H19 antisense RNA can up-regulate igf2 transcription by activation of a novel promoter in mouse myoblasts
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Abstract

It was recently shown that a long non-coding RNA (lncRNA), that we named the 91H RNA (i.e. antisense H19 transcript), is overexpressed in human breast tumours and contributes in trans to the expression of the Insulin-like Growth Factor 2 (IGF2) gene on the paternal chromosome. Our preliminary experiments suggested that an H19 antisense transcript having a similar function may also be conserved in the mouse. In the present work, we further characterise the mouse 91H RNA and, using a genetic complementation approach in H19 KO myoblast cells, we show that ectopic expression of the mouse 91H RNA can up-regulate IGF2 expression in trans despite almost complete unmethylation of the Imprinting-Control Region (ICR). We then demonstrate that this activation occurs at the transcriptional level by activation of a previously unknown IGF2 promoter which displays, in mouse tissues, a preferential mesodermic expression (Pm promoter). Finally, our experiments indicate that a large excess of the H19 RNA can counteract 91H-mediated IGF2 activation. Our work contributes, in conjunction with other recent findings, to open new horizons to our understanding of lncRNA gene regulation and functions of the 91H/H19 RNAs in normal and pathological conditions.

Introduction

Long non-coding RNAs (lncRNAs) are major components of the mammalian transcriptome (for a review, see ref. [1]). Recent efforts to better characterize such transcripts revealed that they play important roles in both oncogenic and tumour suppressive pathways [2]. LncRNAs display a myriad of molecular functions [3], from chromatin remodelling (ARV1L, HOTAIR, Xist) [4,5,6,7] and modulation of alternative splicing (Zeb2/Sip1 gene locus) [8], to RNA metabolism (11/2-sbs and HULC RNAs) [9,10] and generation of micro- and small-RNAs (MEG3/Gtl2 and MALAT1 transcripts) [11,12]. They also have a great variety of forms: most of them are generated by the RNA polymerase II, but some are synthesized by the RNA Pol III (BC200 RNA) [13]; moreover, while most are poly-adenylated, many lncRNAs remain unpoly-adenylated [14] like, for example, the natural Sense-Antisense transcripts (SAT) [15] which are known to overlap each other and are co-ordinately expressed [16].

Several lncRNA are also produced from imprinted genes, whose expression is depending on the parental origin of the chromosome. Among them, the Airn and Kcnq1ot1 transcripts have been shown to “coat” the imprinted locus on the paternal chromosome from which they are expressed. Interestingly, both transcripts are known to interact with the histone H3 Lysine 9 methyltransferase G9a and to repress multiple genes in cis on the paternal chromosome [17]. Finally, two genes encoding imprinted lncRNAs map downstream the Insulin-like Growth Factor 2 gene (IGF2): one is the recently described PIBiH1 [10] and another is the H19 gene.

Since its discovery, twenty years ago [19], the function of the H19 gene remains enigmatic. H19 gene silencing is associated with the appearance of Wilms’ tumours in the Beckwith-Wiedemann syndrome [20,21]. Furthermore, ectopic expression of the H19 gene in human embryonic tumour cell lines leads to loss of clonogenicity and reduced tumourigenicity in nude mice [22]. It was recently confirmed that, in the mouse, H19 acts as a tumour suppressor [23]. However, several studies have also shown that the H19 RNA can accumulate in cancer cells and tumours [24,25,26,27,28] and it has been considered as an oncofetal RNA by some authors [29]. The gene encodes an untranslated RNA which is expressed only when maternally inherited.
Monoallelic expression of H19, like that of the neighbouring oppositely imprinted Insulin-like growth factor-2 (Igf2) gene, depends on the paternal-specific DNA methylation of an Imprinting-Control Region (ICR) located between 2 and 4 kb upstream of the H19 gene [30]. This methylation is acquired during male gametogenesis and prevents the binding of CTCF, an insulator protein. On the unmethylated maternal allele, CTCF is bound to the ICR and creates a boundary which prevents interactions between enhancers, located downstream of the H19 gene, and the Igf2 gene [31].

While the mechanisms of imprinting at the Igf2/H19 locus have focused much attention, very little is known about transcriptional regulation of the expressed Igf2 and H19 alleles. The two genes are tightly co-regulated during mouse embryonic development and are repressed shortly after birth in most tissues. Both genes belong to a network of coexpressed imprinted genes (Imprinted Gene Network, IGN) that may control embryonic growth in the mouse [32]. Recently, the non-coding H19 RNA was shown to contribute to the trans regulation of at least 9 genes of the IGN [33]. However, whether the H19 transcript acts through direct or indirect mechanisms and which step of gene expression is affected by such a regulation have not yet been investigated. Interestingly, we recently discovered in human that an antisense H19 transcript, named the H19 RNA (or H19os for “H19 opposite strand” transcript), augments in trans the paternal Igf2 expression which is known to favour tumour progression. In agreement with this notion, the H19 RNA is a large nuclear transcript which accumulates in breast tumours by RNA stabilization [34]. Preliminary experiments indicated that the antisense H19 transcript is evolutionarily conserved and expressed during the perinatal period in the mouse. In this work, we present further insights about the function of the mouse H19 and H19 transcripts. Using a genetic inactivation/complementation approach in cultured murine myoblasts, we suspected that this truncated H19 RNA would not be functional and that Igf2 expression may be affected in this cell line. Remarkably, we showed that, as for the classical C2C12 myoblast line, H19 KO myoblasts can differentiate into myotubes upon 3 days of serum starvation (Figure S1). Using the mE and mF PCR amplons (Figure 1), we then showed that the endogenous truncated H19 RNA displays a similar level of expression compared to the native H19 RNA in C2C12 myoblasts (Figure S2A). As found for tissues [34], the endogenous truncated H19 RNA levels are very low as compared to that of the sense H19 transcript (about 10^7-10^8 lower than H19 RNA levels observed in C2C12 myoblasts). Interestingly, in early passages of cell culture, H19 KO myoblasts displayed substantial Igf2 expression levels, as observed in the muscle of H19KO animals [36] (data not shown). However, in later passages, we found that Igf2 expression levels were very weak in undifferentiated cells and could only be detected by RT-qPCR (Figure S2B) but neither inrun-on (Fig. 3A) nor in Northern-Blot (Fig. 3B) experiments.

Run-on and Northern-blot experiments were also performed on myoblasts derived from mice having a paternal inheritance of the H19KO deletion (control paternal heterozygous myoblasts: +/−). These cells display the same genetic background than H19KO myoblasts and they were cultivated under identical experimental conditions (late passages of cell culture). However, they harbour accurate Igf2 transcription (Figure 3A) and regular steady state Igf2 mRNA levels (Figure 3B). Finally, Igf2 downregulation in H19KO myoblasts was correlated with a progressive loss of DNA methylation of the H19 ICR (CTCF site 2) observed upon
increasing passages in cell culture (Figure 3C). Therefore, loss of \textit{Igf2} expression in this cell line appears to be linked to ICR demethylation that may itself result, directly or indirectly, from the deletion of the \textit{H19} transcription unit.

Isolation of \textit{H19} KO Myoblast Clones that Express Ectopic \textit{91H} and \textit{H19} Transcripts

To investigate the possibility that the effect of the \textit{H19} transcription unit on \textit{Igf2} expression (Figure 3) may depend on antisense sequences complementary to the \textit{H19} gene [34,37], we then transfected the \textit{H19} KO myoblasts with constructs containing this region with the \textit{H19} gene under the control of a strong promoter (CMV promoter). Unfortunately, despite intensive efforts, such mini-constructs were systematically unable to express significant levels of the transgenes in \textit{H19} KO myoblast cells. However, transfection of a 16 kb \textbf{BamHI-BamHI} restriction fragment encompassing the \textit{H19} endodermic enhancers and the whole \textit{H19} gene. PCR amplicons used to quantify the ectopic RNAs are indicated (\textit{H19} RNA, m1-m3, m2 and mC). DNA methylation is indicated by black lollipops and RNAs are depicted in red. Positions of restriction sites and PCR amplicons used for real-time PCRs are indicated relative to the \textit{H19} transcription start site. The mA and mB PCR amplicons have been used in a previous study [34] and are indicated here solely for clarity of our PCR nomenclature. For primer sequences see Table S1.

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Figure 1. Map of the mouse \textit{H19/91H} region showing the PCR amplicons used in RT-qPCR experiments. The region corresponding to the sequence removed by the \textit{H19} deletion in the \textit{H19} KO myoblasts (see below) is indicated in blue. The \textit{H19/91H} insert transfected into the \textit{H19} KO myoblast cells is also shown in the figure (green lane). The insert is a 16 kb \textbf{BamHI-BamHI} fragment encompassing the \textit{H19} endodermic enhancers and the whole \textit{H19} gene. PCR amplicons used to quantify the ectopic RNAs are indicated (\textit{H19} RNA, m1-m3, m2 and mC). DNA methylation is indicated by black lollipops and RNAs are depicted in red. Positions of restriction sites and PCR amplicons used for real-time PCRs are indicated relative to the \textit{H19} transcription start site. The mA and mB PCR amplicons have been used in a previous study [34] and are indicated here solely for clarity of our PCR nomenclature. For primer sequences see Table S1.

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Ectopic Expressions of \textit{91H} and \textit{H19} RNAs are Oppositely Linked to Induced \textit{Igf2} Transcription

To investigate gene expression at the transcriptional level, we performed nuclear run-on experiments on undifferentiated and differentiated transfected \textit{H19} KO myoblasts corresponding to the whole cell population or to the isolated clones (Figure 5A). Using a phosphorImager, we then quantified the relative \textit{Igf2} transcription levels from the autoradiographies shown in Figure 5A. These experiments showed that \textit{Igf2} transcription is undetectable in untransfected \textit{H19} KO myoblasts, while all transfected clones re-expressed \textit{Igf2} at significant levels (Figure 5B). No correlation was found between \textit{Igf2} transcription levels and the \textit{91H}/\textit{H19} deletion of the \textit{H19} gene (Figure 1), displayed some ectopic expression (Figure 3D). Therefore, loss of \textit{Igf2} expression in this cell line appears to be linked to ICR demethylation that may itself result, directly or indirectly, from the deletion of the \textit{H19} transcription unit.

Isolation of \textit{H19} KO Myoblast Clones that Express Ectopic \textit{91H} and \textit{H19} Transcripts

To investigate the possibility that the effect of the \textit{H19} transcription unit on \textit{Igf2} expression (Figure 3) may depend on antisense sequences complementary to the \textit{H19} gene [34,37], we then transfected the \textit{H19} KO myoblasts with constructs containing this region with the \textit{H19} gene under the control of a strong promoter (CMV promoter). Unfortunately, despite intensive efforts, such mini-constructs were systematically unable to express significant levels of the transgenes in \textit{H19} KO myoblast cells. However, transfection of a 16 kb \textbf{BamHI-BamHI} restriction fragment encompassing the \textit{H19} endodermic enhancers and the whole \textit{H19} gene (Figure 1), displayed some ectopic expression (Figure 4). This fragment includes the native \textit{H19} and the \textit{91H} endodermic promoters and starts at a \textbf{BamHI} restriction site located 1.8 kb upstream of the \textit{H19} start site, thus excluding the Imprinting Control Region (ICR) of the locus (Figure 1). This fragment was co-transfected with a hygromycin-resistance plasmid into \textit{H19} KO myoblast cells. Interestingly, after hygromycin selection, in addition to ectopic \textit{91H}/\textit{H19} RNAs, we recovered \textit{Igf2} expression in the whole population of transfected cells (Figure 4A).

We then isolated 16 clones among which 15 displayed expression from the \textit{H19} construct and chose 6 clones for further characterisation (Figure 4B). All transfected clones displayed high ectopic \textit{H19} and \textit{91H} transcript levels (Figure 4B, left panel). For comparison, in wild-type C2C12 myoblast cells, \textit{91H} RNA levels were dramatically lower than \textit{H19} RNA levels (Figure 4B, right panel). Clearly, ectopic \textit{91H} RNA is overexpressed in all transfected cells analysed. However, contrary to the situation in the wild-type C2C12 cells (Figures 4B, right panel), neither the ectopic \textit{H19} nor the ectopic \textit{91H} transcripts are up-regulated during myoblast differentiation (Figure 4B, left panel) suggesting that some regulatory elements are probably missing in the construct used for ectopic expression. 5'RACE experiment performed on total RNA from one of the clone (clone 4) mapped the same TSS found in liver for the endogenous \textit{91H} RNA (see Figure 2) as well as two minor upstream start sites (Figure S3). Finally, using actinomycin D treatments, we definitively validated our experimental system of cellular complementation by showing that, mimicking their endogenous counterparts (Figure S4 and ref. [34,36]), the ectopic \textit{H19} RNA is very stable, while the ectopic \textit{91H} transcript is much more labile (Figure S4B).
transgene copy number ($R^2 = 0.0317$, data not shown). This demonstrates that $Igf2$ trans-activation occurs at the transcriptional level and suggests that this trans-activation depends on ectopic RNA expression.

In agreement with our previous findings in the human [33,34], $Igf2$ trans-activation is strongly correlated to the ectopic $91H$ RNA ($R^2 = 0.6918$) (Figure 6A). We also observed a positive correlation between $Igf2$ transcription and ectopic $H19$ RNA ($R^2 = 0.5315$) levels (Figure 6B). Both RNAs are produced from the same ectopic copies and therefore, it is not surprising that both display a positive correlation with $Igf2$ transcription levels. This indicates that at least one is truly correlated with $Igf2$ transcription. p values indicate that the correlation with $91H$ RNA is more significant ($p = 6.10^{-5}$) than that with $H19$ RNA ($p = 10^{-3}$). This result suggests that it is essentially the ectopic $91H$ RNA up-regulates $Igf2$ transcription in trans. Moreover, very large amounts of ectopic $H19$ RNA, as observed in clones 4, 11 and 12ND (see Figure 4B), can counteract this effect. Indeed, by plotting $Igf2$ transcription levels versus the ratio of $91H/H19$ ectopic RNA levels, we observed a clear negative effect of ectopic $H19$ RNA on $Igf2$ transcription in these clones where the $91H/H19$ ratio was inferior to 0.2 (Figure 6C, black diamonds). Finally, in all other clones, where $H19$ RNA is much lower (in which the $91H/H19$ ratio was superior to 0.2), the levels of the $H19$ RNA do not display any significant effect on $Igf2$ transcription levels (Figure 6C, white diamonds).

We thus propose that the relative sense/antisense ectopic $H19$ RNA levels are able to control $Igf2$ trans-activation in our complementation assay.

Figure 2. Characterisation of TSS of the endogenous mouse $91H$ RNA. 5’RACE experiment was performed on unpolyadenylated and capped RNA from 7 days-old mouse liver. (A) The RT Primer was designed in the mFb region (Figure 1) and a band was successfully amplified by nested PCRs. The RT primer corresponds with the forward primer of PCRa and nested PCR reactions were performed using the GeneRacer DNA oligonucleotide as reverse primer. (B) Ethidium bromide staining of an agarose gel showing PCRs product obtained from amplifications indicated above (MW: Molecular Weight). Sequencing of PCRa and PCRe products showed that these bands correspond essentially to unspecific amplifications while PCRb correspond to the TSS of the $91H$ RNA. (C) Electrophoregram of the sequenced 5’RACE product amplified from the capped RNA fraction (PCRb). This sequence identified a unique Cap site located in the endodermic enhancer 2 at position chr7:149,755,206 or chr7:149,755,207 on mouse July 2007/ mm9 Assembly. Due to the presence of a C residue at the end of the GeneRacer RNA oligonucleotide primer and/or the possibility that the last C residue may derive from the cap of the RNA, the exact position of the TSS remains ambiguous between two consecutive C residues found in the mouse genome sequence. (D) The sequence of the endodermic enhancer 2 is indicated in bold. The position of the TSS of the $91H$ RNA is indicated (black arrow).

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Igf2 Trans-activation by Ectopic 91H RNA can Occur Without H19 ICR Re-methylation

We then assessed whether Igf2 up-regulation in transfected H19 KO myoblasts is accompanied by re-methylation of the H19 ICR. In addition, we also investigated the methylation levels of the other Differentially Methylated Regions (DMRs) of the locus. In order to determine DMR methylation levels at the Igf2/H19 locus, we used digestions by methylation-sensitive restriction enzymes of DNA from untransfected and transfected H19 KO myoblasts as well as control myoblasts (paternal heterozygous) (Figure S6). These experiments confirm that untransfected H19 KO myoblasts are poorly methylated on
the \(H19\) ICR (Figure S6A) and showed that \(Igf2\) DMR1 also becomes unmethylated (Figure S6B) while \(Igf2\) DMR2 remains highly methylated (Figure S6C). Low methylation levels are also found at the \(H19\) promoter (Figure S7A). However, the IgDMR at the \(Dlk1/Gtl2\) locus on chromosome 12 remains methylated (Figure S7B) indicating that the unmethylation observed at the \(H19\) ICR is not a general phenomenon, since it is not found at another imprinted locus.

In transfected clones, the ICR (Figure S6A) and \(Igf2\) DMR1 (Figure S6B) show low DNA methylation levels while \(Igf2\) DMR2 remains largely methylated (Figure S6C). We conclude that ectopic \(91H\) and \(H19\) RNA expressions do not convincingly change DNA methylation patterns observed in untransfected \(H19\) KO myoblasts.

Finally, bisulfite-sequencing experiments confirmed that \(H19\) KO myoblasts are indeed very poorly methylated on the \(H19\) ICR (Figure S6E) and that the transfected \(H19\) KO myoblasts that displays the highest \(Igf2\) expression level (clone 4) is not re-methylated at the \(H19\) ICR (Figure S6F). Oppositely, the \(H19\) ICR is highly methylated in control myoblasts (\(+/-\) and C2C12 cells) (Figure S6D and S6G). These results clearly indicate that \(Igf2\) trans-activation by ectopic \(91H\) RNA occurs without \(H19\) ICR re-methylation.

**Figure 4. Quantifications of \(91H\) and \(H19\) ectopic RNAs and endogenous \(Igf2\) RNA levels in transfected \(H19\) KO myoblast cell lines.**

(A) The \(91H/H19\) insert (see Figure 1) have been co-transfected with a hygromycin-resistance plasmid into \(H19\) KO myoblast cells. Ectopic \(91H\), \(H19\) and endogenous \(Igf2\) mRNA (\(Igf2mRNA\) PCR amplicon) levels were determined by RT-qPCR in undifferentiated \(H19\) KO myoblasts untransfected or transfected (whole hygromycin-resistant transfected cell population) with the \(91H/H19\) insert. Results were normalized to \(Gapdh\) expression levels. (B) Comparison between the \(H19\) and \(91H\) ectopic RNA levels. RT-qPCR quantifications were performed in undifferentiated (ND) or differentiated (D). The whole hygromycin-resistant transfected cells ("whole") and 6 isolated clones (left panel), as well as C2C12 myoblasts (right panel), were analysed. The ectopic \(H19\) RNA expression levels (open bars) were assessed by using \(qPCR\) primers located at \(H19\) exon-exon junctions (\(H19\) RNA PCR amplicon). The ectopic \(91H\) RNA levels (black bars) were quantified with the \(mC\) and \(m1-m3\) PCR amplicons. Error bars correspond to s.e.m. of quantifications obtained with \(mC\) and the mean of \(m1\) PCR amplicons. Detailed data are shown in figure S5 (see also Materials & Methods section and Table S1). Please note that the other PCR amplicons shown in Figure 1 also target the endogenous truncated \(91H\) RNA produced from the endogenous locus and therefore they could not be used to quantify ectopic transcripts. Sample names and transgene copy numbers are indicated below the histogram. doi:10.1371/journal.pone.0037923.g004

\(Igf2\) Trans-activation by Ectopic \(91H\) RNA Occurs Through Up-regulation of a Novel \(Igf2\) Promoter

Since \(Igf2\) trans-activation by ectopic \(91H\) RNA can occur without \(H19\) ICR re-methylation, we decided to investigate in...
Figure 5. Nuclear Run-on experiments. (A) Autoradiographies of nuclear Run-on experiments on transfected and untransfected H19 KO myoblasts. Nuclear Run-on experiments were performed as previously described [38] and the α-32P UTP labelled transcripts were hybridized on filters to denatured plasmids containing the insert DNA of genes indicated on the figure. 91H/H19 transcription was assayed using an insert corresponding to the H19 sequence and Igf2 with a genomic 2.4 kb BamHI-BamHI DNA fragment encompassing the exon 4-exon 6 region. Such nuclear run-on experiments were performed on undifferentiated (ND) and differentiated (D) cells either on the whole hygromycin-resistant transfected H19 KO myoblast cell population (“Whole”) and transfected clones. (B) The same filters as those used for the autoradiographies shown in A were used for PhosphorImager quantifications. The ectopic 91H/H19 transcription levels (open bars) were compared to the endogenous Igf2 transcription levels (black bars). For each hybridized filter, the relative transcription levels were determined for each gene by normalizing to the Gapdh transcription level.

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Figure 6. Comparison between the endogenous Igf2 transcription levels and the steady state levels of the ectopic RNAs. In these graphs, we compared, for each transfected H19 KO clones, Igf2 transcription data shown in Figure 5B with the steady state 91H and H19 ectopic RNA levels shown in Figure 4B. (A) Igf2 transcription versus 91H RNA levels. (B) Igf2 transcription versus H19 RNA levels. In untransfected H19 KO myoblasts (−/−), both 91H and H19 are not expressed (RNA levels = 0) and Igf2 transcription level is below the “empty plasmid” background (see Fig. 5A) which is inferior to 0.11. (C) Igf2 transcription level versus the ratio of 91H/H19 RNA levels. Clones expressing large amount of ectopic H19 RNA (clones 4, 11 and 12ND, black diamonds) (Figure 4B) were analysed separately from the others (ratio of 91H/H19 RNA levels >0.2; open diamonds). In clones expressing high H19 RNA levels (black diamonds), the ectopic H19 RNA level relative to the 91H RNA level (which leads to a decrease of the 91H/H19 RNA ratio) is inversely proportional to Igf2 transcription levels (R² = 0.8173).

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more detail regulation of Igf2 mRNA expression in transfected clones. Surprisingly, promoter usage analyses showed that the strongest P2/P3 Igf2 promoters were not up-regulated in transfected H19 KO myoblasts (Figure 7A, left panel). Since, the P0 and P1 promoters were not significantly expressed (data not shown), we hypothesized that Igf2 up-regulation may occur by activation of an unknown Igf2 promoter. To assess this possibility, we performed 5’ RACE experiments in clone 4 that displays the highest Igf2 transcriptional activity (see Figure 5B). We identified a novel capped Igf2 mRNA which contains a new exon which is 1411x long and is spliced to the exon 4 common to all known Igf2 mRNAs (Figure 7B/C). This new transcript is initiated from a novel TSS located in the DMR1 (position chr7: 149,852,285 on mouse July 2007 mm9 Assembly). Using primers specific of the new Igf2 exon, we determined the levels of this novel Igf2 mRNA in different mouse tissues and showed that, while being poorly abundant, it is more expressed in mesodermic tissues (kidney, tongue and heart) than in liver or brain. We therefore called it “mesodermic” promoter (Pm) (Figure 8A).

We then analysed the Pm transcript in all our myoblastic cell lines. It turns out that this mRNA, like the other Igf2 transcripts, was down-regulated in the H19 KO myoblasts compared to control myoblasts (+/-) (Figure 8B). Remarkably, opposite to all the other Igf2 transcripts, it was trans-activated in all transfected H19 KO myoblasts except clone 11 which displays the lowest DMR2 methylation levels in addition to ICR hypomethylation (see Figure S6C and S6A respectively). Globally, the mean Pm Igf2 mRNA level showed 10 fold up-regulation in transfected clones compared to untransfected H19 KO myoblasts and reached P3 Igf2 mRNA levels (Figure 7A, right panel). Therefore, we conclude that Igf2 trans-activation by ectopic H19 RNA occurs essentially through up-regulation of the Pm Igf2 promoter.

Discussion

We recently contributed to show that, in the human, a large antisense H19 transcript (91H RNA) regulates Igf2 mRNA levels [34] whereas, in the mouse, the H19 RNA is a negative trans- regulatory of Igf2 mRNA levels [33]. In the present work, we derived an H19 KO myoblast cell line from mice carrying a deletion of the H19 transcription unit (H19H) [36] in which the Igf2 gene is repressed. Remarkably, loss of Igf2 transcription in H19 KO myoblasts correlates with a loss of H19 ICR methylation. It is therefore very likely that the CTCF protein binds to the ICR on both parental alleles leading to an almost complete insulation of the regular P2/P3 Igf2 promoters from the enhancers. Using a genetic complementation approach (introduction of the H19 sequence in H19 KO myoblasts), we investigated steady-state levels and halves-lives of ectopic 91H and H19 RNAs, as well as endogenous Igf2 transcriptional activity, and we show (i) that strong ectopic expression of antisense H19 transcripts synthesized from the enhancer 2 region can release Igf2 silencing in mouse myoblasts (ii) that this Igf2 reactivation takes place at the transcriptional level by targeting a previously unknown Igf2 promoter and (iii) that a large amount of ectopic H19 RNA can counteract Igf2 trans-activation by ectopic 91H RNA. Strikingly, we show that trans-activation of this novel Pm Igf2 promoter occurs without H19 ICR re-methylation indicating that this promoter is able to by-pass the insulator function of the unmethylated ICR. It thus remains possible that Pm activity also occurs on the maternal allele. This effect may potentially rely on the activity of the DMR2 that remains largely methylated in our experimental system (Figure S6C) and is known in mouse to favour Igf2 transcription on the methylated paternal allele [39]. This possibility would be reminiscent to some human pancreatic tumors like insulinomas where Igf2 DMR2 is hypermethylated while the H19 ICR is monoallelically methylated and where Igf2 becomes also expressed from the unmethylated maternal allele (loss of imprinting) [40].

All together, our inactivation/complementation approach, in conjunction with other recent findings [33,34], reveals that the mouse H19 antisense RNA favours Igf2 transcription and activates the Igf2 Pm promoter while large amounts of the H19 transcript counteract this effect, suggesting that these two transcripts are antagonistic riboregulators (Figure 9). Therefore, in cells like the C2C12 myoblasts where we observe very low amounts of 91H RNA and large amounts of H19 RNA, one can expect that the endogenous H19 RNA exerts a strong Igf2 transcriptional repression at least on the Pm promoter.

Our experiments also agrees with the pioneer work by Wilkin et al. which suggested that, in human, a partial H19 cDNA construct could activate IGF2 when expressed in the antisense orientation while H19 RNA can repress transcription from the Igf2 P3 promoter [37]. However, at that time, the endogenous antisense H19 RNA was unknown and its effect in this work remained enigmatic.

Our results reveal a functional relationship between H19 and 91H RNAs. Consequently, depending on the cell context, the functional relevance of the H19 transcriptional unit for Igf2 gene control will depend on the relative expression levels of the sense and antisense H19 transcripts. This finding is particularly relevant for a better understanding of the conflicting data obtained for H19 gene expression in cancer cells and tumours. Indeed, 91H RNA levels should be taken into consideration as this transcript is a good marker of tumourigenesis in breast cancer cells [34]. In summary, the 91H RNA could be assumed to be oncogenic by favouring Igf2 transcription while H19, which counteracts this effect, would act as a tumour suppressor [23]. Consistently, normal breast tissues display high H19 and very low 91H RNA levels, while the opposite is observed in cancerous breast tissues [34]. Interestingly, the effect of the 91H RNA on Igf2 derepression observed here in complementation studies, may explain the Igf2 derepression occurring in many tumours where 91H RNA was found to accumulate while the H19 gene is maintained in a repressed state [34]. One can note that an H19 antisense transcript called H19 opposite tumor suppressor (HOTS) was recently found in human [41]. It extends from 2.8 kb downstream of H19 to 1 kb upstream and is encoding a nucleolar protein which is not conserved in the mouse. An evolutionarily conserved microRNA miR675 has been also described in the H19 exon 1 [42,43]. Recently, this H19-derived miR-675 was shown to regulate tumor suppressor RB in human colorectal cancer favoring its progression [44]. An interesting possibility is that this miRNA may also be directly involved in controlling levels of 91H RNA. An open question is why, in vivo, so much H19 RNA would be required to produce this miRNA and to control such small amounts of 91H RNA? This may be due to the fact that the H19 RNA is mainly cytoplasmic while the 91H transcript is nuclear [34]. Therefore, only a small sub-set of nuclear H19 RNA may be involved in this process. Furthermore, the miRNA production does not appear to significantly affect H19 RNA levels and therefore this process should not interfere with other functions that the H19 RNA may have in the cytoplasm where it is known to localize with the polysomes [38,45]. Alternatively, the opposite effects of the 91H and H19 RNAs on Igf2 transcription may occur through more indirect mechanisms involving for example the Igf2 DMRs.

Here, upon isolation of H19 KO mouse myoblasts, the Igf2 gene expression was strongly decreased after passages in cell culture probably due to the observed loss of H19 ICR DNA

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methylation. It is formally possible that methylation levels have changed as a consequence of cell culture. Alternatively, this may also result from the deletion of the \( H19 \) transcriptional unit. In the mouse mesoderm-derived tissues, and more particularly in the postnatal muscle, maternal inheritance of the \( H19 \) deletion is known to lead to loss of \( Igf2 \) imprinting and re-expression of this gene from the maternal allele [23,36]. In the physiological context of this tissue, the presence of other cell types, such as for example satellite cells, may strongly contribute to maintain normal \( Igf2 \) levels by signalling through intercellular pathways which may control myoblast cell differentiation [46].

In the present study, we reactivated \( Igf2 \) transcription in \( H19 \) KO myoblasts by ectopic \( 91H \) RNA expression without \( H19 \) ICR re-methylation. Although we could not investigate whether this reactivation is monoallelic or biallelic, both parental alleles are largely unmethylated since methylation levels are very low (Figure S6) indicating that \( Igf2 \) reactivation could occur on both parental alleles. It now would be of interest to modify in the animal the \( 91H \) RNA levels independently of \( H19 \) RNA levels, as performed above.

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**Figure 7. Characterization of a novel \( Igf2 \) Pm transcript.** (A) Comparison of \( Igf2 \) transcripts produced from the P2/P3 (left panel) or Pm (right panel) promoters in untransfected (-/-) and transfected (“clones”) \( H19 \) KO myoblasts. Transcript levels are given relative to the P3 transcript level in untransfected \( H19 \) KO myoblasts (-/-) (100%). (B) Characterization of the TSS of the endogenous \( Igf2 \) Pm transcript. 5’ RACE experiment was performed on total capped RNA from transfected \( H19 \) KO myoblasts (clone 4). The primer used for RT is the E4 AS3 and PCR reactions were performed using the Gene Racer primer and the E4 AS2 and then the E4 AS1 primers for nested PCR. The ethidium bromide staining of an agarose gel shows PCR products obtained from nested PCR amplifications. Sequencing of PCR product (Pm band) showed that it corresponds to a novel TSS of the \( Igf2 \) gene (position chr7: 149,852,285 on mouse July 2007/mm9 Assembly). The smaller band in the gel corresponds to the \( Igf2 \) P3 transcript (data not shown). On the left is shown a 100bp-molecular weight ladder (C) The sequence of the novel \( Igf2 \) Pm exon is given in blue and the novel splice site with exon 4 (purple) is indicated below. Intronic sequences are indicated in red. SD = Splice Donor; SA = Splice Acceptor; The indicated CT dinucleotide corresponds to the splice acceptor site of an intron of a \( Igf2 \) antisense RNA [58]. (D) Map of the \( Igf2 \) gene showing the \( Igf2 \) Pm promoter (blue rectangle). The sense and antisense \( Igf2 \) exons are shown as black and white rectangles respectively. The first intron of the Pm \( Igf2 \) mRNA is indicated in red.

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**Figure 8. \( Igf2 \) Pm mRNA levels in mouse tissues and \( Igf2 \) promoter usage in transfected \( H19 \) KO myoblasts.** (A) Pm \( Igf2 \) mRNA levels relative to total \( Igf2 \) mRNA level (100%) in different mouse tissues. Total \( Igf2 \) mRNA levels were determined by RT-qPCR using a PCR primer pair (Igf2exo6 PCR amplicon) located in the exon 6 common to all \( Igf2 \) transcripts. (B) \( Igf2 \) promoter usage in untransfected/transfected \( H19 \) KO myoblasts. Relative mRNA levels (%) are calculated relative to the P3 \( Igf2 \) transcript level in the control cells (+/-) (100%).

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in the myoblast H19 KO cell line. Unfortunately, such an experiment is tricky to do in vivo since both RNAs possess identical expression patterns and are both produced from the maternal allele. Furthermore, the investigations should be performed at the transcriptional level (nuclear run-on assays) to exclude any potential post-transcriptional effects. Finally, a transgenic line that would display high ectopic H19 RNA but low ectopic H19 RNA has not yet been produced. Such a transgenic mouse strain would most probably in the sequence inverted in 91H region for muscle specific enhancers and, in mesodermic tissues, H19 RNAs in myoblastic cells. However, H19 RNA expression should be altered in the minute mouse mutant. Indeed, Davis et al. [47] have shown that the minute (Mn) mutation is an inversion that disrupts a candidate region for muscle specific enhancers and, in mesodermic tissues, 91H RNA is initiated downstream of the endodermic enhancers, most probably in the sequence inverted in minute mice. However, we cannot rule out that the H19 antisense RNA expression may persist in Mn mice by activation of some propitious transcription start sites. Therefore, it may be interesting to investigate 91H and Igf2 Pm transcript levels in this mouse mutant. Since we previously demonstrated that the 91H RNA acts in trans on Igf2 mRNA levels in human cells, one could hypothesize that this RNA may act exclusively at the post-transcriptional level. The present work clearly demonstrates that this is not the case, and that the 91H RNA augments Igf2 expression by acting at the transcriptional level. This finding raises the question about the mechanisms involved in such a regulation. Igf2 transcriptional regulation is known to be controlled through long-range interactions between regulatory elements, such as the Differentially Methylated Regions (DMRs) and the enhancers located downstream of H19 [18,48,49]. We could therefore propose that the 91H RNA can up-regulate tissue-specific Igf2 transcription by contributing, directly or indirectly, to higher-order chromatin architecture of this locus. For example, it may favour interactions between the Igf2 gene and specific enhancers since our experiments show that, in myoblastic cells, the 91H RNA can reactivate the Pm Igf2 promoter which is used in mesodermic tissues. Alternatively, the 91H RNA could also titrate factors such as transcriptional repressors, targeting Igf2 as well as some other genes of the Imprinted Gene Network (IGN) [32]. Finally, the 91H RNA is produced in liver from the endodermic enhancers (Figure 2) that themselves control the H19 expression levels in cis [50]. It is also able to act in trans to control Igf2 transcription (Figure 5) and to up-regulate the Pm transcript (Figure 8). Therefore, this lncRNA appears as a novel important player for co-regulation of genes at the Igf2/H19 locus.

**Materials and Methods**

**Ethics Statement**

All experimental designs and procedures are in agreement with the guidelines of the animal ethics committee of the French “Ministère de l’Agriculture”. Our animal unit has been registered at the departmental office for population protections (Direction départementale de la protection des populations) at the “Hérault préfecture” (Agreement N˚34-172-16). All the experimental protocols (mouse dissections) have been specifically approved by an inspector in charge of the veterinary public health from the same office at the “Hérault préfecture” (Agreement N˚34-31).

**Isolation of H19 KO Myoblasts**

Primary cultures were prepared from the thigh muscles of H19KO mice as previously described [51]. Primary cells (H19 KO and control paternal heterozygous myoblasts) were serially passaged for analysis. Using qPCR on genomic DNA we checked that, as expected, the isolated H19 KO myoblasts were devoid of the H19 transcription unit and that the Igf2 gene was in an identical copy number in H19 KO myoblasts as in C2C12 myoblast cells, suggesting that no aberrant loss or duplication of chromosome 7 occurred in the H19 KO myoblast cell line (data not shown).

**Cell Culture and Transfections**

H19 KO (−/−) and control paternal heterozygous (+/−) myoblasts were cultured in DMEM/MCDB 1:1, containing 20% FCS and 2% Ultroser (Gibco). Cells were differentiated into myotubes upon 3 days of serum starvation. The 16 kb BamHI-BamHI fragment corresponding to the H19 gene locus (Figure 1) was cloned into the Not I site of the pBlueScript plasmid using appropriate linkers. The construct was digested with Not I and the insert was gel-purified before being co-transfected with a hygromycin-resistance plasmid into H19 KO myoblast cells using lipofectamin (Gibco) according to the recommendations of the manufacturer. Actinomycin D at a final concentration of 3 μg/ml was added to the cell culture medium for the times indicated in figure legends.

**RNA Isolation, Northern-blot and RT-qPCR Analyses**

Total RNA was isolated from mouse tissue samples or from myoblastic cells by the guanidinium thiocyanate procedure as previously described [38]. Non-polyadenylated RNAs were prepared by using the PolyA Tract mRNA isolation system III® (Promega). The Igf2 and H19 RNAs were analysed in Northern-blot as previously described [52]. Reverse transcriptions and real-time quantitative PCRs were performed as previously described [34,53] using a qPCR mix described in Luttfalla et al. [54] with some modifications given in Court et al. [55]. The Igf2 steady-state mRNA levels were quantified using a PCR ampiclon which targeted the messenger RNA (for primer sequences see Table S1). The ectopic H19 RNA levels were quantified using primers.
located at H19 exon-exon junctions while the ectopic 91H transcript was quantified either in the intergenic region between the endodermic enhancers and the H19 gene (mC', mD, mE, mF and mJ PCR amplicons) or within the H19 introns (mI1, mI2 and mI3 PCR amplicons) (Figure 1 and Table S1). Indeed, since the levels and the half-lives of the RNAs quantified by the intronic PCR amplicons are similar to those quantified by the mC' PCR amplicon, we assume that the H19 intron sequences essentially account for the ectopic 91H RNA in transfected H19 KO myoblasts (Figure S3). Throughout this work RNA levels determined by RT-qPCR were expressed relative to Gapdh mRNA levels. Igf2 promoter usage was assessed by quantifying transcripts on each promoter-specific exon (first exons).

5'RACE

Rapid Amplification of 5' complimentary DNA Ends (5'RACE) was performed on non-polyadenylated d7 mouse liver RNAs (endogenous 91H RNA) or transfected H19 KO myoblast RNAs (clone 4) (ectopic 91H RNA and endogenous Igf2 Pm transcript) according to manufacturer's instructions (GeneRacer® Kit from Invitrogen ref. L1502). RT and nested PCR Primer sequences are given in Table S1.

Transgene Copy-number Determination

Transgene copy-numbers were determined by qPCR relative to the endogenous Igf2 gene.

Nuclear Run-on

Isolation of nuclei and nuclear run-on experiments were performed as previously described [38,53].

DNA Methylation Analyses

Each sample was digested by the StyI restriction enzyme (20 units) to eliminate potential PCR bias due to the reduced accessibility of primers on undigested genomic DNA [56]. For H19 ICR methylation analyses (CTCF site 2), half of each samples was then additionally digested by the BcaI methylation-sensitive enzyme (4 Units/reaction) and qPCR quantifications were performed on BcaI-digested and undigested fractions after normalization against a loading control (242C19 primer pair). A similar approach was followed using the methylation-dependent McrBC enzyme to determine methylation levels in the Ig-DMR (Dlk1/Gli2 locus on mouse chromosome 12) [57] or methylation-sensitive enzymes (Ncle for Ig2 DMR1 and HpaII for Ig2 DMR2). Methylation levels of the CpG residues studied for the H19 ICR and Ig2 DMRs are known to be representative of DNA methylation levels of the whole DMRs [52]. Primer sequences are available in Table S1.

Bisulfite Treatments

Genomic DNA was prepared from myoblastic cells and conversion with sodium bisulfite was performed with the Epitect® kit (Qiagen) following the manufacturer’s instructions. PCR fragments were cloned using a PCR cloning Kit from Qiagen. Clones with strictly identical patterns of conversion were removed from the results (since they are likely to represent identical molecules). We used the MethPrimer software to design primers on bisulfite treated DNA. Primer sequences are available in Table S1.

Supporting Information

Figure S1 Differentiation of H19 KO myoblast cells. The figure shows pictures of the H19 KO myoblasts under the optical microscope (20× enhancement) during the myogenic differentiation process (ND = undifferentiated; d1, d2 and d3 correspond to 1, 2 or 3 days of differentiation). The transcriptional levels of the myogenin, a myogenic marker, are up-regulated during differentiation of H19 KO myoblast cells with the same amplitude (6–7 fold) as observed in C2C12 myoblasts (data not shown).

Figure S2 Quantifications of the intact or truncated endogenous 91H RNA levels (A) and of Igf2 mRNA (B) relative to gapdh mRNA levels in myoblast cell lines. (A) Comparison between the intact (black bars) and truncated (grey bars) endogenous 91H RNA levels determined by RT-qPCR in C2C12 myoblasts and H19 KO myoblasts respectively. (B) Quantification of Igf2 mRNA levels during differentiation of H19 KO myoblasts (late passage cells) (ND = undifferentiated; D = differentiated). One can note that, as observed for the endogenous truncated 91H RNA (Figure S2A), the low Igf2 levels observed in H19 KO myoblasts were strongly up-regulated (by at least 20-fold) during myogenic differentiation (Figure S2B). This suggests that the H19 transcription unit is dispensable to Igf2 up-regulation processes observed during myogenic differentiation.

Figure S3 Characterisation of TSS of the ectopic mouse 91H RNAs. 5’RACE experiments were performed on total capped RNA from transfected H19 KO myoblasts (clone 4). (A) Map of the enhancer region showing the primers used for RT and PCR reactions. The RT was initiated from the forward primer of PCRa. (B) Ethidium bromide staining of an agarose gel showing PCRs product obtained from amplifications as indicated in Figure 2B) (MW: Molecular Weight). Sequencing of PCRs product showed that this band corresponds essentially to unspecific amplification while PCRb correspond to the major TSS of the 91H RNA (position chr7:149,755,206 or chr7:149,755,207 on mouse July 2007/ mm9 Assembly) and PCRe contains two minor TSS initiated within the endodermic enhancer 2 sequence upstream of the major TSS. These minor TSS could be identified in this experiment probably because ectopic 91H RNA is overexpressed compared to its endogenous counterpart. (C) The sequence of the endodermic enhancer 2 is indicated in bold. The positions of the minor and major TSS are indicated by black arrows. Due to the presence identical nucleotide sequences at the end of the GeneRacer RNA oligonucleotide primer and at the TSS, the exact position of the major and one minor TSS remain ambiguous.

Figure S4 Endogenous vs ectopic 91H/H19 RNA half-lives. (A) Stability of the endogenous 91H and H19 RNAs in C2C12 myoblasts. C2C12 myoblast cells were treated with Actinomycin D and relative RNA levels were determined by real time RT-qPCR at the indicated times (in hours). Data were normalized to Gapdh expression levels. H19 (H19 RNA PCR amplicon), 91H (RT-qPCR quantifications with the mC' PCR amplicon) and H19 precursor (intron 2, mI2 PCR amplicon) RNA levels are shown. Note that the half-life of the 91H RNA (middle panel) is similar to that of an unspliced H19 precursor RNA (right panel). (B) Stability of the ectopic 91H and H19 RNAs were determined in transfected H19 KO myoblasts using the same PCR amplicons as above. The whole hygromycin-resistant transfected H19 KO myoblast cell population was treated with Actinomycin D as described above and the ectopic H19 and ectopic 91H RNA levels were quantified as indicated above. Note that the ectopic 91H RNA appears to be more stable than the endogenous 91H transcript in C2C12 cells (compare Figure S4A with Figure S4B). This may be due to the
1000-overexpression of the ectopic 91H RNA found in transfected H19 KO myoblasts relative to the endogenous levels observed in C2C12 cells (Figure 4B, compare right and left panels). Since, in transfected KO myoblasts, the ectopic 91H RNA is found in similar amounts as the ectopic H19 RNA (Figure 4B, left panel) despite its low stability (Figure S4B), we should conclude that ectopic H19 transcription is much higher than that of the ectopic H19.

**Figure S7** RT-qPCR quantifications of 91H RNAs. Note that, in C2C12 cells, quantifications by the m1-m3 PCR amplicons (blue bars) account for the endogenous H19 precursor RNA level but not the endogenous 91H transcript which is much lower as shown using the mC' PCR amplicon (red bar). In the opposite, in transfected H19 KO myoblasts, quantifications using the m1-m3 PCR amplicons, as well as with the mC' PCR amplicon, account for the ectopic 91H RNA level which is very high. The 91H RNA levels shown in Figure 4B corresponds to the mean of quantifications using mC' and m1-m3 PCR amplicons.

**References**

1. Kapranov P, Willingham A, Gingeras T (2007) Genome-wide transcription and the implications for genomic organization. Nat Rev Genet 8: 413–423.
2. Gibb E, Brown C, Lam W (2011) The functional role of long non-coding RNA in human carcinomas. Mol 10: 38.
3. Wiusu J, Sunwoo H, Spector D (2011) Long noncoding RNAs: functional surprises from the RNA world. Genes Dev 23: 1494–1504.
4. Chaumeil J, Le Baccon P, Wutz A, Heard E (2006) A novel role for Xist RNA in DNA methylation-sensitive positive regulator of the INK/ARF locus. Biochem Biophys Res Commun 392: 129–134.
5. Gupta R, Shah N, Wang K, Kim J, Horlings H, et al. (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 464: 1071–1076.
6. Kotake Y, Nakajova T, Kitagawa K, Suzuki S, Liu N, et al. (2011) Long noncoding RNA ANRIL is required for the PRC2 recruitment to and silencing of the p15(INK4B) tumor suppressor gene. Oncogene 30: 1956–1962.
7. Rodriguez C, Borgel J, Court F, Cathala G, Forne T, et al. (2010) CTCF is a DNA methylation-sensitive positive regulator of the INK/ARF locus. Biochem Biophys Res Commun 392: 129–134.
8. Beltrán M, Puig I, Pena C, Garcia J, Alvarez A, et al. (2008) A natural antisense transcript regulates Zeb2/Sip1 gene expression during Smad-induced epithelial-mesenchymal transition. Genes Dev 22: 756–769.
9. Gong C, Maquat L (2011) long non-coding RNA transactivates CTCF around the 3'UTR of 91H RNA (Figure 4B, left panel) as demonstrated by the expression of the reporter construct by RT-qPCR. Genes Dev 25: 2223–2237.
10. Wang J, Liu X, Wu H, Ni P, Gu Z, et al. (2010) CREB up-regulates long non-coding RNA: RNA Biol 6: 100–106.
11. Court F, Baniol M, Hage`ge H, Petit J, Lebaccon P, et al. (2011) Long-range chromatin interactions at the mouse Igf2/H19 locus reveal a novel paternally expressed long non-coding RNA. Nucleic Acids Res 39: 5895–5903.
12. Pachnis V, Braumann C, Tijhuis M S (1986) The structure and expression of a novel gene activated in early mouse embryogenesis. EMBO J 7: 673–681.
13. Moulton T, Crenshaw T, Hsu Y, Moosikasuwan J, Lin N, et al. (1994) Epigenetic lesions at the H19 locus in Wilms' tumour patients. Nat Genet 7: 440–447.
14. Taniguchi T, Sullivan M, Ogawa O, Reeve A (1995) Epigenetic changes encompassing the Igf2/H19 locus associated with relaxation of Rgf2 imprinting and silencing of H19 in Wilms tumor. Proc Natl Acad Sci U S A 92: 2139-2146.
15. Kiyosawa H, Mise N, Iwase S, Hayashizaki Y, Abe K (2005) Disclosing hidden transcripts: mouse natural sense-antisense transcripts tend to be poly(A) negative and nuclear localized. Genome Res 15: 463–474.
16. Watanabe Y, Numata K, Murata S, Osada Y, Saito R, et al. (2010) Genome-wide analysis of expression modes and DNA methylation status at sense-antisense transcript loci in mouse. Genomics 96: 333–341.
17. Latus P, Barlow D (2009) Regulation of imprinted expression by macro non-coding RNAs: RNA Biol 6: 100–106.
18. Court F, Baniol M, Hagege H, Petit J, Lelam-Taha M, et al. (2011) Long-range chromatin interactions at the mouse Igf2/H19 locus reveal a novel paternally expressed long non-coding RNA. Nucleic Acids Res 39: 5895–5903.
19. Automatisms and nuclear localized. Genome Res 15: 463–474.
20. Taniguchi T, Sullivan M, Ogawa O, Reeve A (1995) Epigenetic changes encompassing the Igf2/H19 locus associated with relaxation of Rgf2 imprinting and silencing of H19 in Wilms tumor. Proc Natl Acad Sci U S A 92: 2139–2146.
21. Hsu Y, Crenshaw T, Moulton T, Newcomb E, Tycko B (1993) Tumour-suppressor activity of H19 RNA. Nature 365: 764–767.
22. Moulton T, Crenshaw T, Hsu Y, Moosikasuwan J, Lin N, et al. (1994) Epigenetic lesions at the H19 locus in Wilms' tumour patients. Nat Genet 7: 440–447.
23. Taniguchi T, Sullivan M, Ogawa O, Reeve A (1995) Epigenetic changes encompassing the Igf2/H19 locus associated with relaxation of Rgf2 imprinting and silencing of H19 in Wilms tumor. Proc Natl Acad Sci U S A 92: 2139–2146.
24. Adriaenssens E, Dumont L, Lottin S, Bolle D, Lepretre A, et al. (1998) H19 is a novel gene activated in early mouse embryogenesis. EMBO J 7: 673–681.
25. Cooper M, Fischer M, Komitowski D, Shevelev A, Schulze E, et al. (1996) Developmentally imprinted genes as markers for bladder tumor progression. J Urol 155: 2120–2127.
26. Cooper M, Fischer M, Komitowski D, Shevelev A, Schulze E, et al. (1996) Developmentally imprinted genes as markers for bladder tumor progression. J Urol 155: 2120–2127.
27. Lottin S, Adriaenssens E, Depres S, Bertaux N, Montpellier C, et al. (2002) Overexpression of an ectopic H19 gene enhances the tumorigenic properties of breast cancer cells. Carcinogenesis 23: 1885–1895.

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**Author Contributions**

Conceived and designed the experiments: GC TF. Performed the experiments: VGT M-NL-T F. Carbonell F. Court EA AD NA LM GC. Analyzed the data: JP MW. Contributed reagents/materials/analysis tools: CP DM LD. Wrote the paper: TF GC.
20. Lustig-Yariv O, Schulze E, Komitowski D, Erdmann V, Schneider T, et al. (1997) The expression of the imprinted genes H19 and IGF-2 in chorionicc- 
noma cells line. Is H19 a tumor suppressor gene? Oncogene 15: 169–177.
21. Ariel I, Ayesh S, Perlman E, Pines G, Tano O, et al. (1997) The product of the 
imprinted H19 gene is an oncodel RNA. Mol Pathol 50: 34–44.
22. Thoevaladan J, Duran K, Bartolomei M (1998) Deletion of the H19 differentially 
methylated domain results in loss of imprinted expression of H19 and IGF2. 
Genes Dev 12: 3693–3702.
23. Hark A, Schoenherr C, Katz D, Ingram R, Levorse J, et al. (2000) CTCF 
mediates methylation-sensitive enhancer-blocking activity at the H19/IGF2 locus. 
Nature 405: 486–489.
24. Vazquez S, Gueydac T, Delalbre A, Bellmann A, Housami S, et al. (2006) 
Zac1 regulates an imprinted gene network critically involved in the control of 
embryonic growth. Dev Cell 11: 711–722.
25. Gabory A, Ripoche M, Le Digarcher A, Watrin F, Zyyat A, et al. (2009) H19 
acts as a trans regulator of the imprinted gene network controlling growth in 
mice. Development 136: 3413–3421.
26. Berteaux N, Apel N, Cathala G, Genton C, Coll J, et al. (2008) A novel H19 
antisense RNA overexpressed in breast cancer contributes to paternal IGF2 
expression. Mol Cell Biol 28: 6731–6745.
27. Schoenfelder S, Smits G, Fraser P, Reik W, Paro R (2007) Non-coding 
transcripts in the H19 imprinting control region mediate gene silencing in 
transgenic Drosophila. EMBO Rep 8: 1068–1073.
28. Ripoche M, Kress C, Fouvier F, Dandolo L. (1997) Deletion of the H19 
transcription unit reveals the existence of a putative imprinting control element. 
Genes Dev 11: 1596–1604.
29. Wilkin F, Paquette J, Ledru E, Hamelin C, Pollak M, et al. (2000) H19 sense 
and antisense transcripts modify insulin-like growth factor-II mRNA levels. 
Eur J Biochem 267: 4020–4027.
30. Milligan L, Antoine E, Weber M, Brunel C, et al. (2000) H19 gene 
imprinted Dlk1/Gtl2 locus. J Biol Chem 283: 18612–18620.
31. Murrell A, Heeson S, Antoine E, Brunel C, et al. (2006) CTCF 
mediates methylation-sensitive enhancer-blocking activity at the H19/IGF2 locus. 
Genes Dev 12: 3693–3702.
32. Varrault A, Gueydan C, Delalbre A, Bellmann A, Hemonnot B, et al. (2002) Turnover 
of primary transcripts is a major step in the regulation of mouse H19 gene 
expression. EMBO Rep 3: 774–779.
33. Milligan L, Antoine E, Bishal G, Weber M, Brunel C, et al. (2000) H19 gene 
expression is up-regulated exclusively by stabilization of the RNA during muscle 
cell differentiation. Oncogene 19: 5010–5016.
34. Murrell A, Heeson S, Bowden L, Smith P, Dean W, et al. (2002) Disruption of 
mesodermal enhancers for IGF2 in the minute mutant. Development 129: 
1657–1668.
35. Kurukuti S, Tisvari V, Tavosuidan G, Pagacheva E, Murrell A, et al. (2006) 
CTCF binding at the H19 imprinting control region mediates maternally 
inherited higher-order chromatin conformation to restrict enhancer access to 
IGF2. Proc Natl Acad Sci U S A 103: 10684–10689.
36. Ripoche M, Kress C, Fouvier F, Dandolo L. (1997) Deletion of the H19 
transcription unit reveals the existence of a putative imprinting control element. 
Genes Dev 11: 1596–1604.
37. Wilkin F, Paquette J, Ledru E, Hamelin C, Pollak M, et al. (2000) H19 sense 
and antisense transcripts modify insulin-like growth factor-II mRNA levels. 
Eur J Biochem 267: 4020–4027.
38. Court F, Miro J, Braem C, Lelay-Taha M, Brisebarre A, et al. (2011) Modulated 
contact frequencies at gene-rich loci support a statistical helix model for 
mammalian chromatin organization. Genome Biology 12: R42.
39. Court F, Miro J, Braem C, Lelay-Taha M, Brisebarre A, et al. (2011) Modulated 
contact frequencies at gene-rich loci support a statistical helix model for 
mammalian chromatin organization. Genome Biology 12: R42.
40. Weber M, Hagege H, Lafuitta G, Dandolo L, Brunel C, et al. (2003) A real-time 
polymere chain reaction assay for quantification of allele ratios and correction of 
amplification bias. Anal Biochem 320: 252–258.
41. Court F, Miro J, Braem C, Lelay-Taha M, Brisebarre A, et al. (2011) Modulated 
contact frequencies at gene-rich loci support a statistical helix model for 
mammalian chromatin organization. Genome Biology 12: R42.
42. Court F, Miro J, Braem C, Lelay-Taha M, Brisebarre A, et al. (2011) Modulated 
contact frequencies at gene-rich loci support a statistical helix model for 
mammalian chromatin organization. Genome Biology 12: R42.
43. Court F, Miro J, Braem C, Lelay-Taha M, Brisebarre A, et al. (2011) Modulated 
contact frequencies at gene-rich loci support a statistical helix model for 
mammalian chromatin organization. Genome Biology 12: R42.