Long noncoding RNA-mediated intrachromosomal interactions promote imprinting at the Kcnq1 locus

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Kcnq1ot1 is a long noncoding ribonucleic acid (RNA; lncRNA) that participates in the regulation of genes within the Kcnq1 imprinting domain. Using a novel RNA-guided chromatin conformation capture method, we demonstrate that the 5' region of Kcnq1ot1 RNA orchestrates a long-range intrachromosomal loop between KvDMR1 and the Kcnq1 promoter that is required for maintenance of imprinting. PRC2 (polycomb repressive complex 2), which participates in allelic repression of Kcnq1, is also recruited by Kcnq1ot1 RNA via EZH2. Targeted suppression of Kcnq1ot1 lncRNA prevents the creation of this long-range intrachromosomal loop and causes loss of Kcnq1 imprinting. These observations delineate a novel mechanism by which an lncRNA directly builds an intrachromosomal interaction complex to establish allele-specific transcriptional gene silencing over a large chromosomal domain.

Introduction

Although most of the mammalian genome is transcribed into RNA, the majority of this RNA does not code for proteins (Prasanth and Spector, 2007). Long noncoding RNAs (lncRNAs) represent a very large subgroup of the mammalian transcriptome. Unlike most mRNAs, lncRNAs are usually intronless and are often transcribed in the antisense orientation with respect to associated protein-coding genes (Lee, 2012). A recent study has demonstrated that some lncRNAs are involved in the epigenetic regulation in cis and in trans of a cluster of genes within large chromosomal domains by forming scaffolds to recruit and interact with the chromatin-modifying machinery (Nagano and Fraser, 2011).

Kcnq1ot1, which is transcribed into a 91-kb noncoding RNA, is imprinted and expressed exclusively from the paternal chromosome in an antisense direction with respect to Kcnq1 transcript; the majority of this RNA does not code for proteins (Smilinich et al., 1999; Pandey et al., 2008; Redrup et al., 2009).

A more detailed mapping of transcription products suggests that in stem cells, the Kcnq1ot1 may be transcribed as a 471-kb lncRNA (Golding et al., 2011). In the mouse, Kcnq1ot1 maps to the Kcnq1 domain located at the distal end of chromosome 7. Its human orthologue is located on human chromosome 11, and epimutations of this region have been implicated in the Beckwith–Wiedemann syndrome, a disorder of prenatal overgrowth, and a predisposition to embryonal malignancies, such as Wilms' tumor (Lee et al., 1999). In close to 50% of individuals with Beckwith–Wiedemann syndrome, loss of maternal-specific methylation at KvDMR1, the putative imprinting control region (ICR) within intron 10 of the Kcnq1 gene, leads to expression of the normally silent maternal allele of the Kcnq1ot1 transcript (Lee et al., 1999; Smilinich et al., 1999; Fitzpatrick et al., 2002).

Kcnq1ot1 RNA is a bidirectional silencer that regulates genes in cis over ~1 Mb in the Kcnq1 imprinting domain, which contains 8–10 maternally expressed protein coding genes (Mancini-DiNardo et al., 2003; Thakur et al., 2004). The Kcnq1ot1 promoter is located in a differentially methylated ICR (Kcnq1 ICR, called KvDMR1) in intron 10 of the Kcnq1 gene, and its
transcriptional activity is controlled by DNA methylation and a variety of histone modifications. Because its promoter on the maternal chromosome is methylated, Kcnq1ot1 is expressed only from the paternal chromosome.

Although Kcnq1ot1 RNA regulates allelic expression of both ubiquitously and placenta-specific imprinted genes (Kanduri, 2011), the mechanisms for allelic silencing of these two classes of genes over long distances remain enigmatic. KvDMR1, the region that carries the imprinting signal to control imprinting, is ∼200 kb away from the Kcnql promoter, one of its target genes, and it is not known how this imprinting control message is delivered over this long distance. Several investigators have shown that Kcnq1ot1 interacts with chromatin, and it is possible that this relationship underlies the ability of the IncRNA to control the imprinting of genes in this region (Murakami et al., 2007; Terranova et al., 2008). In this communication, we report the use of a novel RNA-guided chromosome conformation capture (3C; R3C) approach to delineate the role of Kcnq1ot1 in long-range chromatin interactions. Using standard 3C (Dekker et al., 2002), we found that Kcnq1ot1 IncRNA functions as a necessary scaffold molecule to orchestrate a long-range intrachromosomal loop between KvDMR1 and the Kcnq1 promoter, recruiting a variety of proteins, including PRC2 (polycomb repressive complex 2), to maintain monoallelic expression of the cluster of genes in the Kcnq1 imprinting domain.

Results

A novel R3C approach to detect the RNA–DNA interaction

To explore the mechanism by which Kcnq1ot1 IncRNA regulates the imprinting of genes in cis over several hundred kilobases of DNA, we devised a novel method called R3C to detect RNA–DNA interactions (Fig. 1 A). In this approach, the DNA-binding RNA is first reverse transcribed into double-stranded cDNA with biotin labeling. After digestion with restriction enzyme EcoRI, the IncRNA-converted double-strand cDNA is ligated to adjacent genomic DNAs by T4 DNA ligase. The biotinylated double-strand cDNA is then separated from other DNA–DNA products by streptavidin pull-down using paramagnetic Dynabeads and analyzed by PCR using RNA–DNA interaction-specific primers.

To demonstrate the feasibility of the method, we set up controls to validate each critical step of the R3C assay. First, we determined whether the restriction enzyme EcoRI was active on biotin-labeled double-strand cDNAs, as the enzyme activity can be inhibited by overlapping methylated CpGs. We used PCR to amplify three known DNA fragments with overlapping CpGs at the EcoRI site (cGAATTCC, or GAATTCC; capital letters are the EcoRI site, and the lowercase letters are the EcoRI-overlapping CGs that can be methylated in the genome; Fig. S1 A) with 1:10 biotin–deoxy-CTP (dCTP)/dCTP added in the PCR reaction. The biotinylated PCR DNAs were then used as substrates to determine the enzymatic activity of EcoRI. We found that the DNA fragments that were labeled with 10% biotin-dCTP did not interfere with the activity of EcoRI (Fig. S1 A, lanes 2, 4, and 6).

Next, we tested whether Kcnq1ot1 IncRNA could be reverse transcribed and amplified by PCR after it was cross-linked with chromatin. The Kcnq1ot1-associated chromatin fragments were fixed by formaldehyde and digested by EcoRI to shear the genomic DNA. The cross-linked Kcnq1ot1 fragments were pulled down using histone H3 antibody–protein G magnetic beads to allow the subsequent reactions, such as reverse transcription (RT) and ligation, to take place on solid substrate-associated chromatin. After the treatment with DNase I to digest the DNA, the Kcnq1ot1 IncRNA was reverse transcribed in the presence of either Kcnq1ot1-specific or Kcnq1-specific RT primers (Fig. S1 B). We detected Kcnq1ot1 cDNA products but not Kcnq1 cDNA products, demonstrating the availability of chromatin cross-linked RNA for the RT-PCR reaction.

Finally, we determined whether the second-strand cDNA could be synthesized from the chromatin-linked Kcnq1ot1 IncRNA without losing its cross-link to the chromatin DNA after digestion with the RNase H in the reaction. The chromatin-bound IncRNA was pulled down using anti–histone H3 antibody–protein G magnetic beads and was reverse transcribed by ThermoScript transcriptase using a Kcnq1ot1-specific primer, #R4A (Fig. S1 C). After washing away the remaining primer and RT enzyme, the second-strand cDNA was synthesized on protein G magnetic beads in the presence of 1:10 biotin-dCTP/dCTP. The biotinylated double-strand cDNAs were released from the protein G beads and were separated from genomic DNAs by streptavidin beads for PCR detection. We detected PCR products for the Kcnq1ot1 second-strand cDNA from the chromatinbeads using the #R4A downstream PCR primers (H147/H148; Fig. S1 C, lane 1), indicating the presence of an intact double-strand cDNA–chromatin complex. No PCR products were detected using the #R4A upstream primers (H145/H263; Fig. S1 C, lane 6), excluding the possibility of contamination of the biotin nick-labeled genomic DNA during the replacement reaction of the second-strand cDNA synthesis. These experiments demonstrate that the R3C enzyme digestion and ligation can occur on the chromatin DNA–IncRNA–cDNA complex.

Capturing Kcnq1ot1 IncRNA-genomic DNA interactions by R3C

We then used the R3C approach to detect the ligated Kcnq1ot1 IncRNA–DNA product using a PCR primer set consisting of one primer from the Kcnq1ot1 cDNA and the other primer from the DNA region with which it interacts and mapped the RNA–DNA interaction using primer sets covering the entire Kcnq1ot1 IncRNA (Fig. 1 B). We detected the direct interaction of Kcnq1ot1 noncoding RNA (Ra and Rb sites) with both the Kcnq1 promoter (D1 and D2 sites; Fig. 1 B, left, lanes 1 and 2) and the Kcnq1 ICR (KvDMR1; D5 and D6 sites; Fig. 1 B, right, lanes 5 and 6). No interactions were detected in nonbiotin-labeled samples (Fig. 1 B, lanes 3, 4, 7, and 8). We also tested the specificity using a series of negative controls. No R3C products were observed when the sample was pretreated with RNase A to destroy Kcnq1ot1 IncRNA or when R3C was performed in the absence of RT (Fig. S2, RT−) or at the off-target EcoRI sites located 10 kb downstream of the KvDMR1 on chromosome 7 (Fig. S2). R3C products were confirmed by sequencing.
A novel RNA 3C assay is developed to detect the interaction of Kcnq1ot1 with Kcnq1 promoter and KvDMR1. (A) The schematic diagram shows the procedure for the R3C assay. Red dots, biotin; blue ovals, cross-linked protein; horizontal arrows above the ligated DNA show primers used to detect the R3C products. (B) R3C detects the Kcnq1ot1 lncRNA–DNA interaction. PCR detection sites were labeled as D1–D8 for genomic DNA and Ra–Rf for Kcnq1ot1 lncRNA. M, 100-bp marker. Note the interaction of Kcnq1ot1 lncRNA at regions (Ra and Rb) with the Kcnq1 promoter (D1 and D2; left) and with KvDMR1 (D5 and D6; right). (C) R3C products are confirmed by DNA sequencing. The R3C products derived from the Kcnq1 promoter–Kcnq1ot1 lncRNA interaction (D2/Rb) and the KvDMR1–Kcnq1ot1 lncRNA interaction (D6/Ra) were cloned and sequenced. Note that the R3C products contain the EcoR1 (5’-GAATTC-3’) that was flanked on both sides by chromatin DNA and Kcnq1ot1 cDNA. (D) The Kcnq1ot1 RNA–chromatin interaction is allele specific. After PCR, the two parental alleles were distinguished using the PvuII restriction enzyme. Input, genomic DNA collected before biotin pull-down. D1 and Ra, PCR primers used to detect the lncRNA–DNA interaction.
and confirmed the identity of the ligated Kcnq1 promoter/Kcnq1ot1 cDNA (Fig. 1 C, left) and the ligated Kcnq1 promoter/KvDMR1 (Fig. 1 C, right).

To determine whether this RNA–DNA interaction is allele-specific, we used a mouse fibroblast cell line (MBW2) derived from an F1 cross between Mus musculus and a C57B/6 mouse. We sequenced a 292-bp genomic DNA covering the EcoRI site and found an A/G single nucleotide polymorphism (SNP) on the PvuII site that distinguishes the two parental alleles (Fig. 1 D, top). Using this SNP to separate the allelic R3C products, we sequenced a 292-bp genomic DNA covering the EcoRI site and confirmed the identity of the ligated Kcnq1 promoter (Fig. 1 D, bottom, lanes 1 and 2).

**Loss of the RNA–DNA interaction after Kcnq1ot1 silencing**
To confirm this allelic RNA–DNA interaction, we specifically silenced the paternal Kcnq1ot1 lncRNA using a de novo DNA methylation approach (Zhang et al., 2011) based on the known regulatory mechanism of this imprinting locus. The allelic expression of Kcnq1ot1 is regulated by allele-specific DNA methylation. The mouse Kcnq1 ICR (KvDMR1) contains two CpG islands: CpG1 and CpG2 (Fig. 2 A; Paulsen et al., 2005). CpG1 island, located ~200 kb downstream of the Kcnq1 promoter, overlaps with the Kcnq1ot1 promoter, and contains two critical CTCF binding sites (CTS1 and CTS2; Fig. 2 B). The CpG1 DNA is paternal unmethylated and maternally methylated, thereby allowing the exclusive expression of Kcnq1ot1 from the paternal chromosome. CTCF binds to the unmethylated paternal allele and may participate in the regulation of the expression of Kcnq1ot1 (Fitzpatrick et al., 2007).

We reasoned that paternal Kcnq1ot1 expression would be abolished if the KvDMR1 CpG island was de novo methylated to the level seen in the maternal promoter. For this purpose, we synthesized a recombinant CTCF analogue (zinc finger [ZF]-Sss1) by replacing the functional C terminus of CTCF with CpG methylase Sss1, while maintaining the ZF DNA-binding domain that binds to CTS1 and CTS2. When this fusion protein binds to CTCF binding sites, it has the potential to methylate nearby CpG dinucleotides. With this approach, we attempted to silence the paternal Kcnq1ot1 lncRNA interacted exclusively with the paternal Kcnq1 promoter (Fig. 1 C, right).

We then used RT-PCR to examine whether the targeted DNA methylation would suppress the expression of Kcnq1ot1 lncRNA. In control cells, Kcnq1ot1 lncRNA was detected by three primer sets covering three different regions of the Kcnq1ot1 lncRNA (Fig. 2 C, lanes 1 and 2) at sites A, E, and B. In ZF-Sss1–expressing cell clones, Kcnq1ot1 expression was significantly reduced in association with de novo DNA methylation (Fig. 2 C, lanes 3 and 4). These data demonstrate that the recombinant protein ZF-Sss1 is able to methylate CpG dinucleotides in the paternal Kcnq1ot1 promoter region, leading to silencing of paternal Kcnq1ot1 gene expression. We will refer to these cells as Kcnq1ot1 methylation “silenced” cells to distinguish them from the gene deletion knockout and shRNA depletion cells in the following text.

We then examined whether Kcnq1ot1 silencing would affect the Kcnq1ot1 RNA–DNA interaction. Using R3C, we show that in MBW2 control cells, Kcnq1ot1 directly interacted with the Kcnq1 promoter (Fig. 2 D, left, lanes 1 and 2). In Kcnq1ot1-silenced cells, however, no interaction was detected (Fig. 2 D, left, lanes 5 and 6). Similarly, no interaction was detected at the KvDMR1 in the Kcnq1ot1-silenced cells (Fig. 2 D, right, lanes 5 and 6).

We validated this finding by examining Kcnq1ot1 knockout mouse embryonic fibroblasts (MEFs), in which the 2.8-kb KvDMR1 fragment containing the Kcnq1ot1 promoter had been deleted (Fitzpatrick et al., 2002). In both the wild-type cells and in cells in which the deletion was maternally inherited (Fig. 2 E, −(M)/+(M)), we detected interactions of Kcnq1ot1 noncoding RNA with the Kcnq1 promoter DNA (Fig. 2 E, D2/Rb) and with KvDMR1 (Fig. 2 E, left, lanes 1–3, D5/Rb). These interactions were completely abolished in those cells in which the deletion was paternally inherited (Fig. 2 E, left, lanes 7 and 8, +/(P)).

We further confirmed this finding in a third Kcnq1ot1 deletion model using a standard RNAi approach. Three shRNAs were used to target the 5′ region (4 kb; A-11), the middle (41 kb; D-11), and the 3′ end (71 kb; G-12) of Kcnq1ot1 (Fig. 2 A). These shRNAs were packaged into lentiviruses and transduced into F1 mouse fibroblasts (MBW2) that maintain normal Kcnq1ot1 imprinting. Stable cell clones were selected using puromycin, and the knockdown of Kcnq1ot1 by shRNA was confirmed using both RT-PCR and quantitative PCR (Fig. S3, A–C). Using R3C, we show that in both the wild-type and control shRNA cells (shK1, shH19, and shNespas), Kcnq1ot1 directly interacted with the Kcnq1 promoter (Fig. 2 F, D2/Rb) and KvDMR1 (Fig. 2 F, D5/Rb). However, these interactions were lost in the Kcnq1ot1 knockdown A11 and G-12 cells (Fig. 2 F, right top and middle, lanes 11 and 13). This lncRNA–DNA interaction was also confirmed by a chromatin oligonucleotide-affinity precipitation (ChOP) method (Fig. S4; Mariner et al., 2008; Pandey et al., 2008). Collectively, our data from these three distinct models demonstrate an allele-specific interaction between the Kcnq1ot1 lncRNA and KvDMR1–Kcnq1 promoter DNA regions.

**Kcnq1ot1 lncRNA orchestrates a long-range intrachromosomal loop between the Kcnq1 promoter and the KvDMR1**

Because Kcnq1ot1 lncRNA interacts with both the Kcnq1 promoter and the KvDMR1, which are 200 kb apart, we hypothesized that the Kcnq1ot1 lncRNA scaffolds these long-distance DNA regions to form an intrachromosomal structure, which is critical for the maintenance of allelic expression of genes in this
Figure 2. The lncRNA–chromatin interaction is lost after targeted DNA methylation-mediated knockdown of Kcnq1ot1. (A) The diagram shows the allelic expression of genes in the Kcnq1 imprinting domain on mouse chromosome 7. 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). 890 SD, 890-kb silencing domain. (B) Bisulfite DNA sequencing confirmed the induced DNA methylation in the Kcnq1ot1 promoter. After sodium bisulfite treatment, genomic DNA was amplified by PCR primers (#151/152 and #153/154), cloned in the TA vector (Invitrogen), and sequenced. A total of 13 CpG dinucleotides at the KvDMR1 was examined. Each line represents a single sequenced PCR molecule. Black circles represent methylated CpG dinucleotides, and white circles represent unmethylated CpG dinucleotides. Control, wild-type cells; second and third rows, Kcnq1ot1-silenced cells. (C) Targeted DNA methylation silences the Kcnq1ot1 lncRNA in F1 mouse fibroblast cells. GAPDH was used as a control. Sites A, E, and B are nucleotides 999–1,299, 22,445–22,745, and 31,019–31,315 from the Kcnq1ot1 transcription start site, respectively. (D) Kcnq1ot1 lncRNA silencing by targeted DNA methylation induces loss of the lncRNA–DNA interaction. The Kcnq1ot1 lncRNA–chromatin interactions were detected at the D1/Ra and D2/Rb sites for the Kcnq1 promoter and the D6/Ra and D5/Rb sites for KvDMR1 as shown in Fig. 1B. (E) The lncRNA–DNA interaction is abolished in Kcnq1ot1 knockout MEFs. WT, wild type; −(M)/+, maternally inherited mutants; +/(P), paternally inherited mutants. (F) Kcnq1ot1 lncRNA depletion by shRNA abrogates the lncRNA–DNA interaction. The Kcnq1ot1 lncRNA–chromatin interactions were detected at the 2/b site for the Kcnq1 promoter and the 5/b site for KvDMR1. NS, nonsilencing control. A-11 and G-12, shRNA. M, 100-bp marker.
Figure 3. **The Kcnq1 promoter and KvDMR1 interact through intrachromosomal looping.** (A) Schematic diagram shows the EcoRI sites in the Kcnq1 imprinting locus for the 3C assay. R1–R5, EcoRI sites. (B) The 3C assay detects the long-range and local chromatin interactions. (C) Long-range intrachromosomal interactions between the Kcnq1 promoter and KvDMR1 are abrogated in Kcnq1ot1 DNA methylation-silenced cells, knockout mutant mouse, and shRNA-depletion cells. Silenced, Kcnq1ot1-suppressed cells by targeted DNA methylation; control, wild-type cells. Knockout: WT, wild-type cells; −/[M]/+, maternally inherited mutant; +/[P], paternally inherited mutant. shRNA depletion: CT, control; NS, nonsilencing shRNA control; A11 and G12, Kcnq1ot1 shRNAs. (D) The chromatin interactions at the Kcnq1 locus are allele specific. After PCR, the parental alleles were distinguished by PvuII polymorphic restriction enzyme. M, 100-bp marker.
imprinted region. We therefore used 3C (Dekker, 2006; Li et al., 2008) to assess the potential chromatin interactions between these two DNA regions.

Cells were fixed with 2% formaldehyde, digested with restriction enzyme EcoRI, and then ligated with T4 DNA ligase to examine the remote interaction between the Kcnq1 promoter and KvDMR1 that are 200 kb apart (Fig. 3 A). In control mouse fibroblasts that maintain normal Kcnq1ot1 expression, we found that the KvDMR1 region (R4 and R5) directly interacted with the Kcnq1 promoter (R1 and R2; Fig. 3 B, top left, lanes 1–3), suggesting the presence of a long-range intrachromosomal loop. Local chromosomal interactions within KvDMR1 and within the Kcnq1 promoter regions were also detected (Fig. 3 B, top right, lanes 1 and 2, R4/R5 and R1/R2). These intrachromosomal interaction PCR products were confirmed by TA cloning and sequencing (Fig. 3 B, bottom). However, the long-range intrachromosomal interaction was abolished in the Kcnq1ot1 ZF-Ss1–silenced cells (Fig. 3 C, lanes 3 and 4, left middle sections for three EcoRI ligated products: R1/R5, R2/R4, and R2/R5). Similarly, this long-range intrachromosomal interaction was lost in our other two models: the paternally inherited KvDMR1-deleted MEFs (Fig. 3 C, middle, lanes 3 and 4, R1/R5 and R2/R5) and the shRNA knockdown cells (Fig. 3 C, right, lanes 3 and 4, R1/R5 and R2/R5). Of note, the local interactions were not affected by Kcnq1ot1 depletion. These data indicate that the lncRNA Kcnq1ot1 is directly involved in and may be necessary for the maintenance of the long-range intrachromosomal loop between KvDMR1 and Kcnq1 promoter.

Figure 4. Genomic imprinting of Kcnq1 and Cdkn1c is abolished in Kcnq1ot1-silenced cells. [A] Physical map of the Kcnq1 locus shows the placental-specific and ubiquitously imprinted genes. [B–G] The imprinting status of H19, Igf2, Cd81, Kcnq1, Cdkn1c, and Osbp15 was determined by polymorphic restriction enzymes Fox1 (B), DpnII (C), RsaI (D), EcoRV (E), AvaI (F), and BfaI (G), respectively. gDNA, genomic DNA used as the positive control; mock, empty lentiviral vector; control, untreated cells; NS, nonsilencing shRNA control; A-11 and G-12, Kcnq1ot1 shRNAs. Lanes 1–21 in B and C represent the different puromycin-selected ZF-Ss1 colony RNAs transcribed for the analysis of Igf2 (B) and H19 imprinting (C). M, 100-bp marker.
The role of Kcnq1ot1 IncRNA 5’ fragments is examined in shRNA-depleted cells. (A) Schematic diagrams show the 5’ Kcnq1ot1 fragments. Three 5’ subfragments were used in the functional mapping: ER6K (6,020 bp), ER1 (1,950 bp), ER2 (2,030 bp), and ER3 (2,040 bp). (B) RT-PCR shows the expression of exogenous constructs of 5’ Kcnq1ot1 subfragment clones in the MBW2 fibroblast. RT-PCR product sizes: ER6K = 203 bp, ER1 = 483 bp, ER2 = 138 bp, and ER3 = 127 bp. (C) Long-range chromatin interactions are restored by ectopically expressed Kcnq1ot1 subfragments. The chromatin loop between the Kcnq1 promoter (R2) and KvDMR1 (R4) mediated by Kcnq1ot1 IncRNA was detected by 3C. Control, normal MBW2 cells; D11 and G12, Kcnq1ot1 shRNAs. (D) R3C confirms the interaction of the virally expressed 5’ Kcnq1ot1 IncRNA subfragment (Ra) with the Kcnq1 promoter (D1) and KvDMR1 [D6]. M, 100-bp marker.

Kcnq1ot1 IncRNA is required for the maintenance of Kcnq1 and Cdkn1c imprinting

We then examined whether the abolition of the intrachromosomal loop between the Kcnq1 promoter and KvDMR1 after Kcnq1ot1 silencing would affect allelic expression of genes in the Kcnq1 locus (Fig. 4 A). Using PCR, we detected monoallelic expression of Kcnq1 in control and mock-transfected mouse fibroblast cells (Fig. 4 E, lanes 5 and 6); however, in cells that lack Kcnq1ot1, imprinting was lost (Fig. 4 E, lanes 1–4), and Kcnq1 became biallelically expressed. Similarly, Cdkn1c also became biallelically expressed when Kcnq1ot1 was silenced (Fig. 4 F, lanes 1–4). These data thus demonstrate the importance of the Kcnq1ot1-mediated intrachromosomal interaction in the maintenance of allelic expression in this imprinting locus.

Cd81 (Fig. 4 D) and Osbp15 (Fig. 4 G), genes that are normally only imprinted in the placenta, were biallelically expressed in the control cells as well as in the cells that lacked Kcnq1ot1. Imprinting of the far upstream genes Igf2 (Fig. 4 C) and H19 (Fig. 4 B) was not altered, and there was no alteration in the DNA methylation of the Igf2/H19 ICR (Fig. S3 D).

Restoration of intrachromosomal interaction by ectopic expression of 5’ Kcnq1ot1 RNA

Next, we were interested in exploring the mechanism by which Kcnq1ot1 noncoding RNA coordinates the long-range chromatin interaction in cis and, consequently, allelic regulation. We focused on the role of the 5’ Kcnq1ot1 IncRNA in vivo by taking advantage of shRNA knockdown cells that are deficient in Kcnq1ot1 IncRNA and that show an absence of the intrachromosomal interaction and the loss of genomic imprinting. To delineate the specific regions that interact with chromatin DNA, we cloned a 6-kb 5’ Kcnq1ot1 fragment (ER6K) and three 2-kb subfragments (ER1, ER2, and ER3; Fig. 5 A) and virally expressed them in two shRNA knockdown cell clones (G-12 and D-11). The A-11 knockdown cell clone (Fig. 2 A) was excluded from this study as it covers a region that is very close to the 5’ Kcnq1ot1 region. RT-PCR assay showed that these Kcnq1ot1 fragments were expressed in virally transduced cells but not in the control cells (Fig. 5 B).
Long noncoding RNA regulates Kcnq1 imprinting • Zhang et al.

Kcnq1ot1 lncRNA regulates Kcnq1 imprinting

Next, we were interested in exploring trans-chromatin–modifying factors that coordinate the Kcnq1ot1-mediated long-range chromatin interaction and allelic regulation. For this purpose, we focused on PRC2. We first used chromatin RNA immunoprecipitation to examine whether Kcnq1ot1 lncRNA recruits the methyltransferase EZH2, a component of PRC2, in guiding allelic gene regulation. The 5' terminus of Kcnq1ot1 contains an 890-bp silencing region (890 silencing domain; Mohammad et al., 2008) and a conserved repeat motif (MD1 repeats) region, whereas the 3' terminus has a LINE (long interspersed nuclear element) element-rich region (Pandey et al., 2008). We used an anti-EZH2 antibody to immunoprecipitate chromatin complexes containing nuclear RNA. After digestion of genomic DNA and RT of RNA into cDNA, PCR was used to map the binding sites of Kcnq1ot1 to EZH2 (Fig. 6A). We found that in control cells, EZH2 interacted with the 890-bp silencing domain region at the 5' terminus of Kcnq1ot1 (Fig. 6B, top, lane 1). No EZH2 interaction was detected at the central (B and C sites) or 3' terminus (D site; Fig. 6B, top, lanes 2–4). In Kcnq1ot1-silenced cells, the 5' terminal interaction was lost (Fig. 6B, top, lanes 5–8). Using the same approach, we also observed the loss of the EZH2 recruitment in the 5' region of the lncRNA in cells in which the Kcnq1ot1 was knocked down by G-12 shRNA (Fig. 6C).

Kcnq1ot1 lncRNA guides allelic H3-K27 methylation at the Kcnq1 promoter

We also performed RNA FISH and DNA FISH assays simultaneously to examine this chromatin interaction. As previously described (Redrup et al., 2009), we detected the colocalization of the endogenous Kcnq1ot1 RNA with the maternal Kcnq1 chromatin DNA in MBW2 fibroblasts (Fig. S5A). However, the viral 6-kb Kcnq1ot1 lncRNA was abundantly expressed in shRNA knockdown cells, making it hard to detect the single molecule interaction of the viral lncRNA with Kcnq1 chromatin DNA.
Figure 7. Kcnq1ot1 IncRNA guides histone H3-K27 methylation in the Kcnq1 promoter. (A) Schematic diagram shows the size of H3-K27 ChIP products in the Kcnq1 imprinting domain. The letters (a–e) mark the location of the ChIP-specific primers. (B–F) ChIP assay detects the recruitment of Ezh2, CTCF, and histone H3-K27 methylation at the Kcnq1 promoter in Kcnq1ot1-silenced cells (B, D, and F) and shRNA-depleted cells (C and E). Cross-linked DNA–protein
The Kcnq1 promoter and KvDMR1 regions (Fig. 7 B, top and middle, lanes 1–5, blue arrows); however, in Kcnq1ot1-silenced cells, there was far less EZH2 binding at those regions (Fig. 7 B, top and middle, lanes 6–10, red arrows). Similarly, the binding of EZH2 to the promoter and KvDMR1 was lost in the Kcnq1ot1 shRNA knockdown cells (Fig. 7 C). Together, these data suggest that Kcnq1ot1 lncRNA must be present to recruit PRC2 to the targeted DNA regions.

We then examined whether the recruitment of the PRC2 complex would induce the H3-K27 methylation suppression mark in the Kcnq1 promoter. In control cells, we observed H3-K27 hypermethylation at the Kcnq1 promoter and KvDMR1 region (Fig. 7 D, middle, lanes 1–8, blue arrows). In cells that lack Kcnq1ot1, in contrast, there was a decrease in H3-K27me3 in the promoter (Fig. 7 D, middle, lanes 9–16, red arrows), consistent with a loss of EZH2 binding. In support of this finding, we also observed that H3-K27 methylation suppression was abolished in the Kcnq1ot1 shRNA–knocked down cells (Fig. 7 E).

We also examined CTCF binding in these regions. In control cells, CTCF bound only at the KvDMR1 region and not at the Kcnq1 promoter (Fig. 7 D, top, lane 7, blue arrow). In Kcnq1ot1 methylation silenced cells, no CTCF binding was detected (Fig. 7 D and F). These data suggest that CTCF may not directly participate in this long-distance intrachromosomal interaction. Loss of CTCF binding may simply be related to the exclusion of CTCF after DNA methylation at the CTCF site in KvDMR1.

To identify whether EZH2 binding and histone H3-K27 methylation at the Kcnq1 promoter is allele specific, we sequenced the immunoprecipitated DNA. Using a BsmFI SNP site in the Kcnq1 promoter to distinguish the parental alleles, we found that EZH2 binding and H3-K27 methylation occurred in a paternal allele-specific manner (Fig. 7 G). However, this parent-specific EZH2 binding and H3-K27 methylation did not occur in shRNA-mediated Kcnq1ot1 depletion cells (Fig. 7 H). These data thus suggest that Kcnq1ot1 lncRNA guides allelic H3-K27 methylation at the Kcnq1 promoter to induce the allelic imprinting.

**Discussion**

We have begun to unravel the mechanisms by which the lncRNA Kcnq1ot1 regulates allelic expression of a cluster of genes in the Kcnq1 imprinting domain. Using two complementary approaches, we demonstrate that Kcnq1ot1 lncRNA interacts directly with chromatin to form a 200-kb intrachromosomal loop between the downstream Kcnq1 promoter and the KvDMR1 where the Kcnq1ot1 promoter is located.

First, we developed an R3C method to examine this RNA–chromatin DNA interaction. The Kcnq1ot1 lncRNA that was interacting with chromatin was reverse transcribed into double-stranded cDNA. After restriction enzyme digestion, the sticky ends from the Kcnq1ot1 cDNA and the interacting chromatin DNA were ligated, and the chimeric products were identified after PCR amplification. To confirm this lncRNA–DNA interaction, we also used a ChOP method (Mariner et al., 2008; Pandey et al., 2008) to detect enrichment of Kcnq1ot1 lncRNA at the Kcnq1 promoter and KvDMR1 regions. The Kcnq1ot1 lncRNA was pulled down with biotin-labeled antisense oligonucleotides, and the interacting chromatin DNA was amplified by PCR primers. Using these complementary techniques, we demonstrate that Kcnq1ot1 lncRNA interacts with chromatin DNA through its 5′-terminal region, which has been termed the silencing domain (Fig. S4). The R3C methodology can be used to examine specific RNA–DNA interactions throughout the genome.

To demonstrate the role of the lncRNA in regulating this imprinted region, we inhibited Kcnq1ot1 lncRNA synthesis by methylating the CpG island on the paternal allele, thereby silencing the lncRNA’s promoter. This was achieved by constructing a fusion protein containing the ZF DNA binding portion of CTCF connected to Sss1, a DNA methyltransferase. This protein binds to CTCF binding sites and has the ability to methylate nearby CpG moieties in some but not all genes (Zhang et al., 2011). In cells transfected with a lentivirus delivering this fusion construct, we demonstrated that CpG1 was methylated by this fusion protein. Using this novel approach, we showed that the expression of Kcnq1ot1 was greatly decreased in these cells and that no interactions of this lncRNA at KvDMR1 or the Kcnq1 promoter could be detected. These findings were validated in a KvDMR1 knockout mutant mouse cell line (Fig. 2 E; Fitzpatrick et al., 2002) as well as in an shRNA-mediated RNAi depletion model (Fig. 2 F).

The interaction of Kcnq1ot1 with the KvDMR1 and with Kcnq1 loci that are 200 kb apart suggests the presence of a DNA loop that juxtaposes the two regulatory regions (Murrell et al., 2004). Using a standard 3C approach, we showed that the two regions do in fact interact with each other but that this interaction no longer occurs when the Kcnq1 expression is silenced by targeted DNA methylation or by shRNA or is knocked out in the paternally inherited mutant fetal mice (Fig. 3 C), indicating that this lncRNA is a necessary component of this three-dimensional structure. The suppression of Kcnq1ot1 leads to loss of imprinting in this region, as well, suggesting that this long-range chromatin interaction is required for allele-specific gene expression.
Chromatin loops are built and stabilized with a host of nuclear interactions. CTCF has been implicated in numerous long-range interactions, and as a result, it has been called the master weaver of the genome (Phillips and Corces, 2009). CTCF regulates allelic expression of mouse Ig2 by forming a long-range intrachromosomal loop (Murrell et al., 2004; Li et al., 2008; Qiu et al., 2008). Although CTCF binds to KvDMR1, it does not bind to the Kcnq1 promoter, suggesting that CTCF is not involved in the loop formation at the Kcnq1 locus. In this study, however, we have identified a totally novel, RNA-based mechanism for organizing intrachromosomal looping. The R3C data show that only the 5′ terminus of the Kcnq1ot1 IncRNA is necessary for establishing the Kcnq1ot1–DNA interaction. By expressing fragments of the 5′ end of Kcnq1ot1 ectopically, we have localized the DID to a ~6-kb region (Fig. 5). This DID domain binds to both the Kcnq1 promoter and KvDMR1 DNAs and helps establish a long-distance intrachromosomal interaction.

Although the precise mechanisms governing genomic imprinting are not fully understood, it is now clear that each imprinted chromosomal domain contains its ICR, in which the imprinting signal coordinates the imprinting of multiple genes over hundreds of kilobases of DNA (Leighton et al., 1995; Wutz et al., 1997; Thorvaldsen et al., 1998; Fitzpatrick et al., 2002). In the Kcnq1 imprinting domain, the ICR has been identified as a conserved, differentially methylated CpG island within intron 10 of Kcnq1 in both human (De Marzo et al., 1999; Lee et al., 1999) and mouse (Smilinich et al., 1999), and deletion of EZH2 supports a role for EZH2 in imprinting repression and higher-order genomic contraction along the entire parental Kcnq1 cluster (Terranova et al., 2008). In this case, however, it is not the ZF protein CTCF but rather the nuclear noncoding RNA Kcnq1ot1 that conveys the imprinting message from the ICR to the Kcnq1 promoter, which is 200 kb away (Fig. S5 B).

Many questions remain regarding the mechanisms underlying the formation and maintenance of this intrachromosomal looping. Our data are consistent with a model in which the Kcnq1ot1 IncRNA may function as a molecular hinge, using different segments of the 5′-terminal region to link the paternal Kcnq1 promoter and KvDMR1 DNAs together and scaffold a long-range intrachromosomal loop (Fig. S5 B). With this spatial proximity, the imprinting signal in the KvDMR1 can be delivered to the Kcnq1 promoter. The IncRNA then recruits PRC2 to the target Kcnq1 promoter, where allele-specific histone H3-K27 methylation turns off expression of the paternal Kcnq1. On the maternal chromosome or in Kcnq1ot1-deficient cells, failure to form an intrachromosomal loop leads to biallelic expression of Kcnq1. However, it should be noted that the chromatin DNA in imprinted loci, such as the Kcnq1 region, is highly condensed. The compaction of the silenced chromatin may also increase the proximity of the IncRNA with the Kcnq1 promoter as detected by R3C. Further studies are needed to validate the role of Kcnq1ot1 IncRNA in the formation of an intrachromosomal loop by excluding the involvement of condensed chromatin.

In summary, formation of intrachromosomal looping may constitute a common mechanism underlying the long-range cis-regulation of clustered genes. We present a model whereby the Kcnq1ot1 IncRNA participates in long-distance chromatin interaction through its 5′-terminal region. EZH2 is then recruited to the Kcnq1 promoter by the nuclear factor interaction domain of the IncRNA (Fig. S5 B). We suggest that the Kcnq1ot1 IncRNA acts as a scaffold molecule to juxtapose the Kcnq1 promoter region to the KvDMR1 region on the paternal chromosome. This concept is supported by the fact that the long-range chromosomal interaction conformation was lost after the silencing of Kcnq1ot1 by targeted DNA methylation. This long-range intrachromosomal interaction is essential for the maintenance of imprinting of genes in the Kcnq1 region. Thus, long-range chromatin interactions may play an important role in the allelic regulation of gene expression and genomic imprinting. Because many imprinted genes have noncoding RNAs associated with them, it is likely that these IncRNAs may play an important role in regulating chromatin structure to direct gene expression (Spitale et al., 2011).

Materials and methods

Cell lines
Mouse fibroblast MBW2 cells were cultured from an F1 newborn mouse derived from breeding an M. spretus male with a C57B/6 female in our laboratory as previously described (Hu et al., 1996, 1997). These cells maintain normal monoallelic expression for imprinted genes (Hu and Hoffman, 2001), including Kcnq1ot1 and Kcnq1. 293SF-PacLV cells were provided by R. Gilbert from the National Research Council (Ottawa, Ontario, Canada) and were cultured as previously described (Broussau et al., 2008). The Kcnq1ot1 knockout MEFs were cultured from embryonic day 15.5–16.5 fetuses, which carry the deletion of a 2.8-kb KvDMR1 fragment containing the Kcnq1ot1 promoter (Fitzpatrick et al., 2002). Both MBW2 and Kcnq1ot1 knockout MEFs were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin.

Methylation targeting vector construction
The CTCF analogue ZF-Sxs1 was synthesized as previously described (Zhang et al., 2011). In brief, Sxs1 DNA methyltransferase DNA was amplified from Spiroplasma monobiae strain MQ1 (33825; ATCC) genomic DNA, and the cDNA fragment encoding the ZF domain was generated from the pOB7/CTCF vector by PCR amplification. These two DNA fragments were then linked by SV40 NSL and a short linker sequence to produce the ZF-Sxs1 construct, which was then cloned into the Nhel-BamHI sites in pCDH-CMV-MCS-5′-F1-Puro lentivirus vector (System Biosciences, Inc.).

Lentiviral transduction
The lentiviruses were generated in 293SF-PacLV packing cells that stably express VSVG, Gag-Pol, and Rev packaging genes (Broussau et al., 2008). In brief, cells were transfected with the lentiviral expression vector. 12 h after transfection, culture medium was replaced by fresh medium containing 1 μg/μl doxycycline and 30 μg/μl cume. The virus-containing supernatants were collected at 48 and 72 h. The pooled viral supernatants were filtered through a 0.35-mm filter, concentrated by a PEG-ii kit (System Biosciences, Inc.), and stored at −80°C. Mouse MBW2 fibroblasts were seeded at 1.0 × 104 cells per well in 6-well plates 24 h before transduction. The medium was replaced with virus-containing supernatant containing 5 μg/ml polybrene (Sigma-Aldrich) and incubated overnight. 24 h after transduction, the virus-containing medium was replaced with fresh medium. 4 d later, the infected cells were harvested by trypsinization and replated on a new 100-mm dish. When cell confluence reached 20–30%, the culture medium was replaced by fresh medium containing 1 μg/ml puromycin for colony selection. The medium was changed every 3–4 d. After selection, colonies were chosen and expanded for further analyses.

DNA methylation analysis
Total nucleic acids were extracted from normal and transformed MBW2. As previously described (Yao et al., 2003; Chen et al., 2006), total nucleic acid was treated with sodium bisulfate, and PCR was performed using DNA methylation–specific primers designed for CTCF binding sites (Table S1). To examine the status of DNA methylation in every CpG site in the two
CTCF binding regions of KvDMR1, the amplified PCR DNAs were cloned into the TA vector (Invitrogen) and sequenced. DNA in the third CTCF binding site of the Igf2/H19 differentially methylated region was amplified and separated by BeStUl to determine methylated and unmethylated DNAs.

3C

The 3C assay was performed as previously described (Dekker, 2006; Zhang et al., 2011). In brief, cells were cross-linked with 2% formaldehyde and lysed with cell lysis buffer. An aliquot of nuclei (2 x 10^4) was digested with 5 U EcoRI at 37°C overnight. Chromatin DNA was diluted with ligation buffer (New England Biolabs, Inc.) and ligated with 4,000 U T4 DNA ligase. DNA was extracted with phenol-chloroform and used for PCR amplification using primers in Table S2.

R3C

Cells were fixed with 2% formaldehyde and lysed with cell lysis buffer (10 mM Tris, pH 8.0, 10 mM NaCl, 0.2% NP-40, and protease inhibitors). Nuclei were suspended in 100 µl cDNA synthesis buffer in the presence of 0.3% SDS. After incubated at 37°C for 1 h, Triton X-100 was added to a final concentration of 1.8% to sequester the SDS. The first-stand cDNAs were synthesized in an 80-µl reaction [3 µl random primer, 3 µl gene-specific primer, 10 mM deoxy-ATP [1.5 µl], 10 mM deoxy-GTP [1.5 µl], 10 mM deoxy-TP [1.5 µl], 0.4 mM dCTP-biotin [3.75 µl], 10 mM dCTP [1.35 µl], 6 µl RT enzyme, 6 µl RNase inhibitors, 0.1 M DTT [6 µl], and 18 µl 5x cDNA synthesis buffer]. The second-strand cDNA was synthesized in a 150-µl reaction using cDNA Synthesis Kit (Agilent Technologies). A 50-µl aliquot was digested with 600 U EcoRI at 37°C overnight. After stopping the reaction by adding 1.6% SDS and incubating the mixture at 65°C for 20 min, chromatin DNA was diluted with NEB ligation reaction buffer and then ligated with 4,000 U T4 DNA ligase at 16°C for 4 h (final DNA concentration of 2.5 µg/ml). After treatment with 10 mg/ml proteinase K at 65°C overnight to reverse cross-links and with 0.4 µg/ml RNase A for 30 min at 37°C, DNA was extracted with phenol–chloroform, ethanol precipitated, and then purified by streptavidin beads (Invitrogen). Purified DNA was used for PCR amplification using primers in Table S3. Synthetic oligonucleotides were used as positive controls. The R3C products were cloned into plasmid vector (Thermo Fisher Scientific) and sequenced to confirm the IncRNA–chromatin DNA interaction.

R3C control assays

We first examined whether the biotin-labeled DNA fragments were able to be digested by restriction enzyme EcoRI. Biotinylated DNAs were prepared by PCR amplification of plasmid vectors pcDH1 (System Biosciences, Inc.) and pEGFPC1 (Takara Bio Inc.) in the presence of 1 µl of 0.4 mM dCTP-biotin (Invitrogen). Amplified PCR products were digested by EcoRI enzyme. We then examined whether the crosslinked chromatin RNA were able to be reverse transcribed. As described in the previous R3C section, the EcoRI-digested sample was immunoprecipitated by 5-10 µg histone H3 antibody (EMD Millipore), pulled down by protein G magnetic beads (EMD Millipore), and pretreated by DNase I for 30 min at 37°C. The first-stand cDNA was synthesized using a cDNA kit (Agilent Technologies). After cross-linking reversal, PCR was used to examine the synthesized cDNA by specific PCR primers: pKcnq1I (H6/H9) and Kcnq1ot1 (H1.45/H1.46).

Finally, we examined whether the second-strand cDNA can be synthesized on cross-linked chromatin IncRNA. The first-strand cDNA was synthesized on the anti-histone H3 pull down protein G magnetic beads using a strand-specific RT primer R4A (Table S2). The second-strand cDNA was synthesized using the cDNA kit (Agilent Technologies) in the presence of 3.75 µl of 0.4 mM dCTP-biotin. After washing beads three to five times with 500 µl PBS buffer with 0.1% Tween 20, the synthesized second-strand cDNA was released from the beads and purified by biotin-streptavidin pull-down (Invitrogen). PCR reactions were performed to distinguish double-strand cDNA and genomic DNA: double-strand cDNA (H1.45/H1.48) and genomic DNA (H1.45/H263; H263 sequence, 5'-TAGAGATCCGGGCTCGGAGCCGACT3').

ChiP

ChiP assays were performed as described previously (Zhang et al., 2011). In brief, cells were fixed with 1% formaldehyde and sonicated on ice with a Branson sonicator. 150 µl of sonicated chromatin was immunoprecipitated using 2-5 µl of specific antiseraum and 60 µl protein G-agarose. Antibodies to SMCT1, CTCF, SUZ12, Ezh2, trimethyl-H3-K27 (lysine 27 of histone H3), and dimethyl-H3-K4 (lysine 9 of histone H3) were obtained from Abcam. The DNA was extracted for PCR using primers listed in Table S4.

RNA ChiP

RNA immunoprecipitation assays were performed as described by Rinn et al. (2007). In brief, cells were fixed with 1% formaldehyde, treated with DNase I, and sonicated using a sonicator (Vibra-Cell VCX130; Sonic & Materials, Inc.). Sonicated samples were immunoprecipitated with protein G-agarose, and antibodies were raised against SMCT1, SUZ12, Ezh2, and IgG (Abcam). The precipitated RNA was released, and cDNA was synthesized. After proteinase K treatment, DNA was precipitated, and Kcnq1ot1 and GAPDH were detected by semi-quantitative PCR using the primers listed in Table S5.

ChOP

The ChOP assays were performed according to published protocol (Mariner et al., 2008; Pandey et al., 2008). In brief, nuclei were lysed in nuclear lysis buffer, sonicated, incubated with 50 pmol biotin-labeled antisense oligonucleotide, and pulled down according to the manufacturer’s protocol (Invitrogen). The enrichment of Kcnq1ot1-interacting genomic DNA regions was analyzed by PCR using primers derived from the Kcnq1 locus (Table S6).

Determination of genomic imprinting

Total RNA extraction and cDNA synthesis were performed as previously described (Hu et al., 1995). Allelic expression of Kcnq1, Cdkn1c, Osbpl5, and Cdh1 was examined by PCR in cDNA samples using primers specific for polymorphic restriction enzymes. Allelic expression of Igf2 and H19 was assessed by polymorphic restriction enzyme DpnII and Foxl, respectively. PCR primers used to assess allelic expression are listed in Table S7.

RNA extraction and RT-PCR analysis

Total RNA was extracted by TRI Reagent (Sigma-Aldrich), and cDNA was synthesized using RNA RT. PCR was performed using KlenTaq I mix, and amplified PCR products were quantified and normalized using GAPDH as a control (Tables S5 and S8).

shRNA lentivirus and selection

Kcnq1ot1, SMCT1, Kcnq1, and H19, and NespAS shRNA were purchased from Thermo Fisher Scientific (Table S9). Selection was performed with 3 µg/ml puromycin, and the clones were screened for GFP expression.

5'-end Kcnq1ot1 constructs

Four fragments of the 5'-terminus of Kcnq1ot1, including a full 6kb sequence (ER6K; 6,020 bp), ER1 (1,950 bp), ER2 (2,030 bp), and ER3 (2,040 bp) sequences, were amplified from bacterial artificial chromosome (BAC) clone RP23-400C11 and cloned into the Nhel–NotI sites in pCDH-CMV-MCS-E1–Puro lentivirus vector (Table S10).

RNA–DNA FISH

Cells were fixed with 4% formaldehyde/10% acetic acid and stored overnight in 70% ethanol. For RNA FISH, fluorescence-labeled single-strand DNA probes (Stellaris) were synthesized by Biosearch Technologies and were hybridized according to manufacturer’s protocol. To increase stability of RNA FISH, RNA signals were detected with a tyramide–Alexa Fluor 488 signal amplification kit (Invitrogen). For DNA FISH, BAC probes labeled with DNP-dUTP were denatured and hybridized overnight. Detection of BAC probes was performed with rabbit anti-DNP (Invitrogen) and secondary goat anti-rabbit conjugated to Alexa Fluor 594. After labeling, fluorescence was detected using a microscope (BX41; Olympus). Optical sections of 0.2 µm were collected with SlideBook 5.0 (Intelligent Imaging Innovations, Inc.).

Statistical analysis

All experiments were performed in triplicate, and the data are expressed as means ± SD. The comparative threshold cycle method was applied in the quantitative real-time RT-PCR assay according to the ΔΔ threshold cycle method.

Online supplemental material

Fig. S1 shows the control assays that validate each critical step in the R3C approach (Table S8). Fig. S2 shows the data of negative controls for R3C method. Fig. S3 demonstrates shRNA knockdown of Kcnq1ot1 IncRNA. Fig. S4 validates the Kcnq1ot1 IncRNA–chromatin DNA interaction by ChOP assay. Fig. S5 shows the interaction of Kcnq1ot1 IncRNA with Kcnq1 promoter DNA by RNA–DNA FISH and the proposed model for Kcnq1ot1 IncRNA-mediated Kcnq1 imprinting. Table S1 shows primers used for the DNA methylation assay. Table S2 shows primers used for 3C at the Kcnq1 locus. Table S3 shows primers used for R3C at the Kcnq1 locus.

Statistical analysis

All experiments were performed in triplicate, and the data are expressed as means ± SD. The comparative threshold cycle method was applied in the quantitative real-time RT-PCR assay according to the ΔΔ threshold cycle method.

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Table S4 shows primers used for the ChIP assay. Table S5 shows primers used for the RNA ChIP assay. Table S6 shows oligonucleotides and PCR primers used for the ChOP assay. Table S7 shows primers for allelic expression of imprinted genes. Table S8 shows primers used to amplify DNA fragments with the EcoRI site. Table S9 shows primers and shRNA sets for RNAi. Table S10 shows primers for the KvDmr1-5’-end deletion assay. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201304152/DC1.

We thank the Protein and Nucleic Acid Facility at the Stanford University School of Medicine for oligonucleotide synthesis. This work was supported by a California Institute of Regenerative Medicine grant (RT2-01942), Jila International Collaboration grant (2011-20720), and a Natural Science Foundation of China grant (81102323) to J.-F. Hu; the National Natural Science Foundation of China grant (81272294) to J.-F. Hu; the 973 Program grant (RT2-01942), Jilin International Collaboration grant (20120720), and a Shanghai Leading Academic Discipline Project (XDA01040411) to S. Ge; and a Shanghai Leading Academic Discipline Project the National Natural Science Foundation of China grant (81172323) and the 973 Program grant (86473032) to M. Lee, M.P., M.R. DeBaun, K. Mitsuya, H.L. Galonek, S. Brandenburg, M. Oshimura, and M.J. Higgins. 2002. Regional loss of methylation of imprinting control region. Development 129:675–684.

References

Submitted: 23 April 2013
Accepted: 25 November 2013

Lee, M.P., M.R. DeBaun, K. Mitsuya, H.L. Galonek, S. Brandenburg, M. Oshimura, and A.P. Feinberg. 1999. Loss of imprinting of a paternal expressed transcript, with antisense orientation to KVLQT1, occurs frequently in Beckwith-Wiedemann syndrome and is independent of insulin-like growth factor II imprinting. Proc. Natl. Acad. Sci. USA. 96: 5203–5208. http://dx.doi.org/10.1073/pnas.96.9.5203

Leighton, P.A., R.S. Ingram, J. Eggenschwiler, A. Elfratadiat, and S.M. Tilghman. 1995. Disruption of imprinting caused by deletion of the H19 gene region in mice. Nature. 375:34–39. http://dx.doi.org/10.1038/375030a0

Li, T., J.F. Hu, X. Qiu, J. Ling, H. Chen, S. Wang, A. Hou, T.H. Vu, and A.R. Hoffman. 2008. CTCF regulates allelic expression of Igf2 by orchestrating a promoter-polycomb repression complex 2 intrachromosomal loop. Mol. Cell. Biol. 28:6473–6482. http://dx.doi.org/10.1128/MCB.00204-08

Mancini-DiNardo, D., S.J. Steele, R.S. Ingram, and S.M. Tilghman. 2003. A differentially methylated region within the gene KvDmr1 functions as an imprinting promoter and silencer. Hum. Mol. Genet. 12:283–294. http://dx.doi.org/10.1093/hmg/ddg024

Mariner, P.D., R.D. Walters, J. Beroukhim, R.R. Pandey, S. Ardlund, S.D. Wagner, J.F. Kugel, and J.A. Goodrich. 2008. Human Alu RNA is a modular transcriptional repressor of mRNA transcription during heat shock. Mol. Cell. 29:499–509. http://dx.doi.org/10.1016/j.molcel.2007.12.013

Mohammad, F., R.R. Pandey, T. Nagano, L. Chakalova, T. Mondal, P. Fraser, and C. Kanduri. 2008. Kcnq1ot1/Lit1 noncoding RNA mediates transcriptional silencing by targeting to the perinucleolar region. Mol. Cell. Biol. 28:3713–3728. http://dx.doi.org/10.1128/MCB.02263-07

Murakami, K., M. Oshimura, and H. Kugoh. 2007. Suggestive evidence for chromosomal localization of non-coding RNA from imprinted LIT1. J. Hum. Genet. 52:926–933. http://dx.doi.org/10.1007/s10038-007-0196-4

Murrell, A., S. Heeson, and W. Reik. 2004. Interaction between differentially methylated regions partitions the imprinted genes Igf2 and H19 into parent-specific chromatin loops. Nat. Genet. 36:889–893. http://dx.doi.org/10.1038/ng1402

Nagano, T., and P. Fraser. 2011. No-nonsense functions for long noncoding RNAs. Cell. 145:178–181. http://dx.doi.org/10.1016/j.cell.2011.03.014

Pandey, R.R., T. Mondal, S. Enroth, L. Redrup, J. Morsomro, 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

Paulsen, M., T. Khare, C. Burgard, S. Tierling, and J. Walter. 2005. Evolution of the Beckwith-Wiedemann syndrome region in vertebrates. Genome Res. 15:146–153. http://dx.doi.org/10.1101/gr.2689805

Phillips, J.E., and V.G. Corces. 2009. CTFC: master weaver of the genome. Cell. 137:1194–1211. http://dx.doi.org/10.1016/j.cell.2009.06.001

Prasanth, K.V., and D.L. Spector. 2007. Eukaryotic regulatory RNAs: an answer to the ‘genome complexity’ conundrum. Nat. Rev. Genet. 8:3855–3860.

Rinn, J.L., M. Kertesz, J.K. Wang, S.L. Squazzo, X. Xu, S.A. Brummage, L.H. Goodnough, J.A. Helms, P.J. Farnham, E. Segal, and H.Y. Chang. 2007. Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs. Cell. 129:1311–1323. http://dx.doi.org/10.1016/j.cell.2007.05.022

Smilde, A.J., C.D. Day, G.V. Fitzgerald, G.M. Caldwell, A.C. Lossie, P.R. Cooper, A.C. Smallwood, J.A. Joyce, P.N. Schofield, W. Reik, et al. 1999. A methylated CpG island in KVLQT1 is associated with an antisense paternal transcript and loss of imprinting in Beckwith-Wiedemann syndrome. Proc. Natl. Acad. Sci. USA. 96:8064–8069. http://dx.doi.org/10.1073/pnas.96.14.8064

Spitale, R.C., M.C. Tsai, and H.Y. Chang. 2011. RNA templating the epigenome: long noncoding RNAs as molecular scaffolds. Epigenetics. 6:539–543. http://dx.doi.org/10.4161/epi.6.5.15221

Terranova, R., S. Yokobayashi, M.B. Stadler, A.P. Ote, M. van Lohuizen, S.H. Orkin, and A.H. Peters. 2008. Polycomb group proteins Ezh2 and Rnf2 distinctively silence noncoding RNA from the Beckwith-Wiedemann syndrome region in vertebrates. Proc. Natl. Acad. Sci. USA. 129:1311–1323. http://dx.doi.org/10.1073/pnas.1200003

Thakur, N., V.K. Tiwari, H. Thomassini, R.R. Pandey, M. Kanduri, A. Gondor, T. Grange, R. Ohlsson, and C. Kanduri. 2004. An antisense RNA regulates the bidirecational silencing property of the Kcnq1 imprinting control region. Mol. Cell. Biol. 24:7855–7862. http://dx.doi.org/10.1128/MCB.24.18.7855-7862.2004
Thorvaldsen, J.L., K.L. Duran, and M.S. Bartolomei. 1998. Deletion of the H19 differentially methylated domain results in loss of imprinted expression of H19 and Igf2. *Genes Dev.* 12:3693–3702. http://dx.doi.org/10.1101/gad.12.23.3693

Wutz, A., O.W. Smrzka, N. Schweifer, K. Schellander, E.F. Wagner, and D.P. Barlow. 1997. Imprinted expression of the Igf2r gene depends on an intronic CpG island. *Nature.* 389:745–749. http://dx.doi.org/10.1038/39631

Yao, X., J.F. Hu, M. Daniels, H. Shiran, X. Zhou, H. Yan, H. Lu, Z. Zeng, Q. Wang, T. Li, and A.R. Hoffman. 2003. A methylated oligonucleotide inhibits IGF2 expression and enhances survival in a model of hepatocellular carcinoma. *J. Clin. Invest.* 111:265–273.

Zhang, H., B. Niu, J.F. Hu, S. Ge, H. Wang, T. Li, J. Ling, B.N. Steelman, G. Qian, and A.R. Hoffman. 2011. Interruption of intrachromosomal looping by CCCTC binding factor decoy proteins abrogates genomic imprinting of human insulin-like growth factor II. *J. Cell Biol.* 193:475–487. http://dx.doi.org/10.1083/jcb.201101021