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Mechanisms of long range silencing by imprinted macro non-coding RNAs

Florian M Pauler, Denise P Barlow and Quanah J Hudson

Non-coding (nc) RNA silencing of imprinted genes in extra-embryonic tissues provides a good model for understanding an underexamined aspect of gene regulation by macro or long ncRNAs, that is their action as long-range *cis*-silencers. Numerous long intergenic ncRNAs (lincRNAs) have been recently discovered that are thought to regulate gene expression, some of which have been associated with disease. The few shown to regulate protein-coding genes are suggested to act by targeting repressive or active chromatin marks. Correlative evidence also indicates that imprinted macro ncRNAs cause long-range *cis*-silencing in placenta by targeting repressive histone modifications to imprinted promoters. It is timely, however, to consider alternative explanations consistent with the published data, whereby transcription alone could cause gene silencing at a distance.

Address
CeMM Research Center for Molecular Medicine of the Austrian Academy of Science, Lazarettgasse 14, AKH-BT 25.3, 1090 Vienna, Austria

Corresponding author: Hudson, Quanah J (qhudson@cemm.oeaw.ac.at)

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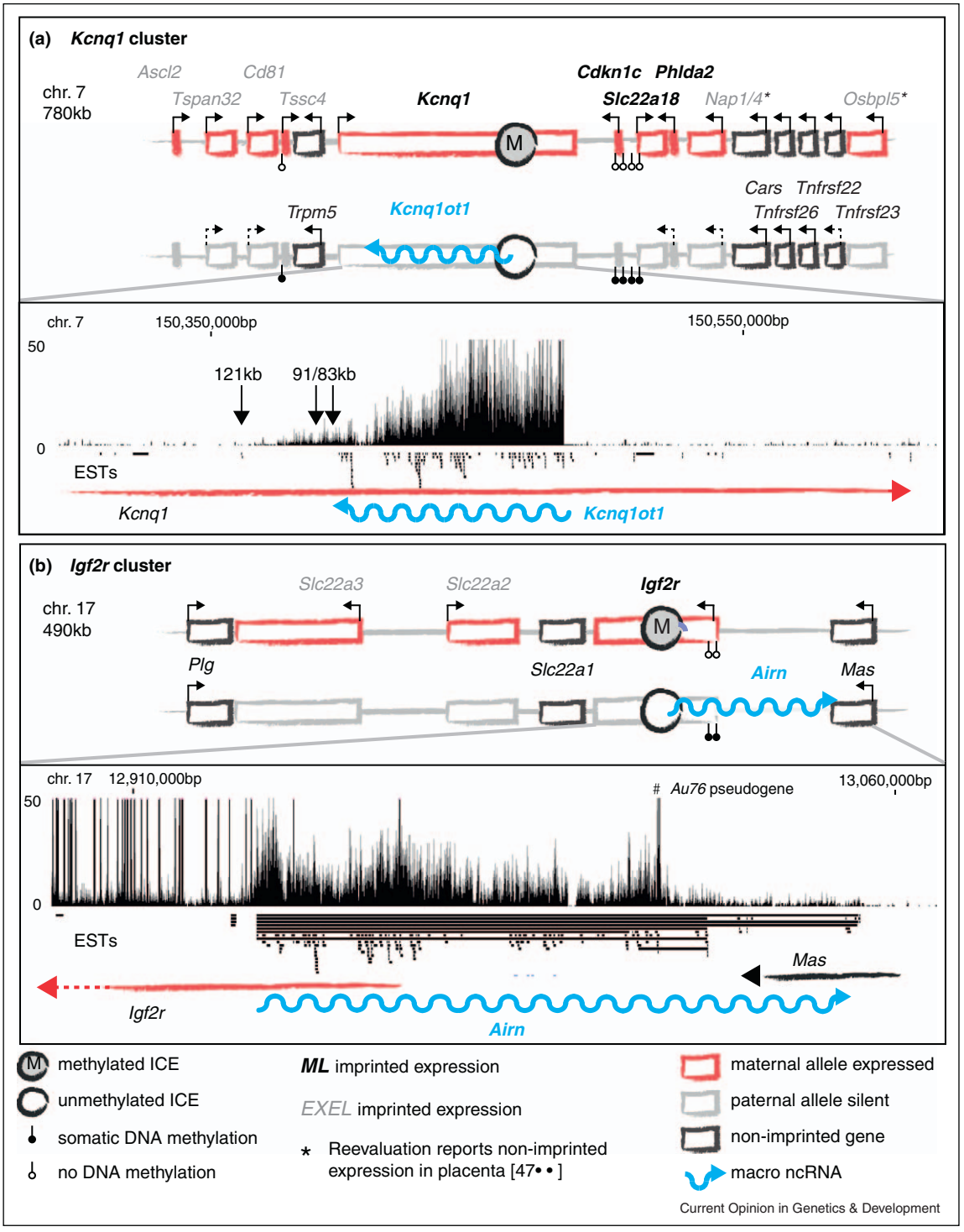
Genomic imprinting is an epigenetic process that controls parent-of-origin expression of an estimated 1–2% of genes in the mammalian genome [1,2•]. Although few in number, many imprinted genes play important roles in development and growth, often in a dose-dependent manner [3]. Imprinted genes mostly occur in clusters in the genome controlled by a CpG rich region known as an Imprint Control Element (ICE). This ICE shows differential DNA methylation, which is established in the germ cells of one parent and maintained on this parental chromosome throughout life. The ICE on the other parental allele remains unmethylated. The unmethylated ICE activates a macro non-coding (nc) RNA *in cis*, while methylation prevents activation on the other allele. Macro ncRNAs are inefficiently processed long ncRNAs whose main product is unspliced [1]. In three of four cases where the function of the imprinted macro ncRNA has been tested, it acts as a *cis*-silencer to prevent upregulation of flanking imprinted genes in the cluster [4–6,7••]. A hallmark of imprinted genes is that they show developmental and tissue-specific regulation of imprinted expression [8]. For example, the *Dlk1* gene is paternally expressed and plays a dose-dependent role in regulating growth of the embryo, but switches to biallelic expression in neural stem cells and niche astrocytes where it is required for normal postnatal neurogenesis [9,10••]. Imprinted genes can be divided into two groups based on their tissue-specific imprinted expression pattern. Multi-lineage (ML) genes show imprinted expression in both the embryo and extra-embryonic tissues, while extra-embryonic lineage-specific (EXEL) genes show imprinted expression restricted to specific cell lineages in the placenta and visceral yolk sac. EXEL genes are an example of long-range *cis*-silencing by a macro ncRNA, as they are located in the outer region of an imprinted cluster at a greater distance from the macro ncRNA than ML genes (Figure 1) [11••].

Long ncRNAs are widespread throughout the genome and include a group known as long intergenic ncRNAs or lincRNAs, which are defined by an H3K4me3-H3K36me3 chromatin signature [12,13]. Some lincRNAs are associated with long-range *cis*-activation of neighbouring genes [14]; for example, *HOTTIP* and *Mistral* activate nearby, but not distant, genes in the *HOXD* and *HOXA* clusters by recruiting the H3K4me3 methyltransferase MLL1 [15,16•]. Other lincRNAs are implicated in gene silencing. Approximately 20% of lincRNAs are associated with polycomb complex 2 (PRC2), which deposits the repressive H3K27me3 modification [17]. The human lincRNA *HOTAIR* expressed from the *HOXC* cluster acts *in trans* by targeting PCR2 to the *HOXD* cluster and causing gene silencing [18]; however, this function is not conserved in mouse [19••]. The function of most lincRNAs remains unknown, but the example of imprinted macro ncRNAs indicates that some may regulate nearby genes by long-range *cis*-silencing. Another example of long-range *cis*-silencing by a long ncRNA is X chromosome inactivation, which is regulated by *Xist* ncRNA [20]. However, X-inactivation results in silencing of a whole chromosome whereas imprinted macro ncRNAs silence a more limited domain of protein-coding genes, making them the more appropriate model to understand how long-range *cis*-silencing by lincRNAs may work [21•].

Figure 1



Long-range silencing by macro ncRNAs in two imprinted clusters. **(a)** Top: The imprinted *Kcnq1* cluster on mouse chromosome 7 spans 780 kb. In this cluster ten genes are repressed on the paternal allele by the macro ncRNA *Kcnq1ot1*. *Kcnq1ot1* is repressed on the maternal allele by a DNA methylation mark on the imprint control element (ICE) acquired in the oocyte. Of the ten maternally expressed genes, four show multi-lineage (ML) imprinted expression (bold font) and six show extra-embryonic lineage specific (EXEL) imprinted expression (grey font). Genes reported to be non-imprinted in placenta by a recent reevaluation are indicated (*) [45••]. *Kcnq1ot1* is located entirely within the protein-coding gene *Kcnq1* and is necessary for the silencing of all genes in the cluster [5,25]. Bottom: The *Kcnq1* gene is shown visualizing all transcripts in the region by Illumina RNA-Sequencing of mouse fetal head 14.5 days post coitum, a tissue in which *Kcnq1ot1*, but not *Kcnq1*, is expressed (pileup of sequencing reads, cut-off at 50 reads, data from [28]). Annotated minus strand unspliced ESTs (same strand as *Kcnq1ot1*) are also shown as short horizontal bars. Arrows

Imprinted macro ncRNAs as a model of long-range *cis*-repressors

Two types of *cis*-silencing can be mediated by macro ncRNAs: short-range silencing occurs when the ncRNA transcript fully or partially overlaps the regulated gene, while long-range silencing refers to regulation of non-overlapped genes. This review concentrates on recent findings on the mechanism of long-range *cis*-silencing by ncRNAs. A fundamental question is whether macro ncRNA silencing of gene expression requires the ncRNA product or if transcription alone is responsible for silencing. This question arises because features of imprinted macro ncRNAs, including the lack of sequence conservation, a low splicing rate and their unusually large size do not indicate a function for the RNA product [22,23]. The role of long ncRNAs in regulating genes in the surrounding imprinted cluster has been tested in four cases. The *H19* ncRNA is fully spliced and thus not a macro ncRNA, and it is also not responsible for *cis*-silencing in the *Igf2* cluster, but instead has been reported to regulate imprinted genes *in trans*, a function that may relate to its role as a micro RNA host transcript [24]. By contrast, truncation of the *Airn* and *Kcnq1ot1* macro ncRNAs in the *Igf2r* and *Kcnq1* clusters, respectively, by insertion of polyadenylation cassette resulted in re-expression of all the genes in these clusters from their normally silent paternal allele [5,6,25]. A similar strategy was used recently to show that *Nespas* macro ncRNA in the *Gnas* cluster silences *Nesp*, but the impact of the ncRNA truncation on the other promoters in the cluster has not yet been reported [7••].

Genes showing ML imprinted expression may or may not be overlapped by the regulating macro ncRNA. However, all genes showing EXEL imprinted expression are not overlapped and lie further away from the ncRNA, making them a better model to understand long-range *cis*-silencing by ncRNAs. EXEL imprinted expression is restricted to certain cell types in extra-embryonic tissues, meaning studies of EXEL gene regulation can be compromised when using an intact organ like placenta that contains non-EXEL embryonic cell types as well as maternal endothelial and blood tissue. We have recently shown that visceral endoderm, an EXEL cell type, can be efficiently isolated from visceral yolk sac providing a homogenous cell population to study EXEL gene regulation *in vivo* [11••]. This review examines recent findings that provide information on how imprinted macro ncRNAs may cause long-range *cis*-silencing of EXEL genes, focusing on silencing by *Airn* and *Kcnq1ot1* in the *Igf2r* and *Kcnq1* clusters (Figure 1).

The *Kcnq1ot1* macro ncRNA product may be necessary for long-range silencing

The *Kcnq1ot1* macro ncRNA is transcribed from the unmethylated paternal ICE located in intron 10 of *Kcnq1* and silences four ML genes and six EXEL genes on the paternal allele (Figure 1a). Using quantitative polymerase chain reaction (qPCR) assays, it was recently reported that *Kcnq1ot1* is 471 kb long in all examined tissues, and therefore overlaps all downstream genes, including EXEL genes [26]. However, this finding conflicts with previous reports using qPCR and RNase protection assays that mapped *Kcnq1ot1* to be 91 kb or 121 kb [22,27]. In addition, our own RNA sequencing data and the distribution of reported ESTs are consistent with the earlier studies, mapping *Kcnq1ot1* to be between 83 and 121 kb, meaning that it would only overlap *Kcnq1* introns 10–11 (Figure 1a) [28]. The *Kcnq1ot1* RNA is reported to have different behaviour in embryonic versus extra-embryonic tissues. The *Kcnq1ot1* RNA fluorescence *in situ* hybridization (FISH) signal is larger in placenta than in embryo, correlating with the greater number of genes silenced in the placenta [22]. *Kcnq1ot1* also shows a greater association with chromatin in placenta than embryo, implying an association between the ncRNA product and the chromosome in placenta [27]. In Trophoblast Stem (TS) cells, the precursor of EXEL cell types in the placenta, *Kcnq1ot1* colocalises with a contracted chromosome compartment containing the entire *Kcnq1* imprinted cluster and the repressive chromatin modifications H3K9me3, H2A119u1 and H3K27me3 [29••]. In addition, the PRC1 and PRC2 complexes, responsible for depositing H2A119u1 and H3K27me3, also colocalise with this putative repressive compartment, and the loss of either complex affects the imprinted expression of both the ML gene *Cdkn1c* and EXEL genes *Cd81* and *Tssc4* in extra-embryonic tissues only [29••]. By contrast, loss of the H3K9methyltransferase EHMT2 affects imprinted expression of EXEL genes only [30]. Although a direct connection has not been shown, these results imply that the *Kcnq1ot1* ncRNA product targets repressive chromatin modifying complexes to imprinted genes in extra-embryonic tissues causing silencing. A recent study reported that RNAi knockdown of *Kcnq1ot1* in embryonic (ES), trophoblast (TS) and extra-embryonic endoderm

(Figure 1 Legend Continued) indicate the reported end of *Kcnq1ot1* at 83 kb (Refseq annotation), 91 kb [27] and 121 kb [22]. **(b)** Top: The imprinted *Igf2r* cluster spans 490 kb on mouse chromosome 17. The *Airn* macro ncRNA represses three genes on the paternal chromosome, while *Airn* is repressed on the maternal chromosome by oocyte acquired DNA methylation of the ICE. On the paternal chromosome *Airn* silences *in cis* the overlapped ML gene *Igf2r* (short-range silencing), and the non-overlapped EXEL genes *Slc22a2* and *Slc22a3* whose promoters lie 157 kb and 234 kb upstream respectively (long-range silencing). Bottom: Part of the *Igf2r*/*Airn* region is shown visualizing all transcripts in the region by RNA-Sequencing (as above). *Airn* is covered continuously with sequence reads and also by plus strand (same strand as *Airn*) unspliced (short horizontal bars) and spliced (long horizontal bars) ESTs over its annotated 118 kb length. The exons of *Igf2r* are visible as focal peaks cut-off at 50 reads. Signal from the *Rangap1* pseudogene *Au76* is indicated (#). The key is given below the Figure. For both loci the names of imprinted genes are above the maternal chromosome, and the names of non-imprinted genes are below.

(XEN) stem cells had no effect on the maintenance of imprinted expression raising the possibility that the ncRNA product plays no role in silencing [26]. However these results need to accommodate the finding that *Kcnq1ot1* is a nuclear localised ncRNA and it is uncertain if RNAi can act in the mammalian nucleus [27,31].

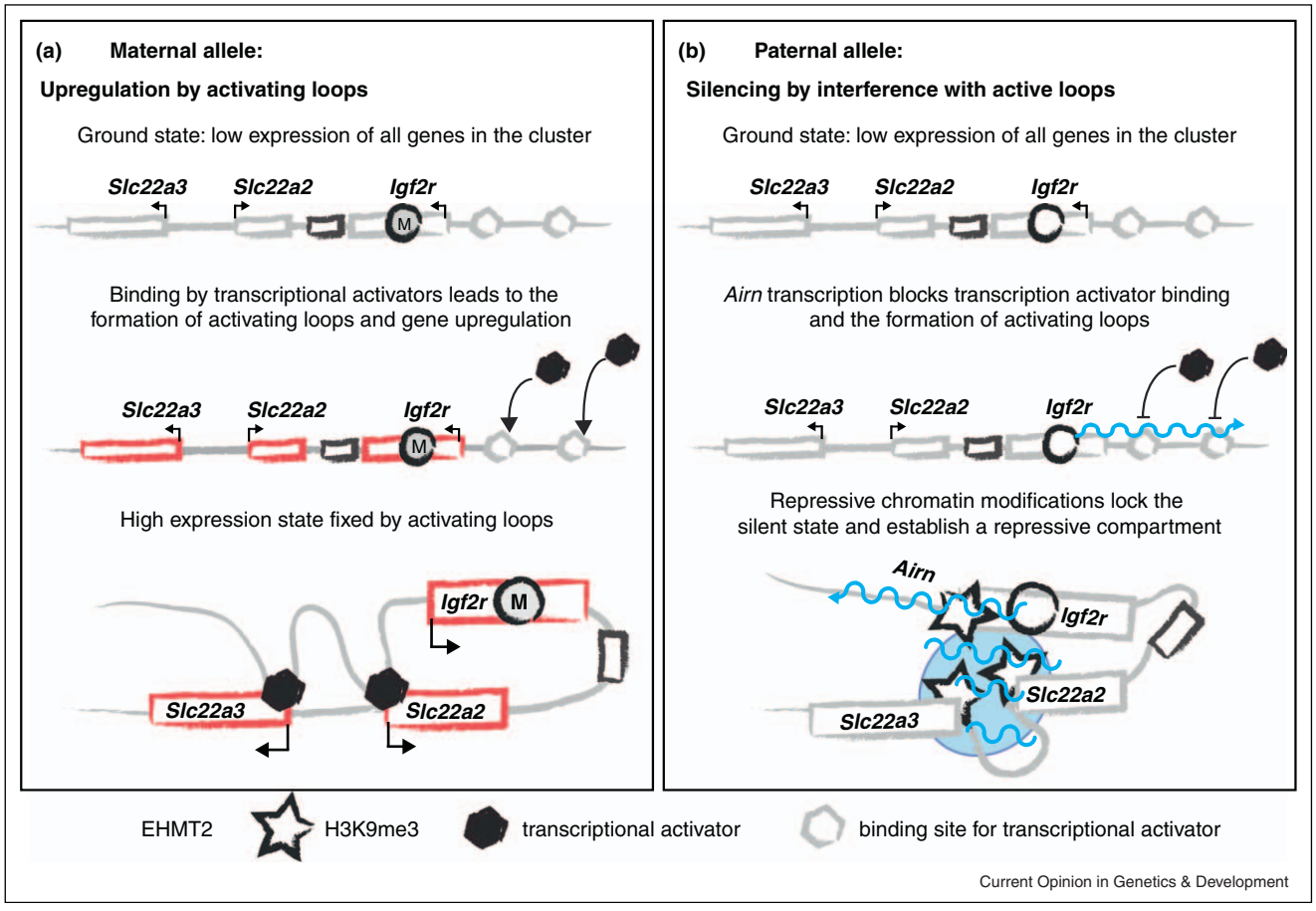
Airn macro ncRNA may silence by transcription alone

The concept that transcription, rather than the macro ncRNA product, may regulate overlapped imprinted genes is emerging for the *Igf2r*, *Gnas*, and *Copg2* imprinted gene clusters. Transcriptional interference, where one transcriptional process interferes with another without the involvement of a mature RNA, is a well-established *cis*-silencing mechanism in non-mammalian organisms like bacteria, yeast, and *Drosophila*, and has been suggested to occur in mammals [32••]. In both the *Igf2r* and the *Gnas* clusters, the macro ncRNA overlaps the promoter of a protein-coding gene in an antisense orientation. Truncation of the macro ncRNAs *Airn* and *Nespas*, so that the *Igf2r* and *Nesp* promoters are not overlapped, respectively, leads to a loss of repression of both protein-coding genes, indicating that repression may result from transcriptional interference; however, these data do not exclude a role for the ncRNA product [6,7••,33]. In the *Copg2* cluster, alternative polyadenylation of the paternally expressed *Mest* gene produces a longer form of this gene called *MestXL*, specifically in the mouse nervous system. *MestXL* overlaps the 3′ end of *Copg2* in antisense orientation correlating with paternal repression of *Copg2*, and this repression is lost when *MestXL* is truncated [34]. This result shows that variants of protein-coding genes can also act like macro ncRNAs to regulate other genes, and was interpreted as silencing by transcriptional interference, which would indicate that transcription across the promoter is not required. However, truncation experiments do not exclude a role for the ncRNA product in silencing, as both transcription and the ncRNA product are lost downstream of the truncation site. In the case of *Airn*, two aspects of its RNA biology, a short half-life and inefficient splicing [23], make it less likely that the mature ncRNA product is involved in silencing *Igf2r* in the embryo.

Transcriptional interference of overlapped genes by imprinted macro ncRNAs is consistent with what is known from other species, but long-range *cis*-silencing of non-overlapped genes by transcription interference is conceptually more difficult to imagine. The *Airn* and *Kcnq1ot1* genes are up to several hundred kilobases away from the EXEL genes they regulate (Figure 1a and b), and in both cases correlative evidence suggests that the ncRNA product is causing repression at a distance, as described for *Kcnq1ot1* above. In the placenta, the *Airn* macro ncRNA product is located in close proximity to the silent paternal promoter of the EXEL gene *Slc22a3* that also carries a repressive H3K9me3 histone mark [35••]. Silencing of *Slc22a3* depends on the lysine methyltransferase EHMT2 [35••] whose main activity is to catalyse H3K9me2, but which can also catalyse H3K9me3 at some loci [30,36,37]. As *Airn* also associates with EHMT2 in placenta, it is possible that the *Airn* ncRNA product is responsible for the recruitment of EHMT2 to the *Slc22a3* promoter and therefore for its silencing. The Tagging and Recovery of Associated Proteins (TRAP) method that is dependent on detecting the ncRNA by *in situ* hybridization was used to detect the close proximity of the *Airn* ncRNA and the *Slc22a3* promoter [35••]. Interestingly this technique was initially used to discover a chromosome loop connecting enhancers in the *β-globin* locus control region with the *β-globin* promoter [38]. Applying the same concept to the *Airn* TRAP data implies that the *Slc22a3* promoter is close to the *Airn* transcription unit in three-dimensional space. With this in mind we propose a model consistent with the published data, where the mature ncRNA product is not responsible for silencing genes at a distance, but rather *Airn* transcription blocks the binding of transcriptional activators that are required to facilitate chromosomal looping and activation of *Slc22a2* and *Slc22a3* expression.

In this model, early development is defined by a ground state chromatin conformation that allows low-level biallelic expression of protein-coding genes on both parental alleles (Figure 2a and b, top). This ground state is well established for *Igf2r* in pre-implantation embryos [39,40], and for *Slc22a2* and *Slc22a3*, which are not upregulated until post-implantation [11••]. In this ground state *Airn* is not made, because DNA methylation of the ICE prevents *Airn* expression on the maternal chromosome [11••] and most probably essential transcription factors are not yet expressed to activate the paternal allele [41]. In the post-implantation embryo, following the binding of transcriptional activators, activating loops form on the maternal chromosome between enhancers and the promoters of *Slc22a2* and *Slc22a3*, causing their upregulation (Figure 2a, middle and bottom). On the paternal allele, *Airn* transcription prevents upregulation of *Igf2r* by transcription interference, and blocks the binding of transcriptional activators and the formation of activating loops within the *Airn* gene body, preventing upregulation of *Slc22a2* and *Slc22a3* (Figure 2b, middle). In a secondary step, EHMT2 is recruited to the *Slc22a2* and *Slc22a3* promoters and is required to maintain repression of these genes [35••]. The repressed genes then attract PRC1 and PRC2 to catalyse the H2A119u1 and H3K27me3 modifications causing chromatin compaction and the formation of a repressive compartment (Figure 2b bottom). This compaction brings the *Airn* macro ncRNA, the *Slc22a2* and *Slc22a3* promoters and EHMT2 in close physical proximity that can be detected by sensitive techniques like TRAP and RNA immunoprecipitation. This model is supported by the formation of a repressive compartment

Figure 2



Long-range silencing by interference with active loops. A model for transcription mediated long-range *cis*-silencing by a macro ncRNA using the example of *Airn* regulated imprinted expression in the *Igf2r* cluster. In the ground state, all protein-coding genes show a low level of expression from both the maternal and paternal allele (**a** and **b**, top). On the maternal allele binding of transcriptional activators leads to the formation of activating loops and upregulation of *Slc22a2* and *Slc22a3* (**a**, middle). The activating loops maintain high expression levels (**a**, bottom). On the paternal allele *Airn* transcription prevents *Igf2r* upregulation by transcriptional interference, and blocks transcription activator binding and the formation of activating loops preventing *Slc22a2* and *Slc22a3* upregulation (**b**, middle). The silent promoters attract EHMT2 to deposit H3K9me3, and polycomb complexes then deposit H3K27me3 and H2A119u1 locking the silent state and causing chromatin compaction. This compaction establishes a repressive compartment bringing *Airn* ncRNA in close proximity to the silenced promoters, the repressive histone modifications and their histone modifying complexes (**b**, bottom). The key is given below the Figure. The gene colour code is the same as for Figure 1.

on the paternal chromosome containing *Airn* ncRNA, a contracted *Igf2r* cluster, PRC1 and PRC2 and the repressive H2A119u1, H3K27me3 and H3K9me3 modifications [29••].

Concluding remarks

Recent reports have highlighted the importance of long ncRNAs in disease. Overexpression of the lincRNA *HOTAIR* in breast and colorectal cancers is associated with increased PRC2 activity and an altered H3K27me3 distribution, and correlates with metastasis and a poor prognosis [42,43•]. The prostate cancer associated long ncRNA, *PCAT-1*, is correlated with aggressive prostate cancer, and appears to have a prostate specific role in regulating cell proliferation [44•]. The many long ncRNAs that have been recently discovered are likely to play a role in gene regulation and misregulation in disease, demonstrating the need for well-characterised model systems to understand their different mechanisms of action. Understanding the mechanism of imprinted macro ncRNA action may reveal new drug targets and enable improved therapy for diseases where macro ncRNAs play a role.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Barlow DP: **Genomic imprinting: a mammalian epigenetic discovery model**. *Annu Rev Genet* 2011, **45**:379-403.

2. • Williamson CM, Blake A, Thomas S, Beechey CV, Hancock J, Cattanach BM, Peters J: **World Wide Web Site – Mouse Imprinting Data and References MRC Harwell, Oxfordshire**. In http://www.har.mrc.ac.uk/research/genomic_imprinting/. 2011.

A comprehensive database of verified mouse imprinted genes integrating information on chromosome regions associated with imprinted phenotypes with imprinted genes in these regions, and also containing imprinted genes in regions not associated with known imprinted phenotypes.

3. Ferguson-Smith AC: **Genomic imprinting: the emergence of an epigenetic paradigm**. *Nat Rev Genet* 2011, **12**:565-575.

4. Bell AC, Felsenfeld G: **Methylation of a CTCF-dependent boundary controls imprinted expression of the Igf2 gene**. *Nature* 2000, **405**:482-485.

5. Mancini-Dinardo D, Steele SJ, Levorse JM, Ingram RS, Tilghman SM: **Elongation of the Kcnq1ot1 transcript is required for genomic imprinting of neighboring genes**. *Genes Dev* 2006, **20**:1268-1282.

6. Sleutels F, Zwart R, Barlow DP: **The non-coding Air RNA is required for silencing autosomal imprinted genes**. *Nature* 2002, **415**:810-813.

7. •• Williamson CM, Ball ST, Dawson C, Mehta S, Beechey CV, Fray M, Teboul L, Dear TN, Kelsey G, Peters J: **Uncoupling antisense-mediated silencing and DNA methylation in the imprinted Gnas cluster**. *PLoS Genet* 2011, **7**:e1001347.

This study demonstrates that a macro ncRNA or its transcription can silence an overlapped protein-coding gene independent of DNA methylation. This finding contributes to understanding the sequence of events in epigenetic silencing by macro ncRNAs.

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

9. da Rocha ST, Charalambous M, Lin SP, Gutteridge I, Ito Y, Gray D, Dean W, Ferguson-Smith AC: **Gene dosage effects of the imprinted delta-like homologue 1 (Dlk1/Pref1) in development: implications for the evolution of imprinting**. *PLoS Genet* 2009, **5**:e1000392.

10. •• Ferron SR, Charalambous M, Radford E, McEwen K, Wildner H, Hind E, Morante-Redolat JM, Laborda J, Guillemot F, Bauer SR *et al.*: **Postnatal loss of Dlk1 imprinting in stem cells and niche astrocytes regulates neurogenesis**. *Nature* 2011, **475**:381-385.

This study shows that biallelic expression of the imprinted gene *Dlk1* is required for correct neurogenesis. This demonstrates that developmental and tissue-specific regulation of imprinted expression can be important for imprinted gene function.

11. •• Hudson QJ, Seidl CI, Kulinski TM, Huang R, Warczok KE, Bittner R, Bartolomei MS, Barlow DP: **Extra-embryonic-specific imprinted expression is restricted to defined lineages in the post-implantation embryo**. *Dev Biol* 2011.

This study defines the extra-embryonic lineages that show EXEL imprinted expression and demonstrates that maternal contribution to the placenta and biallelic expression in non-EXEL cell types can complicate the interpretation of studies into the regulation of EXEL genes. A new technique is presented enabling efficient isolation of visceral endoderm from visceral yolk sac, providing an improved model of EXEL imprinted expression that lacks maternal and non-EXEL cell types.

12. Guttman M, Amit I, Garber M, French C, Lin MF, Feldser D, Huarte M, Zuk O, Carey BW, Cassady JP *et al.*: **Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals**. *Nature* 2009, **458**:223-227.

13. Wright MW, Bruford EA: **Naming 'junk': human non-protein coding RNA (ncRNA) gene nomenclature**. *Hum Genomics* 2011, **5**:90-98.

14. Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q *et al.*: **Long noncoding RNAs with enhancer-like function in human cells**. *Cell* 2010, **143**:46-58.

15. Bertani S, Sauer S, Bolotin E, Sauer F: **The noncoding RNA Mistral activates Hoxa6 and Hoxa7 expression and stem cell differentiation by recruiting MLL1 to chromatin**. *Mol Cell* 2011, **43**:1040-1046.

16. • Wang KC, Yang YW, Liu B, Sanyal A, Corces-Zimmerman R, Chen Y, Lajoie BR, Protacio A, Flynn RA, Gupta RA *et al.*: **A long noncoding RNA maintains active chromatin to coordinate homeotic gene expression**. *Nature* 2011, **472**:120-124.

This study is the first example where the mechanism of *cis*-activation by a long ncRNA is shown. Chromosome looping brings the lincRNA *HOTTIP* in close proximity to its target genes in the *HOXA* cluster. *HOTTIP* RNA binds the WDR5 adaptor protein and targets the WDR5/MLL complex to the adjacent region of the *HOXA* cluster driving H3K4me3 modification and transcription.

17. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, Rivea Morales D, Thomas K, Presser A, Bernstein BE, van Oudenaarden A *et al.*: **Many human large intergenic noncoding RNAs associate with chromatin-modifying complexes and affect gene expression**. *Proc Natl Acad Sci USA* 2009, **106**:11667-11672.

18. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, Brugmann SA, Goodnough LH, Helms JA, Farnham PJ, Segal E *et al.*: **Functional demarcation of active and silent chromatin domains in human HOX loci by noncoding RNAs**. *Cell* 2007, **129**:1311-1323.

19. •• Schorderet P, Duboule D: **Structural and functional differences in the long non-coding RNA hotair in mouse and human**. *PLoS Genet* 2011, **7**:e1002071.

In human cells the *HOTAIR* lincRNA recruits PRC2 and H3K27me3 *in trans* to the *HOXD* cluster causing gene silencing. This study shows that this function is not conserved in the mouse, demonstrating that lincRNAs can be fast evolving.

20. Wutz A: **Gene silencing in X-chromosome inactivation: advances in understanding facultative heterochromatin formation**. *Nat Rev Genet* 2011, **12**:542-553.

21. • Bateman A, Agrawal S, Birney E, Bruford EA, Bujnicki JM, Cochrane G, Cole JR, Dinger ME, Enright AJ, Gardner PP *et al.*: **RNAcentral: A vision for an international database of RNA sequences**. *RNA* 2011, **17**:1941-1946.

An important proposal to create a comprehensive database including all types of ncRNA sequences.

22. Redrup L, Branco MR, Perdeaux ER, Krueger C, Lewis A, Santos F, Nagano T, Cobb BS, Fraser P, Reik W: **The long noncoding RNA Kcnq1ot1 organises a lineage-specific nuclear domain for epigenetic gene silencing**. *Development* 2009, **136**:525-530.

23. Seidl CI, Stricker SH, Barlow DP: **The imprinted Air ncRNA is an atypical RNAPII transcript that evades splicing and escapes nuclear export**. *EMBO J* 2006, **25**:3565-3575.

24. Gabory A, Ripoche MA, Le Digarcher A, Watrin F, Ziyyat A, Forne T, Jammes H, Ainscough JF, Surani MA, Journot L *et al.*: **H19 acts as a trans regulator of the imprinted gene network controlling growth in mice**. *Development* 2009, **136**:3413-3421.

25. Shin JY, Fitzpatrick GV, Higgins MJ: **Two distinct mechanisms of silencing by the KvDMR1 imprinting control region**. *EMBO J* 2008, **27**:168-178.

26. Golding MC, Magri LS, Zhang L, Lalone SA, Higgins MJ, Mann MR: **Depletion of Kcnq1ot1 non-coding RNA does not affect imprinting maintenance in stem cells**. *Development* 2011, **138**:3667-3678.

27. Pandey RR, Mondal T, Mohammad F, Enroth S, Redrup L, Komorowski J, Nagano T, Mancini-Dinardo D, Kanduri C: **Kcnq1ot1 antisense noncoding RNA mediates lineage-specific transcriptional silencing through chromatin-level regulation**. *Mol Cell* 2008, **32**:232-246.

28. Huang R, Jaritz M, Guenzl P, Vlatkovic I, Sommer A, Tamir IM, Marks H, Klampfl T, Kralovics R, Stunnenberg HG *et al.*: **An RNA-Seq strategy to detect the complete coding and non-coding transcriptome including full-length imprinted macro ncRNAs**. *PLoS ONE* 2011, **6**:e27288.

29. Terranova R, Yokobayashi S, Stadler MB, Otte AP, van
•• Lohuizen M, Orkin SH, Peters AH: **Polycomb group proteins Ezh2 and Rnf2 direct genomic contraction and imprinted repression in early mouse embryos**. *Dev Cell* 2008, **15**:668-679.
This paper shows that in the *Kcnq1* and *Igf2r* clusters the silent allele is compacted and within a putative repressive compartment, colocalising with repressive histone modifications and the macro ncRNA. The PRC1 and PRC2 complexes, that colocalise with this repressive compartment together with the modifications they catalyse, H3K27me3 and H2A119u1, are shown to be required for imprinted expression of *Kcnq1* cluster genes in placenta.

30. Wagschal A, Sutherland HG, Woodfine K, Henckel A, Chebli K, Schulz R, Oakey RJ, Bickmore WA, Feil R: **G9a histone methyltransferase contributes to imprinting in the mouse placenta**. *Mol Cell Biol* 2008, **28**:1104-1113.

31. Ideue T, Hino K, Kitao S, Yokoi T, Hirose T: **Efficient oligonucleotide-mediated degradation of nuclear noncoding RNAs in mammalian cultured cells**. *RNA* 2009, **15**:1578-1587.

32. Palmer AC, Egan JB, Shearwin KE: **Transcriptional interference
•• by RNA polymerase pausing and dislodgement of transcription factors**. *Transcription* 2011, **2**:9-14.
This review provides an excellent overview of the evidence for transcriptional interference in different organisms, and discusses models explaining how transcriptional interference may work.

33. Pauler FM, Koerner MV, Barlow DP: **Silencing by imprinted noncoding RNAs: is transcription the answer?** *Trends Genet* 2007, **23**:284-292.

34. MacIsaac JL, Bogutz AB, Morrissy AS, Lefebvre L: **Tissue-specific alternative polyadenylation at the imprinted gene Mest regulates allelic usage at Copg2**. *Nucleic Acids Res* 2011 doi: 10.1093/nar/gkr871.

35. Nagano T, Mitchell JA, Sanz LA, Pauler FM, Ferguson-Smith AC,
•• Feil R, Fraser P: **The Air noncoding RNA epigenetically silences transcription by targeting G9a to chromatin**. *Science* 2008, **322**:1717-1720.
This work in placenta shows an association between *Airn* and the *Slc22a3* promoter as well as an association between *Airn* and the EHMT2 H3K9methyltransferase. EHMT2 and H3K9me3 are enriched on the *Slc22a3* promoter, and EHMT2 is shown to be required for *Slc22a3* imprinted expression.

36. Kubicek S, O'Sullivan RJ, August EM, Hickey ER, Zhang Q, Teodoro ML, Rea S, Mechtler K, Kowalski JA, Homon CA *et al.*: **Reversal of H3K9me2 by a small-molecule inhibitor for the G9a histone methyltransferase**. *Mol Cell* 2007, **25**:473-481.

37. Tachibana M, Ueda J, Fukuda M, Takeda N, Ohta T, Iwanari H, Sakihama T, Kodama T, Hamakubo T, Shinkai Y: **Histone methyltransferases G9a and GLP form heteromeric complexes and are both crucial for methylation of euchromatin at H3-K9**. *Genes Dev* 2005, **19**:815-826.

38. Carter D, Chakalova L, Osborne CS, Dai YF, Fraser P: **Long-range chromatin regulatory interactions in vivo**. *Nat Genet* 2002, **32**:623-626.

39. Lerchner W, Barlow DP: **Paternal repression of the imprinted mouse Igf2r locus occurs during implantation and is stable in all tissues of the post-implantation mouse embryo**. *Mech Dev* 1997, **61**:141-149.

40. Szabo PE, Mann JR: **Allele-specific expression and total expression levels of imprinted genes during early mouse development: implications for imprinting mechanisms**. *Genes Dev* 1995, **9**:3097-3108.

41. Latos PA, Stricker SH, Steenpass L, Pauler FM, Huang R, Senergin BH, Regha K, Koerner MV, Warczok KE, Unger C *et al.*: **An in vitro ES cell imprinting model shows that imprinted expression of the Igf2r gene arises from an allele-specific expression bias**. *Development* 2009, **136**:437-448.

42. Kogo R, Shimamura T, Mimori K, Kawahara K, Imoto S, Sudo T, Tanaka F, Shibata K, Suzuki A, Komune S *et al.*: **Long noncoding RNA HOTAIR regulates polycomb-dependent chromatin modification and is associated with poor prognosis in colorectal cancers**. *Cancer Res* 2011, **71**:6320-6326.

43. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ,
• Tsai MC, Hung T, Argani P, Rinn JL *et al.*: **Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis**. *Nature* 2010, **464**:1071-1076.
See annotation to Ref. [44•].

44. Prensner JR, Iyer MK, Balbin OA, Dhanasekaran SM, Cao Q,
• Brenner JC, Laxman B, Asangani IA, Grasso CS, Kominsky HD *et al.*: **Transcriptome sequencing across a prostate cancer cohort identifies PCAT-1, an unannotated lincRNA implicated in disease progression**. *Nat Biotechnol* 2011, **29**:742-749.
References [43•] and [44•] show a role for two lincRNAs in cancer, demonstrating the importance of understanding the different mechanisms of action that long ncRNAs can have on gene regulation.

45. Okae H, Hiura H, Nishida Y, Funayama R, Tanaka S, Chiba H,
•• Yaegashi N, Nakayama K, Sasaki H, Arima T: **Re-investigation and RNA sequencing-based identification of genes with placenta-specific imprinted expression**. *Hum Mol Genet* 2011.
This study reevaluates reported placenta-specific imprinted genes using embryo transfer between mouse strains to control for maternal contribution to placenta. The results provide a reliable list of EXEL genes in placenta as a reference for future studies of imprinted gene regulation in this organ.