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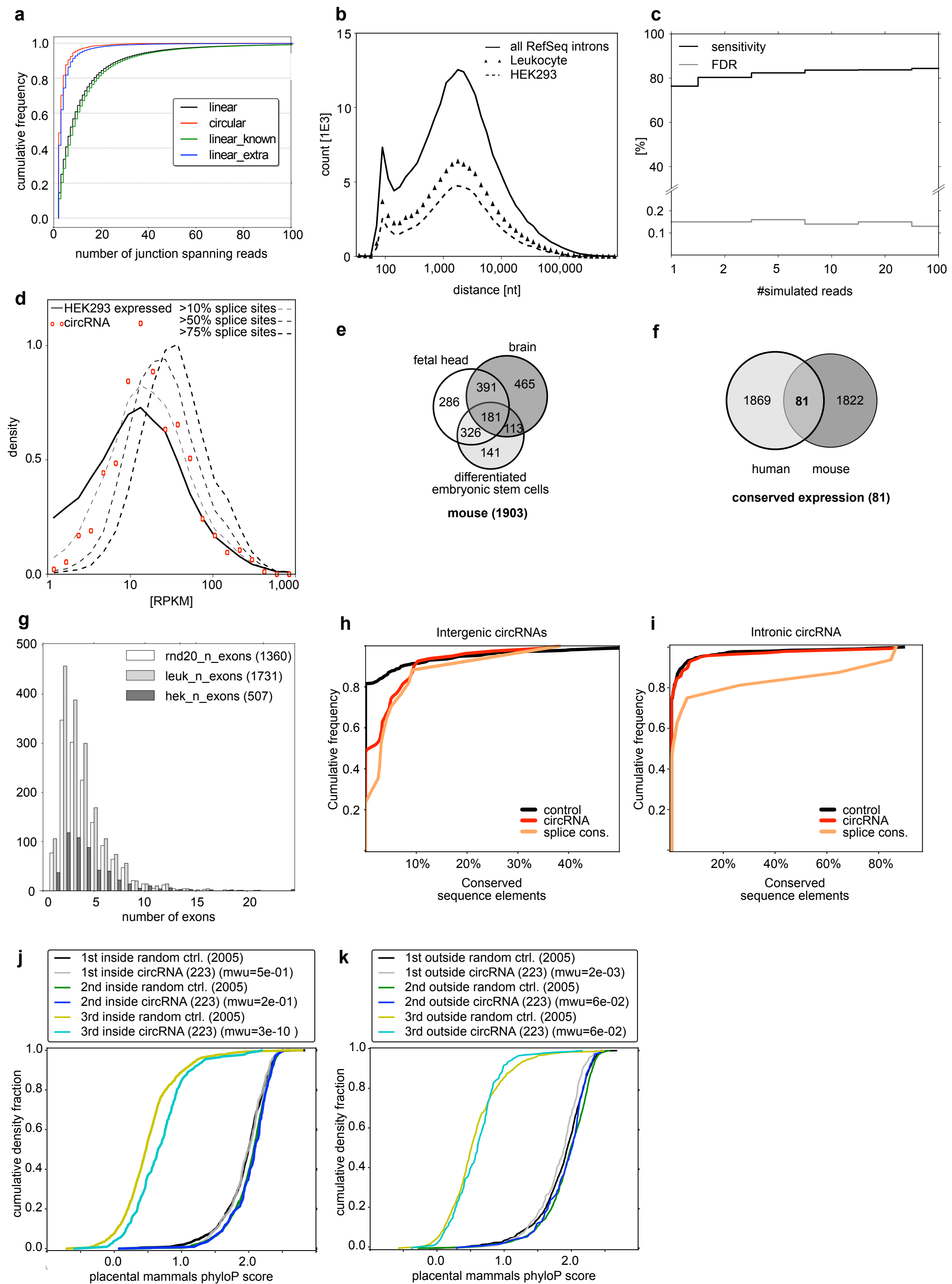
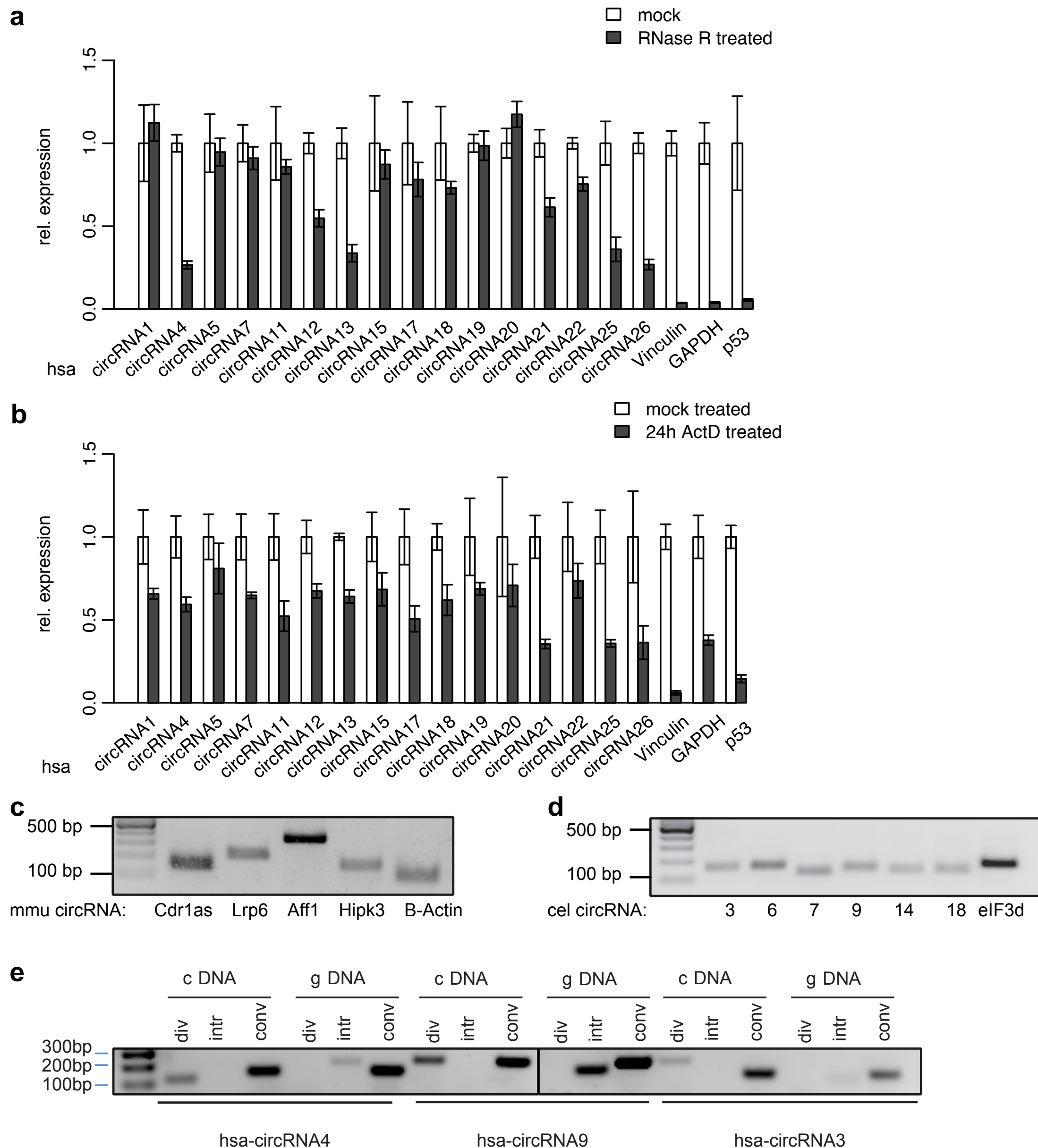


Supplementary Figure 1 Computational screen and statistical analysis
a, Cumulative frequency of the number of reads that span linear and circular splice sites

At the same cutoffs that were used to call the reported 1950 human circRNAs, our screen identifies 101,456 linear exon-exon junctions. Of these, 89% correspond to annotated RefSeq and non-coding RNA exon-exon junctions. By this comparison we find additional 11,521 exon-exon junctions that may be found in more sensitive but less stringently validated gene annotations.
The circular splice junction candidates that we identify in our screen are generally covered by much less reads than linear junctions (about ten times less on average).
b, The algorithm recovers annotated introns.
The logarithmic length histogram of detected linearly spliced introns in HEK293 (dashed lines) and leukocyte data (triangles) recapitulates the RefSeq intron length distribution (solid line).
c, Sensitivity and false discovery rate estimates
Using chains of randomly chosen, consecutive internal exons as shown in **g)**, we simulated reads spanning the circularizing splice junction. Performing the complete analysis on these synthetic data (~10.000 simulated circRNAs) yields a sensitivity (recovered simulated circRNAs over total simulated circRNAs) of > 75%, even for low coverage of the splice site. The false discovery rate (reported circRNAs that were not simulated divided by the number of all reported circRNAs) is below 0.2%.
d, The sensitivity of splice site detection depends only weakly on gene expression.
Histograms of gene expression levels obtained from polyA+ RNA sequencing in HEK293 cells. The number of reads per kilobase of exon per million mapped reads (RPKM) reflects mRNA abundance. Genes that are predicted to give rise to circRNAs (red circles) are not specifically enriched for high expression, (solid line: all genes). circRNAs from lowly expressed genes are detected less frequently, comparable to the loss of sensitivity observed for linear splicing (black dashed line: genes with > 75% of annotated splice sites recovered, gray dashed line: >50%, light gray: >10%).
e, Mouse circRNAs. Venn diagram of circRNAs detected in mouse tissues.
f, Conserved circularization. We find 81 circRNAs with orthologous splice sites, detected independently in human and mouse data.
g, Exon number distribution for human circRNAs (gray:leukocytes, dark gray:HEK293 cells) and matched random exon controls, used for **(c)** (white).
h, Conserved sequence element **(Methods)** content of intergenic human circRNAs (red) significantly exceeds random intergenic controls (black), P < 5e-10, Mann-Whitney-U, *n*=81. Same for circRNAs with conserved splice sites (orange, P < 1e-6, Mann-Whitney-U, *n*=17). **i, Intronic circRNAs** show enrichment of conserved sequence elements if flanking splice sites are conserved (orange, P < 0.07, Mann-Whitney-U, *n*=16), but not significant for the complete set (red, P > 0.5, Mann-Whitney-U, *n*=215). Controls (black) randomly drawn from same introns.
j, Coding sequence phyloP conservation score distributions of first and second codon positions match between circRNAs and controls, in contrast the 3rd codon position is significantly more conserved in circRNAs (P < 3e-10 *n*=223 Mann-Whitney-U (mwu)(also main Fig1. f). **k,** The conservation score distributions in the remaining parts of the CDS (outside the circRNA or control) do not differ significantly for codon positions two and three. For the first codon position, the controls are actually more conserved, P < 2e-3 *n*=223 Mann-Whitney-U (mwu)), therefore conservative.



Supplementary Figure 2 Validation of circRNAs

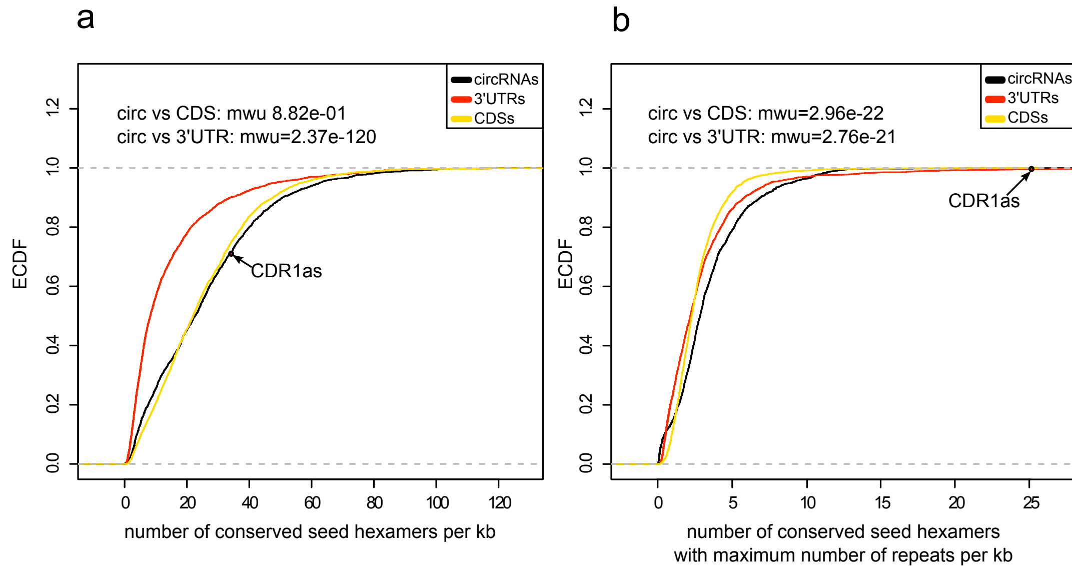
a, circRNAs are RNase R resistant.

16 human (hsa) circRNA candidates were tested in a RNase R assay for exonuclease resistance. VINCULIN, GAPDH and p53 specific primers were used as linear controls, error:stdev.

b, circRNA expression after transcriptional block. HEK293 cells were Actinomycin D treated and circRNA expression was assayed by qPCR (controls as in a)

c,d. Expression of mouse and *C. elegans* circRNAs. RT-PCRs using divergent primers detect circRNAs in mouse (mmu) and *C. elegans* mixed stage (cel).

e, RT-PCR of human circRNA candidates. RT-PCR was performed on cDNA (gDNA as control) using divergent or convergent primers for three circRNAs that span at least one intron. Additionally convergent intron specific primers were used **(Methods)**. hsa-circRNA 9 corresponds to AFF1 (Fig. 1e).



Supplementary Figure 3 Human circRNAs are significantly enriched for repetitions of conserved miRNA seed matches.
a, Empirical cumulative distribution function (ECDF) of the number of conserved miRNA seed matches (hexamers, miRNA position 2-7) per kilobase of inferred spliced sequence **(Methods)** of circRNAs (black). A seed match is considered to be conserved when it is present in 5/5 different mammals spanning the evolutionary distance from man to dog **(Methods)**. Analogous ECDFs were plotted for coding exons (n=3873) and 3'UTRs (n=3182) with similar length distributions as human circRNAs (n=1950). For the statistical comparisons Mann-Whitney-U test was used. **b,** Similar to (**a**) but counting only the number of those miRNA seed matches that are repeated most often. CDR1as is marked as a circle.

```
                  +0
Human/1           AGGGTTTCCGATGGCACCTGTGTCAAGGTCTTCCAACAACTC-CGG-----GTCTTCCAGCGACTTCAAG-------------------TCTTCCAATAATCTCAAGG-----TCTTCCAG-AT-----AATCCTGAGCTTCCAGAAAAT
Gorilla/1-1511    AGGGTTTCCGATGGCGCCAGTGTCAAGGTCTTCCAACAACTC-CGG-----GTCTTCCAGCGACTTCAAG-------------------TCTTCCAATAATCTCAAGG-----TCTTCCAG-AT-----AATCCTGAGCTTCCAGAAAAT
Macaque/1-1582    AGGGTTTCCAATGGCGCCAGTGTCAAGGTCTTCCAACAACTC-CGG-----GTCTTCCAGCGACTTCAAG-------------------TCTTCCAACAGTCTCAAGG-----TCTTCCAG-AT-----AATCCTGAGCTTCCAGAAAAT
Guinea_Pig/1-1236 AGGGTTTCCAATGGCGCCAGTGTCAAGATCTTCCAGCAAATC-GAA-----GTCTTCCAGCGATTTCAAG-------------------------------------G-----TCTTCCAG-AC-----AATCTCGGGCTTCCAGAAAAT
Cow/1-893         -----------------------------------------------------------------------------------------------------------------------------------------TCTTCCAGAAAAT
Naked_mole/1-1145 AGGGTTTCCAGTGGCTTCAGTGTCAAGGTCTTCCAGCAACTCACAG-------------------------------------------TCTTCCAACAATCTCAAGG-----TCTTCCAG-AC-----AATCTCGGTCTTCCAGAAAAT
Rabbit/1-1572     AGGGTTTCCAGTGGCGCCAGTGTCAATGTCTTCCAACAACTC-CAG-----GTCTTCCATCAACTCCAAG-------------------TCTTCCAAAAACCTCAAAG-----TCTTCCAG-AC-----AATCCTGGTCTTCCAGAAAAT
Elephant/1-1371   AGGGTTTCCAATGGAGCCGGTGTAAAGGTCTTCTGACAACTC-TGG-----ATCTTCCAGCGACTCCAAGTCTTCCAACTAACTCCACGTCTTCCAACAATCTCAAGG-----TCTTCCAG------CCAATCCTGGTCTTCCAGGAGAT
Marmoset/1-1311   AGGGTTTCCACTGGCGCCAGTGTCAACGTCTTCCAGCAACTC-CAG-----GTCTTCCAGCGACTTCAAG-------------------TCTTCCAACAATCTCAAGG-----TCTTCCAG-AC-----AATCCTGAGCTTCCAGAAAAT
Pig/1-1652        AGGGTTTCTGATGGCGCCAGTGTCAAGGTCTTCCAGTGACTC-CAG-----GTCTTCCAGCAATTCCAAG-------------------TCTTCCAACAACATCAAGG-----------------------------TCTTCCAGCTAAC
Dog/1-1356        AGGGTTTCCAATGGCGCCAGTGTCAAGGTCTTCCAACAACTC-CAG-----GTCTTCCAGCAACTCCAAG-------------------TCTTCCAACAAATTCCAAG-----TCTTCCAACAG-----CATCAAGGTCTTCCAGCTAAT
Horse/1-1309      AGGGTTTCGACTGGTGCCAGTGTCAAGGTCTTCCTACAACAC-CAG-----GTCTTCCAGAAACTCCAAG-------------------TCTTCCAACTAATTTCAAG-----CCTTCCAACAA-----CATCAAGGTCTTCCAGAAGAT
Lamprey/1-1177    ----TTCCAGAAGATCCCAGTTTT---GTCTTCCAGATGATC-CCATATTTATCTTCCAGAAGATTCCAGATTTGT-------------TCTTCCAGAAGACTGCAGATTTGTTCTTCCAGAAGATGCCAGATTTGTTCTTCGAGAAGAT

                  +150
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Gorilla/1-1511    CC-----ACATCTTCCAGACA-----ATCCACGTCTTCCG-GACAATCCA----TGTCTTCCAAGAAGCTCCAAGTCTTCCAGTAAATCAAGTCTTCCAGCAAATCCAGTCTTCCAGCAATT-----ATAGGTCTTCCACCAAATCGAG-
Macaque/1-1582    CA-----ACATCTTCCAGACA-----ATCCATGTCTTCCG-GACAATCCA----CGTCTTCCAAGAAGCTCCAAGTCTTCCAGT-----------------AAATCCAGTCTTCCAGCAATT-----ATAGGTCTTCCACCAAATACAG-
Guinea_Pig/1-1236 TC-----ATGTCTGCCAGGAA-----ATCCATGTCTTCCA-GAAAATTAA----TGTCTTCCCGANNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Cow/1-893         CC-----TCGTCTTCCAATGA-----AGGTGCACCTTCCAGGAAAATCCA----CGTCTTCC------CTCC-----------------------------AAATCTCGTCTTCCGATGAAG-----GTGCGCCTTCCAGGAAATCCA--
Naked_mole/1-1145 CC-----ATGTCTTCCGGAAA-----ATTAATGTCTTCCACAATCCTGCA----TGTCTTCCAGC------------------------------------AAATCTCGTCTTCCG--------------------CAAGCAAATCCAG-
Rabbit/1-1572     CC-----TCGTCTCCCAAATA-----ATATATGTCTTCCAAAAGTCTCCA----AGTCTTCCAGG------------------------------------AAATCCAGTCTTCCGGCAGTT-----ATATGTCTTCCACAAAATCCAG-
Elephant/1-1371   CC-----CAGTCTTCCAGACA-----AACCACATCTTCCG-GACAACCCA----TGTCTTCCAACGACCTCCAAGTCTTTCAGC-----------------AAATCCGTTCTTCCAGCAACT-----ATAGATCTTCCACAAAATCTAA-
Marmoset/1-1311   CC-----ACGTCTTCCAGACA-----ATCCACGTCTTCCA-GGCAATCCA----TGTCTTCCAAGAACCTCCAAGTCTTCCAGT-----------------AAATCCAGTCTTCCAGCAATT-----ATAGGTCTTCCAACAAATCCAG-
Pig/1-1652        CC------AGTCTTCCAGAA------ATCTATGTCTTCCG-GACAATCCA----TGTCTTCCAGG------------------------------------AAATCCAGTCTTCCAGCAATT-----ATATGTCTTCCACAAAATCCAT-
Dog/1-1356        CC-----TAGTCTTCCAGAAA-----ATTCACGTCTTCCA-GATAATCCA----CGTCTTCCA-----------------------------------------------------GTAATT-----CTCGGTCTTCCACAAAATCCAT-
Horse/1-1309      CT-----ACGTCTTCCAGACA-----ATTCACGTCTTCCA-GACAATCCA----TGGCTTCCAGT-----------------------------------AAATCCAGTCTTCCAGCAATT-----ACGGGTCTTCCACAAAATCCAT-
Lamprey/1-1177    CCTAGATTAGTCTTCAAGAAGTACCCAGATTTGTCTTCCAGAAGATTCCAGATTTGTCTTCCAAAAGATTCC-----------------------------AGATTTGTTCTTCCAGAAGATCCCAGATTTGTCTTCCAGAAGATCCCAG

                  +300
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Gorilla/1-1511    ----ATCTTCCAGGAAAATCCACGTCTTCCAG-GAAATCCATGTCTTCCAATAATTTCAAGGTCTTCCATCAAATAC-------------------AGATCTTCCAGCTAATCCATG------TCTTCCAGAAAAATCTG----TGTCTT
Macaque/1-1582    ----ATCTTCCAGGAAAATCCACGTCTTCCAG-AAAATACGTGTCTTCCAATAATTTCAAGGTCTTCCATCAAATAC-------------------AGATCTTCCAGCTAATCCATG------TCTTCCAGAAAAATCTA----CGTCTT
Guinea_Pig/1-1236 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN------NNNNNNNNNNNNNNNNNNNNNNNNNNN
Cow/1-893         ------------------------------------------------------------------------------------------------------------------------------------------------CGTCTT
Naked_mole/1-1145 ----ATCTTCCAGGAAAATCCAGATCTTCCAGAAAAATCCATGTCTTCCAACAATTTCA-------------------------------------ACGTCTTCCTATGAGTCTGTG------TCTTCCAGGAAAATCTG----TGTCTT
Rabbit/1-1572     ----ATCTTCCAGGAAAACATACGTCTTCCAG-AAAATCCATGTCTTCCA---GTTTTGAGGTCTTCCTGCCAATCTACATCTTCCAACAATTTCAAGGTCTTCCCGCCAATTCATG------TCTTCCAGAAAAATCTG----TGTCTT
Elephant/1-1371   ----ATCTTCCAGGAAAATCCATGTCTTCCAGTAATTTCAAGATCTTCCACCAAACTC--------------------------------------AGGTCTTCCAACCAATCCACG------TCTTCTGGAAAAACCTA----TGTCTT
Marmoset/1-1311   ----ATCTTCCAGGAAAATCCACGTCTTCCAG---------------------------------------------------------------------------CTAATCCATG------TCTTCCAGAAAAATA------TGTCTT
Pig/1-1652        ----AGCTTCCAGGAAAATCCACGTCTTCCAG-AAAATCCATGTCTTCCAACAATTTCA-------------------------------------AGGTCTTCCAGCCAATCCACG------TCTTCCAGAAAAATCTA----TGTCTT
Dog/1-1356        ----ATCTTCCAGGAAAATCCACGTCTTCCAG-AAAATCTACGTCTTCCAATGATTTCAAGGTCTTCCAGACAATCCACGTCTTCCAGCAATTTCAAGGTCTTCCAGCCAATCCACG------TCTTCCAGAAAAAATTA----TGTCTT
Horse/1-1309      ----ATCTTCCAGGAAAATCCATGTCTTCCAGAAAAATCCATGTCTTCCAATGGTTTCAAGGTCTTCCAGACAATCCACGTCTTCCAACAATTTCAAGGTCTTCCGGCTAATCCACG------TCTTCCAGAAAAATC------TGTCTT
Lamprey/1-1177    ATTTGTCTTCCAGAAGATTCCAGATTT----------------------------------------------------------------------GTTCTTCCAGAAAATTCCAGATTTGTTCTTCCAGAAGATTCTAGATTTGTCTT

                  +450
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Gorilla/1-1511    CCACCAAATCCAAGTCTTCCAGTAAATCTAGTTCTTCCAG------------------AA------------------AAATCTAGATCTTCCAGTCAATCAGTGTCTTCCAGAAAGAAATCCAG----GTCTTCCAGTCAATCA-----
Macaque/1-1582    CCACCAAATCCAAGTCTTCCAGTAAATCTAGTTCTTCCAG------------------AA------------------AAATCTAGATCTTCCAGTCAATCAGTGTCTTCCAGAAAGAAATCCAG-------------------------
Guinea_Pig/1-1236 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Cow/1-893         CCAGAAAATCCTAGTCTTCCAATGAAGGTGCGCCTTCCAG------------------GA------------------------------------------------------------------------------------------
Naked_mole/1-1145 CCACCAAATCCAAGTCTTCCAATGAATTCAGGTCTTCCAG------------------AA------------------AAATCCAGGTCTTCCAGCAAACT-------------------------------------------------
Rabbit/1-1572     CCACCAAATCCAAGTCTTCCAATCAATCTGGTTCTTCCAG------------------AA------------------AAGTCCAAGTCTTCC----AAACAATGTCTTCCAGAAAGAAATCCAG----GTCTTCCAGGCAGCCC-----
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Marmoset/1-1311   CCACCAAATCCAAGTCTTCCAATAAATCTAGTGCTTCCGG------------------AA------------------ATATCCAGATCTTCCAGTCAATCAGTGTCTTCCAGAAAGAAATCCAG----GTCTTCCAGTCA---------
Pig/1-1652        CCGCCAAATCTAAGTCTTCCAGCTAATTTACGTCTTCCAG------------------AA------------------AAATCCAAGTCTTCCAGCCAATCTCTGTCTTCCAGAAAGAAATCCAG----GTCTTCCACTCAGTCC-----
Dog/1-1356        CCACCAAATCCAAGTCTTCCAATCAATTCAAGTCTTCCAGCTAATTTATGTCTTCCGGAA------------------AAATCCAGGTCTTCCAGTCAGTCGATGTCTTCCGGAAAGAAATCCAG----GTCTTCCACTCAGTCC-----
Horse/1-1309      CCGCCAAATCCAAGTCTTCCAGT-------------------------------------------------------CAATCCAGGTCTTCCAGCTAATTTATGTCTTCCAGAAAGAAATCCAG----GTCTTCCACTCAGTCT-----
Lamprey/1-1177    CCAGAAGATCCCAGTTTT-------------ATCTTCCAG------------------AA------------------GATTCCAGATTTGT-------------TCTTCCAGAAG--ATCCCAGTTTTGTCTTCCAGATGATCCCATAT

                  +600
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Gorilla/1-1511    GTGTCTTCCAGAAAGA-----------------------------------------------------------------------AATCCAGGTCTTCCAG---TCAGTCAGTGTCTTCCAGA--AATATCTATGTC-----TTCCAC
Macaque/1-1582    ----------------------------------------------------------------------------------------------GTCTTCCAG---TCAGTCAGCGTCTTCCAGA--AAAATCTATGTC-----TTCCAC
Guinea_Pig/1-1236 NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN-----------------------------------------NNNNNN
Cow/1-893         ---------------------------------------------------------------------------------------AATCCACGTCTTCCAG------------------------AAAATCCTCGTC-----TTCCAA
Naked_mole/1-1145 ATGTCTTCCA---------------------------------------------------------------------------CCAAATCAAGTCTTCCAG---TCAACTCCTGTCTTCCAGAC-AAAATCTGTGTC-----TTCCAC
Rabbit/1-1572     ATGTCTTCCAGAAAAATCTACGTCTTCCTCCAAATCCAAGTTTTCCAATCAATCCAGTTCTTCCGGAAATGTTCAAGTCTTCCAGTCAATCAGTGTCTTCCAGAAAGAAATCCAGGTCTTCCAGA--AAAATCTACGTC-----TTCCAC
Elephant/1-1371   ACGTCTCCCCAGAA-------------------------------------------------------------------------CATCCAGGTCTTCCAG---ACAATCTACGTCTTCCAGG--AAAATCCATGTC-----TTTCAC
Marmoset/1-1311   GTGTCTTCCAGAATGA-----------------------------------------------------------------------AATCCAGGTCTTCCAG---TCAGTCAGTGTCTTCCAGA--AAAATCTACGTC-----TTCCAC
Pig/1-1652        ATGTCTTCCGGAGAAATCTGTGTCTTCC------------------AGAAAAATCCAGGTCTTCCAACCAACCCACGTCTTCCATCAAATCCA-GTCTTCCAG---TGCATTTATGTCTTCCAGACAAAAATCTATGTC-----TTCCAC
Dog/1-1356        ACGTCTTCCAGAAAAATCTATGTCTTCCGCCAAATCCATATCTTCCGGAAAAATCCAGGTCTTCCAACCAATCCATGTCTTCCACCGAATCCA-GTCTTCCAG---TCAATCCACGTCTTCCAGAC-AAAATTTATGTC-----TTCCAC
Horse/1-1309      GTGTCTTCCAGGAACATCTACGTCTTCCACCAA-------------------------------------ATCCAGATCTTCCACCAAATCCA-GTCTTCCAG---CCAATCCACGTCTTCCAGAG-AAAATCTATGTC-----TTCCAC
Lamprey/1-1177    TTATCTTCCAGA----------------------------------------------------------------------------------ATATTCCAG------ATTTGT-TCTTCCAGA--AGACTCCAGATTTGTTATTCCAG

                  +750
Human/1           ----CAAATCCAGG------TCTTCCAGTCAATCC-----ACATCTTCCGGAAAAAATCCAGGTCTTCCAGCCA-----------------------------------------------------ATATATGTCTTCCTGAAGATCCA
Gorilla/1-1511    ----CAAATCCAGG------TCTTCCAGTCAATCC-----ACATCTTCCGGAAAAAATCCAGGTCTTCCAGCCA-----------------------------------------------------ATATATGTCTTCCTGAAGATCCA
Macaque/1-1582    ----CAAATCCAGG------TCTTCCAGTCAATCC-----ACGTCTTCCGGAAAAAATCCAGGTCTTCCAGCCA-----------------------------------------------------ATATATGTCTTCCTGAAGATCCA
Guinea_Pig/1-1236 NNNNAAGACTCAAG------TCTGCCAGGAAATCC-----ATGACTTCCAG--AAAATTCATGTCTTGCATGAA----------------------------------------------------------------------------
Cow/1-893         ----TGAAGGTGCA------CCTTCCAGGAAATCC-----ACGTCTTCCAG--AAAATCCCTGTCTTCCAATGAA----------------------------------------------------AGGTGCGCCTTCCAGGAAATCCA
Naked_mole/1-1145 ----TAAATCCAGC------TCTTCCAGTAAAGCC-----ATGTCTTCCAG--AAAATTCATGTCTTCCAGTAA----------------------------------------------------------------------TCTCCA
Rabbit/1-1572     ----AAAACCCAGG------TCTTCCAGTCAATCC-----ATGTCTTCTGG-AAAAATCCAGGTCTTCCAACCAATCTCTCTTCCACCAAATCAAGTCTTCCAATCAACCCGTGTCTTCCTGATAAAATGTCTGTCTTCCACTAAATCTG
Elephant/1-1371   ----TAAATCCTG-------TCTTCTGGTCAGTCC-----ACATCTTCCAGACAAAATCTGTGTCTTCCACCAA-----------------------------------------------------------------------ATCCA
Marmoset/1-1311   ----CAAATCCAGG------TCTTCCAGTTAATCC-----ACGTCTTCCGGAAAAAATCCAGGTCTTCCAGCCA-----------------------------------------------------ATATATGTCTTCCTGAAGATCCA
Pig/1-1652        ----TGAATCCAGG------TCTTCCGGTAAATCT-----ACGTCTTCCAG--AAAATTCATGTCTTCCACTAA-----------------------------------------------------ATTCAGGTCTTCCAGCAAATTTA
Dog/1-1356        TAAATAAATCCAGG------TCTTCCAAAAAAGCC-----ACGTCTTCCAG--AAAATTTATGTCTTCCACTAA-----------------------------------------------------ATCCAAGTCTTCCAACAAATATA
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Gorilla/1-1511    CGTCTTCCAGAAAATCCATGTCTTCCAG-AAAATCCAT-------------------GTCTTCCAGTAACCTCCCA----GTCTTCCA--------------GAAAATCCA-----CGTCTTCCCAAC-AATCCAGG-----TCTTCTGG
Macaque/1-1582    CGTCTTCCTGAACATCCATGTCTTCCAG-AAAATCCATGTCTTCCAGTAACCTCCCAGTCTTCCAGTAACCTCCCA----GTCTTCCA--------------GAAAATCCG-----CGTCTTCCCAAC-AATCCAGG-----TCTTCCGG
Guinea_Pig/1-1236 --------------------CCTTCCCA----------------------------------------------------GTCTTCCA--------------GCAAATCAA-----TGTCTTCCAAAATAATCCAGG-----TCTTCCGG
Cow/1-893         CGTCTTCCAGAAAATCCCGGTCTTCCAA-TGAAGGTGC------------------------------------------GCCTTCCA--------------GGAAATCCA-----CGTCTTCCAAAA-AATCCCTG-----TCTTCCAA
Naked_mole/1-1145 GTTCTTCCAGCCAATGTATGTCTTCCTG-AAGATTCAA------------------------------------------GTCTTCCA--------------GAAAATCCA-----CGTCTTCCAGAA-AATTCATG-----TCTTCCAT
Rabbit/1-1572     TATCTTCCAATTCAGCCAAGTCTTCCAG-AAAATCCT-------------------TGTCTTCCAATAATCTCCAG----GTCTTCCAGTCAATGTCTTCCTGAAGATCTA-----TGTCTTCCAGAA-TATCCGTA-----TCTTCCTG
Elephant/1-1371   AGTCTTCCAATAAAGCCATGTCTTCCAG-AAAATTCAT-------------------GTCTTCCAAGAACCTCCAG----GTCTCCCT--------------GGAGATCCA-----CGTCGTCCAGAA-AATCCACG-----TCTTCCAG
Marmoset/1-1311   CGTCTTCCAGAAAGTCCATGTCTTCCAG-AAAATTCGT-------------------GTCTTCCAGTAACCTCCCA----GTCTTCCA--------------GAAAATCCA-----CGTCTTCCAAAC-AATCCAGG-----TCTTCCGG
Pig/1-1652        TGTCTTCCTAGAGAT---TGTCTTCCAGAAAAATTCAT------------------------------------------GTCTTCCA--------------GCAAATCTG-----TGTCTTCCAAAT-AATCCAGG-----TCTTCCGG
Dog/1-1356        CGTCTTCCTAGAGATCCACGTCTTCCAG-AAAATTCAT-------------------GTCTTCCAGTAACCTCCAG----GTCTTCCA--------------GCAAATCGA-----CGTCTTCCAAAC-AATCCAGG-----TCTTCCGG
Horse/1-1309      AGTCTTCCAGCAAATATATGTCTTCCAG-AAAATCCAT-------------------GTCTTCCAGTAACCTCCAG----GTCTTCCA--------------GCAAATCTA-----CGTCTTCCAAAC-AACCCAGG-----TCTTCCAG
Lamprey/1-1177    AGATTT------------TTTCTTCCAG-AAGATTCCAGATTT-------------GTTCTTCTAGAAGATCCCAGTTTTGTCTTCCA--------------GAAGATCCCAGATTTGTCTTCCAGAAGATTCCAGATTTGTTCTTCCAG
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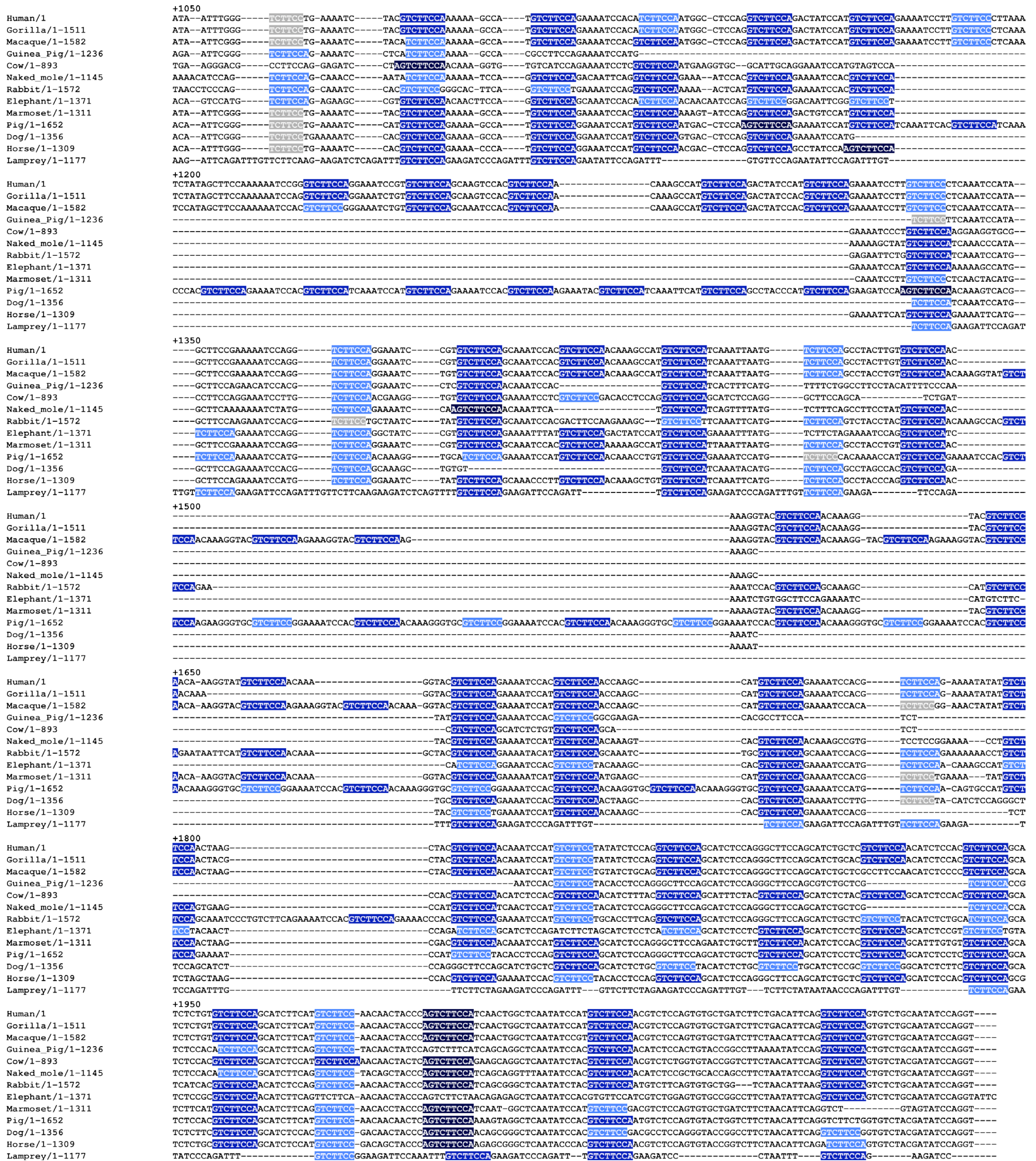
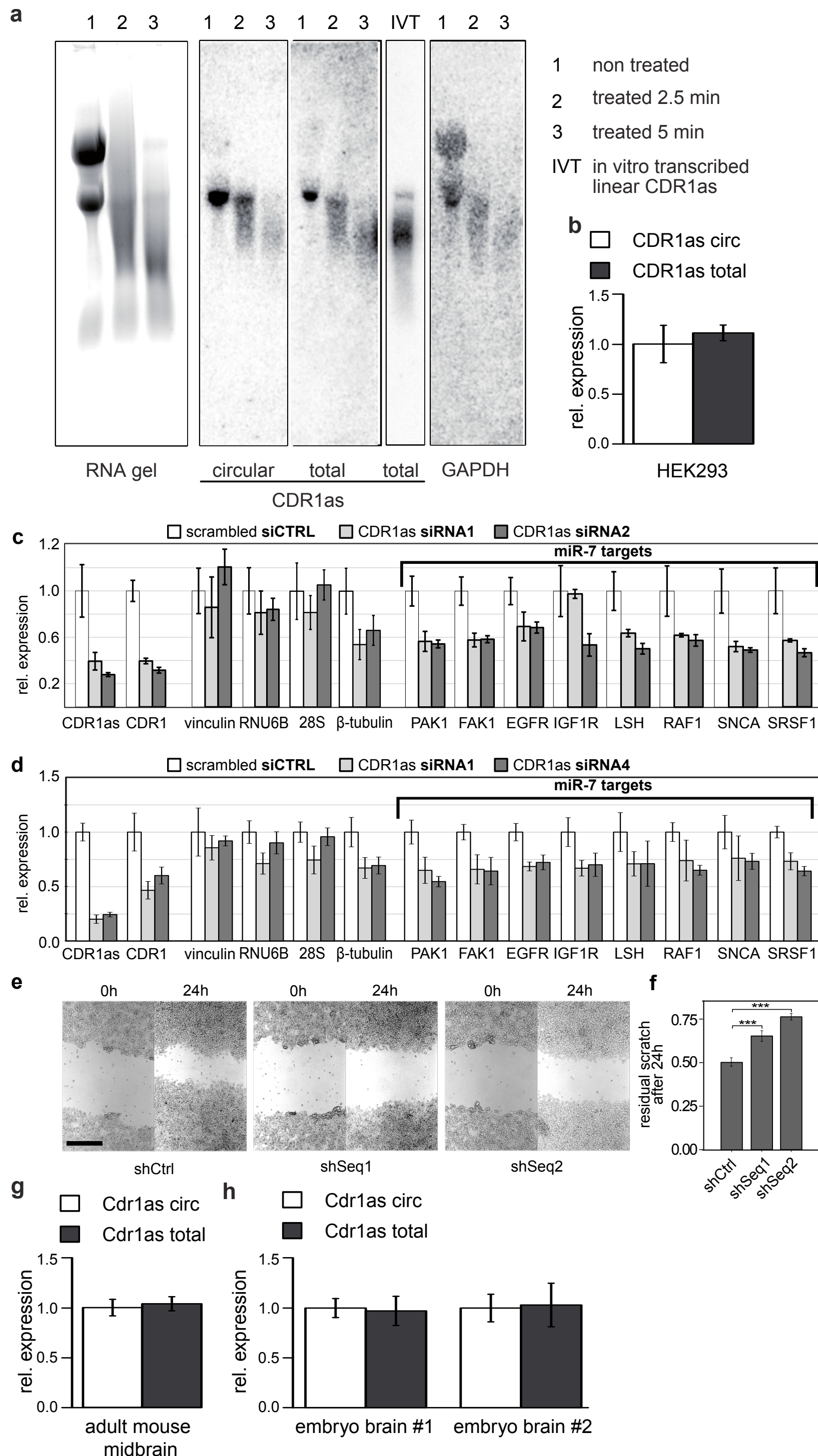


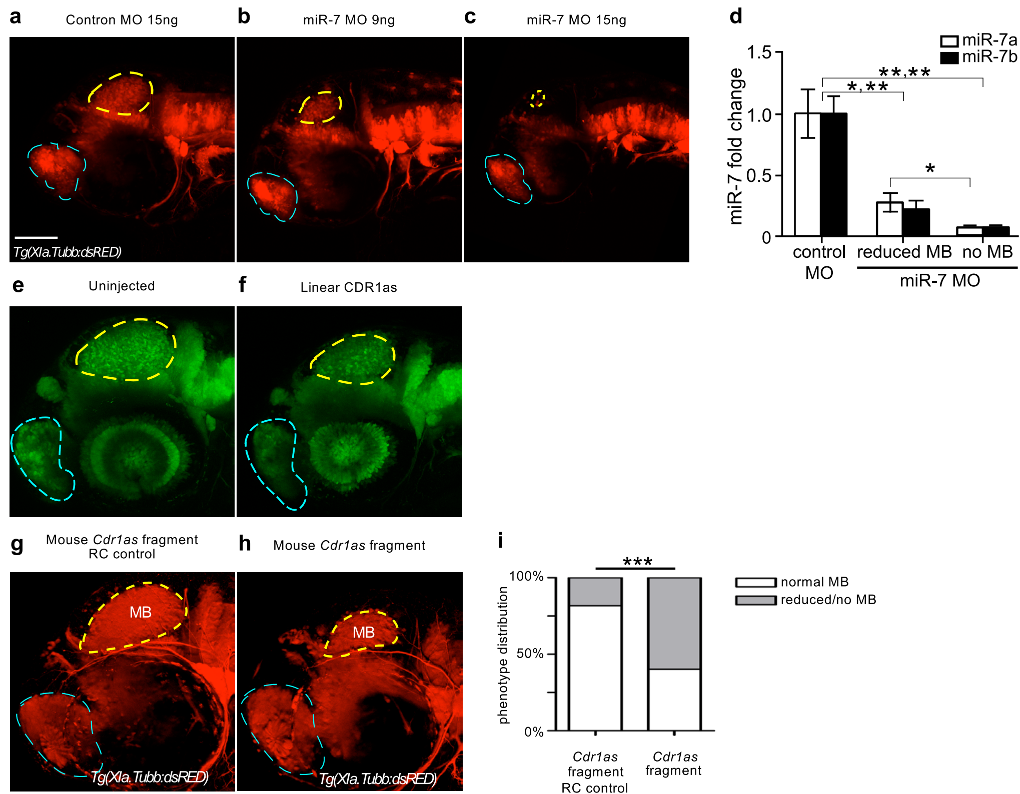
Supplementary Figure 4 CDR1as multiple species alignment.
We obtained sequences homologous to the human CDR1as sequence using BLAT and the UCSC Genome browser. If the reverse BLAT hit human CDR1as, we kept the sequence for further analysis. A multiple species alignment was built with MUSCLE. miR-7 seed matches are color coded by their strength (gray: 6mer, light blue: 7mer, dark blue: 8mer, black: 9mer).



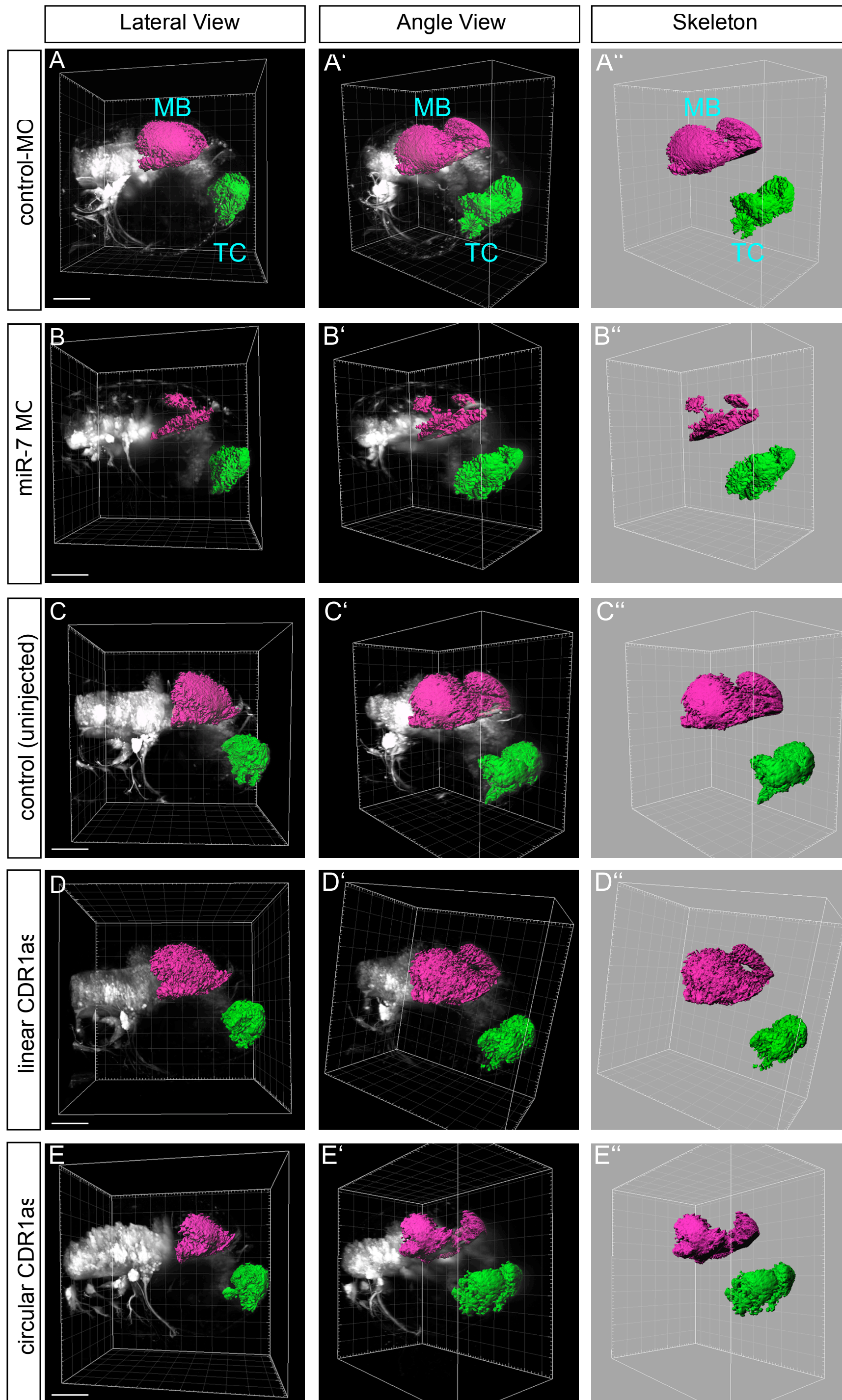
Supplementary Figure 5 circular CDR1as nicking, expression and knock down in HEK293 cells and circular *Cdr1as* expression in mouse brain

a, RNA nicking assay. Total RNA from HEK293 was subjected to RNA nicking (**Methods)** for the indicated time points. The "circular" probe spans CDR1as head-to-tail junction, "total" can detect linear as well as circular CDR1as. GAPDH: control. A linear IVT product of CDR1as serves as positive control.

b, qPCR analysis of CDR1as circularization in HEK293 (error:stdev).
c, siRNA depletion of CDR1as induces repression of miR-7 target genes. siRNA knock down reduces levels of CDR1as and CDR1 mRNA by ~60%. Some house keeping genes are slightly affected, while 8/8 tested miR-7 targets are repressed, consistent with a release of miR-7 loaded RISC from CDR1as to the cytoplasm. **d,** A replicate of the experiment in **(c)** is shown with siRNA1 and the additional siRNA4.
e, Stable CDR1 knock down causes a cellular migration defect *in vitro*. 24 hours after disruption of a confluent cell monolayer, two cell lines derived from HEK293 cells with stable CDR1as knock downs (shSeq1, shSeq2) were significantly impaired in migrating into the scratch compared to control cell lines. Scalebar is 1mm.
f, Quantification of the migration defect. Three experiments were performed in quadruplicates and one in duplicate. Errorbars are standard deviation (P=0.002 shSeq1 vs shCtrl, P=1.2E-5 shSeq2 vs shCtrl, two-tailed T-test, unequal variance, *n*=14).
g,h, *Cdr1as* is circular in adult midbrain and two mouse embryonic whole brains (error:stdev).

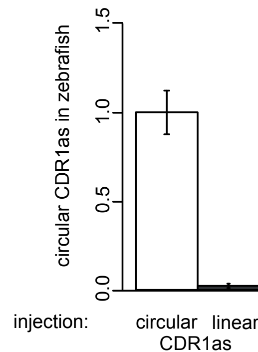


Supplementary Figure 6 miR-7 MO/CDR1as phenotype in zebra fish a-c, Representative confocal images of Tg(*Xia.Tubb:dsRED*) embryos at 48hpf injected with 15ng control MO **a**, 9ng miR-7 MO **b**, or 15ng miR-7 MO **c,** scale bar represents 100 µm. Yellow dashed line indicates midbrain, blue dashed line indicates the telencephalon in **a-h)**. **d,** qPCR analysis of *miR-7a* and *miR-7b* expression in control and *miR-7* morphants showing reduced midbrain (MB) or no MB. error:stdev **P* < 0.05; ***P* < 0.01 in Student's *t*-test **e,f,** Representative confocal images of *Tg(huC:egfp)* animals after injection of a plasmid coding for linear CDR1as or unjected controls. **g,** Confocal z-stack projection from Tg(*Xia.Tubb:dsRED*) embryos at 48hpf in lateral view, after injection of mouse *Cdr1as* reverse complement (RC) control RNA or mouse *Cdr1*as RNA **h**. Yellow dashed line indicates midbrain, blue dashed line telencephalon. **i,** phenotype distributions of fish shown in **(g,h)**, ****P* < 0.001; in Student's *t*-test.



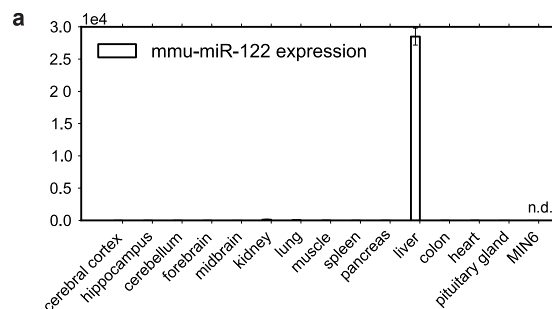
Supplementary Figure 7 Volume measurement of zebra fish embryo midbrain and telencephalon based on confocal 3D stacks. A-E: Midbrain and telencephalon volumes were calculated using Imaris 64x7.6.1 software based on high resolution 3D stacks obtained from Tg(*Xia.Tubb:dsRED*) embryos. Confocal images were taken at steps of 2µm, line scan 4 times, and 200 images were acquired per embryo. Midbrain (MB) was pseudo-colored in pink, telencephalon (TC) in green; other neuronal structures are in white. For clarity 3D projections are presented in lateral view **(A-E)**, after rotation about the *z*-axis **(A'-E')**, and isolated from other brain structures **(A''-E'')**. Embryos were injected with 15ng control MO **(A)**, 9ng miR-7 MO **(B)**. In a separate experiment, uninjected control embryos **(C)**, were compared with embryos injected with plasmid expressing linear CDR1as **(D)**, or circular

CDR1as **(E)**. Note: the telencephalon structures (green) appear intact in miR-7 MO and CDR1as treated embryos. Dorsal is up, anterior to the right, posterior to the left. Scale bar:100μm. MB, midbrain; TC, telencephalon.

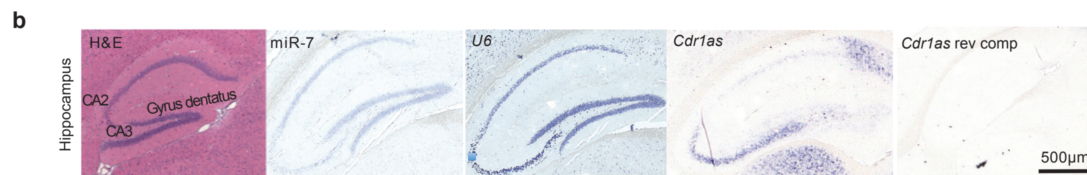


Supplementary Figure 8 circularized CDR1as in injected zebra fish
qPCR analysis of CDR1as transcripts in zebra fish 48hpf after injection of a plasmid coding for linear CDR1as or a plasmid coding for a transcript that can be circularized. PCR was performed using divergent primers, the signal from CDR1as linear injected fish is at background level. Data are normalized to β-actin (error:stdev).

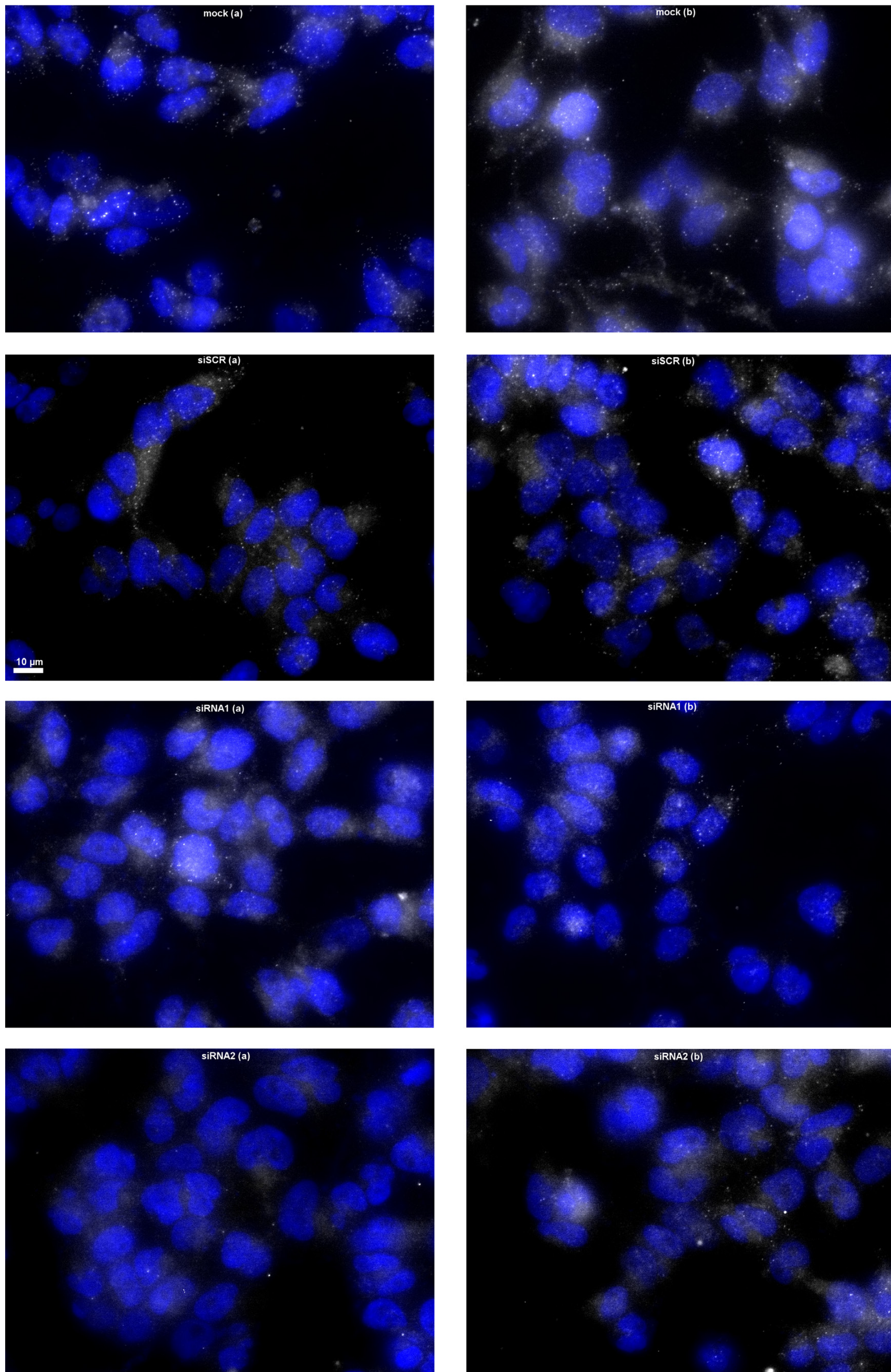
a



b



Supplementary Figure 9 miR-122 expression across mouse tissues and MIN6 cells, *Cdr1as* in mouse brain
a, miR-122 expression in 14 mouse tissues and MIN6 cells. As expected, miR-122 is highly and specifically expressed in liver. **b,** In situ hybridizations (ISH) were performed on paraffin sections showing *Cdr1as* expression in brains of adult C57Bl6/129Sv mice. A probe specific for the circular *Cdr1as* reverse complement serves as negative control. A histological staining (H&E) depicts the highly nucleated structures of the hippocampus (the areas of within the hippocampus CA2, CA3 neuronal layer and the Gyrus dentatus are labeled). ISH using double Digoxigenin labeled LNA probes detects miR-7 expression specifically in the hippocampus but not in the surrounding tissues. A U6 specific probe serves as positive control. The *Cdr1as* expression is most pronounced in the CA2 and CA3 area of the hippocampus whereas a scrambled LNA serves as negative control and yields no signal.



Supplementary Figure10 smRNA FISH of CDR1as in HEK293

species	#miR-7 seed matches	length[nt]	6mer	7mer_1	7mer_2	8mer_1	8mer_2	9mer_1	11mer_1	other matches
human	74	1529	1	6	7	52	1	7		
gorilla	72	1511	1	6	7	51		7		
rhesus macaque	76	1582	1	6	11	51		7		
marmoset	62	1311		2	7	47		6		
mouse	139	2954	9	7	16	26	73	8		
rat	36	814	1	3	4	24		4		
naked mole-rat	52	1145		6	6	33		7		
guinea pig	22	744		3	6	11		2		
rabbit	74	1572	2	6	11	44	1	10		
pig	83	1652		1	15	58		9		
cow	34	893		1	3	27		1	2	
horse	61	1309		3	8	43		7		
dog	65	1356	1	1	13	42		7	1	
elephant	54	1371	1	12	6	32		3		
lamprey	38	1177		19	1	18				

Supplementary Table 4 miR-7 seed matches in vertebrates.
We obtained sequences homologous to the human CDR1as sequence using BLAT and the UCSC Genome browser. If the reverse BLAT hit human CDR1as, we kept the sequence for further analysis. We counted miR-7 seed matches independently in the species used for our multiple species alignment.

smFISH CDR1as knock down control

	CDR1as relative expression	stdev
siSCR(control)	1.00	0.18
siRNA1	0.34	0.14
siRNA2	0.34	0.06

Supplementary Table5 qPCR quantification of CDR1as in smFISH samples.
CDR1as expression was quantified by qPCR from the samples used for single molecule fluorescent *in situ* hybridization.

CDR1as expression
in stable knock down cell lines

	CDR1as relative expression	stdev
shCtrl	1.00	0.21
shRNA1	0.47	0.06
shRNA2	0.34	0.07

Supplementary Table7 qPCR quantification of CDR1as in shCDR1as knock down cells.
CDR1as expression was quantified by qPCR from cell lines stably expressing shCDR1as shown in Supplementary Fig 5e.