# Appendix E-1

## Satellite Cell Isolation and Flow Cytometry

Satellite cells were isolated from gastrocnemius and rotator cuff muscles, using a method previously described[18] and modified on the basis of discussions with Dr. Charlotte A. Peterson. $Pax7^{CreERT2}$:$R26R^{tdTomato}$ mice were previously used to genetically label satellite cells with tdTomato, with a recombination efficiency of 99.7% for gastrocnemius muscles[17]. Freshly isolated muscles were washed with media consisting of Ham's F-10 with 10% horse serum (HS) and 1% antibiotic-antimycotic (AbAm) (ThermoFisher Scientific). Tendons, adipose tissue, and other connective tissue were removed, and muscles were finely minced and then transferred to separate conical tubes containing a dissociation buffer consisting of wash media and 800 units/mL collagenase II (ThermoFisher Scientific). Each tube was incubated for 40 minutes at 37°C with vigorous mixing. Cold wash media was then added to each tube and inverted to mix, and samples were centrifuged at 500 ×g for 5 minutes. The supernatant was then aspirated, and the cell pellet was resuspended in a solution of 1,000 units/mL collagenase II and 11 units/mL of dispase in phosphate-buffered saline (PBS) solution and incubated for 20 minutes at 37°C. Samples were then aspirated and passed through an 18-gauge needle 10 times, wash media was added, and samples were centrifuged at 500 ×g for 5 minutes. Next, the supernatant was aspirated, the pellet was resuspended in wash media, and sequentially run through 100-µm and 40-µm tissue strainers. Samples were again centrifuged at 500 ×g for 5 minutes, supernatant was removed, and pellets were resuspended in 1 mL of PBS solution containing 1:1,000 DAPI (4′,6-diamidino-2-phenylindole) to stain for nuclei of cells that had been disrupted.

Flow cytometry was performed next. The sorting baseline was established with the use of wild type C57BL/6 mice that have no endogenous fluorescent proteins. During sorting of the experimental samples, cells positive for DAPI, which indicates nonviability due to cell membrane compromise, were removed. Cells were sorted in a Synergy Flow Cytometer system (iCyt) based on forward scatter area (FSC-A) cell size and tdTomato fluorescence, and tdTomato$^{+}$ cells were collected into growth media consisting of low glucose (1 g/L) Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and 1% AbAm (ThermoFisher Scientific). To determine the purity of isolated samples following fluorescence-activated cell sorting (FACS), an aliquot of the cells was placed on a coverslip and analyzed for the presence of tdTomato by using both phase contrast and red fluorescent filters in an EVOS FL Imaging System (ThermoFisher Scientific). Approximately 90% of the sorted cells were viable and expressed tdTomato. The remaining cells were either cultured and used for in vitro experiments or used for DNA sequencing.

## Cell Culture

Sorted cells were plated on tissue culture dishes (Corning) coated with growth factor-reduced Matrigel (Corning) in a 1:100 dilution. Cells were expanded in growth media containing low glucose DMEM with 10% FBS and 1% AbAm (ThermoFisher Scientific) for 2 passages, and then on reaching 70% confluence, were switched to adipogenic media to trigger adipogenesis[14,19]. First, cells were incubated in an adipogenic growth media consisting of low-glucose DMEM with 10% FBS, 1% AbAm, 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 1 µM dexamethasone, 850 nM insulin, 1 nM triiodothyronine (T3), and 1 µM rosiglitazone (Sigma). After 2 days, cells were switched to adipogenic differentiation media

consisting of low-glucose DMEM, 1% AbAm, 10% FBS, 850 nM insulin, 1 nM T3, and 1 μM rosiglitazone, and cultured in this media for 5 days prior to analysis. For all experiments, media was changed every 2 days.

### DNA Methylation Analysis

DNA was isolated from freshly sorted tdTomato$^{+}$ satellite cells, and DNA methylation analysis was performed by the University of Michigan Epigenomics Core using enhanced reduced representative bisulfite sequencing (ERRBS), as previously described[24,25]. Genomic DNA was digested with the methylation-insensitive restriction enzyme MspI, followed by end-repair, A-tailing, and ligation of methylated adapters. Fragments between 150 to 500 bp were selected, and bisulfite conversion of methylated sequences was performed prior to polymerase chain reaction (PCR) amplification. Each sample was sequenced as a single lane on a HiSeq 2500 system (Illumina). FASTQC (version 0.11.3; Babraham Bioinformatics) was used to assess the overall quality of each sequenced sample and identify specific reads and regions that may benefit from trimming. Trim Galore! (version 0.4.0; Babraham Bioinformatics) was used to trim low-quality bases (quality score of <20), adaptor sequences (stringency 6), and end-repair bases from the 3′ end of reads. Bismark (version 0.14.3; Babraham Bioinformatics)[51] was used for alignment and methylation calling; this is an integrated alignment and methylation call program that performs unbiased alignment by converting residual cytosines to thymines prior to alignment in both reads and the reference. Reads were aligned to the reference genome (University of California, Santa Cruz mm10) using Bowtie 2 (version 2.2.1; Benjamin Langmead, Johns Hopkins University)[52] with default parameter settings except for maximum number of mismatches in seed alignment (N), which was set to 1, and length of seed substrings (L), which was set to 20. Methylation calls were reported for all nucleotides with a read depth of at least 10 sites; those with >500 read depth were removed because of the likelihood that they were biased by duplicates from PCR amplification. Then, the methylSig R package (0.4.4; The Sartor Lab, University of Michigan)[27] assessed the overall quality of methylation calls and coverage, and identified differentially methylated positions, as well as regions, by tiling the methylation data across windows of 25 bases. In addition, information from nearby CpG sites was used to improve variance estimates (local window size of 200 bases). For each pairwise comparison, methylSig uses a beta-binomial approach to calculate differential methylation statistics, accounting for variation among replicates within each group.

The p values were adjusted for multiple testing using the false discovery rate (FDR) approach, and sites were considered to be differentially methylated when they had a percent change in methylation of at least 20% and an FDR-adjusted p value of <0.05. Finally, sites and regions were annotated using the UCSC Genome Browser annotations for CpG islands, promoters, and other genomic regions[53]. iPathwayGuide software (Advaita Bioinformatics) was used to perform gene enrichment and pathway analysis. All sequencing data have been deposited at the National Institutes of Health Gene Expression Omnibus (NIH GEO) repository (GSE112867).

TABLE E-1 Primer Sequences Used for Quantitative Polymerase Chain Reaction

| Gene | Description | Forward Primer | Reverse Primer | Size *(bp)* | Reference Sequence |
|---|---|---|---|---|---|
| Adipoq | Adiponectin | TATCGCTCAGCGTTCAGTGT | AGAGTCCCGGAATGTTGCAG | 142 | NM_009605.5 |
| B2M | Beta-2-microglobulin | ATGGGAAGCCGAACATACTG | CAGTCTCAGTGGGGGTGAAT | 177 | NM_009735.3 |
| Cebpa | CCAAT/enhancer binding protein (C/EBP), alpha | AATGGCAGTGTGCACGTCTA | CCCCAGCCGTTAGTGAAGAG | 109 | NM_007678.3 |
| Des | Desmin | GACGCTGTGAACCAGGAGTT | TAGTTGGCGAAGCGGTCATT | 83 | NM_010043.2 |
| Fabp4 | Fatty acid binding protein 4, adipocyte | CGATGAAATCACCGCAGACG | CCAGCTTGTCACCATCTCGT | 131 | NM_024406.2 |
| Myf6 | Myogenic factor 6/MRF4 | CTTGAGGGTGCGGATTTCCT | TCCACGTTTGCTCCTCCTTC | 107 | NM_008657 |
| Pparg | Peroxisome proliferator activated receptor gamma | TTCGCTGATGCACTGCCTAT | GGAATGCGAGTGGTCTTCCA | 129 | NM_011146.3 |
| Tmem8c | Transmembrane protein 8C, myomaker | GCCTTTACCACCTTCTCCCCA | GCCTCCATGTAGAAACGCCTC | 144 | NM_025376.3 |