



HHS Public Access

Author manuscript

Biochem Biophys Res Commun. Author manuscript; available in PMC 2017 July 29.

Published in final edited form as:

Biochem Biophys Res Commun. 2016 July 29; 476(3): 121–126. doi:10.1016/j.bbrc.2016.05.042.

H19ICR mediated transcriptional silencing does not require target promoter methylation

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Abstract

Transcription of the reciprocally imprinted genes *Insulin-like growth factor 2 (Igf2)* and *H19* is orchestrated by the 2.4-kb *H19* Imprinting Control Region (*H19ICR*) located upstream of *H19*. Three known functions are associated with the *H19ICR*: (1) it is a germline differentially methylated region, (2) it is a transcriptional insulator, and (3) it is a transcriptional silencer. The molecular mechanisms of the DMR and insulator functions have been well characterized but the basis for the ICR's silencer function is less well understood. In order to study the role the *H19ICR* intrinsically plays in gene silencing, we transferred the 2.4-kb *H19ICR* to a heterologous non-imprinted location on chromosome 5, upstream of the *alpha fetoprotein (Afp)* promoter. Independent of its orientation, the 2.4-kb *H19ICR* silences transcription from the paternal *Afp* promoter. Thus silencing is a function intrinsic to this DNA element. Further, ICR mediated silencing is a developmental process that, unexpectedly, does not occur through DNA methylation at the target promoter.

Keywords

Genomic Imprinting; gene silencing; H19

INTRODUCTION

Genomic imprinting is an epigenetic mechanism that regulates transcription of about 100 genes in mammals [¹]. Imprinted genes display parent-of-origin specific gene expression and only one of the two parental alleles is expressed while the other becomes silenced. Imprinted genes are usually located in clusters scattered throughout the genome. One such imprinted cluster is the *Igf2/H19* locus on mouse chromosome 7 (Fig. 1A). Loss of imprinting defects at this locus are associated with Beckwith Wiedemann and Russell-Silver syndromes and with many cancers [²].

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Igf2 and *H19* are 80-kb apart and share enhancers that are located downstream of the *H19* gene (Fig. 1A)^[3, 4]. Transcription of both genes is regulated in *cis* by a 2.4-kb DNA element, the *H19* imprinting control region (*H19*ICR)^[5–7]. The ICR is located between the two genes, just upstream of the *H19* promoter. Deletion and mutational studies at the endogenous locus have demonstrated three functions for the region. First, the ICR is the only germ line differentially methylated region (gDMR) at the locus and thus is responsible for establishing all the differences in DNA methylation that distinguish maternal and paternal chromosomes in later development [8–10]. Second, when non-methylated, as on the maternal chromosome, the ICR binds CTCF protein and forms a transcriptional insulator that blocks activation of the maternal *Igf2* promoter by the shared downstream enhancers [11]. Third, a paternally inherited, methylated ICR is required for the developmentally regulated silencing of the adjacent paternal *H19* promoter [6, 12].

To test whether these three functions are intrinsic to the 2.4-kb *H19*ICR we generated insertion mutation mouse models that carry the ICR at the non-imprinted *alpha-fetoprotein* (*Afp*) location on mouse chromosome 5 (Fig. 1B) [13]. The *Afp* gene is highly expressed in fetal liver but rapidly repressed after birth. Mice with one functioning copy of *Afp* are healthy and fertile.

We first looked at the DNA methylation patterns of maternally and paternally inherited ICR insertions and saw that the 65 CpGs within the *H19*ICR became methylated but only on paternal chromosomes. *That is, there is no detectable methylation upon maternal inheritance but upon paternal inheritance, the ICR is fully protected from digestion by methylation sensitive enzymes.* Thus the 2.4-kb *H19*ICR has intrinsic gDMR activity [13].

We next looked at transcriptional insulation. When inserted at the *Afp* location and maternally inherited, the ICR is not methylated, binds the protein CTCF, and *therefore* insulates the maternal *Afp* promoter from interacting with its enhancers so that maternal *Afp* expression is not detected [14]. In fact, multiple studies in transgenic mice and cell culture models support the idea that insulator function is entirely intrinsic to the *H19*ICR [11].

Our understanding of the *methylated* ICR as a transcriptional silencer is more limited. At the endogenous *Igf2/H19* locus, silencing of the *H19* promoter is a developmental process. Expression of the *H19* gene is bi-allelic in early embryos. Repression of the paternal *H19* allele in the epiblast correlates with the “spread” of DNA methylation from the ICR to the adjacent CpG-rich *H19* promoter/exon 1 region [10, 15]. Sometime after the *H19* promoter has become methylated, its silencing is permanent and independent of the continued presence of the paternal *H19*ICR [6, 9, 12]. For these reasons, it has been assumed that DNA methylation spreading is the mechanism that silences the paternal *H19* gene.

In this study, we test whether gene silencing is an intrinsic property of the 2.4-kb *H19*ICR and whether the spread of DNA methylation is essential for the ICR’s function as a silencer. Unlike the *H19* promoter, the *Afp* promoter does not harbor any CpG-rich motifs but only a few scattered CpG dinucleotides [16]. However, when upstream of the *Afp* gene, the *H19*ICR successfully silenced the paternal *Afp* allele without changing *Afp* promoter methylation. DNA methylation is therefore likely not the mechanism that is used by the ICR

to silence nearby promoters. However, we present developmental analyses that are consistent with the idea that DNA methylation contributes to the stability of gene silencing.

MATERIALS AND METHODS

Mice

Animal research was approved by the NICHD Animal Care and Use Committee and done according to NIH guidelines. Key mouse lines are depicted in Figure 1. ICR^{fllox} and ICR are from Srivastava [6]. *Afp*-A, *Afp*-B and *Afp*-D are from Park [13]. *Afp*-A^{fllox} was generated for this study and is similar to *Afp*-A except that the 2.4-kb *Bgl*II fragment carrying the *H19*ICR is flanked with loxP sites. To generate *Afp*-loxP animals, *Afp*-A^{fllox} males were crossed to females transgenic for *Ella-Cre* (Jackson Labs 003724). *Albumin*-cre [17] and *Sox2*-cre [18] mice were backcrossed into FVB to introduce a single nucleotide polymorphism that distinguishes FVB and SvJ129 alleles of *Afp*.

Parent-of-origin specific gene expression at *Afp* and *H19*

Total RNA was extracted from liver, converted into cDNA, and maternal and paternal specific transcription of *Afp* and *H19* was quantitated using DNA melting curve analyses [19]. Data were analyzed using the unpaired t-test.

Bisulfite sequencing

Methods to quantitate DNA methylation are detailed in the legend to Supplemental Figure 1.

RESULTS

The 2.4-kb *H19*ICR silences the exogenous *Afp* promoter

When paternally inherited, ICR insertions at the *Afp* locus are methylated at CpG dyads [13]. To test the ability of these methylated *H19*ICR elements to silence *Afp* expression we quantitated allele-specific expression of *Afp* upon paternal inheritance of each of the chromosomes described in Fig. 1B. In each experimental animal, the maternal *Afp* chromosome is wild type and served as an internal reference to normalize expression of the paternal *Afp* chromosome. Thus a value of 1 would indicate equal levels of expression from maternal and paternal chromosomes while lower values would demonstrate reduced expression of the paternal chromosomes.

In wild type animals, the paternal and maternal chromosomes are comparably expressed ($Afp/Afp = 0.91 \pm 0.04$, $n=3$) consistent with the fact that *Afp* is not an imprinted gene (Fig. 2A). However, insertion of the 2.4-kb *H19*ICR in either orientation results in >40-fold reduction in paternal *Afp* expression ($Afp/Afp-A = 0.02 \pm 0.01$, $n=6$; $Afp/Afp-B = 0.03 \pm 0.01$, $n=5$). We do not think that this loss in paternal *Afp* expression is due to an insertion mutation that disrupts key *Afp* regulatory elements because the paternal *Afp*-loxP chromosome expresses wild type levels of *Afp* (0.88 ± 0.05 , $n=3$).

It is interesting to see that the *Afp*-D insertion has only a mild phenotype (0.42 ± 0.05 , $n=3$; see also [14]). The *Afp*-D chromosome has the 2.4-kb *H19*ICR in the same orientation as

Afp-B but carries an additional 4.8-kb that separate the inserted ICR from the *Afp* promoter (Fig. 1B). The mild *Afp*-D phenotype suggests a role for proximity of the ICR to its target promoter.

As additional controls we quantitated expression upon maternal inheritance of each of the alleles described in Fig. 1B. *From previous studies we know that maternally inherited H19ICR elements are not methylated and therefore bind CTCF. CTCF binding organizes the maternal chromosome into structures that prevent physical interactions of the Afp enhancer and promoter* [14]. In Fig. 2B, we see effects on expression that are consistent with these biochemical analyses. Insertions of the *H19ICR* in either orientation prevent activation of the maternal promoter (*Afp*-A/*Afp* = 0.07±0.02, n=10; *Afp*-B/*Afp* = 0.14±0.02, n=6) compared to wild type controls (*Afp*/*Afp* = 0.93±0.03, n=5). Most importantly for this study, the *Afp*-D insertion is highly effective in insulating the maternal *Afp* promoter from enhancer activation (*Afp*-D/*Afp* = 0.02±0.00, n=3). *This contrast with the phenotype associated with paternal Afp-D inheritance emphasizes that repression of Afp by insulation (unmethylated maternal ICR) and by silencing (methylated paternal ICR) are distinct processes.*

The *Afp* promoter becomes silenced without DNA methylation

The *H19* promoter and the 5' end of *H19* exon 1 are embedded within a 5-kb very CpG-rich region. Silencing of the paternal *H19* promoter at gastrulation correlates with the spread of DNA methylation from the *H19ICR* through the promoter [15]. We chose the *Afp* gene as the model locus for this study because it is depleted of CpG di-nucleotides. Even using non stringent criteria the nearest CpG-rich regions are 32-kb downstream of the *Afp* transcriptional start site and thus far outside the locations of known *Afp* regulatory sequences [20]. Given the prevailing models for silencing at the *H19* locus and the overall dearth of CpG residues in the *Afp* promoter region, we were surprised to see that the ectopic *H19ICR* was able to completely abrogate transcription from the paternal *Afp* promoter.

We therefore looked more carefully to see if there were any changes in DNA methylation of *Afp* gene sequences that were associated with paternal inheritance of the *H19ICR* insertion and that might explain *Afp* transcriptional silencing. Transgenic analyses have identified 150-bp upstream of the *Afp* transcriptional start site that are necessary and sufficient for *Afp* promoter function [20]. We concentrated on the three CpGs within this region and on the four CpGs at the 5' end of *Afp* exon 1 (Fig. 3A). Using bisulfite sequencing, we determined their methylation patterns in E13.5 embryonic livers collected from wild type progeny or from embryos carrying an ICR inserted on the paternal chromosome (*Afp*/*Afp*-A). There were no differences between the degrees or the patterns of methylation in these samples (Fig. 3B, SFig.1 and STable 1).

Transcriptional silencing depends on developmental processes

To better understand the mechanism for *H19ICR* mediated silencing, we wanted to test whether the ICR was required only to establish gene silencing or if its continued presence was necessary to prevent expression of the paternal *Afp* promoter. Therefore, we deleted the *H19ICR* at different points in development, using *Sox2-cre* [18] and *Albumin-cre* (*Alb-cre*)

[17] to achieve recombination by E6.5 and E8.5, respectively. In each case, we measured gene activity in livers isolated from E16.5 embryos and compared expression profiles to littermates which did not carry the *cre* transgene (“Never Deleted” in Fig. 4) and also to age-matched animals where the ICR had been deleted in the germ line (“Always Deleted” in Fig. 4).

To assure the efficacy of our model system, we first studied the effect of deletion on *Afp* expression when the *H19*ICR was maternally inherited (Fig. 4A). The maternal chromosomes serve as good controls because it is well established that transcriptional insulation is absolutely dependent upon the continual presence of the CTCF-bound ICR to prevent promoter-enhancer interactions. Here we show that *cre*-mediated deletion of the *H19*ICR from chromosome 5 at E6.5 or at E8.5 results in high levels of maternal *Afp* expression (*Sox2-cre* = 0.91 ± 0.02 , n=13; *Alb-cre* = 0.43 ± 0.07 ; n=3) compared with control animals that did not carry the *cre* transgene (0.10 ± 0.06 ; n=5). Similarly, we saw high levels of maternal *Igf2* expression when the *H19*ICR was deleted with *Sox2-cre* or with *Alb-cre* using the ICR^{fllox} chromosome depicted in Fig. 1A (data not shown). Together, these results demonstrate the relative effectiveness of the two *cre* transgenes in removing the *H19*ICR at both the endogenous *H19* and the exogenous *Afp* loci. Note that at both *Igf2* and at *Afp*, the *Alb-cre* is only partially effective in recombination.

We next studied the effect of *cre* recombination on silencing of the endogenous *H19* gene (Fig. 4B). Previous studies have shown that silencing of the paternal *H19* promoter is a developmental process: the *methylated* ICR is needed to establish silencing but once silencing is established the ICR becomes superfluous and can be removed without re-activation of the paternal promoter [6, 12]. Consistent with these earlier findings, silencing of the paternal *H19* is fully maintained when the ICR is removed by *Alb-cre* (Never deleted = 0.02 ± 0.02 , n=2; Deleted E8.5 = 0.02 ± 0.01 , n=8). However, deletion at E6.5 via *Sox2-cre* results in a phenotype that is indistinguishable from that seen in mice where the ICR was already removed in the germ line (germ line or “always deleted” = 0.30 ± 0.04 , n=5; Deleted E6.5 = 0.32 ± 0.02 , n=8). That is, having the ICR present only until E6.5 is no better than never having it there at all. These data show that the epigenetically stable silent state of the paternal *H19* promoter is established between E6.5 and E8.5.

Our main interest was in the stability of silencing at the *Afp* locus. These results are depicted in Fig. 4C. Deletion of the *H19*ICR at E6.5 results in complete loss of silencing just as it does at the endogenous *H19* locus (germ line or “always deleted” = 0.91 ± 0.03 , n=21; Deleted E6.5 = 0.89 ± 0.02 , n=6). However, deletion via *Alb-cre* at E8.5 has an intermediate phenotype (0.22 ± 0.03 , n=4) with the promoter showing increased activity relative to the “Never Deleted” littermates (0.05 ± 0.01 , n=3, $p < 0.01$) but also showing repression relative to the “Always Deleted” control animals ($p < 0.001$).

DISCUSSION

The *H19*ICR is essential for imprinting of the *Igf2/H19* locus [5–7] and for organizing maternal and paternal chromosome domains into distinct 3D structures that promote high levels of monoallelic expression of *H19* and *Igf2*, respectively [14, 21–23]. Deletion of the

endogenous *H19ICR* results in maternal and paternal chromosomes that are functionally equivalent and that lack the long-range interactions characteristic of wild type chromosomes.

We tested the intrinsic properties and functionality of the ICR by transferring it to ectopic locations and assaying its ability to keep track of its own parental origin and to regulate the expression of its new neighboring genes. Others and we have shown that the 2.4-kb *BglIII* fragment is sufficient to act as a DMR in whatever genomic context it has been tested [13, 24, 25]. There is one interesting caveat: the exact timing for acquisition of paternal specific methylation varies dependent upon the site of integration. At the endogenous locus, the *H19ICR* is fully methylated during spermatogenesis while at ectopic locations, DNA methylation is not established until after fertilization.

We have already shown that maternal (non-methylated) *H19ICR* insertions at the *Afp* locus bind *CTCF* and prevent physical interactions between the *Afp* promoter and its distal enhancers through *CTCF-mediated processes* just as the endogenous *H19ICR* prevents physical interactions between the maternal *Igf2* promoters and their distal enhancers [14]. These results are consistent with numerous transgene studies in mice and in tissue culture that show that the *H19ICR*'s function as a transcriptional insulator is universal and dependent only on its location between any enhancer and its target promoters [11].

In this study we focus on the paternally inherited ICR and its ability to repress transcription of the adjacent Afp promoter. H19ICR mediated repression of paternal Afp is highly efficient and reduces promoter activity by 40-fold. Thus silencing activity of the H19ICR is intrinsic to the ICR and not dependent upon special features of the H19 promoter, its normal target. Upon paternal inheritance, the H19ICR inserted at Afp is methylated and cannot bind CTCF. Thus mechanisms for repression seen in Figure 2A (paternal inheritance) and Figure 2B (maternal inheritance) must be distinct. In this study, we refer to the repression of Afp mediated by the paternally inherited ICR as silencing, consistent with terminology used to described repression of the paternal H19 promoter.

Results in Figure 2 provide clear genetic evidence that two distinct mechanisms are used on the two parental chromosomes. Most importantly, the Afp-D insertion completely insulates Afp expression upon maternal inheritance but has only a minimal effect upon paternal inheritance. In fact, a comparison of expression from Afp-B and Afp-D chromosomes suggests that proximity of the ICR to its target promoter is necessary for effective gene silencing on the paternal chromosome but is not important for insulation on the maternal chromosome.

Altogether we see that each of the *H19ICR*'s key functions and properties are intrinsic to the 2.4-kb *BglIII* fragment. The ability of the *H19ICR* to organize chromosomal structures and to establish gene expression patterns can be predicted on its own intrinsic properties and its activities are not dependent on unique features of the *Igf2/H19* locus. Thus while many research labs, including our own, have identified and emphasized the critical significance of specific long-range interactions mediated by the *H19ICR* at the *Igf2/H19* locus, ([11, 26], it is also important to keep in mind that the ICR is promiscuous in its ability to find suitable partners in regulating gene activity.

We note that we were actually surprised to see *H19*ICR-mediated silencing of the paternal *Afp* promoter given that the *Afp* gene does not have any CpG-rich regions and in fact, is overall quite deficient in CpG dyads. In fact, silencing by the *H19*ICR was not accompanied by any measurable changes in CpG methylation within the *Afp* sequences yet *Afp* repression is as efficient as that noticed for paternal *H19*.

The spread of DNA methylation from the ICR to the *H19* promoter has seemed like an excellent explanation for *H19* silencing. DNA methylation has been long associated with gene repression and especially CpG island (CGI) methylation is associated with robust repression [27]. Paternal *H19* promoter methylation occurs in epiblasts and correlates with loss of paternal *H19* expression. Moreover, genetic studies have clearly demonstrated that *Dnmt1 methyltransferase* activity is essential for normal *H19* imprinting [28]. In contrast, our analyses of repression of paternal *Afp* suggest that DNA methylation is not essential for transcriptional silencing per se and that a paternally inherited *H19*ICR has the ability to repress transcription from an adjacent promoter without changing methylation frequency or patterns. We suggest that the developmental studies in Fig. 4B might address this paradox. By E8.5, silencing of *H19* is epigenetically stable and continues even after removal of the *H19*ICR. In contrast, silencing at *Afp* is unstable. Altogether these results are consistent with the idea that other changes such as chromatin are the initial signals that direct promoter repression while CpG methylation functions as a stable cell memory for transcriptional state [27, 29].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the Eunice Kennedy Shriver National Institute of Child Health and Human Development, Division of Intramural Research (ZIA HD 001804). Steve Coon and Tianwei Li generated the deep sequencing.

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Highlights

The H19ICR can silence a heterologous promoter

Silencing by the H19ICR does not depend on promoter methylation.

Silencing by the H19ICR is a developmental process.

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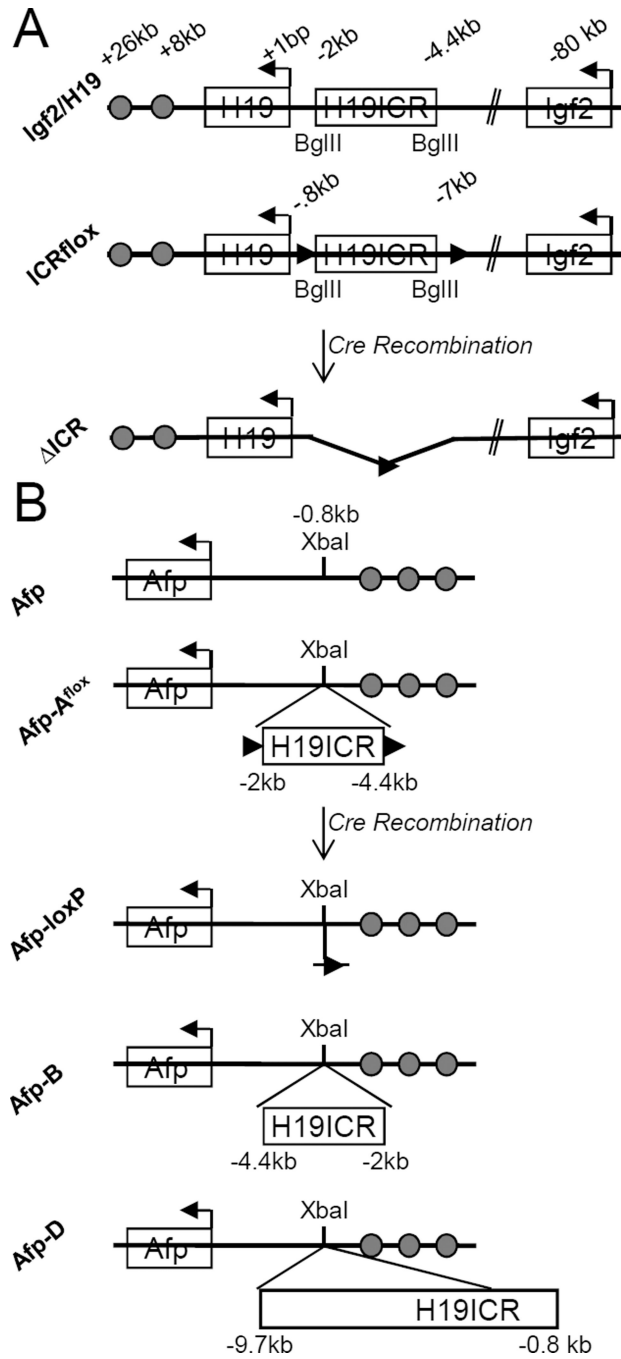


Figure 1. Mouse models used in this study

(A) *Igf2/H19* wild type and mutant alleles. The relative locations of the *H19* and *Igf2* genes and the *H19ICR* are depicted along with the shared endodermal and mesodermal enhancers (filled circles at +8 and +26 kb, respectively). All coordinates are relative to the *H19* start site. The *ICR^{lox}* allele [6] carries *loxP* insertions at -0.8 and -7-kb. Thus *cre*-mediated recombination results in deletion of the entire *H19ICR* but leaves the *H19* promoter in tact. (B) *Afp* wild type and insertion alleles. The relative locations of the *Afp* gene and its three enhancer elements (located between -1 and -7.6-kb) are depicted along with the *XbaI* site

where *H19ICR* sequences were inserted. These coordinates are relative to the *Afp* transcriptional start site. *Afp-A^{fllox}* has the *H19ICR* carried on a 2.4-kb *BglIII* fragment inserted at the *XbaI* site. *Cre*-mediated recombination of *Afp-A^{fllox}* removes the *H19ICR* but a residual 194-bp including a single loxP site remains. *Afp-B* differs from *Afp-A* in the orientation of the inserted ICR. *Afp-D* carries the *H19ICR* inserted in the same orientation as *Afp-B* but has an additional 4.8-kb of sequences that separate the *H19ICR* from the *Afp* promoter. For *Afp-A^{fllox}*, *Afp-B*, and *Afp-D*, the numbers below the ICR delineate the endogenous coordinates of the *H19ICR* fragment.

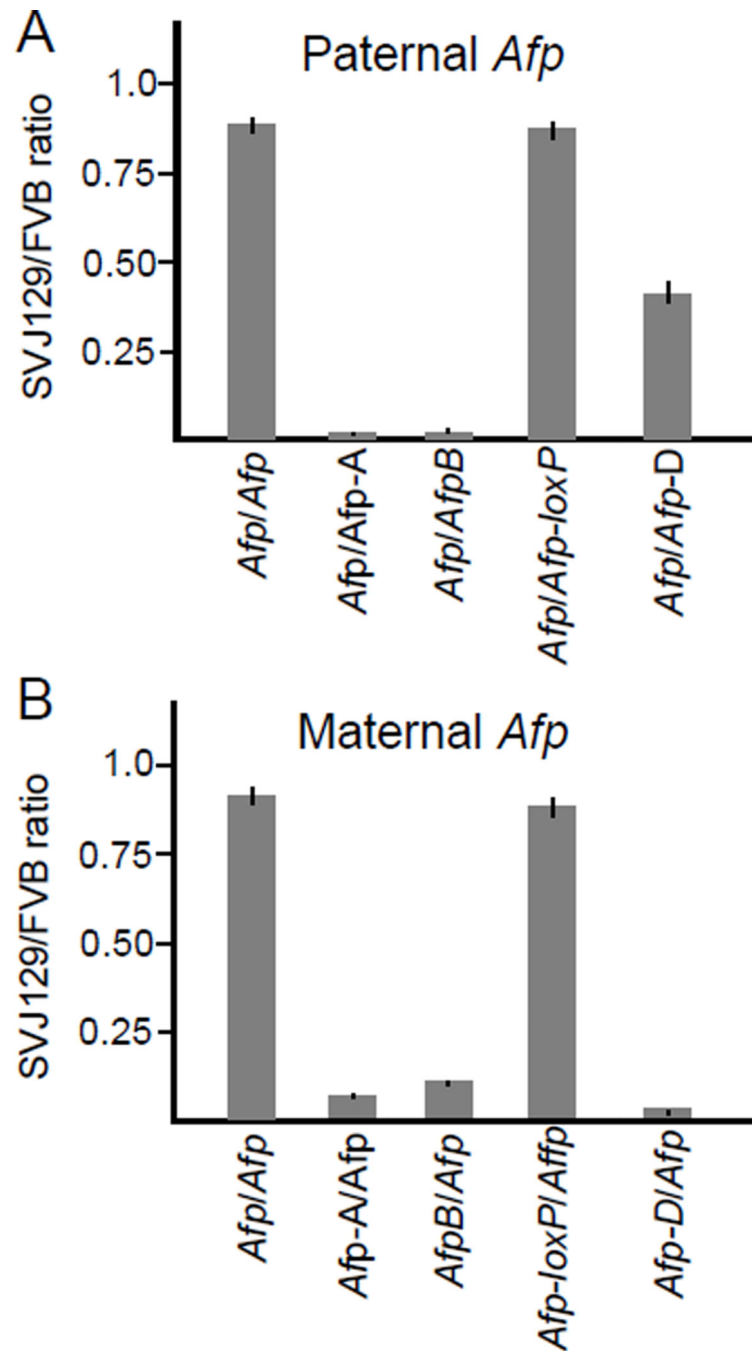


Figure 2. Effect of H19ICR insertional mutations on *Afp* expression

(A) The 2.4-kb H19ICR functions as a transcriptional silencer upon paternal inheritance. Crosses were set up to generate pups so that maternal chromosome 5 was always FVB and wild type in origin while paternal chromosome 5 was always SvJ129 in origin and was either wild type or mutant as described on the X-axis. RNAs were isolated from neonatal livers and assayed for allele specific expression of *Afp*. Results are depicted as the ratio of SVJ129 to FVB *Afp* RNAs. (B) The 2.4-kb H19ICR functions as a transcriptional insulator upon maternal inheritance. Crosses were set up similarly to those described in panel A

except that the paternal chromosome 5 was always FVB and wild type in origin while maternal *Afp* was always SvJ129 and was either wild type **or** mutant as indicated on the X-axis. Allele specific expression is reported as the ratio of 129 to FVB *Afp* RNAs. Bar graphs depict mean \pm SEM with N values indicated in the text.

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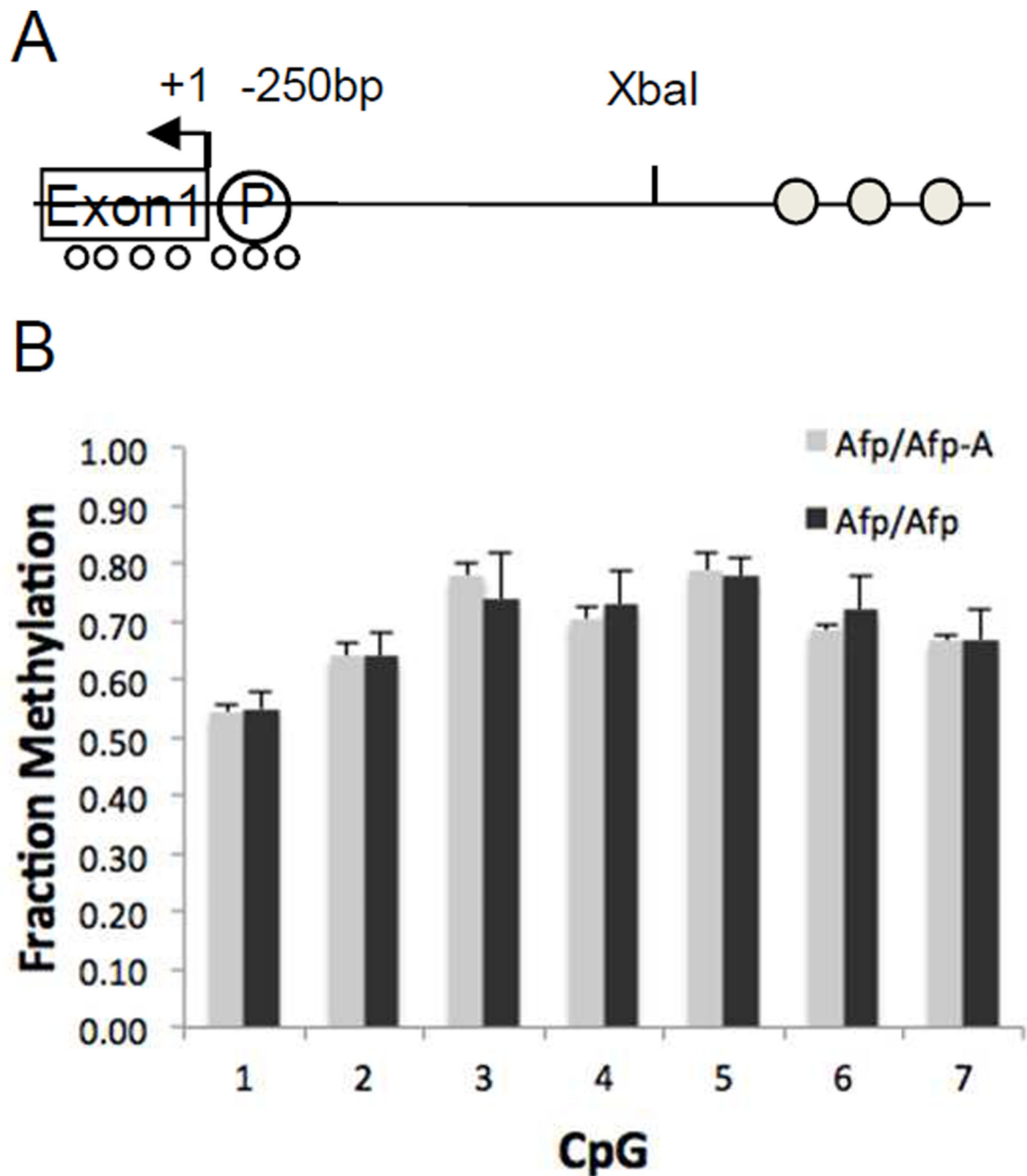


Figure 3. *Afp* promoter CpG methylation does not change in response to the *H19*ICR insertion (A) Schematic depiction of the *Afp* locus showing the four CpGs within exon 1 and the three CpGs within the 250-bp *Afp* promoter that were analyzed. (B) Methylation frequencies for DNA isolated from livers of late gestation wild type mice (*Afp/Afp*), mice in which the inserted 2.4-kb *H19*ICR was paternally inherited (*Afp/Afp-A^{fllox}*), and mice in which the 2.4-kb *H19*ICR was maternally inherited (*Afp-A^{fllox}/Afp*). Methylation fractions were calculated from at least 50,000 reads from each of 10 samples as detailed in Sup Fig 1.

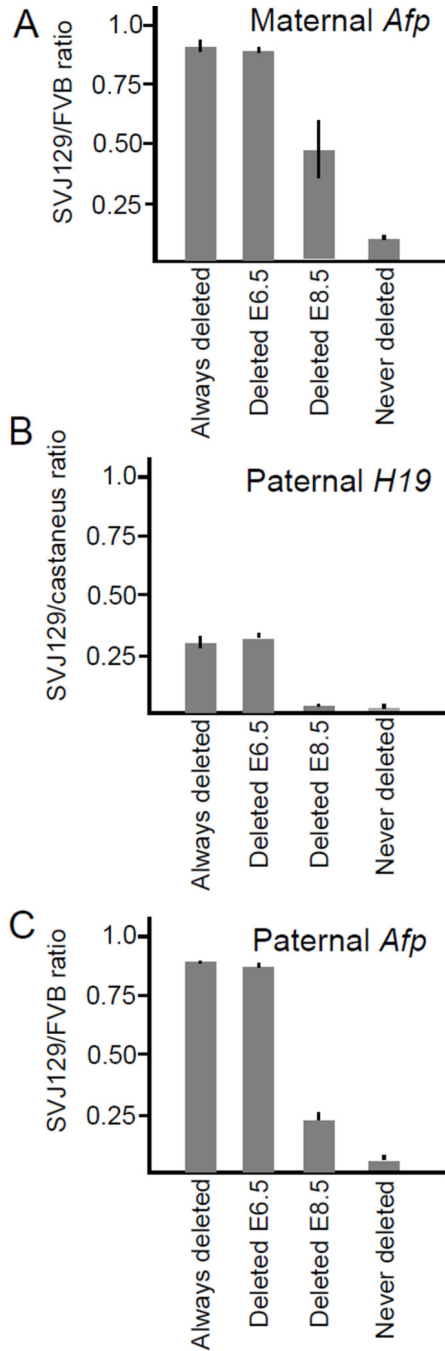


Figure 4.

A. Stability of transcriptional insulation after removal of the *H19*ICR from the maternal chromosome. Expression of the maternal *Afp* allele was quantitated as described in Fig. 2B in *Afp-loxP/Afp* pups (“Always deleted”), in *Afp-A^{flox}/Afp* pups carrying the *Sox2-cre* transgene (“Deleted E6.5”), in *Afp-A^{flox}/Afp* pups carrying the *Alb-cre* transgene (“Deleted E8.5”), and *Afp-A^{flox}/Afp* pups that did not carry a *cre* transgene (“Never deleted”). **B.** Stability of transcriptional silencing at the *H19* locus. Expression of the paternal *H19* allele (SvJ129) was quantitated relative to the expression of the maternal *H19* allele that was

always wild type and *Mus castaneus* in origin. “Always deleted” = $H19^+/H19^{ICR}$; “Deleted E6.5” = $H19^+/H19^{CRflox} + Sox2-cre$ transgene; “Deleted E8.5” = $H19^+/H19^{CRflox} + Alb-cre$ transgene; “Never deleted” = $H19^+/H19^+$. **C. Stability of transcriptional silencing at the *Afp* locus.** Crosses were set up as described for Fig. 2A. Expression of paternal *Afp* (SvJ129 allele) was quantitated relative to the expression of wild type maternal FVB *Afp* in *Afp/Afp-loxP* pups (“Always deleted”), in *Afp/Afp-A^{flox}* pups carrying the *Sox2-cre* transgene (“Deleted E6.5”), in *Afp/Afp-A^{flox}* pups carrying the *Alb-cre* transgene (“Deleted E8.5”), and in *Afp/Afp-A^{flox}* pups that did not carry a *cre* transgene (“Never deleted”).