Long noncoding RNA-mediated maintenance of DNA methylation and transcriptional gene silencing

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SUMMARY
Establishment of silencing by noncoding RNAs (ncRNAs) via targeting of chromatin remodelers is relatively well investigated; however, their role in the maintenance of silencing is poorly understood. Here, we explored the functional role of the long ncRNA Kcnq1ot1 in the maintenance of transcriptional gene silencing in the one mega-base Kcnq1 imprinted domain in a transgenic mouse model. By conditionally deleting the Kcnq1ot1 ncRNA at different stages of mouse development, we suggest that Kcnq1ot1 ncRNA is required for the maintenance of the silencing of ubiquitously imprinted genes (UIGs) at all developmental stages. In addition, Kcnq1ot1 ncRNA is also involved in guiding and maintaining the CpG methylation at somatic differentially methylated regions flanking the UIGs, which is a hitherto unknown role for a long ncRNA. On the other hand, silencing of some of the placental-specific imprinted genes (PIGs) is maintained independently of Kcnq1ot1 ncRNA. Interestingly, the non-imprinted genes (NIGs) that escape RNA-mediated silencing are enriched with enhancer-specific modifications. Taken together, this study illustrates the gene-specific maintenance mechanisms operational at the Kcnq1 locus for tissue-specific transcriptional gene silencing and activation.

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

INTRODUCTION
During the past few years, a large number of studies have focused on the establishment of epigenetic states during development, but how these epigenetic states are maintained and accurately perpetuated through successive cell divisions is less well explored. DNA methylation is considered to be a stable epigenetic mark, which is passed on from one cell generation to the other by the maintenance DNA methyltransferase, Dnmt1 (Hermann et al., 2004). By contrast, histone modifications are considered to be more labile in nature, a property that is used by the cellular regulatory networks to quickly change the transcriptional state of genes. For example, in embryonic stem cells many genes have both active and repressive histone modifications, i.e. H3K4me3 and H3K27me3, respectively, and this bivalent chromatin state provides a flexibility of acquiring active or silent states during differentiation and development (Bernstein et al., 2006; Cedar and Bergman, 2009). Prc2 complex members Eed and Ezh2 have been implicated in the heritable maintenance of H3K27me3 during cell division (Hansen et al., 2008; Margueron et al., 2009). An interesting dimension to the propagation of this heterochromatic environment is provided by barrier elements that resist this spreading of silencing to maintain the active transcriptional status of neighboring regions (Gaszner and Felsenfeld, 2006). These barriers can be either physical in nature, such as DNA-associated protein complexes, or active chromatin features, such as hyperacetylation of histone tails conferred by chromatin remodelers (Donze and Kamakaka, 2002). The maintenance of silenced or active epigenetic memory must occur through individual or coordinated efforts of maintenance mechanisms operating in a tissue- and stage-specific manner at a given genomic locus.

Global genomic and transcriptomic analyses have uncovered a surprising abundance of noncoding RNA (ncRNA) (Kapranov et al., 2007; Prasanth and Spector, 2007; Mercer et al., 2009; Wilusz et al., 2009; Huang et al., 2011). One of the areas in which ncRNAs are fast emerging as key players is transcriptional gene silencing by modifying the chromatin structure (Bernstein and Allis, 2005; Whitehead et al., 2009; Mondal et al., 2010; Beisel and Paro, 2011). Over the last few years using Xist, Kcnq1ot1, Hottair and Airn ncRNAs as model systems, it has been shown that these ncRNAs, by interacting with DNA and chromatin-modifying enzymes, establish transcriptional gene silencing over chromosomal domains or chromosomes (Sleutels et al., 2002; Rinn et al., 2007; Nagano et al., 2008; Pandey et al., 2008; Terranova et al., 2008; Zhao et al., 2008; Ferguson-Smith, 2011). In all the above-mentioned instances, the role of ncRNA in establishing transcriptional silencing has been very well investigated. However, in the case of Xist, it has been shown that the RNA is required only during the initial phase of X inactivation, but not at later stages (Wutz and Jaenisch, 2000).

In the current investigation, we have chosen Kcnq1ot1 ncRNA as a model system to understand the functional role of long ncRNAs in the maintenance of the transcriptional silencing of genes. The promoter of the Kcnq1ot1 ncRNA maps to intron 10 of the Kcnq1 gene in the one mega-base Kcnq1 locus, located at the distal end of the mouse chromosome 7. It is 91 kb long, nuclear localized, and its exclusive expression on the paternal chromosome correlates in cis with the paternal silencing of eight maternally expressed imprinted genes (Smilichnik et al., 1999; Pandey et al., 2008). The Kcnq1 locus is of special interest, as genes within the locus show imprinted silencing in a tissue-specific manner. Another interesting aspect of this locus is that it has several biallelically
expressed [non-imprinted genes (NIGs): Nap1l4 and Cars] flanking the eight imprinted genes. Based on the transcriptional status, the imprinted genes within the Kcnq1 locus can be further classified into two categories: ubiquitously imprinted genes (UGs: Kcnq1, Cdhn1c, Slc22a18, Phlda2) and placental-specific imprinted genes (PIGs: Ascl2, Cd81, Tsc24, Osphl3). The UIGs, as the name implies, show imprinted silencing in both placental and embryonic tissues, whereas PIGs show silencing on the paternal chromosome only in placenta. Kcnq1ot1 RNA has been implicated in the transcriptional silencing of both UIGs and PIGs. The silenced state of these two classes of imprinted genes, together with the active NIGs in the Kcnq1 locus, provides an attractive model system to study the RNA-mediated initiation and maintenance of transcriptional silencing. From recent studies, it is evident that Kcnq1ot1 RNA is required for establishing the lineage-specific transcriptional silencing of the imprinted genes in the Kcnq1 locus via interacting with chromatin remodelers such as EzH2 and G9a (Ehmt2 – Mouse Genome Informatics) and the maintenance methyltransferase Dnmt1 (Pandey et al., 2008; Redrup et al., 2009; Mohammad et al., 2010). However, it is not clear how this lineage-specific transcriptional silencing is maintained. Another important question that has not been explored is how NIGs maintain transcription against the silencing effects of Kcnq1ot1 RNA.

Here, we investigated the role of Kcnq1ot1 RNA in the maintenance of gene silencing at the Kcnq1 locus by conditionally deleting the Kcnq1 imprinting control region (ICR), encompassing the Kcnq1ot1 promoter, at different developmental stages of the mouse embryo. We found that the gene-silencing mechanisms operating over the UIGs are labile in nature and require continuous expression of Kcnq1ot1 RNA during embryonic development to at least embryonic day (E) 8.5. Interestingly, in the case of the PIGs, silencing once established is maintained independently of RNA. When we investigated the mechanisms that maintain transcriptional activation of the NIGs in the Kcnq1 locus, we found that the genes that show active transcription are enriched with the enhancer-specific modifications H3K4me1 and H3K27ac, indicating that these chromatin marks may act as the active barriers to spreading of RNA-mediated silencing. Taken together, current investigation provides insights into mechanisms by which the Kcnq1ot1 long ncRNA maintains transcriptional silencing in a spatiotemporal manner, and also highlights the mechanisms that prevent the spreading of RNA-mediated silencing into the neighboring non-imprinted regions.

**MATERIALS AND METHODS**

**Generation of transgenic mouse with Kcnq1 ICR flanked with loxP sequences**

The targeting vector was generously provided by Prof. Wolf Reik (Babraham Institute, Cambridge, UK) and modified to add longer 5' flanking sequence and a neomycin resistance gene flanked by flippase recognition target (FRT) sequences before electroporating into the mouse R1 embryonic stem (ES) cells. The electroporated ES cells were selected with the drug G418 and DNA was prepared from the individual ES cell clones and genotyped using Southern hybridization, as shown in Fig. 1. The ES cells with correct homologous recombination were injected into the C57BL/6 blastocysts, which were then transferred to C57BL/6 pseudopregnant foster mothers to generate chimeras. The chimeric males were then crossed to C57BL/6 females and their progeny were PCR genotyped for germline transmission (PCR primers are described in supplementary material Table S1). The heterozygous transgenic mice were then crossed with mice expressing Flp recombinase to remove the neomycin resistant gene and the heterozygous mice were crossed to produce homozygous ICR2lox/2lox mice.

**Mice strains, crosses and tamoxifen treatment**

The homozygous ICR2lox/2lox mice have a mixed genetic background of 129 (129X1/SvJ × 129S1) and C57BL6/J (B6). We received Mec5Cre/+ and Er5Cre/+ mice from The Jackson Laboratory and backcrossed with C57D7 mice (a congenic strain containing distal chromosome 7 of Mus musculus background) to get Mec5Cre/+ and ER5Cre/+ mice in C57D7 background, which were then crossed with ICR2lox/2lox mice. Backcrossing various Cre strains with C57D7 allowed us to distinguish the parental alleles based on single nucleotide polymorphisms (SNPs).

In the crosses, which involved mice displaying tamoxifen-inducible Cre recombinase activity (ER5Cre/+), the pregnant females were force fed one dose of tamoxifen (TM) (5 mg in sunflower oil) at E8.5 to induce the recombination and the deletion of the Kcnq1 ICR.

**RNA isolation, reverse transcription and allele-specific quantitative RT-PCR**

Total RNA was isolated from the tissues using Trizol reagent (Invitrogen). The RNA was treated with DNase I (RQ1, Promega) and then 2 μg of treated RNA was used as a template to make cDNA using random primers and Superscript II Reverse transcriptase (Invitrogen). CDNA was diluted and used for qRT-PCR using allele-specific primers as described previously (Mohammad et al., 2010).

**DNA methylation analysis**

Allele-specific DNA methylation analyses of E11.5 whole embryo, E13.5 placenta and fetal liver tissues were performed as described by Mohammad et al. (Mohammad et al., 2010). The primers and SNPs used for allelic analysis were described previously (Mohammad et al., 2010).

**Chromatin immunoprecipitation and chromatin oligonucleotide affinity precipitation assays**

Chromatin immunoprecipitation (ChIP) and chromatin oligo affinity precipitation (ChOP) analyses were performed according to the protocols described by Pandey et al. (Pandey et al., 2008). qPCR was performed on the purified ChIP material using previously published primers (Mohammad et al., 2010) for both H3K27me3 ChIP and Dnmt1 ChIP. Primers for allele-specific ChIP are listed in supplementary material Table S1. The antibodies used in the respective ChIP experiments were H3K27me3 (17-622, Millipore), H3K27ac (ab4729, Abcam), H3K4me1 (ab 8895, Abcam), Dnmt1 (IMG-261A, IMGENEX). EzH2 antibody was generated in the laboratory of Prof. Kristian Helin (Biotech Research and Innovation Centre, University of Copenhagen, Denmark) and was used as has been described earlier (Pasini et al., 2010).

ChOP assay was performed with a biotinylated oligo antisense to Kcnq1ot1 or with a scrambled oligo. In the ChOP assay by transfection, either 200 pM of biotinylated Kcnq1ot1 antisense oligonucleotide or scrambled oligonucleotide was transfected into mouse embryonic fibroblast (MEF) cells. Cells were harvested 36 hours after transfection. Transfected cells were fixed with 1% formaldehyde followed by chromatin preparation and the sonicated chromatin was used directly for the capturing of the biotinylated oligo. The enrichment of Kcnq1ot1 interacting regions was analyzed with primers specific to Slc22a18 and Cdkn1c somatic differentially methylated regions (DMRs) or with the negative control primers designed against the distal regions of the somatic DMRs as has been described earlier above. Data represented as the enrichment with Kcnq1ot1 antisense oligonucleotide over the scrambled oligonucleotide.

**Analysis of the histone modifications over the X-linked genes**

ChIP-seq data on mouse H3K27ac and H3K4me1 for liver was taken from Creighton et al. (Creighton et al., 2010). ChIP-on-chip data on H3K27me3 and genes escaping X-inactivation in mouse were collected from Yang et al. (Yang et al., 2010). Aggregated signal plots over the transcription start sites (TSS) were generated using the SICITIN programs (Enroth et al., 2010). In the ChIP-seq data, the sequencing depth was normalized such that the average coverage over the X-chromosome was 1. For the ChIP-on-chip data, only the locations with probe measurements were used, e.g. no extrapolation between probes was carried out in the raw data. Otherwise, the data was used as given by the original investigators. Gene sets were (intersections, set differences) generated using BEDTools (Quinlan and Hall, 2010) and plots were generated using custom R-scripts.
Enhancer assay using luciferase vector

Primers were designed (sequences provided in the supplementary material Table S1) covering the regions with ChIP-on-chip signal for H3K27ac and H3K4me1 to amplify approximately 1300-1500 base pairs from the genomic DNA. PCR fragments were cloned into the PGL3 promoter vector (Promega, E 176A). The PGL3 cloned fragments were transfected into Hela cells using Lipofectamine 2000 reagent (Invitrogen) and assayed for luciferase activity 48 hours post-transfection with the Promega luciferase assay system (E1501).

RESULTS

To delete the Kcnq1ot1 RNA conditionally in vivo, we have generated a transgenic mouse, in which the Kcnq1 ICR encompassing the Kcnq1ot1 ncRNA promoter is flanked with loxp sequences (ICRloxP), using homologous recombination in mouse ES cells. The ES cells were screened for correct homologous recombination using Southern hybridization (Fig. 1A,B). The ES cells with correct homologous recombination were used to generate transgenic mice. The endogenous locus after targeting was also shown. The sites for the restriction enzymes (H, HindIII; X, Xhol; Bst, BstBI; Bsr, BsrGI) and the position of the probes used in Southern analysis (P1 and P2) are also shown. (B) Southern analysis to identify ES cell clones with correct homologous recombination performed using two different probes, P1 and P2. WT, wild type.

Conditional removal of Kcnq1ot1 RNA in early embryonic lineages leads to the relaxation of imprinting of the UIGs

Imprinted silencing of the UIGs was noted as early as E3.5 embryos (supplementary material Fig. S1A), whereas the PIGs appear to be silenced between E3.5 and E5.5 embryos. Both UIG and PIGs are silenced in a heterochromatic compartment triggered by Kcnq1ot1 RNA. As only UIGs show imprinted silencing in embryonic tissues, we first addressed the stability of the Kcnq1ot1 RNA conditionally in vivo, we have generated a transgenic mouse, in which the Kcnq1ot1 RNA is required to suppress transcription of the Cdkn1c gene, the Never2 gene, the Meox gene, and the PIGs are silenced in a heterochromatic compartment triggered by Kcnq1ot1 RNA. As only UIGs show imprinted silencing in embryonic tissues, we first addressed the stability of the Kcnq1ot1 RNA-mediated silencing of the UIGs in early embryonic tissues by crossing the male ICRloxP mouse with the female MeoxCre mouse that expresses Cre recombinase in the epiblast lineage which gives rise to embryonic tissues later in development. Conditional deletion of the ICR at E5.5 in embryonic lineages on the paternal chromosome in the mutant embryos (ICR1loxM) resulted in the relaxation of imprinted UIGs in these tissues (supplementary material Fig. S1C). Additionally, we performed imprinting analyses in brain and heart at E11.5 and found relaxation of imprinted silencing of UIGs in these tissues (supplementary material Fig. S1B). Additionally, we performed imprinting analyses in brain and heart at E11.5 and found relaxation of imprinted silencing of UIGs in these tissues (supplementary material Fig. S1C).

The loss of imprinting of all the genes at the Kcnq1 locus upon germline deletion of the Kcnq1 ICR, encompassing the Kcnq1ot1 promoter, has previously been associated with growth retardation in mice (Fitzpatrick et al., 2002). We addressed whether conditional deletion of the ICR at E5.5 has a similar effect on growth as the zygotic deletion. To this end, we measured the weight of 4-week-old ICR2loxM and ICR1loxM mice. The mutant ICR1loxM mice showed significantly reduced weight compared with wild-type (ICR2loxM) animals (Fig. 2A), indicating that Kcnq1ot1 RNA is required to maintain imprinting of the UIGs (Fig. 2B,C; supplementary material Fig. S1B). Additionally, we performed imprinting analyses in brain and heart at E11.5 and found relaxation of imprinted silencing of UIGs in these tissues (supplementary material Fig. S1C). Imprinting analysis was performed on the mutant embryos (ICR1loxM) as well as their wild-type littermates (ICR2lox) and after deletion of the Kcnq1 ICR (ICRloxP), is also shown. The sites for the restriction enzymes (H, HindIII; X, Xhol; Bst, BstBI; Bsr, BsrGI) and the position of the probes used in Southern analysis (P1 and P2) are also shown. (B) Southern analysis to identify ES cell clones with correct homologous recombination performed using two different probes, P1 and P2. WT, wild type.
Conditional deletion of Kcnq1ot1 RNA at E8.5 affects the silencing of the UIGs but not the PIGs

As we investigated the stability of UIGs in embryonic tissues in the ICR1loxM mice at E5.5, we wanted to investigate whether there is any tissue and developmental-specific difference in the requirement of Kcnq1ot1 RNA for the maintenance of the silencing of UIGs. In addition, we also wanted to explore the stability of the imprinted silencing of the PIGs in response to conditional deletion of Kcnq1ot1 RNA in placenta. To address these issues, we conditionally deleted the Kcnq1 ICR at E8.5 by crossing the male ICR 2lox mouse with female ERCre/+ mouse that expresses TM-inducible Cre-recombinase driven by constitutively expressed cytomegalovirus (CMV) promoter. We fed the pregnant female with TM at E8.5, and analyzed the stability of the silencing of the UIGs and PIGs in mutant embryos (ICR1loxER8.5) as well as their wild-type littermates (ICR2loxER8.5) at E13.5. TM treatment led to the deletion of the Kcnq1 ICR in embryonic as well as in placental tissues (Fig. 3 A). The loss of Kcnq1ot1 RNA expression occurred only if the ICR is conditionally deleted on the paternal chromosome (Fig. 3B). Conditional loss of the Kcnq1ot1 expression from the paternal chromosome at E8.5 resulted in loss of silencing of the UIGs (Fig. 3C; supplementary material Fig. S2D), indicating that Kcnq1ot1 RNA is required to maintain the silencing of the UIGs even at later developmental stages. By contrast, the silencing of the PIGs was variably maintained in response to conditional removal of Kcnq1ot1 RNA: Ascl2 showed loss of imprinted silencing whereas Cdh1, Tssc4 and Osbpl5 maintained silencing even in the absence of the RNA in the ICR1loxER8.5 offspring (Fig. 3C). The conditionally deleted
ICR\textsuperscript{1loxM} allele showed no effect on the imprinted silencing of the genes at the \textit{Kcnq1} locus when inherited maternally (data not shown).

\textbf{\textit{Kcnq1ot1} RNA is required for maintaining DNA methylation at somatic differentially methylated regions (somatic DMRs)}

Previously, two somatic DMRs have been mapped in the vicinity of \textit{Cdkn1c} and \textit{Slc22a18} genes (Yatsuki et al., 2000; Lewis et al., 2004). Both the somatic DMRs attain methylation by E3.5 in the preimplantation stage of embryonic development (supplementary material Fig. S2B,C), and methylation at the DMRs correlates with the silencing of \textit{Cdkn1c} and \textit{Slc22a18} genes. In addition, both \textit{Cdkn1c} and \textit{Slc22a18} genes showed loss of imprinted silencing in \textit{Dnmt1} mutant embryos (Lewis et al., 2004) and this DNA methylation-dependent imprinted gene silencing has recently been shown to involve the interaction of \textit{Kcnq1ot1} RNA with \textit{Dnmt1} (Mohammad et al., 2010). We then analyzed the effect of conditional deletion of \textit{Kcnq1ot1} RNA on the maintenance of \textit{CpG} methylation at the \textit{Cdkn1c} and \textit{Slc22a18} somatic DMRs in the ICR \textit{2loxM}, ICR\textsuperscript{1loxER8.5} and ICR\textsuperscript{1loxM} embryos using bisulfite sequencing. Both \textit{Cdkn1c} and \textit{Slc22a18} DMRs showed a complete loss of methylation over the paternal allele in the embryonic tissues from the ICR\textsuperscript{1loxER8.5} offspring compared with the ICR\textsuperscript{1loxM}.
Pandey et al., 2008) on cell lysates from the MEFs obtained from this end, we performed a ChOP assay as described by Pandey et al. for these somatic DMRs at later stages of development, i.e. E14.5. To investigate whether RNA is continuously present at DMRs, we assayed the enrichment of Dnmt1 at the somatic DMRs (Fig. 4A). As previously (Pandey et al., 2008), we obtained similar results when we performed ChOP using an alternative method in which MEFs were transfected with Kcnq1ot1 antisense oligonucleotides or scrambled oligonucleotides, and pulled-down the Kcnq1ot1 associated chromatin as described previously (Pandey et al., 2008). (C) ChOP assay using an alternative method in which MEFs were transfected with Kcnq1ot1 antisense oligonucleotides or scrambled oligonucleotides, and pulled-down the Kcnq1ot1 associated chromatin as mentioned in Fig. 4B. The same set of primers mapping to Slc22a18 and Cdkn1c DMRs along with negative control primers NC1 and NC2 were used in the ChIP and ChOP assays. The data represent values from two independent experiments plotted over IgG. *P<0.05.

and ICR2loxER8.5 offspring, respectively (Fig. 2E and Fig. 3D). Cdkn1c and Slc22a18 DMRs also showed loss of methylation in extra-embryonic tissues in the ICR1loxER8.5 offspring compared with the ICR2loxER8.5 offspring (Fig. 3D). The DMR sequences on the maternal allele remain unmethylated upon conditional removal of the Kcnq1ot1 promoter (data not shown). These results indicate the requirement of Kcnq1ot1 RNA for the maintenance of DNA methylation at the Cdkn1c and Slc22a18 DMRs.

**Loss of methylation at the somatic DMRs is associated with loss of Dnmt1 enrichment**

Loss of methylation of the somatic DMRs upon conditional loss of Kcnq1ot1 RNA expression highlights the role of Kcnq1ot1 RNA in the maintenance of methylation at the somatic DMRs. Moreover, loss of imprinted silencing of Slc22a18 and Cdkn1c genes was detected in different Dnmt1 mutant backgrounds (Lewis et al., 2004; Weaver et al., 2010). To gain further insights into the role of Kcnq1ot1 and Dnmt1 interaction in the maintenance of CpG methylation at the somatic DMRs, we assayed the enrichment of Dnmt1 and Kcnq1ot1 ncRNA at the somatic DMRs by using ChIP and ChOP techniques, respectively, in embryonic and extra-embryonic tissues of the ICR1loxER8.5 and ICR2loxER8.5 litters. ChIP analysis using a Dnmt1 antibody indicated that there is a significant loss of enrichment of Dnmt1 at the Cdkn1c and Slc22a18 DMRs in embryonic and extra-embryonic tissues upon conditional removal of Kcnq1ot1 RNA at E8.5, which correlates with the loss of DNA methylation at the DMRs (Fig. 4A). As Kcnq1ot1 RNA availability determines the Dnmt1 enrichment at the somatic DMRs, we investigated whether RNA is continuously present at these somatic DMRs at later stages of development, i.e. E14.5. To this end, we performed a ChOP assay as described by Pandey et al. (Pandey et al., 2008) on cell lysates from the MEFs obtained from the ICR2loxER8.5 mice at E14.5. We found significant enrichment of the somatic DMR sequences in the pull-down Kcnq1ot1-RNA-associated chromatin material, indicating that Kcnq1ot1 RNA is specifically enriched at the somatic DMRs (Fig. 4B). We obtained similar results when we performed ChOP using an alternative approach, wherein we transfected both Kcnq1ot1-specific and scrambled biotin-labeled oligos into E14.5 MEFs from the ICR2loxER8.5 mice (Fig. 4C) and then quantified the DMR sequences from the pulled-down Kcnq1ot1 associated chromatin material. These observations suggest the continuous requirement of Kcnq1ot1 RNA at the somatic DMRs of Cdkn1c and Slc22a18 for the stable maintenance of CpG methylation.

**Repressive histone modifications remain unchanged over the Kcnq1 domain upon conditional deletion of Kcnq1ot1 RNA**

Kcnq1ot1 ncRNA interacts with the Pre2 complex member Ezh2 (Pandey et al., 2008) and mediates contraction of the Kcnq1 locus via the lysine 27 residue of histone H3 (H3K27me3) (Terranova et al., 2008). H3K27me3 repressive modification is more enriched at the promoters of PIGs than UIG promoters (Unlaufl et al., 2004; Pandey et al., 2008). As we observed differential relaxation of imprinted gene silencing among the placental-specific imprinted genes, we measured the enrichment of Ezh2 as well as H3K27me3 across the Kcnq1 domain using ChIP on chromatin prepared from E13.5 placenta and liver tissues of mutant (ICR1loxER) and control (ICR2loxER) backgrounds (Lewis et al., 2004; Pandey et al., 2008). When we calculated Pearson correlation coefficient (r2) between the Ezh2 and H3K27me3 ChIP-qPCR and found no significant difference in the H3K27me3 profiles between the mutant and their wild-type littermates ICR2loxER8.5 at E13.5. The ChIP material from the extra-embryonic tissue was hybridized to the NimbleGen high-resolution oligonucleotide tiling array. ChIP-on-chip data profiling of H3K27me3 and EZH2 across the Kcnq1 domain showed that H3K27me3 and EZH2 occupancy remain unchanged over the entire domain upon conditional deletion of Kcnq1ot1 RNA (Fig. 5A,B). When we calculated Pearson correlation coefficient (r2) between the Ezh2 and H3K27me3 ChIP experiments, we found a high correlation between the Ezh2 and H3K27me3 patterns across the Kcnq1 domain in both wild-type ICR2loxER8.5 and mutant embryos (Fig. 5C). We further verified the ChIP-on-chip data with ChIP-qPCR and found no significant difference in the H3K27me3 profiles between the mutant and their wild-type littermates (supplementary material Fig. S3A-C). Maintenance of H3K27me3 across the Kcnq1 domain upon conditional deletion of Kcnq1ot1 RNA at E8.5 developmental stage correlates with the maintenance of silencing of some of the PIGs.
Enhancer-specific histone marks maintain active transcription at the NIGs in the Kcnq1 locus by evading Kcnq1ot1-mediated silencing

In the Kcnq1 domain, PIGs and UIGs are flanked with non-imprinted, biallelically expressed genes. It is not known how Kcnq1ot1 RNA distinguishes imprinted genes from NIGs for transcriptional silencing. In the current study we tried to understand how NIGs maintain their active transcription while their imprinted counterparts undergo transcriptional silencing by ncRNA. As Kcnq1ot1 RNA executes its actions via chromatin structure, there should be probable signatures specific to imprinted and non-imprinted genes that are interpreted by Kcnq1ot1 RNA. Consistent with this notion, analysis of the repressive histone modification H3K27me3 by ChIP-on-chip over the entire Kcnq1 domain showed that genes such as Nap114 and Cars that escape silencing lack H3K27me3, whereas the neighboring silenced imprinted genes are.
enriched with this repressive histone modification (Fig. 6A). Based on this data, we hypothesized that the presence of active histone marks may counteract the ncRNA-dependent silencing and thus maintain these regions free of repressive chromatin marks. Recent studies have shown that H3K27me3 and the enhancer-specific modification H3K27ac are distributed in a mutually exclusive manner across the genome (Pasini et al., 2010). As we did not detect any H3K27me3 over the NIGs in our ChIP-on-chip data, we hypothesized that the enhancer-specific modifications H3K4me1 and H3K27ac (Heintzman et al., 2009; Creyghton et al., 2010; Rada-Iglesias et al., 2010) could be present over these regions. To this end, we have profiled H3K27ac and H3K4me1 along the one mega-base *Kcnq1* domain, using ChIP on chip. Interestingly, we found the regions enriched with H3K27ac and H3K4me1 overlapping the H3K27me3-free regions containing NIGs (Fig. 6A). We have also found moderately enriched regions with H3K27ac and H3K4me1 marks surrounding the *Kcnq1ot1* promoter region, which evades the silencing effects of its own transcriptional product (Fig. 6A). We validated H3K27ac and H3K4me1 ChIP-on-chip data with ChIP-qPCR and found correlation between the two experiments over the enriched regions (supplementary material Fig. S4A).

Previously, we have shown that H3K27me3 is distributed in a lineage-specific fashion over the *Kcnq1* domain between fetal liver and placental tissues and this correlates with the PIGs showing imprinted silencing in placenta and biallelic expression in fetal liver. Chromatin for the ChIP analyses was prepared from E13.5 placenta, dissected from embryos born to a cross between wild-type B6 male and SD7 female mice. ChIP analysis was performed using antibodies against H3K27ac, H3K4me1 and H3K27me3 and the ChIP material was hybridized to a NimbleGen high-resolution oligonucleotide tiling array. Two rectangles indicate H3K27me3-free regions enriched with H3K27ac and H3K4me1 enhancer-specific chromatin marks. (B) Model depicting *Kcnq1ot1* ncRNA-mediated maintenance mechanisms at the UIGs and PIGs during mouse embryonic development. The mechanisms that maintain silencing of UIGs at both early (E3.5) and late (E8.5) stages of development are labile and require the continuous presence of *Kcnq1ot1* RNA. DNA methylation at the somatic DMRs, which controls the imprinted silencing of CDKN1C and SLC22A18, is also maintained in a *Kcnq1ot1*-RNA-dependent manner at all developmental stages. In the case of the PIGs, silencing is maintained independently of *Kcnq1ot1* RNA via recruitment of chromatin remodelers. Presence of the enhancer-specific chromatin marks H3K4me1 and H3K27ac at the actively expressed genes (*Kcnq1ot1* and NIGs) prevents the RNA-mediated repression of these genes.
liver. We investigated whether biallelic expression of PIGs in fetal liver correlates with the presence of H3K27ac and H3K4me1 marks. ChIP-on-chip analyses with H3K4me1 and H3K27ac antibodies on chromatin from fetal liver revealed that the PIGs that show biallelic expression in fetal liver (except *AscI2*, which is biallelically repressed in liver) are specifically enriched with H3K4me1 and H3K27ac marks (supplementary material Fig. S5A). We also found similar enrichment of enhancer-specific modifications in MEFs where PIGs are biallelically expressed (supplementary material Fig. S5B). This observation further supports the functional role of enhancer-specific histone marks in conferring resistance to RNA-mediated silencing.

p300 is a histone acetyltransferase, that is often used as a marker to identify enhancers on a genome-wide scale (Visel et al., 2009). It is known to be involved in acetylation of H3K27 (Pasini et al., 2010). Hence we analyzed the p300 presence over the H3K4me1- and H3K27ac-enriched regions using ChIP with a p300 antibody followed by qPCR. We found all the tested H3K27ac-enriched regions were also enriched with p300, suggesting that acetylation of H3K27 in the *Kcnq1* domain may be carried out by p300 (supplementary material Fig. S4B). We then analyzed in more detail the H3K27ac- and H3K4me1-enriched regions with respect to the *Nap1l4* and *Cars* promoters (supplementary material Fig. S5C). We found the overlapping H3K27ac and H3K4me1 peaks are located distally to the promoter, suggesting that they might be putative enhancers, activating the expression of the NIGs. We tested some of the regions showing enrichment for both H3K4me1 and H3K27ac for their enhancer activity using a luciferase-based assay and found that all of the tested fragments showed significant luciferase activity in comparison with negative controls (supplementary material Fig. S6A). However, our ChIP-on-chip analysis showed a few NIGs, such as *Tspan32*, *R74862* and *Trpm5*, are devoid of H3K27ac and H3K4me1. We have looked into the expression levels of these genes using available placental microarray data (F.M. and C.K., unpublished) and found that these are expressed at a background level in the placental tissue (supplementary material Fig. S6B), and we further verified this data by qRT-PCR (supplementary material Fig. S6C). This low level of expression of NIGs is consistent with their high enrichment of H3K27me3 (supplementary material Fig. S7A), whereas the expressed NIGs were devoid of H3K27me3 modification (supplementary material Fig. S7B).

In addition to addressing the possible role of enhancer-specific modifications in evading the ncRNA-mediated silencing, we also looked into the distribution of chromatin insulator protein CTCF-binding sites along the *Kcnq1* domain. As chromatin insulator elements act as barriers to the spreading of chromatin-mediated silencing (Gaszner and Felsenfeld, 2006), we presume that the presence of CTCF-binding sites bordering the NIGs could provide protection against the ncRNA-mediated silencing. Using publicly available data sets, we mapped CTCF targets in the *Kcnq1* domain in mouse ES cells and MEFs (supplementary material Fig. S7C). We found CTCF targets were uniformly distributed along the *Kcnq1* domain and no specific enrichment of CTCF targets over the NIGs, indicating that CTCF targets are unlikely to play a critical role in the protection against ncRNA-mediated silencing.

We presume that the presence of H3K27ac- and H3K4me1-mediated maintenance of active transcription of the NIGs could be a general mechanism and that the X-linked genes that escape inactivation on the inactive X chromosome could use such mechanisms to evade *Xist* ncRNA-mediated silencing. To this end, we have analyzed the enrichment of H3K27me3, H3K27ac and H3K4me1 chromatin marks over 13 X-linked genes that have been shown to escape *Xist*-mediated silencing using publicly available data sets (Creighton et al., 2010; Yang et al., 2010). Consistent with earlier observations (Yang et al., 2010), we have found that the genes which escape X inactivation are less enriched with H3K27me3 in comparison with the genes that undergo inactivation (supplementary material Fig. S8). We also noted high enrichment of H3K27ac along with a moderate enrichment of H3K4me1 over the TSS of the active genes in comparison to the silent genes on the inactive X-chromosome. This suggests that H3K27ac could provide protection against the *Xist*-mediated repression, as in the case of *Kcnq1ot1*. Like the NIGs in the *Kcnq1* domain, the distal regions of the active genes on the inactive X chromosome were also enriched with both H3K27ac and H3K4me1 (supplementary material Fig. S9A-G), suggesting that they might have role in maintenance of the active transcription over the inactive X chromosome. Interestingly, similar to the *Kcnq1ot1* promoter, the *Xist* promoter, which escapes silencing from its own transcriptional product *Xist* ncRNA, also harbors distal H3K27ac- and H3K4me1-enriched regions (supplementary material Fig. S9A). Taken together, by using the mouse *Kcnq1*-imprinted domain and inactive X chromosome as model systems, we have shown that enhancer-specific chromatin modifications appear to play an important role in protection against ncRNA-mediated silencing.

**DISCUSSION**

Transcriptional gene silencing at individual loci can be distinct, ranging from monolayer regulation to multilayered pathways, operational in a developmental-stage and tissue-specific manner. These silencing mechanisms, comprising histone modifications and DNA methylation, are established by epigenetic modifiers such as histone methyltransferases, histone deacetylases and DNA methyltransferases. This establishment of epigenetic memory has recently been shown to involve ncRNA. Here we address two outstanding issues concerning the long ncRNA-mediated transcriptional silencing: firstly, using *Kcnq1ot1* ncRNA as a model system, we investigated how the ncRNA maintains tissue-specific silencing of imprisoned genes within the *Kcnq1* locus during early embryonic development. Secondly, we characterized the plausible mechanisms by which certain NIGs escape the silencing effects of long ncRNAs.

**Continuous expression of Kcnq1ot1 RNA is required to maintain the silencing status of the UIGs**

Loss of silencing of the UIGs upon deletion of the *Kcnq1* ICR at E5.5 and E8.5 suggests that *Kcnq1ot1* RNA is not only required for the establishment of silencing but also takes part in its maintenance throughout the development (Fig. 6B). This is a surprising observation given that the UIGs are silenced on the paternal chromosome in all tissues and that some of the UIGs (*Cdkn1c*) are stably silenced at all developmental stages (Caspary et al., 1998; Gould and Pfeifer, 1998). In addition, the UIGs *Cdkn1c* and *Slc22a18* are flanked by somatic DMRs, which become methylated on the paternal chromosome in a *Kcnq1ot1*-RNA-dependent manner (Lewis et al., 2004). Loss of DNA methylation at the DMRs of both the *Slc22a18* and *Cdkn1c* genes in the E5.5 and E8.5 conditional deletions point towards a relatively new facet of RNA-mediated regulation in which the *Kcnq1ot1* ncRNA is required not only for the establishment of methylation but also for the continuous maintenance of methylation through successive generations.
Our data pose an interesting question as to why Kcnq1ot1 RNA is required during each cell cycle to pass on the stable methylation imprint. From the observations that Kcnq1ot1 interacts with the maintenance methylation machinery Dnmt1 and that both Dnmt1 and Kcnq1ot1 are enriched at the somatic DMRs, it can be assumed that the RNA works as a recruiting platform for the continuous presence of the Dnmt1 for maintaining the labile silencing of the UIGs via DNA methylation. This result is contrary to the general view of DNA methylation as a stable epigenetic mark that once established is successfully maintained through generations during DNA replication via Dnmt1-Pcna-Uhrf1 (Bostick et al., 2007). As the DNA methylation pattern at the somatic DMRs is allele specific compared with DNA methylation patterns at other genomic loci, which are methylated in a non-allele-specific manner, it is possible that maintenance of DNA methylation at the somatic DMRs may utilize a different mechanism. Recently, it has been shown that methylation of selected promoters involves cooperation between Dnmt1 and transcription factors such as Sp1 or p53 (Esteve et al., 2005; Hervouet et al., 2010). Kcnq1ot1-ncRNA-mediated maintenance of methylation seems to be synergistic with the Dnmt1-Pcna-Uhrf1 mechanism, in which where Dnmt1 partners with DMR-associated Kcnq1ot1 ncRNA in a manner similar to the transcription factors such as p53 or Sp1. In addition, these observations attain significance in view of the fact that transcription is required to establish methylation at the differentially methylated regions across the genome (Chotalia et al., 2009). Furthermore, triple helical structures resulting from interactions between RNA and DNA have been shown to form the basis for de novo methylation (Schmitz et al., 2010).

Kcnq1 and Phlda2 lack any known DMRs, yet we observed loss of silencing in the E5.5 and E8.5 conditional deletions. Both Kcnq1 and Phlda2 genes show loss of imprinted silencing in Dnmt1−/− embryos: Kcnq1 shows relaxation only in embryonic tissues, whereas Phlda2 showed loss of silencing in both embryonic and extra-embryonic tissues (Lewis et al., 2004). This lack of DMRs in the vicinity of the Kcnq1 and Phlda2 genes, and differential loss of silencing of the Kcnq1 and Phlda2 genes in Dnmt1 mutant embryos, indicate that RNA-dependent methylation together with other synergistic and non-redundant mechanisms may maintain the labile silencing of these two genes. As the relaxation of imprinting of genes within the Kcnq1 locus remains similar between the deletions encompassing Kcnq1 ICR (3.0 kb), Kcnq1ot1 promoter (246 bp) or the truncation of the Kcnq1ot1 transcript that retains 3.0 kb ICR (Mancini-Dinaro et al., 2006), the observed loss of transcriptional silencing of UIGs is mainly due to conditional depletion of Kcnq1ot1 RNA rather than other functional elements within the Kcnq1 ICR. In a recent investigation, by post-transcriptionally depleting Kcnq1ot1 RNA using a short hairpin RNA (shRNA) in cultured ES cells it was demonstrated that Kcnq1ot1 ncRNA is not required to maintain imprinted silencing of the UIGs (Golding et al., 2011). As these experiments were performed in cultured cells, and for a snapshot of time, it is not clear how well they represent the in vivo situation.

Maintenance of silencing of PIGs is independent of Kcnq1ot1 expression

Like UIGs, PIGs are silenced in a Kcnq1ot1-RNA-dependent manner. Based on histone modification profiles at the PIG promoters and their loss of silencing in various chromatin modifier mutants such as Eed, Ezh2 and G9a, it has been suggested that histone modifications play a critical role in silencing of PIGs (Mager et al., 2003; Pandey et al., 2008; Terranova et al., 2008; Wagschal et al., 2008). Maintenance of PIG silencing (Cd81, Tssc4 and Osbpl5) upon conditional depletion of Kcnq1ot1 at E8.5, suggests that Kcnq1ot1 RNA may be required to establish silencing but not for their maintenance (Fig. 6B). This observation indicates that distinct mechanisms could be involved in the maintenance of silencing of the PIGs and UIGs. However, loss of imprinted silencing of placental-specific gene Ascl2 upon conditional depletion of Kcnq1ot1 ncRNA at E8.5 indicates that Ascl2 seems to behave more like UIGs.

A multilayered pathway, comprising different repressive histone modifications such as H3K19ub1, H3K27me3 and H3K9me3 (Lewis et al., 2004; Umlauf et al., 2004; Pandey et al., 2008; Terranova et al., 2008), has been proposed to explain the regulation of imprinted silencing of the UIGs and PIGs. These histone modifications represent different layers of synergistic and/or redundant mechanisms and are suggested to occur in a progressive manner during early embryonic development (Terranova et al., 2008). Germline deletion of the Kcnq1ot1 promoter resulted in loss of imprinted silencing PIGs (Mancini-Dinaro et al., 2006). However, maintenance of silencing of PIGs Cd81, Tssc4 and Osbpl5 upon the conditional removal of RNA at E8.5 points towards heritability of heterochromatin states comprising histone modifications. Consistent with the latter notion, it has been shown that the repressive histone modification of H3K27me3, once established over silent genes, is passed on to next cell generation by the Eed subunit of the Pre2 complex by allosterically activating the methyltransferase activity of the Pre2 complex (Margueron et al., 2009). In addition, another Pre2 complex member, Ezh2, has been implicated in the heritability of H3K27me3 marks (Hansen and Helin, 2009). The observations that presence of H3K27me3 and Ezh2 along the Kcnq1 domain upon conditional depletion of Kcnq1ot1 RNA and significant correlation between the profiles of H3K27me3 and Ezh2 explains in part why H3K27me3 levels are maintained.

Enhancer-specific chromatin signatures at the NGIs provide protection against Kcnq1ot1 RNA-mediated silencing

The Kcnq1 imprinted domain harbors NIGs, which are actively transcribed from both parental alleles, while the neighboring imprinted genes undergo transcriptional silencing on the paternal chromosome (Fig. 6B). Similar to the Kcnq1 domain, a subset of genes on the mouse inactive X chromosome also escape RNA-mediated transcriptional gene silencing. It is currently not clear how ncRNA distinguishes targets from non-target genes. Previously, we have observed complete lack of repressive H3K27me3 over the NIGs in comparison with the imprinted genes (Pandey et al., 2008), indicating that chromatin structure could be the determinant of ncRNA actions. Enrichment of enhancer-specific chromatin marks H3K27ac, together with H3K4me1 and Ezh2 explains in part why H3K27me3 levels are maintained.
enriched with H3K27ac and H3K4me1, indicating a role for H3K27ac- and H3K4me1-enriched putative enhancers in resisting ncRNA-mediated silencing. Collectively, H3K27ac over TSS together with enrichment of H3K27ac and H3K4me1 at the distal regions could maintain active transcription in a repressive environment. In particular, these observations have implications in understanding how NIGs in other imprinted domains escape RNA-mediated silencing. However, further experiments need to be performed to implicate enhancer-specific modifications in evading RNA-mediated silencing.

In summary, this comprehensive investigation was aimed at providing deeper understanding of how a long ncRNA maintains lineage-specific transcriptional silencing of multiple genes in a chromosomal domain at different developmental stages.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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References
Gene regulation by noncoding RNA


(A) Blastocyst expression

(B) Meox Cre

(C) Expression (%)
H3K27me3 enrichment

(A)

Relative enrichment over IgG

(B)

Relative enrichment over IgG

(C)

Relative enrichment over IgG

Ubiquitously imprinted genes
Placental specific imprinted genes
Non-imprinted genes

Placenta ICR
Placenta ICR
Placenta ICR
Liver ICR
Liver ICR

H3K27me3 enrichment

Ascl2-P
Ascl2-M
Tssc4-P
Tssc4-M

Cdkn1c-P
Cdkn1c-M
Slc22a18-P
Slc22a18-M
(A) Liver H3K27ac

(B) MEF H3K4me1

(C) Placenta peaks analysis

Scale:
chr7: 2.41 _

K27ac_Placenta_Peaks

K4me1_Placenta_Peaks

Nap1l4
Cars
(A) Relative Luciferase activity

(B) Relative expression

(C) Relative expression
(A) H3K27me3

Log2 ratio

0

20 kb

Tspan32

R74862

Cd81

Trpm3

(B) H3K27me3

Log2 ratio

0

20 kb

Nap1l4

Cars

(C) CTCF ChIP seq data from ENCODE

ES cells

MEF
Genes escape X chromosome inactivation

Genes undergo X chromosome inactivation