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ARTICLE

Preferential reciprocal transfer of paternal/maternal *DLK1* alleles to obese children: first evidence of polar overdominance in humans

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DLK1 is part of the Notch signalling pathway that controls various developmental processes. A functional role for DLK1 in adipogenesis is suggested by several animal models. Interestingly, the *DLK1* gene is imprinted in eutherian mammals. To study whether variations in *DLK1* affect body weight in humans, we analysed 32 polymorphisms in a 109 kb genomic region encompassing *DLK1* on human chromosome 14. In a study sample of 1025 French and German trio families comprised of both parents and extremely obese offspring we found a single nucleotide polymorphism (rs1802710) associated with child and adolescent obesity. Analysis of the allelic transmission pattern indicated the existence of polar overdominance, an unusual mode of non-mendelian inheritance in humans previously known from the callipyge mutation in sheep.

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Introduction

The delta-like 1 homologue (*Drosophila*) gene (*DLK1*), also known as *PREF1*, *FA1* or *pG2*, encodes a transmembrane protein containing epidermal growth factor-like repeats homologous to the Notch/Delta/Serrate family of genes, which are involved in cell fate determination. Delta-like genes are conserved throughout the animal kingdom. Imprinting of *DLK1* was probably acquired in eutherian mammals,[1] which express only the paternal allele.[2] While *DLK1* is widely expressed in embryogenesis, expression is turned off after birth in most tissues. DLK1 activity is important for many developmental processes including adipocyte differentiation. For example, it is highly expressed in 3T3-L1 preadipocytes but not detectable in differentiated adipocytes.[3] Accordingly, adipogenesis is

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inhibited by constitutive expression of *Dlk1* in 3T3-L1 cells,[4] and increased by antisense RNA-mediated down-regulation of *Dlk1*.[5]

These *in vitro* data are supported by rodent models. Mice expressing a *Dlk1* transgene in adipose tissue are lean and show a decreased fat pad weight.[6] Conversely, *Dlk1* knockout mice are obese in addition to displaying other phenotypes such as growth retardation and skeletal malformations.[7] The same phenotypes are observed in heterozygous animals if the knockout allele is inherited from the father, reflecting silencing of the maternal copy by imprinting. Heterozygotes having inherited the maternal null allele were normal.

Another interesting aspect of the *DLK1* phenotypic spectrum has been discovered in sheep. Lambs, which express the callipyge phenotype, show a decreased fat mass and muscle fibre hypertrophy.[8] The phenotype is caused by an apparent single locus mutation located distal to *Dlk1*,[9] which is characterized by an unusual, non-mendelian mode of inheritance termed 'polar overdominance'.[10] Only heterozygous animals, which have inherited the callipyge mutation (CLPM) from their father ($CLPG^{pat}/+^{mat}$ genotype) show 'visible' muscle hypertrophy when compared to the other three genotypes ($+^{pat}/+^{mat}$, $+^{pat}/CLPG^{mat}$, and $CLPG^{pat}/CLPG^{mat}$). While in animals carrying the latter three genotypes, Dlk1 protein is not detectable in muscle tissue of 8-week-old lambs, the callipyge animals ($CLPG^{pat}/+^{mat}$) do express Dlk1 in muscle suggesting an association between Dlk1 protein expression and the callipyge phenotype.[11] A causative role of *Dlk1* expression with respect to this phenotype has been shown by ectopic expression of *Dlk1* in mice. That is, transgenic mice expressing *Dlk1* under the control of the myosin promoter in skeletal muscle exhibit muscular hypertrophy resembling the callipyge phenotype of sheep.[11]

Evidence of polar overdominance has also been reported in pigs.[12] Analysis of the parental origin of a silent polymorphism in *Dlk1* revealed that paternal inheritance of one allele and maternal inheritance of the other allele in F2 offspring of two intercrossed lines is associated with decreased fatness and increased lean body mass.

Based on this evidence, we decided to analyse *DLK1* with respect to a potential role in human body weight regulation. More specifically, we were interested in whether there would be parent-of-origin effects and, in particular, whether a pattern of polar overdominance would be detectable in humans. Therefore, we genotyped polymorphisms at the human *DLK1* locus in two independent study samples and investigated a potential association with obesity. In order to be able to detect parent-of-origin effects, we chose to study trio families (two parents and one obese child) and employed statistical methods adapted to both detect imprinting effects and polar overdominance.

Materials and methods

Study subjects

The family-based association and parent-of-origin studies in German samples were based on trios comprising unrelated children and adolescents with extreme obesity and both biological parents. The ascertainment strategy for these trio samples has been described previously.[13] French trios were all Caucasians recruited in the Pediatric Unit of Jeanne de Flandre Hospital in Lille or through a national media campaign.[14] The initial sample, comprising 359 obese children and adolescents (207 females; (mean ± SD) body mass index (BMI) $31.77 \pm 6.07\,kg/m^2$; age 13.72 ± 3.10 years) and both biological parents, was genotyped for initially 3 and subsequently 29 polymorphisms.

The independent confirmatory sample comprised German and French trios. For both groups, the same phenotype definition was applied (BMI ≥ 99th percentile according to www.mybmi.de) allow for a better comparability of the samples. The German families consisted of 288 trios with obese children and adolescents (155 females; BMI, $33.93 \pm 5.39\,kg/m^2$; age 13.12 ± 2.62 years). The French sample comprised 378 trios with obese offspring (219 females; BMI, $31.99 \pm 6.32\,kg/m^2$; age 11.32 ± 3.37 years). In total, the initial and the independent confirmatory sample comprised 1025 obese children and adolescents (581 females; BMI, $32.46 \pm 6.05\,kg/m^2$; age 12.67 ± 3.25 years) and both biological parents. Screening for sequence variations was performed in a subgroup of the initial sample comprising 180 obese children and adolescents (101 females; BMI, $33.77 \pm 6.41\,kg/m^2$; age 14.38 ± 2.82 years). Written informed consent was given by all participants and, in the case of minors, by their parents. The ethics committee of the Universities of Marburg and Duisburg-Essen approved genotyping of obese subjects and their parents. The genetic study in French families was approved by ethical committee of the Centre Hospitalier Régional Universitaire in Lille.

Genotyping of polymorphic variants

In the initial sample three polymorphic variants were genotyped by PCR, following diagnostic restriction fragment length polymorphism (PCR-RFLP; Table 1). In total, 29 polymorphic variants were genotyped by PCR followed by a MassEXTEND reaction and subsequent matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS) analysis according to the manufacturer's protocol (hME; Sequenom, San Diego, CA, USA; Table 1). The German trios in the confirmatory sample were genotyped by MALDI-TOF MS. In addition to MALDI-TOF MS genotyping, the polymorphic variant rs1802710 was genotyped by tetra-primer amplification refractory mutation system[16] in a subgroup of the confirmatory sample. The French trios in the confirmatory sample were genotyped using Taqman assays (Applied Biosystems). Double genotyping was performed in 250 subjects for the

Table 1 Genotyped polymorphic variants in a region encompassing 109 kb including 25–76 kb 3′ of the human *DLK1* gene

Nos.	ID of polymorphic variants[a]	Position[b]	Gene	Alleles[c]	Intermarker distance (bp)	Distance DLK1 (bp)	Observed heterozygosities (minor allele frequency)[d]
1	rs17099615	100238191		T	3746	24848	0
2	rs7140792	100241936		C>T	874	21103	0.19 (0.11)
3	rs9635184	100242809		C>T	2743	20230	0.25 (0.15)
4	rs10151519	100245551	put exon 1 *DLK1*	G>A	170	17488	0.34 (0.20)
5	rs10873520	100245720	put exon 1 *DLK1*	A>G	803	17319	0.33 (0.20)
6	rs1555405	100246522	put exon 1 *DLK1*	G>A	1467	16517	0.39 (0.25)
7	rs1004573	100247988		C>G	3371	15051	0.26 (0.15)
8	rs7155649	100251358		C>A	7127	11681	0.23 (0.13)
9	rs7155375	100258484		C>T	3569	4555	0.46 (0.40)
10	rs3759556	100262052		A	542	987	0
11	rs11622172	100262593	put exon 1 *DLK1*	C	551	446	0
12	rs10139403	100264314	*DLK1* intron 1	G>A	384		0.42 (0.34)
13	rs6575799	100268170	*DLK1* exon 4	G	10		0
14	rs2273607	100268179	*DLK1* exon 4	G	14		0
15	rs1058006	100268192	*DLK1* exon 4	A	303		0
16	rs2273608	100268494	*DLK1* intron 4	C>T	1905		0.16 (0.08)
17	rs1802710	100270398	*DLK1* exon 5	C>T	216		0.48 (0.46)
18	rs1058009	100270613	*DLK1* exon 5	G>A	3344		0.11 (0.06)
19	rs876374	100273956	DAT	C>A	4085	2742	0.48 (0.44)
20	rs1956742	100278040		T>A	2871	6826	0.43 (0.33)
21	rs927257	100280910		A>G	3837	9696	0.44 (0.33)
22	rs2104070	100284746		A>G	5959	13532	0.24 (0.15)
23	rs11160604	100290704		T>C	494	19490	0.24 (0.15)
24	rs2400938	100291197		G>A	3479	19983	0.12 (0.06)
25	rs2400940	100294675		A>G	8325	23461	0.30 (0.19)
26	rs1190820	100302999		A>G	6156	31785	0.50 (0.48)
27	rs941571	100309154		C>T	18355	37940	0.49 (0.46)
28	rs6575803	100327508	197 bp 5′ of CLPM	C>T	2861	56294	0.18 (0.10)
29	rs8004581	100330368	2665 bp 3′ of CLPM	C>T	4552	59154	0.27 (0.16)
30	rs4906013	100334919		T>C	11638	63705	0.45 (0.41)
31	rs4906014	100346556	IG-DMR	A>G	495	75342	0.31 (0.20)
32	rs1884539	100347539	IG-DMR	G>A		76325	0.46 (0.38)

Abbreviations: CLPM, callipyge mutation in sheep; IG-DMR, intergenic differentially methylated region; Nos., numbers.
[a]dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).
[b]Human reference genome at the University of California Santa Cruz (UCSC) genome browser (http://genome.ucsc.edu/cgi-bin/hgGateway) (hg17).
[c]Major allele > minor allele.
[d]In parents of the initial sample.
Except for rs9635184 (with $P = 0.05$) no evidence for deviations from Hardy–Weinberg equilibrium in the founder data was obtained by PEDSTATS 0.6.3.[15] (all exact $P \gg 0.10$).

polymorphic variant rs1802710 and the concordance rate was 100%. In addition, no recurrent mendelian inconsistencies were detected in both the German and the French pedigrees using the PEDCHECK (version 1.1) program.[17]

Screening for sequence variations

For analysis of 223 bp of the human genomic region, which is orthologous to the ovine callipyge region containing the CLPM, single-strand conformation polymorphism analysis (SSCP) was performed as described previously.[18] All amplicons were electrophoresed on 15% acrylamide gels (37.5:1; Q-BIOgene, Heidelberg, Germany). Gels were run at room temperature for 5 h at 500 V and additionally at 4°C for 17.5 h at 400 V. Gels were silver stained. PCR products of individuals showing aberrant SSCP patterns were re-sequenced by a service laboratory (Seq/Lab, Göttingen, Germany).

Statistical analysis

Single marker family-based association analyses were performed using UNPHASED (version 3.0.5)[19] and its implementation of the classical transmission disequilibrium test (TDT).[20] The parent-of-origin effects were investigated by the parent-of-origin likelihood ratio test (PO-LRT[21]). This test allows for a comparison of paternal and maternal transmission rates while statistically controlling for prenatal (presumably non-genetic) maternal transmission effects. The parameters in the model (I_M, S) were obtained by log-linear regression as implemented in SAS (version 8.2; SAS Institute Inc., Cary, NC, USA.). Under the global null hypothesis all parameters are expected to be 1 and a *P*-value for the global test can be derived (Table 2, column 4). Evidence for a paternally expressed copy is obtained by demonstrating that $I_M < 1$ meaning that a paternally derived copy of the allele is associated with a greater increase in risk than a maternally derived copy.

Table 2 Analysis of parent-of-offspring effects by the PO-LRT parent-offspring likelihood ratio test (Weinberg[21]) of genotyped polymorphic variants in 359 German obesity trio families

Nos.	*Polymorphic Variants*[a]	*Reference Allele*	*LRT*		I_M				*S*			
			χ^2	P-*value*	*Estimate*	*95% CI*		P *(exact)*	*Estimate*	*95% CI*		P *(exact)*
2	rs7140792	C	2.20	0.33	1.37	0.58	3.25	0.55	0.68	0.38	1.19	0.20
3	rs9635184	C	0.10	0.95	0.92	0.42	1.99	0.95	1.02	0.61	1.71	1.00
4	rs10151519	A	0.21	0.90	1.01	0.50	2.05	1.00	1.06	0.66	1.69	0.90
5	rs10873520	A	0.12	0.94	0.96	0.47	1.97	1.00	1.07	0.67	1.71	0.86
6	rs1555405	A	0.15	0.93	0.96	0.47	1.97	1.00	1.06	0.68	1.66	0.88
7	rs1004573	C	0.13	0.94	1.08	0.51	2.32	0.97	0.92	0.55	1.53	0.82
8	rs7155649	A	6.07	0.05	0.59	0.27	1.28	0.20	1.82	1.08	3.13	0.02
9	rs7155375	C	0.40	0.82	1.13	0.58	2.21	0.81	0.89	0.60	1.32	0.61
12	rs10139403	A	0.89	0.64	0.80	0.40	1.58	0.59	1.20	0.81	1.79	0.40
16	rs2273608	C	5.97	0.05	2.20	0.85	5.90	0.11	0.47	0.23	0.91	0.02
17	rs1802710	C	8.39	0.02	0.43	0.22	0.82	0.01	1.66	1.13	2.47	0.01
18	rs1058009	C	0.38	0.83	1.15	0.40	3.33	0.96	0.84	0.44	1.59	0.66
19	rs876374	A	5.76	0.06	2.07	1.08	4.01	0.03	0.67	0.45	0.99	0.04
20	rs1956742	A	1.47	0.48	1.16	0.58	2.30	0.78	1.05	0.70	1.60	0.87
21	rs927257	A	1.20	0.55	0.87	0.44	1.71	0.78	0.96	0.64	1.45	0.92
22	rs2104070	A	1.30	0.52	0.67	0.32	1.41	0.34	1.20	0.76	1.92	0.47
23	rs11160604	C	1.30	0.52	1.49	0.71	3.15	0.34	0.83	0.52	1.32	0.47
24	rs2400938	A	2.44	0.29	2.03	0.74	5.71	0.20	0.64	0.31	1.28	0.23
25	rs2400940	A	3.76	0.15	1.81	0.91	3.61	0.09	0.69	0.44	1.06	0.09
26	rs1190820	A	4.08	0.13	1.27	0.66	2.47	0.54	0.74	0.50	1.08	0.12
27	rs941571	C	3.72	0.16	0.86	0.44	1.71	0.77	1.30	0.89	1.91	0.19
28	rs6575803	C	2.34	0.31	1.48	0.65	3.44	0.41	0.99	0.57	1.74	1.00
29	rs8004581	C	0.91	0.63	1.23	0.61	2.48	0.65	1.00	0.62	1.60	1.00
30	rs4906013	C	0.36	0.84	0.84	0.43	1.62	0.68	1.06	0.73	1.56	0.81
31	rs4906014	A	0.04	0.98	0.95	0.49	1.86	1.00	1.04	0.70	1.55	0.92
32	rs1884539	A	0.13	0.94	0.93	0.49	1.77	0.93	1.07	0.72	1.59	0.80

Abbreviations: I_M, parent-of-origin risk estimate ($=1$ no effect, <1 paternal effect; >1 maternal effect); LRT, likelihood ratio test; Nos., numbers; S, relative risk estimates for the prenatal maternal effects assuming a co-dominant model.
[a]dbSNP (http://www.ncbi.nlm.nih.gov/SNP/).
Monomorphic variants are not listed in this table.

Table 3 Modelling of genotype relative risks in all investigated obesity trios samples for rs1802710 in exon 5 of *DLK1*. Likelihood ratio test *P*-values for each model, GRR point and interval estimates (95% confidence interval) and the respective *P*-values for comparison to the reference category are presented

Model	*Initial sample of 359 German trios*			*Confirmatory sample of 666 trios*			*Combined sample of 1025 trios*		
	LRT (P-*value*)	*Estimate*	*95% CI*	*LRT* (P-*value*)	*Estimate*	*95% CI*	*LRT* (P-*value*)	*Estimate*	*95% CI*
No parent-of-origin effects									
T/T	0.93	1	—	0.34	1	—	0.73	1	—
C/T		0.96	(0.66–1.40)		1.21	(0.90–1.62)		1.08	(0.84–1.39)
C/C		1.03	(0.68–1.55)		1.27	(0.90–1.78)		1.12	(0.84–1.49)
Imprinting effects									
T^{mat}/T^{pat}	0.07	1	—	0.13	1	—	0.03	1	—
T^{mat}/C^{pat}		1.47	(0.89–2.43)		1.44	(1.02–2.05)		1.39	(1.03–1.89)
C^{mat}/T^{pat}		0.67	(0.42–1.07)		0.93	(0.62–1.40)		0.78	(0.55–1.09)
C^{mat}/C^{pat}		1.05	(0.69–1.59)		1.22	(0.86–1.71)		1.08	(0.81–1.45)
Polar overdominance[a]									
T^{mat}/T^{pat} and C^{mat}/C^{pat}	0.03	1	—	0.12	1	—	0.01	1	—
T^{mat}/C^{pat}		1.43	(0.93–2.20)		1.32	(0.97–1.80)		1.34	(1.02–1.75)
C^{mat}/T^{pat}		0.65	(0.43–0.98)		0.82	(0.58–1.16)		0.74	(0.55–0.99)

Abbreviations: CI, confidence interval; LRT, likelihood ratio test.
A one-parameter model (with dummies coding 1 for T^{mat}/C^{pat} and -1 for C^{mat}/T^{pat} and 0 else) leads to an estimate of 1.35 (95% CI 0.74–1.10) and a *P*-value of 0.003 in the combined sample.
[a]Reciprocal heterozygous genotypes express different phenotypes while homozygous genotypes express similar (normal) phenotypes.

Values of $I_M > 1$ indicate a maternally expressed copy. The estimate of the parameter S denotes the relative risks for maternally mediated genetic effects on the fetus assuming that the risk for a mother with one copy of the variant allele is midway between that for carriers of two or no copies.[22]

In the presence of some evidence for parent-of-origin effects, we decided to investigate whether there is support for a polar overdominance mode of inheritance. This was done within the conditional logistic regression framework suggested by Cordell *et al*,[23] which allows for an estimation of genotype relative risks (GRRs) conditional on parent-of-origin being inferable for the alleles at each locus. Note that GRR modelling differs from PO-LRT modelling due to differences in modelling; for an example, the parameter 'S' will not be estimated and as a result the global likelihood ratio test *P*-values of the GRR models will not be identical to those obtained by PO-LRT. For coarse model comparisons, Akaike's information criterion (AIC) was used.

The analysis of between-marker linkage disequilibrium (LD) was done by use of Haploview version 3.2,[24] applying the definition of 'haplotype blocks' as described by Gabriel *et al*.[25] Parent-of-origin effects in haplotypes were investigated by FAMHAP version 16[26] using the 'PAT' option, which allows to consider all combinations of tightly linked markers in nuclear families while incorporating corrections for multiple testing.

If not explicitly pointed out, all reported *P*-values are two sided and nominal. While exploration of the first sample lead to an obvious multiple testing problem, the second sample was only investigated for one single nucleotide polymorphism (SNP) by PO-LRT and the significance level for this test was $\alpha = 0.05$ (two sided). In the initial sample, a transmission rate of 0.57 was observed in the informative fathers, that is in about 36% of all genotyped fathers. The power of the PO-LRT to confirm a parent-of-origin effect is mainly related to these parameters such that a sample size of 666 independent trios is estimated to yield a power of about 67% to detect a true transmission rate of 0.57 among an expected number of $0.36 \times 666 = 240$ informative fathers (one-sided binomial test with $\alpha = 0.05$ using StatXact (version 5.0.3)).

Results

Study sample of 359 German obesity trio families

To get an initial idea if *DLK1* might be involved in body weight control in humans we initially genotyped three polymorphisms in 359 obesity trio families. One variant is located in the putative promoter region (rs3759556) and two in the translated region of exon five (rs1802710, rs1058009). rs3759556 was monomorphic in our study sample. When neglecting the parental origin of the transmitted alleles, the TDT revealed no evidence of linkage and allelic association with obesity for rs1802710 and rs1058009. However, for rs1802710, a coding synonymous variation, stratification based on parental origin showed a more frequent paternal transmission of the C-allele to obese children (75 transmitted *versus* 56 non-transmitted among heterozygous fathers). Consequently, we applied the PO-LRT, which is suitable for detection of parent-of-origin effects.[21] For rs1802710, this test indicated evidence for an association with obesity ($P = 0.015$) with an increased paternal transmission of the C-allele to the affected offspring ($I_M = 0.43$; 95% CI, 0.22–0.82, see 'Materials and methods' and Table 2 for details) and some evidence for maternally mediated genetic effects as well (S = 1.66; 95% CI, 1.13–2.47). No such pattern was found for rs1058009 (Table 2).

To detect signs of polar overdominance, we calculated the GRRs for rs1802710 taking the T^{mat}/T^{pat} genotype as reference category (based on the PO-LRT results). Table 3 shows a summary of these calculations. The results for the heterozygous genotypes T^{mat}/C^{pat} and C^{mat}/T^{pat} are consistent with the imprinting hypothesis for *DLK1*, that is only the paternally transmitted allele is expressed, and are an alternative illustration of the PO-LRT results reported above. Note however that the relative risk for carriers of the homozygous C^{mat}/C^{pat} genotype is not increased compared to the reference genotype. In the case of polar overdominance though, the increased risk would not be present for C^{mat}/C^{pat} genotype carriers. Thus, the observed pattern of inheritance in our sample is congruent with the model of polar overdominance and reminiscent of the callipyge pattern originally observed in sheep. This result prompted us to further investigate the *DLK1* genomic region including 5′- and 3′-flanking regions.

In order to identify potentially causative mutations which are in LD with rs1802710, we analysed 29 additional SNPs in a 109 kb region encompassing *DLK1* (Figure 1). In total, 10 of these SNPs were located in the 5′-region of *DLK1*, 5 SNPs in the transcribed region including introns and 14 SNPs in the 3′-region. The 3′-region also contains the sequences homologous to the ovine callipyge region[27] and the murine intergenic differentially methylated region (IG-DMR).[28] The observed heterozygosities and the minor allele frequencies of all analysed SNPs are listed in Table 1. Five of the presumed polymorphisms were found to be monomorphic in our study sample. For the remaining 24 SNPs, the calculation of PO-LRT did not reveal strong evidence for an association with obesity and parent-of-origin effects (rs7155649 $P = 0.048$, all other $P > 0.05$; Table 2).

Screening of the callipyge homologous region

In sheep, the CLPM is located 56 kb 3′ of *Dlk1*, and, interestingly, the adjacent sequence is highly conserved between human and sheep (Figure 2a). To find out whether a callipyge (CLPG)-like mutation occurs in our patient sample we analysed an 86 bp genomic fragment homo-

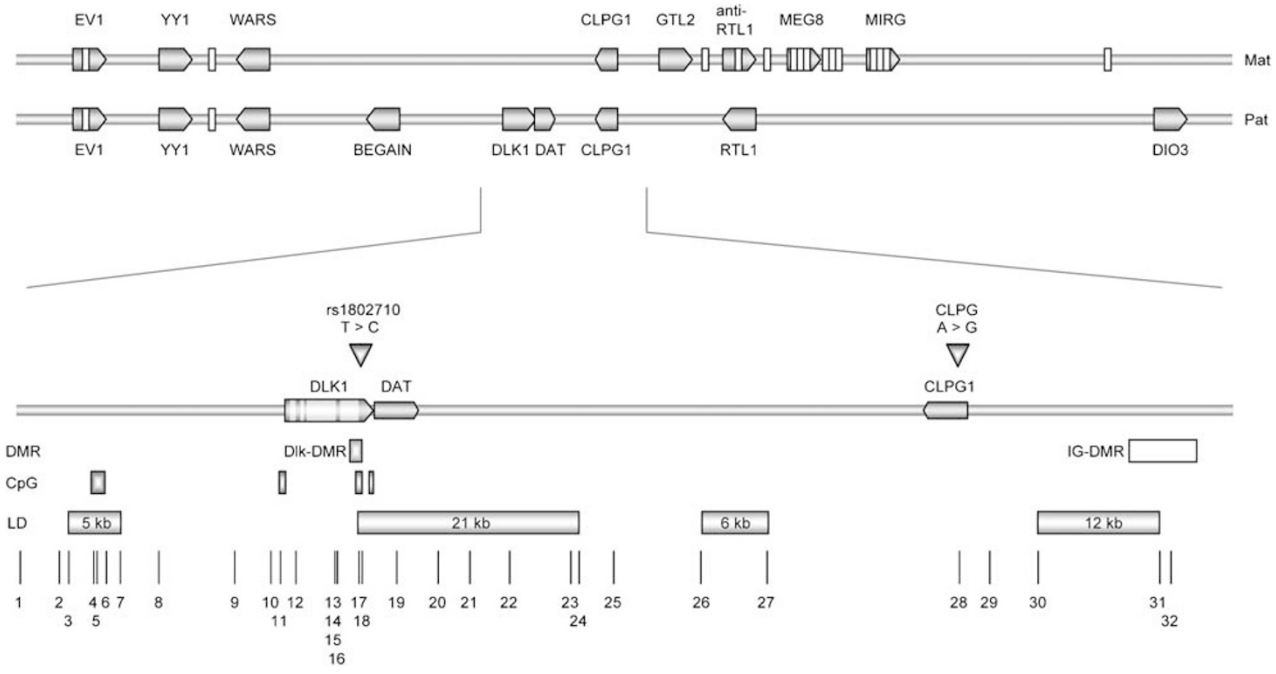

Figure 1 (Top) Schematic representation of the imprinted chromosomal region surrounding the human *DLK1* gene. (Bottom) *DLK1* region analysed in the current study. Arrows show transcription units (lighter areas within *DLK1* depict introns). White boxes indicate single or clustered snoRNAs or miRNAs. Triangles point to the positions of the rs1802710 polymorphism and of the callipyge (CLPG) mutation, respectively. Numbered vertical lines represent the polymorphisms as listed in Table 1. DMR, differentially methylated region; CpG, CpG island; LD,linkage disequilibrium block.

a
```
Ovis orientalis    1 tgtcctggtctatttttcgggcctctgctgagagcgcaggaatcca 45
                     ||||||||||||||||| |||||||||||| |||| ||||||||||
Homo sapiens       1 tgtcctggtctatttttagggcctctgctgtgagcacaggaatcca 45

                                                CLPG A>G exchange
Ovis orientalis   46 ggcgaaggggcccgagggctgggaccacctgtcagatcctt 86
                     |||   | |||||||  |||||||||||||||||||||| |||||
Homo sapiens      46 ggcatatgggcccaggggctgggaccacctgtcaggtcctt 86
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b
```
Homo sapiens         GGCGTCTGCACTGACATT/CGGGGGCGACTTCCGCTGCCGGT
                           | |  |  |  | ||||||  || ||      |  |
Sus scrofa           ACAAGCCGTGCCTG-AAT/CGGGGGCACCTGCCTGCAGCACT
```

Figure 2 (**a**) Alignment of human and ovine 86-bp genomic sequences surrounding the callipyge (CLPG) mutation. (**b**) Sequence similarity of fragments encompassing the exon 5 polymorphism in pig[12] and rs1802710 in humans. The positions of the CLPG mutation and the exon 5 polymorphisms are indicated in bold.

logous to the ovine CLPG region by SSCP analysis. However, screening of 180 obese patients did not reveal any mutations.

Linkage disequilibrium and haplotype analyses

The haplotype structure of the 109 kb genomic region encompassing *DLK1* was analysed in 359 obesity trios. Four relatively short 'LD blocks' were identified. rs1802710 was located at the edge of LD block 2 (Figure 1). None of the relevant haplotypes showed stronger evidence for an obesity association or parent-of-origin effect than rs1802710 alone. Furthermore, testing parent-of-origin effects in all combinations of possible haplotypes among SNPs within LD block 2 revealed that the top 4% (sorting by nominal *P*-values) of all 255 combinations always included rs1802710.

Confirmatory sample of German and French obesity trio families

The results in our first study sample indicated a small effect size and suggested that a very large sample would be required to confirm the initial finding. In that light the paucity of available obesity trios was challenging especially, because case-control designs cannot detect the

observed mode of inheritance. Therefore, in an effort to test the hypothesis of parent-of-origin effects and of polar overdominance, which was based on the animal data and our initial results for rs1802710, we gathered and combined two additional independent sample sets comprising 666 trios of European descent with extremely obese offspring from France and Germany (see 'Materials and methods' for a detailed description).

A similar transmission pattern as in the first sample was observed in the German and French trios. As found in the first study sample, the standard TDT indicated no association with obesity ($P=0.19$). Again, stratification by parental origin revealed a more frequent paternal transmission of the C-allele (133 transmitted and 100 non-transmitted). The PO-LRT (LRT, $P=0.04$; $I_M=0.62$, 95% CI, 0.40–0.97) again supported the initial observation of parent-of-origin effects while the previously observed maternally mediated genetic effects was now absent ($S=1.16$, 95% CI, 0.90–1.51). In addition, the GRRs (with T^{mat}/T^{pat} as reference genotype) displayed a similar pattern as in the first sample (Table 3).

In order to obtain a more precise estimate of allele transmissions and the related obesity risks, we pooled the data of the first and confirmatory sample totalling in 1025 obesity trios, calculated the PO-LRT for rs1802710 and determined the GRR. The *P*-value of the global LRT was 0.002 for the PO-LRT, due to a preferential transmission of paternal C-alleles to obese offspring ($I_M=0.55$, 95% CI, 0.38–0.78). The results of the GRR calculations are presented in Table 3 and indicated a relative risk for carriers of the T^{mat}/C^{pat} genotype of 1.34 and for carriers of the C^{mat}/T^{pat} genotype of 0.74 (global $P=0.006$). Our data can be best described (smallest AIC) by assuming a polar overdominance mode of inheritance.

Discussion

Several model systems support an association of the imprinted *DLK1* gene with adiposity. Adipogenesis of 3T3-L1 cells is inhibited by *Dlk1* expression[4] and enhanced by antisense RNA-mediated downregulation of *Dlk1*.[5] Knockout mice lacking both or only the paternally inherited *Dlk1* gene are obese. CLPG sheep display muscle hypertrophy and reduced fatness.[8] In pig, analysis of a silent polymorphism in exon 5 of *Dlk1* suggested the existence of polar overdominance too. Animals having inherited one allele of this polymorphism from the father and the other allele from the mother were less fat and had increased lean body mass compared with all other genotypes.[12]

In our study in humans, a synonymous polymorphism (rs1802710) in the fifth exon of *DLK1*, only 93 bp 5′ of the relevant polymorphism in the orthologous pig sequence, was associated with extreme obesity. This was revealed by a hypothesis-driven analysis assuming an inheritance pattern compatible with polar overdominance, which was originally reported in sheep. Initially, genotyping was done in a study sample of 359 obesity trios and multiple genetic markers and multiple genetic models were evaluated even though one may argue that based on the animal data there is a hierarchy among the tests performed (that is the test for polar overdominance should be the most appropriate one). To address the problem of overfitting, a second and independent sample of 666 obesity trios was genotyped for the relevant marker (rs1802710) to validate the initial finding. The combined sample ($n=1025$) represents one of the largest obesity trio samples to be analysed until today. Analyses of the transmission pattern of rs1802710 alleles to the affected offspring in both independent trio samples revealed a significant parent-of-origin effect reflecting the known silencing of the maternally inherited copy by imprinting.[2] Unexplainable by imprinting alone, we also observed a transmission pattern that is best explained on the basis of polar overdominance.

Due to the lack of body composition data we are unable to assess potential effects of SNP rs1802710 on fat mass and lean body mass separately. Based on the sheep and pig data, the polymorphism could even be associated with opposed values of fat and lean body mass that could obscure the phenotype when looking solely at BMI. Certainly, further confirmation in independent study samples would be desirable to reduce the risk of a false positive finding. The analysed 1025 German and French obesity trios, however, represented all trios with extremely obese offspring available to us. Because of the special mode of inheritance a replication cannot be done by case-control designs. Furthermore, family samples for a powerful replication need to be of substantial size as we estimated a rather small genetic effect size, which holds true for other common polymorphisms involved in body weight regulation. Assuming that our finding is not a false positive one, how then can our result be explained and integrated into existing theories?

Whether the T>C exchange at rs1802710 has functional relevance is unclear. The exchange is silent and analysis of the fifth exon of *DLK1* with the ESEfinder program (release 2.0) did not provide evidence for impaired or newly created splice sites by any of the analysed polymorphic variants. Theoretically, it is conceivable that rs1802710 is linked to a proximate mutation changing amino acids. However, this seems rather unlikely since it is difficult to imagine how changes at the amino-acid level could evoke a phenomenon such as polar overdominance characterized by phenotypically normal homozygous mutation carriers.

A look at callipyge sheep suggests alternatives to explain the observed rs1802710 results. The relationship between different models of gene action and the slaughter phenotypes of callipyge lambs[8] suggested that the inheritance of CLPG is best explained by polar overdominance:

Reciprocal heterozygous genotypes express different phenotypes (polar), and the two homozygous genotypes produce similar, normal phenotypes (overdominance).[29] Although the exact molecular activities underlying the CLPG phenotype are still not understood, the identification of the CLPG mutation[9] allowed to better analyse its molecular effects. The CLPG mutation is an A>G transition in the intergenic region between *Dlk1* and *Gtl2*. The mutation changes the expression level of many of the genes in the imprinting region (Figure 1). However, the only known molecular marker that is perfectly correlated with the callipyge muscle phenotype is Dlk1 protein.[11] Only in muscles of the $+^{mat}$/CLPG^{pat} genotype Dlk1 protein is expressed whereas in muscles of all other genotypes (including CLPG^{mat}/CLPG^{pat}) no Dlk1 protein was detectable. These findings resulted in a polar overdominance model, in which CLPG^{pat} expresses a muscle hypertrophy effector (Dlk1) and CLPG^{mat} expresses a repressor that inhibits translation of Dlk1. This way, only the $+^{mat}$/CLPG^{pat} genotype would produce Dlk1 protein. The repressor would likely be one of the non-coding RNAs transcribed from the maternal chromosome, possibly a microRNA, which would bind to *DLK1* RNA and prevent its translation.[11] Of course, alternative models cannot be excluded at this point.

Because of the callipyge example, we had screened the highly conserved human region corresponding to the region flanking the CLPG mutation in sheep for sequence variations that might be in LD with rs1802710. In 360 analysed human chromosomes, we could not detect any sequence variation. Furthermore, genotyping of SNPs located close to the CLPG orthologous position did not reveal any parent-of-offspring effects and association with obesity. This is in accordance with our observation that all other SNPs that we genotyped in the *DLK1* region (Figure 1) and all analysed haplotypes did not show better obesity association than rs1802710 alone. A similar situation exists in pigs. Here, a silent polymorphism was detected in the fifth exon of *Dlk1*, which is associated with decreased fat deposition and increased lean muscle mass. Again, sequencing of pig DNA fragments homologous to the *Dlk1-Gtl2* region did not reveal additional polymorphisms.[12] This suggests that in both pig and human the exon 5 polymorphisms are either in LD with a causative mutation most likely outside of the analysed region or that the altered DNA sequences are causative themselves.

An alignment of pig and human *DLK1* sequences shows that both polymorphisms are located closely together, only 93 bp apart. More interestingly, the sequences immediately adjacent to both SNPs are very similar (Figure 2b). Both polymorphisms are part of a TGGGGGC stretch and the T>C exchange creates a new CpG dinucleotide. In human, this region is located inside of a CpG island (Figure 1). The sequence identity around the polymorphisms suggests that this site could be the target site of transcriptional or post-transcriptional regulator(s) as postulated in the aforementioned polar overdominance model. Obviously, it would be desirable to measure expression levels of *DLK1* in adipose tissue *in vivo*. However, in humans this task is not easily accomplished. A sufficient number of high quality tissue probes grouped into the four different genotypes would be necessary for this experiment. Acquisition of such adipocyte probes is not trivial, particularly if children are involved. Furthermore, the known biological roles of DLK1 suggest that it functions early during development, when access to suitable tissue probes is not readily possible.

Despite the many open questions, further analysis of the potential link between imprinting controlled gene regulation and obesity could improve our understanding of this complex phenotype. Mice generated by somatic cloning showed increased body fat and body size, possibly caused by imprinting defects induced during the cloning process.[30] Analysis of gene expression in cloned mice revealed that *Dlk1* is one of the genes with consistently and significantly reduced expression levels.[31] Interestingly, even *in vitro* fertilized mice are heavier than their background stock mice.[30] In view of the sporadic reports about increased incidence of imprinting defects (eg Angelman or Beckwith–Wiedemann syndrome) in children born after *in vitro* fertilization,[32] it may be indicated also to monitor long-term development such as increased adiposity at later ages of *in vitro* fertilized humans.

In conclusion, we have shown that the human *DLK1* gene is associated with extreme early-onset obesity. This association is only apparent when imprinting of the gene is considered. The allelic transmission pattern supports the existence of polar overdominance in humans. We hope that our finding encourages other groups to attempt a replication.

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