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Non-coding transcripts in the *H19* imprinting control region mediate gene silencing in transgenic *Drosophila*

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The imprinting control region (ICR) upstream of *H19* is the key regulatory element conferring monoallelic expression on *H19* and *Igf2* (*insulin-like growth factor 2*). Epigenetic marks in the ICR regulate its interaction with the chromatin protein CCCTC-binding factor and with other control factors to coordinate gene silencing in the imprinting cluster. Here, we show that the *H19* ICR is biallelically transcribed, producing both sense and antisense RNAs. We analyse the function of the non-coding transcripts in a *Drosophila* transgenic system in which the *H19* upstream region silences the expression of a reporter gene. We show that knockdown of *H19* ICR non-coding RNA (ncRNA) by RNA interference leads to the loss of reporter gene silencing. Our results are, to the best of our knowledge, the first to show that ncRNAs in the *H19* ICR are functionally significant, and also indicate that they have a role in regulating gene expression and perhaps epigenetic marks at the *H19/Igf2* locus.
Keywords: gene silencing; *H19*; imprinting control region; non-coding RNAs

INTRODUCTION

Genomic imprinting is an epigenetic phenomenon that results in the parent of origin-dependent monoallelic expression of a particular set of mammalian genes (Reik & Walter, 2001). The imprinted *Igf2* (*insulin-like growth factor 2*) and *H19* genes are expressed monoallelically from the paternal and maternal alleles, respectively. Two crucial regulatory elements control the mono-allelic expression of *H19* and *Igf2* in endodermal tissues: a set of enhancers downstream of the *H19* gene and the imprinting

control region (ICR) upstream of *H19* (Thorvaldsen *et al*, 1998). The *H19* ICR is methylated on the paternal chromosome, whereas the insulator protein CCCTC-binding factor (CTCF) binds to the element on the maternal chromosome. CTCF establishes a chromatin boundary on the maternal allele, restricting the enhancer to act on the maternal *H19* gene (Bell & Felsenfeld, 2000; Hark *et al*, 2000; Kaffer *et al*, 2000).

A growing body of evidence indicates an essential role for non-coding RNAs (ncRNAs) in the regulation of eukaryotic gene expression (Prasanth & Spector, 2007). The paternally expressed antisense *Igf2r* RNA (*Air*) and *Kcnq1ot1* genes produce two ncRNAs with an essential role in genomic imprinting. *Air* represses the transcription of the paternal alleles of the imprinted genes *Igf2r*, *Slc22a2* and *Slc22a3* (Sleutels *et al*, 2002). Similarly, *Kcnq1ot1* mediates transcriptional repression of multiple genes within the *Kcnq1* imprinting locus (Mancini-DiNardo *et al*, 2006). Furthermore, in three other imprinting loci, deletion of ncRNA promoters leads to the loss of silencing of associated protein-coding genes, indicating that gene silencing mediated by ncRNAs might represent a widespread mechanism in imprinted clusters (Chamberlain & Brannan, 2001; Lin *et al*, 2003; Williamson *et al*, 2006). However, so far, the ICRs themselves have not been systematically analysed to see whether they are transcribed. This is of interest because several regulatory sequences in the genome, including polycomb response elements and locus control regions, are also transcribed and this is important for their function (Gribnau *et al*, 2000; Schmitt *et al*, 2005). Some preliminary observations indicate that there might be transcripts in the *H19* ICR; however, they have not yet been characterized and nothing is known about their function (Drewell *et al*, 2002). Here, we show that in the mouse, the *H19* ICR is biallelically transcribed, resulting in both sense and antisense transcripts. The non-coding *H19* ICR transcripts are coexpressed with the *H19* and *Igf2* genes, and are retained in the nucleus. To address the potential function of the non-coding *H19* ICR RNAs, we used a *Drosophila* transgenic *H19* locus in which expression of the reporter gene *mini-white* is silenced by the *H19* upstream region (Lyko *et al*, 1997). Remarkably, expression of an RNA interference (RNAi) hairpin construct directed against the transcribed *H19* ICR relieved transcriptional repression of the reporter gene in the *Drosophila* transgene. Derepression of reporter gene transcription

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correlated with downregulation of the level of *H19* ICR transcripts, indicating a role for ncRNAs in regulating gene silencing at the *H19–Igf2* locus.

RESULTS AND DISCUSSION

The *H19* ICR produces sense and antisense transcripts

ICRs have been shown to harbour promoters for ncRNAs that control monoallelic gene expression (Smilinich *et al*, 1999; Sleutels *et al*, 2002). In addition, it has been estimated that more than 80% of all imprinted transcription units produce sense–antisense transcript pairs (Katayama *et al*, 2005). These findings prompted us to search for transcripts in the *H19* ICR. Using reverse transcription reactions, we detected transcripts with all primer pairs that we had chosen to analyse the transcriptional status of the *H19* upstream regulatory region, including those specific for the *H19* ICR and the silencer region (Fig 1A). The RNA for the reverse transcription reactions was isolated from F1 generation fetal livers obtained from a cross between the C57Bl6 and SD7 strains, therefore single nucleotide polymorphisms between the strains allowed us to determine the parental origin of the transcripts. Sequencing of the complementary DNA PCR products showed that the *H19* upstream region is biallelically transcribed (Fig 1B, PCRs 1–10), whereas a PCR product covering a part of the *H19* transcribed region confirmed its monoallelic expression (Fig 1B, PCR 11). Next, we carried out quantitative chromatin immuno-precipitation (ChIP) experiments using an antibody raised against the transcribing form of RNA polymerase II (Pol II) to analyse Pol II occupancy within the *H19* upstream region in mouse fetal liver cells. The Pol II antibody and an unspecific control antibody were used to pull down crosslinked fragments. The immunoprecipitates were then analysed using real-time PCR. We detected only a weak Pol II enrichment in the region upstream of the *H19* ICR, whereas, surprisingly, the *H19* silencer region was four- to fivefold enriched in the Pol II immunoprecipitates compared with the control immunoprecipitates (Fig 1C). Pol II binding to *H19* silencer sequences was only twofold lower than to the *H19* gene itself, and approximately 30-fold lower than to the highly transcribed α-globin gene. Next, we sought to determine the subcellular localization of the non-coding *H19* ICR transcripts. We separated nuclear and cytoplasmic RNA fractions from mouse embryonic fibroblasts. *Igf2* and *H19* gene transcripts were found both in the nucleus and in the cytoplasm, whereas the ncICR transcripts were almost exclusively detected in the nuclear fraction (Fig 1D), which is consistent with a function in gene expression. We then addressed the question of the orientation of the transcripts by carrying out reverse transcription reactions with strand-specific primers in the cDNA synthesis. Sense and antisense transcripts were detected in the *H19* ICR and the silencer region, originating from both parental chromosomes, as determined by sequencing of the cDNA PCR products (Fig 2A,E). To analyse whether the ncICR transcripts are expressed in the same cells as the genes encoding *Igf2* and *H19*, we carried out double-labelled RNA fluorescence *in situ* hybridization (RNA-FISH) experiments on mouse fetal liver cells using strand-specific probes against the ncICR transcripts in combination with probes against the paternal *Igf2* gene transcripts. Cells with monoallelic *Igf2* expression and ICR transcripts were grouped into three categories: expression from the maternal chromosome (Fig 2B,F: one ICR signal non-overlapping with the

Igf2 signal), expression from the paternal chromosome (Fig 2C,G: one ICR signal overlapping with the *Igf2* signal) and biallelic expression (Fig 2D,H: two ICR signals, one of which overlaps with the *Igf2* signal). Sense and antisense ICR transcripts were detected at comparable frequencies originating from both parental chromosomes. We confirmed these results by RNA-FISH experiments using probes against the ncICR transcripts in combination with a probe against the maternal *H19* gene transcript (supplementary Fig 1 online). Together, our results indicate an unexpectedly high transcriptional activity within the *H19* ICR.

Transcription at a *Drosophila H19* ICR transgene

To characterize the molecular function of the ncRNAs in the *H19* ICR, we used a transgenic fly line carrying an *H19* ICR transgene (Lyko *et al*, 1997). In this fly strain, expression of the eye colour reporter gene *mini-white* is controlled by the 3.8 kb *H19* upstream regulatory element, including the *H19* ICR. Flies carrying the *H19* ICR transgene show a yellow eye colour, which indicates a partial silencing of *mini-white*. Using *Drosophila* genetics, we have previously identified a silencer element within the *H19* ICR (Lyko *et al*, 1997). The *H19* silencer represses transcription of reporter genes in *Drosophila* (Lyko *et al*, 1997). Deletion of the silencer from the endogenous mouse locus results in loss of silencing of the paternal *H19* allele (Drewell *et al*, 2000). This indicates that the *Drosophila* transgene can uncover evolutionarily conserved regulatory elements of the mice *H19* ICR. Here, we first investigated whether the *Drosophila* transgene recapitulates the transcriptional activity of the ICR at the endogenous locus. Indeed, we found that the *H19* ICR is bidirectionally transcribed in *H19* ICR transgenic *Drosophila* (supplementary Fig 2 online), mirroring the transcriptional activity at the endogenous mouse locus. The finding that both the silencing activity and the transcriptional status of the *H19* upstream region are conserved between mice and trans-genic *Drosophila* strongly indicates a common underlying epigenetic mechanism and enables us to use the *Drosophila* ICR transgene as a model system to study the molecular function of the non-coding *H19* ICR transcripts.

Silencing at the *H19* ICR transgene is RNAi-independent

RNAi pathways are known to mediate transcriptional gene silencing in the nucleus (Matzke & Birchler, 2005). However, despite the presence of sense and antisense RNAs, we were unable to detect small interfering RNAs (siRNA) from the *H19* ICR, nor did mutations in RNAi pathway genes relieve *H19* ICR-conferred reporter gene repression. This argues against an RNAi mechanism mediating transcriptional repression at the transgenic *Drosophila H19* ICR locus (supplementary Fig 3 online).

RNA-mediated silencing at the transgenic *H19* ICR locus

To eliminate the non-coding transcript in the ICR to potentially uncover its function, we decided to use the RNAi technique. We expressed a 430-bp fragment from the *H19* ICR as an inverted repeat, which leads to the formation of a double-stranded RNA that is subsequently cleaved to siRNAs targeting the ncICR transcripts (supplementary Fig 3 online). We found that expression of the RNAi hairpin vector led to a substantially reduced level of *H19* ICR transcripts (Fig 3C,D). Semiquantitative reverse trans-cription PCR (RT–PCR) showed that the level of *H19* ICR RNA was
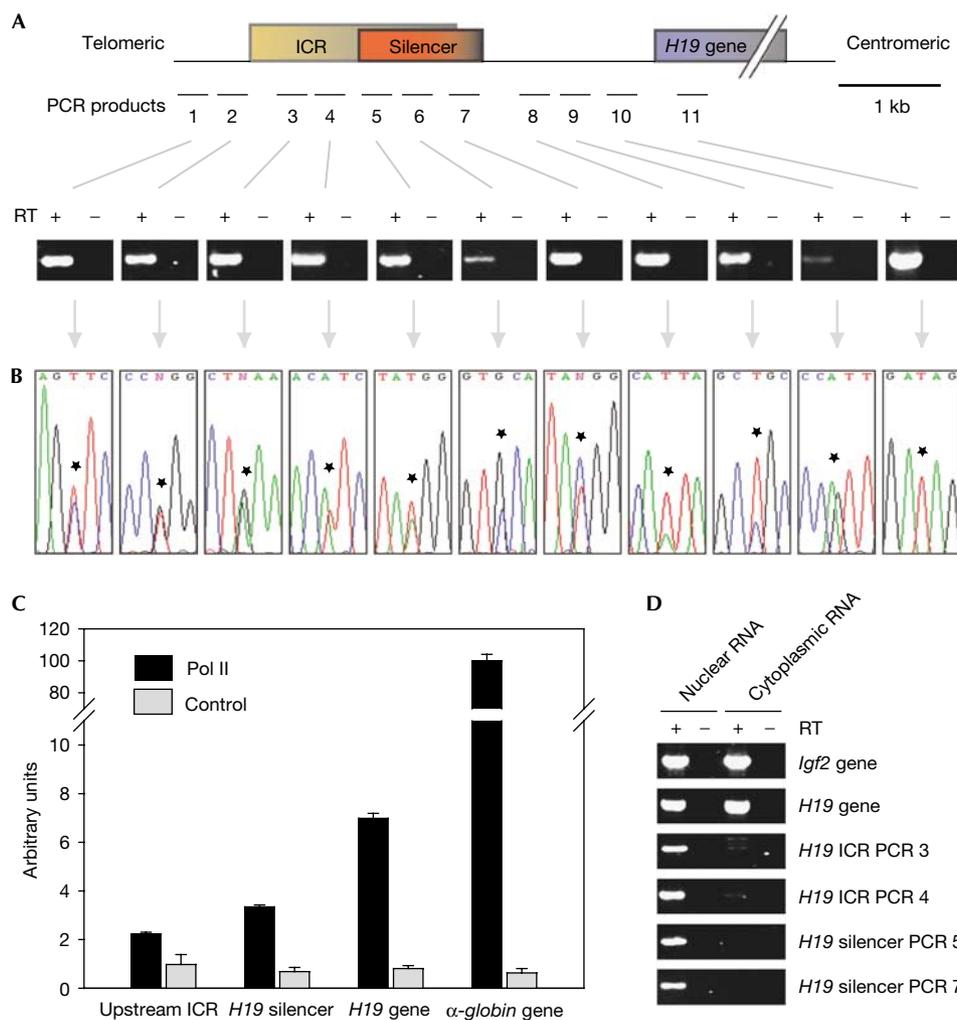
Fig 1 | The mouse *H19* upstream regulatory region is biallelically transcribed. (**A**) Schematic representation of the mouse *H19* locus and the PCR amplicons used in reverse transcription reactions. The imprinting control region (ICR) extends from −4 to −2 kb in relation to the *H19* gene transcription start site, overlapping with the silencer (−2.9 to −1.7 kb relative to the *H19* transcription start site). PCR products shown below were amplified from complementary DNA obtained from an F1 generation fetal liver (embryonic day (E)15.5) of a C57Bl6/SD7 cross using random primers, with or without reverse transcriptase (RT). (**B**) Selected sequence traces of PCR products shown in (**A**). Asterisks indicate SNPs between the C57Bl6 and SD7 strains. (**C**) Pol II occupancy at the *H19* region. Real-time PCR of ChIP material obtained with an antibody against Pol II or a control antibody. Sequences correspond to amplicons 2 (upstream ICR), 5 (*H19* silencer) and 11 (*H19* gene), and a control fragment from the α-globin gene. (**D**) Analysis of the subcellular localization of the ICR transcripts. RNA from mouse embryonic fibroblasts was fractionated and reverse transcription reactions were carried out on the nuclear and the cytoplasmic RNA pools, respectively. ChIP, chromatin immunoprecipitation; Pol II, RNA polymerase II; SNP, single-nucleotide polymorphism.

reduced by more than 50% upon GAL4-induced expression of the RNAi vector, whereas it was not significantly reduced in *H19* ICR transgenic flies in which expression from the RNAi vector was not induced (Fig 3C,D).

Remarkably, expression of the RNAi hairpin construct directed against the transcribed *H19* ICR in *Drosophila* relieves transcriptional repression of the reporter gene *mini-white*, which is controlled by the *H19* ICR (Fig 3A,B). Flies carrying the *H19* ICR transgene have a yellow eye colour, which indicates silencing of *mini-white* (Fig 3B, left). Combining the *H19* ICR transgene with the RNAi vector transgene did not alter the expression of

mini-white, as the RNAi vector transgene fails to be expressed in the absence of the transactivator GAL4 (Fig 3B, middle). However, on GAL4-induced expression of the RNAi transgene in flies carrying the *H19* ICR transgene, *mini-white* silencing is lost resulting in orange- to red-eyed flies (Fig 3B, right). Quantification of *mini-white* expression by photometric pigment measurements indicated that expression of the RNAi vector leads to a more than fivefold increase of *mini-white* gene function. We showed that this effect is specific as it depends on the presence of the *H19* ICR sequence targeted by the RNAi vector (supplementary Fig 4 online).
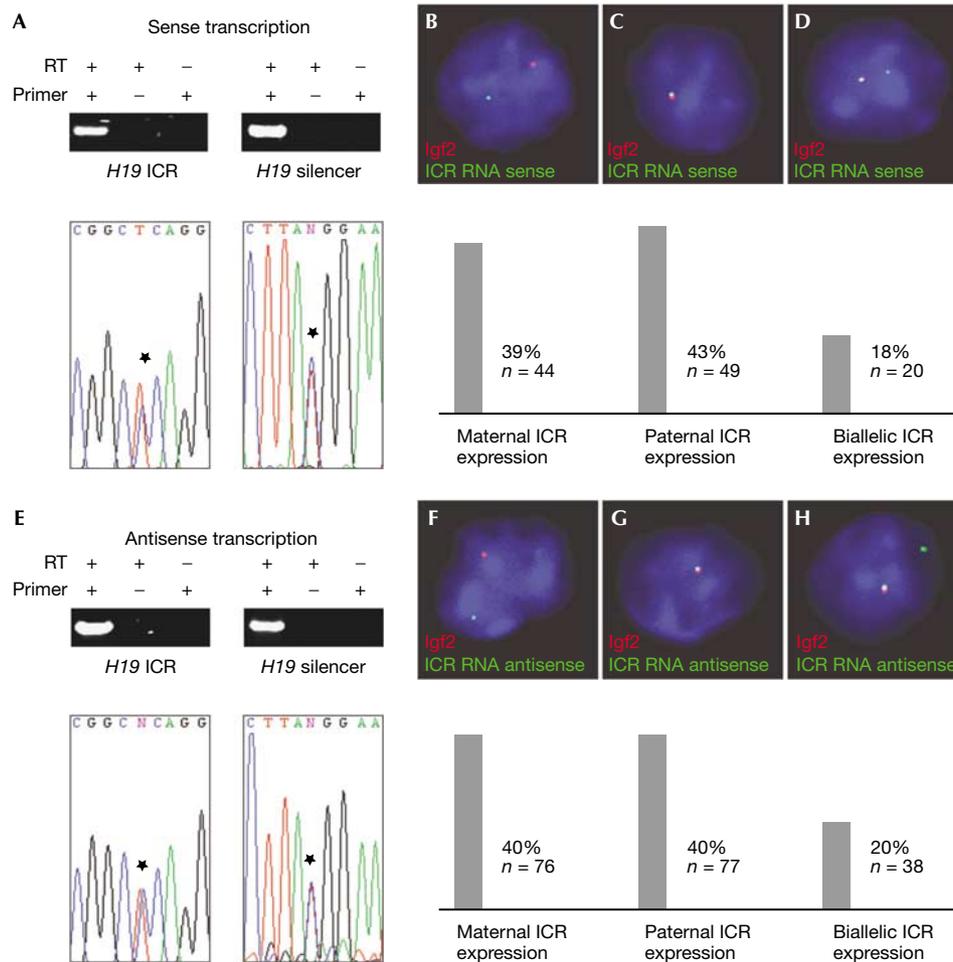
Fig 2 | The *H19* imprinting control region produces sense and antisense transcripts. Reverse transcription reactions with RNA obtained from mouse embryonic day (E)15.5 fetal liver cells, using primers specific for the sense (**A**) or antisense (**E**) ICR transcripts. Complementary DNA synthesis reactions were carried out with or without reverse transcriptase (RT), and with or without strand-specific primers. PCR products correspond to amplicons 4 (*H19* ICR) and 7 (*H19* silencer) in Fig 1. Sequence traces of PCR products are shown below the gel pictures, with asterisks indicating SNPs. Detection of *Igf2* in combination with ICR sense (**B–D**) and antisense (**F–H**) transcripts in mouse E14.5 fetal liver cells by double-labelled RNA-FISH. Green signals are ICR transcripts, red signals are *Igf2* gene transcripts. DAPI staining is blue. Bars below the pictures show frequencies of the respective signals. DAPI, 4,6-diamidino-2-phenylindole; FISH, fluorescence *in situ* hybridization; ICR, imprinting control region; SNP, single-nucleotide polymorphism.

In conclusion, the transgenic expression of an RNAi vector directed against the transcribed region of the *H19* ICR leads to the loss of silencing of an *H19* ICR-controlled reporter gene in *Drosophila*. Furthermore, the loss of transcriptional repression correlates with a marked reduction in the level of RNA from the *H19* ICR. These findings indicate an active role for non-coding transcripts from the *H19* ICR in the transcriptional repression of a reporter gene controlled by the *H19* upstream regulatory region in transgenic *Drosophila*.

The expression status of the transgenic locus in *Drosophila* is most comparable with that of the paternal allele in the mouse. Here, the *H19* gene is repressed, which requires DNA methylation of the ICR. Importantly, even in the presence of methylation, deletion of the region that corresponds to the *Drosophila* silencer element (and is transcribed in mice as shown here) relieves

repression of *H19* (Drewell *et al*, 2000). Therefore, the ncRNAs might be required for heterochromatic silencing in conjunction with DNA methylation, involving potentially H3K9 methylation. Other ncRNAs that direct gene silencing to imprinted regions have recently been shown to attract repressive histone modifications to adjacent genes (Lewis *et al*, 2004; Umlauf *et al*, 2004). We have previously found that mutations in some genes encoding heterochromatin components, such as the histone methyltransferase Su(var)3–9, partly relieve ICR-mediated silencing of *mini-white* in ICR transgenic flies (Schoenfelder & Paro, 2004), indicating that the *H19* ICR confers silencing to reporter genes through the formation of a heterochromatin-like structure that could be targeted by the ncRNAs (supplementary Fig 5 online). The recent observation of strikingly similar patterns of repressive histone marks at the endogenous paternal *H19* ICR and at pericentric
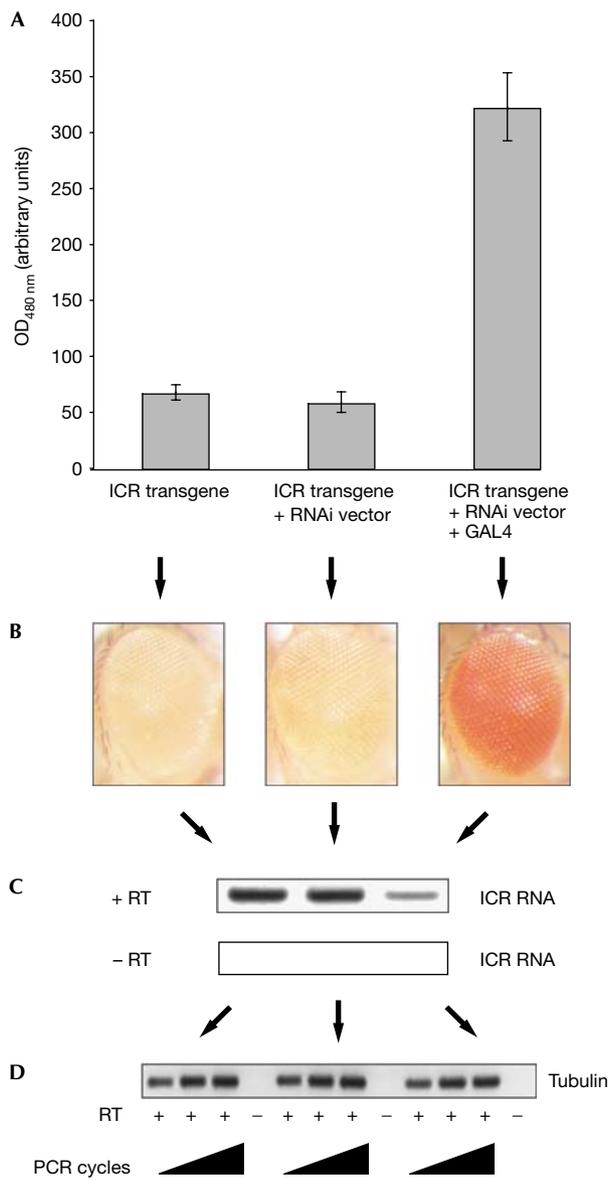
Fig 3 | RNA interference-mediated downregulation of *H19* imprinting control region non-coding RNA leads to loss of reporter gene silencing. (**A**) Quantification of reporter gene expression by photometric measurement of eye colour pigmentation in *Drosophila* lines carrying the transgene combinations indicated below the respective bars. (**B**) Shown here are the eyes of the *Drosophila* lines carrying the *H19* ICR transgene (left panel), the *H19* ICR transgene combined with the RNAi vector (middle panel), and the *H19* ICR transgene combined with the RNAi vector and GAL4 (right panel). (**C**) Reverse transcription (RT) reaction with primers in the *H19* ICR with (+RT, upper panel) or without (−RT, lower panel) reverse transcriptase. RNA samples are obtained from heads of the respective *Drosophila* lines shown in (**B**). (**D**) Reverse transcription reaction with primers against β-tubulin. RNA samples used for the reverse transcription reaction are the same as in (**C**). ICR, imprinting control region; OD, optical density; RNAi, RNA interference.

heterochromatin has highlighted the molecular parallels between both chromosomal regions (Delaval *et al*, 2007). Remarkably, an RNA component has been shown to contribute to the maintenance of the higher-order structure at pericentric heterochromatin (Maison *et al*, 2002). A related mechanism controls gene expression in a ribosomal gene cluster in which ncRNAs are required to maintain a silent, heterochromatin-like state (Mayer *et al*, 2006). Experimental downregulation of the repressive ncRNAs leads to increased expression of the neighbouring ribosomal genes in the locus (Mayer *et al*, 2006), which is strikingly similar to the effect on reporter gene transcription that we describe here on knockdown of the ncRNAs in the *H19* transgene system.

The ncRNAs are also expressed from the maternal allele. On the maternal allele, the ICR is unmethylated, binds to CTCF and *H19* is transcribed. Notably, deletion of the CTCF-binding sites or point mutations within the *H19* ICR that abolish CTCF binding lead to DNA methylation of the maternal ICR and result in a marked reduction of *H19* expression (Pant *et al*, 2003; Schoenherr *et al*, 2003; Fedoriw *et al*, 2004; Engel *et al*, 2006). One scenario we could predict is that CTCF acts to neutralize the action of the ncRNAs in attracting silencing. In addition, we cannot exclude a functional role of the maternal transcripts, for example in the establishment of an open chromatin conformation at the locus or in loop formation between the ICR and DMR1 in *Igf2* (Murrell *et al*, 2004; Kurukuti *et al*, 2006).

Our results are the first, to our knowledge, that describe nuclear non-coding transcripts in the *H19* ICR, and indeed in any paternally methylated ICR, and provide evidence that such transcripts have a role in gene silencing. Further insight into their function in the mouse requires detailed developmental studies and genetic manipulation.

METHODS

RNA isolation, reverse transcription and PCR. Livers were collected from C57BL6/SD7 F1 progeny at an age of embryonic day (E)15.5. RNA was obtained by double purification with Qiagen (Crawley, UK) columns and dual DNase I treatment. cDNA was synthesized with Superscript III (Invitrogen, Paisley, UK). Nuclear and cytoplasmic RNA fractions were obtained from E14.5 mouse embryonic fibroblasts using Paris columns (Ambion, Ambion/ Applied Biosystems, Warrington, UK). PCRs were carried out with *Taq* DNA polymerase and PCR optimizer buffers (Invitrogen).

RNA-FISH. RNA-FISH was carried out as described previously (Osborne *et al*, 2004). ICR transcripts were visualized with digoxigenin-labelled single-stranded DNA probes and fluorescein isothiocyanate detection. Dinitrophenol-labelled single-stranded DNA probes combined with Texas Red detection were used to visualize *H19* and *Igf2* transcripts.

Chromatin immunoprecipitation. ChIP was carried out as described previously (Umlauf *et al*, 2004). Antibodies used were Abcam (Cambridge, UK) Ab5131 (against RNA Pol II) and Rabbit anti-Goat IgG (G4018; Sigma, Gillingham, UK) as control.

***Drosophila* stocks and genetic crosses.** The *H19* ICR transgenic and the *H19* ICR transgene Δ silencer line were originally described as P(hzh) and P(hzh)Δ4, respectively (Lyko *et al*, 1997). The *H19* ICR RNAi transgene plasmid was obtained by cloning a 430-bp fragment from the *H19* ICR (−2915 to −2486 relative to the *H19* transcriptional start site) in inverted orientation into

P(UASyellow). The *H19* ICR RNAi transgenic line was generated using standard techniques. To express the ICR RNAi transgene, transgenic flies were crossed to flies carrying the P(GMR-GAL4w-) transgene, which drives strong GAL4 expression in the developing and adult *Drosophila* eye.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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