THE REGULATION OF EXERCISE-MEDIATED SKELETAL MUSCLE FUNCTION: THE ROLE OF PROTEIN UBIQUITYLATION

by

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Abstract

Skeletal muscle is a dynamic tissue responsible for maintaining healthy functioning of the human body. Failure in maintaining skeletal muscle health can cause deleterious effects such as metabolic diseases. Ubiquitylation is a post-translational modification known to regulate most biological processes in eukaryotic cells through both degradative and non-degradative mechanisms. Evidence suggests that ubiquitylation plays a fundamental role in protein degradation, controlling protein quality control in skeletal muscle. However, ubiquitylation currently remains a poorly established process with respect to its complexity. Exercise is a well-established physiological stimulus for improving skeletal muscle health and preventing, attenuating or offsetting impaired skeletal muscle function. Therefore, understanding how ubiquitylation is mediated by exercise will help to identify novel therapeutic strategies to improve skeletal muscle health. In this thesis, I will review some existing knowledge on exercise-mediated ubiquitylation in skeletal muscle. Previous work has indicated that exercise can promote protein degradation. As a result, the majority of research investigating exercise-mediated ubiquitylation has focused on ubiquitin-mediated protein degradation. These studies have commonly utilised targeted approaches by investigating individual proteins. Recent efforts have harnessed mass spectrometry-based proteomics for studying exercise-mediated ubiquitylation in skeletal muscle, providing an unbiased and more comprehensive investigation of this post-translational modification. However, the current proteomic methods to detect protein ubiquitylation in primary tissue sample, such as skeletal muscle, lacks sensitivity and so requires further improvements. Therefore, we will finally suggest improved methods for analysing exercise-mediated ubiquitylation in whole skeletal muscle proteome alongside providing some of the ongoing research I have conducted to validate their effectiveness.

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List of Abbreviations:

cAMP - Cyclic adenosine monophosphate

diGLY - diglycine

DUBs - De-ubiquitylating enzymes

Fbxl22 - F-box and leucine-rich protein 22

HECT - Homologous to E6AP C-terminus

HIIT - High intensity interval training

MAPK - Mitogen-activated protein kinase

MHC - Myosin Heavy Chain

MICT - Moderate intensity continual training

MPB - Muscle protein breakdown

MPS - Muscle protein synthesis

MuRF1 - Muscle RING-finger protein-1

MUSA1 - Muscle ubiquitin ligase of SCF complex in atrophy-1

MAFbx - Muscle atrophy F-box

Nedd4-1 - Neural precursor cell expressed developmentally down-regulated protein 4

PTM - Post-translational modification

RBR - RING-in-between RING

RING - Really Interesting New Gene

SMART - Specific of Muscle Atrophy and Regulated by Transcription

TR-TUBE - Trypsin resistant tandem ubiquitin binding entity

TUBE - Tandem ubiquitin-binding entity

UPS - Ubiquitin proteasome system

1. Introduction

1.1 Importance of skeletal muscle

Skeletal muscle is an integral tissue within the human body and plays a crucial role not only in locomotion but also in metabolism. Skeletal muscle compromises ~40% of our body weight and is capable of generating force for movement, posture and breathing. Due to its large mass and high demands, skeletal muscle is also essential for energy metabolism through utilising, storing and supplying key nutrient substrates such a glucose and amino acids. Importantly, skeletal muscle is a dynamic tissue that undergoes constant remodelling in response to its demands. This plasticity enables skeletal muscle to adapt to various internal and external stimuli such as nutrient availability and exercise to cope or excel in a challenging environment. Due to the critical role of skeletal muscle for whole body functioning, it is not surprising that failure to maintain this tissue results in deleterious consequences. When skeletal muscle mass declines (sarcopenia) and its metabolic functions are impaired, this can result in diseases such as type 2 diabetes and heart diseases (Srikanthan and Karlamangla, 2011; Hong *et al.*, 2017; Xia *et al.*, 2020). Loss of skeletal muscle mass is associated with the reduction of longevity in individuals (Srikanthan and Karlamangla, 2014), which is also the case in diseased states such as cancer (Caan *et al.*, 2018). Not only does impaired skeletal muscle negatively impact an individual's quality of life, but also has economic burdens to healthcare systems with muscle wasting resulting in over £2.5 billion UK healthcare costs annually (Pinedo-Villanueva *et al.*, 2019). Altogether, this demonstrates the importance of this tissue throughout our lifespan, therefore maintaining healthy skeletal muscle is vita

1.2 Role of post-translational modifications

In order to maintain healthy skeletal muscle, post-translational modifications (PTMs) are an essential biological process. Their role is to modify intracellular proteins after they have been synthesised in order to alter their activity, location, interactions and abundance in the cell. As a result, PTMs regulate protein function and influence the majority of cellular processes, including cell cycle control (Cuijpers and Vertegaal, 2018), apoptosis (Zamaraev *et al.*, 2017) and cell signalling (Deribe, Pawson and Dikic, 2010). PTMs are essential in all cells and tissues, providing them with an expansive and complex array of protein species (proteome) essential for tight regulation of cellular processes (Virág *et al.*, 2020). Within skeletal muscle, PTMs are known to regulate key processes such as muscle growth, through modifying signalling pathways required for muscle protein synthesis (von Walden *et al.*, 2016) and muscle fibre development (Maggs *et al.*, 2000). Moreover, aberrant PTMs can hinder skeletal muscle function, for instance age-related impaired motor function (Li *et al.*, 2015) and sarcopenic phenotype (Wei *et al.*, 2018). This illustrates the regulatory role of PTMs and the downstream implications this has in skeletal muscle, crucial for healthy functioning.

Proteins can be modified by a complex array of PTMs, each of which contribute towards the expansion of the proteome. Analysis of PTMs has identified over 200 different types (de Hoog and Mann, 2004), with certain ones being more commonly researched such as phosphorylation, ubiquitylation, methylation, acetylation, hydroxylation, carbonylation and glycosylation. These modifications each have their own roles (Hu, Guo and Li, 2006; Karve and Cheema, 2011), however proteins can also be modified simultaneously by a

combination of different PTMs via crosstalk (Venne, Kollipara and Zahedi, 2014). In addition, proteins can be separately modified by various PTMs, owing to the reversible nature of certain PTMs such as phosphorylation and ubiquitylation (Prabakaran *et al.*, 2012). As a result of the complexity involved with PTMs, much remains to be understood with regard to their physiological relevance. Together, with the importance of PTM in skeletal muscle it is crucial that we expand our knowledge of PTMs, especially those that are ill-defined.

1.3 Ubiquitylation

1.3.1 Importance of ubiquitylation

Ubiquitylation is less well defined than many other PTMs yet is recognised as an essential process within eukaryotic cells. This limited understanding is largely attributed to the versatile effects this PTM can exert on proteins, arising from its ability to create functionally distinct modifications on proteins, signalling for different biological processes within the cell. Accordingly, deciphering protein ubiquitylation has been a challenging task. Nevertheless, as a result of its versatility, ubiquitylation impacts most biological processes, including DNA regulation (Ghosh and Saha, 2012), cell cycle control (Skaar and Pagano, 2009; Emanuele and Enrico, 2019) and protein turnover (Hershko and Ciechanover, 1998). The latter is where ubiquitylation has been most famously recognised, illustrated by a Nobel prize award in 2004 (Lecker, Goldberg and Mitch, 2006) for discovering the role of ubiquitylation in degrading cellular proteins (Ciechanover, Hod and Hershko, 1978; Hershko *et al.*, 1980; Hershko, Ciechanover and Rose, 1981). As a result of its widespread contribution in regulating an array of biological processes, it is not surprising that dysfunctional ubiquitylation is deleterious. Within humans, dysfunctional ubiquitylation leads to multiple diseases such as cancer, neurodegeneration and muscular disorders

(Kitada *et al.*, 1998; Sandri *et al.*, 2013; Rape, 2017). This demonstrates that ubiquitylation is a key determinant of healthy cell functioning, therefore investigating this PTM is important.

1.3.2 Protein ubiquitylation process

Ubiquitylation is a complex process that modifies proteins through covalent binding of a ubiquitin molecule (molecular weight of ~8.6 kDa). This system involves a ubiquitin activating (E1), conjugating (E2) and ligating (E3) enzyme that work in tandem to regulate this process. In the human genome, there are 2 E1s, over 38 E2s and more than 600 E3s (Ye and Rape, 2009). E1 enzymes use adenosine triphosphate (ATP) to activate ubiquitin, initiating the downstream pathway that proceeds. E2 enzymes interact with the ubiquitin bound to E1 and then with a specific E3 enzyme allowing the transfer of ubiquitin. E3 enzymes (hereafter referred to as E3 ligases) bring the substrate and ubiquitin together enabling protein ubiquitylation to occur (Fig.1). Ubiquitin commonly tags its substrate via isopeptide bond formation in which the C-terminal carboxylate group of ubiquitin is bound to the lysine residue of its substrate. Ubiquitin tagging can occur in a variety of ways from a single ubiquitin molecule (monoubiquitylation), multiple single ubiquitin molecules (multiubiquitylation) or branches of ubiquitin molecules (polyubiquitylation) which result in varying chain types and lengths (Swatek and Komander, 2016). During ubiquitin chain formation, the C-terminus of ubiquitin forms covalent bonds with another ubiquitin molecule through either of its seven lysine residues (isopeptide) or N-terminus methionine (peptide). Therefore, polyubiquitylation is capable of forming eight diverse polyubiquitin chain types (K6, K11, K27, K29, K33, K48, K63 and M1). The binding of ubiquitin is a dynamic and transient process as the process of ubiquitylation can be reversed deubiquitylating enzymes (DUBs). DUBs cleave ubiquitin modifications from substrates or

even between ubiquitin molecules (Mevissen and Komander, 2017). Together, this illustrates the complexity of protein ubiquitylation and the numerous enzymes involved in this process.

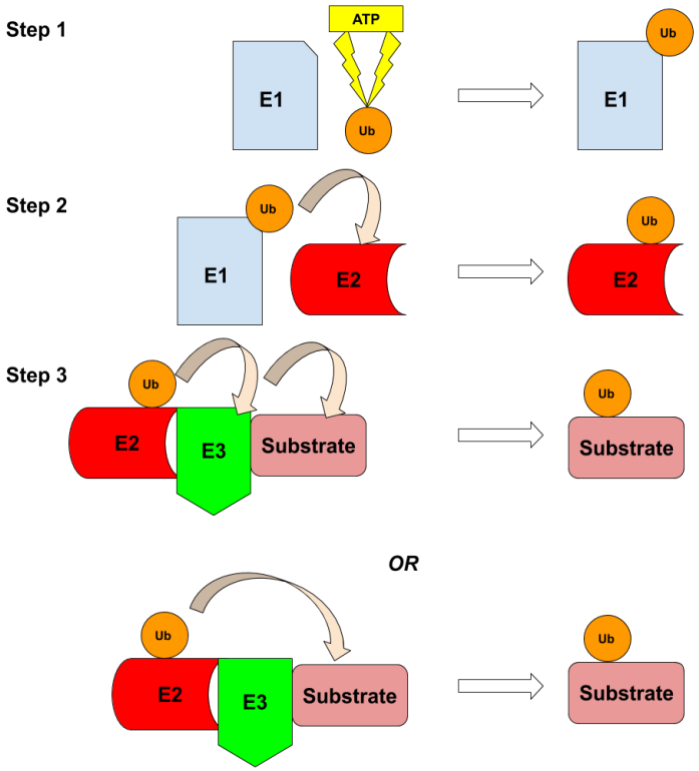

Figure 1: Schematic representation of protein ubiquitylation. Step 1: E1 forms a thioester bond with free ubiquitin using ATP. Step 2: The E1-bound ubiquitin is transferred over to an E2, which also interact using a thioester bond. Step 3: The E2-bound ubiquitin cooperates with an E3 in order to form covalent-bound ubiquitin on the target substrate.

1.3.3 Fate of protein ubiquitylation

1.3.3.1 Ubiquitin code

Once a protein has been ubiquitylated, there are various cellular processes that this modification can signal for. This is because the different types of protein ubiquitylation signal for alternative biological outcomes (Akutsu, Dikic and Bremm, 2016; Swatek and

Komander, 2016). As a result, ubiquitylation functions on a coded system that informs the cell what appropriate action needs to be taken. Consequently, ubiquitylation must be tightly regulated to ensure the correct response is carried out. Importantly, the structural diversity of ubiquitin chains allows ubiquitin binding domains - which recognise and bind to ubiquitin, to distinguish them from one another (Sadowski *et al.*, 2012). The most extensively researched ubiquitin chain types are K48 and K63 ubiquitin chains, well characterised in degradative and non-degradative roles respectively (Komander and Rape, 2012; Swatek and Komander, 2016). Due to methodological limitations, current analysis of the other 'atypical' ubiquitin chain types is lacking. Recently, research has begun to decipher the complexity of the ubiquitin code through utilising more advanced approaches such as genetic analysis and proteomics, uncovering roles of certain atypical chain types (Huang and Zhang, 2020), for instance K11 chains have since been associated with cell cycle control (Gutierrez *et al.*, 2018). Together, our understanding of protein ubiquitylation is continually expanding, providing us with a better idea of the ubiquitin code.

1.3.3.2 Key Ubiquitin enzymes

The fate of protein ubiquitylation is dependent on the specific ubiquitin enzymes harnessed. There are 3 sub-types of E3 ligase that are characterised by their domains: Really Interesting New Gene (RING), Homologous to E6AP C-terminus (HECT) and RING-in-between RING (RBR) (Morreale *et al.*, 2016). Importantly, RING domains - which form the vast majority of E3 ligases, act as a scaffold protein bringing the ubiquitin bound E2 and substrate together to allow protein ubiquitylation and therefore have no catalytic activity (Metzger *et al.*, 2014). Therefore, in this case E2 enzymes transfer the ubiquitin over to the substrate and subsequently determine the ubiquitin linkage type and thus the fate of protein ubiquitylation (van Wijk and Timmers, 2010). This concept has been

supported by studies demonstrating chain type variability depending on the E2 bound to the RING domain (Christensen, Brzovic and Klevit, 2007) and chain type specificity when utilising the same E2 (Petroski and Deshaies, 2005). Alternatively, if the E3 ligase harnessed is a HECT or RBR type, the transfer of ubiquitin onto the substrate is performed by these enzymes so they determine the fate of protein ubiquitylation. Nevertheless, regardless of the sub-type, E3 ligases select the substrate to be ubiquitylated, for instance E3 ligases target certain substrates for protein degradation (Hershko *et al.*, 1986). Therefore, although they may not always determine the fate of protein ubiquitylation, E3 ligases always determine which protein is being ubiquitylated despite the fact that less than 10% of all known E3 ligases have recognised substrates (Dikic *et al.*, 2014).

1.3.3.3 Ubiquitin-Proteasome System

The most extensively researched outcome of ubiquitylation is protein degradation via the ubiquitin proteasome system (UPS). The UPS degrades ubiquitylated proteins in a highly targeted fashion, utilising an array of subunits that work in tandem to allow this complex ubiquitin-regulated machinery to function. This degradation mechanism consists of a 19S cap, consisting of a base and a lid, and 20S core (Fig.2), each with distinct roles. The 19S cap contains three major ubiquitin binding subunits in the base subcomplex: Rpn10 (S5), Rpn13 and Rpn1, crucial for the recognition of ubiquitylated proteins tagged for degradation (Deveraux *et al.*, 1994; Husnjak *et al.*, 2008; Shi *et al.*, 2016). Recently these subunits have emerged as being a versatile binding platform with specific roles for certain ubiquitin chains (Martinez-Fonts *et al.*, 2020). Once these 19S subunits bind to ubiquitin, the tagged protein must then be unfolded in an ATP-dependent manner before being degraded in the 20S core (Weber-Ban *et al.*, 1999; Collins and Goldberg, 2017). It is the role of the 19S base subcomplex to unfold proteins through its ATPase subunits (Rpt1-6),

which require ATP hydrolysis (Liu *et al.*, 2006). Importantly, ubiquitin is not degraded, but rather recycled through three DUBs: Rpn11, Usp14 and Uch37, which remove polyubiquitin chains from the substrate (Reyes-Turcu, Ventii and Wilkinson, 2009). Rpn11 DUB is located in the 19S lid and is the most highly conserved subunit in this subcomplex (Yao and Cohen, 2002). Interestingly, this deubiquitylating process appears to directly regulate substrate translocation into the 20S core for degradation (Zhu *et al.*, 2005). The 20S core is the central catalytic part of the UPS which contains alpha and beta (β) subunits. The catalytic activity comes from three beta-subunits: β1, β2 and β5 which cleave peptide bonds, causing the protein to be degraded (Fig.2) (Tanaka, 2009). Although β1, β2 and β5 subunits confer the same capabilities, they have distinct mechanisms for degrading proteins which are peptidyl-glutamyl peptide-hydrolysing (PGPH), trypsin-like and chymotrypsin-like activity, respectively (Heinemeyer *et al.*, 1997). The distinct β1, β2 and β5 subunits cleave peptide bonds at specific C-terminal amino acids, namely acidic, basic and hydrophobic residues, respectively (Nussbaum *et al.*, 1998; Tanaka, 2009). In summary, the UPS is composed of two main cores which contain multiple subunits with specific roles, all contributing towards the degradation of ubiquitylated proteins.

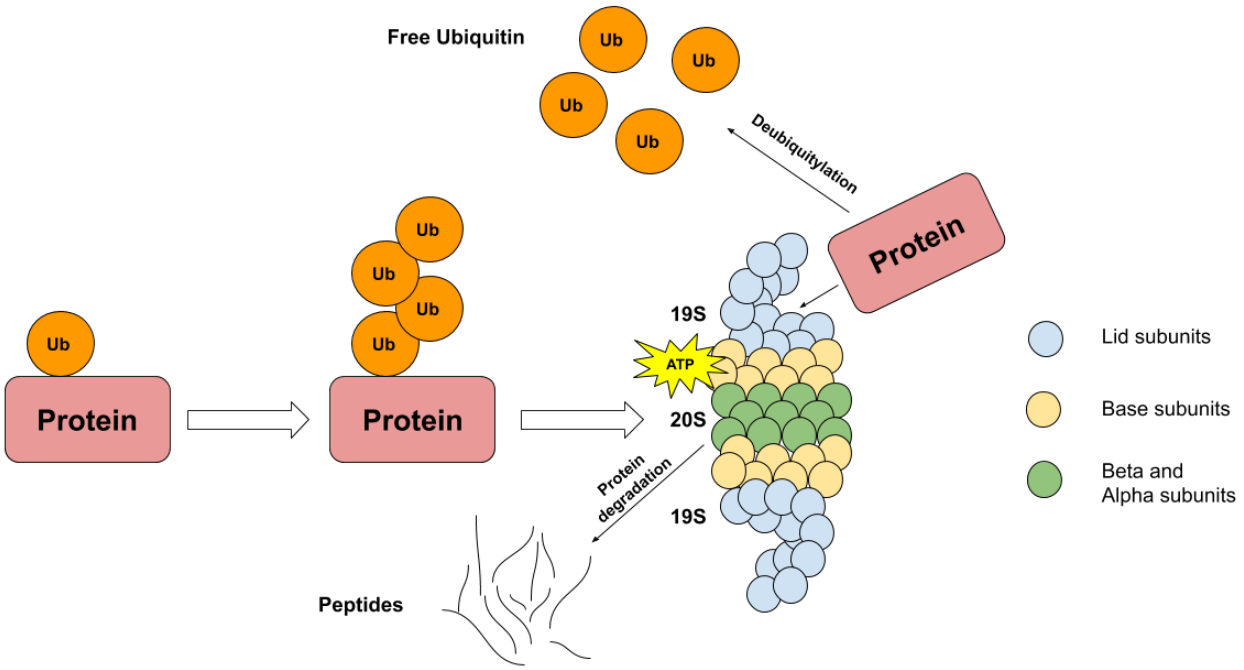

Figure 2: Schematic representation of UPS-mediated protein degradation. A protein substrate is tagged with a ubiquitin molecule multiple times, forming a polyubiquitin chain. The polyubiquitylated substrates are then recognised by UPS subunits on the 19S cap which contain ubiquitin binding domains. The ubiquitin molecule is removed by DUBs and the substrate is unfolded using a ring of ATPase subunits on the entrance of the 20S core. Once entered into the 20S core, the unfolded substrate is degraded into peptides using the catalytic β-subunits.

The UPS is a quality control mechanism crucial for to maintaining a healthy cell environment. Proteins are inherently unstable in comparison to other molecules such as DNA and thus require favourable conditions in order to sustain their structure. The lifespan of cellular proteins can vary; thus, proteins are often considered as either short or long-lived. The majority of cellular proteins are long-lived and ~2% of them are degraded per hour in mammalian cells, compared to ~11% in short-lived proteins (Gronostajski, Pardee and Goldberg, 1985; Goldberg, 2003). Both short and long-lived proteins are thought to be degraded by the UPS in an ATP-dependent manner (Gronostajski, Pardee and Goldberg, 1985). The decay of long-lived proteins is thought to be triggered by random events

(Goldberg, 2003), perhaps causing them to lose functionality and so the UPS can target and remove them so they don't hinder normal cell functioning. Some proteins never attain a functional structure and immediately become defective, thus known as short-lived proteins. Remarkably, they account for approximately 30% of newly synthesised proteins and the UPS is heavily attributed towards degrading these misfolded proteins (Schubert *et al.*, 2000). Alongside this housekeeping role of removing damaged and misfolded proteins, the UPS is also essential for regulating various cellular processes. These processes include mediating transcription (Zimmermann *et al.*, 2000; Muratani and Tansey, 2003), DNA repair (Falaschetti *et al.*, 2011; Karpov *et al.*, 2013) and cell cycle control (Emanuele and Enrico, 2019; Kito *et al.*, 2020). These can be altered as a result of protein degradation via the UPS, demonstrating the downstream effects of this catalytic machinery. Transcription for instance, is critical for the correct regulation of gene expression through the controlled timing, location and abundance of transcription proteins. UPS-dependent protein degradation is important for removing transcriptional activators, preventing uncontrolled gene expression (Salghetti *et al.*, 2001; Muratani and Tansey, 2003), but also for removing transcription repressors providing a feed-forward mechanism to upregulate gene expression when required (Yun and Lee, 2003; Durairaj and Kaiser, 2014). Remarkably, the UPS is thought to be involved in more than 75% of protein degradation in mammalian cells (Craiu *et al.*, 1997), highlighting its pivotal role for both maintaining protein homeostasis alongside regulating a plethora of key cellular processes.

1.3.3.4 UPS-independent protein ubiquitylation

Ubiquitylation has alternative functions besides signalling for protein degradation. Such functions include protein trafficking, kinase signalling and endocytosis which are thought to be largely regulated through either monoubiquitylation and K63-linked polyubiquitin chains

(Terrell *et al.*, 1998; Deng *et al.*, 2000; Haglund and Dikic, 2005; Mukhopadhyay and Riezman, 2007; Erpapazoglou, Walker and Haguenauer-Tsapis, 2014). This non-proteolytic regulation has been established for a while, for instance kinase signalling through IkB kinase is activated by ubiquitylation (Chen, Parent and Maniatis, 1996) which occurs through K63 chains independent of the UPS (Deng *et al.*, 2000). K63 chains are widely documented not to interact with the UPS, unless being branched with other chains that are associated with the UPS such as K48 chains (Ohtake *et al.*, 2018). Importantly, they are the second most abundant chain type after K48 chains in mammalian cells (Ohtake *et al.*, 2016), suggesting an active role for UPS-independent protein ubiquitylation. Protein degradation via the UPS is primarily thought to require polyubiquitin chains (Thrower *et al.*, 2000), thus monoubiquitylated proteins are not seen as a target of the UPS. Interestingly, research has demonstrated a role for monoubiquitylation in substrate recruitment for the UPS (Isasa *et al.*, 2010), however their roles have mainly been linked to non-proteolytic functions such as endocytosis (Haglund, Di Fiore and Dikic, 2003). Further support of UPS-independent protein ubiquitylation comes from UPS inhibition in human non-muscle cell lines, resulting in negligible increases K63 ubiquitin chains (W. Kim *et al.*, 2011). Moreover, certain less well characterised ubiquitin chain types such as K6, K27, K33 have not been linked to the UPS, although their precise roles are still being investigated. It is therefore evident that ubiquitylation is not limited to protein degradation and in doing so has highlighted alternative roles of ubiquitylation.

1.4 Ubiquitylation in skeletal muscle

1.4.1 Role of protein ubiquitylation in skeletal muscle

Within skeletal muscle, research investigating ubiquitylation has predominantly focused on its degradative role. As a highly metabolic tissue, skeletal muscle undergoes constant

protein turnover which is regulated in part by protein breakdown. Not only is protein degradation essential for removing unwanted proteins, but also for promoting amino acid recycling for the synthesis of new proteins which is crucial for muscle growth. The degradation of proteins within skeletal muscle is particularly important because this tissue is the largest protein reservoir of the body (Béchet *et al.*, 2005; Argilés *et al.*, 2016) and so maintaining protein homeostasis is crucial. Although there are different mechanisms that regulate protein breakdown including lysosomal, calcium-dependent calpains and the UPS (Lecker *et al.*, 1999), the UPS is seen as one of the main catalysts for protein breakdown skeletal muscle, demonstrating a key role in myotube cells (Zhao *et al.*, 2015). Together, this has contributed towards an emphasis on ubiquitin-mediated protein degradation research in skeletal muscle.

1.4.2 Ubiquitin chain types in skeletal muscle

Investigation into ubiquitin chain types in skeletal muscle has recently advanced, progressing our understanding of protein ubiquitylation in this tissue. Due to their well-established role in protein degradation via the UPS (Wilkinson *et al.*, 1995), K48-linked ubiquitin chains have been the more extensively studied ubiquitylation chain type in skeletal muscle (Ramirez-Martinez *et al.*, 2017; VerPlank *et al.*, 2019). Interestingly, although in most tissues K48 chains appear to be the most dominant ubiquitin chain type, a new study has demonstrated that in skeletal muscle K33 ubiquitin chains make up ~76% of total ubiquitin chains and K29 ubiquitin chains are also highly present when compared to liver and lung tissues (Heunis *et al.*, 2020). Both of these ubiquitin chain types are linked with non-degradative roles such as protein trafficking (Yuan *et al.*, 2014) and signal transduction (Fei *et al.*, 2013). Importantly, when analysing which proteins these atypical chain types ubiquitylated in mouse skeletal muscle, mitochondrial proteins were

highlighted and so perhaps these ubiquitin chains types play a role in mitochondrial function and metabolism (Heunis *et al.*, 2020). Together, these observations show that protein ubiquitylation in skeletal muscle extends much further than K48-linked chains, emphasising the need for further investigation into the roles of ubiquitin chain types in skeletal muscle.

1.4.3 Ubiquitin E3 ligases in skeletal muscle

Over the last few decades, there has been a surge of interest towards E3 ligases selectively expressed in skeletal muscle. The role of E3 ligases in skeletal muscle has gained substantial interest since their discovery as ‘atrogenes’ (atrophy-regulated genes) implicated with regulating skeletal muscle atrophy (wasting) (Gomes *et al.*, 2001). Muscle RING-finger protein-1 (MuRF1) and muscle atrophy F-box (MAFbx) have been the main focus of skeletal muscle E3 ligase research since their identification in skeletal muscle atrophy models in rodents (Bodine *et al.*, 2001) and later in humans (Chen *et al.*, 2007; de Boer *et al.*, 2007; Gustafsson *et al.*, 2010). In addition to these, other E3 ligases have since been associated with skeletal muscle atrophy, including: muscle ubiquitin ligase of SCF complex in atrophy-1 (MUSA1) (Sartori *et al.*, 2013), Specific of Muscle Atrophy and Regulated by Transcription (SMART) (Milan *et al.*, 2015), Neural precursor cell expressed developmentally down-regulated protein 4 (Nedd4-1) (Nagpal *et al.*, 2012) and F-box and leucine-rich protein 22 (Fbxl22) (Hughes *et al.*, 2020). This signifies a significant function of E3 ligases in skeletal muscle mass regulation. However, it is worth noting that although the discovery of E3 ligases involved in skeletal muscle atrophy is increasing, how they operate to facilitate this process is mostly unknown. For instance, despite compelling evidence surrounding the expression of certain E3 ligases such as MuRF1 during skeletal muscle atrophy, combined with structural and contractile proteins they target (Witt *et al.*,

2005; Cohen *et al.*, 2009), their molecular interactions and signalling pathways remain largely unidentified.

Although E3 ligases expressed in skeletal muscle play a well-established role in muscle atrophy, such enzymes are not limited to this process. One particular E3 ligases that has recently come into the spotlight is the HECT-type ligase, UBR5. This ligase has been heavily associated with K48 chain assembly for UPS-mediated protein degradation (Ohtake *et al.*, 2018). Surprisingly, it has emerged that this E3 ligase may be involved in skeletal muscle hypertrophy and recovery from atrophy, displaying different mRNA expression patterns to MuRF1 and MAFbx in rodents (Seaborne *et al.*, 2019). Further investigation has suggested that UBR5 mRNA expression is a positive regulator of skeletal muscle mass and is activated downstream of mechano-transduction signalling in mouse skeletal muscle, associated with hypertrophy and anabolism (Turner *et al.*, 2020). Ubiquitylation has also been linked to the maintenance of skeletal muscle through protein stabilisation, rather than its degradation. The ubiquitylation of a Cullin-RING E3 ligase adaptor protein called KLHL41 regulates the stabilisation of Nebulin, a sarcomeric protein (Ramirez-Martinez *et al.*, 2017). This process appears independent of protein degradation as nebulin stabilisation occurred without functional UPS or autophagy (lysosomal-based protein degradation) (Ramirez-Martinez *et al.*, 2017). Certain E3 ligases expressed in skeletal muscle have established themselves in processes other than controlling skeletal muscle mass, including TRIM72 (Lee *et al.*, 2010; Yi *et al.*, 2013) and Parkin (Gouspillou *et al.*, 2018) regulating myogenesis and mitochondrial function respectively. Notably, there are many other E3 ligases expressed in skeletal muscle and their roles within skeletal muscle remain to be clarified. Therefore, our current understanding of E3 ligases expressed in skeletal muscle is poor. However, what current research has managed to

demonstrate is that they are not confined to regulating skeletal muscle atrophy but seem to be important for regulating the maintenance, remodelling and growth of skeletal muscle.

1.4.4 Current understanding of protein ubiquitylation in skeletal muscle

Overall, the current literature on protein ubiquitylation in skeletal muscle is ill-defined. This is illustrated by the limited discovery of important regulators and their corresponding substrates, alongside the inadequate research conducted on ubiquitin chain types and more specifically those involved in UPS-independent processes within skeletal muscle. Nevertheless, what studies have managed to demonstrate is: a) compelling evidence that ubiquitylation plays an integral role in skeletal muscle b) UPS-mediated protein degradation is an important process in skeletal muscle and c) a brief insight into key ubiquitin chain types and E3 ligases expressed in skeletal muscle. Together, it is evident that the regulation of biological processes through appropriate ubiquitylation is essential for maintaining healthy skeletal muscle.

1.5 Effect of exercise on skeletal muscle

1.5.1 Importance of exercise on skeletal muscle function

Exercise is a physiological stimulus that provides numerous benefits towards skeletal muscle. Resistance exercise predominantly regulates myofiber number, size and type, which subsequently improves muscle mass and strength (Gonyea, 1980; Macdougall *et al.*, 1980; Tesch, 1988). On the other hand, endurance exercise improves energy production through increasing mitochondrial biogenesis, resulting in greater oxidative capacity in skeletal muscle (Booth *et al.*, 2015). This classical view of exercise adaptation is still widely accepted, however the benefits of exercise towards skeletal muscle function is complex and so these exercise types are not confined to these adaptations. For

instance, acute resistance exercise has been shown to increase mitochondrial biogenesis (Wilkinson *et al.*, 2008; Burd *et al.*, 2012; Donges *et al.*, 2012) and endurance exercise can increase skeletal muscle growth (Konopka and Harber, 2014). Accordingly, a lack of exercise can lead to impaired skeletal muscle function and subsequent deleterious consequences. Physical inactivity is a major risk factor in a host of metabolic diseases such as diabetes and obesity (Dowse *et al.*, 1991; Manson *et al.*, 1992; Pietiläinen *et al.*, 2008) which can develop as a consequence of impaired skeletal muscle functioning such as reduced glucose transport into the muscle (Stanford and Goodyear, 2014)**.** Moreover, physical inactivity can cause muscle weakness and lead to physical disorders such as frailty and sarcopenia (Marzetti *et al.*, 2017). Importantly, in patients with debilitating conditions exercise can improve the health of skeletal muscle, acting as a secondary prevention of several diseases. Metabolic syndrome is particularly prevalent in modern society, thought to occur in approximately 25% of the world's population (Saklayen, 2018). Endurance exercise training in patients with metabolic syndrome improves mitochondrial biogenesis and expression of insulin and glucose receptors in skeletal muscle (Stuart *et al.*, 2013). Together, these studies demonstrate that c

1.5.2 Importance of exercise-regulated ubiquitylation in skeletal muscle

The beneficial effects of exercise on skeletal muscle function are reliant on ubiquitylation to respond to the altered cellular conditions fashioned. During exercise, the cellular environment in skeletal muscle is drastically altered as a result of triggering a structural and metabolic remodelling. Resistance exercise elicits a dynamic shift in muscle protein balance, contributing towards increased muscle protein breakdown (MPB) in humans (Biolo *et al.*, 1995; Phillips *et al.*, 1997, 1999; Tipton, Hamilton and Gallagher, 2018). Although there is currently less evidence, there are studies suggesting MPB is also

increased following endurance exercise in humans (Carraro *et al.*, 1990) and rodents (Dohm *et al.*, 1980; Rennie and Kevin D. Tipton, 2000). This response is critical not only to respond to the immediate muscle protein damage created through mechanical, oxidative and heat stress for instance, but also for later remodelling and subsequent adaptation observed following exercise. The importance of the catabolic response to exercise alongside the role of ubiquitylation for eliciting the rapid degradation of damaged proteins, means the ubiquitin signalling response to exercise is crucial. One major function of the UPS is to degrade proteins that have undergone signal-dependent ubiquitylation in response to physiological processes, reinforcing the integral role of ubiquitylation in response to exercise. Importantly, the physiological states fashioned as a result of exercise training have been shown to alter key degradative markers of protein ubiquitylation, such as increased mRNA expression of MuRF1 and MAFbx in high oxidative fibres (Van Wessel *et al.*, 2010) and increased UPS activity during skeletal muscle hypertrophy (Baehr, Tunzi and Bodine, 2014; Bell, Al-Khalaf and Megeney, 2016). With this in mind, it is imperative that we delineate the effects of exercise on protein ubiquitylation in skeletal muscle and explore what existing literature has found. This is necessary because exercise-regulated ubiquitylation is likely to have important implications for the development of therapeutic strategies designed to recapitulate the effects of exercise (exercise mimetics) to restore skeletal muscle health. Notably, due to the vast array of molecular signalling that follows exercise in skeletal muscle, coupled with the range of cellular processes that ubiquitylation controls, our understanding of exercise-regulated ubiquitylation in skeletal muscle is still in its pioneering days. Therefore, advancing our current perception of exercise-regulated ubiquitylation is also essential.

2. Exercise-regulated ubiquitylation in skeletal muscle

2.1 Key proteins investigated

The complexity of ubiquitylation means studies investigating exercise-regulated ubiquitylation in skeletal muscle tend to focus on a few select markers. Interestingly, measuring the levels of protein ubiquitylation in response to exercise is often a poor assessment of exercise-regulated ubiquitylation. This is because studies measure this output to report changes in the rates of protein ubiquitylation, however their levels are also altered by UPS-mediated protein degradation. As a result, many studies measuring protein ubiquitylation have seen no changes (Cunha *et al.*, 2012; Jamart *et al.*, 2012; Moberg *et al.*, 2017; Cui *et al.*, 2019) or even reductions (H. J. Kim *et al.*, 2011) in response to exercise. This can be misleading because it may appear that exercise does not increase the rates of protein ubiquitylation, when in fact these results could be due to a similar increase in UPS-mediated protein degradation. Instead, the importance of the UPS and the growing interest regarding E3 ligases expressed in skeletal muscle, has prompted their investigation in response to exercise. An important role of the UPS has been linked with skeletal muscle hypertrophy (Baehr, Tunzi and Bodine, 2014) and regeneration through myogenesis (Gardrat *et al.*, 1997; Abu Hatoum *et al.*, 1998; Bell, Al-Khalaf and Megeney, 2016). This suggests the UPS may be important for muscle growth and repair in response to exercise. Accordingly, efforts have been made to examine changes in UPS expression (Willoughby, Rosene and Myers, 2003; Willoughby, Taylor and Taylor, 2003) and activity levels (Cunha *et al.*, 2012; VerPlank *et al.*, 2019) following exercise. Since the discovery of MuRF1 and MAFbx in muscle atrophy models, the mRNA expression levels of these E3 ligases has been extensively researched in response to exercise (Yang, Jemiolo and Trappe, 2006; Louis *et al.*, 2007; Nedergaard *et al.*, 2007; Mascher *et al.*, 2008; Stefanetti

et al., 2015). This is because they are thought to be involved in the protein breakdown pathway, so whilst exercise provides a level of protection against muscle atrophy, the immediate catabolic response could increase their expression. As a result, both the UPS and these muscle specific E3 ligases have contributed towards the majority of literature investigating exercise-regulated ubiquitylation in skeletal muscle.

2.1.1 UPS response to exercise in skeletal muscle

Recently, it has become evident that UPS activity is altered in response to exercise in skeletal muscle. In particular, eccentric exercise is commonly used when measuring the response of the UPS due to the heightened muscle damage induced by this exercise bout. Initially, UPS-mediated protein degradation was thought to be solely regulated by protein ubiquitylation levels. Accordingly, early studies had primarily focused on ubiquitin conjugated proteins to examine changes in UPS-mediated protein degradation in human skeletal muscle following eccentric exercise, finding increases in their protein content (Thompson and Scordilis, 1994; Stupka *et al.*, 2001). However, this provides limited information and so certain studies began directly measuring exercise-regulated changes in the UPS, initially focusing on their mRNA and protein expression levels which increased post-eccentric exercise in human skeletal muscle (Willoughby, Rosene and Myers, 2003; Willoughby, Taylor and Taylor, 2003). Later studies then began to measure UPS activity (β1, β2, and β5) and found that post-endurance exercise in mice, there was a significant rise in β5 subunit activity levels in skeletal muscle after a single bout and following 8 weeks of training (Cunha *et al.*, 2012). Interestingly, recent studies have begun uncovering the molecular mechanisms regulating UPS activity. Importantly, it has been shown that UPS activity can be increased through Cyclic adenosine monophosphate (cAMP) (Lokireddy, Kukushkin and Goldberg, 2015). cAMP is a signalling messenger than can be

activated by epinephrine (adrenaline), a hormone released with exercise (Kjaer *et al.*, 1986) and so this UPS-activating pathway has since been applied into the context of exercise (VerPlank *et al.*, 2019). In the Lokireddy et al study, cAMP induced UPS activity via phosphorylation of the 19S subunit Rpn6, therefore this measurement was harnessed as a marker of UPS activation (VerPlank *et al.*, 2019). They found that following the cessation of a single bout of endurance exercise, Rpn6 phosphorylation and β5 subunit activity increased and K48 ubiquitylated protein levels were reduced in human skeletal muscle (VerPlank *et al.*, 2019). From this they concluded that exercised-induced epinephrine secretion, stimulated cAMP mediated increases in UPS activation, causing a reduction in ubiquitylated proteins targeted for degradation. However, raising UPS activity through cAMP activation only promotes the degradation of short-lived and not long-lived proteins (Lokireddy, Kukushkin and Goldberg, 2015). Therefore, this model cannot explain how the UPS degrades long-lived proteins following exercise. It must be noted that this is just one axis of exercise-induced UPS activation that has been examined, therefore it is reasonable to suggest that there is much more to be uncovered. Nevertheless, the recent identification of signalling molecules that orchestrate UPS activity, which are enhanced following exercise, provides valuable information as to how this ubiquitin-mediated degradation system is regulated in response to exercise.

On the other hand, there is evidence to suggest that exercise does not always activate the UPS in skeletal muscle. The activity of the UPS is not maintained at a constant high level in skeletal muscle, suggesting that elevated UPS activity is not always beneficial and perhaps even detrimental to skeletal muscle. Interestingly, some studies have found that exercise bouts carried out in an energy deficit state do not increase UPS activity in human skeletal muscle. For instance, a prolonged low-intense field exercise program resulting in

an energy deficit state, led to no changes in the activity of any of the β-subunits in the UPS (Moberg *et al.*, 2017). Furthermore, studies have also shown decreases in UPS activity following prolonged periods of endurance exercise, for instance after a 200km run there was a reduction in the β5 subunit activity (H. J. Kim *et al.*, 2011). One explanation as to why UPS activity may not increase, or even decrease, is due to protein sparring in order to conserve skeletal muscle tissue following these energy demanding exercises. Interestingly, it appears that a null response in UPS activity is not limited to energy demanding exercises because a moderate intensity 45-minute run also caused no increase in β5 subunit activity (Carbone *et al.*, 2014). However, due to the limited number of studies assessing UPS activity in response to exercise, it is hard to draw definitive conclusions. Moreover, it is important to consider that studies have found the activity of β-subunits within the UPS do not always respond similarly to exercise. For instance, following an ultramarathon, β2 subunit activity increased whereas β1 and β5 subunits remained unchanged in human skeletal muscle (Jamart *et al.*, 2012). Therefore, depending on the β-subunit being measured, their results may differ and subsequently influence their conclusions. Nonetheless, these studies draw attention to the fact that UPS activity does not always increase after exercise, most likely when performing endurance exercise in an energy deficit state.

2.1.2 E3 ligase response to exercise in skeletal muscle: MuRF1 and MAFbx

In response to exercise, certain factors appear to influence the mRNA and protein expression levels of MuRF1 and MAFbx in skeletal muscle. Although, their precise roles within skeletal muscle have yet to be resolved, MuRF1 and MAFbx have so far been solely associated with the selective removal of proteins through ubiquitylation signalling. Therefore, their expression levels may be expected to compliment the extent of MPB

triggered by exercise. Notably, after training skeletal muscle becomes adapted to cope with the exercise demands. Following resistance exercise training, this results in a diminished catabolic response (Phillips *et al.*, 1999). Interestingly, resistance exercise training also appears to reduce MuRF1 and MAFbx mRNA expression levels in human skeletal muscle (Churchley *et al.*, 2007; Mascher *et al.*, 2008) and in rat skeletal muscle (Ribeiro *et al.*, 2017). This supports the concept that MuRF1 and MAFbx are increased to respond to the catabolic demands of exercise. However, both MuRF1 mRNA and MAFbx mRNA/protein expression have shown significant increases following 8 weeks of resistance exercise training in human skeletal muscle (Léger *et al.*, 2006), illustrating that exercise training does not necessarily negate their elevated response to resistance exercise. Interestingly, the response of MuRF1 in human skeletal muscle towards energy demanding exercises appears greater than towards muscle damaging exercises. For instance: endurance exercise training increased MuRF1 mRNA expression unlike with resistance exercise training (Stefanetti *et al.*, 2015), performing strength exercise inhibited the increased MuRF1 mRNA expression induced shortly after endurance exercise (Lysenko *et al.*, 2016) and concentric exercise increased MuRF1 mRNA expression following 3 hours of exercise cessation which did not occur following eccentric exercise (Nedergaard *et al.*, 2007) - an exercise which requires less energy (Lastayo *et al.*, 2000). Moreover, in rat skeletal muscle, MuRF1 protein expression increased more following high-intensity interval training (HIIT) than moderate-intensity continuous training (MICT) (Cui *et al.*, 2019). This finding supports the concept that MuRF1 responds more profoundly to high energy demanding exercises. One possibility for the enhanced response from these exercises is that FoxO proteins known to regulate MuRF1 (Milan *et al.*, 2015) also seem to follow this trend (Louis *et al.*, 2007; Nedergaard *et al.*, 2007; Stefanetti *et al.*,

2015). Alongside this enhanced response, increased MuRF1 mRNA expression also appears more prolonged after endurance exercise, remaining elevated 24 hours post-endurance exercise compared to 8 hours after resistance exercise in human skeletal muscle (Louis *et al.*, 2007). However, not all studies align with these trends, for instance mRNA expression of both MuRF1 and MAFbx displayed no differences between concentric and eccentric exercise in rat skeletal muscle (Ato *et al.*, 2017). Nevertheless, from the majority of literature it is reasonable to conclude that the expression of these E3 ligases in skeletal muscle can be altered by exercise, but the outcome might be different depending on the conditions of the exercise performed.

From the literature it appears that these E3 ligases (MuRF1 and MAFbx) have temporal roles post exercise. It may be expected that their response following exercise would be transient and acute, in order to rapidly turnover damaged proteins without reducing muscle mass. To capture their response over time, a time-course study which examined the effects of either resistance or endurance exercise on both MuRF1 and MAFbx mRNA expression was produced (Louis *et al.*, 2007). They found that both types of exercise caused a significant increase in MuRF1 mRNA expression following 1 to 4 hours of exercise cessation, whereas this temporal response for MAFbx mRNA expression was only seen following endurance exercise (Louis *et al.*, 2007). There are many studies that support the acute mRNA response of MuRF1 following either endurance exercise (Nedergaard *et al.*, 2007; Stefanetti *et al.*, 2015; Lysenko *et al.*, 2016) or resistance exercise (Mascher *et al.*, 2008). This acute response is also seen at the protein level (using a proteomic approach) in which MuRF1 protein increased 2h and 5h post endurance exercise (Parker *et al.*, 2020). However, there is a lack of literature available to support these findings and so time-course studies examining the protein response of

MuRF1 and MAFbx following exercise are needed. Interestingly, a similar acute response is seen with the mRNA expression of inflammation markers e.g. TNF-α and IL-6 which increased between 2 and 24 hours following exercise (Louis *et al.*, 2007). Analogous to that of MuRF1 and MAFbx, IL-6 and TNF-α are involved in regulating muscle protein breakdown (Goodman, 1991, 1994; Tsujinaka *et al.*, 1995) and chronic elevation of these cytokines appears to promote skeletal muscle wasting which is observed in diseased states such as cancer cachexia (Fong, Moldawer and Michael, 1989; Haddad *et al.*, 2005; Carson and Baltgalvis, 2010). However, the acute increase of inflammatory cytokines, such as IL-6, can provide a number of functional responses towards skeletal muscle e.g., satellite cell proliferation (Cantini *et al.*, 1995), beneficial for the regeneration of damaged myofibers following exercise. Therefore, it is perhaps not unreasonable to postulate that similar to these inflammation cytokines, the transient and acute expression of MuRF1 and MAFbx following exercise is crucial in order to respond to exercise-induced stress and damage without causing skeletal muscle atrophy.

It is important to note that MuRF1 and MAFbx mRNA expression do not appear concurrent in response to exercise. Following a single bout of resistance exercise, MuRF1 mRNA expression is largely upregulated in human skeletal muscle (Yang, Jemiolo and Trappe, 2006; Louis *et al.*, 2007; Mascher *et al.*, 2008; Murton, Constantin and Greenhaff, 2008; Borgenvik, Apró and Blomstrand, 2012; Fry *et al.*, 2013). On the other hand, despite some evidence of increased MAFbx mRNA expression (Deldicque *et al.*, 2008) MAFbx has often been reported to remain unchanged (Mascher *et al.*, 2008; Borgenvik, Apró and Blomstrand, 2012; Fry *et al.*, 2013) or even downregulated (Yang, Jemiolo and Trappe, 2006; Kostek *et al.*, 2007) in human skeletal muscle following a single bout of resistant

exercise. Unlike with resistance exercise, both MuRF1 and MAFbx mRNA expression appear to increase in human skeletal muscle after a single bout of endurance exercise (Louis *et al.*, 2007; Pasiakos *et al.*, 2010; H. J. Kim *et al.*, 2011; Stefanetti *et al.*, 2015) and following endurance training (Stefanetti *et al.*, 2015). However, unlike in human skeletal muscle, their response following endurance exercise training has shown to be divergent in mouse skeletal muscle (Cunha *et al.*, 2012). Together, this illustrates that the mRNA expression levels of these E3 ligases can be dissimilar in human skeletal muscle following resistance exercise specifically. This observation hints towards a divergent regulation of these E3 ligases following resistance exercise. An interesting hypothesis emerging is that despite the acknowledgement that the Akt-FoxO signalling node can regulate their expression (Sandri *et al.*, 2004; Milan *et al.*, 2015) their response to resistance exercise in skeletal muscle may stem from different molecular pathways. In support, FoxO appears to be predominantly expressed in different muscle fibre types to MuRF1 and MAFbx (Van Wessel *et al.*, 2010) and its mRNA expression has been shown to increase after the rise in MuRF1 and MAFbx in mouse skeletal muscle following hypertrophy (Baehr, Tunzi and Bodine, 2014). Examples of potential alternative mechanisms involved with exercise could be through mitogen-activated protein kinase (MAPK), known to regulate MAFbx in skeletal muscle (Li *et al.*, 2005) and NF-kB which regulates MuRF1 (Cai *et al.*, 2004). Another explanation for the differential regulation following resistance exercise is the substrates that MuRF1 and MAFbx target. Unlike MAFbx, MuRF1 has been reported to associate with structural and contractile proteins such as titin (Witt *et al.*, 2005) and myosin heavy chain (Cohen *et al.*, 2009) perhaps giving MuRF1 a mechanosensory regulation, a response that is highly relevant with respect to resistance exercise. However, this explanation is speculative because MuRF1 and MAFbx targets following exercise have yet to be

established and those attempting to investigate potential targets have displayed null results (Stefanetti *et al.*, 2015). Currently more questions are being asked than answered regarding the expression of these E3 ligases following exercise. Nonetheless, these studies do demonstrate that MuRF1 and MAFbx can respond differently following resistance exercise at the transcriptional level in human skeletal muscle.

2.1.3 Relationship between UPS and MuRF1/MAFbx in skeletal muscle following exercise

Despite their association, it is unclear as to the relationship between UPS activity and the expression of MuRF1 and MAFbx in skeletal muscle following exercise. The association is not surprising due to their degradative nature, which has led to MuRF1 and MAFbx often being referred to as molecular markers of the UPS pathway (Stefanetti *et al.*, 2015). Both MuRF1 mRNA expression and β5 subunit activity have reported increases immediately after an endurance exercise session and following training in mouse skeletal muscle (Cunha *et al.*, 2012). However, in human skeletal muscle, an ultra-endurance exercise increased both MuRF1 and MAFbx mRNA expression, with no rise in β5 subunit activity (H. J. Kim *et al.*, 2011). Similarly, it appears that in response to mechanical loading they may also be regulated independently. This has been illustrated in mouse skeletal muscle whereby functional overload causing muscle hypertrophy led to increased β5 subunit activity throughout the 14-day period, whereas MuRF1 and MAFbx mRNA expression returned to baseline by 3 days (Baehr, Tunzi and Bodine, 2014). Despite not being an exercise intervention per se, this model is often harnessed to replicate the effects resistance exercise has on skeletal muscle. Altogether, these studies suggest that a rise in UPS activity following exercise does not necessarily equate to a rise in MuRF1 and MAFbx

mRNA expression and vice versa in skeletal muscle. Accordingly, MuRF1 and MAFbx expression should not be used as markers for UPS activity nor protein degradation. To understand the role of MuRF1 and MAFbx in skeletal muscle following exercise, it is imperative that studies begin to investigate their substrates and the fate of these substrates in response to exercise.

2.1.4 Limitations with exercise-regulated ubiquitylation analysis

It is evident that the current literature has often failed to establish conclusive evidence of exercise-regulated ubiquitylation. A major reason for this is due to the methodological approaches used in the vast majority of these studies. Research investigating MuRF1 and MAFbx often only measure mRNA expression levels using PCR-based methods in order to analyse their response to exercise (Yang, Jemiolo and Trappe, 2006; Kostek *et al.*, 2007; Mascher *et al.*, 2008; Fry *et al.*, 2013). Given that mRNA expression only correlates ~40% of protein expression (De Sousa Abreu *et al.*, 2009; Vogel and Marcotte, 2012) their mRNA levels do not provide a good indication of protein expression. For instance, Stefanetti et al., 2015 found no changes in MuRF1 protein expression despite increases in mRNA expression following single bout and exercise training. As a result, mRNA expression has little physiological relevance and so current literature studying the effects of MuRF1 and MAFbx following exercise have failed to establish the impact of their response to exercise. For instance, increases in MuRF1 mRNA expression following resistance exercise were not accompanied by increases in MPB rates (Reitelseder *et al.*, 2014). Although measuring mRNA expression provides very limited information, measuring protein expression of these E3 ligases also has issues. MuRF1 has many

commercially available antibodies for detecting this protein, however they have demonstrated a lack of specificity in skeletal muscle lysate (Bodine, 2020). Therefore, studies using these antibodies to measure MuRF1 protein expression may in fact be detecting other proteins, producing invalid data. Furthermore, until this year, the methodology used to study exercise-regulated ubiquitylation only employed targeted approaches which look at specific proteins, in most cases MuRF1, MAFbx and UPS subunits. As a consequence, this has provided no further insight into the role of other proteins involved in exercise-regulated ubiquitylation for example other E3 ligases, which no doubt play an important role. Overall, it is apparent that there has been limited progress made in relation to exercise-regulated ubiquitylation. In order to advance our understanding, it is important to address these issues and utilise more effective methods going forward.

2.2 Exercise-regulated Ubiquitylome

2.2.1 Proteomic approach investigating exercise-regulated ubiquitylation

Recently, our understanding of exercise-regulated ubiquitylation in skeletal muscle has expanded in a new profound manner, addressing some of the issues with previous research. This recent development involves a new methodological approach utilising mass spectrometry-based proteomics to analyse protein ubiquitylation. Proteomic analysis of protein ubiquitylation (hereafter referred to as the ubiquitylome) provides a global identification of ubiquitylated proteins and their modification sites. In doing so, this can reveal uncharacterised ubiquitylated proteins that otherwise would be masked when using targeted approaches. In addition, identifying their modification sites allows researchers to create site-specific mutations, so they become resistant to ubiquitylation, which allows

further analysis of this modification to reveal its importance. Furthermore, modification sites from ubiquitin chains can also be quantified, revealing the types of ubiquitylation occurring. As a result of this comprehensive profiling and unbiased analysis, investigating the ubiquitylome allows for a more in-depth examination of protein ubiquitylation. This approach has recently been implemented for the first time in the context of exercise (hereafter referred to as the exercise-regulated ubiquitylome) in human skeletal muscle (Parker *et al.*, 2020). After performing an acute bout of high-intense endurance exercise, they analysed both the ubiquitylome and proteome of human skeletal muscle lysate (Parker *et al.*, 2020). This study focused on the mechanisms regulating UPS activation and they concluded that NEDDylation – a PTM analogous to ubiquitylation, is a mediator for maintaining UPS-mediated protein degradation post-exercise through promoting E3 ligase activity and subsequent protein ubiquitylation following cAMP activation (Parker *et al.*, 2020). Alongside this finding, they also provided an array of data regarding exercise-regulated protein ubiquitylation, identifying ~400 ubiquitylated peptides and exposing potential key regulators such as MuRF1 which increased 2-5 hours after exercise (Parker *et al.*, 2020). Additionally, this study quantified each ubiquitin chain and discovered only K27 chains increased during exercise (Parker *et al.*, 2020). This atypical chain is not associated with regulating protein degradation, thus prompting further investigation into the role of UPS-independent ubiquitylation in skeletal muscle during exercise. Altogether, this pioneering study has progressed our understanding of exercise-regulated ubiquitylation in addition to providing a platform for future research.

2.2.2 Shortfalls of current exercise-regulated ubiquitylome understanding

Whilst this study has provided a more comprehensive overview of exercise-regulated protein ubiquitylation, there is still much more to elucidate. During their investigation, they utilised ubiquitylome analysis to investigate PTM crosstalk (Parker *et al.*, 2020). Despite being an intriguing signalling response to exercise, the role of PTM crosstalk following exercise was poorly examined. For instance, over 40 proteins were regulated by both ubiquitylation and phosphorylation, yet no further work was carried out investigating the function of this PTM crosstalk (Parker *et al.*, 2020). Additionally, although NEDDylation was examined further, they attempted to validate its role for increasing protein ubiquitylation levels using pharmacological activation in non-muscle cell lines (Parker *et al.*, 2020) which has little physiological relevance when investigating the exercise response in human skeletal muscle. Consequently, further research examining this proposed model in more appropriate applications is required to solidify this concept. Our understanding of key ubiquitin ligase-substrate interactions that occur with exercise, crucial for determining the fate of ubiquitylation, has not advanced following this study. For instance, despite reporting increased MuRF1 protein levels post exercise, no further work was carried out examining the specific substrates targeted or the effects this had on biological functions within the muscle (Parker *et al.*, 2020). In order to investigate these interactions, further analysis is required which harness appropriate techniques capable of analysing select proteins in more depth such as co-immunoprecipitation. Moreover, employing a more prolonged intense exercise could reveal a more profound ubiquitylation response. As previously mentioned, energy demanding exercises seems to increase the mRNA and protein expression of characterised muscle E3 ligases such as MuRF1 (Nedergaard *et al.*, 2007; Cui *et al.*, 2019). Therefore, future experiments could look to examine the

ubiquitylome in response to different exercise conditions. Overall, whilst the first exercise-regulated ubiquitylome study has revealed interesting findings, unsurprisingly there is much more to be discovered.

2.2.3 Issues with current exercise-regulated ubiquitylome analysis

Although exercise-regulated ubiquitylome analysis is undoubtably a vast improvement on the techniques previously available, this approach does not come without its issues. The low stoichiometry of protein ubiquitylation, partly due to the transient and potential degradative nature of this PTM, means ubiquitylome analysis often requires large sample input and extensive analysis in order to achieve sufficient detection of ubiquitylated proteins (Hansen *et al.*, 2020; Udeshi *et al.*, 2020). An obvious problem occurs when the amount of sample available is limited, often the case in primary tissue samples such as skeletal muscle. Therefore, large-scale ubiquitylome profiling is often limited to cell studies which can generate larger amounts of lysate needed for proteomic analysis. In order to circumvent this issue, mass spectrometry-based proteomics needs to be able to process small amounts of sample for large-scale analysis of the ubiquitylome. Subsequently, there is a need to improve the sensitivity of ubiquitylome analysis, crucial for deeper analysis of exercise-regulated ubiquitylation in skeletal muscle.

3. **Improved Ubiquitylome analysis**

Recently, the capability for mass spectrometry-based proteomics to detect ubiquitylated proteins has increased. This is due to advanced methodology that has been employed for improved ubiquitylome analysis. Using mass spectrometry to analyse the ubiquitylome is complex and requires multiple steps. Firstly, the ubiquitylated proteins need to be enriched so that they can be detected by mass spectrometry. Without prior enrichment, detection of ubiquitylated proteins is challenging due to their low stoichiometry. As a result, two different enrichment processes are frequently employed for ubiquitylated substrates before ubiquitylome analysis, both at the protein and peptide level, named tandem ubiquitin-binding entity (TUBE)-based pull-down and diglycine (diGLY) antibody-based immunoprecipitation respectively. Taking advantage of these enrichment techniques, studies have developed novel methods to improve the sensitivity of ubiquitylome analysis, named trypsin-resistant tandem ubiquitin binding entity (TR-TUBE) (Yoshida *et al.*, 2015) and UbiFast (Udeshi *et al.*, 2020) respectively. Once enriched, ubiquitylated peptides are analysed by mass spectrometry which is an imperfect approach limited by the ability to detect the entire set of ubiquitylated peptides. To maximise the capture of ubiquitylated peptides detected by mass spectrometry, studies have recently moved away from data-dependent acquisition (DDA) and have begun harnessing a more sensitive workflow, named data-independent acquisition (DIA) (Hansen *et al.*, 2020; Steger *et al.*, 2020). Therefore, in this section we will provide more detail on these methodological advancements and explain how they have developed upon more commonly used techniques for ubiquitylome analysis.

3.1 Tandem ubiquitin-binding entity-based pull-down

A common approach utilised to enrich ubiquitylated proteins prior to ubiquitylome analysis is TUBE-based pull-down. TUBE is a ubiquitin trap which take advantage of ubiquitin binding domains present on proteins that bind to ubiquitin such as ubiquitin enzymes, DUBs and UPS subunits (Hicke, Schubert and Hill, 2005; Hjerpe *et al.*, 2009). Ubiquitin binding domains have a low affinity for ubiquitin due to their non-covalent binding resulting in transient interactions, critical for the rapid and reversible cellular responses that occur. However, the tetra-ubiquitin structure of TUBE means that unlike the endogenous ubiquitin binding domains, they have a much higher affinity for ubiquitin, in particular with polyubiquitin chains (Hjerpe *et al.*, 2009). As a result of this high affinity, TUBE has been employed for enriching ubiquitylated proteins whereby TUBE-bound beads undergo a pull-down method which separates ubiquitylated proteins bound to TUBE from unbound non-ubiquitylated proteins (Fig.3). Notably, TUBE displays protective capabilities towards ubiquitylated proteins from DUBs and the UPS, preserving ubiquitylated proteins during this enrichment process (Hjerpe *et al.*, 2009) (Fig.3). Although inhibitors are commonly used to prevent DUBs and the UPS from working during pull-down to minimise loss of ubiquitylated proteins, TUBE has shown to prevent de-ubiquitylation to a higher extent than certain inhibitors (Hjerpe *et al.*, 2009). Moreover, although the heightened affinity towards polyubiquitylated chains means that monoubiquitylated protein enrichment is relatively weak, it does mean that unbound ubiquitin is also enriched less. This is useful when using TUBE-based pull-down in skeletal muscle lysate, because like in other major tissues, skeletal muscle contains mainly unconjugated ubiquitin (Heunis *et al.*, 2020). Altogether, this results in a greater abundance of ubiquitylated proteins in comparison to total protein content, essential for their detection via proteomics. TUBE-based pull-down

also has a function for identifying specific protein ubiquitylation by western blot. Importantly, this can be used as confirmation that a certain protein identified by ubiquitylome analysis is in fact ubiquitylated. Accordingly, TUBE-based pull-down has become a useful approach for enriching ubiquitylated proteins when performing ubiquitylome analysis.

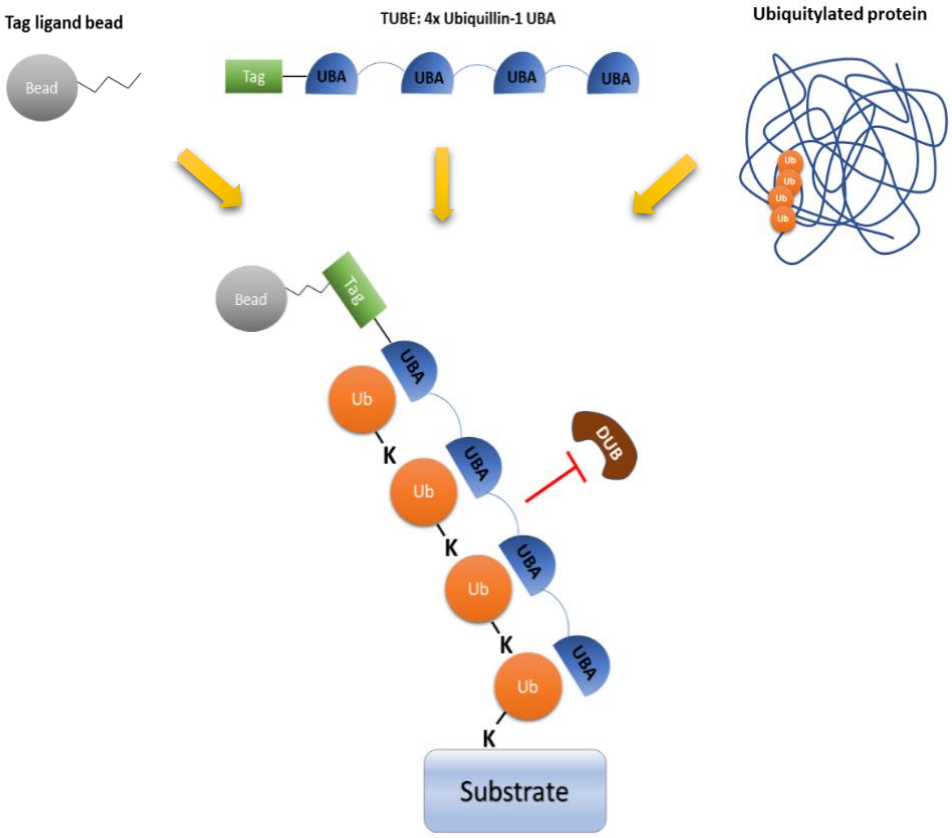

Figure 3: Schematic representation displaying the function of TUBE. This protein ubiquitylation enrichment strategy uses TUBE protein which consists of tandem ubiquitin associated (UBA) domain repeats from the protein Ubiquilin-1. These UBA repeats allow TUBE to bind to ubiquitylated proteins with high affinity. In order to enrich the TUBE-bound ubiquitylated proteins by pull-down, TUBE contains a tag which covalently binds to beads containing a tag ligand. Once mixed together, they form a complex with one another and can be isolated by pull-down using centrifugation to isolate ubiquitylated proteins. Following pull-down, the enriched sample of ubiquitylated proteins can be analysed by western blot or used for proteomic analysis.

3.2 Trypsin resistant tandem ubiquitin-binding entity

Building upon the ability of TUBE for enriching ubiquitylated proteins, this ubiquitin binding entity has been modified to make it more suitable for ubiquitylome analysis. The amino acid sequence of TUBE has been mutated so that arginine (Arg) residues were replaced by alanine (Ala), meaning TUBE becomes resistant to trypsin digestion, termed trypsin resistant TUBE (TR-TUBE) (Yoshida *et al.*, 2015). Before any mass spectrometry-based proteomic analysis can be performed, proteins need to be converted into peptides using a protease like trypsin. Subsequently, during proteolysis, TUBE itself is not digested. This prevents noise signals from TUBE-derived peptides that would otherwise be present during mass spectrometry analysis. In doing so, this maximises the detection of ubiquitylated substrates and thus increases the sensitivity of ubiquitylome analysis. The use of TR-TUBE also removes the need for in-gel trypsin digestion whereby ubiquitylated proteins are subject to separation via gel electrophoresis, removed and digested prior to mass spectrometry. This is beneficial because in-gel digestion has been shown to produce additional contaminants to the sample which effects the accuracy of mass spectrometry analysis (Duncan *et al.*, 2003; Swart *et al.*, 2018). Moreover, TR-TUBE has shown to provide a level of protection towards bound ubiquitin during proteolysis, reducing ubiquitin-derived peptides and thus enhancing the proportion of peptides derived from ubiquitylated substrates (Yoshida *et al.*, 2015). Although this will subsequently reduce the identification of ubiquitin chain types, ubiquitin chain-derived peptides have shown to be the most abundant ubiquitylated peptides in human cell lines (Hansen *et al.*, 2020). Thus, reducing their abundance will still allow for ubiquitin chain type identification, whilst maximising the identification of ubiquitylated substrates. Yoshida et al used TR-TUBE directly in cells where the ubiquitylated proteins were captured, then enriched in vitro using

immunoprecipitation (antibody-based pull-down) before mass spectrometry. They found that harnessing TR-TUBE to capture ubiquitylated proteins in cell before being enriched at the peptide level, improved the ratio of ubiquitylated peptides to total peptides captured by mass spectrometry compared to when only enriched at the peptide level (Yoshida *et al.*, 2015). Although the application of TR-TUBE for enriching ubiquitylated proteins in skeletal muscle lysate has not been published, TUBE-based pull-down has been effective at enriching ubiquitylated proteins in skeletal muscle lysate (Ryder *et al.*, 2015). Therefore, it is highly conceivable that TR-TUBE-based pull-down will also be successful for in vitro use in this tissue. As such, this presents an opportunity to implement TR-TUBE-based pull-down to improve our understanding of the exercise-regulated ubiquitylome in skeletal muscle.

3.2.1 Applying TR-TUBE approach in vitro

Before conducting any exercise-regulated ubiquitylome experiments that employ this technique to enrich ubiquitylated proteins in skeletal muscle lysate, it is imperative that it has been validated for use in vitro capture of ubiquitylated proteins. Consequently, we sought about determining whether the TR-TUBE enrichment approach could be adapted from in cell capture of ubiquitylated proteins, to acting as bait during in vitro pull-down in skeletal muscle lysate. Before testing its capabilities for enriching ubiquitylated proteins from skeletal muscle lysate, it is first important to compare the effectiveness of TR-TUBE-based pull-down alongside the conventional TUBE-based pull-down technique in vitro. Without this comparison, future work harnessing TR-TUBE cannot confidently know whether they are utilising the most effective ubiquitylated protein enrichment method available. Therefore, in order to examine both the relative effectiveness of TR-TUBE in

vitro and its capability within skeletal muscle tissue lysate, we conducted two separate experiments.

3.2.1.1 TR-TUBE in C2C12 cell lysate

To investigate the relative effectiveness of TR-TUBE in vitro, we designed an experiment utilising TR-TUBE as a bait protein for ubiquitin pull-down from C2C12 muscle cell lysate. Within this experiment, we also compared TR-TUBE with two other TUBE-based proteins acting as positive and negative controls to provide the most conclusive evidence of its ability for enriching ubiquitylated proteins in vitro. TUBE was employed as a positive control because this bait protein has been well-established as an effective method for pull-down of ubiquitylated proteins in cell lysate (Hjerpe *et al.*, 2009). As the negative control, we harnessed a deficient TR-TUBE – mutation at leucine residue causing defective ubiquitin binding (Yoshida *et al.*, 2015). By employing these control samples, we can confirm whether or not our experiment ran correctly, verifying whether the results seen from TR-TUBE-based pull-down are valid. After conducting the procedures necessary to examine the ubiquitin enrichment capabilities of these TUBE-based proteins, it was evident that TR-TUBE was equally as effective at enriching ubiquitylated protein as TUBE (Fig.4). The abundance of ubiquitylated proteins present after pull-down represents the capability of these TUBE-based proteins to isolate these proteins from cell lysate. It appears that TR-TUBE-based pull-down is equally capable at isolating ubiquitylated proteins as conventional TUBE-based pull-down (Fig.4). To confirm these isolated ubiquitylated proteins have been enriched from skeletal muscle lysate, we employed a total lysate sample in which no enrichment procedure was used. The abundance of ubiquitylated proteins after TR-TUBE-based pull-down was greater than total lysate (Fig.4), confirming the enrichment of ubiquitylated proteins. In order to analyse the

proportion of ubiquitylated proteins enriched, a flow-through analysis was conducted containing all the proteins not bound to these TUBE-based proteins. From this, it is apparent that TR-TUBE is just as effective as TUBE for enriching the majority of ubiquitylated proteins because few remained in the flow-through of both (Fig.4). As expected, deficient TR-TUBE was incapable of enriching ubiquitylated proteins because more ubiquitylated proteins were present in total lysate and a substantial amount remained in the flow-through (Fig.4). This data also demonstrates that TR-TUBE is effective at enriching ubiquitylated proteins rather than free unbound ubiquitin (~8.5Kd) which seem to appear in the flow-through lanes (Fig.4). Minimising the presence of unbound ubiquitin is useful for ubiquitylome analysis because this will increase the proportion of ubiquitylated proteins relative to total protein, enhancing their identification by mass spectrometry. Overall, this figure provides evidence that TR-TUBE can effectively pull-down ubiquitylated proteins in vitro, similar to that of TUBE.

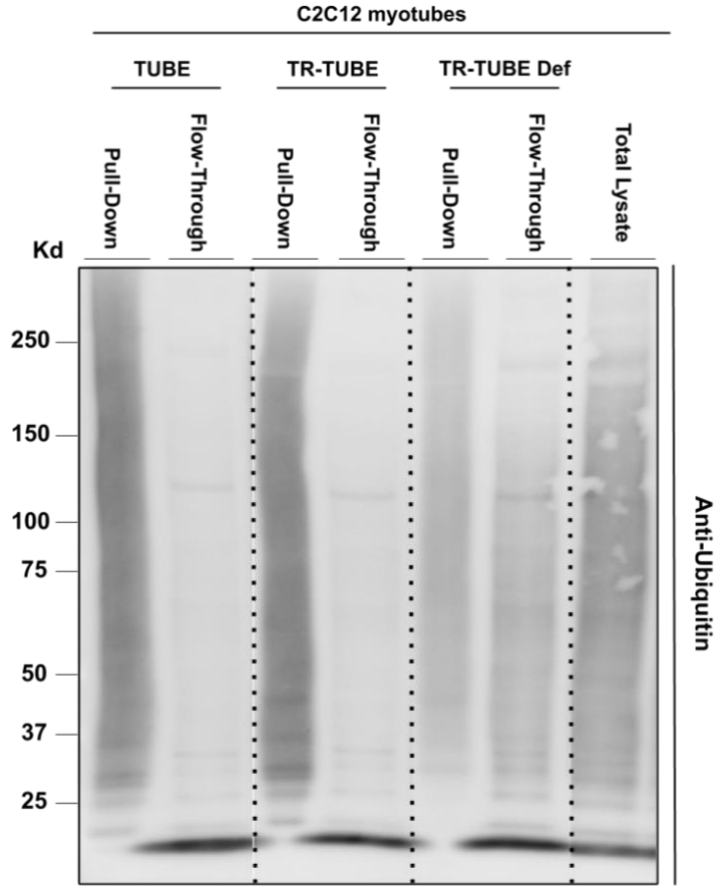

Figure 4: TR-TUBE in vitro pull-down effectively enriches ubiquitylated proteins in C2C12 myotubes. C2C12 myoblasts were differentiated into myotubes and lysed. Cell lysates were incubated with ubiquitin-binding resins containing 50µg of various ubiquillin1 UBA domain tetramer (UBA^{UBQLN1}) proteins: his-halo-TUBE, halo-TR-TUBE or halo-TR-TUBE deficient. Following pull-down, captured proteins were eluted and subject to SDS-PAGE and western blotting alongside flow-through and total lysate control samples. Ubiquitylated proteins were detected with an antibody specific to total ubiquitin (Anti-Ubiquitin #646302). N.B. Dotted lines show where lanes have been merged together.

3.2.1.2 TR-TUBE in skeletal muscle lysate

Before utilising TR-TUBE for proteomic analysis of the exercise-regulated ubiquitylome in skeletal muscle, the next experiment aimed to confirm its ability for enriching ubiquitylated proteins present in skeletal muscle tissue. Here we conducted an experiment utilising human mixed skeletal muscle tissue samples to analyse the effectiveness of TR-TUBE for ubiquitin pull-down in this lysate. In this study we also aimed to evaluate the capacity of TR-TUBE for enriching ubiquitylated proteins, by loading different amounts of protein

lysate. Subsequently, this provides an insight into the ratio of TR-TUBE bait to skeletal muscle protein lysate required before saturation. In doing so, TR-TUBE enrichment can be optimised because too much TR-TUBE may cause unspecific binding of non-ubiquitylated proteins, whereas too little would lead to incomplete enrichment of ubiquitylated proteins. Through conducting this experiment, we were able to confirm the ability of TR-TUBE-based pull-down for enriching ubiquitylated proteins in skeletal muscle lysate and begin to determine an optimal ratio between TR-TUBE bait and skeletal muscle protein lysate to refine this enrichment process (Fig.5). Firstly, we can see that TR-TUBE-based pull-down can isolate ubiquitylated proteins from skeletal muscle lysate (Fig.5). Importantly, these isolated proteins are enriched from the muscle lysate demonstrated by a higher abundance of ubiquitylated proteins in comparison to total lysate (Fig.5). It is apparent that TR-TUBE-based pull-down can thoroughly enrich ubiquitylated proteins as negligible ubiquitylated proteins remain in the flow-through lanes (Fig.5). Remarkably, it appears as though even 8mg of protein does not saturate 50ug of TR-TUBE protein (Fig.5), indicating its high capacity for enriching ubiquitylated proteins. As a result, future experiments can increase the amount of total protein lysate present during TR-TUBE-based pull-down in order to determine its saturation ratio, needed to optimise this enrichment process. Again, this data also demonstrates that TR-TUBE is effective at enriching ubiquitylated proteins and not ubiquitin that isn't bound to a substrate, illustrated by the flow-through lanes whereby unbound ubiquitin (~8.6Kd) and ubiquitin dimers (17kD) appear present. Overall, this figure provides evidence that TR-TUBE-based pull-down in vitro is an effective method for enriching ubiquitylated proteins in skeletal muscle lysate. Therefore, it will be interesting to apply this technique prior to mass spectrometry to analyse the exercise-regulated ubiquitylome in human skeletal muscle.

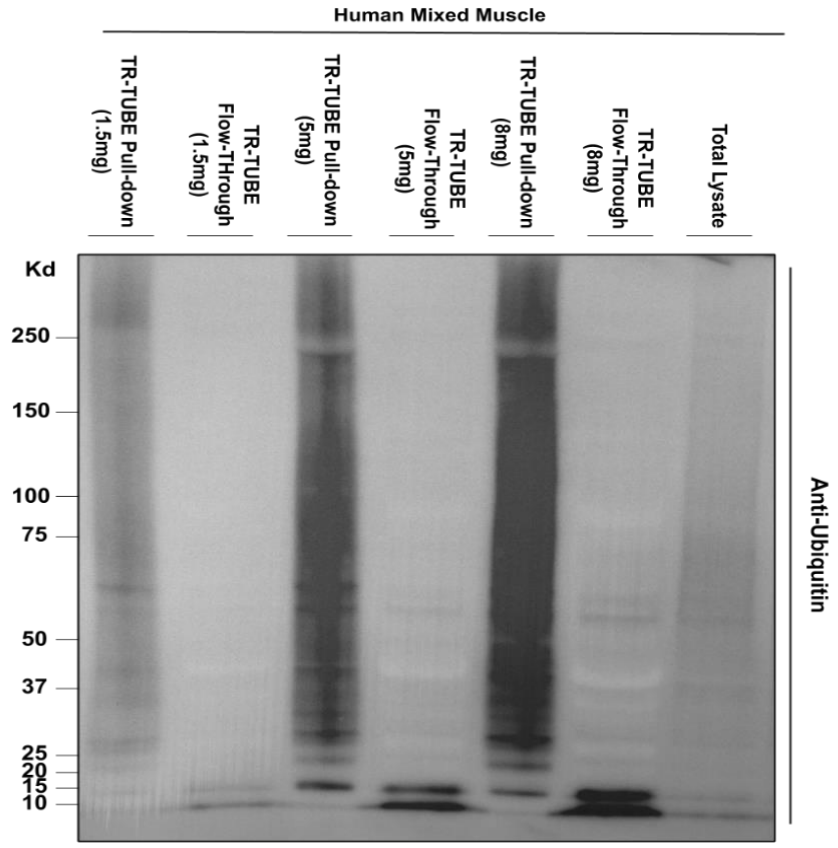

Figure 5: TR-TUBE pull-down effectively enriches ubiquitylated proteins in skeletal muscle tissue. Mixed human skeletal muscle lysates of various protein concentrations (1.5mg, 5mg and 8mg) were incubated with ubiquitin-binding resins containing 50µg halo-TR-TUBE protein. Following pull-down, captured proteins were eluted and subject to SDS-PAGE and western blotting alongside flow-through and total lysate control samples. Ubiquitylated proteins were detected with an antibody specific to total ubiquitin (Anti-Ubiquitin #646302).

3.3 diglycine antibody-based immunoprecipitation

In order to enrich ubiquitylated peptides prior to proteomic analysis, studies often take advantage of a unique motif present on ubiquitylated proteins after proteolysis. Trypsin cuts at both lysine (Lys) and arginine (Arg) residues and because of the amino acid sequence of ubiquitin, this leaves a diglycine (diGLY) remnant on its C-terminal (Fig.6) (Goldknopf and Busch, 1977). Proteins that have been ubiquitylated are bound to the C-

terminal of ubiquitin, therefore they will contain this diGLY remnant on the lysine that has been ubiquitylated. Importantly, this ubiquitylated lysine will not be cleaved due to the presence of the side chain ubiquitin modification. The diGLY remnant is a unique motif with a monoisotopic mass of 114.04Da, which can be recognised using mass spectrometry. Accordingly, ubiquitin-remnant-containing peptides can be identified based on this unique mass change, revealing proteins that have been ubiquitylated. Importantly, an antibody that binds to this diGLY remnant on lysine residues of ubiquitylated peptides has been generated (Xu, Paige and Jaffrey, 2010). Consequently, diGLY antibodies (K-ε-GG) have been employed for immunoprecipitation, to enrich ubiquitylated peptides prior to mass spectrometry (Fig.6). Importantly this process has shown to enrich ubiquitylated peptides with a near 100% yield (Xu, Paige and Jaffrey, 2010). Remarkably, over 70% of ubiquitylated proteins that this antibody-based peptide enrichment technique identified were previously unknown at the time (Xu, Paige and Jaffrey, 2010). As a result, diGLY antibody-based immunoprecipitation has since been commonly employed to enrich ubiquitylated peptides in ubiquitylome studies prior to mass spectrometry-based proteomics.

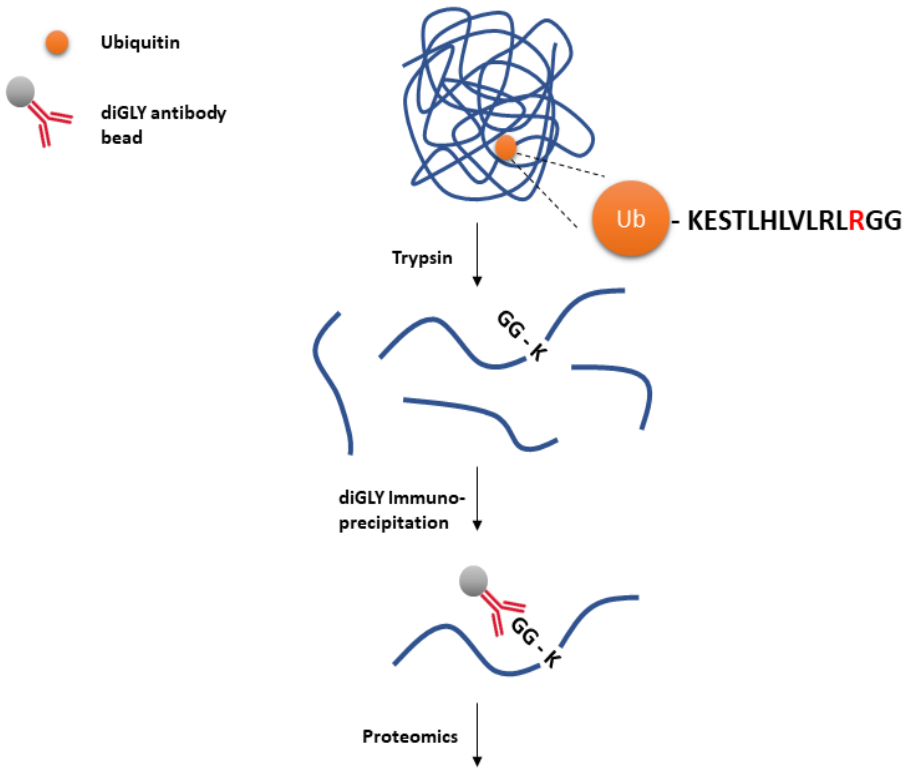

Figure 6: Schematic representation of diGLY-based enrichment of ubiquitylated proteins. This enrichment strategy takes advantage of the diGLY remnant present on ubiquitylated peptides following trypsin digestion, cleaving ubiquitin at Arg74 present on the C-terminus. A monoclonal antibody designed to bind to this exposed diGLY remnant present on the Lys residue of ubiquitylated peptides is used for immunoprecipitation. Following this procedure, ubiquitylated peptides are enriched and proteomic analysis can be performed by which diGLY peptides are identified based on their unique monoisotopic mass (114Da).

3.4 UbiFast

Recently, a novel diGLY-antibody based method for ubiquitylome profiling has been developed which enhances the sensitivity of ubiquitylation site identification. The development of this more sensitive approach, known as UbiFast, came from taking advantage of an antibody-bound epitope method used in mass spectrometry. The binding of the antibody towards the substrate protected the antibody binding site (epitope) from

proteolysis, which could then be detected using mass spectrometry (Suckau *et al.*, 1990). Encouraged by the protective capacity of antibodies towards their epitope, UbiFast applies a diGLY antibody-bound approach to protect the exposed diGLY remnant on ubiquitylated peptides from labelling prior to mass spectrometry (Udeshi *et al.*, 2020). Labelling ubiquitylated peptides through isobaric chemical tags such as tandem mass tag (TMT) is commonly used to enable their precise quantification during mass spectrometry. TMT labels are amine-reactive and so can bind to the primary amine present on diGLY remnants (Xu, Paige and Jaffrey, 2010; Udeshi *et al.*, 2020). By utilising antibody-bound labelling, TMT labels cannot access the diGLY remnant and instead bind to amine groups present on the N-termini or on other lysine residues. Therefore, after the ubiquitylated peptides are eluted from the antibody, the diGLY remnant remains exposed for identification during mass spectrometry. As a result, when comparing this approach to the original in-solution labelling method, UbiFast protocol resulted in the detection of a greater level of both total ubiquitylated peptides and their relative abundance (Udeshi *et al.*, 2020). Remarkably, UbiFast identified over 13,000 diGLY peptides from only 500µg of peptides per sample derived from human tumour tissue (Udeshi *et al.*, 2020). Moreover, relative to previous SILAC labelling methods (Udeshi *et al.*, 2013), UbiFast requires 6-12 times less peptide input (Udeshi *et al.*, 2020). Therefore, this approach is suitable for small amounts of primary tissue sample, crucial for human skeletal muscle ubiquitylome analysis where sample availability is often limited. Accordingly, through improving sample preparation before conducting mass spectrometry analysis on ubiquitylated peptides, this approach may enhance our understanding of the exercise-regulated ubiquitylome in skeletal muscle.

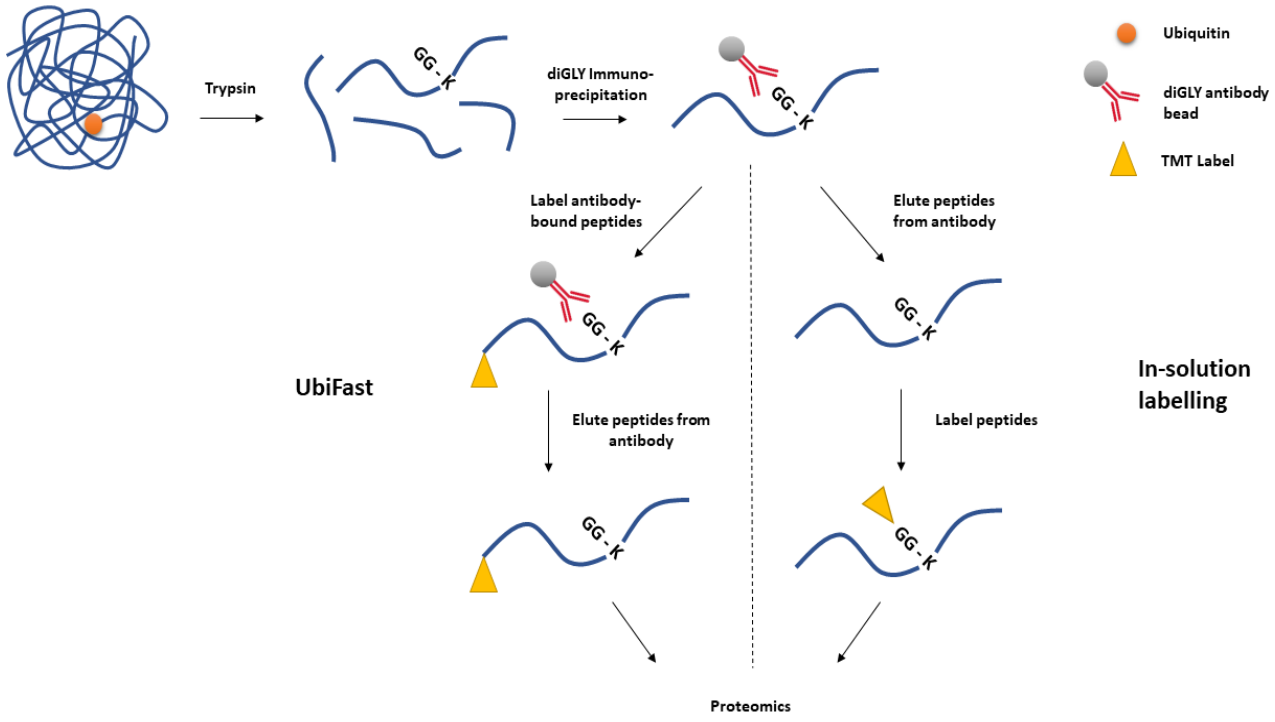

Figure 7: Schematic representation of UbiFast approach compared to conventional in-solution labelling method. UbiFast approach employs an antibody labelling method whereby the diGLY enriched ubiquitylated peptides are subject to TMT labelling whilst still bound to the diGLY antibody. Unlike the in-solution labelling method, TMT labels cannot bind to the diGLY remnant, reducing the level of TMT contaminant side-products blocking the diGLY site. The UbiFast approach subsequently increases the sensitivity of mass spectrometry-based proteomic methods for identifying ubiquitylated peptides.

3.5 Data-independent acquisition

Increased ubiquitylome sensitivity of diGLY enriched proteins has also emerged from a unique method of analysing ubiquitylated proteins during mass spectrometry. Until lately, ubiquitylome analysis has employed data-dependent acquisition (DDA) which detects ubiquitylated proteins through intensity-based selection, which limits the sensitivity of mass spectrometry. Alternatively, data-independent acquisition (DIA) identifies peptides simultaneously within a pre-defined mass-to-charge window (Venable *et al.*, 2004).

Subsequently, DIA-based approach leads to improved peptide quantification accuracy during mass spectrometry analysis alongside reducing the number of peptides missed over a large dynamic range. Therefore, this approach was recently employed for ubiquitylome analysis in the hope that it may detect more ubiquitylated proteins which are otherwise missed in the conventional DDA method (Hansen *et al.*, 2020; Steger *et al.*, 2020). When applying an optimised DIA approach to identify enriched diGLY peptides from human non-muscle cell lines, they identified over 30,000 diGLY peptides in a single shot measurement from as little as few hundred µg of peptides (Hansen *et al.*, 2020; Steger *et al.*, 2020). Remarkably, this is double the amount identified from a single shot in the UbiFast approach (Udeshi *et al.*, 2020). It must be noted that in order to detect ubiquitylated proteins using this DIA method, a comprehensive peptide spectral library was required, serving as a template for the identification of ubiquitylated peptides. This approach works most effectively when peptide spectral libraries are obtained from project-specific samples (Hansen *et al.*, 2020), which may not be feasible when analysing lysate from limited human skeletal muscle tissue. Whether non-project specific spectral libraries can be used for the DIA-based approach to improve exercise-regulated ubiquitylome profiling in skeletal muscle, remains to be seen. On the other hand, library-free DIA approaches would be an effective alternative for analysing exercise-regulated ubiquitylome in skeletal muscle, but these are yet to be developed. Nonetheless, applying this DIA method when analysing the exercise-regulated ubiquitylome in skeletal muscle may still identify previously uncharacterised ubiquitylated proteins due to its high sensitivity.

4. Conclusion

In summary, our current understanding of exercise-regulated ubiquitylation in skeletal muscle remains in its infancy. Here we have outlined literature that has shaped our understanding of exercise-regulated ubiquitylation in skeletal muscle, highlighted recent progressions made in the field and finally, suggested future directions that studies can take for further exploration. Importantly, despite previous research providing very limited information, they have helped to lay down the foundations with respect to exercise-regulated ubiquitylation in skeletal muscle. For now, it seems as though UPS regulation plays a fundamental role, with research beginning to identify key mediators involved in regulating its activity following exercise. Although little is known about MuRF1 and MAFbx protein expression in skeletal muscle following exercise, it seems as though they can act independently not only of one another, but also of the UPS. Therefore, this has stressed the importance of elucidating their roles alongside their substrate targets in skeletal muscle following exercise. The emergence of the exercise-regulated ubiquitylome has exploited key pathways and proteins involved, thus presenting a multitude of avenues to be explored. Moving forward, applying the recently developed methods that facilitate more accurate and precise measurements of ubiquitylome analysis, in the context of exercise, will help to expand our knowledge of exercise-regulated ubiquitylation in skeletal muscle. Therefore, by revealing the potential for these newly developed methods, the hope is that future investigations will employ them to identify novel ubiquitin-mediated signalling pathways that regulate skeletal muscle metabolism and function following exercise.

5. Methods

Mutagenesis

To construct a bacterial vector for TR-TUBE, we used site-directed mutagenesis (Liu and Naismith, 2008) to mutate all arginine residues to Alanine on each of the 4 ubiquitin binding domains of TUBE in the pET28a His-Halo-TUBE vector. To construct a ubiquitin-binding–deficient TR-TUBE mutant, two conserved Leucine residues of the TR-TUBE construct, were replaced with Alanine in four positions simultaneously (Yoshida *et al.*, 2015).

Protein purification

Plasmids (pET28a His-Halo-TUBE, pET28a His-Halo-TR-TUBE and pET28a His-Halo-TR-TUBE deficient) were transformed into BL21 E.Coli. A colony of each was selected and grown into LB media, expanding to 2000 mL at 37°C. The cells were grown to OD 600 of 0.6 before adding 250 µM ITPG and left to express overnight at 18°C. Cultures were pelleted and resuspended in lysis buffer (50mM Tris-HCL pH 8.0, 150mM NaCL, 50mM Imidazole, 0.5mM TCEP, 1mM PMSF) and then lysed using Emulsiflex C3 Cell Disruptor (Avestin Europe, Mannheim, Germany). Recombinant proteins were purified by the use of HIS-Trap (GE Healthcare) as per the manufacturer's instructions. The concentration was measured by nanodrop and the purified protein was then stored at -80°C.

Cell Culture

Mouse skeletal muscle C2C12 myoblast cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were seeded and cultured in DMEM

containing GlutaMAX, 25 mM glucose and 1 mM sodium pyruvate, supplemented with 10% (v/v) foetal bovine serum (GE Healthcare, Buckinghamshire, UK) and 1% (v/v) Penicillin-Streptomycin (10,000 Units/mL-ug/mL). Myoblasts were differentiated (for 4-6 days) into myotubes at 90% confluency in DMEM containing GlutaMAX, 25 mM glucose and 1 mM sodium pyruvate, supplemented with 2% horse serum (Sigma-Aldrich, Cambridgeshire, UK) and 1% (v/v) Penicillin-Streptomycin (10,000 Units/mL-ug/mL). Cultures were maintained in a humidified incubator at 37°C with an atmosphere of 5% CO2 and 95% air.

Cell lysis

Cells were lysed in ice-cold sucrose lysis buffer containing: 250 mM sucrose, 50 mM Tris-base (pH 7.5), 50 mM sodium fluoride, 10 mM sodium β-glycerolphosphate, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM EGTA, 1 mM benzamidine, 1 mM sodium orthovanodate, 1 x complete Mini EDTA-free protease inhibitor cocktail, 1% Triton X-100 and 100 mM 2-chloroacetamide. Lysates were centrifuged at 4°C for 15 min (13,000 rpm) and the supernatant was collected for protein measurement. Protein concentration was determined by Bradford protein assay (ThermoScientific) using BSA as standards.

Muscle homogenisation

40-200mg mixed human muscle samples were ground to powder before being homogenised in 12.5ul/mg of ice-cold sucrose lysis buffer (see ‘Cell lysis’), using a polytron homogenizer to obtain a homogenous solution. Lysates were centrifuged at 4°C for 15 min (13,000 rpm) and the supernatant was collected for protein measurement. Protein concentration was determined by Bradford protein assay (ThermoScientific) using BSA as standards.

Ubiquitin pull-down

The ubiquillin1 UBA domain tetramer (UBA^{UBQLN1}) proteins: his-halo-TUBE, his-halo-TR-TUBE or his-halo-TR-TUBE deficient were expressed in E. coli BL21 cells and purified (see 'Protein Purification'). In each ubiquitin pull-down experiment, 45µl of HaloLink resin (Promega, Hampshire, UK) was used (containing ~10µl of Halo beads) per sample number. The total volume of resin was pre-washed with PBS and resuspended in 750µl of binding buffer: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% NP-40, 1 mM dithiothreitol (DTT). The solution was incubated with 50µg of the selected TUBE protein per 10µl of Halo beads overnight at 4 °C. Conjugated HaloLink resin and TUBE protein was washed in binding buffer and resuspended into sucrose lysis buffer (see 'Cell lysis'). TUBE proteins bound with conjugated HaloLink resin were incubated with 300-8000µg of protein from lysed cell or homogenised human muscle samples overnight at 4°C. After 3 washes in sucrose lysis buffer (plus 150 mM NaCl), the enriched ubiquitin and poly-ubiquitin chains were eluted with 1x NuPAGE LDS sample buffer (ThermoFisher Scientific, Leicestershire, UK). Samples were left to denature overnight at room temperature in 1.5% 2-mercaptoethanol.

Western Blot

10% of lysate after pull-down was loaded into 4-12% BIS-Tris gels prior to SDS-PAGE. Gels were run in 1x MOPS buffer for approximately 80 minutes at 150V. Proteins were transferred onto PVDF membranes (GE Healthcare Life Sciences; 10600021) for 1h at 100V. Each membrane was blocked in 5% Milk diluted in Tris-buffered saline Tween-20 (TBS-T): 150 mM NaCl, 20 mM Tris-base 7.5 pH, 0.1% Tween-20 for 1 h and then washed in TBS-T (3x10 min) before being incubated overnight at 4°C with a ubiquitin antibody

(1:2000 #P4D1; Biolegend, London UK). Membranes were washed in TBS-T (3x10 min) prior to incubation in horseradish peroxidase-conjugated mouse secondary antibodies (1:10 000) at room temperature for 1h. Membranes were then washed in TBS-T (3x10 min) prior to antibody detection using enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Hertfordshire, UK). Imaging was undertaken using a G:Box Chemi-XR5 (Syngene, Cambridgeshire, UK).

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