Elongation of the *Kcnq1ot1* transcript is required for genomic imprinting of neighboring genes

Debora Mancini-DiNardo, Scott J.S. Steele, John M. Levorse, Robert S. Ingram, and Shirley M. Tilghman[1]

Department of Molecular Biology, Princeton University, Princeton, New Jersey 08544, USA

The imprinted gene cluster at the telomeric end of mouse chromosome 7 contains a differentially methylated CpG island, KvDMR, that is required for the imprinting of multiple genes, including the genes encoding the maternally expressed placental-specific transcription factor ASCL2, the cyclin-dependent kinase CDKN1C, and the potassium channel KCNQ1. The KvDMR, which maps within intron 10 of *Kcnq1*, contains the promoter for a paternally expressed, noncoding, antisense transcript, *Kcnq1ot1*. A 244-base-pair deletion of the promoter on the paternal allele leads to the derepression of all silent genes tested. To distinguish between the loss of silencing as the consequence of the absence of transcription or the transcript itself, we prematurely truncated the *Kcnq1ot1* transcript by inserting a transcriptional stop signal downstream of the promoter. We show that the lack of a full-length *Kcnq1ot1* transcript on the paternal chromosome leads to the expression of genes that are normally paternally repressed. Finally, we demonstrate that five highly conserved repeats residing at the 5′ end of the *Kcnq1ot1* transcript are not required for imprinting at this locus.

Mammalian development requires the genetic contribution of both parental genomes (McGrath and Solter 1984; Surani et al. 1984). This requirement can be accounted for by the existence of ~80 imprinted genes whose expression is monoallelic based on the parent of origin (http://www.mgu.har.mrc.ac.uk/research/imprinting/imprin-viewdata.html). The precise mechanisms governing genomic imprinting are not fully understood, yet the propensity of imprinted genes to exist in clusters suggests that regulatory mechanisms that act over large genomic distances are likely to be involved. Indeed, studies of several imprinted chromosomal domains have identified imprinting control regions (ICRs) that coordinate the imprinting of multiple genes over hundreds of kilobases of DNA (Leighton et al. 1995; Wutz et al. 1997; Thorvaldson et al. 1998; Bielinska et al. 2000; Fitzpatrick et al. 2002; Lin et al. 2003).

The regulatory elements governing the imprinting of the genes at the distal end of mouse chromosome 7 have been the subject of extensive investigation (Fig. 1A). The more centromeric *H19*, *Igf2*, and *Ins2* genes are coordinately regulated by a single ICR located 5′ of *H19* (Leighton et al. 1995; Thorvaldson et al. 1998). On the maternal chromosome the ICR binds CTCF, a zinc-finger protein, which mediates the activity of an enhancer blocker that inhibits the access of enhancers 3′ of *H19* to *Igf2* and *Ins2* (Bell and Felsenfeld 2000; Hark et al. 2000; Kaffer et al. 2000; Kanduri et al. 2000). On the paternal chromosome DNA methylation at the ICR, acquired during spermatogenesis, inhibits CTCF binding and spreads after fertilization to the *H19* promoter to inhibit its expression. Deletion of the ICR eliminates imprinting of *H19*, *Igf2*, and *Ins2*, but has no effect on the imprinting of genes that lie telomeric to *Ins2* (Leighton et al. 1995; Caspary et al. 1998; Thorvaldson et al. 1998).

The telomeric region contains at least eight maternally expressed genes that include *Ascl2*, which encodes an essential placenta-specific transcription factor (Guillemot et al. 1995), *Cdkn1c*, encoding a cyclin-dependent kinase inhibitor (Hatada and Mukai 1995), and *Kcnq1*, encoding a potassium channel (Gould and Pfeifer 1998). However, the extent of the imprinting of these genes is highly variable. For example, *Cdkn1c* is expressed exclusively from the maternal allele in all expressing tissues and at all stages of development, while *Kcnq1* is imprinted early in embryogenesis in a variety of tissues but becomes fully biallelic in adult tissues (Caspary et al. 1998; Gould and Pfeifer 1998), and expression and imprinting of *Ascl2* is restricted to the placenta (Guillemot et al. 1995).

The region also harbors a gene encoding a single pa-
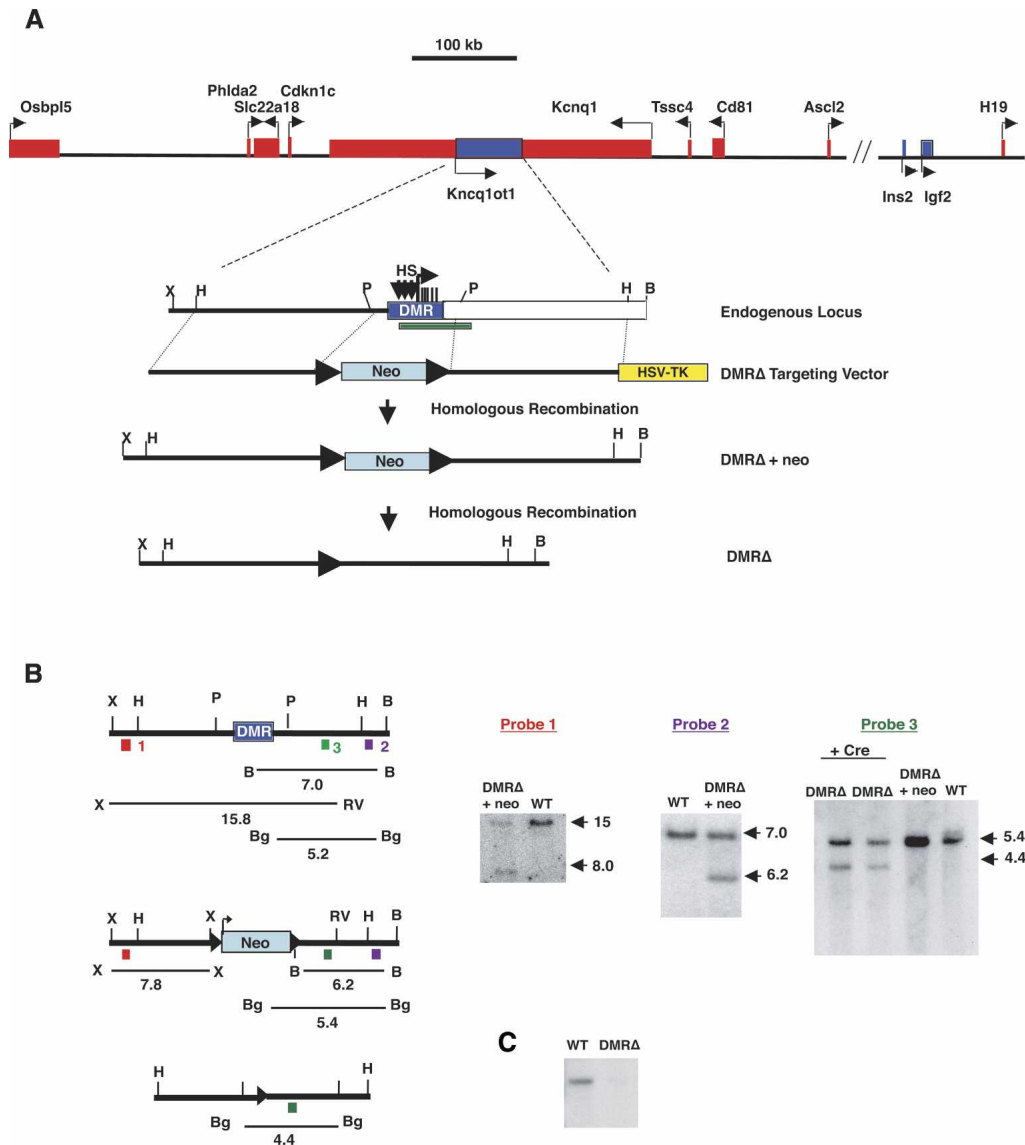
Figure 1. Deletion of the KvDMR. (*A*) The *top* line represents a physical map of the imprinted domain in mouse distal chromosome 7, with maternally expressed genes represented by red boxes and paternally expressed genes shown as blue boxes. Depicted *below* is an enlargement of the KvDMR (blue box), illustrating the three DNase I hypersensitivity sites (HS; vertical arrows), the *Kcnq1ot1* transcription start site (horizontal arrow), and the five repeats (vertical bars). The location of the KvDMR deletion described by Fitzpatrick et al. (2002) is depicted by the green rectangle. The targeting vector is shown *below*, in which the neomycin resistance gene (*neo*) flanked by *loxP* sites (closed triangles) replaces 3.6 kb of the DMR. The herpes simplex virus thymidine kinase gene (HSV-TK) was included to provide negative selection. The structures of the targeted loci, before and after the deletion of the neo gene, are indicated. (*B*) Southern blot analysis of DNAs prepared from wild-type (WT), heterozygous DMRΔ +*neo*, and heterozygous DMRΔ (−*neo*) mice after digestion with restriction enzymes and hybridization with the probes 1–3 as indicated in the diagrams. The diagrams of the endogenous and targeted loci depict the positions and sizes of the fragments detected by the probes. (*C*) RNA protection analysis of RNAs isolated from wild type and mice inheriting DMRΔ paternally using probe 3. (P) PstI; (X) XhoI; (H) HindIII; (B) BamHI; (RV) EcoRV; (Bg) BglI.

ternally expressed transcript, *Kcnq1ot1*, whose promoter is contained within intron 10 of the 320-kb-long *Kcnq1* gene (Smilinich et al. 1999). The ~60-kb transcript is unspliced and is transcribed in the direction opposite to that of *Kcnq1*. Its promoter lies within an extensive CpG island that is methylated selectively on the maternal chromosome (KvDMR) (Mancini-DiNardo et al. 2003). Paternal deletion of 2.8 kb of the KvDMR in the mouse

germline demonstrated that it was required for the silencing of at least six neighboring genes on the paternal chromosome (Fitzpatrick et al. 2002).

From the outset it seemed unlikely that the mechanism of action of the KvDMR resembled that at the *H19* ICR. Enhancer blockers are highly position dependent, in that they must reside between the gene and the enhancer to inhibit transcription. The complexity of the arrange-

ment of the genes on both sides of the KvDMR and their varied expression patterns make an enhancer-blocking model difficult to orchestrate. Indeed using in vitro transfection assays, the KvDMR behaved as a silencer rather than as an enhancer blocker (Mancini-DiNardo et al. 2003; Thakur et al. 2003). The other well-established mechanism for silencing imprinted genes is the RNA-dependent silencing by an antisense transcript, as occurs at the *Igf2r* locus in the mouse (Sleutels et al. 2002).

In this report, we show that silencing by the KvDMR requires transcription of *Kcnq1ot1*, and it is either the transcript itself or its transcriptional elongation that is critical, not its initiation. We also test the possibility that five highly conserved repeat elements located at the 5′ end of the *Kcnq1ot1* transcript are required for silencing, as has been shown for similar repeats in the *Xist* noncoding transcript that are required for X chromosome inactivation (Wutz et al. 2002). In the case of *Kcnq1ot1*, the repeats are dispensable for silencing.

Results

Deletion of the KvDMR

Fitzpatrick et al. (2002) previously reported that a 2.8-kb deletion of the KvDMR leads to disruption of silencing of six genes located on both sides of the deletion. We have confirmed their result and refined the mapping of the KvDMR by using homologous recombination in embryonic stem (ES) cells to generate a 3.6-kb deletion that includes an additional 1.7 kb 5′ to the first deletion, but leaves 875 base pairs (bp) 3′ of the deletion intact (Fig. 1A). The region that is deleted contains the entire differentially methylated CpG island, three hypersensitive sites that coincide with the promoter of *Kcnq1ot1*, and five repeats that lie within the transcript itself. Correctly targeted ES cells were introduced into C57BL/6 blastocysts, and the resulting males were bred to C57BL/6 females to yield germline transmission of the mutation and the removal of the neomycin cassette (Fig. 1B). To confirm that the deletion eliminated expression of *Kcnq1ot1* we examined its expression by a ribonuclease protection assay. After paternal inheritance of the DMRΔ, the noncoding *Kcnq1ot1* transcript was not detected (Fig. 1C).

We tested the effect of the deletion on the imprinting of six maternally expressed genes by crossing F_1 mutant mice to congenic mice that harbor *Mus spretus* alleles of all genes on the distal end of chromosome 7. RNAs were prepared from embryos at embryonic day 13.5 (E13.5), a time in development when the imprinting of these genes is still intact, and allele-specific RT–PCR assays were used to assess the expression of the imprinted genes. Paternal transmission of the deletion led to biallelic expression of *Ascl2*, *Kcnq1*, *Cdkn1c*, *Slc22a18*, *Phlda2*, and *Osbpl5* (Fig. 2). For each PCR reaction, we conducted control experiments in which the two parental alleles were represented in ratios of 1:1, 3:1, and 1:3. For *Kcnq1*, *Cdkn1c*, *Osbpl5*, and *Slc22a18*, there was a bias in favor of the *M. spretus* allele, and for *Ascl2* there was a C57BL/6 bias. When these biases are taken into account,
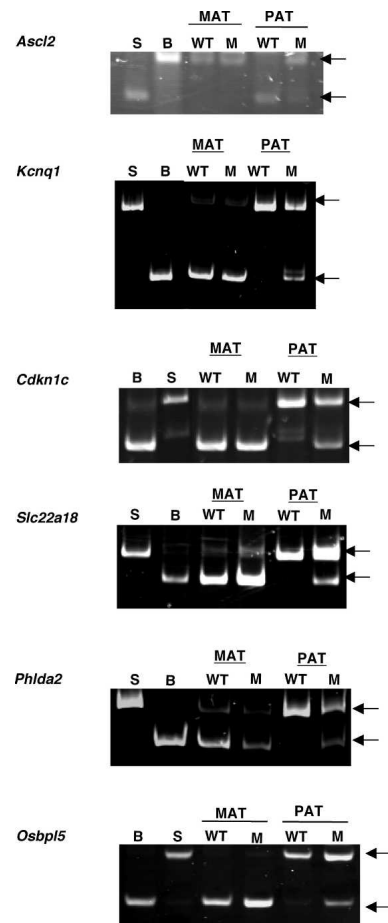
Figure 2. Effect of the KvDMR deletion on imprinting. Allele-specific RT–PCR assays were used to analyze the expression of the genes listed on the *left* using primers specific for each transcript (Table 2). Analysis was performed on E13.5 placental RNA isolated from offspring of reciprocal crosses between heterozygous DMRΔ and *M. spretus* mice (M) as well as their wild-type littermates (WT). (MAT) DMRΔ × *M. spretus*; (PAT) *M. spretus* × DMRΔ; (B) C57BL/6; (S) *M. spretus*. The arrows on the *right* refer to the allele-specific products.

the expression of the two parental alleles in the paternally inherited mutations is roughly equal.

In contrast, these genes displayed normal imprinted expression when the KvDMR deletion was inherited maternally. Our results confirm that the KvDMR functions as an ICR, and by showing that the most telomeric gene, *Osbp15*, falls under the influence of KvDMR, we significantly extend the size of the domain to at least 446 kb centromeric and 330 kb telomeric to KvDMR. We have also further delimited the essential sequences to 1.925 kb within the KvDMR. The deletion has no impact on the imprinting of *Igf2*, *H19*, and *Ins2*, which are regulated by a separate ICR (Fitzpatrick et al. 2002; data not shown).

We examined the effect of paternal inheritance of the DMR deletion on the growth of heterozygotes as compared with their wild-type littermates. We observed a 10%–20% decline in weight, consistent with the find-

ings of Fitzpatrick et al. (2002). No other gross phenotypic changes were noted.

DMRΔ rescues lethality of a Cdkn1c loss of function mutant

The absence of *Cdkn1c* has been shown to affect the ability of cells to exit the cell cycle, and *Cdkn1c* null mice show a variety of phenotypes including renal dysplasia, lens and gastrointestinal abnormalities, and skeletal defects (Yan et al. 1997; Zhang et al. 1997). Approximately 10% of these mice die in utero, with the remainder dying within 2 wk of birth. Since the deletion of KvDMR results in expression of *Cdkn1c* from the normally silent paternal allele, we asked if the level of paternally provided CDKN1C in DMRΔ mice could rescue a *Cdkn1c* loss of function mutation. We crossed *Cdkn1c* mutant females with DMRΔ males and looked for *Cdkn1c* mutant progeny that lived beyond 2 wk of age. Of the animals that lived to adulthood we observed 10 *Cdkn1c*/DMRΔ double mutants, six wild type, six DMRΔs, and, as expected, no *Cdkn1c* single mutants. Northern analysis confirmed wild-type levels of *Cdkn1c* mRNA in double-mutant animals (data not shown). These results demonstrate that the deletion of KvDMR results in expression of *Cdkn1c* from the paternal allele that is sufficient to rescue the *Cdkn1c* mutation, although it is formally possible that biallelic expression of other genes on the paternal allele may contribute to the survival of these mice.

Deletion of the Kcnq1ot1 promoter

We previously demonstrated that the promoter of the antisense transcript *Kcnq1ot1* is contained with the KvDMR (Mancini-DiNardo et al. 2003). To test whether regulation of imprinting at this locus requires the transcription of *Kcnq1ot1*, we used homologous recombination in ES cells to generate a 244-bp deletion of the *Kcnq1ot1* promoter (Fig. 3A). This deletion removes two of the three allele-specific DNase I hypersensitive sites within the promoter region and the transcription start site itself (Mancini-DiNardo et al. 2003), but leaves most of the CpG island intact. Once heterozygous mice containing the promoter deletion and missing the neomycin cassette were obtained (Fig. 3B,C), we used an RNase protection assay to ascertain whether paternal *Kcnq1ot1* transcription was eliminated. Using a probe derived from 481 bp downstream of the transcription start site, we show in Figure 3D that no transcript was detected when the deletion was inherited paternally.

We then tested the effect of the *Kcnq1ot1* promoter deletion on the imprinting of seven maternally expressed genes using E11.5 embryos and placentae (Fig. 4). Paternal transmission of the *Kcnq1ot1* promoter deletion activated the normally silent paternal alleles of all genes tested, while proper expression was maintained when the *Kcnq1ot1* promoter was deleted maternally. As with the KvDMR deletion, removal of the *Kcnq1ot1* promoter does not affect *Igf2* imprinting (data not shown). This mutation further delineates the sequences that are nec-

essary for the paternal silencing of the genes in the region and demonstrates that transcription of *Kcnq1ot1* is required for imprinting.

Premature termination of Kcnq1ot1 transcript

Deletion of the *Kcnq1ot1* promoter abolishes both the act of transcription as well as the transcript itself. As such, it is not possible to determine which of these is critical for the mechanism of imprinting control. Experiments exploiting an artificial episomal transfection system suggested that the transcript itself might play a role in gene silencing (Thakur et al. 2004). To assess in the genomic context whether the *Kcnq1ot1* transcript is involved in the silencing of flanking genes, we used homologous recombination in ES cells to insert a poly(A)-based transcription stop element ~1.5 kb downstream of the *Kcnq1ot1* start site (Fig. 5A). With this strategy, *Kcnq1ot1* transcription should be initiated, but a significantly shorter transcript produced in place of the ~60-kb *Kcnq1ot1* transcript.

Once mice with germline transmission of the mutation were obtained (Fig. 5B,C), we verified that initiation of transcription at *Kcnq1ot1* was not perturbed by the insertion of the stop signal by employing an RNase protection probe that spanned the site of insertion of the element (Probe D in Fig. 5). RNAs prepared from tissues of animals that inherited the mutation paternally protected a smaller fragment compared with wild-type animals, indicating that transcription was preserved at the locus. The absence of a protected fragment upon maternal transmission showed that the stop signal had no effect on the silencing of the transcript on the maternal chromosome. When a 261-bp probe (probe E) located downstream of the stop signal was used in the same assay, no transcript was detected from the paternally inherited mutant allele (Fig. 5E). This establishes that the stop signal was effective at terminating transcription.

We tested the effect of the premature termination of *Kcnq1ot1* on the imprinting of seven maternally expressed genes using E11.5 embryos and placentae (Fig. 6A). On the paternal allele, premature termination of the *Kcnq1ot1* transcript led to the derepression of the paternal copies of all seven genes. In contrast, the insertion of the stop signal on the maternal chromosome had no impact on their expression.

RNase protection analysis was also employed to more accurately assess the degree of derepression of a representative gene in the cluster, *Tssc4*. As shown in Figure 6B, when the mutant C57BL/6 allele is inherited paternally, *Tssc4* is expressed at levels comparable to the *M. spretus* allele. Thus we conclude that either the *Kcnq1ot1* transcript itself, or the elongation of its transcription beyond the termination signal inserted 1.5 kb downstream of its promoter, is necessary for imprinting in the region.

Deletion of Kcnq1ot1 5′ repeats

We next sought to determine whether there are specific sequences within the *Kcnq1ot1* transcript that are nec-
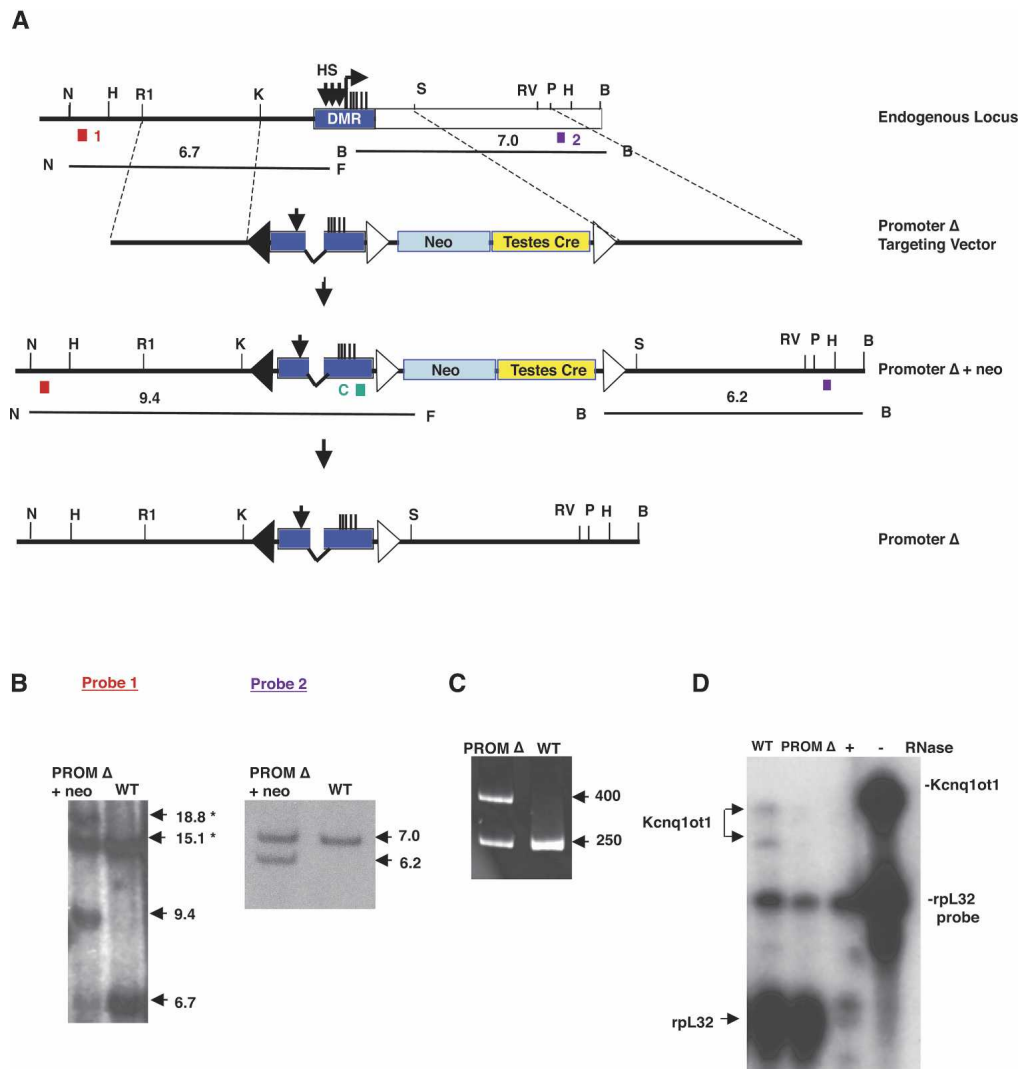
Figure 3. Deletion of the *Kcnq1ot1* promoter. (*A*) The endogenous locus is depicted as in Figure 1. The promoter Δ targeting vector contains a 244-bp deletion of the *Kcnq1ot1* minimum promoter region and two DNase I hypersensitive sites, as well as the neomycin resistance gene (*neo*) and the Cre recombinase gene driven by a testes-specific promoter (testes-*cre*), flanked by *lox*P sites (open triangles). The *lox*P site at the 5′ end of the KvDMR (closed triangle) contains point mutations that reduce its recombination frequency with the downstream sites. The positions of the probes used in *B* (numbered colored boxes) and the fragments that are detected are indicated. (H) HindIII; (R1) EcoRI; (K) KpnI; (F) FspI; (P) PstI; (S) SspI; (RV) EcoRV; (B) BamHI; (N) NheI. (*B*) Southern blot analysis of DNAs prepared from wild-type (WT) and heterozygous PromΔ +*neo* mice after digestion with restriction enzymes and hybridization with the external probes as indicated in *A*. The asterisks denote the presence of additional bands that are the result of methylation of FspI sites. In wild-type cells, a 15.1-kb band derives from methylation of the maternal allele and the 6.7-kb band from the unmethylated paternal allele. In targeted ES cells, the targeted paternal allele produces both a new 9.4-kb band created by a FspI site in the neo gene and an additional 18.8-kb band when that site is methylated. (*C*) DNA from PromΔ (–*neo*) and wild-type (WT) mice was amplified by PCR using primers that span the *neo* and testes cre sequences that yield a 250-bp product derived from amplification of the nontargeted allele and a 400-bp product from the PromΔ (–*neo*) allele (Table 1). (*D*) RNA protection of placental RNA from wild-type (WT) and *M. spretus* × PromΔ mice (PromΔ) was performed using probe C located at the 3′ end of the DNA repeats in KvDMR and an rpL32 ribosomal protein RNA probe as a control. Radiolabeled probes were incubated in the presence (+) and absence (–) of RNase and yeast RNA.

essary for imprinting control at this locus. We previously identified five evolutionarily conserved 30-bp repeated sequences at the 5′ end of the *Kcnq1ot1* transcript (Mancini-DiNardo et al. 2003). Repeated DNA elements are common features of imprinted gene clusters, and in the case of X chromosome inactivation, repeated sequences

at the 5′ end of the *Xist* noncoding RNA have been implicated in gene silencing (Wutz et al. 2002). To test the role of the repeated sequences in the 5′ end of the *Kcnq1ot1* transcript in imprinting, we deleted a 657-bp region of the *Kcnq1ot1* gene containing these repeats. In the previous experiment, the repeats are upstream of the
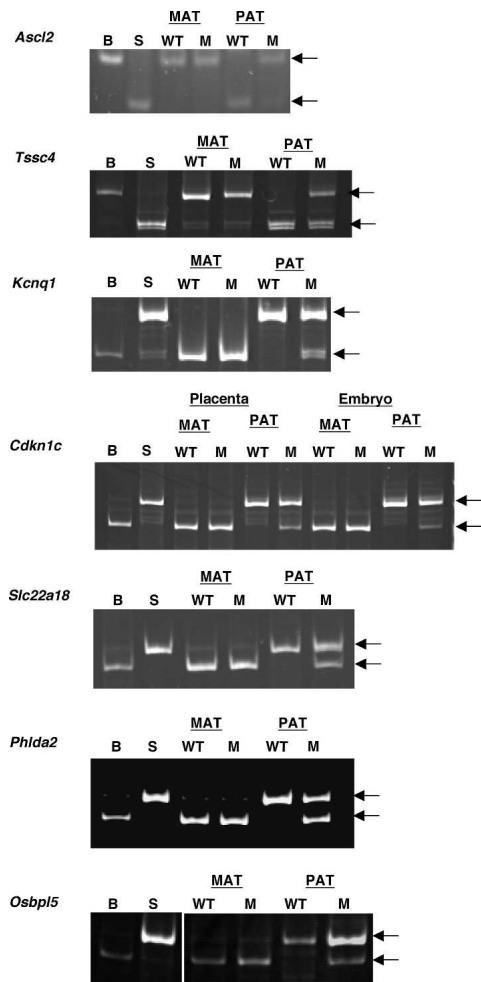
Figure 4. Effect of *Kcnq1ot1* promoter deletion on imprinting. Allele-specific RT–PCR assays were used to analyze the expression of the genes listed on the *left* using primers specific for each transcript (Table 2). Analysis was performed on E11.5 placental RNAs and in embryo RNAs (for *Cdkn1c*), isolated from offspring of reciprocal crosses between heterozygous Prom∆ and *M. spretus* mice (M) as well as their wild-type littermates (WT). (MAT) Prom∆ × *M. spretus*; (PAT) *M. spretus* × Prom∆; (B) C57BL/6; (S) *M. spretus*. The arrows on the *right* refer to the allele-specific products.

stop signal and as such are contained within the truncated *Kcnq1ot1* transcript. Thus, if these elements are necessary, they cannot be sufficient for imprinting. Following the correct targeting in ES cells and the germline transmission of the mutant allele (Fig. 7A–C), we tested whether the deletion affected expression of the *Kcnq1ot1* transcript using an RNase protection probe that spanned the 3′ end of the deletion. As shown in Figure 7D, a shortened *Kcnq1ot1* transcript was detected, indicating that the loss of the repeats had no effect on the production or stability of the RNA. When we used allele-specific RT–PCR assays to examine the expression of the imprinted genes in the region, no disruptions in imprinting were noted on either the maternal or paternal chromosomes (Fig. 8).

## Tissue distribution of Cdkn1c imprinting

Cdkn1c is one of the best-studied imprinted genes in this cluster. It is involved in embryonic growth control and is likely to have a role in the etiology of Beckwith Wiedemann syndrome in humans (Hatada et al. 1996; O'Keefe et al. 1997; Lam et al. 1999; Maher and Reik 2000). *Cdkn1c* is faithfully imprinted in all mouse tissues in which it is expressed, from early embryonic development through adulthood. In contrast, the majority of the other imprinted genes in the cluster show tissue-specific imprinting that is often lost in neonates (Hatada and Mukai 1995; Caspary et al. 1998; Gould and Pfeifer 1998). We have examined the imprinting status of *Cdkn1c* in multiple tissues in newborn and adult mice bearing each of three mutations. We determined that when the DMR∆, Prom∆, and Term mutations were inherited paternally, every tissue analyzed (brain, heart, liver, lung, spleen, kidney) displayed biallelic expression of *Cdkn1c* (data not shown).

## Methylation analysis of the Kcnq1ot1 promoter

The critical role of gametic methylation in the establishment and maintenance of genomic imprinting has been well established (Li et al. 1993; Bourc'his et al. 2001; Hata et al. 2002; Kaneda et al. 2004). A number of ICRs acquire their differential methylation during gametogenesis and preserve the pattern after fertilization at a time when the zygotic genome is undergoing significant epigenetic reprogramming (Kafri et al. 1992; Stoger et al. 1993; Tremblay et al. 1995; Davis et al. 2000). Furthermore the loss of methylation in mutant mice that lack *Dnmt1* and *Dnmt3a*, genes encoding DNA methyltransferases, has been shown to disrupt imprinting of multiple genes (Li et al. 1993; Bourc'his et al. 2001).

In wild-type mice, the *Kcnq1ot1* promoter within the KvDMR is heavily methylated selectively on the maternal chromosome, and that methylation is acquired during oogenesis (Engemann et al. 2000). The paternal allele is unmethylated, allowing for the transcription of *Kcnq1ot1*. Thus it is formally possible that the loss of silencing of the genes in the Term mutants could be occurring indirectly through inappropriate acquisition of DNA methylation on the paternal KvDMR allele, and not because of the truncation of the transcript. To assess this possibility we undertook sodium bisulfite analysis of DNA methylation of the KvDMR in Term mice and in Rep∆ mice as a control. To distinguish the parental chromosomes, we sequenced progeny from crosses between mutant and congenic *M. spretus* mice.

As shown in Figure 9, a 537-bp subregion of the KvDMR containing 23 CpG dinucleotides was unmethylated in E13.5 embryos on the Term paternal allele, in a manner indistinguishable from that in Rep∆ mice, where imprinting is unaffected. As expected, the *M. spretus* maternal alleles in both instances are heavily methylated. Thus we conclude that it is the loss of the full-length *Kcnq1ot1* transcript, and not a change in the methylation pattern of the KvDMR, that is responsible for the loss of imprinting in Term mice.
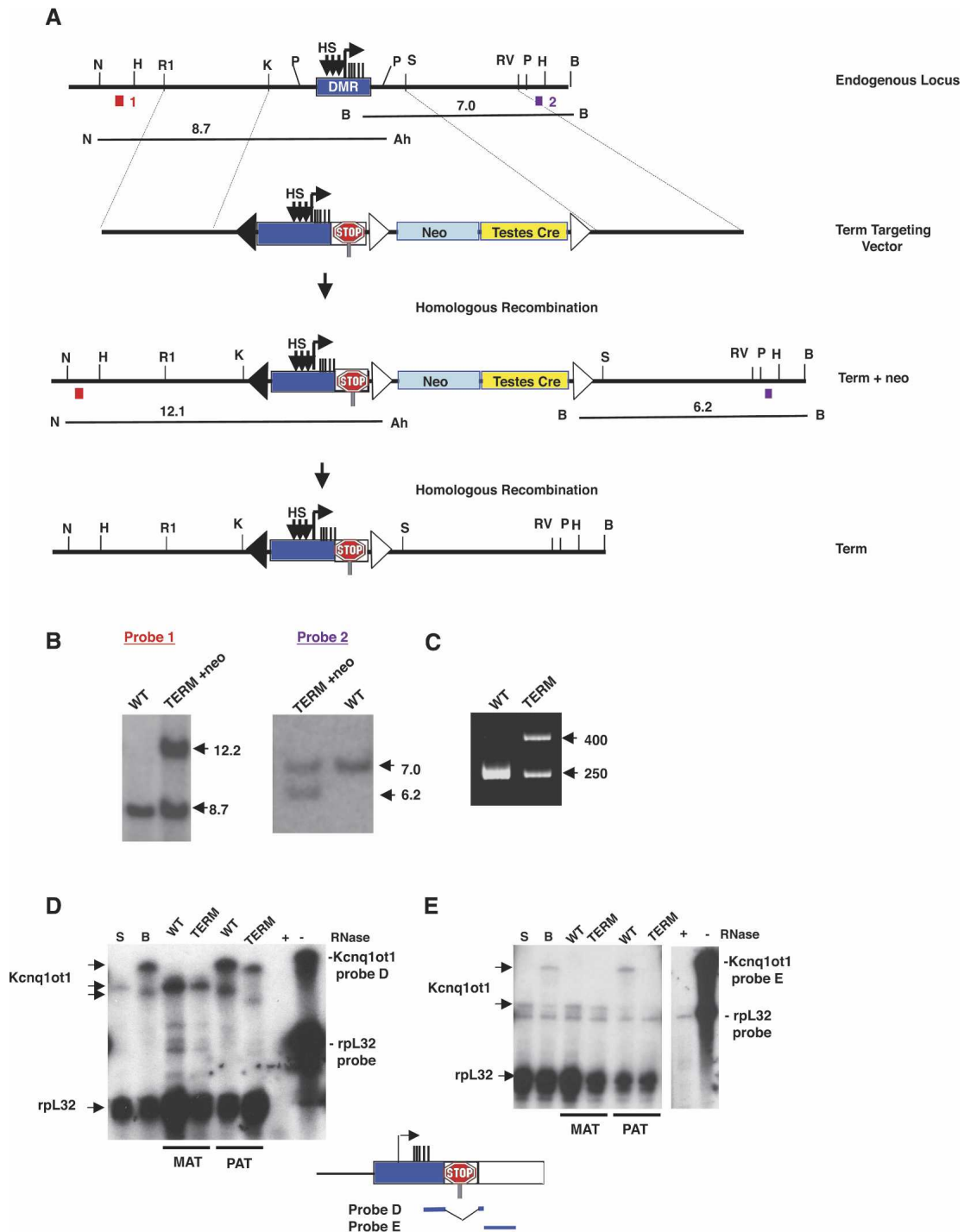
Figure 5. Termination of the *Kcnq1ot1* transcript. (*A*) The endogenous locus is depicted as in Figure 1. The Term targeting vector contains a poly(A)-based transcriptional stop element (stop sign) that is inserted 1.5 kb downstream of the *Kcnq1ot1* transcription start site. The rest of the vector is described in the legend for Figure 3. The positions of the probes used in *B* and the positions of the restriction fragments that are detected are indicated. (N) NheI; (H) HindIII; (R1) EcoRI; (K) KpnI; (P) PstI; (S) SspI; (RV) EcoRV; (B) BamHI; (Ah) AhdI. (*B*) Southern blot analysis of DNAs prepared from wild-type (WT) and Term +*neo* mice after digestion with restriction enzymes and hybridization with the external probes as indicated in *A*. (*C*) DNA from Term (−*neo*) and wild-type (WT) cells was amplified by PCR using primers as described in the legend for Figure 3C. (*D,E*) RNase protection of E13.5 placental RNAs of wild-type (WT) and heterozygous Term embryos. Probe D spans the integration site of the poly(A) terminator and probe E is derived from downstream of the poly(A) terminator (see diagram). An rpL32 ribosomal protein RNA probe was included as a control. Radio-labeled probes were incubated in the presence (+) and absence (−) of RNase and yeast RNA.
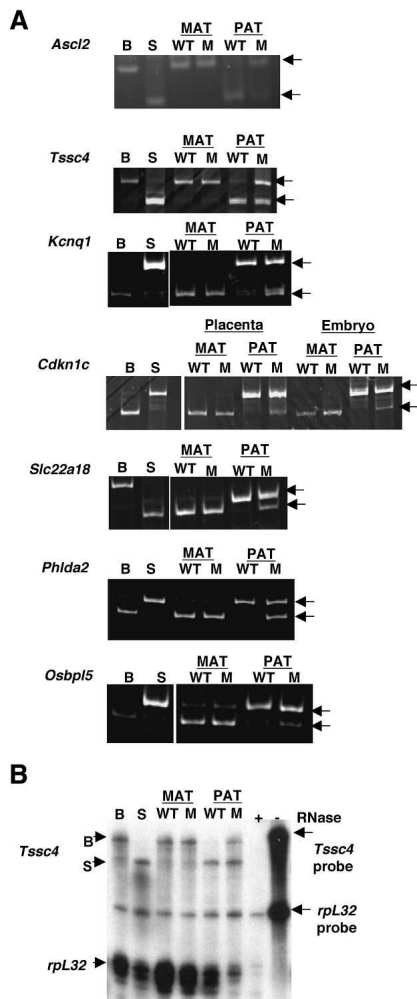
Figure 6. Effect of premature termination of the *Kcnq1ot1* transcript on imprinting. (*A*) Allele-specific RT–PCR assays were used to analyze the expression of the genes listed on the *left* using primers specific for each transcript (Table 2). Analysis was performed on E11.5 placental RNAs and in embryo RNAs (for *Cdkn1c*), isolated from offspring of reciprocal crosses between heterozygous TermΔ and *M. spretus* mice (M) as well as their wild-type littermates (WT). (MAT) TermΔ × *M. spretus*; (PAT) *M. spretus* × TermΔ; (B) C57BL/6; (S) *M. spretus*. The arrows on the *right* refer to the allele-specific products. (*B*) Allele-specific RNase protection analysis was used to determine the levels of *Tssc4* RNA in E11.5 and E13.5 placentae isolated from offspring of heterozygous Term × *M. spretus* mice (M) as well as their wild-type littermates (WT). An rpL32 ribosomal protein RNA probe was included to control for RNA amount. Radiolabeled probes were incubated in the presence (+) and absence (−) of RNase and yeast RNA. (MAT) TermΔ × *M. spretus*; (PAT) *M. Spretus* × TermΔ.

Discussion

Two distinct mechanisms for silencing imprinted genes have been described to date. The first mechanism was identified at the *H19/Igf2* locus, where a bifunctional ICR that lies between the paternally expressed *Igf2* gene and the maternally expressed *H19* gene acts as a nucleus for the spread of DNA methylation on the paternal allele

and a chromatin insulator on the maternal allele (Bell and Felsenfeld 2000; Hark et al. 2000; Kaffer et al. 2000; Kanduri et al. 2000). The activity adopted by the ICR is determined by its methylation status. The second mechanism for silencing was first described for the *Igf2r* imprinted gene cluster on mouse chromosome 17. In this case the ICR is a large maternally methylated CpG island that serves as a promoter for a noncoding RNA, *Air*, that is paternally expressed and contained within an intron of the maternally expressed *Igf2r* gene (Stoger et al. 1993; Wutz et al. 1997). Using a strategy very similar to the one employed in this study, Sleutels et al. (2002) showed that synthesis of the *Air* transcript itself is required to mediate repression of *Igf2r*, as well as other genes in the cluster in *cis*. The authors postulated that the *Air* transcript may have a direct role in establishing a chromatin state on the paternal chromosome that represses gene expression bidirectionally.

In this report we provide strong evidence that the imprinting mechanism at the *Cdkn1c–Kcnq1–Ascl2* imprinted gene cluster falls into the second class. As in the case of the *Igf2r* ICR, the KvDMR is maternally methylated and serves as a promoter for a paternally expressed noncoding transcript (Smilinich et al. 1999). When a large portion of the KvDMR was deleted in the mouse germline, imprinting of neighboring genes was eliminated, thus establishing the central role of the element in imprinting (Fitzpatrick et al. 2002). We have extended this finding by examining the consequences of deleting just 244 bp of the DMR coinciding with the minimal *Kcnq1ot1* promoter. The loss of imprinting of all genes in the cluster identified the production of the noncoding transcript as a critical element in the imprinting mechanism. By inserting a strong transcriptional stop signal into the *Kcnq1ot1* transcription unit, we established that the imprinting mechanism requires either the production of the transcript itself or its elongation past the engineered termination signal that is 1.5 kb downstream of the transcription start site. This finding is consistent with previous results using a stable transfection system that suggested that elongation of a segment of *Kcnq1ot1* was required for silencing reporter genes (Thakur et al. 2004). By showing that premature termination of the *Kcnq1ot1* transcript results in the loss of imprinting of at least seven genes normally silent on the paternal allele, including *Osbpl5*, which was not assessed in the earlier report (Fitzpatrick et al. 2002), we extend the regulatory influence of the KvDMR to a region encompassing at least 775 kb from *Ascl2* to *Osbpl5*.

One way to distinguish between a role for the transcript itself, versus the extent of its elongation, is to show that specific sequences within the transcript are required for gene silencing. For example, Wutz et al. (2002) identified a distinct domain within the noncoding *Xist* RNA that is required for the inactivation of genes on the X chromosome in *cis*. The *Xist* silencing domain resides at the 5′ end of the RNA and contains seven or eight copies of a 25-bp repeated sequence that is conserved between mice and humans (Brown et al. 1992; Hendrich et al. 1993). The authors demonstrated that a
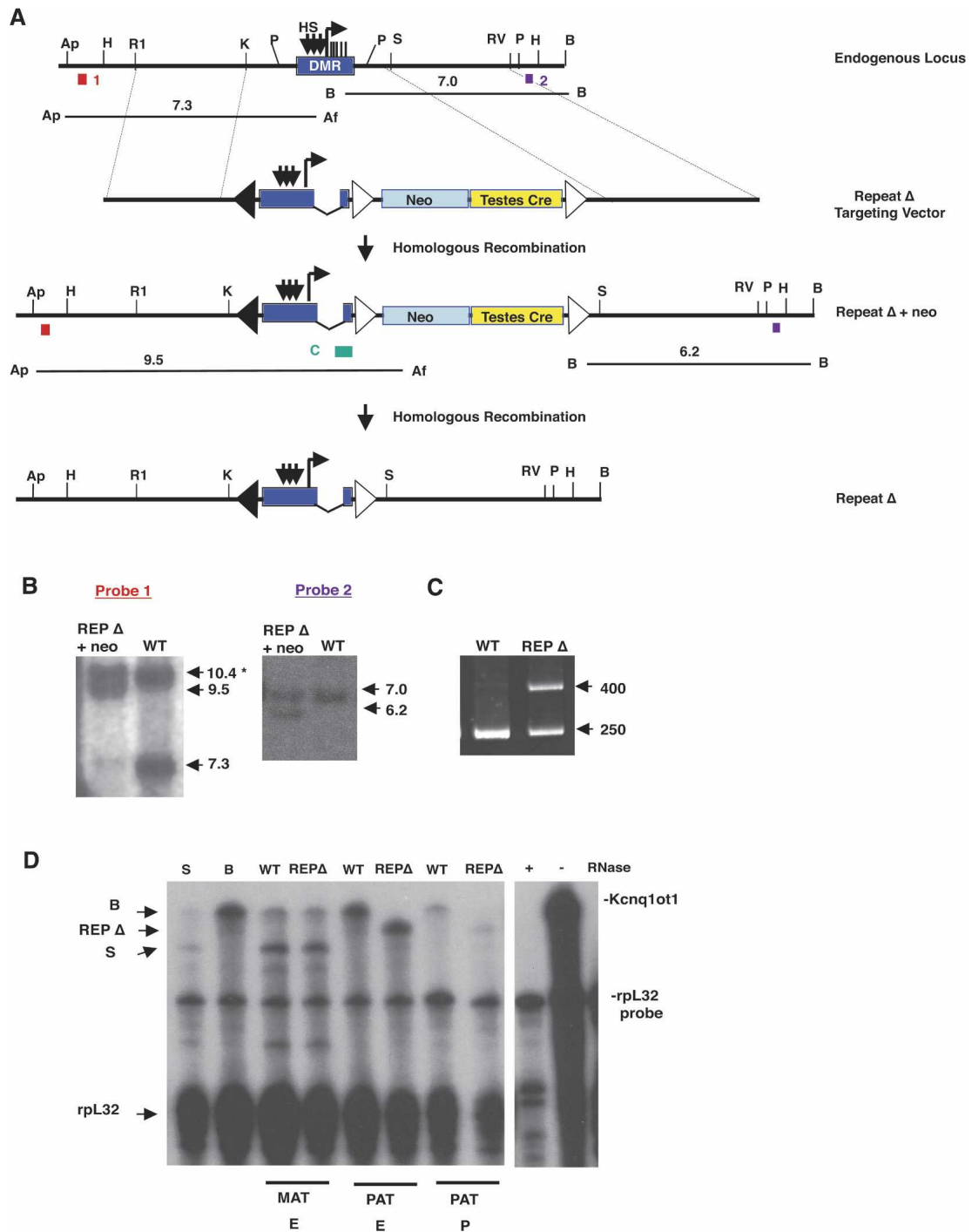
Figure 7. Deletion of DNA repeats in the *Kcnq1ot1* transcript. (*A*) The endogenous locus is depicted as in Figure 1. The RepΔ targeting vector contains a 657-bp deletion of five repeats within the *Kcnq1ot1* transcript. The rest of the vector is described in the legend for Figure 2. The positions of the probes used in *B* and the sizes and positions of the restriction fragments that are detected are indicated. (Ap) ApaLI; (H) HindIII; (R1) EcoRI; (K) KpnI; (P) PstI; (Af) AfeI; (S) SspI; (RV) EcoRV; (B) BamHI. (*B*) Southern blot analysis of DNAs prepared from wild-type (WT) and RepΔ +neo mice after digestion with restriction enzymes and hybridization with the external probes as indicated in *A*. The asterisk denotes the presence of a 10.4-kb band that results from the methylation of an AfeI site on the untargeted maternal chromosome. (*C*) DNA from RepΔ (–*neo*) and wild-type (WT) cells was amplified by PCR using primers as described in the legend for Figure 3C. (*D*) RNase protection of placental (P) and embryo (E) RNAs of wild-type (WT) and RepΔ embryos in which the RepΔ is inherited either maternally (MAT) or paternally (PAT). Probe C spans the 3′ end of the repeat region that is deleted. An rpL32 ribosomal protein RNA probe was included to control for RNA quantity. Radiolabeled probes were incubated in the presence (+) and absence (–) of RNase and yeast RNA.
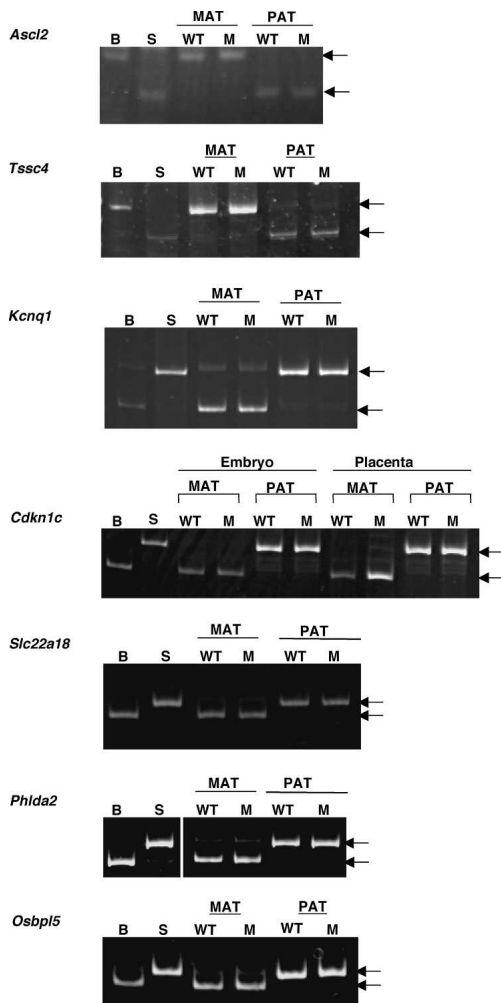
Figure 8. Effect of deletion of DNA repeats in the *Kcnq1ot1* transcript on imprinting. Allele-specific RT–PCR assays were used to analyze the expression of the genes listed on the *left* using primers specific for each transcript (Table 2). Analysis was performed on E11.5 placental RNA and in embryo RNAs (for *Cdkn1c*), isolated from offspring of reciprocal crosses between heterozygous RepΔ and *M. spretus* mice (M) as well as their wild-type littermates (WT). (MAT) RepΔ × *M. spretus*; (PAT) *M. spretus* × RepΔ; (B) C57BL/6; (S) *M. spretus*. The arrows on the *right* refer to the allele-specific products.

deletion of the repeated sequences resulted in a transcript that was able to coat the X chromosome destined to be inactivated, but was no longer able to repress transcription. We previously identified the presence of five repeats located at the 5′ end of the *Kcnq1ot1* transcript that are highly conserved in human, mouse, and galago (Mancini-DiNardo et al. 2003; Paulsen et al. 2005). Arguing by analogy to *Xist*, these repeats were potential candidates for a *cis*-acting silencing element within the *Kcnq1ot1* transcript. However, as shown in Figure 8, the loss of these 5′ repeated sequences does not affect the imprinting of any of the genes in the cluster. Thus, although repeated sequences seem to be a common feature of imprinting gene domains, in only two instances have

they been shown to play a mechanistic role (Reinhart et al. 2002; Yoon et al. 2002).

It remains to be established how noncoding RNAs like *Air* and *Kcnq1ot1* might affect gene silencing in *cis.* The simplest model, whereby the RNAs destablilize sense transcripts through the formation of double-stranded RNAs, is difficult to reconcile with the genomic organization of the imprinted gene clusters. In the case of *Kcnq1ot1*, this model could explain the silencing of *Kcnq1*, but it cannot explain the silencing of *Cdkn1c* and the other more telomeric genes that are transcribed in the same direction of *Kcnq1ot1*. It is also difficult to explain by this model the transient imprinting of *Kcnq1*, which is only imprinted during embryonic and fetal development, but becomes biallelic in tissues that continue to express *Kcnq1ot1* later in development (Mancini-DiNardo et al. 2003). An alternative RNA-dependent silencing mechanism is RNA interference (RNAi), in which the noncoding transcript is processed into small RNAs, some fraction of which would base-pair with the mRNA or DNA of the imprinted genes in the cluster (Dykxhoorn et al. 2003). Such a model would have to incorporate an additional constraint to restrict silencing in *cis.*

There are a number of transcription-dependent models that are also plausible candidates for the silencing of genes in the *Cdkn1c*–*Kcnq1*–*Ascl2* cluster. For example, the repression could occur via the movement of RNA polymerase through the length of the 60-kb *Kcnq1ot1* gene, leading to topological changes that attract repressive chromatin proteins in *cis.* In this model the actual sequences transcribed are not relevant, only the production of a full-length transcript. Such a model must accommodate the fact that embedded in the 775-kb cluster are several genes that escape silencing on the paternal chromosome, for example, *Trpm5* between *Kcnq1* and *Tssc4* (Enklaar et al. 2000). However, there is ample precedent for this; for example, there are numerous genes known to escape inactivation on the inactive X chromosome (Carrel and Willard 2005) and silencing by *Drosophila* heterochromatin (Hoskins et al. 2002). Alternatively *Kcnq1ot1* could participate in an RNA-dependent transcriptional silencing mechanism in which RNAs attract both DNA methyltransferases and repressive chromatin proteins (Matzke and Birchler 2005). Such a mechanism has been proposed for the role of *Xist* RNA in X chromosome inactivation, where it has been shown to bind to and coat the entire inactive X chromosome prior to gene inactivation (Ogawa and Lee 2002).

Whatever the specific details of how *Kcnq1ot1* transcription sets up the imprinting of the genes in the cluster, it is likely that a variety of well-known mechanisms are then exploited to maintain and replicate the silent state at each cell division. An extensive analysis of DNA methylation in the region has revealed that other than KvDMR, only *Cdkn1c* and *Slc22a18* are associated with substantial allele-specific methylation in their vicinities (Yatsuki et al. 2002; Lewis et al. 2004). This finding is consistent with reports that the imprinting of genes such as *Osbpl5*, *Kcnq1*, *Tccs4*, and *Ascl2* in extraembryonic
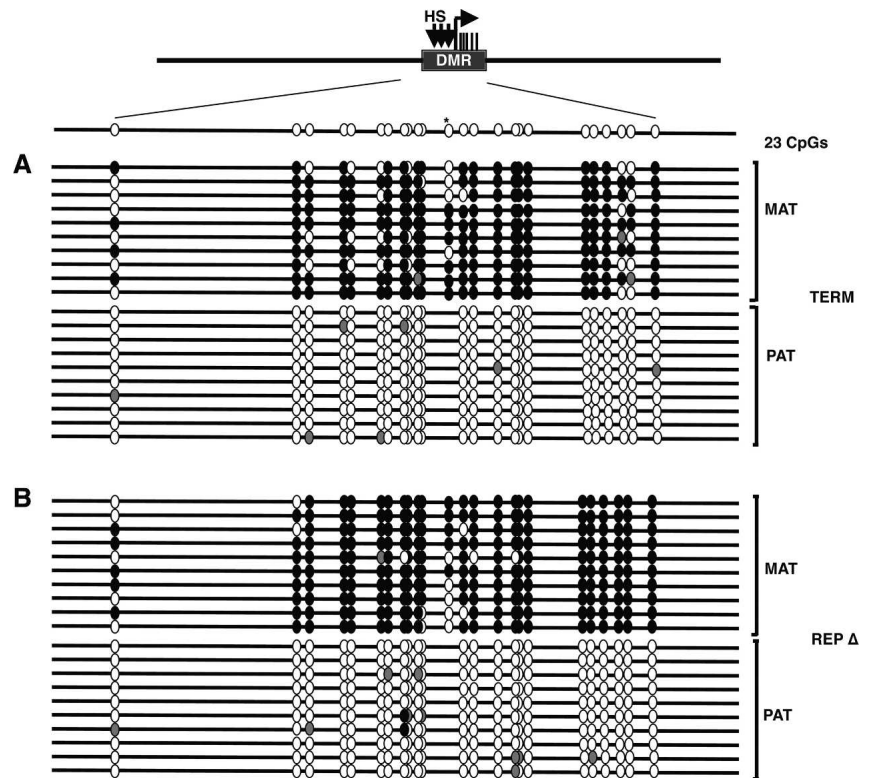
Figure 9. CpG methylation analysis at KvDMR. A 537-bp region spanning the *Kcnqt1ot1* promoter, which includes 23 CpG dinucleotides, was amplified from E11.5 and E13.5 embryos and yolk sac following treatment with sodium bisulfite. The asterisk refers to a polymorphism between *M. spretus* and C57BL/6 that was used to distinguish the parental alleles. DNA from *M. spretus* × Term embryos (*A*) and *M. spretus* × RepΔ embryos (*B*). Black ovals indicate methylated CpGs, open ovals indicate unmethylated CpGs, and gray ovals represent unreadable sequence.

tissues is unaffected by the loss of DNA methylation in mice that are mutant for the maintenance DNA methyltransferase, *Dnmt1* (Caspary et al. 1998; Tanaka et al. 1999; Lewis et al. 2004). Interestingly the imprinting of *Kcnq1* was lost in the embryo but not the trophoblast of *Dnmt*−/− embryos, suggesting tissue-specific differences in the relative importance of DNA methylation to imprinting. These findings suggest that somatic differential DNA methylation can only partially explain the imprinting of genes in the cluster. Indeed even for *Cdkn1c*, its paternal-specific methylation is not inherited from the gametes but is established post-implantation during the wave of de novo genome methylation (Engemann et al. 2000). Analysis of preimplantation embryos revealed maternal-specific expression of *Cdkn1c* prior to the establishment of its differential methylation, implying that methylation does not contribute to the establishment of the imprinted state at *Cdkn1c*, but adds another level of stability, effectively reinforcing the imprinted state within the embryo (Bhogal et al. 2004).

The *Cdkn1c–Kcnq1–Ascl2* cluster is characterized by extensive differential histone modifications in placenta, but not embryo, with the exception of the KvDMR and *Cdkn1c*, which are differentially associated with histones in both tissues (Lewis et al. 2004; Umlauf et al. 2004). Histone modifications form part of a complex histone code, in which transcriptionally inactive genes are associated with deacetylated histones H3 and H4 as well as dimethylation at Lys 9 and trimethylation at Lys 27 on histone H3 (Nielsen et al. 2001). In contrast, active genes display histone acetylation at Lys 9 and Lys 14 of histone H3 as well as dimethylation of Lys 4 of histone H3. As expected, upon paternal deletion of the KvDMR, the paternal alleles of several genes in the cluster became associated with histone H3 acetylated at Lys 9 and methylated at Lys 4, characteristic of an active transcriptional state (Lewis et al. 2004).

In addition to the histones themselves, repressive chromatin contains other proteins that are critical to establishing the repressed state. One such class of proteins is the Polycomb group (PcG), which promote gene silencing via histone methyltransferase activity as well as through their association with histone deacetylases. PcG proteins are able to repress gene activity over a considerable distance, likely by spreading a repressive chromatin state (Wang et al. 2004). The Polycomb group protein complex, which includes embryonic ectoderm development (Eed), enhancer-of-zeste homolog-2 (Ezh2), and suppressor-of-zeste-12 (Suz12) is known to methylate Lys 27 of histone H3 (Cao and Zhang 2004), and associates with the silent paternal alleles of *Ascl2* and *Cdkn1c* in placenta, but not the silent promoter of *Kcnq1ot1* (Umlauf et al. 2004) This is consistent with the report that *Eed*-deficient embryos displayed partial loss of imprinting of these genes, but not *Kcnq1ot1* (Mager et al. 2003), which presumably is primarily regulated by its extensive gametic methylation.

In summary, our current model for the imprinting of the genes within the *Cdkn1c–Kcnq1–Ascl2* gene cluster proposes that the differential gametic DNA methylation of KvDMR establishes the initial difference between the parental chromosomes and leads to paternal-specific

transcription of *Kcnq1ot1*. By a mechanism that remains to be determined, the transcription of *Kcnq1ot1* early in development leads to the bidirectional recruitment of silencing factors in *cis* that confer DNA methylation and histone modifications that do not occur on the maternal chromosome. The challenge now becomes to connect at the molecular level the transcription of *Kcnq1ot1* to the presence of these epigenetic modifications in the region.

Materials and methods

Generation of DMRΔ, PromΔ, Term, and RepΔ mice

To generate the DMRΔ targeting vector, a selection cassette consisting of the neomycin resistance gene (*neo*) was inserted in place of a 3.6-kb PstI fragment spanning the *Kcnq1ot1* promoter and KvDMR CpG island (Fig. 1A). To create the PromΔ, Term, and RepΔ targeting vectors, modified versions of a 3.5-kb KpnI–SspI fragment that spans the KvDMR were inserted between the two 5′ *lox*P sites in the vector described in Schoenherr et al. (2003) (Figs. 3A, 5A, 7A). The modifications were, in the PromΔ vector, deletion of a 244-bp BsrGI–SfoI fragment to disrupt the *Kcnq1ot1* promoter; in the RepΔ targeting vector, deletion of a 657-bp SfoI–AvrII fragment to delete the repeats; and in the Term vector, a transcriptional stop element composed of four tandem SV40 poly(A) sequences (GenBank accession no. AF316141.1) derived from a 3.4-kb XhoI–EcoRI fragment within the pBS. DAT–LoxStop vector (kindly provided by Tyler Jacks' laboratory at Massachussetts Institute of Technology, Cambridge, MA) was placed at an EcoRI site 1.5 kb downstream of the transcription start site.

The targeting vectors were then individually electroporated into CJ7 mouse ES cells. The cells were grown on neomycin-resistant primary mouse embryonic fibroblasts for a period of 7–10 d under selective media. Surviving colonies were expanded in 96-well plates, and DNA was prepared and digested with restriction enzymes for Southern blot analysis to identify correctly targeted cells. For all constructs, a 3′ external probe was used to detect a 7-kb BamHI fragment in wild-type cells and a 6.2-kb fragment in correctly targeted cells that exploits a BamHI site in the vector. For the DMRΔ targeted allele we used as a probe a 340-bp PCR product generated from cloned genomic DNA (probe 2: nucleotides 51907–52247, NR001461; Fig. 1B), and for the PromΔ, Term, and RepΔ targeted alleles we used a 379-bp PstI–HindIII fragment (probe 2; Figs. 3B, 5B, 7B). To detect the 5′ end of the PromΔ, Term, and RepΔ targeted alleles, a

1.2-kb AseI–HindIII fragment (probe 1) was employed with a variety of enzymes, depending on the construct (Figs. 3B, 5B, 7B). For the DMRΔ targeted allele, we used a 600-bp XhoI–HindIII fragment (probe 1) (Fig. 1B).

Correctly targeted clones were injected into C57BL/6 blastocysts and implanted into pseudopregnant mice. Male chimeras were bred to C57BL/6 females and their agouti progeny were genotyped by PCR using DNA isolated from tails (see Table 1 for primers). In DMRΔ mutants, the *neo* selectable marker was subsequently removed in F₂ animals by microinjecting Cre recombinase into the fertilized eggs of F₁ mutant animals. Proper excision was confirmed by Southern analysis on genomic DNA from F₂ animals using a 1.1-kb PstI fragment 3′ of the deletion as probe (probe 3) (Fig. 1B). The PromΔ, Term, and RepΔ targeting vectors contain the Cre recombinase (*cre*) gene under the control of a testes-specific promoter (Schoenherr et al. 2003). As such, the *neo* and *cre* genes were removed in the germline of male chimeras by testes-specific expression of Cre recombinase. A third variant Lox site was included 5′ of the KvDMR to provide a future option for homologous recombination within the region in intact mice. To confirm excision, a PCR-based assay was utilized that yields an ~400-bp band after excision of the cassette (Table 1).

F₁ and F₂ mutants were crossed to C57BL/6 congenic mice harboring *M. spretus* sequences at the distal chromosome 7 to provide allelic differences in parental alleles.

Allele-specific RT–PCR analysis

Total RNA from E11.5–E13.5 embryos and placentae as well as newborn mouse tissues was isolated using Trizol (Sigma) according to the manufacturer's instructions. The RNA was treated with DNase I (Stratagene) for 30 min at 37°C followed by phenol:chloroform (1:1) extraction and ethanol precipitation. The RNA was transcribed into cDNA using SuperScript First Strand (Invitrogen) and amplified using primers specific to each gene tested (Table 2). The PCR products were digested with restriction enzymes that selectively digested either the *M. spretus* or C57BL/6 allele. The products were analyzed on 10% polyacrylamide gels.

Allele-specific RNase protection assay

RNase protection assays were carried out using the RPAIII kit (Ambion). Antisense RNAs were synthesized from cDNA templates cloned into either pBluescript II KS+ or TA cloning vectors in the presence of [³²P]CTP and T3, T7, or Sp6 polymerases.

Table 1. *Primers used for genotyping PCR*

Targeting vector	Primer sequence
DMRΔ	FOR 5′-CAATGGCAGGAAGCAGGTGC-3′
DMRΔ (WT)	REV 5′-CGAGAGCCAGTCCTGTGAACAG-3′
DMRΔ (MUT)	REV 5′-CTTGTGTAGCGCCAAGTGC-3′
DMRΔ Neo Out	FOR 5′-CAATGGCAGGAAGCAGGTGC-3′
	REV 5′-GATCCTTCTAGCTGGAGCACAAATCC-3′
PromΔ	FOR 5′-CTCAGGTATCTGCCCTGTACTGCATTG-3′
	REV 5′-CGTAACCACGCCGAGGAGAAGCCCACCGAAGTA-3′
Term	FOR 5′-GTGTGCCTAGGACACCGGCTCAGGCCTTCGG-3′
	REV 5′-CCTTCACAAAGATCCCTCGAGCCCAATTCC-3′
RepΔ	FOR 5′-CTCAGGTATCTGCCCTGTACTGCATTG-3′
	REV 5′-CTTGCGACTTGTGCCGTGCTGACTC-3′
PromΔ, Term, and RepΔ	FOR 5′-GGAGTGCTTTCCGCTGTAACCTTTCTGGGC-3′
Neo out	REV 5′-GCCTGAGGATGGCTGTGTATTAGG-3′

Table 2. *Primers and restriction sites used for allele-specific RT–PCR*

Gene	Primer sequence	Polymorphic enzyme
Osbpl5 (*Obph1*)	FOR 5′-TGGACGAAGCTGTGGTGTG-3′ REV 5′-CGTCTGATTCAGAAGCGGC-3′	BfaI
Phlda2 (*Tssc3*, *Ipl*)	FOR 5′-ACTGCGTGGAGCACACCTCT-3′ REV 5′-ACACGGAATGGTGGGTTGGA-3′	BlpI
Slc22a18 (*Impt1*)	FOR 5′-TGTCTGCCTGGGATGTCTG-3′ REV 5′-GCCCGCCAGGAAGGAGAG-3′	HpaII
Cdkn1c (*p57^{Kip2}*)	FOR 5′-TTCAGATCTGACCTCAGACCC-3′ REV 5′-AGTTCTCTTGCGCTTGGC-3′	AvaI
Kcnq1 (*Kvlqt1*)	FOR 5′-GATCACCACCCTGTACATTGG-3′ REV 5′-CCAGGACTCATCCCATTATCC-3′	PvuII
Tssc4	FOR 5′-CTGTGCGCGCTAGGATACCCTTTAA-3′ REV 5′-AGGTCCAGCGAGATGGGCTAGCTCC-3′	HpyCH4IV
Ascl2 (*Mash2*)	FOR 5′-TGGGCTGCTCTGAGCCTACC-3′ REV 5′-AAGTCCTGATGCTGCAAGGT-3′	BstNI

Alternative names of the genes are indicated in parentheses.

In cases where T3 or T7 polymerase was used, samples were incubated at 37°C for 1 h. Probes synthesized using Sp6 were incubated at 42°C for 1 h. The probes were treated with RNase-free DNase for 15 min at 37°C and purified on 8 M urea/5% polyacrylamide gels. Each probe (20,000–80,000 cpm) was hybridized to 10 µg of total RNA for at least 16 h at 55°C. Following RNase digestion with a 1:50 dilution of the RNase A/T1 mix, the samples were separated on 7 M urea/7.5% polyacrylamide gels. Gels were then exposed to X-ray film and quantitated with a Molecular Dynamics PhosphorImager. The following probes were used for the allele-specific RPAs: *Tssc4* (450 bp: nucleotides 566–1016; NM020285), Term probe D (410 bp: nucleotides 6536–6946; AF119385), Term probe E (261 bp: nucleotides 8287–8548; AF119385), and probe C, (441 bp: nucleotides 5935–6376; AF119385).

Sodium bisulfite analysis

Genomic DNA was isolated from E11.5 and E13.5 embryos and yolk sacs using the DNeasy kit (Qiagen) according to the manufacturer's instructions. The DNA was subsequently modified using the sodium bisulfite conversion protocol (Andrews et al. 1996; Mancini et al. 1998). We amplified the promoter region of *Kcnq1ot1* using primers previously described (Yatsuki et al. 2002). All PCR amplifications were performed in 20-µL reactions containing ~150 ng of bisulfite-treated genomic DNA. The amplification conditions were 94°C for 3 min followed by 30 cycles at 94°C (30 sec), 58°C (30 sec), and 72°C (1 min). A final 7-min incubation at 72°C was also performed. Nested PCR reactions were carried out using 1 µL of the previous PCR amplifications for 30 cycles under the same conditions. PCR products were gel-purified and cloned into the pCRII-TOPO vector (Invitrogen), transformed, purified, and sequenced using fluorescence cycle sequencing.

Acknowledgments

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