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DISSERTATION

Titel der Dissertation

Onset and maintenance of *Airn* non-coding RNA mediated imprinted expression in an *in vitro* embryonic stem cell model

Verfasserin

Federica Santoro, M.Sc.

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Betreuerin:	Prof. Denise P. Barlow, PhD

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1. ZUSAMMENFASSUNG

Genomische Prägung ist ein epigenetisches Phänomen, welches die Expression bestimmter Gene in Säugetieren von nur einem der beiden elterlichen Allele darstellt. Geprägte Gene treten meistens in Clustern auf welche sowohl protein-kodierende als auch lange nicht-kodierende RNA (lnkRNA) Gene beinhalten. In dem geprägten *Igf2r* Cluster der Maus bewirkt die väterlich exprimierten lnkRNA *Airn* das Stilllegen von *Igf2r* am selben Chromosom, dem eine Anreicherung von DNA Methylierung am unterdrückten *Igf2r* Promoter folgt. Es ist jedoch weder bekannt ob *Airn* oder die DNA Methylierung die genomische Prägung von *Igf2r* bewirkt noch ob die stilllegende Funktion von *Airn* an ein Zeitfenster während der Entwicklung gebunden ist. Für meine Untersuchungen habe ich embryonale Stammzellen (ESZ) verwendet, da während deren Differenzierung der Beginn der genomischen Prägung von *Airn* und *Igf2r* nachvollzogen werden kann. Durch gezielte Genmodifikationen habe ich zwei induzierbare Systeme entwickelt, mit denen ich die Transkription von *Airn* während der ESZ Differenzierung an- und abschalten kann. Die erste Zelllinie beinhaltet zwei *loxP*-Stellen die den Promoter von *Airn* flankieren. Durch Verwendung einer Tamoxifen induzierbaren CreER Rekombinase kann die Promotersequenz entfernt und die Transkription von *Airn* während der ESZ Differenzierung abgeschaltet werden. Mit dieser Methode konnte ich zeigen, dass die kontinuierliche Transkription von *Airn* notwendig ist um die Stilllegung von *Igf2r* beizubehalten, jedoch nur bis der väterliche *Igf2r* Promoter DNA methyliert wird. Weiters konnte ich beobachten, dass diese Methylierung unabhängig von *Airn* am Promoter erhalten bleibt. Die zweite Zelllinie exprimiert eine verkürzte, nicht funktionelle Form von *Airn*, wobei das Abbruchsignal wiederum von zwei *loxP*-Stellen flankiert wird. In diesem Fall wird durch CreER die durchgehende, funktionsfähige Form von *Airn* während der ESZ Differenzierung wiederhergestellt. Mit diesen Zellen konnte ich zeigen, dass die unterdrückende Aktivität von *Airn* gegenüber *Igf2r* an keinerlei Zeitfenster während der Differenzierung gebunden ist. Außerdem konnte ich beobachten, dass die Stilllegung von *Igf2r* ohne DNA Methylierung auftreten kann. Zusammengefasst zeigen die Ergebnisse meiner Arbeit, dass während ESZ Differenzierung die lnkRNA *Airn* unerlässlich aber auch ausreichend für die Stilllegung von *Igf2r* ist. Darüberhinaus repräsentiert DNA Methylierung eine zusätzliche epigenetische Ebene um die Unterdrückung von *Igf2r* zu gewährleisten.

1. ABSTRACT

Genomic imprinting is an epigenetic phenomenon controlling parental-specific monoallelic expression of some mammalian genes. Imprinted genes generally occur in clusters, containing both protein-coding and long non-coding RNA (lncRNA) genes. In the mouse imprinted *Igf2r* cluster, expression of the *Airn* lncRNA is necessary to initiate paternal-specific silencing of *Igf2r*, followed by gain of DNA methylation on the repressed *Igf2r* promoter. However, it is unknown if *Airn* and DNA methylation are necessary to maintain stable *Igf2r* repression or if the repressive effects of *Airn* are confined to a developmental 'window of opportunity'. Here, I used an embryonic stem (ES) cell differentiation system that mimics the developmental onset of *Igf2r* and *Airn* imprinted expression and gene targeting technology, to establish two inducible systems to turn *Airn* on or off during ES cell differentiation. First, I created an ES cell line in which *loxP* sites flank the *Airn* promoter. I then used the tamoxifen-inducible CreER recombinase to delete the promoter, turning *Airn* transcription off, during ES cell differentiation. By using this tool, I was able to show that continuous *Airn* expression is needed to maintain *Igf2r* silencing, but only until the paternal *Igf2r* promoter gains DNA methylation. I could also show that this methylation mark is maintained independently of the *Airn* lncRNA. Next, I established an ES cell line expressing a truncated, non-functional form of *Airn* in which *loxP* sites flank the truncation signal. In this case, activating CreER restores full-length *Airn* transcription during differentiation. With this tool, I showed that the silencing activity of *Airn* is not limited to a developmental 'window of opportunity', as *Airn* can silence *Igf2r* in both early and late differentiated ES cells. Moreover, I observed that *Igf2r* repression could be maintained in the absence of DNA methylation. Together, the results presented in this thesis show that the *Airn* lncRNA is both necessary and sufficient to silence *Igf2r* throughout ES cell differentiation and that DNA methylation probably adds an extra layer of epigenetic information to safeguard the silent *Igf2r* allele.

2. INTRODUCTION

2.1. Genomic imprinting: an epigenetic *cis*-silencing mechanism

Diploid organisms such as mammals possess two homologous sets of autosomal chromosomes and they inherit one set from their mother and one from their father. The presence of two copies of each gene is generally considered beneficial, as the deleterious effects of recessive mutations in one allele will be masked by the presence of the functional wild-type allele (Otto and Goldstein, 1992). Not all mammalian genes, however, are expressed equally from both alleles. Monoallelic gene expression was first observed for X-chromosome linked genes (Lyon, 1961). In female mammals, one X chromosome is inactivated to equalize X-linked gene expression between the two sexes. The process is random, as it does not depend on the parental origin of the X chromosome. Some autosomal genes also display random monoallelic expression. This phenomenon was originally described for immunoglobulin, T cell and odorant receptor genes, but it seems to be more widespread than previously thought. In fact, recent studies have shown that surprisingly large numbers of human and mouse autosomal genes (~5-10% in humans and more than 10% in mice) exhibit random monoallelic expression (Gimelbrant et al., 2007; Zwemer et al., 2012). Imprinted genes also display monoallelic expression, but in a parent-of-origin specific fashion (Fig. 1). This phenomenon, that causes some genes to be expressed from the maternally or the paternally derived allele only, is known as genomic imprinting.

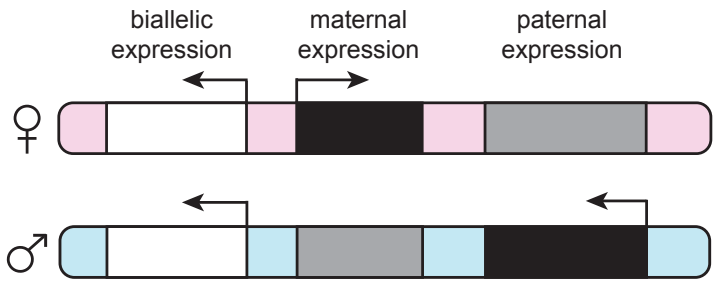

Fig. 1. Imprinted genes show parental-specific monoallelic gene expression. The majority of mammalian genes are expressed biallelically from both parental chromosomes (white boxes). Imprinted genes instead are expressed from one allele, depending on its parental origin (black boxes), and repressed on the other one (grey boxes). Maternal and paternal chromosomes are depicted in pink and blue, respectively. Arrows indicate transcription.

Genomic imprinting has been reported in angiosperm plants and in mammals, among which it occurs in marsupials and placental eutherians, but not egg-laying

monotremes (Jiang and Kohler, 2012; Renfree et al., 2009). The term 'imprinting' was first used to describe the preferential elimination of paternally derived X chromosomes in sciarid flies (Crouse, 1960). Similarly, imprinted X inactivation was found to occur in marsupials and in extraembryonic tissues of the mouse (Cooper et al., 1971; Takagi and Sasaki, 1975). In the 1970s, the first autosomal parent-of origin effects were reported: the deletion of a specific mouse chromosomal region was shown to cause embryonic lethality upon maternal, but not paternal, transmission (Johnson, 1974). The confirmation that the two parental genomes are not equivalent came only a decade later, when scientists attempted to generate biparental embryos by fusing two maternal or two paternal pronuclei. All attempts resulted in early postimplantation lethality, with bimaternal or parthenogenetic embryos exhibiting scarce extraembryonic tissues and bipaternal or androgenetic ones displaying abundant extraembryonic tissues but severe embryo growth retardation (Barton et al., 1984; McGrath and Solter, 1984; Surani et al., 1984). Additional insight into autosomal parental-specific effects came from the analysis of mice carrying uniparental disomies (UPDs), in which the paternal or maternal chromosome is duplicated, with concomitant loss of the opposite parental homolog. When viable, the mice displayed growth or behavioral abnormalities and genetic analysis led to the identification of subchromosomal regions for which both maternal and paternal copies are needed to ensure proper development (Cattanach and Kirk, 1985) (see also Fig. 2). However, it was not until another decade that the first imprinted genes were identified. In 1991, the *Igf2r*, *Igf2* and *H19* mouse genes were reported to show parental-specific monoallelic expression (Barlow et al., 1991; Bartolomei et al., 1991; DeChiara et al., 1991; Ferguson-Smith et al., 1991). Since then, many more imprinted genes have been identified (Williamson et al., 2012). Apart from a few cases that seem to have no obvious function, imprinted genes regulate processes such as embryonic and/or neonatal growth, placentation, brain development, social behavior and metabolism.

An important feature of genomic imprinting is its epigenetic nature. An epigenetic trait is commonly defined as "*a stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence*" (Berger et al., 2009). Laboratory mouse strains are inbred and therefore carry genetically identical parental autosomes. The observation that inbred mice can express identical DNA sequences in a parental-specific fashion led to the conclusion that genomic imprinting must arise from an epigenetic, rather than a genetic, mechanism.

Moreover, the two parental alleles of an imprinted gene co-exist in the same nuclear environment, but silencing is restricted to one allele without affecting expression of the other allele *in trans* (i.e., on the other chromosome). Genomic imprinting thus represents an invaluable model to understand epigenetic *cis*-silencing (adapted from (Santoro et al., 2013)). The establishment of a stable epigenetic state requires initiators, which define its chromosomal coordinates, and maintainers, that sustain the epigenetic state but might not be sufficient to initiate it (Berger et al., 2009). DNA methylation, histone modifications and long non-coding (lnc) RNAs are the main factors involved in the initiation and/or maintenance of epigenetic states, and their contribution to imprinted gene silencing will be discussed in this thesis.

2.2. The evolution of genomic imprinting

As mentioned above, the advantage of diploidy over haploidy is that recessive mutations in one copy of a gene can be rescued by the backup copy on the other chromosome. Imprinted genes lack this advantage, as their monoallelic expression means they are functionally haploid. The cost of genomic imprinting is exemplified by a number of human disorders that arise when imprinted gene expression is lost, such as Prader-Willi syndrome (PWS), Angelman syndrome (AS), Beckwith-Wiedemann syndrome (BWS) and Silver-Russell syndrome (SRS). Despite its cost, genomic imprinting persisted through 125 million years of mammalian evolution, indicating it must have provided an evolutionary advantage to the organisms in which it arose (Renfree et al., 2009). However, the nature of this advantage remains a question of debate. Several hypotheses have been proposed to explain why genomic imprinting arose in mammals. Although none of them provide a unifying explanation for the evolution of all imprinted genes, their arguments fit reasonably well to specific imprinted loci, suggesting there might have been different driving forces during evolution to select for different imprinted genes.

One of the earliest hypotheses states that genomic imprinting evolved to prevent parthenogenesis, based on the observation that parthenogenetic species occur in nature, but never among mammals (Solter, 1988). If they cannot reproduce asexually via parthenogenesis, diploid organisms must employ sexual reproduction, in which the two parental chromosome sets undergo recombination. By allowing recombination to occur, sexual reproduction provides two important advantages over asexual reproduction: faster adaptation and the possibility to remove deleterious

mutations (Engelstadter, 2008). Genomic imprinting might therefore have evolved to ensure that mammalian species reproduce sexually rather than asexually. Recently, parthenogenetic mice have been generated by engineering the maternally silent *Dlk1* and *Igf2* imprinted genes to display paternal expression patterns, thus formally demonstrating that the only barrier to mammalian parthenogenesis is represented by genomic imprinting (Kawahara et al., 2007; Kono et al., 2004). However, the 'prevention of parthenogenesis' hypothesis does not fully explain why genomic imprinting evolved, as inactivating maternal genes alone could prevent parthenogenesis, but many imprinted genes are silenced on their paternally derived alleles.

Another early hypothesis attempting to explain the origin of genomic imprinting is known as the 'ovarian time-bomb' hypothesis (Varmuza and Mann, 1994). During mammalian embryogenesis, only the cells of the inner cell mass give rise to the embryo proper, whereas trophoblast cells invade the uterus wall of the mother to form the placenta, a specialized organ which ensures nutrient and gas exchange between the mother and the developing fetus. If unfertilized oocytes were to undergo spontaneous activation, many parthenogenetic embryos could develop and invade the maternal uterus, posing threats to the health of the mother. The ovarian time-bomb hypothesis states that genes responsible for trophoblast development are inactivated in oocytes to prevent invasive, oocyte-derived tumors from arising. In support of this hypothesis, parthenogenetic embryos derived from nuclear transfer experiments exhibit reduced trophoblast-derived tissues (Surani et al., 1984). Interestingly, occasional ovarian teratomas arising from spontaneously activated oocytes are benign tumors, as they lack the paternal genes necessary for trophoblast invasion. Unfortunately, this hypothesis does not provide any explanation for the imprinted status of genes that are not involved in trophoblast development, dismissing them all as 'innocent bystanders'. Another caveat of the hypothesis is that it does not explain why genomic imprinting exists in species that lack invasive placentas, such as marsupials and sheep (Wilkins and Haig, 2003).

Unlike the hypotheses discussed so far, the 'host defence hypothesis' focuses on the origin of the molecular mechanisms underlying genomic imprinting and links them to host defence strategies against foreign DNA elements (Barlow, 1993; Kaneko-Ishino and Ishino, 2010). Eukaryotic genomes contain large numbers of mobile transposable elements, most of which have been silenced during evolution to

prevent extensive genome damage. The host defence hypothesis proposes that insertion of such transposable elements in the genome, followed by DNA methylation-mediated silencing, might have triggered the evolution of genomic imprinting. The basic assumptions of the hypothesis are that a major function of DNA methylation is host defence and that genomic imprinting arose as a side effect for genes containing sequences that make them look like foreign DNA (Barlow, 1993). Indeed, the cell uses the same machinery (the *de novo* DNA methyltransferase DNMT3A and its cofactor DNMT3L) to establish genomic imprinting and to silence transposons, but in a sexually dimorphic fashion (Barlow, 1993; Bourc'his and Bestor, 2004; Bourc'his et al., 2001; Yoder et al., 1997). In female germ cells, DNMT3L is required to set maternal imprints but not for genome-wide methylation (Bourc'his et al., 2001), whereas in male germ cells it is needed for global methylation but not for paternal imprint establishment (Bourc'his and Bestor, 2004). These observations suggest that host defence against transposable elements is triggered differently in oocytes and spermatocytes, thus providing a possible explanation as to why different genes are imprinted in the two sexes (Bestor and Bourc'his, 2004). The host defence hypothesis is also supported by the existence of retrotransposon-derived imprinted genes, such as *Peg10* and *Rtl1*, for which insertion into the genome coincided with gain of imprinted expression at the locus of insertion during evolution (Edwards et al., 2008; Suzuki et al., 2007). Additionally, it has been observed that the increasing number in the genome of some classes of repeats, such as LTRs and DNA elements, correlates with the acquisition of genomic imprinting during evolution (Pask et al., 2009). However, the hypothesis does not explain why many retrotransposed elements are not imprinted (Youngson et al., 2005).

To date, the most widely accepted hypothesis in the field is the ‘parental conflict’ or ‘kinship hypothesis’, which states that genomic imprinting arose as a result of evolutionary conflict between maternally and paternally derived alleles (Moore and Haig, 1991; Moore and Mills, 2008). In mammalian species, where all the resources for offspring growth and development are of maternal origin, the interest of the father is to obtain large and strong offspring, whereas the interest of the mother is to reduce the amount of resources allocated per pregnancy, so that she can produce more offspring during a lifetime. As a consequence, paternally derived genes that enhance nutrient transfer to the fetus or neonate and maternally derived genes that suppress fetal or neonatal growth should have been selected for imprinted

expression during evolution. Several experimental observations are in line with the predictions of the hypothesis: imprinted genes are generally expressed during embryonic and early postnatal development; many, but not all, mutations in imprinted regions tend to cause growth and/or behavioral abnormalities; many paternal genes enhance fetal and placental growth, whereas many maternal ones suppress them; some brain-specific imprinted genes influence feeding behavior in the young (Williamson et al., 2012). However, not all imprinted genes fall into these categories and can find justification in the kinship hypothesis. Moreover, as different fathers can compete with each other for slightly better alleles, the hypothesis predicts that paternally expressed imprinted genes would evolve faster than maternally expressed ones - but this is not seen. Finally, the hypothesis is difficult to reconcile with the molecular mechanisms that control the establishment of imprinted expression patterns, as control over the paternally expressed genes is achieved in the female germline by silencing the maternal alleles and *vice versa*.

Recently, the 'coadaptation hypothesis' has been proposed, according to which genomic imprinting evolved to coadaptively regulate embryo development and reproductive behavior in mammals (Keverne and Curley, 2008). Parent and infant coadapt by coexpressing imprinted genes in the placenta and the hypothalamus. The hypothesis is based on the observation that some imprinted genes are paternally expressed - therefore maternally silenced - in both hypothalamus and placenta and that mutations of these genes in the maternal hypothalamus, the fetal hypothalamus or the placenta determine similar phenotypes. A paradigmatic example is *Peg3*, a paternally expressed gene that is essential for normal food intake, maternal care, milk letdown and sucking behavior in mother and pup (Curley et al., 2004).

2.3. Features of imprinted genes

2.3.1. Imprinted genes are clustered

So far, 150 imprinted genes have been identified in the mouse genome and many of them also show imprinted expression in humans (Morison et al., 2001; Williamson et al., 2012). Although a few of them represent 'solo' imprinted genes, the majority is located in clusters containing both maternally and paternally expressed genes (Fig. 2). Clustering allows efficient control over multiple genes through a single master

regulator, known as the imprint control element (ICE) or imprint control region (ICR), as described below.

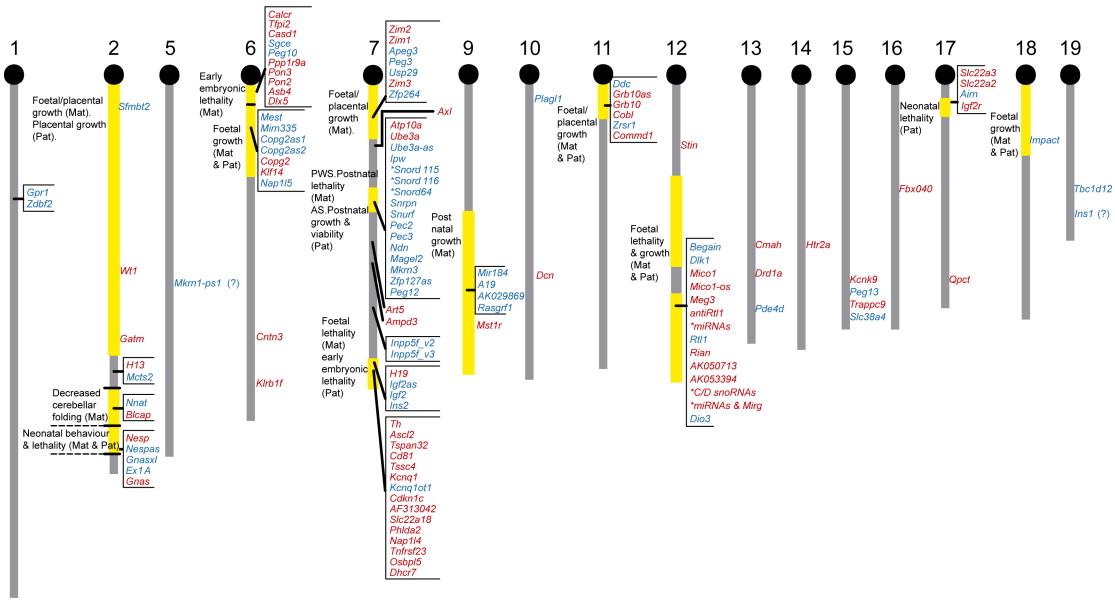

Fig. 2. Map of imprinted regions in the mouse genome and associated phenotypes. Vertical grey bars indicate mouse chromosomes 1-19. Regions in yellow show abnormal imprinting phenotypes upon maternal (Mat) or paternal (Pat) duplication. Maternally expressed genes are in red, paternally expressed genes in blue. *: imprinted small nucleolar RNAs and microRNAs. (?): conflicting data. Adapted from http://www.har.mrc.ac.uk/research/genomic_imprinting.

2.3.2. Imprinted clusters are differentially methylated and DNA methylation controls activity of the imprint control element

Imprinted gene clusters display allele-specific DNA methylation that is restricted to discrete regions. DNA methylation is an epigenetic modification that occurs predominantly on CpG dinucleotides in mammalian cells and is usually associated with transcriptional repression (Schubeler, 2012). CpG dinucleotides are not distributed equally through the genome, but are often found in CpG-rich sequences called CpG islands (CGIs). CGIs are associated with approximately 70% of annotated gene promoters and are usually unmethylated, regardless of the expression state of the associated gene (Deaton and Bird, 2011). Imprinted CGIs instead are peculiar, as they gain DNA methylation in a parental-specific fashion, giving rise to differentially methylated regions (DMRs). Compared to non-imprinted control regions, imprinted DMRs contain more tandem direct repeats (Neumann et al., 1995), which in a few cases have been shown to be necessary for the differentially methylated state (Koerner et al., 2012; Reinhart et al., 2006).

Depending on the time of establishment, imprinted DMRs can be distinguished into gametic or gDMRs and somatic or sDMRs. gDMRs acquire parental-specific methylation in the germline and maintain it thereafter, with the exception of a few gDMRs that gain DNA methylation on the other allele in a tissue-specific manner (Ferron et al., 2011; Proudhon et al., 2012). As differential methylation is established in the gametes, at a time when the two parental genomes are physically separated and before imprinted expression arises, gDMRs represent potential ICEs. Of the 23 imprinted gDMRs identified so far, 19 are maternally methylated and mostly overlap imprinted gene promoters (Chotalia et al., 2009; Proudhon et al., 2012). By contrast, only four gDMRs are paternally methylated and they all map to intergenic regions (Chotalia et al., 2009; Kobayashi et al., 2009). Unlike gDMRs, sDMRs are established in somatic tissues after fertilization. Acquisition of differential DNA methylation at these sites depends on the presence of gDMRs, but not all 23 gDMRs direct the establishment of sDMRs in their vicinity. Indeed, sDMRs are extremely rare in the mouse genome and modify the silent alleles of only a handful of imprinted genes. Moreover, they are established only after imprinted expression arises, indicating that they are not responsible for the initiation of imprinted expression (John and Lefebvre, 2011).

To date, nine imprinted gDMRs have been functionally tested by targeted deletions in mice. Of these, eight qualified as ICEs, as their deletion on the unmethylated allele caused derepression *in cis* of all imprinted genes in the cluster (Fitzpatrick et al., 2002; Lin et al., 2003; Shiura et al., 2009; Thorvaldsen et al., 1998; Williamson et al., 2006; Wutz et al., 1997; Yang et al., 1998; Yoon et al., 2002) (Figs. 3-4). Imprinted expression was not affected when the ICE was deleted on the methylated allele, indicating that the ICE is a methylation-sensitive *cis*-acting repressor. Given the importance of DNA methylation in controlling ICE activity, it is not surprising that disruption of the maintenance DNA methyltransferase enzyme *Dnmt1* results in biallelic ICE activation and therefore biallelic silencing of imprinted protein-coding genes (Li et al., 1993). The only tested gDMR whose role as an ICE has not yet been convincingly proven is the *Peg3* gDMR, as its deletion did not disrupt imprinted expression of all genes in the cluster (Kim et al., 2012).

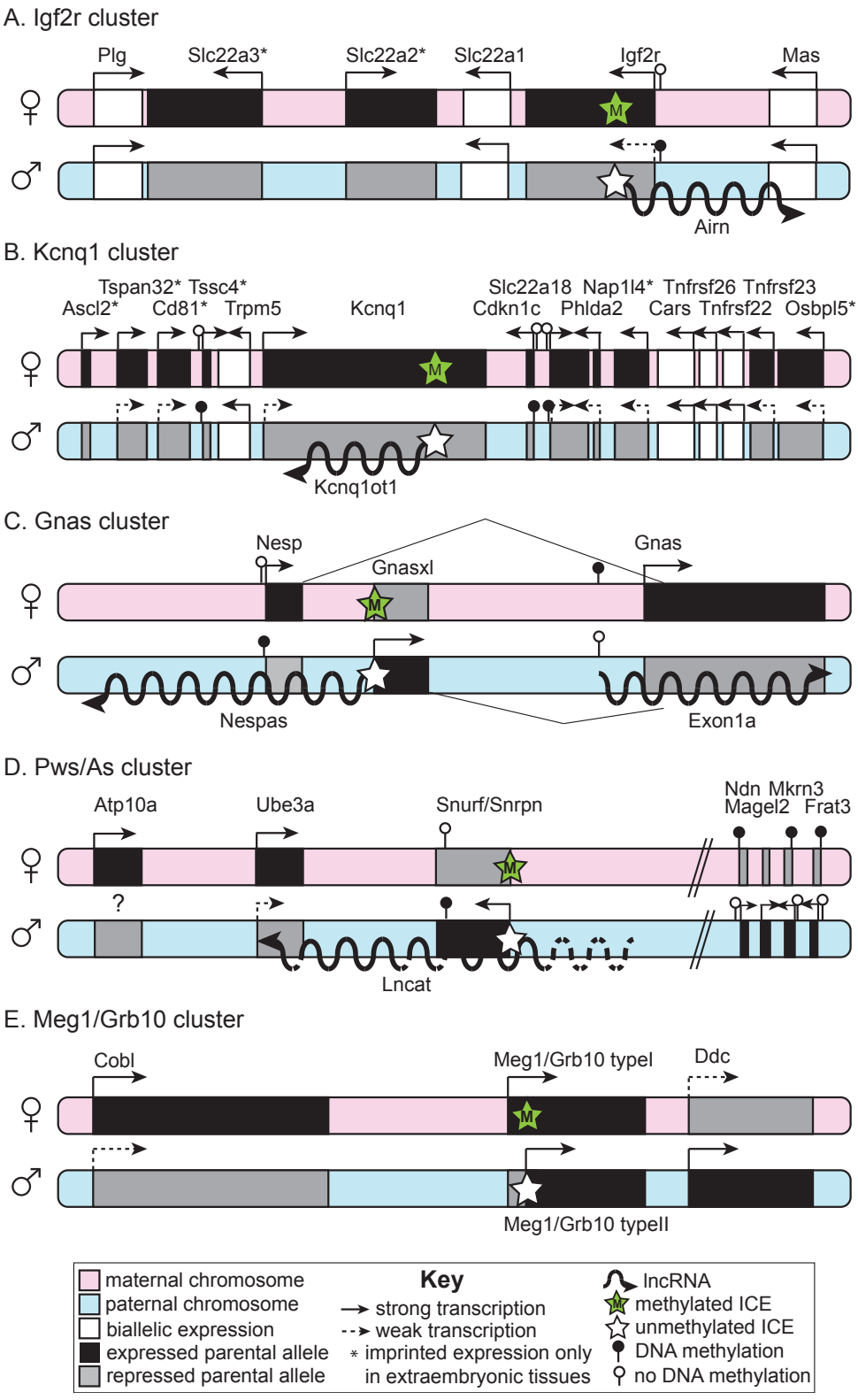

Fig. 3. The five imprinted clusters containing a maternally methylated ICE. For all five clusters, the ICE was defined by genetic deletion experiments in the mouse. See text for additional details. Adapted from (Santoro and Barlow, 2011).

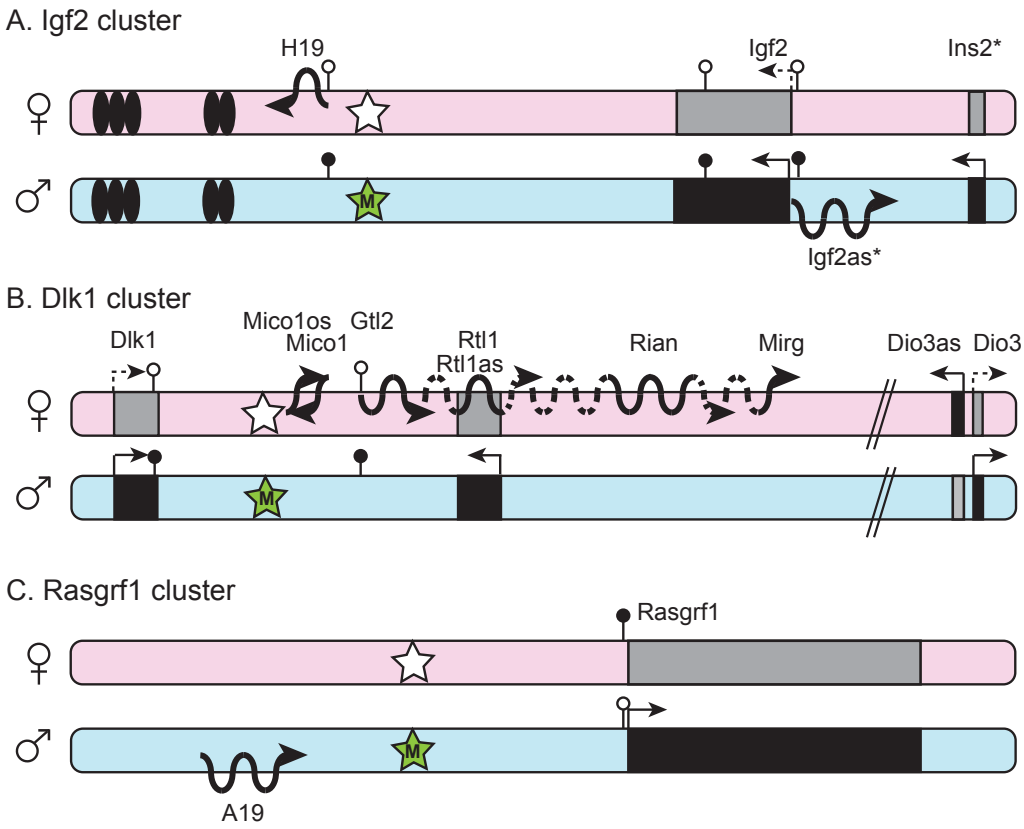

Fig. 4. The three imprinted clusters containing a paternally methylated ICE. For all three clusters, the ICE was defined by genetic deletion experiments in the mouse. See text for additional details. Key as in Fig. 3. Black ovals: enhancers.

2.3.3. Imprinted clusters display allele-specific histone modifications

In addition to differential DNA methylation, imprinted regions also display allele-specific post-translational histone modifications, which are generally restricted to short regions containing promoters, enhancers or the ICE itself. ChIP-chip and ChIP-Seq studies have shown that ICEs are typically marked by active H3K4me3 (histone H3 lysine 4 trimethylation) on the unmethylated allele and repressive H3K9me3 (histone H3 lysine 9 trimethylation) and H4K20me3 (histone H4 lysine 20 trimethylation) on the methylated allele (McEwen and Ferguson-Smith, 2010). Similarly, H3K4me3 and H3K9 acetylation mark the expressed alleles of imprinted genes, whereas focal repressive heterochromatin (H3K9me3, H4K20me3) marks their repressed alleles. H3K27me3 (histone H3 lysine 27 trimethylation) can sometimes be found as well. It is absent from the *Igf2r* cluster in embryonic fibroblasts (Regha et al., 2007), but it is present at the *Kcnq1* cluster in both embryo and placenta (Lewis et al., 2006; Lewis et al., 2004; Umlauf et al., 2004).

The presence of both active and repressive focal marks is a characteristic of imprinted regions and chromatin features can be used to identify novel imprinted genes and ICEs (Mikkelsen et al., 2007; Singh et al., 2011). However, unlike DNA methylation, which plays a fundamental role in the imprinting process, it is unclear whether histone modifications are required to establish imprinted expression. Allele-specific chromatin marks might merely correlate with allele-specific expression at imprinted loci or they could contribute to the maintenance, rather than the establishment, of specific epigenetic states. Upon deletion of the Polycomb protein EED, which is required to establish H3K27me3, only four out of 18 tested imprinted genes, located in three different clusters, were reported to lose imprinted expression in perigastrulation embryos (Mager et al., 2003). Interestingly, most imprinted genes were not derepressed in the absence of EED but maintained correct imprinted expression. The EHMT2 histone methyltransferase, which catalyzes H3K9me2 (histone H3 lysine 9 dimethylation), is needed to repress some imprinted genes in the *Kcnq1* and *Igf2r* clusters in placenta but not in the embryo, where imprinted expression is maintained in the absence of EHMT2 (Nagano et al., 2008; Wagschal et al., 2008). The only histone mark that so far seems to play an instructive role in the establishment of imprinted states is H3K4me3. Experiments conducted in female germ cells have shown that H3K4 must be demethylated for *de novo* DNA methylation to occur at some ICEs (Ciccone et al., 2009). This result is in line with a previous report that showed that the *de novo* methyltransferase DNMT3A-DNMT3L complex binds H3K4me3-modified DNA with lower affinity (Ooi et al., 2007). In conclusion, histone modifications seem to contribute to the establishment of DNA methylation imprints in the germline, but additional work is needed to determine the causal role, if any, of histone marks in establishing imprinted expression in somatic tissues.

2.3.4. Imprinted clusters contain long non-coding RNAs

In addition to protein-coding genes, many imprinted clusters also express long non-coding (lnc) RNAs, non-protein-coding transcripts longer than 200 bp in length whose function does not depend on processing to smaller RNAs (Koerner et al., 2009). Global transcriptome analyses in recent years have revealed that lncRNAs are not unique features of imprinted clusters, as many thousands are found throughout the mammalian genome (Derrien et al., 2011). Their abundance, tissue-specific expression and developmental regulation indicate that, far from being mere

'transcriptional noise', many lncRNAs may play important cellular roles, especially in the regulation of gene expression (Guttman and Rinn, 2012). However, compared to the multitude of lncRNAs known to date, only few have been functionally analyzed. Imprinted lncRNAs were the first autosomal lncRNAs shown to have a gene silencing function (Barlow, 2011). Many imprinted lncRNAs possess atypical features – such as inefficient splicing, extreme length, high repeat content, lack of conservation and short half-life – that set them apart from the majority of lncRNAs identified so far. Due to the greater abundance of its unspliced form, this type of lncRNA has also been termed 'macro' (Guenzl and Barlow, 2012) (adapted from (Santoro et al., 2013) and (Santoro and Pauler, 2013)).

In the maternally imprinted *Igf2r*, *Kcnq1* and *Gnas* clusters, the macro lncRNAs *Airn*, *Kcnq1ot1* and *Nespas*, respectively, are expressed from a promoter located inside the ICE and overlap one imprinted protein-coding gene in the cluster in antisense orientation (Smilinich et al., 1999; Wroe et al., 2000; Wutz et al., 1997) (Fig. 3A-C). Similarly, the ~1000 kb-long *Lncat* macro lncRNA in the Pws/As cluster overlaps the *Ube3a* gene in antisense direction (Landers et al., 2004) (Fig. 3D). In the paternally imprinted *Igf2*, *Dlk1* and *Rasgrf1* clusters, the macro lncRNAs *H19*, *Gtl2* and *A19*, respectively, are transcribed from intergenic promoters located some distance from the ICE (de la Puente et al., 2002; Pachnis et al., 1984; Tierling et al., 2006) (Fig. 4). *Gtl1* overlaps the *Rtl1* protein-coding gene, whereas the *H19* and *A19* lncRNAs do not overlap any other gene in their clusters. Interestingly, with the exception of *A19*, all imprinted macro lncRNAs mentioned above share two properties: firstly, they are expressed from the chromosome carrying the unmethylated ICE and secondly, they show reciprocal patterns of expression with most imprinted protein-coding genes in the cluster (Koerner et al., 2009).

2.4. Mechanisms regulating imprinted expression

As mentioned above, monoallelic expression in imprinted gene clusters is controlled by the ICE, a methylation-sensitive *cis*-silencing DNA element. So far, three mechanisms have been described to explain how the unmethylated ICE silences genes in its vicinity (Fig. 5). The ICE can act as a methylation-sensitive insulator element to restrict access of imprinted gene promoters to their enhancers. In the *Igf2* cluster, for example, the CTCF insulator protein binds the unmethylated ICE on the maternal chromosome and mediates higher-order chromatin loops which facilitate

interactions between the *H19* lncRNA promoter and downstream enhancers (Kurukuti et al., 2006). The formation of CTCF-dependent chromatin looping relies on the presence of cohesin, which is recruited by CTCF to its target sites (Nativio et al., 2009; Wendt et al., 2008), and prevents access of the *Igf2* and *Ins2* promoters to the same enhancers. This results in maternal-specific *Igf2* and *Ins2* silencing, independently of the *H19* lncRNA itself (Schmidt et al., 1999). On the paternal chromosome instead, CTCF cannot bind the methylated ICE and the *Igf2* and *Ins2* promoters have free access to the enhancers (Bell and Felsenfeld, 2000; Hark et al., 2000). As a consequence, the paternal allele expresses *Igf2* and *Ins2*, but not *H19* (Fig. 5A). The *Dlk1* cluster also contains an intergenic ICE, but imprinted expression is unlikely to depend on a similar mechanism because the ICE does not contain CTCF binding sites and the *Dlk1* and *Gtl2* genes do not share the same enhancers (da Rocha et al., 2008). The *Rasgrf1* ICE instead is a methylation-sensitive CTCF-dependent insulator and, similarly to *Igf2* and *Ins2*, *Rasgrf1* is only expressed from the paternal allele, where CTCF cannot bind the methylated ICE (Yoon et al., 2005).

Rather than by binding insulator proteins, the ICE can exert its silencing function by activating the expression of *cis*-silencing lncRNAs. In the *Igf2r* cluster, for example, imprinted expression of the *Igf2r*, *Slc22a2* and *Slc22a3* protein-coding genes is controlled by the *Airn* macro lncRNA, whose methylation-sensitive promoter lies within the ICE (Wutz et al., 1997). *Airn* is exclusively transcribed from the paternal allele, as the gametic DNA methylation imprint represses its maternal promoter (Fig. 5B). Upon truncation of the *Airn* lncRNA to 3% of its length, all three protein-coding genes switch from maternal-specific to biallelic expression, showing that *Airn* is required for their paternal-specific silencing (Sleutels et al., 2002). Similar experiments have shown that the *Kcnq1ot1*, *Nespas* and *Ube3a-as* lncRNAs control silencing of protein-coding genes in the *Kcnq1*, *Gnas* and Pws/As clusters, respectively (Mancini-Dinardo et al., 2006; Meng et al., 2012; Williamson et al., 2011). These functional imprinted lncRNAs represent invaluable epigenetic models to understand how lncRNAs repress genes *in cis* (adapted from (Santoro and Barlow, 2011) and (Santoro et al., 2013)).

Finally, another way to establish imprinted expression is by regulating alternative polyadenylation. This mechanism was first described for the *H13* imprinted cluster, in which maternal-specific expression of the *H13* gene depends on an intronic, maternally methylated gDMR. Methylation inactivates the gDMR, which contains the

promoter for the retrotransposon-derived *Mcts2* gene, resulting in maternal-specific *Mcts2* silencing. On the paternal allele instead, the unmethylated gDMR drives *Mcts2* transcription and this correlates with premature *H13* polyadenylation (Wood et al., 2008) (Fig. 5C). The same mechanism seems to operate at the *Herc3* cluster, in which expression of the *Nap1l5* retrogene, from an unmethylated gDMR located inside an intron of *Herc3*, correlates with premature *Herc3* polyadenylation on the paternal allele (Cowley et al., 2012). However, it is currently unknown whether the unmethylated gDMR or expression of the retrogene is needed to interfere with full-length transcription of the host gene.

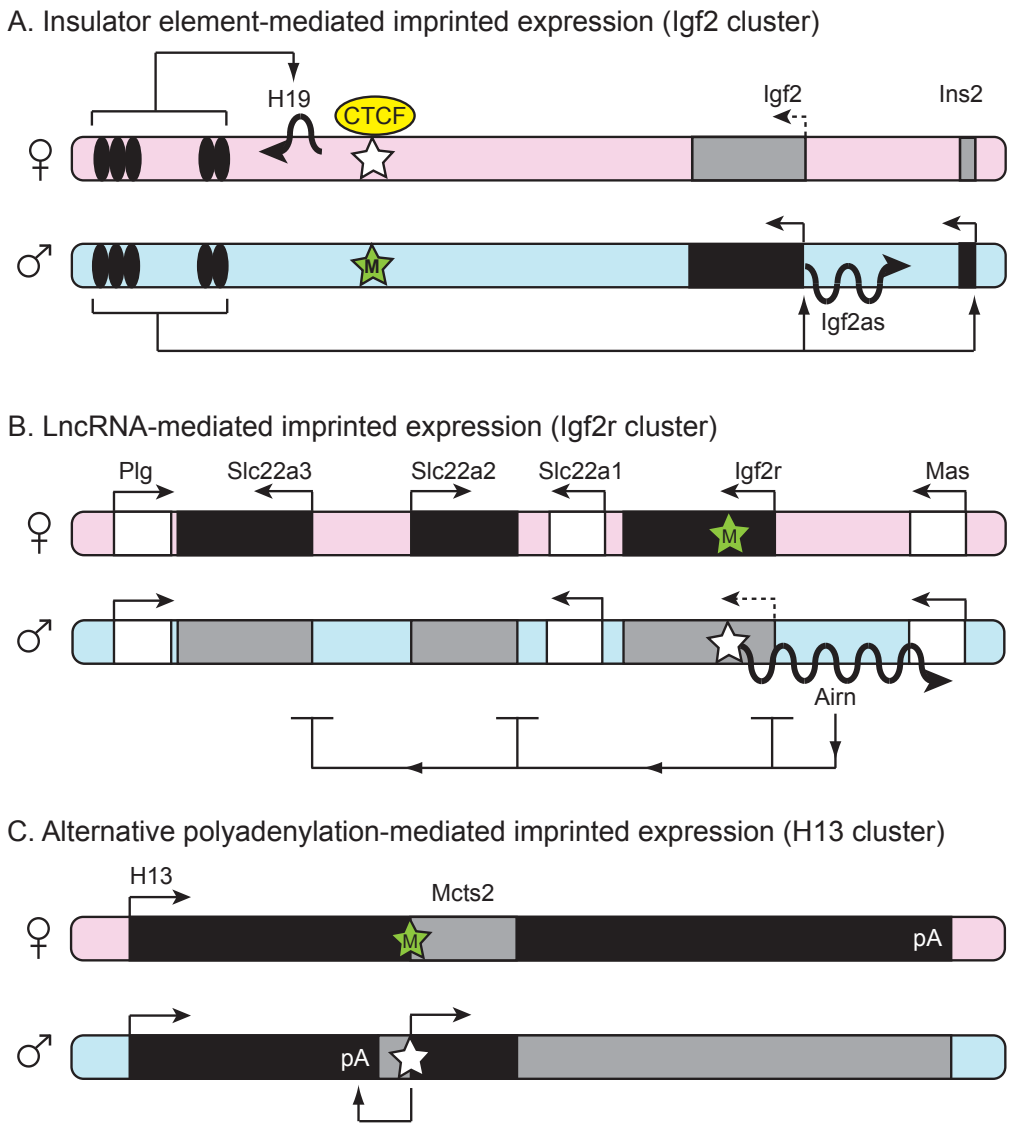

Fig. 5. Mechanisms controlling imprinted expression. The ICE can control imprinted expression by acting as a methylation-sensitive insulator element, as in the *Igf2* cluster (**A**), or by driving expression of a lncRNA that silences protein-coding genes *in cis*, as in the *Igf2r* cluster (**B**). Imprinted expression can also be established via alternative polyadenylation (pA), as in the *H13* cluster (**C**). See text for additional details. Key as in Fig. 3. Black ovals: enhancers.

2.5. Developmental regulation of imprinted expression: initiation and maintenance

As discussed earlier, many imprinted genes regulate embryonic and/or neonatal growth and normal mammalian development requires appropriate imprinted expression patterns (Bartolomei and Ferguson-Smith, 2011). The link between genomic imprinting and development is strengthened further by the fact that imprinting itself consists of a developmentally regulated cycle of events. To ensure transmission of the proper sex-specific imprints to the next generation, the ICE methylation patterns have to be reset in the germline of every organism (Fig. 6). Imprints are erased during the global wave of DNA demethylation that occurs between embryonic day E11.5 and E12.5 in primordial germ cells (PGCs) of the developing embryo (Smallwood and Kelsey, 2012). Imprints are then re-established in a sex-specific manner by the *de novo* methyltransferase activity of the DNMT3A-DNMT3L complex. In female oocytes, *de novo* methylation occurs only after birth, whereas in male sperm it takes place in late fetal development (Hackett and Surani, 2013).

How the methylation machinery recognizes ICE sequences and marks them differently in the male and female germ cells remains an open question in the imprinting field. Histone modifications may play a role, as binding of the DNMT3A-DNMT3L complex to chromatin is inhibited by H3K4 methylation and the KDM1B H3K4 demethylase is required to establish *de novo* DNA methylation at maternally imprinted gDMRs (Ciccone et al., 2009; Ooi et al., 2007). Transcription across gDMRs has been implicated in the establishment of some maternal-specific imprints in oocytes and has been suggested to act by maintaining open chromatin environments for *de novo* DNA methylation to occur (Chotalia et al., 2009). Small RNA pathways might be involved in the male germ line, as *de novo* methylation of the *Rasgrf1* ICE in sperm has been shown to depend on piRNAs (Watanabe et al., 2011). Lastly, tandem direct repeats may be required for acquisition and/or maintenance of ICE DNA methylation, as recently shown for the *Igf2r* ICE (Koerner et al., 2012).

Once established in the germ line, the imprints must be maintained to ensure proper imprinted expression in the developing organism. This means that firstly, they need to be propagated through cell divisions and secondly, they need to survive the

dramatic epigenetic reprogramming that occurs after fertilization in the zygote (Fig. 6). Propagation of DNA methylation patterns across cell divisions is ensured by the DNMT1 maintenance methyltransferase (Li et al., 1993). In preimplantation embryos, where *Dnmt1* is expressed at very low levels, this likely occurs through the concerted action of the oocyte-specific DNMT1O and low levels of somatic DNMT1 (Hirasawa et al., 2008).

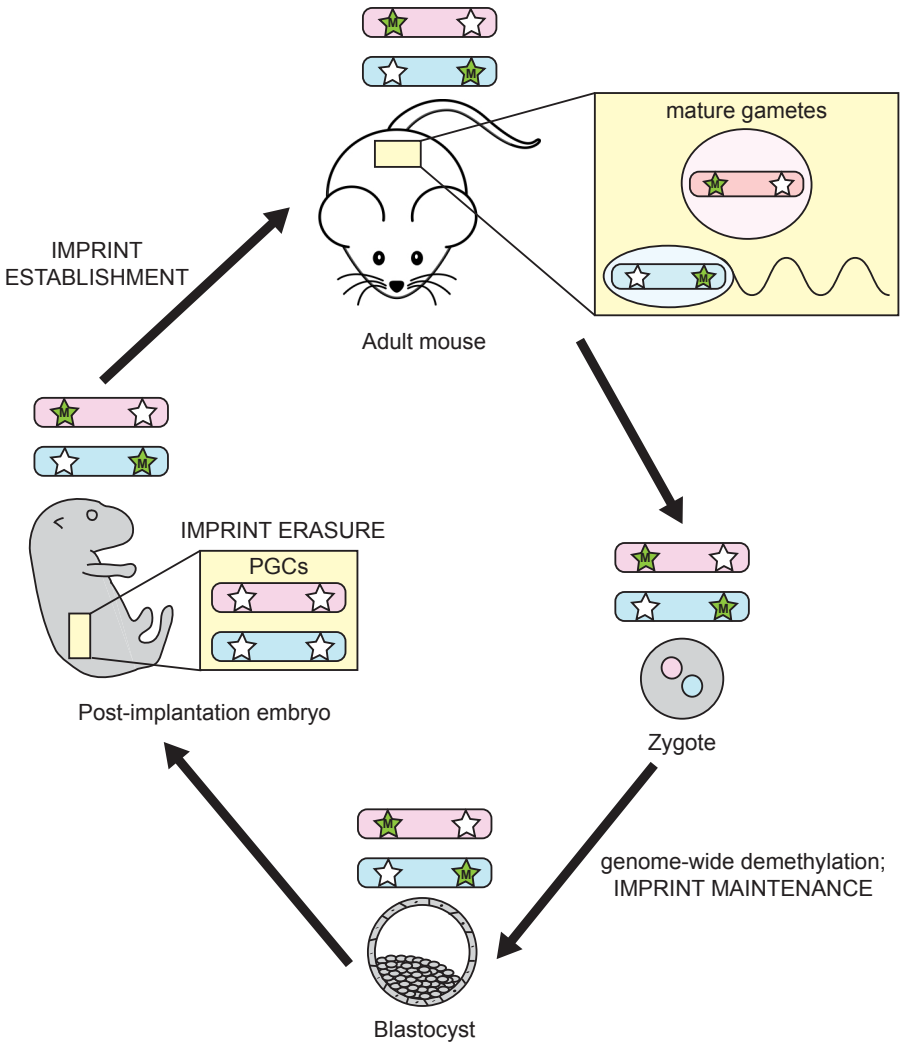

Fig. 6. The imprint lifecycle. DNA methylation imprints are established in the germline in a sex-specific fashion: methylation is acquired at maternally methylated ICEs in oocytes (pink circle) and at paternally methylated ICEs in sperm cells (blue oval). After fertilization, the imprints are maintained in the diploid zygote, despite the genome-wide demethylation that occurs at this stage, and they are preserved during subsequent stages of embryonic development. Imprints are only erased in primordial germ cells (PGCs) of the developing embryo, to allow correct resetting of the methylation mark in the gametes. The maternally and paternally derived chromosomes are depicted as pink and blue bars, respectively. White star: unmethylated ICE. Green star: methylated (M) ICE.

After fertilization, the zygotic genome is reprogrammed into a pluripotent state via massive epigenetic remodelling that includes removal of DNA methylation marks. Interestingly, this demethylation occurs with parental-specific dynamics: whereas the maternal genome is passively demethylated with each cell cycle, the paternal genome undergoes active demethylation, probably through hydroxymethylcytosine intermediates (Gu et al., 2011; Wossidlo et al., 2011). Gametic imprints are protected from this wave of DNA demethylation by several different proteins, including the maternal factor PGC7/Stella and the KRAB zinc-finger protein ZFP57 (Li et al., 2008; Nakamura et al., 2007). Except for a few imprinted gDMRs that lose differential methylation in a tissue-specific fashion, the majority retains parental-specific methylation throughout life in all somatic tissues (Ferron et al., 2011; Proudhon et al., 2012). Finally, imprints are selectively erased in the germ cells so a new imprinting cycle can begin (Fig. 6).

Given the near-ubiquitous nature of the imprint, a given gene could in theory display imprinted expression everywhere and anytime within an organism. In most cases, however, imprinted expression is restricted to specific tissues, cell types or developmental stages (Prickett and Oakey, 2012). This apparent paradox is explained by considering that the imprint alone does not initiate imprinted expression, which only arises in the presence of additional factors - the imprint 'readers' (Efstratiadis, 1994). Examples of imprint readers include the insulator-binding CTCF protein and transcription of silencing lncRNAs such as *Airn* and *Kcnq1ot1*. A consequence of the distinction between the imprint and its readers is that spatio-temporal regulation of imprinted gene expression can be achieved by modulating expression of the readers themselves. At the *Igf2r* cluster for example, despite the ubiquitous presence of the paternally unmethylated ICE, *Igf2r* only displays paternal-specific silencing when and where the *Airn* lncRNA is transcribed (Latos et al., 2009; Yamasaki et al., 2005). *Airn* is therefore the developmentally regulated imprint reader responsible for developmentally regulated *Igf2r* imprinted expression. However, not all imprinted genes in the *Igf2r* cluster show imprinted expression in the presence of *Airn*. The *Slc22a2* and *Slc22a3* genes, which only show imprinted expression in some extraembryonic lineages, become insensitive to the effects of *Airn* in some late embryonic stages and adult tissues (Zwart et al., 2001b). Therefore, imprint readers such as lncRNAs might not be the only developmentally regulated players in the establishment and maintenance of imprinted expression, but are likely to require additional, differentially expressed

cofactors to perform their silencing function (adapted from (Santoro and Barlow, 2011)).

The silent alleles of imprinted genes are sometimes marked by promoter DNA methylation. These differentially methylated regions are known as somatic or secondary DMRs (sDMRs), as they are not present in germ cells and are only detected in postimplantation embryos and adult somatic tissues. DNA methylation at promoter CGIs is generally associated with long-term transcriptional repression, but it is still not clear whether methylation is a cause or consequence of gene silencing (Jones, 2012). At imprinted loci, experimental evidence argues against a role for sDMRs in gene silencing initiation. Firstly, DNA methylation only marks the repressed alleles of a minority of imprinted protein-coding genes (John and Lefebvre, 2011). Secondly, somatic methylation imprints are often gained only after imprinted silencing has occurred. Thirdly, imprinted protein-coding genes can be repressed in the absence of DNA methylation (Li et al., 1993). However, even though dispensable for initiation of imprinted silencing, DNA methylation could play a role in its long-term maintenance.

2.6. The imprinted *Igf2r* cluster: a model system to understand gene silencing by lncRNAs

2.6.1. The Airn lncRNA silences three genes in cis

The *Igf2r* cluster on mouse chromosome 17 includes three maternally expressed protein-coding genes - *Igf2r*, *Slc22a2* and *Slc22a3* - and one paternally expressed macro lncRNA called *Airn* (antisense to *Igf2r* RNA non-coding) (Fig. 3A). *Igf2r* was the first imprinted gene to be discovered in the mouse genome (Barlow et al., 1991). It encodes the insulin-like growth factor 2 receptor, a scavenging receptor that binds *Igf2* at the cell surface, provoking its internalization and lysosomal degradation (Lau et al., 1994; Wang et al., 1994). *Igf2r* is also known as the cation-independent mannose 6-phosphate (M6P) receptor, as it binds M6P-labeled ligands in the Golgi network and shuttles them to the lysosomes. The latter must have been in fact its ancestral function, as M6P binding sites are found in all investigated vertebrates, whereas the *Igf2* binding site is only present in marsupials and eutherians (Killian et al., 2000). Interestingly, the appearance of the binding pocket for *Igf2*, also an imprinted gene, coincided with the emergence of *Igf2r* imprinted expression during

evolution. The *Slc22a2* and *Slc22a3* genes instead encode membrane-spanning solute carriers and, unlike *Igf2r*, are not essential for mouse development, viability or fertility (Jonker et al., 2003; Wang et al., 1994; Zwart et al., 2001a). *Igf2r* imprinted expression is widespread and can be detected in all embryonic, extraembryonic and adult mouse tissues, with the exception of preimplantation stage embryos and postmitotic neurons (Lerchner and Barlow, 1997; Szabo and Mann, 1995; Yamasaki et al., 2005). Imprinted expression of *Slc22a2* and *Slc22a3* instead is restricted to some extraembryonic lineages, such as placenta and visceral yolk sac endoderm (Hudson et al., 2011; Zwart et al., 2001b). *Slc22a2* imprinted expression is maintained throughout embryonic development, whereas *Slc22a3* becomes biallelically expressed by E16.5 (Hudson et al., 2011) (adapted from (Santoro et al., 2013).

Imprinted expression of all three protein-coding genes is controlled by the paternally expressed *Airn* macro lncRNA, the first autosomal lncRNA for which a silencing function was shown (Sleutels et al., 2002). The *Airn* lncRNA promoter lies inside *Igf2r* intron 2, within a 4 kb region genetically defined as the ICE (Lyle et al., 2000; Wutz et al., 1997). On the maternal chromosome, the ICE methylation imprint silences the *Airn* promoter, allowing expression of *Igf2r*, *Slc22a2* and *Slc22a3* (Wutz et al., 1997; Zwart et al., 2001b). On the paternal chromosome, the unmethylated ICE drives expression of the 118 kb-long *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 their silencing (Sleutels et al., 2002). Of the three genes silenced by *Airn*, *Igf2r* is the only one to gain DNA methylation on the silenced paternal promoter (Stoger et al., 1993; Zwart et al., 2001b). This somatic imprint is gained late in development and is not required for silencing initiation (Li et al., 1993; Seidl et al., 2006) (adapted from (Santoro et al., 2013)).

The *Airn* lncRNA is poorly conserved among mammalian species. In opossums, *Igf2r* imprinted expression occurs in the absence of the intron 2 CGI, of the *Airn* lncRNA and of differential methylation of the silent *Igf2r* promoter, indicating that different regulatory mechanisms evolved to control *Igf2r* imprinted expression in marsupials and rodents (Weidman et al., 2006). In humans, the *IGF2R* intron 2 CGI is maternally methylated as in mice (Smrzka et al., 1995), but *IGF2R*, *SLC22A2* and

SLC22A3 are biallelically expressed in adults and only show polymorphic imprinted expression (i.e., in a subset of individuals) in fetal tissues, placenta and Wilms' tumors (Monk et al., 2006; Xu et al., 1993; Xu et al., 1997). Human *AIRN* is expressed from the intron 2 CGI in mouse transgenic assays and in some Wilms' tumor samples, but it is not known whether it is responsible for *IGF2R* silencing as in mice (Yotova et al., 2008). Interestingly, the b*AIRN* lncRNA was recently identified in cattle and the onset of its expression correlated with gain of *IGF2R* imprinted expression during bovine embryonic development (Farmer et al., 2013).

2.6.2. Transcript or transcription?

Since the discovery of *Airn*, the mouse *Igf2r* cluster has provided an excellent model system to study how lncRNAs silence genes *in cis*. After observing that imprinted silencing of all three protein-coding genes in the cluster is lost upon *Airn* truncation, two hypotheses were formulated to explain how *Airn* might silence its target genes. In the RNA-directed targeting model, the *Airn* lncRNA coats the paternal chromosome and induces its heterochromatinization. This model is based on the example of the *Xist* (X-inactive specific transcript) lncRNA, which coats one X chromosome in female mammals and recruits chromatin-modifying repressor complexes to inactivate it (Brockdorff, 2011). In the transcriptional interference model instead, *Airn* transcription interferes with the activity of promoter or enhancer elements required for *Igf2r*, *Slc22a2* and *Slc22a3* expression, with no role for the RNA product itself (Pauler et al., 2007; Pauler et al., 2012) (adapted from (Santoro and Pauler, 2013)).

In placenta, the *Airn* lncRNA product has been shown to maintain *Slc22a3* silencing by recruiting the H3K9 histone methyltransferase EHMT2 to the paternal *Slc22a3* promoter (Nagano et al., 2008). *Airn* interacts with both EHMT2 and *Slc22a3* promoter chromatin in placenta and its accumulation at the *Slc22a3* promoter correlates with local acquisition of H3K9me3 and transcriptional silencing, which are lost in the absence of EHMT2 (Nagano et al., 2008). *Igf2r* silencing however is independent of both EHMT2 and the *Airn* lncRNA product but requires *Airn* transcriptional overlap to interfere with RNA polymerase II (RNAPII) recruitment to the *Igf2r* promoter (Latos et al., 2012; Nagano et al., 2008). Together, these experiments indicate that the *Airn* lncRNA can operate via an RNA-directed targeting

mechanism to silence *Slc22a3* but *Igf2r* is silenced via transcriptional interference (adapted from (Santoro et al., 2013)).

2.6.3. Is Airn silencing activity restricted to a 'window of opportunity'?

An unresolved question concerning the initiation of *Igf2r* silencing by *Airn* is whether *Airn* transcription is sufficient or if it requires a special chromatin environment or specific cofactors to be functional. *Igf2r* imprinted expression is developmentally regulated and established after embryonic implantation, concomitantly with the onset of *Airn* expression (Lerchner and Barlow, 1997; Szabo and Mann, 1995). If *Airn* requires additional factors, their expression may be restricted to the same developmental window during which *Airn* establishes *Igf2r* silencing. Testing if *Airn*-mediated silencing is limited to a permissive time frame or 'window of opportunity' during development is the first step towards identifying such factors (adapted from (Santoro and Barlow, 2011) and (Santoro et al., 2013)).

The idea of a 'window of opportunity' for *Airn* is analogous to the one described for the *Xist* lncRNA. Random X chromosome inactivation (XCI) occurs when *Xist* is transcribed from one of the two female X chromosomes, inducing transcriptional silencing over the whole chromosome. In females, *Xist* is constantly expressed from the two-cell stage, but there is only a specific time during which it can mediate XCI. With the use of an inducible *Xist* transgene in differentiating mouse embryonic stem (ES) cells, it was shown that XCI is only induced if the *Xist* lncRNA is expressed within 48 hours of ES cell differentiation, indicating that *Xist*-mediated silencing can only occur during a specific period of early development (Wutz and Jaenisch, 2000). Although *Xist* cannot silence genes in differentiated somatic cells, its silencing function is transiently reestablished in committed precursors of the hematopoietic system (Savarese et al., 2006), suggesting that *Xist* activity critically depends on the epigenetic context of the cell (adapted from (Santoro and Barlow, 2011)).

By comparing gene expression profiles of *Xist*-responsive and *Xist*-resistant cells, the nuclear protein SATB1 was identified as an initiation factor for XCI (Agrelo et al., 2009). SATB1 expression is developmentally regulated and coincides with XCI permissive time frames during ES cell and lymphocyte differentiation. Moreover, its ectopic expression is sufficient to enable *Xist* activity in mouse embryonic fibroblasts that are normally unresponsive to *Xist* induction (Agrelo et al., 2009). The role of

SATB1 in XCI initiation is currently unknown and data from SATB1 knockout mice, which have no defects in XCI, indicate that SATB1 is only one of several redundant factors regulating XCI initiation (Nechanitzky et al., 2012; Wutz and Agrelo, 2012). If *Airn* possesses a 'window of opportunity' like *Xist*, similar analyses could be performed to identify the cofactors *Airn* requires to establish *Igf2r* imprinted expression.

2.6.4. Is Airn required to maintain Igf2r silencing?

Airn expression is necessary to initiate *Igf2r* silencing – but is it also needed to maintain it? Once its expression is turned on during embryonic development, *Airn* is transcribed continuously where *Igf2r* shows imprinted expression. However, it is unknown if continuous lncRNA expression or additional repressive factors are required to maintain *Igf2r* silencing. The *Xist* lncRNA is also constantly present in the developing and adult mouse, but it is dispensable for maintaining the inactive X chromosome. Gene silencing along the X chromosome depends on *Xist* expression before 48 hours of ES cell differentiation. After this time, *Xist*-mediated silencing becomes irreversible and independent of continuous *Xist* expression (Wutz and Jaenisch, 2000), consistent with mouse experiments showing that *Xist* is not needed to maintain the inactive state in adult somatic cells (Csankovszki et al., 1999). Gene repression on the inactive X chromosome seems to be maintained by a synergistic combination of epigenetic mechanisms, which include DNA methylation, histone H4 hypoacetylation and chromosomal late replication (Csankovszki et al., 2001) (adapted from (Santoro et al., 2013) and (Santoro and Barlow, 2011)).

In order to fulfill its function and ensure faithful transmission of the silent state, a putative maintenance factor would have to be stably inherited through cell divisions and perpetuate itself in a lncRNA-independent fashion. The most obvious candidate for such a mark is DNA methylation, the only known heritable repressive epigenetic modification. At the *Igf2r* cluster, *Igf2r* is the only imprinted protein-coding gene whose promoter is methylated on the repressed paternal allele (Zwart et al., 2001b). This methylation mark, however, seems to play no active silencing role, as *Igf2r* can still be silenced in the absence of DNA methylation in early postimplantation embryos (Li et al., 1993; Seidl et al., 2006). Although dispensable for silencing initiation, the *Igf2r* somatic imprint could nevertheless play a maintenance role. Histone modifications have been suggested to play a more important role than DNA

methylation in maintaining imprinted gene silencing and allele-specific active and repressive histone marks have been identified in the *Igf2r* cluster (Regha et al., 2007). Epigenetic marks indicative of repressive heterochromatin, such as H3K9me3 and H3K27me3, play a role in the placenta but are not required to maintain embryonic imprinted expression, as discussed above. Moreover, it is currently unclear how or if histone modifications are propagated during cell division (Probst et al., 2009) (adapted from (Santoro and Barlow, 2011)).

2.7. ES cell models to study the developmental regulation of genomic imprinting

Mouse ES cells are pluripotent stem cells deriving from the inner cell mass of the preimplantation blastocyst (Evans and Kaufman, 1981; Martin, 1981). Under appropriate culture conditions, ES cells maintain the ability to self-renew and can be propagated indefinitely *in vitro* whilst retaining pluripotency. In the presence of appropriate stimuli however, they can be induced to differentiate into multiple cell types (Williams et al., 2012). As their differentiation mimics peri- and postimplantation stages of embryonic development, ES cells represent a cheaper and quicker tool, compared to mouse models, to study the molecular events occurring during early mouse development (Niwa, 2010). For example, ES cells and their *in vitro* differentiated counterparts are frequently used as a model system for X chromosome inactivation studies, as XCI *in vivo* occurs during embryo implantation (Navarro and Avner, 2010). ES cells are increasingly appreciated for genomic imprinting studies as well (Kohama et al., 2012; Latos et al., 2009).

It has been previously shown that ES cell differentiation recapitulates the onset of *Igf2r* imprinted expression and the gain of repressive epigenetic modifications at the silent *Igf2r* promoter observed during embryo development (Latos et al., 2009) (Fig. 7). In undifferentiated ES cells, as in the preimplantation mouse embryo, *Igf2r* is expressed at low levels from both alleles (Latos et al., 2009; Lerchner and Barlow, 1997). At this stage, no *Airn* lncRNA is detected. Its expression is first seen after embryo implantation and in differentiating ES cells, where the onset of its transcription determines the onset of imprinted *Igf2r* expression. As the *Slc22a2* and *Slc22a3* genes only show imprinted expression in extraembryonic lineages, they cannot be analyzed in differentiated ES cells and none of the tested *in vitro* models

of extraembryonic development have convincingly recapitulated extraembryonic specific imprinted expression (Hudson et al., 2010).

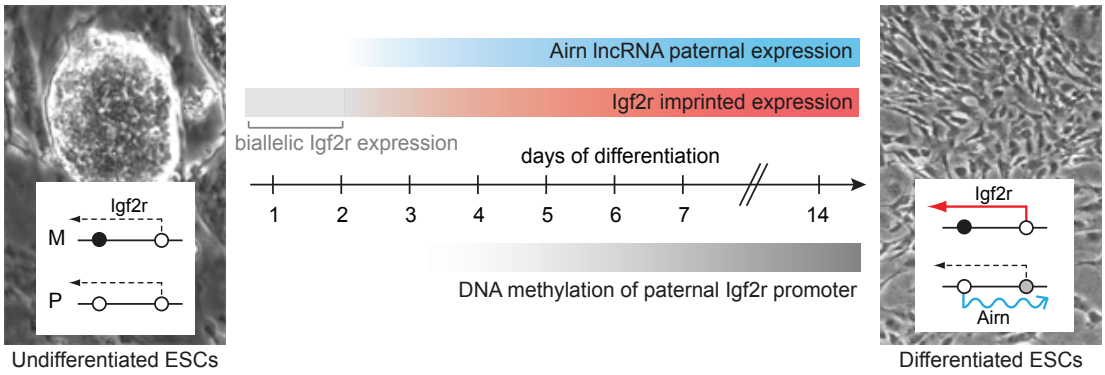

Fig. 7. Developmental regulation of *Igf2r* imprinted expression. Undifferentiated ES cells show low-level biallelic *Igf2r* expression (dashed arrow) and *Airn* is not expressed. Starting from day 2-3 of differentiation, expression of the maternal (M) *Igf2r* promoter is upregulated up to 20-fold (red bar/arrow). The *Airn* macro lncRNA is expressed from the paternal (P) chromosome with the same kinetics as maternal *Igf2r* upregulation (blue bar/wavy arrow). 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, but it gains DNA methylation (grey bar/circle) and low-level H3K9me3. White circle indicates an unmethylated CpG island. Figure taken and legend adapted from (Santoro et al., 2013).

2.8. Inducible gene expression systems

2.8.1. Tetracycline-inducible systems

Inducible systems allow spatio-temporal control over gene expression. The most popular inducible expression system in mammalian cells is the Tetracycline (Tet)-regulated one, of which two variants exist, TetOff and TetOn (Stieger et al., 2009). Both versions employ (1) a tetracycline-controlled transcriptional activator (tTA), under the control of either a ubiquitous or a cell-type specific promoter, and (2) a tetracycline-responsive promoter (TetP), located upstream of a gene of interest. In the TetOff variant, the target gene is on by default and is only shut off by treating with the inducer drug. In the absence of tetracycline or its derivative doxycycline, the tTA transactivator binds TetP and activates transcription of the downstream gene, whereas in the presence of doxycycline tTA dissociates from TetP, resulting in transcriptional repression (Gossen and Bujard, 1992). Point mutations were introduced in the tTA transactivator to generate the TetOn system, in which the rtTA (reverse tTA) only binds TetP and activates target gene expression in the presence of doxycycline (Gossen et al., 1995) (Fig. 8A). The TetOn system was optimized further by developing improved rtTA proteins, such as rtTA2^{S}-M2, which show higher

sensitivity to doxycycline and less background activity in its absence (Urlinger et al., 2000).

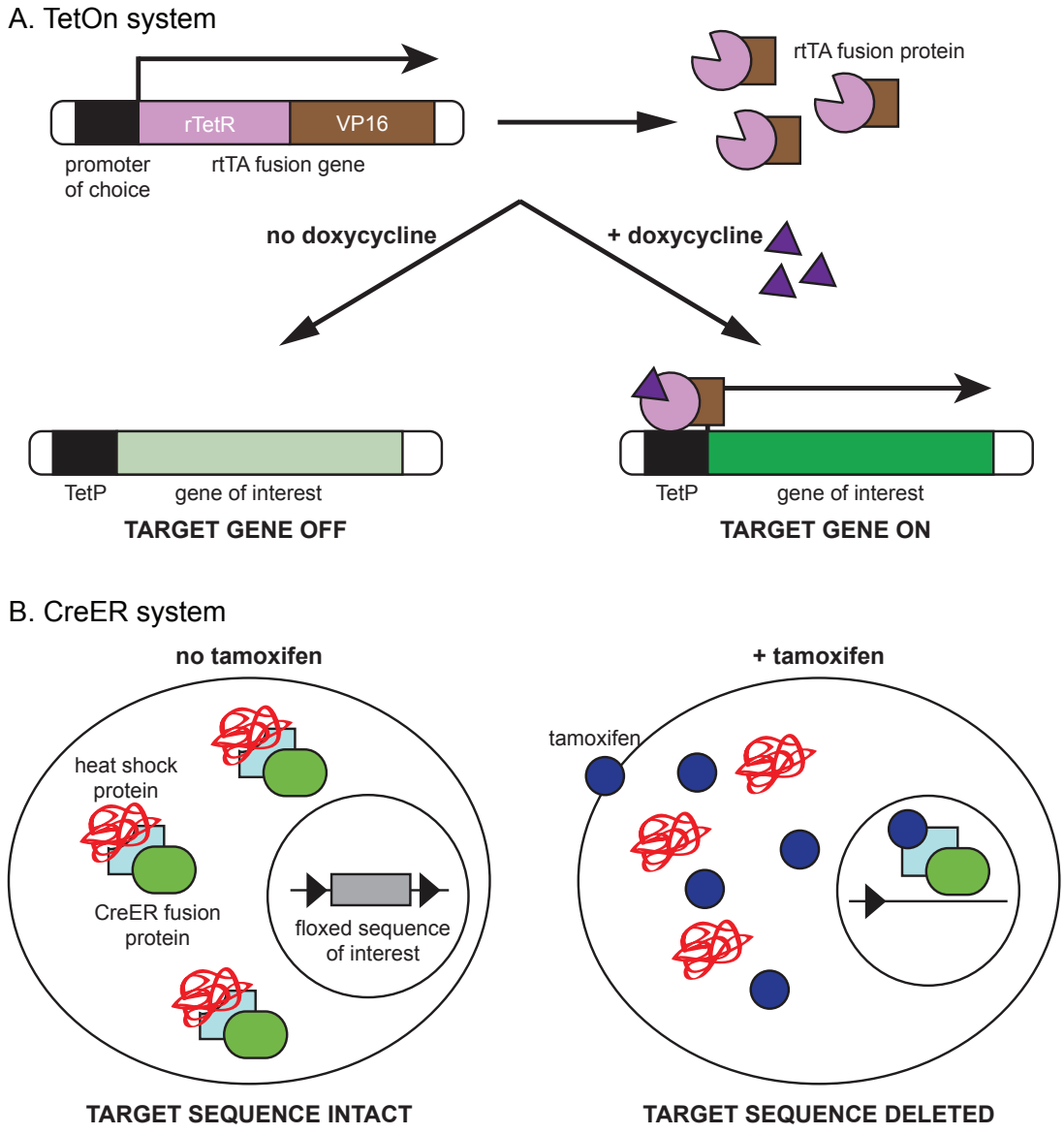

Fig. 8. Inducible systems to control gene expression. (**A**) In the TetOn system, the tetracycline-analog doxycycline is used to activate transcription of a gene of interest. The target gene is placed under the control of a tetracycline-responsive promoter (TetP) and the rtTA protein, consisting of the reverse tetracycline-dependent repressor (rTetR) fused to the VP16 transcriptional activator, is expressed from a promoter of choice. In the absence of doxycycline, rtTA cannot bind TetP and the target gene is transcriptionally silent. In the presence of doxycycline, rtTA binds TetP and activates transcription of the target gene. Transcriptional activation can be reversed by removing doxycycline. (**B**) In the CreER system, the gene or sequence of interest is 'floxed' (i.e. flanked by *loxP* sites, black triangles) and the CreER fusion protein, consisting of the Cre recombinase (green) fused to the ligand binding domain of an estrogen receptor (turquoise), is expressed from a promoter of choice. In the absence of tamoxifen, inhibitory heat shock proteins (red) bind the estrogen receptor domain and sequester CreER in the cytoplasm. The floxed target gene in the nucleus is therefore intact (left). In the presence of tamoxifen, CreER dissociates from the heat shock proteins and translocates to the nucleus, where it causes recombination between the *loxP* sites and excision of the intervening sequence (right).

2.8.2. Inducible recombination-based systems

Site-specific recombinase technology is a powerful tool to insert, delete or invert DNA sequences and has revolutionized mouse genetics ever since its introduction (Branda and Dymecki, 2004). Cre, FLP and the recently established Dre recombinases bind DNA at specific target sites (*loxP*, *FRT* and *rox*, respectively) and recombine the intervening sequences without the need for additional cofactors. The Cre-*loxP* system is the most widely used, owing to its remarkable recombination efficiency, whereas FLP continues to be less efficient than Cre, despite several optimization attempts (Buchholz et al., 1998; Raymond and Soriano, 2007). Dre recombinase was shown to be as efficient as Cre (Anastassiadis et al., 2009). However, due to its recent development, Dre has not replaced Cre as optimal site-specific recombinase for genome engineering.

Ligand-regulated versions of all three recombinases have been developed to allow temporal control of recombination activity. The most successful inducible forms of Cre and FLP involve fusion to a mutated estrogen receptor (ER) ligand-binding domain, which is insensitive to endogenous estrogens but highly responsive to the synthetic estrogen antagonist 4-hydroxytamoxifen (TAM) (Feil et al., 1996; Logie and Stewart, 1995). The recombinase-ER fusion proteins are sequestered in the cytoplasm in the absence of ligand, but are rapidly shuttled to the nucleus upon TAM treatment, where they mediate recombination of their target sites (Fig. 8B). By placing the sites at strategic positions inside the gene body or its regulatory regions, expression of a gene of interest can be conditionally altered. Inducible recombinases thus provide an alternative way to modulate gene expression in a temporal-specific fashion.

2.9. Aim of the study

In this work, I investigate developmental control of *Igf2r* silencing by using two inducible Cre-*loxP* systems to alter the timing of *Airn* expression during mouse ES cell differentiation. By conditionally switching *Airn* expression off, I find that eliminating *Airn* transcription in differentiated ES cells reverses *Igf2r* silencing, unless the paternal *Igf2r* promoter is methylated. This indicates that continuous *Airn* expression is required to maintain *Igf2r* silencing, but only in the absence of DNA methylation. I also show that the methylation mark on the silent *Igf2r* promoter is maintained independently of *Airn*, indicating no role for *Airn* in its propagation. By conditionally activating *Airn* function, I find that *Airn* can initiate *Igf2r* silencing in early and late differentiated ES cells, although with decreasing efficiency, indicating a 'window of opportunity' does not limit its repressive effects. Finally, I show that *Igf2r* repression is maintained in the absence of DNA methylation. Together, my results indicate that *Airn* acts alone to silence *Igf2r* and that the somatic methylation imprint on the paternal *Igf2r* promoter, while dispensable for silencing initiation and maintenance, may play a reinforcing role (adapted from (Santoro et al., 2013)).

N.B.: Work from this thesis contributed to one research paper for which I wrote the text and obtained data for all figures (Santoro et al., 2013), and to another research paper for which I obtained data for two figures (Stricker et al., 2008). During my PhD, I also published one review article for which I wrote the text and prepared figures (Santoro and Barlow, 2011), and a second review article for which I wrote the text only (Santoro and Pauler, 2013). I adapted these published texts and figures for use in this thesis and indicated this by the statement 'adapted from'. All four papers are included in the appendix.

3. RESULTS

The majority of the data presented here have been published in (Stricker et al., 2008) or (Santoro et al., 2013), as indicated in each figure legend. Daniela Mayer, a student who completed her diploma thesis in the lab under my supervision, performed some of the experiments described below. Data on Cre recombination, *Airn* expression, *Igf2r* expression, Southern blot analysis of *Igf2r* methylation and proliferation marker expression in the *Airn* CKO cell line are therefore included, in modified form, in her diploma thesis (Daniela Mayer, "Control of *Igf2r* imprinted silencing in embryonic stem cell differentiation", submitted to Universität Wien on February 15th, 2013, diploma awarded on March 22nd, 2013).

3.1. The tetracycline-inducible *Airn* allele

I originally attempted to control *Airn* transcription in differentiating ES cells by establishing a tetracycline-inducible *Airn* system (Fig. 9). Stefan Stricker had previously generated APD-Tet (*Airn* promoter deletion-tetracycline) cells, in which a tetracycline-responsive TetP promoter replaced the endogenous *Airn* promoter (Stricker et al., 2008). Together with Florian Pauler, I targeted the tetracycline-dependent transactivator rtTA2^S-M2 into the *ROSA26* (*R26*) locus in APD-Tet cells, thus generating APD-Tet-Rolo (*Airn* promoter deletion-tetracycline-Rosa locus) cells (Fig. 10). The ubiquitously expressed *R26* locus was chosen to ensure expression of the rtTA2^S-M2 transactivator throughout ES cell differentiation (Beard et al., 2006; Zambrowicz et al., 1997).

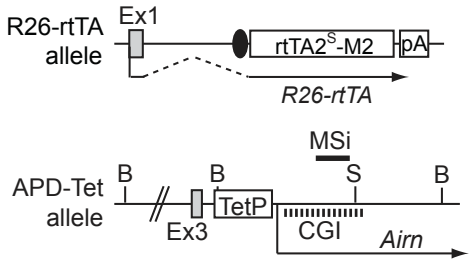

Fig. 9. The tetracycline-inducible *Airn* system. The tetracycline-dependent transactivator rtTA2^S-M2 was targeted into the ubiquitously expressed *ROSA26* (*R26*) locus (top) and the endogenous *Airn* promoter was replaced with a tetracycline-inducible promoter (TetP, bottom) (Stricker et al., 2008). The *Airn* downstream CpG island (CGI) is maintained in the APD-Tet (*Airn* promoter deletion-tetracycline) allele. Ex, exon; pA, polyadenylation signal; B, BamHI; S, SfuI; black oval, splice acceptor site; MSi, Southern blot probe. Adapted from (Santoro et al., 2013).

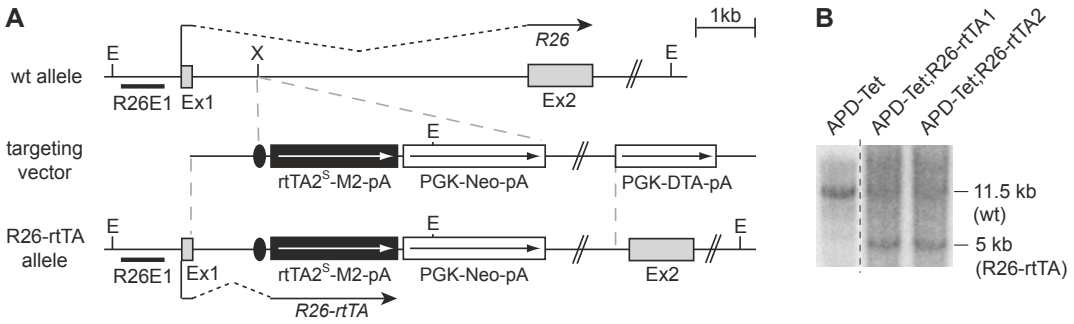

Fig. 10. Generation of the R26-rtTA allele. (**A**) Targeting strategy (details as in Fig. 9). Top: wild-type (wt) *ROSA26* (*R26*) allele on mouse chromosome 6. Below: targeting vector used to insert the rtTA2^S-M2 coding sequence (rtTA2^S-M2-pA) and a neomycin selection cassette (PGK-Neo-pA) into the XbaI (X) site in *R26* intron 1. A diphtheria toxin A gene (PGK-DTA-pA), located outside the homology arms, was used for negative selection. The R26-rtTA allele was generated by homologous recombination in APD-Tet ES cells, which carry a paternal APD-Tet allele. E, EcoRV; R26E1, Southern blot probe. (**B**) Southern blot genotyping for homologous recombination performed on two independently targeted clones (APD-Tet;R26-rtTA1,2) and their parental cell line (APD-Tet). DNA was digested with EcoRV and hybridized to probe R26E1. Dotted line, boundary between juxtaposed lanes from same gel. The 5 kb band from the R26-rtTA allele confirms correct homologous recombination. These targeted cells are referred to in the text as APD-Tet-Rolo (*Airn* promoter deletion-tetracycline-Rosa locus) (Stricker et al., 2008).

Differentiating APD-Tet-Rolo cells in the presence of the tetracycline-analogue doxycycline (Dox) induced *Airn* expression to wild-type levels (Fig. 11A). However, when Florian Pauler and I analyzed *Igf2r* transcription in these cells by RNA FISH, we observed only a partial gain of imprinted expression (Fig. 11B). Following its transcriptional silencing in differentiated ES cells, the paternal *Igf2r* promoter normally gains DNA methylation (Latos et al., 2009). In differentiated APD-Tet-Rolo cells however, we detected very little DNA methylation on the paternal *Igf2r* promoter, consistent with its incomplete silencing (Fig. 11C). I then examined the DNA methylation status of the *Airn* CpG island (CGI), which is located downstream of its promoter and is normally unmethylated on the paternal allele to allow *Airn* expression. Surprisingly, I detected abnormal DNA methylation on the paternal *Airn* CGI in APD-Tet-Rolo cells (Fig. 11D). Together, these data suggest that during differentiation only the subpopulation of cells carrying an unmethylated APD-Tet allele are able to induce *Airn* and gain *Igf2r* imprinted expression, thus accounting for the incomplete *Igf2r* silencing observed in the entire APD-Tet-Rolo population.

In an effort to rescue the system, I then isolated unmethylated cells by subcloning the APD-Tet-Rolo cell line, screening clones for *Airn* CGI methylation and selecting those with an unmethylated APD-Tet-Rolo allele (subclone 2, Fig. 12A). However, when I cultured the selected subclone in the presence of doxycycline, I again observed a strong gain of DNA methylation on the *Airn* CGI (Fig. 12B).

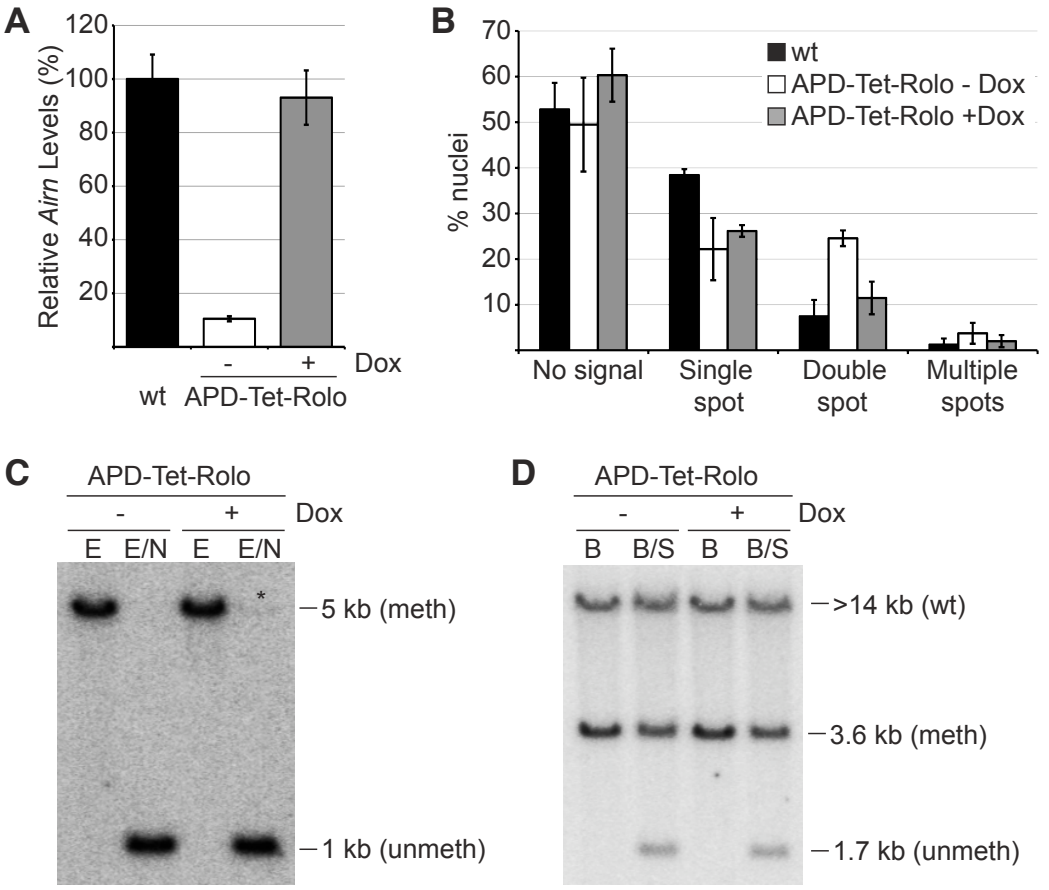

Fig. 11. The Tet-*Airn* allele gains DNA methylation and silences *Igf2r* incompletely. All data shown here, with the exception of panel C, have been published in (Stricker et al., 2008). (**A**) RT-qPCR with Airn-middle primers shows that *Airn* expression is induced to wt levels in day 5-differentiated APD-Tet-Rolo cells treated with doxycycline (Dox). Relative *Airn* levels were set to 100 in wt cells. Data are mean±s.d. of three technical replicates. Adapted from (Stricker et al., 2008) using data prepared by myself. (**B**) Quantification of *Igf2r* allelic transcription by RNA FISH using intronic probe FP1 in day 5-differentiated APD-Tet-Rolo cells. Numbers of counted nuclei are plotted against number of *Igf2r* transcription signals. No signal: cells with no visible RNA FISH spots. Single spots indicate monoallelic or stochastic biallelic expression. Double spots indicate biallelic expression. Multiple spots indicate unspecific signals. Wt cells show more single than double spots, as expected for *Igf2r* imprinted expression. Control non-induced APD-Tet-Rolo cells (-Dox) show an increase in cells with double spots (24%), a pattern typical of *Igf2r* biallelic expression in the absence of *Airn*. Induction of *Airn* expression (+Dox) decreases the number of double spots (12%) but not to wt levels (7%), suggesting incomplete *Igf2r* silencing. Data are mean of three independent counts, two performed blind. The large number of nuclei lacking a signal results from intronic RNA FISH probes underestimating the number of expressing alleles due to discontinuous transcription of active genes (Osborne et al., 2004). Figure adapted from (Stricker et al., 2008) using data prepared by myself, legend adapted from (Santoro et al., 2013). (**C**) *Igf2r* promoter methylation assayed by Southern blot analysis of a diagnostic methyl-sensitive NotI site, containing two CpG dinucleotides, in day 14-differentiated APD-Tet-Rolo cells. DNA was digested with EcoRI (E) or EcoRI + NotI (E/N) and hybridized to probe NEi. meth, methylated; unmeth, unmethylated. Very little *Igf2r* methylation is gained in Dox-treated APD-Tet-Rolo cells that express *Airn* (*). Legend adapted from (Santoro et al., 2013). (**D**) *Airn* promoter CGI methylation assayed by Southern blot analysis of a diagnostic methyl-sensitive MluI site, containing one CpG dinucleotide, in day 14-differentiated APD-Tet-Rolo cells. DNA was digested with BamHI (B) or BamHI + SfuI (B/S) and hybridized to probe MSi. Maternal and paternal alleles can be distinguished due to the presence of an additional BamHI site in the TetP sequence (see Fig. 9). As a result, both methylated 3.6 kb and unmethylated 1.7 kb fragments derive from the paternal allele. The APD-Tet-Rolo allele gains abnormal DNA methylation on the *Airn* CGI, both in the presence or absence of Dox. Adapted from (Stricker et al., 2008) using data prepared by myself.

Upon differentiation in the presence of Dox, these cells induced *Airn* to wild-type levels (Fig. 12C). Nevertheless, they showed a very mild gain of *Igf2r* imprinted expression compared to wild-type cells, as shown by the high proportion of cells expressing biallelic *Igf2r* (Fig. 12D) and by the low level of DNA methylation on the paternal *Igf2r* promoter (Fig. 12E). In conclusion, the tetracycline-inducible *Airn* allele did not allow efficient manipulation of *Airn* expression in differentiating ES cells and was not suitable for further experiments.

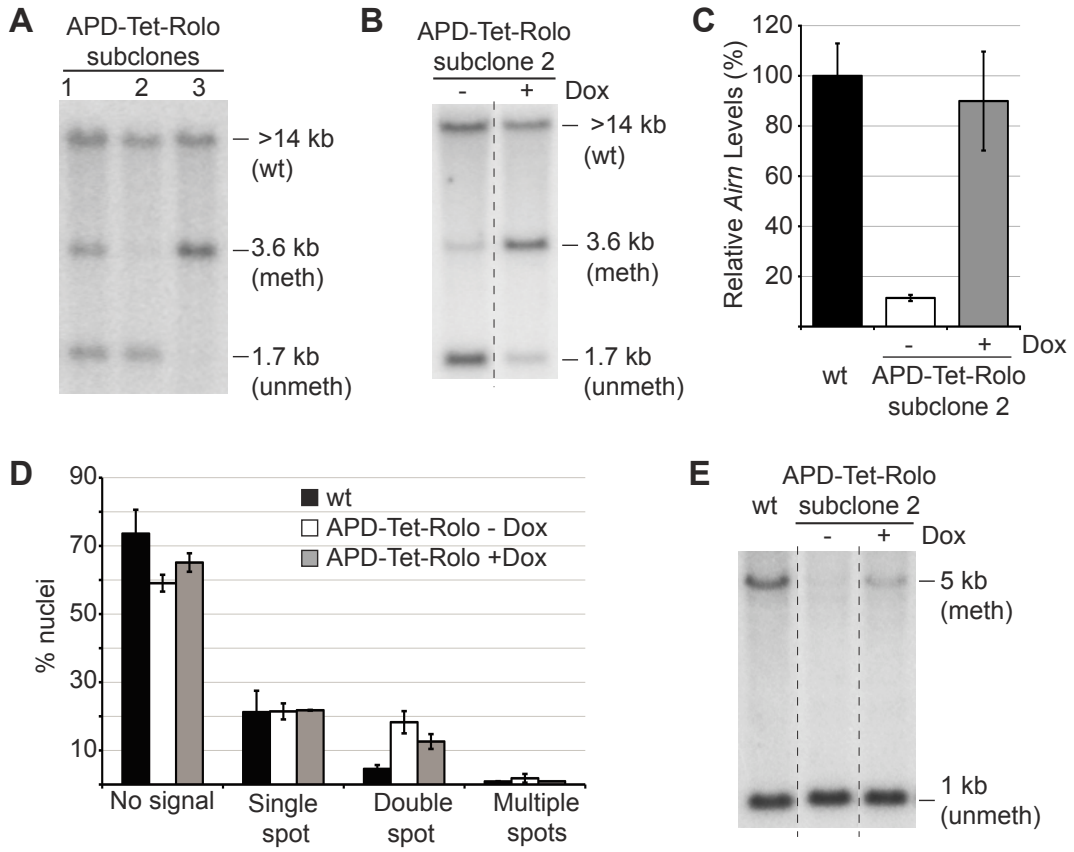

Fig. 12. Subcloning unmethylated Tet-*Airn* clones does not rescue the inducible system. (**A-B**) Southern blot analysis of *Airn* CGI methylation, as in Fig. 11D, in three APD-Tet-Rolo subclones obtained from low-density plating of APD-Tet-Rolo ES cells. (A) The APD-Tet-Rolo allele gains abnormal DNA methylation on the *Airn* CGI (see Fig. 11D), which prevents full activation of Tet-inducible *Airn*. To rescue the system, cells were subcloned and screened for lack of CGI methylation (absence of the 3.6 kb band). 1, 2, 3: representative subclones with partial, no or complete methylation, respectively. (B) Subclone 2 cultured with doxycycline (+Dox) gains more DNA methylation compared with the untreated control (-Dox). Dotted line as in Fig. 10B. Figures taken and legends adapted from (Santoro et al., 2013). (**C**) RT-qPCR as in Fig. 11A shows that *Airn* expression is induced to wt levels in day 5-differentiated APD-Tet-Rolo subclone 2 treated with doxycycline (Dox). Relative *Airn* levels were set to 100 in wt cells. Data are mean±s.d. of three technical replicates. (**D**) Quantification of *Igf2r* allelic transcription as in Fig. 11B in day 5-differentiated APD-Tet-Rolo subclone 2. Induction of *Airn* expression (+Dox) decreases the number of double spots slightly, suggesting incomplete gain of *Igf2r* imprinted expression. Data are mean of two independent counts, one performed blind. Figure taken and legend adapted from (Santoro et al., 2013). (**E**) *Igf2r* promoter methylation assayed as in Fig. 11C in day 14-differentiated APD-Tet-Rolo subclone 2. Dotted lines as in Fig. 10B. Low levels of *Igf2r* methylation are gained in Dox-treated cells that express *Airn*.

3.2. Two inducible Cre-*loxP* systems to control *Airn* lncRNA expression

I developed an alternative genetic system to control *Airn* expression during ES cell differentiation using a D3 ES cell 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. 7). To ensure expression throughout ES cell differentiation, I inserted the CreER^{T2} gene into the *R26* locus (Fig. 13, top; Fig. 14A-C). I verified CreER expression at mRNA and protein levels (Fig. 14D,E) and designated the cells 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 to parental S12/+ cells are referred to as wild type. Using S12^{RC}/+ ES cells, I then modified the *Airn* locus to generate *Airn* promoter conditional knockout (CKO) and *Airn* expression conditional rescue (CRes) cell lines (Fig. 13) (adapted from (Santoro et al., 2013)).

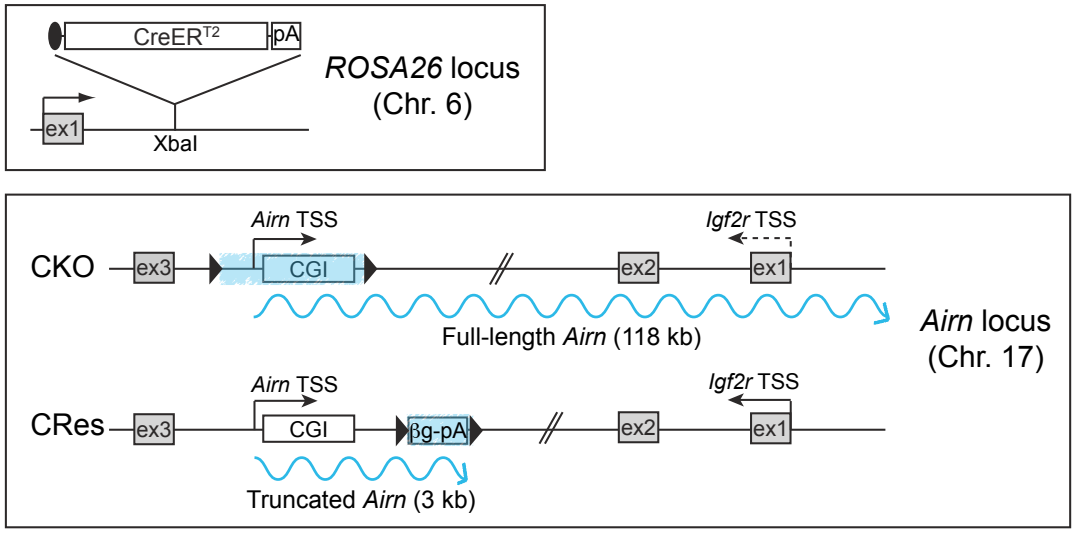

Fig. 13. Two inducible Cre-*loxP* systems to control *Airn* lncRNA expression. Top: a tamoxifen-inducible Cre recombinase gene (CreER^{T2}) was targeted into the ROSA26 locus in S12/+ ES cells that carry a SNP to distinguish maternal and paternal *Igf2r* expression. Below: inducible Cre-*loxP* strategies. 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). Cre recombination during ES cell 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 ES cell differentiation removes the polyA signal, rescuing full-length functional *Airn* transcription. Adapted from (Santoro et al., 2013).

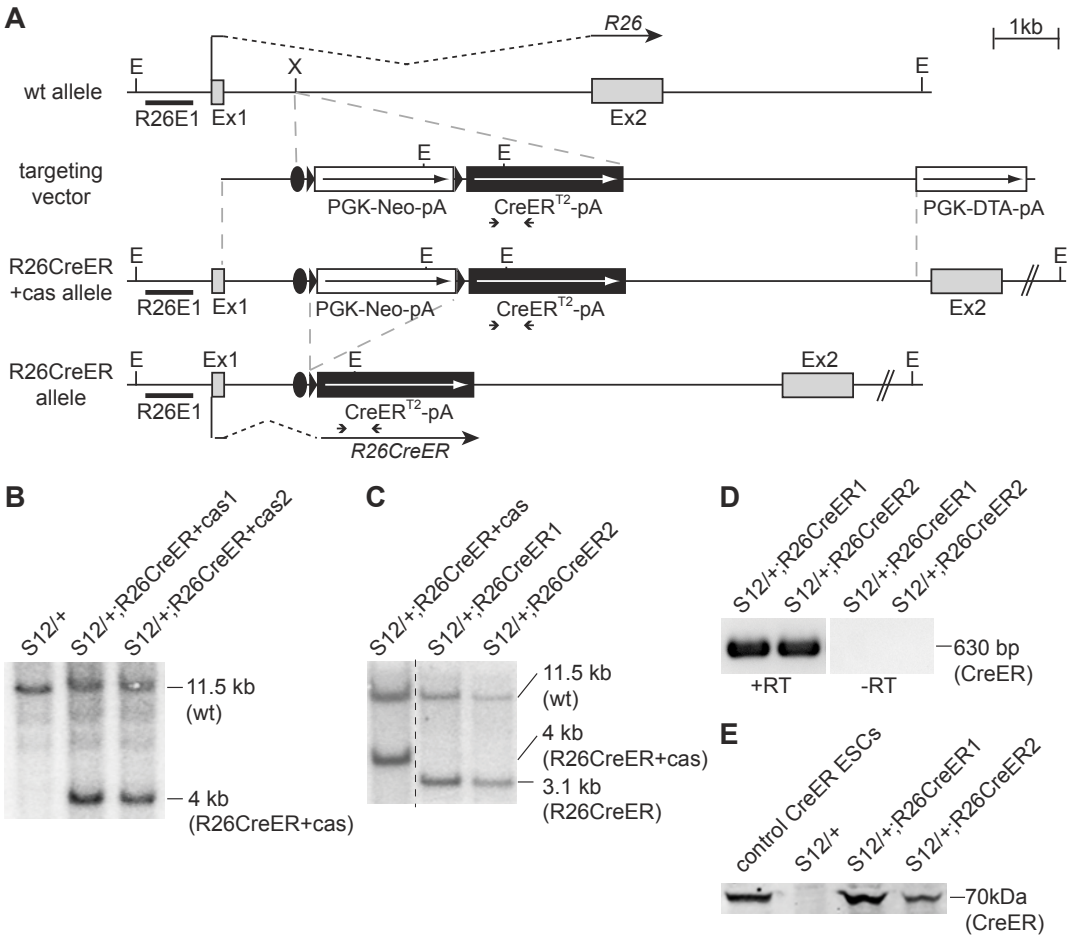

Fig. 14. Generation of R26CreER ES cells. (**A**) Targeting strategy, details as in Fig. 10A. Top: wt *R26* allele. Below: targeting vector used to insert the CreER^{T2} coding sequence (CreER^{T2}-pA) and a 'floxed' neomycin selection cassette (PGK-Neo-pA) into the XbaI (X) site in *R26* intron 1. The R26CreER+cas allele was generated by homologous recombination in S12/+ ES cells. Transient transfection of Cre recombinase was used to delete the neomycin resistance gene and obtain the R26CreER allele. Arrowheads, RT-PCR primers used in D; E, EcoRV. Figure taken and legend adapted from (Santoro et al., 2013). (**B**) Southern blot genotyping for homologous recombination as in Fig. 10B, performed on two independently targeted clones (S12/+;R26CreER+cas1,2) and their parental cell line (S12/+). The 4 kb band from the R26CreER+cas allele confirms correct homologous recombination. Figure taken and legend adapted from (Santoro et al., 2013). (**C**) Southern blot genotyping for selection cassette removal. DNA from targeted clones before (S12/+;R26CreER+cas) and after Cre recombination (S12/+;R26CreER1,2) was assayed as in Fig. 10B. Loss of the 4 kb band and gain of the 3.1 kb band confirms selection cassette removal. These targeted cells are referred to in the text as S12^{RC}/+ and show wt *Igf2r* and *Airn* expression. Dotted line as in Fig. 10B. Figure taken and legend adapted from (Santoro et al., 2013). (**D**) RT-PCR to detect CreER mRNA expression in two targeted clones using Cre primers shown in A. +/-RT, plus/minus reverse transcriptase. Both clones express the CreER mRNA. Figure taken and legend adapted from (Santoro et al., 2013). (**E**) Western blot analysis with a Cre-specific antibody to detect CreER protein in whole-cell lysates from two targeted clones and control ES cells. The 70kDa CreER fusion protein is detected in control ES cells that express a CreER transgene (lane1) and in both R26CreER clones (lanes 3-4), but not in the parental cell line that carries a wt *R26* allele (lane 2). Figure taken and legend adapted from (Santoro et al., 2013).

3.3. Turning *Airn* off: *Airn* conditional knockout (CKO) cells

3.3.1. Generating and testing Airn CKO ES cells

I generated *Airn* CKO ES cells by introducing *loxP* sites flanking a 1.9 kb region that contains the *Airn* promoter and CGI (Fig. 15A). As 5' boundary, I selected a PacI site 580 bp upstream of the *Airn* TSS and 385 bp from *Igf2r* exon 3. The same PacI site had been previously used for the 5' boundary of the 4 kb R2Δ deletion, which genetically identified the *Igf2r* cluster ICE (Wutz et al., 1997; Wutz et al., 2001). As 3' boundary, I chose an NsiI site 1.3 kb downstream of the *Airn* TSS and outside the CGI. Two independent clones ($S12^{RC}/CKO^{FI}$+cas1,2; Fig. 15B) were targeted on the paternal allele that carries the unmethylated ICE and expresses the *Airn* lncRNA (Fig. 15C). Preferential targeting of the paternal allele in the region between the *Airn* and *Igf2r* promoters has been reported previously (Koerner et al., 2012; Latos et al., 2012; Stricker et al., 2008). Interestingly, a targeting vector containing the selection cassette in opposite orientation, but still upstream of the *Airn* promoter, generated no homologously targeted clones in 1127 picked clones (Table 1). Selection cassette removal generated clones $S12^{RC}/CKO^{FI}$1,2 (Fig. 15D). CKO^{FI} cells were TAM treated to delete the *loxP*-flanked *Airn* promoter, thus generating the CKO^{Δ} allele (Fig. 16A). I tested CreER-mediated excision efficiency in undifferentiated ES cells (Fig. 16B). Independent of TAM dose, >80% of CKO^{FI} alleles undergo recombination by 24 hours, with complete excision by 48 hours (Fig. 16C). Moreover, CKO cells differentiate normally, both in the presence and absence of a functional *Airn* promoter, as shown by downregulation of pluripotency markers and upregulation of differentiation markers (Fig. 17) (adapted from (Santoro et al., 2013)).

Table 1. Effect of selection cassette orientation on targeting efficiency. Adapted from (Santoro et al., 2013).

Selection cassette orientation relative to *Airn* promoter	Number of screened clones	Number of homologously targeted clones
Same (as in Fig. 15A)	152	9
Opposite (not shown)	1127	0

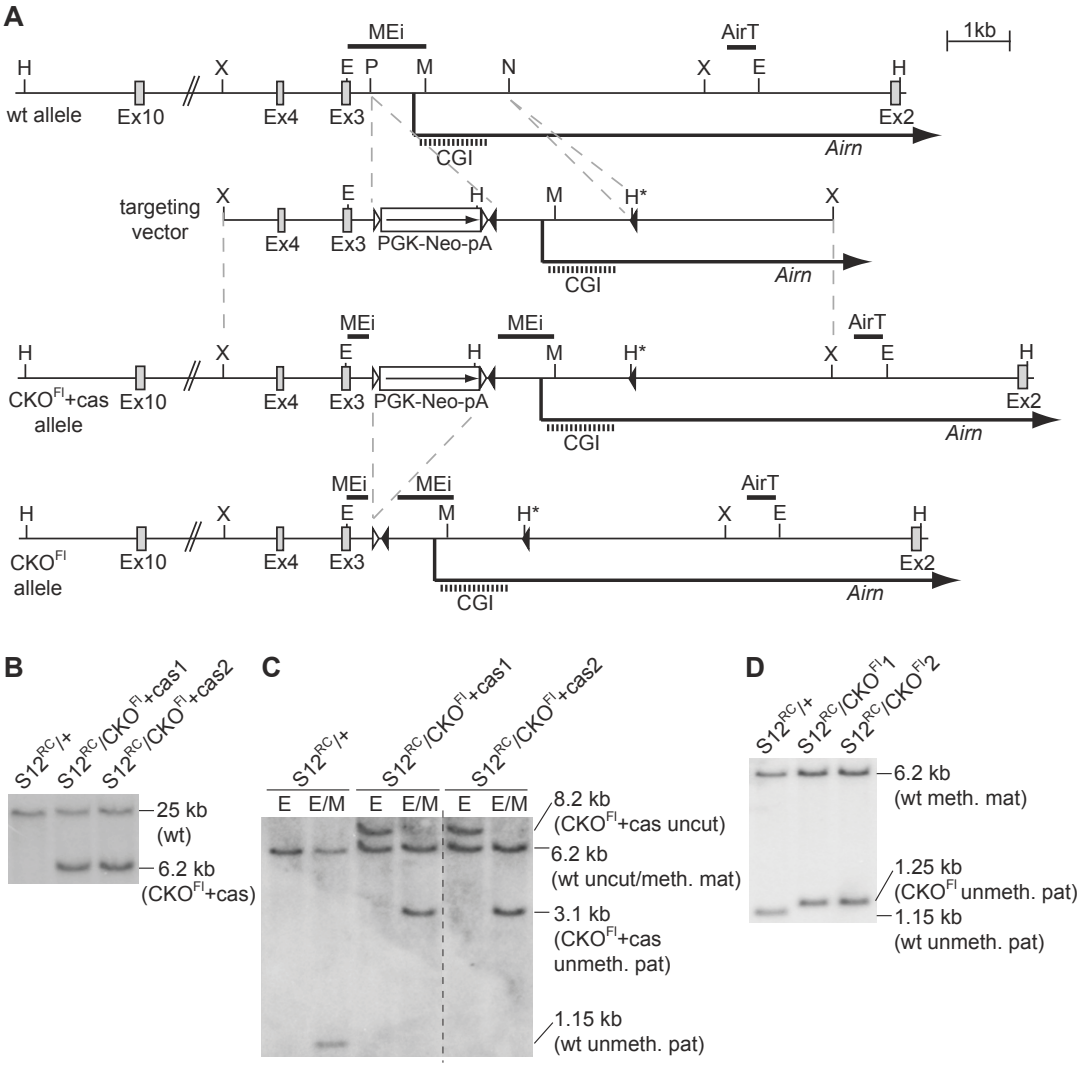

Fig. 15. Generation of *Airn* promoter conditional knockout (CKO) ES cells. (**A**) Top: wt allele showing *Airn* transcript overlapping *Igf2r* intron 2. Below: construct used to insert *loxP* sites (black triangles) flanking 1.9 kb containing the *Airn* promoter CGI (dashed bar). A selection cassette (PGK-Neo-pA) flanked by *FRT* sites (white triangles) with one *loxP* site was inserted into a PacI (P) site (chr17:12,740,792, UCSC build GRCm38/mm10). A second *loxP* site with a diagnostic HindIII site (H*) was inserted into an NsiI (N) site (chr17:12,742,677). Homologous recombination in $S12^{RC}$/+ ES cells generated the CKO^{Fl}+cas allele. Transient transfection of FLP recombinase deleted the selection cassette to generate the CKO^{Fl} allele. Fl, 'floxed'; Ex, *Igf2r* exons; MEi, AirT, Southern blot probes; E, EcoRI; H, HindIII; M, MluI; X, XbaI. Figure taken and legend adapted from (Santoro et al., 2013). (**B**) Southern blot genotyping of independently targeted clones ($S12^{RC}$/CKO^{Fl}+cas1,2). The $S12^{RC}$/+ parental cell line contains a PstI SNP in *Igf2r* exon 12 (Latos et al., 2012) and R26CreER (Fig. 14). Probe AirT hybridized to HindIII-digested DNA identifies a 6.2 kb correctly targeted band in CKO^{Fl}+cas. Figure taken and legend adapted from (Santoro et al., 2013). (**C**) Southern blot to identify parental origin of the targeted alleles. Samples from B were digested with EcoRI or EcoRI + MluI (E/M) and hybridized to probe MEi. Loss of a 1.15 kb and gain of a 3.1 kb band in CKO^{Fl}+cas cells shows targeting of the paternal allele, which contains an unmethylated MluI site (Stoger et al., 1993). Dotted line as in Fig. 10B. Figure taken and legend adapted from (Santoro et al., 2013). (**D**) Southern blot genotyping for selection cassette removal. DNA from parental $S12^{RC}$/+ cells and $S12^{RC}$/CKO^{Fl}1,2 targeted clones after FLP recombination was digested with EcoRI + MluI and hybridized to probe MEi. Loss of the 3.1 kb (seen in C) and gain of a 1.25 kb band confirms selection cassette removal. Figure taken and legend adapted from (Santoro et al., 2013).

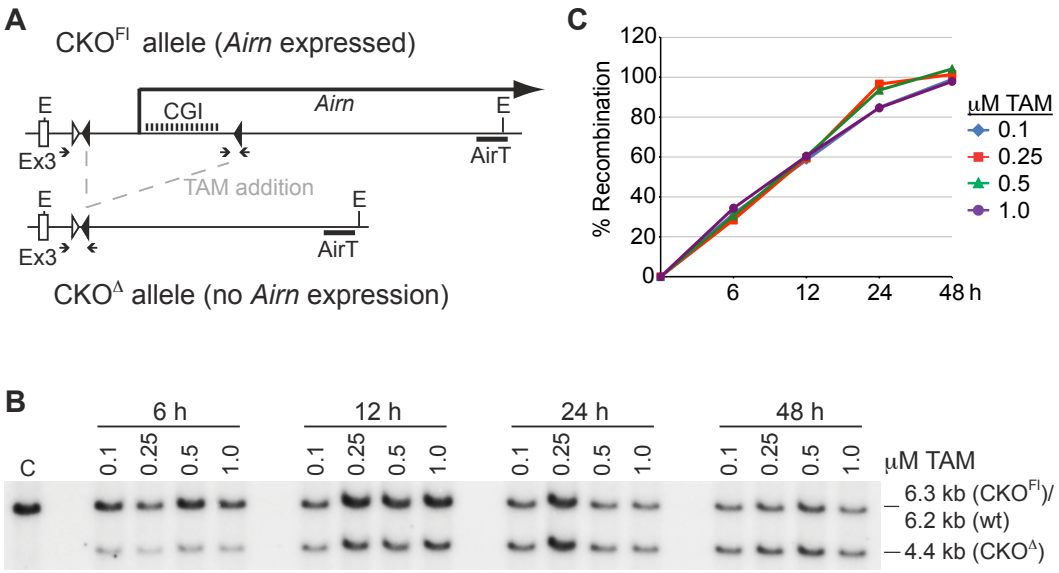

Fig. 16. Recombination efficiency and kinetics in CKO ES cells. (**A**) Southern blot strategy, details as in Fig. 15A. Arrowheads indicate qPCR primers used in Fig. 19B. Figure taken and legend adapted from (Santoro et al., 2013). (**B**) Southern blot analysis showing Cre-mediated excision efficiency and kinetics of the CKO^{Fl} allele in response to increasing concentrations of 4-hydroxytamoxifen (TAM). DNA from undifferentiated CKO ES cells, treated with the indicated amounts of TAM for the indicated number of hours, was digested with EcoRI and hybridized to probe AirT. Top band: wt 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$^{\Delta}$ 4.4 kb). Control (C) cells were treated with vehicle only and harvested after 48 hours. Figure taken and legend adapted from (Santoro et al., 2013). (**C**) ImageQuant quantification of recombination efficiency. Independently of the amount of TAM used, complete recombination of the CKO^{Fl} allele is achieved between 24 and 48 hours in undifferentiated CKO ES cells. Figure taken and legend adapted from (Santoro et al., 2013).

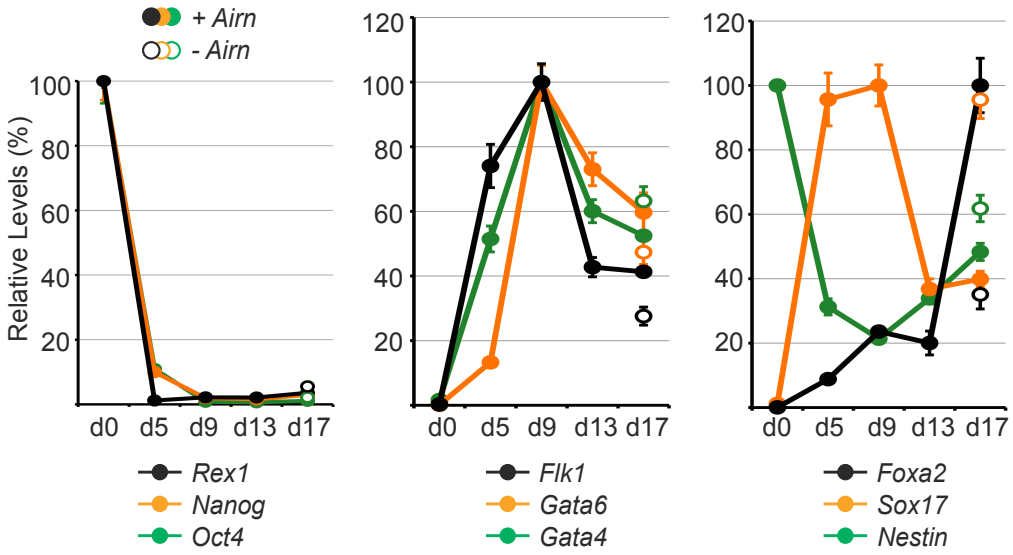

Fig. 17. CKO cells differentiate normally. RT-qPCR with primers specific for pluripotency (*Rex1*, *Nanog*, *Oct4*), meso-endodermal differentiation (*Flk1*, *Gata6*, *Gata4*), definitive endoderm differentiation (*Foxa2*, *Sox17*) and neuro-ectodermal differentiation (*Nestin*) markers. Marker expression was assayed in embryoid body differentiated CKO cells, in the presence (+*Airn*) or in the absence (-*Airn*) of a functional *Airn* promoter. Differentiated CKO cells show downregulation of pluripotency markers and upregulation of differentiation markers, with little or no difference between +*Airn* and –*Airn* samples in the majority of cases. In each series, the highest value was set to 100. Data are mean±s.d. of three technical replicates. Figure taken and legend adapted from (Santoro et al., 2013).

3.3.2. The Airn promoter is deleted efficiently throughout EB, but not RA, differentiation

Imprinted *Igf2r* expression arises between days 2 and 3 of ES cell differentiation (Fig. 7). To test whether *Airn* expression is needed to maintain *Igf2r* silencing after it is initiated, I differentiated CKO ES cells using retinoic acid (RA), then I deleted the *Airn* promoter at day 5, 9 or 13 by TAM addition, and I harvested cells 4 days later (Fig. 18). *Airn* has a half-life of less than 2 hours and transcripts are absent ~10 hours after promoter deletion (Seidl et al., 2006).

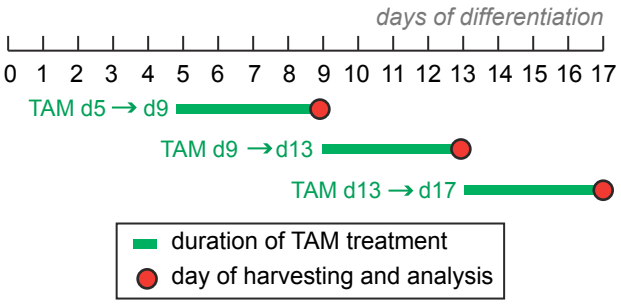

Fig. 18. Experimental strategy to turn *Airn* off during ES cell differentiation. Taken from (Santoro et al., 2013).

I first quantified Cre-mediated excision of the CKO^{Fl} allele by Southern blot (Fig. 19A, left). In contrast to undifferentiated ES cells (Fig. 16), the *Airn* promoter showed 88-91% recombination at day 5 of differentiation, which was reduced to 51-72% by day 9 or day 13 (Fig. 19A, left). I then developed a qPCR assay that uses forward primers specific for either the unrecombined CKO^{Fl} allele or the recombined CKO^{Δ} allele, in combination with a common reverse primer (Fig. 16A). qPCR quantification shows 83% recombination at day 5 and 59-63% at day 9 or day 13 (Fig. 19B, left). To test whether Cre recombination improves in a different lineage, I performed the same experiment on CKO^{Fl} cells differentiated by embryoid body (EB) formation. As shown by Southern blot (Fig. 19A, right) and qPCR quantification (Fig. 19B, right), the *Airn* promoter is deleted more efficiently in EB differentiated ES cells, with only 19-26% residual unrecombined alleles (adapted from (Santoro et al., 2013)).

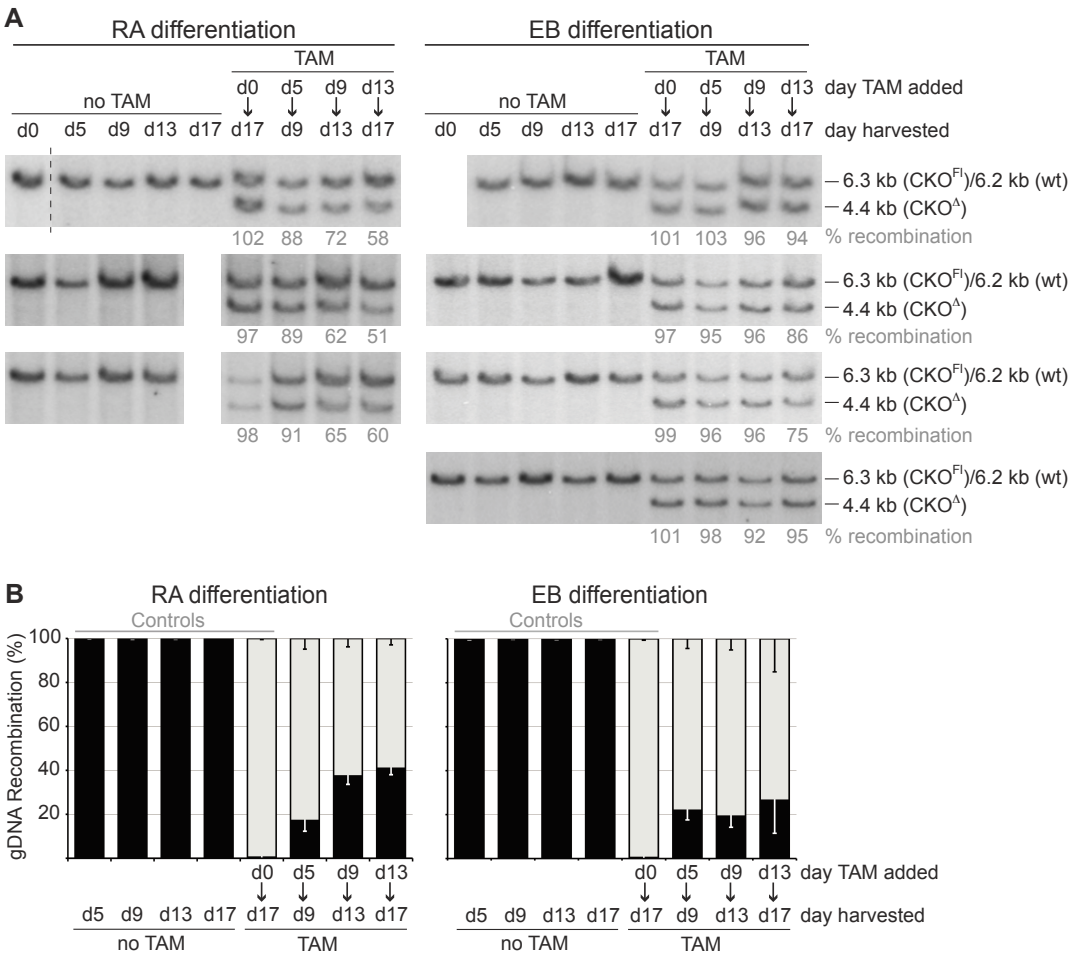

Fig. 19. The *Airn* promoter is deleted efficiently throughout EB, but not RA, differentiation. (**A**) Southern blot analysis of Cre recombination, as in Fig. 16B, in retinoic acid (RA) or embryoid body (EB) differentiated CKO cells shows high recombination efficiency in EB differentiated cells and in early, but not late, RA differentiated cells. Lanes 1-5, control no TAM; lane 6, TAM treatment prior to differentiation; lanes 7-9, TAM added during differentiation. The percentage recombination {4.4 kb band/[(6.2 + 4.4 kb band)/2]} is shown underneath. Data from three RA and four EB replicates are shown. Dotted line as in Fig. 10B. Adapted from (Santoro et al., 2013). (**B**) Quantification of recombined (grey) and unrecombined (black) alleles using samples in A amplified with allele-specific primers (Fig. 16A). Combined recombined and unrecombined levels were set to 100. Bars show the percentage occupied by each allele as mean±s.d. of three or four biological replicates for RA and EB differentiated cells, respectively. Figure taken and legend adapted from (Santoro et al., 2013).

3.3.3. Airn expression is suppressed upon promoter deletion

I assessed the effect of the conditional promoter deletion on *Airn* steady-state levels by RT-qPCR (Fig. 20). As expected, *Airn* is upregulated in differentiated CKO cells carrying an intact promoter (Fig. 20, 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. 20, 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. 20, right, bars 7-9). Higher residual levels of *Airn*, seen when

the deletion is induced during late RA differentiation (Fig. 20, left, bars 8-9), are explained by inefficient recombination of the CKO^{FI} allele. The data show that promoter deletion during ES cell differentiation can eliminate *Airn* expression (adapted from (Santoro et al., 2013)).

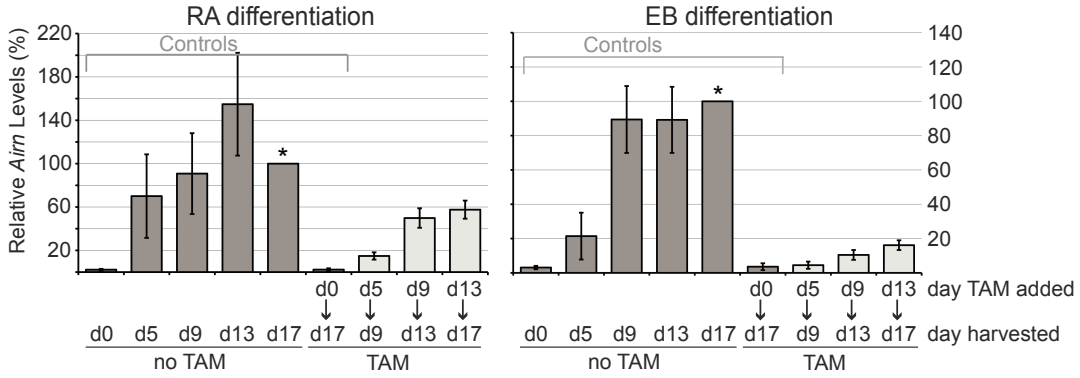

Fig. 20. *Airn* expression is suppressed upon promoter deletion. RT-qPCR with Airn-middle primers. Relative *Airn* levels were set to 100 in untreated day 17 cells (*) that retain the *Airn* promoter. Data are mean±s.d. of three or four biological replicates for RA and EB differentiated cells, respectively. Dark bars, control samples; pale bars, *Airn* promoter deletion induced during differentiation. Figure taken and legend adapted from (Santoro et al., 2013).

3.3.4. Igf2r silencing requires continuous Airn expression

To determine the effect of *Airn* removal after *Igf2r* silencing is initiated, I examined *Igf2r* imprinted expression in differentiated CKO cells. I first assayed allele-specific *Igf2r* expression non-quantitatively using the maternal-specific SNP in exon 12 that destroys a PstI site (Fig. 21A). PstI digestion of amplified cDNA from undifferentiated ES cells, which express *Igf2r* biallelically, yields an undigested maternal band and two paternal PstI-cut fragments (Fig. 21A, sample 1). Reduced paternal *Igf2r* fragments relative to the maternal fragment in differentiated cells that express *Airn* indicate maternal-specific *Igf2r* upregulation (Fig. 21A, samples 2-5). When the *Airn* promoter is deleted from CKO cells at day 0, *Igf2r* expression remains biallelic with visible paternal-specific bands throughout differentiation (Fig. 21A, sample 6), 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, I 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. 21A, samples 7-9), indicating that *Igf2r*

silencing is not maintained in the absence of *Airn* (adapted from (Santoro et al., 2013)).

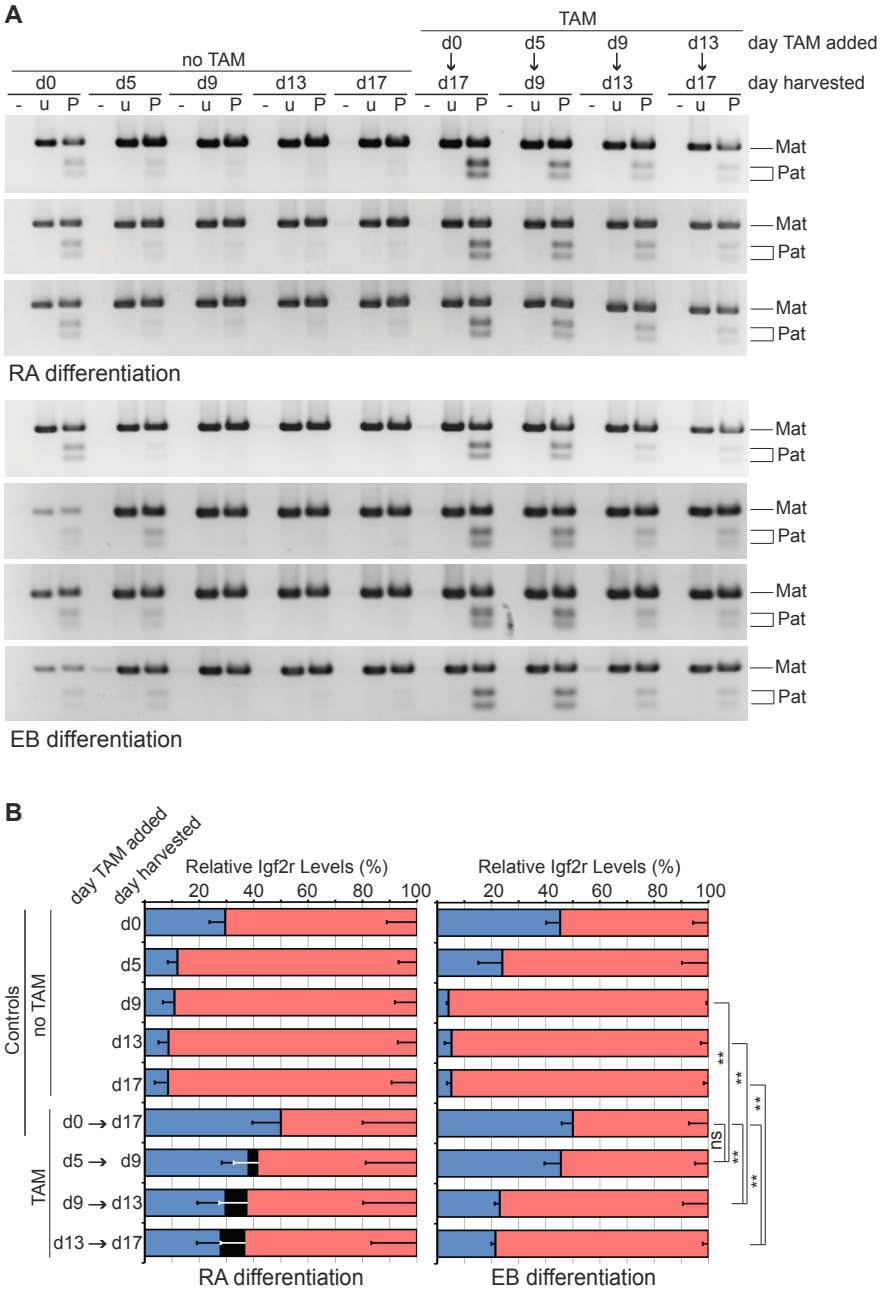

Fig. 21. *Igf2r* silencing requires continuous *Airn* expression. (**A**) Allele-specific *Igf2r* expression in RA or EB differentiated CKO cells, assayed by RT-PCR + PstI digest of a paternal-specific restriction site. Maternal *Igf2r* expression yields a 541 bp product, paternal expression yields two 318+223 bp products. Data from three RA and four EB replicates are shown. -, minus RT; u, undigested; P, PstI digested; Mat, maternal; Pat, paternal. Adapted from (Santoro et al., 2013). (**B**) Allele-specific RT-qPCR as in A. Maternal (red) and paternal (blue) *Igf2r* levels are displayed as percentage of total *Igf2r* expression with mean±s.d. of three biological replicates for RA differentiated cells and four biological replicates for EB differentiated cells. 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. 19B) 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 seen in day 0 untreated cells, which retain the *Airn* promoter, most likely arises from a low degree of spontaneous differentiation, leading to a small amount of paternal *Igf2r* silencing by *Airn* expression. Figure taken and legend adapted from (Santoro et al., 2013).

Next, I 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) (Fig. 21B). Control differentiated cells that lack the *Airn* promoter and express *Igf2r* biallelically were used to set the maternal:paternal ratio to 50:50 (Fig. 21B, 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. 21B, bars 2-5). Confirming results from Fig. 21A, the qPCR assay shows that 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. 21B, left, bar 7, blue bar), but is reduced to ~30% when *Airn* is removed at day 9 or day 13 (Fig. 21B, 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. 21B, 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. 21B, 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. 21B, 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 (adapted from (Santoro et al., 2013)).

3.3.5. Igf2r methylation is maintained in the absence of Airn

Igf2r silencing by *Airn* during embryonic development and ES cell differentiation is marked by a late gain of DNA methylation on the paternal *Igf2r* promoter CGI (Latos et al., 2009; Stoger 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. I tested *Igf2r* promoter methylation in differentiated CKO cells by Southern blot analysis of a methyl-sensitive NotI site diagnostic of the methylation status of the *Igf2r* CGI (Stoger et al., 1993) (Fig. 22).

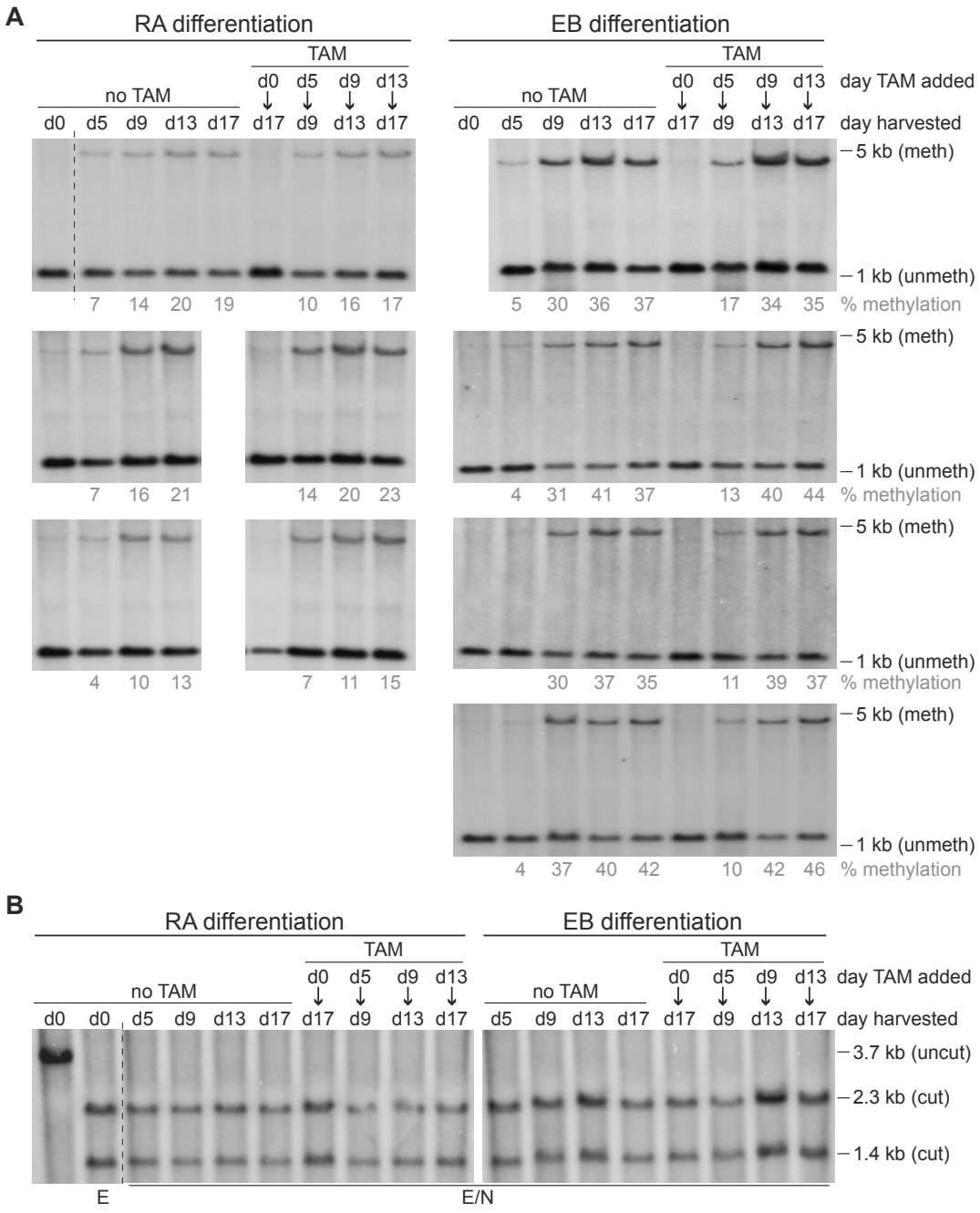

Fig. 22. *Igf2r* methylation is maintained in the absence of *Airn*. (**A**) *Igf2r* promoter methylation assayed as in Fig. 11C in CKO cells differentiated with RA or EB formation. Data from three RA and four EB replicates are shown. Paternal *Igf2r* methylation [% methylated/(methylated+unmethylated)] is shown below each blot. Maximum methylation levels are 50%, as only the paternal allele is methylated. Dotted line as in Fig. 10B. Adapted from (Santoro et al., 2013). (**B**) Southern blot analysis to check for blot transfer efficiency and complete NotI digestion, as a control for the *Igf2r* DNA methylation analysis shown in A. Data for two representative replicates are shown. Blots were rehybridized with the Htf9 probe, which contains an unmethylated NotI site (Lavia et al., 1987). The absence of the 3.7 kb NotI-uncut band and the presence of the 2.3 kb and 1.4 kb NotI-cut bands in all EcoRI/NotI treated samples (E/N) confirm complete NotI digestion. The equal intensity of the large and small bands confirms efficient blot transfer in all lanes. E, EcoRI only digest. Dotted line as in Fig. 10B. Adapted from (Santoro et al., 2013).

In differentiated control cells lacking the *Airn* promoter, the paternal *Igf2r* promoter is expressed and lacks DNA methylation, as shown by the presence of the single NotI-digested 1 kb band (Fig. 22A, lane 6). 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, NotI-undigested 5 kb band (Fig. 22A, lanes 2-5). I saw maximum methylation levels of ~20% in RA differentiation (Fig. 22A, left, lane 5) and of ~40% in EB differentiation (Fig. 22A, right, lane 5). 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. 22A, right, compare lanes 7-9 to lanes 2-5). This was not due to cell cycle arrest, as both RA and EB differentiated cells continued to proliferate throughout the observation period and each S-phase would require the action of DNMT1 to maintain the methylated state (Fig. 23). In conclusion, the data show that DNA methylation on the paternal *Igf2r* promoter is maintained independently of the *Airn* lncRNA (adapted from (Santoro et al., 2013)).

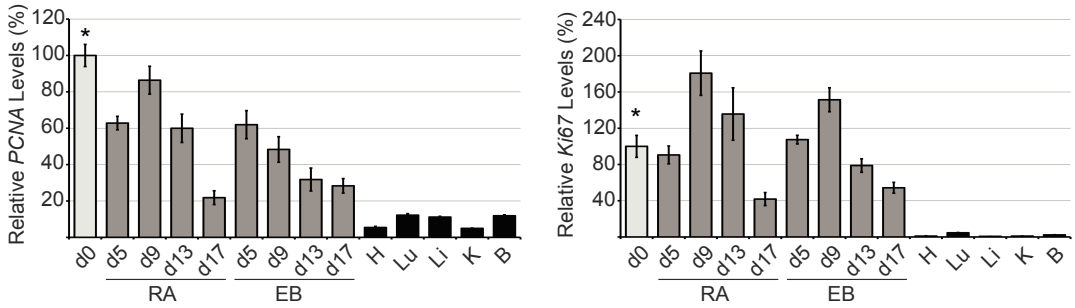

Fig. 23. CKO cells proliferate throughout differentiation. RT-qPCR with primers specific for proliferation markers *PCNA* (left) and *Ki67* (right) to measure relative proliferation levels in RA and EB differentiated CKO cells. Undifferentiated (pale grey) or differentiated (dark grey) CKO ES cells were compared with adult mouse tissues (black). Relative *PCNA* and *Ki67* levels were set to 100 in undifferentiated ES cells (*). ES cells differentiated via RA or EBs show similar proliferation levels, which are higher than in adult tissues even after 17 days of differentiation (d17). Data are mean±s.d. of three technical replicates. H, heart; Lu, lung; Li, liver; K, kidney; B, brain. Figure taken and legend adapted from (Santoro et al., 2013).

3.4. Turning *Airn* on: *Airn* expression conditional rescue (CRes) cells

3.4.1. The FLP-ER-T16 cells

To test if *Airn* can silence *Igf2r* at any differentiation stage, I established a cell line in which the silencing function of *Airn* can be switched on during differentiation. Paulina Latos had previously generated AirnT16 cells, in which *Airn* is truncated to a non-functional length that cannot silence *Igf2r*, by introducing an *FRT*-flanked polyA signal 16 kb after the *Airn* TSS (Latos et al., 2012). To conditionally delete the truncation signal and restore *Airn* to its full length, I then inserted a FLPe-ER^{T2} transgene in the AirnT16 cell line, thus generating FLP-ER-T16 cells (Fig. 24A). As no specific antibody against FLPe is available (Susan Dymecki and Francis Stewart, personal communication), I screened FLP-ER-T16 transgenic clones by Northern blot analysis and selected three clones expressing the highest levels of the FLPe-ER^{T2} transcript (Fig. 24B).

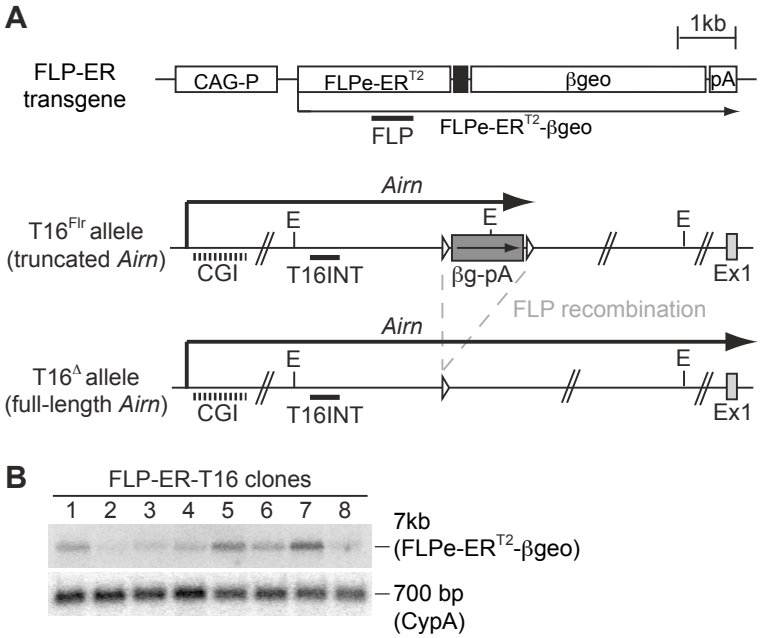

Fig. 24. Generation of FLP-ER-T16 ES cells. (**A**) Top: map of the vector used to insert the FLPe-ER^{T2} transgene in AirnT16 ES cells, which carry a paternal *Airn* 16 kb truncation allele (shown below). Due to the presence of the IRES (internal ribosome entry site, black box), a FLPe-ER^{T2}-βgeo fusion transcript is made. CAG-P, CMV early enhancer/chicken β-actin promoter; βgeo, β-galactosidase + neomycin resistance fusion gene for positive selection; FLP, Northern blot probe. Middle and bottom: the T16^{Flr} allele was generated by introducing a rabbit β-globin polyadenylation signal (βg-pA) in *Igf2r* intron 2 to truncate *Airn* 16 kb after its TSS (Latos et al., 2012). The truncation signal is 'flrted', or flanked by *FRT* sites (white triangles). FLP-mediated recombination deletes the truncation signal (T16^{Δ} allele) and restores full-length *Airn* expression. T16INT: Southern blot probe. Additional details as in Fig. 15A. (**B**) Northern blot analysis to quantify FLPe-ER^{T2}-βgeo mRNA expression in eight FLP-ER-T16 transgenic clones. Total RNA was hybridized to probe FLP and to probe CypA for normalization. Clones 1, 5 and 7 express the highest levels of FLPe-ER^{T2}-βgeo.

I then tested FLP-ER-mediated excision efficiency in undifferentiated ES cell clones (Fig. 25). Unfortunately, even after 48 hours treatment with up to 4 μM TAM, I detected no recombination in FLP-ER-T16 transgenic clones. Transfecting AirnT16 cells with a constitutively expressed FLP recombinase, however, resulted in polyA excision, indicating that the *FRT* sites in the AirnT16 allele are functional (Fig.25, lane 2). As I did not observe *FRT* recombination of the truncated *Airn* allele with the inducible FLP-ER recombinase, I decided to employ a CreER-*loxP* strategy instead.

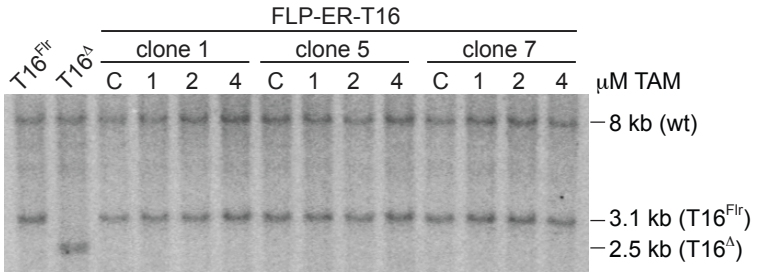

Fig. 25. No recombination is induced in FLP-ER-T16 cells. Southern blot analysis to detect FLP-mediated excision of the $T16^{Flr}$ allele. Lane 1, parental AirnT16 cell line used to generate FLP-ER-T16 clones; lane 2, control AirnT16 cells transiently transfected with a constitutive form of FLP recombinase; lanes 3-14, FLP-ER-T16 clones 1, 5 and 7 (see Fig. 24B) treated for 48 hours with increasing amounts of TAM or with vehicle only as a control (C). DNA was digested with EcoRI and hybridized to probe T16INT. Successful recombination is indicated by the presence of a 2.5 kb fragment and is only detected in control $T16^{\Delta}$ cells, but in none of the FLP-ER-T16 clones.

3.4.2. Generating and testing Airn CRes ES cells

I introduced a *loxP*-flanked polyA signal into $S12^{RC}$/+ cells, at a BamHI site located 3 kb after the *Airn* TSS (Fig. 26A), to create a conditional version of an *Airn* 3 kb truncation allele that cannot silence *Igf2r* (Latos et al., 2012; Sleutels et al., 2002). I confirmed paternal targeting of two independently targeted clones ($S12^{RC}$/$CRes^{Fl}$+cas1,2; Fig. 26B,C) and removed the selection cassette to generate clones $S12^{RC}$/$CRes^{Fl}$1,2 (Fig. 26D). Deletion of the *loxP*-flanked polyA signal in the $CRes^{Fl}$ allele generated the $CRes^{\Delta}$ allele (Fig. 27A). Compared with CKO^{Fl} cells (Fig. 16), recombination is faster in undifferentiated CRes cells, which have *loxP* sites further downstream of the *Airn* promoter. I observed more than 80% recombination 12 hours after TAM treatment and complete excision by 24 hours (Fig. 27B,C)]. As CKO cells, CRes cells differentiate normally too, both in the presence and absence of the *Airn* truncation signal (Fig. 28) (adapted from (Santoro et al., 2013)).

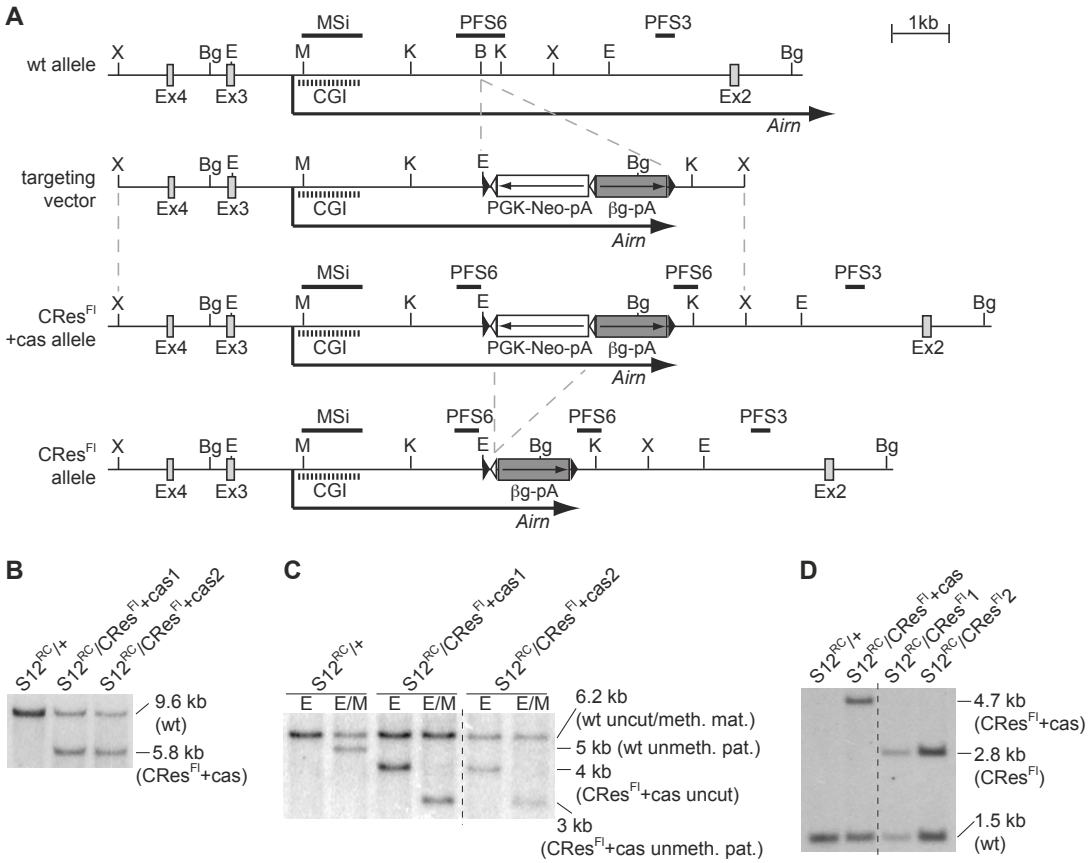

Fig. 26. Generation of *Airn* expression conditional rescue (CRes) ES cells. (**A**) Targeting strategy, details as in Fig. 15A. Top: wt *Airn* allele. Below: targeting vector used to truncate *Airn* 3 kb after its TSS. The same selection cassette as in Fig. 15A and a 'floxed' βg-pA truncation signal were inserted into a BamHI (B) site (chr17:12,744,359) in *Igf2r* intron 2. Homologous recombination in $S12^{RC}$/+ ES cells generated the $CRes^{Fl}$+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. MSi, PFS3, PFS6, Southern blot probes; Bg, BglII; K, KpnI. Figure taken and legend adapted from (Santoro et al., 2013). (**B**) Southern blot genotyping of two independently targeted clones ($S12^{RC}$/$CRes^{Fl}$+cas1,2) and the $S12^{RC}$/+ parental cell line shows correct homologous recombination ($CRes^{Fl}$+cas 5.8 kb). DNA was digested with BglII and hybridized to probe PFS3. Figure taken and legend adapted from (Santoro et al., 2013). (**C**) Southern blot to identify parental origin of the targeted allele. Samples from B were digested with EcoRI or EcoRI + MluI (E/M) and hybridized to probe MSi. Loss of a 5 kb and gain of a 3 kb band in $CRes^{Fl}$+cas cells shows the paternal allele was targeted. Dotted line as in Fig. 10B. Figure taken and legend adapted from (Santoro et al., 2013). (**D**) Southern blot genotyping confirms selection cassette removal, as shown by loss of a 4.7 kb and gain of a 2.8 kb band. DNA from the $S12^{RC}$/+ parental cell line and from targeted cells before ($S12^{RC}$/$CRes^{Fl}$+cas) and after ($S12^{RC}$/$CRes^{Fl}$1,2) FLP recombination was digested with KpnI and hybridized to probe PFS6. Dotted line as in Fig. 10B. Figure taken and legend adapted from (Santoro et al., 2013).

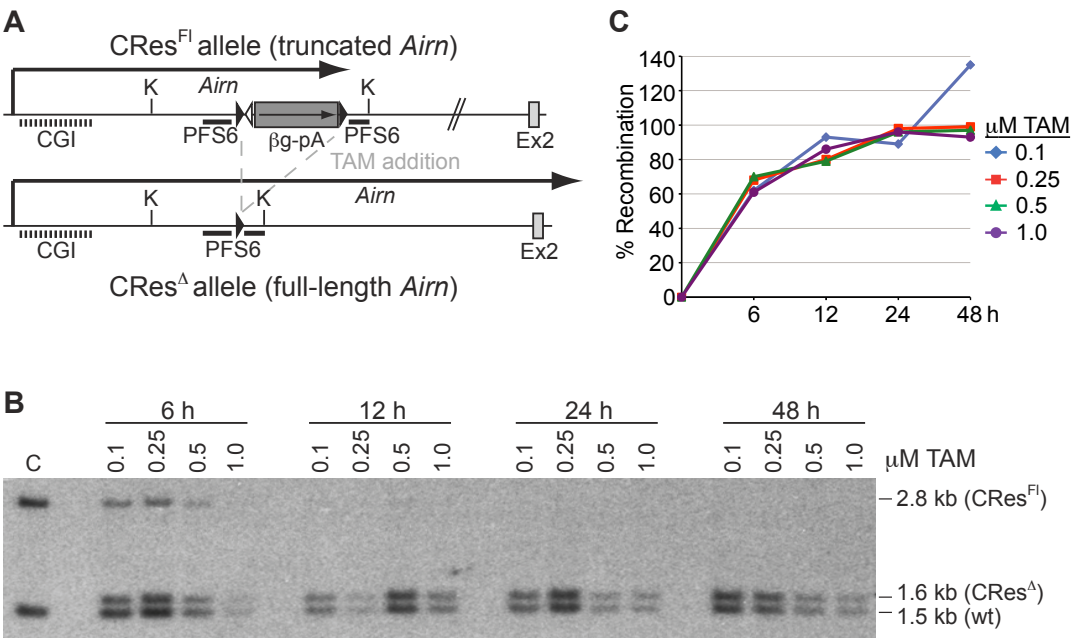

Fig. 27. Recombination efficiency and kinetics in CRes ES cells. (**A**) Southern blot strategy, details as in Fig. 26A. Taken from (Santoro et al., 2013). (**B**) Southern blot analysis showing Cre-mediated excision efficiency and kinetics of the CRes^{Fl} allele in response to increasing TAM concentrations. DNA from undifferentiated CRes ES cells, treated with the indicated amounts of TAM for the indicated number of hours, was digested with KpnI and hybridized to probe PFS6. Cre-mediated recombination converts the 2.8 kb 'floxed' paternal CRes^{Fl} allele to 1.6 kb (CRes$^{\Delta}$). The wt maternal allele is 1.5 kb. Control (C) cells were treated with vehicle only and harvested after 48 hours. Figure taken and legend adapted from (Santoro et al., 2013). (**C**) ImageQuant quantification of recombination efficiency. Independently of the amount of TAM used, complete recombination of the CRes^{Fl} allele is achieved already between 12 and 24 hours in undifferentiated CRes ES cells. Figure taken and legend adapted from (Santoro et al., 2013).

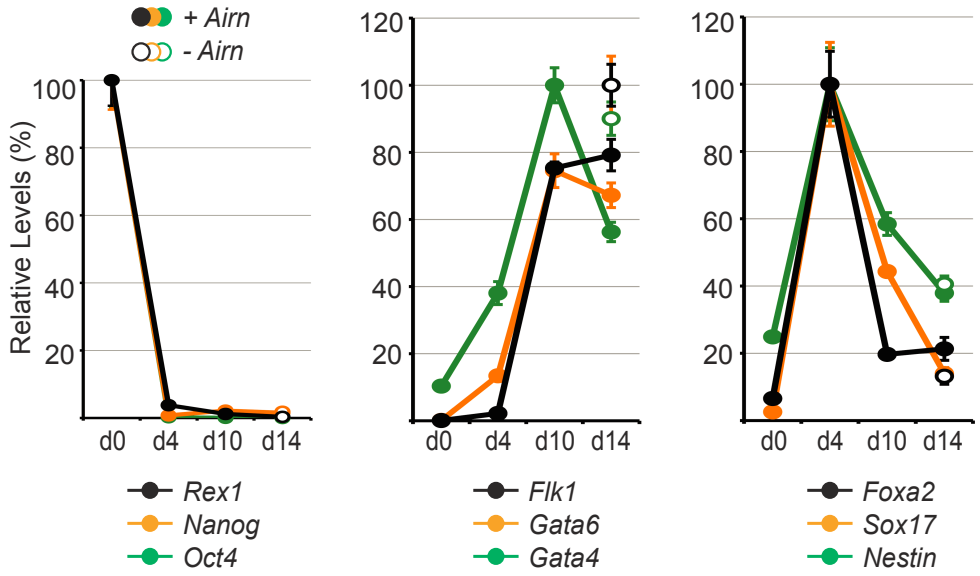

Fig. 28. CRes cells differentiate normally. RT-qPCR with primers specific for pluripotency and differentiation markers, as in Fig. 17. Marker expression was assayed in retinoic acid differentiated CRes cells, in the presence (+*Airn*) or in the absence (-*Airn*) of full-length *Airn* expression. Differentiated CRes cells show downregulation of pluripotency markers and upregulation of differentiation markers, with little or no difference between +*Airn* and –*Airn* samples in the majority of cases. Figure taken and legend adapted from (Santoro et al., 2013).

3.4.3. Deleting the truncation signal rescues Airn expression to wild-type levels

To test whether removing the polyA signal restores full-length *Airn* transcription to wild-type levels, I induced RA differentiated CRes cells to delete the polyA signal daily between day 1 and day 10 and harvested them after 3-4 days (Fig. 29). As a control for wild-type *Airn* levels, I codifferentiated CRes$^{\Delta}$ cells (TAM treated at day 0) for 4-14 days. Cre-mediated excision, monitored by Southern blot, showed the CRes^{FI} allele is recombined efficiently (over 85%) throughout RA differentiation (Fig. 30). I did not detect full-length *Airn* in differentiated cells carrying the unrecombined CRes^{FI} allele (Fig. 31, bar 8), confirming the polyA signal truncates *Airn*. *Airn* is strongly upregulated during differentiation in control CRes$^{\Delta}$ cells, showing that truncation of *Airn* is reversible (Fig. 31, 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. 31). Overall, the data show that the CRes system efficiently rescues full-length *Airn* transcription during ES cell differentiation, allowing a switch from a short, non-functional *Airn* to its longer, functional form at any time (adapted from (Santoro et al., 2013)).

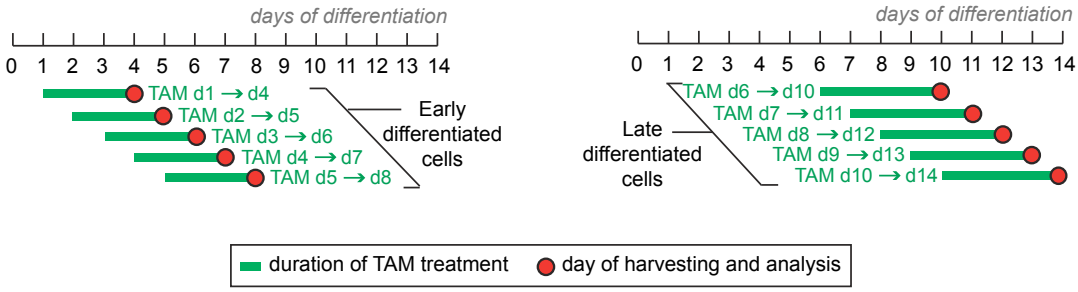

Fig. 29. Experimental strategy to turn *Airn* on during ES cell differentiation. Taken from (Santoro et al., 2013).

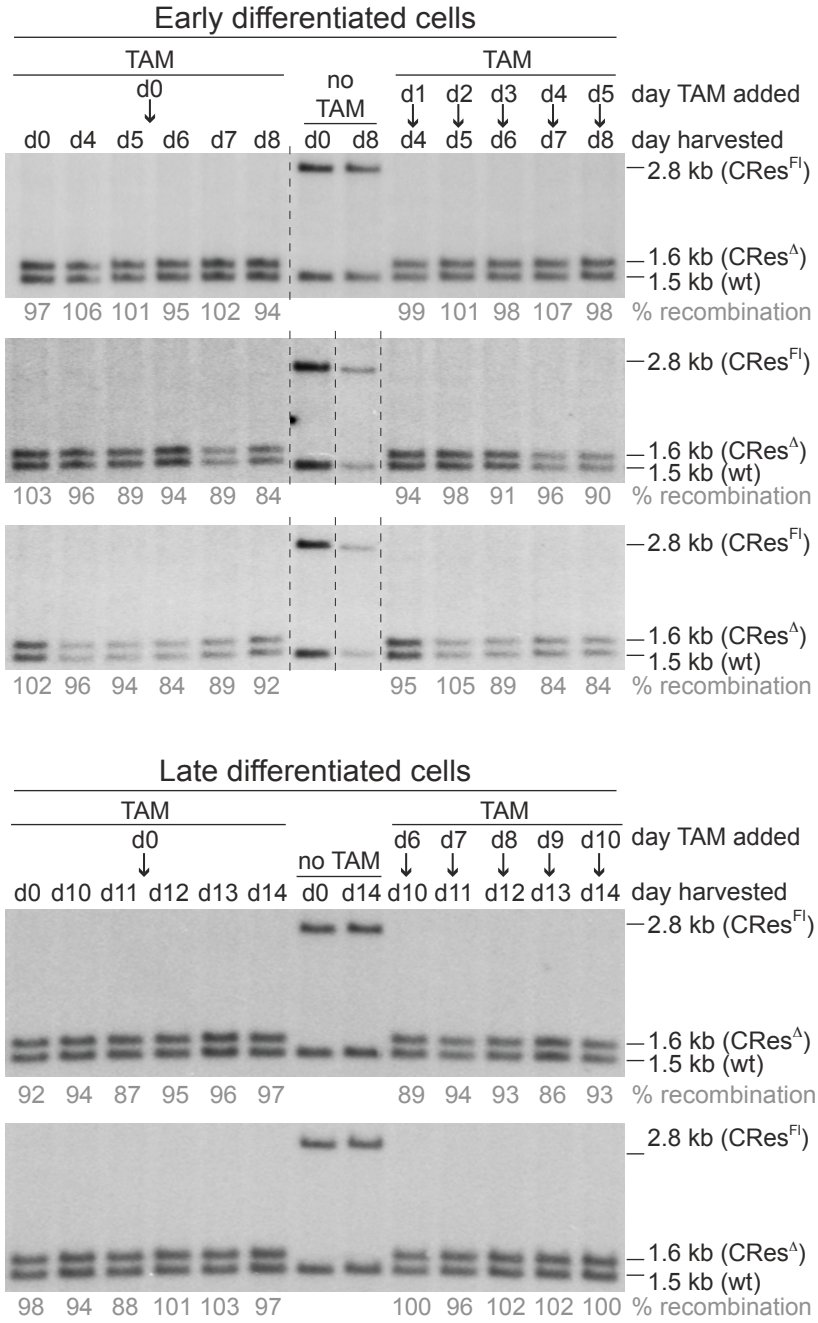

Fig. 30. The *Airn* truncation signal is deleted efficiently throughout ES cell differentiation. Southern blot analysis of Cre recombination, as in Fig. 27B, in CRes cells treated with RA for up to 14 days shows efficient recombination in early and late differentiated cells. Top: early differentiated day 4-8 cells. Bottom: 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. Recombination efficiency (% $CRes^{\Delta}$/wt bands) is shown underneath. Data from three early and two late differentiation replicates are shown. Dotted lines as in Fig. 10B. Adapted from (Santoro et al., 2013).

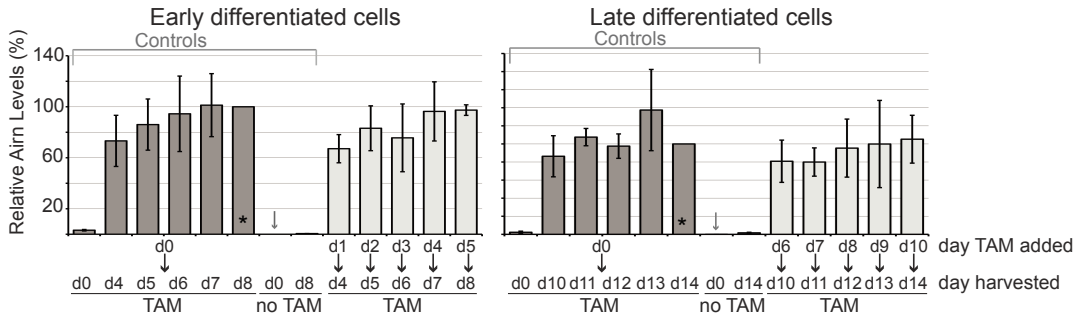

Fig. 31. Deleting the truncation signal rescues *Airn* expression to wild-type levels. RT-qPCR with Airn-middle primers, lying 49 kb downstream of the inserted polyA, shows that deleting the truncation signal during early and late differentiation restores full-length *Airn* expression to wt levels (pale bars). Relative *Airn* levels were set to 100 in control day 8 or day 14 cells (*) in which the polyA signal was removed prior to differentiation (dark bars). Data are mean±s.d. of three biological replicates (left) and mean±max/min of two biological replicates (right). Figure taken and legend adapted from (Santoro et al., 2013).

3.4.4. Airn expression can silence Igf2r at any time during ES cell differentiation

To test whether *Airn* can silence *Igf2r* at any time or whether its effects are restricted to a developmental window, I examined *Igf2r* imprinted expression in CRes cells using the PstI assay (Fig. 32A). In agreement with mouse studies (Sleutels et al., 2002), differentiated CRes^{FI} cells carrying the truncated *Airn* allele fail to establish *Igf2r* imprinted expression and display maternal- and paternal-specific bands throughout differentiation (Fig. 32A, no TAM day 8, day 14). By contrast, control CRes$^{\Delta}$ cells display wild-type gain of *Igf2r* imprinted expression during differentiation (Fig. 32A, left). I next restored full-length *Airn* expression at 24-hour intervals, testing early (Fig. 32A, top right) and late (Fig. 32A, bottom right) differentiation time points. Compared with the truncated *Airn* control that does not silence *Igf2r*, I observed *Igf2r* repression at all time points (Fig. 32A, compare samples 9-13 to sample 8 in each row). However, paternal-specific bands were more visible compared with wild-type controls, especially at late differentiation time points (Fig. 32A, compare samples 9-13 to samples 2-6 in each row) (adapted from (Santoro et al., 2013)).

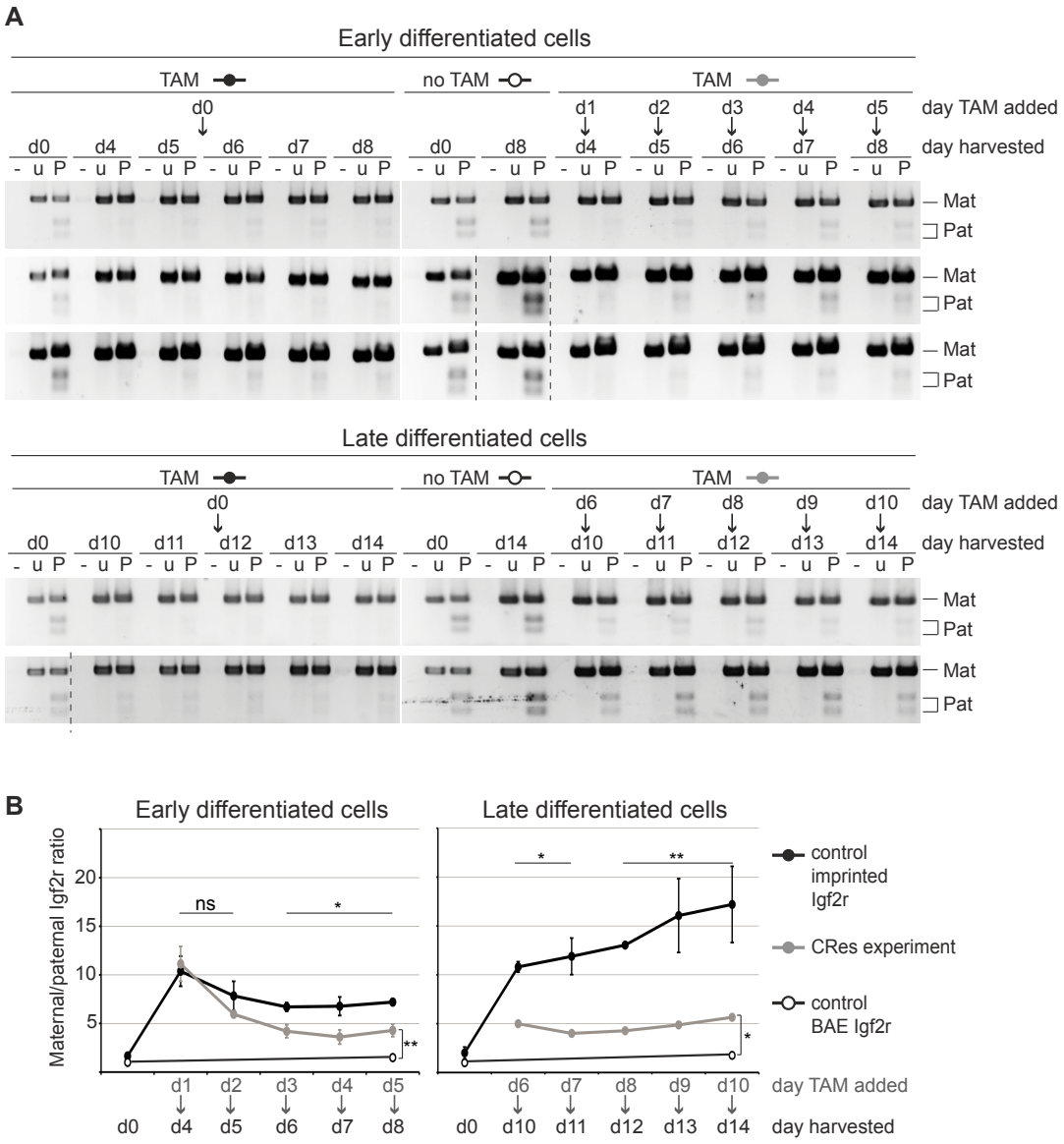

Fig. 32. *Airn* expression can silence *Igf2r* at any time during ES cell differentiation. (**A**) Allele-specific *Igf2r* expression in early or late differentiated CRes cells assayed as in Fig. 21A. Untreated (no TAM) day 8 or day 14 cells show full biallelic *Igf2r* expression. All cells treated at 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. Data from three early and two late differentiation replicates are shown. Dotted lines as in Fig. 10B. Adapted from (Santoro et al., 2013). (**B**) Allele-specific *Igf2r* RT-qPCR as in Fig. 21B. Maternal/paternal *Igf2r* ratios are plotted over time. Control imprinted *Igf2r* (black circles): CRes cells with the truncation signal deleted prior to differentiation show wt 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) show biallelic expression (BAE) of *Igf2r* throughout differentiation and were used to set the maternal/paternal ratio to 1 at day 0. Data are mean±s.d. of three biological replicates (left) and mean±max/min of two biological replicates (right). 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]. Figure taken and legend adapted from (Santoro et al., 2013).

I quantified allele-specific *Igf2r* expression (Fig. 32B) setting to 1 the ratio between maternal and paternal expression in undifferentiated control cells that carry the *Airn* truncation and express *Igf2r* biallelically (Fig. 32B, 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$^{\Delta}$ cells 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. 32B, control imprinted *Igf2r*). When *Airn* is turned on between day 1 and day 10 of differentiation, I observe a gain of *Igf2r* imprinted expression at all time points, with maternal:paternal ratios between 4 and 11 (Fig. 32B, CRes experiment). This ratio is similar to control cells when the polyA signal is removed at day 1 or day 2 (Fig. 32B, 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. 32B, 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 (adapted from (Santoro et al., 2013)).

3.4.5. Igf2r silencing in late differentiation does not depend on gain of DNA methylation

I next analyzed DNA methylation of the *Igf2r* promoter CGI by Southern blot (Fig. 33). Undifferentiated ES cells or differentiated control cells that express truncated *Airn* and show biallelic *Igf2r* lack DNA methylation, as shown by the single 1 kb band (Fig. 33A, lanes 1, 7 and 8). 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. 33A, lanes 2-6). Unexpectedly, when *Airn* function is rescued during differentiation, I observed little or no DNA methylation on the *Igf2r* promoter (Fig. 33A, lanes 9-13). I observed methylation levels comparable with wild-type controls only when the polyA signal is removed at day 1 (Fig. 33A, left, compare lane 9 and lane 2). When *Airn* length is functionally restored between day 2 and day 4, I detected low methylation. Rescuing at day 6 or later results in no detectable (nd) DNA methylation on the *Igf2r* promoter (Fig. 33A, right, compare lanes 9-13 and lanes 2-6).

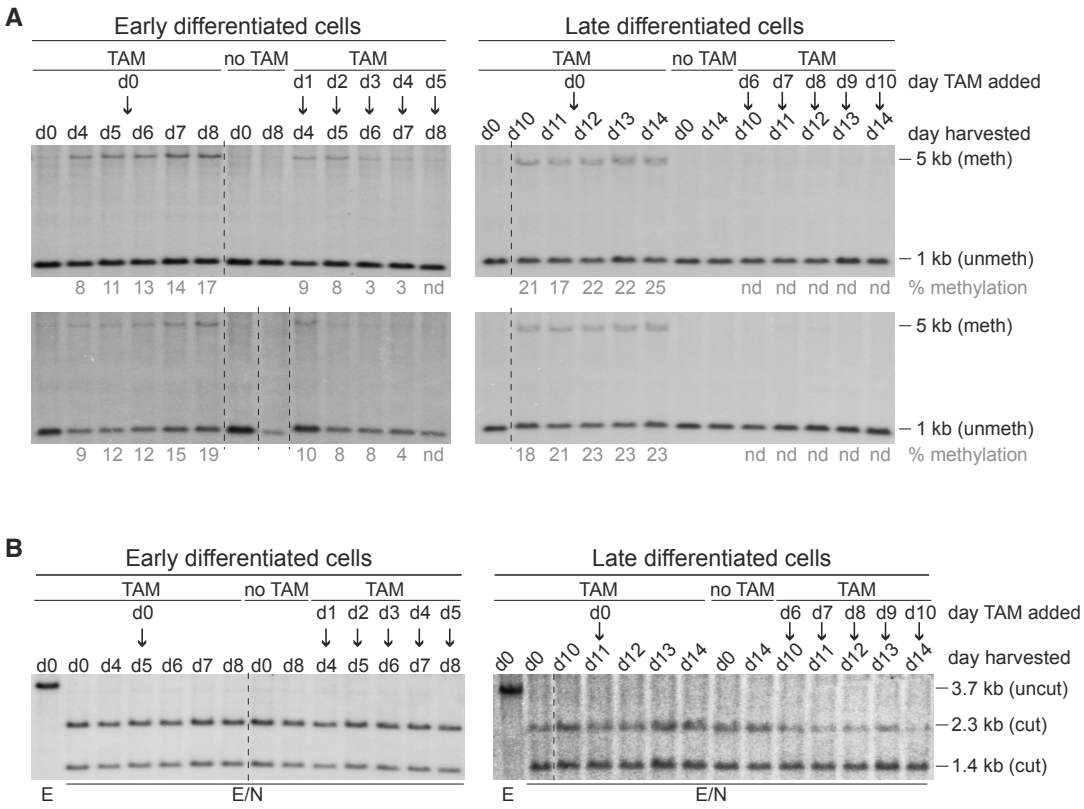

Fig. 33. *Igf2r* silencing in late differentiation does not depend on gain of DNA methylation. (**A**) *Igf2r* promoter methylation assayed as in Fig. 11C, in early or late differentiated CRes cells. Data from two early and two late differentiation replicates are shown. The *Igf2r* promoter gains up to 25% DNA methylation (5 kb band) by day 14 when full-length *Airn* is expressed throughout ES cell differentiation (right, lane 6). Lower methylation gain (3-10%) is seen when full-length *Airn* is rescued during early differentiation from day 1-4 (left, lanes 9-12). No gain of DNA methylation is seen when full-length *Airn* is expressed after day 6 (right, lanes 9-13; nd, not detected). Dotted lines as in Fig. 10B. Adapted from (Santoro et al., 2013). (**B**) Southern blot analysis to check for blot transfer efficiency and complete NotI digestion, as a control for the *Igf2r* DNA methylation analysis shown in A. Data for two representative replicates are shown. Details as in Fig. 22B. Figure taken and legend adapted from (Santoro et al., 2013).

Bisulfite sequencing of the *Igf2r* CGI supports these observations (Fig. 34). 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 (Fig. 35). However, low levels of repressive H3K9me3 modification are gained at the *Igf2r* promoter when *Airn* function is restored at day 10 (Fig. 36). 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 (adapted from (Santoro et al., 2013)).

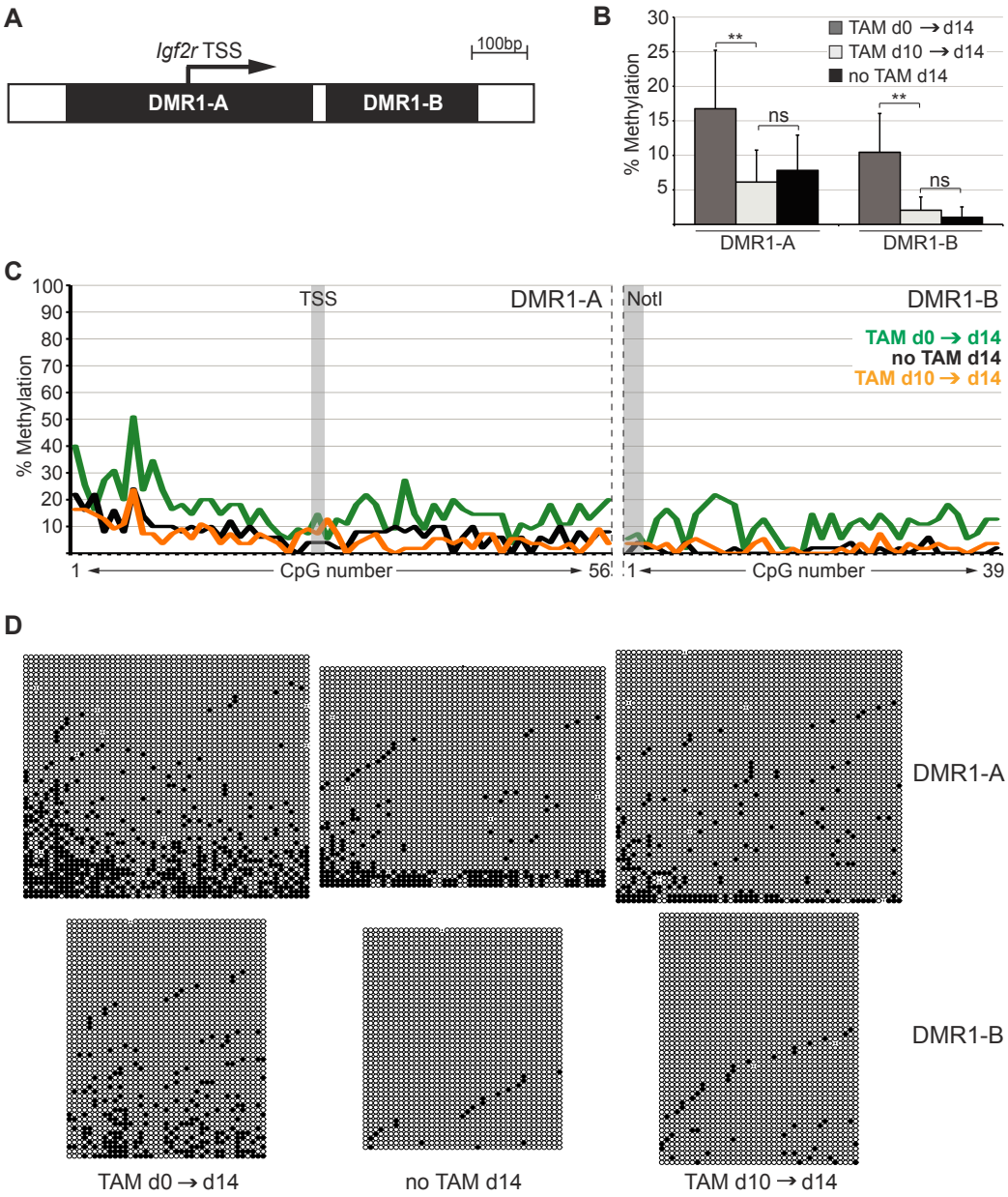

Fig. 34. Bisulfite sequencing analysis of *Igf2r* promoter methylation in late differentiated ES cells. Bisulfite sequencing analysis of the *Igf2r* CGI confirms that *Igf2r* silencing in late differentiated CRes cells is not accompanied by gain of DNA methylation. Figures taken and legends adapted from (Santoro et al., 2013). (**A**) The *Igf2r* CGI (chr17:12,769,059-12,770,009). The TSS and two regions assayed by bisulfite sequencing (DMR1-A and B) are indicated. The direction of *Igf2r* transcription is displayed in opposite orientation to previous figures. DMR1-A spans 433 bp (chr17:12,769,475-12,769,907) and contains 56 CpG dinucleotides. DMR1-B spans 268 bp (chr17:12,769,184-12,769,451) and contains 39 CpG dinucleotides. (**B**) Bisulfite sequencing analysis of DMR1-A and DMR1-B 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±s.d. of methylation levels in each region. Samples were compared using an unpaired *t*-test [**P<0.001; ns (not significant), P>0.01]. (**C**) Methylation levels at each CpG position within DMR1-A and B, in the absence of full-length *Airn* (black), in the presence of full-length *Airn* throughout differentiation (green) or in the presence of full-length *Airn* from day 10 onwards (orange). Grey bars mark the positions of the TSS and of the NotI restriction site analyzed in Fig. 33A. (**D**) Lollipop-style representation of data in C. Each column represents a CpG dinucleotide, each row one allele. White circles, unmethylated CpGs; black circles, methylated CpGs.

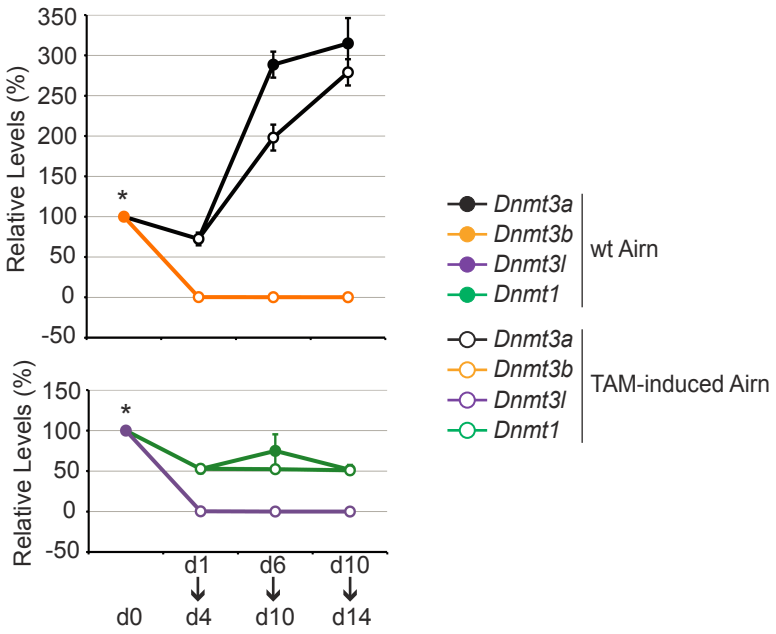

Fig. 35. Methyltransferase gene expression during ES cell differentiation. RT-qPCR with primers specific for the *de novo* methyltransferases *Dnmt3a* and *Dnmt3b*, their cofactor *Dnmt3l* and the maintenance methyltransferase *Dnmt1* show their expression levels vary during ES cell differentiation. Differentiated CRes cells were assayed in the presence of full-length *Airn* from day 0 (wt *Airn*) or upon full-length *Airn* induction during differentiation (TAM-induced *Airn*). Relative levels were set to 100 in undifferentiated ES cells (*). Data are mean±s.d. of three technical replicates. As ES cells differentiate, *Dnmt3a* expression is upregulated three-fold and *Dnmt1* expression is downregulated two-fold. *Dnmt3b* and *Dnmt3l* expression is lost early in differentiation. Filled and empty circles for *Dnmt3b* and *Dnmt3l* overlap completely. Figure taken and legend adapted from (Santoro et al., 2013).

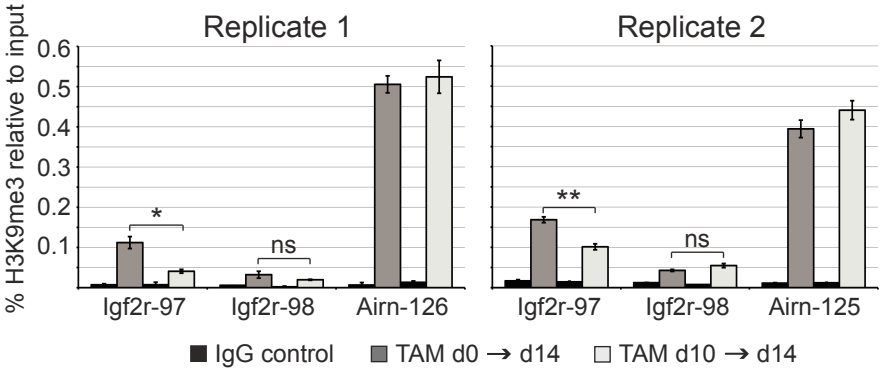

Fig. 36. *Igf2r* silencing correlates with gain of H3K9me3. ChIP-qPCR analysis of H3K9me3 in day 14 differentiated CRes cells. Airn-126 and Airn-125 assays map to the ICE and serve as positive control. The Igf2r-98 assay located inside the *Igf2r* gene body serves as negative control. The Igf2r-97 assay maps 257 bp upstream of the *Igf2r* TSS and shows that the *Igf2r* promoter gains H3K9me3 upon *Airn*-induced silencing in differentiated cells. Compared with control cells expressing full-length *Airn* throughout differentiation (dark grey, TAM treatment at day 0), cells that only restore full-length *Airn* from day 10 gain lower levels of H3K9me3 (pale grey, TAM treatment at day 10). H3K9me3 enrichment is plotted relative to input. Data for two biological replicates are shown as mean±s.d. of three technical replicates. Samples were compared using an unpaired *t*-test [**P<0.001; *P=0.001-0.01; ns (not significant), P>0.01]. Figure taken and legend adapted from (Santoro et al., 2013).

4. DISCUSSION

4.1. Summary of results

By using inducible ES cell systems that control endogenous *Airn* lncRNA expression, I investigated here the developmental regulation of imprinted *Igf2r* silencing. *Airn* is a well-established example of a *cis*-repressing lncRNA. *Airn* transcription silences the paternal *Igf2r* allele in a manner that is independent of the lncRNA product (Latos et al., 2012) and subsequently the silenced paternal *Igf2r* promoter gains a somatic DNA methylation imprint (Latos et al., 2009; Stoger et al., 1993). Using two inducible systems, I tested whether *Airn* expression is continuously needed to maintain *Igf2r* silencing and its somatic DNA methylation imprint, and whether *Airn* silencing is restricted to a 'window of opportunity' during ES cell differentiation. The data show that although *Airn* expression is both necessary and sufficient to initiate and maintain *Igf2r* silencing at any stage during ES cell differentiation, DNA methylation adds an extra layer of epigenetic information that may act to safeguard the silent state (adapted from (Santoro et al., 2013)).

4.2. Inducible ES cell systems to control endogenous gene expression

4.2.1. ES cell differentiation is a good model system to study developmental regulation of imprinted expression

ES cells are frequently used as models for X chromosome inactivation (Navarro and Avner, 2010; Wutz, 2007) and are becoming more appreciated for genomic imprinting studies (Kohama et al., 2012). Here, I modified the previously established S12/+ ES cell line, an *Igf2r* imprinting model that faithfully recapitulates the developmental onset of *Igf2r* imprinted expression (Latos et al., 2009). Although *Airn* expression is also necessary to silence the paternal *Slc22a2* and *Slc22a3* alleles in extraembryonic tissues, ES cells derived from the blastocyst inner cell mass cannot yet be differentiated into these tissues. In differentiated ES cells, the *Slc22a2* and *Slc22a3* genes show low-level non-imprinted expression, typical of embryonic tissues (Hudson et al., 2010; Latos et al., 2009; Zwart et al., 2001b). Both genetically modified ES cell lines derived here (CKO and CRes) differentiated normally in response to retinoic acid (RA) treatment or embryoid body (EB) formation, as shown by downregulation of the *Rex1*, *Nanog* and *Oct4* pluripotency genes and by

upregulation of meso-, endo- and neuro-ectodermal differentiation markers. Upon differentiation in the presence of wild-type *Airn* expression, both cell lines established proper *Igf2r* imprinted expression and gained DNA methylation on the paternal *Igf2r* promoter. Interestingly, compared to RA differentiated cells, I observed twice as much *Igf2r* promoter methylation in EB differentiated cells. An ES cell study of the *Kcnq1* imprinted cluster demonstrated that *Cdkn1c* was silenced during RA differentiation without acquiring the promoter DNA methylation seen in mouse embryos (Wood et al., 2010). However, I could show that the *Cdkn1c* promoter acquires ~20% methylation after EB differentiation (Santoro et al., 2013). My results confirm the utility of ES cell models for studying some aspects of epigenetic silencing of imprinted genes, but demonstrate that differentiation protocols require careful consideration (adapted from (Santoro et al., 2013)).

4.2.2. The tetracycline-inducible Airn allele gains abnormal DNA methylation

I initially attempted to control endogenous *Airn* expression using a TetOn system. The modified Tet-*Airn* allele, in which a Tet-inducible promoter replaced the paternal *Airn* promoter, expressed low levels of *Airn* that could be upregulated, upon activation of a Dox-dependent rtTA transactivator, to silence *Igf2r*. However, the Tet-driven *Airn* promoter was modified by abnormal DNA methylation and the effects of inducing *Airn* expression could only be assayed in a subset of cells. I then subcloned these Tet-inducible ES cells and obtained some clones with an unmethylated Tet promoter, but they rapidly became methylated in response to doxycycline treatment and rtTA activation. Although the TetOn system is widely used to drive transgene expression in mammalian cells (Stieger et al., 2009), little is known about the ability of a Tet promoter targeted to an endogenous gene to attract DNA methylation. The silent wild-type paternal *Airn* promoter is slightly methylated in undifferentiated ES cells, but this methylation is lost as the *Airn* promoter is expressed during differentiation (Koerner et al., 2012). It is possible that sequences within the Tet promoter interfere with this process, as recent work mapping DNA methylation in different mouse strains has identified a strong dependence on adjacent bases in attracting *de novo* methylation (Xie et al., 2012) (adapted from (Santoro et al., 2013)).

4.2.3. The inducible FLP-FRT system does not rescue the Airn truncation

Due to the methylation problem with the Tet-inducible *Airn* allele, I switched strategies and decided to employ inducible site-specific recombinases to control *Airn* expression during ES cell differentiation. A conditional *Airn* truncation allele, in which *FRT* sites flank the polyA signal, had already been established in the lab and I attempted to rescue the truncation using a TAM-inducible FLP-ER transgene (Hunter et al., 2005). However, even upon treatment with high TAM doses, I observed no excision of the *Airn* truncation signal. Expressing a constitutive form of the FLP recombinase resulted in efficient *FRT* recombination, indicating that the *FRT* sites are functional but FLP-ER is not. I detected FLP-ER mRNA expression in all transgenic ES cell clones, but I could not verify whether the FLP-ER protein is also present, as no specific anti-FLPe antibody is available (Susan Dymecki and Francis Stewart, personal communication). Therefore, the inability of FLP-ER to induce *FRT* recombination upon TAM treatment could be due to either protein absence or malfunction.

4.2.4. The inducible Cre-loxP system allows efficient control over Airn expression

Given the inadequacy of both the Tet-inducible and the FLP-*FRT* systems, I created two inducible Cre-*loxP* systems – CKO and CRes – to manipulate *Airn* expression kinetics during ES cell differentiation. The CKO system used *loxP* sites flanking the *Airn* promoter to delete it during ES cell differentiation, whereas the CRes system used *loxP* sites flanking a polyA signal to functionally elongate *Airn* during ES cell differentiation. In a previous study, different CreER variants were introduced into the *ROSA26* locus in mouse ES cells and systematically tested for recombination efficiency (Hameyer et al., 2007). The $CreER^{T2}$ variant was shown to be the most responsive to ligand induction, as after 48 hours near-complete recombination of the target sequence was obtained with as little as 0.2 μM TAM (Hameyer et al., 2007). Consistent with these observations, I showed here that low levels of TAM (0.1 μM) were efficient in inducing Cre-mediated recombination of the *Airn* locus in undifferentiated ES cells and that the number of recombined alleles increased over time in a dose-independent manner. Recombination was complete by 48 hours in the CKO system and by 24 hours in the CRes system. The faster recombination observed in the CRes system could be explained by the shorter target sequence (1.2

kb, versus 1.9 kb in CKO cells) or by some interference from the *Airn* promoter or CGI with Cre activity in the CKO system (adapted from (Santoro et al., 2013)).

I tested the effect of deleting or inducing functional *Airn* during differentiation by assaying *Igf2r* imprinted expression 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, I observed reduced recombination efficiency in RA compared with EB differentiated cells and therefore based my 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 (Santoro et al., 2013). The greater abundance of transcription complexes at the highly expressed *Airn* promoter in RA differentiated cells might interfere with Cre recombinase binding to the *loxP* sites, resulting in reduced recombination efficiency. In EB differentiated cells, in which the *Airn* promoter was excised efficiently throughout differentiation, I detected less than 20% of wild-type *Airn* levels upon TAM treatment. Importantly, the levels of Cre recombination in this ES cell system are comparable to those obtained in studies using mouse embryos. For example, a conditional knockout strategy similar to the one reported here was recently used to conditionally delete the promoter of the imprinted *Kcnq1ot1* lncRNA in mouse embryos (Mohammad et al., 2012). Residual *Kcnq1ot1* levels, indicating incomplete recombination, ranged from 10 to 20%, which are similar to the residual *Airn* levels following Cre-mediated recombination of the *Airn* promoter during EB differentiation. In the CRes system, Cre-mediated excision of the polyA signal was extremely efficient and rescued full-length *Airn* expression to wild-type levels at all tested time points. In conclusion, the inducible Cre-*loxP* strategy proved a valid alternative to both the Tet-inducible and the FLP-*FRT* systems (adapted from (Santoro et al., 2013)).

4.3. How is imprinted gene silencing maintained?

4.3.1. The role of the ICE in maintaining imprinted silencing

As described in the introduction, the ICE, genetically defined for the imprinted *Igf2r* cluster as a 4 kb DNA fragment containing the *Airn* promoter and CGI (Wutz et al., 1997), is essential to initiate parental-specific expression of imprinted genes (Koerner and Barlow, 2010). However, the role of the ICE in maintaining imprinted

expression during development is less understood, as conditional ICE deletion alleles have been established for only a few imprinted clusters so far. The first study to address this issue reported a conditional deletion of the *Igf2* cluster ICE in neonatal muscle (Srivastava et al., 2000). A similar experiment was later performed in neonatal liver (Thorvaldsen et al., 2006). Conditional deletion of the unmethylated maternal ICE caused re-expression of the silent *Igf2* allele, indicating that the CTCF-mediated insulator activity of the ICE is continuously required to silence the maternal copy of *Igf2* (Srivastava et al., 2000; Thorvaldsen et al., 2006). In the absence of the insulator element, the downstream enhancers gain access to the *Igf2* promoter and reactivate its expression. Conditional deletion of the methylated ICE on the paternal chromosome, instead, did not relieve silencing of the *H19* lncRNA, indicating that the methylated ICE is dispensable for *H19* silencing maintenance (Srivastava et al., 2000; Thorvaldsen et al., 2006). Interestingly, unlike *Igf2*, the *H19* lncRNA gains DNA methylation on the promoter of its silent allele during embryonic development (Bartolomei et al., 1993; Ferguson-Smith et al., 1993). This methylation mark is preserved after conditional deletion of the ICE, indicating that, although needed to establish *H19* promoter methylation in the first place, the ICE is not required to maintain it (Srivastava et al., 2000; Srivastava et al., 2003; Thorvaldsen et al., 2006). Importantly, this also suggests that the somatic DNA methylation imprint can maintain *H19* silencing independently of the ICE.

Recently, a conditional deletion of the Pws-ICE in the Pws/As cluster has been reported (DuBose et al., 2012). The unmethylated paternal ICE is thought to act as a positive regulator to promote paternal-specific expression of the *Ndn*, *Magel2*, *Mkrn3* and *Frat3* genes in brain. Germline deletion of the ICE leads to so-called maternalization of the paternal allele, with transcriptional silencing of all four genes and gain of DNA methylation on the *Ndn* and *Mkrn3* promoters (Bielinska et al., 2000; Dubose et al., 2011; Yang et al., 1998). However, conditional deletion of the ICE between E10.5 and E12.5 in neuronal precursor cells does not affect expression or methylation of these genes, indicating that the Pws-ICE, although necessary to initiate parental-specific expression and methylation patterns, is not needed to maintain them (DuBose et al., 2012). Unfortunately, the authors did not test whether conditional deletion of the methylated maternal ICE causes re-expression of the silent alleles, so it is currently unknown whether the Pws-ICE plays any role in maintaining gene silencing on the maternal chromosome.

In another recent study, maintenance of imprinted silencing in the *Kcnq1* cluster was analyzed by conditionally deleting the ICE at different stages of mouse development (Mohammad et al., 2012). The *Kcnq1* ICE contains the promoter for the *Kcnq1ot1* macro lncRNA, which controls imprinted expression of both the ubiquitous and the placental-specific imprinted genes in the cluster (Mancini-Dinardo et al., 2006). Conditional deletion of the paternally expressed *Kcnq1ot1* promoter relieved silencing of the ubiquitous imprinted genes in all tested tissues and at all tested developmental stages, indicating that continuous *Kcnq1ot1* expression is required to maintain silencing of these genes in both embryo and placenta. By contrast, conditional removal of the *Kcnq1ot1* promoter did not affect silencing of the placental-specific imprinted genes. Following their transcriptional silencing, the paternal alleles of the ubiquitous imprinted genes *Cdkn1c* and *Slc22a18* gain promoter DNA methylation (Bhogal et al., 2004). Interestingly, these somatic methylation marks were lost in both embryonic and placental tissues upon conditional deletion of the *Kcnq1ot1* promoter (Mohammad et al., 2012). In a previous study, *Kcnq1ot1* had been reported to bind the maintenance methyltransferase enzyme *Dnmt1* (Mohammad et al., 2010) and together the data would support a role for the *Kcnq1ot1* transcript in silencing some of its target genes by guiding and maintaining DNA methylation at their promoters.

Here, I investigated maintenance of imprinted *Igf2r* silencing by the *Airn* lncRNA, whose promoter lies within the *Igf2r* cluster ICE. By deleting the *Airn* promoter during ES cell differentiation, I showed 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 ES cell 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. The data therefore show that, similar to *Kcnq1ot1*, continuous *Airn* expression is necessary to maintain *Igf2r* silencing (Fig. 37A), but unlike *Kcnq1ot1*, only until the *Igf2r* promoter gains DNA methylation (Fig. 37B). Establishment of the *Igf2r* somatic methylation imprint thus determines a switch from *Airn*-dependent to *Airn*-independent 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 ES cell

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. The results therefore show that, unlike the *Cdkn1c* and *Slc22a18* sDMRs, the *Igf2r* somatic imprint is maintained in a lncRNA-independent fashion, most likely through the hemi-methyltransferase activity of DNMT1 (Ooi et al., 2009) (adapted from (Santoro et al., 2013)).

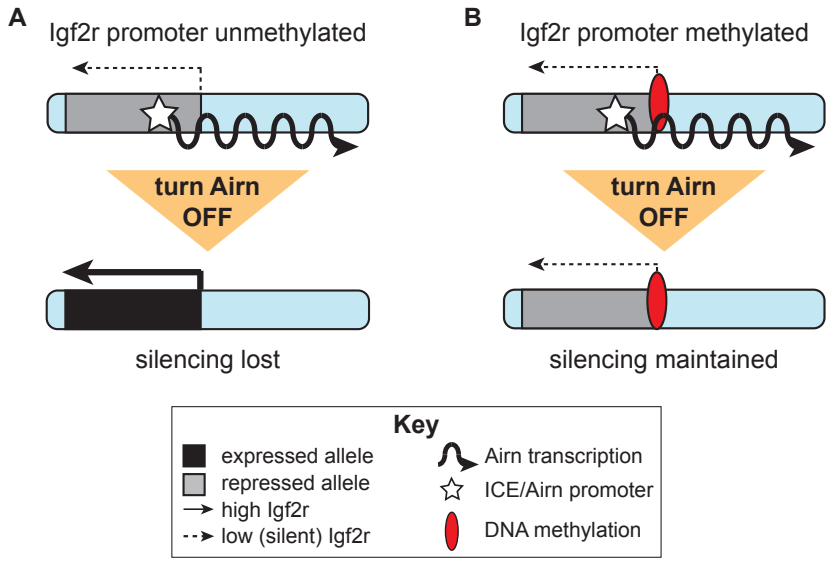

Fig. 37. Continuous *Airn* expression or DNA methylation maintains *Igf2r* silencing. In the absence of DNA methylation on the *Igf2r* promoter, as in RA or early EB differentiation, *Igf2r* silencing is lost when *Airn* expression is turned off, indicating that continuous *Airn* expression is needed to maintain the silent state (**A**). However, the *Igf2r* promoter gains DNA methylation in late EB differentiation and this methylation mark is sufficient to maintain *Igf2r* silencing in the absence of *Airn* (**B**). Note that only the paternal allele is shown.

In conclusion, different mechanisms seem to have evolved to maintain parental-specific expression of imprinted genes. In some cases, maintenance of imprinted expression requires the ICE to be continuously active, as either an insulator element or lncRNA promoter. In other cases, the ICE becomes dispensable during development and imprinted silencing is maintained by repressive epigenetic modifications, such as DNA methylation. The implications of my findings in the *Igf2r* cluster for our general understanding of how epigenetic silencing is maintained are discussed below.

4.3.2. Continuous lncRNA expression during development: a safeguarding mechanism against reactivation of epigenetically silenced genes

My results raise several questions concerning the developmental regulation of *Igf2r* silencing by *Airn* transcription (Latos et al., 2012). Once its expression is activated in embryonic development, *Airn* is transcribed continuously in all tissues in which the paternal *Igf2r* promoter is silenced and methylated (Pauler et al., 2005; Stoger et al., 1993; Yamasaki et al., 2005). However, if *Airn* is dispensable to maintain *Igf2r* silencing once DNA methylation is established, as my results in early development show, it is unclear why *Airn* needs to be expressed continuously throughout life (adapted from (Santoro et al., 2013)).

At the onset of X inactivation, the *Tsix* lncRNA is transcribed in antisense orientation to *Xist* on the future active X chromosome (Xa) (Lee et al., 1999a). Similar to *Airn* and *Igf2r*, it has been shown that *Tsix* transcription through the *Xist* promoter is required to silence *Xist in cis* (Luikenhuis et al., 2001; Ohhata et al., 2008; Sado et al., 2001; Shibata and Lee, 2004). The *Tsix* lncRNA interacts with the *de novo* methyltransferase enzyme DNMT3A and the EZH2 subunit of the PRC2 repressive complex, thus recruiting DNA methylation and H3K27me3 to the silent *Xist* promoter (Sun et al., 2006; Zhao et al., 2008). Unlike *Airn* however, *Tsix* expression is lost in late development and other repressive mechanisms, including DNA methylation, maintain *Xist* silencing on the Xa in the absence of *Tsix* (Barr et al., 2007; Beard et al., 1995). If DNA methylation can also maintain *Igf2r* silencing alone, why then is *Airn* expression not switched off during development like *Tsix*?

Similar to *Airn*, the *Xist* lncRNA is continuously expressed in mouse tissues. In females, random XCI occurs only once during development and each cell then propagates the same inactive X chromosome (Xi) through subsequent divisions. Whereas XCI initiation is critically dependent on *Xist* (Marahrens et al., 1997), the Xi seems to be maintained independently of *Xist* in both differentiated ES cells and somatic cells. This was originally shown by deleting *Xist in vitro* in post-XCI fibroblasts, which did not cause reactivation of the *Pgk1* and *Hprt* X-linked genes (Csankovszki et al., 1999), and was later confirmed in ES cells carrying an inducible autosomal *Xist* transgene, where switching off *Xist* expression after silencing had been established did not lead to gene reactivation (Wutz and Jaenisch, 2000). And yet, despite not being required for XCI maintenance, *Xist* continues to be expressed

long after silencing on the Xi has been established. Human cancers are frequently associated with supernumerary active X chromosomes (Pageau et al., 2007), suggesting that reactivation of X-linked genes could have deleterious effects on the organism. Continuous *Xist* expression could thus serve as one of several redundant safety measures to ensure Xi stability throughout life.

Indeed, upon deeper examination, the conditional *Xist* deletion in embryonic fibroblasts was shown to induce reactivation, albeit at very low frequency, of an X-linked transgene and of the endogenous *Hprt* gene (Csankovszki et al., 2001). The reactivation frequency of these two genes was further increased upon inhibition of DNA methylation and histone deacetylation (Csankovszki et al., 2001), indicating that *Xist* acts synergistically with DNA methylation and histone hypoacetylation to maintain the remarkable stability of the Xi. The effect of a conditional loss of *Xist* on XCI maintenance was also tested in immortalized skin fibroblasts (Zhang et al., 2007). After several passages in culture, the authors reported loss of H3K27me3 from the Xi and stochastic reactivation of a few X-linked genes, confirming that, although not the only mechanism through which stable XCI is ensured, continuous *Xist* expression reinforces Xi stability. Recently, *Xist* was deleted in hematopoietic stem cells, which arise at E10.5 of mouse development after XCI has already been established (Yildirim et al., 2013). Surprisingly, deleting *Xist* in the blood compartment severely disrupted long-term XCI maintenance, which in turn led to the development of aggressive and lethal hematological cancers. These results therefore indicate that, at least in a fast replicating compartment such as the blood, the *Xist* lncRNA is required not only to initiate but also to maintain XCI and that *Xist* functions as a potent suppressor of hematological cancers *in vivo* (Yildirim et al., 2013). Interestingly, although *Xist* can initiate XCI only in early development (Wutz and Jaenisch, 2000), the permissive context for XCI initiation is transiently reestablished in hematopoietic precursor cells (Savarese et al., 2006). Together, these observations highlight the peculiar chromatin environment of blood cells, which renders them – unlike any other somatic cell tested so far - both sensitive to and dependent on the silencing activity of *Xist* for XCI.

Here, I showed that *Airn* expression is dispensable for *Igf2r* silencing once the *Igf2r* promoter is methylated. However, similar to *Xist*, continuous *Airn* expression may be important to maintain stable *Igf2r* silencing throughout life. I only tested the effect of *Airn* removal on *Igf2r* imprinted expression until day 13 of ES cell differentiation. At

this stage, I observed ~35% DNA methylation on the *Igf2r* promoter in EB differentiated cells and removing *Airn* transcription caused partial re-expression of the paternal *Igf2r* allele. As DNA methylation at promoter regions is associated with transcriptional silencing, I assume that paternal *Igf2r* is only re-expressed in cells with no or little DNA methylation, whereas switching *Airn* expression off in cells with a highly methylated *Igf2r* promoter should not affect *Igf2r* silencing. However, I did not prove this on a single-cell level. As single-cell assays of this kind are technically challenging, one could alternatively determine whether removing *Airn* transcription in an adult organ that displays full *Igf2r* promoter methylation has any effect on the stability of *Igf2r* silencing. If *Airn* expression is completely dispensable in the presence of DNA methylation, then no changes in *Igf2r* imprinted expression should be observed in a fully methylated tissue. If however, similar to *Xist* for the maintenance of XCI, continuous *Airn* expression is needed for the long-term stability of *Igf2r* silencing, then *Airn* removal could result in derepression of the silent *Igf2r* allele over time. To assay the effect of the conditional *Airn* deletion after several rounds of cell divisions, either primary cells from an actively dividing adult tissue or immortalized cells from a terminally differentiated one would have to be employed for this experiment.

4.3.3. DNA methylation: an extra layer of repressive epigenetic information to maintain the silent state

As discussed so far, the need for life-long *Airn* expression is puzzling, considering that *Igf2r* silencing seems to be maintained in the absence of *Airn* once DNA methylation on the repressed *Igf2r* promoter is set. On the other hand, given that *Airn* is continuously transcribed, the need for DNA methylation on the *Igf2r* promoter is equally puzzling. 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 amount of 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. Some genes, such as *Ube3a*, *Copg2* and *Murr1*, maintain imprinted expression in the adult in the absence of promoter DNA methylation (John and Lefebvre, 2011),

indicating that methylation is not strictly required for continuous imprinted silencing. Nevertheless, it could represent a means to ensure stable epigenetic repression of essential imprinted genes throughout life (adapted from (Santoro et al., 2013)).

In the *Igf2r* and *Kcnq1* clusters, somatic imprints only mark the repressed promoters of the genes that show life-long imprinted expression - *Igf2r*, *Cdkn1c* and *Slc22a18*. Parental-specific DNA methylation is acquired on the repressed alleles of these genes only after imprinted silencing is established, indicating that silencing initiation does not require DNA methylation (Bhogal et al., 2004; Stoger et al., 1993). Moreover, both *Igf2r* and *Cdkn1c* are silenced in *Dnmt1* knockout embryos that lack maintenance DNA methylation. Loss of DNMT1 causes ICE demethylation and biallelic expression of the *Airn* and *Kcnq1ot1* lncRNAs. This results in biallelic silencing of both *Igf2r* (Li et al., 1993; Seidl et al., 2006) and *Cdkn1c* (Green et al., 2007; Hudson et al., 2011; Lee et al., 2002), indicating that *Airn* and *Kcnq1ot1* can exert their silencing function in the absence of DNA methylation. It must be noted that earlier studies reported biallelic expression of *Cdkn1c* in the absence of DNA methylation, thus concluding that *Kcnq1ot1* is not able to repress *Cdkn1c* in the absence of its somatic imprint (Bhogal et al., 2004; Caspary et al., 1998; Lewis et al., 2004). However, these studies employed non-quantitative assays to detect allele-specific *Cdkn1c* expression, whereas quantitative assays show that, compared to wild-type samples, *Cdkn1c* expression is reduced on both alleles in *Dnmt1* mutants that express *Kcnq1ot1* biallelically (Green et al., 2007; Hudson et al., 2011). The paternal *Cdkn1c* allele was also shown to maintain its silent state in the absence of DNA methylation in RA differentiated ES cells, further supporting the idea that *Cdkn1c* silencing by *Kcnq1ot1* does not require DNA methylation (Wood et al., 2010).

Other lines of evidence, however, indicate that DNA methylation does play a role in *Cdkn1c* silencing. The *Kcnq1ot1* lncRNA was shown to bind DNMT1 and to recruit it to the *Cdkn1c* promoter (Mohammad et al., 2010). Moreover, as discussed in the previous section, both *Cdkn1c* paternal-specific silencing and the somatic methylation imprint are lost upon conditional deletion of *Kcnq1ot1* (Mohammad et al., 2012). *Cdkn1c* silencing and methylation also require the LSH protein, a member of the SNF2 family of chromatin remodeling proteins, which reinforces DNA methylation at Polycomb target sites (Fan et al., 2005). Together, the data suggest that DNA methylation at the *Cdkn1c* promoter is established and maintained in a lncRNA-

dependent manner and that, although not necessary for *Cdkn1c* silencing, the methylation imprint may be important to maintain the silent state in the long term. Interestingly, DNA methylation plays no role in silencing the human *CDKN1C* gene, which is not modified by a somatic methylation imprint on the repressed paternal allele (Chung et al., 1996; Monk et al., 2006).

Igf2r silencing is unaffected in the absence of both DNMT1 and LSH, indicating that *Airn* expression can maintain silencing alone in the absence of DNA methylation (Fan et al., 2005; Li et al., 1993; Seidl et al., 2006). On the other hand, my results with the conditional *Airn* allele show that, in contrast to the *Cdkn1c* somatic methylation imprint, DNA methylation at the *Igf2r* promoter is established in a lncRNA-independent manner and can maintain silencing in the absence of *Airn*. Therefore, even though dispensable for *Igf2r* silencing, DNA methylation seems to add an extra layer of repressive epigenetic information to reinforce the silent state of the paternal *Igf2r* promoter. Whether *Airn* expression and DNA methylation have a redundant or a synergistic effect on the maintenance of *Igf2r* silencing is currently unknown and it would be interesting to test whether removing DNA methylation in adult tissues that express *Airn* would destabilize *Igf2r* silencing.

Similar to the *Igf2r* somatic imprint, methylation of the X-linked *Hprt* promoter follows XCI by several days and this early observation originally suggested that DNA methylation could be involved in maintaining, rather than initiating, gene silencing on the Xi (Lock et al., 1987). Like *Airn*, *Xist* can also silence in the absence of DNA methylation (Panning and Jaenisch, 1996; Sado et al., 2004), but the two mechanisms act synergistically to maintain XCI over time, as shown by the increase in reactivation frequency of some X-linked genes in *Xist* mutant cells after treatment with DNA methylation inhibitors (Csankovszki et al., 2001). Consistent with a role for DNA methylation in maintaining XCI, patients suffering from ICF (immunodeficiency, centromeric instability, facial anomalies) syndrome, which lack a functional DNMT3B enzyme and have virtually no CGI methylation on the Xi, show unstable silencing (Hansen et al., 2000). However, only some loci on the Xi are reactivated in these patients, indicating that additional repressive mechanisms compensate for the reduced methylation levels. DNA methylation also contributes to the stable repression of the *Xist* promoter on the Xa. As mentioned earlier, once *Tsix* expression is switched off in late development, other silencing mechanisms prevent *Xist* reactivation on the active X chromosome (Barr et al., 2007; Beard et al., 1995).

DNA methylation is among these mechanisms and is important for long-term silencing of *Xist*, as shown by *Dnmt3a/3b* or *Dnmt1* knockout cells, which only show significant *Xist* reactivation after prolonged time in culture (Panning and Jaenisch, 1996; Sado et al., 2004).

In addition to DNA methylation, the inactive X chromosome is enriched for repressive histone modifications, including H2Aub1 (monoubiquitination of histone H2A), H3K27me3 and H4K20me1, and most of these modifications depend on *Xist* expression to be deposited on the Xi (Wutz, 2011). Similar to DNA methylation, repressive histone marks seem to be dispensable for XCI but important for 'locking in' the silent state. H3K27me3 and H4K20me1 are not sufficient to trigger gene silencing on the Xi in the presence of a silencing-incompetent *Xist* mutant (Kohlmaier et al., 2004) and the PRC2 complex member EED is not required for either initiation or maintenance of random XCI in embryonic tissues (Kalantry and Magnuson, 2006). Similarly, *Tsix*-mediated *Xist* repression is not affected in EED-deficient ES cells (Schoeftner et al., 2006). Repressive chromatin marks may play a more important role in gene silencing in extraembryonic tissues, where EED is needed to maintain imprinted XCI (Wang et al., 2001). As described in the introduction, repressive histone modifications also mark the silent alleles of many imprinted genes, but their relevance to the establishment and maintenance of imprinted expression is unclear (Barlow, 2011). The repressed *Igf2r* promoter lacks H3K27me3 but is marked by focal enrichment of H3K9me3 (Latos et al., 2009; Regha et al., 2007). However, *Igf2r* silencing is unaffected not only in the absence of EED, which is required to establish H3K27me3, but also in the absence of the EHMT2 histone methyltransferase, which catalyzes H3K9me2 (Mager et al., 2003; Nagano et al., 2008). As for X inactivation, repressive chromatin modifications may be dispensable for imprinted gene silencing, but important for its stable maintenance, although determination of their exact role at the *Igf2r* and other imprinted clusters will require further investigation.

In conclusion, multiple layers of repressive epigenetic mechanisms may act cooperatively to maintain stable repression of target genes and prevent their reactivation during development (Payer and Lee, 2008). The presence of multiple levels of control likely explains the remarkable stability of the inactive X chromosome throughout life and in general, the "*stacking of imperfect repressive tendencies may be an evolutionary strategy to ensure leak-proof gene silencing"* (Barr et al., 2007).

Based on the results presented here, I suggest that a similar strategy operates at the *Igf2r* locus, where continuous expression of the *Airn* lncRNA and DNA methylation on the repressed *Igf2r* promoter cooperatively maintain imprinted silencing of the *Igf2r* gene throughout life.

4.3.4. Why does imprinted expression persist in the adult?

The presence of multiple silencing mechanisms to ensure stable gene repression on the Xi probably reflects the importance of dosage compensation for normal functioning of the organism. Perhaps not surprisingly, the inability to perform XCI in the embryo or in extraembryonic tissues results in lethality (Marahrens et al., 1997; Takagi and Abe, 1990) and reactivation of the Xi in the blood compartment causes aggressive hematological cancers (Yildirim et al., 2013). Similarly, the presence of multiple repressive mechanisms to ensure continuous *Igf2r* imprinted expression suggests that it is important for the organism to control *Igf2r* dosage throughout adulthood. But is it really so? As the debate on the evolutionary significance of genomic imprinting is mainly based on the different reproductive strategies between mammals and other vertebrates (Haig, 2004), the function of imprinted genes has been investigated mostly in early life. Some studies however suggested that imprinted genes are important in the adult as well (John and Lefebvre, 2011). *Nesp55* seems to control exploratory behavior and the ability to react properly to novel environments (Plagge et al., 2005), whereas *Cdkn1c* affects the maturation of a specific subtype of neuronal cell and may therefore have an impact on social behavior (Joseph et al., 2003). Finally, alterations in the dosage of *Igf2* in the adult were shown to affect adiposity (Da Costa et al., 1994; Jones et al., 2001).

Regarding *Igf2r*, it is currently unknown whether gene dosage alterations have any impact on adult functions. In general, *Igf2r* seems to be more important in early life than in adulthood. A complete knockout of the gene results in neonatal lethality, indicating that its function is essential in development (Wang et al., 1994), whereas it becomes dispensable in the adult, as tissue-specific knockout mice in which the gene is disrupted in liver, heart or skeletal muscle are viable and show no obvious phenotype (Wylie et al., 2003). However, the relevance of *Igf2r* imprinted expression in the adult has not been addressed so far. *Igf2r* gene dosage needs to be tightly controlled during development to ensure normal birth weight, as mice that express *Igf2r* biallelically show a 20% reduction in body weight that is first observed in late

embryonic development and persists through adulthood (Wutz et al., 2001). The conditional *Airn* knockout allele described here could help determine whether monoallelic *Igf2r* expression also plays a role in the adult. However, it must be noted that since DNA methylation can repress *Igf2r* in the absence of *Airn*, the re-establishment of biallelic *Igf2r* expression in neonatal or adult tissues would require erasure of the *Igf2r* somatic imprint in addition to *Airn* deletion.

4.4. How do lncRNAs initiate gene silencing?

4.4.1. 'Windows of opportunity' for lncRNA silencing activity

Despite the growing numbers of novel lncRNAs discovered in the mammalian genome, to date only few have been functionally characterized (Guttman and Rinn, 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 in which essential cofactors or chromatin environments are present. For example, such 'windows of opportunity' have been described for the *Xist* and *Tsix* lncRNAs (adapted from (Santoro et al., 2013)).

The idea that the silencing activity of *Xist* may be temporally restricted stemmed from the observation that, although ectopic *Xist* expression from the Xa can silence X-linked genes in ES cells and early mouse embryos (Panning and Jaenisch, 1996), it is not able to do so in somatic mouse/human hybrids, despite proper *Xist* expression and localization (Clemson et al., 1998). This indicates that *Xist* alone is not sufficient for silencing and requires additional factors that are not present in somatic cells. The 'window of opportunity' for *Xist* silencing activity was then defined by analyzing the ability of an autosomal *Xist* transgene to initiate chromosome-wide silencing during ES cell differentiation (Wutz and Jaenisch, 2000). As *Xist* can only induce gene silencing within 48 hours of ES cell differentiation, the transition from an *Xist*-sensitive to an *Xist*-insensitive environment *in vivo* most likely occurs during implantation (Wutz and Jaenisch, 2000) (Fig. 38). 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). Based on its expression profile, which is restricted to the developmental contexts in which XCI occurs, the chromatin remodeler SATB1 was identified as one of the factors required for *Xist*-mediated silencing (Agrelo et al., 2009). Similar to *Xist*, *Tsix* silencing function also

depends on a lineage-specific environment and is restricted to a specific 'window of opportunity' in some extraembryonic tissues. In the parietal endoderm, *Tsix* can repress *Xist* throughout development, whereas it can only silence until E9.5 in trophoblast giant cells and the spongiotrophoblast (Ohhata et al., 2011).

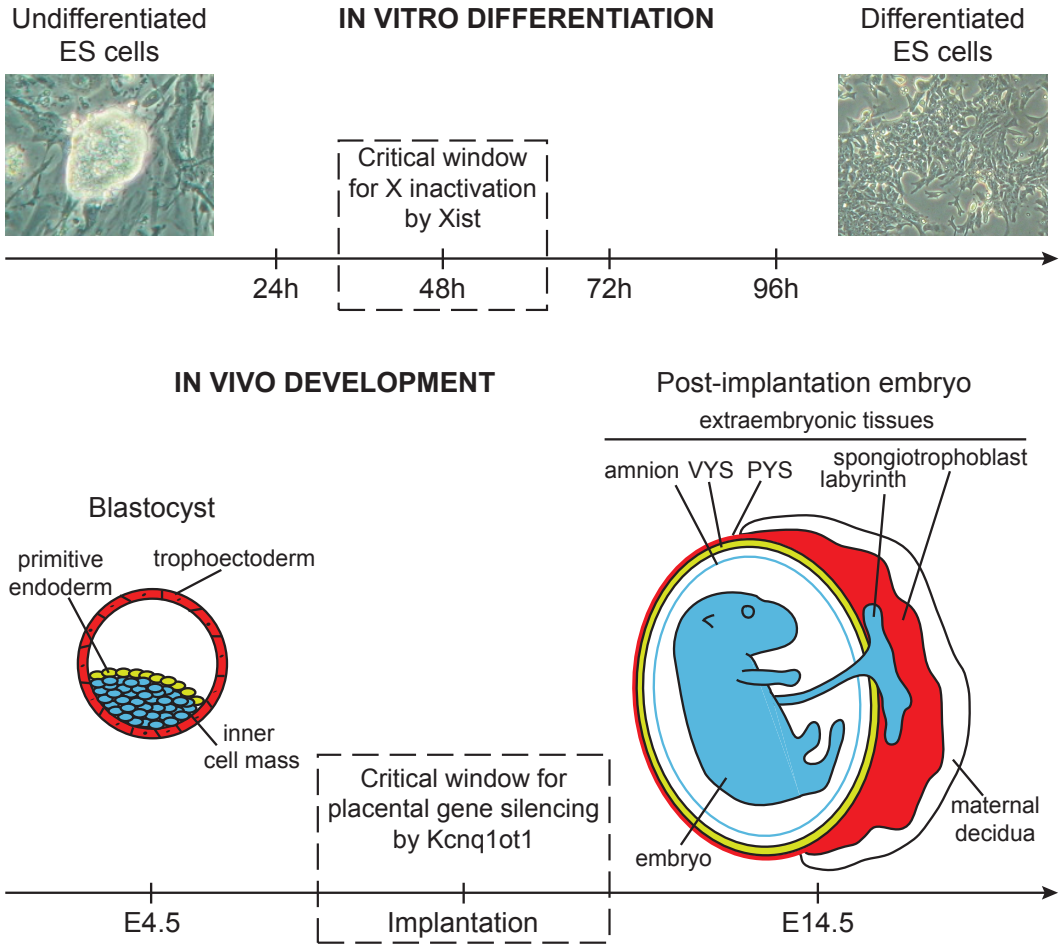

Fig. 38. 'Windows of opportunity' of lncRNAs during mouse development. (Top) The 'window of opportunity' for the silencing activity of the *Xist* lncRNA was identified in an *in vitro* ES cell differentiation system. *Xist* must be expressed within 48 hours of ES cell differentiation in order to cause X chromosome inactivation. (Bottom) The 'window of opportunity' for the silencing activity of the imprinted *Kcnq1ot1* lncRNA was identified during *in vivo* mouse development. *Kcnq1ot1* can silence extraembryonic-specific imprinted genes only before implantation occurs. See text for additional details. Tissues of the postimplantation embryo derived from the blastocyst inner cell mass, primitive endoderm and trophoectoderm components are indicated in blue, yellow and red, respectively. Figure taken and legend adapted from (Santoro and Barlow, 2011).

So far, a developmental 'window of opportunity' for an imprinted lncRNA has only been described for *Kcnq1ot1*. By using two different *Dnmt1* knockout mouse models, in which DNA methylation is lost at different stages of embryo development, *Kcnq1ot1* expression was induced from the normally silent maternal allele either before or after implantation. Biallelic *Kcnq1ot1* expression before implantation

disrupts imprinted expression of both ubiquitous and placental-specific imprinted genes in the cluster, indicating that the maternally expressed *Kcnq1ot1* lncRNA can silence all genes during the preimplantation stage (Green et al., 2007). However, when *Kcnq1ot1* is biallelically expressed in placenta after implantation, it can silence the ubiquitous, but not the placental-specific, imprinted genes (Green et al., 2007). The critical window for *Kcnq1ot1 cis*-silencing activity thus seems to coincide, as for *Xist*, with the implantation stage of development (Fig. 38). The 'window of opportunity' does not seem to apply to genes showing ubiquitous imprinted expression in the *Kcnq1* cluster, as they can be silenced both before and after implantation, giving further support to the idea that ubiquitous and placental-specific imprinted genes are silenced via different mechanisms (Hudson et al., 2010). It must be noted, however, that these findings may need to be reinterpreted, given the recent demonstration that the *Dnmt1* somatic isoform is active from the two-cell stage, earlier than previously thought and prior to the onset of imprinted expression in the *Kcnq1* cluster (Hirasawa et al., 2008) (adapted from (Santoro and Barlow, 2011)).

Here, by turning *Airn* on at different time points of ES cell differentiation, I asked whether I could identify a 'window of opportunity' for *Airn* similar to the ones described above. In contrast to *Xist*, I find that *Airn* can initiate *Igf2r* silencing throughout ES cell differentiation. *Airn* is normally upregulated between days 2 and 3 of ES cell differentiation (Latos et al., 2009) and activating functional *Airn* after day 3 represses paternal *Igf2r* expression at all time points, showing that *Airn* silencing activity is not restricted to a window. Moreover, this indicates that *Airn*-mediated *Igf2r* silencing is unlikely to depend on developmentally regulated factors (adapted from (Santoro et al., 2013)).

4.4.2. Gene silencing by lncRNAs: active repression or prevention of activation?

Although *Airn*-mediated silencing is observed throughout ES cell differentiation, the data show that *Igf2r* repression after day 3 is less efficient than in the continuous presence of *Airn*. This is also shown by the lower gain of H3K9me3 on the repressed *Igf2r* promoter when *Airn* is activated in late differentiation. It is noteworthy that *Airn* and *Igf2r* share the same *cis*-regulatory elements and show similar expression kinetics in mouse tissues and differentiated ES cells (Latos et al., 2009; Pauler et al., 2005). *Igf2r* is expressed at very low levels in undifferentiated ES cells but its

maternal expression increases sharply between days 2 and 3 of differentiation, concomitantly with paternal *Airn* upregulation. This could indicate that *Airn* repressor activity is limited by higher *Igf2r* promoter activity and that, rather than repressing an active promoter, *Airn* silences *Igf2r* by preventing its upregulation during development (Latos et al., 2009). Transcriptional interference, whereby one transcriptional process suppresses another one *in cis*, has been shown to act at the *Igf2r* locus (Latos et al., 2012). *Airn* transcription through the *Igf2r* promoter interferes with expression of the latter by dislodging transcription initiation complexes (Latos et al., 2012). Transcriptional interference depends, among other factors, on the relative strength of the two promoters, the strong interfering one and the weak sensitive one whose expression is reduced (Palmer et al., 2011). It has been previously shown that *Airn* needs to be expressed from a strong promoter in order to silence *Igf2r* (Stricker et al., 2008). 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 strongly expressed, are in agreement with a transcriptional interference model and indicate that *Airn* acts by preventing *Igf2r* upregulation (Fig. 39). This is contrast to *Tsix*, which can repress a fully activated *Xist* promoter at any time in some extraembryonic lineages, indicating that it does not simply prevent *Xist* upregulation but uses an active repression mechanism to silence *Xist* (Ohhata et al., 2011) (adapted from (Santoro et al., 2013)).

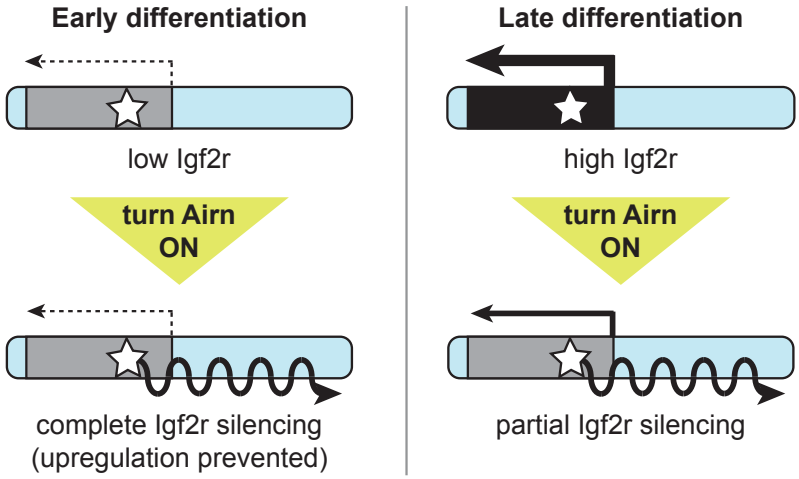

Fig. 39. *Airn* silencing efficiency inversely correlates with *Igf2r* promoter strength. *Airn* expression can silence *Igf2r* at any time during ES cell differentiation. However, silencing is maximal when *Airn* expression is turned on in early differentiation, when *Igf2r* is expressed at low levels (left). In the absence of *Airn*, *Igf2r* is expressed at high levels in late differentiation and can be silenced only partially by turning *Airn* expression on at this developmental time point (right). Note that only the paternal allele is shown. Key as in Fig. 37. Adapted from (Santoro and Pauler, 2013).

4.4.3. Is DNA methylation necessary for silencing?

Although *Igf2r* silencing is usually followed by gain of DNA methylation (Latos et al., 2009; Stoger et al., 1993), activating *Airn* after day 3 results in very little gain of DNA methylation and none is detected when *Airn* is activated after day 5. As for H3K9me3, this may reflect the less efficient *Igf2r* repression. On the other hand, it also correlates with decreased levels of the *de novo* methyltransferase DNMT3B and the DNMT3L cofactor during ES cell differentiation, suggesting that, due to developmental regulation of these factors, *de novo* DNA methylation on the *Igf2r* promoter can only be established within an early developmental window. Does the inability to methylate the *Igf2r* promoter during development affect the ability of *Airn* to repress it? In other words, is DNA methylation necessary for efficient *Igf2r* silencing? Earlier studies suggest this is not the case. In the absence of DNA methylation, *Igf2r* silencing can be established and maintained up to E8.5, as shown by *Dnmt1* knockout mice that upregulate *Airn* two-fold and silence *Igf2r* biallelically (Li et al., 1993; Seidl et al., 2006). This indicates that DNA methylation, although able to maintain the silent state in the absence of *Airn* as discussed above, is not necessary for either silencing initiation or maintenance. The decrease in *Airn* silencing efficiency during ES cell differentiation is therefore more likely to depend on the concomitant increase in *Igf2r* promoter strength, rather than the absence of DNA methylation. Similar to *Airn*, both *Xist* and *Tsix* can silence their target genes in the absence of DNA methylation (Panning and Jaenisch, 1996; Sado et al., 2004), but methylation reinforces lncRNA-mediated silencing over time. In conclusion, my data support the idea that *Airn* expression alone is required to initiate and maintain *Igf2r* silencing during development and that, similar to other loci, DNA methylation adds an additional repressive layer to ensure stable maintenance of the silent state (adapted from (Santoro et al., 2013)).

4.5. Developmental control of lncRNA-mediated silencing: clinical implications

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, especially those associated with abnormal gene silencing in human disease (Wang and Chang, 2011). For example, several lncRNAs were recently

shown to be involved in cancer. These include the *ANRIL/p15AS* lncRNA that is antisense to the *p15* tumor suppressor gene (Yap et al., 2010; Yu et al., 2008), a long lncRNA antisense to *p21* (Morris et al., 2008) and the *HOTAIR* lncRNA that was shown to promote cancer invasiveness and metastasis in a PRC2-dependent manner (Gupta et al., 2010). In all these cases, disease therapy could benefit from strategies that relieve the dormant alleles and lncRNAs with a role in establishing or maintaining gene silencing therefore represent attractive therapeutic targets. The same holds true for human imprinting syndromes arising from aberrant expression of imprinted genes or loss of the parental allele expressing the protein-coding gene. The majority of patients affected by Beckwith-Wiedemann syndrome, an overgrowth disease, show maternal hypomethylation of the DMR found at the human *KCNQ1* cluster on chromosome 11 (Lee et al., 1999b). The rest usually have paternal UPD of this chromosome. In both cases, the *KCNQ1OT1* lncRNA is biallelically expressed, causing biallelic silencing of the neighboring *CDKN1C* tumor suppressor gene, which suppresses cell proliferation. Novel therapeutic strategies specifically targeting the *KCNQ1OT1* lncRNA may be useful to restore expression of the repressed *CDKN1C* gene. The Angelman syndrome is a neurological disorder caused by loss of expression of the maternally expressed *UBE3A* gene that shows neuronal-specific imprinted expression. Angelman patients with paternal UPD of this chromosome or an imprinting defect that results in a paternal-only methylation pattern have biallelic expression of the *UBE3A-ATS* lncRNA and no expression of *UBE3A*. It was recently shown that the mouse *Ube3a-ats* lncRNA is responsible for silencing the *Ube3a* gene *in cis* (Meng et al., 2012), indicating that if a similar mechanism operates in humans, *UBE3A-ATS* may become a useful therapeutic target to restore *UBE3A* expression. Interestingly, topoisomerase inhibitors have recently been used to reactivate the silent *Ube3a* gene and this correlated with downregulation of the antisense *Ube3a-as* lncRNA (Huang et al., 2012) (adapted from (Santoro and Barlow, 2011) and (Santoro et al., 2013)).

The data presented in this thesis, 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. Destroying a lncRNA to relieve silencing of its target genes only makes sense under some conditions: (1) the transcript and not its transcriptional activity are responsible for gene silencing and (2) the lncRNA is constantly required to maintain the silent state

(Fig. 40A). If the lncRNA causes gene silencing via transcriptional interference with an overlapped promoter or enhancer, then strategies to abolish lncRNA transcription rather than to destroy the transcript would be required. For example, exogenously administered siRNAs complementary to specific promoter sequences were shown to block transcription of human genes (Malecova and Morris, 2010). If the lncRNA continues to be expressed but plays no role in maintaining gene silencing, it will be necessary to regain expression of the gene of interest by interfering with the repressive epigenetic marks that keep it silent (Fig. 40B). Several epigenetic drugs, with a broad and largely unknown mode of action, have been approved for use in clinical practice (Agrelo and Wutz, 2010). The DNA demethylating agents 5-azacytidine (Vidaza®) and its deoxy-derivative Decitabine or 5-aza-2'deoxycytidine (Dacogen®) are used for the treatment of myelodysplastic syndrome. The histone deacetylase inhibitor Vorinostat (Zolinza®) was also approved for clinical use in the treatment of cutaneous T cell lymphoma. Unfortunately, since the action of these drugs is genome-wide, their anti-proliferative effects on neoplastic cells are likely to result from a general effect on cell viability. Therefore, where possible, a more specific therapy directed at the lncRNA that initiates the epigenetic modifications is a desirable alternative. Of course, if the lncRNA and the repressive epigenetic modifications maintain silencing in a cooperative fashion, as shown here for the *Igf2r* locus, then removing either one or the other would not relieve silencing and therapeutic strategies that target both would have to be employed (Fig. 40C) (adapted from (Santoro and Barlow, 2011) and (Santoro et al., 2013)).

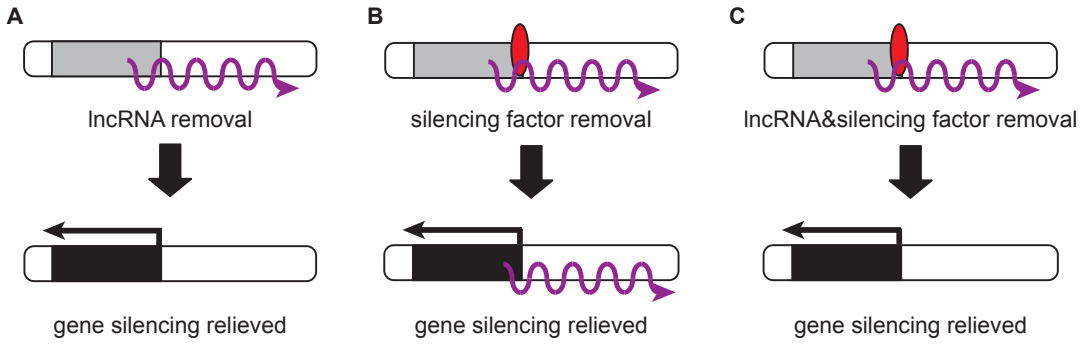

Fig. 40. Strategies to reactivate epigenetically silenced genes. In disease, aberrant lncRNA expression can result in epigenetic silencing of genes. (**A**) If the lncRNA alone is needed to maintain silencing, strategies to remove the RNA transcript or its transcription can be adopted to relieve expression of the gene of interest. (**B**) If the lncRNA is not needed to maintain silencing, RNA-targeting strategies will not be useful and repressive epigenetic factors (red oval) responsible for silencing maintenance will have to be targeted. (**C**) If lncRNA and repressive epigenetic modifications cooperatively maintain silencing, strategies to remove both of them will have to be devised, as removing either one or the other will not relieve silencing. Adapted from (Santoro and Barlow, 2011).

5. MATERIALS AND METHODS

5.1. Chemical transformation of $CaCl_2$ competent bacteria

DH5α $CaCl_2$ competent *E. coli* were thawed for 30 min on ice. After addition of DNA (1 µl of circular plasmid or 20 µl of a ligation solution), the bacteria were incubated on ice for additional 30 min, heat-shocked by placing in a 42°C water bath for 90 sec and incubated again on ice for 2 min. 900 µl of LB broth medium, prewarmed to 37°C, were then added. The bacteria were incubated for 45 min at 37°C with shaking and plated on circlegrow-agar plates containing 50 µg/ml ampicillin. For blue-white selection, 100 µl of 0.1 M IPTG and 20 µl of 50 mg/ml X-Gal in N, N'-dimethyl-formamide were spread over the circlegrow-agar plate and incubated for 30 min at 37°C before use. Plates were incubated overnight at 37°C.

5.2. Induction of FLP or Cre recombination in EL250 and EL350 bacteria

Single colonies of EL250 or EL350 DH10β-derived *E. coli* strains were inoculated in 5 ml LB broth medium and cultured overnight at 32°C. On the next day, 1 ml of the overnight culture was inoculated to 10 ml LB and cultured at 32°C for 2-3 hrs, until the optical density (OD600) was 0.5. After adding 100 µl of 10% L-arabinose to induce FLP or Cre recombinase expression, bacteria were cultured for 1 hr at 32°C and then centrifuged for 30 min at 4.3 krpm at 4°C. The supernatant was discarded and the pellet resuspended in 1 ml ice-cold H_2O and transferred to a 1.5 ml microfuge tube on ice. Cells were spun for 20 sec at 13.2 krpm at 4°C in a microcentrifuge and washed with 1 ml ice-cold H_2O four more times. The bacteria were then resuspended in 50 µl ice-cold H_2O and transferred to a 0.1 cm-gap electroporation cuvette. After adding 1 ng of plasmid DNA and incubating for 1 min on ice, the bacteria were electroporated in a Gene Pulser (BioRad), using 1.75 kV, 25 µF and pulse controller set at 200 Ω. 1 ml LB broth medium prewarmed to 32°C was added, the bacteria were incubated for 1 hr at 32°C with shaking and 100 µl of the culture was plated on circlegrow-agar plates containing 50 µg/ml ampicillin. Plates were incubated overnight at 32°C. Single colonies were then screened for FLP- or Cre-mediated recombination by mini prep and restriction digest of plasmid DNA.

5.3. Mini prep of plasmid DNA from bacteria

Single colonies were inoculated in 3 ml LB broth medium containing 50 µg/ml ampicillin and cultured overnight at 37°C with shaking. On the next day, 1.5 ml of the liquid culture were transferred into 1.5 ml microfuge tubes and spun for 1 min at 13.2 krpm at room temperature in a microcentrifuge. The supernatant was discarded and the pellet resuspended in 100 µl of ice-cold Alk-1 solution. 200 µl of Alk-2 solution were added to the suspension, mixed by inversion and incubated for 5 min on ice. The lysis reaction was stopped by adding 150 µl of Alk-3 solution, inverting to mix and incubating for 5 min on ice. After 5 min centrifugation at 13.2 krpm at 4°C, the supernatant was transferred to a fresh 1.5 ml microfuge tube. Plasmid DNA was precipitated by adding 0.6 volumes isopropanol, inverting and incubating for 2 min at room temperature, followed by centrifugation for 10 min at 13.2 krpm at 4°C. The DNA was washed once with 70% ethanol and dissolved in 50 µl of TE buffer.

5.4. Midi and maxi prep of plasmid DNA from bacteria

Single colonies were inoculated in a 2-step liquid culture of LB broth medium containing 50 µg/ml ampicillin. In the morning, a 3 ml starter culture was inoculated and incubated for 8-10 hrs at 37°C with shaking. These 3 ml cultures were then diluted into a larger culture, with a final volume of 50 ml (midi) or 250 ml (maxi), which were incubated overnight at 37°C with shaking. Plasmid DNA was isolated using the QIAFilter Plasmid Midi Kit or the EndoFree Plasmid Maxi Kit, according to the manufacturer's instructions.

5.5. Restriction digests

1-35 µg of DNA were digested using the appropriate enzyme (number of units varied depending on the incubation time), in the supplied buffer for 2 hrs or overnight at 37°C.

5.6. DNA electrophoresis

DNA was loaded on 0.8%-2% agarose gels together with a DNA ladder. Electrophoresis was performed in 1x TAE at 7 V/cm. Gels were stained in 1 mg/l ethidium bromide and photographed.

5.7. Enzymatic DNA modifications for cloning of DNA

For blunt-ending of 5' protruding ends, DNA was incubated for 10 min at 37°C with 0.05 mM dNTP mix and 4U of Klenow fragment, in the supplied buffer or a compatible restriction enzyme buffer. The reaction was stopped by heating for 10 min at 75°C. For blunt-ending of 3' protruding ends, DNA was incubated for 20 min at 11°C with 0.1 mM dNTP mix and 5U of T4 DNA polymerase, in the supplied buffer or a compatible restriction enzyme buffer. The reaction was stopped by heating for 10 min at 75°C. To dephosphorylate 5'-termini of vector backbones, 1U of calf intestine alkaline phosphatase was used, in the supplied buffer or a compatible restriction enzyme buffer. The reaction was incubated for 30 min at 37°C and stopped by heating for 15 min at 85°C.

5.8. Gel elution

DNA elution from agarose gels was performed using the Wizard SV Gel and PCR Clean-Up System, according to the manufacturer's instructions.

5.9. Ligation

Ligation reactions were performed using either the pGEM-T Easy Vector System, according to the manufacturer's instructions, or by incubating the DNA with 5U of T4 DNA ligase and the supplied buffer in 20 µl volume reactions, overnight at 16°C. Ligations were set with vector-insert molar ratios of 1:1 and 1:3.

5.10. DNA sequencing

DNA was sequenced at LGC Genomics (Berlin, Germany) or Microsynth AG (Balgach, Switzerland) using standard (M13F, M13R) or custom sequencing primers.

5.11. PCR

PCR primers, listed in section 5.37, were designed using Primer3 (Rozen and Skaletsky, 2000) and synthesized by VBC-Biotech Service GmbH (Vienna, Austria) or Sigma-Aldrich Handels GmbH (Vienna, Austria). The lyophilized primers were dissolved in TE buffer to a stock concentration of 100 pmol/µl and 1:10 dilutions

used as working solutions. PCR reactions were performed in 50 µl final volume with 0.1 µl GoTaq DNA polymerase (5U/µl), 1 µl dNTP mix (10 mM), 10 µl GoTaq Flexi Buffer (5x), 5 µl $MgCl_2$ (25 mM), 4 µl forward primer (10 pmol/µl), 4 µl reverse primer (10 pmol/µl) and 4 µl betaine (5 M). 10 ng of cosmid DNA or 100 ng of genomic DNA were used as a template. PCR conditions were as follows: initial denaturation at 94°C for 5 min; 35 cycles of 94°C for 30 sec, 58°C for 1 min and 72°C for 1 min/kb; final extension step of 72°C for 5 min.

5.12. Cloning of Southern/Northern blot probes

Genomic DNA of S12/+ ES cells (for probes Cdkn1c, R26E1 and T16INT), cos940PS cosmid DNA (for probes PFS3 and PFS6) or pCAG-FLPe-ER^{T2}-IRES-βgeo plasmid DNA (for probe FLP) was amplified with PCR primers listed in section 5.37. PCR conditions were as described above. Plasmids were generated by cloning the PCR products into pGEM-T Easy (see above).

5.13. Cloning of pCAG-FLPe-ER^{T2}-IRES-βgeo transgenic construct

The 2.7 kb FLPe-ER^{T2} coding sequence was cut out from pBKC-FLPe-ER^{T2} using SacI and KpnI (Hunter et al., 2005), blunt-ended using T4 DNA polymerase and inserted upstream of the IRES sequence in pCAG-IRES-βgeo, previously linearized with NotI, blunt-ended using Klenow fragment and dephosphorylated. Before electroporation into ES cells, the pCAG-FLPe-ER^{T2}-IRES-βgeo construct was linearized with AvrII.

5.14. Cloning of pAirnCKO targeting construct

The pBSXbaXba plasmid, used to generate both pAirnCKO and pAirnCRes targeting constructs, was cloned by Paulina Latos. A 7.3 kb XbaI-XbaI fragment, containing the *Airn* transcription start site and exons 3 and 4 of *Igf2r*, was subcloned from cos940PS into pBSIIKS(-), previously digested with BamHI and SmaI and blunt-ended. The resulting pBSXbaXba plasmid contains a 129Sv homology region corresponding to chr17:12,738,432-12,745,760 (UCSC build GRCm38/mm10). To generate pAirnCKO, I flanked a 1.9 kb PacI-NsiI region (chr17:12,740,792-12,742,677) with *loxP* sites. Firstly, the *loxP*-PGKprom-Neo^R-PGKpA-*loxP* selection cassette was cut out from pKSloxPNT using EcoRI and SalI, blunt-ended using

Klenow fragment and ligated into the NsiI site (blunt-ended using T4 DNA polymerase and dephosphorylated) of pBSXbaXba. The resulting plasmid, called pBSXbaXba-floxNeoNsi, was transformed into the Cre recombinase-expressing EL350 *E. coli* strain (see above) to remove the floxed selection cassette and obtain pBSXbaXba-loxNsi, which contains a single *loxP* site at the NsiI position. To generate the final targeting construct, the *FRT*-PGKprom-Neo^R-PGKpA-*FRT-loxP* selection cassette was excised from pK-II using ApaI and SacII, blunt-ended using T4 DNA polymerase and ligated into the PacI site (blunt-ended using T4 DNA polymerase and dephosphorylated) of pBSXbaXba-loxNsi. The functionality of *loxP* and *FRT* sites was checked by transforming pAirnCKO into EL350 and EL250 *E. coli* strains, respectively (see above). Before electroporation into ES cells, the targeting construct was linearized with SacI.

5.15. Cloning of pAirnCRes targeting construct

To obtain the CRes construct, a 1.2 kb rabbit β-globin pA cassette (βg-pA) (Sleutels et al., 2002) was inserted into a BamHI site inside *Igf2r* intron 2, at chr17:12,744,359. Iveta Yotova generated plasmid pLpA1.2 by excising the βg-pA sequence from plasmid pβG-BS with BamHI and SalI and subcloning it into the HindIII site of pBSKS-Lp (a pBluescript vector containing one *loxP* site) by blunt-end ligation. I subcloned the *FRT*-PGKprom-Neo^R-PGKpA-*FRT-loxP* selection cassette (see above) into the dephosphorylated EcoRV site of pLpA1.2, generating plasmid pFRTNeo-LpA1.2. To obtain the final targeting construct, the entire floxed sequence containing the βg-pA and the selection cassette was excised from pFRTNeo-LpA1.2 using KpnI and SacII, blunt-ended using T4 DNA polymerase and ligated into the BamHI site (blunt-ended using Klenow fragment and dephosphorylated) of pBSXbaXba (see above). The functionality of *loxP* and *FRT* sites was checked by transforming pAirnCRes into EL350 and EL250 *E. coli* strains, respectively (see above). Before electroporation into ES cells, the targeting construct was linearized with SacI.

5.16. Southern blotting

5.16.1. Genomic DNA isolation

Cells were lysed overnight at 55°C in a suitable amount of DNA lysis buffer (400 µl for ES cell clones from 24-well plates, 1 ml for cells from 10 cm dishes, 2 ml for

embryoid bodies from T75 flasks). To precipitate cell debris, 300 µl of a saturated NaCl solution were added, followed by centrifugation for 10 min at 13.2 krpm in a microcentrifuge at room temperature. The supernatant was transferred to a fresh microfuge tube and mixed with 0.6 volumes isopropanol to precipitate the DNA. After centrifugation for 10 min at 13.2 krpm at 4°C, the DNA was washed with 70% ethanol and dissolved in the appropriate amount of TE buffer overnight at 55°C.

5.16.2. Digests, electrophoresis and blotting

15-20 µg of genomic DNA (for ES cell screening: 15 µl of 35 µl DNA solution) were digested overnight at 37°C with 20 U of the appropriate restriction enzyme in the supplied buffer. Digests were loaded onto a 0.8% agarose gel together with a DNA ladder and electrophoresis performed in 1x TBE at 5.3 V/cm. After staining in 1 mg/l ethidium bromide, the gel was photographed and washed twice for 30 min in denaturing solution to denature the DNA. The gel was then placed upside down on 3MM chromatography paper folded over a glass plate with its ends immersed in denaturing solution. A Hybond-XL nylon membrane was soaked for 1 min in ddH_2O, then in denaturing solution and placed onto the gel. To avoid short circuits, areas of the gel not covered by membrane were covered with plastic stripes. Two more sheets of 3MM chromatography paper soaked in denaturing solution were added on top. Finally, a staple of paper towels, a glass plate and a blot weight were placed on top of the blot. Capillary transfer was allowed to proceed for at least 18 hrs, after which the blot was disassembled. The nylon membrane was neutralized by soaking in 20 mM Na_2HPO_4 for 5 min.

5.16.3. Probe labeling

Southern blot probes, listed in section 5.37, were labeled by random priming. After denaturation by heating at 99°C for 5 min, followed by 2 min on ice, 20 ng of probe fragment were mixed to 18 µl of LS buffer, 5.5 µl of CTG mix and H_2O, to a final volume of 37.5 µl. To this mix, 2-3 µl of α^{32}P-dATP (10 µCi/µl) and 2U of Klenow fragment were added and the reaction incubated at room temperature for at least 6 hrs to overnight. The labeled probe was cleaned to remove unincorporated radioactive nucleotides, using a Sephadex G-50 spin column.

5.16.4. Membrane hybridization

The membrane was prehybridized in Church buffer in a rotating hybridization tube for 30 min-2 hrs at 65°C. The labeled probe was denatured (see above) and added

to the prehybridized membrane in fresh Church buffer. The membrane was kept in the rotating hybridization tube for at least 18 hrs at 65°C. After hybridization, the membrane was washed twice for 30 min in Church wash, prewarmed to 65°C, then sealed in plastic and exposed to a PhosphorImager screen. Screens were scanned in the Typhoon Trio Scanner (Amersham).

5.16.5. Membrane stripping for rehybridization

Membranes were stripped by shaking twice for 30 min at room temperature in 40 mM NaOH. After rinsing three times with ddH_2O, membranes were neutralized in 20 mM Na_2HPO_4 and hybridized as above.

5.17. Establishment of primary MEFs

Wildtype FVB or DR4 (Tucker et al., 1997) mice were crossed and pregnant mice were sacrificed at 13.5 dpc. Whole embryos were dissected, minced through a 20G Sterican needle and seeded on a 15 cm cell culture dish (three embryos per dish). The primary MEFs were grown in MEF medium at 37°C in 5% CO_2 atmosphere. After 3 days, cells were trypsinized and frozen in 50% MEF medium, 40% FBS and 10% dimethyl sulfoxide (DMSO).

5.18. Feeder cell preparation for ES cell culture

Primary MEFs were thawed and expanded in MEF medium. Every third day, they were passaged at a 1:3 ratio by washing once in prewarmed D-PBS, trypsinizing for 3 min at 37°C and resuspending in fresh medium. After three passages, cells were harvested, γ-irradiated for 6 min with 5 Gy/min in the Gammacell 3000 Elan (MDS Nordion) and frozen (see above).

5.19. ES cell culture and differentiation

ES cells were grown in ES cell medium at 37°C in 5% CO_2 atmosphere. Medium was replaced daily and cells were passaged every second or third day, according to their density. ES cells were kept on irradiated feeder cells in gelatinized dishes. Wildtype feeders were used for normal expansion and DR4 feeders for G418 selection. Before differentiation, cells were feeder-depleted for 20 min and passaged at least once on feeder-free gelatinized dishes. Monolayer differentiation was

induced by LIF withdrawal and addition of 0.27 µM retinoic acid. Cells were seeded on 10 cm dishes at appropriate densities to be confluent on the day of harvesting. Embryoid body differentiation was induced by LIF withdrawal and ES cell aggregation in AggreWell plates for 8 hrs, followed by culture on ultra-low adherence T75 flasks. Differentiating cells were fed every day or every other day, depending on the experimental setup.

5.20. Doxycycline and tamoxifen treatment of ES cells

Doxycycline was dissolved in embryo transfer H_2O to a stock concentration of 10 mg/ml and diluted 1:10 to obtain working dilutions. To activate the Tet-inducible promoter, doxycycline was diluted in the cell medium to a final concentration of 1 µg/ml and fresh doxycycline was added with every medium change. 4-hydroxytamoxifen was dissolved in 96% ethanol to a 10 nM stock concentration and diluted 1:10 to obtain working dilutions. For use in cell culture, tamoxifen was diluted in medium to the desired concentration. 0.1-1 µM concentrations were used to test Cre recombination efficiency in undifferentiated ES cells and 1µM concentration was used for all differentiations. Fresh tamoxifen was added with every medium change and untreated control cells were fed with medium containing an equal volume of 96% ethanol.

5.21. ES cell targeting

5.21.1. Electroporation and selection

ES cells were feeder-depleted and $8x10^6$ cells in 800 µl of D-PBS were electroporated with 35 µg of linearized targeting construct in a 0.4 cm-gap electroporation cuvette. A Gene Pulser II (BioRad) was used with 0.24 kV and 500 µF. Cells were seeded onto DR4 feeders and G418 selection was started 24 hrs after electroporation, by feeding the cells with ES cell medium supplemented with 400 µg/ml (for APD-Tet cells) or 250 µg/ml (for $S12^{RC}$/+ cells) G418. The selection medium was replaced every day for 6-8 days. Single G418-resistant colonies were picked, trypsinized, transferred to a 24-well plate on wildtype feeder cells and grown for 2-3 days in non-selective medium. Clones were then trypsinized and half of the cell suspension was frozen in a 96-well plate with 50% ES cell media, 40% FBS and 10% DMSO. The second half was seeded onto gelatinized 24-well plates, grown for

3-6 days till confluent and harvested for DNA isolation and genotyping by Southern blotting (see above).

5.21.2. Selection cassette removal by transient transfection

Cells were electroporated as above with 50 µg pMC-Cre or pCAGGS-FLPe plasmid, diluted 1:3000 to 1:10000 and seeded onto wildtype feeders. Colonies were grown for 5-7 days in non-selective medium. Picking, freezing and expansion for DNA isolation were performed as above.

5.21.3. Subcloning of ES cells

Confluent cells from one 10 cm dish were harvested in growth medium, diluted 1:500 to 1:10000 and plated onto wildtype feeders. Colonies were grown for 5-7 days. Picking, freezing and expansion for DNA isolation were performed as above.

5.22. RNA isolation

Cells were lysed in a suitable amount of TRI reagent (1 ml for cells from 10 cm dishes, 2 ml for embryoid bodies from T75 flasks) and total RNA was isolated according to the manufacturer's instructions. RNA pellets were dissolved in the appropriate amount of RNA storage solution and stored at -80°C.

5.23. DNaseI treatment of RNA

To remove contaminating DNA prior to reverse transcription, RNA samples were treated with DNaseI for 30 min. The DNA-free Kit was used according to the manufacturer's instructions.

5.24. Reverse transcription of RNA

Reverse transcription of 2-4 µg of DNaseI-treated RNA samples (1-2 µg each for plus and minus reverse transcriptase reactions) was performed using the RevertAid First Strand cDNA Synthesis Kit with random hexamer primers, according to the manufacturer's instructions.

5.25. RT-PCR

RT-PCR reactions were performed in 50 µl final volume with 0.2 µl GoTaq DNA polymerase (5U/µl), 1 µl dNTP mix (10 mM), 10 µl GoTaq Flexi Buffer (5x), 4 µl $MgCl_2$ (25 mM), 2.5 µl forward primer (10 pmol/µl), 2.5 µl reverse primer (10 pmol/µl). 2 µl of cDNA or 2 µl of the –RT reaction (cDNA reaction in the absence of reverse transcriptase) were used as template. PCR conditions were as follows: initial denaturation at 95°C for 3 min, 35 cycles of 95°C for 45 sec, 52°C (Cre) or 59°C (Ex12cDNA) for 45 sec and 72°C for 45 sec; final extension step of 72°C for 5 min. Primers are listed in section 5.37.

5.26. Real-time qPCR

PrimerExpress (Applied Biosystems) or Primer3 (Rozen and Skaletsky, 2000) were used to design primers and Taqman probes, listed in section 5.37. Primers were synthesized by VBC-Biotech Service GmbH (Vienna, Austria) or Sigma-Aldrich Handels GmbH (Vienna, Austria) and probes by Microsynth AG (Balchag, Switzerland). qPCR runs were performed on a 7900HT Fast Real Time PCR (Applied Biosystems). For Taqman assays, 900 nM primers, 200 nM probe and qPCR Mastermix Plus were used. For SybrGreen assays, 100 nM primers and Mesa Green qPCR Master Mix Plus were used. PCR conditions were as follows: 2 min 50°C, 10 min 95°C, 40 cycles of 15 sec 95°C and 1 min 60-65°C. Serial dilutions of cDNA or plasmid DNA were used to calculate the standard curve. Relative quantification and statistics were performed according to the manufacturer's instructions.

5.27. RNA electrophoresis

Gel chamber, tray and combs were soaked in ddH_2O + 1/1000 (v/v) DEPC for 30 min. To check quality of RNA templates before FISH probe preparation, 18 µl of formaldehyde loading dye were added to 200 ng of RNA probe template dissolved in RNase-free H_2O. For Northern blot analysis, 18 µl of formaldehyde loading dye were added to 15 µg of total RNA dissolved in RNA storage solution. Samples were heated at 65°C for 15 min, then placed on ice until loading. Denaturing 1% agarose gels were prepared using NorthernMax denaturing gel buffer, according to the manufacturer's instructions, and electrophoresis was performed in NorthernMax

running buffer at 6.6 V/cm at 4°C. Gels were stained in 1 mg/l ethidium bromide and photographed.

5.28. Northern blotting

Blots were assembled as described in section 5.16.2 using 50 mM Na_2HPO_4 + 1/1000 (v/v) DEPC. After blotting, the nylon membrane was dried at 55°C for 15 min and crosslinked in the UV Stratalinker 1800 (Stratagene) for 18 sec with 120000 μJ/cm^2. To visualize blotted RNA, the membrane was stained with methylene blue, destained in 25 mM Na_2HPO_4 and scanned to record the image. Probes were labeled as described in section 5.16.3. Hybridization tubes were treated for 30 min with ddH_2O + 1/1000 (v/v) DEPC. Membrane pre-hybridization, hybridization, washing and scanning were performed as described in section 5.16.4.

5.29. RNA fluorescence *in situ* hybridization (FISH)

5.29.1. Probe preparation

The AIFP1 probe (see section 5.37 for chromosomal coordinates) was PCR-amplified from cosOT1 cosmid DNA, with one of the PCR primers carrying a T7 RNA polymerase promoter sequence (5'-TAATACGACTCACTATAGGG-3'). The PCR product was then used as a template for *in vitro* transcription, performed with the MegaScript Kit according to the manufacturer's instructions. The RNA was cleaned with the RNeasy Mini Kit, according to the manufacturer's instructions, and run on a denaturing agarose gel to check size and integrity of the RNA product (see above). To synthesize the digoxigenin (DIG)-labeled cDNA probe, 4 μg of RNA in 2 μl RNase-free H_2O were added to 1.5 μl hexamers (5U/μl) and denatured for 5 min at 70°C. The following reagents were then added on ice: 4 μl Superscript buffer (5x), 2 μl DTT (0.1M), 7.5 μl DIG dNTP mix, 1 μl RNasin. The mixture was incubated for 5 min at 25°C, 2 μl Superscript RT were added and the reaction was incubated for 10 min at 25°C, followed by 90 min at 42°C and 10 min at 70°C. The RNA template was destroyed by adding 2 μl NaOH (4 M) and incubating for 30 min at 37°C, followed by addition of 2 μl HCl (4 M) to neutralize the reaction. The probe was cleaned with the Nucleotide Removal Kit, eluted in 30 μl H_2O and 1 μl of probe was used per slide.

5.29.2. Cell fixation

Differentiated ES cells were harvested by trypsinization and resuspended in D-PBS at a concentration of $2x10^6$/ml. 20 μl of this suspension were applied per microscope slide and allowed to settle down for 2 min. To permeabilize the nuclei, the slides were incubated for 5 min at 4°C in CSK buffer containing 0.5% (v/v) Triton X-100 and 5% (v/v) ribonucleoside vanadyl complexes. Nuclei were then fixed for 10 min at 4°C in PFA (4% in PBS, pH=7.2). The slides were washed three times for 5 min at room temperature in 70% EtOH and stored in 70% EtOH at -20°C or hybridized immediately (see below).

5.29.3. Hybridization

For each slide, 1 μl probe and 1 μl mouse Cot-1 DNA (1 μg/μl) were mixed in 15 μl Fraser hybridization buffer. The probe was denatured by heating at 80°C for 5 min and pre-annealed at 37°C for at least 15 min. The slides were dehydrated by washing at room temperature for 5 min in 70% EtOH, 3 min in 90% EtOH and 3 min in 97% EtOH. The probe mix was applied on the air-dried dehydrated slides, which were then covered with coverslips and incubated overnight at 37°C in a humidified chamber containing hybridization solution.

5.29.4. Washing and detection

Slides were washed three times for 10 min at 39.5°C in wash solution and three times for 10 min at 39.5°C in 2x SSC. Both solutions were prewarmed to 39.5°C before use. Slides were then washed once for 5 min at room temperature in TST buffer, covered with 100 μl of TSB buffer under coverslips and incubated overnight at 37°C in a humidified chamber containing TST buffer. After blocking, a FITC-conjugated sheep α-DIG antibody, diluted 1:1000 in TSB buffer, was applied under a coverslip and the slides incubated for at least 30 min at room temperature in the humidified chamber, as above. Slides were washed twice for 5 min at room temperature in TST buffer. 1:200 dilutions of the second antibody (a FITC-conjugated rabbit anti-sheep) and the third antibody (a FITC-conjugated goat anti-rabbit) were applied and washed as described for the first antibody. Finally, slides were washed for 5 min at room temperature in TS buffer, dehydrated (see above) and air-dried. Mounting media containing DAPI was applied under a coverslip, which was then sealed with nail polish to prevent evaporation.

5.29.5. Image acquisition and counting

Slides were observed with an Axioplan2 fluorescence microscope (Zeiss), using filters for FITC (495/519 nm) or DAPI (359/461 nm). RNA FISH signals were counted by three people (F. Pauler, F. Santoro, S. Stricker) and at least one of the counts was performed blind.

5.30. Bisulfite sequencing

10 µg genomic DNA were digested with 20U EcoRI and treated with 10 µg RNaseA overnight at 37°C. Digested DNA was purified from the reaction mixture with the QIAquick PCR Purification Kit, according to the manufacturer's instructions. Bisulfite conversion was performed on 1 µg purified DNA, using the EpiTect Bisulfite Kit, according to the manufacturer's instructions. Bisulfite sequencing PCR primers (listed in section 5.37) were designed using MethPrimer (Li and Dahiya, 2002) or Zymo Research Bisulfite Primer Seeker (http://www.zymoresearch.com/tools/bisulfite-primer-seeker). PCR reactions were performed in 50 µl final volume with 2.5 µl JumpStart REDTaq DNA polymerase (1U/µl), 1 µl dNTP mix (10 mM), 5 µl JumpStart PCR Buffer (10x), 1.25 µl forward primer (10 pmol/µl) and 1.25 µl reverse primer (10 pmol/µl). 1 µl of bisulfite converted genomic DNA was used as a template. PCR conditions were as follows: initial denaturation at 94°C for 1 min; 40 cycles of 94°C for 1 min, 58°C for 30 sec and 72°C for 1 min; final extension step of 72°C for 5 min. PCR products were gel-purified and ligated into pGEM-T Easy (see above). Plasmid DNA from single colonies was purified using the QIAprep Spin Miniprep Kit, according to the manufacturer's instructions, and analyzed by restriction digest (see above). Positive clones were sequenced at LGC Genomics, using standard primers (see above). Ruth Klement helped with PCR, cloning and plasmid preps.

5.31. Chromatin immunoprecipitation (ChIP)

ChIP was performed by Florian Pauler using published protocols and antibodies (Regha et al., 2007). Immunoprecipitated DNA was analyzed by qPCR, using primers listed in section 5.37.

5.32. Protein isolation

Cells were washed once in ice-cold D-PBS, lysed on ice in Frackelton buffer (200 µl for a 10 cm dish) and transferred to a 1.5 ml microfuge tube. After 5 min incubation on ice, the cell extract was cleared by centrifugation for 5 min at 13.2 krpm in a microcentrifuge at 4°C. The supernatant was transferred to a fresh microfuge tube and mixed with an equal volume of 2x Laemmli buffer. Samples were heated at 95°C for 10 min, placed on ice and loaded on a gel for Western blot analysis (see below).

5.33. Western blotting

Western blotting was performed by Nina Gratz. 10 µl of 200 µl protein lysate were loaded onto a 10% polyacrylamide gel (a 4% polyacrylamide gel was used for stacking) together with a protein ladder. Electrophoresis was carried out in running buffer at 25 V/cm. A semi-dry blot was then assembled by stacking the following between cathode and anode of the transfer apparatus: six sheets of 3 MM chromatography paper soaked in cathode buffer, the gel, an Optitran nitrocellulose membrane soaked in anode II buffer, three sheets of 3MM chromatography paper soaked in anode I buffer and five sheets soaked in anode I buffer. Proteins were transferred at 20 V (60 mA) for 2 hrs, after which the blot was disassembled. The nitrocellulose membrane was stained with Ponceau S solution to visualize transferred proteins, destained in TBST buffer and blocked with 15% milk in TBST for 1 hr at room temperature. After washing three times for 10 min with TBST, a rabbit α-Cre antibody was added. The antibody was diluted 1:1000 in TBST containing 1% BSA and 0.05% NaN_3 and incubated overnight at 4°C. The membrane was then washed three times for 10 min with TBST and incubated with a 1:20000 dilution of an IRDye800-conjugated anti-rabbit IgG antibody for 30 min at room temperature. Membranes were scanned with an Odyssey Infrared Imaging System (LI-COR).

5.34. Bioinformatics and statistical analysis

The December 2011 (GRCmm38/mm10) assembly of the UCSC genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) was used to retrieve mouse genomic sequences. The genomic localization of constructs, primers and probes was visualized through BLAT search (http://genome.ucsc.edu/cgi-

bin/hgBlat?hgsid=295650607&command=start). Interspersed repeats were identified using RepeatMasker (http://www.repeatmasker.org/). The intensity of Southern blot bands was quantified using the ImageQuant (Amersham) software. Analysis and quality control of bisulfite sequencing data were performed using BiQAnalyzer and standard settings (Bock et al., 2005). *P*-values were calculated using analysis of variance (ANOVA) in R statistical environment (r-project.org) or unpaired Student's *t*-test (http://www.graphpad.com/quickcalcs/). ANOVA tests were run by Alexey Stukalov.

5.35. Materials

Materials	Source/Reference
Mice	
FVB (wildtype)	
DR4	(Tucker et al., 1997)
ES cells	
D3 (129Sv/129Sv)	gift from Erwin Wagner
Bacteria	
DH5α	(Taylor et al., 1993)
EL250	gift from Alexander Stark
EL350	gift from Alexander Stark
Plasmids/cosmids	
pBKC-FLPe-ER^{T2}	gift from Susan Dymecki
pBSIIKS(-)	Stratagene
pCAGGS-FLPe	gift from Erwin Wagner
pCAG-IRES-βgeo	gift from Austin Smith
pGEM-T Easy	Promega
pK-II	gift from Maria Sibilia
pKSloxPNT	gift from Maria Sibilia
pMC-Cre	gift from Erwin Wagner
pR26CreER^{T2}	gift from Austin Smith
pR26/N-rtTA2^{S}-M2	gift from Anton Wutz
cos940PS	(Stoger et al., 1993)
cosOT1	(Lyle et al., 2000)
Chemicals and other materials	
20G Sterican needle	Braun
3MM chromatography paper	Whatman
6-aminocaproic acid	Sigma
α-^{32}P-dATP	PerkinElmer
β-mercaptoethanol	Sigma

β-mercaptoethanol for cell culture	Gibco
Acetic acid	VWR
Agar	AppliChem
Agarose for DNA work	Biozym
Agarose for RNA work	Ambion
AggreWell plates	Stemcell Technologies
Ammonium persulfate (APS)	Roth
Ampicillin	Roche
Betaine	Sigma
Blocking reagent	Roche
Boric acid	AppliChem
Bovine serum albumin (BSA)	QBIOgene
Bromophenol blue	Sigma
Cell culture dishes	Nunc
Circlegrow broth	MP Biomedicals
Cot-1 DNA	Invitrogen
dCTP, dTTP, dGTP for Southern blot probes	Bioron
Denhardt's solution	QBIOgene
Diethyl pyrocarbonate (DEPC)	Sigma
DIG-dNTP mix	Roche
Dimethyl sulfoxide (DMSO)	Sigma
Dithiothreitol (DTT)	Sigma
DNA ladder, 100 bp	Fermentas
DNA ladder, 1 kb	Fermentas
dNTP mix (10 mM)	Fermentas
Doxycycline hyclate	Sigma
Dulbecco's Modified Eagle Medium (DMEM)	Gibco
Dulbecco's Modified Eagle Medium (DMEM)+HEPES	Gibco
Dulbecco's Phosphate Buffered Saline (D-PBS)	Gibco
Electroporation cuvettes	BioRad
Ethanol 96%	Merck
Ethidium bromide	Merck
Ethylenediaminetetraacetic acid (EDTA)	Merck
Fetal bovine serum (FBS), ES cell tested	PAA
Formaldehyde loading dye	Ambion
Formamide (FA)	Fluka
G418	Gibco
Gelatin	Sigma
Gentamicin	Gibco
Glucose	Gibco
Glycerol	QBIOgene

Glycine	Sigma
HCl	Merck
HEPES	Roth
Hybond-XL nylon membrane	Amersham
Isopropanol	Merck
Isopropyl-β-D-thiogalactopyranoside (IPTG)	AppliChem
KAc	Sigma
L-(+)-arabinose	Sigma
L-glutamin	Gibco
LB broth medium	Lab M Limited
MEM non-essential amino acids	Gibco
Mesa Green qPCR Mastermix Plus	Eurogentec
Methanol	Roth
Methylene blue	Merck
$MgCl_2$	Sigma
$MgCl_2$ (25 mM, for PCR)	Fermentas
Mounting media, with DAPI	Vectashield
Mouse Cot1-DNA	Invitrogen
N,N'-dimethyl-formamide	Sigma
NaCl	AppliChem
NaH_2PO_4	Merck
Na_2HPO_4	Merck
NaF	Sigma
NaOH	AppliChem
$Na_4P_2O_7$	Sigma
NorthernMax 10x denaturing gel buffer	Ambion
NorthernMax 10x running buffer	Ambion
Optitran nitrocellulose membrane	Whatman
PAGE Ruler	Fermentas
Paraformaldehyde (PFA)	Sigma
Phosphate buffered saline (PBS)	QBIOgene
PhosphorImager screen	Fuji Photo Film
PILLE protease inhibitors	Roche
PIPES	Sigma
qPCR Mastermix Plus	Eurogentec
Random hexamer primers for RNA FISH probes	Roche
Random hexamer primers for Southern blot probes	Pharmacia
Retinoic acid	Sigma
Ribonucleoside vanadyl complex	New England BioLabs
RNA millennium marker	Ambion
RNA storage solution	Ambion

RNasin	Promega
Rotiphorese gel 30	Roth
Salmon sperm DNA	Invitrogen
Sephadex G-50	Amersham
Sodium citrate	Sigma
Sodium dodecyl sulfate (SDS)	AppliChem
Sodium pyruvate	Sigma
Sucrose	Sigma
Superfrost PLUS microscope slides	Roth
TEMED	Roth
TRI reagent	Sigma
Tris	AppliChem
Triton X-100	Sigma
Trypsin-EDTA	Gibco
Tween-20	Sigma
Ultra-low adherence T75 flasks	Corning
Vectashield with DAPI	Vector Labs
Water for embryo transfer	Sigma
X-Gal	Roth
Xylenol orange	Sigma
(Z)-4-Hydroxytamoxifen	Sigma
Enzymes and buffers	
Calf intestine alkaline phosphatase (CIAP)	Fermentas
GoTaq DNA polymerase	Promega
GoTaq Flexi buffer	Promega
Klenow Fragment	Fermentas
Proteinase K	QBIOgene
Restriction enzymes	Fermentas/Roche
RNaseA	Fermentas
Superscript II reverse transcriptase	Invitrogen
T4 DNA ligase	Fermentas
T4 DNA polymerase	Invitrogen
Antibodies	
FITC-conjugated sheep α-DIG antibody	Roche
FITC-conjugated rabbit anti-sheep antibody	Calbiochem
FITC-conjugated goat anti-rabbit antibody	Calbiochem
IRDye800-conjugated anti-rabbit IgG antibody	Rochland
Rabbit α-Cre antibody	Covance
Kits	
DNA-free Kit	Ambion
EndoFree Plasmid Maxi Kit	Qiagen

EpiTect Bisulfite Kit	Qiagen
MegaScript Kit	Ambion
Nucleotide Removal Kit	Qiagen
pGEM-T Easy Vector System	Promega
QIAFilter Plasmid Midi Kit	Qiagen
QIAprep Spin Miniprep Kit	Qiagen
QIAquick PCR Purification Kit	Qiagen
RevertAid First Strand cDNA Synthesis Kit	Fermentas
RNeasy Mini Kit	Qiagen
Wizard SV Gel and PCR Clean-Up System	Promega

5.36. Solutions

Alk-1

50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA pH 8.0

Alk-2

200 mM NaOH, 1% SDS

Alk-3

3 M KAc, 11.5% acetic acid

TE buffer

10 mM Tris-HCl pH 8.0, 1 mM EDTA

DNA loading buffer

0.5% xylenol orange, 30% glycerol, 1x TAE

TAE buffer

40 mM Tris, 20 mM acetic acid, 1 mM EDTA pH 8.0

TBE buffer

90 mM Tris, 90 mM boric acid, 2 mM EDTA pH 8.0

DNA lysis buffer

1x TEN pH 9.0, 1% SDS, 0.5 mg/ml proteinase K

5x TEN pH 9.0

250 mM Tris pH 9.0, 100 mM EDTA pH 8.0, 200 mM NaCl

CTG mix for radioactive probes

100 µM dCTP, 100 µM dTTP, 100 µM dGTP, 2 mg/ml BSA

LS buffer for radioactive probes

25 ml 1 M HEPES pH 6.6, 25 ml TM (250 mM Tris-HCl pH 8.0, 25 mM $MgCl_2$, 50 mM β-mercaptoethanol), 1 ml 30 OD U/ml random hexamer primers in TE, pH 8.0

Denaturing solution for Southern blots

0.5 M NaOH, 1.5 M NaCl

Church buffer

250 mM Na_2HPO_4, 7% SDS, 1 mM EDTA

Church wash

20 mM Na_2HPO_4, 1% SDS

ES cell medium

DMEM-HEPES (L-glutamine, 4500 mg/l D-glucose, 25 mM HEPES buffer), 15% FBS, 50 µg/ml gentamicin, 2 mM L-glutamin, 1× MEM (non-essential amino acids), 1 mM sodium pyruvate, 0.1 mM β-mercaptoethanol, LIF

MEF medium

DMEM (L-glutamine, 4500 mg/l D-glucose, 110 mg/l sodium pyruvate), 10% FBS, 50µg/ml gentamicin, 2mM L-glutamin

CSK buffer

3 mM $MgCl_2$, 100 mM NaCl, 0.1% sucrose, 10 mM PIPES

20x SSC

3 M NaCl, 300 mM sodium citrate, adjust to pH 7.0 with HCl

Fraser hybridization buffer

50% FA, 2x SSC, 200 ng/µl sheared salmon sperm DNA, 5x Denhardt's solution, 500mM NaH_2PO_4/Na_2HPO_4 pH 7.0, 1 mM EDTA

RNA FISH hybridization solution

25% FA, 2x SSC

RNA FISH wash solution

50% FA, 2x SSC

TST buffer for RNA FISH

100 mM Tris-HCl pH 7.5, 145 mM NaCl, 0.5% (v/v) Tween20

TSB buffer for RNA FISH

100 mM Tris-HCl pH 7.5, 145 mM NaCl, 1x blocking reagent

TS buffer for RNA FISH

100 mM Tris-HCl pH 7.5, 145 mM NaCl

Frackelton buffer

10 mM Tris-HCl pH 7.1, 50 mM NaCl, 50 mM NaF, 30 mM $Na_4P_2O_7$, 1% Triton X-100, 1 mM DTT, PILLE protease inhibitors

10% polyacrylamide running gel

10% Rotiphorese gel 30, 375 mM Tris pH 8.8, 0.1% SDS, 0.002% TEMED, 0.06% APS

4% polyacrylamide stacking gel

4% Rotiphorese gel 30, 375 mM Tris pH 6.8, 0.1% SDS, 0.002% TEMED, 0.06% APS

2x Laemmli buffer

125 mM Tris-HCl pH 6.8, 20% glycerol, 4% SDS, 10% β-mercaptoethanol, 0.004% bromophenol blue

PAGE running buffer

250 mM Tris, 1.92 M glycine, 1% SDS

Cathode buffer

40 mM 6-aminocaproic acid, 20% methanol, 0.01% SDS

Anode I buffer

300 mM Tris pH 10.4, 20% methanol

Anode II buffer

2.5 mM Tris pH 10.4, 20% methanol

TBST buffer for Western blot

10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween20

5.37. Primers and probes

PCR Primers			
PCR assay	**Primer name**	**Sequence (5'-3')**	**Reference**
AIFP1	AIFP1-F	GCTGGTCCTTACCTTGTGGA	(Stricker et al., 2008)
	AIFP1-R	GCAAGACCACATCACACACC	
Cdkn1c	Cdkn1c-F	ACAGACTCGCTGTCCACCTC	(Santoro et al., 2013)
	Cdkn1c-R	ACTGAGAGCAAGCGAACAGG	
CypA	CypA-F	GTCTCCTTCGAGCTGTTTGC	(Seidl et al., 2006)
	CypA-R	TTCACCTTCCCAAAGACCAC	
FLP	FLP-F	GCATCTGGGAGATCACTGAG	(Hunter et al., 2005)
	FLP-R	CCCATTCCATGCGGGGTATCG	
PFS3	PFS3-F	GAGCCTGGACTACAGGACCA	(Santoro et al., 2013)
	PFS3-R	CTCTTGGAAGCGATGGTGTT	
PFS6	PFS6-F	CCCTACTCACCTCTCCCTGA	(Santoro et al., 2013)
	PFS6-R	CCCTTCCTGTATGGGACTCA	
R26E1	R26E1-F	CCAGTCCGCCAACACAGTAG	(Stricker et al., 2008)
	R26E1-R	GGCGTTCAGGAAGATTATGG	
T16INT	T16INT-F	GGCTCCAGCTACAACTCCAG	unpublished
	T16INT-R	CCAGGAGGAAGCTGTCTGTC	
RT-PCR assay	**Primer name**	**Sequence (5'-3')**	**Reference**
Cre	Cre-F	ATCCGAAAAGAAAACGTTGA	(Kim et al., 2004)
	Cre-R	ATCCAGGTTACGGATATAGT	

Igf2r Ex12-non-qSNP	Ex12cDNA-F	TTCACAGGTGAGGTGGACTG	(Koerner et al., 2012)
	Ex12cDNA-R	CCGTGCAGTTCTCTCCTTCT	
RT-qPCR assay	**Primer/Taqman probe name**	**Sequence (5'-3')**	**Reference**
Airn-middle	Airn-TQ-F	GACCAGTTCCGCCCGTTT	(Koerner et al., 2012)
	Airn-TQ-R	GCAAGACCACAAAATATTGAAAAGAC	
	Airn-TQ-P	FAM-TACAAGTGATTATTAACTCCACGCCAGCCTCA-TAMRA	
CyclophilinA	CypA-F	AGGGTTCCTCCTTTCACAGAATT	(Koerner et al., 2012)
	CypA-R	GTGCCATTATGGCGTGTAAAGTC	
	CypA-P	FAM-TCCAGGATTCATGTGCCAGGGTGG-TAMRA	
Dnmt1	Dnmt1-F	CCTAGTTCCGTGGCTACGAGGAGAA	(Feng et al., 2010)
	Dnmt1-R	TCTCTCTCCTCTGCAGCCGACTCA	
Dnmt3a	Dnmt3a-F	GCCAAGAAACCCAGAAAGAG	(Santoro et al., 2013)
	Dnmt3a-R	TGAGGCTCCCACATGAGATA	
Dnmt3b	Dnmt3b-F	TTCAGTGACCAGTCCTCAGACACGAA	(Anier et al., 2010)
	Dnmt3b-R	TCAGAAGGCTGGAGACCTCCCTCTT	
Dnmt3l	Dnmt3l-F	CGTGGCAGAGACTACCAGAA	(Santoro et al., 2013)
	Dnmt3l-R	CTGACTTGGGCTTGCAGATA	
Flk1	Flk1-F	GGGATGGTCCTTGCATCAGAA	(Ishitobi et al., 2011)
	Flk1-R	ACTGGTAGCCACTGGTCTGGTTG	
Foxa2	Foxa2-F	TACGCCAACATGAACTCGAT	(Santoro et al., 2013)
	Foxa2-R	GTGTAGCTGCGTCGGTATGT	
Gata4	Gata4-F	CGCTGTGGCGTCGTAATG	(Latos et al., 2009)
	Gata4-R	GGAACCCCATGGAGCTTCAT	
Gata6	Gata6-F	ACCATCACCCGACCTACTCG	(Cho et al., 2012)
	Gata6-R	CGACAGGTCCTCCAACAGGT	
Igf2r Ex12-qSNP	WtSeFCG	TGGCCTTGCCCTCCTGC	(Koerner et al., 2012)
	MutSeFCG	CTGGCCTTGCCCTCCTGT	
	GeSeR2	GCTATGACCTGTCTGTGTTGGCT	
Ki67	Ki67-F	CAGTACTCGGAATGCAGCAA	(Vinuesa et al., 2008)
	Ki67-R	CAGTCTTCAGGGGCTCTGTC	
Nanog	Nanog-F	CCTCCATTCTGAACCTGAGC	(Santoro et al., 2013)
	Nanog-R	GGATGCTGGGATACTCCACT	
Nestin	Nestin-F	CAACTGGCACACCTCAAGAT	(Santoro et al., 2013)
	Nestin-R	GTGTCTGCAAGCGAGAGTTC	
Oct4	Oct4-F	ACCTTCAGGAGATATGCAAATCG	(Latos et al., 2009)
	Oct4-R	TTCTCAATGCTAGTTCGCTTTCTCT	
	Oct4-P	FAM-AGACCCTGGTGCAGGCCCGG-TAMRA	
Pcna	Pcna-F	AATGGGGTGAAGTTTTCTGC	(Vinuesa et al., 2008)
	Pcna-R	CAGTGGAGTGGCTTTTGTGA	

Rex1	Rex1-F	CTAGCCGCCTAGATTTCCAC	(Santoro et al., 2013)
	Rex1-R	CCACGTGTCCCAGCTCTTA	
Sox17	Sox17-F	CAGAACCCAGATCTGCACAA	(Glover et al., 2006)
	Sox17-R	GCTTCTCTGCCAAGGTCAAC	
qPCR assay	**Primer/Taqman probe name**	**Sequence (5'-3')**	**Reference**
Airn-125	Airn-125-F	CTGAGCTTTCCCTTCCCTTTC	(Regha et al., 2007)
	Airn-125-R	CGGAGCAATTCCGGTTGT	
	Airn-125-P	FAM-ACCGCAACTCAGCACAACCAAGGATC TAMRA	
Airn-126	Airn-126-F	GGCGGTGCTGTGCTTCTT	(Regha et al., 2007)
	Airn-126-R	TGCCGAGGCTTCAACATTATATC	
	Airn-126-P	FAM-CTGCCCGCTAGAGCAAGGAGGGAT TAMRA	
Igf2r-97	Igf2r-97-F	CACTTGCAACACTAAACATCAACCT	(Regha et al., 2007)
	Igf2r-97-R	CGCTTCCTAACTCTCTCTTCTTCA	
	Igf2r-97-P	FAM-ACTCCATCTCGGCCACCGTACTGGTC TAMRA	
Igf2r-98	Igf2r-98-F	CTCTGGGATCCAAGGTTGTATAATTT	(Regha et al., 2007)
	Igf2r-98-R	TCCCTAGGCCCACAAGTCTGT	
	Igf2r-98-P	FAM-CAGTCCTGTCGAAGTTTGTTGGTGTTGG TAMRA	
CKO recombination	CKO^{Fl}-F	AGGGTTTGGCGCTATCCT	(Santoro et al., 2013)
	CKO^{Δ}-F	TTGAACACATGGGATGGAGT	
	CKO-R	CACCCTCAATTCCGATCAT	
Bisulfite Sequencing PCR assay	**Primer name**	**Sequence (5'-3')**	**Reference**
DMR1-A	DMR1-A -F	GGAAATTGAGGTTTGGTTTTGAG	(Santoro et al., 2013)
	DMR1-A -R	CCAAAAACAACAACAACAAAAAC	
DMR1-B	DMR1-B -F	GTTTTTGTTGTTGTTGTTTTTGG	(Santoro et al., 2013)
	DMR1-B -R	AACCTTAACTCTACCCCCTACAACT	

Southern blot/Northern blot/RNA FISH probes			
Probe name	**Chromosome/ Accession Number**	**Start (bp)**	**End (bp)**
AIFP1	17	12,764,671	12,767,328
AirT	17	12,746,083	12,746,525
Cdkn1c	7	143,460,442	143,460,955
CypA	X52803	99	439
FLP	U46493	805	1562
Htf9	16	18,247,103	18,250,845
MEi	17	12,740,364	12,741,518
MSi	17	12,741,515	12,742,529
NEi	17	12,768,435	12,769,450

PFS3	17	12,747,398	12,747,722
PFS6	17	12,743,786	12,744,602
R26E1	6	113,077,486	113,078,168
T16INT	17	12,755,429	12,756,089

The bp positions for start and end refer to the GRCm38/mm10 assembly from UCSC Genome Browser or to the indicated GenBank accession numbers.

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7. CURRICULUM VITAE

Personal Information

Name:	Federica Santoro
Date of birth:	26.01.1984
Place of birth:	Naples, Italy
Nationality:	Italian

Education

2005-2007	Masters degree in Medical Biotechnology Federico II University, Naples, Italy Thesis: 'Regulation of the Glycyl-tRNA synthetase gene by Notch' (Supervisor: Prof. Tommaso Russo) CEINGE, Naples, Italy
2002-2005	Bachelor degree in Biotechnology Federico II University, Naples, Italy Thesis: '*In situ* hybridization on mouse embryo sections' (Supervisor: Prof. Roberto Di Lauro) CEINGE, Naples, Italy
1997-2002	High school Jacopo Sannazaro Gymnasium, Naples, Italy
1989-1997	Elementary and middle school Anglo-Italian Montessori School, Naples, Italy

Employment

2007-2013	Doctoral studies as part of the Vienna Biocenter and DK-RNA Biology international PhD program Thesis: 'Onset and maintenance of *Airn* non-coding RNA mediated imprinted expression in an *in vitro* embryonic stem cell model' (Supervisor: Prof. Denise Barlow) CeMM, Vienna, Austria

Publications

Santoro F, Mayer D, Klement RM, Warczok KE, Stukalov A, Barlow DP, Pauler FM. Imprinted Igf2r silencing depends on continuous Airn lncRNA expression and is not restricted to a developmental window. *Development*. 2013 Mar; 140(6): 1184-1195.

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Stricker SH*, Steenpass L*, Pauler FM*, Santoro F, Latos PA, Huang R, Koerner MV, Sloane MA, Warczok KE, Barlow DP. Silencing and transcriptional properties of the imprinted Airn ncRNA are independent of the endogenous promoter. *EMBO J*. 2008 Dec 3; 27(23): 3116-3128.
*equal contribution

Conferences

Keystone Symposia's 2012 Meeting on Non-Coding RNAs
Snowbird, Utah, USA
March 31-April 5, 2012
(poster presentation)

3rd EMBO Meeting
Vienna, Austria
September 10-13, 2011
(poster presentation)

1st EMBO Meeting
Amsterdam, Netherlands
August 29-September 1, 2009
(poster presentation)

5th Annual Meeting of the Epigenome Network of Excellence
Edinburgh, United Kingdom
July 1-4, 2009
(poster presentation)

Theoretical course on RNA Structure and Function
Trieste, Italy
March 30-April 2, 2009
(oral presentation)

8. ACKNOWLEDGMENTS

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bureaucracy; Aleksandra Kornienko, for teaching me that tea is only worth drinking if scorching hot and that a woman can never own enough shoes; Daniel Andergassen, Wolfgang Allhoff, Irena Vlatkovic, Ru Huang, Basak Senergin, Stephan Hütter, Simon Hör, Lucas Rudigier, Renping Qiao, Mathew Sloane, Stefan Stricker and Paulina Latos, for opening my eyes to so many different cultures and for being wonderful colleagues. I am also grateful to Angelika Eisner and Xenia Weissmann, for their patience whenever I would run to them seeking help with bureaucratic, administrative or everyday-life-in-Austria matters.

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By helping out in the bad times and celebrating the good times, superb friends kept me sane all along. Therefore, a very warm thanks goes to them - all my beer-drinking, cake-eating, coffee-breaking, pub-quizzing, theatre-playing, party-dancing companions.

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9. APPENDIX

I included here a copy of the publications I contributed to during my PhD and whose figures and text were adapted for use in this thesis:

Santoro F, Mayer D, Klement RM, Warczok KE, Stukalov A, Barlow DP, Pauler FM. Imprinted Igf2r silencing depends on continuous Airn lncRNA expression and is not restricted to a developmental window. *Development*. 2013 Mar; 140(6): 1184-1195.

Santoro F, Pauler FM. Silencing by the imprinted Airn macro lncRNA: Transcription is the answer. *Cell Cycle*. 2013 Mar 1; 12(5): 711-712.

Santoro F, Barlow DP. Developmental control of imprinted expression by macro non-coding RNAs. *Semin Cell Dev Biol*. 2011 Jun; 22(4): 328-335.

Stricker SH, Steenpass L, Pauler FM, Santoro F, Latos PA, Huang R, Koerner MV, Sloane MA, Warczok KE, Barlow DP. Silencing and transcriptional properties of the imprinted Airn ncRNA are independent of the endogenous promoter. *EMBO J*. 2008 Dec 3; 27(23): 3116-3128.

Development 140, 1184-1195 (2013) doi:10.1242/dev.088849

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

Federica Santoro, Daniela Mayer, Ruth M. Klement, Katarzyna E. Warczok, Alexey Stukalov, Denise P. Barlow* and Florian M. Pauler*

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

CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences, Lazarettgasse 14, AKH BT 25.3, 1090 Vienna, Austria.

*Authors for correspondence (dbarlow@cemm.oeaw.ac.at; fpauler@cemm.oeaw.ac.at)

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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

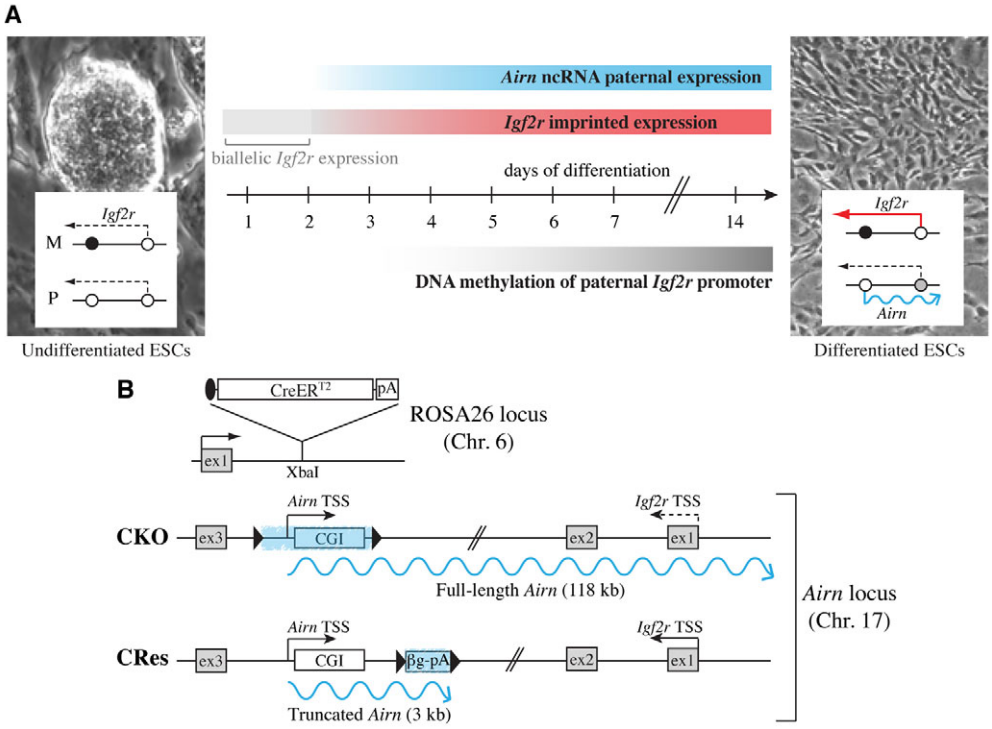

Fig. 1. Inducible systems to control *Airn* lncRNA. (**A**) Undifferentiated ESCs show low-level biallelic *Igf2r* expression; *Airn* is not expressed. Starting from day 2-3 of differentiation, expression of the maternal (M) *Igf2r* promoter is upregulated up to 20-fold. The *Airn* macro lncRNA is expressed 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

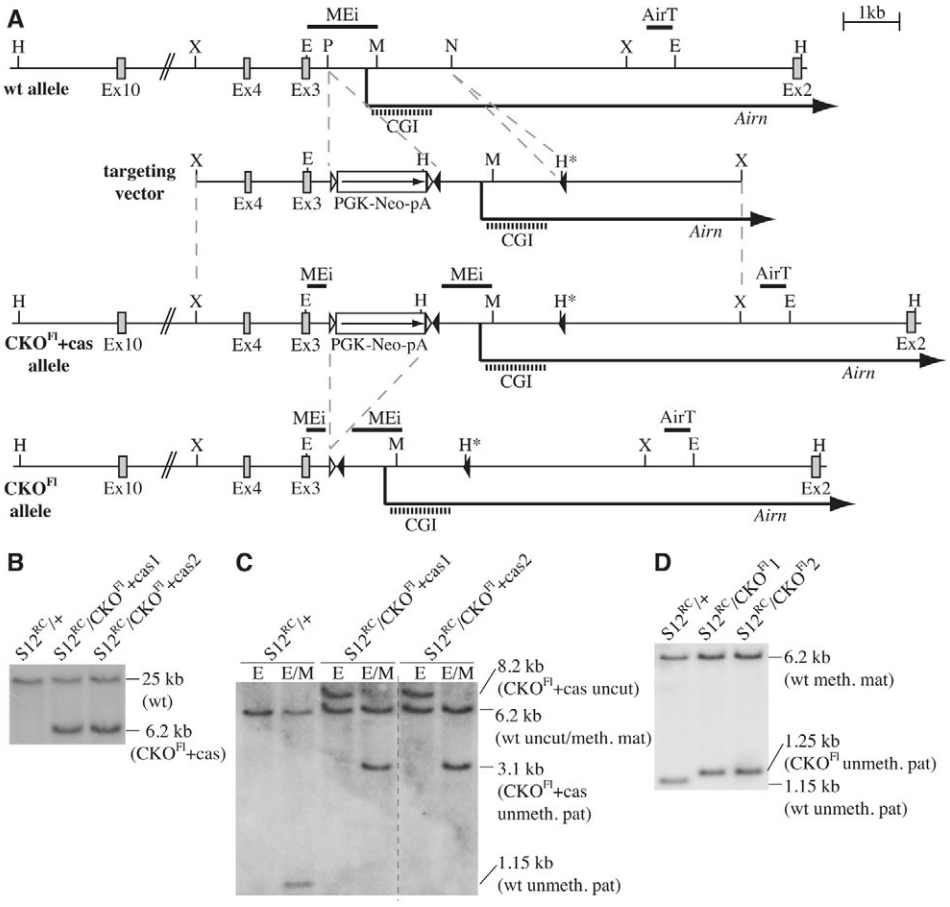

Fig. 2. *Airn* promoter conditional knockout (CKO) ESCs. (**A**) Top: wild-type allele showing *Airn* transcript overlapping *Igf2r* intron 2. Below: construct used to insert loxP sites (black triangles) flanking 1.9 kb containing the *Airn* promoter CGI (dashed bar). A selection cassette (PGK-Neo-pA) 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

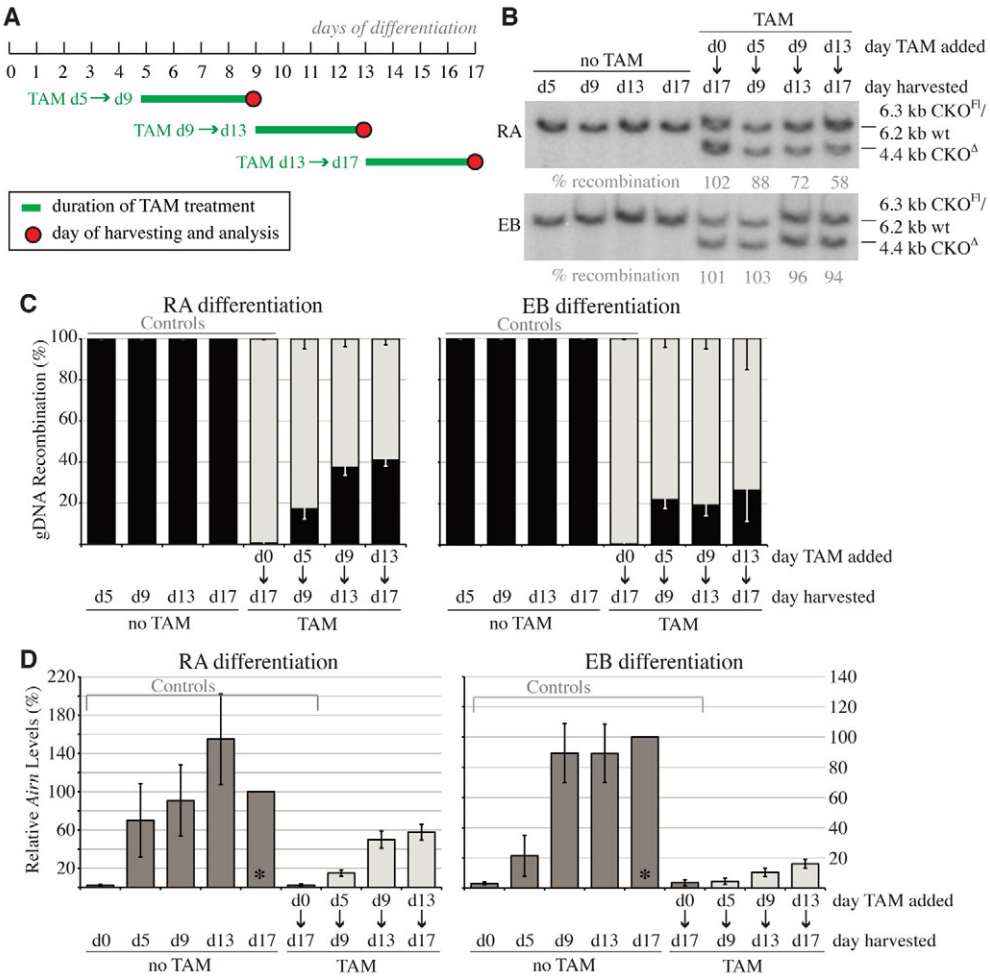

Fig. 3. Conditional *Airn* promoter deletion. (**A**) Experimental strategy to turn *Airn* off during ESC differentiation. (**B**) Southern blot of Cre recombination in retinoic acid (RA) or embryoid body (EB) differentiated CKO cells (supplementary material Fig. S3 shows strategy and replicates). DNA 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

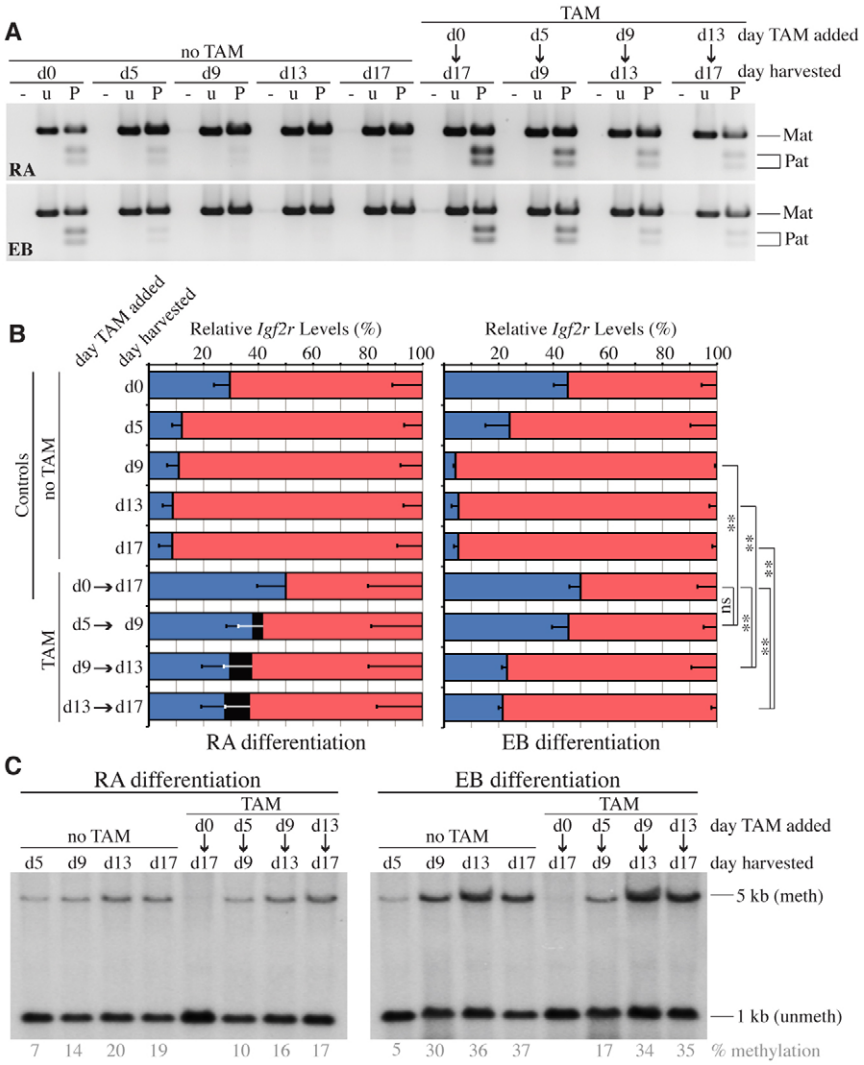

Fig. 4. *Igf2r* silencing requires continuous *Airn* expression. (**A**) Allele-specific *Igf2r* expression in RA (top) or EB (bottom) differentiated CKO cells, assayed by RT-PCR + *Pst*I digest of a paternal-specific restriction site. Maternal *Igf2r* expression generates 541 bp, paternal expression 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

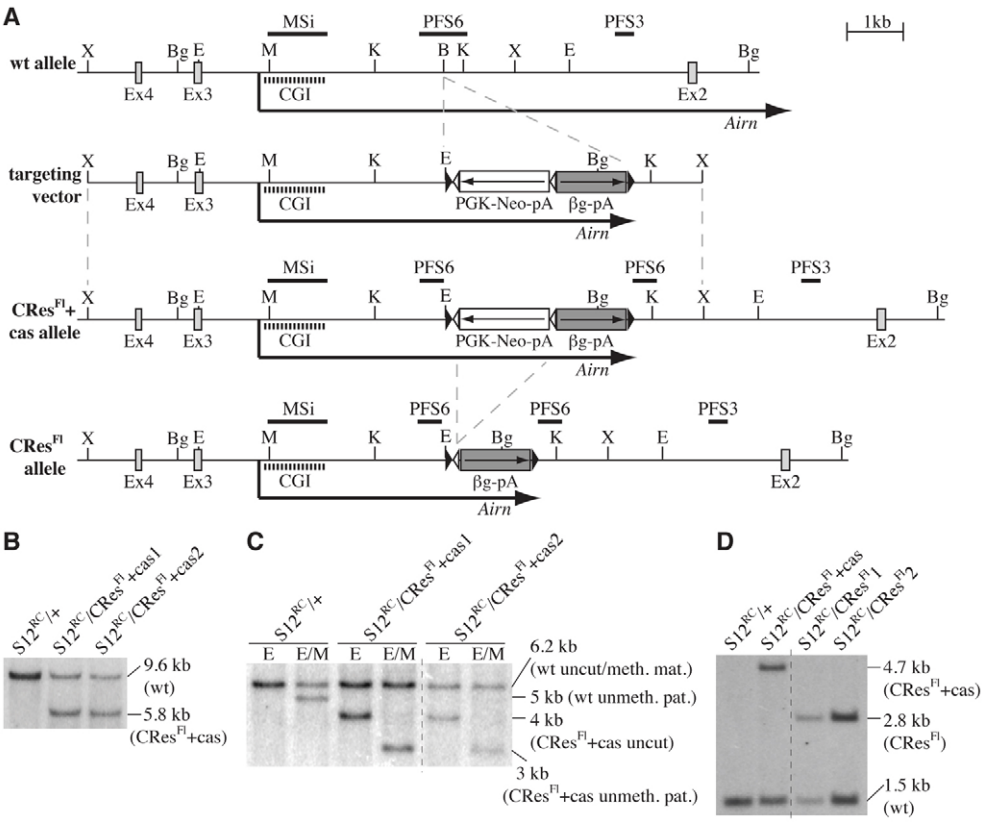

Fig. 5. *Airn* expression conditional rescue (CRes) ESCs. (**A**) Targeting strategy (details as Fig. 2A). Top: wild-type *Airn* allele. Below: targeting vector used to truncate *Airn* 3 kb after its TSS. The same selection cassette as Fig. 2A and a floxed rabbit β-globin polyadenylation signal (βg-pA) 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^{FI}+cas allele. Transient transfection of FLP recombinase deleted the selection cassette to obtain the CRes^{FI} 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^{FI}+cas1,2 and the S12^{RC}/+ parental cell line using *Bgl*II-digested DNA hybridized to probe PFS3 shows correct homologous recombination (CRes^{FI}+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^{FI}+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^{FI}+cas) and after (S12^{RC}/CRes^{FI}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^{FI} 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

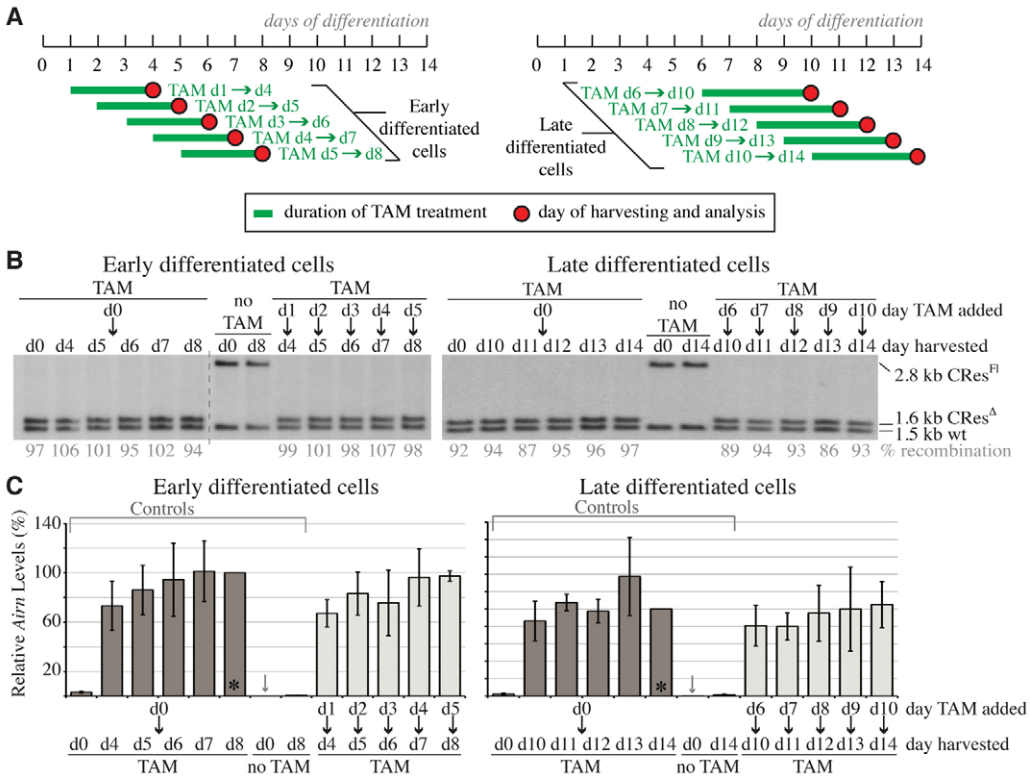

Fig. 6. Conditional deletion of an *Airn* truncation signal. (**A**) Experimental strategy to turn *Airn* on during ESC differentiation. (**B**) Southern blot to detect Cre recombination (supplementary material Fig. S6B shows replicates) in undifferentiated (day 0) or differentiated (days 4-14) CRes 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

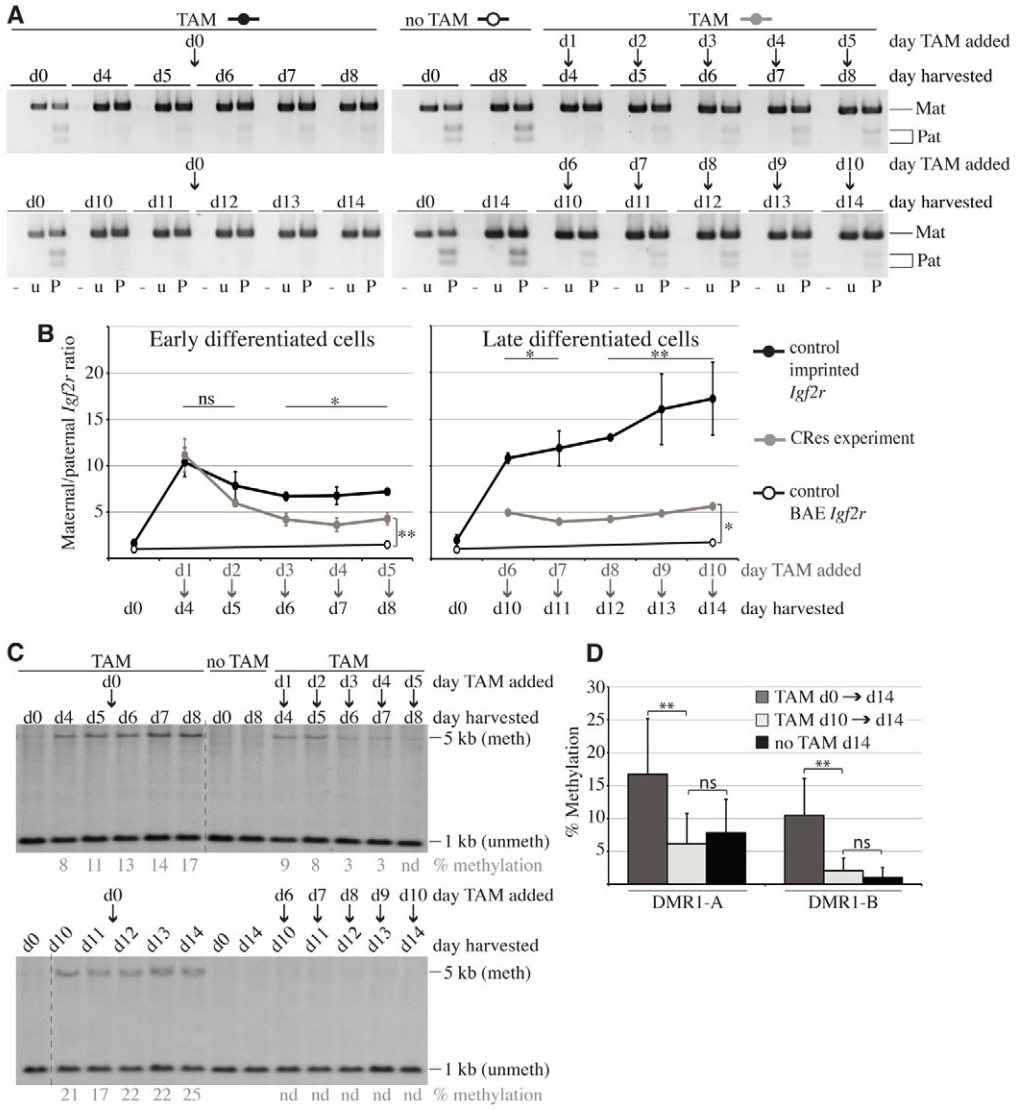

Fig. 7. *Airn* expression can silence *Igf2r* at any time during ESC differentiation. (**A**) Allele-specific *Igf2r* expression in early (top) or late (bottom) differentiated CRes cells (supplementary material Fig. S7A shows replicates) assayed as in Fig. 4A. Untreated (no TAM) day 0-14 cells show full 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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Silencing by the imprinted *Airn* macro lncRNA

Transcription is the answer

Federica Santoro and Florian M. Pauler*

CeMM Research Center for Molecular Medicine of the Austrian Academy of Sciences; Vienna, Austria

Abbreviations: ESC, embryonic stem cell; lncRNA, long non-coding RNA; polyA, polyadenylation; TSS, transcription start site

The majority of the mammalian genome is transcribed into long non-coding (lnc) RNAs, transcripts longer than 200 nucleotides and devoid of protein-coding potential. Recent genome-wide mapping efforts led to the identification of thousands novel lncRNAs, but so far only few have been functionally analyzed.[1] Fifteen years ago, our group discovered *Airn* (antisense to Igf2r RNA non-coding), the first autosomal lncRNA shown to have a silencing function.[2] *Airn* is an imprinted gene transcribed from the paternal chromosome, in antisense orientation to the imprinted but maternally expressed *Igf2r* gene. *Airn* expression controls *Igf2r* imprinted expression by silencing it in cis on the paternal chromosome,[2] but how *Airn* exerts its silencing function has long been a mystery.[3,4] Now, a study published in *Science* shows that *Airn* silences *Igf2r* through transcription alone and not via its RNA product.[5]

The road leading to this discovery was long and winding. When we first identified *Airn* and deleted its expressed, paternal promoter, we observed de-repression of the normally silent paternal *Igf2r* allele.[6] After this landmark experiment, three hypotheses were formulated to explain how *Airn* may silence *Igf2r*:[3] (1) the *Airn* promoter competes with the *Igf2r* promoter for common transcription factors or enhancers; (2) the *Airn* lncRNA coats the paternal chromosome, inducing its heterochromatization; (3) *Airn* transcription through the *Igf2r* promoter interferes with its expression. The promoter competition model was ruled out some years later by an experiment that prematurely terminated the 118 kb-long *Airn* transcript by inserting a polyadenylation (polyA) cassette 3 kb after its transcription start site (TSS).[2] Similarly to the *Airn* promoter deletion, the *Airn* truncation relieved paternal *Igf2r* silencing, indicating that either the *Airn* transcript or its transcription through the locus, but not the *Airn* promoter, is needed to silence *Igf2r*.

At that time, no examples of transcriptional interference had been reported in mammals. The lncRNA field was dominated by one exemplary cis-silencing transcript, the *Xist* lncRNA, which coats one X chromosome in female mammals and recruits chromatin-modifying repressor complexes to inactivate it. We reasoned that if *Airn* silences *Igf2r* in an *Xist*-like fashion, broad domains of allele-specific repressive chromatin should cover the silenced chromosomal region. However, when we analyzed the distribution of repressive histone marks at the *Igf2r* locus, we found no broad heterochromatic domains, but only focal enrichment at the silent paternal *Igf2r* promoter.[7] If *Airn* does not behave like *Xist*, might then mere transcription through the *Igf2r* promoter, rather than the transcript, be the answer?[4] The transcriptional interference hypothesis, by implying no role for the RNA product, would also account for some unusual, "macro" lncRNA-like features of *Airn*, which, unlike *Xist*, is a predominantly unspliced, repeat-rich and highly unstable transcript.[4]

We therefore set out to test our hypothesis, reasoning that if transcription alone is important, then no *Airn*-specific sequences should be required to silence *Igf2r*. To perform genetic manipulations of the *Airn* locus and monitor their effects on *Igf2r* imprinted expression during embryonic development, we established an embryonic stem cell (ESC) differentiation system. By inserting a polyA cassette at four different positions from the *Airn* TSS, we then truncated the *Airn* transcript at its 3' end, either before or after the *Igf2r* promoter. Interestingly, only the longer versions of *Airn*, overlapping the *Igf2r* promoter, were able to silence it (**Fig. 1**). Next, to exclude a silencing role for the *Airn* sequence located at its 5' end, we moved the *Airn* promoter immediately before the *Igf2r* promoter. This modified *Airn* allele could also silence *Igf2r* (**Fig. 1**). Having excluded *Airn* sequences located both before and after the *Igf2r* promoter, the only region that remained to test was the one overlapping the *Igf2r* promoter itself. However, we had previously shown that this sequence can be replaced with an exogenous promoter, with no sequence or structural similarity with the *Igf2r* promoter, and still undergo *Airn*-mediated silencing.[5] Together, this led us to conclude that *Igf2r* silencing does not require any part of the *Airn* lncRNA, but only transcription through the *Igf2r* promoter. Since we detected less capped *Igf2r* transcripts, indicative of transcription initiation, we propose that the *Airn*-transcribing RNA polymerase prevents *Igf2r* expression by running through its promoter and disrupting transcription initiation complex assembly.[5]

Transcriptional interference models predict a strong promoter interfering with activity of a weak promoter. In accordance with this model, in another recent study we report that, although *Airn* transcription can repress *Igf2r* at any time, it does

*Correspondence to: Florian M. Pauler; Email: fpauler@cemm.oeaw.ac.at
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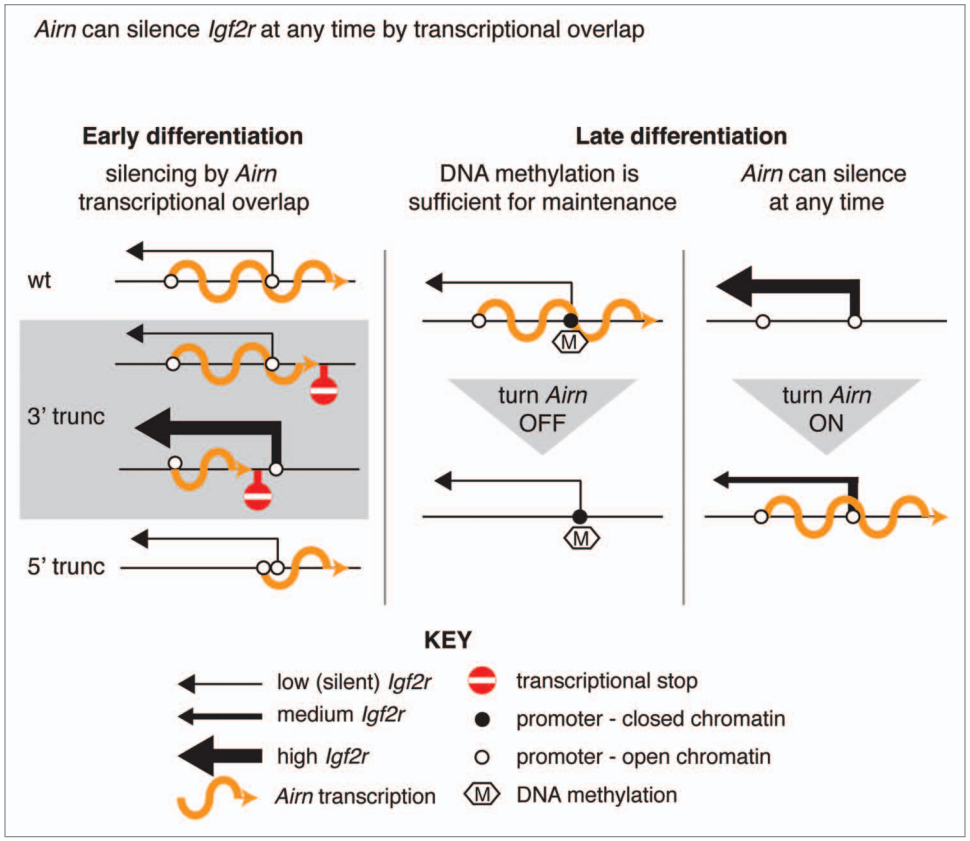

Figure 1. *Airn* can silence *Igf2r* at any time by transcriptional overlap. Left: early embryonic stem cell (ESC) differentiation. *Airn* silences *Igf2r* on the paternal allele and the *Igf2r* promoter is in an open chromatin conformation (top). Truncation of *Airn* from the 3' end (3' trunc) after but not before the *Igf2r* promoter maintains *Airn*-mediated silencing (middle). To truncate *Airn* from the 5' end (5' trunc), the *Airn* promoter was moved close to the *Igf2r* promoter: *Igf2r* was silenced in this case too (bottom). Together, these truncations show that *Airn* only needs to overlap the *Igf2r* promoter to silence it and that all 3' and 5' sequences are not necessary. Right: late ESC differentiation. *Airn*-mediated silencing causes the late acquisition of closed chromatin and DNA methylation on the *Igf2r* promoter, which maintains the silent state when *Airn* is turned off (left). In the absence of *Airn*, *Igf2r* is expressed at high levels and can only be partially silenced by turning *Airn* on at this developmental time point (right). Note that only the paternal allele is shown.

so less efficiently when paternal *Igf2r* expression is high, as in late ESC differentiation (**Fig. 1**).[8] Once silenced, the paternal *Igf2r* promoter normally gains DNA methylation. Interestingly, the same study shows that *Igf2r* silencing requires continuous *Airn* expression, but only until DNA methylation is established, at which point *Airn* becomes dispensable, indicating a role for DNA methylation in safeguarding lncRNA-mediated silencing (**Fig. 1**).[8]

In conclusion, 15 years after the first report of the *Airn* lncRNA, the cumulated evidence indicates that transcription is indeed the answer.[5] We hope our work will act as a "wake-up call" for the entire lncRNA field, as many more lncRNAs may act via their transcription alone.

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Review

Developmental control of imprinted expression by macro non-coding RNAs

Federica Santoro, Denise P. Barlow*

CeMM Research Center for Molecular Medicine of the Austrian Academy of Science, Lazarettgasse 14, AKH-BT25.3, 1090 Vienna, Austria

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ABSTRACT

Genomic imprinting is a developmentally regulated epigenetic phenomenon. The majority of imprinted genes only show parent-of-origin specific expression in a subset of tissues or at defined developmental stages. In some cases, imprinted expression is controlled by an imprinted macro non-coding RNA (ncRNA) whose expression pattern and repressive activity does not necessarily correlate with that of the genes whose imprinted expression it controls. This suggests that developmentally regulated factors other than the macro ncRNA are involved in establishing or maintaining imprinted expression. Here, we review how macro ncRNAs control imprinted expression during development and differentiation and consider how this impacts on target choice in epigenetic therapy.

Contents

1. Introduction

Mammals are diploid organisms and while the majority of genes are equally expressed from both chromosomes, a subset are subject to genomic imprinting and show maternal- or paternal-specific expression [1]. To date, 143 imprinted mouse genes are known that are mostly grouped into clusters [2]. Imprinted expression within a cluster is controlled by epigenetic mechanisms that act *in cis* (i.e.: on the same chromosome). The master regulator controlling expression of all genes in a cluster is the imprint control element (ICE) [3]. Each ICE is epigenetically marked on either the maternal or the paternal allele by a DNA methylation "imprint" acquired during gametogenesis and maintained on the same parental chromosome in diploid cells of the embryo after fertilization. The six best-studied mouse imprinted gene clusters in which the ICE was identified by deletion experiments are the *Igf2r*, *Kcnq1*, *Igf2*, *Gnas*, *Dlk1* and *Pws*/*As* clusters (reviewed in [4]).

All defined ICEs control expression of a macro or long non-protein-coding RNA (ncRNA) (defined here as a ncRNA >200 bp that is not processed to smaller RNAs). However, allele-specific silencing occurs by different mechanisms. For example, imprinted expression at the *Igf2* cluster arises because the ICE acts as a methylation-sensitive insulator interacting with CTCF and cohesin only on the unmethylated maternal allele that exclusively expresses the *H19* ncRNA (reviewed in [4]). Imprinted expression at the *Igf2r* and *Kcnq1* clusters (Fig. 1) is controlled, respectively, by the *Airn* and *Kcnq1ot1* macro ncRNAs, whose methylation-sensitive promoter lies in the ICE [5,6]. *Airn* and *Kcnq1ot1* are exclusively transcribed from the paternal allele as the gametic DNA methylation imprint represses the maternal promoter. Truncation experiments in mice that shortened these ncRNAs to 5% of their length show that they control paternal silencing of all genes in their cluster in embryonic and placental tissues [7,8].

Macro ncRNAs are now known to be widespread in the mammalian genome and are thought to function as transcriptional regulators although few have been studied in detail [9]. Here, we review the developmental regulation of imprinted macro ncRNAs, focusing on the *Airn* and *Kcnq1ot1* ncRNAs that play a functional silencing role. We consider how ncRNA developmental regulation

* Corresponding author. Tel.: +43 1 40160 70031.
E-mail address: dbarlow@cemm.oeaw.ac.at (D.P. Barlow).

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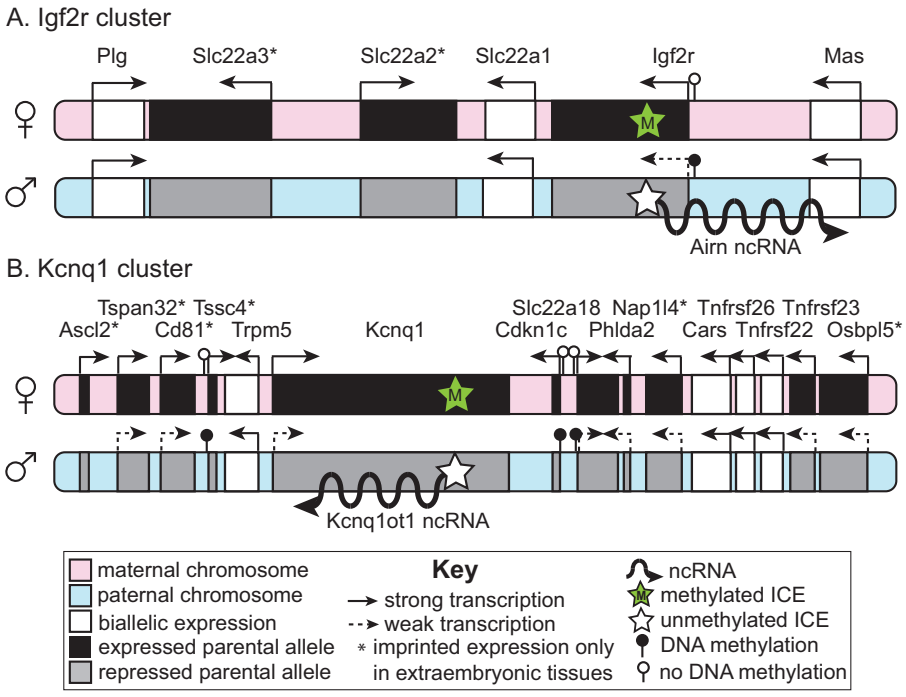

Fig. 1. Two mouse imprinted gene clusters containing a regulatory macro ncRNA. Genomic organization of the mouse *Igf2r* (A) and *Kcnq1* (B) imprinted gene clusters.

impacts on imprinted expression of flanking protein-coding genes. Finally, we discuss the choice of macro ncRNAs as targets in epigenetic therapy.

2. Imprinted macro ncRNAs are developmentally regulated

Genome-wide studies of macro ncRNAs revealed developmental and tissue-specific expression patterns, suggesting they are not unspecific "transcriptional noise" [9]. The expression of imprinted macro ncRNAs is also highly regulated (Table 1), and in most cases, imprinted expression of flanking protein-coding genes in the cluster correlates with ncRNA expression. The paternally expressed *Kcnq1ot1* macro ncRNA displays the most widespread expression pattern: it is found already in preimplantation embryos from the two-cell stage and maintained throughout mouse development, correlating with imprinted expression of flanking protein-coding genes [10,11]. Production of the *Airn* macro ncRNA correlates with *Igf2r* imprinted expression. *Airn* is absent from post-mitotic

Table 1
Developmental- and tissue-specific regulation of imprinted macro ncRNAs.

Imprinted cluster	ncRNA	Developmental-specific expression	Tissue-specific expression
Igf2r	*Airn*	From implantation stage; differentiated ESCs [24]	Ubiquitous, except neurons [12]
Kcnq1	*Kcnq1ot1*	From 2-cell stage [10]	Ubiquitous [11]
Igf2	*H19*	From blastocyst stage; differentiated ESCs [53]	Embryonic and extraembryonic tissues [15,53]; adult: skeletal muscle, thymus, heart, lung [53]
	Igf2as	From E10.5 till postnatal day 4 [54]	Skull, muscle, placenta [54]
Gnas	*Nespas*	E10.5 and E15.5[a]; adult [17–19]	Widespread [17–19]
	Exon1A	n.a.	Ubiquitous [55]
Dlk1	*Gtl2*	Continuous [56]	Widespread in fetus [21]; adult: brain, testis, spinal cord, skeletal muscle [57]
	Rtl1as	From E8.5[a] [58]	Brain [57]
	Rian	From E9.5 [57]	Extraembryonic tissues; embryo: brain, somites and cartilage; adult: brain, testis, skin, heart, muscle [57]
	Mirg	From E11.5 [57]	Embryonic and extraembryonic tissues [59]; adult: mainly brain, limbs, tongue, skin, testis [57]
	Mico1	From E8.5[a] [60]	Embryo: brain, heart, branchial arches; adult: mainly brain, pituitary, spleen, kidney, uterus [60]
	Mico1os	From E8.5[a] [60]	Embryo: brain, heart, branchial arches; adult: mainly brain, pituitary, spleen, kidney, uterus [60]
Pws/As	*Lncat*[b]	From E10.5 [16]	Post-mitotic neurons [16]

Abbreviations: n.a.: not analyzed; ESCs: embryonic stem cells; E: embryonic day; ubiquitous: expressed in all tested tissues.
[a] Not tested earlier.
[b] Includes U exons, *Ube3a-ats*, *Ipw* and *Pwcr1* ncRNAs.

neurons that express *Igf2r* biallelically [12] and transcribed after embryo implantation when imprinted *Igf2r* expression is first observed [13]. The *H19* macro ncRNA is generally co-expressed with the *Igf2* gene [14]. Interestingly, an early report found that in pre-gastrulation post-implantation embryos, *H19* and *Igf2* exhibit biallelic and monoallelic expression respectively, rather than the reciprocal monoallelic modes usually observed [13]. The opposite situation was described in fetal tissues of neuroectodermal origin where only *H19* was found to be monoallelically expressed, whereas *Igf2* exhibited biallelic expression [15]. These findings are in line with the non-functional role of the *H19* ncRNA in *Igf2* imprinted expression and suggest that monoallelic expression of the two genes is controlled by distinct mechanisms in different tissues or developmental stages.

In the *Pws*/*As* cluster [4], the *Ube3a* gene shows maternal-specific expression in neurons only and is biallelically expressed in glial cells and neural progenitor cells. The paternally expressed *Ube3a-ats* macro ncRNA overlaps *Ube3a* in antisense orientation and is found exclusively in neurons, however its function has not been directly tested. Expression of this ncRNA was only found in neuronal subtypes showing *Ube3a* biased imprinted expression, but not in those showing complete silencing of the *Ube3a* paternal allele [16]. This may indicate that the *Ube3a-ats* ncRNA does not functionally silence the *Ube3a* gene or is dispensable for maintaining silencing.

The paternally expressed *Nespas* ncRNA in the *Gnas* cluster [4] is transcribed in antisense orientation to the *Nesp* maternally expressed protein-coding gene. *Nespas* function is not yet known, but *Nesp* and *Nespas* show the same spatio-temporal expression pattern [17]. Studies analyzing tissue-specific expression of the *Nespas* ncRNA in adult mice have found that it is widely expressed, however some tissues appear to maintain *Nesp* imprinted expression despite absence of the overlapping ncRNA [18,19]. As with the *Ube3a-ats* ncRNA described above, this may indicate a lack of a functional role for *Nespas* or that it is dispensable for maintaining silencing. The *Gnas* gene itself, like *Nesp*, displays maternal-specific expression, although only in a subset of tissues (e.g. brown adipose tissue, renal proximal tubes). If the *Nespas* ncRNA turns out to be responsible for silencing *Gnas in cis*, it will also be interesting to find out how, despite being widely expressed, its activity can be restricted to specific tissues [20].

In the *Dlk1* cluster [4], the *Gtl2* ncRNA is expressed in a reciprocal imprinted fashion to the protein-coding *Dlk1* gene. Apart from a few cases, *Gtl2* and *Dlk1* are generally not co-expressed in tissues and cell types in which they both show imprinted expression [21]. Notably, *Dlk1* exhibits paternal-specific expression in tissues where the *Gtl2* ncRNA is not detected, suggesting that, at least in these tissues and at the developmental stages analyzed, the *Gtl2* ncRNA is not involved in *cis*-repression of the maternal *Dlk1* allele.

3. Imprinted gene expression: how does it begin?

An early hypothesis regarding the mechanisms underlying genomic imprinting postulated the existence of imprint "printers", that add a covalent modification or imprint to a specific DNA sequence (imprinting box), and of imprint "readers", that can recognize the imprint and execute its function on transcriptional regulation [22]. We know now that *de novo* DNA methyltransferases serve as printers, by adding a DNA methylation mark to the CpG-rich sequences within the ICEs. Imprinting is, strictly speaking, the introduction of the primary gametic imprint. The imprint alone, however, does not initiate imprinted expression, which only arises in the presence of additional factors – the imprint readers. One example of an imprint reader is the insulator-binding CTCF protein, which regulates imprinted expression at the *Igf2* cluster by

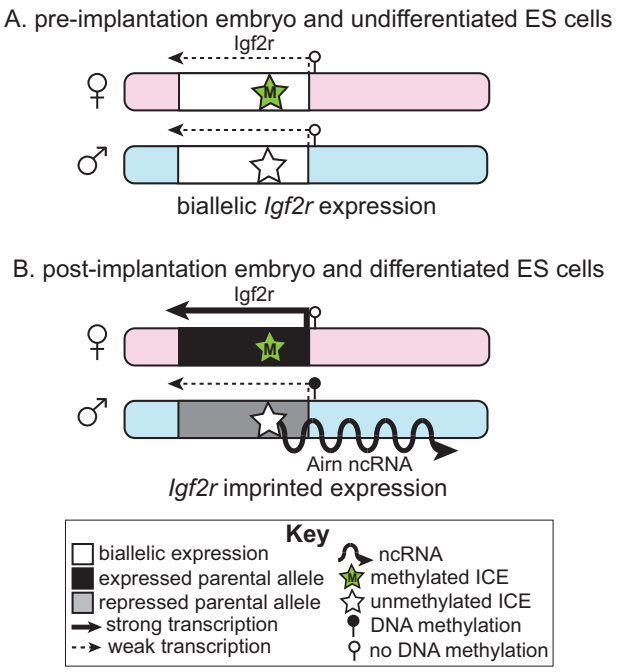

Fig. 2. Developmental regulation of *Igf2r* imprinted expression. Expression of *Igf2r* and *Airn* before (A) and after (B) embryo implantation and embryonic stem (ES) cell differentiation. (A) In the early pre-implantation embryo and in undifferentiated ES cells, no *Airn* ncRNA is transcribed. *Igf2r* is biallelically expressed at low levels at this stage. (B) In the post-implantation embryo and in differentiated ES cells, the *Airn* ncRNA is paternally transcribed. *Igf2r* imprinted expression is established, with strong expression from the maternal allele and residual weak expression from the paternal allele. Establishment of *Igf2r* imprinted expression is accompanied by gain of a somatic DNA methylation mark on the paternal *Igf2r* promoter.

binding the unmethylated but not the methylated ICE. The other clear examples are the *Airn* and *Kcnq1ot1* ncRNAs, both of which are only expressed from the chromosome carrying the unmethylated ICE and whose repressive activity is thus restricted to that chromosome.

A consequence of the distinction between the imprint and its readers is that spatio-temporal regulation of imprinted gene expression can be achieved by modulating expression of the readers themselves. Once it is established, the gametic imprint is faithfully propagated from one cell division to the next by the DNMT1 maintenance DNA methyltransferase [23]. Except for the developing gametes, in which it is erased and reset, the imprint is present in every somatic cell of an individual throughout its life cycle. So potentially, a given gene could display imprinted expression everywhere and anytime within an organism. However, as discussed above, in most cases imprinted expression is restricted to specific tissues, cell types or developmental stages. Given the ubiquitous nature of the imprint, the developmental regulation of imprinted expression can only be explained by assuming that the imprint readers and/or additional factors required for establishing or maintaining imprinted expression are themselves differentially expressed.

The *Igf2r* cluster provides a good example of how developmentally regulated expression of a macro ncRNA affects expression of the imprinted genes it controls (Fig. 2). *Igf2r* displays near ubiquitous maternal-specific expression. In the pre-implantation mouse embryo and, *in vitro*, in undifferentiated embryonic stem (ES) cells, *Igf2r* is expressed at low levels from both alleles, despite the presence of the ICE methylation mark on the maternal ICE. At this stage, no *Airn* ncRNA is detected. Its expression is first seen after embryo implantation and in differentiating ES cells where the onset of its transcription determines the onset of imprinted *Igf2r* expression

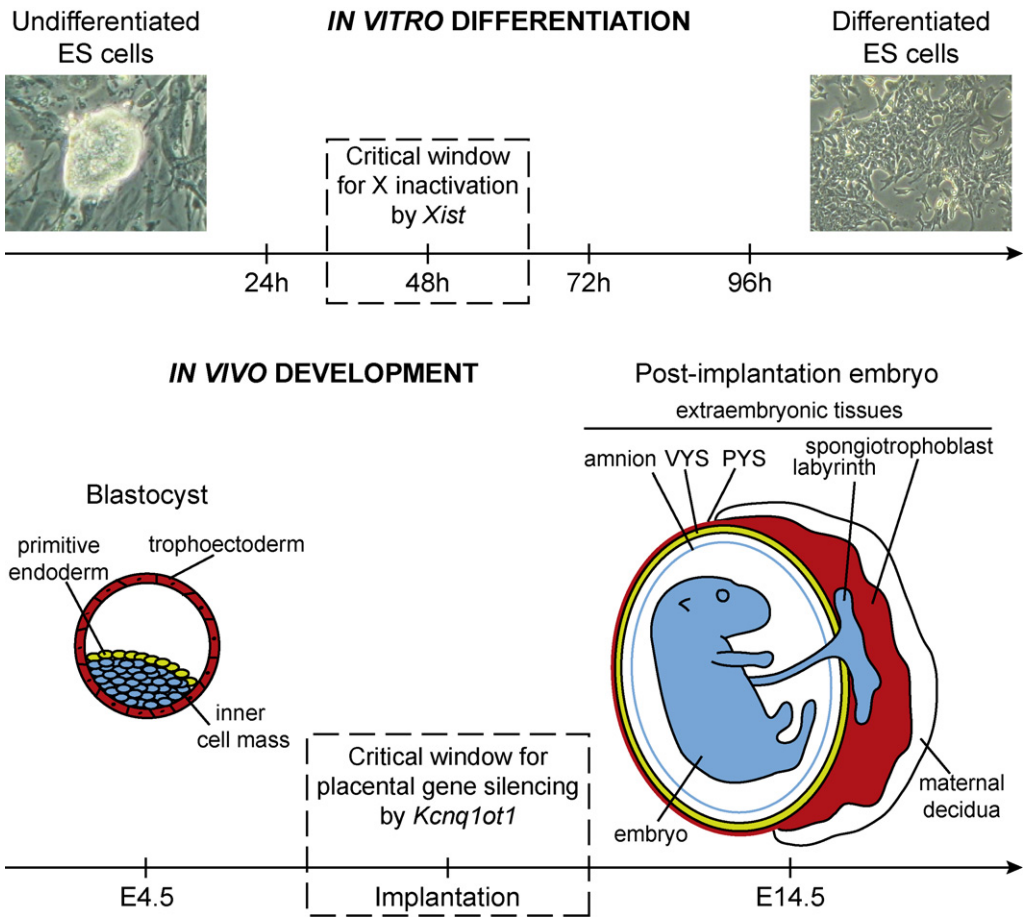

Fig. 3. Windows of opportunity of macro ncRNAs during mouse development. *In vitro* and *in vivo* systems used to identify developmental windows of opportunity for macro ncRNAs. (Top) The *in vitro* differentiation ES cell system in which the window of opportunity for the *Xist* ncRNA was defined. The *Xist* ncRNA must be expressed within 48 h of ES cell differentiation in order to cause X chromosome inactivation. (Bottom) *In vivo* mouse embryo development before and after implantation stage. The *Kcnq1ot1* ncRNA can silence placental-specific imprinted genes in placenta only before implantation occurs. See text for additional details. Tissues of the post-implantation embryo derived from the blastocyst inner cell mass, primitive endoderm and trophoectoderm components are indicated in blue, yellow and red, respectively.

[24]. From this stage, the *Airn* ncRNA is always present when *Igf2r* shows maternal-specific expression; where *Igf2r* is biallelically expressed as in neurons (see above), no *Airn* ncRNA is transcribed [12]. The *Airn* ncRNA is therefore the developmentally regulated imprint reader responsible for developmentally regulated *Igf2r* imprinted expression. However, not all imprinted genes in the *Igf2r* cluster show imprinted expression in the presence of *Airn*. The *Slc22a2* and *Slc22a3* genes, that only show imprinted expression in the placenta where it is controlled by the *Airn* ncRNA (Fig. 1), are insensitive to its effects in some stages and tissues. *Slc22a3* loses imprinted expression in placenta after E15.5 and together with *Slc22a2* shows biallelic expression in adult tissues [25]. In the *Kcnq1* cluster (Fig. 1), where the *Kcnq1ot1* ncRNA displays ubiquitous imprinted expression from the two-cell stage on and is responsible for inducing imprinted expression of all genes in this cluster [8,10,11], only the *Cdkn1c* gene shows imprinted expression in all tissues and at all developmental stages examined. The *Kcnq1* gene loses imprinted expression already by E14.5 of embryonic development [11]. Several genes in the cluster show imprinted expression in placental tissues but are biallelically expressed in embryo (*Osbp15*, *Cd81*, *Tssc4*) [26]. Interestingly, these placental-specific imprinted genes only show imprinted expression after implantation, even though the *Kcnq1ot1* ncRNA is present since earlier stages [10].

In conclusion, the *Airn* and *Kcnq1ot1* ncRNAs can initiate imprinted expression, but additional levels of regulation must exist to explain why some genes escape ncRNA-mediated silencing in certain tissues or at defined developmental stages. The ncRNAs might not be the only developmentally regulated players in the establishment of imprinted expression and they are likely to require additional, differentially expressed cofactors to perform their silencing function.

4. Windows of opportunity for macro ncRNAs

The *Airn* and *Kcnq1ot1* ncRNAs control imprinted expression of several protein-coding genes but, as described above, some genes in these clusters lack imprinted expression in the presence of the ncRNA. This may indicate that these macro ncRNAs, even though necessary to establish imprinted expression, might not always be sufficient to maintain it. Despite being ubiquitously expressed, the ncRNAs may require a special chromatin environment or specific cofactors to be functional, restricting their activity to those stages or tissues in which such favourable conditions are present. In order to understand what is needed to establish imprinted expression, it is therefore essential to identify the permissive time frame, or "window of opportunity", for ncRNA activity during development.

The idea of a window of opportunity for imprinted macro ncRNAs is analogous to the one described for the *Xist* macro ncRNA (Fig. 3, top). Random X inactivation occurs when the *Xist* ncRNA is transcribed from one of the two female X chromosomes inducing transcriptional silencing over the whole chromosome. Imprinted ncRNAs and *Xist* share some common features, as they are developmentally-regulated long non-coding transcripts that

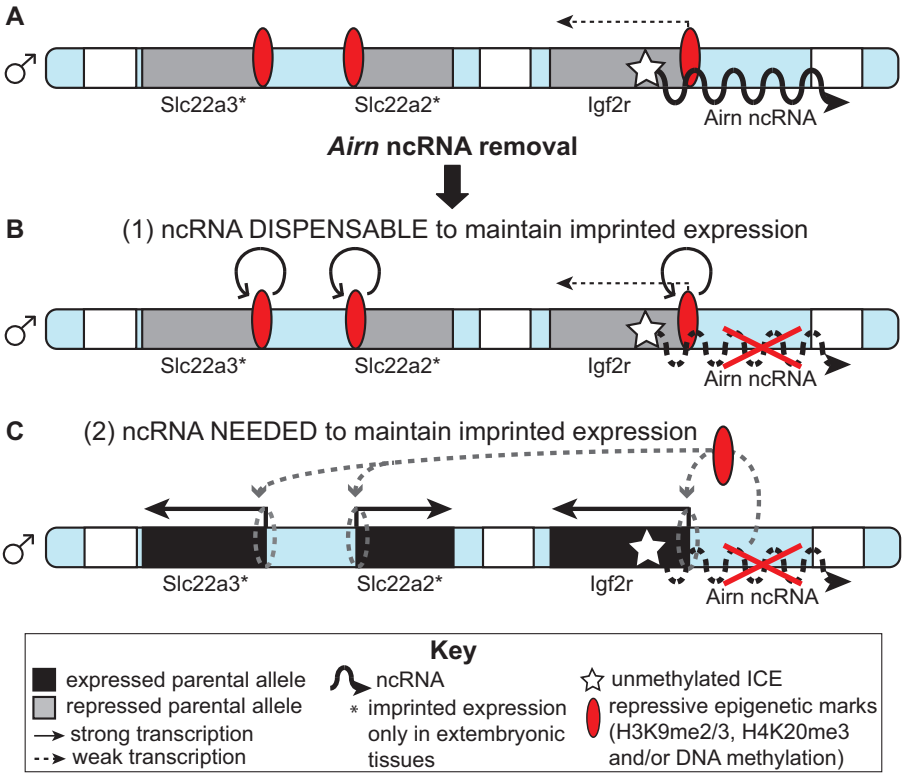

Fig. 4. Maintenance of imprinted expression at the *Igf2r* cluster. The role of imprinted macro ncRNAs in maintaining imprinted expression can be tested by removing the ncRNA after imprinted expression has been established. (A) "Steady-state" imprinted expression at the *Igf2r* cluster: the *Airn* ncRNA is paternally transcribed and the protein-coding genes are silenced *in cis*. Removing the *Airn* ncRNA once "steady-state" imprinted expression has been established can cause either no change in imprinted expression (B) or loss of imprinted expression (C). The first outcome would suggest that the ncRNA is dispensable to maintain imprinted expression. Self-perpetuating repressive epigenetic marks (circular arrow over red ellipses) might be involved in maintaining the repressed state independently of the ncRNA. The second outcome would suggest that the ncRNA is constantly needed to maintain imprinted expression, either through the constant ncRNA-mediated recruitment of repressive marks to target genes (dashed grey arrows and lines) or through constant ncRNA-mediated transcriptional interference.

silence flanking genes *in cis* and, in some cases, recruit repressive chromatin marks [27–29]. In females, *Xist* is constantly expressed from the two-cell stage, but there is only a specific time during which it can mediate X inactivation. With the use of an inducible *Xist* transgene in differentiating ES cells, it was shown that if the *Xist* ncRNA is expressed within 48 h of ES cell differentiation, inactivation of the surrounding genes is observed. However, if *Xist* is expressed at later stages, no inactivation is induced [30], indicating that *Xist*-mediated silencing can only occur during a specific period of early development. Importantly, as X inactivation *in vivo* occurs during implantation, the study confirms the utility of ES cells and their differentiated *in vitro* derivatives to study X chromosome inactivation. We have reported the utility of ES cells in imprinting studies as well as their recapitulation of epigenetic events seen in the developing embryo [24].

A developmental window of opportunity for an imprinted macro ncRNA has been described so far only for the *Kcnq1ot1* ncRNA (Fig. 3, bottom). The *Kcnq1ot1* ncRNA is normally silenced on the maternal allele, where its promoter is embedded within the methylated ICE. By using two different *Dnmt*1 knockout mouse models in which DNA methylation is lost at different stages of embryo development, expression of the maternal *Kcnq1ot1* allele was induced before and after implantation. When *Kcnq1ot1* is biallelically expressed before implantation, imprinted expression of genes showing ubiquitous and placental-specific imprinted expression is disrupted, indicating that maternal *Kcnq1ot1* can silence all genes *in cis* during the pre-implantation stage [26]. However, when *Kcnq1ot1* is biallelically expressed after implantation in placenta, it can silence ubiquitously but not placental-specific imprinted genes [26]. The critical window for *Kcnq1ot1 cis*-silencing activity seems to coincide, as for *Xist*, with the implantation stage of development. The window of opportunity does not seem to apply to genes showing ubiquitous imprinted expression in the *Kcnq1* cluster, as they can be silenced both before and after implantation, giving further support to the idea that ubiquitous and placental-specific imprinted genes are silenced via different mechanisms [31]. It must be noted, however, that the window of opportunity for *Kcnq1ot1* may need to be reinterpreted, given the recent demonstration that the *Dnmt1* somatic isoform is active from the 2-cell stage, earlier than previously thought and prior to the onset of imprinted expression in the *Kcnq1* cluster [32].

5. Silencing by imprinted macro ncRNAs: initiation or maintenance?

The imprinted *Airn* and *Kcnq1ot1* ncRNAs are necessary to silence their target genes *in cis* – but are they also needed to maintain silencing once it has been established? Mice that express a short, truncated form of either the *Airn* or *Kcnq1ot1* ncRNA lack imprinted expression of the surrounding genes [7,8]. These experiments highlight the critical function of these macro ncRNAs in establishing imprinted gene expression, but do not distinguish between an initiation and a maintenance role of the ncRNAs. The expression of *Airn* and *Kcnq1ot1* from early stages of development and throughout adult life does not mean that they are constantly needed to maintain imprinted gene silencing. The *Xist* ncRNA is also constantly present in the developing and adult mouse, but it has been clearly shown that it is dispensable for maintaining the inactive X chromosome. Gene silencing along the X chromosome depends on *Xist* expression before 48 h of ES cell differentiation.

After this time, *Xist*-mediated silencing becomes irreversible and independent of continuous *Xist* expression [30], consistent with mouse experiments showing that *Xist* is not needed to maintain the inactive state in adult somatic cells [33]. Gene repression on the inactive X chromosome seems to be maintained by a synergistic combination of epigenetic mechanisms, including DNA methylation, histone H4 hypoacetylation and chromosomal late replication [34].

Similar studies have yet to be performed for imprinted macro ncRNAs and will be crucial to elucidate their exact role in imprinted gene silencing. Fig. 4 shows the possible outcomes, using the imprinted *Igf2r* cluster as an example, of experiments in which ncRNA expression or the transcript itself is abolished in a conditional fashion. If the ncRNA were dispensable for imprinted expression maintenance (Fig. 4B), imprinted expression would be maintained even though the ncRNA is no longer present. This would imply that similar to X inactivation, additional repressive epigenetic factors maintain the silent state once it has been established. In order to fulfil its function and ensure faithful transmission of the silent state, the maintenance factor and/or modification would have to be stably inherited through cell divisions and perpetuate itself independently of the macro ncRNA. The most obvious candidate for such a mark is DNA methylation, the only known heritable repressive epigenetic modification. However, it must be noted that DNA methylation rarely modifies the repressed alleles of imprinted genes. This was first noted at the silent maternal copy of the *Igf2* gene [35] and has since been confirmed for a subset of other imprinted genes. At the *Igf2r* cluster, for instance, the *Igf2r* gene is the only one whose promoter is methylated on the repressed paternal allele. This methylation mark, however, seems to play no active silencing role, as *Igf2r* can still be silenced in the absence of DNA methylation in early post-implantation embryos [23,36]. At the *Kcnq1* cluster, DNA methylation modifies the repressed copies of only three out of eleven imprinted genes and is similarly dispensable for silencing [26]. Given these observations, it is unlikely that DNA methylation is the repressive mark that maintains imprinted expression in the absence of the *Airn* and *Kcnq1ot1* ncRNAs. Histone modifications have been suggested to play a more important role than DNA methylation in maintaining imprinted gene silencing and allele-specific active and repressive histone marks have been identified in the *Igf2r* and *Kcnq1* clusters [37,38]. Repressive epigenetic marks indicative of repressive 'heterochromatin' such as histone H3 lysine 9 dimethylation (H3K9me2) and histone H3 lysine 27 trimethylation (H3K27me3) play a role in the placenta but they are not required for maintaining embryonic imprinted expression (reviewed in [4]). Histone modifications could therefore be responsible, at least in placental tissues, for maintaining imprinted expression. However, it is currently unclear how or if histone modifications are propagated during cell division [39].

If the ncRNA were indispensable for imprinted expression (Fig. 4C), loss of imprinted expression would strictly correlate with absence of the macro ncRNA. Repressive histone modifications could be required, as discussed above, to keep the repressed state, but they might be unable to maintain a stable epigenetic memory on their own. The ncRNA could be needed to constantly guide histone-modifying enzymes to specific positions within the imprinted cluster, to ensure that repressive marks are maintained for the desired amount of time. Indeed, in placenta but not in embryo, the *Airn* and *Kcnq1ot1* ncRNAs can both bind histone methyltransferases and localize to chromatin and thereby target repressive histone marks to the imprinted cluster [28,29]. How this targeting mechanism operates and is subject to the observed developmental regulation described above is currently unclear. Possible mechanisms may involve regulating macro ncRNA affinity for histone-modifying enzymes. Contrary to placenta, repressive histone marks found on embryonic and adult imprinted genes seem to have no clear regulatory role, although current experiments have not directly tested if these marks play a maintenance role. It is also possible that some repressive chromatin modifications simply mark transcriptionally repressed genes without playing an active role in maintaining the silent state.

An alternative model that has been proposed to explain how the *Airn* macro ncRNA regulates embryonic imprinted expression is based on transcriptional interference. This model postulates that ncRNA transcription through the promoter or an enhancer element of the target gene, rather than the transcript itself, is responsible for silencing the gene *in cis* [40]. Transcriptional interference that maintains constant transcription across a susceptible promoter could also explain the loss of imprinted expression observed upon ncRNA removal late in development, as imagined in Fig. 4C. At our current level of understanding, a transcriptional interference model does not easily explain how imprinted expression can be regulated in the presence of a ubiquitously transcribed ncRNA. A test of the transcriptional interference model would ideally require a post-transcriptional knockdown strategy. Conventional small interfering RNA-mediated RNA interference (RNAi), that takes advantage of the endogenous RNAi machinery of the cell to induce cleavage of cytoplasmic mRNAs, cannot be applied to the nuclear-localized *Airn* and *Kcnq1ot1* ncRNAs [41]. Surprisingly, some studies do report RNAi activity in the nucleus [42,43]. A more efficient method to degrade nuclear ncRNAs in mammalian cell lines is to make use of antisense oligonucleotides that bind the target RNA and cause its degradation by inducing endogenous RNaseH activity [44].

6. Developmental control of imprinted gene silencing: clinical implications

Of the 30% of imprinted genes that control embryonic and neonatal growth, those that are paternally expressed act as growth promoters, while those that are maternally expressed act as growth repressors [1,2]. Alterations in the expression of imprinted genes that cause them to be biallelically expressed or silenced can therefore lead to overgrowth or growth defects. To date, nine human imprinting syndromes have been recognized: Angelman syndrome (AS), Beckwith-Wiedemann syndrome (BWS), Prader-Willi syndrome (PWS), Russell-Silver syndrome (RSS), maternal and paternal uniparental disomy of chromosome 14 (matUPD14, patUPD14), pseudohypoparathyroidism type 1b (PHP-1b), transient neonatal diabetes (TND) and maternal hypomethylation syndrome [45]. The molecular mechanisms underlying each one of them are diverse but they can be grouped into two classes: genetic or epigenetic, based on the underlying cause. Genetic causes include deletions or duplications of regions containing imprinted genes, mutations in imprinted genes or in the ICE and uniparental disomy (UPD). Epigenetic mechanisms involve epimutations, i.e. alterations in the DNA methylation and/or histone modification patterns with no changes in the DNA sequence. The majority of patients with imprinting syndromes show large chromosomal rearrangements, UPDs or epigenetic alterations, with point mutations in imprinted genes accounting for a small number of cases [45]. The fact that mutations in imprinted genes are rare highlights the importance of understanding how imprinted expression is established and developmentally controlled, as a prerequisite for devising strategies to treat imprinting syndromes.

With this perspective, imprinted ncRNAs with a function in establishing or maintaining imprinted expression can become attractive therapeutic targets. The majority of patients affected by BWS, an overgrowth syndrome, show maternal hypomethylation of the DMR found at the human *KCNQ1* cluster on chromosome 11 [46]. The rest usually have paternal UPD of this chromosome. In

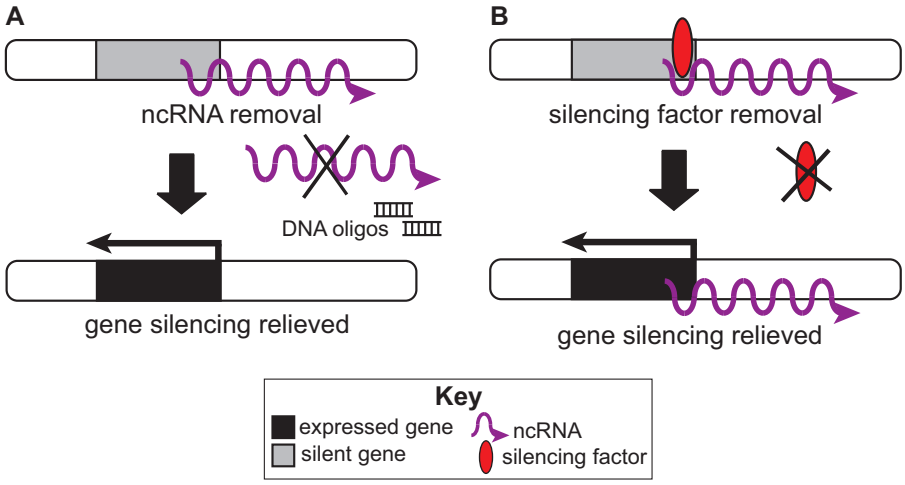

Fig. 5. Clinical implications for developmental regulation of macro ncRNAs. Therapeutic strategies to relieve ncRNA-mediated silencing of a gene of interest. In disease, aberrant macro ncRNA expression can result in epigenetic silencing of genes. (A) If the ncRNA is needed to maintain epigenetic silencing, RNA-targeting strategies can be adopted to relieve expression of the gene of interest. (B) If the ncRNA is not needed to maintain epigenetic silencing, RNA-targeting strategies will not be useful and repressive epigenetic factors responsible for maintaining the silencing might have to be targeted.

both cases, the *KCNQ1OT1* ncRNA is biallelically expressed causing biallelic silencing of the neighbouring *CDKN1C* tumour suppressor gene that negatively regulates cell proliferation. Novel therapeutic strategies specifically targeting the *KCNQ1OT1* ncRNA might be useful in this case to restore expression of the repressed *CDKN1C* gene. The AS is a neurological disorder caused by loss of expression of the maternally expressed *UBE3A* gene that shows neuronal-specific imprinted expression. AS patients with paternal UPD of this chromosome or an imprinting defect that results in a paternal-only methylation pattern have biallelic expression of the *UBE3A-ATS* ncRNA and no expression of *UBE3A*. Unlike the *Kcnq1ot1* ncRNA, there is no evidence yet that the *Ube3a-ats* ncRNA can silence the *Ube3a* gene *in cis*. However, if a functional role for the antisense ncRNA is found, *UBE3A-ATS* may become a useful therapeutic target to restore *UBE3A* expression. Imprinted macro ncRNAs belong to a larger class of long non-coding transcripts putatively involved in epigenetic regulation of protein-coding genes and it may be expected that deregulation of non-imprinted ncRNAs also plays a role in complex human diseases. Recently, some long or macro ncRNAs have been suggested to be involved in cancer. Examples include the *ANRIL*/*p15AS* ncRNA that is antisense to the *p15* tumour suppressor gene [47,48], a long ncRNA antisense to *p21* [49] and the *HOTAIR* ncRNA that was recently shown to promote cancer invasiveness and metastasis in a PRC2-dependent manner [50].

Given the involvement of macro ncRNAs in disease and the advances in technology to silence genes in animals, targeting therapies have been proposed to destroy ncRNAs in a specific manner. Destroying a macro ncRNA to relieve silencing of its target genes (Fig. 5A) only makes sense under some conditions: (1) the transcript and not its transcriptional activity are responsible for gene silencing and (2) the ncRNA is constantly required to maintain the silent state. If the ncRNA causes transcriptional gene silencing via transcriptional interference with an overlapped promoter or enhancer, then strategies to abolish ncRNA transcription rather than to destroy the transcript would be required. For example, exogenously administered siRNAs complementary to specific promoter sequences may block transcription of human genes [51]. If macro ncRNAs continue to be expressed but play no role in maintaining gene silencing it will be necessary to regain expression of the gene of interest by interfering with the repressive epigenetic marks that keep it silent (Fig. 5B). Several epigenetic drugs that appear to have a broad and largely unknown mode of action have been approved for use in clinical practice [52]. The DNA demethylating agents 5-azacytidine (Vidaza®) and its deoxy derivative decitabine or 5-aza-2′deoxycytidine (Dacogen®) are used for the treatment of myelodysplastic syndrome. The histone deacetylase inhibitor Vorinostat (Zolinza®) was also approved for clinical use in the treatment of cutaneous T cell lymphoma. Since the action of these drugs is genome-wide their therapeutic effects if any, are likely to result from a general effect on cell viability. Therefore where possible, a more specific therapy directed at a macro ncRNA that initiates epigenetic modifications is a desirable alternative.

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

Stefan H Stricker[1,2], Laura Steenpass[1], Florian M Pauler[1], Federica Santoro, Paulina A Latos[2], Ru Huang, Martha V Koerner, Mathew A Sloane[3], Katarzyna E Warczok and Denise P Barlow*

CeMM, Research Center for Molecular Medicine of the Austrian Academy of Sciences, Vienna, Austria

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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*Corresponding author. CeMM, Research Center for Molecular Medicine of the Austrian Academy of Sciences, c/o Dr Bohr-Gasse 9/4, Vienna Biocenter Campus, 1030 Vienna, Austria. Tel.: +43 1 4277 54 610; Fax: +43 1 4277 95 46; E-mail: denise.barlow@univie.ac.at
[1]These authors contributed equally to this work
[2]Present address: Wellcome Trust Centre for Stem Cell Research, University of Cambridge, Cambridge CB2 1QR, UK
[3]Present address: CeMM c/o, AKH, Leitstelle 5H.J2.09, Währinger Gürtel 18–20, 1090 Vienna, Austria

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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

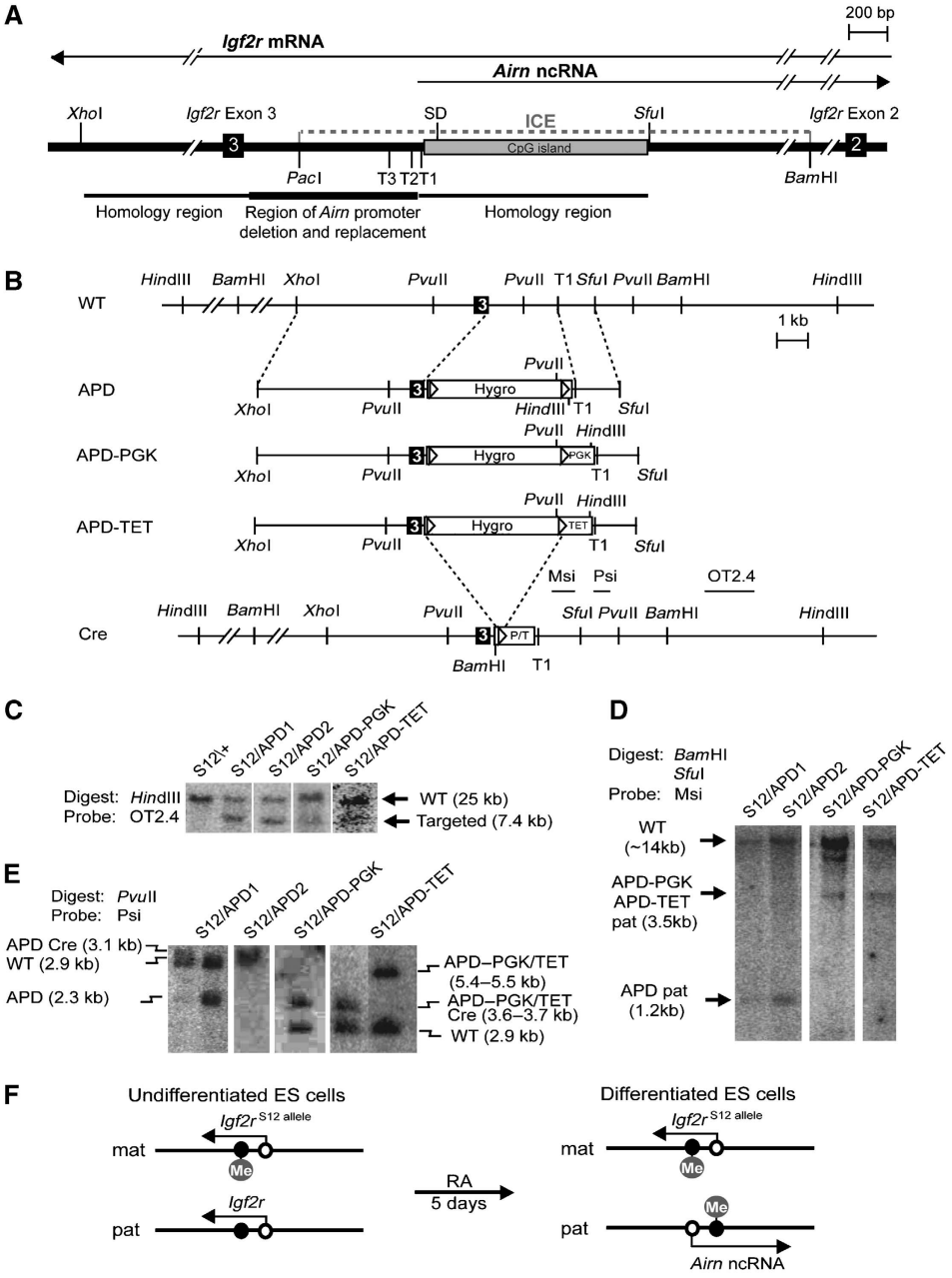

Figure 1 Deletion and replacement of *Airn* promoter constructs. (**A**) The *Airn* promoter lies in an antisense orientation in *Igf2r* intron 2. A 959-bp fragment is deleted in the APD, APD-PGK and APD-TET alleles (thick line). The main transcription start site (T1) and downstream CpG island are unchanged. 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

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

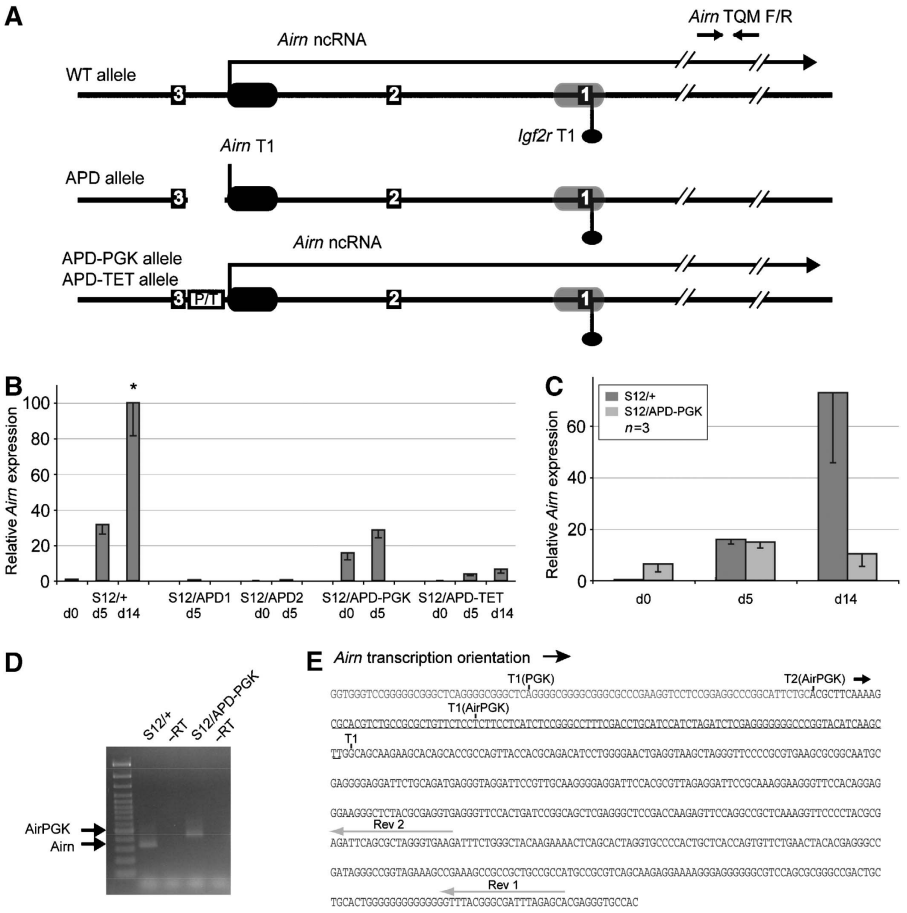

Figure 2 *Airn* expression changes in promoter deletion and replacement cells. (**A**) Map of the wild-type *Airn* promoter (WT), the *Airn* promoter deletion (APD) and replacement alleles (APD-PGK and APD-TET). *Airn* TQM F/R: QPCR assay used in (**B**, **C**). Numbered black boxes: *Igf2r* exons. Black 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:

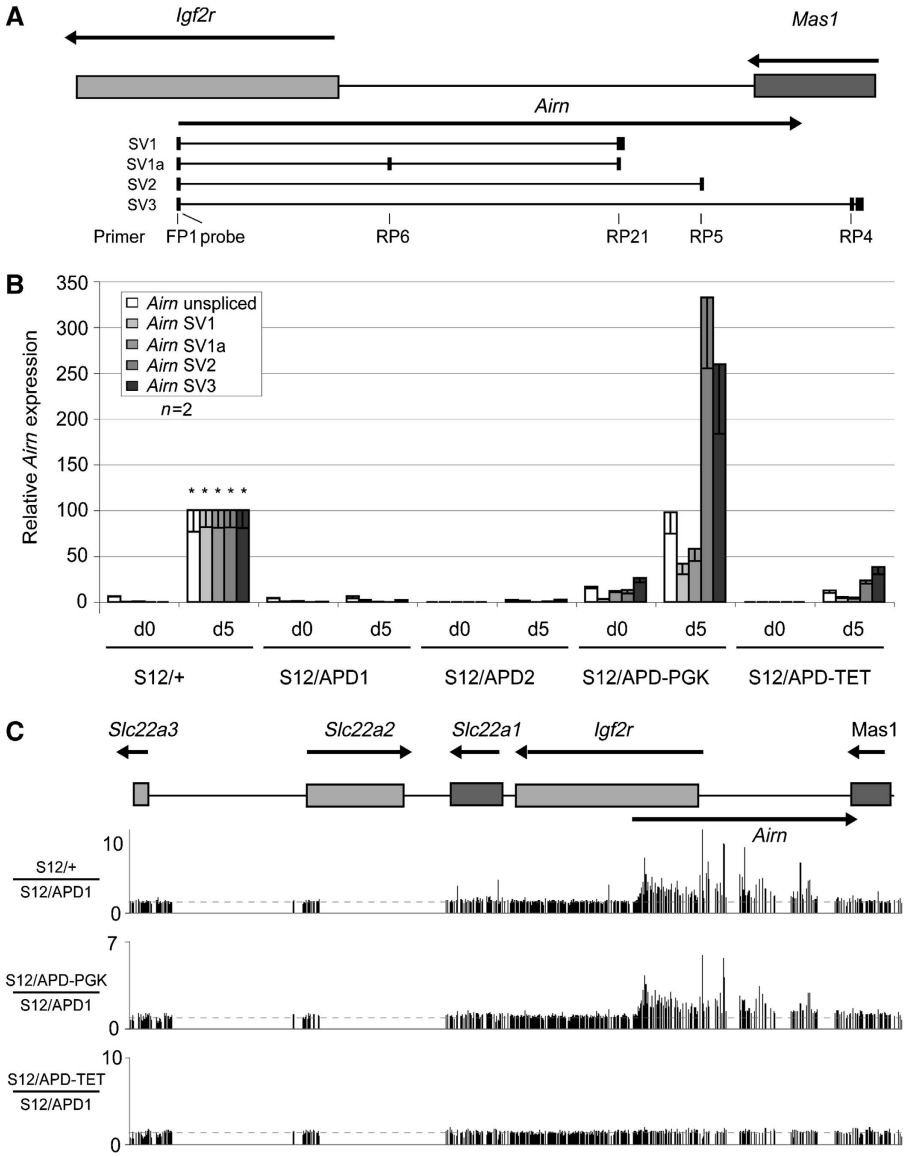

Figure 3 The PGK promoter expresses full-length *Airn*. (**A**) Map showing unspliced *Airn* (black arrow) and the known *Airn* splice variants and primers used in the splice variant-specific QPCR. The unspliced and spliced transcripts use the same transcription start sites and all four spliced variants 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

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.

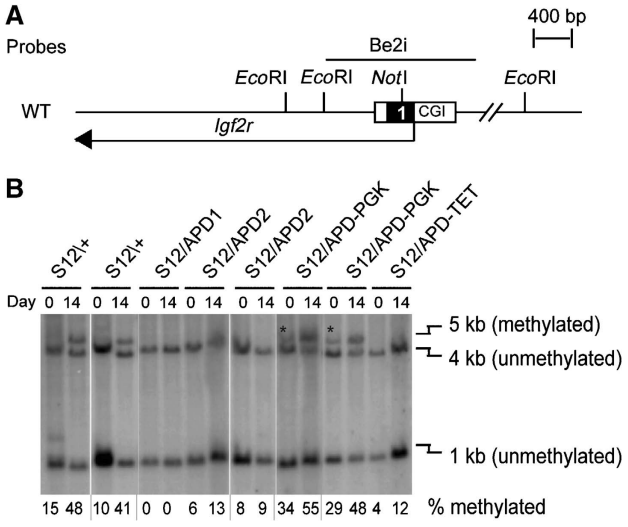

Figure 4 *Igf2r* promoter DNA methylation. (**A**) Map of an 8-kb region containing the *Igf2r* promoter (black arrow: transcription orientation). White box: CpG island; black box 1: *Igf2r* exon 1. Be2i: Southern blot probe. (**B**) Southern blot on genomic DNA of days 0 and 14 RA differentiated ES 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*.

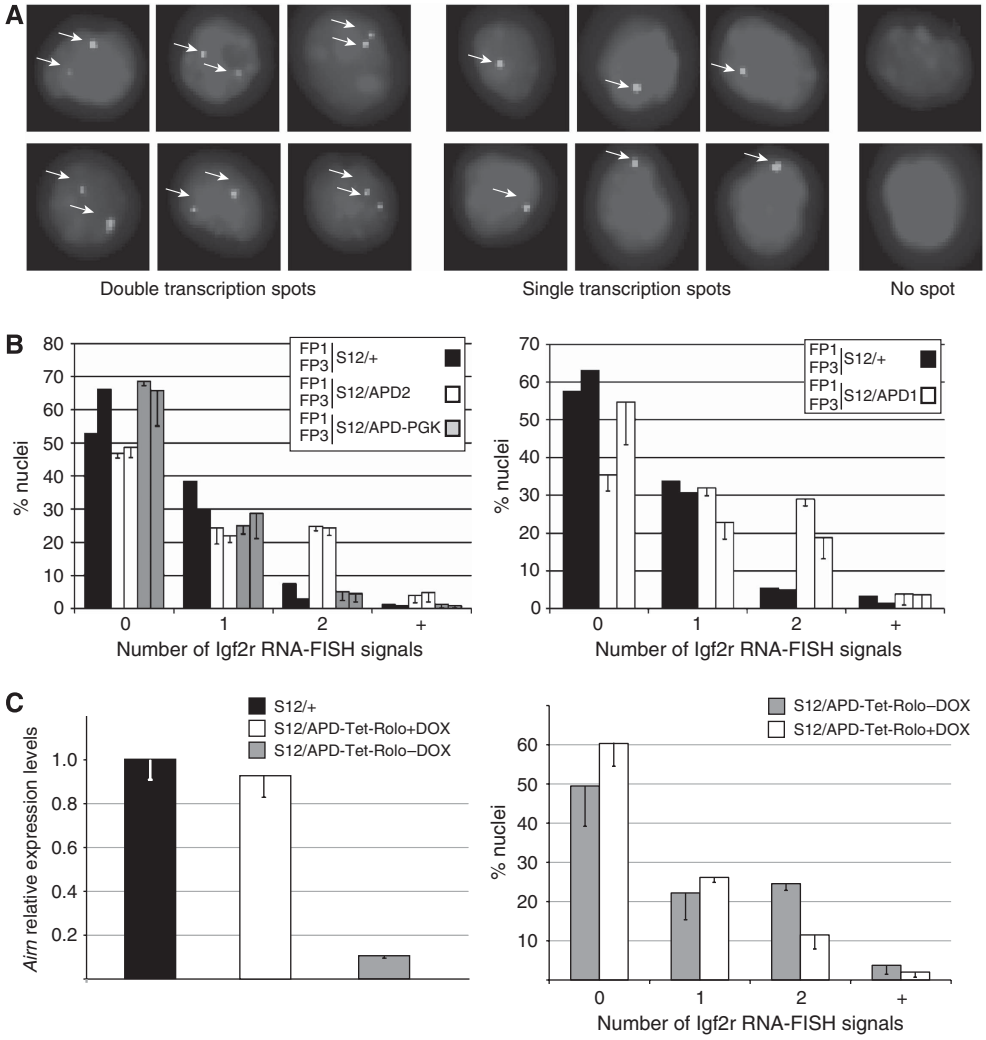

Figure 5 PGK-*Airn* and induced TET-*Airn* silence *Igf2r in cis*. (**A**) RNA FISH with the *Igf2r* intronic FP1 strand-specific probe in S12/APD ES cells. Representative examples of day 5 RA differentiated cells with double, single and no transcription spots are shown. (**B**) Left panel: quantification 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

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

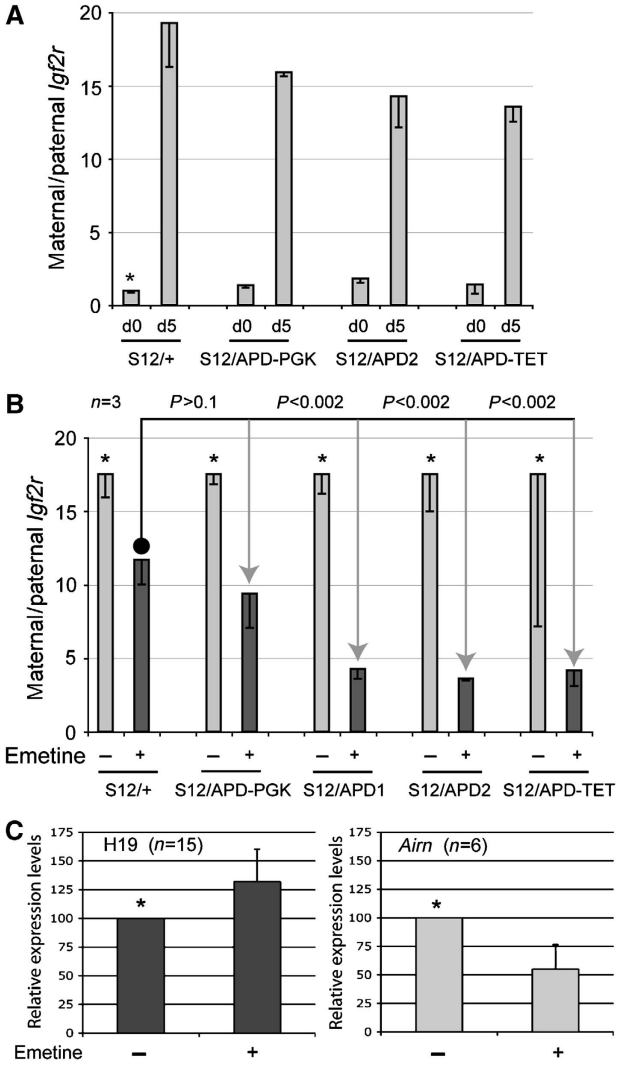

Figure 6 PGK promoter-driven *Airn* silences paternal *Igf2r*. (**A**) Allele-specific QPCR showing the ratio of maternal/paternal *Igf2r* expression in undifferentiated and differentiated wild-type and targeted ES cells. Numbers are ratios of maternal (S12) to paternal (+) allele. The ratio at day 0 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.

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.

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.

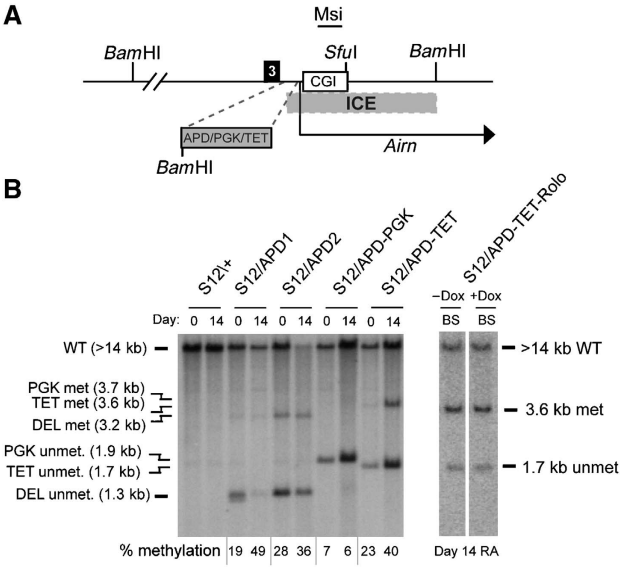

Figure 7 *Airn* expression protects the *Airn* CpG island from DNA methylation. (**A**) Map of a 20-kb region containing the *Airn* promoter. White box labelled CGI: CpG island; dark grey box: *Airn* promoter deletion or replacement cassette, which introduced a *Bam*HI site. ICE, details in Figure 1A, 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

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

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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