Complete Biallelic Insulation at the *H19/Igf2* Imprinting Control Region Position Results in Fetal Growth Retardation and Perinatal Lethality

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Abstract

**Background:** The *H19/Igf2* imprinting control region (ICR) functions as an insulator exclusively in the unmethylated maternal allele, where enhancer-blocking by CTCF protein prevents the interaction between the *Igf2* promoter and the distant enhancers. DNA methylation inhibits CTCF binding in the paternal ICR allele. Two copies of the chicken β-globin insulator (ChβGI)² are capable of substituting for the enhancer blocking function of the ICR. Insulation, however, now also occurs upon paternal inheritance, because unlike the *H19* ICR, the (ChβGI)² does not become methylated in fetal male germ cells. The (ChβGI)² is a composite insulator, exhibiting enhancer blocking by CTCF and chromatin barrier functions by USF1 and VEZF1. We asked the question whether these barrier proteins protect the (ChβGI)² sequences from methylation in the male germ line.

**Methodology/Principal Findings:** We genetically dissected the ChβGI in the mouse by deleting the binding sites USF1 and VEZF1. The mutation of the maternal versus normal (ChβGI)² significantly increased from 11% to 32% in perinatal male germ cells, suggesting that the barrier proteins did have a role in protecting the (ChβGI)² from methylation in the male germ line. Contrary to the *H19* ICR, however, the mutant (mChβGI)² lacked the potential to attain de novo methylation in the germ line and to maintain methylation in the paternal allele in the soma, where it consequently functioned as a biallelic insulator. Unexpectedly, a stricter enhancer blocking was achieved by CTCF alone than by a combination of the CTCF, USF1 and VEZF1 sites, illustrated by undetectable *Igf2* expression upon paternal transmission.

**Conclusions/Significance:** In this in vivo model, hypomethylation at the ICR position together with fetal growth retardation mimicked the human Silver-Russell syndrome. Importantly, late fetal/perinatal death occurred arguing that strict biallelic insulation at the *H19/Igf2* ICR position is not tolerated in development.

Introduction

Enhancers are capable of activating gene promoters from great distances. It is the role of insulators in the genome to inhibit promiscuous long range activation of promoters [1,2,3]. Insulator action can manifest in enhancer blocking and chromatin barrier functions [2,4]. Enhancer blocking means that an insulator is located between enhancer and promoter elements and prevents their communication. Chromatin barriers function to demarcate active and repressive chromatin domains. CTCF binding factor (CTCF) [5,6,7] is the major insulator protein in vertebrates [5]. The enhancer-blocking role of the CTCF protein has been confirmed in various in vitro and in vivo transgenic assays and in genetic studies in the mouse [8,9]. In the context of the genome, in vivo CTCF binding is often associated with sharp chromatin transitions, indicative of the presence of chromatin barriers [10,11]. CTCF, however, does not have barrier function [12]. Chromatin barrier function has recently been attributed to upstream stimulatory factor 1 (USF1) [13] and to vascular endothelial zinc finger 1 (VEZF1), also called beta globin protein 1 (BGP1) [14,15,16,17,18,19,20] in transgenic mouse experiments [21,22].

The chicken β-globin insulator (ChβGI) and the *H19/Igf2* imprinting control region (ICR) are two well-studied insulator regions. Both regions exhibit very strong insulation between an enhancer and promoter elements and their insulator function depends on CTCF binding. There is, however, a major difference between these two insulators in that the insulator activity of the *H19/Igf2* ICR depends on parental origin [23,24,25,26]. The 2.4 kb long ICR [27,28,29,30] is methylated in the sperm, but is unmethylated in the egg. This primary methylation difference (genomic imprint) is passed into the zygote, maintained during embryogenesis and determines the activity status of the ICR in the soma. The ICR is responsible for maternal allele specific expression of *H19* and for paternal allele specific expression of *Igf2* [28]. In the soma the maternally inherited unmethylated allele binds CTCF at four sites in vivo [26,31,32,33,34], resulting in insulation [34,35,36,37,38,39] between the insulin-like growth
binding sites 1, 3 and 5 (blue circle): VEZF1 (BGP1); binding site 2: CTCF; and binding site 4 (pink oval): USF1. (D) Structure of the mutant chicken β-globin insulator duplex (mChβGI)₂. Only the CTCF binding site (thick underlining) remains in each unit after deleting (x) binding sites 1, 3, 4 and 5 using site-directed mutagenesis. (E) Confirmation of the site-directed mutagenesis by DNA sequencing. Arrows indicate the positions of the deleted binding sites (deleted sequences shown underneath) and light underlining shows added nucleotides at footprint 1. Novel restriction sites, Scal, Stul and NheI, marked above, were generated to aid the screening of mutant colonies. One out of two Smal sites remained at the footprint 3 deletion.
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Figure 1. Imprinted versus non-imprinted insulation at the H19/Igf2 locus by two distinct insulators. (A) Imprinted insulation at the H19/Igf2 imprinted domain by the ICR. Maternal chromosome (M): unmethylated (white lollipops) ICR (shaded area) is inherited from the egg. CTCF (yellow ovals) imparts insulator activity (bracekt) between the Igf2 promoters and the shared, downstream enhancers (orange oval). Initiation of H19 expression depends on an unmethylated ICR during embryogenesis. Paternal chromosome (P): methylated (black lollipops) ICR is inherited from the sperm, CTCF cannot bind, hence ICR has no insulator activity. Igf2 promoters and enhancers can interact. Early in postimplantation development, the H19 promoter is inactivated by an ICR-dependent mechanism (horizontal arrow). (B) Non-imprinted insulation at the H19/Igf2 locus by the chicken β-globin insulator duplex (ChβGI)₂ [44]. The (ChβGI)₂ is unmethylated and insulates the Igf2 promoter from the shared enhancers when substituted for the ICR and transmitted maternally (not shown) or paternally (P), with 10% Igf2 activity remaining. H19 is overactivated 1.5-fold by the (ChβGI)₂ sequences in the paternal allele (bold arrow). (C) Structure of the (ChβGI)₂ with the five in vitro footprints of the core insulator [8]:

factor 2 (Igf2) promoters and the shared downstream enhancers [40]. In contrast, in the paternally inherited ICR allele DNA methylation inhibits CTCF binding/enhancer blocking function, hence Igf2 is expressed (Figure 1A). The paternally inherited ICR is also required for inactivating H19 during early embryo development by methylation spreading [41]. Inactivation of the CTCF binding sites in the maternal allele results in the loss of enhancer-blocking activity in the maternal allele, biallelic Igf2 expression and large fetus size [34,35,36,37,38,39]. CTCF binding in the maternally inherited ICR is also required in the early embryo for initiation of H19 expression [35], and for maintaining hypomethylation of the ICR in the soma [34,35,36,37,38,39]. CTCF binding, however, is not responsible for the germ line events that establish the methylation differences at the ICR between egg and sperm. The CTCF site-mutant ICR was correctly unmethylated in female fetal germ cells [39] and ovulated oocytes [35,38,39], and it was correctly methylated in fetal male germ cells [39] and in sperm [35].

The 1.2 kb long ChβGI is located in the constitutive DNaseI hypersensitive site 4 between a 12 kb heterochromatin stretch and the β-globin locus in the chicken. In transgenic mice two copies of the 1.2 kb can protect transgenes from position effects [42,43]. Most of the insulator activity resides in a 250 bp “core element” which contains five in vitro footprints (Figure 1C) [8]. Insulator function has been attributed to footprint 2 (CTCF) whereas chromatin barrier activity was associated with footprints 1, 3, 5 (BGP1/VEZF1) and 4 (USF) [12]. The barrier protein, USF is required for maintaining euchromatin features including histone 3 lysine 4 dimethylation (H3K4me2) and histone hyperacetylation of the ChβGI and the ChβGI-surrounded transgene sequences [22]. BGP1 (VEZF1) is important for maintaining euchromatin at the insulated transgene [22] and for maintaining DNA hypomethylation at the ChβGI and along the ChβGI-surrounded transgene [21].

We previously substituted the ICR with two copies of the chicken β-globin insulator, (ChβGI)₂ [44]. The (ChβGI)₂ lacks homology to the ICR except for the two CTCF sites. We found that due to CTCF binding, upon maternal transmission the (ChβGI)₂ sequences substituted for the ICR function of the ICR: in fetal organs Igf2 expression was very low in the mutant allele. Upon paternal transmission, however, the (ChβGI)₂ failed to undergo de novo methylation in the male germ line and remained unmethylated in the soma, resulting in biallelic insulation (Figure 1B). Igf2 expression was reduced to 10% and fetus size was 50–61% of normal siblings. H19 expression was biallelic and the paternal allele’s expression was overactivated, it accounted for 77% of total H19 expression in fetal livers and kidneys [44].

We now asked the question whether the regulatory elements that provide the (ChβGI)₂ with barrier activity are responsible for the non-imprinted behavior of the (ChβGI)₂ at the H19 ICR position. We hypothesized specifically that, due to their euchro-
matin-maintaining potential, the USF and VEZF1 sites may protect the (ChβGI)2 from de novo methylation in the male germ line. This could occur if the genomic locus outside the H19/Igf2 ICR carried clues for directing de novo methylation to the endogenous ICR or to an introduced DNA fragment, such as (ChβGI)2 at the ICR position. We tested this hypothesis by deleting the USF and VEZF1 binding sites from the (ChβGI)2 and used two copies of this mutant β-globin insulator (mChβGI2) to substitute for the H19/Igf2 ICR. In this way enhancer blocking activity was maintained at the ICR position due to two intact CTCF sites but barrier activity was abolished because six VEZF1 and two USF sites were absent. We expected that with these barrier proteins were removed, the (mChβGI2) could become methylated in the male germ line. If this methylation is maintained in the paternal allele in the soma, it would result in parental-allele specific H19 and Igf2 expression.

We found that the (mChβGI)2 attained significantly more methylation in fetal male germ cells than the normal (ChβGI), suggesting that the boundary proteins provided protection from methylation in the male germ line. This methylation, however, was not maintained in the paternal allele, indicating that the (mChβGI)2 lacked the capacity for methylation maintenance in the soma. Therefore, similarly to the (ChβGI)2, the (mChβGI)2 was a biallelic insulator. Its paternal transmission resulted in biallelic H19 expression and undetectable Igf2 expression. The enhancer blocking function was, unexpectedly, stronger by CTCF alone than by using a combination of CTCF, USF and VEZF1 sites. Our results argue that complete biallelic enhancer blocking function was, unexpectedly, stronger by CTCF than by USF and VEZF1.

Two copies of the mChβGI were inserted into the Sac I and the EcoRI – SphI positions of the acceptor plasmid, pGEM4Z-Link2. The acceptor plasmid was generated by ligating the annealed Link2 oligos (5'-AATTGAGACGATTCGAGATCGACTAG-3' and 5'-AGCTTGGATCCGATCGAAC-3') into HindIII and EcoRI double digested pGEM4Z. The orientation of the inserts was verified by SodI analytical digestion.

Gene targeting to produce mouse lines with the ICR substitution

The 2.2 kb long BsmHI fragment of (mChβGI)2 from pGEM4Z-Link2 was ligated into the BglII site of the H19 ICR targeting vector [45]. The direction of the insert was verified by MluI digestion. Gene targeting was done in ES cells as before [39,44,45]. 96 neo positive ES cell clones were screened by PCR and verified by Southern blot hybridization (Figure 2). Probe a was a 0.5 kb PCR fragment made with primers 5'-GGTGGCATCAGCTTACGAC-3' and 5'-CTGCTTAGATGACCATGGCACAG-3'; probe b was a BsmHI-EcoRI restriction fragment and probe c was a SodI-EcoRI restriction fragment. From 26 PCR-positive clones 24 clones underwent conservative recombination. Four ES cell lines were injected into 8-cell muras and 2, 3, 1 and 9 chimeras were obtained from ES cell lines #1, #2, #22 and #29, respectively. None of the male chimeras produced viable mutant offspring. Male chimeras from independent ES cell clones did transmit the mutation, because we found fetuses positive for the mutation at 18.5 and 19.5 dpc in females pregnant from one chimera of ES#2 and three chimeras of ES#29 origin. One male chimera from ES#29 had a litter of 5 dead newborns, all positive for the mutation. Fetuses fathered by one other male chimera of ES#29 origin were systematically investigated (Table 1). Two chimeras from two independent ES cell lines (#22 and #29 origin) were female and produced male and female live F1. The neo cassette was removed by mating the female chimaeras with Hprt-Cre males of 129S1 genetic background [46]. Removal of the neo cassette was verified by the presence of a 0.24 kb PCR fragment spanning the remaining loxp site using primers 5'-GGCCACCAAGCTGCTAGGCA-3' and 5'-CCTAGGAGAATTCGAGGACCTAATAAC-3'. Male F1 mutant did not produce live mutant offspring whereas female mutants transmitted the mutation. The Hprt-Cre gene was removed by mating of the F1 females with 129S1 males and was confirmed by PCR. The mutation from ES#29 was kept in the 129S1 strain in M/+ form. Male mutants from this line were bred to females of different genetic background including 129S1, FVB inbred lines and C57 outbred mice. Mutant pups never survived beyond day 1 after birth. Positive mice were identified by PCR ChβGLU: 5'-TCTGGCAGTGGTAGAGTAACA-3' and probe c was a SodI-EcoRI restriction fragment.

Materials and Methods

The experiments involving mice had been approved by the IACUC of the City of Hope. Housing and care of the animals has been consistent with the Public Health Service Policy, the NIH “Guide for the Care and Use of Laboratory Animals” and the Animal Welfare Act.

Site-directed mutagenesis

The mutator plasmid, pGEM4Z-link3, was generated by ligating annealed Link 3 polynucleotide oligos (5'-AATTCCGAGCTTCGGTACCCTTCCTTATTCACTTGCCTATC-3' and 5'-AGCTTGGATCCGAGCTGGAGCTAGCG-3') into HindIII and EcoRI double-digested pGEM4Z. The direction of the insert was verified by MluI digestion. The mutator plasmid, pGEM4Z-link3, was generated by ligating the annealed Link 2 polynucleotide oligos (5'-GCCCACCAA and inserting TACT using the 5'-CTAGAGGGACAGTACTCAGGATATT-3' oligo containing a SodI site. The mutant clones were identified by restriction digestion and verified by DNA sequence analysis.
Allele-specific gene expression by Sequenom SNuPE

Allele-specific H19 and Igf2 RNA gene expression analysis was based on SNPs between inbred 129S1 (129) and CAST/Ei (CS) mouse strains and was analyzed by reverse-transcription PCR SNuPE assays [44,47], except mass spectrometry quantified the extension primers [EP] based on molecular mass difference between alleles [48,49]. Primers were designed using MassArray Assay v3.1. H19: 5'-ACGTTTGATGATGGACGACGTTGCTCGAGTATGAGTCTGCTCTTTC-3' and 5'-AGCAGACCTGCATCCAGTCTGCTGGG-3'. Igf2: 5'-ACGTTTGATGATGGACGACGTTGCTCGAGTATGAGTCTGCTCTTTC-3' and 5'-AGCAGACCTGCATCCAGTCTGCTGGG-3'.

RNA Isolation and RT-PCR

RNA was isolated from using RNA-Bece (Tel-Test). Contaminating DNA was removed with the DNA-free Kit (Ambion). Reverse transcription was performed using equal amount of RNA using the Superscript III Random Primer Synthesis kit (Invitrogen). RT-PCR primers and probes were: Igf2 exon 2–3: 5'-GAGCAGGCGTCTCAGTCT-3' and 5'-AAGCTGCTCGTTGGAAGGGGCTT-3'; Gapdh exon 4–5: 5'-ATTGCAGCCTGCTCGGAGCTG-3', 5'-AATGTGGTGCTGATG-3' and 5'-CAACCTGGTCCTCAGTCTGCTGAG-3'.

Methylation analysis by Southern hybridization

DNA was digested with BamHI and EcoRI and with control MspI (methylation non-sensitive) or HpaII (methylation sensitive) enzymes. The mChβGI was labeled for hybridization probe. After HpaII digestion, the probe visualized four bands: 1.45 kb (weak band due to short overlap with the probe), 800 bp, 700 bp and 350 bp, the same bands as after MspI digestion. Therefore, the mChβGI was unmethylated.

Bisulphite genomic sequencing

200 ng genomic DNA from fetal organs or 10,000–23,000 flow-sorted germ cells were used per bisulphite reaction performed in a MassArray Compact mass spectrometer (Sequenom) using the Spectroacquire program (Sequenom) and was analyzed by MassArray Typer v3.4. We applied skew correction using a true heterogeneous DNA sample to correct for any allelic imbalance in the SNP allele products. The % expression of each allele in the total expression was calculated at each given SNP.

Results

Replacing the H19/Igf2 ICR with two copies of the mutant chicken β-globin insulator

We deleted the VEZF1 and USF1 binding sites from the 1.2 kb (ChbGI) using site-directed mutagenesis. The correctly mutagenized (mChβGI) was identified by restriction digestion and verified
by DNA sequencing (Figure 1E). Two copies of this mutant insulator, (mChβGI2) were introduced into mice to replace the H19/Igf2 ICR by gene targeting (Figure 2). The (mChβGI2) still harbored two functional CTCF binding sites and a high density of methylatable CpGps.

Maternal inheritance of the (mChβGI2)

Maternal inheritance resulted in normal and viable mice. The size of the (M)/+ fetuses was normal (Table 1). The parental allele-specific expression patterns of H19 and Igf2 were normal. H19 was expressed from the maternal allele in livers and kidneys of (M)/+ 18.5 days post coitum (dpc) fetuses (Figure 3C) whereas Igf2 was paternally expressed (data not shown) in the same samples. The (mChβGI2) therefore perfectly substituted for the insulation function of the ICR, just as the (ChβGI2) did. The (mChβGI2) DNA, including the two CTCF binding sites, was unmethylated in somatic organs, kidneys and livers, of perinatal (M)/2 fetuses (Figure 4A and B) similarly to the maternally inherited ICR (Figure 4C). The (mChβGI2) correctly did not attain de novo methylation in the germ cells of female (+/−(P) fetuses (Figure 5A). Therefore, the USF1 and VEZF1 binding sites were not required for protecting the (ChβGI2) sequences from methylation in the fetal female germ line and in the maternal allele in the soma.

Paternal inheritance of the (mChβGI2)

Out of 19 litters in different genetic backgrounds, we obtained 101 +/+ weanlings (Table 2). The expected number of +/+ (P) weanlings was 101, but 0 was found. We inspected each cage on the day of birth. A total of 13 +/+ (P) pups were found dead or died within a few hours after birth, consistent with a late fetal/perinatal lethality phenotype. Despite maternal attention, milk was not found in the stomach of the live newborns, indicating inability for feeding. Detailed histology was performed on coronal sections of the head and longitudinal sections of the body. Apart from the small size of body and organs there was no abnormality present in +/+ (P) newborns (data not shown). 18.5 dpc +/+ (P) fetuses were small (Table 1), but of normal appearance (Figure 3A). The weight of +/+ (P) fetuses was 44% or 50% of the +/+ siblings depending on whether the mother was of CS or TgOG2 mouse strain. Placenta weight was also reduced (Table 1). Independently targeted ES cells gave similar results: +/− (P) fetuses from a male chimera (ES#2) or from male descendants of a female chimera (ES#29) were small (Table 1). The phenotype of +/(mChβGI2) was more severe than that of +/(ChβGI2) [44], where fetus weight at 18.5 dpc was 62% and 50–61% of ++ littermates in the respective CS and TgOG2 crosses, and Igf2 levels were 10%. Igf2 expression was undetectable in +/(mChβGI2) fetuses (Figure 3B) indicating that enhancer blocking by (mChβGI2) was more complete in the absence of the USF1 and VEZF1 binding sites at the ICR position.

Similarly to the (ChβGI2) [44], the paternally inherited (mChβGI2) was unmethylated in fetal organs (Figure 4A and B), indicating that the USF1 and VEZF1 sites were dispensable for hypomethylation of the (mChβGI2) in the soma. The (mChβGI2) DNA was more methylated than the (ChβGI2) in male germ cells (32% versus 11% of CpGps methylated) (Figure 5B) [44], suggesting that the USF1 and/or VEZF1 proteins contributed to protecting the (ChβGI2) sequence from de novo methylation in the male germ line. The (mChβGI2), however, was less methylated than the normal ICR [52,53] (Figure 5C), suggesting that it lacks the sequences that trigger full methylation of the ICR in spermatogonia. The fact that partial methylation was attained in the male germ line (Figure 5B) but it was not observed in the soma (Figure 4A and B) demonstrates that the (ChβGI2) DNA lacks the potential to maintain methylation in the paternal allele.

In the kidney and liver of +/−(P) perinatal fetuses, H19 levels were more than 2-fold than in normal siblings (Figure 3B) and H19 was biallelically expressed (Figure 3C) indicating that, unlike the fully methylated ICR, the hypomethylated (mChβGI2) was not capable of inactivating the H19 promoter in the paternal allele during post-fertilization development. Contrary to the (ChβGI2), the (mChβGI2) did not overactivate the H19 in the paternal allele relative to the maternal allele. The paternal and maternal H19 alleles each contributed 50% of total H19 expression (Figure 3C). This suggests that USF and VEZF1 proteins in the (ChβGI2) were responsible for overactivating H19 in cis.

**Discussion**

In this study we dissected the insulator and barrier functions of the (ChβGI) by deleting the USF and VEZF1 binding sites from

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**Table 1. Weight of 18.5 dpc fetuses on maternal and paternal inheritance of the (mChβGI).**

|          | Mean wet weight (g) ± s.d. (n) (%) of +/+ weight |
|----------|--------------------------------------------------|
|          | fetus                                             | placenta                                      |
| +/+      | 1.313 ± 0.098 (24) (1.166–1.530)                  | 0.666 ± 0.009 (24) (0.503–0.956)              |
| (M)/+    | 1.293 ± 0.155 (24) (0.871–1.511) (98.5%)          | 0.600 ± 0.008 (24) (0.047–0.077) (90.9%)      |
| +/+      | 1.436 ± 0.128 (13) (1.233–1.593)                  | 0.057 ± 0.014 (13) (0.046–0.101)              |
| +/+ (P)  | 0.635 ± 0.055 (9) *(0.571–0.718) (44.2%)          | 0.032 ± 0.007 (9) *(0.024–0.044) (56.1%)      |
| +/+ (P)  | 1.465 ± 0.117 (19) (1.267–1.692)                  | 0.062 ± 0.022 (19) (0.038–0.109)              |
| +/+ (P)  | 0.653 ± 0.068 (16) *(0.497–0.755) (44.5%)         | 0.046 ± 0.019 (16) *(0.022–0.083) (74.2%)     |
| +/+      | 1.126 ± 0.194 (14) (0.873–1.339)                  | 0.077 ± 0.011 (14) (0.057–0.092)              |
| +/+ (P)  | 0.564 ± 0.071 (18) *(0.486–0.705) (50.1%)         | 0.038 ± 0.006 (17) *(0.030–0.051) (49.4%)     |

* Sibling from -(M) × +/+ × +/+ matings.
* Sibling from +/+ × +/+ × (P) × +/+ matings.
* Sibling from +/+ × +/+ × (P) × +/+ matings. +/+ females were from transgenic line TgOG2.
* P < 0.0001.
* P < 0.025.
(M), Maternal allele; (P), Paternal allele.

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the (ChbGI)2 and used two copies of this mutant chicken β-globin insulator (mChbGI)2 to substitute for the H19/Igf2 ICR. Our results have implications for understanding insulator function and imprint establishment. The observed lethality phenotype argues that strict biallelic insulation at the ICR position is not tolerated in mouse development.

On Insulator Function

Insulators are often complex, harboring enhancer blocking and chromatin barrier activities. We genetically dissected the (ChbGI)2, and tested whether its barrier function is required for substituting the H19/Igf2 ICR. The (mChbGI)2 insulated the Igf2 promoter from the shared enhancers, suggesting that CTCF binding is sufficient and the VEZF1 and USF barrier proteins are dispensable for insulation at the H19/Igf2 ICR position. Whereas 10% Igf2 residual expression remained in livers and kidneys of +/(ChbGI)2 fetuses and 75 and 95% of this was from the paternal allele, respectively (Szabó, PE and Mann, JR, unpublished) indicating incomplete insulation, Igf2 RNA was undetectable in +/(mChbGI)2 fetuses, indicating complete insulation. A stronger enhancer blocker function was, therefore, achieved by CTCF alone than by using a combination of CTCF, USF and VEZF sites.

Barrier proteins, USF and VEZF1, do not insulate enhancers from promoters but protect surrounded transgenes from the invasion of heterochromatin: they maintain active chromatin by recruiting histone acetyltransferases (HATs) and also protect the DNA from de novo methylation. USF directly recruits HATs p300/CBP and PCAF and H3K4 methyltransferase Set7/9 to enforce active chromatin [22,42]. VEZF1 is important for maintaining euchromatin [22] and DNA hypomethylation at the ChbGI and along the ChbGI-surrounded transgene [21]. CTCF protein, apart from its enhancer blocking function, has very similar activities. CTCF maintains ICR hypomethylation in somatic cells [34,35,37,38,39]. CTCF can recruit the HAT, CHD8 to the ICR [54]. In the maternal allele CTCF recruits active histone tail modification marks to the ICR and to the H19 gene [31] and also recruits at a distance, Polycomb-mediated H3K27me3 repressive marks at the Igf2 promoter and at the Igf2 DMRs [31,33]. Further studies will be required to fully understand the molecular mechanisms of chromatin barrier versus enhancer blocking functions. It will be interesting to see if chromatin barriers exist in vertebrates without CTCF. It will be interesting for example, to compare the in vivo occupancy of CTCF binding sites with VEZF1 and USF1 sites in a genome-wide study. CTCF alone may insulate by enhancer blocking but in combination with barrier proteins it may insulate by forming chromatin barriers. Because VEZF1 and USF barrier proteins were dispensable for insulation at the ICR position, chromatin barrier formation in the maternal allele may not be required at all for proper regulation of imprinted genes at the ICR position.

We find it interesting that whereas CTCF is required for protecting the ICR from methylation in the soma [35,38,39], it doesn’t protect from methylation imprint establishment the male germ line [35,39]. USF and VEZF1 sites, on the other hand, are not required for protecting the (ChbGI)2 from methylation in the soma but contribute to its protection in the male germ line at the ICR position. In fetal male germ cells CTCF protein may not bind

Figure 3. Phenotype of 18.5 dpc fetuses inheriting the (mChbGI)2. (A) Representative +/+ and +/-P fetuses are shown. (B) Expression of Igf2 and H19 was measured by real-time RT-PCR in kidneys. RNA from two +/–(P) and -(M)/+ fetuses (samples 3–4 and 7–8, respectively) and their +/+ littermates (samples 1–2 and 5–6) was analyzed. (C) Allele-specific expression of the H19 in kidneys and livers of the same fetuses was measured by RT-PCR SNuPE. The % expression of the maternal (M) and paternal (P) allele in the total expression is shown. doi:10.1371/journal.pone.0012630.g003
in the ICR whereas USF and/or VEZF1 proteins may bind in the (ChbGI)_2. Chromatin analysis in fetal germ cells will be needed to follow up on these possibilities.

On Imprint Establishment

The mechanism that targets DNA methylation imprint establishment to the H19/Igf2 ICR in fetal male germ cells is still unknown. Tandem repeats in this domain have no role in methylation targeting [55,56,57]. Mutagenesis of specific protein binding sites had no effect on DNA methylation imprint establishment at the ICR: methylation was undisturbed in mutant male germ cells and lack of methylation was undisturbed in mutant female germ cells [45,58,59,60]. Whereas the ICR became methylated in male germ cells in a randomly integrated 150 kb H19 transgene, it did not attain DNA methylation or accumulated only partial methylation in the male germ line when introduced to genomic locations other than at the H19 locus [61,62,63,64]. These results suggest that the genomic location is important for methylation imprint establishment of the ICR in the male germ line.

Our study provides indirect clues to the question whether the genomic locus or the H19/Igf2 ICR sequence determines DNA methylation imprint establishment in the germ line. We found that the (mChbGI)_2 became partially (32%) methylated in male germ cells but remained unmethylated in female germ cells. This male germ cell-specific methylation of the (mChbGI)_2 is consistent with the possibility that the genomic locus carries "methylator elements" that target Dnmt3a and Dnmt3L to the ICR position. Parental specific methylation can occur on integrated transgenes [65,66,67,68]. The genomic location could target de novo methylation to the ICR for example by an RNA that is transcribed across the ICR specifically in male fetal germ cells, similarly to the Gnas DMRs in growing oocytes [69]. The (mChbGI)_2 attained 3-fold higher CpG methylation in prospermatogonia than the (ChbGI)_2, suggesting that the boundary proteins USF1 and VEZF1 provided the (ChbGI)_2 protection from de novo methylation in the male germ line. Unlike the endogenous ICR sequence, however, the mutant (mChbGI)_2 was not fully methylated, indicating that the (mChbGI)_2 may be missing sequence elements that target methylation to the ICR in prospermatogonia.

Whereas the (mChbGI)_2 attained 32% de novo male germ cell-specific methylation, it was unmethylated in the paternally inherited allele in fetal somatic organs. The (mChbGI)_2, therefore, lacks the potential of methylation imprint maintenance at the H19/Igf2 locus. CpG methylation is likely lost during the global wave of epigenetic remodeling events in the embryo. A methylation maintaining role of 9 CpGs in the 4 CTCF sites has been confirmed in the ICR [36], but 2 CpG-s in the (mChbGI)_2 CTCF sites did not fulfill this role. Alternatively, the level of methylation has to be over a threshold at the ICR position to be recognized for maintenance.

On the Lethality Phenotype

Paternal inheritance of (mChbGI)_2 resulted in a more severe phenotype than that of (ChbGI)_2, causing not only smaller fetus size but also perinatal death. The lethality phenotype cannot be explained by the absence of the paternally inherited ICR, because paternal deletion of the endogenous ICR [28] or its substitution with the (ChbGI)_2 [44] does not cause lethality.
The insulator function of the (ChβGI)2 became even stronger in the absence of the barrier proteins as illustrated by the levels of Igf2 expression. IGF2 is an embryonic and fetal mitogen [70,71] and is also important for placenta development [72]. Therefore, the difference in Igf2 expression (10% versus 0%) likely accounts for the weight difference between the +/(ChβGI)2 and the +/(mChβGI)2 fetuses (50–61% versus 44–50%) and placentas (60% versus 56%), respectively. The lethality phenotype, however, cannot be explained by lack of Igf2, because although Igf2+/−(P) and Igf2−/− mice are small (50–62%), they are viable [70,71,73]. Our data argue that biallelic strict insulation at the ICR position is the cause of lethality in +/(mChβGI)2 pups by causing misexpression of at least one gene in addition to Igf2.

Figure 5. DNA methylation of the (mChβGI)2 in 18.5 dpc fetal germ cells. Bisulfite sequencing results are shown from +/−(P) fetuses. (A) The paternally inherited (mChβGI)2 allele in female germ cells. (B) The paternally inherited (mChβGI)2 allele in male germ cells. (C) The maternally inherited ICR sequences in male germ cells are shown as controls. The percentage of methylated CpGs is indicated for each allele. The bar above indicates the position of the previously analyzed CpGs [44] with the % of methylated CpGs in this subset. Chromosomes from independent bisulfite reactions are grouped. Other details are as in Figure 4.

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Table 2. Paternal transmission results in late fetal/neonatal lethality phenotype.

| Mother      | Offspring from m(ChβGI)2 fathers |
|-------------|----------------------------------|
|             | +/+                              |
|             | +/−(P)                           |
| CF1         | 3                                |
|             | 0 (1*)                           |
| 9           | 0                                |
| 8           | 0                                |
| 4           | 0                                |
| 10          | 0 (7††)                          |
| 7           | 0                                |
| 8           | 0                                |
| FVB         | 4                                |
|             | 0                                |
| 7           | 0                                |
| 4           | 0 (3*)                           |
| 2           | 0                                |
| 4           | 0                                |
| 5           | 0                                |
| 129S1       | 3                                |
|             | 0                                |
| 4           | 0                                |
| 5           | 0 (2†)                           |
| 5           | 0                                |
| 5           | 0                                |
| Total       | 101                              |
|             | 0 (13†)                          |

Normal (+/+ outbred CF1 and inbred, 129S1 and FVB, mothers were crossed with m(ChβGI)2/+ fathers and the offspring was genotyped at weaning. The number of wild type +/+ and mutant +/−(P) heterozygous young from each litter is given per row. Numbers in parentheses †indicate dead pups of greatly reduced size, found on the day of birth.  ††doi:10.1371/journal.pone.0012630.0002
Allele-specific insulation by the ICR likely affects a number of transcripts in the imprinted domain apart from \( H19 \) and \( Igf2 \) in the fetus and \( Insulin 2 \) (\( Ins2 \)) in the placenta [74]. These transcripts, \( H19 \) microRNA (\( Mir675 \)) [75], \( Igf2 \) antisense RNAs (\( Igf2as \)) [76,77] and \( Mir483 \) within an intron of \( Igf2 \) [78] could be also misregulated by biallelic insulation. Bi-maternal misexpression of one or more of these transcripts (too much \( H19 \) or \( Mir675 \) or missing \( Ins2 \), \( Mir483 \) or \( Ins2 \) or other, yet unidentified ICR-controlled transcripts, by strict biallelic insulation must contribute to the death of +/-\( (\text{mCh}\beta\text{GI})_2 \) pups. The \( H19 \) noncoding RNA has been suggested to regulate an imprinted gene network [79].

Maternal duplication of chromosome 7 distal to the T9H translocation breakpoint (MatDup.dist7) [80,81,82] exhibits small fetus weight (about 40\%), undetectable \( Igf2 \) expression and late fetal/perinatal lethality [81,83]. In MatDup.dist7 fetuses, bi-maternal misexpression of imprinted genes occur within the influence of the ICR [81], also called imprinting control center 1 (IC1) and outside the influence of the ICR for example under the control of the KvDMR1 or imprinting control center 2 (IC2) [84,85]. Yet, none of the tested bi-maternal misexpressions causes death [81]. The lethality phenotype of MatDup.dist7 was completely rescued by maternal transmission of the mutant \( H19/\text{Igf2} \) ICR that lacks CTCF binding and, therefore, lacks insulator function [81], suggesting that correction of biallelic ICR insulation to monoallelic insulation at the IC1 is sufficient to rescue the perinatal lethality of the MatDup.dist7 genotype (Figure 6A).

The reciprocal experiment, introducing biallelic insulation at the IC1 did not, at first, cause death [44], suggesting then that biallelic insulation by IC1 and additional misexpressions in distal chromosome 7 are responsible for the MatDup.dist7 lethality. In the present study, by substituting the paternal ICR with the \((\text{mCh}\beta\text{GI})_2 \) lacking the USF and VEZF1 binding sites, a complete biallelic insulation was achieved at the IC1 and this resulted in lethality in the +/-\( (\text{mCh}\beta\text{GI})_2 \) genotype (Figure 6B). Our present experiment, therefore, is consistent with the explanation that the lethality of the MatDup.dist7 genotype is caused by misregulation of \( Igf2 \) and something else under the control of the IC1 and is not dependent on genes outside of the control of IC1. Similarly, bi-maternal insulation by the IC1 can explain the perinatal lethality of bi-maternal \( ng\Delta12/\text{fg} \) fetuses produced from a non-growing oocyte genome carrying an IG-DMR deletion in chromosome 12 and a fully grown oocyte genome [86].

The present mouse mutation will be a useful animal model for understanding the severe form of Silver-Russell syndrome (SRS) (OMIM 180860) [87]. SRS is characterized by intrauterine and postnatal growth retardation and in the majority of cases is associated with hypomethylation of the ICR. The severity of low birth weight phenotype in SRS correlates with the level of ICR hypomethylation [88] and likely correlates with insulator strength, because CTCF binding in the ICR is methylation sensitive [23,24,25,26]. In our mouse models, the decision between life and death depended on insulator strength. The barrier proteins, VEZF1 and USF can rescue lethality by reducing the insulator strength at the IC1 position by 10\%. It is not known if small fetus/placenta weight per se causes stillbirth in humans, but intrauterine growth restriction/placental insufficiency was diagnosed in 23\% of human stillbirth cases in a recent study [89]. We predict that the most severe cases of SRS—which would be expected to have a complete lack of methylation at the ICR and strict biallelic insulation—do not survive to term or die around birth.

**Figure 6. Lethality is caused by strict biallelic insulation at the IC1 in mouse chromosome 7.** (A) Maternal (pink) duplication of distal chromosome 7 (MatDup.dist7) fetuses carry biallelic insulation (STOP signal) at the imprinting control center 1 (IC1) and die. The lethality phenotype is rescued by maternal transmission of one copy of the mutant IC1 that lacks CTCF binding (x) and insulator function [81]. The imprinting control center 2 (IC2) is bi-maternal. Correction of biallelic ICR insulation to monoallelic insulation is sufficient to rescue perinatal lethality of the MatDup.dist7 genotype. (B) Our present experiments provide the reciprocal argument: introducing strict biallelic insulation to the IC1 causes lethality. By substituting the paternal chromosome’s (light blue) methylated (black lollipop) ICR with the unmethylated (\( \text{Ch}\beta\text{GI})_2 \) or the \((\text{mCh}\beta\text{GI})_2 \) genotype \([44]\). By removing the USF and VEZF1 binding sites from the (\( \text{Ch}\beta\text{GI})_2 \) genotype, biallelic insulation has become complete, causing death in the +/-\( (\text{mCh}\beta\text{GI})_2 \) genotype. doi:10.1371/journal.pone.0012630.g006

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Author Contributions

Conceived and designed the experiments: PES. Performed the experiments: DHL PS WT PES. Analyzed the data: DHL PS PES. Contributed reagents/materials/analysis tools: WT. Wrote the paper: PES.
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