Downregulation of NR4A3 During Inactivity Alters Glucose Metabolism and Impairs Translation in Human Skeletal Muscle

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Background: *Physical activity promotes health, whereas inactivity is associated with metabolic impairment. The transcription factor nuclear receptor subfamily 4 group A member 3 (NR4A3) is a pleiotropic regulator of skeletal muscle exercise adaptation and metabolism. However, the consequence of lower NR4A3 expression remains largely unexplored. We investigated the impact of NR4A3 downregulation on human skeletal muscle metabolism.*

Methods: *Published transcriptomic datasets from human bed rest and limb immobilisation studies were curated to meta-analyse the effect of physical inactivity on skeletal muscle NR4A3 levels. In primary human skeletal muscle myotubes, siRNA and lentivirus were used to silence and overexpress NR4A3, respectively. Basal and stimulated (insulin ± leucine) signal transduction was determined by immunoblot analysis. Effects on glucose, fatty acid, and protein metabolism were measured using radiolabelled substrate assays. Lactate production was assessed in culture supernatant by colourimetry. Cell morphology was analysed by immunocytochemistry and gene expression was quantified by RT-qPCR.*

Results: *Physical inactivity decreased skeletal muscle NR4A3 (-27%), concomitant with pathways related to mitochondrial function, cytoskeleton organization, chromatin regulation, protein synthesis and degradation. Silencing of nuclear-localised NR4A3 protein (-38%) decreased glucose oxidation (-18%) and increased lactate production (+23%) in vitro. This coincided with greater signalling downstream of AMPK and elevated rates of basal (+26%) and FCCP-stimulated (+55%) fatty acid oxidation. NR4A3 downregulation lowered protein synthesis (-25%), and impaired mTORC1 signalling. Alternatively, overexpression of the canonical NR4A3 protein isoform (+290%) augmented translation (+152%) and total cellular protein content, while partial restoration of NR4A3 levels rescued glucose oxidation in NR4A3-silenced cells. NR4A3 depletion reduced myotube area (-48%) and further altered protein and gene expression of key contractile elements in skeletal muscle.*

Conclusions: *Our study connects reduced NR4A3 expression with physical inactivity and indicates that NR4A3 downregulation in human skeletal muscle has adverse effects on glucose metabolism and protein synthesis. Thus, decrements in NR4A3 abundance could be causal in the deleterious health consequences resulting from sedentary lifestyles and targeting NR4A3 may offer new avenues for combating conditions such as disuse muscle atrophy.*

Keywords Skeletal muscle, inactivity, metabolism, mitochondria, protein synthesis

Introduction

Engagement in physical activity initiates a cascade of integrative responses related to skeletal muscle contraction, such as increased energy expenditure, haemodynamics, and oxygen consumption [1]. Depending on the modality, intensity, and volume of work performed, these homeostatic processes trigger a myriad of beneficial downstream events that serve to maintain or enhance aspects of human physiology, including cardiovascular function, skeletal muscle mass, and peripheral insulin sensitivity [1]. Conversely, inactivity impairs skeletal muscle size and composition. These alterations have adverse effects on local tissue function and metabolism, which in turn impacts systemic health. Inactivity diminishes physical performance [2, 3], whole-body metabolic flexibility [4], and is associated with reduced quality of life [5] and increased risk of non-communicable diseases like type 2 diabetes [6].

Changes in skeletal muscle mass are determined by rates of myofibrillar protein synthesis versus degradation. Physical inactivity favours signalling and transcriptional programmes that promote sustained periods of negative protein balance and muscle loss. Indeed, detectable muscle atrophy occurs within just four days of disuse [2] and involves a complex molecular interplay. One of the major regulators of translation is the mammalian target of rapamycin complex 1 (mTORC1), which permits myofibrillar protein synthesis and muscle hypertrophy [7]. However, mTORC1 acts in tandem with ribosomal content (among other processes) to regulate muscle translation [8]. Detriments in both mTORC1 [9] and ribosomal abundance [10] are observed in human skeletal muscle with inactivity. Alongside insulin- and amino acid- resistance [2, 9, 11, 12], this downregulation of translational machinery drives initial losses in muscle mass through dampened myofibrillar protein synthetic responses [2, 12]. As a transducer of the phosphatidylinositol-3-kinase (PI3K)-AKT pathway, mTORC1 is also central to muscle proteostasis via temporal coordination of anabolic and catabolic processes [13].

Disturbances in skeletal muscle metabolism during unloading further compounds disuse atrophy. Physical inactivity rapidly reduces skeletal muscle mitochondrial content [14] and oxidative

capacity [15], contributing towards an accumulation of inter- and intra- muscular lipids (refs. [15] and [3]). Ectopic lipid depots secrete factors, such as inflammatory cytokines, that foster insulin resistance [16]. Similarly, bioactive lipid species from both inter- and intra- muscular lipid compartments can impede insulin signalling (refs. [15] and [16]) and mitochondrial function [17]. In turn, chronically elevated production of reactive oxygen species (ROS) from dysfunctional mitochondria can inflict damage upon cellular components [18], and upregulate proteolytic pathways [18, 19] while dampening AKT-mTORC1 signalling [19], exacerbating loss of skeletal muscle mass [18, 19].

Exercise training and physical inactivity are not mere opposing ends of a linear spectrum, but rather multifaceted processes with distinct underlying mechanisms. Understanding the dichotomy between exercise and inactivity necessitates recognising that they engage both common and distinct signalling and transcriptional pathways [20]. The NR4A family of stress-responsive orphan nuclear receptors are commonly induced by exercise. However, only *NR4A3* (also known as *NOR1*) shows opposite regulation to exercise and inactivity [20]. Notably, all members of the NR4A family have ties to skeletal muscle metabolism, development, or remodelling. Depletion of NR4A3 in primary human [20] or mouse C2C12 [21] skeletal muscle cells reduces mitochondrial oxidative capacity. Alternatively, transgenic overexpression of *Nr4a3* [22] or *Nr4a1* (also known as *Nur77*) [23] shifts skeletal muscle composition towards an oxidative phenotype, suggesting functional redundancy between these NR4A family members through shared NGFI-B [24] or Nur [25] response elements. Furthermore, overexpression of *Nr4a3* [22], *Nr4a1* [23], or *Nr4a2* (also known as *Nurr1*) [26] in skeletal muscle enhances physical performance, while deletion of *Nr4a1* impairs muscle size [27]. Silencing of *Nr4a3* in mouse C2C12 myotubes reduces mTORC1 signalling and global myosin heavy chain gene expression [21] but the molecular mechanisms underlying the metabolic, anabolic, and transcriptomic roles of NR4A3 remain largely unknown in human skeletal muscle.

Here, we investigated the impact of NR4A3 on metabolism and post-mitotic growth processes. We provide evidence that *NR4A3* is reduced during muscle disuse and that downregulation of *NR4A3* in human skeletal muscle impairs glucose metabolism and protein synthesis, contributing towards atrophy of skeletal muscle fibres.

Results and Discussion

Downregulation of *NR4A3* early during inactivity correlates with transcripts involved in myogenic and metabolic pathways.

NR4A3 mRNA is induced by exercise and repressed during inactivity in human skeletal muscle [20]. We previously showed that gene expression of primary human skeletal myotubes with *NR4A3* silencing best reflects the human muscle transcriptome after periods of bedrest and limb immobilisation [20]. To build upon this observation and gain further insight into the regulation of *NR4A3*, we analysed eight published transcriptomic studies of the human skeletal muscle response to inactivity. The meta-analysis revealed that physical inactivity reduced *NR4A3* mRNA by 27%, but this decrease was not evident in all studies (**Figure 1A**). Grouping studies based on the duration of inactivity demonstrated that skeletal muscle *NR4A3* expression is transiently repressed during the first days of disuse before returning to baseline levels after 2 weeks (**Figure 1B**). Correlation of *NR4A3* with all other transcripts present in the 8 studies allowed for the characterisation of pathways co-regulated with *NR4A3* during inactivity (**Figure 1C**). Gene set enrichment analysis showed that *NR4A3* was positively associated with mitochondrial function and negatively associated with pathways related to cytoskeleton organisation, chromatin regulation, protein synthesis and degradation (**Figure 1C**). Furthermore, combined analysis of two datasets exploring the restoration of ambulatory behaviour after inactivity showed that reloading re-established *NR4A3* mRNA to pre-inactivity levels (**Figure 1D**), consistent with its role of as a contraction-responsive transcription factor in muscle [20].

Silencing *NR4A3* attenuates glucose oxidation by diverting glucose towards lactate and increases fatty acid oxidation in primary human skeletal myotubes.

To mimic the decreased levels observed in human skeletal muscle after inactivity, *NR4A3* was experimentally downregulated using RNA interference in differentiated primary human skeletal muscle cells. Silencing of *NR4A3* in human myotubes was associated with modest compensatory increases in other NR4A family members *NR4A1* (*NUR77*) and *NR4A2* (*NURR1*) (**Figure 2A**). NR4A3 protein was almost exclusively localised to the nucleus in myotubes and silencing efficiently reduced nuclear protein abundance (**Figure 2B**). This depletion of NR4A3 lowered basal and FCCP-stimulated (uncoupled) glucose oxidation (**Figure 2C**) independent of glucose transport. Rather, phosphorylation of the canonical insulin signalling transducer AKT was potentiated at site thr308 by silencing of *NR4A3*

(**Supplementary Figure 1A**). Furthermore, no impairments in basal or insulin-mediated glucose uptake and incorporation into glycogen were observed (**Supplementary Figures 1B, 1C**).

Upon *NR4A3* silencing lactate production and release into culture medium was augmented (**Figure 2D**), indicating a shift in glucose fate that was also reported in mouse C2C12 myotubes [28]. Accordingly, mRNA expression of the alpha isoform of lactate dehydrogenase (*LDHA*) increased after *NR4A3* interference, while expression of the beta isoform decreased (*LDHB*) (**Figure 2E**). Despite no change in total LDH protein (**Supplementary Figure 1D**), this modification of LDH isoform composition indicates preferential production of lactate from glycolysis and/or less conversion of lactate to pyruvate [29]. Lactate release upon NR4A3 depletion could be further supported by the reduction of pyruvate kinase muscle 2 (*PKM2*) mRNA (**Figure 2E**), as inhibiting noncanonical PKM2 activity increased lactate release from C2C12 myoblasts [30]. For glucose to be oxidised in the mitochondria, pyruvate must be metabolised to acetyl coenzyme A (acetyl-CoA) via the pyruvate dehydrogenase (PDH) complex. *NR4A3* silencing upregulated pyruvate dehydrogenase kinase 4 (*PDK4*) gene expression (**Figure 2E**) consistent with attenuated glucose oxidation [31] (**Figure 2C**). However, the inhibitory PDK4-target phosphorylation site on PDH E1 component subunit alpha (PDHA1^{ser293}) was unaltered (**Figure 2F**) indicating no differences in PDH activity.

We previously reported that mitochondrial respiration (in the presence of glucose, glutamine, and pyruvate) and protein subunits of the electron transport chain were diminished by *NR4A3* silencing in primary human skeletal myotubes [20]. This aligns with findings in C2C12 myotubes, where *Nr4a3* depletion decreased mitochondrial membrane potential in the absence of changes in mitochondrial DNA [21]. Instead, downregulation of *Nr4a3* negatively affected the expression of several respiratory complex genes, as well as transcripts for mitochondrial ribosomal proteins [21]. Additionally, *Nr4a3* silencing reduced the mRNA abundance of mitofusin-2 (*Mfn2*) but increased dynamin 1 like expression (*Dnm1l*; also known as *Drp1*) [21]. These data suggest that loss of NR4A3 might attenuate mitochondrial fusion dynamics (instead favouring fission), mitochondrial reticulum connectivity, and assembly of electron transport chain complexes in a manner separate from alterations in mitochondrial abundance. A maintained myotube response to the mitochondrial uncoupler FCCP after *NR4A3* RNA interference further supports not only retained mitochondrial content, but also functional mitochondria (**Figure 2C)**. Thus, the attenuation of glucose oxidation in NR4A3-depleted myotubes appears predominantly a consequence of greater diversion of glucose towards lactate, which would reduce glucose-derived acetyl-CoA entry into the tricarboxylic acid (TCA) cycle. However, aspects of mitochondrial dysfunction might also play a role.

Skeletal muscle cells are highly glycolytic in culture, preferentially oxidising glucose at rates 100-fold faster than long-chain lipids [32]. Therefore, we explored the consequence of NR4A3-dependent reductions in glucose oxidation on lipid metabolism in primary human skeletal myotubes. NR4A3 depletion upregulated basal and FCCP-stimulated rates of fatty acid oxidation (**Figure 3A**) without altering the intracellular composition of select lipid species (**Figure 3B**). Whereas the ratio of triacylglycerides, diacylglycerides, free fatty acids, and other lipids were modified by FCCP, *NR4A3* RNA interference had no effect (**Figure 3B**). Compatible with greater fatty acid oxidation, *NR4A3* silencing increased phosphorylation of the AMP-activated protein kinase α subunit (AMPKα^{thr172}) and its substrates acetyl-CoA carboxylase (ACC^{ser79}) and TBC domain family member 1 (TBC1D1^{ser237}) (**Figure 3C**). This signalling coalesced with activating phosphorylation of hormone-sensitive lipase (HSL^{ser660}), reduced LIPIN1 protein abundance (**Figure 3D**), and downregulation of total ACC protein (**Figure 3C**) as well as the mRNA of its cytosolic isoform (*ACCα*) (**Figure 3E**). Further profiling of transcripts involved in fatty acid metabolic pathways revealed that NR4A3 depletion altered the composition of AMPKα isoforms and fatty acid transport genes (**Figure 3E**). Compared to control, *NR4A3* silencing increased the expression of fatty acid transport protein 1 (*FATP1*) and reduced the expression of fatty acid binding protein 3 (*FABP3*) (**Figure 3E**). Together, the protein and transcriptional changes induced after RNA interference implicate NR4A3 in physiological mechanisms compensating for energy stress due to impaired glucose oxidation.

***NR4A3* silencing in primary human myotubes downregulates translation, mTORC1 signalling, and pre-rRNA abundance.**

Metabolic assays are commonly normalised to protein content to account for differences in seeding density. We observed that silencing of *NR4A3* consistently reduced the total protein and RNA abundance of cultured human myotubes (**Figures 4A**, **4B**). Over 80% of cellular RNA is ribosomal (rRNA) and rates of translation are proportional to total RNA content in skeletal muscle [33]. These observations, combined with the association of reduced *NR4A3* levels during bed rest and limb immobilisation inactivity (**Figure 1**), led us to explore the putative effects of *NR4A3* silencing on protein synthesis. Relative rates of translation can be measured in vitro by the surface

sensing of translation (SUnSET) method, which detects puromycin incorporation into nascent peptide chains by immunoblot analysis [34]. Using this technique, we observed reduced puromycilation of proteins upon *NR4A3* silencing both at baseline and after insulin plus leucine stimulation (**Figure 4C**), suggesting impaired protein synthesis with NR4A3 downregulation.

The efficiency and total capacity for translation in skeletal muscle is determined by mTORC1 signalling and ribosomal content, respectively [8, 10]. In vitro, AMPK impedes mTORC1 activity through phosphorylation of tuberous sclerosis complex 2 (TSC2) [35] and raptor [36]. Consistent with energy-stress dependent upregulation of AMPK (**Figure 3C**), the ratio of phosphorylated-to-total protein of AMPK target sites on TSC2 (ser1387) and raptor (ser792) were increased by NR4A3 depletion (**Figures 4D**, **4E**; **Supplementary Figure 2A**) indicative of AMPK interference of mTORC1. Furthermore, *NR4A3* silencing reduced the protein content of raptor, mTOR, and ribosomal protein S6 (RPS6) (**Figures 4D**, **4E**; **Supplementary Figures 2B, 2C**). These collective AMPK-related and unrelated changes likely coalesced to inhibit mTOR^{ser2448} phosphorylation and activation of downstream mTORC1-substrates, including RPS6$^{ser235/236}$ phosphorylation, with negative consequences for skeletal muscle protein synthesis following NR4A3 depletion (**Figures 4D**, **4E**; **Supplementary Figures 2B, 2C**).

In agreement with diminished translation, gene expression analyses confirmed that *NR4A3* RNA interference decreased mRNA abundance of *RPS6*, as well as eukaryotic translation initiation factor 4E (*EIF4E*) and 45S pre-ribosomal RNA (5' external transcribed spacer; *5' ETS*) (**Figure 4F**). The 45S pre-rRNA polycistronic precursor is transcribed by RNA polymerase I (Pol I) and gives rise to the 18S, 5.8S, and 28S rRNAs [37]. These rRNAs are subsequently assembled with Pol II-dependent ribosomal proteins and Pol III-transcribed 5S rRNA into the functional 40S (small) and 60S (large) ribosomal subunits [37]. As such, Pol I activity seems to be the rate-limiting step in ribosomal biogenesis. Due to the short half-life of the ETS region [38], measurement of the 45S pre-RNA 5' ETS is a reliable indicator of Pol I-mediated ribosomal transcription [37]. Thus, our results imply that lower levels of total RNA from *NR4A3*-silenced myotubes (**Figure 4B**) are a consequence of attenuated ribosomal biogenesis, resulting in reduced ribosomal mass, and that NR4A3 downregulation impedes both translational efficiency (i.e. mTORC1) and capacity (i.e. ribosomal abundance).

Despite an overall reduction in protein synthesis compared to control (**Figure 4C**), *NR4A3*-depleted myotubes still responded to insulin plus leucine stimulation, as suggested by the interaction between *NR4A3* silencing and treatment for mTOR^{ser2488}, RPS6$^{ser235/236}$, and the inhibitory AKT-target phosphorylation site on TSC2 (thr1462) (**Figure 4D**; **Supplementary Figures 2B-D**). AKT^{thr308} levels were greater upon insulin treatment (**Supplementary Figure 1A**) and tended to increase with insulin plus leucine stimulation (**Figure 4D**; **Supplementary Figure 2E**). Furthermore, mRNA of the large neutral amino acid transporter small subunit 1 (*LAT1*; also known as *SLC7A5*) was upregulated by *NR4A3* silencing (**Figure 4F**). LAT1 is the dominant antiporter for leucine uptake into cells [39]. The rise in intracellular leucine is then detected through biochemical sensing mechanisms, including disassociation of sestrin-2 (SESN2) with GATOR2 [40] and activation of leucyl-tRNA synthetase 1 (LARS1) [41], with subsequent activation of mTORC1. As such, increased LAT1 could feasibly support higher intramuscular leucine concentrations under *NR4A3*-silenced conditions, which could have offset any detrimental effects of increased *SESN2* or reduced *LARS1* mRNA (**Figure 4F**). Hence, canonical insulin signalling, combined with enhanced leucine transport, might mediate retained anabolic sensitivity of NR4A3-depleted myotubes via the AKT-mTORC1 pathway.

We next investigated the impact of NR4A3 downregulation on markers of protein degradation. Although the contribution of protein breakdown towards disuse muscle atrophy is unclear in humans [2, 12], the ubiquitin-proteasomal (UPS) and autophagy-lysosomal systems are markedly induced during rodent models of inactivity [18, 19]. Additionally, skeletal muscle-specific *NR4A3* transgenic mice display greater autophagic flux [42]. Both major proteolytic pathways use ubiquitination as a signal for select protein degradation [43]. However, in contrast to the stark impairment of protein synthesis, *NR4A3* silencing did not alter global protein ubiquitination in primary skeletal myotubes (**Supplementary Figure 2F**). This was despite elevated mRNA expression of transcription factor forkhead box O3 (*FOXO3*) and the muscle-enriched E3 ubiquitin ligase muscle ring-finger 1 (*MuRF1* or *TRIM63*) (**Supplementary Figure 2G**). *MuRF1* is a downstream target of FOXO3 [44] that, when overexpressed, is sufficient to induce ubiquitination and murine muscle atrophy [45]. *NR4A3* interference also had mixed effects on transcripts associated with the catalytic core particle of the proteasome, increasing proteasome 20S Subunit Alpha 1 (*PSMA1*) and decreasing proteasome 20S Subunit Beta 2 (*PSMB2*) (**Supplementary Figure 2G**). Furthermore, inflammation-endoplasmic reticulum stress can exacerbate muscle atrophy. NR4A3 depletion

reduced IkBα protein abundance (**Supplementary Figure 2H**), and upregulated activating transcription factor 3 (*ATF3*) and DNA damage-inducible transcript 3 (*DDIT3*; also known as *CHOP*) mRNA (**Supplementary Figure 2G**). Nevertheless, other surrogates of inflammatory nuclear factor kappa B (NF-kB) and endoplasmic stress signalling, such as interleukin 6 (*IL-6*) and *ATF4*, were unaltered (**Supplementary Figure 2G**).

Altogether, silencing *NR4A3* in skeletal myotubes triggered AMPK- dependent and independent inhibition of mTORC1, reducing ribosomal biogenesis and protein translation. Pathways related to proteolysis were negligibly affected, suggesting NR4A3 downregulation primarily reduces skeletal muscle protein content via multi-level attenuation of protein synthesis.

Overexpression of the canonical *NR4A3* isoform increases protein synthesis in primary human myotubes.

The *NR4A3* gene produces four transcripts encoding three isoforms of the NR4A3 protein. The RNA interference method described in previous figures targeted an exon common to all isoforms, precluding the analysis of differential effects of select *NR4A3* variants. Thus, we explored lentiviral overexpression of the canonical *NR4A3* isoform (*NR4A3-203*; NM_006981.4) in primary human skeletal myotubes. Overexpression of the *NR4A3-203* (*NR-203*) transcript by >140-fold increased total NR4A3 protein levels by >4.5-fold without altering the abundance of other NR4A family members (**Figure 5A**) or effecting aspects of glucose and fatty acid metabolism (**Supplementary Figures 3A-D**). However, consistent with the effects of *NR4A3* silencing, *NR4A3-203* overexpression enhanced basal protein synthesis, with no further stimulatory effect of insulin plus leucine treatment (**Figure 5B**). This result was further supported by assessment of radiolabelled phenylalanine incorporation into protein (**Figure 5C**). Here, canonical *NR4A3* overexpression increased rates of translation under both unstimulated conditions and after foetal bovine serum (FBS) plus leucine treatment when combined with proteasomal blockade (lactacystin) or mTORC1-inhibition (rapamycin) (**Figure 5C**). That total cellular protein content of *NR4A3-203* overexpressing myotubes was greater in these same conditions implies the canonical NR4A3 isoform may upregulate overall protein turnover at least somewhat independently from mTORC1 (**Figure 5D**). Indeed, no differences in total RNA concentrations or mTORC1 signalling were observed after *NR4A3-203* overexpression (**Supplementary Figures 3E, 3F**), suggesting that *NR4A3* silencing and overexpression phenotypes manifest through different mechanisms. This hypothesis was further substantiated by the failure of *NR4A3-203* overexpression to modulate transcripts affected by silencing (**Supplementary Figure 3G**). Nevertheless, overexpression of the canonical *NR4A3* isoform in the presence of siRNA targeting all *NR4A3* variants (**Figure 5E**) partially restored glucose oxidation (**Figure 5F**). Thus, the *NR4A3-203* isoform appears to majorly underly the negative metabolic outcomes associated with NR4A3 downregulation.

***NR4A3* silencing alters primary human myotube size and structure.**

NR4A3 is decreased during inactivity associated with muscle disuse (**Figure 1A, 1B**). We therefore tested how changes in translation and protein synthesis induced by *NR4A3* silencing impact on myotube size and contractile apparatus. Immunostaining for fast-type myosin heavy chain isoforms (MYH1/2; type-IIX/type-IIA) revealed a striking decrease in myotube size (**Figure 6A**). This difference was driven by a reduction in myotube area (**Figure 6B**) without any change in the number of nuclei (**Figure 6C**) or the ability of myoblasts to fuse into myotubes (**Figure 6D**).

NR4A3 depletion reduced the protein abundance of fast (MYH1/2) and slow (MYH7) myosin heavy chain isoforms (**Figure 6E**; **Supplemental Figure 4**), and altered the expression of numerous transcripts encoding for proteins integral to skeletal muscle contraction and structure. This included downregulation of *MYH1*, titin (*TTN*), capping actin proteins of muscle Z-line (*CAPZ* family), and troponin isoforms *TTNT2* and *TTNT3* (**Figure 6F**). Altogether, the protein and mRNA levels of major structural and contractile proteins of skeletal muscle cells were disrupted by *NR4A3* silencing, associated with diminished myotube size.

Conclusions

NR4A3 is markedly induced following a single exercise bout, but not after exercise training [20]. Similarly, *NR4A3* is transiently downregulated during reduced physical activity and quickly upregulated upon reloading, but not changed after prolonged immobilisation (>2 weeks). The onset of muscle fibre atrophy occurs rapidly with disuse [2] and is most aggressive across the initial two weeks of unloading [46], which corresponds with the lowest levels of *NR4A3* mRNA. This expression pattern suggests a crucial role for NR4A3 in regulating muscle function during acute transition phases associated with intense tissue remodelling. The first two weeks of unloading correspond to lower postprandial [11] and total daily [9] rates of myofibrillar protein synthesis. Here, we provide evidence that NR4A3 can directly influence translation in muscle. Furthermore, NR4A3 downregulation in skeletal muscle cells

recapitulated adverse effects observed in skeletal muscle tissue following inactivity. This includes impaired glucose metabolism, reduced protein synthesis, altered expression of contractile apparatus, and diminished myotube size. Thus, our results shed light on the involvement of NR4A3 in the control of skeletal muscle protein synthesis and metabolism in disuse atrophy.

There are several lines of evidence from both *in vitro* and *in vivo* studies that NR4A3 is associated with metabolic responses in skeletal muscle. Rats bred for fitness have skeletal muscle enrichment of NR4A3, associated with higher mitochondrial content and increased running capacity [47]. In mice, overexpression of *Nr4a3* promotes skeletal muscle remodelling towards an oxidative phenotype [22], suggesting that NR4A3 plays a causal role in metabolic reprogramming of skeletal muscle. Observations from *in vivo* studies corroborate with results from *in vitro* experiments in mouse C2C12 and primary human myotubes demonstrating that silencing of NR4A3 changes gene expression profiles towards anaerobic programmes [28] and impairs mitochondrial function [20, 28]. Such effects may account for the lower adenosine triphosphate (ATP) concentrations in C2C12 myotubes after *Nr4a3* silencing [28]. Our data add mechanistic evidence that NR4A3 downregulation profoundly impacts substrate utilisation, shifting metabolism towards increased lactate production and fatty acid oxidation, while decreasing glucose oxidation. Additionally, we corroborate findings in C2C12 [21] that the mTORC1 complex is a major signalling node inhibited in response to NR4A3 depletion. Activation of AMPK led to the phosphorylation of TSC2 and raptor at sites that blunt mTORC1 transduction. However, we also observed decreased mTOR and RPS6 protein abundance, a phenomenon unlikely triggered exclusively by the activation of AMPK. Indeed, rates of translation and ribosomal biogenesis were attenuated in response to *NR4A3* RNA interference, indicating that reductions in NR4A3 impair cellular metabolism by obstructing cell-wide anabolic pathways. In agreement, rescuing protein synthesis via overexpression of the canonical NR4A3 isoform restored glucose oxidation in *NR4A3*-silenced myotubes.

Our study connects lower levels of NR4A3 with inactivity paradigms and indicates that attenuation of NR4A3 in human skeletal muscle has adverse effects on protein synthesis and metabolic responses. Muscle mass and strength are important predictors of mortality in intensive care [48]. Therefore, NR4A3 may be a key molecular transducer, downregulation of which contributes to the deleterious health consequences associated with sedentary lifestyles. Understanding and addressing NR4A3 regulation could have far-reaching implications for global health by potentially mitigating the adverse effects of inactivity, aging, and metabolic diseases.

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Methods

Transcriptomic meta-analysis. Transcriptomic studies of inactivity in human trials were selected using the MetaMEx database [20]. Studies were annotated with the sex and age of participants, as well as the total duration of inactivity. Statistics were performed individually for each study and the mean, variance and *n* size were used to fit a random effects model to the data. The restricted maximum-likelihood (REML) method was applied to the data using the R package metaphor. Obtained p-values were adjusted using the Benjamini & Hochberg method. To evaluate the effect of inactivity duration, all transcriptomic studies were merged by gene name and split in three groups: less than one week, one-two weeks or more than two weeks. A linear model was used to test the overall effect of inactivity duration, as well as pair-wise comparisons to the pre-inactivity group using Empirical Bayes Statistics for Differential Expression with the limma package [49]. Gene set enrichment analysis was performed using ClusterProfiler [50] on genes ranked based on Spearman correlation with *NR4A3*. Gene ontology biological processes were considered significant at FDR<0.05. The effect of reloading was estimated with the same method as above after merging GSE21496 and GSE24215.

Primary human cell culture. Primary cells were isolated from *vastus lateralis* skeletal muscle biopsies derived from ten healthy volunteers (age: 36 ± 15 years; BMI: 24.0 ± 1.9 $Kg.m^{-2}$; biological sex: eight males, two females). The ethical committee at Karolinska Institutet approved protocols. Cells were cultured and differentiated as described previously [32]. Successful myotube formation was monitored under the microscope and cells were used for terminal experiments ten days after the initiation of differentiation. The absence of mycoplasma contamination was routinely confirmed by PCR.

Silencing by RNA interference. On days six and eight of differentiation, myotubes were transfected with 10 nM of either Silencer Select siRNA Negative Control No. 2 (no. 4390847) or Silencer Select siRNA s15542 targeting *NR4A3* (Life Technologies, Foster City, CA). Transfections were performed for 5 h in OptiMEM reduced serum media with Lipofectamine RNAiMAX transfection reagent (Invitrogen, Carlsbad, CA). Terminal experiments were performed ≈48 h after the second transfection.

Plasmid transformation, expansion, and purification. cDNA of the canonical *NR4A3* transcript (NR4A3-203; NM_006981.4) was synthesised into a modified pLenti CMV Puro DEST (w118-1) vector (Addgene plasmid #17452) by GENEWIZ (Azenta Life Science, Leipzig, Germany). One Shot TOP10 Chemically Competent *E. coli* cells (Invitrogen, ThermoFisher Scientific) were used for bacterial transformation and expansion. Modified pLenti CMV Puro DEST (w118-1) empty vector (EV) and NR4A3-overexpressing (*NR-203*^{Oex}) plasmid DNA was then purified using endotoxin-free buffers (Cat. No. 19048) and columns (Cat. No. 10083) from Qiagen, before assessing DNA concentration and purity by spectrophotometry.

Lentivirus production. HEK293T cells were grown in DMEM media (Gibco #10569) supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin, and 500 µg/mL geneticin (G418). Twenty-four hours prior to transfection, HEK293T cells were seeded into T-225 flasks coated with poly-L-lysine. Once ≈90% confluent, lentiviral harvest was conducted by transfecting each T-225 with a transfection reagent mix consisting of 69 µg of EV or *NR-203*^{Oex} plasmid DNA, second generation packaging vectors (69 µg of psPAX2 DNA and 45 µg of pMD2.G DNA), 549 µg polyethylenimine (PEI; 3 µg per µg of total plasmid DNA) and PBS at a final volume of 4.5 mL. The transfection mix was then added to 40.5 mL of pre-warmed growth medium (without geneticin) and added to HEK293T cells for 12-14 h. At this point, the transfection media was removed and replaced with fresh medium for 2 h before switching to new growth media supplemented with 5 mM sodium butyrate. Thirty hours later, viral media was collected and passed through a 0.45 µM low-protein binding filter (Millipore) and concentrated by low-speed centrifugation (3300 g for 30 min at 4°C) using 100 kDa molecular weight cut-off Centricon Plus-70 cartridges (UFC710008). Three volumes of concentrated virus medium were subsequently mixed with one volume of Lenti-X concentrator (Takara Bio) and rotated at 4°C overnight before centrifugation (1500 g for 45 min at 4°C). Supernatant was discarded and the pellet resuspended in PBS, aliquoted, and stored at -80°C. Virus concentration was determined using Lenti-X GoStix Plus (Takara Bio) after one freeze-thaw cycle to be consistent with experimental conditions.

Transduction of primary skeletal myotubes. On day 7 of differentiation, myotubes were transduced with either EV or *NR-203*^{Oex} lentivirus

(concentration equivalent to 16.5 ng of p24.mL^{-1} per 9.6 cm^2 of cells) in post-fusion medium supplemented with 5 ug.mL^{-1} of polybrene. After 18-20 h, viral medium was removed, myotubes were washed once with PBS and then switched to normal post-fusion media [32]. Terminal experiments were performed ≈48 h later.

RNA extraction and analysis. RNA was isolated from cultured muscle cells using TRIzol-cholorform extraction according to manufacturer's instruction (Invitrogen). RNA concentration and purity were determined by spectrophotometry. All equipment, software, and reagents for performing reverse transcription and RT-qPCR were from Thermo Fisher Scientific. The High-Capacity cDNA Reverse Transcription kit was used for cDNA synthesis according to manufacturer's instructions. RT-qPCR was performed on a StepOne Plus system using Taqman or Sybr Green technologies (probe sequences for all analysed genes are available upon request). Relative gene expression was calculated by the comparative ΔΔCt method using the geometric mean of at least two of the following reference genes: *18S*, *B2M*, *GUSB*, *HPRT1* or *TBP*. The most stable reference gene combination was determined within experimental conditions.

Glucose uptake. Myotubes were washed once with sterile PBS and incubated for 4 h in un-supplemented, serum-free low-glucose Dulbecco's Modified Eagle Medium (DMEM) (5.5 mM glucose, Gibco #21885) (i.e. serum starvation medium). Cells were then switch to fresh serum starvation medium in the absence or presence of insulin (120 nM) for 1 h. Myotubes were washed once with warm glucose- and serum-free DMEM (Gibco #11966) and glucose uptake was measured in the same medium by adding 1 uL.mL^{-1} of 2-[1,2-^{3}H]Deoxy-D-glucose (MT911, Moravek) and 10 µM unlabelled 2-Deoxy-D-glucose in for 15 min [32]. Cell monolayers were washed three times with ice-cold PBS and lysed in 1 mL 0.03% SDS. 0.5 mL of the cell lysate was counted in a liquid scintillation counter (TRI-CARB 4910TR, PerkinElmer) and protein content of the remaining lysate was measured for normalisation of results (BCA Protein Assay Kit; #23225, Thermo Fisher Scientific, Rockford, IL).

Glycogen synthesis. Incorporation of glucose into glycogen was performed using similar methods detailed elsewhere [32]. Briefly, myotubes were nutrient-starved as in glucose uptake experiments and then treated with 0, 10, or 120 nM of insulin in fresh serum-starvation medium for 30 min before adding 2 µL.mL^{-1} D-[^{14}C(U)] glucose (NEC042B005MC; Perkin Elmer) for a further 90 min. Cells were washed three times with ice-cold PBS and lysed in 0.5 mL 0.03% SDS. Thereafter, [^{14}C]-labelled glycogen was extracted from 0.4 mL of the lysate and counted in a liquid scintillation counter [32]. Cellular protein concentration was determined by bicinchoninic acid (BCA) assay from the remaining 0.1 mL of cell lysate and used to normalize results.

Glucose oxidation. Glucose oxidation was performed using methods as described [32]. Myotubes were washed with PBS and incubated with D-[^{14}C(U)] glucose in serum starvation medium in the presence or absence of 2 µM carbonyl cyanide p-trifluoro methoxyphenylhydrazone (FCCP) for 4 h. Thereafter, [^{14}C]-CO_2 was then released from the medium by adding of 150 µL 2 M HCl per mL of medium and captured in 300 µL 2 M NaOH for 1 h. The captured [^{14}C]-CO_2 was counted using a liquid scintillation counter. Cells were washed three times with ice-cold PBS and homogenised in 0.4 mL 0.5 M NaOH. After complete lysis, 0.1 mL 2 M HCl was added to neutralise pH and protein was assessed using the BCA assay for normalisation of results.

Lactate assay. Lactate-released into culture supernatant was measured in post-fusion medium [32] under after 48 h of basal or (2 µM) FCCP-stimulated conditions using a colourimetric assay as described [32]. Assay buffer contained Tris (50 mM pH 8), NAD (7.5 mM), N-methylphenazonium methyl sulfate (250 µM), p-iodonitrotetrazolium violet (500 µM), and lactate dehydrogenase (4 U.mL^{-1}). Medium was passed through 3 kDa filter by centrifugation (14000 g for 15 min at 4°C) and 50 µL of sample was mixed with 150 µL of assay buffer. After a 20-min incubation the absorbance was read at 490 nm. Results are normalised to total cellular protein content as measured by BCA assay after lysis in 0.03% SDS.

Fatty acid oxidation. [^{14}C(U)] palmitic acid (NEC534050UC; Perkin Elmer) oxidation was performed using the same experimental procedure as glucose oxidation, except that 1 µL.mL^{-1} of isotope was added to base glucose-free DMEM (Gibco #11966) supplemented with 25 µM of unlabelled BSA-conjugated palmitate.

Thin-layer chromatography (TLC). Myotubes were incubated for 6 h in the same medium as described for [^{14}C(U)] palmitic acid oxidation. Cells were then washed three times with ice-cold PBS and hydrophobic lipids were extracted as detailed elsewhere [51]. The resulting lipid pellet was eluted in 50 µL of a methanol:chloroform solution (1:1) and loaded on a TLC plate (Silica Gel G 250 µm 20×20 cm, Analtech, DE, USA). Lipid species were separated for 30 min in a loading chamber filled with 100 mL of a hexane:diethylether:acetic acid mixture (80:20:3). The loaded TLC plate was then transferred to an exposure cassette (GE Healthcare) and exposed to an X-ray film for four weeks at -80˚C before developing in an X-ray developer machine. Identification of the

appropriate bands was determined against standards for 1,2-Dioctanyl [1-^{14}C] rac-glycerol (1,2-DAG), 1,3-Dioleoyl-rac-glycerol [oleoyl-1-^{14}C] (1,3-DAG) (American Radiolabeled Chemicals Inc.), [^{14}C(U)] palmitic acid (Perkin Elmer), and [^{14}C] triolein (TG). Densitometric quantification was performed using Image Lab software (Bio-Rad).

Phenylalanine incorporation into protein. Myotubes were washed once with PBS and incubated in low-glucose base DMEM for 4 h. After starvation, media was changed to low-glucose DMEM supplemented with an additional 25 μM unlabelled phenylalanine and 4 μL of [^{14}C]phenylalanine (NEC284E050UC; Perkin Elmer) per mL for 6 h, with or without 20% FBS plus 10 mM leucine, and in the presence or absence of 100 nM rapamycin or 10 μM lactacystin. Cells were then washed in three times with ice-cold PBS and lysed in 0.5 mL 0.03% SDS. Protein concentration was measured from 0.1 mL of cell lysate by BCA protein assay. Protein from the remaining cell lysate was precipitated in 50% Trichloroacetic acid with 1% BSA overnight at −20°C, followed by centrifugation (12000 g for 15 min at 4°C). Supernatant was discarded and the protein pellet was washed twice in acetone with centrifugation after each wash (12000 g for 15 min at 4°C). Acetone was discarded and the protein pellet dissolved in 0.5 M NaOH for 1 h in a heating block at 65°C with shaking. The dissolved pellet homogenate was transferred to scintillation vials and the amount of [^{14}C] phenylalanine incorporation determined by scintillation counting.

Subcellular fractionation. Separation of sarcoplasmic and nuclear fractions was performed on fresh cells, without freezing. Myotubes were washed once in ice-cold PBS and scraped in cell lysis buffer (10 mM HEPES pH 7.5, 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, 0.5% NP-40) supplemented with 1 mM PMSF, 0.5 mM Na_3VO_4 and 1x Protease Inhibitor Cocktail Set 1 (PIC) (Calbiochem). Lysates were mixed by pipetting and rotated at 4°C for 20 min then subjected to centrifugation (12000 g for 10 min at 4°C). The sarcoplasmic fraction (supernatant) was collected and stored at -80°C. The remaining pellet was washed three times in ice-cold cell lysis buffer by inverting the Eppendorf multiple times per wash and then resuspended in nuclear extraction buffer (20 mM HEPES pH 7.5, 400 mM NaCl, 1 mM EDTA, 1 mM DTT) supplemented with 1 mM PMSF, 0.5 mM Na_3VO_4 and 1x PIC. Resuspension was performed in the same volume collected for the sarcoplasmic fraction to retain the sarcoplasmic to nuclear ratio of proteins. Samples were next rotated at 4°C for 30 min and debris pelleted by centrifugation (12000 g for 15 min at 4°C). The nuclear fraction supernatant was collected and stored at -80°C until lysate preparation for immunoblotting.

Surface sensing of translation (SUnSET) assay. Relative rates of in vitro protein synthesis were determined using the SUnSET method as described [34]. Briefly, myotubes were washed once in PBS and incubated in low-glucose base DMEM for 4 h. Media was then changed to fresh low-glucose vehicle DMEM or low-glucose DMEM supplemented with 120 nM insulin and 10 mM leucine. After 1 h of stimulation, puromycin (1 μM) was added to the medium and cells were incubated for a further 30 min before collection. Medium was aspirated and cells were washed once in ice-cold PBS. Samples were then harvested in protein lysis buffer (137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, 20 mM Tris pH 7.8, 10 mM NaF, 1 mM EDTA, 1 mM PMSF, 0.5 mM Na_3VO_4 and 1x PIC), rotated for 30 min at 4 °C and subjected to centrifugation (12,000 g for 15 min at 4 °C). Supernatants were then stored at -80°C until protein determination by BCA assay and subsequent sample preparation for immunoblotting.

Insulin stimulation for assessment of signalling. For interrogation of the canonical insulin signalling cascade, myotubes were incubated in serum starvation medium for 4 h. Cells were then stimulated with 0, 10, or 120 nM of insulin in fresh serum starvation medium for 20 min and lysed as mentioned for SUnSET assay samples.

Immunoblot analysis. Samples were prepared for SDS-PAGE with Laemmli buffer (60 mM Tris pH 6.8, 2% w/v SDS, 10% v/v glycerol, 0.01% w/v bromophenol blue, 1.25% v/v β-mercaptoethanol). Equal amounts of protein were loaded and separated on Criterion XT Bis-Tris Gels (Bio-Rad, Hercules, CA) and transferred to polyvinylidene fluoride membranes (Merck, Germany). Membranes were stained with Ponceau S to confirm transfer quality and even loading of samples. Membranes were then blocked with 5% non-fat milk in Tris-buffered saline supplemented with Tween-20 (TBST; 20 mM tris-HCl pH 7.6, 137 mM NaCl, 0.02% Tween-20) for 1 h at room temperature, before an overnight incubation with primary antibodies at 4°C with gentle rocking. Primary antibodies were diluted in TBS plus 0.1% w/v bovine serum albumin and 0.1% w/v NaN_3 or 5% non-fat milk in TBST (specific antibodies and dilutions are available upon request). Membranes were next washed with TBST and incubated with species-appropriate horseradish peroxidase-conjugated secondary antibody (1:25000 in TBST with 5% non-fat milk). Proteins were visualised by enhanced chemiluminescence (Amersham ECL Western Blotting Detection Reagent, UK) and quantified by densitometry (QuantityOne or Image Lab software, Bio-Rad).

Total protein and RNA. Unless stated otherwise, total protein concentrations reported in figures are from BCA protein assay measurements of basal conditions in metabolic assays. Compared to sample lysis for immunoblot analysis, homogenisation of cell monolayers for normalisation of metabolic assays was performed in larger volumes of buffer. Thus, these measurements more accurately reflect total protein concentration per well. In cases where total protein and RNA was determined for multiple experiments using primary cells from the same donor, values were log2 transformed, Z-scored and the average Z-score across assays was used for statistical analysis.

Immunocytochemistry. Myotubes were immuno-stained for fast myosin heavy chain isoforms (MYH1/2, 1:250; sc-53088, Santa Cruz Biotechnology) using methods as described [32], with the addition of nuclear counterstaining. After 90 min incubation with Alexa Fluor 594 goat anti-mouse (A-11005) secondary antibody (1:500) at room temperature with gentle rocking, cells were washed twice with 0.025% Tween-20 in PBS and counterstained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) in PBS for 5 min at room temperature. Myotubes were then washed twice in PBS and kept in PBS protected from light at 4°C until imaging. MYH1/2 and nuclei images were taken using 20x magnification on a Zeiss Axio Vert.A1 inverted fluorescent microscope, equipped with ZEN software (Carl Zeiss Microscopy GmbH, Germany). Images were obtained from three random fields of view per well across 3 technical replicate wells, producing nine images per condition for each donor. MYH1/2 images were converted to 8-bit binary and quantification of MYH1/2 area (μm^2) per field was automated using ImageJ software (Fiji) to prevent measurement bias. Nuclei were counted manually using the ImageJ cell-counter plugin. Fused myonuclei were nuclei counted within the MYH1/2 area automatically detected by ImageJ software in the MYH1/2 analysis and fusion index calculated as the percentage of fused versus total nuclei per field of view.

Statistics. Analyses were performed using either R 4.1.0 (www.r-project.org) or GraphPad Prism 10.0.3 software (GraphPad Software Inc.). Exact p-values are specified for comparisons where probability met $p<0.1$. Data are presented as box-and-whisker plots with Tukey distribution and pairing by donor unless specified otherwise in figure legends. Normality was tested using Shapiro-Wilk test before applying appropriate parametric or non-parametric tests. In 2-way ANOVA analyses, Tukey ladder transformation was used when residuals violated assumptions of normality. Statistical tests and sample sizes are described in figure legends. Bioinformatic analyses are described in their respective method sections.

Main Text Figures

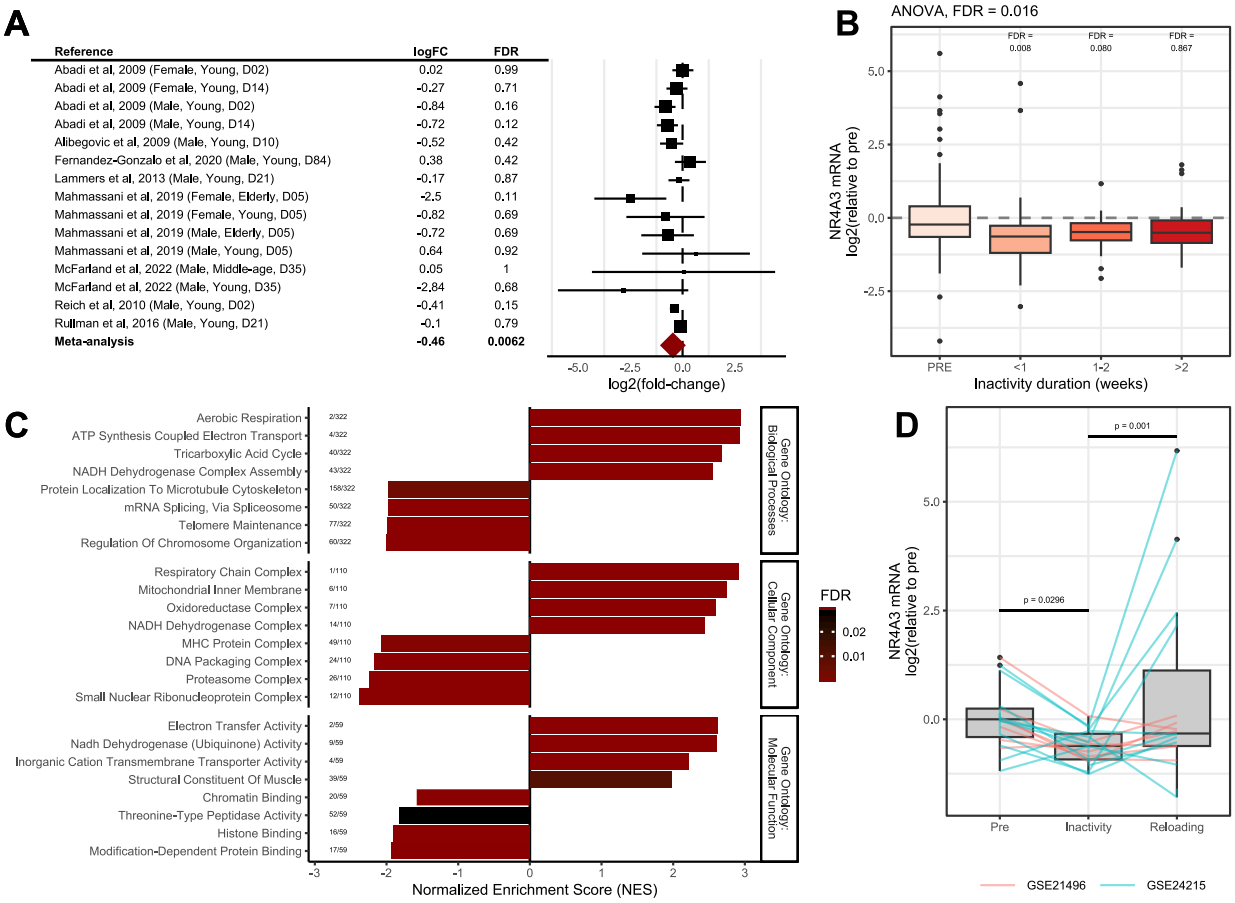


Figure 1. Downregulation of *NR4A3* early during inactivity correlates with transcripts involved in myogenic and metabolic pathways. A. Meta-analysis of transcriptomic inactivity studies in human skeletal muscle. **B.** Transcriptomic studies were pooled by the duration of inactivity protocols: less than one week, one-two weeks or more than two weeks. Datasets were normalized as described in methods. **C.** Gene set enrichment analysis with the Gene Ontology dataset was performed on genes ranked on Spearman correlation with *NR4A3* across all transcriptomic studies of inactivity. **D.** The two studies that explored effects of reloading after inactivity were merged and analysed as described in methods.

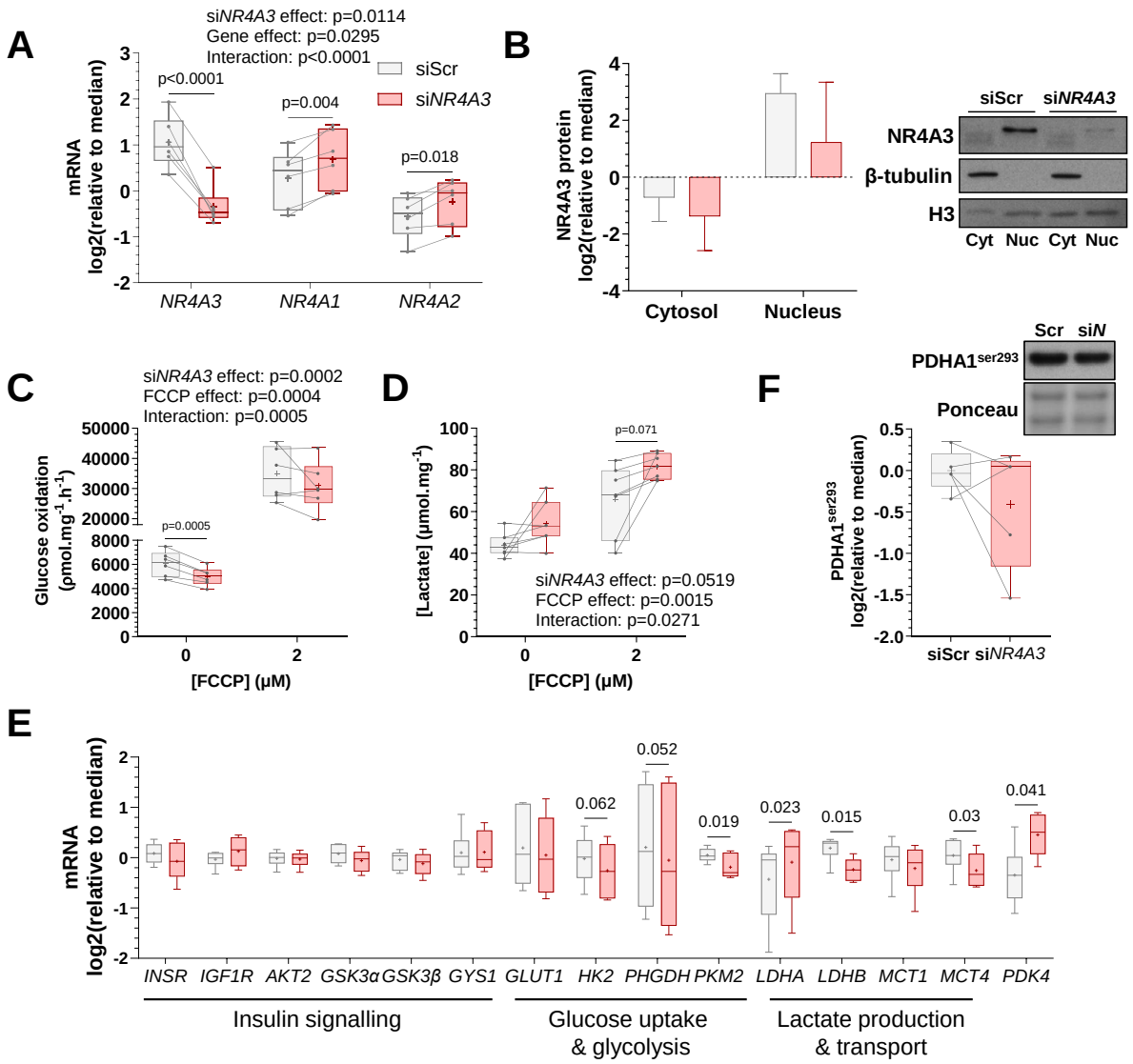


Figure 2. *NR4A3* silencing in primary human myotubes attenuates glucose oxidation by diverting glucose towards lactate production. Primary skeletal muscle cells were exposed to a control scramble sequence (siScr) or a silencing RNA targeting *NR4A3* (si*NR4A3*). **A.** mRNA expression of NR4A family members *NR4A3*, *NR4A1* and *NR4A2* measured by RT-qPCR. n = 6, 2-way ANOVA (silencing x gene) with Šidák correction. **B.** Protein level of NR4A3 in cytosolic and nuclear fractions assessed by immunoblot analysis. Results are mean ± SEM, n = 2. **C.** Rates of radiolabelled glucose oxidation under basal and (2 µM) FCCP-stimulated conditions over 4 h. n = 6, 2-way ANOVA (silencing x FCCP) with Šidák correction. **D.** Lactate concentration in cell supernatant measured after 48 h of basal or (2 µM) FCCP-stimulated conditions. n = 7, 2-way ANOVA (silencing x FCCP) with Šidák correction. **E.** mRNA expression of genes involved in glucose metabolism measured by RT-qPCR. Results are box-and-whisker plots with Tukey distribution. n = 6, paired t-tests with FDR correction. **F.** Representative immunoblot and quantification of pyruvate dehydrogenase E1 Subunit Alpha 1 (PDHA1) phosphorylation at ser^{293}. n = 5, 2-way ANOVA (silencing x insulin + leucine). Only basal data are shown.

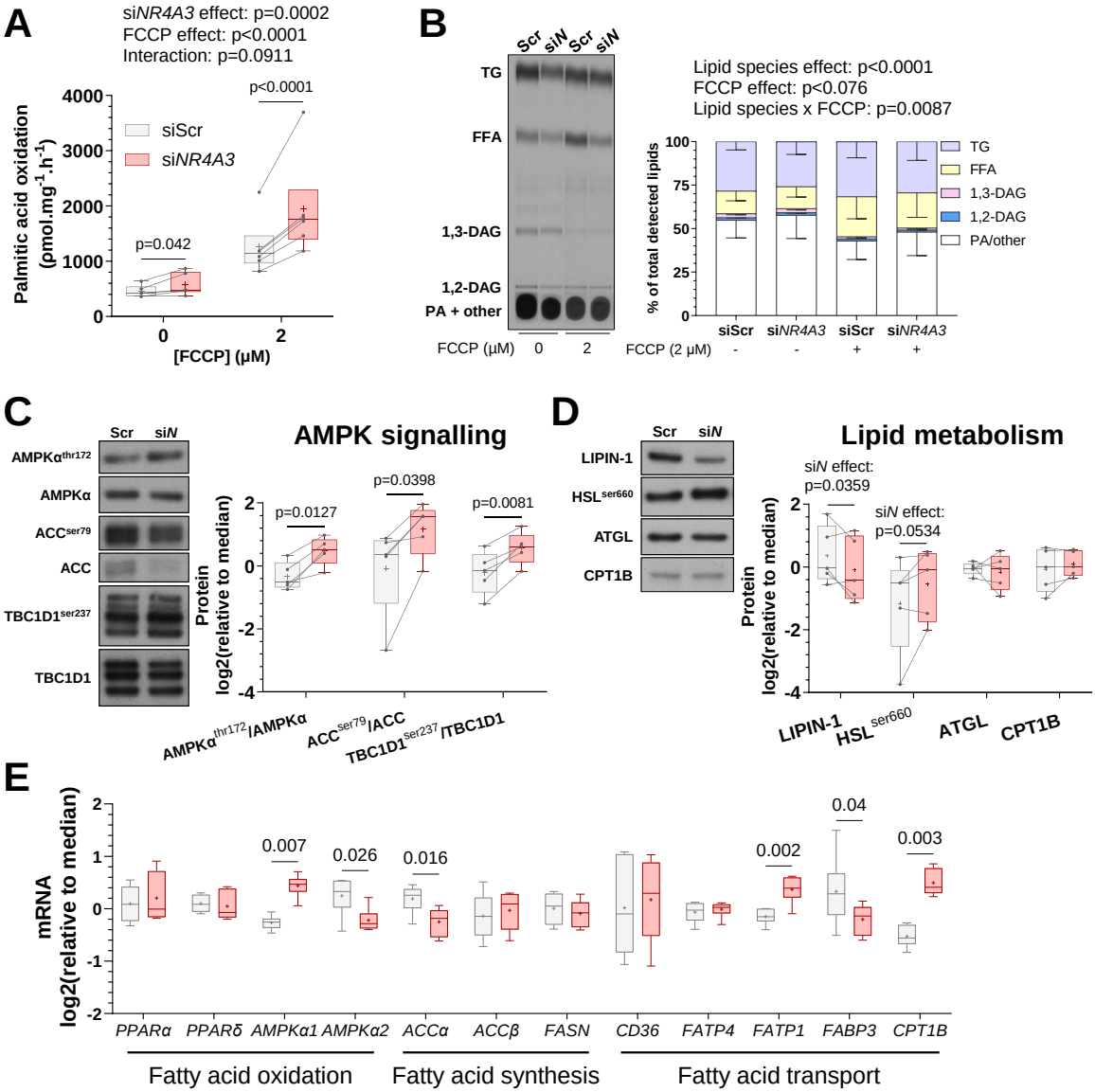


Figure 3. NR4A3 depletion upregulates fatty acid oxidation and AMPK signalling. Primary skeletal muscle cells were exposed to a control scramble sequence (siScr) or a silencing RNA targeting *NR4A3* (si*NR4A3*). **A.** Rates of radiolabelled ^{14}C palmitic acid oxidation under basal and (2 µM) FCCP-stimulated conditions over 4 h. n = 6, 2-way ANOVA (silencing x FCCP) with Šidák correction. **B.** Detection of select lipid species by thin layer chromatography (TLC) of lipid extracts from myotubes after 6 h of basal or (2 µM) FCCP-stimulated conditions. TG = triacylglycerides, FFA = free fatty acids, DAG = diacylglycerol, PA + other = phosphatidic acid plus all other non-migrating lipid species. Results are the mean ± SEM of each lipid species as a percentage of total detected lipid species within condition. n = 5, 3-way ANOVA (silencing x lipid species x FCCP). **C.** Immunoblot of AMPK-signalling proteins from a representative donor and quantification of phosphorylated-to-total protein ratios. n = 5, 2-way ANOVA (silencing x insulin + leucine) with Šidák correction. Only basal data are shown. **D.** Representative immunoblot and quantification of proteins involved in lipid storage, mobilisation, and transport. n = 5, 2-way ANOVA (silencing x insulin + leucine) with Šidák correction. Only basal data are shown and overall effects of silencing in the model are presented (p<0.1). **E.** mRNA expression of genes involved in lipid metabolism measured by RT-qPCR. Results are box-and-whisker plots with Tukey distribution. n = 6, paired t-tests with FDR correction.

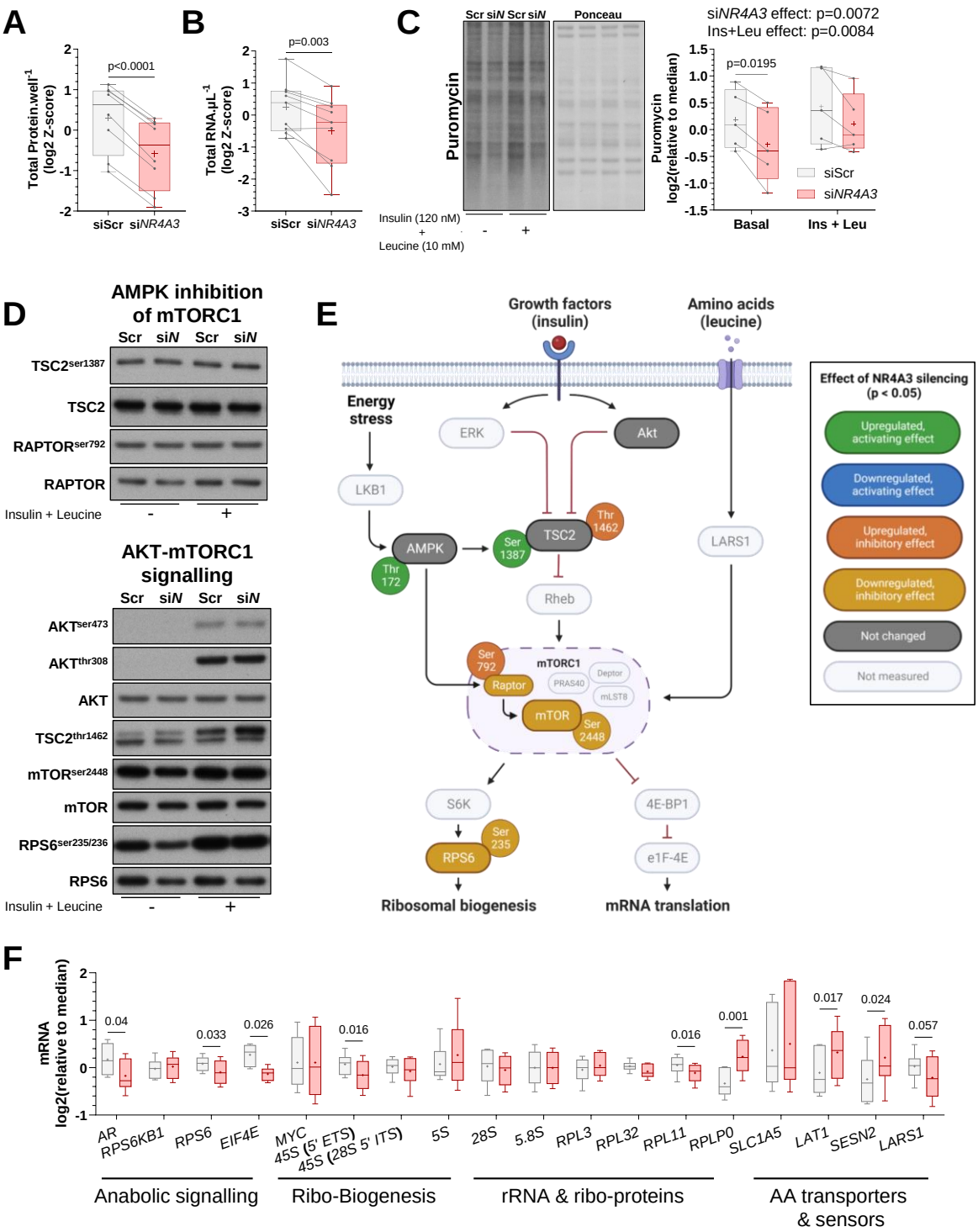


Figure 4. *NR4A3* silencing impairs mTORC1 signalling, ribosomal biogenesis, and muscle translation. Primary skeletal muscle cells were exposed to a control scramble sequence (siScr) or a silencing RNA targeting *NR4A3* (si*NR4A3*). **A.** Total protein per well and **B.** RNA per µL. Results are the average Z-score of log2-transformed values across experiments. n = 8 for protein and n = 9 for RNA, paired t-test. **C.** Representative immunoblot and quantification of cellular protein synthesis assessed by protein puromycilation (i.e. SUnSET method). n = 5, 2-way ANOVA (silencing x insulin + leucine) with Šidák correction. **D.** Immunoblotting of proteins involved in AMPK-mediated mTORC1 inhibition and AKT-mTORC1 pathways from a representative donor. **E.** Schematic representation of immunoblot analysis. n = 5, 2-way ANOVA (silencing x insulin + leucine) with Šidák correction. Quantification of each depicted signalling event can be found in **Supplementary Figure 2**. **F.** mRNA expression of genes involved in amino acid (AA) metabolism and protein synthesis measured by RT-qPCR. Results are box-and-whisker plots with Tukey distribution. n = 6, paired t-tests with FDR correction. 'Ribo-' = ribosomal.

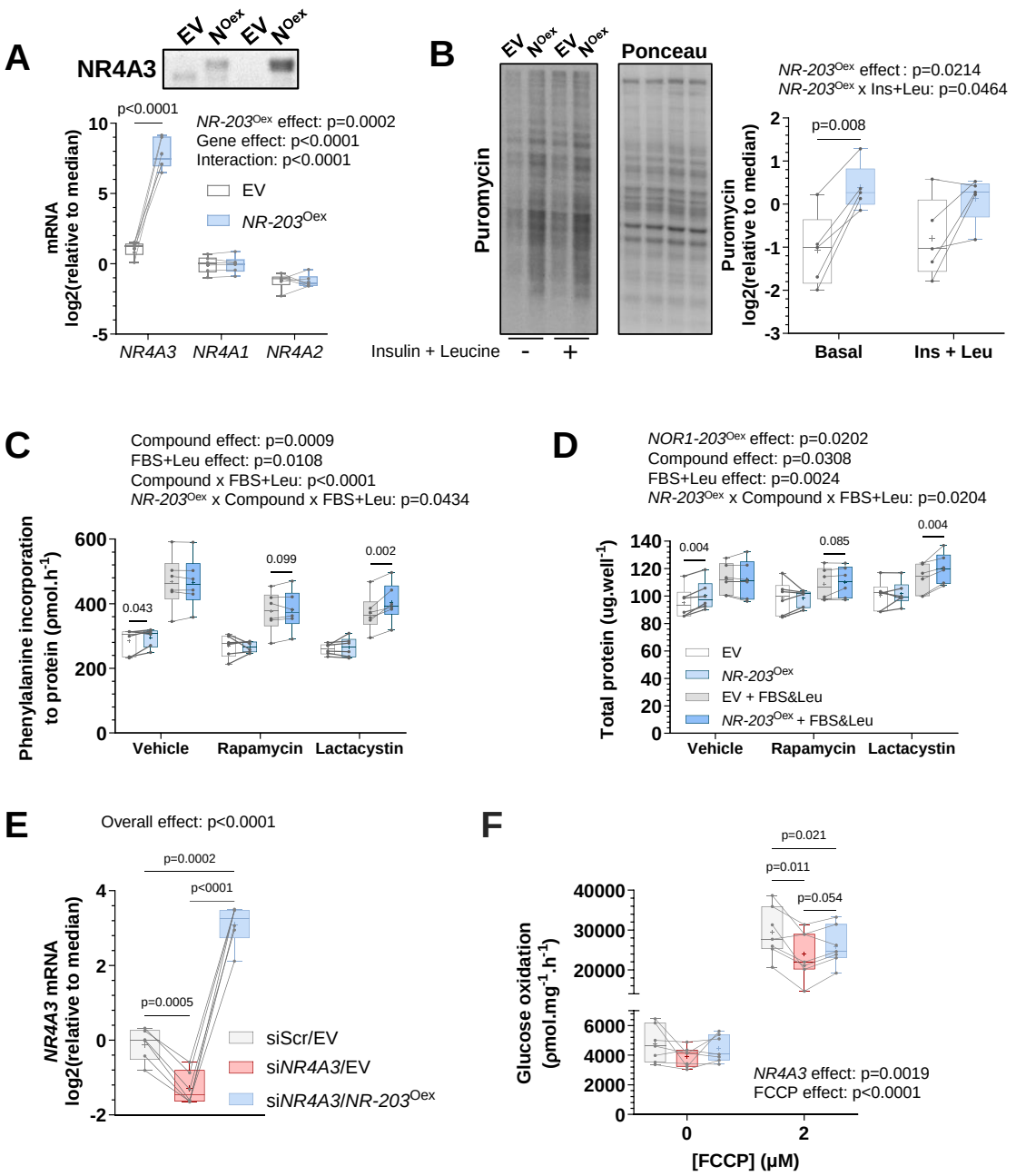


Figure 5. Overexpression of the canonical *NR4A3* isoform increases muscle protein synthesis and partially restores glucose oxidation in NR4A3-deplete myotubes. Primary skeletal muscle cells were transduced with an empty vector control plasmid (EV) or a plasmid containing variant *NR4A3-203* (*NR-203*^{Oex}). **A.** mRNA expression of NR4A family members *NR4A3*, *NR4A1* and *NR4A2* measured by RT-qPCR. n = 6, 2-way ANOVA (overexpression x gene) with Šidák correction. Also, protein level of NR4A3 from two indicative donors. **B.** Representative immunoblot and quantification of cellular protein synthesis assessed by protein puromycilation (i.e. SUnSET method). n = 5, 2-way ANOVA (overexpression x insulin+leucine) with Šidák correction. **C.** Rates of radiolabelled phenylalanine incorporation to protein under basal and (20%) FBS plus (10 mM) leucine-stimulated conditions over 6 h, with or without mTORC1- (rapamycin) or proteasomal- (lactacystin) inhibition. n = 6, 3-way ANOVA (overexpression x FBS+Leucine x compound) with FDR correction. **D.** Total protein per well from same experiment as C. **E.** *NR4A3* mRNA measured by RT-qPCR. n = 6, 1-way ANOVA with Tukey correction. **F.** Rates of radiolabelled glucose oxidation under basal and (2 µM) FCCP-stimulated conditions over 4 h. n = 7, 2-way ANOVA (overexpression x FCCP) with Fisher's LSD post-test.

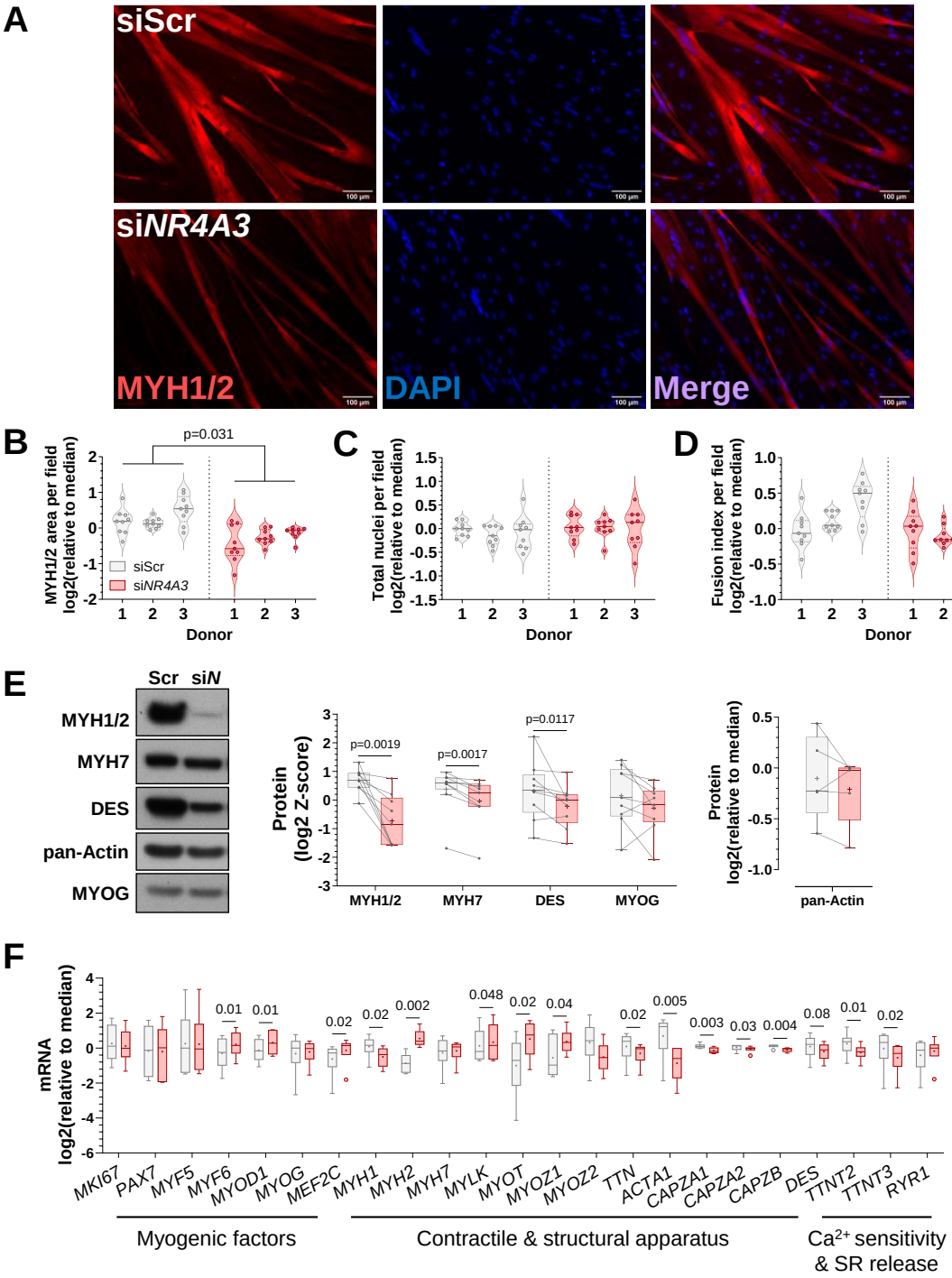
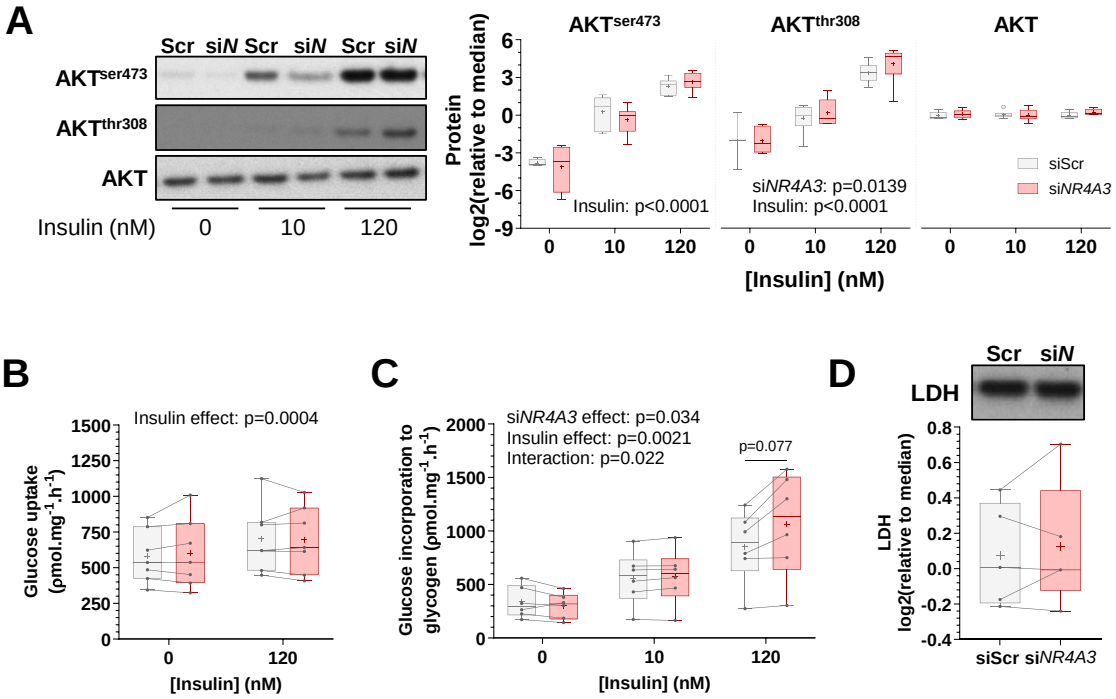
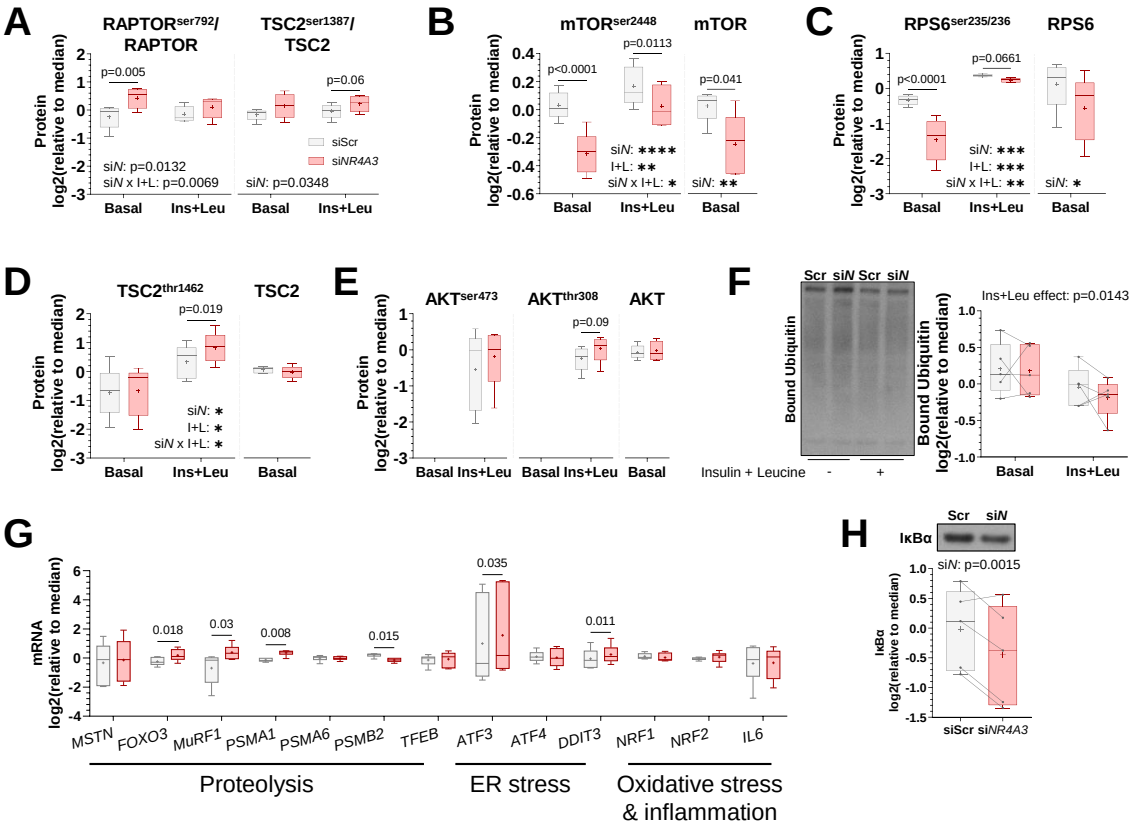


Figure 6. NR4A3 downregulation reduces myotube size and alters the expression of muscle contractile and structural apparatus. Primary skeletal muscle cells were exposed to a control scramble sequence (siScr) or a silencing RNA targeting *NR4A3* (si*NR4A3*). **A.** Immunocytochemistry of fast myosin heavy chain isoforms (MYH1/2, red) and nuclei (DAPI, blue) from a representative donor. Scale bar = 100 µm. Quantification of immunocytochemistry for **B.** MYH1/2 area, **C.** total nuclei, and **D.** fusion index per field of view. Results are violin plots with median and interquartile range. Circles represent measurements from different fields of view across three technical replicates per donor. n=3, nested paired t-test. **E.** Representative immunoblot and quantification of proteins involved in contraction, structure, and myogenic transcription. Results for MYH1/2, MYH7, DES, and MYOG are the average Z-score of log2-transformed values across experiments (n = 9, paired t-test). Results for pan-Actin are n = 5, 2-way ANOVA (silencing x insulin + leucine) with only basal data shown. **F.** mRNA expression of genes involved in myogenesis, muscle structure and contraction measured by RT-qPCR. Results are box-and-whisker plots with Tukey distribution. n = 6, paired t-tests with FDR correction. 'SR' = sarcoplasmic reticulum.

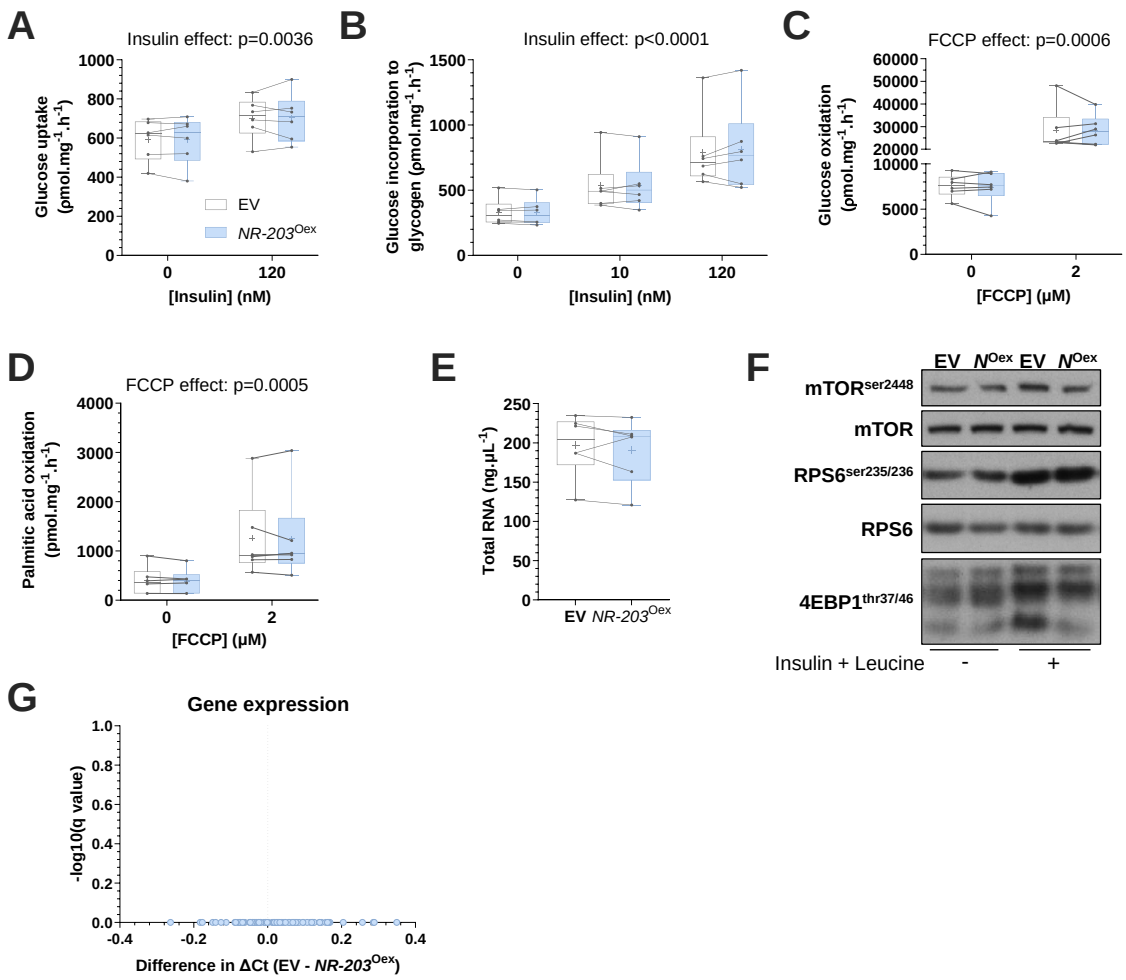
Supplementary Figures



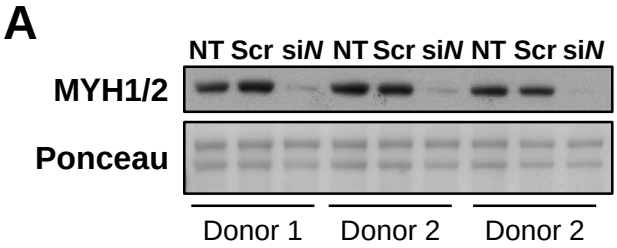
Supplementary Figure 1. NR4A3-depleted myotubes retain responsiveness to insulin stimulation. Primary skeletal muscle cells were exposed to a control scramble sequence (siScr) or a silencing RNA targeting *NR4A3* (si*NR4A3*). **A.** Representative immunoblot and quantification of AKT phosphorylation at sites ser473 and thr308. n = 6, 2-way ANOVA (silencing x insulin) with Šidák correction. **B.** Rates of radiolabelled 2-Deoxy-D-glucose uptake (n=7) and **C.** glycogen synthesis (n=6) under basal and insulin-stimulated conditions. 2-way ANOVA (silencing x insulin) with Šidák correction. **D.** Representative immunoblot and quantification of total lactate dehydrogenase (LDH). n = 5, 2-way ANOVA (silencing x insulin + leucine). Only basal data are shown.



Supplementary Figure 2. *NR4A3* silencing attenuates mTORC1 signalling with inconsistent effects on protein degradation pathways. Primary skeletal muscle cells were exposed to a control scramble sequence (siScr) or a silencing RNA targeting *NR4A3* (si*NR4A3*). **A.** Immunoblot quantification of phosphorylated-to-total protein ratios for AMPK target sites on raptor (ser792) and TSC2 (ser1387). **B.** Immunoblot quantification of AKT-mTORC1 activating phosphorylation sites and total protein levels for mTOR, **C.** RPS6, **D.** TSC2, and **E.** AKT. **F.** Representative immunoblot and quantification of cellular protein ubiquitination. **G.** mRNA expression of genes related to the negative regulation of muscle mass, endoplasmic reticulum (ER) stress, oxidative stress, and inflammation measured by RT-qPCR. Results are box-and-whisker plots with Tukey distribution. n = 6, paired t-tests with FDR correction. **H.** Representative immunoblot and quantification of IκBα. All immunoblot analyses are n = 5, 2-way ANOVA (silencing x insulin + leucine) with Šidák correction. Only basal data are shown for total mTOR, RPS6, TSC2, AKT, and IκBα. ******** = p<0.0001, ******* = p<0.001, ****** = p<0.01, ***** = p<0.05.



Supplementary Figure 3. Overexpression of the canonical *NR4A3* isoform increases muscle translation without altering glucose and fatty acid metabolism, RNA abundance, or mTORC1 signalling. Primary skeletal muscle cells were transduced with an empty vector control plasmid (EV) or a plasmid containing variant *NR4A3-203* (*NR-203*^{Oex}). **A.** Basal and stimulated rates of radiolabelled 2-Deoxy-D-glucose uptake, **B.** glycogen synthesis, **C.** glucose oxidation, and **D.** ^{14}C palmitic acid oxidation. n = 6, 2-way ANOVA (silencing x stimulation). **E.** Total RNA concentration (ng.µL^{-1}). n = 6, paired t-test. **F.** Immunoblotting of proteins in the mTORC1 signalling cascade from a representative donor. No difference in phosphorylation or total protein levels were detected with *NR-203*^{Oex}. n = 5, 2-way ANOVA (overexpression x insulin + leucine). **G.** Volcano plot of all mRNA transcripts assessed after *NR-203*^{Oex} measured by RT-qPCR. Genes were the same as analysed in *NR4A3*-silenced conditions. n = 6, paired t-tests with FDR correction.



Supplementary Figure 4. Effect of *NR4A3* silencing on contractile proteins is not an artefact of siRNA negative control. Primary skeletal muscle cells were exposed to a control scramble sequence (Scr) or a silencing RNA targeting *NR4A3* (si*N*). **A.** Representative immunoblot of myosin heavy chain isoforms IIX (MYH1) and IIA (MYH2) from three independent donors. 'NT' = non-transfected condition.