ORIGINAL PAPER

Six1 and Six1 cofactor expression is altered during early skeletal muscle overload in mice

Bradley S. Gordon · Diana C. Delgado Díaz ·
James P. White · James A. Carson ·
Matthew C. Kostek

Abstract Six1 is a transcription factor that, along with cofactors (Eya1, Eya3, and Dach2), regulates skeletal muscle fiber-type and development. SIX1 (human) gene expression decreases after overload, but the time course of Six1 expression, if protein is affected, and if the response differs between muscles with differing phenotypes, is not known. Our purpose was to examine Six1 gene and protein expression and co-factor gene expression during the initiation of muscle overload, and determine if the muscle phenotype altered this response. The plantaris and soleus were functionally overloaded by synergistic ablation of the gastrocnemius, and Six1 gene and protein, and Six1 cofactor gene expression was measured. Six1 gene expression decreased at 1 day of overload 48 ± 9 and $47 \pm 20\%$ ($p < 0.01$) in the plantaris and soleus. After 3 days of overload, Six1 protein expression increased 73 ± 17 and $168 \pm 57\%$ in the plantaris and soleus ($p < 0.05$). After 1 day of overload, Dach2 gene expression decreased 56 ± 9 and $35 \pm 3\%$ in both muscles ($p < 0.001$), while Eya1 decreased $33 \pm 5\%$ only in the soleus ($p < 0.01$). Eya3 gene expression increased $127 \pm 26\%$ ($p < 0.05$) and $76 \pm 16\%$ ($p < 0.05$) in the plantaris and soleus, while Dach2 gene expression decreased $71 \pm 4\%$ ($p < 0.05$) in the soleus after 3 days of overload. Six1 and Six1 co-factor expression is responsive to muscle overload in both fast and slow muscles. This indicates that this molecular program may affect overload adaptation regardless of muscle phenotype.

Keywords Synergistic ablation · Eya1 · Eya3 · Dach2

B. S. Gordon · D. C. Delgado Díaz · J. P. White ·
J. A. Carson · M. C. Kostek (✉)
Department of Exercise Science, Public Health Research Center,
University of South Carolina, 3rd Floor, 921 Assembly Street,
Columbia, SC 29208, USA
e-mail: mkostek@mailbox.sc.edu

B. S. Gordon
e-mail: gordonbs@mailbox.sc.edu

D. C. Delgado Díaz
e-mail: delgadoc@email.sc.edu

J. P. White
e-mail: james_white1@dfci.harvard.edu

J. A. Carson
e-mail: carsonj@mailbox.sc.edu

D. C. Delgado Díaz
Escuela de Fisioterapia, Universidad Industrial de Santander,
Bucaramanga, Colombia

Introduction

The plasticity of skeletal muscle is both morphologic and physiologic, and these adaptations occur through changes in gene and protein expression. Skeletal muscle overload leads to an increase in muscle size (hypertrophy), an increase in force production, and improvements in overall skeletal muscle function resulting in enhanced work capacity [1, 2]. These favorable adaptations are beneficial to a wide range of individuals (e.g., from elite athletes to individuals with chronic disease) [3, 4]; however, the molecular processes leading to these beneficial changes are not completely understood. A better understanding of these processes will enhance therapies designed to improve skeletal muscle function.

Characterization of a skeletal muscle phenotype can be done by examining a muscle's contractile and metabolic

properties [5, 6]. Both slow-contracting, highly oxidative fibers and fast-contracting, highly glycolytic fibers adapt favorably to overload [7]; however, there is evidence that fast and slow fibers differ in the rate and magnitude of response when subjected to overload [5, 7]. Additionally, fast and slow muscles may not respond similarly to the same overload because of differences in recruitment or loading, resulting in an altered adaptive response [5, 8]. Therefore, knowledge of the fiber type-specific response to muscle overload is important in designing more effective training or rehabilitation programs.

Whole genome expression profiling can be used to identify candidate genes that control the adaptation of skeletal muscle to stress [9]. Recently, our laboratory utilized this technique in humans at multiple early time points following acute muscle overload to identify novel transcriptional and regulatory factors [10]. Out of thousands of gene probes on the profiling chip, only two were identified as having altered expression at more than one time point. Of the two genes identified, SIX1 gene expression was decreased 3 and 6 h following overload, which was further verified by qRT-PCR. Some limitations of this study are that there was no quantification of Six1 protein or Six1 cofactors, only one heterogeneous (fiber type) muscle biopsy was examined, and no measures were made beyond 24 h. Thus, while intriguing, few conclusions could be made regarding the role of Six1 or cofactors in the muscle overload response.

Six1 is an embryonic homeobox developmental gene homologous to *sine oculus* (without eyes) first identified in *Drosophila*, and is necessary for proper embryonic development of many tissues and organs, including skeletal muscle [11–15]. Indeed, homozygous deletion of Six1 is embryonic lethal and results in a severe lack of muscle formation [16]. In addition to whole body muscle formation, Six1 is also necessary for the specific development of fast twitch muscles during embryogenesis [17, 18]. At the molecular level, Six1 acts upstream of the myogenic regulatory factors (MRF's) during development, controlling their expression through the MEF3 sequence of the MRF's promoter [11, 19–23]. In vitro, overexpression of Six1 in skeletal muscle satellite cells enhances myoblast fusion and increases the number of nuclei per myotube [24]. Conversely, reducing the expression of Six1 during differentiation of C2C12 myoblasts significantly reduces the formation of myotubes [25]. This myoblast fusion is a similar process to that which occurs during muscle hypertrophy in adult skeletal muscle after overload [26, 27]. Though these cell culture and embryonic developmental studies clearly show the necessity and importance of Six1 in the formation of skeletal muscle, little is known of Six1's function in healthy adults where Six1 is expressed exclusively in skeletal muscle [19].

The study of Six1 is further complicated as it can repress and activate gene expression depending on its interaction with cofactors. This has been demonstrated in the *Xenopus* embryo where Six1 over expression led to both enhancement and repression of the expression of various genes [12]. In mice, a combination of Six1 and Dach2 represses the myogenin promoter, while in vitro, a combination of Six1 and Eya3 activates the myogenin promoter [23, 28, 29]. In a separate study, a combination of Six1 and Eya1 elicited a slow to fast fiber-type shift in the predominantly slow soleus muscle of mice, though Six1 alone was not a sufficient stimulus [22]. These findings further demonstrate that the transcriptional activity of Six1 is dependent on its cofactors [22].

Six1 has the potential to regulate many cellular and molecular processes that lead to favorable physiological outcomes, it is expressed in adult human muscle, and its expression is altered in response to overload. However, the one report of Six1 after muscle overload was in a heterogeneous human muscle biopsy where protein was not measured. Because of the limited knowledge of Six1 and its cofactors in adult skeletal muscle, and their potential impact on so many physiologic variables, we chose to examine whether the expression of Six1 and its cofactors is altered in both slow oxidative and fast glycolytic muscles during the initiation of muscle overload in an animal model. We hypothesized that Six1 gene expression, Six1 protein expression, and co-factor expression would be altered at 1 and 3 days of overload. These results will lead to a better understanding of Six1 and its cofactors during muscle overload, which will inform future mechanistic studies related to improvements in muscle growth and function.

Materials and methods

Animal care and functional overload

The University of South Carolina IACUC approved all protocols used in this study. Mice were housed and cared for in the animal facility at The University of South Carolina, kept on a 12:12 h light-dark cycle, and given ad libitum access to water and Purina chow. Functional overload of the plantaris and soleus muscles was produced by the bilateral surgical ablation of the gastrocnemius muscle as previously described [30]. This model of overload was used as it allows simultaneous investigation of changes in both slow oxidative and fast glycolytic muscles following overload [31]. Bilateral synergistic ablation was used instead of unilateral synergistic ablation to ensure maximal hypertrophy of the ablated limbs (as unilateral ablation could allow preferential use of the sham limb,

reducing the hypertrophy of the ablated limb). It also ensured adequate tissue would be available for both gene and protein expression measurements from the same animal. This model also exhibits similar cellular, molecular, and physiological adaptations that occur in humans following overload, including hypertrophy and a fast-to-slow fiber type shift [4, 27, 30, 32–34]. Briefly, C57BL/6 male mice ($n = 6$ mice per experimental and control group/time point analyzed) were anesthetized with a subcutaneous injection of a cocktail containing ketamine/xylazine/acepromazine (1.4 ml/kg bodyweight). Under sterile conditions, the gastrocnemius muscles were exposed through a posterior longitudinal incision through the skin of the lower hind limb. After the soleus and plantaris muscles were separated from the gastrocnemius muscle at the Achilles tendon, the distal two-thirds of the heads for each gastrocnemius muscle were excised. Incisions were closed using sutures. A sham operation consisted of the same procedure as outlined above, except for the gastrocnemius excision. The sham operation group served as a control. Mice from both groups were housed in individual cages until killed at 1, 3, and 21 days post-functional overload or sham surgery. On the day of tissue collection, mice were anesthetized as described above. Muscles were harvested, weighed, immediately snap frozen in liquid nitrogen, and stored at −80 °C until they were processed.

Total RNA extraction and cDNA synthesis

RNA was extracted using the TRIzol method (Invitrogen, Carlsbad, CA) as previously described [35]. RNA quantity and purity were assessed spectrophotometrically (Thermo Fisher Scientific, Wilmington, DE). RNA integrity was examined by visualization of the 28s and 18s ribosomal subunits after electrophoresis in a 1.1 % agarose gel; 2 µg of total RNA was added to DEPC H_2O totaling 11 µl. Then 1 µl of Oligo dT (Invitrogen) was added to all samples and incubated at 70 °C for 10 min followed by incubation on wet ice for 2 min. Reverse transcription of RNA into cDNA was carried out using 4 µl buffer, 2 µl DTT, 1 µ deoxyNTPs, and 1 µl Superscript III Reverse Transcriptase (Invitrogen) per sample. Reverse transcription was carried out for 1 h at 50 °C followed by 5 min at 70 °C to stop the reaction.

Real-time PCR

Gene expression for Six1, Eya1, Eya3, Dach2, and myogenin was determined by quantitative Real-Time Polymerase Chain Reaction using SyberGreen® Master Mix (Applied Biosystems, Foster City, CA). Amplification was carried out with 1 µl cDNA (20 ng), 12.5 µl SyberGreen® Master Mix, 1 µl Forward Primer (200 nM), 1 µl Reverse Primer (200 nM), and 9.5 µl RNase-free H2O in a final volume of 25 µl/well. Forward and Reverse custom designed primer sequences for all genes analyzed are outlined in Table 1.

RT-PCR was performed in triplicate on an Applied Biosystems 7300 thermocycler. Glyceraldehyde 3-phosphate dehydrogenase (Gapdh) was used as an internal control. Gapdh was chosen as it has been previously used as an internal control with this model of muscle overload, and it is a reliable internal control for the possible increase in total mRNA during hypertrophy [36]. A dissociation curve was performed to verify specific amplification, and the PCR products were also run on a 2 % agarose gel to verify specific amplification and proper amplicon size. Relative gene expression fold change was calculated using the delta delta Ct method. The delta Ct was determined for each sample by the equation ($Ct_{experimental\ gene} - Ct_{internal\ control}$). The delta delta Ct (ddCt) was calculated by the equation (delta $Ct_{ablation\ sample} - $ mean delta $Ct_{sham\ operation\ group}$). Fold change relative to the sham group was calculated by the equation (fold change = 2^{-ddCt}). Six1, Eya1, Eya3, and Dach2 gene expression of the ablation group is reported as fold change relative to the sham group.

SDS PAGE and Western blot

Whole muscle protein was extracted by manual tissue disruption in RIPA buffer consisting of 50 mM Tris-HCl, pH 8.0, with 150 mM sodium chloride, 1.0 % Igepal CA-630 (NP-40), 0.5 % sodium deoxycholate, and 0.1 % sodium dodecyl sulfate and protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Protein concentration was determined immediately prior to beginning Western blotting by the Bradford method [37] using BSA as a protein standard. Standards and samples were run in triplicate with all samples and standards showing a coefficient of variation <5 %. Crude muscle homogenate (100 µg) was fractionated on a BIO-RAD 12.5 % Criterion™ Precast Gel (Hercules, CA). Six1 peptide-positive control was also run with every gel as a molecular weight marker verifying the localization of Six1 on each blot. Protein was transferred to PVDF membranes with equal protein-loading qualitatively assessed using Ponceau staining (or Gapdh when possible). Membranes were then blocked in 5 % milk in phosphate-buffered saline with 0.1 % Tween-20 (PBS-T). Primary antibody for Six1 (ABNOVA, Taiwan) was incubated at a 1:500 dilution overnight at 4 °C in 5 % milk in PBS-T. Secondary anti-mouse Ig horseradish peroxidase-linked antibody was incubated in 5 % milk in PBS-T for 1 h at room temperature with the membranes at a 1:1,000 or 1:2,000 dilutions for the plantaris and soleus, respectively. Enhanced chemiluminescence (GE Healthcare Life Sciences, Piscataway, NJ) was used to visualize the antibody–antigen

Table 1 Primer sequences used to amplify Six1, Eya1, Eya3, Dach2, Myogenin and Gapdh

Gene	Forward	Reverse
Six1	5′-CGA GGC CAA GGA AAG GGA G-3′	5′-ACT CCT CTT CTG AGC TGG ACA TG-3′
Eya1	5′-GTA CAG CTA CCA GAT GCG TAG CAG-3′	5′-ATG TAG TGT GCT GGA TAC GGC GAG-3′
Eya3	5′-AAT GTG GGT GGC CTC CTC AG-3′	5′-AAC ATT CGC ACA GTT CTT TCG AG-3′
Dach2	5′-ACT GAA AGT GGC TTT GGA TAA-3′	5′-TTC AGA CGC TTT TGC ATT GTA-3′
Myogenin	5′-GAG CAT TGT CCA GGC CAG GTC-3′	5′-GAG TTG CAT TCA CTG GGC ACC ATG-3′
Gapdh	5′-GGA GCC AAA CGG GTC ATC AT-3′	5′-TCA CGC CAC ATC TTT CCA GA-3′

interactions and developed by autoradiography. Digitally scanned blots were analyzed by measuring the optical density of each band using digital imaging software (Image J Software, Bethesda, MD). Six1 protein for both the plantaris and soleus are expressed as pixel density fold change relative to the sham group.

Statistical analysis

Muscle weights at each time point analyzed are expressed as mean $\pm$ SE. Gene expression and Western blot results are reported as mean $\pm$ SE. Gene expression fold changes for each gene at each time point were analyzed using independent Student's t tests. Western blot pixel densities between the sham and overload groups at each time point were analyzed using independent Student's t test. Bonferroni corrections were performed for each gene analyzed to account for multiple comparisons error. Microsoft Excel software and Statistical Analysis Software (SAS) were used to calculate the Student's t tests. Statistical significance was set at $p < 0.05$ for all analysis for protein. Bonferroni adjusted p values were 0.025 for all gene expression analysis.

Results

Muscle weights

Table 2 illustrates the wet weights of the plantaris and soleus muscles 1 and 3 days post-ablation surgery. The soleus increased its muscle wet weight 31.4 ± 9 % ($p < 0.05$) after 1 day of overload. Both the soleus and plantaris increased in wet weight 40.9 ± 7 and 6.6 ± 2 %, respectively ($p < 0.05$), after 3 days of overload. Since the initial gain in muscle weight early in this model is due to inflammation and edema [31], we measured the muscle weight of the plantaris 21 days post-ablation surgery to verify hypertrophy, which is one adaptation that is known to occur in this overload model [36]. The plantaris increased its wet weight 43.2 ± 6 % ($p < 0.05$) after 21 days of overload, verifying our model as hypertrophy results from chronic overload in this model [30].

Six1 gene expression

We investigated the Six1 gene expression in the plantaris and soleus at 1 and 3 days post-ablation surgery. Figure 1 demonstrates the Six1 gene expression in the plantaris and soleus after 1 and 3 days of overload. Six1 gene expression decreased 48 ± 9 % ($p < 0.01$) in the plantaris after 1 day of overload compared to sham. There was no difference in plantaris Six1 gene expression 3 days post-ablation surgery ($p = 0.1$).

Six1 gene expression in the soleus decreased 47 ± 20 % ($p < 0.01$) after 1 day of overload compared to sham. There was no difference in Six1 gene expression in the soleus 3 days post-ablation surgery.

Six1 protein expression

To further characterize the expression of Six1 during muscle hypertrophy, we examined the Six1 protein expression in the plantaris and soleus 1 and 3 days post-ablation surgery (Fig. 2). There was no change in Six1 protein expression in the plantaris 1 day post-ablation surgery (Fig. 2a), whereas the Six1 protein expression in the plantaris increased 73 ± 17 % ($p < 0.05$) in the overload group 3 days post-ablation surgery compared to sham (Fig. 2b). There was no change in Six1 protein expression in the soleus 1 day post-ablation surgery (Fig. 2c). However, Six1 protein expression in the soleus increased 168 ± 57 % ($p < 0.05$) in the overload group 3 days post-ablation surgery compared to sham (Fig. 2d). The Six1 positive control in Fig. 2c became visible only after additional exposure time of the remainder of the blot. Therefore, we cut and pasted the positive control band in its correct location from the exposed blot and placed it in the current figure for illustration purposes.

Myogenin, Eya1, Eya3, and Dach2 gene expression

To verify our model of overload and to contextualize our Six1 expression results, we measured myogenin gene expression after 3 days of overload (Fig. 3). Myogenin gene expression increased $1,140 \pm 200$ % in the plantaris and 260 ± 38 % in the soleus after 3 days of overload relative to

Table 2 Soleus and plantaris muscle wet weights

Muscle (days post-surgery)	n	Absolute weight (mg)		% Increase
		Sham	Overload	
Soleus (1)	6	6.9 ± 0.5	$9.1 \pm 0.6^*$	31.4 ± 9
Plantaris (1)	6	13.6 ± 0.7	13.3 ± 0.9	No change
Soleus (3)	6	7.8 ± 0.3	$10.9 \pm 0.5^*$	40.9 ± 7
Plantaris (3)	6	16.4 ± 0.4	$17.5 \pm 0.3^*$	6.6 ± 2
Plantaris (21)	4–6	20.3 ± 0.7	$29.1 \pm 1.2^*$	43.2 ± 6

Values are mean $\pm$ SE

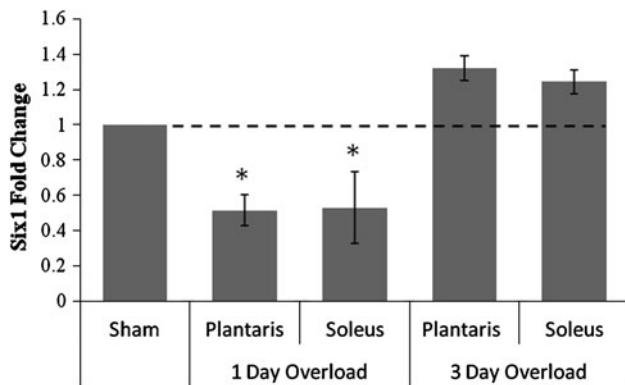* Significance at $p < 0.05$ compared to sham operation at the same time point

Fig. 1 Six1 gene expression of the plantaris and soleus after 1 and 3 days of overload. Six1 gene expression in the plantaris decreased 48 ± 9 % after 1 day of overload relative to sham. There was no difference between sham and overload at 3 days. Six1 gene expression in the soleus significantly decreased 47 ± 20 % after 1 day of overload relative to sham. *Asterisk* indicates significance at $p < 0.01$

sham ($p < 0.025$). To further investigate the events related to Six1 during the early stages of muscle overload, we measured Eya1, Eya3, and Dach2 gene expression in both the plantaris and soleus after 1 and 3 days of overload (Fig. 4). Figure 4a illustrates the gene expression results after 1 day of overload in both the plantaris and soleus. Eya1 gene expression in the soleus decreased 33 ± 5 % ($p < 0.01$) after 1 day of overload compared to sham, while there was no change in Eya1 gene expression in the plantaris at this time point. Eya3 gene expression did not change in either the plantaris or the soleus after 1 day of overload. Dach2 gene expression decreased 56 ± 9 and 35 ± 3 % in both the plantaris and soleus, respectively ($p < 0.001$) after 1 day of overload relative to sham. Figure 4b illustrates the Eya1, Eya3, and Dach2 gene expression results after 3 days of overload in both the plantaris and soleus. There was no difference in Eya1 gene expression in the plantaris or soleus after 3 days of overload. Eya3 gene expression increased 127 ± 26 % ($p < 0.025$) in the plantaris and 76 ± 16 % ($p < 0.025$) in the soleus after 3 days of overload relative to sham. Dach2 gene expression decreased 71 ± 4 %

($p < 0.025$) in the soleus after 3 days of overload relative to sham, while there was no difference in Dach2 gene expression in the plantaris after 3 days of overload.

Discussion

The primary aims of this project were to examine if Six1 gene expression, Six1 protein expression, and Six1 cofactor gene expression were altered at the onset of overload in both fast and slow mouse hindlimb muscles. This study reports the novel finding of a decrease in Six1 gene expression after 1 day of overload in both slow and fast muscles, while Six1 protein expression increases in both fast and slow muscles after 3 days of overload. After 1 day of overload, Dach2 gene expression decreases in both the fast and slow muscles, while Eya1 gene expression decreases in slow muscle only after 1 day of overload. Additionally, after 3 days of overload, Eya3 gene expression was increased in both fast and slow muscles, while Dach2 gene expression was decreased only in the slow muscle. This study is the first to expand on our previous findings that SIX1 gene expression decreases following acute overload in humans [10], hereby characterizing the Six1 and Six1 cofactor expression changes in both fast and slow muscles during overload.

The decrease in Six1 mRNA from our original work in humans [10] is perplexing considering the important role of Six1 during muscle development, and here we noted this same decrease in Six1 mRNA in both the soleus and plantaris. Yet, Six1 protein expression did not decrease after 1 day of overload, which is not surprising considering its known importance. A change in Six1 protein (translation), however, is likely to lag behind the change in the gene transcript suggesting that a reduction in Six1 protein occurs at a later time point. Contrary to this supposition, we report an increase in Six1 protein after 3 days of overload with no difference in Six1 mRNA between the overload and sham groups in either muscle analyzed.
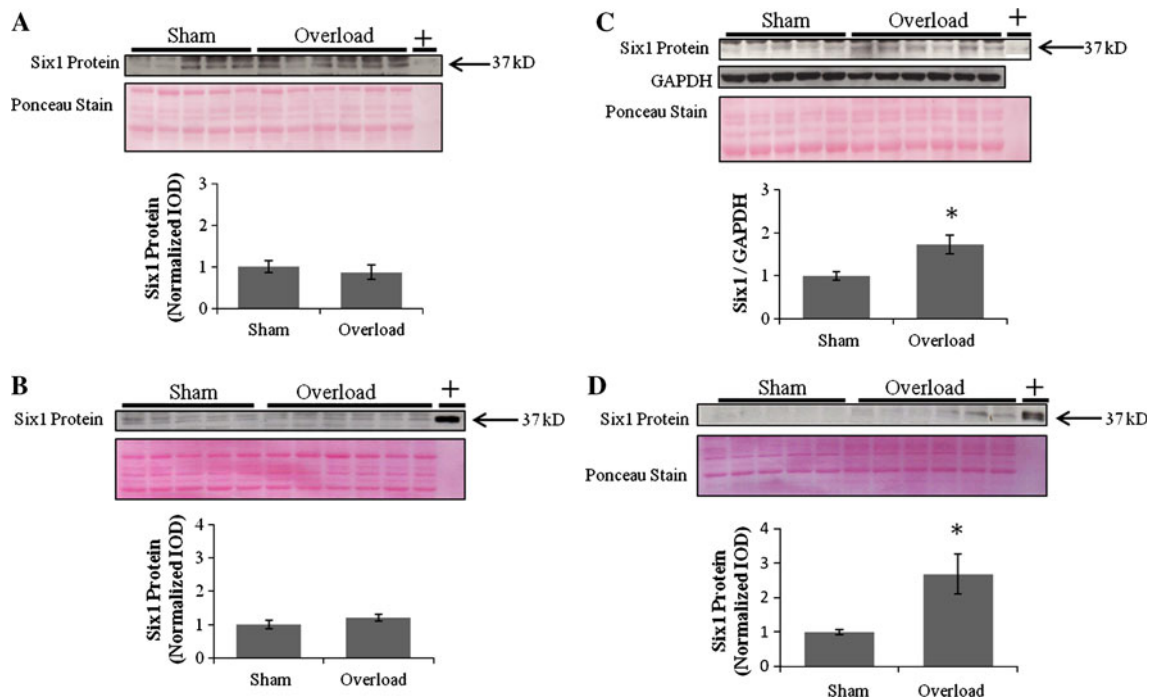
Fig. 2 Six1 protein expression in the plantaris and soleus after 1 and 3 days of overload. There was no difference in Six1 protein expression after 1 day of overload in the plantaris or soleus relative to sham (**a**, **b**, respectively). Six1 protein increased 73 ± 17 % in the plantaris (**c**), and Six1 protein increased 168 ± 57 % in the soleus (**d**) after 3 days of overload relative to sham. *Plus symbol* indicates the Six1 peptide (positive control). *Asterisk* indicates significance at $p < 0.05$
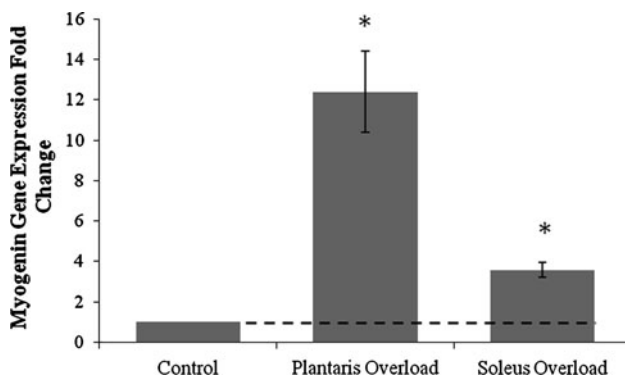

Fig. 3 Myogenin gene expression in the plantaris and soleus after 3 days of overload. Myogenin gene expression increased 1,139 ± 200 % in the plantaris and 258 ± 38 % in the soleus after 3 days of overload relative to sham. *Asterisk* indicates $p < 0.05$

Despite the differential regulation of Six1 mRNA and protein expression, increased Six1 protein expression during this process is consistent with Six1's necessary role in muscle development and the expression of MRF's, such as myogenin [16, 19]. Therefore, this increase of Six1 protein may be necessary for the subsequent myogenin expression that occurred in our study as well as previously reported in this model of overload [30]. This is also consistent with a previous report regarding Six1's role in the fusion of muscle satellite cells during muscle formation as this model of muscle overload results in significant

hypertrophy, partially because of the activation and fusion of myogenic precursor cells [24, 33].

Similar to Six1 gene/protein expression, the Six1 cofactor expression pattern was similar between slow and fast muscle. The gene expression responses of Dach2 after 1 day of overload and Eya3 after 3 days of overload were similar in both muscles, and in concordance with the theoretical induction of myogenin mRNA that occurs with overload, in that a decrease in Dach2 expression would be expected as a previous report showed co-expression of Dach2 with Six1 significantly represses the myogenin promoter [28]. Conversely, while overexpression of Six1 alone represses the myogenin promoter, coexpression of Six1 with Eya3 greatly enhances the activity of the myogenin promoter [23]. To this extent, we observed significant increases of Eya3 gene expression in both the plantaris and soleus after 3 days of overload, corresponding with increased myogenin gene expression. Despite these similar responses, Eya1 gene expression decreased only in the soleus after 1 day of overload, and Dach2 gene expression was only decreased in the soleus after 3 days of overload. Though the majority of the responses occurred in both the slow and fast muscle, indicating the possibility that this gene program performs similar functions in both types of muscles, some differential responses were observed. Other reports also show differential responses of genes between these two muscles during synergistic
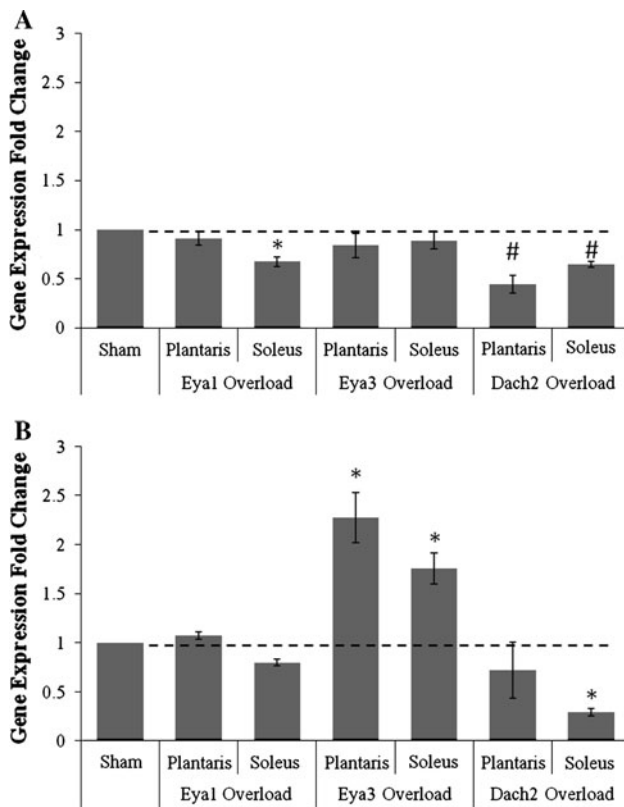
Fig. 4 Eya1, Eya3, and Dach2 gene expression in the plantaris and the soleus after 1 and 3 days of overload. **a** One day of overload: there was no difference in Eya1 gene expression in the plantaris after 1 day of overload. Eya1 gene expression in the soleus decreased 33 ± 5 % (*asterisk*) after 1 day of overload. There was no difference in Eya3 gene expression in the plantaris or the soleus after 1 day of overload. Dach2 gene decreased 56 ± 9 % (*asterisk*) and 35 ± 3 % (*asterisk*) in the plantaris and soleus, respectively, after 1 day of overload. **b** Three days of overload: there was no difference in Eya1 gene expression in the plantaris or soleus after 3 days of overload. Eya3 gene expression increased 127 ± 26 % in the plantaris and 76 ± 16 % in the soleus after 3 days of overload relative to sham. Dach2 gene expression decreased 71 ± 4 % in the soleus after 3 days of overload relative to sham. *Asterisk* indicates significance at $p < 0.025$. *Hash symbol* indicates significance at $p < 0.001$

overload with a possible explanation for this occurrence being the different recruitment or loading pattern of these two muscles as it is known that slow twitch muscles are recruited more often than fast twitch muscles, possibly altering the rate and magnitude of cellular processes [5, 8]. Yet, this differential response in gene expression could also be due to inherent molecular differences (e.g., epigenetics) between the muscles.

While our study further described the expression of Six1 and its cofactors during the overload process in fast and slow muscles, we note limitations. The first is that we did not test a potential mechanism surrounding Six1 and its cofactors. Yet, this comparative study was necessary to gain further insight into the expression of Six1 and its cofactors to inform future mechanistic investigations. A second limitation is that we do

not measure the protein levels of the Six1 co-factors. As the first report to examine these cofactors during the overload process we chose to focus on gene expression, though future studies should now examine protein levels and protein interaction with Six1. Thirdly, although our model elicits physiological changes similar to that in humans with muscle overload, the adaptations that occur in this model are due to chronic overload of the muscle. This may alter our interpretation as the physiological changes in humans with overload are not due to chronic stimulation. Additionally, we note an anatomical difference between the muscles; the plantaris crosses the knee joint, whereas the soleus does not, which will affect both loading and activation patterns. Though not a limitation, analyzing the gene and protein content minutes (instead of days) after overload, as well as at other time points, may uncover more details regarding Six1 and its cofactors. Lastly, there was difficulty finding a suitable Six1 antibody for Western blotting. Therefore, we used Six1 peptide as a positive control for all Western blots to verify our results.

This study represents a significant and necessary step toward identifying a role for the Six1 molecular pathway in the regulation of adult skeletal muscle plasticity by demonstrating that changes of Six1 and its cofactors occur in both fast and slow muscles during muscle overload. The previous findings regarding Six1 and its cofactors ability to enhance muscle fiber formation and modify fiber type make these genes a novel target for therapies aimed at a wide range of individuals such as an elite athlete rehabilitating an injury and an elderly individual trying to maintain muscle size and function to perform daily tasks. Additionally, Six1 itself is an intriguing ubiquitous homeobox gene that is both necessary for drosophila compound eye formation and critical for rib, inner ear, and kidney development in mice; while in humans, SIX1 is a potential cause of congenital deafness, a form of kidney disease, and some late-life cancers [38, 39]. Furthermore, Six1 has been suggested to belong to a core set of genes that supports all characteristics of being an animal [40]. Indeed, the primordial representative of Six1 is present in the sponge, perhaps the simplest and oldest (from an evolutionary perspective) animal life form on earth [40, 41]. The fact that this evolutionarily conserved developmental gene remains expressed after development almost exclusively in skeletal muscle, and that Six1 and its cofactors change expression during a morphologic and physiologic adaptation in adult mammals, adds to the curiosity of their role in skeletal muscle. Yet, further descriptive studies are not likely to be extremely informative; thus, future studies should investigate the role of Six1 and its cofactors during the hypertrophy process by manipulating their expression. These experiments are currently underway in our laboratory.

In conclusion, this study demonstrates that Six1 and its cofactors alter their expression in both fast and slow muscles after overload. These results suggest that the Six1 gene/protein network may have a role in the molecular and cellular processes that occur with muscle overload regardless of muscle phenotype. Understanding the role of this gene network during physiologic adaptation in muscle aids our understanding of the adaptation process, and further understanding of this network is likely to aid rehabilitation interventions in those suffering from a muscle loss due to disease, disuse, injury, and aging.

References

1. Brown AB, McCartney N, Sale DG (1990) Positive adaptations to weight-training in the elderly. J Appl Physiol 69:1725–1733
2. Hather BM, Tesch PA, Buchanan P, Dudley GA (1991) Influence of eccentric actions on skeletal-muscle adaptations to resistance training. Acta Physiol Scand 143:177–185
3. Fletcher IM, Hartwell M (2004) Effect of an 8-week combined weights and plyometrics training program on golf drive performance. J Strength Cond Res 18:59–62
4. Geisler SBC, Schiffer T, Kreutz T, Bloch W, Brixius K (2011) The influence of resistance training on patients with metabolic syndrome-significance of changes in muscle fiber size and muscle fiber distribution. J Strength Cond Res 25:2598–2604
5. Carlson CJ, Fan ZQ, Gordon SE, Booth FW (2001) Time course of the MAPK and PI3-kinase response within 24 h of skeletal muscle overload. J Appl Physiol 91:2079–2087
6. Williamson DL, Gallagher PM, Carroll CC, Raue U, Trappe SW (2001) Reduction in hybrid single muscle fiber proportions with resistance training in humans. J Appl Physiol 91:1955–1961
7. Morgan MJ, Loughna PT (1989) Work overload induced changes in fast and slow skeletal-muscle myosin heavy-chain gene-expression. FEBS Lett 255:427–430
8. Hennig R, Lomo T (1985) Firing patterns of motor units in normal rats. Nature 314:164–166
9. Allen DL, Bandstra ER, Harrison BC, Thorng S, Stodieck LS, Kostenuik PJ, Morony S, Lacey DL, Hammond TG, Leinwand LL, Argraves WS, Bateman TA, Barth JL (2009) Effects of spaceflight on murine skeletal muscle gene expression. J Appl Physiol 106:582–595
10. Kostek MC, Chen YW, Cuthbertson DJ, Shi R, Fedele MJ, Esser KA, Rennie MJ (2007) Gene expression responses over 24 h to lengthening and shortening contractions in human muscle: major changes in CSRP3, MUSTN1, SIX1, and FBXO32. Physiol Genomics 31:42–52
11. Giordani J, Bajard L, Demignon J, Daubas P, Buckingham M, Maire P (2007) Six proteins regulate the activation of Myf5 expression in embryonic mouse limbs. Proc Natl Acad Sci USA 104:11310–11315
12. Brugmann SA, Pandur PD, Kenyon KL, Pignoni F, Moody SA (2004) Six1 promotes a placodal fate within the lateral neurogenic ectoderm by functioning as both a transcriptional activator and repressor. Development 131:5871–5881
13. Fougerousse F, Durand M, Lopez S, Suel L, Demignon J, Thornton C, Ozaki H, Kawakami K, Barbet P, Beckmann JS, Maire P (2002) Six and Eya expression during human somitogenesis and MyoD gene family activation. J Muscle Res Cell Motil 23:255–264
14. Brodbeck S, Englert C (2004) Genetic determination of nephrogenesis: the Pax/Eya/Six gene network. Pediatr Nephrol 19:249–255
15. Stierwald M, Yanze N, Bamert RP, Kammermeier L, Schmid V (2004) The Sine oculis/Six class family of homeobox genes in jellyfish with and without eyes: development and eye regeneration. Dev Biol 274:70–81
16. Laclef C, Hamard G, Demignon J, Souil E, Houbron C, Maire P (2003) Altered myogenesis in Six1-deficient mice. Development 130:2239–2252
17. Niro C, Demignon J, Vincent S, Liu YB, Giordani J, Sgarioto N, Favier M, Guillet-Deniau I, Blais A, Maire P (2010) Six1 and Six4 gene expression is necessary to activate the fast-type muscle gene program in the mouse primary myotome. Dev Biol 338:168–182
18. Bessarab DA, Chong SW, Srinivas BP, Korzh V (2008) Six1a is required for the onset of fast muscle differentiation in zebrafish. Dev Biol 323:216–228
19. Spitz F, Demignon J, Porteu A, Kahn A, Concordet JP, Daegelen D, Maire P (1998) Expression of myogenin during embryogenesis is controlled by Six/sine oculis homeoproteins through a conserved MEF3 binding site. Proc Natl Acad Sci USA 95:14220–14225
20. Ikeda K, Watanabe Y, Ohto H, Kawakami K (2002) Molecular interaction and synergistic activation of a promoter by Six, Eya, and Dach proteins mediated through CREB binding protein. Mol Cell Biol 22:6759–6766
21. Zhang H, Stavnezer E (2009) Ski regulates muscle terminal differentiation by transcriptional activation of Myog in a complex with Six1 and Eya3. J Biol Chem 284:2867–2879
22. Grifone R, Laclef C, Spitz F, Lopez S, Demignon J, Guidotti JE, Kawakami K, Xu PX, Kelly R, Petrof BJ, Daegelen D, Concordet JP, Maire P (2004) Six1 and Eya1 expression can reprogram adult muscle from the slow-twitch phenotype into the fast-twitch phenotype. Mol Cell Biol 24:6253–6267
23. Li X, Oghi KA, Zhang J, Krones A, Bush KT, Glass CK, Nigam SK, Aggarwal AK, Maas R, Rose DW, Rosenfeld MG (2003) Eya protein phosphatase activity regulates Six1-Dach-Eya transcriptional effects in mammalian organogenesis. Nature 426:247–254
24. Yajima H, Motohashi N, Ono Y, Sato S, Ikeda K, Masuda S, Yada E, Kanesaki H, Miyagoe-Suzuki Y, Takeda S, Kawakami K (2010) Six family genes control the proliferation and differentiation of muscle satellite cells. Exp Cell Res 316:2932–2944
25. Liu YCA, Chakroun I, Islam U, Blais A (2010) Cooperation between myogenic regulatory factors and SIX family transcription factors is important for myoblast differentiation. Nucleic Acids Res 38:6857–6871
26. Phelan JN, Gonyea WJ (1997) Effect of radiation on satellite cell activity and protein expression in overloaded mammalian skeletal muscle. Anat Rec 247:179–188
27. McCarthy JJ, Mula J, Miyazaki M, Erfani R, Garrison K, Farooqui AB, Srikuea R, Lawson BA, Grimes B, Keller C, Van Zant G, Campbell KS, Esser KA, Dupont-Versteegden EE, Peterson CA (2011) Effective fiber hypertrophy in satellite cell-depleted skeletal muscle. Development 138:3657–3666
28. Tang HB, Goldman D (2006) Activity-dependent gene regulation in skeletal muscle is mediated by a histone deacetylase (HDAC)-Dach2-myogenin signal transduction cascade. Proc Natl Acad Sci USA 103:16977–16982

29. Tang HB, Macpherson P, Marvin M, Meadows E, Klein WH, Yang XJ, Goldman D (2009) A histone deacetylase 4/myogenin positive feedback loop coordinates denervation-dependent gene induction and suppression. Mol Biol Cell 20:1120–1131

30. White JP, Reecy JM, Washington TA, Sato S, Le ME, Davis JM, Wilson LB, Carson JA (2009) Overload-induced skeletal muscle extracellular matrix remodelling and myofibre growth in mice lacking IL-6. Acta Physiol 197:321–332

31. Armstrong RB, Marum P, Tullson P, Saubert CW (1979) Acute hypertrophic response of skeletal muscle to removal of synergists. J Appl Physiol 46:835–842

32. Timson BF, Bowlin BK, Dudenhoeffer GA, George JB (1985) Fiber number, area, and composition of mouse soleus muscle following enlargement. J Appl Physiol 58:619–624

33. Adams GR, Haddad F, Baldwin KM (1999) Time course of changes in markers of myogenesis in overloaded rat skeletal muscles. J Appl Physiol 87:1705–1712

34. Darr KC, Schultz E (1987) Exercise induced satellite cell activation in growing and mature skeletal muscle. J Appl Physiol 63:1816–1821

35. Delgado-Diaz DGB, Dompier T, Burgess S, Dumke C, Mazoue C, Caldwell T, Kostek MC (2011) Therapeutic ultrasound affects IGF-1 splice variant expression in human skeletal muscle. Am J Sports Med 39(10):2233–2241

36. Carson JA, Nettleton D, Reecy JM (2001) Differential gene expression in the rat soleus muscle during early work overload-induced hypertrophy. Faseb J 15:207–209

37. Bradford MM (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal Biochem 72:248–254

38. Ruf RG, Xu PX, Silvius D, Otto EA, Beekmann F, Muerb UT, Kumar S, Neuhaus TJ, Kemper MJ, Raymond RM, Brophy PD, Berkman J, Gattas M, Hyland V, Ruf EM, Schwartz C, Chang EH, Smith RJH, Stratakis CA, Weil D, Petit C, Hildebrandt F (2004) SIX1 mutations cause branchio-oto-renal syndrome by disruption of EYA1-SIX1-DNA complexes. Proc Natl Acad Sci USA 101:8090–8095

39. Coletta RDCK, Micalizzi DS, Jedlicka P, Varella-Garcia M, Ford HL (2008) Six1 overexpression in mammary cells induces genomic instability and is sufficient for malignant transformation. Cancer Res 68:2204–2213

40. Kumar JP (2009) The sine oculis homeobox (SIX) family of transcription factors as regulators of development and disease. Cell Mol Life Sci 66:565–583

41. Hill A, Boll W, Ries C, Warner L, Osswalt M, Hill M, Noll M (2010) Origin of Pax and Six gene families in sponges: single PaxB and Six1/2 orthologs in *Chalinula loosanoffi*. Dev Biol 343:106–123