# Abnormal postnatal maintenance of elevated *DLK1* transcript levels in callipyge sheep

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## Abstract

The underlying mechanism of the callipyge muscular hypertrophy phenotype in sheep (*Ovis aries*) is not presently understood. This phenotype, characterized by increased glycolytic type II muscle proportion and cell size accompanied by decreased adiposity, is not visibly detectable until approximately three to eight weeks after birth. The muscular hypertrophy results from a single nucleotide change located at the telomeric end of ovine Chromosome 18, in the region between the imprinted *MATERNALLY EXPRESSED GENE 3* (*MEG3*) and *DELTA, DROSOPHILA, HOMOLOG-LIKE 1* (*DLK1*) genes. The callipyge phenotype is evident only when the mutation is paternally inherited by a heterozygous individual. We have examined the pre- and postnatal expression of *MEG3* and *DLK1* in sheep of all four possible genotypes in affected and unaffected muscles as well as in liver. Here we show that the callipyge phenotype correlates with abnormally high *DLK1* expression during the postnatal period in the affected sheep and that this elevation is specific to the hypertrophy-responsive fast-twitch muscles. These results are the first to show anomalous gene expression that coincides with both the temporal and spatial distribution of the callipyge phenotype. They suggest that the effect of the callipyge mutation is to interfere with the normal postnatal downregulation of *DLK1* expression.

Callipyge (Greek for "beautiful buttocks") was the name given to the phenotype observed in certain descendants of a ram named "Solid Gold," born to a sheep (*Ovis aries*) flock in Oklahoma in 1983. Callipyge sheep exhibit muscular hypertrophy characterized by an elevation in number and size of type IIb glycolytic (fast-twitch) muscle fibers (Koohmaraie et al. 1995), including those in the *longissimus dorsi* and *biceps femoris* muscles. This is not a global muscle attribute, in that other muscles within the affected sheep (e.g., *infraspinatus*) are not subject to hypertrophy. The callipyge phenotype is inherited in a manner that is similar to that observed for imprinted genes; however, callipyge is apparent only in heterozygotes in which the mutation is inherited from the sire ($N^{MAT}C^{PAT}$, where $N$ indicates the wild-type allele, $C$ denotes the mutant allele, and the superscripts $MAT$ and $PAT$ indicate maternally and paternally derived, respectively). Unexpectedly, sheep homozygous for the mutation ($C^{MAT}C^{PAT}$) are phenotypically normal despite paternal inheritance of the mutated allele. This mode of inheritance was given the designation "polar overdominance" (Cockett et al. 1996) because the alternate heterozygotes exhibit divergent phenotypes (polar) and both homozygote genotypes are phenotypically normal (overdominance). Although highly suggestive of an imprinting component, the incomplete penetrance in the homozygous $C^{MAT}C^{PAT}$ individuals is not consistent with a phenotype strictly mediated by altered imprinting.
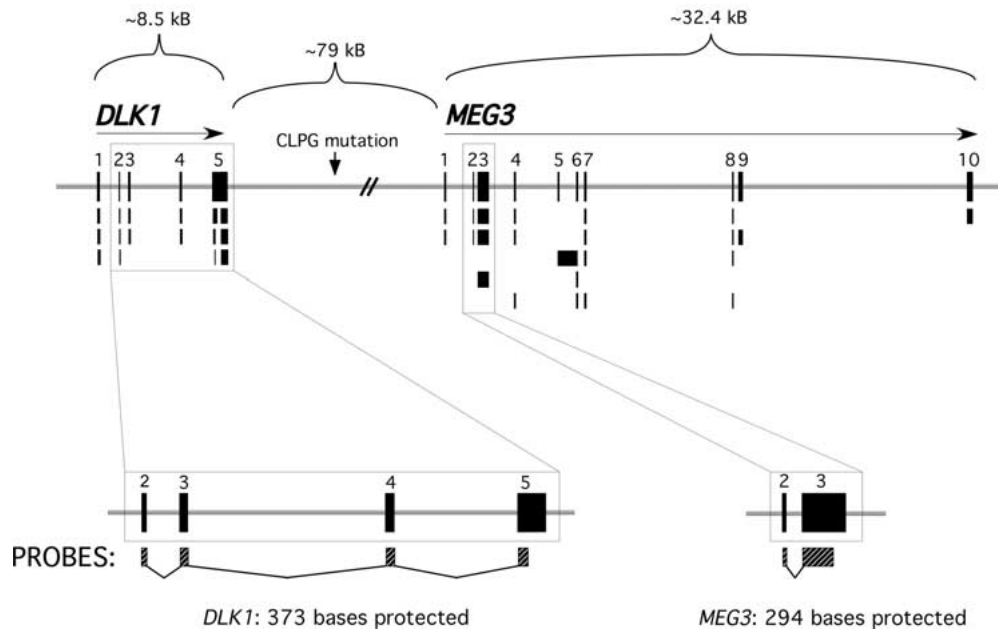
**Fig. 1.** Schematic representation of the *DLK1/MEG3* locus on ovine Chromosome 18. The direction of transcription is indicated by the arrows. Paternally expressed *DLK1* and maternally expressed *MEG3* are separated by approximately 79 kb and the relative position of the causative callipyge mutation is shown. The position of riboprobes designed to detect *DLK1* and *MEG3* [variant A (Bidwell et al. 2001)] transcripts is indicated below. Exon numbering for *DLK1* is based on information for the bovine (Fahrenkrug et al. 1999) and ovine (Charlier et al. 2001b) *DLK1* genomic structure. *MEG3* exons represent all known splice variants (Bidwell et al. 2001).

The nearly 10-year search for the mutation that causes the callipyge phenotype in sheep recently concluded with the identification of a single nucleotide change located within an intergenic region of the *DLK1* (*DELTA, DROSOPHILA, HOMOLOG-LIKE 1*) and *MEG3* (*MATERNALLY EXPRESSED GENE 3*, also referred to as *GTL2*) imprinted domain (Fig. 1) (Freking et al. 2002; Smit et al. 2003). In spite of the identification of this mutation, two things remain unclear: first, the molecular mechanism that allows a single nucleotide change in an apparently intergenic region to give rise to callipyge and, second, why the homozygous callipyge animals are seemingly unaffected.

There are several other imprinted genes in addition to *DLK1* and *MEG3* in the vicinity of the callipyge mutation. Charlier et al. (2001a) reported that the imprint status of these genes, including *DAT* (*DLK1-Associated Transcript*), *PEG11* (*Paternally Expressed Gene 11*), *MEG8* (*Maternally Expressed Gene 8*), and the aforementioned genes *DLK1* and *MEG3* was not altered in any of the callipyge genotypes evaluated. We independently confirmed the monoallelic expression of these genes in both fetal

and adult sheep (unpublished data). The callipyge mutation therefore does not alter the imprinting *per se* of nearby genes; this suggests instead that other aspects of the regulated expression of these genes may be affected.

The muscular hypertrophy of $N^{MAT}C^{PAT}$ animals occurs only in fast-twitch muscles and does not become apparent until after birth, indicating temporal regulation of the causative gene. We therefore predicted that the altered expression pattern of such a gene or genes would (1) be specific to sheep demonstrating the callipyge phenotype ($N^{MAT}C^{PAT}$ sheep) and not be observed in animals with other genotypes, (2) be specific to muscles affected by the mutation, and (3) be concurrent with the appearance of the phenotype.

The known biological activities of DLK1 strongly suggest that this protein contributes to the etiology of the callipyge phenotype. DLK1 is similar to the *Drosophila* neurogenic protein Delta (Laborda et al. 1993) and belongs to the epidermal growth factor-like family of proteins. DLK1 functions in regulating adipogenesis and cellular growth and differentiation (Laborda et al. 1993; Laborda 2000), including neu-

roblast differentiation (Van Limpt et al. 2000, 2003). *Dlk1* knockout mice exhibit growth retardation, rapidly develop obesity, and are unable to maintain appropriate levels of lipid metabolites, demonstrating important antiadipogenic and growth roles for murine Dlkl (Moon et al. 2002). *DLK1* is indeed highly expressed in preadipocytes and inhibits their differentiation, and is suppressed once the differentiation process begins (Smas and Sul 1993). Carcasses from callipyge sheep are characterized by decreased adiposity with approximately 24.3% fat and 71.3% fat-free lean compared with 31.5% and 64.0% for normal carcasses at typical industry slaughter weights (Freking et al. 1998b). The close proximity of sheep *DLK1* to the callipyge mutation, together with the roles of DLK1 in growth and differentiation processes, led us to previously propose that this gene is an excellent candidate for involvement in development of the callipyge phenotype (Fahrenkrug et al. 1999). Based on the known functions of DLK1, altered expression due to paternal inheritance of the callipyge mutation may be responsible for the observed lean phenotype of the callipyge sheep.

As an initial step in studying gene expression that may be affected by the callipyge mutation, we chose to examine *DLK1* and *MEG3*. *MEG3* encodes RNA that lacks a significant open reading frame and whose function remains unknown. There are multiple alternatively spliced transcripts of *MEG3* in mice (Croteau et al. 2003), humans (UCSC Genome Browser, July 2003; http://genome.ucsc.edu/), and sheep (Bidwell et al. 2001) without preservation of potential coding sequence, suggesting that *MEG3* functions as a noncoding RNA. *DLK1* and *MEG3* may exhibit coregulated expression, since they share many features of the coordinately regulated *IGF2/H19* imprinted gene pair that also influence growth and development (Wylie et al. 2000; Paulsen et al. 2001; Takada et al. 2002). Furthermore, *MEG3* has been postulated to regulate *DLK1* expression in *trans* (Georges et al. 2003), as has *H19* also been implicated as a *trans* regulator of *IGF2* (Forne et al. 1997; Li et al. 1998; Runge et al. 2000). We therefore examined the pre- and postnatal profile of gene expression for *MEG3* and *DLK1* to determine whether the presence of the mutation contributes to deregulated transcription of these genes in a tissue-specific and/or ontogenic manner.

## Materials and Methods

***Tissues.*** Sheep muscle tissue (*longissimus dorsi, biceps femoris, infraspinatus*) and liver were obtained from a resource population produced and maintained at the United States Department of Agriculture Meat Animal Research Center in Clay Center, Nebraska (Freking et al. 1998a). Tissues were stored at –80°C.

***Nucleic acid purification.*** Total RNA was isolated from frozen tissues using RNA Stat-60 according to the manufacturer's directions (TelTest), resuspended in nuclease free water, and either used immediately or stored at –80°C prior to use.

***Quantitative RNA analysis.*** 3.0 $\mu$g of total RNA from fetal (97–133 days gestation; see Freking et al. 1998a) [$N^{MAT}N^{PAT}$ ($n = 4$), $C^{MAT}N^{PAT}$ ($n = 3$), $N^{MAT}C^{PAT}$ ($n = 4$), and $C^{MAT}C^{PAT}$ ($n = 2$)] and adult (233–240 days) sheep [$N^{MAT}N^{PAT}$ ($n = 2$), $C^{MAT}N^{PAT}$ ($n = 2$), $N^{MAT}C^{PAT}$ ($n = 4$), and $C^{MAT}C^{PAT}$ ($n = 2$)] was analyzed using RNase protection assays (RPAII kit; Ambion, Austin, TX). Probes for *DLK1* and *MEG3* were prepared by PCR amplification using oligo dT-primed sheep cDNA with oligonucleotide primers for *DLK1*: DLKX2F (5′-GAA TGC TTC CCG GCC TGC CAC C-3′) and DLKX5R (5′ CCC TCA TCG TCC ACG CAG C-3′) and for *MEG3*: MEG3F1 (5′-GAC <u>A</u>CC TTC CGT CTG CCT TCC-3′) and MEG3R9 (5′ CAG AAG CAG ACG <u>C</u>AC ATA GAA AAG C-3′). The *MEG3* primers were designed based on the known bovine sequence because the ovine sequence including *MEG3* (Accession No. AF354168) was not available at the time these studies were initiated. The underlined residues in the *MEG3* primer sequences represent mismatches between the ovine and the bovine sequence, but these did not hinder the ability to amplify the *MEG3* sequence from ovine cDNA.

The amplicons were cloned into pGemT-Easy vectors (Promega) followed by nucleotide sequence confirmation from insert-containing colonies. The 373-nt *DLK1* probe sequence (within the 448-nt total length probe) corresponds to positions 54,868–61,074 of accession No. AF354168, and the 311-nt *MEG3* probe sequence (which protects a 294-nt fragment due to mismatches in the primer sequences within the 397-nt total length probe) corresponds to positions 140,792–141,315 of Accession No. AF354168. Both cDNA probes were designed to contain sequence from multiple exons. Antisense probes were generated by *in vitro* transcription (MaxiScript, Ambion) from linearized templates in the presence of $\alpha^{32}$P-[rCTP] using the Ambion Maxiscript kit. 18S rRNA was used as an internal control with $\alpha^{32}$P-[rCTP]-labeled probes generated from the pTRI-RNA-18S vector using the MEGAscript kit (Ambion). The protected *DLK1*, *MEG3*, and 18S rRNA fragments were separated on 6% denaturing polyacrylamide gels and quantified using a Molecular

Dynamics Storm PhosphorImager System (Amersham Biosciences, Piscataway, NJ). Relative levels of the protected *DLK1* and *MEG3* transcripts were normalized to the protected 18S rRNA fragments present in each sample.

Statistical analysis of the expression levels for each gene was performed using the unpaired Student's *t*-test with a two-tailed distribution assuming heteroscedastic variance. Unless otherwise specified, comparisons were made relative to normal sheep. Probability values less than 0.05 were considered significant.

### Results

We predicted that a callipyge effector gene(s) would exhibit altered patterns of expression among the callipyge genotypes that correlate with the presence and absence of the phenotype. To this end, we used RNase protection assays (Fig. 1) to measure expression of *MEG3* and *DLK1* in the hypertrophy-responsive *longissimus dorsi* and *biceps femoris* muscles, nonhypertrophic *infraspinatus* muscle, and liver from sheep of all four genotypes ($N^{MAT}N^{PAT}$, $C^{MAT}N^{PAT}$, $N^{MAT}C^{PAT}$, and $C^{MAT}C^{PAT}$).

During prenatal development, we found low levels of *MEG3* transcripts in *lonsissimus dorsi* muscle of $N^{MAT}N^{PAT}$ and $N^{MAT}C^{PAT}$ animals, with a modest increase in fetuses with maternal inheritance of the callipyge mutation (Fig. 2A). This trend is maintained in the adult sheep, with an overall elevation in *MEG3* transcript levels relative to those in the fetal sheep of the same genotype (Fig. 2A). The increased level of *MEG3* mRNA observed in the fetal $C^{MAT}N^{PAT}$ and $C^{MAT}C^{PAT}$ sheep is even more prominent in the adults, but these differences were not statistically significant.

To determine the expression levels of *DLK1*, we measured *DLK1* transcripts in fetal and adult *longissimus dorsi*. *DLK1* expression is high prenatally in all four genotypes with the maximum level of expression in the $C^{MAT}N^{PAT}$ animals (Fig. 3A, 3B). Postnatally, there is an approximate threefold decrease in the expression of *DLK1* in the $N^{MAT}N^{PAT}$, $C^{MAT}N^{PAT}$, and $C^{MAT}C^{PAT}$ sheep, but strikingly, the $N^{MAT}C^{PAT}$ sheep maintain elevated levels of *DLK1* transcripts ($p = 0.013$ compared to that of the $N^{MAT}N^{PAT}$ animals; Fig. 3A, 3C). Our results indicate that $N^{MAT}C^{PAT}$ sheep do not repress postnatal transcription of *DLK1*.

To examine whether these patterns of expression are specific to muscles containing predominantly fast-twitch fibers (the muscles affected in the callipyge sheep), we evaluated *MEG3* and *DLK1* expression in two other muscle types in adult sheep. *Biceps*

*femoris*, another hypertrophy-responsive muscle, exhibits a very similar profile of *MEG3* expression to *longissimus dorsi*, with elevated *MEG3* levels in sheep with a maternally inherited callipyge mutation (Fig. 4A). Interestingly, this pattern is also seen in *infraspinatus* (Fig. 4B), which is composed primarily of slow-twitch muscle fibers and is unaffected in the callipyge phenotype. Although the levels of *infraspinatus MEG3* transcripts are higher in sheep inheriting the callipyge mutation on the maternal allele compared to normal sheep, these differences were not significant. Fetal and adult $N^{MAT}C^{PAT}$ sheep do not express significantly altered levels of *MEG3* relative to $N^{MAT}N^{PAT}$ sheep in any of the hypertrophic muscle types analyzed. These results indicate that the influence of the callipyge mutation on expression of *MEG3* is not restricted to fast-twitch muscle fibers and therefore suggest that *MEG3* expression is not directly responsible for the callipyge phenotype.

*DLK1* expression is increased in *biceps femoris* of the $N^{MAT}C^{PAT}$ animals approximately fourfold over that observed in the $N^{MAT}N^{PAT}$ ($p = 0.042$) and $C^{MAT}N^{PAT}$ animals ($p = 0.039$) (Fig. 4A). *Biceps femoris* in the $C^{MAT}C^{PAT}$ animals exhibits slightly higher levels of *DLK1* transcripts relative to the $N^{MAT}N^{PAT}$ and $C^{MAT}N^{PAT}$ sheep, but the expression is much lower ($\sim$40%) than in $N^{MAT}C^{PAT}$ sheep ($p = 0.114$). Although *biceps femoris* is an affected muscle in the callipyge sheep, our results indicate that the increase in *DLK1* expression in this particular muscle is not as high as that observed in *longissimus dorsi*. However, *DLK1* expression in *biceps femoris* in the $N^{MAT}C^{PAT}$ adult sheep is distinctly elevated relative to the other three genotypes.

In contrast to the elevated *DLK1* gene expression profile seen in adult $N^{MAT}C^{PAT}$ fast-twitch muscles, *DLK1* is expressed at much lower levels in *infraspinatus* of the same adult sheep (Fig. 4B). We observed small elevation in expression in the $C^{MAT}N^{PAT}$ (1.8-fold), $N^{MAT}C^{PAT}$ (1.6-fold), and $C^{MAT}C^{PAT}$ (1.7-fold) adult animals relative to the $N^{MAT}N^{PAT}$ sheep. In spite of this small elevation in *DLK1* levels across all three genotypes carrying the mutated allele(s), the differences in expression for *both DLK1* and *MEG3* between $N^{MAT}C^{PAT}$ and $N^{MAT}N^{PAT}$ adult sheep are statistically significant ($p = 0.027$ and $p = 0.031$, respectively). This finding is likely due to the greater precision of testing differences between a larger number of $N^{MAT}C^{PAT}$ individuals ($n = 4$) relative to the other genotypes ($n = 2$ for each), since $C^{MAT}N^{PAT}$ and $C^{MAT}C^{PAT}$ both express higher averaged levels of *DLK1* and *MEG3* than do the $N^{MAT}C^{PAT}$ animals. These results indicate that the abnormal elevation of *DLK1* expression in the adult $N^{MAT}C^{PAT}$ animals
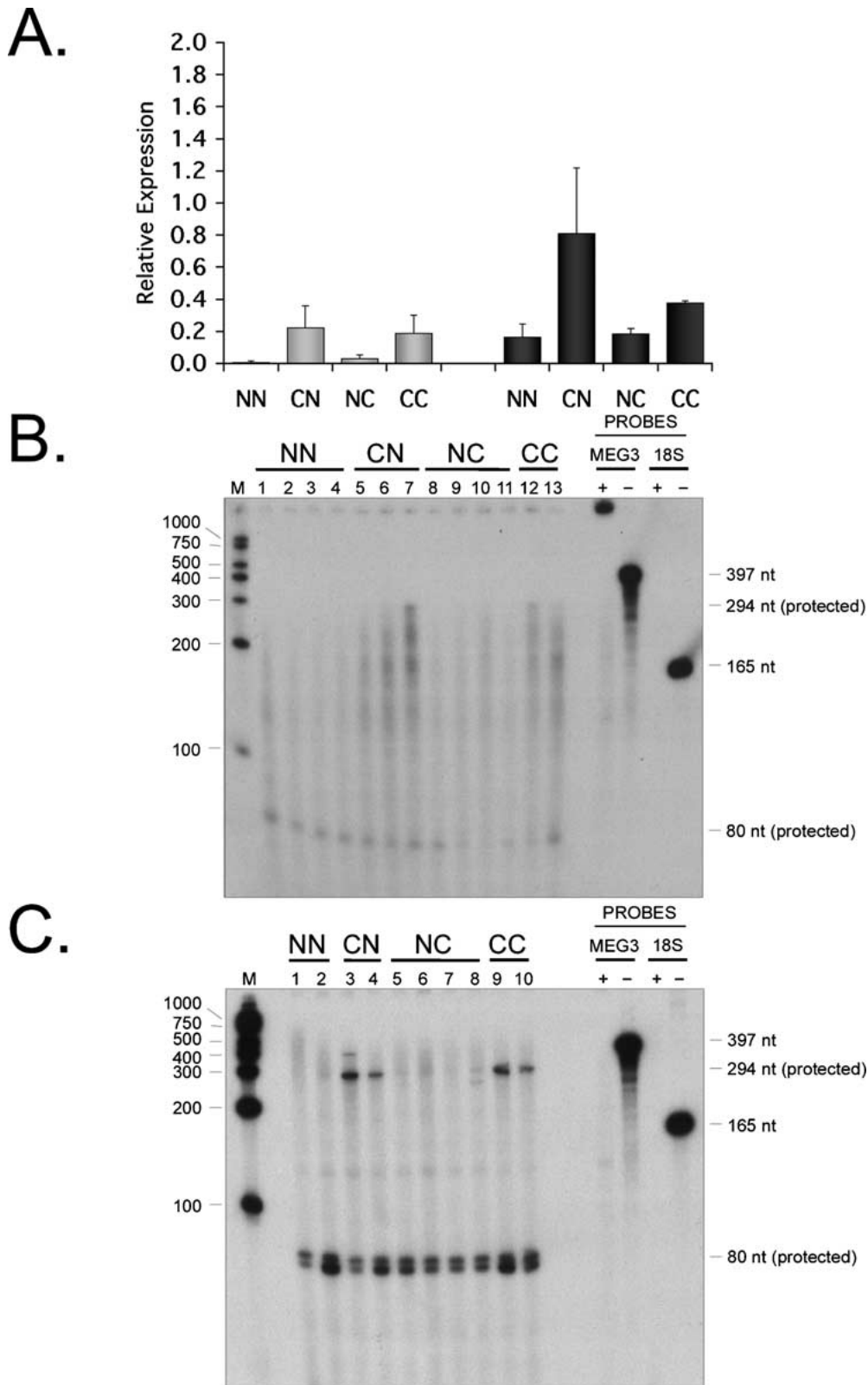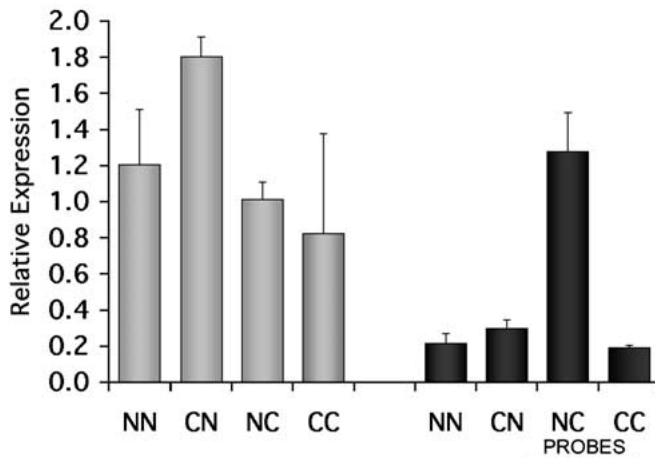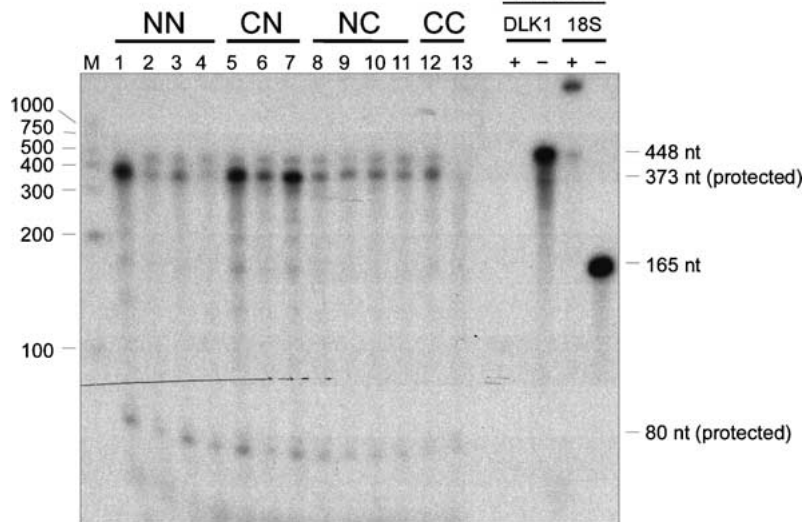
**Fig. 2.** Quantitative analysis of *MEG3* expression in *longissimus dorsi* of fetal and adult sheep. Total RNA from *longissimus dorsi* muscle was analyzed using RNase protection and normalized to 18S rRNA present in each sample to give the relative level of gene expression. (**A**) Relative expression of *MEG3* in fetal *longissimus dorsi* (gray bars) from $N^{MAT}N^{PAT}$ ($n$ = 4), $C^{MAT}N^{PAT}$ ($n$ = 3), $N^{MAT}C^{PAT}$ ($n$ = 4), and $C^{MAT}C^{PAT}$ ($n$ = 2) sheep and adult *longissimus dorsi* (black bars) from $N^{MAT}N^{PAT}$ ($n$ = 2), $C^{MAT}N^{PAT}$ ($n$ = 2), $N^{MAT}C^{PAT}$ ($n$ = 4), and $C^{MAT}C^{PAT}$ ($n$ = 2) sheep. Error bars, standard error of the mean (SEM). (**B, C**) RNase protection assays used to generate the histogram in panel (**A**) showing expression of *MEG3* in fetal (**B**) and adult (**C**) *longissimus dorsi*. Probes were run in the presence (+) or absence (−) of RNase. The *MEG3* probe protects 294 nucleotides of *MEG3* variant A (Bidwell et al. 2001), while the 18S rRNA probe protects a doublet
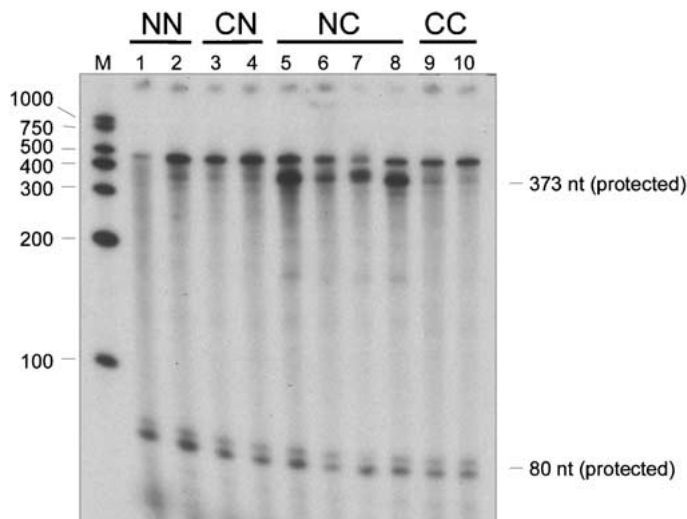
**Fig. 3.** Quantitative analysis of *DLK1* expression in *longissimus dorsi* of fetal and adult sheep. Total RNA from *longissimus dorsi* muscle was analyzed using RNase protection and normalized to 18S rRNA present in each sample to give the relative level of gene expression. (**A**) Relative expression of *DLK1* in fetal *longissimus dorsi* (gray bars) from $N^{MAT}N^{PAT}$ ($n = 4$), $C^{MAT}N^{PAT}$ ($n = 3$), $N^{MAT}C^{PAT}$ ($n = 4$), and $C^{MAT}C^{PAT}$ ($n = 2$) sheep and adult *longissimus dorsi* (black bars) from $N^{MAT}N^{PAT}$ ($n = 2$), $C^{MAT}N^{PAT}$ ($n = 2$), $N^{MAT}C^{PAT}$ ($n = 4$), and $C^{MAT}C^{PAT}$ ($n = 2$) sheep. Error bars-SEM. (**B, C**) RNase protection assays used to generate the histogram in panel (**A**) showing expression of *DLK1* in fetal (**B**) and adult (**C**) *longissimus dorsi*. Probes in (**B**) were run alone in the presence (+) or absence (−) of RNase. The *DLK1* probe protects 373 nucleotides, while the 18S rRNA probe protects a doublet migrating at approximately 80 nucleotides. M-marker lane.
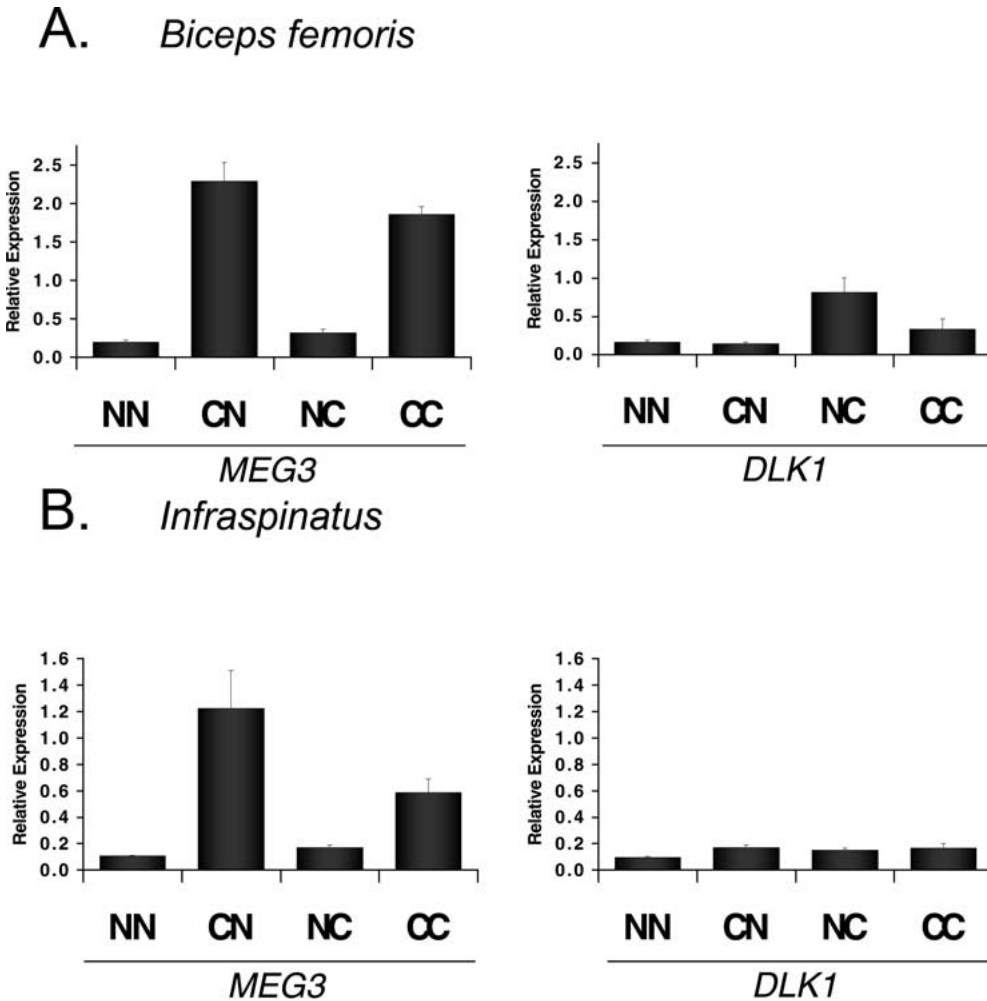
**Fig. 4.** Quantitative analysis of *MEG3* and *DLK1* expression in *biceps femoris* and *infraspinatus* muscles. Total RNA from *biceps femoris* muscle was analyzed using RNase protection and normalized to 18S rRNA present in each sample to give the relative level of gene expression. (**A**) Relative expression of *MEG3* (left) and *DLK1* (right) in adult *biceps femoris* from $N^{MAT}N^{PAT}$ (n = 2), $C^{MAT}N^{PAT}$ (n = 2), $N^{MAT}C^{PAT}$ (n = 4), and $C^{MAT}C^{PAT}$ (n = 2) sheep. (**B**) Relative expression of *MEG3* (left) and *DLK1* (right) in adult *infraspinatus* from $N^{MAT}N^{PAT}$ (n = 2), $C^{MAT}N^{PAT}$ (n = 2), $N^{MAT}C^{PAT}$ (n = 4), and $C^{MAT}C^{PAT}$ (n = 2) sheep. Error bars-SEM.

specifically occurs in fast-twitch muscle fibers, i.e., those affected by callipyge.

Little is known about the expression of *DLK1* and *MEG3* in tissues other than muscle in the callipyge sheep. To investigate whether the patterns of expression for *MEG3* and *DLK1* in mesodermal-derived tissues of the sheep harboring the callipyge mutation extend into tissues from a different embryonic lineage, we performed RNase protection assays in endodermal tissue using the same *MEG3* and *DLK1* probes (Figs. 5 and 6). In both fetal and adult liver, the relative level of expression of both genes is low compared to muscle (note Y-axis), and the overall level of *MEG3* is greatly decreased in the adults relative to that found in fetal sheep (7.9-fold averaged across genotypes). Interestingly, in spite of

the low RNA levels, *MEG3* expression does vary significantly between the $N^{MAT}N^{PAT}$ and $C^{MAT}N^{PAT}$ (p = 0.031) genotypes in adult liver and expression is lower (although not statistically significant) in the $C^{MAT}C^{PAT}$ adults as well (Fig. 5A, Table 1). This pattern of expression, with higher levels of *MEG3* in the $N^{MAT}N^{PAT}$ and $N^{MAT}C^{PAT}$ is opposite that observed in the fetal liver and in muscle tissues, indicating independent regulatory mechanisms for *MEG3* expression in tissues of mesodermal and endodermal origin.

*DLK1* expression is also higher in the fetal liver samples versus adult liver (6.6-fold when averaged across genotypes), and there is a tendency toward higher levels of expression of *DLK1* where the callipyge mutation is maternally inherited ($C^{MAT}N^{PAT}$
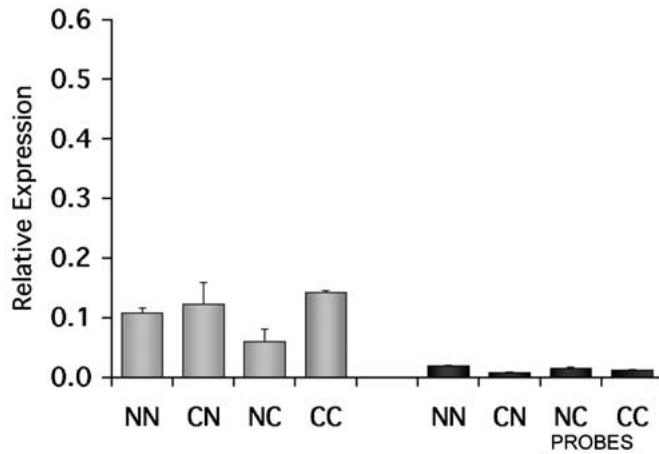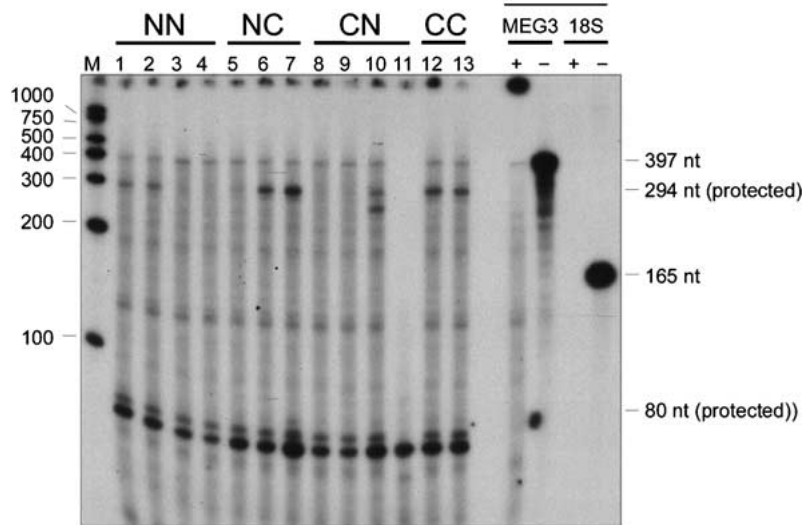
**Fig. 5.** Quantitative analysis of *MEG3* expression in liver of fetal and adult sheep. Total RNA from liver tissue was analyzed using RNase protection and normalized to 18S rRNA present in each sample to give the relative level of gene expression. (**A**) Relative expression of *MEG3* in fetal liver (gray bars) from $N^{MAT}N^{PAT}$ ($n$ = 4), $C^{MAT}N^{PAT}$ ($n$ = 3), $N^{MAT}C^{PAT}$ ($n$ = 4), $C^{MAT}C^{PAT}$ ($n$ = 2) sheep and adult liver (black bars) from $N^{MAT}N^{PAT}$ ($n$ = 2), $C^{MAT}N^{PAT}$ ($n$ = 2), n$N^{MAT}C^{PAT}$ ($n$ = 4), and $C^{MAT}C^{PAT}$ ($n$ = 2) sheep. Error bars-SEM. (**B, C**) RNase protection assays used to generate the histogram in panel (**A**) showing expression of *MEG3* in fetal (**B**) and adult (**C**) liver. Probes were run alone in the presence (+) or absence (−) of RNase. The *MEG3* probe protects 294 nucleotides, while 18S rRNA probe protects a doublet migrating at approximately 80 nucleotides. M-marker lane.
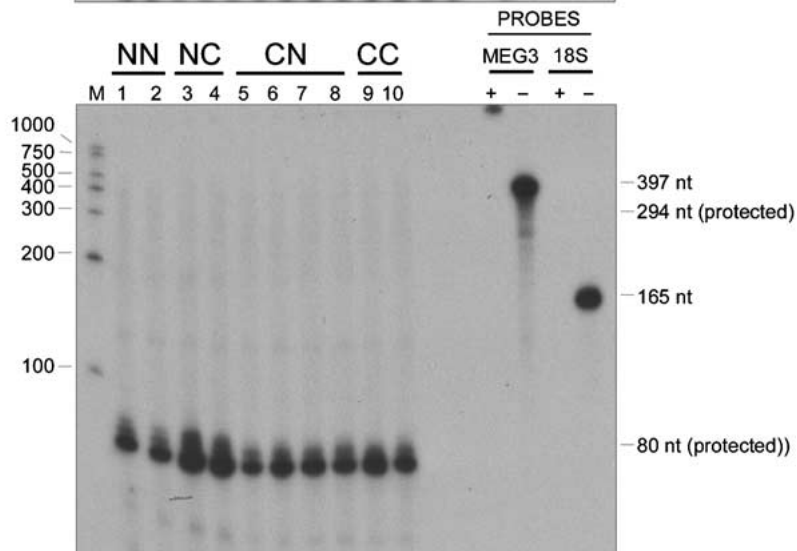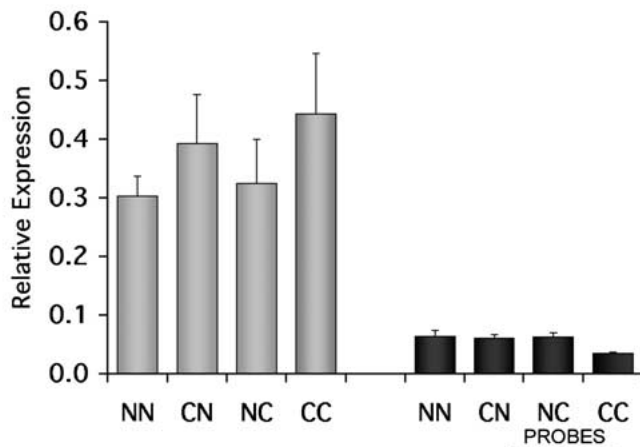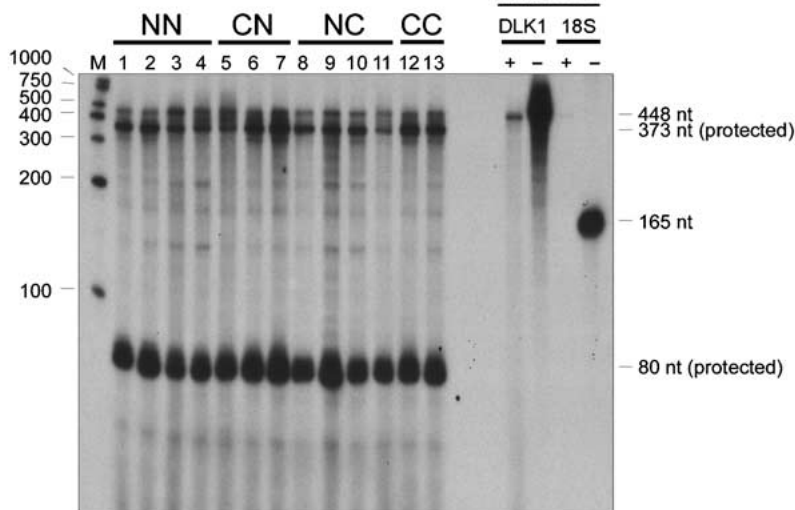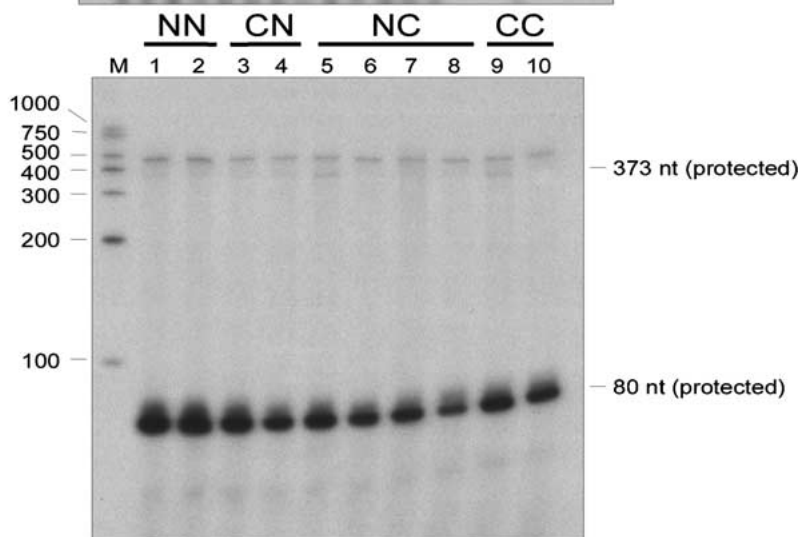
**Fig. 6.** Quantitative analysis of *DLK1* expression in liver of fetal and adult sheep. Total RNA from liver tissue was analyzed using RNase protection and normalized to 18S rRNA present in each sample to give the relative level of gene expression. (**A**) Relative expression of *DLK1* in fetal liver (gray bars) from $N^{MAT}N^{PAT}$ ($n$ =4), $C^{MAT}N^{PAT}$ ($n$ = 3), $N^{MAT}C^{PAT}$ ($n$ = 4), and $C^{MAT}C^{PAT}$ ($n$ = 2) sheep and adult liver (black bars) from $N^{MAT}N^{PAT}$ ($n$ = 2), $C^{MAT}N^{PAT}$ ($n$ = 2), $N^{MAT}C^{PAT}$ ($n$ = 4), and $C^{MAT}C^{PAT}$ ($n$ = 2), sheep. Error bars-SEM. (**B, C**) RNase protection assays used to generate the histogram in panel (**A**) showing expression of *DLK1* in fetal (**B**) and adult (**C**) liver. Probes in (**B**) were run alone in the presence (+) or absence (−) of RNase. The *DLK1* probe protects 373 nucleotides, while the 18S rRNA probe protects a doublet migrating at approximately 80 mucleotides. M-marker lane.

**Table 1.** Relative gene expression values for individual sheep[a].

| Sheep | Genotype | Longissimus dorsi | | | | | Biceps femoris | | | | | Infraspinatus | | | | | Liver | | | | |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| | | DLK1 | p[b] | MEG3 | p[b] | DLK1:MEG3[c] | DLK1 | p[b] | MEG3 | p[b] | DLK1:MEG3[c] | DLK1 | p[b] | MEG3 | p[b] | DLK1:MEG3[c] | DLK1 | p[b] | MEG3 | p[b] | DLK1:MEG3[c] |
| **FETAL** | | | | | | | | | | | | | | | | | | | | | |
| 34 | NN | 2.077 | R | 0.000 | R | 114.9 | | | | | | | | | | | 0.337 | R | 0.092 | R | 2.8 |
| 51 | | 0.987 | | 0.007 | | | | | | | | | | | | | 0.349 | | 0.132 | | |
| 63 | | 1.112 | | 0.021 | | | | | | | | | | | | | 0.322 | | 0.106 | | |
| 73 | | 0.646 | | 0.014 | | | | | | | | | | | | | 0.201 | | 0.103 | | |
| 35 | CN | 1.910 | 0.146 | 0.028 | 0.269 | 8.2 | | | | | | | | | | | 0.229 | 0.397 | 0.051 | 0.733 | 3.2 |
| 44 | | 1.583 | | 0.144 | | | | | | | | | | | | | 0.503 | | 0.169 | | |
| 46 | | 1.916 | | 0.491 | | | | | | | | | | | | | 0.447 | | 0.148 | | |
| 48 | NC | 0.799 | 0.586 | 0.000 | 0.295 | 29.2 | | | | | | | | | | | 0.468 | 0.802 | 0.079 | 0.090 | 5.4 |
| 49 | | 0.978 | | 0.010 | | | | | | | | | | | | | 0.201 | | 0.077 | | |
| 62 | | 1.025 | | 0.084 | | | | | | | | | | | | | 0.442 | | 0.084 | | |
| 72 | | 1.253 | | 0.045 | | | | | | | | | | | | | 0.186 | | 0.000 | | |
| 31 | CC | 1.376 | 0.618 | 0.299 | 0.348 | 4.4 | | | | | | | | | | | 0.546 | 0.385 | 0.141 | 0.023 | 3.1 |
| 50 | | 0.274 | | 0.078 | | | | | | | | | | | | | 0.341 | | 0.145 | | |
| **ADULT** | | | | | | | | | | | | | | | | | | | | | |
| 23806 | NN | 0.160 | R | 0.244 | R | 1.3 | 0.204 | R | 0.230 | R | 0.8 | 0.088 | R | 0.110 | R | 0.9 | 0.053 | R | 0.021 | R | 3.3 |
| 23885 | | 0.272 | | 0.082 | | | 0.127 | | 0.164 | | | 0.104 | | 0.104 | | | 0.074 | | 0.018 | | |
| 23963 | CN | 0.252 | 0.379 | 1.217 | 0.347 | 0.4 | 0.169 | 0.685 | 1.981 | 0.090 | 0.1 | 0.150 | 0.129 | 0.934 | 0.161 | 0.1 | 0.055 | 0.874 | 0.007 | 0.031 | 7.5 |
| 24054 | | 0.344 | | 0.407 | | | 0.118 | | 2.594 | | | 0.190 | | 1.510 | | | 0.067 | | 0.009 | | |
| 23829 | NC | 1.746 | 0.013 | 0.136 | 0.842 | 7.0 | 1.287 | 0.042 | 0.266 | 0.103 | 2.6 | 0.162 | 0.027 | 0.166 | 0.031 | 0.9 | 0.042 | 0.309 | 0.022 | 0.223 | 3.1 |
| 23833 | | 0.870 | | 0.261 | | | 0.823 | | 0.283 | | | 0.183 | | 0.174 | | | 0.064 | | 0.015 | | |
| 23884 | | 0.966 | | 0.119 | | | 0.347 | | 0.266 | | | 0.125 | | 0.210 | | | 0.048 | | 0.009 | | |
| 24036 | | 1.530 | | 0.218 | | | 0.782 | | 0.451 | | | 0.131 | | 0.129 | | | 0.031 | | 0.013 | | |
| 23835 | CC | 0.203 | 0.747 | 0.388 | 0.225 | 0.5 | 0.192 | 0.436 | 1.960 | 0.026 | 0.2 | 0.133 | 0.264 | 0.483 | 0.134 | 0.3 | 0.034 | 0.222 | 0.012 | 0.074 | 2.8 |
| 23951 | | 0.182 | | 0.366 | | | 0.466 | | 1.751 | | | 0.199 | | 0.688 | | | 0.036 | | 0.013 | | |

a) Genotypes are listed with the maternally-inherited allele first; N, wild-type allele; C, mutated allele. Light grey denotes animals with the callipyge phenotype. *Biceps femoris* and *infraspinatus* values were not determined for fetal sheep. b) *p* value from Student's t-test, two-tailed distribution (unequal variance) using the NN sheep as referent (R). Dark grey shading indicates significant *p* values. c) Ratio of averaged *DLK1* to *MEG3* expression for each group.

and $C^{MAT}C^{PAT}$ animals), although this difference is not statistically significant (Fig. 6A). Like *MEG3* in adult liver, this pattern of expression is contrary to the profile observed in muscle. These subtle differences in *DLK1* expression are not evident postnatally, with a genotype-independent decreased level of *DLK1* expression in adult sheep liver (Fig. 6A, C). The lack of correlation between the expression of *MEG3* and *DLK1* in endodermal tissue with the phenotype of the callipyge sheep underscores that the abnormal patterns of expression of *DLK1* are unique to mesodermal tissues in the affected sheep; specifically, the fast-twitch muscles. Taken together, these results suggest that the callipyge phenotype is a result of deregulation of *DLK1* expression that occurs postnatally and that this effect is highly tissue-specific.

## Discussion

To date, much speculation has focused on the mechanism underlying callipyge and the gene(s) responsible. We have quantified expression of the two genes immediately adjacent to the callipyge mutation, *MEG3* and *DLK1*, in an attempt to determine their potential contribution to this unusual phenotype. We used RNase protection assays with gene-specific probes on fetal and adult sheep tissues and have discounted a direct role for *MEG3* as a mediator of the callipyge phenotype and provided strong evidence that *DLK1* expression has a causal role.

Our results for *MEG3* agree with those previously reported by Charlier et al. (2001a) and Bidwell et al. (2001) in that there is an increase in maternally expressed *MEG3* from alleles carrying the mutation. In the Charlier et al. study, *MEG3* transcripts were elevated in *longissimus dorsi* of unaffected 8-week-old $C^{MAT}N^{PAT}$ and $C^{MAT}C^{PAT}$ sheep. Bidwell et al. (2001) evaluated all four genotypes at day 56 postnatal in the hypertrophy-responsive *semimembranosus* muscle, and their results are similar to those shown here for *longissimus dorsi* of adult sheep, in that the $C^{MAT}N^{PAT}$ and $C^{MAT}C^{PAT}$ sheep exhibit higher levels of *MEG3* expression compared to the $N^{MAT}N^{PAT}$ and $N^{MAT}C^{PAT}$ animals. In contrast to the Bidwell et al. study (2001), we found that *MEG3* expression during prenatal development was lower across all four genotypes than in the adult sheep. This discrepancy may be due to the smaller number of animals analyzed in their study (one each of the $N^{MAT}N^{PAT}$ and $N^{MAT}C^{PAT}$ at each time point) or differences in RNA loading. Nevertheless, because the altered expression patterns of the *MEG3* transcripts are not

unique to the affected $N^{MAT}C^{PAT}$ sheep, these results together suggest that *MEG3* is unlikely to be a direct mediator of the muscle hypertrophy phenotype.

Our data clearly show persistent elevation of *DLK1* expression in postnatal $N^{MAT}C^{PAT}$ sheep, specifically in callipyge-affected muscles. In another study, *DLK1* was found to be elevated in *longissimus dorsi* muscle of both $N^{MAT}C^{PAT}$ and $C^{MAT}C^{PAT}$ eight-week-old lambs by Northern blot, with decreased levels in the $N^{MAT}N^{PAT}$ and $C^{MAT}N^{PAT}$ sheep (Charlier et al. 2001a). In our study, the average age of the adult sheep was approximately 33 weeks, and at this age the callipyge phenotype is fully evident. The elevated *DLK1* in eight-week-old $C^{MAT}C^{PAT}$ lambs suggests that there may be a delay in downregulation of *DLK1* expression in the homozygous mutant sheep postnatally, and this delay is apparently not sufficient to give rise to muscular hypertrophy. Combined, these results suggest that the normal attenuation of *DLK1* expression takes place perinatally but further work will be required to determine the precise timing of this event.

One mechanism that could account for the normal reduction of *DLK1* expression in postnatal sheep is via a *trans*-regulatory effect (Charlier et al. 2001a; Georges et al. 2003; Charlier 2004). In this scenario, another gene that is reciprocally expressed with *DLK1* (e.g., *MEG3*) may modulate *DLK1* expression. Our results indicate that *DLK1* expression is normally reduced postnatally, and this reduction appears to be concomitant with an increase in *MEG3* expression. Table 1 shows the ratio of the average expression of these two genes for each group of animals in this study. In support of a *trans*-regulatory role for *MEG3*, the ratio of *DLK1* to *MEG3* is high during fetal development in all four genotypes (at a time when the phenotype is not evident), while in adult sheep this ratio remains elevated only in the affected $N^{MAT}N^{PAT}$ animals, and this pattern is observed in the affected muscles. We and others (Charlier et al. 2001a) have noted that expression of genes linked *in cis* to the callipyge mutation is elevated; as such, the lack of a phenotype in the $C^{MAT}C^{PAT}$ animals may result from the ability of increased *MEG3* from the maternal allele to "control" *DLK1* that is expressed from the paternal allele. This type of *trans* effect might occur at the transcriptional or translational level. The results shown in Fig. 3 suggest that this type of regulatory effect may indeed be at the level of transcription since *DLK1* transcripts are not elevated in the $C^{MAT}C^{PAT}$ sheep, but more work will be required to test this postulate.

Together with the known biological activities of the DLK1 protein, the combined studies make sheep DLK1 the strongest candidate to date for mediating the effects of the callipyge mutation. Further support for *DLK1* as the callipyge effector gene comes from several lines of evidence. First, pigs also exhibit polar overdominance associated with growth, adiposity, and muscle fiber composition; this is linked to paternal inheritance of *DLK1* (Kim et al. 2004). Second, the presence of DLK1 protein was recently shown to be highly specific to fast-twitch muscle fibers in postnatal $N^{MAT}C^{PAT}$ sheep (Davis et al. 2004). Lastly, transgenic mice overexpressing Dlk1 display generalized muscular hypertrophy, linking Dlk1 to the key characteristic of the callipyge phenotype (Davis et al. 2004). The signaling pathways through which DLK1 acts are currently not well understood. Given the role of DLK1 in specifying alternative differentiation fates *in vitro*, and the increased number of fast-twitch muscle fibers and reduced adiposity in the callipyge sheep, it is possible that sheep DLK1 functions in mesodermal differentiation processes, specifically in the choice between myoblast versus adipocyte cell lineages. Appropriate *in vitro* and *in vivo* model systems will be required to investigate this aspect of DLK1 function.

Imprinted genes other than *MEG3* and *DLK1* are located in the same region and might also contribute to the callipyge phenotype (Charlier et al. 2001b). These include *DAT* and *MEG8*, whose human sequence was recently shown to encode snoRNAs (small nucleolar RNAs) (Cavaille et al. 2002). *PEG11* is also located in this region and encodes a gag and pol-like polyprotein of unknown function. *PEG11* is expressed in sheep skeletal muscle (Charlier et al. 2001a) and is therefore potentially involved in callipyge. *PEG11* transcripts are indeed very highly expressed in the affected muscles of postnatal $N^{MAT}C^{PAT}$ sheep compared to the other genotypes, consistent with a specific effect resulting from the presence of the callipyge mutation that correlates with phenotype (Bidwell et al. 2004). However, it is unknown whether PEG11 protein is produced, or how *PEG11* may functionally contribute to muscular hypertrophy. In addition, temporal differences in *PEG11* expression between fetal and adult sheep remain to be demonstrated. Seitz et al. (2003) established that in mice the previously reported *anti-PEG11* (Charlier et al. 2001b) in fact produces microRNAs, and they postulated that these microRNAs may be involved in regulating expression of PEG11. Whether this applies to sheep and whether other microRNAs are present within this imprinted domain that contribute to callipyge muscular hypertrophy is presently unknown. The callipyge mutation is located in a region that is transcribed in sheep to produce *CLPG1*, an RNA that has the potential to encode protein (Freking et al. 2002).

However, the open reading frame of this putative protein is not well-conserved across species and, therefore, it is presently unresolved whether *CLPG1* has a direct role in the callipyge phenotype. Quantitative expression studies of all of these genes, similar to those described in the current study, are required to answer these questions.

In conclusion, we have provided evidence that the callipyge phenotype correlates with altered temporal expression of the *DLK1* gene. Specifically, the appearance of the phenotype coincides with abnormally elevated *DLK1* expression in the affected sheep; this is in direct contrast to the downregulation of *DLK1* in all of the nonaffected genotypes, including the homozygous $C^{MAT}C^{PAT}$ sheep that are phenotypically normal. Furthermore, substantial *DLK1* mRNA transcript elevation is not seen in the nonaffected *infraspinatus* muscle or in liver of the $N^{MAT}C^{PAT}$ animals; therefore, this variance does not represent a global deregulation of this gene. This evidence, together with the role of *DLK1* in cellular differentiation, strongly suggests that *DLK1* is an effector of callipyge and that the callipyge mutation is somehow negating the ability to downregulate expression of *DLK1* postnatally. Further investigation will be required to determine how the callipyge mutation mechanistically influences the expression of the imprinted genes in the callipyge region.

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