JYU DISSERTATIONS 208

Jaakko Hentilä

Muscle Physiology and Proteostasis

Effects of Changes in Muscle Size and Exercise

UNIVERSITY OF JYVÄSKYLÄ
FACULTY OF SPORT AND
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ABSTRACT

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Proteostasis results from an equilibrium between the synthesis of functional and degradation of dysfunctional proteins. It is regulated by biological processes, including unfolded protein response (UPR) and autophagy. UPR tries to relieve endoplasmic reticulum (ER) stress that results from protein misfolding in ER and autophagy degrades cellular organelles and proteins. Autophagy and UPR have a pivotal role for skeletal muscle function, but the current knowledge how they are regulated by different conditions influencing muscle quality is limited. This thesis elucidated the effects of muscle wasting, hypertrophy and exercise on the markers of autophagy and UPR by measuring protein and mRNA expression in skeletal muscle. To elucidate the effects of muscle wasting, *mdx* and colon carcinoma 26 (C26) tumor-bearing mice were used to study muscular dystrophy and cancer cachexia, respectively. Additionally, the effects of muscle hypertrophy induced by blocking activin receptor ligands in healthy, cancer cachectic and dystrophic *mdx* mice were examined. Voluntary wheel running was also studied in *mdx* mice. In addition to experimental animal models, the acute (1 h and 48 h) and long-term effects (21 weeks) of resistance exercise and training (RE and RT, respectively) in young (26 ± 4 years) and older (61 ± 6 years) previously untrained men were elucidated. Furthermore, the effects of 20-week experimental training period (EX), in which strength training was integrated with sprint training, in master sprinter men (40–76 years) were studied. The main results of this thesis were that UPR is induced by muscular dystrophy, as well as by a single RE bout in young and older men. Additionally, muscle hypertrophy induced by activin receptor ligand blocking increased UPR markers in healthy mice, while this increase in UPR markers was not observed in muscle hypertrophy induced by the 21-week RT period in young and older men. Autophagosome content, marked by lipidated LC3 protein (LC3II), was increased in C26 cancer cachexia and by RE and RT in previously untrained young men, as well as in masters sprinters conducting long-term strength training in conjunction with sprint training. These results indicate that, as an adaptation to muscle hypertrophy, wasting and exercise UPR and autophagy are regulated distinctly in skeletal muscle depending on the context. These results may be applied in the future as a scientific basis to develop new strategies to prevent and treat muscle wasting and in offering evidence based exercise recommendations.

Keywords: skeletal muscle, autophagy, proteostasis, hypertrophy, atrophy, ER stress, unfolded protein response, redox balance

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Proteostaasissa eli proteiinihomeostaasissa vallitsee tasapaino toiminnallisten proteiinien muodostuksen ja toimimattomien proteiinien hajotuksen välillä. Proteostaasia säätelevät biologiset järjestelmät, kuten laskostumattomien proteiinien vaste [unfolded protein response, UPR] ja autofagia. UPR pyrkii vähentämään endoplasmisen kalvoston [endoplasmic reticulum, ER] stressiä (ER-stressi), joka aiheutuu väärinlaskostuneiden ja laskostumattomien proteiinien kasaantumisesta endoplasmiselle kalvostolle. Autofagia puolestaan hajottaa ja kierrättää soluelimiä ja proteiineja. Autofagia ja UPR säätelevät lihaksen toimintaa. Niiden roolia eri tilanteissa, jotka vaikuttavat lihaksen laatuun ja kokoon, ei täysin tunneta. Tämän väitöskirjan tarkoitus oli tutkia lihaskadon, -kasvun ja liikunnan vaikutuksia UPR:ään ja autofagiaan analysoimalla näiden ilmiöiden merkkiaineiden proteiini- ja mRNA-pitoisuuksia luustolihaksessa. Lihasdystrofian vaikutuksia tutkittiin *mdx*-hiirimallilla. Syöpään liittyvää rajua lihaskatoa ja painonpudotusta eli kakeksiaa tutkittiin kokeellisella paksusuolisyövän (C26) hiirimallilla. Lisäksi tarkasteltiin aktiviinireseptorihoidon aikaansaaman lihaskasvun vaikutuksia terveessä, dystrofisessa ja syöpäkakektisessa lihaksessa. *mdx*-hiirillä tutkittiin juoksupyöräjuoksun vaikutuksia dystrofisessa lihaksessa. Aiemmin harjoittelemattomilla nuorilla (26 ± 4 vuotta) ja vanhemmilla miehillä (61 ± 6 vuotta) tarkasteltiin voimaharjoittelun akuutteja (1 ja 48 tuntia harjoituksen jälkeen) ja pitkäaikaisia (21 viikkoa harjoittelua) vaikutuksia. Lisäksi tutkittiin pikajuoksuharjoittelun rinnalle lisätyn voima- ja nopeusvoimaharjoittelun pitkäaikaisia vaikutuksia (20 viikkoa) veteraanimiespikajuoksijoilla (40–76 vuotta). UPR merkkiaineet lisääntyivät lihasdystrofiassa ja 48 tuntia yksittäisen voimaharjoituksen jälkeen nuorilla ja vanhemmilla aiemmin harjoittelemattomilla miehillä. Terveillä hiirillä aktiviinireseptorihoidolla aiheutettu lihaskasvu lisäsi UPR-merkkiaineita, mutta nousua ei huomattu voimaharjoittelulla saavutetussa lihaskasvussa. Autofagosomien määrää kuvastavan LC3-proteiinin (LC3II) rasvoittunut muoto lisääntyi syövän aiheuttamassa lihaskadossa sekä pidempiaikaisen voimaharjoittelun jälkeen nuorilla miehillä ja veteraanipikajuoksijoilla. Tulokset osoittavat, että sekä autofagiaa että UPR:ää säädellään lihaksessa liikunnan, lihaskasvun ja -kadon seurauksena eri tavoin tilanteen mukaan proteostaasin säilyttämiseksi. Näitä tuloksia voidaan hyödyntää esimerkiksi liikuntasuositusten laatimisessa sekä pohjana uusien lihaskatohoitomuotojen kehittämisessä.

Asiasanat: luurankolihas, autofagia, proteostaasi, hypertrofia, atrofia, ER stressi, laskostumattomien proteiinien vaste, hapetus-pelkistys tasapaino

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on following original publications that are referred by their Roman numerals in the text of this thesis.

I Hulmi JJ, **Hentilä J**, DeRuisseau KC, Oliveira BM, Papaioannu KG, Autio R, Kujala UM, Ritvos O, Kainulainen H, Korkmaz A, Atalay M. 2016. Effects of muscular dystrophy, exercise and blocking activin receptor IIB ligands on the unfolded protein response and oxidative stress. Free Radical Biology and Medicine 99: 308–322. https://doi.org/10.1016/j.freeradbiomed.2016.08.017

II **Hentilä J**, Nissinen TA, Korkmaz A, Lensu S, Silvennoinen M, Pasternack A, Ritvos O, Atalay M, Hulmi JJ. 2019. Activin receptor ligand blocking and cancer have distinct effects on protein and redox homeostasis in skeletal muscle and liver. Frontiers in Physiology 18 (9): 1917. https://doi.org/10.3389/fphys.2018.01917

III **Hentilä J**, Ahtiainen JP, Paulsen G, Raastad T, Häkkinen K, Mero AA, Hulmi JJ. 2018. Autophagy is induced by resistance exercise in young men, but unfolded protein response is induced regardless of age. Acta Physiologica 224 (1): e13069. https://doi.org/10.1111/apha.13069

IV **Hentilä J**, Hulmi JJ, Laakkonen EK, Ahtiainen JP, Suominen H, Korhonen MT. Sprint and strength training modulates autophagy and proteostasis in aging sprinters. Medicine & Science in Sports & Exercise: September 2020 - Volume 52 – Issue 9 – p 1948 - 1959. https://doi.org/10.1249/MSS.0000000000002340

I planned the studies that are included in this thesis together with my supervisor Juha Hulmi in the publications I - IV. The original data collection were designed by Juha Hulmi (I-III), Marko Korhonen and Harri Suominen (IV) as well as Juha Hulmi, Tuuli Nissinen and I (II). I conducted all the biochemical analyses in the publications III and IV and most of the Western blot and RT-qPCR analyses in I and II. I conducted all the statistical analyses in the publications II–IV and for the western blot results in the publication I. In the publication II, I conducted the C26 cancer *in vivo* experiments with Tuuli A. Nissinen and participated in the tissue collection. I had the major responsibility of writing the manuscripts II–IV and wrote the publication I together with Juha Hulmi.

ABBREVIATIONS

ACVR	Activin receptor
ACVR2B	Activin receptor type 2b
AKT	RAC-alpha serine/threonine-protein kinase
AMPK	Adenosine monophosphate activated protein kinase
ALS	Amyotrophic lateral sclerosis
ATF4	Activating transcription factor 4
ATF6	Activating transcription factor 6
ATF6f	Cytosolic domain of activating transcription factor 6
ATG7	Autophagy-related protein 7
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BCL-2	Apoptosis regulator BCL-2
BMI	Body mass index
BMP-11	Bone morphogenetic protein 11
BSA	Bovine serum albumin
CASA	Chaperone assisted selective autophagy
cDNA	Complementary DNA
CHO	Chinese hamster ovary
CHOP	C/EBP homologous protein
CKD	Chronic kidney disease
COPD	Chronic pulmonary obstructive disease
CSA	Cross-sectional area
C26	Colon carcinoma 26
DAPC	Dystrophin associated protein complex
DMD	Duchenne muscular dystrophy
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Egtazic acid
eIF2α	Elongation initiation factor alpha
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum associated protein degradation
FBS	Fetal bovine serum
FCS	Fetal calf serum
FOXO	Forkhead box proteins
GA	Gastrocnemius
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDF-8	Growth differentiation factor 8/Myostatin
GDF-15	Growth differentiation factor 15
GRP78	Glucose regulated protein 78
GHSR-1a	Growth hormone segretagogue receptor 1a
GSH	Glutathione (reduced form)

GSSG	Glutathione disulfide (oxidized form)
GSSG/GSH	Ratio of glutathione disulfide to glutathione
GR	Glutathione reductase
HEPES	N-2-hydroxyethylpiperazine-N-ethanesulfonic acid
HF	Heart failure
HRP	Horse radish peroxidase
HSP47	Heat shock protein 47
IFNγ	Interferon gamma
IgG1 Fc	Crystallizable fragment of the immunoglobulin class G1
IL-1	Interleukin 1
IL-6	Interleukin 6
IRE1α	Inositol requiring protein 1 alpha
i.p.	Intraperitoneal
JAK	Janus kinase
JNK	c-Jun terminal kinase
kDa	kilo Dalton
LC3	Microtubule-associated protein 1 light chain 3
LC3I	Cytosolic form of Microtubule-associated protein 1 light chain 3
LC3II	Microtubule-associated protein 1 light chain 3 containing PE conjugate
LLC	Lewis lung carcinoma
LIF-1	Leukemia inhibitory factor 1
mdx	Mouse model for Duchenne muscular dystrophy
MEK	Mitogen-activated extracellular signal-regulated kinase
$MgCl_2$	Magnesium chloride
mRNA	Messenger RNA
mTOR	Mechanistic target of rapamycin
NADPH	Reduced form of nicotinamide adenine dinucleotide phosphate
Na_3VO_4	Sodium orthovanadate
Na4P2O7 ·10H2O	Tetrasodium pyrophosphate decahydrate
NaF	Sodiumfluoride
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PARKIN	E3 ubiquitin-protein ligase parkin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDI	Protein disulfide-isomerase
PE	Phosphatidylethanol
PERK	PRKR-like endoplasmic reticulum kinase
pH	Power of hydrogen - indicator of hydrogen concentration
PINK1	PTEN-induced putative kinase protein 1
PI3K	Phosphoinositide 3-kinase

PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene fluoride
P62/SQSTM1	Sequestome 1
RE	Resistance exercise
REDOX	Reduction - oxidation
RM	Repetition maximum
RNA	Ribonucleic acid
RT	Resistance training
sACVR2B-Fc	Recombinant soluble activin receptor type 2B
sACVR	Recombinant soluble activin receptor type 2B
SDS-PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
Ser	Serine
shRNA	Short hairpin RNA
SIRT	Sirtuin
SR	Sarcoplasmic reticulum
STAT	Signal transducer and activator of transcription
TA	Tibialis anterior
TB	Tumor-bearing
TBS-T	Tris-buffered saline containing tween
TFEB	Transcription factor EB
TGF-β	Transforming growth factor beta
Thr	Threonine
TNF-α	Tumor necrosis factor alpha
TUDCA	Tauroursodeoxycholic acid
TWEAK	Tumor necrosis factor ligand superfamily member 12
Tyr	Tyrosine
ULK	Unc-51 like kinase
UPR	Unfolded protein response
UPS	Ubiquitin-proteasome-system
VL	Vastus lateralis
VO_2 max	Maximum oxygen uptake
WB	Western blot
wt	Wild-type
XBP1	X-box binding protein 1
Xbp1s	Spliced variant of X-box binding protein 1 mRNA
Xbp1t	Unspliced X-box binding protein 1 mRNA

CONTENTS

1 INTRODUCTION

Skeletal muscle is a highly plastic post-mitotic tissue that is connected to bones by tendons and is together with nervous system responsible of our locomotion. In addition to locomotion, skeletal muscle is involved in thermoregulation and due to its high metabolic activity, it has a key role in the prevention of metabolic diseases (Frontera and Ochala 2015). Furthermore, muscle wasting for example in cancer is an independent bad prognosis factor (Martin et al. 2013; Choi et al. 2015) and the loss of muscle function in the most severe forms of muscular dystrophies lead to premature death (Mercuri and Muntoni 2013). Furthermore, greater muscle function and strength are associated with reduced mortality (Cooper et al. 2010) and better survival from catastrophic situations, for example after hip fracture (Rantanen et al. 2002), while some early rodent studies even suggest that maintaining muscle mass in cancer improves survival (Zhou et al. 2010). Additionally, sufficient muscle strength in the elderly enables independent lifestyle (Kell et al. 2001; Reid et al. 2008).

Since skeletal muscle mass and function are important for mobility, wellbeing and health, as described above, it is important to understand the underlying mechanisms regulating muscle mass and function in different conditions that lead to changes in muscle quality. This knowledge would provide a profound basis to treat muscle in different conditions ultimately leading to better muscle function. In athletes, this could mean improved performance, whereas in frail populations improved mobility and enhanced quality of life. Despite huge progress made in the research field of muscle physiology, the current knowledge of the underlying mechanisms regulating muscle function is still limited (Mukund and Subramaniam 2020).

Accumulating evidence suggest that the maintenance of protein homeostasis, which results from an equilibrium between the synthesis of functional and degradation of dysfunctional proteins, is vital for the cells (Schneider and Bertolotti 2015). To maintain the vital proteostasis, a network of integrated processes including autophagy and unfolded protein response (UPR), among others, has evolved for cells (Sandri 2010; Powers and Balch 2013; Hetz et al. 2015). Of these two protein quality control processes, autophagy recycles and degrades

dysfunctional proteins and organelles (Sandri 2010), whereas UPR is activated to relieve endoplasmic reticulum (ER) stress that is induced by increased protein misfolding in ER (Hetz et al. 2015). Recent evidence from knockout animal models demonstrate that these two processes are vital for muscle function and may also play a role in eliciting adaptations to exercise (Masiero et al. 2009; Masiero and Sandri 2010; Wu et al. 2011; Lira et al. 2013; Bohnert et al. 2016). In addition, previous studies in humans have shown that these processes are associated with muscle wasting conditions such as muscular dystrophies and are induced upon exercise (Kim et al. 2011a; Moorwood and Barton 2014; Ogborn et al. 2014, 2015). However, the current knowledge of their role in different conditions that lead to changes in muscle quality is not comprehensive. The long-term exercise training effects in humans are especially lacking. In addition, the effects of activin receptor ligand blocking – a potent pharmacologic way to increase muscle mass – on muscle proteostasis in naïve and muscle wasting conditions, is not known. Thus, this thesis has diversely elucidated the effects of different conditions that lead to changes in muscle quality on the markers of UPR induced by ER stress and autophagy. In conjunction with UPR and autophagy, redox balance was addressed in muscle wasting conditions and in response to blocking activin receptor ligands because redox balance influences protein homeostasis (Cao and Kaufman 2014). The focus was on the effects of muscle wasting, hypertrophy and exercise, which were studied in preclinical animal models and human populations that differed in age and training status.

2 LITERATURE REVIEW

2.1 Skeletal muscle protein homeostasis

During cellular protein homeostasis (i.e. proteostasis), there is an equilibrium between the synthesis of correctly folded functional proteins and the degradation of misfolded, damaged or long-lived dysfunctional proteins (Clausen et al. 2019). Since proteins are involved in a vast majority of the processes that occur in cells, the maintenance of a functional proteome is paramount in keeping a biological organism functional (Schneider and Bertolotti 2015). In the worst-case scenario, disrupted proteostasis may induce, for example, aggregation of cytotoxic proteins that are suggested to cause or be associated with several pathological conditions (Balch et al. 2008). In addition, there is an age-related decline in the proteostasis control that is suggested to contribute, for example, to age-related muscle atrophy, that is, sarcopenia (Fernando et al. 2019). In order to maintain proteostasis under normal and various stress conditions, a network of several integrated biological processes responsible for intricate protein quality control has evolved for cells (Powers and Balch 2013; Schneider and Bertolotti 2015). The most relevant ones of these quality control processes for this thesis are described in more detail in the following sections.

2.1.1 Autophagy lysosome pathway

For the past few decades, autophagy, which literally means "self-eating" (Yang and Klionsky 2010), has been a hot research topic. In 2016, for example, the Nobel prize in Physiology or Medicine was awarded to Yoshinori Ohsumi who, together with his research group, discovered the key mechanisms of autophagy (Dlugonska 2017). In normal conditions, this constantly ongoing process degrades and recycles dysfunctional cellular organelles and proteins (Rabinowitz and White 2010; Yin et al. 2016). Autophagy thus acts as a quality control process

that is vital for maintaining cell homeostasis. As an indicator of its importance in homeostasis regulation, many pathologic conditions are associated with impaired autophagy, which may lead to the accumulation of cytotoxic protein aggregates and dysfunctional cellular organelles (Levine and Kroemer 2019). In addition, basal autophagy is suggested to decline during aging, which might partially explain the aging phenotype and the pathogenesis of many age-related diseases (Rubinsztein et al. 2011; Martinez-Lopez et al. 2015).

Autophagy can also be induced during various stress conditions in order to maintain cellular homeostasis and viability (Yin et al. 2016). For example, during starvation, the breakdown of cellular material through autophagy provides energy for essential cellular reactions. It can also provide building blocks such as amino acids for the vital cellular proteins during amino acid deprivation (Rabinowitz and White 2010). Furthermore, stress conditions that increase damage to organelles and proteins, such as increased oxidative stress (Lee et al. 2012) heat (Swanlund et al. 2008; Kumsta et al. 2017) and mechanical stress (King et al. 2011) induce autophagy.

Autophagy can be divided into three subcategories: (i) macroautophagy, (ii) microautophagy and (iii) chaperone-assisted selective autophagy (CASA). The most studied and best understood of these subcategories is macroautophagy, hereafter referred to simply as autophagy. It is a catabolic process in which a bulk of cytoplasmic material such as cellular organelles and proteins are engulfed into double-membrane vesicles called autophagosomes and are transported to lysosomes for degradation (Mizushima 2007; Yin et al. 2016). The membrane source for autophagosomes is still under debate, but it has been suggested that there are several possible donor candidates, including endoplasmic reticulum, mitochondria and plasma membrane (Reggiori and Ungermann 2017; Yu et al. 2018).

Steps of autophagy. Autophagosome formation is the initial step in the whole autophagy process and it can be divided into three steps: (i) initiation, (ii) nucleation and (iii) expansion of the isolation membrane. The initiation step is regulated by the unc-51 like kinase (ULK) complex, which consists of several proteins, including ULK1 (Kaur and Debnath 2015). The activity of this complex, and thus the initiation of autophagy, is regulated by various post-translational modifications of ULK1 (Zachari and Ganley 2017). For example, during anabolic conditions, the mechanistic target of rapamycin (mTOR) is able to phosphorylate ULK1 at ser757, thus inhibiting the ULK complex's activity, whereas during catabolic conditions, such as energy deprivation, AMP-activated protein kinase (AMPK) phosphorylates ULK1 at ser555, which leads to the activation of the ULK complex (Kim et al. 2011b). The next step in autophagosome formation is the nucleation of the autophagosome membrane. During this phase, the growing autophagosome is called the phagophore. To induce nucleation, the activated ULK complex activates a class III phosphoinositide 3-kinase (PI3K) complex that is composed of several proteins, including beclin-1 (Kaur and Debnath 2015). This step can be antagonized by apoptosis regulator BCL-2 that inhibits beclin-1 (Pattingre et al. 2005). After the nucleation step, the phagophore elongates and expands into a complete double-membrane vesicle called the autophagosome. This expansion

and completion phase requires, for example, the conjugation of phosphatidylethanolamine (PE) to the microtubule-associated protein 1 light chain 3 (LC3) (Kaur and Debnath 2015). The PE-conjugated LC3 is called LC3II. It resides on internal and external autophagosome membranes and is thus used as a marker to measure autophagosome content (Kabeya et al. 2000; Klionsky et al. 2016). The PE-conjugation of LC3 is required for the autophagosome membrane expansion and for the fusion of autophagosome with lysosomes (Kaur and Debnath 2015). In addition, it is needed for the selection of disposable autophagic cargo because of its ability to interact with sequestome 1 (p62), a protein that recognizes and binds to cellular material that is to be degraded through autophagy (Bjorkoy et al. 2005). After these complex steps, the complete autophagosomes dock and fuse with lysosomes to form another vacuole structure called the autolysosome. Finally, after the fusion, autophagosomes release their disposable cargo into lysosomes where the cellular material is degraded by hydrolase enzymes that are active in low pH (Kaur and Debnath 2015).

In addition to non-selective "bulk" autophagy described above, there are special types of autophagy, such as mitophagy, that selectively degrade damaged mitochondria. When mitochondria are damaged and they lose their membrane potential, PTEN-induced putative kinase protein 1 (PINK1) accumulates around mitochondria and subsequently recruits E3 ubiquitin-protein ligase parkin (PARKIN). Then PARKIN ubiquitylates mitochondrial proteins, which induces engulfment of damaged mitochondria by isolation membranes. Once these vacuole structures are complete, they fuse with lysosomes where mitochondria become degraded (Youle and Narendra 2011).

Muscle. Autophagy plays an important role for skeletal muscle homeostasis regulation since skeletal muscle is metabolically a very active post-mitotic tissue that encounters various stressors (e.g. mechanical, heat and oxidative stress) (Sandri 2010; Vainshtein and Hood 2016). To give an example of its importance in muscle homeostasis regulation, the muscle-specific deletion of a crucial autophagy gene called autophagy-related protein 7 (ATG7) induced substantial muscle atrophy that was accompanied by the accumulation of abnormal cellular organelles and disorganization of sarcomere structure in mice (Masiero et al. 2009). In addition, in another study, the same mouse model manifested a shorter life-span and deterioration of normal muscle-nerve interaction (Carnio et al. 2014). Furthermore, impairments in autophagy are associated with several myopathies and muscle dystrophies (Sandri et al. 2013), and excess levels of autophagy are associated with muscle atrophy in cancer cachexia (Penna et al. 2013), sepsis (Mofarrahi et al. 2012) and chemotherapy treatment (Smuder et al. 2011).

Exercise. In 1984, Salminen and Vihko observed for the first time that autophagic vacuolar structures were increased after a bout of strenuous aerobic exercise in rat muscles (Salminen and Vihko 1984). Since then, autophagy in the context of exercise has been investigated in a growing body of studies. Data obtained from transgenic mouse studies have suggested that autophagy is crucial for the maintenance of muscle glucose homeostasis during exercise (He et al. 2012), to elicit muscle remodeling in response to endurance training (Lira et al.

2013) and to maintain a functional pool of mitochondria after repeated exercise bouts (Lo Verso et al. 2014). In addition, markers of autophagy and especially LC3II as a marker of autophagosome content have been shown to increase acutely after endurance exercise in rodents (Grumati et al. 2010; He et al. 2012; Vainshtein et al. 2015), suggesting autophagy induction. Furthermore, as an adaptation to chronic endurance training, LC3II was increased in mouse muscle (Hulmi et al. 2013b; Lira et al. 2013). However, the data obtained from human endurance exercise studies differ from rodent studies. In human muscle, autophagosome content (LC3II) has either decreased (Moller et al. 2015; Schwalm et al. 2015; Fritzen et al. 2016) or remained unchanged (Masschelein et al. 2013) acutely after bouts of endurance exercise.

Resistance exercise. Regarding resistance exercise (RE), the autophagosome content has been reported to decrease acutely after a bout of RE, but may increase a few days after the RE bout in human muscle (Fry et al. 2013; Ogborn et al. 2015). As an addition to studies investigating only macroautophagy, chaperone-assisted selective autophagy (CASA) was reported to be activated after a strenuous bout of RE in humans (Ulbricht et al. 2015). This induction of CASA has been suggested to be initiated by mechanical strain that caused damage to the cytoskeletal proteins. The studies investigating the effects of long-term resistance training on muscle macroautophagy are currently limited to one rodent study that suggested chronic resistance training increases basal muscle autophagy in rats (Luo et al. 2013) but the flux of autophagy was not measured. In addition, a previous study suggested that proteins involved in CASA are increased after four weeks of progressive resistance training as an adaptation to cope with mechanical stress (Ulbricht et al. 2015). Even though the intervention studies examining the chronic effects of exercise training on basal muscle autophagy are lacking in humans, cross-sectional studies suggest that life-long participation in mainly endurance-type exercise prevents age-related decline in basal autophagy (Carnio et al. 2014). Thus, long-term studies examining the effects of RE on basal autophagy are warranted.

2.1.2 Endoplasmic reticulum stress and unfolded protein response

Endoplasmic reticulum (ER) is a multifunctional cellular organelle that provides intracellular calcium storage, and is a site for lipid biosynthesis in which at least one third of all the proteins translated are folded and post-translationally modified into their functional structure. These folding and post-translational modification steps are assisted and conducted by an ER-resident protein folding machinery that consists of chaperones and different enzymes. Despite the folding quality control system, many proteins fail to fold to their functional three-dimensional structure, which results in protein misfolding. During ER stress, protein misfolding in ER is increased due to the disturbances in ER homeostasis that consequently may also perturb the function of the folding machinery. Various stressors, including hypoxia, nutrient deprivation, disrupted redox balance and increased protein synthesis, can cause ER stress. Furthermore, point-mutations in the proteins that are folded in ER and disruptions in the ER calcium homeostasis

also increase protein misfolding, and thus induce ER stress (Hetz et al. 2015; Schwarz and Blower 2016).

Upon ER stress, a compensatory stress response, called unfolded protein response (UPR), is activated. It attempts to restore the proteostasis in ER through three distinct signaling pathways that, once activated, expand the volume of ER, increase ER resident chaperone mass, blunt the overall protein translation, induce autophagy and increase ER-associated protein degradation (ERAD), among other effects (Figure 1). However, if cells cannot adapt to severe and prolonged ER stress, UPR will activate apoptotic signaling pathways as a final solution (Hetz et al. 2015; Schwarz and Blower 2016). Accumulating evidence suggests that ER stress plays a role in the pathogenesis of several diseases, including neurodegenerative and muscle wasting diseases (Bohnert et al. 2017; Lindholm et al. 2017).

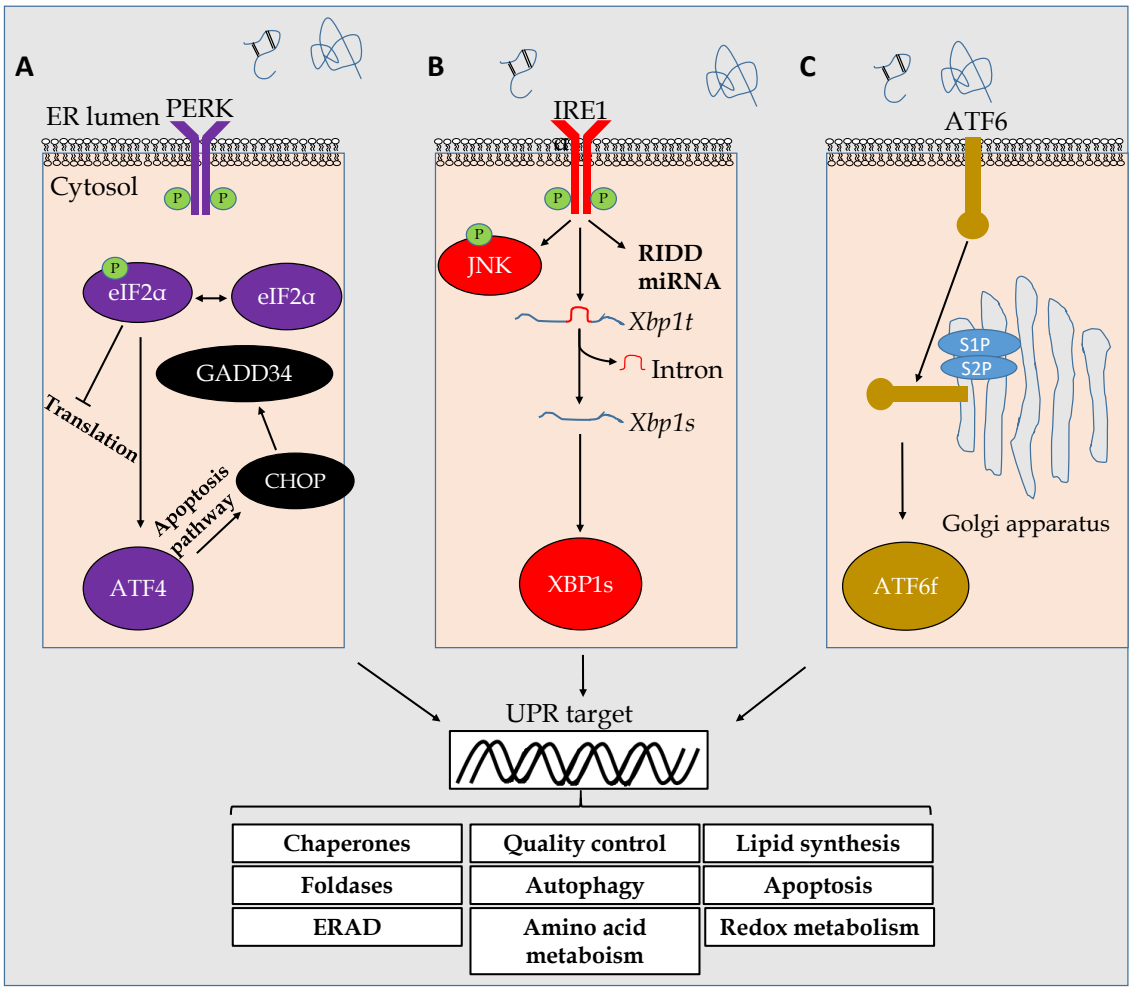

FIGURE 1 The three unfolded protein response (UPR) signaling pathways activated upon endoplasmic reticulum (ER) stress. (A) During ER stress, PRKR-like endoplasmic reticulum kinase (PERK) is autophosphorylated leading to its activation. Activated PERK phosphorylates elongation initiation factor alpha (eIF2α) at ser51. This leads to the blunting of the overall protein translation but increases the gene expression of the transcription factor activating transcription factor 4 (ATF4). ATF4 increases the gene expression of selected genes (listed in white boxes) to restore ER homeostasis. During prolonged ER stress, ATF4 increases the gene expression of the pro apoptotic transcription factor called C/EBP homologous protein (CHOP), which leads to a cascade of apoptotic events. (B) Inositol requiring protein 1 alpha (IREα) is also autophosphorylated during ER stress, leading to its activation. This leads to c-Jun terminal kinase (JNK) phosphorylation and splicing of XBP1 mRNA and other mRNAs. The spliced Xbp1 (*Xbp1s)* produces a transcription factor (XBP1) that increases the expression of selected genes to restore homeostasis in ER (listed in white boxes). (C) Upon ER stress, the activating transcription factor 6 (ATF6) migrates from the ER membrane to the Golgi apparatus where it is cleaved by S1 and S2 proteases. This cleavage produces a cytosolic domain of ATF6 (ATF6f) that acts as a transcription factor inducing the transcription of genes listed in white boxes to restore ER homeostasis (adapted from Hetz et al. 2015).

Muscle and exercise. A specialized form of ER called sarcoplasmic reticulum (SR) is located in skeletal muscle (Rossi et al. 2008). It plays an important role in intracellular calcium transient regulation because muscle contraction requires the release of calcium from SR to cytosol and muscle relaxation requires calcium reuptake from cytosol to SR (Rossi and Dirksen 2006). The structure of skeletal muscle ER/SR differs from other eukaryotic cells. For example, ER in other eukaryotic cells can easily be divided into at least three sub-compartments: (1) rough ER, (2) transitional ER and (3) smooth ER. However, in muscle cells, the distinction between these compartments is not that clear (Rossi et al. 2008). Even though ER in muscle is different than in other eukaryotic cells, accumulating evidence shows that ER stress/UPR plays a role in the regulation of skeletal muscle function and remodeling. For example, mice that lack in skeletal muscle the activating transcription factor 6 (ATF6), which is an upstream regulator of UPR (ATF6$\alpha^{-/-}$), does not improve their endurance running capacity similar to their wild-type littermates after repeated bouts of treadmill running (Wu et al. 2011). This suggests that UPR not only restores ER homeostasis, but that it is also involved in mediating skeletal muscle remodeling in response to aerobic exercise training. In addition, recently, muscle-specific targeted ablation of PRKR-like endoplasmic reticulum kinase (PERK), which is another upstream regulator of UPR signaling, was shown to decrease muscle mass and strength in mice (Gallot et al. 2018). Furthermore, the silencing of spliced variant mRNA of X-box binding protein (*Xbp1s*), which produces a transcription factor that induces genes involved in restoring ER homeostasis, by short hairpin RNA (shRNA) decreased myotube size in C_2C_{12} myotubes (Bohnert et al. 2019). These findings further highlight the importance of UPR for muscle function.

In addition to gene knockout and silencing studies, according to previous findings, UPR is activated in skeletal muscle acutely after a bout of endurance and RE in rodents and humans (Kim et al. 2011a; Wu et al. 2011; Ogborn et al. 2014), which suggests that exercise may be added to the list of ER stress inducers. Furthermore, ER stress/UPR was shown to be induced in muscle by a high-fat diet in mice (Deldicque et al. 2010, 2013), cancer cachexia in Lewis lung carcinoma (LLC) tumor-bearing mice (Bohnert et al. 2016) and rapid muscle hypertrophy induced by synergist ablation in rats (Hamilton et al. 2014). However, the regulatory role of UPR for muscle physiology, pathology and adaptation is currently not completely understood and further studies are needed. Regarding exercise studies, particularly the long-term RE studies investigating basal UPR in humans are lacking.

2.1.3 Oxidative stress

Multiple cellular metabolic events consist of reduction and oxidation reactions (redox) that are defined as gaining and losing electrons, respectively. Some of these redox reactions, such as those involved in the electron transport chain, are susceptible to producing oxidants as byproducts. In addition to the endogenous factors increasing oxidant production, several exogenous factors, including pollutants and radiation, also contribute to oxidant production. Oxidants can react

with different cellular components, including lipids, proteins and DNA, to cause oxidative damage. To prevent this damage, an endogenous antioxidant system has evolved in living organisms that is complemented by exogenous antioxidants provided by dietary intake (Trachootham et al. 2008). In homeostasis, there is an equilibrium between oxidants and antioxidants, but during oxidative stress, this equilibrium is imbalanced in favor of oxidants, leading to increased molecular damage and aberrant redox signaling (Sies and Jones 2007). This imbalance may be the result of increased oxidant production or due to a deficiency in the antioxidant system (Trachootham et al. 2008).

Oxidative stress has a substantial effect on protein homeostasis, because oxidants can induce several deleterious modifications to the proteins, which are the major target of oxidants due to their high cellular abundance. These modifications occur either in the peptide backbone or amino acid residues and may ultimately result in, for example, protein fragmentation, aggregation and unfolding. (Davies 2016). One of the most commonly occurring harmful oxidative protein modifications is carbonylation, in which oxidants react with protein amino acid residues to form ketone, aldehyde or lactam derivatives (Fedorova et al. 2014). If cells cannot remove carbonylated proteins, they may form highly insoluble and cytotoxic aggregates that are associated with several human diseases, including Alzheimer's and Parkinson's disease, as well as with muscle dystrophies and cancer cachexia (Dalle-Donne et al. 2003, 2006; Laviano et al. 2007; Terrill et al. 2013).

As mentioned above, endogenous and exogenous antioxidants, which are further divided into enzymatic and non-enzymatic categories, prevent cellular constituents, including proteins, from oxidant-induced damage (Sies 1997). One of the most important endogenous antioxidants is a peptide called glutathione (GSH), which consists of glutamate, cysteine and glycine (Sies 1999; Wu et al. 2004). It scavenges or removes cellular oxidants such as free radicals (Galano and Alvarez-Idaboy 2011) and peroxides (Day 2009) through redox reactions. In these reactions, GSH is oxidized into glutathione disulfide (GSSG) whereas oxidants are reduced. GSSG is then reduced back to GSH in another redox reaction where a reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) acts as an electron donor and glutathione reductase (GR) as a catalyzing enzyme (Day 2009). On top of directly reacting with oxidants, GSH also interacts with other non-enzymatic antioxidants such as vitamins E & C by reducing them and thus replenishing the cellular antioxidant pool (Forman et al. 2009). Because GSH and GSSG are a major cellular redox couple, the ratio of their abundance is used as an estimate of redox balance and oxidative stress. Typically, during oxidative stress, the ratio of GSSG/GSH increases (Jones 2002).

Exercise. Physical exercise is a potent inducer of oxidative stress because single bouts of exercise have been shown to acutely increase the production of oxidants and induce oxidative modifications to the cellular constituents in muscle. To prevent the harmful effects of exercise-induced oxidative stress, adaptive responses are induced. These responses include the increased expression of antioxidants, which enables more efficient removal of exercise-induced oxidants. As

the second line of defense, the capacity to recycle oxidatively modified cellular constituents is improved. This adaptation process is called hormesis and may be beneficial for the overall viability and function of cells (Radak et al. 2008; Powers et al. 2011, 2016). Interestingly, in exercise intervention studies those participants who received high doses of supplemental antioxidants did not improve their endurance performance (Gomez-Cabrera et al. 2008) or gain muscle mass (Bjornsen et al. 2015) as much as the participants that were in the group receiving a placebo. However, in another resistance-training study, supplemental antioxidants did not hamper muscle hypertrophy, even though anabolic signaling was blunted after a single bout of exercise (Paulsen et al. 2014). Thus, even though excessive production of oxidants during exercise may be harmful, some quantity of oxidants is needed as a signal transducer to elicit adaptations in response to exercise.

2.2 Muscle hypertrophy

Postnatal muscle hypertrophy is primarily a result of the enlargement of single muscle fibers, which is mainly achieved through positive net accretion of proteins and water. Thus, in order to achieve hypertrophy, muscle protein synthesis must be greater than degradation over a longer period of time (Schiaffino et al. 2013). Muscle hypertrophy is often accompanied by increased content of myonuclei (provided by satellite cells) and ribosomes to parallel the increased necessity of protein synthesis (Brook et al. 2019) and may also elicit epigenetic modulation of DNA (Seaborne et al. 2018). At the molecular level, several positive and negative signaling pathways regulating muscle size have been identified (Glass 2003; Schiaffino et al. 2013). An essential regulatory protein complex in these pathways is the mechanistic target of rapamycin (mTOR), which is a conserved energy sensor regulating cellular growth. It is able to activate pathways leading to increased protein synthesis, concurrently halting degradation pathways, such as autophagy (Ma and Blenis 2009). Postnatal muscle hypertrophy occurs during normal maturation as an adaptation to mechanical overloading (e.g. resistance exercise (RE)) and as a response to an administration of exogenous anabolic stimulants such as anabolic hormones (Schiaffino et al. 2013; Francaux and Deldicque 2019). The next two sections summarize RE and blocking activin receptor ligands as strategies to increase muscle growth.

2.2.1 Resistance training

Functional overloading induced by repeated bouts of RE with sufficient energy and protein intake as well as recovery between the RE sessions is capable of increasing muscle hypertrophy (Tesch 1988; Morton et al. 2015; Ahtiainen et al. 2016) that results primarily from increased muscle fiber size (Mero et al. 2013; Haun et al. 2019). It is suggested that significant muscle hypertrophy (approximately 3%–5%) is observable approximately after three to four weeks of heavy resistance training (RT) (Seynnes et al. 2007; DeFreitas et al. 2011). To induce a

hypertrophic stimulus, a variety of loads (40%–80% of the maximum load that can be lifted once, in other words, one repetition maximum, 1RM) with multiple sets can be used, given that the sets are conducted at least close to concentric failure (Schoenfeld et al. 2014, 2017). The primary stimulus to induce muscle hypertrophy seems to be mechanical stress, even though some evidence suggests that the accumulation of RE-induced metabolites or in other words metabolic stress may also have a contributing role. However, the intracellular mechanosensitive factors initiating the adaptations processes have yet to be identified (Wackerhage et al. 2019).

In addition to muscle hypertrophy, RT is capable of increasing muscle strength, which in previously untrained individuals is only partially explained by gains in muscle mass (Moritani and deVries 1979; DeFreitas et al. 2011; Ahtiainen et al. 2016). Typically, the rate of muscle strength gain is rapid in untrained populations, who are typically responsive to almost any kind of RT program. Eventually, after the phase of rapid gain in muscle strength, a plateau is reached and more attention must be paid to the planning of progressive overload (Kraemer and Ratamess 2004). The most important underlying factor contributing to the increased muscle strength, besides muscle mass, is overall improved neural drive to the muscles. This RE-induced improved neural input results in better coordination of agonist-antagonist muscles as well as more frequent and synchronized contraction of muscle fibers, which ultimately increases force production (Gabriel et al. 2006). To maximize the RE-induced stimulus on muscle strength, loads representing >70% of 1RM are recommended (Schoenfeld et al. 2017). Improved muscle force production is not only beneficial for athletic performance (Paavolainen et al. 1999; Bolger et al. 2015), but it also enables independent lifestyle for older people whose maximal force-generating capacity may be close to the force required to stand up from a chair or to climb stairs (Peterson et al. 2011; Larsson et al. 2019).

2.2.2 Blocking activin receptor ligands

In most cases to date, RT is the most efficient way to increase and maintain muscle mass and strength, which is accompanied by several health benefits (Westcott 2012; Morley 2016). However, the increased knowledge of molecular mechanisms underlying muscle mass regulation has motivated the investigation and development of pharmacological aids to increase and maintain muscle mass as a treatment option for populations that are affected by inevitable and detrimental muscle wasting (Rantanen et al. 2002; Martin et al. 2013; Morley 2016; Furrer and Handschin 2019). One of the pharmacological options to increase muscle mass that has been investigated recently is the blockade of activin receptor (ACVR) ligands.

Myostatin (GDF-8), which belongs to the superfamily of transforming growth factor-β (TGF-β) proteins, was discovered by Se Jin Lee, who observed myostatin null mice to have two to three times bigger muscle mass compared with their wild-type littermates (McPherron et al. 1997). Furthermore, a dysfunc-

tional mutated form of myostatin was found in two breeds of cattle with remarkably large muscle mass (McPherron and Lee 1997). In addition to these findings in animals, myostatin loss-of-function mutation has been found in an otherwise healthy human infant who was exceptionally muscular already at a very early age (Schuelke et al. 2004). The role of myostatin as a negative regulator of muscle mass was further confirmed in another study in which systemic overexpression of myostatin induced severe muscle and fat wasting in mice (Zimmers et al. 2002). These findings provoked an interest to investigate whether myostatin could be pharmacologically targeted to increase muscle mass. Indeed, the administration of a neutralizing antibody against myostatin was reported to be able to increase muscle mass and strength in adult mice (Whittemore et al. 2003).

Myostatin was found to exert its function via binding to type 2 activin receptors, especially to type 2B (ACVR2B) (Lee and McPherron 2001), while these receptors were found to be expressed in skeletal muscle by the group of Olli Ritvos (Hilden et al. 1994). Based on the finding that myostatin binds to ACVR2B, a soluble activin receptor 2B (sACVR2B) that could be systemically administered to living organisms to increase muscle mass was developed (Lee et al. 2005). Since the first report (Lee et al. 2005), the administration of this receptor has been shown to induce remarkable muscle hypertrophy in a strikingly short time in mice (Cadena et al. 2010; Zhou et al. 2010; Pistilli et al. 2011; Hoogaars et al. 2012; Hulmi et al. 2013a). Interestingly, the administration of sACVR2B increased muscle mass more than neutralizing antibodies against myostatin in myostatin knock-out mice (Lee et al. 2005). This finding suggested that there could be other negative regulators of muscle mass besides myostatin that signal through activin receptor 2B. Indeed, activins A, B, AB and bone morphogenetic protein-11 (BMP-11) have been shown to bind both type 2 activin receptors A and -B. Furthermore, these ACVR2B ligands were suggested to be capable of regulating muscle mass (Souza et al. 2008). Later the role of activin A, which was originally found by Wylie Vale (1986 as reported, in Bloise et al. 2019), as a negative regulator of muscle mass has been confirmed (Lee et al. 2010; Chen et al. 2014).

The effects of sACVR2B administration in pre-clinical animal experiments have been promising. For example, its administration has prevented muscle loss (Benny Klimek et al. 2010) and improved survival in tumor-bearing mice while it had no effect on systemic inflammation or tumor size (Zhou et al. 2010). However, the underlying mechanisms remain unknown. Furthermore, it has been shown to exert beneficial effects on mouse models of muscle amyotrophic lateral sclerosis (ALS) (Morrison et al. 2009) and osteogenesis imperfecta (DiGirolamo et al. 2015). However, features of increased muscle fatigue, myopathy and decreased markers of oxidative capacity have been observed in response to long-term sACVR2B administration in dystrophic mice (Relizani et al. 2014; Kainulainen et al. 2015). Additionally, myostatin knockout mice have exhibited lower muscle specific force generating capacity compared with wild-type littermates despite larger muscles (Amthor et al. 2007). These findings warrant further research on the effects of ACVR2B ligand blocking on muscle function and quality including its effects on proteostasis.

2.3 Muscle atrophy

Adequate muscle mass is not only important for locomotion, but it plays a role in, for example, the prevention of metabolic diseases due to its high metabolic rate. Additionally, muscle tissue is a major amino acid reservoir during nutrient and energy deficit (Wolfe 2006). Muscle atrophy, meaning a reduction in muscle mass and fiber size, occurs when protein degradation is greater than synthesis and it happens due to, among other factors, energy and nutrient restriction, aging, cancer, muscular dystrophies, mechanical unloading, loss of neural input and administration of exogenous catabolic stimulants. Protein degradation in skeletal muscle is achieved via ubiquitin-proteasome-system (UPS), autophagy-lysosome pathway and calpain mediated proteolysis. At the molecular level, several catabolic signaling pathways that upregulate the activity of these degradation pathways and inhibit anabolic pathways have been found. These include, for example, SMAD and non-SMAD pathways that are activated by TGF-β family members (e.g. myostatin and activins). In addition, forkhead box protein (FOXO) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) transcription factors upregulated by, for example, AKT and cytokines, respectively, mediate muscle atrophy (Schiaffino et al. 2013). In the sections 2.3.1 to 2.3.3, the conditions causing muscle atrophy that are most relevant to this thesis are summarized.

2.3.1 Duchenne muscular dystrophy

Muscular dystrophies are a group of heterogeneous, inherited myogenic disorders that feature progressive skeletal and sometimes heart muscle wasting and weakness (Emery 2002; Mercuri and Muntoni 2013). They often lead to a dependent lifestyle and significantly impair the patients' quality of life, with the most severe forms leading to premature death (Mercuri and Muntoni 2013). Even though most of the mutated genes causing different dystrophies have been identified, there is currently no clinical treatment that could fully prevent the detrimental muscle wasting progression (Mercuri and Muntoni 2013; Furrer and Handschin 2019).

Duchenne muscular dystrophy (DMD) is the most common of the lethal muscle dystrophies affecting approximately 1 in 3,600 to 6,000 men (Bushby et al. 2010). It is a disorder linked to the X chromosome that is caused by mutations in the gene that encodes a protein called dystrophin (Hoffman et al. 1987). Dystrophin is a cytoskeletal protein that is a component of the dystrophin associated protein complex (DAPC), which links the internal cytoskeleton to the extracellular matrix. Thus, it stabilizes the cellular and especially sarcolemmal integrity during, for example, muscle stretch and series of contraction-relaxation cycles (Petrof et al. 1993). However, in DMD patients, the mutated dysfunctional dystrophin results in destabilized DAPC, making the sarcolemma susceptible to damage and leakage when encountering mechanical strain (Davies and Nowak

2006). This consequently induces inevitable muscle degeneration that is suggested to be mediated by, for example, oxidative stress (Terrill et al. 2013), hypoxia (Sander et al. 2000; Kobayashi et al. 2008), ER stress (Moorwood and Barton 2014), aberrant calcium signaling (Hopf et al. 2007) and impaired autophagy (De Palma et al. 2014). The muscle degeneration induces satellite cell activation to regenerate muscle fibers but the repetitive cycles of degeneration–regeneration exhaust the muscle regeneration capacity by depleting the satellite cell reservoir (Rahimov and Kunkel 2013). In addition to their muscle regeneration inability, DMD patients exhibit muscle tissue replacement by connective fibrous and fat tissue that contribute to the loss of muscle function. Consequently, DMD patients are typically bound to a wheelchair by the age of 12 and die in their twenties due to respiratory or heart failure (Nowak and Davies 2004; Davies and Nowak 2006).

The primary treatment for DMD would be gene therapy that would permanently replace the dysfunctional dystrophin with a functional one. However, despite significant progress in this field, it is still unachievable in humans (Verhaart and Aartsma-Rus 2019). Thus, other strategies to mainly ameliorate the symptoms and decelerate the muscle wasting process have been used and investigated. The commonly used treatment for DMD patients is the administration of glucocorticoids that inhibit the progression of muscle wasting and weakness but has adverse side-effects when used in the long-term (Furrer and Handschin 2019). In *mdx* mice, an animal model for DMD (Sicinski et al. 1989; Yucel et al. 2018), the activin receptor ligand blocking by sACVR2B-Fc (Pistilli et al. 2011; Hoogaars et al. 2012) and other blocking strategies (Bogdanovich et al. 2002, 2005; Morine et al. 2010) have increased muscle mass. However, sACVR2B-Fc administered *mdx* mice have also displayed adverse side effects, mainly features of decreased muscle oxidative capacity (Relizani et al. 2014; Kainulainen et al. 2015). Thus, more research is needed to elucidate the effects of sACVR2B-Fc on dystrophic muscle.

In addition to pharmacological treatment options, the possibility of exercise as a DMD treatment option has been investigated in *mdx* mice. These studies have suggested that voluntary-wheel running may improve limb muscle oxidative capacity (Kainulainen et al. 2015) and force production (Baltgalvis et al., 2012.; Hde et al., 2013.; Selsby et al., 2013.) but may have adverse side effects on the diaphragm (Selsby et al. 2013) and heart (Hde et al., 2013.). However, chronic forced high-intensity exercise worsens the muscle wasting progression in *mdx* mice (De Luca et al. 2003). Furthermore, perhaps surprisingly, improved muscle force generating capacity has been observed in *mdx* mice after high-intensity eccentric contractions if the recovery period is sufficient (Call et al. 2011). Altogether, further exercise studies on *mdx* mice and human DMD patients are warranted in order to provide an evidence-based exercise prescription for DMD patients at different stages of disease progression (Markert et al. 2011). Finally, because the decrease in the markers of oxidative capacity by sACVR2B-Fc was restored by voluntary wheel running (Kainulainen et al. 2015) the combined effects of these two candidate treatments need to be elucidated.

2.3.2 Cancer cachexia

Cancer-associated cachexia is a debilitating condition that is characterized by systemic inflammation and weight loss, which occurs primarily through muscle and fat atrophy that cannot be prevented by nutrition treatments alone (Fearon et al. 2013; Argiles et al. 2014). In addition to cancer, cachexia is associated with chronic pulmonary obstructive disease (COPD), end-stage chronic heart failure (HF), chronic kidney disease (CKD), severe rheumatoid arthritis and sepsis (von Haehling and Anker 2014). Approximately 40%–80% of all cancer patients are affected by cachexia depending on the tumor type, and it indirectly accounts for approximately 20 per cent of all the deaths among cancer patients. Despite mainly having visible effects on fat and muscle tissue, cancer cachexia is a multifactorial syndrome that affects several metabolic pathways in various tissues and organs. Cachexia symptoms in cancer patients include muscle weakness, anorexia, anemia and fatigue that greatly reduce the quality of everyday life (Argiles et al. 2014).

Muscle loss is an inevitable occurrence in cachexia that is in part a result of abnormal metabolism induced by an extensive systemic inflammatory process. In addition, reduced food intake and physical deconditioning and tumor treatments such as chemotherapy or radiotherapy contribute to the muscle wasting (Fearon et al. 2013; Argiles et al. 2014; Baracos et al. 2018). Importantly, muscle atrophy in many types of cancers is an independent bad prognosis factor among cancer patients (Martin et al. 2013; Choi et al. 2015). The exact mechanisms leading to muscle wasting in cancer cachexia are not fully known but it seems to be primarily mediated by increased protein degradation via the ubiquitin proteasome system (UPS), autophagy-lysosome-pathway and calpain-mediated cleavage of myofilaments. These protein degradation pathways seem to target mainly myofibrillar proteins (actin and myosin), the loss of which contributes to the development of muscle weakness. In addition to increased protein degradation, muscle regeneration and protein synthesis have been reported to be downregulated in cachectic muscles (Argiles et al. 2014; Baracos et al. 2018). Furthermore, cachectic muscles in human cancer patients and experimental mouse models exhibit increased levels of autophagy markers (LC3II, P62, Beclin-1), suggesting that autophagy is a major contributor to the muscle atrophy in cachexia (Penna et al. 2013; Aversa et al. 2016). Furthermore, the autophagy flux was reported to be upregulated in C26 tumor-bearing mice (Penna et al. 2013).

At the molecular level, pro-inflammatory cytokines and other circulatory factors either secreted directly from tumors or other sites such as immune cells contribute to the muscle atrophy in cancer-associated cachexia. Of the pro-inflammatory cytokines, TNF-α, IL-1, IL-6, IFNγ, LIF-1, GDF-15 and TWEAK have been recognized to mediate muscle atrophy in pre-clinical animal models (Baracos et al. 2018). However, targeting just one of the listed cytokines at a time by pharmacological agent, has failed to cure cachexia, suggesting a more complicated phenomenon (Aversa et al. 2017). The factors that may induce muscle wast-

ing in cachexia bind to their corresponding receptors, which activate downstream signaling pathways and ultimately induce the expression of components belonging to autophagy-lysosome pathway and UPS. Moreover, cytokines may also suppress AKT activity, which leads to the downregulation of mTOR and thus protein synthesis. Of the non-inflammatory circulating factors, TGF-β family members such as activin A and myostatin have been reported to be upregulated in some cancers with cachexia and are also suggested to contribute to the cachexia-induced muscle wasting (Baracos et al. 2018). Furthermore, cachectic muscles display increased oxidative stress (Laviano et al. 2007) and impaired mitochondrial function (Argiles et al. 2014), which also have a role in the muscle wasting process. Finally, there is also some evidence from pre-clinical animal models (Bohnert et al. 2016) that cachectic muscle exhibits ER stress that may play a role in the muscle wasting process, but further confirmative studies are still required.

The main treatment in cancer is to treat the primary cause of the disease, that is, the tumor. However, chemotherapy *per se* often exacerbates muscle wasting and induces muscle weakness and fatigue that may reduce the physical activity levels, thus aggravating the muscle loss (Nissinen et al. 2016; Aversa et al. 2017). As already mentioned, muscle loss predicts independently shorter survival time in cancer patients and cachexia greatly impairs cancer patients' quality of life (Fearon et al. 2013; Martin et al. 2013; Choi et al. 2015). In addition, diminished muscle mass reduces the capacity to tolerate cancer treatments. Thus, there is a great need for practical pharmacological and lifestyle strategies to treat cachexia, which would improve the efficacy of the primary treatment. Several pharmacological approaches (e.g. ACVR2B ligand blocking, proteasome inhibitors, β_2-agonists, MEK and JAK/STAT inhibitors) have shown beneficial effects on pre-clinical animal models, but these drugs have either failed in clinical trials or have yet to enter them. Furthermore, anamorelin – a selective agonist of the ghrelin receptor GHSR-1a, is one of the most promising drugs so far. In phase III trials, it has increased muscle mass but not function (Aversa et al. 2017). Because no single drug that could cure cachexia exists, further studies that examine the mechanisms of cachexia are clearly warranted. In addition, the mechanisms underlying the improved survival in pre-clinical animal models for example by sACVR2B-Fc administration (Zhou et al. 2010) need to be determined.

2.3.3 Aging related muscle loss

Aging is associated with an unavoidable loss of skeletal muscle mass and, most importantly, of strength that cannot be fully prevented by lifestyle choices. This age-related degenerative process is exceedingly complex and heterogeneous among people and can be either accelerated or decelerated by several intrinsic and extrinsic factors (Larsson et al. 2019). On average, muscle strength reaches its maximum at the age of 30 while the accelerative decline in muscle strength that starts approximately between the ages of 40 and 50 is not completely explained by age-related reduction in muscle size (Larsson et al. 1979; Kallman et al. 1990). However, based on the world records of masters athletes who have many years

of intensive training experience in track and field sprint events, a linear decline in muscle power starts already at the age of 30 and further accelerates at approximately the age of 70 (Reaburn and Dascombe 2008; Lazarus and Harridge 2017). Age-related muscle atrophy is the sum of the reduction in muscle fiber size and number while the first is due to a net loss of proteins and the latter cell loss due to apoptosis. In aging muscle, apoptosis targets primarily the fast type II fibers, which primarily results from the loss of neural input from motoneurons. Thus, the proportion of slow oxidative muscle fibers with low force-generating capacity increases with aging. This shift in the type of muscle fiber contributes to the age-related decline in muscle strength together with reduced fiber size and other changes in muscle quality (Larsson et al. 2019).

The intracellular mechanisms contributing to the age-related muscle loss have not been fully elucidated. However, aging is associated with a decline in protein quality control processes that may also contribute to age-related muscle atrophy (Labbadia and Morimoto 2015; Larsson et al. 2019). For example, a decline in key autophagy markers (LC3II and ATG7) was found in muscles of old mice compared with young mice. In the same study, a similar decline in key autophagy markers was observed in older sedentary sarcopenic humans compared with sedentary young ones (Carnio et al. 2014). This age-related decline in autophagy may result from excess activity of mTOR signaling, which has been found in older humans and mice, because mTOR is able to block autophagy induction through inhibitory phosphorylation of ULK1 (Castets et al. 2013; Markofski et al. 2015; Joseph et al. 2019). Furthermore, ATF4, a transcription factor that is typically induced by ER-stress–activated UPR, was suggested to mediate muscle atrophy in aging mice (Ebert et al. 2015). However, inconsistent findings regarding UPR components have been found in aging muscle and other tissues (Estebanez et al. 2018). Further research to elucidate the role of UPR in aging muscle is therefore needed.

Exercise. The age-related loss in muscle mass and strength can be decelerated but not totally avoided by regular exercise, which should be accompanied by proper nutrition, especially adequate protein intake (Gaffney-Stomberg et al. 2009; Larsson et al. 2019). Of the exercise modalities, resistance exercise has been shown to be the most efficient way to increase muscle mass and strength and consequently improve everyday functionality, such as gait speed, in older people (Landi et al. 2014). In addition, strength training may also be able to increase sprint performance in trained masters athletes (Reaburn and Dascombe 2008). Furthermore, endurance exercise also elicits beneficial muscle remodeling, including improvements in mitochondrial function and oxidative capacity that can slow down the age-related muscle loss (Landi et al. 2014). Because both resistance and aerobic training decelerate the loss of muscle mass and function but have different underlying mechanisms, their combination may be the best general recommendation for aging populations (Mcleod et al. 2019). Interestingly, human cross-sectional studies suggest that regular participation in life-long physical activity, mainly in endurance-type exercise, is capable of counteracting the age-related decline in autophagy and overall protein quality control (Carnio et al. 2014;

Mancini et al. 2019). This could be one of the many underlying mechanisms mediating the beneficial effects of exercise to counteract age-related muscle loss. However, there is a lack of long-term exercise intervention studies comprehensively examining protein quality control processes including UPR and autophagy among older humans.

3 PURPOSE OF THE STUDY

The purpose of this thesis was to elucidate the effects of different conditions that lead to changes in muscle quality on biological processes that regulate protein homeostasis (proteostasis) in skeletal muscle. The focus was on the effects of muscle wasting, muscle hypertrophy and exercise training. To achieve this purpose, data obtained from human and experimental animal models was analyzed. Of the protein homeostasis regulatory processes, this thesis focused on unfolded protein response (UPR) induced by endoplasmic reticulum (ER) stress and autophagy. In addition, muscle redox balance was investigated because it influences protein homeostasis.

The specific aims were to study:

1. The effects of muscular dystrophy as well as individual and combined effects of activin receptor ligand blocking induced muscle hypertrophy and voluntary wheel running on UPR markers induced by ER stress as well as redox balance markers in *mdx* mice. (I).
2. The effects of rapid muscle hypertrophy and atrophy on muscle UPR, redox balance and autophagy markers in mice. The rapid hypertrophy stimulus was induced by blocking activin receptor ligands, whereas the atrophy stimulus was experimental C26 cancer induced cachexia. In addition, the effects of muscle wasting alleviation by blocking activin receptor ligands on these same processes were elucidated in C26 cancer cachexia (II).
3. The acute and prolonged effects of resistance exercise on muscle autophagy and UPR markers induced by ER stress in young (26 ± 4 years) and older (61 ± 6) previously untrained healthy men (III).
4. The effect of a new long-term training stimulus that consisted of maximal and explosive strength training integrated to conventional sprint training regimen on muscle autophagy and UPR markers in middle-aged and older (40–76 years) men masters sprinters (IV).

4 MATERIALS AND METHODS

4.1 Animal experiments (I & II)

4.1.1 Animals and cells (I & II)

Muscular dystrophy and its treatments (I). 6–7-week-old male healthy control (C57Bl/10ScSnJ) and *mdx* mice (an animal model for human Duchenne muscular dystrophy) that originated from the same strain (Jackson Laboratories, Bar Harbor, Maine, USA) were used in the experiments.

sACVR2B-Fc administration on healthy muscle (II). For the acute and short-term effects of sACVR2B-Fc administration (see 4.1.3) on healthy muscle, 6–7-week-old male C57Bl/10SnJ (Jackson Laboratories, Bar Harbor, Maine, USA) mice were used in the experiments.

C26 cancer experiments (II). In the C26 cancer experiments, 5–6-week-old male BALB/cAnCrl mice that were purchased from Charles River Laboratories were used. Colon 26 carcinoma (C26) cells (Corbett et al. 1975) were maintained in Complete Dulbecco's Modified Eagle's Medium (DMEM, high glucose, GlutaMAX™ Supplement pyruvate, Gibco™, Life Technologies) supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) and 10% FBS. Dr. Fabio Penna provided the C26 cells that he had originally received from Professor Mario P. Colombo.

All the mice were housed under standard conditions (temperature approximately 22° C and a 12 h light/dark cycle) and had a free access to water and food pellets (R36, 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labfor, Stockholm, Sweden).

The animals were treated in strict accordance with European convention for the protection of vertebrate animals used for experimental and other scientific purposes. The protocols used in the studies were approved by the Finnish National Animal Experiment Board (Permit number ESLH-2009-08528/Ym-23 in

mdx and acute sACVR2B-Fc experiments and Permit number ESAVI/10137/04.10.07/2014 in cancer experiments).

4.1.2 Experimental designs (I & II)

Muscular dystrophy and its treatments (I). The effects of 7-week voluntary wheel running and activin receptor ligand blocking by sACVR2B-Fc administration alone and combined were elucidated on UPR and redox balance markers in dystrophic *mdx* muscle. In addition, the effects of muscle dystrophy *per se* were examined. For this purpose, *mdx* mice were randomly divided into four groups of dystrophic mice while wild-type mice served as controls. The groups were: (1) healthy wild-type controls (n = 5, smaller sample size in wild-type (wt) mice was due to the expected lower variation compared with *mdx* mice), (2) PBS (PBS administered sedentary, n = 8), (3) sACVR (sACVR2B-Fc administered sedentary, n = 8), (4) PBS Run (PBS administered voluntary wheel running, n = 8), (5) sACVR RUN (sACVR2B-Fc administered voluntary wheel running, n = 8). sACVR2B-Fc (5 mg/kg) or PBS was administered with an intraperitoneal (i.p.) injection once per week for seven weeks. Mice had a free access to running wheels throughout the experiment, except for the last two days to elucidate the long-term, not acute effects of running. All the mice were euthanized approximately at the age of 14 weeks by cervical dislocation and muscles were collected (Figure 2). To examine the effects of muscular dystrophy, wt mice were compared with PBS administered sedentary *mdx* mice.

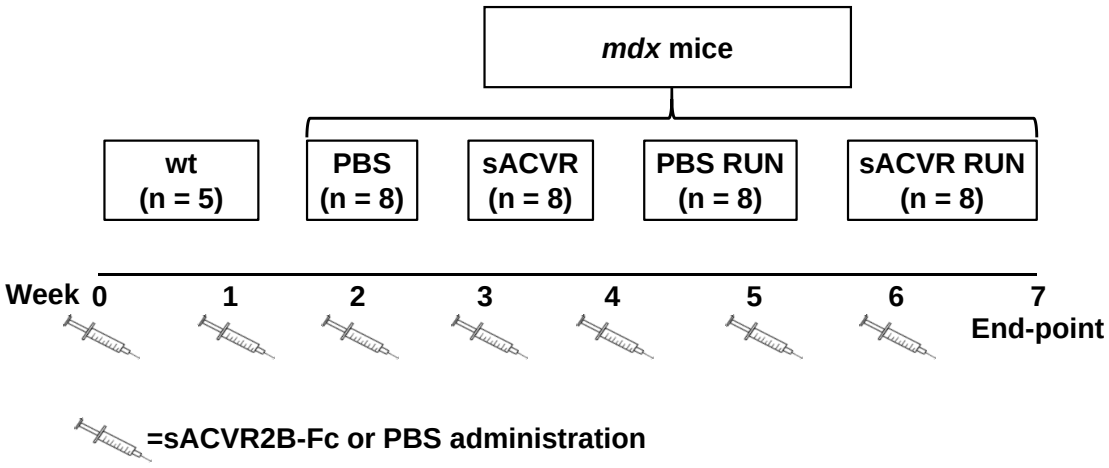

FIGURE 2 Illustration of the experimental design in the muscular dystrophy experiment (I). RUN = voluntary wheel running. sACVR/sACVR2B-Fc = soluble recombinant protein of activin receptor type 2B. PBS = phosphate buffered saline (vehicle).

sACVR2B-Fc administration on healthy muscle (II). To elucidate the acute effects of activin receptor ligand blocking, mice were divided into three groups (n = 6–7) that were euthanized one or two days after a single i.p. injection of sACVR2B-Fc

(10 mg/kg) or PBS (Figure 3A). To elucidate the 2-week effects, mice were divided into two groups that were administered with sACVR2B-Fc (n = 11) or PBS (n = 6) (i.p., once or twice per week) and were euthanized two weeks after the first injection (Figure 3B).

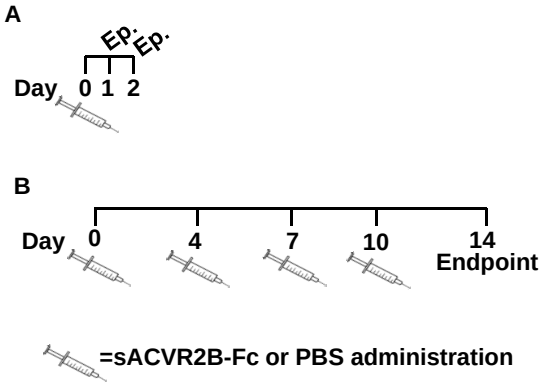

FIGURE 3 Illustration of the design in the A) acute and B) 2-week sACVR2B-Fc administration experiments on healthy mice. sACVR2B-Fc = soluble recombinant protein of activin receptor type 2B. PBS = phosphate buffered saline (vehicle). Ep = endpoint when muscle were collected.

C26 cancer experiments (II). To elucidate the effects of muscle atrophy and its alleviation by sACVR2B-Fc during experimental cancer cachexia, mice were randomly divided into four weight-matched groups. These four groups were: (1) CTRL (n = 7, vehicle-treated (PBS) healthy control mice) (2) C26 + PBS (n = 7, C26 tumor-bearing (TB) mice administered with a vehicle (PBS)), (3) C26 + sACVR/b (n = 7, C26 TB mice administered with sACVR2B-Fc before the C26 tumor formation and replaced by a vehicle (PBS) after the tumor formation), and (4) C26 + sACVR/c (n = 8, C26 TB mice continuously administered with sACVR2B-Fc throughout the experiment). The rationale for the sACVR/b group was to examine the prophylactic effects of increased muscle mass before the atrophying stimulus. The TB groups were subcutaneously (s.c.) inoculated with C26 colon carcinoma cells (5×10^5 cells in ~ 120 µl PBS) into their interscapular region. Mice were administered with PBS or sACVR2B-Fc (5 mg/kg, i.p.) every fourth day and mice were euthanized 11 days after the C26 cell inoculation to represent the onset phase of cachexia (Figure 4).

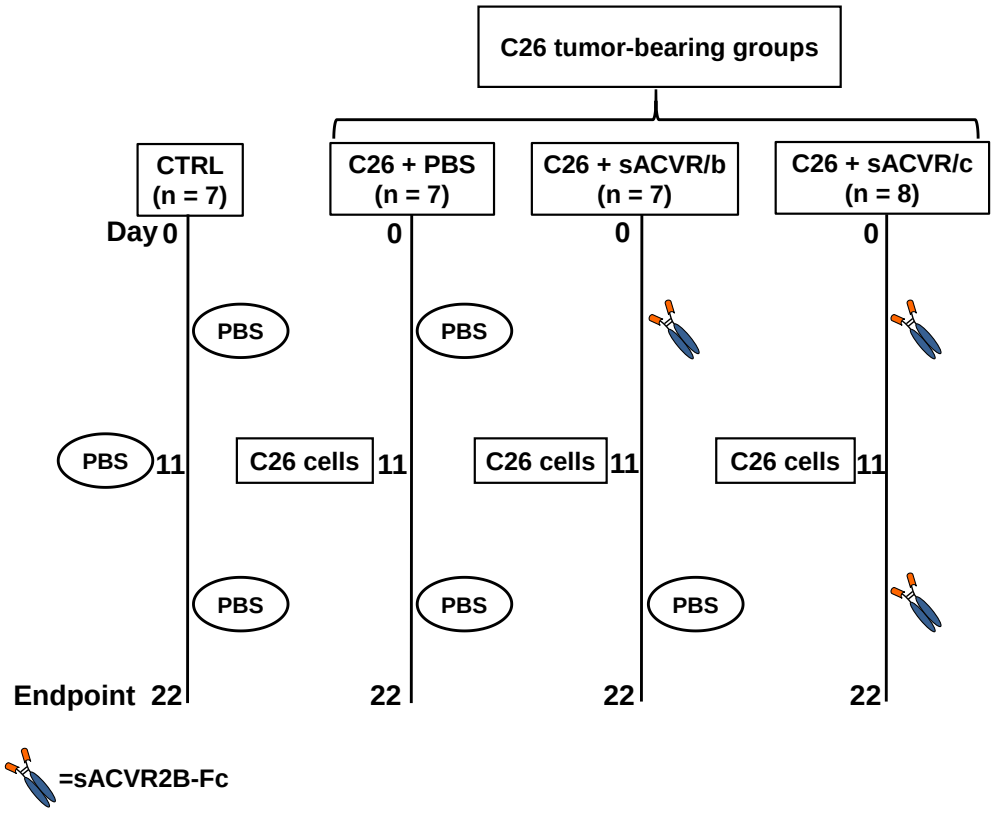

FIGURE 4 Illustration of the design in the C26 cancer experiment. sACVR/sACVR2B-Fc = soluble recombinant protein of activin receptor type 2B. C26 cells = Colon 26 carcinoma cells. PBS = phosphate buffered saline (vehicle).

4.1.3 sACVR2B-Fc production (I & II)

The recombinant soluble type 2B activin receptor (sACVR2B-Fc) was produced in house by Dr. Arja Pasternack in the laboratory of Dr. Olli Ritvos. Firstly, a human sACVR2B ectodomain was amplified by PCR using the following primers: 5′-GGACTAGTAACATGACGGCGCCCTGG-3′ and 5′-CCAGATCTGCGGTGGGGGCTGTCGG-3′ from a plasmid that contained the human ACVR2B sequence (in pCR-Blunt II-TOPO AM2-G17 ACVR2B, IMAGE clone number 40005760, The IMAGE Consortium). Secondly, a human a crystallisable fragment of the immunoglobulin class G1 (IgG1 Fc) domain with a COOH-terminal His6 tag was amplified by PCR using the following primers 5′-GCAGATCTAATCGAAGGTCGTGGTGATCCCAAATCTTGTGAC-3′ and 5′-TCCCTGTCTCCGGGTAAACACCATCACCATCACCATTGAGCGGCCGCTT-3′ from the plgPlus expression plasmid. Thirdly, these amplified PCR products were subcloned into pGEM-T easy (Promega) vectors that were then sequenced and fused before they were cloned into the expression vector pEFIRES-p. Fourthly, to induce the sACVR2B-Fc protein production, Chinese hamster ovary cells (CHO) were transfected with the ACVR2Becd-FcHis6-pEFIRES-p expression vector via lipofection (Fugene 6; Roche) and selected with puromycin (Sigma Aldrich). During the selection phase, CHO cells were maintained in DMEM sup-

plemented with 2 mmol/l L-glutamine, 100 µg/ml streptomycin, 100 IU/ml penicillin, and 10% FCS. In order to harvest the final protein product, supernatant of the cell culture was filtrated through a 0.22-µm membrane (Steritop; Millipore). Then, NaCl and imidazole were added to the filtrated supernatant solution that was subsequently pumped through a Ni^{2+}-loaded HiTrap Chelating column (GE Healthcare Life Sciences, Uppsala, Sweden) at 4° C. To elute the final protein product, the imidazole concentration of the solution was increased, dialysis against PBS was conducted and finally the protein was concentrated with Amicon Ultra concentrator (30000 MWCO; Millipore). The purity of the sACVR2B-Fc was confirmed by silver-stained SDS-PAGE.

4.1.4 Voluntary wheel running (I)

The *mdx* mice had free access to custom-made running wheels (diameter 24 cm, width 8 cm) that were placed in their home cages throughout the 7-week-experiment except for the last two days of the experiment. Sedentary animals were housed in similar cages without running wheels.

4.1.5 Measurement of grip strength (I)

The forelimb grip strength in *mdx* and wild-type mice was measured using a suggested protocol on the day that preceded euthanization (web-link: http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD_M.2.2.001.pdf. Grip strength was measured five times and the best result was reported.

4.1.6 Measurement of muscle cross-sectional area (I)

To measure muscle cross-sectional area (CSA) in *mdx* and wild-type mice, cross-sections from gastrocnemius muscle were cut using a cryomicrotome. Subsequently, muscle membranes were stained by an antibody that bound to caveolin 3 (ab2912, Abcam). The muscle cross-sections were imaged with Olympus BX-50 fluorescent microscopy. For the analysis, 10 x magnification was used, while in the randomly picked areas, where the sample quality was high, the average fiber number was 342.8 ± 30.5 fibers per section. ImageJ software (U.S. National Institutes of Health, Bethesda, MD, USA) was used to quantify the fiber CSA.

4.1.7 Tissue collection (I & II)

In the *mdx* (I) as well as acute and 2-week sACVR2B-Fc administration experiments (II), mice were euthanized by cervical dislocation and gastrocnemius muscles (GA) were dissected, weighed and flash frozen in liquid nitrogen and stored at -80° C until homogenization and further processing.

In the C26 cancer experiments (II), mice were euthanized by cervical dislocation after heart puncture under anesthesia (ketamine (Keminol®) ~ 110–120 mg/kg + xylazine (Rompun®): 15–16 mg/kg). Subsequently, tibialis anterior (TA)

and GA muscles were dissected, weighed and flash frozen in liquid nitrogen and were stored at -80° C until homogenization and further processing.

4.2 Human experiments (III & IV)

4.2.1 Participants and the ethical statements (III & IV)

Untrained young and older men (III). In these experiments, previously collected and homogenized muscle samples were used. Based on the muscle homogenate sample availability, healthy, normal weight or a bit overweight young (n = 16, 26 ± 4 years, BMI: 19.6–28.9) and older men (n = 8, 61 ± 6 years, BMI: 23.4–28.8) without previous resistance training experience were selected from a larger group of participants from previous studies (Hulmi et al. 2009a; Mero et al. 2013) based on the availability of muscle biopsy samples. Young men were originally randomized to either a protein group (26 ± 6 years, n = 6 participants with biopsy samples available for this study), a placebo group (27 ± 2 years, n = 6 in this study) or into non-exercise control group (26 ± 4 years, n = 4 in this study). The protein supplementation did not have a significant effect on the measured variables ($P > 0.05$, comparison of percentage changes (PRE to 1 h, PRE to 48 h and PRE to 21 weeks) between the protein and placebo groups by Holm-Bonferroni adjusted t-test or Mann Whitney U)). Thus, the participants in the placebo and protein groups were pooled to improve statistical power for the RE/RT effects and to simplify the study design.

Masters athletes (IV). In this study, previously collected and homogenized muscle samples from a previous randomized controlled trial were used. For the original randomized controlled trial, masters athletes (≥ 40 years) living all around Finland with a long-term training background and a successful competition history in sprinting events (100–400 m) were recruited from a previous cross-sectional study that served as baseline measurements (Korhonen et al. 2006). In total, 72 healthy participants without a previous systematic strength training background were included to the original study and were subsequently randomly divided into experimental (EX, n = 40) and control groups (CTRL, n = 32). All the participants from the EX group of whom muscle samples were available were included to this study (n = 16). Consequently, 16 participants from the original CTRL group were chosen for this study based on muscle sample availability and to age-match the groups. Based on the per protocol analysis, three participants from the CTRL group were excluded because the reported weekly strength training volume in these three subjects exceeded the average strength training volume of EX group. Furthermore, one subject in the EX group had a poor adherence to the experimental training program and was removed from this study. Thus, 15 participants in the EX group and 13 in the CTRL group were included to this study (Table 1).

The Ethical committee of the University of Jyväskylä approved all the human studies included to this thesis. Every participant was carefully informed of

the study design, discomfort and possible risks related to the experiments and signed a written informed consent. The studies were conducted according to the Declaration of Helsinki.

TABLE 1 Masters athletes characteristics in the baseline (IV). Values are represented as mean ± SD

	Experimental group (EX; n = 15)	Control group (CTRL; n = 13)
Age (years)	60 ± 12	61 ± 11
Training background (years)	34 ± 14	34 ± 18
Height (cm)	175 ± 6	175 ± 8
Body mass (kg)	73.5 ± 9.5	77.3 ± 10.4
Body fat (%)	14.6 ± 4.3	15.8 ± 4.8

4.2.2 Experimental designs (III & IV)

Untrained young men (III). In this experiment, the acute effects of an unaccustomed resistance exercise (RE) bout as well as long-term effects of resistance training (RT) on muscle autophagy and unfolded protein response (UPR) markers were investigated from muscle biopsies obtained from young previously untrained men. Participants performed a single experimental RE bout and muscle biopsies were obtained 30 minutes before, 1 h after and 48 h after the RE bout. Muscle biopsies were also obtained from participants who did not conduct the RE bout (No RE) at the same time-points and of these 4 were included to the study based on the muscle sample availability. Then participants conducted a 21-week RT period and muscle biopsies were obtained (Figure 5A).

Untrained older men (III). In this experiment, the acute and long-term effects of RE and RT, respectively, on muscle autophagy and UPR markers were investigated from biopsies obtained from older previously untrained men. In addition, the acute effects of RE were investigated after the RT period. Older men performed a single experimental RE bout before and after a 21-week RT period and muscle biopsies were obtained before and 48 h after the RE bout at pre- and post-RT (Figure 5B).

Masters athletes (IV). In this experiment the effect of 20-week experimental training program on muscle autophagy and UPR markers were investigated. This effect was studied from muscle biopsies obtained from masters athletes with a long-term training background and successful competition history in sprint-run events (100–400 m) but had no previous experience of consistent heavy strength training. The participants in the experimental group (EX) completed a periodized 20-week training program in which maximal and explosive strength training was integrated to running-based sprint training routines. The participants in the control (CTRL) group were told to continue their accustomed training regimen that mainly consisted of sprint training. The main laboratory-tests were conducted before and immediately after the 20-week intervention period

on two consecutive days. On the first day, several performance tests and anthropometric measurements were carried out. On the following day, muscle biopsies were obtained (Figure 5C). Participants were told to prepare for the measurements as they would for an important competition and to restrain from vigorous exercise 1–2 days before the first measurement day.

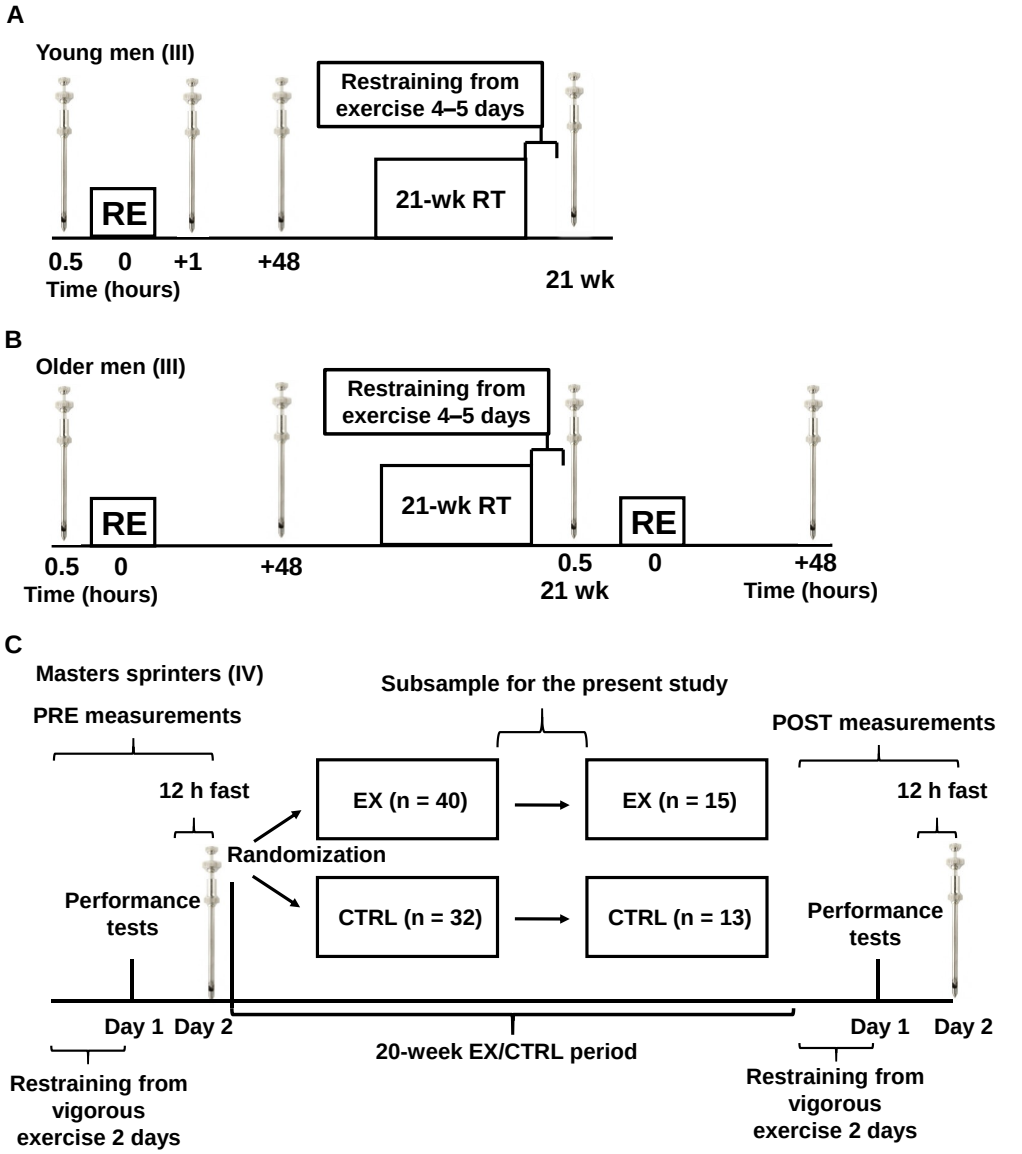

FIGURE 5 Illustration of the experimental designs in the A) young previously untrained men (III), B) older previously untrained men (III) and C) masters athletes in sprinting events (IV). Needles depict time-points when muscle biopsies were obtained from the vastus lateralis muscle. RE = 5 X 10 RM in leg press device. 21-wk RT = 21-week whole-body progressive resistance training program planned to increase muscle strength and hypertrophy. EX = 20-week experimental training period, in which maximal and explosive strength exercises were integrated to masters sprinters' sprint training routines. Experiment A contained also few non-exercise controls and experiment C a control group (CTRL) of masters sprinters that continued their sprint training as they were used to.

4.2.3 Resistance exercise bout and resistance training (III)

The young and older previously untrained men conducted a similar resistance exercise (RE) bout and completed a similar 21-week resistance-training (RT) period. The RE bout was five sets of 10 repetition maximums (RM) on a leg press device (David 210) with a recovery time of 2 minutes between sets. The leg press started from the flexed-knee position (~70°), which was followed by concentric leg extension to full extension (180°) and eccentric lowering to the starting position. Due to the accumulating fatigue, the lifted loads were adjusted in order to enable participants to complete the ten repetitions in each set. Participants were slightly assisted in the last repetitions if the load was too heavy to complete the ten repetitions.

During the 21-week RT period, which was designed to increase muscle mass and strength, participants conducted two whole body RE sessions per week under supervision. The training program consisted of three 7-week training phases that differed in volume and intensity. All the loads were individually determined throughout the RT-period. In the first phase, muscle strength endurance training was conducted by using light loads (40–60% of 1RM) with multiple repetitions (10–20 RM) and short rest intervals between the sets. In the second phase, the training was planned to especially increase muscle size as well as strength and thus loads progressively increased (60–80% of 1RM) and concurrently the number of repetitions decreased (8–12 RM). In the last phase, the training was planned to especially increase maximal strength simultaneously still increasing muscle size. Hence, loads became heavier (70–90% of the 1RM) while the number of repetitions decreased (5–8 RM) and rest intervals between sets became longer. During the RT period, there was at least two days between each exercise session. The training program particularly focused on knee extensor muscles including vastus lateralis (VL) because it was the muscle from which muscle biopsies were obtained and cross-sectional area was measured. Bilateral leg press, knee extension and knee flexion were included to every RE session of which leg press and knee extension especially targeted the VL muscle. Additionally, chest, shoulder, upper back, trunk extensor and flexor, upper arm, ankle extensor, hip abductor and adductor muscle groups were exercised during the RT period.

4.2.4 Experimental training program (IV)

The 20-week experimental training program was a combination of heavy and explosive strength training that was integrated to the conventional running-based sprint training. The training program was designed to improve explosive force production in muscle groups crucial for acceleration and maximum speed sprinting (quadriceps femoris, hamstrings, gluteus, calves, hips and core muscles) and consequently sprint performance. The training program consisted of two similar 9- and 11-week periods that were further divided into three 3–4-week phases differing in intensity, volume and type of training. The strength training started in the first phase by strength endurance and muscle hypertrophy exercises (3–4 sets

x 8–12 repetitions, 50–70% of 1RM). After the first phase, strength training progressed to the next two phases and mainly consisted of maximal strength training exercises (2–3 sets x 4–6 repetitions, 70%–85% 1RM), explosive-type of weight lifting (2–3 sets x 4–6 repetitions, 35%–60% of 1RM) and plyometric exercises (2–3 sets of 3–10 repetitions). The first 9-week period was followed by a rather similar 11-week training period that involved a progressive increase in training intensity and a reduction in volume. Two strength and plyometric training sessions, which were separated by at least one day, were conducted per week while plyometric exercises were always done before sprint training. Leg press and/or half squat on machines, clean pull (from knee height) and/or stiff-legged deadlift using free weights were the main strength exercises incorporated to the training program. Additionally, core and upper body exercises as well as dynamic hip extension, hip flexion, knee flexion, knee extension and ankle plantar flexion that were conducted on machines, were included as supplementary exercises. The plyometric and sprint training progressed from lower-intensity vertical jumps and speed endurance training to higher intensity horizontal jumps and maximal speed and sprint acceleration exercises, respectively. The strength training program was slightly different (lighter loads, more repetitions) for older (≥ 65 years; n = 6) participants compared with younger participants, to avoid injuries. The participants in the control group were told to continue their sprinting based training regimen as they were used.

The training programs accompanied by detailed instructions regarding exercises and the overall training program were mailed to the participants in EX group. All the participants in the both groups were told to complete detailed training logs in order to monitor training adherence and subsequently training mode specific volumes were calculated as training h and sessions per week (Table 2).

TABLE 2 The reported training h during the 20-week intervention period. The only statistical difference in training mode specific volume between the groups was in strength training ($P < 0.001$) which were reported as training h/week. "Other exercises" included for example recreational ball games, aerobic exercise, stretching, bodyweight gymnastic exercise, household physical activity. Data is presented as means ± SD.

	Experimental group (EX; n = 15)	Control group (CTRL; n = 13)
Total training (h/week)	4.6 ± 1.2	3.9 ± 1.7
Strength training (h/week)	1.8 ± 0.6 ***	0.8 ± 0.4
Plyometrics (h/week)	0.3 ± 0.2	0.3 ± 0.6
Sprint training (h/week)	1.6 ± 0.6	1.4 ± 0.6
Other exercises (h/week)	0.9 ± 0.9	1.4 ± 1.8

4.2.5 Strength and performance tests (IV)

In masters athletes, to assess sprinting performance, the subjects ran two 60-meter trials on a synthetic indoor track and times were recorded by double-beam photocell-gates that were connected to an electronic timer. To examine explosive strength production, participants squatted on a contact mat, hands on their hips, to an approximately 90° knee angle, stopped and rapidly jumped as high as possible and landed with their legs extended. Jump height was calculated from the flight time.

4.2.6 Muscle biopsies (III & IV)

The muscle biopsies were obtained with a similar needle biopsy technique with suction in all the experiments included into this thesis. Muscle biopsies (approximately 100–150 mg) were obtained from the vastus lateralis (VL) muscle using a 5 mm needle under local anesthesia (1% lidocaine-adrenalin) from a spot that was midway between the patella and greater trochanter at a depth of ~1.5–2.5 cm below the surface of the skin. Muscle biopsies were excised from visible blood, adipose and connective tissue, flash frozen in liquid nitrogen and subsequently stored in -80° C until homogenization and further analysis.

Untrained young and older men. In young and older previously untrained men, the pre-, post-RE 48 h and 21-week RT biopsies were taken from the same thigh, whereas the post-RE 1 h biopsy in young men was from another thigh. The post-RE 48 h biopsy was taken approximately 3 cm above the scar that was induced by the pre-biopsy obtainment in order to avoid possible residual effects involved in the obtainment of repeated biopsies. The subjects were told to fast for three h preceding the pre-, post-RE 48 h and 21-week RT biopsies. In addition, young men were not allowed to eat before the post-RE 1 h biopsy. The pre-biopsies were obtained 30 minutes before the RE bout. Furthermore, the biopsies after the 21-week RT period were obtained 4–5 days after the last RE session to represent long-term training effects.

Masters athletes (IV). The baseline and post-intervention biopsies were obtained from the same dominant leg in the morning after an overnight fast (12 h). The morning when the biopsies were taken, was preceded by a first measurement day during which several performance and anthropometric measurements were conducted.

4.3 Biochemical analyses (I–IV)

4.3.1 Protein extraction (I–IV)

Animal experiments (I & II). In the muscular dystrophy (I) and the acute and 2-week sACVR2B-Fc experiments (II), gastrocnemius muscle was pulverized in liquid nitrogen and the powder was homogenized in ice-cold buffer (pH 7.4, 20 mM

HEPES, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% NP-40, 0.2% sodium deoxycholate), with protease and phosphatase inhibitors (3%, P 78443; Pierce, Rockford, IL) with a dilution of 15 µl/mg of muscle weight. The muscle homogenates were rotated for 30 minutes at 4° C and in order to remove cell debris centrifuged at 10000 g for 10 minutes at 4° C. The supernatants were then collected and total protein concentration was measured using the bicinchoninic acid protein assay (BCA). In the cancer cachexia experiments (II), protein extraction was conducted similarly as described above except that tibialis anterior muscle was used for the protein extraction and it was homogenized manually by glass rods.

Young and older previously untrained men (III). In young men, muscle biopsy specimens were manually homogenized in ice-cold buffer (pH 7.4, 20 mM HEPES, 1 mM EDTA, 5 mM EGTA, 10 mM $MgCl_2$, 100 mM β-glycerophosphate, 1 mM Na_3VO_4, 2 mM DTT, 1% Triton X-100, 0.2% sodium deoxycholate, 30 µg/ml leupeptin, 30 µg/ml aprotinin and 60 µg/ml PMSF) including phosphatase inhibitors (1%, P 2850; Sigma, St. Louis, USA)) at a dilution of 15 µl/mg of muscle weight. The total protein concentration was determined by BCA-method. The muscle biopsy specimens of older men were manually homogenized slightly differently compared with young men in ice-cold buffer (pH 7.4, 50 mM HEPES, 0.1% Triton X-100, 4 mM EGTA, 10 mM EDTA, 15 mM $Na_4P_2O_7 \cdot 10H_2O$, 100 mM β-glycerophosphate, 25 mM NaF, 1 mM Na_3VO_4, 0.5 µg/ml leupeptin, 0.5 µg/ml pepstatin and 0.3 µg/ml aprotinin) at a 4% (w/v) solution. The homogenates were centrifuged at 10000 g for 15 minutes and supernatants were collected. Total protein concentrations were measured using Lowry based method with bovine serum albumin (BSA) as a standard reference.

Masters athletes (IV). The muscle biopsy specimens were manually homogenized in Eppendorf tubes with plastic rods (VWR Disposable Pestle, 1.5 ml, Cat. No. 47747-358) in ice-cold buffer (pH 7.4, 20 mM HEPES, 10 mM NaCl, 1.5 mM $MgCl_2$, 20% glycerol, 0.1% Triton X-100, 1 mM dithiothreitol) at a dilution of 15 µl/mg of muscle weight. Before homogenization, Hans buffer was supplemented with protease and phosphatase inhibitors including 1mM pepstatin and 0.5 mM EDTA-containing HALT Protease and phosphatase inhibitor cocktail (Pierce, 78440). The homogenate was centrifuged at 1000 rpm for 1 minute at 4° C and the supernatant containing the cytoplasmic fraction of the proteins was collected. The remaining pellet was re-suspended to 360 µl of inhibitor-supplemented Hans lysis buffer. At this point, the NaCl concentration of Hans buffer was increased by adding 40 µl of 3 M NaCl. Suspension was subsequently rotated for 1 h in 4° C and centrifuged at 14000 rpm for 15 minutes in 4° C. Supernatants containing the nuclear fraction of the proteome were collected. The total protein concentration was measured with BCA.

4.3.2 Western blot (I–IV)

The western blot (WB) protocol was conducted similarly in the publications I–III for most of the measured proteins. Muscle homogenates were mixed (1:1) with 2 x Laemmli sample buffer (Bio-Rad #161-0737) containing β-mercaptoethanol

(5%), which was followed by heating at ~95° C for 10 minutes. Approximately 30 µg of total protein was separated by SDS-PAGE using Criterion TGX 4–20% Precast Gels (Bio-Rad Laboratories). Subsequently, proteins were transferred to a PVDF membrane (Amersham™ Hybond™ P 0.45, GE Healthcare, Life-Sciences) in a blotting chamber filled with transfer buffer (pH 8.3, 2.5 mM Tris Base, 19 mM glycine, 10% methanol) with a constant electric current (300 mA, ~2 h 40 minutes) on ice at 4° C. The membranes were then blocked (5% (w/v) fat-free milk in TBS-T)) for two h in room temperature. After the blocking, membranes were incubated with primary antibodies (Table 3) overnight at 4° C. Then, membranes were washed with TBS-T (4 x 5 minutes with a moderate rocking) and incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) for 1 h at room temperature with gentle rocking. The incubation with secondary antibodies was followed by washing (5 x 5 minutes in moderate rocking). Then, proteins were visualised by enhanced chemiluminescence (SuperSignal West Femto maximum sensitivity substrate, Pierce Biotechnology, Rockford, IL, United States) using a ChemiDoc XRS device and quantified with Quantity One software (version 4.6.3. Bio-Rad Laboratories, Hercules, CA, United States). The quantification was always conducted at least twice and average values were used. The quantified signal was normalized to the average value of Ponceau S (strong band at ~42 kDa) and GAPDH. In publications I and II, the samples were pipetted on the gel electrophoresis as sets. In the *mdx* experiment the sets were organized in the following order: PBS, sACVR, PBS RUN, sACVR RUN and wt. In the acute sACVR2B-Fc experiment the sets were organized in the following order: PBS, sACVR1D and sACVR2D and in the 2-week sACVR2B-Fc experiment in the following order: PBS, sACVR2Wk and sACVR2Wk. In the cancer cachexia experiment the sets were organized in a following order CTRL, C26 + PBS, C26+sACV/b and C26+sACVR/c. Within each set, quantified signal normalized to the mean of Ponceau S and GAPDH was further normalized to control mouse (PBS + *mdx*, PBS1-2D, PBS 2Wk or C26 + PBS, depending on the experiment) that was thus set to be 1. This was done to reduce the variation in background noise from left to right on the membrane caused by chemiluminescence and Ponceau S staining. In addition, the sets were organized so that within each set the mice next to each other on the gel were euthanized consecutively and thus the effect of time of the day was reduced. For the figures, standard error of mean for the normalized group that was set to be 1 (PBS + mdx, PBS1-2D, PBS 2Wk or C26 + PBS, depending on the experiment) was calculated after normalizing the calculated signal to the mean of the normalized group. In the publication III, the signal normalized to the mean of GAPDH and Ponceau S was further normalized to PRE condition which was set to be 1 for the statistics but the calculated signal normalized to the mean of GAPDH and Ponceau S are shown in figures as arbitrary units.

In the muscular dystrophy (I) and the acute and 2-week sACVR2B-Fc experiments (II), GRP78 and HSP47 were measured in another laboratory with a previously described protocol (Lappalainen et al. 2009) with some slight modifi-

cations. In brief, 20 µg of total protein was separated with SDS-PAGE and subsequently transferred to a nitrocellulose membrane (Millipore, Bedford, MA, United States). After the transfer, membranes were blocked with 5% (w/v) fat-free milk solution at 37° C for 1 h and thereafter incubated with primary antibodies (Table 3) over-night at 4° C. After the incubation with fluorescence dye conjugated secondary antibodies, proteins were visualized by Odyssey (LI-COR Biosciences Inc., Lincoln, NB, United States) and quantified by Odyssey Software. To confirm equal protein loading and transfer efficiency, membranes were stained with Ponceaus S and re-probed with a primary antibody that bound to Actin.

For the masters athletes (IV), the WB protocol differed slightly from the one used in publications I–III (described first in this section). For the WB analysis, cytosolic and nuclear fractions were combined because the interest was in the changes in the total protein content and not in their subcellular localization and because the fractionation protocol was not thoroughly reliable (e.g. the cytoplasmic eIF2α was observed in nuclear fraction, not shown). The protocol was similar to the one that was described first in this section, except for the following parts. Approximately 12 µg of protein was separated by SDS-PAGE using Criterion™ TGX Stain-Free™ 4–20% Precast Gels (Bio-Rad Laboratories). Thereafter, proteins were transferred to a PVDF membrane (Trans-Blot® Turbo™ midi-size LF, Bio-Rad Laboratories, Inc) using a Trans-Blot® Turbo™ Transfer system (Bio-Rad Laboratories) with built-in transfer protocols (2.5 A, 25 V, 7–10 minutes) and recommended transfer buffer (20% 5 x Trans-Blot® Turbo™ Transfer Buffer, 20% ethanol, 60% ddH_2O). The proteins were visualized by ChemiDoc MP device (Bio-Rad Laboratories) and the signal was quantified by using Image Lab software and normalized to the whole-lane signal of the stain-free blot.

Protein carbonyls. In the muscular dystrophy (I) and the acute and 2-week sACVR2B-Fc experiments (II), protein carbonyls were derivatized using 2,4-dinitrophenyl hydrazine as described previously (Shacter et al. 1994; Gordillo et al. 2002) before the separation by SDS-PAGE using 12.5% gels. The nitrocellulose membranes containing the separated proteins were incubated with a primary antibody against 2,4-dinitrophenol (Zymed Laboratories, San Francisco, CA, USA) overnight at 4° C. The immunoblots were then visualized by Odyssey (Li-Cor Biosciences Inc., Lincoln, NB, USA) and the signal was quantified using Odyssey Software. Protein carbonyls were measured in C26 cancer (II) and human resistance training experiments (III) with same principle but with commercial OxyBlot Protein Oxidation Detection kit (Merck Millipore, S1750) according to the manufacturer's instructions. Briefly, 10 µg of total protein was used for the derivatization reaction and blots were visualized similarly as in the protocol used in publications I–III. In both protein carbonyl measurement protocols, the whole-lane signal was quantified and normalized to either Actin/total protein loading (*mdx* and sACVR2B-Fc acute and 2-week studies (I & II) or Ponceau S (C26 cancer and resistance training studies (II & III).

TABLE 3 List of primary antibodies used to detect proteins by western blot.

Protein	Primary antibody
ATF4 (III & IV)	Cell Signaling #11815
Beclin-1 (II, III & IV)	Cell Signaling #3738
BCL-2 (II, III & IV)	Cell Signaling #3498
eIF2α (I–IV)	Cell Signaling #5324
p-eIF2α at ser51 (I–IV)	Cell Signaling #3398
GAPDH (I–IV)	AbCam #9485
GRP78 (II (C26), III & IV)	Cell Signaling #3177
GRP78 (I & II)	Enzo Life Sciences SPA-826
HSP47 (I & II)	Enzo Life Sciences SPA-470
IRE1α (I–IV)	Cell Signaling #3294
JNK (II & III)	Cell Signaling #9252
p-JNK at Thr183/Tyr185 (II & III)	Cell Signaling #4668
LC3I & LC3II (II, III & IV)	Sigma-Aldrich L7543
PDI (I–III)	Cell Signaling #3501
P62/SQSTM1 (II, III & IV)	Cell Signaling #5114
PERK (I & II)	Cell Signaling #3192
PERK (III & IV)	Cell Signaling #5683
TFEB (IV)	Cell Signaling #37785
ULK1 (II, III & IV)	Cell Signaling #8054
p-ULK1 at ser555 (III)	Cell Signaling #5869
p-ULK1 at ser757 (II, III & IV)	Cell Signaling #14202

4.3.3 RNA extraction and cDNA synthesis (I–III)

Muscular dystrophy and its treatments (I). RNA was extracted from gastrocnemius muscle using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The possible DNA-remnants were degraded with DNAse kit (TURBO DNA-free™ Kit, Applied Biosystems by Life Technologies, South San Francisco, CA, USA) before the mRNA analyses were conducted. The purity and integrity of the yielded RNA was confirmed by spectrophotometry (Nanodrop ND-1000, Thermo Fisher Scientific, Inc., Waltham MA, USA) and agarose gel electrophoresis, respectively. To produce cDNA, approximately three µg of total RNA was reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, USA) according to the instructions provided by the manufacturer.

C26 cancer experiment (II). The RNA extraction and cDNA protocols differed from the ones described above by following parts. To extract and purify total RNA, QIAzol and RNeasy Universal Plus kit (Qiagen) were used, respectively. For the cDNA reverse-transcription, iScript™ Advanced cDNA Synthesis Kit (Bio-Rad Laboratories) was used. All the steps were conducted following the instructions provided by the manufacturers.

Young previously untrained men (III). The RNA extraction and cDNA synthesis from muscle biopsy specimens was conducted similarly as in the protocol used in publication I, except that DNAse kit was not used.

4.3.4 Real-time RT-qPCR (I–III)

In the publications I–III, the mRNA expression levels were analyzed using real-time quantitative polymerase chain reaction (RT-qPCR) with iQ™ SYBR® Green Supermix and CFX96 Real-Time PCR detection system with primers listed in table 4 unless otherwise stated. The protocol for the spliced variant of X-box binding protein (*Xbp1s*) in the publications I and II was initiated at 95° C for 3 minutes, which was followed by 39 cycles of denaturation at 95° C for 10 seconds, annealing at 61° C for 30 seconds and extension at 68° C for 30 seconds. The protocol for *Xbp1t* and *Xbp1s* in the publication III was initiated at 95° C for 3 minutes, which was followed by 40 cycles of denaturation at 95° C for 10 seconds, annealing at 62° C for 15 seconds and extension at 72° C for 30 seconds. When pre-designed and validated primers were used (Bio-Rad PrimePCR™), the analysis was conducted according to the recommended protocol provided by the manufacturer (activation at 95° C for 2 minutes followed by 40 cycles of denaturation at 95° C for 5 seconds and annealing/extension at 60° C for 30 seconds. The mRNA expression data was normalized to *Gapdh* (I & III) or *36b4* (II) which acted stably as previously reported for the publications II and III (Hulmi et al. 2007; Nissinen et al. 2018). To measure *Gapdh* in the publication I, Abi 7300 Real-time PCR System (Applied Biosystems, Foster City, CA, USA) with a Taqman probe (Table 4) and a following protocol was used: activation at 50° C for 2 minutes and 95° C for 10 minutes followed by 40 cycles of denaturation at 95° C for 15 seconds and annealing and extension at 60° C for 1 minute. *Gapdh* acted stably also in the publication I. The mRNA expressions were analyzed from the exponential amplification phase using the delta delta CT method (ΔΔCt).

TABLE 4 List of primers used in RT-qPCR.

Gene	Primer
Atf6b (I)	Pre-designed (qMmuCID0022729; Bio-Rad PrimePCR™)
Chop (I & II)	Pre-designed (qMmuCID0005629; Bio-Rad PrimePCR™)
Gapdh (I)	Taqman probe: Mm99999915_g1
Lc3b (II)	Pre-designed (qMmuCED0048150; Bio-Rad PrimePCR™)
P62/SQSTM1(II)	Pre-designed (qMmuCID0024517; Bio-Rad PrimePCR™)
Xbp1s (I & II)	FWD: TGCTGAGTCCGCAGCAGGTG; REV: CTGATGAGGTCCCCAC-TGACAGA
Xbp1s (III)	FWD: TGCTGAGTCCGCAGCAGGTG, REV: GCTGGCAGGCTCTGGGGAAG
Xbp1t (III)	FWD: AGGAGAAGGCGCTGAGGAG-GAAACT REV: ACCACTTGCTGTTCCAGCTCAC-TCA

4.3.5 Glutathione analysis (I–II)

Glutathione analyses were conducted in the University of Eastern Finland by Dr. Mustafa Atalay and his research group as previously described (Lappalainen et al. 2009). Briefly, in all experiments (I–II), gastrocnemius muscle was homogenized on ice by an Ultra-Turax homogenizer (Janke and Kunkel, Germany) in a 1:10 (w/v) dilution of ice-cold 5% metaphosphorus acid. Thereafter, homogenate was centrifuged at 10000 g for 15 minutes at 4° C and supernatant was collected and stored at -80° C until the measurement day. On the measurement day, homogenates were diluted to distilled water. Subsequently, total glutathione (TGSH) and its oxidized form (GSSG) were measured spectrophotometrically using a GSSG reductase recycling method. The reduced form of glutathione (GSH) was calculated as GSH=TGSH-GSSG and all values were normalized to the weight of the muscle specimen.

4.4 Statistics

Publication (I). To analyze the effect of muscular dystrophy, t-test or Mann-Whitney U test were used (PBS treated sedentary *mdx* vs. non-treated wild-type control). To analyze the effects of the treatments in *mdx* mice, 2 x 2 ANOVA followed by Tukey's post hoc tests was used.

Publication (II). To analyze the main effect (sACVR2B-Fc 1–2 days, and treatments on C26 tumor-bearing mice), one-way ANOVA or Kruskal-Wallis test, followed by Holm-Bonferroni corrected LSD or Mann Whitney U post-hoc tests, depending on the data distribution, were used. For the 2-week sACVR2B-Fc effect, t-test or Mann Whitney U were used. The C26 cancer effect (CTRL vs. C26 + PBS and CTRL vs. C26 pooled) was analysed by t-test or Mann Whitney U.

Publication (III). For the both experiments (young and older men) the main effect of RE/RT was investigated with a General Linear Model with repeated measures followed by post-hoc tests. For the post hoc tests, RE- and RT induced fold changes from pre-values were analyzed by Holm-Bonferroni adjusted t-test or Wilcoxon-signed rank test, depending on the data-distribution. For the young previously untrained men, three comparisons were included to the Holm-Bonferroni adjusted tests (PRE vs 1 h, PRE vs. 48 h and PRE vs. 21 weeks). For the older men, the comparisons were (PRE vs 48 h, PRE vs. post 21 weeks (PRE RT), and PRE vs. 48 h (POST RT).

Publication (IV). The effect of the experimental training period was analyzed by repeated measures ANOVA. Within group effects in CTRL and EX groups were analyzed by paired t-test or a related samples Wilcoxon signed rank-test depending on the data distribution.

In all publications, statistical analyses were conducted by PASW statistics or Excel software. The level of statistical significance was set at $P \leq 0.05$.

5 RESULTS

5.1 Muscular dystrophy, voluntary wheel running and activin receptor ligand blocking (I)

5.1.1 Background results

This study was a follow up to previously published work. Briefly, *mdx* muscles contained centrally nucleated and fibrotic fibers as well as exhibited great amount of proliferating non-muscle cells. Activin receptor ligand blocking by soluble recombinant activin receptor type 2B (sACVR2B-Fc) increased muscle mass in *mdx* mice and further decreased the markers of aerobic metabolism. Voluntary wheel running increased the markers of aerobic metabolism towards healthy wild-type sedentary controls and restored decreased aerobic metabolism in sACVR2B-Fc-treated mice (Hulmi et al. 2013b; Kainulainen et al. 2015). The present thesis shows that *mdx* mice exhibited decreased muscle strength and greater amount of small (<1000 μm^2) and very small muscle fibers (<600 μm^2) ($P < 0.05$, Figures 6A–C). Treatments did not increase muscle strength in *mdx* mice (Figure 6D) but running shifted the fiber size distribution further towards smaller fibers (Figure 6E, $P < 0.05$).

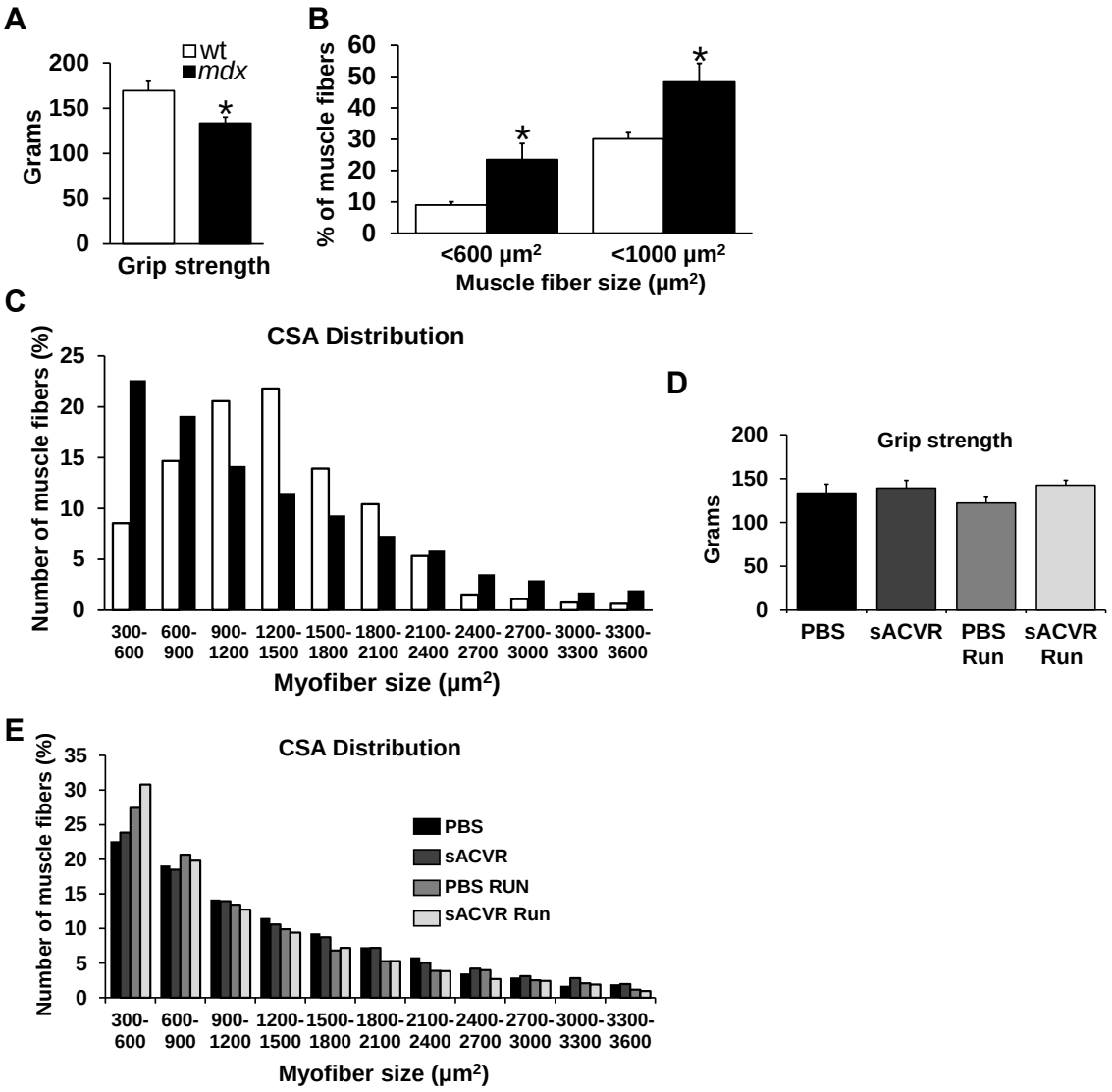

FIGURE 6 Effects of muscular dystrophy, voluntary wheel running and activin receptor ligand blocking (alone and combined) on muscle grip strength and muscle fiber cross sectional area (CSA). A) Grip strength and B) the proportion (%) of small (<1000 µm²) and very small muscle fibers (<600 µm²) in *mdx* and wild-type (wt) mice. C) Myofiber CSA distribution (%) in *mdx* and wt mice. D) Grip strength and E) myofiber CSA area distribution (%) in treated *mdx* mice. In D–E, the *mdx* groups were, PBS = PBS administered sedentary, sACVR = sACVR2B-Fc administered sedentary, PBS Run = PBS administered voluntary wheel running, sACVR RUN = sACVR2B-Fc administered voluntary wheel running. In *mdx* groups n = 8 and in wt, n = 5. In A, B & D values are means ± S.E.M, whereas in C and E only means. * = $P < 0.05$.

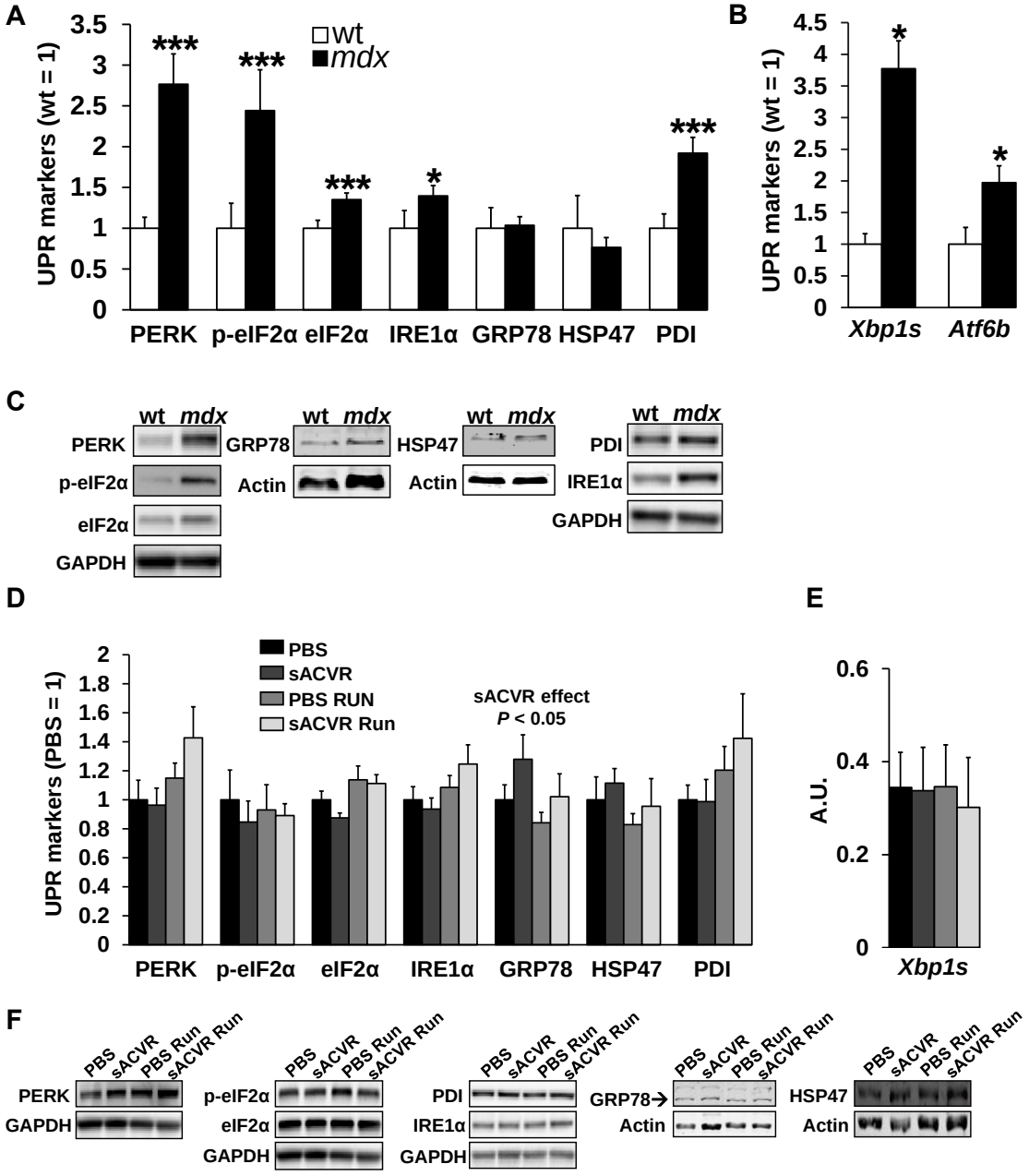

FIGURE 7 Effects of muscular dystrophy, voluntary wheel running and activin receptor ligand blocking (alone and combined) on ER stress/UPR markers in *mdx* muscle. A) PERK, p-eIF2α at ser51, eIF2α, IRE1α, GRP78, HSP47 and PDI as well as B) mRNA of *Xbp1s* and *Atf6b* in *mdx* and wild type (wt) mice. C) Representative immunoblots of *mdx* and wt mice. D) PERK, p-eIF2α at ser51, eIF2α, IRE1α, GRP78, HSP47 and PDI as well as E) *Xbp1s* mRNA in treated *mdx* groups. F) Representative immunoblots of the treated *mdx* groups. In D–F, the *mdx* groups were, PBS = PBS administered sedentary, sACVR = sACVR2B-Fc administered sedentary, PBS Run = PBS administered voluntary wheel running, sACVR RUN = sACVR2B-Fc administered voluntary wheel running. In *mdx* groups n = 7–8 and in wt, n = 4–5. Values are means ± S.E.M * = $P < 0.05$, *** = $P < 0.001$. 2 X 2 ANOVA-effects are shown as text above the bars.

5.1.2 ER stress and unfolded protein response

Effect of muscular dystrophy. The markers of all three canonical unfolded protein response (UPR) signaling pathways were upregulated in *mdx* muscle (Figures 7A & B).

Treatment effects. sACVR2B-Fc administration increased endoplasmic reticulum resident chaperone GRP78 (2 x 2 ANOVA sACVR2B-Fc-effect, $P < 0.05$, Figure 7D) while other UPR markers were unaltered by the treatments (7 weeks of voluntary wheel running and sACVR2B-Fc administration alone and combined) (Figures 7D–E).

5.1.3 Redox balance

Effect of muscular dystrophy. Oxidized form of glutathione (GSSG) was increased ($P < 0.05$) in *mdx* muscle compared with wild-type controls, while there were no differences in reduced form of glutathione (GSH), the ratio of GSSG to GSH (GSSG/GSH) and protein carbonyl content (Figures 8A–D).

Treatment effects. Seven weeks of voluntary wheel running increased GSSG, GSSG/GSH and protein carbonyl content in *mdx* muscle (2 x 2 ANOVA running-effect, $P < 0.05$, Figures 8E, G & H). In addition, there was a significant sACVR2B-Fc and running interaction effect on protein carbonyl content (2 x 2 ANOVA interaction effect, $P < 0.05$, Figure 8H). Furthermore, protein carbonyl content was significantly greater in sACVR RUN group compared with sACVR group ($P < 0.05$, Figure 8H).

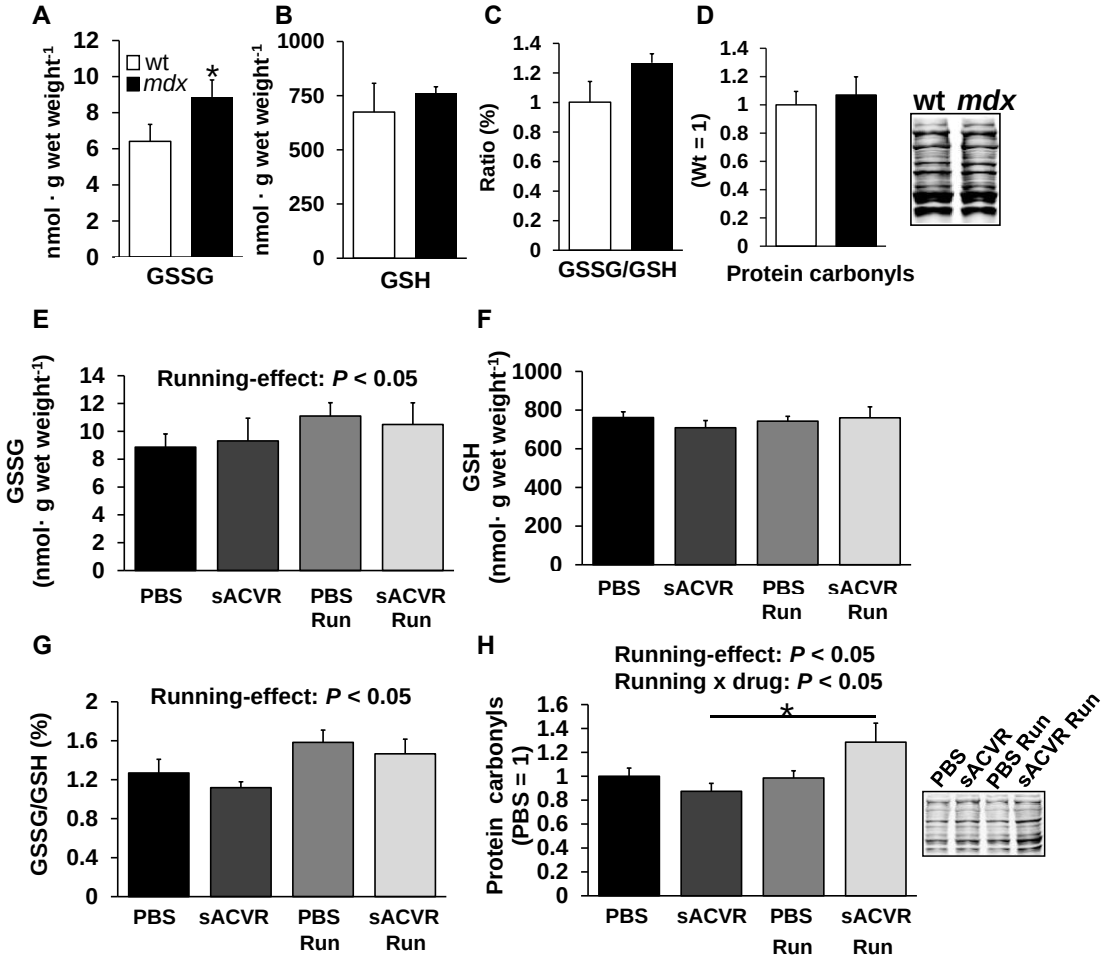

FIGURE 8 Effects of muscular dystrophy, voluntary wheel running and activin receptor ligand blocking (alone and combined) on redox balance markers in *mdx* muscle. Concentration of A) oxidized form of glutathione (GSSG), B) reduced form of glutathione (GSH), C) their ratio (GSSG/GSH) and D) protein carbonyl content in *mdx* and wild type (wt) control mice. Concentration of E) GSSG, F) GSH, G) their ratio (GSSG/GSH) and H) protein carbonyl content and the representative immunoblot in the treated *mdx* groups. In E–H, the *mdx* groups were, PBS = PBS administered sedentary, sACVR = sACVR2B-Fc administered sedentary, PBS Run = PBS administered voluntary wheel running, sACVR RUN = sACVR2B-Fc administered voluntary wheel running. In *mdx* groups n = 7–8 and in wt, n = 5 Values are means ± S.E.M. * = $P < 0.05$. 2 X 2 ANOVA-effects are shown as text above the bars.

5.2 Activin receptor ligand blocking in healthy mice (II)

5.2.1 Background results

This study was a follow up for previously published work (Hulmi et al. 2013a). Briefly, the previously published results showed that a single dose of recombinant soluble activin receptor type 2B (sACVR2B-Fc) induced muscle protein synthesis and mTOR signaling one and two days after the administration in healthy mice. This led to increased muscle mass at two weeks of sACVR2B-Fc administration.

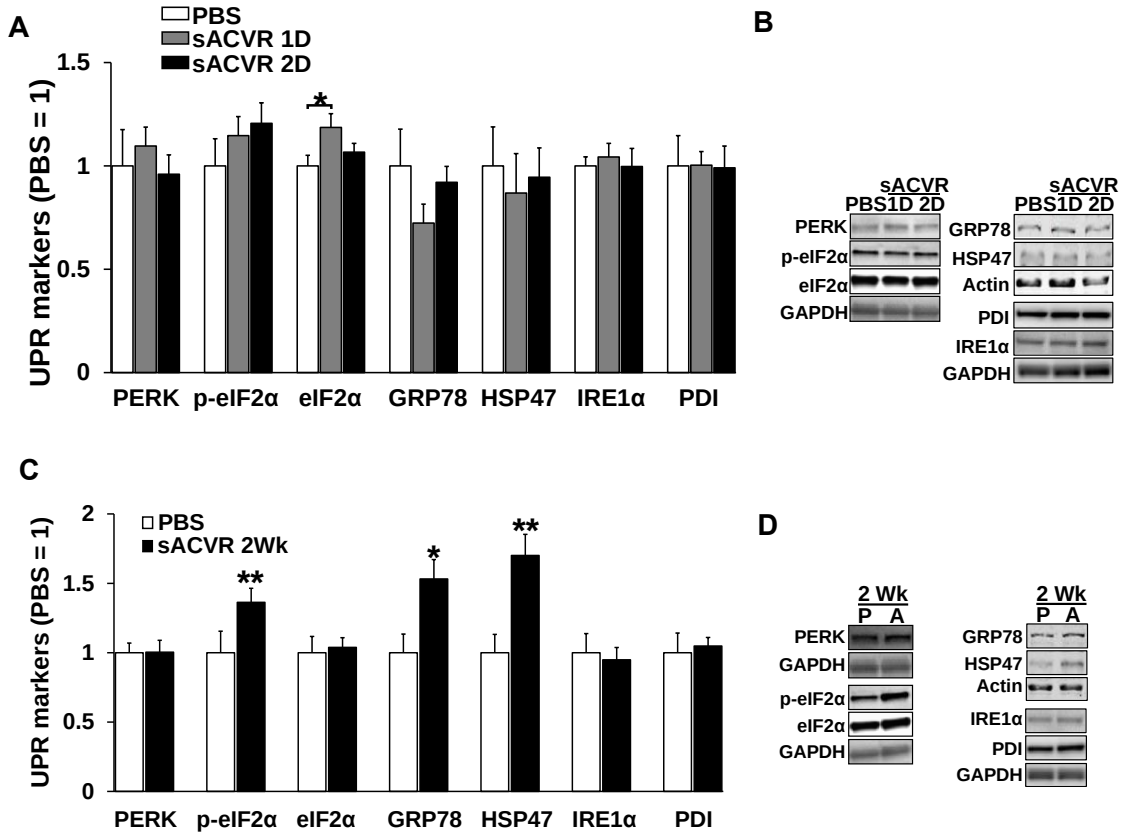

FIGURE 9 Skeletal muscle ER stress/UPR markers in healthy mice that were administered with sACVR2B-Fc or PBS. A) PERK, p-eIF2α at ser51, eIF2α, GRP78, HSP47, IRE1α and PDI as well as their B) representative immunoblots at one (sACVR 1D) and two (sACVR 2D) days after a single administration of sACVR2B-Fc or PBS. C) PERK, p-eIF2α at ser51, eIF2α, GRP78, HSP47, IRE1α and PDI as well as their D) representative immunoblots at two weeks after sACVR2B-Fc (sACVR 2Wk) or PBS administration. n = 6–7 in all groups except n = 9–11 in sACVR 2 Wk group. * = $P < 0.05$, ** = $P < 0.01$. P = PBS, A = sACVR2B-Fc Values are means ± S.E.M.

5.2.2 ER stress and unfolded protein response

A single sACVR2B-Fc administration had no consistent effect on muscle ER stress/UPR markers at one or two days in healthy mice (Figure 9A). However, two weeks of sACVR2B-Fc administration increased phosphorylated eIF2α at ser51 ($P < 0.01$), GRP78 ($P < 0.05$) and HSP47 ($P < 0.01$) while the rest of the analyzed markers were unchanged (Figure 9C).

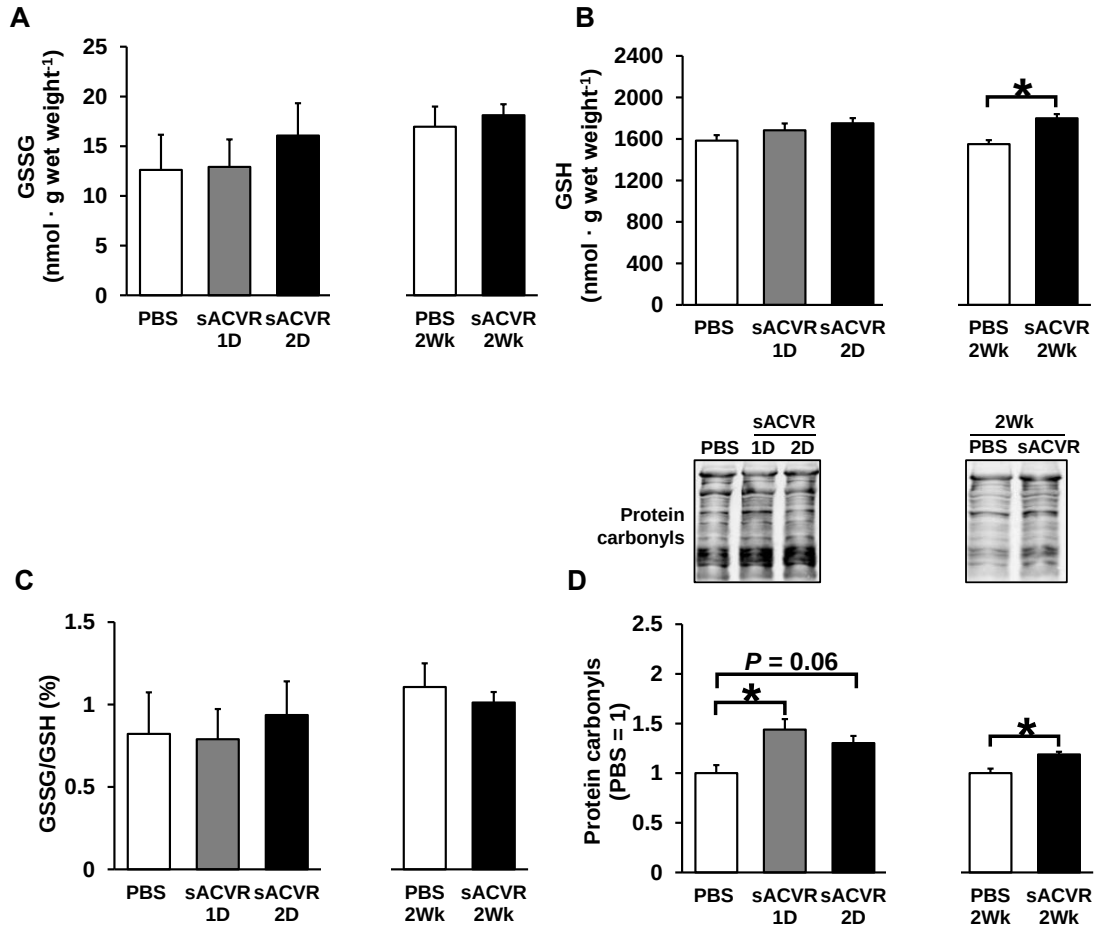

FIGURE 10 Skeletal muscle redox balance markers in healthy mice that were administered with sACVR2B-Fc or PBS. Concentration of A) oxidized form of glutathione (GSSG), B) reduced form of glutathione (GSH), C) their ratio (GSSG/GSH) and D) protein carbonyl content and representative immunoblots. Muscles were collected one or two days after a single dose of sACVR2B-Fc or PBS (sACVR 1–2D) or two weeks of administration (sACVR 2Wk). Values are means ± S.E.M. n = 5–6 in all groups except n = 8–11 in sACVR 2 Wk group. * = $P < 0.05$.

5.2.3 Redox balance

Skeletal muscle protein carbonyl content increased acutely by a single sACVR2B-Fc administration and by the 2-week administration (Day 1: $P < 0.05$, Day 2: $P =$

0.06, 2-week: $P < 0.05$), while at 1 and 2 days after the single administration oxidized form of glutathione (GSSG), reduced form of glutathione (GSH) and their ratio (GSSG/GSH) were unaltered (Figures 10A–D). Two weeks of sACVR2B-Fc administration increased GSH concentration ($P < 0.05$), whereas GSSG and GSSG/GSH were unaltered (Figures 10A–D).

5.3 Experimental cancer and activin receptor ligand blocking (II)

5.3.1 Background results

This study was a follow up for previously published work (Nissinen et al. 2018). Briefly, previously published results showed that C26 tumor-bearing mice exhibited muscle and fat wasting that were accompanied by decreased muscle protein synthesis, and increased markers of ubiquitin-proteasome-system. Furthermore, continuous sACVR2B-Fc administration before and after the C26 cell inoculation prevented muscle wasting and improved survival without an effect on tumor mass.

5.3.2 ER stress and unfolded protein response

Effects of C26 cancer. Of the skeletal muscle ER stress/UPR markers, phosphorylated eIF2α at ser51, phosphorylated JNK54 at thr183/tyr185 and HSP47 were decreased by C26 cancer ($P < 0.05$, Figure 11A). Other ER stress/UPR markers were unaltered by C26 cancer (Figures 11A & B).

sACVR2B-Fc administration in C26 cancer. The continued activin receptor ligand blocking after the tumor formation by sACVR2B-Fc (sACVR/c) increased ER-resident chaperone GRP78 compared with PBS administered tumor-bearing (TB) mice ($P < 0.05$). The rest of the analyzed ER stress/UPR markers were unaffected by sACVR2B-Fc administration (Figures 11A & B).

5.3.3 Autophagy

Effects of C26 cancer. The PE-conjugated (i.e. lipidated) form of LC3 as a marker of autophagosome content (LC3II, $P = 0.051$), the ratio of LC3II to LC3I ($P < 0.05$), Beclin-1 ($P < 0.01$) and P62 ($P < 0.01$) were upregulated in skeletal muscle by C26 cancer (Figure 11D). Other autophagy markers were unaltered (Figure 11D). In addition, *Lc3b* mRNA was significantly increased by cancer ($P < 0.05$), while the *p62* mRNA was unchanged (Figure 11E).

sACVR2B-Fc administration in C26 cancer. The discontinued sACVR2B-Fc treatment protocol increased LC3II/LC3I compared with other TB groups ($P < 0.01$) without other effects of sACVR2B-Fc treatment protocols (Figures 11D & E).

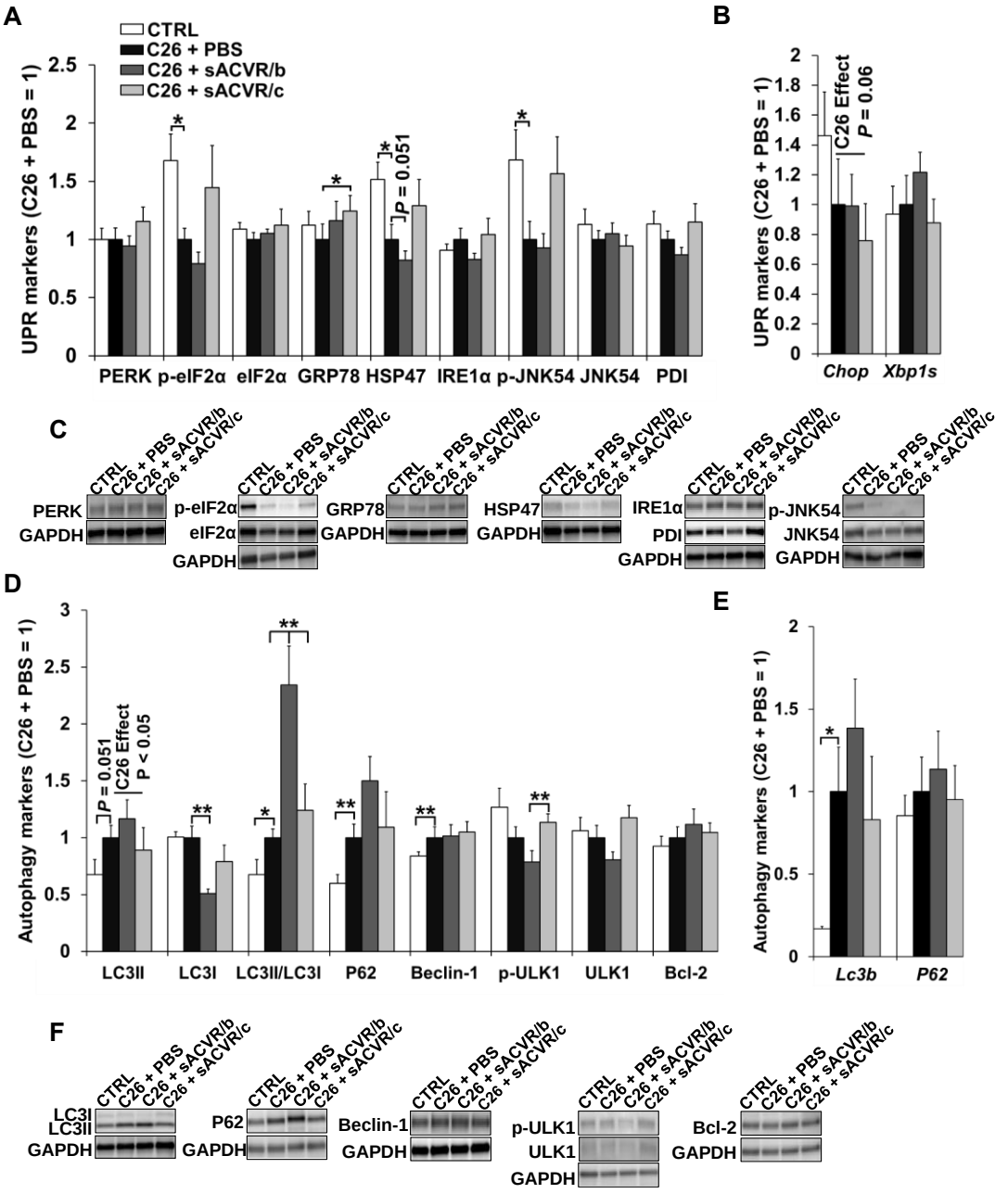

FIGURE 11 ER stress/UPR and autophagy markers in skeletal muscle of C26 tumor-bearing (TB) mice. A) UPR markers (PERK, p-eIF2αs51, eIF2α, GRP78, HSP47, IRE1α, p-JNK54 at thr183/tyr185, JNK54, and PDI). B) mRNA levels of UPR markers (*Chop* and *Xbp1s*). C) The representative immunoblots of ER stress/UPR protein markers. D) The autophagy markers (LC3II, LC3I, LC3II/LC3I, P62, Beclin-1, p-ULK1 at ser757, ULK1 and BCL-2). E) The mRNA levels of autophagy markers (*Lc3b* and *p62*). F) The representative immunoblots of autophagy protein markers. CTRL = vehicle-treated (PBS) control mice, C26 + PBS = C26 TB mice administered with a vehicle (PBS). C26 + sACVR/b = C26 TB mice administered with sACVR before the tumor formation and replaced by a vehicle (PBS) after the tumor formation, C26 + sACVR/c = C26 TB mice continuously administered with sACVR throughout the experiment. In all groups and variables n = 7–8. Values are means ± S.E.M. * = $P < 0.05$, ** = $P < 0.01$. For the C26-effect all the mice in tumor-bearing groups were pooled.

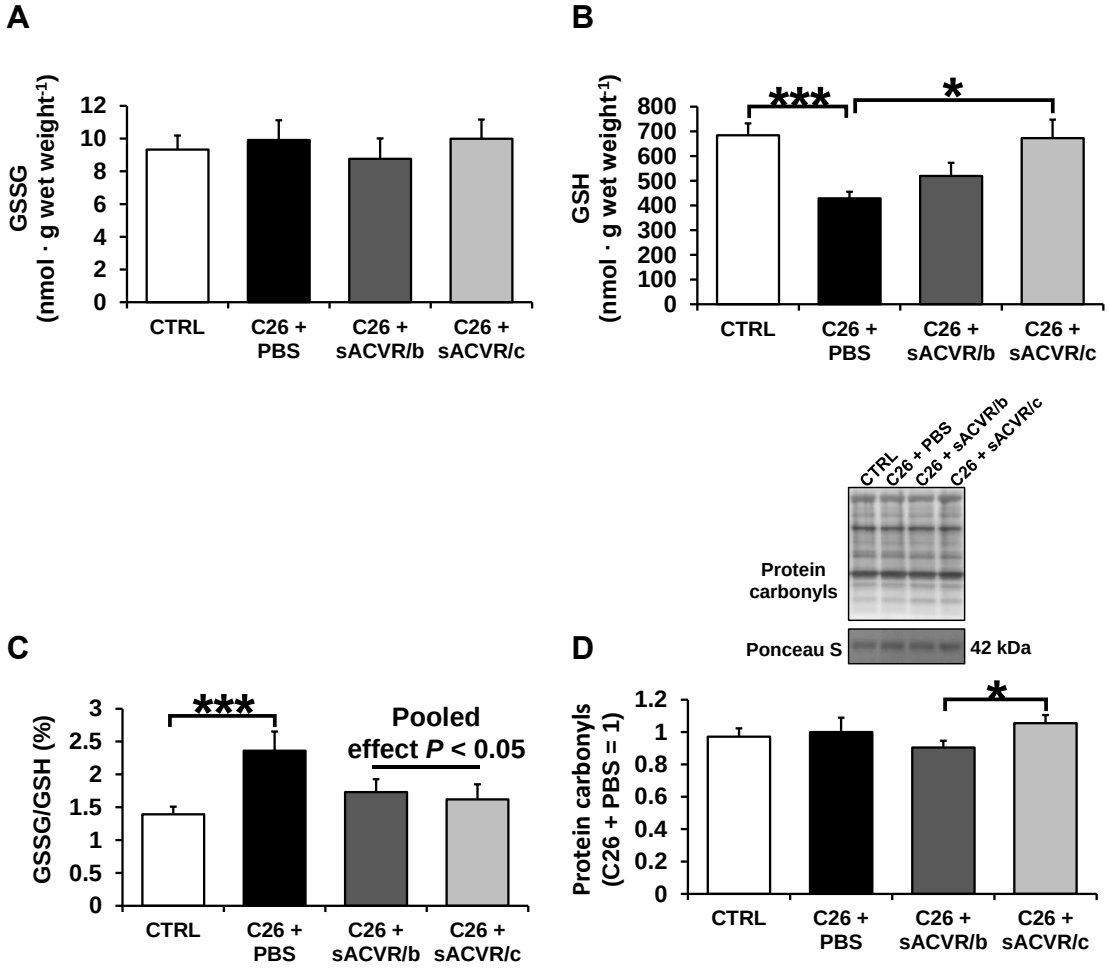

FIGURE 12 Skeletal muscle redox balance markers in C26 tumor-bearing (TB) mice. Concentration of A) oxidized form of glutathione (GSSG), B) reduced form of glutathione (GSH), C) their ratio (GSSG/GSH) and D) protein carbonyl content and its representative immunoblot. CTRL = vehicle-treated (PBS) control mice, C26 + PBS = C26 TB mice administered with a vehicle (PBS), C26 + sACVR/b = C26 TB mice administered with sACVR before the tumor formation and replaced by a vehicle (PBS) after the tumor formation. C26 + sACVR/c = C26 TB mice continuously administered with sACVR throughout the experiment. In all groups and variables n = 7–8. Values are means ± S.E.M. * = $P < 0.05$, *** = $P < 0.001$. For the sACVR-effect, mice in C26 + sACVR/b and C26 + sACVR/c groups were pooled.

5.3.4 Redox balance

Effects of C26 cancer. In muscle, the reduced form of glutathione (GSH) and the ratio of oxidized form of glutathione (GSSG) to GSH (GSSG/GSH) were increased by C26 cancer ($P < 0.001$, Figures 12B & C). Protein carbonyls and GSSG were unaffected by C26 cancer (Figures 12A & D).

sACVR2B-Fc and C26 cancer. The continued activin receptor ligand blocking (C26 + sACVR/c) prevented the C26-induced decline in muscle GSH concentration ($P < 0.05$, Figure 12B). Furthermore, compared with PBS administered TB

mice (C26 + PBS), GSSG/GSH was decreased when sACVR2B-Fc administered groups were pooled (pooled sACVR2B-Fc effect, $P < 0.05$; Figure 12C).

5.4 Resistance exercise and training in young men (III)

5.4.1 Background results

This study was a follow up for previously published work. Briefly, 21 weeks of progressive heavy resistance training (RT) increased leg extensor strength and muscle fiber cross-sectional in young men (26 ± 4 years) (Mero et al. 2013). These results were also statistically significant in this current thesis with a smaller sample size (1RM in concentric leg press: PRE 167.9 ± 29.6 kg to POST 198.8 ± 29.4 kg, $P < 0.001$, Isometric leg press: PRE 3608 ± 1134 N to POST 4388 ± 1458 N, $P < 0.001$, Average CSA of type I and II fibers: PRE 4606 ± 519 μm^2 to POST 6590 ± 1020 μm^2, $P < 0.001$). In addition, mTOR signaling was induced at 1 h after an unaccustomed RE bout (Hulmi et al. 2009b).

5.4.2 ER stress and unfolded protein response

Effect of RE bout at post 1 h. Of the ER stress induced UPR markers, phosphorylated JNK at thr183/tyr185 ($P < 0.01$) and its ratio to total JNK (p-JNK/JNK) ($P < 0.05$) increased in vastus lateralis muscle (VL) at 1 h after the RE bout (Figure 14D). Other UPR markers were unchanged (Figures 13 & 14).

Effect of RE bout at post 48 h. The protein content of PERK, ATF4, GRP78, p-JNK at thr183/tyr185 and p-JNK/JNK increased at 48 h after the RE bout ($P < 0.05$, Figures 13A, B & 14C & D). In addition, spliced variant *Xbp1s* and full length *Xbp1t* mRNA increased ($P < 0.05$, Figures 14A & B). Other UPR markers were unaltered (Figures 13C & D). GRP78 protein seemed to increase systematically also in the non-exercised participants but this effect was not observed in other UPR markers (Figures 13 & 14).

Effect of 21-week RT period. All the analyzed UPR markers (Figures 13 & 14) were unchanged by a 21-week resistance training (RT) period.

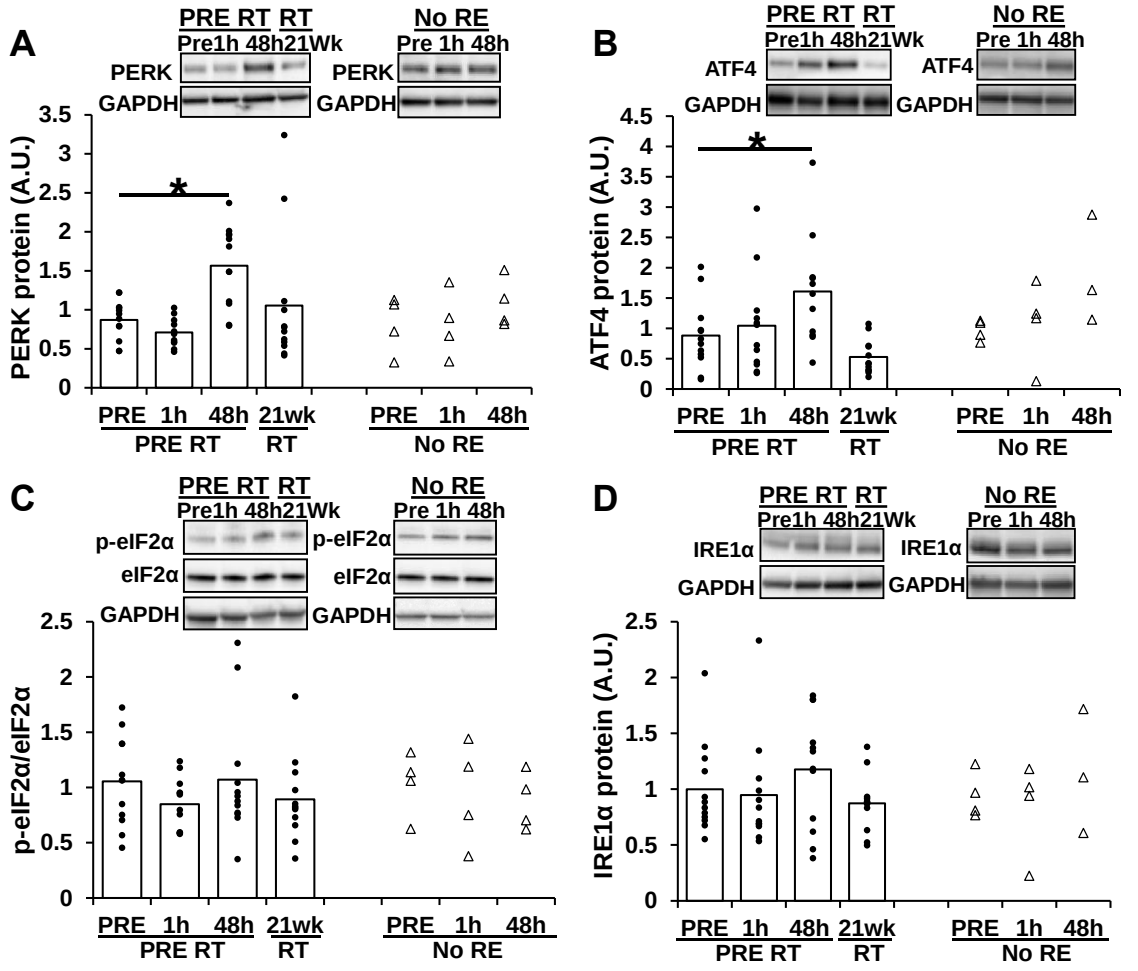

FIGURE 13 Muscle ER stress/UPR markers before, at 1 h and at 48 h after the RE bout (PRE RT) as well as after the RT period in young men. A) PERK , B) ATF4, C) the ratio of p–eIF2α at ser51 to eIF2α D) IRE1α, E) spliced variant of XBP1 mRNA (Xbp1s), F) total XBP1 mRNA (Xbp1t), G) GRP78 and H), the ratio of p–JNK at thr183/tyr185 to JNK (46 and 54 kDa averaged). No RE = Non-exercised control subjects. Open bars depict means. Circles and triangles depict individual values. * = $P < 0.05$. n = 10–12 in all RE & RT and n = 3–4 in all No RE. Representative immunoblots are placed on the top of their corresponding graphs.

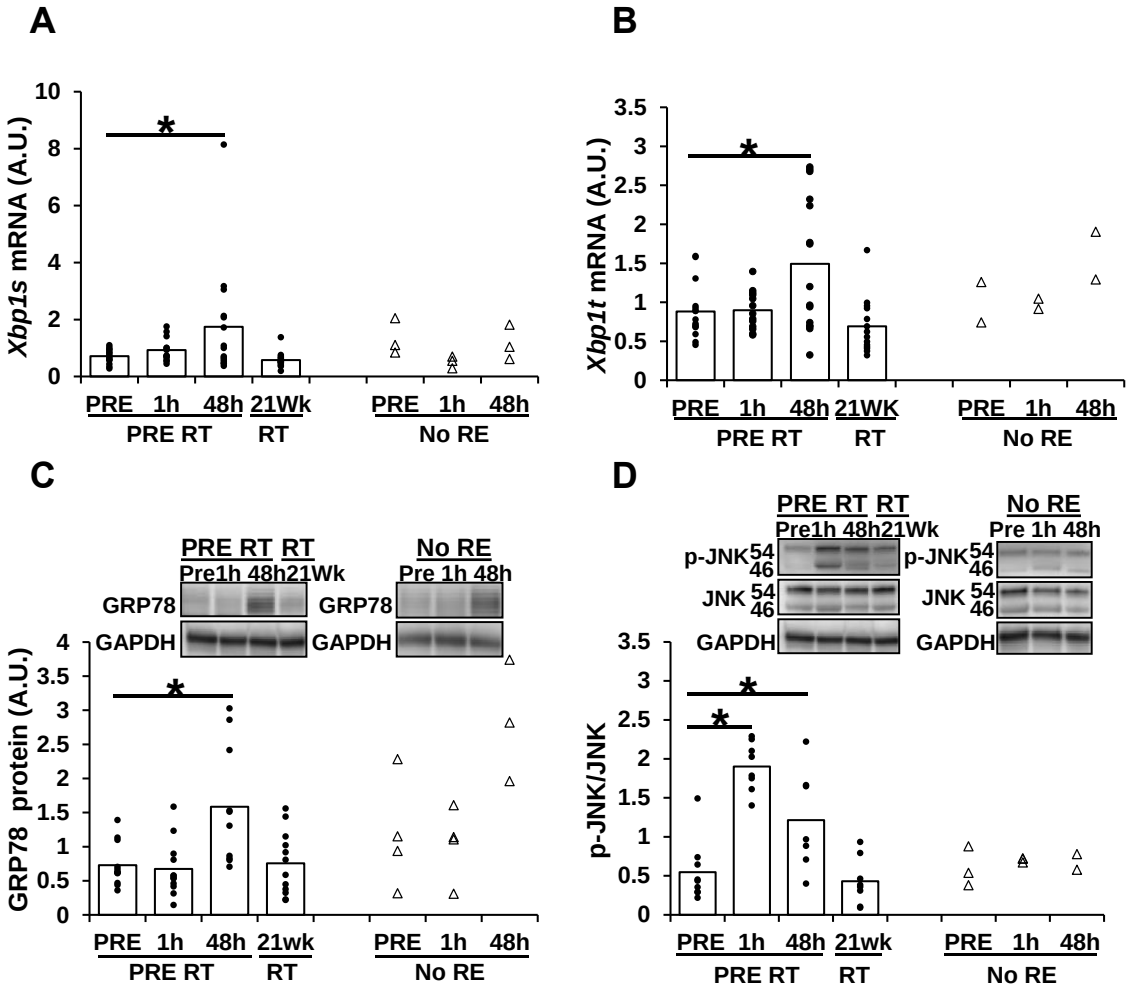

FIGURE 14 Muscle ER stress/UPR markers before, at 1 h and at 48 h after the RE bout (PRE RT) as well as after the RT period in young men. A) spliced variant of XBP1 mRNA (*Xbp1s*), B) total XBP1 mRNA (*Xbp1t*), C) GRP78 and D), the ratio of p–JNK at thr183/tyr185 to JNK (46 and 54 kDa averaged). No RE = Non-exercised control subjects. Open bars depict means. Circles and triangles depict individual values. * = $P < 0.05$. n = 10–12 in all RE & RT and n = 3–4 in all No RE except in *Xbp1s* and *Xbp1t* (n = 13–15 in RE & RT and n = 2–3 in No RE) and p–JNK/JNK (n = 7–9 in RE & RT and n = 2–4 in No RE). Representative immunoblots are placed on the top of their corresponding graphs.

5.4.3 Autophagy

Effect of RE bout at post 1 h. As an indicator of autophagosome content, the PE-conjugated form of LC3 (LC3II) decreased at 1 h after the RE bout ($P < 0.01$; Figure 15A). In addition, phosphorylated ULK1 at ser555 decreased ($P < 0.05$, Figure 16B). Other autophagy markers were unchanged (Figures 15 & 16).

Effect of RE bout at post 48 h. LC3II, LC3I and P62 were increased ($P < 0.05$) and Beclin-1 tended to increase ($P = 0.06$) at 48 h after the RE bout (Figures 15A–D). Other autophagy markers were unchanged (Figures 16A–C).

Effect of 21-week RT period. LC3II increased after the 21-week resistance training (RT) period ($P < 0.01$, Figure 15A). In addition, the ratio of p-ULK1 at ser757 to total ULK1 increased ($P < 0.05$, Figure 16A), whereas p-ULK1 at ser555 decreased ($P < 0.05$, Figure 16B). In addition, BCL-2 content was decreased ($P < 0.05$, Figure 16C). Other autophagy markers were unchanged (Figures 15B–D).

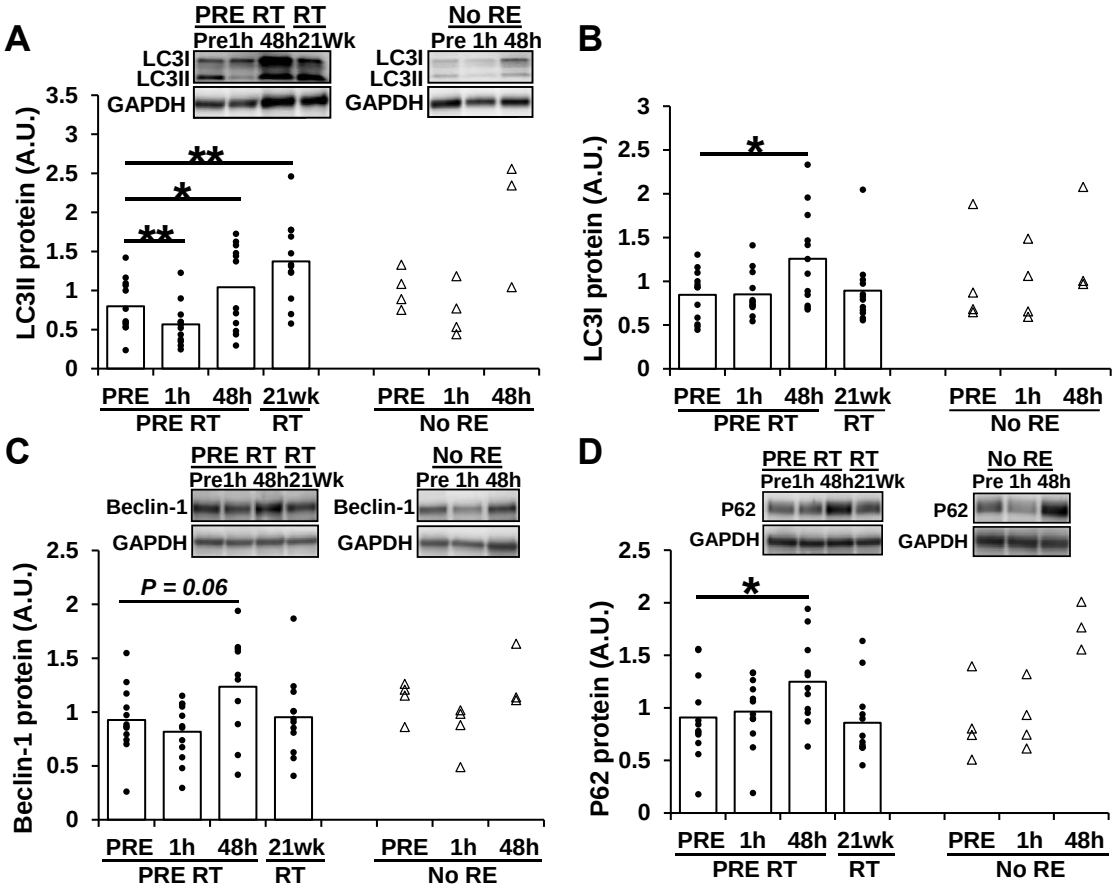

FIGURE 15 Muscle autophagy markers before, at 1 h and at 48 h after the RE bout (PRE RT) as well as after the RT period in young men: A) LC3II, B) LC3I, C) Beclin-1 D) P62. No RE = Non-exercised control subjects. Open bars depict means whereas circles and triangles depict individual values. * = $P < 0.05$. ** = $P < 0.01$. n = 10–12 in all RE & RT and n = 3–4 in all No RE. Representative immunoblots are placed on the top of their corresponding graphs.

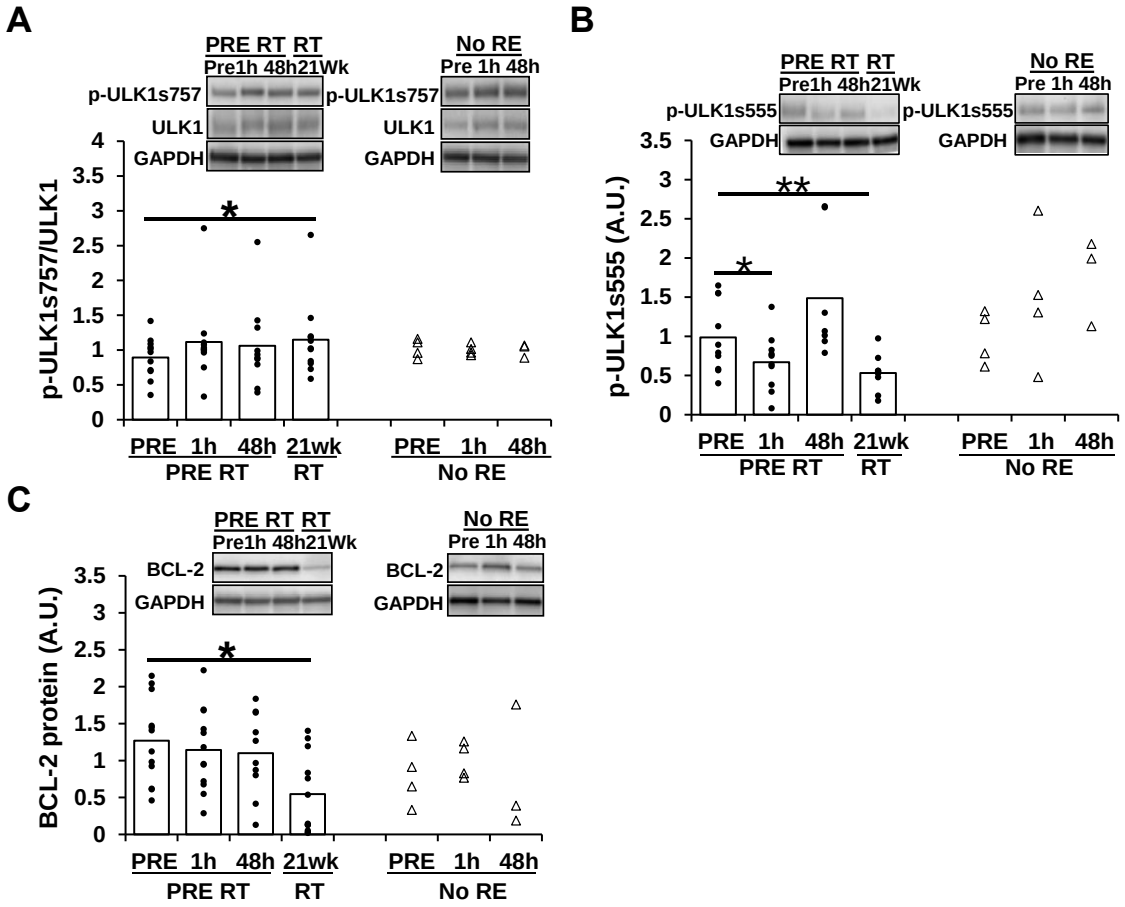

FIGURE 16 Muscle autophagy markers before, at 1 h and at 48 h after the RE bout (PRE RT) as well as after the RT period in young men: A) the ratio of p-ULK1 at ser757 to total ULK1 B) p-ULK1 at ser555 and C) BCL-2. No RE = Non-exercised control subjects. Open bars depict means whereas circles and triangles depict individual values. * = $P < 0.05$. ** = $P < 0.01$. n = 10–12 in all RE & RT and n = 3–4 in all No RE except in p-ULK1 at ser555 (n = 7–10 in RE & RT and 2–4 in No RE). Representative immunoblots are placed on the top of their corresponding graphs.

5.5 Resistance exercise and training in older men (III)

5.5.1 Background results

This study was a follow up for previously published work. Briefly, 21 weeks of progressive heavy resistance training (RT) increased leg extensor strength and muscle fiber cross-sectional in older (61 ± 4 years) men (Mero et al. 2013). These results were also statistically significant in this thesis with a smaller sample size (1 RM in concentric leg press: PRE 155.0 ± 24.2 kg to POST 186.3 ± 29.6 kg, $P <$

0.001, Isometric leg press: PRE 2727 ± 714 N to POST 2989 ± 496 N, $P < 0.05$, Average CSA of type I and II fibers: PRE 4906 ± 635 μm^2 to POST 6178 ± 923 μm^2, $P < 0.01$).

5.5.2 ER stress and unfolded protein response

Effect of RE bout at post 48 h. Of the muscle ER stress/UPR markers, ATF4 increased ($P < 0.05$) at 48 h after a RE bout in VL muscle that was conducted before the 21-week RT period (Figures 17A & B). Additionally, GRP78 content increased at 48 h after the RE bout that was conducted after the 21-week RT period ($P < 0.05$; Figure 15A), while ATF4 was unchanged (Figure 15B). Other ER stress/UPR markers were unchanged (Figures 17C–E).

Effect of 21-week RT period. All the measured UPR markers were unchanged by the 21-week RT period in older men (Figures 17A–E).

5.5.3 Autophagy

All the measured muscle autophagy markers were unchanged at 48 h after the resistance exercise (RE) bout and after the 21-week RT period (Figures 18A–E).

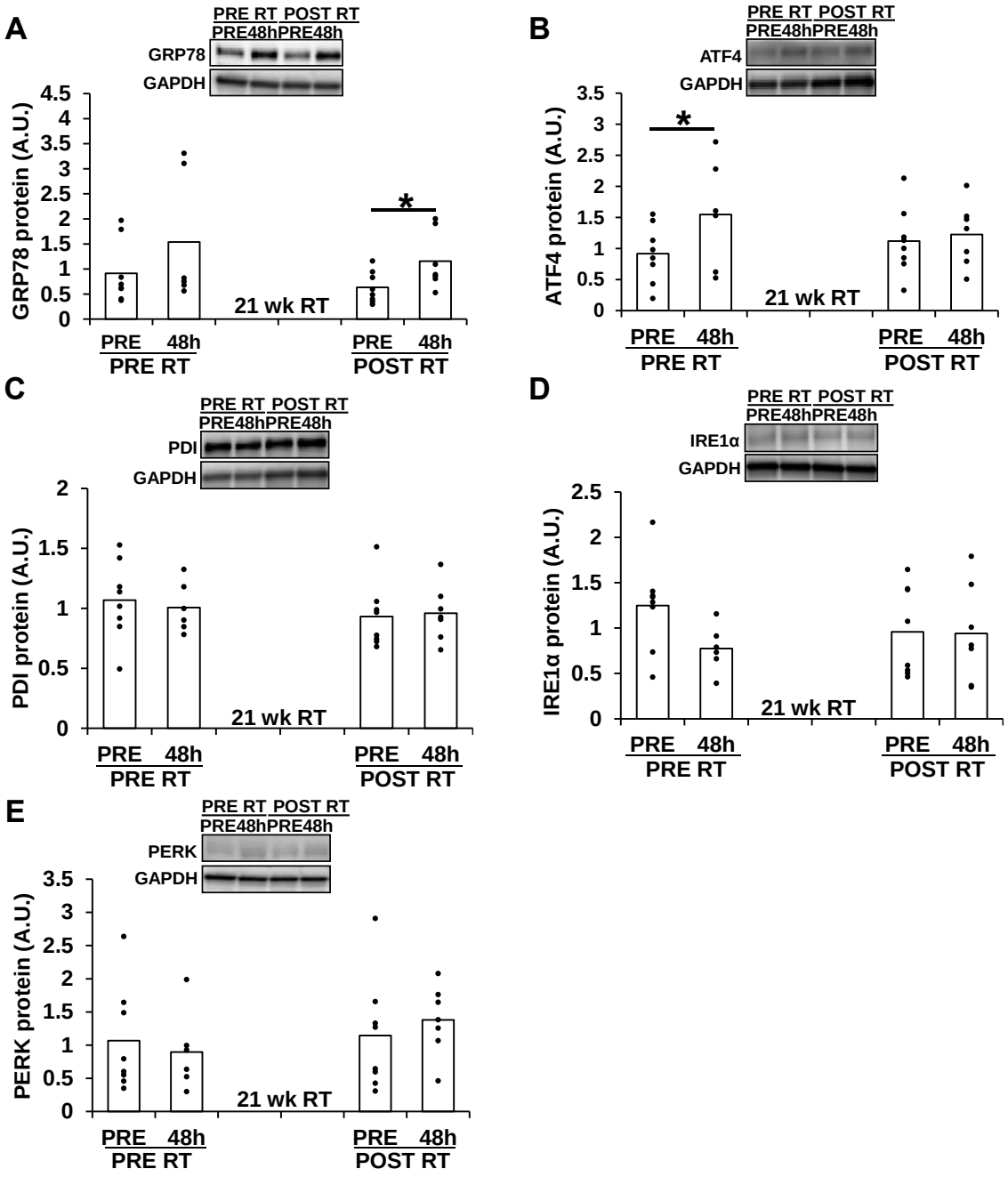

FIGURE 17 Muscle ER stress/UPR markers before and at 48 h after a RE bout (PRE RT) as well as before and at 48 h after a RE bout conducted after a 21-wk resistance training (RT) period (POST RT) in older men. Protein content of A) GRP78, B) ATF4, C) PDI, D) IRE1α, E) PERK. Open bars depict means. Circles and triangles depict individual values.* = $P < 0.05$. In all the variables and time-points n = 6–8. Representative immunoblots are placed on the top of their corresponding graphs.

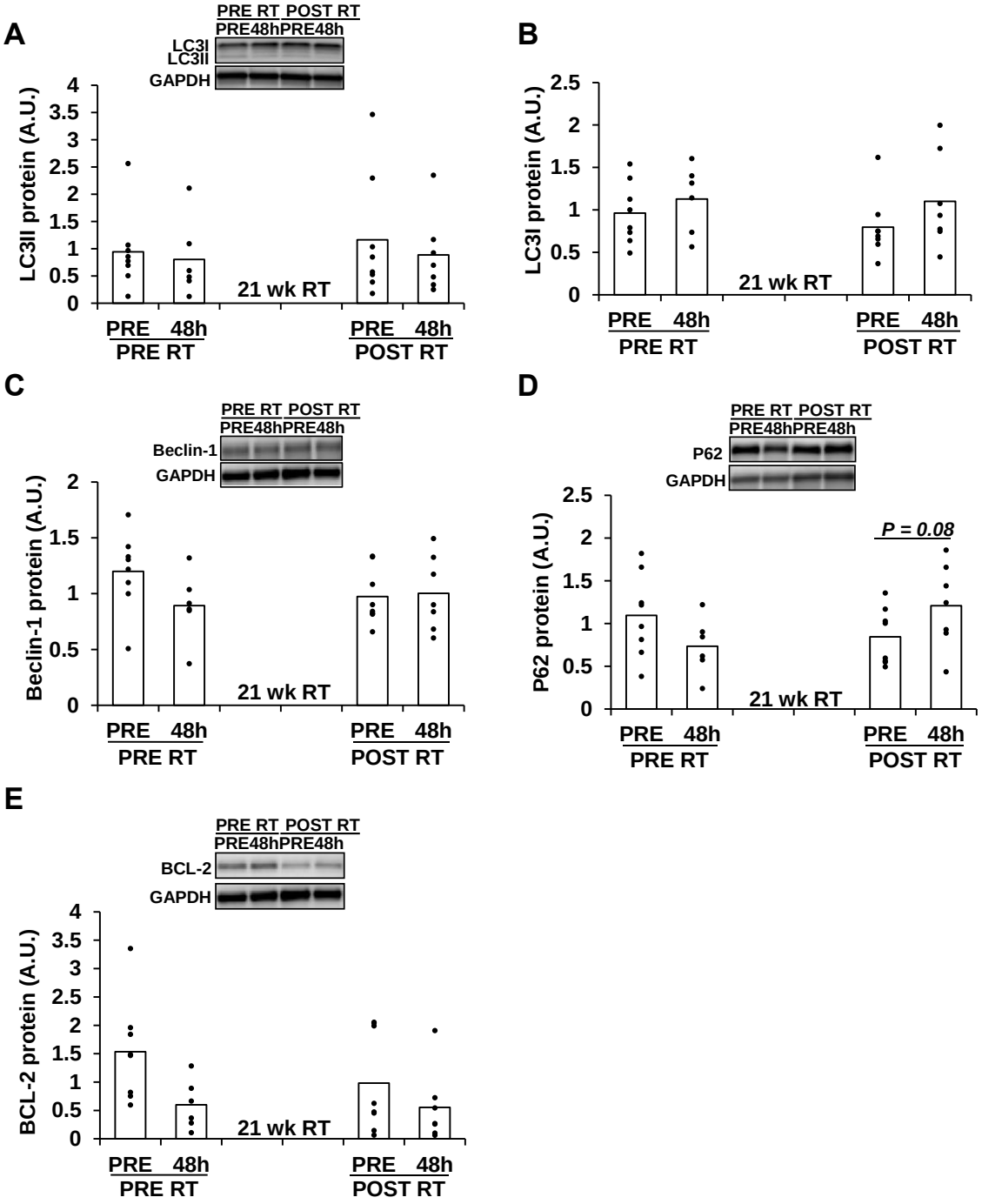

FIGURE 18 Muscle autophagy markers before and at 48 h after a RE bout (PRE RT) as well as before and at 48 h after a RE bout conducted after a 21-wk RT period (POST RT) in previously untrained older men. Protein content of A) LC3II, B) LC3I, C) Beclin-1, D) P62, E) BCL-2. Open bars depict means. Circles and triangles depict individual values. In all the variables and time-points n = 6–8. Representative immunoblots are placed on the top of their corresponding graphs.

5.6 Combined strength and sprint training in masters athletes (IV)

5.6.1 Performance

The 20-week experimental training program (EX), in which maximal and explosive strength exercises were integrated to sprint training regimen, improved 60 meter sprint performance from 8.52 ± 0.54 s to 8.40 ± 0.58 s ($P < 0.05$), whereas it declined in controls from 8.37 ± 0.63 s to 8.48 ± 0.62 s ($P < 0.05$, time x group-effect: $P < 0.01$). Furthermore, squat jump height tended to improve in EX group from 28.86 ± 5.59 cm to 30.75 ± 8.24 after the 20-week intervention period ($P = 0.076$) while it was unchanged in CTRL (PRE 27.94 ± 5.4 cm, POST 28.05 ± 5.47, $P = 0.85$, time x group effect $P = 0.140$). Vastus lateralis muscle (VL) thickness was unchanged in both groups (EX: PRE 2.03 ± 0.38 cm to POST 2.00 ± 0.40 cm, $P = 0.590$; CTRL: PRE 1.97 ± 0.34 cm to POST 1.92 ± 0.37 cm, $P = 0.533$, time x group effect $P = 0.880$).

5.6.2 ER stress and unfolded protein response

Of the ER stress induced UPR markers, ATF4 protein content decreased in EX group ($P < 0.01$, Figure 19A). In addition, phosphorylated eIF2α at ser51 tended to increase ($P = 0.067$) and total eIF2α increased ($P < 0.05$) in the EX group after the intervention period (Figures 19B & C). Other UPR markers were unchanged in EX group but there was a time x group effect in IRE1α (Figures 19D–F).

5.6.3 Autophagy

The LC3II increased in EX group after the intervention period suggesting increased autophagosome content ($P < 0.05$, Figure 20A) while LC3I was unchanged (Figure 20B). Furthermore, P62 protein was decreased in EX group after the intervention period ($P < 0.01$, Figure 20C). TFEB, a transcription factor that regulates lysosome biogenesis was increased in EX group ($P < 0.05$, Figure 20D). The rest of the analyzed autophagy markers were unchanged (Figures 20D-F).

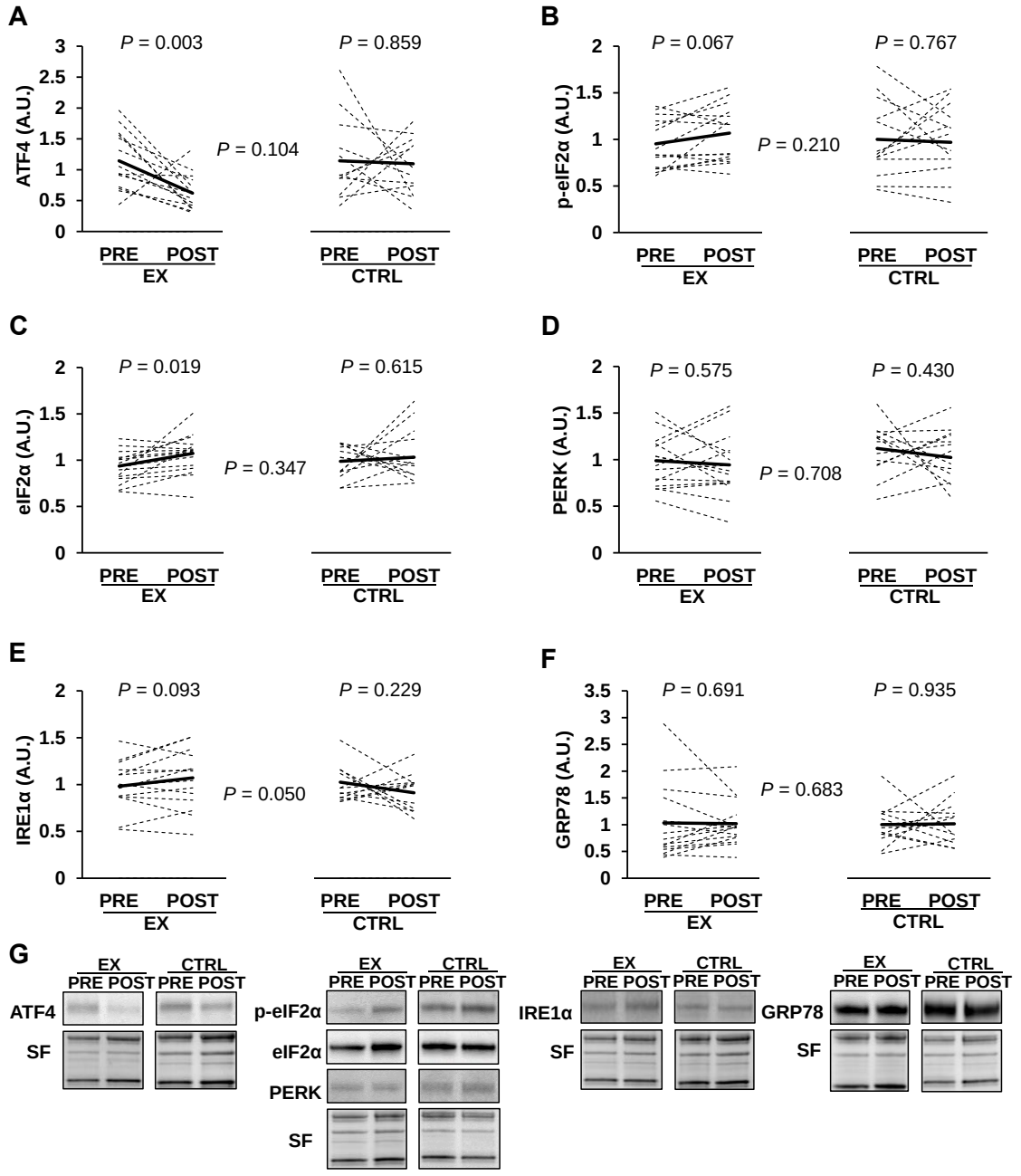

FIGURE 19 Muscle ER stress/UPR markers before (PRE) and after (POST) the 20-week intervention period in experimental (EX) and control (CTRL) groups. Protein content of A) ATF4, B) phosphorylated eIF2α at ser51, C) total eIF2α, D) PERK, E) IRE1α and F) GRP78 and their G) representative immunoblots. n = 15 in EX and n = 13 in CTRL. Dashed lines depict individual values, whereas bolded line represent the average of the group. *P* values above the dashed lines depict the statistical significance within a group (PRE vs POST, paired t-test or Mann Whitney U) whereas *P* values between the groups of dashed lines depict the time x group effect (repeated measures ANOVA). Stain-free blots in G are cropped at 25–42 kDa to save space, but the whole lane was quantified. In F, repeated measures ANOVA was conducted with logarithmic corrected values because the data was not normally distributed.

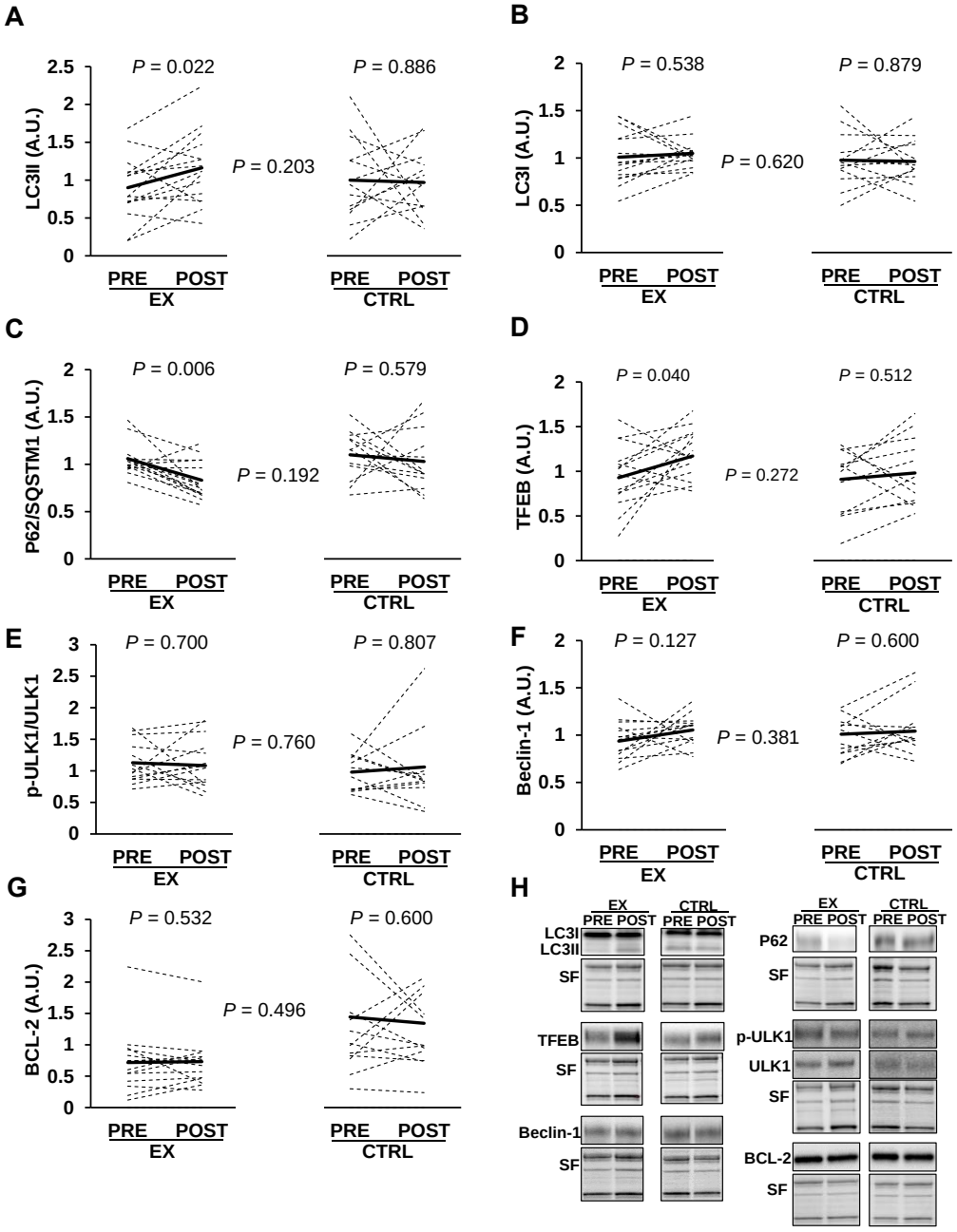

FIGURE 20 Muscle autophagy markers before (PRE) and after (POST) the 20-week intervention period in experimental (EX) and control (CTRL) groups. Protein content of A) LC3II, B) LC3I, C) sequestome1/P62, D) TFEB E) the ratio of p-ULK1 at ser757 to total ULK1, F) Beclin-1, G) BCL-2 and their H) representative immunoblots. In all n = 15 in EX and n = 13 in CTRL. Dashed lines depict individual values, whereas bolded line represents the average of the group. *P* values above the dashed lines depict the statistical significance within a group (PRE vs POST, paired t-test or Mann Whitney U) whereas *P* values between the groups of dashed lines depict time x group effect (repeated measures ANOVA). Stain-free blots in H are cropped at 25–42 kDa to save space but the whole lane was quantified. In C, E and F, repeated measures ANOVA was conducted with logarithmic corrected values because the data was not normally distributed.

6 DISCUSSION

The main findings of this thesis were as follows:

1. Long-term voluntary running and muscle hypertrophy induced by activin receptor ligand blocking (sACVR2B-Fc) have an overall neutral effect on unfolded protein response (UPR) in dystrophic *mdx* mice that displayed increased ER stress. However, voluntary running increased oxidative stress in dystrophic muscle (I).
2. Rapid muscle atrophy induced by experimental C26 cancer cachexia alters the redox balance in the oxidative direction, increases markers of autophagy but is not associated with ER stress or UPR (II).
3. Muscle hypertrophy induced by sACVR2B-Fc is accompanied by increased muscle GSH content and activation of UPR. In addition, the alleviation of muscle atrophy by sACVR2B-Fc prevents the cachexia-induced depletion of muscle GSH, but does not have an effect on autophagy markers (II).
4. A bout of resistance exercise induces UPR with a latency of two days in young and older previously untrained men (III). However, muscle hypertrophy induced by resistance training is accompanied by unaltered content of UPR components in young and older men (III).
5. Resistance exercise increases autophagosome content acutely with a latency of two days and after five months of training in previously untrained young but not in older men (III).
6. A new prolonged training stimulus that comprised explosive and maximal strength training may increase autophagosome content and may modulate the content of UPR components in middle aged and older men sprinters despite long training history (IV).

6.1 Muscular dystrophy and cancer cachexia

ER stress and UPR. All three canonical branches of UPR were activated in dystrophic hind limb muscle, suggesting elevated ER stress in *mdx* mice (an animal model for Duchenne muscular dystrophy). This finding of increased muscle ER stress in dystrophic muscle supports previous studies that have reported increased muscle ER stress markers in *mdx* mice and human DMD patients (Moorwood and Barton 2014) as well as in other muscle dystrophies such as myotonic dystrophy type 1 (Botta et al. 2013), sporadic inclusion body myositis (Vattemi et al. 2004) and tibial muscular dystrophy (Screen et al. 2014). In *mdx* mice, the amelioration of ER stress either by crossbreeding an ER-resistant caspase 12 null *mdx* mouse (Moorwood and Barton 2014) or by administering TUDCA, which is a bile acid known to relieve ER stress, showed beneficial effects on muscle function and characteristics (Pauly et al. 2017). Hence, it has been suggested that ER stress is not only associated with DMD, but also contributes to the muscle degeneration process.

Redox balance. In conjunction with ER stress, redox balance and oxidative stress were also assessed in dystrophic muscle because oxidative stress is a potent ER stress inducer (Cao and Kaufman 2014) and has also been associated with DMD pathogenesis (Terrill et al. 2013). The oxidized form of glutathione (GSSG) was upregulated in *mdx* muscle while the reduced form of glutathione (GSH) and protein carbonyls were unchanged. These findings suggest a shift in redox balance in the oxidative direction, which may be one of the ER stress inducers together with other triggering candidates such as aberrant calcium homeostasis (Pauly et al. 2017), increased protein synthesis (MacLennan and Edwards 1990) and the misfolding of mutated dystrophin *per se* in dystrophic muscle (Marshall et al. 2012). However, oxidative damage, at least to proteins, was not upregulated, which is contrary to another study that reported increased protein carbonyl content in three-month-old *mdx* mice that were approximately at the same age as the mice studied in the present thesis (Kaczor et al. 2007).

This thesis also elucidated the role of ER stress in conjunction with redox balance in skeletal muscle in another muscle wasting condition – cancer cachexia. Unlike in dystrophic muscle, ER stress/UPR markers were either unchanged (PERK, IRE1α, GRP78 and *Xbp1s*) or downregulated (HSP47, p-eIF2α and p-JNK) by C26 cancer cachexia at 11 days after the tumor cell inoculation when cancer cachexia had already occurred. The observation that muscle UPR markers are not elevated in C26 tumor-bearing (TB) mice is in line with another study that reported decreased content of p-eIF2α in C26 TB mice (Penna et al. 2010). However, it contradicts two other experimental cancer cachexia models – Lewis lung carcinoma (LLC) TB and $Apc^{Min/+}$ mice (Bohnert et al. 2016) – as well as human lung cancer patients (Aniort et al. 2019) that have exhibited increased muscle ER stress / UPR markers. Notably, the activation of UPR and especially the PERK signaling branch seem to have a protective role against further muscle wasting (Bohnert et al. 2016; Gallot et al. 2018), while the IRE1α branch may mediate the

muscle atrophy during cachexia at least in LLC TB mice (Bohnert et al. 2019). Even though the C26 TB mice did not exhibit increased ER stress in the present thesis, the downregulated UPR markers (HSP47, p-eIF2α and p-JNK) correlated positively with the change in body mass between the last 2 days of the experiment and negatively with the tumor mass (data not shown), suggesting that they were associated with the severity of the cachexia. The reason for the downregulation of muscle UPR markers in C26 TB mice may be due to the decreased protein synthesis that was reported in these same mice (Nissinen et al. 2018). In theory, the downregulated protein synthesis would reduce the necessity for protein-folding capacity in ER. The endpoint in this cancer experiment represented the onset phase of rapid muscle atrophy, so it cannot be ruled out that ER stress is elevated either earlier or later in the cachexia progression in C26 cancer cachexia as well. Due to these discrepant findings, more research on the role of ER stress in cachectic muscle is warranted especially in humans during different stages of cachexia as well as in different cancers.

Of the redox balance markers, the vehicle-treated C26 TB mice manifested decreased muscle GSH content and an increased ratio of oxidized glutathione (GSSG) to GSH (GSSG/GSH), which suggest oxidative stress and depletion of the antioxidant pool, respectively. These findings are in agreement with previous studies that have reported increased oxidative redox balance and depletion of anti-oxidants including GSH in pre-clinical cancer cachexia models (Der-Torossian et al. 2013; Ham et al. 2014). However, as in *mdx* muscle, oxidized proteins were not increased by C26 cancer, which could be explained by increased protein degradation because both autophagy markers in the present thesis as well as markers of the ubiquitin proteasome system previously in these same mice were upregulated (Nissinen et al. 2018). The finding of increased autophagy markers in C26 TB mice is in accordance with other studies with experimental cancer cachexia models (C26 and LLC mice as well as AH-130 hepatoma rats) (Penna et al. 2013; Molinari et al. 2017) and human cachexia patients (Aversa et al. 2016). In addition to autophagy markers, the autophagy flux was also reported to be upregulated in C26 TB mice (Penna et al. 2013), further supporting the pivotal role of autophagy in the muscle atrophy process in cachexia.

6.2 Muscle hypertrophy and alleviation of muscle wasting by blocking activin receptor ligands

Blocking myostatin and activins by soluble activin receptor type 2B (sACVR2B-Fc) was reported to alleviate muscle atrophy (Benny Klimek et al. 2010) and to improve survival in pre-clinical experimental cancer cachexia models (Zhou et al. 2010; Nissinen et al. 2018), yet the underpinning mechanisms remain unclear. In addition, sACVR2B-Fc was reported to increase muscle hypertrophy in wild-type (Lee et al. 2005; Cadena et al. 2010) as well as *mdx* mice (Pistilli et al. 2011; Hoogaars et al. 2012), including the mice studied in the present thesis (Hulmi et

al. 2013a, 2013b). However, blocking the normal signaling of activins and myostatin, which results in substantial and rapid hypertrophy, may also induce adverse side-effects on muscle quality but the underlying mechanisms are not fully known (Amthor et al. 2007; Relizani et al. 2014; Kainulainen et al. 2015). This thesis further elucidated the effects of sACVR2B-Fc administration on protein homeostasis and redox balance markers in dystrophic, cachectic and healthy muscle.

Redox balance. Blocking activin receptor ligands increased muscle GSH content in healthy mice and interestingly prevented the decline of GSH in C26 TB mice. These findings support a previous study that reported increased muscle GSH content in myostatin knockout mice, which was accompanied by increased ROS tolerance, suggesting that the absence of myostatin signaling may have antioxidative effects (Ploquin et al. 2012). However, the increment in GSH by sACVR2B-Fc treatment was not observed in *mdx* mice, which may be explained by the muscular dystrophy or by the difference in the administration duration (7 weeks vs. 2–3 weeks). In addition, the increase in GSH by sACVR2B-Fc may also be a compensatory mechanism to increased oxidative stress because a single dose of sACVR2B-Fc increased protein carbonyls and this increment also persisted after two weeks of blocking in healthy muscle. However, protein carbonyls were unaltered in dystrophic and cachectic muscle by sACVR2B-Fc administration, which may be explained by the increased protein turnover observed previously in dystrophic (MacLennan and Edwards 1990) and cachectic muscle (Nissinen et al. 2018)

ER stress and UPR. Previously, sACVR2B-Fc increased muscle protein synthesis at one and two days after the administration in the healthy mice that were further examined in this thesis (Hulmi et al. 2013a). Despite the increased protein synthesis and protein oxidation observed at these time-points, UPR markers were unchanged. Nevertheless, after the two-week blocking period when the muscle hypertrophy had occurred (Hulmi et al. 2013a), phosphorylated eIF2α at ser51, HSP47 and GRP78 of the UPR markers were upregulated. Of these changed UPR markers, p-eIF2α halts protein synthesis, while the others are ER resident chaperones (Mala and Rose 2010; Hetz et al. 2015). This suggests that the resulting increment in ER-resident chaperones may compensate the increased necessity to fold proteins while p-eIF2α blunts the overall protein synthesis concurrently inhibiting the muscle growth. This latter claim of UPR acting as a break to inhibit muscle growth is supported by previous studies that reported inhibition of protein synthesis and mTOR signaling in C_2C_{12} myogenic cells with elevated ER stress (Deldicque et al. 2010). Furthermore, ER stress markers have been upregulated by another hypertrophy stimulus, synergist ablation, which induces substantial and rapid muscle growth somewhat similarly as sACVR2B-Fc administration (Hamilton et al. 2014). Notably, based on the body mass gain curves in the sACVR2B-Fc administered mice studied in the present thesis, the rapid and linear muscle growth seemed to reach a plateau at day 10 after the first administration (Hulmi et al. 2013a). This suggests that the plateau in muscle hypertrophy may be partially mediated by UPR.

In the two muscle wasting conditions examined in the present thesis (cachexia and dystrophy), sACVR2B-Fc increased the GRP78 of the UPR markers, while the others were not significantly altered. This finding suggests that muscle hypertrophy induced by sACVR2B-Fc is accompanied by increased content of this multifunctional ER resident-chaperone (Wang et al. 2009) ubiquitously during different conditions, to meet the increased demand to fold and process nascent polypeptides. Additionally, even though components of UPR signaling branches were upregulated by muscular dystrophy, sACVR2B-Fc did not further up- or downregulate these altered markers. These findings suggest that sACVR2B-Fc has an overall neutral effect on the upregulated ER stress in dystrophic muscle, except for the increased GRP78 in *mdx* mice.

Autophagy. Blocking activins and myostatin decreased lipidated LC3 (LC3II), which is an indicator of autophagosome content, in the same *mdx* and healthy mice that were further studied in the present thesis (Hulmi et al. 2013b). However, despite these findings in healthy and dystrophic muscles, the upregulated autophagy markers in cachectic C26 TB mice were not altered by the continuous sACVR2B-Fc administration protocol, which has been reported to improve survival and alleviate muscle atrophy (Nissinen et al. 2018). Blocking activins and myostatin, therefore, is not enough to downregulate the pathologically high levels of autophagy during cancer-induced muscle atrophy.

6.3 Exercise training

6.3.1 Voluntary wheel running in muscular dystrophy

In *mdx* mice, the effects of voluntary wheel running alone and combined with sACVR2B-Fc on ER stress/UPR and redox balance were examined. Typically, in healthy organisms repeated bouts of exercise training lead to improved tolerance against oxidative stress (Radak et al. 2008). However, in the *mdx* mice studied in the present thesis, exercise increased GSSG, GSSG/GSH and protein carbonyls, with protein carbonyls being especially increased when running was combined with sACVR2B-Fc administration. This finding suggests increased oxidative stress. Thus, even though voluntary wheel running resulted in beneficial upregulation of oxidative capacity and autophagy markers in these same mice as previously reported (Hulmi et al. 2013b; Kainulainen et al. 2015), the voluntary exercise was probably not fully tolerated due to the observed oxidative stress. In addition, running altered the muscle fiber cross-sectional area towards smaller fibers and did not improve grip strength, which may be partially due to increased oxidative stress.

In previous studies, exercise has either had pro- or anti-oxidative effects on *mdx* muscle. More specifically, 8 weeks of very low intensity treadmill running resulted in a decrease of oxidative stress manifested as decreased muscle protein carbonyl and malondialdehyde content in three-month-old *mdx* mice (Kaczor et

al. 2007). Furthermore, 4 weeks of low-intensity swimming decreased the carbonylation of proteins involved in energy metabolism and muscle contraction, which were identified by proteomics-based analysis (Hyzewicz et al. 2015). However, increased markers of oxidative stress have been reported in two-month-old *mdx* mice after 4 weeks of treadmill running (Schill et al. 2016), in which the intensity was greater than in the study by Kazcor and colleagues (2007). Taken together, these studies suggest that exercise, including voluntary wheel running, may be pro-oxidative in a dystrophic muscle if the exercise intensity and volume are not regulated.

Even though voluntary exercise resulted in increased oxidative stress in *mdx* muscle, it did not further increase or decrease the content of UPR markers. For this reason, it is suggested that voluntary exercise in dystrophic muscle has a neutral effect on ER stress, similarly to sACVR2B-Fc administration. In addition to muscular dystrophies, muscle ER stress has also been reported to be induced by high-fat feeding in mice (Deldicque et al. 2013). Interestingly, 6 weeks of treadmill running was reported to further increase the UPR markers in these mice. UPR markers, it seems, are regulated differently in response to prolonged exercise training in a muscle that manifests increased ER stress depending on the exercise protocol and the origin of the elevated ER stress.

6.3.2 The effects of resistance training in human participants

In addition to voluntary wheel running in dystrophic mice, this thesis examined the effects of resistance exercise (RE) and training (RT) on muscle UPR and autophagy in humans. First, the acute and prolonged effects of resistance exercise were studied in young and older previously untrained men.

6.3.2.1 Unfolded protein response

This thesis showed that the markers of UPR were not systematically induced at 1 h after an unaccustomed heavy RE bout that consisted of 5 x 10 repetitions until failure in young men. However, several UPR markers were induced at 48 h after the RE bout, suggesting that the heavy RE bout was able to disrupt ER homeostasis and, as a compensatory mechanism, UPR signaling was activated to restore the homeostasis. Thus, it seems that part of the muscle remodeling after resistance exercise bout is mediated by UPR signaling. To support this claim, mice lacking ATF6, which is an upstream regulator of UPR, do not recover from repeated exercise bouts and show greatly impaired running performance already after four consecutive days of treadmill running sessions (Wu et al. 2011). The observation of increased UPR after an RE bout is in accordance with a previous study that also reported increased UPR markers in untrained young and older men at one and two days after the RE bout but not acutely at three h after (Ogborn et al. 2014).

The direct age-effect on the magnitude of UPR protein expression was not investigated in the present study due to slightly different homogenization procedures of the biopsies between young and older men. Instead, only relative

changes were independently investigated in both age groups. Nevertheless, in agreement with Ogborn et al. (2014), older men in the present thesis also showed an increase in UPR markers (GRP78 and ATF4) at two days after the RE bout, suggesting that UPR is induced regardless of age in a delayed fashion. However, a recent study reported attenuated expression of several UPR transcripts in older participants (65–85 years) when compared with young participants (18–35 years) at 18 h after an RE bout (Hart et al. 2019). This suggests that UPR signaling in response to acute resistance exercise may be attenuated in very old populations.

Previously, a human study utilizing micro array data and a rodent study have suggested that acute UPR activation is attenuated by chronic exercise training (Wu et al. 2011; Gordon et al. 2012). Indeed, in the present thesis, ATF4 was not induced at 48 h in older men after the RE bout that was conducted after the 21-week RT period, while it was induced before the training period. This suggests that some components of UPR signaling may be induced differently by a bout of RE depending on the training status and may at least in part be a homeostatic response to RE (Damas et al. 2016). Indeed, in the study by Wu and colleagues (2011), *Atf4* mRNA was especially downregulated acutely after an exercise bout that was conducted after a training period, whereas it was induced before the training period (Wu et al. 2011).

Even though several UPR markers were induced by RE at 48 h after the training bout, their content in the resting state was unaltered by the 21-week RT period that increased muscle fiber size in both previously untrained young and older men. It seems that components of UPR signaling as well as ER-resident chaperones increase to a similar extent as other muscle proteins do during RT-induced muscle hypertrophy. This is because these proteins were related to the mean signal of GAPDH and Ponceau S staining, which was not significantly altered by training and muscle hypertrophy.

Comparison of hypertrophy induced by sACVR2B-Fc and RT on UPR. When the two stimuli to induce muscle hypertrophy that were investigated in the present thesis (resistance training and blocking activin receptor ligands) are compared, their effect on UPR components and ER-resident chaperones differ. It seems that resistance exercise induces UPR acutely in a delayed fashion, whereas during muscle hypertrophy achieved by activin receptor ligand blocking, UPR signaling is activated when the substantial muscle hypertrophy has already occurred. This difference may be explained by the difference in the rate of muscle growth that is far more rapid during activin receptor ligand blocking compared with resistance training (Seynnes et al. 2007; Hulmi et al. 2013a) and thus induces greater demand on protein folding machinery in ER. On the other hand, RE-induced stress, that is, mechanical loading and alterations in the metabolic milieu, seem to challenge ER homeostasis to a greater extent than the inhibition of myostatin and activins at a few days after the hypertrophic stimuli (sACVR2B-Fc administration or RE). The finding that UPR components and ER-resident chaperones are not altered in the resting state by prolonged exercise training in previously untrained young and older men is in line with the findings in *mdx* mice in the present thesis.

This thesis also examined the effect of a new long-term training stimulus on muscle UPR in middle-aged and older (40–76 years) men masters sprinters with a long training background and a successful competition history. The new training stimulus comprised of explosive and maximal strength exercises that were integrated into the running-based training regimens. Of the UPR markers, ATF4 content decreased in the EX group, while it was unchanged in the control (CTRL) group that were told to continue their running-based training routines as they were used to. The finding of decreased ATF4 protein in the EX group was somewhat surprising because its upstream regulators where either unchanged (PERK), upregulated (eIF2α) or tended to be upregulated (p-eIF2α). The change in ATF4 content thus occurred without downregulation of its upstream regulators, at least at the time-point when the muscle biopsies were collected. Similar discrepant findings of PERK signaling branch components were also observed in the livers of C26 TB mice studied in this thesis. (Note: liver results were not included). More specifically, decreased ATF4 content was observed even though PERK and p-eIF2α were concomitantly upregulated.

The finding of decreased ATF4 in the EX group could be considered as positive adaptation because ATF4 has been associated with muscle atrophy (Ebert et al. 2010, 2015; Aniort et al. 2019) and it may depict decreased basal ER stress. Previously, *Atf4* mRNA and GRP78 protein have been reported to be downregulated in an exercise intensity and volume-dependent fashion (Kim et al. 2014). In more detail, these UPR markers were only downregulated when the rats exercised with high intensity (80%–85% VO_2 max), which also resulted in a greater training volume compared with the low-intensity training regimen (50%–55% VO_2 max). Based on this finding, it is suggested that the difference in UPR component changes after a prolonged training period in masters athletes compared with untrained participants may be explained by a greater total training volume in masters athletes (2 times per week vs. 4 times per week) even though the strength training was performed twice per week in both training programs. In addition, the difference in the type of strength training may also explain the different results because the training programs had different aims and outcomes, for example, on muscle hypertrophy. Taken together, the findings in the present thesis combined with the previous literature suggest that prolonged exercise has an overall neutral effect on UPR markers at a resting state, but some UPR components may change depending on the training volume and intensity.

6.3.2.2 Autophagy

In conjunction with UPR induced by ER stress, this thesis examined the effects of resistance and strength exercise on muscle autophagy markers. In previously untrained young men, the RE bout resulted in decreased LC3II content acutely at 1 h after the exercise bout, which was accompanied by decreased phosphorylated ULK1 at ser555. These results suggested decreased autophagosome content and decreased autophagosome formation, respectively (Kim et al. 2011b; Klionsky et al. 2016). The decrease in muscle autophagosome content as well as the downregulation in autophagy precursor formation at this time-point may be explained

by increased mTOR signaling, which was observed previously in these participants (Hulmi et al. 2009b), because mTOR is able to inhibit the ULK1 complex that mediates the initiation of autophagosome precursor formation (Kim et al. 2011b). The decrease in LC3II content acutely after an RE bout is in accordance with previous studies on human resistance exercise that have reported either decreased (Fry et al. 2013; Dickinson et al. 2017) or unchanged (Glynn et al. 2010) LC3II content in the first few h following a single RE bout.

Like the UPR induction at 48 h after the RE bout, several autophagy markers were upregulated at this time-point, including LC3II, LC3I, Beclin-1 and P62. These results collectively suggest that an unaccustomed RE bout not only increases autophagosome content (LC3II) but also increases the protein content of several proteins that are involved in the regulation of autophagy process (P62 and Beclin-1). The increase in autophagy markers, and especially in P62 and LC3II a few days after the RE bout, supports a previous study, in which total LC3B as well as P62 increased at 48 h after a bout of RE (Ogborn et al. 2015). The primary reason for the autophagy induction a few days after an RE bout can only be speculated. Nevertheless, it may be a homeostatic response to the RE-induced damage on muscle protein structures and organelles that consequently are degraded by the autophagy-lysosome pathway. To indirectly support this claim, a maximal eccentric resistance exercise has been reported to disrupt Z-disc orientation, which was accompanied by a co-localization of LC3II with filamin C, a protein that resides at Z-discs and unfolds upon mechanical loading (Ulbricht et al. 2015).

In young men, the LC3II content was also upregulated in the a resting state after the 21-week RT period suggesting increased autophagosome content that either results from increased autophagosome formation or a reduction in their degradation. In humans, it is currently not possible to kinetically measure the rate of autophagosome removal, that is, the autophagy flux. However, this flux is commonly indirectly assessed by measuring P62 content, which is a protein linking the autophagic cargo and autophagosomes and is degraded in lysosomes with the disposable cargo during the autophagy process. Typically, the simultaneous increase in P62 with LC3II is suggested to be an indicator of decreased autophagosome removal (Bjorkoy et al. 2009). Because the P62 protein content was unaffected and BCL-2, which is a negative regulator of autophagosome formation (Pattingre et al. 2005), was downregulated by the RT period, it is suggested that prolonged resistance training resulting in muscle hypertrophy is accompanied by increased autophagosome content without any evidence of impairment in the autophagosome degradation. Thus, in addition to UPR markers, the RT and sACVR2B-Fc induced muscle hypertrophy have a different outcome on the autophagosome content as well, because LC3II was previously reported to decrease by sACVR2B-Fc administration (Hulmi et al. 2013a)The physiological meaning of the increased autophagosome content by resistance training can only be speculated. However, given that autophagy is involved in the recycling of dysfunctional organelles and proteins (Masiero et al. 2009; Sandri 2010), increased autophagosome content may be interpreted as an improved capacity to

recycle dysfunctional cellular constituents that are formed during exercise and normal metabolism. In parallel with the findings of the present thesis, components of chaperone-assisted selective autophagy (CASA) have been reported to increase after four weeks of resistance training in young participants (Ulbricht et al. 2015). Furthermore, 8 weeks of endurance training resulted in increased LC3I but not LC3II content in moderately trained young men subjects, while the authors interpreted the results as improved autophagy capacity (Brandt et al. 2018). In addition, rodent studies have indicated that chronic endurance training increases LC3II (Lira et al. 2013) and also the flux of autophagy is increased by chronic contractile activity (Carter et al. 2018) and a period of long-term swimming training (Ju et al. 2016).

Even though the autophagy markers were increased by resistance exercise and training in young previously untrained men, none of the measured autophagy markers were systematically altered in older previously untrained men at any time-point. This finding suggested that aging may interfere with the muscle's capacity to induce autophagy in response to RE. However, previous studies have not reported age-related changes in autophagy markers acutely after a single bout of resistance exercise (Fry et al. 2013; Ogborn et al. 2015), and a period of long-term RT has been suggested to increase basal autophagy in older rats, but the flux was not directly measured (Luo et al. 2013). In addition, cross-sectional studies have suggested that life-long participation in ball games and endurance exercise have prevented the age-related decline in autophagy markers (Carnio et al. 2014; Mancini et al. 2019). Due to these discrepant results, the effect of prolonged strength training on muscle autophagy was further studied in this thesis in older (40–76 years) men masters sprinters. Interestingly, the prolonged experimental training, in which maximal and explosive strength exercises were integrated into running-based training routines, increased LC3II and decreased P62, suggesting increased content of autophagosomes again without any evidence of impaired autophagosome removal. In addition, TFEB, which is a transcription factor that regulates, for example, lysosomal biogenesis (Settembre et al. 2011), was increased in the EX group after the intervention period. This finding further suggested that the components of the autophagy lysosome pathway may be increased in the resting state by prolonged strength training that also improved sprinting performance. In addition, the change in autophagy markers (LC3II and P62) did not correlate significantly with age in masters athletes (Note: the correlations are not shown in this thesis), suggesting that the increase in components of autophagy in response to prolonged strength training is preserved in a relatively wide age-spectrum and despite a long-term training history. Taken together, prolonged resistance or strength training seems to increase basal autophagosome content and this effect may be more consistent in younger individuals. However, due to the limitations in the study designs used in this thesis (see 6.4), more research is needed to confirm these results and also to elucidate the underlying mechanisms. This could be achieved by creating a study design which would enable the straight comparison of young and older men in response to acute and prolonged training.

6.4 Strengths and limitations

This thesis comprehensively examined the role of UPR induced by endoplasmic reticulum stress and autophagy in skeletal muscle in conjunction with redox balance during conditions that lead to changes in muscle quality and size. Regarding muscle hypertrophy, the strength of this thesis is that it examined both pharmacologically induced muscle hypertrophy as well as resistance training induced muscle hypertrophy. For muscle atrophy, experimental cachexia as well as muscle dystrophy in conjunction with possible treatment modalities were examined, a further strength of this thesis. In addition, in regards to resistance exercise experiments, this thesis examined both the acute and prolonged effects in different populations that differed in age and training status. Furthermore, the resistance training periods in all experiments were sufficient (approximately 5 months) to observe long-term training adaptations. Notably, the major novel findings in the present thesis were the prolonged effects of resistance and strength training on the markers of UPR and autophagy in humans.

This thesis provided a comprehensive and broad overview of how different UPR and autophagy markers are regulated at the protein and mRNA level in muscle during hypertrophy, atrophy and exercise. However, the methods used also have their limitations and only the changes in total mRNA or protein content together with some post-translational modifications were measured. To improve this thesis, the immunostaining of muscle cross sections could have provided more information about the LC3II or P62 localization in the muscle after the acute RE and RT period. Based on this information, one could have observed which structures are targeted by autophagy in response to RE and RT. In addition, measuring mRNA levels of P62 would have enabled more comprehensive interpretation of the decreased P62 protein content observed in masters sprinters. Furthermore, measuring enzyme activities of lysosomal proteases in response to prolonged resistance training would have also been additive to this thesis.

In cancer cachexia, autophagy flux could have been measured in addition to markers of autophagosome content and regulation. Redox balance and oxidative stress were assessed in this thesis only based on glutathione metabolites and protein carbonyls. Given that redox balance regulation is multifactorial and oxidative damage also targets other cellular constituents in addition to proteins, the conclusions regarding oxidative stress would have been stronger if these processes were investigated more diversely.

The major limitation in the experiments involving young and older previously untrained men was that only a few biopsy samples from controls were available for the acute RE protocol. Thus, the possible residual effects of repeated biopsy obtainment at 48 h (biopsy obtained approximately 3 cm above the PRE biopsy) could not be thoroughly assessed as in other studies published on autophagy and UPR in humans (Ogborn et al. 2014, 2015). In addition, the effect of possible normal day-to-day fluctuation in, for example, autophagy markers in

long-term adaptations could not be fully addressed. On the other hand, participants in the control group might also change their behavior during the long intervention period, which might explain some small changes observed in the controls. In the masters athletes experiment, for example, the training in both groups was not fully supervised, even though both groups completed training logs and the only significant difference in the reported training mode specific volumes between the groups was in strength training. It may be that some of the competitive masters athletes belonging to the control group also changed their training routines during the intervention period knowing that the experimental group did so, which might have increased variation in the results. In addition, the POST measurements were close to the competition period whereas the PRE measurements were in training period and thus the type of training preceding the PRE and POST measurements most probably differed in CTRL group. These above described factors may increase variance to results of the CTRL group and may explain why there was no difference between groups in most of the variables even though a systematic and significant difference was observed in the EX group but not in the CTRL group. Additionally, bigger sample size would have enabled more accurate direct comparison between the EX and CTRL group.

In the human experiments of this thesis, previously collected and homogenized muscle samples were analyzed based on muscle sample availability. These biopsies were homogenized with slightly different protocols. Thus, only relative changes were independently examined in young and older previously untrained men, which removed the direct comparison between the different age groups. In addition, the relatively small sample size in older previously untrained men (6–8) may have been underpowered for a reliable straight comparison.

For muscle wasting experiments, the limitations in these experiments was that only one time-point was assessed and thus the effect of the muscle wasting process itself on muscle protein homeostasis could not be fully addressed. In addition, exercise in *mdx* mice was voluntary running on a wheel, which may not fully represent exercise in dystrophic humans. In addition, the pathologic conditions were limited to experimental animal models because conducting these experiments in humans is still currently very challenging. Finally, in cancer experiments, the primary treatment of the tumor was not included.

6.5 Future directions

Accumulating evidence indicates that the biological processes that control protein homeostasis have an important role for muscle function. This thesis elucidated further that UPR and autophagy of these processes are modulated during muscle hypertrophy, atrophy and exercise. However, the findings of this study were mostly based on gene and protein expression data that cannot fully depict, for example, the rate of autophagosome formation and turnover. In addition, the rate of protein misfolding in endoplasmic reticulum, that is, ER stress could not be measured. Instead, ER stress was assessed by measuring the gene and protein expression of

components that are involved in its compensatory stress response (UPR) and chaperones that reside in ER. New methods to investigate ER stress and autophagy in a more kinetic fashion, especially in humans, would thus provide more accurate evidence of their function in muscle during different conditions.

Regarding the human exercise studies, this thesis focused on resistance training on previously untrained populations and strength training combined with sprint training in older trained populations, while the effect of other exercise modalities was not assessed. Thus, future studies should examine the effects of other exercise modalities with an emphasis on long-term adaptations, because there is more existing data regarding acute effects of, for example, endurance exercise on muscle UPR and autophagy (Martin-Rincon et al. 2017; Estebanez et al. 2018). Particularly interesting would be longitudinal studies, in which the effects of different exercise modalities (e.g. resistance and endurance exercise) could be studied in parallel and compared. This would enable the deduction of whether the long-term adaptations of exercise on, for example, autophagosome content are specific to the training mode. In addition, different training modalities such as volumes and intensities should be elucidated. Furthermore, the interaction of different nutritional states and gender differences would be important areas of future research in humans.

On average, the aging populations studied in this thesis were not very old (approximately 60 years). To further elucidate the impact of aging on muscle proteostasis adaptability in response to exercise, people older than 70 years should be studied because the muscle function starts to decline in an accelerative manner at the age of 70 (Reaburn and Dascombe 2008).

Regarding the muscle atrophy conditions, only data from pre-clinical animal models was examined. Given that C26 tumor–bearing and *mdx* mice do not completely reflect human cachexia and Duchenne muscular dystrophy, respectively, the observations found in these animal models should be recapitulated in humans. The recombinant protein that was administered to block activin receptor ligands may not be perfectly suitable, because one clinical trial had to be terminated due to non-muscle related side-effects (Campbell et al. 2017). Therefore, other compounds having a more muscle-specific effect should also be examined in the future regarding their effect on muscle protein homeostasis.

Finally, muscle proteostasis and especially the specific function of different components regulating these integrated processes are still far from being completely understood. For this reason, many gain- and loss-of-function studies on animal and cell culture models would be called for in the future. Consequently, the relevance of these findings needs to be recapitulated in humans. The comprehensive and profound understanding of the proteostasis network would enable an optimal treatment in different conditions that would result in better tissue function and ultimately more wellbeing.

7 MAIN FINDINGS AND CONCLUSIONS

Endoplasmic reticulum stress and the activation of its compensatory response (unfolded protein response, UPR) can be induced by muscular dystrophy that is a devastating condition leading to severe impairment in muscle function – as well as by a stimulus that leads to a beneficial increase in muscle strength and mass, in other words, by resistance exercise (RE). However, in contrast to prolonged and unresolved ER stress in muscular dystrophy, the activation of UPR following a single RE bout is transient and capable of restoring homeostasis regardless of age in healthy individuals. Thus, the repeated RE bouts leading to muscle hypertrophy are not accompanied by increased UPR in the basal state. However, a completely different hypertrophic stimulus, activin receptor ligand blocking, results in UPR activation later when muscle hypertrophy has occurred, showing a difference in these two hypertrophic stimuli. In addition, although exercise has many positive effects, it may also have some negative effects on dystrophic muscle such as increased oxidative stress even if exercise is voluntary and conducted with light intensity.

This thesis also showed that autophagy can be regulated somewhat similarly during rapid muscle atrophy and as an adaptation to a new acute and prolonged stress stimulus – resistance exercise, because in both conditions lipidated LC3 (LC3II) increased indicating increased muscle autophagosome content. However, it is postulated that in response to a single bout of resistance exercise and exercise training, the increase in autophagosome content reflects a homeostatic response in order to recycle damaged cellular compartments, whereas during cachexia, the increased autophagosome content results from pathologically high muscle protein and organelle degradation in which autophagy plays a contributing role. However, the increment in autophagy markers in response to resistance exercise was not observed in previously untrained older men, which may be explained by aging. More studies comparing young and older participants will possibly confirm whether the autophagic response to exercise training is blunted in aging muscle and its underlying mechanisms.

Myostatin/activin receptor ligand blocking has shown promising effects in preclinical animal models of cancer cachexia and to some extent also on muscle

dystrophy. The results of this thesis suggest that the underlying mechanisms of this therapeutic protein are not mediated through alterations in ER stress or autophagy. Thus, other strategies to treat muscle that results in the relief of ER stress in dystrophic muscle and inhibits pathologically high levels of autophagy in cachectic muscle need to be developed. However, the significance of a possible antioxidative effect of blocking activin receptor ligands in cachectic muscle warrants further studies in which oxidative stress is more thoroughly assessed and compared with other antioxidative treatments.

As a conclusion, the results of this thesis show that as an adaptation to muscle hypertrophy, wasting and exercise, unfolded protein response and autophagy are regulated distinctly in skeletal muscle depending on the context. The results of this thesis expand the current knowledge of muscle physiology at the molecular level that may be applied in the future for example to develop new strategies to treat muscle wasting. In addition, it provides new evidence how muscle responds to a new acute and prolonged exercise stimulus and in particular resistance exercise which may be applied when planning and offering evidence based exercise recommendations.

TIIVISTELMÄ (FINNISH SUMMARY)

Luustolihas on mukautuva kudos, joka ei pelkästään vastaa meidän liikkumisestamme. Se osallistuu myös esimerkiksi lämmönsäätelyyn ja ehkäisee aineenvaihdunnallisten sairauksien kuten aikuisiän diabeteksen syntyä. Lisäksi riittävä lihasmassa ja -voima ovat yhteydessä pienempään kuolleisuusriskiin ja parempaan selviytymistodennäköisyyteen katastrofitilanteista, jotka vaativat pidempää vuodelepoa. Lisäksi vanhuksilla riittävä lihasvoima mahdollistaa itsenäisen elämäntyylin. Lihasmassalla ja -voimalla on tärkeä rooli liikkumiskyvyn, hyvinvoinnin ja terveyden kannalta. Siksi olisi tärkeä ymmärtää lihaksen toimintaa säätelevät biologiset mekanismit eri tilanteissa, jotka vaikuttavat lihaksen laadullisiin muutoksiin ja kokoon. Näitä tilanteita ovat esimerkiksi lihaskasvu, lihaskato ja liikunnan aikaansaamat muutokset lihaksessa. Perusteellisen tiedon pohjalta voitaisiin kehittää tapoja hoitaa potilaita lihaskatotilanteissa sekä antaa näyttöön perustuvia liikunta- ja harjoittelusuosituksia eri väestöryhmille.

Lähes kaikkia kehomme toimintoja, mukaan lukien lihasten toimintaa, säätelevät proteiinit, jotka koostuvat pääasiassa aminohappojen muodostamista pitkistä ketjuista. Lihaksen toiminnan kannalta on tärkeää, että proteiinit toimivat oikein. Tämä vaatii sen, että aminohappoketjut asettuvat oikeanlaiseen kolmiulotteiseen järjestykseen. Tätä järjestäytymistä kutsutaan laskostumiseksi. Mikäli proteiinit laskostuvat väärin, ne saattavat kasaantua soluun ja olla haitallisia niiden toiminnan kannalta. Suuri osa solujen proteiineista laskostuu solulimakalvostolla (endoplasmic reticulum). Jos proteiinit laskostuvat väärin, siitä seuraa stressitila soluille. Tätä stressitilaa varten soluihin on kehittynyt stressivaste (unfolded protein response, UPR), joka pyrkii vähentämään proteiinien väärinlaskostumista. Mikäli stressi ei mene ohi, solun toiminta saattaa häiriintyä ja viimeisenä vaihtoehtona on ohjattu solukuolema. Väärinlaskostumisen lisäksi proteiinit voivat vahingoittua, jolloin niiden rakenne saattaa muuttua ja toiminta heikentyä. Tällöin niistä saattaa tulla haitallisia solun toiminnan kannalta. Proteiinien hajotusta varten on kehittynyt erilaisia biologisia järjestelmiä, joista erittäin tärkeä solujen toiminnan kannalta on autofagia. Se ei hajota ja kierrätä pelkästään proteiineja, vaan myös soluelimiä.

Viimeaikaisten tutkimusten mukaan autofagialla ja UPR-reitillä on erittäin tärkeä rooli lihaksen toiminnan kannalta. Esimerkiksi geenimanipuloiduilla jyrsijöillä tehdyissä tutkimuksissa näiden reittien toiminnan estäminen lihaksessa heikensi lihasvoimaa. Lisäksi jyrsijät eivät joko palautuneet liikuntaharjoittelusta normaalilla tavalla tai niiden lihaksissa ei tapahtunut samanlaisia suorituskykyä parantavia muutoksia kuin terveessä lihaksessa. Nämä kaksi proteiinien laadusta vastaavaa järjestelmää näyttäisivät osallistuvan lihaskadon säätelyyn lihasdystrofioissa sekä syöpään liittyvässä rajussa painonlaskussa (kakeksia). Näiden ilmiöiden tuntemus eri tilanteissa, jotka vaikuttavat lihaksen toimintaan ja laatuun, on kuitenkin vielä rajallista ja tarvitsee lisää tutkimusta. Tämän takia tämän väitöskirjan tarkoituksena oli tutkia liikunnan sekä lihasten surkastumisen ja kasvun vaikutuksia UPR-reittiin ja autofagiaan luustolihaksessa.

Hiirillä tutkittiin lihaksen toimintaa merkittävästi heikentävän geneettisen sairauden (Duchennen lihasdystrofia) vaikutuksia sekä syöpään liittyvää rajua lihaskatoa eli kakeksiaa. Lisäksi tarkasteltiin biologisen lääkkeen (liukoinen aktiviinireseptori) aikaansaaman lihaskasvun vaikutuksia sekä terveessä että lihaksen toimintaa rappeuttavissa tilanteissa (lihasdystrofia ja syöpään liittyvä kakeksia). Lihasdystrofiasta kärsivillä hiirillä tutkittiin myös juoksupyöräjuoksun itsenäistä ja yhdistettyä vaikutusta lihasmassaa lisäävän biologisen lääkkeen kanssa.

Koe-eläinmallien lisäksi tutkittiin voimaharjoittelun akuutteja (1 ja 48 tuntia harjoituksen jälkeen) ja pitkäaikaisia (21 viikkoa harjoittelua) vaikutuksia nuorilla (26 ± 4 vuotta) ja vanhemmilla miehillä (61 ± 6 vuotta). Näillä koehenkilöillä ei ollut aiempaa voimaharjoittelutaustaa. Tämän lisäksi veteraanimiespikajuoksijoilla (40–76 vuotta) tutkittiin pikajuoksuharjoittelun rinnalle lisätyn pitkäaikaisen (20 viikkoa) voima- ja nopeusvoimaharjoittelun vaikutuksia.

Tämän väitöskirjan perusteella havaittiin, että proteiinien väärinlaskostumisesta solulimakalvostolla johtuva stressivaste (UPR-reitti) oli koholla lihasdystrofiassa sekä kaksi vuorokautta yksittäisen voimaharjoituksen jälkeen nuorilla ja vanhemmilla miehillä. Terveillä hiirillä biologisella lääkkeellä aiheutettu nopea lihaskasvu sai aikaan UPR-reitin aktivoitumisen. Se ei kuitenkaan ollut kohonnut pidemmän voimaharjoitusjakson jälkeen, jolla saavutettiin lihaskasvua nuoremmilla ja vanhemmilla miehillä. Tämän perusteella voimaharjoittelun ja biologisen lääkkeen aikaansaamalla lihaskasvulla näyttäisi olevan erilainen vaikutus UPR-reittiin. Lisäksi yksittäisen voimaharjoituksen aikaansaama UPR-reitin aktivoituminen näyttäisi olevan väliaikaista. Tällä saattaa olla rooli lihaksen mukautumisessa voimaharjoitteluun.

Lisäksi havaittiin, että autofagiaprosessiin liittyvien hajotuskoneistojen (autofagosomien) määrä lisääntyi syöpäkakeksiaan liittyvässä lihaskadossa. Näiden koneistojen määrä lisääntyi myös pidempiaikaisen voimaharjoittelun jälkeen nuorilla miehillä ja veteraanipikajuoksijoilla. Vanhemmilla aiemmin harjoittelemattomilla miehillä autofagosomien määrä ei kuitenkaan lisääntynyt voimaharjoittelun seurauksena. Näin ollen ikääntyminen saattaa vaikuttaa siihen, miten lihaksen autofagiakoneisto mukautuu voimaharjoitteluun. Voimaharjoittelun aikaansaamaa autofagiakoneistojen määrän lisääntymistä voidaan pitää hyödyllisenä, koska tällöin vaurioituneen ja täten haitallisen solumateriaalin kierrätys toimii tehokkaammin. Tällä mukautumisella saattaa olla positiivinen vaikutus lihaksen toiminnan kannalta. Syöpään liittyvässä lihaskadossa autofagiakoneiston määrän lisääntyminen näyttäisi kuitenkin osallistuvan lihaskatoprosessia lisäävänä tekijänä.

Tämän väitöskirjan tulokset osoittavat, että proteiinien toimintaan osallistuvat UPR-reitti ja autofagia aktivoituvat lihaksessa liikunnan, lihaskasvun ja -kadon seurauksena eri tavoin tilanteen mukaan. Tämän väitöskirjan tuloksia voidaan hyödyntää esimerkiksi liikuntasuositusten laatimisessa, pohjana uusien lihaskatohoitomuotojen kehittämisessä ja uusien tutkimushypoteesien luomisessa.

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ORIGINAL PAPERS

I

EFFECTS OF MUSCULAR DYSTROPHY, EXERCISE AND BLOCKING ACTIVIN RECEPTOR IIB LIGANDS ON THE UNFOLDED PROTEIN RESPONSE AND OXIDATIVE STRESS

by

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Original article

Effects of muscular dystrophy, exercise and blocking activin receptor IIB ligands on the unfolded protein response and oxidative stress

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ABSTRACT

Protein homeostasis in cells, proteostasis, is maintained through several integrated processes and pathways and its dysregulation may mediate pathology in many diseases including Duchenne muscular dystrophy (DMD). Oxidative stress, heat shock proteins, endoplasmic reticulum (ER) stress and its response, i.e. unfolded protein response (UPR), play key roles in proteostasis but their involvement in the pathology of DMD are largely unknown. Moreover, exercise and activin receptor IIB blocking are two strategies that may be beneficial to DMD muscle, but studies to examine their effects on these proteostasis pathways are lacking. Therefore, these pathways were examined in the muscle of *mdx* mice, a model of DMD, under basal conditions and in response to seven weeks of voluntary exercise and/or activin receptor IIB ligand blocking using soluble activin receptor-Fc (sAcvR2B-Fc) administration. In conjunction with reduced muscle strength, *mdx* muscle displayed greater levels of UPR/ER-pathway indicators including greater protein levels of IRE1α, PERK and *Atf6b* mRNA. Downstream to IRE1α and PERK, spliced *Xbp1* mRNA and phosphorylation of eIF2α, were also increased. Most of the cytoplasmic and ER chaperones and mitochondrial UPR markers were unchanged in *mdx* muscle. Oxidized glutathione was greater in *mdx* and was associated with increases in lysine acetylated proteome and phosphorylated sirtuin 1. Exercise increased oxidative stress when performed independently or combined with sAcvR2B-Fc administration. Although neither exercise nor sAcvR2B-Fc administration imparted a clear effect on ER stress/UPR pathways or heat shock proteins, sAcvR2B-Fc administration increased protein expression levels of GRP78/BiP, a triggering factor for ER stress/UPR activation and TxNIP, a redox-regulator of ER stress-induced inflammation. In conclusion, the ER stress and UPR are increased in *mdx* muscle. However, these processes are not distinctly improved by voluntary exercise or blocking activin receptor IIB ligands and thus do not appear to be optimal therapeutic choices for improving proteostasis in DMD.

Abbreviations: AcvR2B, activin receptor IIB; sAcvR2B-Fc, ligand blocking using soluble activin receptor-Fc; AMPK, 5′ adenosine monophosphate-activated protein kinase; CSA, cross-sectional area; DMD, Duchenne muscular dystrophy; *mdx*, DMD mouse model; eIF2α, eukaryotic initiation factor 2 subunit α; ER, endoplasmic reticulum; GPX, glutathione peroxidase; GRD, glutathione reductase; GRP78, glucose regulated protein 78; GSEA, gene set enrichment analysis; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione S-transferase; HSP, heat shock protein; IRE1α, inositol-requiring enzyme 1α; PDI, protein disulfide isomerase; PERK, protein kinase R-like ER protein kinase; ROS, reactive oxygen species; SDH, succinate dehydrogenase; TPOR, thiol protein oxidoreductase; TRX, thioredoxin; TxNIP, thioredoxin-interacting protein; UPR, unfolded protein response; XBP1, X-box binding protein 1

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1. Introduction

Duchenne muscular dystrophy (DMD) is a disease characterized by progressive wasting of skeletal muscle [1]. The absence of functional dystrophin is a major reason for perturbations to cellular changes including abnormal Ca^{2+} homeostasis, inflammatory cell infiltration, fibrosis, necrosis, regeneration [2] and in turn protein homeostasis (proteostasis) in DMD and its animal model, *mdx* mice. The restoration of dystrophin expression in all of the muscles and for all of the different mutations is currently unattainable [3]. Therefore, other strategies are being developed that may complement dystrophin restoration approaches.

Oxidative stress is a disruption of thiol redox circuits that results in impaired cell signaling and dysfunctional redox-control [4,5]. It is linked to several pathological processes including dysfunction of proteostasis and the accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER), resulting in ER stress [6]. Notably, secondary consequences of dystrophin deficiency include the loss of skeletal muscle calcium homeostasis and hypoxia [7,8] as well as deficiency in nitric oxide synthase NOS [2] that can trigger oxidative stress [9,10] and in theory, ER stress. Furthermore, accumulation of improperly folded dystrophin in *mdx* mice [11] may also cause ER stress. The unfolded protein response in ER (UPR_{ER}) resolves ER stress and consists of several branches of signaling pathways aimed to recover proteostasis by increasing the protein folding machinery (chaperones), suppressing the overall translation of proteins and increasing the ER associated protein degradation (ERAD) [12]. Additionally, mitochondrial UPR (UPR_{mt}) [13] and cytoplasmic chaperones including heat shock proteins (HSP) [14] prevent accumulation of unfolded or incorrectly folded proteins. When ER stress is too severe or chronic, or the UPR and HSP responses are impaired and unable to cope with the protein-folding defects needed to maintain proteostasis, pro-apoptotic signaling pathways are activated in the cell [12]. Indeed, a recent study showed that glucose regulated protein 78 (GRP78/BiP), which is a triggering factor for ER stress/UPR activation, was associated with ER-related apoptosis signaling in human DMD muscle and/or *mdx* mice [15]. A more thorough understanding of these processes in muscular dystrophy would provide further insight into the role these factors may play in mediating the disease pathology in order to develop new therapeutic tools.

Type IIb activin receptor (AcvR2B) ligands myostatin and activins inhibit muscle hypertrophy [16,17]. Blockade of AcvR2B ligands can be achieved, e.g. by using the soluble ligand binding domain of type IIb activin receptor fused to the Fc domain (sAcvR2B-Fc) to effectively increase muscle size [18–21]. Blocking these proteins using various strategies has been shown to attenuate dystrophic pathology of the *mdx* mouse in some [18,22], but not in all studies [20,23]. However, the effect of AcvR2B ligand blocking on ER stress and UPR in dystrophic muscle is currently unknown.

Muscular dystrophy is associated with a reduced skeletal muscle oxidative capacity [24]. Exercise improves muscle oxidative capacity in *mdx* mice [25,26], which as an adaptation could increase resistance to the dystrophic pathology [7]. Exercise training may decrease markers of oxidative stress in *mdx* mice, but this response may depend on the dose, type, intensity and duration of exercise, and possibly the disease status [9,10]. Decreased levels of oxidative stress would be beneficial since ER stress and oxidative stress can work in a positive feed-forward loop in a manner that disrupts cell function and induces pro-apoptotic signaling [6,27]. Therefore, the performance of regular, tolerable exercise alone or in combination with other therapeutic tools may positively modulate pathways involved in proteostasis that could alleviate skeletal muscle pathologies.

The overall purpose of this experiment was twofold. One purpose was to investigate for the first time the effects of muscular dystrophy on oxidative stress concurrently with ER stress, UPR and HSP defense. The second purpose was to examine these same physiological states in response to AcvR2B ligand blocking and voluntary exercise training as these interventions may elicit beneficial effects on *mdx* muscle by altering muscle proteostasis.

2. Materials and methods

2.1. Animals

Six- to seven-week-old male *mdx* mice and C57Bl/10ScSnJ controls originating from the same strain (Jackson Laboratories, Bar Harbor, Maine, USA) were used in the experiments. The mice were housed under standard conditions (i.e., 22 °C, 12 h light:dark cycle) and had free access to tap water and food pellets (R36; 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labfor, Stockholm Sweden).

The treatment of animals was in strict accordance with the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes. The protocol was approved by the National Animal Experiment Board (Permit Number: ESLH-2009-08528/Ym-23).

2.2. Experimental design

Two experimental designs were used in this investigation: 1) effect of the *mdx* phenotype on ER stress/UPR and oxidative stress; and 2) exercise and blocking activin receptor ligands on the same parameters in *mdx* mice. In the first experiment, *mdx* mice (n=8) and wild type mice from the same strain (C57Bl/10ScSnJ) (n=5) were compared. In the second experiment, 7-week old *mdx* mice were randomly divided into four groups in a 2×2 design (n=8 animals/group): 1) sedentary control injected with PBS (vehicle); 2) running wheel and injection with PBS; 3) sedentary injected with sAcvR2B-Fc; and 4) running wheel and injection with sAcvR2B-Fc. sAcvR2B-Fc (5-mg/kg) or PBS was injected intraperitoneally once per week for seven weeks with or without voluntary wheel running exercise. To allow treatments to take effect, the mice were prevented from exercising by locking the running wheels for two days at the start of the experiment. In order to study only long-term effects of exercise the mice did not have access to running wheels on the last two days of the experiment. During the experiments all the conditions were standardized. At ~14 weeks of age all the mice were euthanized by cervical dislocation and muscle samples were collected. Forelimb grip strength was measured the day before the sacrifice using the protocols of TREAT-nmd (web-link: http://www.treat-nmd.eu/downloads/file/sops/dmd/MDX/DMD_M.2.2.001.pdf). The measurements were conducted five times with the highest score (absolute force) taken as the final result.

2.3. sAcvR2B-Fc production

The recombinant fusion protein was produced and purified *in house* as described previously [19]. The protein is similar, but not identical with that originally generated by Se-Jin Lee [21]. In short, the fusion protein contains the ectodomain (ecd) of human sAcvR2B and a human IgG1 Fc domain. The protein was expressed in Chinese hamster ovary (CHO) cells grown in suspension culture.

2.4. Voluntary wheel running

The mice were housed in cages where they had free access to custom-made running wheels (diameter 24 cm, width 8 cm) 24 h/

day. Sedentary animals were housed in similar cages without the running wheel.

2.5. Muscle Immunohistochemistry

Gastrocnemius muscle cross-sections were cut using a cryomicrotome and stained for membrane (caveolin 3 (ab2912, Abcam, UK), dilution 1:100) and captured with Olympus BX-50 fluorescent microscopy. 10x magnification was used and the average fiber number in randomly selected fields of high quality was 342.8 ± 30.5 fibers per section. Image analysis of fiber CSA was performed with specific software (ImageJ, U.S. National Institutes of Health, Bethesda, MD, USA).

2.6. Muscle sampling and homogenization

The gastrocnemius was immediately removed from the hind limb, weighed, and frozen. The muscle was pulverized and one-half of the powder was allocated for protein analysis and one-half to RNA isolation. The powder for protein analysis (except enzyme analysis) was homogenized in ice-cold buffer with protease inhibitors (Pierce Biotechnology, Rockford, IL, USA) and total protein measured using the bicinchoninic acid protein assay (Pierce Biotechnology) with an automated KoneLab analyzer (Thermo Scientific, Vantaa, Finland).

2.7. Western immunoblot analyses

Western blots were conducted in two laboratories and thus two slightly different protocols are provided below. Unless otherwise stated, all chemicals and reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) and were of analytic grade or the highest grade available.

2.7.1. PDI, IRE-1α, PERK, p-eIF2α/eIF2α, Sirtuins, AMPK, lysine acetylation

Muscle homogenates mixed in Laemmli sample buffer were heated at 95 °C to denature proteins and processed as described previously [19,25]. In short, protein was separated by SDS-PAGE and transferred to a PVDF membrane, blocked and incubated overnight at 4 °C with primary antibodies. The membrane was then washed and incubated with secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 h followed by washing. Proteins were visualized by ECL (SuperSignal west femto maximum sensitivity substrate, Pierce Biotechnology, Rockford, IL, USA) and quantified using a ChemiDoc XRS device with Quantity One software (version 4.6.3. Bio-Rad Laboratories, Hercules, CA, USA). The uniformity of the protein loading was confirmed by staining the membrane with Ponceau S and by re-probing the membrane with an antibody against GAPDH (Abcam, Cambridge, UK).

2.7.2. Heat shock proteins, GRP78, TRX and TxNIP

After SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Millipore, Bedford, Mass., USA) as previously described [28]. The membranes were blocked and after that treated with antibodies overnight at 4 °C. Immunoblots were visualized using an Odyssey Imaging System (Li-Cor Biosciences Inc., Lincoln, NB, USA) and quantified using Odyssey Software.

2.7.3. Protein carbonyls

Protein carbonyls were derivatized using 2,4-dinitrophenyl hydrazine immediately before electrophoresis as previously described [29,30]. Protein extracts (20 ug protein/lane) were electrophoresed on 12.5% SDS-PAGE. A rat monoclonal primary antibody raised against 2,4-dinitrophenol (Zymed Laboratories, San Francisco, CA, USA) was incubated overnight at 4 °C. After secondary antibody incubation, the immunoblots were visualized using an Odyssey Imaging System (Li-Cor Biosciences Inc., Lincoln, NB, USA) and quantified using Odyssey Software.

2.7.4. Primary antibodies

The antibodies for inositol-requiring enzyme 1α (IRE1α, #3294), protein disulfide isomerase (PDI, #3501), lysine acetylated proteins (#9441), protein kinase R-like ER protein kinase (PERK, #3192), eukaryotic initiation factor 2 subunit α (eIF2α, #5324) and its phosphorylated form at ser51 and phosphorylated sirtuin 1 at ser46 in mouse (#3398) and AMPK total (#2603) and phosphorylated at Thr172 (#4188) were purchased from Cell Signaling Technology. Antibodies against GAPDH (ab9485), sirtuin 1 (ab28170), 3 (ab118334) and 6 (ab62739) were from Abcam. Antibodies that recognize the inducible forms of heat shock protein 70 (HSP70, SPA-810), heat shock protein 60 (HSP60, SPA-806), heat shock protein 90 (HSP90, SPA-835), heat shock protein 25 (HSP25, SPA-801), heat shock protein 47 (HSP47, SPA-470) and glucose-regulated protein 78 (GRP78, SPA-826) and 75 (GRP75, SPS-825) were purchased from Enzo Life Sciences Inc., (Farmingdale, NY, USA). Antibody against thioredoxin (ATRX-06) was purchased from IMCO Corp (Stockholm, Sweden), TxNIP (thioredoxin-interacting protein, VDUP-1, # K0205-3) from MBL (Medical and Biological laboratories Co. Ltd, Nagoya, Japan) and actin (A-2066) and HSP10 (SAB4501465) from Sigma-Aldrich (St. Louis, MO, USA).

2.8. Assays for glutathione levels and antioxidant enzyme activity

2.8.1. GSSG/TGSH

Gastrocnemius muscle was homogenized on ice in brief bursts by an Ultra-Turax homogenizer (Janke and Kunkel, Germany) in a 1:10 (w:v) dilution of ice-cold 5.0% metaphosphorous acid. Resultant homogenates were centrifuged at 10,000*g* for 15 min (4 °C), and the supernatant was stored at −70 °C. On the day of measurement the supernatant was diluted with distilled water and TGSH was measured spectrophotometrically by a GSSG reductase recycling method as described earlier [31]. The rate of change in absorbance at 412 nm was monitored with double-beam spectrophotometer at room temperature and tissue concentrations were estimated by linear regressions from the standard curve.

2.8.2. GPX, GRD, GST and TPOR

Total glutathione peroxidase (GPX) activity was determined with cumune hydroperoxide as substrate. Glutathione reductase (GRD) activity was determined in the presence of 50 mM Tris-HCl buffer with 1 mM EDTA, 2 mM NADPH, and 20 mM GSSG by monitoring the decrease in absorbance per minute due to the oxidation of NADPH at 340 nm. GST activity was also assayed at 340 nm with 1,2-dichloro-4-nitrobenzene as substrate. Activities of muscle GPX, GRD, and GST were determined from the cytosolic supernatant spectrophotometrically, as described previously [32]. Thiol-Protein Oxidoreductase Activity (TPOR) to demonstrate PDI activity was assayed as earlier [33], in which the enzyme-catalyzed reduction of the disulfide bonds of insulin by GSH is linked to the reduction of GSSG to GSH, by NADPH and glutathione reductase; the linked reaction is monitored by spectrophotometer at 340 nm.

2.9. RNA and DNA isolation and cDNA synthesis

Gastrocnemius muscle was pulverized and homogenized in liquid nitrogen after which 65–75 mg of muscle powder was placed into Trizol reagent (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted according to the manufacturer's guidelines and analyzed in duplicate using a Nanodrop ND-1000 (Thermo Fisher Scientific Inc., Waltham MA, USA). Any possible remaining DNA was further

degraded using DNAse kit (TURBO DNA-free™ Kit, Applied Biosystems by Life Technologies, South San Francisco, CA, USA) prior to mRNA analysis. The quality of RNA was confirmed spectrophotometrically (OD_{260}/OD_{280} ratio of 1.9-2.0), by agarose gel electrophoresis as well as the Agilent Bioanalyzer (Agilent Technologies Inc., Santa Clara, CA, USA).

2.10. Microarray analysis

RNA samples from *mdx* mice (n=5 mice/group) and the control (n=4) were analyzed with a Illumina Sentrix MouseRef-8 v2 Expression BeadChip containing approximately 25,600 annotated transcripts and over 17,900 unique genes (Illumina Inc., San Diego, CA, USA) by the Finnish Microarray and Sequencing Centre at the Turku Centre for Biotechnology according to the manufacturer's instructions. Raw data were normalized with quantile normalization (including log2-transformation of the data) and data quality was assessed using Chipster software (IT Centre for Science, Espoo, Finland) [34] and R with Limma package software [35]. The normalized gene expression data were imported into Excel spreadsheets (Microsoft Corp., Redmond, WA, USA). From the genes represented by multiple probes, the probe with the highest fold-change ratio was used for further analysis. MIAME guidelines were followed during array data generation, preprocessing, and analysis.

2.11. Real-time qRT-PCR

Three μg of total RNA were reverse transcribed to synthesize cDNA using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, Ca, USA). The mRNA expression levels were quantified with Real-time qPCR according to standard procedures using iQ SYBR Supermix (Bio-Rad Laboratories) and CFX96 Real-Time PCR Detection System (Bio-Rad Laboratories). Real-time qRT-PCR was conducted under standard PCR conditions as recommended by the manufacturer (Applied Biosystems, Foster City, CA, USA) in triplicate. The spliced variant of X-box binding protein 1 (*sXbp1*) was analyzed using SYBR green primers: forward: TGCTGAGTCCGCAGCAGGTG and reverse: CTGATGAGGTCCCCACTGACAGA with product length of 251 bp (496 – 746) (Invitrogen, USA). qPCR for C/EBP homologous protein (Chop/Ddit3, assay ID qMmuCID0020314), activating transcription factor 6b (ATF6b, qMmuCID0022729) and caseinolytic peptidase (*ClpP*, qMmuCID0005629) were conducted using pre-designed Bio-Rad PrimePCR™ SYBR® Green Assays. The gene expressions were normalized to the expression of stably acted Gapdh (Taqman probe: Mm99999915_g1). The analysis was conducted using the delta delta Ct (ΔΔCt) method and quantification was performed in the exponential amplification phase.

2.12. Data analysis

All data, with the exception of microarray, were evaluated by analysis of variance (ANOVA) followed by Tukey's post-hoc test (treatments) or t-test (*mdx* vs. control). The level of significance in these analyses was set at $P < 0.05$. Data are expressed as means ± SE. Correlations were analyzed using Pearson's Product Moment Coefficient.

The list of differentially expressed genes generated between the *mdx* and wild type sample groups from the microarray data were detected utilizing linear modeling and empirical Bayes methods of the Limma package of R. The resulting raw p-values were adjusted using the Benjamini and Hochberg method. The genes with an adjusted $P \leq 0.05$ and absolute fold change higher than 1.5 were considered to be differentially expressed. Enrichment of functionally related genes in three different gene set collections was first performed using a non-biased method by Gene Set Enrichment Analysis software (GSEA; Version 2.0). For this analysis, ranking lists representing different ratio combinations of the normalized data were created by averaging the group results for each gene. The enriched gene sets were ranked based on their fold-change ratios. The collection used was the Canonical Pathways collection (1452 gene sets, C2: CP, version 3.0) (http://www.broadinstitute.org/gsea/msigdb/collections.jsp). The number of permutations by gene set was set to 1000 and gene sets with at least five, and no more than 500 genes were taken into account in each analysis. Each analysis was carried out at least five times and all the results were averaged into a single value. The statistical significance was calculated using false discovery rate (FDR). The level of significance was set at FDR < 0.05.

Further, the differentially expressed genes were uploaded to DAVID functional annotation tool [36] to analyze the effects of the phenotype. The enrichment analysis was run against the default gene groups including gene ontologies and KEGG-pathways. The Disulfide bond was highly enriched within the DAVID results, and thus the genes within this pathway with most striking regulation, i.e. having an absolute fold change ≥3 and $P < 0.05$, were more carefully studied with hierarchical clustering. The expression values of these genes were analyzed with hierarchical clustering and illustrated with a heatmap using MATLAB. Within clustering, the Euclidean distance and Ward's minimum variance criterion linkage method was used.

3. Results

3.1. Reported skeletal muscle characteristics

This study is a follow-up to work that was previously published [25,26]. In brief, muscles of *mdx* mice typically contained centrally nucleated fibers and a large content of fibrosis and proliferative cells other than muscle fibers. sAcvR2B-Fc administration increased the mass of all muscles weighed. Wheel running exercise performed by the *mdx* mice enhanced markers of muscle aerobic capacity (e.g. citrate synthase and SDH activities as well as gene sets of aerobic metabolism in microarray) to levels similar to, or higher than those observed in healthy mice.

3.2. Effects of mdx phenotype on muscle morphology and grip strength

No difference in mean fiber cross-sectional area (CSA) was observed in *mdx* mice when compared to wildtype mice ($P=0.70$, Fig. 1A), but fiber size distribution tended to show differences (Fig. 1B). Statistically, this could be observed as a greater percentage of very small ($< 600\ \mu m^2$) or small ($< 1000\ \mu m^2$) fibers in *mdx* mice ($P < 0.05$), which represents newly regenerating fibers (Fig. 1C). Along with the higher percentage of very small fibers, lower grip strength presented as absolute values (Fig. 1D) or normalized to body weight (not shown) was also observed among *mdx* mice ($P < 0.05$).

3.3. Effects of mdx phenotype on redox balance

Recent studies showed increased oxidative stress as one candidate mechanism that may mediate the pathology of dystrophin deficiency [9,10]. Dystrophic *mdx* mice at the age of ~14 weeks showed significantly greater levels of oxidized glutathione (GSSG) ($P < 0.05$) compared to healthy wild type animals (Fig. 2A–C). No effect of muscular dystrophy was seen on protein content of redox-active proteins, TxNIP and TRX (Fig. 2D).

Next, a gene clustering analysis was conducted from microarray

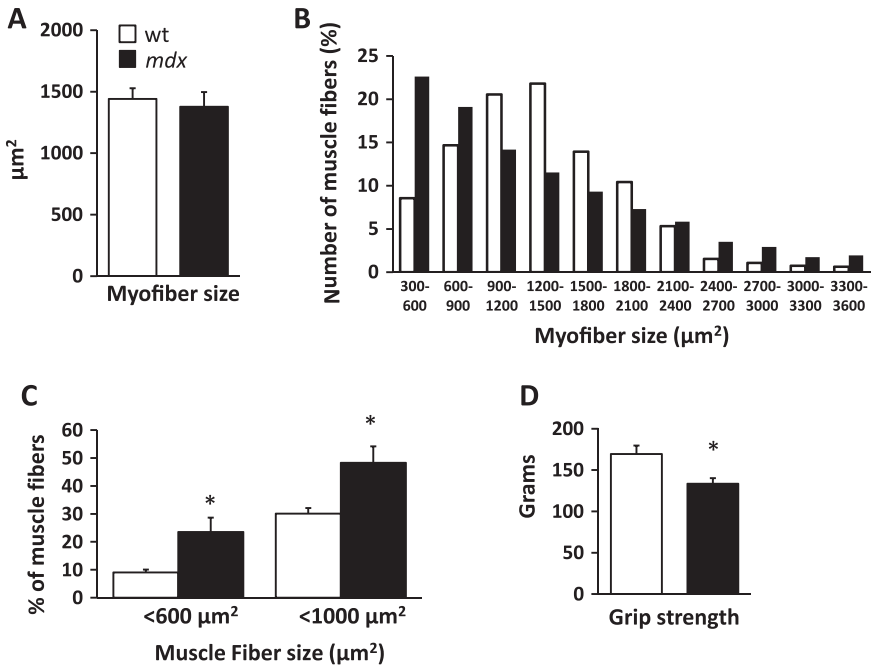

Fig. 1. The effects of muscular dystrophy on muscle size and strength. A) Myofiber size in gastrocnemius muscle at age of 14 weeks. No differences in mean fiber cross-sectional area (CSA) in *mdx* mice, but B-C) the percentage of very small ($<600\ \mu m^2$) or small ($<1000\ \mu m^2$) fibers was greater than in healthy wildtype (wt) controls ($P<0.05$). D) Healthy wild type mice had greater grip strength ($P<0.05$) than *mdx* mice. Data are mean ± s.e.m; n=8 for *mdx* and n=5 for wt.

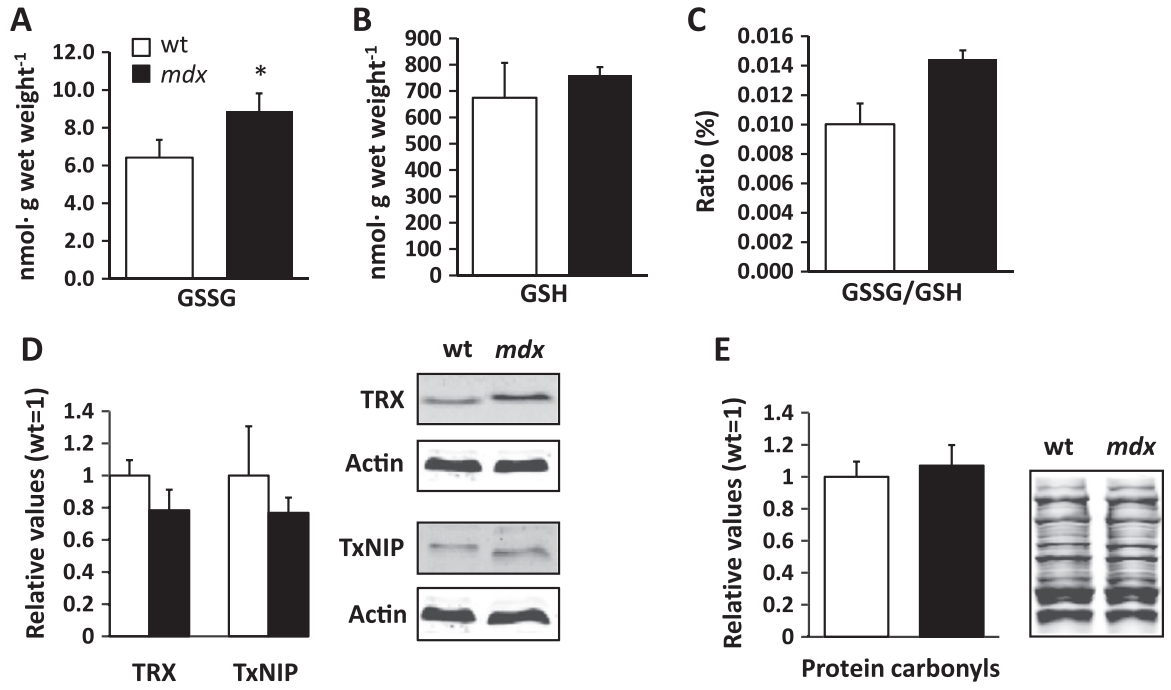

Fig. 2. The effects of muscular dystrophy on glutathione. A) Increased GSSG ($P<0.05$) in *mdx* mice without significant differences in B) GSH, C) GSSG/GSH, D) TRX and TXNIP, or E) protein carbonyls. Data are mean ± s.e.m; n=8 for *mdx* and n=5 for wt except n=4 for glutathione results.

data with a gene set enrichment analysis (GSEA) that is designed to find physiologically meaningful results from large gene sets with <20% average changes in individual gene expression [37]. A trend for decreased pathways of glutathione (*FDR*=0.07) metabolism was observed in *mdx* mice compared to healthy mice (Fig. S1 and Table S1), which further supports the presence of altered redox balance in *mdx* mice.

In addition, anti-oxidative enzyme activities related to redox balance and glutathione metabolism (glutathione reductase (GRD), glutathione S-transferase (GST), glutathione peroxidase (GPX) and

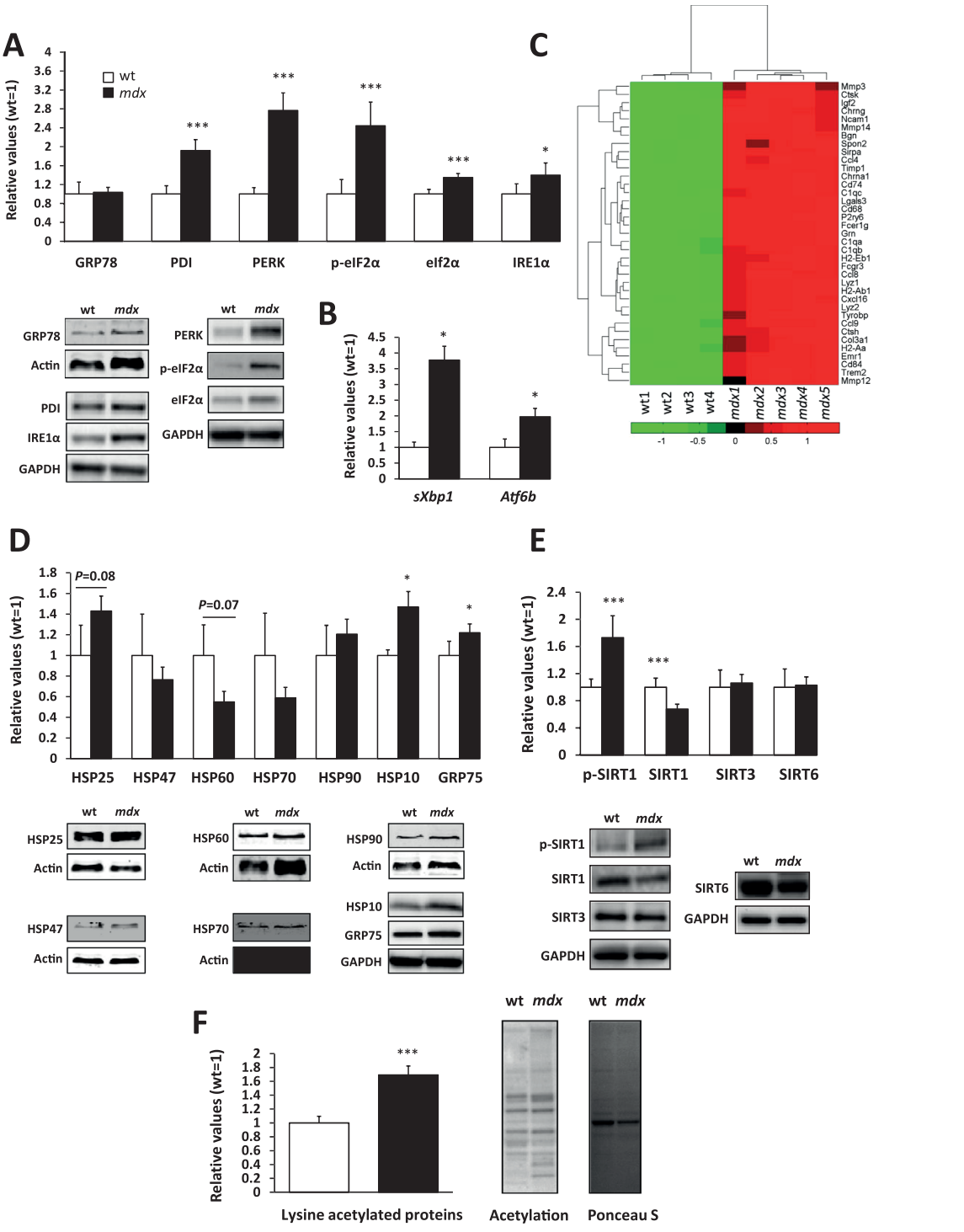

Fig. 3. The effects of muscular dystrophy on ER stress, UPR activation, sirtuins and acetylated proteins. A) Most UPR/ER stress markers (PDI, PERK, p-eIF2α, eIF2α, and IRE1α proteins were upregulated ($P < 0.05$-0.001) in muscle from *mdx* mice. B) Spliced *Xbp1* (*sXbp1*) and *Atf6* mRNA. C) Hierarchical clustering of the gene-wise standardized logarithmic expression values of the genes within disulfide bond pathway that were up-regulated (FC ≥ 3 and $P < 0.05$) in dystrophic *mdx* muscle vs. wildtype muscle. D) No major differences were noticed in most of the HSPs between *mdx* and wt mice except mitochondrial proteins HSP10 and GRP75 which were increased in *mdx* mice ($P < 0.05$). E) Higher p-SIRT1, but lower total SIRT1 in *mdx* mice ($P < 0.001$), but no changes in other sirtuins ($P > 0.05$). F) Increased acetylated lysine residues in *mdx* mice ($P < 0.001$). Data are mean ± s.e.m; n=8 for *mdx* and n=5 for wt.

thiol protein oxidoreductase (TPOR)) were measured in order to assess antioxidant capacity, the oxidative protein folding and protein disulfide isomerase (PDI) activity in mdx mice (Fig. S2). There were, however, no significant differences in any of the four measured enzyme activities. Similarly, no effect of the *mdx* phenotype on protein carbonyls, a marker of oxidative damage to proteins, was observed (Fig. 2E).

3.4. Greater ER stress and UPR_{ER} is observed in mdx muscle, without systematic changes in HSP proteins or UPR_{mt}

In order to achieve a more comprehensive view of the muscle cellular stress state of *mdx* mice, ER stress and UPR_{ER} were analyzed. Regardless of no significant effect of muscular dystrophy on GRP78/BiP (Fig. 3A), all three canonical branches of the UPR_{ER} and their downstream responses were upregulated in *mdx* mice. More specifically, higher protein content of PDI ($P<0.001$) and PERK ($P<0.001$) were detected in *mdx* muscle. In addition, downstream to PERK, phosphorylated eIF2α at serine 51 ($P<0.001$), and total eIF2α protein ($P<0.001$) as well as p-eIF2α per total eIF2α ($P<0.05$) were significantly increased in *mdx* mice (Fig. 3A). Another UPR pathway component IRE1α ($P<0.05$; Fig. 3A) and downstream to it spliced *Xbp1* (*sXbp1*) mRNA ($P<0.05$) were also increased in *mdx* mice (Fig. 3B). In addition, mRNA levels of the third UPR sensor *Atf6b* were also upregulated in *mdx* muscles in microarray (1.4-fold, adjusted $P<0.001$) and this was confirmed by qPCR (Fig. 3B). In addition, the third-most upregulated gene set in the microarray DAVID analysis was disulfide bond (fold enrichment 2.13, FDR$=4\times10^{-24}$, Fig. 3C); a set of genes related to protein folding and thus to ER stress, since ER is one of the major sites for disulfide bond formation. To further understand the role of UPR proteostasis in ER, apoptotic pathway components downstream to PERK, *Atf4-Chop-Gadd34* were quantified with microarray and qPCR. In microarray *Atf4* (1.3-fold), *Chop* (1.3-fold and qPCR NS. Fig. S3) and *Gadd34* (1.4-fold) were unaltered or marginally decreased ($P<0.05$). This suggests that UPR through PERK-eIF2α cascade and translational halt can override PERK-ATF4-CHOP-GADD34 apoptosis pathway at least at age of week 14 in mdx mice.

In addition, several HSPs were measured because of their key role as chaperones that assist with the proper folding of polypeptides into functional proteins and prevent the aggregation of misfolded proteins mainly in the cytosol compartment, but also in other cellular organelles including mitochondria. However, unlike responses of the UPR_{ER} our results did not display significantly altered levels of HSP-proteins (Fig. 3D). Next, UPR in mitochondria, i.e., UPR_{mt} and mitochondrial chaperones were examined. In microarray only marginal (1.1–1.3 fold) changes were observed in the UPR_{mt} markers *Hsp60*, *Clpp*, *Jnk2*, *Timm17a*, *Hsp10*, *Yme1l1*, *Cebpb*, *Jun*, *Hsp74* and *Pmpcb* (data available at GEO: http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE52766) of which Clpp (Fig. S3) and HSP60 (Fig. 3D) were confirmed with qPCR or western blotting, respectively. However, the protein levels of UPR_{mt} marker HSP10 and mitochondrial chaperone GRP75 were slightly increased in *mdx* mice (Fig. 3D).

3.5. mdx muscle shows alteration of p-SIRT1/SIRT1 and increased total lysine acetylated proteins

Phosphorylated SIRT1 and AMPK (p-SIRT1 and p-AMPK), and protein levels of SIRT1, SIRT3, SIRT6 and AMPK were measured due to their redox, heat shock response and UPR_{mt} properties and possible effects on muscle dystrophy [7,38]. Increased p-SIRT1 and decreased total SIRT1 were observed in *mdx* (Fig. 3E) mice leading to increased ratio of phosphorylated SIRT1 to total SIRT1 ($P<0.001$). No effect on total sirtuins 3 or 6 (Fig. 3E) or phosphorylated AMPK (Fig. S4) was observed. In addition, overall proteome lysine residue acetylation analysis revealed that *mdx* mice had higher levels of acetylated protein lysine residues ($P<0.001$; Fig. 3F).

3.6. Effects of exercise and blocking AcvR2B ligands on muscle morphology and grip strength

Blockade of AcvR2B ligands using sAcvR2B-Fc or placebo (PBS) was administered for seven weeks with or without voluntary running exercise in young male *mdx* mice. Increased muscle mass by sAcvR2B-Fc, as reported earlier [25,26], did not translate into greater gastrocnemius muscle fiber size or grip strength either presented as absolute values (Fig. S5) or normalized to body weight (not shown). In addition, no effect of exercise was noticed on grip strength, but exercise shifted the CSA distribution further towards smaller fibers (Fig. S5), along with smaller gastrocnemius mass after running as previously published [25]. No statistically significant difference was noted in the percentage mean count of small fibers ($<600\ \mu m^2$) by running vs. no running (2×2 ANOVA $P>0.29$) and thus the decreasing effect of exercise was throughout the fiber size range.

3.7. Redox balance and oxidative stress are affected by running and blocking AcvR2B ligands

Oxidized glutathione (GSSG and GSSG/GSH ratio) in gastrocnemius muscle was increased by running (2×2 ANOVA running effect $P<0.05$; Fig. 4A–C). No difference in reduced glutathione (GSH) was observed between the treatments. In addition, microarray data showed that combination of sAcvR2B-Fc and exercise significantly increased gene sets of glutathione metabolism ($FDR<0.01$) with a smaller increasing effect of running or sAcvR2B-Fc alone (Fig. S1 and Table S1).

Thiol-Protein Oxidoreductase Activity (TPOR), which reflects the major PDI function, showed a sAcvR2B-Fc and running interaction effect in gastrocnemius muscle ($P<0.05$) (Fig. 4D). There was no consistent effect of the interventions on the enzyme activities of GRD, GST or GPX (Fig. S6).

Exercise increased protein carbonyl levels (2×2 ANOVA running effect $P<0.05$; Fig. 4E). Furthermore, the exercise response also interacted with administration of sAcvR2B-Fc (2×2 ANOVA sAcvR2-Fc and running interaction effect, $P<0.05$; Fig. 4E). The interaction effect was further seen in a post hoc test as running increased protein carbonyls only in the group administered with sAcvR2-Fc (sAcvR2-Fc +running vs. sAcvR2-Fc alone, $P<0.05$; Fig. 4E).

3.8. sAcvR2B-Fc increased GRP78/BiP and TxNIP, but neither treatment altered UPR markers or HSPs

sAcvR2B-Fc increased a marker of ER stress/UPR, GRP78/BiP (2×2 ANOVA sAcvR2B-Fc administration effect, $P<0.05$) (Fig. 5A). TxNIP, an endogenous inhibitor of antioxidant TRX and a protein induced in ER stress [39] was also increased due to sAcvR2B-Fc (2×2 ANOVA drug effect, $P<0.05$; Fig. 5B). Supporting the link between ER stress and TxNIP protein, these two proteins also strongly correlated with each other in *mdx* mice ($r=0.876$, $P<0.001$) (Fig. S9). No effect of the treatments was found on TRX protein (Fig. 5C).

No systematic effect of sAcvR2B-Fc was seen in ER stress or UPR_{ER} (PDI, IRE1α, PERK, p-eIF2α at ser^{51} and eIF2α and total eIF2α) protein levels or *Xbp1* mRNA splicing levels (Fig. 6A–E). However, exercise tended to increase PDI and IRE1α (2×2 ANOVA running-effect, $P=0.07$–$P=0.08$; Fig. 6A–B). According to microarray data, there was no effect of interventions on mRNA expression in ER stress or UPR_{ER} related genes (*Grp78, Perk, eif2α* and

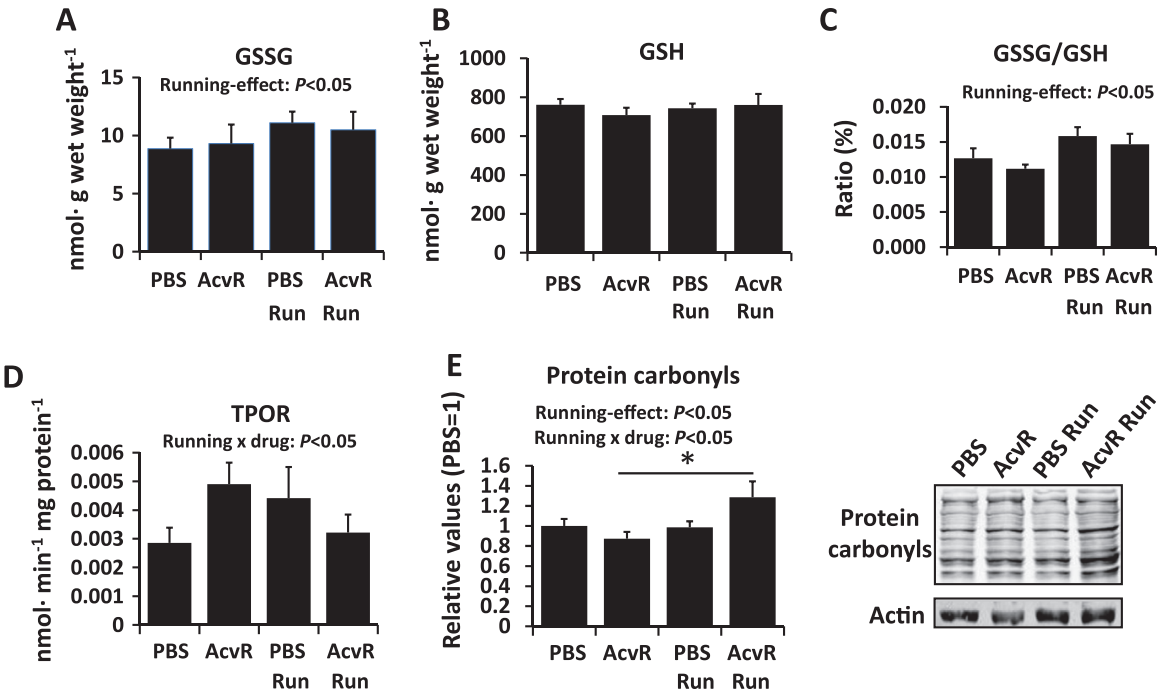

Fig. 4. The effects of exercise and sAcvR2B-Fc on redox markers and oxidative stress in *mdx* muscle. (A–C) Oxidized glutathione (GSSG), reduced glutathione (GSH), ratio of oxidized glutathione (GSSG) and reduced glutathione (GSSG/GSH) levels, (D) TPOR activity and (E) protein carbonyls after the 7-week intervention period. PBS=PBS injected sedentary (n=8), AcvR=sAcvR2B-Fc administered sedentary (n=8), PBS Run=PBS injected voluntary wheel running (n=7) and AcvR Run=sAcvR2B-Fc administered voluntary wheel running (n=7). Data are mean ± s.e.m. 2 × 2 ANOVA (main and interaction effects) results are shown as text above the bars and possible post hoc (Tukey's test) differences between individual groups.

Ire1α, Atf4, Chop, Gadd34, Pdi, Xbp1, and *Atf6*; $P > 0.85$, data not shown). There were also no systematic effects of exercise or sAcvR2B-Fc administration alone or in combination on HSPs (Fig. 7). However, exercise tended to increase HSP70 (P=0.07) (Fig. 7B) and decreased HSP90 protein (2 × 2 ANOVA running effect,$P < 0.05$, Fig. 7C). There were also no effects of treatments on

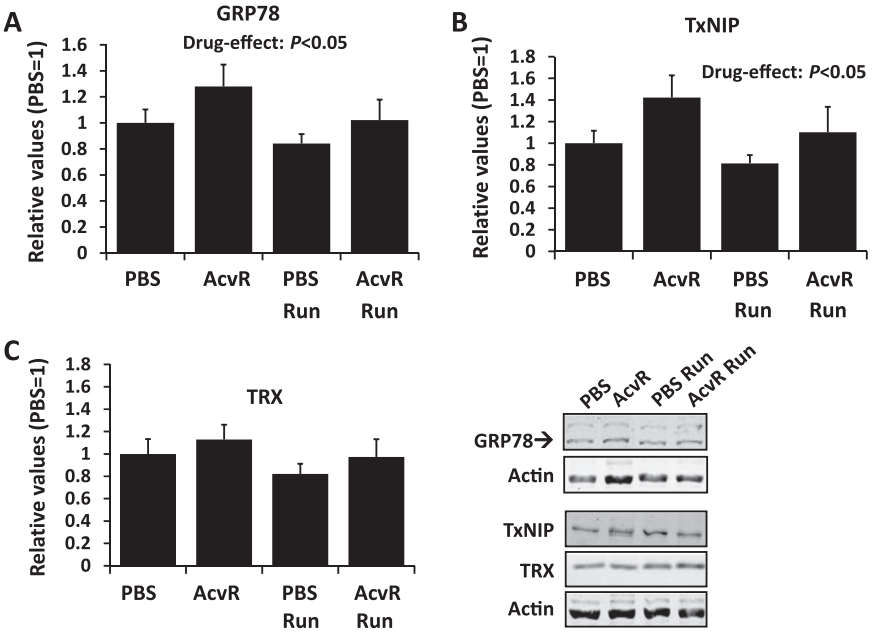

Fig. 5. The effects of exercise and sAcvR2B-Fc on ER chaperones and thioredoxin and its regulation in *mdx* muscle. (A) GRP78/BiP, (B) TxNIP and (C) TRX protein content after the 7-week intervention period. PBS=PBS injected sedentary (n=8), AcvR=sAcvR2B-Fc administered sedentary (n=8), PBS Run=PBS injected voluntary wheel running (n=7) and AcvR Run=sAcvR2B-Fc administered voluntary wheel running (n=7). Data are mean ± s.e.m. 2 × 2 ANOVA (main and interaction effects) results are shown as text above the bars. There were no post hoc (Tukey's test) differences between individual groups.

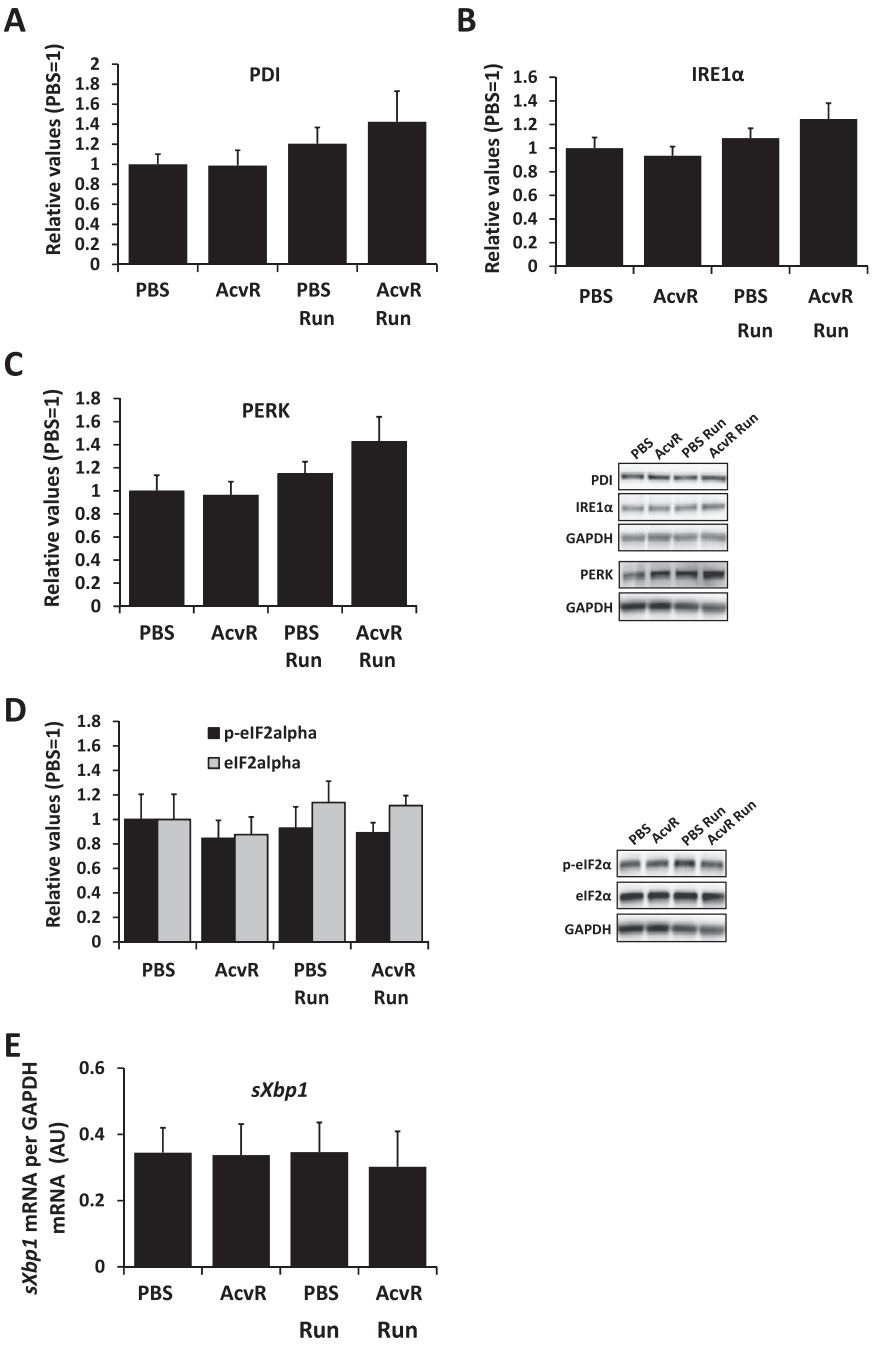

Fig. 6. The effects of exercise and sAcvR2B-Fc on ER stress and UPR in *mdx* muscle. (A) PDI, (B) IRE1α (C) PERK, (D) p-eIF2α (black bars) and total eIF2α (gray bars) protein contents and E) spliced *Xbp1* mRNA after the 7-week intervention period. Abbreviations: PBS=PBS injected sedentary AcvR=sAcvR2B-Fc administered sedentary. PBS=PBS injected sedentary (n=8), AcvR=sAcvR2B-Fc administered sedentary (n=8), PBS Run=PBS injected voluntary wheel running (n=7) and AcvR Run=sAcvR2B-Fc administered voluntary wheel running (n=7). Data are mean ± s.e.m.

$UPR_{mt.}$ markers such as HSP60 protein and in microarray *Clpp, JNK2, Timm17a, HSP10, Yme1l1, Cebpb, Jun, HSP74* and *Pmpcb* mRNA ($P > 0.85$, data not shown).

3.9. AMPK, Sirtuin1 and lysine acetylated proteins are affected by the treatments

No effect of exercise or sAcvR2B-Fc on pAMPKα at Thr^{172}, total AMPK or p-AMPK/AMPK was observed, but p-AMPK/AMPK showed a running x drug interaction effect (2×2 ANOVA, $P < 0.05$; Fig. S7). The phosphorylated sirtuin 1 (p-SIRT1 at Ser^{46}) showed both sAcvR2B-Fc administration (2×2 ANOVA, $P < 0.05$) and running x sAcvR2B-Fc administration interaction effects (2×2 ANOVA, $P < 0.05$) (Fig. S7). This was due to a fact that sAcvR2B-Fc increased p-SIRT1 in the exercised mice ($P < 0.05$) and not in sedentary mice (Fig. S7). The ratio of p-SIRT1 to total SIRT1 (p-SIRT1/

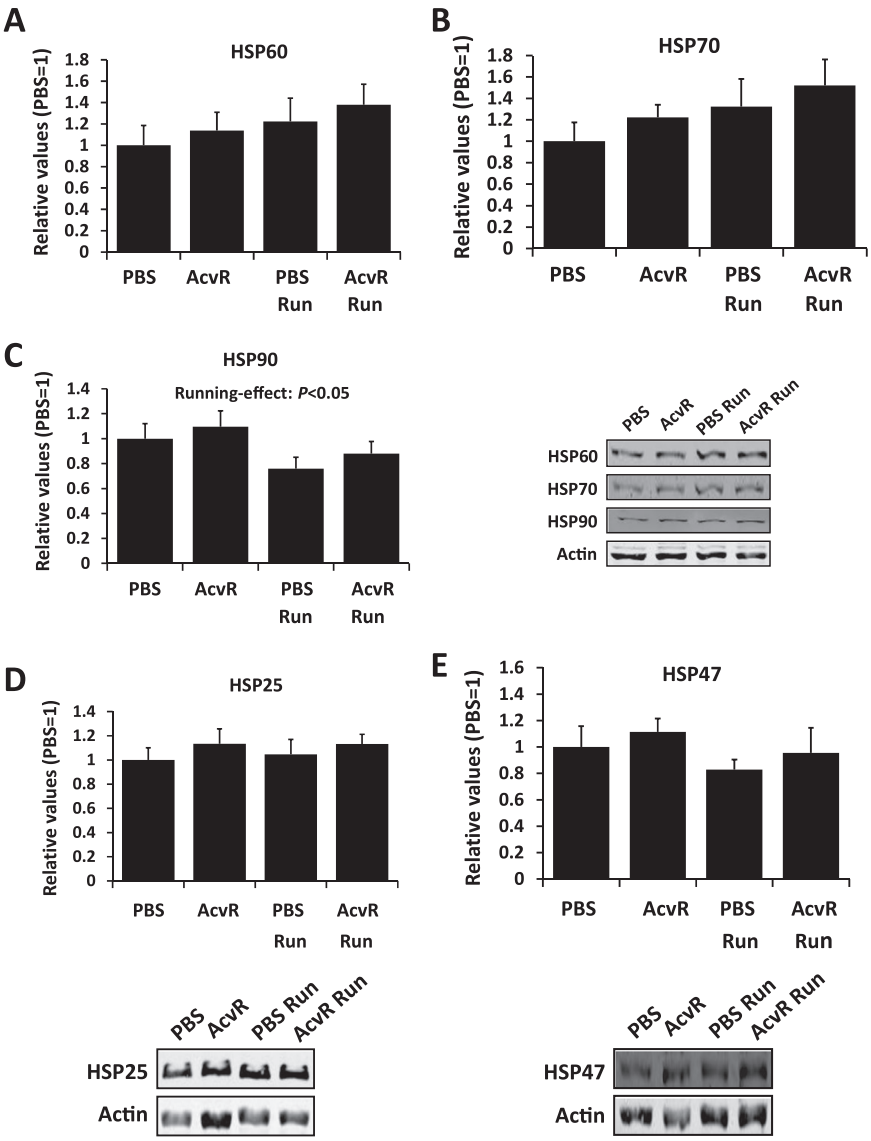

Fig. 7. The effects of exercise and sAcvR2B-Fc on heat shock proteins in *mdx* muscle. (A) HSP60, (B) HSP70, (C) HSP90, (D) HSP25, (E) HSP47. PBS=PBS injected sedentary (n=8), AcvR=sAcvR2B-Fc administered sedentary (n=8), PBS Run=PBS injected voluntary wheel running (n=7) and AcvR Run=sAcvR2B-Fc administered voluntary wheel running (n=7). Data are mean ± s.e.m. 2 × 2 ANOVA (main and interaction effects) results are shown as text above the bars. There were no post hoc (Tukey´s test) differences between individual groups.

SIRT1) was in line with the p-SIRT1 result (not shown). There were no significant differences in the protein expression levels of SIRT3 or SIRT6 between the treatments (Fig. S7). Running increased levels of protein acetyls at lysine residues (2 × 2 ANOVA, $P < 0.05$; Fig. S8).

3.10. Associations between the measured variables

A computationally determined network was created between different variables. For this purpose, biologically similar variables were merged. Katiska/Himmeli software using in GNU Octave program environment (http://www.finndiane.fi/software/katiska/) was used for analysis [40]. Clearly, different HSPs were connected and a link to UPR/ER stress was also evident (Fig. 8A). UPR/ER stress was also linked to acetylated proteins and protein carbonyls to mitochondria count (Fig. 8A). Of individual associations, TxNIP correlated strongly and positively with HSPs in *mdx* mice ($P < 0.01$–$P < 0.001$) (Fig. S9). Especially interesting was the strong correlation between TxNIP and GRP78 as mentioned earlier (Fig. S9). Carbonyls correlated positively and strongly in the sAcvR2B-Fc injected mice with activity index ($r = 0.87$, $P < 0.001$) that was assessed by measuring the whole activity level of mice in the cage (running and non-running) (Fig. S9), a measure reported earlier [26]. Fig. 8B summarizes the main effects of muscular dystrophy on ER stress/UPR/chaperones in the present manuscript.

4. Discussion

The present study provides an extensive analysis of the effects of muscular dystrophy on various types of metabolic stress and two candidate therapeutic strategies to treat dystrophin deficient

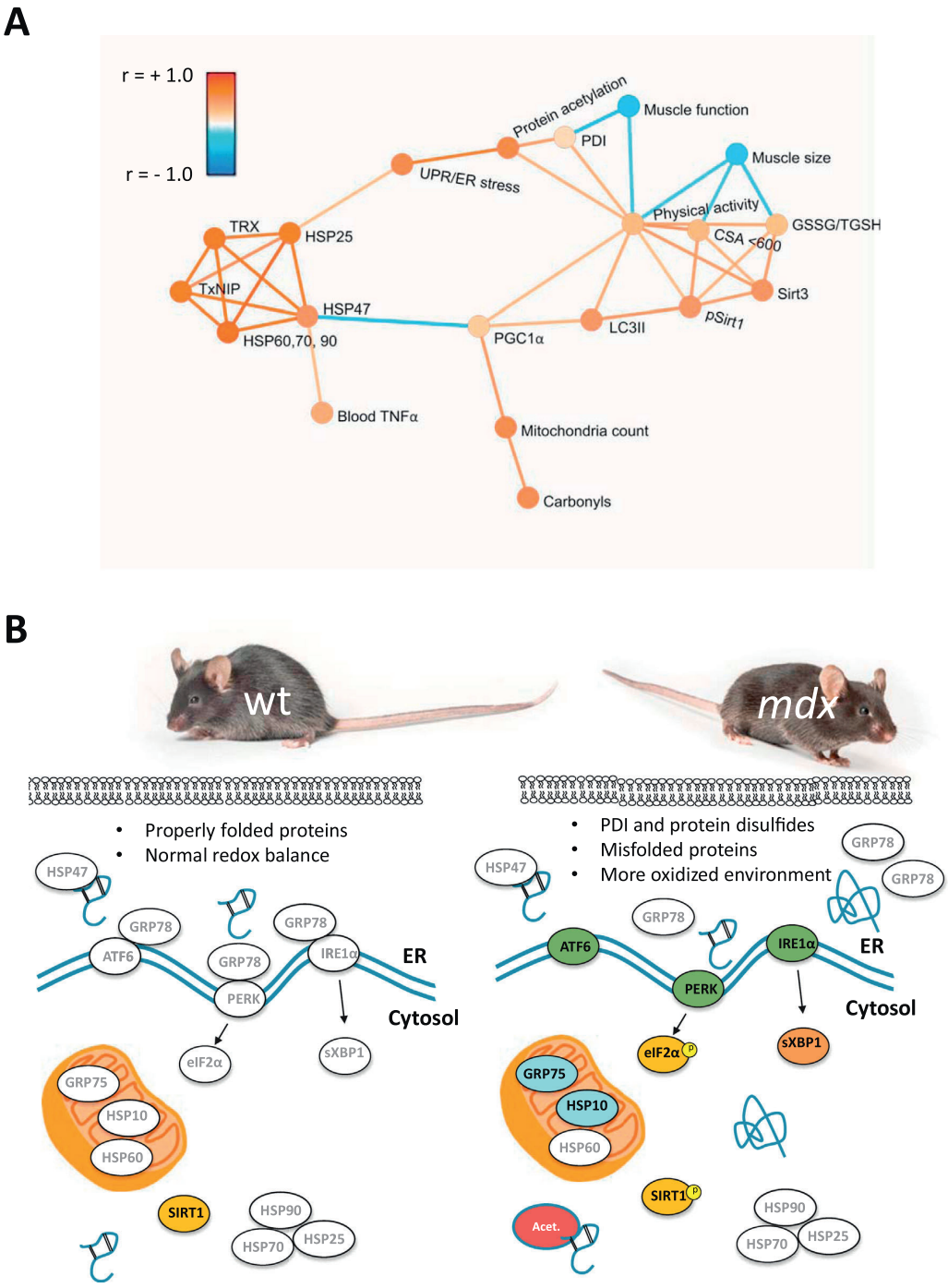

Fig. 8. (A) Visualization of the correlation structure between the measured variables from the present mice. A Katiska/Himmeli software (http://www.finndiane.fi/software/katiska/) was used [40]. As illustrated, different HSPs were connected and a link to UPR/ER stress was evident. UPR/ER stress was linked to acetylated proteins and protein carbonyls to mitochondria count. Mitochondria count = mitochondria count estimate (average of citrate synthase activity, mitochondrial, DNA and cytochrome C protein content) [25]), Muscle function=average of grip strength and hanging wire time, Muscle size=average of gastrocnemius mass and fiber CSA, UPR/ER stress=average of GRP78, spliced *Xbp1*, IRE1α, PERK, peIF2α, Physical activity=all voluntary activity of mice in their cages measured by force platforms measured 3 times within 7 weeks as reported earlier [26]. (B) Summary of the effects of muscular dystrophy on ER stress/UPR/chaperones. UPR in ER is clearly increased with some minor changes in few, but not most mitochondrial, but not cytosolic chaperones. Upregulation in *mdx* vs wild-type control are shown as bolded and in color and no change is shown as gray color.

skeletal muscle. Of these complex processes we focused specifically on proteostasis pathways of ER stress and the UPR, which consists of three canonical branches (PERK, IRE1α and ATF6). Notably, most of the measured components of the three canonical branches (IRE1α protein level, spliced *Xbp1 mRNA*, PERK, p-eIF2α and total eIF2α, and *Atf6* mRNA) in skeletal muscle were greater in

mdx mice compared to wild-type healthy controls. Although a recent report showed increased GRP78/BiP and CHOP proteins in muscle of *mdx* mice and that ER stress-related caspase activity and apoptosis contributes to *mdx* pathology [15], this is the first study to provide insight into all three canonical pathways of UPR activation in conjunction with measures of oxidative stress and heat shock protein defense. Notably, the importance of ER stress to the pathology of *mdx* mice is consistent with other dystrophy studies reporting ER stress in sporadic inclusion body myositis (s-IBM) [41] and tibial muscular dystrophy (TMD) [42]. Running and AcvR2B ligand blocking do not improve or further deteriorate proteostasis in *mdx* mice, but rather seem to impart mostly neutral effect.

Altered redox balance in the ER has been shown to induce ER stress, which in turn induces the production of ROS in the ER and mitochondria [6]. Oxidative protein folding in the ER has been shown to be a major source of oxidative stress, particularly during ER stress [6]. We detected an increased protein expression of PDI along with a markedly induced gene set of disulfide bond formation in *mdx* muscle. These alterations in addition to expansion of the protein folding machinery may result from an elevated muscle protein synthesis previously observed in *mdx* muscle [43]. PDI, an oxidoreductase that belongs to the TRX family, assists the oxidative folding of polypeptides into functional proteins by catalyzing the disulfide bond formation between cysteine residues to stabilize tertiary and quaternary structures [44]. According to microarray data, *mdx* muscle showed an upregulation in the response of a gene set related to glutathione metabolism possibly contributing to UPR and increased oxidized glutathione (GSSG) levels. The glutathione and thioredoxin superfamilies are the most abundant endogenous thiol antioxidants that maintain redox homeostasis and facilitate correct oxidative protein folding, which involve thiol-disulfide exchange reactions [45]. Several ER-resident glutathione and thioredoxin-dependent peroxidases, including peroxiredoxins and certain isoforms of GPX (i.e., GPX 7 and GPX8) play a critical role in oxidative protein folding [44]. We did not observe any changes in thioredoxin, GSH levels, or GPX activity in *mdx* mice.

In addition to the UPR in ER (UPR_{ER}), the cytosolic heat shock response and mitochondrial UPR (UPR_{mt}) were investigated. An association between HSPs with UPR/ER stress was evident in correlation-based network analyses indicating the close association of UPR and stress protein responses. This observation is also in agreement with a previous report which showed that increased skeletal muscle HSP72 expression preserves muscle function and slows the progression of severe muscular dystrophy in *mdx* mice [46]. Moreover, HSP72 provides protection against ER stress by enhancing IRE1α/XBP1 signaling and attenuating ER stress-induced apoptosis [47]. In addition to HSPs, a mitochondrial unfolded protein response (UPR_{mt}) was recently discovered as an adaptation to deficiencies in proteostasis [13]. Unlike ER stress and UPR_{ER}, no systematic changes in heat shock response/adaptation or UPR_{mt} was noticed in *mdx* mice at the age of ~14 weeks except slightly increased protein contents of HSP10 and GRP75. Intriguingly, *mdx* mice showed greater levels of acetylated protein lysine residues. This is interesting because protein hyper-acetylation can be associated with many pathological states [48]. Moreover, because lysine residues can have many different posttranslational modifications, increased acetylation may compete with other posttranslational modifiers [49]. Collectively, these results demonstrate a clear increase in UPR_{ER} in dystrophic *mdx* mice that is accompanied by increases in some indices of oxidative stress and redox regulation including GSSG levels and lysine acetylated proteome, but overall only minor changes in heat shock response/adaptation and UPR_{mt}.

An objective of this study was to also address whether two different, but often investigated treatment options including physical exercise and/or blocking myostatin/activins would improve the status of these pathways in skeletal muscle of *mdx* mice. Administration of sAcvR2B-Fc to block myostatin/activins increased protein expression levels of GRP78 and TxNIP, independent of exercise. GRP78 is an ER-located molecular chaperone belonging to the HSP70 family that is involved in many ER-related cellular processes including sensing ER stress, inducing UPR, the translocation of newly synthesized polypeptides across the ER membrane, facilitation of protein folding, targeting misfolded proteins for degradation and regulation of calcium homeostasis (for a review see [50]). Although markers of UPR_{ER} were unchanged by sAcvR2B-Fc, an increase in GRP78 expression is consistent with a recent study wherein overexpression of decorin, an inhibitor of myostatin [51], increased ER-marker genes including GRP78 in duck muscle cells [52]. Conversely, TxNIP inhibits the thioredoxin system, which is a key antioxidant system that protects cells from oxidative stress through its disulfide reductase activity [53]. It was previously shown that ER stress increases TxNIP levels through induction of IRE1α activity [39]. Interestingly, in the present study GRP78 was strongly associated with TxNIP, a mediator of ER stress-induced inflammation and a protein linking oxidative stress, ER stress and UPR pathways [39]. GRP78 is significantly upregulated after 2 weeks of sAcvR2B-Fc administration, but not yet after single injection (1–2 days) (Hentilä et al. unpublished data) when protein synthesis is acutely already increased [19]. This suggests that the level of disruption to ER homeostasis through increased amounts of newly synthesized proteins by blocking AcvR2B ligands may not be severe enough to trigger a UPR_{ER} response.

HSP levels were not significantly affected by the *mdx* phenotype, nor were levels changed in response to exercise or sAcvR2B-Fc treatment in *mdx* mice. A lack of a well-established effect of regular exercise on increasing HSP levels in *mdx* muscle suggests the presence of an ameliorated stress protein response and disrupted proteostasis in dystrophic muscle. This assumption is in agreement with previous studies showing that the various disease models which may interfere with protein synthesis also blunt HSP responses to physical exercise [54].

Protein carbonyls can be used as an outcome marker of oxidative damage to skeletal muscle proteins. Voluntary running in combination with sAcvR2B-Fc increased protein carbonyl levels. In addition, protein carbonyl levels showed a strong, positive correlation with activity index in the sAcvR2B-Fc injected mice. These results were corroborated by observations showing that voluntary running also increased GSSG, which is also correlated with protein carbonyl levels in sAcvR2B injected mice (Fig. S8). Even if exercise training has been shown to increase the endogenous anti-oxidative capacity in healthy organisms [4], no consistent effects of exercise on the endogenous antioxidant markers (except glutathione metabolism gene set in microarray data) were observed in the present study. Thus, it seems that running alone, and particularly in combination with sAcvR2B-Fc was not completely tolerable and shifted the redox-balance of the functional dystrophin deficient skeletal muscle to more oxidizing direction. Previously, increased oxidative stress was reported after 4 weeks of running in *mdx* mice [55]. However, decreased markers of oxidative stress in *mdx* mice has also been reported after 8 weeks of very low-intensity treadmill running [9] and after 4 weeks of low intensity swimming [10]. Differences in the age of animals, time point of animal euthanization following the last bout of exercise, exercise mode, intensity and volume, and in the basal redox status of *mdx* mice may explain these results.

In addition to elevating protein carbonyls, running further increased lysine acetylated proteins in *mdx* mice. Based on these

findings it is tempting to suggest that the lack of functional dystrophin protein in skeletal muscle cells of *mdx* mice leads to hyperacetylation of proteins that becomes exacerbated when additional stress (e.g., wheel running) is added. However, the significance of this observation to the pathophysiology of DMD can only be speculated and needs to be studied further in the future. Running independently, and in combination with sAcvR2B-Fc did not exert significant effects on protein folding gene set, PDI protein expression, or other ER stress or UPR markers. Thus, low intensity voluntary running independently or combined with AcvR2B ligand blocking does not increase ER stress or UPR in *mdx* mice despite the elevated oxidative stress.

Increased muscle mass by sAcvR2B-Fc was not the result of a statistically significant increase in muscle fiber size, nor was it associated with grip strength. Our muscle strength finding using sAcvR2B-Fc is in agreement with others [20,23], and muscle fiber size by some [23], but not all studies [20]. The reason for this lack of change is unknown, but may be explained by changes in proteostasis in the present study and eventually in muscle quality shown previously in long-term AcvR2B ligand blocking experiment [23] and in myostatin knock-out mice [56], possibly together with altered calcium signaling [57].

Exercise shifted the muscle fiber distribution towards smaller fibers, which may explain why strength endurance was not improved by exercise, although aerobic capacity per volume of muscle mass was increased as reported earlier [25]. Exercise has well-known positive effects on health and also voluntary wheel running can improve skeletal muscle function of *mdx* mice at least in some muscles [58,59]. However, some studies have even shown that certain markers are negatively altered by exercise in *mdx* mice, including e.g. calcium homeostasis if the intensity of the exercise is high [55,60]. Clearly, more research is needed on the dose and type of exercise in dystrophic muscle. Thus, although positive effects of exercise on markers of oxidative capacity was reported earlier [25,26], it can be speculated that partially due to increased oxidative stress, no positive effects was seen in muscle endurance or in muscle histology.

Sirtuins are proteins involved in many important processes, including aerobic metabolism, redox regulation as well as UPR [61,62]. Increased phosphorylation of SIRT1 at ser^{46} and decreased total SIRT1 was observed in *mdx* mice when compared to wild-type mice. Moreover, voluntary wheel running independently, and in combination with sAcvR2B-Fc increased the phosphorylation of SIRT1. This coincided with increased protein carbonyls and oxidized glutathione. Previously, Chalkiadaki et al. [38] showed no difference in SIRT1 protein and mRNA expression between wild-type and *mdx* mice (age 10–12 weeks). This is in contrast with our results and Hourde et al. [58] that reported lower mRNA expression levels of SIRT1 in *mdx* mice that are increased following 4 months of voluntary wheel running. The effects of the altered phosphorylation of SIRT1 at Ser$^{46/47}$ on the activity of the protein are unclear. However, the interventions that increase the enzymatic activity of SIRT1 were suggested to be beneficial for muscular dystrophies [38,63]. On the other hand, even if the increased phosphorylation of SIRT1 at ser^{46} caused by exercise alone or in combination with sAcvR2B-Fc would improve redox regulation, the outcome of the running combined with the sAcvR2B-Fc was a greater amount of protein carbonyls and increased level of oxidized glutathione that are indicators of elevated levels of oxidative stress and damage.

5. Conclusions

Lack of functional dystrophin protein resulted in altered redox regulation, activation of UPR response in ER and increased lysine acetylated proteome in 14-week-old *mdx* mice. These processes may contribute to the pathophysiology of Duchenne muscular dystrophy. However, mitochondrial UPR or heat shock response/adaptation were not consistently altered, thus the muscle proteostasis of *mdx* mice at the age of 14 weeks is not totally dysfunctional. Apart from the observation that sAcvR2B-Fc administration increased protein expression levels of GRP78/BiP and TxNIP, running and activin receptor IIB ligand blocking did not exert a systematic effect on ER stress, UPR or heat shock proteins. A marginal oxidative stress resulting from voluntary wheel running alone or in combination with sAcvR2B-Fc administration does not further compromise the proteostasis of *mdx* mice but has rather neutral effect. Thus, these treatments may be utilized in conjunction with more direct dystrophin restoration approaches in an effort to restore dysfunctional proteostasis in muscular dystrophy.

Competing interests

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

JJH, HK and OR designed the original study, while the present study were mainly designed by JJH, JH and MA. JJH and BMO carried out the in vivo experiments. MS assisted in the in vivo experiments and isolated muscles. JJH and MA drafted the manuscript with the help from JH and KCD. JJH, JH, MA, AK and BMO carried out the analyses. JJH, UMK, HK, KGP and RA were involved in the microarray analysis. OR prepared sAcvR2B-Fc used in the study. All authors read and approved the final manuscript.

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Data availability

The complete data set is publicly available in the NCBI Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/; accession no. GSE52766).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2016.08.017.

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II

ACTIVIN RECEPTOR LIGAND BLOCKING AND CANCER HAVE DISTINCT EFFECTS ON PROTEIN AND REDOX HOMEOSTASIS IN SKELETAL MUSCLE AND LIVER

by

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Activin Receptor Ligand Blocking and Cancer Have Distinct Effects on Protein and Redox Homeostasis in Skeletal Muscle and Liver

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Muscle wasting in cancer cachexia can be alleviated by blocking activin receptor type 2 (ACVR2) ligands through changes in protein synthesis/degradation. These changes in cellular and protein metabolism may alter protein homeostasis. First, we elucidated the acute (1–2 days) and 2-week effects of blocking ACVR2 ligands by soluble activin receptor 2B (sACVR2B-Fc) on unfolded protein response (UPR), heat shock proteins (HSPs) and redox balance in a healthy mouse skeletal muscle. Second, we examined UPR, autophagy and redox balance with or without sACVR2B-Fc administration in muscle and liver of C26 tumor-bearing mice. The indicators of UPR and HSPs were not altered 1–2 days after a single sACVR2B-Fc administration in healthy muscles, but protein carbonyls increased ($p < 0.05$). Two weeks of sACVR2B-Fc administration increased muscle size, which was accompanied by increased UPR markers: GRP78 ($p < 0.05$), phosphorylated eIF2α ($p < 0.01$) and HSP47 ($p < 0.01$). Additionally, protein carbonyls and reduced form of glutathione increased (GSH) ($p < 0.05$). On the other hand, C26 cancer cachexia manifested decreased UPR markers (p-eIF2α, HSP47, p-JNK; $p < 0.05$) and antioxidant GSH ($p < 0.001$) in muscle, whereas the ratio of oxidized to reduced glutathione increased (GSSG/GSH; $p < 0.001$). Administration of sACVR2B-Fc prevented the decline in GSH and increased some of the UPR indicators in tumor-bearing mice. Additionally, autophagy markers LC3II/I ($p < 0.05$), Beclin-1 ($p < 0.01$), and P62 ($p < 0.05$) increased in the skeletal muscle of tumor-bearing mice. Finally, indicators of UPR, PERK, p-eIF2α and GRP78, increased ($p < 0.05$), whereas ATF4 was strongly decreased ($p < 0.01$) in the liver of tumor-bearing mice while sACVR2B-Fc had no effect. Muscle GSH and many of the altered UPR indicators correlated with tumor mass, fat mass and body mass loss. In conclusion, experimental cancer cachexia is accompanied by distinct and tissue-specific changes in proteostasis. Muscle hypertrophy induced by blocking ACVR2B ligands may be accompanied by the induction of UPR and increased protein carbonyls but blocking ACVR2B ligands may upregulate antioxidant protection.

Keywords: cancer cachexia, autophagy, myostatin, activin, unfolded protein response, glutathione, oxidative stress/redox

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INTRODUCTION

Cachexia is characterized by loss of skeletal muscle mass with or without reduction of fat mass and is common in diseases such as cancer, chronic obstructive pulmonary disease and sepsis (Fearon et al., 2011; Argiles et al., 2014; von Haehling and Anker, 2014). Loss of skeletal muscle mass is an independent predictor of bad prognosis in cancer (Martin et al., 2013; Choi et al., 2015). In preclinical animal models, prevention of cancer-induced muscle loss without an effect on tumor growth (Cai et al., 2004; Zhou et al., 2010; Nissinen et al., 2018) suggests possible causality between maintenance of muscle mass and improved survival in cachexia, but the underlying mechanisms are unknown.

Cellular protein homeostasis (i.e., proteostasis) is maintained through several integrated biological processes. For instance, undesirably modified or misfolded proteins are degraded by ubiquitin proteasome or autophagy lysosome pathways (Schneider and Bertolotti, 2015; Sandri, 2016). Additionally, disrupted cellular homeostasis induced by, for example, robustly increased protein synthesis, aberrant redox control or unbalanced endoplasmic reticulum (ER) calcium homeostasis can lead to accumulation of misfolded proteins into the lumen of ER, a process also known as ER stress (Cao and Kaufman, 2014; Hetz et al., 2015). Cells respond to ER stress in order to maintain homeostasis by activating unfolded protein response (UPR). It is a process which restores ER homeostasis by various mechanisms, such as increasing protein folding machinery, degrading misfolded proteins, suppressing protein synthesis and inducing autophagy. If ER stress is not rescued by UPR, metabolic impairments or apoptosis may occur (Hetz et al., 2015).

In skeletal muscle, disrupted protein homeostasis has been observed in wasting conditions and muscular dystrophies. This has been manifested as increased ER stress and oxidative stress in muscular dystrophies (Renjini et al., 2012; Screen et al., 2014; Hulmi et al., 2016), and there is some evidence that ER and oxidative stress are increased in some experimental cancer cachexia models as well (Der-Torossian et al., 2013; Ham et al., 2014; Bohnert et al., 2016). In skeletal muscle, cancer cachexia has been reported to induce ER stress and UPR in 2 experimental animal models: Lewis lung carcinoma (LLC) and cachectic $Apc^{min/+}$ mice (Bohnert et al., 2016). Interestingly, UPR inhibition in cancer cachexia by chemical chaperone 4-PBA accelerated muscle wasting in LLC and $Apc^{min/+}$ mice (Bohnert et al., 2016). Very recently, skeletal muscle specific ablation of PERK induced muscle wasting in healthy mice and further increased muscle wasting in LLC tumor bearing mice (Gallot et al., 2018). In addition to skeletal muscle, increased ER stress in the liver has been observed in cachectic $Apc^{min/+}$ mice (Narsale et al., 2015). Upregulated and/or impaired autophagy has also been reported in cancer cachexia (Penna et al., 2013; Aversa et al., 2016). It is unknown if UPR is ubiquitously induced in other wasting and cachexia models such as in C26 tumor-bearing mice.

Blocking activin receptor ligands using the soluble ligand binding domain of a type 2B activin receptor fused to the Fc domain (sACVR2B-Fc) rapidly increases muscle size in mice (Lee et al., 2005; Zhou et al., 2010; Pistilli et al., 2011; Hoogaars et al., 2012; Hulmi et al., 2013a) and in humans (Attie et al., 2013). This occurs through increased protein synthesis (Hulmi et al., 2013a; Nissinen et al., 2016) but possibly in some situations through decreased protein degradation (Zhou et al., 2010). Increased muscle mass may not, however, always translate into better muscle function (Amthor et al., 2007; Relizani et al., 2014), perhaps in part due to qualitative changes in muscle (Amthor et al., 2007; Relizani et al., 2014; Hulmi et al., 2016; Marabita et al., 2016). However, the effect of rapid muscle hypertrophy induced by an activin receptor ligand blockade on protein homeostasis is currently unknown. We previously reported improved survival in C26 tumor-bearing mice with sACVR2B-Fc treatment (Nissinen et al., 2018). Cachectic mice had decreased protein synthesis in skeletal muscle and increased protein synthesis in the liver, the latter of which was alleviated by sACVR2B-Fc treatment (Nissinen et al., 2018). Thus, we further investigated the effects of cancer cachexia and sACVR2B-Fc treatment on protein homeostasis in muscle and the liver.

The purpose of this study was to elucidate the effects of muscle wasting induced by cancer cachexia, and muscle hypertrophy induced by sACVR2B-Fc treatment on biological processes contributing to the protein and redox homeostasis in skeletal muscle. Furthermore, because skeletal muscle and the liver are known to crosstalk (Whitham et al., 2018), we explored the effects of cancer and sACVR2B-Fc treatment on protein homeostasis in the liver as well. We hypothesized that both rapid atrophy and hypertrophy would alter several integrated processes that regulate protein homeostasis.

MATERIALS AND METHODS

Ethics Statement

The treatment of the animals was in strict accordance with the European convention for the protection of vertebrate animals used for experimental and other scientific purposes. The protocols were approved by the National Animal Experiment Board (Permit No.: ESLH-2009-08528/Ym-23 and ESAVI/10137/04.10.07/2014).

Animals and Cells

Single and 2-Week Administration of sACVR2B-Fc on Healthy Mice

Male, 6–7-week-old C57Bl/10SnJ mice were used as previously described (Hulmi et al., 2013a). The mice were purchased from the Jackson Laboratory (Bar Harbor, ME, United States).

The Cancer Cachexia Experiments

In the cancer cachexia experiments, 5–6-week-old male BALB/*c* (BALB/cAnCrl) mice (Charles River Laboratories) were used. All the mice were housed in standard conditions (temperature 22°C, light from 8:00 AM to 8:00 PM) and had free access to tap water and food pellets (R36, 4% fat, 55.7% carbohydrate, 18.5% protein, 3 kcal/g, Labfor, Stockholm, Sweden).

Tumor Cell Culture

Complete Dulbecco's Modified Eagle's Medium (DMEM, high glucose, GlutaMAX™ Supplement pyruvate, Gibco™, Life Technologies) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% FBS was used for the maintenance of the colon 26 carcinoma cells as previously described (Nissinen et al., 2018).

Experimental Design

This study consisted of three separate experiments (**Figure 1**), which are described in detail below.

Acute sACVR2B-Fc Experiment in Healthy Mice

To study the acute effects of sACVR2B-Fc administration in healthy skeletal muscle, mice were divided into three groups that were euthanized 1 or 2 days after a single injection of PBS (i.p.) or sACVR2B-Fc (10 mg/kg, i.p.) as previously described (Hulmi et al., 2013a; **Figure 1A**).

Two-Week sACVR2B-Fc Experiment in Healthy Mice

To study the short-term effects of sACVR2B-Fc administration in healthy skeletal muscle, mice were randomly divided into mice administered (i.p., 1–2 times per week) with (1) PBS or (2) sACVR2B-Fc as previously described (Hulmi et al., 2013a; **Figure 1B**).

Cancer Cachexia Experiment

Mice were randomized into one of the four weight-matched groups: (1) vehicle-treated (PBS) healthy control mice (CTRL), (2) C26 tumor-bearing mice administered with a vehicle (PBS) (C26 + PBS), (3) C26 tumor-bearing mice administered with sACVR2B-Fc before the C26 tumor formation and replaced by a vehicle (PBS) after the tumor formation (C26 + sACVR/b), and (4) C26 tumor-bearing mice continuously administered with sACVR2B-Fc throughout the experiment (C26 + sACVR/c). Cancer groups were inoculated subcutaneously (s.c.) with C26 colon carcinoma cells (5×10^5 cells in ∼ 120 μl PBS) into their interscapular region. The vehicle (PBS) and sACVR2B-Fc (5 mg/kg in ∼ 100 μl PBS) were administered (i.p.) every fourth day (**Figure 1C**). The weight loss from day 10 to 11 after C26 inoculation strongly predicted survival (Nissinen et al., 2018) and thus the end-point was chosen to be 11 days after the C26 inoculation to represent the onset of cachexia. When the symptoms of cancer started to occur, mice were strictly and carefully monitored according to the end-point guidelines of animal experiments approved by the National Animal Experiment Board. However, none of the mice fulfilled the end-point criteria before the actual end-point.

sACVR2B-Fc Production

The production of the recombinant sACVR2B-Fc, which is similar but not totally identical with the original version generated by Lee et al. (2005), has been described in detail before (Hulmi et al., 2013a). Briefly, we fused a human IgG1 Fc domain with the ectodomain of human ACVR2B and the protein was expressed in Chinese hamster ovary cells grown in suspension culture.

Tissue Collection and Processing

Tissue Collection

In the acute and 2-week experiments the mice were euthanized by cervical dislocation and gastrocnemius muscles were weighed and subsequently flash frozen in liquid nitrogen and stored at −80°C for further analysis. In the cancer experiment mice were euthanized by cervical dislocation after heart puncture under anesthesia [ketamine (Ketaminol®): ∼110–120 mg/kg + xylazine (Rompun®): 15–16 mg/kg]. Gastrocnemius, tibialis anterior (TA) and liver were weighed and flash frozen in liquid nitrogen and were stored at −80°C for further analysis.

RNA Extraction and cDNA Synthesis

In the cancer cachexia experiment, total RNA was extracted from gastrocnemius muscles with QIAzol and were purified with RNeasy Universal Plus kit (Qiagen). The quality of RNA was confirmed by spectrophotometry (NanoDrop; Thermo Fisher Scientific) and agarose gel electrophoresis. iScript™ Advanced cDNA Synthesis Kit (Bio-Rad Laboratories) was used to reverse transcribe the RNA to cDNA. Both steps were conducted according to the manufacturer's guidelines.

Protein Extraction and Content Measurement

Muscle and liver samples were homogenized in ice-cold buffer with protease and phosphatase inhibitors as previously described (Hulmi et al., 2013a; Nissinen et al., 2018). The total protein content was measured using the bicinchoninic acid protein assay (Pierce Biotechnology, Rockford, IL, United States) with an automated KoneLab analyzer (Thermo Fisher Scientific, Vantaa, Finland).

Tissue Analyses

Real-Time-qPCR

mRNA expression levels were analyzed with RT-qPCR following standard procedures using iQ SYBR Supermix (Bio-Rad Laboratories) and CFX96 real-time PCR Detection system. The mRNA levels of *Chop*/*Ddit3* (assay ID qMmuCID0020314), *Lc3b* (assay ID qMmuCED0048150), and *P62* (assay ID qMmuCID0024517) were measured with pre-designed and pre-validated primers (Bio-Rad PrimePCR™ SYBR Green assays). The protocol recommended by the manufacturer was used. The spliced variant mRNA level of X-box binding protein 1 (*Xbp1s*) was analyzed using SYBR green primers: forward: TGCTGAGTCCGCAGCAGGTG and reverse: CTGATGAGGTCCCCACTGACAGA (Invitrogen, United States). The protocol for the *Xbp1s* was initiated at 95°C which was followed by 39 cycles of denaturation at 95°C for 10 s, annealing at 61°C for 30 s and extension at 68°C for 30 s. mRNA expression levels were calculated from the exponential amplification phase using the efficiency corrected ΔΔCT method. *36b4* (Forward primer: 5′-GGCCCTGCACTCTCGCTTTC-3′, Reverse primer: 5′-TGCCAGGACGCGCTTGT-3′) was used as a house-keeping reference gene because it was unaffected by the cancer and the sACVR2B-Fc treatments as previously reported ($p > 0.16$) (Nissinen et al., 2018).

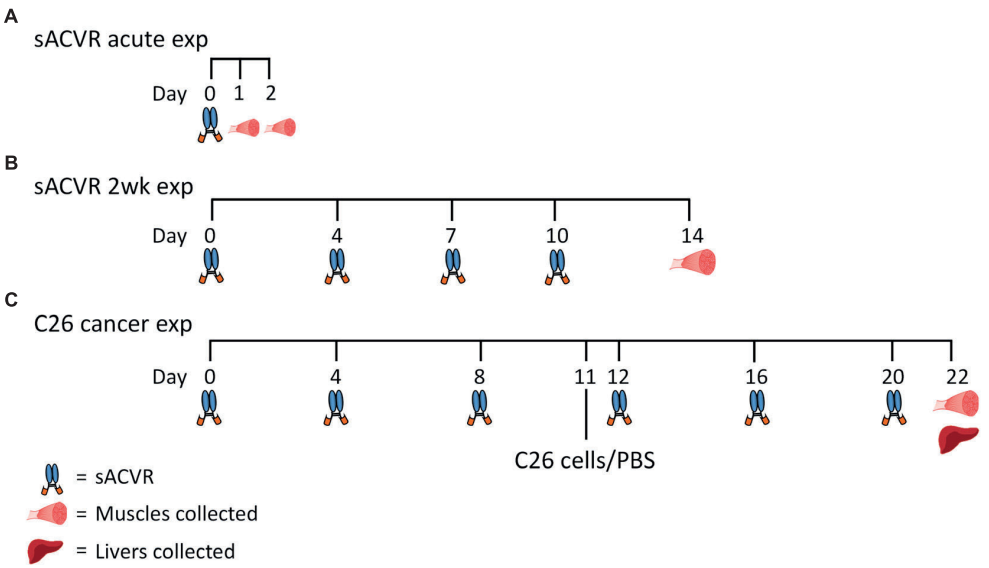

FIGURE 1 | Overall study design. **(A)** Healthy mice were administered (i.p.) with a single dose of vehicle (PBS) or soluble activin receptor type 2B (sACVR) to block ACVR2B ligands. Muscles were collected either 1 or 2 days after the single sACVR or vehicle administration (n = 6–7 per group). **(B)** Healthy mice were administered (i.p.) with vehicle (PBS) or sACVR for 2 weeks (1–2 times per week) and muscles were collected (n = 6 in PBS and n = 11 in sACVR 2wk). **(C)** C26 cancer experiment: (1) CTRL (+PBS, n = 9), (2) C26 + PBS (n = 7), (3) C26 + sACVR/b (n = 7), and (4) C26 + sACVR/c (n = 8). Cancer groups were inoculated subcutaneously (s.c.) with C26 cells on day 11. The vehicle (PBS) and sACVR2B-Fc were administered (i.p.) every fourth day. Muscles and livers were collected 11 days after the C26 cell inoculation.

Western Blot

Western blot analyses were conducted in two laboratories and therefore slightly different protocols are provided below:

LC3B, P62, Bcl-2, Beclin-1, p-ULK1^{ser757}*, ULK1, PERK, p-eIF2α*^{ser51}*, total eIF2α, IRE1α, p-JNK54, JNK54, PDI, ATF4, Cleaved Caspase 3 and 12* as well as *HSP25, GRP78, and HSP47* were analyzed only from the cancer experiment by a protocol previously described in more detail (Hulmi et al., 2013a; Hentila et al., 2018). In short, muscle and liver homogenates mixed with Laemmli sample buffer + β-mercaptoethanol were heated at 95°C to denature proteins. Proteins were separated with SDS–PAGE and transferred to a PVDF membrane, blocked (5% fat-free milk in TBS-T) and incubated overnight at 4°C with primary antibodies. The membrane was then washed and incubated with secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, United States) for 1 h followed by washing. Proteins were visualized by enhanced chemiluminescence (SuperSignal West Femto maximum sensitivity substrate, Pierce Biotechnology, Rockford, IL, United States) using a ChemiDoc XRS device and quantified with Quantity One software (version 4.6.3. Bio-Rad Laboratories, Hercules, CA, United States). The uniformity of the protein loading was confirmed by staining the membrane with Ponceau S and by re-probing the membrane with an antibody against GAPDH (Abcam, Cambridge, United Kingdom). The results were normalized to the mean of Ponceau S (strong band at ∼42 kDa) and GAPDH value.

HSP60, HSP70, HSP90 TRX, and TxNIP as well as *GRP78, HSP25, and HSP47* were analyzed from the acute experiments by a protocol as described previously (Atalay et al., 2004; Lappalainen et al., 2009). Briefly, protein extracts (20 μg protein per well) with molecular weight markers were electrophoresed on SDS/PAGE and transferred to a nitrocellulose membrane (Millipore, Bedford, MA, United States). The uniformity of the protein loading was confirmed by staining the membrane with Ponceau S and by re-probing the membrane with an antibody against Actin (Sigma, A-2066). Membranes were blocked with 5% fat-free milk solution at 37°C for 1 h and treated with monoclonal or polyclonal antibodies overnight at 4°C (StressGen, VIC, Canada; IMCO, Stockholm, Sweden; MBL International, Woburn, MA, United States; Sigma, St. Louis, MO, United States). Immunoblots were visualized by Odyssey (LI-COR Biosciences Inc., Lincoln, NB, United States) and quantified by Odyssey Software.

Antibodies

Antibodies for IRE1α (#3294), PDI (#3501), PERK (#3192), eIF2α (#5324) and its phosphorylated form at ser51 (#3398, p-eIF2α^{ser51}) GRP78 (used in the cancer experiment, #3177), P62 (#5114), Bcl-2 (#3498), p-ULK1^{ser757} (#14202), ULK1 (#8054), p-JNK$^{Thr183/Tyr185}$ (#4668), JNK (#9252), ATF4 (#11815), Beclin-1 (#3495), caspase 12 (#2202), and cleaved caspase 3 (#9661) were purchased from Cell Signaling Technology. GAPDH (ab9485) antibody was purchased from Abcam (Cambridge, United Kingdom). LC3I and LC3II were measured by antibody (L7543) that was purchased from Sigma-Aldrich (St. Louis, MO, United States). Monoclonal primary antibodies were used for the detection of heat shock protein 70 (HSP70, StressGen, SPA-810),

heat shock protein 60 (HSP60, StressGen, SPA-806), heat shock protein 90 (HSP90, StressGen, SPA-835), heat shock protein 47 (HSP47, StressGen, SPA-470) and thioredoxin interacting protein (TXNIP and MBL). Polyclonal primary antibodies were used to detect thioredoxin (TRX, IMCO, and ATRX-06), actin (Sigma, A-2066), heat shock protein 25 (HSP25, StressGen, and SPA-801), glucose-regulated protein 78 (GRP78, StressGen, SPA-826, used in the acute experiments). Horseradish peroxidase conjugated IgG secondary antibodies were used (Jackson ImmunoResearch Laboratories, PA, United States and StressGen and Zymed, San Francisco, CA, United States).

Analysis of Protein Carbonyls

In the acute and 2 weeks experiments protein carbonyls were analyzed by western blot technique after derivatization with 2,4-dinitrophenyl hydrazine immediately before the electrophoresis, as previously described (Hulmi et al., 2016). In the C26 cancer experiment, the principle of the measurement was the same as with acute and 2 weeks experiment. However, the measurement was carried out with a commercial OxyBlot Protein Oxidation Detection kit (Merck Millipore, S1750) according to manufacturer's instructions as previously described (Hentila et al., 2018).

Glutathione Assays

After gastrocnemius muscle homogenization, total glutathione (TGSH) was measured spectrophotometrically by an oxidized glutathione (GSSG) reductase recycling method as described earlier (Lappalainen et al., 2009; Hulmi et al., 2016). The rate of change in absorbance at 412 nm was monitored with a double-beam spectrophotometer at room temperature and tissue concentrations were estimated by linear regressions from the standard curve.

Statistical Methods

The main effect was analyzed by one-way analysis of variance (ANOVA) or the Kruskal-Wallis test, followed by Holm-Bonferroni corrected LSD or Mann-Whitney U *post hoc* tests depending on the distribution of the data (Shapiro-Wilk). The effect of 2-week sACVR2B-Fc (sACV 2wk vs. PBS 2wk) administration was examined with Student's *t* test or Mann Whitney U if data were not normally distributed. The C26 cancer effect (CTRL vs. C26 + PBS and CTRL vs. C26 groups pooled) was analyzed with Student's *t* test or the Mann-Whitney *U*-test when data were not normally distributed. Correlations were analyzed using Pearson's product-moment coefficient.

PASW statistics version 24.0 was used for statistical analyses (SPSS, Inc., Chicago, IL, United States). The level of significance was set at $P \leq 0.05$. Data are expressed as means ± SE.

RESULTS

Background Results

This is a follow-up study on our two previous studies. In brief, in Nissinen et al. (2018) we showed that C26 tumor implantation resulted in muscle and fat wasting and increased hepatic protein synthesis as well as acute phase response, a cytokine-induced early defense mechanism (Cray et al., 2009). Treating mice with sACVR2B-Fc increased muscle mass and protein synthesis in healthy mice (Hulmi et al., 2013a) and prevented muscle loss and prolonged survival in tumor-bearing mice without affecting the tumor size when sACVR2B-Fc was administered continuously before and after the C26 cell inoculation (Nissinen et al., 2018).

Two-Week sACVR2B-Fc Administration Induces Unfolded Protein Response

The protein content of UPR indicators and ER-resident chaperones were unchanged 1 and 2 days after the single sACVR2B-Fc administration (**Figures 2A–G**) when muscle protein synthesis was greatly induced (Hulmi et al., 2013b). Later, after 2 weeks of sACVR2B-Fc administration, the phosphorylation of eIF2α^{Ser51} ($p < 0.05$) was increased without changes in total eIF2α (**Figures 2A,B**). In addition, ER resident chaperones GRP78 ($p < 0.05$) and HSP47 ($p < 0.01$) were increased (**Figures 2C,D**), suggesting partial induction of UPR by 2-week sACVR2B-Fc administration, while other UPR indicators were unchanged (PERK, PDI, and IRE1α) (**Figures 2E–G**).

Protein Carbonylation and the Reduced Form of Glutathione Are Increased by sACVR2B-Fc Administration

As a marker of oxidative damage, protein carbonyls were increased 1 and 2 days after the sACVR2B-Fc administration in skeletal muscle (Day 1: $p < 0.05$, Day 2: $p = 0.06$, **Figure 3A**) and remained increased after 2 weeks of sACVR2B-Fc administration ($p < 0.05$) (**Figure 3A**). Possibly as a delayed response to increased oxidative stress, there was an increase in reduced glutathione ($p < 0.05$, **Figure 3B**) and a trend for increased TRX protein content ($p = 0.10$, **Figure 3E**) after 2 weeks of sACVR2B-Fc administration without changes in the protein content of TxNIP (**Figure 3F**). Oxidized glutathione (GSSG) concentration (**Figure 3C**) and the ratio of oxidized and reduced glutathione (GSSG/GSH) (**Figure 3D**) were unchanged by the sACVR2B-Fc administration in all the time-points. Of the heat shock response indicators, 2-week sACVR2B-Fc administration increased only the protein content of small heat shock protein 25 (HSP25) at 2 weeks ($p < 0.001$, **Figure 4A**) without changes in larger HSPs 60, 70, and 90 at any time-point (**Figures 4B–D**).

Decreased Glutathione Levels in C26 Tumor-Bearing Mice Are Restored by Continued sACVR2B-Fc Administration

Next, we investigated the effects of the C26 tumor that induced cachexia and sACVR2B-Fc administration that alleviated cachexia. As a marker of skeletal muscle redox balance, the reduced form of glutathione was decreased in PBS-treated tumor-bearing mice compared with healthy controls ($p < 0.001$, **Figure 5A**). This was accompanied by increased ratio of oxidized glutathione and reduced glutathione (GSSG/GSH), suggesting increased oxidative stress ($p < 0.001$, **Figure 5B**). Interestingly, sACVR/c administration prevented the decrease in reduced glutathione levels ($p < 0.05$), thus decreasing the GSSG/GSH

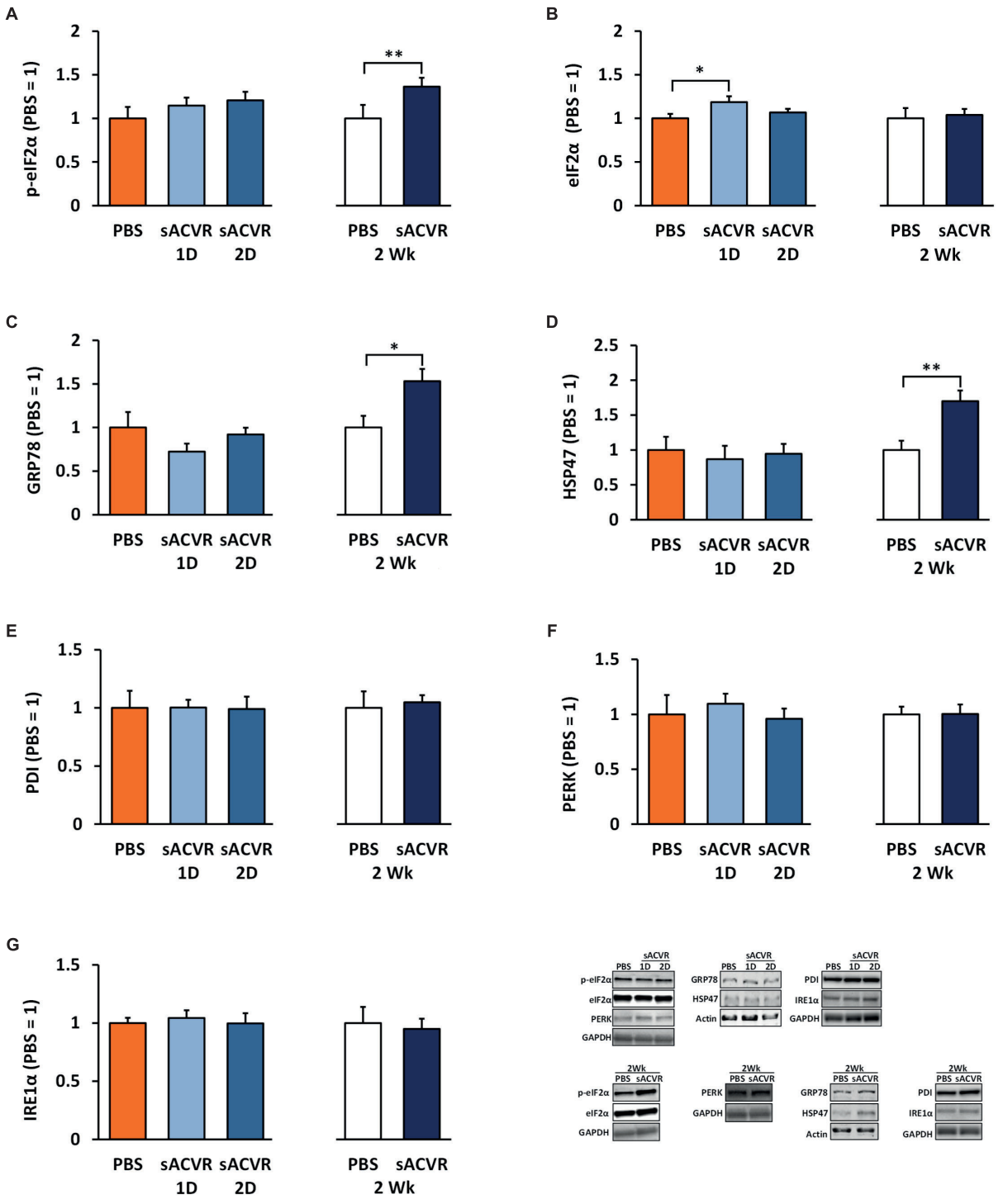

FIGURE 2 | Unfolded protein response (UPR) markers in healthy skeletal muscle in mice that were administered with soluble activin receptor type 2B (sACVR) or vehicle (PBS). Muscles were collected 1 or 2 days after a single sACVR administration or after 2 weeks of administration. **(A)** p-eIF2α^{ser51}, **(B)** total eIF2α protein, **(C)** GRP78 protein, **(D)** HSP47 protein, **(E)** PDI protein, **(F)** PERK protein, **(G)** IRE1α protein; n = 6–7 in all groups except n = 9–11 in sACVR 2 Wk group. The symbol * depicts statistical significance $p < 0.05$ whereas the symbol ** depicts statistical significance $p < 0.01$. Data is expressed as means ± SE. Representative blots were cropped from the original blot images (**Supplementary Figures 6–8**). Representative blots with same IDs and analyzed in the same run are aligned on top of each other and share the same representative control blot (GAPDH or actin).

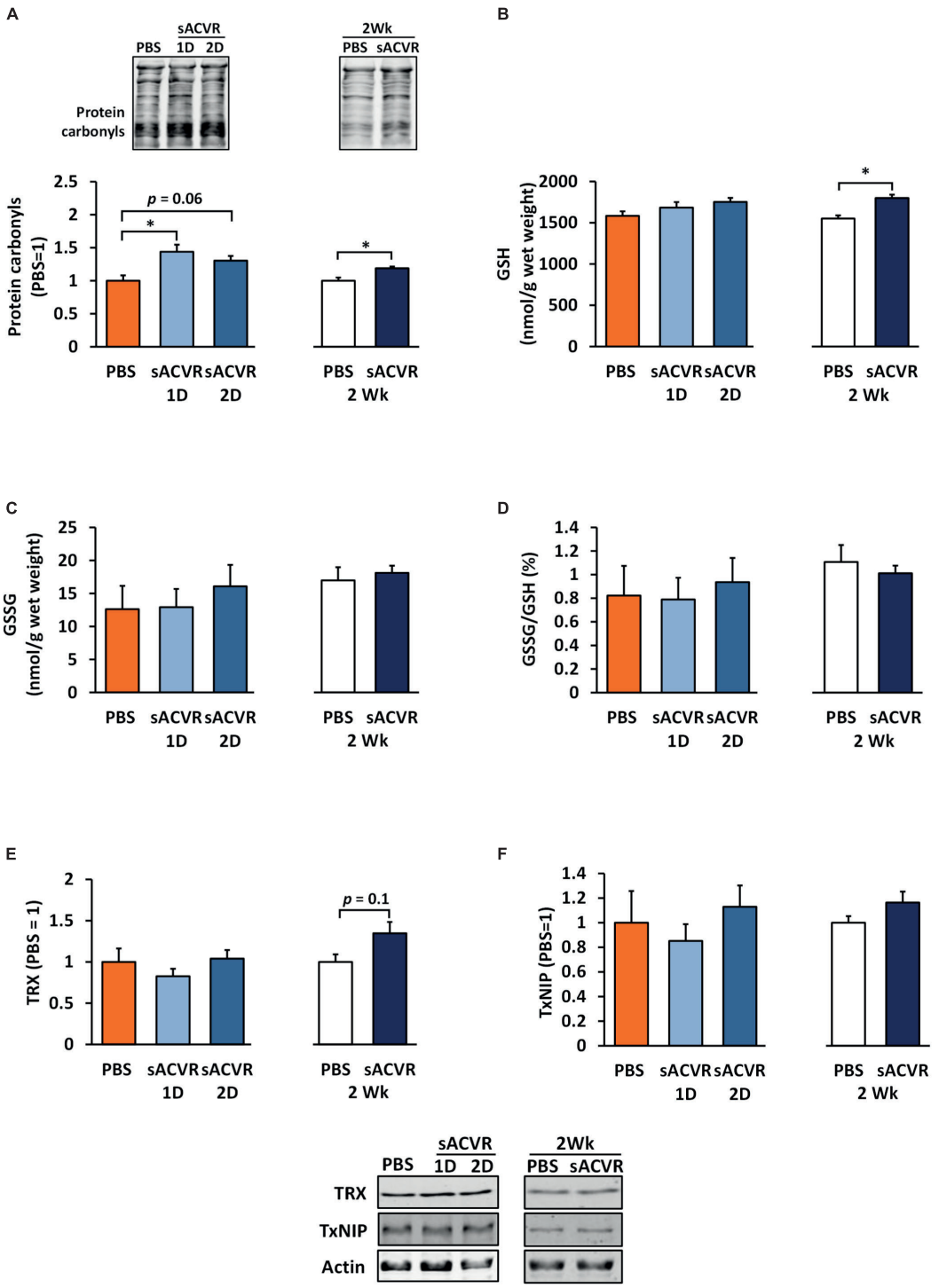

FIGURE 3 | Redox-balance markers in healthy skeletal muscle in mice that were administered with soluble activin receptor type 2B (sACVR) or vehicle (PBS). Muscles were collected 1 or 2 days after a single sACVR administration or after 2 weeks of administration. **(A)** Protein carbonyls, **(B)** reduced glutathione (GSH), **(C)** oxidized glutathione (GSSG), **(D)** ratio of oxidized glutathione to reduced glutathione (GSSG/GSH), **(E)** TRX protein, **(F)** TxNIP protein; n = 5–6 in all groups except n = 8–11 in sACVR 2 Wk group. The symbol * depicts statistical significance $p < 0.05$. Data is expressed as means ± SE. Representative blots were cropped from the original blot images (**Supplementary Figure 7**). Representative blots of TRX and TxNIP share the same IDs and were analyzed in the same run and are thus aligned on top of each other and share the same representative control blot (actin). Protein carbonyls were normalized to total protein loading.

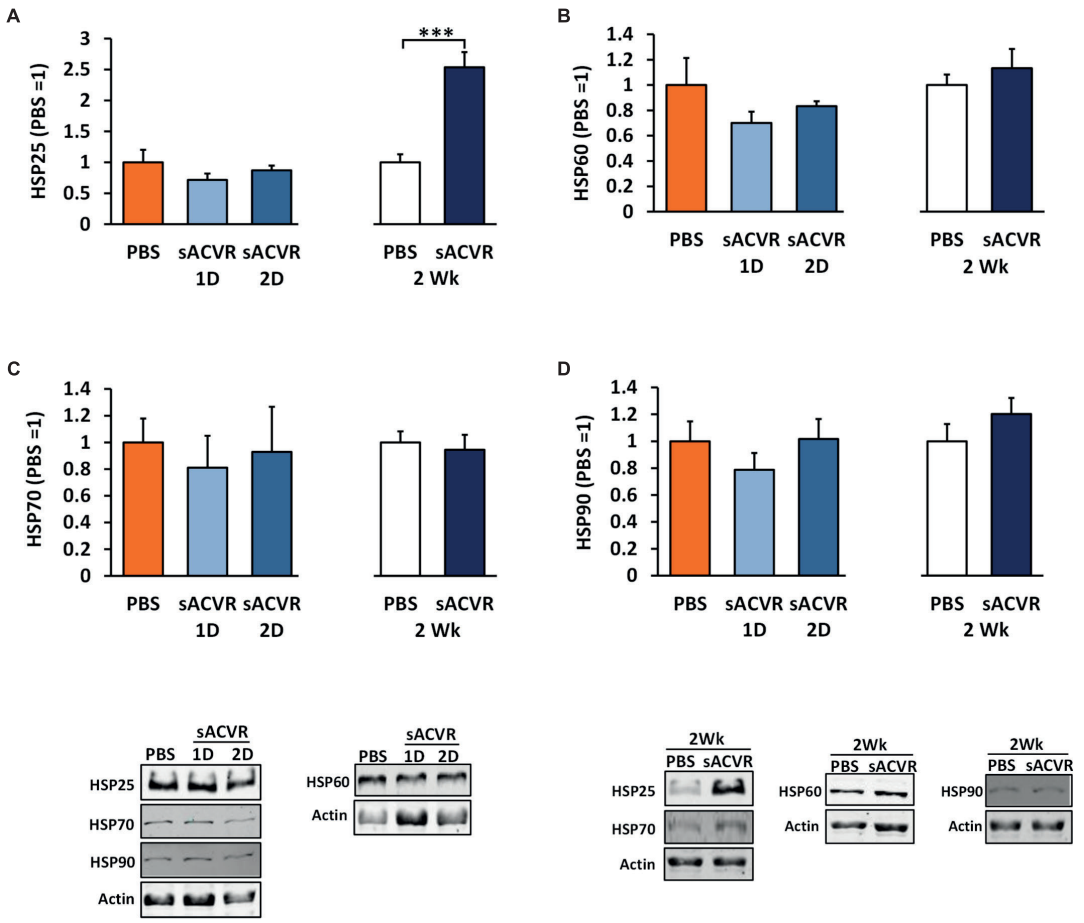

FIGURE 4 | Heat shock protein (HSP) response markers in healthy skeletal muscle in mice that were administered with soluble activin receptor type 2B (sACVR) or vehicle (PBS). Muscles were collected 1 or 2 days after a single sACVR administration or after 2 weeks of administration. **(A)** HSP25, **(B)** HSP60, **(C)** HSP70, and **(D)** HSP90; n = 6–7 in all groups except n = 10–11 in sACVR 2 Wk group. The symbol *** depicts statistical significance $p < 0.001$. Data is expressed as means ± SE. Representative blots were cropped from the original blot images (**Supplementary Figure 8**). Representative blots with same IDs and analyzed in the same run are aligned on top of each other and share the same representative control blot (actin).

ratio (pooled sACVR effect, $p < 0.05$) (**Figure 5B**). Oxidized glutathione concentration (GSSG) (**Figure 5C**) and a marker of oxidative damage/stress, protein carbonyl content were unaltered by cancer and sACVR2B-Fc treatment (**Figure 5D**).

Cancer Cachexia Is Associated With Decreased HSP47, p-eIF2α, and p-JNK54 of the UPR Indicators in Muscle

Of the UPR markers, C26 cancer decreased the phosphorylation of eIF2α at Ser^{51} and the phosphorylation of JNK54 at Thr^{183}/Tyr^{185}, as well as decreased the levels of HSP47 protein in skeletal muscle ($p < 0.05$, **Figure 6A**). In addition, the mRNA level of the pro-apoptotic indicator *Chop* tended to be decreased when tumor-bearing groups were pooled ($p = 0.06$, **Figure 6B**). Other UPR indicators remained unaltered by C26 cancer (**Figures 6A,C**). The continued sACVR2B treatment (sACVR/c) increased GRP78 protein ($p < 0.05$) compared to PBS-administered mice and tended to increase p-eIF2α^{Ser51} ($p = 0.18$), HSP47 ($p = 0.11$), and p-JNK54 ($p = 0.14$) to healthy control levels (**Figure 6A**). A mitochondrial UPR marker HSP10 and HSP25 were unaltered by C26 cancer (**Figures 6D,E**). However, when sACVR2B treated groups were pooled, HSP25 protein was increased compared to PBS-treated C26 tumor-bearing mice ($p < 0.05$, **Figure 6E**).

Selective Hepatic UPR Is Induced in Tumor-Bearing Mice

In the liver, the UPR indicators PERK ($p < 0.05$), p-eIF2α^{Ser51} ($p < 0.01$) (no change in total eIF2α) and GRP78 ($p < 0.001$) were increased by C26 cancer (**Figures 7A–D**). However, ATF4 protein content was greatly decreased ($p < 0.01$, **Figure 7E**) and there was a trend for decrease in IRE1α ($p = 0.053$, **Figure 7F**). Phosphorylation of JNK was unaltered by cancer (**Figure 7G**) but total JNK was increased in tumor-bearing mice

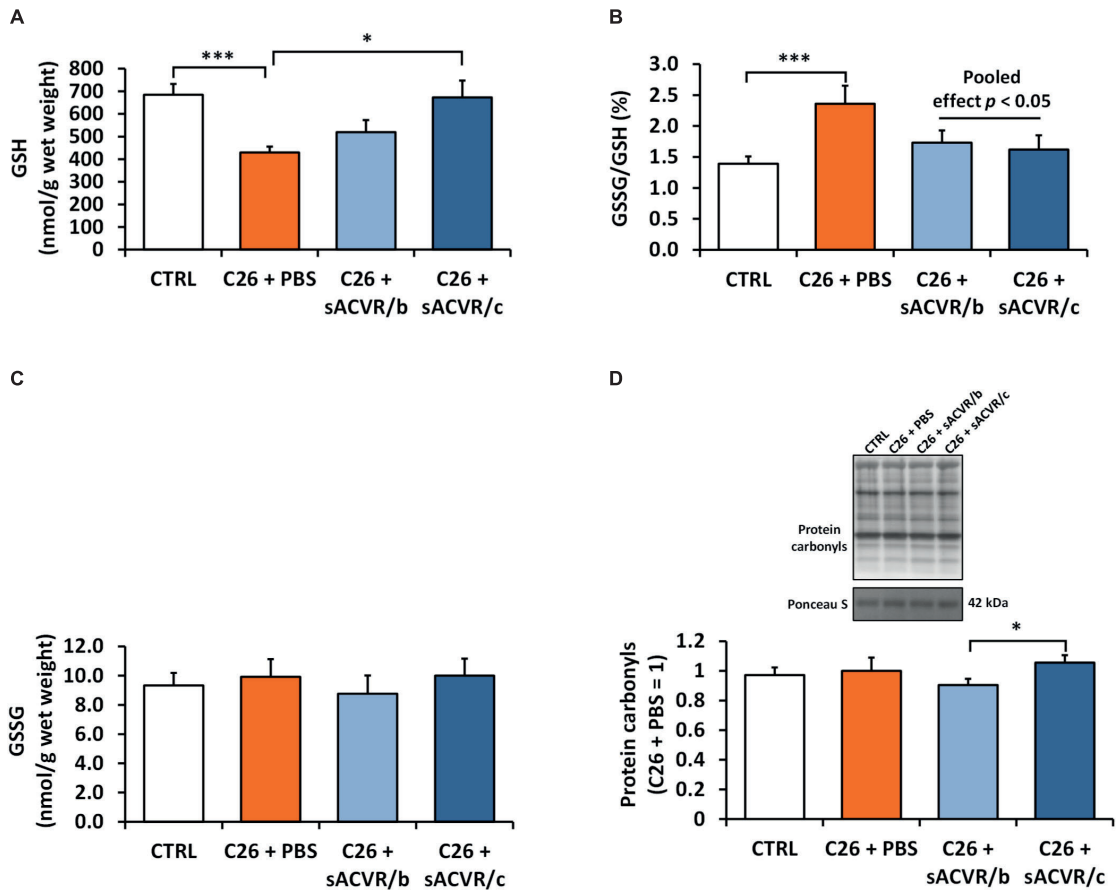

FIGURE 5 | Redox balance markers in skeletal muscle of C26 tumor-bearing mice. **(A)** Reduced glutathione (GSH), **(B)** ratio of oxidized to reduced glutathione (GSSG/GSH), **(C)** oxidized glutathione (GSSG), and **(D)** protein carbonyls. CTRL, vehicle-treated (PBS) healthy control mice, n = 7–9. C26 + PBS = C26 tumor-bearing mice administered with a vehicle (PBS), n = 7. C26 + sACVR/b = C26 tumor-bearing mice administered with sACVR before the tumor formation and replaced by a vehicle (PBS) after the tumor formation, n = 7. C26 + sACVR/c = C26 tumor-bearing mice continuously administered with sACVR throughout the experiment, n = 8. The symbol * depicts statistical significance $p < 0.05$ whereas the symbol *** depicts statistical significance $p < 0.001$. For the pooled effect sACVR administered groups were pooled. Data is expressed as means ± SE. Protein carbonyls were normalized to Ponceau S staining (strongest band at ~42 kDa). Representative protein carbonyl blot was cropped from the original blot image (**Supplementary Figure 10**).

when groups were pooled ($p < 0.05$, **Figure 7H**). sACVR2B-Fc administration had no significant effect on any of the variables. Pro caspase 12 was decreased ($p < 0.05$) by C26 cancer but its cleaved form was unchanged (**Supplementary Figure 1**). In addition, cleaved caspase 3 (**Supplementary Figure 1**) was unchanged, suggesting that apoptosis was not activated at this time point.

Autophagy-Lysosome Pathway Is Induced in Skeletal Muscle and Liver of Tumor-Bearing Mice

In skeletal muscle, lipidated LC3 (LC3II) ($p = 0.051$) and the ratio of LC3II to LC3I ($p < 0.05$) which can be used as a marker of autophagosome content, were both increased by the C26 cancer (**Figure 8A**). In addition, Beclin-1, involved in autophagy induction was increased by C26 cancer ($p < 0.01$, **Figure 8A**). Moreover, the protein content of P62, which acts as an adaptor protein sequestering cellular compartments that are to be degraded by autophagy–lysosome pathway, was also increased by C26 cancer ($p < 0.01$, **Figure 8A**). *Lc3b* mRNA ($p < 0.05$, **Figure 8C**) was increased by cancer whereas the protein content of LC3I, p-ULK1^{Ser757} as well as total ULK1 and Bcl-2 and *p62* mRNA were unaltered (**Figures 8A,B**).

LC3II ($p < 0.05$), LC3II/I ($p < 0.01$), and Beclin-1 ($p < 0.05$) increased in the liver by the C26 cancer without changes in p-ULK1^{Ser757} and P62 protein (**Figure 8D**). Contrary to skeletal muscle, LC3I ($p < 0.05$) increased in the liver by C26 cancer. The discontinued administration of sACVR2B-Fc further increased LC3II/I in skeletal muscle compared to other tumor-bearing groups ($p < 0.01$, **Figure 8A**). Otherwise there were no systematic effects of sACVR2B-Fc administration on the autophagy indicators in skeletal muscle or in the liver (**Figures 8A–D**).

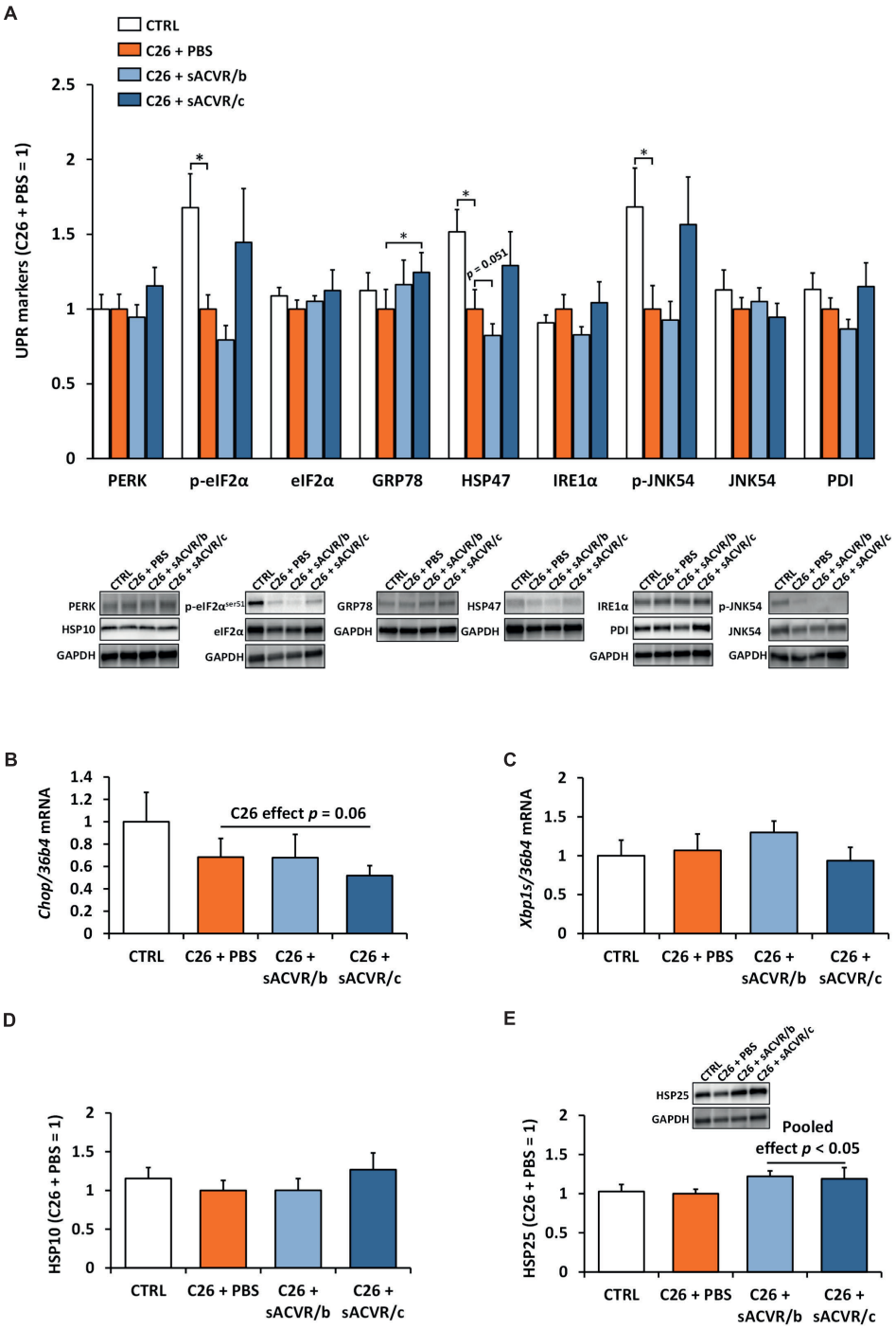

FIGURE 6 | Unfolded protein response (UPR) and heat shock protein (HSP) response markers in skeletal muscle of C26 tumor-bearing mice. **(A)** Protein content of UPR markers (PERK, p-eIF2α^{ser51}, eIF2α, GRP78, HSP47, IRE1α, p-JNK54, JNK54, and PDI), **(B)** mRNA level of *Chop*, and **(C)** of spliced *Xbp1 (Xbp1s)*. **(D)** Protein content of HSP10 and **(E)** HSP25. CTRL, vehicle-treated (PBS) healthy control mice, $n = 7$. C26 + PBS = C26 tumor-bearing mice administered with a vehicle (PBS), $n = 7$. C26 + sACVR/b = C26 tumor-bearing mice administered with sACVR before the tumor formation and replaced by a vehicle (PBS) after the tumor formation, $n = 7$. C26 + sACVR/c = C26 tumor-bearing mice continuously administered with sACVR throughout the experiment, $n = 8$. The symbol * depicts statistical significance $p < 0.05$. For the C26 effect all the tumor-bearing groups were pooled. For the sACVR pooled effect both of the sACVR groups were pooled. Data is expressed as means ± SE. Representative blots were cropped from the original blot images (**Supplementary Figures 9**, **10**). Representative blots with same IDs and analyzed in the same run are aligned on top of each other and share the same representative control blot (GAPDH).

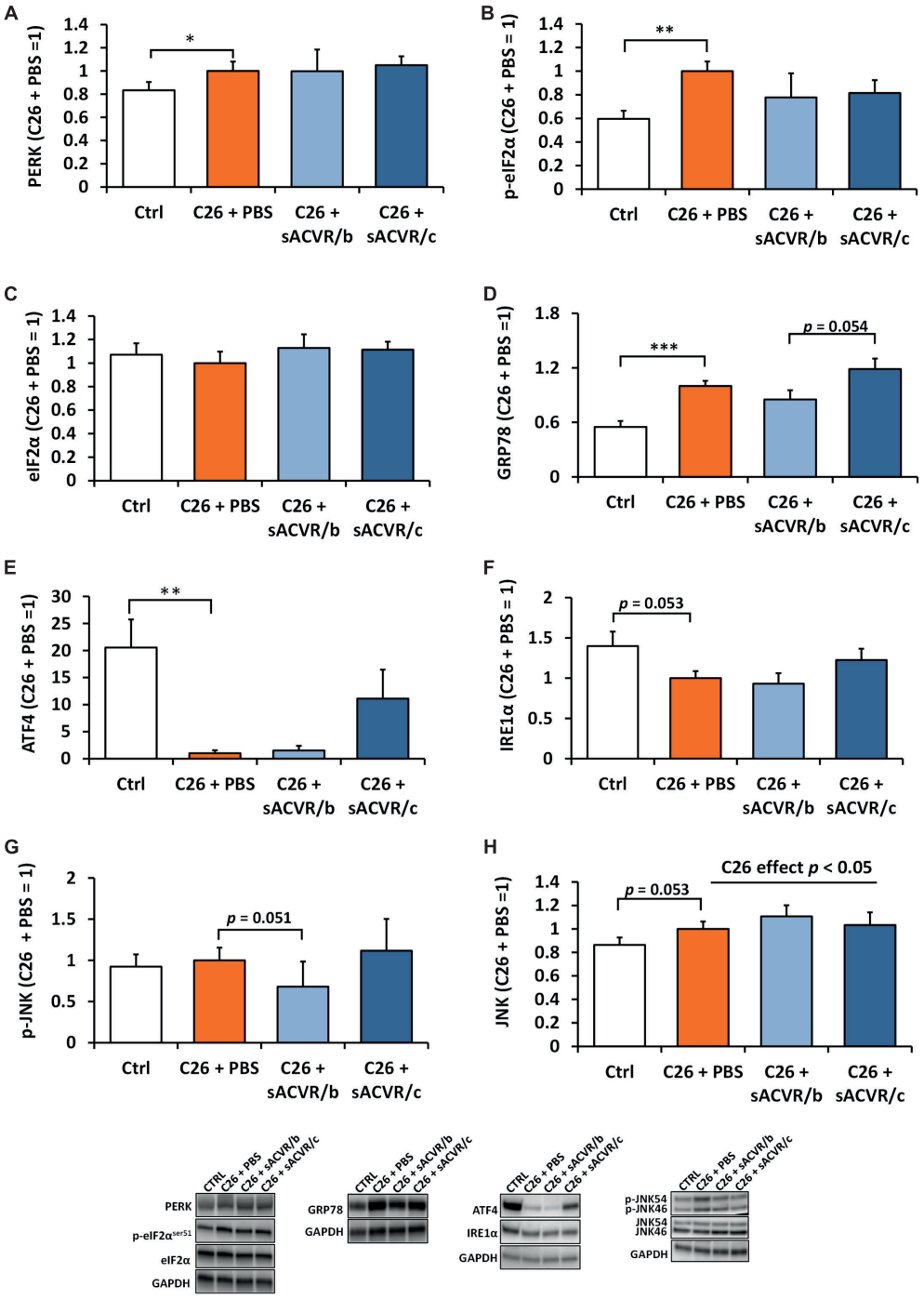

FIGURE 7 | Unfolded protein response (UPR) markers in the liver of C26 tumor-bearing mice. **(A)** PERK protein, **(B)** p-eIF2α^{ser51}, **(C)** eIF2α, **(D)** GRP78 protein, **(E)** ATF4 protein, **(F)** IRE1α protein, **(G)** mean of p-JNK46 and p-JNK54, and **(H)** mean of total JNK46 and JNK54 protein. CTRL, vehicle-treated (PBS) healthy control mice, $n = 9$. C26 + PBS = C26 tumor-bearing mice administered with a vehicle (PBS), $n = 7$. C26 + sACVR/b = C26 tumor-bearing mice administered with sACVR before the tumor formation and replaced by a vehicle (PBS) after the tumor formation, $n = 7$. C26 + sACVR/c = C26 tumor-bearing mice continuously administered with sACVR throughout the experiment, $n = 8$. The symbol * depicts statistical significance $p < 0.05$, the symbol ** depicts statistical significance $p < 0.01$ and the symbol *** depicts statistical significance $p < 0.001$. For the C26 effect, tumor-bearing groups were pooled. Data is expressed as means ± SE. Representative blots were cropped from the original blot images (**Supplementary Figures 11**, **12**). Representative blots with same IDs and analyzed in the same run are aligned on top of each other and share the same representative control blot (GAPDH).

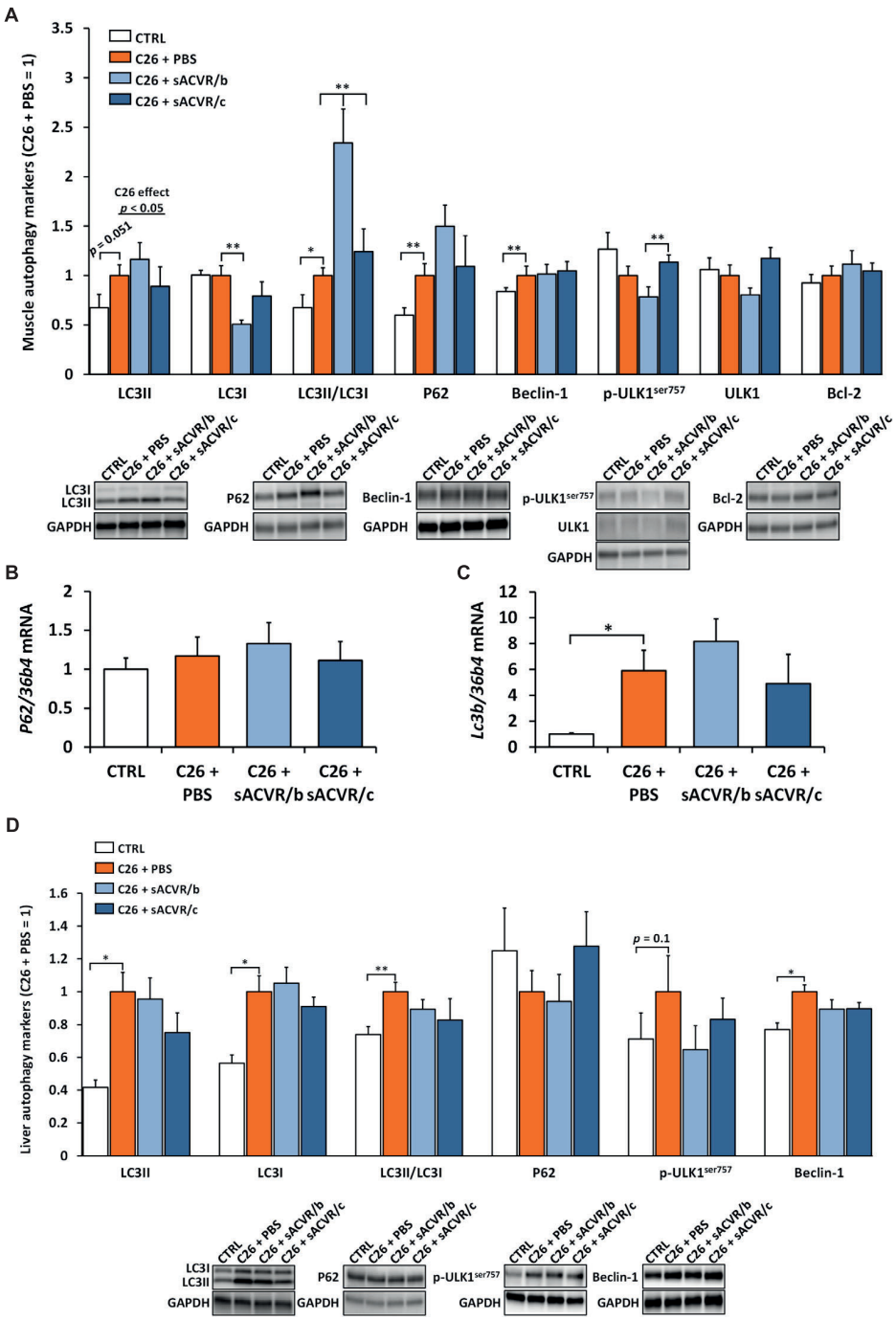

FIGURE 8 | Autophagy markers in skeletal muscle and the liver of C26 tumor-bearing mice. **(A)** Protein content of autophagy markers in skeletal muscle (LC3II, LC3I, LC3II/LC3I, P62, Beclin-1, p-ULK1^{ser757}, total ULK1, and Bcl-2). The mRNA level of **(B)** *P62* and **(C)** *Lc3b* in skeletal muscle. **(D)** Protein content of autophagy markers in the liver (LC3II, LC3I, LC3II/LC3I, P62, p-ULK1^{ser757}, and Beclin-1). CTRL, vehicle-treated (PBS) healthy control mice, n = 7–9. C26 + PBS = C26 tumor-bearing mice administered with a vehicle (PBS), n = 7. C26 + sACVR/b = C26 tumor-bearing mice administered with sACVR before the tumor formation and replaced by a vehicle (PBS) after the tumor formation, n = 7. C26 + sACVR/c = C26 tumor-bearing mice continuously administered with sACVR throughout the experiment, n = 8. The symbol * depicts statistical significance $p < 0.05$ and the symbol ** depicts statistical significance $p < 0.01$. For the C26 effect, tumor-bearing groups were pooled. Data is expressed as means ± SE. Representative blots were cropped from the original blot images (**Supplementary Figures 9, 11**, **12**). Representative blots with same IDs and analyzed in the same run are aligned on top of each other and share the same representative control blot (GAPDH).

DISCUSSION

We demonstrated that muscle hypertrophy induced by blocking ACVR2B ligands increased markers of unfolded protein response (UPR) and the reduced form of glutathione. On the other hand, experimental C26 cancer cachexia was accompanied by decreased levels of reduced glutathione, indicating a decline in antioxidant defense capacity in skeletal muscle. Interestingly, blocking ACVR2B ligands restored this decline. Moreover, we showed that selective UPR was induced in the liver, but not in skeletal muscle during the onset of rapid weight loss in experimental cancer.

We (Nissinen et al., 2018) and others (Cai et al., 2004; Zhou et al., 2010) have previously shown that the prevention of cancer cachexia without any effect on tumor growth improves survival in experimental mouse models. This result suggests a possible causality between the prevention of muscle loss and improved survival in cachexia, but its underlying mechanisms are unknown. More specifically, we showed that a soluble growth factor receptor, namely sACVR2B-Fc, improved survival in C26 cancer cachexia only when the treatment continued after the tumor formation, whereas a discontinued prophylactic treatment had no positive survival effects (Nissinen et al., 2018). In the present study, we further investigated the tumor-bearing mice and elucidated the role of biological processes regulating protein homeostasis. We observed altered redox balance, which shifted toward increased oxidative stress manifested by decreased levels of reduced glutathione (GSH) resulting in an increased ratio of oxidized to reduced glutathione (GSSG/GSH) in the skeletal muscle of tumor-bearing mice. The result is consistent with previous studies in which hallmarks of altered redox state were manifested by decreased levels of GSH (Ham et al., 2014) and a depletion of antioxidant peptides (glutathione, anserine, and carnosine), accompanied by a shift of the ratio of mannitol and mannose toward the oxidized form (Der-Torossian et al., 2013) in the skeletal muscle of tumor-bearing mice. Interestingly, together with improving survival (Nissinen et al., 2018), only the continued blocking of ACVR2B ligands restored glutathione levels in cachectic skeletal muscle in line with the levels in the healthy mice. Moreover, blocking ACVR2B ligands increased the reduced form of glutathione in healthy mice as well, suggesting that the effect is not limited to cachectic muscle only. This supports the earlier study in which the absence of normal myostatin signaling *per se* increased GSH concentration (Ploquin et al., 2012). We also observed that muscle GSH concentration correlated positively with adipose tissue mass, skeletal muscle mass and change in body mass (last 2 days of the experiment) as well as negatively with tumor mass, suggesting that skeletal muscle GSH depletion is associated with the severity of the cachexia (**Supplementary Figures 2A–D**). Indeed, in a previous study, administration of glycine, which is a precursor for glutathione, improved muscle function and inhibited muscle wasting in C26 cachexia (Ham et al., 2014), suggesting that the reduced GSH may have an impact on the cachexia progression and restoring the redox balance may be beneficial.

The increase in GSH may also be a compensatory mechanism for increased oxidative damage/stress after a single or repeated sACVR administration. Unlike in healthy mice, protein carbonyls were, however, unchanged in tumor-bearing mice with or without blocking ACVR2B ligands. The accumulation of oxidatively damaged proteins is also known to induce proteasome activity intended to achieve their removal (Radak et al., 2000). Therefore, no difference in oxidatively modified protein levels may be explained by their increased removal. To support this assumption we observed increased markers of protein degradation (Nissinen et al., 2018) and in the current study increased markers of autophagy. Thus, a possible increase of oxidative protein modification cannot be discarded in tumor-bearing mice. Heat shock proteins are known to be induced, for example, by oxidative and metabolic stress (Niforou et al., 2014). We did not observe induction of the expression HSPs 60, 70, and 90 in healthy skeletal muscle, which does not support the assumption that sACVR2B-Fc administration increases cellular and especially oxidative stress. However, the expression of HSP25, which is also known to protect cells from oxidative stress (Escobedo et al., 2004) was also upregulated in healthy and cachectic sACVR administered mice, the effect being more profound in healthy muscle. This result is in line with previous literature in which skeletal muscle hypertrophy was observed (Huey, 2006; Frier and Locke, 2007; Huey et al., 2010), suggesting that induction of HSP25 regulates muscle homeostasis during rapid muscle hypertrophy. We analyzed only HSP content to assess heat shock response and not their subcellular localization, which is a key feature of their function (Paulsen et al., 2007, 2009). Future studies are warranted to investigate the effect of blocking ACVR2B ligands on oxidative stress and redox regulation as well as HSP localization in more detail.

To further investigate muscle protein homeostasis, we analyzed several indicators of unfolded protein response (UPR), a process that is also known to be induced by ER stress and oxidative stress (Cao and Kaufman, 2014). We observed that ER stress and UPR are not induced at the onset of C26 induced cachexia and, if anything, UPR may instead be downregulated. These observations are in line with decreased p-eIF2α in C26 tumor-bearing mice (Penna et al., 2010). However, they are contrary to a previous study in which UPR in skeletal muscle was upregulated in two other commonly used experimental animal models of cancer cachexia: LLC and $Apc^{min/+}$ mice. Interestingly, UPR seems to protect muscles from further wasting in these experimental cancer models (Bohnert et al., 2016; Gallot et al., 2018). The decreased UPR indicators were associated with the severity of cachexia because the downregulated UPR indicators (p-eIF2α, HSP47 and p-JNK) correlated positively with the change in body mass between the last 2 days of the experiment and negatively with the tumor mass (**Supplementary Figures 3A–F**). Alternatively, the decreased levels of UPR indicators in skeletal muscle that we observed in tumor-bearing mice could possibly be explained by decreased overall protein synthesis (Nissinen et al., 2018), which would reduce the demand of the folding of the nascent newly synthesized proteins. To support this, p-eIF2α, HSP47,

and p-JNK correlated positively with muscle protein synthesis (**Supplementary Figures 4A–C**). Another possible mechanism downregulating these indicators may be a decrease in physical activity that we previously reported (Nissinen et al., 2018) because HSP47 has been shown to decline during muscle unloading (Oguro et al., 2003) and phosphorylation of JNK has been shown to be inducible by resistance exercise (Hentila et al., 2018) and mechanical strain (Martineau and Gardiner, 2001).

Interestingly, many of the downregulated UPR markers in cancer were or tended to be increased in mice that received continued sACVR2B-Fc treatment, compared with other tumor-bearing mice. In healthy mice, 2-week sACVR2B-Fc administration increased the same UPR markers (GRP78, HSP47, and p-eIF2α) that also tended to be upregulated in sACVR2B-Fc administered C26 tumor-bearing mice. This suggests that these UPR indicators are upregulated more ubiquitously by sACVR2B-Fc administration. However, UPR indicators were not induced acutely 1 or 2 days after a single administration of sACVR2B-Fc, even though the protein synthesis was greatly induced, as we previously reported (Hulmi et al., 2013a). This is contrary to another stimulus, increasing protein synthesis, resistance exercise (RE), which has been shown to induce UPR 1 and 2 days after the RE bout (Ogborn et al., 2014; Hentila et al., 2018). Thus, it is suggested that UPR activation following sACVR2B-Fc ligand blocking is delayed compared with a RE bout. Previously, activation of UPR has been shown to inhibit mTORC1 signaling (Deldicque et al., 2010, 2011) and was suggested to act as a molecular break, suppressing protein synthesis during rapid muscle growth (Hamilton et al., 2014). Indeed, based on growth curves, the rate of muscle growth seemed to reach a plateau after 10 days during the sACVR2B-Fc administration (Hulmi et al., 2013a), which might be attributed to the induction of UPR and consequently blunted muscle growth.

We previously reported increased hepatic protein synthesis and induction of acute phase response (APR), suggesting altered protein homeostasis in the liver of tumor-bearing mice (Nissinen et al., 2018). Thus, we also analyzed UPR indicators in the liver of the tumor-bearing mice. In contrast to skeletal muscle, PERK, p-eIF2α, and GRP78 were increased by C26, whereas ATF4 protein content was strongly decreased. These results suggest that specific branches of UPR are activated in the liver of the tumor-bearing mice while some indicators may respond with a strong decrease. In a previous study, specific UPR markers have been associated with the severity of the cachexia: the pro-apoptotic indicators were especially associated with the progression of cachexia (Narsale et al., 2015). To analyze the maladaptive UPR branch that drives apoptosis, we analyzed the content of the ER stress-specific apoptosis marker caspase 12 (Szegezdi et al., 2006) from the livers of tumor-bearing mice. Interestingly, the content of the cleaved, active caspase 12 was unchanged, but the uncleaved pro caspase 12 was decreased in the liver of tumor-bearing mice. In addition, another ER-stress-associated pro-apoptotic indicator, JNK phosphorylation (Urano et al., 2000; Szegezdi et al., 2006) and the apoptosis marker cleaved caspase 3 were unchanged. This suggests that ER-stress-driven apoptosis was not induced in the liver of tumor-bearing mice. Nevertheless, the hepatic UPR markers GRP78 and ATF4 correlated positively with the change in body mass (the last 2 days of the experiment) (**Supplementary Figures 5A,B**) and ATF4 correlated negatively with the tumor mass (**Supplementary Figure 5C**), suggesting that also some of the hepatic UPR indicators may be associated with the severity of cachexia.

As a third biological process that is connected to redox balance (Lee et al., 2012) and UPR (Yorimitsu et al., 2006) and which contributes to the muscle protein homeostasis, we investigated the indicators of the autophagy-lysosome pathway that is responsible for the degradation and recycling of unnecessary and/or damaged proteins and organelles (Sandri, 2013) from the tumor-bearing mice. We observed increased content of lipidated LC3, increased ratio of LC3II and LC3I (LC3II/LC3I), *Lc3b* mRNA and Beclin-1 in the skeletal muscle of C26 tumor-bearing mice, which indicates increased autophagosome content and the induction of autophagy, respectively. These results were accompanied by increased P62 protein and unchanged *P62* mRNA levels, suggesting decreased clearance of autophagosomes (Klionsky et al., 2016). Our results are consistent with the previous studies conducted with experimental animal models and also in cachectic humans (Penna et al., 2013; Aversa et al., 2016; Molinari et al., 2017), clearly indicating that autophagy is induced in cachectic muscle and that the clearance of the autophagosomes may be inhibited. Even though the continued sACVR2B-Fc treatment previously inhibited the muscle wasting in the tumor-bearing mice (Nissinen et al., 2018) and has decreased markers of autophagy in healthy mice (Hulmi et al., 2013b), the novelty of our present study was that the sACVR2B-Fc treatment did not inhibit the increase in autophagy that was observed 11 days after the C26 inoculation. These results suggest that autophagy contributes to the muscle wasting together with the ubiquitin-proteasome system, which was also upregulated in the muscle of the C26 tumor-bearing mice despite the sACVR treatment (Nissinen et al., 2018). In the liver, as in the skeletal muscle, lipidated LC3 content and LC3II/LC3I were increased in C26 tumor-bearing mice but not P62, suggesting increased autophagosome content without impairment in the autophagic flux. Our results suggest that cancer cachexia induces aberration in the hepatic proteostasis, which is compensated by the induction of autophagy and UPR without any evident ER-stress related apoptosis. The induction of autophagy may also be explained by decreased feed intake, a known stimulus for autophagy activation (Sandri, 2010, 2013).

In conclusion, experimental cancer cachexia decreased the anti-oxidant defense capacity manifested by decreased glutathione content in skeletal muscle. However, sACVR2B-Fc administration increased glutathione in both healthy and cachectic muscle, suggesting that it increases anti-oxidant defense capacity in skeletal muscle. Based on the correlations of the data, we suggest that alterations in the processes contributing to protein homeostasis are associated with the severity of cachexia. The induction of UPR in the liver but not in skeletal muscle indicates that the protein homeostasis is altered in a tissue-specific manner in C26 cancer cachexia.

AUTHOR CONTRIBUTIONS

JJH, JH, and TN designed the cancer experiments. JJH and OR designed the acute study. TN and JH carried out the cancer experiments. JJH carried out the acute sACVR2B-Fc experiments with the healthy mice. MS and SL assisted in the *in vivo* experiments. JH drafted the manuscript with the help from JJH, MA, and TN. JH carried out the analysis with the help from TN, MA, and AK. AP and OR designed and produced the recombinant sACVR2B-Fc used in the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2018.01917/full#supplementary-material

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III

AUTOPHAGY IS INDUCED BY RESISTANCE EXERCISE IN YOUNG MEN, BUT UNFOLDED PROTEIN RESPONSE IS INDUCED REGARDLESS OF AGE

by

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ORIGINAL ARTICLE

ACTA PHYSIOLOGICA

Autophagy is induced by resistance exercise in young men, but unfolded protein response is induced regardless of age

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Abstract

Aim: Autophagy and unfolded protein response (UPR) appear to be important for skeletal muscle homoeostasis and may be altered by exercise. Our aim was to investigate the effects of resistance exercise and training on indicators of UPR and autophagy in healthy untrained young men (n = 12, 27 ± 4 years) and older men (n = 8, 61 ± 6 years) as well as in resistance-trained individuals (n = 15, 25 ± 5 years).

Methods: Indicators of autophagy and UPR were investigated from the muscle biopsies after a single resistance exercise bout and after 21 weeks of resistance training.

Results: Lipidated LC3II as an indicator of autophagosome content increased at 48 hours post-resistance exercise ($P < .05$) and after a resistance training period ($P < .01$) in untrained young men but not in older men. Several UPR_{ER} markers, typically induced by protein misfolding in endoplasmic reticulum, were increased at 48 hours post-resistance exercise in untrained young and older men ($P < .05$) but were unaltered after the 21-week resistance training period regardless of age. UPR was unchanged within the first few hours after the resistance exercise bout regardless of the training status. Changes in autophagy and UPR_{ER} indicators did not correlate with a resistance training-induced increase in muscle strength and size.

Conclusion: Autophagosome content is increased by resistance training in young previously untrained men, but this response may be blunted by ageing. However, unfolded protein response is induced by an unaccustomed resistance exercise bout in a delayed manner regardless of age.

KEYWORDS
autophagy, resistance training, unfolded protein response

1 | INTRODUCTION

An acute bout of resistance exercise (RE) with sufficient load and volume induces skeletal muscle remodelling that leads within few months of training to increases in muscle size and strength.[1,2] These adaptations are accompanied by numerous benefits for health and performance,[3,4] yet many of the underlying cellular mechanisms leading to the specific adaptations are not yet fully understood.

Disruptions of cellular homoeostasis (eg due to mechanical, genotoxic and/or heat stress) may induce damage to proteins and cellular organelles.[5,6] To maintain homoeostasis,

the damaged structures are degraded in a stress response.[5] More specifically, disrupted cellular homoeostasis may also induce misfolding of premature proteins in the endoplasmic reticulum (ER), an organelle responsible for protein folding, trafficking and calcium homoeostasis.[7] ER stress activates an unfolded protein response (UPR), which tries to balance the homoeostasis in ER. If ER stress is chronic and cannot be resolved by UPR, metabolic impairments or apoptosis may occur.[8] Rodent studies suggest that UPR plays a regulatory role in skeletal muscle metabolism and possibly also in exercise adaptations.[9,10] Resistance exercise may also induce UPR in humans regardless of age,[11] but the effect of longer periods of resistance training is unknown.

UPR induces—among other biological processes—autophagy,[12] which is a major catabolic route in cells responsible for clearance of proteins and organelles.[13] In the process of macroautophagy (hereafter called autophagy), proteins or organelles are surrounded by double-membraned vesicles that deliver their cargo to lysosomes in which they are degraded.[13] High, and thus, pathological levels of autophagy are associated with muscle-wasting conditions,[6] whereas inhibition of autophagy in skeletal muscles can also lead to diminished muscle mass and dysfunction due to aggregation of damaged cellular organelles and proteins.[14]

Transgenic rodent studies suggest that autophagy is required to induce some exercise adaptations,[15] maintain functional mitochondria after repeated exercise bouts,[16] and regulate muscle glucose metabolism during endurance exercise.[17] Indicators of autophagy have been reported to be induced by a single endurance exercise bout and chronic endurance training in rodent skeletal muscle.[16-22] However, the effect has not been as consistent in humans.[23-25] The limited data from human resistance exercise (RE) studies suggest that autophagy is unchanged or decreased within the first few hours[26-28] and even 24 hours after a RE bout,[29,30] but may increase 48 hours after the RE bout in young and old adults.[30] Decreased markers of autophagy have been reported in ageing rodents and sedentary older males compared with young males.[31] Resistance training may increase autophagy in aged rats,[32] but the long-term effect of resistance training interventions on autophagy is currently unknown in humans.

Our main purpose was to investigate the acute and prolonged effects of resistance exercise on autophagy and UPR in healthy young adults as well as in healthy older individuals. On the basis of the previous literature, we hypothesized that UPR is activated after a resistance exercise bout in a delayed manner but that long-term resistance training has no effect on UPR at resting state. Autophagosome content markers are also hypothesized to be induced by resistance exercise and training.

2 | RESULTS

2.1 | Resistance training increases muscle size and strength (Experiments 1 and 2)

As a result of a 21-week supervised resistance training period (RT), muscle strength and fibre size were significantly increased in 21 young males and 18 older males.[33] The increase in muscle strength and muscle fibre size was also significant with the smaller sample size used in this study for young men (27 ± 4 years, n = 12) (Experiment 1) and for older men (Experiment 2; n = 8, 61 ± 6 years, $P < .05$; data not shown).

2.2 | Resistance exercise (RE) bout leads to acute decrease in muscle force (Experiments 1 and 2)

In the young men of Experiment 1, after an unaccustomed RE bout of 5 × 10 repetition maximum (RM) until failure on a leg press device, isometric bilateral leg extension force significantly decreased from pre-values 3513 ± 1434 N to 2203 ± 765 N ($P < .001$) immediately after the RE bout and to 2748 ± 1063 N ($P < .001$) at 1 hour. Muscle strength remained slightly decreased 48 hours after the RE bout (3265 ± 1382 N, $P < .05$). In the older men of Experiment 2, isometric bilateral leg extension force was significantly decreased from the pre-values 2567 ± 451 N to 1808 ± 334 N ($P < .001$) immediately after the RE bout. After the RT period, the force values were PRE = 2803 ± 349 N and post 48 hours = 2132 ± 125 N respectively ($P < .001$). Isometric muscle force was slightly more decreased in young men compared with older men immediately after the RE bout (young: 36 ± 7% vs older 30 ± 7%, $P < .05$). There was no significant difference in the force decline between the RE bouts that were conducted before and after the RT period in older men.

2.3 | UPR increases 48 hours after the resistance exercise bout in young men (Experiment 1)

Of the UPR indicators, one bout of unaccustomed RE increased PERK, ATF4, GRP78, p-JNK, *Xbp1s* and *Xbp1t* (main effect: $P < .05$). More specifically, acutely 1 hour after the RE bout, the protein content of PERK and IRE1α signalling pathway markers was unchanged (Figure 1A-G). At 48 hours, from the PERK arm of the UPR, PERK and ATF4 proteins increased ($P < .05$; Figure 1A,B). To support the finding that UPR is induced 48 hours after the RE bout, downstream of IRE1α pathway the mRNA level of spliced *Xbp1* (*Xbp1s*) as well as total (*Xbp1t*) were

increased (Figure 1E,F). However, not all of the UPR markers increased at this time point, because the IRE1α protein and the phosphorylation of eIF2α at ser51 were unchanged (Figure 1C,D). The protein content of ER-located chaperone GRP78/BiP increased 48 hours after the RE bout (Figure 1G), but the content of 2 other ER-located chaperones, PDI and calnexin, was unchanged (Figure S1a, b). From the apoptotic arm of the UPR, the phosphorylation of JNK ($P < .01$) and the mRNA level of *Chop* increased already 1 hour after the RE bout. At 48 hours after the RE bout, JNK phosphorylation remained elevated while *Chop* mRNA decreased back to baseline (Figure 1H and Figure S2a).

The post 48-hour biopsy was from the same leg as the PRE-biopsy, although 3 cm above the biopsy scar. In the nonexercised subjects (No RE; n = 4), there was no consistent biopsy effect on PERK, *Xbp1s* or *Xbp1t* (Figure 1A,E, F). However, the protein content of GRP78 seemed to be systematically increased in the No RE subjects 48 hours after the RE bout (Figure 1G).

2.4 | UPR is unaltered by 21 weeks of resistance training in young men

Unlike at 48 hours after the unaccustomed RE bout, the 21-week RT period did not alter relative content of UPR markers or ER chaperones at 4-5 days after the last RE session (Figure 1 and Figure S1).

2.5 | Autophagy markers are increased 48 hours after an unaccustomed resistance exercise bout and remain elevated after 21 weeks of resistance training in young men

Resistance exercise had a consistent effect on the protein content of following indicators (LC3II, LC3I, Beclin-1, P62, BCL-2 and p-ULK1 at ser^{555} (main effect: $P < .05$). More specifically, 48 hours after the RE bout, LC3II, LC3I and P62 were increased ($P < .05$), and Beclin-1 protein tended ($P = .06$) to be elevated (Figure 2A-D). Protein expression levels of LC3I, LC3II and P62 are probably not explained by their transcription/transcript levels, because the mRNA levels of *lc3b* and *p62* were unchanged (Figure S3a,b). BCL-2 and the phosphorylation of ULK^{ser555} and $ULK1^{ser757}$ as well as total ULK1 were unchanged at 48 hours after the RE bout (Figure 2E-G). At 48 hours after the RE bout, the increase in autophagy markers may be in part due to repeated biopsy effect because P62 and LC3II tended to be increased in some No RE subjects as well (Figure 2A,D). At 1 hour after the RE bout, LC3II was decreased ($P < .05$; Figure 2A), whereas p-$ULK1^{ser757}$ tended to be increased ($P = .12$; Figure 2E) and p-$ULK1^{ser555}$ was decreased ($P < .05$; Figure 2F). Additionally, LC3I, Beclin-1, P62, total ULK1, BCL-2 proteins (Figure 2B-E,G) and the mRNA level of *P62* and *lc3b* were unchanged (Figure S3a,b).

After the 21-week RT period, LC3II as a marker of autophagosome content increased ($P < .01$), whereas BCL-2 decreased ($P < .05$; Figure 2A,G). The RT period had no effect on the protein content of LC3I, P62, Beclin-1, p-$ULK1^{ser757}$ and total ULK1 (Figure 2B-E) or *lc3b* and *p62* mRNA levels (Figure S3a,b), but the p-$ULK1^{ser757}$ per total ULK (p-$ULK1^{ser757}$/ULK1) increased at 21 weeks, whereas p-$ULK1^{ser555}$ was decreased ($P < .05$, Figure 2E,F).

2.6 | Unaltered UPRmt and markers of oxidative stress in younger men

From the indicators of oxidative stress, there was no effect of the RE bout or 21 weeks of RT on protein carbonyls or glutaredoxin (Figure 3A and Figure S3c). Heat-shock protein 27 (HSP27) and αB-crystallin significantly decreased 1 hour after the RE bout (Figure S3d,e). From the mitochondrial UPR (UPR_{MT}) markers, glucose-regulated protein 75 (GRP75) and heat-shock protein 10 (HSP10) were analysed. Even though the RE bout seemed to increase ER stress-induced UPR, neither RE nor RT had any effect on GRP75 or HSP10 (Figure 3C,D).

Neither the RE nor the RT had effects on PGC1α1 protein, which is known to be associated with UPR[9] (Figure 3B), or on the marker for AMPK activation, p-ACC, at measured time points (Figure S4a). Similarly, no RT effect was observed on the marker for the mitochondria content cytochrome c (cyt c). However, 48 hours after the RE bout, cytochrome c protein was significantly decreased (Figure S4b).

2.7 | Unaltered markers of autophagy and oxidative stress, but increased markers of UPR in older men (Experiment 2)

As in the young men, some UPR markers were increased at 48 hours after the RE bout in older men. This manifested as increased protein content of ATF4 ($P < .05$) and an increased trend ($P = .1$) in GRP78 after the RE bout conducted before RT (Figure 4A,B). Also as in the young men, the RT period did not influence UPR, which was marked by unchanged levels of ATF4 and GRP78 proteins at 4-5 days after the last RE session (Figure 4A,B). The protein content of GRP78 increased significantly 48 hours after the RE bout that was conducted after the 21-week RT period without changes in ATF4 (Figure 4A,B). There were no RE or RT effects on PDI, IRE1α and PERK (Figure 4C-E).

Unlike in the young men, neither the RE bout nor the RT period had an effect on LC3II, LC3I, P62, Beclin-1 or

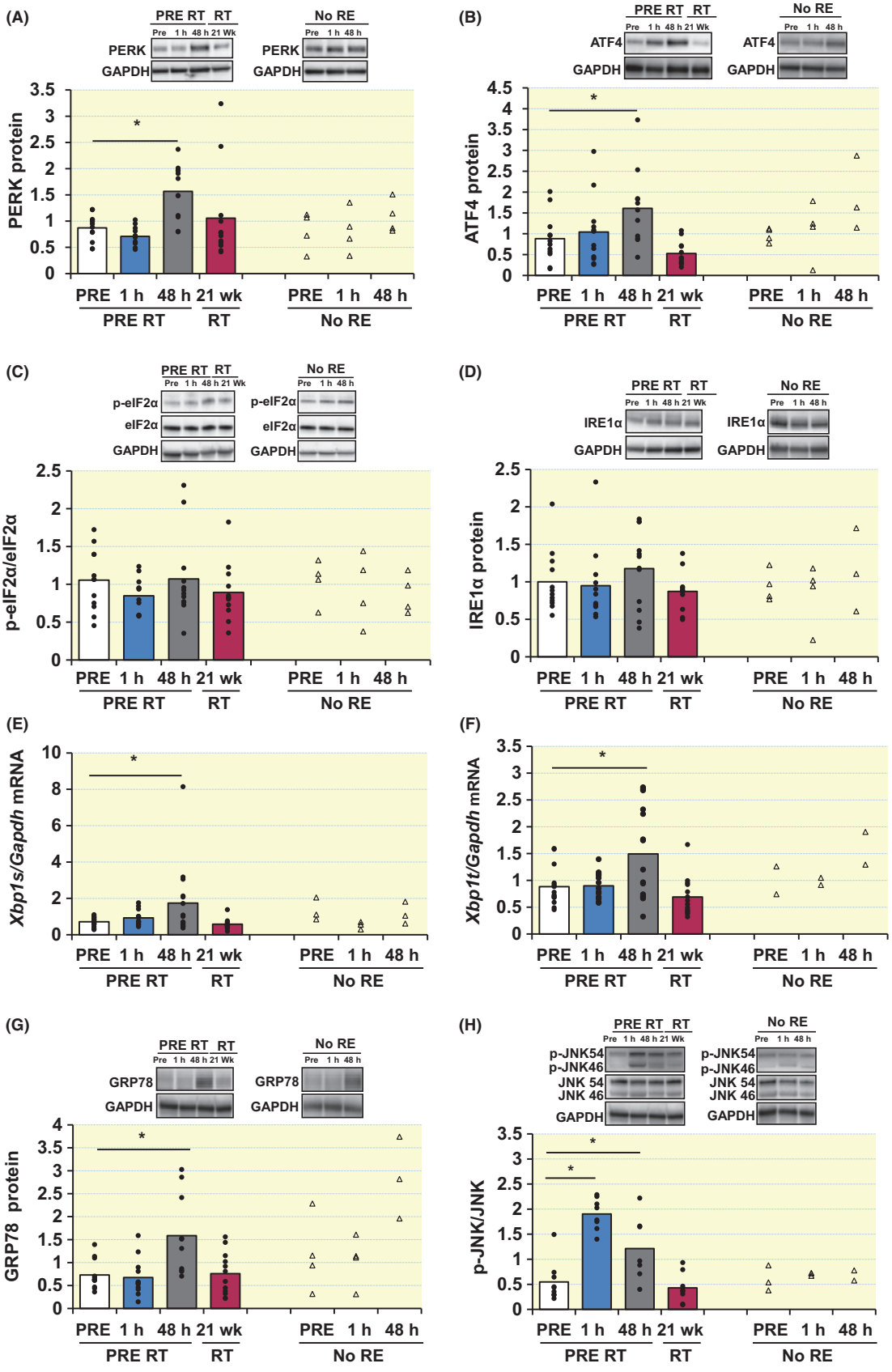

(A)
PRE RT
RT
No RE
Pre 1 h 48 h 21 Wk
PERK
GAPDH
PERK protein
PRE 1 h 48 h 21 wk PRE 1 h 48 h
PRE RT
RT
No RE
(B)
ATF4
GAPDH
ATF4 protein
(C)
p-eIF2α
eIF2α
GAPDH
p-eIF2α/eIF2α
(D)
IRE1α
GAPDH
IRE1α protein
(E)
Xbp1s/Gapdh mRNA
(F)
Xbp1t/Gapdh mRNA
(G)
GRP78
GAPDH
GRP78 protein
(H)
p-JNK54
p-JNK46
JNK 54
JNK 46
GAPDH
p-JNK/JNK

FIGURE 1 Unfolded protein response (UPR) indicators before, 1 and 48 h after an unaccustomed resistance exercise bout (PRE-RT) as well as after 21-wk RT period (RT) and in nonexercised controls (No RE) in young men (Experiment 1). A, PERK protein, B, ATF4 protein, C, the ratio of p-eIF2α and eIF2α, D, IRE1α protein, E, spliced *Xbp1* (*Xbp1s*) mRNA, F, total *Xbp1* (*Xbp1t*) mRNA, G, GRP78 protein and H, the ratio of p-JNK and JNK (46 and 54 kDa averaged). Open bars depict means. Circles and triangles depict individual values. The symbol * depicts the statistical significance $P < .05$ from PRE. n = 10-12 in RT and n = 3-4 in No RE subjects except in Xbp1s and Xbp1t n = 13-15 in RT and n = 2-3 in No RE subjects and in p-JNK/JNK n = 7-9 in RT and n = 2-4 in No RE

BCL-2 proteins (Figure 5A-E). Similar to the young men, neither RE nor RT influenced protein carbonyl (Figure 4F) or PDI protein content (Figure 4C). PGC1-α1 protein content was decreased at 48 hours after the unaccustomed RE bout, but was unchanged after the RT period or 48 hours after the RE bout that was conducted after the RT period (Figure S5a). Neither the RE bout nor the RT period influenced the UPR_{MT} marker HSP10 protein and the marker of mitochondria content cytochrome c (Figure S5b,c).

2.8 | Effects of RE bout in previously strength-trained young individuals (Experiment 3)

To elucidate the acute effects of an RE bout on UPR and autophagy with a slightly longer acute time course and whether previous training background has any effects, we also analysed biopsies from 1.5 to 3 hours after a RE bout from a group of previously trained participants.[34] The RE bout had no effects on LC3II, LC3I protein or p-$ULK1^{ser757}$, but decreased the protein content of Beclin-1 at 150 minutes after the RE bout in these recreationally strength-trained individuals (Figure 6D and Figure S6b,c). Of the UPR_{ER} and UPR_{MT} markers, the bout of RE had no effects on IRE1α, PERK, PDI and p-eIF2α or HSP10 (Figure 6A-C and Figure S6a,d). Nevertheless, p-JNK54 and the ratio of p-JNK54/JNK54 were increased at both time points after the RE bout (Figure 6E).

2.9 | Changes in autophagy and UPR markers are not associated with muscle hypertrophy, strength development and isometric force recovery

The main results of the 3 experiments are summarized in Figure 7. For Experiment 1, a computationally determined network was created between the main outcome variables. For this purpose, biologically similar variables were merged. The software Katiska/Himmeli running in a GNU Octave program environment (http://www.finndiane.fi/software/katiska/) was used for the analysis.[35] Interestingly, neither the change in UPR markers nor the autophagosome content at 48 hours after the RE bout and after the 21-week RT period was associated with improvement in leg press strength or muscle hypertrophy. These results were supported by the lack of correlation between these variables (Figure S7). In addition, the autophagosome content and UPR markers at 48 hours did not correlate with the recovery of isometric muscle force at 48 hours after the RE bout.

3 | DISCUSSION

This study demonstrated, for the first time, that long-term hypertrophic resistance training increases the autophagosome content in previously untrained young men and has no effect on the content of UPR indicators relative to skeletal muscle total protein content at resting state or on ER-located chaperones in skeletal muscle. Additionally, 2 days after an unaccustomed bout of RE, indicators of UPR and autophagy were increased in previously untrained young and older men. However, the changes in autophagy and UPR indicators were not associated with oxidative stress or changes in muscle size and strength.

Autophagy is a complex and dynamic process[36] that can be estimated from human biopsies by measuring lipidated LC3 protein (LC3II) as well as gene and protein expression of key proteins involved in various steps of autophagy.[37] In young men, LC3II was increased 48 hours after the RE bout probably due to post-transcriptional regulation, suggesting increased autophagosome content. Moreover, increased LC3I and Beclin-1 suggest increased autophagic capacity and the induction of autophagy respectively. However, the increased LC3II may also be due to decreased autophagosome degradation in the lysosomes and, thus, decreased autophagic flux.[36] To indirectly estimate autophagic flux, we also analysed the protein content of P62, which acts as an adaptor protein in the autophagosomes and is degraded when the autophagosomes release their cargo to the lysosomes.[38] P62 was increased at 48 hours after the RE bout indicating decreased clearance of the autophagosomes, or alternatively greater synthesis of P62 protein over its degradation. The present results are consistent with the previous research in which total protein of LC3b and P62 was increased 48 hours after an unaccustomed RE bout in young men.[30]

The effects of a long-term RT period on autophagy have never been investigated in human skeletal muscle.[39] In young men, RT increased LC3II, which indicates an

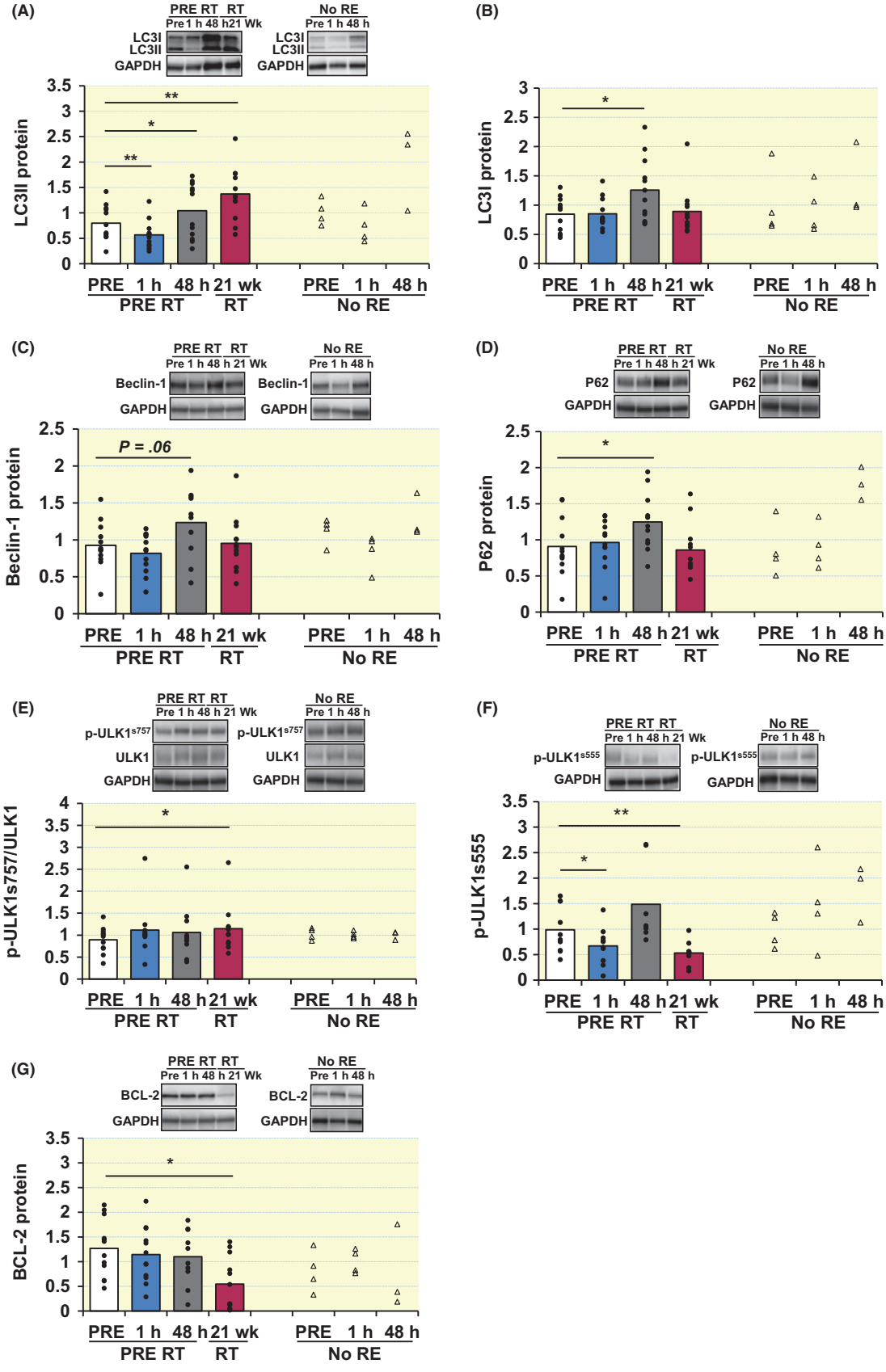

(A)
PRE RT RT
Pre 1 h 48 h 21 Wk
No RE
Pre 1 h 48 h
LC3I
LC3II
GAPDH
LC3II protein
PRE 1 h 48 h 21 wk
PRE RT RT
PRE 1 h 48 h
No RE
(B)
LC3I protein
(C)
Beclin-1
Beclin-1 protein
P = .06
(D)
P62
P62 protein
(E)
p-ULK1s757
ULK1
p-ULK1s757/ULK1
(F)
p-ULK1s555
p-ULK1s555
(G)
BCL-2
BCL-2 protein

FIGURE 2 Autophagy indicators before, 1 and 48 h after an unaccustomed resistance exercise bout (PRE-RT) as well as after 21-wk RT period (RT) and in nonexercised controls (No RE) in young men (Experiment 1). A, LC3II protein, B, LC3I protein, C, Beclin-1 protein, D, P62 protein, E, the ratio of p-ULK1^{ser757} and total ULK1, F, p-ULK1^{ser555} and G, BCL-2 protein. Open bars depict means. Circles and triangles depict individual values. The symbols * and ** depict the statistical significance $P < .05$, $P < .01$; respectively. n = 10-12 in RT and n = 3-4 in No RE for all except p-ULK1^{ser555} n = 7-10 in RT and 2-4 in No RE

increased number of autophagosomes and possibly increased autophagic flux, because the result was accompanied by unchanged LC3I and P62. However, the ratio of phosphorylated ULK1 at an inhibitory cite (ser757) to total ULK1 increased, whereas the phosphorylation of ULK1 at the activation cite at ser555 decreased. This may suggest downregulation of autophagosome precursor formation.[40] However, because after RT, there was no change in Beclin-1, which is an autophagy induction marker, further studies are warranted to investigate the phenomenon

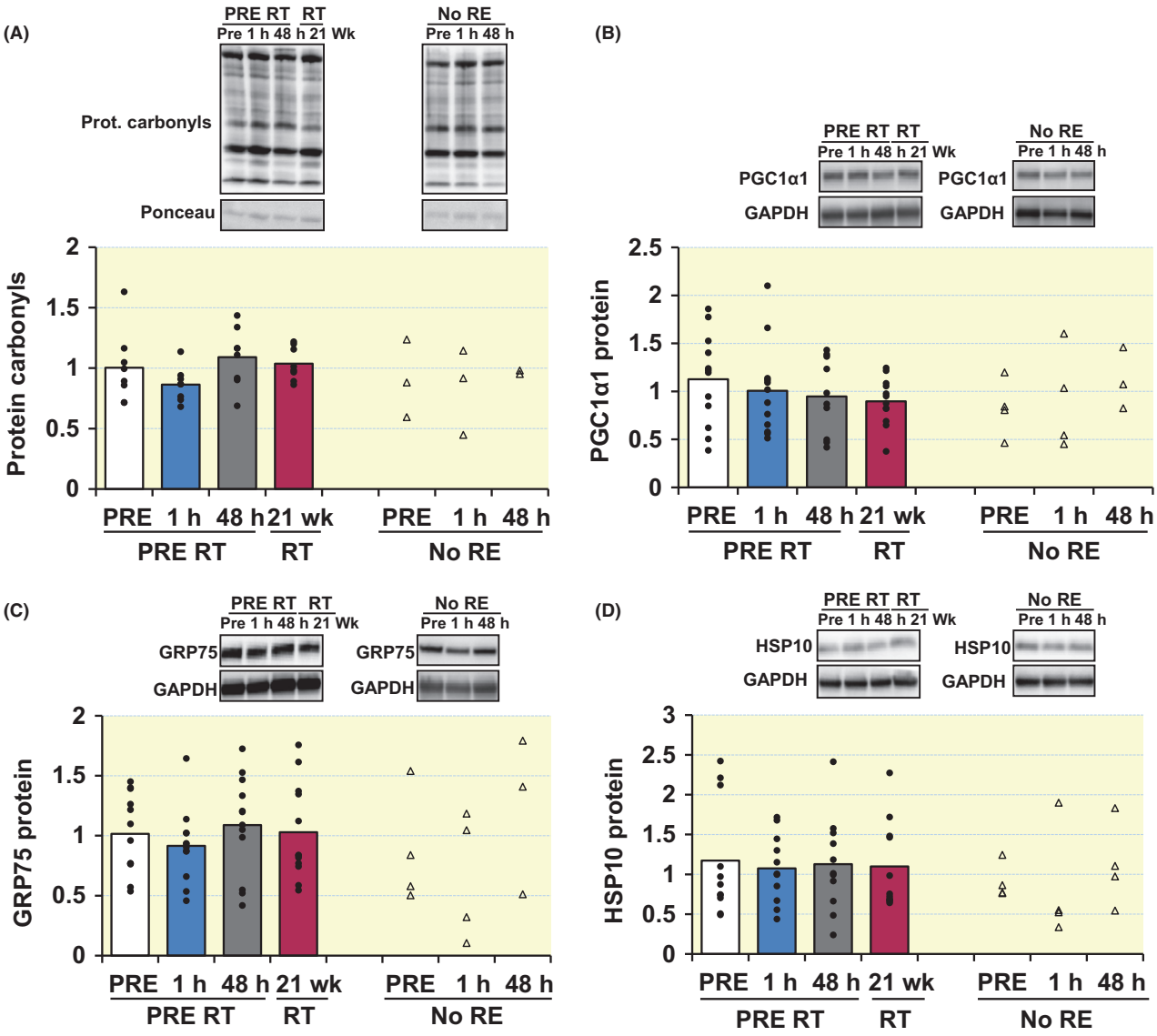


FIGURE 3 Indicators of oxidative stress, aerobic metabolism and mitochondrial unfolded protein response (UPR) before, 1 and 48 h after an unaccustomed resistance exercise bout (PRE-RT) as well as after 21-wk RT period (RT) and in nonexercised controls (No RE) in young men (Experiment 1). A, Protein carbonyl content, B, PGC1α1 protein content, C, GRP75 protein content, D, HSP10 protein content. Open bars depict means. Circles and triangles depict individual values. In protein carbonyls n = 8 in RT and n = 2-3 in No RE. In PGC1α1, GRP75 and HSP10 n = 10-12 in RT and n = 3-4 in No RE

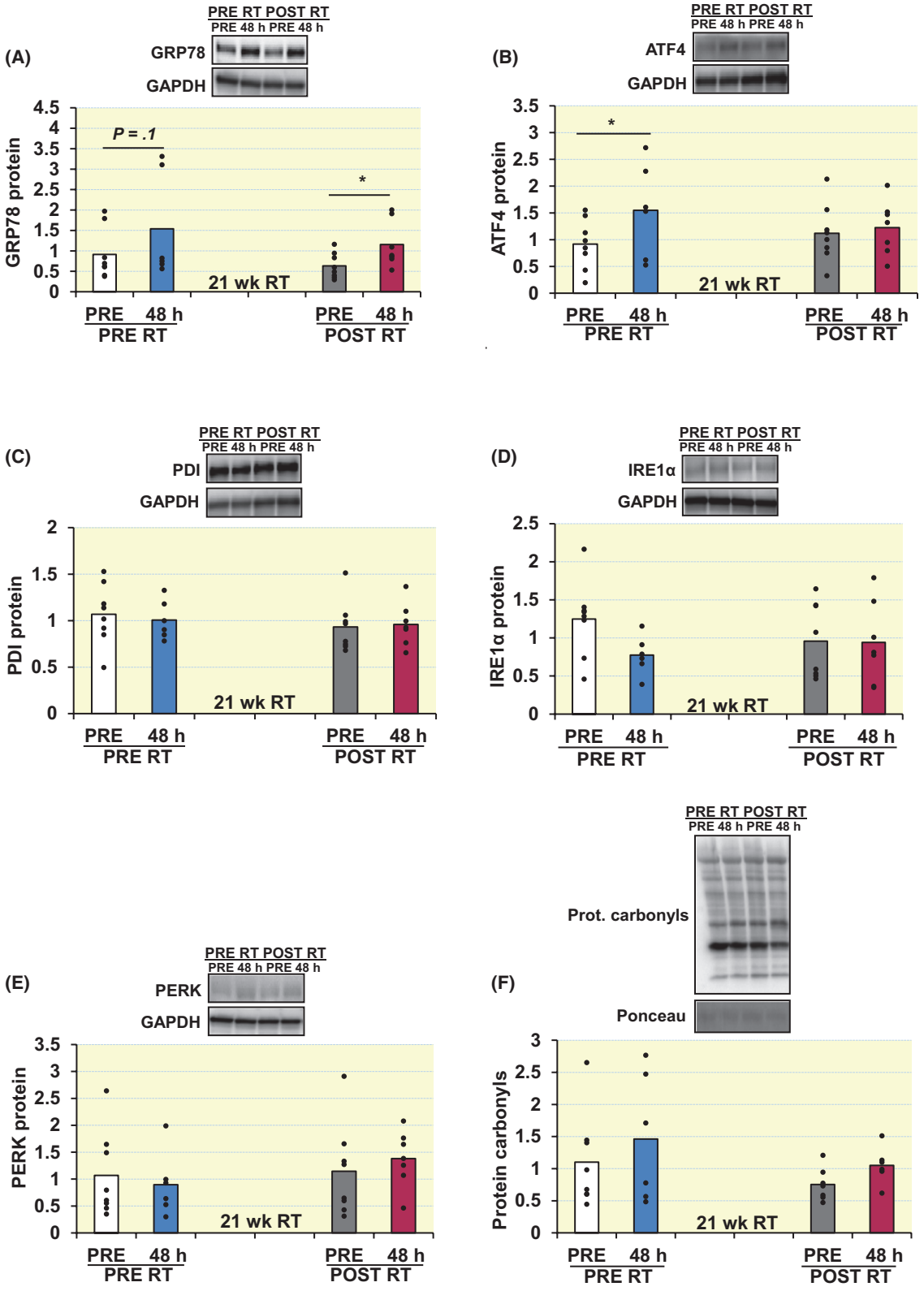


FIGURE 4 Unfolded protein response (UPR) indicators before and 48 h after an unaccustomed resistance exercise bout (PRE-RT) and before and 48 h after a RE bout after 21-wk RT period (POST-RT) in older men (Experiment 2). A, GRP78 protein, B, ATF4 protein, C, PDI protein, D, IRE1α protein, E, PERK protein, F, Protein carbonyls. Open bars depict means. Circles and triangles depict individual values. The symbol * depicts the statistical significance $P < .05$ from PRE state. In all of the variables n = 6-8

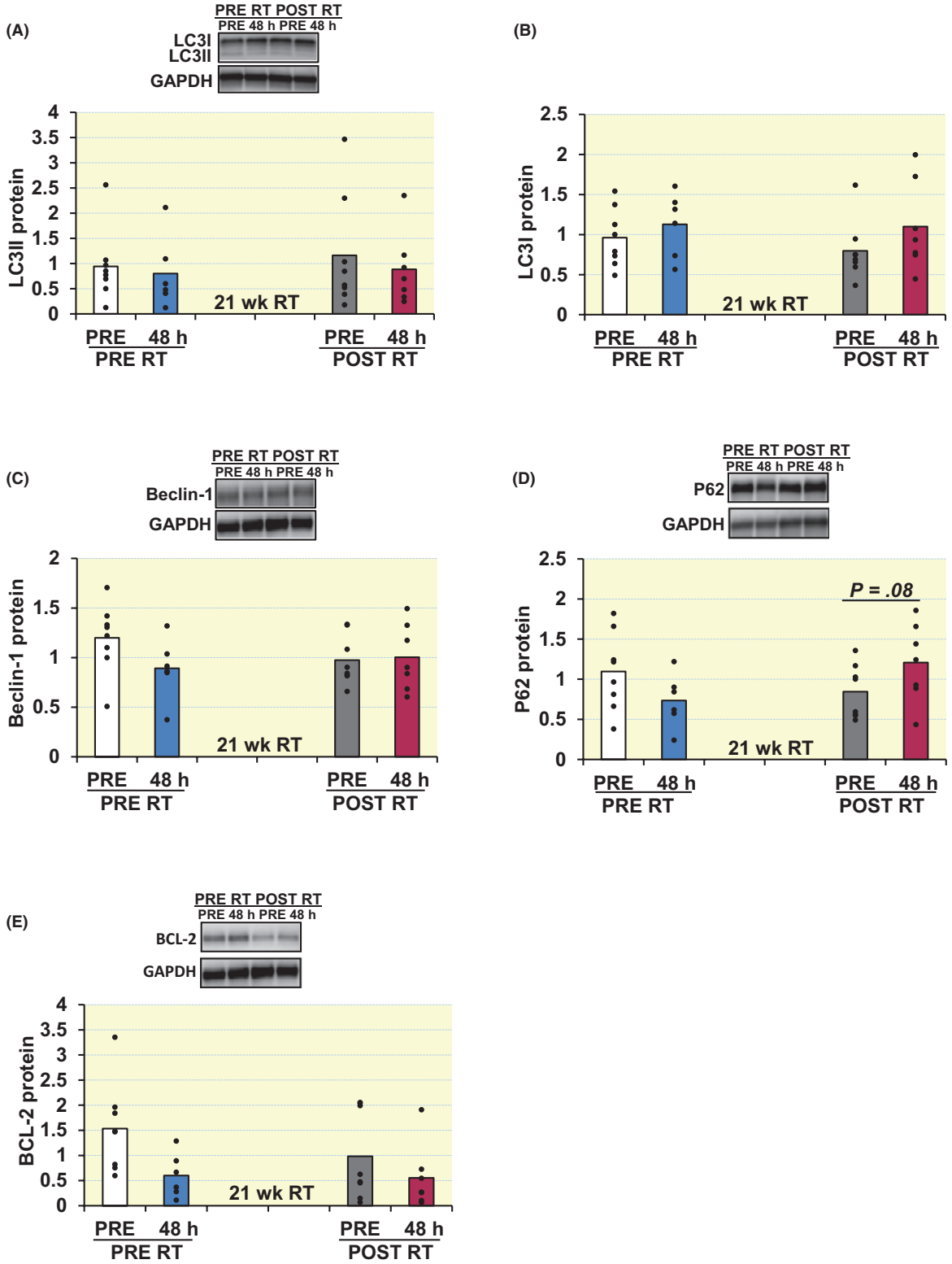


FIGURE 5 Autophagy indicators before and 48 h after an unaccustomed resistance exercise bout (PRE-RT) and before and 48 h after a RE bout after 21-wk RT period (POST-RT) in older men (Experiment 2). A, LC3II protein content, B, LC3I protein content, C, Beclin-1 protein content, D, P62 protein content, E, BCL-2 protein content. Open bars depict means. Circles and triangles depict individual values. In all of the variables n = 6-8

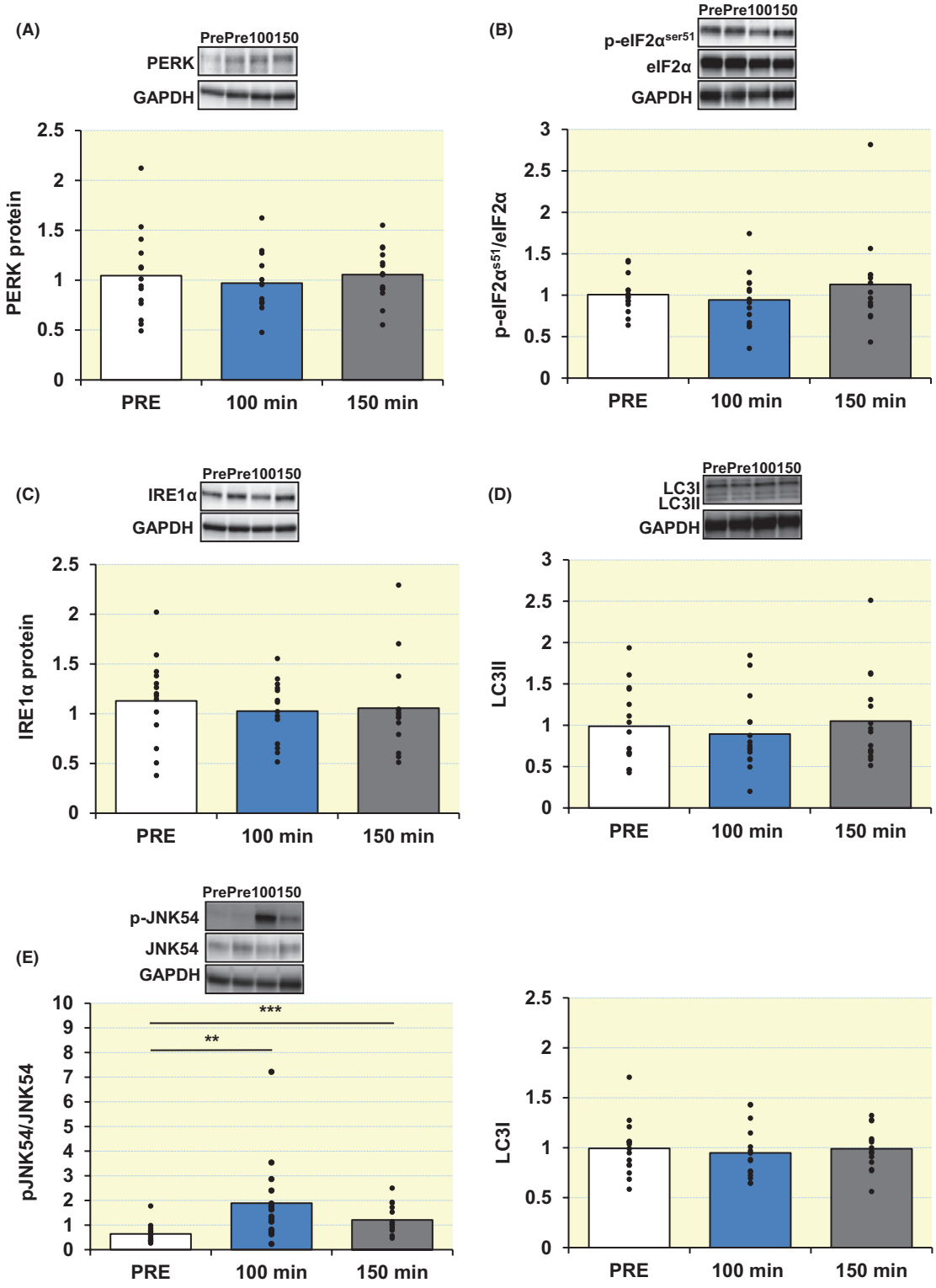


FIGURE 6 Unfolded protein response (UPR) and autophagy indicators before, 100 and 150 min after a resistance exercise bout in recreationally resistance-trained young individuals (Experiment 3). A, PERK protein, B, the ratio of p-eIF2α and eIF2α protein, C, IRE1α protein, D, LC3II and I, E, Ratio of p-JNK54 and total JNK54 protein. PRE value is average of 2 biopsies before the RE bout. Open bars depict means. Circles and triangles depict individual values. The symbol * depicts the statistical significance $P < .05$ from PRE state. n = 14-15

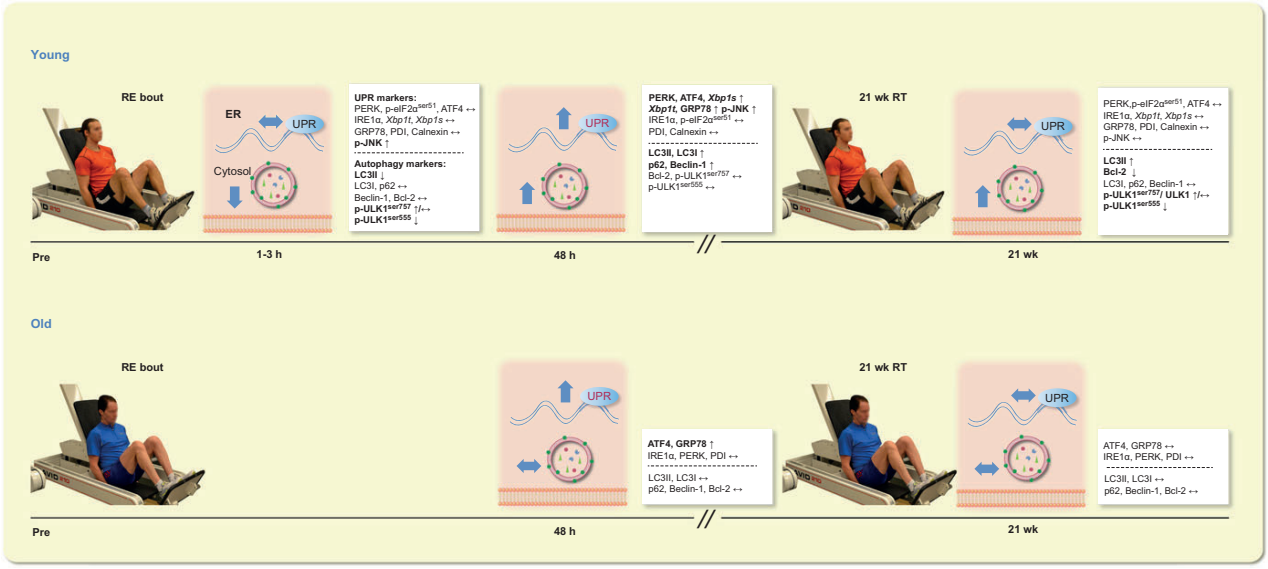


FIGURE 7 Summary figure of the resistance exercise (RE) and resistance training (RT) induced changes in unfolded protein response (UPR) and autophagy markers in young previously untrained (Experiment 1) and previously recreationally strength-trained (Experiment 3) as well as older previously untrained individuals (experiment 2). The symbol of autophagy (autophagosome) is depicting autophagosome content, whereas the ellipse entitled: "UPR" depicts ER stress-induced overall unfolded protein response. Autophagy indicators were either decreased or unchanged acutely after the RE bout. In addition, UPR indicators were unaltered acutely after the RE bout. Several autophagy markers increased at 48 h after the RE bout in young men indicating activation of autophagy and an increase in autophagosome content (LC3II). LC3II content increased after the RT period in untrained young men indicating increased autophagosome content and possibly autophagic flux (unchanged LC3I and P62). The ratio of phosphorylated ULK1 at an inhibitory cite (ser757) to total ULK1 increased, whereas the phosphorylation of ULK1 at the activation cite at ser555 decreased. This would suggest inhibition of autophagy initiation but the suggestion is not supported by unchanged content of Beclin-1. Autophagy markers were unaltered by RE and RT in older individuals suggesting that ageing may blunt the resistance training induced increase in autophagy. Several UPR markers, typically induced by protein misfolding in endoplasmic reticulum, were increased at 48 h after RE bout in untrained individuals but were unaltered after the 21-wk RT period regardless of age. The arrows in the boxes depict changes in UPR and autophagy markers; ↓ relative decrease ↑ relative increase ↔ no change. Markers that were changed are highlighted as bolded

further. The physiological increase in the autophagosome content may be considered as a positive adaptation[41] if we assume a greater recycling capacity of damaged organelles and proteins at rest or during recovery from a strenuous exercise bout. Previously in mice, 4-5 weeks of voluntary wheel running increased autophagic flux, as indicated by increased LC3II and decreased P62 content.[15] In the same study, in Atg6$^{+/-}$ (a critical protein in autophagy) mice, there was no increased autophagy flux, improved endurance performance, increased angiogenesis nor increased mitochondrial biogenesis as a result of 4-5 weeks of voluntary wheel running. This suggests that increased autophagy is needed to elicit endurance training adaptations. In contrast to the present finding, pharmacological ways to increase muscle mass, such as blocking activins and myostatin, may not induce autophagy.[21]

The present study consisted of 3 separate experiments, and only the relative changes induced by RE and RT within each experiment were analysed. Nevertheless, in contrast to the young men, in which 11/12 of the subjects showed an increase in LC3II content after the RT period, in older healthy men 3 of 8 subjects showed an increase in LC3II content and 5 of 8 subjects showed a decrease, showing the clear difference in the response. In addition, several autophagy markers were induced 48 hours after an acute exercise bout in the young men but not in the older men. This suggests that ageing may interfere with the RE/RT-induced increase in the regulation of autophagosome content. Previously, ageing has been shown to decrease the markers of autophagy (ie ATG7 and LC3II/LC3I) in the skeletal muscle of sedentary humans.[31] They also reported that life-long participation in mainly endurance type of exercise prevented the decreases in the markers of autophagy, but the role of life-long exercise vs genomic effects remains speculative. Nevertheless, if autophagy or its response to exercise is interfered by ageing, this may negatively affect cellular metabolism.[31,42] Our data from the acute loading are contrary to the previous study by Ogborn et al, which showed that autophagy was similarly induced within 2 days in both young and older men.[30] Furthermore, in older rodents, RT enhanced markers of autophagy.[32] Therefore, more studies are needed to elucidate the effects

of different types, intensities/loads, and volumes of RE and RT on autophagy, especially in humans at different ages.

There was a decrease in the content of lipidated LC3 (LC3II) 1 hour after the unaccustomed RE bout, a finding that supports earlier studies in humans.[23-25,27-29] This may be explained by increased mTOR signalling that was previously observed in our subjects.[43] mTOR is known to inhibit autophagy by phosphorylating ULK1 at ser^{757},[44] and there was a nonsignificant trend for increase in p-$ULK1^{ser757}$ content at 1 hour after the RE bout. In addition, p-$ULK1^{s555}$, which activates autophagosome precursor formation, decreased at 1 hour after the RE bout. The decrease in LC3II did not occur in recreationally strength-trained individuals at 100-150 minutes after the RE bout, even though increased mTOR signalling was observed,[34] suggesting that the LC3 lipidation after an RE bout may vary by training status. However, the difference may also be due to the different RE protocols and the timing of the biopsies.

While the UPR markers in young individuals were unresponsive to the RE bout within the first 1-3 hours regardless of previous strength training background, many of the ER stress-induced UPR indicators were increased 48 hours after the unaccustomed RE bout in young and older previously untrained men. This is consistent with a recent study reporting increased UPR markers at 24 and 48 hours after an unaccustomed RE bout in young and older men.[11] Thus, probably part of the muscle fibre remodelling following an unaccustomed RE bout may be mediated by UPR regardless of age. To support this, knockout mice lacking an upstream regulator of one UPR branch ($ATF6\alpha^{-/-}$ mice) exhibit impaired ability to recover from exhaustive treadmill running and become exercise intolerant after repeated treadmill running bouts.[9] On the other hand, UPR has been associated with acting as a molecular brake in rodents during rapid supraphysiological muscle hypertrophy.[10] The observed changes in UPR markers may be linked to the increased phosphorylation of JNK, which is suggested to occur during ER stress by IRE1α pathway. JNK phosphorylation has been suggested to promote apoptosis during ER stress[45,46] and has also been associated with mechanotransduction.[47]

Decreased muscle force and decreased protein content of cytochrome c were observed at 48 hours post-RE in young men, which previously has been related to mild muscle damage.[48] Unaccustomed exercise can cause disruption of the cytoskeleton structure of muscle fibres,[49] leading to the remodelling of muscle fibres,[50] a process in which integrins may have a role.[51,52] Interestingly, the integrin-derived adaptations may involve activation of UPR,[53] but making firmer conclusions on this require further research. Unlike UPR_{ER}, mitochondrial UPR (UPR_{MT} based on the protein contents of GRP75 and HSP10[54]) was unchanged. This suggests that an unaccustomed RE bout with predominant anaerobic energy production and a heavy load that induces mechanical strain on muscle tissue challenges the protein folding preferably in ER over mitochondria. To the authors' knowledge, this is the first publication examining the long-term effects of RT on UPR signalling and ER-resident chaperones. We did not observe changes in the UPR indicators or ER-resident chaperones in young and older previously untrained men after the 21-week RT period. This suggests that the content of these proteins is increased to the same extent as the overall muscle protein content when muscle size is increased after a long-term RT period. In previous studies, high-intensity endurance training has decreased the UPR markers in rodent skeletal muscle,[55] whereas low-intensity endurance exercise did not.[55,56] These results suggest that exercise training may suppress ER stress at resting state in some but not in all conditions.

Both autophagy[15,17] and UPR[9,10] have been suggested to mediate part of the adaptation to exercise. However, the lack of significant correlations observed in the present study in young previously untrained young men suggests that the greater induction of UPR or autophagy markers did not translate into greater or minor muscle hypertrophy nor into strength in response to long-term resistance training. In addition, they were not associated with the recovery of maximal isometric force of the loaded muscles following the RE bout. Instead, when combined with the previous literature,[9,14,57] we suggest that these processes may be more likely related to the maintenance of skeletal muscle homoeostasis than to increase or decrease muscle hypertrophy and/or strength. Because in our study, protein carbonyls were unchanged, it is suggested that oxidative stress may not be the main contributor to the UPR induction[7] or autophagy[58] following RE.

In young men, the changes in the autophagy and UPR markers were more robust and more consistent in the RE group than they were in the individual responses in the individuals who did not do RE. However, in human muscle biopsy studies, not all of the acute exercise-induced changes in the gene expression are simply due to the exercise session, but may be induced by repeated biopsy obtainment or due to some unknown nonexercise stimulus.[59] Even though the 48-hour biopsy was obtained approx. 3 cm above the PRE-biopsy to avoid the residual effects of the repeated biopsy obtainment, some of the UPR markers as well as LC3II and P62 proteins were increased in a few nonexercisers as well. Due to this, we analysed normal variation in these proteins between 2 PRE-biopsies obtained from different legs in Experiment 3. Although there was a good correlation between the results from the 2 biopsies at the before RE situation, there were some differences suggesting that part of the changes in the control subjects are just normal variation. The increase in some of the UPR and autophagy markers in nonexercisers

may also be induced by inflammation after repeated biopsies from the same leg.[60-62] Therefore, the present study recommends future studies to always include a nonexercise group or nontraining limb for the acute experiments with multiple biopsies when studying UPR and/or autophagy.

In conclusion, the present study indicated that resistance training increases autophagosome content in the trained skeletal muscle, but this may be interfered with by ageing. Additionally, a single bout of RE induced UPR in a delayed manner, but long-term RT had no effect on the UPR indicators or ER-located chaperone content at resting state regardless of age. Changes in autophagy and UPR indicators were not associated with the increase in muscle size, strength or isometric force recovery. When combined with the previous literature, our data suggest that, in response to resistance exercise, the induction of autophagy and UPR may be more important for maintaining skeletal muscle homoeostasis than it is for regulating muscle growth or strength adaptations.

4 | MATERIALS AND METHODS

4.1 | Ethical approval

The University of Jyväskylä Ethical committee approved Experiments 1 and 2, and the Regional Ethics Committee for Medical and Health Research of South-East Norway approved Experiment 3. All the subjects were carefully informed of the design, possible risks and discomfort related to the study and signed a written informed consent to participate. All the studies were conducted according to the Declaration of Helsinki.

4.2 | Overall study design

The present study consisted of 3 separate experiments. The first 2 experiments investigated the effects of the resistance exercise (RE) and resistance training (RT)-induced changes on UPR and autophagy in healthy previously untrained young men (Experiment 1) and older men (Experiment 2; Figure 8A,B). Experiment 3 examined the RE-induced acute changes on UPR and autophagy markers in healthy previously trained subjects with a slightly longer acute time course than in Experiment 1 (Figure 8C). In all of these experiments, previously collected muscle samples were analysed.

4.3 | Subjects

4.3.1 | Experiments 1 and 2

Healthy, normal weight or slightly overweight and untrained young men (26 ± 4 years, n = 16 BMI: 19.6-28.9) and older men (61 ± 6 years, n = 8, BMI: 23.4-28.8) were selected from a larger group of subjects from previous studies[33,63] based on muscle sample availability. In Experiment 1, the young men were originally randomized to either a whey protein group (26 ± 6 years, n = 6 in this study), a placebo group (27 ± 2 years, n = 6 in this study) or into nonexercise control group (26 ± 4 years, n = 4).[63] There were no significant effects of protein supplementation on the measured variables ($P > .05$). More specifically, the differences in the major variables were small, and the P value was >.2 in more than 97% of the results, including the autophagy and UPR findings reported in the present study. Thus, for the sake of simplicity and to improve the statistical power for the RE/RT effects, the groups were pooled (27 ± 4 years, n = 12). The subjects have previously been described in more detail elsewhere.[33,63] Subjects were excluded from the study if they had previous regular strength training experience or if they had cardiovascular or pulmonary diseases, malfunctions of the thyroid gland, diabetes, were overweight (body mass index >30) or were unable to perform the exercise training or tests due to some other disease. In addition, medication known to influence the endocrine system, heart rate, cardiovascular or neuromuscular performance were used as exclusion criteria.

4.3.2 | Experiment 3

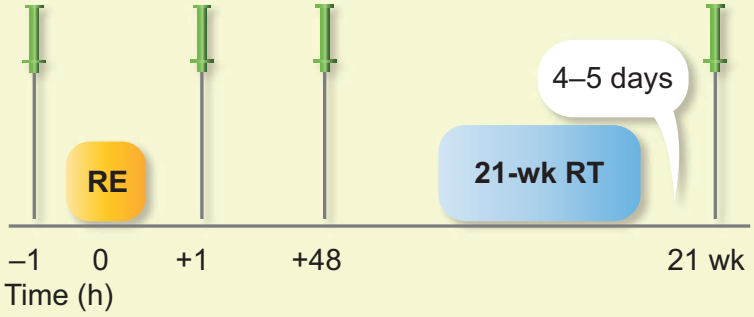
To elucidate the effects of acute resistance exercise with a slightly longer time course for the biopsy obtainment, with a slightly different exercise protocol than in Experiment 1, and if previous training status has an effect in the acute experiment, we also analysed muscle samples from another group of young, healthy and normal weight recreationally strength-trained subjects (5 females and 10 males) (25 ± 5 years, n = 15).[34] Originally subjects were randomized to either a placebo (24 ± 2 years, n = 8, in this study) or antioxidant (26 ± 9 years, n = 7, in this study) group. There was no antioxidant effect on measured variables ($P > .05$). To simplify the study and to improve statistical power, groups were pooled.

4.4 | Experiments 1 and 2

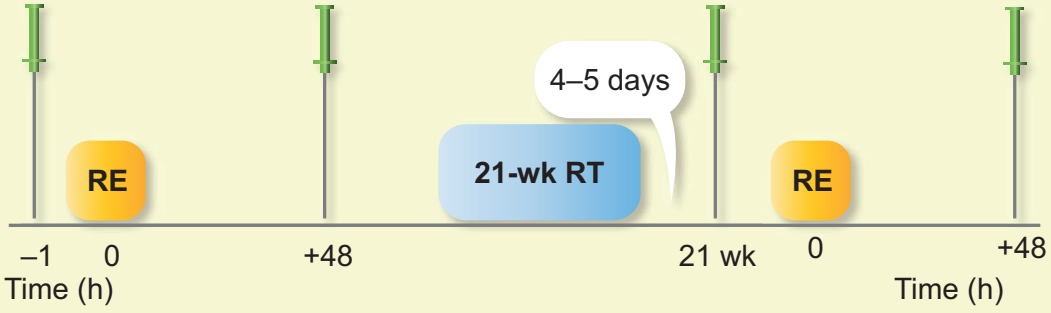
4.4.1 | Experimental resistance exercise (RE)

In Experiments 1 and 2, a resistance exercise bout (RE) was 5 × 10 RM performed on a leg press device, as previously described.[63,64] Maximal isometric bilateral leg extension force was measured before and after the last set with an electrodynamometer with a knee angle of 107°. Additionally, in Experiment 1, isometric leg extension force was also measured 1 hour after the RE bout as well as 48 hours after the bout.

Young men (Experiment 1)



Older men (Experiment 2)



(Experiment 3)

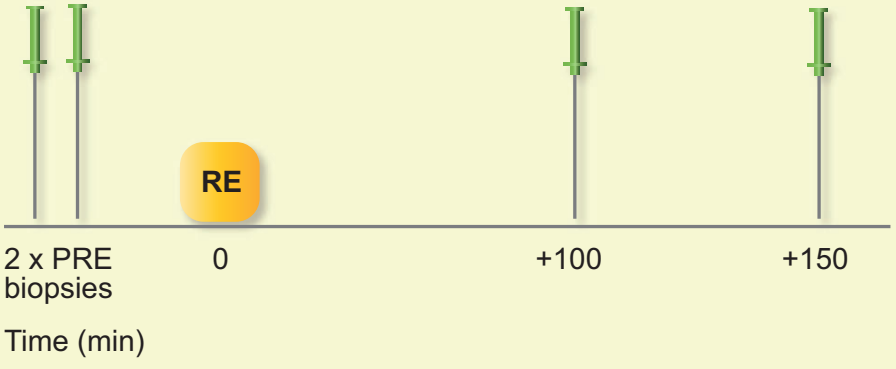


FIGURE 8 Study design of the 3 experiments. A, In young previously untrained (Experiment 1) and B, older previously untrained men (Experiment 2), resistance exercise (RE) was 5 × 10 repetitions in leg press until failure. Resistance training (RT) was 21 wk of supervised heavy whole body resistance training and was conducted similarly in young and older men. C, In previously recreationally strength-trained subjects (Experiment 3), RE was 4 × 10 RM of both leg press and knee extension, with 1 min of rest between sets and 3 min between exercises. Needles in the figure depict the time points when biopsies were obtained from the vastus lateralis (VL) muscle. The biopsy after the RT period (Experiment 1 and 2) was obtained 4-5 d after the last RE session to represent resting state and to avoid the acute effects of the last RE session of RT period

4.4.2 | Resistance training (RT)

The resistance training was conducted in the same manner in Experiments 1 and 2. During the 21-week RT period, participants conducted 2 RE sessions per week as previously described.[33,63] All the RE sessions were supervised by experienced trainers, and there was at least 2 days between each session. The training programme focused especially on knee extensor muscles (ie vastus lateralis, VL) because the muscle cross-sectional area (CSA) was measured and biopsies were obtained from that muscle. In each RE session, bilateral leg press, knee extension and bilateral knee flexion were conducted. In addition, exercises for chest, shoulder, upper back, trunk extensor and flexor, upper arm, ankle extensor, hip abductor and adductor muscle groups were performed during the RT period. Of these exercises, leg press and knee extension activate the vastus lateralis (VL) muscle, which was the muscle mainly studied in the present study.

4.5 | Resistance exercise in Experiment 3

The participants were tested in an acute experiment at a time point where they had conducted 4-6 weeks of RT intervention. The acute exercise sessions included 4 × 10 RM of both leg press and knee extension, with 1 minute of rest between sets and 3 minutes between exercises, as previously described.[34]

4.6 | Muscle biopsies

4.6.1 | Experiments 1 and 2

Biopsies were obtained from the VL muscle with a 5-mm Bergström biopsy needle, midway between the patella and greater trochanter as previously described.[63,64] In both experiments, the subjects fasted similarly for 3 hours before the morning biopsies (pre, 48 hours and pre-RE biopsy at post 21 weeks) obtained and were not allowed to eat before the 1 hour post-RE biopsy. In Experiment 1, muscle biopsies were obtained 30 minutes before (PRE), 1 hour after and 48 hours after the first RE bout as well as 4-5 days after the last RE session of the 21-week RT period (Figure 8A). In Experiment 2, biopsies were obtained before the RE bout and 48 hours after at both pre- and post-RT (Figure 8B). The resting state post-RT biopsy was obtained 4-5 days after the last RE session in both experiments. In both experiments, the PRE and 48-hour biopsies were obtained from the same leg, and the post 1-hour biopsy was from the other leg. To avoid the residual effects of repeated biopsies, the 48-hour biopsy was obtained approx. 3 cm above the PRE-biopsy scar. The 21-week biopsy was taken from the same leg as the PRE and 48-hour biopsies.

4.6.2 | Experiment 3

Muscle biopsies were obtained from the VL muscle twice before the acute exercise bout (2 PRE-samples from different legs) and twice after at 100 and 150 minutes (Figure 8C), as previously described.[34] The repeated biopsies were always obtained approx. 3 cm proximal to the previous insertion.

In all the experiments, muscle biopsies were flash frozen in liquid nitrogen after being rinsed of blood and any visible connective and adipose tissue. Muscle biopsies were stored at −80°C for future protein and mRNA analysis.

4.7 | Protein extraction

In Experiments 1 and 3, muscle biopsies were hand-homogenized in ice-cold buffer (20 mmol L^{-1} HEPES (pH 7.4), 1 mmol L^{-1} EDTA, 5 mmol L^{-1} EGTA, 10 mmol L^{-1} $MgCl_2$, 100 mmol L^{-1} β-glycerophosphate, 1 mmol L^{-1} Na_3VO_4, 2 mmol L^{-1} DTT, 1% Triton X-100, 0.2% sodium deoxycholate, 30 μg mL^{-1} leupeptin, 30 μg mL^{-1} aprotinin, 60 μg mL^{-1} PMSF and 1% phosphatase inhibitor cocktail [P 2850; Sigma, St. Louis, MO, USA]) at a dilution of 15 μl mg^{-1} of wet weight muscle as previously described.[43] To remove cell debris, homogenates were rotated for 30 minutes at 4°C, centrifuged at 10 000 *g* for 10 minutes at 4°C and subsequently stored at −80°C for future analysis. Bicinchonic acid protein assay (Pierce Biotechnology, Rockford, IL, USA) and Bio-Rad DC protein microplate assays (Bio-Rad, Hercules, CA, USA) were used to determine total protein amount.

In Experiment 2, muscle biopsies were hand-homogenized on ice with a buffer that is rather similar, but not identical as in Experiments 1 and 3: 50 mmol L^{-1} Hepes

pH 7.4, 0.1% Triton X-100, 4 mmol L^{-1} EGTA, 10 mmol L^{-1} EDTA, 15 mmol L^{-1} $Na_4P_2O_7 \cdot 10H_2O$, 100 mmol L^{-1} β-glycerolphosphate, 25 mmol L^{-1} NaF, 1 mmol L^{-1} Na_3VO_4, 0.5 μg mL^{-1} leupeptin, 0.5 μg mL^{-1} pepstatin and 0.3 μg mL^{-1} aprotinin) in a 4% (w/v) solution. Lowry-based method was used (Bio-Rad) to determine protein concentrations. As no phosphatase inhibitor cocktails were used for the older men samples, only total protein levels were analysed from these samples.

4.8 | RNA extraction and cDNA synthesis

In Experiment 1, another part of the muscle biopsy was homogenized with FastPrep (Bio101 Systems, USA) for the RNA-analysis. Tubes and total RNA were extracted using TRIzol-reagent (Invitrogen, Carlsbad, CA, USA) as previously described.[63] The yielded RNA extraction was un-degraded and DNA free based on an OD260/OD280 ratio (1.8-2.0) and electrophoresis respectively. RNA (3 μg) was reverse transcribed to cDNA by High Capacity cDNA Archive Kit (Archive Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

4.9 | Western immunoblot analyses

For all the 3 experiments, muscle homogenates were mixed with Laemmli sample buffer and were heated at 95°C for 10 minutes to denaturize proteins. Approx. 30 μg of total protein was separated by SDS-PAGE and was transferred to a PVDF membrane. The membrane was blocked with 5% milk (TBS including tween 0.1%) for 2 hours in room temperature and incubated overnight with primary antibodies in 4°C. Membrane was washed with TBS-T and incubated with secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 1 hour in RT. After secondary antibody incubation, membranes were washed with TBS-T, and proteins were visualized with ECL (SuperSignal west femto maximum sensitivity substrate, Pierce Biotechnology). The chemiluminescent signal was quantified by ChemiDoc XRS device with Quantity One software (version 4.6.3. Bio-Rad laboratories). The average of Ponceau-S staining and GAPDH (Abcam, Cambridge, UK) was used to normalise the protein loading and blotting efficiency. There were 2 PRE-samples in Experiment 3, and the CV (%) and intraclass correlations are shown in Table 1.

4.9.1 | Protein carbonyls

As a marker of oxidative stress, protein carbonyl content was measured with Oxyblot Protein Oxidation Detection kit (Merck Millipore, S1750) according to manufacturer's instructions with 10 μg of total protein used for the derivatization reaction. The blots were visualised and quantified as described above. The membrane was stained with Ponceau-S to normalise protein loading and blotting efficiency.

TABLE 1 Coefficient of variation (CV %) and intraclass correlation values of the results between 2 PRE muscle biopsies from different legs in Experiment 3

Protein	CV (%)	Intraclass correlation (average measures)
PERK	24	$R = 0.91$, $P < .001$
IRE1α	29	$R = 0.81$, $P < .01$
PDI	18	$R = 0.65$, $P < .05$
p-eIF2α	23	$R = 0.55$, $P = .08$
eIF2α	10	$R = 0.72$, $P < .05$
HSP10	27	$R = 0.805$, $P < .01$
LC3I	17	$R = 0.78$, $P < .01$
LC3II	20	$R = 0.84$, $P < .01$
p-ULK1ser757	23	$R = 0.81$, $P < .01$
p-JNK54	22	$R = 0.85$, $P < .01$
p-JNK46	38	$R = 0.11$, $P = .42$
JNK54	13	$R = 0.68$, $P < .05$
JNK46	22	$R = 0.57$, $P = .07$
Beclin-1	15	$R = 0.85$, $P < .01$

4.9.2 | Primary antibodies

Antibodies were purchased from several companies. The antibodies from Cell Signaling Technology (Danvers, MA, USA) were as follows: inositol-requiring enzyme 1α (IRE1α, #3294, 1:1000), protein disulphide isomerase (PDI, #3501, 1:3000), protein kinase R-like endoplasmic reticulum protein kinase (PERK, #5683, 1:1000), eukaryotic initiation factor 2 subunit α (eIF2α, #5324, 1:1000) and its phosphorylated form at ser51 (p-eIF2α, #3398, 1:1000), glucose-regulated protein 78 (GRP78, #3177, 1:1000), calnexin (#2679, 1:1000), serine/threonine protein kinase (ULK1, #8054, 1:1000) and its phosphorylated form at serine 757 (p-ULK1, #14202, 1:1000) and at ser555 (p-ULK1, #5869, 1:1000), Sequestome-1/P62 (P62, #5114, 1:2000) Beclin-1 (#3738, 1:1000) apoptosis regulator BCL-2 (BCL-2, #3498, 1:1000) activating transcription factor 4 (ATF4, #11815, 1:1000), jun-amino-terminal-kinase (JNK, #9252, 1:1000) and its phosphorylation at Thr183/Tyr185 (p-JNK, #4668, 1:1000), crystallin-αB (CRYAB, #45844; 1:3000) and phosphorylated acetyl-CoA carboxylase at ser79 (p-ACC, #8578, 1:2000). Antibody against GAPDH (ab9485, 1:10 000) was from Abcam. Antibodies that recognize the inducible forms of heat-shock protein 27 (HSP25, SPA-801, 1:1000) and glucose-regulated protein 75 (GRP75, SPS-825, 1:1000) were from Enzo Life Sciences (Farmingdale, NY, USA). Antibody against HSP10 (SAB4501465, 1:1000) was from Sigma-Aldrich

(St. Louis, MO, USA). For measuring protein levels of the nontruncated full-length splice variants of PGC-1α, the antibody (1:5000, Calbiochem, Merck KGaA, Darmstadt, Germany) against C-terminus of protein (amino acids 777-797) was used. LC3I and LC3II protein levels were measured using an antibody (L7543, 1:1000) from Sigma-Aldrich. Cytochrome C was measured with an antibody (SC-8385, 1:1000) from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Dallas, USA). Antibodies against glutaredoxin HSP27 and GRP75 were kindly provided by Dr. Mustafa Atalay.

4.10 | RT-qPCR

RT-qPCR with standard procedures using iQ SYBR Supermix (Bio-Rad laboratories) and CFX96 real-time PCR Detection system (Bio-Rad laboratories) were used to measure mRNA expression level in triplicates. Total X-box binding protein (*Xbp1t*) and spliced variant (*Xbp1s*) were analysed using SYBR green primers: *Xbp1s*: forward: tgctgagtccgcagcaggtg, reverse: gctggcaggctctggggaag, product length: 169 bp and *Xbp1t*: forward: aggagaaggcgctgaggaggaaact, reverse: accacttgctgttccagctcactca, product length: 100 bp. The protocol of *Xbp1s* and *Xbp1t* was initiated at 95°C for 3 minutes, which was followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 62°C for 15 seconds and extension at 72°C for 30 seconds. *Xbp1s* and *Xbp1t* end-products were verified by DNA electrophoresis. qPCR for C/EBP homologous protein (*Chop/Ddit3*, assay ID (qHsaCED0056908), *Lc3b* (assay ID qHsaCED0038576) and sequestome 1 (*P62/SQSTM1*, assay ID qHsaCED0045925) were conducted by using pre-designed and validated Bio-Rad PrimePCRtm SYBR Green Assays using the recommended protocol by the manufacturer. GAPDH was used to normalise the mRNA expression levels because it was unaffected by RE and RT and was better than 18SRNA as previously described.[65] Delta delta Ct (ddCt) method was used to measure gene expression levels at the exponential amplification phase.

4.11 | Statistical analysis

For all 3 experiments, the main effect of RE/RT was investigated with a General Linear Model with repeated measures (IBM SPSS statistics version 24). For the post hoc tests, the RE- and RT-induced fold changes from the PRE condition were evaluated by a Holm-Bonferroni adjusted *t* test or a related samples Wilcoxon rank-test (when not normally distributed). In Experiment 1, 3 comparisons were included in the Holm-Bonferroni adjustments (PRE vs 1 hour, PRE vs 48 hours and PRE vs 21 weeks). In Experiment 2, the comparisons were PRE vs 48 hours, PRE vs post 21 weeks (PRE-RT) and PRE vs 48 hours (POST-RT). In Experiment 3, the comparisons were PRE (an average of 2 PRE-samples) vs 100 and PRE (an average of 2 PRE-samples) vs 150.

Due to the small sample size in the No RE group in Experiment 1 (n = 2-4), statistical tests were not performed for it and instead the individual values are shown. Due to the nature of separate experiments with minor differences between the experiments, direct comparisons were not conducted between the experiments. Pearson's product-moment coefficient was used to analyse associations between the variables. In the figures, open bars depict the mean of that time point whereas circles and triangles depict individual values. The statistical significance was set at $P < .05$.

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AUTHORS' CONTRIBUTION

Experiments 1 and 2 were designed and carried out by JJH, JPA, AAM and KH, while the present study was designed by JH, JJH and JPA. Experiment 3 was originally designed and carried out by GP and TR. JH analysed the data in the present study with the supervision of JJH. The first version of the manuscript was drafted by JH, which was then commented by other authors and revised by JH, JJH and JPA. All the authors read and approved the final manuscript.

COMPETING INTERESTS

No conflicts of interest, financial or otherwise, are declared by the authors.

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IV

SPRINT AND STRENGTH TRAINING MODULATES AUTOPHAGY AND PROTEOSTASIS IN AGING SPRINTERS

by

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