Kcnq1ot1 noncoding RNA mediates transcriptional gene silencing by interacting with Dnmt1

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SUMMARY
A long noncoding RNA, Kcnq1ot1, regulates the expression of both ubiquitously and tissue-specific imprinted genes within the Kcnq1 domain. Here, we have generated a knockout mouse with a deletion encompassing an 890-bp silencing domain (Δ890) downstream of the Kcnq1ot1 promoter. Maternal transmission of the Δ890 allele has no effect on imprinting, whereas paternal inheritance of the deletion leads to selective relaxation of the imprinting of ubiquitously imprinted genes to a variable extent in a tissue-specific manner. Interestingly, the deletion affects DNA methylation at somatically acquired differentially methylated regions (DMRs), but does not affect the histone modifications of the ubiquitously imprinted genes. Importantly, we found that Kcnq1ot1 recruits Dnmt1 to somatic DMRs by interacting with Dnmt1, and that this interaction was significantly reduced in the Δ890 mice. Thus, the ubiquitous and placental-specific imprinting of genes within the Kcnq1 domain might be mediated by distinct mechanisms, and Kcnq1ot1 RNA might mediate the silencing of ubiquitously imprinted genes by maintaining allele-specific methylation through its interactions with Dnmt1.

KEY WORDS: Noncoding RNA, Epigenetics, Chromatin, Genomic imprinting, Kcnq1ot1

INTRODUCTION
Genomic imprinting is an epigenetic phenomenon in mammals whereby the expression of a subset of autosomal genes is restricted to one of the parental chromosomes such that they are expressed either from the maternal or the paternal chromosome. So far more than 80 imprinted genes have been identified and most often they are organized in clusters. The short stretches of differentially methylated DNA sequences known as imprinting control regions (ICRs) play a crucial role in regulating the imprinting of multiple genes through various mechanisms (Bartolomei, 2009). Interestingly, some of the ICRs that map to introns of protein coding genes act as promoters for long noncoding RNAs (ncRNAs); for example, for Kcnq1ot1 and Airn. Transcription of these long ncRNAs is functionally linked to the epigenetic silencing of multiple genes in cis (Latos and Barlow, 2009; Lewis and Reik, 2006; Mercer et al., 2009; Whitehead et al., 2008).

Kcnq1ot1 is 91.5 kb ncRNA, which maps to the 1 Mb Kcnq1 imprinted cluster located at the distal end of mouse chromosome 7 (Pandey et al., 2008; Redrup et al., 2009). The Kcnq1ot1 promoter maps to the Kcnq1 ICR and is expressed only from the paternal chromosome, as the ICR is methylated on the maternal chromosome (Smilinich et al., 1999). The expression of Kcnq1ot1 on the paternal chromosome is functionally linked to the silencing of eight to ten genes in cis (Mancini-Dinardo et al., 2006). The genes that are located near the Kcnq1ot1 promoter (Kcnq1, Cdkn1c, Slc22a18 and Phlda2) are imprinted both in the embryo and in the placenta, and hence they are considered to be ubiquitously imprinted genes. By contrast, the genes that are located far from the Kcnq1ot1 promoter (Ascl2, Cd81, Tssc4 and Osbp5) are imprinted only in the placenta and are considered to be placental-specific imprinted genes (Fig. 1A). Although Kcnq1ot1 RNA regulates both ubiquitously and placental-specific imprinted genes, the mechanisms regulating these two classes of genes remain unclear.

Long ncRNAs such as Kcnq1ot1 and Airn interact with members of the polycomb repressive complexes (PRC1 and PRC2) and/or with G9a, and recruit these proteins to the promoters of flanking genes to establish repressive chromatin marks (Nagano et al., 2008; Pandey et al., 2008; Terranova et al., 2008; Wagschal et al., 2008). However, the functional sequences within these long ncRNAs that mediate the silencing of cis-linked imprinted genes remain unknown. By using an episome-based system, we have previously shown that an 890 base pair (bp) silencing domain, 610 bp downstream of the Kcnq1ot1 transcription start site, plays a central role in bidirectional silencing (Mohammad et al., 2008). Here, we show that a targeted deletion of the 890-bp silencing domain in mouse resulted in a selective relaxation of imprinting of the ubiquitously imprinted genes. We found that the 890-bp region regulates the imprinting of ubiquitously imprinted genes by maintaining DNA methylation of the somatic DMRs through interacting with Dnmt1.

MATERIALS AND METHODS
Generation of the Δ890 mouse
The targeting construct was generated by PCR, by amplifying the flanks from the mouse genomic DNA and ligating them to the 3.6-kb Kcnq1 ICR, which lacks the 890-bp silencing domain (Mohammad et al., 2008). The loxP-flanked neomycin cassette containing a testis-specific Cre and the neomycin resistance gene was inserted at the point of the deletion. The targeting construct was electroporated into R1 ES cells. After selection with G418, the drug resistant colonies were expanded. Genomic DNAs from the expanded colonies were screened by Southern hybridization for the neomycin-resistance marker.

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Two independently targeted ES cell clones were injected into C57BL/6 (B6) blastocysts, which were then transferred to B6 pseudopregnant foster mothers. Male chimeras were mated to B6 mice and their progeny were genotyped using PCR to identify germ-line transmission of the \(890\) allele, using the primers listed in Table S1 in the supplementary material. The neomycin cassette was removed in the F1 progeny by the Cre recombinase expressed in the testes of chimeric mice during germline transmission. All animal experiments were performed according to Swedish Animal Board guidelines (application number: C48/8).

**Allele-specific expression analysis**

RNA was extracted from E13.5 and E11.5 placenta and liver tissues, dissected from mutant as well as wild-type littermates from crosses between heterozygous mutant mice in the Mus musculus (B6) background and wild-type Mus spretus (SD7), using the Trizol reagent (Invitrogen). Random primed cDNA was synthesized with Superscript II Reverse Transcriptase (Invitrogen) using 2 \(\mu\)g of DNase I-treated RNA as a template. cDNA was used for quantitative PCR (qPCR) using allele-specific primers (Terranova et al., 2008) (see Table S2 in the supplementary material). Alternatively, RT-PCR was performed using the primers listed in Table S3 in the supplementary material. Products were directly sequenced. Allele-specific expression analysis was performed using a PCR approach (primers are listed in Table S4 in the supplementary material). (A) Southern hybridization of DNA derived from the wild-type and the heterozygous \(890 + \text{Neo}\) ES cells. Genomic DNA was digested with BamHI or Asel, and hybridized with the P1 and P2 probes. (C) Genotyping of mice using a PCR approach (primers are listed in Table S1 in the supplementary material). (D) The effect of an 890-bp deletion on Kcnq1ot1 RNA expression, analyzed by qRT-PCR. No significant difference between wild-type and mutant tissues was found (P>0.05). Data represent mean±s.d. of three independent biological replicates. (E) CpG island methylation of the Kcnq1 ICR of wild-type and \(890\) crosses, analyzed by bisulphite sequencing using DNA from E13.5 placentas.

**DNA methylation analysis**

Bisulphite sequencing was performed by converting 1.0 \(\mu\)g of genomic DNA using the EpiTest Bisulphite kit (Qiagen). Fragments encompassing the Cdkn1c and Slc22a18 DMRs were amplified using the primers listed in Table S4 in the supplementary material. PCR products were cloned into the pGEM-T Easy vector (Promega) and individual clones were sequenced. Methylation statistics were generated for 10 to 25 unique clones with a conversion rate above 96% using the BiQ Analyzer software (Bock et al., 2005). SNPs that were used to distinguish parental alleles are shown in Fig. S8 in the supplementary material. For Southern hybridization (20 \(\mu\)g) and qPCR-based quantification of Cpg methylation (5.0 \(\mu\)g), genomic DNA from E13.5 placenta and liver tissues was digested with appropriate restriction endonucleases. Southern hybridization was carried out as described previously (Chandrasekhar and Raman, 1997). qPCR-based analysis of methylation was performed using primers flanking the methylation-sensitive restriction sites.

**Chromatin immunoprecipitation (ChIP)**

ChIP analysis was performed as previously described (Kanduri et al., 2006). Chromatin was immunopurified using antibodies specific for H3K27me3 (Upstate) and Dnmt1 (IMGENEX). qPCR was performed on the purified ChIP material using the primers listed in Table S5 in the supplementary material.

**Chromatin RNA immunoprecipitation (ChRIP)**

ChRIP and RIP were performed as described previously (Pandey et al., 2008).

**Chromatin conformation capture (3C)**

The 3C experiment was performed according to the published protocol (Hagege et al., 2007), using the Trizol reagent (Invitrogen). Bisulphite sequencing was performed by converting 1.0 \(\mu\)g of DNase I (RQ1, Promega)-treated DNA using the EpiTest Bisulphite kit (Qiagen). Fragments encompassing the \(890\) region were amplified using the primers listed in Table S5 in the supplementary material. PCR products were cloned into the pGEM-T Easy vector (Promega) and individual clones were sequenced. Methylation statistics were generated for 10 to 25 unique clones with a conversion rate above 96% using the BiQ Analyzer software (Bock et al., 2005). SNPs that were used to distinguish parental alleles are shown in Fig. S8 in the supplementary material. For Southern hybridization (20 \(\mu\)g) and qPCR-based quantification of Cpg methylation (5.0 \(\mu\)g), genomic DNA from E13.5 placenta and liver tissues was digested with appropriate restriction endonucleases. Southern hybridization was carried out as described previously (Chandrasekhar and Raman, 1997). qPCR-based analysis of methylation was performed using primers flanking the methylation-sensitive restriction sites.
The homozygous Δ890 mice appeared normal and were fertile. The 890-bp deletion had no effect on Kcnq1ot1 expression or on its promoter methylation (Fig. 1D,E; see also Fig. S1 in the supplementary material). There was no difference in the Kcnq1ot1 RNA sub-cellular localization or half-life in the Δ890 mice when compared with wild-type mice (see Fig. S2A,B in the supplementary material).

**Targeted deletion of the 890-bp region affects the imprinting of ubiquitously imprinted genes in a lineage-specific manner**

We analyzed the effect of the 890-bp deletion on the imprinting of eight maternally expressed genes by crossing the mutant mice (in a *M. musculus* background) with *M. spretus* mice (SD7, which is a congenic strain containing the distal chromosome 7 of *M. spretus* in the *M. musculus* background). Although the Δ890 mice have a mixed genetic background consisting of 129 (129X1/SvJ × 129S1) and B6, we designated these mice as B6 for simplicity. Allele-specific quantitative RT-PCR analyses were performed to analyze the imprinting pattern at the Kcnq1 locus (Fig. 2; see also Table S2 in the supplementary material). All of the allele-specific primers used were able to measure *M. musculus* or *M. spretus* RNA specifically, as well as quantitatively (Fig. 2, control panel; see also Fig. S3 in the supplementary material). In addition, allele-specific expression analyses were performed by direct sequencing of the RT-PCR products (see Fig. S4 in the supplementary material). Imprinting assays were also performed in mice with the Kcnq1ot1 promoter...
development as a control, because the promoter deletion affects both ubiquitously and placental-specific imprinted genes (Mancini-Dinardo et al., 2006).

Paternal transmission of the \( \Delta890 \) allele resulted in the loss of allele-specific expression of the ubiquitously imprinted genes \( Kcnq1, Cdkn1c, Slc22a18 \) and \( Phlda2 \), whereas the genes that are imprinted only in placenta (\( Ascl2, Osbp5 \) and \( Tsc3 \)) retained maternal-specific expression in the placenta, indicating that the 890-bp deletion affects primarily the imprinting of ubiquitously imprinted genes (Fig. 2, second panel; see also Fig. S4 in the supplementary material). By contrast, maternal-specific expression of all eight imprinted genes was noted when the \( \Delta890 \) allele was inherited maternally (Fig. 2, third panel; see also Fig. S4 in the supplementary material). In wild-type mice, \( Kcnq1 \) showed partial relaxation of imprinting by E13.5 and, hence, the imprinting analysis was performed using E11.5 mice, where \( Kcnq1 \) showed complete imprinting. Interestingly, \( Kcnq1 \) showed relaxation of imprinting both in placenta and in fetal liver, whereas \( Cdkn1c \) and \( Phlda2 \) showed relaxation of imprinting only in the placenta, and \( Slc22a18 \) showed relaxation of imprinting only in the fetal liver (Fig. 2A,B). \( Cd81 \) showed biallelic expression with maternal bias in the wild-type placenta and fetal liver, which is consistent with the previously published observations (Lewis et al., 2004). We did not observe any loss of this maternal-biased expression in either the promoter deletion or \( \Delta890 \) mice (Fig. 2). The loss of imprinting of \( Phlda2 \) was specific to placenta, but not to fetal liver, in both the \( Kcnq1 \) promoter deletion and the \( \Delta890 \) mice, indicating that RNA-independent mechanisms regulate imprinting at the \( Kcnq1 \) domain in the liver. Taken together, these observations indicate that the 890-bp region is an important functional region of the \( Kcnq1 \) RNA and that its deletion affects the paternal repression of ubiquitously imprinted genes to variable extents in a lineage-specific manner.

The effects of the 890-bp deletion on imprinting are very specific, because a 657-bp deletion that encompasses a region between the \( Kcnq1 \) promoter and the 890-bp region, and that contains conserved MD1 repeats, has no effect on the imprinting of genes within the \( Kcnq1 \) domain, which indicates that the conserved MD1 repeats have no functional role in the establishment or maintenance of imprinting in the \( Kcnq1 \) domain (Mancini-Dinardo et al., 2006).

**The 890-bp region regulates CpG methylation levels of somatically acquired differential methylated regions (somatic DMRs)**

Selective loss of the imprinting of the ubiquitously imprinted genes in \( \Delta890 \) mice is comparable to that in \( Dnmt1 \) mutant mice, where loss of imprinting of the ubiquitously imprinted but not placental-specific imprinted genes was observed (Lewis et al., 2004), suggesting that the 890-bp region might function by regulating DNA methylation levels. \( Cdkn1c \) and \( Slc22a18 \) have somatic DMRs and are methylated on the paternal, but not on the maternal, chromosome in both placenta and embryo (Lewis et al., 2004). The methylation patterns of these DMRs were analyzed using bisulphite sequencing. In the \( \Delta890 \) mice, a decrease in DNA methylation was found over both the DMRs in placenta as well as in fetal liver (Fig. 3A-D). We confirmed the bisulphite sequencing results using two complementary approaches: qPCR-based quantification of methylation and Southern blotting (see Fig. S5A,B in the supplementary material). Although loss of imprinting of the \( Cdkn1c \) and \( Slc22a18 \) DMRs occurred in both placenta and fetal liver, relaxation of imprinting was detected only in the placenta for

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**Fig. 3. The effect of the 890-bp deletion on DNA methylation at the Kcnq1 locus.** (A-D) Methylation analysis of \( Cdkn1c \) (A,B) and \( Slc22a18 \) (C,D) DMRs was performed on E13.5 placenta and liver from wild-type and \( \Delta890 \) crosses. The data were collected from two independent biological replicates. The extent of methylation is shown by the percentage for paternal (Pat) and maternal (Mat) alleles, as calculated by the BiQ Analyzer. Single nucleotide polymorphisms (SNPs) between SD7 and B6 were used to distinguish parental alleles. The asterisk indicates the SNP that has affected the CpG in the \( Slc22a18 \) DMR. Solid black circles represent methylated CpGs, whereas empty circles represent unmethylated CpGs. The vertical lines indicate missing CpGs.

\( Cdkn1c \) and only in fetal liver for \( Slc22a18 \), indicating that DNA methylation is one of multiple levels of regulation of genomic imprinting at the \( Kcnq1 \) locus and that affecting one of these levels results in a partial relaxation of imprinting.
No DNA methylation at the Kcnq1 and Phlda2 promoters in either the wild-type or the Δ890 mice was detected by Southern hybridization (data not shown). Although imprinting of Kcnq1 and Phlda2 is disrupted in Dnmt1Δ/Δ mice, the promoters of these two genes are not methylated (Yatsuki et al., 2002). It is currently unclear how loss of Dnmt1 results in the loss of imprinting of Kcnq1 and Phlda2. It is possible that methylation of these promoters is established in early stage embryos, thus establishing a transcriptional memory that could be maintained in the latter stages of embryogenesis in a DNA methylation-independent manner.

The 890-bp region mediates the interaction of Kcnq1ot1 RNA with chromatin

Kcnq1ot1 RNA promotes transcriptional silencing through directly interacting with chromatin and, subsequently, epigenetically modifying the associated chromatin (Pandey et al., 2008). Using an episomal system, we have shown that the chromatin interaction was dependent on the 890-bp region (Mohammad et al., 2008). To address whether the 890-bp deletion modulates the interaction of Kcnq1ot1 RNA with chromatin in vivo, we performed chromatin RNA immunoprecipitation (ChRIP) on cross-linked chromatin isolated from E13.5 placental tissues of wild-type and Δ890 mice using an anti-H3K27me3 antibody. H3K27me3 (a repressive chromatin mark) is highly enriched along the Kcnq1 domain on the paternal chromosome (Pandey et al., 2008; Umlauf et al., 2004). Immunoprecipitation of the Kcnq1ot1 RNA was reduced by 50% in the Δ890 mice, compared with in the wild-type mice (Fig. 4A), indicating that the 890-bp region mediates the interaction of the Kcnq1ot1 RNA with chromatin.

The 890-bp deletion does not affect the repressive histone modification patterns of the Kcnq1 domain

Chromatin immunoprecipitation (ChIP) was performed on tissues from wild-type and Δ890 mice, using anti-H3K27me3. We found no noticeable difference in the enrichment of H3K27me3 over the imprinted genes within the Kcnq1 locus between wild-type and Δ890 mice (Fig. 4C), indicating that the 890-bp deletion does not significantly compromise the repressive histone modification levels over the imprinted genes. Consistent with this observation, the 890-bp deletion did not affect the interactions of the histone methyltransferases Ezh2 and G9a with the Kcnq1ot1 RNA in the E13.5 placenta (Fig. 4B), as detected by RNA immunoprecipitation (RIP). These results are consistent with the lack of major effects on the chromatin status in the Δ890 mice.
The 890-bp region mediates the interaction between Kcnq1ot1 RNA and Dnmt1

Our data indicate that the 890-bp region is an important feature of the Kcnq1ot1 RNA that is required to maintain DNA methylation over some of the ubiquitously imprinted genes, suggesting that the 890-bp region acts through interacting with Dnmt1. Indeed, in our RIP assays, Dnmt1 interacted with wild-type Kcnq1ot1 RNA, but interaction with the mutant Kcnq1ot1 RNA was reduced by several fold. These results indicate that the deletion of the 890-bp region probably affects the crucial secondary structure in Kcnq1ot1 RNA that is required for Dnmt1 interactions (Fig. 4D). The nature of the Kcnq1ot1 RNA interactions with the DNA methylation machinery is not clear. Although the RIP studies point to a direct interaction between RNA and the methylation machinery, the possibility that RNA controls the imprinting of ubiquitously imprinted genes via the gene promoters containing somatic DMRs, indicates that the methylation and Dnmt1 enrichment at some of the ubiquitously imprinted Ezh2 and G9a, to the promoters. In the ∆890 mice, loss of Dnmt1 interaction with the Kcnq1ot1 RNA, and the corresponding loss of methylation and Dnmt1 enrichment at some of the ubiquitously imprinted gene promoters containing somatic DMRs, indicates that the Kcnq1ot1 RNA controls the imprinting of ubiquitously imprinted genes via the recruitment of Dnmt1 to somatic DMRs. Notably, the imprinting patterns of ubiquitously imprinted genes but not of placental-specific imprinted genes are conserved between mouse and human (Monk et al., 2008), indicating that DNA methylation could be the conserved silencing mechanism regulating ubiquitously imprinted genes.

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Competing interests statement
The authors declare no competing financial interests.

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