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(54) **Title:** METHODS OF REDUCING EXPRESSION OF X-INACTIVATION ESCAPEE GENES AND AUTOSOMAL GENES

(57) **Abstract:** Inhibitory nucleic acids, e.g., antisense oligonucleotides (ASO) against PAR-TERRA RNA or other chromosome-specific TERRA transcripts (i.e., inclusive of chromosome-specific subtelomeric sequences), and methods of use thereof to downregulate expression of escapee genes on the inactive X chromosome, expression from the active X chromosome, subtelomeric autosomal loci (e.g., FSHD locus), or expression of autosomal genes involved in growth control and apoptosis, e.g., in cells and subjects with supernumerary X chromosomes and/or cancer and other human diseases.



Methods of Reducing Expression of X-inactivation Escapee Genes and Autosomal Genes

CLAIM OF PRIORITY

This application claims the benefit of U.S. Provisional Patent Application
5 Serial No. 62/261,698, filed on December 1, 2015. The entire contents of the
foregoing are hereby incorporated by reference.

FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with Government support under Grant No. R01-
GM58839 awarded by the National Institutes of Health. The Government has certain
10 rights in the invention.

TECHNICAL FIELD

Described herein are inhibitory nucleic acids, e.g., antisense oligonucleotides
(ASO) against PAR-TERRA RNA and TERRA of autosomal origin, and methods of
use thereof to downregulate: (i) expression of escapee genes on the inactive X
15 chromosome (Xi), e.g., in cells and subjects with supernumerary X chromosomes, (ii)
expression from the active X chromosome (Xa), (iii) expression a network of
autosomal genes involved in growth control and apoptosis, and (iv) expression of the
genetic locus associated with FSHD (facioscapulohumeral muscular dystrophy) and
other subtelomeric autosomal genes.

BACKGROUND

The mammalian genome is ubiquitously transcribed and the ends of telomeres
are no exception. In spite of their heterochromatic properties, telomeric ends actively
synthesize a heterogeneous population of long noncoding RNAs dubbed "TERRA"
(Azzalin et al., 2007; Schoeftner and Blasco, 2007; Zhang et al., 2009). TERRA
25 transcripts range in size from 100 bases up to >9 kb and contain the canonical
telomeric repeat sequence, UUAGGG, as well as sequences unique to the sub-
telomeric region of each chromosome. The function of TERRA has generated major
interest in light of its association with human diseases, such as cancer and the ICF
syndrome (immunodeficiency, centromere instability, and facial anomalies)(Maicher
30 et al., 2012; Azzalin and Lingner, 2015). Elegant studies have pointed to a number of

telomere-associated functions. Telomeres are well-defined nucleoprotein complexes that cap the physical ends of linear chromosomes and protect them from unprogrammed shortening and genetic rearrangements (Blackburn et al., 2006; Sfeir and de Lange, 2012; Bernardes de Jesus and Blasco, 2013; Doksani and de Lange, 2014; Azzalin and Lingner, 2015). The reverse transcriptase activity of the RNA-containing telomerase complex enables regeneration of chromosomal ends that are lost with every DNA replication (Lingner et al., 1997). However, TERRA's activity does not appear to be directly related to telomerase activity (Schoeftner and Blasco, 2007; Redon et al., 2010; Redon et al., 2013). Rather, TERRA seems to keep telomere length in check (Sandell et al., 1994; Luke et al., 2008; Maicher et al., 2012; Pfeiffer and Lingner, 2012; Pfeiffer et al., 2013; Wang et al., 2015), regulate recombination (Balk et al., 2013; de Silanes et al., 2014; Yu et al., 2014), and serve as a scaffold for recruitment of HP1, histone methyltransferases, and shelterins to telomeric heterochromatin (Deng et al., 2009). Thus, TERRA is an integral part of the telomeric architecture.

Cytological studies indicate that only about half of detectable TERRA transcripts are localized to telomeres (Le et al., 2013). The remaining half is presumed to be "free" in the nucleoplasm. Nevertheless, investigation into TERRA function has focused almost exclusively on telomeres, though early observations noted a large cluster of TERRA transcripts near the inactive X-chromosome (Xi) of somatic female cells (Schoeftner and Blasco, 2008; Zhang et al., 2009). TERRA RNA is also concentrated next to the Y-chromosome (Zhang et al., 2009).

SUMMARY

Telomeric repeat-containing RNAs (TERRA) are highly conserved long non-coding RNAs transcribed from telomeric ends of eukaryotic chromosomes. TERRA has so far only been ascribed function in telomere biology. Genome-wide binding sites for TERRA have now been identified, and show that TERRA localization is not cis-limited, nor is TERRA function confined to telomeres. Transcriptomic analysis shows that TERRA depletion results in dysregulation of TERRA target genes.

Described herein is a subclass of TERRA transcripts specific to the sex chromosomes. Dubbed PAR-TERRA, these transcripts originate within the pseudoautosomal region (PAR) and mediate two special sex-linked processes. First, in somatic cells, PAR-TERRA prevents spreading of Xist RNA away into genes that escape silencing on the

inactive X (Xi). PAR-TERRA renders X-linked escapee genes immune to Xist RNA. Depleting PAR-TERRA leads to downregulation of escapees. We also show that depleting PAR-TERRA reduces expression of the Xa gene and various target autosomal genes, especially those involved in apoptosis and cell cycle regulation.

5 Thus, the methods can also be applied to downregulate a network of autosomal genes involved in growth control and apoptosis. Provided herein is evidence that PAR-TERRA sets up a specialized privileged compartment that aids in boosting transcriptional activity specific genes across the genome. Thus, PAR-TERRA may be targeted to turn down (i) expression of escapee genes on the inactive X chromosome
10 (Xi), e.g., in cells and subjects with supernumerary X chromosomes, (ii) expression from the active X chromosome (Xa), or (iii) expression a network of autosomal genes involved in growth control and apoptosis. The present methods include using inhibitory nucleic acids, e.g., antisense oligonucleotides (ASO) against PAR-TERRA RNA to downregulate expression of these classes of genes.

15 Thus, provided herein are isolated inhibitory nucleic acids targeting PAR-TERRA, preferably wherein the inhibitory nucleic acid is modified, and compositions comprising the isolated nucleic acids.

Also provided are methods for decreasing expression of an Xi escapee gene in a cell, preferably a cell of a subject have a supernumerary X chromosome. The
20 methods include administering to the cell an inhibitory nucleic acid targeting PAR-TERRA, preferably wherein the inhibitory nucleic acid is modified.

Further, provided are methods for decreasing expression of Xa genes in a cell, preferably a cell of a subject having a supernumerary X chromosome. The methods include administering to the cell an inhibitory nucleic acid targeting PAR-TERRA,
25 preferably wherein the inhibitory nucleic acid is modified.

Also provided are methods for treating a subject who has a disorder of sex chromosome aneuploidy associated with a supernumerary X chromosome. The methods include administering to the subject an inhibitory nucleic acid targeting PAR-TERRA, preferably wherein the inhibitory nucleic acid is modified.

30 Also provided is a composition described herein, e.g., comprising an inhibitory nucleic acid targeting PAR-TERRA, for treating a subject who has a disorder of sex chromosome aneuploidy associated with a supernumerary X chromosome.

In some embodiments described herein, the subject has 46,XY, 47,XXY, 48,XXYY, 48,XXXY, 47,XXX, 48,XXXX or 49,XXXXX aneuploidy.

In some embodiments described herein, the cell is from a subject who has 46,XY, 47,XXY, 48,XXYY, 48,XXXY, 47,XXX, 48,XXXX or 49,XXXXX aneuploidy.

Also provided are methods for decreasing expression of X-linked, autosomal growth control or apoptosis genes, and sub-telomeric autosomal genes in a cell (e.g., out D4Z4, DUX4, FRG1, and FRG2 for FSHD, from Chr4). The methods include administering to the cell an inhibitory nucleic acid targeting PAR-TERRA, PAR, or TERRA, preferably wherein the inhibitory nucleic acid is modified.

In addition, provided are methods for decreasing expression of autosomal genes in a cell. The methods include administering to the cell an inhibitory nucleic acid targeting PAR-TERRA or an autosome-specific TERRA (e.g., TERRA species originating with the subtelomeric region of an autosome and comprising autosome-specific 5' sequences), preferably wherein the inhibitory nucleic acid is modified. In some embodiments, the inhibitory nucleic acid targets Chr4-specific TERRA. In some embodiments, expression of FRG1, FRG2, DUX4, and the long noncoding RNAs of forward and reverse orientations from the macrosatellite repeat, D4Z4 is decreased. In some embodiments, the cell is from or in a subject who has facioscapulohumeral muscular dystrophy (FSHD). Thus, in a specific example the methods include targeting the Chr4 region associated with facioscapulohumeral muscular dystrophy (FSHD), which is located in the subtelomeric region of human Chr4 and contains coding genes FRG1, FRG2, DUX4, and the long noncoding RNAs of forward and reverse orientations from the macrosatellite repeat, D4Z4. FSHD is caused by ectopic expression of these genes when the D4Z4 repeat contracts and becomes "activated". Thus, PAR-TERRA or Chr4-specific TERRA can be targeted to downregulated the associated subtelomeric genes. Other subtelomeric genes, e.g., as shown in Fig 4A,B, from 5 chromosomes are shown (Chr1,3,8,18,19), can also be targeted.

In some embodiments described herein, the inhibitory nucleic acid does not comprise three or more consecutive guanosine nucleotides or does not comprise four or more consecutive guanosine nucleotides.

In some embodiments described herein, the inhibitory nucleic acid is 8 to 30 nucleotides in length.

In some embodiments described herein, at least one nucleotide of the inhibitory nucleic acid is a nucleotide analogue.

5 In some embodiments described herein, at least one nucleotide of the inhibitory nucleic acid comprises a 2' O-methyl, e.g., wherein each nucleotide of the inhibitory nucleic acid comprises a 2' O-methyl.

In some embodiments described herein, the inhibitory nucleic acid comprises at least one ribonucleotide, at least one deoxyribonucleotide, or at least one bridged
10 nucleotide.

In some embodiments described herein, the bridged nucleotide is a LNA nucleotide, a cEt nucleotide or a ENA modified nucleotide.

In some embodiments described herein, each nucleotide of the inhibitory nucleic acid is a LNA nucleotide.

15 In some embodiments described herein, one or more of the nucleotides of the inhibitory nucleic acid comprise 2'-fluoro-deoxyribonucleotides and/or 2'-O-methyl nucleotides.

In some embodiments described herein, one or more of the nucleotides of the inhibitory nucleic acid comprise one of both of ENA nucleotide analogues or LNA
20 nucleotides.

In some embodiments described herein, the nucleotides of the inhibitory nucleic acid comprise comprising phosphorothioate internucleotide linkages between at least two nucleotides, or between all nucleotides.

In some embodiments described herein, the inhibitory nucleic acid is a gapmer
25 or a mixmer.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Methods and materials are described herein for use in the present invention; other, suitable methods and materials known in the art can also
30 be used. The materials, methods, and examples are illustrative only and not intended to be limiting. All publications, patent applications, patents, sequences, database entries, and other references mentioned herein are incorporated by reference in their

entirety. In case of conflict, the present specification, including definitions, will control.

Other features and advantages of the invention will be apparent from the following detailed description and figures, and from the claims.

5

REFERENCE TO SEQUENCE LISTING

This application includes a sequence listing submitted herewith in electronic format. The entire content of this files is hereby incorporated by reference.

DESCRIPTION OF DRAWINGS

Figures 1A-H. Telomeric RNAs produced by the sex chromosomes

10

A. TERRA RNA FISH followed by PAR DNA FISH using P34567 probes, which are subsets of BAC RP24-500I4 DNA. Higher exposure of TERRA RNA FISH revealed that multiple TERRA foci were sparsely distributed across the nucleus in mES cells. DAPI detects nuclear DNA.

15

B. Map of the PAR and relative positions of BAC clones, RP24-143B12 and RP24-500I4. Locations of internal (TTAGGG) repeats shown in red. Dotted purple lines, incompletely assembled regions.

20

C. TERRA RNA FISH followed by PAR DNA FISH (n=204) using P34567 probes in ES cells. Lower exposure of TERRA RNA FISH showed that the dominant TERRA foci were colocalized with PAR DNA. 80-90% of TERRA signals localized to ChrX and Y.

D. Percent colocalization of PAR and TERRA signals. n=139 (male); 209 (female).

25

E. Two color RNA FISH detecting TERRA (Alexa488, green) and PAR transcripts (Cy3, red) in ES cells. Nick-translated BAC DNA was used to detect PAR RNA.

30

F. Top panel: Map of sub-BAC probes and PCR amplicons. Left panel: Northern blot analysis of PAR-TERRA RNA using either TERRA or 36K oligo probes in ES cells on differentiation days 0-12, as indicated. GAPDH, loading control. Right panel: Primer extension using an antisense TERRA oligo probe with PCR amplification using PAR-specific primer pairs located at 33, 36, and 39 k (kb) from the end of BAC RP24-500I4TERRA. +, with RT; -, without RT.

G. Northern blot analysis of PAR-TERRA in ES cells using TERRA-specific or PAR-specific oligo probes, as shown in panel F.

H. Higher exposure RNA FISH indicating colocalization of TERRA and PAR signals at both large and small foci in ES cells. Three-color RNA FISH (upper panel):
 5 TERRA oligo probe; PAR-specific probes, 47k and 22k. Two-color RNA FISH (lower panel): TERRA oligo probe; PAR specific probe, 31k. DAPI was used for nuclear staining. Right graph, quantitation of colocalization.

Figures 2A-I. Mapping genomic PAR-TERRA binding sites by ChIRT-seq

A. To capture the PAR-TERRA transcripts, five DNA oligo probes were used:
 10 22k, 31k, 34k, 36k, and 47k. Each probe has multiple alignments to the RP24-500I4 BAC DNA around the telomeric repeats.

B. RNA slotblot analysis showing that TERRA-AS and PAR-31-AS probes specifically captured TERRA RNA by ChIRT. Total RNA was extracted from beads after ChIRT hybridization without RNase H elution.

15 C. Quantitative PCR showing the enrichment of PAR DNA in TERRA-AS ChIRT and PAR ChIRT, but not TERRA-S ChIRT in ES cells. TERRA-AS ChIRT used antisense DNA oligos against TERRA, TERRA-S ChIRT used TERRA sense probes as a control, and PAR ChIRT used PAR probes for the PAR transcripts.

D. Enrichment of PAR DNA following TERRA ChIRT was observed only
 20 when elution was performed with RNaseH. Enrichment was abolished in the RNase A pre-treated control (Pre-RNaseA).

E. Table of ChIRT results indicating the number of PAR and TERRA binding sites in ES cells on different days of differentiation and in MEFs. Different normalization methods produced similar results, as shown.

25 F. Scatterplot analysis comparing log2 coverages of TERRA and PAR ChIRT in indicated samples. Pearson's r shown. ChIRT results were normalized to input unless otherwise indicated.

G. TERRA ChIRT-seq showed enrichment for telomeric repeats DNA in female ES cells. Samples captured by TERRA-AS or TERRA-S. No-RNaseH for the
 30 TERRA-AS capture is also shown as a control.

H. Pie charts show relative representation of various genomic regions in TERRA (top) and PAR (bottom) ChIRT-seq experiments in female ES cells.

I. CEAS analysis shows significant over-representation of introns and noncoding regulatory regions. Exons are under-represented. ***, $P < 0.001$ (one-sided binomial test). The genome reference was obtained from the ChIRT-seq input.

Figures 3A-D. X-linked PAR-TERRA RNA binds in cis and in trans to multiple chromosomes.

A. ChIRT-seq tracks showing PAR-TERRA enrichment at the ends of various chromosomes in female ES cells (top) and MEFs (bottom). TERRA ChIPT-seq data was normalized to input (TERRA/input), no-RNase H control (TERRA/no RNase H), or the sense control (TERRA/sense).

B. Magnified views of the female ES PAR-TERRA ChIRT-seq results for the pseudoautosomal regions of ChrX and ChrY.

C. Female ES ChIRT-seq tracks showing PAR-TERRA enrichment on multiple autosomes. Red bars, internal TTAGGG repeats. Grey bars, sequence gaps.

D. ChIRT-seq tracks of female ES cells showing PAR-TERRA binding to non-telomeric autosomal regions.

Figures 4A-G. Transcriptome analysis of PAR-TERRA-depleted cells.

A. Northern blot analysis of TERRA RNA shows depletion by treatment with gapmer LNA against TERRA or PAR in ES cells. Control, scramble LNA gapmer (Scr KD).

B. RNA FISH detecting TERRA (Alexa-488, green), or PAR (Cy5, cyan blue) after LNA knockdown in ES cells.

C. Venn diagram of genes affected by TERRA versus PAR KD. Number of genes in each circle and overlapped region is indicated. 56 genes are shared between TERRA and PAR KD in female ES cells; 36 in MEF.

D. Heatmap of differentially expressed genes in TERRA KD, PAR, and Scr KD. 56 shared genes were examined for ES cells; 36 shared genes for MEF. Scale in Log₁₀ FPKM.

E. Heatmap of differentially expressed genes in TERRA KD, PAR KD, and Scr KD ES cells or MEFs. 8 genes were shared in both ES cells and MEFs.

F. Probability density function for the 565 genes with and 14,724 genes without PAR-TERRA binding sites in the structural gene ± 10 Kb of flanking sequence, with respect to their likelihood of changing gene expression following PAR-TERRA KD. Log₂ fold-change (Δ FPKM) is plotted. After PAR-TERRA KD, there is a net

downregulation for the group of genes with PAR-TERRA binding sites. Kolmogorov-Smirnoff (KS) test, $P < 0.0001$.

G. Cumulative fraction that genes with or without PAR-TERRA sites would be up- or down-regulated following PAR or TERRA KD, as indicated. P-values determined by χ^2 analyses.

Figures 5A-F. PAR-TERRA protects escapees and genes of the subtelomeric/pseudoautosomal regions from silencing.

- A. RNA-seq shows downregulation of subtelomeric genes following TERRA KD in ES cells. ChIRT-seq and post-KD RNA-seq tracks are shown.
- 10 B. RT-qPCR confirms that Tmx3 and Wls are downregulated upon TERRA KD in MEFs.
- C. Whole-ChrX view of PAR-TERRA binding sites. Two regions (boxes) show high-level binding. Escapee genes shown below the chart.
- D. Table showing the numbers of total and ChrX PAR-TERRA binding sites in female ES cells and in MEFs.
- 15 E. Probability density functions for escapees ($n=15$), Xi genes ($n=438$) subject to XCI. Escapee genes have higher PAR-TERRA binding densities relative to genes subject to XCI ($P < 0.001$ for TERRA density, $P < 0.05$ for PAR density, KS test).
- F. RT-qPCR of pseudoautosomal genes following PAR or TERRA KD. P-values determined by the Student t-test.
- 20

Figures 6A-I. TERRAs regulate the gene expression on the PAR

- A. Dynamics of Xist RNA spread following PAR-TERRA KD in female MEFs. Shown are tracks for Xist CHART-seq after Scr, TERRA, or PAR KD, and tracks for PAR-TERRA ChIRT-seq. Yellow-shaded region corresponds to the PAR-TERRA and Xist boundaries within Mid1.
- 25 B. Metagene analysis of PAR-TERRA density across XCI-repressed ($n=438$) and escapee genes ($n=15$). Relative PAR-TERRA density from PAR or TERRA ChIRT in MEFs was produced by CEAS analysis.
- C. Scatterplot analysis comparing Xist coverage ($\log_2$ scale) in PAR-TERRA KD female MEFs relative to Scr KD on ChrX. The transcriptomic profiles are highly similar (Pearson's $r > 0.90$). Outliers (dots) map to the "borders" of pseudoautosomal genes. Xist coverage files were normalized to the corresponding
- 30

ChrX median values, and individual dots in the scatterplot represents an average of two biological replicates.

D. Metagene analysis of Xist density across XCI-repressed (n=438) and escapee genes (n=15) after TERRA KD or Scr KD in MEFs.

5 E. RNA FISH detecting TERRA (Alexa-488) and Xist (Cy3, red) in MEFs cells. 87% show colocalization (n=139).

F. 3D DNA FISH to determine the colocalization frequency of PAR (Cy3), the Xic (Ftx-Jpx probe; Cy5) and Hprt (FITC). A colocalization event is scored when two signals show overlapped pixels in 3D space. N=276 nuclei. P, determined
10 by two-tailed Fisher's exact test.

G. 3D DNA FISH to determine the frequency of PAR-Xic colocalization after PAR-TERRA KD. A colocalization event is scored when two signals show overlapped pixels. N = 256-272 nuclei. P, determined by two-tailed Fisher's exact test.

H. 2D model: PAR-TERRA protects escapees from Xist silencing by
15 setting up a privileged compartment and walling off Xist at the 5' end of escapee genes. When PAR-TERRA is depleted, Xist spreads into the privileged compartment.

I. 3D Model: PAR-TERRA as an organizing center. PAR-TERRA forms a privileged nuclear compartment next to the Xist cloud. The Xi is partitioned spatially into a silent domain and an active domain for escapees.

20 Figures 7A-I. TERRAs regulate Xic pairing in mES cells

A. PAR-TERRA ChIRT-seq tracks of the Xic pairing center (red bar) in MEFs and in ES cells on d0, d3, and d7 of differentiation. Note prominent ES-specific PAR-TERRA peaks at the pairing center.

B. Cumulative frequency curves of inter-allelic differences measured
25 between Xic-Xic, telomere-telomere, and Hprt-Hprt (bottom). Measurements were taken on DNA FISH experiments (representative DNA FISH image is shown) which detected Xic (Xist), TeloX (RP23-461E16, ChrX telomeric BAC), and Hprt (Cy5). ES cells on d0 and d4 shown. Normalized distance (ND) = distance /d, where d = 2 X (nuclear area/ π)^{0.5}. ND 0.0-0.2 are shown. n=109-120. P values were determined
30 using the KS test.

C. Cumulative frequency curves for inter-allelic telomeric distances for ChrX (TeloX) or Chr2 (Telo2) on day 4 of ES differentiation. n=120-158. P values were determined using the KS test.

D. PAR-to-PAR pairing during female and male ES cell differentiation. n=246-385. P values were determined using the KS test.

E. Cumulative frequency of paired PAR DNA (TERRA RNA signals) in male ES cells on d0 versus d4. DNA FISH shows that, on d4 of differentiation, the PAR's of ChrX and ChrY were frequently colocalized (one dot) or very close in 3D space (2 neighboring dots of <0.2 ND). N = 149 (d0); 176 (d4). P value was determined using the KS test.

F. Cumulative frequency curves show that TERRA knockdown disrupted telomeric pairing in both female and male ES cells at 6 hr post-transfection at d4 of differentiation. P values were determined using the KS test. n=235-336.

G. Cumulative frequency curve shows that TERRA knockdown disrupted Xic-Xic pairing in female ES cells at 3 hr post-transfection on d4 of differentiation. P=0.001 (KS test). n=326-377.

H. Cumulative frequency curves indicate an increase in Xic-telo distances after 3 hours of TERRA KD in d4 female ES cells. P=0.009 (KS test). n=174-214.

I. Model: Without wishing to be bound by theory, it is believed that PAR-TERRA forms an organizing center to facilitate X-X pairing. (1) Prior to cell differentiation, the two female X-chromosomes are separated. (2) During early cell differentiation, trans-interactions between two telomeres bring the sex chromosomes in close proximity. (3) PAR-TERRA also drives the intra-chromosomal interactions between the Xic and the telomere in cis. (4) These events bring the Xic pairing center to the same juxta-telomeric compartment, accelerating the homology search between the two Xic pairing centers by the reduced effective search space. The pairing event induces initiation of XCI in female cells. In male cells, the telomeric pairing interaction also occurs, but is not followed by Xic pairing; thus, XCI is not initiated.

Figures 8A-E. Cytological analysis of PAR-TERRA transcripts. This figure relates to Figures 1A-H.

A. DNA FISH detecting PAR DNA using P34568 sub-probes of BAC RP24-500I4 DNA (Cy3, red), and X chromosomes (FITC labeled X painting probes, green) on metaphase spread in female ES cells. P34568 probes mark on the end of X chromosomes.

B. Electrophoresis of PCR products amplified from BAC RP24-500I4 DNA. The pools of P3, P4, P5, P6, and P8 PCR products were used for generating P34568 sub-probes to detect PAR DNA in DNA FISH experiments.

C. RNA FISH detecting TERRA RNA in various human (lower panel) and mouse (upper panel) cell lines.

D. PAR-TERRA RNA is localized next to the Xist cloud. RNA FISH detecting TERRA (Alexa-488, green), I4-31k (Cy5, cyan blue), and Xist (Cy3, red) in MEFs (upper panel). Image of the overexposed TERRA foci (green) to display moderate intensity of TERRA foci was shown in the lower panel. False color for I4-31k (red, lower panel).

E. RNA FISH detecting TERRA (Cy5, cyan blue), Xist (FITC, green), the PAR transcripts with DNA oligo probes: I4-47k (Alexa-488, green) and I4-22k (Cy3, red) in MEFs (hybrid strain cas/mus, Xist clouds specifically on mus alleles), female ES cells (hybrid strain, cas/mus) and male ES cells (mus/mus). I4-22k probes only mark on cas alleles.

Figures 9A-C. ChIRT-seq statistics. This figure relates to Figure 2.

A. Quantitative PCR showing the enrichment of PAR DNA following ChIRT using oligo probes TERRA-AS (AS) that targets to TERRA transcripts or sense probes. Various detergents (0.1% NP40, or 0.1% SDS, or 0.1% N-lauryol Sarcosine) were added separately during the final DNA elution. NP40 retains RNase H activity better than other detergents in ChIRT elution.

B. Scatterplot comparing log2 coverages of biological replicates for PAR and TERRA ChIRT-seq analysis in ES cells. Pearson's r shown. Replicate 1 (Rep1) was normalized with input. Replicate 2 (Rep2) was normalized to RNaseA pre-treated control.

C. Read statistics for two biological replicates of the PAR-TERRA ChIRT-seq analysis.

Figure 10. PAR-TERRA RNA binds subtelomeric regions of select autosomes.

This figure relates to Figure 3. ChIRT-seq tracks showing PAR-TERRA enrichment at the subtelomeric regions of Chr19, 8, and 16 in ES cells.

Figures 11A-C. PAR-TERRA knockdown by LNA gapmers. This figure relates to Figure 4.

A. LNA gapmers efficiently knocked down PAR-TERRA in ES cells after 1 to 48 hours.

B. RNA FISH detecting TERRA (Alexa-488, green) after LNA transfection at various time points in ES cells.

5 C. As shown by scatterplots comparing log2 FPKM values, there is good correlation between biological replicates of RNA-seq biological replicates after PAR-TERRA KD in ES cells and MEFs. Pearson's r as shown.

Figures 12A-B. Analysis of gene expression following PAR-TERRA knockdown. This figure relates to Figure 5.

10 A. ChIRP-seq tracks (red) for PAR-TERRA binding sites near differentially downregulated genes after PAR-TERRA KD in ES cells. RNA-seq coverage are FPM-normalized and tracks are shown in blue.

B. ChIRP-seq tracks (red) for PAR-TERRA binding sites near differentially upregulated genes after PAR-TERRA KD in ES cells. RNA-seq
15 coverage are FPM-normalized and tracks are shown in blue.

Figures 13A-B. Telomeric pairing analysis: Whole distributions of inter-PAR distances. This figure relates to Figure 7.

A. Distributions of PAR-PAR distances in female ES cells on days 0, 4, and 8 of differentiation. Normalized distance (ND) = PAR-PAR distance / d, where d =
20 $2 \times (\text{nuclear area} / \pi)^{0.5}$.

B. Distributions of PAR-PAR distances in male ES cells on days 0, 4, and 8 of differentiation.

DETAILED DESCRIPTION

XCI is an epigenetic pathway that results in silencing of one X-chromosome in
25 the female cell to compensate for unequal X-chromosome number between male (XY) and female (XX) cells (Starmer and Magnuson, 2009; Lee, 2011; Wutz, 2011; Disteche, 2012). The pathway is controlled by long noncoding RNAs (lncRNA) of the X-inactivation center (Xic). During early development and as recapitulated by differentiating embryonic stem (ES) cells, the X-to-autosome ratio is assessed and the
30 XCI pathway is induced only when there is more than one X-chromosome in a diploid nucleus. This "counting" mechanism has been proposed to involve a titration of the X-linked Jpx lncRNA and autosomally encoded CTCF protein (Sun et al., 2013). In

parallel, a transient interaction (“pairing”) between two female X-chromosomes mediates the mutually exclusive choice of Xi and Xa (active X) (Bacher et al., 2006; Xu et al., 2006), with the subsequent action of Tsix lncRNA blocking XCI on the designated Xa (Lee et al., 1999) and the action of Xist lncRNA inducing whole-
 5 chromosome silencing on the designated Xi (Brown et al., 1992; Penny et al., 1996). Xist spreads along the Xi and recruits silencing complexes (Zhao et al., 2008; Wutz, 2011). With the exception of a small class of genes that escape XCI, nearly all 1000 genes on the Xi are subject to silencing. Although significant progress has been made, many aspects of XCI mechanism continue to elude understanding.

10 TERRA’s affinity for sex chromosomes led the present inventors to hypothesize that TERRA might have non-telomeric functions; based on its association with sex chromosomes, possible roles surrounding the process of X-chromosome inactivation (XCI) were investigated. TERRA’s association with the X-chromosome provides a new and potentially relevant avenue for exploration. Here we generate a
 15 map of TERRA’s genomic binding sites, identify multiple non-telomeric targets, and interrogate the relationship of X-linked target sites to sex chromosome biology.

Here we have shown that TERRA function is not confined to telomeres, nor is it cis-limited in action. TERRA is predominantly expressed from the sex chromosomes and originates at least in part from the sub-telomeric region known as
 20 the pseudoautosomal region (PAR). Several lines of evidence argue that X- and Y-linked “PAR-TERRA” is a continuous transcript and that PAR-TERRA comprises a major subclass of telomeric RNAs. First, similar results are obtained by Northern blot analysis using PAR and TERRA oligo probes (Fig. 1F,G). Second, primer extension indicates that TERRA and PAR RNA sequences are physically continuous (Fig. 1F).
 25 Third, RNA FISH experiments using TERRA and PAR probes demonstrate overlapping RNA signals (Fig. 1A,C,E,H). Fourth, ChIRT analysis indicates that nearly all TERRA-binding sites are also PAR-binding sites (Fig. 3,S3). Furthermore, knocking down PAR sequences using LNA gapmers results in TERRA depletion as well (Fig. 4A,B). FISH analysis demonstrates that PAR-TERRA establishes a
 30 compartment next to the telomeric ends of each sex chromosome, with a large PAR-TERRA RNA focus localizing next to but not overlapping the Xist RNA domain.

We postulate that PAR-TERRA is as an organizing center for two XCI-related processes (Fig. 6H-I, 7I). First, PAR-TERRA regulates gene regulation on a global

scale (Fig. 4). Altogether, we identified hundreds of TERRA-binding sites throughout the genome in MEFs, of which 30-94 are X-linked. High PAR-TERRA coverage occurs near escapee genes, including genes of the pseudoautosomal region (Fig. 5). Perturbation experiments demonstrate that X-linked PAR-TERRA sites promote expression of escapee genes on the Xi. In the pseudoautosomal region, PAR-TERRA appears to protect genes from telomeric position effects. Analysis of Xist RNA localization indicated that Xist RNA is often enriched within defined peaks near escapees (Simon et al., 2013), such as the prominent peaks seen at *Mid1* (Fig. 6A). These peaks suggest that Xist RNA may be sequestered at “boundaries” near escapee genes and be prevented from entering privileged loci. The idea of a boundary near escapees has been explored previously, with CTCF emerging as a candidate regulator (Filippova et al., 2005; Horvath et al., 2013). The loss of these Xist-enriched boundaries following PAR-TERRA depletion argues that PAR-TERRA also aids in formation of the Xist boundaries. We therefore propose a model in which PAR-TERRA holds Xist RNA in check and brings escapee genes into a privileged juxta-telomeric compartment that is permissive of transcription (Fig. 6H-I).

Methods of Reducing Expression of X-linked Escapee Genes

The present methods include using antisense oligonucleotides (ASO) against PAR-TERRA RNA to downregulate expression of escapee genes. In humans, all chromosomes may have the capacity to produce TERRA transcripts, each from their own subtelomeric regions. These subtelomeric regions are chromosome-specific; therefore, the X and Y subtelomeric region (also called pseudoautosomal region) are distinct from autosomes. Sex-chromosome-specific effects can be achieved by targeting the PAR end of the telomeric transcript. Without wishing to be bound by theory, knocking down PAR, TERRA, or PAR-TERRA with an inhibitory nucleic acids, e.g., an ASO, may disrupt the organizing center and thereby induce escapee gene downregulation. These inhibitory nucleic acids can therefore be used to treat disorders of sex chromosome aneuploidy, e.g., Klinefelter Syndrome (XXY) and Triple X Syndrome (XXX), or any other condition that results in extra copies of all or part of the X-chromosome (e.g., unbalanced X-autosome translocations). While individuals with extra X chromosomes are mostly dosage compensated due to the counting mechanism (XXX women have two Xi's; XXY men have 1 Xi), they have uncompensated dosage of the 15 % of X-linked genes that escape XCI.

Escapee genes include those listed in Table A.

TABLE E Escapee Genes	
Human Escapee Genes	
PR48; CALB3; SYAP1; HDHD1A; T54860; BC014382; AA348446; DKFZP564I1922; PRKX; Hs.431292; ITM2A; SRPX2; KIAA1817; MDS031; FLJ23018; HSU24186; Hs.271686; WBP5; TRPC5; TNFSF5; Hs.122516; Hs.404298; ARMCX4; FLJ11016; Hs.333016; DOCK11; LOC203427; CITED1; PLP1; PLCXD1; SLC25A6; LOC375793; ASMTL; DHRSX; FLJ43159; FLJ39679; CD99; XG; GYG2; ARSD; ARSE; Hs.399941; FLJ43700; AA971220; NLGN4X; FLJ12417 ; STS; Hs.186498; Hs.495638 ; PNPLA4; Hs.495641; Hs.348675 ; MGC17403; RAB9A; SEDL; Hs.41434; AA952971; FAM51A1; PIR; TMEM27; CA5BL; CA5B; AP1S2; Hs.121592; Hs.431654; CTPS2; Hs.431102; CXORF15; RBBP7; EIF1AX; EIF2S3; ZFX; Hs.458197; Hs.128084; USP9X; Hs.282780; Hs.86849 ; Hs.229338; DDX3X; MAOA; DUSP21; Hs.232417; AA130835; UBE1; INE1; JARID1C; A009X24; KIAA0522; Hs.87752; RPS4X; XIST; FLJ31610; F03810; PLXNB3 ; AVPR2; IKBKG; N74477; GPM6B; MGC39350; FUNDC1; SH3BGRL; L1CAM; GAB3; Hs.86443 ; TBL1X; GPR143; SMC1L1; RIBC1; Hs.258828; FLJ38564; NAP1L3; ZD89B07; SYTL4; Hs.527551~; ARHGAP4 ; RENBP; PCTK1; GRPR; CHM; HEIL2; HCFC1; OFD1; CRSP2; CLCN4; Hs.157695; MORF4L2; MYCL2; BRS3; ARD1; CXORF12; AF069137; Hs.108029 ; SH3KBP1; USP11; WAS; XEDAR; MAGEE1; ATP7A; Hs.445729; NXF3; LOC340544; PLS3; CUL4B; DXYS155E; MKRN4; 23809; MSL3L1; ASB11; NHS; PHEX ; TIMP1; MLLT7 ; PIN4; COX7B; RAB40A; COL4A6; FLJ36576; UTP14A; COVA1; PLAC1; LOC159090; MAGEA8; ABCD1; C6.1A; CLIC2; PDZK10; REPS2; CDKL5; Hs.435570 ; Hs.446513; RS1; PHKA2; N53651; Hs.444490; ACATE2; TAB3; Hs.177986; BCoR; SYP; CCNB3; LOC51248; FLJ20105; Hs.37464; ABCB7; Hs.182171; BTK; RPL36A; GLA; BEX1; FLJ21174; NXT2; FLJ22679; Hs.425072; AMMECR1; Hs.61094; PAK3; LHFPL1; FLJ22965; UPF3B; MCTS1; GPC4; PHF6; MOSPD1; Hs.436787 ; CDR1; SLITRK2; LOC347512~; ZNF185; M78874; HCA127; FLJ34366; FLJ12525 ; FMR1; IRAK1; TKTL1; VBP1; Hs.522189~; KIAA1280; MID1; Hs.187608; ARHGAP6; H48827; M62076; GLRA2; EUROIMAGE 35971; CXorf23; SAT; AA601738; DMD; AA461044; FLJ42925; TM4SF2; FLJ43479; ATP6AP2; ZC35F11; SLC9A7; RGN; SLC38A5; GATA1; KCND1; GRIPAP1; FLJ21687; HADH2; UREB1; DT1P1A10; FGD1; Hs.13041; LOC90736; MAGEH1; W68846; DKFZp686L07201; LOC92249; Hs.38448; MSN; STARD8; EFNB1; PJA1; ACRC; GPR23; FLJ13042; TM4SF6; ARMCX1; ARMCX2; Hs.53997; MGC23947; RAB9B; FLJ33516 ; CLDN2; AI650369; H66935; PRPS1; PSMD10; APG4A; CHRDL1; KLHL13; NKAP; PEPP-2~; ODZ1; XPNPEP2; CXorf9; Hs.269127; FHL1 ; Hs.205436; FLJ38034; ATP11C; Hs.112784; Hs.127679; Hs.31542; LDOC1; CD99L2; PNMA5; SYBL1; G06389; TIMM8A; HPRT1; FAM9C; AW448933; UBQLN2 ; FLJ31204; PGPL; SHOX; ZBED1; KAL1 ; IL9R; IL9R	
Mouse Escapee Genes	
1810030O07Rik; 5530601H04Rik; 5730416F02Rik; Abcb7; Aff2; Bgn; Car5b; Col4a6; Cox7b; Cxx1b; Ddx3x; Dkc1; Dmd; Dusp9; Eda; Eif2s3x; Fgf13; Firre; Frmpd4; Ftx; Gm5124; Gyk; Hmgb3; Il1rap11; Irak1; Jpx; Kdm5c; Kdm6a; Kif4; Lage3; Mageb16; Mageb18; Mbtps2; Mid1; Msn; Naa10; Ndufb11; Ngfrap1; Nono; Pbdcl; Pdhal; Plp2; Polal; Praf2; Prickle3; Rbbp7; Rbm10; Reps2; Rpl39; Rps4x; Sept6; Shroom4; Slc16a2; Slc25a5; Usp11; Wbp5; Xist; Flna; Ikbkg; Hcfc1; Huwel; Maged1; Ogt; Asmt; and Erdr1	

305 escapees in human, and 65 escapees in mouse

human escapees defined as $X_i/X_a > 0.1$ based on allele specific PCR in this paper:
(Nature, 2005, Laura Carrell & Huntington F. Willard) X-inactivation profile reveals
extensive variability in X-linked gene expression in females
#mouse escapees defined as $X_i/X_a > 0.1$ or X_i read counts > 5 in MEF RNA-seq

- 5 Table B provides a list of escapee genes in the human pseudoautosomal region (PAR) of the X chromosome. Annotated gene name and full gene description are shown. Whether or not there is a Y-chromosome homologue is noted in the third column.

Table B. Human PAR genes or Y orthologues or pseudogene

PR48	Protein phosphatase 2A 48 kDa subunit	Pseudoautosomal; Y identity
PLCXD1	Phosphatidylinositol phospholipase C, X domain 1	Pseudoautosomal; Y identity
SLC25A6	Solute carrier family 25, member 6	Pseudoautosomal; Y identity
LOC375793	Hypothetical protein with EST support	Pseudoautosomal; Y identity
ASMTL	Acetylserotonin O-methyltransferase-like	Pseudoautosomal; Y identity
DHRX	Dehydrogenase/reductase (SDR) family	Pseudoautosomal; Y identity
FLJ43159	mRNA of unknown function	Pseudoautosomal; Y identity
FLJ39679	mRNA of unknown function	Pseudoautosomal; Y identity
CD99	CD99 antigen	Pseudoautosomal; Y identity
DXYS155E	Lymphocyte surface protein	Pseudoautosomal; Y identity
PGPL	Pseudoautosomal GTP-binding protein-like	Pseudoautosomal; Y identity
SHOX	Short stature homeobox	Pseudoautosomal; Y identity
ZBED1	zinc finger, BED domain containing 1	Pseudoautosomal; Y identity
SYBL1	Synaptobrevin-like 1	Pseudoautosomal; Y identity
IL9R	Interleukin 9 receptor	Pseudoautosomal; Y identity
RPS4X	Ribosomal protein S4, X isoform	Y orthologue
UBE1	Ubiquitin-activating enzyme E1	Ancestral Y homolog
JARID1C	Jumonji, AT rich interactive domain 1C	Y orthologue
RAB9A	Ras-related GTP-binding protein	Y homology by BLAST
SEDL	Spondyloepiphyseal dysplasia, late	Y pseudogene
CXORF15	Chromosome X open reading frame 15	Y orthologues
EIF1AX	Eukaryotic translation initiation factor 4C	Y orthologue
ZFX	Zinc finger protein X-linked	Y orthologue
USP9X	Ubiquitin specific protease 9, X isoform	Y orthologue
DDX3X	DEAD/H box 3, X-linked	Y orthologue
DUSP21	Dual specificity phosphatase 21	Y orthologue
OFD1	Oral-facial-digital syndrome 1 gene	Y pseudogene
CRSP2	Cofactor required for Sp1 transcriptional activation	Y pseudogene
TAB3	TAK1-binding protein 3	Y pseudogene
BCoR	BCL6 co-repressor	Y pseudogene
FAM9C	Family with sequence similarity 9, member C	Y homology by BLAST
HDHD1A	Haloacid dehalogenase-like hydrolase domain 1	Y pseudogene
NLGN4X	Neurologin 4	Y orthologue
STS	Steroid sulfatase	Y pseudogene
TBL1X	Transducin (beta) like 1	Y orthologue

Table B. Human PAR genes or Y orthologues or pseudogene

GPR143	G protein-coupled receptor 143	Y pseudogene
KAL1	Kallmann syndrome 1 sequence	Y pseudogene
AA348446	ESTs	Y homology by BLAST
DKFZP564I1922	Adlican	Y pseudogene
PRKX	Protein kinase, X-linked	Y orthologue
XG	Xg blood group	Y pseudogene
GYG2	Glycogenin 2	Y pseudogene
ARSD	Arylsulfatase D	Y pseudogene
ARSE	Arylsulfatase E	Y pseudogene
Hs.399941	ESTs	Y homology by BLAST
FLJ43700	Hypothetical protein with mRNA & EST support	Y homology by BLAST

The sequence of human PAR-TERRA is provided herewith as SEQ ID NO:1.

The sequence of mouse PAR-TERRA is provided herewith as SED ID NO:2. In some embodiments, the sequence of an oligo targeting TERRA is 5'-TAA CCC TAA CCC TAA C-3' (SEQ ID NO:5); or PAR-TERRA is 5'-TCT CTG TCT CTG TCG C-3' (SEQ ID NO:6).

Method of Reducing expression of from non-Xi genes

TERRA forms a special compartment for gene activation, not only for escapees on the Xi but also for autosomal genes and subtelomeric genes on autosomal ends, of which Chr 1, 3, 4, 8, 18, and 19 are shown herein (see, e.g., Figs. 4, 5A-B).

PAR-TERRA and various Chromosome-specific TERRAs (produced from the subtelomeric regions) are also used to regulate genes outside of the Xi. The present methods can be applied to downregulate a network of Xa and subtelomeric autosomal genes involved in growth control and apoptosis, and other processes relevant to human disease. Two examples include active X (Xa) genes and the FSHD subtelomeric region of human Chromosome 4. The Xa also produces PAR-TERRA from its pseudoautosomal region and has multiple targets outside of its pseudoautosomal region. Targeting PAR, TERRA, or PAR-TERRA can reduce expression from the Xa. PAR-TERRA and TERRA also target thousands of autosomal sites with closely linked genes. A specific example include the Chr4 region associated with facioscapulohumeral muscular dystrophy (FSHD), which is located in the subtelomeric region of human Chr4 and contains coding genes FRG1, FRG2, DUX4, and the long noncoding RNAs of forward and reverse orientations from the macrosatellite repeat, D4Z4. FSHD is caused by ectopic expression of these genes when the D4Z4 repeat contracts and becomes "activated". Thus, PAR,

TERRA, or PAR-TERRA or Chr4-specific TERRA could be targeted to downregulated the associated subtelomeric genes.

In addition, PAR-TERRA knockdown resulted in downregulation of genes enriched for cell cycle and apoptosis genes (see Example 5). Thus, targeting PAR, TERRA, or PAR-TERRA transcripts can be an effective method of treating cancer and other human diseases where the X-chromosome and various growth control genes are frequently overexpressed. The sequence of human Chr4 FSHD region is provided herewith as SEQ ID NO:3.

Disorders of Sex Chromosome Aneuploidy

The present methods can be used to reduce expression of escapee genes in subjects with disorders of sex chromosome aneuploidy in which at least one extra X chromosome is present (referred to herein as a supernumerary X chromosome). The term Klinefelter syndrome (KS) describes a group of disorders in which at least one extra X chromosome is present in addition to a normal male karyotype, referred to in standard genetics nomenclature as 46,XY. Related to the KS group is 47,XXY aneuploidy, which is the most prevalent disorder of sex chromosomes in humans with a prevalence of about 1:500. Rarer sex chromosome aneuploidies include 48,XXYY and 48,XXXY (about 1:17,000 to 1:50,000); 49,XXXXY (about 1:85,000 to 1:100,000) births. See, e.g., Visootsak and Graham, Orphanet J Rare Dis. 2006; 1: 42; Targaltia et al., Acta Paediatr. 2011 Jun;100(6):851-60). Triple X syndrome (47,XXX) is a disorder in which at least one extra X chromosome is present in addition to a normal female karyotype; 48,XXXX and 49,XXXXX have also been described (Schoubben et al., Eur J Pediatr. 2011 Oct;170(10):1325-7). Conditions resulting from unbalanced X-autosome translocations or cancers (and other human diseases) with X-chromosomal aneuploidies may be treated similarly using the technology. These conditions can result in a large number of deleterious physical, psychological, and intellectual effects in affected individuals (see, e.g., Visootsak and Graham, Orphanet J Rare Dis. 2006; 1: 42; Schoubben et al., Eur J Pediatr. 2011 Oct;170(10):1325-7; Targaltia et al., Acta Paediatr. 2011 Jun;100(6):851-60).

Inhibitory Nucleic Acids Targeting PAR-TERRA or other chromosome-specific TERRA

The methods and compositions described herein can include nucleic acids such as a small inhibitory RNA (siRNA) or LNA that targets (specifically binds, or is
 5 complementary to) PAR, PAR-TERRA, or other chromosome-specific TERRA (e.g., Chr4-specific, as produced from the subtelomeric region of human Chr4 which is associated with facioscapulohumeral muscular dystrophy (FSHD)) RNA. Inhibitory nucleic acids useful in the present methods and compositions include antisense oligonucleotides, ribozymes, external guide sequence (EGS) oligonucleotides, siRNA
 10 compounds, single- or double-stranded RNA interference (RNAi) compounds such as siRNA compounds, molecules comprising modified bases, locked nucleic acid molecules (LNA molecules), antagomirs, peptide nucleic acid molecules (PNA molecules), and other oligomeric compounds or oligonucleotide mimetics which hybridize to at least a portion of the target nucleic acid and modulate its function. In
 15 some embodiments, the inhibitory nucleic acids include antisense RNA, antisense DNA, chimeric antisense oligonucleotides, antisense oligonucleotides comprising modified linkages, interference RNA (RNAi), short interfering RNA (siRNA); a micro, interfering RNA (miRNA); a small, temporal RNA (stRNA); or a short, hairpin RNA (shRNA); small RNA-induced gene activation (RNAa); small activating
 20 RNAs (saRNAs), or combinations thereof. See, e.g., USSN 62/010,342, WO 2012/065143, WO 2012/087983, and WO 2014/025887. However, in some embodiments the inhibitory nucleic acid is not an miRNA, an stRNA, an shRNA, an siRNA, an RNAi, or a dsRNA.

In some embodiments, the inhibitory nucleic acids are 10 to 50, 10 to 20, 10 to
 25 25, 13 to 50, or 13 to 30 nucleotides in length. One having ordinary skill in the art will appreciate that this embodies inhibitory nucleic acids having complementary portions of 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, or 50 nucleotides in length, or any range therewithin. In some embodiments, the inhibitory nucleic
 30 acids are 15 nucleotides in length. In some embodiments, the inhibitory nucleic acids are 12 or 13 to 20, 25, or 30 nucleotides in length. One having ordinary skill in the art will appreciate that this embodies inhibitory nucleic acids having complementary portions of 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30

nucleotides in length, or any range therewithin (complementary portions refers to those portions of the inhibitory nucleic acids that are complementary to the target sequence).

5 The inhibitory nucleic acids useful in the present methods are sufficiently complementary to the target RNA, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect. "Complementary" refers to the capacity for pairing, through hydrogen bonding, between two sequences comprising naturally or non-naturally occurring bases or analogs thereof. For example, if a base at one position of an inhibitory nucleic acid is capable of hydrogen bonding with a base at
10 the corresponding position of a RNA, then the bases are considered to be complementary to each other at that position. 100% complementarity is not required.

 Routine methods can be used to design an inhibitory nucleic acid that binds to the target sequence with sufficient specificity. In some embodiments, the methods include using bioinformatics methods known in the art to identify regions of
15 secondary structure, e.g., one, two, or more stem-loop structures, or pseudoknots, and selecting those regions to target with an inhibitory nucleic acid. For example, "gene walk" methods can be used to optimize the inhibitory activity of the nucleic acid; for example, a series of oligonucleotides of 10-30 nucleotides spanning the length of a target RNA can be prepared, followed by testing for activity. Optionally, gaps, e.g.,
20 of 5-10 nucleotides or more, can be left between the target sequences to reduce the number of oligonucleotides synthesized and tested. GC content is preferably between about 30-60%. Contiguous runs of three or more Gs or Cs should be avoided where possible (for example, it may not be possible with very short (e.g., about 9-10 nt) oligonucleotides).

25 In some embodiments, the inhibitory nucleic acid molecules can be designed to target a specific region of the RNA sequence. For example, a specific functional region can be targeted, e.g., a region comprising a known RNA localization motif (i.e., a region complementary to the target nucleic acid on which the RNA acts). Alternatively or in addition, highly conserved regions can be targeted, e.g., regions
30 identified by aligning sequences from disparate species such as primate (e.g., human) and rodent (e.g., mouse) and looking for regions with high degrees of identity. Percent identity can be determined routinely using basic local alignment search tools

(BLAST programs) (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656), e.g., using the default parameters.

Once one or more target regions, segments or sites have been identified, e.g., within a sequence known in the art or provided herein, inhibitory nucleic acid
5 compounds are chosen that are sufficiently complementary to the target, i.e., that hybridize sufficiently well and with sufficient specificity (i.e., do not substantially bind to other non-target RNAs), to give the desired effect.

In the context of this invention, hybridization means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between
10 complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. Complementary, as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a RNA
15 molecule, then the inhibitory nucleic acid and the RNA are considered to be complementary to each other at that position. The inhibitory nucleic acids and the RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridisable" and "complementary" are terms
20 which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the inhibitory nucleic acid and the RNA target. For example, if a base at one position of an inhibitory nucleic acid is capable of hydrogen bonding with a base at the corresponding position of a RNA, then the bases are considered to be complementary to each other at that position.

25 100% complementarity is not required.

It is understood in the art that a complementary nucleic acid sequence need not be 100% complementary to that of its target nucleic acid to be specifically hybridisable. A complementary nucleic acid sequence for purposes of the present methods is specifically hybridisable when binding of the sequence to the target RNA
30 molecule interferes with the normal function of the target RNA to cause a loss of activity, and there is a sufficient degree of complementarity to avoid non-specific binding of the sequence to non-target RNA sequences under conditions in which specific binding is desired, e.g., under physiological conditions in the case of in vivo

assays or therapeutic treatment, and in the case of in vitro assays, under conditions in which the assays are performed under suitable conditions of stringency. For example, stringent salt concentration will ordinarily be less than about 750 mM NaCl and 75 mM trisodium citrate, preferably less than about 500 mM NaCl and 50 mM trisodium citrate, and more preferably less than about 250 mM NaCl and 25 mM trisodium citrate. Low stringency hybridization can be obtained in the absence of organic solvent, e.g., formamide, while high stringency hybridization can be obtained in the presence of at least about 35% formamide, and more preferably at least about 50% formamide. Stringent temperature conditions will ordinarily include temperatures of at least about 30° C, more preferably of at least about 37° C, and most preferably of at least about 42° C. Varying additional parameters, such as hybridization time, the concentration of detergent, e.g., sodium dodecyl sulfate (SDS), and the inclusion or exclusion of carrier DNA, are well known to those skilled in the art. Various levels of stringency are accomplished by combining these various conditions as needed. In a preferred embodiment, hybridization will occur at 30° C in 750 mM NaCl, 75 mM trisodium citrate, and 1% SDS. In a more preferred embodiment, hybridization will occur at 37° C in 500 mM NaCl, 50 mM trisodium citrate, 1% SDS, 35% formamide, and 100 µg/ml denatured salmon sperm DNA (ssDNA). In a most preferred embodiment, hybridization will occur at 42° C in 250 mM NaCl, 25 mM trisodium citrate, 1% SDS, 50% formamide, and 200 µg/ml ssDNA. Useful variations on these conditions will be readily apparent to those skilled in the art.

For most applications, washing steps that follow hybridization will also vary in stringency. Wash stringency conditions can be defined by salt concentration and by temperature. As above, wash stringency can be increased by decreasing salt concentration or by increasing temperature. For example, stringent salt concentration for the wash steps will preferably be less than about 30 mM NaCl and 3 mM trisodium citrate, and most preferably less than about 15 mM NaCl and 1.5 mM trisodium citrate. Stringent temperature conditions for the wash steps will ordinarily include a temperature of at least about 25° C, more preferably of at least about 42° C, and even more preferably of at least about 68° C. In a preferred embodiment, wash steps will occur at 25° C in 30 mM NaCl, 3 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps will occur at 42° C. in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS. In a more preferred embodiment, wash steps

will occur at 68° C in 15 mM NaCl, 1.5 mM trisodium citrate, and 0.1% SDS.

Additional variations on these conditions will be readily apparent to those skilled in the art. Hybridization techniques are well known to those skilled in the art and are described, for example, in Benton and Davis (Science 196:180, 1977); Grunstein and
5 Hogness (Proc. Natl. Acad. Sci., USA 72:3961, 1975); Ausubel et al. (Current Protocols in Molecular Biology, Wiley Interscience, New York, 2001); Berger and Kimmel (Guide to Molecular Cloning Techniques, 1987, Academic Press, New York); and Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York.

10 In general, the inhibitory nucleic acids useful in the methods described herein have at least 80% sequence complementarity to a target region within the target nucleic acid, e.g., 90%, 95%, or 100% sequence complementarity to the target region within an RNA. For example, an antisense compound in which 18 of 20 nucleobases of the antisense oligonucleotide are complementary, and would therefore specifically
15 hybridize, to a target region would represent 90 percent complementarity. Percent complementarity of an inhibitory nucleic acid with a region of a target nucleic acid can be determined routinely using basic local alignment search tools (BLAST programs) (Altschul et al., J. Mol. Biol., 1990, 215, 403-410; Zhang and Madden, Genome Res., 1997, 7, 649-656). Inhibitory nucleic acids that hybridize to an RNA
20 can be identified through routine experimentation. In general the inhibitory nucleic acids must retain specificity for their target, i.e., must not directly bind to, or directly significantly affect expression levels of, transcripts other than the intended target.

For further disclosure regarding inhibitory nucleic acids, please see US2010/0317718 (antisense oligos); US2010/0249052 (double-stranded ribonucleic
25 acid (dsRNA)); US2009/0181914 and US2010/0234451 (LNAs); US2007/0191294 (siRNA analogues); US2008/0249039 (modified siRNA); and WO2010/129746 and WO2010/040112 (inhibitory nucleic acids), as well as WO 2012/065143, WO 2012/087983, and WO 2014/025887 (inhibitory nucleic acids targeting non-coding RNAs/supRNAss), all of which are incorporated herein by reference in their entirety.

30 *Antisense*

In some embodiments, the inhibitory nucleic acids are antisense oligonucleotides. Antisense oligonucleotides are typically designed to block expression of a DNA or RNA target by binding to the target and (without wishing to

be bound by theory) halting expression at the level of transcription, translation, or splicing. Antisense oligonucleotides of the present invention are complementary nucleic acid sequences designed to hybridize under stringent conditions to an RNA. Thus, oligonucleotides are chosen that are sufficiently complementary to the target, i.e., that hybridize sufficiently well and with sufficient specificity, to give the desired effect.

siRNA/shRNA

In some embodiments, the nucleic acid sequence that is complementary to a target RNA can be an interfering RNA, including but not limited to a small interfering RNA ("siRNA") or a small hairpin RNA ("shRNA"). Methods for constructing interfering RNAs are well known in the art. For example, the interfering RNA can be assembled from two separate oligonucleotides, where one strand is the sense strand and the other is the antisense strand, wherein the antisense and sense strands are self-complementary (i.e., each strand comprises nucleotide sequence that is complementary to nucleotide sequence in the other strand; such as where the antisense strand and sense strand form a duplex or double stranded structure); the antisense strand comprises nucleotide sequence that is complementary to a nucleotide sequence in a target nucleic acid molecule or a portion thereof (i.e., an undesired gene) and the sense strand comprises nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. Alternatively, interfering RNA is assembled from a single oligonucleotide, where the self-complementary sense and antisense regions are linked by means of nucleic acid based or non-nucleic acid-based linker(s). The interfering RNA can be a polynucleotide with a duplex, asymmetric duplex, hairpin or asymmetric hairpin secondary structure, having self-complementary sense and antisense regions, wherein the antisense region comprises a nucleotide sequence that is complementary to nucleotide sequence in a separate target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion thereof. The interfering can be a circular single-stranded polynucleotide having two or more loop structures and a stem comprising self-complementary sense and antisense regions, wherein the antisense region comprises nucleotide sequence that is complementary to nucleotide sequence in a target nucleic acid molecule or a portion thereof and the sense region having nucleotide sequence corresponding to the target nucleic acid sequence or a portion

thereof, and wherein the circular polynucleotide can be processed either in vivo or in vitro to generate an active siRNA molecule capable of mediating RNA interference.

In some embodiments, the interfering RNA coding region encodes a self-complementary RNA molecule having a sense region, an antisense region and a loop region. Such an RNA molecule when expressed desirably forms a “hairpin” structure, and is referred to herein as an “shRNA.” The loop region is generally between about 2 and about 10 nucleotides in length. In some embodiments, the loop region is from about 6 to about 9 nucleotides in length. In some embodiments, the sense region and the antisense region are between about 15 and about 20 nucleotides in length.

Following post-transcriptional processing, the small hairpin RNA is converted into a siRNA by a cleavage event mediated by the enzyme Dicer, which is a member of the RNase III family. The siRNA is then capable of inhibiting the expression of a gene with which it shares homology. For details, see Brummelkamp et al., Science 296:550-553, (2002); Lee et al, Nature Biotechnol., 20, 500-505, (2002); Miyagishi and Taira, Nature Biotechnol 20:497-500, (2002); Paddison et al. Genes & Dev. 16:948-958, (2002); Paul, Nature Biotechnol, 20, 505-508, (2002); Sui, Proc. Natl. Acad. Sci. USA, 99(6), 5515-5520, (2002); Yu et al. Proc Natl Acad Sci USA 99:6047-6052, (2002).

The target RNA cleavage reaction guided by siRNAs is highly sequence specific. In general, siRNA containing a nucleotide sequences identical to a portion of the target nucleic acid are preferred for inhibition. However, 100% sequence identity between the siRNA and the target gene is not required to practice the present invention. Thus the invention has the advantage of being able to tolerate sequence variations that might be expected due to genetic mutation, strain polymorphism, or evolutionary divergence. For example, siRNA sequences with insertions, deletions, and single point mutations relative to the target sequence have also been found to be effective for inhibition. Alternatively, siRNA sequences with nucleotide analog substitutions or insertions can be effective for inhibition. In general the siRNAs must retain specificity for their target, i.e., must not directly bind to, or directly significantly affect expression levels of, transcripts other than the intended target.

Ribozymes

Trans-cleaving enzymatic nucleic acid molecules can also be used; they have shown promise as therapeutic agents for human disease (Usman & McSwiggen, 1995

Ann. Rep. Med. Chem. 30, 285-294; Christoffersen and Marr, 1995 J. Med. Chem. 38, 2023-2037). Enzymatic nucleic acid molecules can be designed to cleave specific RNA targets within the background of cellular RNA. Such a cleavage event renders the RNA non-functional.

5 In general, enzymatic nucleic acids with RNA cleaving activity act by first binding to a target RNA. Such binding occurs through the target binding portion of a enzymatic nucleic acid which is held in close proximity to an enzymatic portion of the molecule that acts to cleave the target RNA. Thus, the enzymatic nucleic acid first recognizes and then binds a target RNA through complementary base pairing, and
10 once bound to the correct site, acts enzymatically to cut the target RNA. Strategic cleavage of such a target RNA will destroy its ability to direct synthesis of an encoded protein. After an enzymatic nucleic acid has bound and cleaved its RNA target, it is released from that RNA to search for another target and can repeatedly bind and cleave new targets.

15 Several approaches such as in vitro selection (evolution) strategies (Orgel, 1979, Proc. R. Soc. London, B 205, 435) have been used to evolve new nucleic acid catalysts capable of catalyzing a variety of reactions, such as cleavage and ligation of phosphodiester linkages and amide linkages, (Joyce, 1989, Gene, 82, 83-87; Beaudry et al., 1992, Science 257, 635-641; Joyce, 1992, Scientific American 267, 90-97;
20 Breaker et al, 1994, TIBTECH 12, 268; Bartel et al, 1993, Science 261 :1411-1418; Szostak, 1993, TIBS 17, 89-93; Kumar et al, 1995, FASEB J., 9, 1183; Breaker, 1996, Curr. Op. Biotech., 1, 442). The development of ribozymes that are optimal for catalytic activity would contribute significantly to any strategy that employs RNA-cleaving ribozymes for the purpose of regulating gene expression. The hammerhead
25 ribozyme, for example, functions with a catalytic rate (kcat) of about 1 min⁻¹ in the presence of saturating (10 mM) concentrations of Mg²⁺ cofactor. An artificial "RNA ligase" ribozyme has been shown to catalyze the corresponding self-modification reaction with a rate of about 100 min⁻¹. In addition, it is known that certain modified hammerhead ribozymes that have substrate binding arms made of DNA catalyze RNA
30 cleavage with multiple turn-over rates that approach 100 min⁻¹.

Modified Inhibitory Nucleic Acids

In some embodiments, the inhibitory nucleic acids used in the methods described herein are modified, e.g., comprise one or more modified bonds or bases. A

number of modified bases include phosphorothioate, methylphosphonate, peptide nucleic acids, or locked nucleic acid (LNA) molecules. Some inhibitory nucleic acids are fully modified, while others are chimeric and contain two or more chemically distinct regions, each made up of at least one nucleotide. These inhibitory nucleic acids typically contain at least one region of modified nucleotides that confers one or more beneficial properties (such as, for example, increased nuclease resistance, increased uptake into cells, increased binding affinity for the target) and a region that is a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. Chimeric inhibitory nucleic acids of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures comprise, but are not limited to, US patent nos. 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, each of which is herein incorporated by reference.

In some embodiments, the inhibitory nucleic acid comprises at least one nucleotide modified at the 2' position of the sugar, most preferably a 2'-O-alkyl, 2'-O-alkyl-O-alkyl or 2'-fluoro-modified nucleotide. In other preferred embodiments, RNA modifications include 2'-fluoro, 2'-amino and 2' O-methyl modifications on the ribose of pyrimidines, abasic residues or an inverted base at the 3' end of the RNA. Such modifications are routinely incorporated into oligonucleotides and these oligonucleotides have been shown to have a higher T_m (i.e., higher target binding affinity) than; 2'-deoxyoligonucleotides against a given target.

A number of nucleotide and nucleoside modifications have been shown to make the inhibitory nucleic acid into which they are incorporated more resistant to nuclease digestion than the native oligodeoxynucleotide; these modified oligos survive intact for a longer time than unmodified inhibitory nucleic acids. Specific examples of modified inhibitory nucleic acids include those comprising modified backbones, for example, phosphorothioates, phosphotriesters, methyl phosphonates, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. Most preferred are inhibitory nucleic acids with

phosphorothioate backbones and those with heteroatom backbones, particularly CH₂ - NH-O-CH₂, CH₂-N(CH₃)-O-CH₂ (known as a methylene(methylimino) or MMI backbone], CH₂ -O--N (CH₃)-CH₂, CH₂ -N (CH₃)-N (CH₃)-CH₂ and O-N (CH₃)-CH₂ -CH₂ backbones, wherein the native phosphodiester backbone is represented as

5 O- P-- O- CH₂); amide backbones (see De Mesmaeker et al. *Ace. Chem. Res.* 1995, 28:366-374); morpholino backbone structures (see Summerton and Weller, U.S. Pat. No. 5,034,506); peptide nucleic acid (PNA) backbone (wherein the phosphodiester backbone of the inhibitory nucleic acid is replaced with a polyamide backbone, the nucleotides being bound directly or indirectly to the aza nitrogen atoms of the

10 polyamide backbone, see Nielsen et al., *Science* 1991, 254, 1497). Phosphorus-containing linkages include, but are not limited to, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotriesters, methyl and other alkyl phosphonates comprising 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates

15 comprising 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates having normal 3'-5' linkages, 2'-5' linked analogs of these, and those having inverted polarity wherein the adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'; see US patent nos. 3,687,808; 4,469,863;

20 4,476,301; 5,023,243; 5, 177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455, 233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563, 253; 5,571,799; 5,587,361; and 5,625,050.

Morpholino-based oligomeric compounds are described in Dwaine A. Braasch

25 and David R. Corey, *Biochemistry*, 2002, 41(14), 4503-4510); *Genesis*, volume 30, issue 3, 2001; Heasman, J., *Dev. Biol.*, 2002, 243, 209-214; Nasevicius et al., *Nat. Genet.*, 2000, 26, 216-220; Lacerra et al., *Proc. Natl. Acad. Sci.*, 2000, 97, 9591-9596; and U.S. Pat. No. 5,034,506, issued Jul. 23, 1991.

Cyclohexenyl nucleic acid inhibitory nucleic acid mimetics are described in

30 Wang et al., *J. Am. Chem. Soc.*, 2000, 122, 8595-8602.

Modified inhibitory nucleic acid backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside

linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These comprise those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts; see US patent nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference.

One or more substituted sugar moieties can also be included, e.g., one of the following at the 2' position: OH, SH, SCH₃, F, OCN, OCH₃, OCH₃, O(CH₂)_n CH₃, O(CH₂)_n NH₂ or O(CH₂)_n CH₃ where n is from 1 to about 10; C₁ to C₁₀ lower alkyl, alkoxyalkoxy, substituted lower alkyl, alkaryl or aralkyl; Cl; Br; CN; CF₃; OCF₃; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; SOCH₃; SO₂ CH₃; ONO₂; NO₂; N₃; NH₂; heterocycloalkyl; heterocycloalkaryl; aminoalkylamino; polyalkylamino; substituted silyl; an RNA cleaving group; a reporter group; an intercalator; a group for improving the pharmacokinetic properties of an inhibitory nucleic acid; or a group for improving the pharmacodynamic properties of an inhibitory nucleic acid and other substituents having similar properties. A preferred modification includes 2'-methoxyethoxy [2'-O-CH₂CH₂OCH₃, also known as 2'-O-(2-methoxyethyl)] (Martin et al, *Helv. Chim. Acta*, 1995, 78, 486). Other preferred modifications include 2'-methoxy (2'-O-CH₃), 2'-propoxy (2'-OCH₂CH₂CH₃) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the inhibitory nucleic acid, particularly the 3' position of the sugar on the 3' terminal nucleotide and the 5' position of 5' terminal nucleotide. Inhibitory nucleic acids may also have sugar mimetics such as cyclobutyls in place of the pentofuranosyl group.

Inhibitory nucleic acids can also include, additionally or alternatively, nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include adenine (A), guanine (G), thymine (T), cytosine (C) and uracil (U). Modified nucleobases

include nucleobases found only infrequently or transiently in natural nucleic acids, e.g., hypoxanthine, 6-methyladenine, 5-Me pyrimidines, particularly 5-methylcytosine (also referred to as 5-methyl-2' deoxycytosine and often referred to in the art as 5-Me-C), 5-hydroxymethylcytosine (HMC), glycosyl HMC and gentobiosyl HMC, as well
5 as synthetic nucleobases, e.g., 2-aminoadenine, 2- (methylamino)adenine, 2- (imidazolylalkyl)adenine, 2-(aminoalkylamino)adenine or other heterosubstituted alkyladenines, 2-thiouracil, 2-thiothymine, 5-bromouracil, 5- hydroxymethyluracil, 8- azaguanine, 7-deazaguanine, N6 (6-aminohexyl)adenine and 2,6- diaminopurine. Kornberg, A., DNA Replication, W. H. Freeman & Co., San Francisco, 1980, pp75-
10 77; Gebeyehu, G., et al. Nucl. Acids Res. 1987, 15:4513). A "universal" base known in the art, e.g., inosine, can also be included. 5-Me-C substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C. (Sanghvi, Y. S., in Crooke, S. T. and Lebleu, B., eds., Antisense Research and Applications, CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions.

15 It is not necessary for all positions in a given inhibitory nucleic acid to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single inhibitory nucleic acid or even at within a single nucleoside within an inhibitory nucleic acid.

In some embodiments, both a sugar and an internucleoside linkage, i.e., the
20 backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an inhibitory nucleic acid mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an inhibitory nucleic acid is replaced with
25 an amide containing backbone, for example, an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds comprise, but are not limited to, US patent nos. 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by
30 reference . Further teaching of PNA compounds can be found in Nielsen et al, Science, 1991, 254, 1497-1500.

Inhibitory nucleic acids can also include one or more nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein,

"unmodified" or "natural" nucleobases comprise the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U).

Modified nucleobases comprise other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudo-uracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine.

Further, nucleobases comprise those disclosed in United States Patent No. 3,687,808, those disclosed in 'The Concise Encyclopedia of Polymer Science And Engineering', pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, 1990, those disclosed by Englisch et al., Angewandte Chemie, International Edition', 1991, 30, page 613, and those disclosed by Sanghvi, Y. S., Chapter 15, Antisense Research and Applications', pages 289-302, Crooke, S.T. and Lebleu, B. et al., CRC Press, 1993.

Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, comprising 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-

1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds, 'Antisense Research and Applications', CRC Press, Boca Raton, 1993, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications. Modified nucleobases are described in US patent nos.

3,687,808, as well as 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540;

5,587,469; 5,596,091; 5,614,617; 5,750,692, and 5,681,941, each of which is herein incorporated by reference.

In some embodiments, the inhibitory nucleic acids are chemically linked to one or more moieties or conjugates that enhance the activity, cellular distribution, or

cellular uptake of the inhibitory nucleic acid. Such moieties comprise but are not limited to, lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett., 1994, 4, 1053-1060), a thioether, e.g., hexyl-S- tritylthiol (Manoharan et al, Ann. N. Y. Acad. Sci., 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem. Lett., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Kabanov et al., FEBS Lett., 1990, 259, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49- 54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1 ,2-di-O-hexadecyl- rac-glycero-3-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol chain (Mancharan et al., Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochim. Biophys. Acta, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-t oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923-937). See also US patent nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552, 538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486, 603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762, 779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082, 830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5, 245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391, 723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5, 565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599, 928 and 5,688,941, each of which is herein incorporated by reference.

These moieties or conjugates can include conjugate groups covalently bound to functional groups such as primary or secondary hydroxyl groups. Conjugate groups of the invention include intercalators, reporter molecules, polyamines, polyamides, polyethylene glycols, polyethers, groups that enhance the pharmacodynamic properties of oligomers, and groups that enhance the pharmacokinetic properties of oligomers. Typical conjugate groups include cholesterol, lipids, phospholipids, biotin, phenazine, folate, phenanthridine, anthraquinone, acridine, fluoresceins,

rhodamines, coumarins, and dyes. Groups that enhance the pharmacodynamic properties, in the context of this invention, include groups that improve uptake, enhance resistance to degradation, and/or strengthen sequence-specific hybridization with the target nucleic acid. Groups that enhance the pharmacokinetic properties, in the context of this invention, include groups that improve uptake, distribution, metabolism or excretion of the compounds of the present invention. Representative conjugate groups are disclosed in International Patent Application No.

PCT/US92/09196, filed Oct. 23, 1992, and U.S. Pat. No. 6,287,860, which are incorporated herein by reference. Conjugate moieties include, but are not limited to, lipid moieties such as a cholesterol moiety, cholic acid, a thioether, e.g., hexyl-5-tritylthiol, a thiocholesterol, an aliphatic chain, e.g., dodecandiol or undecyl residues, a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethylammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate, a polyamine or a polyethylene glycol chain, or adamantane acetic acid, a palmityl moiety, or an octadecylamine or hexylamino-carbonyl-oxy cholesterol moiety. See, e.g., U.S. Pat. Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941.

Locked Nucleic Acids (LNAs)

In some embodiments, the modified inhibitory nucleic acids (including ASOs) used in the methods described herein comprise locked nucleic acid (LNA) molecules, e.g., including [alpha]-L-LNAs. LNAs comprise ribonucleic acid analogues wherein the ribose ring is "locked" by a methylene bridge between the 2'-oxygen and the 4'-carbon – i.e., inhibitory nucleic acids containing at least one LNA monomer, that is, one 2'-O,4'-C-methylene-β-D-ribofuranosyl nucleotide. LNA bases form standard Watson-Crick base pairs but the locked configuration increases the rate and stability of the basepairing reaction (Jepsen et al., *Oligonucleotides*, 14, 130-146 (2004)).

LNAs also have increased affinity to base pair with RNA as compared to DNA. These properties render LNAs especially useful as probes for fluorescence in situ hybridization (FISH) and comparative genomic hybridization, as knockdown tools for miRNAs, and as antisense oligonucleotides to target mRNAs or other RNAs, e.g.,
5 RNAs as described herein.

The LNA molecules can include molecules comprising 10-30, e.g., 12-24, e.g., 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in each strand, wherein one of the strands is substantially identical, e.g., at least 80% (or more, e.g., 85%, 90%, 95%, or 100%) identical, e.g., having 3, 2, 1, or 0
10 mismatched nucleotide(s), to a target region in the RNA. The LNA molecules can be chemically synthesized using methods known in the art.

The LNA molecules can be designed using any method known in the art; a number of algorithms are known, and are commercially available (e.g., on the internet, for example at exiqon.com). See, e.g., You et al., *Nuc. Acids. Res.* 34:e60
15 (2006); McTigue et al., *Biochemistry* 43:5388-405 (2004); and Levin et al., *Nuc. Acids. Res.* 34:e142 (2006). For example, "gene walk" methods, similar to those used to design antisense oligos, can be used to optimize the inhibitory activity of the LNA; for example, a series of inhibitory nucleic acids of 10-30 nucleotides spanning the length of a target RNA can be prepared, followed by testing for activity. Optionally,
20 gaps, e.g., of 5-10 nucleotides or more, can be left between the LNAs to reduce the number of inhibitory nucleic acids synthesized and tested. GC content is preferably between about 30-60%. General guidelines for designing LNAs are known in the art; for example, LNA sequences will bind very tightly to other LNA sequences, so it is preferable to avoid significant complementarity within an LNA. Contiguous runs of
25 more than four LNA residues, should be avoided where possible (for example, it may not be possible with very short (e.g., about 9-10 nt) inhibitory nucleic acids). In some embodiments, the LNAs are xylo-LNAs.

For additional information regarding LNAs see U.S. Pat. Nos. 6,268,490; 6,734,291; 6,770,748; 6,794,499; 7,034,133; 7,053,207; 7,060,809; 7,084,125; and
30 7,572,582; and U.S. Pre-Grant Pub. Nos. 20100267018; 20100261175; and 20100035968; Koshkin et al. *Tetrahedron* 54, 3607-3630 (1998); Obika et al. *Tetrahedron Lett.* 39, 5401-5404 (1998); Jepsen et al., *Oligonucleotides* 14:130-146

(2004); Kauppinen et al., *Drug Disc. Today* 2(3):287-290 (2005); and Ponting et al., *Cell* 136(4):629–641 (2009), and references cited therein.

Making and Using Inhibitory Nucleic Acids

The nucleic acid sequences used to practice the methods described herein,
5 whether RNA, cDNA, genomic DNA, vectors, viruses or hybrids thereof, can be isolated from a variety of sources, genetically engineered, amplified, and/or expressed/ generated recombinantly. Recombinant nucleic acid sequences can be individually isolated or cloned and tested for a desired activity. Any recombinant expression system can be used, including e.g. in vitro, bacterial, fungal, mammalian,
10 yeast, insect or plant cell expression systems.

Nucleic acid sequences of the invention can be inserted into delivery vectors and expressed from transcription units within the vectors. The recombinant vectors can be DNA plasmids or viral vectors. Generation of the vector construct can be accomplished using any suitable genetic engineering techniques well known in the art,
15 including, without limitation, the standard techniques of PCR, oligonucleotide synthesis, restriction endonuclease digestion, ligation, transformation, plasmid purification, and DNA sequencing, for example as described in Sambrook et al. *Molecular Cloning: A Laboratory Manual*. (1989)), Coffin et al. (*Retroviruses*. (1997)) and “RNA Viruses: A Practical Approach” (Alan J. Cann, Ed., Oxford
20 University Press, (2000)). As will be apparent to one of ordinary skill in the art, a variety of suitable vectors are available for transferring nucleic acids of the invention into cells. The selection of an appropriate vector to deliver nucleic acids and optimization of the conditions for insertion of the selected expression vector into the cell, are within the scope of one of ordinary skill in the art without the need for undue
25 experimentation. Viral vectors comprise a nucleotide sequence having sequences for the production of recombinant virus in a packaging cell. Viral vectors expressing nucleic acids of the invention can be constructed based on viral backbones including, but not limited to, a retrovirus, lentivirus, adenovirus, adeno-associated virus, pox virus or alphavirus. The recombinant vectors capable of expressing the nucleic acids
30 of the invention can be delivered as described herein, and persist in target cells (e.g., stable transformants).

Nucleic acid sequences used to practice this invention can be synthesized in vitro by well-known chemical synthesis techniques, as described in, e.g., Adams

(1983) J. Am. Chem. Soc. 105:661; Belousov (1997) Nucleic Acids Res. 25:3440-3444; Frenkel (1995) Free Radic. Biol. Med. 19:373-380; Blommers (1994) Biochemistry 33:7886-7896; Narang (1979) Meth. Enzymol. 68:90; Brown (1979) Meth. Enzymol. 68:109; Beaucage (1981) Tetra. Lett. 22:1859; U.S. Patent No. 4,458,066.

Nucleic acid sequences of the invention can be stabilized against nucleolytic degradation such as by the incorporation of a modification, e.g., a nucleotide modification. For example, nucleic acid sequences of the invention includes a phosphorothioate at least the first, second, or third internucleotide linkage at the 5' or 3' end of the nucleotide sequence. As another example, the nucleic acid sequence can include a 2'-modified nucleotide, e.g., a 2'-deoxy, 2'-deoxy-2'-fluoro, 2'-O-methyl, 2'-O-methoxyethyl (2'-O-MOE), 2'-O-aminopropyl (2'-O-AP), 2'-O-dimethylaminoethyl (2'-O-DMAOE), 2'-O-dimethylaminopropyl (2'-O-DMAP), 2'-O-dimethylaminoethoxyethyl (2'-O-DMAEOE), or 2'-O--N-methylacetamido (2'-O--NMA). As another example, the nucleic acid sequence can include at least one 2'-O-methyl-modified nucleotide, and in some embodiments, all of the nucleotides include a 2'-O-methyl modification. In some embodiments, the nucleic acids are "locked," i.e., comprise nucleic acid analogues in which the ribose ring is "locked" by a methylene bridge connecting the 2'-O atom and the 4'-C atom (see, e.g., Kaupinnen et al., Drug Disc. Today 2(3):287-290 (2005); Koshkin et al., J. Am. Chem. Soc., 120(50):13252-13253 (1998)). For additional modifications see US 20100004320, US 20090298916, and US 20090143326.

Techniques for the manipulation of nucleic acids used to practice this invention, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook et al., Molecular Cloning; A Laboratory Manual 3d ed. (2001); Current Protocols in Molecular Biology, Ausubel et al., eds. (John Wiley & Sons, Inc., New York 2010); Kriegler, Gene Transfer and Expression: A Laboratory Manual (1990); Laboratory Techniques In Biochemistry And Molecular Biology: Hybridization With Nucleic Acid Probes, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

Pharmaceutical Compositions

The methods described herein can include the administration of pharmaceutical compositions and formulations comprising an inhibitory nucleic acid that targets PAR-TERRA RNA and other chromosome-specific TERRA RNAs.

5 In some embodiments, the compositions are formulated with a pharmaceutically acceptable carrier. The pharmaceutical compositions and formulations can be administered parenterally, topically, orally or by local administration, such as by aerosol or transdermally. The pharmaceutical compositions can be formulated in any way and can be administered in a variety of
10 unit dosage forms depending upon the condition or disease and the degree of illness, the general medical condition of each patient, the resulting preferred method of administration and the like. Details on techniques for formulation and administration of pharmaceuticals are well described in the scientific and patent literature, see, e.g., Remington: The Science and Practice of Pharmacy, 21st ed., 2005.

15 The inhibitory nucleic acids can be administered alone or as a component of a pharmaceutical formulation (composition). The compounds may be formulated for administration, in any convenient way for use in human or veterinary medicine. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents,
20 sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

Formulations of the compositions of the invention include those suitable for intradermal, inhalation, oral/ nasal, topical, parenteral, rectal, and/or intravaginal administration. The formulations may conveniently be presented in unit dosage form
25 and may be prepared by any methods well known in the art of pharmacy. The amount of active ingredient (e.g., nucleic acid sequences of this invention) which can be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration, e.g., intradermal or inhalation. The amount of active ingredient which can be combined with a carrier
30 material to produce a single dosage form will generally be that amount of the compound which produces a therapeutic effect, e.g., an antigen specific T cell or humoral response.

Pharmaceutical formulations can be prepared according to any method known to the art for the manufacture of pharmaceuticals. Such drugs can contain sweetening agents, flavoring agents, coloring agents and preserving agents. A formulation can be admixed with nontoxic pharmaceutically acceptable excipients which are suitable for manufacture. Formulations may comprise one or more diluents, emulsifiers, preservatives, buffers, excipients, etc. and may be provided in such forms as liquids, powders, emulsions, lyophilized powders, sprays, creams, lotions, controlled release formulations, tablets, pills, gels, on patches, in implants, etc.

Pharmaceutical formulations for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in appropriate and suitable dosages. Such carriers enable the pharmaceuticals to be formulated in unit dosage forms as tablets, pills, powder, dragees, capsules, liquids, lozenges, gels, syrups, slurries, suspensions, etc., suitable for ingestion by the patient. Pharmaceutical preparations for oral use can be formulated as a solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable additional compounds, if desired, to obtain tablets or dragee cores. Suitable solid excipients are carbohydrate or protein fillers include, e.g., sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose such as methyl cellulose, hydroxypropylmethyl-cellulose, or sodium carboxy-methylcellulose; and gums including arabic and tragacanth; and proteins, e.g., gelatin and collagen. Disintegrating or solubilizing agents may be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate. Push-fit capsules can contain active agents mixed with a filler or binders such as lactose or starches, lubricants such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active agents can be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycol with or without stabilizers.

Aqueous suspensions can contain an active agent (e.g., nucleic acid sequences of the invention) in admixture with excipients suitable for the manufacture of aqueous suspensions, e.g., for aqueous intradermal injections. Such excipients include a suspending agent, such as sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, sodium alginate, polyvinylpyrrolidone, gum tragacanth and gum acacia, and dispersing or wetting agents such as a naturally

occurring phosphatide (e.g., lecithin), a condensation product of an alkylene oxide with a fatty acid (e.g., polyoxyethylene stearate), a condensation product of ethylene oxide with a long chain aliphatic alcohol (e.g., heptadecaethylene oxycetanol), a condensation product of ethylene oxide with a partial ester derived from a fatty acid and a hexitol (e.g., polyoxyethylene sorbitol mono-oleate), or a condensation product of ethylene oxide with a partial ester derived from fatty acid and a hexitol anhydride (e.g., polyoxyethylene sorbitan mono-oleate). The aqueous suspension can also contain one or more preservatives such as ethyl or n-propyl p-hydroxybenzoate, one or more coloring agents, one or more flavoring agents and one or more sweetening agents, such as sucrose, aspartame or saccharin. Formulations can be adjusted for osmolarity.

In some embodiments, oil-based pharmaceuticals are used for administration of nucleic acid sequences of the invention. Oil-based suspensions can be formulated by suspending an active agent in a vegetable oil, such as arachis oil, olive oil, sesame oil or coconut oil, or in a mineral oil such as liquid paraffin; or a mixture of these. See e.g., U.S. Patent No. 5,716,928 describing using essential oils or essential oil components for increasing bioavailability and reducing inter- and intra-individual variability of orally administered hydrophobic pharmaceutical compounds (see also U.S. Patent No. 5,858,401). The oil suspensions can contain a thickening agent, such as beeswax, hard paraffin or cetyl alcohol. Sweetening agents can be added to provide a palatable oral preparation, such as glycerol, sorbitol or sucrose. These formulations can be preserved by the addition of an antioxidant such as ascorbic acid. As an example of an injectable oil vehicle, see Minto (1997) J. Pharmacol. Exp. Ther. 281:93-102.

Pharmaceutical formulations can also be in the form of oil-in-water emulsions. The oily phase can be a vegetable oil or a mineral oil, described above, or a mixture of these. Suitable emulsifying agents include naturally-occurring gums, such as gum acacia and gum tragacanth, naturally occurring phosphatides, such as soybean lecithin, esters or partial esters derived from fatty acids and hexitol anhydrides, such as sorbitan mono-oleate, and condensation products of these partial esters with ethylene oxide, such as polyoxyethylene sorbitan mono-oleate. The emulsion can also contain sweetening agents and flavoring agents, as in the formulation of syrups and elixirs. Such formulations can also contain a demulcent, a preservative, or a

coloring agent. In alternative embodiments, these injectable oil-in-water emulsions of the invention comprise a paraffin oil, a sorbitan monooleate, an ethoxylated sorbitan monooleate and/or an ethoxylated sorbitan trioleate.

The pharmaceutical compounds can also be administered by in intranasal,
5 intraocular and intravaginal routes including suppositories, insufflation, powders and aerosol formulations (for examples of steroid inhalants, see e.g., Rohatagi (1995) J. Clin. Pharmacol. 35:1187-1193; Tjwa (1995) Ann. Allergy Asthma Immunol. 75:107-111). Suppositories formulations can be prepared by mixing the drug with a suitable non-irritating excipient which is solid at ordinary temperatures but liquid at body
10 temperatures and will therefore melt in the body to release the drug. Such materials are cocoa butter and polyethylene glycols.

In some embodiments, the pharmaceutical compounds can be delivered transdermally, by a topical route, formulated as applicator sticks, solutions, suspensions, emulsions, gels, creams, ointments, pastes, jellies, paints, powders, and
15 aerosols.

In some embodiments, the pharmaceutical compounds can also be delivered as microspheres for slow release in the body. For example, microspheres can be administered via intradermal injection of drug which slowly release subcutaneously; see Rao (1995) J. Biomater Sci. Polym. Ed. 7:623-645; as biodegradable and
20 injectable gel formulations, see, e.g., Gao (1995) Pharm. Res. 12:857-863 (1995); or, as microspheres for oral administration, see, e.g., Eyles (1997) J. Pharm. Pharmacol. 49:669-674.

In some embodiments, the pharmaceutical compounds can be parenterally administered, such as by intravenous (IV) administration or administration into a body
25 cavity or lumen of an organ. These formulations can comprise a solution of active agent dissolved in a pharmaceutically acceptable carrier. Acceptable vehicles and solvents that can be employed are water and Ringer's solution, an isotonic sodium chloride. In addition, sterile fixed oils can be employed as a solvent or suspending medium. For this purpose any bland fixed oil can be employed including synthetic
30 mono- or diglycerides. In addition, fatty acids such as oleic acid can likewise be used in the preparation of injectables. These solutions are sterile and generally free of undesirable matter. These formulations may be sterilized by conventional, well known sterilization techniques. The formulations may contain pharmaceutically

acceptable auxiliary substances as required to approximate physiological conditions such as pH adjusting and buffering agents, toxicity adjusting agents, e.g., sodium acetate, sodium chloride, potassium chloride, calcium chloride, sodium lactate and the like. The concentration of active agent in these formulations can vary widely, and
5 will be selected primarily based on fluid volumes, viscosities, body weight, and the like, in accordance with the particular mode of administration selected and the patient's needs. For IV administration, the formulation can be a sterile injectable preparation, such as a sterile injectable aqueous or oleaginous suspension. This suspension can be formulated using those suitable dispersing or wetting agents and
10 suspending agents. The sterile injectable preparation can also be a suspension in a nontoxic parenterally-acceptable diluent or solvent, such as a solution of 1,3-butanediol. The administration can be by bolus or continuous infusion (e.g., substantially uninterrupted introduction into a blood vessel for a specified period of time).

15 In some embodiments, the pharmaceutical compounds and formulations can be lyophilized. Stable lyophilized formulations comprising an inhibitory nucleic acid can be made by lyophilizing a solution comprising a pharmaceutical of the invention and a bulking agent, e.g., mannitol, trehalose, raffinose, and sucrose or mixtures thereof. A process for preparing a stable lyophilized formulation can include
20 lyophilizing a solution about 2.5 mg/mL protein, about 15 mg/mL sucrose, about 19 mg/mL NaCl, and a sodium citrate buffer having a pH greater than 5.5 but less than 6.5. See, e.g., U.S. 20040028670.

The compositions and formulations can be delivered by the use of liposomes. By using liposomes, particularly where the liposome surface carries ligands specific
25 for target cells, or are otherwise preferentially directed to a specific organ, one can focus the delivery of the active agent into target cells *in vivo*. See, e.g., U.S. Patent Nos. 6,063,400; 6,007,839; Al-Muhammed (1996) J. Microencapsul. 13:293-306; Chonn (1995) Curr. Opin. Biotechnol. 6:698-708; Ostro (1989) Am. J. Hosp. Pharm. 46:1576-1587. As used in the present invention, the term "liposome" means a vesicle
30 composed of amphiphilic lipids arranged in a bilayer or bilayers. Liposomes are unilamellar or multilamellar vesicles that have a membrane formed from a lipophilic material and an aqueous interior that contains the composition to be delivered. Cationic liposomes are positively charged liposomes that are believed to interact with

negatively charged DNA molecules to form a stable complex. Liposomes that are pH-sensitive or negatively-charged are believed to entrap DNA rather than complex with it. Both cationic and noncationic liposomes have been used to deliver DNA to cells.

5 Liposomes can also include "sterically stabilized" liposomes, i.e., liposomes comprising one or more specialized lipids. When incorporated into liposomes, these specialized lipids result in liposomes with enhanced circulation lifetimes relative to liposomes lacking such specialized lipids. Examples of sterically stabilized liposomes are those in which part of the vesicle-forming lipid portion of the liposome comprises
10 one or more glycolipids or is derivatized with one or more hydrophilic polymers, such as a polyethylene glycol (PEG) moiety. Liposomes and their uses are further described in U.S. Pat. No. 6,287,860.

The formulations of the invention can be administered for prophylactic and/or therapeutic treatments. In some embodiments, for therapeutic applications,
15 compositions are administered to a subject who is need of reduced triglyceride levels, or who is at risk of or has a disorder described herein, in an amount sufficient to cure, alleviate or partially arrest the clinical manifestations of the disorder or its complications; this can be called a therapeutically effective amount. For example, in some embodiments, pharmaceutical compositions of the invention are administered in
20 an amount sufficient to decrease serum levels of triglycerides in the subject.

The amount of pharmaceutical composition adequate to accomplish this is a therapeutically effective dose. The dosage schedule and amounts effective for this use, i.e., the dosing regimen, will depend upon a variety of factors, including the stage of the disease or condition, the severity of the disease or condition, the general state of
25 the patient's health, the patient's physical status, age and the like. In calculating the dosage regimen for a patient, the mode of administration also is taken into consideration.

The dosage regimen also takes into consideration pharmacokinetics parameters well known in the art, i.e., the active agents' rate of absorption,
30 bioavailability, metabolism, clearance, and the like (see, e.g., Hidalgo-Aragones (1996) J. Steroid Biochem. Mol. Biol. 58:611-617; Groning (1996) Pharmazie 51:337-341; Fotherby (1996) Contraception 54:59-69; Johnson (1995) J. Pharm. Sci. 84:1144-1146; Rohatagi (1995) Pharmazie 50:610-613; Brophy (1983) Eur. J. Clin.

Pharmacol. 24:103-108; Remington: The Science and Practice of Pharmacy, 21st ed., 2005). The state of the art allows the clinician to determine the dosage regimen for each individual patient, active agent and disease or condition treated. Guidelines provided for similar compositions used as pharmaceuticals can be used as guidance to
5 determine the dosage regimen, i.e., dose schedule and dosage levels, administered practicing the methods of the invention are correct and appropriate.

Single or multiple administrations of formulations can be given depending on for example: the dosage and frequency as required and tolerated by the patient, the degree and amount of therapeutic effect generated after each administration (e.g.,
10 effect on tumor size or growth), and the like. The formulations should provide a sufficient quantity of active agent to effectively treat, prevent or ameliorate conditions, diseases or symptoms.

In alternative embodiments, pharmaceutical formulations for oral administration are in a daily amount of between about 1 to 100 or more mg per
15 kilogram of body weight per day. Lower dosages can be used, in contrast to administration orally, into the blood stream, into a body cavity or into a lumen of an organ. Substantially higher dosages can be used in topical or oral administration or administering by powders, spray or inhalation. Actual methods for preparing parenterally or non-parenterally administrable formulations will be known or apparent
20 to those skilled in the art and are described in more detail in such publications as Remington: The Science and Practice of Pharmacy, 21st ed., 2005.

Various studies have reported successful mammalian dosing using complementary nucleic acid sequences. For example, Esau C., et al., (2006) Cell Metabolism, 3(2):87-98 reported dosing of normal mice with intraperitoneal doses of
25 miR-122 antisense oligonucleotide ranging from 12.5 to 75 mg/kg twice weekly for 4 weeks. The mice appeared healthy and normal at the end of treatment, with no loss of body weight or reduced food intake. Plasma transaminase levels were in the normal range ($AST \frac{3}{4} 45$, $ALT \frac{3}{4} 35$) for all doses with the exception of the 75 mg/kg dose of miR-122 ASO, which showed a very mild increase in ALT and AST levels. They
30 concluded that 50mg/kg was an effective, non-toxic dose. Another study by Krützfeldt J., et al., (2005) Nature 438, 685-689, injected anatgomirs to silence miR-122 in mice using a total dose of 80, 160 or 240 mg per kg body weight. The highest dose resulted in a complete loss of miR-122 signal. In yet another study, locked

nucleic acids ("LNAs") were successfully applied in primates to silence miR-122. Elmen J., et al., (2008) Nature 452, 896-899, report that efficient silencing of miR-122 was achieved in primates by three doses of 10 mg kg⁻¹ LNA-antimiR, leading to a long-lasting and reversible decrease in total plasma cholesterol without any
 5 evidence for LNA-associated toxicities or histopathological changes in the study animals.

In some embodiments, the methods described herein can include co-administration with other drugs or pharmaceuticals, e.g., compositions for providing cholesterol homeostasis. For example, the inhibitory nucleic acids can be co-
 10 administered with drugs for treating or reducing risk of a disorder described herein.

EXAMPLES

The invention is further described in the following examples, which do not
 15 limit the scope of the invention described in the claims.

Experimental Procedures

The following materials and methods were used in the Examples below.

FISH

Cells were cytopun onto glass slides and permeabilized with CSK buffer
 20 containing 0.5% Triton X-100, and fixed in 4 % paraformaldehyde. DNA oligos probes for RNA FISH were ordered from Integrated DNA Technologies. For TERRA: (TAACCC)₇-Alexa488-3' and 5'-Cy5-(TAACCC)₇. For I4 oligos: I4-47k 5'-Alexa488-TGC ACT GAC GTC CTG TGG CCA CTG GGT GGC GCC AGA GCAT (SEQ ID NO:7); I4-22k: 5'-Cy3-taa tet gaa tat ctg ggc ctc cgt gtg cag acc tga ggt t
 25 (SEQ ID NO:8); I4 31k: 5'-Cy5-gtc tet gtg tet gtc tet ctg tct ctg tcg cta act cta t (SEQ ID NO:9). DNA oligo probes for RNA-FISH were mixed at the final concentration 0.5 pmol/μl in hybridization solution (50% formamide, 2×SSC, 2 mg/ml BSA, 10% Dextran Sulfate-500K). BAC DNA probes and PCR-PAR probes were labeled with fluorophore-dUTP using nick translation, used 1 ng/μl for RNA-FISH and 50 ng/μl
 30 for DNA FISH at the final concentration in hybridization buffer. Hybridization was carried out at 42°C overnight for RNA FISH. Slides were washed with 2×SSC/50% formamide for 5 min three times at 44°C, and then wash with 2×SSC for 5 min twice

at 44°C. For DNA FISH, slides were treated with 0.4 mg/ml RNase A in PBS at 37°C for 1 hr, washed with PBS, incubated with 0.1 N HCl for 10 min. Slides were washed in PBST (0.2% Tween 20 in 1 X PBS) at RT for 5 mins, and then the denaturation was carried out in 70% formamide/2X SSC at 80°C for 15 mins. Slides were then washed with PBS, dehydrated with EtOH, and air dried. Hybridization was carried out at 37°C overnight for DNA FISH, and washing condition was the same as for RNA-FISH with additional wash in 0.1×SSC for 5 min at 44°C. For metaphase spread, cells were incubated with 50 ng/ml Colcemid for 2 hr, harvested, washed with PBS, incubated in cold 0.056M KCl on ice for 30 min and fixed in methanol/acetic acid (3:1). Metaphase spread chromosomes were spread on glass slides, air dried and fixed in 4% formaldehyde.

For pairing assay, digital images were obtained with the Nikon and processed using Volocity software (PerkinElmer). In brief, z sections were captured at 0.2 μm intervals and 3D images were projected on a single two-dimensional plane. Distance of Xic-Xic, PAR-PAR (x), and the nuclear areas (A) was analyzed using Volocity software. Only nuclei with two resolvable X signals were scored (single dots were excluded). ‘Normalized distance’ (ND) is defined as x/d , where d is the nuclear diameter, defined as $2(A/\pi)^{0.5}$. PCR-PAR PCR primer pairs were used as follows:

Primer	Sequence	SEQ ID NO:
P3-F:	CTCAGAGCCCAGTGTCAATCAC,	10
P3-R:	CACGACCGCTTAGAAGAACCGG	11
P4-F:	GAGACGGCCTACCATGTGCTTC,	12
P4-R:	GTGAGTGCTGTGAACTCGGCTG	13
P5-F:	CAGGGCCTGATTTGGCTTGAAAC	14
P5-R:	GAAGAGTAGTCTGACCTCATCTC	15
P6-F:	CAGGGCATGATATCCTCTTTGG	16
P6-R:	CATTCAATGGTGTGATGATGGTAC	17
P8-F:	GGTTAGAATACAGCGCGGACATTCA	18
P8-R:	GTGAATCTCCGAGGCAACTGTC	19

ChIRT-seq analysis

The PAR-TERRA ChIRT protocol was modified from the original ChIRP and CHART protocols (Chu et al., 2011; Simon et al., 2011) as follows: (i) We used a

minimum number of capture probes to reduce off-target effects. (ii) We also increased the shearing size to 0.5 – 3 kb to preserve integrity of long noncoding RNAs. (iii) Because we observed that RNaseH is not active in SDS buffer, we used NP40 instead of SDS or N-lauroyl sarcosine in the final DNA elution; we used a lower
5 concentration of NP40 detergent to better preserve RNaseH activity (Fig. 9A).

Specifically, mouse ES cells were grown to 80% confluency and feeder cells were removed. 15 millions of cells were spun down and washed with PBS once. Cells were resuspended in 10 ml of PBS and then another 10 ml of 2% of glutaraldehyde were added to fix cells at room temperature for 10 min. Crosslinking was then
10 quenched with 0.125 M glycine for 5 min. Cells were then spun down at 2000g for 5 min at 4°C. Cells were then washed with cold PBS and then spun down again. Cell pellets were immediately frozen in liquid nitrogen and stored at -80°C. Mouse ES cells at embryonic body stages (Day3, Day7) were trypsinized, filtered with cell strainers (40 µm). The following steps were prepared as the same as undifferentiated
15 ES cells. Cells were thaw out on ice, and were resuspended in 1 ml of swelling buffer (0.1 M Tris pH 7.0 10 mM KOAc, 15 mM MgOAc, 1% NP40, 1mM DTT, 1mM PMSF, 100 U/ml Suprase-In[Ambion]) for 10 min on ice. Cells were then dounced and pelleted at 2500 g for 5 min. Nuclei was further lyzed in nuclear lysis buffer (50 mM Tris pH7.0, 10 mM EDTA, 1 % SDS, 1mM DTT, 1mM PMSF, protease
20 inhibitor, 100 U/ml Suprase-In) on ice for 10 min, and sonicated using Bioruptor until DNA size 0.5-3 kb (it usually takes 1.5 hr and depends on the cell numbers). Cell lysates were then spun down at 13,000 rpm for 5 min to remove insoluble debris. Cell lysates were then frozen in liquid nitrogen and stored in -80°C. Streptavidin-magnetic C1 (Life Technologies) beads were blocked with 500 ng/ul yeast total RNA,
25 and 1mg/ml BSA for 1 hr at 37°C, and resuspended in 1X hybridization buffer (1 volume of lysis buffer plus 2 volume of 2X hybridization buffer). Cell lysates were diluted in two times volume of 2X hybridization buffer (750 mM NaCl, 1% SDS, 50 mM Tris pH 7.0, 1 mM EDTA, 15% Formamide, 1 mM DTT, PMSF, protease inhibitor, and 100 U/ml Suprase-in), and were preclean with Streptavidin- magnetic
30 C1 beads at 37°C for 1 hr (100 µl of beads for 1 ml lysates). Precleaned lysates were incubated with pooled probes (100 pmol for 3 ml of diluted cell lysates) at 37°C for 3 hr. Three hundred microliters washed/blocked C1 beads were added per 100 pmol of probes, and the whole reaction was mixed for another 1 hr at 37°C. DNA probes for

ChIRT were ordered from Integrated DNA Technologies and labeled with 3' biotin-TEG. PAR DNA probe sequences were listed as follows: 36K:

gagcgctcagtggtgcaaatct (SEQ ID NO:20), 47K:

ACTGGGTGGCGCCAGAGCAT (SEQ ID NO:21), 22K: ctccgtgtgcagacctgaggtt

5 (SEQ ID NO:22), 34K: ccctacctaccctccagaga (SEQ ID NO:23), 31K:

tctctgtctctgtcgtaac (SEQ ID NO:24). TERRA-AS probe sequence:

TAACCCTAACCCTAACCCTA (SEQ ID NO:25). TERRA-sense probe sequence:

TTAGGGTTAGGGTTAGGGTT (SEQ ID NO:26). Beads:biotin-

probes:RNA:chromatin adducts were captured by magnets, washed five times at 37°C

10 for 5 min with wash buffer (2X SSC, 0.5% SDS, 1 mM DTT, 1 mM PMSF), and then

washed twice for 5 min at room temperature with 0.1% NP40 buffer (150 mM NaCl,

50 mM Tris pH8.0, 3 mM MgCl₂, 10 mM DTT, 0.1% NP40). DNA was then eluted

twice for 20 min in 450 µl of 0.1% NP40 buffer with 200 U/ml RNase H (NEB) at

room temperature. DNA for no RNase H controls was eluted in 0.1% NP40 buffer

15 without RNaseH. Eluted DNA was treated with RNase A (1 mg/ml) at 37°C for 1 hr,

and then was treated with proteinase K (1 mg/ml) and supplied addition of SDS to

0.5% at final concentration at 55°C for 16 hr. DNA was extracted with

phenol/chloroform using phase lock gel tubes. For pre-RNaseA treatment control, cell

lysates were treated with RNase A at 37°C overnight before hybridization. For RNA

20 elution after hybridization, beads:biotin-probes:RNA:chromatin adducts were washed

5 time in wash buffer, then treated with proteinase K in PK buffer (100 mM NaCl,

Tris pH 7.0, 1 mM EDTA, 0.5% SDS) at 55°C for 30 min. Beads suspension was

boiled at 90°C for 5 min, and then RNA was extracted using TRIzol (Invitrogen).

Primer pairs for q-PCR were used as followed: PAR-DNA-F:

25 TGGAGGTAAACGATTATTTATCTGC (SEQ ID NO:27), PAR-DNA-R:

ACGAGTTTCCAAGGTGCTG (SEQ ID NO:28); Hprt-F:

CTGCTACTTCAACTCCTGGTGTGC (SEQ ID NO:29), Hprt-R:

AGGCGAATTGGGATGTAGCTCAG (SEQ ID NO:30).

PAR-TERRA ChIRT-seq analysis

30 PicoGreen (Life Technologies) was used to estimate the concentration of eluted DNA. Before library construction, equal amount of lambda DNA (0.015 pg of PCR products, ~250bp) was added as spike-in control into eluted DNA samples. The PCR primers sequences for lambda DNA are as follows: Lambda 5-F, 5'-GCA TAT

GTT GTG TTT TAC AG-3' (SEQ ID NO:31); Lambda 5-R, 5'-GCA ACA AAT TGA TAA GCA-3' (SEQ ID NO:32). Following the removal of adaptor sequences and PCR duplicates, paired-end 50 bp sequencing data was aligned to mouse reference genome (GRCm38/mm10 and NCBI37/mm9) using the software Novoalign (v3.00.02) (Li H. (2013) Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv:1303.3997v1 [q-bio.GN]). The coverage files were generated using R software library SPP software (Kharchenko et al., 2008) with smoothing using 500 bp bins with a 100 bp step size to generate control-subtracted, normalized read densities. Controls include input, sense-ChIRT, and TERRA-ChIRT without RNase H elution (no RNase H). These data were visualized using IGV software to display all tracks with a mean windowing function and scales indicated in each figure. Other methods to generate normalized coverage files, including the generation of conservative enrichment and maximum likelihood estimates, resulted in similar distribution patterns. Scatter plots for correlation analysis used input-normalized coverage produced by SPP, windowed by 3 kb bins and filtered out unenriched bins with an averaged density smaller than 4. Peaks were called by MACS (1.4.2)(Zhang et al., 2008) software using normalization to indicated controls (e.g., input, sense, no RNaseH or pre-RNaseA), and filtered by peak length greater than 1 kb. Metagene profiles were produced by software CEAS (0.9.9.7) (Shin et al., 2009) using 2 fold enriched over input wig files and bed files produced by MACS peak calling.

TERRA knockdown

Mouse ES cells (female, 16.7, cas/mus hybrid) were grown to 70% confluency, and then trypsinized, and feeder cells were removed. A total of 2×10^6 mES cells were transfected with LNA gapmer oligos at a concentration of 2~8 μ M in 100 μ l nucleofector solution using A30 program (nucleofector kits, Lonza). A total of 2 ml of feeders-conditional medium (medium from feeders grown in mES medium for 6-18 hr) was added to the cells, and the cells were plated on gelatinized plates. LNA gapmers were designed and synthesized by Exiqon with modified LNA bases and phosphothiolated backbone modification. The LNA sequences were as follows: Scr, 5'-CAC GTC TAT ACA CCA C-3' (SEQ ID NO:4); TERRA, 5'-TAA CCC TAA CCC TAA C-3' (SEQ ID NO:5); PAR, 5'-TCT CTG TCT CTG TCG C-3'

(SEQ ID NO:6). SV40T transformed MEFs (cas/mus hybrid) were used for TERRA LNA knockdown.

RNA-seq analysis

Total RNA was isolated using TRIzol (Invitrogen), depleted of DNA by DNase treatment (TURBO DNase, Ambion), depleted of ribosomal RNA (Ribominus Eukaryote Kit v2, Invitrogen), purified greater than 200 nucleotides using mirVana RNA extraction kit (Ambion), and fragmented in first strand synthesis buffer (NEB) containing magenisium at 95 °C for 10 min to a median size 150-200 bp. cDNA were reversed transcribed with random primers (with Actinomycin D) using Superscript III (Invitrogen) at 50°C for 30 min. The following steps such as second strand synthesis, end repaired, dA-tailing, adaptor ligation, USER enzyme digestion, double size selection (0.6x-1.2x AMPure XP beads), and library amplification were performed according to NEBUltra Directional RNA library preparation protocol for Illumina (NEB). Sequencing of purified libraries was carried out on an Illumina HiSeq instrument for paired 50 nucleotides reads. After removal of adaptor sequences by Trim Galore, reads were aligned to mouse genomes (GRCm38/mm10 and NCBI37/mm9) using Tophat2. After removal of PCR duplicates, data was analyzed using either Cuffdiff 2 (Trapnell et al., 2013). Differential expression was called using Cuffdiff 2 with a threshold of q-Value < 0.05. Coverage of RNA-seq was normalized by per million mapped reads as FPM value shown in the tracks. Allelic RNA-seq analysis was described previously (Simon et al., 2013). Briefly, reads were aligned to allele-specifically to 129S1/SvJm (mus) and CAST/EiJ (cas) using Tophat2. All reads mapping to gene bodies were summed for cas, mus and comp tracks, and PCR duplicates were removed. Differential expression between sets of genes in KD samples was analyzed using R library EdgeR (3.4.2) within by HOMER (4.8) software (Heinz et al., 2010) using function analyseRepeates.pl to generate count numbers on mus tracks for gene expression on Xi (mus).

Northern blotting analysis

DNA sequences for Northern probes were listed as follows:

Probe	Sequence	SEQ ID NO.
TERRA	TAACCCTAACCCTAACCCTAACCCTAACCC	33
GAPDH	GTAGACCCACGACATACTCAGCACCGGCCTCACCCATT	34

I4-15k	aaggccagccgcgggtccagacctgcgggtgcggccgtgtc	35
I4-22k	taatctgaatatctgggcctccgtgtgcagacctgaggtt	36
I4-27k	ttgggggcgtgtctcagagcaggaggggtgtggtctggca	37
I4-31k	gtctctgtgtgtctctctgtctctgtcctaactctat	38
I4-34k	aaagccaccaggcctctaataccctaccctccagaga	39
I4-42k	cctggagaaatcaagtctgcgaagatccaaaaattaaaat	40
I4-47k	TGCACTGACGTCCTGTGGCCACTGGGTGGCGCCAGAGCAT	41
I4-53k	CTGACCACCAGGCTACAGTGTCTCTGTAACCGCCAGGCATA	42

All oligo probes were end labeled using T4 polynucleotide kinase. I4-31k oligos were used for PAR-TERRA transcripts in Figure 4B. Total RNA was extracted using TRIzol followed by acid phenol extraction. Total RNA (5 µg) was loaded in each lane. Hybridization was carried out at 42°C overnight using ULTRAhyb-Oligo hybridization buffer (Ambion).

Quantitative RT-PCR

Total RNA was isolated using TRIzol (Invitrogen), treated with TURBO DNase (Ambion), and reverse-transcribed with random primers using Superscript III reverse transcriptase (Invitrogen). qRT-PCR was performed using iQ SYBR Green Supermix (Bio-Rad). Expression levels were normalized to GAPDH levels. Primer pairs were used as follows:

Primer	Sequence	SEQ ID NO:
GAPDH-F	CGTCCCGTAGACAAAATGGT	43
GAPDH-R	TTGATGGCAACAATCTCCAC	44
Erdr1-F	CACAGTGATGTACCCACGA	45
Erdr1-R	GTGAGAATCGCTCCGTCCTG	46
Mid1-intron1-F	GGACGAGAGGGGACAAAGGA	47
Mid1-intron1-R	GGTCAAACCTGGACTCTGGCA	48
Asmt-F	GAAGTGGGACAGGAAGTGAG	49
Asmt-R	CGGGAACAGGAAGTGGC	50
Wls-F	CCAGTCTAATGGTGACCTGGG	51
Wls-R	TGAGAGTCAGCATGCACCAG	52

Tmx3-F	TACCGAGGACCACGGACTAA	53
Tmx3-R	AATACACGGTGCCTCTTCCG.	54

XIST CHART-seq analysis

The Xist CHART was modified from the original XIST CHART protocols (Simon et al., 2011). We used 7 oligo probes to target Xist RNA:

Oligo	Sequence	SEQ ID NO:
Xist-503	CAGGTATCCATGGCCCCGATGGGC	55
Xist-1895	CTCGGTCTCTCGAATCGGATCCGAC	56
Xist-3322	GAGTTATGGGCACTGCATTTTAGCA	57
Xist-5799	TTGTTAAACGCAGGCTAGATCCTGA	58
mXist-1240	CGCCATTTTATAGACTTCTGAGCAG	59
mXist-935	CCtaattcttggcgtaactggctcg	60
mXist-5651	ATGCTTAGGAAGAGGGACAAATGCA	61

5 In detail, 20 million cells were crosslinked by with 1% formaldehyde for 10 min at room temperature. Crosslinking was then quenched with 0.125 M glycine for 5 min. After washing 3 times with PBS, crosslinked cells were re-suspended in 2 ml of sucrose buffer (0.3 M sucrose, 1% Triton-X-100, 10 mM HEPES pH 7.5, 100 mM KOAc, 0.1 mM EGTA), dounced 20 times with a tight pestle, and kept on ice for 10 min. The following steps were using polystyrene tubes, glass pipettes, and DNA LoBind microtubes (Eppendorf) to avoid cell clumps sticking onto the walls of tubes or pipettes. Nuclei were collected by centrifugation at 1,500g for 10 min on top of a cushion of 5 ml glycerol buffer (25% glycerol, 10 mM HEPES pH7.5, 1 mM EDTA, 0.1 mM EGTA, 100 mM KOAc). Nuclei were further crosslinked with 3% formaldehyde for 30 min at room temperature. After washing three times with PBS, nuclei were extracted once with 50mM HEPES pH7.5, 250 mM NaCl, 0.1mM EGTA, 0.5% N-lauroylsarcosine, 0.1% sodium deoxycholate, 5mM DTT, 100 U ml⁻¹ SUPERasIN (Invitrogen) for 10 min on ice, and centrifuged at 400g for 5 min at 4 uC. Nuclei were resuspended in 270 µl of sonication buffer (50 mM HEPES pH 7.5, 75 mM NaCl, 0.1 mM EGTA, 0.5% N-lauroylsarcosine, 0.1% sodium deoxycholate, 5 mM DTT, 10 U/ml SUPERasIN, and sonicated in microtubes using Covaris S2 sonicator at 10% duty cycle, 200 bursts per cycle, intensity 3 for 5 min. The size of chromatin fragments was 0.2~3 kb. Fragmented chromatin was subjected to

hybridization immediately. Hybridization, washing and elution were performed similarly to TERRA-ChIRP protocol. In brief, beads were blocked by yeast tRNA and BSA. 320 µl of 2X hybridization buffer (750 mM NaCl, 1% SDS, 50 mM Tris pH 7.0, 1 mM EDTA, 15% Formamide, 1 mM DTT, PMSF, protease inhibitor, and 100 U/ml Suprase-in) was added into 160 µl lysates, and then this 1X hybridization lysate was precleaned by 60 µl of blocked beads at room temperature for 1 hr. After removal of the beads, 7 probes (labeled with 3' biotin-TEG) for Xist RNA (3.66 pmol/per probe) were added into the 1X hybridization lysate and incubate at room temperature for overnight. Beads:biotin-probes:RNA:chromatin adducts were captured by magnets, washed once with 1X hybridization buffer at 37°C for 10 min, washed four times at 37°C for 5 min with wash buffer (2X SSC, 0.5% SDS, 1 mM DTT, 1 mM PMSF), and then washed twice for 5 min at room temperature with 0.1% NP40 buffer (150 mM NaCl, 50 mM Tris pH8.0, 3 mM MgCl₂, 10 mM DTT, 0.1% NP40). DNA was then eluted twice for 20 min in 450 µl of 0.1% NP40 buffer with 200 U/ml RNase H (NEB) at room temperature.

Metagene analysis

Escapee genes are as previously described (Carrel and Willard, 2005; Yang et al., 2010; Pinter et al., 2012). *Xist* itself is excluded as an escapee in the metagene analysis. "Repressed" genes are all other genes on the Xi which have an FPKM >1.0 on the Xa. The normalized coverage files produced from SPP were used for metagene analysis with CEAS software.

Example 1. Identification of sex-linked PAR-TERRA transcripts

RNA fluorescence in situ hybridization (FISH) using TERRA oligo probes showed that TERRA can be seen, in high-exposure and higher contrast images, as multiple foci in the nuclei of ES cells (Fig. 1A). Consistent with our previous report (Zhang et al., 2009), two of the speckles were especially prominent. To confirm their colocalization next to the sex chromosomes, we performed serial RNA-DNA FISH using probes from the pseudoautosomal region (PAR) of the sex chromosomes. The PAR represents the only homologous region between chromosomes (Chr) X and Y. Because PAR genes are shared between the sex chromosomes, these genes are not subject to XCI. At the commencement of this project, the most distally mapped X- and Y-linked gene was the PAR gene, *Mid1* (*Erdr1* and *Asmt* were partially

assembled in recent months). We obtained two BAC clones mapping to *Mid1* — the 15 kb BAC RP24-143B12 and the ~146 kb RP24-500I4. RP24-500I4 contains several internal telomeric repeats, two of 40 bp and the third of 314 bp (Fig. 1B). To isolate PAR-specific probes, we subcloned the BACs, generated unique PCR fragments, and identified a set of unique probes consisting of P3, P4, P5, P6, and P8 (P34568; Fig. 8A,B). Serial RNA-DNA FISH showed that the large TERRA foci indeed mapped to the PAR in ES cells. Quantitation of RNA FISH intensities indicated that sex chromosome-associated TERRA RNA accounted for ~80-90% of total TERRA transcripts and the finer speckles ~10-20% of detected signals (Fig. 1A,C).

Although cytological analysis shows that TERRA RNA localizes to the ends of most, if not all chromosomes (Azzalin et al., 2007; Schoeftner and Blasco, 2008), the origin of TERRA transcription is not fully known. TERRA may be transcribed by all telomeres and retained in cis, or it may be transcribed by only a few loci but localized in trans to multiple distant sites. A murine transcriptomic study indicates that TERRA is synthesized predominantly from the end of Chr18 (de Silanes et al., 2014). Because sub-telomeric sequences of some chromosomes, including Chr X and Y, have not been fully sequenced or assembled, determining additional transcriptional origins for TERRA is possible.

To determine whether the sub-telomeric region of ChrX and ChrY could contribute to sex-linked TERRA transcription, we carried out RNA FISH to compare signals arising from PAR versus TERRA probes. The PAR and TERRA RNA clusters looked nearly identical in male and female ES cells (Fig. 1D,E), raising the possibility that PAR and TERRA may extend — at least in a fraction of total transcription — as a single long noncoding RNA. On Northern blot analyses, an antisense TERRA oligo probe detected a smear of signals from 100 bp to >9kb (Fig. 1F, left panel), consistent with TERRA being of heterogeneous size (Azzalin et al., 2007; de Silanes et al., 2014). However, we observed a dominant species in ES cells of >>9 kb, indicating that some TERRA transcripts may originate much further upstream in relation to the telomeric repeats. Primer extension using an antisense TERRA oligo probe gave positive amplification by RT-PCR using PAR-specific primer pairs located at 33, 36, and 39 kb from the end of BAC RP24-500I4 (Fig. 1F, right panel), demonstrating that PAR-initiated RNAs is physically contiguous with at least a fraction of TERRA-containing RNA. We then repeated Northern analysis using sub-BAC walking probes

to verify the physical contiguity and observed smears of high molecular weight transcripts similar to those observed with TERRA probes (Fig. 1F,G). The pattern was especially similar with probes 22k, 27k, 34k, 36k, and 47k. Two-color RNA FISH using BAC sub-probes showed that >90% of large RNA clusters were coincident with TERRA foci in ES cells (Fig. 1H) as well as in MEF (Fig. 8C). In MEFs, ~93% of PAR and TERRA clusters (n=285) localized next to, but generally did not overlap with, the Xist cloud (Fig. 8D). Henceforth, we refer to the TERRA transcripts of PAR origin as “PAR-TERRA”, to distinguish them from TERRA transcripts intrinsic to the TTAGGG repeats and to those originating within sub-telomeric regions of autosomes.

TERRA foci were similarly observed in various human cells (Fig. 8C). In mice, PAR-TERRA expression showed some strain-specific variation. For example, PAR-TERRA signals were detectable with the 22k probe in hybrid ES and MEF cells of mixed *Mus musculus* (mus) and *Mus castaneus* (cas) origin. Using the 22k probe, however, PAR-TERRA could not be detected in cells of pure *musculus* origin (Fig. 8E). This is consistent with the PAR being variable in sequence between mouse strains (Soriano et al., 1987). As a consequence, the two large TERRA foci were often asymmetric in size in hybrid 16.7 female ES cells, with *Mus musculus* cluster being smaller than the *Mus castaneus* cluster. In J1 male ES cells, TERRA RNAs on both Chr X and Y were usually smaller, consistent with their being of *Mus musculus* origin. We conclude that a substantial fraction of sex-linked TERRA transcripts originates in the pseudoautosomal region (PAR-TERRA). The X-linked origin could have been missed previously (de Silanes et al., 2014) because pseudoautosomal sequences and assembly were not available until recently.

Example 2. Mapping genome-wide targets of TERRA and PAR RNA by ChIRT-seq

Although RNA FISH showed that >90% localized in cis to Chr X and Y, additional foci throughout the nucleus were clearly evident (Fig. 1H). Because the probes were PAR-specific, this finding indicated that PAR-TERRA could diffuse away from the sex chromosomes and possibly localize elsewhere in the nucleus. To identify genome-wide targets of TERRA and PAR RNA, we captured RNA-bound genomic sites by merging elements of ChIRP (Chu et al., 2011) and CHART (Simon et al., 2011) to achieve high specificity of chromatin pulldown (henceforth “ChIRT”). Because capture probes could potentially interact with DNA rather than RNA, we

included an RNaseH elution step (Fig. 9A). Several DNA-based capture probes were designed: (i) TERRA antisense (TERRA-AS), to capture transcripts containing UUAGGG, (ii) PAR, to capture PAR-TERRA transcripts, and (iii) TERRA sense (TERRA-S, the reverse complement), to control for background. Slot blot analysis showed that both TERRA-AS and 31k-PAR-AS probes captured TERRA RNA after ChIRT, whereas the corresponding sense probes did not (Fig. 2B). Quantitative PCR indicated that PAR sequences were enriched relative to *Hprt* sequences after ChIRT using TERRA-AS and PAR-AS probes, but not when the TERRA-S probe was used (Fig. 2C). The enrichment was dependent on RNaseH treatment and was abolished by RNase A treatment (Fig. 2D), indicating that the pulldown was mediated by interaction between the DNA capture probes and RNA targets.

We then performed deep sequencing of ChIRT-seq pulldowns to identify genome-wide binding sites. To rule out artifacts due to direct probe hybridization to genomic DNA rather than the intended RNA target, we sequenced two critical controls: (i) an RNaseH- control in which RNaseH was omitted in the elution step, which would in principle preclude elution of RNA-dependent interactions; and (ii) a TERRA-S control, which would not hybridize to TERRA RNA but could potentially pull down contaminating DNA (in addition to any potential antisense-TERRA transcripts). We collected ES cells on differentiation days 0, 3, 7 (d0, d3, d7) and MEFs for ChIRT-seq. Approximately 30 million 50-bp paired-end reads were obtained for each library. After removing PCR duplicates, >70% of reads uniquely mapped to the mouse genome. Biological replicates showed a high degree of correlation (Fig. 9B,C).

We used MACS software to call statistically significant enrichment peaks signifying genomic binding sites of TERRA and PAR transcripts (Fig. 2E). To call enrichment peaks, we normalized ChIRT reads to (i) input library, (ii) TERRA-S library, or (iii) no-RNaseH library. The results were highly similar with each method of normalization (Fig. 2F), and MACS called a similar number of peaks (Fig. 2E). Major enrichment at telomeric repeat DNA in the TERRA-AS pulldown relative to TERRA-S and no-RNaseH controls provided a validation of our ChIRT method (Fig. 2G). Across the genome, we observed >2,000 peaks of TERRA binding in day 0 and day 3 ES cells, ~1,800 peaks in d7 differentiating ES cells, and ~500 peaks in MEFs (Fig. 2E). Peaks called in the sense ChIRT samples did not overlap those called for

TERRA or PAR (Fig. 2F), and could represent either background or binding of a putative antisense TERRA transcript. Similarly for PAR RNA, we observed thousands of binding sites in ES cells and hundreds in MEFs. Overall, PAR-TERRA binding sites were enriched in noncoding space, including upstream regulatory regions, introns, and intergenic space, whereas binding in exons was depleted (Fig. 2H,I).

There was considerable overlap between TERRA and PAR ChIRT profiles, with high Pearson's r values in correlation plots for d0 and d3 ES cells and for MEFs, but not for comparisons to sense-ChIRT controls (Fig. 2F). There was also a high degree of similarity between biological replicates (Fig. 9B,C). In ES cells and MEFs, whole-genome views demonstrated strongest overall enrichment of both TERRA and PAR RNAs at telomeric ends, inclusive of sub-telomeric regions — regions with unique sequence that enabled unambiguous alignment of paired-ends TERRA reads to the specific chromosome ends (Fig. 3A). Intriguingly, the X-linked RNAs could diffuse to autosomes and bind their telomeric ends (Fig. 3A, PAR track). In MEFs, the number of TERRA and PAR targets decreased overall, but telomeric ends remained enriched. Control TERRA-S pulldowns did not resemble TERRA-AS pulldowns and showed no significant enrichment at telomeres. These results demonstrated the specificity of the TERRA and PAR pulldowns and argued against artifacts of genomic DNA hybridization. Taken together, our findings demonstrate that telomeric RNAs (i) are indeed produced from the sex chromosomes and (ii) bind both in cis and in trans to their site of synthesis.

Table 1 provides a list of X-linked genes with the highest PAR-TERRA binding. Top binders are defined as the top quartile in terms of PAR-TERRA density over the gene. There are 452 active genes in the MEF cell line. Mm10 coordinates are used. Start and stop positions of the gene target, along with the gene name and transcribed strand are shown. RNA-seq FPKM refers to gene expression in frequency per kilobase per million reads. Mean TERRA coverage over the gene is also shown.

Table 1: PAR-TERRA binding sites on the mouse X-chromosome in MEFs

mm10 assembly	Start	End	gene name	FPKM	strand	Mean of TERRA coverage
chrX	169685246	169990797	Mid1	1.39743	+	203.232
chrX	169311530	169320343	Hccs	27.2857	-	64.2334
chrX	167207093	167209218	Tmsb4x	454.052	-	24.1368
chrX	152336851	152342484	Tspyl2	28.3268	-	13.6914
chrX	166499814	166510478	Tceanc	2.77384	-	13.1733

Table 1: PAR-TERRA binding sites on the mouse X-chromosome in MEFs

mm10 assembly	Start	End	gene name	FPKM	strand	Mean of TERRA coverage
chrX	74270815	74273135	Rpl10	82.0002	+	12.988
chrX	74270815	74273135	Snora70	201.378	+	12.988
chrX	74273216	74282333	Dnase1l1	1.20938	-	12.9053
chrX	134601285	134607054	Hnrnp2	44.022	+	11.0383
chrX	74369218	74373349	Slc10a3	10.7721	-	10.2314
chrX	153498231	153501558	Ubqln2	81.5338	+	10.2078
chrX	73673132	73682500	Slc6a8	57.6063	+	10.028
chrX	168795098	169304435	Arhgap6	1.7761	+	9.93581
chrX	134588168	134601005	Gla	10.9353	-	9.78217
chrX	168654117	168673902	Msl3	7.40496	-	9.71429
chrX	134686518	134697772	Armxc4	9.21521	+	9.7044
chrX	152233229	152274354	Kdm5c	101.365	+	9.38192
chrX	101449108	101453541	Itgb1bp2	1.18288	+	8.99717
chrX	101429650	101448593	Nono	123.217	+	8.59172
chrX	74313032	74320149	Fam50a	52.844	+	8.43926
chrX	94636068	94638561	Gspt2	18.0414	+	8.38342
chrX	166457251	166479867	Rab9	44.5493	-	8.34863
chrX	74282696	74290151	Taz	20.6918	+	8.29363
chrX	99136129	99148991	Efnb1	8.80092	+	8.27655
chrX	152004583	152016295	Ribc1	8.85901	-	8.25976
chrX	101254527	101260873	Foxo4	35.4845	+	7.89614
chrX	152144267	152225236	Iqsec2	3.55372	+	7.89114
chrX	13281021	13293983	Ddx3x	202.014	+	7.79031
chrX	74365717	74368548	Ubl4	27.821	-	7.72789
chrX	152001895	152004442	Hsd17b10	58.1856	+	7.67299
chrX	101532734	101601789	Taf1	47.7176	+	7.64038
chrX	152016427	152061973	Smc1a	57.1067	+	7.62341
chrX	152294827	152327493	2900056M20Rik	1.73042	-	7.3519
chrX	150571506	150588149	Apex2	30.0146	-	7.27157
chrX	151803281	151935417	Huwe1	161.224	+	7.14147
chrX	20688492	20699877	Cdk16	89.5491	+	6.96555
chrX	101404383	101420685	Zmym3	53.5806	-	6.93208
chrX	103560909	103623754	Ftx	6.13111	-	6.83641
chrX	134748454	134751419	Armxc6	1.47279	-	6.69088
chrX	8061170	8074760	Suv39h1	10.5102	-	6.59123
chrX	109095406	109162467	Sh3bgrl	11.0008	+	6.35196
chrX	94535473	94542074	Maged1	150.771	-	6.24733
chrX	96096044	96168553	Msn	125.765	+	6.23976
chrX	73483634	73495936	Bgn	131.985	+	6.19272
chrX	163909159	163929546	Ap1s2	22.2628	+	6.12689
chrX	106015699	106022450	Cox7b	81.8186	+	6.02631
chrX	74013913	74023936	Irak1	54.9015	-	5.97292
chrX	74013913	74023936	Mir5132	28310	-	5.97292
chrX	166523006	166585716	Egfl6	2.75673	-	5.89232
chrX	101274090	101298934	Med12	16.9166	+	5.74426
chrX	74223460	74246534	Flna	119.321	-	5.66957
chrX	139779680	139782353	Ripply1	9.2593	-	5.66923
chrX	94188708	94212651	Eif2s3x	13.3916	-	5.62242
chrX	140539528	140600522	Tsc22d3	4.01031	-	5.60397

Table 1: PAR-TERRA binding sites on the mouse X-chromosome in MEFs

mm10 assembly	Start	End	gene name	FPKM	strand	Mean of TERRA coverage
chrX	74254838	74257747	Emd	131.791	+	5.52669
chrX	94074630	94123407	Zfx	14.4577	-	5.457
chrX	8138974	8147963	2900002K06Rik	2.48279	+	5.45401
chrX	8138974	8147963	Rbm3	21.0491	-	5.45401
chrX	151047232	151096543	Fgd1	81.8043	+	5.44937
chrX	151047232	151096543	Tsr2	23.1259	-	5.44937
chrX	57383347	57393036	RbmX	15.7016	-	5.4457
chrX	73916869	73921944	Naa10	94.1038	-	5.41574
chrX	74297096	74304721	Atp6ap1	57.5415	+	5.33398
chrX	100622905	100625907	Pdzd11	67.6827	-	5.24963
chrX	166440824	166452543	Trappc2	5.95827	+	5.23482
chrX	101640063	101684351	Ogt	82.85	+	5.19793
chrX	74329065	74344689	Plxna3	6.81077	+	5.15815
chrX	73716596	73738287	Abcd1	9.8525	+	5.05484
chrX	7884243	7894492	Slc35a2	8.80274	+	5.05244
chrX	7919821	7928607	Eras	53.4302	-	5.03256
chrX	7919821	7928607	Pcsk1n	14.3249	+	5.03256
chrX	73437314	73459029	Haus7	37.766	-	5.03236
chrX	7894518	7899269	Pqbp1	101.762	-	4.95918
chrX	140948424	140956711	Psmc10	16.0633	-	4.86065
chrX	160502165	160598878	Phka2	10.5119	+	4.82787
chrX	159627407	159975917	Sh3kbp1	13.5148	+	4.81242
chrX	9654269	9662983	Dynl1t3	8.16616	-	4.77036
chrX	60891365	60893430	Sox3	5.66273	-	4.76974
chrX	73778962	73786897	Idh3g	186.084	-	4.72145
chrX	142317992	142390535	Acsl4	25.1009	-	4.71533
chrX	164373547	164402647	Figf	2.67737	+	4.70077
chrX	7823842	7836503	Kcnd1	5.67212	+	4.6921
chrX	163935442	163958666	Zrsr2	32.212	-	4.68589
chrX	159372194	159385699	Eif1ax	160.42	+	4.667
chrX	150983132	151017322	Gnl3l	79.5544	-	4.63998
chrX	7722248	7728201	Wdr45	57.6147	+	4.57451
chrX	7762660	7775202	Tfe3	88.3365	+	4.55791
chrX	101377336	101385624	Gjb1	7.30102	+	4.55117
chrX	155213138	155216409	Sat1	31.7602	-	4.49062
chrX	164419786	164433915	Piga	14.5205	+	4.46376
chrX	73686182	73716175	Bcap31	136.655	-	4.34133
chrX	7728570	7731063	Praf2	45.3074	+	4.32855
chrX	136139044	136140437	Bex4	54.8305	+	4.31599
chrX	134585653	134588062	Rpl36a	1.66059	+	4.30142
chrX	134804141	134809221	Armxc2	20.2464	-	4.29013
chrX	73853779	73880834	L1cam	1.33532	-	4.26027
chrX	58030627	58036630	Zic3	68.0886	+	4.24685
chrX	159532667	159593081	A830080D01Rik	43.9242	+	4.19267
chrX	7899397	7907652	Timm17b	31.5895	+	4.06939
chrX	142853473	142966728	Ammecr1	13.3866	-	4.01434
chrX	164070702	164076049	Siah1b	49.6673	-	3.99607
chrX	150806420	150814339	Maged2	15.8384	-	3.94816
chrX	100626064	100727271	Kif4	34.3476	+	3.93177

Table 1: PAR-TERRA binding sites on the mouse X-chromosome in MEFs

mm10 assembly	Start	End	gene name	FPKM	strand	Mean of TERRA coverage
chrX	75095853	75130949	Dkc1	136.814	+	3.90988
chrX	75095853	75130949	Mpp1	38.769	-	3.90988
chrX	37091833	37110322	Upf3b	39.8344	-	3.88822
chrX	12936872	12938541	AA414768	7.27643	+	3.8628
chrX	48411048	48463132	Elf4	16.0656	-	3.8481
chrX	7959259	7967910	Gata1	1.29963	-	3.77677
chrX	134308162	134362639	Cenpi	16.8886	+	3.77197
chrX	134059348	134086821	Cstf2	45.5011	+	3.75403
chrX	51003913	51018018	Rap2c	14.0536	-	3.69198
chrX	8238667	8252406	Ftsj1	29.4	-	3.68661

Top 25% of TERRA mean coverage over gene

Total active gene 452

Example 3. PAR-TERRA localizes in cis and in trans

5 Although TERRA and PAR ChIRT profiles were very similar, ChIRT analysis revealed heterogeneity in the telomeric RNAs. Some binding sites were dominated by TERRA RNA (e.g., telomeric ends of Chr 3,4,5, etc), while others showed prominent peaks of both TERRA and PAR RNA (Fig. 3). A distinct PAR-TERRA species was further supported by the nearly identical ChIRT profiles at these sites of overlap

10 (examples shown in Fig. 3B-D), as well as by the above molecular analyses indicating a physical contiguity (Fig. 1F,G). PAR-TERRA binding was especially notable at the subtelomeric end (pseudoautosomal region) of the sex chromosomes (Fig. 3B). *Midl*, *Erdr1*, and *Asmt* are tens of kilobases away from the TERRA DNA sequence [(TTAGGG)*n* telomeric repeat], but nevertheless demonstrated prominent TERRA

15 peaks. The intronic regions and 3' end of *Midl* contained some of the strongest PAR-TERRA peaks in the genome. The gene body of *Asmt* was also a strong binding site. By far the strongest peaks were found in *Erdr1*, which itself contains two short stretches of (TTAGGG)*n* repeats. [Note: It should be emphasized that these TERRA reads could be unambiguously assigned to the *Erdr1* repeats — and not telomeric

20 repeats — because pair-end sequencing enabled utilizing the unique sequence at one end to align the other repetitive end.] These results demonstrated that PAR-TERRA not only localizes in cis to the telomeres of sex chromosomes, but also spreads locally in cis to encompass genes of the pseudoautosomal region.

Intriguingly, PAR-TERRA also targeted sites in trans. Magnified views showed strong PAR-TERRA binding peaks in the sub-telomeric regions of Chr 2, 9, 13, and 18 (Fig. 3C), and more moderate binding peaks in the sub-telomeric regions of Chr 8 and 19 (Fig. 10). Additionally, PAR-TERRA targeted internal regions of autosomes. Many binding peaks occurred within genes, especially within introns, as exemplified by *Abcb10*, *Uchl10s*, and *Hes3* (Fig. 3D). PAR-TERRA also targeted internal (TTAGGG)_n telomeric repeats which occur at a number of locations throughout the genome (one example shown in Fig. 3C; NOTE: The reads could be assigned unambiguously to the internal repeats because of pair-end sequencing, in which the unique end is used to align the repeat end). We conclude that TERRA and PAR-TERRA transcripts are not confined to telomeric ends of mouse chromosomes.

Example 4. Epigenomic regulation by PAR-TERRA

Because PAR-TERRA accounts for the vast majority of TERRA transcripts in the nucleus and has both X-linked and autosomal targets, we examined its effect on gene expression on a genome-wide basis. We perturbed PAR-TERRA expression using knockdown (KD) approaches to avoid undesirable consequences of genetically deleting telomeres. Neither siRNA nor shRNA resulted in knockdown. On the other hand, single-stranded antisense oligonucleotide (ASO) locked nucleic acids (LNA) gapmers led to substantial depletion of TERRA after 12 hours in ES cells, as shown by Northern blot analysis (Fig. 4A, 11A) and RNA FISH (Fig. 4B, 11B). ASO's to TERRA sequences and PAR-specific sequences (31k) achieved similar results. At optimal LNA concentrations, at least 75-90% of PAR-TERRA were degraded as quantitated by Northern analysis (Fig. 4A, 11A). RNA FISH showed a substantial depletion of both TERRA and PAR signals in >86% of nuclei (n=217) after 6 hours (4B, 11B). Notably, treating with 31k-PAR gapmers dramatically reduced TERRA RNA FISH signals (Fig. 4B). Conversely, administering TERRA gapmers also reduced PAR FISH signals. These data provide strong support for the idea of a continuous PAR-TERRA transcript and the conclusion that PAR-TERRA accounts for the majority (~90%) of telomeric transcripts in ES cells.

We then carried out transcriptomic analysis on two biological replicates of ES cells after 12 hours of PAR-TERRA depletion (Fig. 4, 11C). Analysis of the biological replicates of TERRA-specific and PAR-specific KD's revealed overlapping transcriptomic changes (Fig. 4C). Analysis using Cuffdiff2 uncovered 126

differentially expressed genes after TERRA KD and 324 after PAR KD in ES cells, among which 56 genes were shared between the TERRA- and PAR-specific KD cells. In MEFs, TERRA KD led to 137 significant changes and PAR KD led to 309, among which 36 genes were shared. Among the shared genes for each cell type, the changes in gene expression after PAR and TERRA KD were very similar (Fig. 4D). Among 8 genes that were shared between female ES cells and MEFs, the expression changes were also highly similar (Fig. 4E).

On the other hand, there was a significantly increased probability that genes with PAR-TERRA binding sites would be downregulated by PAR-specific KD (Fig. 4F). The probability density function for 565 genes with PAR-TERRA binding sites was significantly different from that for the 14,724 genes without a PAR-TERRA site (Fig. 4F; KS test, $P < 0.0001$). PAR KD resulted in net downregulation of genes with PAR-TERRA binding sites (left-shift of red distribution). Among all downregulated genes after PAR KD, the degree of downregulation was significantly more pronounced for those with PAR-TERRA sites (Fig. 4G, right panels; χ^2 test, $P < 0.001$), possibly indicative of a direct effect on PAR-TERRA target genes. In contrast, among the upregulated genes, there was no significant difference between genes with and without a PAR-TERRA site (χ^2 test, $P = 0.26$), suggesting that upregulation may be an indirect consequence. These data argue that PAR-TERRA RNA exerts a direct positive effect on target genes.

The non-overlapping transcriptomic changes for TERRA- versus PAR-specific knockdowns may suggest the presence of a gene class targeted by TERRA-intrinsic transcripts (containing only UUAGGG repeats), rather than by PAR-TERRA. Consistent with this, we observed a number of autosomal genes — particularly those within sub-telomeric regions — where TERRA RNA was the predominant bound form (Fig. 5A). At such genes, TERRA KD consistently resulted in gene downregulation, as shown by RNA-seq and confirmed by RT-qPCR (Fig. 5B). Such autosomal sub-telomeric genes might therefore be controlled by TERRA transcripts produced in cis, perhaps in addition to some contribution from X-linked PAR-TERRA in trans. We proposed that genes responsible for facioscapulohumeral muscular dystrophy (FSHD) can be controlled in this way. The FSHD locus is located in the subtelomeric region of human Chr4 and contains coding genes FRG1, FRG2, DUX4, and the macrosatellite repeat, D4Z4. FSHD is caused by ectopic expression

of these genes when the D4Z4 repeat contracts and becomes “activated”. Thus, PAR-TERRA or Chr4-specific TERRA could be targeted to downregulate the associated subtelomeric genes.

Example 5. PAR-TERRA protects pseudoautosomal genes and escapees from XCI

ChrX has a large number of PAR-TERRA sites (Fig. 3A). In d0 ES cells, ChrX harbors 84-86 PAR-TERRA sites; in MEFs, ChrX harbors 30-94 sites, with one broad domain at the distal end of ChrX, another around the X-inactivation center, and several additional hotspots in more proximal regions of ChrX (Fig. 5C,D).

Intriguingly, PAR-TERRA densities were greater at escapees than at genes subject to XCI (Fig. 5E; $P < 0.05$). Escapees play important roles in human disease (e.g., XO Turner Syndrome) and generally have ChrY homologues that render XCI unnecessary for these genes (Berletch et al., 2010; Berletch et al., 2011; Deng et al., 2014).

Escapee genes are located throughout ChrX, and may occur singly or in clusters (Berletch et al., 2011; Lopes et al., 2011). In humans, 15% of X-linked genes escape silencing, whereas approximately a dozen or so genes escape XCI in mice (Carrel and Willard, 2005; Yang et al., 2010). Their association with PAR-TERRA raised the possibility that PAR-TERRA could regulate escape from XCI.

To test this idea, we performed quantitative RT-PCR. Significantly, PAR-TERRA KD resulted in downregulation of all pseudoautosomal genes, including *Mid1*, *Erdr1*, and *Asmt* (Fig. 5F). This downregulation was confirmed by RNA-seq analysis and was similar to the downregulation at sub-telomeric regions of autosomes after TERRA KD (Fig. 5A,B). Thus, one of TERRA’s cis functions may be to protect sub-telomeric genes from position effects of telomeric heterochromatin. To examine Xi-specific changes, we performed allele-specific RNA-seq analysis using a hybrid MEF clonal cell line in which the Xi is always of *Mus musculus* origin and the Xa is of *Mus castaneus* origin (Pinter et al., 2012). RNA-seq analysis confirmed downregulation of PAR genes and additional known escapees outside of the PAR, such as *Shroom4*, *Kdm6a*, *1810030O07Rik*, as well as escapees (*Fgf13*, *Mbtps2*, *Huwe1*, *Sept6*, *Aifm1*, and *Kif4*) observed in our cell line. We concluded that PAR-TERRA promotes expression of escapee genes on the Xi.

We next investigated the mechanistic relationship between PAR-TERRA and escapees. While escapee gene bodies generally have low Xist coverage, their flanking

regions are often marked by high Xist coverage suggestive of a boundary that sequesters Xist and prevents it from entering escapee loci (Fig. 6A, *Midl* shown)(Simon et al., 2013). Intriguingly, metagene analysis revealed that PAR-TERRA was highly enriched at the transcriptional start sites (TSS) of escapee genes, where the Xist boundary occurred (Fig. 6B). For example, *Midl* was marked by a sharp border of Xist RNA beyond which Xist coverage dropped off dramatically (Fig. 6A). To determine how PAR-TERRA affected Xist spreading near escapees, we carried out CHART-seq of Xist following knockdown of PAR-TERRA for 6 hours. Scatterplot analysis of Xist coverages in 40-kb windows showed that Xist binding changed very little for nearly all Xi loci (Fig. 6C, Pearson's $r > 0.90$), with the notable exception of outliers corresponding to some escapees (Fig. 6C, red dots). Upon PAR-TERRA KD, we observed decreased accumulation of Xist at the TSS and a shift to flanking regions (Fig. 6D). Taken together, these data suggest that PAR-TERRA protects escapees from silencing by sequestering Xist at the TSS of escapees, thereby preventing the spread of Xist into upstream regulatory regions and into the TSS regions of escapees.

Given that ~90% of PAR-TERRA foci occurred next to but did not overlap the Xist cloud (Fig. 6E; 8D)(Zhang et al., 2009), we considered the possibility that PAR-TERRA might organize a privileged, transcription-permissive compartment next to the Xi. We noted that PAR has a higher colocalization rate with other escapees, such as *Ftx* and *Jpx*, than with genes subject to XCI, such as *Hprt* (Fig. 6F; $P < 0.0001$). Depleting PAR-TERRA resulted in a statistically significant decrease in colocalization frequency (Fig. 6G; $P < 0.02$, 0.001). These data are consistent with the idea of a privileged compartment that is geographically close to the pseudoautosomal region.

Gene ontology (GO) analysis was performed, restricted to differentially expressed genes in TERRA KD (P adjusted value < 0.05 in DEseq2 analysis). The top 10 enriched biological process terms are listed in Table 2, in the upregulated gene set or in downregulated gene set in MEFs.

Table 2

MEF up-regulated				
Category	Term	Count	PValue	Benjamini
GOTERM BP FAT	GO:0007155~cell adhesion	25	3.26E-06	0.011919699
GOTERM BP FAT	GO:0022610~biological adhesion	25	3.54E-06	0.006157583
GOTERM BP FAT	GO:0030029~actin filament-based process	12	1.12E-04	0.047233272
GOTERM BP FAT	GO:0034330~cell junction organization	6	1.35E-04	0.042637379
GOTERM BP FAT	GO:0030036~actin cytoskeleton organization	11	2.94E-04	0.073349276
GOTERM BP FAT	GO:0034329~cell junction assembly	5	2.95E-04	0.051751534
GOTERM BP FAT	GO:0010810~regulation of cell-substrate adhesion	5	3.97E-04	0.070796403
GOTERM BP FAT	GO:0034621~cellular macromolecular complex subunit organization	13	5.34E-04	0.082874247
GOTERM BP FAT	GO:0001944~vasculature development	13	8.40E-04	0.087939921
GOTERM BP FAT	GO:0034622~cellular macromolecular complex assembly	12	8.75E-04	0.083776541

MEF down-regulated				
Category	Term	Count	PValue	Benjamini
GOTERM BP FAT	GO:0007049~cell cycle	27	2.17E-06	2.99E-05
GOTERM BP FAT	GO:0051726~regulation of cell cycle	14	2.06E-06	0.001425534
GOTERM BP FAT	GO:0007346~regulation of mitotic cell cycle	10	2.42E-06	0.00111689
GOTERM BP FAT	GO:0008219~cell death	21	3.42E-06	0.001182773
GOTERM BP FAT	GO:0012501~programmed cell death	20	4.72E-06	0.001304329
GOTERM BP FAT	GO:0016265~death	21	4.88E-06	0.001129723
GOTERM BP FAT	GO:0005915~apoptosis	19	1.41E-05	0.002783689
GOTERM BP FAT	GO:0051130~positive regulation of cellular component organization	10	1.74E-05	0.00390798
GOTERM BP FAT	GO:0033043~regulation of organelle organization	11	1.81E-05	0.002793488
GOTERM BP FAT	GO:0022402~cell cycle process	17	2.36E-05	0.00325365

Example 6. TERRA facilitates homologous sex chromosome pairing

In mammals, pairing between two homologous chromosomes rarely occurs outside of meiosis. An exception is the X-chromosome, which undergoes transient homologous interactions prior to the initiation of XCI in ES cells (Bacher et al., 2006; Xu et al., 2006). Their transient pairing via the noncoding *Tsix* and *Xite* loci is proposed to result in mutually exclusive selection of Xa and Xi and, thereby, to ensure upregulation of Xist RNA from a single ChrX (Xu et al., 2006). To date, only a few regulatory factors have been identified, including a 15-kb “pairing center” from which the noncoding *Tsix* and *Xite* RNAs are produced (Xu et al., 2007). *Tsix* and *Xite* RNAs work together with the chromosomal architectural protein, CTCF, to establish the paired state (Kung et al., 2015). How the two X-chromosomes search and identify each other during the pairing process is not known.

Interestingly, PAR-TERRA ChIRT revealed strong PAR-TERRA binding sites in *Xite* and *Tsix* (Fig. 7A). These peaks grabbed our attention because they occurred within the pairing center and were specific to ES cells. To test a role in pairing, we knocked down TERRA and measured interchromosomal distances in a 3D DNA FISH assay (Xu et al., 2006; Kung et al., 2015). We measured inter-allelic distances at the telomere, the X-inactivation center (*Xic*), and a distant locus, *Hprt*. Using *Xic* probes mapping to *Tsix/Xist*, we observed a left shift in the cumulative

frequency curve between days 0 and 4 of differentiation, indicating an increase in the number of nuclei displaying inter-allelic distances of <0.1 nuclear diameters (ND) or <1 micron (Fig. 7B, 13A), a distance implying the occurrence of pairing, as previously determined (Bacher et al., 2006; Xu et al., 2006). By contrast, the number of such events increased minimally between *Hprt* alleles. Curiously, however, when measured between the telomeres (TeloX) or between PAR, there was also a significant increase in allelic colocalization events. The degree of left shift indicated a very robust telomeric pairing (Fig. 7B). Telomeric pairing was X-specific and was not observed between homologous autosomal telomeres (Fig. 7C, Chr2 telomere shown). Moreover, the time window of telomeric pairing overlapped with that for *Xic-Xic* pairing.

This unexpected relationship raised the possibility that the X-telomeres may be involved in somatic X-X pairing. Since the 1920's, the telomere has been suspected to facilitate homology searching during meiotic chromosome pairing, where a single telomeric "bouquet" (a clustering of telomeric ends of all chromosomes) nucleates synapsis and enables synaptic extension along the length of homologous pairs (Maguire, 1984; Rockmill and Roeder, 1998; Reig-Viader et al., 2013; Xiang et al., 2014). During male meiosis, Chr X and Y also pair in spite of their limited homology, and they do so via their pseudoautosomal region — the only region of homology between the two sex chromosomes.

We explored the possibility that telomeric clustering could also mediate sex chromosome pairing in non-meiotic cells. First, given the occurrence of meiotic X-Y pairing, we asked whether somatic telomeric pairing extends to the X and the Y. Indeed, during differentiation, the inter-PAR and inter-telomeric distances between Chr X and Y shifted to the left, much in the same way as those observed for X-X telomeric pairing (Fig. 7D, 13B). This colocalization of telomeres/PARs was also transient, occurring on day 4, but not on day 0 or 12. Serial RNA/DNA FISH showed that, while the paired telomeric signals of the sex chromosomes were very close ($<1\mu\text{m}$ between two dots) or overlapped (one dot), the rest of the two chromosomes were not paired, as visualized by Chr X and Y painting probes (Fig. 7E). These data demonstrate that somatic telomeric pairing is not limited to the two female X-chromosomes. Transient homologous chromosome pairing also occurs between Chr X and Y during differentiation of male ES cells.

We asked whether X-X and X-Y pairing might be controlled by PAR-TERRA in cis. To examine telomeric pairing, we assessed pairing frequencies between telomeres on d4 after treating cells with LNA gapmers to degrade TERRA RNA. Significantly, we observed a right shift of inter-telomeric (PAR-PAR) distances in
 5 TERRA KD cells relative to control cells that were administered a scrambled (Scr) LNA. These results demonstrated a loss of telomeric colocalization when TERRA RNA was depleted (Fig. 7F). Telomeric X-X pairing in female cells and X-Y pairing in male cells were both affected. Therefore, TERRA RNA is required for somatic telomeric pairing of sex chromosomes. Given the presence of PAR-TERRA binding
 10 sites at the pairing center, we investigated whether *Xic-Xic* pairing in female cells might also be affected by the loss of TERRA. We treated differentiating female ES cells with TERRA LNAs and measured inter-*Xic* distances at day 4. Relative to cells treated with the control Scr LNA, there was a significant right shift of colocalization frequencies between the *Xic* alleles after TERRA KD (Fig. 7G), indicating a loss of
 15 pairing. Thus, both types of trans-interactions — telomeric pairing and *Xic-Xic* pairing — require the function of TERRA.

These data lead to the notion that PAR-TERRA RNA could facilitate homologous interchromosomal interactions by inducing co-clustering of crucial pairing sites. Because the pairing center (Fig. 7A) and the pseudoautosomal region
 20 (Fig. 2H) are both major hotspots of PAR-TERRA binding, we examined the possibility that intra-chromosomal cis-interactions between the *Xic* and telomere could bring the *Xic* to the juxta-telomeric compartment, which in turn would bring the two *Xic*'s in close proximity due to the action of telomeric pairing. Indeed, DNA FISH using *Xic* and sub-telomeric (PAR) probes showed that the *Xic* and telomere
 25 frequently colocalized on day 4 (Fig. 7H). This co-clustering depended on TERRA RNA, as knocking down TERRA abolished the *Xic*-telomeric interactions (Fig. 7H). We conclude that both (i) cis-interactions between the *Xic* and the telomere and (ii) trans-interactions between two telomeres require TERRA RNA. We propose that TERRA RNA tethers the ends of sex chromosomes to facilitate inter-chromosomal
 30 interactions between the *Xic*'s (Fig. 7I).

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Schoeftner, S., and Blasco, M.A. (2008). Developmentally regulated transcription of mammalian telomeres by DNA-dependent RNA polymerase II. *Nature cell biology* 10, 228-236

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OTHER EMBODIMENTS

It is to be understood that while the invention has been described in
conjunction with the detailed description thereof, the foregoing description is intended
15 to illustrate and not limit the scope of the invention, which is defined by the scope of
the appended claims. Other aspects, advantages, and modifications are within the
scope of the following claims.

WHAT IS CLAIMED IS:

1. A composition comprising an isolated inhibitory nucleic acid targeting PAR-TERRA, preferably wherein the inhibitory nucleic acid is modified.
2. A method of decreasing expression of an Xi escapee gene in a cell, preferably a cell of a subject have a supernumerary X chromosome, the method comprising administering to the cell an inhibitory nucleic acid targeting PAR-TERRA, preferably wherein the inhibitory nucleic acid is modified.
3. A method of decreasing expression of Xa genes in a cell, preferably a cell of a subject having a supernumerary X chromosome, the method comprising administering to the cell an inhibitory nucleic acid targeting PAR-TERRA, preferably wherein the inhibitory nucleic acid is modified.
4. A method of treating a subject who has a disorder of sex chromosome aneuploidy associated with a supernumerary X chromosome, the method comprising administering to the subject an inhibitory nucleic acid targeting PAR-TERRA, preferably wherein the inhibitory nucleic acid is modified.
5. A method of decreasing expression of autosomal genes in a cell, the method comprising administering to the cell an inhibitory nucleic acid targeting PAR-TERRA or an autosome-specific TERRA, preferably TERRA species originating within the subtelomeric region of an autosome and comprising autosome-specific 5' sequences, preferably wherein the inhibitory nucleic acid is modified.
6. The method of claim 5, wherein the inhibitory nucleic acid targets Chr4-specific TERRA.
7. The method of claim 6, wherein expression of FRG1, FRG2, DUX4, and the long noncoding RNAs of forward and reverse orientations from the macrosatellite repeat, D4Z4, is decreased.
8. The method of claims 6-7, wherein the cell is from or in a subjection who has facioscapulohumeral muscular dystrophy (FSHD).

9. The composition of claim 1, for treating a subject who has a disorder of sex chromosome aneuploidy associated with a supernumerary X chromosome.
10. The composition or methods of claims 4 or 9, wherein the subject has 46,XY, 47,XXY, 48,XXYY, 48,XXXY, 47,XXX, 48,XXXX or 49,XXXXX aneuploidy.
11. The method of claim 2, wherein the cell is from a subject who has 46,XY, 47,XXY, 48,XXYY, 48,XXXY, 47,XXX, 48,XXXX or 49,XXXXX aneuploidy.
12. A method of decreasing expression of X-linked or autosomal growth control or apoptosis genes in a cell, the method comprising administering to the cell an inhibitory nucleic acid targeting PAR-TERRA, PAR, or TERRA, preferably wherein the inhibitory nucleic acid is modified.
13. The composition or method of claims 1-7, 9, 11 or 12, wherein the inhibitory nucleic acid does not comprise three or more consecutive guanosine nucleotides or does not comprise four or more consecutive guanosine nucleotides.
14. The composition or method of claims 1-7, 9, 11 or 12, wherein the inhibitory nucleic acid is 8 to 30 nucleotides in length.
15. The composition or method of claims 1-7, 9, 11 or 12, wherein at least one nucleotide of the inhibitory nucleic acid is a nucleotide analogue.
16. The composition or method of claims 1-7, 9, 11 or 12, wherein at least one nucleotide of the inhibitory nucleic acid comprises a 2' O-methyl, e.g., wherein each nucleotide of the inhibitory nucleic acid comprises a 2' O-methyl.
17. The composition or method of claims 1-7, 9, 11 or 12, wherein the inhibitory nucleic acid comprises at least one ribonucleotide, at least one deoxyribonucleotide, or at least one bridged nucleotide.
18. The composition or method of claim 17, wherein the bridged nucleotide is a LNA nucleotide, a cEt nucleotide or a ENA modified nucleotide.
19. The composition or method of claims 1-7, 9, 11, or 12, wherein each nucleotide of the inhibitory nucleic acid is a LNA nucleotide.

20. The composition or method of claim 19, wherein one or more of the nucleotides of the inhibitory nucleic acid comprise 2'-fluoro-deoxyribonucleotides and/or 2'-O-methyl nucleotides.
21. The composition or method of claims 1-7, 9, 11, or 12, wherein one or more of the nucleotides of the inhibitory nucleic acid comprise one of both of ENA nucleotide analogues or LNA nucleotides.
22. The composition or method of claims 1-7, 9, 11, or 12, wherein the nucleotides of the inhibitory nucleic acid comprise comprising phosphorothioate internucleotide linkages between at least two nucleotides, or between all nucleotides.
23. The method or compositions of claims 1-7, 9, 11, or 12, wherein the inhibitory nucleic acid is a gapmer or a mixmer.

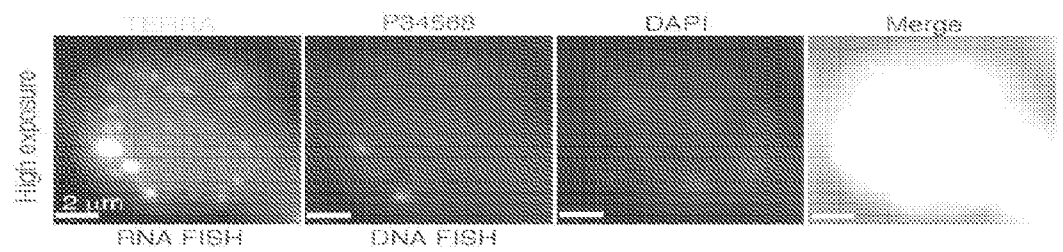


FIG. 1A

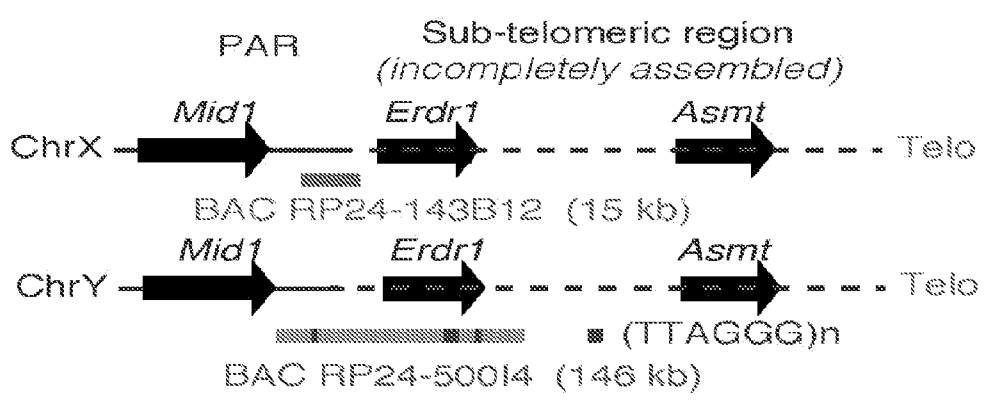


FIG. 1B

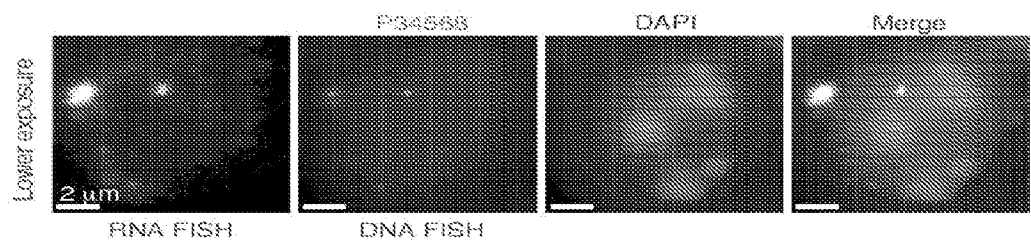


FIG. 1C

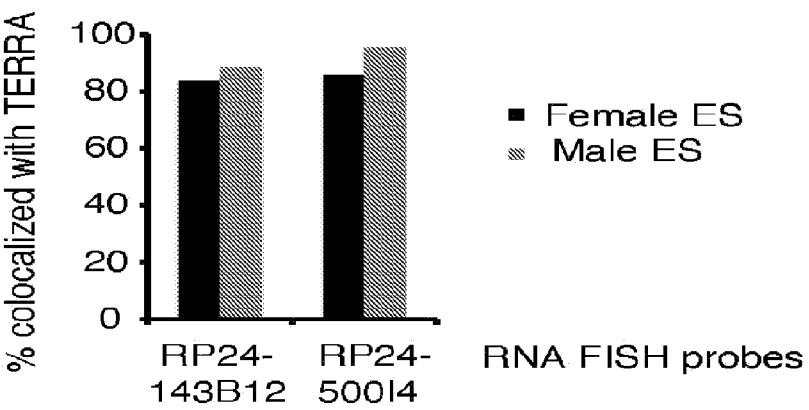


FIG. 1D

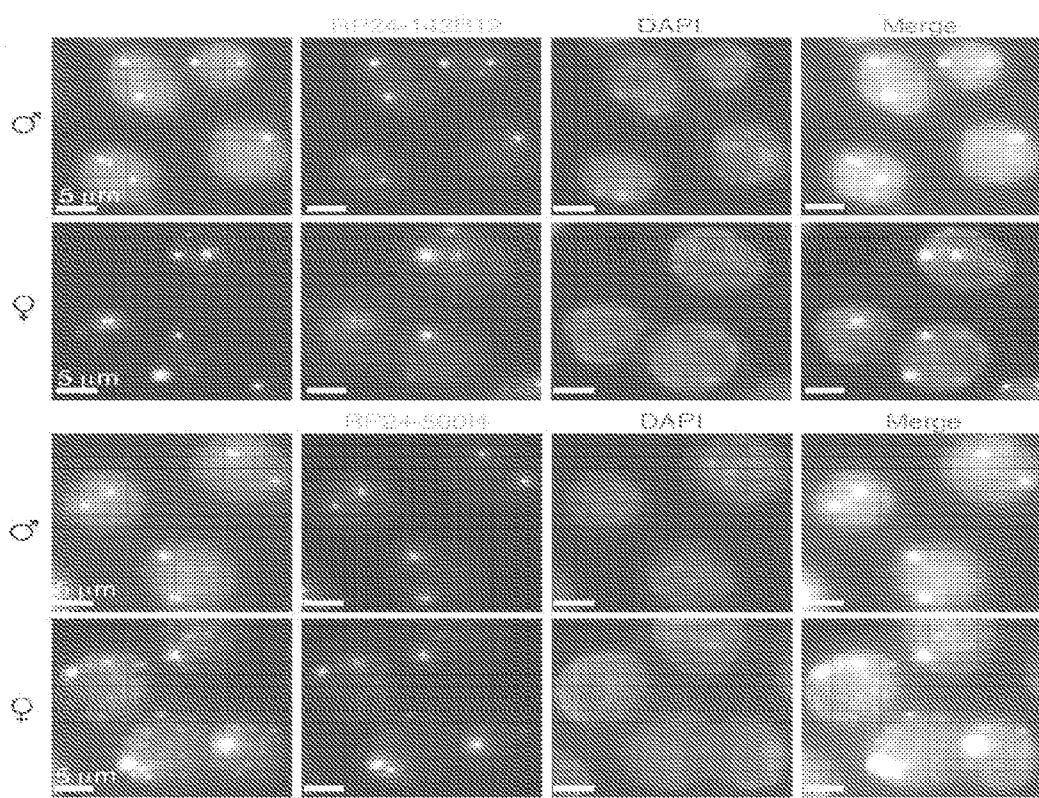


FIG. 1E

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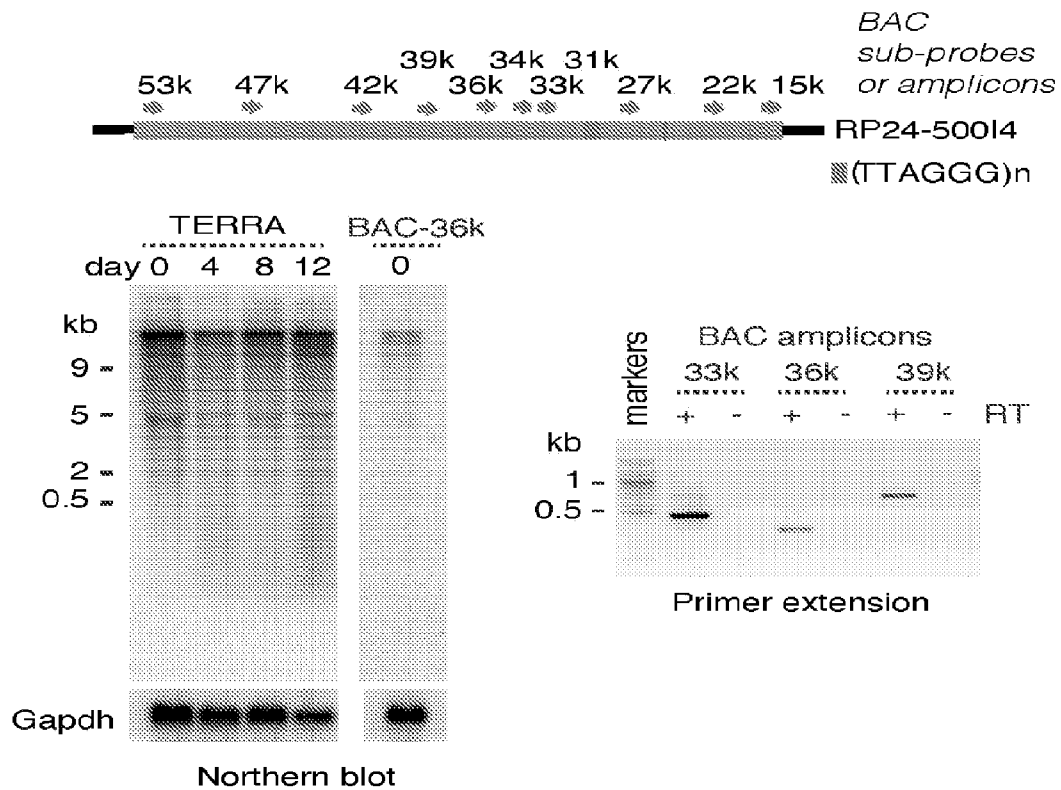


FIG. 1F

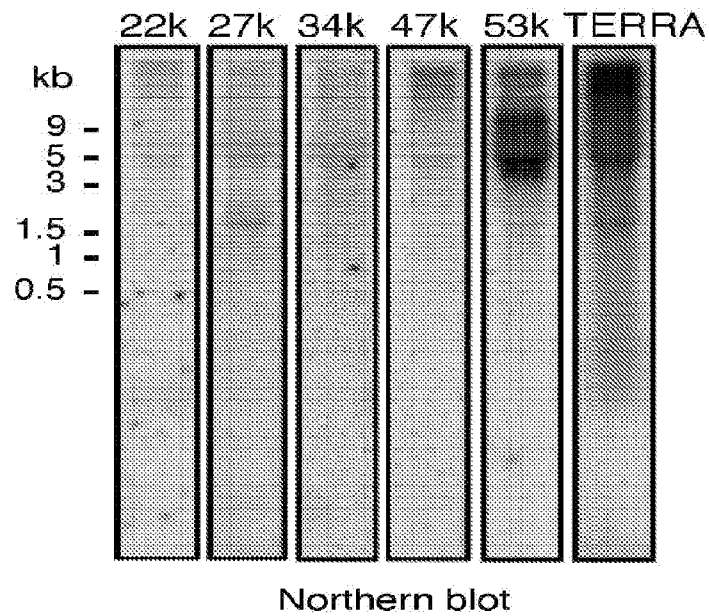


FIG. 1G

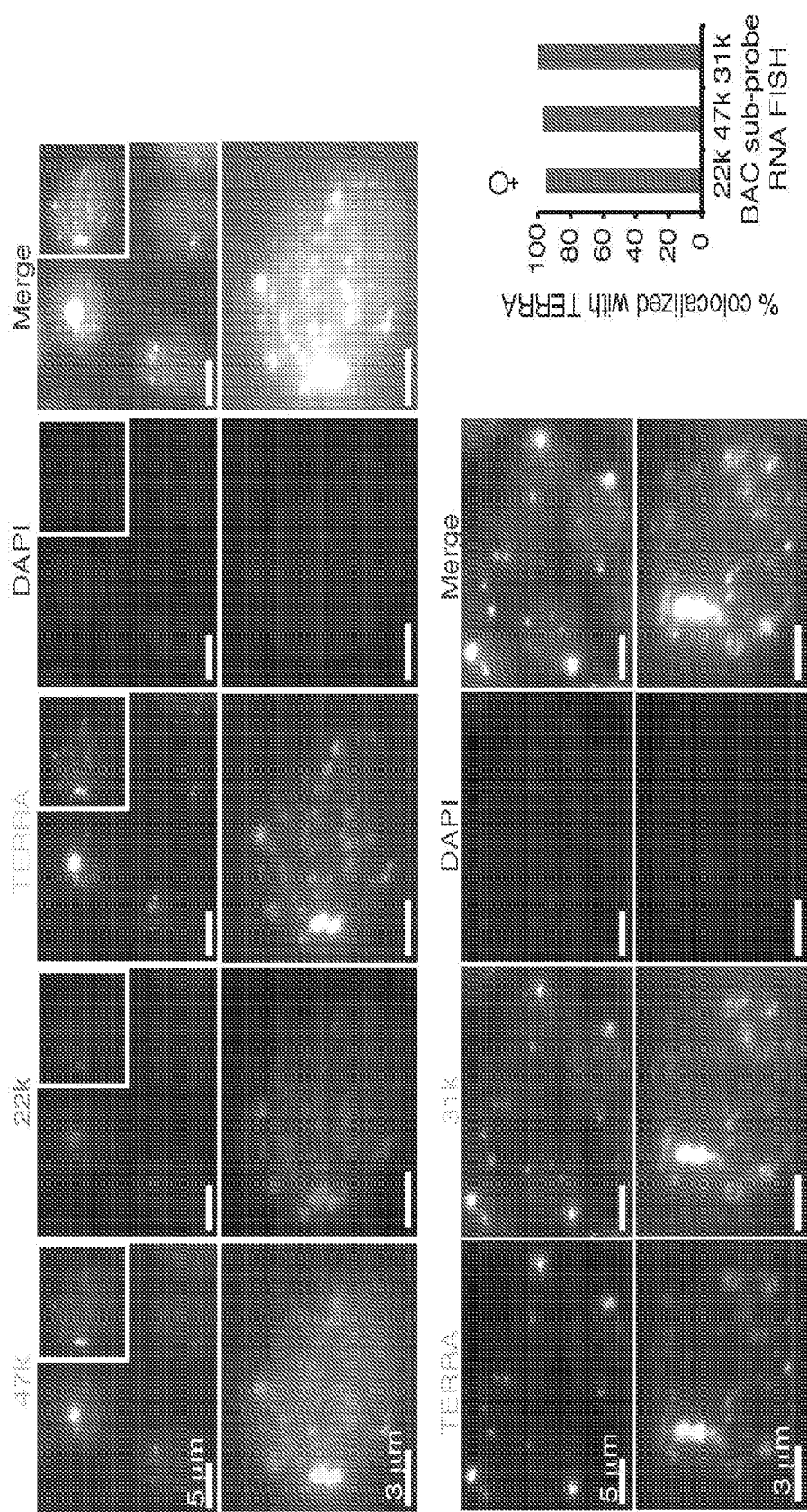


FIG. 1H

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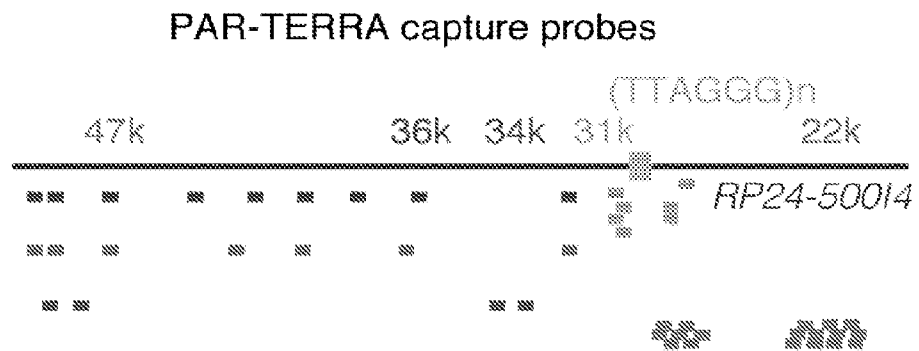


FIG. 2A

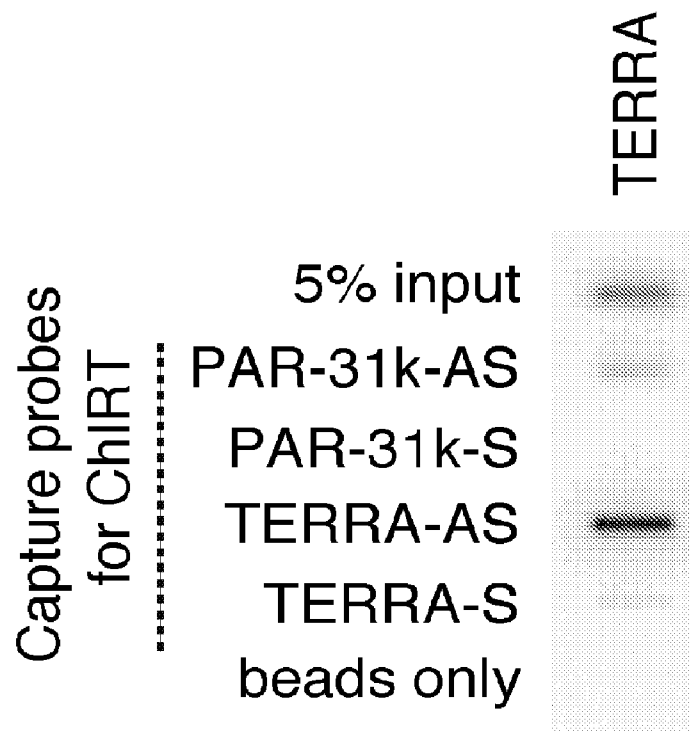


FIG. 2B

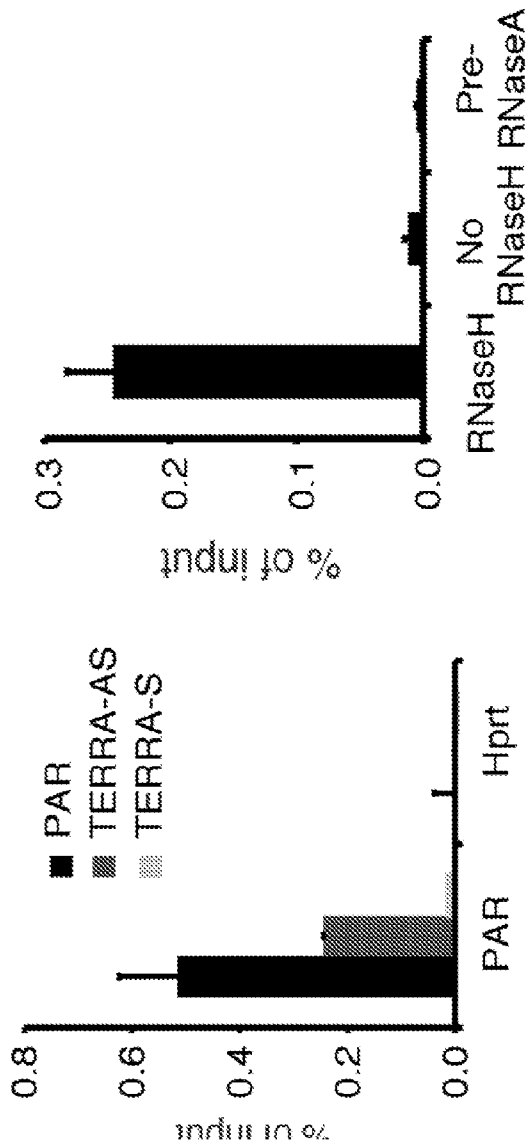


FIG. 2C

FIG. 2D

Number of PAR-TERRA binding sites											
	ES_d0			ES_d3			ES_d7			MEF	
	TERRA	PAR	Sense	TERRA	PAR	Sense	TERRA	PAR	Sense	TERRA	Sense
Normalized to input	4054	2304	78	2125	5373	1884	3199	500	440	544	
Normalized to sense	4076	-	-	-	-	-	-	492	-	-	-
Normalized to no-RNase H	3880	-	-	-	-	-	-	492	-	-	-

FIG. 2E

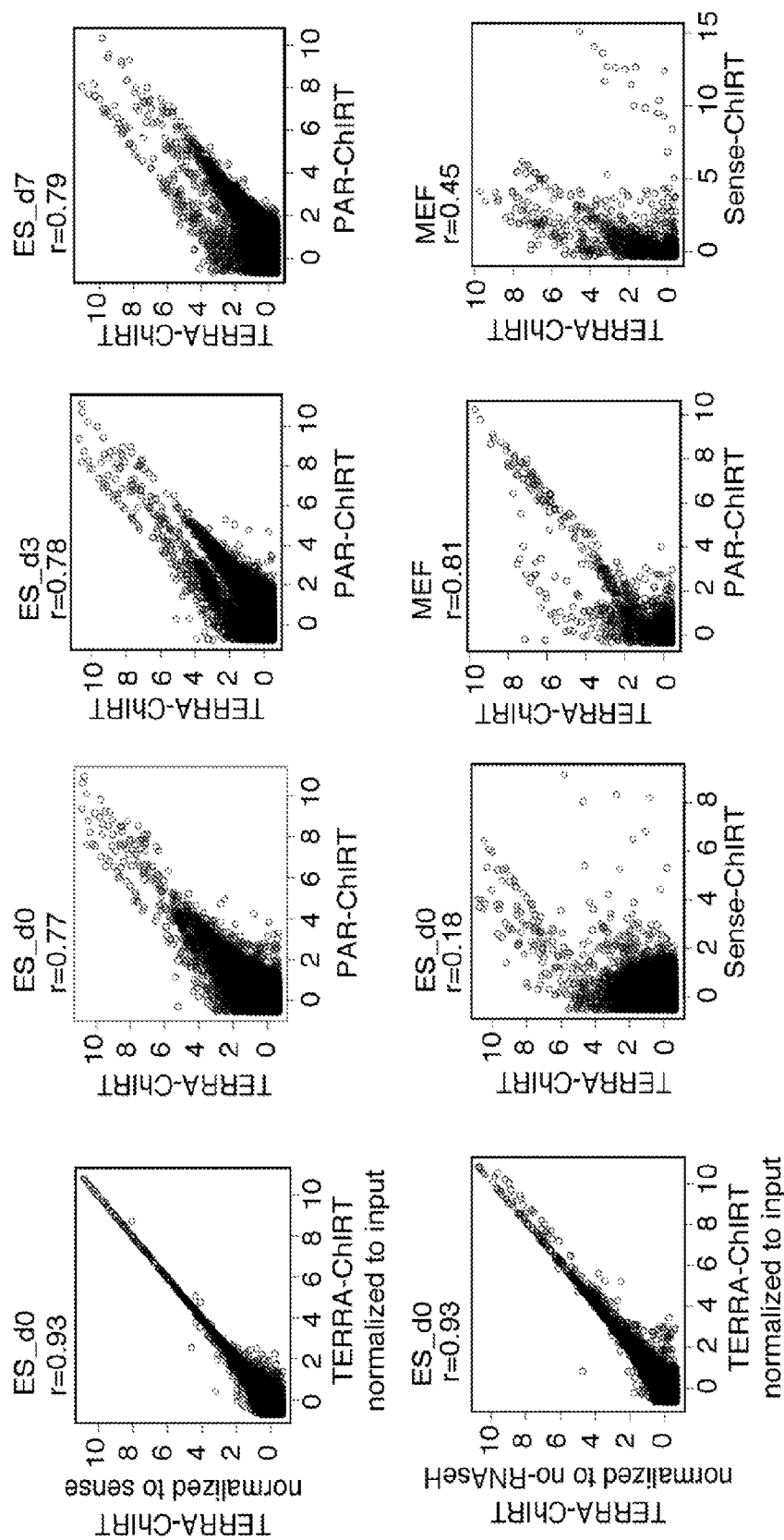


FIG. 2F

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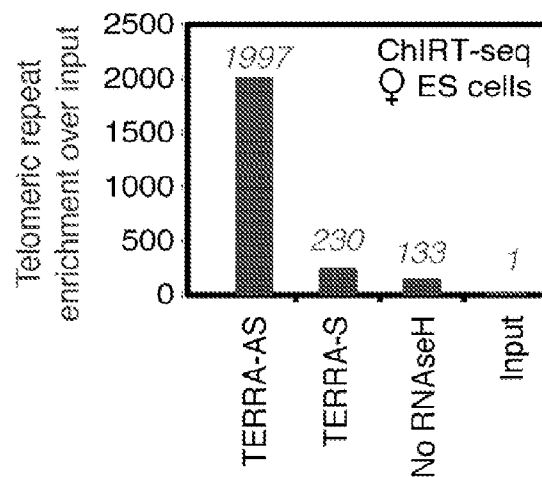
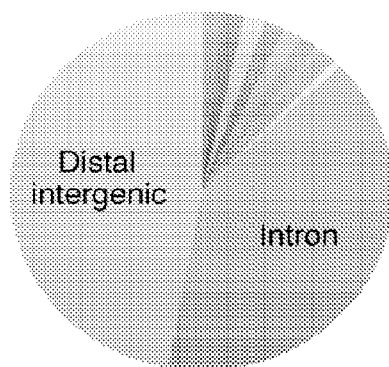


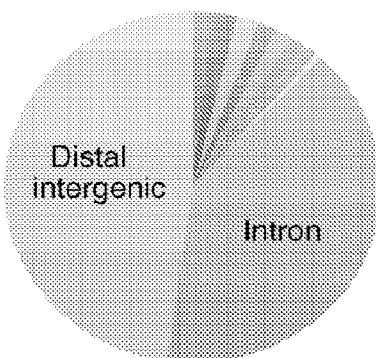
FIG. 2G

TERRA ChIRT-seq



- Promoter (≤ 1000 bp): 1.8 %
- Promoter (1000-2000 bp): 1.8 %
- Promoter (2000-3000 bp): 1.8 %
- Downstream (≤ 1000 bp): 1.7 %
- Downstream (1000-2000 bp): 1.6 %
- Downstream (2000-3000 bp): 1.4 %
- 5'UTR: 0.6 %
- 3'UTR: 1.4 %
- Coding exon: 0.7 %
- Intron: 40.1 %
- Distal intergenic: 47.2 %

PAR ChIRT-seq



- Promoter (≤ 1000 bp): 1.8 %
- Promoter (1000-2000 bp): 2.0 %
- Promoter (2000-3000 bp): 1.9 %
- Downstream (≤ 1000 bp): 1.6 %
- Downstream (1000-2000 bp): 1.6 %
- Downstream (2000-3000 bp): 1.3 %
- 5'UTR: 0.3 %
- 3'UTR: 0.9 %
- Coding exon: 0.4 %
- Intron: 40.4 %
- Distal intergenic: 47.8 %

FIG. 2H

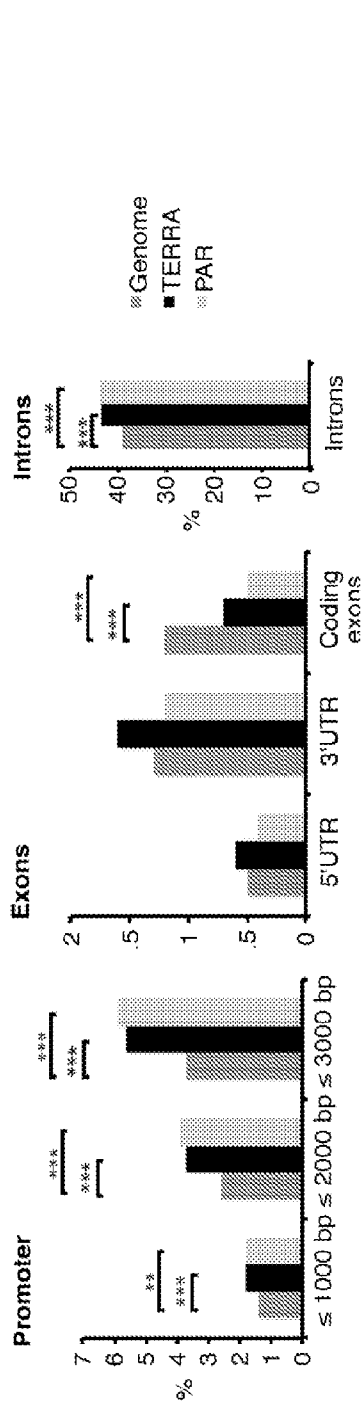


FIG. 21

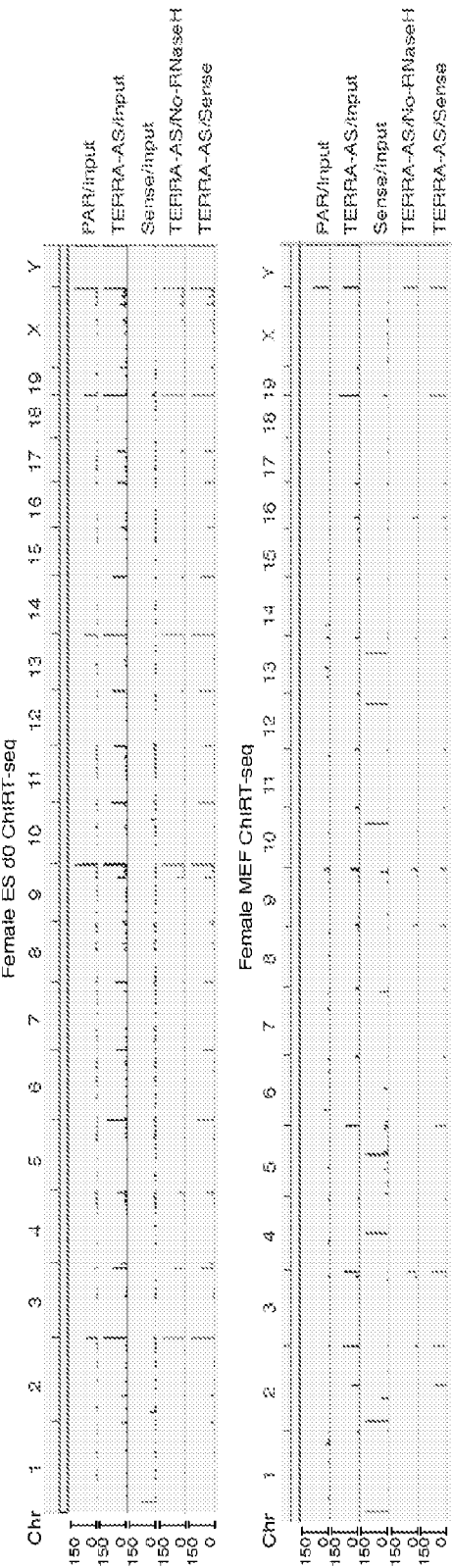


FIG. 3A

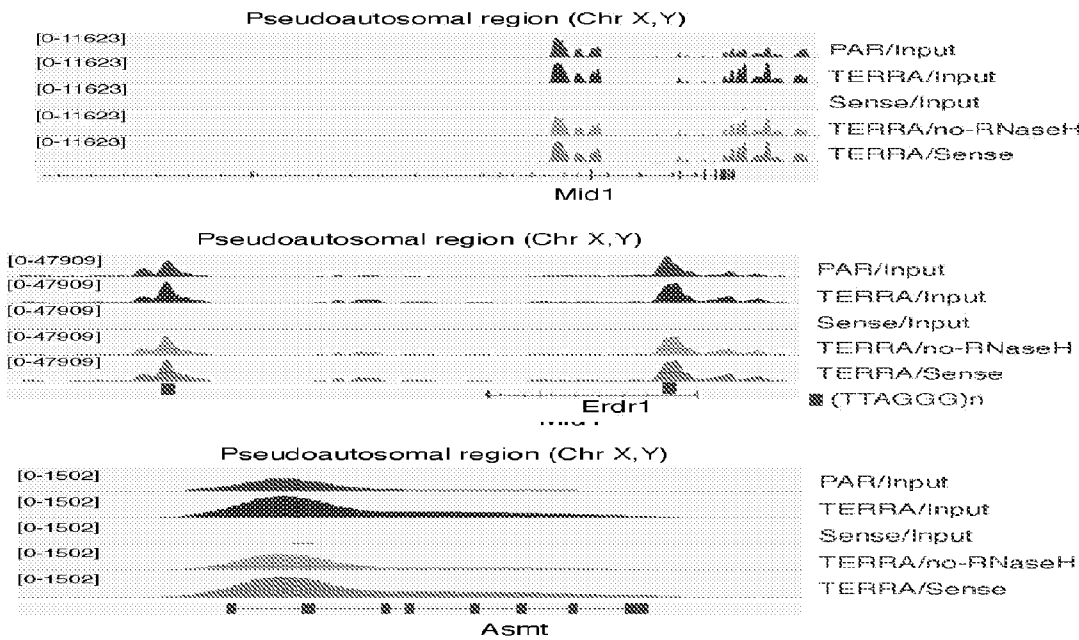


FIG. 3B

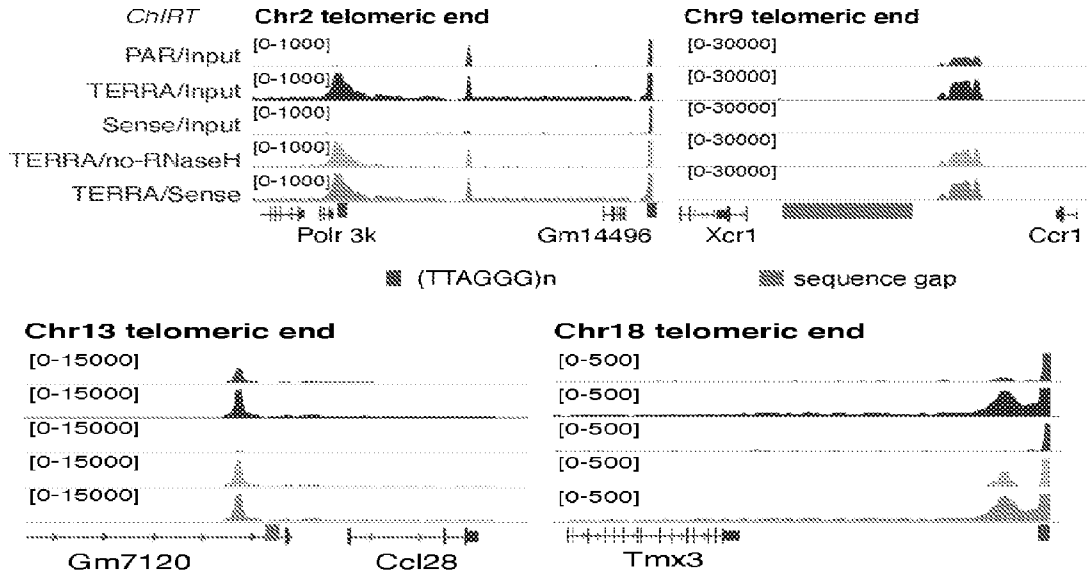


FIG. 3C

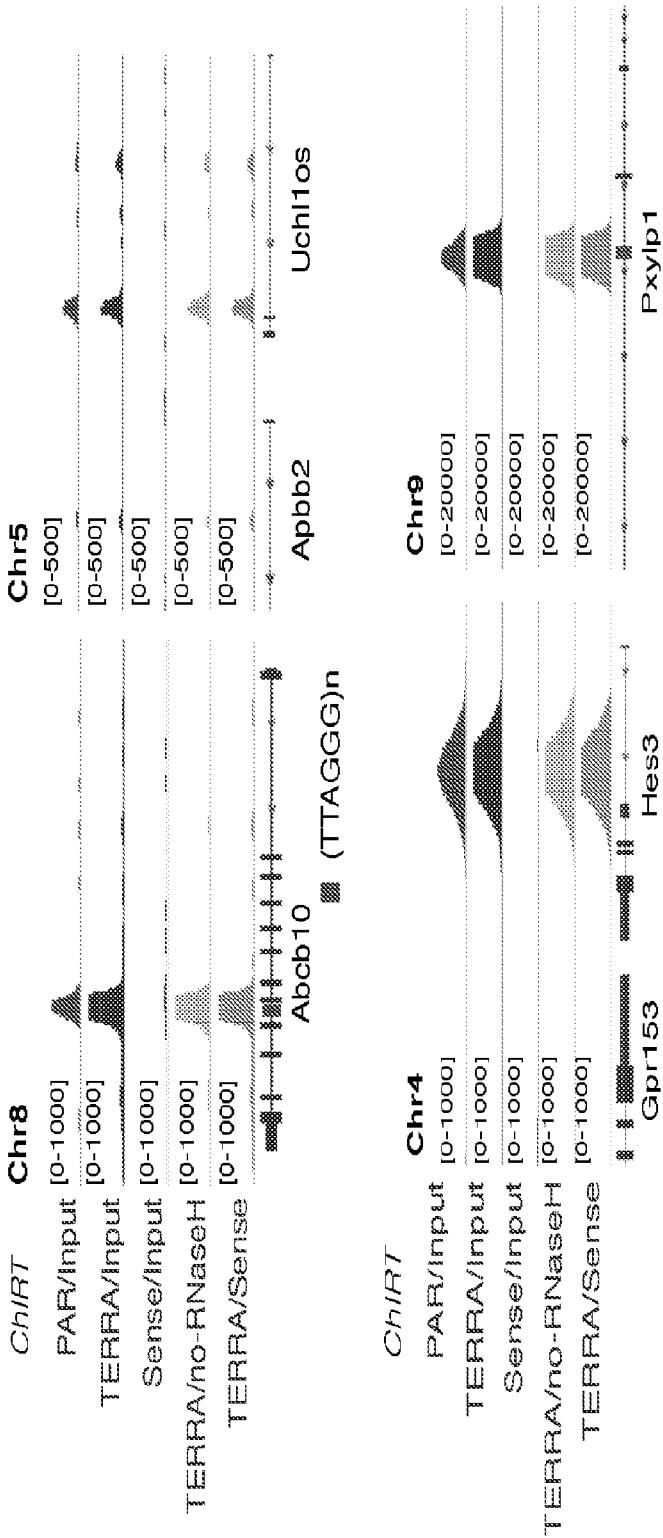


FIG. 3D

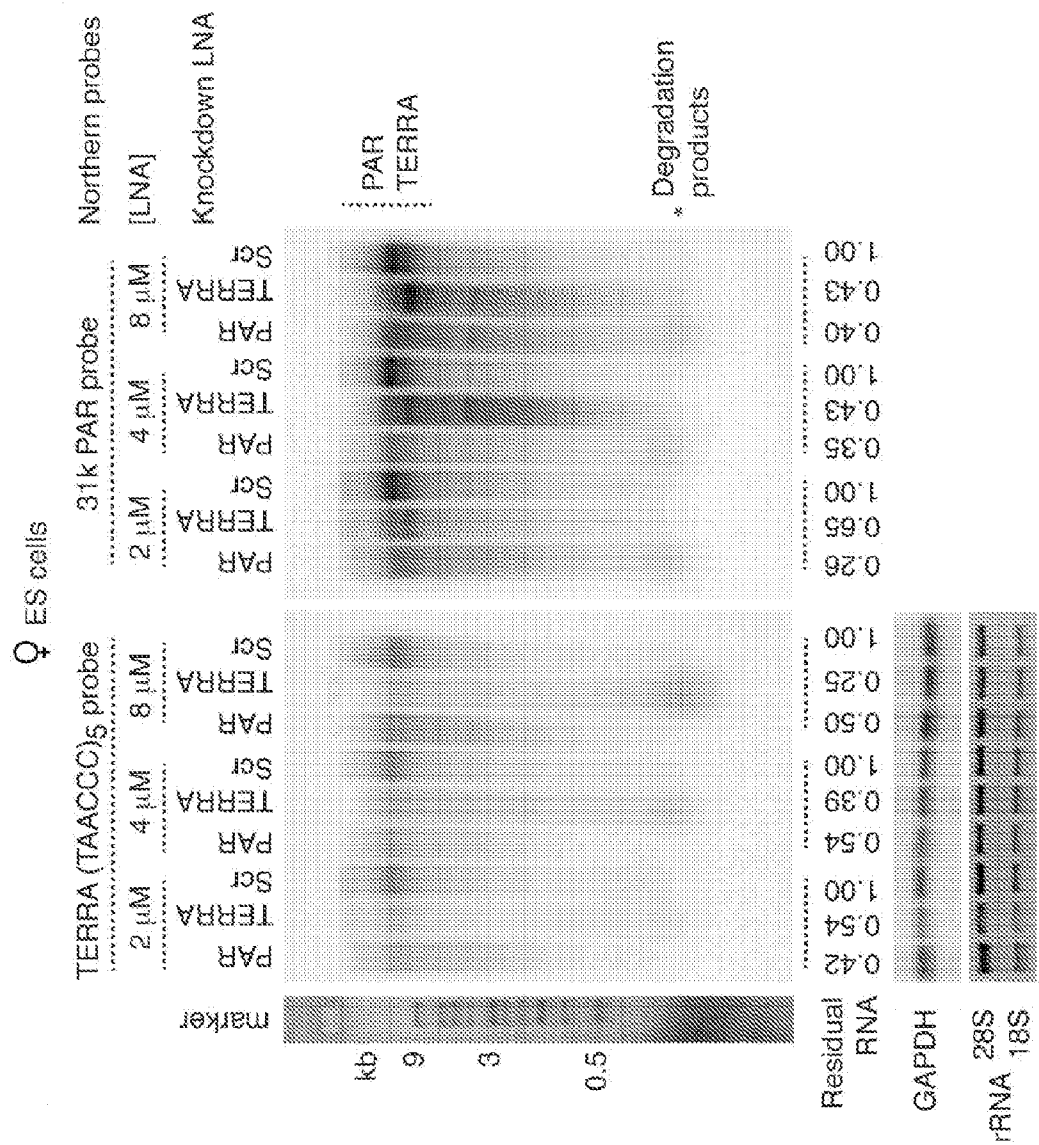


FIG. 4A

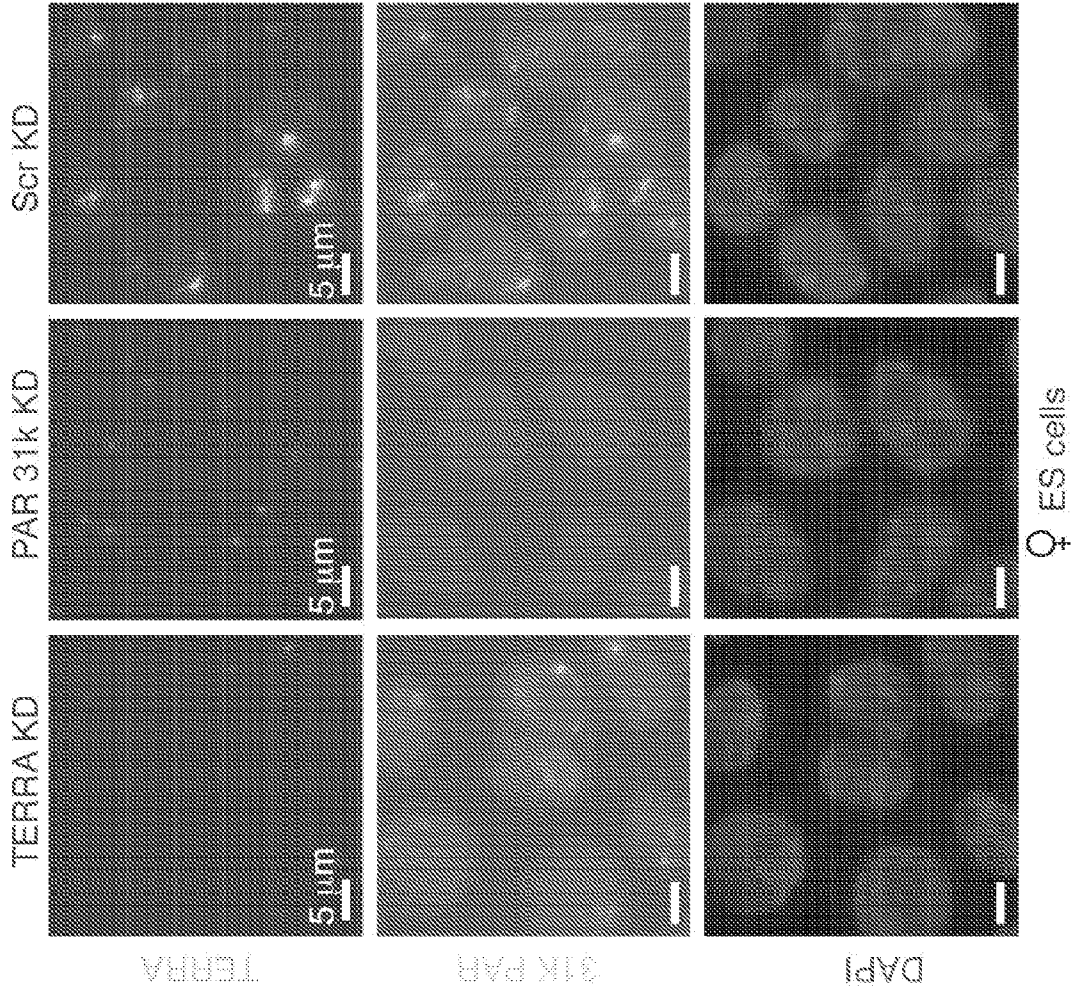


FIG. 4B

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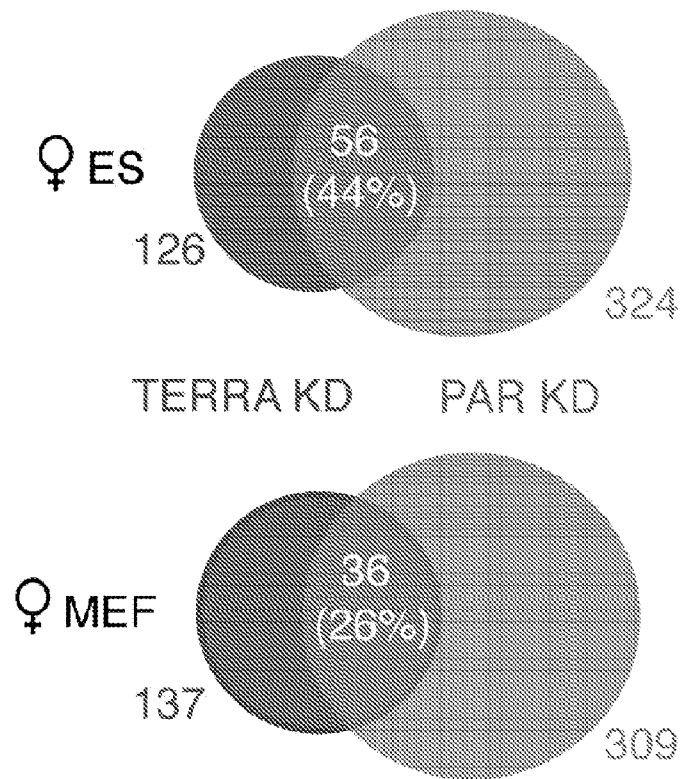


FIG. 4C

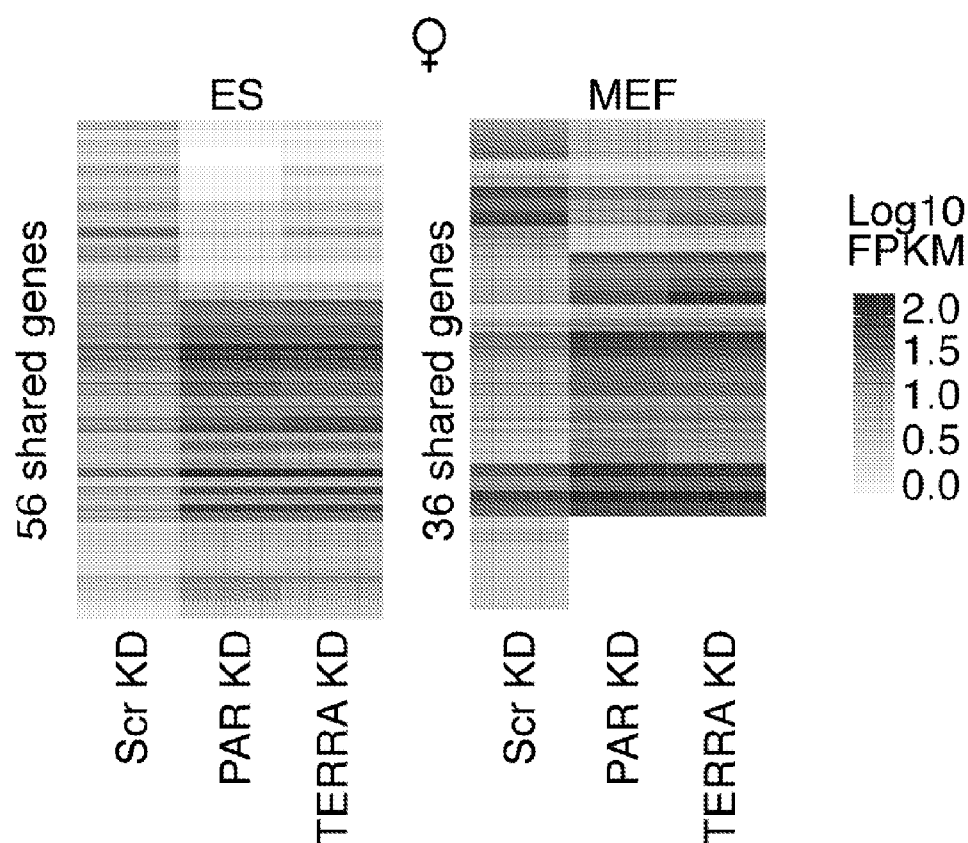


FIG. 4D

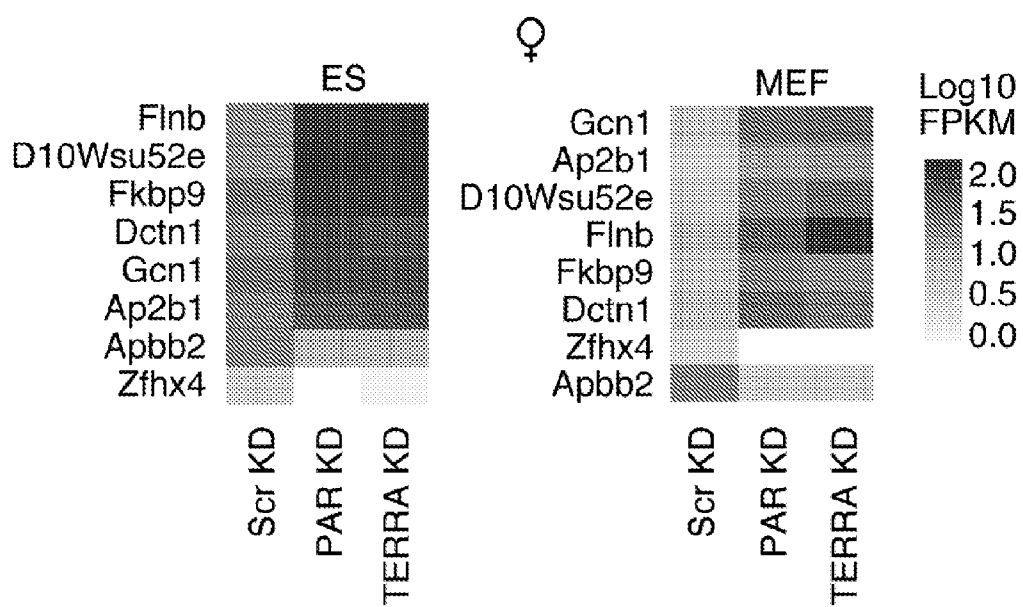


FIG. 4E

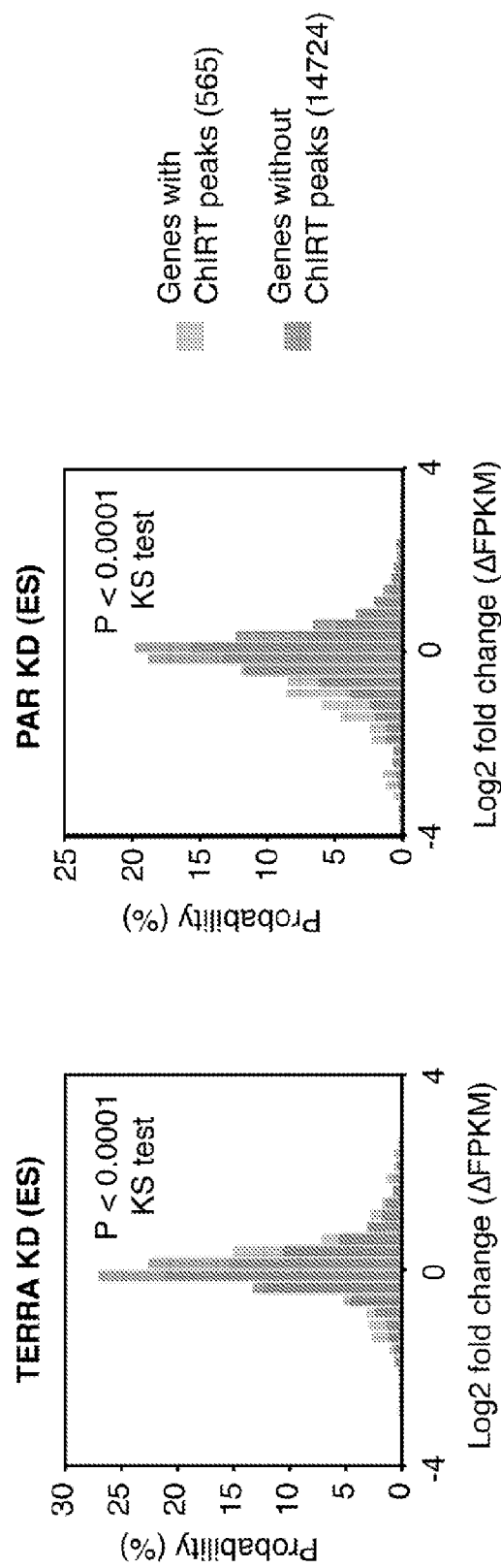


FIG. 4F

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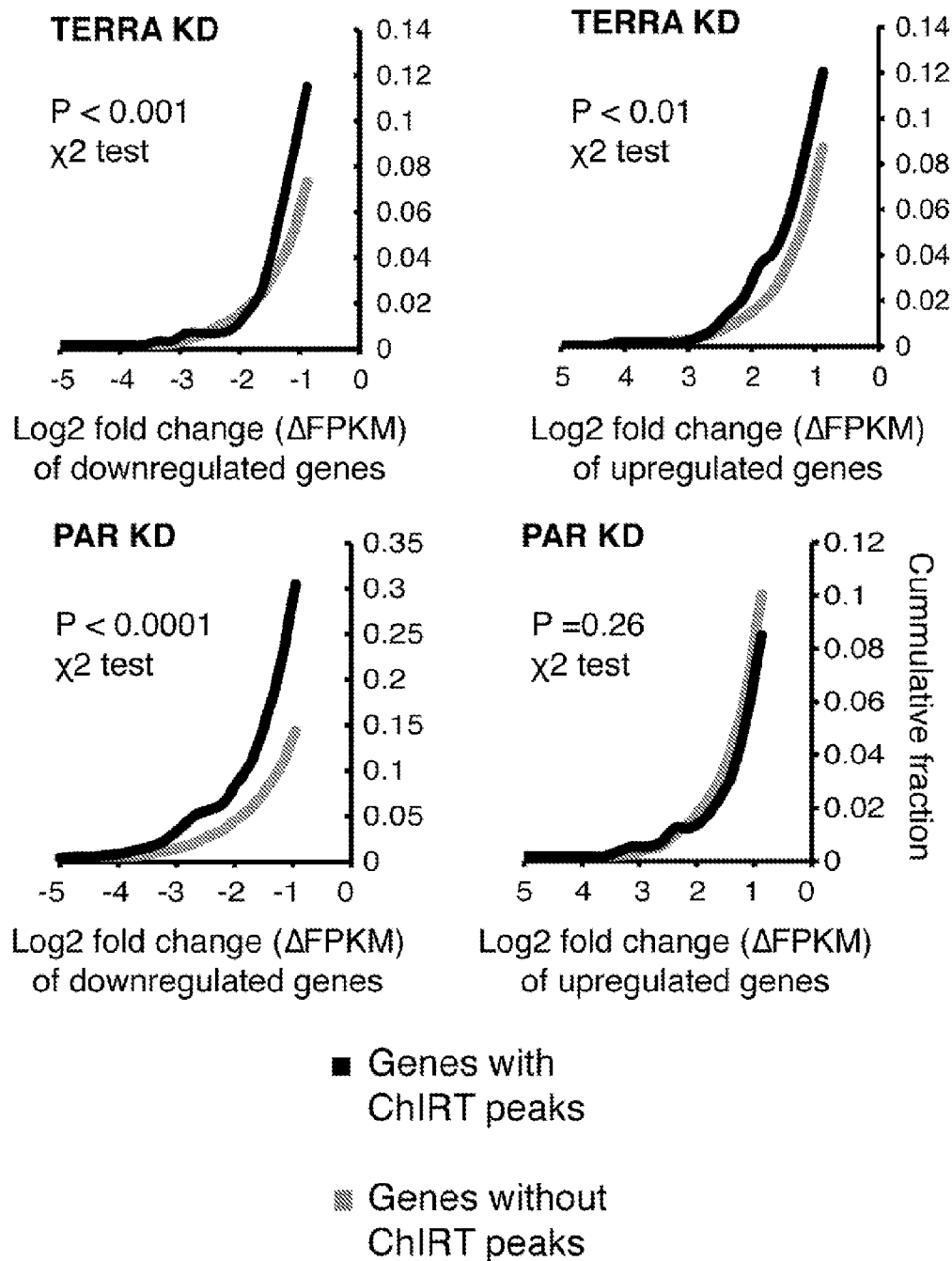


FIG. 4G

Sub-telomeric regions

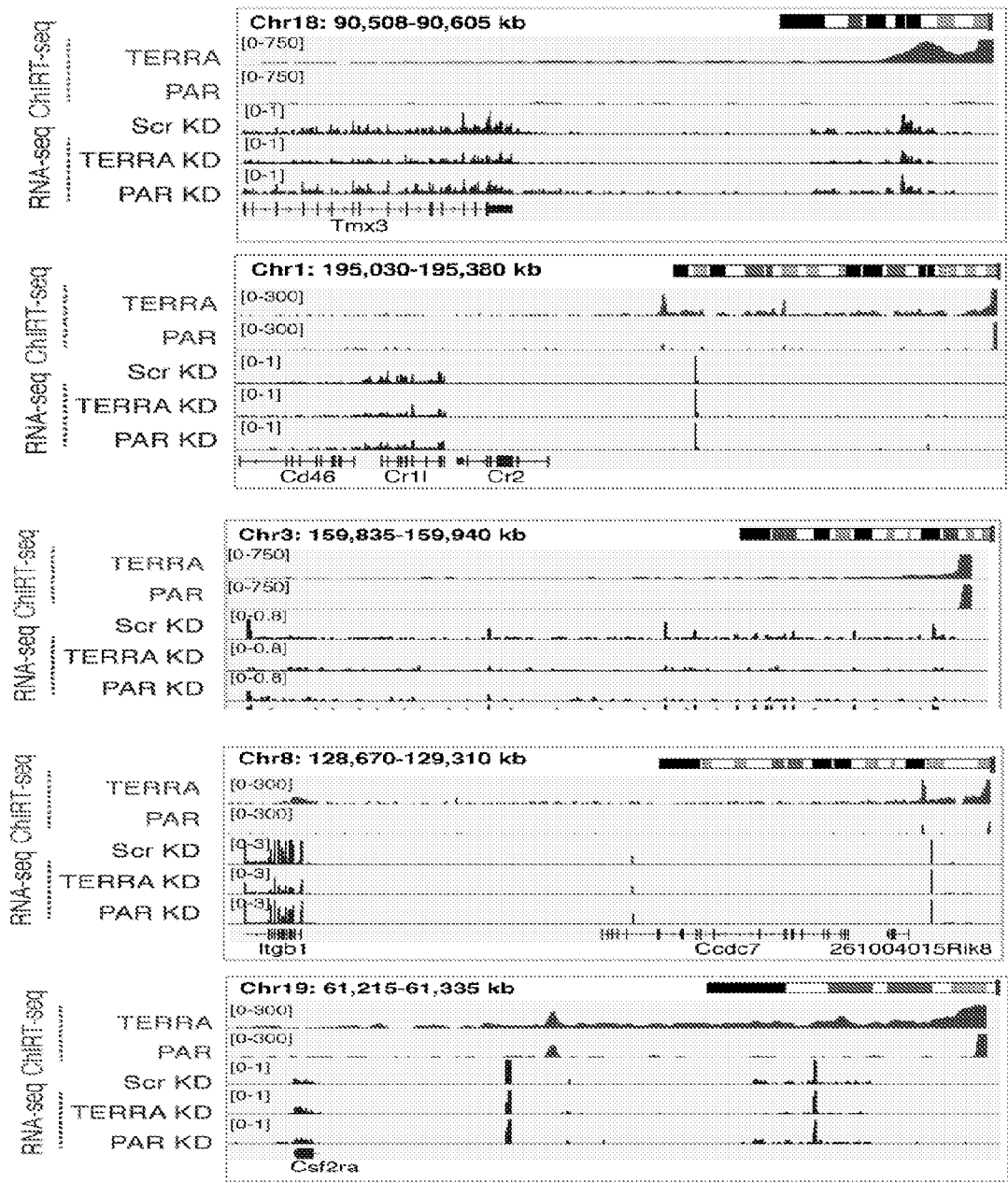


FIG. 5A

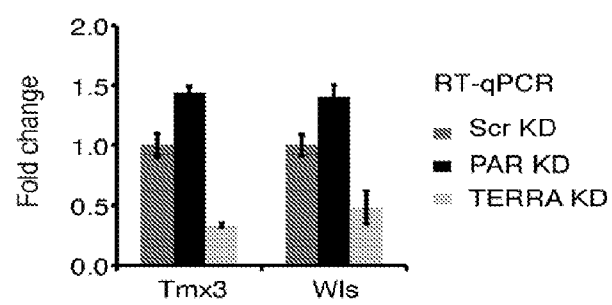


FIG. 5B

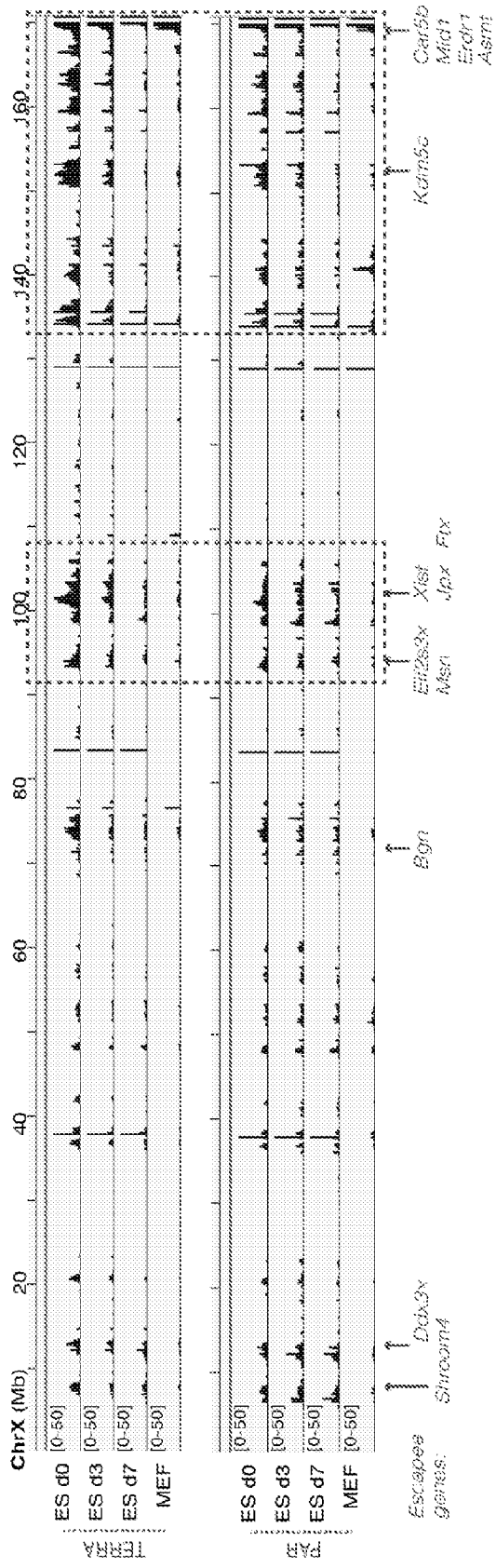


FIG. 5C

Number of binding sites		
ES d0	total	ChrX
TERRA	4054	86
PAR	2304	84
MEF	total	ChrX
TERRA	500	94
PAR	440	30

FIG. 5D

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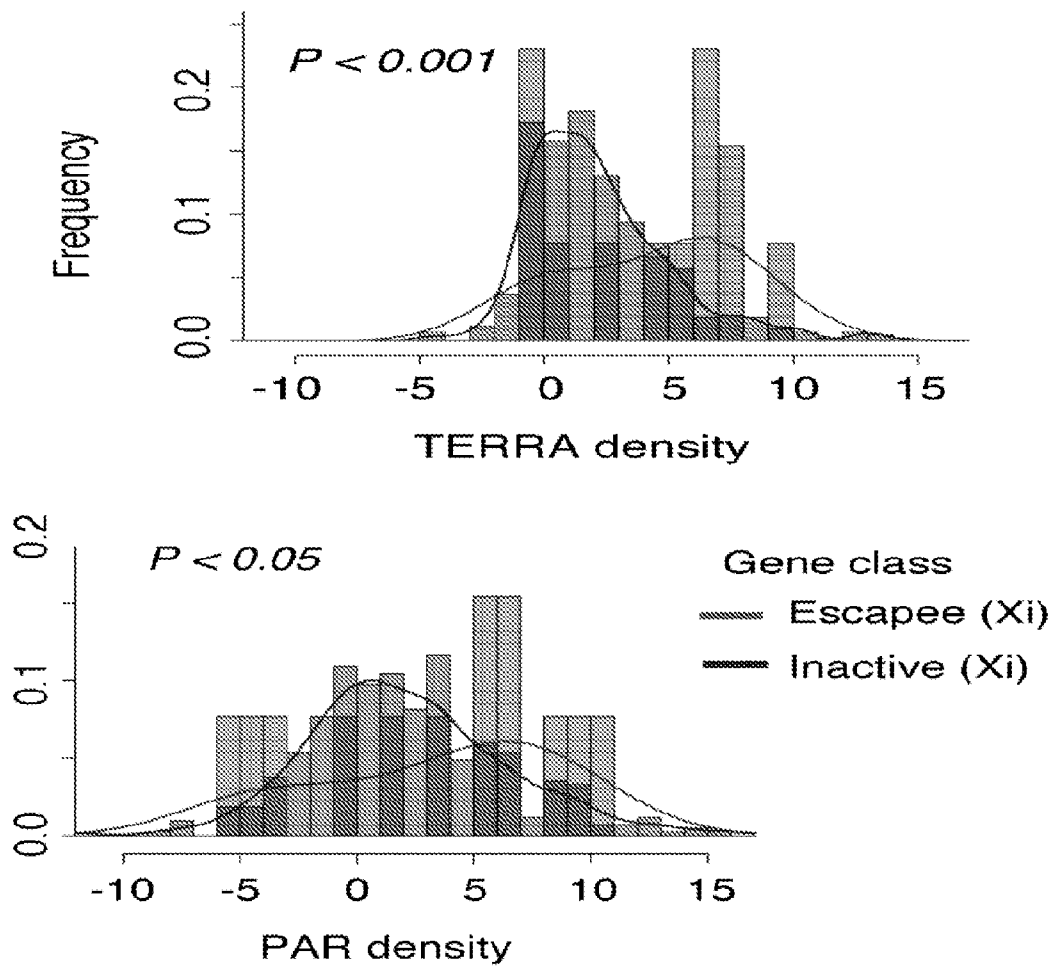


FIG. 5E

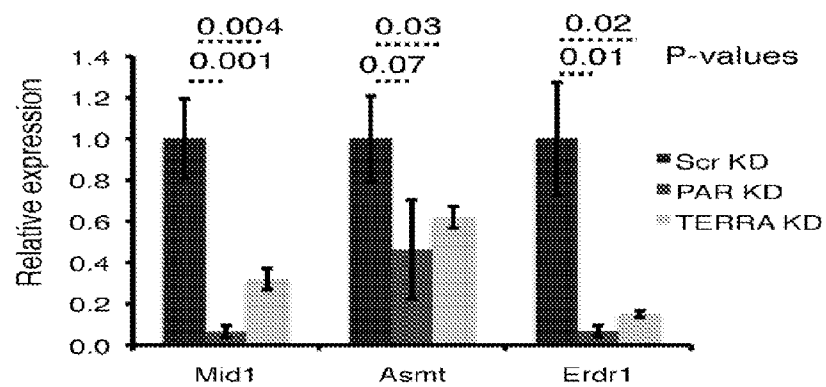


FIG. 5F

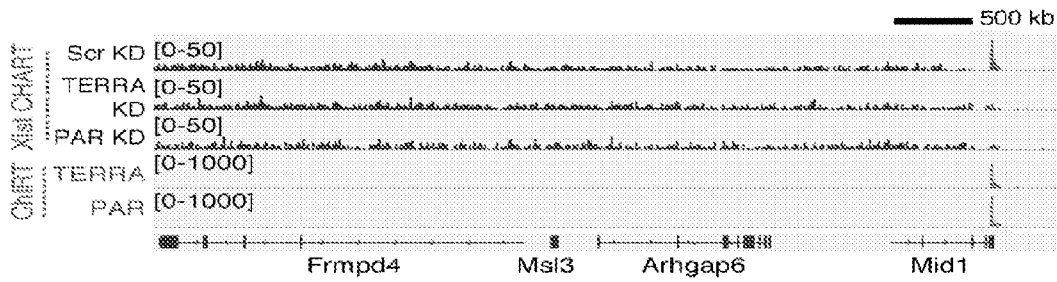


FIG. 6A

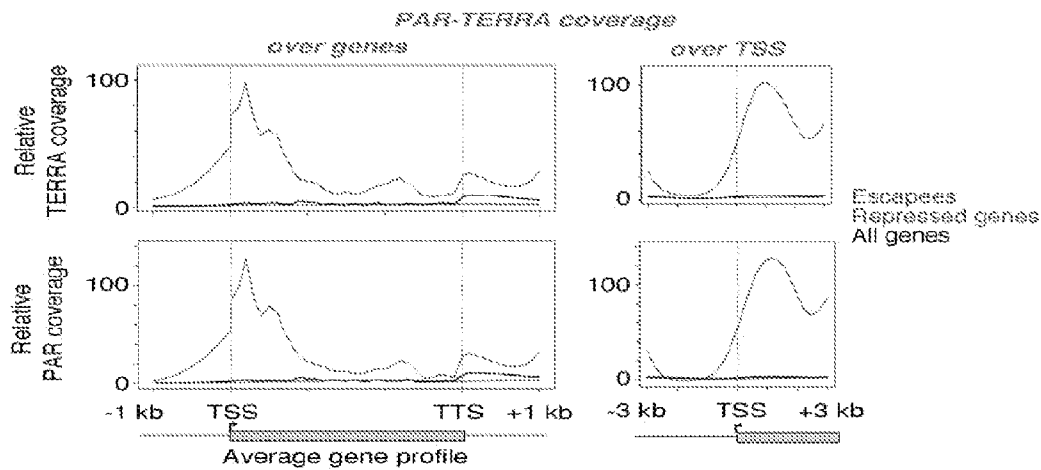


FIG. 6B

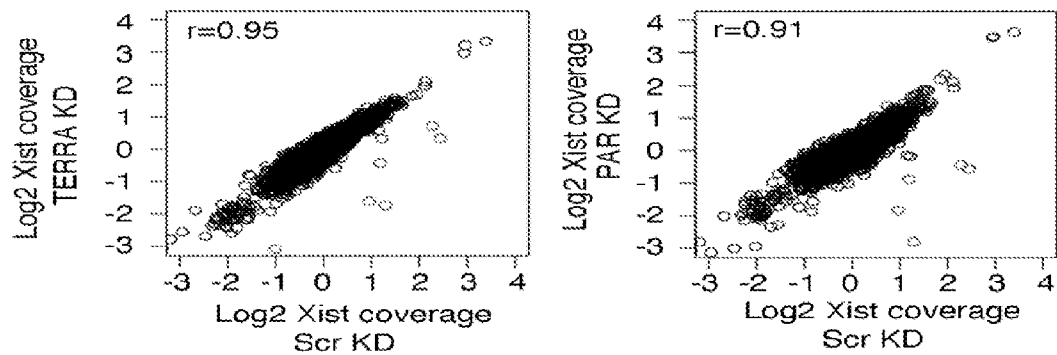


FIG. 6C

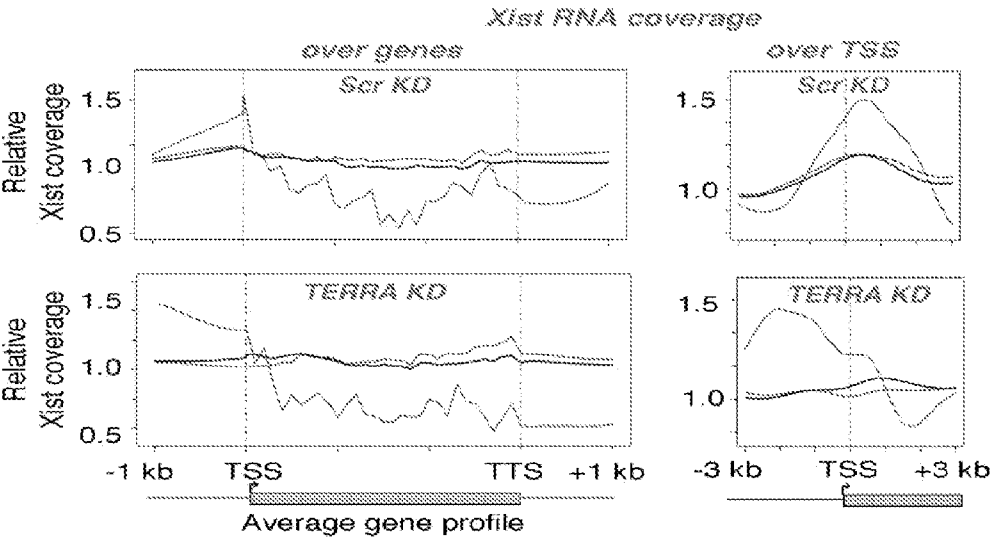


FIG. 6D

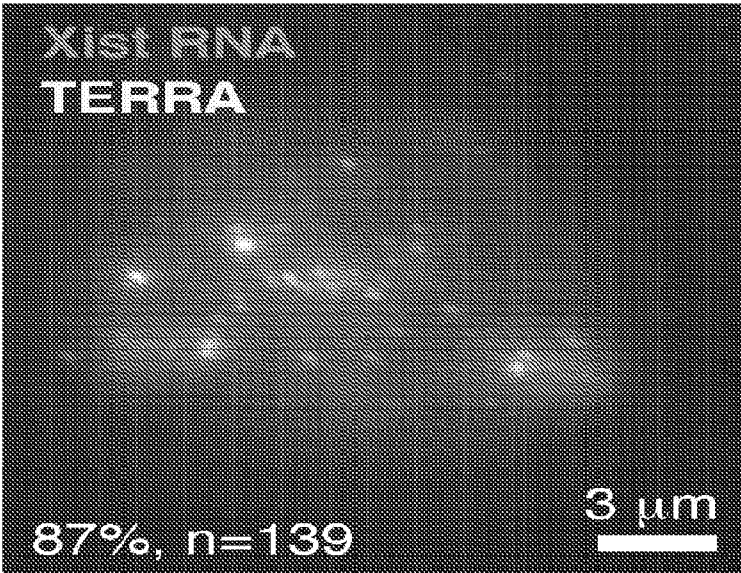


FIG. 6E

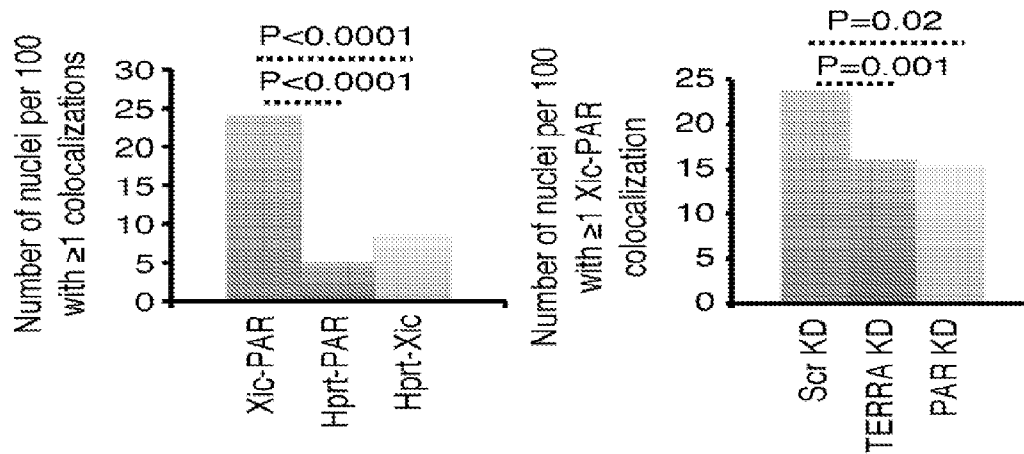


FIG. 6F

FIG. 6G

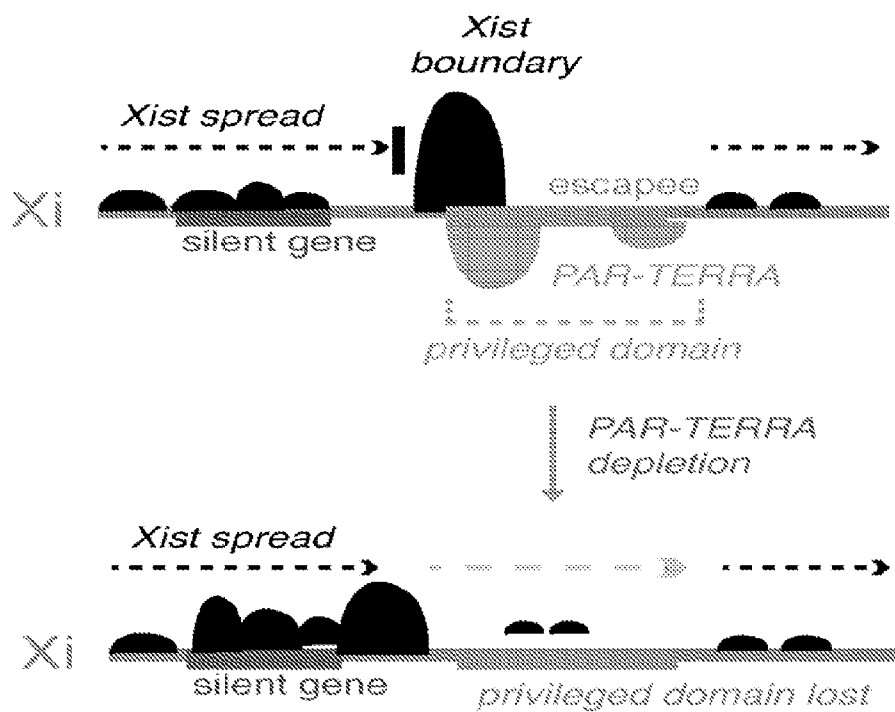


FIG. 6H

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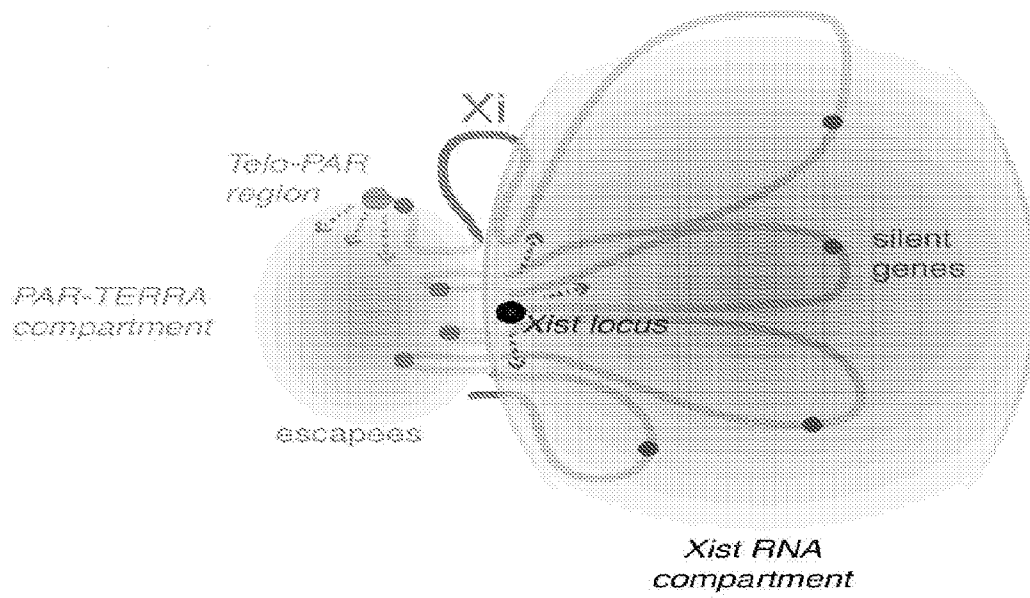


FIG. 6I

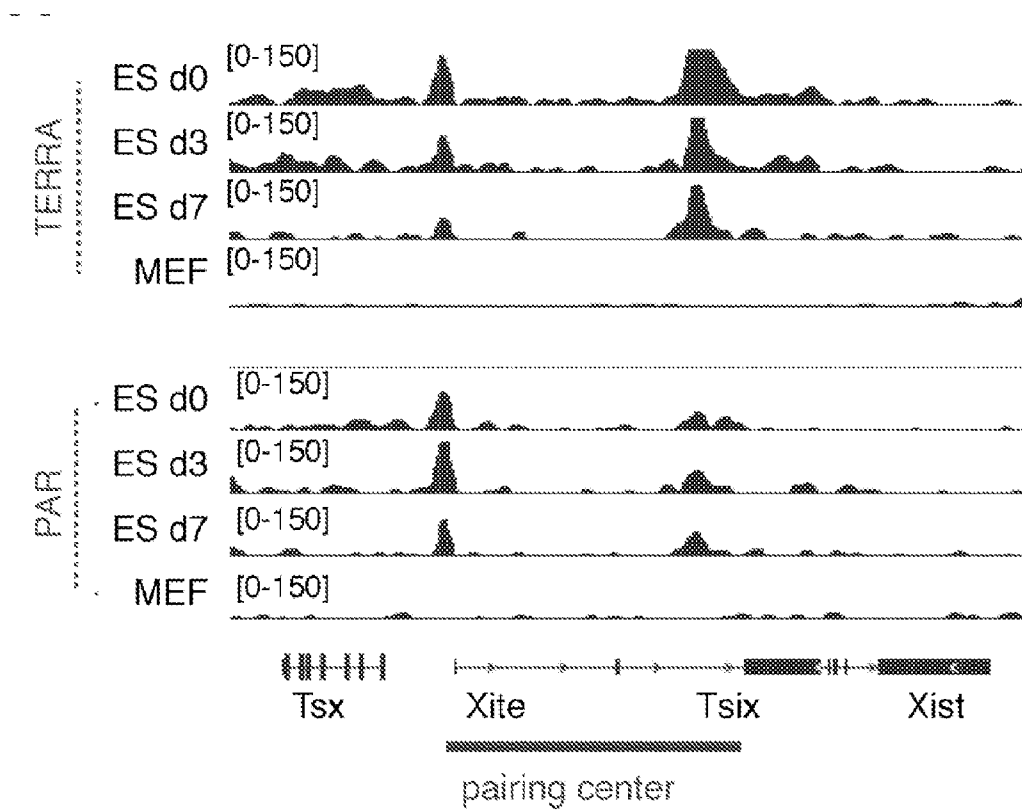


FIG. 7A

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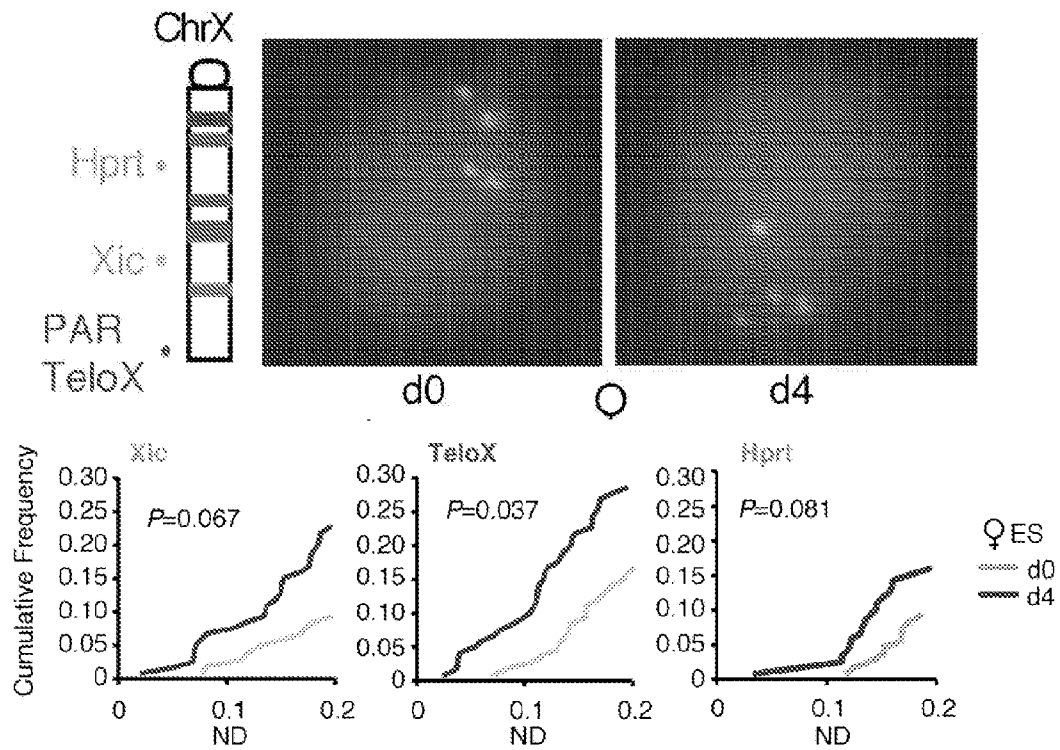


FIG. 7B

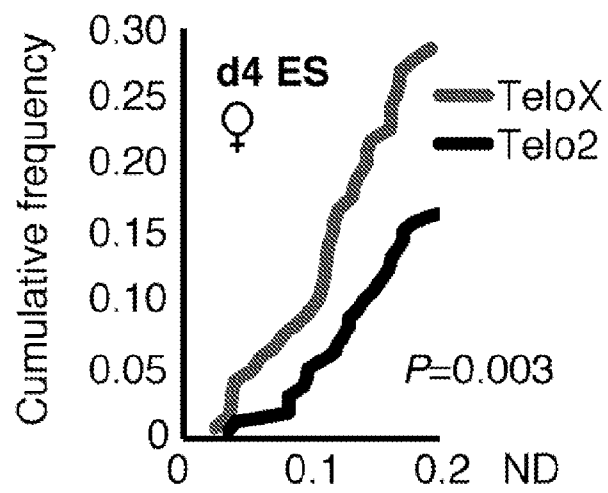


FIG. 7C

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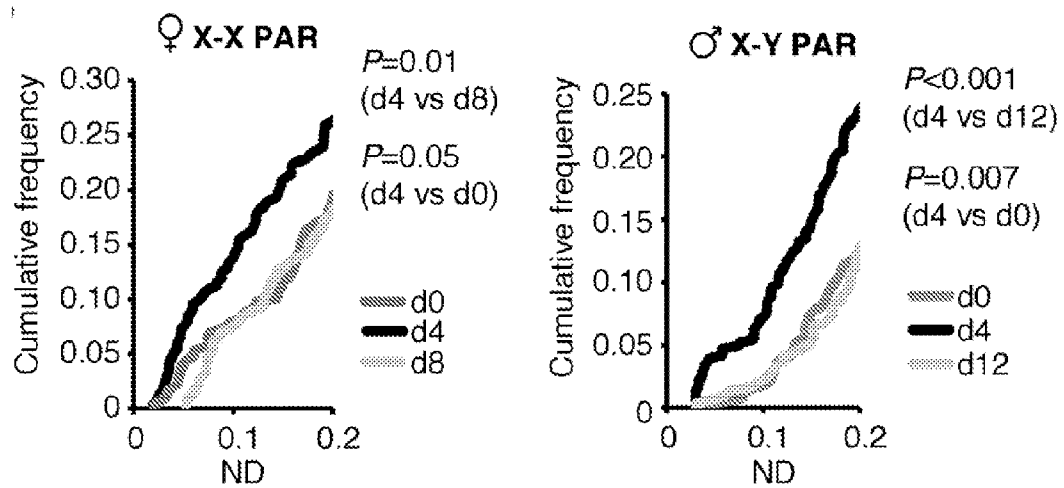


FIG. 7D

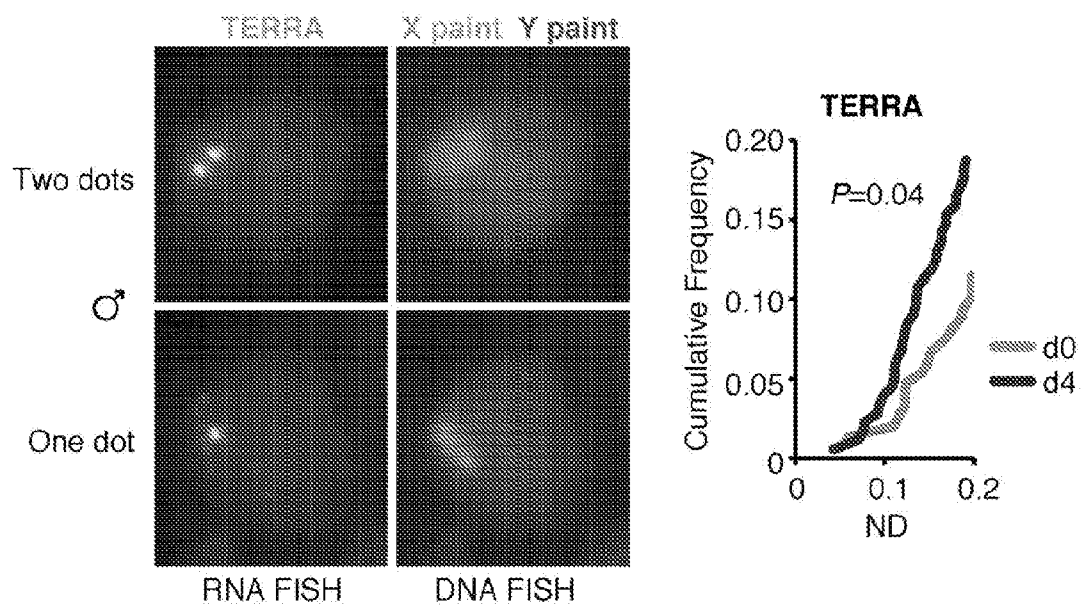


FIG. 7E

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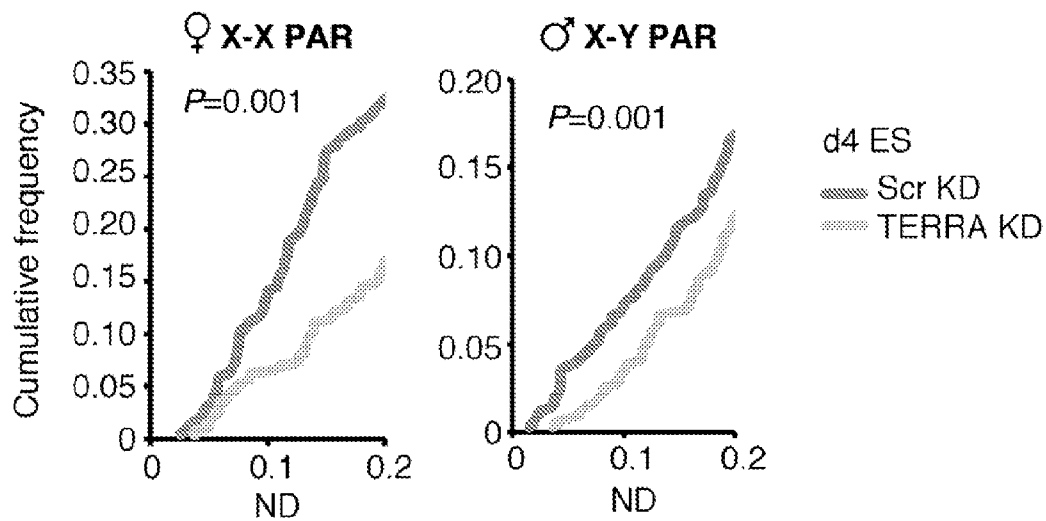


FIG. 7F

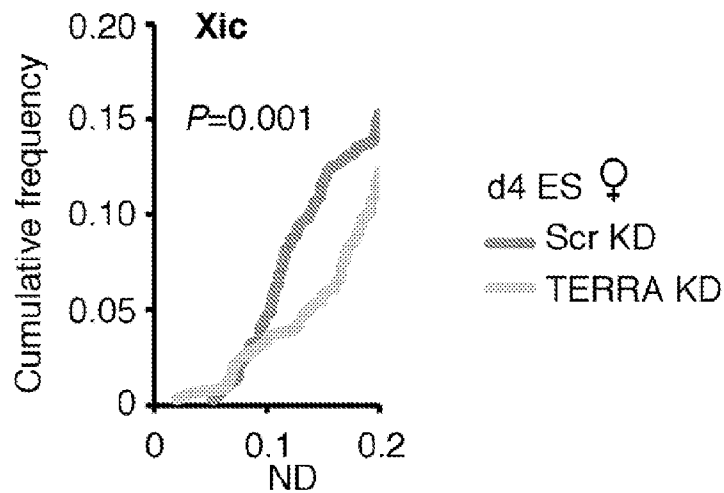


FIG. 7G

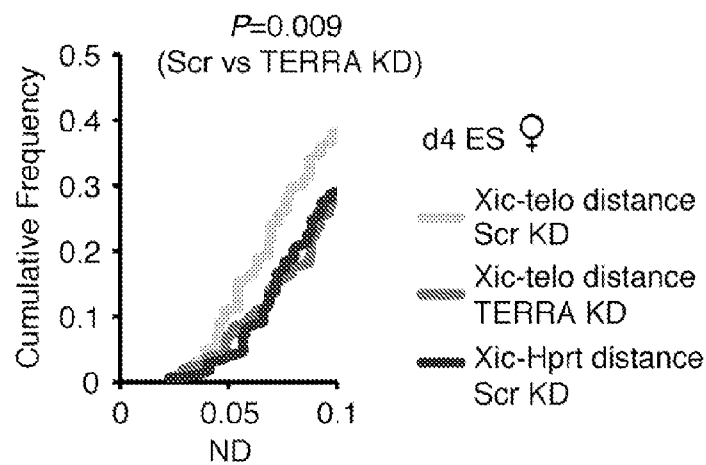


FIG. 7H

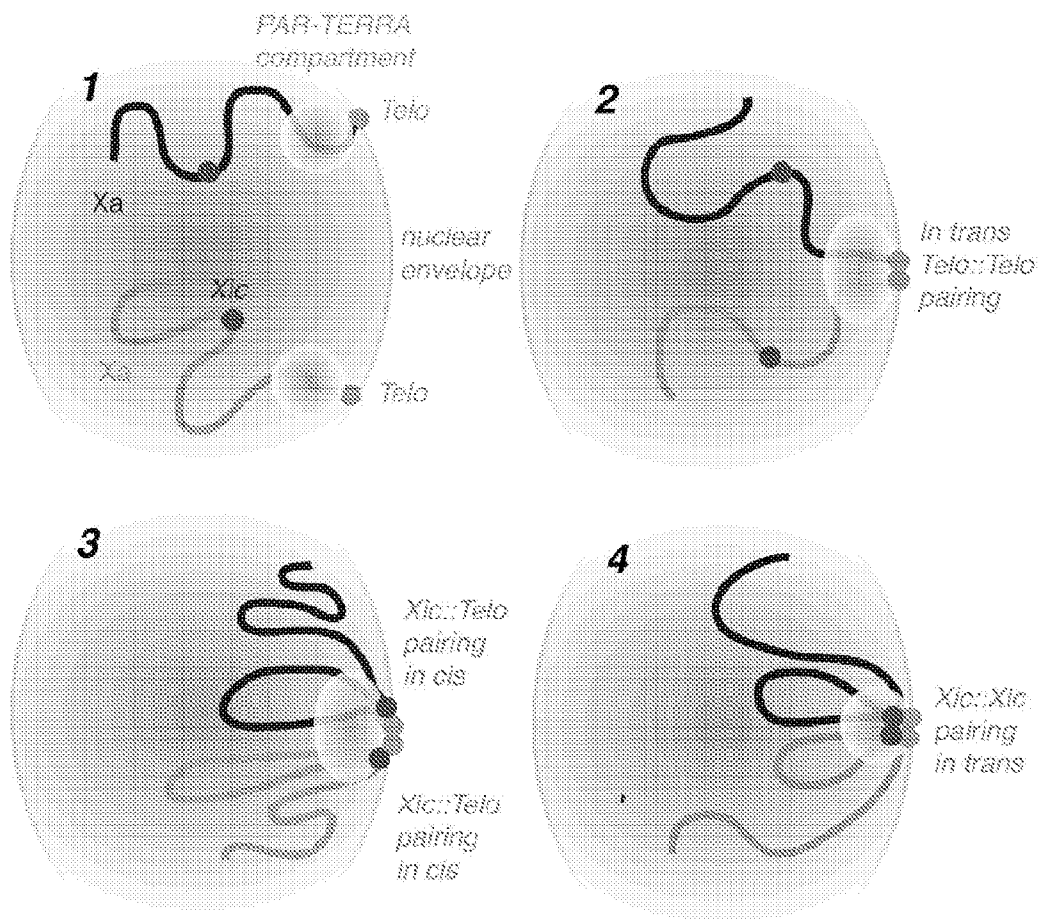


FIG. 7I

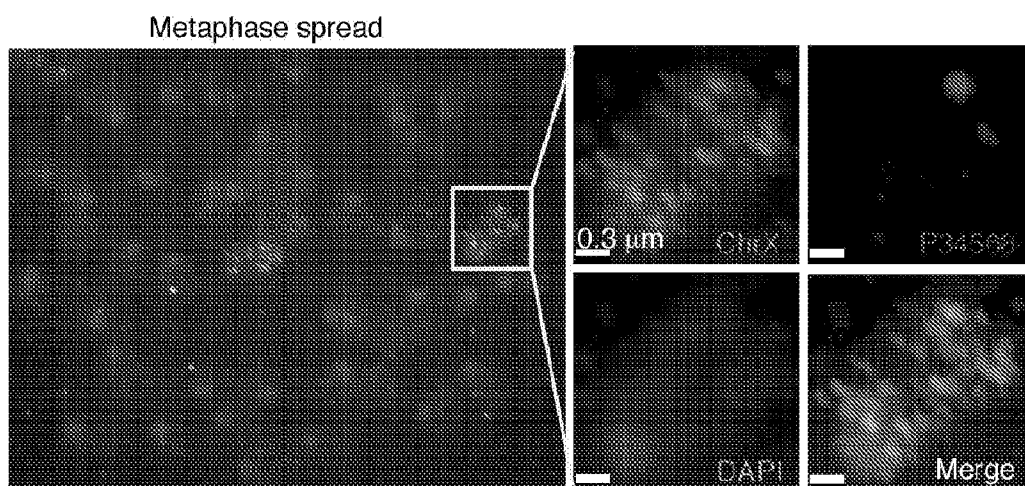


FIG. 8A

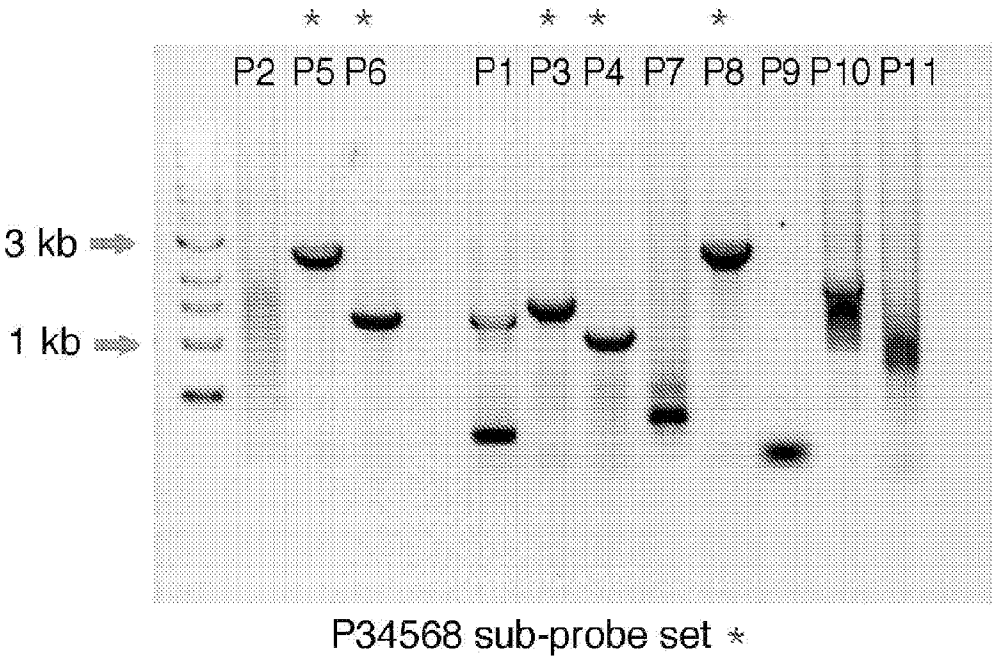


FIG. 8B

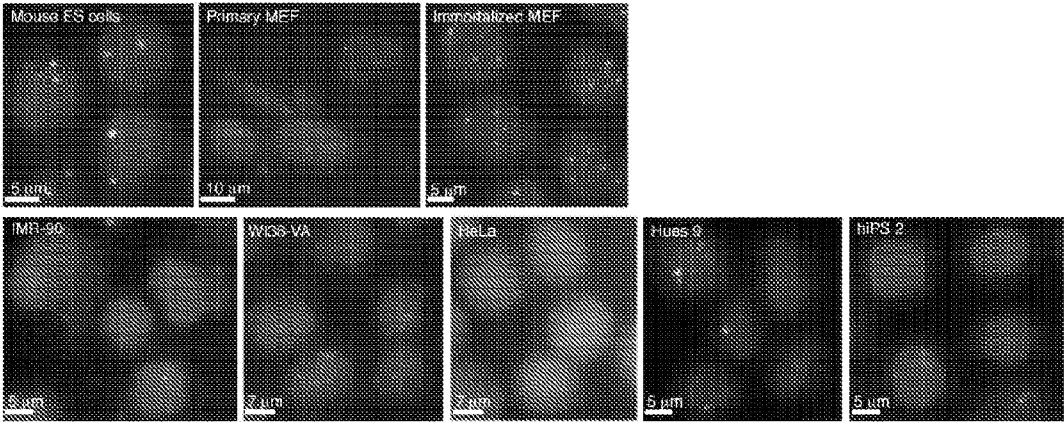
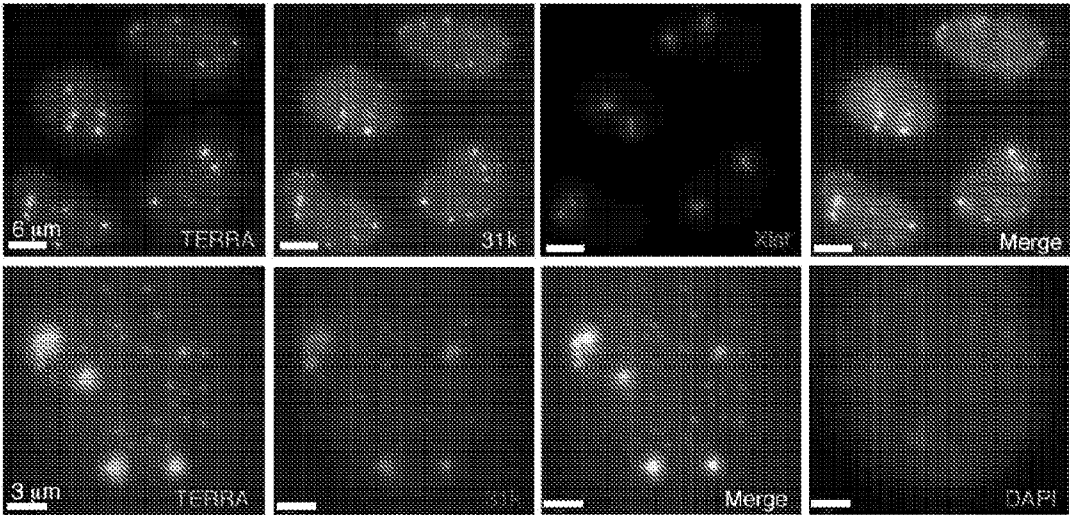


FIG. 8C



Immortalized female MEF (tetraploid)

FIG. 8D

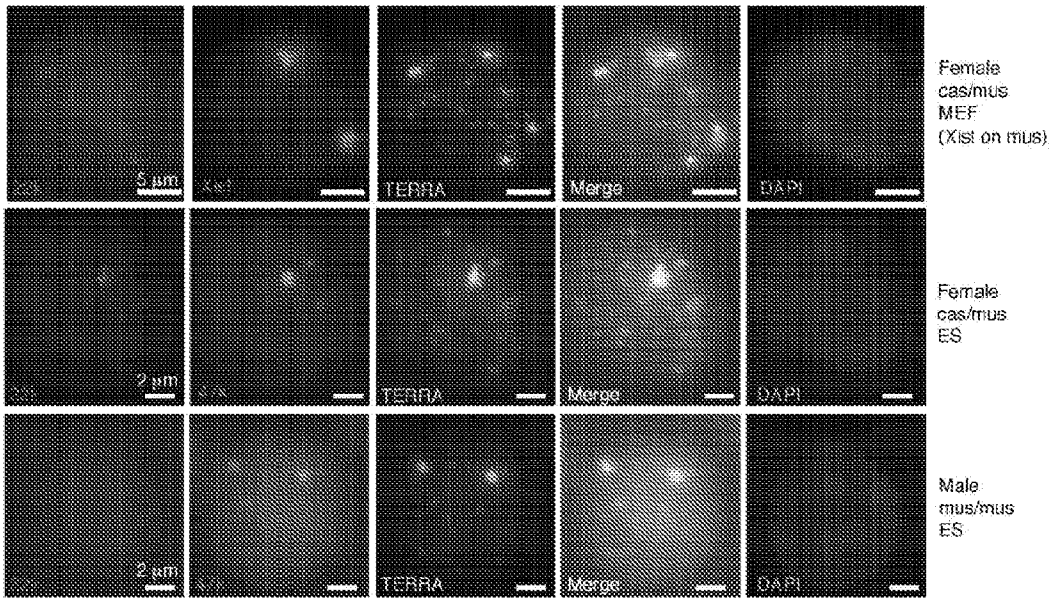


FIG. 8E

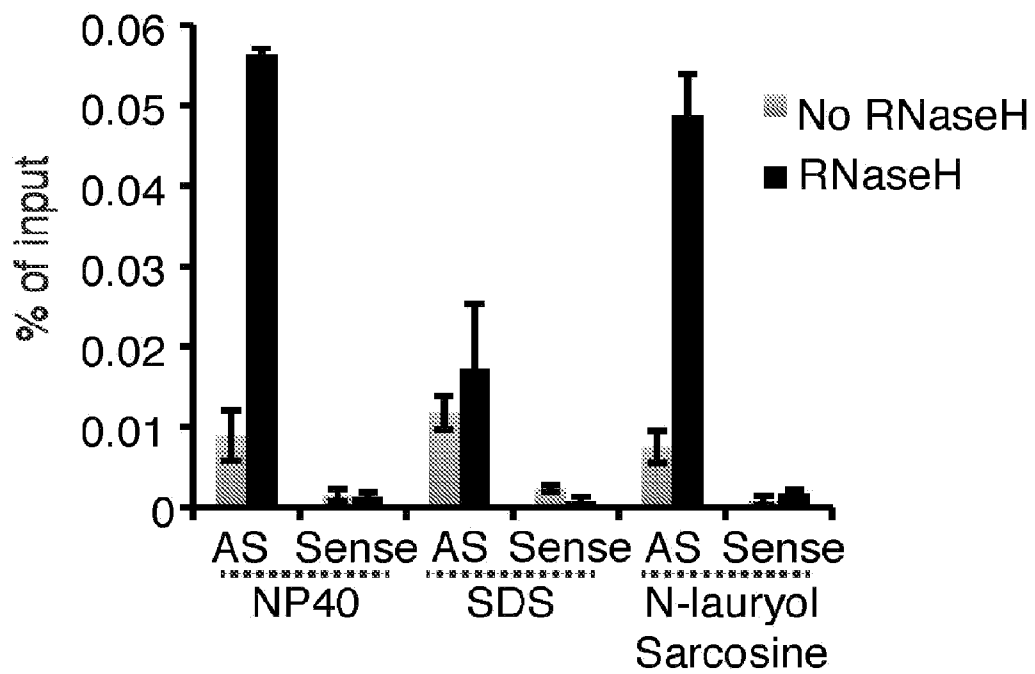


FIG. 9A

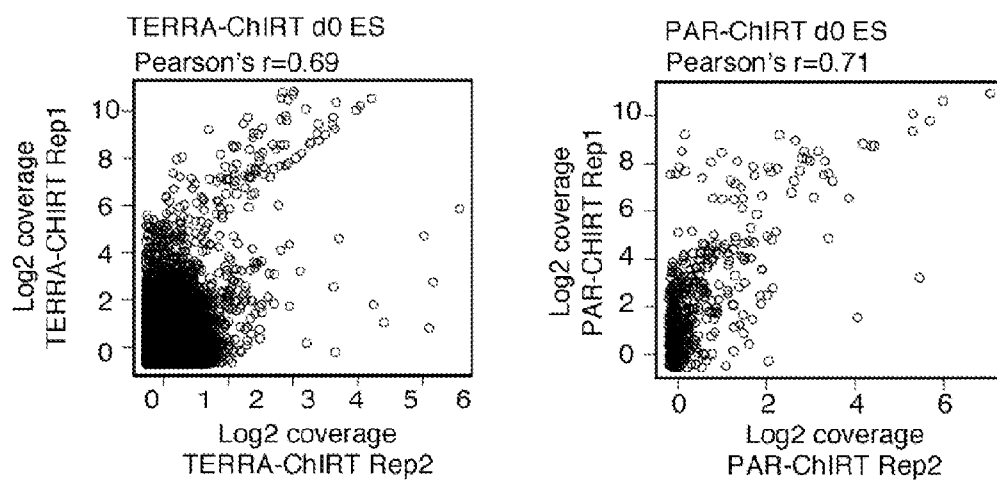


FIG. 9B

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CHIRT Replicate 1

Name of library	Total reads	Reads after PCR duplicates removal	Uniquely mapped reads in mm10	% Uniquely mapped reads in mm10
ES_d0 PAR-AS	32464373	31255733	30472507	97.49
ES_d0 TERRA-AS	28932181	23905938	22631527	94.67
ES_d0 TERRA-S	46282449	44747817	43894751	98.09
ES_d0 TER (no RNaseH)	41852461	34807415	32453318	93.24
ES_d0 input	45396054	41983022	41545697	98.96
ES_d3 input	30065363	27270315	26488296	97.13
ES_d3 PAR-AS	41668524	35614418	28292145	79.44
ES_d3 TERRA-AS	33458866	27658177	23664357	85.56
ES_d7 input	37132369	35901389	12171677	33.90
ES_d7 PAR-AS	36815663	34240958	32758921	95.67
ES_d7 TERRA-AS	33045601	27014673	23824033	88.19
MEF PAR-AS	31682605	28971390	25213047	87.03
MEF TERRA-AS	35059769	32329303	7773238	24.04
MEF TERRA-S	38609175	32433255	19840775	61.17
MEF TERRA (no RNaseH)	43253571	33402120	26037320	77.95
MEF input	46619652	40321599	39755914	98.60

CHIRT Replicate 2

Name of library	Total reads	Reads after PCR duplicates removal	Uniquely mapped reads in mm10	% Uniquely mapped reads in mm10
ES_d0 PAR	11537196	7807554	6826109	87.43
ES_d0 PAR RNase A	15826305	14859144	12993461	87.44
ES_d0 TERRA	14094905	12515985	10725993	85.70
ES_d0 TERRA RNaseA	13627224	12411472	10985881	88.51
ES_d0 sense	14950599	13133452	10864427	82.72
ES_d0 sense RNase A	13903763	12050175	10459053	86.80
ES_d0 TERRA no Rnase H	13666488	10053384	8013263	79.71
MEF PAR	17210856	13251314	11195046	84.48
MEF PAR RNase A	14849691	12367944	9990314	80.78
MEF TERRA	13684453	8039976	6620225	82.34
MEF TERRA RNaseA	12206274	6923577	5602706	80.92
MEF sense	12117053	8649137	7467591	86.34
MEF sense RNase A	15981875	11504737	9553319	83.04
MEF TERRA no Rnase H	12542949	8715062	6604865	75.79

FIG. 9C

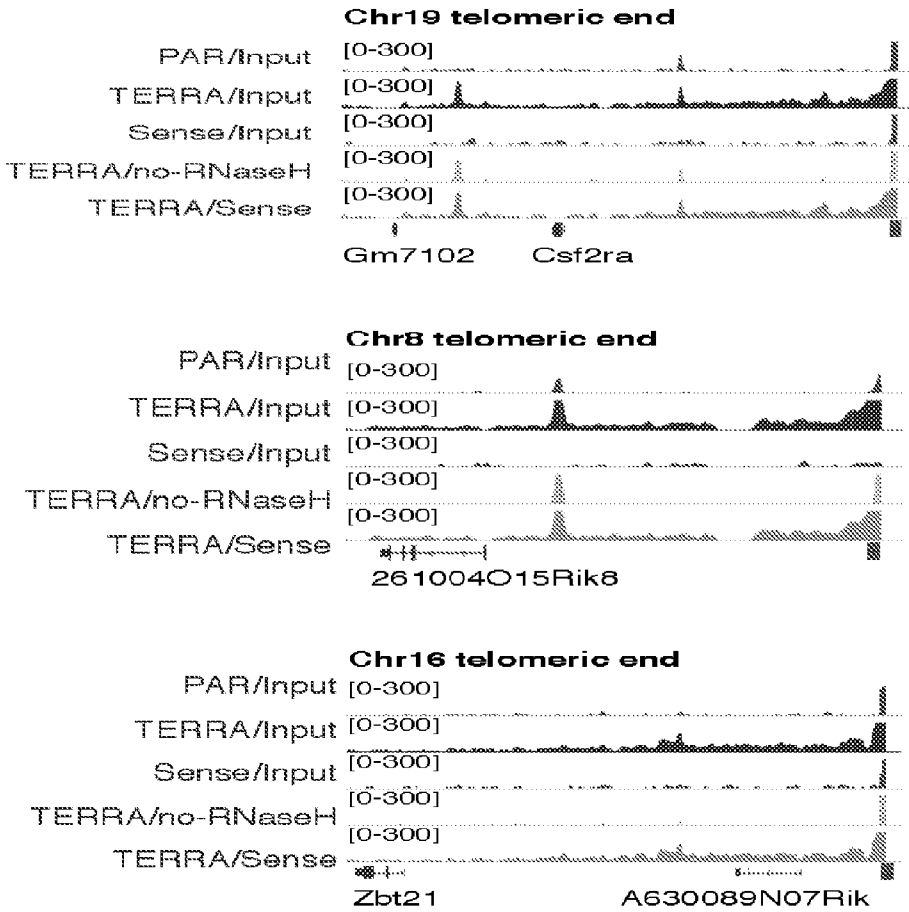


FIG. 10

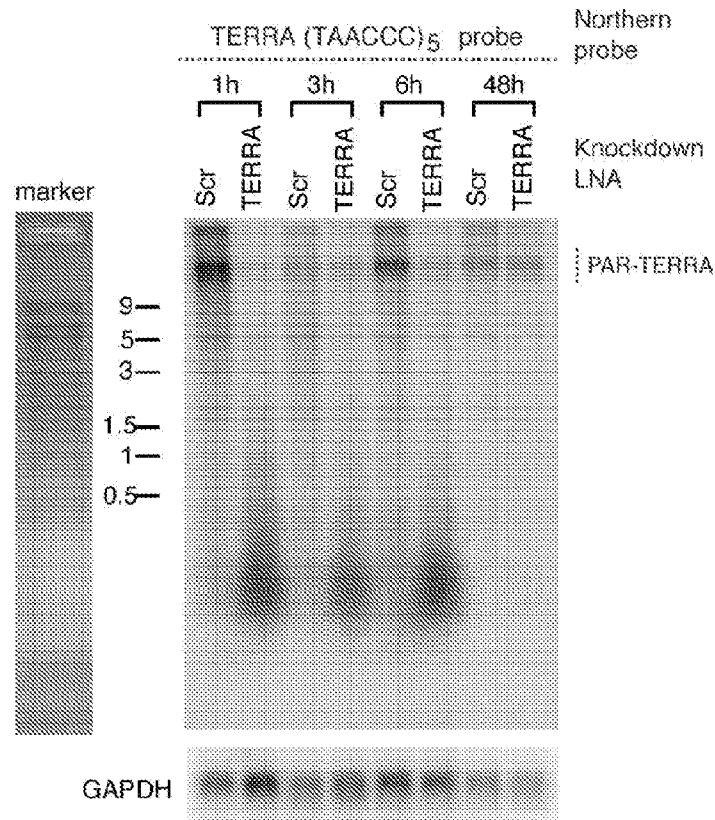
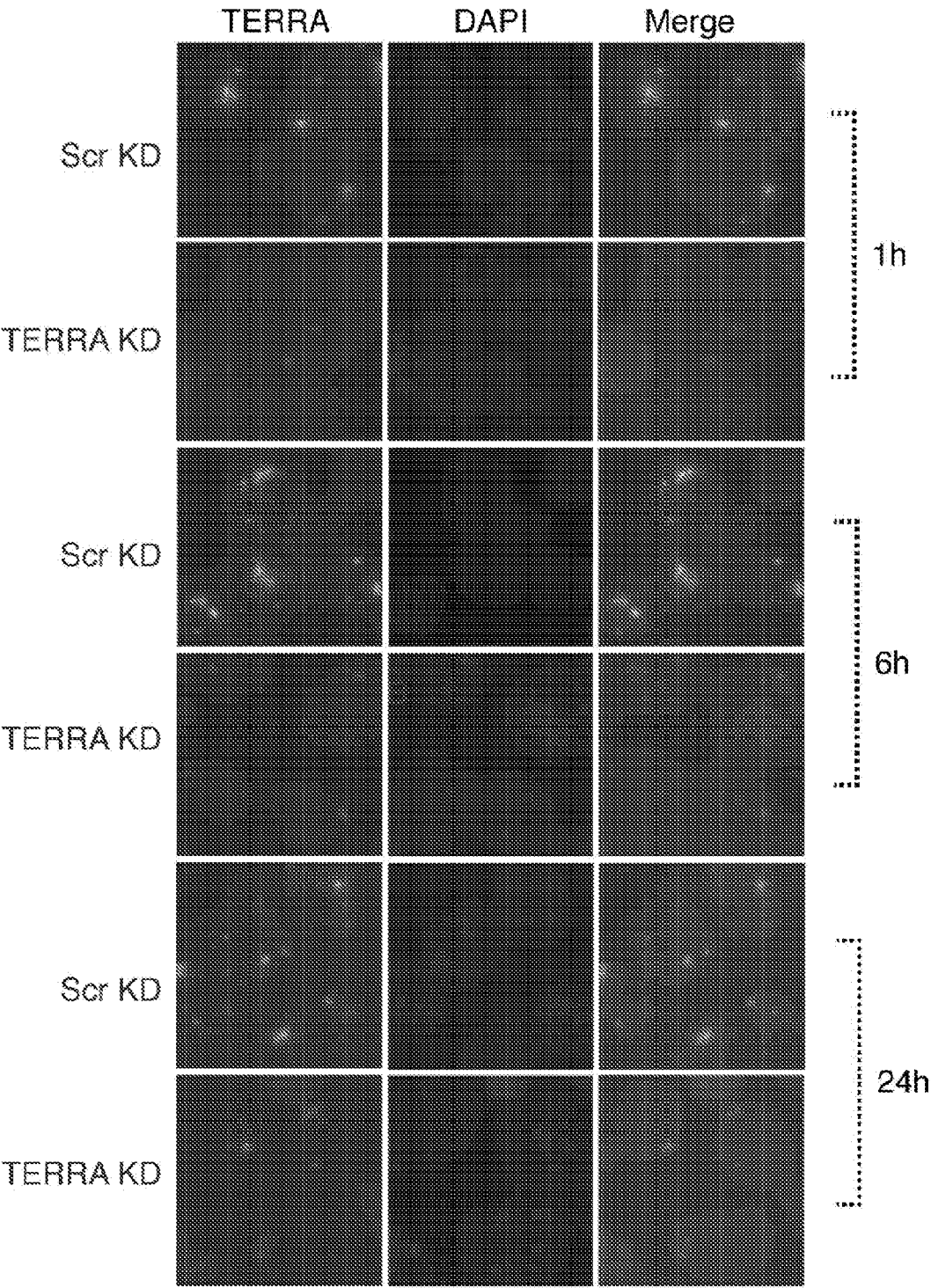


FIG. 11A



♀ ES cells

FIG. 11B

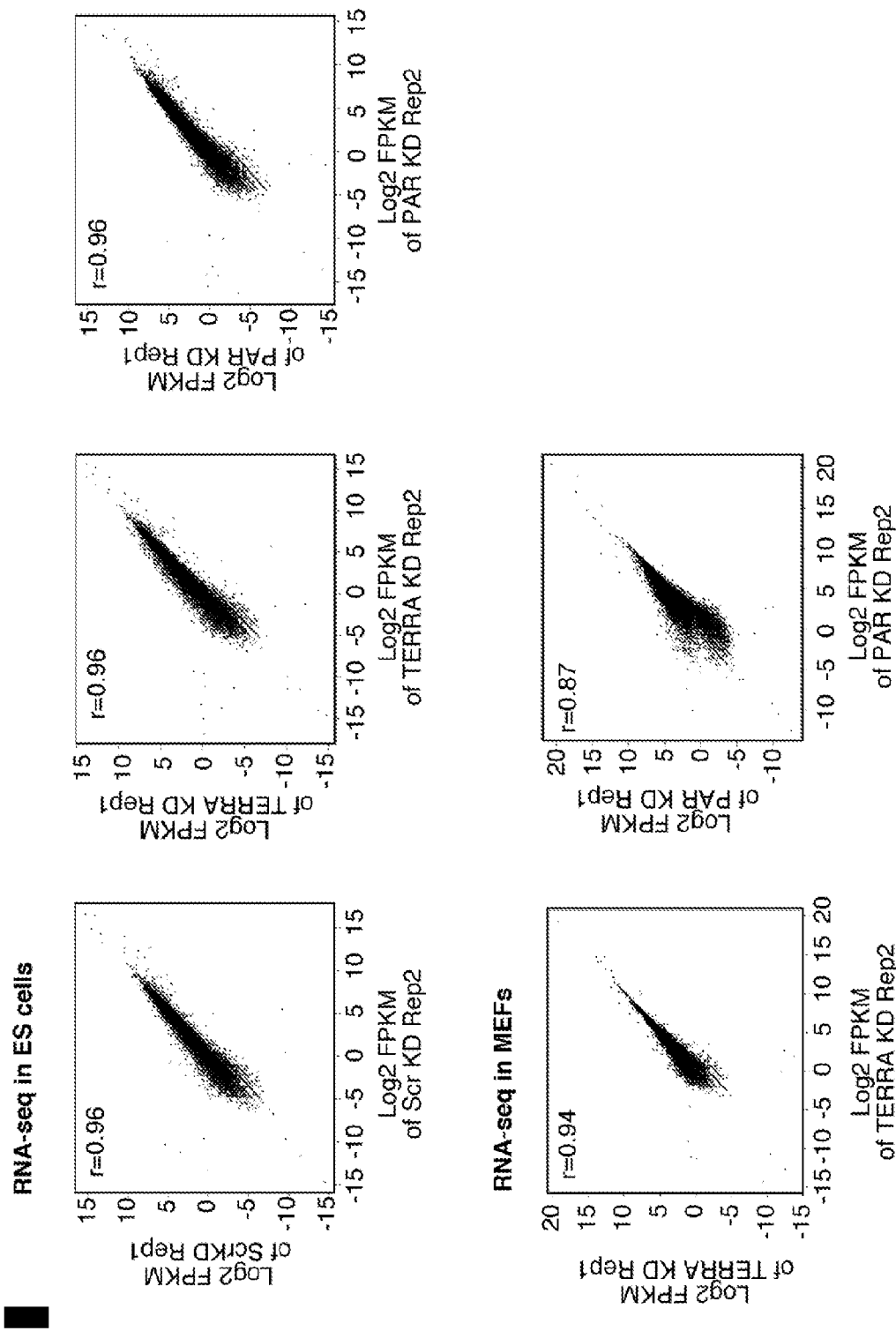


FIG. 11C

FIG. 12A

[illegible]

FIG. 12B

Upregulated genes					
    	PARP1	Chr3: 39,547-39,608 kb	Chr3: 79,340-79,580 kb	Chr3: 3,100-3,350 kb	
	TEFRA1	10-1501	10-3001	10-3001	10-1501
	SESN1	10-1501	10-3001	10-3001	10-1501
	SESN2	10-1501	10-3001	10-3001	10-1501
	TEFRA2	10-1501	10-3001	10-3001	10-1501
    	PARP1	Chr3: 39,547-39,608 kb	Chr3: 79,340-79,580 kb	Chr3: 3,100-3,350 kb	
	TEFRA1	10-1501	10-3001	10-3001	10-1501
	SESN1	10-1501	10-3001	10-3001	10-1501
	SESN2	10-1501	10-3001	10-3001	10-1501
	TEFRA2	10-1501	10-3001	10-3001	10-1501
    	PARP1	Chr3: 39,547-39,608 kb	Chr3: 79,340-79,580 kb	Chr3: 3,100-3,350 kb	
	TEFRA1	10-1501	10-3001	10-3001	10-1501
	SESN1	10-1501	10-3001	10-3001	10-1501
	SESN2	10-1501	10-3001	10-3001	10-1501
	TEFRA2	10-1501	10-3001	10-3001	10-1501
    	PARP1	Chr3: 39,547-39,608 kb	Chr3: 79,340-79,580 kb	Chr3: 3,100-3,350 kb	
	TEFRA1	10-1501	10-3001	10-3001	10-1501
	SESN1	10-1501	10-3001	10-3001	10-1501
	SESN2	10-1501	10-3001	10-3001	10-1501
	TEFRA2	10-1501	10-3001	10-3001	10-1501

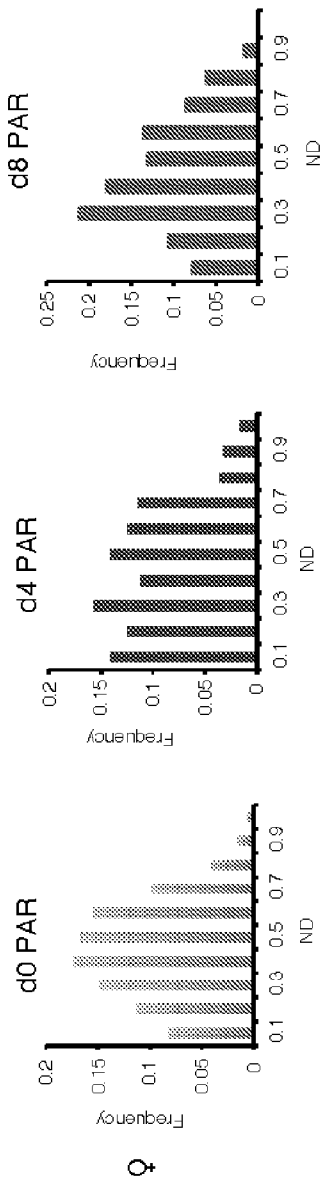


FIG. 13A

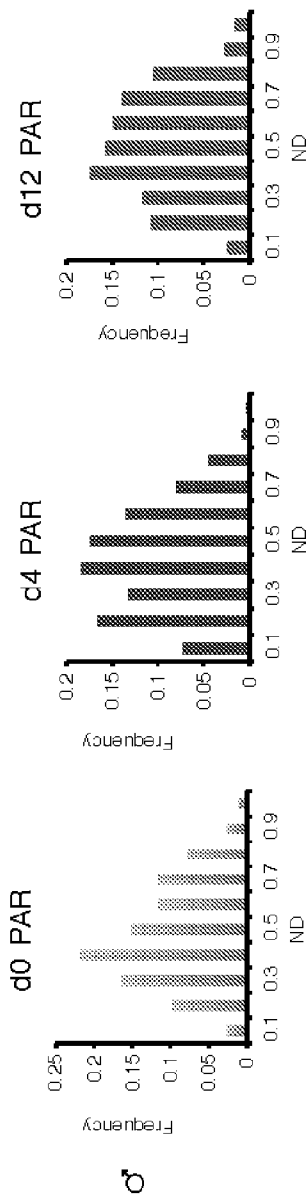


FIG. 13B