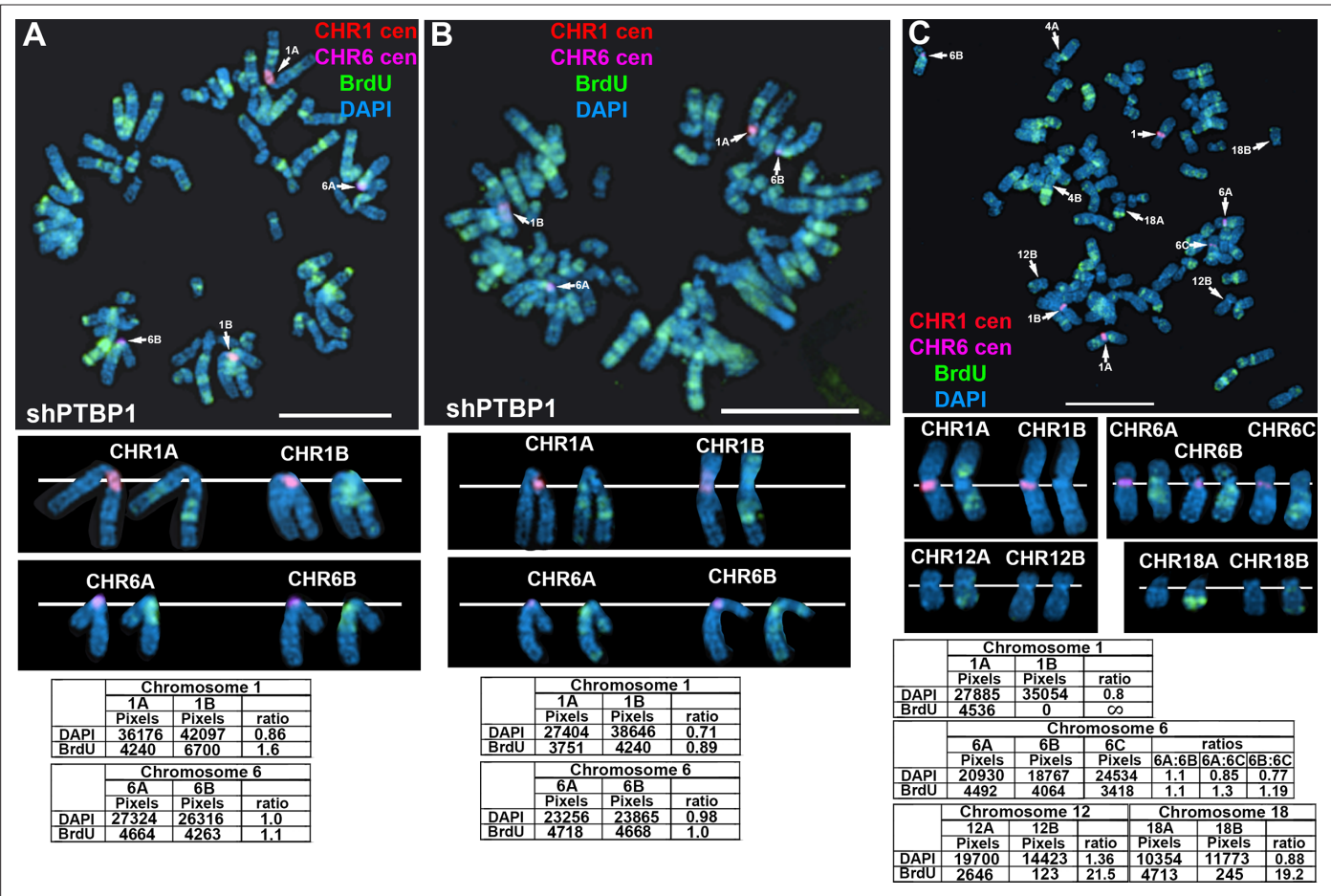


**Figure 6.** Depletion of RBPs results in asynchronous replication on autosome pairs. **(A)** BrdU incorporation in parental HTD114 cells. Cells were exposed to BrdU for five hours and processed for BrdU incorporation using an antibody against BrdU. This panel represents chromosomes from multiple mitotic spreads showing representative BrdU incorporation in chromosome pairs. Both homologs of autosome pair were captured from the same mitotic cell, and each pair displays a typical BrdU incorporation pattern that is consistent with synchronous replication timing. **(B)** HTD114 cells were transfected with the *HNRNPU* shRNA expression vector, exposed to BrdU for five hours, and processed for BrdU incorporation. This panel represents chromosomes from multiple mitotic spreads showing representative BrdU incorporation in pairs of autosomes. Both homologs of autosome pairs were captured from the

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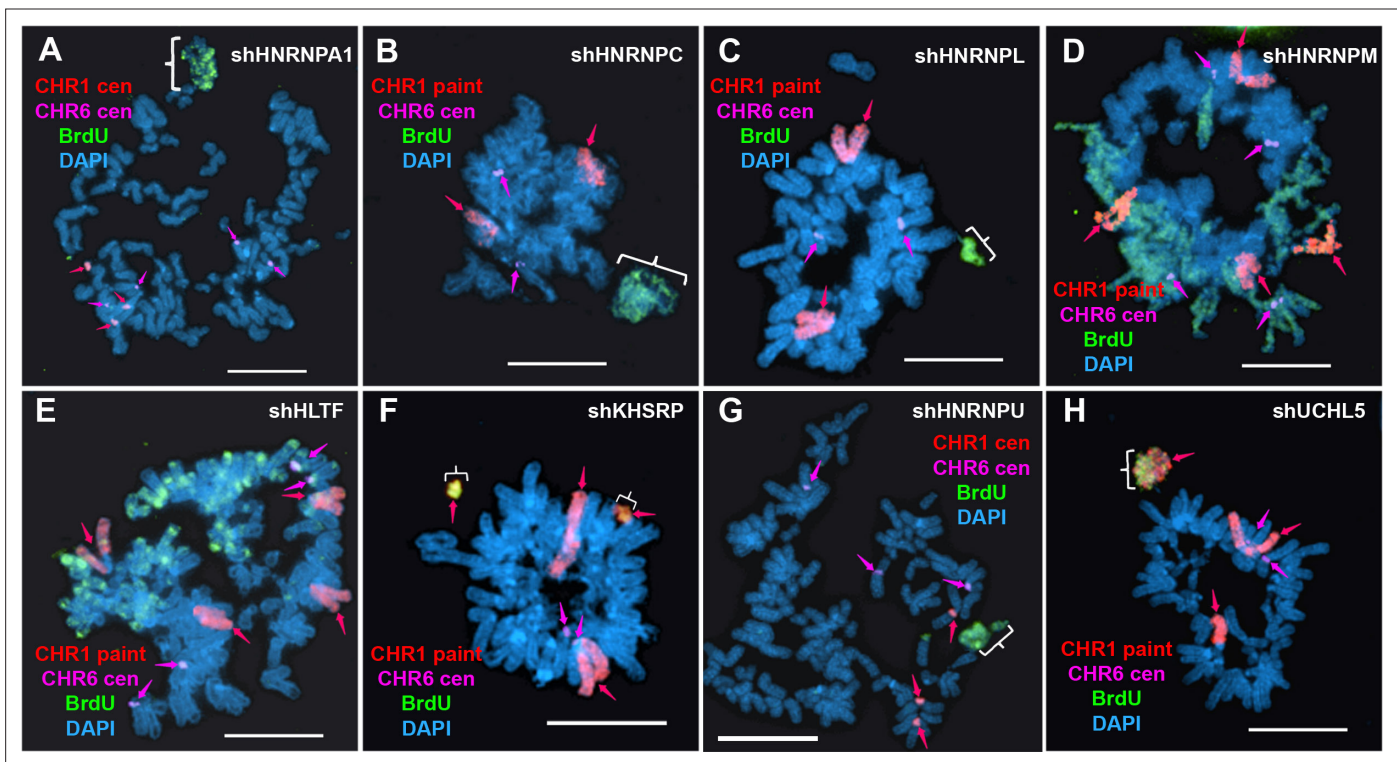
## Figure 6 continued

same mitotic cell, and each pair displays a differential BrdU incorporation pattern that is consistent with asynchronous replication timing. **(C)** Shows two chromosome 4 homologs (CHR4A and CHR4B) side by side. **(D)** Shows the BrdU incorporation in chromosome 18 homologs (CHR18A and CHR18B). For each chromosome pair the inverted DAPI staining (black and white), BrdU incorporation (green), and DAPI staining (blue) are shown. **(E and F)** Quantification of BrdU incorporation in multiple cells depleted for HNRNPU, HNRNPUL1, HTLF, KHSRP, UCHL5, HNRNPA1, MATR3, PTBP1, PTBP2, SAFB, and SAFB2. Box plots indicate mean (solid line), standard deviation (dotted line), 25th, 75th percentile (box), 5th and 95th percentile (whiskers) and a minimum of 10 individual cells (single points) are indicated. p Values were calculated using the Kruskal-Wallis test (**Kruskal, 1964**). **(G)** DRT/DMC on chromosome 6 following HNRNPU depletion. HTD114 cells were transfected with the *HNRNPU* shRNA expression vector, exposed to BrdU for 5 hr, harvested for mitotic cells, and processed for BrdU incorporation (green) and DNA FISH using a chromosome 6 centromeric probe (red). **(H)** DRT/DMC on chromosome 1 following UCHL5 depletion. HTD114 cells were transfected with the *UCHL5* shRNA expression vector, exposed to BrdU for 5 hr, harvested for mitotic cells, and processed for BrdU incorporation (green) and DNA FISH using a chromosome 1 paint as probe (red). The arrow marks the chromosome 1 with DRT/DMC, and the inset shows only the DAPI staining of the chromosome 1 highlighting the DMC.

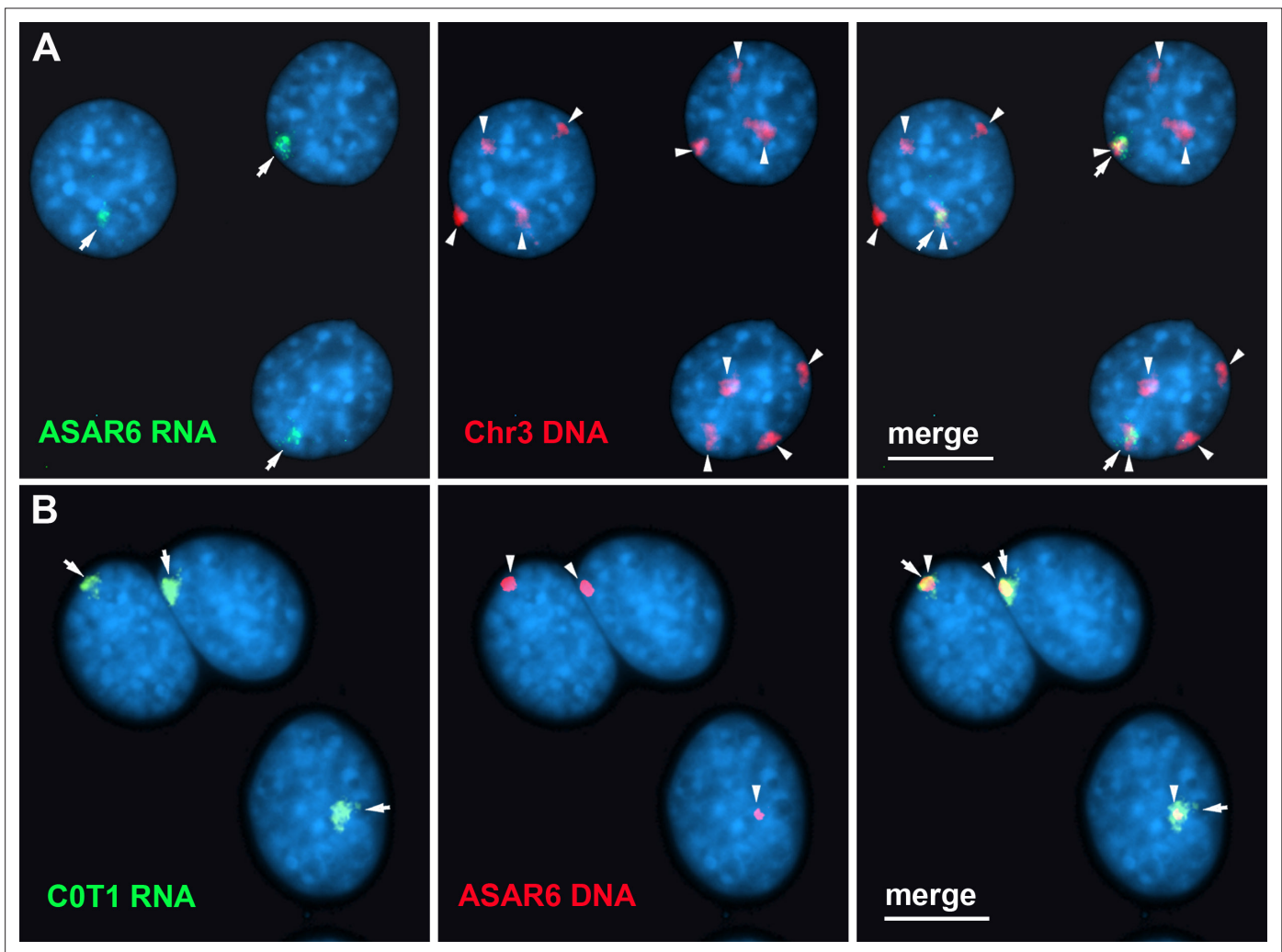


**Figure 6—figure supplement 1.** Replication timing in cells transfected with shRNA expression vectors. **(A and B)** HTD114 cells were transfected with *PTBP1* shRNA expression vector, exposed to BrdU for 5 hr, harvested for mitotic cells, and processed for BrdU incorporation (green) and DNA FISH using a chromosome 1 (A, red) or 6 (B, magenta) centromeric probe. Cells were exposed to BrdU for five hours, processed for BrdU incorporation using an antibody against BrdU. The ratio of BrdU incorporation in chromosome 1 and chromosome 6 homologs are shown in the bottom panels of A and B, respectively. **(C)** HTD114 cells were transfected with shRNA expression vector for *HNRNPU*, exposed to BrdU for 5 hr, harvested for mitotic cells, and processed for BrdU incorporation (green) and DNA FISH using centromeric probes for chromosome 1 (red) or 6 (magenta). The ratio of BrdU incorporation in chromosome homologs for 1, 6, 12, and 18 are shown in the bottom panels. DNA was stained with DAPI, and scale bars are 10  $\mu$ m.





**Figure 6—figure supplement 2.** Replication timing alterations in shRNA depleted cells. HTD114 cells were transfected with shRNA expression vectors for *HNRNPA1* (A), *HNRNPC* (B), *HNRNPL* (C), *HNRNPM* (D), *HLTF* (E), *KHSRP* (F), *HNRNPU* (G), or *UCHL5* (H), exposed to BrdU for 5 hr, harvested for mitotic cells, and processed for BrdU incorporation (green) and DNA FISH using a chromosome 1 centromeric probe (A and G; red, arrows) or chromosome 1 paint (B, C, D, E, F and H; red, arrows) plus a chromosome 6 centromeric probe (A-H; magenta, arrows). The brackets mark chromosomes with DRT/DMC. DNA was stained with DAPI, and scale bars are 10  $\mu$ m.



**Figure 7.** Human C0T-1 DNA detects ASAR6 RNA. **(A and B)** RNA-DNA FISH on mouse cells containing an ASAR6 BAC transgene integrated into mouse chromosome 3 *Donley et al., 2013*. **(A)** RNA-DNA FISH using an ASAR6 fosmid probe to detect ASAR6 RNA (green; arrows) plus a mouse chromosome 3 paint probe to detect DNA (red; arrowheads). **(B)** RNA-DNA FISH using human C0T-1 DNA to detect RNA (green; arrows) plus the ASAR6 BAC (red arrowheads) to detect DNA.