High-Intensity Interval Training Remodels the Proteome and Acetylome of Human Skeletal Muscle

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23 **Abstract**

24 Exercise is an effective strategy in the prevention and treatment of metabolic diseases. Alterations in
 25 the skeletal muscle proteome, including post-translational modifications, regulate its metabolic
 26 adaptations to exercise. Here, we examined the effect of high-intensity interval training (HIIT) on the
 27 proteome and acetylome of human skeletal muscle, revealing the response of 3168 proteins and 1263
 28 lysine acetyl-sites on 464 acetylated proteins. We identified global protein adaptations to exercise
 29 training involved in metabolism, excitation-contraction coupling, and myofibrillar calcium
 30 sensitivity. Furthermore, HIIT increased the acetylation of mitochondrial proteins, particularly those
 31 of complex V, likely via non-enzymatic mechanisms. We also highlight the regulation of exercise-
 32 responsive histone acetyl-sites. These data demonstrate the plasticity of the skeletal muscle proteome
 33 and acetylome, providing insight into the regulation of contractile, metabolic and transcriptional
 34 processes within skeletal muscle. Herein, we provide a substantial hypothesis-generating resource to
 35 stimulate further mechanistic research investigating how exercise improves metabolic health.

36

Introduction

Exercise training is the most effective means to improve cardiovascular fitness and metabolic health, both being predominant determinants of life expectancy and all-cause mortality¹. Alterations to the proteome of skeletal muscle underpin its adaptations to exercise training, including glucose and fatty acid metabolism, insulin sensitivity, mitochondrial respiration, immune function and excitation-contraction coupling²⁻⁸. Furthermore, proteome-wide post-translational modifications play an important role in regulating metabolism via modulating signaling, protein stability and enzyme activity^{9,10}, and are sensitive to exercise stimuli¹¹⁻¹³.

The application of mass-spectrometry-based proteomics has vastly expanded the catalog of post-translationally modified proteins, including acetylated proteins, and in doing so has revealed novel insights into both histone and non-histone acetylation¹⁴⁻¹⁷. Nevertheless, the understanding of the acetylome in human skeletal muscle is still incomplete and remains to be studied in response to exercise training. Numerous cellular processes are regulated by protein acetylation, including transcription, metabolism, apoptosis, growth and muscle contraction among others¹⁶. Lysine acetylation is an evolutionarily conserved post-translational modification, whereby lysine acetyltransferases catalyze the transfer of an acetyl group from acetyl-CoA to the ϵ -amino acid side chain of lysine and deacetylases remove acetyl groups from lysine residues. Alternatively, acetyl groups can be non-enzymatically transferred to lysine from acetyl-CoA. In human *vastus lateralis* muscle biopsies from male athletes, 941 acetylated proteins containing 2811 lysine acetylation sites have been identified¹⁸. Mitochondria were the cellular component of human skeletal muscle exhibiting the greatest proportion of acetylated proteins¹⁸. Furthermore, the sensitivity of the acetylome to physiological stimuli has been demonstrated in rodents in response to acute fasting and

chronic caloric restriction in liver¹⁹⁻²¹ as well as acute exercise¹² and high-fat diet²² in skeletal muscle¹².

Continual advances in mass spectrometry-based proteomic technologies and approaches are increasing the depth and coverage of proteomic analyses. Nevertheless, proteome and proteome-wide post-translational modification analyses of skeletal muscle tissue remain highly challenging due to a wide dynamic range of protein expression²³. Single-shot data-dependent acquisition (DDA) analyses are often limited in their protein identifications and are characterized by relatively poor data completeness (i.e. high number of missing values)²⁴. However, the relatively recent development of data-independent acquisition (DIA) is helping to vastly reduce the proportion of missing data while simultaneously improving proteome depth in single-shot analyses of complex tissues²⁵. Nonetheless, DDA in combination with peptide-level fractionation remains a useful approach to overcome sample complexity, particularly when performing post-translational modification proteomics.

In this study, untrained men undertook five weeks of high-intensity interval training (HIIT) and *vastus lateralis* muscle biopsies were collected before and after HIIT for proteomic and lysine-acetylation analyses. We produced the largest single-shot human skeletal muscle proteome to date and undertook the first investigation of the acetylomic adaptations to exercise training within human skeletal muscle. HIIT remodeled skeletal muscle favoring mitochondrial biogenesis, but induced changes in protein expression indicative of reduced calcium sensitivity. Furthermore, HIIT increased acetylation, particularly of the mitochondria and enzymes of the tricarboxylic acid (TCA) cycle.

Results and Discussion

Physiological adaptations to HIIT

Eight untrained men (23–38 years of age) completed a HIIT regimen (Fig. 1A) that consisted of five weeks of supervised cycling, performed as 4 – 5 × 4-min intervals at a target heart rate of >90%-max interspersed by 2 mins of active recovery, undertaken three times weekly. Participants were healthy, non-smokers and occasionally active, but otherwise untrained. Characteristics of the participants are presented in Fig. 1B. Participants experienced a 14% and 17% improvement in $\dot{V}O_{2\max}$ and incremental peak power output, respectively, during HIIT (Fig. 1B and C; $p < 0.001$), which compares favorably to typical HIIT-induced adaptations²⁶. In addition, participants gained an average of 1.0 ± 0.3 kg lean mass during HIIT training, without changes in fat mass (Fig. 1B). The rate of fat oxidation during submaximal cycling (50-150 W) increased following HIIT (Fig. 1D), while no apparent changes were observed for carbohydrate oxidation (Fig. 1E).

HIIT increases mitochondrial respiratory capacity of skeletal muscle

Resting muscle biopsies were collected from the *vastus lateralis* before and three days after the final training session. Mitochondrial respirometry analyses were immediately performed on the freshly excised muscle samples using a substrate-uncoupler-inhibitor titration protocol²⁷ on a high-resolution O2K respirometer (Oroboros, Innsbruck, Austria). In line with previous findings²⁸⁻³³, HIIT increased mitochondrial respiration across a range of respiratory states, increasing CI+II coupled respiration by 30% ($p < 0.001$) and electron transport system capacity by 15% ($p = 0.049$) (Fig. 1F). Furthermore, HIIT induced a robust increase in citrate synthase activity by 42% ($p = 0.002$) (Fig. 1G), a validated marker of mitochondrial volume in human skeletal muscle³⁴.

Elevated mitochondrial respiration is underpinned by increased expression of mitochondrial proteins following HIIT

107 Skeletal muscle biopsy samples were prepared for liquid-chromatography tandem mass spectrometry
 108 and measured using single-shot DIA²⁵ (Fig. 1H). We obtained extensive proteome coverage
 109 identifying 3343 proteins (Table S1), the largest human single-shot skeletal muscle proteome to date.
 110 After applying a filter of 4 valid values out of the 8 participants in at least one time point, 3168
 111 proteins were quantified within skeletal muscle (Table S2). Due to the nature of DIA, few missing
 112 values required imputation in each sample (Fig. S1A; mean proteins quantified per sample: $2853 \pm$
 113 33). Good correlation (median correlation: 0.88) between biological replicates (participants at the
 114 same time point) was apparent (Fig. S1B). Furthermore, the proteome contained good coverage across
 115 multiple cellular compartments (Fig. S1C). Thus, our DIA proteome analysis displayed extensive
 116 coverage and data completeness.

117
 118 HIIT regulated 126 proteins (permutation-based FDR<0.05). Of these, 102 proteins were upregulated,
 119 while 24 were downregulated (Fig. 2A, Table S2). These included the upregulation of classical
 120 endurance exercise training-responsive mitochondrial proteins (e.g. cytochrome c oxidase subunit 5b;
 121 COX5B & NADH dehydrogenase 1 alpha subcomplex subunit 7; NDUFA7), as well as proteins
 122 involved in NAD⁺ metabolism (e.g. nicotinamide phosphoribosyltransferase; NAMPT), branched-
 123 chain amino acid metabolism (branched-chain alpha-ketoacid dehydrogenase kinase; BCKDK) and
 124 ubiquinone biosynthesis (5-demethoxyubiquinone hydroxylase; COQ7), the latter of which we have
 125 recently described in exercise-trained mouse skeletal muscle³ (Fig. 2A). We also identified novel
 126 exercise-training regulated proteins, including, but not limited to, glutaminyl-tRNA synthase (QARS)
 127 and rab GDP dissociation inhibitor alpha (GDI1). QARS, which catalyzes the glutamate
 128 aminoacylation of tRNA and thus plays a central role in translation (Deutscher, 1984), was amongst
 129 the most significantly upregulated proteins within the proteome. In addition, GDI1, which inhibits

insulin-stimulated glucose uptake by preventing the dissociation of GDP from Rab10³⁵, was lowered, providing a novel potential mechanism for the insulin-sensitizing effects of exercise training.

After filtering for differentially regulated proteins, enrichment analysis using Fisher's exact test indicated the predominant regulation of mitochondrial proteins (e.g. GOCC: mitochondrial part and mitochondrial inner membrane) (Fig. 2B, Table S3). Hierarchical clustering analysis on z-scored differentially regulated proteins identified that the mitochondrial terms were enriched within the upregulated proteins (Fig. 2C). Summed protein abundances also demonstrated mitochondrial biogenesis (Fig. 2D), including upregulated protein content of electron transport chain complexes (Fig. 2E and S2), mitochondrially-encoded proteins (Fig. 2F) and proteins containing a mitochondria-targeting transit peptide (Fig. 2G). Thus, the increase in the proteome mirrors the functional increase in mitochondrial respiratory capacity of skeletal muscle after HIIT (Fig. 1F).

HIIT regulates proteins involved in skeletal muscle excitation-contraction coupling

We next investigated whether HIIT influenced the fiber-type proportions of skeletal muscle. While HIIT did not change skeletal muscle fiber-type composition in terms of myosin heavy chain isoforms (Fig. 3A) and myosin light chain proteins (Fig. 3B), a coordinated regulation of proteins modulating myosin light chain phosphorylation was identified (Fig. 3C). Expression of myosin light chain kinase 2 (MYLK2) decreased concomitantly with a reduction in smoothelin-like protein 1 (SMTNL1), an inhibitor of the myosin phosphatase complex³⁶ and an increase in myosin phosphatase-targeting subunit 1 (PPP1R12A), the myosin-targeting regulatory subunit of protein phosphatase 1³⁷ (Fig. 3C). Reduced MYLK2 expression was confirmed by immunoblotting (Fig. S3A). Furthermore, we have previously detected these adaptations in slow- and fast-twitch muscle fibers following moderate-intensity continuous cycling training². Given that phosphorylation of myosin light chains promotes

154 myofibrillar calcium sensitivity³⁸, the reduction in both MYLK2 and SMTNL1 alongside an increase
 155 in PPP1R12A indicates an adaptation towards reduced myosin phosphorylation and lowered calcium
 156 sensitivity following exercise training. Indeed, myofibrillar calcium sensitivity decreases following
 157 high-intensity sprint training in untrained males, particularly at a low pH representing physiological
 158 exercising conditions³⁹. Although the functional importance of such adaptations remains elusive,
 159 knockdown of Smtnl1 augments exercise training-induced improvements in endurance performance,
 160 while sedentary Smtnl1^{-/-} mice display a fiber-type switch mimicking endurance exercise training³⁶.
 161 Although we did not observe any concomitant myosin fiber-type switching (Fig. 3A), the changes
 162 observed in proteins regulating myosin phosphorylation following HIIT could result in a right-shift
 163 of the Ca²⁺-force relationship, meaning that a higher myoplasmic calcium concentration would be
 164 required for a given force production. This decline in myofibrillar calcium sensitivity may, however,
 165 support faster off-kinetics of calcium from troponin-C to be re-sequestered in the sarcoplasmic
 166 reticulum. Therefore, the changes observed in proteins regulating myosin phosphorylation following
 167 HIIT provide an intriguing mechanism for a HIIT-induced reduction in calcium sensitivity and
 168 warrants further attention.

169
 170 Providing a further link to the altered calcium handling following HIIT³⁹, we identified a
 171 downregulation of L-type calcium channel subunits, otherwise known as the dihydropyridine receptor
 172 (DHPR) (Figs. 3 D and E). DHPR controls the coupling of membrane depolarization and
 173 sarcoplasmic reticulum Ca²⁺-release in skeletal muscle via its interaction with ryanodine receptor 1
 174 (RYR1)⁴⁰. Conversely to DHPR, HIIT did not influence the expression of RYR1 (Fig. 3F) or the
 175 sarcoplasmic/endoplasmic reticulum calcium ATPases 1-3 (ATP2A1-3 (SERCAs); Fig. S3B). The
 176 observation that DHPR abundance declined following HIIT points to a ‘slowing’ of muscle fibers.
 177 Indeed, a period of training can induce rapid changes in the expression of proteins with importance

for calcium handling, irrespective of changes in fiber type distribution⁴¹⁻⁴⁵. Fast-twitch fibers have greater sarcoplasmic reticulum volume, faster calcium release and re-uptake kinetics, and a greater content of DHPR than slow-twitch fibers^{2,46,47}. Thus, the HIIT-induced reduction of DHPR expression may represent a slowing of muscle fibers independently of their myosin heavy chain expression.

Lysine acetylomics of skeletal muscle identifies the predominant acetylation of mitochondrial and acetyl-coA metabolic proteins

Skeletal muscle biopsy samples were prepared for lysine acetylomics using a PTMscan Acetyl-Lysine Motif kit (Cell Signaling Technology)¹⁷ and measured via liquid-chromatography tandem mass spectrometry using DDA. Due to limited sample availability from one participant, lysine acetylomics was performed for 7 of the participants. We identified a total of 1990 acetyl-sites on 664 proteins (Table S4). While this is fewer than the 2811 acetyl-sites on 941 proteins identified in the largest human skeletal muscle acetylome to date¹⁸, we identified 1073 acetyl-sites that were not identified in the analysis of Lundby et al (Fig. S4A), thus substantially extending the human skeletal muscle acetylome. After applying a filter of 4 valid values, out of the 7 participants in at least one time point we quantified 1263 acetylated sites on 464 proteins within skeletal muscle (Figs. 1G & 4A, Table S5). Six and 42 acetyl-sites were quantified exclusively in the pre- and post-HIIT samples, respectively. The mean number of acetyl-sites quantified in each sample was 976 (Fig. S4B). Median correlation between biological replicates (between participants at the same time point) was 0.76 (Fig. S4C), indicating greater variation in the human acetylome than the proteome, as would be expected¹⁸. The acetylome also displayed extensive coverage across multiple cellular compartments (Fig. S4D), which largely reflected the abundance distribution of the proteome (Fig. S1C), albeit with a slight relative increase in the mitochondrial identifications. There was considerable overlap with proteins

quantified in the proteome, with 421 proteins quantified in both (Fig. 4A). Of the quantified acetylated sites (1263), the majority (1232) were located on these 421 proteins (Fig. 4A). Single acetyl-sites were quantified on the majority of proteins, with a trend for decreasing frequency with additional acetyl-sites (Fig. 4B). However, several proteins were quantified with a large number of acetylation sites (e.g. ≥ 15 acetyl-sites) (Fig. 4B), examples of which include the contractile proteins titin (TTN) and slow-twitch myosin beta (MYH7).

After summing the median intensities of acetyl peptides from each protein in the pre-HIIT samples, we ranked the abundance of acetylated proteins within skeletal muscle (Table S6) and highlighted the top 10 proteins with the highest acetyl-intensity (Fig. 4C). Histone H4 (HIST1H4A) had the highest acetylation intensity, making up approximately 10% of the total acetylome intensity, supporting the regulatory role of histone acetylation in modulating transcription^{48,49}. The majority of the remaining top acetylated proteins were metabolic enzymes, including the TCA cycle proteins malate dehydrogenase (MDH2) and fumarate hydratase (FH), as well as subunits of complex V (ATP5H and ATP5O) within the electron transport chain. Superoxide dismutase (SOD2), a canonical acetylated enzyme involved in mitochondrial reactive oxygen species handling^{50,51}, also displayed high acetylation intensity. We further compared the protein abundance rank for these high-intensity acetylated proteins within the proteome (Fig. 4D). These proteins did not appear to be the highest intensity acetylated proteins simply due to their relative protein abundance. While they all fell within the top 318 proteins in the proteome, they did not make up the most abundant proteins, except for creatine kinase M-type (CKM), which is extremely abundant in skeletal muscle (Fig. 4D). To identify systematic trends in protein acetylation, we performed a one-dimensional enrichment analysis⁵² on the summed protein acetylation intensities, which ranked the proteins by acetylation intensity and identifies Gene Ontology (GO) annotations that are systematically over-represented with higher

intensities (positive enrichment factor) and lower intensities (negative enrichment factor). This identified mitochondrial terms (e.g. GOCC: mitochondrial part and mitochondrial matrix) as enriched for high acetylation intensity (Fig. 4E and Table S7). In particular, proteins of carboxylic acid metabolic processes (e.g. TCA cycle) and monovalent inorganic cation transport (e.g. electron transport chain complex V proteins) display systematically high acetylation intensity (Fig. 4E and Table S7). Furthermore, the Uniprot keyword term “muscle protein” was also enriched for high acetylation intensity, a term that mainly encompasses the contractile machinery of skeletal muscle (e.g. myosins).

In order to examine acetylation stoichiometry within skeletal muscle, we estimated the relative stoichiometries of acetyl-sites using abundance-corrected intensities (ACI) (Table S8). ACIs were calculated by dividing acetyl-peptide intensities by the intensities of their corresponding protein, with ACI values correlating with stoichiometry¹⁵. After ranking acetyl-site ACI for the pre-HIIT samples, we highlighted the top 10 acetyl-sites with the highest ACI (Fig. 4F). Trifunctional enzyme subunit alpha (HADHA), FH, HIST1H4A and 3- ketoacyl-CoA thiolase (ACAA2), which were identified as among the highest intensity acetylated proteins (Fig. 4C), all contain high stoichiometry acetyl-sites (Fig. 4F). However, nicotinamide nucleotide transhydrogenase (NNT) K70 had the highest ACI within skeletal muscle (Fig. 4F). NNT is an inner mitochondrial membrane protein, which uses the proton motive force to maintain high levels of NADPH (Hoek and Rydström, 1988) and in doing so can regulate metabolism^{53,54}. NNT is also highly acetylated in cardiac muscle⁵⁵, although the effect of acetylation on NNT activity remains to be determined. Nonetheless, NNT can regulate acetylation via NADPH-mediated regulation of histone deacetylase 1 (HDAC1) activity⁵⁴, thus acetylation of NNT may represent a feedback mechanism controlling cellular acetylation and metabolism.

250 To identify systematic trends in acetyl-site stoichiometry, we performed a one-dimensional
 251 enrichment analysis⁵² on acetyl-site ACI using the leading protein ID for relative enrichment⁵⁶. We
 252 identified the mitochondria (e.g. GOCC: mitochondrial part and mitochondrial inner membrane) and
 253 carboxylic acid catabolic processes to be enriched within higher stoichiometry acetylated proteins
 254 (Figs. 4G, 4H & Table S9). This extends previous analyses by not only showing that mitochondrial
 255 proteins make up the majority of acetylated proteins in skeletal muscle¹⁸, but also by identifying
 256 mitochondrial proteins as having relatively high stoichiometry acetyl-sites – an observation that is
 257 consistent with analyses in HeLa cells, rodent liver and yeast^{15,21,57}. Enriched for proteins with low
 258 stoichiometry acetyl-sites were proteins involved in contraction (e.g. GOCC: contractile fiber part;
 259 Figs. 4G, 4H & Table S9), including the Uniprot keyword term “muscle protein” (Fig. 4G), which,
 260 conversely, was enriched for high protein acetylation intensity (Fig. 4E). Myosin displayed the
 261 greatest enrichment for low acetylation stoichiometry (Fig. 4G). Thus, despite contractile proteins
 262 displaying high protein acetylation intensity and making up a substantial number of known acetylated
 263 proteins within skeletal muscle¹⁸, they contain acetylation sites of relatively low stoichiometry. The
 264 high acetylation intensity of these proteins is therefore likely due to high protein abundance and a
 265 large number of acetylation sites per protein. Proteins annotated to the cytosol and plasma membrane
 266 also displayed low acetyl-site ACI (Fig. 4G). This highlights differences in the inter-organelle
 267 acetylation levels of skeletal muscle and can likely be explained by subcellular compartmentalization
 268 of acetyl-CoA⁵⁸.

269 **HIIT induces acetylation of mitochondrial and TCA cycle proteins**

271 HIIT increased the mean number of acetyl-sites quantified per sample by over 100 sites (Pre: 915 ±
 272 44, Post: 1037 ± 51; $p = 0.015$; Fig. S4B). Using a stringent statistical approach of permutation-based
 273 FDR corrected paired t-tests, we identified 20 upregulated acetyl-sites and 1 downregulated site (FDR

274 < 0.05; Fig. 4A). In addition, by applying a validated, albeit less stringent, significance score (Π -
 275 value), which combines the statistical significance (p-value) with the fold change⁵⁹, we extended this
 276 to identify 257 upregulated and 26 downregulated acetyl-sites following HIIT ($\Pi < 0.05$; Fig. 5A and
 277 Table S5). In general, there was a trend for increased acetylation following HIIT (Fig. 4A), which
 278 could not be explained by changes in protein content as the fold changes in acetylation generally
 279 exceeded those of protein abundance (Fig. 5B). Therefore, we did not normalize the acetylome data
 280 to protein abundance. Within the increased sites, acetyl-sites on metabolic enzymes isocitrate
 281 dehydrogenase (IDH2), succinate CoA ligase subunit alpha (SUCLG1) and ATP synthase coupling
 282 factor 6 (ATP5J) were apparent (Fig. 5A), while hyperacetylation of SOD2 occurred at K68, 75, 122
 283 and 130 in response to HIIT (Fig. 5A). Included within the downregulated sites were acetyl-sites on
 284 annexin A6 (ANXA6), laminin (LMNA) and p38 mitogen-activated protein kinase γ (MAPK12) (Fig.
 285 5A). Interestingly, Histone H1.5 (HIST1H1B) K49 showed increased acetylation, despite
 286 significantly reduced protein abundance (Fig. 5B).

287
 288 A Fisher's exact test of HIIT-regulated acetyl-sites ($\Pi < 0.05$), using leading protein ID for relative
 289 enrichment, indicated the predominant regulation of the TCA cycle pathway, mitochondrial proteins
 290 (e.g. GOCC: mitochondrial part and mitochondrial inner membrane) and, in particular, electron
 291 transport chain complex V proteins (GOCC: mitochondrial proton-transporting ATP synthase
 292 complex) (Fig. 5C, Table S10). These GO terms were strikingly similar to the terms enriched for high
 293 acetylation intensity and stoichiometry in the pre-HIIT samples (Figs. 4E and G), indicating that
 294 proteins that are highly acetylated in the basal state are most susceptible to increased acetylation
 295 following HIIT. To further explore the regulation of HIIT-induced acetylation in skeletal muscle, we
 296 looked for consensus sequences around the acetylated lysine residues using iceLogo⁶⁰. We identified
 297 a sequence with a predominance of proximal cysteine residues as well as an aspartic acid residue in

the +1 position (Fig. 5D). The aspartic acid at +1 is a feature of mitochondrial acetylated proteins¹⁸ and its enrichment is likely due to the over-representation of mitochondrial proteins in the upregulated acetyl-sites (Fig. 5C). Proximal cysteine residues are associated with promoting non-enzymatic lysine acetylation^{15,61} via initial cysteine acetylation followed by transfer of the acetyl group to lysine⁶¹. Indeed, non-enzymatic acetylation increases alongside elevated fatty acid oxidation²⁰, as is apparent in skeletal muscle mitochondria after HIIT (Figs. 1E). This likely occurs through changes in acetyl-CoA concentration and/or increased acetyl-CoA flux²⁰. Although acetyl-CoA content within resting skeletal muscle does not change in response to exercise training⁶², acetyl-CoA levels and TCA cycle flux increase during exercise⁶²⁻⁶⁵, which may result in an accumulation of lysine acetylation during repeated bouts of exercise. Conversely, acute exercise decreases skeletal muscle acetylation in exercising rats¹², but whether this is the case for humans remains to be determined. Nonetheless, we identify a robust increase in skeletal muscle acetylation, predominantly of mitochondrial proteins, following five weeks of HIIT in humans.

Because HIIT altered the skeletal muscle acetylome, we examined proteins regulating deacetylases following HIIT. Sirtuin 1 (SIRT1), a nuclear and cytosolic deacetylase, was not quantified by mass spectrometry, and a small but non-significant trend for increased expression of the mitochondrial-localized sirtuin 3 (SIRT3) was apparent (Table S2). To further investigate this, we immunoblotted for SIRT1 and SIRT3. Expression of SIRT1 did not change, while SIRT3 increased in abundance following HIIT (Fig. 5E). Thus, mitochondrial acetylation increased concomitantly with an increase in the mitochondrial deacetylase (Figs. 5B and 5E). Indeed, SIRT3 appears to play a major role in suppressing non-enzymatic mitochondrial acetylation²¹. Therefore, the elevated SIRT3 abundance following exercise training might be interpreted as a mechanism to suppress excess acetylation, either to preserve the activity of mitochondrial enzymes⁶⁶⁻⁶⁸ or to scavenge acetyl groups for reintegration

into acetyl-CoA and energy production. Altogether, the data contained within Fig. 5 indicate a predominantly non-enzymatic acetylation of mitochondrial proteins during HIIT.

Given the enrichment of mitochondrial terms and particularly of complex V proteins in the HIIT-regulated acetylated proteins, we filtered for proteins annotated to the different complexes of the electron transport chain using the HUGO database⁶⁹ and highlighted the regulation of each acetyl-site on these proteins alongside the changes in protein abundance (Fig. 6A). While proteins of complexes I, II, III and IV showed a mixed acetylation response to exercise training, almost every quantified complex V protein had at least one acetyl-site that increased following HIIT (Fig. 6A). In fact, ATPase inhibitor (ATPIF1), one of the two complex V proteins that do not show elevated acetylation, is a negative regulator of complex V and is unlikely to be constitutively bound to the ATP synthase complex⁷⁰. Why complex V displays elevated acetylation levels both in the basal (Fig. 4E) and exercise trained states is unclear (Figs. 5C and 6A). However, as a large proportion of ATP synthase extends into the mitochondrial matrix, many subunits are likely to be more exposed to acetyl-CoA than other membrane-embedded electron transport chain complexes. In support, we detected acetylation on all of the subunits of complex V that reside or extend into the mitochondrial matrix (ATP5A1, ATP5B, ATP5C1, ATP5D, ATP5E, ATP5F1, ATP5H, ATP5J and ATP5O), while we only detect acetylation on two of the membrane-embedded subunits (ATP5L and MT-ATP8), despite detecting a further four membrane-embedded subunits in the proteome (ATP5I, ATP5J2, MT-ATP6 and USMG5). Indeed, the majority of acetylated proteins quantified on each electron transport chain complex (CI-CV) are exposed to either the mitochondrial matrix or the intermembrane space. Similarly, TCA cycle enzymes, which are localized to the mitochondrial matrix, displayed high acetylation intensity and stoichiometry in the basal state and HIIT augmented levels of acetylation at every stage of the cycle (Fig. 6B), again likely due to their proximity to acetyl-CoA. Conversely,

cytosolic proteins regulating skeletal muscle contraction showed fewer upregulated acetyl-sites with a number of downregulated sites following HIIT (Fig. 6C). Thus, proteins in close proximity to acetyl-CoA appear to be more susceptible to acetylation.

Despite widespread HIIT-induced acetylation of mitochondrial proteins, these data should be considered in the context of acetylation stoichiometry, which is generally low²¹. Median acetylation stoichiometry in rodent liver has been calculated at only 0.05%, which increases slightly to 0.11% for mitochondrial proteins²¹. Although acetylation is tissue-dependent¹⁸, skeletal muscle levels are unlikely to be vastly different. The median log₂-fold change for mitochondrial acetyl-sites following HIIT was 0.9 (the corresponding value in the proteome was 0.3), indicating that median post-HIIT stoichiometry would have remained at less than 1%. Even for the acetyl-sites with the greatest fold change in our dataset, which display log₂-fold changes in the order of 4-5 (Figs. 5A and 5B), post-HIIT stoichiometry will likely be in the single figure percentages. Thus, the levels of HIIT-induced changes in acetylation may not be large enough to influence gross activity on most metabolic enzymes. Indeed, hyperacetylation has limited impact on mitochondrial bioenergetics within murine skeletal muscle, although severe hyperacetylation increases long-chain fatty acid-supported respiration²². These data are consistent with the concomitant increase in acetylation (Fig. 5A) and mitochondrial respiratory capacity (Fig. 1F) following HIIT.

HIIT increases histone acetylation

Aside from metabolic enzymes and contractile proteins, histones are regulated by acetylation^{48,49} and may represent a more specifically regulated pool of proteins, owing to the nuclear localization of acetyltransferases⁷¹. Increases in certain acetylated histone residues are well characterized to augment transcription, with exercise known to acutely regulate H3 acetylation (e.g. H3 K4, H3 K9/14 & H3

K36)⁷²⁻⁷⁵. Here we identified 18 distinct acetylated peptides from histones, with acetylated peptides from H1.5 (HIST1H1B K48), H2B type 2F (HIST2H2BF K16/20) and H3.3 (H3F3B K23) being elevated following HIIT (Figs. 7A-D). H1.5 K48 was exclusively quantified in post-HIIT samples, however, it was only quantified in 4 out of 7 of the post-HIIT samples, which is our minimum cut off for valid values. The regulatory roles of these HIIT-regulated histone acetyl-sites are unknown. However, acetylation of the N-terminal of H2B, including K16 & K20, has been linked to transcriptional activation^{76,77}, including the expression of NAD-metabolic genes in yeast⁷⁷. The role of H3 K23 is also obscure, however, its acetylation contributes to the development of cancer through the recruitment of the transcription factor transcription intermediary factor 1-alpha (TRIM24) and the nuclear receptor estrogen receptor alpha (ERS1)^{78,79}, augmenting expression of phosphatidylinositol 4,5-biphosphate 3-kinase catalytic subunit alpha (PIK3CA), a subunit of phosphoinositide-3-kinase (PI3K), and phosphorylation of protein kinase B (AKT1)⁷⁸. Indeed, TRIM24 promotes the expression of glycolytic and TCA cycle genes in transforming human mammary epithelial cells⁸⁰, while ERS1 regulates mitochondrial biogenesis in skeletal muscle⁸¹. Whether H3 K23 regulates TRIM24, ERS1, PI3K, and AKT1 activity and influences metabolism in skeletal muscle remain unclear. Further studies are warranted to understand the transcriptional and metabolic implications of exercise training-induced acetylation on these histone sites.

387

388 **Conclusion**

389 Here, we leveraged advances in DIA mass spectrometry to produce the largest single-shot human
390 skeletal muscle proteome to date and applied this approach to ascertain exercise training adaptations.
391 Furthermore, we provide the first investigation of the human acetylome in response to exercise
392 training. In the proteome, we highlight known and novel exercise-responsive proteins, including a
393 potential mechanism to explain altered skeletal muscle calcium sensitivity following HIIT. In the

acetylome, we describe the predominant acetylation of mitochondrial proteins after HIIT, particularly of TCA cycle proteins and subunits of electron transport chain complex V, as well as highlighting the regulation of novel exercise-responsive histone acetyl-sites. Altogether, we provide a substantial hypothesis-generating resource, identifying novel exercise-regulated proteins and acetyl-sites with the aim of stimulating further mechanistic research investigating how exercise improves metabolic health.

Methods

Participants

Eight men gave their informed consent after receiving written and oral information about the study and potential risks associated with the experimental procedures. Inclusion criteria were healthy males, 18-40 years old, maximal oxygen consumption ($\dot{V}O_{2\max}$) between 45-55 mL $\cdot$ min⁻¹ $\cdot$ kg⁻¹ or 3000-4000 mL $\cdot$ min⁻¹ and BMI between 19-26 kg $\cdot$ m⁻². Exclusion criteria were abnormal electrocardiogram, chronic disease, ongoing medical treatment, and smoking. Subject characteristics are presented in Table 1. The study was approved by the National Committee on Health Research Ethics (H-17004045) and registered at clinicaltrials.gov (NCT03270475). The study complied with the Declaration of Helsinki (2013).

Experimental trials

The study consisted of two experimental days separated by five weeks of high-intensity interval training (HIIT). At each experimental day, body composition was measured by dual-energy X-ray absorptiometry (DXA) scans (Lunar iDXA, GE Healthcare, GE Medical systems, Belgium). Then, subjects rested in a supine position for 15 min and two 4 mm incisions separated by 2 cm were made

in the above the *vastus lateralis* muscle under local anaesthesia (Xylocaine 20 mg · mL⁻¹ without epinephrine, AstraZeneca, London, UK) and a muscle biopsy was taken from each incision using a Bergström needle with suction. Afterwards, $\dot{V}O_{2\max}$ was determined by indirect calorimetry using a breath-by-breath gas analyser (Oxycon Pro, CareFusion, San Diego, USA) during an incremental test on a bike ergometer (Monark LC4, Monark Exercise, Vansbro, Sweden). The incremental test consisted of 5 min rest followed by 4 min stages at 50, 100, 150 W, after which the resistance was increased in increments of 30 W · min⁻¹ until exhaustion. Time to exhaustion was used to calculate incremental peak power output (iPPO) accounting for time spent at the last increment. Subjects completed the experimental protocol the same time of day before and after the intervention and were instructed to eat a small meal with 500 mL water two hours before arriving at the laboratory. In addition, subjects abstained from alcohol, caffeine, and physical activity for 48 h before each experimental day and were instructed to maintain their activity level and diet during the study. The experimental days were performed three days before and after the first and last training, respectively.

Body composition

At each experimental day, two scans were performed to reduce scan-to-scan variation⁸². Before the first scanning, subjects rested in a supine position for 10 min to allow fluid distribution reducing intra-scan variation^{83,84}. Whole-body and leg composition were calculated using software (enCORE Forma v. 15, GE Healthcare Lunar, Buckinghamshire, UK).

Substrate utilization

Gas exchange measurements during rest and submaximal exercise were used to calculate fat and glucose oxidative as previously described⁸⁵ and corrected for protein oxidation⁸⁶. Fat oxidation and carbohydrate oxidation were calculated as:

$$Fat\ Oxidation\left(\frac{mg}{min}\right) = (1.695 \cdot \dot{V}O_2 - 1.701 \cdot \dot{V}CO_2) - \frac{1.92 \cdot 0.14 \cdot BM}{1440}$$

$$Carbohydrate\ Oxidation\left(\frac{mg}{min}\right) = (4.344 \cdot \dot{V}CO_2 - 3.061 \cdot \dot{V}O_2) - \frac{2.87 \cdot 0.14 \cdot BM}{1440}$$

where $\dot{V}O_2$ and $\dot{V}CO_2$ are in $L \cdot min^{-1}$ and BM (*Body mass*) is in kg.

Muscle biopsies

Immediately after collection, muscle biopsies were rinsed in ice-cold saline. A small piece (~15 mg) of the muscle was transferred into ice-cold preservation solution (BIOPS) for analysis of mitochondrial respiratory capacity. The remaining muscle tissue was snap-frozen in liquid nitrogen and stored at $-80^{\circ}C$. The frozen muscle samples were freeze-dried for 48 h and dissected free from connective tissues, fat and blood under a microscope in a humidity-controlled room (~25% humidity) before storage at $-80^{\circ}C$ for later analyses.

High intensity interval training

Subjects underwent a five-week high-intensity interval training intervention consisting of three weekly training sessions with $4-5 \times 4$ min intervals interspersed by two min active recovery on indoor spinning bikes. The training volume increased from four intervals during the first two weeks to five intervals during the last three weeks. During each interval, subjects were instructed to reach >90% of maximal heart rate (HR_{max}). All training sessions were supervised and subjects received verbal encouragement during all intervals. The mean HR_{max} during intervals was $96 \pm 1\%$ of HR_{max} .

Mitochondrial profiling

Mitochondrial respiratory capacity was determined in duplicate by high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) at $37^{\circ}C$ using 1-3 mg wet weight muscle

fibers per chamber in MiR06. Oxygen concentrations were kept between 200 and 450 μ M throughout the experiment. The muscle sample in BIOPS was prepared for high-resolution respirometry as previously described⁸⁷. In short, muscle fibers were dissected free from connective tissue and fat followed by permeabilization in BIOPS with saponin and washed in mitochondrial respiration buffer (MiR06). A substrate-uncoupler-inhibitor titration protocol was used to assess different respiratory states as previously described²⁷. Leak respiration (L_N) in the absence of adenylates was induced by the addition of malate (2 mM) and octanoylcarnitine (0.2 mM). Fatty acid oxidation (FAO) was determined after titration of ADP (2.5 mM). Submaximal CI and CI+II-linked respiration (CI_D & P_D) was measured in the presence of glutamate (10 mM) and succinate (10 mM), respectively. Oxidative phosphorylation capacity (P) was assessed after another titration of ADP (2.5 mM). The mitochondrial outer membrane integrity was then tested with the addition of cytochrome C (10 μ M). The average change in oxygen flux was 3.8% (range: 0.2-9.4%). Respiratory electron transfer-pathway capacity (E) was measured during step-wise (0.5 μ M) titration of carbonyl cyanide p-trifluoro-methoxyphenyl hydrazine (FCCP). Succinate-supported electron transfer-pathway capacity (E_{CI}) was assessed by the addition of rotenone (0.5 μ M). Residual oxygen consumption was determined after the addition of malonic acid (5 mM), myxothiazol (0.5 μ M) and antimycin A (2.5 μ M) and subtracted from each respiratory state.

Maximal enzyme activity of citrate synthase was determined from muscle homogenate using fluorometry (Fluoroscanner Ascent, Thermo Fisher Scientific, Waltham, USA) at 25°C as previously described⁸⁸.

Proteomic sample preparation

Human skeletal muscles were lysed in a buffer consisting of 1% Sodium Deoxycholate (SDC), 10 mM Tris (2-carboxyethyl) phosphine (TCEP), 40 mM Chloroacetamide (CAA) and 100 mM of Tris (pH 8.5). The muscles were homogenized with an Ultra Turrax homogenizer (IKA). Lysates were boiled at 95°C for 10 min and sonicated using a tip sonicator. Lysates were then centrifuged at 16000g for 10 min. Proteins were digested using the endoproteinases LysC and trypsin (1:100 w/w) at 37°C overnight with shaking. Digested peptides were acidified using 1% Trifluoroacetic acid (TFA) and precipitated SDC was removed by centrifugation. Peptides were purified using Sep-pak C18 cartridges (Waters) and were eluted in 50% acetonitrile. 20 µg of peptides were saved for total proteome analysis while 3 mg peptides were saved for enrichment of acetylated peptides.

Acetylated peptide enrichment

The acetyl peptide enrichment was performed on 3 mg of digested peptides using PTMscan Acetyl-Lysine Motif kit (Cell Signaling #13416). The peptides were mixed with 100 µL of 10x IP buffer (500 mM MOPS, pH 7.2, 100 mM Na-phosphate, 500 mM NaCl, 5% NP-40). The acetonitrile was removed and the volume was reduced to ~1mL by vacuum centrifugation. The final volume was adjusted with water to a concentration of 3 mg·mL⁻¹. The peptides were clarified by centrifugation at 20000g for 5 min. 20 µL of anti-acetylated lysine antibody was washed 3 times in 1 mL IP buffer and then mixed with the peptides. Peptides were enriched overnight at 4°C, washed 3 times in 1 mL ice-cold (4°C) IP buffer, 4 times in 1 mL ice-cold IP buffer without NP-40, and once in 1 mL water. All wash buffer was removed via aspiration. Acetylated peptides were eluted with 100 µL 0.15% TFA. The elution procedure was repeated three times. The eluted peptides were de-salted on C18 stage-tips⁸⁹, vacuum concentrated and resuspended in buffer A* (5% acetonitrile, 0.1% trifluoroacetic acid).

High pH reversed-phase fractionation

To generate a deep proteome library for data-independent acquisition (DIA), 50 µg of peptide was pooled from pre- and post-HIIT samples, separately, and fractionated using high pH reversed-phase chromatography⁹⁰. 24 fractions (per pool) were automatically concatenated using a rotor valve shift of 90 s. Approximately 0.3 µg of each fraction were subjected to LC-MS/MS measurements via data-dependent acquisition (DDA). To obtain comprehensive coverage of the acetylome, acetylated peptides from each sample were fractionated into 8 fractions using a rotor valve shift of 90 s. For the first subject (both pre and post samples) all 8 fractions were analysed separately. After observing lower than expected peptide intensities for the first subject, the neighboring two fractions were pooled together for each of the remaining samples (total four fractions/subject/timepoint) before LC-MS analysis.

Mass spectrometry

Peptides were measured using LC-MS instrumentation consisting of an Easy nanoflow HPLC system (Thermo Fisher Scientific, Bremen, Germany) coupled via a nanoelectrospray ion source (Thermo Fischer Scientific, Bremen, Germany) to a Q Exactive HF-X mass spectrometer. Purified peptides were separated on a 50 cm C18 column (inner diameter 75 µm, 1.8 µm beads, Dr. Maisch GmbH, Germany). Peptides were loaded onto the column with buffer A (0.5% formic acid) and eluted with a 100 min linear gradient increasing from 2-40% buffer B (80% acetonitrile, 0.5% formic acid). After the gradient, the column was washed with 90% buffer B and re-equilibrated with buffer A.

For the deep proteome library generation, mass spectra were acquired from each fraction via DDA with automatic switching between MS and MS/MS using a top 15 method. MS spectra were acquired in the Orbitrap analyzer with a mass range of 300-1750 m/z and 60,000 resolutions at m/z 200 with a target of 3×10^6 ions and a maximum injection time of 25 ms. HCD peptide fragments acquired at

27 normalized collision energy were analyzed at 15000 resolution in the Orbitrap analyzer with a target of 1×10^5 ions and a maximum injection time of 28 ms. The mass spectra for the acetylome experiments were also acquired from each fraction via DDA with a similar MS method used for deep proteome library generation, except the maximum injection time for the fragment ion spectra was set to 250 ms. A DIA MS method was used for the total proteome measurements in which one full scan (300 to 1650 m/z , resolution = 60,000 at 200 m/z) at a target of 3×10^6 ions was first performed, followed by 32 windows with a resolution of 30000 where precursor ions were fragmented with higher-energy collisional dissociation (stepped collision energy 25%, 27.5%, 30%) and analyzed with an AGC target of 3×10^6 ions and maximum injection time at 54 ms in profile mode using positive polarity.

Data processing

Raw MS files from the experiments measured in DDA mode (muscle proteome library & acetylome), were processed using MaxQuant⁹¹. MS/MS spectra were searched by the Andromeda search engine (integrated into MaxQuant) against the decoy UniProt-human database (downloaded in December 2017) with forward and reverse sequences. In the main Andromeda search precursor, mass and fragment mass were matched with an initial mass tolerance of 6 ppm and 20 ppm, respectively. The search included variable modifications of methionine oxidation and N-terminal acetylation and fixed modification of carbamidomethyl cysteine. For the raw files from the acetylome experiments, acetylated lysine was also added as a variable modification. Acetylated peptides were filtered for a minimum Andromeda score of 40, as per the default settings for modified peptides. The false discovery rate (FDR) was estimated for peptides and proteins individually using a target-decoy approach allowing a maximum of 1% false identifications from a reversed sequence database. Raw files acquired in the DIA mode (total proteome) were processed using Biognosys Spectronaut

software version 13⁹². A single peptide library was generated in Spectronaut using the combined MaxQuant search results for the DDA runs from both of the fractionated muscle samples. The experimental DIA runs were then analyzed in Spectronaut using default settings.

Bioinformatics

Bioinformatic analyses were performed in the Perseus software⁵⁶. Categorical annotations were supplied in the form of Gene Ontology (GO) biological process (BP), molecular function (MF), and cellular component (CC), as well as UniProt Keywords, KEGG and REACTOME pathways. All annotations were extracted from the UniProt database. Quantified proteins were filtered to have at least 4 valid values in at least one time point. Missing data were imputed by drawing random numbers from a Gaussian distribution with a standard deviation of 30% and a downshift of 1.8 standard deviations from the mean. The imputed values have been tuned in order to simulate the distribution of lowly abundant proteins. To identify differentially regulated proteins and acetyl-sites, two-tailed paired *t*-tests were performed with a permutation-based false discovery correction applied (FDR = 0.05). In the acetylome, an additional analysis was performed to expand the identification of differentially expressed proteins, whereby an *a posteriori* information fusion scheme combining the biological relevance (fold change) and the statistical significance (p-value), was implemented, as previously described⁵⁹. A Π -value significance score cut-off of 0.05 was selected. Alterations to organelles were assessed using summed total protein abundances⁹³ and two-tailed paired *t*-tests ($p < 0.05$). Comparisons between individual proteins measured via immunoblotting were performed using two-tailed paired *t*-tests ($p < 0.05$). Hierarchical clustering analysis was performed on z-scored differentially regulated proteins using Euclidean distance. Enrichment analyses were performed on differentially regulated proteins and acetyl-sites using Fisher's exact tests and the application of a Benjamini-Hochberg false discovery correction (FDR = 0.02). Motif enrichment was performed on

upregulated acetyl-sites using iceLogo ($p < 0.01$)⁶⁰. One-dimensional enrichment analyses were performed as previously described⁵². To analyze the relative stoichiometries of acetylated proteins in Fig. 4 abundance corrected intensities (ACIs) were calculated for each acetyl-peptide by dividing the acetyl-peptide intensity by the intensity of the corresponding protein in the proteome. Enrichment analyses were performed against a background of all quantified proteins or acetyl-sites, except for hierarchical clustering analyses where clusters were compared within the z-scored differentially regulated matrix. To visualize complexes and functional protein-interactions in Fig. 6, protein interaction networks were mapped using the STRING database, using an overall confidence cutoff of 0.7 without limiting the number of interactions⁹⁴, and further processed with Cytoscape (www.cytoscape.org).

Immunoblotting

A fresh batch of ice-cold homogenization buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β -glycerophosphate, 2 mM Na_3VO_4 , 10 mM NaF, 2 mM PMSF, 1 mM EDTA (pH 8), 1 mM EGTA (pH 8), 10 $\mu\text{g}\cdot\text{mL}^{-1}$ Aprotinin, 10 $\mu\text{g}\cdot\text{mL}^{-1}$ Leupeptin and 3 mM Benzamidine) was prepared and 80 μL added to 1 mg of freeze dried and dissected muscle tissue. The tissue was homogenized for 1 min at 28.5 Hz in a TissueLyser (Qiagen TissueLyser II, Retsch GmbH, Germany) before samples were rotated end over end for 1 hour at 4°C and centrifuged at 18320g for 20 min at 4°C. The pellet was discarded and the supernatant was taken and used for immunoblotting analyses. Total protein concentration in the samples was determined with a standard BSA kit (Millipore). 15 μL of the sample was diluted with 60 μL ultrapure water in order to keep the concentration within the linear range of the calibration curve (0.2 to 2 $\mu\text{g}\cdot\mu\text{L}^{-1}$). The protein concentration of each sample was adjusted by the addition of 6 $\times$ Laemmli buffer (7 mL 0.5M Tris-base, 3 mL glycerol, 0.93 g DTT, 1 g SDS and 1.2 mg bromophenol blue) and ultrapure water

607 to reach equal concentrations ($1.5 \mu\text{g total protein} \cdot \mu\text{L}^{-1}$). Human skeletal muscle standard samples
608 (Ctrl) were obtained as a pool of all samples included in the experiment.

609

610 Equal amounts of total protein were loaded on 10% TGX Stain-Free or 16.5% Criterion Tris-
611 Tricine/Peptide gels (Bio-Rad) alongside two protein markers (Precision plus all blue and dual color
612 standards, Bio-Rad), three human skeletal muscle control samples and a four-point standard curve
613 (skeletal muscle samples of increasing volume). After standard SDS-page gel electrophoresis proteins
614 were semi-dry transferred to a PVDF membrane. Membranes were blocked with either 2% skimmed
615 milk or 3% BSA in Tris-buffered saline with 0.1% Tween-20 (TBST) before overnight incubation
616 with primary antibody. Afterwards, the membranes were washed in TBST, incubated for 1 hour in
617 HRP conjugated secondary antibody at room temperature and washed $3 \times 15 \text{ min}$ in TBST before the
618 bands were visualized with an enhanced chemiluminescent reaction (Immobilon Forte Western HRP
619 substrate, Millipore) and signals recorded with a digital camera (ChemiDoc MP Imaging System,
620 Bio-Rad). Densitometry quantification of the immunoblotting band intensities was done using Image
621 Lab version 4 (Bio-Rad) and determined as the total band intensity adjusted for the background
622 intensity. The standard curve on each gel was used to confirm that the loaded amount of protein in
623 samples was capable of determining differences between samples by the signal intensity being on the
624 linear part of the standard curve. The variation of the triplicate human standard sample signal loaded
625 was used to evaluate equal transfer of proteins across each gel. Coomassie staining was conducted to
626 confirm equal loading and transfer of total protein from the different samples.

627

628 Listed are the primary antibodies used and their size of migration. MFN2: #11925, Cell Signaling
629 Technologies, 80 kDa; MYLK2: PA5-29324, Invitrogen, 65 kDa; NAMPT: A300-779A, Bethyl
630 Laboratories, 56 kDa; OXPHOS antibody cocktail: ab110411, Abcam, 15-55 kDa; SIRT-1: 07-131,

631 Millipore, 80 kDa; SIRT-3: #5490, Cell Signaling Technologies, 28 kDa; SIRT-5: #8782, Cell
 632 Signaling Technologies, 30 kDa; HRP conjugated goat anti-rabbit (4010-05, SouthernBiotech) and a
 633 goat anti-mouse (P0447, DAKO Denmark) were used as secondary antibodies.

634

635 *Statistical analyses*

636 For non-omics analyses, two-tailed paired t-tests were applied to assess the effect of HIIT, except for
 637 substrate oxidation analyses whereby a mixed linear model was performed ($p < 0.05$).

638

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Author contributions

Conceptualization: M.H., A.S.D., A.K.L. and J.B. **Data curation:** A.S.D., M.H., A.K.L. and B.S. **Formal analysis:** B.S., A.K.L., M.H., M.T., J.K.L. and A.S.D. **Funding acquisition:** A.S.D., M.H. and J.B. **Investigation:** B.S., A.K.L., M.H., M.T., A.G., J.K.L. and A.S.D. **Methodology:** M.H., A.S.D., A.K.L., B.S., J.K.L., A.G. and J.B. **Project administration:** M.H. and A.S.D. **Resources:** A.S.D., M.H. and J.B. **Supervision:** A.S.D. and M.H. **Validation:** M.T., B.S., A.K.L., M.H. and A.S.D. **Visualization:** B.S. **Writing – Original draft:** B.S., M.H., A.K.L. and A.S.D. **Writing – review and editing:** M.H., A.K.L., B.S., A.G., J.K.L. M.T., B.W., J.B. and A.S.D.

Competing interests

The authors declare no competing interests.

Materials & Correspondence

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664 **Supplementary Tables**

665 ***Table S1. Identified proteins.***

666

667 ***Table S2. Quantified proteins and their regulation by HIIT.***

668

669 ***Table S3. Fisher's exact test of HIIT-regulated proteins ($FDR < 0.05$).***

670

671 ***Table S4. Identified acetyl-sites.***

672

673 ***Table S5. Quantified acetyl-sites and their regulation by HIIT.***

674

675 ***Table S6. Summed acetylation intensity per protein.***

676

677 ***Table S7. One-dimensional enrichment analysis of summed acetylation intensity.***

678

679 ***Table S8. Abundance corrected intensities (ACIs) of acetyl-sites pre-HIIT.***

680

681 ***Table S9. One-dimensional enrichment analysis of pre-HIIT acetyl-site ACI (Leading protein IID***
 682 ***was used for relative enrichment).***

683

684 ***Table S10. Fisher's exact test of HIIT-regulated ($\Pi < 0.05$) acetyl-sites (Leading protein IID was***
 685 ***used for relative enrichment).***

686

687 <https://drive.google.com/drive/folders/13Hx0NcA66gY6Fl-5L3NjFov0F4KoH36H?usp=sharing>

688 **Figure Legends**

689 ***Figure 1. Physiological adaptations to HIIT***

690 **A.** Experimental overview. **B.** Subject characteristics. **C.** Five weeks of HIIT increased maximal
691 oxygen uptake ($\dot{V}O_{2\max}$) and incremental peak power output (iPPO). **D** HIIT increased whole-body
692 fat oxidation during submaximal exercise (50-150 W) without altering **E.** carbohydrate oxidation. **F.**
693 HIIT increased mitochondrial respiration in skeletal muscle (L_N : leak respiration, FAO : fatty acid
694 oxidation, CI_D : submaximal CI respiration, P_D : submaximal CI+II respiration, P : oxidative
695 phosphorylation capacity, E : electron transport system capacity, E_{CII} : succinate-supported electron
696 transport system capacity). **G.** HIIT increased skeletal muscle citrate synthase (CS) activity. **H.**
697 Analytical workflow. Summary statistics are mean $\pm$ SEM (n = 8). * $p < 0.05$, ** $p < 0.01$, *** $p <$
698 0.001.

700 ***Figure 2. HIIT increases mitochondrial proteins and reduces a subset of contractile fiber*** 701 **associated proteins**

702 **A.** Volcano plot displaying 102 upregulated and 24 downregulated proteins following HIIT (FDR <
703 0.05). **B.** Fisher's exact tests identified the enrichment of mitochondrial terms within the differentially
704 regulated proteins (enrichment analysis FDR < 0.02). **C.** Hierarchical clustering and enrichment
705 analysis (enrichment analysis FDR < 0.02) on differentially regulated proteins identified that
706 mitochondrial terms are enriched within the upregulated proteins, while cytoplasm (UniProt
707 Keyword) is enriched amongst the downregulated proteins. **D.** Summed total protein abundances for
708 different organelles (UniProt Keyword) shows upregulation of the mitochondrial protein content. **E.-**
709 **G.** Summed total protein abundances display upregulation of electron transport chain complexes (**E.**),
710 mitochondrially-encoded proteins (**F.**) and proteins with a transit peptide (**G.**). Summary statistics are
711 mean $\pm$ SEM (n = 8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

712

713 **Figure 3. HIIT regulates proteins involved in skeletal muscle calcium sensitivity and handling**

714 HIIT did not alter the expression of myosin heavy chain (MYH1: MyHC2x, MYH2: MyHC2a,
715 MYH4: MyHC2x, MYH7: MyHC β ; **A.**) or light chain (**B.**) isoforms. **C.** HIIT regulates proteins
716 controlling myosin phosphorylation. **D.** HIIT reduces expression of subunits of the dihydropyridine
717 receptor. **E.** Summed total protein abundances display downregulation of the dihydropyridine
718 receptor. **F.** HIIT does not alter the expression of ryanodine receptor 1. Summary statistics are mean
719 $\pm$ SEM (n = 8). ^ FDR < 0.05. * p < 0.05, ** p < 0.01, *** p < 0.001.

720

721 **Figure 4. The human skeletal muscle acetylome displays higher stoichiometry on mitochondrial**
722 **proteins and lower stoichiometry on contractile proteins**

723 **A.** We quantified 1263 acetyl-sites corresponding to 464 proteins (n = 7). Of these, 421 proteins were
724 also quantified in the proteome with 1232 of the quantified acetyl-sites located on these 421 proteins.
725 **B.** Distribution of acetyl-sites on proteins within the pre-HIIT acetylome. **C.** Top 10 highest intensity
726 acetylated proteins (acetyl-peptide intensities were summed for each protein) within the pre-HIIT
727 acetylome. **D.** Cumulative protein abundance and rank within the proteome (the top 10 highest
728 intensity acetyl-proteins are highlighted). **E.** One-dimensional enrichment analysis of acetylated
729 protein intensity identified mitochondrial proteins, particularly those involved in carboxylic acid
730 metabolism and monovalent inorganic cation transport (e.g. complex V) as having systematically
731 high acetylation intensities (enrichment analysis FDR < 0.02). **F.** Top 10 acetyl-sites with highest
732 abundance-corrected intensities (ACIs). **G.** One-dimensional enrichment analysis of acetyl-site ACIs
733 identified mitochondrial and carboxylic acid catabolic proteins as higher stoichiometry (positive
734 enrichment factor), while contractile fiber cytosolic and plasma membrane proteins were enriched
735 as lower stoichiometry (negative enrichment factor; (enrichment analysis FDR < 0.02; enrichment

performed on leading protein IDs). **H.** Histogram depicting the ACI distribution of the total acetylome (blue), the mitochondrial acetyl-sites (green) and the contractile fiber acetyl-sites (yellow). Mitochondrial proteins were distributed at higher ACI values and contractile fiber proteins at lower ACI values than the total acetylome.

Figure 5. HIIT increases acetylation of mitochondrial and TCA cycle proteins concomitantly with an increase in SIRT3 expression

A. Volcano plot displaying 20 upregulated (filled red circles) and 1 downregulated (filled blue circle) acetyl-sites following HIIT at an FDR < 0.05, while 257 acetyl-sites were upregulated (red circles) and 26 downregulated (blue circles) at $\Pi < 0.05$ ($n = 7$). **B.** Scatter plot indicating that HIIT-induced changes in acetyl-site intensity typically exceeded that of the corresponding protein. **C.** Fisher's exact tests identified the enrichment of mitochondrial and TCA cycle terms within the differentially regulated acetyl-proteins (enrichment analysis FDR < 0.02; enrichment performed on leading protein IDs). **D.** IceLogo motif enrichment ($p < 0.01$) for the upregulated sites displayed a predominance of proximal cysteine residues relative to the acetylated lysine (position 0). **E.** Immunoblotting analysis identified the upregulation of SIRT3, but no change in SIRT1, following HIIT ($n = 8$). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6. Individual acetyl-site regulation of mitochondrial, TCA cycle and contractile proteins following HIIT

Regulation of acetyl-sites on **A.** electron transport chain complex subunits (annotated by HUGO), **B.** TCA cycle proteins (annotated by KEGG) and **C.** muscle contraction proteins (annotated by REACTOME).

Figure 7. HIIT increases acetylation on specific histone acetyl-sites

HIIT increased acetylation on **A.** H1.5 K48, **B.** H2B type 2F K16/20 and **C.** H3 K23. **D.** HIIT did not alter H4 acetylation. Summary statistics are mean $\pm$ SEM (n = 7). $\dagger$ $\Pi < 0.05$.

Figure S1. Proteome quality control

A. Quantified proteins from each sample. **B.** Representative Pearson correlation between biological replicates (Pre 4 v Pre 5). **C.** Cellular compartment (GOCC) coverage of the proteome.

Figure S2. HIIT increases electron transport chain subunits

Immunoblotting analyses confirmed the upregulation of electron transport chain proteins. Summary statistics are mean $\pm$ SEM (n = 8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure S3. HIIT decreases MYLK2 abundance

A. Immunoblotting analysis confirms the downregulation of MYLK2. **B.** No change in the expression of sarcoplasmic/endoplasmic reticulum calcium ATPases 1-3 (SERCAs). Summary statistics are mean $\pm$ SEM (n = 8). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure S4. Acetylome quality control

A. Overlap between identified acetyl-site with those identified in human skeletal muscle by Lundby et al¹⁸. **B.** Quantified acetyl-sites from each sample. **C.** Representative Pearson correlation between biological replicates (Pre 4 v Pre 5). **D.** Cellular compartment (GOCC) coverage of the acetylome.

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Figure 3

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Figure S1

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Figure S2

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