SUPPLEMENTAL FIGURE LEGENDS

Supplemental Figure 1. CoT-1 RNA signal is brighter than poly-A RNA signals in Tig-1 and hybrid cells, and it is removed with RNase treatment. (Related to Figure 1). All blue signals are DAPI DNA. A) 3D deconvolved image of CoT-1 RNA signal in a field of cells from frozen renal tissue. B-G) RNase A treatment removes the majority of CoT-1 RNA and XIST RNA signals in all treated cells (E-G) compared to controls (B-D). The RNA signals are separated in images C-D & F-G. All images were taken at same exposure. H) High degree of co-localization measured for XIST RNA and Chr4 DNA paint signals in a 3D single cell (G3 XIST transgene cell line (Hall et al., 2002)) (R=0.878). All scale bars are 5μm.

Supplemental Table 1. Mammalian cell types positive for CoT-1 RNA. (Related to Figure 1). 13 human cancer lines, 7 non-cancer human lines (all fibroblasts are primary lines and not immortalized), 4 normal human tissue sections and 4 mouse cell lines were examined. All were positive for CoT-1 RNA.

Supplemental Movie 1. CoT-1 RNA is co-localized over the human chromosome territory (rotating). (Related to Figure 3). CoT-1 RNA (green) paints the human Chromosome 4 (Chr4) territory delineated by CoT-1 DNA (red) in a hybrid cell nuclei (DAPI DNA, blue).

Supplemental Movie 2. CoT-1 RNA is co-localized over the human chromosome territory (through the Z-axis of nucleus). (Related to Figure 3). CoT-1 RNA (green) moves with the human Chr4 territory delineated by CoT-1 DNA (red) through the Z-axis of the nucleus (DAPI DNA, blue).

Supplemental Movie 3. XIST RNA is co-localized over the inactive chromosome territory (rotating). (Related to Figure 3). XIST RNA (green) paints the inner core (Barr body) of the inactive human Chr4 labeled with a chromosome library probe (green).

Supplemental Figure 2. CoT-1 RNA remains localized, is more stable than XIST or collagen in interphase, and is re-synthesized after release from DRB. (Related to Figures 3, 4

& 5). All blue signals are DAPI DNA. A) XIST RNA is localized to the inactive chromosome and delineates the DAPI dense Barr body (BB). Close up of XIST covered chromosome separated into DNA and XIST RNA channels at right. White line is region in linescan. F) A linescan shows a defined XIST RNA border over the DAPI dense BB. C) Mouse (mCoT-1) and human CoT-1 RNA do not co-localize, and remain associated with their respective chromosomes, making a "hole" in the mCoT-1 RNA signal (arrow) over the human chromosome territory. Close up of region separated into mCoT-1 and hCoT-1 RNA channels at right D-E) Human Chr4 library probe non-specifically cross-hybridizes with other chromosomes in a metaphase spread (DNA stain of chromosomes not shown), due to the presence of residual CoT-1 repeats in the probe (D), which are effectively repressed with unlabeled CoT-1 competition (E). F-G) Col1A1 makes very large transcription foci in many fibroblast nuclei. H-I) Most cells lack collagen RNA after 5 hours of transcriptional inhibition, although a low number (18%) still show a residual signal (arrows). Images F-I are at the same exposure. J-K) CoT-1 RNA signal is fairly uniform in interphase Tig-1 cells treated with DRB for 5 hours, but the XIST RNA signal is highly variable in this same treatment, with over half the population showing significant reduction or no XIST signal remaining (arrows). XIST was more variable with DRB than we had seen before. L-M) All G1 daughter cells from mitotic shake-off show full repression of CoT-1 re-synthesis in the transcriptional inhibitor DRB, and within 1 hour of DRB removal CoT-1 expression has recovered in all cells. All scale bars are 5μm.

Supplemental Figure 3. CoT-1 RNA is stable under long-term transcriptional inhibition, some is mislocalized by ActD and evidence suggests RNAPII may transcribe some of it. (Related to Figure 5). All blue signals are DAPI DNA. A-H) The DNA intercalating inhibitor, Actinomycin-D, sometimes affected localization of both CoT-1 RNA (A-D) (A-B is a control cell) and XIST RNA (E-H) (E-F is a control cell). RNA channels are separated in black & white. I-J) α-amanatin was effective in preventing re-synthesis of CoT-1 RNA in 92% of mitotic shake-off (G1d enriched) cells. Control cells in insert. K) Even high concentrations of α-amanitin for 16 hours did not affect localized CoT-1 RNA levels in interphase Tig-1 and hybrid cells (~93% of cells had localized CoT-1 RNA). Insert shows the intensity of the CoT-1 RNA territory in treated cells compared to the bead standard. Bead is 2.5μm. L-M) RNAP I transcribed 18s rRNA is present in nucleoli of all control (L) and DRB-treated (M) G1 daughter cells (arrows). N-Q)

RNAP III transcribed 5s rRNA is present in nucleoli of all control (N-O) and DRB treated (P-Q) post-mitotic, G1 daughter cells. All scale bars are 5μm.

Supplemental Figure 4. Use of fluorescent beads for in situ standardization/quantitative comparison. (Related to Figures 4 & 6). We use standardized microscope intensity calibration beads of uniform intensity (~3% relative intensity) and size (2.5μm) (Invitrogen/Molecular Probes InSpeck Green (505/515) Microscope Image Intensity Calibration Kit) to provide standardization for quantitative comparisons between different samples hybridized under identical conditions in the same color. These kits contain a series of well-defined fluorescent intensity levels covering the range of intensities and fluorescence spectra (red, green & blue) commonly encountered in microscopy applications. Appropriately colored beads (same Fluorescence Ex/Em spectra as labeled probed to be measured) are included in the mounting media on the slides with the cells. Calibration of different sample images is done at the time of photographing (all exposure settings adjusted to bead intensity), or can be accomplished later by manipulating image enhancement with software such as Photoshop, ensuring that all beads in the images are of equal intensity (A-D). This allows you to compare signal intensities across different experiments that may not have been taken at the same exposure or on the same day. It also provides an internal "scale bar" of 2.5μm.

The beads also allow us to measure relative probe signal intensities more reliably between different samples. For example, linescans (E-F) or area measurements (G-H) (including whole nuclei area measurements as in Figure 2 E-H) can be used to measure the signal intensities of the same labeled probe on different samples, normalized to the bead. White line in (E) is region measured in linescan (F). Max pixel intensity for green bead and cell signal indicated by arrows. Signal intensities measured in nucleoli (if signal to be measured is not present there) or on the glass slide are subtracted as background.

Supplemental Table 2. List of probes and primer sequences used. (Related to Experimental Procedures)

Supplemental Figure 5. L1 ORF2 RNA is expressed in different cell types, and is not aberrantly expressed in hybrid cells. (Related to Figure 6). All blue signals are DAPI DNA. A)

Full-length LINE elements are 6 kb long and have two open reading frames (ORF1 and ORF2), encoding a reverse transcriptase and an endonuclease. Four different probes were used to detect L1 RNA or DNA by FISH. Two plasmids containing either the entire ORF1 or ORF2 regions of L1, or two PCR-generated probes to the 3' or 5' UTRs (see Table S2 for primer sequences). B-G) L1 ORF2 RNA is expressed as a large component of CoT-1 RNA in all cell types examined, including (B-C) primary female fibroblasts, (D-E) cancer cell lines and (F-G) human ES cells. Exposures are the same in all images, and XIST RNA is included in the fibroblasts for comparison. H-I) The intensity of CoT-1 RNA signal is similar between normal Tig-1 fibroblasts and mouse/human hybrid cells, suggesting the hybrid cells do not aberrantly over-express this RNA (cells were hybridized together on the same slide). J-K) As expected, the intensity of L1 DNA signal between the single human chromosome in the hybrid cells is similar to the average human signal in fibroblasts, although some regions of the human nucleus are brighter. L-M) The L1 ORF2 RNA from the human chromosome in the hybrid cells is not aberrantly over expressed and may even be slightly lower than the average level in the human nucleus.

Supplemental Table 3. L1 versus Alu RNA levels normalized to their DNA signal in both cell lines. (Related to Figure 6) Since DNA is double-stranded and the RNA is single stranded, the ratio of signals is ~6-8 times more RNA than DNA per L1 ORF2 sequence element.

Supplemental Figure 6. L1 RNA arises predominantly from the 3' end of the element, in the sense direction, and it is heterogeneous in size, while Alu RNA is expressed at a low level. (Related to Figure 6). All blue signals are DAPI DNA. A) Quantification of signal intensity for L1 ORF1 and ORF2 DNA and RNA signal in hybrid cells reveals that very little ORF1 RNA is expressed from its corresponding DNA signal, confirming previous reports that full-length L1 elements are silenced in normal cells. B-E) Because of L1 sequences are divergent this made it difficult to design an oligo to broadly detect L1 sequences as was done for Alu. Instead we used 200bp PCR-generated probes for the 3' and 5' UTR of L1 to confirm more 3' expression (B-C) compared to 5' (D-E) in hybrid and fibroblast cells. All images are at the same exposure, except for the insert in D (RNA signal in black & white), which has been increased for visibility. F) The 3' and 5' UTR RNA levels were measured using digital morphometrics and normalized to the bead. G) Most L1Hs deep sequencing reads from pancreatic cancer (Ting et

al., 2011) map to the 3' end of the element and are predominantly in the sense direction. H) Northern blot analysis shows L1 ORF2 RNA extracted by Trizol is low in abundance but heterogeneous in size. I-P) Because Alu shows more conservation than L1, oligo probes succesfully detected Alu DNA sequences comparable to full length (~200nt) Alu plasmid probes. Here, using an oligo probe, Alu DNA (I-J) signal is much brighter than Alu RNA (K-L) in Tig-1 fibroblasts. Similarly, Alu DNA (M-N) signal is brighter than Alu RNA (O-P) in hybrid cells. All images are at the same exposure, normalized to the bead. Alu RNA is visible by eye through the microscope in hybrid cells, but it is too low to be seen in the image (P) when normalized to the bead. All beads are 2.5μm.

Supplemental Figure 7. XIST and CoT-1 RNA are both retained on the nuclear matrix and released with the SAF-A C280 mutant, and the RNA may resist standard RNA extraction. (Related to Figure 7). All blue signals are DAPI DNA. A-D) Like CoT-1 RNA, L1 Orf2 RNA is localized across the interphase chromosome territory of its parent chromosome in control cells (A-B), and remains attached following removal of ~90-95% of DNA and chromatin proteins using ammonium sulfate and DNase (C-D) (Clemson et al 1996). Scale bar 5μm. E-H) This is also true for XIST RNA, which remains equally localized in all control (E-F) and matrix-digested (G-H) hybrid interphase nuclei. All treated and control images are taken at the same exposure showing little to no diminishment of the RNA signal after chromatin digestion. I) Diagram of full length SAF-A/HNRNPU and the C280 SAF-A deletion mutant. J-K) RNA from the 3'UTR of L1 is released from Chr 4 in hybrid cells transfected with the C280 mutant, while untransfected cells in the same field are not affected. L) Number of cells showing mis-localized CoT-1 RNA was scored in cells transfected with C280 or full length SAF-A. M-N) In Tig-1 cells transfected with C280, CoT-1 RNA is released and is degraded over time, while untransfected cells in the same field still contain CoT-1 RNA. O) L1 ORF2 DNA:RNA ratio and β-actin DNA:RNA ratio by qPCR from fibroblasts (corrected for cell input number, see Supp Methods). Shown in log scale. P-Q) Comparison of L1 and XIST RNA levels in normal cells measured either by digital fluorimetry of RNA FISH in fibroblast cell line (P) or by RNA deep-sequencing reads in normal uterine tissue (Q). Data was normalized to account for size of the probe. Deep-seq reads from normal uterus (Ting et al., 2011) were mapped to the same sequence used as probes for RNA FISH (L1Hs ORF2 and XIST G1A) by local BLAT. R-U) A chromosome 21

transgene (Jiang et al., 2013) demonstrates that XIST RNA can act on, and silence, Cot-1 repeat RNA on an autosome, which is coincident with Barr body formation. Separated channels are shown in white (S-U).

Supplemental Figure 8. RNase digestion and transcriptional inhibition causes changes in chromatin structure. (Related to Figure 7). A) Control DMSO treated G1 daughter cells exhibit a normal nuclear phenotype B) DRB treated G1 daughter cells show condensed chromatin clumps by EM. Scale bar 1μm. C) Enlarged normal G1 daughter to show chromatin distribution D) Enlarged DRB treated G1 daughter to show aberrant chromatin clumps E) Example of aberrant darkly staining structure with spindle-like fibers radiating outwards (arrow), which are found in all DRB treated G1d cells (2-8 per nucleus). Scale bar 0.5μm. F) High resolution EM analysis shows large clumps of dense material in DRB treated G1d cells (arrow), present in ~70% of G1d nuclei. Scale bar 0.5μm. G-H) Paired nuclei with condensed chromatin clumps (arrows) were about twice as common in transcriptionally inhibited cultures (H) compared to controls (G). Control = 11%, DRB = 23% & ActD = 25%. I-L) Nucleoplasmic chromatin proteins showed a more "lumpy" distribution in DRB treated G1d nuclei than in control G1d nuclei. Some delineated the condensed chromatin (HP1 and H3K9me3), while others were excluded from the chromatin clumps (Rad21). M-N) Linescan measurements through the nuclear DNA of two representative cells show a broader range of DAPI DNA pixel intensity in a transcriptionally inhibited G1d nucleus compared to a similarly treated interphase nucleus. O) The DNA pixel intensity was measured in ten 3D images of transcriptionally inhibited G1d and neighboring interphase cells and the highest 3/5ths (pixel intensity>129) of the DAPI signal was compared. Box and whisper plot of high intensity DAPI staining calculated as percent area of nuclei for DRB treated G1 and interphase cells (n=10). Horizontal lines on boxes indicate lower, median and upper quartile values. P-Q) Human Chr4 is delineated by CoT-1 DNA hybridization (P) and the regions of the chromosome territories (white lines) are transferred to the DAPI DNA channel (Q) to determine if individual chromatin clumps comprise a single chromosome. R-S) All CoT-1 RNA is removed with RNase A treatment in unfixed cells. All blue signals are DAPI DNA in colored images, and scale bars are 5μm.

Supplemental Table 1: (Related to Figure 1).

Cell types positive for nucleoplasmic CoT-1 RNA

HUMAN CANCER LINES
U2OS - Osteosarcoma
SAOS-2 - Osteosarcoma
PC3 - Prostate Adenocarcinoma
HEP-G2 - Hepatocellular carcinoma
HT-1080 - Fibrosarcoma
MCF-7 - Breast Adenocarcinoma
HCC-1937 - Breast Ductal Carcinoma
T47D - Breast Ductal Carcinoma
MDA-MB-436 - Breast Adenocarcinoma
MDA-MB-231 - Breast Adenocarcinoma
SUM-149pt - Inflammatory breast cancer
JAR - Choriocarcinoma
HELA - Cervical Adenocarcinoma
HUMAN NON-CANCER LINES
MCF-10A - Breast Fibrocystic Disease
IMR-90 – Primary Lung Fibroblast
WS1 – Primary Embryonic Skin Fibroblast
TIG-1 – Primary Fetal Lung Fibroblast
Wi38 –Primary Fetal Lung Fibroblast
HFF – Primary Foreskin Fibroblast
HSMM – Myoblasts & Myotubes
HUMAN NORMAL TISSUES
1880N – Renal
2081N – Fallopian Tube
2312N – Pancreas
2632N – Thyroid
MOUSE LINES
NIH 3T3 - Fibroblasts
MEFs – Embryonic Fibroblasts
3X female line
GM11667 – Mouse-Human Hybrid cells

Supplemental Table 2: (Related to Experimental Procedures)

Probe Sequences:

Probe Name	**Sequence**	**Label**	**Reference**
Sat2-LNA (Oligo)	5'ATTCCATTCAGATTCCATTCGATC	5' Biotin	Exiqon, Product # 200501-03
Sat3 (Oligo)	5'CCATTCCATTCCATTCCATT	3' Biotin	Prosser et al., 1986 (Invitrogen)
HuAlphaSat (Oligo)	5' CCT TTT GAT AGA GCA GTT TTG AAA CAC TCT TTT TGT AGA ATC TGC AAG TGG ATA TTT GG	3'& 5' FITC	Biosource & Invitrogen
HuAlu (Oligo)	CCC AAA GTG CTG GGA TTA CAG GCG TGA GCC ACC	3'& 5' FITC	Biosource
DMPK intron 9 (primers)	Fwd: 5'GAGTTGCAGGATCAGTCTTGGA Rev: 5'TGCTGATTCTCTGGTGGAGAAC	Biotin or Dig PCR label	
L1 5' UTR (primers)	Fwd: 5' GAACAGCTCCGGTCTACAGC Rev: 5'TCACCCCTTTCTTTGACTCG	Biotin or Dig PCR label	
L1 3' UTR (primers)	Fwd: 5'TGATGAGTTCATATCCTTTGTAGGG Rev: 5'GATATTCCCCTTCCTGTGTCC	Biotin or Dig PCR label	
Poly A (oligo)	T…(54)	5' FITC	
5s rRNA	CCTGCTTAGCTTCCGAGATCAGACGAG ATCGGGCGCGTTCAGGGTGGTATGGCC G	Alexa 594	
COL1A1 Intron 6	Fwd: 5'GTTCTGCTTGTGTCTGGGTTTC Rev: 5' AGTAAGTTCAACCTTCCCCCTCC		
XIST Intron 1	Fwd: 5'TCCCATCACTGTTCAATGCC Rev: 5'ATTGTGGACCCAGCTCTATGC		
GADPH Intron 1	Fwd: 5'ATGGAGGTCCTCTTGTGTCCC Rev: 5'AGACAAGAGGCAAGAAGGCAT		

Supplemental Table 3. Ratio of RNA to DNA signal for L1 and Alu. (Related to Figure 6).

	RNA	DNA
L1 Fibroblast	4	1
L1 Hybrid	4.5	1
Alu Fibroblast	0.25	1
Alu Hybrid	<0.1	1

EXTENDED EXPERIMENTAL PROCEDURES

Extended fixation and hybridization protocols:

Fixation: As detailed previously (Johnson et al., 1991; Tam et al., 2002), cells are permeabilized in CSK buffer, 5% triton, and VRC (vanadyl ribonucleoside complex) for 1-3 min, then fixed in 4% Paraformaldehyde for 10 min, and stored in 1XPBS or 70% EtOH.

Hybridization: *RNA:* In order to detect RNA exclusively, all RNA hybridizations were carried out under non-denaturing conditions, preventing DNA accessibility to the probe. All DNA probes (1µg/reaction) were nick-translated using biotin-11-dUTP or digoxigenin-16-dUTP (Roche), and 50ng were used per hybridization (in 2XSSC, 20% VRC and 50% formamide at 37°C overnight). Oligos were usually hybridized at 15% formamide. Unlabeled Human CoT-1 DNA (10-15µg) was included in the hybridization buffer to block non-specific background in all RNA FISH reactions except for CoT-1, L1 or Alu RNA hybridizations. Posthybridization washes: 50% formamide in 2xSSC for 20 min (37°C); 2xSSC for 20 min (37°C); 1xSSC for 20 min (rm temp); 4xSSC for 5 min (rm temp). Detection: add appropriate labeled secondary (e.g Fitc anti-digoxigenin or Dylight 488 or 594 streptavidin) in 1%BSA, 4xSSC for 1 hr at 37°C. Post-detection washes: 4xSSC; 4xSSC with 0.1% Triton; and 4xSSC, for 10 min (rm temp).

DNA: Fixed cells were denatured in 70% formamide, 2xSSC, at 80°C for 2 min, before ethanol dehydration and air-drying. Hybridization and detection was carried out as described above for RNA.

Northern blot

Total RNA was extracted from Tig-1 fibroblast cell pellets using Trizol (Invitrogen) per manufacturer's instructions. 10µg total RNA was DNase-treated (Promega RQ1 RNase-free

DNase) then electrophoresed on a 1% agarose denaturing gel and transferred to a nylon membrane (Ambion Brighstar Plus) using a semi-dry electroblotter at 400mA for 1 hour and UV crosslinked. 10ng Biotin-labeled L1ORF2 plasmid was hybridized overnight in UltraHyb buffer (Ambion Northernmax) in a rotating hyb oven at 37°C and washes were performed according to manufacturer's instructions (Ambion Brightstar Biodetect kit).

3D co-localization analysis

0.5μm Z-stacks were deconvolved using Zeiss Axiovision or Volocity (Perkin Elmer) Restoration package. Images were quick projected for publication quality images or co-localization analysis was performed in 3D using Volocity co-localization analysis. A thresholded Pearson's correlation coefficient was generated from 3D images to take into account background levels for individual fluorophor channels. Digital image movies were generated with Volocity.

qPCR

L1 qPCR: Trizol-extracted Tig-1 fibroblast RNA was reverse transcribed with IScript Supermix (Bio-Rad) and then RT-qPCR for L1 was performed and analyzed as per (Carone DM, 2013). Expression levels obtained with primer sets for L1 5' UTR: Fwd: 5' GAACAGCTCCGGTCTACAGC Rev: 5'TCACCCCTTTCTTTGACTCG and L1 3'UTR: Fwd: 5'TGATGAGTTCATATCCTTTGTAGGG Rev: 5'GATATTCCCCTTCCTGTGTCC were normalized to levels for β-ACTIN: Fwd: 5'AGCGAGCATCCCCCAAAGTT Rev: 5'GGGCACGAAGGCTCATCATT.

DNA/RNA qPCR: DNA or RNA was extracted from $1.4X10^5$ Tig-1 fibroblast cells with phenol-chloroform or Trizol, respectively. RNA was then reverse transcribed as above and qPCR was performed from Tig-1 cDNA and genomic DNA with the primer sets as above. Dilution calculations were taken into account to normalize for the amount of DNA or RNA going into the initial PCR reaction (to account for amount subjected to reverse transcription and dilution of DNA). qPCR analysis was performed as above. Results shown (Supp Figure 7) are in log scale.

Transmission electron microscopy (TEM) and fixation

Mitotic shake-off cell cultures grown for 2 hours in the presence of 40ug/mL DRB or 4uL/mL DMSO (control) in 6 well plates were fixed by adding 2.5% glutaraldehyde in 0.1 M Na Cacodylate buffer (pH 7.2) to the culture plates one drop at a time until the initial volume of media was doubled. The cell cultures were allowed to stabilize in this solution for 10 min, then all the media/glutaraldehyde was removed and fresh 2.5% glutaraldehyde in the same buffer was added and the cells were allowed to fix for 60 min. at room temperature. After this primary fixation, the cells were rinsed three times in fresh fixation buffer for 10 min. each time and were secondarily fix with 1.0% osmium tetroxide in ddH2O for 30 min at room temperature. The cell cultures were then washed again three times in ddH2O and subjected to a tertiary fixation with 1% Uranyl Acetate in ddH2O for another 30 min. After two more washes with ddH2O the cells were dehydrated through a graded series of ethanol (10% to 100%; 3 changes), and then transferred to ethanol 100%: SpiPon 812/Araldite 502 resin (50:50 / V:V) for 12 h at room temperature. Following infiltration in the resin, the cells were transferred through 3 changes of pure SpiPon 812/Araldite 502 epoxy resin each 1 hr long. The cells were then changed to a final step of embedding resin mixture and were polymerized for two days at 70° C in their original culture dishes. Each individual culture plate was separated from the others, and then the rims of

each dish were sawed off. The individual sawn plates were then placed in liquid nitrogen to separate the bottom of the dish from the cells embedded in the resin. Pieces of the embedded cultures were then mounted onto blank stubs with a drop of super glue and the blocks were trimmed and sectioned (100 nm thick). Sections were collected onto 200 mesh copper support grids and imaged using both a Philips CM 10 and Tecnai 12 BT Transmission under 80Kv accelerating voltage. Images were recorded with a Gatan Erlangshen CCD Digital camera.

Supplemental References

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