Assessment of Commercial Versus Custom-made MuRF1 Antibodies

by

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A thesis submitted to the University of Birmingham for the degree of MASTER OF SCIENCE by RESEARCH

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September 2019

Abstract

Muscle wasting is the result of muscle protein breakdown occurring at a greater rate than muscle protein synthesis, resulting in a net loss of muscle mass. Muscle protein breakdown is contributed to by the ubiquitin proteasome system, where E3 ubiquitin ligases facilitate the degradation of proteins; MuRF1 has been identified as an E3 ubiquitin ligase that targets muscle proteins as a substrate. The role of MuRF1 function is still under investigation and there is no published data on the efficacy of immunoblotting based techniques used to identify this protein. The aim of this study was to compare a novel custom-made vs commercial MuRF1 antibodies in for the purpose of western blots and immunoprecipitation. Preliminary dot blots showed that rabbit sera stimulated to produce MuRF1 antibodies were able to detect MuRF1 specific peptides. Western blots revealed that purified custom-made antibodies were able to detect recombinant MBP-MuRF1 but not MuRF1 in overexpressed cell lysate; Santa Cruz offered the most sensitive and specific commercial MuRF1 antibody for western blot. Custom-made antibodies were the most effective at enriching MuRF1 in immunoprecipitation. This provides an important set of tools to characterise MuRF1, explore muscle atrophy signalling, and produce therapies to treat chronic muscle pathologies.

Acknowledgements

Many people have been directly and indirectly responsible in my career and in this research. I would like to acknowledge some of them here:

- I would like to acknowledge the support of my close friends and family without who, I would not have been able to pursue education as a mature student.

- I give special thanks to my partner, Natalie, who has taken the risks of this journey and believed in me, and my parents who have always offered their care and support.

- Thanks to Jodie and Katie Hemmings-Trigg for being great friends and cheerleaders on my academic journey.

- Thanks to Sam Lord, for your superb data collection that made this work possible.

- Dr Yu-Chiang Lai – Thank you for your mentorship and persistent belief in my potential as a researcher and pushing me to have a high standard of work. I literally would not have made it this far without your support and encouragement.

- Dr Thomas Solomon, thank you for channelling my enthusiasm for science into research - Without this first step, I would not be where I am now.

- Dr Jonathan Barlow, thank you for your time and patience to show me the real-world of research as an undergraduate student and support in my PGR career.

- Dr Leigh Breen, thank you for seeing my potential and being supportive of my research career.

- Thanks to the Physiological Society who provided funding for research experience and the opportunity to learn molecular techniques.

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1. Introduction

1.1 Importance of Skeletal Muscle Mass

Skeletal muscle acts a protein reservoir for the body, providing amino acids and energy during times of nutritional poverty. The maintenance of human muscle protein is achieved by balancing muscle protein synthesis (MPS) and muscle protein breakdown (MPB), if MPB exceeds MPS over time there is net loss of muscle protein, termed atrophy. The loss of large amounts of muscle mass are associated with cancer, diabetes, sepsis, and heart failure as well as myopathies and muscular dystrophies (Bonaldo and Sandri, 2013).

1.2 Muscle Loss (Atrophy)

1.2.1 Sarcopenia

Sarcopenia (From the greek ‘sarx’ meaning flesh and ‘penia’ meaning deficiency; Penia was a Greek god who personified poverty) is the progressive loss of muscle mass and function through age, the most common form of muscle atrophy observed in humans. This loss of strength reduces the independence of older individuals who are unable to rise from a chair, bathe themselves, prepare food etc, thereby lowering the quality of life; The loss of strength in older age means that individuals are at greater risk of falls and, with muscle mass being related to bone density, they are more likely to break bones and recover slower (Marty *et al.*, 2017). Additionally, muscle mass is a predictor of mortality in humans beyond quantification of total body mass. Metter et al (2002) performed a longitudinal study over 25 years of 1071 males, measuring grip strength, muscle mass (using 24hr creatinine secretion), and questionnaire obtained physical activity profiles. In all cases they showed that these markers decreased with age and found that lower levels of muscle mass, strength, and declines in strength have a linear correlation with the mortality of the participants of the 25-year study (Metter *et al.*, 2002).

1.2.2 Cancer Cachexia

Cancer cachexia (From the greek ‘kakus’ meaning bad, ‘hexis’ meaning active state) is a wasting disease characterised by patients suffering from anorexia (reduced appetite and food consumption), asthenia (weakness), weight loss, and anaemia (reduction in healthy red blood cells). The weight loss in cancer cachexia is due to an equal loss of both adipose and skeletal muscle tissue, beyond the

majority loss of adipose tissue common in weight loss from starvation - This loss of muscle mass via cancer cachexia is associated with decreased survival in cancer patients (Dewys *et al.*, 1980; Argilés *et al.*, 2010). Currently there is a lack of understanding regarding the molecular mechanisms within patients sharing the same pathological type of cancer (Baracos, Mazurak and Bhullar, 2019).

1.2.2 Understanding Muscle Loss

More focus should be given to maintaining muscle mass and strength with age and reduce the situation in which declining strength and muscle mass can occur, such as inactivity, hospital bedrest, atrophic pathologies, and exposure atrophy inducing substances. It would be of great benefit to be able to develop tools to measure the signalling process that facilitates sarcopenia so that more effective therapies can be developed.

The understanding of the molecular mechanisms of muscle protein degradation will provide a solid grounding to tackling these pathologies. There are many different pathways that instigate MPB, however all these terminate with protein degradation (Sartorelli and Fulco, 2004). In their review of protein degradation (Proteolytic) pathways Lecker et al (1999) identifies the Ubiquitin-proteasome system (UPS), which degrades the bulk of intracellular proteins via the 26S proteasome, as a mediator of muscle proteolysis. The UPS is directly implicated in muscle loss for most atrophy models, including cancer cachexia (Attaix *et al.*, 2008), sepsis (Tiao *et al.*, 1997), starvation and denervation (Wing, Haas and Goldberg, 1995), and limb unweighting (Taillandier *et al.*, 2003). Furthermore, the inhibition of UPS attenuates proteolysis in atrophy (Fareed *et al.*, 2006; Houston, Hain and Dodd, 2018).

Therefore, understanding of the UPS is crucial to characterising mechanisms of muscle atrophy.

1.3 The Ubiquitin Proteasome system

1.3.1 Ubiquitination

Ubiquitination is the post-translational modification of a protein by the addition of a ubiquitin molecule. Ubiquitin is attached by its C-Terminal glycine residue to an ε-amino group situated in the lysine residue of a substrate (Komander, 2009). This can mark the protein for degradation in the proteasome, alter their location, modulate their activity, or alter their protein interactions.

1.3.2 Forms of Ubiquitination

There are 3 ways in which protein can be ubiquitinated (Fig 1.3.2): Monoubiquitination is the addition of a single ubiquitin molecule to a substrate; multiubiquitination is the addition multiple single parallel ubiquitin molecules to a substrate; polyubiquitination is the addition of a ubiquitin polymer, a series of attached ubiquitin together forming chains (Komander, 2009).

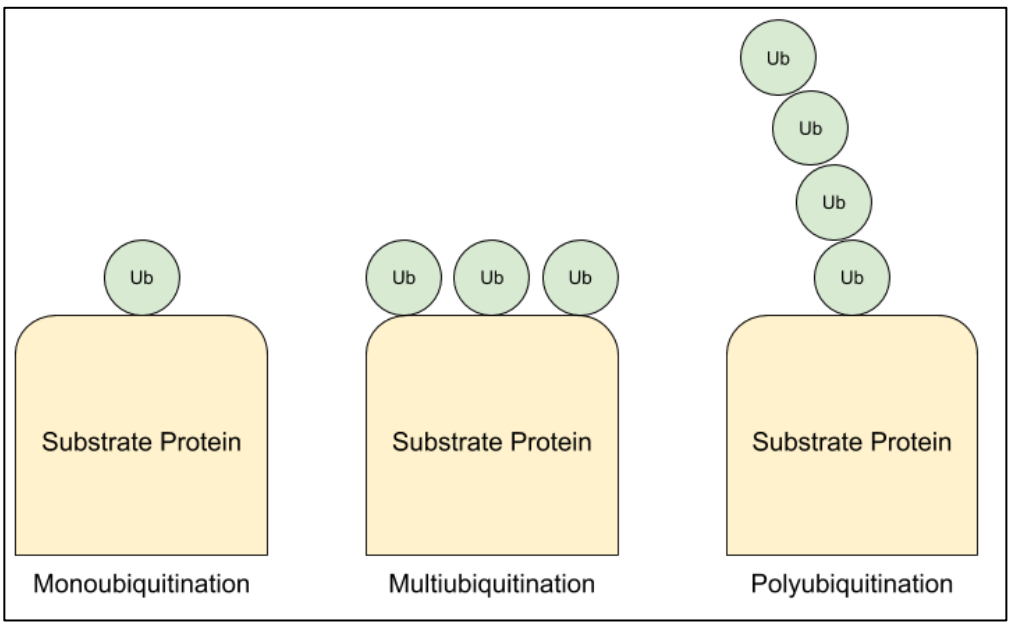


Fig 1.3.2. Illustration showing of types of ubiquitination

1.3.3 Polyubiquitin Chains

Ubiquitin can form chains on seven lysine residues and its C-terminus: K6, K11, K27, K29, K33, K48, K63 and M1. The different polyubiquitin chains ultimately change the effect that ubiquitination has on the substrate - K48 chains have been well established as mediating proteasomal degradation, K63 are associated with signal transduction and endocytosis, however recent evidence suggests that K63 and K48 branched chains also facilitate associated to the proteasome (Ohtake *et al.*, 2018). The function of the other chains is still being elucidated (Komander, 2009; Xu *et al.*, 2009).

1.3.3 Mechanism of Ubiquitination

Ubiquitin is attached in a three enzymatic stage process:

E1 Ubiquitin Activating Enzyme; Only one functioning E1 enzyme has been discovered in mammals (Handley *et al.*, 1991); E1 is an abundant enzyme that utilises ATP to create a reactive form of ubiquitin (Haas and Rose, 1982). *E2 Ubiquitin Conjugating Enzyme*; There are 16-35 different E2 enzymes present in eukaryotic cells, 35 have been identified in humans. E2s are characterised by the presence of a highly conserved ubiquitin conjugating domain (UBC domain), which accommodate the, now ATP-activated, ubiquitin from E1. They function by creating selective protein-protein interactions with E1 and E3 enzymes. E2s dictate the specific lysine, on the substrate, where the ubiquitin chain will be formed, determining the final state of the substrate protein (Van Wijk and Timmers, 2010). *E3 Ubiquitin Ligase*; E3s are responsible for substrate recognition and create a protein-protein interaction between the substrate and E2 to transfer the ubiquitin

molecule to the substrate. Additionally, some E3s can also generate unanchored polyubiquitin chains without a substrate (Rajsbaum and Bharaj, 2016).

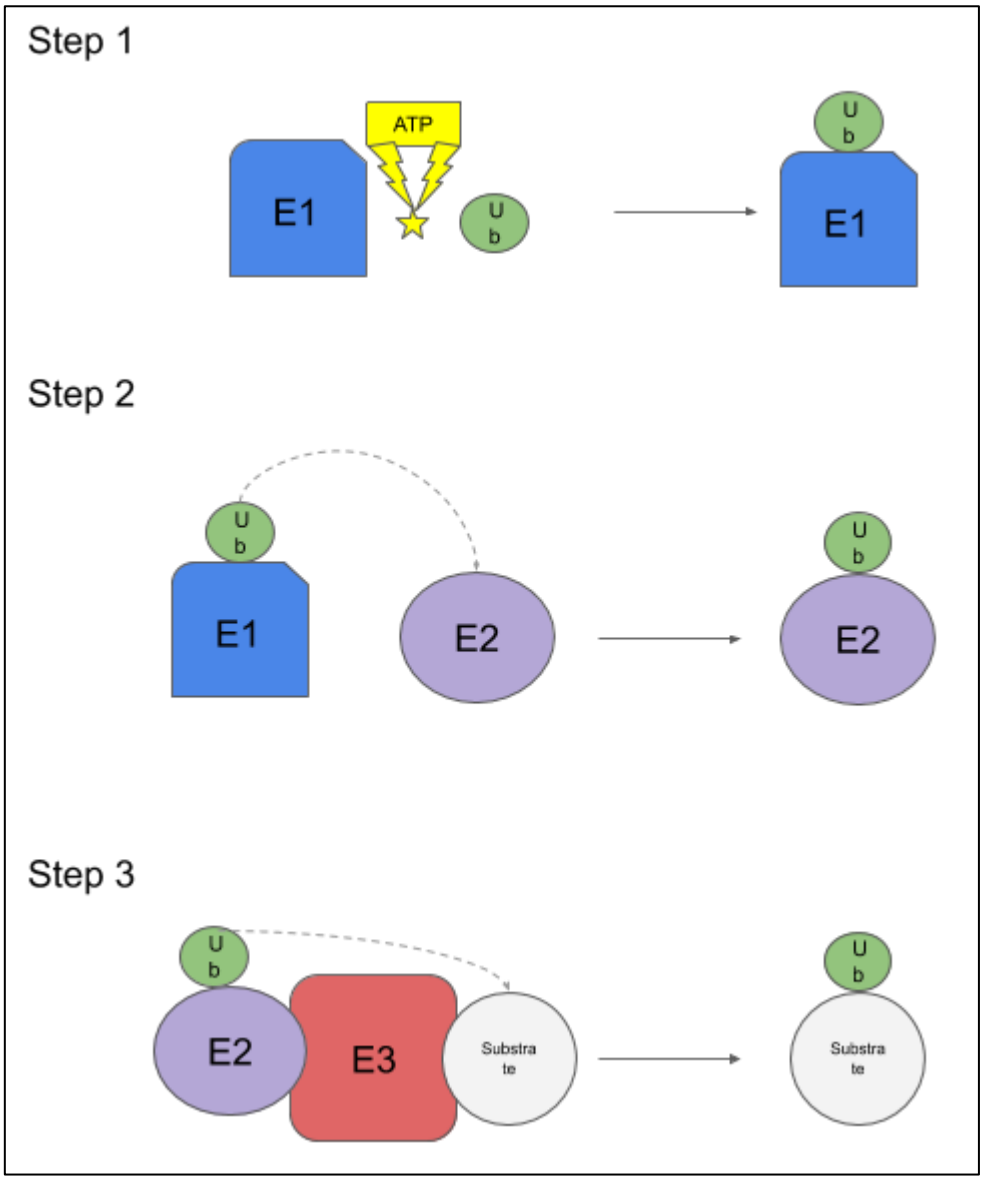


Fig 1.3.3. Illustration of the enzymatic process of ubiquitination

1.3.4 Types E3 Ubiquitin Ligases

There are 3 types of E3 ligases characterised by their domains and mechanism of ubiquitin-substrate interaction: RING (Really Interesting New Gene) E3s is the most abundant E3 ligase, these act as a scaffold to transfer the ATP-activated ubiquitin

directly from the E2 to the substrate; HECT (Homologous to E6AP Carboxyl Terminus) E3s. The catalytic domain of these E3s follow a 2-step reaction of transferring the ATP-activated ubiquitin to a catalytic cystine on the E3, and then transferring the ubiquitin directly to the substrate; RING -between- RING (RBR) E3 ligases are characterised by having 2 RING domains with an in-between RING domain (IBR). Their function is analogous to HECT by transferring the ATP-activated ubiquitin to a catalytic cystine on the E3 and then transferring the ubiquitin directly to the substrate (Morreale and Walden, 2016).

1.4 MuRF1 Discovery and Implications

1.4.1 Identification of MuRF1 As an E3 Ligase

Bodine et al (2001) aimed to identify genes implicated in skeletal muscle atrophy. To begin they used an immobilization model in the gastrocnemius muscle of rats, one leg that is immobilised for 3 days and the other free limb as mobile control. The mRNA from these muscles were analysed using gene-tag differential display to show the profiles of gene expression in immobilised vs control muscle and investigated genes expressing a >3-fold higher amount in the immobilised sample. They then compared these profiles to those in samples of rats that were denervated, immobilised, and unweighted from 1-14 days, models that show equal amounts of muscle atrophy. They discovered that denervation and immobilisation have similar perturbation of genes, but the unweighting samples genes were largely unaffected. Two genes were found to be upregulated in all models of atrophy: Muscle RING (Really interesting new gene) Type 1 (MuRF1) and Muscle Atrophy F-Box (MAFBx). To elucidate if these were more universal atrophy markers, they then analysed the expression of these genes in 2 further atrophy models: treatment with Interleukin-1

(IL-1) treatment, a cachectic cytokine, and treatment with the glucocorticoid dexamethasone. Both treatments resulted in over a 10-fold expression of MuRF1 and MAFBx.

Following identification of expression of MuRF1, they proceeded to characterise the proteins function as a potential E3 Ubiquitin Ligase by performing an in-vitro assay (incubating E1, UBc5C (UBE2), GST-MuRF1, Ubiquitin, and ATP). Western blot detection of ubiquitin confirmed ubiquitin polymerisation was MuRF1 dependant.

Their next experiment was to explore the in-vivo functions of MuRF1. Mice were genetically engineered by replacing genomic DNA for MuRF1 with LacZ/Neomycin cassette, allowing simultaneous gene disruption and the expression of a reporter for attempted gene expression at this locus. Denervation of the of the tibialis anterior and gastrocnemius muscles resulted in upregulation of LacZ expression, indicating that denervation directly causes upregulation of MuRF1 expression. The MuRF1 knockout also provided a muscle sparing effect against denervation sparing 36% of muscle mass at 14 days compared to wild-type controls. This study offers a clear causal relationship between the expression of MuRF1 in muscle atrophic conditions and its facilitation of muscle loss.

1.4.2 Discovery of MuRF1 Association with Muscle Protein

MuRF1 was first identified as an interactor of muscle protein when exploring the titin-kinase domain of titin (Centner *et al.*, 2001) by performing yeast two-hybrid screening with baits corresponding with the A167 and M2 region of titin to extract any interactors with this domain. 22 interacting clones were identified out of those screened, 4 of which could be confirmed using beta-galactosidase assays.

Sequencing revealed that 2 of these 4 clones were MuRF1. Using systematic deletion of constructs for titin showed that A168/A169 was necessary for the interaction of MuRF1 and titin. The use of a screening human heart and skeletal muscle revealed the full-length cDNA sequence of MuRF1, which allowed for the systematic deletion of MuRF1 constructs. Further two-yeast hybrid screens demonstrated that a central 144-residue segment of MuRF1 facilitated interaction with titin A168-A169. Finally, the use of a GST pulldown assay on titin A168/A169 demonstrated interaction of MuRF1 and titin by western blot. This is a seminal study that demonstrates the existence of MuRF1 as interactor of the muscle protein titin, but at the time of its publication there was no characterisation of MuRF1 function.

1.5 Characterisation of MuRF1

1.5.1 MuRF1 Structure and Function

MuRF are a subgroup of proteins of the Tripartite Motif (TRIM) family, characterised by their NH_2-Terminal tripartite motif containing: RING, Zing-finger B-Box, and coil-coil domains (Bodine and Baehr, 2014). There are 3 known MuRF proteins, MuRF1, MuRF2, MuRF3, that share a highly homologous structure (Fig 1.5.1) (Spencer *et al.*, 2000; Centner *et al.*, 2001). MuRF1 is the only protein of this family that highly expresses in atrophy models, whereas little is known about the function of MuRF2 and MuRF3.(Bodine and Baehr, 2014).

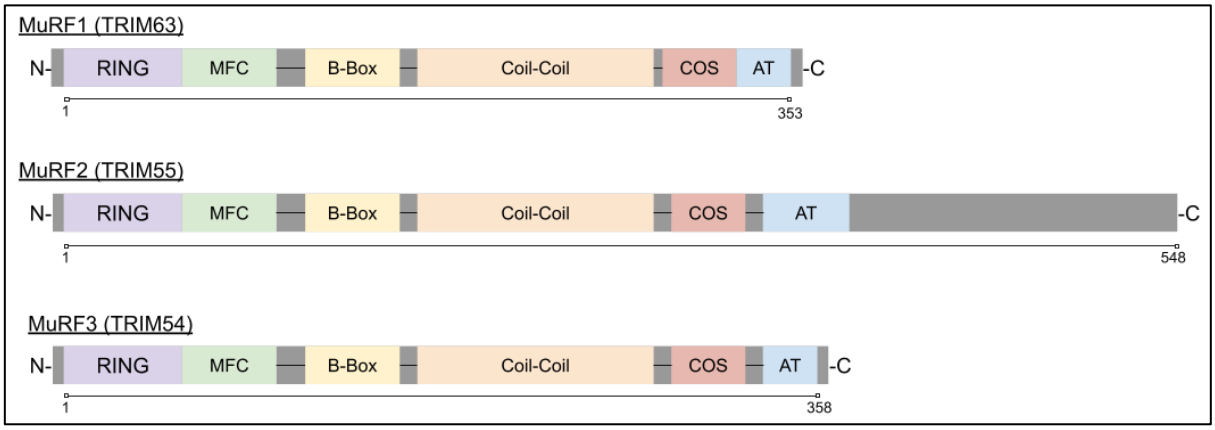


Fig 1.5.1. MuRF family protein structures. Consisting of a RING, Murf Family Conserved (MFC), B-Box, Coil-coil, COS, and Acidic Region (AR) (Adapted from Witt *et al.*, 2005; Bodine and Baehr, 2014; Nowak *et al.*, 2019)

1.5.2 Knockout MuRF1

Baehr et al (2011) generated MuRF1 knockout (M1KO) and MAFBx (MAFKO) mice and created an atrophy model by treating them with the glucocorticoid dexamethasone (Baehr, Furlow and Bodine, 2011). Over the 14 days of the study the dexamethasone treatment was shown to induce atrophy in wild type littermates, but this effect was attenuated by M1KO (but not MAFKO). This was confirmed by

measuring muscle cross-sectional area, tibialis anterior mass, triceps surae mass, and body mass. Exploring dexamethasone induced expression of MuRF1 (Using rtPCR) and MAFbx (Using Northern blot) revealed that expression of both were increased >2-fold vs controls in wild-type mice. Using ELISA, total and polyubiquitin levels in wild-type and M1KO samples were measured, this showed that M1KO mice had less total ubiquitin and polyubiquitin levels compared to wild-type. One can conclude that MuRF1 and MAFBx expression are induced by dexamethasone treatment, but it is MuRF1 that is responsible for muscle degradation, as its ablation attenuates the atrophic phenotype even when MAFBx is still expressed.

1.5.3 Transgenic MuRF1

As MuRF1 is not exclusive in skeletal muscle but also in cardiac muscle, Willis *et al* (2009) investigated the impact of overexpressing MuRF1 on heart structure and function. This was explored using MuRF1 transgenic (MTg) mice and performing transaortic constriction (TAC) (ligation of the artery) to stimulate heart failure. At baseline (Before TAC) MTg mice cardiac function was impaired and there was significant thinning of the left ventricular walls (Anterior Wall = 27.6% and Posterior Wall = 32.5% less than wild type mice during diastole). The MTg were also adversely affected by the TAC, with it causing progressive decreases in fractional shortening (70% lower than wild type) and not being subject the hypertrophy of LV wall found in wild type. This implicates MuRF1 in the breakdown of cardiac muscle tissue and modulates the response to induced heart failure.

1.6 Limitations of Current MuRF1 Research

In order to fully understand the role of a protein in the physiology of a cell there needs to be a systematic approach to studying it. For comprehensive exploration of the gene driving a phenotype one would need to explore:

mRNA → Protein Expression → Protein-Protein Interaction / Post Translational Modification → Phenotypic Changes.

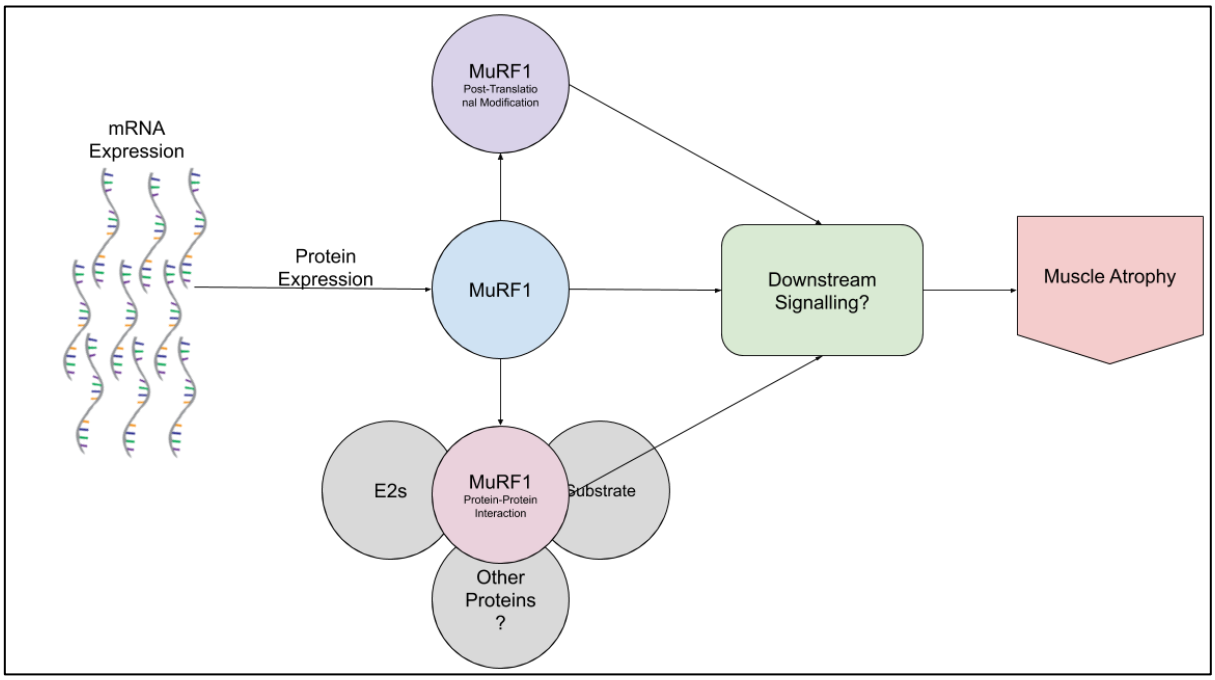


Fig 1.6. Gross schematic of the molecular process of gene expression to phenotypic change for MuRF1

There are several issues with the current study of MuRF1. First, the majority of atrophy models in which MuRF1 is implicated gets justified by inferring protein expression from mRNA expression .This is criticised by Bodine (2014) as a major limitation of MuRF1 research and can be seen across the MuRF1 literature (Bodine *et al.*, 2001; Lecker *et al.*, 2004; NIKAWA *et al.*, 2004; Urso *et al.*, 2007; de Boer *et al.*, 2007; Raue *et al.*, 2007; Witt *et al.*, 2008; Willis *et al.*, 2009; Altun *et al.*, 2010;

Lomonosova, Shenkman and Nemirovskaya, 2012; Hanson *et al.*, 2013; Oakley *et al.*, 2018; Seaborne *et al.*, 2019). Second, the presence of MuRF1 studies that measure mRNA and protein expression can offer contradictory results placing doubt on the usefulness of mRNA data. For example, Drummond et al (2008) measured MuRF1 in the soleus of paraplegic rats in which the mRNA was no different to controls but protein expression was significantly higher ($p<0.05$) in paraplegic versus healthy controls. Thirdly, the selective use of mRNA expression and protein expression in one paper throws the results into doubt. For example, Bowen et al (2017) alternates between measuring protein and mRNA expression of MuRF1 between experiments within the same paper, inmplying MuRF1s regulation of cachexia in mice. To validate dexamethasone induced atrophy in cell culture they use mRNA data but in cachexia-induced mice they demonstrate ubiquitination and measure MuRF1 protein expression instead. This dissociated use of methods to explore one protein can lead to incongruent data or, at worst, leave room for selective use of experimental data to falsely support a hypothesis.

The characterisation of a MuRF1 antibody would provide a standard with which future research could be used to explore MuRF1s role systematically: Does mRNA correlate to protein expression? Does protein expression correlate to proteasomal activity? Does MuRF1 dependant proteasomal activity correlate to atrophy? Following this broad understanding of MuRF1, experiments can be designed to fully detail the mechanisms of action, e.g. Which E2s facilitate MuRF1 induced proteolysis? What substrate/s does MuRF1 target and how do they interact? Are there any post-translational modifications of MuRF1? Does MuRF1 offer any cooperative effect, specifically with other E3s? A full mechanistic understanding of MuRF1 would make it possible to identify all the biochemical and molecular

mechanisms for MuRF1s atrophic function and offer a nuanced approach to deterring these effects.

1.7 MuRF1 Assay Techniques

As previously discussed, there seems to be an abundance of data examining MuRF1 gene expression (Bodine *et al.*, 2001; Lecker *et al.*, 2004; NIKAWA *et al.*, 2004; Urso *et al.*, 2007; de Boer *et al.*, 2007; Raue *et al.*, 2007; Witt *et al.*, 2008; Willis *et al.*, 2009; Altun *et al.*, 2010; Lomonosova, Shenkman and Nemirovskaya, 2012; Hanson *et al.*, 2013; Oakley *et al.*, 2018; Seaborne *et al.*, 2019), but a scant number of research using protein quantification. In the few papers that use MuRF1 protein measurements, antibodies are often shared between research groups without any published validation (Adams *et al.*, 2007; Willis *et al.*, 2009; Labeit *et al.*, 2010; Mearini *et al.*, 2010).

To date there is no published methodological data comparing the efficacy of current techniques used in the detection of MuRF1. This can lead to incongruities between the data produced by different labs and cost time and resources of each research group having to validate their methodology. The characterisation of MuRF1 antibodies that can be used in multiple techniques will set a standard either to begin research or start a discussion in the peer-reviewed literature into the topic.

1.8 Antibody Characterisation

1.5.1 Antibody characteristics

Antibody based techniques offer a wide range of possible techniques to explore the abundance, location, and interaction of different proteins in biological sample. Before an antibody can be used it must first be validated as effective. In their review on antibody validations, Bordeaux et al (2010) state that to validate an antibody it must be demonstrated that an antibody be specific, selective, and its use provide reproducible results. Specificity is defined as the antibodies ability to recognise and associate with the target protein; selectivity is the characteristic of the antibody to preferentially target a specific protein in the presence of other proteins, and reproducibility is the consistency with which the antibody will provide the results over multiple assays.

1.5.2 Approaches

Uhlen et al (2016) suggest the use of at least one of the following five strategies for validating antibodies:

1) Genetic: Reducing or eliminating the expression of a target protein through gene editing or RNA interference

2) Orthogonal: Running a parallel method of protein detection with the antibody-based technique. E.g. Mass spectrometry (MS) together with western blot

3) Independent antibodies: Quantifying the expression of a protein using antibodies targeting non-overlapping epitopes.

4) Tagged protein expression: Expressing the target protein with a tag and correlating this tag expression to the target protein.

5) Immunocapture Mass Spectrometry (IMS): The use of the antibody to precipitate the target protein is followed by quantitative MS to validate its efficacy.

1.5.3 Challenges

For the characterisation of a MuRF1 antibody there is an issue of objectively measuring antibody against a standard. As there is currently no reliable data on MuRF1 protein abundance in cell types, no validated antibodies, and mRNA expression not being a reliable indicator protein expression; therefore. Reviewing the five strategies for antibody validation previous it will not be possible to all genetic or independent antibody-based techniques.

1.9 Aims

This study has 4 key aims:

1) To generate a custom-made antibody raised against MuRF1-specific peptides

2) Validate commercially available and custom-made MuRF1 antibodies using a western blot

3) Validate the which MuRF1 antibody will be most effective for immunoprecipitation (IP)

4) Having found an effective combination of antibodies for IP and western blot; characterise the specificity of these antibodies to MuRF1.

1.10 Hypothesis

MuRF1 is understudied due to the lack of ability to measure protein expression. Antibodies need to be identified and validated for both western blotting and IP if MuRF1 is to be studied further. It is hypothesised here that custom made MuRF1 antibodies raised against multiple MuRF1-specific peptides will offer greater specificity and sensitivity to MuRF1 that commercially available antibodies.

2. Methods

2.1 Generation and Validation of Custom-made antibodies

2.1.1 Custom-made Antibody Production

Custom made antibodies were obtained from 21st Century Biochemicals (Marlboro, MA, USA) who generated them by inoculating two rabbit subjects (6149 and 6150) with three MuRF1-specific peptides in order to produce somatic hypermutation response to MuRF1 and thereby creating antibodies. Sera was collected at multiple time points: pre-bleed (before peptide inoculation), and 1, 2, 3 and 4 weeks after peptides were inoculated (Fig 2.1.1).

The use of this method provides a more targeting approach than inoculating rabbits with whole MuRF1 proteins (Trier, Hansen and Houen, 2019). The target peptides are more specific and can be used to both test the rabbit sera for the presence of antibodies and to then purify out the requisite antibodies. This approach minimises the number of non-specific antibodies purified from sera.

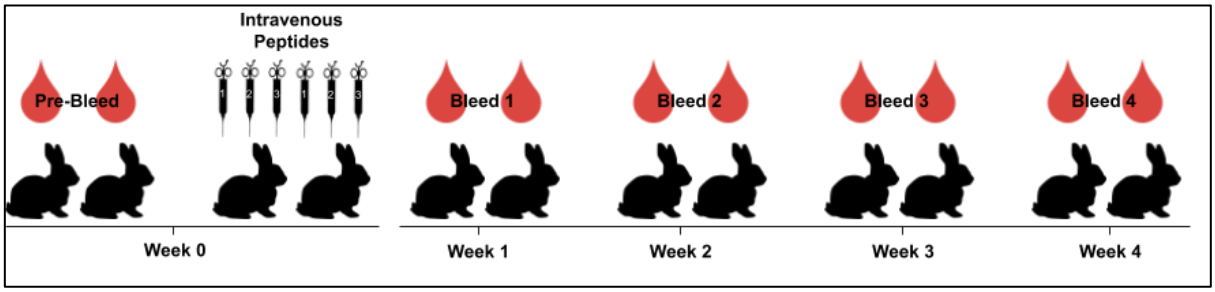


Fig 2.1.1 Timeline of custom-made antibody production

2.1.2 Custom-made Antibody Validation – Dot blotting using Sera

Antibodies were validated using a dot blotting method with MuRF1-specific peptides used as sample to assay the rabbit sera. A range of peptide concentrations were

dot blotted. A correlation between concentration and densitometry signal would indicate antibody binding and non-specific data.

2.1.3 Custom-made Antibody Validation – Western blotting using Sera

Western blots were then conducted using rabbit sera to detect recombinant MuRF1 proteins and cell samples. This was to ensure that the detection ability of antibodies translate to whole MuRF1 proteins.

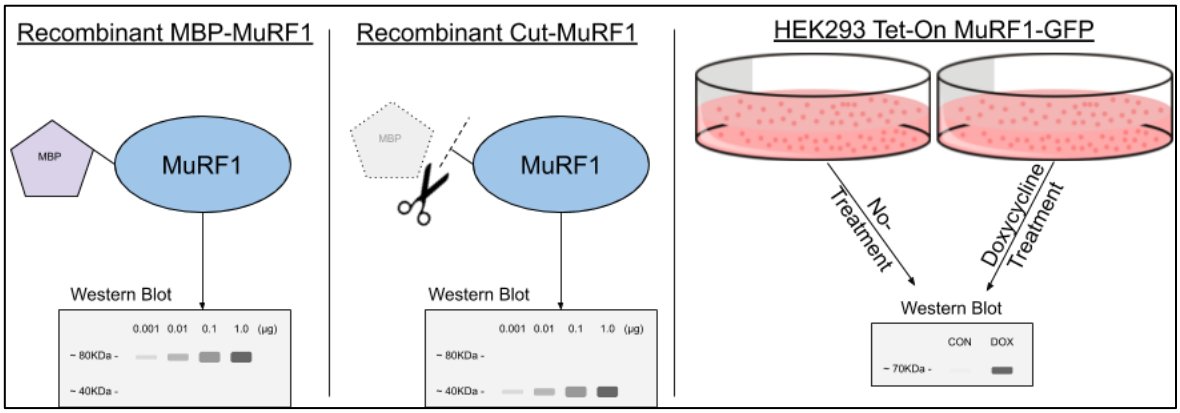


Fig 2.1.3 Diagram representing sample types used for whole MuRF1 antibody validation. Western blots are predicting the use of an effective MuRF1 antibody

Three whole MuRF1 protein samples were generated (Fig 2.1.3). Recombinant MBP-MuRF1; MuRF1 with an N-Terminal maltose-binding-protein (MBP) tag. MBP-MuRF1 molecular mass ~80KDa. Cut MuRF1; MBP-MuRF1 is generated and the MBP tag is cleaved using HRV3c protease. MuRF1 molecular mass ~40kDa. HEK293 (Human Embryonic Kidney) Cell Lysate; HEK293 with a knock-in MuRF1-GFP containing Tet-on gene (Tetracycline-controlled transcriptional activation) were used to generate a cell model. HEK293 are not known to express MuRF1 natively, so untreated cells act as controls. Doxycycline (Dox) induction of this knock-in cell line causes expression of MuRF1-GFP. MuRF1-GFP molecular mass ~70KDa.

2.1.4 Validation of samples and commercial antibodies

To validate the samples used in 2.1.3, commercially available GFP and MBP antibodies as controls to ensure presence of epitope tagged MuRF1 proteins. Commercial MuRF1 antibodies were then tested at this point to determine the best to use for western blotting.

2.1.5 Purification and testing of rabbit antibodies

Having determined the sensitivity of the rabbit antibodies to MuRF1-specific peptides and their affinity to whole MuRF1 proteins, the antibodies were purified from the rabbit sera. This was conducted by the antibody manufacturer (21st Century Biochemicals), using the MuRF1-specific peptides shown to be detectible during the dot blot 2.1.1.

Once the antibodies for MuRF1 were purified from the rabbit sera they were tested by western blot using the previous whole MuRF1 samples (2.1.3).

2.2 Comparing different MuRF1 antibodies for IP

A range of commercial antibodies were then compared for their effectiveness in performing IP. HEK293 cell lysate expressing MuRF1-GFP was used (As per 2.1.3) as sample. 4 commercial antibodies and 4 custom-made antibodies were used to IP. Western blots were for MuRF1 (Santa Cruz) and GFP (Control)

2.3 Determining the specificity of the most effective MuRF1 antibodies

Once the most effective MuRF1 antibodies were determined for both IP and western blot, their specificity and selectivity were evaluated. This was done by using lysate of transiently transfected HEK293 with GFP-MuRF1, -MuRF2, and -MuRF3 genes plus a wild-type vehicle control.

As MuRF1, 2, and 3, have some homologous structures and amino acid sequences these could act as off-target proteins for the MuRF1 antibodies. These samples were first western blotted using the most effective antibodies and GFP antibodies (To show the presence of GFP-MuRF proteins). Following this an IP was performed on the samples, using the most effective MuRF1 IP antibody determined in 2.2. It is expected that a western blot will not show any bands other than MuRF1 (Fig 2.3)

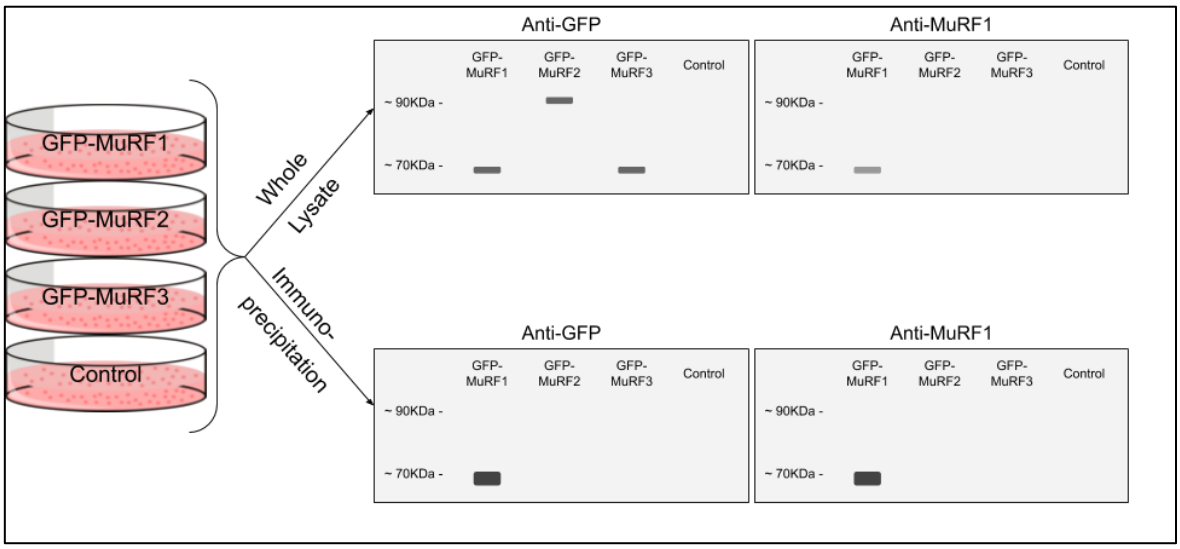


Fig 2.1.3 Diagram representing sample types used to determine the selective and specific nature of MuRF1 antibodies by western blot. GFP-MuRF1 and GFP-MuRF3 are ~70KDa and GFP-MuRF2 are ~70KDa.

2.4 Methodological Protocols

2.4.1 Cloning of MBP-MuRF1, -2, and -3 Constructs

A donor plasmid (pcDNA3.1) containing either TRIM63, TRIM55, and TRIM54 (The MuRF1, 2, and 3 genes respectively) were obtained from GenScript and pMEX3Cb-MBP was selected as a recipient vector (MRC PPU, University of Dundee); pMEX3Cb-MBP is a bacterial expression vector that contains a sequence for the maltose binding protein (MBP) on the N-terminal of the inserted sequence. Inserts with restriction site overhangs were generated by PCR (Q5 high-fidelity DNA polymerase (NEB, UK) then digested, along with the recipient vector (pMEX3Cb) using BglII and BamHI restriction enzymes (NEB, UK) as per manufacturer's instructions and ligated using T4 DNA Ligase (NEB, UK). The vector was then transformed into DH5a E.Coli competent cells and plated on LB Agar treated with ampicillin overnight at 37 °C. Successful colonies were selected and inoculated overnight for mini-prep the following day as per manufacturer's instructions (Miniprep, QIAgen, USA). Clones were validated by DNA sequencing (Source Biosciences, UK).

2.4.2 Expression and purification of recombinant MBP-MuRF1, -2, and -3 Proteins

MBP-MuRF1, 2, and 3 constructs were transformed into BL21 E. Coli competent cells and plated on ampicillin treated LB Agar and grown overnight at 37 °C. Colonies were selected and pre-cultured in 20ml of ampicillin-treated LB media overnight, the next day were transferred into 1000ml of ampicillin-treated LB media containing 200uM $ZnSO_4$ until OD600 reached >0.6. At this point 250uM IPTG was added to induce protein expression and media was left overnight at 18 °C. Cells were pelleted and resuspended in lysis buffer (50mM TRIS-HCl pH 7.5, 150mM NaCl, 5% Glycerol, 1mM DTT, 1mM AEBSF) and lysed using Emulsiflex C3 Cell Disruptor (Avestin Europe, Mannheim, Germany), clarified at (25,000 RPM, 1 hour), and filtered in 0.45 um vacuum filter.

Protein was purified from lysate by adding samples to amylose beads (To associate MBP-tagged proteins out of solution) and rotated at 4 °C overnight. The total solution was then filtered through a gravity flow column, washed with high-salt (500mM NaCl), then low-salt (150mM NaCl) lysis buffer before eluting with lysis buffer containing 10mM Maltose. Samples were checked by SDS-page and Coomassie blue stain for presence of target proteins and quantified by nanodrop and stored at -80 °C.

2.4.3 HEK293 Cell Culture and Lysis Protocol

Human Embryonic Kidney (HEK293) cells with a stably transfected MuRF1-GFP gene (Provided by Yu-Chiang Lai, University of Birmingham) were seeded into culture media (DMEM-GlutaMax, 10% FBS, 100U/ml Penicillin & 100µg/ml Streptomycin, 1mM Sodium Pyruvate) and grown to 85% confluence and had MuRF1-GFP expressed using doxycycline (0.2 µg/ml) for 24 hours before lysing

2.4.4 Dot Blotting Protocol

Nitrocellulose membranes (Protran) were dotted with 2 µl of pure peptide samples of each peptide in a range of total peptide mass (4, 2, 1, 0.5, 0.25, 0.1, 0.01, 0.001ug).and allowed to dry for 1 hour. These membranes were then blocked using 5% skim milk powder diluted in TBST for 1 hour, rinsed in TBST and incubated in the requisite rabbit sera sample (resuspended in ddH2O 1:100) for 1 hour, followed by incubation in horseradish peroxidase conjugated rabbit secondary antibody (1:10,000) for 1 hour. Membranes were finally washed in TBST 3 x 10mins and then exposed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) as per manufacturer's instructions and imaged using SynGene G-Box and quantification of densitometry was conducted using Fiji (ImageJ).

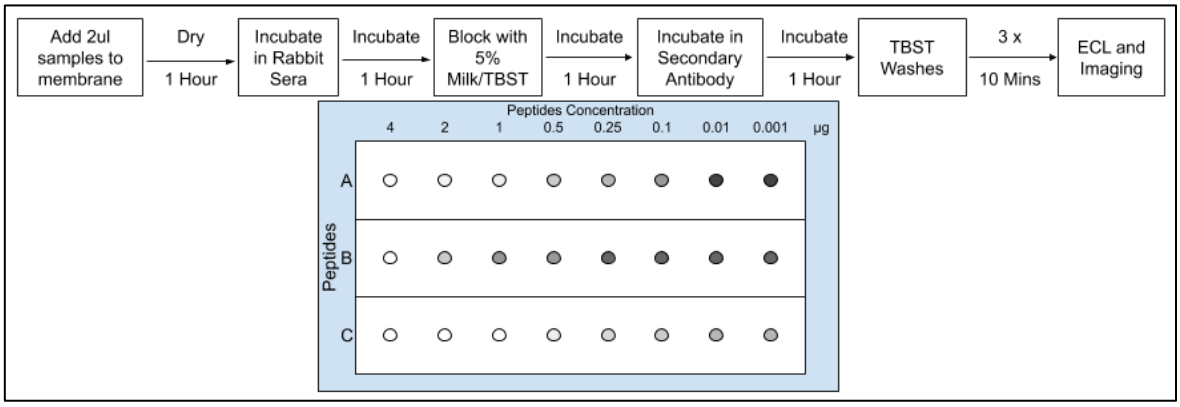


Fig 2.4.4 Dot blotting method schematic

2.4.5 Western Blot Protocol

Proteins were separated using SDS-Page (10% TRIS-Glycine gel) before transfer onto PVDF membrane (GE Life Sciences). Membranes were blocked with TBS-T buffer (Tris-Buffered Saline containing 0.1% Tween-20) containing 5% (w/v) non-fat skimmed milk powder for 1h at room temperature. Membranes were then incubated in requisite primary antibodies (Anti-MuRF1: Santa Cruz SC-398608, Proteintech 55456-1-AP, ECM MP3401, Abcam ab172479; Anti-MBP: NewEnglandBiolabs

E8038S; Anti-GFP: Chromotek 3H9-100) overnight at 4C. The following day, membranes were washed 3x10mins in TBS-T followed by incubation in requisite secondary antibodies diluted into TBS-T containing 3% w/v bovine serum albumin (BSA). Membranes were exposed using Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) as per manufacturer's instructions and imaged using SynGene G-Box and quantification of densitometry was conducted using Fiji (ImageJ) with detected bands being normalised against image background to compensate for varying exposures.

2.4.6 Immunoprecipitation Protocol

Washing

10 µl Protein A beads (NEB) were washed twice in PBS and equilibrated in lysis buffer (50mM TRIS-HCl pH 7.5, 1mM EDTA, 1mM EGTA, 10mM Na-B-Glycerophosphate, 5mM Na-Pyrophosphate, 1mM Benzamidine, 250mM Sucrose, 50mM NaF, 1mM Na_3VO_4, 1x Protease Inhibitor Cocktail, 1% Triton) and loaded with 500µg of cell lysate. Each sample/bead mixture had undiluted purified antibodies added: 4µl of Custom Antibodies (21st Century Biochemicals) and 2µl of all commercial antibodies (Anti-MuRF1: Santa Cruz SC-398608, Proteintech 55456-1-AP, ECM MP3401, Abcam ab172479; Anti-MBP: NewEnglandBiolabs E8038S; Anti-GFP: Chromotek 3H9-100) - This mixture was then rotated overnight at 4°C.

The following day beads were washed using 700µl of lysis buffer plus NaCl (*150mM NaCl)* 3 times with the final pellet being resuspended in 400µl lysis buffer before eluting using 20 µl of 1x Lithium dodecyl sulfate (LDS) in a filter column and adding of 3 µl of 10% beta-mercaptoethanol to the final eluted sample.

3. Results

3.1 Inoculated rabbits produce sera containing Anti-MuRF1 peptide antibodies.

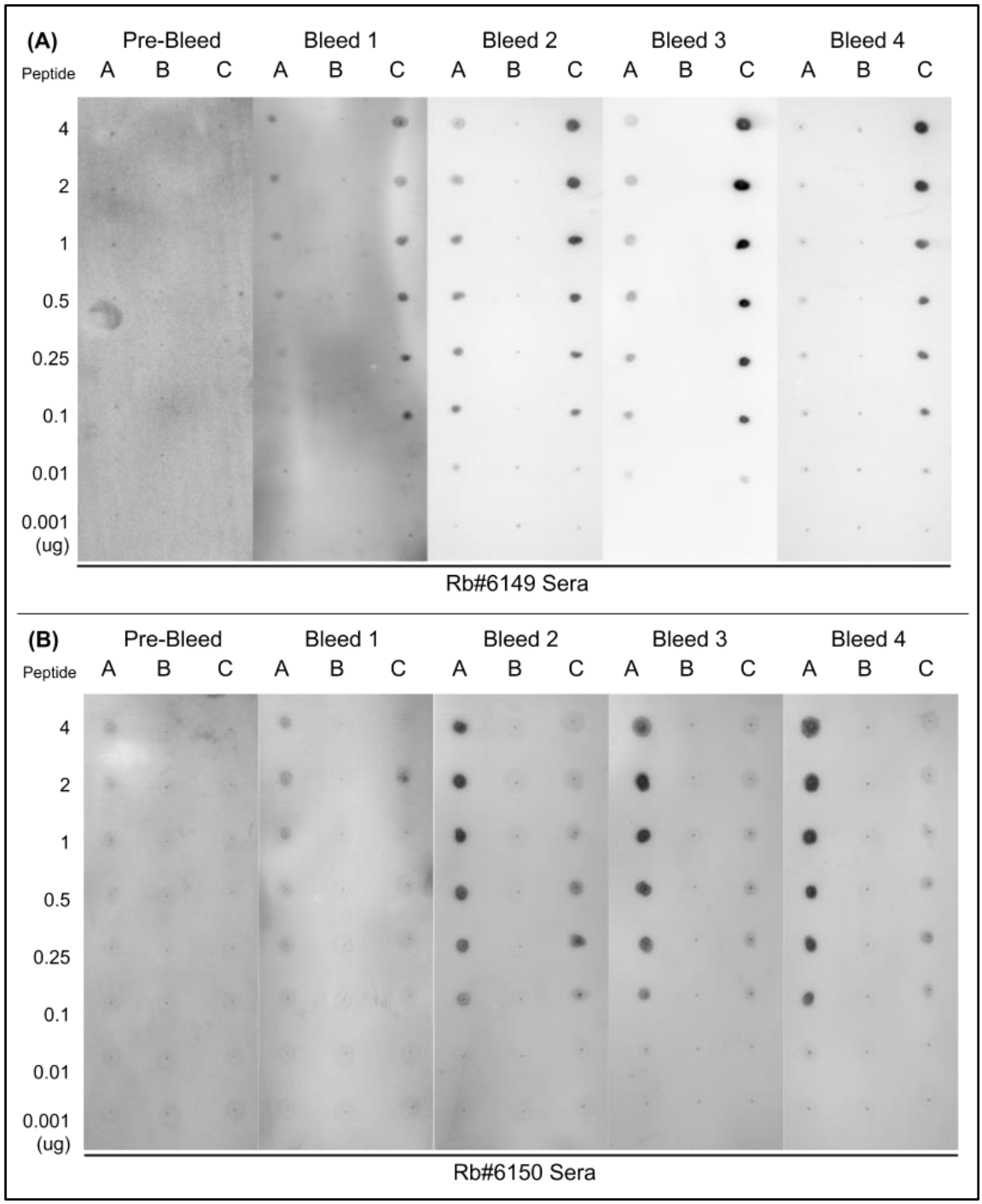


Fig 3.1 Dot blot detection of MuRF1 specific peptides using sera from rabbits 6149 (A) and 6150 (B) inoculated with MuRF1 specific peptides.

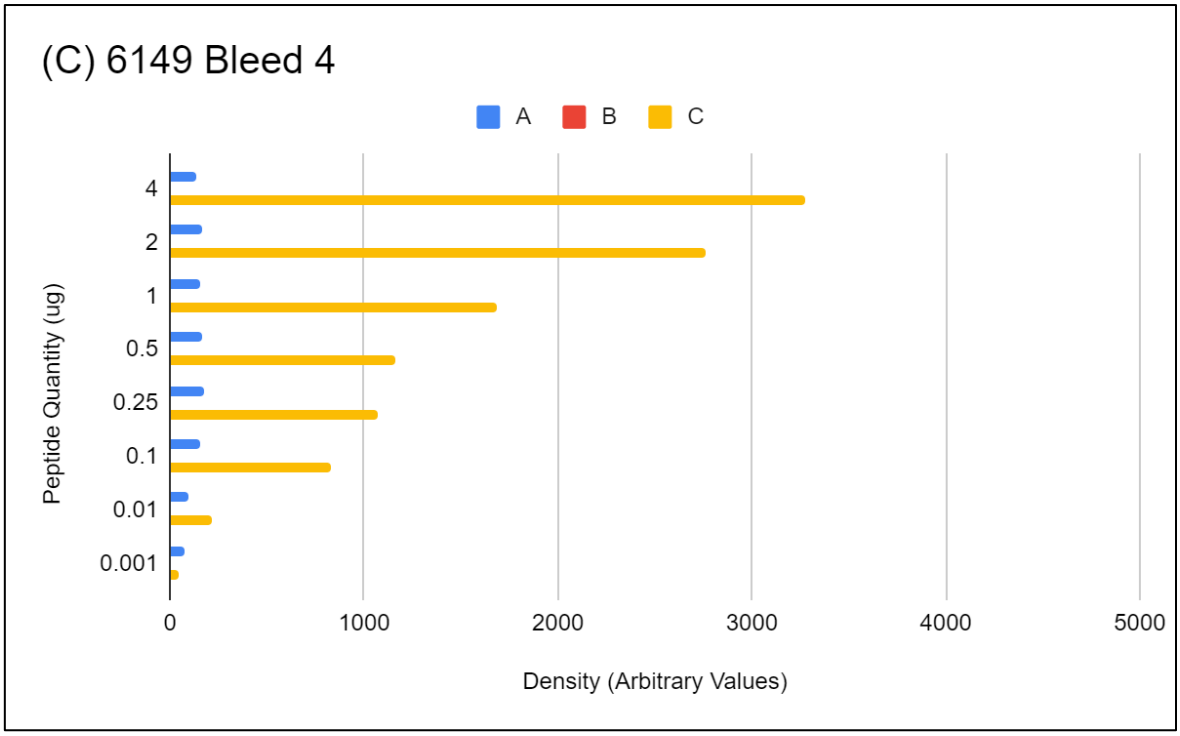


Fig 3.1C. Quantitative comparison of peptide sensitivity of rabbit 6149 sera at bleed 4 to MuRF1 specific peptides. Peptide B was not detectable.

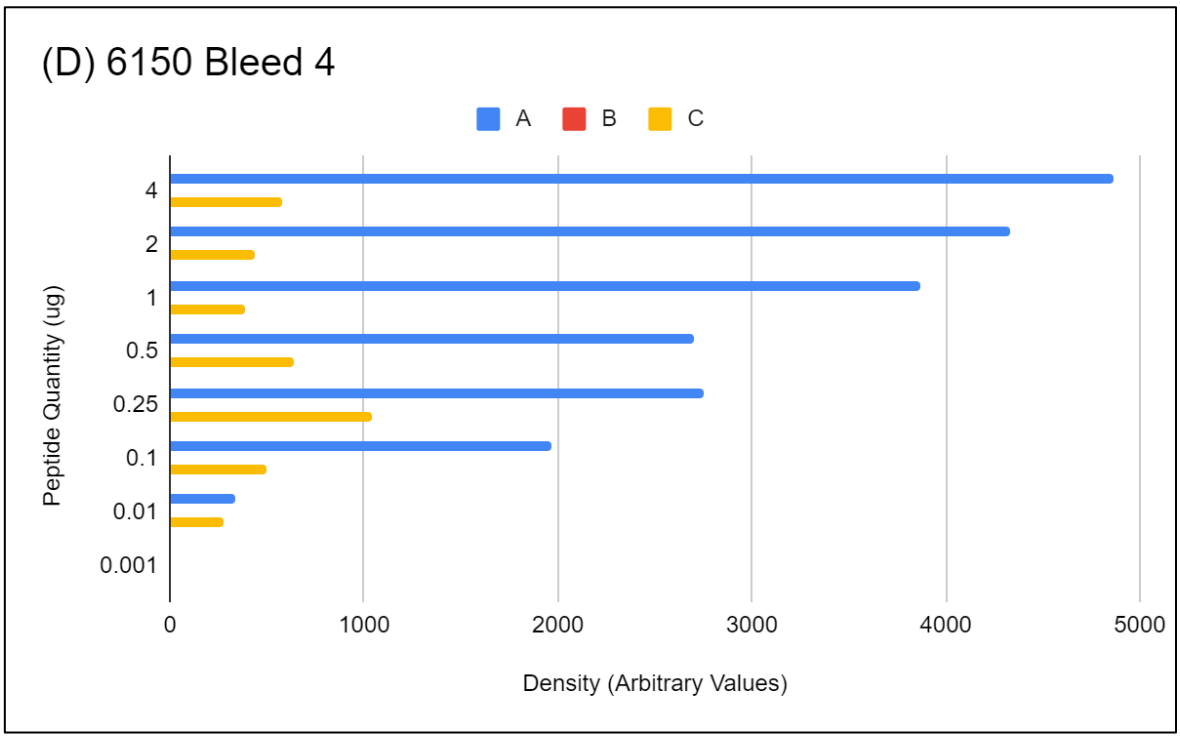


Fig 3.1D. Quantitative comparison of peptide sensitivity of rabbit 6150 sera at bleed 4 to MuRF1 specific peptides. Peptide B was not detectable. No detectible densitometry was possible at 0.001ug of any peptide.

To verify that the rabbit subjects had generated antibodies specific to MuRF1 peptides, a dot blot was performed. Using three MuRF1-specific peptides, rabbits were inoculated to stimulate production of anti-MuRF1 antibodies. A gradient of concentrations was added to a nitrocellulose membrane before incubating in rabbit sera taken at weekly timepoints following peptide inoculation. The signal increase between pre-bleed and bleed 4 demonstrates the presence of antibodies were as a result of the peptide inoculation.

Both rabbits showed sensitivity to peptides A and C but no detection could be made of antibodies specific to peptide B. A different profile of antibody specificity was seen between subjects; 6149 was more sensitive to peptide C and 6150 was more sensitive to peptide A. This is seen by the dose dependant relationship between peptide concentration and dot blot density seen in Fig 3.1 C & D.

3.2 Rabbit 6149 sera contains antibodies more sensitive to lower concentrations of MuRF1-specific peptides.

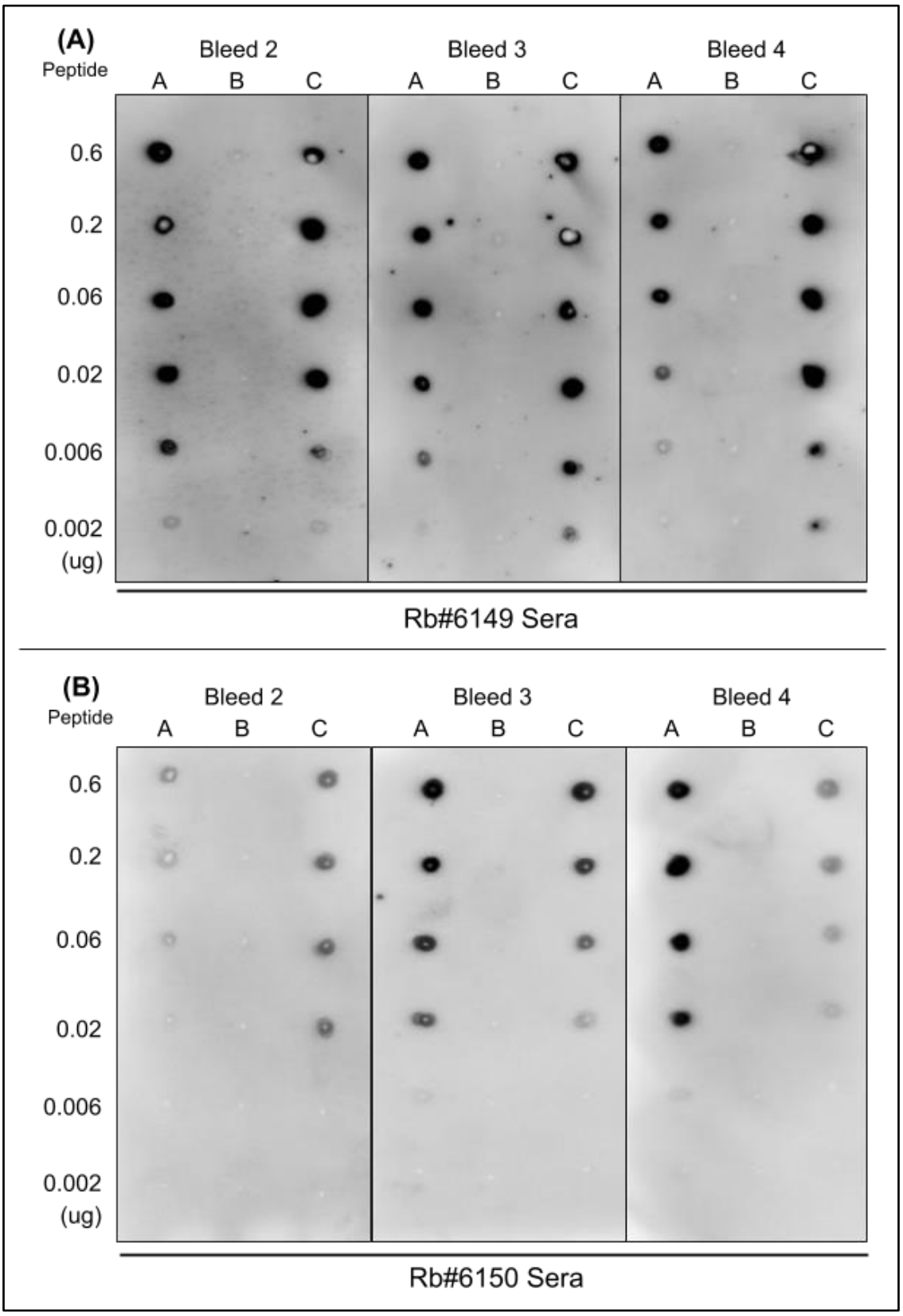


Fig 3.2 Dot blot detection of MuRF1 specific peptides using sera from rabbits 6149 (A) and 6150 (B) inoculated with MuRF1 specific peptides.

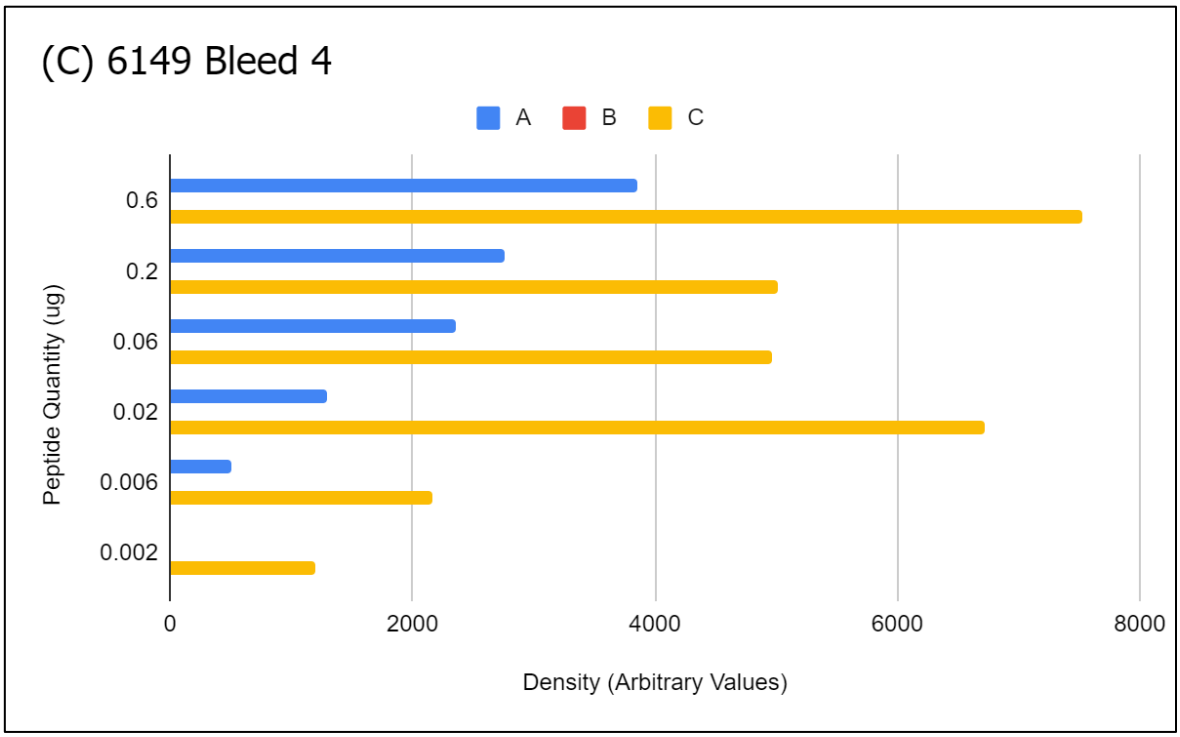


Fig 3.2C. Quantitative comparison of peptide sensitivity of rabbit 6149 sera at bleed 4to MuRF1 specific peptides. Peptide B was not detectable.

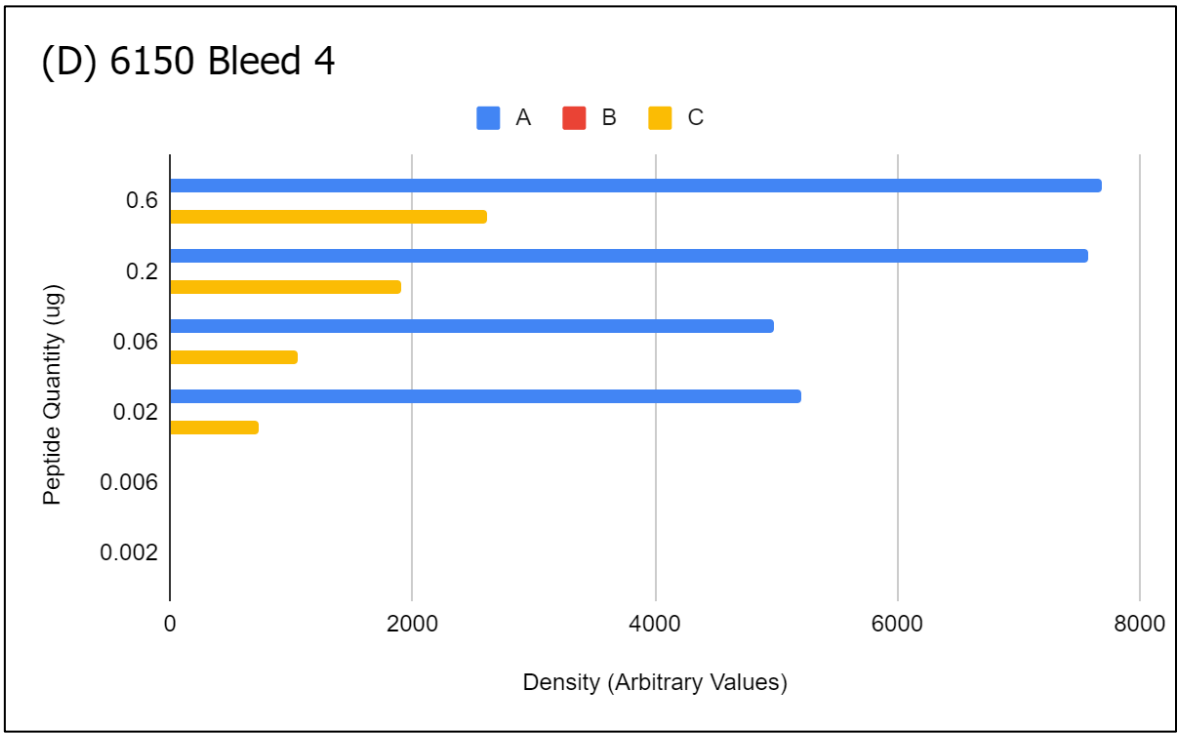


Fig 3.2D. Quantitative comparison of peptide sensitivity of rabbit 6150 sera at bleed 4 to MuRF1 specific peptides. Peptide B was not detectable.

Dot blot assays were performed by fixing nitrocellulose membrane with MuRF1 specific peptides (A, B, and C) in increasing quantities and probed using sera from 2 different rabbits: 6149 (Fig A & C) and 6150 (Fig B & D)

It was necessary to determine how sensitive each subject was to the 3 peptides. Using the data from Fig 3.1, peptides at a narrower range of concentrations near the threshold of sensitivity, 0.002-0.6ug of peptide, were dot blotted and detected using rabbit sera.

This data reveals that rabbit 6149 is more sensitive to MuRF1 specific peptides than 6150. 6149 was able to detect peptide C from 0.006ug and peptide A from 0.002ug whereas 6150 was only sensitive above 0.02ug of peptides A and C.

3.3 Custom-Made Antibody Rabbit Sera Can Detect Recombinant MBP-MuRF1 but not Overexpressed Cell Lysate MuRF1-GFP

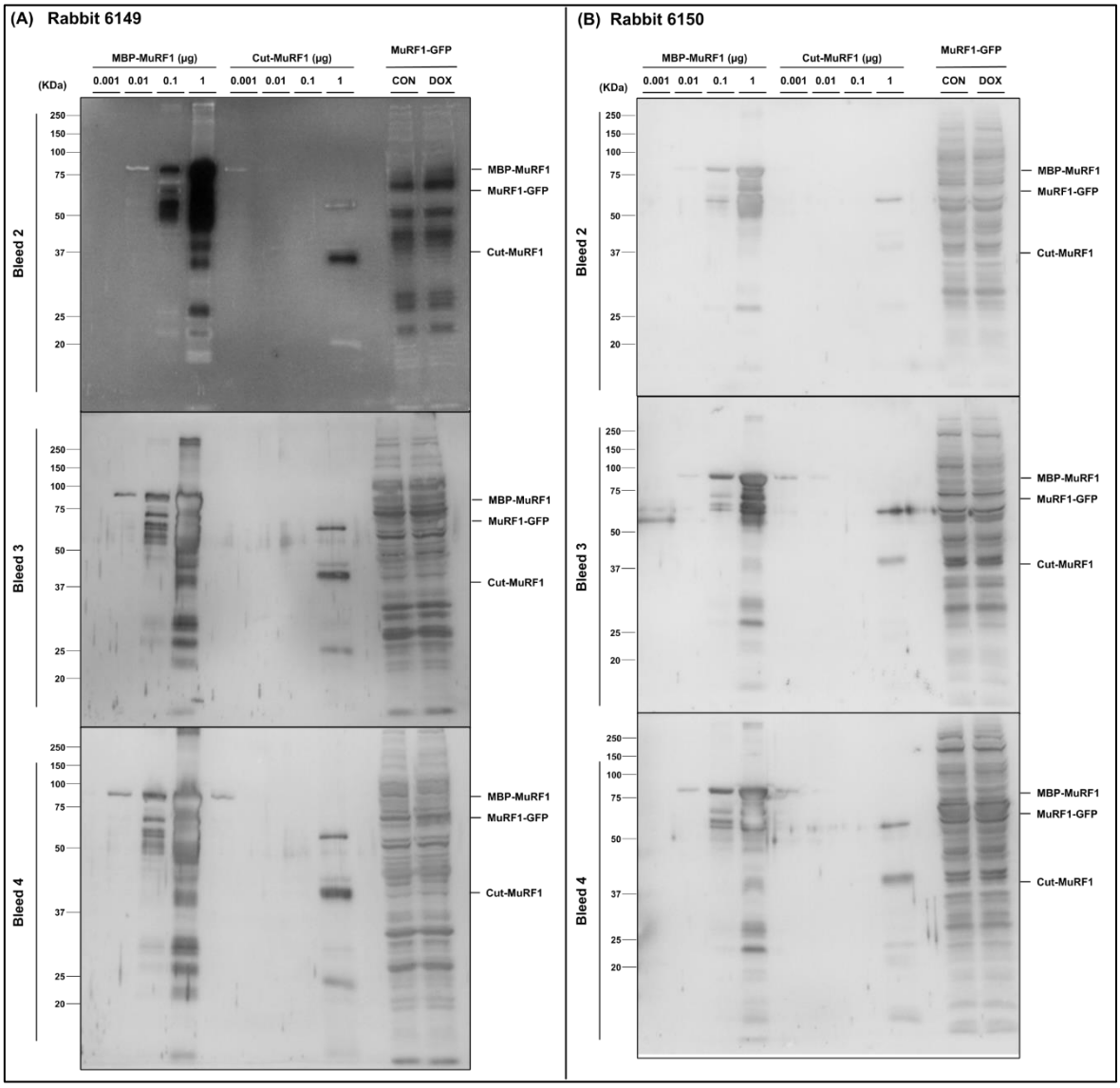


Fig 3.3. Western blot detection of MBP-MuRF1, Cut-MuRF1 (MBP Cleaved), and HEK293 lysate with (DOX) or without (CON) doxycycline induced MuRF1-GFP expression. Blotted using rabbit sera in place of primary antibodies from 6149 (A) and 6150 (B)

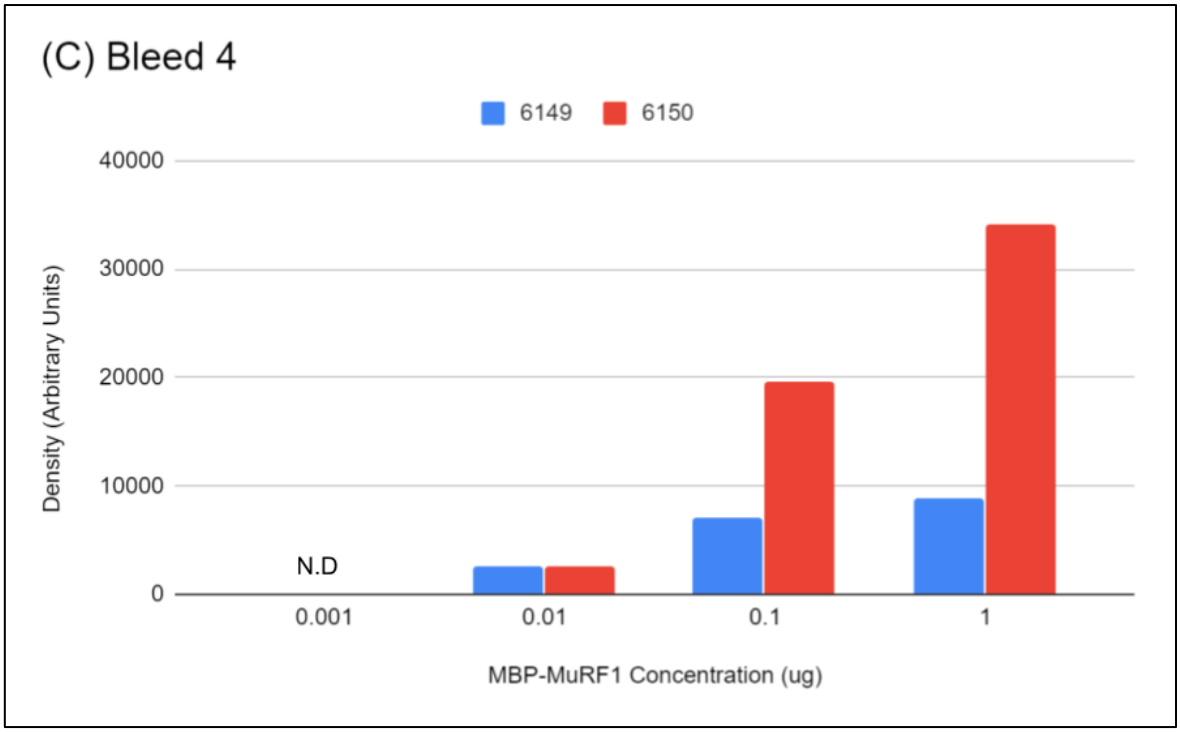


Fig 3.3 C Comparison of densitometry between rabbit sera in the detection of increasing MBP-MuRF1 concentrations. N.D = Not detectable.

Having established the effectiveness of the rabbit subject sera to the peptides used to generate antibodies, we proceeded to characterise the efficacy of using sera as a primary antibody for western blot to detect MuRF1. Using recombinant MuRF1 proteins: MBP-MuRF1 and Cut-MuRF1 (Cleaved MBP-MuRF1), and HEK293 cell lysate overexpressing MuRF1-GFP (Induced by doxycycline treatment).

Both 6149 and 6150 are sensitive to >0.01ug of recombinant MBP-MuRF1 and >1g of cut MuRF1, with the levels of non-specific binding increasing with protein concentration. MuRF1-GFP lysate with and without doxycycline treatment showed non-specific binding but no discernible MuRF1-GFP band. While both sets of antibodies can detect MBP-MuRF11 to similar concentrations, quantification of the blots (Fig 3.3 C) reveal that 6150 has a stronger affinity for MBP-MuRF1 over 6149.

3.4 Comparison of commercial antibody western blot efficacy: Santa Cruz MuRF1 antibodies can detect recombinant and overexpressed MuRF1-GFP in cell lysate; Proteintech antibodies are only able to detect recombinant MuRF1.

(A)

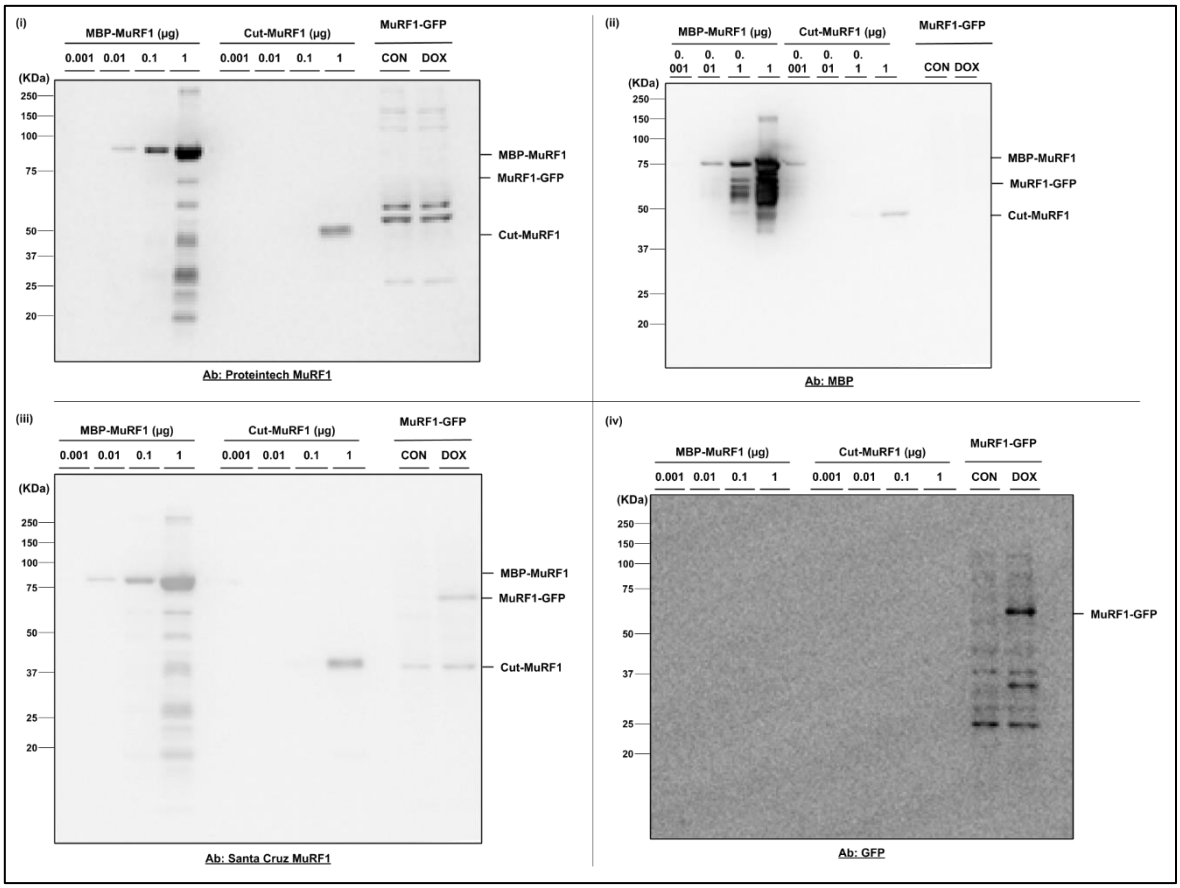


Fig 3.4. Western blots using commercially available MuRF1 antibodies (i & iii) plus validation using MBP (ii) and GFP (iv) antibodies. Antibodies tested against samples containing recombinant MBP-MuRF1 at gradient of concentrations, Cut-MuRF1 (MBP-MuRF1 with the tag cleaved), and cell lysate from HEK293 cells that have been induced to express MuRF1-GFP with doxycycline (Dox) or untreated controls (Con).

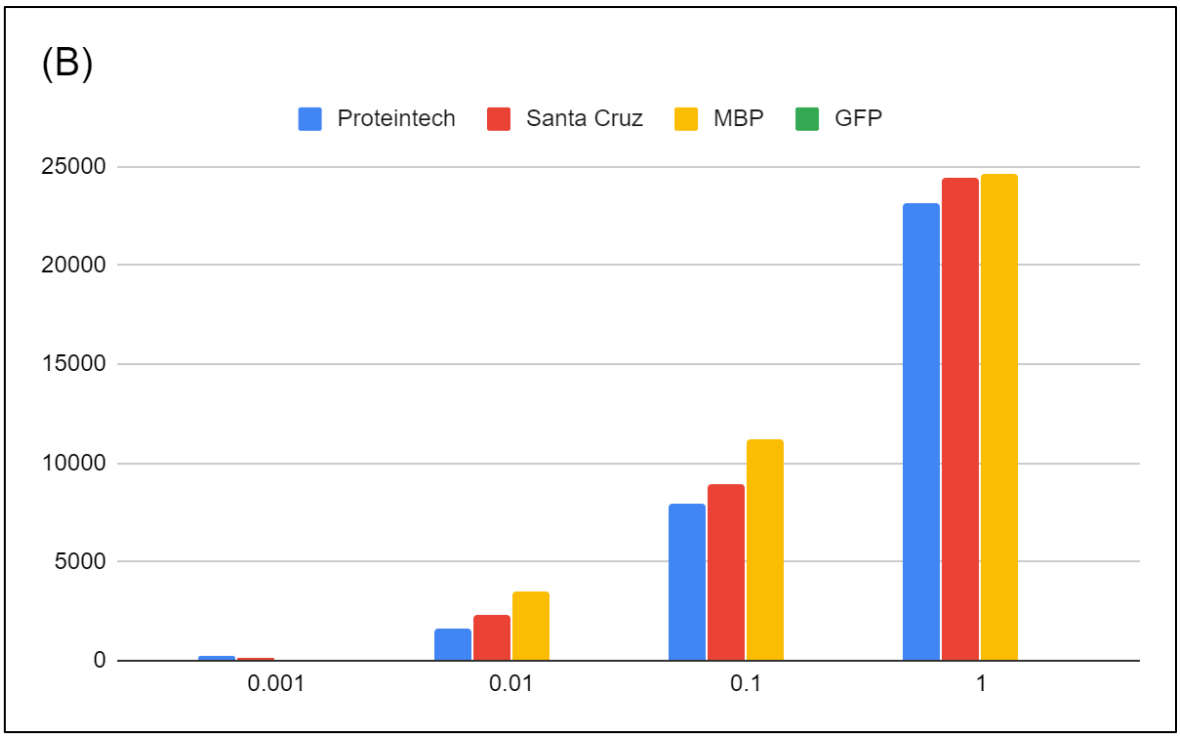


Fig 3.4b. Quantification of MBP-MuRF1 band densities from western blots (in Fig 3.3.2a) comparing different commercial antibodies

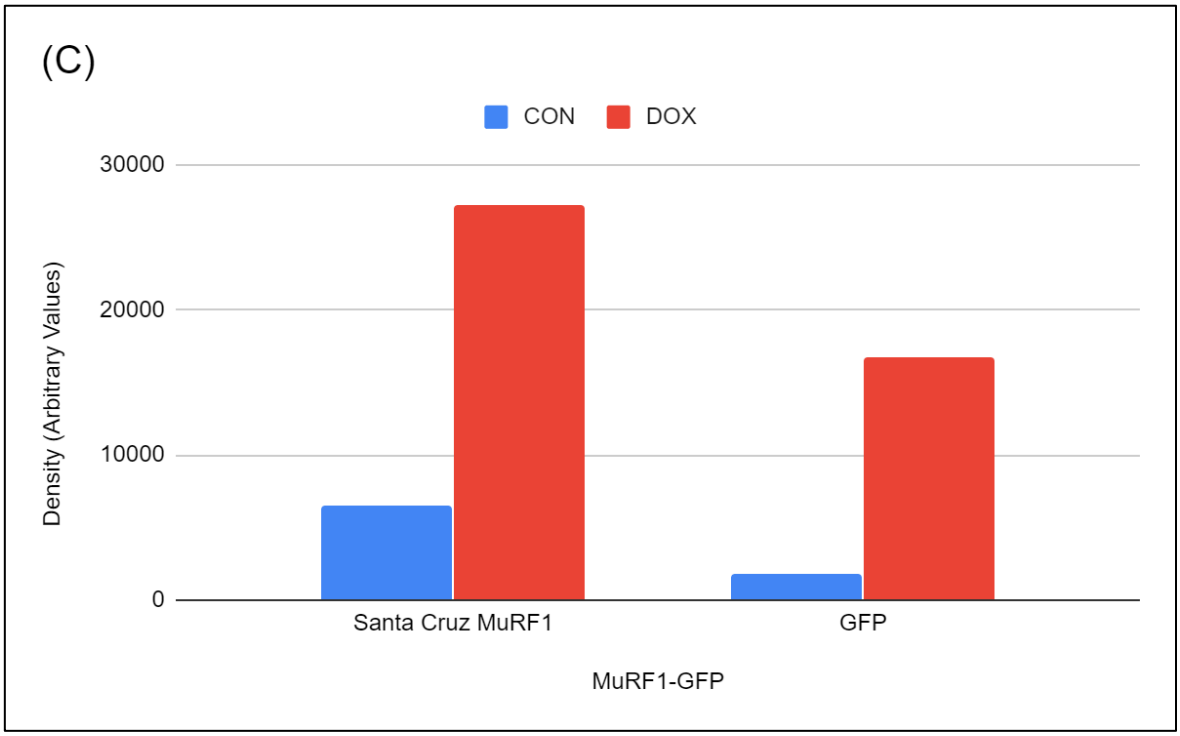


Fig 3.4c. Quantification of HEK293 MuRF1-GFP expression levels from western blots comparing Santa Cruz MuRF1 and GFP antibodies

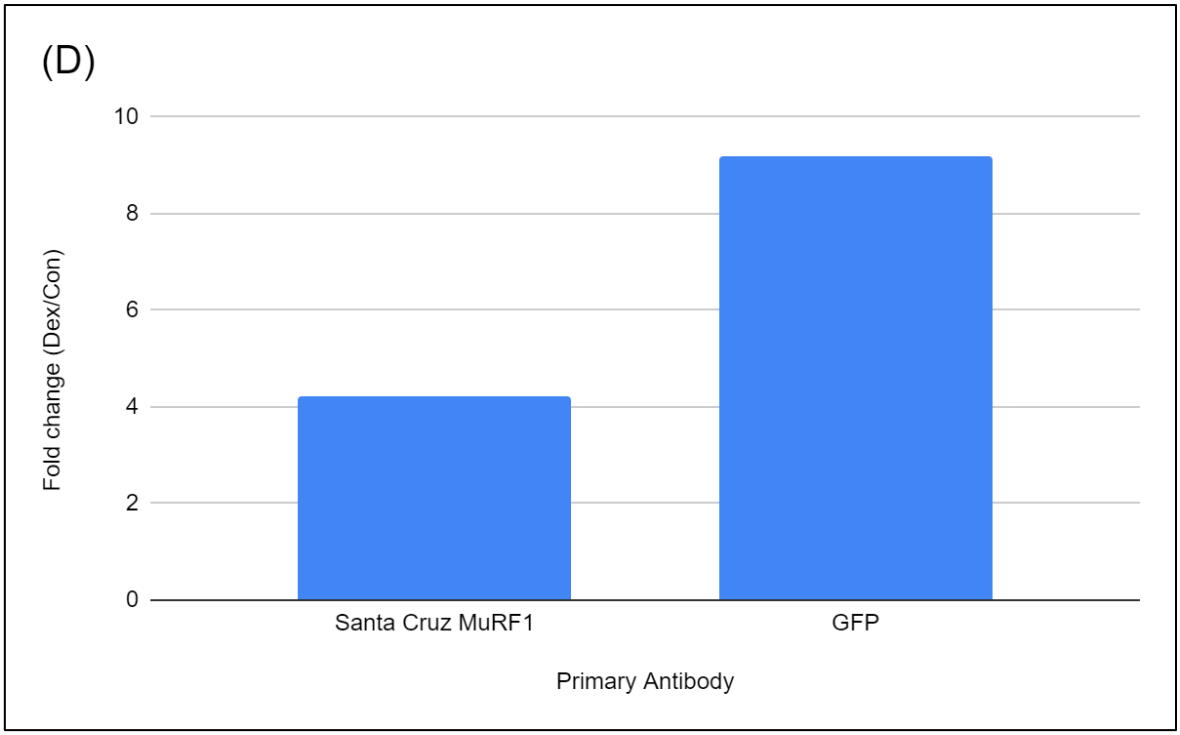


Fig 3.3.2d. Fold change of MuRF1-GFP expression over control (Dex/Con) to compare Santa Cruz MuRF1 and GFP commercial antibodies.

To validate the MuRF1 samples western blots were performed using 2 commercial MuRF1, an MBP, and GFP antibodies. MBP and GFP specific antibodies validate the presence of recombinant MBP-MuRF1, and expression of MuRF1-GFP induced by doxycycline (DOX) but not in control (CON). Detection of cut-MuRF1 at 1ug using the MBP antibody indicate issues with the Cut-MuRF1 samples and were therefore these samples were disregarded.

Proteintech and Santa Cruz MuRF1 antibodies are sensitive to the same concentrations of recombinant MBP-MuRF1 (0.01-1ug). Santa Cruz is more specific to MuRF1 in cell lysate, detecting expressed MuRF1-GFP at >4-fold greater in Dox vs Con. Proteintech is unable to detect overexpressed MuRF1-GFP in cell lysate compared to control (CON). The results of this experiment prompted the use of Santa Cruz MuRF1 antibodies for Western Blot assays moving forward.

3.5 Purified Custom-Made Antibodies Can Detect Recombinant but not Overexpressed Cell Lysate MuRF1

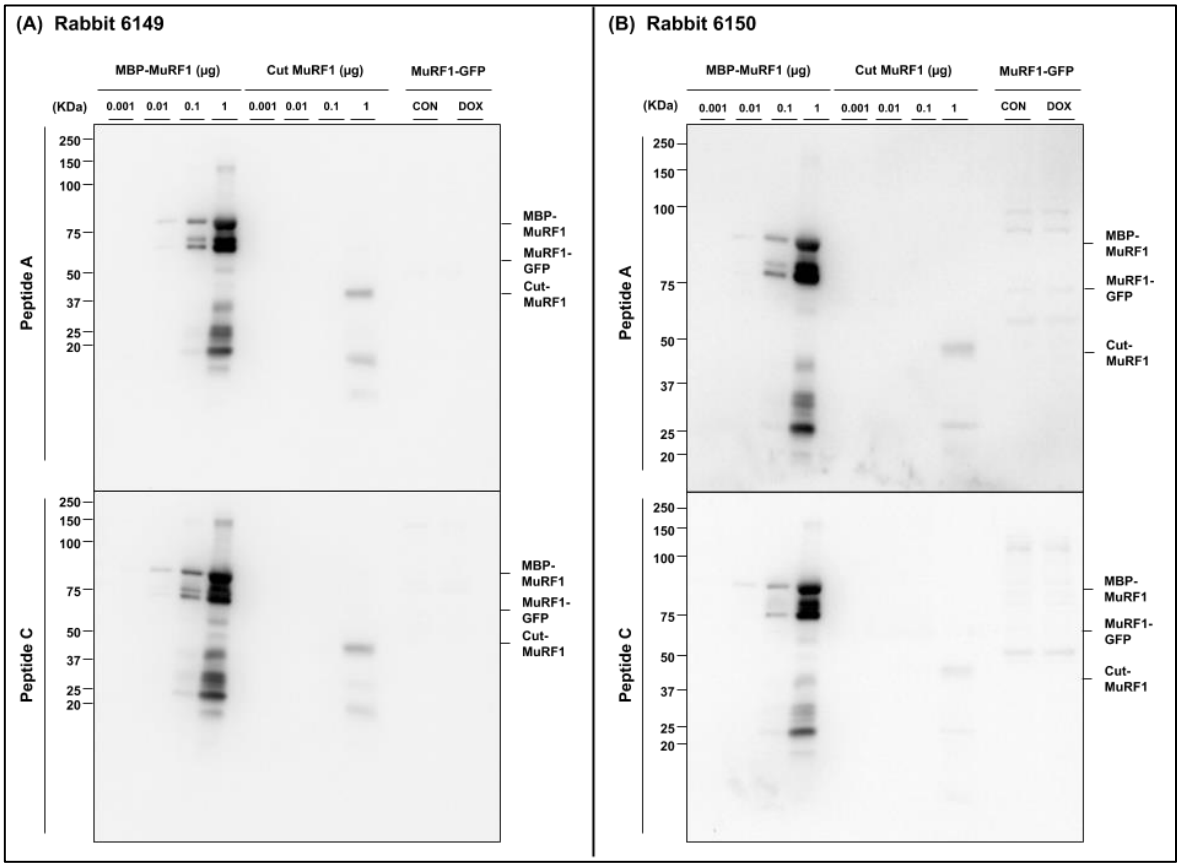


Fig 3.5 Evaluation of purified custom-made antibodies sensitivity and specificity to recombinant MBP-MuRF1 and overexpressed cell MuRF1-GFP. Antibodies from 6149 (A) and 6150 (C) were purified from sera using peptides A and C and used to western blot samples: MBP-MuRF1, Cut-MuRF1 (MBP tag cleaved) and MuRF1-GFP inducible cell lysate with (DOX) and without (CON) doxycycline induced expression

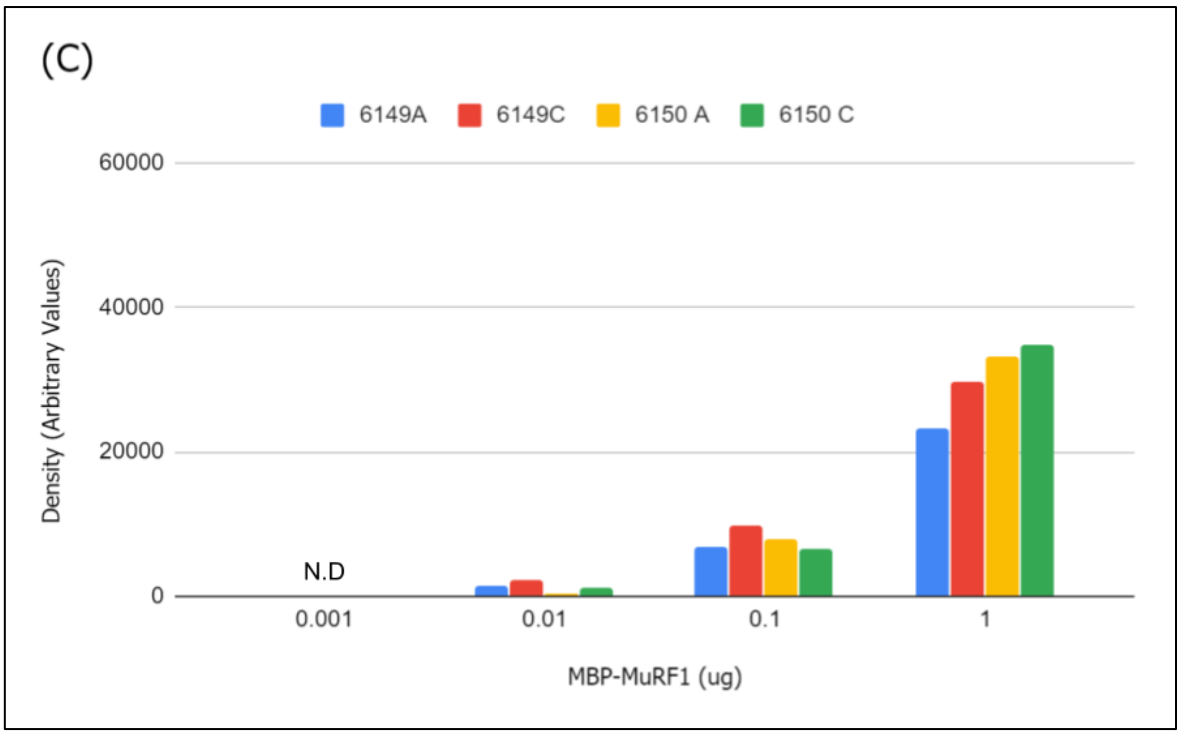


Fig 3.5 Quantification of MBP-MuRF1 band densities from western blots (in Fig 3.5 A&B) comparing different custom-made antibodies. N.D = Not detectable.

Having validated the presence of MuRF1-specific antibodies in the rabbit sera and explored the inter-rabbit variability, MuRF1-specific peptides were used to purify requisite antibodies from the rabbit sera. These were then tested in a western blot using the same recombinant and cell lysate samples from section 3.3.

Both subjects were sensitive from 0.01 to 1ug of MBP-MuRF1 and as with whole sera, neither subjects' antibodies were able to detect MuRF1 in cell lysate.

3.6 Evaluation of IP Efficacy of Commercial and Custom Antibodies to enrich overexpressed MuRF1-GFP from HEK293 cells

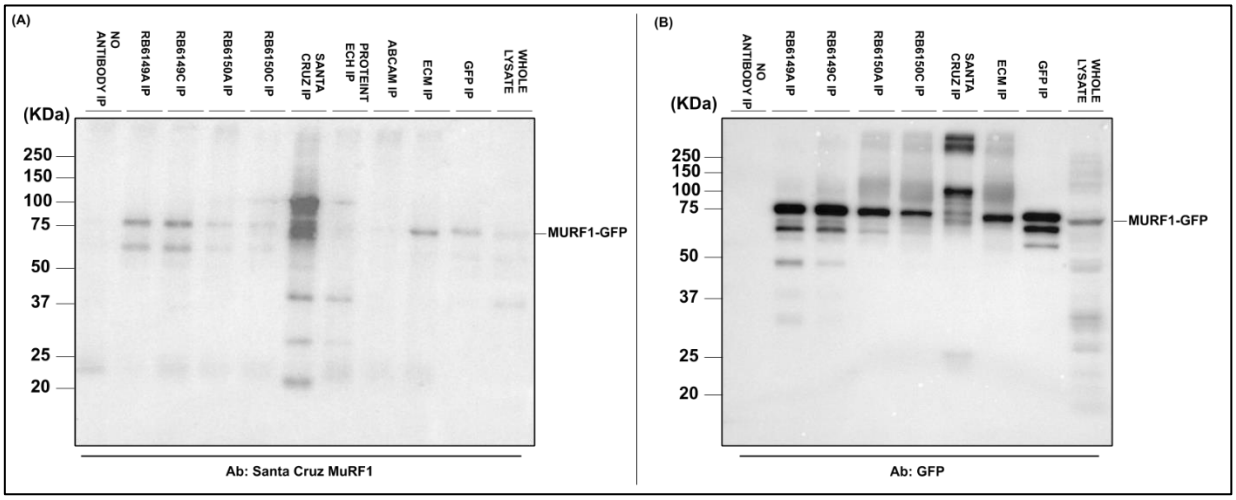


Fig 3.6. Comparison of IP efficacy of different MuRF1 antibodies. Overexpressed HEK293 MuRF1-GFP lysate was subject to IP using different antibodies and western blotted with Santa Cruz MuRF1 antibody (A) and GFP antibody (B)

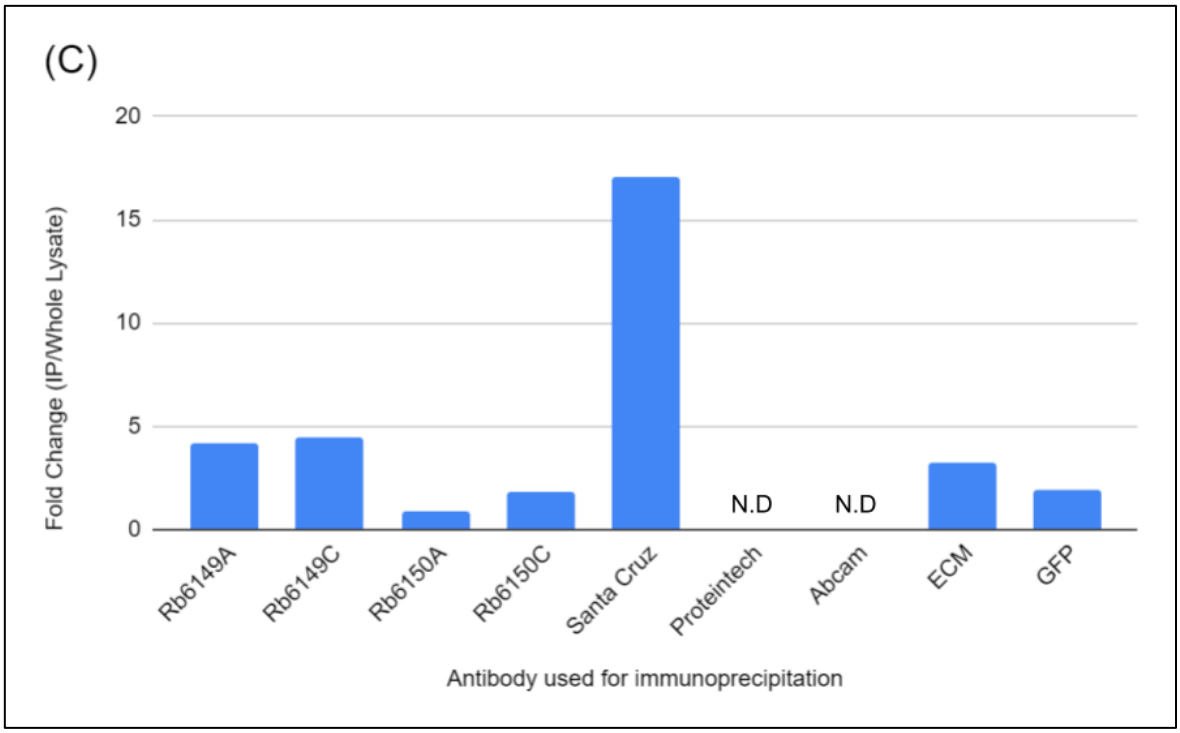


Fig 3.6 C. Quantification of commercial antibody IP efficacy. IP performed using different commercial MuRF1 antibodies and western blotted with

Santa Cruz MuRF1 (Fig 3.6 A). Expressed as fold change of IP over whole lysate. N.D = Not detectable. Note: Santa Cruz MuRF1 is non-discernible from surrounding bands and is therefore disregarded.

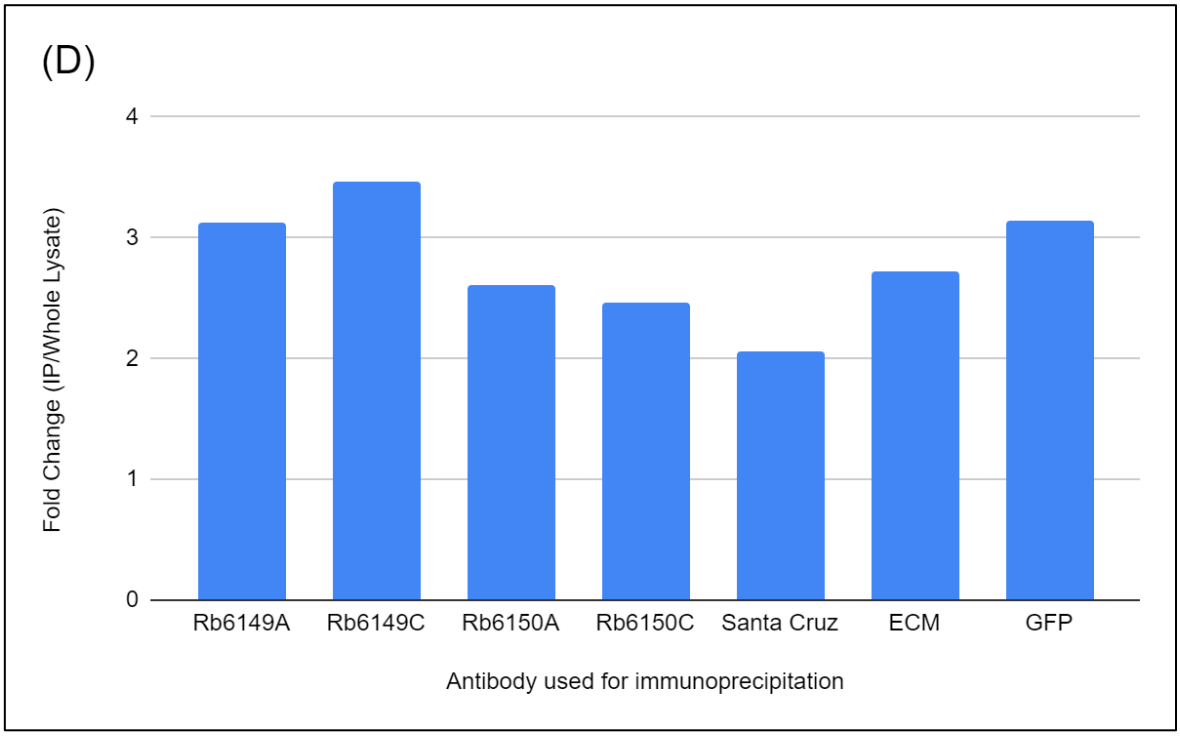


Fig 3.6 D. Quantification of commercial antibody IP efficacy. IP performed using different commercial MuRF1 antibodies and western blotted with GFP (Fig 3.6 B). Expressed as fold change of IP over whole lysate.

Antibodies were then tested for the ability to IP MuRF1-GFP. As Santa Cruz antibodies were the most effective for western blot, this was used as primary antibody to blot the IP samples and GFP was used for validation blots of HEK293 overexpressed MuRF1-GFP lysate.

GFP control blots (B) validate that MuRF1-GFP was present in cell lysate and that IP enriched a greater concentration of MuRF1 GFP from lysate. Abcam and Proteintech antibodies were unable to IP MuRF1-GFP to detectable levels in these

samples (Fig 3.6 C), for this reason these IP samples were removed from future blots.

Santa Cruz IP provides a large concentration of non-specific binding, making the MuRF1-GFP detection indeterminate. ECM and custom-made antibodies were able to IP MuRF1 with Rb6149 enriching the greatest amount of MuRF1-GFP from lysate. The blot using GFP antibody confirms the data provided by the Santa Cruz antibody; This data also shows that Santa Cruz was unable to enrich MuRF1-GFP proteins to a detectable level.

3.7 Neither Custom-Made nor Santa Cruz MuRF1 Antibodies Can Detect MuRF2 or MuRF3 in Western Blot

(A)

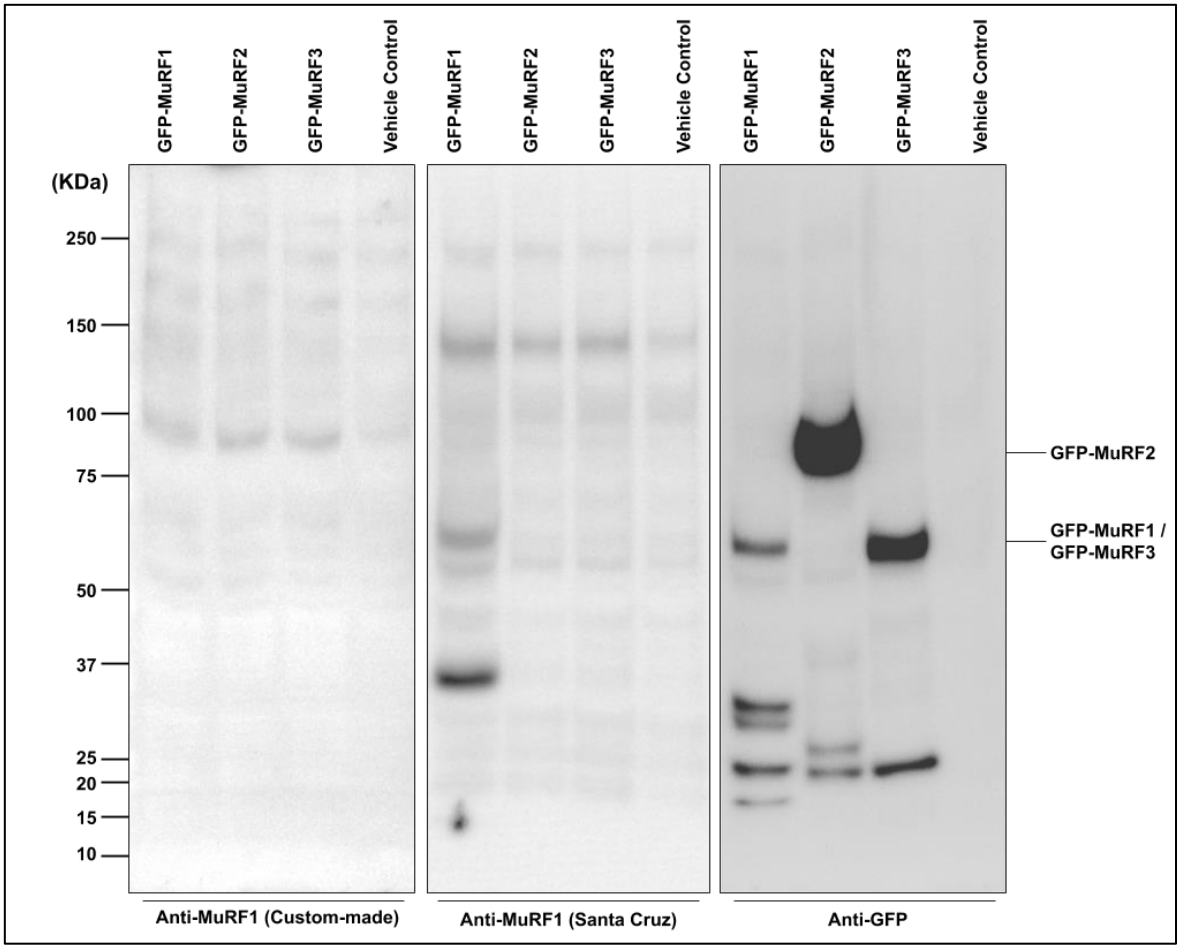
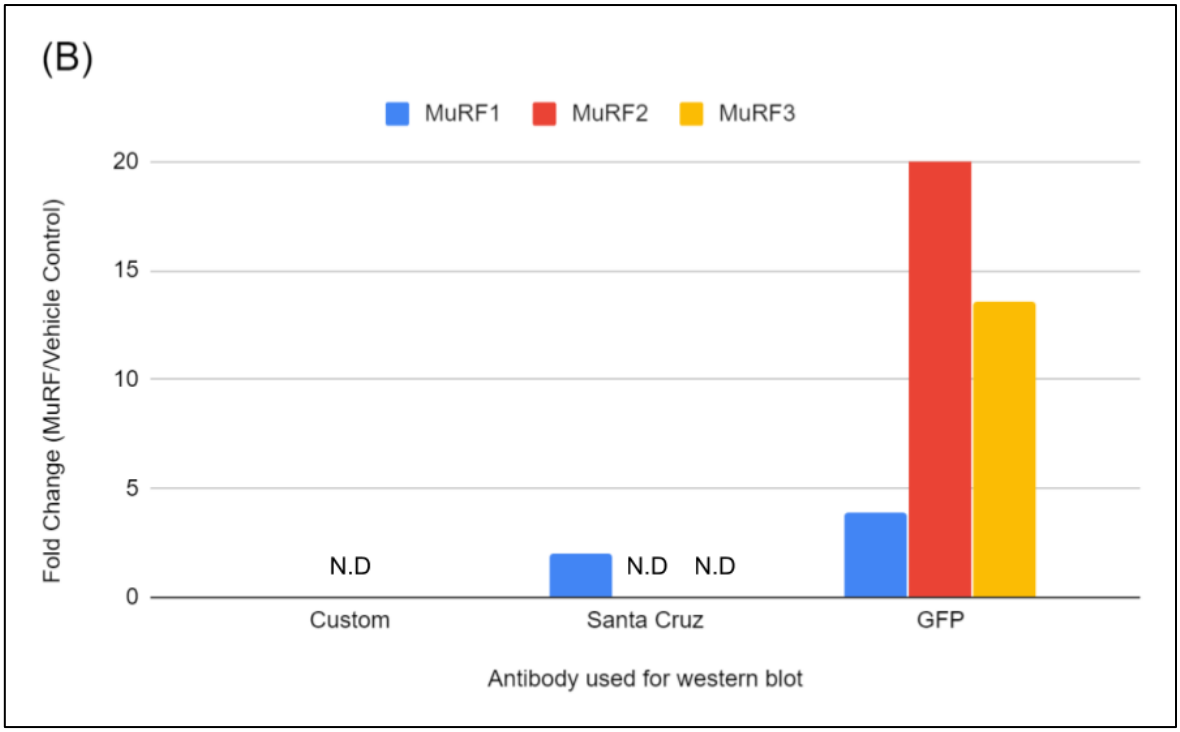


Fig 3.7 A. Comparison of specificity of MuRF1 antibodies versus MuRF2 and MuRF3. HEK293 cell lysate overexpressing MuRF1-GFP, MuRF2-GFP, or MuRF3-GFP were analysed by western blot using GFP, and Santa Cruz MuRF1 antibody.



3.7 B. Quantification of MuRF1 antibodies ability to specifically detect MuRF1 and not MuRF2 or MuRF3. Data expressed as fold change of MuRF protein band over vehicle control.

Following the establishment of the most effective IP and western blot antibodies, the next experiments were designed to test the specificity of these antibodies by using samples containing other MuRF family proteins - MuRF2 and MuRF3. HEK293 cells transiently transfected with MuRF1-GFP, MuRF2-GFP, and MuRF-3 GFP plasmids were used.

The GFP control blot revealed that there was expression of the 3 proteins at the weights expected and there were no detectable proteins found in the non-transfected control sample. Blotting of these whole cell lysates revealed similar results to previous cell lysate blots; custom rabbit antibodies were unable to detect MuRF1 in any of the cell lysate samples, but Santa Cruz was able to detect MuRF1-GFP. Neither MuRF1 antibody was able to detect proteins at the expected weights

for MuRF2-GFP or MuRF3-GFP Santa Cruz was able to detect MuRF1-GFP in cell lysate.

3.8 IP Using Custom-Made Antibody Only Enrich MuRF1, and not MuRF2 and MuRF3.

(A)

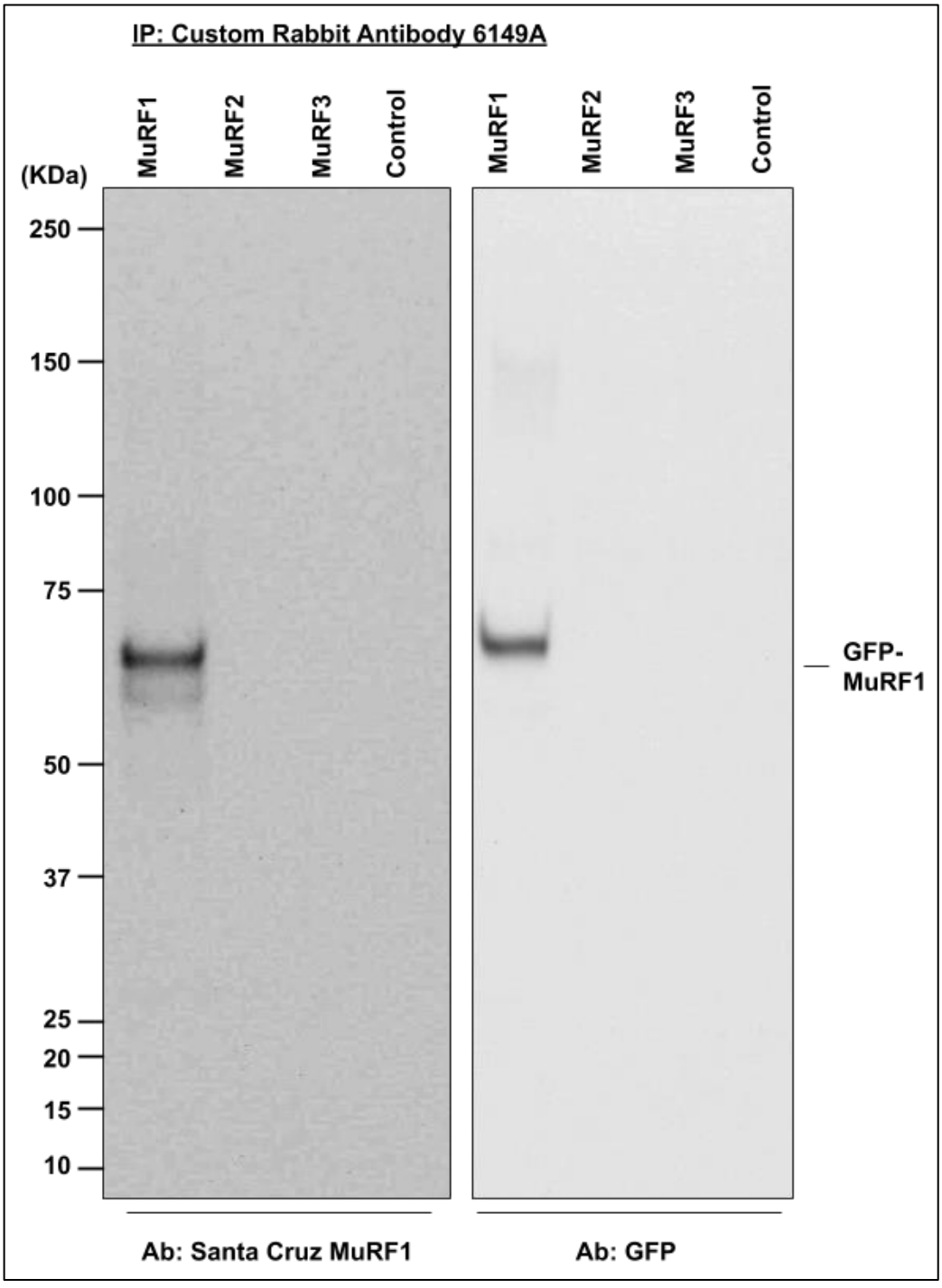


Fig 3.8 A. Comparison of specificity of custom antibody IP followed by blotting with Santa Cruz. HEK293 cell lysate overexpressing MuRF1-GFP,

MuRF2-GFP, or MuRF3-GFP were enriched using IP with custom-made antibodies and analysed by western blot using Santa Cruz MuRF1, and GFP antibodies.

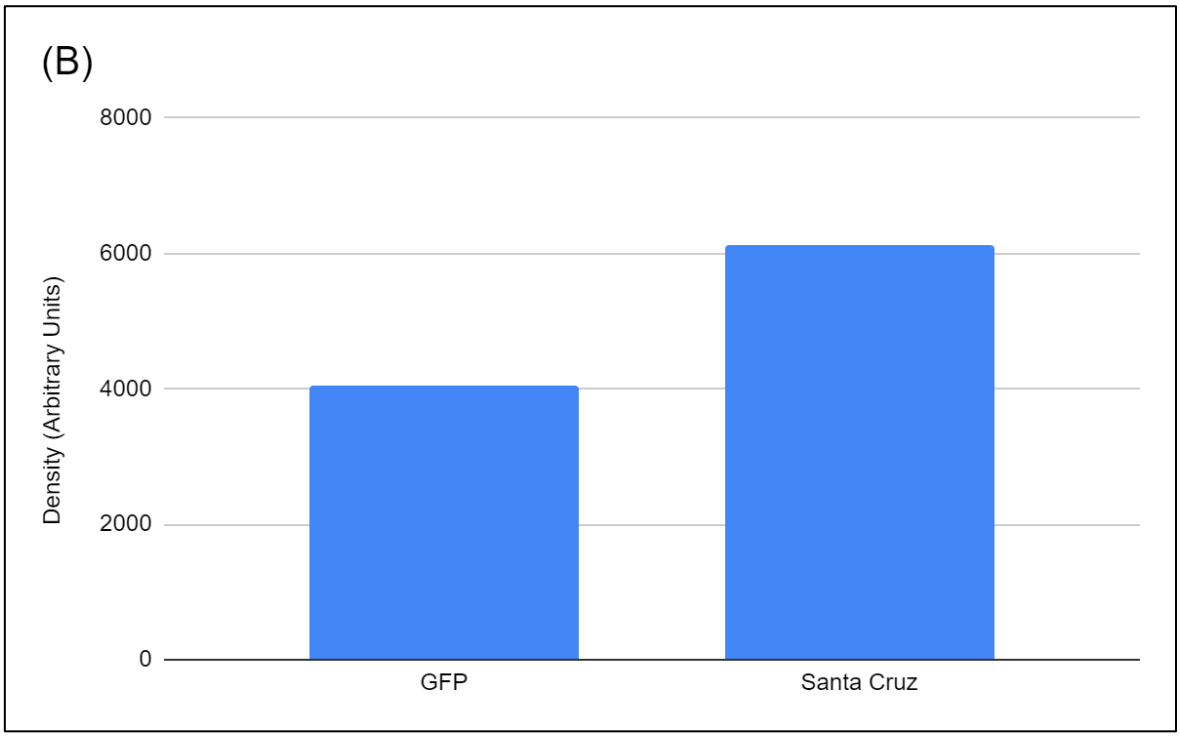


Fig 3.8 B. Quantification of western blot (Fig 3.7 A) evaluating the effectiveness of Santa Cruz MuRF1 antibody to detect GFP-MuRF1 immunoprecipitated by custom-made antibodies.

The final experiment was to test if the custom antibody was specific to MuRF1 in performing IP. Custom-made MuRF1 antibody (6149A) was used to IP cell lysate from the previous experiment 3.7. This was followed by western blot with Santa Cruz MuRF1 and GFP antibodies.

This data is very clear with no detectable bands in the control, MuRF2, or MuRF3 lanes when blotting with GFP or Santa Cruz antibodies; MuRF1 was successfully enriched and able to be blotted when using this antibody protocol. This data

conclusively demonstrates that using custom antibodies to IP MuRF1 and blotting with Santa Cruz is the most effective method for detecting MuRF1 in cell lysate.

4. Discussion

4.1 - Conclusions of Findings

In this study a custom-made MuRF1 antibody was characterised and found to be inferior to commercial antibodies in detecting MuRF1 by western blot. However, the custom-made antibodies provided superior IP ability over commercial antibodies. This knowledge sets a standard for western blotting MuRF1 and provides the ability to enrich MuRF1 from samples and explore in-vivo and cell culture exactly the mechanisms in how it functions.

4.2 Analysis of Findings

The main finding of this experiment is the high efficacy of using a custom-made antibody IP MuRF1 from cell lysate samples. Additionally, using the MuRF1 antibody from Santa Cruz for western blotting was most effective. Interestingly the inverse use of these techniques (Santa Cruz IP and Custom-made western blot) provided no useful data on MuRF1 protein abundance. This could be explained by the nature of antibody-antigen interactions. Antibodies interacting with a target protein is driven by the antigen-antibody complex that is formed. The size of an antigen can vary and if the area is small then it can become buried in the 3D structure of a large protein, making the antigen inaccessible to the antibody (Wilson and Stanfield, 1993). During a western blot the protein is denatured, changing its structure prior to applying the antibody. The denaturing unfolds the protein, making it more linear, and thereby offering a different range of potential antigens. In an IP the protein has not been subject to denaturing conditions and therefore has a different structure to that of a western blot prepared sample. This difference in

structure would account for the variance in efficacy of the same antibody used across these different techniques.

4.3 Implications of findings

With the tools of IP and western blot developed in this study, it is now possible to validate previous research using mRNA expression, then translate this to protein expression. We can also now use this characterised antibody that can IP to study protein-protein interactions and deploy more comprehensive characterisation of MuRF1. This will benefit this field to better understand how MuRF1 functions in muscle atrophy.

4.4 Further Study

Further research can be conducted on other antibody-based techniques for MuRF1 such as enzyme-linked immunosorbent assays (ELISA) and immunohistochemistry. With regards to the characterisation of MuRF1 antibodies, we would need to perform IP using Santa Cruz on MuRF1-GFP and analyse the bands above the expected weight for MuRF1-GFP. This can be completed by running an SDS-page gel, extracting these bands, and performing mass-spectrometry proteomics to identify if these bands are MuRF1. If these are modified MuRF1, then identification of modifications or protein interactions can be identified.

To exploring MuRF1 further using these tools, there are several broad areas that can be explored:

E2 ubiquitin-conjugating enzymes

The use of tools developed in this paper, it is now possible to fully elucidate the E2 interactions that facilitate MuRF1 ubiquitination and proteolysis. As an E3 Ligase,

MuRF1 requires interaction with E2's to facilitate ubiquitin transfer to the substrate. Currently, there are no data published on the interaction of MuRF1 with different E2's and what effect this has on the ubiquitination function of MuRF1. Fletcher et al (2015) identified TRIM5α (MuRF1 is alternatively named Trim63 and is in the same family of genes as TRIM5α), an E3 ubiquitin ligase responsible for ubiquitinating retroviral capsids (providing an antiviral mechanism within cells) and wanted to identify the enzymology of this E3 ligase. Using Ha-tagged TRIM5α they were able to demonstrate a 2-step process in which UBE2W mono-ubiquitinates TRIM5α and then UBE2N+V1 causes anchored polyubiquitin chains to form. Preliminary data from our lab suggests MuRF1 reacts similarly with UBE2W and UBE2N+V1 in-vitro. As MuRF1 is an analogue of TRIM5α it will be possible to explore similarly the interactions and types of ubiquitination that MuRF1 mediates and then using the IP method outlined it will be possible to explore this mechanism in cell – Following in-vitro E2 characterisation, one can then systematically knockout E2s in the cell, induce expression of MuRF1, and measure proteasomal activity or breakdown to confirm if this mechanism is physiologically relevant.

Substrates

The use of IP in-vivo or cell culture can be used to explore MuRF1-Substrate interaction.

MuRF1 will ultimately interact with a substrate that is destined for degradation via ubiquitination. Mrosek et al (2007) performed meticulous research into the structure of MuRF1 and its interaction with the sarcomeric protein titin. Here they were able to isolate the specific 4-residue sequence in which titin associates to MuRF1. After isolating the domains of titin that bind to MuRF1 they then used X-ray

crystallography to visually observe their interaction. This type of research is crucial to fully understanding the 3d structure of proteins, however, with the use of IP of MuRF1 the same conclusion could be made using mass-spectrometry proteomics followed by strategic mutation of interacting residues.

E3 Ligases

MuRF1 is not the only E3 ligase implicated in atrophy. MuRF2 (Witt, 2008), MuRF3 (Bodine, 2014), MAFBx (Bodine, 2001), UBR5 (Seaborne, 2019), DCAF8 (Nowak, 2019) and TRIM72 (Manring, 2016) are all implicated in skeletal muscle atrophy but little research has been conducted into any potential interactive or cumulative effect of these ligases.

With the ability to IP MuRF1 effectively, it will now be possible to enrich MuRF1 from different sample types and, using techniques such as mass-spectrometry proteomics, identify any potential E3 interaction, cooperative effect, or independent function and design experiments to explore the impact of these ligases.

Post-translational modifications

Once the levels of protein expression and the impact of the difference of protein expression is elucidated. Then the next stage to measuring MuRF1 would be to investigate if the effect of MuRF1 can be modulated via post-translational modification. For example, MuRF1 must be degraded in some manner and may therefore require ubiquitination – How and when this occurs could provide a key therapeutic tool in treating atrophy driven by upregulation of MuRF1 by increasing its own degradation, rather than impacting expression.

Characterisation of E3 Ligases

Exploration of the mechanism of MuRF1 may also parse out any difference of mechanisms in different tissues. An interesting one of which would be cardiac versus skeletal muscle; following Witt et al (2007) experiments on MuRF1 knockout, it was shown that a detrimental side-effect of MuRF1 ablation was the hypertrophying of the heart. If it was the case that the mechanism is different between cardiac and skeletal muscle, it would provide a new avenue of therapy to target skeletal MuRF1 induced muscle atrophy only and not heart muscle MuRF1.An example of another E3 Ligase, Parkin, was studied by Matsuda et al (2005) - They were able to characterise ubiquitination, showing exactly which E2 instigates substrate ubiquitination, and which Parkin domains were essential for interaction with their substrate, and the ability to ubiquitinate proximal proteins as pseudo-substrates. As Parkin is an analogue of MuRF1 this experiment could easily be repeated on MuRF1 using an effective antibody for immunoblotting.

4.5 Final Conclusion

In conclusion, we identified antibodies that were able to perform western blot and IP of MuRF1, obtain reliable results, this will open many opportunities to further explore a protein implicated in skeletal muscle atrophy, it's mechanisms, and potential treatment. This has exciting implications for the understanding of muscle pathologies, offering therapeutic treatments for cancer, ageing, diabetes, sepsis, and myopathies. This will increase the health and longevity of a large population of people and relieve burden from the healthcare system.

References

Adams, V. *et al.* (2007) ‘Myocardial expression of Murf-1 and MAFbx after induction of chronic heart failure: Effect on myocardial contractility’, *Cardiovascular Research*, 73(1), pp. 120–129. doi: 10.1016/j.cardiores.2006.10.026.

Altun, M. *et al.* (2010) ‘Muscle wasting in aged, sarcopenic rats is associated with enhanced activity of the ubiquitin proteasome pathway’, *Journal of Biological Chemistry*, 285(51), pp. 39597–39608. doi: 10.1074/jbc.M110.129718.

Argilés, J. M. *et al.* (2010) ‘Optimal management of cancer anorexia-cachexia syndrome’, *Cancer Management and Research*, 2(1), pp. 27–38.

Attaix, D. *et al.* (2008) ‘Role of the ubiquitin-proteasome pathway in muscle atrophy in cachexia.’, *Current opinion in supportive and palliative care*, 2(4), pp. 262–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19069311.

Baehr, L. M., Furlow, J. D. and Bodine, S. C. (2011) ‘Muscle sparing in muscle RING finger 1 null mice: Response to synthetic glucocorticoids’, *Journal of Physiology*, 589(19), pp. 4759–4776. doi: 10.1113/jphysiol.2011.212845.

Baracos, V. E., Mazurak, V. C. and Bhullar, A. S. (2019) ‘Cancer cachexia is defined by an ongoing loss of skeletal muscle mass’, *Annals of palliative medicine*, 8(1), pp. 3–12. doi: 10.21037/apm.2018.12.01.

Bodine, S. C. *et al.* (2001) ‘Identification of ubiquitin ligases required for skeletal Muscle Atrophy’, *Science*, 294(5547), pp. 1704–1708. doi: 10.1126/science.1065874.

Bodine, S. C. and Baehr, L. M. (2014) ‘Skeletal muscle atrophy and the E3 ubiquitin ligases MuRF1 and MAFbx/atrogin-1’, *American Journal of Physiology - Endocrinology and Metabolism*, 307(6), pp. E469–E484. doi: 10.1152/ajpendo.00204.2014.

de Boer, M. D. *et al.* (2007) ‘The temporal responses of protein synthesis, gene expression and cell signalling in human quadriceps muscle and patellar tendon to disuse’, *Journal of Physiology*, 585(1), pp. 241–251. doi: 10.1113/jphysiol.2007.142828.

Bonaldo, P. and Sandri, M. (2013) ‘Cellular and molecular mechanisms of muscle atrophy’, *Disease Models & Mechanisms*, 6(1), pp. 25–39. doi: 10.1242/dmm.010389.

Bordeaux, J. *et al.* (2010) ‘Antibody validation’, *BioTechniques*, 48(3), pp. 197–209. doi: 10.2144/000113382.

Bowen, T. S. *et al.* (2017) ‘Small-molecule inhibition of MuRF1 attenuates skeletal muscle atrophy and dysfunction in cardiac cachexia’, *Journal of Cachexia, Sarcopenia and Muscle*, 8(6), pp. 939–953. doi: 10.1002/jcsm.12233.

Centner, T. *et al.* (2001) ‘Identification of muscle specific ring finger proteins as potential regulators of the titin kinase domain’, *Journal of Molecular Biology*, 306(4), pp. 717–726. doi: 10.1006/jmbi.2001.4448.

Dewys, W. D. *et al.* (1980) ‘Prognostic effect of weight loss prior tochemotherapy in cancer patients’, *The American Journal of Medicine*, 69(4), pp. 491–497. doi: 10.1016/S0149-2918(05)80001-3.

Fareed, M. U. *et al.* (2006) ‘Treatment of rats with calpain inhibitors prevents sepsis-induced muscle proteolysis independent of atrogin-1/MAFbx and MuRF1 expression’, *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, 290(6), pp. 1589–1597. doi: 10.1152/ajpregu.00668.2005.

Haas, A. L. and Rose, I. A. (1982) ‘The mechanism of ubiquitin activating enzyme. A kinetic and equilibrium analysis.’, *The Journal of biological chemistry*, 257(17),

pp. 10329–37. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6286650.

Handley, P. M. *et al.* (1991) ‘Molecular cloning, sequence, and tissue distribution of the human ubiquitin-activating enzyme E1’, *Proceedings of the National Academy of Sciences of the United States of America*, 88(1), pp. 258–262. doi: 10.1073/pnas.88.1.258.

Hanson, A. M. *et al.* (2013) ‘Longitudinal characterization of functional, morphologic, and biochemical adaptations in mouse skeletal muscle with hindlimb suspension’, *Muscle and Nerve*, 48(3), pp. 393–402. doi: 10.1002/mus.23753.

Houston, F. E., Hain, B. A. and Dodd, S. L. (2018) ‘Inhibition of the proteasome partially attenuates atrophy in botulinum neurotoxin treated skeletal muscle’, *Toxicon*. Elsevier Ltd, 144, pp. 48–54. doi: 10.1016/j.toxicon.2018.01.023.

Komander, D. (2009) ‘The emerging complexity of protein ubiquitination’, *Biochemical Society Transactions*, 37(5), pp. 937–953. doi: 10.1042/BST0370937.

Labeit, S. *et al.* (2010) ‘Modulation of muscle atrophy, fatigue and MLC phosphorylation by MuRF1 as indicated by hindlimb suspension studies on MuRF1-KO mice’, *Journal of Biomedicine and Biotechnology*, 2010. doi: 10.1155/2010/693741.

Lecker, S. H. *et al.* (1999) ‘Muscle Protein Breakdown and the Critical Role of the Ubiquitin-Proteasome Pathway in Normal and Disease States’, *The Journal of Nutrition*, 129(1), pp. 227S-237S. doi: 10.1093/jn/129.1.227s.

Lecker, S. H. *et al.* (2004) ‘Multiple types of skeletal muscle atrophy involve a common program of changes in gene expression’, *FASEB Journal*, 18(1), pp. 39–51. doi: 10.1096/fj.03-0610com.

Lomonosova, Y. N., Shenkman, B. S. and Nemirovskaya, T. L. (2012) ‘Attenuation of unloading-induced rat soleus atrophy with the heat-shock protein inducer 17-

(allylamino)-17-demethoxygeldanamycin', *FASEB Journal*, 26(10), pp. 4295–4301. doi: 10.1096/fj.12-204412.

Marty, E. *et al.* (2017) 'A review of sarcopenia: Enhancing awareness of an increasingly prevalent disease', *Bone*. Elsevier Inc., 105, pp. 276–286. doi: 10.1016/j.bone.2017.09.008.

Mearini, G. *et al.* (2010) 'Atrogin-1 and MuRF1 regulate cardiac MyBP-C levels via different mechanisms', *Cardiovascular Research*, 85(2), pp. 357–366. doi: 10.1093/cvr/cvp348.

Metter, E. J. *et al.* (2002) 'Skeletal muscle strength as a predictor of all-cause mortality in healthy men', *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 57(10), pp. B359–B365. doi: 10.1093/gerona/57.10.B359.

Morreale, F. E. and Walden, H. (2016) 'Types of Ubiquitin Ligases', *Cell*. Elsevier, 165(1), pp. 248-248.e1. doi: 10.1016/j.cell.2016.03.003.

NIKAWA, T. *et al.* (2004) 'Skeletal muscle gene expression in space-flown rats', *The FASEB Journal*, 18(3), pp. 522–524. doi: 10.1096/fj.03-0419fje.

Nowak, M. *et al.* (2019) 'DCAF8, a novel MuRF1 interaction partner, promotes muscle atrophy', *Journal of Cell Science*, (September), p. jcs.233395. doi: 10.1242/jcs.233395.

Oakley, R. H. *et al.* (2018) 'Muscle-specific regulation of right ventricular transcriptional responses to chronic hypoxia-induced hypertrophy by the muscle ring finger-1 (MuRF1) ubiquitin ligase in mice', *BMC Medical Genetics*. BMC Medical Genetics, 19(1), pp. 1–17. doi: 10.1186/s12881-018-0670-1.

Ohtake, F. *et al.* (2018) 'K63 ubiquitylation triggers proteasomal degradation by seeding branched ubiquitin chains', *Proceedings of the National Academy of Sciences of the United States of America*, 115(7), pp. E1401–E1408. doi:

10.1073/pnas.1716673115.

Rajsbaum, R. and Bharaj, P. (2016) ‘Unanchored Polyubiquitin Chains and Innate Immune Sensing of Influenza Virus Infection’, *The Journal of Immunology*, 196(1 Supplement), pp. 63.1 LP-63.1. Available at: http://www.jimmunol.org/content/196/1_Supplement/63.1.abstract.

Raue, U. *et al.* (2007) ‘Proteolytic gene expression differs at rest and after resistance exercise between young and old women’, *Journals of Gerontology - Series A Biological Sciences and Medical Sciences*, 62(12), pp. 1407–1412. doi: 10.1093/gerona/62.12.1407.

Sartorelli, V. and Fulco, M. (2004) ‘Molecular and cellular determinants of skeletal muscle atrophy and hypertrophy.’, *Science’s STKE : signal transduction knowledge environment*, 2004(244), pp. 1–10.

Seaborne, R. A. *et al.* (2019) ‘UBR5 is a novel E3 ubiquitin ligase involved in skeletal muscle hypertrophy and recovery from atrophy’, *The Journal of Physiology*, 597(14), pp. 3727–3749. doi: 10.1113/JP278073.

Spencer, J. A. *et al.* (2000) ‘Regulation of microtubule dynamics and myogenic differentiation by MURF, a striated muscle RING-finger protein’, *Journal of Cell Biology*, 150(4), pp. 771–784. doi: 10.1083/jcb.150.4.771.

Taillandier, D. *et al.* (2003) ‘Regulation of proteolysis during reloading of the unweighted soleus muscle’, *International Journal of Biochemistry and Cell Biology*, 35(5), pp. 665–675. doi: 10.1016/S1357-2725(03)00004-9.

Tiao, G. *et al.* (1997) ‘Sepsis is associated with increased mRNAs of the ubiquitin-proteasome proteolytic pathway in human skeletal muscle’, *Journal of Clinical Investigation*, 99(2), pp. 163–168. doi: 10.1172/JCI119143.

Trier, N., Hansen, P. and Houen, G. (2019) ‘Peptides, antibodies, peptide

antibodies and more', *International Journal of Molecular Sciences*, 20(24), pp. 1–22. doi: 10.3390/ijms20246289.

Uhlen, M. *et al.* (2016) 'A proposal for validation of antibodies', *Nature Methods*. Nature Publishing Group, 13(10), pp. 823–827. doi: 10.1038/nmeth.3995.

Urso, M. L. *et al.* (2007) 'Alterations in mRNA expression and protein products following spinal cord injury in humans', *Journal of Physiology*, 579(3), pp. 877–892. doi: 10.1113/jphysiol.2006.118042.

Van Wijk, S. J. L. and Timmers, H. T. M. (2010) 'The family of ubiquitin-conjugating enzymes (E2s): Deciding between life and death of proteins', *FASEB Journal*, 24(4), pp. 981–993. doi: 10.1096/fj.09-136259.

Willis, M. S. *et al.* (2009) 'Cardiac Muscle Ring Finger-1 Increases Susceptibility to Heart Failure In Vivo', *Circulation Research*, 105(1), pp. 80–88. doi: 10.1161/CIRCRESAHA.109.194928.

Wilson, A. and Stanfield, R. L. (1993) 'Antibody-antigen interactions', pp. 113–118.

Wing, S. S., Haas, A. L. and Goldberg, A. L. (1995) 'Increase in ubiquitin-protein conjugates concomitant with the increase in proteolysis in rat skeletal muscle during starvation and atrophy denervation', *Biochemical Journal*, 307(3), pp. 639–645. doi: 10.1042/bj3070639.

Witt, C. C. *et al.* (2008) 'Cooperative control of striated muscle mass and metabolism by MuRF1 and MuRF2', *EMBO Journal*, 27(2), pp. 350–360. doi: 10.1038/sj.emboj.7601952.

Witt, S. H. *et al.* (2005) 'MURF-1 and MURF-2 target a specific subset of myofibrillar proteins redundantly: Towards understanding MURF-dependent muscle ubiquitination', *Journal of Molecular Biology*, 350(4), pp. 713–722. doi: 10.1016/j.jmb.2005.05.021.

Xu, P. *et al.* (2009) ‘Quantitative Proteomics Reveals the Function of Unconventional Ubiquitin Chains in Proteasomal Degradation’, *Cell*, 137(1), pp. 133–145. doi: 10.1016/j.cell.2009.01.041.