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Silencing and transcriptional properties of the imprinted *Airn* ncRNA are independent of the endogenous promoter

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The *Airn* macro ncRNA is the master regulator of imprinted expression in the *Igf2r* imprinted gene cluster where it silences three flanking genes *in cis*. *Airn* transcription shows unusual features normally viewed as promoter specific, such as impaired post-transcriptional processing and a macro size. The *Airn* transcript is 108 kb long, predominantly unspliced and nuclear localized, with only a minority being variably spliced and exported. Here, we show by deletion of the *Airn* ncRNA promoter and replacement with a constitutive strong or weak promoter that splicing suppression and termination, as well as silencing activity, are maintained by strong *Airn* expression from an exogenous promoter. This indicates that all functional regions are located within the *Airn* transcript. DNA methylation of the maternal imprint control element (ICE) restricts *Airn* expression to the paternal allele and we also show that a strong active promoter is required to maintain the unmethylated state of the paternal ICE. Thus, *Airn* expression not only induces silencing of flanking mRNA genes but also protects the paternal copy of the ICE from *de novo* methylation.

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Introduction

The classical view of gene regulation has been challenged in recent years by genome-wide studies that simultaneously investigated whole cell transcriptomes (Carninci *et al*, 2005; Cheng *et al*, 2005). The most surprising result of these studies was the high proportion of long non-protein coding transcripts (macro ncRNAs). Indeed, non-coding transcription units, excluding those regulating mRNA processing and translation, outnumber coding transcription units. The abundance and specific regulation of ncRNAs have been taken as an indication that many may have functional roles, most likely in the regulation of flanking genes. Most members of this new class of ncRNA are transcribed by RNAPII but they can show unusual transcriptional properties compared with mRNAs as they are often antisense to coding transcripts and enriched in the nuclear, non-polyadenylated and unspliced fraction (Mattick, 2005; Kapranov *et al*, 2007; Yasuda and Hayashizaki, 2008).

For a small number of macro ncRNAs, a functional role in gene silencing has been shown. These examples include *Xist*, the macro ncRNA required for X chromosome inactivation in female mammalian cells, *Airn* the repressive macro ncRNA of the *Igf2r* imprinted gene cluster (formerly known as *Air* but now renamed *Airn* by the HUGO Gene Nomenclature Committee), and *Kcnq1ot1* the repressive macro ncRNA of the *Kcnq1* imprinted gene cluster (Penny *et al*, 1996; Sleutels *et al*, 2002; Mancini-Dinardo *et al*, 2006). We focus here on the *Igf2r* imprinted gene cluster in which paternal-specific expression of the *Airn* ncRNA silences three genes *in cis* spread over a 300-kb region. The *Airn* ncRNA promoter lies in an antisense orientation in *Igf2r* intron 2 and drives a 108 kb mainly unspliced ncRNA that overlaps the *Igf2r* promoter; however, the two other silenced genes (*Slc22a2* and *Slc22a3*) that lie 80–150 kb upstream are not overlapped, nor do they share sequence homology with the *Airn* ncRNA (Pauler *et al*, 2007).

The *Airn* ncRNA is maternally repressed by a DNA methylation imprint that is set in oocytes on a CpG island that is part of a 3.65-kb region genetically defined as the imprint control element (ICE) for the *Igf2r* imprinted cluster (Stoger *et al*, 1993; Wutz *et al*, 2001; Seidl *et al*, 2006). The *Airn* ncRNA promoter lies on the immediate 5′ side of the CpG island, thus all *Airn* ncRNA transcripts run through the CpG island (Figure 1A). The identical 3.65-kb region on the paternal chromosome is not methylated during spermatogenesis and also remains unmethylated in diploid embryonic cells, thus the *Airn* ncRNA promoter is only active on the paternal chromosome. It is unknown why the ICE is unmethylated in sperm and diploid embryos, as both these stages experience waves of *de novo* DNA methylation (Li, 2002). The unmethylated state of the paternal ICE in embryonic stages may be a passive process that results from the

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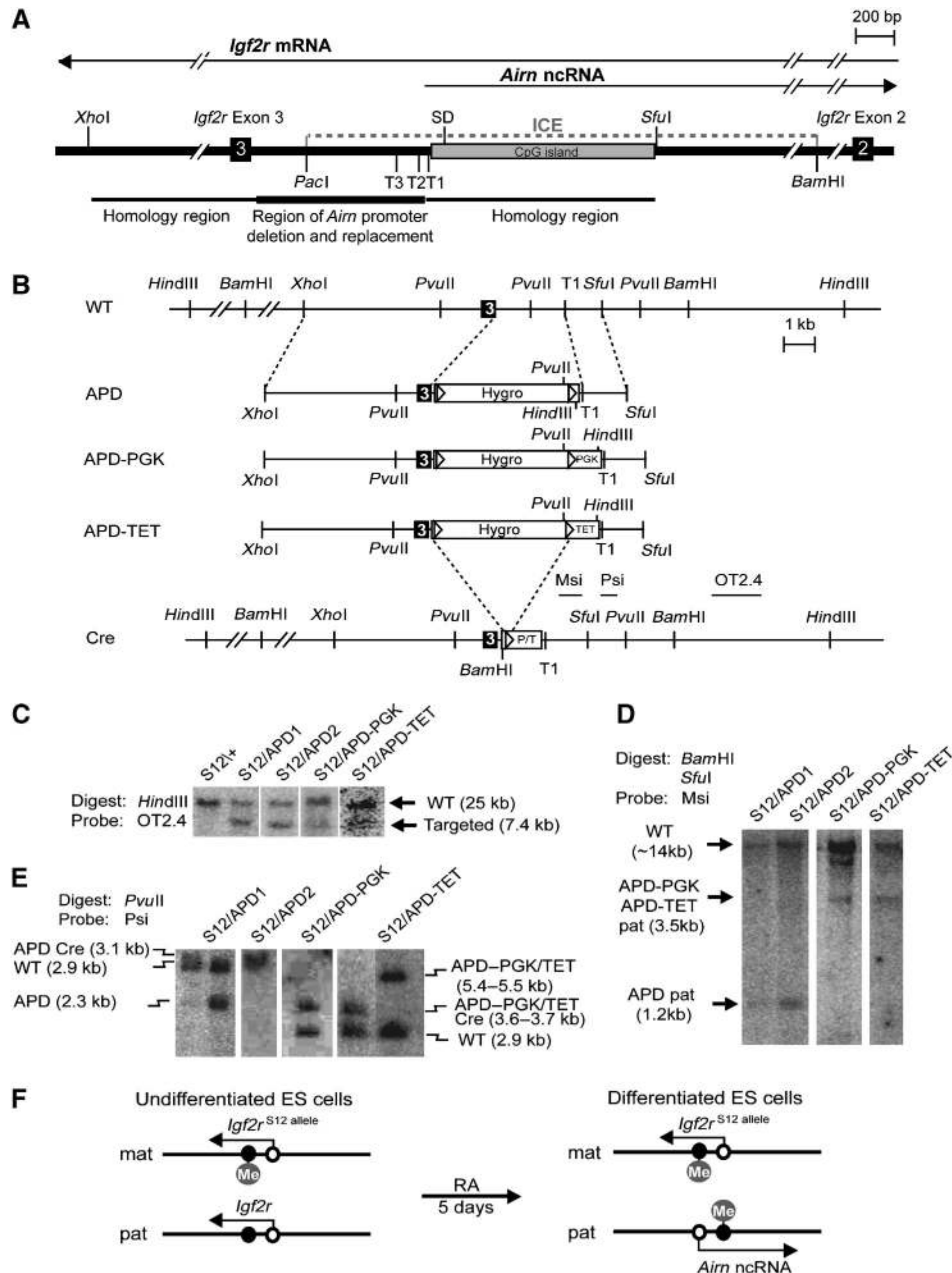

Figure 1 Deletion and replacement of *Airn* promoter constructs. (**A**) The *Airn* promoter lies in an antisense orientation in *Igf2r* intron 2. A 959-bp fragment is deleted in the APD, APD-PGK and APD-TET alleles (thick line). The main transcription start site (T1) and downstream CpG island are unchanged. SD: *Airn* splice donor. A 3.65-bp *Pac*I–*Bam*HI fragment that has been genetically defined as the imprint control element (ICE) for the *Igf2r* imprinted cluster is indicated by the dotted line (Wutz *et al*, 2001). (**B**) Three constructs were used for targeting the *Airn* promoter deletion (APD) and the two promoter replacement alleles containing a PGK promoter (APD-PGK) or a TET promoter (APD-TET). Msi, Psi, OT2.4: Southern blot probes; hygro: hygromycin cassette. (**C**) Southern blot showing homologous targeting indicated by a 7.4-kb band. (**D**) Southern blot using the *Sfu*I methyl-sensitive enzyme shows paternal targeting indicated by a 1.2 kb (APD) or 3.5 kb (APD-PGK, APD-TET) band. (**E**) Southern blot shows successful Cre recombination by loss of the unrecombined allele (APD 2.3 kb, APD-PGK 5.5 kb and APD-TET 5.4 kb) and gain of the recombined allele (APD 3.1 kb, APD-PGK 3.7 kb, APD-TET 3.6 kb). (**F**) Undifferentiated ES cells show biallelic expression of *Igf2r* and no expression of *Airn*. The *Airn* CpG island is methylated (Me) on the maternal allele (mat). Differentiation with retinoic acid (RA) leads to *Airn* upregulation, repression of *Igf2r* and methylation of the *Igf2r* CpG island on the paternal allele (pat) (PAL, manuscript submitted).

failure to attract the *de novo* methylation machinery in the paternal germ line and the availability only of maintenance methylation enzymes in embryonic stages, which only copy existing methylation patterns. However, it has been reported that short sequences in the paternal ICE actively protect it from *de novo* DNA methylation (Birger *et al*, 1999). More recently, it has also been suggested that active transcription might protect CpG island promoters from *de novo* DNA methylation. This suggestion arose from observations that weakening of promoters by mutating transcription factor-binding sites leads to promoter DNA methylation (Brandeis *et al*, 1994; Macleod *et al*, 1994).

The majority (>95%) of transcripts from the *Airn* ncRNA promoter are unspliced and we have suggested that splicing suppression is the key feature of the *Airn* ncRNA, as other transcriptional properties such as its macro size and nuclear

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localization could depend on this (Seidl *et al*, 2006). In support of this model, retention at the site of transcription has been shown to be a consequence of impaired splicing and 3′ end processing in a human beta globin transgene (Custodio *et al*, 1999). Splicing and 3′ end processing leading to transcript termination are thought to occur co-transcriptionally (Kornblihtt *et al*, 2004; Bentley, 2005). This indicates that the *Airn* ncRNA promoter itself should regulate its transcriptional properties and thus its ability to silence genes in the *Igf2r* gene cluster. Here, we show by deletion and replacement of the *Airn* ncRNA promoter in an *in vitro* ES cell imprinting model that *Airn* ncRNA transcriptional properties, as well as *Igf2r* silencing, are independent of the endogenous *Airn* ncRNA promoter. All attributes of imprinted silencing, including DNA methylation and transcriptional silencing of the overlapped paternal *Igf2r* promoter, can be induced by *Airn* ncRNA expression driven by a strong exogenous promoter. However, a low expression level of the *Airn* ncRNA driven by a weak exogenous promoter is not sufficient for *Igf2r* silencing. Splicing suppression and termination are unchanged in the *Airn* promoter replacement allele, indicating that functional regions of the *Airn* ncRNA are located within its transcribed gene body not in its promoter. Surprisingly, ES cells lacking an *Airn* ncRNA promoter or containing a weakly expressed promoter gain DNA methylation of the paternal ICE. This indicates that the unmethylated state of the paternal ICE requires transcriptional run-through for protection from *de novo* methylation.

Results

Establishment of Airn promoter deletion and promoter replacement by ES cell targeting

Three targeting constructs were produced to modify the *Airn* ncRNA promoter that lies in an antisense orientation in *Igf2r* intron 2, close to exon 3 (Figure 1). Construct 1 generated a deletion (named APD: *Airn* promoter deletion) of the *Airn* ncRNA promoter that spans 959 bp, starting 3 bp upstream of the 3′ splice acceptor site of *Igf2r* exon 3 and ending 1 bp upstream of the *Airn* main transcriptional start site (T1) (126236–127195 bp in accession number AJ249895). Constructs 2 and 3 were used to insert the ubiquitously expressed mouse phosphoglycerate kinase promoter (APD-PGK) or a tetracycline-inducible promoter (APD-TET) into the APD in the same orientation as the endogenous *Airn* promoter.

The APD, APD-PGK and APD-TET targeting constructs were electroporated into D3 ES cells previously modified on the maternal allele to carry a single nucleotide polymorphism (SNP) in *Igf2r* exon 12 (PAL, manuscript submitted). This exon 12 modified allele is named S12, and ES cells carrying a maternally modified allele are labelled S12/+ (note that the maternal allele is written on the left side throughout the text). Southern blot analysis of 800 hygromycin-resistant colonies revealed homologous targeting for nine APD, six APD-PGK and eight APD-TET constructs (Figure 1C and data not shown). Except for one maternally targeted APD construct, all other homologous recombination events occurred on the paternal allele (Figure 1D and data not shown). A paternal-specific targeting bias in ES cells of unknown cause has been reported earlier for the *Igf2r* and *Airn* promoter regions (Wang *et al*, 1994; Sleutels *et al*, 2002, 2003). The selection cassette was removed by transient transfection with a Cre recombinase expression plasmid (Figure 1E). Four homologously targeted ES cell lines were used in this study: S12/APD-1 and S12/APD-2 (two independent APD clones that lack a paternal copy of the *Airn* promoter), S12/APD-PGK (that replaces paternal copy of the endogenous *Airn* promoter with the ubiquitously expressed PGK promoter) and S12/APD-TET (that replaces paternal copy of the endogenous *Airn* promoter with an inducible TET promoter). The sequence of the APD-PGK and APD-TET replacement promoters was amplified from the targeted ES cell genomic DNA, which shows the correct targeting of the replacement promoters (Supplementary Figure 1). Note that in all these cells the maternal chromosome carries the introduced *Igf2r* exon 12 SNP and the paternal chromosome carries the *Airn* promoter modification.

APD and replacements change Airn expression in ES cells

Airn expression is undetectable in undifferentiated ES cells, which show biallelic *Igf2r* expression. Imprinted expression of *Igf2r* arises during ES differentiation coincident with paternal *Airn* ncRNA expression (Figure 1F and PAL, manuscript submitted). Therefore, the four ES cell lines were differentiated by withdrawal of LIF and addition of retinoic acid (RA) to assay the expression of the *Airn* ncRNA. After 2 days, ES cultures gained visual signs of differentiation and were virtually free of undifferentiated cells after 5 days as assessed by phenotypic appearance.

To test whether the APD is sufficient to eliminate *Airn* expression, cDNA from different time points of ES cell differentiation was produced and expression was analysed by QPCR using a Taqman probe located 54 kb downstream of the *Airn* promoter (Figure 2A). Figure 2B shows that undifferentiated control ES cells (S12/+/d0), as well as undifferentiated ES cells with a deletion of the paternal promoter (S12/APD2/d0), lack detectable *Airn* ncRNA expression. After differentiation for up to 14 days, control S12/+ ES cells showed strong *Airn* expression; however, differentiated ES cells with a deletion of the paternal promoter (S12/APD1/d5 and S12/APD2/d5) showed only background *Airn* expression levels (<1% of wild type; Figure 2B). The data show that the 959 bp deleted region contains essential parts of the *Airn* promoter. These findings were confirmed by RNase Protection Assay, which showed a complete absence of *Airn* ncRNA in differentiated APD-1 ES cells using a probe located 25.8 kb downstream of the *Airn* promoter (data not shown).

Figure 2B shows *Airn* expression in S12/APD-PGK and S12/APD-TET cells in which the *Airn* promoter is replaced by the ubiquitously expressed PGK promoter or the non-induced TET promoter. The PGK promoter should be active in undifferentiated and differentiated ES cells, whereas the TET promoter is predicted to be silent in the absence of the transactivator. In undifferentiated ES cells, the APD-PGK allele expressed *Airn* at ~50% of the level found in wild-type ES cells differentiated for 5 days. In ES cells differentiated for 5 days, the APD-PGK allele showed increased *Airn* expression equivalent to that seen in wild-type cells differentiated for a similar period; however, in ES cells differentiated for 14 days the APD-PGK allele produces significantly less *Airn* (Figure 2C). This shows that an exogenous PGK

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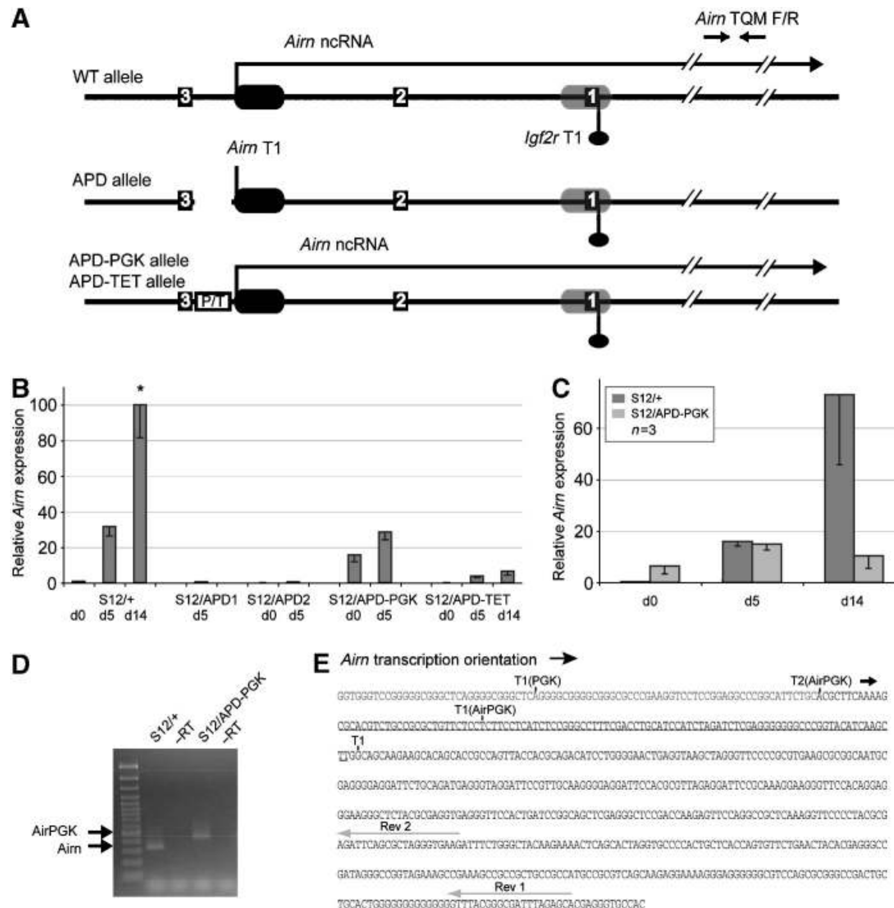

Figure 2 *Airn* expression changes in promoter deletion and replacement cells. (**A**) Map of the wild-type *Airn* promoter (WT), the *Airn* promoter deletion (APD) and replacement alleles (APD-PGK and APD-TET). *Airn* TQM F/R: QPCR assay used in (**B**, **C**). Numbered black boxes: *Igf2r* exons. Black oval: *Airn* CpG island, grey oval: *Igf2r* CpG island. The positions of the *Airn* and *Igf2r* transcription start sites with respect to the CpG island were determined by 5′RACE (Seidl *et al*, 2006). (B) QPCR showing *Airn* expression in targeted ES cells differentiated for 0, 5 or 14 days. Control undifferentiated ES cells (S12/+) lack *Airn* expression (day 0). Differentiation for 5 or 14 days increases *Airn* expression strongly. S12/APD cells lack *Airn* expression at all time points. S12/APD-PGK cells show moderate *Airn* expression (50% of 5 day control) in undifferentiated cells (d0) that increases to WT levels by day 5. S12/APD-TET cells show weak *Airn* expression under non-induced conditions but only in differentiated cells (3.8% at 5 days, 6.5% at 14 days relative to 14 day control). *Set to 100. Error bars represent standard deviation of three technical replicates (one biological replica). (C) QPCR comparing *Airn* expression in three biological replicates of S12/+ and S12/APD-PGK ES cells, differentiated for 0, 5 or 14 days, shows equal *Airn* expression at 5 days in S12/+ and S12/APD-PGK that is reduced at 14 days to 15% of control levels. Details as in (B). (**D**) 5′RACE products to visualize *Airn* transcription starts in 14-day differentiated S12/+ and S12/PGK cells. (**E**) Sequence showing locations of published *Airn* (T1) and PGK (T1(PGK)) transcription start sites and two transcription start sites of PGK-driven *Airn* (T1(AirPGK) and T2(AirPGK)). Rev 1, Rev 2: RACE primers; underlined: PGK promoter sequence; black font: PGK-*Airn* transcript; black arrow: transcript direction.

promoter can drive expression of the *Airn* ncRNA in undifferentiated ES cells and be upregulated during early differentiation to a similar level as the endogenous promoter. However, in contrast to the endogenous promoter, the PGK promoter is not further upregulated at later differentiation stages (Figure 2C). The non-induced APD-TET allele was not expressed in undifferentiated ES cells, but early and later differentiated ES cells expressed low amounts of *Airn* equivalent to ~7% of the level seen in wild-type differentiated cells (Figure 2B). We next used 5′RACE to map the transcription start site of the APD-PGK transcript using nested primers located downstream from the *Airn* T1 transcription start (Figure 2D and E). The APD-PGK 5′RACE product was larger than the wild-type product and sequencing revealed that the PGK promoter did not use its standard transcription start but instead used two new transcription start sites that added 106 or 70 bp to the endogenous *Airn* T1 transcription start site.

Replacement of the Airn promoter does not abolish splicing suppression of the Airn transcript

To test whether splicing suppression of the *Airn* ncRNA is regulated by its endogenous promoter, we quantified by QPCR the expression levels of the unspliced *Airn* and of the four known *Airn* splice variants (SV1, SV1a, SV2 and SV3) in wild-type S12/+, APD, APD-PGK and APD-TET ES cells differentiated for 5 days (Figure 3A and B). The results show that APD cells carrying a paternal APD lack all *Airn* spliced variants, thus the spliced products share the same essential promoter elements as the unspliced product. The APD-PGK and APD-TET alleles produced all four spliced *Airn* variants in addition to unspliced *Airn* with a moderately changed ratio. The amount of unspliced *Airn* produced by the APD-PGK and APD-TET alleles was 98 and 12% of wild-type levels, respectively. Spliced variants SV1 and SV1a were decreased (APD-PGK: 42% (SV1), 58% (SV1a); APD-TET:

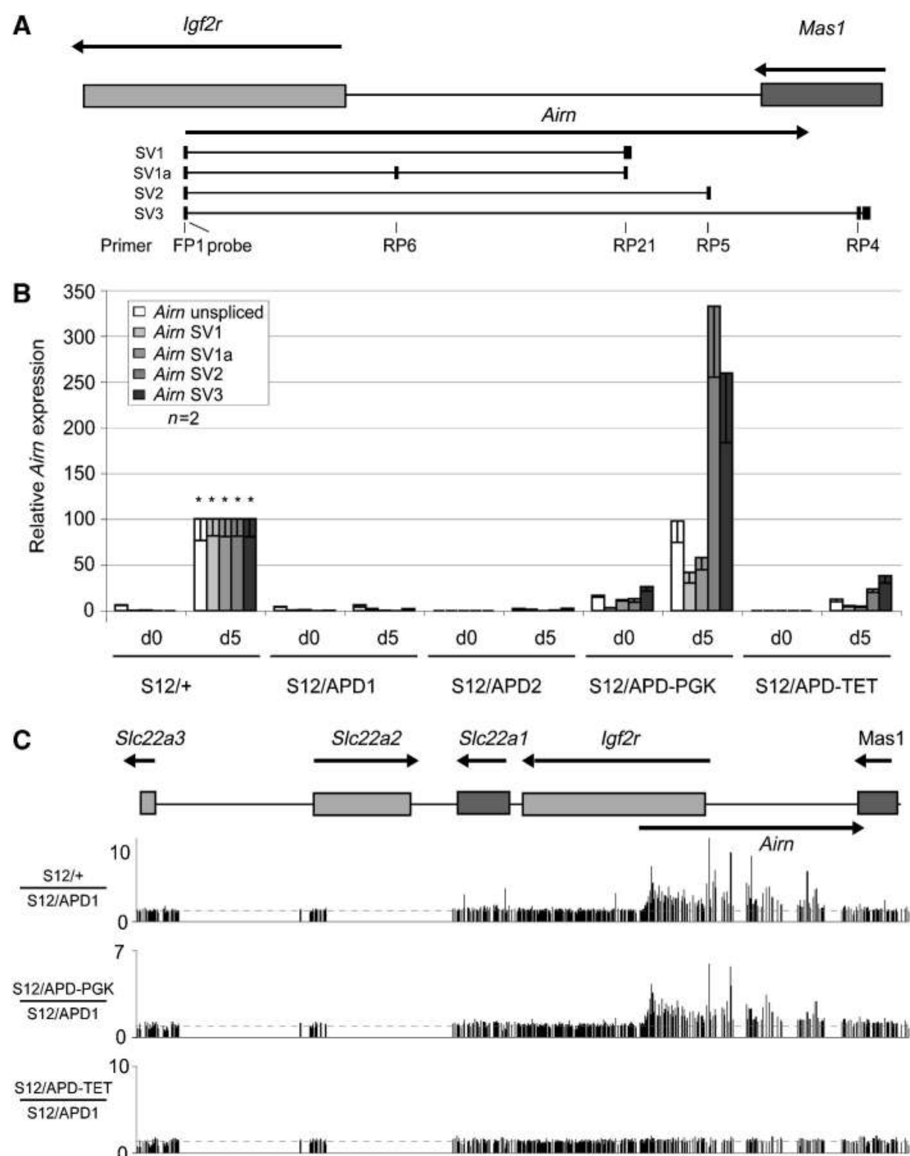

Figure 3 The PGK promoter expresses full-length *Airn*. (**A**) Map showing unspliced *Airn* (black arrow) and the known *Airn* splice variants and primers used in the splice variant-specific QPCR. The unspliced and spliced transcripts use the same transcription start sites and all four spliced variants use the same 53 bp exon 1. SV3 extends beyond the mapped end of the unspliced *Airn* transcript. A common forward primer (FP1) and Taqman probe (Probe) are combined with different reverse primers (RP21 (SV1), RP6 (SV1a), RP5 (SV2) and RP4 (SV3)). (**B**) Splice variant-specific QPCR. S12/+ control cells mostly lack expression of all splice variants at day 0 and show maximum expression at day 5. Spliced variants represent <5% of total *Airn* transcripts (Seidl *et al*, 2006); however, for purposes of comparison with the targeted alleles, the expression levels of unspliced and splice variants transcripts in S12/+ cells at day 5 is set to 100 (*). S12/APD cells and S12/APD-TET lack *Airn* splice variants at day 0, whereas S12/APD-PGK shows low expression levels at day 0. Expression of splice variants increases in S12/APD-PGK and S12/APD-TET ES cells at day 5. Mean values and standard deviations of two biological replicates are shown. (**C**) cDNA hybridization experiment on a custom PCR genomic tiling array (Regha *et al*, 2007). Top: map of the *Igf2r* imprinted cluster contained on the tiling array (note that only the promoter regions of *Slc22a2* and *Slc22a3* were included on the array). The three lower tracks on the *y* axis show RNA signal ratios of S12/+, S12/APD-PGK, S12/APD-TET relative to S12/APD1 in 5 day differentiated cells. Outside the *Airn* transcription unit the mean ratio is close to 1 (dashed line), indicating equal expression in the experimental (S12/+, S12/APD-PGK and S12/APD-TET) and reference cell lines (S12/APD1). S12/+ cells show elevated levels inside the *Airn* transcription unit that is reproduced by S12/APD-PGK, indicating no significant gain of splicing or premature termination in this replacement allele. Weak *Airn* expression from S12/APD-TET was not detected.

5% (SV1) and 5% (SV1a)); however, spliced variants SV2 and SV3 were expressed at higher levels relative to unspliced *Airn* (APD-PGK: 332% (SV2), 260% (SV3); APD-TET: 23% (SV2), 38% (SV3)). As it has been shown earlier that only a small minority of *Airn* ncRNA transcripts (<5%) are spliced (Seidl *et al*, 2006), this 2- to 3-fold upregulation of two of the four splice variants in the *Airn* promoter replacement alleles does not indicate a significant loss of splicing suppression.

To gain a more complete view of the ratio of unspliced and spliced *Airn* transcripts, we used a custom genome tiling array prepared from PCR amplicons spanning the complete *Igf2r*/*Airn* region (Regha *et al*, 2007). In Figure 3C, the genome tiling array was hybridized with two cDNA populations, one labelled with Alexa 555 was prepared from S12/APD differentiated ES cells that lack *Airn* expression, the other labelled with Alexa 647 was prepared from either

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S12/+ (top row), S12/APD-PGK (middle row) or S12/APD-TET (bottom row) differentiated ES cells. The vertical bars in Figure 3C show signal intensity ratios between the cell lines. Regions showing similar expression in both cell lines (such as *Slc22a1*) have ratios close to one (dashed grey line). However, throughout the *Airn* transcription unit S12/+ and S12/APD-PGK show significantly higher signal ratios (75 and 93% of signals are elevated), the signal variance within *Airn* is related to probe lengths on the tiling array. The pattern of wild-type *Airn* transcription (top row) closely resembles that of PGK-*Airn* (middle row), indicating no major gain of splicing or premature termination in S12/APD-PGK cells. In S12/APD-TET cells, the lowly expressed TET-*Airn* transcript is not detected by the tiling array.

Can Airn expressed from an exogenous promoter silence Igf2r?

We have shown earlier that expression of *Airn* leads to silencing *in cis* of the paternal *Igf2r* promoter and gain of DNA methylation on the *Igf2r* promoter CpG island (Sleutels *et al*, 2002). To test whether *Airn* driven from an exogenous promoter will induce gain of DNA methylation on the *Igf2r* promoter, genomic DNA was extracted from undifferentiated and differentiated ES cells and the methylation status of several methyl-sensitive restriction enzyme sites was analysed by Southern blot (Figure 4 and data not shown). In wild-type S12/+ ES cells, DNA methylation of a *Not*I site, which is diagnostic of the methylation status of the *Igf2r* promoter CpG island (Stoger *et al*, 1993), was absent in undifferentiated cells and reached a level of almost 50% at day 14, which indicates full methylation of the paternal allele in diploid cells. Differentiated APD1 and APD2 cells that lack the paternal *Airn* promoter as well as differentiated APD-TET cells with a replacement TET promoter, all failed to gain methylation of the *Igf2r* promoter CpG island. In contrast, APD-PGK cells with the replacement PGK promoter gained normal levels of DNA methylation on the *Igf2r* promoter CpG island in differentiated cells. In addition, APD-PGK cells show a low level of *Igf2r* promoter DNA methylation in undifferentiated ES cells (*). These data indicate that high levels of *Airn* driven from the exogenous PGK promoter can silence *Igf2r in cis*, whereas low levels of *Airn* driven from an exogenous non-induced TET promoter cannot.

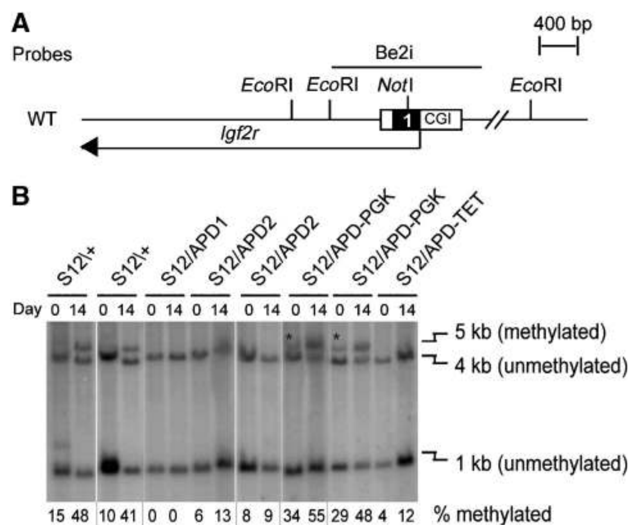

Figure 4 *Igf2r* promoter DNA methylation. (**A**) Map of an 8-kb region containing the *Igf2r* promoter (black arrow: transcription orientation). White box: CpG island; black box 1: *Igf2r* exon 1. Be2i: Southern blot probe. (**B**) Southern blot on genomic DNA of days 0 and 14 RA differentiated ES cells using *Eco*RI and the methyl-sensitive *Not*I enzyme and probe Be2i. One of two biological replicates is shown. S12/+ ES cells at day 14 show DNA methylation on *Not*I that is indicative of general methylation levels on the CpG island on the paternal allele (Stoger *et al*, 1993), whereas S12/APD1 and S12/APD-TET lack methylation. S12/APD-PGK cells show partial *Not*I methylation at day 0 (29–34%, asterisks) and full methylation at day 14 (48–55%, note that only the paternal allele is methylated (Stoger *et al*, 1993)). Numbers below indicate the relative intensities of methylated bands (ImageJ).

PGK promoter-driven expression of Airn leads to transcriptional silencing of Igf2r

RNA FISH (fluorescence *in situ* hybridization) using intronic probes allows visualization of nascent transcription on a single-cell level, and was used to investigate *Igf2r* imprinted expression. Figure 5A shows typical images using a strand-specific hybridization probe located in *Igf2r* intron 1 where each fluorescent spot indicates nascent transcription of *Igf2r*. Using intronic probes, only 32–47% of control differentiated S12/+ cells show a fluorescent signal for *Igf2r*. The lack of fluorescent signal in every cell in the population likely arises from stochastic gene expression where at any one time point, only a proportion of nuclei are transcribing *Igf2r* even though all cells may contain the gene product.

Figure 5B (left panel) shows RNA FISH analysis using strand-specific *Igf2r* hybridization probes located either in intron 1 (FP1, left bar) or intron 5 (FP3, right bar). For each data set, at least 100 cells with a fluorescent signal were counted by visual inspection and the percentage of cells with no spot (0), a single spot (1), two spots (2) or multiple spots (+) was determined. An independent counter with no knowledge of the cell genotype repeated the analysis and error bars indicate differences between these two counters. In wild-type S12/+ cells, 53–66% of cells lack any fluorescent spot signal, 30–38% show a single spot and 3–7% show double spots. Double spots represent biallelic expression of *Igf2r*, thus the majority of S12/+ differentiated ES cells show monoallelic *Igf2r* expression. In S12/APD cells that have a paternal deletion of the *Airn* promoter, 47–49% of cells lack any fluorescent spot signal, 22–24% show a single spot and 24–25% show double spots. Thus, APD cells show an increase of approximately five-fold in the number of nuclei showing two spots, indicating biallelic expression of *Igf2r*. This finding is confirmed by analysis of a second independent clone containing the APD (S12/APD1; Figure 5B, right panel). The similar numbers of nuclei with single spots in wild-type and APD cells was unexpected, and we interpret this as stochastic gene expression leading to an underestimation of the percentage of APD cells with double spots. In contrast to S12/APD cells, the APD-PGK cells in which the PGK promoter drives *Airn* expression showed a fluorescent spot signal distribution similar to the wild type (66–69% of cells lack any fluorescent spot signal, 25–29% show a single spot and 5% show double spots). These data indicate that *Airn* expressed from an exogenous PGK promoter is sufficient for transcriptional silencing of *Igf2r*.

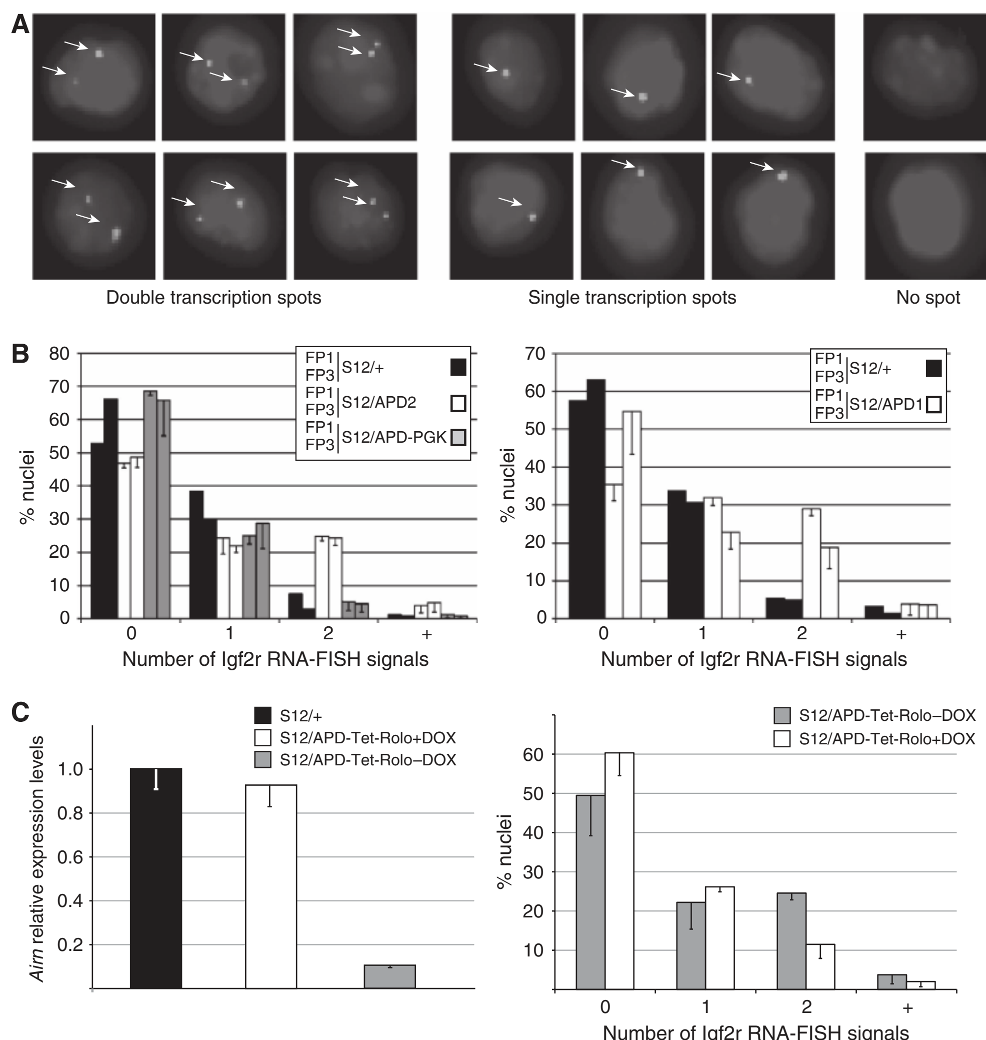

Figure 5 PGK-*Airn* and induced TET-*Airn* silence *Igf2r in cis*. (**A**) RNA FISH with the *Igf2r* intronic FP1 strand-specific probe in S12/APD ES cells. Representative examples of day 5 RA differentiated cells with double, single and no transcription spots are shown. (**B**) Left panel: quantification of *Igf2r* transcription by FISH using intronic probes FP1 (left bar) and FP3 (right bar). Double spots (2) indicate biallelic expression, single spots (1) monoallelic or stochastic biallelic expression. +: multiple spots indicate unspecific signals. Control S12/+ cells (dark grey bars) show mainly single spots. *Airn* promoter deletion S12/APD2 (white bars) shows an increase in cells with double spots, indicating a loss of imprinted expression. S12/APD-PGK (light grey bars) cells show mainly single spots consistent with imprinted *Igf2r* expression. Error bars represent means of two independent counts (one performed blind). Right panel: as left panel, but using an independently targeted APD allele (S12/APD1). (**C**) Left panel shows induced *Airn* expression levels assayed by QPCR as described in Figure 3 in APD-TET-Rolo ES clones carrying a transactivator (rtTA) gene targeted into the ROSA26 locus (Beard *et al*, 2006). Treatment of S12/APD-TET-Rolo cells with Doxycycline during 5d of ES cell differentiation leads to induction of the *Airn* ncRNA comparable to wild-type levels. Right panel shows an RNA FISH analysis of *Igf2r* transcription in S12/APD-TET-Rolo cells induced to express high levels of *Airn*. Uninduced S12/APD-TET-Rolo cells show a transcription pattern indistinguishable from S12/APD cells (double spots: 24%, −Dox). Induction of *Airn* ncRNA expression decreases biallelic *Igf2r* expression (double spots: 12%, +Dox). Error bars represent means of three independent counts (two were performed blind).

High TET-Airn levels can induce Igf2r silencing

The analysis of the APD-TET promoter in Figures 3B and 4B showed that this promoter replacement allele expressed low levels of *Airn* that were insufficient to induce DNA methylation of *Igf2r* in differentiated ES cells. In contrast, Figures 3B, 4B and 5B show the APD-PGK allele produced high levels of *Airn* that were sufficient for transcriptional silencing of *Igf2r*. To test whether transcriptional activity or the promoter sequence of the replacement *Airn* promoter is related to its ability to silence *Igf2r*, we induced expression of the APD-TET promoter. We targeted a tetracycline-inducible rtTA transactivator gene into the ubiquitously expressed ROSA26 locus of S12/APD-TET cells that allows rtTA expression to be driven by the ROSA26 promoter (Beard *et al*, 2006). The resultant cell line (S12/APD-TET-Rolo) was differentiated for 5 days in the presence of doxycycline and showed a nine-fold induction of *Airn* that is similar to wild-type levels at day 5 (Figure 5C, left panel). Figure 5C (right panel) shows an RNA FISH analysis of *Igf2r* transcription in the same S12/APD-TET-Rolo cells analysed in Figure 5C (left panel). The results show that induction of APD-TET promoter causes a significant reduction of cells expressing *Igf2r* biallelically compared with the untreated control (26% minus doxycycline and 12% plus doxycycline). This reduction, although significant (unpaired *t*-test: $P<0.005$), is less than that observed in Figure 5B with wild-type or APD-PGK promoters that showed

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only 5–7% double spots. We therefore examined DNA methylation at an *Sfu*I site that is diagnostic for the methylation status of the *Airn* CpG island (Stoger *et al*, 1993) in the APD-TET-Rolo cells and found in contrast to the wild-type allele, that it was methylated in differentiated ES cells despite continuous doxycycline induction (see below and Figure 7). This indicates that only a sub-population of cells is responsible for producing high APD-TET expression following doxycycline treatment, thus the proportion of cells showing a loss of biallelic *Igf2r* is reduced. The gain of imprinted *Igf2r* expression in induced APD-TET-Rolo cells was also accompanied by a slight gain of DNA methylation (data not shown) on the *Not*I site that is diagnostic for the methylation status of the *Igf2r* CpG island (Stoger *et al*, 1993). Thus, the data shows that induced expression of *Airn* from the APD-TET promoter is able to silence *Igf2r*, indicating that the expression level of the *Airn* ncRNA is a key factor in *Igf2r* silencing.

PGK-Airn silences the paternal Igf2r promoter

The RNA FISH experiments described above demonstrated that PGK-driven *Airn* was sufficient to induce imprinted *Igf2r* expression. However, RNA FISH cannot identify the parental alleles in a diploid nucleus. We therefore used the SNP introduced into *Igf2r* exon 12 (named S12) to determine whether PGK-driven *Airn* was able to mimic the wild-type *Airn* ncRNA and specifically silence the paternal *Igf2r* promoter. Using an allele-specific QPCR assay (PAL, manuscript submitted), the expression status of the two parental *Igf2r* alleles was analysed in S12/APD, S12/APD-PGK and in non-induced S12/APD-TET differentiated ES cells. In Figure 6A, control undifferentiated wild-type S12/+ ES cells were assigned a 1:1 ratio of maternal and paternal expression in agreement with earlier reports that *Igf2r* shows biallelic expression in undifferentiated ES cells (Wang *et al*, 1994). After 5 days, differentiated S12/+ cells show a maternal/paternal ratio of 19:1, indicating that wild-type differentiated ES cells have gained imprinted maternal-specific *Igf2r* expression. A similar increased maternal/paternal ratio from 1.4:1 in undifferentiated cells to 16:1 in differentiated cells was seen in S12/APD-PGK differentiated cells, indicating maternal-specific *Igf2r* expression. Surprisingly, S12/APD and S12/APD-TET differentiated cells also showed a similar increased maternal/paternal ratio in differentiated ES cells (from 1.8:1 to 14:1 and from 1.4:1 to 14:1). This result is surprising because it contradicts the RNA FISH and the DNA methylation analyses described above, which showed that the APD and non-induced APD-TET alleles did not silence the paternal *Igf2r* promoter. To explain how the paternal *Igf2r* allele appears to lack transcription silencing yet fails to produce a stable transcript that can be quantified by QPCR, we considered that the *Airn* promoter 959 bp deletion may have disturbed the splice acceptor of *Igf2r* exon 3 specifically in differentiated ES cells. We performed a non-quantitative PCR using primers spanning *Igf2r* exons 2–4 on cDNA from differentiated S12/APD cells and identified mis-splicing on the APD allele from exons 2 to 4 (data not shown), which would introduce multiple premature stop codons in the first 500 bp of *Igf2r* and be predicted to reduce transcript stability through nonsense-mediated RNA decay (NMD).

As NMD requires active translation, we treated ES cells differentiated for 5 days with the translation inhibitor emetine. Figure 6B shows that the maternal/paternal *Igf2r* ratio in emetine-treated wild-type S12/+ differentiated cells (that show *Igf2r* imprinted expression) is 12:1. A similar maternal/paternal ratio was obtained from emetine-treated S12/APD-PGK differentiated cells. This indicates that a PGK-driven *Airn* transcript is able to specifically repress the paternal *Igf2r* promoter in agreement with results obtained above from RNA FISH and DNA methylation analyses.

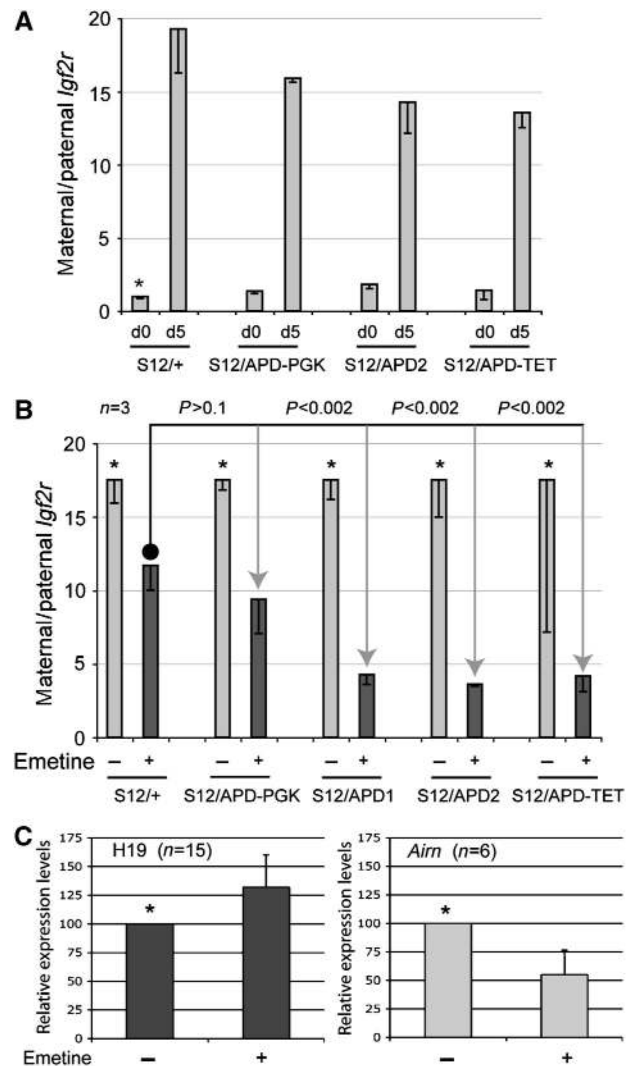

Figure 6 PGK promoter-driven *Airn* silences paternal *Igf2r*. (**A**) Allele-specific QPCR showing the ratio of maternal/paternal *Igf2r* expression in undifferentiated and differentiated wild-type and targeted ES cells. Numbers are ratios of maternal (S12) to paternal (+) allele. The ratio at day 0 in S12/+ cells was set to 1 (asterisk) and an increased ratio at day 5 indicates higher expression of the maternal relative to the paternal allele. The ratios of all targeted ES cell clones (S12/APD, S12/APD-PGK and S12/APD-TET) at day 5 are not significantly different from control cells (S12/+). Error bars represent standard deviation of three technical replicates (one biological replica). (**B**) As (A), but cells were cultured at day 5 for 10 h with or without (+/−) emetine. Mean values and standard deviations of three biological replicates are shown. *Set to 17.5, the value obtained from day 5 S12/+ cells shown in (A). A Student's *t*-test (two-tailed, equal variance) shows that S12/APD and S12/APD-TET cells show a significant lower ratio of maternal to paternal *Igf2r* under emetine treatment compared with S12/+ cells, whereas S12/APD-PGK cells do not ($P>0.1$). (**C**) QPCR showing changes in *Airn* and *H19* expression after emetine treatment in targeted ES cells differentiated for 5 days. Mean values and standard deviations of 6 (*Airn*) or 15 (*H19*) biological replicates are shown. *Set to 100.

In contrast, the maternal/paternal ratio from emetine-treated S12/APD and non-induced S12/APD-TET differentiated cells is significantly lowered, to approximately 4:1 ($P<0.002$; Student's *t*-test). This indicates emetine treatment stabilized the paternal APD-*Igf2r* allele and that these cells express paternal and maternal *Igf2r*, in agreement with data obtained above from RNA FISH and DNA methylation analyses. The maternal/paternal ratio does not reach 1:1 in S12/APD and S12/APD-TET ES cells, most likely due to incomplete NMD inhibition by emetine. However, the ratio is significantly lower than in S12/+ and S12/APD-PGK cells (Figure 6B). Thus the allele-specific QPCR assay shows that the APD-PGK allele represses the paternal *Igf2r* promoter.

The maternal/paternal ratio in emetine-treated wild-type S12/+ differentiated cells was 33% lower than that observed in untreated S12/+ cells (12:1 compared with 19:1). This reduction could be explained by a reduction in *Airn* expression upon emetine treatment and was of potential interest because of a previous report that the NMD pathway stabilizes expression of some spliced ncRNAs such as *Xist* and the imprinted *H19* ncRNA (Ciaudo *et al*, 2006). We used QPCR to quantify *Airn* and *H19* ncRNA expression in all samples following emetine treatment. Figure 6C shows that *Airn* expression is reduced by 25–50% after emetine treatment; however, the *H19* ncRNA shows an increase of 25–50%. These results indicate that any stabilization effects by the NMD pathway are not translation dependent, and perhaps shows that factors needed for full expression or turnover of ncRNAs are short-lived proteins and lost after emetine treatment.

Transcription is required to protect the paternal Airn CpG island from DNA methylation

The *Airn* promoter is located just upstream of a CpG island that is methylated during oocyte maturation on the maternal chromosome, whereas the paternal copy lacks methylation (Seidl *et al*, 2006). To test whether the absence of paternal *Airn* CpG island methylation is a passive process or requires *Airn* transcription, we assayed the methylation status of the paternal *Airn* CpG island in ES cells carrying an APD or exogenous *Airn* promoter replacement on the paternal allele. Figure 7 shows that ES cells with an active strong exogenous *Airn* promoter (S12/APD-PGK) are unmethylated at an *Sfu*I site that is diagnostic for the methylation status of the paternal *Airn* CpG island (Stoger *et al*, 1993). In contrast, ES cells lacking the *Airn* promoter (APD) or with a lowly expressed *Airn* promoter (non-induced APD-TET) gain paternal *Airn* CpG island methylation in undifferentiated ES cells, which increases slightly during differentiation. DNA methylation on the paternal *Airn* CpG island in differentiated ES cells was similar in induced and non-induced APD-TET-Rolo cells (Figure 7), indicating that the presence of an activated rtTA transactivator does not change established methylation patterns. Taken together, this indicates that the unmethylated status of the paternal *Airn* CpG island is an active process requiring transcriptional run through from a strong upstream promoter.

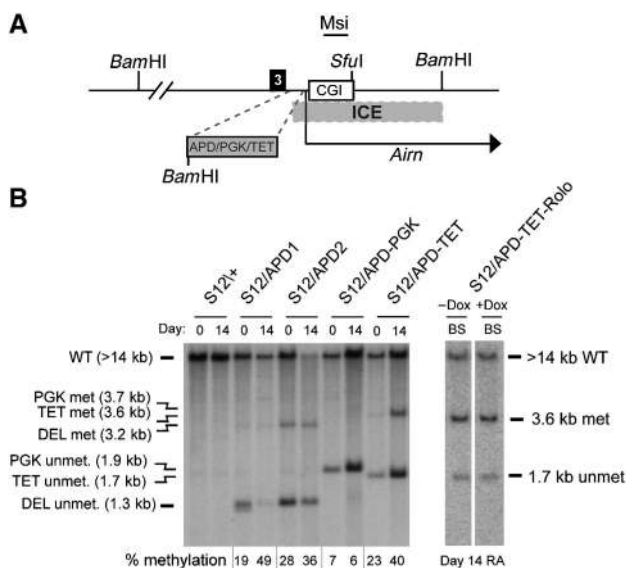

Figure 7 *Airn* expression protects the *Airn* CpG island from DNA methylation. (**A**) Map of a 20-kb region containing the *Airn* promoter. White box labelled CGI: CpG island; dark grey box: *Airn* promoter deletion or replacement cassette, which introduced a *Bam*HI site. ICE, details in Figure 1A, Msi Southern blot probe, *Sfu*I: methyl-sensitive site assayed in (**B**). (B) Left panel: Southern blot on genomic DNA of days 0 and 14 RA differentiated ES cells digested with *Bam*HI+*Sfu*I and probed with fragment Msi. Wild-type fragments from S12/+ are too large to separate and migrate together above 14 kb, all fragments below this arise from the paternally targeted allele. In S12/APD and S12/APD-TET cells, no or weak *Airn* transcription leads to gain of DNA methylation on the paternal *Airn* CpG island (met). DNA methylation is prominent in differentiated ES cells at day 14 (36–49% methylation); however, some DNA methylation is also present in undifferentiated ES cells (19–28% methylation). In contrast, S12/APD-PGK that shows strong *Airn* expression lacks DNA methylation on the *Airn* CpG island (unmet). Numbers below indicate the relative intensities of methylated bands (ImageJ). Right panel: the same methylation assay performed on S12/APD-TET-Rolo cells differentiated for 14 days minus (−) or plus (+) doxycycline (Dox), shows that in both conditions the *Airn* CpG island gains similar levels of DNA methylation.

Discussion

We show, by deletion of the *Airn* ncRNA promoter in ES cells and its replacement with a strong PGK promoter or a non-induced or induced TET promoter, that all functional regions regulating the transcriptional features and the silencing properties of the *Airn* ncRNA are located within its 108 kb transcribed gene body. Although transcription of *Airn* above a critical level is necessary for silencing and DNA methylation of the paternal *Igf2r* promoter, we also demonstrate a role for *Airn* transcription in protecting its own CpG island from *de novo* DNA methylation.

The APD deletion contains essential parts of the Airn promoter

Deletion of 959 bp upstream of the *Airn* ncRNA main transcriptional start site on the paternal chromosome led to loss of all *Airn* transcripts, including all *Airn* splice variants. This shows that an essential part of the *Airn* promoter in this region controls both unspliced and spliced *Airn* transcription. It is notable that the *Airn* promoter, defined in this and in a previous transient transfection study (Lyle *et al*, 2000), is immediately upstream of a CpG island that contains a paternal-specific DNase1 hypersensitive site and 13 predicted GC boxes and 12 consensus sites for Myc-associated zinc fingers (http://www.genomatix.de). Thus, the *Airn* CpG island is not

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part of the promoter lying upstream to the transcription start, although it may contribute in an as yet undefined manner to regulate *Airn* transcription initiation or elongation. Earlier reports that have mapped promoters to be inside the 5′ part of CpG islands have considered the CpG island to be part of the promoter; however, its specific role in initiation or elongation of transcription has not been studied (Antequera, 2003).

NMD of Igf2r in differentiated ES cells carrying the APD allele

The 959 bp APD ended 3 bp upstream of the 3′splice acceptor site of *Igf2r* exon 3. In differentiated ES cells, this deletion induced mis-splicing of *Igf2r* such that exon 2 spliced to exon 4 and the resultant transcript that contained multiple premature stop codons was not detected in stable mRNA populations. The stabilization of the mis-spliced *Igf2r* transcript by translation inhibitors such as emetine indicates but does not prove a role for the NMD pathway (Behm-Ansmant *et al*, 2007). It is possible that *Igf2r* exon 2–3 splicing elements such as a pyrimidine tract, a branch site or an intronic splice enhancer (Kim *et al*, 2008; Seth *et al*, 2008) are located in the 959 bp *Airn* promoter region. Notably in undifferentiated APD ES cells, paternal *Igf2r* mRNA accumulation was not affected. The NMD pathway is active in undifferentiated ES cells (Shigeoka *et al*, 2005), this could indicate that mis-splicing is a feature of high levels of gene expression as differentiated cells express 15- to 20-fold more *Igf2r* than undifferentiated cells (Figure 6). The transcriptional elongation rate is thought to be a crucial regulator of alternative and mis-splicing events (Hicks *et al*, 2006; Lavelle, 2007). Therefore, it is possible that low *Igf2r* expression in undifferentiated cells favours correct splicing events through slow elongation rates, whereas upregulation favours exon skipping.

Airn expression protects its own CpG island from DNA methylation

Although the DNA methylation mark on the maternal ICE that contains most of the *Airn* promoter and the CpG island is set in oocytes, the paternal ICE stays unmethylated in spermatogenesis and throughout development. It was suggested that short sequences inside a 113 bp 'imprinting box' in the *Airn* CpG island protect the paternal allele from *de novo* methylation (Birger *et al*, 1999). However, these sequences are retained in all APD and replacement alleles studied here and they did not protect the paternal ICE from *de novo* methylation in ES cells. Instead, our analysis indicates that the methylation-free state of the paternal ICE is maintained by active transcription from the upstream *Airn* promoter. Moreover, only *Airn* expression from the endogenous promoter or from the strong PGK replacement promoter, but not weak expression from the non-induced TET promoter could maintain the *Airn* CpG island in a methylation-free state, indicating that a certain expression threshold level is required. Interestingly, high expression of the TET transactivator during ES cell differentiation failed to reverse the DNA methylation of the APD-TET *Airn* CpG island, indicating that once the methylation mark is gained it is not reversed by high levels of the activated rtTA transactivator. A protective role for transcription against DNA methylation is in agreement with current suggestions that DNA methylation may not silence active promoters, but affects genes already silenced (Bird, 2002). It is also in agreement with results from diverse areas of research. For example, experiments deleting transcription factor-binding sites induce DNA methylation on CpG islands (Brandeis *et al*, 1994; Macleod *et al*, 1994), and the *de novo* methyltransferase-regulating factor *DNMT3L* cannot bind histone H3 modified by K4 trimethylation, a mark for expressed promoters (Ooi *et al*, 2007). It is surprising that undifferentiated ES cells without detectable *Airn* transcripts gain DNA methylation on the *Airn* CpG island only when a strong promoter is lacking. However, it was recently found that short initiating transcripts are found on promoters in undifferentiated human ES cells even when the associated gene is not expressed. These promoters contained H3K4me3 and bound the initiating form of RNAPII, but the gene did not show H3K36me3 or full-length transcripts (Guenther *et al*, 2007). This might represent a genome-wide transcription mechanism to protect CpG island promoters from *de novo* methylation. Interestingly, H3K4me3 is found on the *Airn* promoter in undifferentiated ES cells that lack expression of *Airn* (http://www.broad.mit.edu/seq_platform/chip/).

The Airn promoter does not suppress Airn ncRNA splicing

The *Airn* macro ncRNA differs from mammalian RNAPII mRNA transcripts in transcriptional and post-transcriptional features. Unspliced *Airn* is relatively unstable and nuclear localized, and its termination appears imprecise as two widely spaced 3′ polyadenylation sites, have been identified (Seidl *et al*, 2006). However, its most remarkable feature is that 95% of *Airn* ncRNA transcripts are unspliced despite *in silico* prediction of multiple splice sites throughout its 108 kb length (http://www.fruitfly.org/cgi-bin/seq_tools/splice.html). This indicates that splicing is actively suppressed. Splicing suppression could occur at the level of the promoter or arise from specific splicing silencer sequences (Kornblihtt, 2005, 2006; Wang *et al*, 2006). The data presented here exclude a role for the endogenous promoter, as PGK-driven *Airn* transcripts remain mainly unspliced. However, both the PGK and TET promoters slightly favoured splicing to downstream exons, which led to moderate downregulation of splice variants 1 and 1a, and upregulation of splice variants 2 and 3. Although this may indicate a role for the *Airn* promoter in modulating alternative splicing events, it excludes a role for the promoter in determining the unspliced to spliced ratio.

The silencing activity of the Airn ncRNA depends on a critical expression level

We show here that a PGK promoter can produce a functional *Airn* transcript with silencing activity that abolishes *Igf2r* transcription and induces gain of DNA methylation in differentiated ES cells. In contrast, the weakly expressed non-induced TET promoter lacks the ability to silence *Igf2r in cis.* However, induction of high *Airn* ncRNA expression from the TET promoter is able to repress *Igf2r,* albeit in an incomplete manner due to the gain of methylation on the *Airn* CpG island in the APD-TET allele. Thus, the silencing activity of *Airn* in differentiated ES cells depends not just on an active promoter expressing *Airn* but on a critical expression level. *Airn* is normally not expressed in undifferentiated

ES cells that consequently show biallelic *Igf2r* expression (Wang *et al*, 1994; Braidotti *et al*, 2004). However, the PGK promoter used here is a ubiquitously expressed promoter and PGK-*Airn* was expressed in undifferentiated ES cells to approximately 50% of the level seen in differentiated ES cells (Figure 2). This led to a low level of DNA methylation on the *Igf2r* promoter, which was not obviously correlated with *Igf2r* repression as measured by the allele-specific QPCR assay (Figures 4 and 6). This may indicate this assay lacks the sensitivity to detect a small difference in expression between the maternal and paternal *Igf2r* alleles (that are expressed at relatively low levels in undifferentiated ES cells) or that *Airn* cannot induce silencing in undifferentiated ES cells. This question will be further investigated by generating *Airn* alleles with stronger expression in undifferentiated ES cells.

Models of Airn-mediated gene silencing

One of the most intriguing aspects of *Airn*-dependent gene silencing is the ability of *Airn* transcription to silence neighbouring genes *in cis*, although its own promoter is unaffected. In these studies, a mouse PGK promoter is able to silence *Igf2r* in differentiated ES cells, without being itself affected. Currently, two models have been suggested to explain the silencing activity of the *Airn* ncRNA. The RNA-directed targeting model is based on parallels to X chromosome inactivation and proposes a function for the *Airn* ncRNA itself. This model proposes that the *Airn* ncRNA coats the silenced region and recruits effector proteins that induce widespread repressive epigenetic modifications (Pauler *et al*, 2007). Intuitively, this model implies a special ability of the *Airn* promoter to resist silencing as the induced epigenetic changes on the paternal allele silence *Igf2r*, *Slc22a2* and *Slc22a3*, but not the *Airn* promoter. Our findings argue against this model, as the PGK promoter in the APD-PGK allele was not affected by *Airn* transcription. The PGK promoter used here comes from the X-linked *Pgk1* gene that is known to be susceptible to X chromosome inactivation and DNA methylation, so it is not intrinsically resistant to epigenetic silencing (Pfeifer *et al*, 1989). A further argument against an RNA-directed targeting model operating in embryonic cells comes from analysis of parental-specific histone modifications, which found that repressive histone modifications that are dependent on *Airn* ncRNA expression do not spread throughout the silenced region, but instead are restricted to the silent *Igf2r* promoter and to one pseudogene element inside the *Airn* gene body (Regha *et al*, 2007).

The second silencing model called the transcriptional interference model proposes that the ncRNA itself is not necessary, instead the function of *Airn* ncRNA transcription is sufficient because it blocks the interaction of effector proteins to transcriptional activators within the *Airn* gene body (Seidl *et al*, 2006). Expression of large numbers of mammalian genes has been shown to be controlled by transcriptional interference (Shearwin *et al*, 2005; Petruk *et al*, 2006; Abarrategui and Krangel, 2007; Racanelli *et al*, 2008). In all these examples, a non-coding RNA overlaps the silenced gene promoter. In the *Igf2r* imprinted gene cluster, *Airn* silences three genes *in cis* but only overlaps *Igf2r*. The other two silenced genes *Slc22a2* and *Slc22a3* lie more than 80 kb upstream to *Airn* and lack any sequence homology (these genes show only imprinted expression in placental trophoblast and so could not be assayed in ES cells that cannot differentiate into placental cells). However, a transcriptional interference model can explain silencing of non-overlapped genes if transcription interferes with a common regulatory element (Seidl *et al*, 2006). Our studies are in agreement with a transcriptional interference model operating at the *Igf2r* imprinted cluster. Transcription interference has been shown to depend on the promoter strength of an overlapping transcription pair (Shearwin *et al*, 2005). Hence, it is feasible that the strongly expressed PGK promoter and the induced TET promoter, but not the weakly expressed non-induced TET promoter, are able to functionally replace the *Airn* promoter in *Igf2r* silencing. Taken together, the results presented here exclude a role for the *Airn* promoter in the silencing process, but highlight the importance of the *Airn* transcriptional unit and thus further support the transcriptional interference model of *Igf2r* silencing.

Materials and methods

Construction of targeting plasmids

The homology region of 7.6 kb for all targeting vectors was constructed by joining restriction fragments with tailored PCR products leading to homology fragments reaching from *Xho*I to the splice acceptor of *Igf2r* exon 3 and from *Airn* transcription start T1 to the next *Kpn*I site. A loxP511-flanked hygromycin and thymidine kinase cassette was used for selection. In the APD construct, the selection cassette replaced 959 bp containing the *Airn* promoter (126236–127195 bp; AJ249895). For APD-PGK, a 518-bp fragment of the PGK promoter was cut out with *Eco*RI and *Xho*I from pPGK-Hygro (gift from A Wutz), for APD-TET the 438 bp tetOP promoter from pTET-Splice (Invitrogen) was cut with *Xho*I and *Sac*II, and ligated to the selection cassette. Electroporation and hygromycin selection were performed using standard conditions. Cassette removal was performed by electroporation of the pMC-Cre plasmid. All targeted alleles were sequenced from PCR of genomic DNA using primers: F: TGGCAGCCCATAGTGGTGTTGA and R: CTCGCA TTGCCGCGCTTCAC. PCR fragments were cloned into pGEM-T Easy (Promega). Two clones from each allele were sequenced from both ends (Supplementary Figure 1).

Generation of APD-TET ES cells carrying a transactivator gene targeted into the ROSA26 locus

APD-TET ES cells with a tetracycline-dependent transcriptional activator under the control of the ROSA26 promoter were generated by introducing the *Cla*I linearized M2rtTA construct (Beard *et al*, 2006) into APD-TET cells. Screening for homologous recombination was performed by DNA blotting of *Eco*RV-digested DNA and a PCR-amplified probe (5′-GCACCGGCCAATAAGTGT-3′, 5′-GTAGG-CAATACCCAGGCAAA-3′). Single integration and the integrity of the recombined allele were checked on the same DNA blot with a 0.7 kb *Eco*RI–*Bam*HI fragment (from the M2rtTA construct) as a probe.

ES cell culture and differentiation assays

ES cell lines were grown in standard culture conditions on irradiated primary embryonic fibroblast feeders with the IPdel/Thp genotype (maternal *Igf2r* promoter deletion/paternal Thp deletion; Sleutels *et al*, 2003) that lack maternal and paternal copies of *Igf2r* and carry a repressed DNA methylated maternal *Airn* promoter. Differentiation was induced by withdrawal of LIF, depletion of feeder cells and addition of 0.08 μg/ml all trans RA (Sigma). The tetracycline-responsive activator was induced by the presence of 1 μg/ml doxycycline (Sigma) continuously for the differentiation period.

RNA and DNA analyses

For translation inhibition, ES cells were differentiated with RA for 5 days and emetine hydrochloride hydrate (100 μg/ml; Sigma) was added for 10 h. RNA was treated with DNaseI before reverse transcription. RNA FISH was performed using standard protocols and strand-specific intronic *Igf2r* probes FP1 (AIFP1F 5′-GCTGGTCCTTACCTTGTGGA-3′; AIFP1R 5′-GCAAGACCACATCA

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CACACC-3′) and FP3 (AIFP3F 5′-TCCTCAGGTACCATGCTATGC-3′; AIFP3R 5′-GGCAGGTTCTCTTGTTGAGG-3′). Fluorescent spots were counted by two people and one count was performed blind. 5′RACE was performed with the FirstChoiceRLM-RAC (Ambion) and primers 5′-GCTCTAAATCGCCCGTAAAC-3′ and 5′-TTCACCCTAGCG CTGAATCT-3′. Real-time QPCR and conventional PCR used the following primers not described earlier (Seidl *et al*, 2006): Igf2rex1: 5′-GCCGTTCAGCTGGGACC-3′; Igf2rex4: 5′-GGCTGCAG TCCTCCATT-3′. *Igf2r* allele-specific PCR assay (MutSEF: 5′-CTGGCCTTCCCCTCCTGT-3′; WtSEF: 5′-TGGCCTTCCCCTCCTGC-3′, GESER2: 5′-GCTATGACCTGTCTGTGTTGGCT-3′). DNA methylation probes Msi (AJ249895: 124993–126087 bp), Psi (AJ249895: 124370–124992 bp), OT2.4 (AJ249895: 120967–123159 bp), Be2i (AJ249895: 97091–99081 bp).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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