Park7 Expression Influences Myotube Size and Myosin Expression in Muscle

Hui Yu1, Jolena N. Waddell1, Shihuan Kuang1,2, Christopher A. Bidwell1,4
1 Department of Animal Sciences, Purdue University, West Lafayette, Indiana, United States of America, 2 Center for Cancer Research, Purdue University, West Lafayette, Indiana, United States of America

Abstract
Callipyge sheep exhibit postnatal muscle hypertrophy due to the up-regulation of DLK1 and/or RTL1. The up-regulation of PARK7 was identified in hypertrophied muscles by microarray analysis and further validated by quantitative PCR. The expression of PARK7 in hypertrophied muscle of callipyge lambs was confirmed to be up-regulated at the protein level. PARK7 was previously identified to positively regulate PI3K/AKT pathway by suppressing the phosphatase activity of PTEN in mouse fibroblasts. The purpose of this study was to investigate the effects of PARK7 in muscle growth and protein accretion in response to IGF1. Primary myoblasts isolated from Park7 (+/+ ) and Park7 (−/−) mice were used to examine the effect of differential expression of Park7. The Park7 (+/+ ) myotubes had significantly larger diameters and more total sarcomeric myosin expression than Park7 (−/−) myotubes. IGF1 treatment increased the mRNA abundance of Myh4, Myh7 and Myh8 between 20-40% in Park7 (+/+ ) myotubes relative to Park7 (−/−). The level of AKT phosphorylation was increased in Park7 (+/+ ) myotubes at all levels of IGF1 supplementation. After removal of IGF1, the Park7 (+/+ ) myotubes maintained higher AKT phosphorylation through 3 hours. PARK7 positively regulates the PI3K/AKT pathway by inhibition of PTEN phosphatase activity in skeletal muscle. The increased PARK7 expression can increase protein synthesis and result in myotube hypertrophy. These results support the hypothesis that elevated expression of PARK7 in callipyge muscle would increase levels of AKT activity to cause hypertrophy in response to the normal IGF1 signaling in rapidly growing lambs. Increasing expression of PARK7 could be a novel mechanism to increase protein accretion and muscle growth in livestock or help improve muscle mass with disease or aging.

Introduction
Callipyge sheep exhibit postnatal muscle hypertrophy, with higher rates of protein accretion and lower rates of fat deposition compared to normal sheep [1,2]. The muscle hypertrophy phenotype is most prominent in the loin and hind-quarters at 4–6 weeks of age due to increased muscle fiber diameter and percentage of fast-twitch, glycolytic muscle fibers, [3–6]. The callipyge mutation is a single nucleotide polymorphism in the imprinted gene cluster [7,8] that causes up-regulation of Delta-like 1 (DLK1) and Retransposon-like 1 (RTL1) in hypertrophied muscles [9–13]. Transgenic mice over-expressing Dlk1 exhibited increased muscle mass and myofiber diameter [14]. Muscle-specific gene ablation of Dlk1 in the mouse resulted in reduced body weight and skeletal muscle mass due to reductions in myofiber numbers [15]. Conversely, over-expression of Dlk1 in culture was shown to inhibit myoblast proliferation and enhance myotube differentiation [15]. Microarray analysis of gene expression identified 199 genes that were differentially expressed in semimembranosus muscle of callipyge and normal lambs [16]. Parkinson Protein 7 (PARK7; also known as DJ-1) expression was up-regulated in hypertrophied muscles. PARK7 encodes a ubiquitously expressed, highly conserved protein that has been shown to be involved in diverse biological processes including oxidative stress response, transcriptional regulation and cell survival modulation. A mutation causing a loss of function of PARK7 was found to be responsible for a recessive early-onset form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17]. PARK7 protects neurons and somatic cells from oxidative stress by oxidizing itself to a more acidic form of Parkinson’s disease [17].
regulate cell proliferation [36], cell survival [37] and protein synthesis [38].

The PI3K/AKT pathway is known to positively regulate muscle growth [39,40]. The binding of insulin-like growth factor 1 (IGF1) to its receptor initiates this pathway and activates AKT. Addition of IGF1 into culture medium induced hypertrophy in C2C12 myotubes through enhanced activation of AKT [40]. Muscle-specific over-expression of IGF1 caused muscle hypertrophy in mice [41] and conversely muscle-specific inactivation of the IGF1 receptor impaired muscle growth due to reduced muscle fiber number and size [42]. It also had been well demonstrated that the activation of AKT is sufficient to induce hypertrophy. Over-expression of activated Akt in muscle fibers results in significantly larger fiber size [39,43]. Transgenic mice expressing a constitutively active form of Akt in muscle exhibit rapid skeletal muscle hypertrophy [44]. Conversely, genetic depletion of Akt in mice leads to a smaller body size and shorter life span [45].

Though extensively studied, a role for PARK7 in muscle growth had not been reported until recently. In addition to callipyge lambs [16], transcriptomic and proteomic analyses in quadriceps muscles in myostatin-null mice, which exhibit a muscle hyperplasia phenotype, found elevated levels of PARK7 and phosphorylation of AKT [46]. Further work by the same group in double-muscled cattle also showed higher PARK7 expression in double-muscled fetuses compared to normal controls [47]. In food animal agriculture, there is a need to identify genes that can increase muscle growth and protein accretion. The identification of elevated expression of PARK7 in hyper-muscular animals suggests a hypothesis that increasing PARK7 gene expression can increase muscle growth and protein accretion. Differential expression of PARK7 was examined in this study using Park7 (+/+ and Park7 (−/−) mouse models to investigate the effects of PARK7 gene expression in muscle growth and protein accretion in response to IGF1.

Results

PARK7 is up-regulated in hypertrophied muscle of callipyge lambs

Semitendinosus and supraspinatus muscle samples were collected from 3 callipyge and 3 normal lambs at 30–35 days of age when muscle hypertrophy is detectable by muscle mass. The semitendinosus from the pelvic limb undergoes the largest magnitude of hypertrophy and supraspinatus from the thoracic limb does not become hypertrophied [1,48]. Western blots (Figure 1A) showed that PARK7 protein expression was elevated about 3-fold (P=0.027; Figure 1B) in the semitendinosus (SM) of callipyge lambs (+/C) relative to normal lambs (+/+), but no difference in PARK7 or DLK1 protein levels in the supraspinatus (SS). B: Quantitative analysis of PARK7 protein expression was significantly higher in the SM of callipyge lambs. α-Tubulin was the control for protein loading. Data shown are mean signal intensity ± SE for the PARK7 without normalization using three animals per genotype. The p-values for comparisons between genotypes for each muscle are shown.

Figure 1. PARK7 protein expression level in sheep skeletal muscles. A: There were higher levels of PARK7 and DLK1 proteins in the semitendinosus (SM) of callipyge lambs (+/C) relative to normal lambs (+/+), but no difference in PARK7 or DLK1 protein levels in the supraspinatus (SS). B: Quantitative analysis of PARK7 protein expression was significantly higher in the SM of callipyge lambs. α-Tubulin was the control for protein loading. Data shown are mean signal intensity ± SE for the PARK7 without normalization using three animals per genotype. The p-values for comparisons between genotypes for each muscle are shown.

doi:10.1371/journal.pone.0092030.g001

Effects of Park7 genotype on myosin heavy chain gene expression

The initial Park7 (−/−) mouse was obtained from Jackson Laboratory and back crossed with C57BL6 mice to generate Park7 (+/+), (+/−) and (−/−) littermate mice. Live animal body weights were monitored and collected from 3 – 6 weeks of age. Mice were euthanized at 6 weeks and carcass (muscle and skeleton) weight and internal organs weights were collected. The phenotypic data from male and female mice was analyzed by linear regression separately to account for gender differences. No significant differences were found between the three genotypes for analysis of live weight by age, carcass weight by live weight, or organ weight by live weight analysis in males or females (Figure S1 and Table S1).

The expression of PARK7 protein in the vastus lateralis muscle was examined to verify the loss of expression in Park7 (−/−) mice (Figure 2). The expression of PARK7 was easily detectable in the muscles in Park7 (+/+ and Park7 (+/−) animals, but no PARK7 protein was detectable in Park7 (−/−) muscles. Primary myoblasts were isolated from Park7 (+/+ and Park7 (−/−) animals and fused into myotubes in order to model the effect of PARK7 levels on growth and myosin expression. The relative sizes of myotubes were determined after 72 hours of differentiation in fusion media (Figure 3A). The fusion index was calculated to determine the proportion of myoblasts that fused into myotubes.

Figure 2. PARK7 protein expression in vastus lateralis. PARK7 protein expression was not detectable in vastus lateralis in Park7 (−/−) mice in two replicated experiments of two animals per genotype. α-Tubulin was used as a control to show equal protein loading.

doi:10.1371/journal.pone.0092030.g002
myotubes, and myotube diameters were measured to estimate relative myotube size. There were no differences (p = 0.4103) in the fusion index between the Park7\(^{+/+}\) and Park7\(^{-/-}\) cultures (Figure 3B). Park7\(^{+/+}\) myotubes were significantly larger (1.6-fold; p < 0.0001) than Park7\(^{-/-}\) myotubes (Figure 3C). An in vitro ELISA assay was performed to detect total sarcomeric myosin protein accretion to test the hypertrophy mechanism. IGF1 treatments were included due to its well-established role in regulating muscle hypertrophy [39,40]. Two-way analysis of variance indicated the overall effect of genotype was significant (p = 0.0471) with the Park7\(^{+/+}\) myotubes having about 10% higher myosin expression than Park7\(^{-/-}\) myotubes, regardless of IGF1 treatment (Figure 4). All IGF1 treatments had a significant effect compared to no added IGF1 (p = 0.0015), with 34% higher myosin expression in IGF1 treated myotubes. The genotype by IGF1 interaction effect was not significant (p = 0.5208) but the effect IGF1 treatment appeared to reach saturation at lower concentrations for Park7\(^{+/+}\) myotubes (25 ng/mL) relative to Park7\(^{-/-}\) myotubes (100 ng/mL).

Quantitative PCR was used to detect the effect of Park7 genotype on Myh4, Myh7, Myh8 and Myh3 expression in IGF1 treated mature myotubes (Figure 5). The mRNA abundance of Myh4 was 40% greater (p = 0.0032) in Park7\(^{+/+}\) myotubes, relative to Park7\(^{-/-}\) myotubes (Figure 5A). The addition of IGF1 in culture induced significantly higher Myh4 expression with

Figure 3. Comparison of myotube size between Park7\(^{-/-}\) and Park7\(^{+/+}\) genotypes. A: Primary myoblasts were induced to differentiate for 72 hours and stained with an antibody for total myosin heavy chain (red: MF20) and DNA (blue: DAPI). B: No significant differences were detected between the two genotypes for fusion index. C: The distributions of myotube diameters for the two genotypes are shown. The mean diameter for Park7\(^{+/+}\) (1.70 ± 0.24) myotubes were significantly larger (P < 0.0001) than Park7\(^{-/-}\) (1.05 ± 0.025) myotubes.

doi:10.1371/journal.pone.0092030.g003

Figure 4. Detection of total sarcomere myosin expression in myotubes by ELISA. There were significant effects for genotype and IGF1 treatment on total myosin, but there was no significant interaction. The Park7\(^{+/+}\) myotubes had significantly more total sarcomere myosin expression than Park7\(^{-/-}\) myotubes and addition of IGF1 at all concentrations induced significant higher sarcomere myosin compared to no added IGF1.

doi:10.1371/journal.pone.0092030.g004
1.6-fold increase at 10 ng/mL, 1.9-fold increase at 25 ng/mL and 1.3-fold increase at 50 ng/mL of IGF1 respectively. The Myh7 and Myh8 genes (Figure 5 B and C respectively) had similar expression patterns as Myh4 but with smaller changes in gene expression. The mRNA abundance of Myh7 and Myh8 were increased 20% (p = 0.0018) and 30% (p = 0.0001) respectively in Park7 (+/+ /−/) myotubes. IGF1 also stimulated Myh7 and Myh8 mRNA expression, but only at the lower concentration (10 ng/mL) with 23% increase in Myh7 and 10% increase in Myh8 respectively, relative to the treatment without IGF1. At the high concentration of IGF1 (50 ng/mL), Myh7 expression dropped 12% and Myh8 expression dropped 16% compared to the treatment without IGF1. Myh3 was the only isoform measured that showed no genotype effect or IGF1 effect. There was no significant genotype by IGF1 interactions for the four myosin isoforms.

Effect of Park7 genotype on phosphorylation level of AKT (S473)

In order to explore the mechanism of Park7 regulation on myotube size and myosin gene expression, a series of experiments were conducted to assess the activation of AKT by phosphorylation at S473 in the presence or absence of Park7. It has been demonstrated that PARK7 can suppress PTEN phosphatase activity, which would have the effect of increasing receptor tyrosine kinase signaling through AKT [33]. IGF1 was used to test the effect of Park7 genotype on the phosphorylation of AKT. The level of phosphorylation of AKT was elevated in Park7 (+/+ /−/) myotubes relative to Park7 (−−/) myotubes in 5% horse serum differentiation media without added IGF1 (Figure 6). The phosphorylation of AKT was substantially elevated in Park7 (+/+ /−/) myotubes at all levels of added IGF1. To investigate if the activity of PARK7 could be mimicked by inhibition of PTEN function, VO-OHpic trihydrate, a known effective PTEN inhibitor [49] was introduced in this study. Myotubes were first treated with IGF1 (5 ng/mL) for 15 minutes to induce increased phosphorylation of AKT. Different concentrations of VO-OHpic trihydrate were then added for an additional 15 minutes after removing IGF1. The addition of VO-OHpic trihydrate at 800 nM increased the level of AKT phosphorylation in Park7 (+/+ /−/) myotubes and eliminated the differences between the two Park7 genotypes (Figure 7A). There were no discernable differences in

Figure 6. Phosphorylation of AKT (S473) protein in Park7 (+/+ /−/) and Park7 (−−/) myotubes. Myoblasts were induced to differentiation for 72 hours and treated with IGF1 at the indicated concentrations for 15 minutes. There was increased phosphorylation of AKT (P-AKT) in Park7 (+/+ /−/) myotubes relative to total AKT (AKT) in myotubes in fusion medium (5% horse serum). There was a substantial increase in AKT phosphorylation in Park7 (+/+ /−/) myotubes at all IGF1 concentrations. α-Tubulin was the control for protein loading.
doi:10.1371/journal.pone.0092030.g006
PTEN protein levels (Figure 7A) or RNA expression (Figure 7B, p = 0.6057) between Park7 (+/+) and Park7 (-/-) myotubes.

Since increased phosphorylation of AKT was found in Park7 (+/+) myotubes, the timing and duration of the elevated phospho-AKT was tested. Myotubes were treated with IGF1 for 15 minutes and protein was collected at several time points up to 12 hours. Western blots showed increased phosphorylation of AKT in Park7 (+/+) myotubes (Figure 8A). Quantitative analysis of relative signal intensity based on the ratio of phospho-AKT to total AKT indicated a 2-fold higher level of P-AKT in Park7 (+/+) myotubes relative to Park7 (-/-) myotubes with no added IGF1 (Figure 8B). The level of AKT phosphorylation in Park7 (+/+) myotubes reached its maximum at 45 minutes at 3-fold higher signal intensity than Park7 (-/-) myotubes after removal of IGF1. Park7 (-/-) myotubes had maximum phosphorylation at 90 minutes and the decreases in phosphorylation over time were similar with Park7 (+/+) myotubes, generally maintaining approximately 20% higher signal intensity through 12 hours.

Discussion

Overall, the present results indicate that differential expression of the PARK7 protein can result in increased myosin gene expression, protein accretion and myotube growth through an altered response to IGF1/AKT signaling. This study focused on Park7’s effect on AKT phosphorylation due to its prominent role in regulating muscle growth, other reported biological activities in oxidative stress, anti-apoptosis and several regulatory pathways [17–21,29–32] may also contribute to improved muscle growth. The Park7 (-/-) mice are viable and lack obvious developmental abnormalities. No differences were found between Park7 (+/+) and Park7 (-/-) animals in vivo when comparing body weight, carcass weight and internal organ weight which indicate that Park7 is not a dominant regulator of live animal growth. These results were consistent with other findings, which indicated no significant differences in body weights between the two genotypes from birth to 12 months [50]. Skeletal muscle growth in live animals is stimulated by several hormones such as IGF1, androgens and β-adrenergic agonists to initiate multiple signaling pathways to regulate growth.
A previous study showed elevated PARK7 expression in callipyge sheep at the transcriptional level [16] and the present results confirmed the increased expression of PARK7 in hypertrophied muscles at the protein level. An in vitro cell culture model was used in this study to investigate the effects of differential expression of Park7 on myogenesis. The in vitro comparison of myotube size showed Park7 (+/+), myotubes had a bigger size and higher myosin expression than Park7 (−/−) myotubes, but no significant change in the fusion index, which indicates Park7 expression affects myotube hypertrophy (increased myofiber diameter), not hyperplasia (increased myofiber number). These results support the proposed models that elevated PARK7 expression is part of the physiological mechanism for increased muscle growth in callipyge lambs and myostatin null mice and cattle [16,46,47].

The PI3K/AKT pathway is one of the primary targets of IGF1 signaling and has been well characterized for its positive regulation on muscle growth [39,40]. The postnatal muscle mass and myofiber size generally reflects protein accretion. AKT stimulates protein synthesis by activating mammalian target of rapamycin (mTOR) and its downstream effectors. This study showed that the Park7 (+/+), myotubes had increased phosphorylation of AKT compared to Park7 (−/−) after IGF1 stimulation, which induced significantly more total sarcromeric protein accretion. Notably, this increase of protein accretion reached saturation at lower IGF1 concentrations in Park7 (+/+), relative to Park7 (−/−) myotubes. The expression of Myh4 showed the largest increases although Myh7 and Myh8 mRNA expression were also elevated in Park7 (+/+), myotubes indicating that both myosin transcription and translation were affected by Park7 expression. Muscle contractile characteristics partially depend on the expression of myosin heavy chain isoforms. The hypertrophied muscles in callipyge sheep have an increased size of fast twitch glycolytic myofibers along with increased MYH4 expression and a decreased frequency of oxidative fibers [4,6]. Over-expression of constitutively active Akt had been reported to promote myofiber growth with no effect on fiber specification in regenerating skeletal muscle [43]. The use of a doxycycline-inducible Akt transgene in mice resulted in the hypertrophy of glycolytic myofibers, but not oxidative myofibers [51]. A recent study supported this result by identifying a regulatory cascade of AKT activation to drive the metabolic and contractile specification of fast-twitch glycolytic fibers as well [52]. Similarly, the results presented here are congruent with the up-regulation of Myh4 and the enhanced activation of AKT in the presence of Park7. Interestingly, double-muscled cattle, in which the expression of Park7 gene is also elevated, have an increased proportion of fast-twitch glycolytic fibers as well [53].

The differences in phosphorylation of AKT in Park7 (+/+) and Park7 (−/−) myotubes was eliminated when treated with higher concentrations of IGF1.
concentrations of PTEN inhibitor, but the total amount of PTEN had not been changed, suggesting PARK7 acts through inhibition of PTEN phosphatase activity, not total PTEN expression. This result was consistent with the findings of Kim et al. [33,54]. In their studies, they also examined the phosphorylation level of PTEN at S380, T382, T383 and S385 in Park7 transfected NIH3T3 cells, but no change was detected; indicating the inhibition of PTEN phosphatase activity by PARK7 is not affected by phosphorylation of PTEN. The mechanism by which PARK7 inhibits PTEN phosphatase activity remains unclear. Some studies have proposed that PARK7 can directly bind to PTEN to confer an inactive conformation by forming a disulfide bond between C71 and C124 of PTEN upon oxidative stress [54–56]. It has also been speculated that the interaction of PARK7 with PTEN may accelerate the conformation by forming a disulfide bond between C71 and C124 in PTEN phosphatase activity, not total PTEN expression. This had not been changed, suggesting PARK7 acts through inhibition of PTEN phosphatase activity, not total PTEN expression.

Conclusions

The present study provides a novel insight into a role for Park7 in the regulation of skeletal muscle growth. Park7 expression can modulate IGF1/AKT signaling, increase myosin gene expression and protein synthesis and increase myotube size. These results support the hypothesis that elevated expression of PARK7 is part of the physiological mechanism for muscle hypertrophy in callipyge lambs and increased muscle mass in double muscled cattle. Elevated expression of PARK7 in the muscles of callipyge lambs could lead to increased muscle growth in response to the normal IGF1 signaling present in young growing lambs. The prominent increase in Myh4 gene expression in myotubes was also consistent with changes in myosin fiber types in callipyge muscle. DLK1 is one of the causative genes for the callipyge phenotype due to the inheritance of the mutation [9,12]; therefore elevated PARK7 expression could be considered a downstream response to DLK1 signaling. Identifying how PARK7 gene expression is regulated in callipyge induced muscle hypertrophy could provide new mechanisms to increase livestock muscle growth and production efficiency or prevent the loss of muscle mass due to disease or aging.

Materials and Methods

Ethics Statement

Sheep were maintained at the Purdue Animal Sciences Research and Education Center under standard agricultural housing and management. Lambs were euthanized for dissection of muscle tissue for the growth trial through pneumothorax for dissection of muscle tissue for the growth trial sional personnel. Mice were euthanized by cervical dislocation and humane procedures were followed. Mice were euthanized by cervical dislocation and humane procedures were followed. Lambs were euthanized for tissue sampling using three lethal procedures of captive bolt stunning, exsanguination and pneumothorax. This procedure was approved by Purdue Animal Care and Use Committee (protocol #1112000493). All mice were fed with standard chow food and water, and housed at 22–23°C with nesting material with 12 hour light/dark cycle. Cage bedding was changed weekly by professionals. Cells were seeded in growth media (F-10 Ham’s addition of fusion media consisting of DMEM supplemented with 5% horse serum, 0.292 mg/ml of L-glutamine, and 4 ng/ml basic fibroblast growth factor) on rat-tail collagen (Roche Applied Science)-coated dishes with preplating on non-coated plates for 45 minutes to deplete fibroblasts as previously described [15,58]. Myoblasts were differentiated into myotubes after plating cells at approximately 80% confluency on Matrigel (BD Biosciences, San Jose, CA USA) coated plates and the addition of fusion media consisting of DMEM supplemented with 5% horse serum, 100 units of penicillin, 100 µg of streptomycin, 0.292 mg/ml of L-glutamine, and 0.025% trypsin for 10 minutes with agitation. Cells were seeded in growth media (F-10 Ham’s medium supplemented with 20% fetal bovine serum, 100 units of penicillin, 100 µg of streptomycin, 0.292 mg/ml of L-glutamine, and 4 ng/ml basic fibroblast growth factor) on rat-tail collagen (Roche Applied Science)-coated dishes with preplating on non-coated plates for 45 minutes to deplete fibroblasts as previously described [15,58]. Myoblasts were differentiated into myotubes after plating cells at approximately 80% confluency on Matrigel (BD Biosciences, San Jose, CA USA) coated plates and the addition of fusion media consisting of DMEM supplemented with 5% horse serum, 100 units of penicillin, 100 µg of streptomycin, and 0.292 mg/ml of L-glutamine. LongR3 IGF1 (Sigma-Aldrich Co, St Louis, MO USA) was added to the fusion medium for 15 minutes at 3 days after differentiation. Cells were collected for analysis of protein expression.

DNA Isolation and Quantitative PCR Analysis

RNA from cultured cells was extracted and purified using Nucleo Spin RNA II columns (Machery-Nagel Inc., Easton, PA USA). First strand cDNA was synthesized from RNA using random hexamer and oligo dT priming and moloney murine leukemia virus reverse transcriptase (MMLV, Life Technologies) to produce random primed cDNA. Quantitative PCR measurements were performed using the SA Bioscience SYBR Green Supermix (QIAGEN, Valencia, CA USA) reagents on an iCycler Real-Time PCR Detection System (Bio-Rad Inc., Hercules, CA USA). Each reaction was carried out in 15 µl reaction volumes of SA Bioscience SYBR Green Supermix with 5 pM of each primer and diluted first-strand cDNA. All cDNA samples were measured in duplicate. Ribosomal protein large protein 38 (Rpl38) was used as the housekeeping gene control for ΔC_T calculation [ΔC_T = (C_T of the target gene) – (average C_T of housekeeping genes)]. Primer sequences were listed in Table S2. Fold change expression values were calculated using 2ΔΔC_T methods [57], where ΔΔC_T = (ΔC_T of the treatment sample) – (ΔC_T of control treatment samples) with no added IGF1 as control treatment and normalized to 1.

Primary Myoblast Isolation and Culture

Primary myoblasts were isolated from hind limb skeletal muscles at 3–5 weeks of age. Muscles were washed with Dulbecco’s Phosphate-Buffered Saline (DPBS), minced and digested in type I collagenase and dispase B mixture (Roche Applied Science, Indianapolis, IN USA). The digested muscle pulp was then filtered through a 100 µm filter to remove large muscle fiber debris and then plated on culture dishes. In 3 days, the cells together with the small debris were collected and digested with 0.025% trypsin for 10 minutes with agitation. Cells were seeded in growth media (F-10 Ham’s medium supplemented with 20% fetal bovine serum, 100 units of penicillin, 100 µg of streptomycin, 0.292 mg/ml of L-glutamine, and 4 ng/ml basic fibroblast growth factor) on rat-tail collagen (Roche Applied Science)-coated dishes with preplating on non-coated plates for 45 minutes to deplete fibroblasts as previously described [15,58]. Myoblasts were differentiated into myotubes after plating cells at approximately 80% confluency on Matrigel (BD Biosciences, San Jose, CA USA) coated plates and the addition of fusion media consisting of DMEM supplemented with 5% horse serum, 100 units of penicillin, 100 µg of streptomycin, and 0.292 mg/ml of L-glutamine. LongR3 IGF1 (Sigma-Aldrich Co, St Louis, MO USA) was added to the fusion medium for 15 minutes at 3 days after differentiation. Cells were collected for analysis of protein expression.
was transferred into ultracentrifuge tubes and centrifuged at 60,000 g for 15 minutes at 4°C. The supernatant was transferred into ultracentrifuge tubes and centrifuged at 50,000 × g for 15 minutes at 4°C. Proteins were quantified by BCA assay (Thermo Scientific, Rockford, IL USA) per manufacturer’s recommendation. A total of 60 μg of protein from each muscle sample was diluted in SDS loading buffer, denatured by heating for 5 minutes in boiling water and loaded in the 6%-18% gradient SDS-PAGE gel. The gel was transferred to PVDF membrane by semi-dry transfer using 20% methanol, 40 mM glycine, 0.15% ethanolamine, 0.2% SDS at 180 mA for 1 h 45 min. PVDF membrane was blocked overnight with agitation in 5% sodium caseinate in PBS with 0.1% Tween 20. Antibodies used for blotting were goat polyclonal anti-Park7 (sc-27006, Santa Cruz Biotechnology, Santa Cruz, CA USA), anti-bovine DLK1 (provided by Dr. Ross L. Tellam, CSIRO, St. Lucia, QLD Australia), mouse monoclonal anti-tubulin (G67, Developmental Studies Hybridoma Bank, Iowa City IA USA) and secondary antibodies were rabbit anti-goat antibody conjugated HRP, goat anti-rabbit conjugated HRP, or goat anti-mouse conjugated HRP respectively (Thermo Scientific Pierce, Rockford, IL USA). The blots were incubated in primary antibody 2 hours at room temperature with a dilution of 1:1000. After washing 3 times with PBDS, the blots were incubated with secondary antibody with dilution of 1:20000. Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific Pierce) was used to detect HRP on immunoblots. The blots were visualized using Gel Logic 2200 PRO (Carestream Molecular Imaging System) with exposure times of 2-6 minutes. Carestream Molecular Imaging Software was used to quantify the chemiluminescent signal intensity in arbitrary units. The signal intensities for PARK7 were compared using a t-test without normalization.

Analysis of Myotube Differentiation and Size
Primary myotubes were fixed in 4% formaldehyde and permeabilized in 0.5% Triton X-100. Cells were blocked in 1% bovine serum albumin (BSA) in PBS for 1 hour at room temperature and stained with 1:1000 anti-mysin heavy chain antibody (MF20, Developmental Studies Hybridoma Bank, Iowa City, IA USA) for 2 hours. Cells were washed in PBS, and then incubated in 1:100 Alexa Fluor 594-conjugated donkey anti-mouse IgG secondary antibodies (Life Technologies) for 1 hour. Finally, cells were mounted with Prolong Gold anti-fade reagent with DAPI (Life Technologies). The IP Lab software (Scanalytics Inc., Madison, WI USA) and Leica DM6000 microscope were used to acquire pictures.

The fusion index was defined as the ratio of nuclei within the myotubes (>2 nuclei) to the total number of nuclei. The average number of nuclei per myotube was determined by counting over 500 nuclei from randomly chosen MHC-positive cells. Myotube diameters were measured at 5 points along the entire tube. A total of 150 myotubes were examined for each genotype in the experiment.

In situ ELISA Quantification of Myosin Expression
Primary myoblasts were put into Matrigel coated-96-well plate at a high density with four replicate wells for each genotype by IGF1 treatment combination. The myoblasts were fused into myotubes for 3 days in fusion media with different concentration (0, 12.5, 25, 50, 100 and 200 ng/mL) of IGF1. The mature myotubes were fixed in 4% formaldehyde in PBS for 5 minutes at room temperature, washed 3 times with PBS and permeabilized with 0.5% TritonX100 for 5 minutes. After washing 3 times with PBS, the cells were blocked for 1 hour in 1% BSA in PBS. Cells were incubated with anti-mysin heavy chain antibody (MF20, Developmental Studies Hybridoma Bank) diluted in 0.1% BSA overnight at 4°C, and washed 5 times in PBS. Secondary antibody (Donkey anti-mouse IgG, Life Technologies) were diluted in 0.1% BSA and filtered through 0.2 μM filters. Cells were incubated in secondary antibody for 1 hour and washed 5 times with PBS. The fluorescence signals were read on Tecan Genios Pro (Tecan Group Ltd., Morrisville, NC USA) plate reader.

AKT Phosphorylation in Primary Myotubes
Cultured primary myoblasts or myotubes were used to examine AKT phosphorylation. Different concentrations of IGF1 were added into culture for 15 minutes to induce phosphorylation of AKT. For the PTEN inhibitor experiment, different concentrations (0, 100 nM and 800 nM) of VO-OHpic trihydrate (Sigma-Aldrich) were added into culture and incubated with myotubes for 15 minutes after removal of IGF1. For testing sustained activity of phosphorylation of AKT, IGF1 was removed after 15 minutes incubation with myotubes. The cells were incubated in normal differentiation conditions and cell lysis was collected at the indicated time points (0, 45, 90, 180, 360, 720 minutes). Cells attached to plates were rinsed with ice-cold DPBS, and scraped on ice in cell lysis buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 20 mM sodium fluoride, 1% Triton 100, 1 X phos-stop (Roche Applied Science) and 1 X complete protease inhibitor cocktail. Cells were collected in 1.5 mL microcentrifuge tubes, rotated for 45 minutes, and centrifuged at 12,000 × g for 10 minutes at 4°C to remove insoluble debris. A total of 45 μg of protein from each cell sample was diluted in SDS loading buffer, and loaded in the 6%-18% gradient SDS-PAGE gel. The gel electrophoresis and transfer to PVDF membranes were the same as described above. The PVDF membranes were blocked in 5% non-fat milk in Tris-Buffered Saline (TBS) with 0.1% Tween 20 for 3 hours. Antibodies used for blotting were rabbit polyclonal anti-AKT (Cat #: 4685, Cell Signaling Technology, Danvers, MA USA), anti-phospho-AKT (Ser473) (Cat #: 4695 Cell Signaling Technology), anti-PTEN (Cat #: 9188 Cell Signaling Technology). The primary antibody was incubated at 4°C overnight at a dilution of 1:1000. The goat anti-rabbit conjugated HRP was used as secondary antibody and was incubated for 1 hour at 4°C at a dilution of 1:20000. The visualization of blots was the same as described above.

Statistical Analysis Methods
All of the statistical analysis presented in this work was conducted using SAS 9.2 software (Statistical Analysis Systems Institute, Cary NC, USA) with assistance from the Statistical Consulting Service at Purdue University. The linear regression models for pairwise comparisons of the three Park7 genotypes used in the mouse growth trials are provided in Supplementary Table S1. Assays that compared two genotypes, including the western blot data for sheep genotypes used in the mouse growth trials are provided in Supplementary Table S1. Assays that compared two genotypes, including the western blot data for sheep genotypes used in the mouse growth trials are provided in Supplementary Table S1. Assays that compared two genotypes, including the western blot data for sheep genotypes used in the mouse growth trials are provided in Supplementary Table S1. Assays that compared two genotypes, including the western blot data for sheep genotypes used in the mouse growth trials are provided in Supplementary Table S1. Assays that compared two genotypes, including the western blot data for sheep genotypes used in the mouse growth trials are provided in Supplementary Table S1. Assays that compared two genotypes, including the western blot data for sheep genotypes used in the mouse growth trials are provided in Supplementary Table S1.

Supporting Information

Figure S1 Analysis on live body weight by age in *Park7* (+/+) (+/-) and (-/-) mice. Twenty females with 4 *Park7* (+/+),
12 (+/-) and 4 (-/-) animals and twenty-five males with 4 Park7 (+/+) and 11 (+/-) and 10 (-/-) were examined to analyze the live animal growth. Animals live body weights were collected from 3 weeks to 6 weeks old. Live body weights were regressed on age. Equations were given for each genotype. There were no genotype effect on the slopes and intercepts of any regression lines in both male and female.

**Table S1** Regression analysis of growth in Park7 (+/+), (+/-) and (-/-) mice.

| Variable | Regression Analysis |
|----------|---------------------|
| Age | Slopes and intercepts |

**References**

1. Jackson SP, Miller MF, Green RD (1997) Phenotypic characterization of rambouillet sheep expressing the callipyge gene: II. Carcass characteristics and retail yield. J Anim Sci 75: 125–132.

2. Freking BA, Keele JW, Beattie CW, Kappes SM, Smith TP, et al. (1998) Evaluation of the ovine callipyge locus: I. Relative chromosomal position and gene action. J Anim Sci 76: 2062–2071.

3. Dockett SK, Snowder GD, Cockett NE (2000) Effect of the callipyge gene on muscle growth, calpastatin activity, and tenderness of muscles across the growth curve. J Anim Sci 78: 2836–2841.

4. Carpenter E, Rice OD, Cockett NE, Snowder GD (1996) Histology and composition of muscles from normal and callipyge lambs. J Anim Sci 74: 308–319.

5. Jackson SP, Miller MF, Green RD (1997) Phenotypic characterization of rambouillet sheep expression the callipyge gene: III. Muscle weights and muscle weight distribution. J Anim Sci 75: 133–136.

6. Carden CE, Cockett NE (2000) Histology of longissimus muscle from 2-week-old and 8-week-old normal and callipyge lambs. J Anim Sci 80: 511–514.

7. Freking BA, Murphy SK, Wylie AA, Rhodes SJ, Keele JW, et al. (2002) Identification of the single base change causing the callipyge muscle hypertrophy phenotype, the only known example of polar overdominance in mammals. Genome Res 12: 1496–1506.

8. Sun M, Seeger K, Carrascosa LG, Shay T, Baraldi F, et al. (2003) Mosaicism of SoliGold supports the causality of a noncoding A-to-G transition in the determination of the callipyge phenotype. Genetics 163: 453–456.

9. Bidwell CA, Carrascosa LG, Shay T, Baraldi F, et al. (2003) Expression of PEG11 and PEG11AS transcripts in normal and callipyge sheep. PLoS ONE 5: e15055.

10. Fleming-Waddell JN, Wilson LM, Olbricht GR, Vuocolo T, Byrne K, et al. (2007) Analysis of gene expression during the onset of muscle hypertrophy in callipyge lambs. Anim Genet 38: 25–36.

11. Murphy SK, Nolan CM, Huang Z, Kucera KS, Freking BA, et al. (2006) Callipyge mutation affects gene expression in cis: a potential role for chromatin structure. Genome Res 16: 340–346.

12. Charlier C, Segers K, Karim L, Shay T, Gyapay G, et al. (2001) The callipyge mutation enhances the expression of coregulated imprinted genes in cis without affecting their imprinting status. Nat Genet 27: 367–369.

13. Perkins AC, Kramer LN, Scharlock DM, Hadfield TS, Cockett NE, et al. (2006) Postnatal changes in the expression of genes located in the callipyge region in sheep skeletal muscle. Anim Genet 37: 535–542.

14. Davis E, Jensen GH, Schroder HD, Farnir F, Shay-Hadfield T, et al. (2004) Ectopic expression of DLK1 protein in skeletal muscle of padmanal heterozygotes causes the callipyge phenotype. Curr Biol 14: 1858–1862.

15. Waddell JN, Zhang P, Wen Y, Gupta SK, Yeovdychenko A, et al. (2010) Dlk1 Is Necessary for Proper Skeletal Muscle Development and Regeneration. PLoS ONE 5: e15055.

16. Fleming-Waddell JN, Olbricht GR, Taxe TM, White JD, Vuocolo T, et al. (2009) Effect of DLK1 and RTL1 but Not MEG3 or MEG8 on Muscle Gene Expression in Callipyge Lambs. PLoS ONE 4.

17. Bondai V, Rizzo P, van Baren MJ, Schaap O, Breedveld GJ, et al. (2003) Mutations in the Dlk1 gene associated with autosomal recessive early-onset parkinsonism. Science 299: 256–259.

18. Mitsumoto A, Nakagawa Y (2001) DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 33: 885–893.

19. McNally RS, Davis BK, Clements CM, Accavitti-Loger MA, Mak TW, et al. (2011) DJ-1 enhances cell survival through the binding of Cezanne, a negative regulator of NF-kappaB. J Biol Chem 286: 4098–4106.

20. Takahashi K, Taira T, Niki T, Seino G, Iuchi-Ariga SM, et al. (2002) DJ-1 positively regulates the androgen receptor by impairing the binding of Piasalpha to the receptor. J Biol Chem 276: 37535–37536.

21. Zhong N, Kim CY, Rizzo P, Geula C, Porter DR, et al. (2006) DJ-1 transcriptionally up-regulates the human tyrosine hydroxylase by inhibiting the

**Checklist S1**

**Author Contributions**

We want to thank Jun Wu for managing the mice colony, Gerald Kelly for his management of the sheep flocks and Weiyi Liu for his input in experimental techniques.

**References**

Conceived and designed the experiments: CAB. SK. Performed the experiments: HY. JNW. CAB. Analyzed the data: HY. JNW. Contributed reagents/materials/analysis tools: CAB. SK. Wrote the paper: HY. SK. CAB.
42. Mavalli MD, DiGirolamo DJ, Fan Y, Riddle RC, Campbell KS, et al. (2010) Distinct growth hormone receptor signaling modes regulate skeletal muscle development and insulin sensitivity in mice. J Clin Invest 120: 4007–4020.
43. Pallafacchina G, Calabria E, Serrano AL, Kalhoff JM, Schiaffino S (2002) A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. Proc Natl Acad Sci USA 99: 9213–9218.
44. Lai KM, Gonzalez M, Poueymirou WT, Kline WO, Na E, et al. (2004) Conditional activation of akt in adult skeletal muscle induces rapid hypertrophy. Mol Cell Biol 24: 9295–9304.
45. Chen WS, Xu PZ, Gottlob K, Chen ML, Sokol K, et al. (2001) Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev 15: 2203–2208.
46. Chell I, Meunier B, Picard B, Reece MJ, Chevalier C, et al. (2009) Molecular profiles of Quadriceps muscle in myostatin-null mice reveal PI3K and apoptotic pathways as myostatin targets. BMC Genomics 10: 196.
47. Chell I, Picard B, Hocquette JF, Casas-Malek I (2011) Myostatin inactivation induces a similar muscle molecular signature in double-muscled cattle as in mice. Animal 5: 278–286.
48. Koohmaraie M, Shackelford SD, Wheeler TL, Lonergan SM, Doumit ME (1995) A muscle hypertrophy condition in lamb (callipyge): characterization of effects on muscle growth and meat quality traits. J Anim Sci 73: 3586–3607.
49. Kwon J, Lee SR, Yang KS, Ahn Y, Kim YJ, et al. (2004) Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. Proc Natl Acad Sci USA 101: 16419–16424.
50. Liu W, Wen Y, Bi P, Lai X, Liu XS, et al. (2012) Hypoxia promotes satellite cell self-renewal and enhances the efficiency of myoblast transplantation. Development 139: 2857–2865.