

# Ectopic Expression of DLK1 Protein in Skeletal Muscle of Padumnal Heterozygotes Causes the Callipyge Phenotype

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## Summary

The callipyge (*CLPG*) phenotype is an inherited skeletal muscle hypertrophy described in sheep. It is characterized by an unusual mode of inheritance (“polar overdominance”) in which only heterozygous individuals having received the *CLPG* mutation from their father ( $+^{MAT}/CLPG^{PAT}$ ) express the phenotype [1].  $+^{MAT}/CLPG^{PAT}$  individuals are born normal and develop the muscular hypertrophy at approximately 1 month of age. The *CLPG* mutation was identified as an A to G transition in a highly conserved dodecamer motif located between the imprinted *DLK1* and *GTL2* genes [2, 3]. This motif is thought to be part of a long-range control element (LRCE) because the *CLPG* mutation was shown, in postnatal skeletal muscle, to enhance the transcript levels of the *DLK1*, *PEG11*, *GTL2*, and *MEG8* genes in *cis* without altering their imprinting status [4]. As a result, the  $+^{MAT}/CLPG^{PAT}$  individuals have a unique expression profile thought to underlie the callipyge phenotype: an overexpression of the paternally expressed protein encoding *DLK1* (Figure 1A) and *PEG11* transcripts in the absence of an overexpression of the maternally expressed noncoding *GTL2* and *MEG8* transcripts [4]. However, the way in which this distinct expression profile causes the callipyge muscular hypertrophy has remained unclear. Herein, we demonstrate that the callipyge phenotype is perfectly corre-

lated with ectopic expression of *DLK1* protein in hypertrophied muscle of  $+^{MAT}/CLPG^{PAT}$  sheep. We demonstrate the causality of this association by inducing a generalized muscular hypertrophy in transgenic mice that express *DLK1* in skeletal muscle. The absence of *DLK1* protein in skeletal muscle of *CLPG/CLPG* animals, despite the presence of *DLK1* mRNA, supports a *trans* inhibition mediated by noncoding RNAs expressed from the maternal allele.

## Results and Discussion

To further clarify the relationship between the observed transcript profile and the callipyge (*CLPG*) phenotype, we monitored the expression of *DLK1* at the protein level by immunohistochemistry. Two individuals of each of the four possible genotypes ( $+/+$ ,  $+^{MAT}/CLPG^{PAT}$ ,  $CLPG^{MAT}/+^{PAT}$ , and  $CLPG/CLPG$ ) were studied at three developmental stages (2 weeks before birth, 2 weeks after birth, and 8 weeks after birth) for a total of 24 animals. For each of these, we examined the pancreas (positive control), *Longissimus dorsi* (a skeletal muscle with hypertrophy in callipyge animals), and *Supraspinatus* (a skeletal muscle without hypertrophy in callipyge animals). As expected, all analyzed individuals showed positive *DLK1* staining in the islets of Langerhans, irrespective of *CLPG* genotype and developmental stage. In phenotypically “wild-type”  $+/+$ ,  $CLPG^{MAT}/+^{PAT}$ , and  $CLPG/CLPG$  animals, *DLK1* could neither be detected in *Longissimus dorsi* nor in *Supraspinatus*, irrespective of developmental stage. Likewise, negative results were obtained in skeletal muscle of the two  $+^{MAT}/CLPG^{PAT}$  animals sampled 2 weeks before birth, a developmental stage at which they do not yet express the callipyge phenotype. At 2 weeks of age, still before expressing the callipyge phenotype, one of the  $+^{MAT}/CLPG^{PAT}$  animals was negative in both analyzed muscles while the other showed weak staining in *Longissimus dorsi*. However, both 8-week-old  $+^{MAT}/CLPG^{PAT}$  callipyge animals showed marked *DLK1* staining in *Longissimus dorsi* (Figure 1B), whereas remaining negative in *Supraspinatus*.

Whether at 2 or 8 weeks of age, only a subset of the  $+^{MAT}/CLPG^{PAT}$  myotubes in *Longissimus dorsi* stained positive for *DLK1*. Knowing that the callipyge phenotype is due to an increase in the proportion and diameter of type II muscle fibers [5], we determined which myofibers were expressing *DLK1* by using fiber-specific anti-myosin heavy-chain (MHC) antibodies. None of the type I fibers expressed *DLK1*, but 51% of the type II fibers did ( $p < 0.05$ ) (Figure 1B). Regression analysis revealed a significant ( $p = 0.004$ ) inverse relationship between fiber type diameter and level of staining (none, intermediate, or strong) (Figure 1C).

To test the hypothesis of a direct role of ectopic *DLK1* expression in the determinism of the callipyge phenotype, we produced transgenic mice. We generated a construct to express ovine *DLK1* under the dependence

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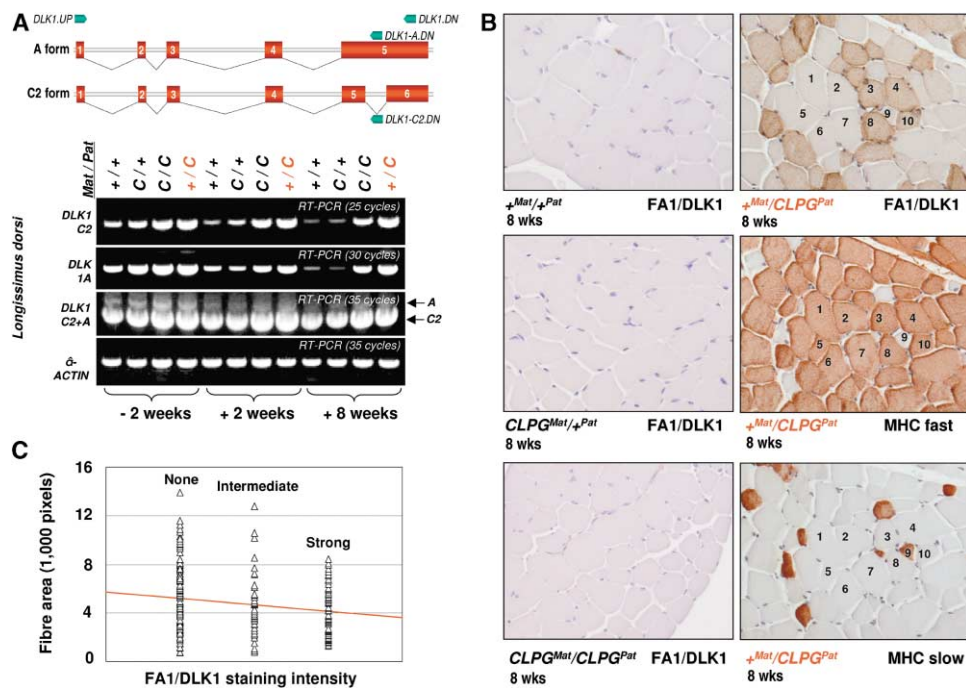


Figure 1. Expression Pattern of *DLK1* at the mRNA and Protein Levels in Sheep

(A) Schematic representation of the structure of the A and C2 alternatively spliced *DLK1* transcripts found in ovine skeletal muscle, as well as the approximate position of the primers used to amplify either the A, C2, or both species simultaneously. The C2 form is assumed to encode a membrane bound version of the *DLK1* protein, whereas the A form would undergo proteolytic cleavage to release a soluble *DLK1* species. RT-PCR amplification of the C2 (primers *DLK1.UP* and *DLK1-C2.DN*), A (primers *DLK1.UP* and *DLK1-A.DN*), and A+C2 (primers *DLK1.UP* and *DLK1-DN*) *DLK1* transcripts from total RNA extracted from *Longissimus dorsi* of individuals representing the four possible genotypes at the *CLPG* locus sampled 2 weeks before birth, 2 weeks after birth, and 8 weeks after birth. It can be seen that the membrane bound C2 form is by far the most abundant one in *Longissimus dorsi* and that both forms are downregulated after birth in  $+^{MAT}/+^{PAT}$  and  $CLPG^{MAT}/+^{PAT}$  individuals but not in  $+^{MAT}/CLPG^{PAT}$  and  $CLPG^{MAT}/CLPG^{PAT}$  animals.  $\beta$ -actin amplification was used to control for the equality of the engaged amounts of RNA amongst samples.

(B) Immunohistochemical analyses of transversal sections of *Longissimus dorsi* of 8-week-old sheep, representing the four possible genotypes at the callipyge locus, via anti-human FA1/*DLK1*, anti-MHC(fast), and anti-MHC(slow) antibodies, showing the detection of *DLK1* protein in fast-twitch (Type II) fibers of  $+^{MAT}/CLPG^{PAT}$  animals only.

(C) Regression analysis showing the inverse relationship between fiber area and intensity of anti-FA1/*DLK1* labeling (none, intermediate, strong). The linear regression line is shown in red ( $p = 0.004$ ).

of the murine myosin light chain 3F promoter and 2E enhancer (Figure 2A), previously shown to drive high-level expression in type IIB muscle fibers throughout pre- and postnatal development [6]. We selected the C2 membrane-bound form of *DLK1* because this splice variant is most abundant in skeletal muscle of callipyge animals (Figure 1A).

We generated two transgenic lines expressing *DLK1* at the mRNA and protein levels (lines A and D). Although lines A and D were shown to have each integrated two tandem copies of the transgene, the expression level was more pronounced in skeletal muscle of line D than of line A. This was the case whether the expression level was monitored by RT-PCR (Figure S1 in the Supplemental Data available with this article online) or immunohistochemistry (Figure 2B). The expression of the transgene was shown by RT-PCR to be restricted to skeletal muscle in line A, whereas being parallel to the expression of the endogenous myosin light chain (*MLC*) gene in line D, i.e., expressed mainly in skeletal muscle and to a lesser extent in heart and lung (Figure S1). As could be evaluated by immunohistochemistry, the expression level in *Longissimus dorsi* of 8-week-old callipyge sheep

was intermediate between the expression levels observed in *Quadriceps femoris* of 6-week-old  $+/T$  mice of lines A and D, respectively (Figures 1B and 2B).

We generated F2 pedigrees counting 107 (line A) and 113 (line D) offspring. The three genotypes were shown to segregate in Mendelian proportions for line A ( $p = 0.80$ ) but not for line D ( $p = 6.5 \times 10^{-9}$ ) in which *T/T* individuals were absent. F2 animals were reared until 25 weeks of age for line A and 11 weeks of age for line D, then killed and dissected. Throughout this period, the transgene did not have a significant effect on live weight in line A, but there was a negative effect in line D, especially in males (Figure S2). It remains unknown whether the absence of *T/T* individuals and growth effects observed in line D are related due to the insertional inactivation of an essential gene or to dosage-sensitive *DLK1* toxicity.

Notably, the transgene increased relative muscle mass in both lines, whether measured in the rear (weight of *Quadriceps femoris*/carcass weight) (Figure 2C) or front (weight of *Triceps brachii*/carcass weight) legs (data not shown). In line A, the relative weight of the *Quadriceps femoris* was increased by 9.3% ( $p < 0.0001$ )

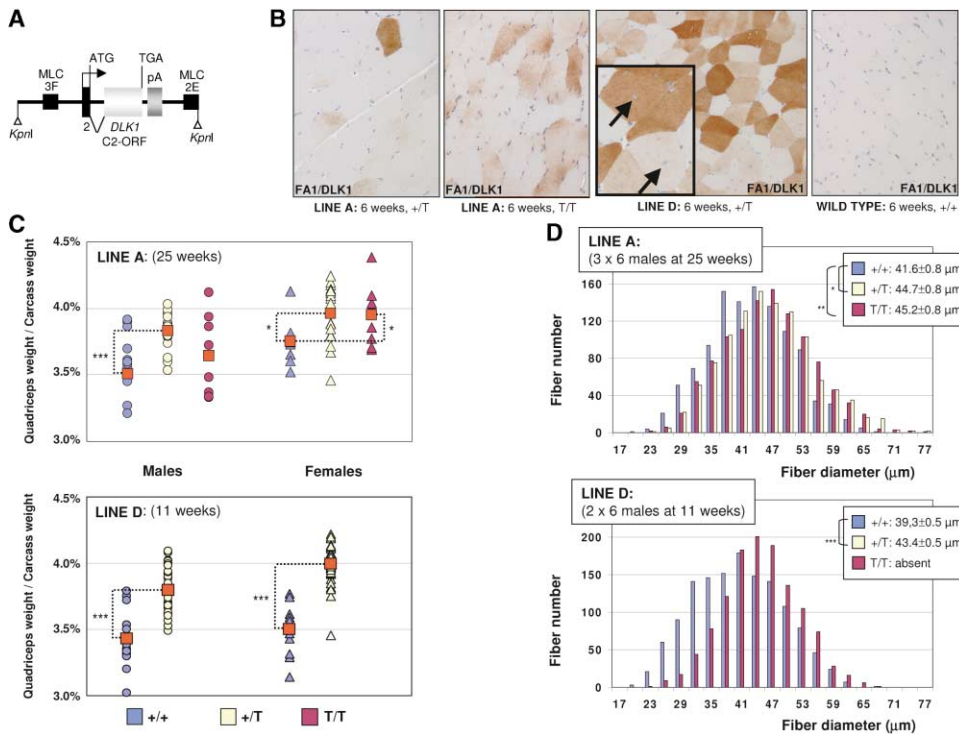


Figure 2. Phenotypic Characterization of the A and D Transgenic Lines

(A) Schematic representation of the transgene construct showing in black: *MLC* components including the 3F promoter, exon II (including a start codon), intron 2, and the *MLC* 2E enhancer bordering the construct at the 3' end; in gray: the bovine *GH* polyadenylation site; and in white: the ovine *DLK1* C2-ORF. The construct was excised from the plasmid vector by using the shown *KpnI* restriction sites.

(B) Immunohistochemical analyses of transversal sections of the *Quadriceps femoris* of a 6-week-old +/T mouse of line A, a T/T mouse of line A, a +/T mouse of line D, and a +/+ wild-type control via anti-human FA1/DLK1 antibodies, showing a moderate level of ectopic *DLK1* expression in the +/T animal of line A, an intermediate level in the T/T animal of line A, and a high level in the +/T animal of line D. The arrows in the inset point toward centrally located nuclei found at high frequency in myofibers of line D.

(C) Effect of the transgene on relative muscle mass (*Quadriceps femoris* weight divided by carcass weight) of animals sorted by line, sex, and transgene genotype, showing the hypertrophic effect of ectopic *DLK1* expression at the macroscopic level. Significance of the contrasts between genotypes, estimated within line and sex by General Linear Model Procedure (PROCGLM, SAS Institute), are given as \* ( $p < 0.05$ ) or \*\*\* ( $p < 0.0001$ ). The absence of an increase in phenotypic variance in the +/T class (including both individuals having inherited the transgene from the father and from the mother) when compared to the homozygous classes indicates that the effect of the transgene is not influenced by its parental origin. Indeed, if the parental origin had an effect on the expression of the transgene, one would expect the phenotypic average of the +<sup>Mat</sup>/T<sup>Pat</sup> class to be different from that of the T<sup>Mat</sup>/+<sup>Pat</sup> class, which would manifest itself by an increase in the phenotypic variance of the heterozygotes when compared either to the +/+ or to the T/T class.

(D) Frequency distribution of myofiber diameter in *Quadriceps femoris* of male mice of line A (25 weeks) and D (11 weeks) sorted by transgene genotype. Each genotype class is represented by six individuals, each contributing on average 195 measurements collected in nine distinct sectors of the *Quadriceps femoris*. The inset provides the mean fiber diameter ( $\pm$  standard error) for each genotype class, estimated by Mixed Model Procedure (PROC MIXED, SAS Institute) with a model including fixed genotype and sector effects as well as a random animal effect accounting for the covariance between repeated measurements estimated with the compound symmetry structure [16]. Statistical significance of the contrasts between genotypes within lines are given as \* ( $p < 0.05$ ), \*\* ( $p < 0.01$ ), or \*\*\* ( $p < 0.0001$ ).

and 5.5% ( $p = 0.0164$ ) in +/T males and +/T females, respectively, when compared to their +/+ counterparts. Unexpectedly, the corresponding figures were only +4.0% ( $p = 0.1428$ ) and +5.3% ( $p = 0.0388$ ) when comparing T/T males and females to the corresponding +/+ animals. Although neither T/T males nor T/T females differed significantly from their +/T counterparts, these results nevertheless suggest that in line A, the transgene effect is more pronounced in heterozygotes than in homozygotes. Why this is so remains unknown. We cannot exclude that it is related to the deleterious transgene effects observed in line D (see above). In line D, the relative weight of the *Quadriceps femoris* was very significantly increased by 10.5% ( $p < 0.0001$ ) and 14% ( $p <$

0.0001) in +/T males and females, respectively, when compared to +/+ contemporaries.

We measured the distribution of myofiber diameter in both F2 populations sorted by transgene genotype. The average fiber diameter was clearly increased in transgenic animals in both lines (Figure 2D). In line A, the average fiber diameter in *Quadriceps femoris* was increased by 7.4% and 8.7% in +/T and T/T animals when compared to +/+ animals ( $p = 0.0116$  and 0.0045, respectively). The fiber diameter did not differ significantly between +/T and T/T animals ( $p = 0.6490$ ), corroborating the absence of significant difference in relative weight of *Quadriceps femoris* between +/T and T/T genotype (Figure 2C). In line D, average fiber diameter was

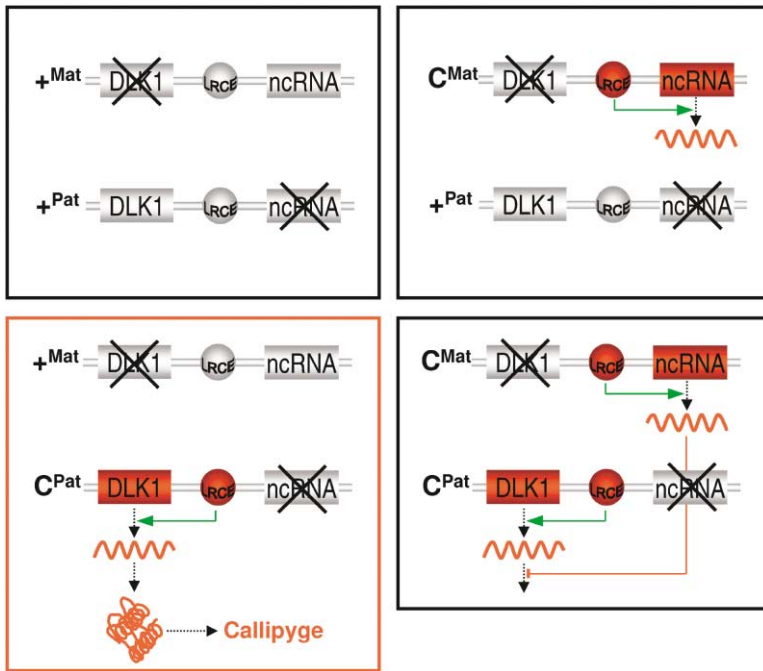


Figure 3. Model for Polar Overdominance at the Callipyge Locus

Working model for polar overdominance involving a paternally expressed growth promoter (*DLK1*), a maternally expressed *trans*-acting (red arrow) growth repressor thought to correspond to one or more noncoding RNA genes (*ncRNA*), and a *cis*-acting (green arrow) *LRCF* affected by the *CLPG* mutation. The crosses correspond to the parental imprinting effects. The functional status in post-natal skeletal muscle is illustrated for the four *CLPG* genotypes: active elements are shown in red, inactive elements in white. Only the  $+^{Mat}/C^{Pat}$  individuals express the callipyge phenotype (boxed in red) due to ectopic expression of the *DLK1* protein.

very significantly increased by 10.4% in  $+/T$  animals relative to  $+/+$  animals ( $p < 0.0002$ ). Transgenic animals of line D also displayed centrally located nuclei in a majority of myofibers, a sign of abnormal myofiber maturation (Figure 2B).

Altogether, these results indicate that ectopic expression of *DLK1* in skeletal muscle directly contributes to the muscular hypertrophy of callipyge sheep. The function of this member of the *EGF* domain-containing *Notch/Delta/Serrate* protein family remains poorly understood. It has been implicated in adipogenesis, haematopoiesis, lymphopoiesis, neuroendocrine differentiation, as well as tumorigenesis [7]. *Dlk1* null mice display accelerated adiposity as well as symptoms shared with *mUPD12* mice and *mUPD14* humans: growth retardation, blepharophimosis, and skeletal abnormalities [8]. A possible involvement of *Dlk1* in myogenesis is suggested by the myofiber hypertrophy and delayed maturation observed in murine *pUPD12* fetuses expressing a double dose of *Dlk1* [9] and by the known involvement of *Notch* signaling in myogenesis [e.g., 10, 11]. The observation of an inverse relationship between *DLK1* expression level and myofiber diameter suggests that ectopic *DLK1* expression causes both the hypertrophy and its own down regulation. Assuming that *DLK1* operates as a ligand in a *Notch*-related pathway, the ligand-receptor interaction could either be intercellular (in *trans*) or cell-autonomous (in *cis* [12]).

In addition to *DLK1*, the *DLK1-GTL2*-imprinted domain encompasses three protein-encoding genes that are preferentially expressed from the paternal allele in at least some tissues: *PEG11*, *BEGAIN*, and *DIO3*. *BEGAIN* and *DIO3* are weakly expressed in skeletal muscle, and their expression profile is not affected by the *CLPG* mutation (M. Smit et al., submitted). Therefore, it seems unlikely that these might play a role in the determinism of the callipyge phenotype. However, we cannot exclude

a role for *PEG11*, whose mRNA levels are influenced by the *CLPG* mutation in a manner analogous to *DLK1* [4]. Note that no evidence is available as of yet, supporting the translation of the corresponding mRNA. Experiments are underway to test whether ectopic expression of *PEG11* may contribute to the callipyge as well.

The presence of *DLK1* protein in skeletal muscle of  $+^{MAT}/CLPG^{PAT}$  animals only, despite comparable (albeit slightly lower) mRNA levels in *CLPG/CLPG* animals (Figure 1A), strongly supports a previously hypothesized posttranscriptional *trans* inhibition of *DLK1* mediated by one or more of the noncoding RNA genes expressed exclusively from the maternal *CLPG* allele [13]. It is intriguing in this regard that several maternally expressed miRNA genes were recently identified in the *DLK1-GTL2* imprinted domain [14]. We hypothesize that some of these may be directed toward *DLK1* transcripts interfering with their translation. The slight reduction of *DLK1* mRNA in *CLPG/CLPG* individuals when compared to  $+^{MAT}/CLPG^{PAT}$  animals (as well as the parallel reduction of *PEG11* mRNA) [4] could be explained by an associated RNAi activity. Alternatively, the hypothesized posttranscriptional *trans* inhibition might be mediated by cytoplasmic proteins binding both *DLK1* and noncoding RNA transcripts in a manner analogous to the *IMP* proteins binding both to *IGF2* and *H19* transcripts [15]. Note that because of the observed reduction of *DLK1* and *PEG11* mRNA in *CLPG/CLPG* versus  $+^{MAT}/CLPG^{PAT}$  individuals, a *trans* inhibition operating at the transcriptional level cannot totally be ruled out.

Figure 3 summarizes the main features of the present working model for polar overdominance at the callipyge locus. It includes a paternally expressed growth promoter identified in this work as being *DLK1*, a maternally expressed *trans*-acting *DLK1* suppressor hypothesized to be one or more of the noncoding RNAs, and a *cis*-

acting long-range control element (LRCE) affected by the *CLPG* mutation.

#### Supplemental Data

Supplemental Data including Experimental Procedures and two additional figures are available at <http://www.current-biology.com/cgi/content/full/14/20/1858/DC1/>.

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