

**Abstract**

Human chromosome 14q32.2 harbors the germline-derived primary *DLKI-MEG3* intergenic differentially methylated region (IG-DMR) and the postfertilization-derived secondary *MEG3-DMR*, together with multiple imprinted genes. Although previous studies in cases with microdeletions and epimutations affecting both DMRs and paternal/maternal uniparental disomy 14 (upd(14)pat/mat)-like phenotypes argue for a critical regulatory function of the two DMRs for the 14q32.2 imprinted region, the precise role of individual DMR remains to be clarified. We studied an infant with upd(14)pat body and placental phenotypes (patient 1) and the mother with upd(14)mat-like body phenotype (patient 2), and a neonate with upd(14)pat body, but no placental, phenotype (patient 3). Structural analysis showed a familial 8,558 bp microdeletion involving the IG-DMR alone in patients 1 and 2, and a *de novo* 4,303 bp microdeletion involving the *MEG3-DMR* alone in patient 3. Methylation and expression analyses revealed that loss of the hypomethylated IG-DMR of maternal origin in patient 1 was associated with epimutation (hypermethylation) of the *MEG3-DMR* in the body and caused paternalization of the imprinted region in examined body and placental tissues, whereas loss of the hypomethylated *MEG3-DMR* of maternal origin in patient 3 permitted normal methylation pattern of the IG-DMR and resulted in maternal to paternal epigenotypic alteration in examined body tissues. The imprinting status appeared normal in patient 2. These results, together with the finding that the IG-DMR remains as a DMR and the *MEG3-DMR* exhibits a non-DMR in the placenta, imply that the IG-DMR and the *MEG3-DMR* function as imprinting control centers in the placenta and the body, respectively, with a hierarchical interaction for the methylation pattern in the body. In addition, the phenotype of patient 2 may suggest the presence of a *cis*-acting regulatory element for *DLKI* expression around the IG-DMR.

**Author Summary**

Human chromosome 14q32.2 imprinted region harbors the germline-derived primary *DLK1-MEG3* intergenic differentially methylated region (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR, together with multiple imprinted genes. Consistent with this, paternal and maternal uniparental disomy 14 (upd(14)pat and upd(14)mat) causes distinct phenotypes. Here, we show that the IG-DMR acts as an upstream regulator for the methylation pattern of the *MEG3*-DMR in the body but not in the placenta, and that the IG-DMR and the *MEG3*-DMR function as imprinting control centers in the placenta and the body, respectively. To our knowledge, this is the first study demonstrating not only different roles between the primary and the secondary DMRs at a single imprinted region, but also an essential regulatory function for the secondary DMR. Thus, the results provide significant advance in the clarification of underlying mechanisms involved in the imprinting regulation at the 14q32.2 region and the development of upd(14)pat/mat phenotype. In addition, we also suggest the presence of a *cis*-acting regulatory element for the *DLK1* expression around the IG-DMR.

## Introduction

Human chromosome 14q32.2 carries a cluster of protein-coding paternally expressed genes (*PEGs*) such as *DLK1* and *RTL1* and non-coding maternally expressed genes (*MEGs*) such as *MEG3* (alias, *GTL2*), *RTL1as* (*RTL1* antisense), *MEG8*, *snoRNAs*, and *microRNAs* [1,2]. Consistent with this, paternal uniparental disomy 14 (upd(14)pat) results in a unique phenotype characterized by facial abnormality, small bell-shaped thorax, abdominal wall defects, placentomegaly, and polyhydramnios [2,3], and maternal uniparental disomy 14 (upd(14)mat) leads to less-characteristic but clinically discernible features including growth failure [2,4].

The 14q32.2 imprinted region also harbors two differentially methylated regions (DMRs), i.e., the germline-derived primary *DLK1-MEG3* intergenic DMR (IG-DMR) and the postfertilization-derived secondary *MEG3*-DMR [1,2]. Both DMRs are hypermethylated after paternal transmission and hypomethylated after maternal transmission in the body, whereas in the placenta the IG-DMR alone remains as a DMR and the *MEG3*-DMR is rather hypomethylated [1,2]. Furthermore, previous studies in cases with upd(14)pat/mat-like phenotypes have revealed that epimutations (hypermethylation) and microdeletions affecting both DMRs of maternal origin cause paternalization of the 14q32.2 imprinted region, and that epimutations (hypomethylation) affecting both DMRs of paternal origin cause maternalization of the 14q32.2 imprinted region, while microdeletions involving the DMRs of paternal origin have no effect on the imprinting status [2,5–8]. These findings, together with the notion that parent-of-origin specific expression patterns of imprinted genes are primarily dependent on the methylation status of the DMRs [9], argue for a critical regulatory function of the two DMRs for the 14q32.2 imprinted region, with possible different effects between the body and the placenta.

However, the precise role of individual DMR remains to be clarified. Here, we report that the IG-DMR and the *MEG3*-DMR show a hierarchical interaction for the methylation pattern in the body, and function as imprinting control centers in the placenta and the body, respectively. To our knowledge, this is the first study demonstrating not only different roles between the primary and secondary DMRs at a single imprinted region, but also an essential regulatory function for the secondary DMR.

## Results

### Clinical reports

We identified familial cases, a proband (patient 1) and the mother (patient 2), and a sporadic case (patient 3). Detailed phenotypes of patients 1 and 3 are summarized in Table 1 (see also Figure 1), and those of patient 2 are summarized in Table 2. In brief, patient 1 was delivered by a caesarean section at 33 weeks of gestation due to progressive polyhydramnios despite amnioreduction at 28 and 30 weeks of gestation, whereas patient 3 was born at 28 weeks of gestation by a vaginal delivery due to progressive labor without discernible polyhydramnios. Placentomegaly was observed in patient 1 but not in patient 3. Patients 1 and 3 were found to have characteristic face, small bell-shaped thorax with coat hanger appearance of the ribs, and omphalocele. Patient 1 received surgical treatment for omphalocele immediately after birth and mechanical ventilation for several months. At present, she is 5.5 months of age, and still requires intensive care including oxygen administration and tube feeding. Patient 3 died at four days of age due to massive intracranial hemorrhage, while receiving intensive care including mechanical ventilation. Thus, upd(14)pat body phenotype was unequivocally exhibited by patients 1 and 3, whereas upd(14)pat placental phenotype, which has invariably been identified by 28 weeks of gestation in upd(14)pat patients [2,3], was present in patient 1 and absent from patient 3. Patient 2 was noticed to have upd(14)mat-like body phenotype including short stature, obesity, and small hands, through familial studies. The father of patient 1 and the parents of patient 3 were clinically normal.

### Sample preparation

We isolated genomic DNA (gDNA) and transcripts (*mRNAs*, *snoRNAs*, and *microRNAs*) from fresh leukocytes of patients 1 and 2, the father of patient 1, and the parents of patient 3, from fresh skin fibroblasts of patient 3, and from formalin-fixed and paraffin-embedded placental samples of patient 1 and similarly treated pituitary and adrenal samples of patient 3 (although multiple body tissues were available in patient 3, useful gDNA and transcript samples were not obtained from other tissues probably due to drastic post-mortem degradation). We also made metaphase spreads from leukocytes and skin fibroblasts. For comparison, we obtained

control samples from fresh normal adult leukocytes, neonatal skin fibroblasts, and placenta at 38 weeks of gestation, and from fresh leukocytes of upd(14)pat/mat patients and formalin-fixed and paraffin-embedded placenta of a upd(14)pat patient [2,3].

#### Structural analysis of the imprinted region

We first examined the structure of the 14q32.2 imprinted region (Figure 2). Upd(14) was excluded in patients 1–3 by microsatellite analysis (Table S1), and FISH analysis for the two DMRs identified a familial heterozygous deletion encompassing the IG-DMR alone in patients 1 and 2 and a *de novo* heterozygous deletion encompassing the *MEG3*-DMR alone in patient 3 (Figure 2). The microdeletions were further localized by SNP genotyping for 66 loci (Table S1) and quantitative real-time PCR (q-PCR) analysis for four regions around the DMRs (Figure S1A), and serial direct sequencing for the long PCR products harboring the deletion junctions successfully identified the fusion points of the microdeletions in patients 1–3 (Figure 2). According to the NT\_026437 sequence data at the NCBI Database (Genome Build 36.3) (<http://preview.ncbi.nlm.nih.gov/guide/>), the deletion size was 8,558 bp (82,270,449–82,279,006 bp) for the microdeletion in patients 1 and 2, and 4,303 bp (82,290,978–82,295,280 bp) for the microdeletion in patient 3. The microdeletion in patient 3 also involved the 5' part of *MEG3* and five of the seven putative CTCF binding sites A–G [13], and was accompanied by insertion of a 66 bp sequence duplicated from *MEG3* intron 5 (82,299,727–82,299,792 bp on NT\_026437). Direct sequencing of the exonic or transcribed regions detected no mutation in *DLK1*, *MEG3*, and *RTL1*, although several cDNA polymorphisms (cSNPs) were identified (Table S1). Oligoarray comparative genomic hybridization identified no other discernible structural abnormality (Figure S1B).

#### Methylation analysis of the two DMRs

We next studied methylation patterns of the previously reported IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7) [2] and those of the seven putative CTCF binding sites, using bisulfite treated gDNA samples (Figure 3A). Bisulfite sequencing and combined bisulfite restriction analysis using body samples revealed a hypermethylated IG-DMR and *MEG3*-DMR

in patient 1, a hypomethylated IG-DMR and differentially methylated *MEG3*-DMR in patient 2, and a differentially methylated IG-DMR and hypermethylated *MEG3*-DMR in patient 3, and bisulfite sequencing using placental samples showed a hypermethylated IG-DMR and rather hypomethylated *MEG3*-DMR in patient 1 (Figure 3B). Furthermore, bisulfite sequencing revealed that, of the seven putative CTCF binding sites, sites C and D exhibited methylation patterns comparable to those of CG7 (Figure 3C).

#### Expression analysis of the imprinted genes

Finally, we performed expression analyses, using standard reverse transcriptase (RT)-PCR and/or q-PCR analysis for multiple imprinted genes in this region (Figure 4A–C). For leukocytes, weak expression was detected for *MEG3* and *SNORD114-29* in a control subject and patient 2 but not in patient 1. For skin fibroblasts, although all *MEGs* but no *PEGs* were expressed in control subjects, neither *MEGs* nor *PEGs* were expressed in patient 3. For placentas, although all imprinted genes were expressed in control subjects, *PEGs* only were expressed in patient 1. For the pituitary and adrenal of patient 3, *DLK1* expression alone was identified.

Expression pattern analyses using informative cSNPs revealed monoallelic *MEG3* expression in the leukocytes of patient 2 (Figure 4D), and biparental *RTL1* expression in the placenta of patient 1 (no informative cSNP was detected for *DLK1*) and biparental *DLK1* expression in the pituitary and adrenal of patient 3 (*RTL1* was not expressed in the pituitary and adrenal) (Figure 4E), as well as maternal *MEG3* expression in the control leukocytes and paternal *RTL1* expression in the control placentas (Figure S2). Although we also attempted q-PCR analysis, precise assessment was impossible for *MEG3* in patient 2 because of faint expression level in leukocytes and for *RTL1* in patient 1 and *DLK1* in patient 3 because of poor quality of mRNAs obtained from formalin-fixed and paraffin-embedded tissues.

#### Discussion

The data of the present study are summarized in Figure 5. Parental origin of the microdeletion positive chromosomes is based on the methylation patterns of the preserved

DMRs in patients 1–3 as well as maternal transmission in patient 1. Loss of the hypomethylated IG-DMR of maternal origin in patient 1 was associated with epimutation (hypermethylation) of the *MEG3*-DMR in the body and caused paternalization of the imprinted region and typical upd(14)pat body and placental phenotypes, whereas loss of the hypomethylated *MEG3*-DMR of maternal origin in patient 3 permitted normal methylation pattern of the IG-DMR in the body and resulted in maternal to paternal epigenotypic alteration and typical upd(14)pat body, but no placental, phenotype. In this regard, while a 66 bp segment was inserted in patient 3, this segment contains no known regulatory sequence [14] or evolutionarily conserved element [15] (also examined with a VISTA program, <http://genome.lbl.gov/vista/index.shtml>). Similarly, while no control samples were available for pituitary and adrenal, the previous study in human subjects has shown paternal *DLKI* expression in adrenal as well as monoallelic *DLKI* and *MEG3* expressions in various tissues [14]. Furthermore, the present and the previous studies [2] indicate that this region is imprinted in the placenta as well as in the body. Thus, these results, in conjunction with the finding that the IG-DMR remains as a DMR and the *MEG3*-DMR exhibits a non-DMR in the placenta [2], imply the following: (1) the IG-DMR functions hierarchically as an upstream regulator for the methylation pattern of the *MEG3*-DMR on the maternally inherited chromosome in the body, but not in the placenta; (2) the hypomethylated *MEG3*-DMR functions as an essential imprinting regulator for both *PEGs* and *MEGs* in the body; and (3) in the placenta, the hypomethylated IG-DMR directly controls the imprinting pattern of both *PEGs* and *MEGs*. These notions also explain the epigenotypic alteration in the previous cases with epimutations or microdeletions affecting both DMRs (Figure S3).

For the *MEG3*-DMR, the CTCF binding sites C and D may play a pivotal role in the imprinting regulation. The methylation analysis indicates that the two sites reside within the *MEG3*-DMR, and it is known that the CTCF protein with versatile functions preferentially binds to unmethylated target sequences including the sites C and D [13,16–18]. In this regard, all the *MEGs* in this imprinted region can be transcribed together in the same orientation and show a strikingly similar tissue expressions pattern [1,15], whereas *PEGs* are transcribed in different directions and are co-expressed with *MEGs* only in limited cell-types [1,19]. It is possible, therefore, that preferential CTCF binding to the grossly unmethylated sites C and D

activates all the *MEGs* as a large transcription unit and represses all the *PEGs* perhaps by influencing chromatin structure and histone modification independently of the effects of expressed *MEGs*. In support of this, CTCF protein acts as a transcriptional activator for *Gtl2* (the mouse homolog for *MEG3*) in the mouse [20].

Patient 2 had upd(14)mat-like body phenotype. This may be co-incidental, because her clinical features are not specific to upd(14)mat. Indeed, consistent with the notion that loss of the paternally derived IG-DMR does not affect the imprinted status [2,21], *MEG3* showed normal monoallelic expression in the presence of the differentially methylated *MEG3*-DMR. However, since upd(14)mat phenotype is primarily ascribed to loss of functional *DLK1* with an additional effect of loss of functional *RTL1* (Figure S3B) [2,22,23], the microdeletion involving the IG-DMR may have affected a *cis*-acting regulatory element for *DLK1* expression (Figure 5). In this case, the microdeletion is predicted to affect *DLK1* expression on the paternalized chromosome of maternal origin in patient 1. However, this is not inconsistent with the typical upd(14)pat phenotype in patient 1, because patient 1 had clear biparental *RTL1* expression in the absence of *MEGs* expression. Indeed, typical upd(14)pat body and placental phenotype has primarily been ascribed to markedly elevated *RTL1* expression, which is explained by the synergic effect of two active copies of *RTL1* and the absence of functional *RTL1as* as a repressor for *RTL1* [21,23–25], rather than to doubled *DLK1* expression (Figure S3A) [2].

This imprinted region has also been studied in the mouse. Clinical and molecular findings in wildtype mice [1,26,27], mice with PatDi(12) (paternal disomy for chromosome 12 harboring this imprinted region) [28–30], and mice with targeted deletions for the IG-DMR ( $\Delta$ IG-DMR) [21,26] and for the *Gtl2*-DMR (the mouse homolog for the *MEG3*-DMR) ( $\Delta$ *Gtl2*-DMR) [31] are summarized in Table 3. These data, together with human data, provide several informative findings. First, in both the human and the mouse, the IG-DMR is differentially methylated in both the body and the placenta, whereas the *MEG3/Gtl2*-DMR is differentially methylated in the body and exhibits non-DMR in the placenta. Second, the IG-DMR and the *MEG3/Gtl2*-DMR show a hierarchical interaction on the maternally derived chromosome in both the human and the mouse bodies. Indeed, the *MEG3/Gtl2*-DMR is epimutated in patient 1 and mice with maternally inherited  $\Delta$ IG-DMR, and the IG-DMR is



normally methylated in patient 3 and mice with maternally inherited  $\Delta Gtl2$ -DMR. Third, the function of the IG-DMR is comparable between human and mouse bodies and different between human and mouse placentas. Indeed, patient 1 has upd(14)pat body and placental phenotypes, whereas mice with the  $\Delta IG$ -DMR of maternal origin have PatDi(12)-compatible body phenotype and apparently normal placental phenotype. It is likely that imprinting regulation in the mouse placenta is contributed by some mechanism(s) other than the methylation pattern of the IG-DMR, such as chromatin conformation [26,32,33].

Unfortunately, however, the data of  $\Delta Gtl2$ -DMR mice appears to be drastically complicated by the retained neomycin cassette in the upstream region of *Gtl2*. Indeed, it has been shown that the insertion of a *lacZ* gene or a neomycin gene in the similar upstream region of *Gtl2* causes severely dysregulated expression patterns and abnormal phenotypes after both paternal and maternal transmissions [34,35], and that deletion of the inserted neomycin gene results in apparently normal expression patterns and phenotypes after both paternal and maternal transmissions [35]. (In this regard, although a possible influence of the inserted 66 bp segment can not be excluded formally in patient 3, phenotype and expression data in patient 3 are compatible with simple paternalization of the imprinted region.) In addition, since the apparently normal phenotype in mice homozygous for  $\Delta Gtl2$ -DMR is reminiscent of that in sheep homozygous for the callipyge mutation [36], a complicated mechanism(s) such as the polar overdominance may be operating in the  $\Delta Gtl2$ -DMR mice [37]. Thus, it remains to be clarified whether the *MEG3/Gtl2*-DMR has a similar or different function between the human and the mouse.

In summary, the results show a hierarchical interaction and distinct functional properties of the IG-DMR and the *MEG3*-DMR in imprinting control. Thus, this study provides significant advance in the clarification of underlying mechanisms involved in the imprinting regulation at the 14q32.2 imprinted region and the development of upd(14)pat/mat phenotype.

## Methods

### Ethics Statement

This study was approved by the Institutional Review Board Committees at National Center for Child health and Development, University College Dublin, and Dokkyo University School of Medicine, and performed after obtaining written informed consent.

### Primers

All the primers utilized in this study are summarized in Table S2.

### Sample preparation

For leukocytes and skin fibroblasts, genomic DNA (gDNA) samples were extracted with FlexiGene DNA Kit (Qiagen), and RNA samples were prepared with RNeasy Plus Mini (Qiagen) for *DLK1*, *MEG3*, *RTL1*, *MEG8* and *snoRNAs*, and with mirVana™ miRNA Isolation Kit (Ambion) for *microRNAs*. For paraffin-embedded tissues including the placenta, brain, lung, heart, liver, spleen, kidney, bladder, and small intestine, gDNA and RNA samples were extracted with RecoverAll™ Total Nucleic Acids Isolation Kit (Ambion) using slices of 40 µm thick. For fresh control placental samples, gDNA and RNA were extracted using ISOGEN (Nippon Gene). After treating total RNA samples with DNase, cDNA samples for *DLK1*, *MEG3*, *MEG8*, and *snoRNAs* were prepared with oligo(dT) primers from 1 µg of RNA using Superscript III Reverse Transcriptase (Invitrogen), and those for *microRNAs* were synthesized from 300 ng of RNA using TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems). For *RTL1*, cDNA samples were synthesized with *RTL1*-specific primers that do not amplify *RTL1as*. Control gDNA and cDNA samples were extracted from adult leukocytes and neonatal skin fibroblasts purchased from Takara Bio Inc. Japan, and from a fresh placenta of 38 weeks of gestation. Metaphase spreads were prepared from leukocytes and skin fibroblasts using colcemide (Invitrogen).

### Structural analysis

Microsatellite analysis and SNP genotyping were performed as described previously [2].

For FISH analysis, metaphase spreads were hybridized with a 5,104 bp FISH-1 probe and a 5,182 bp FISH-2 probe produced by long PCR, together with an RP11-566I2 probe for 14q12 used as an internal control [2]. The FISH-1 and FISH-2 probes were labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the RP11-566I2 probe was labeled with biotin and detected by avidin conjugated to fluorescein isothiocyanate. For quantitative real-time PCR analysis, the relative copy number to RNaseP (catalog No: 4316831, Applied Biosystems) was determined by the Taqman real-time PCR method using the probe-primer mix on an ABI PRISM 7000 (Applied Biosystems). To determine the breakpoints of microdeletions, sequence analysis was performed for long PCR products harboring the fusion points, using serial forward primers on the CEQ 8000 autosequencer (Beckman Coulter). Direct sequencing was also performed on the CEQ 8000 autosequencer. Oligoarray comparative genomic hybridization was performed with 1x244K Human Genome Array (catalog No: G4411B) (Agilent Technologies), according to the manufacturer's protocol.

#### Methylation analysis

Methylation analysis was performed for gDNA treated with bisulfite using the EZ DNA Methylation Kit (Zymo Research). After PCR amplification using primer sets that hybridize both methylated and unmethylated clones because of lack of CpG dinucleotides within the primer sequences, the PCR products were digested with appropriate restriction enzymes for combined bisulfite restriction analysis. For bisulfite sequencing, the PCR products were subcloned with TOPO TA Cloning Kit (Invitrogen) and subjected to direct sequencing on the CEQ 8000 autosequencer.

#### Expression analysis

Standard RT-PCR was performed for *DLK1*, *RTL1*, *MEG3*, *MEG8*, and *snoRNAs* using primers hybridizing to exonic or transcribed sequences, and one  $\mu$ l of PCR reaction solutions was loaded onto Gel-Dye Mix (Agilent). Taqman real-time PCR was carried out using the probe-primer mixtures (assay No: Hs00292028 for *MEG3* and Hs00419701 for *MEG8*; assay ID: 001028 for *miR433*, 000452 for *miR127*, 000568 for *miR379*, and 000477 for *miR154*) on

the ABI PRISM 7000. Data were normalized against *GAPDH* (catalog No: 4326317E) for *MEG3* and *MEG8* and against *RNU48* (assay ID: 0010006) for the remaining *miRs*. The expression studies were performed three times for each sample.

To examine the imprinting status of *MEG3* in the leukocytes of patient 2, direct sequence data for informative cSNPs were compared between gDNA and cDNA. To analyze the imprinting status of *RTL1* in the placental sample of patient 1 and that of *DLK1* in the pituitary and adrenal samples of patient 3, RT-PCR products containing exonic cSNPs informative for the parental origin were subcloned with TOPO TA Cloning Kit, and multiple clones were subjected to direct sequencing on the CEQ 8000 autosequencer. Furthermore, *MEG3* expression pattern was examined using leukocyte gDNA and cDNA samples from multiple normal subjects and leukocyte gDNA samples from their mothers, and *RTL1* expression pattern was analyzed using gDNA and cDNA samples from multiple fresh normal placentas and leukocyte gDNA from the mothers.

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**Author Contributions**

Conceived and designed the experiments: MK ACF-S TO. Performed the experiments: MK MF KM FK. Contributed reagents/materials/analysis tool: MJO AJG YW OA NM KM TO. Wrote the paper: TO.

**Competing Interests**

The authors have declared that no competing interests exist.

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## Figure Legends

**Figure 1.** Clinical phenotypes of patients 1 and 3 at birth.

Both patients have bell shaped thorax with coat hanger appearance of the ribs and omphalocele. In patient 1, histological examination of the placenta shows proliferation of dilated and congested chorionic villi, as has previously been observed in a case with upd(14)pat [2]. The horizontal black bar indicates 100  $\mu$ m.

**Figure 2.** Physical map of the 14q32.2 imprinted region and the deleted segments in patients 1–3 (shaded in gray). *PEGs* are shown in blue, *MEGs* in red, and the IG-DMR (CG4 and CG6) and the *MEG3*-DMR (CG7) in green. It remains to be clarified whether *DIO3* is a *PEG*, although mouse *Dio3* is known to be preferentially but not exclusively expressed from a paternally derived chromosome [12]. For *MEG3*, the isoform 2 with nine exons (red bars) and eight introns (light red segment) is shown (Ensembl; <http://www.ensembl.org/index.html>). Electrochromatograms represent the fusion point in patients 1 and 2, and the fusion point accompanied by insertion of a 66 bp segment (highlighted in blue) with a sequence identical to that within *MEG3* intron 5 (the blue bar) in patient 3. Since PCR amplification with primers flanking the 66 bp segment at *MEG3* intron 5 has produced a 194 bp single band in patient 3 as well as in a control subject (shown in the box), this indicates that the 66 bp segment at the fusion point is caused by a duplicated insertion rather than by a transfer from intron 5 to the fusion point (if the 66 bp is transferred from the original position, a 128 bp band as well as a 194 bp band should be present in patient 3) (the marker size: 100, 200, and 300 bp). In the FISH images, the red signals (arrows) have been identified by the FISH-1 probe and the FISH-2 probe, and the light green signals (arrowheads) by the RP11-566I2 probe for 14q12 used as an internal control. The faint signal detected by the FISH-2 probe in patient 3 is consistent with the preservation of a ~1.2 kb region identified by the centromeric portion of the FISH-2 probe.

**Figure 3.** Methylation analysis. Filled and open circles indicate methylated and unmethylated cytosines at the CpG dinucleotides, respectively.

(A) Structure of CG4 and CG6 (the IG-DMR) and CG7 (the *MEG3*-DMR), and sequence of the putative CTCF binding sites [13]. Pat: paternally derived chromosome; and Mat: maternally derived chromosome. The PCR products for CG4 (311 bp) harbor 6 CpG dinucleotides and a G/A SNP (*rs12437020*), and are digested with *Bst*UI into three fragments (33 bp, 18 bp, and 260 bp) when the cytosines at the first and the second CpG dinucleotides and the fourth and the fifth CpG dinucleotides (indicated with orange rectangles) are methylated. The PCR products for CG6 (428 bp) carry 19 CpG dinucleotides and a C/T SNP (*rs10133627*), and are digested with *Taq*I into two fragments (189 bp and 239 bp) when the cytosine at the 9th CpG dinucleotide (indicated with an orange rectangle) is methylated. The PCR products for CG7 harbor 7 CpG dinucleotides, and are digested with *Bst*UI into two fragments (56 bp and 112 bp) when the cytosines at the fourth and the fifth CpG dinucleotides (indicated with orange rectangles) are methylated. These enzymes have been utilized for combined bisulfite restriction analysis (COBRA). For the putative CTCF binding sites A–G, the consensus CTCF binding motifs are shown in red letters; the cytosine residues at the CpG dinucleotides within the CTCF binding motifs are highlighted in blue, and those outside the CTCF binding motifs are highlighted in green.

(B) Methylation analysis of CG4, CG6, and CG7. Left part shows bisulfite sequencing data. The SNP typing data are also denoted for CG4 and CG6. The circles highlighted in orange correspond to those shown in Figure 3A. The relatively long CG6 was not amplified from the formalin-fixed and paraffin-embedded placental samples, probably because of the degradation of genomic DNA. Note that CG4 is differentially methylated in a control placenta and is massively hypermethylated in a upd(14)pat placenta, whereas CG7 is rather hypomethylated in a upd(14)pat placenta as well as in a control placenta. Right part shows COBRA data. U: unmethylated clone specific bands (311 bp for CG4, 428 bp for CG6, and 168 bp for CG7); and M: methylated clone specific bands (260 bp for CG4, 239 bp and 189 bp for CG6, and 112 bp and 56 bp for CG7). The results reproduce of the bisulfite sequencing data, and delineate normal findings of the father of patient 1 and the parents of patient 3.

(C) Methylation analysis of the sites A–G. Left part shows bisulfite sequencing data, using

leukocyte genomic DNA samples. Since PCR products for the site B contain a C/A SNP (*rs11627993*), genotyping data are also indicated. The circles highlighted in blue correspond to those shown in Figure 3A. The sites C and D exhibit clear DMRs. Right part indicates the results of the sites C and D using leukocyte and/or placental genomic DNA samples. The findings are similar to those of CG7.

**Figure 4. Expression analysis.**

- (A) Reverse transcriptase (RT)-PCR analysis. L: leukocytes; SF: skin fibroblasts; and P: placenta. The relatively weak *GAPDH* expression for the formalin-fixed and paraffin-embedded placenta of patient 1 indicates considerable mRNA degradation. Since a single exon was amplified for *DLK1* and *RTL1*, PCR was performed with and without RT for the placenta of patient 1, to exclude the possibility of false positive results caused by genomic DNA contamination.
- (B) Quantitative real-time PCR (q-PCR) analysis of *MEG3*, *MEG8*, and *miRNAs*, using fresh skin fibroblasts (SF) of patient 3 and four control neonates. Of the examined *MEGs*, *miR433* and *miR127* are encoded by *RTL1as*.
- (C) RT-PCR analysis for the formalin-fixed and paraffin-embedded pituitary (Pit.) and the adrenal (Ad.) in patient 3. The bands for *DLK1* are detected in the presence of RT and undetected in the absence of RT, thereby excluding contamination of genomic DNA.
- (D) Monoallelic *MEG3* expression in the leukocytes of patient 2. The three cSNPs are present in a heterozygous status in gDNA and in a hemizygous status in cDNA. D: direct sequence.
- (E) Biparental *RTL1* expression in the placenta of patient 1 and biparental *DLK1* expression in the pituitary and adrenal of patient 3. D: direct sequence; and S: subcloned sequence. In patient 1, genotyping of *RTL1* cSNP (*rs6575805*) using gDNA indicates maternal origin of the “C” allele and paternal origin of the “T” allele, and sequencing analysis using cDNA confirms expression of maternally as well as paternally derived *RTL1*. Similarly, in patient 3, genotyping of *DLK1* cSNP (*rs1802710*) using gDNA denotes maternal origin of the “C” allele and paternal origin of the “T” alleles, and sequencing analysis using cDNA confirms expression of maternally as well as paternally inherited *DLK1*.