TITLE: Nucleus Type-Specific DNA Methyonomics Reveals Epigenetic “Memory” of Prior Adaptation in Skeletal Muscle

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RUNNING TITLE: Nuclear Analysis of Muscle Epi-Memory

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ABSTRACT

Using a mouse model of conditional and inducible *in vivo* fluorescent myonuclear labeling (HSA-GFP), sorting purification of nuclei, low-input reduced representation bisulfite sequencing (RRBS), and a translatable and reversible model of exercise (progressive weighted wheel running, PoWeR), we provide the first nucleus type-specific epigenetic information on skeletal muscle adaptation and detraining. Adult (>4 month) HSA-GFP mice performed PoWeR for 8 weeks then detrained for 12 weeks; age-matched untrained mice were used to control for the long duration of the study. Myonuclei and interstitial nuclei from plantaris muscles were isolated for RRBS. Relative to untrained, PoWeR caused similar myonuclear CpG hypo- and hypermethylation of promoter regions and substantial hypomethylation in interstitial nuclear promoters. Over-representation analysis of promoters revealed a larger number of hyper- versus hypomethylated pathways in both nuclear populations after training and evidence for reciprocal regulation of methylation between nucleus types, with hypomethylation of promoter regions in Wnt signaling-related genes in myonuclei and hypermethylation in interstitial nuclei. After 12 weeks of detraining, promoter CpGs in documented muscle remodeling-associated genes and pathways that were differentially methylated immediately after PoWeR were persistently differentially methylated in myonuclei, along with long-term promoter hypomethylation in interstitial nuclei. No enduring gene expression changes in muscle tissue were observed using RNA-sequencing. Upon 4 weeks of retraining, mice that trained previously grew more at the whole muscle and fiber type-specific cellular level than training naïve mice, with no difference in myonuclear number. Muscle nuclei have a methylation epi-memory of prior training that may augment muscle adaptability to retraining.
INTRODUCTION

Skeletal muscle, comprised of diverse cell types, may possess a long-term CpG DNA methylation “memory” of prior stimuli such as exercise training \(^1,2\) and inactivity \(^3\) that could have consequences for future muscle adaptability. The largest cells by volume in muscle tissue are post-mitotic multi-nuclear muscle fibers, but muscle fiber nuclei (myonuclei) may only represent ~50-60% of all nuclei \(^4\). The remaining nuclei are from resident and infiltrating mononuclear cells, the proportion of which can change with time and condition \(^4\). To understand how skeletal muscle is epigenetically regulated \textit{in vivo}, and whether muscle fibers have a memory of prior chronic contractile activity, the analysis of purified myonuclei is imperative. In this investigation, we leveraged a muscle-specific inducible and conditional genetically modified mouse model \(^5\), nucleus type-specific low-input representation bisulfite sequencing (RRBS) \(^4,6\), and a translatable murine model of hypertrophic high-volume endurance and resistance-type voluntary exercise training recently developed by our laboratory (progressive weighted wheel running, PoWeR) \(^7-11\) to define the myonuclear and interstitial nuclear-specific methylome with adult muscle adaptation. We then evaluated the presence of a nucleus type-specific CpG methylation “epi-memory” following a prolonged cessation from training and tested whether previously trained adult muscle adapts more readily to re-training at the whole muscle and cellular levels.

RESULTS

Human skeletal actin promoter reverse tetracycline transactivator tetracycline response element driven histone 2B green fluorescent protein mice (termed HSA-GFP) were generated to label resident myonuclei at the onset of experimentation \(^4,6,8,11\). A 4-month-old cohort of these mice were subjected to PoWeR for 8 weeks (6 months old when euthanized, 48 hour wheel lock and 24 hour fasted, 6M PoW, or “trained”), and another cohort was trained then detrained for 12 weeks to reverse training adaptations (9 months old when euthanized, 9M PoW+DT, or “detrained”), as described previously \(^7\); age-matched untrained mice served as controls (6M UT and 9M UT) (Figure 1). To isolate interstitial nuclei upon completion of PoWeR without contamination from satellite cell derived myonuclei acquired during training (non-GFP labeled) \(^7,8,11,12\), we labeled myonuclei toward the end of training. Mouse characteristics, evidence of detraining that corroborates our previous PoWeR studies \(^7,8\), and flow cytometry for nuclear isolations via FACS are shown in Supplemental Figure 1. Plantaris muscle nuclei (GFP+ myonuclei and mixed cell-type GFP- interstitial nuclei) were isolated via FACS and DNA from both populations was analyzed using low-input RRBS \(^4,6\).
Nucleus type-specific promoter methylation reveals reciprocal regulation of resident myonuclei and interstitial nuclei with muscle adaptation

In general, promoter methylation inversely regulates transcription. In myonuclei, 1696 differentially methylated CpG sites in promoter regions (defined as 1000 bp upstream of the transcription start site, TSS) were identified between trained and untrained (6M PoW versus 6M UT, q value false discovery rate [FDR] <0.05), slightly more of which were hypomethylated (869 CpGs) (Figure 1B). In interstitial nuclei, 1452 DM CpGs were identified, the majority of which were hypomethylated (942 unique sites) (Figure 1B). Global hypomethylation at the muscle tissue level with exercise training may therefore be driven by interstitial nuclei, which emphasizes the importance of nucleus type-specific analyses for understanding muscle fiber adaptation.

Our previous investigation revealed that the myonuclear methylome after acute plantaris overload (72 h) was defined by hypomethylation in promoters of genes associated with mTOR and autophagy. With PoWeR, at least one CpG in the promoter of the mTOR pathway-related gene Ring1 (FDR=0.00013) and autophagy-related gene Csnk2a2 (FDR=0.00016) were hypomethylated similar to acute overload (Supplemental Table 1). A CpG in the promoter of the mitochondrial biogenesis regulator Pgc1α (FDR=0.00012) was also hypomethylated in myonuclei in trained relative to untrained, and is similarly modified by acute endurance-type exercise in human muscle tissue (Supplemental Table 1). Pathway over-representation analysis identified NFκB and Wnt signaling gene promoters as hypomethylated in myonuclei of trained plantaris muscles (Figure 1C); previous research in muscle samples of humans after resistance training similarly reported hypomethylation in growth-related pathways. Promoter sites in genes such as Gadd45b (FDR=0.022) and Fzd5 (FDR=0.036) were hypomethylated in trained versus untrained (Figure 1D&E). The top over-represented pathways for genes with at least one hypermethylated CpG in the promoter after PoWeR were cytoplasmic ribosomal proteins and phosphatidylinositol (PI) metabolism (Figure 1F&G). Hypermethylation of ribosomal protein genes in myonuclei is congruent with down-regulation of ribosomal protein gene expression with PoWeR analyzed using single myonucleus RNA-sequencing. A CpG site in the promoter of Gde1 was highly hypermethylated after PoWeR (FDR=0.000002, Figure 1H); the role of this gene in muscle is undefined, but the glycerophosphodiester phosphodiesterase family member Gde5 is negatively associated with muscle mass. A CpG site in the promoter
region of Pten, which negatively associates with muscle mass and insulin sensitivity \(^{28,29}\), was also modestly hypermethylated by PoWeR (FDR=0.03, Figure 1H).

In interstitial nuclei, pathway analysis of hypomethylated promoter sites revealed over-representation of fatty acid metabolism (a comparatively small pathway), Ub-C terminal hydroxylase (UCH) proteinases, and mRNA processing genes with PoWeR (Figure 2A-D). Promoter region hypermethylation was primarily over-represented by cancer-related and Wnt signaling pathways (Figure 2E-F), the latter of which is inversely methylated in myonuclei. Additional genes that were inversely methylated in promoters compared to myonuclei were Gadd45b and Fzd5, which were hypermethylated in interstitial nuclei with PoWeR (Supplemental Table 2). In trained versus untrained, 104 of the same genes had at least one hypomethylated promoter CpG in both nuclear populations and 58 genes had similarly hypermethylated promoter CpGs, while 52 (hypomethylated in myonuclei and hypermethylated in interstitial nuclei) and 76 genes (hypermethylated in myonuclei and hypomethylated in interstitial nuclei) were oppositely regulated; these gene lists are reported in Supplemental Table 3.

Collectively, inverse methylation of CpGs in myonuclei versus interstitial nuclei points to coordinated reciprocal regulation of promoters between nucleus types at the individual gene and pathway levels with training. Furthermore, global promoter hypomethylation characterized both nuclear populations with PoWeR, but a larger number of pathways were affected by promoter hypermethylation in the trained state (Figure 1B).

**Persistent promoter methylation changes 12 weeks after PoWeR in myonuclei**

To assess whether there was a myonuclear CpG site-specific memory of prior exposure to training, we specifically looked for CpG sites in promoter regions of annotated genes that were differentially methylated in one direction (hypo- or hyper-methylated, FDR<0.05) in 6M PoW (trained) and 9M PoW+DT (detrained) versus their respective controls (FDR<0.05). According to these criteria, a CpG in the promoter region of Asrgl1 (site 9113043, FDR=0.01 6M PoW versus 6M UT and FDR=0.04 9M PoW+DT versus 9M UT) remained hypomethylated after detraining in resident myonuclei, while Rps13 (CpG site 115933918, FDR=0.0004 and 0.02) and Pkd2l1 (CpG site 44235606, FDR=0.000006 and 0.007) remained hypermethylated after detraining (Figure 3A). Pkd2l1 deficiency was recently shown to drive cardiac hypertrophy \(^{30}\). Differentially
methylated region (DMR) analysis of promoters revealed persistent hypomethylation of Med19, and hypermethylation of Erh, Gm36144, and Gm34222 (p<0.05, data not shown).

Using less stringent criteria across promoter regions (i.e. at least one differentially methylated CpG within a given promoter regardless of base pair location), there were persistent changes from prior training across a variety of unannotated and known genes, with more genes maintaining hypermethylation (Supplemental Table 4). A promoter CpG in Gdf10, recently identified as a negative regulator of adipogenesis in skeletal muscle 31, was hypomethylated after training and detraining in myonuclei (FDR=0.006 6M PoW versus 6M UT and FDR=0.04 9M PoW+DT versus 9M UT) (Figure 3B). CpG sites in the promoter of Pitx1, over-expression of which causes dystrophies and is negatively associated with muscle mass 32, was hypermethylated after training and detraining (FRD=0.04 and 0.049) (Figure 3C). The promoter of nuclear anchoring protein Sun2 (FDR=0.03 and FDR=0.002), a lack of which causes cardiac hypertrophy via enhanced AKT/MAPK signaling in the absence of fibrosis 33, also remained hypermethylated following detraining, as did Usp43 (FDR=0.0007 and FDR=0.006), which strongly suppresses cell growth 34 (Figure 3C). Sites in the promoter of the highly conserved translational initiation factor Eif1a, which positively associates with muscle protein synthesis 35 (FDR=0.036 and FDR=0.022), and the muscle hypertrophy-inducing protein Ski 36-40 (FDR=0.005 and FDR=0.01) (Figure 3C) remained hypermethylated after detraining; while perhaps opposite of expected, it is worth noting that Ski negatively regulates Gdf10 in skeletal muscle 41. At the pathway level (i.e. comparing all genes differentially methylated in 6M versus 9M timepoints), there was a memory of promoter hypermethylation in genes associated with intracellular signaling by second messengers, Pip3k activates Akt signaling, Mapk1/Mapk3 signaling, Runx1 interactions, signaling by receptor tyrosine kinases, and breast cancer (Figure 3D). There was no persistent promoter hypomethylation at the pathway level in myonuclei following detraining.

**Evidence for persistent methylation changes in interstitial nuclei after detraining**

Using the aforementioned criteria in interstitial nuclei, promoter CpG sites in Ippk (CpG site 49574364, FDR=0.000006 6M PoW versus 6M UT and FDR=0.004 9M PoW+DT versus 9M UT), Kdm4b (56632760, FDR=0.0008 and FDR=0.03x10^-7), Map7d1 (126000000, FDR=0.015 and FDR=0.0489M), and Snord58b (75133958, FDR=0.000066 and FDR=0.03) were hypomethylated after training and detraining; DMR analysis revealed persistent hypomethylation in one unannotated gene (LOC118567611, p<0.05, data not shown). No specific CpG sites in
promoters of annotated genes had a memory of hypermethylation after detraining. Not accounting for specific CpGs, more genes (many of which unannotated) had a memory of hypomethylation across promoter regions (88 genes), while 36 genes had a memory of hypermethylation, which is the opposite trend of what occurred in myonuclei (Supplemental Table 5). Although promoters of numerous individual genes displayed persistent changes after training, pathway analysis did not reveal persistent methylation changes at a broader level of regulation. When directly comparing the lists of persistently altered promoter CpGs after detraining in myonuclei versus interstitial nuclei, sites in promoters of GM15723 and Myl12b were hypomethylated in both populations, while Pitx1 was oppositely methylated (hypermethylated in myonuclei and hypomethylated in interstitial nuclei) (FDR<0.05) (Supplemental Table 5). Interestingly, over-expression of Pitx1 in satellite cells promotes differentiation 42. The inverse nucleus type-specific methylation profile of Pitx1 with detraining may somehow be protective or advantageous for muscle adaptation depending on the cellular location and stimulus on the muscle (e.g. acute exercise or retraining).

**Methylation of myonuclear ribosomal DNA (rDNA) with training and detraining**

Myonuclear rDNA methylation is modified by acute mechanical overload of the plantaris 6, concomitant with ribosome biogenesis that associates positively with muscle hypertrophy 43-46. Similar to what occurs with acute overload, differential methylation occurs along the myonuclear rDNA repeat (CpG sites 8387 and 8599 downstream of the rDNA TSS, FDR=0.00006 and 0.05, respectively) as well as interstitial nuclear rDNA (site 2180, FDR=0.005; 7923, FDR=0.05; 8604,FDR=0.007; 9413, FDR=0.049; 10962, FDR=0.049; and 12618, FDR=0.016) in trained versus untrained, without differences in rDNA promoter methylation 6; however, we did not observe rDNA CpG site-specific changes that persisted with detraining in either nuclear population.

**Gene expression changes from training do not persist following detraining**

We performed RNA-sequencing on the same muscles that nucleus type-specific methylation analyses were performed. After PoWeR, 179 genes were up-regulated and 118 genes were down-regulated (FDR<0.05), consistent with predominant promoter hypomethylation observed with training. The PoWeR transcriptome was most enriched for ECM-related processes (Supplemental Figure 2A&B), while some genes involved in SMAD signaling were down-regulated (Supplemental Figure 2C&D). Exercise training responsiveness is linked to the magnitude of ECM-related gene up-regulation 47, so enrichment of ECM genes with PoWeR
seems indicative of a robust endurance and/or resistance training stimulus\textsuperscript{47-53} and is in agreement with our recent work in PoWeR mice\textsuperscript{9}. Inhibition of SMAD signaling is associated with muscle hypertrophy, also consistent with the literature\textsuperscript{54-56}. A subset of genes with hypo- or hyper-methylated promoter regions in myonuclei or interstitial nuclei after PoWeR had concomitant up- or down-regulated gene expression in muscle respectively, with relatively greater contribution from myonuclei (Supplemental Table 6). Modest agreement between RRBS and RNA-seq is likely in part due to the somewhat limited coverage of low-input nucleus type-specific RRBS. There were no genes that were altered by PoWeR that remained differentially expressed following detraining.

**Accelerated whole muscle and muscle fiber hypertrophy in previously trained muscle**

We report a DNA methylation memory of PoWeR in myonuclei, which builds on our previous work showing a long-lasting epigenetic MyomiR-1 mediated memory of PoWeR training that could facilitate retraining adaptation independent from myonuclear number\textsuperscript{8}. We therefore asked whether muscles previously exposed to PoWeR would adapt more rapidly than untrained muscles. We trained and detrained mice as described above (see Supplemental Figure 1 and our previous work for muscle detraining characteristics)\textsuperscript{7,8} then retrained the mice for 4 weeks (PoW+DT+RT, n=5, "retrained"); age-matched untrained mice also trained for 4 weeks to compare to the previously trained group (4 wk PoW, n=5) (Figure 3E). Running volume (km/night) (Figure 3F) and body weight (Figure 3G) were not different between groups, but plantaris muscle mass (absolute and normalized) (Figure 3H&I) was significantly greater in the retrained group ($p=0.0014$ and $0.0044$, respectively). Plantaris tissues was flash-frozen for future molecular analyses, but we previously reported that the size of myosin heavy chain (MyHC) 2A fibers of the gastrocnemius muscle tracks with plantaris fiber size after PoWeR and detraining\textsuperscript{7,8}. We analyzed gastrocnemius MyHC 2A fiber size by immunohistochemistry and found retrained fibers were 10% larger than 4-week trained control fibers ($p=0.03$, Figure 3J), with no difference in MyHC 2A fiber type percentage or myonuclear number between groups (Figure 3K-M).

**DISCUSSION**

In trained plantaris muscles, promoters of genes associated with Wnt signaling were hypomethylated in myonuclei and hypermethylated in interstitial nuclei. Wnt signaling is implicated in hypertrophic muscle adaptation\textsuperscript{57,58}, and our data suggest that epigenetic
regulation may occur in different nuclear types in muscle in a reciprocal fashion. PoWeR caused global hypomethylation in myonuclei and interstitial nuclei but was also associated with promoter region hypermethylation broadly across pathways in both nuclear populations. Highly conditioned muscle demonstrates a more “refined” and targeted transcriptional response to a bout of exercise relative to untrained. Wide-ranging promoter hypermethylation may “shut-down” unnecessary pathways and contribute to the molecular characteristics of a well-trained phenotype. After 12 weeks of detraining (~10% of the murine lifespan), we found evidence for an epigenetic memory of prior adaptation with a bias toward promoter hypermethylation in myonuclei and hypomethylation in interstitial nuclei. In myonuclei, we report long-term DNA methylation changes in the promoters of genes associated with striated muscle adaptation and/or growth, specifically Pkd2l1, Gdf10, Pitx1, Sun2, Eif1a, and Ski. We also observed maintenance of hypermethylation at the broader pathway level after detraining, but did not observe persistent changes at the mRNA level, consistent with findings after detraining from chronic single-leg kicking exercise in humans. Persistent methylation changes in promoters of a range of genes in interstitial nuclei may also contribute to “muscle memory”. The benefit of long-lasting epigenetic changes from exercise training for future muscle adaptability is likely most attributable to targeted or “focused” transcriptional responsiveness upon acute contraction and/or prolonged retraining, and not persistent alterations to resting gene expression.

Finally, previously trained adult mice had a more rapid hypertrophic response to retraining, similar to resistance-trained adult humans, and we confirm these findings at the cellular level. We speculate that enduring epigenetic modifications from training that occur within skeletal muscle fibers, such as myonuclear DNA methylation and/or miRNA alterations, in concert with potential motor learning/neuromuscular factors and interstitial nuclear adaptations, collectively contribute to accelerated hypertrophy in previously trained muscle.

Our data illustrate how DNA from terminally differentiated myonuclei sustain long-term epigenetic changes from environmental exposures, extending our understanding of the molecular bases of “muscle memory”. Future investigations may: 1) analyze nucleus type-specific epigenetic responses to short-term exercise training in previously trained versus training-naïve muscle, as distinct differences at the methylation level have been observed in human muscle samples that correspond with accelerated growth at this time point, and 2) focus on the mechanisms and consequence of DNA methylation regulation within adult muscle fibers, as our understanding of this area is in its infancy.
METHODS

Animal procedures were approved by the University of Kentucky IACUC. Mice were housed in temperature and humidity-controlled rooms, maintained on a 14:10-h light-dark cycle, and food and water were provided ad libitum. Wheels were locked for PoWeR mice 48 hours before being euthanized, and all mice were fasted overnight prior to tissue collection. Animals were euthanized in the morning via a lethal dosage of sodium pentobarbital injected intraperitoneally, followed by cervical dislocation. Plantaris muscles were harvested, flash frozen, and stored at -80°C until nuclear isolations. Gastrocnemius muscles were prepared and frozen in liquid nitrogen-cooled isopentane for immunohistochemistry, as previously described.

Male HSA+/−-GFP+/− mice were generated by crossing homozygous human skeletal actin reverse tetracycline transactivator (HSA-rtTA) mice generated by our laboratory with homozygous tetracycline response element histone 2B green fluorescent protein mice (TetO-H2B-GFP) obtained from the Jackson Laboratory (005104). Mice (biological triplicate per group) were treated with low-dose doxycycline in drinking water (0.5 mg/ml with 2% sucrose) for one week as previously described (during acclimation to the running wheel for PoWeR mice and controls, and 2 months prior to euthanization in 9M mice). A subset of mice was treated for 72 hours during the last week of training to isolate a pure population of interstitial nuclei. Mice were subjected to PoWeR as previously described, and myonuclei and interstitial nuclei were isolated according to von Walden et al. from mice that ran similar volume. Briefly, frozen muscle samples were minced and Dounce homogenized in a physiological buffer, filtered through 40 µm strainer, stained with propidium iodide, then purified via FACS. Nuclear DNA was isolated using the QIAamp DNA micro kit (Qiagen), and Msp1 RRBS was performed by Zymo Research. RNA from 5-10 mg of muscle was extracted from the remaining muscle using TRIzol, a bead homogenizer, and the Zymo microprep kit.

For RRBS, data were processed as previously described. A custom genome assembly to interrogate rDNA methylation was generated by adding the consensus rDNA repeat sequences, BK000964.3, as a separate chromosome to the GRCm39 mouse (RefSeq Accession:...
GCF_000001635.27) reference genome assembly. Adapter- and quality-trimmed reads were realigned to the custom mouse reference genome using Bismark 0.19.0. Methylated and unmethylated read totals for each CpG site were collected using the Methylation Extractor tool. Methylation levels of each sampled cytosine were estimated as the number of reads reporting a “C”, divided by the total number of reads reporting a “C” or “T”. Differential methylation analyses were performed using MethylSig v1.0.0, optimized for sample sizes of three or more and accounts for both read coverage (minimum set to 10x) and biological variation. The data were analyzed using a beta-binomial distribution, and sites where a CpG was present in every sample were included for analysis. CpG sites were annotated relative to chromosomal locations of genes provided by NCBI, and promoters were defined as within 1 kb upstream of transcription start sites. Immunohistochemistry for dystrophin and MyHC 2A fibers was performed as previously described by us, and was analyzed using MyoVision. RNA-sequencing was performed by Novogene as previously described by us. FASTQ files were processed using Partek with a minimum read cutoff of 50, mapped to mm10 and annotated using ENSEMBL, normalization and differentially expressed genes were determined using DESeq with the FDR step-up procedure. Pathway analysis was performed using KEGG, Reactome, and Wikipathways databases in ConcensusPathDB with default settings. To determine pathway-level “memory”, all differentially methylated promoters in 6M PoW versus 6M UT and 9M PoW versus 9M UT were compared, irrespective of whether specific CpGs and genes were persistently different after detraining.

Statistics

RRBS data were analyzed in MethylSig using a generalized linear model accounting for all groups. False discovery rate for all sequencing (FDR, reported as the Benjamini-Hochberg adjusted p value) was controlled at <0.05. For muscle phenotyping data, unpaired t-tests and ANOVAs were utilized with p<0.05, and statistics and figures were generated in GraphPad Prism (GraphPad, San Diego, CA, USA).
**Figure 1** Myonuclear CpG promoter region DNA methylation changes in response to progressive weighted wheel running (PoWeR).  

**A** Study design schematic showing myonuclear labeling using the HSA-GFP mouse, PoWeR training (6M PoW) and detraining (9M PoW+DT) and age-matched untrained controls (6M UT and 9M UT), doxycycline treatment time points,
and fluorescent activated cell sorting (FACS) of myonuclear and interstitial nuclear populations for downstream reduced representation bisulfite sequencing (RRBS) analysis. **B** Myonuclear and interstitial nuclear methylation in promoter regions after 8 weeks of PoWeR. **C** Pathway analysis of hypomethylated promoters in myonuclei after PoWeR (relative to 6M UT). **D** Myonuclear genes with hypomethylated promoters in the NFκβ signaling pathway after PoWeR (FDR<0.05). **D** Myonuclear genes with hypomethylated promoters in the Wnt signaling pathway after PoWeR (FDR<0.05). **F** Pathway analysis of hypermethylated promoters in myonuclear after PoWeR (relative to 6M UT). **G** Myonuclear ribosomal protein genes with hypermethylated promoters after PoWeR (FDR<0.05). **H** Myonuclear genes with hypermethylated promoters in the phosphatidylinositol (PI) metabolism pathway (FDR<0.05). Gene names listed multiple times = multiple CpG sites, which can be found in Supplemental Tables.
Figure 2 Interstitial CpG promoter region DNA methylation changes in response to progressive weighted wheel running (PoWeR). A Pathway analysis of hypomethylated promoters in interstitial nuclei after PoWeR (relative to 6M UT). B Interstitial nuclear genes with hypomethylated promoters in the fatty acid (FA) metabolism signaling pathway after PoWeR (FDR<0.05). C Interstitial nuclear genes with hypomethylated promoters in the Ub-C terminal hydrosylase (UCH) proteinases pathway after PoWeR (FDR<0.05). D Interstitial nuclear genes
with hypomethylated promoters in the mRNA Processing pathway after PoWeR (FDR<0.05). E Pathway analysis of hypermethylated promoters in interstitial nuclei after PoWeR (relative to 6M UT). F Interstitial nuclear genes with hypomethylated promoters in the Cancer and Wnt signaling pathways after PoWeR (FDR<0.05).
Figure 3  Myonuclear promoter region methylation memory of prior PoWeR training may facilitate retraining adaptations. A Memory of promoter site-specific CpG methylation in
myonuclear *Pkd2l1* (FDR<0.05). B Memory of promoter region-specific hypomethylation in myonuclear *Gdf10*. C Memory of promoter region-specific hypermethylation in myonuclear *Eif1a, Pitx1, Sun2, Usp43*, and *Ski* (FDR<0.05). D Evidence for promoter region CpG methylation memory of previous PoWeR at the pathway level in myonuclei. E Study design schematic showing how mice were subjected to PoWeR for 8 weeks, detrained for 3 months, the retrained for 4 weeks (PoW+DT+RT); age-matched mice that only trained for 4 weeks served as controls (4 wk PoW). F Average nightly running volume during 4 weeks of retraining. G Body weight at the time of being euthanized. H Absolute plantaris muscle weight in milligrams (mg) (*p<0.05, directional t-test). I Plantaris muscle weight (mg) normalized to body weight in grams (g) (*p<0.05, directional t-test). J Myosin heavy chain (MyHC) 2A cross sectional area (CSA) of gastrocnemius muscle fibers (*p<0.05, directional t-test). K MyHC 2A proportion. L MyHC 2A myonuclear number measured using dystrophin and DAPI. M Representative image of dystrophin, MyHC 2A fibers, and nuclei in 4 wk PoW and PoW+DT+RT muscles. Scale bar = 50 µm.
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Author Contributions
K.A.M designed experiments. K.A.M, C.M.D, and F.V.W performed experiments. Y.W. and K.A.M analyzed and interpreted data. K.A.M wrote the manuscript and prepared the figures with input from Y.W. K.A.M. provided funding support and supervised the study. C.M.B and T.V. assisted with mouse breeding, colony management, and data analysis. All authors edited and approved the final manuscript.

Declaration of Interests
The authors have no conflicts to declare.

Data Availability
The data underlying this article are available in GEO GSE180433 (RRBS) and GSE180877 (RNA-seq)
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