

# Differentially methylated regions in maternal and paternal uniparental disomy for chromosome 7

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**Abbreviations:** DMRs, differentially methylated regions; UPD, uniparental disomy; matUPD7, maternal uniparental disomy; patUPD7, paternal uniparental disomy; SRS, Silver-Russell syndrome; CGIs, CpG islands; TSS, transcription start sites; ICR, imprinting control center; TNDM, transient neonatal diabetes mellitus; BWS, Beckwith-Wiedemann syndrome; AS, Angelman syndrome; PWS, Prader-Willi syndrome; LOM, loss of methylation; lncRNA, long non-coding RNA; TUCP, transcript of uncertain coding potential; IGB, Integrated Genome Browser; qRT-PCR, quantitative reverse transcriptase-PCR; MIM, Mendelian Inheritance in Man; UCSC, University of California, Santa Cruz

DNA methylation is a hallmark of genomic imprinting and differentially methylated regions (DMRs) are found near and in imprinted genes. Imprinted genes are expressed only from the maternal or paternal allele and their normal balance can be disrupted by uniparental disomy (UPD), the inheritance of both chromosomes of a chromosome pair exclusively from only either the mother or the father. Maternal UPD for chromosome 7 (matUPD7) results in Silver-Russell syndrome (SRS) with typical features and growth retardation, but no gene has been conclusively implicated in SRS. In order to identify novel DMRs and putative imprinted genes on chromosome 7, we analyzed eight matUPD7 patients, a segmental matUPD7q31-qter, a rare patUPD7 case and ten controls on the Infinium HumanMethylation450K Bead-Chip with 30017 CpG methylation probes for chromosome 7. Genome-scale analysis showed highly significant clustering of DMRs only on chromosome 7, including the known imprinted loci *GRB10*, *SGCE/PEG10*, and *PEG/MEST*. We found ten novel DMRs on chromosome 7, two DMRs for the predicted imprinted genes *HOXA4* and *GLI3* and one for the disputed imprinted gene *PON1*. Quantitative RT-PCR on blood RNA samples comparing matUPD7, patUPD7, and controls showed differential expression for three genes with novel DMRs, *HOXA4*, *GLI3*, and *SVOPL*. Allele specific expression analysis confirmed maternal only expression of *SVOPL* and imprinting of *HOXA4* was supported by monoallelic expression. These results present the first comprehensive map of parent-of-origin specific DMRs on human chromosome 7, suggesting many new imprinted sites.

## Introduction

Methylation of the 5'-cytosines in CpG dinucleotides is an epigenetic mark that can affect gene expression. Clusters (approximately 1 kb) of multiple CpGs form CpG islands (CGIs), which are usually unmethylated when located at gene transcription start sites (TSS), but are also found within coding regions, at 3' ends, as well as in intra- and inter-genic regions.<sup>1</sup> Methylation near TSSs blocks transcription initiation, but

methylation within gene bodies may increase expression and influence splicing.<sup>1</sup> Methylation of promoter CGIs is rare and found in genes where expression is permanently repressed, as for imprinted genes, genes on the inactive X chromosome, and genes exclusively expressed in germ cells.<sup>1</sup> Moreover, CpG methylation is a hallmark of imprinted genes, which are expressed exclusively from the maternal or paternal allele and most of which play crucial roles in growth and development.<sup>2</sup> Imprinted genes generally reside in clusters where an imprinting control center (ICR) and

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other additional differentially methylated regions (DMRs) control the expression of the imprinted genes.<sup>3</sup> In human, approximately 32 imprinting clusters and over 70 imprinted genes have been described (see Catalogue of Imprinted Genes and Parent-of-origin Effects in Humans and Animals, <http://igc.otago.ac.nz>).<sup>4</sup>

The normal balance of imprinted genes can be disrupted by uniparental disomy (UPD), the inheritance of both chromosomes of a chromosome pair exclusively either from the mother (maternally, matUPD) or the father (paternally, patUPD).<sup>5</sup> UPD can lead to imprinting syndromes where loss or gain of methylation at a specific DMR/ICR leads to the syndrome phenotype, e.g., patUPD for chromosome 6 (patUPD6) in transient neonatal diabetes mellitus (TNDM [MIM 601410]), matUPD7 in Silver-Russell Syndrome (SRS [MIM 180860]), patUPD11 in Beckwith-Wiedemann syndrome (BWS [MIM 130650]), matUPD14 and patUPD14 [MIM 608149] as well as patUPD15 in Angelman syndrome (AS [MIM 105830]), and matUPD15 in Prader-Willi syndrome (PWS [MIM 176270]).<sup>5-10</sup> However, aberrant methylation is not always confined to only one locus, and multilocus loss of methylation (LOM) at multiple imprinted loci has been reported for TNDM, PWS, and a matUPD7q patient with also hypomethylation at the paternally imprinted *DLK1/MEG3* locus at 14q32 (MIM 176290).<sup>11,12</sup>

MatUPD7 is found in approximately 10% of SRS patients and hypomethylation of the ICR1 of *IGF2-H19* (MIM 147470, MIM 103280) on chromosome 11p15 in 20–60%.<sup>13,14</sup> SRS is characterized by severe pre- and postnatal growth restriction, macrocephaly, skeletal asymmetry, a triangular face and other variable dysmorphic features.<sup>15</sup> Conversely, patUPD7 does not affect growth and development.<sup>16-18</sup> An imprinted gene on chromosome 7 has been suggested to cause SRS, but none of the known imprinted genes at three suggested domains (7p12: *GRB10* [MIM 601523], 7q21.3: *TFPI2* [MIM 600033], *SGCE* [MIM 604149], *PEG10* [MIM 609810], *PPP1R9A* [MIM 602468], and 7q32.2: *CPA4* [MIM 607635], *MEST* [MIM 601029], *MESTIT1* [MIM 607794], *COPG2IT1* [MIM 610581], *KLF14* [MIM 609393]) have been conclusively linked with SRS.<sup>19-23</sup> Segmental maternal duplications spanning the imprinted gene *GRB10* on 7p12.2 and rare cases of segmental matUPD7q31-qter, matUPD7q, and a mosaic matUPD7q21-qter in SRS patients have however narrowed down the candidate SRS regions.<sup>24-28</sup>

Imprinted genes can be identified through parent-of-origin specific difference in methylation and expression.<sup>29</sup> High-throughput genome wide methylation profiling has enabled systematic identification of multiple new loci.<sup>30</sup> Whole genome methylation has already been used to identify new imprinted genes by profiling between matUPD15 and patUPD15 cases and in rare reciprocal genome-wide UPD samples.<sup>31,32</sup> Global methylation arrays with 1505 and 27 500 CpG sites in SRS patients with/without ICR1 hypomethylation at *IGF2-H19* did not reveal any significant common associations outside the *H19* promoter.<sup>33,34</sup> However, individual ICR1 hypomethylated patients showed increased methylation changes at separate loci.<sup>34</sup> No matUPD7 patients were included in these studies and to our knowledge high-throughput methylation profiling of matUPD7 has not been reported.

To identify novel DMRs on chromosome 7, we compared DNA methylation status of matUPD7 cases to controls and a rare patUPD7 case with the Illumina Infinium HumanMethylation450K BeadChip methylation assay.<sup>35</sup> We found 17 DMRs on chromosome 7, of which 14 are novel and three are the known DMR/ICRs of *GRB10* and the *SGCE/PEG10* and *PEG/MEST* clusters. Imprinted expression was suggestive for three genes with novel DMRs, *HOXA4* (MIM 142953), *GLI3* (MIM 165240), and *SVOPL* (MIM 611700) by qRT-PCR. Allele specific expression of *SVOPL* confirmed it as a novel imprinted gene and monoallelic expression of *HOXA4* supported imprinting. These results present to our knowledge the first comprehensive map of parent-of origin specific DMRs on human chromosome 7, suggesting novel imprinted domains.

## Results

### Genome-wide methylation in UPD7

To identify DMRs between maternal and paternal chromosomes 7, we performed genome-wide comparisons of the methylation of individual CpG sites between nine matUPD7 cases, including one segmental matUPD7q31-qter, ten controls and one patUPD7 case. We used the Infinium HumanMethylation450K BeadChip (Illumina), which measures methylation of 485 512 individual CpG sites, of which 30 017 are located on chromosome 7. Comparison between matUPD7 and controls showed that the differentially methylated CpGs mapped exclusively to chromosome 7 (Fig. 1), as expected for matUPD7. None of the CpGs outside chromosome 7 exceeded the genome-wide significance level ( $P = 5 \times 10^{-8}$ ), but single CpGs exceeding suggestive significance level ( $P = 1 \times 10^{-5}$ ) were observed on chromosomes 2, 4, 5, 6, 14, 16, 17, and 20. None of these CpGs coincide with known imprinted gene clusters or genes previously reported to show LOM in SRS patients with *H19* hypomethylation or other syndromes.<sup>11,33,36,37</sup>

Comparing the single patUPD7 sample with controls, the strong clustering of differentially methylated CpGs was also observed on chromosome 7. Because only a single patUPD7 sample was available, the statistical analysis was disturbed by noise, and scattered CpGs above the significance threshold emerged genome-wide (data not shown). Although the information provided by the patUPD7 had limitations in statistical analyses, the sample was highly informative when analyzing imprinted loci in greater detail.

### Differentially methylated regions on chromosome 7

We screened chromosome 7 for DMRs by using a step-by-step filtering process for all 30 017 CpG sites on chromosome 7 (Fig. 2). The process included two separate tracks, one screening for maternally hypermethylated CpGs and the other for maternally hypomethylated CpGs. The first step of the filtering process was based on identifying a pattern where the median M-value of the different groups (matUPD7, controls, and patUPD7) would differ in the way that would be expected for an imprinted locus, i.e., methylation in controls is midway and matUPD7s and patUPD7 diverge in opposite directions. For maternally hypermethylated CpGs, we set the filter to include all CpGs where

the median M-value of matUPD7s was larger than that of the controls, and the median M-value of the controls was larger than that of the patUPD7. For the maternally hypomethylated genes, we set the filter to include all CpGs where the median M-value of the patUPD7 was larger than that of the controls, and the median M-value of the controls was larger than that of the matUPD7s. Step two excluded all CpGs where the methylation difference between matUPD7s and controls did not reach nominal significance ( $P$  value < 0.05). A nominal threshold was applied in order to avoid false negative findings. The significance level of differential methylation between controls and patUPD7 was disregarded for having only a single patUPD7 sample. The third step was set to include only those CpGs where a consecutive row of three or more CpGs had passed steps one and two in the filtering process. We excluded single and only two adjacent significant CpGs because these were judged more likely to be false positives. We excluded CpGs with poor signals and chose only adjacent CpGs with a good signal across the entire locus to increase the likelihood of obtaining true positive results. Our strategy may have left some loci undetected, but should have a low false positive rate.

We found 338 single maternally hypermethylated CpGs scattered along chromosome 7 and 150 CpGs with at least two adjacent CpGs clustered to nine genes (*MEST*, *SGCE*, *GRB10*, *HTR5A* [MIM 601305], *RPS2P32*, *SVOPL*, *IQCE*, *HOXA5* [MIM 142952], and *HOXA6* [MIM 142951]; note that six of these genes also contained a stretch of three or more consecutive CpGs with differential methylation) and two intergenic regions (Table S1). For maternally hypomethylated CpGs, we found 609 single CpGs and 98 adjacent CpGs in 20 genes and seven intergenic regions (Tables S2 and S3).

We found significant differential methylation that passed our three step filtering process for 204 CpGs at 17 DMRs/CGIs spread along chromosome 7, localizing to 14 genes and two intragenic regions at long non-coding RNAs (lncRNA) (Table 1; Tables S4 and S5; Fig. 3). The majority of the DMRs were maternally hypomethylated (11/17, 65%) and six maternally hypermethylated (Table 1; Tables S4 and S5; Fig. 3). As expected, the three known DMRs of the imprinted domains of *GRB10* on 7p12.2, *SGCE/PEG10* on 7q21.3, and *MEST/MESTIT1* on 7q32 passed our filter (Table 1).<sup>23,38,39</sup> The well-conserved CGI2 upstream of *GRB10* was entirely differentially methylated, with four CpGs encompassing the CGI2 and two CpGs in the North shore covering 113 bp (Table 1). The DMR in the promoter of *MEST* has been proposed to act as an ICR for the whole imprinted cluster spanning from *CPA4* to *KLF14*.<sup>22,40</sup> We identified 63 maternally hypermethylated CpGs spanning the entire *MEST* ICR, with 50 over the CGI and 13 CpGs located to the North shore covering 793 bp (Table 1). We found 55 maternally hypermethylated CpGs in the *SGCE/PEG10* ICR with 41 located in the CGI and 14 in the South shore covering 715 bp (Tables 1 and 2). The *SGCE/PEG10* CGI was not completely differentially methylated as 662 bp in the 5' end lacked a DMR. CGI shores are defined as the region 0–2 kb upstream (North) or downstream (South) from the CGIs and shelves as the region 2–4 kb from the CGIs (Illumina, based on UCSC predictions).

Altogether ten DMRs located to genes with no prior imprinting status, two to previously predicted imprinted genes (*HOXA4* and *GLI3*),<sup>41</sup> and one to *PONI* (MIM 168820), with a disputed imprinting status (<http://igc.otago.ac.nz>) (Table 1). The DMR of *PONI* was the only new DMR found in a known imprinting cluster (*SGCE/PEG10*). Previously, only one DMR, in an ICR situated between *PEG10* and *SGCE*, has been found in this cluster out of all the CGIs ranging from *CALCR* (MIM 114131) to *PPP1R9A* (MIM 602468).<sup>23,42</sup>

The intergenic DMR at 7p21.1 partially overlapped the promoter and 5' region of a possible long non-coding RNA (lncRNA), defined as a transcript of uncertain coding potential (TUCP) by the UCSC (University of California, Santa Cruz) genome browser (<http://genome.ucsc.edu/>) (TCONS\_I2\_00026389, 710 bp at 16 625 596–16 626 306 bp) and the second intergenic DMR at 7q36.2 was located 659 bp upstream of the TSS of another TUCP (TCONS\_I2\_00027011, 135 475 bp at 156 264 552–156 400 027 bp). Several imprinting clusters contain at least one lncRNA located in the proximity or partially overlapping a protein-coding gene, e.g., *MESTIT1* overlapping *MEST* at the 7q32 imprinted cluster.<sup>43,44</sup> Imprinted lncRNAs, e.g., *Air* (antisense *Igf2r* RNA, [MIM 604893]) and *Kcnq1ot1* (*Kcnq1* overlapping transcript 1, [MIM 604115]), have also been shown to play a major role in silencing multiple genes in the imprinted clusters. The ICR can lie within the lncRNA, a few kb upstream of the promoter or at the lncRNA promoter.<sup>43</sup>

Interestingly, *RPS2P32* at 7p15.3 is itself a 1025 bp lncRNA, with the maternally hypermethylated DMR located to the TSS and gene body (Table 1). *MAD1L1* (MIM 602686) at 7p22 overlaps in its 3' end with the antisense lncRNA AK127048 (4632 bp at 1 884 936–1 889 567 bp). *HTR5A* and its DMR overlap with an uncharacterized non-coding RNA LOC100128264 (4489 bp at 154 858 779–154 863 267 bp) and *HOXA4* overlaps with an antisense hypothetical protein LOC285943 (6842 bp at 27 161 538–27 168 379 bp). We checked up to 500 kb up- and down-stream of each DMR for lncRNAs on the UCSC browser and found at least two up to 28 lncRNAs and TUCPs for each (not analyzed further here). The two closest protein coding genes of the intergenic DMR at 7p21.1 were *LRRRC72* located 5 kb upstream at 16 566 505–16 621 114 bp and *ANKMY2* 13 kb downstream at 16 639 401–16 685 442 bp. The two closest protein coding genes of the intergenic DMR at 7q36.2 are *RNF32* (MIM 610241) 32 kb downstream at 156 433 353–156 469 820 bp and *LMBR1* (MIM 605522) 72 kb also downstream at 156 473 570–156 589 186 bp. The closest protein coding genes to *RPS2P32* are *IGF2BP3* (MIM 608259) 20 kb upstream at 23 349 828–23 509 995 bp and *TRA2A* (MIM 602718) 13 kb downstream at 23 544 401–23 571 656 bp.

The lengths of the DMRs ranged from 196 bp (*GLI3*) to 3164 bp (*MEST/MESTIT1*) (Table 1). All significant CpGs defining DMRs located primarily to CGIs and their shores (Table 1). For *MAD1L1* and *GLI3* all significant CpGs located only to shores and the DMR for *CARD11* (MIM 607210) did not hit a predicted CGI nor the shores or shelves of a CGI. *SH2B2* (MIM 605300) had two separate DMRs in two independent CGIs.

**Table 1.** DMRs identified on chromosome 7

Gene	Locus	DMR bp (length)	CGI bp (length)	DMR CpGs	TSS	5'UTR/1st exon	Gene body	3'UTR	MatUPD7 methylation	Known imprinting status
<i>MAD1L1</i>	7p22	1 883 226–1 883 901 (675 bp)	1 885 033–1 885 402 (369 bp)	5			5		hypo	-
<i>CARD11</i>	7p22	3 019 134–3 019 407 (273 bp)	No CGI	4		4			hypo	-
Intergenic	7p21.2	16 626 199–16 626 555 (356 bp)	16 626 148–16 626 531 (383 bp)	3					hypo	-
<i>RPS2P32</i>	7p15.3	23 529 999–23 530 810 (811 bp)	23 530 434–23 530 690 (256 bp)	6	1		5		hyper	-
<i>HOXA4</i>	7p15.2	27 169 649–27 171 179 (1530 bp)	27 169 572–27 170 638 (1066 bp)	19	12	6	1		hypo	predicted <sup>41</sup>
<i>PRR15</i>	7p14.3	29 605 599–29 606 374 (775 bp)	29 605 827–29 606 290 (463 bp)	6		3	2	1	hypo	-
<i>GLI3</i>	7p13	42 277 925–42 278 121 (196 bp)	42 276 003–42 277 850 (1847 bp)	5	5				hypo	predicted <sup>41</sup>
<i>GRB10</i>	7p12.2	50 849 639–50 850 870 (1231 bp)	50 849 752–50 850 871 (1119 bp)	6		6			hyper	maternal <sup>69,70</sup>
<i>SGCE/PEG10</i>	7q21.3	94 285 520–94 287 242 (1722 bp)	94 284 858–94 286 527 (1669 bp)	55	52	3			hyper	maternal <sup>71,72</sup>
<i>PON1</i>	7q21.3	94 953 628–94 954 227 (599 bp)	94 953 769–94 953 971 (202 bp)	9	4	3	2		hypo	disputed (www.otago.ac.nz/IGC)
<i>SH2B2 area 1</i>	7q22	101 944 250–101 944 581 (331 bp)	101 943 785–101 944 557 (772 bp)	3			3		hypo	-
<i>SH2B2 area 2</i>	7q22	101 961 771–101 962 148 (377 bp)	101 961 741–101 962 226 (485 bp)	3			1	2	hypo	-
<i>MEST/MESTIT1</i>	7q32	130 129 946–130 133 110 (3164 bp)	130 130 739–130 133 111 (2372 bp)	63	60	3			hyper	maternal <sup>40</sup>
<i>SVOPL</i>	7q34	138 348 749–138 349 468 (719 bp)	138 348 962–138 349 444 (482 bp)	4	3	1			hyper	-
<i>HTR5A</i>	7q36.1	154 862 745–15 486 340 (661 bp)	154 862 680–15 486 345 (565 bp)	6		5	1		hyper	-
<i>RARRES2</i>	7q36.1	150 038 477–150 038 923 (446 bp)	150 037 459–150 039 031 (1572 bp)	3	1	2			hypo	-
Intergenic	7q36.2	156 400 686–15 640 015 (329 bp)	156 400 018–15 640 712 (694 bp)	4					hypo	-

DMR coordinates are based on MAPINFO for Illumina probes. CGI loci are according to USCS. “DMR CpGs” shows the number of consecutive CpGs, indicating a DMR, and the following four columns show the position(s) of a DMR with regard to the TSS, transcription start site, and gene. “MatUPD7 methylation” shows the degree of methylation of a DMR in matUPD7s compared with controls and patUPD7.

Otherwise, all genes had a DMR located to a single CGI and its shores, but none extended into the shelves.

#### Differentially methylated regions and gene regulatory elements

The location of a DMR in regard to, e.g., promoters, enhancers, TSSs, and CTCF binding sites may affect gene expression.<sup>1,45</sup> The identified DMRs were predominantly in the 5' UTRs, TSSs, and first exons of the genes (Table 1). Only for two genes (*MAD1L1*, *SH2B2*) the DMRs located to the body of the gene or the 3' UTR (Table 1). For *HOXA4* and *PON1* the DMRs were spread from the TSS to the gene body and for *PRR15* from the 5'UTR to the 3'UTR. However, *PRR15* is a small gene of 1717 bp

(NM\_175887) with only two exons. Methylation at the promoter and TSS is usually associated with decreased gene expression while gene body or 3' UTR methylation increases expression and can affect splicing.<sup>1</sup> Altered methylation of CTCF binding sites is known for some imprinted genes, e.g., *H19*, where one of seven CTCF binding sites shows parent-of-origin specific methylation.<sup>1</sup> Methylation prevents the binding of CTCF that acts as an insulator, and the unblocked enhancers are able to drive the promoters to upregulate transcription of the target gene. We observed a strong signal for CTCF-binding sites in 4/6 (67%) of our maternally hypermethylated genes, including the CGI2 of *GRB10*, and in 3/10 of the maternally hypomethylated domains (Table 2).

**Table 2.** Differential expression of genes and location of DMRs in relation to gene expression regulatory elements

Gene	Locus	MatUPD7 methylation	Expression	MatUPD7 ddCT	Control ddCT	T-test P value	Promoter	Enhancer	CTCF	TFBS	lncRNA/TUCP
<i>MAD1L1</i>	7p22	hypo	bip	2.30	2.81	0.51	-	-	-	1	AK127048, antisense
<i>CARD11</i>	7p22	hypo	mat	8.16	6.56	0.47	1/4	4/4	++	-	-
Intergenic	7p21.2	hypo	nt	nt	nt	nt	-	-	+	-	TCONS_12_00026389
<i>RPS2P32</i>	7p15.3	hyper	pat	1.88	2.32	0.40	3/6	4/6	+	-	<i>RPS2P32</i> is a lncRNA
<i>HOXA4</i>	7p15.2	hypo	mat	<b>5.91</b>	<b>2.19</b>	<b>0.0021</b>	15/19	14/19	+	11	LOC285943, antisense
<i>PRR15</i>	7p14.3	hypo	mat	4.57	2.02	0.42	-	-	+	1	-
<i>GLI3</i>	7p13	hypo	pat	<b>0.44</b>	<b>1.56</b>	<b>9.39 x 10<sup>-5</sup></b>	-	-	+	1	-
<i>GRB10</i>	7p12.2	hyper	nt	nt	nt	nt	5/6	1/6	++	8	-
<i>SGCE/PEG10</i>	7q21.3	hyper	pat	<b>0</b>	<b>5.49</b>	<b>0.0017</b>	55/55	-	-	-	-
<i>PON1</i>	7q21.3	hypo	nr	nr	nr		-	9/9	+	-	-
<i>SH2B2, area 1</i>	7q22	hypo	mat	2.39	2.03	0.61	-	-	++	1	-
<i>SH2B2, area 2</i>	7q22	hypo	nt	nt	nt	nt	-	1/3	++	1	-
<i>MEST/MESTIT1</i>	7q32	hyper	nt	nt	nt	nt	43/63	11/63	-	-	<i>MESTIT1</i> , antisense
<i>SVOPL</i>	7q34	hyper	mat	5.92	2.64	<b>0.016</b>	-	-	++	-	-
<i>HTR5A</i>	7q36.1	hyper	nr	nr	nr	nr	-	-	++	-	LOC100128264, antisense
<i>RARRES2</i>	7q36.1	hypo	bip	0.94	0.62	0.46	-	1/3	+	-	-
Intergenic	7q36.2	hypo	nt	nt	nt	nt	-	-	++	-	TCONS_12_00027011 659bp upstream of TSS

Genes with significant Student *t* test *P* values < 0.05, for expression difference between matUPD7s and controls are highlighted in bold. Hypo, hypomethylation in matUPD7s compared with controls and patUPD7; hyper, hypermethylation in matUPD7s compared with controls and patUPD7; bip, biparental expression; mat, maternal expression; pat, paternal expression; nt, not tested; nr, no result. Average ddCT values calculated with the comparative *C<sub>T</sub>* method for matUPD7s and controls. Promoter, enhancer; number of CpGs/DMR according to Illumina annotations. CTCF binding sites: -, no binding sites; +, weak to moderate; ++, strong, according to UCSC. TFBS, transcription factor binding site, the number of TFBS located in the DMR, according to UCSC. lncRNA, long non-coding RNAs or TUCPS, transcript of unknown coding potential, overlapping the gene, according to UCSC.

Methylation of CpG sites in the recognition sequences of transcription factors can strongly influence transcription factor binding by complex mechanisms.<sup>1,46</sup> Conserved transcription factor binding sites were observed for 7/17 (41%) of the DMRs (Table 2).

#### Validation of methylation status with pyrosequencing

We chose three DMRs (*HTR5A*, *RPS2P32*, and *HOXA4*) for validation with pyrosequencing. There was a high correlation of methylation levels between the Infinium HumanMethylation450K BeadChip and pyrosequencing: 94% for the *HTR5A* CpG located at 154 862 969 (hg19 build) and 95% for the *HOXA4* CpG located at 27 170,892 (hg19 build) (Fig. 4), suggesting that the Infinium microarray results were highly accurate. Pyrosequencing for *RPS2P32* failed with two different primer sets.

#### Data visualization

The methylation level of each CpG in each sample in the chromosome 7 DMRs and surrounding regions was visualized in detail with the Integrated Genome Browser (IGB, <http://bioviz.org/igb/>).

The full data set is available at [http://publications.scilifelab.se/kere\\_j/imprinting7](http://publications.scilifelab.se/kere_j/imprinting7) as raw data files and IGB compatible files. As expected, *GRB10*, *SGCE/PEG10*, and *MEST/MESTIT1* showed a clear parent-of-origin methylation pattern, with the methylation level of matUPD7s and patUPD7 deviating in opposite directions from the controls (Fig. 5). *HTR5A*, *PON1*, and *RPS2P32* also showed a similar parent-of-origin specific methylation. For *SVOPL*, one matUPD7 case deviated from the others, suggesting individual variation (Fig. 5). *HOXA4* showed clear hypomethylation for the matUPD7s, but the patUPD7 was indistinguishable from the controls (Fig. 5). For the remaining DMRs, clustering of the different groups was less apparent despite statistically significant group differences, suggesting more subtle methylation differences (data not shown).

#### Expression study of genes with DMRs

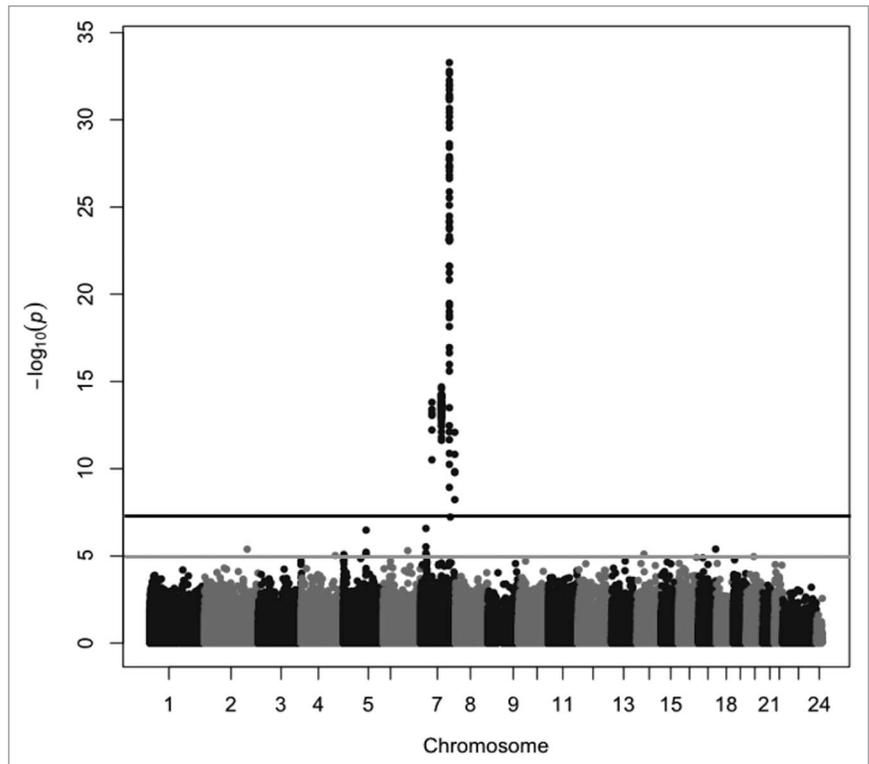
To define possible imprinted expression of the genes displaying DMRs, we performed quantitative reverse transcriptase-PCR

(qRT-PCR) on freshly drawn blood cells. Differential expression between matUPD7s, controls, and patUPD7, compatible with imprinting, was seen for 7/11 (64%) of the genes with novel DMRs studied (Table 2). *PEG10* was studied as a positive control for imprinting and displayed the expected paternal expression, with a significant difference between matUPD7s and controls, *t* test *P* value 0.0017 (Fig. 6D and H).

A significant difference in expression between matUPD7s and controls was found for three genes; *HOXA4* at 7p15.2, *GLI3* at 7p13, and *SVOPL* at 7q34 (Table 2; Fig. 6). *HOXA4* (Fig. 6A) and *SVOPL* (Fig. 6C) showed significantly increased expression in matUPD7s compared with controls (*t* test *P* values 0.008 and 0.017, respectively) and the patUPD7 showed markedly lower expression compared with the controls and matUPD7s (Fig. 6). *GLI3* showed significantly decreased expression in matUPD7s compared with controls (*t* test *P* value 0.049) and the one patUPD7 showed higher expression compared with the controls and to matUPD7 (Fig. 6B). Significant expression differences were also confirmed by performing a linear regression analysis, including the single patUPD7 case (Fig. 6E–G). Maternal hypomethylation of the *HOXA4* promoter region (Fig. S1A) appears to result in increased expression in matUPD7s, and hypermethylation in decreased expression in the patUPD7. However, for *GLI3* maternal hypomethylation at the TSS (Fig. S1B) resulted in decreased expression in matUPD7s compared with controls and patUPD7. For *SVOPL*, maternal hypermethylation at the TSS and 5'UTR (Fig. S1C) results in increased maternal expression. Overall, *HOXA4* and *SVOPL* showed maternal expression while *GLI3* was paternally expressed.

In addition, *CARD11*, *PRR15*, and *SH2B2* showed a clear pattern of increased maternal expression compared with controls and decreased expression in the patUPD7 suggestive of maternal expression, and *RPS2P32* of decreased maternal expression indicative of paternal expression, but the difference between matUPD7s and the controls was not statistically significant for any of these genes (data not shown). For *MAD1L1* and *RARRES2* (MIM 601973) we did not identify differential expression indicative of imprinting and for *HTR5A* and *PONI*, expression was undetectable in lymphocytes. We did not study the lncRNAs or genes close to the two intergenic DMRs (Table 1).

To confirm the imprinting of *HOXA4*, *GLI3*, and *SVOPL*, we studied parent-of-origin specific expression by sequencing selected exonic SNPs in nine parent-child trios. Four SNPs with high expected heterozygosity were chosen for *HOXA4* (rs17471888, rs4722660, rs1801085, rs2158218), five for *GLI3* (rs3735361, rs3823720, rs2051935, rs929387, rs846266) and three for *SVOPL* (rs1614641, rs3734944, rs2305816) (Table S6). Genomic DNA

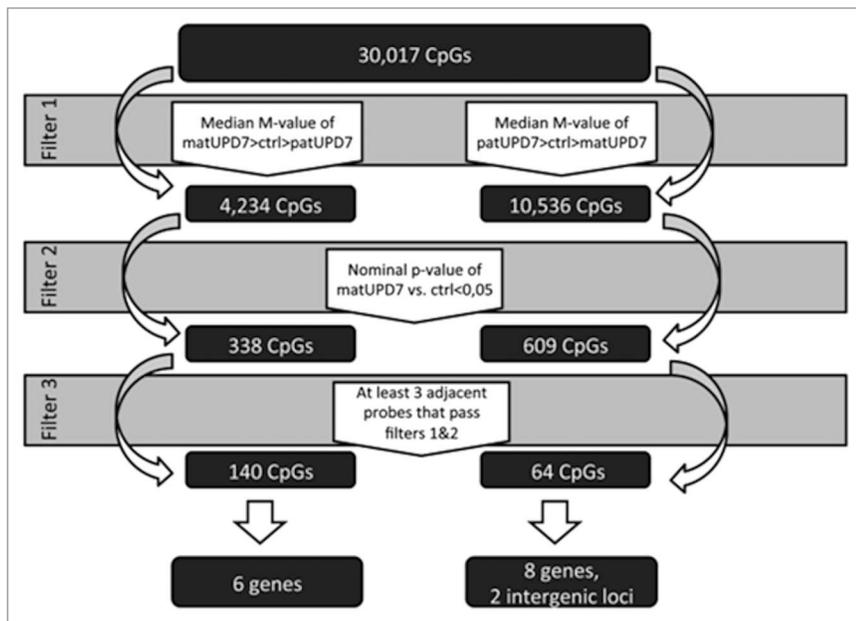


**Figure 1.** Manhattan plot of the differentially methylated CpG sites between matUPD7 and controls. The black line indicates the genome-wide significance level of  $P = 5 \times 10^{-8}$  and the gray line indicates the suggestive significance level of  $P = 1 \times 10^{-5}$ .

from the children and parents was first sequenced for all SNPs to find children heterozygous for a SNP and parents either both homozygous for different alleles or at least one parent homozygous for one allele. The child's cDNA was then sequenced from the trios with informative SNP alleles to see whether the maternally or paternally inherited allele was expressed. For *SVOPL*, two trios were informative and both children showed expression of only the maternal allele for rs2305816, confirming the maternal expression as seen in the qPCR (Fig. S2A). Only one child showed two heterozygous SNPs for *HOXA4*, but both parents were also heterozygous. Sequencing of the child's cDNA for rs2158218 showed monoallelic expression thus supporting imprinting (Fig. S2C). Because both parents share the same allele it is not possible to discern which parental allele is expressed. The other heterozygous SNP rs4722660 failed to give a readable cDNA sequence. For *GLI3* we failed to obtain conclusive data due to high PCR failure rate. Further analyses in larger sample sets are warranted to confirm the parent-specific expression.

## Discussion

We performed a genome-wide methylation study for 450 000 CpG sites to identify DMRs on chromosome 7 in matUPD7 and patUPD7 cases in comparison to biparental controls. We used a three step filtering approach to identify the most significant CpGs in terms of parent-of-origin specific methylation. We identified 17 distinct DMRs spread along chromosome 7 localizing



**Figure 2.** Schematic presentation of the filtering process for the DMRs.

to 14 genes and two intragenic regions at lncRNAs, including the known DMRs at the *SGCE/PEG10*, *GRB10*, and *MEST/MESTIT1* imprinted domains, and also two previously predicted imprinted genes, *HOXA4* on 7p15.2 and *GLI3* on 7p13.<sup>41</sup> Novel DMRs were located near eight protein coding genes and two lncRNAs, none previously implicated in imprinting, and *SVOPL* was confirmed as imprinted by parent-specific expression.

The majority of the DMRs were maternally hypomethylated (65%) and they were predominantly located in the promoter regions of the genes. This is contradictory to the current knowledge that the majority of imprinted genes are maternally hypermethylated. Maternally methylated DMRs and ICRs of imprinted genes are typically located at the promoters.<sup>45</sup> DMRs and ICRs of imprinted genes may also be situated within the genes and gene body methylation is known to enhance expression and possibly affect splicing.<sup>1</sup> For *MAD1L1* and *SH2B2* the maternally hypomethylated DMRs were found only in the gene bodies. However, *SH2B2* showed increased maternal expression, although non-significant, and *MAD1L1* biparental expression whereas gene body maternal hypomethylation would have been expected to result in decreased maternal expression in both genes. We did not study differential splicing for these genes. Paternally methylated DMRs are preferentially located in intergenic regions,<sup>45</sup> and consistently both of the DMRs we found in intergenic regions were maternally hypomethylated. The CpGs in the DMRs located to CpG islands and shores but not shelves.

CTCF-binding sites, which act as insulators between promoters and enhancers, are commonly found at imprinting clusters.<sup>45,48</sup> All of the DMRs found here, except for *MAD1L1*, *SGCE/PEG10*, and *MEST/MESTIT1*, co-localized with CTCF-binding sites. Differential methylation at CTCF-binding sites can affect the imprinted expression of the genes regulated by the insulators, e.g., *H19*.<sup>1</sup> Several imprinting clusters contain also at least

one antisense lncRNA located in the proximity or partially overlapping a protein coding gene.<sup>43,44</sup> Imprinted lncRNAs are necessary for the imprinted expression of the genes in the cluster, but can also regulate small clusters of autosomal genes in cis.<sup>44</sup> *XIST* (Inactive X-specific transcript, [MIM 314670]) and *TSIX* (Inactive-specific transcript, antisense [MIM 300181]) are the best-known functional lncRNAs required for the epigenetic X chromosome inactivation in female mammals.<sup>44</sup> Both of the two intergenic DMRs identified here were in close proximity to possible lncRNAs defined TUCPs, suggesting that these TUCPs may have imprinted regulation by the DMRs and potentially the DMRs and lncRNAs may be part of larger imprinted domains with surrounding genes. In addition, we observed antisense lncRNAs overlapping a few genes with DMRs, namely *HTR5A* and *MAD1L1*, and an antisense hypothetical protein overlapping *HOXA4*. Interestingly, *RPS2P32* at 7p15.3 is itself a

lncRNA. More than 80% of imprinted genes reside in clusters of several imprinted genes.<sup>3</sup> It remains to be seen whether the DMRs identified here act as ICRs by regulating the imprinted expression of other genes nearby, in addition to the genes that the DMRs lie closest to and have been studied here. In depth methylation and expression analysis of the neighboring genes and lncRNAs is thus warranted, but is beyond the scope of this study.

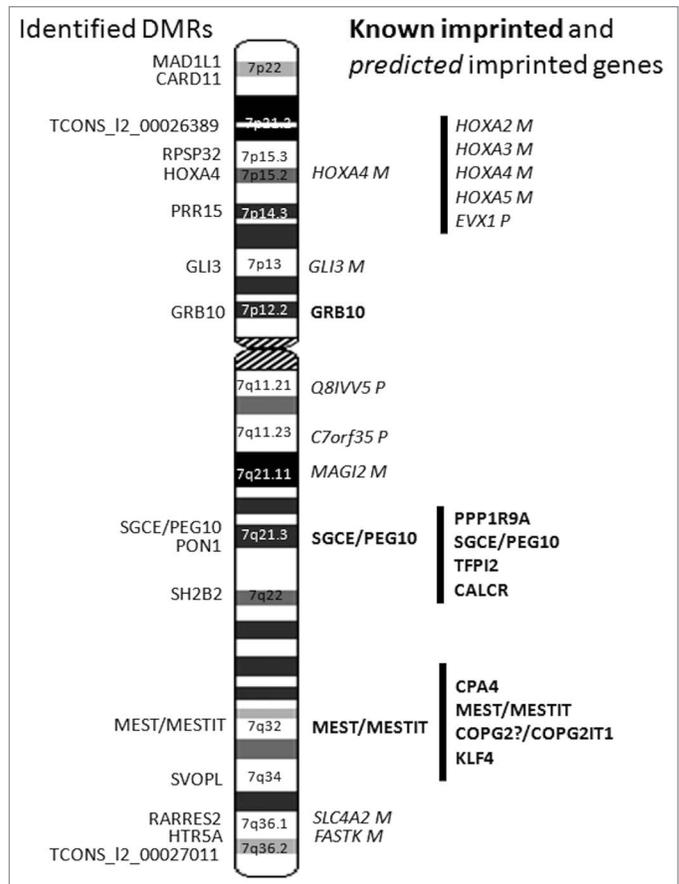
Our results give further insight to the extent of the DMRs at the *SGCE/PEG10*, *GRB10*, and *MEST/MESTIT1* imprinted domains. The entire CGIs and parts of their North shores of *GRB10* and *MEST/MESTIT1* proved to be DMRs, but only 61% of the *SGCE/PEG10* CGI turned out to be a DMR that extended well into the South shore. We also identified a novel DMR in the CGI in the promoter and first exon of *PONI* in the *SGCE/PEG10* cluster. Parent-of-origin specific expression of *PONI* has not been shown, although paternal expression of *PONI* has been reported in mouse hybrids containing a single maternal or paternal human chromosome 7.<sup>49,50</sup> We did not detect *PONI* expression in lymphocytes and could thus not study its possible parent of origin specific expression. *PONI* is located between *PPP1R9A*, imprinted in both humans and mice, and *PON2* (MIM 602447) and *PON3* (MIM 602720), both imprinted in mouse, but biallelically expressed in humans.<sup>21,23</sup> The ICR for *PPP1R9A* is unknown, as a CGI in the first exon of *PPP1R9A* did not show differential methylation between matUPD7 and patUPD7 lymphoblastoid cell lines or fetal placenta, liver or muscle,<sup>21</sup> nor was it picked up through our screening. The *PONI* DMR identified here might act as an ICR for also *PPP1R9A*, although it is situated over 9 kb downstream from the 3' end of *PPP1R9A*. We did not observe additional DMRs for any of the other imprinted genes in the *MEST/MESTIT1* cluster (*CPA4*, *COPG2IT1*, *COPG2* [disputed, MIM 604355] and *KLF14*). No DMRs were identified for any genes near *GRB10*. *DDC* (MIM 107930) and *COBL* (MIM

610317) adjacent to *GRB10* have been shown to be imprinted in mouse, but the DMR of *GRB10* appears to control imprinting of all three genes in mouse.<sup>51</sup> However, in human *DDC* and *COBL* were biparentally expressed in multiple fetal tissues.<sup>52</sup> *DLX5* (MIM 60028) at 7q21.3 was initially shown to be maternally only expressed in human lymphoblasts,<sup>49</sup> but subsequently biallelic expression has been reported.<sup>23,53</sup> We did not find DMRs in or near *DLX5* which would indicate imprinting.

Several predicted imprinted loci at 7q11.21, 7q11.23, 7q21.11, and 7q36.1 failed to show differential methylation, suggesting either tissue or developmental stage specific imprinting beyond the blood cells that we studied, or imprecision of the predictions.<sup>41</sup> As all CpGs are not covered by the Infinium HumanMethylation450K BeadChip, some DMRs might have been missed by the study. We set our filtering process to exclude single and two adjacent CpGs, assuming that these CpGs were more likely to be false positives, and therefore some DMRs might have been ignored. We excluded the possibility of blood lineage-specific alternative methylation as a potential source of bias by verifying all loci for the absence of such differences.<sup>46</sup> Among the detected DMRs only *GRB10*, *MEST*, and *PON1* have been reported to show age-dependent methylation level changes using the same microarray method.<sup>54</sup> Thus, the methylation results for the remaining DMRs should not be affected by the age of the subjects studied.

Only loci on chromosome 7 showed genome-wide significant differences in methylation. Consistent with our findings, there have been no reports of multilocus LOM associated with UPDs for other chromosomes. Thus, our data support the finding that matUPD7 is not associated with multilocus LOM.<sup>55</sup> However, up to 73% of SRS patients with *H19* hypomethylation have been reported to show multilocus LOM, suggesting a generalized defect in establishing imprints in these patients.<sup>34,48</sup> We found suggestive loci on seven other chromosomes, but these loci did not hit known imprinted domains. A small proportion of them might be cross-reactive probes that have co-hybridized to both chromosome 7 and other chromosomes. We have not explored these single CpG sites further.

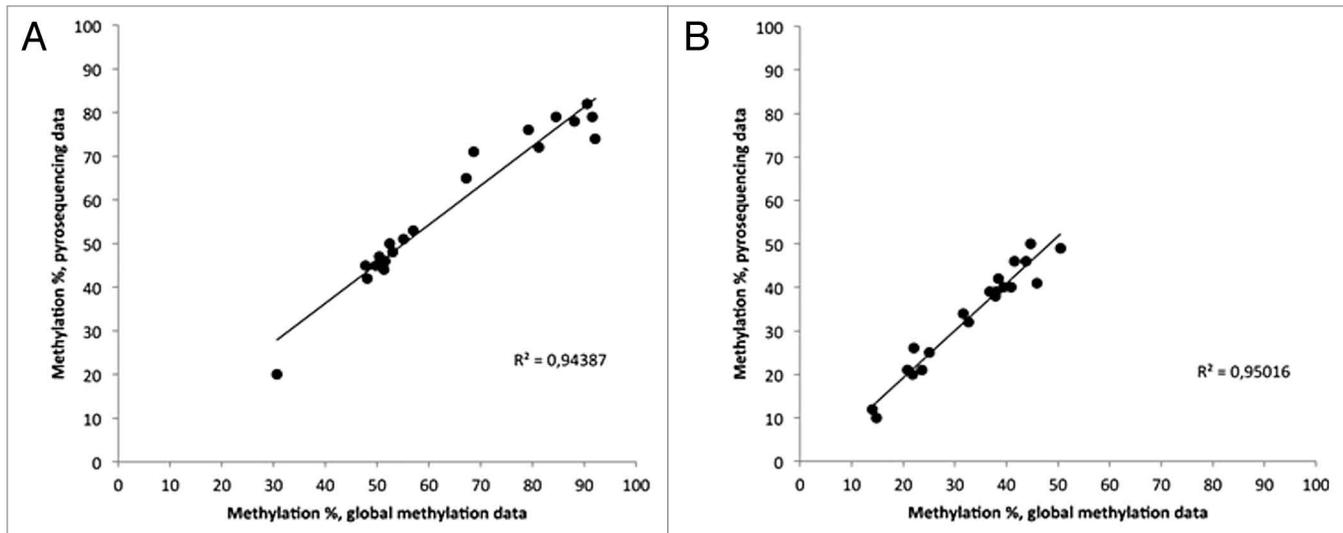
Our data supported the imprinted status of *HOXA4* at 7p15.2, *GLI3* at 7p13, and *SVOPL* at 7q34 by showing differential expression between matUPD7s and patUPD7 and a significant expression difference between matUPD7s and controls. Concordant with the qRT-PCR results, allele-specific expression studies revealed maternal only expression of *SVOPL*, thus confirming it as an imprinted gene. *HOXA4* showed monoallelic expression supporting imprinting, but because the child and both parents were heterozygous for the SNP we could not discern which parental allele was expressed. We failed to confirm imprinting of *GLI3* because of high PCR failure rate. Further studies are needed to confirm imprinting. Hypomethylation of the promoter of *HOXA4* in matUPD7s associated with increased expression of *HOXA4* compared with controls, while hypermethylation in patUPD7 resulted in decreased expression. *HOXA4* belongs to the cluster of *HOXA* genes on 7p15.2, which is a family of homeodomain containing transcription factors key during embryonic development.<sup>56</sup> Several other genes in the *HOXA* cluster



**Figure 3.** DMRs identified on chromosome 7. The DMRs identified in this study are shown on the left of the chromosome 7 ideogram. Known imprinted genes are highlighted in bold and predicted imprinted genes are highlighted in italics right of the chromosome 7 ideogram.

are also predicted to be imprinted (Fig. 3).<sup>41</sup> The *HOXA* cluster has two lncRNAs known to regulate *HOXA* gene expression: *HOTAIRM1* at the 3' end regulating *HOXA1* (MIM 142955), *HOXA4*, and *HOXA5* at least in myeloid cell development and *HOTTIP* (MIM 614060) at the 5' end regulating *HOXA9* (MIM 142956), *HOXA10* (MIM 142957), *HOXA11* (MIM 142958), and *HOXA13* (MIM 142959).<sup>57,58</sup> Also an antisense lncRNA, *HOXA11\_AS* (27 225 027–27 228 912, 3886 bp) (MIM 607530), lies 192 bp from the 5' end of *HOXA11*. lncRNAs are found in many imprinted gene clusters and have been found to be crucial for the imprinted expression of genes in the imprinted clusters.<sup>44</sup> Further studies on the other *HOXA* genes and lncRNAs in the *HOXA* cluster are needed to clarify if this is a new imprinting cluster and also if the DMR at the promoter of *HOXA4* would also act as an ICR in this region.

For *GLI3*, maternal hypomethylation of the DMR at the TSS resulted in decreased maternal expression. *GLI3* encodes a zinc finger transcription factor that functions as a transcriptional activator and a repressor of the sonic hedgehog signaling pathway, and plays a role in early development.<sup>59</sup> Defects in *GLI3* are found in disorders affecting limb development, resulting in



**Figure 4.** Correlation of methylation levels between pyrosequencing and the Infinium HumanMethylation450K BeadChip. Dots indicate individual samples. (A) *HTR5A*, (B) *HOXA4*.

pre- and postaxial polydactyly and syndactyly of fingers and toes, and variable other malformations of other organs.

For *SVOPL*, maternal hypermethylation at the TSS and 5' UTR resulted in increased maternal expression. Several imprinted genes have methylated DMRs on the active allele, where it is proposed that the methylation inactivates silencing factors.<sup>2</sup> *SVOPL* was found through a sequence similarity search for SLC22 anion transporters, and it shows sequence similarity with the synaptic vesicle protein SVOP, but otherwise little is known of *SVOPL*.<sup>60</sup>

*CARD11*, *PRRI5*, and *SH2B2* showed increased maternal expression and *RPS2P32* of decreased maternal expression, implicating imprinted expression, but we did not see a statistically significant difference between matUPD7s and the controls for these genes. Lack of statistical significance can be due to the small sample sets available for this study and also inter-individual variation seen within the sample sets. *CARD11* acts as a critical signal transducer for NF-kappaB activation in both B and T lymphocytes and plays a crucial role in the antigen-specific immune response in human. The function of *PRRI5* is poorly understood in human, but in the mouse, the expression pattern of *Prr15* closely resembles that of a number of important negative cell cycle regulators and it is suggested that *Prr15* could be involved in controlling cellular proliferation and/or differentiation.<sup>61</sup> *SH2B2* encodes an adaptor protein SH2B2, also known as APS, which belongs to the SH2B protein family, that regulates several signaling pathways and participates in various physiological responses and developmental processes.<sup>62</sup> SH2B2 interacts with insulin receptor substrate 1 (IRS1), IRS2, or Janus kinase 2 (JAK2) to regulate insulin, leptin, and growth hormone signaling. *MAD1L1* and *RARRES2* appeared to be biparentally expressed, as expression was similar in matUPD7s, controls and patUPD7. Imprinting is not completely excluded for these genes, as other tissues, specific isoforms or developmental time points might still reveal parent-of-origin specific differences.<sup>45</sup> For *PONI* and *HTR5A* we did not detect any expression in the blood

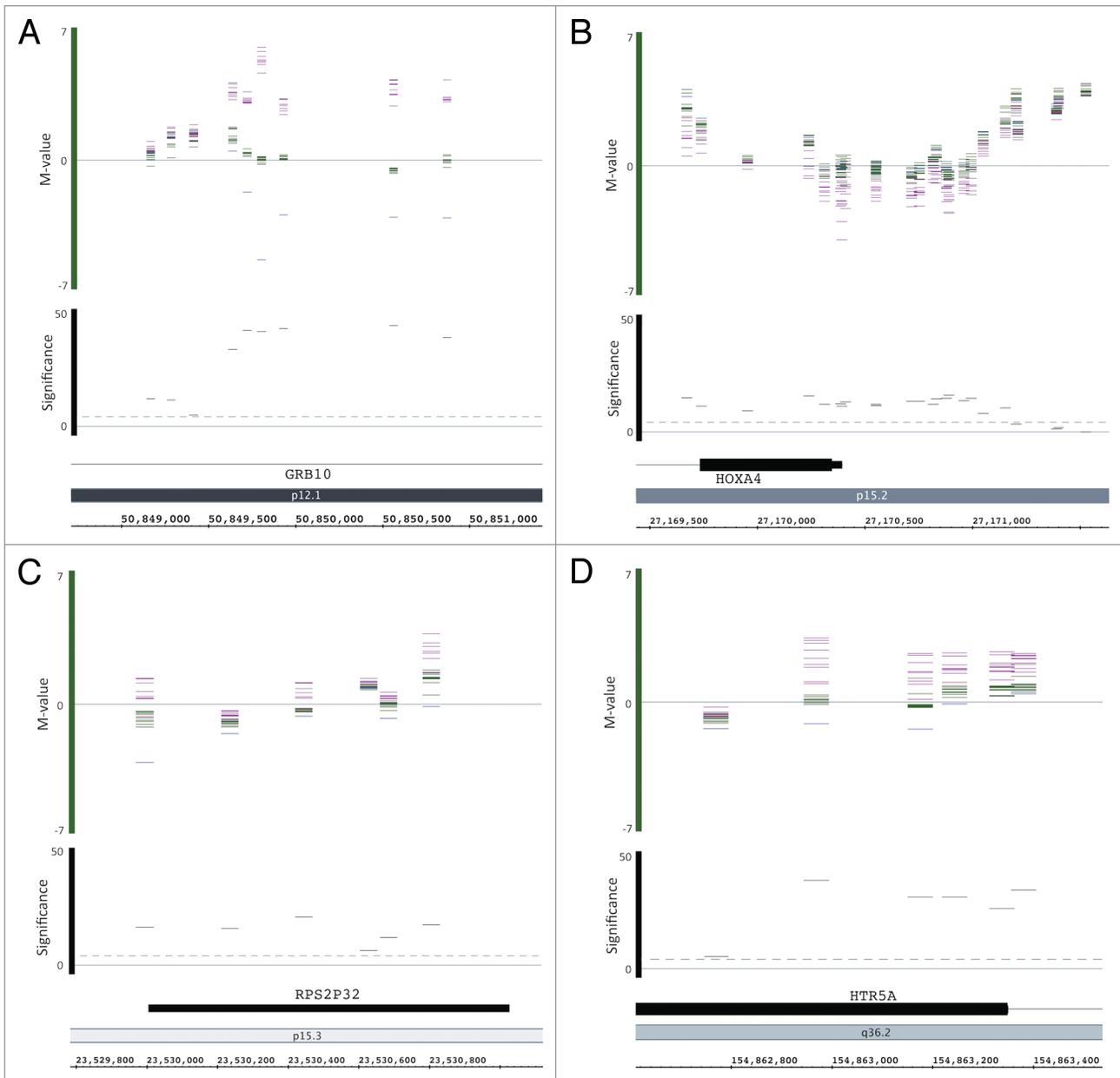
samples available and therefore could not make any conclusion on the effects of the DMRs on their expression.

In conclusion, we have identified 14 novel parent-of origin specific DMRs on human chromosome 7 using a genome wide methylation microarray. Expression study identified a novel imprinted gene *SVOPL* that was confirmed by parent-specific expression and supported the previously suggested genes *HOXA4* and *GLI3* as being imprinted. Imprinted genes on human chromosome 7 are implicated in the etiology of the matUPD7 phenotype of SRS. Interestingly, *HOXA4* and *GLI3* and many of genes close to the novel DMRs have known functions in cellular growth and development, which make them appealing as putative SRS genes in future studies.

## Materials and Methods

### Patients

We obtained fresh blood samples from eight SRS patients with matUPD7, of whom six have been reported before.<sup>14,63-65</sup> and two are first reported in this study as well as a SRS patient with segmental matUPD7q31-qter<sup>26</sup> and one individual with patUPD7.<sup>16</sup> SRS patients were recruited from the Hospital for Children and Adolescents, Helsinki University Central Hospital, Finland. Additionally three patients were referred from the Oulu University Central Hospital and one from the Päijät-Häme Central Hospital, Finland. All patients were seen by a pediatric endocrinologist and diagnosis of SRS was based on clinical features and matUPD7 was verified by microsatellite markers as described before.<sup>63,64</sup> Control samples from ten unrelated normal height adults were obtained. For parent-of-origin allele-specific expression analysis we obtained fresh blood samples from nine parent-child trios. All children were from our growth retardation study cohort and had previously been excluded from having matUPD7, as described before.<sup>64</sup> Seven children had H19 hypomethylation and SRS,<sup>14</sup> one was a normally growing sister of a

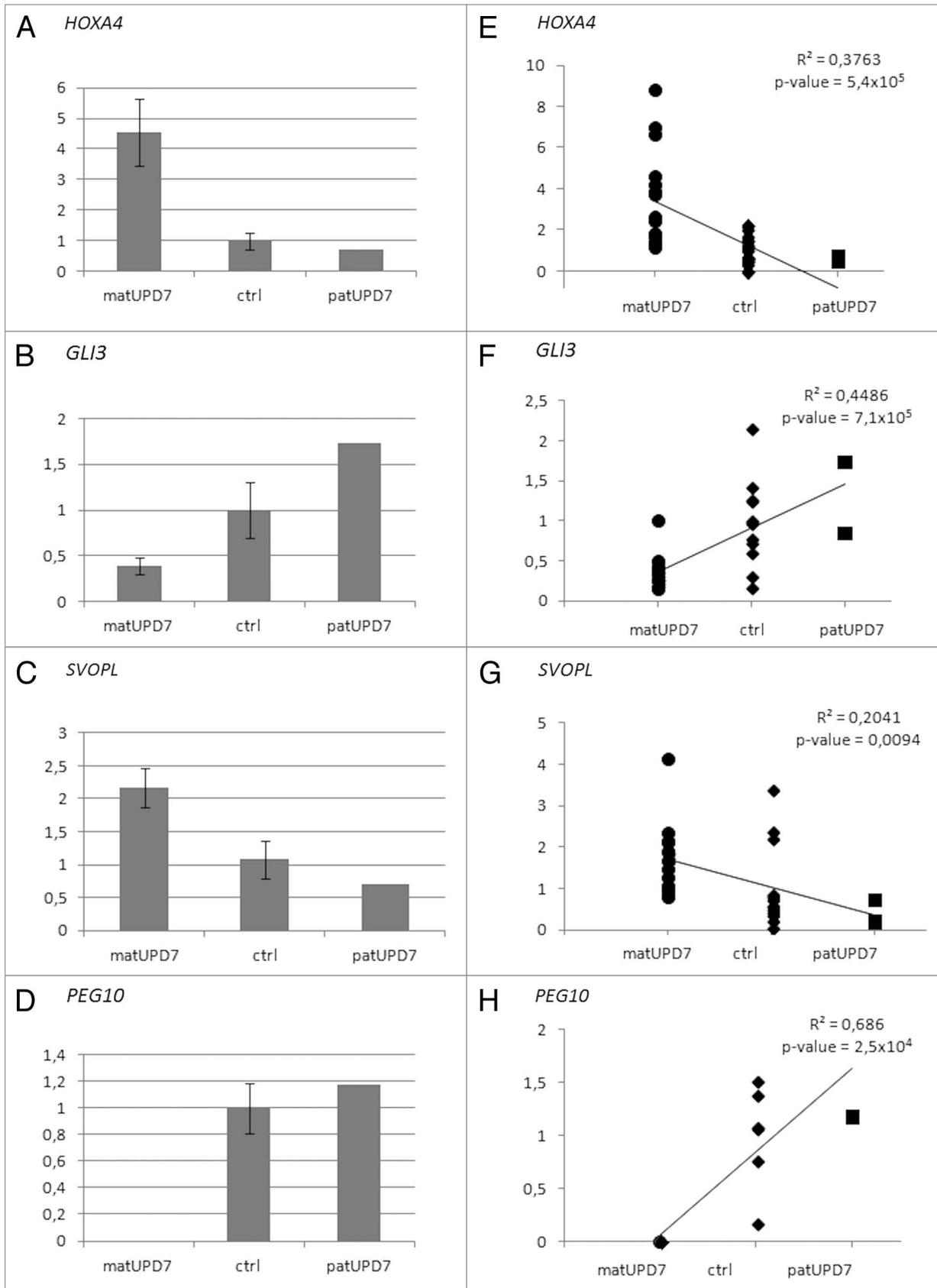


**Figure 5.** Infinium HumanMethylation450K BeadChip methylation levels visualized by the Integrated Genome Browser (IGB) for (A) *GRB10*, (B) *HOXA4*, (C) *RPS2P32*, and (D) *HTR5A*. Bars show the M-values of individual samples for each CpG in the DMRs along the chromosome (horizontal axis). Bar color indicates sample type: purple, matUPD7s; green, controls; and blue patUPD7. M-value 0 indicates 50% methylation, positive values hypermethylation and negative values hypomethylation. The significance level (-log<sub>10</sub>) of matUPD7s vs. controls for each CpG is shown by the black bars. The dashed line indicates the significance threshold of *P* value 0.05. Figures were modified from the IGB output, and the CpG locations are approximations showing a 50 bp region calculated +/-25 bp from the Illumina annotation file coordinate corresponding to the genomic position of the C in CG dinucleotide, according to Genome build 37. The partial matUPD7 sample was excluded for genes located outside the partial matUPD7 region: *GRB10*, *HOXA4*, and *RPS2P32*.

matUPD7 patient and one had postnatal growth retardation with no dysmorphic features. This study was approved by the Ethical Review Board of the Hospital for Children and Adolescents, Helsinki University Central Hospital, Helsinki, Finland. Written informed consent was obtained from all study participants. All clinical investigations have been conducted according to the principles expressed in the Declaration of Helsinki.

#### Genome-wide methylation analysis with the Infinium HumanMethylation450K BeadChip

Genomic DNA was extracted from fresh EDTA-blood samples with the FlexiGene DNA Kit (Qiagen) according to the manufacturer's instructions. 500 ng of DNA from each subject was bisulfite converted with the EZ-96 DNA Methylation Kit (Zymo research Corporation) according to the manufacturer's instructions. Array-based specific DNA methylation analysis was performed



**Figure 6.** Expression differences of differentially methylated genes. (A) *HOXA4* (B) *GLI3*, (C) *SVOPL*, and (D) *PEG10*. Error bars indicate standard error of the mean for the matUPD7s and controls. PatUPD7 is a single sample. Regression plots for (E) *HOXA4*, (F) *GLI3*, (G) *SVOPL*, and (H) *PEG10*. Combined data from two replicate Taqman qPCRs are included for *HOXA4*, *GLI3*, and *SVOPL*. Only one Taqman qPCR was run for *PEG10*.

with the Infinium HumanMethylation450K BeadChip technology (Illumina). All samples were analyzed for more than 450 000 CpG sites at single nucleotide resolution with 99% coverage of RefSeq genes and 96% coverage of CGIs. The CpGs were distributed in CGI shelves, CGI shores, CGIs, promoter regions 5'UTRs, first exon, gene body, and 3'UTRs. Bisulfite-treated genomic DNA was whole-genome amplified and hybridized to the HumanMethylation450 BeadChips (Illumina) and scanned using the Illumina iScan at the Bioinformatic and Expression Analysis (BEA) Core Facility of the Karolinska Institutet. The intensity of the images was extracted with the GenomeStudio Methylation Software Module (v 1.9.0, Illumina).

#### Quality Control analysis and data validation

Quality control was conducted in GenomeStudio software (v2011.1) using the methylation module (v1.9.0) according to the manufacturer's recommendations (Illumina). Briefly, the controls included assessment of DNP and Biotin staining, hybridization, target removal, extension, bisulfite conversion, G/T mismatch, and negative and non-polymorphic controls. The various controls indicated overall good quality of DNA preparations and chip performance.

#### Bioinformatics analysis of Infinium HumanMethylation-450K BeadChip data

The analysis of the HumanMethylation450 BeadChips was performed as previously described.<sup>46</sup> Briefly, the raw data were analyzed with the BioConductor package lumi in R v. 2.13.<sup>66</sup> The data was adjusted for color channel imbalance and background noise, and normalized according to the quantile method. Probe-wise differential methylation was assayed by linear model followed by pair-wise comparisons by empirical Bayes *t* test on the normalized M values.<sup>67</sup> The M-value is calculated as the log<sub>2</sub> ratio of the intensities of methylated probe vs. unmethylated probe and describes a measurement of how much more a probe is methylated compared with unmethylated.<sup>68</sup> A value close to 0 indicates a similar intensity between the methylated and unmethylated probes, which means the CpG site is about half-methylated.<sup>68</sup> Positive M-values mean that more molecules are methylated than unmethylated, while negative M-values mean that more molecules are unmethylated. The M values give higher resolution than the  $\beta$  values for extreme hyper- and hypomethylation levels, whereas at middle-range methylation levels they are collinear.

#### Process of filtering for differentially methylated regions

All 30 017 CpGs on chromosome 7 were filtered according to a three-step process. For maternally hypermethylated CpGs, step-1 of the filtering process was passed if the median M-value of all eight matUPD7s was larger than that of the ten controls and median M-value of controls was larger than the M-value of the patUPD7 sample. No specific threshold was set and the filter was passed even with a minimal difference between the groups. For maternally hypomethylated CpGs, step-1 of the filtering process was passed if M-value of the patUPD7 sample was larger than the median M-value of the controls, and median M-value of the controls was larger than that of the matUPD7 samples. Step-2 of the filtering process excluded all CpGs, where the differential methylation between matUPD7s and controls did not

reach the empirical Bayes significance (nominal *P* value < 0.05). The step-3 in the filtering process was set to include only those adjacent CpGs which were included in a consecutive row of at least three CpGs which also had passed steps one and two of the filtering process. Adjacent CpGs were considered as CpGs consecutively mapped in a given chromosomal region after one another (i.e., with no other probes in between them). A genomic distance was not used to define adjacency.

#### CpGs in regard to CGIs, genes, TSS, Promoters, CTCF binding sites

The loci of the CpGs in regard to CGIs, TSSs, promoters, and CTCF binding sites were obtained from the Infinium Human Methylation450K BeadChip annotation files and from the UCSC genome browser. Localization of DMRs to lncRNAs was determined according to UCSC genome browser.

#### Data visualization

Genome-wide visualization of the global methylation data was performed with the IGB version 6.5.1/6.5.1\_5.<sup>47</sup>

#### Pyrosequencing

Two micrograms of DNA was bisulfite-converted with EpiTect Bisulfite Kit (Qiagen) from all matUPD7, patUPD7, and controls. An amount of 10–20 ng of bisulfite modified DNA was used for PCR amplification. Primers were designed by PyroMark Assay Design SW 2.0 to encompass the DMR regions identified by the Infinium 450K Bead chip for *HTR5A*, *RPS2P32*, and *HOXA4*. Specific primer sequences will be given upon request. PCR was performed in standard conditions with 10  $\mu$ M primers and successful PCR was verified by 1% agarose gel electrophoresis. Pyrosequencing was performed on the PyroMark Q24 system (Qiagen), according to the manufacturer's instructions. Methylation level of the CpGs was analyzed with the PyroMark Q24 software (Qiagen).

Correlation between the pyrosequencing and the Infinium HumanMethylation450K Bead chip results was done by converting the M-values of the representative CpG sites obtained from the Infinium 450K Bead chip to Beta-values, using an approximation method.<sup>68</sup> Beta-values of the CpG sites were then compared with the methylation percentage derived from pyrosequencing experiments.

#### Quantitative PCR

Fresh whole blood samples were collected from all matUPD7, patUPD7, and controls into PAXgene Blood RNA tubes (PreAnalytiX, GmbH). RNA was extracted with the PAXgene Blood miRNA Kit (PreAnalytiX, GmbH), according to the manufacturer's instructions. RNA quality was checked with Bioanalyzer (Agilent Technologies) and all samples had a RIN (RNA integrity number) value above eight.

Reverse transcription (RT) was performed using TaqMan RT reagents according to standard protocols (Applied Biosystems). qPCR reactions were run in triplicate 10  $\mu$ l reactions, using 10 ng of reverse transcribed RNA and the standard reaction conditions for TaqMan Fast Advanced Master Mix and the TaqMan Gene Expression Assays: *MAD1L1* (Hs002069119\_m1), *CARD11* (Hs00269119\_m1), *RPS2P32* (Hs01010823\_g1), *HOXA4* (Hs01573270\_m1), *PRR15* (Hs00828414\_m1), *GLI3* (Hs00609233\_n1), *PEG10* (Hs01122880\_m1), *PONI*

(Hs00166557\_m1), *SH2B2* (Hs00184134\_m1), *SVOPL* (Hs00736451\_m1), *HTR5A* (Hs00225153\_m1), and *RARRES2* (Hs00161209\_g1) were used (Applied Biosystems). The PCR runs were performed with the 7900HT Sequence Detection System (Applied Biosystems) and the results were calculated with the comparative  $C_T$  method with standard protocols.

The Student's T-test was used to calculate significance between matUPD7s and controls. ANOVA was used to calculate significance for regression plots. The partial matUPD7q31-qter was included in the matUPD7 group only for genes in the 7q31-qter region, *SVOPL1* and *RARRES2*, otherwise the sample was excluded from the analyses.

#### Parent-of-origin allele-specific expression analyses

The HapMart data mining tool was used to select exonic SNPs within *HOXA4*, *GLI3*, and *SVOPL*, that had a minor allele frequency greater than zero reported for the CEPH (CEU) population. Primers were designed by PrimerZ and Primer-BLAST. Specific primer sequences are available upon request.

Genomic DNA from nine parent-child trios was extracted from fresh EDTA-blood samples with the FlexiGene DNA Kit (Qiagen), according to the manufacturer's instructions. Fresh whole blood samples from the children were obtained in PAXgene Blood RNA tubes (PreAnalytiX, GmbH). RNA was extracted with the PAXgene Blood miRNA Kit (PreAnalytiX, GmbH), according to the manufacturer's instructions. Reverse

transcription was performed by High Capacity Reverse Transcription Kit (Applied Biosciences), according to the manufacturer's protocol. Genomic DNA and cDNA were amplified by PCR using Phusion High Fidelity DNA polymerase (Thermo Scientific) by standard protocols. Products were cleaned with EXO-SAP IT® (USB) and sequenced. Genotypes were viewed using FinchTV (1.5.0, Geospiza).

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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#### Supplemental Materials

Supplemental materials may be found here: [www.landesbioscience.com/journals/epigenetics/article/27160](http://www.landesbioscience.com/journals/epigenetics/article/27160)

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