Circular RNAs are large class of animal RNAs with regulatory potency

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Circular RNAs (circRNAs) in animals are an enigmatic class of RNA with unknown function. To explore circRNAs systematically, we sequenced and computationally analysed human, mouse and nematode RNA. We detected thousands of well-expressed, stable circRNAs, often showing tissue/developmental-stage-specific expression. Sequence analysis indicated important regulatory functions for circRNAs. We found that a human circRNA, antisense to the cerebellar degeneration-related protein 1 transcript (CDR1as), is densely bound by microRNA (miRNA) effector complexes and harbours 63 conserved binding sites for the ancient miRNA miR-7. Further analyses indicated that CDR1as functions to bind miR-7 in neuronal tissues. Human CDR1as expression in zebrafish impaired midbrain development, similar to knocking down miR-7, suggesting that CDR1as is a miRNA antagonist with a miRNA-binding capacity ten times higher than any other known transcript. Together, our data provide evidence that circRNAs form a large class of post-transcriptional regulators. Numerous circRNAs form by head-to-tail splicing of exons, suggesting previously unrecognized regulatory potential of coding sequences.

Mature messenger RNAs are linear molecules with 5′ and 3′ termini that reflect start and stop of the RNA polymerase on the DNA template. In cells, different RNA molecules are sometimes joined together by splicing reactions (trans-splicing), but covalent linkage of the ends of a single RNA molecule to form a circular RNA (circRNA) is usually considered a rare event. circRNAs were discovered in plants and shown to encode subviral agents1. In unicellular organisms, circRNAs mostly stem from self-splicing introns of pre-ribosomal RNA2, but can also arise from protein-coding genes in archaea3. In the few unambiguously validated circRNAs in animals, the spliceosome seems to link the 5′ and downstream 3′ ends of exons within the same transcript4–10. Perhaps the best known circRNA is antisense to the mRNA transcribed from the SRY (sex-determining region Y) locus and is highly expressed in testses6. Evidence from computational analyses of expression data in Archaea and Mammalia suggests that circRNAs are more prevalent than previously thought11–13; however, it is unknown whether animal circRNAs have any biological function.

In comparison to circRNAs, miRNAs are extremely well studied. miRNAs are ~21-nucleotide-long non-coding RNAs that guide the effector protein Argonaute (AGO) to miRNAs of coding genes to repress their protein production11–14. In humans, miRNAs directly regulate expression of most mRNAs15–18 in a diverse range of biological functions. However, surprisingly little is known about how and if mRNAs can escape regulation by a miRNA. A recently discovered mechanism for miRNA removal in a sequence-specific manner is based on target sites acting as decoys or miRNA sponges19,20. RNA with miRNA binding sites should, if expressed highly enough, sequester away the miRNA from its target sites. However, all reported mammalian miRNA sponges have only one or two binding sites for the same miRNA and are not highly expressed, limiting their potency19,20.

To identify circRNAs across animal cells systematically, we screened RNA-seq data for circRNAs. Compared to previous approaches16–18 our computational pipeline can find circRNAs in any genomic region, takes advantage of long (~100 nucleotides) reads, and predicts the acceptor and donor splice sites used to link the ends of the RNAs. We do not rely on paired-end sequencing data or known splice sites. Using published10,25,26 and our own sequencing data, our method reported thousands of circRNAs in human and mouse tissues as well as in different developmental stages of Caenorhabditis elegans. Numerous circRNAs appear to be specifically expressed across tissues or developmental stages. We validated these data and showed that most tested circRNAs are well expressed, stable and circualized using the predicted splice sites. circRNA sequences were significantly enriched in conserved nucleotides, indicating that circRNAs compete with other RNAs for binding by RNA binding proteins (RBPs) or miRNAs. We combined biochemical, functional and computational analyses to show that indeed a known human circRNA, CDR1 antisense (CDR1as)9, can function as a negative regulator of miR-7, a miRNA with perfect sequence conservation from annelids to humans. Together, our data provide evidence that circRNAs form an important class of post-transcriptional regulators.

circRNAs have complex expression patterns

To comprehensively identify stably expressed circRNAs in animals we screened RNA sequencing reads for splice junctions formed by an acceptor splice site at the 5′ end of an exon and a donor site at a downstream 3′ end (head-to-tail) (Fig. 1a). As standard RNA expression profiling enriches for polyadenylated RNAs, we used data generated after ribosomal RNA depletion (ribominus) and random priming. Such data were used before to detect scrambled exons in mammals10 (see Methods for comparison). However, this approach was not specifically designed to detect circRNAs and (1) only used existing exon-intron annotations, thus missing RNAs transcribed from introns or unannotated transcripts; (2) did not explicitly identify

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from at least two independent junction-spanning reads (Fig. 1b). The expression of genes predicted to give rise to circRNAs was only slightly shifted towards higher expression values (Supplementary Fig. 1d), indicating that circRNAs are not just rare mistakes of the spliceosome. We also identified 1,903 circRNAs in mouse (brains, fetal head, differentiation-induced embryonic stem cells; Supplementary Fig. 1e)\(^{15,20}\); 81 of these mapped to human circRNAs (Supplementary Fig. 1f). To explore whether circRNAs exist in other animal clades, we used sequencing data that we produced from various C. elegans developmental stages (Stoeckius, M. et al., manuscript in preparation) (Methods) and detected 724 circRNAs, with at least two independent reads (Fig. 1c).

Numerous circRNAs seem to be specifically expressed in a cell type or developmental stage (Fig. 1b, c and Supplementary Fig. 1e). For example, hsa-circRNA 2149 is supported by 13 unique, head-to-tail spanning reads in CD19\(^+\) leukocytes but is not detected in CD34\(^+\) leukocytes (which were sequenced at comparable depth; Supplementary Table 1), neutrophils or HEK293 cells. Analogously, a number of nematode circRNAs seem to be expressed in oocytes but absent in 1- or 2-cell embryos.

We annotated human circRNAs using the ReFeSe database and a catalogue of non-coding RNAs\(^{27–29}\). 85% of human circRNAs align sense to known genes. Their splice sites typically span one to five exons (Supplementary Fig. 1g) and overlap coding exons (84%), but only in 65% of these cases are both splice sites that participate in the circularization known splice sites (Supplementary Table 2), demonstrating the advantage of our strategy. 10% of all circRNAs align antisense to known transcripts, smaller fractions align to UTRs, introns, unannotated regions of the genome (Fig. 1d). Examples of human circRNAs are shown in Fig. 1e.

We analysed sequence conservation within circRNAs. As genomic sequence is subject to different degrees of evolutionary selection, depending on function, we studied three subtypes of circRNAs. Intergenic and a few intronic circRNAs display a mild but significant enrichment of conserved nucleotides (Supplementary Fig. 1h, i). To analyse circRNAs composed of coding sequence and thus high overall conservation, we selected 223 human circRNAs with circularization known splice sites (Supplementary Table 2), demonstrating the advantage of our strategy. 10% of all circRNAs align antisense to known transcripts, smaller fractions align to UTRs, introns, unannotated regions of the genome (Fig. 1d). Examples of human circRNAs are shown in Fig. 1e.

Characterization of 50 predicted circRNAs

We experimentally tested our circRNA predictions in HEK293 cells. Head-to-tail splicing was assayed by quantitative polymerase chain reaction (qPCR) after reverse transcription, with divergent primers and Sanger sequencing (Fig. 2a, b). Predicted head-to-tail junctions of 19 out of 23 randomly chosen circRNAs (83%) could be validated, demonstrating high accuracy of our predictions (Table 1). In contrast, 5 out of 7 (71%) candidates exclusively predicted in leukocytes could not be detected in HEK293 cells, validating cell-type-specific expression.

Head-to-tail splicing could be produced by trans-splicing or genomic rearrangements. To rule out these possibilities as well as potential PCR artefacts, we successfully validated the insensitivity of human circRNA candidates to digestion with RNase R—an exonuclease that degrades linear RNA molecules\(^{25}\)—by northern blotting with probes which span the head-to-tail junctions (Fig. 2c). We quantified RNase R resistance for 21 candidates with confirmed head-to-tail splicing by
qPCR. All of these were at least 10-fold more resistant than GAPDH (Fig. 2d and Supplementary Fig. 2a). We reasoned that circRNAs should generally turn over more slowly than mRNAs. Indeed, we tested mouse circRNAs with human orthologues in mouse brains (Supplementary Fig. 2d and Supplementary Table 3). In C. elegans (Fig. 2d and Supplementary Fig. 2a), we discovered that the known human circRNA CDR1as harbours 74 miR-7 seed matches of which 63 are significantly enriched compared to coding sequences ($P < 2.96 \times 10^{-22}$, Mann–Whitney U-test, $n = 3,873$) or 3’ UTR sequences ($P < 2.76 \times 10^{-23}$, Mann–Whitney U-test, $n = 3,182$) (Supplementary Fig. 3a, b).

As an extreme case, we discovered that the known human circRNA CDR1as (ref. 9) harboured dozens of conserved miR-7 seed matches. To test whether CDR1as is bound by miRNAs, we analysed biochemical, transcriptome-wide binding-site data for the miRNA effector AGO proteins. We performed four independent PAR-CLIP (photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation) experiments for human AGO (Methods) and analysed them together with published, lower-depth data15. PAR-CLIP12–14 is based on ultraviolet crosslinking of RNA to protein and subsequent sequencing of RNA bound to a RBP of interest. The ~1.5-kilobase (kb) CDR1as locus stood out in density and number of AGO PAR-CLIP reads (Fig. 3a), whereas nine combined PAR-CLIP libraries for other RBPs gave virtually no signal. Of note, there is no PAR-CLIP read mapping to the sense coding transcript of the CDR1 gene, which was originally identified as a target of autoantibodies from patients with paraneoplastic cerebellar degeneration15.

Sequence analysis across 32 vertebrate species revealed that miR-7 is the only animal miRNA with conserved seed matches that can explain the AGO binding along the CDR1as transcript (Methods). Human CDR1as harbours 74 miR-7 seed matches of which 63 are 10-fold more resistant than GAPDH.

Figure 2 | CircRNAs are stable transcripts with robust expression. a, Human (hsa) ZRANB1 circRNA exemplifies the validation strategy. Convergent (divergent) primers detect total (circular) RNAs. Sanger sequencing confirms head-to-tail splicing. b, Divergent primers amplify circRNAs in cDNA but not genomic DNA (gDNA). GAPDH, linear control, size marker in base pairs. c, Northern blots of mock (−) and RNase R (+) treated HEK293 total RNA with head-to-tail specific probes for circRNAs. GAPDH, linear control. d, e, circRNAs are at least 10-fold more resistant than GAPDH mRNAs (d) and stable after 24 h transcription block (e) (qPCR; error bars indicate standard deviation).

Table 1 | Summary of the validation experiments

<table>
<thead>
<tr>
<th>Sample</th>
<th>Validation experiment</th>
<th>Validation success</th>
</tr>
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<tbody>
<tr>
<td>Human (HEK293)</td>
<td>Head-to-tail splicing</td>
<td>19 of 23</td>
</tr>
<tr>
<td></td>
<td>Circularity</td>
<td>21 of 21</td>
</tr>
<tr>
<td></td>
<td>Expression &gt; 3%</td>
<td>12 of 21</td>
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<tr>
<td></td>
<td>Expression specificity</td>
<td>5 of 7</td>
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<tr>
<td></td>
<td>(leukocyte specific)</td>
<td></td>
</tr>
<tr>
<td>Mouse (adult brain)</td>
<td>Head-to-tail splicing</td>
<td>3 of 3</td>
</tr>
<tr>
<td></td>
<td>Circularity</td>
<td>3 of 3</td>
</tr>
<tr>
<td></td>
<td>Expression &gt; 1%</td>
<td>2 of 3</td>
</tr>
<tr>
<td>C. elegans</td>
<td>Head-to-tail splicing</td>
<td>15 of 20</td>
</tr>
<tr>
<td></td>
<td>Circularity</td>
<td>13 of 13</td>
</tr>
<tr>
<td></td>
<td>Expression &gt; 1%</td>
<td>12 of 15</td>
</tr>
</tbody>
</table>

Most experimentally tested circRNAs are validated.

Figure 3 | The circRNA CDR1as is bound by the miRNA effector protein AGO, and is cytoplasmic. a, CDR1as is densely bound by AGO (red) but not by unrelated proteins (black). Blue boxes indicate miR-7 seed matches. b, c, miR-7 sites display reduced nucleotide variability across 32 vertebrate genomes (b) and high base-pairing probability within seed matches (c). d, CDR1as RNA is cytoplasmic and disperse (white spots; single-molecule RNA FISH; maximum intensity merges of Z-stacks). siSCR, positive; siRNA1, negative control. Blue, nuclei (DAPI); scale bar, 5 μm (see also Supplementary Fig. 10 for uncropped images). e, Northern blotting detects circular but not linear CDR1as in HEK293 RNA. Total, HEK293 RNA; circular, head-to-tail probe; circ+lin, probe within splice sites; IVT lin., in vitro transcribed, linear CDR1as RNA. f, Circular CDR1as is highly expressed (qPCR, error bars indicate standard deviation). g, CDR1as. Blue, seed matches; dark red, AGO PAR-CLIP reads; bright red, crosslinked nucleotide conversions.
conserved in at least one other species (Supplementary Fig. 4). Interspaced sequences were less conserved, indicating that miR-7 binding sites are probably functional (Fig. 3b). Secondary structure analysis of predicted circRNA–miRNA duplexes (Methods) showed reduced base-pairing of miR-7 beyond the seed (Fig. 3c). None of the ~1,500 miR-7 complementary sites across 32 vertebrate sequences was complementary beyond position 12 of miR-7 (only three could form an 11-nucleotide duplex) (Supplementary Table 4). Slicing by mammalian Argonaute requires complementarity of positions 10 and 11 and depends on extended complementarity beyond position 12 (ref. 36). Thus, CDR1as seems optimized to be densely bound but not sliced by miR-7.

Single-molecule imaging (Methods) revealed disperse and mostly cytoplasmic CDR1as expression (HEK293 cells), consistent with miRNA sponge function (Fig. 3d and Supplementary Table 5). CDR1as circularization was assayed by northern blotting (Fig. 3e). Nicking experiments confirmed that CDR1as circRNA can be linearized and degraded (Supplementary Fig. 5a). In RNA from HEK293 cells, circularized but no additional linear CDR1as was detected (Supplementary Fig. 5b). Circular expression levels were quantified by qPCR with divergent primers calibrated by standard curves (Supplementary Table 6). CDR1as was highly expressed (~15% to ~20% of GAPDH expression, Fig. 3f). Estimating GAPDH mRNA copy number from HEK293 RNA-seq data (~1,400 molecules per cell, data not shown) suggests that CDR1as may bind up to ~20,000 miR-7 molecules per cell (Fig. 3g).

If CDR1as functions as a miR-7 sponge, its destruction could trigger downregulation of miR-7 targets. We knocked down CDR1as in HEK293 cells and monitored expression of published miR-7 targets by qPCR with externally spiked-in standards (Methods and Supplementary Fig. 5c, d). All eight miR-7 targets assayed, but also housekeeping genes, were downregulated. Nanostring technology additionally indicated downregulation of many genes (data not shown). Furthermore, stable loss of CDR1as expression by virally delivered small hairpin RNAs led to significantly reduced migration in an in vitro wound closure assay (Methods, Supplementary Fig. 5e, f and Supplementary Table 7). Thus, knockdown of CDR1as affects HEK293 cells, but we could not delineate miR-7-specific effects, potentially because of indirect or miR-7-independent CDR1as function (see below).

**Co-expression of miR-7 and CDR1as in brain**

If CDR1as indeed interacts with miR-7, both must be co-expressed. miR-7 is highly expressed in neuronal tissues, pancreas and pituitary gland. Apart from HEK293 cells, a cell line probably derived from neuronal precursors in embryonic kidney, we quantified miR-7 and CDR1as expression across mouse tissues and pancreatic-island-derived MIN6 cells (Methods and Fig. 4a). CDR1as and miR-7 were both highly expressed in brain tissues, but CDR1as was expressed at low levels or absent in non-neuronal tissues, including tissues with very high miR-7 expression. qPCR suggested that CDR1as is exclusively and abundantly expressed in brain tissues (Supplementary Fig. 5g, h). Thus, CDR1as and miR-7 seem to interact specifically in neuronal tissues. Indeed, when assaying CDR1as and miR-7 in mouse brains by in situ hybridizations (Methods), we observed specific, similar, but not identical, expression patterns in the brain of mid-gestation (embryonic day 13.5 (E13.5) embryos) (Fig. 4b). Specifically, CDR1as and miR-7 were highly co-expressed in areas of the developing midbrain (mesencephalon) 40,41. Thus, CDR1as is highly expressed, stable, cytoplasmic, not detectable as a linear RNA and shares expression domains with miR-7. Together with extensive miR-7 binding within CDR1as, CDR1as has hallmarks of a potent circular miR-7 sponge in neuronal tissues.

**Effects of miR-7 and CDR1as in zebrafish**

It would be informative to knock out CDR1as in an animal model system. However, a knockout would also affect CDR1 protein, with unknown consequences. This problem is circumvented when using zebrafish (Danio rerio) as an animal model. According to our bioinformatic analyses (not shown) zebrafish has lost the cdr1 locus, whereas miR-7 is conserved and highly expressed in the embryonic brain 42. Thus, we can test whether miR-7 has a loss-of-function phenotype and if this phenotype can be induced by introduction of mammalian CDR1as. We injected morpholinos to knock down mature miR-7 expression in zebrafish embryos (Methods). At a dose of 9 ng of miR-7 morpholino, the embryos did not show overall morphological defects but reproducibly, and in two independent genetic backgrounds (Supplementary Fig. 6a–c), developed brain defects (Fig. 5a, b). In particular, ~70% showed a consistent and clear reduction in midbrain size, and an additional ~5% of animals had almost completely lost their midbrains. Of note, the telencephalon at the anterior tip of the brain was not affected in size. Brain volumes were also measured based on confocal three-dimensional stacks (Fig. 5c and Supplementary Fig. 7). Reduction of the midbrain size correlated with miR-7 inhibition in the respective animals (Supplementary Fig. 6d). These data provide evidence that miR-7 loss-of-function causes a specific reduction of midbrain size.

To test whether CDR1as can function as a miR-7 sponge in vivo, we injected embryos with plasmid DNA that expressed a linear version of the full-length human CDR1as sequence (Supplementary Fig. 6e, f) or a plasmid provided by the Kjems laboratory that can produce circular CDR1as in human cells (Fig. 5d, e). qPCR analysis detected circular RNA in zebrafish embryos injected with the latter plasmid (Supplementary Fig. 8), which reproducibly and in independent genetic backgrounds lead to reduced midbrain sizes (Fig. 5g, h). Similarly, animals injected with *in vitro*-transcribed partial mouse CDR1as RNA, but not with RNA from the other strand, showed significant midbrain reduction (Supplementary Fig. 6g–i). Thus, the phenotype is probably caused by CDR1as RNA and not by an unspecified effect of RNA or DNA injection. These results provide evidence that human/mouse CDR1as transcripts are biologically active in vivo and impair brain development similarly to miR-7 inhibition. The midbrain reduction could be partially rescued by injecting miR-7 precursor (Fig. 5f, g), arguing that the biological effect of CDR1as expression is caused at least in part by interaction of CDR1as with miR-7.

**Discussion**

We have shown that animal genomes express thousands of circRNAs from diverse genomic locations (for example, from coding and non-coding exons, intergenic regions or transcripts antisense to 5' and 3' UTRs) in a complex tissue-, cell-type- or developmental-stage-specific manner. We provided evidence that CDR1as can act as a...
post-transcriptional regulator by binding miR-7 in brain tissues: (1) CDR1as is densely bound by miRNA effector molecules; (2) CDR1as harbours 74 miR-7 seed matches, often deeply conserved; (3) CDR1as is expressed highly, stably and mostly cytoplasmic; (4) CDR1as and miR-7 share specific expression domains in mouse embryonic brain; (5) human/mouse CDR1as is circularized in vivo and is not detectable as a linear molecule; (6) human/mouse CDR1as sequences, when injected into zebrafish, and miR-7 knock down have similar phenotypes in brain. While zebrafish circularization of human CDR1as may be incomplete, the midbrain phenotype was stronger compared to expressing linear CDR1as RNA that lacks circularization splice sites. Although the two DNA plasmids used carry identical promoters and expressing linear CDR1as RNA that lacks circularization splice sites.

The phenotype induced by CDR1as expression in zebrafish was only partially rescued by expressing miR-7, indicating that CDR1as could have functions beyond sequestering miR-7. This idea is supported by in situ hybridization in mouse adult hippocampus (Supplementary Fig. 9b) where areas staining for CDR1as but not miR-7 were observed. What could be additional functions of circRNAs beyond acting as sponges? As a single-stranded RNA, CDR1as could, for example, bind in trans 3’ UTRs of target miRNAs to regulate their expression. It is even possible that miR-7 binds CDR1as to silence these trans-acting activities. Alternatively, CDR1as could be involved in the assembly of larger complexes of RNA or protein, perhaps similar to other low-complexity molecules.

How many other circRNAs exist? In this study, we identified approximately 2,000 human, 1,900 mouse and 700 nematode circRNAs from sequencing data, and our validation experiments confirmed most of the 50 tested circRNAs. However, we analysed only a few tissues/developmental stages with stringent cutoffs. Thus, the true number of circRNAs is almost certainly much larger. Although CDR1as is an extreme case, many circRNAs have conserved seed matches. For example, circRNA from the Sry locus has seed sites for murine miRNAs. Therefore, circRNAs probably compete with other RNAs for miRNA binding. Sequence analyses indicated that coding exons serve additional, presumably regulatory functions when expressed within circRNAs, whereas intergenic or intronic circRNAs generally showed only weak conservation. Because we detected thousands of circRNAs, it is appealing to speculate that occasional circularization of exons is easy to evolve and may provide a mechanism for rapid evolution of stably and well expressed regulatory RNAs. Of note, we detected multiple seed matches for viral miRNAs within human circRNAs (not shown). However, there is no reason to think that circRNAs function predominantly to bind miRNAs. As known in bacteria, the decoy mechanism underlying miRNA sponges could be important also for RBPs. Similarly, circRNAs could function to store, sort, or localize RBPs. In summary, our data suggest that circRNAs form a class of post-transcriptional regulators which compete with other RNAs for binding by miRNAs and RBPs and may generally function in modulating the local free concentration of RBPs, RNAs, or their binding sites.

Note added in proof: While this paper was under review, circular RNAs in fibroblasts were described.

**METHODS SUMMARY**

**Computational pipeline for predicting circRNAs from ribonuclues sequencing data.** A detailed description of the computational methods is given in the Methods.

**Cell culture and treatments.** HEK293, HEK293TN and HEK293 Flp-in 293 T-REx (Life Technologies) were cultured following standard protocols. Transcription was blocked by adding 2 μg/ml actinomycin D (Sigma). RNase R (Epigenetix Biotechnologies) treatment (0.5 μg/ml) was performed on total RNA (5 μg) at 37 °C for 15 min. qPCR primers are listed in Supplementary Table 8.

**Single-molecule RNA fluorescence in situ hybridization (smRNA FISH).** Stellaris Oligonucleotide probes complementary to CDR1as were designed using the Stellaris Probe Designer (Biosearch Technologies). Probe pools were obtained from BioCat GmbH as conjugates coupled to Quasar 670. Probes were hybridized at 125 nM at 37 °C. Images were acquired on an inverted Nikon Ti microscope.

**Mouse strains and in situ hybridization.** In situ hybridization (ISH) was performed on paraffin tissue sections from B6129SF1/J wild-type mice as described using locked nucleic acid (LNA) probes or RNAs obtained by in vitro transcription on PCR products.

**Zebrafish methods.** Tg(huc:egfp) and Tg(Xia:ThubMedRED) transgenic zebrafish lines were used. Morpholino antisense oligomers were injected into the yolk of single-cell-stage embryos. Furthermore, two pCS2+ plasmids coding for full-length linear CDR1as or CDR1as plus upstream and downstream sequence that can express circular CDR1as in human cells (courtesy of the Kjems laboratory)
were injected. Confocal imaging was performed using Carl Zeiss MicroImaging. Reduced midbrain development was defined as >50% smaller than the mean size of controls. Each experimental group was evaluated in at least three independent experiments; a minimum of 80 individual embryos per group was examined.

Full Methods and any associated references are available in the online version of the paper.

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Supplementary Information is available in the online version of the paper.
METHODS

Computational pipeline for predicting circRNAs from riboninus sequencing data. Reference genomes (human hg19 [February 2009, GRCh37], mouse mm9 [July 2007, NB137/mm9], C. elegans c6 [May 2008, WormBase v. WS190]) were downloaded from the UCSC genome browser (http://genome.ucsc.edu)\(^2\). In a first step, reads that aligned contiguously and full length to the genomes were discarded. From the remaining reads we extracted 20mers from both ends and aligned them independently to find unique anchor positions within spliced exons. Anchors that aligned in the reversed orientation (head-to-tail) indicated circRNA splicing (compare main Fig. 1a). We extended the anchor alignments such that ambiguous breakpoints were discarded. We used the short-read mapper Bowtie 2 (ref. 52). Initially, ribominus reads were aligned in end-to-end mode to the genome: Bowtie2-p16 -very-sensitive --phred64 --mm -M20 -score-min=-C,-15,0 -q -x <index> -U reads.qfa 2>> bowtie2.1og | samtools view -hbu5 - | samtools sort -sample_vs_genome.gz

The unmapped reads were separated and run through a custom script to split the reads as indicated in Fig. 1a to obtain 20-nucleotide anchors from both ends of the read:

\[
\text{s samtools view -lh sample_vs_genome.bam | samtools view -Sb - > unmapped_sample.bam}
\]

Here is an example of two anchor pairs in the FASTQ format; the original read was kept as part of the first anchors identifier to simplify downstream analysis:

\[
\text{@0_8_1_0001_qseq_14_A_NCCCCGCCTCACCAGGGTCAGTGAGAAAAGCA}
\text{TCAGATGAGGGTCTCTCCGCGGGCCGCCTCGC}
\text{NCGCCCTTCACCAGGTCTAGT}
\text{+}
\text{#BB@8AB8;} = @B@B8058 (}
\text{@s_8_1_0001_qseq_14_B}
\text{CCCAGGGCCCGCCGGCTCGC}
\text{+}
\text{.;.;(>)>0; 8888888}
\]

Next the anchors were aligned individually to the reference, keeping their paired ordering. The resulting alignments were read by another custom script that jointly evaluates compatible anchor alignments belonging to the same original read, performs extensions of the anchor alignments, and collects statistics on splice sites. After the run completes, the script outputs all detected splice junctions (linear and circular) in a UCSC BED-like format with extra columns holding quality statistics, read counts etc. The original full-length reads that support each junction are written to stderr:

\[
\text{s bowtie2-p16 -reorder --mm -M20 -score-min=-C,-15,0 -q -x sample_vs_genome -U sample_anchors.qfa.gz} \text{/find circ.pyc -S hg19-p sample_ -s sample/sites.log > sample/sites.bed} 2>> sample/sites/reads
\]

The resulting BED-like file is readily filtered for minimal quality cutoffs to produce the reported circRNA candidates. In particular, we demanded the following: (1) GU/AG flank the splice sites (built in); (2) unambiguous breakpoint detection; (3) a maximum of two mismatches in the extension procedure; (4) the breakpoint cannot reside more than 2 nucleotides inside an anchor; (5) at least two independent reads (each distinct sequence only counted once per sample) support the junction; (6) unique anchor alignments with a safety margin to the next-best alignment of at least one anchor above 35 points (~more than two extra mismatches in high-quality bases); and (7) a genomic distance between the two splice sites of no more than 100 kb (only a small percentage of the data). As the ribosomal DNA cluster is part of the C. elegans genome assembly (c6) and ribosomal pre-RNAs could give rise to circular RNAs by mechanisms independent of the spliceosome, we discarded 130 candidates that mapped to the rDNA cluster on chr1:15,060,286-15,071,020.

Permutation testing. To test the robustness of the circRNA detection pipeline we altered the sequence of real sequencing reads in different ways at the step of anchor generation. We (1) reversed either anchor; (2) reversed the complete read; (3) randomly reassigned anchors between reads; or (4) reverse complemented the read (as a positive control). Although the reverse complement recovered the same output as expected, the various permutations led to only very few candidate predictions, well below 0.2% of the output with unpermuted reads and in excellent agreement with the results from simulated reads (Supplementary Fig. 1c).

HEK293 RNA seq after rRNA depletion (RibonimusSeq). Total HEK293 RNA was isolated using Trizol as recommended by the manufacturer. Ribosomal RNA was depleted from total RNA using the Ribonimus kit (Invitrogen). A CDNA library was generated by rRNA-depleted RNA according to the Illumina RNA-seq protocol. The cDNA library was sequenced on an Illumina GAIIx by a 2 x 76 bp run.

C. elegans oocyte isolation. Oocytes were isolated from worms carrying a temperature-sensitive (TS) allele for fem-1 (unovulated oocytes BA171(fem-1[1ch17s])) strain) and spe-9 (partially ovulated oocytes BA671(spe-9[db88ts]) as described previously\(^3\). Oocytes were washed at least four times in PBS containing protease inhibitors (Sigma-Aldrich) to separate from worm debris. Oocyte purity was observed under the dissection scope (Zeiss). Oocytes were extracted from young adults to enrich for non-endomitotic oocytes, which was also checked by fluorescence microscopy (Zeiss) with a nuclear dye. Oocytes isolated from fem-1 or spe-9 mutant background worms are hereafter referred to as fem-1 oocytes and spe-9 oocytes, respectively.

C. elegans sperm isolation. Sperm was isolated in principle as described previously\(^4\) from male worms obtained from a fog-2(q71) mutant background. Males were cut in cold PBS containing protease inhibitors (Sigma-Aldrich). Sperm was subsequently purified by filtration (3 x 40 μm nylon mesh, 2 x 10 μm nylon mesh) and a series of differential centrifugations (30 min 300 g, 10 min 450 g) and washed twice in cold PBS. Sperm was subsequently activated by incubation in PBS containing 200 μg/ml \(^{-1}\) Pronase (Sigma-Aldrich) for 30 min at 25°C. Sperm purity is around 70% spermatids and spermatozoa contaminated with around 30% primary and secondary spermatocytes, as observed under oil immersion microscope.

Riboninus RNA preparation from C. elegans samples. We used a kit that was developed for human and mouse samples, but still performs sufficiently to enrich mRNAs up to 30% in C. elegans. Most of the remaining reads mapped to ribosomal RNAs. 1 μg of total RNA per sample was depleted from RNAs with the Riboninus Transcriptome kit (Invitrogen) according to the manufacturer’s instructions involving the modification that annealing of LNA probes to total RNA was performed in a thermocycler (Eppendorf) with a temperature decrease from 70 to 37°C at a rate of 1°C per min. Depletion of rRNAs was validated by capillary gel electrophoresis on a Bioanalyzer (Agilent). The riboninus RNA was then processed for sequencing library preparation according to the Illumina protocol.

Clustering generation and sequencing of C. elegans libraries. Cluster generation as well as sequencing of the prepared libraries was performed on the Illumina cluster station (Illumina) and sequenced on the HiSeq2000 according to the manufacturer’s protocols (Illumina).

Human gene models. We obtained gene models for RefSeq transcripts (12 December 2011), non-coding RNAs\(^5\), and the rnaGene and rRNA tracks from the UCSC table browser (23 April 2012)\(^7\).

Intersection of circRNAs with known transcripts. Our computational screen identifies only the splice sites that lead to circularization but not the internal exon/intron structure of circular RNAs. To perform analyses of the sequence content of circRNAs we therefore inferred as much as possible from annotated transcripts. Intron structure of circular RNAs. To perform analyses of the sequence content of circular RNAs we therefore inferred as much as possible from annotated transcripts. Intron structure of circular RNAs. To perform analyses of the sequence content of circular RNAs we therefore inferred as much as possible from annotated transcripts.
into an intron or beyond transcript boundaries, the closest exon was extended to match the circRNA boundaries. circRNA start/end coordinates were never altered. If no annotated exons overlapped the circRNA we assumed a single-exon circRNA. The resulting annotation of circRNAs is based on the best matching transcript and may in some cases not represent the ideal choice. Changing the annotation rules, however, did not substantially change the numbers in Fig. 1d.

Finding circRNAs conserved between human and mouse. We reasoned that when comparing two species, the cutoff of two independent reads in each of them could be determined, as orthologous circRNAs would automatically be supported by two independently produced reads via the intersection. We therefore mapped all mouse circRNA candidates with less stringent filtering to human genome coordinates using the UCSC liftOver tool37. The mapped mouse circRNAs were compared with independently identified human circRNAs, yielding 229 circRNAs with precisely orthologous splice sites between human and mouse. Of these, 232 were composed exclusively of coding exons and were subsequently used for our conservation analysis (Fig. 1c). When intersecting the reported sets of circRNAs supported by two independent reads in each species, we found 81 conserved circRNAs (supported by at least 4 reads in total).

Conserved element counting. We downloaded genome-wide human (hg19) phyloP conservation score tracks derived from genome alignments of placental mammals from UCSC37. We interrogated the genome-wide profile inside circRNAs in two different ways. (1) Intergenic and intronic circRNAs. We read out the conservation scores along the complete circRNA and searched for blocks of at least 6-nucleotide length that exceeded a conservation score of 0.3 for intergenic and 0.5 for intronic circRNAs. The different cutoffs empirically adjust for the different background levels of conservation and were also used on the respective controls. For each circRNA, we computed the cumulative length of all such blocks and normalized it by the genomic length of the circRNA. Artefacts of constant positive conservation scores in the phyloP profile, apparently caused by missing alignment data, were removed with an entropy filter (this did not qualitatively affect the results). circRNAs annotated as intronic by the best-match procedure explained above that had any overlap with exons in alternative transcripts on either strand (five cases) were removed from the analysis. The resulting distributions are shown in Supplementary Fig. 1b, i. (2) Coding exon circRNAs. We used the best-match strategy outlined above to construct an estimated ‘exon-chain’ for the circRNAs that overlapped exclusively coding sequence. Using this chain we in silico ‘spliced’ out the corresponding blocks of the conservation score profile. We kept track of the frame and sorted the conservation scores into separate bins for each of the mature miRNA families. A putative target site of a miRNA is a sequence of at least 6-nucleotide length that exceeded a conservation score of 0.3 for circRNAs that overlapped exclusively coding sequence (‘outside’ the circRNA) as a control. However, we observed that the level of conservation is systematically different between internal parts of the coding sequence and the amino- or carboxy-terminal parts (not shown). We therefore randomly generated chains of internal exons, mimicking the exon-number distribution of real circRNAs, as a control. When analysing the circRNAs conserved between human and mouse, it became furthermore apparent that we also needed to adjust for the higher level of overall conservation. High expression generally correlates with conservation and thus, an expression cutoff was enforced on the transcripts used as controls.

RNA preparation. To this end 5–10% mixed stage worms by two rounds of freeze–thaw lysis in Trizol LS reagent (Invitrogen) according to the manufacturer’s protocol. RNA was extracted from about 7,000 C. elegans human and mouse miRNAs. Fasta files with C. elegans, human and mouse miRNAs were obtained from miRBase release 16 (ref. 62). Only mature miRNAs were disected and tissue samples were collected directly into ice-cold Trizol for RNA preparation. Caenorhabditis elegans RNA was isolated from about 7,000 mixed stage worms by two rounds of freeze–thaw lysis in Trizol LS reagent (Invitrogen) according to the manufacturer’s protocol. RNA was extracted from aqueous phase with phenol:chloroform (Ambion). RNA was precipitated with isopropanol and GlycoBlue (Ambion) overnight at −20 °C for 30 min at −80 °C, respectively. Reverse transcription was performed using M-MLV (Promega) or Superscript III with oligo(dT) primer (all Invitrogen) or random primer (Meta- biosystems). cDNA was synthesized using SYBR-Green Fluorescin (Thermo Scientific, Fermentas) and a StepOnePlus PCR System (Applied Biosystems). Expression data in CDR1as locus9. Additionally, circRNAs from exons of the genes annotated rules, however, did not substantially change the numbers in Fig. 1d. If no annotated exons overlapped the circRNA we assumed a single-exon into an intron or beyond transcript bounds, the closest exon was extended to be used. Mouse expression data were normalized to Actb. miRNA expression levels were assayed using TaqMan microRNA assays (Applied Biosystems) and normalized to sno-234. Expression levels of circRNAs described in this study were measured by qPCR using divergent primers. A list of primer sequences is available in Supplementary Table 8.

PCR amplification and Sanger sequencing. DNA templates were PCR amplified using BioRad Mastercycler and ThermoScientific DreamTag Green PCR Master Mix according to the manufacturer’s protocol. We performed 35 cycles of PCR. PCR products were visualized after electrophoresis in 2% ethidium bromide-stained agarose gel. To confirm the PCR results, the PCR products were purified using Agencourt AMPure XP PCR purification kit. Direct PCR product Sanger sequencing was performed by LGC Genomics Ready2 Run services. Primer P1 was provided for sequencing the product for each candidate.

Primer design. Divergent primers were designed for each candidate (P1, P2) to anneal at the distal ends of its sequence. As negative controls we used divergent primers for GAPDH and ATRT linear transcript in HEK293 cells, and eIF-3.D in C. elegans. As a further negative control for divergent primers, we used genomic DNA extracted through Qiagen DNA Blood & Tissue kit. As positive controls, we used convergent primers for the corresponding linear transcripts or for housekeeping genes (eIF-3.D for C. elegans).

RNA N rape treatment. HEK293 DNase-treated total RNA (5 μg) was incubated 15 min at 37 °C with or without 3 U μg−1 of RNase R (Epicentre Bio-technologies). RNA was subsequently purified by phenol-chloroform extraction, retro-transcribed through Superscript III (Invitrogen) according to the manufacturer’s protocol, and used in qPCR.

RNA nicking assay. For partial alkaline hydrolysis (nicking) 1 μg μl−1 of HEK293 total RNA was incubated in 50 mM NaHCO3, for 2.5 or 5 min at 90 °C or 5 min on ice for controls. After incubation the samples were immediately suspended in denaturing RNA sample buffer and analysed on northern blots.

Northern blotting. Total RNA (10–20 μg) was loaded on a 1.2% agarose gel containing 1% formaldehyde and run for 2–2.5 h in MOPS buffer.

Caenorhabditis elegans Human and mouse miRNAs. Fasta files with C. elegans, human and mouse miRNAs were obtained from miRBase release 16 (ref. 62). Only mature miRNAs were considered for the seed analysis. According to miRBase 16 a mature miRNA is the predominant miRNA between the two species arising from the two arms of the precursor hairpin (information that is not included in more recent versions). The miRNAs were grouped into families that share a common seed (nucleotides 2–7). There are 117,751 and 723 miRNA families for C. elegans, human and mouse, respectively.

Detecting putative miRNA seed matches. The C. elegans, human and mouse multiple species alignments were scanned for putative conserved miRNA target sites for each of the mature miRNA families. A putative target site of a miRNA is a
6-nucleotide-long sequence in the genome that is the reverse complement of nucleotides 2–7 of the mature miRNA sequence. A putative target site is called conserved if it is found in C. elegans, C. briggsae and C. remanei in the first case or in human, mouse, rat, cow and dog in the latter.

AGO PAR-CLIP. Generation and growth conditions of human embryonic kidney (HEK) 293 cells and HEK293 stably expressing Flag-AGO1 and HA/HA–AGO2 were reported previously65. Stably transfected and parental HEK293 cells were labelled with 100 μM 4-thiouridine for 16 h. After labelling, procedures followed the PAR-CLIP protocol as described23. Briefly, ultraviolet-irradiated cells were lysed in NP-40 lysis buffer. Immunoprecipitation was carried out with protein G magnetic beads (Invitrogen) coupled to anti-Flag antibody (Sigma) and to anti-AGO2 antibody48 from extracts of stably transfected and parental HEK293 cells, respectively, for 1 h at 4°C. Beads were treated with calf intestinal phosphatase (NEB) and radioactively end-labelled by T4 polynucleotide kinase (Fermentas). The crosslinked protein–RNA complexes were resolved on 4–12% NuPAGE gel (Invitrogen), and a labelled protein–RNA complex of close to 100kDa was excised. The protein–RNA was isolated by electroelution. RNA was isolated by proteinase K treatment and phenol-chloroform extraction, reverse transcribed and PCR-amplified. The amplified cDNA was sequenced on a GAIIx (Illumina) with 36 cycles.

Human Argonaute PAR-CLIP Analysis. We obtained Argonaute PAR-CLIP reads from ref. 32. We additionally produced 4 PAR-CLIP libraries. In total, we analysed the following PAR-CLIP data sets: AGO1_4su–1 (SRR048973), AGO3_4su–1 (SRR048976) from ref. 32; AGO1_4su–ML_MM–6, AGO2–4su–ML_MM–7, AGO2_4su–ML_MM–8, and AGO2_4su–3_ML–LG (our own data, published under GEO accession GSE43574).

Redundant reads were collapsed (such that each distinct read sequence appears only once), aligned to the human genome (assembly hg19) using bwa 0.6.1-r104 (ref. 65), and analysed by our in-house PAR-CLIP analysis pipeline (Jens, M., et al., unpublished), essentially as described in ref. 33. Briefly, reads uniquely aligning to the genome are grouped into clusters continguously covering the reference, assigning each cluster a number of quality scores (T conversions, number of independent reads, etc.). Clusters with less than 3 reads from 3 of 6 independent AGO PAR-CLIP libraries or lacking T conversions were discarded. Remaining clusters are annotated against a comprehensive list of transcript models (see below) and collected into ‘only sense’, ‘only antisense’ and ‘intergenic/overlapping transcription’ categories based on their annotation. As PAR-CLIP sequencing preserves the directionality of RNA fragments we assume ‘only antisense’ clusters to predominantly represent false positives due to mapping artefacts (PAR-CLIP RNA is mutated and fragments are often short), and choose quality cutoffs for all clusters such that the fraction of kept ‘only antisense’ clusters is reduced to below 5%. Remaining ‘only antisense’ clusters were discarded. For Fig. 3a, uniquely aligning, collapsed reads are shown.

AGO Binding Sites in C. elegans. Sequencing reads from the Zisoulis Alg-1 HITS-Clip data were obtained from http://yeolab.ucsd.edu/yeolab/Papers_files_ALG1_MT_WT_raw.tar.gz (ref. 66). The raw sequencing data of the wild-type Alg-1 HITS-Clip was pre-processed and mapped with the mapper module from miRDeep2 (ref. 74). The pre-processed reads were mapped with bowtie version 0.12.7 (ref. 67) to the C. elegans genome (ce6). All reads that overlapped when mapped to the genome were merged into bigger regions (islands). Read counts were averaged. This resulted in 24,910 islands in the C. elegans genome.

Analysis of Sequence Conservation in CDR1as. Genome alignments of 32 vertebrates were downloaded from the UCSC database (hg19)27 and analysed for the CDR1as locus. Pристate species other than human were discarded to not bias the analyses. The one species (cow) with more than 50% gaps in the CDR1as locus was also discarded. The alignments for the seed regions were then corrected. Specifically, bases that would clearly align with the seed but had been separated in the alignment by runs of gaps were re-aligned. These corrections were necessary in less than 2% of cases.

For an in-depth analysis we BLAT-ed the human CDR1as sequence with 20-nucleotide flanking region against all vertebrate genomes in the UCSC genome browser and kept only hits that in turn aligned best to the human locus. The resulting sequences were used to build a multiple species alignment with MUSCLE69. The same corrections were applied as described above. This alignment was also used for Supplementary Fig. 4. Entropy was calculated in log, units and averaged across all alignment columns bracketing each human seed site by maximally 8 nucleotides.

RNABinding within CDR1as. RNAfold60 was used to co-fold miR–7 with each of the 74 binding regions within CDR1as defined as the miR–7 seed match TCTTCC and the next 16 bases upstream.

Single-Molecule RNA fluorescence in situ hybridization (smRNA FISH). 48 oligonucleotide probes (20 nucleotides length; spacing 2 nucleotides) complementary to the CDR1as transcript were designed using the Stellaris Probe Designer version 2.0 (Biosearch Technologies) with a masking level of 4 on the human genome to achieve high probe specificity (Supplementary Table 8). Stellaris probe pools were obtained from BioCat GmbH as conjugates coupled to Quasar 670 (a Cy 5 replacement). Flip-In T-Rex 293 cells (Life Technologies) were grown exponentially and seeded into LabTek 4-well chambered coverslips (1 to 2 × 105 cells per well). Hybridizations were performed according to the manufacturer’s instructions with 50 ng/mL DAPI as nuclear counterstain; Stellaris probes were hybridized at 125 nM concentration with a stringency of 10% formamide, an overnight hybridizations at 37°C. Images were acquired on an inverted Nikon Ti microscope with a Hamatsu ORCA R2 CCD camera, a 60× NA 1.4 oil objective and Nikon NIS-Elements Ar software (version 4), using an exposure time of 50 ms for DAPI and 1–1.5 s for Quasar 670. Groups of cells for imaging were chosen in the DAPI channel; Z-stacks were acquired in the Quasar 670 channel using 0.3 μm spacing and comprised a total depth of 6.5 μm (5 μm below and 1.5 μm above the middle of the nucleus) and merged using maximum intensity.

Mouse strains and in situ hybridizations. All mice were bred and maintained in the animal facility of the Max Delbrück Centrum under specific pathogen-free conditions, in plastic cages with regular chow and water ad libitum. All aspects of animal care and experimental protocols were approved by the Berlin Animal Review Board (REG 0441/09). B6129SFi/J wild-type adult, newborns (postnatal day 1) or pregnant females (plug detection at day 0.5; embryo collection at day 13.5) were used, as indicated for each experiment, to obtain the tissues needed for RNA analysis and in situ hybridizations (ISH). After death, embryos or tissues were immediately frozen in liquid nitrogen and stored at −70°C, or fixed for ISH.

Mouse brain structures were collected and named according to the anatomical guidelines of the Gene Expression Nervous System Atlas of the Rockefeller University (http://www.genat.org) and the Mouse Brain Atlas (http://www. mouseembryonicbrainatlas.html).

For the RNA analysis and to clone CDR1as-specific RNA probes, two adult 1-year-old mice of both sexes were dissected, total RNA prepared and analysed. If embryos or newborns were sectioned, a minimum of two specimens were evaluated; in some instances up to 5 specimens were used.

For ISH, samples were fixed in formalin (1× PBS; 4% formaldehyde) for 12 h and post-fixed (70% ethanol, 18 h) before dehydration and paraffin-embedding. Next, the organs were perfused with a standard protocol using a Shandon XP Hypercentre. For ISH mouse embryos or organs were cut in RNase-free conditions at 6 μm and ISH was performed as described48 with digoxigenin (DIG)-labelled RNA probes. All DIG–RNA probes were hybridized at 58°C overnight. A total of 600 ng of the labelled probes was used per slide.

To amplify Cdr1 sense and antisense sequences for ISH probe preparation a standard PCR amplification was performed using mouse cerebellum cDNA. Three Cdr1as amplicons were generated, two of which are probes are meant for the detection of both linear and circular forms using mmuCdr1_1f 5′–TGCGACTGACCCGCTCAGCCC–3′ and mmuCdr1_1r 5′–TTTTCTGTGTGAGATGTCA–3′, as well as mmuCdr1_2f 5′–CCAGAATTTACGTGATCT–3′ and mmuCdr1_2r 5′–ATCTTGGCCTGGAAAGACTTGG–3′. In addition a probe was generated, specific to the circular probe, using the divergent primers mmuCdr1_3f 5′–CTCTCCCTCAGCATCTTT–3′, and mmuCdr1_3r 7′–TGATGCTCTTGGAGAAGACAA–3′ (CDR1_as head to tail probe). All ensuing fragments were subcloned into pCR-BluntII-TOPO (Invitrogen) and verified by sequencing. Linearized plasmids were amenable for in vitro transcription using the T7 (antisense) or SP6 (sense) polymerase and a DIG-labeled nucleic acid mixture according to manufacturer’s instructions (Roche Applied Science).

LNA ISHs were performed according to a protocol suggested by the manufacturer (Exiqon) with minor modifications. For individual LNAs, specific protocols were run at 51°C (miR-7: 3845–15) or 58°C (miR-124: 88066–15) on an InsituPro VS robot (Intavis). A pre-hybridization step was added, which consisted in an incubation loaded and 1.5°C lower than the hybridization temperature for 30 min using hybridization buffer. The antibody-blocking step was performed in the presence of 1% blocking reagent (Rock 11096176001) and 10% sheep serum. The LNA probes were used at the following concentrations: miR–7 40 nM, miR–124 20 nM, U6 snRNA 1 nM; scrambled 40 nM, as suggested by miCURY LNA microRNA ISH Optimization kit (Exiqon; 90004). Before detection all slides were washed 4× in NTMB including 1 μM Levamisole. The doubly DIG-labelled LNAs were detected by the alkaline phosphatase using the substrate BM-purple (Rock; 11422074001) at 37°C. siRNA- and shRNA-mediated knock down. CDR1as was knocked down using custom designed sRNA oligonucleotides (Sigma) and Lipofectamine RNAiMax (Invitrogen). 2 × 106 HEK293 cells were transfected with 10 nM siRNA duplex following the manufacturer’s protocol. After 12–16 h cells were harvested and subjected to RNA analysis. For stable knock down of CDR1as, 293TN cells were co-transfected with the packaging plasmids pLIP, pLP2 and the VSV-G plasmid.
Expression of miR-7 in zebrafish embryos at 48 hours post fertilization was normalized to expression of β-actin. The miR-7 morpholino group, only embryos with a midbrain phenotype were used for the RNA expression analysis. For measuring the expression of dre-miR-7a/b we used Applied Biosystems TaqMan miR assays (ID0000268, ID001088).