Zhang et al., http://www.jcb.org/cgi/content/full/jcb.201304152/DC1

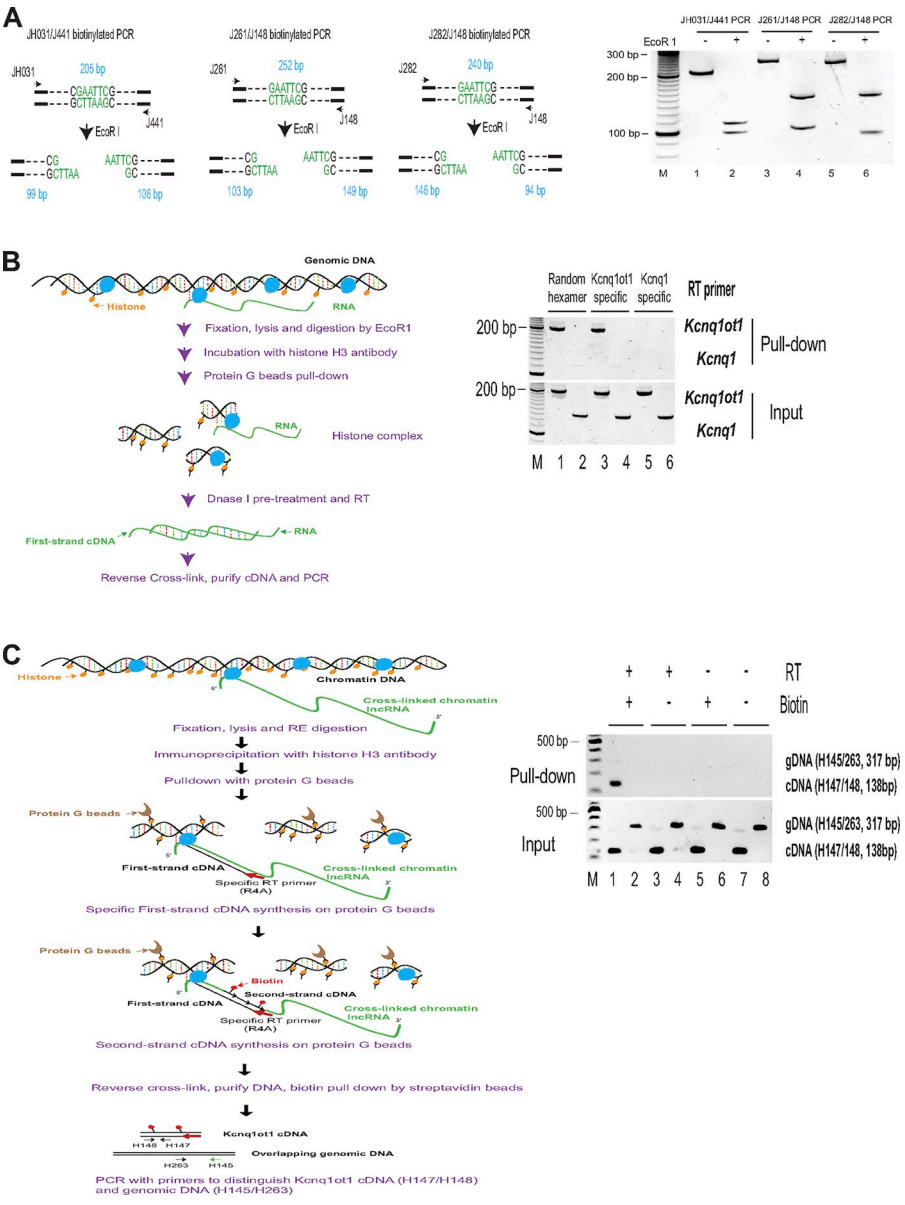

Figure S1. **Control assays that validate each critical step in the R3C approach.** Three critical steps in the R3C assay are validated: (A) The biotin-labeled DNA fragment is digested by EcoRI. (left) Schematic diagram of three PCR fragments with overlapping CpGs at the EcoRI site. (right) Digestion of biotinylated PCR DNA fragments by EcoRI. DNA templates with three EcoRI sites as shown above were amplified by PCR in the presence of 10% biotin-dCTP. After digestion by EcoRI, biotinylated DNAs were separated on 8% polyacrylamide gel. Note the complete digestion of biotinylated DNAs by restriction enzyme EcoRI (lanes 2, 4, and 6). Amplified PCR products of the expected size were digested by EcoRI enzyme (Table S8). (B) RT-PCR is performed on the cross-linked chromatin RNAs. (left) Schematic diagram of detection of RT-PCR products from cross-linked chromatin RNAs. (right) *Kcnq1ot1* reverse transcripts from chromatin cross-linked lncRNA. RT was conducted on histone H3 pull-down chromatin RNAs using either a *Kcnq1ot1*-specific primer or a *Kcnq1*-specific primer. M, 100-bp marker. Note the detection of only the *Kcnq1ot1* RT products (lanes 1 and 3, top). PCR reactions were performed by specific PCR primers: *pKcnq1* (H6/H9) and *Kcnq1ot1* (H145/H146; Tables S4 and S7). (C) The second-strand cDNA is synthesized on cross-linked chromatin lncRNA. (left) Schematic diagram of synthesis of second-strand cDNA from cross-linked chromatin lncRNA. The cross-linked *Kcnqt1ot1* lncRNA was immunoprecipitated with a histone H3 antibody, and the first-strand cDNA was synthesized using specific primer #R4A on protein G magnetic beads. After washing with PBS, the second-strand cDNA was synthesized in the presence of biotin-dCTP. During the "replacement" synthesis, RNase H produces nicks and gaps only in the noncoding RNA stand of the cDNA: noncoding RNA hybrid, creating a series of noncoding RNA fragments that serve as primers for *Escherichia coli* DNA polymerase I in the synthesis of the second strand of cDNA. It is assumed that the lncRNA/cDNA hybrid is still attached to the cross-linked chromatin, as RNase H will not degrade the unhybridized RNA. The newly synthesized double-strand cDNA was separated from genomic DNA by biotin-streptavidin beads and used for PCR detection. Two pairs of primers were used to distinguish Kcnq1ot1 lncRNA cDNA from genomic DNA: H147/H148 for Kcnq1ot1 cDNA (downstream of RT primer #R4A) and H145/H263 for genomic DNA (upstream of RT primer #R4A). (right) Detection of the biotin-labeled *Kcnq1ot1* double-strand cDNA synthesized on histone H3 chromatin–protein G magnetic beads. RT, reverse transcription; gDNA, genomic DNA. (lanes 1, 3, 5, and 7) *Kcnq1ot1* PCR; (lanes 2, 4, 6, and 8) genomic DNA PCR. (lane 2) The undetectable PCR by primer set H145/H263 excludes the contamination of biotin-labeled genomic DNA that was nick translated during the second-strand cDNA synthesis.

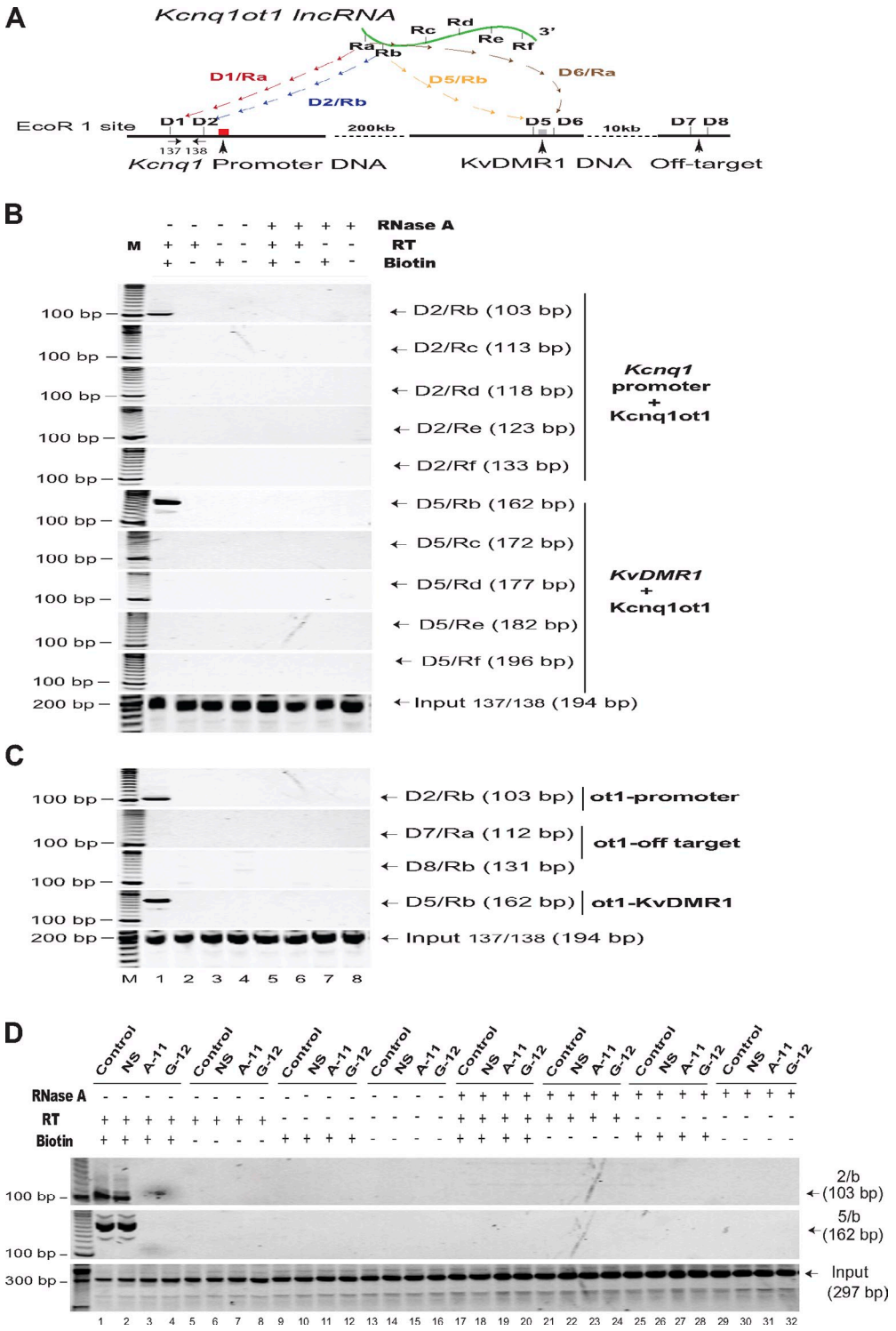

Figure S2. **The specificity of R3C is examined by testing negative controls.** (A) The location of interaction sites are shown in *Kcnq1ot1* lncRNA and chromatin DNAs. (B) R3C is performed with RNase A pretreatment, RT-negative, and biotin-negative controls. RT, reverse transcription. (C) The R3C control is performed for the off-target site. (D) Negative controls are performed in *Kcnq1ot1* shRNA knockdown cells. To evaluate the specificity of the RNA–DNA interactions using this approach, we set up a series of controls to exclude the presence of PCR products derived from the incorporation of biotin-CTP into nicked genomic DNAs during the second-strand cDNA synthesis. First, we prepared the R3C input samples in the presence or absence of RT and/or biotinylated nucleotides. In the absence of RT-initiated first-strand cDNA synthesis, no RNA–DNA interaction was detected (B, lanes 3, 4, 7, and 8). Second, we pretreated the cross-linked samples with RNase A to destroy the RNA and then proceeded with the R3C assay. We did not detect any interaction products in RNase A–treated cells, regardless of the presence of RT and biotin labeling (lanes 5–8). As expected, the positive interaction PCR products were only detected in the 5′region of *Kcnq1ot1* (a and b sites) with the *Kcnq1* promoter (D2/Rb) and the *Kcnq1* ICR (KvDMR1, D5/rb) in the presence of all R3C assay components (C, rows 1 and 4, lane 1). No RNA–DNA interaction signals were observed for the off-target EcoRI sites located 10 kb downstream of the KvDMR1 on chromosome 7 (second and third blots, lanes 1–8). NS, nonsilencing control; M, 100-bp marker.

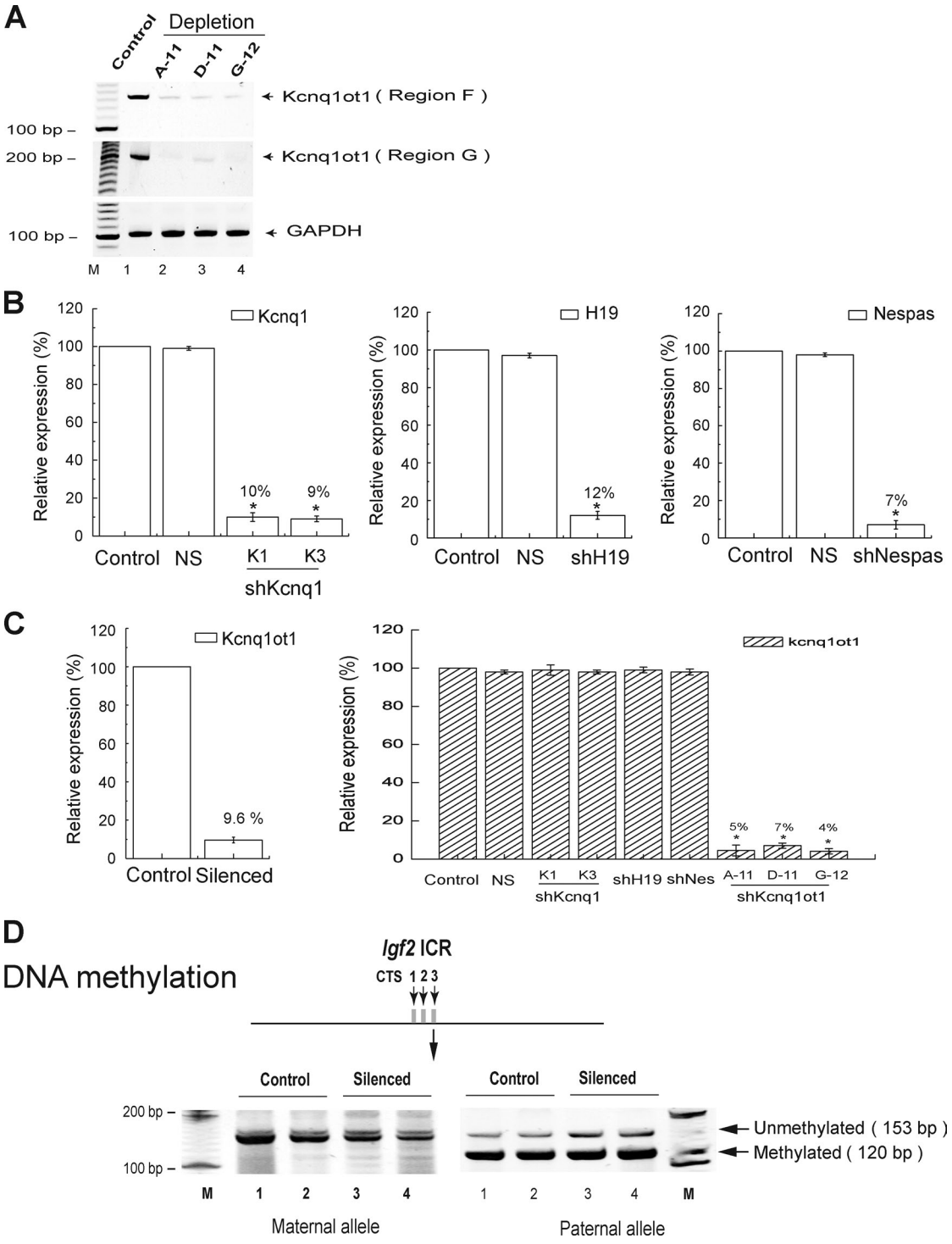

Figure S3. ***Kcnq1ot1* lncRNA is knocked down by shRNA in F1 mouse fibroblast cells.** (A) *Kcnq1ot1* knockdown by three shRNAs (A-11, D-11, and G-12) is quantitated by RT-PCR. A-11, D-11, and G-12 refer to synthetic shRNAs used to knockdown *Kcnq1ot1*. Regions F and G, PCR detection sites of the lncRNA as shown in Fig. 1 A. (B) Quantitative PCR is used to measure the knockdown of control genes. H19 and Nespas shRNAs are used as the imprinted gene controls. GAPDH is used as internal control to normalize gene expression. shK1, Kcnq1 shRNA; shH19, H19 shRNA; shNes, Nespas shRNA. (C) The specificity of Kcnq1ot1 shRNA knockdown is examined by control shRNAs. Cells were treated with shRNAs, and the mRNA transcripts were quantitated by quantitative PCR using *GAPDH* as the control. (D) DNA methylation is examined at the CTCF ICR in the *Igf2/H19* imprinting domain. Restriction enzyme analysis of CpG dinucleotides near the third CTCF binding site of the *IGF2/H19* ICR. Control, wild-type cells; CTS, CTCF binding sites; knockdown, *Kcnq1ot1*-depleted cells. Genomic DNA was treated with sodium bisulfite. After amplification with primers specific for CTCF binding region, PCR products were digested with BstUI to separate methylated (120 bp) and unmethylated (153 bp) DNA. The DNA was methylated on the paternal allele (B) and unmethylated on the maternal allele (A). Results show means ± SD. M, 100-bp marker; NS, nonsilencing control.

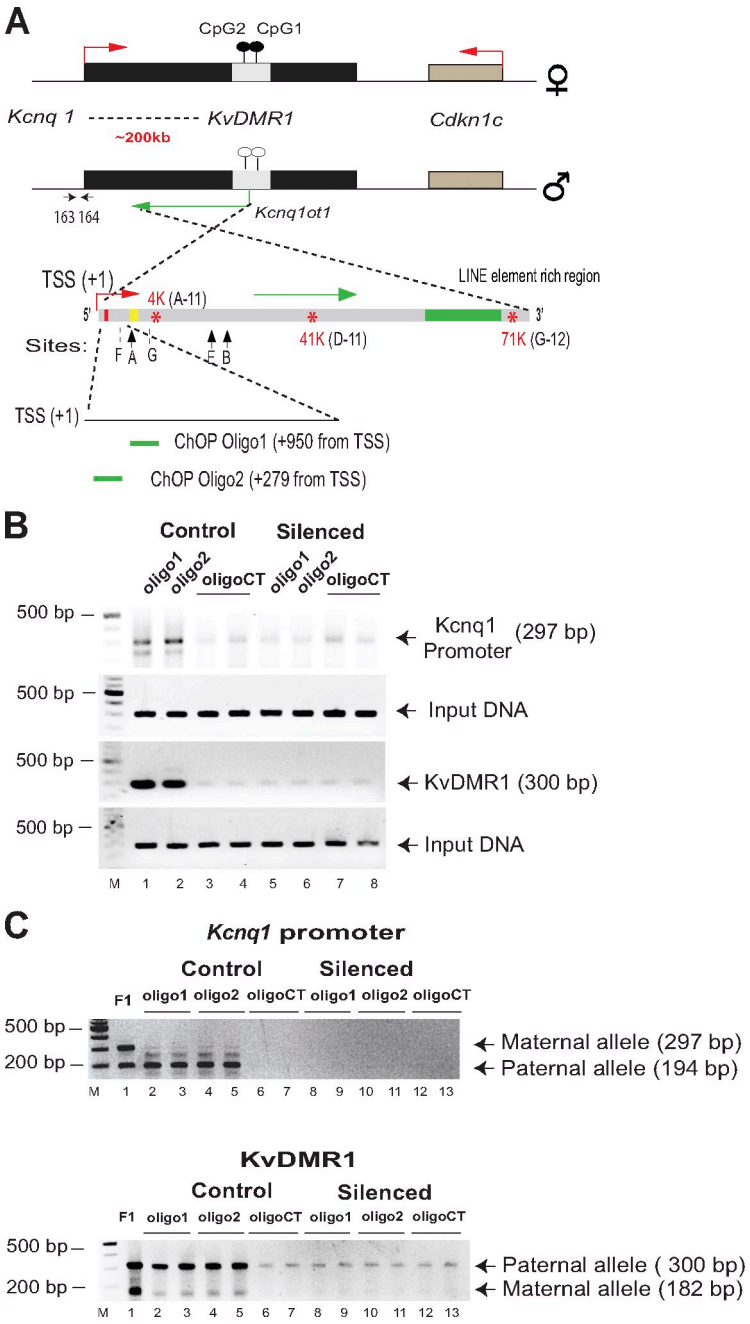

Figure S4. **The *Kcnq1ot1* lncRNA–DNA interaction is validated by ChOP.** Input, genomic DNA collected before biotin pull-down. (A) Diagrams show the location of the ChOP oligonucleotides in *Kcnq1ot1*lncRNA. TSS, the transcription start site of *Kcnq1ot1*lncRNA; red/green arrows, direction of allelic expression; vertical arrows (A, E, B, F, and G), PCR detection of *Kcnq1ot1* lncRNA; stars, location of shRNA targeting sites (A-11, D-11, and G-12); ovals, CpG islands (black, methylated; white, unmethylated). (B and C) PCR shows the altered allelic interaction of *Kcnq1ot1*lncRNA with KvDMR1 chromatin DNA (B) and *Kcnq1ot1* promoter DNA (C) in shRNA knockdown cells. Oligo1 and 2, two oligonucleotides used for the ChOP pull-down assay. oligoCT, random oligonucleotide used for ChOP control. M, 100-bp marker.

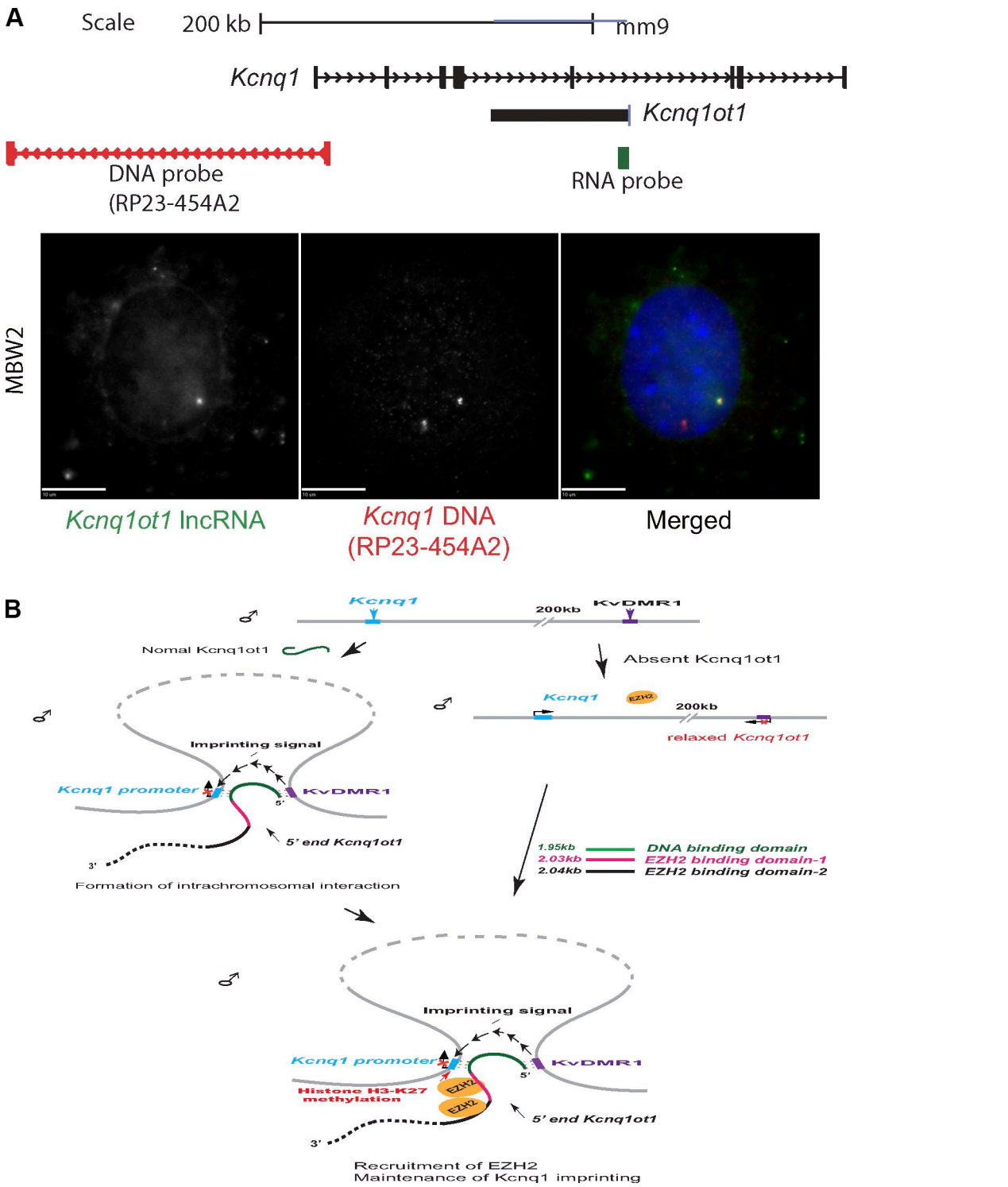

Figure S5. **RNA/DNA FISH detects the *Kcnq1ot1* lncRNA–DNA interaction.** (A) Combined RNA FISH and DNA FISH assays approves the monoallelic co-localization of *Kcnq1ot1* lncRNA and the *Kcnq1* promoter chromatin DNA. Black bar, *Kcnq1ot1* lncRNA gene; red line, BAC probe RP23-454A2 that extends over the *Kcnq1* promoter; green bar, oligonucleotide probes specific to sequences in the first 6 kb of the *Kcnq1ot1* lncRNA. The *Kcnq1ot1* RNA probe overlaps with only single allele of *Kcnq1* in the MBW2 cell line (DAPI, blue). Single 0.2-µm optical z-axis sections were shown. Bars, 10 µm. (B) The diagram shows the model of the *Kcnq1* imprinting region. Aberrant *Kcnq1* imprinting induced through silencing of lncRNA *Kcnq1ot1*. In cells that maintain normal *Kcnq1* imprinting, *Kcnq1ot1* orchestrates an intrachromosomal interaction by binding to both the KvDMR1 and to the *Kcnq1* promoter on the paternal allele. *Kcnq1ot1* then interacts with EZH2, thereby guiding the K27 methylase EZH2 to the *Kcnq1* promoters, where it methylates histone H3 and causes the allele-specific suppression of the paternal promoters. When *Kcnq1ot1* is absent, and the *Kcnq1* promoters/KvDMR1 intrachromosomal architecture is not created. Without *Kcnq1ot1*, EZH2 is not recruited to methylate H3-K27 in the *Kcnq1* promoters, resulting in the reactivation of the normally suppressed paternal allele.

Table S1. **Primers used for DNA methylation assay**

ICR	Primer	Sequence (5′ → 3′)	Tm	Polymorphic site
			°C	
CTCF binding site 1 of KvDMR1 at *Kcnq1* locus	151	ATTYGTTGTYGTTAGGAGGAATAGTTGT	65	–
	152	ACCTAACTAAACCAAAATACACCATCA		
CTCF binding site 2 of KvDMR1 at *Kcnq1* locus	153	GGTTTTTTAYGGTGAGGTTATATTAGTTAG	65	–
	154	ACCTCACCATAAAAATCTAAACTCAAAAC		
Third CTCF binding site of maternal allele at *IGF2/H19* locus	1445	TTGTGTTTTTGGAGGGGGTTTTTTGGTTA	63	BstUI
	1443	AAACCACRATATATAAAAATATACTACCAC		
Third CTCF binding site of paternal allele at *IGF2/H19* locus	1444	TTGTGTTTTTGGAGGGGGTTTTTTGGTTT	63	BstUI
	1443	AAACCACRATATATAAAAATATACTACCAC		

Minus signs indicate no restriction enzyme site. Tm, melting temperature.

Table S2. **Primers used for 3C at the *Kcnq1* locus**

Primer	Sequence (5′ → 3′)	Polymorphic site
R1A	ACTCATGGCAATCCTCCTGCCTCAG	PuvII
R1B	ACTCATGGCAATCCTCCTGCCTCAGCGT	PuvII
R2A	CCAGGTTGGCCTTGAACTTACTATG	–
R2B	CCAGGTTGGCCTTGAACTTACTATGTCG	
R4A	GGTTAAGAACCCACTGCAACCACTG	–
R4B	GGTTAAGAACCCACTGCAACCACTGCGG	
R5A	TAAGGGTCAATGTCAATGCTATGTC	–
R5B	TAAGGGTCAATGTCAATGCTATGTCTTA	

The optimal 68°C PCR annealing temperature was used for PCR primers listed above. Minus signs indicate no restriction enzyme site.

Table S3. **Primers used for R3C at the *Kcnq1* locus**

Primer	Sequence (5′ → 3′)	Polymorphic site
1A	ACTCATGGCAATCCTCCTGCCTCAG	PuvII
1B	ACTCATGGCAATCCTCCTGCCTCAGCGT	PuvII
2A	CCAGGTTGGCCTTGAACTTACTATG	–
2B	CCAGGTTGGCCTTGAACTTACTATGTCG	
5A	TAAGGGTCAATGTCAATGCTATGTC	–
5B	TAAGGGTCAATGTCAATGCTATGTCTTA	
6A	CCTCTATTGTGGGCACTGTGTGACT	–
6B	CCTCTATTGTGGGCACTGTGTGACTGTT	
7	TGCTTTGTCTTTGTCTGAGAGCAAAC	–
8	ATATGTACAAAACCTGGGTTTGA	
aA (~1.5 kb from TSS)	GGTTAAGAACCCACTGCAACCACTG	–
aB (~1.5 kb from TSS)	GGTTAAGAACCCACTGCAACCACTGCGG	
bA (~6 kb from TSS)	CACCAAATAACACCAAGGTGGCAGT	–
bB (~6 kb from TSS)	CACCAAATAACACCAAGGTGGCAGTTAA	
c (~30 kb from TSS)	GTTTCTCTTTTCCAGGGAGGCCCGCTG	–
d (~46 kb from TSS)	ATGGACAGAGAATAACAGGAAACTTGT	
e (~66 kb from TSS)	CATTTAAAATACTCATAGAGTTTTCTAT	–
f (~82 kb from TSS)	TCGGTTTATATAAAAAAAATAGCAA	

The optimal 68°C PCR annealing temperature was used for PCR primers listed above. TSS, RNA transcription start site. Minus signs indicate no restriction enzyme site.

Table S4. **Primers used for the ChIP assay**

Genomic location	Primer	Sequence (5′ → 3′)
Kcnq1 promoter(a)	H135	TGTGGAGGTCAGAGGACAAGTTAGG
−4,283 bp	H136	ATATACTCATAACTGTCCAGGTCTG
Kcnq1 promoter(b)	H137	CTTAACACTAGTCTTCATGCTTGAG
−2,302 bp	H138	AAGTTCAAGGCCAACCTGGGCTATG
Kcnq1 promoter(c)	H139	GAGCAGGTAACCCGCTGGCTGAGCA
−144 bp	H140	AGCTGAGGTGAAGGCAGCGCAGAGC
Kcnq1 promoter(d)	H141	GGATAGATTAGCAGGTGCACTGGTG
835 bp	H142	AGTGAGGGCCAGGTGCAGCTCAGCT
Kcnq1 promoter(e)	H143	TGGGCAGCCTTCAGACACTTGCATG
1,754 bp	H144	AAGCACAGTGTAAGCACAGACTTCC
KvDMR1 (f)	H145	GACCTGATTCTGACTCTGCAGGTCT
	H146	CAGTTACTCATGTTGTGGTGACCTC
KvDMR1 (g)	H147	TGCTGAGGCAGATCGGACCATATCG
	H148	CAGTCCCGACTAGCCATCCTCAGTG
KvDMR1 (h)	H149	TCTACGCACCACCCTGTATCTAGCA

The optimal 66°C PCR annealing temperature was used for PCR primers listed above. Base pairs are from the translation start site. a–h, the location of ChIP primers as shown in Fig. 7.

Table S5. **Primers used for the RNA ChIP assay**

Location	Primer	Sequence (5′ → 3′)
Kcnq1ot1 (site A)	H157	CTCAGTTCCACGATACCCTTCC
	H158	CTTACAGAAGCAGGGGTGGTCT
Kcnq1ot1 (site B)	H161	TTTTCAACTGGAAGCCTCAACA
	H162	GGGTCCAGGAGAAAGTTGAAGA
Kcnq1ot1 (site C)	H291	TCAAAATGTGGTATGTTTGCCTGTG
	H292	AGCTTGGGAAGTAGGAGCTCTGTGT
Kcnq1ot1 (site D)	H295	AGCACTTCAGAGGCAGACAAGAAGA
	H296	GCTGGGATTAAAGGCATATGCTACC
Kcnq1ot1 (site E)	H159	ACCTTGACTGCAGGATCTGAAA
	H160	GGGTCCTCACTTCTCCCTACTG
GAPDH	H179	CTGGAGAAACCTGCCAAGTATGATG
	H180	GAGACAACCTGGTCCTCAGTGTAGC
H19 ICR	H414	TGATGGAGAGGACAGAAGGG
	H415	TTGATTCAGAACGAGACGGAC
Xist	H410	GCCACGGATACCTGTGTGTC
	H411	CCGATGGGCTAAGGAGAAGA

The optimal 65°C PCR annealing temperature was used for PCR primers listed above.

Table S6. **Oligonucleotides and PCR primers used for ChOP assay**

Gene	Primer	Sequence (5′ → 3′)	Polymorphic site
KvDMR1	157	CTCAGTTCCACGATACCCTTCC	BsmFI
	158	CTTACAGAAGCAGGGGTGGTCT	
Kcnq1ot1 promoter	163	TGTCCCTTCTCACTGGAGCTG	MscI
	164	GGTTACTCACACGGTGAAGTGG	
Control-oligo	191[a]	GCCAAGTGTTAAAGGCCAAACTACGTTGAGAGA	–
Biotin-*Kcnq1ot1*-oligo1 (5′ terminal)	193[a]	ACGTGGACGCAAAATACGAGAACTGAGCCA	950–979 from TSS[b]
Biotin-*Kcnq1ot1*-oligo2 (5′ terminal)	192[a]	CCAAAAGAACTGTGGACAAATATGCTGAGGCTG	279–311 from TSS[b]

The optimal 65°C PCR annealing temperature was used for PCR primers listed above. The minus sign indicates no restriction enzyme site.
[a]PCR primers were derived from Pandey et al. (2008).
[b]Location of oligonucleotides from the transcription start site (TSS).

Table S7. **Primers for allelic expression of imprinted gene**

Gene	Primer	Sequence (5′ → 3′)	Tm	Polymorphic site
			°C	
Osbpl5	H167	TGGACGAAGCTGTGGTGTG	68	BfaI
	H168	CGTCTGATTCAGAAGCGGC		
Cd81	H175	AGCCATTGTGGTAGCTGTC	59	RsaI
	H176	CATTGAAGGCATAACAGGGCTTAC		
Kcnq1	H9	GCCAAGCCACTGGCCTGGATC	65	EcoRV
	H6	GTTCCAGCCATCAGAAGAGC		
Cdkn1c	H7	GGCTTCAGATCTGACCTCAG	65	AvaI
	H10	AGACCTGCTCAGGGACCTGT		
Igf2	MII184	CTTGTGCTGGATCGCTGCTTACGG	65	DpnII
	MII219	CTGCGACGGTTGGCACGGCTTGA		
H19	4025	TAAGTCGATTGCACTGGTTTGGAGT	65	FoxI
	4026	TGATGGAACTGCTTCCAGACTAG		

Table S8. **Primers used to amplify DNA fragments with the EcoRI site**

Primer	Sequence (5′ → 3′)
JH031	TACGGTGGGAGGTCTATATAAGC
J441R	CAACTTCTCGGGGACTGTGGGCGAT
J261F	GCGATCACATGGTCCTGCTGGA
J281F	CTGAGCACCCAGTCCGCCCTGAGCA
J148R	GCATTCATTTTATGTTTCAGGTTCAG

The optimal 65°C PCR annealing temperature was used for PCR primers listed above.

Table S9. **Primers and shRNA sets for RNAi**

Gene	shRNA or primer	Sequence (5′ → 3′) or catalog number from Thermo Fisher Scientific
Kcnq1ot1	A-11	V2LMM_95755
Kcnq1ot1	D-11	V2LMM_187732
Kcnq1ot1	G-12	V2LMM_97735
Nonsilencing shRNA control	NS	RHS4346
Kcnq1	K1	V3LMM_453826
Kcnq1	K3	V3LMM_453824
H19	H19	V2LMM_4348
Nespas	Nespas	V2LMM_97652
Nespas	H412	TGGCGGTAGGCTAACTCACT
Nespas	H413	CTCGTCTGCATCGGAGCAGT

The optimal 65°C PCR annealing temperature was used for PCR primers listed above.

Table S10. **Primers for *Kcnq1ot1* 5′-end deletion assay**

Name	Primer	Sequence (5′ → 3′)
E6K (6,020 bp)	H400	TAGAAGATTCTAGAGCTAGCGAGGAACAGTTGCCTCA
	H405	ATCGCAGATCCTTCGCGGCCGCTTACTTTCTCACTGA
ER1 (1,950 bp)	H400	TAGAAGATTCTAGAGCTAGCGAGGAACAGTTGCCTCA
	H403	ATCGCAGATCCTTCGCGGCCGCACCTCAGACCATTAT
ER2 (2,030 bp)	H401	TAGAAGATTCTAGAGCTAGCAGGGATCAGGACTGAAG
	H404	ATCGCAGATCCTTCGCGGCCGCCTTTGATCACATGTC
ER3 (2,040 bp)	H402	TAGAAGATTCTAGAGCTAGCTGGCACCACATATCAGT
	H405	ATCGCAGATCCTTCGCGGCCGCTTACTTTCTCACTGA
E6K-RT (203 bp)	H190	CAACTTCTCGGGGACTGTGG
	H290	TAGGGTTAGCACTCTGTAGGCATCT
ER1-RT (483 bp)	H190	CAACTTCTCGGGGACTGTGG
	H146	CAGTTACTCATGTTGTGGTGACCTC
ER2-RT (138 bp)	H407	CTTTAAATCAACTCTGACCCACATG
	H409	ATCCACGCTGTTTTGACCTCCATAG
ER3-RT (127 bp)	H408	TCTAAGGACTCTGAGTTCACAGGAT
	H409	ATCCACGCTGTTTTGACCTCCATAG

The optimal 65°C PCR annealing temperature was used for PCR primers listed above.

Reference

Pandey, R.R., T. Mondal, F. Mohammad, S. Enroth, L. Redrup, J. Komorowski, T. Nagano, D. Mancini-Dinardo, and C. Kanduri. 2008. *Kcnq1ot1* antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation. *Mol. Cell.* 32:232–246. http://dx.doi.org/10.1016/j.molcel.2008.08.022