Supporting Information

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SI Experimental Procedures

ChIP. For ChIP experiments, 5×10^6–1×10^7 cells were cross-linked with 1% formaldehyde for 10 min, after which glycine (125 mM final concentration) was added for 5 min to quench the cross-linking. The cells were washed twice and then were collected in cold PBS. After centrifugation (1,000 × *g* for 5 min), cells were lysed with 750 μL lysis buffer [50 mM Hepes·KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA (pH 8), 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, protease inhibitors]. Cells then were sonicated 10 times (15-s pulses, 33% amplitude, 10 s between each pulse) using a Sonics Vibra-Cell sonicator while submerged in ice water. The cell debris was pelleted (10,000 × *g* for 5 min), and 50 μL of supernatant was removed to serve as the input. The remainder was immunoprecipitated by adding RIPA buffer (750 μL), primary antibody (5 μg), and protein G magnetic beads (20 μL; 1004D Novex) with overnight incubation on a roller at 4 °C. After five washes, the samples were eluted (1% SDS, 100 mM $NaHCO_3$). The cross-linking in the immunoprecipitated and input samples was reversed by incubation at 65 °C for 5 h, with the addition of 2 μL RNase A (0.5 mg/mL), before purification using the PCR purification kit (28106; Qiagen). For Pitx1 ChIP, αT3-1 cells were transfected with GFP or HA-Pitx1–encoding plasmids for 24 h before harvest, and samples then were processed similarly, except that they were eluted with HA peptide (Sigma). Real-time qPCR then was used to measure the levels of DNA, and the levels in the immunoprecipitated samples were normalized to those of the input. All primers used for qPCR amplification are given in Table S2.

Antibodies used in the ChIP experiments included H3K4me1 (AB8895), H3K4me3 (AB1012), H3K9me3 (AB8898), H3 (AB1791), and H3K27ac (AB4729), all from Abcam. In addition, the following were used: H3K27me3 (07-449; Millipore), CHD1 (4351; Cell Signaling), HA (HA.11 clone, MMS-101P; Covance), and Ago 1/2/3 (clone 2A8; Active Motif).

Chromatin Conformation Capture Assay. The chromatin conformation capture (3C) assay was carried out according to the published protocol (1). Gonadotrope precursor αT3-1 cells (7×10^7 in 10 × 100 mm plates, 70% confluent, for each 3C assay) were cultured as in ref. 2 and then were serum-starved overnight before some were treated with GnRH (100 nM, 2 h, in fresh serum-starved MEM medium). The medium was replaced with 10 mL fresh medium at room temperature before the addition of formaldehyde to a final concentration of 2% and incubation for 10 min at room temperature on a shaking platform. Glycine was added to a final concentration of 0.125 M, and the cells were incubated for another 5 min at room temperature on a shaking platform before holding on ice for 15 min. The medium was removed, and cells were collected into PBS, centrifuged (800 × *g* for 10 min at 4 °C) before 1 mL of lysis buffer [10 mM Tris (pH 8), 10 mM NaCl, 0.2% Nonidet P-40] with 300 μL of protease inhibitor mixture (P8340, Sigma) was added to the pellet. After 15 min on ice, the cells were Dounce homogenized (pestle B, on ice, 15 strokes, 1 min on ice, and 15 more strokes on ice), transferred to Eppendorf tubes, and spun down (2,000 × *g* for 5 min at 4 °C). The pellet was washed with Dpn2 ×1 restriction buffer (New England Biolabs) and was spun again in the same way before being suspended with 350 μL of Dpn2 ×1 restriction buffer and distributed to seven 50-μL tubes. Dpn2 ×1 restriction buffer (312 μL) and 1% SDS (38 μL) were added to each tube and were incubated for 10 min at 65 °C. Triton X-100 (10%: 44 μL) was added to each tube and was pipetted up and down before the addition of Dpn2 (400 μL; New England Biolabs) and incubation at 37° overnight on a roller. On the next day, 10% SDS (86 μL) was added to each tube and was incubated for 30 min at 65 °C. Contents from tubes containing control or GnRH-treated cells were then combined separately into 50-mL tubes, to which were added 745 μL of Triton X-100, 745 μL of 10× ligation buffer, 80 μL of 10 mg/mL BSA, 6.04 mL of double-distilled water, and 7.5 μL T4 ligase (400 U/μL). Tubes were incubated at 16 °C for 2 h before the addition of proteinase K (50 μL of 10 mg/mL) and incubation overnight at 65 °C. On the next day, the same amount of proteinase K was added and incubated for 2 h at 42 °C; then 8 mL of phenol was added, vortexed for 2 min, and spun down (2,500 × *g* for 5 min at room temperature). The upper phase was taken for phenol/chloroform extraction, vortexed for 2 min, and spun down (2,500 × *g* for 5 min at room temperature). This upper phase was transferred to five Corex tubes (Corning), and 1/10 volume of 3 M Na acetate (pH 5.2) was added with 2.5 volumes of 100% ethanol. After incubation overnight at −80°, the tubes were centrifuged (12,000 × *g* for 20 min at 4 °C), and the pellet was washed with 70% ethanol and was suspended in 400 μL Tris EDTA (TE) before transfer to Eppendorf tubes for phenol/chloroform extraction and ethanol precipitation as before. Finally the pellet was washed five times with 70% ethanol, air dried, and resuspended in 100 μL TE. RNase A (10 mg/mL; 1 μL) was added for 15 min at 37 °C. Then the tubes were combined, and these libraries were diluted to 15 ng/μL in double-distilled water.

PCR was carried out to detect the chimeric fragments using 1 μL of the library per 50-μL reaction, nested forward primers targeting −239 and −221 bp upstream of the *Cga* TSS, and sets of primers targeting various parts of the upstream region spanning the enhancer, as shown in Fig. S7 and detailed in Table S2. The first round of PCR was performed at 55 °C over 35 cycles, and the second round was performed at 58 °C over 21 cycles. The control 3C on the *Fshβ* gene was performed on the same libraries under identical conditions, with primers shown in Table S2. PCR products were resolved by gel electrophoresis, and the amplicons were verified by sequencing. Chimeric amplicons were measured by qPCR in two steps, the first as above, using the −239 bp primer and an upstream primer. The amplicons were purified using PCR purification kit (Qiagen), diluted 1:1,000, and 2 μL was taken to a 60-μL qPCR reaction (60 °C over 40 cycles), with the −221 bp proximal primer and the same upstream primer. Levels were quantitated according to standard curves using the same chimeric fragment that had been cloned into pGEM, and averages were made after repeating (*n*-value) both rounds of the PCR. Values are expressed relative to these standard curves in picograms of the chimeric fragment-containing plasmids.

Stable Transfection and Selection. Cells ($\sim 1 \times 10^6$) were transfected with linearized pSUPER-GFP/neo plasmid containing the targeting sequence, using GenePorter2 (Genlantis). After 48 h, cells were trypsinized, diluted 1:50, and sown on 24-well plates. The following day, G418 was added to a final concentration of 500 μg/mL, and this selection was maintained (3). The sequences used for targeting the shRNA are shown in Table S1. Notably the sequences do not match any other Ref-Seq genes in the database (BLAST; National Center for Biotechnology Information) except for sh120 which potentially could target *Cst6,* a secreted protease inhibitor; *Gngt1,* a noncanonical G protein involved in cell-cycle progression in neuronal cells (4); *Gdpgp1*, a predicted glucose phosphorylase; or a noncoding RNA (6330549D23Rik) for which no information is available. It also matches a sequence in a gene predicted to encode a 660-like zinc finger (LOC102639251) and in two other miscellaneous RNAs (Gm 19343 and LOC102631952).

1. Gheldof N, Leleu M, Noordermeer D, Rougemont J, Reymond A (2012) Detecting long-range chromatin interactions using the chromosome conformation capture sequencing (4C-seq) method. *Methods Mol Biol* 786:211–225.
2. Pnueli L, Luo M, Wang S, Naor Z, Melamed P (2011) Calcineurin mediates the gonadotropin-releasing hormone effect on expression of both subunits of the follicle-stimulating hormone through distinct mechanisms. *Mol Cell Biol* 31(24):5023–5036.
3. Wijeweera A, et al. (2015) Gonadotropin gene transcription is activated by menin-mediated effects on the chromatin. *Biochim Biophys Acta* 1849(3):328–341.
4. Yeh C, et al. (2013) IGF-1 activates a cilium-localized noncanonical Gβγ signaling pathway that regulates cell-cycle progression. *Dev Cell* 26(4):358–368.

Fig. S1. Partial knockdown of *Pitx1* leads to a reduction in eRNA and *Cga* mRNA levels. αT3-1 cells were transiently transfected with pSUPER containing shRNA targeting *Pitx1*, and cells were harvested after 48 h. RNA levels were assessed by qPCR and are shown after normalization to *Gapdh* mRNA levels, relative to the levels in WT control cells. Data are shown as mean ± SEM, $n = 2$.

Fig. S2. Comparison of the effects of shRNAs targeting different regions of the eRNAs. αT3-1 cells were stably transfected with constructs expressing shRNAs designed to target various regions of the eRNAs, as shown. After antibiotic selection, RNA levels were assessed by qPCR and are shown after normalization to *Gapdh* mRNA levels, relative to the levels in WT control cells. Data are shown as mean ± SEM, $n = 2–5$.

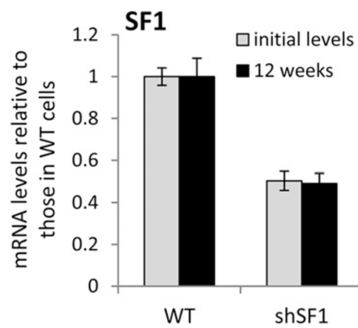


Fig. S3. Stable knockdown of SF1 does not progressively reduce *Sf1* mRNA levels beyond its initial effect. αT3-1 cells were stably transfected with shRNA targeting *Sf1* mRNA (1) as described in *Experimental Procedures*. After completion of the initial selection and 12 wk later, the levels of mRNA were measured and compared with those in WT cells. Values normalized to mRNA levels of *Rplp0* are shown relative to those in WT cells. Data are shown as mean ± SEM, $n = 2–4$.

1. Wijeweera A, et al. (2015) Gonadotropin gene transcription is activated by menin-mediated effects on the chromatin. *Biochim Biophys Acta* 1849(3):328–341.

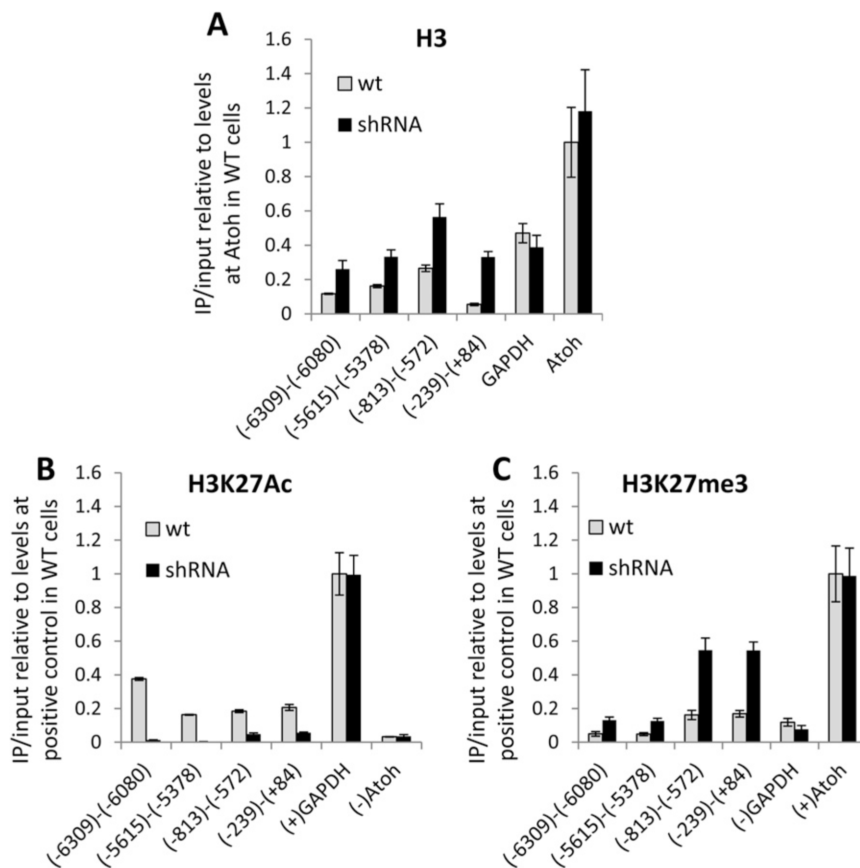


Fig. S4. Knockdown of the eRNA already affects the association and modifications of H3 at the enhancer and *Cga* promoter at 11 wk. Histone H3 (*A*) and its acetylation (*B*) or trimethylation (*C*) on Lys 27 were measured in WT and eRNA-knockdown (shRNA) cells after 11 wk using the sets of primers shown. The levels of immunoprecipitated DNA are presented relative to those at the positive controls in WT cells, after normalization to input samples. Data are shown as mean ± SEM, $n = 4$.

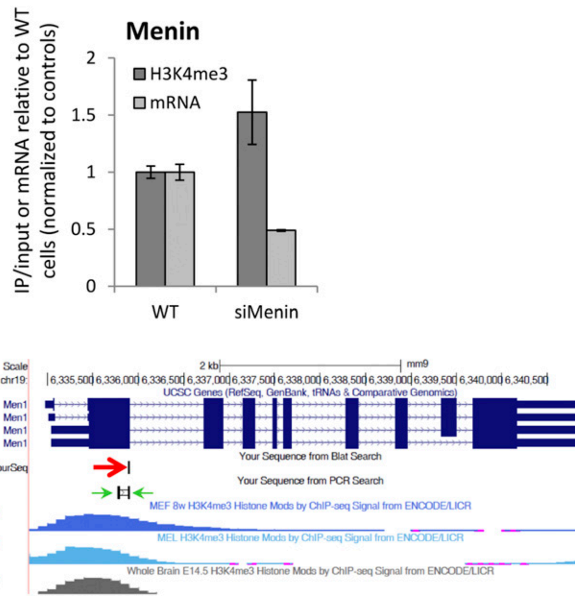


Fig. S5. Long-term stable transfection of shRNA targeting the 5′ end of *Menin* mRNA does not alter H3K4me3 at this gene. αT3-1 cells were stably transfected with shRNA targeting *Menin* mRNA (1), as described in *Experimental Procedures*. (*Upper*) At 10 wk after completion of the initial selection, the levels of H3K4me3 at the 5′ end of the gene and levels of mRNA were measured and compared with those in WT cells. Data are shown as mean ± SEM, $n = 2–4$. (*Lower*) The position of the mRNA target of the shRNA (large red arrow) and the ChIP PCR primers (pair of smaller green arrows), relative to the position of the gene and the peaks of H3K4me3 in various tissues and cell lines.

1. Wijeweera A, et al. (2015) Gonadotropin gene transcription is activated by menin-mediated effects on the chromatin. *Biochim Biophys Acta* 1849(3):328–341.

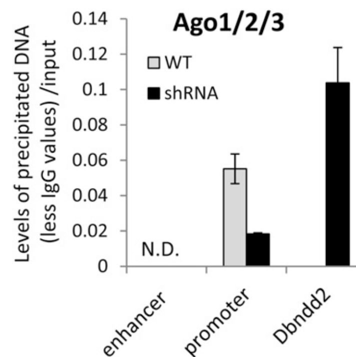
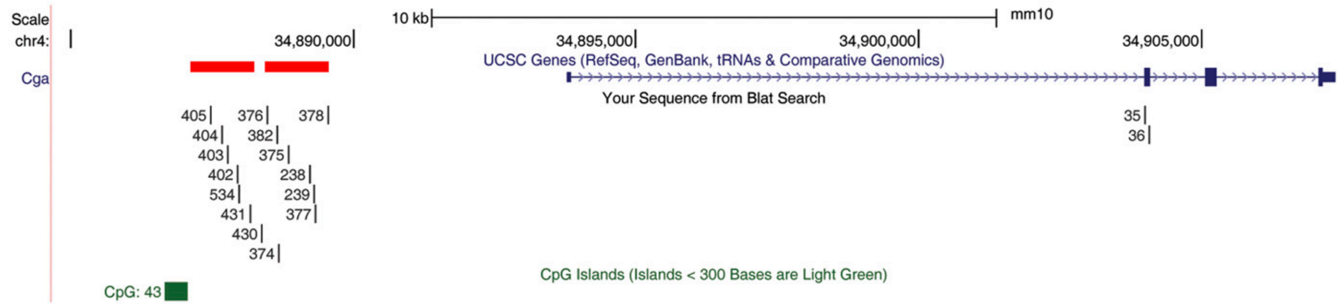


Fig. S6. Ago does not appear to accumulate at the region of the enhancer targeted by the shRNA. ChIP for Ago 1/2/3 was carried out in WT and eRNA-knockdown (shRNA) cells. The levels of immunoprecipitated *Cga* proximal promoter DNA are shown after subtraction of background (IgG) values and normalization to input samples; the distal enhancer was not detected (N.D.) above background in either cell line. Also shown are the levels at the *Dbndd2* gene promoter which was reported to associate with human Ago1 (1). Data are shown as mean ± SEM, $n = 2$.

1. Huang V, et al. (2013) Ago1 Interacts with RNA polymerase II and binds to the promoters of actively transcribed genes in human cancer cells. *PLoS Genet* 9(9):e1003821.

RT-PCR and RACE primers



3C primers

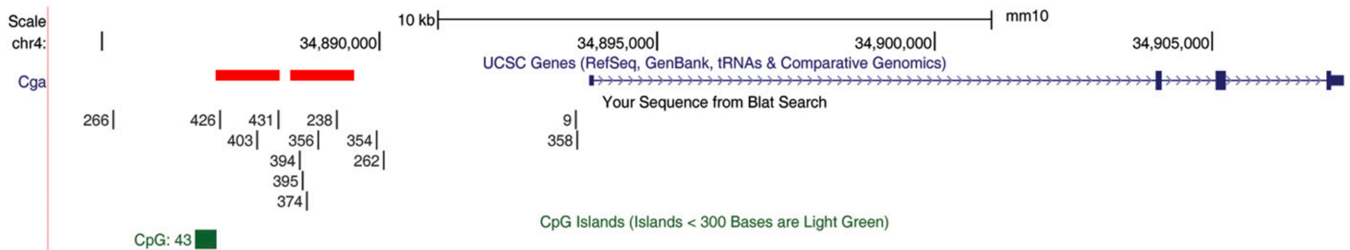


Fig. S7. Location of RT-PCR and RACE primers (*Upper*) and 3C primers (*Lower*) relative to the *Cga* gene. Numbers refer to primer numbers in Table S2. The red bars mark the transcribed region of the enhancer.

Table S1. Sequences targeting shRNA knockdown

Target	Sequence
eRNA-forward (*sh180*)	CAAAGTGGGTACTGGGAAT
eRNA-forward (*sh1000*)	GCACATAAATTCAGTCATA
eRNA-opposite (*sh120*)	GAGAAATCCTGTCTTGAAA
eRNA-opposite (*sh632*)	GGTGAAGGACAGTAGGAAA
Pitx1	CAACATCAACAACCTCACC

Table S2. Primers used in this study

Primer number	Position	Sequence (5′ to 3′)
RT and qPCR		
76	GAPDH F	ATGGTGAAGGTCGGTGTGAA
77	GAPDH R	TCCTGGAAGATGGTGATGGG
35	Cga 97 F	ATGGATTACTACAGAAAATATGCAG
36	Cga 196 R	CCTGAATAATAAAGTCTCCATCAGG
238	(−4559) F Cga	TAGGTAAACGGTACCCTGCT
239	(−4460) R Cga	GATGCTAACTGGAGGCTGCA
374	(−5100) F Cga	TAACCCTGGAAGAGTAGCCTG
375	(−4908) R Cga	TAGCCCTCCAGAAGCAGAGG
376	(−5300) F Cga	CTCTGATCACAAATTGAACA AGA
377	(−4460) F Cga	CTCAAAGTGACTCAGTGGAAG
378	(−4200) R Cga	AACGGTGAAAGTTAAGCCAGAG
382	(−5139) R Cga	GCGTTCACAATAACTTGTAACC
402	(−5800) R Cga	CCTAAGTTGATGTCCTAGAATG
403	(-−6000) F Cga	TGCACAGGCTCTATCAACTAG
404	(−6080) R Cga	GCACGCCAATTCAACTACGC
405	(−6309)F Cga	ATGTTTGATTTGAACTCCGTGG
430	(−5378) R Cga	CCTATGGGTTGTGACCCCTT
431	(−5615) F Cga	AAATTTCCATGAAGCTTATAGACC
534	(−5816) F Cga	CTAGGACATCAACTTAGGAAAT
535	(−5630) R Cga	GCATGCCTTTAATCCCAGCA
184	RPLP0 196 F	GCGACCTGGAAGTCCAACTA
185	RPLP0 296 R	ATCTGCTTGGAGCCCACAT
1	β actin F	GCCATGTACGTAGCCATCCA
2	β actin R	ACGCTCGGTCAGGATCTTCA
522	USP44 ORF 736 F	CCTACTTCGGAAGACAGGAC
523	USP44 ORF 885 R	GCCTTTCTCTTTCTCTTGCC
326	Tet1 5086 F	GAGCCTGTTCCTCGATGTGG
327	Tet1 5287 R	CAAACCCACCTGAGGCTGTT
294	Tet3 3393 F	GAGCACGCCAGAGAAGATCAA
295	Tet3 3492 R	CAGGCTTTGCTGGGACAATC
550	SF1 1348 F	AAATTCCTGAACAACCACAGC
551	SF1 1570 R	GCATCTCAATGAGAAGGTTG
923	Pitx1 exon3 F	TGGAGGCCACGTTCCAAAG
924	Pitx1 exon3 R	GTTCTTGAACCAGACCCGCAC
13	c-jun 1633 F	CTGAAGGAAGAGCCGCAGACC
14	c-jun 1870 R	GTTCCCTGAGCATGTTGGCCG
80	ERα 745 F	CTGTGCCGTGTGCAATGACT
81	ERα 924 R	CATGCCCACTTCGTAACACT
778	GnRHR 471 F	GTCTATGATCAGCCTGGCCT
779	GnRHR 610 R	ACTGTGGAAAGCTGCAGTGG
415	GAPDH(−226) F	GGAAGCAGCATTCAGGTCTC
416	GAPDH (−148) R	CAGGATAGGACTCAGGGAATACAG
286	Atoh1 (−355) F	CCCTCACTCAGGTCGCCTG
287	Atoh1 (−148) R	CGTGCGAGGAGCCAATCA
121	Crystallin (1735) R	GTGAATCTCCACAAAATCCT
122	Crystallin (1381) F	AGGACACACCAGCTTTCTTG
1,129	gtbp1 +185 F	AGCTGGACCTCACCAGCAAG
1,130	gtbp1 +323 R	TGCATCCCTTCAGCGCTCC
205	Fshβ (−161) F	ttctgctctgtggcatttaga
38	Fshβ (−66) R	CCAATACCAACATAAAGCCTGCTG
571	Lhβ (−980) R	CACAGGCTCACCTTGTGAAG
549	Lhβ (−1229) F	CCACTAAGTGTTTGGATAGG
612	Lhβ (−3180) R	TGATCTACTCTACTCGCACAC
546	Lhβ (−3359) F	TTGAGTGTTTTTGCCTGCATG
624	Tet1 enhancer F	GTGTAAAGCCACAAGCCAGT
625	Tet1 enhancer R	GCATATCCCAAGGTAAGATAG
1,147	Major Satellites F	GACGACTTGAAAAATGACGAAATC
1,148	Major Satellites R	CATATTCCAGGTCCTTCAGTGTGC
1,145	Menin ORF F (ChIP)	TATCCTCGAGAGGGAGGTGTT
1,146	Menin ORF R (ChIP)	CTGTGATGAAGCTGAAGAGGGA
449	Men CDS (ORF 1082) R	GTAGATCTCCTCATCCTCCC
452	Men CDS (ORF 908) F	ACCCACTCACCCTTTATCAC

Table S2. Cont.

Primer number	Position	Sequence (5′ to 3′)
1,141	Dbndd2 F	AACTTGGGTGCCCTCCT
1,142	Dbndd2 R	AGGGAAATGGAGAACGAACG
RACE primers		
406	For 5′ forward eRNA (−4794) R	CTGTATGCAACACAGTAAGTGGTTC
400	5′ forward inner primer (−5070) R	GGTAAGAGCACAGGCTACTC
407	For 3′ forward eRNA (−4525) F	TGCAGCTGATCCGCCTGACCAAG
381	3′ forward inner primer (−4200) F	TTGTTACCAGCTTGCAGGGTC
412	For 5′ opposite eRNA (−6309) F	ATGTTTGATTTGAACTCCGTGGCAGAA
403	5′ opposite inner primer (−6000) F	TGCACAGGCTCTATCAACTAG
411	For 3′ opposite eRNA (−6080) R	GCACGCCAATTCAACTACGCTACAG
413	3′ opposite inner primer (−6170) R	AATTAGCGATGGCTTAGTTG
3C PCR primers–*Cga*		
9	(−239) F	GCCAAATGCTCTCTCTTCATAAGC
238	(−4559) F	TAGGTAAACGGTACCCTGCT
262	(−3721) F	CTGCAGTCTAGGAGATTTGAAC
266	(−8599) F	TTGCTAGTTTTCCAGAAGACCA
354	(−3847) F	CTTCAGCAATCTTCTTCAGACTC
356	(−4908) F	AGAATTACAATCACAGCACACC
358	(−221) F	CATAAGCTGTCCTTGAGGTCAC
374	(−5100) F	TAACCCTGGAAGAGTAGCCTG
394	(−5220) F	GATGTTTTACCTGCATGTATGTC
395	(−5170) F	AGTGGCTAGAAGAGGACATC
403	(−6000) F	TGCACAGGCTCTATCAACTAG
426	(−6665) F	CCGCCGCCTCATCTGCTAAAA
431	(−5615) F	AAATTTCCATGAAGCTTATAGACC
3C PCR primers–*Fshb*		
236	Proximal–nested	GAAGCACCTCTGTGTGGTAA
237	Proximal	CTTACCATTGGCCCTAAATTAG
601	Upstream	GGGTGTGTGTCTGTGACCTC
Bisulfite sequencing primers		
748	(−6903) F BS left	GAGTTGTTTTAGTTGGTTATATAGTT
749	(−6672) F BS right	CCCAAATCTCCAATCTTCTTAAATAC
750	(−7175) F BS left	TGGTATTATAAGGGTTTTTAGGTAGT
757	(−6903) F BS right	AACTATATAACCAACTAAAACAACTC

F, forward; R, reverse.