

# The long noncoding RNA *Kcnq1ot1* organises a lineage-specific nuclear domain for epigenetic gene silencing

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Long noncoding RNAs are implicated in a number of regulatory functions in eukaryotic genomes. The paternally expressed long noncoding RNA (ncRNA) *Kcnq1ot1* regulates epigenetic gene silencing in an imprinted gene cluster in cis over a distance of 400 kb in the mouse embryo, whereas the silenced region extends over 780 kb in the placenta. Gene silencing by the *Kcnq1ot1* RNA involves repressive histone modifications, including H3K9me2 and H3K27me3, which are partly brought about by the G9a and Ezh2 histone methyltransferases. Here, we show that *Kcnq1ot1* is transcribed by RNA polymerase II, is unspliced, is relatively stable and is localised in the nucleus. Analysis of conditional *Dicer* mutants reveals that the RNAi pathway is not involved in gene silencing in the *Kcnq1ot1* cluster. Instead, using RNA/DNA FISH we show that the *Kcnq1ot1* RNA establishes a nuclear domain within which the genes that are epigenetically inactivated in cis are frequently found, whereas nearby genes that are not regulated by *Kcnq1ot1* are localised outside of the domain. The *Kcnq1ot1* RNA domain is larger in the placenta than in the embryo, consistent with more genes in the cluster being silenced in the placenta. Our results show for the first time that autosomal long ncRNAs can establish nuclear domains, which might create a repressive environment for epigenetic silencing of adjacent genes. Long ncRNAs in imprinting clusters and the *Xist* RNA on the inactive X chromosome thus appear to regulate epigenetic gene silencing by similar mechanisms.

**KEY WORDS:** Long noncoding RNA, Imprinting, X inactivation, Epigenetic gene silencing, *Kcnq1ot1*

## INTRODUCTION

Noncoding RNAs (ncRNAs) have key roles in the regulation of complex genome functions and plasticity in multicellular organisms (Amaral and Mattick, 2008). Several classes of short ncRNAs (such as miRNAs, siRNAs and piRNAs) regulate not only mRNA stability and translation, but also transcription through targeting of epigenetic marks, including histone modifications and DNA methylation (Neilson and Sharp, 2008). Long ncRNAs are less well characterised, but include antisense and other intergenic transcripts, pervasive genomic transcription and epigenetic regulators (Umlauf et al., 2008). The long ncRNA *Xist*, which is transcribed from the inactive X chromosome, is a well-studied example of a RNA with a crucial role in epigenetic gene silencing of a chromosome region in cis. The *Xist* RNA is transcribed by RNA polymerase II (Pol II), is spliced and polyadenylated, and located in the nucleus in a defined three-dimensional (3D) domain into which the genes along the inactive X chromosome appear to be recruited. X-linked genes that escape inactivation remain located outside the nuclear domain defined by the *Xist* RNA. Coating of the inactive X chromosome by *Xist* is associated with exclusion of Pol II from the silencing compartment, acquisition of repressive histone modifications and

CpG island methylation, thus establishing a stable and heritable repressive chromatin environment (Heard and Bickmore, 2007; Payer and Lee, 2008; Wutz and Gribnau, 2007).

Long ncRNAs are also found associated with epigenetic regulation outside the X chromosome, namely *Kcnq1ot1*, *Air* and *Nespas*, which are implicated in epigenetic gene silencing in imprinting clusters (Peters and Robson, 2008). These RNAs are paternally expressed and maternally silenced by DNA methylation of their promoters established in the female germline. By insertion of premature polyA stop signals, *Kcnq1ot1* and *Air* have been found to be crucial for inactivation in cis of imprinted genes (Mancini-Dinardo et al., 2006; Shin et al., 2008; Sleutels et al., 2002). Transcription of *Kcnq1ot1* was found to be necessary for the acquisition of repressive epigenetic markers, such as H3K9me2, H3K27me3 and DNA methylation (Fitzpatrick et al., 2002; Lewis et al., 2004; Umlauf et al., 2004). Indeed, in mouse mutants of *Dnmt1*, *Eed* (part of the polycomb repressive complex 2, which includes *Ezh2*) or *G9a*, loss of imprinting of different sets of genes in the *Kcnq1ot1* cluster has been observed (Lewis et al., 2004; Mager et al., 2003; Wagschal et al., 2008), consistent with largely separate roles of DNA and histone methylation, but partly overlapping roles of H3K9 and H3K27 methylation, in epigenetic gene silencing.

In parallel with X inactivation, the *Kcnq1ot1* RNA is first paternally expressed in two-cell embryos, and inactivation of the cis linked genes is initiated in preimplantation and early postimplantation embryos (Lewis et al., 2006; Green et al., 2007). In placentae, the silenced domain extends over 780 kb, whereas in the embryo it is considerably smaller (400 kb; Fig. 1A) (Lewis et al., 2004; Umlauf et al., 2004).

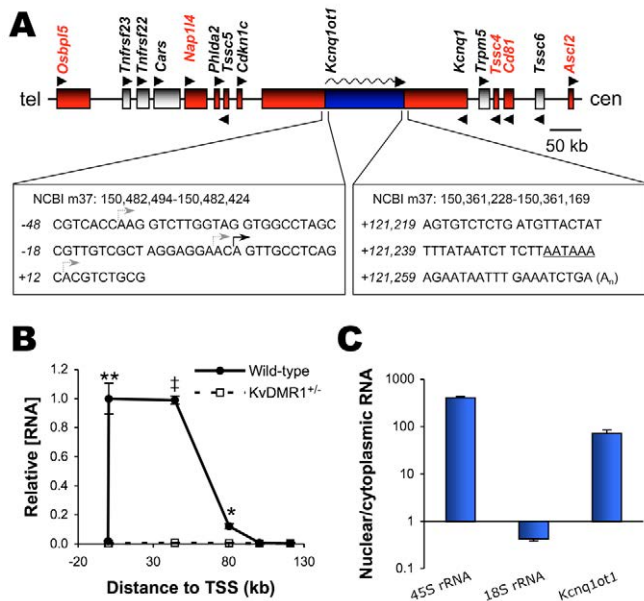
The next key step in our understanding of how imprinted long ncRNAs confer epigenetic gene silencing in cis will come from more precise insights into the molecular pathways involved. It is important to test whether RNAi contributes to imprinted silencing,

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**Fig. 1. The ncRNA *Kcnq1ot1* is 80–121 kb long and localised in the nucleus.** (A) The paternally expressed *Kcnq1ot1* transcript (wavy arrow) is necessary for silencing of adjacent genes on chromosome 7 (red). Genes imprinted solely in the placenta are indicated in red typescript. Four transcription start sites (TSS) were found by 5'-RACE (left box); the predominant TSS is indicated in black. 3'-RACE (right box) identified a polyadenylation site 121 kb downstream of the TSS (polyadenylation signal underlined). (B) qRT-PCR analysis was performed along the length of the *Kcnq1ot1* transcript using wild-type and *KvdDMR1*<sup>-/-</sup> E13.5 embryos. *KvdDMR1*-dependent transcripts can be detected up to at least 80 kb downstream of the TSS (see also Fig. S1 in the supplementary material). \* $P < 0.05$ , \*\* $P < 0.001$ , † $P < 0.0001$ . Error bars represent s.e.m. (C) qRT-PCR analysis of nuclear and cytoplasmic fractions from primary mouse embryonic fibroblasts shows that *Kcnq1ot1* is mainly located in the nucleus. The 45S and 18S rRNAs are included as controls for predominantly nuclear and cytoplasmic RNAs, respectively. Error bars represent s.e.m.

particularly given recent work that has implicated the RNAi pathway in X inactivation (Ogawa et al., 2008). Alternatively, or additionally, long ncRNAs in imprinting clusters may act in the nucleus and establish silencing domains akin to that established by Xist for X inactivation. Here, we test both of these models for the *Kcnq1ot1* imprinting cluster.

## MATERIALS AND METHODS

### Mouse strains and cell lines

All experimental procedures were conducted under licences by the Home Office (UK) in accordance with the Animals (Scientific Procedures) Act 1986. We used C57BL/6J or JolaHsd or SD7 as wild-type strains. SD7 contains the distal region of *Mus spretus* chromosome 7 backcrossed into the F1 (C57BL/6J/CBA/Ca), which provides SNPs to distinguish between parental alleles. *KvdDMR1* mice have been described previously (Fitzpatrick et al., 2002). *Dicer*<sup>lox/lox</sup> mice (Cobb et al., 2005) were crossed with *Meox*<sup>Cre/+</sup> (Tallquist and Soriano, 1999) mice to generate *Meox*<sup>Cre/+</sup>; *Dicer*<sup>Δ/+</sup> mice. These mice were then crossed with *Dicer*<sup>lox/lox</sup>; SD7/SD7 mice to generate *Meox*<sup>Cre/+</sup>; *Dicer*<sup>Δ/Δ</sup>; SD7/B6 embryos. Yolk sac DNA was used for genotyping (primer details can be provided on request). Primary mouse embryonic fibroblasts (MEFs) were obtained from E13.5 C57BL/6JolaHsd embryos and cultured in DMEM (Invitrogen) with 10% FBS, 1% penicillin/streptomycin and 2 mM L-glutamine. For RNA stability

experiments, NIH3T3 cells were cultured in medium containing 0.001% saponin±5 μg/ml α-amanitin, and analysed as previously described (Seidl et al., 2006).

### RNA isolation, reverse transcription, RACE and Sequenom MassArray

Total RNA was isolated from cells and embryos using Trizol (Applied Biosystems) or AllPrep DNA/RNA Mini kit (Qiagen). Nuclear and cytoplasmic RNA was isolated from MEFs using the PARIS kit (Applied Biosystems) and spiked with rabbit α-globin mRNA (Sigma, Dorset, UK) as an internal control. RNA was reverse transcribed using either Superscript II (Invitrogen) or BD Sprint PowerScript (BD Biosciences) using either oligo-dT, random primers (Promega) or specific primers for strand-specific analysis. For miR-16 expression analysis, small RNAs were isolated using the RNeasy MinElute cleanup kit (Qiagen) and cDNA was made using SnoRNA-202 and miR-16 reverse transcription kits (Applied Biosystems). 5' and 3' RACE were carried out using the GeneRacer RACE Ready cDNA Kit (Invitrogen). Allele-specific expression analysis was performed with iPLEX chemistry on the SEQUENOM genotyping platform, as per the manufacturer's instructions. Primer sequence details can be provided on request.

### Quantitative PCR (qPCR)

To analyse the length of the *Kcnq1ot1* transcript, qPCR was performed using Platinum SYBR Green qPCR SuperMix-UDG with Rox (Invitrogen) on an ABI Prism 7700 Sequence Detector. cDNA (5 μl, diluted 1:50) was used per reaction (primer details can be provided on request). Reference RNAs were produced by in vitro transcription of cloned PCR products and quantified using RiboGreen (Invitrogen). The respective cDNAs were used to generate a standard curve allowing quantitative comparison between amplicons. For miR-16 expression analysis, the SnoRNA-202 and miR-16 qPCR kits (TaqMan) were used on an Mx3005 QPCR System (Stratagene).

### Fluorescence in situ hybridisation (FISH) probes

Probes for RNA-FISH (details can be provided on request) were labelled with digoxigenin by nick-translation (Roche Diagnostics). The *Kcnq1* probe is intronic and located outside of *Kcnq1ot1*, and so will only detect *Kcnq1* transcripts, but the *Kcnq1ot1* probe will also detect *Kcnq1*. However, RNA-FISH on *KvdDMR1*-knockout embryo sections using the *Kcnq1ot1* probe showed only background levels of fluorescent signals (not shown). The Xist probe is also capable of detecting both the Xist and Tsix transcripts, but at the developmental time points in our study Tsix is silent (Sado et al., 2001). For DNA-FISH, fosmids (BACPAC Resources; details can be provided on request) were labelled with biotin by nick-translation (Roche).

### RNA-FISH

Frozen E12.5 embryos and placentas were embedded in Cryo-M-Bed (Bright) prior to 14 μm sectioning with a cryostat (Bright). Sections were collected on poly-lysine-coated slides and stored at -80°C. Sections were briefly thawed, permeabilised with 0.5% TX in PBS for 8 minutes on ice, fixed in 4% formaldehyde/5% acetic acid/0.14 M NaCl for 18 minutes at room temperature, washed in PBS, dehydrated in 70–100% ethanol series and air-dried. Probes (2.5 μl) were co-precipitated with 1 μg of mouse Cot1 DNA and resuspended in 40 μl of hybridisation mix (Chakalova et al., 2004). Probe denaturation, hybridisation, washes and probe detection were performed as described previously (Chakalova et al., 2004); the following antibodies were used: sheep anti-digoxigenin (1:1000; Roche) followed by FITC-conjugated rabbit anti-sheep (1:200; Calbiochem) and AlexaFluor 488-conjugated donkey anti-rabbit (1:200; Invitrogen).

### Combined RNA/DNA-FISH

After RNA-FISH as above, antibodies were fixed with 4% formaldehyde in PBS for 30 minutes at room temperature. Sections were treated with 0.1 M HCl for 20 minutes, washed in PBS, equilibrated in 70% formamide/2×SSC before denaturing for 5 minutes at 80°C (in pre-heated 70% formamide/2×SSC/0.1 M phosphate buffer pH 7.0), and then cooled down in ice-cold 50% formamide/2×SSC. Probes were precipitated, re-suspended in hybridisation buffer and denatured as

previously described (Noordermeer et al., 2008), and hybridised to denatured sections overnight at 37°C. Post-hybridisation washes were performed as previously described (Branco and Pombo, 2006). Biotin was detected using an AlexaFluor 555-conjugated streptavidin (Invitrogen; 1:500); blocking, antibody dilutions and washes were carried out in 1% BSA in PSB/0.05% Tween-20.

#### Image collection and analysis

Optical z-stacks were captured using a Zeiss LSM 510 META (63× Plan-Apo 1.40 objective) or Olympus FV1000 (63× Plan-superApo 1.40 objective) confocal microscopes.

For analysis of the RNA-FISH signals, Volocity (v4, Improvion) was used for volume rendering of the image z-stacks using the HR opacity renderer. RNA-FISH signal volumes were also measured in Volocity using intensity-based thresholding; signals not touching DAPI stained objects or with a volume less than 0.1  $\mu\text{m}^3$  were excluded.

Distances between DNA-FISH signals and the edge of the *Kcnq1ot1* domain were measured in 3D using a custom ImageJ (Wayne Rasband, NIH) macro. Briefly, RNA-FISH signals were segmented using an adaptive threshold algorithm and the 3D coordinates of the centre of mass of the DNA-FISH signals were found; the distance from each centre of mass to the nearest RNA domain edge was automatically measured.

## RESULTS AND DISCUSSION

### *Kcnq1ot1* RNA is nuclear and moderately stable

We determined the size and characteristics of the *Kcnq1ot1* transcript by EST searches, 5' and 3' RACE (rapid amplification of cDNA ends) and RT-PCR (Fig. 1A,B; see Fig. S1 in the supplementary material). The RACE experiments provided four transcription start sites within 20 bp and a polyadenylation site 121 kb downstream of the main start site, showing that the *Kcnq1ot1* RNA has both a 5' methyl cap and a polyadenylated tail. All ESTs were contiguous with the genomic sequence, providing no evidence for splicing. We found by quantitative RT-PCR (qRT-PCR) that the main region of transcription extends to about 80 kb from the start site and is dependent on the *Kcnq1ot1* promoter (Fig. 1B), but strand-specific RT-PCR showed that transcription from this promoter can still be detected up to 120 kb into the gene (see Fig. S1 in the supplementary material). The decrease in the level of *Kcnq1ot1* RNA along the length of the transcription unit might be the result of several unmapped polyadenylation sites within the transcript; alternatively, transcription might be impeded by a non-permissive chromatin structure. By comparison, the Air RNA is 108 kb long and largely unspliced (Lyle et al., 2000; Seidl et al., 2006), whereas the Xist RNA is spliced from 23 kb to 17.9 kb, and this splicing may be needed for Xist-mediated epigenetic silencing (Ciaudo et al., 2006).

Quantification of nuclear and cytoplasmic RNA from primary MEFs showed that *Kcnq1ot1* RNA is significantly enriched in the nucleus relative to the cytoplasm (Fig. 1C). To determine the half-life of the *Kcnq1ot1* RNA, NIH 3T3 cells were treated with the fungal toxin  $\alpha$ -amanitin, at a concentration known to inhibit Pol II but not Pols I or III. *Kcnq1ot1* RNA was found to have a half-life of 3.4 hours, whereas the mRNA of the protein-coding gene *Kcnq1* has a half-life of over 20 hours (see Fig. S2 in the supplementary material). The stability of *Kcnq1ot1* is similar to that of the ncRNAs Air and Xist, which have half-lives of 2.1 and 4.6 hours, respectively (Seidl et al., 2006). The stability of the Xist RNA is regulated by the NMD pathway (Ciaudo et al., 2006); whether this is also the case for *Kcnq1ot1* or Air is not known. In summary, *Kcnq1ot1* is transcribed by Pol II, localised in the nucleus and moderately stable, thus resembling other long ncRNAs with epigenetic roles (Umlauf et al., 2008).

### Imprinting in the *Kcnq1ot1* domain is not regulated by the RNAi pathway

Our observation that *Kcnq1ot1* is a nuclear unspliced transcript suggests that the RNA itself may have regulatory roles in epigenetic gene silencing. Recently, it has been suggested that the RNAi pathway plays a role in X inactivation through the nuclear noncoding transcript Xist (Ogawa et al., 2008). We therefore tested genetically whether the RNAi pathway was involved in regulating imprinting at the *Kcnq1ot1* cluster by using mouse mutants for the crucial RNAi enzyme Dicer (Bernstein et al., 2001).

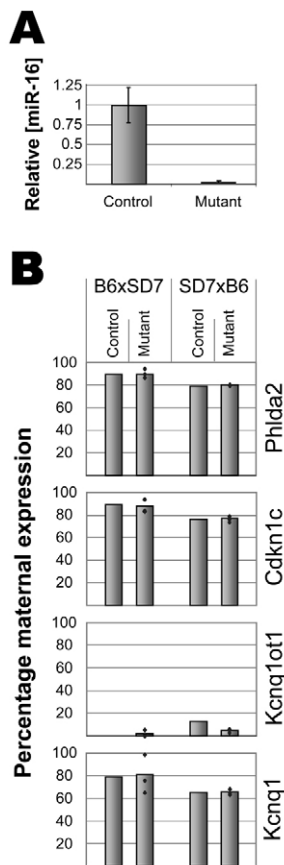
*Dicer* knockout embryos die at around E7.5 (Bernstein et al., 2003), thus hampering a comprehensive analysis of imprinting across the locus. Instead, we made use of a conditional knockout of *Dicer* (Cobb et al., 2005), which we specifically deleted in the epiblast (from E5.5) using a *Cre* transgene driven by the *Meox* promoter (Tallquist and Soriano, 1999). Deletion of *Dicer* in the embryonic lineage resulted in lethality at around E11, with characteristic malformations of the embryonic vasculature and oedema (E.R.P., B.S.C. and Wendy Dean, unpublished). The expression levels of miR-16 were substantially reduced in *Dicer*-null embryos when compared with heterozygous control littermates, showing that Dicer function had been effectively ablated in these knockout embryos (Fig. 2A). We also attempted to delete Dicer in the placental lineage with a *Cyp19Cre* transgene (Wenzel and Leone, 2007), but this did not result in significant deletion of the floxed *Dicer* allele (not shown). If the RNAi pathway is involved in maintaining repression of the maternal *Kcnq1ot1* promoter, we would expect maternal *Kcnq1ot1* expression in the *Dicer* knockout, and perhaps downregulation of the adjacent imprinted genes on the maternal allele. Conversely, if Dicer is needed for silencing via the *Kcnq1ot1* RNA of the imprinted genes in the cluster on the paternal chromosome, we would expect elevated transcription of these genes from their paternal alleles in the knockout.

We analysed the relative allelic expression of four imprinted genes in the cluster, including *Kcnq1ot1*, by RT-PCR followed by Sequenom MassArray, comparing control with mutant embryos (Fig. 2B). Additionally, we also analysed six biallelically expressed genes (not shown). The profile of relative allelic expression determined by MassArray corresponded closely to that determined previously by allele-specific RT-PCR (Lewis et al., 2004). No differences were found between control and mutant embryos, showing that Dicer-dependent siRNA pathways are not involved in maintaining epigenetic gene silencing in the *Kcnq1ot1* cluster (Fig. 2B; data not shown). However, given that Dicer is expressed in the conditional knockout embryos before E5.5, we cannot exclude the possibility that the siRNA pathway is necessary for the establishment of silencing, after which a Dicer-independent maintenance mechanism would take over. Dicer has been implicated in Xist-dependent X inactivation in one study (Ogawa et al., 2008), whereas another study has found no evidence of an involvement of the siRNA pathway in X inactivation (Nesterova et al., 2008).

### The *Kcnq1ot1* RNA establishes a lineage-specific nuclear domain for silenced genes

The Xist RNA has been shown by RNA FISH to produce a large nuclear signal that overlaps with most of the inactive X chromosome, and is thus thought to be coating the chromosome that it inactivates (Clemson et al., 1996; Chaumeil et al., 2006). By contrast, the primary transcripts of protein-coding genes show a small nuclear signal at the site of transcription (Osborne et al., 2004). As we found that *Kcnq1ot1* RNA is localised in the nucleus, and

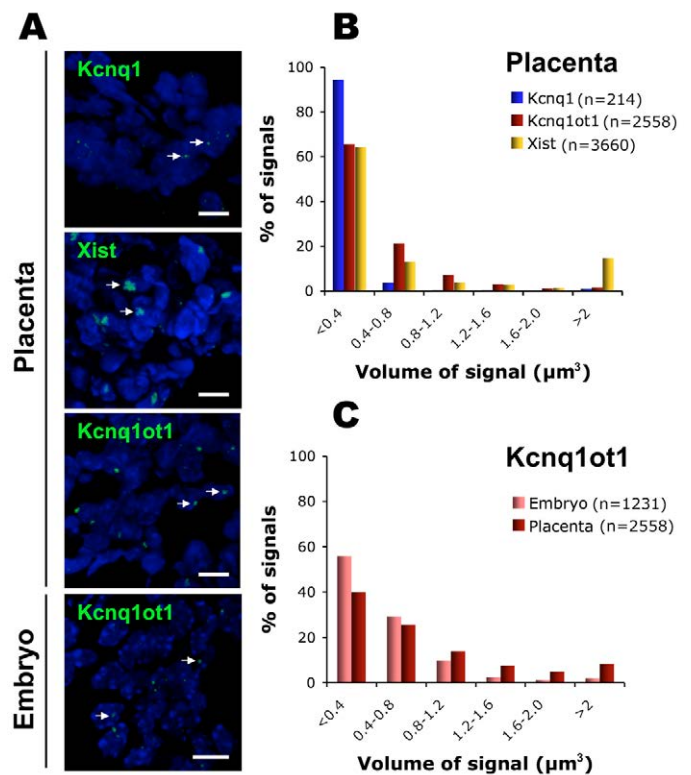




**Fig. 2. Silencing by *Kcnq1ot1* is not dependent on the RNAi pathway.** (A) qRT-PCR analysis of miR-16 abundance in control ( $Dcr^{fllox/\Delta}; Meox^{+/+}$ ) E10.5 and mutant ( $Dcr^{fllox/\Delta}; Meox^{Cre/+}$ ) E10.5 embryos, normalised to SnoRNA-202. The mature form of miR-16 is present in the mutants at a 50-fold lower level, demonstrating that Dicer action has been drastically reduced in the mutants. Error bars represent s.d. (B) RT-PCR and Sequenom MassArray analysis was performed to determine the percentage of maternal expression for the imprinted genes *Phlda2*, *Cdkn1c*, *Kcnq1ot1* and *Kcnq1* in E10.5 embryos. Means are represented as bars and individual data points as blue dots (one control embryo is shown for each cross). Data are shown for both the B6xSD7 and the reciprocal cross to exclude effects due to sequence variations between the B6 and SD7 alleles. *Dicer* mutants did not show loss of imprinting.

apparently does not work through the RNAi pathway, we tested the alternative hypothesis that the RNA might physically coat the chromatin that it silences.

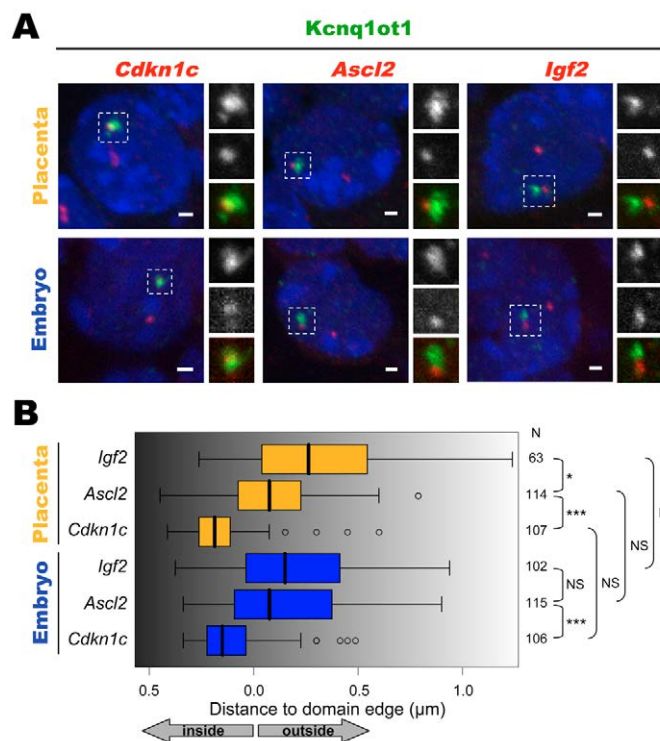
RNA-FISH was performed on E12.5 placental sections using probes for *Kcnq1*, *Kcnq1ot1* and *Xist* (Fig. 3A), and analysed by confocal microscopy. Volumes of fluorescent signals were measured using the 3D imaging software Volocity and grouped into six classes (Fig. 3B). We found significantly different volume distributions for *Kcnq1*, *Kcnq1ot1* and *Xist* ( $P < 0.0001$ ,  $\chi^2$  test; Fig. 3B). As expected, the *Kcnq1* probe produced small signals characteristic of primary transcription (Osborne et al., 2004), whereas the *Xist* probe produced a large signal, which is known to be covering the majority of the inactive X chromosome (Clemson et al., 1996). Interestingly, the *Kcnq1ot1* probe produced signals of intermediate size between *Kcnq1* and *Xist*. Indeed, only 5.6% of the *Kcnq1* probe signal volumes were greater than  $0.4 \mu\text{m}^3$ , whereas 34.4% of the *Kcnq1ot1*



**Fig. 3. The *Kcnq1ot1* transcript establishes a lineage-specific nuclear domain.** (A) RNA-FISH was performed on E12.5 embryo or placental sections using probes for *Kcnq1*, *Xist* and *Kcnq1ot1* (green; arrows). Nuclei were counterstained with DAPI (blue) and imaged by confocal microscopy. Images have been processed using Volocity for volume rendering. Scale bars:  $10 \mu\text{m}$ . (B) After RNA-FISH, the volumes of signals for *Kcnq1*, *Kcnq1ot1* and *Xist* were measured in the placenta, showing significantly different distributions ( $P < 0.0001$ ,  $\chi^2$  test), with the *Kcnq1ot1* signal occupying an intermediate volume between *Kcnq1* and *Xist*. (C) Measurements of *Kcnq1ot1* signal volumes between the embryo and placenta reveal that the *Kcnq1ot1* nuclear domain is larger in the placenta ( $P < 0.0001$ ,  $\chi^2$  test), in line with the larger silencing effect in this tissue.

probe signals and 35.7% of the *Xist* probe signals exceeded a volume of  $0.4 \mu\text{m}^3$ . In addition, 14.6% of the *Xist* probe signals were greater than  $2.0 \mu\text{m}^3$  compared with 1.6% of the *Kcnq1ot1* probe signals. As the *Kcnq1ot1* silenced domain is 780 kb in the placenta and 400 kb in the embryo, we compared the signal volumes of the RNA between the two tissues (Fig. 3C). Strikingly, the volumes occupied by the *Kcnq1ot1* RNA in the embryo were significantly smaller than those in the placenta ( $P < 0.0001$ ,  $\chi^2$  test), consistent with a model in which the RNA establishes a physical compartment in the nucleus associated with the chromosomal domain that is silenced.

In order to test this model more directly, we carried out combined RNA/DNA-FISH experiments on E12.5 embryo and placental sections, in which the *Kcnq1ot1* RNA domain was visualised together with gene loci regulated by *Kcnq1ot1* within the cluster (*Cdkn1c*), at the edge of the cluster (*Ascl2*) and at a locus not



**Fig. 4. Association of genes with the Kcnq1ot1 nuclear domain creates potential for silencing.** (A) After labelling of the Kcnq1ot1 nuclear domain by RNA-FISH (green) on E12.5 embryo and placental sections, DNA-FISH (red) was performed to detect an ubiquitously imprinted gene (*Cdkn1c*), a gene specifically imprinted in the placenta (*Ascl2*) and a gene not regulated by Kcnq1ot1 located ~300 kb away from the gene cluster (*Igf2*). Nuclei were counterstained with DAPI (blue) and imaged by confocal microscopy. Panels to the right of each image are a magnification of the indicated areas, showing the RNA-FISH signal (top), DNA-FISH signal (middle) and the corresponding merged image (bottom). Scale bars: 1 μm. (B) For each DNA signal found close to the Kcnq1ot1 domain, 3D distances between the centre of the DNA signal and the edge of the domain were measured. *Cdkn1c* is found mainly inside and *Igf2* mainly outside the RNA domain in both tissues, with *Ascl2* positioned near the edge. But whereas in the placenta *Ascl2* is closer to the domain than the Kcnq1ot1-unrelated *Igf2* gene, their relative positions are not significantly different in the embryo. \* $P < 0.05$ , \*\*\* $P < 0.001$ ; NS, not significant; Kruskal-Wallis test with Dunn's post-hoc test. Bars on the boxplots represent data points within 1.5 times the interquartile range.

regulated by Kcnq1ot1 located just outside the cluster (*Igf2*) (Fig. 4A). The distance of each of the three DNA probes (~40 kb in size) to the edge of the RNA domain was measured in 3D (Fig. 4B). Strikingly, both in embryo and placenta the RNA signal frequently overlapped the position of *Cdkn1c*, which was found mostly inside of the domain, whereas *Igf2* was predominantly located outside (Fig. 4). In the placenta, the positions of all three genes were significantly different from each other, with *Ascl2* assuming a position close to the edge of the domain. Although we did not find a significant difference in *Ascl2* position between the placenta (where it is imprinted) and the embryo (where it is not imprinted), interestingly, in the latter, the position of *Ascl2* did not differ significantly from that of the adjacent *Igf2* gene, which is not regulated by Kcnq1ot1. This suggests that the local chromatin structure is more compact in the embryo, and that the proximity between *Ascl2* and the Kcnq1ot1

RNA domain in the embryo may not be functionally relevant. Our data suggest that Kcnq1ot1 forms a domain where genes located inside have the potential to be epigenetically inactivated, whereas genes located outside of this domain do not. Importantly, work in human fibroblasts (Murakami et al., 2007) and mouse E14.5 placenta (Pandey et al., 2008) provides evidence that the Kcnq1ot1 RNA associates with chromatin rather than forming an independent RNA cluster.

## Conclusions

Our observations reveal several crucial similarities between gene inactivation by Kcnq1ot1, Air and Xist. In all three situations it appears that the ncRNA is involved in establishing a domain or compartment in the nucleus in which the silenced genes are located (Chaumeil et al., 2006; Nesterova et al., 2008; Nagano et al., 2008) (this study). The Kcnq1ot1 RNA domain may interact with chromatin and also with G9a (Pandey et al., 2008) and Ezh2, resulting in cluster-wide repressive histone marks, gene silencing and DNA methylation of CpG islands in some promoters (e.g. *Cdkn1c*), with methylation being more pronounced in the embryo than in the placenta. Paternal expression of Kcnq1ot1 and Xist begins in the two-cell embryo, and the dynamics of inactivation of individual genes is developmentally regulated and differs between the embryonic and extra-embryonic lineages [most dramatically for the X chromosome, where erasure of imprinted X inactivation in ICM cells in the blastocyst is followed by random inactivation in the epiblast (Mak et al., 2004; Okamoto et al., 2004)], consistent in the case of Kcnq1ot1 with the lineage-specific differences in the organisation of its nuclear silencing domain. Our observations, together with those of others (Pandey et al., 2008; Terranova et al., 2008; Nagano et al., 2008), establish that the epigenetic mechanisms regulating X chromosome inactivation by Xist and clustered genomic imprinting by long ncRNAs share fundamental similarities.

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## Supplementary material

Supplementary material for this article is available at <http://dev.biologists.org/cgi/content/full/136/4/525/DC1>

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