Imprinting disorders (IDs) often affect growth in humans, leading to diseases with overlapping features, regardless of the genomic region affected. IDs related to hypomethylation of the human 14q32.2 region and its DLK1/MEG3 domain are associated with Temple syndrome (TS14). TS14 is a rare type of growth retardation, the clinical signs of which overlap considerably with those of Silver-Russell syndrome (SRS), another ID related to IGF2 down-regulation at 11p15.5 region. We show that 14q32.2 hypomethylation affects expression, not only for genes at this locus but also for other imprinted genes, and especially lowers IGF2 levels at 11p15.5. Furthermore, expression of nonimprinted genes is also affected, some of which are also deregulated in SRS patients. These findings highlight the epigenetic regulation of gene expression at the DLK1/MEG3 domain. Expression profiling of TS14 and SRS patients highlights common signatures, which may account for the clinical overlap observed between TS14 and SRS.
Fig. 1. Overlap of clinical features between SRS, TS14, and PWS and DMRs with parent-specific gene expression from the DLK1/MEG3, IGF2/H19, and SNURF/IPW domains. (A) Schematic representation of the overlapping clinical features in SRS, TS14, and PWS patients. Schematic diagram of the regions imprinted in humans (B) the IGF2/H19 domain on 11p15.5 and (C) the DLK1/MEG3 domain of the 14q32.2 region. The relative positions of hairpin-like [pre-microRNA (miRNA)] structures within the miR-379/miR-410 cluster are indicated in the enlargement in the inset and (D) the SNURF/IPW domain on 15q11-q13. PEGs are shown as blue rectangles, and MEGs are shown as pink rectangles. miRNAs and snoRNAs (small nucleolar RNAs) are depicted as stem loops and ovals, respectively. Arrows indicate the direction of transcription. The DMRs ICR1, IG-DMR, MEG3-DMR, and IC, which control monoallelic expression over the domains, are indicated by closed and open lollipops (methylated and unmethylated, respectively). SGA, small for gestational age.
of IG-DMR hypomethylation has yet to be determined in TS14 patients.

We studied gene expression following the hypomethylation of IG-DMR to characterize the effect of this epigenetic alteration on DLK1/MEG3 domain gene expression. We performed expression profiling for imprinted and nonimprinted genes in human fibroblasts from TS14 (IG-DMR hypomethylation) and SRS (ICR1 hypomethylation) patients (Fig. 2) to identify possible gene expression signatures common to these two IDs, which present a major clinical overlap.

**RESULTS**

**TS14 patients’ molecular diagnostics and collected biological materials**

We collected serum from seven TS14 patients with IG-DMR and MEG3-DMR hypomethylation ($n=5$) or 14q32.2 paternal deletion ($n=2$). We also established fibroblast cell cultures for four TS14 patients with IG-DMR hypomethylation, one SRS/TS14 patient with both 11p15.5 ICR1 and IG-DMR hypomethylation, five SRS patients with ICR1 hypomethylation, and five controls (cells were provided by Coriell Cell Repositories). Clinical data and methylation levels for all patients and controls are listed in tables S1 and S2, respectively.

**DLK1 is absent from the serum of TS14 patients but present in that of age-matched controls**

DLK1 is a single-pass transmembrane protein that can be cleaved by extracellular proteases to release a circulating form (18). We assessed the effect of DLK1/MEG3 domain hypomethylation on DLK1 expression by first measuring the circulating levels of DLK1 in the serum of healthy children ($n=38$, 19 boys and 19 girls) between the ages of 0 and 17 years. We found that serum DLK1 levels decreased considerably after birth, but those patients with paternal deletions or hypomethylation of the DLK1/MEG3 domain had barely detectable levels of DLK1, regardless of their sex, age, or the molecular defect at 14q32.2 (Fig. 3A).

**MEG3, MEG8, and miRNAs are up-regulated because of their expression from the normally silenced paternal allele**

We then investigated the levels of transcription of 14q32.2 MEGs in cultured fibroblasts from TS14 and SRS patients (PEGs were not expressed in fibroblasts). We found that the levels of the IncRNA MEG3 and the ncRNA MEG8 in all TS14 and SRS/TS14 fibroblasts were at least twice those in fibroblasts from controls (Fig. 3, B and C). SRS fibroblasts had normal MEG3 levels, but MEG8 levels were twice those in control fibroblasts (Fig. 3, B and C). In addition to MEG3 and MEG8, we quantified nine miRNAs (encoded by genes distributed throughout the 14q32.2 region), all of which were found

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**Fig. 2. Schematic presentation of the patients, biological materials, and strategies used in the study.** ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative polymerase chain reaction; RNA-seq, RNA sequencing.
Fig. 3. Expression profiling of 14q32.2 genes from the serum and fibroblasts of TS14 patients. (A) DLK1 is absent from the serum of TS14 patients but present in that of age-matched controls. Boys and girls are indicated by open triangles and circles, respectively. TS14 patients are represented by black diamonds. (B to D) MEG3, MEG8, and miRNAs are up-regulated in TS14 patient fibroblasts. Relative levels of expression for MEG3, MEG8, and three miRNAs of the DLK1/MEG3 domain in skin-derived fibroblast cultures from TS14, SRS/TS14, and SRS patients, compared with control fibroblasts. (E and F) MEG3 and H19 are biallelically expressed upon the hypomethylation of 14q32.2 and 11p15.5, respectively. Electropherogram showing the informative SNPs rs11160608 and rs217727, within the MEG3 (E) and H19 (F) coding sequences, respectively, on genomic DNA (gDNA) and cDNA. The data shown are mean values ± SEM for five different passages for the SRS/TS14 patient skin-derived fibroblast cultures, four TS14 patients and five SRS patients, with comparison to five donors as a control. *P ≤ 0.05 and **P ≤ 0.01 versus controls, in Mann-Whitney tests.
to be up-regulated in fibroblasts from TS14 and SRS/TS14 patients relative to SRS patients and controls (Fig. 3D shows data for three of the nine miRNAs). We assessed the stability and passage independence of the overexpression of these genes by quantifying the miRNAs at five different passages for each patient. We found that, in all patients, all the ncRNAs and miRNAs studied were stably overexpressed in all cultures (fig. S1, A and B). Furthermore, single-nucleotide polymorphism (SNP) genotyping (Fig. 3E) provided the first evidence of biallelic expression of MEG3 in fibroblasts from patients with TS14, due to reactivation of the normally silenced paternal allele. No SNP assessment was possible for MEG8 or the miRNAs.

**MLIDs at 14q32.2 and 11p15.5 are associated with multi-imprinting relaxation**

Fibroblasts from a patient with SRS/TS14 displaying hypomethylation at both 14q32.2 and 11p15.5 loci displayed biallelic expression of MEG3 from the DLK1/MEG3 domain. We investigated whether MLID induced multi-imprinting relaxation and biallelic expression at the secondary loci affected by sequencing H19 genomic DNA (gDNA) and complementary DNA (cDNA) from SRS/TS14 fibroblasts. We found that H19 was monoallelically expressed in TS14 fibroblasts, whereas it was biallelically expressed in SRS and SRS/TS14 fibroblasts (Fig. 3F).

**11p15.5 and 15q11-q13 PEGs are down-regulated by 14q32.2 hypomethylation**

We then studied the expression of a large number of imprinted genes from loci involved in growth control in TS14, SRS, and control fibroblasts. For most of the PEGs, expression was barely detectable (PEG3, PEG10, and GNAS-XLA), whereas MEGs were highly variable (H19) or comparable to controls (UBE3A and GRB10) in fibroblast cultures (fig. S1C). We found that the 11p15.5 (IGF2) and 15q11-q13 (SNURF and IPW) PEGs were strongly and stably expressed in cultures of fibroblasts from controls and patients. All TS14 fibroblasts displayed low levels of IGF2 expression, as did SRS and SRS/TS14 fibroblasts. Furthermore, both TS14 and SRS fibroblasts displayed low levels of SNURF expression, whereas only TS14 fibroblasts had low levels of IPW expression (Fig. 4). Similar to the ncRNAs and miRNAs, IGF2, SNURF, and IPW were all stably down-regulated in all fibroblast cultures, at various passages, for all patients (Fig. S1D).

**MEG3 and MEG8 overexpression and knockdown in control fibroblasts are associated with deregulation of the expression of 11p15.5 and 15q11-q13 PEGs**

To study whether the clinical overlap could be explained by the cross-regulation between the three imprinted regions, we investigated the possible role of 14q32.2 ncRNAs MEG3 and MEG8 in the changes in IGF2, SNURF, and IPW expression. For this purpose, we carried out overexpression and knockdown of MEG3 and/or MEG8 in a control primary fibroblast line.

Expression constructs were available only for MEG3. For MEG8, we inserted the full-length MEG8 cDNA obtained from fibroblasts into an expression vector. MEG8 cDNA amplification revealed the presence of two different transcripts: a 497-base pair (bp) transcript corresponding to NR_024149.2 and a previously unreported 639-bp transcript (GenBank KX237564; fig. S2). This new variant has an additional exon, located between exons 1 and 2 of the NR_024149.2 transcript, flanked by donor and acceptor sites for splicing.

We found that the concomitant overexpression of MEG3 and MEG8 in control fibroblast cultures was associated with a moderate but significant down-regulation of IGF2 expression (Fig. 5A). As for the 15q11-q13 PEGs, we found that overexpressing MEG8 (regardless of the variant used for transfection) in control fibroblasts was associated with a down-regulation of SNURF expression, whereas MEG3 overexpression was associated with IPW down-regulation (Fig. 5A). To investigate whether the down-regulation of MEG3 and MEG8 could also lead to deregulation of these PEGs, we knocked down both ncRNA expression with siRNA in the same cell line. We found that IGF2 expression increased following MEG3 and/or MEG8 knockdown, whereas IPW and SNURF were up-regulated by the knockdown of MEG3 and MEG8, respectively (Fig. 5B). These changes in the expression of IGF2, SNURF, and IPW were observed in all transfected cells, despite the low transfection efficiency due to the use of primary fibroblast cultures as the host cells for transfection assays.

**Principal components analysis of overall gene expression in TS14 and SRS fibroblasts**

We investigated the dynamic changes in global gene expression levels and searched for possible gene expression signatures common to these two IDs by performing RNA sequencing (RNA-seq)–based expression profiling on TS14, SRS, and control fibroblasts [Gene Expression Omnibus (GEO) accession number GSE109408]. We first analyzed and compared the overall gene expression patterns of TS14 and SRS fibroblasts. For this purpose, we used principal components analysis (PCA), a statistical technique that summarizes large datasets, reducing the number of dimensions while illustrating relationships between samples based on the covariance of the data considered (19). PC1, PC2, and PC3 accounted for ~50% of the total variance of the original data (Fig. 6A). When all samples (TS14, SRS, and controls) were plotted in a two-dimensional space defined by PC1 and PC2 (accounting for ~35% of all variation), we were able to distinguish two distinct groups, the first clustering all the TS14 and SRS patients and the second encompassing all the controls (Fig. 6B). However, when the samples were plotted in a three-dimensional space defined by PC1, PC2, and PC3, we were able to separate the TS14 + SRS group into two separate groups, one corresponding to TS14 and the other to SRS (Fig. 6C). This analysis provides the first evidence for overlapping but distinguishable gene expression profiles in TS14 and SRS patients.

**RNA-seq reveals that imprinting defects lead to the genome-wide deregulation of gene expression**

We evaluated differential gene expression patterns in TS14 and SRS fibroblasts by grouping our samples based on their imprinting defects: 14q32.2 hypomethylation (four TS14 patients), 11p15.5 hypomethylation (five SRS patients), and controls (four donors). For this analysis, we selected genes with at least a twofold difference in expression between patients and controls and a q value below 0.05. We increased stringency by considering only genes with a mean expression of ≥1 FPKM (fragments per kilobase million) in at least one group. Using these criteria, we identified a total of 11,005, 11,117, and 11,043 expressed genes in control, SRS, and TS14 fibroblasts, respectively. Out of them, 552 genes were differentially expressed genes (DEGs), with expression levels differing between control fibroblasts and fibroblasts obtained from patients with TS14 or SRS (Fig. 6, D and E). Similar numbers of genes were up-regulated...
We found that 61 of the up-regulated genes were up-regulated in both TS14 and SRS fibroblasts, whereas 62 were up-regulated only in SRS fibroblasts, and 148 were up-regulated only in TS14 fibroblasts. By contrast, 49 of the down-regulated genes were down-regulated in both TS14 and SRS fibroblasts, whereas 53 were down-regulated only in SRS fibroblasts, and 179 were down-regulated only in TS14 fibroblasts. Together with the quantitative polymerase chain reaction (qPCR) findings in patients and transfected normal fibroblasts, these results show that imprinting defects induce the deregulation of both imprinted and nonimprinted genes. Moreover, 50% of the DEGs in SRS fibroblasts was also deregulated in TS14 fibroblasts (Fig. 6F). The list of all the DEGs in SRS and TS14 fibroblasts is available in data file S1.

A gene signature common to TS14 and SRS identified by gene set analysis

We then determined whether networks of genes with similar functional annotations were dysregulated in TS14 and SRS patients relative to controls. We analyzed the genes displaying differential expression between these groups using gene ontology pathways from gene set enrichment analysis (GSEA) to annotate genes on the basis of the functional biological processes in which their products are involved. GSEA with a false discovery rate (q value) threshold of q < 0.05, to correct for multiple testing, identified one major pathway as down-regulated and two as up-regulated in both TS14 and SRS (Fig. 6G). 

Allele-specific expression of imprinted genes from TS14 and SRS/TS14 fibroblasts

We previously performed whole-exome sequencing (WES) on DNA extracted from the fibroblasts of two TS14 patients (TS14-1 and TS14-2) (12) and the SRS/TS14 patient. We then used a method combining RNA-seq and WES of these patients to accurately assess allele-specific expression (ASE; fig. S3). On average, 3825 genes were found to be expressed biallelically versus 103 expressed monoallelically (3941, 3638, and 3895 versus 105, 122, and 82 from TS14-1, TS14-2, and SRS/TS14 patients, respectively). We first focused on imprinted human genes from the list of genes available from the geneimprint database (www.geneimprint.com). We compared their ASE to the bulk/single-cell fibroblast ASE recently reported for five healthy individuals (table S3) (20). Consistent with their known imprinted status, we found that four imprinted genes had monoallelic expression in both patient and control fibroblasts (OSBPL5, NDN, ZDBF2, and PEG10). The H19 gene was found to be monoallelically expressed in patient TS14-1, whereas the PRIM2 gene, predicted to be imprinted on the basis of previous studies, also displayed monoallelic expression in both TS14 patients. However, we identified 16 other imprinted genes with biallelic expression in our patients' fibroblasts (table S3). Thirteen of these genes were also biallelically expressed in bulk fibroblast cultures from controls, suggesting that these genes may not be imprinted in fibroblasts. For the remaining three genes, CPA4 was biallelically expressed in all three patients, whereas ZNF597 and H19 (consistent with our Sanger sequencing results for cDNA) were biallelically expressed only in the SRS/TS14 patient.

Fibroblasts from the SRS/TS14 patient displaying hypomethylation at both 14q32.2 and 11p15.5 loci displayed biallelic expression of MEG3 and H19 from the DLK1/MEG3 and IGF2/H19 domains, respectively. We investigated whether the biallelic expression of CPA4 and ZNF597 also resulted from MLID by assessing the methylation levels of the two imprinted regions regulating these genes (the PEG1/MEST and ZNF597-DMRs, respectively). The PEG1/MEST locus was hypomethylated in all three patients, whereas ZNF597 was hypomethylated only in the SRS/TS14 patient, consistent with the biallelic expression profiles of these patients (table S4). These results confirm our previous finding for MLID and multi-imprinting relaxation together with biallelic expression at the affected secondary loci. However, as both CPA4 and ZNF597 are expressed from the
Fig. 5. 11p15.5 and 15q11-q13 PEGs are deregulated upon MEG3 and/or MEG8 overexpression or knockdown in experimental models. (A) Relative levels of expression for IGF2, SNURF, and IPW in a control primary fibroblast line transfected with an empty, MEG3, and/or MEG8 expression vector. (B) Relative levels of expression for IGF2, SNURF, and IPW for a control primary fibroblast line transfected with a negative control, MEG3, and/or MEG8 small interfering RNA (siRNA). The data shown are the mean values ± SEM for four independent transfection assays (with at least four wells transfected per experiment). ***P ≤ 0.001 versus controls (transfected with an empty vector), in unpaired t tests.
Fig. 6. PCA and DEGs from TS14, SRS, and control fibroblasts. (A) Proportion of the variance accounted for by each principal component (PC). (B) Two-dimensional and (C) three-dimensional projection plots of the first three PCs: PC1, PC2, and PC3. (D and E) Volcano plots of the expression of all genes (with FPKM of ≥1) from TS14 and SRS, respectively, relative to controls. Green and red dots represent genes displaying statistically significant underexpression and overexpression, respectively (log₂±2 fold change, with a q value threshold of 0.05). (F) The number of genes differentially regulated in both TS14 and SRS fibroblasts or in one of these types of fibroblasts only. (G) Schematic representation of gene ontology pathways from the GSEA displaying significant up- and down-regulation in patients relative to controls fibroblasts after correction for multiple testing (q < 0.05). GSEA reveals a dysregulated pathway signature common to TS14 and SRS.
methylated maternal allele, their biallelic expression upon hypomethylation of their respective domains is discordant with their parent-of-origin expression. Such discordances have been reported for other imprinted genes but never before for these two genes (20). Last, we looked at the remaining genes of unknown imprinting status displaying monoallelic expression (137 genes; table S4) in our patients. We identified seven putative imprinted genes clustering at four different loci across the genome (fig. S4). These variants were not validated by another targeted sequencing technique.

**DISCUSSION**

IDs often affect growth in humans, leading to diseases with overlapping features, regardless of the genomic region affected (2). We characterized the effect of a loss of methylation at the imprinted DLK1/MEG3 locus on gene expression at the 14q32.2 locus in TS14 patients, and we investigated whether the overlap in clinical phenotypes between TS14 and SRS and PWS might be due to similar patterns of gene deregulation between these syndromes. DLK1 is an important PEG in the 14q32.2 domain. This gene encodes a transmembrane protein that generates both membrane-bound and serum-soluble isoforms through alternative splicing (16). We found that TS14 patients with paternal deletions or hypomethylation of the DLK1/MEG3 domain had no circulating DLK1, whereas serum DLK1 concentration is negatively correlated with age after birth in healthy children [consistent with the rapid decrease in serum DLK1 concentration observed in newborn mice (21)]. Thus, unlike circulating IGF2, which is produced biallelically in the liver after birth, under the control of a specific nonimprinted promoter (22), circulating DLK1 probably continues to be generated by monoallelic expression from the imprinted gene under normal conditions after birth. An analysis of the MEGs from the 14q32.2 domain showed that MEG3, MEG8, and miRNAs were up-regulated in TS14 fibroblasts. We demonstrated that MEG3 overexpression resulted from the reactivation of the normally silenced paternal allele. We hypothesized that the overexpression of MEG8 and miRNA was also due to expression from both parental alleles in TS14 patients. This hypothesis is consistent with the finding of previous studies that DLK1/MEG3 hypermethylation leads to a reactivation of the expression of RTL1 and DLK1 from the maternal allele (17). These findings highlight the parent-of-origin–specific effects of DNA methylation at ICRs on gene expression in the DLK1/MEG3 domain.

We investigated the possible overlap in gene expression patterns between TS14 and SRS and between TS14 and PWS. We found that imprinted genes within the primary affected domains for SRS (IGF2 at 11p15.5 and PWS (SNURF and IPW at 15q11-q13)) were down-regulated in all TS14 fibroblasts and in SRS/TS14 fibroblasts, despite the normal levels of methylation at their imprinted control regions, that are generally altered in SRS and PWS patients (6, 23). We hypothesized that this deregulation of the IGN might affect gene expression throughout the genome. An unsupervised PCA on the RNA-seq data from control and TS14 and SRS patient fibroblast cultures resulted in the clustering of samples into two groups (controls on the one hand and SRS and TS14 patients on the other hand) when the data were projected onto a two-dimensional space. However, the SRS and TS14 patients could be distinguished when the data were projected onto a three-dimensional space. Gene ontology annotations showed that the genes displaying expression alterations belonged to common pathways. Genes encoding products that promote cell and tissue growth were found to be down-regulated in fibroblasts from both TS14 and SRS patients. These results confirm our hypothesis of an overlapping but distinguishable, genome-wide deregulation of the pattern of expression in TS14 and SRS patients, affecting not only other imprinted genes from the IGN but also a number of nonimprinted genes. We showed that hypomethylation at the DLK1/MEG3 domain leads to the down-regulation of other imprinted PEGs, such as IGF2, SNURF, and IPW, mostly genes known to enhance growth (24), and of 87 other nonimprinted genes implicated in promoting growth. Moreover, TS14 fibroblasts had about twice as many deregulated (1.6× up- and 2.2× down-regulated) genes as SRS fibroblasts, with about 50% of the deregulated genes (61 up- and 49 down-regulated) common to both syndromes, regardless of the imprinting defect. This finding may partly explain the phenotypic overlap and also some of the differences between SRS and TS14, such as the much early onset of puberty and greater early weight gain in TS14 than in SRS patients.

Last, we hypothesized that these changes in expression might reflect, directly or indirectly, the involvement of 14q32.2 ncRNAs in this process. ncRNAs have been shown to regulate gene expression both in cis and in trans (25, 26), through association with chromatin modifiers (27). This raises the possibility of 14q32.2 ncRNAs regulating the expression of these genes from the 11p15.5 and 15q11-q13 regions. We found that overexpressing or knocking down the expression of MEG3 and MEG8 in normal fibroblast cultures was associated with deregulation of PEGs from the 11p15.5 and 15q11-q13 regions. We found in our experimental models of normal fibroblasts that (i) overexpressing MEG3 was associated with decreased levels of IPW transcripts, (ii) overexpressing MEG8 was associated with lower levels of SNURF, and (iii) concomitant overexpression of MEG3 and MEG8 was associated with lower levels of IGF2 transcripts. These results are in accordance with observations in TS14 and SRS fibroblasts, where (i) IPW levels were lower only in TS14 fibroblasts where MEG3 was overexpressed; (ii) SNURF levels were lower in TS14 and SRS patients, both having increased levels of MEG8; and (iii) IGF2 levels were lower in TS14 patients overexpressing MEG3 and MEG8 upon DLK1/MEG3 hypomethylation. These results suggest that MEG3 and MEG8 can regulate, in trans, the expression of other imprinted genes. We hypothesize a synergic role of MEG3 and MEG8 to control directly or indirectly the expression of IGF2. Overexpressing one of the two is not enough to increase IGF2 expression, since the other component is missing, while knocking down only one is enough to disrupt the complex and lead to decreased IGF2 expression. Similar patterns of regulation within the IGN have been described before (3, 5), involving IPW and MEG3 in particular (6), for which IPW was found to repress MEG3 expression. Together with our results, this strongly suggests that a system of reciprocal control operates between ncRNAs at imprinted loci and that this system might contribute to the clinical overlap between IDs. However, additional functional studies of the role of MEG3 and MEG8 are required to decipher the mechanisms by which these ncRNAs could regulate gene expression, particularly for imprinted genes. These mechanisms are probably unrelated to gene imprinting status because all TS14 patients have normal methylation levels at the ICRs corresponding to the deregulated imprinted genes (ICR1 for IGF2 and SNRPN-1C for SNURF and IPW). Single-cell RNA-seq has been shown to be more powerful than bulk RNA-seq for studies of the expression of imprinted genes.
genes (20) and therefore would allow the generation of a more extensive map of the deregulated IGN upon hypomethylation of the DLK1/MEG3 and/or IGF2/H19 domains.

In conclusion, we show that 14q32.2 hypomethylation affects the expression not only of genes within this locus but also of other imprinted and nonimprinted genes, some of which are involved in controlling tissue growth. We also observed changes in gene expression common to TS14 and SRS patients, in particular, a diminished IGF2 expression, and to TS14 and PWS patients, with diminished SNURF and IPW expressions (fig. S5). This transcriptomic overlap may account for the clinical overlap between the two syndromes.

**MATERIALS AND METHODS**

**Study approval**

Patients were followed up at Armand Trousseau Children’s Hospital or were referred by other clinical centers for molecular analysis for suspected TS14 or SRS. A geneticist and/or a pediatric endocrinologist examined each patient, and a detailed clinical form was completed. All patients or their parents and the controls included in this study gave written informed consent for participation, in accordance with French national ethics rules for patients recruited in France (Assistance Publique–Hôpitaux de Paris authorization no. 682) and the rules of Institutional Review Board I00000204 of the Mount Sinai School of Medicine for patients recruited in the United States.

**Quantification of serum DLK1 levels**

Serum DLK1 levels from healthy children and TS14 patients were quantified in 96-well plates, with the Quantikine Immunoassay Control for Human Pref-1/DLK1/FAL1 (R&D Systems, France), based on sandwich ELISA (enzyme-linked immunosorbent assay), according to the manufacturer’s instructions.

**Skin-derived fibroblast cultures**

Skin-derived fibroblasts from patients and controls were cultured to confluence in RPMI 1640 (Gibco, Cergy Pontoise, France) supplemented with 10% fetal calf serum and ampicillin (50 μg/ml)/streptomycin (50 μg/ml) at 37°C (number of passages, <10). Cells were then treated with trypsin and centrifuged. The cell pellet obtained was washed twice with 1× phosphate-buffered saline and used for DNA and micro/large-scale RNA extractions.

**Nucleic acid extraction and quantification**

DNA was extracted from blood leukocytes and from skin-derived fibroblast cultures, by an in-house protocol, after cell lysis by a salting-out procedure, as previously described (28, 29). Total RNA and miRNA were extracted from cultured fibroblasts with the NucleoSpin miRNA Kit for the isolation of small and large RNAs (MACHEREY-NAGEL, France). Both DNA and RNA were quantified with a NanoDrop ND-1000 spectrophotometer (Invitrogen, France).

**Reverse transcription and real-time PCR quantification of micro/mRNAs**

We synthesized cDNA from micro/mRNAs isolated from fibroblasts and used it for quantitative PCR with the miScript PCR System (Qiagen, France). Expression in the controls was arbitrarily set to 1, and fold changes (FCs) between two groups were calculated as FC = $2^{-ΔΔCt}$. Details from the reverse transcription and real-time quantification analysis are provided in Supplementary Methods.

**Bisulfite treatment of DNA and methylation analysis**

gDNA (1 μg) was treated with sodium bisulfite, with the EZ DNA Methylation Kit (Zymo Research, Orange, CA), according to the manufacturer’s instructions. The methylation status of the studied loci was assessed by allele-specific methylated multiplex real-time quantitative PCR (ASMM RTQ-PCR), as previously described (30–36). Details for the treatment and technique are provided in Supplementary Methods.

**gDNA and cDNA sequencing**

Two SNPs from the MEG3 and H19 gDNA and cDNA were sequenced by standard Sanger sequencing methods, with the ABI PRISM BigDye Terminator v3.0 Cycle Sequencing Kit and the ABI 3100 Genetic Analyzer. The sequencing products were then analyzed with SeqScape v2.6 (Life Technologies, Courtaboeuf, France).

**Cell cultures and transfection assays**

GM05757 (control human skin-derived fibroblast) cells were cultured in 24-well plates for overexpression and small interfering RNA (siRNA) transfections. Details of culture and transfection conditions and constructs used are described in Supplementary Methods.

**WES, RNA-seq, and bioinformatics analysis**

Library preparations, sequencing, and data analysis were carried out by IntegraGen SA (Evry, France). The sequencing methods and bioinformatics analysis are described in detail in Supplementary Methods.

**RT-qPCR statistical analysis**

For RT-qPCR, we compared data for pairs of groups in Mann-Whitney tests (expression in fibroblasts from patients versus that in control fibroblasts, with n = 5 each) and unpaired t tests (overexpression and siRNA assays with n > 20 per condition). We considered P values below 0.05 to indicate statistical significance. The analyses were performed with GraphPad Prism version 6.00 (GraphPad Software, La Jolla, CA, USA).

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/5/2/eaau9425/DC1

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