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Imprinted *Igf2r* silencing depends on continuous *Airn* lncRNA expression and is not restricted to a developmental window

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SUMMARY

The imprinted *Airn* macro long non-coding (lnc) RNA is an established example of a cis-silencing lncRNA. *Airn* expression is necessary to initiate paternal-specific silencing of the *Igf2r* gene, which is followed by gain of a somatic DNA methylation imprint on the silent *Igf2r* promoter. However, the developmental requirements for *Airn* initiation of *Igf2r* silencing and the role of *Airn* or DNA methylation in maintaining stable *Igf2r* repression have not been investigated. Here, we use inducible systems to control *Airn* expression during mouse embryonic stem cell (ESC) differentiation. By turning *Airn* expression off during ESC differentiation, we show that continuous *Airn* expression is needed to maintain *Igf2r* silencing, but only until the paternal *Igf2r* promoter is methylated. By conditionally turning *Airn* expression on, we show that *Airn* initiation of *Igf2r* silencing is not limited to one developmental 'window of opportunity' and can be maintained in the absence of DNA methylation. Together, this study shows that *Airn* expression is both necessary and sufficient to silence *Igf2r* throughout ESC differentiation and that the somatic methylation imprint, although not required to initiate or maintain silencing, adds a secondary layer of repressive epigenetic information.

KEY WORDS: ES cell differentiation, Genomic imprinting, Long ncRNA

INTRODUCTION

Genomic imprinting is an epigenetic process that causes parental-specific expression of a subset of mammalian genes (Ferguson-Smith, 2011). The two parental alleles of an imprinted gene co-exist in the same nuclear environment, but silencing is restricted to one allele; thus, genomic imprinting is a cis-acting silencing mechanism (Barlow, 2011). To date, 150 mouse imprinted genes have been identified (Williamson et al., 2012), with the majority occurring in clusters. In eight clusters, imprinted expression is controlled by a cis-regulatory DNA sequence – the imprint control element or ICE that acquires a DNA methylation imprint on one parental chromosome during gamete formation (Bartolomei and Ferguson-Smith, 2011). Imprinted protein-coding genes are silenced on the parental chromosome carrying the unmethylated ICE. In six clusters, the unmethylated ICE activates a lncRNA (Koerner et al., 2009) that, in three cases, controls silencing of the clustered protein-coding genes (Mancini-Dinardo et al., 2006; Sleutels et al., 2002; Williamson et al., 2011). These functional imprinted lncRNAs, *Airn*, *Kcnq1ot1* and *Nespas*, represent invaluable epigenetic models for understanding how lncRNAs repress genes in cis. Global transcriptome analyses show that lncRNAs are found throughout the mammalian genome (Derrien et al., 2011). LncRNA abundance, tissue-specific and developmental regulation indicate functional cellular roles that may depend on recruiting chromatin modifiers for trans-regulation (Guttman and Rinn, 2012). Imprinted lncRNAs that silence in cis possess hallmarks – inefficient splicing, high repeat content, low conservation and short half-life – that indicate their transcription is more important than their lncRNA product. This lncRNA class has been termed 'macro' and may exert a silencing function on promoters and enhancers by transcriptional overlap (Guenzl and Barlow, 2012; Pauler et al., 2012).

In this study, we use the mouse *Igf2r* imprinted cluster as a model to investigate developmental regulation of the repressive action of the *Airn* macro lncRNA. *Airn* is paternally expressed and silences three protein-coding genes in cis: *Igf2r*, *Slc22a2* and *Slc22a3* (Sleutels et al., 2002). Of these, only *Igf2r* is essential for development (Wang et al., 1994) and shows imprinted expression in all embryonic, extra-embryonic and adult tissues that co-express *Airn* (Yamasaki et al., 2005). Imprinted expression of *Slc22a2* and *Slc22a3* is restricted to extra-embryonic lineages such as placenta and visceral yolk-sac endoderm (Hudson et al., 2011; Zwart et al., 2001). The *Airn* lncRNA promoter lies in *Igf2r* intron 2 within a 3.7 kb region genetically defined as the ICE (Lyle et al., 2000; Wutz et al., 1997). On the maternal chromosome, an ICE methylation imprint silences the *Airn* promoter, allowing expression of the three protein-coding genes (Wutz et al., 1997; Zwart et al., 2001). On the paternal chromosome, the unmethylated ICE drives expression of the 118 kb *Airn* transcript, a nuclear-localized, mostly unspliced and unstable lncRNA that overlaps the *Igf2r* promoter in antisense orientation (Seidl et al., 2006). Upon truncation of the *Airn* lncRNA to 3 kb, all three protein-coding genes are expressed biallelically, showing that *Airn* is required to initiate silencing (Sleutels et al., 2002). In placenta, the *Airn* lncRNA product has been shown to maintain *Slc22a3* silencing by recruiting EHMT2 histone methyltransferase (Nagano et al., 2008). However, *Igf2r* silencing is independent of both EHMT2 and the *Airn* lncRNA product, but requires *Airn* transcriptional overlap that interferes with RNAPII recruitment to the *Igf2r* promoter (Latos et al., 2012).

An unresolved issue is whether *Airn* transcription is sufficient or whether it requires additional factors to initiate *Igf2r* silencing. *Igf2r* imprinted expression is developmentally regulated and established after embryonic implantation (Lerchner and Barlow, 1997; Szabo and Mann, 1995). This developmental regulation is reproduced in differentiating mouse embryonic stem cells (ESCs) (Latos et al., 2009), where *Igf2r* expression switches from biallelic to monoallelic

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after the onset of *Airn* expression (Fig. 1A). If *Airn* requires additional factors, their expression may be restricted to the same developmental window during which *Airn* establishes *Igf2r* silencing. Testing whether *Airn*-mediated silencing is limited to a developmental window is the first step towards identifying such factors. Another unresolved issue concerns the maintenance of imprinted silencing. Once its expression is turned on, *Airn* is transcribed continuously where *Igf2r* shows imprinted expression. However, it is unknown whether continuous expression is needed to maintain silencing. Among the three genes silenced by *Airn*, *Igf2r* is the only one to gain DNA methylation on the silenced promoter (Zwart et al., 2001). This somatic imprint, gained late in development, is not required for initiation (Li et al., 1993; Seidl et al., 2006) but could play a maintenance role.

Here, we investigate developmental control of *Igf2r* silencing by altering the timing of *Airn* expression, using inducible systems with general applicability to lncRNA genetic studies. We find that eliminating *Airn* transcription in differentiated ESCs reverses *Igf2r* silencing, unless the paternal *Igf2r* promoter is methylated. This shows that *Airn* is continuously required to maintain *Igf2r* silencing, but only in the absence of DNA methylation. This methylation mark is maintained independently of *Airn*, indicating no role for *Airn* in its propagation. Furthermore, *Airn* can initiate *Igf2r* silencing in early and late differentiated ESCs, although with decreasing efficiency, indicating a 'window of opportunity' does not limit its repressive effects. Finally, we show that *Igf2r* repression is maintained in the absence of DNA methylation. Together, our results indicate that *Airn* acts alone to silence *Igf2r* and that the somatic methylation imprint, although dispensable for silencing initiation and maintenance, may play a reinforcing role.

MATERIALS AND METHODS

Targeted ESC generation

The R26CreER^{T2} targeting vector was a gift from Austin Smith (CSCR, Cambridge, UK). CKO and CRes targeting vectors were built using a plasmid with a 7.3 kb 129Sv homology region (chr17:12,738,432-12,745,760, UCSC build GRCm38/mm10). In the CKO construct, a 1.9 kb *Pac*I-*Nsi*I region (chr17:12,740,792-12,742,677) was flanked by loxP sites. First, a loxP-flanked PGK-Neo-pA sequence was subcloned into the *Nsi*I site and the resulting plasmid transformed into EL350 *E. coli*, expressing arabinose-inducible Cre recombinase (a gift from Alexander Stark, IMP, Vienna, Austria). Cre recombination was induced by 0.1% L-(+)-arabinose resulting in Neo excision and generation of a single loxP site at the *Nsi*I position. The second loxP site, together with an FRT-flanked PGK-Neo-pA selection cassette, was subcloned from plasmid pK-II (a gift from Maria Sibilia, ICR, Vienna, Austria) into the *Pac*I site. For the CRes construct, a 1.2 kb rabbit β-globin polyA cassette (Sleutels et al., 2002) and loxP site, plus the same FRT-Neo-FRT+loxP cassette used above, were subcloned into the *Bam*HI site at chr17:12,744,359. Electroporation and neomycin selection were performed under standard conditions. S12/+ cells [a feeder-dependent D3 ESC line carrying a SNP in *Igf2r* exon12 (Latos et al., 2009)] were used to obtain R26CreER ESCs (S12$^{RC/+}$), which were used to obtain CKO and CRes ESCs. The selection cassette was removed by electroporating the pMC-Cre plasmid in R26CreER cells or the pCAGGS-FLPe plasmid in CKO and CRes cells.

ESC culture

ESCs were grown on irradiated primary mouse embryo fibroblasts. Differentiation was induced by feeder-cell depletion, LIF withdrawal and 0.27 μM all-trans RA. Embryoid body formation was induced by ESC aggregation in AggreWell plates (Stemcell Technologies) for 8 hours and culture on ultra-low adherence flasks. The tetracycline-inducible promoter in APD-TET-Rolo cells was induced with 1 μg/ml doxycycline hyclate. Cre recombination in CKO and CRes cells was induced with 1 μM 4-hydroxytamoxifen, unless otherwise stated.

DNA and RNA analysis

Genomic DNA isolation and Southern blots used standard protocols and signal intensities were quantified with ImageQuant. qPCR and RNA FISH were as described previously (Latos et al., 2012). Table S1 in the supplementary material lists primers and probes.

Western blotting

Western blot analysis was performed as described previously (Gratz et al., 2011), using a 1:1000 dilution of the Covance rabbit anti-Cre antibody (a gift from Juergen Knoblich, IMBA, Vienna, Austria).

Bisulfite sequencing

Bisulfite conversion, cloning and sequence analysis were as described previously (Koerner et al., 2012). PCR used primers in supplementary material Table S1 and conditions were 1 minute at 94°C, 30 seconds at 58°C and 1 minute at 72°C for 40 cycles.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was carried out as described previously (Regha et al., 2007).

Statistical analysis

P-values were calculated using analysis of variance (ANOVA) in R statistical environment (r-project.org) or unpaired *t*-test on www.graphpad.com/quickcalcs/.

RESULTS

Two inducible systems to control the *Airn* lncRNA

We previously reported a tetracycline (Tet)-inducible *Airn* allele (Stricker et al., 2008). However, owing to gain of Tet-*Airn* DNA methylation, these cells were not suitable for further experiments (supplementary material Fig. S1). We developed an alternative genetic system to control *Airn* expression during ESC differentiation using a D3 ESC line named S12/+ (the maternal allele is written on the left side throughout the text), which carries an *Igf2r* exon 12 SNP to discriminate maternal and paternal expression, and reproduces the developmental onset of *Igf2r* imprinted expression during differentiation (Latos et al., 2009) (Fig. 1A). The CreER^{T2} gene was inserted into the ROSA26 locus (Zambrowicz et al., 1997) to ensure expression throughout ESC differentiation (Fig. 1B, top; supplementary material Fig. S2A-C). CreER expression was verified at mRNA and protein levels (supplementary material Fig. S2D,E), and the cells were designated S12^{RC}/+. The expressed CreER product remains inactive in the cytoplasm until 4-hydroxytamoxifen (TAM) treatment (Feil et al., 1997). S12^{RC}/+ that carry no additional modification in the *Airn*/*Igf2r* locus compared with parental S12/+ cells are referred to as wild type. Using S12^{RC}/+ ESCs, the *Airn* locus was modified to generate *Airn* promoter conditional knockout (CKO) and *Airn* expression conditional rescue (CRes) cell lines (Fig. 1B).

Airn CKO ESCs

Airn CKO ESCs were generated by introducing loxP sites flanking 1.9 kb containing the *Airn* promoter and CGI (Fig. 2A). The 5′ boundary was a *Pac*I site 580 bp upstream of the *Airn* TSS and 385bp from *Igf2r* exon 3. The 3′ boundary was an *Nsi*I site 1.3 kb downstream of the *Airn* TSS. Two independent clones (S12^{RC}/CKO^{Fl}+cas1,2; Fig. 2B) were targeted on the paternal allele that carries the unmethylated ICE and expresses the *Airn* lncRNA (Fig. 2C). A targeting vector containing the selection cassette in opposite orientation generated no homologously targeted clones (supplementary material Table S2). Selection cassette removal generated clones S12^{RC}/CKO^{Fl}1,2 (Fig. 2D). CKO^{Fl} cells were TAM treated to delete the loxP-flanked *Airn* promoter, thus

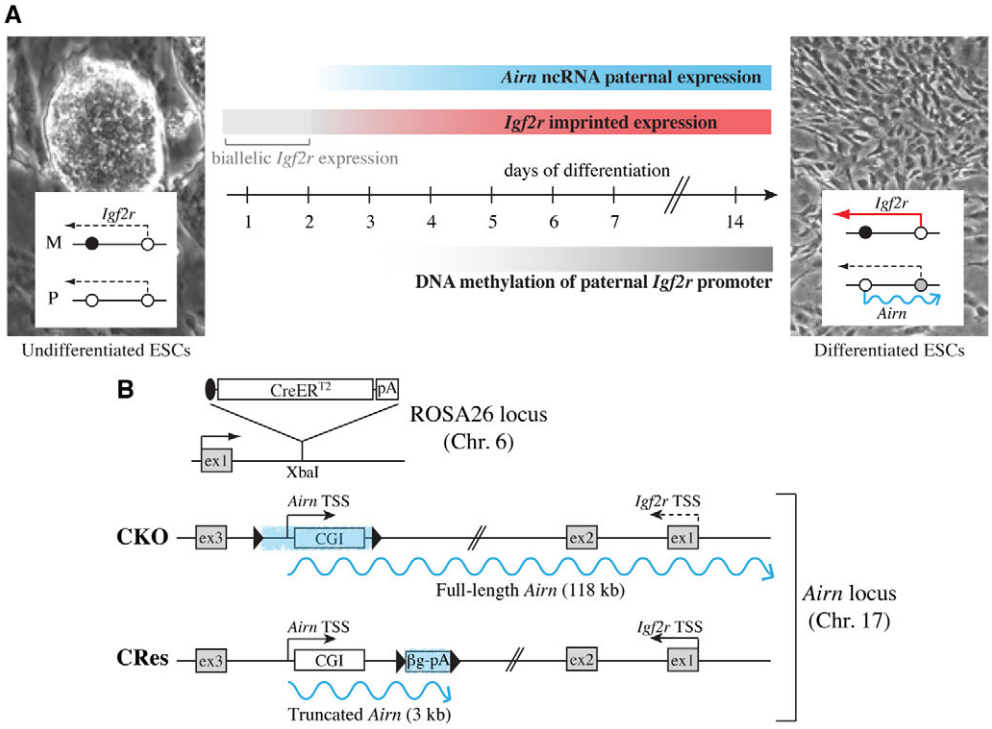

Fig. 1. Inducible systems to control *Airn* lncRNA. (**A**) Undifferentiated ESCs show low-level biallelic *Igf2r* expression; *Airn* is not expressed. Starting from day 2-3 of differentiation, expression of the maternal (M) *Igf2r* promoter is upregulated up to 20-fold. The *Airn* macro lncRNA is expressed from the paternal (P) chromosome with the same kinetics as maternal *Igf2r* upregulation. An oocyte DNA methylation imprint (black circle) silences the maternal *Airn* promoter. The paternal *Igf2r* promoter maintains the same low-level expression found at day 0; however, it gains DNA methylation (grey circle) and low-level H3K9me3. White circle indicates an unmethylated CpG island. (**B**) Top: a tamoxifen-inducible Cre recombinase gene ($CreER^{T2}$) was targeted into the ROSA26 locus in S12/+ ESCs that carry a SNP to distinguish maternal and paternal *Igf2r* expression. Middle and bottom: inducible Cre-loxP strategies. In the *Airn* promoter conditional knockout (CKO) line, loxP sites (black triangles) flank 1.9 kb containing the endogenous *Airn* promoter (TSS, transcription start site; CGI, CpG island). Cre recombination during ESC differentiation deletes this region turning off *Airn* transcription. In the *Airn* expression conditional rescue (CRes) line, loxP sites flank a polyA cassette (βg-pA) that truncates *Airn* to a non-functional length that cannot silence *Igf2r* (Sleutels et al., 2002). Cre recombination during ESC differentiation removes the polyA signal, rescuing full-length functional *Airn* transcription.

generating the CKO^{Δ} allele (supplementary material Fig. S3A). CreER-mediated excision efficiency was tested in undifferentiated ESCs (supplementary material Fig. S3A). Independent of TAM dose, >80% of CKO^{Fl} alleles undergo recombination by 24 hours, with complete excision by 48 hours.

Conditional deletion of the *Airn* promoter

Imprinted *Igf2r* expression arises between days 2 and 3 of ESC differentiation (Fig. 1A). To test whether *Airn* expression is needed to maintain *Igf2r* silencing after it is initiated, CKO ESCs were differentiated using retinoic acid (RA), then the *Airn* promoter was deleted at day 5, 9 or 13 by TAM addition, and cells were harvested 4 days later (Fig. 3A). *Airn* has a half-life of less than 2 hours and transcripts are absent ~10 hours after promoter deletion (Seidl et al., 2006). Cre-mediated excision of CKO^{Fl} was quantified by Southern blot (Fig. 3B, top; supplementary material Fig. S3B). In contrast to undifferentiated ESCs (supplementary material Fig. S3A), the *Airn* promoter showed 88% recombination at day 5 of differentiation, which was reduced to 58-72% by day 9 or day 13 (Fig. 3B, top). qPCR quantification shows 83% recombination at day 5 and 59-63% at day 9 or day 13 (Fig. 3C, left). To test whether Cre recombination improves in a different lineage, we performed the same experiment on CKO^{Fl} cells differentiated by embryoid body (EB) formation. As shown by Southern blot (Fig. 3B, bottom; supplementary material Fig. S3B) and qPCR quantification (Fig. 3C, right), the *Airn* promoter is deleted more efficiently in EB differentiated ESCs, with only 19-26% residual unrecombined alleles.

The effect of the conditional promoter deletion on *Airn* steady-state levels was assessed by RT-qPCR (Fig. 3D). As expected, *Airn* is upregulated in differentiated CKO cells carrying an intact promoter (Fig. 3D, bars 2-5), showing that loxP sites in the CKO^{Fl} allele do not interfere with promoter activity. However, *Airn* is not expressed if its promoter is deleted before differentiation (Fig. 3D, bar 6), confirming that the deletion removes all sequences required for *Airn* transcription. When the promoter is deleted during differentiation, *Airn* steady-state levels are reduced to ~15% of controls in EB differentiated cells (Fig. 3D, right, bars 7-9). Higher residual levels of *Airn*, seen when the deletion is induced during late RA differentiation (Fig. 3D, left, bars 8-9), are explained by inefficient recombination of the CKO^{Fl} allele. The data show that promoter deletion during ESC differentiation can eliminate *Airn* expression.

Igf2r silencing requires continuous *Airn* expression until DNA methylation is acquired

To determine the effect of *Airn* removal after *Igf2r* silencing is initiated, we examined *Igf2r* imprinted expression in differentiated

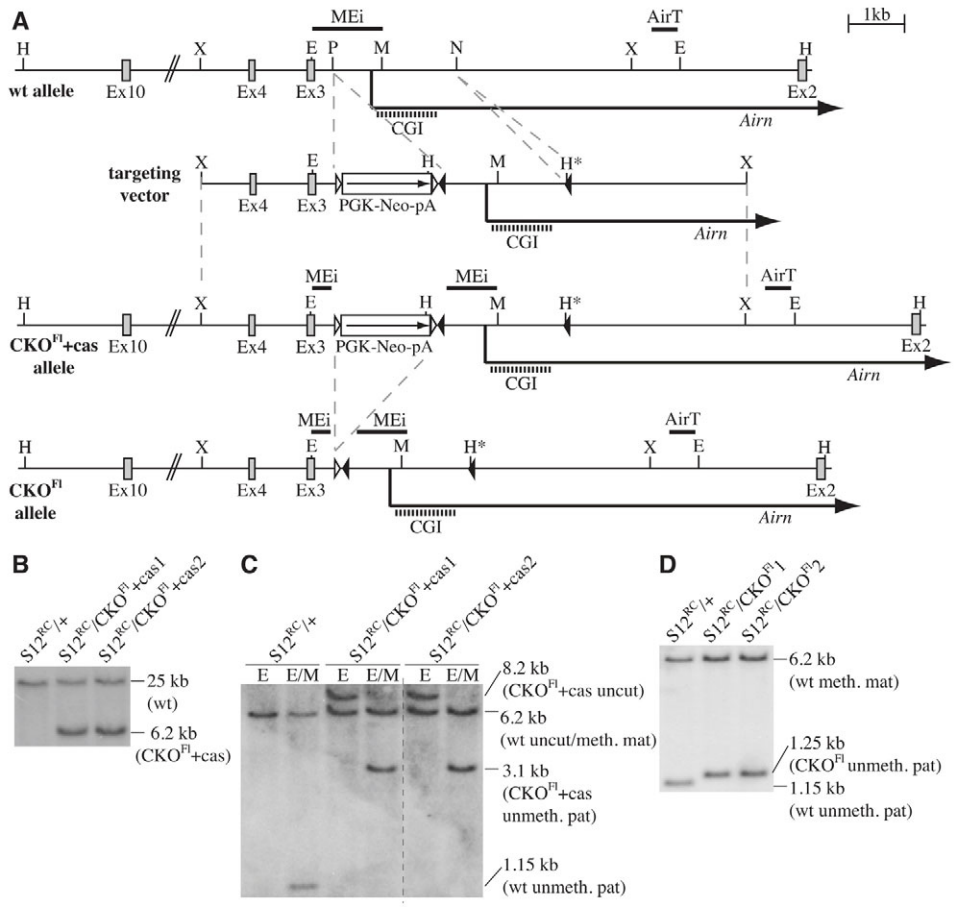

Fig. 2. *Airn* promoter conditional knockout (CKO) ESCs. (**A**) Top: wild-type allele showing *Airn* transcript overlapping *Igf2r* intron 2. Below: construct used to insert loxP sites (black triangles) flanking 1.9 kb containing the *Airn* promoter CGI (dashed bar). A selection cassette (PGK-Neo-pA) flanked by FRT sites (white triangles) with one loxP site was inserted into a *Pac*I (P) site (chr17:12,740,792, UCSC build GRCm38/mm10). A second loxP site with a diagnostic *Hin*dIII site (H*) was inserted into an *Nsi*I (N) site (chr17:12,742,677). Homologous recombination in $S12^{RC}$/+ ESCs generated a CKO^{Fl}+cas allele. Transient transfection of FLP recombinase deleted the selection cassette to generate the CKO^{Fl} allele. Fl, floxed (flanked by loxP sites); Ex, *Igf2r* exons; solid bars, Southern blot probes; E, *Eco*RI; H, *Hin*dIII; M, *Mlu*I; X, *Xba*I. (**B**) Southern blot typing of independently targeted clones ($S12^{RC}$/CKO^{Fl}+cas1,2). The $S12^{RC}$/+ parental cell line contains a *Pst*I SNP in *Igf2r* exon12 (Latos et al., 2009) and R26CreER (supplementary material Fig. S2). Probe AirT hybridized to *Hin*dIII-digested DNA identifies a 6.2 kb correctly targeted band in CKO^{Fl}+cas. (**C**) Southern blot to identify parental origin of targeted alleles. Samples from B were digested with *Eco*RI or *Eco*RI+*Mlu*I (E/M) and hybridized to probe MEi. Loss of a 1.15 kb band and gain of a 3.1 kb band in CKO^{Fl}+cas cells shows targeting of the paternal allele, containing an unmethylated *Mlu*I site (Stöger et al., 1993). Dotted line: boundary between juxtaposed lanes from same gel. (**D**) Southern blot typing for selection cassette removal. DNA from parental $S12^{RC}$/+ cells and $S12^{RC}$/CKO^{Fl}1,2 targeted clones after FLP recombination digested with *Eco*RI+*Mlu*I hybridized to probe MEi. Loss of the 3.1 kb band (C) and gain of a 1.25 kb band confirms selection cassette removal.

CKO cells. Allele-specific *Igf2r* expression was assayed non-quantitatively using the maternal-specific SNP in exon 12 that destroys a *Pst*I site (Fig. 4A; supplementary material Fig. S4). *Pst*I digestion of amplified cDNA from undifferentiated ESCs, which express *Igf2r* biallelically, yields an undigested maternal band and two paternal *Pst*I-cut fragments (Fig. 4A, sample 1; supplementary material Fig. S4). Reduced paternal *Igf2r* fragments relative to the maternal fragment in differentiated cells that express *Airn* indicate maternal-specific *Igf2r* upregulation (Fig. 4A, samples 2-5; supplementary material Fig. S4). When the *Airn* promoter is deleted from CKO cells at day 0, *Igf2r* expression remains biallelic with visible paternal-specific bands throughout differentiation (Fig. 4A, sample 6; supplementary material Fig. S4), in agreement with previous *Airn* promoter deletion alleles that fail to establish *Igf2r* imprinted expression (Stricker et al., 2008; Wutz et al., 2001). To determine whether *Airn* is required to maintain *Igf2r* silencing, we turned *Airn* expression off at day 5, day 9 or day 13 of differentiation, after *Igf2r* silencing has occurred (seen in the untreated 'no TAM' day 5-17 controls). Four days after TAM treatment, re-expression of paternal *Igf2r* occurs at all tested times (Fig. 4A, samples 7-9; supplementary material Fig. S4), indicating that *Igf2r* silencing is not maintained in the absence of *Airn*.

We quantified *Igf2r* allele-specific expression by RT-qPCR using forward primers specific for the wild-type paternal or the SNP-modified maternal *Igf2r* allele and a common reverse primer (Koerner et al., 2012). Control differentiated cells that lack the *Airn* promoter and express *Igf2r* biallelically were used to set the maternal:paternal ratio to 50:50 (Fig. 4B, bar 6). Untreated (no TAM) control cells expressing wild-type levels of *Airn* show maternal-specific *Igf2r* expression, with low-level paternal expression (4-24% of total *Igf2r* levels; Fig. 4B, bars 2-5). Confirming results from Fig. 4A, the qPCR assay shows that

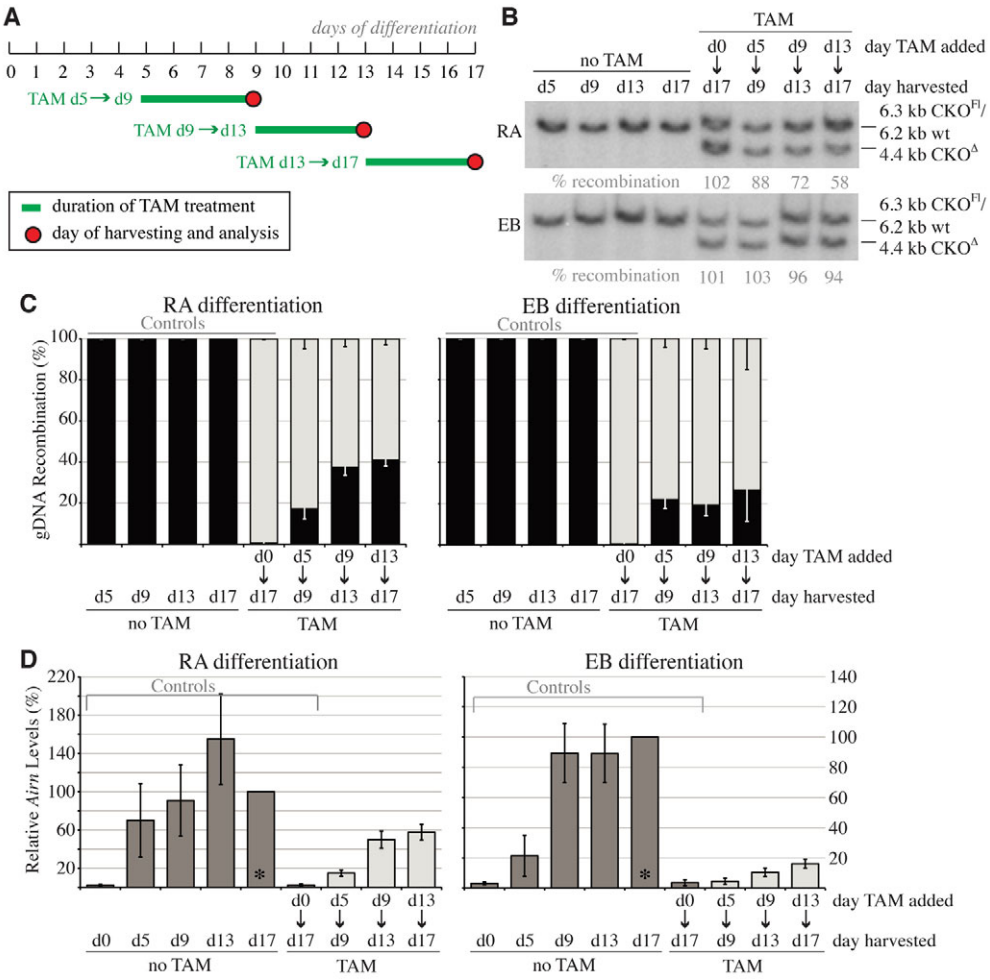

Fig. 3. Conditional *Airn* promoter deletion. (**A**) Experimental strategy to turn *Airn* off during ESC differentiation. (**B**) Southern blot of Cre recombination in retinoic acid (RA) or embryoid body (EB) differentiated CKO cells (supplementary material Fig. S3 shows strategy and replicates). DNA was *Eco*RI digested and hybridized to probe AirT. Lanes 1-4, control no TAM; lane 5, TAM treatment prior to differentiation; lanes 6-8, TAM added during differentiation. Top band: wild-type maternal (6.2 kb) and floxed paternal (CKO^{Fl} 6.3 kb) alleles not separated on this blot. Bottom band: deleted paternal allele after Cre recombination (CKO$^{\Delta}$ 4.4kb). The percentage recombination {4.4 kb band/[(6.2+ 4.4 kb band)/2]} is shown underneath. (**C**) Quantification of recombined (grey) and unrecombined (black) alleles using samples in B and supplementary material Fig. S3B amplified with allele-specific primers (supplementary material Fig. S3A). Combined recombined and unrecombined levels were set to 100. Bars show the percentage occupied by each allele as mean and s.d. of three or four biological replicates for RA (left) and EB (right) differentiated cells, respectively. (**D**) RT-qPCR with Airn-middle primers. Relative *Airn* levels are set to 100 in untreated day 17 cells (asterisk) that retain the *Airn* promoter. Data are mean and s.d. of three or four biological replicates for RA (left) and EB (right) differentiated cells, respectively. Dark bars, control samples; pale bars, *Airn* promoter deletion induced during differentiation.

paternal *Igf2r* silencing is relieved to different extents when *Airn* is turned off during differentiation. In RA differentiated cells, paternal *Igf2r* expression is 38% of total levels when the *Airn* promoter is deleted at day 5 (Fig. 4B, left, bar 7, blue bar), but is reduced to ~30% when *Airn* is removed at day 9 or day 13 (Fig. 4B, left, bars 8 and 9, blue bars). Correcting for recombination efficiency in RA day 9/day 13 differentiated cells, to consider only the subpopulation of cells with no *Airn* promoter, shows that paternal *Igf2r* is re-expressed to ~40% of total levels when the *Airn* promoter is deleted during late differentiation (Fig. 4B, left, black bars). Quantification of allele-specific *Igf2r* expression in EB differentiated cells in which the *Airn* promoter is deleted with higher efficiency shows that when *Airn* is removed at day 5 paternal *Igf2r* is re-expressed to ~45% of total levels (Fig. 4B, right, bar 7). However, when *Airn* is turned off at day 9 or day 13, paternal *Igf2r* re-expression is 21-23% of total levels (Fig. 4B, right, bars 8 and 9). Together, the analysis in RA or EB differentiated cells shows that *Airn* is continuously required to maintain paternal *Igf2r* silencing, but additional factors influence silencing in late differentiated cells.

Igf2r silencing by *Airn* during embryonic development and ESC differentiation is marked by a late gain of DNA methylation on the paternal *Igf2r* promoter CGI (Latos et al., 2009; Stöger et al., 1993). This methylation mark, although not needed to silence *Igf2r* up to 8.5 dpc of embryonic development (Li et al., 1993), could play a later maintenance role. We tested *Igf2r* promoter methylation in differentiated CKO cells by Southern blot analysis of a methyl-sensitive *Not*I site diagnostic of the methylation status of the *Igf2r* CGI (Stöger et al., 1993) (Fig. 4C; supplementary material Fig. S5). In differentiated control cells lacking the *Airn* promoter, the paternal *Igf2r* promoter is expressed and lacks DNA methylation, as shown

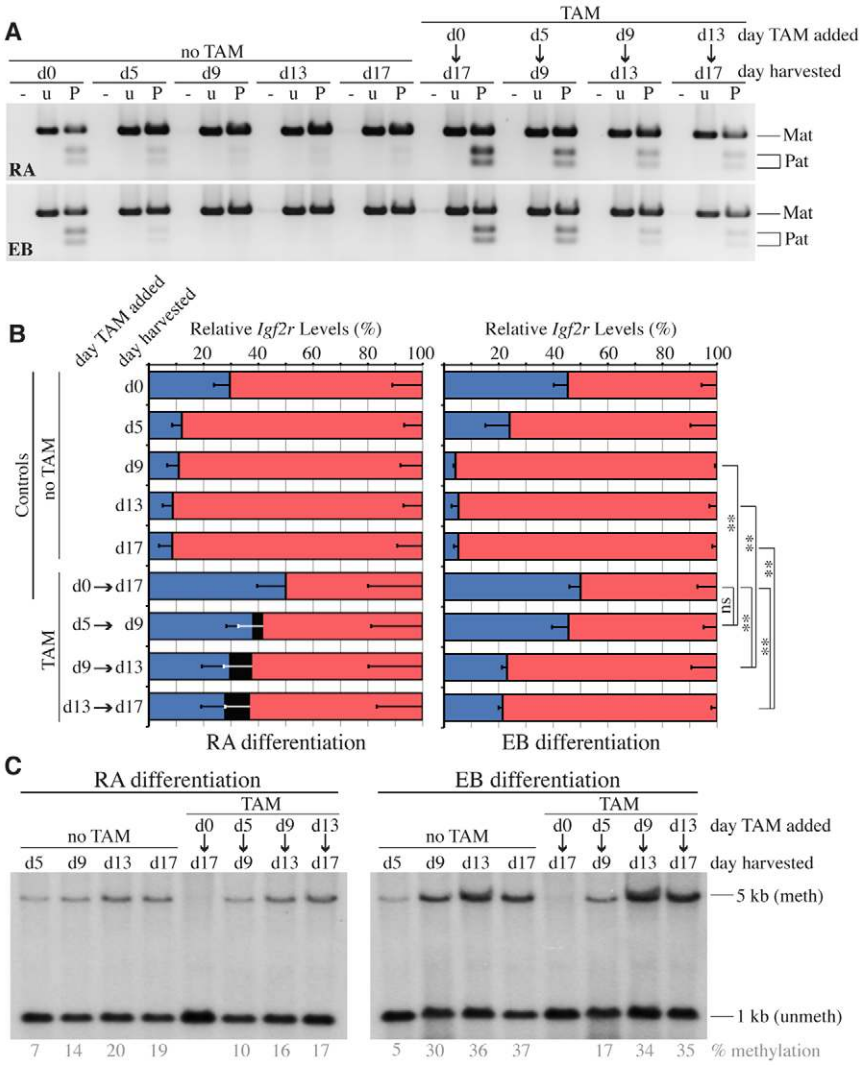

Fig. 4. *Igf2r* silencing requires continuous *Airn* expression. (**A**) Allele-specific *Igf2r* expression in RA (top) or EB (bottom) differentiated CKO cells, assayed by RT-PCR + *Pst*I digest of a paternal-specific restriction site. Maternal *Igf2r* expression generates 541 bp, paternal expression generates 318+223 bp (supplementary material Fig. S4 shows replicates). –, minus RT; u, undigested; P, *Pst*I digested; Mat, maternal; Pat, paternal. (**B**) Allele-specific RT-qPCR as in A. Maternal and paternal *Igf2r* levels are shown as a percentage of total *Igf2r* expression with mean and s.d. of three biological replicates for RA differentiated cells (left) and four biological replicates for EB differentiated cells (right). Maternal:paternal *Igf2r* levels were set to 50:50 in day 17 differentiated cells treated with TAM at day 0. For RA differentiation, data were corrected for Cre recombination efficiency quantified in Fig. 3C to show *Igf2r* expression only in recombined cells (black). EB samples were compared by ANOVA [$^{**}P<0.001$; ns (not significant), $P>0.01$]. The maternally biased *Igf2r* expression in day 0 untreated cells that have an *Airn* promoter most likely arises from a low degree of spontaneous differentiation leading to a small amount of paternal *Igf2r* silencing by *Airn* expression. (**C**) *Igf2r* promoter methylation assayed by Southern blot analysis of a diagnostic methyl-sensitive *Not*I site containing two CpG dinucleotides in CKO cells differentiated with RA (left) or EB formation (right) (supplementary material Fig. S5A shows replicates). DNA was digested with *Eco*RI+*Not*I and hybridized to probe NEi corresponding to the 1 kb unmethylated (unmeth) fragment and included entirely in the 5 kb methylated (meth) fragment (supplementary material Fig. S5B shows complete *Not*I digestion). Paternal *Igf2r* methylation [% methylated/(methylated+unmethylated)] is shown below the blot. Maximum methylation levels are 50%, as only the paternal allele is methylated.

by the presence of the single *Not*I-digested 1 kb band (Fig. 4C, lane 5; supplementary material Fig. S5A). In control-differentiated cells that express *Airn* and establish *Igf2r* imprinted expression, the paternal *Igf2r* promoter is progressively methylated during differentiation, as shown by gain of a methylated, *Not*I-undigested 5 kb band (Fig. 4C, lanes 1-4; supplementary material Fig. S5A). Maximum methylation levels of ~20% were seen in RA differentiation (Fig. 4C, left, lane 4; supplementary material Fig. S5A) and of ~40% in EB differentiation (Fig. 4C, right, lane 4; supplementary material Fig. S5A). Notably, after *Airn* removal and re-expression of the paternal *Igf2r* promoter, the DNA methylation that was gained was maintained despite the absence of *Airn* (Fig. 4C right, compare lanes 6-8 with lanes 1-4; supplementary material Fig. S5A). This shows that DNA methylation on the paternal *Igf2r* promoter is maintained independently of the *Airn* lncRNA.

Airn CRes ESCs

To test whether *Airn* can silence *Igf2r* at any differentiation stage, we established CRes ESCs, in which the silencing function of *Airn* can be switched on during differentiation. We introduced a loxP-flanked polyA signal into $S12^{RC}$/+ cells, at a *Bam*HI site 3 kb after the *Airn* TSS (Fig. 5A), to create a conditional version of an *Airn* 3 kb truncation allele that cannot silence *Igf2r* (Sleutels et al., 2002). Paternal targeting of two independently targeted clones ($S12^{RC}$/$CRes^{Fl}$+cas1,2; Fig. 5B) was confirmed (Fig. 5C) and the selection cassette removed to generate clones $S12^{RC}$/$CRes^{Fl}$1,2 (Fig. 5D). Deletion of the loxP-flanked polyA signal in the $CRes^{Fl}$ allele generated the $CRes^{\Delta}$ allele (supplementary material Fig. S6A). Compared with CKO^{Fl} cells (supplementary material Fig. S3A), recombination is faster in undifferentiated CRes cells, which have loxP sites further downstream of the *Airn* promoter [over 80% recombination 12 hours after TAM treatment and complete excision by 24 hours (supplementary material Fig. S6A)].

Conditional deletion of the truncation signal rescues full-length *Airn* transcription

To test whether removing the polyA signal restores full-length *Airn* transcription to wild-type levels, RA differentiated CRes cells were induced to delete the polyA signal daily between day 1 and day 10 (Fig. 6A), and harvested after 3-4 days (Fig. 6A). $CRes^{\Delta}$ cells (TAM treated at day 0) were co-differentiated for 4-14 days as a control for wild-type *Airn* levels. Cre-mediated excision monitored by Southern blot showed the $CRes^{Fl}$ allele is recombined efficiently (over 85%) throughout RA differentiation (Fig. 6B; supplementary material Fig. S6B). Full-length *Airn* is not detected in differentiated cells carrying the unrecombined $CRes^{Fl}$ allele (Fig. 6C, bar 8), confirming the polyA signal truncates *Airn*. *Airn* is strongly upregulated during differentiation in control $CRes^{\Delta}$ cells, showing

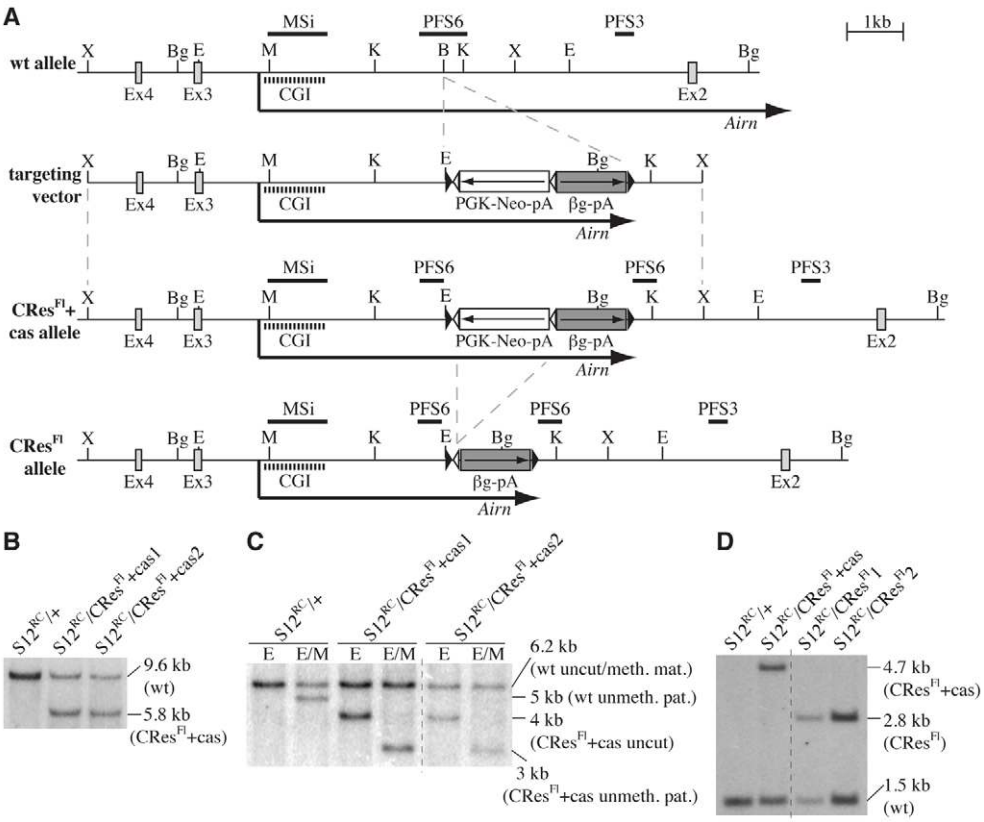

Fig. 5. *Airn* expression conditional rescue (CRes) ESCs. (**A**) Targeting strategy (details as Fig. 2A). Top: wild-type *Airn* allele. Below: targeting vector used to truncate *Airn* 3 kb after its TSS. The same selection cassette as Fig. 2A and a floxed rabbit β-globin polyadenylation signal (βg-pA) were inserted into a *Bam*HI (B) site (chr17:12,744,359) in *Igf2r* intron 2. Homologous recombination in S12^{RC}/+ ESCs generated a CRes^{Fl}+cas allele. Transient transfection of FLP recombinase deleted the selection cassette to obtain the CRes^{Fl} allele, in which loxP sites (black triangles) flank the βg-pA cassette. Bg, *Bgl*II; K, *Kpn*I; X, *Xba*I; M, *Mlu*I; E, *Eco*RI. (**B**) Southern blot of independently targeted clones S12^{RC}/CRes^{Fl}+cas1,2 and the S12^{RC}/+ parental cell line using *Bgl*II-digested DNA hybridized to probe PFS3 shows correct homologous recombination (CRes^{Fl}+cas 5.8 kb). (**C**) Southern blot to identify parental origin of the targeted allele. Samples from B digested with *Eco*RI or *Eco*RI+*Mlu*I (E/M) and hybridized to probe MSi. Loss of 5 kb and gain of 3 kb band in CRes^{Fl}+cas cells shows the paternal allele was targeted. (**D**) Southern blot confirms selection cassette removal (loss of 4.7 kb and gain of 2.8 kb band). DNA from the S12^{RC}/+ parental cell line and targeted cells before (S12^{RC}/CRes^{Fl}+cas) and after (S12^{RC}/CRes^{Fl}1,2) FLP recombination, digested with *Kpn*I and hybridized to probe PFS6. Dotted lines in C,D indicate the boundary between juxtaposed lanes from same gel.

that truncation of *Airn* is reversible (Fig. 6C, bars 2-6). Importantly, when the polyA signal is removed during differentiation, full-length *Airn* expression is restored to levels comparable with wild-type controls (Fig. 6C). Overall, the data show that the CRes system efficiently rescues full-length *Airn* transcription during ESC differentiation, allowing a switch from a short, non-functional *Airn* to its longer, functional form at any time.

Airn expression can silence *Igf2r* at any time during ESC differentiation

To test whether *Airn* can silence *Igf2r* at any time or whether its effects are restricted to a developmental window, we examined *Igf2r* imprinted expression in CRes cells using the *Pst*I assay (Fig. 7A; supplementary material Fig. S7A). In agreement with mouse studies (Sleutels et al., 2002), differentiated CRes^{Fl} cells carrying the truncated *Airn* allele fail to establish *Igf2r* imprinted expression and display paternal-specific bands throughout differentiation (Fig. 7A, no TAM day 8, day 14; supplementary material Fig. S7A). By contrast, control CRes$^{\Delta}$ display wild-type gain of *Igf2r* imprinted expression during differentiation (Fig. 7A, left; supplementary material Fig. S7A). We next restored full-length *Airn* expression at 24-hour intervals, testing early (Fig. 7A, top right; supplementary material Fig. S7A) and late (Fig. 7A, bottom right; supplementary material Fig. S7A) differentiation time points. Compared with the truncated *Airn* control that does not silence *Igf2r*, we observed *Igf2r* repression at all time points (Fig. 7A, compare samples 9-13 and sample 8 in each row; supplementary material Fig. S7A). However, paternal-specific bands were more visible compared with wild-type controls, especially at late differentiation time points (Fig. 7A, compare samples 9-13 and samples 2-6 in each row; supplementary material Fig. S7A).

We quantified allele-specific *Igf2r* expression (Fig. 7B) setting to 1 the ratio between maternal and paternal expression in undifferentiated control cells that carry the *Airn* truncation and express *Igf2r* biallelically (Fig. 7B, day 0 control BAE *Igf2r*). During differentiation, these cells show no gain of *Igf2r* imprinted expression and the maternal/paternal *Igf2r* ratio remains ~1 at day 8 and day 14. Control CRes$^{\Delta}$ express full-length *Airn* and gain wild-type levels of *Igf2r* imprinted expression during differentiation, with maternal:paternal ratios of 6-18 for early and late differentiation (Fig. 7B, control imprinted *Igf2r*). When *Airn* is turned on between days 1-10 of differentiation, we observe a gain of *Igf2r* imprinted expression at all time points, with maternal:paternal ratios between 4 and 11 (Fig. 7B, CRes experiment). This ratio is similar to control

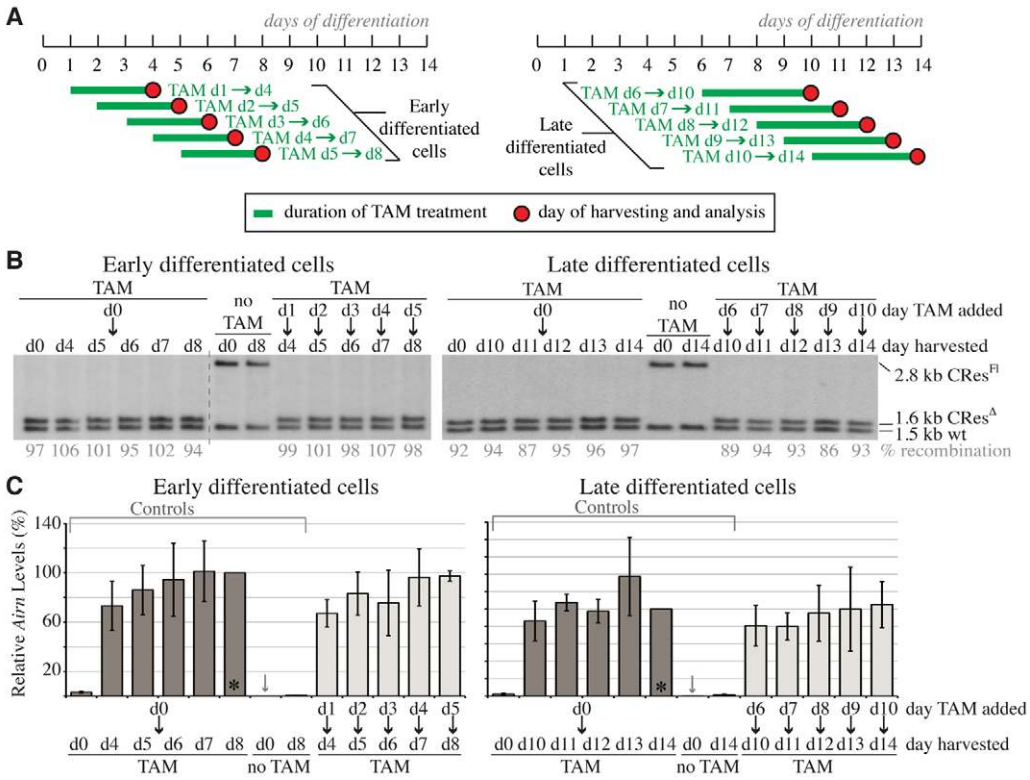

Fig. 6. Conditional deletion of an *Airn* truncation signal. (**A**) Experimental strategy to turn *Airn* on during ESC differentiation. (**B**) Southern blot to detect Cre recombination (supplementary material Fig. S6B shows replicates) in undifferentiated (day 0) or differentiated (days 4-14) CRes cells. DNA was *Kpn*I digested and hybridized to probe PFS6. Left: early differentiated day 4-8 cells. Right: late differentiated day 10-14 cells. Lanes 1-6, TAM treatment prior to differentiation; lanes 7 and 8, untreated controls; lanes 9-13, TAM added during differentiation. Cre-mediated recombination converts the 2.8 kb floxed paternal $CRes^{Fl}$ allele to 1.6 kb ($CRes^{\Delta}$). The wild-type maternal allele is 1.5 kb. Recombination efficiency (% $CRes^{\Delta}$/wild-type bands) is shown underneath. Dotted line indicates the boundary between juxtaposed lanes from same gel. (**C**) RT-qPCR with Airn-middle primers lying 49 kb downstream of the inserted polyA, shows that deleting the truncation signal during early (left) and late (right) differentiation restores full-length *Airn* expression to wild-type levels (pale bars). Relative *Airn* levels were set to 100 in control day 8 or day 14 cells (asterisks) in which the polyA signal was removed prior to differentiation (dark bars). Data are mean and s.d. of three biological replicates (left), and mean and maximum/minimum values of two biological replicates (right).

cells when the polyA signal is removed at day 1 or day 2 (Fig. 7B, left, compare CRes experiment and control imprinted *Igf2r*). When full-length *Airn* is restored after day 3, the maternal:paternal *Igf2r* ratio remains at ~4-5 for all time points (Fig. 7B, compare CRes experiment and control imprinted *Igf2r*). Together, this shows that *Airn* silencing of *Igf2r* is not restricted to one developmental window but silencing is less efficient when functional *Airn* is expressed after day 3.

We next analysed DNA methylation of the *Igf2r* promoter CGI by Southern blot (Fig. 7C; supplementary material Fig. S7B,C). Undifferentiated ESCs or differentiated control cells that express truncated *Airn* and show biallelic *Igf2r* lack DNA methylation, as shown by the single 1 kb band (Fig. 7C, lanes 1, 7 and 8; supplementary material Fig. S7B). Differentiated control cells expressing full-length *Airn* gradually gain *Igf2r* promoter methylation on the repressed paternal allele, as shown by increased intensity of the methylated 5 kb band (Fig. 7C, lanes 2-6; supplementary material Fig. S7B). Unexpectedly, when *Airn* function is rescued during differentiation, we observed little or no DNA methylation on the *Igf2r* promoter (Fig. 7C, lanes 9-13; supplementary material Fig. S7B). Methylation levels comparable with wild-type controls are observed only when the polyA signal is removed at day 1 (Fig. 7C, top, compare lane 9 and lane 2; supplementary material Fig. S7B). When *Airn* length is functionally restored between days 2 and 4, low methylation is detected; rescuing at day 6 or later results in no detectable (nd) DNA methylation on the *Igf2r* promoter (Fig. 7C, bottom, compare lanes 9-13 and lanes 2-6; supplementary material Fig. S7B). Bisulfite sequencing of the *Igf2r* CGI supports these observations (Fig. 7D; supplementary material Fig. S8A,B). The inability of the repressed *Igf2r* allele to gain DNA methylation when *Airn* function is restored in late differentiation correlates with *Dnmt3b* and *Dnmt3l* downregulation (supplementary material Fig. S8C). However, low levels of repressive H3K9me3 modification are gained at the *Igf2r* promoter when *Airn* function is restored at day 10 (supplementary material Fig. S9). Together, the data show that *Igf2r* silencing by *Airn* during late differentiation is accompanied by low-level H3K9me3, but not DNA methylation, on the *Igf2r* promoter.

DISCUSSION

We describe here inducible ESC systems that control endogenous *Airn* lncRNA expression to investigate the developmental regulation of imprinted *Igf2r* silencing. *Airn* is a well-established example of a cis-repressing lncRNA that silences the paternal *Igf2r* allele, which becomes methylated in all embryonic, extra-embryonic and adult tissues where they are co-expressed (Sleutels et al., 2002; Yamasaki

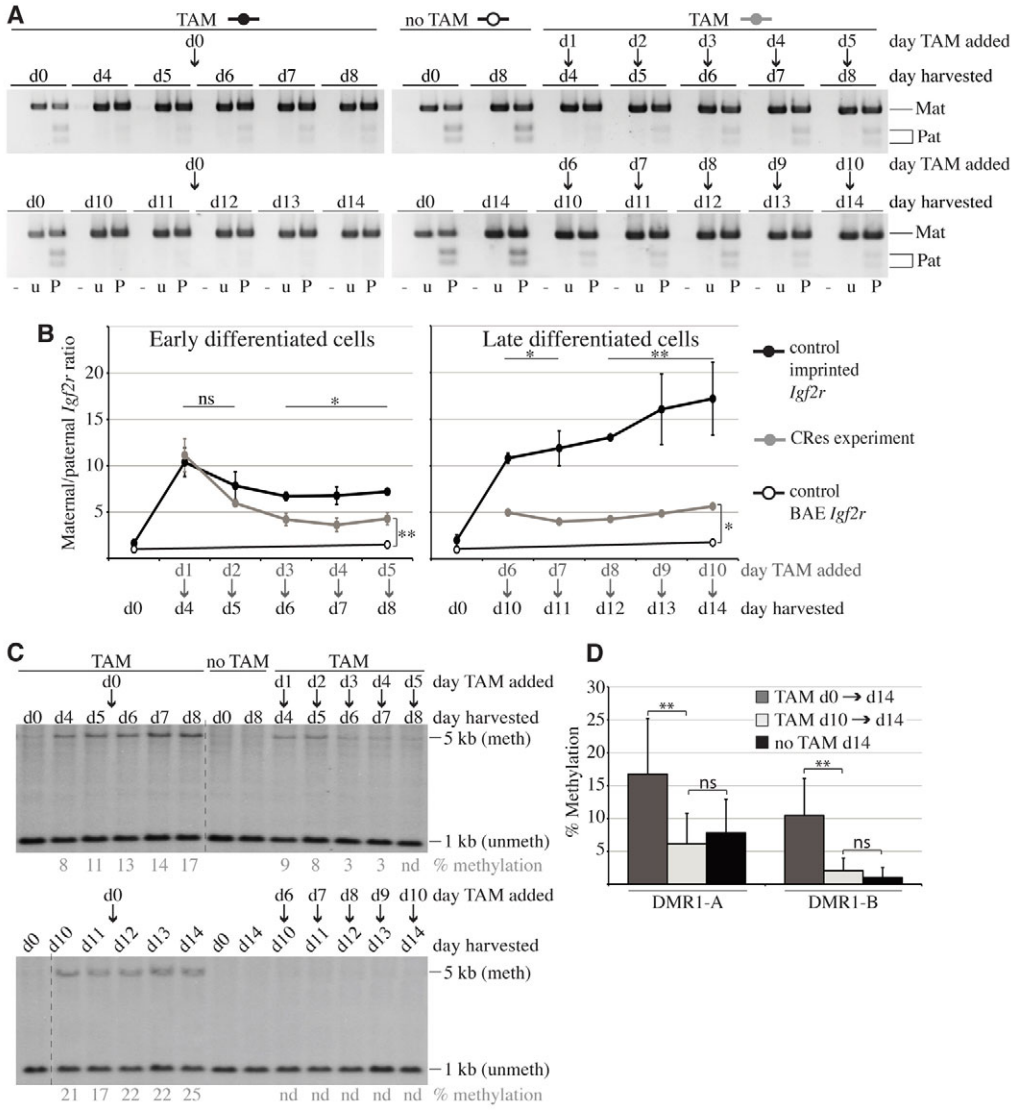

Fig. 7. *Airn* expression can silence *Igf2r* at any time during ESC differentiation. (**A**) Allele-specific *Igf2r* expression in early (top) or late (bottom) differentiated CRes cells (supplementary material Fig. S7A shows replicates) assayed as in Fig. 4A. Untreated (no TAM) day 0-14 cells show full biallelic *Igf2r* expression. All cells treated at day 0 with TAM show silencing of the paternal *Igf2r* allele that is maximal in late differentiated cells. Expressing full-length *Airn* during differentiation represses paternal *Igf2r*, although less efficiently compared with controls treated with TAM at day 0. (**B**) Allele-specific *Igf2r* RT-qPCR as in Fig. 4B. Maternal/paternal *Igf2r* ratios plotted over time [left, early differentiation (mean and s.d. of three biological replicates); right, late differentiation (mean and maximum/minimum values of two biological replicates)]. Control imprinted *Igf2r* (black circles): CRes cells with the truncation signal deleted prior to differentiation show wild-type gain of *Igf2r* imprinted expression. CRes experiment (grey circles): CRes cells with the truncation signal deleted during differentiation show gain of imprinted *Igf2r* expression that is reduced compared with the control above. Control BAE *Igf2r* (white circles): CRes cells that retain the truncation signal (no TAM) and show biallelic expression (BAE) of *Igf2r* throughout differentiation, used to set maternal/paternal ratio to 1 at day 0. CRes experiment and control samples were compared by ANOVA, using data from two subsequent differentiation days to increase statistical power [**P<0.001; *P=0.001-0.01; ns (not significant), P>0.01]. (**C**) *Igf2r* promoter methylation assayed as in Fig. 4C, in early (top) or late (bottom) differentiated CRes cells (supplementary material Fig. S7B shows replicates; Fig. S7C shows complete digestion). The *Igf2r* promoter gains up to 25% DNA methylation (5 kb band) by day 14 when full-length *Airn* is expressed throughout ESC differentiation (bottom, lane 6). Lower methylation gain (3-9%) is seen when full-length *Airn* is rescued during early differentiation from day 1-4 (top, lanes 9-12). No gain of DNA methylation is seen when full-length *Airn* is expressed after day 6 (bottom, lanes 9-13; nd, not detected). Dotted line indicates the boundary between juxtaposed lanes from same gel. (**D**) Bisulfite sequencing analysis of two subregions (DMR1-A spanning 433 bp, DMR1-B spanning 268 bp) of the *Igf2r* CGI in day 14 differentiated CRes cells. Ten to 17% methylation is seen when full-length *Airn* is expressed throughout differentiation (TAM treatment at day 0), but expressing full-length *Airn* from day 10 (TAM treatment at day 10) causes no methylation gain above background levels (no TAM treatment). Untreated cells express truncated *Airn* throughout differentiation. Data are mean and s.d. of methylation levels in each subregion (additional data in supplementary material Fig. S8A,B). Samples were compared using an unpaired *t*-test [**P<0.001; ns (not significant), P>0.01].

et al., 2005). Although *Airn* expression is also necessary to silence the paternal *Slc22a2* and *Slc22a3* alleles in extra-embryonic tissues, ESCs cannot yet be differentiated into these tissues and these genes show low-level non-imprinted expression in differentiated ESCs, typical of embryonic tissues (Hudson et al., 2010; Latos et al., 2009; Zwart et al., 2001). Using two inducible systems, we tested whether

Airn expression is continuously needed to maintain *Igf2r* silencing and whether *Airn* silencing is restricted to a 'window of opportunity' during ESC differentiation. The data show that although *Airn* expression is necessary and sufficient to initiate and maintain *Igf2r* silencing at any stage during ESC differentiation, DNA methylation adds an extra layer of epigenetic information that may act to safeguard the silent state.

Inducible ESC systems to control endogenous gene expression

We have previously characterized an *Igf2r* imprinting model using the S12/+ ESC line, modified here, which faithfully recapitulates the developmental onset of *Igf2r* imprinted expression (Latos et al., 2009). ESCs are frequently used as models for X-chromosome inactivation (XCI) (Navarro and Avner, 2010) and are becoming more appreciated for genomic imprinting studies (Kohama et al., 2012). An ESC study of the *Kcnq1* imprinted cluster demonstrated that *Cdkn1c* was silenced during RA differentiation without acquiring the DNA methylation seen in mouse embryos (Wood et al., 2010). However, we show that the *Cdkn1c* promoter acquires ~20% methylation after EB differentiation (supplementary material Fig. S10A). Our results confirm the utility of ESC models for studying some aspects of epigenetic silencing of imprinted genes, but demonstrate that differentiation protocols need consideration.

We initially attempted to control endogenous *Airn* expression using a TetOn system (Stricker et al., 2008). However, the Tet-driven *Airn* promoter was modified by DNA methylation and the effects of inducing *Airn* expression could be assayed only in a subset of cells. Therefore, we switched strategies and created two inducible Cre-loxP systems, with general applicability for lncRNA genetic studies, to control *Airn* expression during ESC differentiation. The CKO system used loxP sites flanking the *Airn* promoter to delete it during ESC differentiation, whereas the CRes system used loxP sites flanking a polyA signal to functionally elongate *Airn* during ESC differentiation. Both genetically modified ESC lines differentiated normally, as shown by downregulation of pluripotency markers and upregulation of differentiation markers (supplementary material Fig. S10B). The effect of deleting or inducing functional *Airn* was tested 3-4 days after TAM treatment to allow time for chromatin state to change and existing *Igf2r* mRNA to decay. In the CKO system, where loxP sites span the expressed *Airn* promoter, we observed reduced recombination efficiency in RA compared with EB differentiation and therefore based conclusions on experiments with the latter. This difference may be related to promoter activity, as *Airn* was more highly expressed in RA than in EB differentiated cells (supplementary material Fig. S10C). Overall, the inducible Cre-loxP strategy proved a valid alternative to the Tet-inducible system.

Continuous *Airn* expression is necessary for *Igf2r* silencing

By deleting the *Airn* promoter during ESC differentiation, we show that continuous *Airn* expression is needed to maintain *Igf2r* silencing but only in the absence of DNA methylation at the *Igf2r* promoter. Removing *Airn* transcription at day 5 of ESC differentiation, when fewer than 10% of cells have gained *Igf2r* promoter methylation, results in almost complete loss of *Igf2r* silencing. A similar effect is observed when *Airn* is removed at later stages in RA differentiated cells, which gain only ~20% *Igf2r* methylation. However, removing *Airn* in late-differentiated EBs, which gain ~2 fold more *Igf2r* methylation, causes incomplete loss of silencing. Continuous *Airn* expression is therefore necessary for *Igf2r* silencing, but only until DNA methylation is established, determining a switch from *Airn*-dependent to *Airn*-independent *Igf2r* silencing during development. Importantly, the data also show that continuous *Airn* expression is not necessary for DNA methylation to be propagated, as removing *Airn* at any time point during ESC differentiation did not cause loss of the DNA methylation already established on the *Igf2r* promoter. This was not due to cell cycle arrest, as both RA and EB differentiated cells continued to proliferate throughout the observation period (supplementary material Fig. S10D). In a recent mouse study, maintenance of imprinted silencing at the *Kcnq1* cluster was analysed by conditionally deleting the promoter for the *Kcnq1ot1* macro lncRNA that controls this cluster (Mohammad et al., 2012). Similar to observations of *Airn* during ESC differentiation, continuous *Kcnq1ot1* expression is necessary to maintain imprinted silencing of genes in embryos. However, in contrast to *Airn*, DNA methylation at the promoters of two silenced genes is lost in the absence of the *Kcnq1ot1* lncRNA (Mohammad et al., 2012). The results here show that the *Igf2r* somatic imprint is maintained in a lncRNA-independent fashion, most likely through the hemi-methyltransferase activity of DNMT1 (Ooi et al., 2009).

Our results raise questions concerning the developmental regulation of *Igf2r* silencing by *Airn* transcription (Latos et al., 2012). First, if *Airn* is dispensable to maintain *Igf2r* silencing once DNA methylation is established, as our results in early development show, it is unclear why the lncRNA is continuously expressed. Similar to *Airn*, the *Xist* lncRNA responsible for XCI is also continuously expressed in mouse tissues, although XCI is maintained independently of *Xist* in both differentiated ESCs and somatic cells (Csankovszki et al., 1999; Wutz and Jaenisch, 2000). In general, somatic imprints modify the repressed alleles of very few imprinted protein-coding genes and for some of these, methylation is not conserved in humans (John and Lefebvre, 2011). Thus, the role of DNA methylation in maintaining imprinted gene silencing is unclear. In the mouse, many imprinted genes show imprinted expression for only a limited time and switch to biallelic expression during development (Santoro and Barlow, 2011). It is tempting to speculate that the absence of DNA methylation from most silent imprinted gene promoters is due to the need to re-express the silent allele during development. Conversely, DNA methylation could represent a means to ensure stable epigenetic repression of essential imprinted genes throughout life (John and Lefebvre, 2011).

Airn expression can silence *Igf2r* at any time during ESC differentiation

The *Airn* lncRNA is among the few lncRNAs for which a precise function has been described (Guttman and Rinn, 2012; Pauli et al., 2011). It has been recently shown that *Airn* transcription, but not the lncRNA transcript, is responsible for *Igf2r* silencing (Latos et al., 2012). One way to investigate lncRNA mechanism of action is to ask whether its activity is restricted to a permissive developmental context or time frame that contains essential co-factors or chromatin environments. For example, a 'window of opportunity' has been described for the *Xist* lncRNA, which can only initiate XCI within 48 hours of ESC differentiation (Wutz and Jaenisch, 2000). In adult mice, most cells are resistant to *Xist* but permissiveness for XCI is transiently re-established in hematopoietic precursor cells (Savarese et al., 2006). In contrast to *Xist*, *Airn* can initiate *Igf2r* silencing throughout ESC differentiation. *Airn* is normally upregulated between days 2 and 3 of ESC differentiation (Latos et al., 2009) and activating functional *Airn* after day 3 induces paternal *Igf2r* repression at all time points, showing that silencing activity is not

restricted to a window and is unlikely to depend on developmentally regulated factors. Although *Igf2r* silencing is usually followed by gain of DNA methylation (Latos et al., 2009; Stöger et al., 1993), *Igf2r* repression after day 5 is not. This correlates with decreased levels of the *de novo* methyltransferase DNMT3B and of the DNMT3L co-factor during ESC differentiation. Importantly, *Igf2r* silencing can be maintained up to 8.5 dpc in the absence of DNA methylation, as shown by *Dnmt1* knockout mice that silence *Igf2r* biallelically and upregulate *Airn* twofold (Li et al., 1993; Seidl et al., 2006). The data here show that DNA methylation, although able to maintain the silent state, is not necessary for its maintenance and can only be established within an early developmental window.

Although *Airn*-mediated silencing is observed throughout ESC differentiation, the data show that *Igf2r* repression after day 3 is less efficient than in the continuous presence of *Airn*. It is noteworthy that *Airn* and *Igf2r* show similar expression kinetics in mouse tissues and differentiated ESCs (Latos et al., 2009; Pauler et al., 2005). This could indicate that *Airn* repressor activity is limited by higher *Igf2r* promoter activity. Transcriptional interference, whereby one transcriptional process suppresses another one in cis (Palmer et al., 2011) has been shown to act at the *Igf2r* locus (Latos et al., 2012). The data presented here, that *Airn* represses *Igf2r* most efficiently when the latter is weakly expressed and that silencing efficiency decreases when the *Igf2r* promoter is expressed strongly, are in agreement with a transcriptional interference model.

Understanding the order of events that lead to stable silencing of imprinted protein-coding genes by macro lncRNAs is not only relevant for other imprinted clusters, but may be informative for the growing number of lncRNAs identified in the mammalian genome, particularly those associated with abnormal gene silencing in human disease (Wang and Chang, 2011). Human imprinting syndromes arising from aberrant expression of imprinted genes or loss of the parental allele expressing the protein-coding gene can benefit from therapeutic strategies that relieve the dormant alleles. One example is the Angelman syndrome, where topoisomerase inhibitors have recently been used to reactivate the silent *Ube3a* gene, which correlated with downregulation of the antisense *Ube3a-as* lncRNA (Huang et al., 2012). The data here, which show *Airn* expression is continuously required for *Igf2r* silencing until DNA methylation is acquired, underline the importance of understanding how epigenetic silencing is maintained, before strategies to reactivate epigenetically silenced genes can be designed, as removing only DNA methylation or only the lncRNA product would not relieve silencing from similar loci.

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Competing interests statement
The authors declare no competing financial interests.

Supplementary material
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