Genetic Loss of Calcineurin Blocks Mechanical Overload-induced Skeletal Muscle Fiber Type Switching but Not Hypertrophy*

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The serine/threonine phosphatase calcineurin is an important regulator of calcium-activated intracellular responses in eukaryotic cells. In higher eukaryotes, calcium/calmodulin-mediated activation of calcineurin facilitates direct dephosphorylation and nuclear translocation of the transcription factor nuclear factor of activated T-cells (NFAT). Recently, controversy has surrounded the role of calcineurin in mediating skeletal muscle cell hypertrophy. Here we examined the ability of calcineurin-deficient mice to undergo skeletal muscle hypertrophic growth following mechanical overload (MOV) stimulation or insulin-like growth factor-1 (IGF-1) stimulation. Two distinct models of calcineurin deficiency were employed: calcineurin Aβ gene-targeted mice, which show a ~50% reduction in total calcineurin, and calcineurin B1-LoxP-targeted mice crossed with a myosin light chain 1f cre knock-in allele, which show a greater than 80% loss of total calcineurin only in skeletal muscle. Calcineurin Aβ−/− and calcineurin B1-LoxP(flo/fl)-MLC-cre mice show essentially no defects in muscle growth in response to IGF-1 treatment or MOV stimulation, although calcineurin Aβ−/− mice show a basal defect in total fiber number in the plantaris and a mild secondary reduction in growth, consistent with a developmental defect in myogenesis. Both groups of gene-targeted mice show normal increases in Akt activation following MOV or IGF-1 stimulation. However, overload-mediated fiber-type switching was dramatically impaired in calcineurin B1-LoxP(flo/fl)-MLC-cre mice. NFAT-luciferase reporter transgenic mice failed to show a correlation between IGF-1- or MOV-induced hypertrophy and calcineurin-NFAT-dependent signaling in vivo. We conclude that calcineurin expression is important during myogenesis and fiber-type switching, but not for muscle growth in response to hypertrophic stimuli.

Calcineurin, a Ca2+/calmodulin-activated serine-threonine phosphatase, is a heterodimer consisting of a catalytic subunit with a molecular mass of ~59 kDa (CnA), and a regulatory subunit with a molecular mass of 19 kDa (CnB). The CnA subunit contains a phosphatase domain, a CnB-binding domain, a calmodulin-binding domain, and an autoinhibitory loop. The autoinhibitory loop normally obscures the phosphatase domain and is displaced upon binding of CnB and Ca2+/calmodulin to CnA resulting in the full activation of calcineurin (1, 2). There are three major isoforms of CnA (α, β, and γ) and two isoforms of CnB (1 and 2). However, only CnAα, CnAβ, and CnB1 are expressed in skeletal muscle. Downstream targets of calcineurin include members of the nuclear factor of activated T-cells (NFAT) and myocyte enhancer factor 2 (MEF2) family of transcription factors (1, 2). Calcineurin directly binds and dephosphorylates NFAT transcription factors within the cytoplasm, permitting their translocation to the nucleus where they participate in activating gene expression (2). Calcineurin-mediated NFAT and MEF2 activation regulates expression of muscle-specific genes including Myf5, myoglobin, myosin heavy chain, and slow troponin I (3–5).

Skeletal muscle is a highly plastic tissue possessing the ability to adapt to alterations in neuromuscular activity, loading, hormones, substrate supply and environmental stress. Adaptation of skeletal muscle in response to such stimuli involves a molecular reprogramming of gene expression coupled with alterations in protein translational activities and/or protein turnover, thereby facilitating structural remodeling of skeletal muscle (hypertrophy or atrophy) and the transformation of individual muscle fibers. Specific changes involve modifications in expression of the myosin heavy and light chain components of the sarcomere, adaptations in metabolic and oxygen utilization factors, and adaptations in neuromuscular innervation properties (6, 7). For example, slow/oxidative skeletal muscle fibers predominantly express the myosin heavy chain (MyHC I) with some varied proportion of IIA, the slowest of the fast MyHCs. Slow/oxidative fibers also possess metabolic enzymes geared toward sustained energy production, have increased myoglobin content, and have more mitochondria to enhance ATP generation. Fast/glycolytic muscle fibers in rodent skeletal muscle predominantly express two fast isoforms, MyHC IIx

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1 The abbreviations used are: CnA, calcineurin A subunit; CnB, calcineurin B subunit; GH, growth hormone; IGF-1, insulin-like growth factor-1; MLC, myosin light chain; MOV, mechanical overload; MyHC, myosin heavy chain; mTOR, mammalian target of rapamycin; NFAT, nuclear factor of activated T cells; mAb, monoclonal antibody; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MW, muscle weight; TL, tibia length; TRITC, tetramethylrhodamine isothiocyanate; PBS, phosphate-buffered saline.
and IIB, and contain a full complement of metabolic enzymes geared toward rapid substrate utilization (6, 7). Thus, MyHC I fibers are characterized by a low velocity of shortening and high resistance to fatigue, whereas type IIx/B fibers show high velocity of shortening and low resistance to fatigue. The fiber-type program of adult skeletal muscle is highly plastic and dynamically regulated by motor nerve activity, which is associated with altered intracellular calcium handling. Slow/oxidative fibers are induced by a profile of sustained contractile activity and cytosolic Ca++ levels between 100 and 300 nM (8, 9). Fast/glycolytic fibers are characterized by infrequent yet powerful bursts of contractile work, with resting Ca++ levels of \( \leq 50 \text{ nM} \) (10).

Calcineurin is predominantly activated in response to sustained elevations in intracellular Ca++ concentration, consistent with its hypothesized role in regulating skeletal muscle fiber-type switching (1). Indeed, calcineurin has been shown to selectively activate expression of MyHC I (11, 12, 13) and stimulate transcription from slow fiber-specific gene promoters (3). Also, transgenic mice overexpressing an activated form of calcineurin in skeletal muscle show increased numbers of slow/oxidative muscle fibers (14, 15), whereas mice null for either CnAa or CnAb have reduced slow/oxidative fiber content (16). Likewise, administration of calcineurin inhibitors decreases expression of the slow/oxidative fiber-type program (3, 13, 17–20) and reduces MyHC I expression in regenerating muscle (21, 22). In addition to activating the slow/oxidative fiber-type program, calcineurin has also been hypothesized to regulate skeletal muscle hypertrophy. This hypothesis originally stemmed from the observation that expression of activated calcineurin induces a profound hypertrophic response in the heart, another striated muscle (23). Consistent with its role in the myocardium, two groups reported a functional role for calcineurin-NFAT signaling in mediating insulin-like growth factor-1 (IGF-1)-induced skeletal muscle cell hypertrophy/growth (24, 25). Likewise, the calcineurin inhibitor cyclosporine A was observed to reduce compensatory hypertrophy following functional overload in vivo (26), whereas another study showed that plantaris muscle regrowth was mostly inhibited with cyclosporine A (27). However, others have not observed a growth inhibitory effect with cyclosporine A (21, 28), and transgenic mice overexpressing activated calcineurin in skeletal muscle do not show evidence of hypertrophy (14, 29), consistent with the lack of calcineurin up-regulation during hypertrophy (30). More recent studies on skeletal muscle hypertrophy have shown compelling evidence for the importance of an altogether different signaling pathway that utilizes IGF-1-phosphatidylinositol 3'-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) regulatory constituents (19, 30, 31).

Given the controversy surrounding the functional role for calcineurin as a regulator of skeletal muscle hypertrophy, and the issue of nonspecific effects associated with cyclosporine A, we examined skeletal muscle hypertrophy in two distinct models of calcineurin disruption. Whereas calcineurin was observed to be essential for overload-mediated fiber-type switching toward the slow/oxidative phenotype, there was essentially no direct effect on skeletal muscle hypertrophy. These results provide clear evidence that calcineurin expression is not required for skeletal muscle hypertrophy in the adult.

**MATERIALS AND METHODS**

**Animals—**CnAa+/− (16, 32) and CnAb+/− (33, 34) mice have been described previously. CnB1-LoxP mice are described elsewhere (35), and NFAT luciferase reporter mice (NFAT site from the interleukin-4 promoter) have also been previously described (36). Mice expressing Cre recombinase under the control of the myosin light chain 1f (MLC1f) genomic locus (knock-in) were generously provided by Steven Burden (Skirball Institute, NYU) and were previously described (37). \( \beta \)-MyHC-Cre transgenic mice were generated that express the Cre recombinase in fetal and embryonic cardiac muscle. The Cre coding sequence was linked to the 5.6-kb mouse \( \beta \)-myosin heavy chain promoter (38) and SV40 small t-intron and polyadenylation sequences. The transgene was released using SfiI and NotI and used for pronuclear microinjection. All animals had free access to food and water, and all experimentation was performed in the Cincinnati Children’s Hospital Research Foundation animal care facility in accordance with the guidelines of the National Institutes of Health. Experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee. Female mice ~8–10 weeks of age were used for all analyses.

**IGF-1/Growth Hormone (GH) Administration—**IGF-1/GH administration was carried out as previously described (39). Briefly, lypophilized IGF-1 (JRH Biosciences) was reconstituted in saline containing 0.1% bovine serum albumin (Sigma), 0.1 M glacial acetic acid and 150 mM NaOH. IGF-1 and recombinant human GH (Nutropin AQ®, Genentech, Inc.) was administered via subcutaneous injection twice daily, at a dosage totaling 8 mg/kg per day. Some mice were also given an acute injection of IGF-1 and sacrificed 1 h afterward for analysis of phospho-Akt (serine 473). Mice in growth study groups were treated for 14 days.

**Western Blotting—**Extracts were prepared in cell lysis buffer (20 mM sodium phosphate, pH 7.0, 150 mM NaCl, 2 mM MgCl2, 10 mM NaF, 0.1 mM sodium orthovanadate, 1 mM sodium pyrophosphate, 1 mM dithiothreitol, 1% Nonidet P-40, 10% glycerol, 10 \( \mu \)g of leupeptin per ml, 10 \( \mu \)g of aprotinin per ml, 10 \( \mu \)g of N-acetylphenylalanyl chloromethyl ketone (TPCK) per ml, 10 \( \mu \)g of N-acetylphenylalanyl chloromethyl ketone (TLCK) per ml), and proteins were resolved on a sodium dodecyl sulfate-8% polyacrylamide gel (5% for NFAT proteins), transferred to a polyvinylidene difluoride membrane, and immunodetected using an enhanced chemiluminescence kit as specified by the manufacturer (Amersham Biosciences, Piscataway, NJ). The following antibodies were used: calcineurin pan-A rabbit polyclonal antibody (Chemicon International, Inc., Temecula, CA), α-tubulin monoclonal antibody (mAb) (Santa Cruz Biotechnology, Santa Cruz, CA), CnB1 rabbit polyclonal antibody (Affinity BioReagents, Golden, CO), CnB2 goat polyclonal antibody (Santa Cruz Biotechnology), phospho-Akt serine 473 rabbit polyclonal antibody, phospho-Akt (serine 473) rabbit polyclonal antibody Upstate, WAKOD mAb (Research Diagnostics, Inc., Flanders, NJ) and isoform-specific CnA rabbit polyclonal antibodies that were custom synthesized in rabbits and affinity-purified by Zymed Laboratories, Inc., against the CnAa epitope NH2-CSETNGTDSNGSSNSNIQ-COOH and the CnAβ epitope NH2-CHTENHGTGNTTPQCOOH. Western blot reactivity was visualized using a Storm860 PhosphorImager (Molecular Dynamics, Piscataway, NJ) using ImageQuant software. For MyHC Western blotting, extracts were prepared as described previously (16). Briefly, excised muscles were homogenized in Isotris (10 mM Tris-HCl, pH 7.4, 0.9% NaCl) plus 1 mM phenylmethylsulfonyl fluoride, and proteins were resolved by 7.5% sodium dodecyl sulfate-7.5% polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The samples were vortexed, boiled, and centrifuged, and the supernatant was transferred to a fresh tube and used for Western blotting. Proteins were resolved and blotted as stated above. The following antibodies were used: NOQ7 5.4D, MyHC I antibody (Sigma); BF-F3, MyHC IIb mAb; SC-71, MyHCIIA-IX mAb (German Collection of Microorganisms and Cell Cultures).

**Luciferase Assay—**Luciferase assays were performed as described previously (36).

**Immunohistochemistry—**Frozen muscle samples were embedded in O.C.T. compound from Tissue-Tek (Torrence, CA) and sectioned into 7-μm thick slices, dried for 15 min, and washed briefly in PBS. Muscle sections were blocked in 10% horse serum-1× PBS for 1 h at room temperature. The primary antibodies NOQ7 5.4D, MyHC I, BF-F3, and SC-71 mAbs were dilute 1:200 in blocking solution and incubated overnight at 4 °C. Tissue sections were washed three times with PBS for 5 min each, followed by incubation in fluorescein isothiocyanate-conjugated goat anti-mouse secondary antibody. Secondary antibody was diluted 1:400 in blocking solution, and tissue sections were incubated for 1 h at room temperature. Tissue sections were washed three times with PBS and mounted with VectaShield (Vector Laboratories, Burlingame, CA) and coverslips.

**Surgeon Procedure—**The procedure used to induce work overload on the plantaris and soleus muscles of mice has been previously described in detail (40). Sham surgery was performed on all control animals, and mice were compared with the surgical group. The experimental period was conducted over a 6-week time course.

**Fiber Cross-sectional Area—**Plantaris muscles from mice were collected, fixed in 10% formalin containing PBS, and embedded in paraffin. Muscles were cut crosswise through the thickest part of the muscle,
and embedded cut side down. Muscle sections were cut at a thickness of 7 μm. Sections were deparaffinized in xylene and rehydrated in dilutions of ethanol. To visualize fibers, sections were incubated with tetramethyl rhodamine isothiocyanate (TRITC)-labeled wheat germ agglutinin (50 μg/ml in 1× PBS; Sigma) for 1 h, washed three times for 10 min each in PBS, and mounted with VectaShield and coverslips. Fiber cross-sectional areas were determined from ~220 fibers per plantaris muscle per mouse.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)—RNA was isolated from quadriceps muscle from two separate wild-type, CnA−/−, CnB−/−, and CnB1(+/fl)-MLC-cre mice using TRIzol® reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. cDNA synthesis was carried out using SuperScript™ II First-Strand synthesis system for RTPCR (Invitrogen) according to the manufacturer’s protocol. PCR was performed using GeneChoice® Taq DNA polymerase with standard buffer (1.5 mM MgCl₂, final concentration) (PGC Sciences, Frederick, MD). For each reaction, 4 μl of cDNA template was used, and cycling conditions consisted of 96 °C for 25 s, 57 °C for 30 s, and 72 °C for 1 min for 40 cycles. Approximately 9 μl of product was collected every 4 cycles beginning at cycle 23 and run on a 5% acrylamide gel. A 50% of the total calcineurin protein in skeletal muscle (16). The second model consists of a skeletal muscle-specific deletion of the CnB1 gene, which is targeted with LoxP sites, in conjunction with mice harboring a cre recombinase knock-in within the MLC1f locus. It should be noted that whereas the MLC1f locus restricts cre expression to fast/glycolytic fibers; muscle in the mouse is overwhelmingly fast/glycolytic, with the exception of the soleus. CnB1-LoxP(+/fl)-MLC-cre mice showed no reduction in CnB1 protein levels in the brain, kidney, cardiac atria, cardiac ventricle, or the slow/oxidative soleus muscle (Fig. 1A). However, nearly all other muscles examined showed a dramatic loss of CnB1 protein levels; such as a greater than 90% loss in the biceps or gastrocnemius (Fig. 1, A and B). Interestingly, loss of CnB1 led to a stoichiometric reduction in CnA protein levels, whereas α-tubulin Western blotting showed equal protein loading (Fig. 1B). By comparison, mRNA levels for CnAα and CnAβ were not reduced by the loss of CnB1, indicating no change in expression of either gene product (Fig. 1C). Conversely, loss of either CnAα−/− or CnAβ−/− produced a stoichiometric reduction (~50%) in CnB1 protein levels in skeletal muscle, collectively suggesting that CnA and CnB mutually stabilize one another (Fig. 1B). These results also indicate that loss of CnB1 will dramatically compromise overall calcineurin activity in skeletal muscle (more so than either CnAβ or CnAα gene targeting). The CnB2 gene was not expressed in skeletal muscle, nor was it ectopically induced in the absence of CnB1, indicating that loss of CnB1 is fully penetrant (data not shown).

RESULTS

Decreased Expression of CnA or CnB1 In Vivo Leads to Concomitant Reduction in Remaining Calcineurin Protein—Considerable controversy persists regarding the role of calcineurin as a regulator of skeletal muscle hypertrophy, in part because of variables associated with cyclosporine A treatment. To overcome this limitation, here we analyzed the phenotype of two distinct gene-targeted mouse models lacking calcineurin. The first model consists of a global disruption of the CnAβ gene, which we have previously shown constitutes between 40 and 50% of the total calcineurin protein in skeletal muscle (16). The second model consists of a skeletal muscle-specific deletion of the CnB1 gene, which is targeted with LoxP sites, in conjunction with mice harboring a cre recombinase knock-in within the MLC1f locus (35, 37). It should be noted that whereas the

![Fig. 1. Analysis of calcineurin A and B protein levels in CnAβ−/− and CnB1-LoxP-MLC-cre mice.](http://www.jbc.org/)

A. Western blot analysis of whole cell extracts from CnB1-LoxP(+/fl)-MLC-cre tissue demonstrates no loss of CnB1 in brain, kidney, cardiac atria, cardiac ventricle, or soleus, but significant loss in the biceps and gastrocnemius muscles B. Western blot analysis from a fast/glycolytic muscle (Gastroc.) reveals a loss of CnB1 protein and a concomitant loss of total CnA, with a dramatic loss of CnB1 protein levels from two wild-type (W1 and W2) and two CnB1-LoxP(+/fl)-MLC-cre mice (Cre1 and Cre2) from gastrocnemius at the indicated cycle numbers.
mice, with an average increase of muscle weight in most muscles examined from wild-type mice administered IGF-1 for 2 weeks, which induced a significant increase in relative muscle weights in CnAΔ−/− mice, myofiber numbers, and surface areas were calculated from the plantaris. Interestingly, CnAΔ−/− mice exhibited 28% fewer fibers within the plantaris (Fig. 2A), as is evident from representative histological images (Fig. 2B). Fiber numbers were not altered between wild-type and CnB1-LoxP(fl/fl)-MLC-cre mice (Fig. 2, A and B), consistent with a post-differentiation deletion of calcineurin. In addition to analyzing fiber number, the average fiber cross-sectional areas were also examined from the plantaris muscle in each cohort. Average fiber size did not vary between wild-type mice and either CnAΔ−/− or CnB1-LoxP(fl/fl)-MLC-cre mice, indicating that subsequent fiber growth was unaltered (Fig. 2C). Taken together, these results suggest that calcineurin participates in regulating myofiber development during embryogenesis, similar with the phenotype previously characterized in NFATc3 gene-targeted mice (41).

**IGF-1-GH-mediated Hypertrophy Is Not Significantly Compromised in CnAΔ−/− or CnB1-LoxP(fl/fl)-MLC-cre Mice**—Two different models for inducing skeletal muscle hypertrophy in the adult were instituted, either administration of IGF-1-GH (39), or mechanical overload (MOV) of the plantaris muscle by removal of the gastrocnemius (40). In the first model, IGF-1 was co-administered with GH via subcutaneous injection twice daily for 2 weeks, which induced a significant increase in relative muscle weight in most muscles examined from wild-type mice, with an average increase of ~20% (Fig. 3A). Interestingly, both CnAΔ−/− and CnB1-LoxP(fl/fl)-MLC-cre showed a normal hypertrophic growth response, suggesting that calcineurin does not contribute to IGF-1-induced muscle growth in vivo (p < 0.05) (Fig. 3, B and C). To further confirm the effectiveness of the IGF-1-GH treatment regime, Akt phosphorylation was examined as a read-out of IGF-1 signaling. Similar increases of between 120 and 185% were observed in Akt phosphorylation in the three groups treated either acutely with IGF-1 injection (harvested 1 h later) or after 14 days of IGF-1-GH treatment (p < 0.05) (Fig. 3D). GAPDH protein levels did not vary between samples (n = 3 mice in each group, vehicle or treated), nor did IGF-1-GH treatment up-regulate any calcineurin A or B isoform protein expression (data not shown).

**NFAT Reporter Activity Is Not Up-regulated in Response to Chronic IGF-1-GH Administration**—NFAT transcription factor family members are important downstream effectors of calcineurin signaling. Given the difficulties associated with the traditional calcineurin enzymatic assay, we have generated and characterized transgenic mice containing an NFAT-luciferase reporter as a means of assessing calcineurin signaling in vivo (38). Calcineurin activity was previously shown to be increased in cultured C2C12 myoblasts following IGF-1 administration (11, 24). However, other studies have found evidence indicating that this increase in activity is likely associated with a role for calcineurin in myoblast differentiation (5, 11, 42–44). Here we subjected NFAT-luciferase reporter mice to a 2-week regimen of IGF-1-GH administration, which revealed no increase in activity (Fig. 4A), despite the fact that these mice underwent a hypertrophic growth response (Fig. 4B). Mice were also sacrificed 2, 5, 8, 18, 24, and 48 h following IGF-1-GH treatment for measurement of luciferase reporter activity in the event that calcineurin induction only occurred early in the treatment regimen. However, no increases were detected in reporter activity at any time point (data not shown). Western blotting for phosphorylated Akt (serine 473) was also performed to further demonstrate the effectiveness of IGF-1-GH administration (Fig. 4C). Collectively, these data suggest that calcineurin-NFAT signaling does not actively participate in mediating the hypertrophic growth response of adult skeletal muscle.

### Table I

Relative muscle weights in female mice

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Wild type</th>
<th>CnAΔ−/−</th>
<th>CnB1-LoxP(fl/fl)-MLC-cre</th>
</tr>
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<tbody>
<tr>
<td>Gastrocnemius</td>
<td>3.79 ± 0.050</td>
<td>3.19 ± 0.080&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.79 ± 0.090&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Soleus</td>
<td>0.28 ± 0.007</td>
<td>0.23 ± 0.010&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.32 ± 0.017&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Plantaris</td>
<td>0.52 ± 0.008</td>
<td>0.38 ± 0.013&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.55 ± 0.014&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>TA&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.48 ± 0.024</td>
<td>1.36 ± 0.063</td>
<td>1.57 ± 0.050&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Quadriceps</td>
<td>6.15 ± 0.147</td>
<td>4.52 ± 0.053&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.77 ± 0.119&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Triceps</td>
<td>3.13 ± 0.071</td>
<td>2.73 ± 0.123&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.49 ± 0.109&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup>p < 0.01 versus wild type.
<sup>b</sup> TA, tibialis anterior.
Muscle Growth in Response to MOV Is Not Significantly Compromised in CnB1−/− or CnB1-LoxP(fl/fl)-MLC-cre Mice—IGF-1-GH administration is a relatively mild hypertrophic stimulus compared with MOV, which can induce a doubling in plantaris and/or soleus muscle size in the rodent (40, 45–47). Thus, a more robust stimulus might more readily reveal any underlying defect present in either CnB1−/− or CnB1-LoxP(fl/fl)-MLC-cre mice. MOV was performed on each group of mice by removal of the gastrocnemius muscle, leaving the soleus and plantaris muscles intact for a period of 6 additional weeks. Quantitative Western blotting showed that CnB1 and total CnA protein levels remained significantly reduced after MOV, suggesting that pre-existing fast fibers transformed to the slow/oxidative program (Fig. 5A). However, we did observe a subtle increase in CnB1 and CnA protein levels after MOV, suggesting some involvement of satellite cell recruitment (because MLC1f is not expressed in myoblast progenitors, CnB1 is not deleted) (Fig. 5A). The soleus was not analyzed, because the CnB1-LoxP(fl/fl)-MLC-cre mice showed no reduction in calcineurin levels in this muscle.

Analysis of relative plantaris weights from sham-operated and overloaded wild-type, CnAβ−/− and CnB1-LoxP(fl/fl)-MLC-cre mice all showed average increases of 100% or greater (p < 0.05) (Fig. 5B). However, CnAβ−/− mice showed a significant, albeit subtle decrease in the magnitude of the hypertrophic response (p < 0.05), although this difference likely reflects a developmental abnormality in these mice (they have fewer fibers) because the more pervasive deletion with the CnB1-LoxP(fl/fl) strategy did not show a similar effect. Associated with these increases in relative muscle weight were significant increases in fiber number (p < 0.05) (Fig. 5C). Fiber number increases have been seen in previous studies of muscle growth and are thought to occur because of muscle fiber splitting, de novo fiber formation resulting from satellite cell migration, or both (48, 49). Also associated with the observed increase in relative muscle weight was a uniform increase in fiber size. Increased populations of larger fibers were evident in all three groups (Fig. 5D). All plantaris cross-sections were performed at equal depths within the muscle between the groups. Taken together, these data indicate that calcineurin does not appreciably regulate the ability of skeletal muscle to undergo hypertrophic growth following MOV.

Phospho-Akt, and NFAT Reporter Activity Are Up-regulated in Response to MOV—We also subjected NFAT-luciferase reporter mice to MOV to determine if there were any changes in calcineurin-NFAT activity in overloaded muscle. Surprisingly, overloaded plantaris muscle displayed a greater than 3-fold increase in NFAT reporter activity compared with NFAT-lucif-erase sham operated mice (p < 0.05) (Fig. 5C). These reporter mice also demonstrated a nearly 2-fold increase in muscle weight compared with sham-operated mice, verifying the effectiveness of the MOV procedure (Fig. 5D). In parallel, NFAT-luciferase reporter transgenic mice were bred into the CnAβ−/− and CnB1-LoxP(fl/fl)-MLC-cre backgrounds to evaluate any potential reductions in NFAT responsiveness during
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MOV-induced hypertrophy. Remarkably, loss of either CnAβ or a tissue-specific deletion of CnB1 dramatically decreased MOV-induced NFAT activation in the plantaris (p < 0.05) (Fig. 6A). However, phospho-Akt levels were up-regulated normally in the plantaris from all three cohorts following MOV stimulation (Fig. 6C), consistent with a similar degree of hypertrophic growth in each group (data not shown). The observation that NFAT activity was increased in wild-type mice following MOV stimulation, but not in CnAβ−/− or CnB1-deleted mice, despite similar degrees of growth, further suggests that calcineurin-NFAT signaling is not causative in mediating hypertrophy. The increase in NFAT activation following MOV more likely reflects a role in fiber-type switching, as we have previously observed in CnAβ− and CnAα-null mice (16) (see below).

Basal Slow Fiber Populations and MOV-mediated Fiber Type Switching Are Compromised in CnB1-LoxP(fl/fl)-MLC-cre Mice—To further examine the effect of calcineurin disruption on fiber type switching at baseline or following MOV, both Western blotting and immunohistochemistry were performed for MyHC I. Western analysis of the quadriceps muscle of CnB1-LoxP(fl/fl)-MLC-cre mice revealed dramatic deficiencies in both MyHC I and IIA protein expression (Fig. 7A). In parallel, immunohistochemistry for MyHC I revealed a relative decrease in slow fiber numbers in CnAβ−/− and CnB1-LoxP(fl/fl)-MLC-cre muscle (Fig. 7B and data not shown). These results further support the hypothesis that calcineurin signaling regulates the fast versus slow fiber type program in skeletal muscle.

Unfortunately, the MLC-cre knock-in allele is not expressed in slow fibers, so that the role calcineurin might play in maintenance of an existing slow program cannot be addressed with this strategy. However, we attempted to address this issue by generating transgenic mice that express Cre recombinase under the control of the slow fiber-specific β-MyHC promoter. These transgenic mice were crossed with CnB1-LoxP(fl/fl)-MLC-cre mice and data not shown). These results suggest that calcineurin also facilitates maintenance of existing slow fibers.

Finally, the ability of calcineurin to regulate MOV-induced increases in slow fiber composition was also evaluated in the plantaris. Immunohistochemical and Western blot analyses from CnB1-LoxP(fl/fl)-MLC-cre and CnB1−/− mice revealed either significant or minor defects in the ability to up-regulate slow MyHC I expression following overload, respectively (Fig. 7, C and D). For example, CnB1-LoxP(fl/fl)-MLC-cre mice were almost completely devoid in their ability to up-regulate MyHC I following MOV, consistent with a dramatic reduction in total calcineurin protein level. However, loss of CnAβ only partially compromised the ability of the overloaded plantaris to up-

Fig. 5. Plantaris muscle characteristics and calcineurin expression in sham-operated and MOV plantaris muscle. Bilateral removal of the gastrocnemius muscle from wild-type (n = 7), CnAβ−/− (n = 7) and CnB1-LoxP(fl/fl)-MLC-cre (n = 6) mice was performed. A, Western blot analysis of pan-CnA, CnB1, and GAPDH protein expression in CnB1-LoxP(fl/fl)-MLC-cre sham-operated and overloaded plantaris muscles versus wild-type overloaded muscles. B, MW normalized to TL for the indicated groups. *, p < 0.05 versus sham; #, p < 0.05 versus WT MOV. C, fiber number from the indicated groups. *, p < 0.05 versus Sham; D, distribution of fiber cross-sectional areas in the indicated groups quantitated after 6 weeks of overload. Wild-type sham, n = 13; CnAβ−/− sham, n = 8; CnB1-LoxP(fl/fl)-MLC-cre sham, n = 4.

Fig. 6. NFAT-luciferase transgene reporter activity and phospho-Akt levels in sham-operated and MOV plantaris muscle. A, relative NFAT luciferase activity (RLU/μg of protein) following overload in each of the indicated groups. *, p < 0.05 versus sham; #, p < 0.05 versus WT MOV. B, plantaris MW normalized to TL of sham-operated and overloaded plantaris muscles from NFAT luciferase reporter mice. *, p < 0.05. C, Western blot analysis of phospho-Akt (Ser473) in plantaris whole cell extracts from sham or MOV mice that were wild type, CnAβ−/−, or CnB1-LoxP(fl/fl)-MLC-cre. GAPDH is shown as a loading control (n = 3 mice per genotype). The diagram on the right depicts percent increase in phospho-Akt in the MOV mice. *, p < 0.05 versus sham).
regulate slow MyHC I expression, consistent with a less severe reduction in total calcineurin protein (Fig. 7, E and F). Taken together, our results suggest that whereas calcineurin signaling does not appreciably regulate the hypertrophic growth response, it does play a central role in regulating fiber type switching toward the slow/oxidative program.

**DISCUSSION**

Role of Calcineurin in Regulating Skeletal Muscle Hypertrophy—IGF-1 signaling through calcineurin was reported to play a central role in the regulation of myotube formation and its subsequent hypertrophic growth in culture (24, 25). For example, treatment of cultured C2C12 myoblasts, L6 myoblasts, or IGF-1-secreting L6E9 myoblasts with cyclosporine A or a dominant negative calcineurin mutant (or CAIN) attenuated or blocked subsequent myotube formation (24, 25, 43). Moreover, expression of an activated mutant of calcineurin in L6E9 cells, or IGF-1 alone, dramatically enhanced myotube formation and subsequent growth (25, 50). Additionally, in vivo, administration of cyclosporine A to mice was found to prevent the normal increase in plantaris size after 4 weeks of MOV (26). On the surface, these studies all appear to indicate that calcineurin signaling regulates the hypertrophic growth of skeletal myotubes. However, an alternative interpretation of these data is that calcineurin regulates an aspect of myoblast differentiation, and/or the efficiency at which myoblasts fuse to form myotubes. Although calcineurin appears to enhance the size of myotubes in culture, it likely only reflects an accelerated time course of differentiation. For example, we have observed that expression of activated calcineurin enhances differentiation of C2C12 cells and of MyoD-transfected 10T1/2 fibroblasts (11), consistent with similar data reported in L6 myoblast cultures (43). MOV stimulation in vivo also likely utilizes a component of satellite cell recruitment to enhance myonuclear number or the number of myotubes (51–55). Indeed, cyclosporine A administration inhibits muscle regeneration after trauma, a process that requires de novo myoblast differentiation (44). Collectively, the seemingly discordant data initially discussed above can be reconciled if one invokes a primary role for calcineurin in regulating the efficiency of myoblast differentiation, which could be associated with the appearance of myocyte growth in culture. That calcineurin does not play a direct role in regulating skeletal muscle hypertrophy is further supported by the observation that transgene-mediated expression of activated calcineurin in mouse skeletal muscle, driven by the muscle creatine kinase (MCK) promoter, does not produce larger myofibers or overt hypertrophy. However, the MCK promoter only drives maximal expression in committed myotubes, so its role in enhancing differentiation and the ensuing secondary effects on growth cannot be properly evaluated in vivo (14).

Whereas calcineurin is not likely to directly regulate skeletal muscle hypertrophy, a prominent role has been suggested for the IGF-1/Pi3K/Akt/mTOR signaling circuit in this process. For instance, Akt/mTOR is up-regulated during hypertrophy and down-regulated during muscle atrophy, and rapamycin, a selective blocker of mTOR, was shown to block hypertrophy (19, 30). In fact, genetic activation of the Akt/mTOR pathway has been demonstrated to be sufficient to cause hypertrophy and prevent atrophy in vivo, which can even antagonize calcineurin signaling during hypertrophy in vitro (31). Furthermore, constitutively active Akt has been shown to increase fiber size and prevent denervation-induced atrophy in regenerating and adult rat muscles (19). These data suggest that an IGF-1-dependent signaling pathway, but not calcineurin, serves as the focal mediator of skeletal muscle hypertrophy.

A genetic loss-of-function approach for calcineurin has yet to be applied to the ongoing controversy surrounding the signaling pathways that regulate skeletal muscle hypertrophic growth. Here we report the first analysis of the hypertrophic potential of skeletal muscle from mice lacking a single calcineurin isoform (CnB1−/−) or a more significant (>80%), and muscle-specific reduction in calcineurin expression through conditional inactivation of CnB1. Reduction in calcineurin levels had no effect on muscle fiber growth or increase in fiber number following hormonal or MOV stimulation. IGF-1-GH treatment resulted in modest, albeit significant increases in muscle weight associated with elevated fiber sizes (although
Calcineurin Regulates the Slow/Oxidative Fiber Type Program—Slow fibers maintain higher levels of intracellular free calcium compared with fast fibers because of tonic motor nerve activity (8–10). Calcineurin is known to respond only to sustained elevations in intracellular calcium levels, as would be present in slow fibers. Indeed, we have previously reported that loss of either CnAα or CnAβ expression results in decreased expression of MyHC I in unstimulated muscles (16), which is also consistent with the effects attributed to cyclosporine A administration in fiber type composition. Consistent with these previous observations, loss of CnB1 also negatively impacted basal MyHC I expression. Initially, it was uncertain whether a reduction in slow/oxidative fibers would be observed given the fact that cre recombinase expression is directed by the fast type MLC1f genetic locus. However, that a significant reduction in slow fibers was observed, suggests that CnB1 deletion occurs at a developmental time when fibers show more lineage plasticity. In addition to the basal changes in slow fiber expression, we also observed that decreased calcineurin expression reduces or inhibits the normal fast-to-slow fiber type conversions during MOV. Lastly, partial deletion of CnB1 from slow fibers themselves, as directed by the β-MyHC promoter, partially compromised the slow fiber type program and promoted up-regulation of fast MyHC, suggesting that calcineurin participates in maintenance of the slow program once established. An alternate interpretation of our results is that calcineurin functions within satellite cells that are recruited to fibers resulting in affects on the slow versus fast fiber type program. However, this issue was previously addressed by the observations that irradiation, which inhibits cellular proliferation and interferes with satellite cell proliferation, still shows increases in slow MyHC following overload (69). Moreover, CnB1-LoxP(1f/1f)/MLC-cre mice show a profound decrease in the slow type program, despite a lack of CnB1 deletion in satellite cells. Taken together, these observations indicate that calcineurin affects the slow program through a fiber type shift rather than stem cell recruitment and differentiation.

Whereas we did not extensively evaluate the importance of NFAT factors as downstream mediators of calcineurin signaling-induced fiber type transformation, we did observe two interesting correlations. First, mechanically overloaded muscle showed a dramatic up-regulation in NFAT-luciferase transgene activity, coincident with a prominent transformation to the slow/oxidative program. Second, MOV induction of NFAT-luciferase activity was dramatically attenuated in both CnAβ−/− and CnB1-LoxP(1f/1f)/MLC-cre mice. With respect to the slow fiber type program, these results suggest that calcineurin-NFAT signaling is required for this process. This result is also consistent with the observation that slow/oxidative fiber type muscles (soleus) have significantly higher NFAT-luciferase reporter activity compared with fast fiber muscles (16). However, we previously observed that overexpression of activated NFATc3 did not affect fiber type composition in culture (11), possibly suggesting that there may be a specialization among NFAT family members. Indeed, here we observed that MOV induced NFATc1 protein levels in the plantaris of wild-type mice, but not CnB1-LoxP(1f/1f)/MLC-cre mice (data not shown). Thus, we conclude that NFAT activity, whereas not essential for initial slow fiber type determination, may be important for fast-to-slow fiber type transitions through select isoforms.

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Genetic Loss of Calcineurin Blocks Mechanical Overload-induced Skeletal Muscle Fiber Type Switching but Not Hypertrophy

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