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REVIEW

Bone muscle crosstalk targets muscle regeneration pathway regulated by core circadian transcriptional repressors DEC1 and DEC2

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Deletion of proprotein convertase Mbtps1 in bone osteocytes leads to a significant postnatal increase in skeletal muscle size and contractile function, while causing only a 25% increase in stiffness in long bones. Concerns about leakiness in skeletal muscle were discounted since Cre recombinase expression does not account for our findings, and, Mbtps1 protein and mRNA is not deleted. Interestingly, the response of normal skeletal muscle to exercise and the regenerative response of skeletal muscle to the deletion of Mbtps1 in bone share some key regulatory features including a preference for slow twitch muscle fibers. In addition, transcriptional regulators PPAR, PGC-1α, LXR, and repressors DEC1 and DEC2 all occupy central positions within these two pathways. We hypothesize that the age-dependent muscle phenotype in *Dmp1-Cre Mbtps1 cKO* mice is due to bone→muscle crosstalk. Many of the myogenic genes altered in this larger and functionally improved muscle are regulated by circadian core transcriptional repressors DEC1 and DEC2, and furthermore, display a temporal coordination with *Dec1* and *Dec2* expression consistent with a regulatory co-dependency. These considerations lead us to propose that Dmp1-Cre Mbtps1 *cKO* osteocytes activate myogenesis by increased release of an activator of muscle PPAR-gamma, for example, PGE_2 or sphingosine-1-P, or, by diminished release of an inhibitor of LXR, for example, long-chain polyunsaturated fatty acids. We hope that further investigation of these interacting pathways in the *Dmp1-Cre Mbtps1 cKO* model will lead to clinically translatable findings applicable to age-related sarcopenia and other muscle wasting syndromes.

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Introduction

We recently demonstrated dramatic new musculoskeletal functions for ubiquitously expressed proprotein convertase MBTPS1 (SKI-1, S1p, Pcsk8) which cover the vertebrate lifespan. In the embryo, MBTPS1 is required for normal segmentation of somites during spinal development and *Mbtps1* deficient embryos exhibit fused vertebrae and shortened tails as well as hindlimb paralysis.[1] Also, deletion of *Mbtps1* in bone osteocytes leads to a delayed muscle phenotype which is expressed only in mature (or older, JP Gorski, unpublished result) 10- to 12-month-old mice but not at 3 months of age[2] (**Figure 1**). In contrast to aged littermate controls, the muscles of *Dmp1-Cre Mbtps1 cKO* mice, which we have termed 'sumo' mice, are larger in size, display a 30% increase in contractile performance relative to control littermates even when normalized for size, and exhibit morphological characteristics of actively regenerating muscle.[2] Although absolute contractile force for control muscles was lower than wild-type C57Bl6 background strain mice, we believe that the reason may be due to hyperglycemia, which was recently detected in the floxed strain (see below). Thus our findings indicate that deletion of Mbtps1 in bone causes a relative increase of 30% in contractile force. The muscle phenotype was localized to slow twitch (soleus, SOL) muscles expressing type I myosin heavy chain but not to fast twitch (EDL) muscles. As *Mbtps1* is deleted in bone osteocytes and not in skeletal muscle tissues,[2] we have proposed that bone→muscle crosstalk signaling is responsible for the latter age-related muscle phenotype. In an effort to identify the bone-derived crosstalk factor and to understand more fully the nature of the induced metabolic changes, we analyzed the transcriptome of soleus muscles from 'sumo' mice and littermate controls (GSE69985, NCBI).

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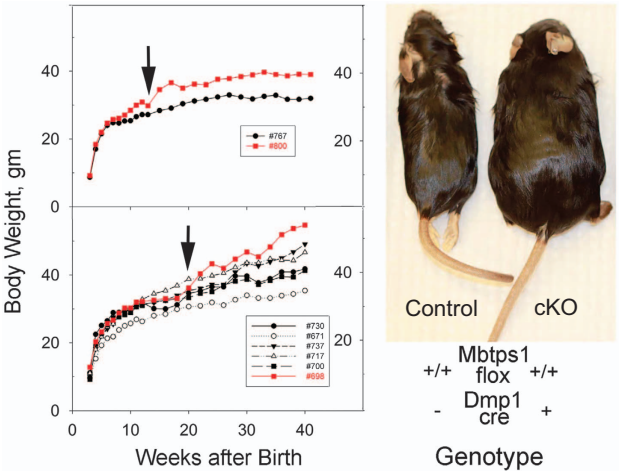

Figure 1 Deletion of *Mbtps1* in bone (red symbols) increases muscle mass after skeletal maturity (arrow, 15–20 weeks.). Left panel, two representative litters are depicted. Right panel, two male littermates at 10 months of age. Fat content (% body wt.) is not substantially different (not shown).

Gene profiling revealed increased expression in pathways mediating EGFR1 signaling, circadian exercise, striated muscle contraction, glycolysis, fatty acid oxidation and adipogenesis (**Table 1**). Briefly, actinin-α3 (*ACTN3*) was elevated in *cKO* SOL; *ACTN3* is a biomarker for increased muscle performance in elite athletes.[3] Other striated muscle contraction genes increased included Mg29, vimentin, desmin and slow twitch muscle restricted troponin. Embryonic and perinatal myosin heavy chains (MYH3 and MYH8), which have been used as biomarkers to identify muscle regeneration,[4–6] were also increased. MYF6, which is associated with satellite cell activation during muscle regeneration,[7] and PAX7, MYOD1 and MYOG, which are required for postnatal growth and regeneration of adult skeletal muscle,[8–10] were also elevated in *Dmp1-Cre Mbtps1 cKO* SOL. Metabolically, enhanced expression of triglyceride lipase in *Mbtps1 cKO* SOL should facilitate release and oxidation of fatty acids. Interestingly, expression of basic helix-loop-helix domain containing transcriptional regulators DEC1 (BHLHE40, STRA-13 and BHLHB3) and DEC2 (BHLHE41, SHARP1 and BHLHB3) was decreased significantly in *Mbtps1 cKO* SOL compared with controls at the time the mice were killed.[2]

DEC1 and DEC2 are core circadian clock genes which like PER and CRY are positively regulated by BMAL and CLOCK;[11,12] in return, expression of DEC1 and DEC2 are subject to negative feedback by transcription factor SREBP-1.[12,13] DEC1 and DEC2 expression in muscle displays a rhythmic 24–28 h cycle[14] which because of negative feedback regulation would be predicted to be offset by ~12–24 h compared with that for BMAL and CLOCK. BMAL expression is required for satellite cell activation in muscle regeneration.[15] DEC1/DEC2, in contrast, were shown previously to repress myogenesis *in vitro* and *in vivo* by blocking transcription of MYOD1, as well by altering expression of a number of contractile and mitochondrial proteins by skeletal muscle.[16–18] By analogy with the age-related onset of the *Dmp1-Cre Mbtps1 cKO* phenotype, DEC2 null mice show no deficit with respect to repair of embryonic muscle, however, as predicted for a repressor of myogenesis, regeneration after injury in post-natal DEC2(−/−) mice is increased.[18] As SOL in *Dmp1-Cre Mbtps1 cKO* mice display characteristics of regenerating muscle, we hypothesized that a cycling circadian expression pattern for myogenic repressors DEC1/DEC2 could stimulate or reduce expression of pro- or anti-myogenic genes, respectively, during their 'down cycle'. Since Lecomte and colleagues have already identified (+) and (−) transcriptional muscle-specific targets of DEC1/DEC2,[16] we asked whether expression of these target genes is temporally correlated with that of transcriptional regulators DEC1/DEC2 in *Dmp1-Cre Mbtps1 cKO* muscle? Importantly, the majority of muscle-specific target genes (79 out of 110) displayed the expected responses[16] as when DEC1/DEC2 are downregulated.[2] As expression of both DEC1 and DEC2 is low in *Dmp1-Cre Mbtps1 cKO* SOL (array data deposited at NCBI, #GSE69985), this is the expected outcome. Although our transcriptional data represents a single time point, and not a systematic timed study of circadian regulation, we still believe the high degree of correlation between predicted and observed target gene expression observed supports a role for DEC1/DEC2 in the regeneration and growth of *Dmp1-Cre Mbtps1 cKO* SOL.[2] The purpose of this article is to justify a hypothetical mechanistic bone→muscle crosstalk signaling pathway that explains the 'sumo mouse' muscle phenotype while incorporating what is known about the control of myogenesis following exercise.

Table 1 Gene profiling reveals signaling pathways activated in Dmp1 cre Mbtps1 cKO soleus muscle

EGFR1
Striated muscle contraction
Membrane trafficking
Fatty acid oxidation
Glycolysis
Circadian exercise
Adipogenesis

Abbreviation: EGFR, epidermal growth factor receptor.

Physical Exercise, Muscle Mass Gain and the Effect with Age

Exercise is currently the only proven method to both inhibit age-related muscle loss and to at least partially restore muscle mass lost via inactivity and age-related sarcopenia.[19,20] In terms of exercise, the two main types of physical exercise affect skeletal muscles differently, for example, endurance and resistance (strength) training. Resistance training works both fast twitch and slow twitch muscles while endurance training primarily works the slow twitch fibers and changes these fibers in terms of increased efficiency and resistance to fatigue.[20,21] Exercise-induced changes in muscles include an increase mitochondrial number and size and in their metabolic capacity to store glycogen and fat, as well as to catabolically use fat and glycogen stores as an energy source. A key similarity between the effect of endurance training and the effect of conditional deletion of Mbtps1 in bone is that they both impact predominantly slow twitch muscle fibers. For example, *Dmp-1-Cre Mbtps1 cKO* SOL muscles (slow twitch), but not EDL muscles, gain mass, alter their metabolic profile, and contract more forcefully. Within soleus muscles, only type I (slow twitch) myosin heavy chain expressing muscle fibers contain centralized nuclei, a morphological characteristic of regeneration. However, not all features are shared with entrained muscle since SOL and EDL from *Dmp1-Cre Mbtps1 cKO* do not

Table 2 Comparison of cross-sectional area of myofibers from Dmp1-Cre Mbtps1 cKO and control SOL muscles

Control (all myofibers)	*Dmp1-Cre Mbtps1 cKO (all myofibers)*	*Dmp1-Cre Mbtps1 cKO (myofibers only with centralized nuclei)*
$1{,}744 \pm 377\ \mu m^2$ STD	$1{,}557 \pm 357\ \mu m^2$ STD $P = 0.274$ (two tailed *t*-test)	$1{,}162 \pm 192\ \mu m^2$ STD $P = 0.006$ (two tailed *t*-test)

Abbreviations: SOL, soleus; STD, standard deviation.

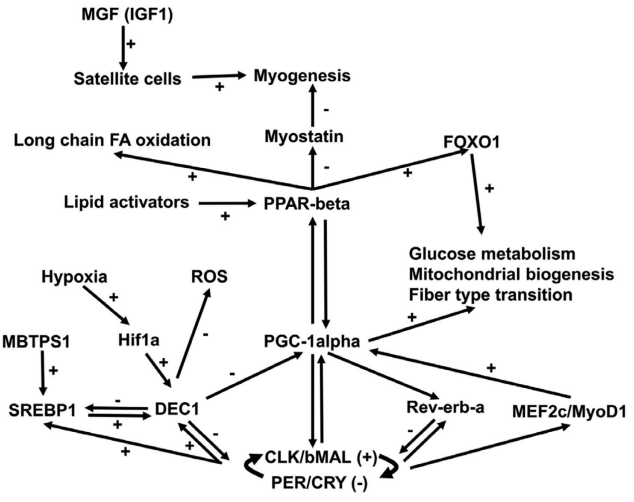

Figure 2 Hypothetical pathway regulating the response of skeletal slow twitch muscle to endurance training: myogenesis, fuel usage, and myosin fiber type change.

display increased resistance to fatigue nor *cKO* EDL exhibit immunochemical evidence of a fast twitch to slow twitch transition.[2,22]

Because historically there is a significant positive correlation of muscle strength and cross-sectional area (CSA), we recently have measured myofiber CSA for our 'sumo' mice (**Table 2**). The data show that average myofiber CSA for the larger SOL muscles in *DMP1-Cre Mbtps1 cKO* mice are not statistically different from control littermates. Actual values, however, are very similar to that for published values for murine SOL muscles (**Table 2**). However, when myofibers within SOL with central nuclei ($\sim$10–15% of total), which we have suggested is a marker for muscle regeneration in our model, were measured it is apparent that the average CSA was significantly smaller than that for total cells (**Table 2**). We believe that this finding is consistent with findings of a large number of publications[23–25] and supports ongoing regeneration within *Dmp1-Cre Mbtps1 cKO* muscle. Specifically, during regeneration of muscle following an injury stimulus, the average CSA of myofibers is initially lower. However, as time elapses during the repair phase, the average CSA increases to reach that of the muscle prior to injury. We believe the rationale is that new myogenic progenitors, stimulated by the injury stimulus, are formed and proceed to 'differentiate through fusion with each other or to damaged fibers to reconstitute fiber integrity and function'.[26]

Although age was once presumed to cause dramatic reductions in optimal muscle performance after endurance training, further research has shown that the rate of decline may be much slower than once believed.[27] Recently, Karsenty and colleagues[28] showed that osteocalcin signaling in myofibers is necessary for maximal adaption to exercise. They also showed that exogenous osteocalcin can dramatically restore the exercise capacity of old mice to that of 3 months old mice.[28] Since no significant difference exists between the osteocalcin content of *Dmp1-Cre Mbtps1 cKO* mice and controls at 12 months of age (23.4 ± 7.1 vs $30.8 \pm 11.6\ ng\ ml^{-1}$, respectively; (Gorski, JP, unpublished result) and osteocalcin did not stimulate muscle growth and regeneration,[28] we do not believe osteocalcin has a prominent role in the *Mbtps1 cKO* muscle phenotype (**Figure 1**).

Exercise and the Signaling Pathway Regulating Slow Twitch Muscle Regeneration

Much work has gone into identifying the signaling pathway that regulates the response of skeletal muscle to endurance training as outlined above. While the individual genes required for exercise-induced increases in myogenesis and in oxidative fuel usage, as well as fiber type transition are generally known, the physiological signals or triggers remain uncertain. However, several key genes mediate changes in myogenesis, in oxidative fuel usage and in fiber type transition: PGC-1α, PPAR-β/δ, MGF (a splice variant of IGF-1), and Rev-erb-α (**Figure 2**).

PGC-1α (PPARγ co-activator 1α) is a transcriptional co-activator that interacts with and regulates the activities of cAMP response element binding protein (CREB). PGC-1α is directly regulated by myogenic regulatory factors (MEF2c and MyoD1).[29] It provides a direct link between external physiological agents and is a master regulator of mitochondrial biogenesis and muscle fiber type determination.[30] One such physiological agent, hypoxia, is a consequence of physical exercise and leads to increased HIF-1α and DEC1 expression[31] (**Figure 2**). DEC1 represses transcription by directly competing with MyoD1 for the same PGC-1α promoter-binding site.[32] Importantly, DEC1 is reciprocally regulated by SREBP1 which requires proteolytic activation by MBTPS1.[16] SREBP-1 is a required transcription factor for most enzymes and receptors mediating lipolysis and lipogenesis and mitochondrial biogenesis, as well as myogenesis.[33] The light/dark entrained mammalian circadian clock is known to control glucose and lipid metabolism and mitochondrial oxidative metabolism. Although widely appreciated, the fact that PGC-1α, SREBP1, DEC1 and MyoD1 are all cyclically expressed in skeletal muscle would seem to provide an explanation.[34]

Peroxisome proliferator activated nuclear receptors (PPARs) function as transcription factors and are so named because they are activated by ligands that induce proliferation of peroxisomes, organelles that mediate oxidation of fatty acids. PPARs can be activated both by dietary and endogenous lipid compounds. Once activated, PPARs can redirect a cell's metabolism. In skeletal muscle, activated PPAR-β/δ induces a switch to form increased numbers of type I muscle fibers.[35] Targeted expression of PPAR-β/δ in skeletal muscle produces a mouse capable of running twice the distance of a wild-type littermate as well conferring resistance to obesity.[35] Lipid ligands capable of activating PPARs include long-chain fatty acids, eicosanoids, and prostaglandins, although no

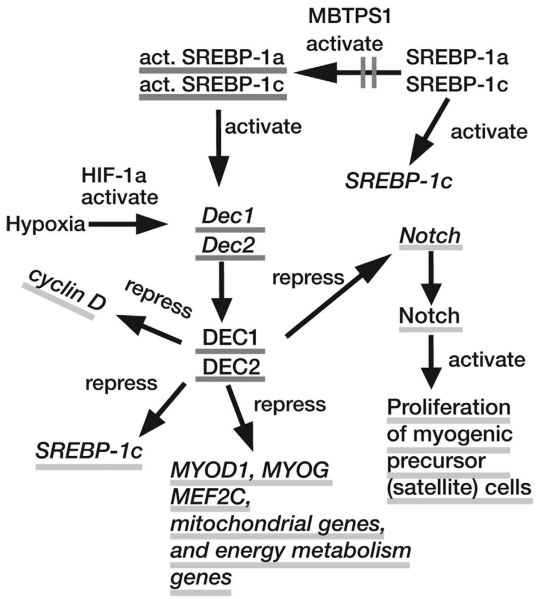

Figure 3 Summary of proposed relationships involving SREBP1 and MBTPS1 and DEC1/DEC2 and their regulation of myogenesis in skeletal muscle.

consensus agreement exists as to the identity of the primary physiological activator.[36,37]

MGF (mechano growth factor) is a splice variant of IGF-1.[38] IGF-1 and MGF are upregulated in exercised[39] and damaged skeletal muscle where MGF has been shown to stimulate proliferation and inhibit differentiation.[40] In general, animal studies have confirmed cell culture studies and confirmed that MGF is a local tissue repair factor which responds to mechanical demands.[38] In a broader sense, different splice variants of IGF-1 display distinct functions within the myogenic pathway wherein isoform IGF-1Eb stimulates satellite cell activation, while isoform IGF-1Ea promotes myoblast proliferation and differentiation into myotubes.[41]

In summary, the above rationale of exercise effects on skeletal muscle suggests several conclusions. The response of skeletal muscle to exercise and its regenerative response to deletion of Mbtps1 in bone shares several key regulatory components. Transcriptional repressors DEC1 and DEC2, PPAR-delta, and PGC-1a are all prominently expressed after exercise and in *Dmp1-Cre Mbtps1 cKO* muscle. While the myogenic actions of PPAR-delta and PGC-1a are well documented, the robust regulatory actions of DEC1 and DEC2 on the proliferation of myogenic satellite cells and the expression of myokines and mitochondrial genes is under appreciated. Implicitly our analysis indicates that bone→muscle crosstalk can modify the metabolism of muscle independent of physical exercise. Although the actual bone-derived crosstalk factor is still under investigation, we have learned much about the regenerative response of skeletal muscle to exercise and to deletion of *Mbtps1* in bone as detailed in the next section. As a result, we wish to emphasize the role of DEC1 and DEC2 in bone muscle crosstalk and exercise-induced muscle regeneration.

Circadian Core Genes DEC1/DEC2 Regulate Myogenesis (and Adipogenesis)

Transcription factor SREBP-1, also termed ADD-1 or adipocyte determination and differentiation-dependent factor 1, is a major positive determinant of lipogenesis in mammals and avian species.[42] SREBP-1 is known to directly regulate the expression of over 200 genes involved in *de novo* cholesterol and fatty synthesis as well as their transport and cellular uptake[33] (**Figure 3**). In view of their common stem cell, the myocyte and adipocyte lineages represent alternative differentiation pathways. Importantly, the work of Lecomte,[16] indicates that while SREBP-1 actively promotes adipocyte differentiation, overexpression of SREBP1 in muscle *in vivo* causes muscle atrophy[16] while *in vitro*, SREBP-1 inhibits myoblast to myotube differentiation. Specifically, overexpression of activated SREBP1 isoforms 1a and 1c in muscle cells altered the expression of over 1300 genes in differentiated human myotubes.[17] Genes experiencing substantial change encoded early myogenic transcription factors MYOD1, MYOG, and MEF2C as well as many muscle contractile apparatus proteins (myosin heavy and light chains, troponins and titin; **Figure 3**). Interestingly, most genes were downregulated in the presence of SREBP-1a or SREBP-1c, however, transcriptional repressors *Dec1* and *Dec2* were induced. Subsequent work demonstrated that *Dec1* and *Dec2* are direct transcriptional targets of SREBP-1a and SREBP-1c and that DEC1 and DEC2 then together mediate the repression of hundreds of muscle genes (**Figure 3**).[16,17] Taken together, this work illustrates an under-appreciated function of transcription factor SREBP-1 (and its target surrogate repressors DEC1 and DEC2), for example, to regulate the differentiation of mesenchymal stem cells to fat and muscle. When present, activated SREBBP-1 stimulates the formation of adipocytes and blocks the differentiation into myocytes.[43] When SREBP-1 is absent, the default fate of mesenchymal stem cells is to differentiate into myocytes. As illustrated in the next sections, we discuss how systemic hormones, environmental factors, circadian cycling, and post-translation processing reactions can determine the nuclear content of activated SREBP-1 (and its surrogates DEC1 and DEC2).

DEC1/DEC2 are Subject to Regulation at the Transcriptional Level by Hormones (Insulin and Retinoic Acid), Environmental Factors (Hypoxia, Oxysterols and Hyperglycemia), and Indirectly via SREBP1

These regulatory pathways are summarized in **Figure 4** and illustrate that expression of *Dec1* and *Dec2* is intimately linked with the metabolic well-being of the host, its access to nutritional sources of fats and glucose, circadian cycling, and its exposure to specific environmental factors. Among these direct regulators, hormones insulin and retinoic acid are prominent. Specifically, Yamada K *et al.*[44] showed that insulin induces *Dec1* via a phosphoinositide-3-kinase pathway. Rexinoid bexarotene induces transcription of *Dec2*, which then can repress cell proliferation by blocking expression of cyclin D1[45] (**Figures 3 and 4**). Interestingly, retinoic acid also indirectly regulates *Dec1* and *Dec2* through SREBP1. Yoshikawa T *et al.*[46] demonstrated that the liver X receptor–retinoid X receptor is an activator of the SREBP1c promoter and that ligands 22-hydroxycholesterol and 9-cis-retinoic acid are able to induce both SREBP-1c mRNA and protein production in cultured cells. As shown in **Figure 4**, SREBP1 and DEC1/DEC2 reciprocally regulate each other at the transcriptional level.[16,47]

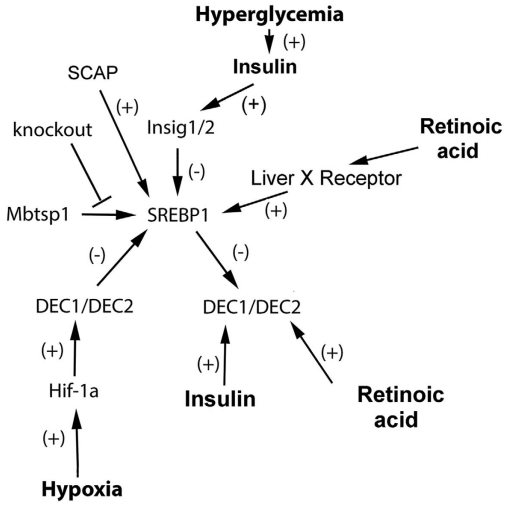

Figure 4 *Dec1* and *Dec2* are subject to transcriptional regulation by environmental agents: systemic hormones, environmental conditions, and SREBP1.

As well as hormones, expression of Dec 1 and Dec2 is also controlled by environmental factors (**Figure 4**). Hypoxia stimulates a general response in all cells that ultimately produces transcription factor HIF-1α which then initiates the hypoxic response. One of the important targets of HIF-1α is the transcriptional repressor pair DEC1 and DEC2. HIF-1α activates transcription of both Dec1 and Dec2 by binding to hypoxia response elements in the transcriptional regulatory regions of both of these genes.[31] Teleologically, HIF-1α induction of *Dec1* and *Dec2* (which would also inhibit cyclin D1 expression) can be viewed as beneficial in terms of conserving resources since a low oxygen environment would not be conducive to cell proliferation. Environmentally, hyperglycemia represents another indirect regulator of *Dec1* and *Dec2* expression. Specifically, hyperglycemia was recently shown to promote N-glycosylation of SCAP, a SREBP-1 transport protein. Under these conditions, the N-glycosylated SCAP protein displays a reduced association with its regulatory protein INSIG-1 which facilitates SREBP1 transport to the Golgi and its subsequent activation/release from the Golgi membrane and nuclear import of the transcriptionally active domain.[48] Finally, oxysterols can also activate SREBP1c transcription by binding to the liver X receptor[49] whereas long chain polyunsaturated fatty acids suppress SREBP1c transcription by inhibiting liver X receptor binding to LXR response elements.[50] An increase in activated nuclear SREBP1 would be predicted to lead to an increase in *Dec1* and *Dec2* transcription (**Figures 3 and 4**).

DEC1 and DEC2 are Subject to Regulation at the Protein Level by Sumoylation, Ubiquitination and Phosphorylation

DEC1 is subject to sumoylation at lysine residues 159 and 279.[51] Interestingly, sumoylation had several consequences evident in cultured cells. First, sumoylation of DEC1 stabilized it's turnover since the alternate post-translational reaction of ubiquitination was inhibited.[52] Second, sumoylation facilitated its repression of circadian core clock genes *Clock* and *Bmal* by recruitment of histone deacetylase1. DEC2 is similarly sumoylated on lysine residues 240 and 255[53] which enhances recruitment of co-repressor G9a (a histone H3 lysine 9 dimethylase). Since mutation of these two SUMO acceptor sites and its degradation by SUMO protease SENP1 both limit the ability of DEC2 to block myogenesis,[18] we assume that sumoylated DEC2 (and DEC1) represent the active penultimate functional forms of these myogenic repressors.

Interestingly, SREBP1a and SREBP1c, but not SREBP2, are hyperphosphorylated within mitotic cells.[54] Hyperphosphorylated SREBP1 was stabilized in G2/M phase and this leads to an increase in its transcriptional activity. However, in general, phosphorylation of SREBP1 in its 'phosphodegron' (a portion of a protein that is important in regulating its degradation) facilitates its degradation by the ubiquitin-proteasome system where Ser-434 may function as a molecular switch.[55]

DEC1 and DEC2 are Subject to Regulation by Circadian Core Proteins

Circadian rhythms are outputs of an internal biological clock that in isolation from environmental cues maintains a highly reproducible cycle with a period of about 24 h. The period and phase of the rhythm are adjusted in nature to those of the earth's rotation by input pathways like the daily light cycle or ingestion of food. The mammalian circadian clock is a circadian transcriptional-translational oscillator driven by rhythmic activation and repression of the CLK/BMAL transcription factor (**Figure 2**).[56] The CLK/BMAL transcription factor binds to an E-box to activate transcription of both core circadian genes as well as many genes that are outputs of the circadian clock. Alternatively, other core circadian elements include *period* (*Per*) and *cryptochrome*s (C*ry*) mRNAs which encode repressor proteins that accumulate with a phase delay relative to their mRNAs because PER protein is phosphorylated by kinases, particularly casein kinases I and II, and then degraded. However, PER and CRY become stabilized when they form a complex that moves into the nucleus, where they inactivate CLK/BMAL, thereby turning off their own transcription which, for each, is activated by CLK/BMAL. This negative feedback loop produces circadian rhythms in CLK/BMAL-dependent gene expression that produce the numerous circadian outputs in muscle and other tissues.[56]

Regulation of transcription factor SREBP1 activation by cholesterol[57] is a well known environmental input to the circadian clock transcriptional circuit. Cholesterol regulates its own synthesis via a homeostatic negative feedback pathway involving SREBP1. Two Golgi-resident proteases are involved in this process, MBTPS1 and Site-2 protease, that sequentially cleave and finally release activated SREBP1 from the Golgi membrane. Once cleaved, the helix-loop-helix motif-containing SREBP1 fragment is transported to the nucleus where it activates numerous genes controlling the synthesis of cholesterol. As the level of cholesterol rises, it eventually downregulates the cleavage of SREBP1 by inducing an INSIG/SCAP interaction, and cholesterol homeostasis is maintained.[57]

The cholesterol homeostatic mechanism interacts with the circadian clock in several ways. First, SREBP1c activates expression of *Dec1*,[16] which represses circadian gene expression by binding to the same E box site that recruits the CLK/BMAL circadian activator protein.[11] As noted below, DEC1 also represses the Notch pathway, thereby providing a link

between metabolism, the clock and muscle stem cell activation (**Figure 3**). Hence, SREBP1 induces a repressor that down regulates transcription of genes controlled by the CLK/BMAL activator. In turn, DEC1 levels oscillate in a circadian manner[11] because they are induced by CLK/BMAL,[12] and DEC1 represses SREBP1 transcription.[47,58] In addition, the circadian nuclear receptor REV-ERBα, that is activated by CLK/BMAL and represses BMAL, also regulates the production of activated SREBP1 because it produces rhythmic transcription of INSIG, a factor that inhibits transport of SREBP1 to the Golgi[59] (**Figure 2**).

Metabolic input to the circadian clock also comes from several metabolic nuclear hormone receptors. The retinoic acid receptors RAR and RXR interact with CLK and its paralog MOP4 in response to binding of retinoic acid to inhibit activation of genes by CLK and MOP4. The Retinoic X Receptor (RXR) and Liver X Receptor (LXR)-RXR heterodimer both bind DEC1 and DEC2, which thereby inhibit the genes that they activate, including sterol-activated genes.[60]

An additional level of metabolic control interfaces with these transcriptional circuits, e.g., control by the insulin receptor and its related downstream kinase signaling pathways.[61] *Dec1* mRNA is induced by insulin receptor signaling via a PI3 kinase pathway, and a high carbohydrate diet induces high levels of *Dec1*.[44] The AMPK kinase interacts with the insulin-signaling pathway to stimulate catabolism. One of the AMPK regulatory subunits (PRKBAB2) shows a circadian oscillation, suggesting that AMPK kinase activity also oscillates in a circadian manner. Catalytically, AMPK phosphorylates the CLK/BMAL repressor CRY, resulting in CRY's degradation;[62] however, AMPK also activates PGC-1a, providing another regulatory interaction with the circadian transcriptional network.

Rationalization of the Role of DEC1 and DEC2 in the 'Muscle Phenotype' of Dmp1-Cre Mbtps1 cKO Mice

Having reviewed the regulatory pathways above which control expression and protein processing of DEC1, DEC2, and SREBP1, we now conclude with a rationalization of how a SREBP1 dependent myogenic pathway in skeletal muscle could be activated transcriptionally by osteocytes. Furthermore, assuming bone→muscle signaling operates systemically, our rationalization will also address how skeletal muscle could respond selectively to a SREBP1 activator while fat tissue does not. At the outset, we also need to provide an explanation for existing data which show the opposite outcome (inhibition of myogenesis) when *Dec1* or *Dec2* are over-expressed in muscle.

We envision that overexpression of *Srebp1* in muscle by an adenoviral vector, as was done by Lecomte *et al.*[16] should result in a continuous high level of expression avoiding on and off cycles, as the adenoviral promoter is not regulated by feedback from SREBP1 or DEC1 or DEC2. As a result, when SREBP1 is over-expressed, we also expect that it will induce persistent expression of *Dec1* or *Dec2* at a high level. Under these conditions, assuming commensurate efficient translation, high levels of DEC1 or DEC2 protein are expected to effectively repress their target genes which include many myogenic growth factors (MyoD1, MyoG and Mef2c), satellite cell markers (Pax7, Myhc3 and Myhc6), and muscle performance genes.[16] We propose that this is the reason that overexpression of SREBP1 in muscle via adenoviral vector

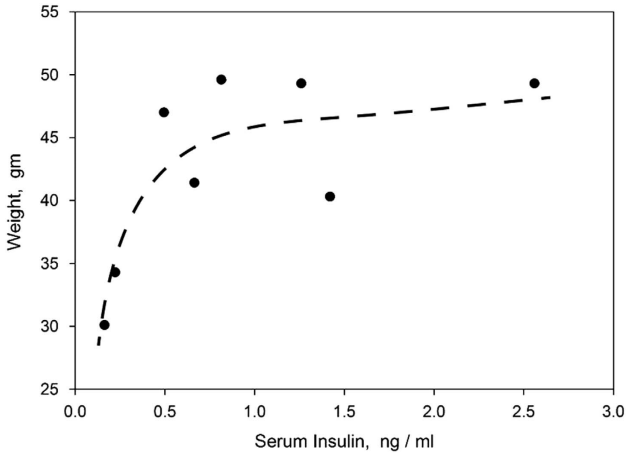

Figure 5 Elevated serum insulin levels appear to be permissive for weight (lean muscle mass) gain in 12-month-old *Dmp1-Cre Mbtps1 cKO* mice.

Table 3 *Dmp-1-Cre Mbtps1 cKO* mice have twofold higher serum leptin concentration than littermate controls

	Dmp1-cre Mbtps1 cKO	*Control littermates*
Leptin	$7176 \pm 2446\ \text{pg ml}^{-1}$ $P = 0.018$	$3575 \pm 1725\ \text{pg ml}^{-1}$

avoids feedback inhibition and leads to complete blockage of myogenesis. On the other hand, *Srebp1*, *Dec1* and *Dec2* are normally expressed on a repeating 24 h on and off circadian cycle.[60] In view of the fact that DEC1 and DEC2 are repressors of *Srebp1* and that SREBP1 is required for transcription of *Dec1*, we envision a reciprocal feedback loop wherein, in the absence of adenoviral mediated overexpression of *Srebp1*, the relative cyclic expression pattern for *Srebp1* should be offset relative to that of its *Dec1*/*Dec2* repressors by approximately 12 h.[16] Because of the complex activation pathway needed to transport SREBP1 protein to the cis-medial Golgi, we presume that *Dmp1-Cre Mbtps1 cKO* mice must necessarily express an activator of the SCAP transport system, for example, hyperglycemia, oxysterol, and/or insulin. Interestingly, recent unpublished data show that *Dmp1-Cre Mbtps1 cKO* male mice are both hyperinsulinemic, as well as hyperleptinemic (Gorski, JP, unpublished data; **Figure 5** and **Table 3**). Although explanations for how remain speculative, concurrent hyperinsulemia and hyperleptinemia conditions would provide a means to both activate *Srebp1* expression systemically while limiting its actions to regeneration of skeletal muscle rather than growth and differentiation of adipocytes and fat. We present our rationale below.

As insulin is an inducer of *Srebp1* transcription,[63–65] we believe this provides a ready explanation for how SREBP1 expression could be activated in hyperinsulemic *Dmp1-Cre Mbtps1 cKO* mice. The latter group showed that insulin action required the presence of SRE (sterol response elements) within the *Srebp1* promoter, while Cagen *et al.*[65] showed that full insulin action required the combinatorial participation of Sp-1, SREBP1, LXR, and NF-Y cis-acting elements. Our preliminary evidence suggests that hyperinsulinemia is permissive for increased skeletal muscle growth. Specifically, serum insulin

concentrations above the normal range ($\sim 0.4\,ng\,ml^{-1}$) are associated with larger body weight (muscle mass) in *Dmp1-Cre Mbtps1 cKO* mice at 10–12 months of age although body weight seems to plateau at ~ 50 g (**Figure 5**). Since full activation of the SREBP1 promoter requires participation by LXR, we speculate that bone osteocytes secrete a lipid-like activator of LXR.[46,66] Osteocytes are known to produce oxysterols which could bind to and activate the LXR receptor. Alternatively, as noted above, PPAR-γ receptor regulates several aspects of muscle regeneration following exercise. Among these ligands sphingosine-1-P and PGE_2 are known to stimulate myogenesis by blocking expression of myogenic repressor myostatin and to induce FOXO1 which then transcriptionally activates fiber type transition and mitochondrial biogenesis (**Figure 2** and **Table 1**). Importantly, sphingosine-1-P and PGE_2 are produced by osteocytes and the amount released can be increased dramatically in response to oscillatory fluid flow (cell stress).[67,68] In this way, we propose that *Dmp1-Cre Mbtps1 cKO* osteocytes are stimulated to release elevated amounts of sphingosine-1-P or PGE_2, and after making its way to the circulation, this bone-derived signaling agent would then activate PPAR-γ receptors expressed on skeletal muscle cells. As noted above, activated PPAR-γ receptors are believed to stimulate myogenesis after exercise according to well established pathways.

Because we have proposed that activation of SREBP1 is required for the muscle phenotype, we have also questioned why SREBP1, also known as ADD-1 (adipocyte development and differentiation factor one) and a master gene of lipid metabolism, does not also stimulate fat production in *Dmp1-Cre Mbtps1 cKO* mice? We hypothesize that an observed 2-fold elevated content of serum leptin (**Table 3**) blocks adipogenesis. Interestingly, when both 10- to 12-month-old controls and knockout mouse data were plotted against body weight, we observed a strong linear relationship ($r^2 = 0.89$) between leptin concentration in serum and total body weight (**Figure 6**). Noticeably, *Dmp1-Cre Mbtps1 cKO* values generally segregated separately from controls, although both seem to lie on the same 95% regression line. Leptin, generally thought to be produced primarily by adipocytes, regulates satiety while also controlling energy expenditure. However, we presume that the most likely source for leptin in *Dmp1-Cre Mbtps1 cKO* mice is skeletal muscle[69–71] since piximus analyses for body fat and direct dissection could not account for the weight gain observed[2] (**Figure 1**) and since a linear relationship exists between body mass (e.g., skeletal muscle) and serum leptin (**Figure 6**). Plasma leptin suppresses AMPK activity in the hypothalamus and restricts food intake.[72] Body weight is determined by the balance between food intake and energy expenditure. It is noteworthy that 10- to 12-month-old male *Dmp1-Cre Mbtps1 cKO* mice consumed the same amount of food as littermate controls over a two day period after acclimatization (**Table 4**) despite the former group weighing an average 3.4 gm more.[2] We interpret this finding as indicating that *Dmp1-Cre Mbtps1 cKO* mice may use energy more efficiently than controls. Consistent with this finding, whole-genome array analyses of SOL from *Dmp1-Cre Mbtps1 cKO* mice have already documented a metabolic shift towards more oxidative metabolic pathways of fat and glucose use (array data deposited at NCBI under accession number GSE69985; Gorski *et al.*[2]).

Hamrick *et al.*[73] showed that aged mice (24 months), but not 12-month-old mice, responded to injection with recombinant leptin by increasing the size and mass of muscles (including EDL), and induction in muscle of several miRNAs known to be associated with regeneration and repair. While it is tempting to presume that increased muscle in *Dmp1-Cre Mbtps1 cKO* mice is solely the result of paracrine signaling by leptin (**Figure 6**), our phenotype is not superimposable since it is evident at 12 months as well as 26 months of age (unpublished result, JP Gorski) and is restricted to SOL (not EDL) muscles.[2] Thus, while the elevated leptin content of *Dmp-Cre Mbtps1 cKO* mice likely contributes positively to the observed muscle phenotype, it does not appear to be the sole determinant.

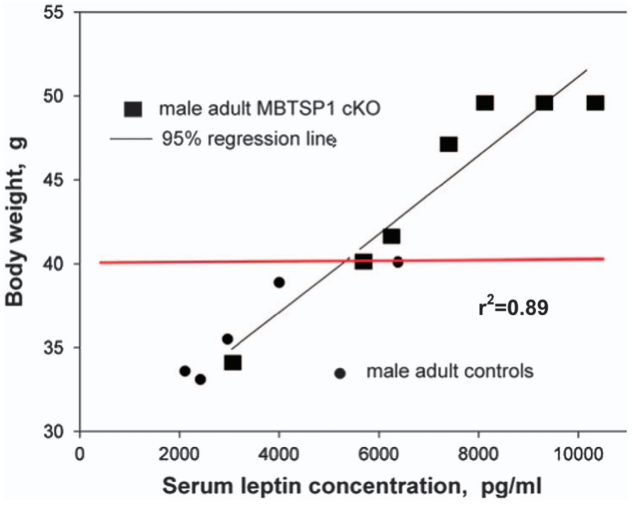

Figure 6 Serum leptin is strongly correlated with total body weight for adult male *Mbtps1 cKO* and control mice. Red line roughly separates control data points from those for *Mbtps1 cKO* values. Black line represents 95% confidence regression line. r^2 value was determined by linear regression analysis using SigmaStat program.

Summary Points

- Conditional deletion of proprotein convertase Mbtps1 in bone osteocytes leads to a dramatic change in skeletal muscle size and contractile function while causing only limited change in the structure and function of long bones. However, we believe that a significant 25% increase in stiffness[2] could be a direct

Table 4 Comparison of food intake and blood glucose values for adult knockout and control littermates

	Food consumption per 2 days	*Blood glucose*
DMP1 cre SKI-1 cKO (males) $n = 8$	9.58 g ± 1.28 STD	239.0 ± 51.5 STD
Control littermates (males) $n = 5$	9.88 g ± 1.45 STD	236.2 ± 52.6 STD
Statistics	$P = 0.697$	$P = 0.911$ (two-tailed *t*-test)

Abbreviation: STD, standard deviation.

response of knockout long bones to the increases in body weight and muscle mass.

- The muscle phenotype is not evident in 3 month old mice, but is detectable at >10 months of age.
- Soleus muscle fibers exhibited significantly more centralized nuclei which were restricted exclusively to type I myosin heavy chain (slow twitch) expressing cells. When combined with increased expression of early myogenic growth factors (MyoD1, MyoG, Mef2c), of embryonic and prenatal myosin heavy chains, of enhanced performance genes (Actinin-3), and of satellite cell biomarkers (Pax7), we conclude that *Dmp1-Cre Mbtps1 cKO* muscle exhibits morphological and transcriptional characteristics of regenerating muscle.
- Concerns about leakiness of the Dmp1-Cre in skeletal muscle were discounted since Cre recombinase expression does not account for our findings, and, Mbtps1 protein and mRNA are not deleted. Based on our findings, the most likely explanation for the muscle phenotype is age-dependent bone$\rightarrow$muscle crosstalk, for example, a bone derived factor that causes skeletal muscle myogenesis.
- Many of the myogenic genes altered in muscle are regulated by circadian core transcriptional repressors DEC1 and DEC2, and, furthermore, display a temporal coordination with *Dec1* and *Dec2* expression which implies a regulatory co-dependency. Importantly, DEC1 and DEC2 are themselves regulated at the transcriptional level by reciprocal feedback loops with SREBP1.
- In order to rationalize a mechanism responsible for the muscle phenotype, we considered both the pattern of gene expression, its shared features with that for entrained muscle, and the identity of known osteocyte-derived mediators of myogenesis. However, in contrast to entrained muscle, SOL and EDL from *Dmp1-Cre Mbtps1 cKO* do not display increased resistance to fatigue nor *cKO* EDL exhibit immunochemical evidence of a fast twitch to slow twitch transition.[2] These considerations lead us to propose that *Dmp1-Cre Mbtps1 cKO* osteocytes activate myogenesis by release an activator of muscle PPAR-γ, for example, PGE_2 or sphingosine-1-P. PPAR-γ plays a similar prominent role in mediating muscle regeneration after exercise. Alternatively, deletion of *Mbtps1* in osteocytes may lead to reduced release of an inhibitor of myogenesis.
- In addition, we believe activation of *Srebp1, Dec1*, and *Dec2* expression in skeletal muscle is also required to obtain the muscle phenotype. At present, we do not have clear explanations for the elevated serum levels of insulin and leptin in *Dmp1-Cre Mbtps1 cKO* mice but we envision that these systemic hormones are critical to establishment and maintenance of the muscle phenotype. Specifically, elevated insulin levels in Mbtps1 *cKO* mice are proposed to induce *Srebp1* expression in skeletal muscle thus leading indirectly to activation over 100 myogenic genes. We propose that elevated levels of leptin serve to block adipogenesis which would normally be a consequence of increased systemic *Srebp1* expression and to contribute positively to the myogenic and regenerative muscle phenotype in *Dmp1-Cre Mbtps1 cKO* mice.
- Taken together, we believe this rationale provides a reasonable explanation for the age-dependent *Dmp1-Cre Mbtps1 cKO* muscle phenotype. However, achieving muscle regeneration and growth with age is a complex process involving signaling pathways controlling satellite cell differentiation, energy utilization, the balance of fat and muscle, and circadian cycling. We hope that further investigation of these interacting pathways identified in the *Dmp1-Cre Mbtps1 cKO* mouse will lead to clinically translatable findings applicable to age-related sarcopenia and other muscle wasting syndromes.

Conflict of Interest

The authors declare no conflict of interest.

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