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1 Screening of patients born small for gestational age with the Silver-Russell syndrome
2 phenotype for *DLKI* variants.

3 Running Title: *DLKI* variants are not involved in Silver Russell syndrome

4

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22 **ABSTRACT**

23 Silver–Russell syndrome (SRS) is a rare imprinting disorder associated with prenatal
24 and postnatal growth retardation. Loss of methylation (LOM) on chromosome 11p15 is
25 observed in 40 to 60% of patients and maternal uniparental disomy (mUPD) for
26 chromosome 7 (upd(7)mat) in approximately 5 to 10%. Patients with LOM or mUPD
27 14q32 can present clinically as SRS. *Delta like non-canonical Notch ligand 1 (DLKI)* is
28 one of the imprinted genes expressed from chromosome 14q32. *Dlk1*-null mice display
29 fetal growth restriction but no genetic defects of *DLKI* have been described in human
30 patients born small for gestational age (SGA). We screened a cohort of SGA patients
31 with a SRS phenotype for *DLKI* variants using a next-generation sequencing (NGS)
32 approach to search for new molecular defects responsible for SRS. Patients born SGA
33 with a clinical suspicion of SRS and normal methylation by molecular testing at the
34 11p15 or 14q32 loci and upd(7)mat were screened for *DLKI* variants using targeted
35 NGS. Among 132 patients, only two rare variants of *DLKI* were identified
36 (p.(Gly35Arg) and p.(His65Arg)). Both variants were inherited from the mother of the
37 patients, which does not favor a role in pathogenicity, as the mono-allelic expression of
38 *DLKI* is from the paternal-inherited allele. We did not identify any pathogenic variants
39 in *DLKI* in a large cohort of SGA patients with a SRS phenotype. *DLKI* variants are
40 not a common cause of SGA.

41

42 **Keywords:** Silver-Russell syndrome, small for gestational age, *DLKI*, next-generation
43 sequencing

44 INTRODUCTION

45 Fetal growth restriction (FGR), defined as the failure of the fetus to reach its genetically
46 determined growth potential, is one of the most common causes of perinatal mortality
47 and morbidity (1). It results from multiple causes, such as genetic and epigenetic
48 alterations, the environment, hormonal dysregulation, or placental vascular dysfunction.
49 More than 150 genetic disorders have been associated with FGR (2).

50 Silver–Russell syndrome (SRS, OMIM #180860) is a rare but well known imprinting
51 disorder (3). Clinical diagnosis of SRS is considered if a patient shows at least four of
52 the six criteria of the Netchine-Harbison clinical scoring system (NH-CSS) (3,4), which
53 includes pre- and post-natal growth retardation, relative macrocephaly at birth, body
54 asymmetry, protruding forehead, and early feeding difficulties. An underlying
55 molecular cause is identified in approximately 60% of patients with SRS (3,5). Among
56 them, loss of methylation at *H19/IGF2:IG-DMR* (also called ICR1) on chromosome
57 11p15 (11p15 LOM) is observed in 40 to 60% and maternal uniparental disomy for
58 chromosome 7 (upd(7)mat) in approximately 5 to 10% (3,5–7). The recent international
59 consensus of SRS recommends additional molecular testing in cases of normal
60 methylation on chromosomes 11 and 7, including screening of *cyclin D kinase inhibitor*
61 *1c* (*CDKN1C*) and *insulin-like growth factor 2* (*IGF2*) genes (8,9). Since the first
62 consensus on SRS, new molecular defects have been identified in *high mobility group*
63 *AT-hook 2* (*HMGA2*) and *pleiomorphic adenoma gene 1* (*PLAG1*) in patients with a
64 clinical presentation of SRS (10,11). The recent use of next-generation sequencing
65 (NGS) for SRS patients has improved the molecular diagnosis (12–16), but more than
66 30% of patients with SRS remain without an identified molecular cause.

67 Temple Syndrome (TS) is another rare cause of prenatal and postnatal growth restriction
68 caused by disruption of the 14q32 imprinted region. In this region, *MEG3/DLK1:IG-*
69 *DMR* is normally methylated on the paternal allele (17), resulting in *Delta like non-*
70 *canonical Notch ligand 1 (DLK1)*, *retrotransposon Gag like 1 (RTL1)*, and
71 *Iodothyronine Deiodinase 3 (DIO3)* expression from the paternal allele (18). In contrast,
72 long noncoding RNAs (*maternally expressed 3 (MEG3)* and *maternally expressed 8*
73 (*MEG8*)), microRNAs, and small nucleolar RNAs are expressed by the unmethylated
74 maternal allele (Figure 1). Maternal uniparental disomy of chromosome 14
75 (*upd(14)mat*), hypomethylation of *MEG3/DLK1:IG-DMR*, and paternal deletion of this
76 region all lead to the phenotype of TS. Clinical overlap between SRS and TS has been
77 described and Geoffron *et al.* reported 73% of patients with 14q32 disruption scoring
78 positively for SRS, with a NHCSS $\geq 4/6$ (19–22). According to Geoffron *et al.*, 14q32
79 disruption may be considered to be an alternative molecular diagnosis of SRS and
80 *MEG3/DLK1:IG-DMR* methylation should be tested in cases of negative results for
81 other molecular testing of SRS patients (3).

82 *DLK1* is widely expressed during fetal development. It encodes a transmembrane
83 glycoprotein with six epidermal growth factor (EGF)-like motifs in its extracellular
84 domain, a juxtamembrane region with a TACE-mediated cleavage site, a single
85 transmembrane domain, and a short cytoplasmic tail (18). The exact function of *DLK1*
86 is uncertain but it is involved in adipogenesis and appears to play an important role in
87 preserving the pool of various progenitor cells until they differentiate (18). With the
88 generation of *Dlk1*-knockout mice, Moon *et al.* demonstrated overlapping phenotypes
89 between *Dlk1*-null mice and human *upd(14)mat*, including growth retardation. They
90 hypothesized that loss of *Dlk1* expression may be responsible for most of the symptoms

91 observed in human upd(14)mat (23). Paternally inherited *DLK1* variants have been
92 recently identified in patients with central precocious puberty (CPP) but no growth
93 retardation (24,25). Genetic defects of *DLK1* have never been described in patients with
94 FGR.

95 We screened *DLK1* variants in a cohort of patients born SGA with a SRS phenotype
96 using an NGS approach to search for new molecular defects responsible for SRS and
97 assess the role of *DLK1* in fetal growth.

98

99 **METHODS**

100 **Population studied.** Patients included were referred to our molecular laboratory
101 because they were born SGA (birth length and/or weight with a standard deviation score
102 (SDS) < -2 (26)) with a clinical suspicion of SRS. Patients born SGA with a NH-CSS ≥
103 4/6 or a NH-CSS = 3/6 with a strong clinical suspicion of SRS (relative macrocephaly
104 and/or protruding forehead), negative molecular testing for *H19/IGF2:IG-DMR*
105 (11p15.5) and *DLK1/MEG3:IG-DMR* (14q32.2) loss of methylation and upd(7)mat, and
106 negative molecular testing for *CDKN1C*, *IGF2*, *HMGA2*, and *PLAG1* variants were
107 included in this study. Written informed consent for participation was received from all
108 patients or parents, in accordance with national ethics rules (Assistance Publique–
109 Hôpitaux de Paris authorization no. 681). Patients were either followed at Armand
110 Trousseau Children’s Hospital or referred by other clinical centers for molecular
111 analysis. Postnatal growth parameters are expressed as SDS according to charts by
112 Sempé and Pedron (27). Blood samples were collected during routine biological follow-
113 up at clinical visits. DNA was extracted in our laboratory from peripheral blood samples

114 using an in-house protocol after cell lysis by a salting-out procedure, as previously
115 described (28,29).

116 **Next-generation sequencing.** *DLKI* was sequenced as a SRS candidate gene using
117 targeted sequencing. Library preparation, gene enrichment, sequencing, and data
118 analysis were performed by IntegraGen SA (Evry, France) or by our laboratory with a
119 pipeline designed by SOPHiA GENETICS (Lausanne, Switzerland).

120 **Sanger sequencing.** Variations of *DLKI* identified through NGS were verified for the
121 probands and their parents by Sanger sequencing using the ABI PRISM Big Dye
122 Terminator v3.0 Cycle Sequencing Kit and an ABI 3100 Genetic Analyzer (Life
123 Technologies, Courtaboeuf, France).

124 **In silico analysis.** The allele frequency was checked in the GnomAD database (online
125 <https://gnomad.broadinstitute.org/>) to predict the functional consequences of any
126 identified *DLKI* variants. Interspecies alignment of *DLKI* was performed using Clustal
127 Omega (online tool from The European Bioinformatics Institute (EMBL-EBI),
128 <http://www.ebi.ac.uk/Tools/msa/clustalo/>) and damage prediction scores were obtained
129 using the Polyphen-2 bioinformatic tool (30). Variants were also classified as benign or
130 likely benign, pathogenic or likely pathogenic, or of uncertain significance following
131 the American College of Medical Genetics and Genomics and the Association for
132 Molecular Pathology (ACMG/AMP) classification of variants (31). Six main categories
133 are evaluated according to these guidelines: population data (prevalence of the variant in
134 control populations), computational in silico predictive data, functional characterization,
135 segregation, de novo data, and allelic data.

136 **Statistical analysis.** Characteristics of the population are described as percentages for
137 qualitative variables or as the SDS and mean (range) for quantitative variables.

138

139 **RESULTS**

140 **Clinical characteristics of patients screened for *DLKI* variants**

141 Samples from 132 patients referred for molecular genetic testing for SRS and without
142 disturbances of 11p15 and 14q32 methylation or upd(7)mat were analyzed using a
143 targeted NSG-based approach (93 by Integragen and 39 using the pipeline of SOPHiA
144 GENETICS). Clinical data for at least four NH-CSS criteria were available for all
145 patients and was complete (all six criteria available) for 61 (46%). All patients were
146 born SGA. Patient characteristics are presented in Tables 1 and 2.

147 **Screening for *DLKI* variants**

148 Among the 132 patients born SGA with a clinical suspicion of SRS, only two rare
149 heterozygous variants of *DLKI* were identified in two independent patients
150 (NM_003836.6:c.103G>C, p.(Gly35Arg) and NM_003836.6: c.194A>G,
151 p.(His65Arg)).

152 **Phenotype of patients with identified *DLKI* variants**

153 Patient 1 was the third female child of two non-consanguineous parents. The proband's
154 parents were healthy. The final height of the father was 176 cm (0.2 SDS) and that of
155 the mother 152 cm (-2.0 SDS). Proband's mother was not born SGA and had no clinical
156 features of SRS. The proband's sisters were healthy, without prenatal or postnatal
157 growth restriction. Patient 1 was born at 36 + 2 weeks of amenorrhea (WA). Her birth
158 weight was 1,930 g (-1.9 SDS), birth length 41 cm (-3.6 SDS), and head circumference

159 31cm (-1.6 SDS). She did not experience catch up growth, with a height of 98.5 cm (-
160 3.3 SDS), weight of 13.5 kg (-3.4 SDS), and head circumference of 49 cm (-1.5 SDS) at
161 six years of age. She had no other remarkable features. She was not treated by growth-
162 hormone (GH) therapy. She had feeding difficulties and a protruding forehead and
163 fulfilled 5 of the 6 criteria of the NH-CSS. NGS sequencing of *DLK1* revealed the
164 heterozygous NM_003836.6: c.103G>C variant located in exon 2, predicting an amino-
165 acid substitution at codon 35 (p.(Gly35Arg)). Gly35 is located within the first EGF-like
166 motif in the extracellular domain of DLK1. This variant was inherited from her healthy
167 mother, who carried the same heterozygous variant (Figure 2).

168 Patient 2 was the first female child of two non-consanguineous parents. The mother's
169 final height was 169 cm (1.0 SDS). The proband's father was born SGA at 35 WA. The
170 father's birth weight and height were 1,290g (-3.4 SDS) and 38 cm (-4.7 SDS). Father's
171 head circumference at birth was unknown. The proband's father had no feeding
172 difficulties during childhood and initially experienced catch-up growth with a height at -
173 1 SDS between 10 and 14 years of age, but his final height was only 163 cm (-2.0 SDS).
174 He was not treated with GH therapy. NH-CSS of proband's father was 1/4. Patient 2
175 was a 29 WA-preterm girl with a birth weight of 920 g (-1.9 SDS), birth length of 34
176 cm (-3.0 SDS), and head circumference of 25 cm (-1.2 SDS). SGA was diagnosed in the
177 second trimester of gestation. She did not develop feeding difficulties but had a
178 protruding forehead. By 16 months of age, she had not experienced catch-up growth,
179 with a height of 68.5 cm (-3.0 SDS) and a head circumference of 44.5 cm (-1.0 SDS).
180 She was suspected of having SRS, with a NH-CSS = 4/6 and fifth finger clinodactyly.
181 At five years of age, she experienced premature adrenarche without precocious puberty,
182 responsible for catch-up growth, with a height of 105.4 cm (-0.8 SDS). NGS sequencing

183 of *DLK1* revealed that she carried a heterozygous NM_003836.6:c.194A>G variation in
184 exon 3 of *DLK1*, predicting an amino-acid substitution at codon 65 (p.(His65Arg)).
185 His65 is located within the second EGF-like motif of the extracellular domain of *DLK1*.
186 This variant was inherited from her healthy mother who carried the same heterozygous
187 variant (Figure 2).

188 **In silico analysis of the two *DLK1* variations**

189 The two variants NM_003836.6:c.103G>C p.(Gly35Arg) and NM_003836.6:
190 c.194A>G p.(His65Arg) are described in GnomAD and dbSNP (rs762558665 and
191 rs147224004) with an allele frequency in the general population of 7.0×10^{-6} and
192 4.7×10^{-4} , respectively. Interspecies alignment of the amino-acid sequences of *DLK1*
193 showed that residue Gly35 is invariant in vertebrates. The variation
194 NM_003836.6:c.103G>C p.(Gly35Arg) is predicted to be probably damaging, with a
195 score of 1.000 by the Polyphen-2 bioinformatic tools of variation damage prediction.
196 This variant is classified as a variant of uncertain significance (class 3) according to the
197 ACMP/AMP classification (PM2-PP3).

198 Residue His65 is conserved only within Pan Troglodytes. Polyphen-2 predicted the
199 variation p.His65Arg to be benign, with a score of 0.215. Variant NM_003836.6:
200 c.194A>G p.(His65Arg) is classified as likely benign (class 1) according to the
201 ACMP/AMP classification (PM2-BP4-BS4).

202 The two variants NM_003836.6:c.103G>C p.(Gly35Arg) and NM_003836.6:
203 c.194A>G p.(His65Arg) were not described in clinvar. We submitted it ... (à
204 completer).

205

206 **DISCUSSION**

207 We found two rare heterozygous variants of *DLK1* in a cohort of 132 SGA patients with
208 clinical suspicion of SRS and no identified molecular defects. The variants have already
209 been reported in databases but with a low frequency. However, caution should be paid
210 about variants frequencies regarding imprinted genes, as such a variant might have a
211 different clinical impact depending on the maternal or parental inheritance. Segregation
212 analysis did not favor a pathogenic effect of these two variants, as they were both on the
213 maternal allele, which is silent due to the maternal imprint of this gene. Thus, we did
214 not identify any pathogenic *DLK1* variants in our cohort.

215 No variants of *DLK1* have been reported in SGA patients. Dauber *et al.* identified a
216 complex defect of *DLK1* (14-kb deletion and 269-bp duplication) in four patients with
217 familial central precocious puberty (CPP). The four patients did not show prenatal or
218 postnatal growth failure, and other classical clinical features of Temple or Silver-Russell
219 syndrome, such as feeding difficulties, facial dysmorphism, precocious obesity, and
220 relative macrocephaly, were excluded (24). Gomes *et al.* identified three frameshift
221 variants of *DLK1* (p.Gly199Alafs*11, p.Val271Cysfs*14, and p.Pro160Leufs*50) in
222 five women from three families with CPP. Among them, three experienced postnatal
223 growth failure, but no data were available about birth weight or length (25). Montenegro
224 *et al.* described a deletion (c.401_404 + 8del) in the splice-site junction of *DLK1* in a
225 girl with sporadic CPP without postnatal growth failure. No data about prenatal growth
226 were available for this patient (32).

227 The role of *DLK1* in fetal growth is not well established. Murine models have suggested
228 a role for *Dlk1* in fetal and postnatal growth. Indeed, *Dlk1*-null mice and heterozygous
229 mice with paternal inheritance of the *Dlk1*-knockout allele showed prenatal and

230 postnatal growth restriction (23,33). By contrast, heterozygous mice with maternal
231 inheritance of the *Dlk1*-knockout allele did not experience growth restriction. Moreover,
232 it has been demonstrated that *Dlk1* promotes *Gh* expression. *Dlk1*-null mice showed
233 reduced pituitary GH content and mice overexpressing *Dlk1* had excessive pituitary and
234 circulating levels of GH (33,34). The modulation of GH levels could explain, at least in
235 part, the postnatal growth failure of mice lacking *Dlk1*, but not the prenatal growth
236 failure. During fetal life, *Dlk1* is expressed in the placenta in the endothelial cells of the
237 placental labyrinth but is not required for its development (18). Mice with a conditional
238 deletion of *Dlk1* in placental endothelial cells did not show FGR (35). Reduced DLK1
239 levels in maternal blood samples have been shown in the second and third trimester of
240 gestation with FGR (36,37). However, a causal relationship between low DLK1 levels
241 and FGR has not been demonstrated or whether DLK1 levels simply reflect fetal
242 weight.

243 To date, less is known about the contribution of individual genes of the 14q32 domain
244 in the TS phenotype and the overlapping features with SRS. FGR could, for example, be
245 explained by the action of several genes of the 14q32 domain in concert with genes in
246 other imprinted domains (38). Indeed, *Abi Habib et al.* demonstrated that
247 overexpression of *MEG3* and *MEG8* in TS patients with 14q32 hypomethylation is
248 associated with downregulation of *IGF2* transcription from the 11p15 imprinting region
249 (28).

250 In conclusion, we did not identify any variants in *DLK1* in a cohort of 132 patients with
251 suspected SRS. Although we screened a large cohort of patients for *DLK1* variants, we
252 cannot rule out the possibility of a role of *DLK1* in fetal growth and the SRS phenotype.

253 However, a frequent contribution of *DLKI* variants among the molecular causes of SRS
254 is unlikely. We did not identify a new molecular cause of SRS by the targeted NGS
255 approach. Whole exome and genome sequencing and characterization of the entire
256 methylome offer promising perspectives for the identification of new molecular causes
257 of SRS.

258

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263

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265

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268 **Contributorship statement**

269 AP: conception of the work, analysis and interpretation of the data, drafting of the
270 manuscript, and final approval of the published version.

271 MLS, DM, EG, FB, and IN: conception of the work, analysis and interpretation of the
272 data, critical revision of the work for important intellectual content, and final approval
273 of the published version.

274 CDN and MLJF: Acquisition of the data and final approval of the published version.

275

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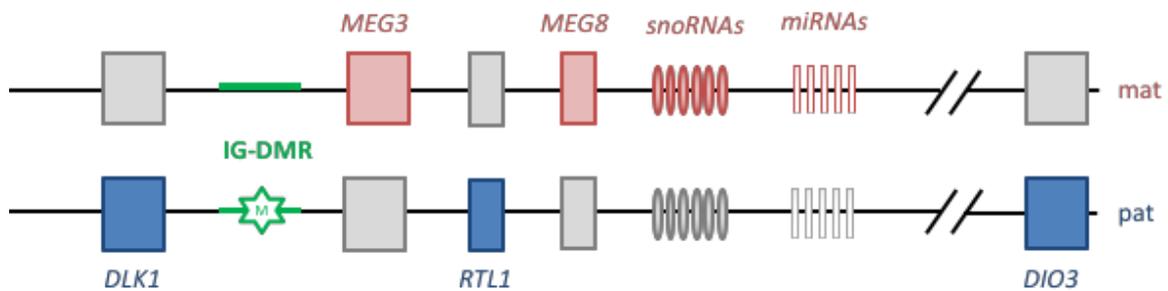
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- 402

403 **Figure Legends**

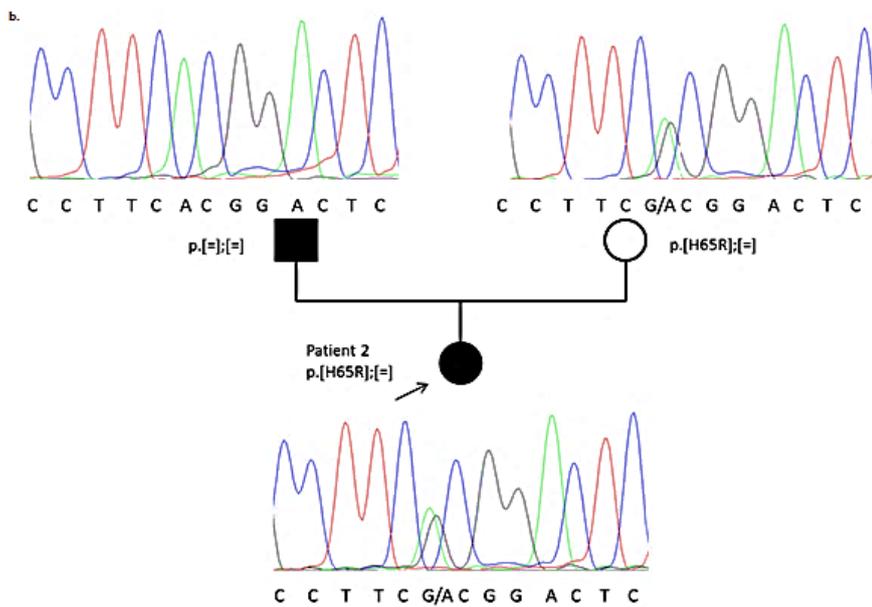
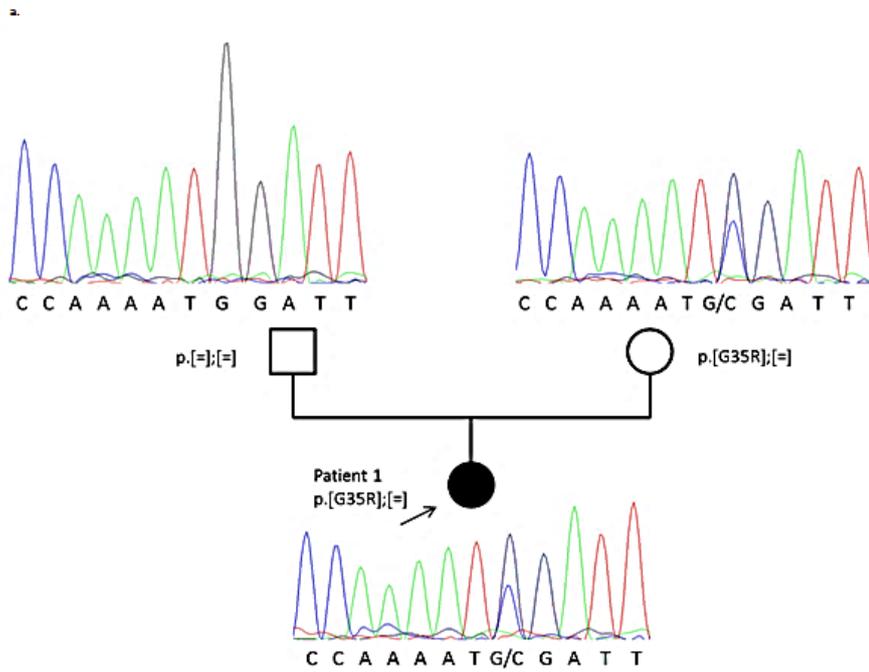
404 **Figure 1.** Schematic representation of the imprinted domain of the 14q32 region. The
405 green line indicates the differentially methylated region (the imprinting control center of
406 14q32, named IG-DMR methylated on the paternal allele). The green star represents
407 methylated DMRs. Blue boxes indicate genes expressed from the paternal (pat) allele
408 (*DLK1*, *RTL1*, and *DIO3*). Red boxes indicate genes expressed from the maternal (mat)
409 allele (the non-coding genes *MEG3* and *MEG8*, and a cluster of snoRNAs and
410 miRNAs).



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413 **Figure 2.** Intrafamilial segregation of variants p.Gly35Arg (p.G35R, c.103G>C,
414 rs762558665) of *DLK1* in family 1 (a) and p.His65Arg (p.H65R, c.194A>G,
415 rs147224004) of *DLK1* in family 2 (b). The two variants were inherited from the mother
416 of the patients. In black, patients born SGA.

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	n	Mean	(Min-Max)	< -2 SDS (%)
Gestational age at birth (weeks)	133	37	(26-42)	
Weight (g)	130	1874	(530-3170)	
Weight (SDS)		-2.6	(-6.0 to 1.8)	89 (68%)
Length (cm)	125	40.9	(30-48)	
Length (SDS)		-3.7	(-8.6 to -1.2)	118 (94%)
Head circumference (cm)	126	30.7	(21-38)	
Head circumference (SDS)		-1.8	(-5.2 to 1.8)	48 (38%)

422 Table 1. Anthropometric data at birth for patients included in the analysis. SDS:
423 standard deviation score.
424

	n (%)
SGA (BW and/or BL \leq -2SDS for gestational age)	132/132 (100%)
Relative macrocephaly at birth	93/128 (73.0%)
Postnatal growth failure	95/105 (90.0%)
Feeding difficulties and/or low BMI	113/128 (88.0%)
Protruding forehead	54/74 (73.0%)
Body asymmetry	7/127 (5.5%)
NH-CSS = 3	60 (45.5%)
NH-CSS = 4	48 (36.4%)
NH-CSS = 5	22 (16.6%)
NH-CSS = 6	2 (1.5%)

425 Table 2. Clinical features and NH-CSS for patients included in the analysis. SGA: small
426 for gestational age, BW: birth weight, BL: birth length, SDS: standard deviation score,
427 BMI: body mass index. NH-CSS: Netchine-Harbison clinical scoring system.

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