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Recurrent training rejuvenates and enhances transcriptome and methylome responses in young and older human muscle

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Abstract

Background The interaction between the muscle methylome and transcriptome is understudied during ageing and periods of resistance training in young, but especially older adults. More information is needed on the role of retained methylome training adaptations in muscle memory to understand muscle phenotypical and molecular restoration after inactivity or disuse.

Methods We measured CpG methylation (microarray) and RNA expression (RNA sequencing) in young (n = 5; age = 22 ± 2 years) and older (n = 6; age = 65 ± 5 years) vastus lateralis muscle samples, taken at baseline, after 12 weeks of resistance training, after training interruption (2 weeks of leg immobilization in young men, 12 weeks of detraining in older men) and after 12 weeks of retraining to identify muscle memory-related adaptations and rejuvenating effects of training.

Results We report that of the 427 differentially expressed genes with advanced age (FDR < 0.1), 71% contained differentially methylated (dm)CpGs in older versus young muscle (FDR < 0.1, M-value difference >0.4). The more dmCpGs within a gene, the clearer the inverse methylation–expression relationship. Around 73% of the age-related dmCpGs approached younger methylation levels when older muscle was trained for 12 weeks. A second resistance training period after training cessation increased the number of hypomethylated CpGs and upregulated genes in both young and older muscle. We found indication for an epi-memory within pro-proliferating *AMOTL1* in young muscle and mechanosensing-related *VCL* in older muscle. For the first time, we integrate muscle methylome and transcriptome data in relation to both ageing and training-induced/inactivity-induced responses and identify focal adhesion as an important pathway herein.

Conclusions This preliminary evidence indicates that previously trained muscle is more responsive to training than untrained muscle at methylome and transcriptome level and recurrent resistance training can partially restore ageing-induced methylome alterations.

Keywords DNA methylation; RNA expression; Muscle ageing; Immobilization; Detraining; Epi-memory

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Introduction

Age-related skeletal muscle decay is the consequence of primary and secondary ageing.[1] The first is described as the inevitable deterioration of cellular structures and biological functions with little chance of reversal and inter-individual differences caused by genetic factors.[2] Secondary ageing, on the other hand, is the result of harmful lifestyle choices and environmental factors. These harmful factors can influence epigenetic mechanisms, defined as events that regulate gene expression without changing the DNA sequence. One of the most studied epigenetic events is DNA methylation,[3] which mostly refers to CpG methylation and implies the binding of a methyl group to a 5′-cytosin-phosphate-guanine-3′ dinucleotide (CpG). Hypermethylation of CpG-dense promoter regions of actively transcribed genes has been associated with transcriptional repression, also known as 'gene silencing'.[4] The methylome of ageing tissues is characterized by global CpG hypomethylation, loci-specific differential methylation and more divergence between DNA methylation patterns of different individuals with the end result leading to aberrant gene expression, reactivation of transposable elements and genomic instability.[5,6] Age-related methylation changes in muscle tissue, on the other hand, appear distinct compared with other tissues.[7] Recently, two genome-wide DNA methylation analyses identified 5518[8] and 17 994[9] differentially methylated CpGs (dmCpGs) between old and young skeletal muscle with most of them hypermethylated in older adults. The integration between age-related muscle methylome and transcriptome changes remains yet to be studied and is a crucial step in understanding the molecular mechanism behind muscle ageing.

As DNA methylation modifications are reversible, adopting a healthy lifestyle could potentially restore the age-associated damage to the muscle methylome and promoting physical activity is one strategy to do this. For instance, endurance exercise interventions are associated with altered methylation levels in genes involved in metabolism, cardiovascular adaptations, inflammatory and immunological processes, and muscle remodelling in young and middle-aged muscle.[10–13] More recently, methylome modifications comparing change of direction to straight line running also identified a more explicit hypomethylated signature for change of direction running in genes within protein binding, MAPK, AMPK, insulin, and axon guidance pathways.[14] These beneficial methylome adaptations could even be passed on to later generations, as has been observed in mice.[15] In older muscle, lifelong physical activity induced promoter hypomethylation of genes involved in energy metabolism, myogenesis, contractile properties, and oxidative stress.[16] The impact of resistance training (RT) on the muscle methylome is less documented in literature, especially in elderly, but it has been suggested that RT has a distinct effect on the methylation status of, among other, growth-related genes.[13,17] Understanding the impact of RT on the muscle methylome is important, as resistance exercises can contribute to regaining muscle mass and strength and should be part of the exercise programme designed to counteract muscle ageing.

Nevertheless, lifelong training without training interruptions seems utopian as training can be occasionally ceased for various reasons. Fortunately, training-related muscle adaptations, and especially strength gains, are preserved longer than the initial training period and are often more rapidly regained following retraining.[18–20] In addition, the basal and acute RT-induced transcription of key genes involved in muscular adaptations appears to be influenced by a previous RT period.[21] This so-called muscle memory has been partially attributed to motor learning[19] and linked to the retention of myonuclei during muscle atrophy,[22] as well as to lasting epigenetic modifications.[17] The slow reversibility of methylation has been, for instance, seen when methylation changes in young skeletal muscle following 5 days of high-fat diet only partially and non-significantly reversed after 6 to 8 weeks.[23] The epigenetic retention of prior external stimuli in a way that re-encountering these stimuli later in life is associated with an enhanced muscle phenotypical response compared with the initial response (i.e. *epi-memory*[24]), has been described in relation to metabolic (e.g. obesity[25]), catabolic (e.g. TNF-α[26]), and anabolic (e.g. RT[17]) stimuli. As DNA methylation modifications are transient in nature after one bout of exercise,[27] the question remains how long beneficial methylation alterations due to an extended period of RT are retained and whether multiple re-encounters are necessary to accumulate and stabilize these changes. Recently, a number of genes were identified in young skeletal muscle with a possible 'epi-memory'.[17,28] These genes displayed hypomethylation after 7 weeks of RT and continued to be hypomethylated during 7 weeks of detraining, leading to enhanced gene expression following retraining. The number of hypomethylated dmCpGs following retraining was also twice the number compared with following training, which coincided with larger muscle growth.[17] Our knowledge on epi-memory is, however, still rudimentary and identifying genes that constitute the epi-memory might provide crucial insights for the future development of therapies that promote muscle regain after inactivity.

Here, we use a training, immobilization and retraining resistance exercise protocol in young men to identify muscle memory-related genes at muscle methylome and transcriptome level. In addition, we test the rejuvenating effect of RT on the older skeletal muscle methylome and whether these beneficial methylome adaptations are maintained during detraining and promote an enhanced transcriptional response during retraining.

Methods

Subjects and experimental design

Nine older men, divided in an exercise group (n = 6, age: 66 ± 5 years, BMI: 26 ± 3 kg/m^2; mean ± SD) and control group (n = 3, age: 70 ± 4 years, BMI: 26 ± 2 kg/m^2), and five younger men (age: 22 ± 2 years, BMI: 24 ± 3 kg/m^2) gave written consent for this study after being fully informed. All men were non-smoking, untrained, healthy individuals (exclusion factors described in detail elsewhere[29]). The experimental design of the study is displayed in *Figure* 1. One younger subject withdrew after the first training period for reasons unrelated to this investigation. Baseline and post-training samples from this subject are included in the analysis. The study protocol was approved by the Medical Ethics Committee of the Catholic University of Leuven (S59380) and is in accordance with the Declaration of Helsinki.

Resistance training programme

A 12 week supervised RT programme was conducted as previously described.[29] Briefly, three times a week, on non-consecutive days, subjects performed eight exercises targeting all major muscle groups of the upper and lower body with load intensity gradually increasing every 4 weeks (65% to 80% of one repetition maximum). The training load was increased for each exercise when the final set comprised more repetitions than the prescribed number. The vastus lateralis muscle was specifically trained with a Signature Series Plate Loaded Linear Leg Press and Insignia Series Leg Extension machine (Life Fitness, Barendrecht, the Netherlands). The same RT programme was used for training and retraining phases with starting loads adapted to the physical state at that time.

Training interruption

Young subjects

The right leg of the young subjects was immobilized with the knee in nearly full extension for 2 weeks using a full leg cast (foot included). Subjects were instructed to walk with crutches during the immobilization phase. To prevent deep vein thrombosis, subjects were screened for risk factors and anticoagulant enoxaparin (40 mg per 0.4 mL per day, Clexane®, Sanofi Belgium, Diegem, Belgium) was administered daily via subcutaneous injection into the abdominal skinfold.

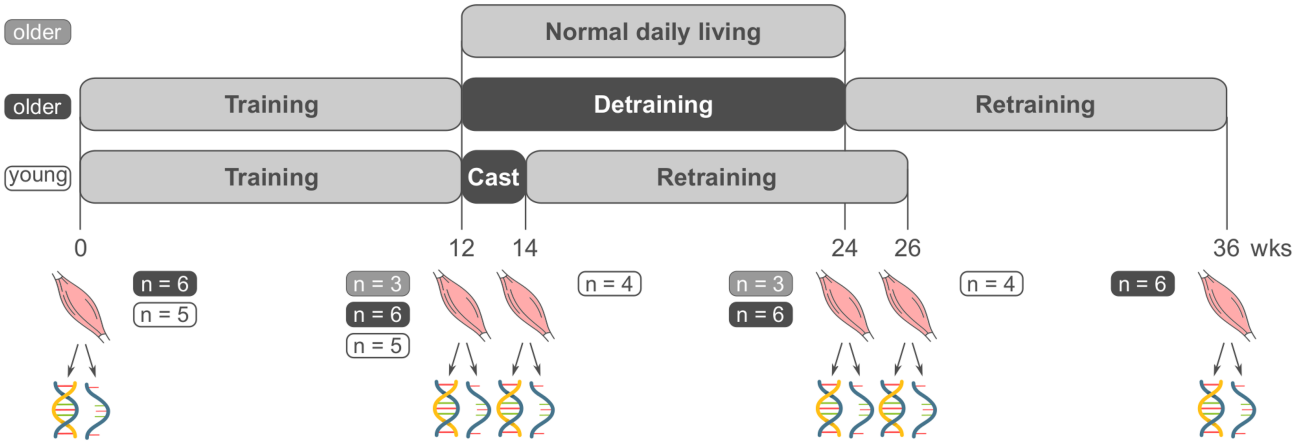
Older subjects in the exercise group

The training programme was interrupted by a detraining phase. Subjects were instructed not to engage in any structured strength or endurance exercises for 12 weeks and to resume normal physical activities from before the start of the study. To verify this, subjects weekly filled in a physical activity diary.

Skeletal muscle biopsy

Muscle biopsies were collected at baseline (left leg), ~72 h after the final RT session of the first training period (left leg), at the end of week 12 from the detraining period (older

Figure 1 Experimental design. Five younger and six older subjects participated in a 12 week RT programme (training). Thereafter, the right leg of the younger subjects was immobilized for 2 weeks (cast), whereas the older subjects ceased training for 12 weeks (detraining). A second 12 week RT-period was provided following the casting/detraining phase (retraining). Muscle biopsies (image of a muscle) were taken before training, after training, after casting/detraining and after retraining. Three older subjects provided a muscle sample from before and after a 12 week period of normal daily living (control group). Part of the biopsy was used to extract DNA and measure DNA methylation (image of a double strand). The other part was used to extract RNA and perform RNA expression analysis (image of a single strand).



subjects, right leg) or immediately after cast removal (younger subjects, right leg) and ~72 h after the final RT session of the retraining period (right leg). The older control subjects provided samples at the post-training (right leg) and post-detraining (left leg) time point of the exercise group. Samples were taken under local anaesthesia from the mid-portion of the m. vastus lateralis with a 5 mm Bergström needle,[30] immediately cleaned of fat and connective tissue and frozen in liquid nitrogen before being stored at −80°C for further analysis.[29] Subjects were asked not to participate in moderate to vigorous physical activity and not to consume alcohol in the 24 h before biopsy collection. Food intake during the 72 h before biopsy collection was comparable between each time point within each subject.

DNA extraction and Illumina methylation assay

Genomic DNA was extracted from 15 to 20 mg muscle tissue with the proteinase K method, which uses proteinase K and lysis buffer. DNA was quantitated with the Qubit 2.0 Fluorometer (Invitrogen). DNA samples were transferred to the Genomics Core facility (UZ/KULeuven, Belgium) to measure DNA methylation level at over 850 000 CpGs using the Illumina Infinium MethylationEPIC BeadChip.

Pre-processing DNA methylation data

IDAT-files, containing raw intensity data from the EPIC BeadChip, were pre-processed in R (version 3.6.1).[31] Firstly, the R/Bioconductor package *minfi* v1.30.0[31] was used to read the IDAT files and perform quality control. None of the samples contained more than 10% of sites with detection *P*-value > 0.05,[32] and all samples were considered good quality samples based upon clustering the log median intensities of raw methylated and unmethylated values. Background correction was performed with Noob dye-bias normalization,[33] implemented in *minfi*. Next, β-values, defined as the ratio of the methylated probe intensity and the overall intensity, with $\beta = 0$ indicating sites are completely unmethylated and $\beta = 1$ expressing fully methylated sites,[34] were extracted from the normalized data. These β-values were subsequently normalized with BMIQ,[35] implemented in R/Bioconductor package *wateRmelon* v1.28.0,[36] to correct for the different design of type I and type II probes in the EPIC BeadChip, as suggested by Liu *et al*.[37] Finally, we filtered out 357 probes that had failed in 20% of the samples based on detection *P*-value > 0.05,[32] 20 655 probes that had failed to hybridize based on a bead count <3 in 5% of the samples,[32] 97 253 probes that are cross-reactive or overlap with genetic variants based on the h19 genome[38] and 206 probes with unknown genomic location. Ultimately, 747 741 probes were considered for downstream analyses, which was performed on M-values, the log2 ratio of the intensities of methylated probe versus unmethylated probe.[39] Principal component analysis (PCA) plots were able to cluster young and older samples based on age, sample ID and timing in the intervention (Supporting Information, *Figures* S1 and S2). To correct for unwanted sources of variation, such as batch effects and cell type heterogeneity within muscle tissue, we used the package *sva* v.3.32.1[40] to perform surrogate variable analysis (SVA) on the M-values of each subject group, while protecting the information coming from the intervention (variable of interest, included in the full model) and the paired subject IDs (adjustment variable, included in the null and full model). The number of surrogate variables per group was estimated with the default 'be' approach.[41] No SVA was performed when comparing young to older muscle methylome, as the function could not handle a design where individuals were nested within groups.

Differential DNA methylation analysis

Differential DNA methylation analysis was performed in Partek Genomics Suite V.7.0 (Partek Inc., St. Louis, MO, USA). To identify age-related dmCpGs, we applied a mixed model with methylation M-values as response variable, 'time' (baseline, post-training) and 'group' (younger subjects, older subjects of the exercise group) as fixed factors and 'subject ID' as random factor. We specifically interpreted the planned contrasts 'younger versus older subjects at baseline' and 'younger subjects at baseline versus older subjects at post-training'. A CpG list was generated using Benjamini-Hochberg's[42] false discovery rate (FDR) < 0.1 and difference in M-values > 0.4[39] as cut-off rules. A histogram of *P*-values for all 747 741 tests for the planned contrast 'younger versus older subjects at baseline' can be found in *Data* S1a. To determine within-group differences over time, three separate mixed models were fitted (one for each subject group). Methylation M-values were entered into the model as response variable, 'time' (all available time points) and surrogate variables as fixed factors and 'subject ID' as random factor. Contrasts were specified to study the different combinations of the various time points. As an example, the histogram of *P*-values for all 747 741 tests for the planned contrast 'casting versus retraining in young subjects' can be found in *Data* S4. Because only one CpG remained after FDR-control and this part of the study is considered explorative in nature,[43] statistical significance was determined at $P < 0.01$ with a M-value difference threshold of 0.4. Probes were annotated based on 'HumanMethylation850' reference, 'MethylationEPIC_v-1-0_B4' annotation file, and hg19 genome build. Overlapping genes and gene regions were determined based on Ensembl Transcripts data release 75. 'Promoter regions' are defined in this study as the combination of TSS1500 (1500–201 base pairs upstream from the

transcription start site), TSS200 (200–0 base pairs upstream from the transcription start site), and the first exon (5′UTR + the first coding sequence). 'Intragenic' will be used as the overlapping term for all exons (except for the first coding sequence), introns, regions between the transcription start and end site of non-coding transcripts and 3′UTR regions. 'Intergenic regions' are regions between genes, not including the promoter region. The relationship to CpG islands (island, shore, shelf, and open sea) was determined with the UCSC genome browser. We furthermore performed pathway enrichment via a Fisher's Exact test in Partek Pathway (Partek Inc., St. Louis, MO, USA) based on the KEGG pathway database. We restricted the analysis to pathways of minimal 10 and maximal 500 genes, as small pathways are redundant with larger pathways and larger pathways overly general (e.g. pathways in cancer), inflating their statistical significance.[44] Finally, to find overlapping CpGs, genes and pathways following each intervention phase, we used the Venn diagram tool available via http://bioinformatics.psb.ugent.be/webtools/Venn/. This tool was also consulted to find overlap between dmCpGs of the present study and comparable studies. Pearson correlations tests and Fisher's exact tests were respectively used to identify correlations and associations between the overlapping datasets (with significance level set at $P < 0.05$).

RNA extraction and sequencing

Total RNA was homogenized from 15 to 20 mg of muscle tissue with silicon beads in 1 mL Trizol reagent (Invitrogen) using a FastPrep-24 5G (MP Biomedicals) and extracted with phase separation reagent 1-Bromo-3-chloropropane. RNA concentration and purity (A260/230) were determined with SimpliNano (Biochrom). RNA integrity was assessed with the Agilent 2100 Bioanalyzer System and was at least 7.7 in all samples. At the Genomics Core facility, sequencing libraries were prepared with the QuantSeq 3′ mRNA-Seq Library Prep Kit FWD (Lexogen) and sequenced on a HiSeq 4000 Sequencing System (Illumina) using the HiSeq3000/4000 SR Cluster and SBS Kit (Illumina).

Pre-processing RNA-sequencing data

Quality control of raw reads was performed with FastQC v0.11.5. Adapters were filtered with ea-utils v1.2.2.18. Splice-aware alignment was performed with the CRAN package *Star* v2.6.1b against the human reference genome hg19 based on annotations from Ensembl release 75. The number of allowed mismatches was two. Reads that mapped to more than one site to the reference genome were discarded. The minimal score of alignment quality to be included in count analysis was 10. Resulting SAM and BAM alignment files were handled with Samtools v0.1.19.24.[45] Quantification of reads per gene was performed with HT-Seq count v0.5.3p3.[46] Further pre-processing was performed in R (version 3.6.1).[47] Data quality assessment and count-based differential expression analysis of the 56 638 sequenced genes were carried out with R/Bioconductor package *DESeq2* v1.26.[48] By inspecting heatmaps of the count matrix and PCA plots, we detected and excluded one outlier within the older exercise group (post-retraining time point) (*Figure* S3). The *sva* package (svaseq-function) was used in a similar way as described earlier to detect unwanted variation in the normalized counts. Low count genes were filtered with *DESeq2*'s independent filtering technique, leaving 23 296, 24 661, and 19 211 genes left for expression analysis in the young exercise, older exercise, and older control groups, respectively. Results were generated on normalized counts, corrected for library size.

Differential RNA expression analysis

To test for any difference over the multiple time points, a likelihood ratio test was performed via *DESeq2*, including 'time', surrogate variables and 'subject ID' in the full model and surrogate variables and 'subject ID' in the reduced model. Pairwise comparisons between time points were analysed with Wald tests. Reported *P*-values were adjusted for multiple testing with the Benjamini–Hochberg procedure[42] after assigning weights using Independent Hypothesis Weighting (*IHW* package v1.6.0[49]). Differentially expressed genes (DEGs) are reported if FDR < 0.1 and the average read count ≥ 5. Overlap between methylation and expression data was determined with the Venn diagram tool (cfr. supra).

Comparison with the MetaMEx meta-analysis

We ran the top 100 DEGs of each analysis in MetaMEx,[50] a tool combining transcriptome data from more than 90 human studies that allows to meta-analyse changes in single genes across exercise and disuse studies. The training and immobilization data of the younger men was, respectively, cross-referenced with RT studies of 10 to 20 weeks (n = 58)[51–56] (*Data* S4h) and immobilization/bed rest studies of 5 to 21 days (n = 42)[10,57–60] (*Data* S4i) in healthy, sedentary/active, lean/overweight, younger men. MetaMEx does not include retraining studies. Therefore, we report genes that display the same expression response as RT studies in MetaMEx or the opposite expression response compared with disuse studies in MetaMEx (*Data* S4j). We cross-referenced the (re-)training data of the older men with RT studies of 12 to 24 weeks in healthy, sedentary and active, lean or overweight, elderly men (n = 66)[51,53,55,61–63] (*Data* S2h and S2j). MetaMEx does not include detraining studies;

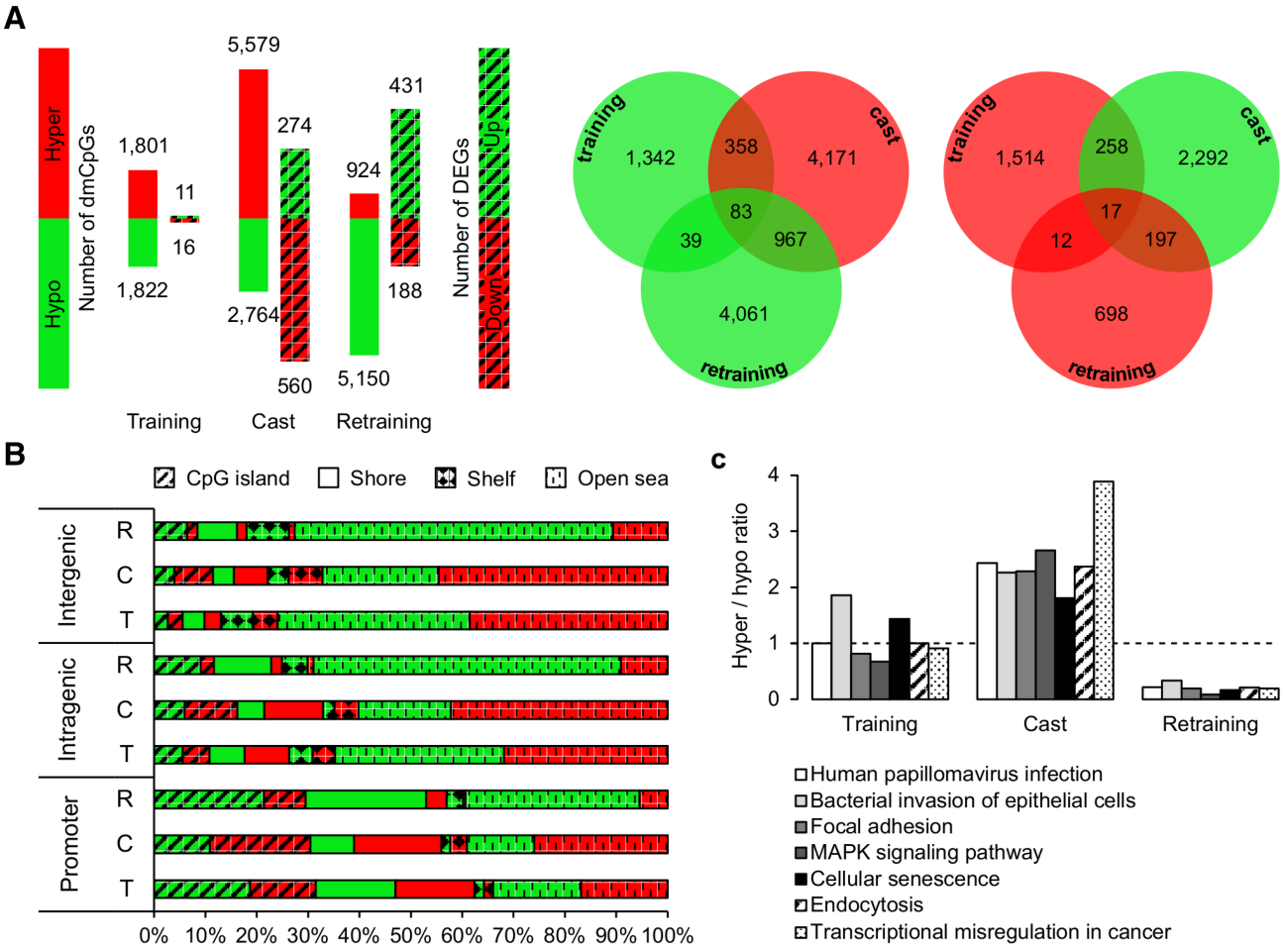
instead, the detraining data of the older men are compared with a 5 day bed rest study in elderly men (n = 10)[57] (*Data* S2i).

Results

More hypomethylated CpGs and upregulated genes in previously trained young muscle

A first training period affected methylation levels of 3623 CpGs in young skeletal muscle ($P < 0.01$, M-value difference > 0.4) with an approximate 50/50-distribution of hypomethylated versus hypermethylated CpGs (1822 vs. 1801) (*Figure* 2A, *Data* S4a). The dmCpGs were not overrepresented or underrepresented in any gene region or structure (*Figure* 2B), thereby mirroring the distribution of the CpGs in the whole genome (*Data* S4d). Interestingly, within promoter-associated CpG islands, a higher number of CpGs were hypomethylated (1326) compared with hypermethylated (909), whereas in other areas, the number of hypomethylated versus hypermethylated CpGs was more equally distributed (*Figure* 2B). At transcriptional level, 11 genes were upregulated, and 16 genes were downregulated following training (FDR < 0.1, average read count ≥ 5) (*Figure* 2A, *Data* S4h). Ten of these 27 genes were differentially expressed in the same direction as comparable training

Figure 2 (A) Number of hypomethylated and hypermethylated CpGs ($P < 0.01$, M-value difference > 0.4; no pattern) versus upregulated and downregulated genes (DEGs) (FDR < 0.1, average read count ≥ 5; dashed pattern) following training (n = 5), casting (n = 4) and retraining (n = 4) compared with previous time point in young muscle. Venn diagrams displaying overlap between dmCpGS following training, casting, and retraining (hypomethylated = green; hypermethylated = red). Alternative overlap patterns are presented in *Data* S4a. (B) Distribution of dmCpGs (% of the total, hypomethylated = green; hypermethylated = red) following training (T), casting (C), and retraining (R) in young muscle across gene regions and gene structures. (C) The hyper/hypo ratio of the seven overlapping pathways following training, casting, and retraining. This ratio is calculated by dividing the number of hypermethylated CpGs with the number of hypomethylated CpGs. The dashed line (ratio = 1) delimitate an equal level of hypermethylated and hypomethylated CpGs within a pathway.



studies (cfr. Supra: Methods) included in the metaMEx meta-analysis (*Data* S4h, 10 showing the same change, 0 showing the opposite change, 13 showing no change, and 4 not found in MetaMEx).

The 2 week casting period reversed methylation levels in 716 of the 3623 dmCpGs following training ($P < 0.01$, M-value difference > 0.4) (*Figure* 2A, *Data* S4b). Using the same threshold criteria, an additional 7627 CpGs unique CpGs were differentially methylated following the 2 weeks of immobilization compared with post-training with the majority being hypermethylated (5138). This immobilization-induced hypermethylation was also reflected within the different gene regions and structures (*Figure* 2B). Two hundred seventy-four genes were upregulated, and 560 genes were downregulated following immobilization compared with post-training (FDR < 0.1, average read count ≥ 5) (*Figure* 2A, *Data* S4i). Immobilization has a clear and distinct effect on the muscle transcriptome, as the top 100 most DEGs following immobilization in the present study corresponded largely to what is found in other disuse studies of the MetaMEx meta-analysis (*Data* S4i, 82 showing the same change, 1 showing the opposite change, 14 showing no change, and 3 not found in MetaMEx). Most genes within this top 100 were downregulated following immobilization and related to metabolism (49) or muscle structure and contraction (17) (*Data* S4i).

A second 12 week training period had a clear hypomethylating effect as 5150 of the 6074 dmCpGs displayed lower methylation levels following retraining compared with post-immobilization ($P < 0.01$, M-value difference > 0.4) (*Figure* 2A, *Data* S4c). Retraining reversed methylation levels in 1264 of the 8343 dmCpGs following immobilization (*Figure* 2A). Furthermore, merely 151 CpGs were differentially methylated in the same direction following retraining as following training (*Figure* 2A). This pattern of similar training–retraining methylation changes is higher than opposite patterns of methylation changes (65 CpGs), as visualised in the alternative patterns in *Data* S4a (Venn diagrams). In both CpG-rich and CpG-poor gene areas, the frequency of hypomethylated CpGs was enhanced compared with following training (*Figure* 2B). A similar enhanced retraining response was reported earlier in young men after 7 weeks detraining.[17] The enhanced retraining response was also reflected at transcriptional level with 431 genes upregulated and 188 downregulated (FDR < 0.1, average read count ≥ 5) (*Figure* 2A, *Data* S4j). Retraining reversed expression levels in 463 of the 834 immobilization-induced transcriptional dysregulated genes. Restoration was observed in, among other, the mitochondrial respiratory chain (16 members of the NADH ubiquinone oxidoreductase complex, 8 members of the ubiquinol-cytochrome c reductase complex, 13 members of the cytochrome c oxidase complex, and 13 members of the ATP synthase complex), in 11 myosin light and heavy chain genes, in 8 genes of the mitochondrial ribosome complex, in 11 ribosomal protein-coding genes, and in 12 genes encoding for solute carrier membrane transport proteins (*Data* S4i-j). Finally, of the top 100 most DEGs following retraining, 32 genes displayed transcriptional changes in the same direction as comparable RT studies (3 in opposite direction, 62 no change) of the metaMEx database and 76 genes in the opposite direction compared with metaMEx disuse studies (1 in opposite direction, 20 no change) (*Data* S4j). Most genes within this top 100 were upregulated and related to metabolism (36), muscle structure and contraction (15) or muscle remodelling (10) (*Data* S4j). This is the first study describing the retraining-related recovery of human muscle after disuse-induced atrophy in relation to the transcriptome and methylome. In rats, denervation-induced muscle atrophy decreased methylation in *MYOG*, *TRIM63*, *FBXO32*, and *CHRNA1* together with increased expression that was returned to control levels following 7 days of recovery.[64] In accordance, *TRIM63*, *FBXO32*, and *CHRNA1* were significantly upregulated following immobilization (FDR ≤ 0.067) in the present study and retraining reversed the RNA expression of all three genes to untrained and trained levels. We could not link this finding to any methylation changes.

Finally, pathway analysis on the methylome data identified 66, 79, and 12 enriched pathways (FDR < 0.1) following training, casting, and retraining, respectively (*Table* 1, *Data* S4e-g), of which 7 enriched during the whole intervention period. The overlapping pathways contained nearly the same number of hypomethylated and hypermethylated CpGs following training, whereas the frequency of hypermethylated CpGs clearly increased and decreased following casting and retraining, respectively (*Figure* 2C). The pathway analysis thereby confirmed that retraining induced more hypomethylation in comparison with training.

Ageing-related hypermethylation in promoter-associated CpG islands and inverse expression levels

Comparing young to older muscle samples at baseline, we identified 50 828 age-related dmCpGs (FDR < 0.1, M-value difference > 0.4), of which 28 443 were hypomethylated and 22 385 were hypermethylated in older muscle tissue (Data S1a). Two earlier genome-wide methylation studies[8,9] that compared young and older human muscle samples found a clear global trend towards hypermethylated aged muscle. We could only confirm this trend in promoter-associated CpG islands, where more than twice as many hypermethylated CpGs (2761) were found compared with hypomethylated ones (1202). In comparison, other regions contained 23 to 45% more hypomethylated CpGs (*Figure* 3A). Promoter-associated dmCpGs in CpG islands were underrepresented compared with CpGs in the whole genome (*Figure* 3B, *Data* S1b), which is a confirmation of the results of Zykovich *et al.*,[8] but in contrast to the findings

Table 1 Top 3 enriched pathways per analysis

	ES	FDR	DMG	Genes in pathway	dmCpGs hypo	dmCpGs hyper
Young muscle following training[a]						
Longevity regulating	11.1	0.005	27	89	9	30
Axon guidance	9.7	0.009	43	182	24	23
Regulation of actin cytoskeleton	9.4	0.009	48	213	35	23
Young muscle following casting[a]						
Focal adhesion	17.8	<0.001	89	199	35	80
Prostate cancer	13.0	<0.001	48	99	17	45
Rap1 signalling	12.1	<0.001	84	206	38	69
Young muscle following retraining[a]						
MAPK signalling	12.2	0.002	91	296	104	9
Focal adhesion	8.5	0.031	61	199	61	12
Endocytosis	8.1	0.032	72	247	75	16
Young versus older muscle						
Focal adhesion	37.4	<0.001	161	199	442	377
Axon guidance	31.3	<0.001	145	182	356	447
Oxytocin signalling	30.7	<0.001	125	153	372	314
Older muscle following training[a]						
Axon guidance	12.9	<0.001	54	182	43	24
Cholinergic synapse	11.0	0.003	36	112	27	20
Calcium signalling	9.7	0.005	51	187	37	31
Older muscle following detraining[a]						
Focal adhesion	19.6	<0.001	85	199	40	69
MAPK signalling	10.7	0.004	102	296	41	88
Endometrial cancer	9.6	0.004	28	59	13	23
Older muscle following retraining[a]						
Platelet activation	14.3	<0.001	42	124	36	14
Phospholipase D signalling	13.9	<0.001	47	147	40	16
Non-small cell lung cancer	12.2	<0.001	26	67	21	11

ES, enrichment score; FDR, false discovery rate-adjusted *P*-value; DMG/dmCpGs, differentially methylated genes/CpG sites determined at the level of FDR < 0.1 for age-related analysis, $P < 0.01$ for other analyses.
[a]Compared with previous time point.

of Turner *et al*.,[9] where most dmCpGs were located in CpG islands. Finally, we found a significant overlap of 2334 CpGs between the age-related dmCpGs of the present study and the age-related dmCpGs of Zykovich *et al*. (P = 2.2e-16) (*Figure* 3C, *Data* S1d). Interestingly, the age-related differences in β-values of the overlapping dmCpGs was nearly identical (r = 0.94, $P < 0.001$) (*Figure* 3D). These overlapping dmCpGs could, therefore, be a representation of an epigenetic clock[5] common between individuals, whereas the dmCpGs uniquely found in each study represent the epigenetic drift[5] between individuals due to stochastic factors and the specific environmental conditions of the population under investigation. To test this assumption, the 2334 overlapping dmCpGs were compared with the 200 CpGs that were recently selected to create a muscle-specific epigenetic clock.[65] We found a significant overlap of 12 CpGs (P = 2.6e-12) (*Figure* 3C, *Data* S1d).

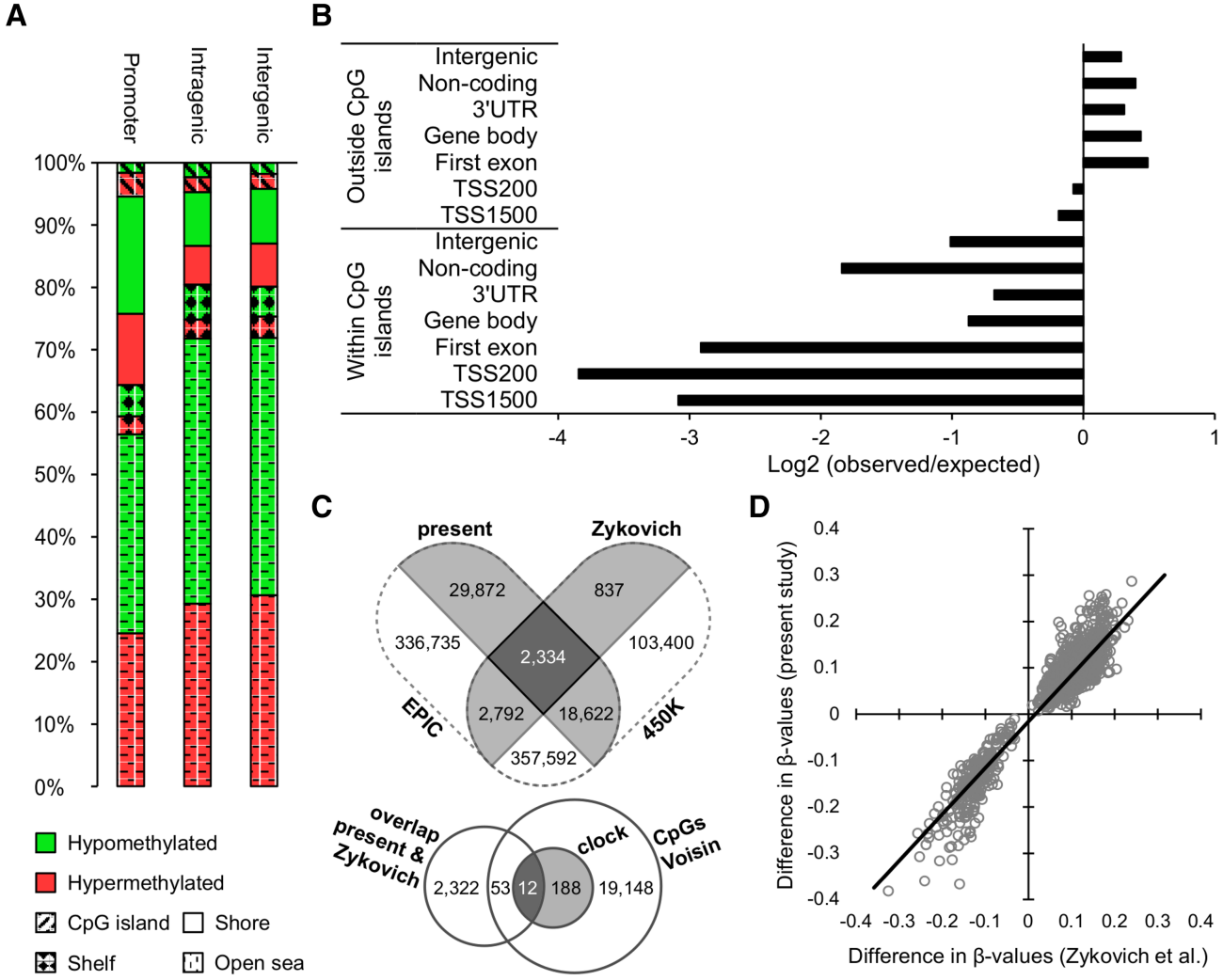
Pathway analysis identified 151 enriched pathways (FDR < 0.1) (top 3 in *Table* 1), belonging to 'organismal systems' (52, e.g. axon guidance), 'human diseases' (44 of which 18 related to cancer), 'environmental information processing' (24, e.g. PI3K-Akt signalling pathway), 'metabolism' (16), 'cellular processes' (13, e.g. focal adhesion), and 'genetic information processing' (2) (*Data* S1c).

In addition, ageing affected expression levels of 427 genes (FDR < 0.1), of which 183 were downregulated and 244 upregulated in older muscle. Remarkably, 71% of these 427 DEGs contained at least one promoter-associated or intragenic dmCpG (FDR < 0.1, M-value difference > 0.4) (*Data* S1e). We selected all DEGs with dmCpGs methylated in the same direction (i.e. all sites hypomethylated or all sites hypermethylated within a specific gene region) (*Figure* 4A, DEGs with differential methylation in promoter regions reported in *Data* S1f). Most of the hypomethylated genes displayed increased expression, whereas expression of hypermethylated genes was generally downregulated. This was independent of the gene region and especially true for genes with more dmCpGs. However, many DEGs included both hypomethylated and hypermethylated CpGs (*Data* S1g), which confirms that the relationship between methylation and expression is more nuanced than a simple inverse relation.[66] Exploring the top 100 differentially methylated and expressed genes between young and old, we found indications of impaired processes important for muscle functioning and remodelling in older muscle (*Table* A1).

Training has rejuvenating effects on the older muscle methylome

Twelve weeks of training in the older men rejuvenated their muscle methylome, as 37 339 of 50 828 age-related dmCpGs at baseline were no longer significantly different between

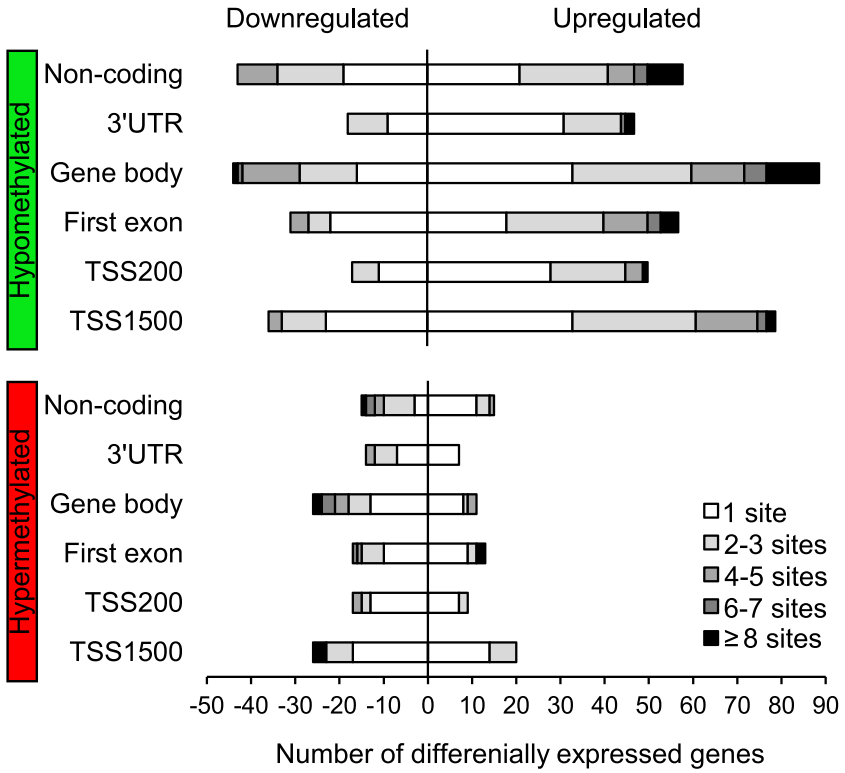
Figure 3 (A) Distribution of age-related dmCpGs (FDR < 0.1, M-value difference > 0.4) (older: *n* = 6, young: *n* = 5) across gene regions and structures, expressed as percentage of the total number per gene region. (B) Distribution of age-related dmCpGs across gene regions and structures (observed) relative to CpG distribution across the whole genome (expected). Negative log values correspond to an underrepresentation of dmCpGs within that area (positive values vice versa). (C) Top Venn diagram displaying the overlap between the present age-related dmCpGs, selected from the MethylationEPIC BeadChip, and the age-related dmCpGs from Zykovich *et al.*,[8] selected from the HumanMethylation450K BeadChip. Bottom Venn diagram displaying the overlap between the overlapping 2334 dmCpGs from the top Venn diagram and the 200 CpGs from the epigenetic clock of Voisin *et al.*,[65] selected from 19 401 CpGs. (D) Grey dots represent the difference between average β-values of older men and younger men per dmCpG (*n* = 2334, cfr. c). The black line is the trend line.



trained older muscle and untrained young muscle (FDR ≥ 0.1 or M-value difference ≤ 0.4) (*Data* S1h). Of these 37 346 CpGs, we selected the ones that changed significantly with training (606 CpGs with $P < 0.01$, M-value difference > 0.4). Based on these 606 CpGs, cluster analysis effectively clustered older post-training samples with younger baseline samples, instead of with older baseline samples (*Figure* 5). Pathway analysis linked four pathways to these CpGs (FDR < 0.1): 'cortisol synthesis and secretion' (ES = 9.6, FDR < 0.001), 'cAMP signalling pathway' (ES = 7.4, FDR = 0.067), 'adrenergic signalling in cardiomyocytes' (ES = 6.8, FDR = 0.067) and 'aldosterone synthesis and secretion' (ES = 6.75, FDR = 0.067). Most dmCpGs in these four pathways were hypermethylated in older compared with young muscle at baseline with a hyper/hypo ratio (i.e. number of hypermethylated CpGs/number of hypomethylated CpGs) between 2.7 and 11. Training had the opposite effect, directing the methylation level towards younger levels.

In total, 2657 CpGs were hypomethylated and 2163 CpGs were hypermethylated after training compared with baseline ($P < 0.01$, M-value difference > 0.4) (*Figure* 6A, *Data* S2a). In comparison, 12 weeks of normal daily living changed the methylation status of merely 255 CpGs in the older control

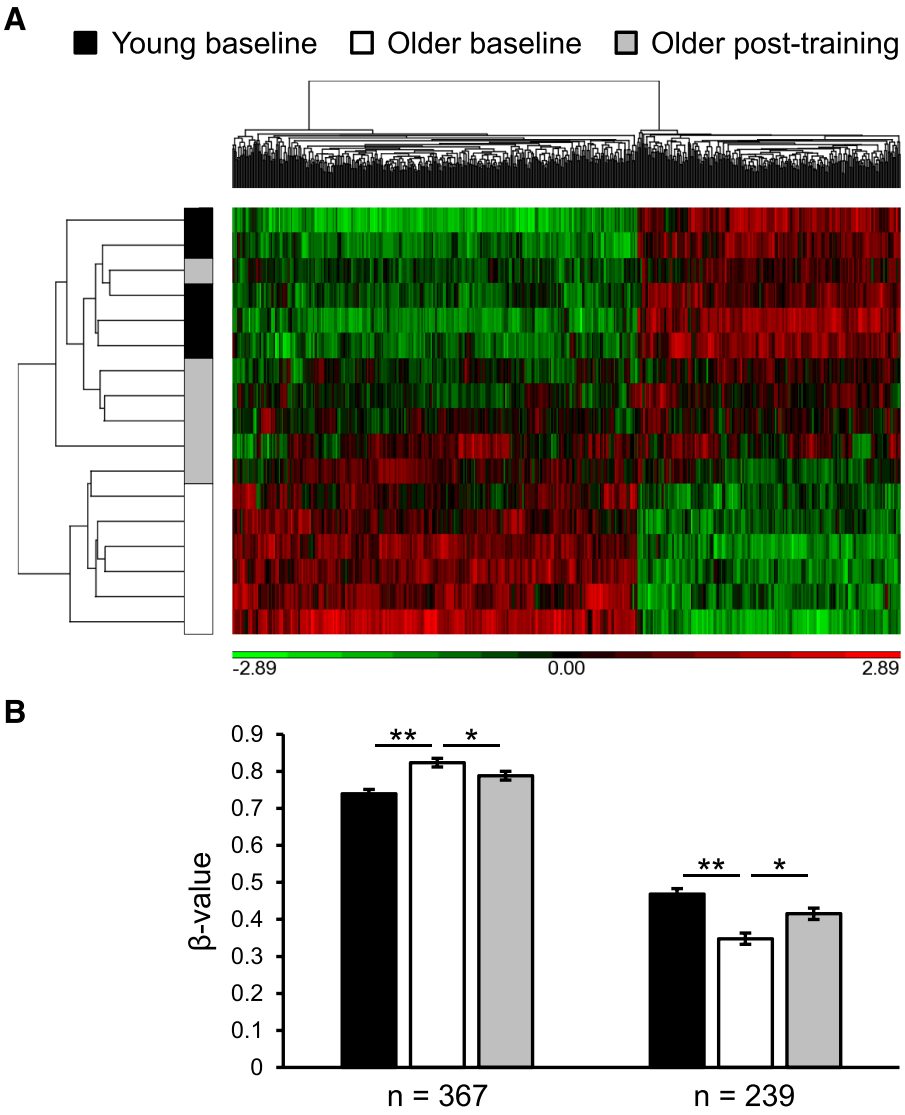
Figure 4 Number of hypomethylated (green) and hypermethylated (red) genes that displayed differential expression between young (n = 5) and older muscle (n = 6) (FDR < 0.1). Left of the Y-axes are downregulated genes, right are upregulated genes. Genes were categorized according to number of dmCpG sites, methylated in the same direction per gene region.



group ($P < 0.01$, M-value difference > 0.4) (*Figure* 6A, *Data* S3a). With respect to CpG islands and shores in these comparisons, training had a hypomethylating effect (*Figure* 6B). Comparable with the results in younger men, the dmCpGs were not overrepresented or underrepresented in any region (*Data* S2d). Of the 20 DEGs following training, only 1 (*TM4SF1*) displayed similar transcriptional changes following training as comparable training studies in older adults in the metaMEx meta-analysis (*Data* S2h, 1 showing the same change, 0 showing the opposite change, 17 showing no change, and 2 not found in MetaMEx). Against expectations, there were more genes downregulated than upregulated following a first training period (*Figure* 6A). Four of these genes (*PPDPFL*, *BBC3*, *PER1*, and *MCL1*) were upregulated in older versus young muscle (FDR ≤ 0.068) and subsequently rejuvenated (i.e. downregulated) following training (FDR ≤ 0.038) (*Data* S1e and S2h). Twelve weeks of detraining, subsequently reverted the training effect and increased expression levels again in *PPDPFL*, *PER1*, and *MCL1* (FDR ≤ 0.040) (*Data* S2i). In addition, detraining reversed training adaptations in RNA expression of seven more genes (*Data* S2i), as well as reverting methylation levels of 628 of the 4820 dmCpGs following training (*Figure* 6A, *Data* S2b). Globally, detraining mainly induced hypermethylation compared with post-training and more genes were downregulated (54) compared with upregulated (40) (*Figure* 6A,B). Eleven of these 94 DEGs were transcriptionally altered in the same direction as a 5 day bed rest study in elderly men,[57] as displayed by the MetaMEx tool (*Data* S2i). On the other hand, RNA expression of 10 genes changed in the opposite direction as this disuse study. In addition, 43 upregulated and 28 downregulated genes in our study did not show significant expression changes by bed-rest in MetaMEx (*Data* S2i). These findings indicate that detraining and bed-rest might have a different effect on certain genes.

Similar to the results in young men, retraining hypomethylated more CpGs compared with training (3077 vs. 2657) (*Figure* 6A), which was observed across all gene regions and structures (*Figure* 6B). Pathway analysis identified 48, 66 and 107 enriched pathways (FDR < 0.1) following training, detraining, and retraining, respectively (*Table* 1, *Data* S2e-g). The majority of the pathways influenced by training were also differentially methylated following detraining and/or retraining, whereas 34 pathways were uniquely affected by retraining (*Figure* 6C), suggesting that the methylome was more responsive to retraining. We found 18 pathways in common between each intervention period (*Figure* 6C). In the majority of the overlapping pathways,

Figure 5 (A) Heat map of normalized β-values (shifted to mean of zero and scaled to SD of one) of CpGs (n = 606) that had significantly different methylation levels between young (n = 5, black) and older men at baseline (n = 6, white) and were reverted to younger levels following training in the older men (n = 6, grey). One CpG per column. Methylation levels are compared between subject groups (rows), with higher methylation coloured red and lower methylation coloured green. CpGs were hierarchically clustered in two categories: CpGs that are hypomethylated (left, n = 367) and CpGs that are hypermethylated (right, n = 239) following training in older men. (B) Average β-values of both clusters in each subject group. Values are mean ± SEM. **FDR < 0.1 and difference between M-values > 0.4, *P < 0.01 and difference between M-values > 0.4.

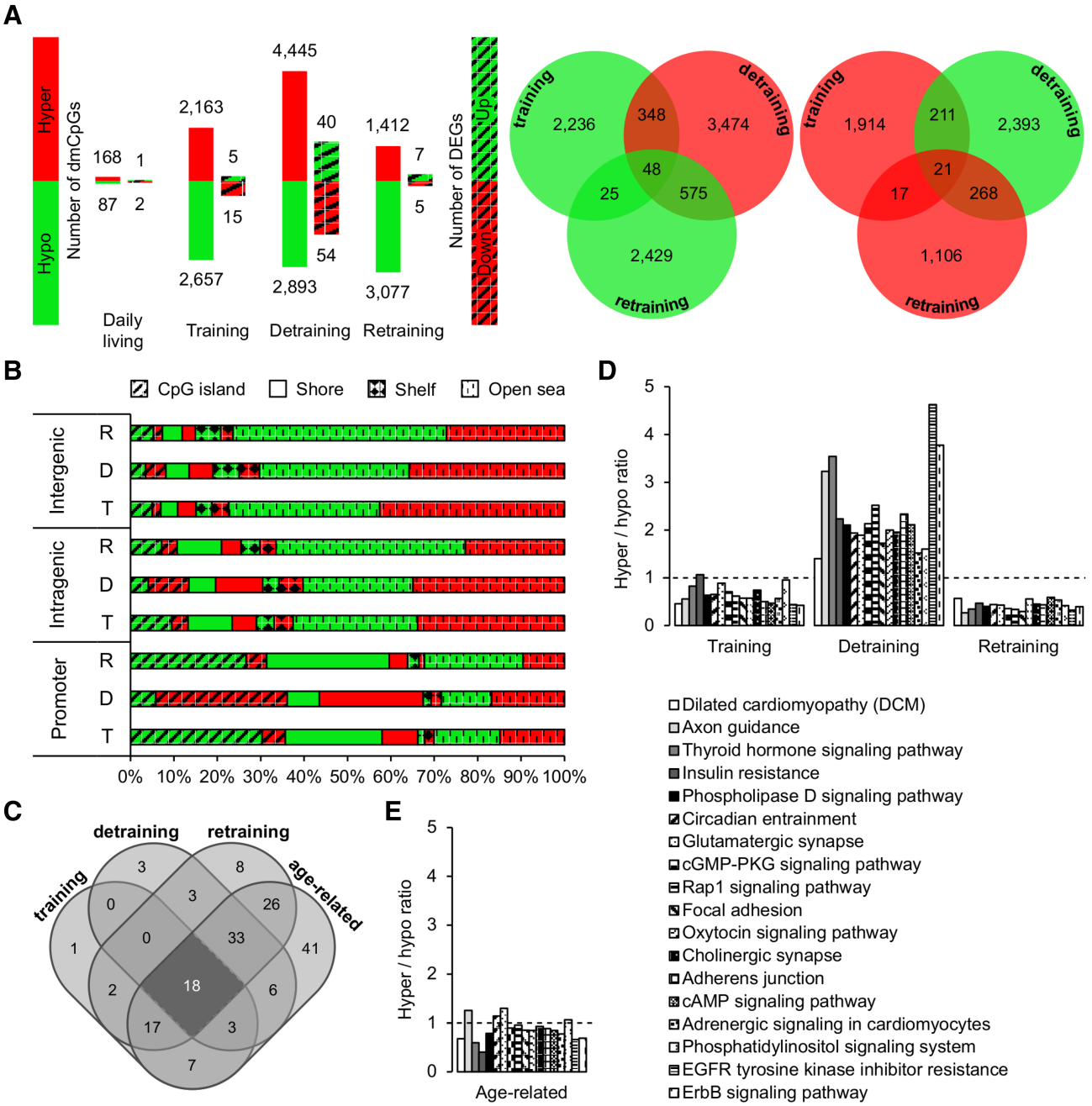


the number of hypomethylated CpGs further increased following retraining compared with training (*Figure* 6D). This could be indicative for an epi-memory within these pathways. Furthermore, these 18 pathways were also enriched when comparing the young and older muscle methylome at baseline (*Figure* 6E). Only 12 genes were transcriptionally altered following retraining (*Figure* 6A, *Data* S2j). However, half of them were also picked up by comparable training studies of the MetaMEx meta-analysis and are involved in inflammation (*IL17D*), muscle growth inhibition (*PLN*), muscle contractions (*TPM3*), mechanosensing (*ANKRD2*), muscle structure (*MYL5*), and energy metabolism (*CARNS1*) (FDR ≤ 0.091) (*Data* S2j). Four other upregulated genes, not picked up by the meta-analysis, are involved in myogenic differentiation (*SOX11*), energy metabolism (*AK1*, *PGK1*), and muscle development and functioning (*PDLIM7*) (FDR ≤ 0.097) (*Data* S2j).

Genes indicative for an epi-memory in young and older skeletal muscle

Of the differentially expressed genes across all intervention phases in young and older muscle (FDR < 0.1, average read count ≥ 5), 11 to 33% contained at least one dmCpG in the

Figure 6 (A) Number of hypomethylated and hypermethylated CpGs ($P < 0.01$, M-value difference > 0.4; no pattern) versus upregulated and downregulated genes (DEGs) (FDR < 0.1, average read count ≥ 5; dashed pattern) following normal daily living ($n = 3$), training, detraining, and retraining ($n = 6/5$, cfr. Outlier RNA expression) compared with previous time point in older muscle. Venn diagrams displaying overlap between dmCpGS following training, detraining and retraining (hypomethylated = green; hypermethylated = red). Alternative overlap patterns are presented in *Data* S2a. (B) Distribution of dmCpGs (% of the total, hypomethylated = green; hypermethylated = red) following training (T), detraining (D), and retraining (R) in older muscle across gene regions and gene structures. (C) Venn diagram displaying the overlap of enriched pathways (FDR < 0.1) following training, detraining, and retraining in older muscle and age-related pathways in older versus young muscle. (D, E) The hyper/hypo ratio of the 18 overlapping pathways identified in 'c'. This ratio is calculated by dividing the number of hypermethylated CpGs with the number of hypomethylated CpGs. The dashed line (ratio = 1) delimitate an equal level of hypermethylated and hypomethylated CpGs within a pathway.



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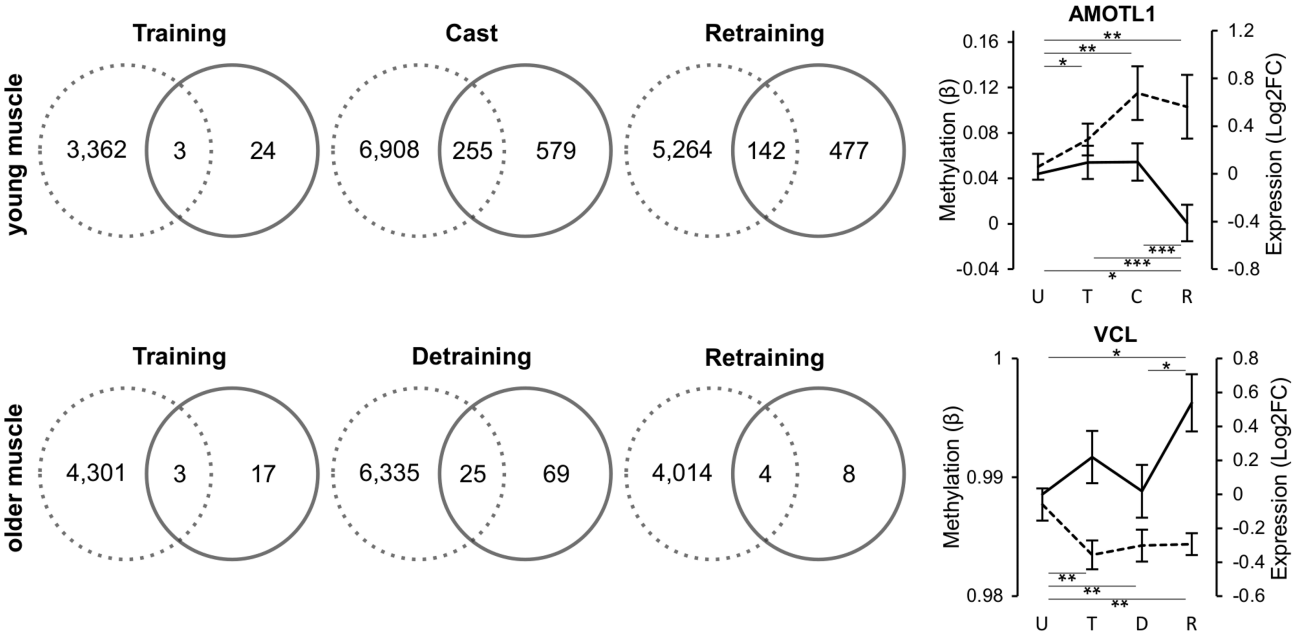
promoter or intragenic region ($P < 0.01$, M-value difference > 0.4) (*Figure* 7). Detailed information on these genes, including plots, is available in *Data* S2l-m and S4l-m. The interaction between the transcriptome and methylome was stronger following retraining compared with training. This might indicate that other epigenetic mechanisms were responsible for the alterations in chromatin accessibility during the first training period, whereas methylation was used during retraining to stabilize these changes.

We were specifically interested in genes constituting the epi-memory. Epi-memory genes can be defined as genes displaying an enhanced transcriptional response to retraining compared with training that could be linked to retained methylation modifications from the first training period (genes with retained methylation levels over time displayed in *Data* S2k and S4k). In young muscle, one CpG (cg15758225) within the open sea area of intron 3 of *AMOTL1* became more methylated following training ($P = 0.006$), which persisted during immobilization ($P < 0.001$) and retraining ($P < 0.001$) (*Figure* 7). As response to the second training period and potentially the hypermethylated state of cg15758225 pre-retraining, *AMOTL1* expression levels decreased (FDR = 0.012). This might indicate an epi-memory within *AMOTL1* and we found confirmation in the muscle biopsy data from Seaborne *et al.*,[17] as the hypermethylated state of two CpGs (cg13554187 and cg06447552) within *AMOTL1* following loading, was retained following unloading and for cg13554187 also following reloading. The investigators found several retraining-upregulated genes themselves indicative for an epi-memory in young muscle (*AXIN1*, *TRAF1*, *GRIK2*, and *CAMK4* in Seaborne *et al.*[17] and *FLNB*, *MYH9*, *SRGAP1*, *SRGN*, and *ZMIZ1* in Turner *et al.*[28]). Despite finding one to three hypomethylated CpGs following retraining in *FLNB*, *MYH9*, *ZMIZ1*, *GRIK2*, and *CAMK4*, we did not find the expected increased expression levels in these genes. We did find several genes with a trend towards enhanced expression levels following retraining and a correlated methylation pattern in young muscle (e.g. *NEXN*, *SERF2*, and *PLCL2*) (*Data* S4m). Seaborne *et al.*[17] also reported on a list of genes (*UBR5*, *RPL35a*, *C12orf50*, *BICC1*, *ZFP2*, *HEG1*, *PLA2G16*, *SETD3*, and *ODF2*) presenting a different pattern of epigenetic memory with hypomethylation responses by loading, no retention of methylation during detraining, followed by a stronger hypomethylation and associated increased gene expression during reloading. Specifically, the role of *UBR5* in muscle atrophy and hypertrophy was further confirmed by *in vivo* and *in vitro* studies in mice and rats by the Sharples group.[67,68] Although not reaching the criteria ($P < 0,01$, M-value difference >0.4) a pattern indicating hypomethylation by training ($P = 0.02$), hypermethylation by casting ($P = 0.01$) and again hypomethylation by retraining ($P = 0.017$) in cg21230538 in the young muscle was supportive of the role of epigenetic changes in *UBR5* due to chronic

Figure 7 Overlap between differentially methylated (dotted) and expressed (full) genes following training (T), casting (C)/detraining (D), and retraining (R) compared with the previous time point in young (n = 5/4, cfr. Drop out after week 12) and older muscle (n = 6/5, cfr. Outlier post-retraining RNA expression). *AMOTL1* and *VCL* are genes indicative for an epi-memory in young and older muscle, respectively. Methylation values (dotted) are model-adjusted means ± SEM. Expression values (full) are model-adjusted log2 fold change values compared with baseline ± SEM. *$P < 0.01$, **$P < 0.001$, ***FDR < 0.1.



training and unloading. This pattern was however not found in older muscle or in other CpGs in this gene region.

In older men, we identified *VCL* as a gene with retained epigenetic modifications (*Figure* 7). Promoter-associated and intron-associated cg15758225, located in open sea area, was less methylated after training, detraining and retraining compared with baseline (all $P < 0.001$). This methylation change might have contributed to the trend towards an enhanced expression level following retraining (FDR = 0.136, $P = 0.002$). In addition, we found several other genes with a trend towards enhanced expression levels following retraining and a correlated methylation pattern in older muscle (e.g. *SOX11*, *PDLIM7*, and *PGK1*) (*Data* S2m).

Discussion

In this study, we identified CpG methylation as a potential upstream mechanism that can explain transcriptional changes in skeletal muscle tissue associated with ageing and training. Interestingly, recurrent RT was accompanied with increasing numbers of hypomethylated CpGs and upregulated genes in young and older skeletal muscle and rejuvenated methylation levels of several CpGs altered by ageing. In addition, we found indications of an epi-memory within *AMOTL1* in young muscle and *VCL* in older muscle.

There have been many studies trying to reveal the age-associated human muscle transcriptome signature,[69–72] but literature lacks the integration of the mechanisms behind these transcriptional changes. We discovered that of the 427 DEGs between young and old, 71% contained at least one dmCpG. This confirms that DNA methylation might be an important regulator of gene expression in muscle tissue. Furthermore, we found that the more dmCpGs methylated in the same direction, the clearer the inverse relationship between DNA methylation and RNA expression and this was regardless of the location of the dmCpG within the gene. It has been reported that a threshold of three CpGs is required to effectively inhibit transcription, with more CpGs and closer proximity to the promoter region associated with a higher percentage of gene repression.[73] This explains why some but not all methylome adaptations over time mediated gene expression alterations in the present study, something that has been observed before in blood tissue.[74,75] In addition, the relationship between methylation and expression is more complex than a simple inverse correlation, as evidenced by the combination of both hypomethylated and hypermethylated CpGs within the different gene regions and structures of some genes. The complex transcriptome–methylome relationship has been described before,[66] but lacked tissue specificity. Therefore, we warrant further investigation into the interplay between methylation and other epigenetic mechanisms in muscle tissue (e.g. histone deacetylation, which works closely together with DNA methylation to silence genes[73]) and their role in the different gene regions and structures. Interestingly, older muscle tissue displayed signs of impaired myogenesis, mitochondrial bioenergetics, autophagy and glucose uptake, as well as indications of muscle atrophy and inflammageing at methylome and transcriptome level. These signs are closely related to cellular senescence,[76] one of the hallmarks of ageing,[77] and might contribute to age-related anabolic resistance and impaired skeletal muscle growth in response to an anabolic stimulus.[78,79] In accordance, we reported earlier that a first training period was unable to induce significant muscle growth in these same older men.[29] In the present study, we were able to link this impaired muscle growth to ageing-induced molecular dysregulation, both at transcriptional and epigenetic level.

Remarkably, 73% of the age-related dmCpGs in untrained older muscle were no longer differentially methylated following 12 weeks of RT compared with young untrained muscle. The ability of resistance exercises to reverse age-related impairments has been extensively described at the muscle phenotypic level,[80–82] proteome level,[53] transcriptome level,[55,61] and other hallmarks of ageing.[83] For the first time, we confirm this finding at methylome level. We are aware of only one study[53] that investigated the effect of RT on the older muscle methylome. However, the investigators were unable to find methylation changes after 12 weeks of RT, probably due to the low sample size, the strict FDR adjustment and the limited number of CpGs tested. We hypothesized that training would reverse the ageing-induced hypermethylation, predominantly found in older muscle tissue in literature,[8,9] based on the hypomethylating effect of aerobic exercise,[10–13] (lifelong) physical activity,[16,84] and RT in young to middle-aged adults.[13,85] However, both in young and in older muscle tissues, we observed that a first training period induced both hypomethylation and hypermethylation. That said, mainly training-induced hypomethylation was found in CpG islands and shores, possibly counteracting the ageing-induced hypermethylation in promoter-associated CpG islands of the older muscle samples. At transcriptional level, training altered 20 genes in older men. Interestingly, *PPDPFL*, *BBC3*, *PER1*, and *MCL1* were upregulated in older versus young muscle, downregulated following training, and upregulated again during detraining in older men. The role of *PPDPFL* in muscular training adaptations is currently unknown. The other three genes, on the other hand, are known to be involved in apoptosis (*BBC3*, BCL2 Binding Component 3, also known as *PUMA*),[86] (*MCL1*, MCL1 Apoptosis Regulator, BCL2 Family Member),[87] and circadian clock-regulated physiological processes (e.g. muscle hypertrophy) (*PER1*, Period Circadian Regulator 1).[88]

Both young and older muscles responded better to a second training period. This was reflected by the higher number of hypomethylated CpGs and upregulated genes following retraining compared to training. In addition, retraining

induced more hypomethylation than training did in the 7 overlapping pathways between training, immobilization and retraining in young men and in the 18 overlapping pathways between training, detraining and retraining in older men. Interestingly, these 18 pathways were also enriched in the age-related analysis, which supports the fact that chronic RT may act on several ageing-related pathways. Accordingly, the indications of inflammageing at baseline in older muscle tissue were only improved by the second training period, and not by the first. We observed nine hypomethylated dmCpGs in proximity to the promoter region of the pro-inflammatory cytokine Interleukin 17D (*IL17D*) when comparing older to young muscle, which was associated with elevated *IL17D* RNA expression in older muscle. *IL17D*, which also activates the acute inflammatory response mediated by NF-κB in the first hours after intense RT,[89] was successfully downregulated following retraining compared to post-detraining, whereas the decline in expression was not significant following the first training period. In addition, we found one hypermethylated dmCpG within *IL17D* following retraining. In agreement, NFKB Inhibitor Alpha (*NFKBIA*), inhibitor of NF-κB signalling, was downregulated following 12 weeks training. *NFKBIA* downregulation could be indicative for a permanent state of inflammation in older exercise-stimulated muscles. Surprisingly, *NFKBIA* expression levels returned to baseline levels during detraining and remained stable during retraining. These results indicate that previously trained older muscle may be less susceptible to exercise-induced chronic inflammation and recurrent RT may in fact improve age-related low-grade inflammation, as has been reported before.[90]

Most interestingly, we identified two genes, indicative for an epi-memory within young and older muscle. In young men, the epi-memory pattern was reflected within the Angiomotin Like 1 (*AMOTL1*) gene. Upon activation of satellite cells, *AMOTL1* activates *YAP1* which triggers pro-proliferating genes and inhibits differentiation of myotubes.[91] The decreased expression following retraining might signal myotube differentiation. Importantly, this result was confirmed when investigating the data of Seaborne *et al*.[17] In older men, an increased expression level in Vinculin (*VCL*) following retraining was linked to the retained hypomethylated state of one CpG during training, detraining and retraining. In striated muscle, vinculin is a structural component of costameres,[92] which connects sarcomeres to the cell membrane to stabilize myofibres during contraction and relaxation. The gene, furthermore, potentiates mechanosensing[93,94] and is upregulated in skeletal muscle tissue following chronic stimulation and disuse.[95] Most interestingly, *VCL* is part of the focal adhesion pathway, which was enriched during both training, detraining and retraining in older men, as well as during training, immobilization and retraining in younger men and the age-related comparison between young and old. Focal adhesion translates the (exercise-induced) cytoskeletal stress signals into cell growth by activating a cascade of signalling pathways.[96] The importance of the focal adhesion pathway in our results might indicate that the rapid regain of training adaptations is related to improved mechanosensing signalling during retraining. To support our results, we investigated other genes related to the mechanosensing pathway and found a trend towards enhanced gene expression and several hypomethylated CpGs following retraining in mechanosensitive transcriptional cofactor-encoding *WWTR1*, stretch-activated ion channel-encoding *PIEZO2* and extracellular-matrix associated *COL1A1* (*Figure* A1).

Finally, we would like to address several limitations and future perspectives. First of all, sample sizes were small. This limited the statistical power and especially the intervention-related analyses might have been prone to type I error. Findings are consequently exploratory and preliminary in nature and should be validated in future studies. On the other hand, findings that were found significant at FDR level of 0.1 despite the limited sample size, should be considered highly interesting. To increase power, a combined analysis of both young and old muscle samples could have been performed for the first training period, as the time*group interaction term was non-significant in the mixed model (data not shown). However, we preferred to test specific hypotheses and contrasts towards the ability of strength training to decrease the large set of age-related dmCpGs between young and old at baseline. Secondly, due to the different unloading modalities (detraining versus casting), comparison of the unloading and retraining phases between young and old was limited. We chose not to apply both situations to each group, because of the complex study design and the risks associated with immobilization in elderly. Nevertheless, by using both unloading modalities, we were able to give additional insights in the differences between a more invasive type of disuse versus long-term training cessation. Thirdly, CpGs may overlap with different regions of different gene transcripts belonging to one gene and it is not clear how each gene transcript is influenced by differential methylation, as RNA expression was only analysed at gene level. Fourthly, although we did perform SVA, we did not directly correct for cell type heterogeneity and fibre type differences between muscle samples. This might have confounded the analysis.[97] Finally, while we do integrate methylome and transcriptome data, we currently lack the translation to the proteome level, which should be the focus in future research. Also needed are similar analyses at single-cell or -nucleus level and the integration of other epigenetic mechanisms to unravel the precise role of epigenetics in the muscle memory phenomenon.

To conclude, these results indicate an enhanced transcriptional training response in previously trained young and older muscle, partially explained by retained and novel methylation adaptations. This supports that we might benefit from earlier training periods and that RT is an effective strategy to rejuvenate our methylome.

Acknowledgements

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Ethical approval

The study protocol was approved by the Medical Ethics Committee of the Catholic University of Leuven (S59380). Our work conformed to the standards set by the latest revision of the Declaration of Helsinki (except for registration in a database) and to the ethical guidelines for authorship and publishing set out by the *Journal of Cachexia, Sarcopenia and Muscle*.[98]

Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Principal component analysis plots of methylation data in the intervention analysis. Plots are depicted post-normalization and post-filtering. **(A)** Plots of young muscles. PC1–3 grouped samples based on sample ID. PC4–6 grouped samples based on timing in the intervention. **(B)** Plots of older muscles. PC1–3 clustered samples based on timing in the intervention. PC4–6 clustered samples based on sample ID.

Figure S2. Principal components analysis plots of methylation data in the age-related analysis. The plot is depicted post-normalization and post-filtering. PCA1–3 clustered samples based on age (old versus young) and timing (baseline versus post-training).

Figure S3. Outlier detection in RNA expression data. (A) Sample distance plot, **(B)** heatmap and **(C)** principal component plot all identified subject P3-t3 (GC069383) of the older exercise group as outlier. In addition, the total number of read counts of this sample was particularly low ($<$ 500,000) compared to the other samples ($>$ 1,000,000). Therefore, we decided to exclude this sample from the expression analysis.

Conflict of interest

The authors report no conflict of interest.

APPENDIX A

Table A1 Important muscle-functioning genes differentially expressed and methylated in older muscle

Gene	Function		Log2fc	SE	FDR	dmCpG		Old β	Old SE	Young β	Young SE	FDR
Impaired myogenesis												
MYF6[a]	Homeostatic role by repressing hypertrophy-related genes	Up	0.96	0.17	<0.001	cg02939404	Hypo	0.34	0.02	0.53	0.03	0.065
						cg08352786	Hypo	0.33	0.01	0.45	0.02	0.045
						cg15166296	Hypo	0.24	0.01	0.35	0.02	0.050
						cg15757530	Hypo	0.42	0.03	0.65	0.03	0.040
						cg17159916	Hypo	0.39	0.01	0.58	0.04	0.067
						cg20171297	Hypo	0.19	0.01	0.33	0.01	0.042
						cg25178519	Hypo	0.45	0.02	0.67	0.02	0.035
						cg26711820	Hypo	0.31	0.01	0.40	0.01	0.063
JAK2	Inhibitor of satellite cell function via JAK/STAT	Up	1.11	0.28	0.009	cg02104682	Hypo	0.35	0.01	0.46	0.02	0.046
IGF2[a]	Autocrine and paracrine growth stimulator	Down	−1.28	0.34	0.011	cg14890224	Hyper	0.75	0.01	0.69	0.01	0.046
						cg17274401	Hyper	0.07	0.01	0.03	0.00	0.062
						cg20728696	Hyper	0.13	0.02	0.06	0.00	0.090
						cg21728792	Hyper	0.93	0.01	0.86	0.01	0.051
						cg01351425	Hypo	0.32	0.01	0.42	0.02	0.064
						cg01921126	Hypo	0.32	0.02	0.48	0.02	0.053
						cg08686462	Hypo	0.22	0.01	0.33	0.01	0.035
						cg10650127	Hypo	0.36	0.01	0.55	0.04	0.076
						cg11005826	Hypo	0.32	0.01	0.40	0.01	0.049
						cg26516510	Hypo	0.17	0.01	0.30	0.03	0.044
						cg27263998	Hypo	0.20	0.01	0.38	0.02	0.035
						cg27331871	Hypo	0.06	0.00	0.10	0.01	0.100
IGFBP2	IGFs modulator and myoblast differentiation stimulator via Akt phosphorylation	Down	−0.90	0.20	0.002	cg06571246	Hyper	0.78	0.01	0.69	0.01	0.060
Impaired mitochondrial bioenergetics												
PERM1[a]	Regulator of mitochondrial biogenesis and oxidative function	Down	−0.90	0.23	0.007	cg00380574	Hypo	0.29	0.01	0.39	0.01	0.035
						cg08843537	Hypo	0.53	0.01	0.63	0.02	0.043
						cg10201533	Hypo	0.23	0.01	0.31	0.01	0.050
						cg10381813	Hypo	0.24	0.01	0.34	0.01	0.035
						cg11143486	Hypo	0.38	0.01	0.46	0.01	0.081
						cg11782208	Hypo	0.39	0.01	0.49	0.01	0.039
						cg15526941	Hypo	0.20	0.01	0.25	0.01	0.078
						cg20869814	Hypo	0.31	0.01	0.39	0.01	0.072
						cg22583825	Hypo	0.18	0.01	0.24	0.00	0.046
						cg22599150	Hypo	0.36	0.00	0.43	0.01	0.044
						cg22699004	Hypo	0.29	0.02	0.42	0.01	0.073

(*Continues*)

Table A1 (continued)

Gene	Function		Log2fc	SE	FDR	dmCpG		Old β	Old SE	Young β	Young SE	FDR
NOS1	Regulator of mitochondrial bioenergetics, excitation-contraction coupling, muscle force generation, auto-regulation of blood flow, calcium homeostasis and myogenesis	Down	−0.61	0.16	0.008	cg01207748	Hyper	0.79	0.01	0.71	0.01	0.066
						cg01686420	Hyper	0.92	0.01	0.84	0.02	0.044
						cg02500231	Hyper	0.69	0.02	0.58	0.02	0.068
						cg02595280	Hyper	0.54	0.02	0.38	0.02	0.036
						cg03019507	Hyper	0.65	0.01	0.57	0.01	0.091
						cg05923508	Hyper	0.60	0.03	0.43	0.04	0.080
						cg07846732	Hyper	0.45	0.02	0.34	0.02	0.071
						cg10914558	Hyper	0.61	0.01	0.50	0.01	0.036
						cg17119907	Hyper	0.60	0.02	0.51	0.02	0.094
						cg18243853	Hyper	0.43	0.01	0.33	0.01	0.051
						cg18947446	Hyper	0.50	0.02	0.41	0.01	0.052
						cg19945354	Hyper	0.67	0.01	0.56	0.03	0.080
						cg20358453	Hyper	0.72	0.01	0.61	0.02	0.045
						cg20840426	Hyper	0.66	0.02	0.55	0.02	0.068
						cg07748838	Hypo	0.40	0.02	0.50	0.02	0.060
						cg13023936	Hypo	0.29	0.02	0.42	0.02	0.049
SAMM50[a]	Role in structural integrity of mitochondria outer membrane via mitochondrial intermembrane space bridging	Down	−0.56	0.15	0.012	cg03373887	Hypo	0.28	0.01	0.37	0.01	0.035
						cg07494691	Hypo	0.43	0.02	0.51	0.01	0.087
						cg15212821	Hypo	0.40	0.02	0.53	0.03	0.041
						cg17161504	Hypo	0.35	0.01	0.49	0.02	0.037
						cg19172487	Hypo	0.29	0.01	0.40	0.01	0.037
COQ10A[a]	Intra-mitochondrial coenzyme in the respiratory chain	Down	−0.70	0.19	0.016	cg03319773	Hypo	0.69	0.01	0.78	0.02	0.095
						cg11631125	Hypo	0.34	0.02	0.55	0.02	0.035
						cg16272957	Hypo	0.32	0.01	0.40	0.01	0.039
						cg19969040	Hypo	0.45	0.01	0.56	0.01	0.035
						cg21147422	Hypo	0.51	0.01	0.65	0.01	0.035
						cg23196303	Hypo	0.47	0.01	0.57	0.01	0.035
Impaired glucose uptake												
ERBB2	Role in homeostasis of glucose via NRG1/ERBB2	Up	0.99	0.22	0.001	cg00459816	Hypo	0.29	0.01	0.37	0.01	0.045
						cg04048710	Hypo	0.42	0.01	0.59	0.02	0.035
						cg13236904	Hypo	0.34	0.02	0.44	0.02	0.051
						cg15227682	Hypo	0.54	0.01	0.70	0.02	0.035
						cg20498199	Hypo	0.89	0.01	0.94	0.01	0.057
Impaired autophagy												
MAP 1 LC3A[a]	Autophagy marker of autophagosome formation	Down	−0.86	0.20	0.003	cg07486199	Hyper	0.67	0.01	0.56	0.02	0.047
						cg15112783	Hyper	0.75	0.01	0.63	0.02	0.039
Signs of atrophy and impaired structural integrity												
AMPD3[a]	Regulator of protein degradation via ATP energetics	Up	1.76	0.44	0.012	cg01116568	Hypo	0.31	0.01	0.44	0.02	0.035
						cg02082273	Hypo	0.37	0.01	0.49	0.02	0.044
						cg02973178	Hypo	0.29	0.01	0.40	0.02	0.044
						cg07268332	Hypo	0.30	0.01	0.42	0.02	0.054
						cg07329251	Hypo	0.26	0.01	0.34	0.01	0.043
						cg12069276	Hypo	0.34	0.02	0.46	0.02	0.058
						cg16118534	Hypo	0.46	0.01	0.57	0.01	0.038
						cg18010131	Hypo	0.32	0.01	0.44	0.01	0.036

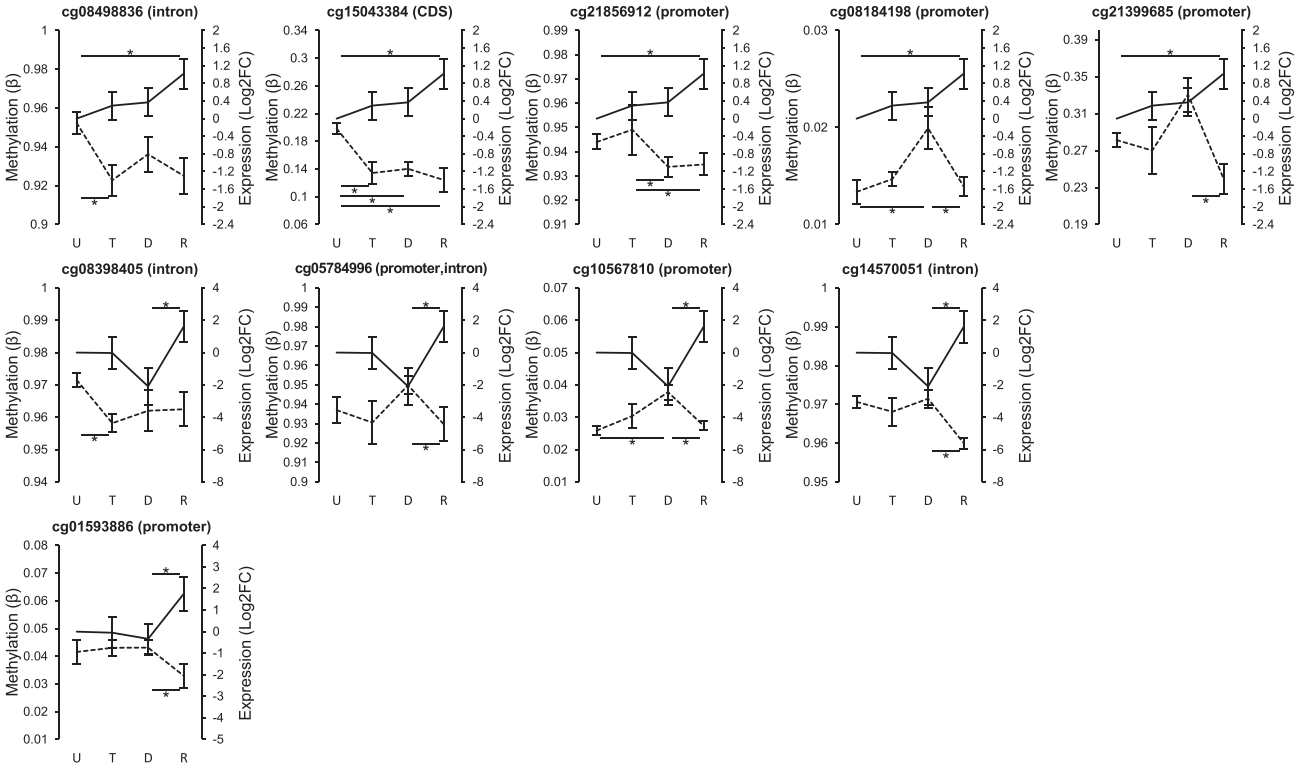
(*Continues*)

Table A1 (continued)

Gene	Function		Log2fc	SE	FDR	dmCpG		Old β	Old SE	Young β	Young SE	FDR
						cg18678492	Hypo	0.34	0.01	0.42	0.01	0.057
						cg19132462	Hypo	0.27	0.01	0.38	0.01	0.040
						cg23341488	Hypo	0.60	0.01	0.51	0.01	0.039
						cg26489413	Hypo	0.33	0.02	0.44	0.02	0.043
						cg24933917	Hyper	0.78	0.02	0.70	0.01	0.095
COL4A3	Structural component of basement membranes	Down	−1.38	0.37	0.016	cg05715709	Hypo	0.54	0.02	0.67	0.01	0.043
						cg24260952	Hypo	0.23	0.01	0.33	0.02	0.039
						cg27243623	Hypo	0.34	0.01	0.45	0.02	0.050
Inflammageing												
IL17D[a]	Stimulator of inflammatory cytokine production	Up	1.63	0.33	<0.001	cg01552711	Hypo	0.25	0.01	0.35	0.02	0.044
						cg02439964	Hypo	0.31	0.02	0.45	0.03	0.044
						cg08371706	Hypo	0.59	0.02	0.68	0.02	0.055
						cg11809038	Hypo	0.37	0.02	0.47	0.02	0.071
						cg12475590	Hypo	0.36	0.02	0.45	0.02	0.089
						cg14133014	Hypo	0.52	0.01	0.60	0.01	0.040
						cg23098661	Hypo	0.31	0.01	0.39	0.01	0.047
						cg24495327	Hypo	0.17	0.01	0.32	0.03	0.035
						cg25710235	Hypo	0.31	0.03	0.44	0.01	0.042
HMGB1[a]	Initiator of inflammatory response via innate immune system activation	Up	0.85	0.20	0.003	cg01504447	Hypo	0.24	0.02	0.36	0.03	0.057
						cg05818394	Hypo	0.19	0.01	0.27	0.01	0.074

Log2fc, log2 fold change of normalized RNA expression counts; SE, standard error; FDR, false discovery rate-adjusted *P*-value; dmCpG, differentially methylated CpG; β, methylation β-value.
Superscript 'a' in the first column denotes significant differentially methylated region based on MetaMeth analysis (https://sarah-voisin.shinyapps.io/MetaMeth/, details presented in *Data* S1).

Figure A1 Enhanced expression following retraining and inversely correlated methylation levels in mechanosensing-related genes *WWTR1*, *PIEZO2*, and *COL1A1*. Data from untrained (U) trained (T), detrained (D) and retrained (R) older muscle samples (n = 6/5, cfr. outlier post-retraining RNA expression). Methylation values (dotted) are model-adjusted means ± SEM. Expression values (full) are model-adjusted log2 fold change values compared with baseline ± SEM. *$P < 0.01$.



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