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Promoter cross-talk via a shared enhancer explains paternally biased expression of *Nctc1* at the *Igf2/H19/Nctc1* imprinted locus

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ABSTRACT

Developmentally regulated transcription often depends on physical interactions between distal enhancers and their cognate promoters. Recent genomic analyses suggest that promoter–promoter interactions might play a similarly critical role in organizing the genome and establishing cell-type-specific gene expression. The *Igf2/H19* locus has been a valuable model for clarifying the role of long-range interactions between *cis*-regulatory elements. Imprinted expression of the linked, reciprocally imprinted genes is explained by parent-of-origin-specific chromosomal loop structures between the paternal *Igf2* or maternal *H19* promoters and their shared tissue-specific enhancer elements. Here, we further analyze these loop structures for their composition and their impact on expression of the linked long non-coding RNA, *Nctc1*. We show that *Nctc1* is co-regulated with *Igf2* and *H19* and physically interacts with the shared muscle enhancer. In fact, all three co-regulated genes have the potential to interact not only with the shared enhancer but also with each other via their enhancer interactions. Furthermore, developmental and genetic analyses indicate functional significance for these promoter–promoter interactions. Altogether, we present a novel mechanism to explain developmental specific imprinting of *Nctc1* and provide new information about enhancer mechanisms and about the role of chromatin domains in establishing gene expression patterns.

INTRODUCTION

Transcription of developmentally regulated or tissue-specific genes often depends on promoter activation by cell-type-specific enhancers. Although promoters and their cognate enhancers may be separated by great distances on the linear chromosome, studies on several model systems confirm that transcriptional activation is invariably associated with the formation of DNA loop structures that bring the promoter and enhancer into close physical proximity (1). Recent genomic analyses suggest that enhancers often regulate multiple promoters simultaneously and indicate that interactions between co-regulated promoters and shared enhancers organize the genome into functional domains (2).

Using genome-wide chromatin interaction analysis, Li *et al.* (3) recently established that cell-type-specific promoter–promoter interactions are also widespread in animal cells. Moreover, their data suggest that these interactions might play important regulatory roles. Specifically, they proposed that promoter–promoter interactions can act cooperatively to activate gene expression and that promoter–promoter interactions might be critical components of the chromosomal structures that underlie coordinated transcription in eukaryotic cells.

The *Igf2/H19* locus has proven to be a particularly useful model system for elucidating the molecular details and functional significance of DNA loop structures in regards to coordinate gene expression via tissue-specific enhancers (4). Expression of *Igf2* and *H19* is each dependent on shared downstream enhancers. The best-characterized enhancers are centered at +92 and +108 kb (all base pairs are given relative to the start site for *Igf2* isoform 1) and drive expression in liver and in skeletal muscle, respectively (Figure 1) (5–11). Additional tissue-specific enhancers are located even further downstream (5,8). Chromatin conformation configuration (3C) analyses of fetal liver provided early support for the importance of physical interactions of promoter and enhancer elements in gene activation (12–14).

Igf2 and *H19* are reciprocally imprinted. *H19* is expressed only when maternally inherited, whereas *Igf2* expression is paternal in origin (15). This imprinting phenotype can be of real practical advantage in transcriptional studies: in a single cell type one can directly

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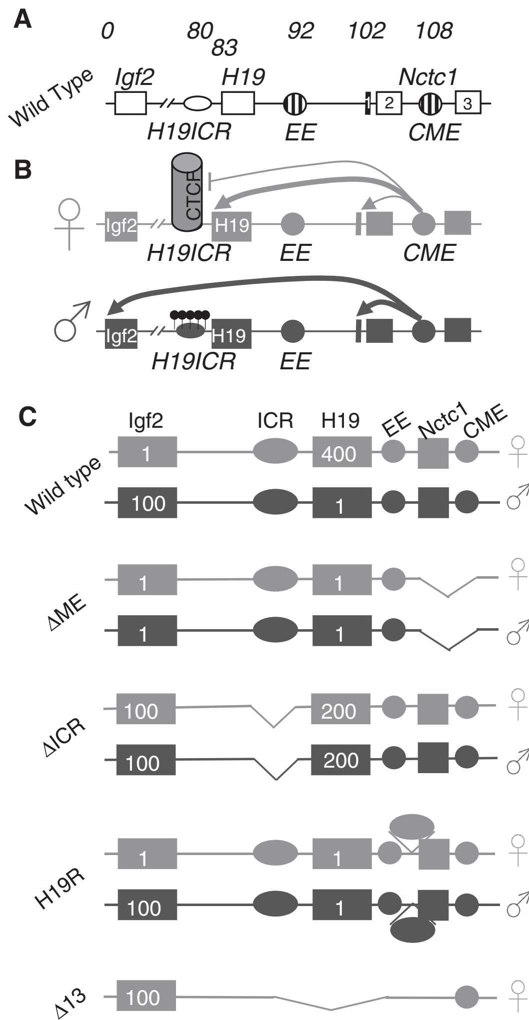

Figure 1. The *Igf2*/*H19*/*Nctc1* locus. (**A**) Organization of the wild-type locus. Open rectangles denote the *Igf2*, *H19* and *Nctc1* genes. Open oval denotes the H19ICR. Striped circles denote the endoderm (EE) and CME. Numbers above the line indicate kilobase relative to the *Igf2* isoform 1 transcriptional start site. (**B**) The insulator model for imprinted expression at the *Igf2*/*H19* locus. On the maternal (gray) chromosome, the H19ICR is not methylated and binds the transcriptional insulator, CTCF, preventing activation of the distal *Igf2* promoters. On the paternal (black) chromosome, methylation of the CpGs (black lollipops) within the H19ICR prevents CTCF binding and thus allows enhancer activation of *Igf2*. In addition, H19ICR epigenetic changes at the adjacent *H19* promoter prevent its expression (32). (**C**) Structures of the ΔME, ΔICR, H19R and Δ13 mutant alleles. Numbers inside the gene boxes indicate the approximate relative expression levels in muscle cells for *H19* and *Igf2* on these maternal (gray) and paternal (black) chromosomes as determined in the references cited above. ΔME (8) carries a deletion that removes the shared CME (41) and exons 1 and 2 of the *Nctc1* gene. ΔICR (32) carries a deletion that removes the 2.4-kb H19ICR but leaves the adjacent *H19* promoter intact. H19R (29) carries an insertion of the 2.4-kb H19ICR at +10 kb, between the endodermal (EE) and mesodermal enhancers (CME). Δ13 (33) carries a deletion that removes the entire *H19* RNA-coding region plus 10 kb of upstream sequences including the *H19* promoter and the H19ICR.

compare active and inactive alleles of the same gene and thus identify epigenetic marks and resultant DNA loop structures and chromatin domains that either promote or disfavor gene expression. In fact, analysis of the

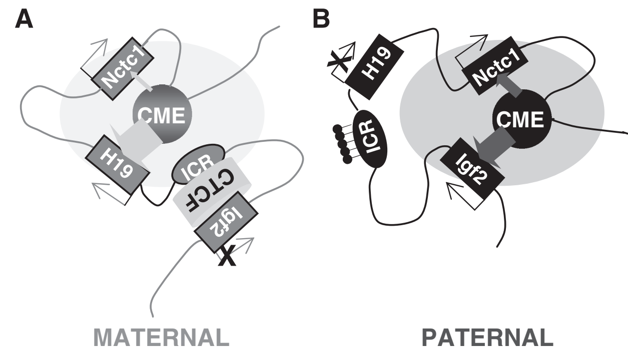

Figure 2. Parent-of-origin specific structures mediate gene expression at the *Igf2*/*H19*/*Nctc1* imprinted locus in muscle cells. (**A**) On the maternal chromosome, a CTCF-dependent insulator organizes the DNA loops between distal *cis*-regulatory elements to favor *H19* expression and to prevent interactions between the *Igf2* promoters and the shared downstream CME. Recent work from Nativio *et al.* (17) demonstrates a critical importance for cohesin in establishing these maternal-specific chromosomal structures. Also, see Zhang *et al.* (25) for detailed mechanisms describing maternal ICR–CTCF–*Igf2* interactions. (**B**) Paternal-specific methylation of CpGs within the ICR prevents CTCF binding, resulting in DNA loop structures that favor *Igf2* promoter interactions with the shared enhancer. The loss of CTCF binding also results in a spread of DNA methylation and heterochromatin from the ICR into the adjacent *H19* promoter region to *H19* transcription. Here, we propose that *Nctc1* levels are regulated by competition with *H19* and *Igf2* promoters for activation by the transcriptional complexes assembling at the shared enhancer. For simplicity, this model does not describe *Igf2* differentially methylated regions (DMRs) located near the *Igf2* promoters. DMR1 is an muscle-specific repressor of *Igf2* expression that is required for complete full postnatal repression of both maternal and paternal chromosomes (62). DMR2 is a tissue non-specific positive regulatory element (63). In muscle, deletion of DMR2 results in modest decreases in *Igf2*. The participation of these two elements in DNA looping structures has been extensively analyzed (12,13,64) but to date, only in endodermal cells.

Igf2/*H19* locus has been especially useful not only in understanding the role of alternative DNA loop structures in regulating gene expression but also in deciphering the molecular and genetic mechanisms that establish these alternative loop domains (Figure 2).

Imprinting at the *Igf2*/*H19* locus is dependent on the 2.4-kb *H19* imprinting control region (H19ICR) that lies between the two genes, just upstream of the *H19* promoter (Figure 1A). Deletion of the H19ICR results in loss of imprinting and biallelic expression of both genes (16). On the maternal chromosome, CTCF protein binds to the ICR and through cohesin (17–19) establishes a transcriptional insulator that organizes the chromosome into loops that favor *H19* expression but block interactions between the maternal *Igf2* promoters and the downstream shared enhancers, thus preventing maternal *Igf2* expression. Upon paternal inheritance, the CpGs within the ICR are methylated, which prevents binding of the CTCF protein so that a transcriptional insulator is not established. Thus, paternal *Igf2* promoters and the shared enhancers do interact via DNA loops and expression of paternal *Igf2* is facilitated. In addition, the methylated ICR drives developmentally programmed changes at the adjacent *H19* promoter to silence its expression (Figures 1B and 2) (9,12,14,20–25).

Not only is the ICR necessary for imprinting at the *Igf2/H19* locus but also the 2.4-kb element is sufficient to establish imprinting at any locus. That is, when inserted into ectopic sites in the genome, the ICR organizes adjacent chromosomal domains into parent-of-origin distinct conformations and thus establishes distinct maternal and paternal expression patterns for proximal genes (26–31).

While parent-of-origin loop structures explain expression of *Igf2* and *H19*, their impact on other genes in the locus has not been investigated. Here, we analyze the expression of the muscle-specific long non-coding RNA, *Nctc1*, that lies downstream of *H19* and is transcribed across the core muscle enhancer (CME) shared by *Igf2* and *H19* (Figure 1A). We show that *Nctc1* expression is also dependent on this CME. Concordantly, the CME physically associates with the *Nctc1* promoter just as it does with the maternal *H19* and paternal *Igf2* promoters. Further, we show that all the promoters in this transcriptional domain physically interact with each other depending on their association with the shared enhancer. Thus, interactions between an enhancer and one promoter do not preclude interactions between that enhancer and another promoter or between promoters. Finally, we provide evidence that these promoter–promoter interactions impact gene expression. Genetic and developmental data support a model wherein the need for the *Nctc1* promoter to share the enhancer and to compete for RNA polymerase complexes assembling at the CME determines *Nctc1* transcription levels and imposes a paternal bias on *Nctc1* expression in neonatal animals. In adult animals, repression of *H19* and *Igf2* eliminates the need for sharing and *Nctc1* imprinting is mitigated. Thus altogether, we provide a novel mechanism to explain developmentally specific imprinting of *Nctc1* and new insights into the role and importance of chromatin domains in regulating gene expression.

MATERIALS AND METHODS

Animal studies

Generation of H19R (29), ΔME (8), ΔICR (32) and Δ13 (33) mutations and of C/C congenic strains (34) has been described. All animal work was done according to the National Institutes of Health (NIH) Policy and was approved by the National Institute of Child Health and Human Development (NICHD) Animal Care and Use Committee.

Quantitative PCR

cDNA samples prepared with and without reverse transcriptase were analyzed using SYBR Green (Roche) on the Roche Cycler 480. Primers are described in Supplementary Table S1.

Primary myoblast culture

Tissues were isolated from p3 to p5 pups and myoblasts isolated as described (35).

Chromatin immunoprecipitation analysis

Myoblasts were isolated from primary neonatal skeletal muscle tissue and differentiated into myotubes in culture for 24 h. Chromatin immunoprecipitation (ChIP) analysis was performed as previously described (14) using anti-Ser-5(P)-RNA polymerase (RNAP) antibody from Abcam (#ab5131) and anti-rabbit IgG antibody from Santa Cruz Biotechnology (#sc2027). ChIP-purified DNA was quantified and normalized as a percentage of input controls by Real-Time quantitative Reverse Transcription PCR (qRT-PCR). Primers are listed in Supplementary Table S1.

Chromatin conformation capture

3C analysis was performed as described (14) using primers and restriction fragment length polymorphisms (RFLPs) described in Supplementary Table S1 and Supplementary Figure S2. Primer efficiencies were tested using bacterial artificial chromosome DNAs as described (36), using clones 198J15 and 11 301 (34) that together cover the entire locus from upstream of *Ins2* to 100 kb downstream of *Nctc1* (Supplementary Figure S2).

Allele-specific assays

Allele-specific expression of *Nctc1* heterogeneous nuclear RNA (hnRNA) was analyzed by melting analysis (37) using a BanI RFLP and PCR products generated with primers described in Supplementary Table S1. DNA melting was performed using a Roche LightCycler 480. The quantitative nature of the assay is demonstrated in Supplementary Figure S4.

DNA methylation analysis

DNA sequences were screened for CpG islands using programs from the European Bioinformatics Institute (http://www.ebi.ac.uk/Tools/emboss/cpgplot/) and the following parameters: window = 100, step = 1, minimum observed to expected ration of C + G to CpG = 0.6, minimum percentage of CpG in a set of 10 windows = 40%, minimum length = 100.

DNA methylation was determined by bisulfite sequencing as described (38,39). Briefly, two independent DNA samples were prepared from primary myocytes isolated from D/C neonates and subjected to cytosine conversion. Two independent nested PCRs were performed (40) on each DNA sample. See Supplementary Table S1 for primer sequences. PCR products were cloned and individual clones sequenced.

RESULTS

Co-regulation of *Nctc1* with *Igf2* and *H19* by a shared muscle-specific enhancer

Nctc1 is a non-coding RNA whose transcript is located within a 12-kb chromosomal region defined by targeted mouse deletions and transgenic studies as the enhancer that is necessary and sufficient for expression of *Igf2* and *H19* in skeletal muscle (Figure 1) (7–9,41,42). Two *Nctc1* isoforms have been identified: a minor isoform includes

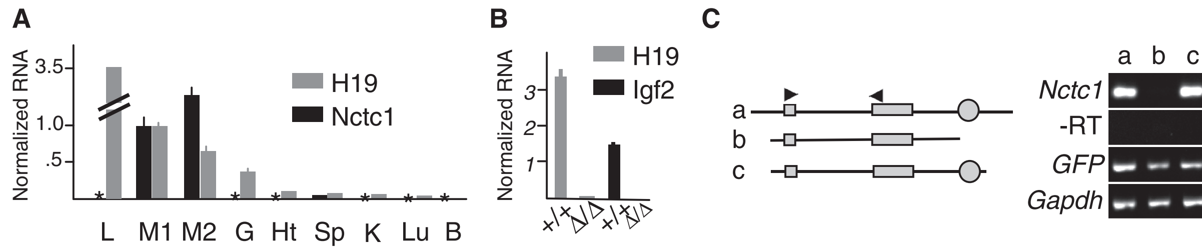

Figure 3. Co-regulation of *Igf2*, *H19* and *Nctc1* in muscle cells. (**A**) Tissue-specific expression of the *Nctc1* gene. RNAs isolated from wild-type neonates were analyzed by qRT-PCR for *Nctc1* and *H19*. Expression in hind limb muscle (M1) was set to 1. Other tissues analyzed were liver (L), tongue (M2), gut (G), heart (Ht), spleen (Sp), kidney (K), lung (Lu) and Brain (B). Asterisk denotes no detectable *Nctc1*. (**B**) The ΔME deletion affects *Igf2* and *H19* expression only in tissues where *Nctc1* is expressed. RNAs from +/+ and from ΔME/ΔME primary myocytes were analyzed by qRT-PCR for expression of *H19* and *Igf2* normalized to *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. Mutant cells display >1000-fold decreases. No effect of the deletion was seen in liver, gut, heart, kidney, lung and brain. (**C**) Expression of *Nctc1* depends on the CME. ΔME/ΔME primary myoblasts were transfected with DNA constructs a, b, or c and a plasmid carrying GFP. After 24-h growth in differentiation media, RNAs were prepared and analyzed by qRT-PCR for *Nctc1, GAPDH* and *GFP*. On construct maps, *Nctc1* exons 1 and 2 are depicted as filled rectangles and the CME is shown as a filled circle.

exons 1, 2 and 3, while the major splice variant includes only exons 1 and 2.

We performed an analysis of *Nctc1* expression using qRT-PCR of RNAs isolated from wild-type neonatal animals using primers that would detect both *Nctc1* isoforms. As already reported for adult tissues (43), *Nctc1* expression in neonates is highly restricted with significant mRNA levels only in skeletal muscle (Figure 3A). These are also the cell types where we see an essential role for the mesoderm enhancer region (as defined by the ΔME chromosomal deletion) in activating *H19* and *Igf2 in vivo* (Figure 3B). Thus, there is a strict correlation between demonstrated activity of the shared mesoderm enhancer *in vivo* and expression of *Nctc1*.

To more precisely map the *Igf2*/*H19* CME, Alzhanov *et al.* (41) used transient transfection analyses and identified a 0.3-kb element (the CME in Figure 1) as necessary and sufficient for enhancer activity in cultured muscle cells. In Figure 3C, we show that this element is also essential for *Nctc1* expression in primary myocytes. Thus altogether, these results indicate that *Nctc1* is part of the *Igf2*/*H19* regulatory complex in that expression of each gene in muscle is dependent on a shared enhancer element, the CME.

Note that, while *Nctc1*, *H19* and *Igf2* transcription are each dependent on the CME, their RNA levels are very different. Altogether, our expression and stability data suggest that the *H19* promoter is somewhat more active (3–4×) than the *Igf2* promoters and significantly more active (∼1000×) than *Nctc1* (Supplementary Figure S1).

Distinct ternary DNA looping structures on maternal and paternal chromosomes mediate imprinting of *Nctc1*

We analyzed chromatin preparations from wild-type primary myocyte cultures using 3C (44) to determine the DNA looping structures associated with the CME. In each experiment, we used reciprocal crosses and chromosomes marked with single-nucleotide polymorphisms so that we could distinguish paternal and maternal chromosomes and therefore follow the effect of parental origin on loop formation. Thus, we can directly compare domain structures for active and inactive alleles. As expected, activation of *Igf2* and *H19* promoters by the CME is invariably associated with physical interactions between the promoters and this enhancer (Figure 4A). Consistent with their imprinted expression, *Igf2* and *H19* promoter–enhancer interactions are restricted to the paternal and maternal chromosomes, respectively. For example, the left panel in Figure 4A analyzes loop formation between the CME and the *Igf2* promoter. In C/D cells (maternal C/paternal D alleles), the amplicon is only D in origin. In D/C cells (maternal D/paternal C alleles), the amplicon is only C in origin. (D and C are each wild-type alleles distinguishable by RFLP analysis as described in Supplementary Figure S2.) Together, these experiments reveal that a DNA loop is formed across the 100 kb between the *Igf2* promoter and the CME but only on the paternal chromosome. Similarly, the *H19* promoter–CME interactions are almost all maternal in origin (Figure 4A, center panel). In contrast, *Nctc1* promoter and enhancer interactions occur on both chromosomes (Figure 4A, right panel).

While the *Nctc1* promoter and CME are only 5.6 kb apart, their physical interaction is specific: 3C analyses do not detect association between the enhancer and comparably distant downstream sites (Supplementary Figure S3).

To gain a clearer understanding of the composition of these DNA loop structures, we next looked for interactions between the three promoter regions. We detected no interactions between the *Igf2* and *H19* promoters (Figure 4B). However, we did note interactions between *Nctc1* and *Igf2* promoters (but only on the paternal chromosome) and between *Nctc1* and *H19* promoters (but only on the maternal chromosome) (Figure 4C). These results are consistent with the presence of two distinct ternary structures containing the CME plus each of the promoters that it activates in *cis*: CME + *H19* promoter + *Nctc1* promoter on the maternal chromosome or CME + *Igf2* promoter + *Nctc1* promoter on the paternal chromosome (Figure 4D). Thus, *Nctc1* transcription is dependent on parent-of-origin distinct chromosomal structures.

To determine if there were consequences to the *Nctc1* promoter's parent-of-origin-specific configurations, we developed assays to quantitate allele-specific expression of

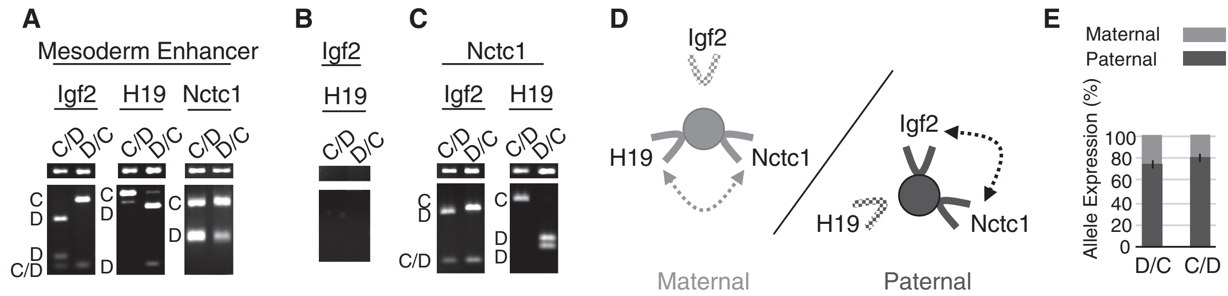

Figure 4. Long-range interactions between the *Igf2*, *H19* and *Nctc1* promoters and the shared muscle enhancer and *Nctc1* imprinting on wild-type maternal and paternal chromosomes. (**A–C**) Chromatin was prepared from wild-type primary myocyte cultures and analyzed for long-range DNA interactions by 3C. (A) Interactions between the mesoderm enhancer and the *Igf2*, the *H19* or the *Nctc1* promoter regions. (B) Interactions between the *Igf2* and *H19* promoters. (C) Interactions between the *Nctc1* promoter and the *Igf2* or *H19* promoter regions. In each case, the narrow top gel displays the PCR amplicon indicative of the DNA loop formation. The bottom gel displays these amplicons after restriction enzyme digestion to reveal the allelic origin(s) of the products as marked along the left margin: C, castaneus; D, domesticus and C/D, castaneus and domesticus. In describing genotypes, we use the convention: maternal allele/paternal allele. Primers and the amplicon sizes before and after digestion are described in Supplementary Table S1 and Supplementary Figure S2. For each analysis at least three independent chromatin preparations were assayed. (**D**) Summary of long-range interactions on maternal (left, gray) or paternal (right, black) chromosomes. On both chromosomes, transcriptionally active promoters (solid lines) interact with the CME (filled circle) and with each other while silenced promoters (stippled lines) do not interact. (**E**) Parent-of-origin specific expression of *Nctc1*. To quantitate the effects of parental origin, allele-specific expression was quantitated for RNAs isolated from D/C and from C/D neonates. Error bars show standard deviations.

Nctc1 (Supplementary Figure S4). In fact, the paternal chromosome accounts for 80% of *Nctc1* RNA transcripts (Figure 4E). Thus, maternal and paternal loop domains are not equivalently active in regard to *Nctc1* transcription. Rather, much more *Nctc1* RNA transcription is coming from CME–*Igf2*–*Nctc1* (paternal) complexes than from CME–*H19*–*Nctc1* (maternal) complexes.

We could not detect any parent-of-origin differences in DNA methylation near the *Nctc1* gene that might account for its imprinting (Supplementary Figure S5). However, *Nctc1* imprinting is dependent on the distant H19ICR (Figure 5A). Surprisingly, either maternal ($n = 4$, $P = 0.004$) or paternal ICR ($n = 4$, $P = 0.004$) deletions each result in loss of imprinting at *Nctc1*. In contrast, the ICR is a chromosome-specific silencer for *Igf2* and for *H19* (9,16).

Chromosome-specific 3C analyses of ΔICR chromosomes confirm that the shared enhancer is capable of interacting with multiple promoters in a way that brings all active promoters within its domain into contact with each other (Figure 5B and D). Thus, the maternal and the paternal ΔICR chromosomes each generate a tertiary looping structure that includes the CME interacting with the *Igf2*, *H19* and *Nctc1* promoters while these promoters are also interacting with each other (Figure 5E). In sum on wild-type and on ΔICR chromosomes, co-expressed promoters interact not only with the shared enhancer but also with each other.

Nctc1 expression inversely correlates with *H19*/*Igf2* promoter activity

It is not straightforward to understand how both maternal and paternal deletion of the H19ICR could each have the same effect on *Nctc1* allelic bias. Given that the *Nctc1* promoter is organized into loop structures that also contain the *Igf2* and *H19* promoters, we considered the possibility that ICR regulation of *Nctc1* imprinting was indirect and secondary to its effects on *H19* and *Igf2* transcriptional activity (see Figure 1C for summary of ICR deletion phenotypes). To first test this hypothesis, we took advantage of the fact that *H19* and *Igf2* are each strongly down-regulated during postnatal development (>30-fold for *H19*, $P < 0.001$ and >200-fold for *Igf2*, $P < 0.001$) (Figure 6A). In contrast, *Nctc1* expression increased 2.5-fold ($P < 0.001$) (Figure 6A) with a significant loss in imprinting (neonate = 79 ± 3% paternal, adult = 60 ± 5% paternal, $P < 0.0001$) or a switch in bias from 4:1 to 1.5:1 (Figure 6B). Thus, decreased *Igf2* + *H19* activation is associated with increased *Nctc1* transcription as well as with *Nctc1* loss of imprinting.

Next, we took a genetic approach and analyzed *Nctc1* expression from the mutant chromosome, H19R, that carries an insertion of the 2.4-kb H19ICR at the +10 kb position (Figure 1C). Maternal inheritance of the ectopic ICR insulates the *H19* promoter from the CME and blocks maternal *H19* expression in muscle (Figure 7A) (14). Thus, the H19R mutation allows us to examine *Nctc1* expression specifically in the absence of *H19* transcription in *cis*. Maternal inheritance of H19R increases total *Nctc1* RNA (Figure 7A) and moreover, abrogates normal *Nctc1* imprinting (Figure 7B, left panel) (+/+ = 26 ± 3% maternal, H19R/+ = 78 ± 4% maternal, $P = < 0.001$). Thus, in the absence of *H19* transcription, expression of *Nctc1* from the maternal chromosome is up-regulated to the point that imprinting is reversed. 3C analyses demonstrate that maternal H19R chromosomes show a binary enhancer looping structure that includes only the CME and the *Nctc1* promoter and not the *H19* promoter (Figure 7C, D and E). That is, the H19R insertion prevents interactions of not only the *H19* promoter and the CME but also of the *H19* and *Nctc1* promoters, consistent with the idea that the promoters interact via the shared enhancer.

Upon paternal inheritance of H19R, the ectopic ICR is methylated so that CTCF does not bind and insulator activity is not established (14). Thus, *H19* and *Igf2* transcription is unaffected. Accordingly, paternal inheritance of H19R has no effect on *Nctc1* imprinting

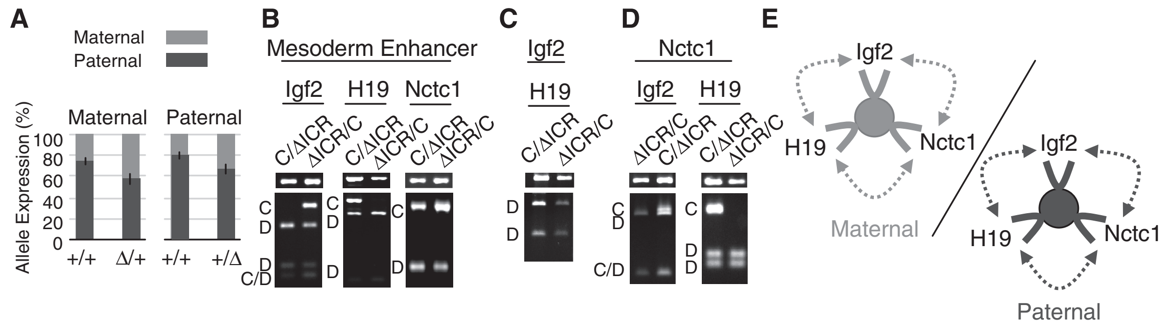

Figure 5. *Nctc1* imprinting and chromatin loop structures are altered on ΔICR chromosomes. (**A**) Parent-of-origin specific expression of *Nctc1*. To quantitate the effects of maternal inheritance, allele-specific expression was measured for RNAs isolated from D/C and of ΔICR/C neonates. To quantitate the effects of paternal inheritance, C/D and C/ΔICR pups were compared. (**B–D**) Chromatin was prepared from primary myocytes isolated from mice carrying a paternal (C/ΔICR) or a maternal deletion (ΔICR/C) of the H19ICR and analyzed for long-range DNA interactions by 3C. (B) Interactions between the mesoderm enhancer and the *Igf2*, the *H19* or the *Nctc1* promoter regions. (C) Interactions between the *Igf2* and *H19* promoters. (D) Interactions between the *Nctc1* promoter and the *Igf2* or *H19* promoter regions. (**E**) Summary of long-range interactions on maternal (left, gray) or paternal (right, black) ΔICR chromosomes. On both chromosomes, *Igf2*, *H19* and *Nctc1* promoters are all active and interact with the CME (filled circle) and with each other. See Figure 4 for additional details.

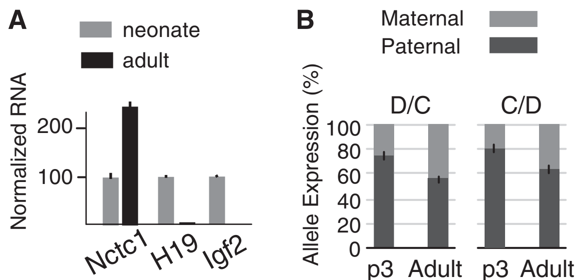

Figure 6. Developmental regulation of *Nctc1*, *H19* and *Igf2* gene expression and of *Nctc1* imprinting. (**A**) RNAs were prepared from hind limbs of neonatal (p3) and of adult (12 weeks) animals and analyzed by qRT-PCR for *Nctc1*, *H19* and *Igf2*. The relative expression for each gene in neonates was set to 100. (**B**) To ascertain the effect of development on *Nctc1* imprinting, allelic frequencies were measured in RNAs isolated from D/C and C/D wild-type animals as described in Figure 4. Error bars show standard deviations.

(+/+ = 80 ± 4% paternal, +/H19R = 82 ± 5% paternal, $P = 0.46$) (Figure 7B, center panel) or on chromosome loop domain structures (data not shown and Figure 7E).

Analysis of the H19R chromosomes indicates that *Nctc1* expression and imprinting is a downstream consequence of *H19* transcriptional levels. We suggest that this is related to the fact that on a wild-type maternal chromosome, the promoters concurrently interact with and share the CME enhancer and are in some sense competing for transcriptional activation. Alleviating this competition by mutation or normal development then results in increased maternal *Nctc1*. However, the maternal H19R phenotype is also consistent with transcriptional interference with *Nctc1* by the *H19* RNA. To distinguish these two mechanisms, we analyzed RNAs isolated from Δ13/+ neonatal muscle. Δ13 is a deletion that removes the entire *H19* gene and also deletes the ICR (Figure 1C) (33). Thus, Δ13 is like H19R in that maternally inherited chromosomes will not express any H19. Therefore, if repression of maternal *Nctc1* is via RNA interference, then Δ13/+ muscle should present the H19R/+ phenotype, i.e. an inversion of normal *Nctc1* imprinting and switch to maternal bias. However, unlike H19R, maternally inherited Δ13 chromosomes express maternal *Igf2* promoters at levels similar to those seen on wild-type paternal chromosomes (Figure 1C). If repression of maternal *Nctc1* is via promoter competition, then Δ13/+ cells should show no imprinting since maternal Δ13 and paternal wild-type chromosomes are equivalent in terms of activities of the upstream *H19* and *Igf2* promoters. What we actually see is the simple loss of imprinting in Δ13/+ animals (Δ13/+ = 46 ± 5% paternal, $n = 4$, $P < 0.01$) (Figure 7B, right panel), predicted by promoter competition.

RNAP II association with *Nctc1* inversely correlates with *H19* transcriptional activation

We wanted to identify molecular correlates that might explain *Nctc1* imprinting and to test if promoter competition occurs at the level of assembly of transcriptional machinery at the *Nctc1* promoters. Therefore, we used ChIP to compare binding of total RNAP II and activated RNAP II [Ser-5(P)-RNAP] to the *Nctc1* promoter in wild-type (+/+) and in H19R/+ myocytes (Figure 8A). Consistent with increased *Nctc1* RNA levels in H19R/+ myocytes, we noted a 3-fold enrichment in RNAP at the promoter in these cells. Moreover, this increase is entirely due to increased assembly on the maternal chromosome (Figure 8B).

We also saw a significant binding of RNAP at the CME that further increases in H19R/+ compared with +/+ cells (Figure 8A). Thus, in H19R/+ cells, the absence of interaction with the *H19* promoter and the consequent loss in *H19* transcription results in an accumulation of RNAP complexes at the CME. As described in the 'Discussion' section, these results are consistent with the idea that enhancers serve as primary recruitment/assembly docks for RNAP that is then dispersed to promoters via complexes we characterize as DNA loops. We suggest that on H19R chromosomes, the removal of the H19 as a competitive gene makes the RNAP complexes more available for *Nctc1* activation.

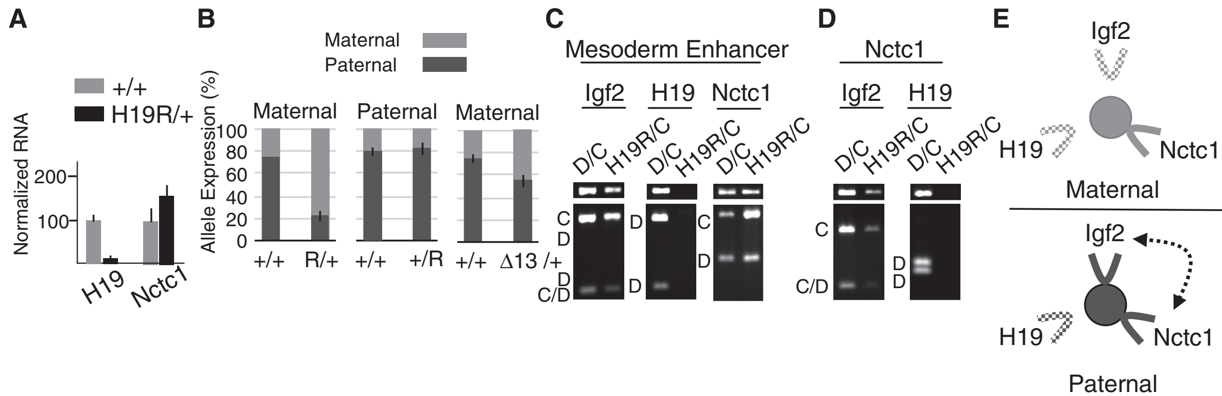

Figure 7. *Nctc1* expression, imprinting and chromatin loop structures are altered on maternal H19R chromosomes. (**A**) Transcription of *H19* and *Nctc1* in H19R/+ muscle cells was quantitated by qRT-PCR and normalized to GAPDH RNA levels. Expression of each gene in wild-type (+/+) cells is set at 100. (**B**) Parent-of-origin specific expression of *Nctc1* on H19R and on Δ13 chromosomes. To quantitate the effects of maternal inheritance of H19R, allele-specific expression was measured for RNAs isolated from D/C and of H19R/C neonates. To quantitate the effects of paternal inheritance of H19R, C/D and C/ΔICR pups were compared. To quantitate the effects of maternal inheritance of Δ13, allele-specific expression was measured for RNAs isolated from D/C and of Δ13/C neonates. (**C–D**) Chromatin was prepared from primary myocytes isolated from wild-type mice (D/C) and from mice carrying a maternally inherited copy of the H19R insertion mutation (H19R/C) and analyzed by 3C. (C) Interactions between the mesoderm enhancer and the *Igf2*, the *H19* or the *Nctc1* promoter regions. (D) Interactions between the *Nctc1* promoter and the *Igf2* or *H19* promoter regions. (**E**) Summary of long-range interactions on maternal (top, gray) or paternal (bottom, black) H19R chromosomes. Maternal inheritance of the ICR insertion prevents *H19* promoter interactions with the CME (filled circle) and with the *Nctc1* promoter. See Figure 4 for additional details.

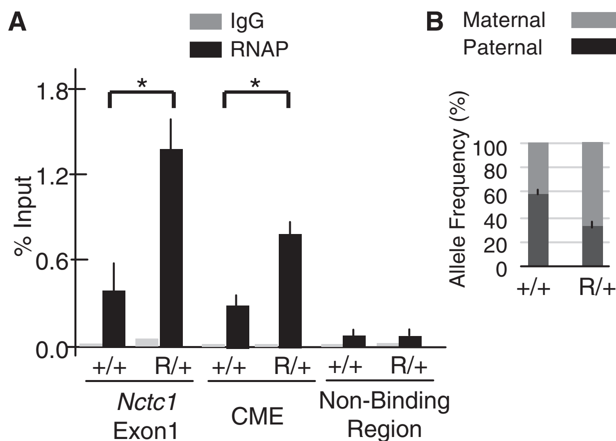

Figure 8. Allele-specific Ser-5(P)-RNAP II binding at the *Nctc1* gene in wild-type versus H19R/+ mice. Primary myoblasts were isolated from wild-type and H19R/+ mice and differentiated in culture for 24 h. (**A**) Total Ser-5(P)-RNAP association at the *Nctc1* gene. ChIP analysis was conducted to measure binding at both *Nctc1* exon1 and the CME in +/+ and in H19R/+ mutant cells. An intergenic region between *H19* and *Igf2* was assayed as a control for non-specific binding. (**B**) Allele-specific frequency of Ser-5(P)-RNAP binding at the *Nctc1* gene. ChIP samples for Nctc1 exon 1 from panel A were further analyzed for allele frequency content using DNA melting analysis. Error bars represent standard errors. $P < 0.05$, $n = 3$.

DISCUSSION

Strong correlative evidence supports the idea that promoter activation by distal enhancers is invariably associated with DNA loop formation between those elements (1,45). Genome-wide analyses indicate that promoter–enhancer interactions are a fundamental aspect of chromosomal organization (2). Independent genomic studies have also recently indicated that promoter–promoter interactions are common and may play a critical role in coordinating gene expression and organizing active transcriptional domains (3,46).

Here, we use a model system approach to assay and analyze the functional role of promoter–enhancer and promoter–promoter interactions in organizing the *Igf2/H19/Nctc1* transcriptional domain. We provide support for the idea that interactions between an enhancer and one promoter do not preclude interactions between that enhancer and other promoters. Instead, as suggested by transcriptional factory models (47), co-regulated promoters are brought into physical proximity to each other via their shared enhancer. We also provide genetic and developmental analyses indicating that these promoter–promoter interactions can have important consequences on gene expression levels. That is, one promoter can regulate another.

Specifically, we characterized expression of the long non-coding RNA, *Nctc1,* which overlaps the muscle-specific enhancer shared by the *Igf2* and *H19* genes and show that *Nctc1* is imprinted in a developmentally regulated fashion. In neonates, expression is biased toward the paternal chromosome, while adult animals show nearly equal levels of paternal and maternal allele activities. Expression of *Igf2*, *H19* and *Nctc1* in muscle is dependent on a shared muscle core enhancer. Genetic and developmental analyses suggest that *Nctc1* imprinting is a side effect of the enhancer sharing. Our data indicate that physical interactions between the *Nctc1*, *Igf2* and *H19* promoters and the CME are not mutually exclusive but that co-regulated promoters can physically interact with each other via their interaction with the shared enhancer. These interactions offer a mechanism for promoters to communicate with and regulate one another. The *Nctc1* allelic bias we see on wild-type and mutant chromosomes can be explained by *Nctc1* promoter competition, especially with *H19*, for the shared enhancer. Thus, loss of

imprinting in adult animals is not because of changes in the imprinting-dependent epigenome, but instead is a side effect of the developmentally programmed decrease in *H19* and *Igf2* gene expression.

To understand the molecular meaning of promoter–promoter interactions, we quantitated the recruitment and activation of RNAP to the *Nctc1* promoter in +/+ cells and H19R/+ cells, where the Nctc1 promoter has sole access to the shared enhancer. ChIP experiments identified an increase in RNAP binding, demonstrating that competition directly affects recruitment and/or assembly of the active basal transcription complex. We were particularly interested to note that polymerase also accumulated at the CME in H19R/+ animals. As suggested in previous reports, it is plausible that the enhancer serves as a recruiting platform or loading dock for RNAP that it transfers to target promoters for transcription initiation (48,49). Precedent for this notion also exists in studies of the β-globin locus, where it is known that multiple enhancers recruit RNAP independent of the globin promoter (50). Later studies indicated that the enhancers transfer RNAP from the enhancers to the globin promoter via loop formation in a manner that can be blocked by a CTCF-bound insulator (51). By this model, the paternal bias at *Nctc1* is mediated by the high efficiency with which the *H19* promoter downloads RNAP at the cost of activation of the maternal *Nctc1* promoter.

There are already several model systems indicating that one promoter can regulate another. The *INS2* promoter (located just upstream of *IGF2*) physically associates with and positively regulates the *SYT8* gene in pancreatic β-cells (52). Negative regulation or promoter competition has been perhaps most extensively studied in regard to vertebrate β-globin transcription, where evidence suggests that competition plays a role in developmental changes in the choice of promoter gene activation (53). Does this competition occur only at the level of loop formation? That is, is the interaction between an enhancer and a promoter exclusive so that it precludes that enhancer's interaction with (and therefore activation of) a second gene? Our report provides indirect but strong evidence that this is not necessarily the case but that at least part of the competition occurs at some step after loop formation. Previous studies already provided evidence that the H19ICR can interact simultaneously with multiple *cis*-elements (54). The DNA loops that we identify and the relative levels of each of these structures are most consistent with the idea that ternary structures or quaternary structures also form around the enhancer. Altogether, analysis of this locus shows that DNA loops are a necessary precondition for gene expression but the rate of loop formation is not sufficient to explain all aspects of gene expression levels. The mechanisms by which long-range interactions activate promoter activity remain largely enigmatic (1,55). This study suggests that rate-limiting steps sometimes occur after loop formation and presents a mechanism for gene regulation likely to affect many co-regulated genes.

The biological function of the *Nctc1* long non-coding RNA was not directly addressed in this study. In mammalian species, only *Nctc1* promoter and not *Nctc1* exonic sequences are conserved (7), suggesting that it is the transcription of the *Nctc1* RNA but not the RNA product itself that is of primary evolutionary significance. In this regard, several recent genome-wide analyses have demonstrated a frequent overlap of long non-coding RNAs and tissue-specific transcriptional enhancers and several authors have hypothesized a functional importance for this association (56–60). In fact, our preliminary data suggest a crucial role for *Nctc1* promoter activity in regulating enhancer function and *Igf2*/*H19* transcription.

In sum, as described in Figure 2, the ICR regulates imprinting at the *Igf2*/*H19* locus by at least two mechanisms. For *Igf2* and *H19*, the ICR generates monoallelic expression directly by preventing physical contact between the shared enhancer and the *Igf2* and *H19* promoters on one of the two parental chromosomes (12–14,61). For *Nctc1*, the ICR acts indirectly by modulating *H19* and *Igf2* promoters, which in turn compete with *Nctc1* for enhancer activation after DNA loop formation.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Table 1 and Supplementary Figures 1–5.

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