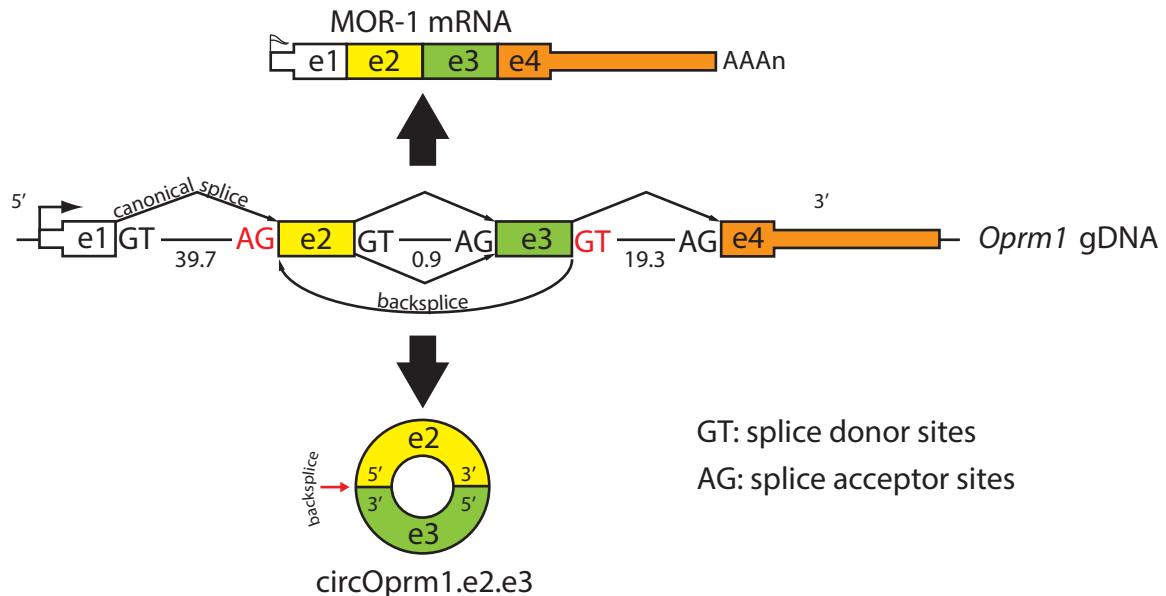
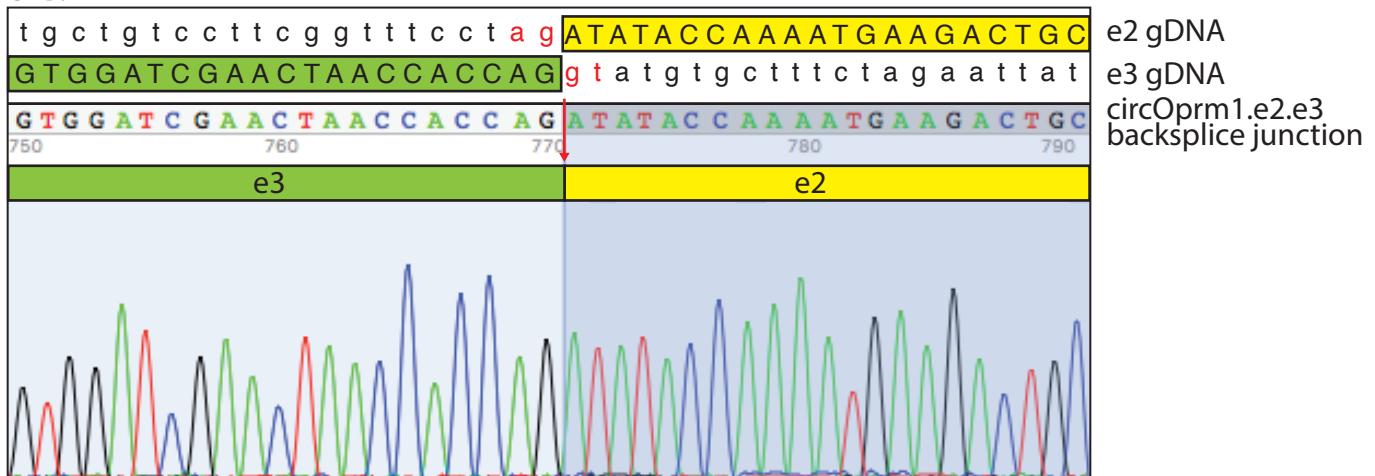


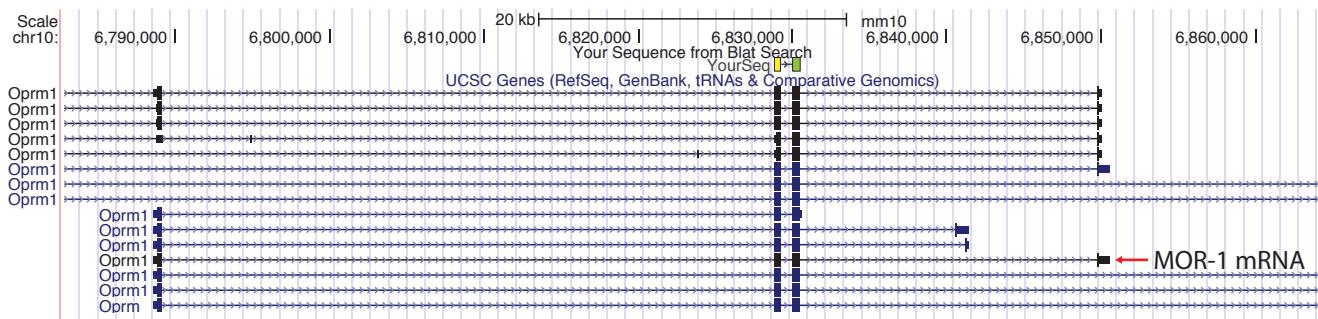
S1A.



S1B.



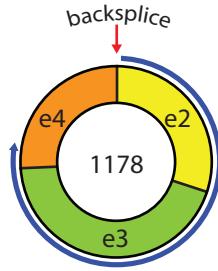
S1C.



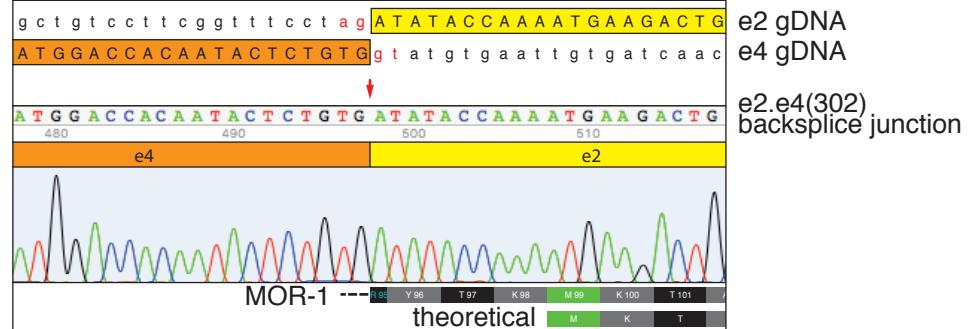
### Supplemental Figure S1. Mouse Oprm1 e2-e3 circRNA.

**S1A.** The mouse *Oprm1* locus is shown with select exons annotated (not to scale). During canonical mRNA splicing, the upstream splice donor sites (GT) and downstream splice acceptor sites (AG) flanking each intron are catalytically joined by the spliceosome following the GT-AG rule. During circOprm1.e2.e3 circRNA formation, the downstream splice donor site (GT in red) and the upstream splice acceptor site (AG in red) are spliced together, resulting in a circular RNA (red arrow designates the backsplice junction). The mRNA is modified with a 5' cap (pennant) and a poly-A tail (AAAn), features missing in circRNAs. **S1B.** A representative sequence chromatogram of the backsplice junction (red arrow) is shown, with genomic sequence from exon2 and exon3 shown above (exons in uppercase, introns in lowercase, splice donor/acceptor nucleotides in red). **S1C.** The UCSC BLAT alignment of the circOprm1.e2.e3 sequence (yellow and green) is shown for scale. The MOR-1 mRNA is labeled (red arrow). The colors and sizes of exonic boxes follow UCSC genome browser conventions; thick boxes represent coding, while thin boxes represent non-coding exon regions.

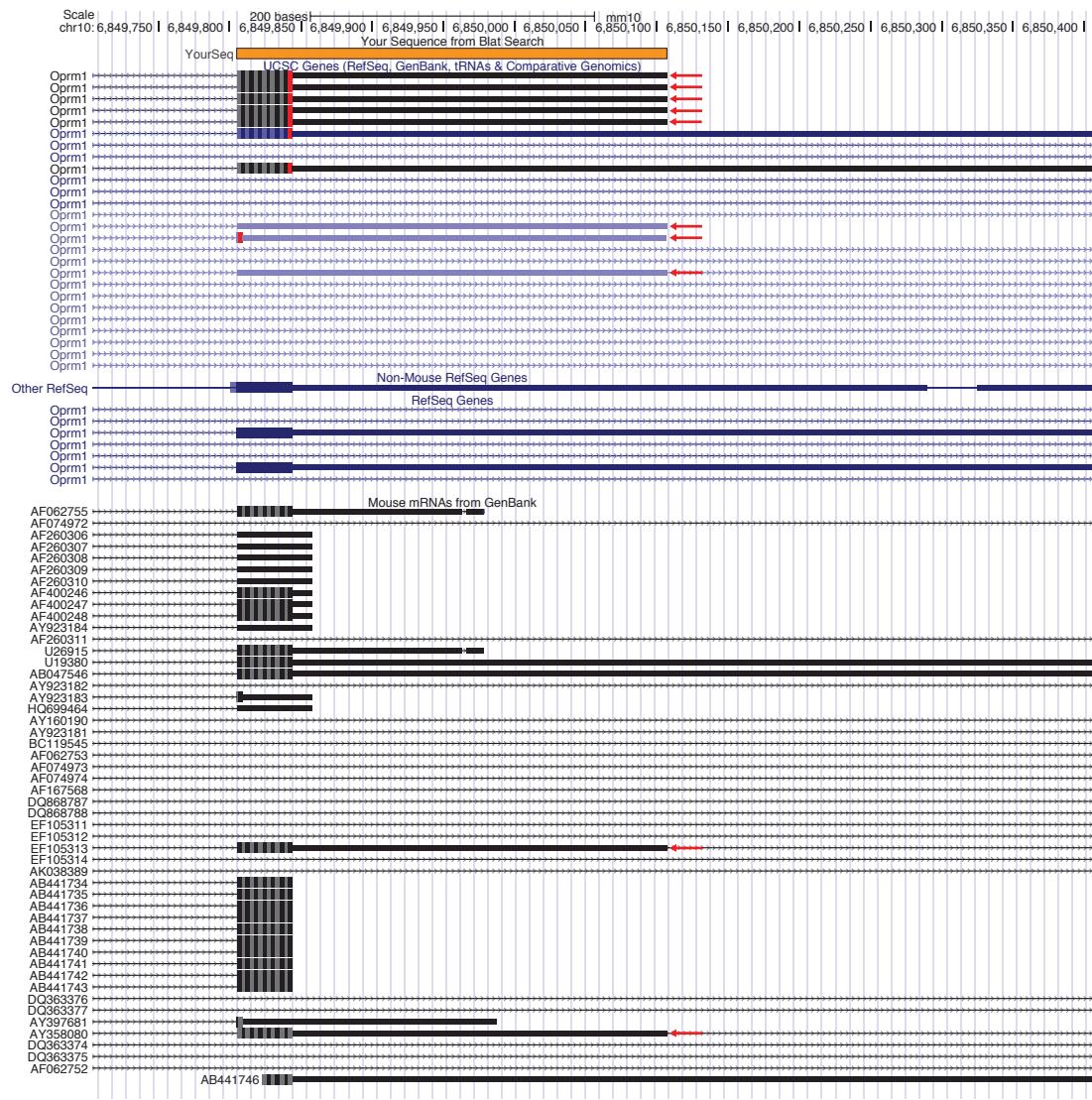
**S2A.**



**S2B.**



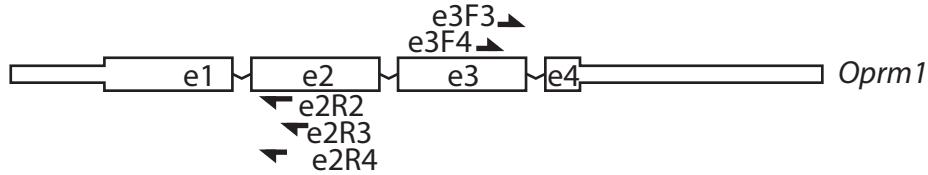
**S2C.**



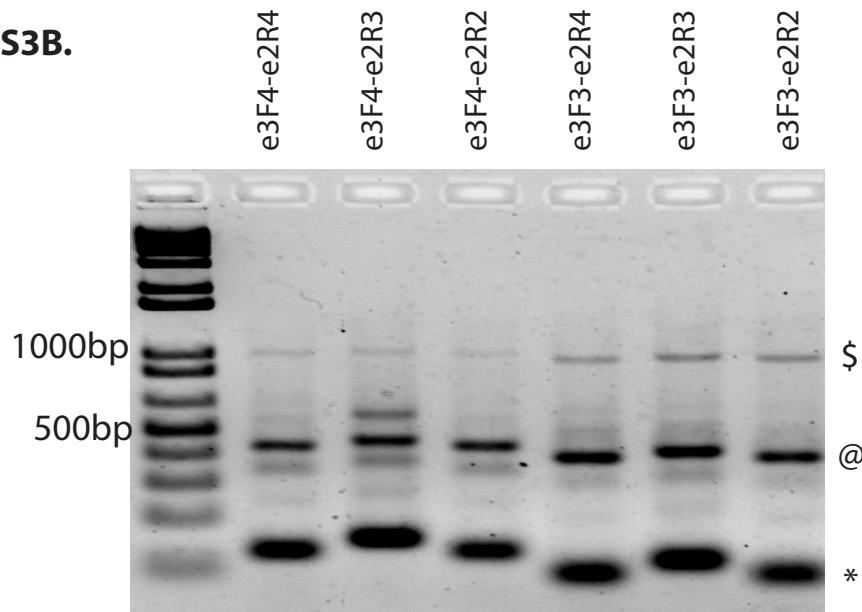
**Supplemental Figure S2. Mouse circOprm1.e2.e3e4(302) circular RNA.**

**(S2A)** The mouse circOprm1.e2.e3e4(302) RNA contains the full exon2 and exon3 (e2 and e3) sequences, and the first 302 bp of *Oprm1* exon4 (e4(302)) backspliced to the 5' end of e2. The red arrow specifies the backsplice junction. The blue circumferential arrow depicts the longest theoretical open reading frame (ORF) that could be encoded on this RNA. **(S2B)** A representative sequence chromatogram of the backsplice junction is shown. For comparison, the genomic sequence (gDNA) at the exon boundaries are shown, with exonic sequence as uppercase and intronic sequence as lowercase. The canonical GT-AG rule splice donor and acceptor sites are shown in red. The amino acid sequence for canonical MOR-1 ORF is shown below the chromatogram, with amino acid numbers referring to position in the polypeptide, and the initial portion of the theoretical ORF depicted in S1A is shown below it. The methionine shared between these two RNAs is also evolutionarily conserved in rat and human. **(S2C)** The *Oprm1* e4(302) sequence (orange) was BLAT aligned in the UCSC genome browser and this span matched perfectly to the e4 portions of several sequence records in the UCSC genome database, as well as the splice sites of some Genbank entries. The e4(302) termini are marked by red arrows. The colors and sizes of exonic boxes follow UCSC genome browser conventions; thick boxes represent coding, thin boxes represent non-coding exon spans, and red boxes are for stop codons at the ends of ORFs.

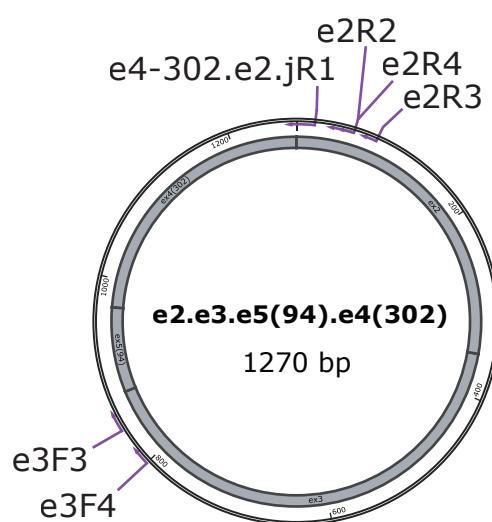
**S3A.**



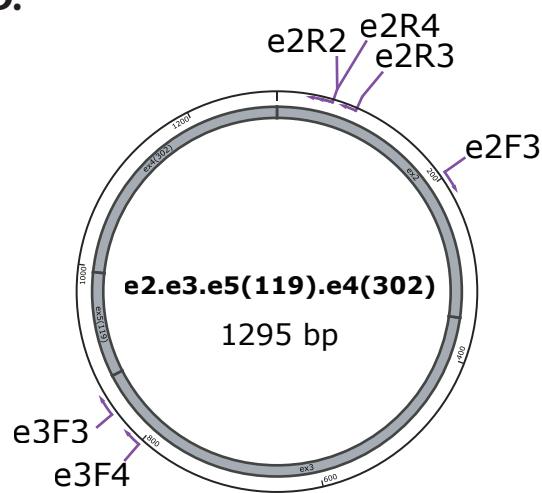
**S3B.**



**S3C.**



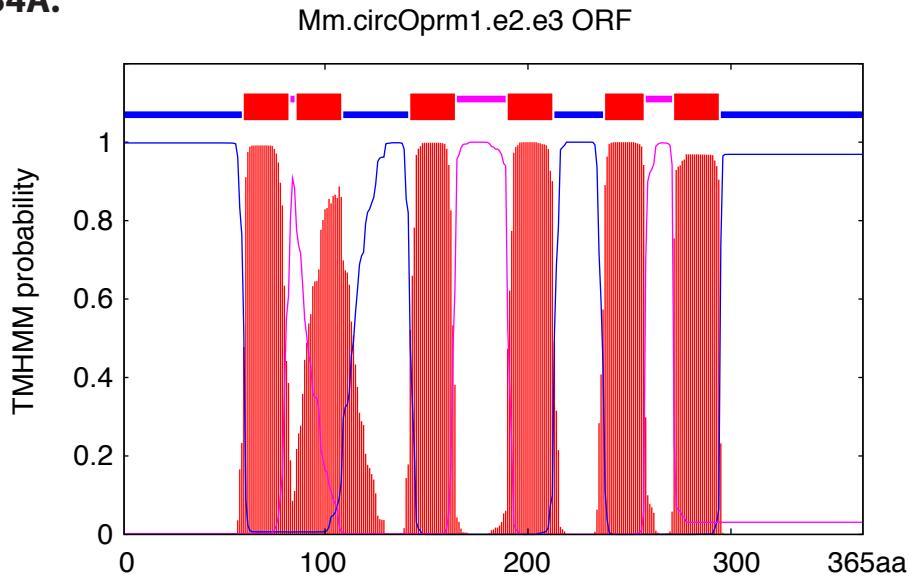
**S3D.**



#### Supplemental Figure S3. Minor variant *Oprm1* circRNA variants detectable in mouse brain.

**S3A.** A cartoon showing the primary mRNA of the *Oprm1* locus encoding the mu opioid receptor (MOR-1) is shown, with exons and primer locations drawn not to scale. Multiple divergent primer pairings within mouse *Oprm1* exon 2 and exon 3 were used for divergent RT-PCR, to search for circRNA variants poorly amplified by the divergent RT-PCR strategy of Figure 1. By pushing primers toward the distal ends of exon 2 and exon 3, in theory, longer circRNA targets could be more efficiently amplified than with the assay of Figure 1. **S3B.** C57BL6 female mouse brain total RNA mock treated with RNaseR was amplified by divergent RT-PCR with 6 primer pairings. Depending upon the primer pairing used, 4-7 bands were amplified. The dominant amplicons correspond to amplification from the e2.e3 and e2.e3.e4(302) circRNAs (\* and @ symbols, respectively). The upper band near 1kb (\$) is the product of rolling circle reverse transcription, which results in a product with tandem repeats of the e2.e3 backsplice variant. **S3C.** An amplicon deriving from an *Oprm1* e2.e3.e5(94).e4(302) circRNA was sequence verified from a sample treated similarly to S3B, amplified with a junction primer spanning the e4(302).e2 backsplice junction paired with the e3F4 primer. This circRNA corresponds to the 556bp product from the e3F4-e2R3 primer pairs. **S3D.** A hippocampal total RNA sample from a CD-1 male mouse implanted subcutaneously with placebo pellets generated amplicons from circRNAs containing e2F3-e2R4.

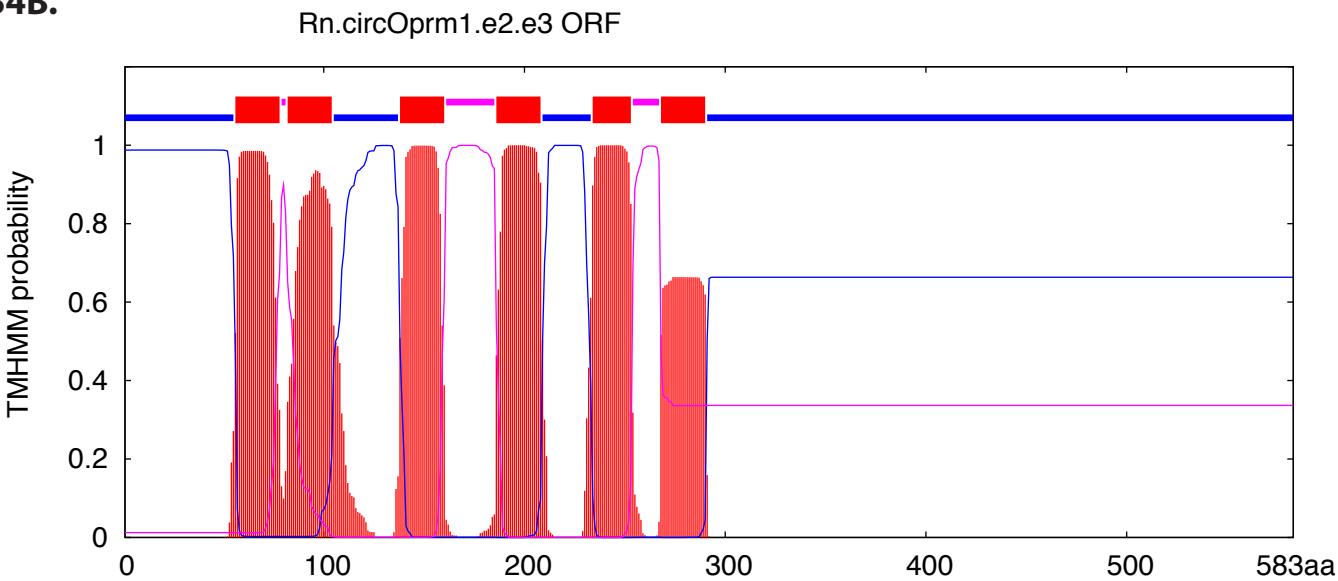
**S4A.**



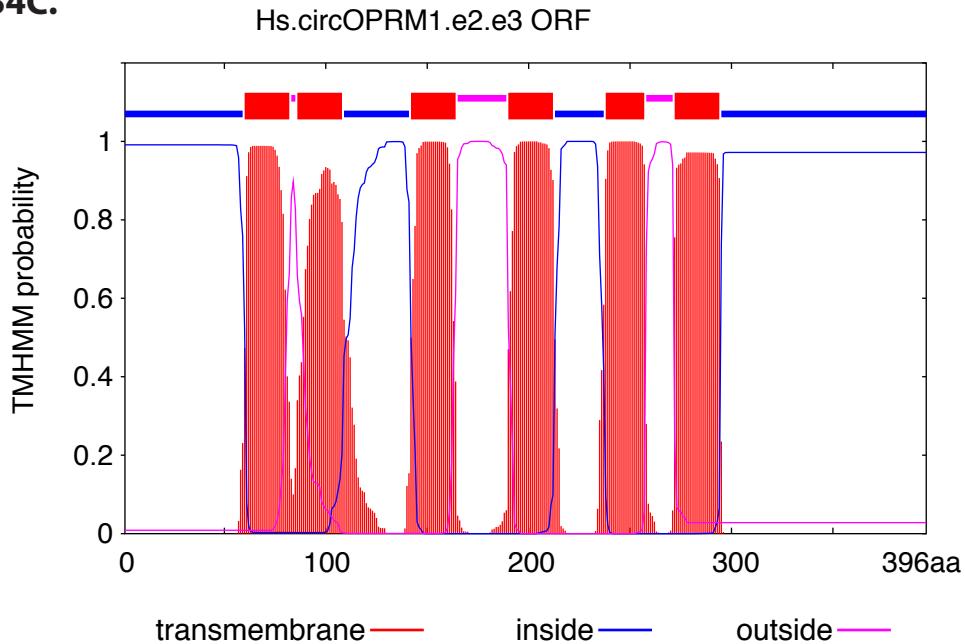
**Supplemental Figure S4. Transmembrane span predictions of potential ORFs of Mouse(Mm), Rat(Rn), and Human(Hs) *Oprm1/OPRM1* e2-e3 circRNAs.**

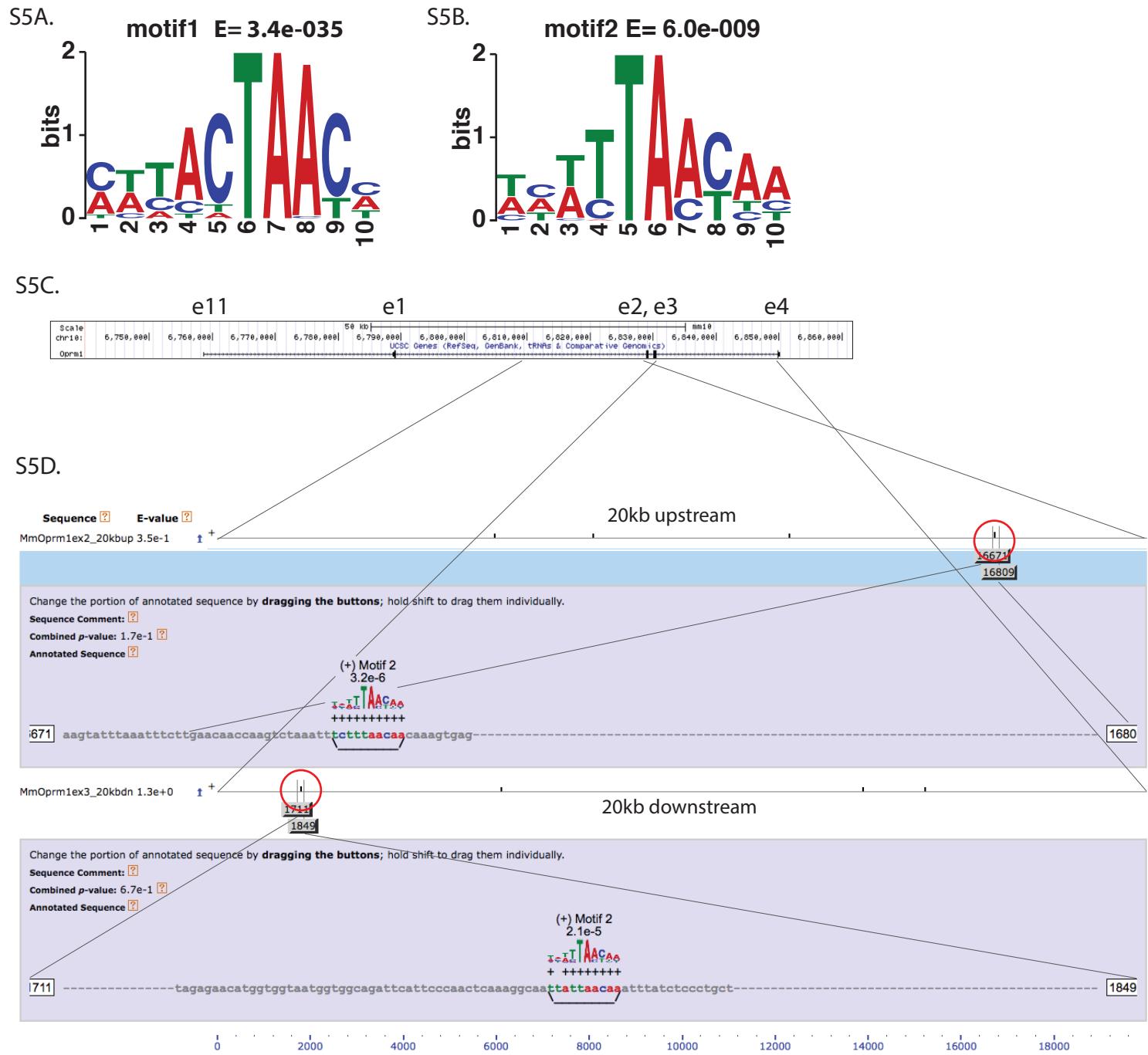
**S4A.** The longest contiguous ORF in the mouse *circOprm1.e2.e3* circRNA is 365aa long, encoded over 1.3 cycles around the circRNA starting in the 3' end of exon 3. TMHMM v2.0 was used to make transmembrane (TM) span predictions. 6TM spans are predicted for this potential ORF. **S4B.** The longest ORF in rat *Oprm1* e2.e3 circRNA is 583aa, encoded over 2.0 cycles around the circRNA, and also has 6 predicted TM spans. **S4C.** The human *OPRM1* e2.e3 circRNA has a longest ORF of 396aa encoded over 1.4 cycles around the circRNA. The red boxes in each box diagram above the TM probabilities plots represent predicted TM spans. The plots are all stretched to the same scale.

**S4B.**



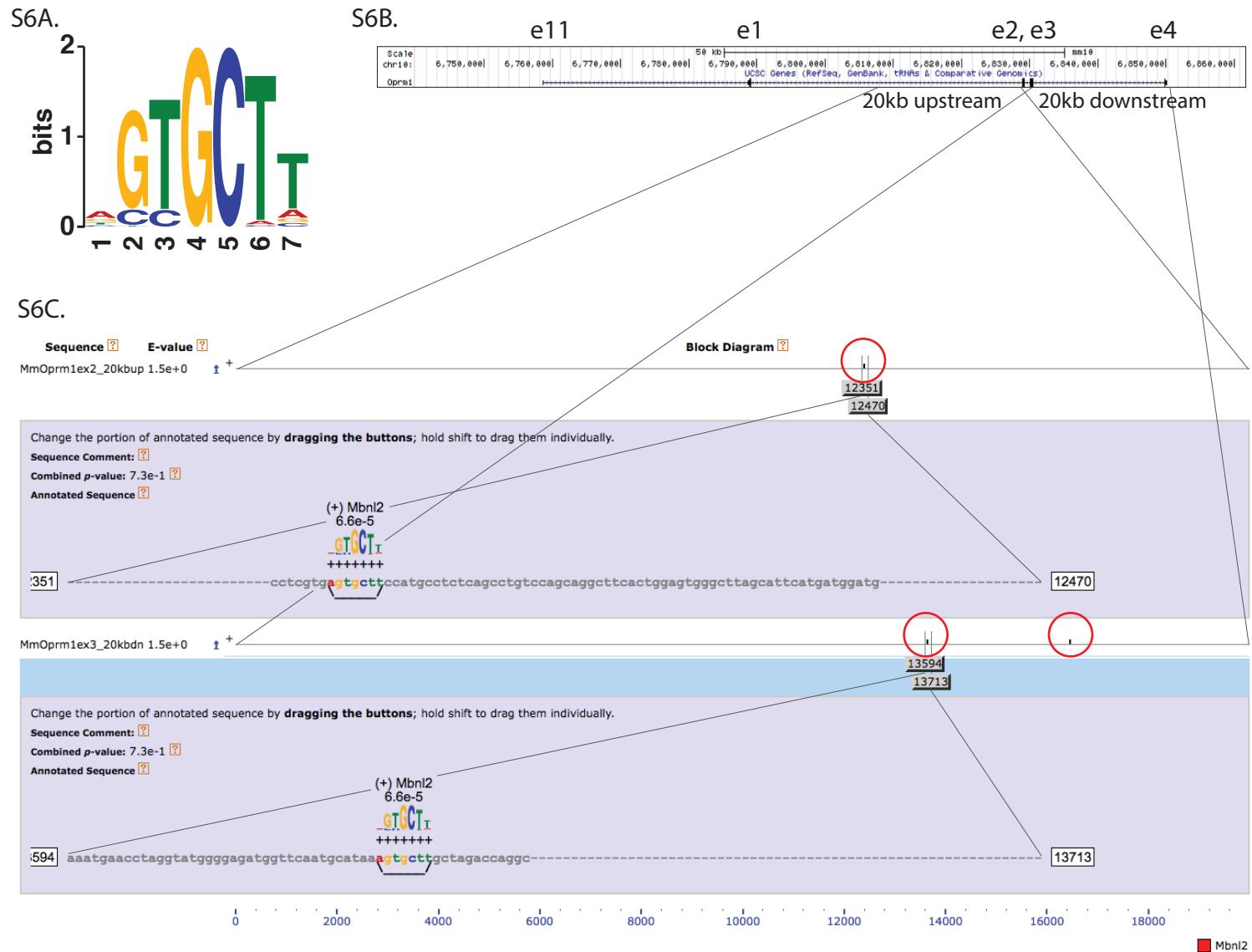
**S4C.**





### Supplemental Figure 5. *In silico* predicted binding sites of sequence specific RNA binding protein Quaking involved in circRNA biogenesis

The Quaking (Qki) binding site motif was reconstructed using MEME on published mouse Qki PAR-CLIP data. Two related motifs scored at E<0.01 (**S5A, S5B**). Part of the mouse *Oprm1* genomic locus is presented for reference (**S5C**); select exons are annotated above the map (for example, e1 is exon 1). The position specific probability matrices for these two motifs were used to search the 20kb of genomic sequence flanking the exons of mouse *Oprm1* e2-e3 circRNA, using MAST (**S5D**). Qki binding site matches were found at 14.1kb, 11.9kb, 7.7kb, and 3.3kb upstream of e2, and also in the intron downstream of e3 at 1.8kb, 6.1kb, 13.9kb, and 15.2kb downstream. Red circles denote locations of motif instances most proximal to included exons, shown blown up to the nucleotide resolution.



### Supplemental Figure 6. *In silico* predicted binding sites of sequence specific RNA binding protein Mbni2

**S6A.** The Mbni2 binding site motif was reconstructed using MEME on published mouse Mbni2 CLIP-seq data. Mbni2 is a brain specific homolog of the *Drosophila* splicing regulator *Muscleblind*, shown to be involved in circRNA biogenesis. **S6B.** A limited view of the mouse *Oprm1* genomic locus is presented for reference; select exons are annotated above the map. Diagonal bars linking from the genomic map to **S6C** demonstrate the location of the 20kb span of sequence input to MAST. **S6C.** The reconstructed Mbni2 position specific probability matrix was used to search the 20kb of genomic sequence flanking the exons of mouse *Oprm1* e2-e3 circRNA, using MAST. A potential Mbni2 binding site was found 7.6kb upstream of e2, while two predicted sites were found in the intron downstream of e3 (at approximately 13.6kb and 16.4kb downstream of e3). Red circles denote locations of these motif instances, and two of them are shown blown up to the nucleotide resolution.

“Opioid receptor circular RNAs.” Irie, T., et al. Molecular Pharmacology. MOL#113977

Figure	Species	target		Target
		topology	RT primer	
1D	Mm	linear & circ	N6	Oprm1 e2F3-e3R2
1D	Mm	circ	N6	Oprm1 e2F3-e2R3
1D	Mm	circ	N6	Oprm1 e3F1-e3R2
1E	Rn	linear & circ	N6	Oprm1 e2F1-e3R3
1E	Rn	circ	N6	Oprm1 e2F1-e2R2
1E	Rn	circ	N6	Oprm1 e3F4-e3R3
1F	Hs	linear & circ	N6	OPRM1 e2F3-e3R2
1F	Hs	circ	N6	OPRM1 e2F3-e2R4
1F	Hs	circ	N6	OPRM1 e3F1-e3R2
2D	Mm	linear	N6	Oprm1 e1F1-e3R2
2D	Mm	circ	N6	Oprm1 e2F3-e2R4
2D	Mm	negative	N6	Oprm1 e2F3-e1R2
2E	Mm	negative	ATGAGTGTAGACCGCTACATTGCCG	Oprm1 e2F3-e2R4
2E	Mm	circ	AAAGGGCAGCGTGCTAGTGG	Oprm1 e2F3-e2R4
3B	Mm	circ	N6	Oprm1 e2F1-e2R2
3B	Mm	circ	N6	Oprm1 e3F1-e3R2
3B	Mm	circ	N6	Oprd1 e2F1-e2R2
3B	Mm	circ	N6	Oprd1 e3F1-e3R2
3B	Mm	circ	N6	Oprk1 e3F1-e3R2
3B	Mm	circ	N6	Oprk1 e4F1-e4R2
3B	Mm	circ	N6	Oprl1 e3F1-e3R2
3B	Mm	circ	N6	Oprl1 e4F1-e4R2
4B	Mm	linear	N6	GAPDH
4B	Mm	circ	N6	Oprm1 e3F1-jR2
S3B	Mm	circ	N6	Oprm1 e3F4-e2R4
S3B	Mm	circ	N6	Oprm1 e3F4-e2R3
S3B	Mm	circ	N6	Oprm1 e3F4-e2R2
S3B	Mm	circ	N6	Oprm1 e3F3-e2R4
S3B	Mm	circ	N6	Oprm1 e3F3-e2R3
S3B	Mm	circ	N6	Oprm1 e3F3-e2R2
S3C	Mm	circ	N6	Oprm1 e3F4-e4-302.e2.jR1
S3D	Mm	circ	N6	Oprm1 e2F3-e2R4

**Supplemental table 1**

Primer sequences and PCR conditions.

“Opioid receptor circular RNAs.” Irie, T., et al. Molecular Pharmacology. MOL#113977

F primer sequence	R primer sequence	cDNA template conc (ng/ul)	annealing temp	number PCR cycles
ATGAGTGTAGACCGCTACATTGCCG	GAGCAGGTTCTCCAGTACCATGTG	1	67	35
ATGAGTGTAGACCGCTACATTGCCG	AAAGGGCAGCGTGCTAGTGG	1	67	35
CCACTTCCAGACTGTTCTGGCA	GAGCAGGTTCTCCAGTACCATGTG	1	67	35
TTCACCCTCTGCACCATGAGCG	CAGTGATGATGAGGACCGGCATGAT	1	67	35
TTCACCCTCTGCACCATGAGCG	AGGATGGTCCGAAGGGCCAT	1	67	35
GTGCTGGTGGTGTGGCTGTATT	CAGTGATGATGAGGACCGGCATGAT	1	67	35
GCCACCCTGTCAAGGCCTAGATT	GGAGTCCAGCAGACGATGAACACAG	5	68	37
GCCACCCTGTCAAGGCCTAGATT	GGTGCAGAGGGTGAATATGCTGGT	5	68	37
CAGCTGCCTCAACCCAGCCTTTAT	GGAGTCCAGCAGACGATGAACACAG	5	68	37
CCATGGTCACAGCCATACCATCAT	GAGCAGGTTCTCCAGTACCATGTG	1	67	35
ATGAGTGTAGACCGCTACATTGCCG	GCATCTGCCAGAGCAAGGTT	1	67	35
ATGAGTGTAGACCGCTACATTGCCG	CTGGTTGCCATCACGTTGGACAAG	1	67	35
ATGAGTGTAGACCGCTACATTGCCG	GCATCTGCCAGAGCAAGGTT	1	67	33
ATGAGTGTAGACCGCTACATTGCCG	GCATCTGCCAGAGCAAGGTT	1	67	33
GTCACCAAGTATCTCACCCCTCTGCACC	CATCTGCCAGAGCAAGGTTAAAAATGTAG	25	68	35
CCACTTCCAGACTGTTCTGGCA	GAGCAGGTTCTCCAGTACCATGTG	25	68	35
GCTACATTGCTGCTGCCATCCTGT	CGCATCAGCAAAGCCAGATTGAAG	25	68	35
TTCTCTACGCCCTCTGGACGAGAA	AGGAACACGCAGATCTGGTCACAG	25	68	35
GATGAGTGTGGACCGCTACATTGCT	TGCATAGCACATCTCCAAAAGGCCA	25	68	35
CCTGGGTTATACCAACAGCAGCCT	TTCCCAGAGCCTCCACCAGGATAAA	25	68	35
CACCTATCCGTGCCCTTGATGTT	GTGTCAGCAAGACCAGGGTATCAGC	25	68	35
GTGTTAGCCAGGTAGTGAGACTGC	CACGAAGTCGTCGAATCATGAGGCT	25	68	35
ACCACAGTCCATGCCATCAC	TCCACCACCTGTTGCTGA	1	65	45
CCACTTCCAGACTGTTCTGGCA	TGGTATATCTGGTGGTAGTCGATCCA	1	66	45
AACTCTGCTCGAACCGTCAAA	GCATCTGCCAGAGCAAGGTT	2.5	65	35
AACTCTGCTCGAACCGTCAAA	AAAGGGCAGCGTGCTAGTGG	2.5	65	35
AACTCTGCTCGAACCGTCAAA	AGGATGGTCCGAAGGGCCAT	2.5	65	35
CCACGGCTAACAGTGGATCGAA	GCATCTGCCAGAGCAAGGTT	2.5	65	35
CCACGGCTAACAGTGGATCGAA	AAAGGGCAGCGTGCTAGTGG	2.5	65	35
CCACGGCTAACAGTGGATCGAA	AGGATGGTCCGAAGGGCCAT	2.5	65	35
AACTCTGCTCGAACCGTCAAA	CAGTCTCATTGGTATATCACAGAGTATTG	1	67	35
ATGAGTGTAGACCGCTACATTGCCG	GCATCTGCCAGAGCAAGGTT	1	67	35