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Report

Global Survey of Genomic Imprinting by Transcriptome Sequencing

Tomas Babak,[1,5] Brian DeVeale,[2,3,5] Christopher Armour,[1] Christopher Raymond,[1] Michele A. Cleary,[1] Derek van der Kooy,[2,3] Jason M. Johnson,[1] and Lee P. Lim[1,4,*]

[1]Rosetta Inpharmatics, LLC, a wholly owned subsidiary of Merck & Co.
401 Terry Avenue North
Seattle, WA 98109
USA

[2]Department of Molecular Genetics
1 King's College Circle
Toronto, ON M5S 1A8
Canada

[3]Terrence Donnelly Centre for Cellular and Biomolecular Research
160 College Street
Toronto, ON M5S 3E1
Canada

Summary

Genomic imprinting restricts gene expression to a paternal or maternal allele. To date, ~90 imprinted transcripts have been identified in mouse, of which the majority were detected after intense interrogation of clusters of imprinted genes identified by phenotype-driven assays in mice with uniparental disomies [1]. Here we use selective priming and parallel sequencing to measure allelic bias in whole transcriptomes. By distinguishing parent-of-origin bias from strain-specific bias in embryos derived from a reciprocal cross of mice, we constructed a genome-wide map of imprinted transcription. This map was able to objectively locate over 80% of known imprinted loci and allowed the detection and confirmation of six novel imprinted genes. Even in the intensely studied embryonic day 9.5 developmental stage that we analyzed, more than half of all imprinted single-nucleotide polymorphisms did not overlap previously discovered imprinted transcripts; a large fraction of these represent novel noncoding RNAs within known imprinted loci. For example, a previously unnoticed, maternally expressed antisense transcript was mapped within the Grb10 locus. This study demonstrates the feasibility of using transcriptome sequencing for mapping of imprinted gene expression in physiologically normal animals. Such an approach will allow researchers to study imprinting without restricting themselves to individual loci or specific transcripts.

Results

Lack of an effective high-throughput screening approach hinders thorough characterization of genomic imprinting. Several methods have been used to characterize imprinting on a global scale, including analysis of differential transcription between parthenogenotes and androgenotes [1–3], computational screens [4, 5], single-nucleotide polymorphism (SNP) microarrays [6, 7], and sequencing and genotyping of DNA associated with histone modifications [8, 9], but no approach yet reported has demonstrated sufficient sensitivity and specificity to comprehensively map imprinting in a practical manner.

We sequenced whole transcriptomes from eight embryos derived from a CAST/EiJ × C57Bl/6J reciprocal cross, four independently sequenced embryonic day 9.5 (E9.5) embryos from each cross. E9.5 is a widely interrogated stage of development and therefore an ideal benchmark for imprinting studies; the divergent *castaneus* strain was selected to maximize SNPs that could be used for inference of allelic expression. Strand-specific cDNA was generated from total RNA with primers devoid of ribosomal complementarity (unpublished data, C.A., T.B., J.M.J., and C.R.). We conducted high-throughput sequencing of this cDNA to obtain over 78 million reads from which SNPs were identified by combining data from both crosses and identifying mismatches to the C57Bl genome (NCBI build 36). To distinguish de novo SNPs from sequencing errors, we used Solexa quality scores, calibrating our approach to the set of *castaneus* SNPs previously identified by Perlegen [10] (see Supplemental Experimental Procedures, available online). At a threshold corresponding to a false-positive rate of 3.8% and sensitivity of 64% (Figure S1A), we detected 160,078 expressed SNPs in the genome, 75% of which had not been previously discovered (Figure S1B). We selected this stringent threshold for SNP calling to reduce the false-positive rate in subsequent allelic-bias calls.

The observed SNPs were then used to detect allelic bias characteristic of imprinting (Figure 1), with the binomial distribution used to calculate the probability of observed allelic counts varying from expected proportions. If the reciprocal crosses agreed with each other on parent-of-origin bias, we computed a conservative "imprinting score" (IS) by using the least significant binomial p value (see Supplemental Experimental Procedures).

To generate a genome-wide map of imprinting, we scanned the genome for clusters of imprinted SNPs, by using a 10 kb sliding window in which allele counts were grouped together to take advantage of proximally imprinted SNPs. The sliding-window method readily detects maternal-specific expression of the mitochondrion and paternal-specific expression of the Y chromosome, as expected (Figure 2). Furthermore, we correctly located 14 of 17 known imprinted loci [1, 11, 12] with $|IS| > 3$ ($p < 0.001$). On the other hand, new loci were not apparent by this method, suggesting that most of the major imprinted loci that are expressed throughout the E9.5 embryo are already accounted for.

To further improve sensitivity and to capitalize on the assay's ability to detect strand specificity, we next used transcript annotations to combine SNPs and assess the likelihood of allelic expression. As before, the combining of allelic counts reduced variance, allowing a more accurate assessment of allelic expression (Figure S8). The p values of transcripts for each cross are plotted in Figures 3A and 3B. Points along the positively sloping diagonal correspond to transcripts

*Correspondence: lee_lim@merck.com
[4]Present address: Sirna Therapeutics, a wholly owned subsidiary of Merck & Co., 1700 Owens Street, San Francisco, CA 94158, USA
[5]These authors contributed equally to this work

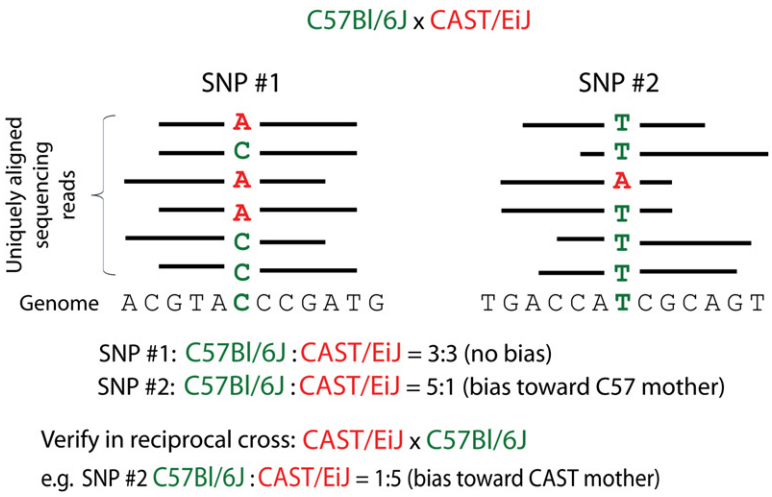

Figure 1. Measuring Allelic Expression by Counting SNPs

Schematic of approach used to detect imprinting. C57Bl/6J polymorphisms are colored in green; CAST/EiJ polymorphisms are in red. Reads were aligned to the genome and SNPs were filtered from sequencing errors by setting Phred score thresholds, calibrated to a set of previously discovered SNPs [10]. Allelic expression bias was measured by counting allele-specific reads that contain SNPs, and parent-of-origin bias was confirmed by the reciprocal cross.

with strain-biased expression (upper right being CAST/EiJ-specific) and comprise 4477 genes ($p < 0.01$) that exhibit allele-biased expression, underscoring the necessity of carrying out a reciprocal cross to distinguish imprinted effects from strain-specific effects. Parent-of-origin-biased transcripts fall on the negatively sloping diagonal and, as expected, are prominently enriched for known imprinted transcripts. Because confidence scores reflect the likelihood that a particular transcript is biased and not necessarily the magnitude of that bias, it is helpful to show the magnitudes of imprinting we observed: Figure 3C shows measurements of parental sex-specific bias made by summing parental alleles for 27 previously known imprinted transcripts as well as 12 transcripts not previously known to be imprinted.

To help visualize transcription and imprinting patterns revealed by the high-throughput sequencing, we incorporated SNPs, read mappings, and allelic-bias data into University of California, Santa Cruz (UCSC) Genome Browser tracks (Document S2). Additional experimental methods were used to confirm biased allelic expression of 20 individual SNPs selected to represent new aspects of the imprinted transcriptome, mainly: (1) extensions of previously known imprinted transcripts, (2) novel imprinted transcripts associated with previously known imprinted loci, and (3) previously unknown imprinted genes (Table 1). We Sanger-resequenced all of these SNPs, and, in cases where common restriction-enzyme sites were available, applied classical cDNA-RFLP [13].

The results of these validation studies show that characterization of known imprinted loci is often incomplete. A striking example of this occurs in the Grb10 locus, where extensive maternally biased transcription occurs immediately upstream and antisense to Grb10, suggesting that an imprinted noncoding transcript originates from the same promoter as the maternally expressed Grb10, but on the opposite strand (Figure 4). A noncoding transcript has not been associated with the Grb10 locus previously.

In the Dlk1 locus, we observed 81 maternally expressed SNPs within a 215 kb region that appears likely to be continuously transcribed from one strand. However, only 40 (49%) of these SNPs overlap any previous transcriptional evidence (mRNAs, expressed-sequence tags, predicted coding genes), demonstrating extensive novel transcription in this locus. This transcription is consistent with extension of a Rian/Meg3 precursor that is processed into the imprinted miRNAs and snoRNAs that are present in this region and that are suggested

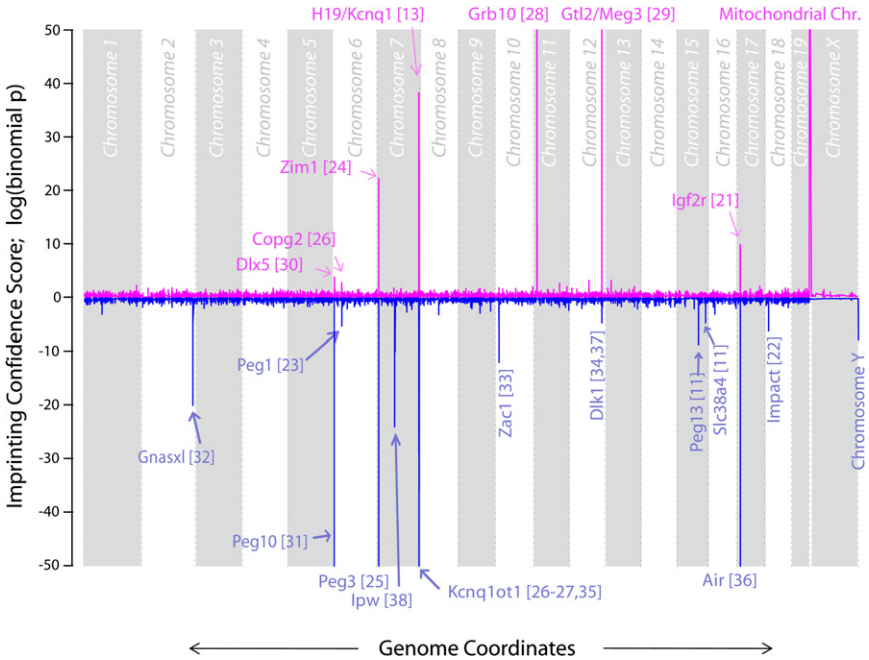

Figure 2. Genome-wide Identification of Imprinted Regions

Imprinting scores were computed for allele counts grouped in 10 kb windows every 2 kb across the genome. Scores were arbitrarily set to negative for paternal bias. Regions are referenced [11, 13, 21–38] by first reported imprinted transcript, or by common literature use. Imprinting on the X chromosome was assessed with data from two reciprocally crossed female embryos to avoid maternal bias from male embryos.

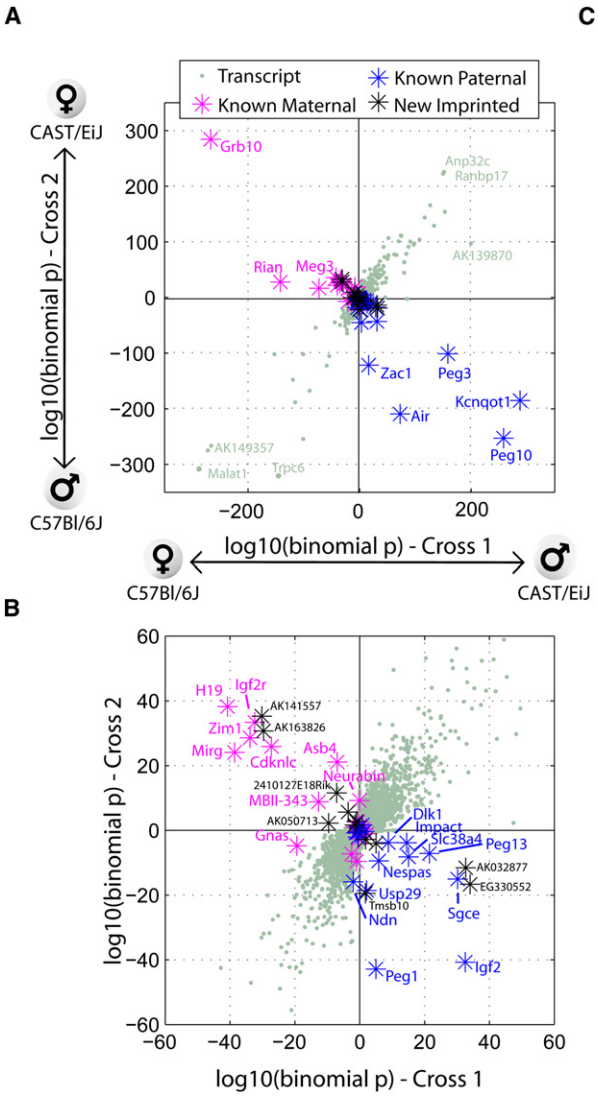

Figure 3. Allele-Biased Expression of Known Transcripts

(A and B) Transcripts comprise UCSC known genes and GenBank mRNAs (outside UCSC known genes) that are expressed, do not map to mitochondrial or sex chromosomes, and contain SNPs (n = 22,932). The plotted allele-bias p values were computed for each cross by use of allele counts across all SNPs within the transcript boundaries (including introns). Transcripts with biased expression toward sex-of-origin map to the negatively sloping diagonal and are enriched for known imprinted genes. Transcripts biased for strain-of-origin map to the positively sloping diagonal.

(C) Parent-of-origin allele bias computed with allele counts from both crosses for selected known and novel imprinted transcripts. Transcripts are UCSC known genes [39] or MGD transcripts [40], as defined in Table 1. All novel imprinted transcripts shown were independently validated as outlined in Table 1.

to be coordinately regulated as a polycistronic locus [14]. The browser view (Figure S3) supports this conclusion because we observe a continuous distribution of reads throughout the locus that is also consistent with the histone methylation patterns observed by chromatin immunoprecipitation in embryonic stem cells [9].

Even in the extensively studied Prader-Willi-Angelman syndrome locus, we find multiple sites of novel imprinted transcription. Imprinted sequencing reads and contiguous transcription up to 3 kb downstream of Frat3 provide evidence for a Frat3 (Peg12) 3′ untranslated region (UTR) extension (Figure S4). We also observe contiguous, paternally biased transcription extending up to 700 kb from Mkrn3 on the negative strand, encompassing transcription antisense to Ndn and Magel2 (Figure S5).

Besides extensive ncRNA transcription, we also identified coding transcripts that had not been previously shown to be imprinted. Within 300 kb of the Mest locus, we observed a maternally expressed gene, mKIAA0265, predicted to encode a protein containing multiple Kelch domains. Adjacent to the paternally expressed Sgce gene of the Peg10 locus, we verified the maternally biased expression of the Casd1 gene, which encodes a glycosyl transferase and is highly expressed in the brain, like other known maternally expressed genes in the cluster such as neurabin and the calcitonin receptor. We also identified three examples of genes that exhibit parent-of-origin sex bias and that are more than 10 Mb from any other known imprinted genes. All three (Zdbf2, Pde4d, and Tbc1d12) exhibited paternal bias (Table 1).

Discussion

We have used high-throughput sequencing to carry out a conceptually simple, genome-wide screen for imprinting in mice that were physiologically normal. Strand-specific total RNA amplification provides an advantage over commonly used methods that rely upon polyA priming and allows the detection of expressed SNPs in entire transcripts, including intronic regions and nonpolyadenylated transcripts. The advantages of sequencing over microarray-based assays include experimental design without a priori knowledge of SNP position or transcript sequence, and digital readout of transcript abundance, which drastically reduces uncertainty associated with microarray probe crosshybridization. The success of this approach is reflected in our ability to detect 14 out of 17 known imprinted mouse loci (six out of six classical regions [15]) that have been identified over the last 30 years.

Interestingly, more than half of imprinted SNPs mapped to locations outside of known imprinted-transcript boundaries (Figure S2, and see Document S1 for a detailed SNP summary by embryo). For example, at an imprinting score threshold of 1.5, 199 of 278 SNPs are outside known imprinted genes. Many of these SNPs occur within noncoding transcripts. Recent research has clearly highlighted the association of imprinting with transcription of noncoding RNAs, and methods that delineate the existence and extent of imprinted ncRNA transcription will enable further advances in the field. Moreover, given that some mechanistic models of imprinting place importance on the act of ncRNA transcription rather than the

Table 1. Summary of Selected Novel Imprinting Features Discovered in This Study

ID	Source	Locus	Novelty Category	Putative Extension of	Representative SNP Coordinates	Expr. Origin	Additional Validation	Imprinting Score (grouped by transcript)	Number of Allele-Specific Reads	Parental Bias (Solexa)	Parental Bias (Sanger)
Zdbf2/ BY714830	UCSC known gene	NA	New Gene	NA	chr1:63235975	P	RFLP, Sanger	−2.1	147	0.67	1.00
D2Ertd173e	MGD	Gnas	Transcript Extension	Nespas	chr2:173919696	P	Sanger	−4	61	0.87	0.95
mKIAA0265	UCSC known gene	Copg2	Gene Associated with Known Locus	NA	chr6:30352727	M	Sanger	7.11	2902	0.58	0.67
Casd1	UCSC known gene	Peg10	Gene Associated with Known Locus	NA	chr6:4567811	M	Sanger	1.78	743	0.57	0.62
A230057D06Rik	MGD	Prader-Willi syndrome	Transcript Extension	Zfp127	chr7:61719694	P	RFLP, Sanger	−4.8	55	0.91	0.97
A330076H08Rik	MGD	Prader-Willi syndrome	Transcript Extension	Zfp127	chr7:61814002	P	Sanger	−2.9	40	0.93	1.00
EG330552	MGD	Prader-Willi syndrome	Transcript Extension	Zfp127	chr7:62069722	P	Sanger	−16	353	0.87	0.86
Frat3-ext	UCSC known gene	Prader-Willi syndrome	Transcript Extension	Frat3	chr7:62339111	P	Sanger	−3.15	68	0.96	1.00
NMIT3	NA	Grb10	Gene Associated with Known Locus	NA	chr11:11974222, 12093735	M	RFLP, Sanger	17.1	213	0.93	1.00
AK018142	GenBank mRNA	Dlk1	Transcript Extension	Meg3	chr12:110010221	M	Sanger	39.9	471	0.94	1.00
AK046138	GenBank mRNA	Dlk1	Transcript Extension	Meg3	chr12:110012068	M	Sanger	13.37	110	0.97	1.00
AK148955	GenBank mRNA	Dlk1	Transcript Extension	Meg3	chr12:110018828	M	Sanger	19.25	262	0.94	1.00
AK086774	GenBank mRNA	Dlk1	Transcript Extension	Rian	chr12:110106925	M	Sanger	5.86	47	0.98	1.00
AK050713	GenBank mRNA	Dlk1	Transcript Extension	Rian	chr12:110116827	M	Sanger	2.23	68	0.88	1.00
AK141557	GenBank mRNA	Dlk1	Transcript Extension	Rian	chr12:110121219	M	Sanger	30.1	292	0.95	1.00
NMIT2	NA	Dlk1	Transcript Extension	Rian	chr12:110124397	M	RFLP, Sanger	1.04	17	1.00	1.00
NMIT1	NA	Dlk1	Transcript Extension	Rian	chr12:110138360	M	RFLP, Sanger	7.01	70	0.96	1.00
Pde4d	UCSC known gene	NA	New Gene	NA	chr13:110471927	P	Sanger	−1.9	108	0.67	0.64
Air	NA	Igf2r-Air	Isoform Definition	Air	chr17:12648421	P	qRT-PCR	−110.51	4247	0.76	NA
Tbc1d12	UCSC known gene	NA	New Gene	NA	chr19:38949154	P	Sanger	−1.8	287	0.60	0.59

Transcripts were identified from various sources as indicated, and imprinting scores were computed with the binomial distribution (see text). Additional validation comprised Sanger sequencing of RT-PCR products and in certain cases RFLP of RT-PCR products containing SNPs within the novel imprinted feature. All coordinates correspond to NCBI build 36 (mm8).

transcript itself and other models place importance on the presence of overlapping transcripts within the nucleus [15], the ability to detect nonpolyadenylated and unspliced transcripts will be critical. We demonstrate here that next-generation sequencing provides a valuable tool for identifying and profiling imprinted ncRNAs; for delineating extensions of ncRNA transcripts such as Nespas, Meg3, and Rian; and for discovering ncRNA transcripts such as those originating from the Peg12 cluster and Grb10.

This study also extended the number and characterization of imprinted coding genes. mKIAA0265 and Casd1 are novel imprinted genes within the Mest and Peg10 locus, respectively, and the Peg12 and Copg2 transcripts were found to extend well beyond their previously characterized 3′ UTRs [16, 17]. Intriguingly, we observed paternally biased expression of several individual genes, far from any previously discovered imprinted loci. These loci may relate to the phenomenon of "microimprinting," which is often associated with single, paternally biased genes that are thought to represent primordial imprints [18]. On the other hand, our study did not detect any new imprinted loci comparable in size or expression bias to such well-known loci as Igf2r and Dlk1.

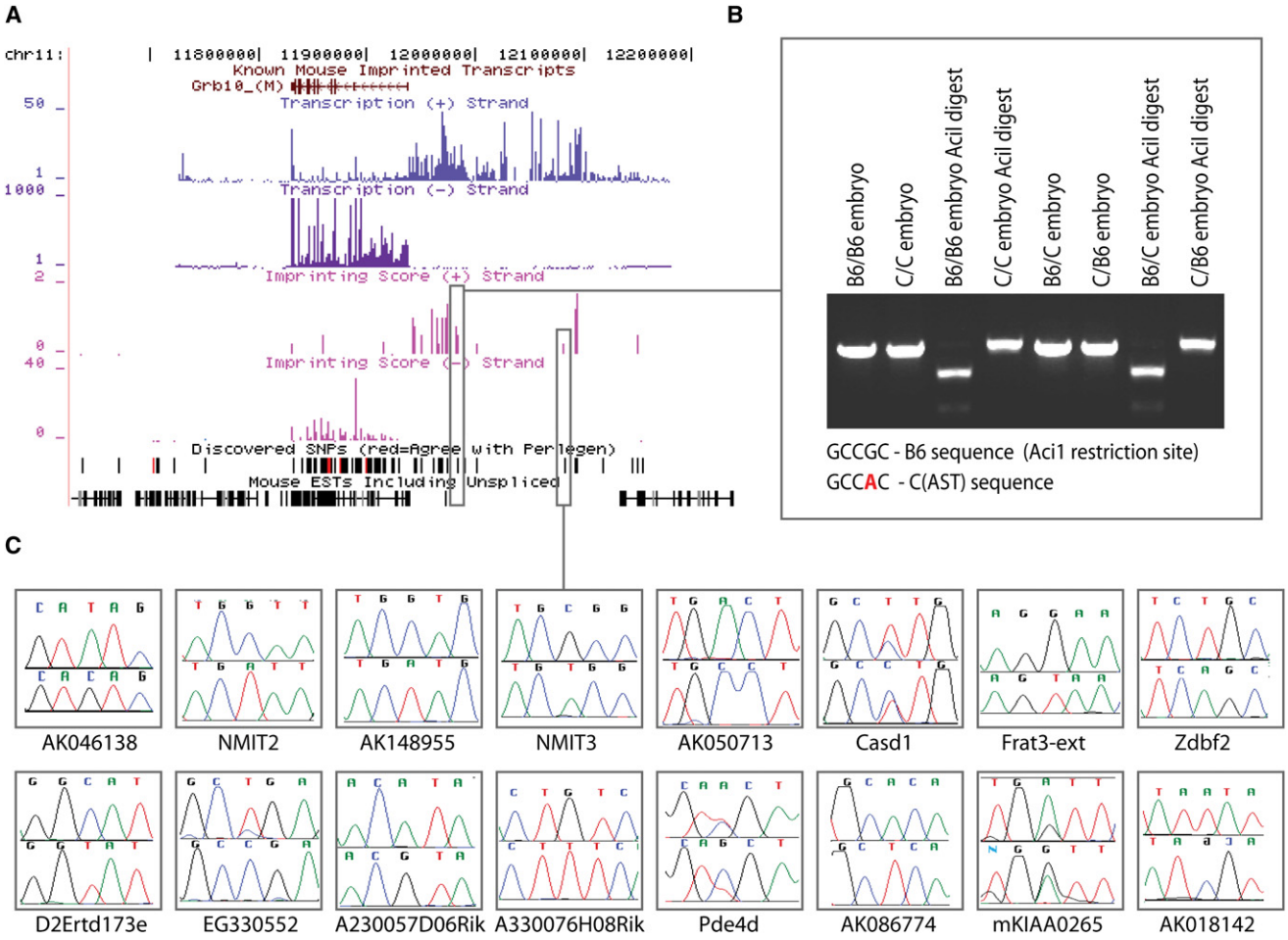

Figure 4. Imprinting beyond Documented Transcriptional Boundaries

(A) UCSC Genome Browser view of novel imprinted transcript (NMIT3) with no previous transcriptional or imprinting evidence. The x axis defines position in genome. Transcription is shown as number of reads overlapping each nucleotide. Imprinting scores are shown at SNPs with sufficient coverage to enable quantification of allelic expression (see Supplemental Experimental Procedures).

(B) Imprinting confirmation of Grb10as by cDNA-RFLP. Additional cDNA-RFLP confirmations of novel regions are shown in Figure S7.

(C) Confirmation of imprinting by Sanger sequencing of RT-PCR products. The top trace of each box corresponds to embryos with C57BL/6J mothers. The base at position 3 is polymorphic. In all cases, direction of allelic bias agrees with sequencing data.

Although future efforts will increase the sensitivity of our screen, it is also possible that the imprinted loci that have yet to be discovered will have different properties from classic loci. For instance, they may have distinct tissue specificities or levels of allelic bias. Recent work that used microarrays to detect widespread monoallelic expression in cell lines [19] suggests an extensive epigenetic landscape with opportunity for evolution of novel imprints.

Nearly all of the known imprinted transcripts that were not detected here either did not contain detectable polymorphisms or were not adequately expressed in the embryo (see Document S3 for results for all known imprinted genes). Of 90 known imprinted transcripts [1, 20], 36 contained SNPs and were sufficiently expressed to make a call on expression bias. Of these 36, 32 (89%) had the expected parent-of-origin bias at a confidence of $p < 0.01$ in one of the two crosses, and 27 (75%) exceeded $p < 0.01$ confidence in both crosses. The three imprinted loci that we did not detect (A19/RasGrf, Murr1, and Xlr3b) only contain seven transcripts total, none of which were abundantly expressed. Screening additional tissues and strains and increasing sequencing reads will ensure wider coverage of expressed SNPs.

The uses of these data should extend beyond mapping imprinted regions to encompass identification and definition of novel transcripts and transcriptional boundaries, identification and usage of splice junctions, and analysis of the extensive strain-specific expression detected in the screen. This sequencing approach can be readily applied to different tissues, species, and developmental stages, and doing so will shed light on a number of outstanding questions in imprinting. What is the spectrum of functions of imprinted transcripts? What forces shape species-specific imprinting? What is the extent of imprinted noncoding transcription? Answering such questions requires a comprehensive account of imprinted transcription in diverse systems.

Supplemental Data

Supplemental Data include SNP coordinates, UCSC Genome Browser tracks, allelic expression data, Supplemental Experimental Procedures,

nine figures, and two tables and can be found with this article online at http://www.current-biology.com/supplemental/S0960-9822(08)01274-8.

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