Muscle development and function in a *Magel2* mouse model of Prader-Willi syndrome

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BIO 489: Independent Research

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ABSTRACT

Background: Prader-Willi syndrome (PWS) is a multigene disorder commonly associated with hyperphagia and obesity. Children with PWS have increased fat mass and decreased lean mass before the onset of hyperphagia and obesity. Severe hypotonia and reduced muscle strength are typically present in PWS infants. Inactivating mutations in one PWS candidate gene, *MAGEL2*, cause a Prader-Willi-like syndrome (Schaaf-Yang syndrome) with neonatal hypotonia and joint contractures, highlighting the importance of loss of *MAGEL2* in PWS phenotypes. The process of autophagy is essential in maintaining musculoskeletal homeostasis, and increased or decreased autophagy can lead to muscle atrophy. Autophagic markers such as ubiquitin (Ub), p62/SQSTM1 (p62) and microtubule associated protein 1-light chain 3 (LC3) are hypothesized to be connected to the MAGE family of proteins that includes MAGEL2, suggesting that loss of *MAGEL2* could modulate autophagy in muscle. **Methods and Results:** Expression of *Magel2* was detected in the murine nervous system and in developing muscle, connective tissue and bone. Neonatal mice lacking Magel2 had reduced muscle mass, increased fat mass and decreased bone mass compared to wild-type mice. Immunohistochemistry (IHC), and immunoblotting were used to determine whether loss of *Magel2* affects the accumulation of autophagic markers or expression of genes associated with muscle atrophy, in neonatal mice. The p62 positive aggregates were increased in muscle from Magel2 mice and atrophy genes were up regulated. **Conclusions:** Abnormal autophagic processes likely contribute to muscle atrophy in mice lacking Magel2. Further studies are needed to determine how the inactivation of *Magel2* causes muscle phenotypes at a cellular level. Our mouse strain carrying loss of *Magel2* provides a model for muscular dysfunction in Prader-Willi syndrome. **Summary:** One of the symptoms of PWS is poor muscle tone, which could be caused by disruption in the autophagy process that regulates skeletal muscle. Autophagy is disrupted and atrophy is increased in muscle from mice lacking Magel2, one of the PWS candidate genes. We propose a mechanism by which loss of MAGEL2 causes hypotonia in children with PWS.

INTRODUCTION

Prader-Willi Syndrome (PWS) was first described in 1956 by Andréa Prader, Alexis Labheart and Heinrich Willi as a genetic disorder affecting multiple body systems caused by the incorrect function of genes on chromosome 15q11-q13 (Cassidy 1997) (Figure 1).

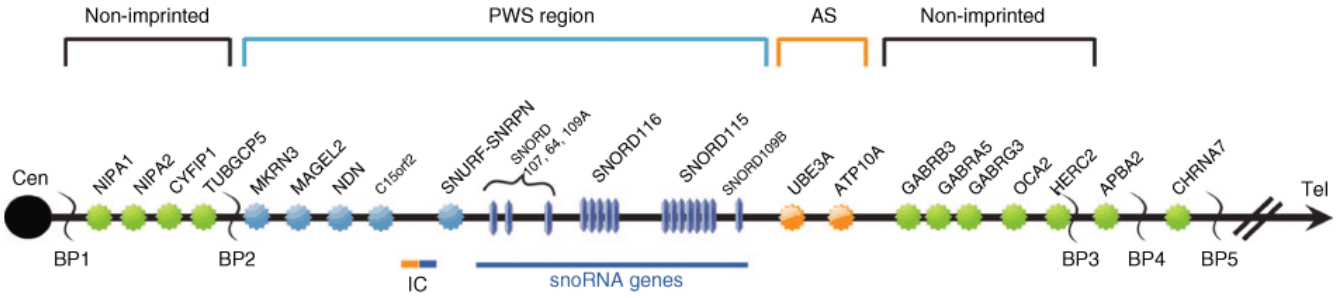


Figure 1: Summary of genetic map of 15q11-q13. The Prader-Willi syndrome (PWS) region (blue) has five paternal-only expressed unique copy genes (*MKRN3*, *MAGEL2*, *NECDIN*, and *SNURF-SNRPN*) and a family of six paternal-only expressed snoRNA genes (http://www.nature.com/gim/journal/v14/n1/full/gim0b013e31822bead0a.html).

The phenotypic expression of PWS is characterized by hypotonia, hypogonadism, hyperphagia and obesity. Other forms of phenotypic expression involve a dysmorphic appearance, short stature, development delay and behavioural issues. One of the candidate genes, *MAGEL2,* and its murine homologue, *Magel2*, have paternal allele-specific expression (Boccaccio and others 1999, Lee and others 2000). Inactivating mutations in *MAGEL2*, cause a Prader-Willi-like syndrome known as Schaaf-Yang syndrome, that results in neonatal hypotonia and joint contractures, which highlights the importance of loss of *MAGEL2* in PWS phenotypes. Schaaf-Yang Syndrome is caused by truncating point mutations of the paternally inherited allele of *MAGEL2* (Fountain and Schaaf 2016). It was discovered when four patients display phenotypic features of PWS including neonatal hypotonia, feeding difficulties, weight gain, developmental delay and hypogonadism, but tested negative for PWS through standard molecular diagnostic testing (Schaaf and others 2013, Fountain and Schaaf 2016). Additional studies have identified more cases of point mutations of *MAGEL2*. A recent study by Soden and others in 2014, identified two more patients, sisters, who displayed decreased fetal movement,

neonatal hypotonia, feeding difficulties, developmental delay, and intellectual disability, but not hyperphagia, obesity and PWS facial features. The sisters were diagnosed with autism spectrum disorder and exhibited joint contractures of the hands. Another study by Mejlachowicz and others in 2015, found an additional three cases, three fetal siblings, who showed joint contractures and decreased fetal movement. All of the additional cases contained point mutations of the paternally inherited *MAGEL2*. *MAGEL2* codes for the MAGEL2 protein that is part of the MAGE, melanoma antigen gene expression, family of proteins. MAGEL2 is expressed in fetal and adult brain cells and is important in neural differentiation. MAGE proteins are believed to form a complex with E3 ubiquitin ligases, leading to the impression that they are involved protein trafficking involving ubiquitination (Doyle and others 2010, Hao and others 2013).

The phenotypes of mice lacking Magel2 mostly reflect the phenotypes displayed in human patients with PWS. Inactivation of the murine Magel2 causes growth abnormalities similar to PWS (Bischof and others 2007). An imprinted copy of Magel2 regulates circadian rhythm (Koslov and others 2007). Magel2-null mice have been shown to have altered serotonin neurochemistry and reduced regions of brain volume resulting in abnormal behaviour (Mercer and others 2009). Loss of Magel2 has also been connected to the impairment of reproductive function in mice (Mercer and Wevrick 2009). Lack of Magel2 has also been connected to impaired hypothalamic regulation of endocrine function and the progressive postnatal decrease in leptin sensitivity of arcuate hypothalamic neurons (Tennese and Wevrick 2011, Pravdivyi and others 2015).

Autophagy, sometimes known as macroautophagy, can be described as a non-selective degradative pathway in eukaryotic cells (Meijer and Codgno 2004). The process begins in the cytoplasm with the formation of an autophagosome that then merges with the (endo) lysosome to form the autolysosome. Autophagy works to maintain cell homeostasis; specifically controlling the amount of protein (Mortimore and Schworer 1977). It can be stimulated as a response to different kinds of stress, specifically starvation, change in cell volume, high levels of misfolded proteins, oxidative stress

and hormonal signaling (Meijer and Codgno 2004). When autophagy occurs the resulting amino acids derived from the catabolism of proteins are used for intermediary metabolism. A review by Neel and others in 2013 illustrated how autophagy in skeletal muscle is important to maintaining functional muscle. Another review by Kirkin et al. in 2009 demonstrated that ubiquitin ligases are connected with autophagy. MAGE proteins are believed to augment the activity of E3 ubiquitin ligases. MAGEL2 is part of the MAGE family of proteins, meaning that there is potential for the presence or absence of MAGEL2 to affect autophagy.

There are three proteins that are under investigation in connection to the process of autophagy and PWS. They include ubiquitin (Ub), p62/SQSTM1 (p62) and microtubule associated protein 1-light chain 3 (LC3) (Kirkin and others 2009, Komatsu and others 2007, Pankiv and others 2007) (Figure 2).

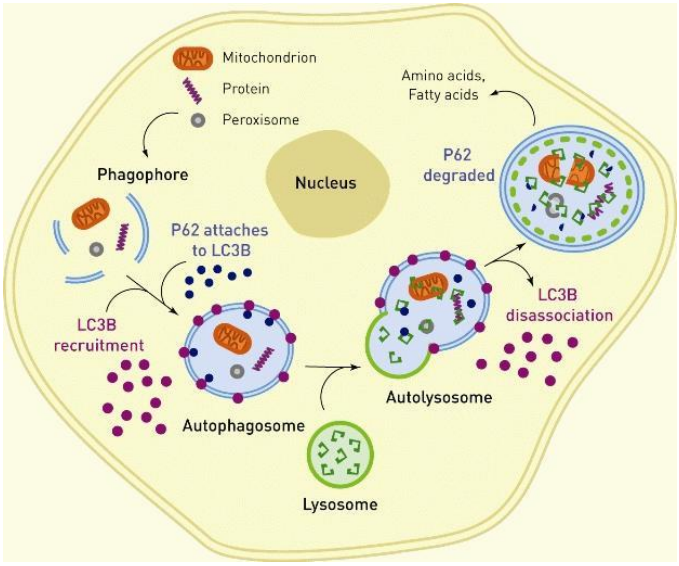


Figure 2: Autophagic process showing involvement of LC3 and p62 (http://www.labroots.com/trending/immunology/2247/macrophage-buildup-exacerbates-atherosclerosis).

Ub is a small, highly conserved protein involved in protein degradation and regulation (Kirkin and Dikic 2007). p62 is an adaptor protein functioning to bind LC3 and Ub in the autophagic-lysosomal pathway (Komatus and others 2007). LC3 is an autophagosome marker that is incorporated in protein aggregates that localizes as the membrane of autophagosomes (Kuma and others 2007, Kabeya and others 2000).

MATERIALS

Division of Labour. M.Sc. student Dila Kamaludin prepared protein lysates form mouse muscle for Figs 6A and 6B. Technician Jocelyn Bischof genotyped the mice by PCR. I collected the tissues from mouse pups and prepared sections, and performed the imunoblotting (Figures 5 and 7).

Mouse model. All animal studies were conducted in accordance with the Canadian Council on Animal Care Guidelines and Policies with approval from the Animal Care and Use Committee: Health Sciences for the University of Alberta. Mice from the strain C57BL/6-Magel2$^{tm1Stw/J}$ mice (The Jackson Laboratory stock #009062) were raised and maintained by the University of Alberta Health Sciences Laboratory Animal Services. Mouse pups between the ages of 3-8 days were used.

Antibodies. Antiserum against SQSTMI/p62, purchased from Abcam, was raised in rabbits, Immunogen affinity purification. Antiserum against ubiquitin, purchased from Dako, was raised in rabbit. Antiserum against β-actin-peroxidase, purchased from Sigma, was raised in mouse. Antiserum against LC3B, purchased from Cell Signaling, was raised in rabbit, and purified via peptide affinity. Antiserum against β-Galactosidase, purchased from Molecular Probes, was raised in rabbit. Polyclonal goat anti-rabbit IgG AlexaFluor®488 green, polyclonal goat anti-mouse IgG AlexaFluor®488 green, polyclonal goat anti-rabbit IgG AlexaFluor®594 red, and polyclonal goat anti-mouse IgG AlexaFluor®594 red were purchased from Life Technologies. ECL™ Anti-rabbit IgG, horseradish peroxidase linked, whole antibody from donkey and Anti-mouse IgG, horseradish peroxidase linked, whole antibody from sheep were purchased from GE Healthcare UK Limited.

Table 1: List of antibodies used in experiments

Primary Antibody	**Species**	**Company**	**Product Number**	**Dilutions**	**Usage**
SQSTMI/p62	Rabbits	Abcam	Ab91526	1:1000	IB/IF
Ubiquitin	Rabbits	Dako	Z0458	1:200	IB/IF
β-actin-peroxidase	Mouse	Sigma	A3854	1:50 000	IB
β-Galactosidase	Mouse	Molecular Probes	A-11132	1:1000(IB) 1:200(IF)	IB/IF
LC3B	Rabbits	Cell Signaling	2775	1:1000(IB) TBD	IB/IF
Secondary Antibody	**Species**	**Company**	**Product Number**	**Dilutions**	**Usage**
anti-Rabbit IgG AlexaFluor®488 green	Goat	Life Technologies	A11054	1:1000	IF
anti-mouse IgG AlexaFluor®488 green	Goat	Life Technologies	A11054	1:1000	IF
anti-rabbit IgG AlexaFluor®594 red	Goat	Life Technologies	A11067	1:1000	IF
anti-mouse IgG AlexaFluor®594 red	Goat	Life Technologies	A11067	1:1000	IF
ECL™ Anti-rabbit IgG	Donkey	GE Healthcare UK Limited	NA934-100UL	1:5000	IB
ECL™ Anti-mouse IgG	Sheep	GE Healthcare UK Limited	NA931-1ML	1:5000	IB

Western Blot development. Immoblin™ Western Chemiluminescent HRP Substrate, purchased from Millipore, was used to develop the Western blots.

Agarose Gel development. SYBR® Safe DNA Gel Stain used in a 1:10 ratio, gel stained after electrophoresis.

Genotyping PCR. Specific primers RW3430, RW4237 and RW4400 from Invitrogen. RW3430-ATGGCTCCATCAGGAGAAC. RW4237-GGGATAGGTCACGTTGGTG. RW4400-GATGGAAAGACCCTTGAGG. *Lacz* reagents and protocols.

Other Materials. Standard reagents in Western blot, Immunohistochemistry, reverse transcriptase PCR, RNA isolation, muscle histology and Crude protein preparation protocols were used (see Appendices).

METHODS

Muscle Collection. Mice were killed by decapitation. Left and right hind leg muscle was collected from postnatal mice ages 3-8 days old (P3-P8). For mice up to 5 days old, bone was collected with muscle. For mice over 5 days old, muscle was separated from bone if the sample collected was going to be used for experiments other than immunohistochemistry. Hypothalamus was collected to be used in lacZ/X-gal staining to determine genotype, either wild type (WT) or mutant lacking *Magel2* (MT).

Muscle Histology. Muscle histology was completed using the standard hematoxylin-eosin staining (H&E) procedures. H&E was undertaken, towards the end of the experiment, to determine the presence or absence of artifacts within the muscle sample slides used in IHC and to confirm where the muscle and bone were located in each section.

Immunohistochemistry for Muscle Sections. Muscle collected from mice ages 3-8 days was embedded in OTC Cryomatrix from Thermo Scientific, and cyrosections of 10-12um were made using a Cryostat machine. The sections were placed on glass slides and stored in the -80 °C freezer. Immunohistochemistry was completed according standard protocol (Appendix A).

RNA Isolation. Isolation of RNA was completed using TRIzol Reagent and according to standard protocol (Appendix A).

Crude Protein Preparation. Preparation of protein samples from collected muscle was done according to standard protocol (Appendix A).

Western Blotting. Western blots were done using 12-14% acrylamide gels made in lab according to standard recipe. Antibody dilutions varied according the different antibodies used. Primary antibody incubation was done mainly overnight at 4°C. For the full protocol see Appendix A.

RT-PCR and Genotyping PCR. RT-PCR was used to make cDNA from the RNA collected through RNA Isolation protocol (Appendix A). Standard protocol and reagents were used for RT-PCR (Appendix A). Specific primers were used to amplify *Magel2* and *LacZ* genes. Primers and primer sequences are listed under Materials. The products of the genotyping PCR were run on 1-2% agarose gels for 30-45 minutes at 100V. The gels were then stained with SYBR Safe Gel Stain in a 1:10 ratio for 30 minutes, room temperature in the dark. After staining, the gels were imaged for 1-5 minutes using the UV light and the SYBR filter on the Kodak camera.

RESULTS

Immunohistochemistry on pup muscle sections using β-Galactosidase, Ubiquitin, p62 and LC3-II.

The knockout mice used by the Wevrick lab have the *Magel2* gene open reading frame replaced with the lacZ gene, which should result in β-Galactosidase expression driven by the endogenous Magel2 promoter. Measuring the levels of β-Galactosidase can be used to determine the location of *Magel2* expression. Immunohistochemistry for β-Galactosidase was completed on sections of muscle from postnatal day 3-5 wild type and *Magel2-null* mice-pups (Figure 3A-B). No expression of β-Galactosidase is expected in tissues from wildtype pups, while expression of β-Galactosidase recapitulating endogenous Magel2 expression is expected in tissues from the Magel2-null pups.

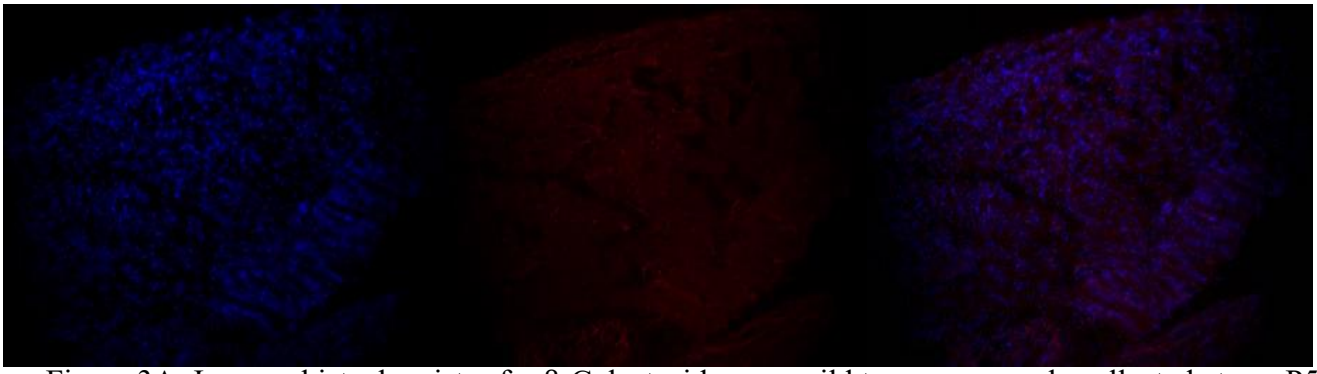


Figure 3A: Immunohistochemistry for β-Galactosidase on wild type pup muscle collected at age P5. Left-DAPI stain (cell nuclei label, blue), Middle- β-Galactosidase, Right-composite of both stains.

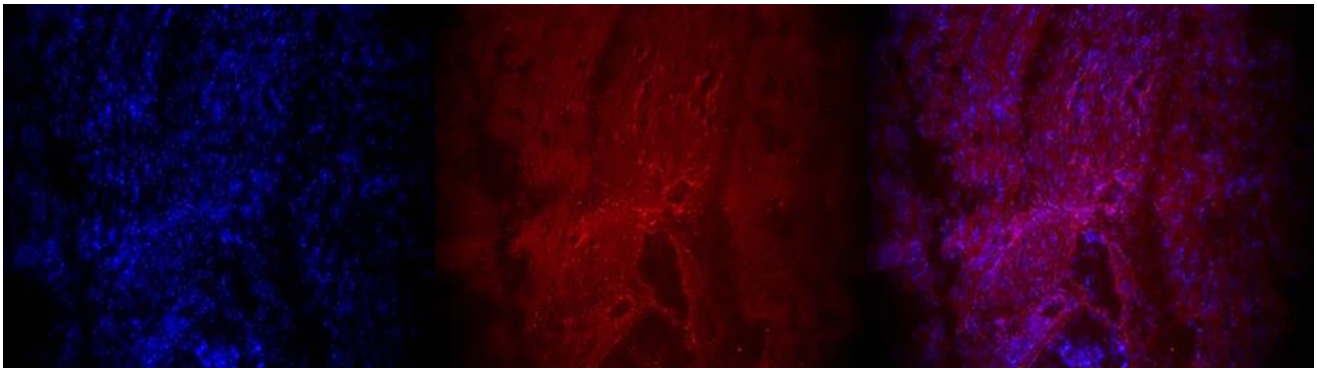


Figure 3B: Immunohistochemistry for β-Galactosidase on mutant, Magel2-null, pup muscle collected at age P5. Left-DAPI stain (cell nuclei label, blue), Middle- β-Galactosidase (red), Right-composite of both stains.

Aberrations in the process of autophagy and protein ubiquitination can be monitored by observing the levels of p62, ubiquitin and LC3-II, proteins that accumulate as protein aggregates inside the cell. Immunohistochemistry was completed for p62, ubiquitin and LC3-II on sections of muscle from postnatal day 3-5 wild type and *Magel2-null* mice-pups. There was a difference in protein expression for Ub, p62 and LC3, specifically with the formation of protein aggregates (p62 and Ub) and changes in localization (LC3), between the wildtype (no aggregates) and the Magel2-null (aggregates) (Figure 4-6).

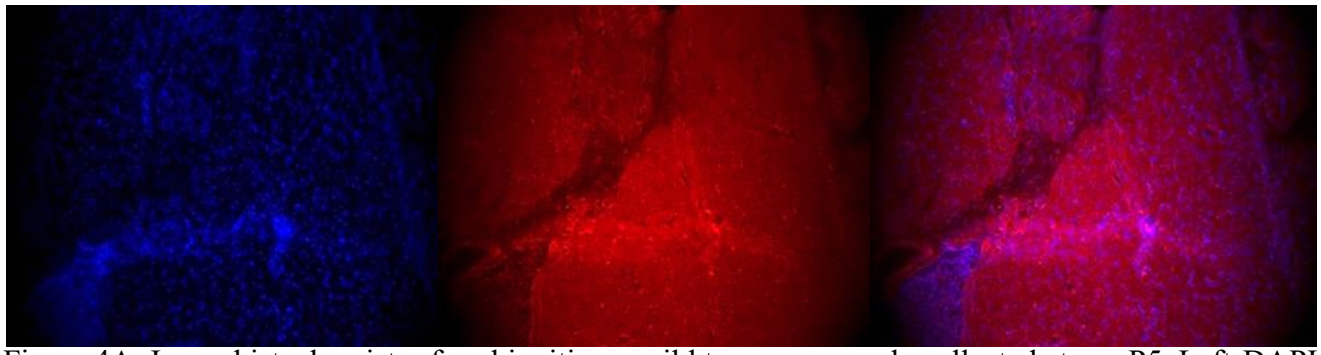


Figure 4A: Imunohistochemistry for ubiquitin on wild type pup muscle collected at age P5. Left-DAPI stain (cell nuclei label (blue), Middle-ubiquitin (red), Right-composite of both stains.

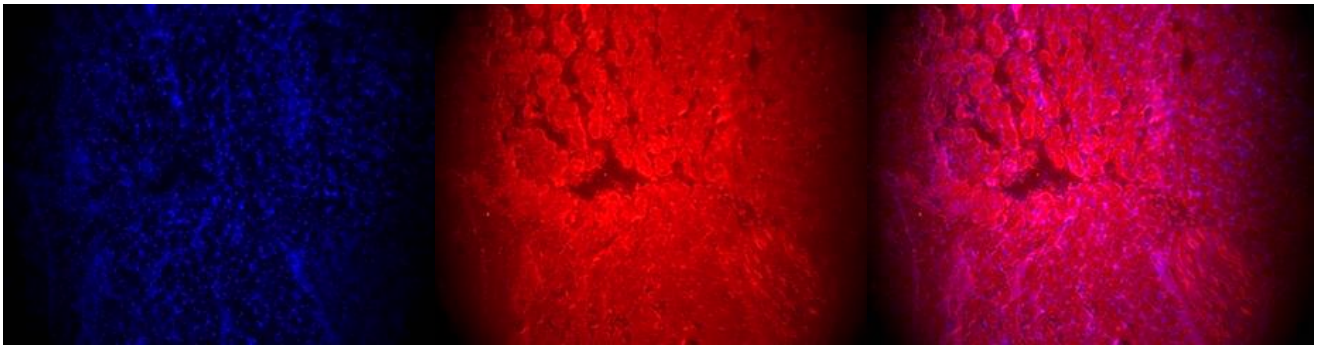


Figure 4B: Immunohistochemistry for ubiquitin on mutant, *Magel2-null,* pup muscle collected at age P5. Left-DAPI stain (cell nuclei label), Middle-ubiquitin, Right-composite of both stains.

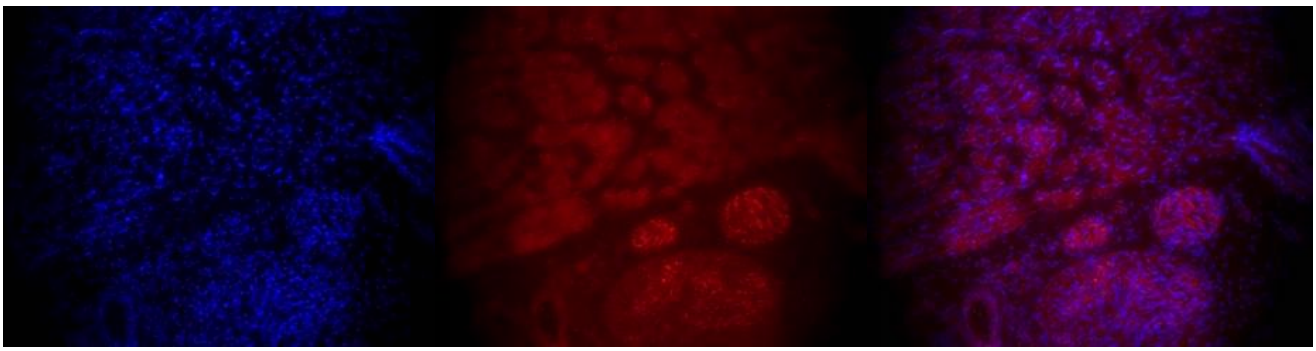


Figure 5A: Immunohistochemistry for p62 on wild type pup muscle collected at age P5. Left-DAPI stain (cell nuclei label), Middle-p62, Right-composite of both stains.

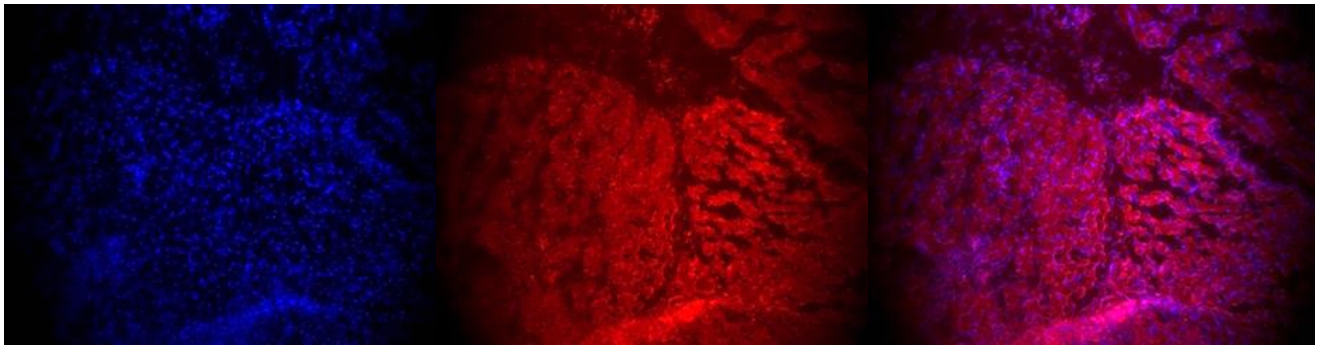


Figure 5B: Immunohistochemistry for p62 on mutant, *Magel2-null*, pup muscle collected at age P5. Left-DAPI stain (cell nuclei label), Middle-p62, Right-composite of both stains.

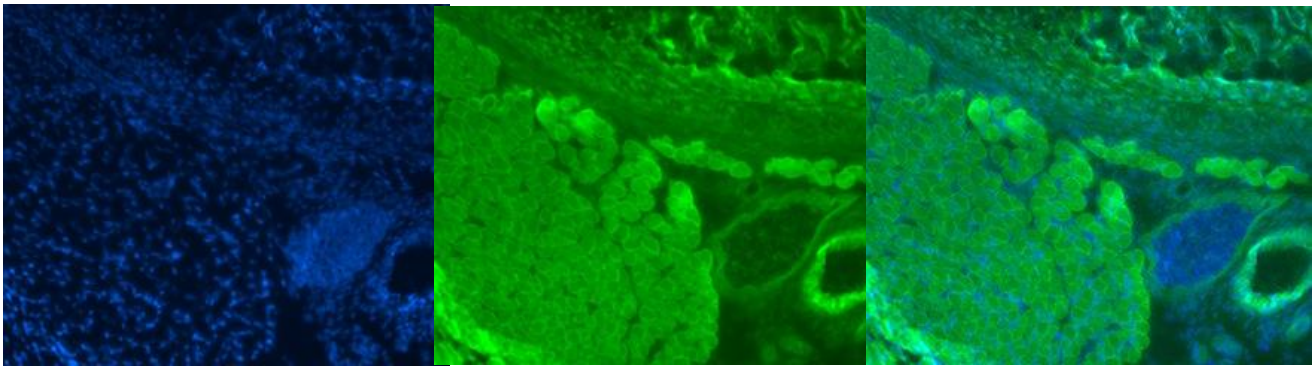


Figure 6A: Immunohistorchemistry for LC3 on wild type pup muscle collected at age P5. Left-DAPI stain (cell nuclei label (blue)), Middle-LC3 (green), Right-composite of both stains.

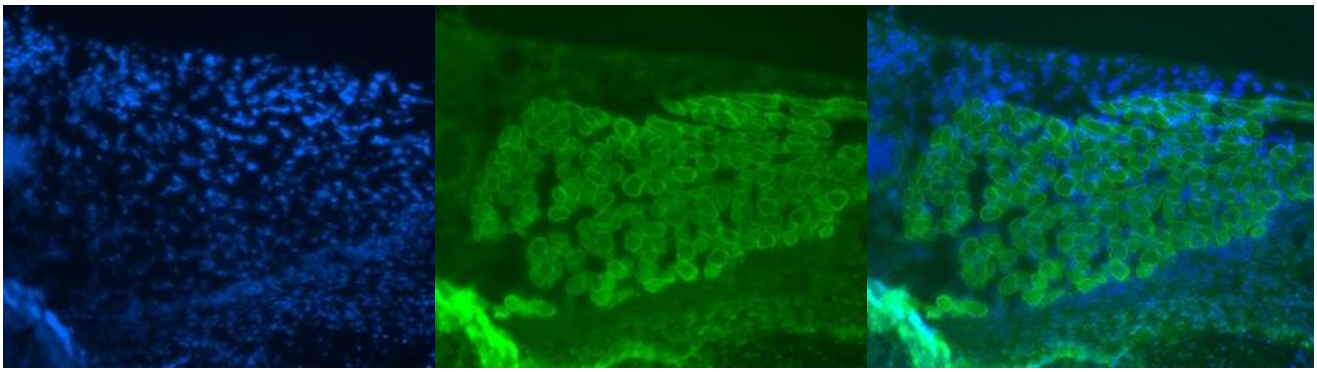


Figure 6B: Immunohistochemistry for LC3 on mutant, *Magel2-null*, pup muscle collected at age P5 Left-DAPI stain (cell nuclei label), Middle-LC3, Right-composite of both stains.

Immunoblotting on pup muscle samples using and β-Galactosidase, p62, ubiquitin, and LC3-II.

In the same way that reporter gene expression can be used to determine patterns of *Magel2* expression by examining β-Galactosidase levels on sections by immunocytochemistry, immunoblotting can also be used to quantify *Magel2* expression by detecting β-Galactosidase using an anti- β-Galactosidase antibody. Immunoblot analysis of protein extracted from muscle from P4 mouse pups was performed to detect the amount of β-Galactosidase in each sample. The muscle samples were from three-four mice of each genotype (wild type and *Magel2-null*). Immunoblot analysis was found to be inconclusive (Figure 7).

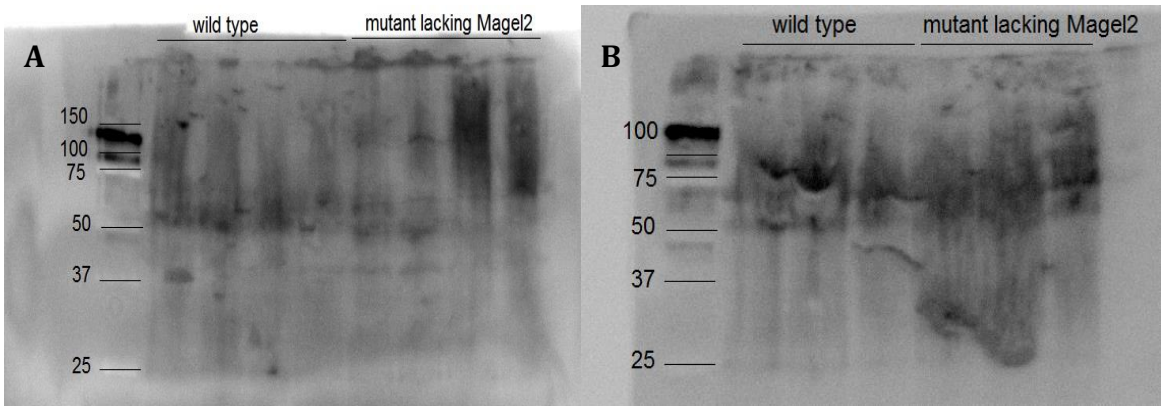


Figure 7: A & B-Immunoblotting for β-Galactosidase on pup muscle collected at age P4.

Immunoblotting was used to quantify the levels of p62, ubiquitin and LC3-II. Immunoblot analysis of protein extracted from muscle from P4 mouse pups was performed to detect the amount of p62, ubiquitin and LC3-II. The muscle samples were from mice of each genotype (wild type and *Magel2-null*). β-actin was used as a control to standardize p62 blots. A protein of the appropriate size for p62 (Figure 8A) and LC3-II (Figure 9) was detected through the immunoblot of neonatal mouse muscle. Bands of the correct size were detected for p62 (62 kDa, Fig. 8A), beta actin (42 kDa, Fig. 8B), and two isoforms of LC3 (14 kDa and 16 kDa). This experiment needs to be repeated to determine whether there are statistically significant differences between genotypes in signal intensity for any of these three proteins.

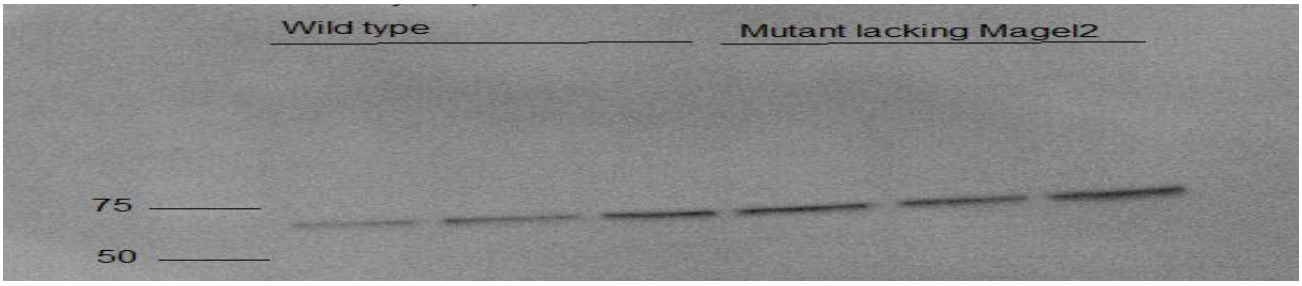


Figure 8A: Immunoblotting for p62 on pup muscle.

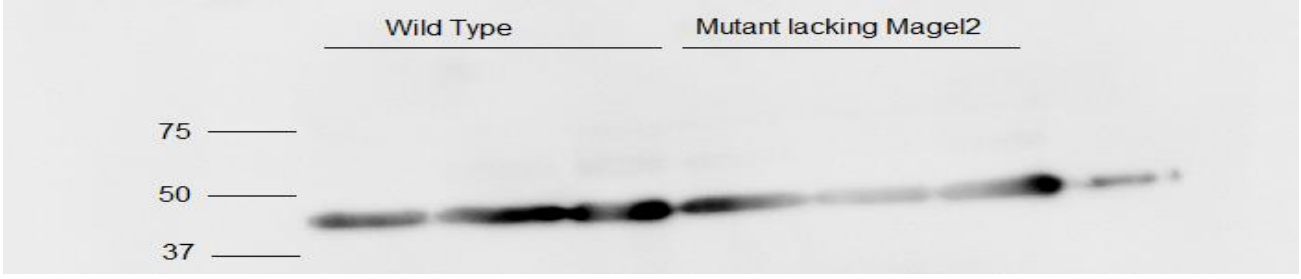


Figure 8B: Immunoblotting for β-actin on pup muscle.

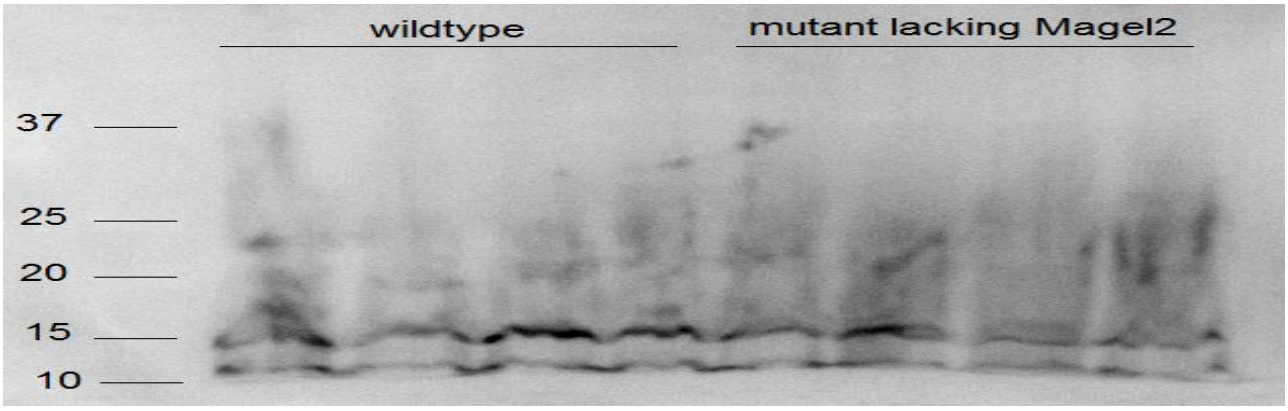


Figure 9: Immunoblotting for LC3 on pup muscle collected at age P4,.

Muscle histology (H and E stain) as a check for artifacts within muscle samples.

H & E staining was performed on both wild type and mutant, *Magel2-null*, pup muscle, to determine the presence or absence of artifacts within the samples. Staining was completed towards the end of experimentation. There were no artifacts present that would inhibit IHC results.

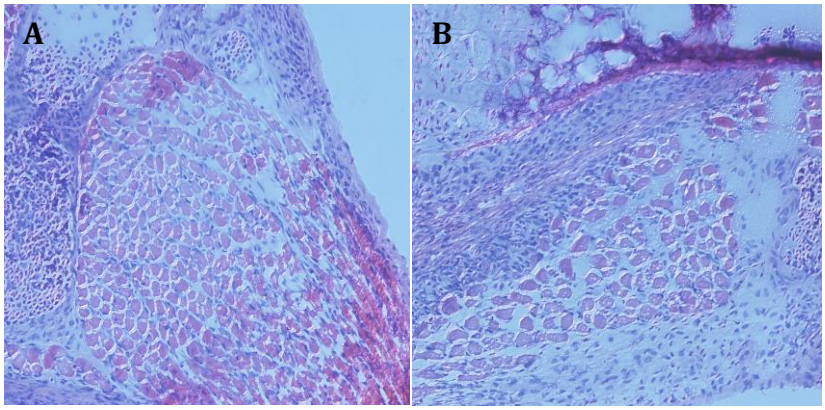


Figure 10: A-H & E staining on wild type pup muscle collected at age P5. B- H & E staining on mutant, *Magel2-null,* pup muscle collected at age P5

DISCUSSION

The immunohistochemistry for β-Galactosidase on pup muscle confirmed the preliminary genotyping of the mice. β-Galactosidase was present in the *Magel2-null* mice pups and absent in the wild type mice pups indicating that the *Magel2* knockout was successfully replaced with β-Galactosidase. Ub, p62 and LC3 are normally found within muscle cells. An abnormal result would result in a change in the quantity of protein with in the cell, a change in protein localization within the

cell, or a change in behaviour of the protein such as the formation of protein aggregates. The results from the immunoblotting were inconclusive and cannot be used to support or reject the connection between Magel2 and the process of autophagy. The H & E staining revealed that there were no artifacts within the muscle sections that interfered with the IHC. The IHC results for Ub, p62 and LC3 in *Magel2-null* muscle (Figure 4B, 5B, 6B) demonstrated a difference in expression behaviour through the formation of protein aggregates and in the case of LC3, localization at the membrane of the cell. This was in contrast to the wild type muscle (Figure 4A, 5A, 6A) that depicted normal localization. Due to the expression difference seen in the *Magel2-null* muscle it is probable that there is error in the process of autophagy because the respective proteins are not being cleared from the cell and are building up to form the protein aggregates. Ub has shown proven connection with the cellular processes of protein degradation and regulation, including the process of autophagy (Kirkin and Dikic 2007). p62 and LC3 are known autophagic markers therefore if there is a change in autophagy there should be an expression in the change of these proteins (Komatus and others 2007, Kuma and others 2007). The probable error in autophagy supports the proposed connection between Magel2 and autophagy. It also can explain the reason for the poor muscle tone in infants with PWS. Autophagy is important to the homeostatic control of skeletal muscle, an increase or decrease in autophagy can cause muscular atrophy (Neel and others 2013). Autophagy has been implicated in other disorders including muscular dystrophy, Pompe's disease and Wolman disease (Neel and others 2013). There are many different proteins involved with the process of autophagy, meaning there are many places in which error can occur to disrupt autophagy (Figure 11).

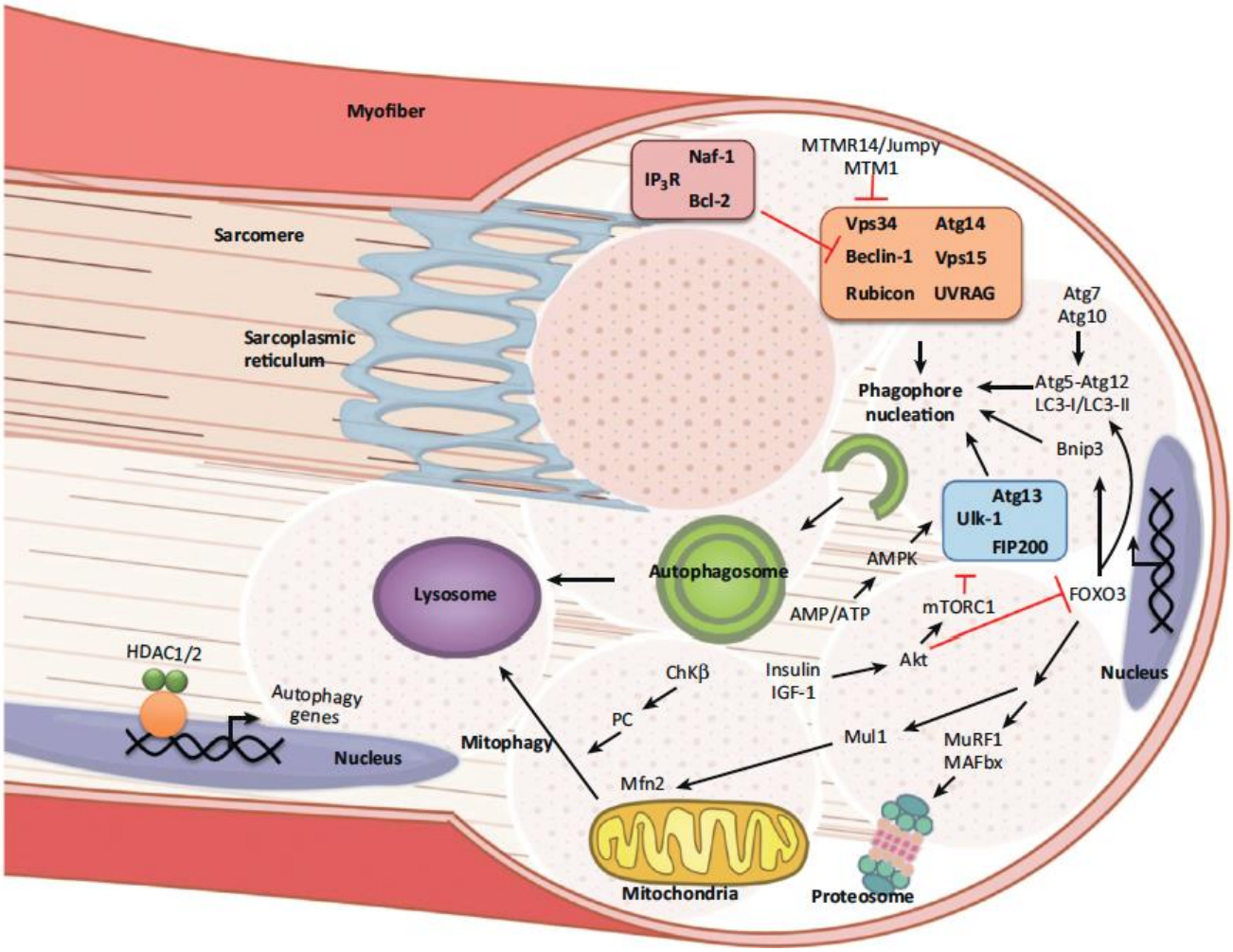


Figure 11: Schematic of autophagy and all involved proteins (Neel and others 2013).

In muscular dystrophy a decrease in levels of beclin-1, bnip3, mTOR and AMPK are responsible for impaired autophagy and skeletal muscle regulation. In Pompe's disease it is a result of a loss of lysosomal glucosidase, GAA, and mTOR, causing the loss of functional lysosomes. In Wolman's disease it is a lack of lysosomal lipase, LIPA, that disrupts autophagy.

CONCLUSION

One of the many symptoms of PWS is hypotonia, poor muscle tone that is especially present in the few months post birth. This hypotonia could be a result of a disruption in the process of autophagy that regulates skeletal muscle. The change in expression of autophagic markers provide support the hypothesis the abnormal autophagic process is likely to contribute to muscular atrophy in *Magel2-null*

mice. Further research is needed to determine how the inactivation of *Magel2* causes muscle phenotypes at a cellular level.

APPENDIX A

Immunohistochemistry Protocol for Sections

Day 1.
1. Warm slides to room temperature for 10-15 min. Outline with PAP pen.
2. Rinse 1 x 5 min. in 1x PBS/0.1% Tween-20 (PBST) at room temperature in a coplin jar.
3. Place slides flat and block with 300 µL per slide 10% normal horse (or goat) serum + 0.3% Triton X-100 in PBS for 1 h at room temperature in a humidified container (tip box lid for slides inside tupperware with PBS wetted filter paper).
4. Incubate with primary antibody in PBS + 1% normal horse (or goat) serum + 0.3% Triton X-100 overnight at room temperature in humidified container.

Day 2.
1. Rinse 3 x 5 min. with PBST at room temperature in coplin jar.

All steps covered in foil from this point!

2. Place slides flat and add 300 µL secondary antibody diluted in PBS with 1% normal horse or goat serum (no Triton). Leave in a humidified chamber for 1 hour at room temperature.
3. Rinse 3 x 5 min. with PBST at room temperature in coplin jar with gentle rocking.
4. Rock 10 min. at room temperature in a coplin jar with Hoechst stain (1:250 dilution in PBST, ie. 2.4 µL per 100 mL).
5. Rinse 1 x 5 min. with PBST at room temperature.
6. Mount with a few 50 µL drops 1:3 glycerol/1X PBS and coverslip. Visualize or store at -20°C. Alternatively aquamount.

RNA Isolation Using TRIzol Reagent
Reagents Required but not Supplied
-Chloroform
-Isopropanol
-75% EtOH (prepared with RNase-free water)
-RNase-free water → to prepare, draw water into RNase-free glass bottles. Add DEPC to 0.01% (v/v). let stand O/N and autoclave.

1. Homogenization

Tissues

-Add 1 ml TRIzol Reagent (fumehood!) per 50-100 mg of tissue to tissue sample in a sterile (RNA only) eppendorf tube. The sample volume should not exceed 10% of the volume of TRIzol reagent used for homogenization.
-grind tissue well with a disposable plastic pestle.

Cells grown in monolayer

-Add 1 ml of TRIzol Reagent to a 3.5 cm diameter dish
-Pass the cell lysate several times through a pipette.
The amount of TRIzol Reagent added is based on the area of the culture dish (1 ml per 10 cm^2) and not on the number of cells present. Insufficient TRIzol reagent may result in contamination of isolated RNA with DNA

Cells grown in suspension

-Pellet cells by centrifugation.
-Lyse cells in TRIzol Reagent by repetitive pipetting. Use 1 ml of the reagent per 5-10 x 10^6 of animal, plant or yeast cells, or per 1 x 10^7 bacterial cells.
Washing cells before the addition of TRIzol Reagent should be avoided as this increases the possibilty of mRNA degradation. Disruption of some yeast and bacterial cells may require the use of a homogenizer

2. Phase Separation
-Incubate homogenized samples for 5 min at RT.
-Add 0.2 ml of chloroform per 1 ml of TRIzol reagent used. Cap tubes securely!!
-Shake tubes vigorously by hand for 15 sec
-Incubate at RT for 2 to 3 min
-Centrifuge at max of 12,000 x g for 15 min at 4°C. Should see lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA is in the aqueous phase

3. RNA precipitation
-Transfer aqueous phase to fresh tube
-Add 0.5 ml (500 µl) isopropanol per 1 ml of TRIzol Reagent used. MIX
-Incubate samples at RT for 10 min
-Centrifuge at max of 12,000 x g for 10 min at 4°C. RNA forms gel-like pellet on side and bottom of tube
4. RNA wash
-Remove supernatant - pour off as much as possible and remove rest with a pipette
-Wash RNA pellet once with 1 ml 75% EtOH per 1 ml of TRIzol Reagent used initially -Vortex sample to mix (get pellet off walls of tube)
-Centrifuge at max of 7500 x g for 5 min at 4°C

5. Redissolving the RNA
-Remove EtOH supernatant by pouring off and using a pipette to remove rest.
-Air-dry the RNA pellet (in fumehood or on bench)
Do NOT dry the RNA by centrifugation under vacuum
-Dissolve RNA in 40-50 µlRNase-free water by pipetting repeatedly
-Incubate for 10 min at 55-60°C (heated sand)
-Store at -80'C

WARNING
TRIzol Reagent is toxic in contact with skin and if swallowed. Causes burns. After contact with skin, wash immediately with plenty of water and detergent. Use gloves and eye protection (shield, safety goggles). Avoid contact with skin or clothing. Use in a chemical fumehood. Avoid breathing vapor.

Crude Protein Preparations From Mammalian Cells/Rapid Protocol
(from Jocelyne/Casey lab)

For TC cells:
•48 h post-transfection (example hEK transfected cells) remove the media from 100 mm petri dish of cells
•carefully wash the cells with 10 mL PBS (do not add PBS directly onto cells), cells should remain adherent if you are careful

(alternatively, cells can be suspended in the PBS, centrifuged at 1000 rpm for 5 min., washed with 10 mL PBS, and centrifuged 1000 rpm for 5 min. as before)
•remove PBS and suspend cells in 500 µL 2X modified sample buffer with scraping and transfer the cells to a microcentrifuge tube…

For tissue (ie. mouse, rat brain):
•prechill Dounce Homogenizer vessels on dry ice (pestles –20°C)
•weigh frozen tissue and then place in dounce homogenizer on ice
•add 1.0 mL of modified 2X sample buffer
•pass through tissue 10X with the loose glass rod followed by 10X with the tight glass rod (let sit on ice for a few minutes to let bubbles settle)
•transfer samples to 1.5 mL microcentrifuge tubes …

•shear DNA by mixing up and down through a 22G needle 10-15X, followed by a 26G needle 10-15X
•incubate 65°C for 5 min.
•centrifuge 10 min., 14000 rpm and transfer crude preparation to new microcentrifuge tube
•divide up the preparation into several small aliquots and freeze on dry ice
•store the protein preparations at –80°C

•thaw protein preparations on ice
(add 2% □ME 2 µL/100 µL and saturated bromphenol blue 1 µL/100 µL to samples before boiling/loading on gel)
•boil 5 min. before loading on gel
•load approx. 20 µg complex mixture/total protein on a mini-gel

Modified 2X Sample Buffer (-□ME, -bromphenol blue)
(□ME interferes with BCA protein assay)

20% glycerol	20 mL/100 mL
4% SDS	4 g
0.13M Tris	1.52 g

•pH to 6.8 with HCl

(add 2% □ME 2 µL/100 µL and saturated bromphenol blue 1 µL/100 µL to samples before boiling/loading on gel)
Infectious/biohazardous: Wear personal protective equipment including gloves, eye protection, lab coat, proper footwear. Use material in a biosafety cabinet or make sure hazard is inactivated before taking to your lab bench. Minimize aerosols. Wash work surfaces down with 70% ethanol, decontaminate any spills with 10% bleach 30 min., decontaminate waste before disposal (ie. autoclave). Wash your hands after you are finished.

Sharps hazard: Do not recap needles. Use scalpel blades with handle. Dispose of only in sharps container!

Chemical hazards are associated with this protocol! Wear personal protective equipment including chemical resistant gloves, eye protection, lab coat, proper footwear. Use in a chemical fumehood! Wash your hands after you are finished. Consult MSDS for further information.

Special considerations:
□-Mercaptoethanol-highly toxic, do not breathe vapor; use only in chemical fumehood
Acrylamide-toxic/causes cancer; do not breathe dust

SDS PAGE
(Laemmli, U.K. (1970). Nature **227**:680-685.
Weber, K. and Osborn, M. (1973). "The Enzymes". 3rd Ed., Vol.1, 179-223.
Ames, G.F-L. (1974) J. Biol. Chem. **249**:634-644.)

Separating gel buffer, 1X

1.5 M Tris	91 g/500 mL
0.4% SDS	2 g

pH to 8.7 with HCl
store at 4°C

Stacking gel buffer, 1X

0.4 M Tris	6.05 g/100 mL
0.4% SDS	0.4 g

pH to 6.8 with HCl
store at 4°C

Reservoir (Running) buffer, 5X

0.125 M Sigma 7-9	15.1 g/ liter
0.960 M glycine	72.0 g
0.5% SDS	5.0 g

Store at room temperature

Acrylamide stock
37.5:1 (30%) acrylamide:bis, BioRad Cat.# 161-0158

Modified 2X Sample Buffer (-□ME, -bromphenol blue)
(□ME interferes with BCA protein assay)

20% glycerol	20 mL/100 mL
4% SDS	4 g
0.13M Tris	1.52 g

pH to 6.8 with HCl
(add 2% □ME 2 µL/100 µL and saturated bromphenol blue 1 µL/100 µL to samples before boiling/loading on gel)

Preparation of PAGE gels
Depending on the size of the protein of interest you may want to use a different concentration of Bis-Acrylamide for each gel.

Protein size (kDa)	Gel percentage (%)
4-40	20
12-45	15
10-70	12.5
15-100	10
25-200	8

Abcam Western Blotting: A Beginner's Guide

1. Prepare **separating gel** as follows:

(half recipe is enough for 2-0.75 mm mini-gels, Mini-Protean 3)

% acrylamide	5	6	7	7.5	8	9	10	12	12.5	
separating gel buffer	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	3.75	mL
MQ H_2O	8.75	8.25	7.75	7.50	7.25	6.75	6.25	5.25	5.00	mL
Acrylamide stock	2.50	3.00	3.50	3.75	4.00	4.50	5.00	6.00	6.25	mL
TEMED	10	10	10	10	10	10	10	10	10	µL
10% APS (prepare fresh)	100	100	100	100	100	100	100	100	100	µL

2. Immediately after adding TEMED and APS pipette ~3.2 mL separating gel into glass plates overlay with water or 70 % Ethanol.
3. Let polymerize 15-30 minutes
4. Prepare **stacking gel** as follows:

(enough for 2-0.75mm Mini-Protean 3 gels)

Stacking gel buffer	1.25 mL
MQ H_2O	3.05 mL
Acrylamide stock	0.67 mL
TEMED	5 µL
10% Ammonium persulfate	25 µL

5. Pour off Ethanol or water after polyerization and pipette stacking gel on top of separating gel, insert combs ensuring no air bubbles.
6. Let polymerize ~30 min.
7. Use or store at 4 °C covered in wet paper towel and seran wrap (zip lock bag)

Sample Preparation

1. Thaw frozen samples on ice
2. Add 2 % βME (2 µL/100 µL) and 1 % saturated bromophenol blue (1 µL/100 µL) to samples
3. Boil samples for 5 minutes (microfuge tubes can be sandwiched shut in metal contraption and boiled in mater on heat block)
4. Assemble chamber and gels and remove combs. Fill chamber and rinse wells with 1X Running Buffer. (160 mL 5X Running Buffer + 640 mL MQ H2O)
5. Load 15-20 µg total protein (10-well comb max Vol. ~30 µL)
6. Warm standards to room temperature and load ~ 8 µL/lane. (Precision Plus BioRad Cat.#161-0373)
7. Run gels

Two mini-gels at either 25mA for ~75 min or 80 mA for ~30
Remove gel and proceed immediately to staining or blotting

Western Blotting

(Towbin, H., Staehelin, T. and Gordon, J. *PNAS* **76**(9):4350-4354 (1979).

Transfer (Transblot) buffer	per liter
25 mM Tris base	3.0275 g

192 mM glycine	14.41 g
20% methanol	200 mL
MQ water	800 mL

Prepare the day before use and store at 4°C.

TBST	per liter
20mM Tris, pH 7.5	2.42 g Tris base
137 mM NaCl	8 g NaCl
0.1% Tween-20	1 mL Tween-20

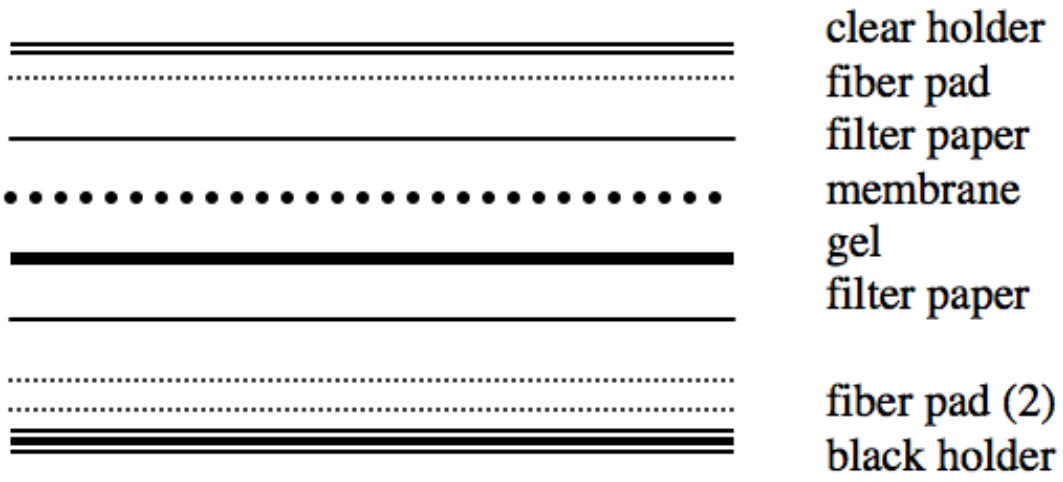
MQ water to 1 L, pH to 7.6 with 1N HCl

10XTBS	per liter
	24.2 g Tris base
	80 g NaCl
	pH to 7.6

TBST-M

TBST containing 5% (w/v) non-fat dry milk powder (eg. Carnation), store at 4°C
(lasts for only a week or so before going bad can be prepared once for all the blotting dilutions)

1. Cut Immobilon-P membrane and filter paper to the size of the gel. Label membrane by either making a mark with a pencil or punching a whole in a corner to be able to determine which side the protein has transferred to. (2 filter papers/gel)
2. Rinse Immobilon-P membrane in Methanol 20-30 sec. and then presoak membrane, filter paper and fiber pad in transfer buffer for ~15 min.
3. Keeping everything wet with transfer buffer, assemble sandwich on blue cafeteria tray near main sink. Open gel holder and assemble sandwich making sure to remove any bubbles between layers using a glass pipette.



4. Put sandwich in transfer chamber with cooling block. (If only running one blot place in the back position.) Fill chamber with transfer buffer submerging fiber pads.
5. Transfer

Running conditions:

Overnight	30V
Rapid (90 min)	100V

With precooled transfer buffer, rapid blotting can be done at RT. Longer transfers should be done in the cold room)

Immunoblotting

1. When transfer is done, immediately place membrane in 20 mL TBSTM with gentle shaking (~50 rpm) for 1 hour to block non-specific binding.

2. Incubate membrane overnight with primary antibody diluted in TBSTM ~10 mL depending on the size of container, must completely cover membrane or blot will dry out an not work properly. Can incubate at RT or 4 °C depending on antibody)

Note: For phosphoproteins incubate primary antibody at 4 °C

3. Wash membrane 3 X 10 min with TBSTM with gentle rocking (50 rpm)
4. Incubate blot with secondary antibody (ex. 2 µL goat anti-Rabbit IgG-HRP/10 mL TBSTM) with gentle rocking (50 rpm)
5. Wash 3 X 10 min with TBSTM

Develop Blot

6. Add equal parts of each of the luminol and peroxidase substrates in the Immobilon chemiluminescent HRP detection kit together and allow to warm to room temperature before use.

Blot Size	Working HRP Substrate Required	
7 × 8.5 cm	6 mL	(3 mL luminol reagent + 3 mL peroxide solution)
10 × 10 cm	10 mL	(5 mL luminol reagent + 5 mL peroxide solution)
8.5 × 13.5 cm	12 mL	(6 mL luminol reagent + 6 mL peroxide solution)

7. Incubate the blot in HRP developer for 5 minutes with the protein side up.
8. Do not let the membrane dry-out
9. Image the blot on Kodak Imager

Fluorescence bulb must be off, filter must be open, filter set to white.
Adjust magnification based on size and desired magnification of blot.
Preview and expose blot for up to 30 min depending on signal

General RT-PCR

1. Mix in PCR tubes: RNA 1 ug (2 µl)
DNAseI(RNAse free) 1 µl
RNAse-free water to 11 ul X µl

DNAse I needed only if primers are within one exon
Also set up 2 control tubes with no RNA
2. Incubate 37˚C 20 min (in PCR machine), 70˚C 10 min.
3. Add 1µl 100 µg/µl random hexamers, incubate 70˚C 5 min, ice 1 min., spin down.
4. Add a mix of 5X first strand buffer 4 µl
0.1M DTT 2 µl
10mM dNTP 1 µl
Superscript 1 µl

As a negative control, omit superscript from 2 tubes (one with RNA and one without RNA)
5. Incubate at 42˚C 45 min., 95˚C 5 min., spin down.
6. Use 2 to 4 µl per 20 µl PCR reaction.

Example PCR

10X	2 µl
2 mMdNTP	2 µl
25 mM Mg	1 µl
1U/µl Taq	0.25 µl
water	8.75-10.75 µl
10 µM primers	1 µl each
cDNA	2-4 µl

95˚C 5 min.
94˚C 30 sec|
50˚C 30 sec| x 30
72˚C 30 sec|
72˚C 5 min.

MageL2 Genotyping PCR
DNA template used is 2ul of elutate from DNEasy Blood and Tissue kit

Components	Invitrogen Catalogue #	Per single 20ul reaction	Per 10 rxn	Per 20 rxn
10X PCR Buffer	10342-020	2ul	20ul	40ul
2mM dNTPs	10297-018	2ul	20ul	40ul
50mM $MgCl_2$	10342-020	0.6ul	6ul	12ul
RW3430 (10mM)		2ul	20ul	40ul
RW4237 (10mM)		1ul	10ul	20ul
RW4400 (10mM)		1ul	10ul	20ul
Taq (Invitrogen)	10342-020	0.1ul	1ul	2ul
MilliQ water		9.3ul	93ul	186ul
Master Mix		18ul		
DNA template		2ul		

Cycling conditions;
Step1 : 95ºC for 5min
Step2 : 95ºC for 30sec
Step3 : 60ºC for 30sec
Step4 : 72ºC for 30sec
Step5 : repeat step2 34 more times
Step6 : 72ºC for 5min
Step7 : 4ºC hold

Primer	Sequence	Info
RW3430	ATG GCT CCA TCA GGA GAA C	MageL2 forward primer
RW4237	GGG ATA GGT CAC GTT GGT GT	LacZ reverse primer
RW4400	GAT GGA AAG ACC CTT GAG GT	MageL2 reverse primer

RW3430 + RW4237 make a band ~400bp
RW3430 + RW4400 make a band ~300bp
Both mutants and WT will have the 300bp band, mutants will also have the 400bp band.

H & E Staining

Let slides warm up to room temperature.

1. Hematoxyline for 10 minutes –Filter hematoxylin with filter paper before use
2. Wash slides in a bucket with running tap water for 15 minutes-Do not attack directly samples
3. Eosin for 10 minutes
4. Dip in 70% EtOH
5. Dip in 90% EtOH
6. 30 seconds in 99% EtOH I
7. 30 seconds in 99% EtOH II
8. Xylene Sustitues I for 5 minutes
9. Xylene Substitues II for 5 minutes
10. Inclusion with Permount Mounting Medium-2 drops per slide then incubate/fix in drawer for 1 hour.

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