The Role of MAGEL2 in Ubiquitination Pathways and its Contribution to Prader-Willi and Schaaf-Yang Syndromes

by

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Abstract

The *MAGEL2* gene is implicated in two neurodevelopmental disorders: Prader-Willi syndrome (PWS) and Schaaf-Yang syndrome (SYS). PWS is characterized by intellectual disability, obesity, poor muscle tone, distinct facial features, excessive daytime sleepiness and nighttime wakening. PWS is a sporadic multigene chromosomal deletion disorder involving the 15q11-q13 region, which encompasses *MAGEL2*. SYS is caused by mutations in *MAGEL2* and has characteristics that overlap with PWS. MAGEL2 belongs to the MAGE (Melanoma Antigen Gene) family of proteins, which are encoded by over 60 genes in the human genome. MAGE proteins are divided into type I that are predominantly expressed in reproductive tissues and tumors, and type II that are expressed in various tissues throughout the human body and have emerging roles in development and disease. Various MAGE proteins have been identified as interacting partners of the ubiquitination system. MAGEL2 acts as a modulator of ubiquitination, a reversible post-translational modification. Ubiquitination is a multistep process that tags proteins with a 76 amino acid protein called ubiquitin. This tag targets proteins for downstream events or for proteasomal degradation. MAGEL2 modifies the ubiquitination levels of components of the ubiquitination system or substrate proteins through interactions with E3 ubiquitin ligases responsible for substrate recognition or with deubiquitinating enzymes. For my thesis, I explored the role of MAGEL2 in ubiquitination and investigated how the loss of MAGEL2 could contribute to symptoms seen in PWS and SYS through its role in the regulation of ubiquitination.

Firstly, I investigated the cellular role of MAGEL2 in ubiquitination of proteins involved in circadian rhythm. Circadian rhythm is the endogenous oscillation of physiological and cellular functions over a roughly 24-hour period. Children with PWS have excessive daytime sleepiness

and experience sleep disturbances. *Magel2* knockout mice have abnormal circadian rhythm. This leads us to our hypothesis that MAGEL2 aids in the regulation of circadian rhythm output. I found that MAGEL2 interacts with the E3 ubiquitin ligase RBX1, the deubiquitinase USP7, and two proteins that are critical for circadian rhythm, Cryptochrome 1 and 2 (CRY1 and CRY2). I determined that MAGEL2 modulates the ubiquitination of CRY1 protein levels and that co-expression of CRY1 and MAGEL2 results in less CRY1 protein in the cytoplasm. The regulation of circadian rhythm relies on the tightly controlled levels of core circadian proteins, including CRY1. The abnormal regulation of CRY proteins through perturbed ubiquitination, secondary to the loss of MAGEL2 could cause circadian rhythm output irregularities in mice and humans with MAGEL2 deficiency.

Secondly, I investigated the role of MAGEL2 in the ubiquitination of proteins in the BBSome. The BBSome is a multi-protein complex involved in the function of cilia, which are organelles that are present on almost all mammalian cells. The BBSome functions in ciliary membrane biogenesis and mediates protein/receptor trafficking to the ciliary and plasma membranes. The genes encoding BBSome proteins are mutated in people with Bardet-Biedl syndrome, a ciliopathy that has many overlapping phenotypes with PWS and SYS, such as obesity, intellectual disability, delayed development, and congenital muscle weakness. MAGEL2 regulates endosomal protein recycling through its interactions with E3 ubiquitin ligases TRIM27 and RNF41 and deubiquitinase USP7. MAGEL2 modified ubiquitination and trafficking of the leptin receptor. The BBSome is also important for recycling of the leptin receptor. I investigated the overlap between the function of MAGEL2 and the BBSome in trafficking of the leptin receptor given the similarities in phenotypes observed when these genes are mutated. Ubiquitination of BBS2, a BBSome component is required for BBSome degradation. I identified TRIM32 and TRIM27 as E3

ubiquitin ligases responsible for the degradation of BBS2, a vital protein in the BBSome complex. I identified MAGEL2 as a modifier of the ubiquitination of BBS2 by TRIM32 and TRIM27. MAGEL2 may reduce ubiquitination of the BBSome and stabilize this protein complex, important for leptin receptor recycling.

Overall, I show MAGEL2 modulates the ubiquitination of substrate proteins, CRY1 and BBS2. Dysregulation of ubiquitinated proteins due to loss of MAGEL2 function could contribute to phenotypes seen in people with PWS, SYS, and related neurodevelopmental disorders such as BBS. My thesis work aids in the understanding of how MAGEL2 (and MAGE proteins in general) can modulate ubiquitination processes to regulate protein stability and fine tune intracellular trafficking.

Preface

Chapter 3 contains original unpublished work primarily conducted by Vanessa Carias. Figure 3.4 and Figure 3.5 were performed by Mercedes Zoeteman who was a summer student under the supervision of Matthea Sanderson and Vanessa Carias.

Chapter 4 contains original unpublished work primarily conducted by Vanessa Carias. Figure 4.6D was performed by Jocelyn Bischof.

Along with Dr. Wevrick, we wrote a review on therapeutics used in preclinical animals models of Prader-Willi syndrome. This review guided the description of the Magel2 mouse models in section 1.3.3.

> Carias, K. V., & Wevrick, R. (2019). Preclinical Testing in Translational Animal Models of Prader-Willi Syndrome : Overview and Gap Analysis. *Molecular Therapy: Methods & Clinical Development*, *13*, 344–358.

I was a contributing author on Wijesuriya et al., 2017, and performed the immunoflouresence experiments of Figure 3A and 3B. Cell lines used in this paper were used in my thesis.

> Wijesuriya, T. M., Ceuninck, L. De, Masschaele, D., Sanderson, M. R., Carias, K. V., Tavernier, J., & Wevrick, R. (2017). The Prader-Willi syndrome proteins MAGEL2 and necdin regulate leptin receptor cell surface abundance through ubiquitination pathways. *Human Molecular Genetics. 26*(21), 4215–4230.

I was also an author on a study where I performed data analysis and helped write the manuscript.

> Karin Vanessa Carias and Rachel Wevrick. (2019). Clinical and genetic analysis of children with dual diagnosis of Tourette syndrome and autism spectrum disorder. *Journal of Psychiatric Research. 111*, 145-153.

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Vanessa Carias

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List of Abbreviations

ACIII	Adenylyl cyclase type 3
AgRP	Agouti-related peptide
ANOVA	Analysis of variance
ARC	Arcuate nucleus
ASD	Autism Spectrum Disorder
BBS	Bardet-Biedl syndrome
BMAL1	Brain and muscle ARNT-like1
BP	Break point
CK1ε	Casein kinase 1 ε
CLOCK	Circadian locomotor output cycles protein kaput
CRL	Cullin-RING ligase
CRY	Cryptochrome
DMEM	Dulbecco's modified Eagle medium
DTT	Dithiothreitol
DUB	Deubiquitinase
E1	Ubiquitin activating
E2	Ubiquitin conjugating
E3	Ubiquitin ligase
EE	Early endosomes
EDS	Excessive daytime sleepiness
EDTA	Ethylenediaminetetraacetic acid
ERC	Endocytic recycling compartment
ESCRT	Endosomal sorting complexes required for transport (ESCRT)
FISH	Fluorescence in-situ hybridization
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
HECT	Homologous to the E6AP carboxyl terminus
HEK 293T	Human embryonic kidney 293T
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	Horseradish Peroxidase

IgG	Immunoglobulin G
K48	Lysine-48
K63	Lysine-63
LepR	Leptin receptor
LGMD2H	Limb girdle muscular dystrophy
LICS	Lung disease immunodeficiency and chromosome breakage syndrome
MAGE	Melanoma Antigen Gene
MAGEL2	Melanoma antigen family member L2
MHD	Mage homology domain
MSB	2 x modified sample buffer
MURF	Muscle-specific ring finger
MVBs	Multivesicular bodies
NDN	Neurally differentiated embryonal carcinoma-derived gene
NLS	Nuclear localization signal
Nse3	Non-SMC-Element-3
OTCS	Opitz-C syndrome
PBS	Phosphate-buffered saline
PER	Period
PIAS1	Protein inhibitor of activated STAT-1
POMC	Pro-opiomelanocortin
PROVEAN	Protein Variation Effect Analyzer
PTM	Post translational modification
PVDF	Polyvinylidene difluoride
PWS	Prader-Willi syndrome
RBR	Ring-Between-Ring
REM	Rapid eye movement
RHT	Retinohypothalamic tract
RING	Really interesting new gene
SCF	Skp1/Cullin/F-box
SCN	Suprachiasmatic nucleus
SERT	Serotonin transporter

SDS	Sodium dodecyl sulfate
SMC	Structural Maintenance of Chromosomes proteins
SNRPN	Small Nuclear Ribonucleoprotein Polypeptide N
SYS	Schaaf-Yang syndrome
TBST	Tris-buffered saline-Tween
TBST-M	Tris-buffered saline-Tween plus milk powder
TRIM	Tripartite motif
Ub	Ubiquitin
UPS	Ubiquitin-protease system (UPS)
WH	Winged helix
WT	Wild-type

1 Chapter 1: Introduction

1.1 Prader-Willi syndrome

1.1.1 Clinical description

Prader-Willi syndrome (PWS) was originally described in 1956 by Prader, Labhart, and Willi. PWS is a complex genetic disorder of the nervous, musculoskeletal, and endocrine systems (OMIM #176270, Bittel & Butler, 2005; Cassidy, Schwartz, Miller, & Driscoll, 2012; Holm, Cassidy, Whitman, & Butler, 1993). PWS occurs in about 1 in 20 000 births (Whittington et al., 2000). PWS can initially present prenatally with reduced fetal activity and a below average size for gestational age in pregnancy (Dudley & Muscatelli, 2007; Whittington, Butler, & Holland, 2008). PWS results in neonatal hypotonia, delayed development, and feeding difficulties in newborns followed by hyperphagia in childhood (Çizmecioğlu et al., 2018). Hyperphagia is characterized by excessive or extreme hunger. Children with PWS present with extreme food seeking behaviours, obsessions with food, and overeating due to lack of satiety (Holland, Treasure, & Dallow, 1995; Holm et al., 1993). Hyperphagia can lead to obesity and further complications such as type 2 diabetes if left unmonitored (Nagail & Mori, 1999; Yang, Kim, Cho, & Jin, 2017). Children with PWS typically exhibit intellectual disability, developmental delay, and may present with autism spectrum disorder (ASD) (Chen et al., 2010; Dykens, Lee, & Roof, 2011; Whittington et al., 2004). PWS can also lead to behavioural problems such as aggression, tantrums, and obsessive-compulsive behaviours like skin picking (Dimitropoulos, 2010). Adolescents present with hypogonadism that results in immature sexual development and eventually infertility (Cassidy et al., 2012). Relative growth hormone deficiency leads to short stature, low muscle mass, and high body fat percentages (Cassidy et al., 2012). PWS can also be accompanied by distinctive facial features such as a narrow forehead, downturned mouth, and small hands and feet. The prevalence of scoliosis in PWS patients is 37.5% (Weiss & Goodall, 2009). Sleeping difficulties

are reported in both children and adults with PWS and include excessive daytime sleepiness (EDS), night-time wakening, and sleep apnea (Weselake & Wevrick, 2012). Other clinical findings include thick saliva, high pain thresholds, and light hair, skin, and eye color. Clinical findings of PWS are summarized in Table 1.

Table 1: Summary of Prader-Willi, Schaaf-Yang, and Bardet-Biedl syndromes - clinical features, implicated genes, mouse models

Disorder	Clinical features	Genes	Mouse models
Prader-Willi syndrome	Fetal akinesia Neonatal hypotonia Feeding difficulties in infancy, failure to thrive Hyperphagia Obesity Developmental delay/Intellectual disability Autism Spectrum Disorder Hypogonadism Short stature Small hands and feet Sleep abnormalities Excessive daytime sleepiness Scoliosis Skin picking	*MKRN3* *MAGEL2* *NDN* *NPAP1* *SNRPN* *SNHG14* (long non-coding RNA): *SNORD116* *IPW* *SNORD115*	Magel2$^{tm1.1Mu}$ (Schaller et al., 2010) Magel2^{tm1Stw} (Kozlov et al., 2007) Ndn$^{tm1.1Mus}$ (Muscatelli et al., 2000) Ndn^{tm1Alb} (Tsai & Armstrong, 1999) Ndn^{tm1Ky} (Kuwako et al., 2005) Ndn^{tm2Stw}(Gérard, Hernandez, Wevrick, & Stewart, 1999) Snrpn^{tm2Cbr} (PWS-ICdel) (Yang et al., 1998) Del(7Ube3a-Snrpn)1Alb (Tsai, Jiang, Bressler, Armstrong, & Beaudet, 1999) B6(Cg)-Snord116$^{tm1.1Uta}$(Ding et al., 2005) Del(7Ipw-Snord116)^{tm1Jbro} (Skryabin et al., 2007)

Schaaf-Yang syndrome	Fetal akinesia Neonatal hypotonia Feeding difficulties in infancy, failure to thrive Hypogonadism Developmental delay/Intellectual disability Autism Spectrum Disorder Small hands and feet Scoliosis Joint contractures Sleep apnea Gastroesophageal reflux	*MAGEL2*	Magel2$^{tm1.1Mu}$ (Schaller et al., 2010) Magel2^{tm1Stw} (Kozlov et al., 2007)
Bardet-Biedl syndrome [a]	Rod-cone dystrophy Vision loss Polydactyly Obesity Developmental delay/Intellectual disability Impaired speech Renal abnormalities Anosmia Male hypogonadism Short stature	*BBS1* *BBS2* *BBS3* (ARL6) *BBS4* *BBS5* *BBS6* (MKKS) *BBS7* *BBS8* (TTC8) *BBS9* *BBS10* *BBS11* (TRIM32) *BBS12* *BBS13* (MKS1) *BBS14* (CEP290/NPHP6/LCA10)	Bbs1$^{M390R/M390R}$ (Davis et al., 2007) LRb^{cre}/Bbs1$^{fl/fl}$ (Guo, Cui, Zhang, Morgan, & Thedens, 2016) Bbs2 $^{-/-}$ (Nishimura et al., 2004) Bbs3$^{-/-}$ (Zhang et al., 2011) Bbs4$^{-/-}$ (Mykytyn et al., 2004) Mkks$^{-/-}$ (Fath et al., 2005)

		BBS15 (WDPCP/FRITZ) *BBS16* (SDCCAG8) *BBS17* (LZTFL1) *BBS18* (BBIP1/10) *BBS19* (IFT27) *BBS20* (AZI1/CEP131) *BBS21* (C8ORF37)	

[a] Mutations found in BBS reviewed in Kaur, de Souza, Gibson & Meyre, 2017

1.1.2 Genetics of Prader-Willi syndrome

PWS is a sporadic multigene disorder caused by the loss of paternally expressed genes on chromosome 15q11-q13. Genes in the PWS region are maternally imprinted and therefore only expressed from the paternally inherited allele in a healthy individual. The majority of PWS cases (65-75%) are caused by a sporadic deletion of genes on the paternally inherited copy of chromosome 15q11-q13. The PWS genomic region has three common breakpoints resulting in two types of deletions: Type I deletion is 6.6 Mb (breakpoint 1 - break point 3) and Type 2 deletion is 5.3 Mb (breakpoint 2 - breakpoint 3) (Figure 1.1, Butler, Fischer, Kibiryeva, & Bittel, 2008; Carias & Wevrick, 2019). The majority of patients with PWS deletions have a Type II deletion (60%) (Bittel & Butler, 2005). Patients with a Type I deletion exhibited more severe behavioural and psychological problems when compared to Type II deletion patients (Butler, Bittel, Kibiryeva, & Talebizadeh, 2004). The second most common cause of PWS (20-30%) is maternal uniparental disomy of chromosome 15. A final subset of PWS cases (2-5%) are caused by sporadic or inherited mutations within the imprinting center that controls expression and methylation of genes in the PWS region.

The PWS region consists of both protein coding genes and non-coding RNAs (Figure 1.1). Several of these genes and non-coding RNAs contribute to the overall clinical presentation of PWS, however the loss of function of the *MAGEL2* gene is thought to play a major role in PWS symptoms. *MAGEL2* is one of several genes inactivated in children with PWS. Mutations in *MAGEL2* alone cause Schaaf-Yang syndrome, a disorder with overlapping phenotypes with PWS (Table 1).

Typically, a physician first suspects PWS in a newborn who exhibits hypotonia with poor suckling and feeding with failure to thrive in the first few days following birth. The physician will then request genetic testing in the form of DNA methylation analysis that targets the 5'CpG island

of the PWS region *SNRPN* gene. DNA methylation analysis can detect all subtypes of PWS, however it cannot distinguish between the subtypes. To determine if PWS is caused by a deletion, usually fluorescence in-situ hybridization (FISH) is used. To diagnose uniparental disomy, a specialized DNA test is performed that requires collection of parental blood samples. However, diagnostic testing for PWS has not been standardized across all clinics due to the wide array of readily available genetic tests (Smith & Hung, 2017).

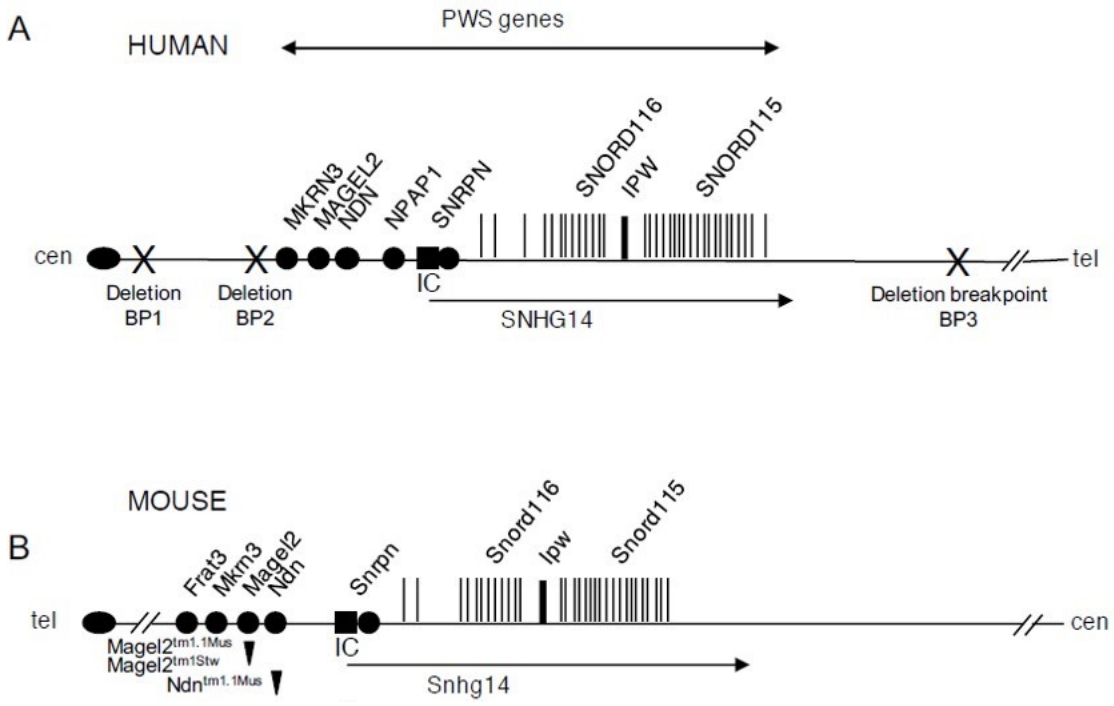


Figure 1.1 Genes implicated in Prader-Willi syndrome (PWS). A) Paternally expressed, imprinted genes located within the PWS deletion region are indicated on a genomic map of human chromosome 15q11-q13. Protein coding genes and non-coding RNAs are indicated as circles and vertical lines, respectively. Common breakpoints (BP; X) are found in cases of PWS by deletion of 15q11-q13. B) The mouse chromosome 7C region has homologous genes with the human PWS region with a few exceptions: mice do not have a homolog of human NPAP1, and Frat3 occurs exclusively in rodents. cen, centromere; IC, imprinting center (black box); tel, telomere. Figure modified from (Carias & Wevrick, 2019).

1.1.3 Therapeutics

Currently, pharmacological treatments for PWS are targeted at the specific symptoms and include hormone replacement therapy and psychiatric medications (Brokamp et al., 2019; McCandless, 2011). Due to the similarities between children with PWS and those with growth hormone deficiency in obesity, higher body fat composition, reduced muscle strength, and short stature, recombinant growth hormone is used to treat PWS (Grugni, Sartorio, & Crinò, 2016). Recombinant human growth hormone therapy is the standard of care for adults and children with PWS (Deal, Tony, Höybye, Allen, & Tauber, 2013). Growth hormone therapy results in increased height, lower body fat composition, increased bone mineral density, and improvements in difficult behaviours (Carrel, Myers, Whitman, & Allen, 1999; Whitman, Myers, Carrel, & David, 2002). Behavioural and physical therapies have also been used with positive outcomes in people with PWS (Dimitropoulos, 2010). However, there are no approved therapies to treat symptoms that affect the daily challenges of children and adults with PWS such as hyperphagia.

1.2 Schaaf-Yang syndrome

1.2.1 Clinical description

Children with a disorder that resembles PWS were found to have loss of function mutations in *MAGEL2*. This condition has been named Schaaf-Yang syndrome (SYS, OMIM #615447, Hamosh, Scott, Amberger, Bocchini, & Mckusick, 2005; Schaaf et al., 2013). SYS shares several phenotypes with PWS, such as neonatal hypotonia, endocrine dysfunction, hypogonadism, developmental delay, sleep apnea, scoliosis, intellectual disability, autism spectrum disorder, and maladaptive behavior (Table 1). Only a subset of children diagnosed with SYS (30-50%) have hyperphagia, whereas hyperphagia is present in almost all cases of PWS (Fountain & Schaaf, 2016; Fountain et al., 2017). Among the SYS cohort, a large range of clinical variability has been reported. Phenotypes range from prenatal arthrogryposis multiplex congenita resulting in death, to

post natal hypotonia with variable degrees of intellectual disability, to normal development (Table 1, Fountain et al., 2017). SYS has several phenotypes distinct from those seen in PWS, such as joint contractures, gastroesophageal reflux, respiratory distress, and abnormal temperature regulation (Mccarthy et al., 2018). Children with SYS also present with more severe intellectual disability scores compared to PWS children and have a higher prevalence of ASD (Thomason et al., 2018). Children with SYS may also exhibit varying facial dysmorphisms, with lack of consistent features across all patients (Fountain & Schaaf, 2016; Fountain et al., 2017).

1.3 MAGEL2

1.3.1 *MAGEL2*

MAGEL2 encodes a 1249 amino acid protein and is a member of the "MAGE" (Melanoma Antigen Gene) protein family. The MAGE protein family shares a ~200 amino acid conserved MAGE homology domain (MHD) (Figure 1.2, Lee & Potts, 2018). MAGEL2 was originally described as a protein of 529 amino acids, but recent data suggests the actual size of MAGEL2 is 1249 amino acids in length, encoded by a single exon (Figure 1.3). Endogenous MAGEL2 protein has yet to be detected using antibody-based techniques. Commercial antibodies have failed to detect MAGEL2 protein due to lack of specificity. Previous attempts made in our lab have failed due to the high sequence similarity of the MHD in various MAGE proteins, as well as the insolubility of the MAGEL2 protein. There are over 60 MAGE proteins expressed in the human body divided into two classes: Type I (45 genes, MAGE A, B, and C subfamilies) and Type II (15 genes, MAGE D, E, F, G, H, L, and Necdin) (Figure 1.2, Bruggen et al., 2019; Chomez et al., 2001; Espantman & Shea, 2010; Pold et al., 1999). Type I MAGE proteins are found in clusters on the X chromosome and are exclusively expressed in malignant tumors, testis, trophoblast, and the placenta (Chomez et al., 2001). Type II MAGE proteins are more widely expressed throughout the human body. Various MAGE genes, specifically Type I MAGEs, have been associated with

many types of tumors (Simpson, Caballero, Jungbluth, Chen, & Old, 2005; Weon & Potts, 2015). However, there are various emerging roles for Type II MAGE genes in genetic disorders: mutations in *MAGED2* cause antenatal Bartter's Syndrome (Laghmani et al., 2016), mutations in *MAGEG1* (NSMCE3) cause lung disease immunodeficiency and chromosome breakage syndrome (LICS) (Crabben et al., 2016), *NDN* in PWS (Macdonald & Wevrick, 1998) and a single case of Smith-Magenis syndrome (Berger, Billington, Fischer, Introne, & Gropman, 2018), and *MAGEL2* in PWS (Lee et al., 2000) and SYS (Schaaf et al., 2013b) (Table 2).

The MHD is approximately 40% conserved among all MAGE proteins (Chomez et al., 2001). MAGE proteins are further classified into subfamilies based on their sequence similarities (Chomez et al., 2001). The MHD consists of two winged-helix motifs (WH-A and WH-B) arranged in tandem, connected by a β-sheet domain (Lee & Potts, 2018; Newman, Cooper, & Roos, 2016). The MHD has been shown to be important for protein-protein interactions, specifically interactions with really interesting new gene (RING)-finger E3 ubiquitin ligases (Doyle, Gao, Wang, Yang, & Potts, 2010; Feng, Gao, & Yang, 2011; Lee & Potts, 2018). Mutations in the MHD alter MAGE protein function and binding abilities (Doyle et al., 2010; Wijesuriya et al., 2017). The dileucine motif in the MHD is conserved across all MAGE proteins and forms part of the hydrophobic core of the WH-A important for protein-protein interactions (Doyle et al., 2010; Newman et al., 2016). Mutations of the dileucine motif to two alanines resulted in failure of MAGE protein, MAGEG1 to bind and activate RING E3 ubiquitin ligase NSE1, and failure of MAGEL2 to bind to the leptin receptor and regulate its cell surface abundance (Doyle et al., 2010; Wijesuriya et al., 2017). Mutations in a conserved arginine residue (R1187) in the mammalian MAGEL2 protein disrupts protein binding and function (Wijesuriya et al., 2017). In another MAGE protein, MAGED2 a mutation of this arginine to a cysteine was deemed pathogenic causing antenatal

kidney disease and was predicted to be very deleterious using predictive software tool PROVEAN (Protein Variation Effect Analyzer) (Choi, Chan, & Craig, 2015; Laghmani et al., 2016). In this thesis, I use MAGEL2 mutant constructs harboring the two previously described MHD mutations to study MAGEL2 protein interactions and function (Figure 1.3, MAGEL2p.LL1031AA and MAGEL2p.R1187C).

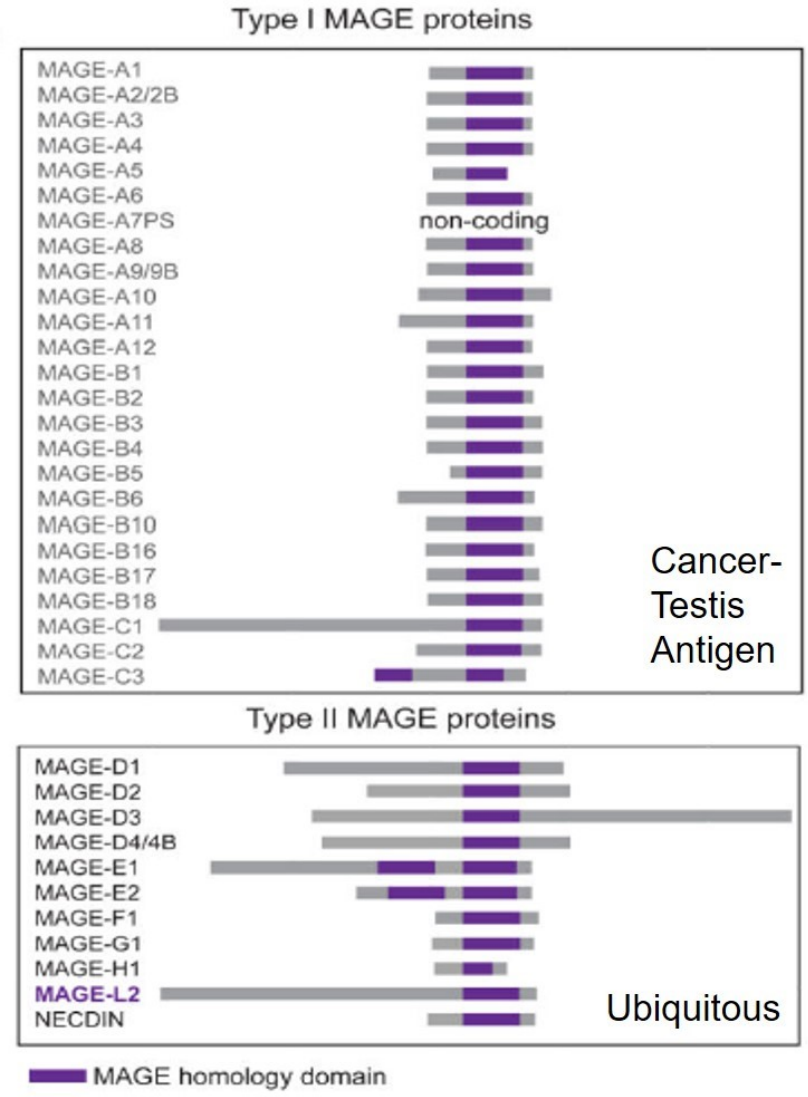


Figure 1.2 The MAGE homology domain (MHD) is shared between Type I and Type II MAGE proteins. There are over 60 MAGE proteins that share an approximate 200 amino acid MHD. The conserved MHD is indicated as a purple rectangle on the representative chromosomes. Type I MAGE proteins are expressed in testis and other reproductive tissues and have been associated with various cancers. Type II MAGE proteins are found ubiquitously throughout the human body. Figure modified from (Tacer & Potts, 2018).

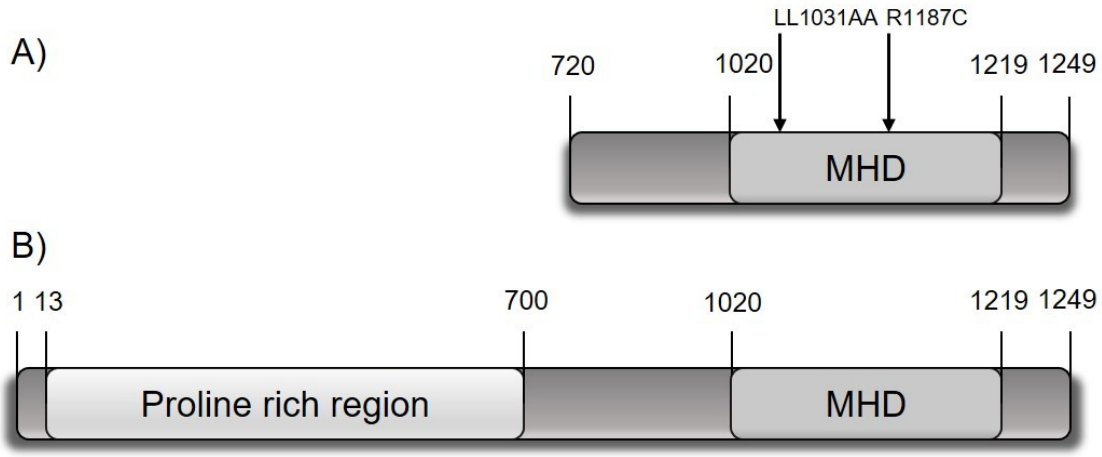


Figure 1.3 Graphical representation of the MAGEL2 protein. A) *MAGEL2* was originally thought to encode a 529 amino acid protein that included the MAGE homology domain. B) Recent data suggests MAGEL2 consists of 1249 amino acids and contains a N terminal proline rich region. Domains are labelled within the protein. Numbers with black lines represent amino acid positions, starting at the N-terminus. Black arrows indicate MAGEL2 MHD mutations used in this study (MAGEL2p.LL1031AA and MAGEL2p.R1187C). Figure adapted from (Urreizti et al., 2017).

Table 2: Overview of MAGE proteins involved in ubiquitination and implicated in genetic disorders

MAGE protein	E3 interacting partner	Deubiquitinase interacting partner	Disorder(s)
MAGEL2	RNF41 TRIM27	USP7/USP8	Prader-Willi syndrome Schaaf-Yang syndrome
Necdin			Prader-Willi syndrome Smith-Magenis syndrome
MAGEA1 [b]	TRIM28 TRIM31		
MAGEA2 [b]	TRIM28 MDM2		
MAGEA3 [b]	TRIM28		
MAGEA4 [b]	TRIM69		
MAGEA6 [b]	TRIM28		
MAGEA11 [b]	Skp2 (CRL) [a]		
MAGEB2 [b]	TRIM28		
MAGEB18 [b]	LNX1		
MAGEC2 [b]	TRIM28 RBX1(CRL) [a]		
MAGED1	TRIM28 Praja-1 XIAP		

MAGED2			Bartter's syndrome
MAGED4B	TRIM27		
MAGEE1	TRIM28		
MAGEF1	NSE1 TRIM27		
MAGEG1	NSE1 Praja-1		Lung disease immunodeficiency and chromosome breakage syndrome (LICS)
MAGEH1			

[a] Proteins are part of a Cullin-RING ligase (CRL) multi-protein ubiquitin ligase complex
[b] MAGE A, B, and C genes are dysregulated in various cancers

MAGEL2 is primarily expressed in the human brain, specifically in the suprachiasmatic nucleus (SCN) and arcuate nucleus (ARC) of the hypothalamus and the cerebral cortex. *MAGEL2* is also expressed in the developing central nervous system, skeletal muscle, adrenal glands, and fetal kidney, liver, and lung tissues (Kamaludin et al., 2016; Lee et al., 2000). In mice, *Magel2* was expressed in late embryonic stages day E11, E15, and E17, as well as in the adult brain (Lee et al., 2000).

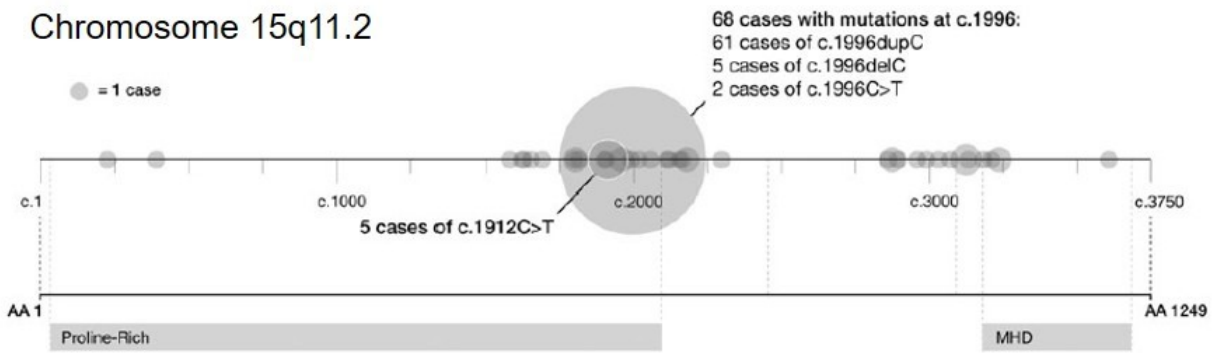
Loss of MAGEL2 expression and function in the hypothalamus could result in many PWS traits, such as growth hormone (GH) deficiency, obesity, and abnormal circadian rhythm. The hypothalamus is a region of the mammalian forebrain and consists of distinct nuclei that control various biological functions. The hypothalamus has a critical role in energy balance and homeostasis and connects the functions of the nervous and endocrine systems through the hypothalamic-pituitary axis. The hypothalamus receives peripheral signals from the nervous system and triggers the release of neurohormones to stimulate release of hormones from the pituitary gland. The hypothalamus regulates body temperature, satiety and hunger, behaviours, reproduction and sexual maturity, and circadian rhythm and sleep. Neurons in the ARC are responsible for the release of growth hormone-releasing hormone (GHRH) that signals to the pituitary gland to stimulate GH secretion. GH regulates body and bone growth, body composition, and metabolism. Children with PWS have GH deficiency resulting in short stature and delayed or halted puberty that could result from the loss of MAGEL2 function in the ARC (Aycan & Baş, 2014; Burman, Ritze, & Lindgren, 2001).

1.3.2 *MAGEL2* mutations

People with deletions of the entire *MAGEL2* gene exhibit milder phenotypes than children with truncating *MAGEL2* mutations (Buiting et al., 2014; Fountain, Tao, & Chen, 2017). This phenomenon could be due to the possible leaky expression of the maternal *MAGEL2* copy when

the entire *MAGEL2* gene and its promoter are deleted (Fountain & Schaaf, 2016; Matarazzo & Muscatelli, 2013). Patients with the c.1996dupC *MAGEL2* mutation had more severe phenotypes than patients with any other mutations identified in the SYS cohort (Mccarthy et al., 2018). Children with the duplication mutation had more severe intellectual disability and developmental delay, and higher rates of joint contractures, feeding difficulties, and respiratory distress (McCarthy et al., 2018). To date, the majority of identified mutations in *MAGEL2* occur at the c.1990-c.1996 nucleotide site hotspot (Figure 1.4, Mccarthy et al., 2018). A *MAGEL2* mutation (*de novo* nonsense mutation p.Q638*) was also identified in a proband diagnosed with Opitz-C syndrome (OTCS) with symptoms that resemble SYS yet exhibits symptoms unique to OTCS (Urreizti et al., 2017). The clinical variability seen in people with *MAGEL2* mutations could be due to the type and location of the mutation. To date there are 115 individuals identified with truncating mutations of *MAGEL2* caused by either a nonsense or frameshift mutation (Figure 1.4, Mccarthy et al., 2018).

Different versions of truncated MAGEL2 protein caused by the various *MAGEL2* mutations identified in SYS are likely to cause the phenotypes unique to children with SYS. Meanwhile, in PWS there is a complete loss of *MAGEL2* and therefore no MAGEL2 protein, and this could result in the PWS specific phenotypes. Since MAGEL2 is encoded by a single exon, in SYS truncated *MAGEL2* mRNA may not be targeted for nonsense mediated decay and would not be degraded. This partial truncated MAGEL2 protein could have gain-of-function that results in the phenotypes seen in SYS but not reported in PWS.



***MAGEL2* mutations by location**

1	c.224delC	1	c.1900G>T	1	c.2113_2114del	1	c.2989G>A
1	c.390delA	1	c.1906C>T	1	c.2118delT	1	c.3031A>T
1	c.1580delC	5	c.1912C>T	1	c.2153C>A	1	c.3070delG
1	c.1621C>T	2	c.1958_1962del5	1	c.2163C>A	3	c.3124C>T
1	c.1628delC	1	c.1990_1991delinsA	2	c.2179_2180del	1	c.3131C>A
1	c.1652delT	61	c.1996dupC	1	c.2296C>T	1	c.3181_3182del
1	c.1690C>G	5	c.1996delC	1	c.2861_2862insG	1	c.3208G>T
1	c.1797_1820del	2	c.1996C>T	2	c.2873G>A	2	c.3235G>T
2	c.1802delC c.1801_1802del	1	c.2015delC	1	c.2894G>A	1	c.3607A>T
1	c.1813G>T	1	c.2056_2066del	1	c.2958delG		

Figure 1.4 MAGEL2 mutations identified to date depicted on a graphical representation of chromosome 15q11.2. The top panel shows a graphical representation of the *MAGEL2* mutations identified to date on Chromosome 15q11.2. All 115 *MAGEL2* mutations are described in the table below. Figure modified from (Mccarthy et al., 2018).

1.3.3 *Magel2* mouse models

The *Magel2* mouse gene is located on chromosome 7C and has high sequence homology with the human *MAGEL2* gene (Figure 1.1). Two mouse models have been developed to study the loss of *Magel2* in a model organism. The Magel2^{tm1Stw} mouse model carries a lacZ insertion replacing the C terminal domain of *Magel2* that encompasses the MHD (JAX stock: 009062, Bischof, Stewart, & Wevrick, 2007; Kozlov et al., 2007). The second *Magel2* mouse line, Magel2$^{tm1.1Mus}$ harbours a deletion of the *Magel2* promoter and most of the open reading frame (Schaller et al., 2010). Most of the phenotypic characterization of Magel2 mutant mice has been performed on the Magel2^{tm1Stw}. Magel2^{tm1Stw} mice displayed neonatal growth retardation, excessive weight gain after weaning, increased adiposity in adulthood, altered circadian rhythm, abnormal brain volume, decreased serotonin and 5-hydroxyindoleacetic acid, reduced motor activity and reduced fertility (Bischof et al., 2007; Kozlov et al., 2007; Mercer & Wevrick, 2009). Magel2^{tm1Stw} mice exhibit sleep disruptions (Mahoney et al., 2017). Assessment of Magel2^{tm1Stw} mice using behavioural tests demonstrated reduced anxiety and lack of preference for novel social interactions compared to wild-type mice (Fountain et al., 2017). The Magel2 $^{tm1.1Mus}$ male mice also had defects in social interaction and recognition and displayed deficits in learning (Meziane et al., 2015). Leptin hormone regulates food intake as previously described, and leptin resistance or deficiency could result in obesity. Magel2^{tm1Stw} mice have significantly impaired POMC neuron activity in response to leptin, abnormal arcuate nucleus development, and are resistant to the effect of peripherally administered leptin hormone (Maillard et al., 2016; Mercer et al., 2013; Oncul et al., 2018). In accordance with the obesity phenotypes seen in children and adults with PWS, Magel2^{tm1Stw} adult mice were leptin resistant, while Magel2^{tm1Stw} pups are sensitive to leptin (Pravdivyi, Ballanyi, Colmers, & Wevrick, 2015). The changes in responses to leptin from pups to adult mice could explain the delayed onset of hyperphagia seen in children with PWS (Pravdivyi, Ballanyi,

Colmers, & Wevrick, 2015). Magel2^{tm1Stw} mice had dopaminergic dysfunction and pathway imbalances (Luck, Vitaterna, & Wevrick, 2016). They also had increased fat mass and decreased muscle mass accompanied by progressive reduction in limb strength, activity, and endurance (Kamaludin et al., 2016; Meziane et al., 2015; Schaller et al., 2010). Magel2^{tm1Stw} mice had reduced bone mass and length compared to their wild-type littermates caused by reduced bone formation and increased bone resorption, bone marrow adiposity and osteoclastogenesis (Baraghithy et al., 2019). The MAGEL2$^{tm1.1Mus}$ mice had suckling defects causing slow growth and lethality in pups as well as deficits in social recognition and interaction with reduced abilities to learn (Schaller et al., 2010, Meziane, et al., 2015). *Magel2* mice recapitulate various phenotypes seen in children with PWS and SYS.

1.4 Ubiquitination

1.4.1 The ubiquitin system

MAGE proteins, including MAGEL2 function as modulators of ubiquitination, a post translational modification (PTM). PTMs are critical for protein and cell function and involve chemical modifications of proteins through the addition of functional groups to amino acid side chains. PTMs introduce a further level of complexity and regulation to the proteome. There are over 200 types of PTMs that influence protein localization, function, activity, and interactions (Deribe, Pawson, & Dikic, 2010).

Ubiquitination is a PTM that involves the addition of a 76 amino acid ubiquitin to a target substrate protein. Ubiquitinated proteins are then targeted towards the 26S proteasome for degradation through the ubiquitin-protease system (UPS) or further downstream modifications such as changes in activity, subcellular localization, protein-protein interactions or signalling. Substrate proteins are mono-, multimono- or poly-ubiquitinated on various lysine residues and chains can be mixed or branched. Ubiquitination plays a role in almost all cell processes including

cell cycle, regulation of transcription, intracellular signalling, endosomal sorting, circadian rhythm, and neuronal development, plasticity, and synapse remodelling.

The ubiquitination process is a three-step cascade that involves three classes of enzymes: ubiquitin activating (E1) enzymes, ubiquitin conjugating (E2) enzymes, and ubiquitin ligase (E3) (Figure 1.5, Deshaies & Joazeiro, 2009). Regulatory proteins that modulate E3 ligase activity also play an important role in the ubiquitination of substrate proteins. In an ATP-dependent process, ubiquitin is first activated by the covalent binding of the E1 enzyme active site cysteine to the C terminus of ubiquitin proteins. E2 enzymes transfer the activated ubiquitin from E1 to E3 enzymes through a thioesterification reaction. E3 enzymes are responsible for substrate recognition and for the transfer of activated ubiquitin from E2 enzymes onto the target protein. There are over 40 E2 ubiquitin conjugating enzymes and over 600 E3 ubiquitin ligases encoded in the human genome (Stewart, Ritterhoff, Klevit, & Brzovic, 2016; Zheng & Shabek, 2017). E3 enzymes recognize and bind to specific substrate proteins to influence their ubiquitination levels and regulation. Protein modifiers, adaptors, and scaffolds also regulate substrate ubiquitination levels and binding. There are three classes of E3 ligase proteins containing different domains: homologous to the E6AP carboxyl terminus (HECT), really interesting new gene (RING)-finger, and Ring-Between-Ring (RBR) (Zheng & Shabek, 2017). The majority of identified E3 ligases belong to the RING-finger E3 ubiquitin ligase family. RING-E3 ligases function by binding to E2 enzymes through their Zinc finger RING domain, which is made of cysteine and histidine residues and mediates direct transfer of ubiquitin from E2 enzymes onto the target protein. The type of ubiquitin chain(s) assembled on a substrate protein determines protein fates.

Ubiquitination of a substrate protein is reversible through the action of deubiquitinating enzymes (DUBs) (Figure 1.5). DUBs catalytically remove mono- or poly-ubiquitin chains from

target proteins and can alter protein fate. There are over 100 DUBs encoded in the human genome that can interact with E2 enzymes and E3 ligases to interrupt and regulate the ubiquitin-substrate loading cascade (Reyes Turcu, Ventii, & Wilkinson, 2009). DUBs can specifically bind to substrates to facilitate removal of ubiquitin molecules directly or through adaptor and scaffold proteins. A further level of substrate specificity and ubiquitination regulation involves the interactions among E3 ligases, DUBS, and adaptor or modulator proteins.

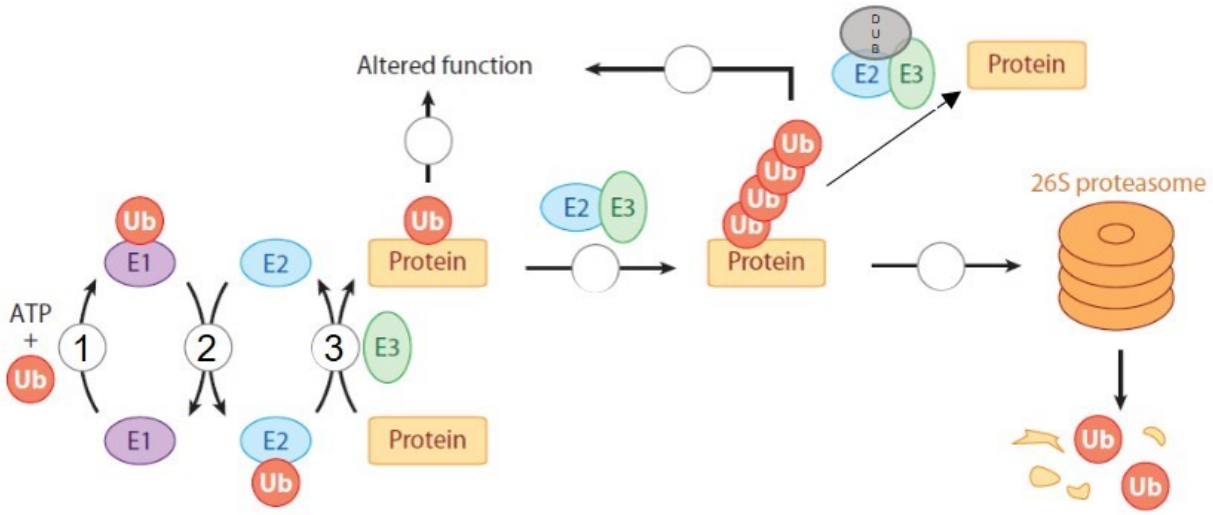


Figure 1.5 The ubiquitin system tags protein with ubiquitin in a step-wise cascade. Ubiquitination involves ubiquitin activating (E1) enzymes, ubiquitin conjugating (E2) enzymes, and ubiquitin ligases (E3). Proteins are tagged with varying chains of ubiquitin molecules and are either targeted for proteasomal degradation through the 26S proteasome or targeted toward an altered function. Deubiquitinating enzymes (DUBs) can then remove ubiquitin from proteins when the ubiquitin signal is no longer needed. Figure modified from (Deshaies & Joazeiro, 2009).

1.4.2 Role of MAGEL2 in ubiquitination

Although MAGE proteins have been primarily investigated for their role in tumor cells, there is emerging evidence of MAGE protein function in ubiquitination mediated processes (Lee & Potts, 2018). The MAGE family of proteins is conserved in all eukaryotes from invertebrates, fungi, plants, to mammals (Donato, Peters, Hussain, & Rodulfo, 2017; Weon & Potts, 2015). For example, the singular MAGE protein found in *Drosophila* is a homolog of the yeast non-SMC-Element-3 (*Nse3*) and necessary in the evolutionary conserved Structural Maintenance of Chromosomes proteins (SMC) complex that regulates genomic stability (Li et al., 2013). Members of the mammalian MAGE family have seen recent rapid evolutionary expansion but can be traced back to protozoa (Lee & Potts, 2018; Weon & Potts, 2015). MAGE proteins interact with RING-zinc finger-type E3 ubiquitin ligases and ubiquitin-specific proteases (deubiquitinases) to influence protein ubiquitination levels (Doyle et al., 2010; Lee & Potts, 2018). MAGE proteins can influence E3 ligase substrate specificity or activity through interactions with the ubiquitin system (Lee & Potts, 2018). Many MAGE proteins have been identified as ubiquitin system interactors reviewed in Table 2. Several of these MAGE proteins, including MAGEL2, are implicated in genetic disorders. Understanding the cellular role of these proteins will be critical in understanding the pathophysiology of these complex syndromes (Table 2).

Until recently, the role of MAGEL2 in the cell had not been elucidated. Endosomal protein trafficking is the movement of proteins from early endosomes to the trans-Golgi network or plasma membrane and is essential to protein recycling and function. Endosomal protein trafficking is facilitated by the WASH complex that binds actin to stimulate filament nucleation essential in protein trafficking (Duleh & Welch, 2010). MAGEL2 was found to interact with RING-zinc finger-type E3 ubiquitin ligase TRIM27 to modify the ubiquitination and stability of the WASH regulatory complex involved in the regulation of retromer-mediated transport (Hao et al., 2013).

The MAGEL2-TRIM27 complex localizes to Retromer-positive endosomes and facilitates lysine 63 ubiquitination of WASH1 to promote F-actin nucleation required for endosome-to-Golgi and endosome-to-plasma membrane protein transport (Hao et al., 2013). Silencing of MAGEL2 or TRIM27 using siRNAs in cultured cells resulted in perturbed protein trafficking through reduced endosomal F-actin levels (Hao et al., 2013). Deubiquitinase USP7 regulates deubiquitination of TRIM27 and the WASH complex to promote stabilization of this protein complex (Hao et al., 2015).

MAGEL2 interacts with another MAGE protein Necdin, which is also deleted in the PWS 15q11-13 chromosomal region. *Neurally differentiated embryonal carcinoma-derived* (*NDN*) encodes the protein Necdin that interacts with various proteins involved in transcription, mitochondria biogenesis, DNA damage response, and cell-membrane receptor signaling (Hasegawa & Yoshikawa, 2008; Hasegawa et al., 2016; Lavi-itzkovitz et al., 2012; Wijesuriya et al., 2017). Necdin functions in neuronal differentiation and survival (Aebischer, Sturny, Andrieu, Rieusset, & Schaller, 2011; Huang, Fujiwara, Minamide, Hasegawa, & Yoshikawa, 2013). Necdin interacts with E3 ubiquitin ligase protein inhibitor of activated STAT-1 (PIAS1) and promoted self ubiquitination and subsequent proteasomal degradation of PIAS1 (Gur, Fujiwara, Hasegawa, & Yoshikawa, 2014). Interactions between Necdin and MAGEL2 may contribute to MAGEL2 function and regulation in cellular processes.

Leptin receptor expressed in the hypothalamus regulates appetite and energy balance. The endosomal sorting complex required for transport (ESCRT) machinery made up of ESCRT subcomplexes (0, I, II, and III) is responsible for LepR trafficking from endosomes to the plasma membrane. LepR levels, sorting, and degradation are regulated by ubiquitination. RING-zinc finger-type E3 RING ubiquitin ligase RNF41 and deubiquitinase USP8 are responsible for LepR

sorting (Ceuninck, Wauman, Masschaele, Peelman, & Tavernier, 2013). MAGEL2 forms a complex with Necdin, RNF41, USP8, and STAM (a component of the ESCRT-0 subcomplex) to regulate leptin receptor abundance at the cell surface (Wijesuriya et al., 2017). MAGEL2 and Necdin act as modifier and scaffold proteins by connecting the leptin receptor to RNF41 and USP8 (Wijesuriya et al., 2017). MAGEL2 increased the expression of the leptin receptor at the cell surface and decreased receptor degradation through its regulation of ubiquitination of RNF41-USP8 and protein stability (Wijesuriya et al., 2017). The role of MAGEL2 and Necdin in leptin receptor trafficking and stability could be a proposed cellular mechanism for the obesity seen in people with PWS. Understanding the role that MAGEL2 plays in ubiquitination pathways of various substrate proteins would lead to a better understanding of neurodevelopmental disorders with MAGEL2 function disruptions.

1.5 Aims and Hypothesis

When I first started my thesis research, the cellular role of MAGEL2 as a modulator of ubiquitination had just been identified (Hao et al., 2013). Up until 2013, the functional role of MAGEL2 at the cellular level had not been fully elucidated. The identification of other MAGE family proteins interacting with E3 ligases made it an intriguing idea that MAGEL2 also modulates ubiquitination. Evidence through mouse models and cell lines pointed to MAGEL2 having an important role in various physiological functions, such as hypothalamic regulation and circadian rhythm, but how MAGEL2 was involved in this regulation at the cellular level remained unknown. The objective of my thesis was to further characterize the role of MAGEL2 at the cellular level. My aim was to determine the role MAGEL2 plays in ubiquitination pathways through its interactions with E3 ubiquitin ligases, deubiquitinases, and substrate proteins. My hypothesis is that MAGEL2 interacts with E3 ligases and deubiquitinases to modulate the ubiquitination of key substrates. When this system is perturbed, this results in the phenotypes seen in people with PWS, SYS or related neurodevelopmental disorders.

In this thesis I will focus on the role of MAGEL2 in two pathways:

1. MAGEL2 and circadian rhythm

Circadian rhythm is the endogenous oscillation of physiological and cellular functions over a roughly 24-hour period seen in many organisms (Reppert & Weaver, 2002). In mammals, the suprachiasmatic nucleus of the hypothalamus is known as the "master clock regulator" and is responsible for circadian rhythm outputs. Circadian rhythm is regulated at the cellular level by transcriptional-translational feedback loops involving core circadian rhythm proteins. MAGEL2 was shown to interact with circadian rhythm genes and proteins and *Magel2* mice have circadian rhythm defects (Devos, Weselake, & Wevrick, 2011; Kozlov et al., 2007). However, the cellular

role of MAGEL2 in circadian rhythm remains to be fully elucidated and will be the objective of Chapter 3.

2. MAGEL2 and the BBSome

The hormone leptin sends signals to leptin receptors (LepR) located in the arcuate nucleus (ARC) of the hypothalamus that are responsible for inhibiting food intake and promoting energy expenditure to regulate body weight. Leptin is produced from adipose tissue in proportion to the amount of adiposity and is a measure of long-term fat stores. Leptin signals to pro-opiomelanocortin (POMC) lateral neurons in the ARC to inhibit appetite. In contrast, agouti-related peptide (AgRP) neurons are responsible for promoting hunger. Leptin signaling in the ARC depolarizes/activates POMC neurons while hyperpolarizing/inhibiting AgRP neurons. Leptin resistance or perturbations to leptin hormone signaling in the hypothalamus causes obesity (Zhou, 2013). Hyperphagia and obesity seen in children with PWS could result from abnormalities of leptin signaling in the brain. Bardet-Biedl syndrome (BBS) is another neurodevelopmental disorder with overlapping phenotypes with PWS such as obesity (Table 1). BBS is caused by mutations in several genes, some of which encode BBSome proteins (OMIM #209900, Hamosh et al., 2005). The BBSome is responsible for cilia biogenesis and function, as well as protein trafficking to the cilia and cell membranes. BBS is caused by mutations in genes involved in the BBSome that also function in LepR trafficking (Guo et al., 2016; Seo et al., 2009). Understanding the interplay between MAGEL2 and BBSome proteins regarding LepR receptor trafficking could explain the overlapping pathologies seen in PWS and BBS and will be the objective of Chapter 4.

In summary, the aim of this thesis was to investigate the cellular role of MAGEL2 in the ubiquitin system that could contribute to phenotypes seen in children with PWS, SYS, or related neurodevelopmental disorders. Specifically, I wanted to determine the role of MAGEL2 in the

regulation of circadian rhythm through interactions with the ubiquitin system and core circadian rhythm proteins (Chapter 3). I also wanted to investigate the role of MAGEL2 in ubiquitination of proteins important in Bardet-Biedl syndrome, a neurodevelopmental disorder that has some phenotypic overlap with Prader-Willi and Schaaf-Yang syndromes (Chapter 4).

2 Chapter 2: Materials and Methods

2.1 Expression plasmids

Various vectors for expression in mammalian cells were created using the Gateway Cloning system. Gateway cloning is a rapid and efficient way to clone the cDNA of a gene of interest into expression vectors, which may express an epitope tag. The entry vectors consist of the gene of interest flanked by recombination sequences attL1 and attL2. The destination vectors carry an cDNA encoding an epitope tag flanked by recombination sequences attR1 and attR2 and an ampicillin resistance gene. The LR clonase enzyme (Thermofisher Scientific #11791020) catalyzes the recombination between the attL sites of the entry vector and the attR sites of the destination vector to create an expression vector. The expression vector carrying the cDNA for an epitope-tagged gene of interest is transformed into One Shot Top10 chemically competent *E. coli* (Thermofisher Scientific #C404010) and selected for using ampicillin treatment. Entry clones with cDNAs for human RBX1, TRIM27, USP7, CRY1, CRY2, MAGEL2, TRIM32, BBS2, and USP8 were obtained from the DNASU plasmid repository (Seiler et al., 2014). Entry clones were recombined into destination vectors (pcDNA-DEST47 vector for GFP, pcDNA3.1nV5-DEST for V5, pDEST-pcDNA5-FLAG for FLAG, or pDEST-pcDNA5-BirA*-FLAG for BirA*-FLAG) to create epitope-tagged cDNA constructs.

HA-Ub (hemagglutinin-ubiquitin) was expressed from pRK5-HA-Ubiquitin-WT, a gift from Ted Dawson (Addgene plasmid # 17608, Lim et al., 2005). Plasmids encoding GFP-WASH1 and GFP-VPS35 were generously provided by Dr. M. Seaman (Cambridge Institute for Medical Research). Mutant MAGEL2 constructs were generated using site-directed mutagenesis of pENTR-MAGEL2 cDNA to create MAGEL2p.R1187C and MAGEL2p.LL1031AA as previously described in Wijesuriya et al., 2017. Mutations were confirmed by sequencing then transferred into pDEST-pcDNA5-FLAG and pDEST-pcDNA5-BirA*-FLAG (Wijesuriya et al., 2017).

pDEST-pcDNA5-FLAG, pDEST-pcDNA5-BirA*-FLAG, and pDEST-pcDNA5-BirA*-FLAG-NLS were generously provided by Dr. A-C. Gingras (Lunenfeld-Tanenbaum Research Institute) (Couzens et al., 2013). TRIM32 mutant constructs (V5-TRIM32.P130S and V5-TRIM32.D487N) were kindly provided by Dr. Gerd Walz at the University Medical Centre (Freiburg, Germany) (Ramachandran et al., 2014). TRIM32iRING (inactive RING) is a mutant form of TRIM32 in which Cys20, Cys39, and His 41 in the RING domain are replaced by alanines (C20A/C39A/H41A), and was kindly provided by Dr. Gerd Walz (University Medical Centre, Freiburg, Germany) (Ramachandran, 2014). pFLAG-BBS2 was provided by Dr. Val Sheffield (University of Iowa).

A partial MAGEL2 protein was used for all experiments within this thesis. The partial MAGEL2 protein contains the C terminal of the MAGEL2 protein which includes amino acids 720 - 1249 which encompasses the MAGE homology domain (Figure 1.3A).

2.2 Cell culture

Tissue culture reagents were from Life Technologies (Carlsbad, CA) unless otherwise stated. Human osteosarcoma (U2OS) cells and human embryonic kidney 293T (HEK 293T) cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% l-glutamine, and 1% penicillin/streptomycin, and cultured at 37°C, in 5% CO_2. U2OS cells were seeded at a density of $2.3x10^5$ cells/well in a 6-well plate, 24 h before transfection of plasmids using Effectene (Qiagen, Mississauga, Canada). Cells were transfected with a ratio of 1:8:10 of DNA: Enhancer: Effectene. Firstly, 0.8µg of plasmid(s) was diluted in 100 µl Buffer EC, enhancer was added then incubated at room temperature for 5 min. Effectene reagent was then added, vortexed, and incubated at room temperature for 10 min. The media was removed from plates, cells were washed with 1 ml phosphate-buffered saline (PBS), and replaced with fresh

media. 600 µl of fresh media was added to the transfection mixture and then added dropwise to each well. Cells were returned to the incubator for 24 h before collection.

HEK 293T cells were seeded at a density of $8.3x10^5$ cells/well in 100 mm dishes, 24 h before transfection of plasmids with Fugene6 (Promega, Madison, WI) at a ratio of 3:1 (Fugene6: DNA). Fugene6 was added to 300 µl of serum free media and incubated at room temperature for 5 min. DNA was then added to the transfection mixture, vortexed, and incubated at room temperature for 15 min. Media was removed from the 100 mm dishes and replaced with fresh media. Transfection mixture was added dropwise to each plate and plates were placed in the incubator.

A stable cell line carrying a tetracycline inducible BirA*-FLAG-MAGEL2 was created in HEK 293T-Rex Flp-In cells (Invitrogen) by selection in 200 µg/ml hygromycin B. Single copy integration of *MAGEL2* is carried out by the Flp recombinase at a unique target site. MAGEL2 expression in these cells is induced by treating cells with 1 µg/ml tetracycline for 24 hours. Control cells are not treated with tetracycline and are uninduced for MAGEL2 expression. Construction and validation of the stable MAGEL2 inducible HEK 293T-Rex Flp-In cells was previously described in (Wijesuriya et al., 2017).

Empty vector plasmids were used as controls and to normalize the amount of plasmid DNA used in each transfection.

2.3 Immunoblotting

Cell lysates were collected 24 h after transfection. Media was removed from the 6 well plates and cells were washed 2 x 5 min with PBS. Cells were spun at 420 g for 6 min and washed with PBS three times then resuspended in 2x modified sample buffer (MSB, 20% glycerol, 4% SDS, 2% beta-mercaptoethanol, 1% bromophenol blue, 130 mM Tris-HCl, pH 6.8) with Complete Mini Protease Inhibitor (Roche Applied Science, Indianapolis, IN), sonicated (3 x 5 sec on/5 sec off), heated to 65°C, spun at 20 800 x g for 10 min, and boiled for 5 min. Protein was quantified using

a BCA protein assay (Pierce, Rockford IL), equal amounts of protein were loaded into each lane, resolved on 8-12% SDS-PAGE gels, transferred to PVDF membranes and immunoblotted. Blots were re-probed with anti-beta-actin to verify equal protein loaded into each lane.

Primary antibodies used were: rabbit anti-HA (Santa Cruz Biotechnology sc-805, 1:500), mouse anti-HA (Thermofisher Scientific Cat #26183, 1:500), mouse anti-V5 (Abcam #ab27671, 1:1000), rabbit anti-FLAG (Sigma #F7425, 1:5000), and chicken anti-GFP (Abcam #ab13970, 1:5000). and HRP-conjugated anti-actin antibodies (Sigma #A3854, 1:50 000) (Table 3). Blots were incubated in primary antibodies overnight at 4°C. Blots were washed 3 x 10 min in Tris-buffered saline-Tween (TBST, 137 mM NaCl, 0.1% Tween-20, 20 mM Tris-HCl, pH 7.5) and incubated in secondary antibody for 1 h at room temperature. Secondary antibodies used were: HRP-linked donkey anti-rabbit IgG (Amersham Pharmacia Biotech #NA934, 1:5000), HRP-linked sheep anti-mouse (Amersham Pharmacia Biotech #RPN4201, 1:5000), and HRP-linked donkey anti-chicken (Thermofisher Scientific #SA1-300, 1:1000) (Table 3). Antibodies were prepared in TBST plus milk powder (TBST-M, 5% non-fat dry milk powder in 137 mM NaCl, 0.1% Tween-20, 20 mM Tris-HCl, pH 7.5). Blots were stripped using Restore western blot stripping buffer (Thermofisher Scientific #21059) and re-probed with anti-actin to normalize for protein amount loaded into each lane.

Blots were incubated at room temperature in Immobilon Western Chemiluminescent HRP substrate (Millipore) for 5 min. Signals on immunoblots were visualized on a Kodak imager and signal intensities were measured by NIH Image J.

2.4 BioID proximity labelling interaction assay

U2OS cells were plated at a density of $3x10^5$ cells/well in 6 well plates. Cells were transfected using Effectene with 0.8 µg of a BirA-expressing plasmid and 0.8 µg plasmid encoding a potentially interacting protein, with 2 wells per transfection. After transfection, biotin was added

to a final concentration of 50 µM. Cells were processed for BioID 24 h after transfection. Media was removed from the wells and washed 3 x 5 min with 1 ml PBS to remove excess biotin. Cells were collected in 400 µl lysis buffer (50 mM Tris HCl, 500 mM NaCl, 0.2% SDS, 2% Triton-X, 1 mM DTT, plus Complete Mini protease inhibitor, pH 7.5). Lysates were sonicated (3 x 5 sec on/5 sec off) and centrifuged at 16 500 x g for 10 min at 4°C. Lysates were then filtered using Amicon Ultra Centrifugal units (Millipore #UFC500324). A 50 µl aliquot was reserved as "input" and added to 50 µl of 2 x MSB.

The remaining filtered lysate was processed by streptavidin sepharose affinity purification. First, 30 µl of streptavidin sepharose beads (GE Healthcare Life Sciences #17-5113-01) were washed with 200 µl of lysis buffer and 200 µl of 50 mM Tris-Cl (pH 7.4) and spun at 800 x g for 2 min. Supernatant was removed from the beads and the remaining filtered lysate was added and incubated overnight on a rotator at 4°C. The following day samples were centrifuged at 800 x g for 2 min and supernatant was discarded. Beads were washed with wash buffer 1 (2% SDS) and incubated at room temperature for 8 min on a rotator. Samples were spun at 800 x g for 2 min and washes were repeated using wash buffer 2 (0.1% deoxycholic acid, 1% Triton-X, 1 mM EDTA, 500 mM NaCl, 50 mM HEPES, pH 7.5) and wash buffer 3 (0.5% deoxycholic acid, 0.5% IGEPAL, 1 mM EDTA, 250 Mm LiCl, 10 mM Tris-Cl, pH 7.4). Finally, beads were resuspended in 30 µl 2 x MSB, 30 µl 50 mM Tris-Cl, and 5 µl of 1 mM Biotin. Samples were analyzed by immunoblotting as described above.

2.5 Immunofluorescence

U2OS cells were grown and transfected on autoclaved coverslips in 6 well plates. 24 h after transfection, media was removed and cells were washed 2 x with PBS for 5 min at room temperature. Cells were then fixed onto coverslips in 4% PFA for 15 min. Cells were blocked in 5% bovine serum albumin in PBSX (PBS, 0.05% Triton X-100) for 15 min. Blocking solution was

removed and coverslips were incubated for 1 h at room temperature in primary antibodies (rabbit anti-FLAG (Sigma #F7425, 1:1000) or mouse anti-V5 (Abcam #ab27671, 1:1000) (Table 3) prepared in 5% bovine serum albumin in PBSX (PBS, 0.05% Triton X-100), and washed in PBSX at 3 x 5 min. Cells were incubated for 1 h at room temperature in secondary antibodies (Alexa Fluor 488 goat anti-rabbit (Thermofisher Scientific #A-11034, 1:1000), Alexa Fluor 594 goat anti-rabbit (Thermofisher Scientific #A-11012, 1:1000), Alexa Flour 488 goat anti-mouse (Thermofisher Scientific #A-11001, 1:1000), or Alexa Fluor 594 goat anti-mouse (Thermofisher Scientific #A-11005, 1:000) prepared in PBSX with 1% normal goat serum (Table 3). Nuclei were counter-stained with Hoechst 33342 for 15 min (Life Technologies, 1:20,000). Coverslips were mounted onto glass slides using ProLong Gold Antifade Mountant (Thermofisher Scientific #P36930) and sealed using clear nail polish. Slides were stored in slide box at -20°C. Transfected cells on coverslips were analyzed using Z-stack imaging taken on a Zeiss LSM 700 confocal microscope with a 40x or 63x oil immersion lens (N. A. 1.4 oil). A Fisher exact test was used to determine the effect of co-expression of MAGEL2 on the subcellular localization of CRY1, with $P<0.05$ deemed to be statistically significant.

2.6 Ubiquitination assay

HEK 293T cells were seeded at $8.3x10^5$ in 100 mm dishes, 24 h before transfection. Cells were transfected using Fugene6 as described above. 24 h after transfection cells were incubated overnight with 5 μM MG132 (Life Technologies) and 25 μM chloroquine (Life Technologies) prepared in serum-free OPTIMEM media. Cells were washed with PBS then incubated for 30 min in lysis buffer (2% SDS, 150 mM NaCl, 10 mM Tris-HCl, 1 mM sodium orthovandate, 1 mM sodium fluoride, 20 mM β-glycerphosphate, 10 mM N-ethylmaleimide with Complete Mini Protease Inhibitor (Roche #11836153001) on a rocker at room temperature before collection. Cell lysates were sonicated (3 x 5 sec on/off), boiled for 10 min, and 50 μl of sample (25% of total

sample) was reserved as “input”. The remaining samples were then diluted (10 mM Tris-HCl, 150 mM NaCl, 2 mM EDTA, 1% Triton-X-100, pH 8.0) and incubated on a rocker at 4°C for 1 h. Samples were centrifuged at 20 000 g for 30 min then precleared with 20 µl Sepharose 4B beads (Sigma #4B200) at 4°C for 1 h on a rocker. Precleared samples were centrifuged at 3000 g for 2 min and the supernatant was transferred to a new tube. Lysates were incubated overnight at 4°C with anti-FLAG M2 Affinity gel (Sigma #A2220) on a rocker. Samples were centrifuged at 3000 g for 2 min and washed with wash buffer (10 mM Tris-HCl, 1 M NaCl, 1 mM EDTA, 1% IGEPAL, pH 8.0) twice. Beads were resuspended in collection buffer (62.5 mM Tris-HCl, 3% SDS, 10% glycerol) then used for immunoblot analysis as described above.

2.7 *Magel2* expression analysis using readily available databases

Images of an adult (56 day old) male C57BL/6J mouse brain, showing *Magel2* expression was downloaded from the Allen Brain Atlas. Under the mouse brain tab, in the ISH database, we searched for the expression of Magel2 in the coronal plane (Probe name RP_071204_04_C02). Image showing *Magel2* RNA expression in the hypothalamus as an *in situ* hybridization was downloaded from the ISH dropdown. The expression mask image showing *Magel2* was downloaded from the expression dropdown. This image is colour coded for cells that have the highest probability of *Magel2* gene expression where red indicates highest expression, yellow indicates moderate expression, and blue indicates low expression. (Image credit: Allen institute)

The Circadian Expression Profiles Database (CircaDB) maps the expression of mammalian clock-controlled genes using Genechip 3.2 hybridization data (Affymetrix, Santa Clara, CA). CircaDB was used to identify the expression profile of *Magel2* in the suprachiasmatic nucleus of the hypothalamus in C57BL/6J mice (Probeset #92681_at) and Clock mutant mice (Probeset #gnf1m13016_a_at) (Panda et al., 2002).

2.8 Statistical analysis

Student t-test was used to test whether there were differences between two groups of triplicate or quadruplicate samples in abundance experiments in U2OS cells, with $P<0.05$ used as a standard for statistical significance. One-Way analysis of variance (ANOVA) with Tukey's Honest Significant Difference post hoc test was used to test whether there were differences between more than two groups of triplicate samples (as in Figure 3.7B), with $P<0.05$ used as a standard for statistical significance. A Fisher exact test was used to determine differences in protein subcellular localization (as in Figure 3.6B), with $P<0.05$ deemed to be statistically significant.

Table 3: List of primary and secondary antibodies used

Antibody	Source	Catalogue number	Immunoblot concentration	Immunofluorescence concentration
Primary antibodies				
HA-probe antibody (Y-11)	Rabbit polyclonal	Santa Cruz Biotechnology Sc-805	1:500	
HA-probe antibody	Mouse monoclonal	Thermofisher Scientific Cat #26183	1:500	
Anti-V5 antibody	Mouse monoclonal	Abcam #ab27671	1:1000	1:1000
Anti-FLAG antibody	Rabbit polyclonal	Sigma-Aldrich #F7425	1:5000	1:1000
Anti-GFP antibody	Chicken polyclonal	Abcam #ab13970	1:5000	
Anti-β-Actin−Peroxidase	Mouse monoclonal	Sigma-Aldrich #A3854	1:5000	
Secondary antibodies				
Horseradish Peroxidase-linked donkey anti-rabbit IgG	Donkey	Amersham Pharmacia Biotech #NA934	1:5000	
Horseradish Peroxidase - linked sheep anti-mouse IgG	Sheep	Amersham Pharmacia Biotech #RPN4201	1:5000	
Horseradish Peroxidase - linked donkey anti-chicken	Donkey	Thermofisher Scientific #SA1-300	1:1000	

IgG				
Goat anti-Rabbit IgG (H+L) Highly Cross-a Absorbed Secondary Antibody, Alexa Fluor 488	Goat	Thermofisher Scientific #A-11034		1:1000
Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Goat	Thermofisher Scientific #A-11012		1:1000
Goat anti-Mouse IgG (H+L) Cross-Adsorbed, Secondary Antibody, Alexa Fluor 594	Goat	Thermofisher Scientific #A-11005		1:1000
Goat anti-Mouse IgG (H+L) Cross-Adsorbed, Secondary Antibody, Alexa Fluor 488	Goat	Thermofisher Scientific #A-11001		1:1000

3 Chapter 3: MAGEL2 regulates CRY1 ubiquitination, a core circadian rhythm protein

3.1 Introduction

Prader-Willi Syndrome (PWS) is a genetic disorder of the nervous and endocrine systems characterized by developmental disabilities, hyperphagia and obesity. Sleep apnea (obstructive and central), poor responses to hypoxia and hypercapnia, night wakening, narcoleptic symptoms, and perturbed circadian rhythm in rapid eye movement (REM) sleep contribute to abnormal sleep structure in individuals with PWS (Clift, Dahlitz, & Parkes, 1994; Sedky, Bennett, & Pumariega, 2014; Weselake & Wevrick, 2012). Excessive daytime sleepiness (EDS) affects 90-100% of adults with PWS, according to parental reports (Clarke, Waters, & Corbett, 1989; Maas et al., 2010; Williams et al., 2008). Although the EDS in the general population could result from obstructive sleep apnea, in PWS sleep apnea does not fully explain the EDS phenotype indicating the function of PWS genes could be contributing factors (Camfferman, Mcevoy, Donoghue, & Lushington, 2008). A subset of people with PWS also exshibited short mean sleep latency and sleep onset rapid eye movement episodes measured by multiple sleep latency testing (Vgontzas et al., 1996). About 35.71% of people with PWS exhibit narcolepsy features such as cataplexy, sleep paralysis, sleep attacks, and hallucinations (Sedky, Bennett, & Pumariega, 2014; Tobias, Tolmie, & Stephenson, 2002). Sleep/wake cycles determined by circadian rhythm are important for normal daily functions and neuronal communication. Endocrine disruption, obesity and EDS are caused by primary central hypothalamic dysfunction (Bruni, Verrillo, Novelli, & Ferri, 2010). Modafinil, a stimulant medication used to treat overt sleepiness in patients with narcolepsy, was effective at reducing daytime sleepiness in an open label pilot study of children and adolescents with PWS but has not yet been tested in a clinical trial setting (De Cock et al., 2011).

Circadian rhythm is the oscillation of physiological and cellular functions, such as sleep and wake, over a 24-hour period (Reppert & Weaver, 2002). The suprachiasmatic nucleus (SCN) of the hypothalamus regulates circadian rhythm and is known as the "master clock", sending signals to peripheral tissue cellular clocks to regulate various bodily functions such as metabolism, sleep and wakefulness, body temperature, and hormone levels (Figure 3.1). The SCN is entrained to the light-dark cycle, receiving light cues and signals from ganglion cells in the retina through the retinohypothalamic tract (RHT). In humans, when light cues are received at the SCN there is rapid transcriptional activation of genes in the neurons of the SCN (Hastings, Maywood, & Brancaccio, 2018).

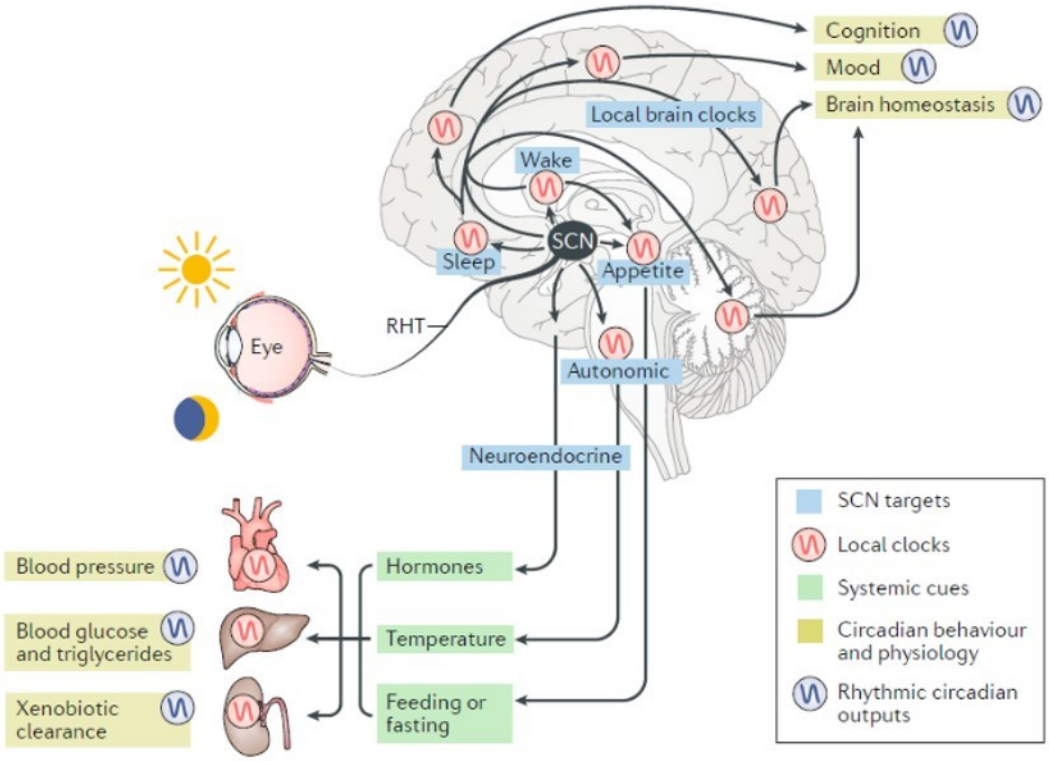


Figure 3.1 The suprachiasmatic nucleus (SCN) of the hypothalamus is known as the master clock regulator in mammals. The SCN receives light input from the retina through the retinohypothalamic tract (RHT) that activates expression of core circadian rhythm genes. The SCN relays signals to various areas of the brain (blue boxes) that then send signals to molecular clocks located in peripheral tissues (green box) such as the heart, kidney, and lung. This activation results in circadian outputs that govern behavior, physiological, and biological functions. Figure modified from (Hastings et al., 2018).

At a molecular level, the circadian clock functions through cell-autonomous, transcriptional and post-translational feedback loops and is controlled by the synthesis, ubiquitination, and degradation of a key set of proteins. A set of bHLH-PAS type transcription factors, including circadian locomotor output cycles protein kaput (CLOCK) and brain and muscle ARNT-like1 (BMAL1) heterodimerize and stimulate transcription from E-box containing promoters. The CLOCK/BMAL1 heterodimer are transcriptional activators responsible for the activation of clock-controlled genes that result in the circadian rhythm output. CLOCK/BMAL1 drive expression of two sets of genes period (*PER*) and cryptochrome (*CRY*) that in turn regulate CLOCK/BMAL1 expression establishing a negative feedback loop (Figure 3.2). CLOCK/BMAL1 act as transcriptional activators by recruiting chromosomal modifiers or co-activators at the promoters and enhancers of clock-controlled genes. CLOCK/BMAL1 drive the rhythmic expression of approximately 15% of the human transcriptome that in turn generate changes in physiology, behaviours and biological functions (Trott & Menet, 2018). The process of circadian rhythm relies on tightly regulated post-translational protein ubiquitination and proteasomal degradation.

Regulation of PER and CRY proteins through ubiquitination and phosphorylation are important in the circadian rhythm feedback loop governing *CLOCK*/*BMAL1* expression levels. Deubiquitinase USP7 interacts with CRY1 and CRY2 and stabilizes these proteins through deubiquitination when high levels of cryptochrome proteins are needed (Hirano, et al., 2016). The Skp1/Cullin/F-box (SCF) protein complex is a Cullin-RING ligase (CRL) that consists of multiple subunits and is ubiquitously expressed in various tissues (Zheng et al., 2002). The SCF complex includes the E3 ubiquitin ligase RBX1, and in the hypothalamus RBX1 promotes polyubiquitination and proteasomal degradation of CRY proteins in the cytoplasm (Figure 3.2,

Yoo et al., 2013). F-box protein FBXL21 targets CRY proteins for degradation in the cytoplasm (Yoo et al., 2013). Conversely, FBXL21 associates with CRY proteins in the nucleus to stabilize and protect CRY from degradation, while F-box protein FBXL3 is responsible for targeting CRY for proteasomal degradation in the nucleus (Yoo et al., 2013). CLOCK/BMAL1 protein levels increase during the subjective day and activate *CRY* and *PER* gene expression (Figure 3.2). CRY protein levels are highest at the end of the subjective day into the beginning of subjective night where they are stabilized by USP7 in the cytoplasm (Hirano et al., 2016). Following protein accumulation, CRY and PER proteins bind and translocate into the nucleus to inhibit CLOCK/BMAL1 transcription. At the end of the subjective night/beginning of subjective day, CRY protein is targeted for proteasomal degradation by the SCF-RBX1 complex to allow for the start of a new circadian cycle (Figure 3.2, Hirano et al., 2013). Consistent with the importance of protein stability in circadian rhythm, a recent genetic study suggested that insomnia is associated with variants in genes involved in ubiquitin-mediated proteolysis and of genes expressed in the brain, skeletal muscle and adrenal glands (Lane et al., 2019).

Of the cluster of genes inactivated in individuals with PWS, one gene, *MAGEL2*, has an expression profile and protein function consistent with a role in EDS and night waking in PWS. Mutations in the *MAGEL2* gene alone cause Schaaf-Yang syndrome, a disorder with overlapping phenotypes with PWS. As the number of people diagnosed with SYS is still low, sleep and circadian rhythm have not been formally investigated in these individuals.

MAGEL2 is highly expressed in the brain, specifically in the dorsal suprachiasmatic nucleus of the hypothalamus, overlapping with vasopressin positive neurons (Kozlov et al., 2007). As previously described, MAGE proteins interact with RING-zinc finger-type E3 ubiquitin ligases and ubiquitin-specific proteases (deubiquitinases) to increase or decrease substrate protein

ubiquitination levels. MAGE family proteins MAGEC2 and MAGEA11 were shown to interact with components of the SCF complex (Hao et al., 2015; Su et al., 2017). MAGEC2 binds to RBX1 to stabilize cyclin E, a protein important for cell cycle progression from G1 to S phase (Hao et al., 2015). MAGEA11 interacts with Skp2 to regulate cyclin A, also involved in cell cycle progression (Su et al., 2017). Interactions between MAGEL2 and SCF components have yet to be identified. However, MAGEL2 interactions with other components of the circadian rhythm cycle have been identified. MAGEL2 interacts with and modulates the stability and activity of circadian rhythm transcription factors CLOCK and BMAL1 and the transcriptional repressor PER2 (Devos et al., 2011). Gene-targeted mice carrying a *Magel2* mutation have a circadian rhythm defect, and while they do entrain to 12:12 lighting conditions, they have reduced total activity with an increase in activity in the light period (subjective night). *Magel2* mice displayed fragmented activity with diminished amplitude when housed in constant darkness (Kozlov et al., 2007). Therefore, people with loss of *MAGEL2* and *Magel2* mutant mice both have circadian rhythm defects and sleep abnormalities.

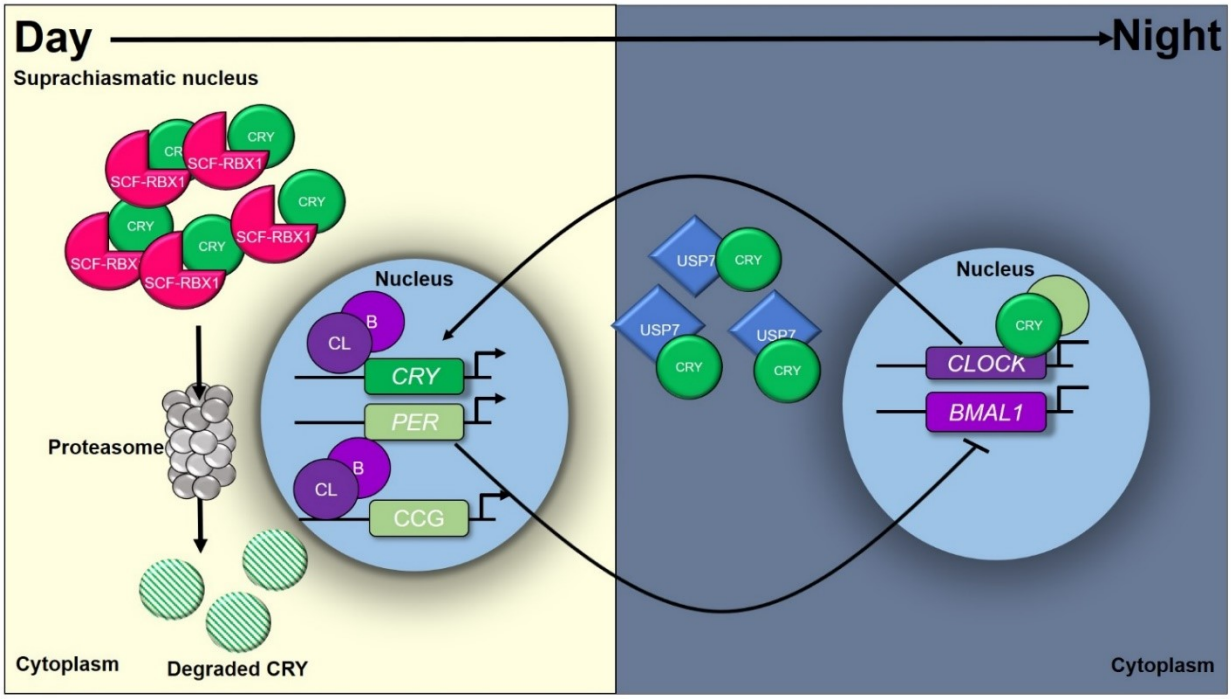


Figure 3.2 Circadian rhythm is regulated by feedback loops and post translational modifications in the suprachiasmatic nucleus of the mammalian hypothalamus. BMAL1 and CLOCK activate transcription of CRY/PER and clock-controlled genes in the nucleus during the subjective day. During the subjective night, CRY and PER protein localize to the nucleus to inhibit *CLOCK* and *BMAL1* gene expression. USP7 stabilizes CRY protein and protects it from proteasomal degradation in the cytoplasm. The SCF-RBX1 complex tags CRY1 with ubiquitin to target it for proteasomal degradation. Scf-RBX1, Skp1/Cullin/F-box – RBX1 protein complex; CL, CLOCK protein; B, BMAL1 protein; CCG, clock-controlled genes.

3.1.1 Aims and hypothesis

MAGE proteins, including MAGEL2 play a role in the regulation of protein ubiquitination. MAGE family proteins, MAGEC2 and MAGEA11 have been shown to bind to components of the SCF complex (Hao et al., 2015; Su et al., 2017). In the suprachiasmatic nucleus of the hypothalamus, the SCF complex is responsible for the regulation of core circadian rhythm proteins. Therefore, we hypothesize that MAGEL2 interacts with and regulates the activity of RING E3 ubiquitin ligases and deubiquitinases towards substrate circadian proteins to regulate the robustness of the mammalian circadian rhythm. In this study, we aim to identify interactions among MAGEL2, the E3 ligase RBX1, the deubiquitinase USP7 and the cryptochrome protein CRY1. We investigate if MAGEL2 modulates the ubiquitination and stability of CRY1 through its interactions with the ubiquitination system responsible for CRY1 degradation and stabilization. We propose that disruption of circadian rhythm in people with Prader-Willi syndrome may be caused by deficiencies in the ubiquitin-dependent regulation of CRY1 levels.

3.2 Results

3.2.1 MAGEL2 has circadian expression and is expressed in the suprachiasmatic nucleus, the area of the brain that controls circadian rhythm

We examined the expression pattern of *Magel2* in the adult mouse brain using the Allen Brain Atlas (Mccarthy, 2006). *Magel2* is highly expressed in the hypothalamus, and most highly in the paraventricular and suprachiasmatic nuclei (SCN) of the hypothalamus (Figure 3.3A, A'). Next, we determined the circadian pattern of *Magel2* expression using data from CircaDB where it was identified as one of the most circadian genes (Figure 3.3B, Panda et al., 2002; Pizarro, Hayer, Lahens, & Hogenesch, 2013). Notably, expression of Magel2 is disrupted in mice carrying a *Clock* gene mutation, indicating that *Magel2* is a clock-controlled gene (Figure 3.3C, Miller et al., 2007).

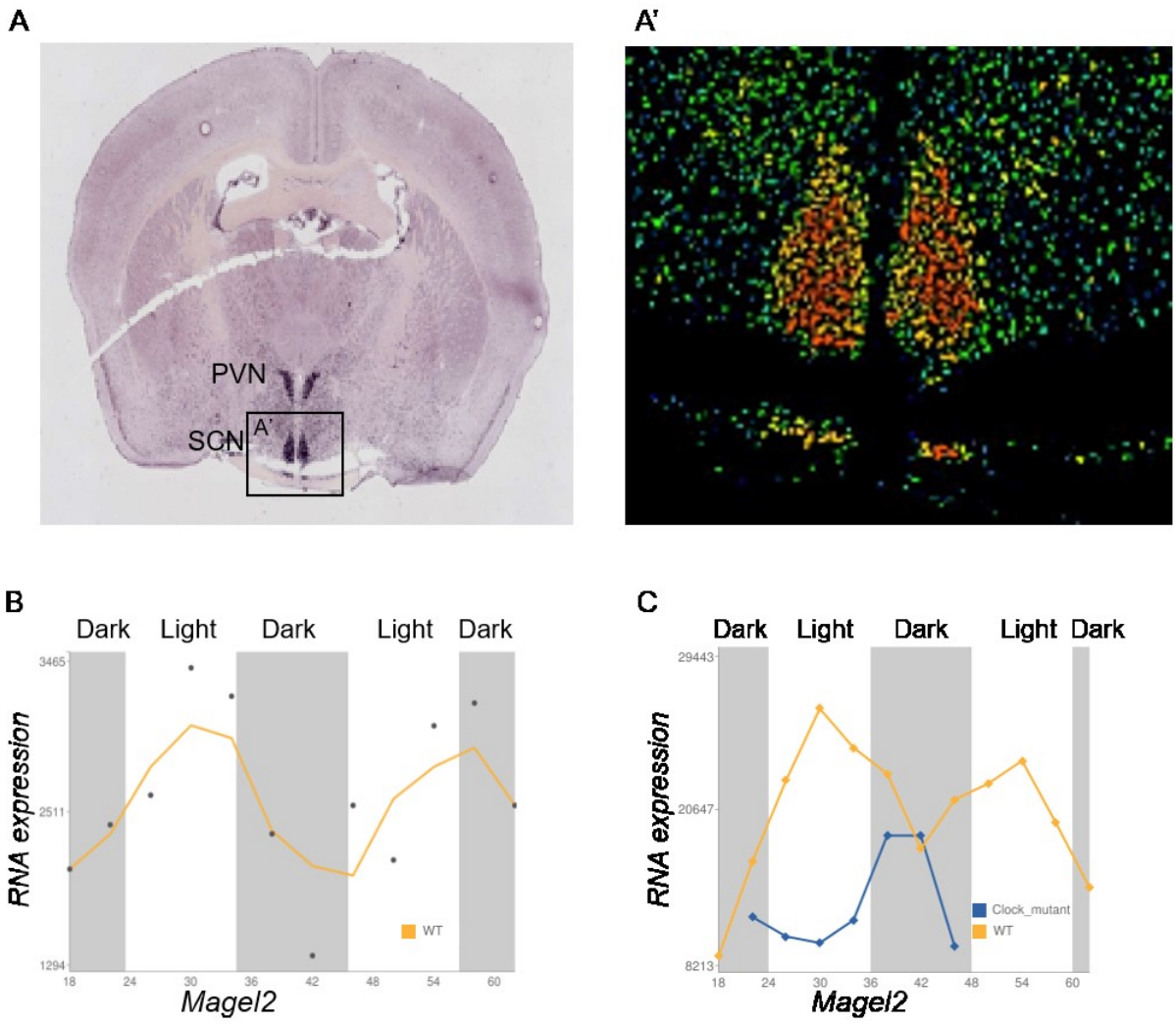


Figure 3.3 Expression of Magel2 is high in the suprachiasmatic nucleus of the hypothalamus and follows a circadian pattern. A) RNA *in situ* hybridization on an adult mouse brain section in the coronal plane, demonstrating high expression of *Magel2* in the hypothalamus (dark blue/purple signal). A') inset shows expression in the suprachiasmatic nucleus (pseudocolored, red signal is highest expression; yellow signal is moderate expression). Data from Allen Brain Atlas. Image credit: Allen Institute. B) Expression of murine *Magel2* follows a highly circadian pattern in the suprachiasmatic nucleus of the hypothalamus. Data from Circadian Expression Profiles Database, CircaDB. (Panda et al., 2002). C) Expression of Magel2 is disrupted in mice carrying a *Clock* gene mutation (blue curve). Data from CircaDB. (Panda et al., 2002).

3.2.2 MAGEL2 interacts with circadian rhythm proteins at the cellular level

MAGEL2 interacts with at least two E3 ligases: TRIM27 to regulate retromer mediated recycling through WASH complex ubiquitination, and RNF41 to regulate leptin receptor cell surface abundance (Hao et al., 2013; Wijesuriya et al., 2017). We therefore hypothesized that MAGEL2 could regulate circadian rhythm proteins through its interaction with RBX1, the E3 ubiquitin ligase component of the cullin-RING ligase complex responsible for ubiquitination and proteasomal degradation of circadian rhythm proteins. We performed proximity-dependent biotin identification (BioID) in U2OS cells transiently co-transfected with a "bait" FLAG-tagged biotin ligase-fusion protein (BirA*-MAGEL2), and a second epitope-tagged protein as prey. After streptavidin-affinity purification of the cell lysate, streptavidin-bound biotinylated proteins were recovered and immunoblotted to detect proteins in proximity to the BirA*-fusion baits *in vivo* (bound). MAGEL2 can biotinylate V5-tagged TRIM27, with both proteins present in the input (detected with anti-V5 and anti-FLAG) and V5-TRIM27 detected in bound fraction of biotinylated proteins (Figure 3.4A). Next, BirA*-MAGEL2 and V5-RBX1 were co-transfected and detected by immunoblotting (input). BirA*-MAGEL2 also biotinylated V5-RBX1, indicating that these two proteins were in proximity to MAGEL2 (Figure 3.4). We then tested whether mutations that alter the MHD of MAGEL2 impair the ability of MAGEL2 to interact with RBX1, in the BioID assay. BirA*-MAGEL2p.R1187C and BirA*-MAGEL2p.LL1031AA carry mutations that disrupt the MHD (Wijesuriya et al., 2017). Unlike with wildtype (WT) BirA*-MAGEL2, V5-RBX1 was not biotinylated by BirA*-MAGEL2p.R1187C or BirA*-MAGEL2p.LL1031AA (Figure 3.4, lanes 2 and 3). As a control, we transfected BirA*-NLS, which carries the biotin ligase cDNA fused to a nuclear localization signal (NLS). BirA*-NLS also did not biotinylate V5-RBX1 (Figure 3.4, lane 4). This demonstrates that wild-type MAGEL2 is in proximity to RBX1 in cultured, co-transfected cells.

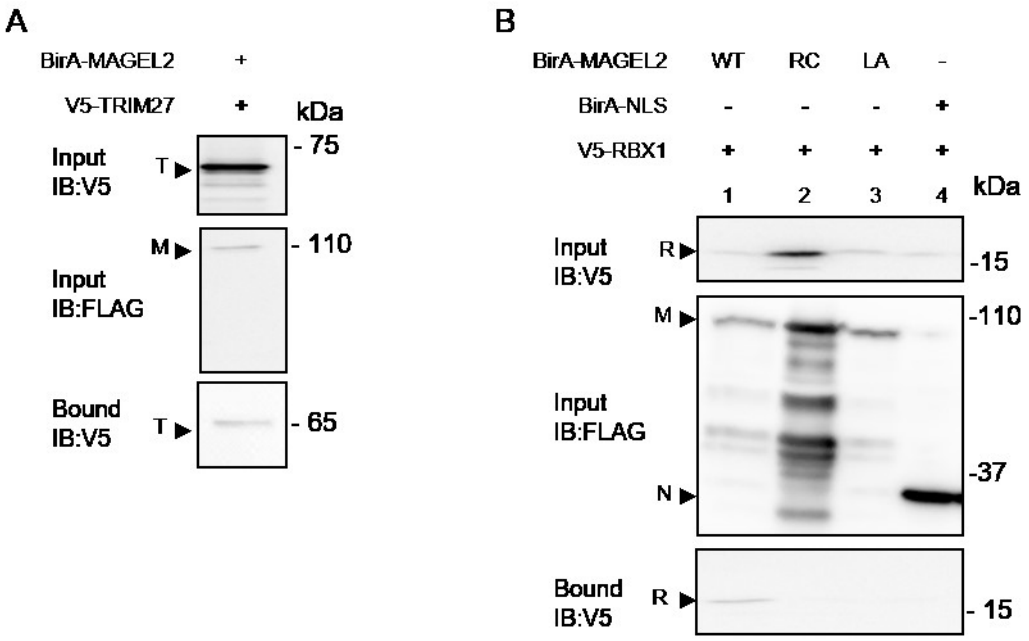


Figure 3.4 MAGEL2 interacts with RING-zinc finger-type E3 ubiquitin RBX1 as detected by proximity-dependent biotinylation (BioID). U2OS cells were transiently transfected with cDNA constructs encoding epitope-tagged proteins and incubated with excess biotin. After 24 h, cell lysates were collected, an aliquot was retained as input, and the remaining sample was processed by streptavidin affinity purification to recover proteins biotinylated by BirA* fusion proteins (bound). Input and bound samples were immunoblotted (IB) to detect recombinant proteins. A) BirA*-MAGEL2 (M) was co-transfected with V5-tagged TRIM27 (T). B) BirA*-MAGEL2 (M, lane 1, wildtype, WT), BirA*-MAGEL2p.R1187C (M, lane 2, RC), BirA*-MAGEL2p.LL1031AA (M, lane 3, LA) or BirA*-NLS (N, lane 4) were co-transfected with V5-tagged RBX1 (R). All BioID experiments were repeated at minimum 3 times, with the exception of RBX1 with mutant MAGEL2 proteins that has only been performed once.

We next tested whether MAGEL2 interacts with the cryptochrome proteins CRY1 and CRY2, which are light-independent inhibitors of the transcription factors CLOCK and BMAL1 (Hirano et al., 2016; Papp et al., 2015). We performed BioID in U2OS cells transiently co-transfected with BirA*-MAGEL2 as bait and GFP-tagged prey proteins. As a positive control, BirA*-MAGEL2 also biotinylated GFP-WASH1 and GFP-VPS35, consistent with their interaction as detected previously by immunoprecipitation and mass spectrometry (Hao et al., 2013) (Figure 3.5A, lanes 3 and 4). However, BirA*-MAGEL2 did not biotinylate GFP alone (Figure 3.5A, lane 5, GFP present in input but not in bound fraction). Next, we tested the effect of mutations in MHD-disrupting mutations in MAGEL2 on the interaction between MAGEL2 and CRY1. GFP-CRY1 was transfected along with BirA*-MAGEL2p.R1187C "R>C" or MAGEL2p.LL1031AA "LL>AA" carrying a mutation in the MAGE homology domain (Figure 3.5B). After transient transfection and BioID, BirA*-MAGEL2 biotinylated GFP-CRY1 and GFP-CRY2 as demonstrated by their presence both in the input fraction and in the biotinylated proteins recovered by streptavidin affinity purification (bound, Figure 3.5A, lane 1 and lane 2). GFP-CRY1 was also recovered after BioID with BirA*-MAGEL2R>C and BirA*-MAGEL2LL>AA (Fig. 3B, lanes 3, 4).

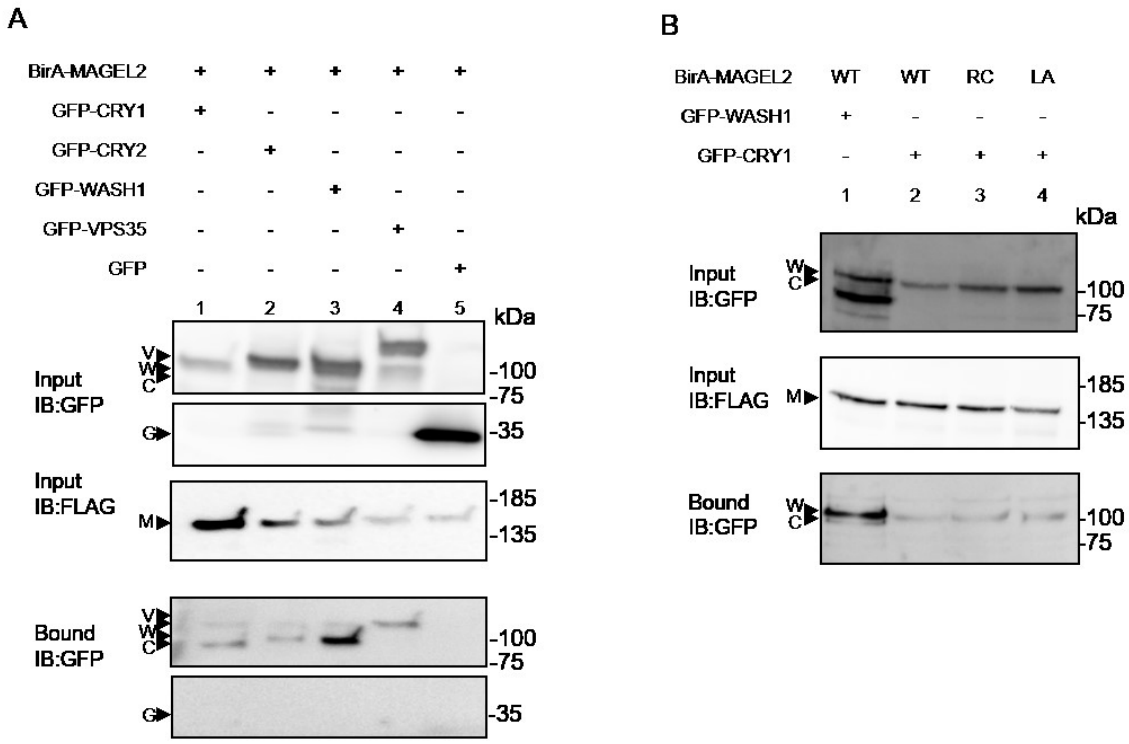


Figure 3.5 MAGEL2 interacts with Cryptochrome 1 (CRY1) and Cryptochrome 2 (CRY2) as detected by BioID. U2OS cells were transiently transfected with cDNA constructs encoding epitope-tagged proteins and incubated with excess biotin. After 24 h, cell lysates were collected, an aliquot was retained as input, and the remaining sample was processed by streptavidin affinity purification to recover proteins biotinylated by BirA* fusion proteins (bound). Input and bound samples were immunoblotted (IB) to detect recombinant proteins. A) BirA*-MAGEL2 (M) was co-transfected with GFP-tagged CRY1 (C) or CRY2 (C). As interacting positive controls BirA*-MAGEL2 (M) was also co-transfected with GFP-WASH1 (W) or GFP-VPS35 (V). GFP alone was used as a non-interacting negative control (G). B) BirA*-MAGEL2 (wildtype, WT), BirA*-MAGEL2p.R1187C (RC), or BirA*-MAGEL2p.LL1031AA (LA) were co-transfected with GFP-CRY1 or GFP-WASH1 (positive control). CRY1-MAGEL2 interactions was repeated, CRY2-MAGEL2 interaction has only been performed once.

3.2.3 MAGEL2 protein expression overlaps with circadian rhythm proteins in the cytoplasm

Co-expression of MAGEL2 has been shown to alter the subcellular localization of E3 ligase complexes to alter protein stability (Wijesuriya et al., 2017). We therefore investigated if MAGEL2 co-localizes with circadian rhythm proteins or affects their subcellular localization. MAGEL2 transfected in U2OS cells has a cytoplasmic distribution, detected by transient transfection and immunofluorescence microscopy (Figure 3.6A, upper panel). V5-RBX1 transfected alone in U2OS cells has a diffuse cytoplasmic distribution (Figure 3.6A, upper panel). When co-transfected, FLAG-MAGEL2 and V5-RBX1 have some overlap in their localization in the cytoplasm (Figure 3.6A, yellow signal in the merged image). V5-CRY1 protein is present in both the cytoplasm and the nucleus of transfected U2OS cells (Figure 3.6B, upper panel). However, in cells co-transfected with FLAG-MAGEL2 and V5-CRY1, more cells appeared to have a nuclear CRY1 signal compared to when *CRY1* was transfected alone. To quantify this effect, we compared the number of transfected cells in which the CRY1 was mostly in the nucleus to cells in which CRY1 was mostly in the cytoplasm, in cells transfected with the CRY1 construct alone or co-transfected with MAGEL2. CRY1 was mostly cytoplasmic, with CRY1 being nuclear in 42 of 102 (41%) of cells when transfected alone. However, CRY1 was mostly nuclear in 41 of 53 cells (77%) of cells when co-transfected with MAGEL2 ($P<0.0001$, Fisher exact test).

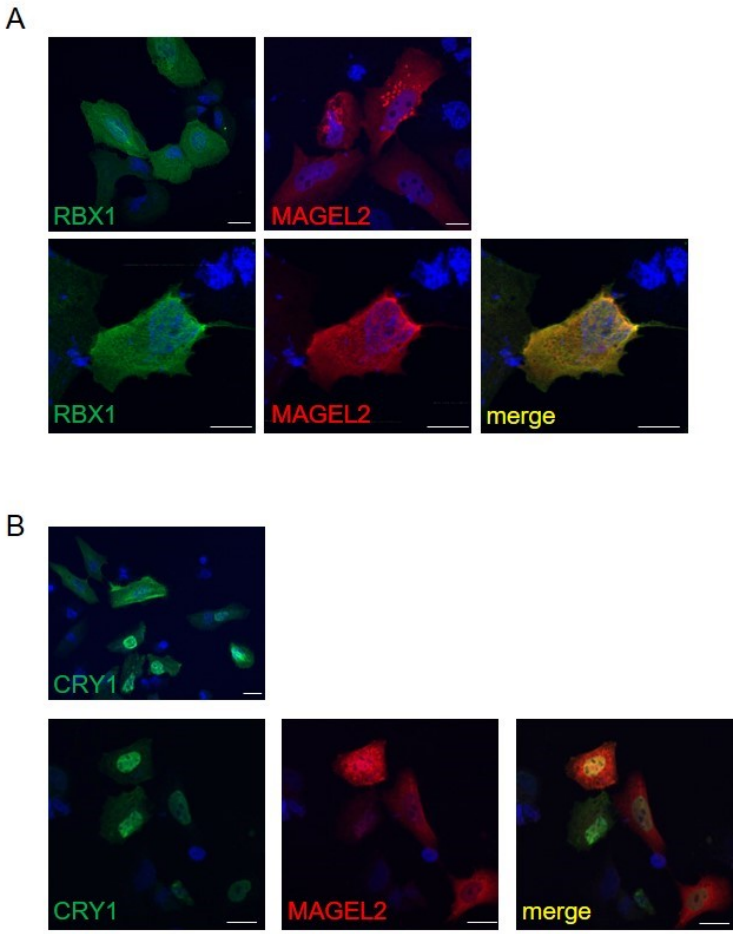


Figure 3.6 MAGEL2 protein expresses similarly to RBX1 in the cytoplasm in transfected U2OS cells, and MAGEL2 modulates the subcellular localization of CRY1. A) Recombinant RBX1 (green) and MAGEL2 (red) were detected in either singly transfected (upper panels) or co-transfected (lower panels) U2OS cells by immunofluorescence microscopy. Yellow signal in merged image indicates where protein expression overlaps in the cell. Nuclei were counterstained blue with Hoechst dye. B) Recombinant CRY1 (green) alone was detected in transfected U2OS cells (upper panel). Recombinant CRY1 (green) and MAGEL2 (red) were detected in transfected U2OS cells (lower panel). Nuclei are counterstained blue with Hoechst dye. Representative cells are shown. Scale bars: 10 µm.

3.2.4 MAGEL2 affects CRY1 protein ubiquitination and protein levels

MAGEL2 affects protein stability and abundance through its role as a modulator of ubiquitination. Having established that MAGEL2 and CRY1 are transiently or lastingly in proximity to each other in cultured cells, we tested whether MAGEL2 could affect the abundance of CRY1. The reduction in cytoplasmic CRY1 in the presence of co-expressed MAGEL2 also suggested that MAGEL2 may promote the proteasomal degradation of CRY1 in the cytoplasm. We tested whether co-expression of MAGEL2 affects CRY protein levels. Cells were transfected with a construct encoding FLAG-CRY1, and increasing amounts of V5-MAGEL2, with empty vector used to equalize the amount of plasmid in all transfections (Figure 3.7A). Immunoblots of cell lysates probed with anti-FLAG demonstrated that increasing amounts of MAGEL2 decreased the abundance of CRY1 protein (Figure 3.7A). This was quantified by performing single- or co-transfection of FLAG-CRY1 and V5-MAGEL2, in triplicate, and measuring the abundance of CRY1 protein by immunoblotting. Co-expression of MAGEL2 reduced the amount of CRY1 detected to 75% of the level detected in lysates from cells transfected with CRY1 alone. We next tested whether MHD mutations affect the ability of MAGEL2 to alter CRY1 levels. When the experiment was repeated using wild-type MAGEL2, MAGEL2 R>C, or MAGEL2 LL>AA, all values were significantly different from no MAGEL2 co-expression by a One-way ANOVA test with Tukey's Honest Significant Difference POST Hoc test. However, when comparing individual experimental conditions, only the MAGEL2 LL>AA was significantly different from no co-expression of MAGEL2, where MAGEL2 LL>AA decreased CRY1 protein levels. MAGEL2 wild-type and MAGEL2R>C had no effect on CRY1 protein levels (data from triplicate transfections, example in Figure 3.7B). Co-expression of wild-type MAGEL2 and LLAA were not significantly different from each other (Figure 3.7B).

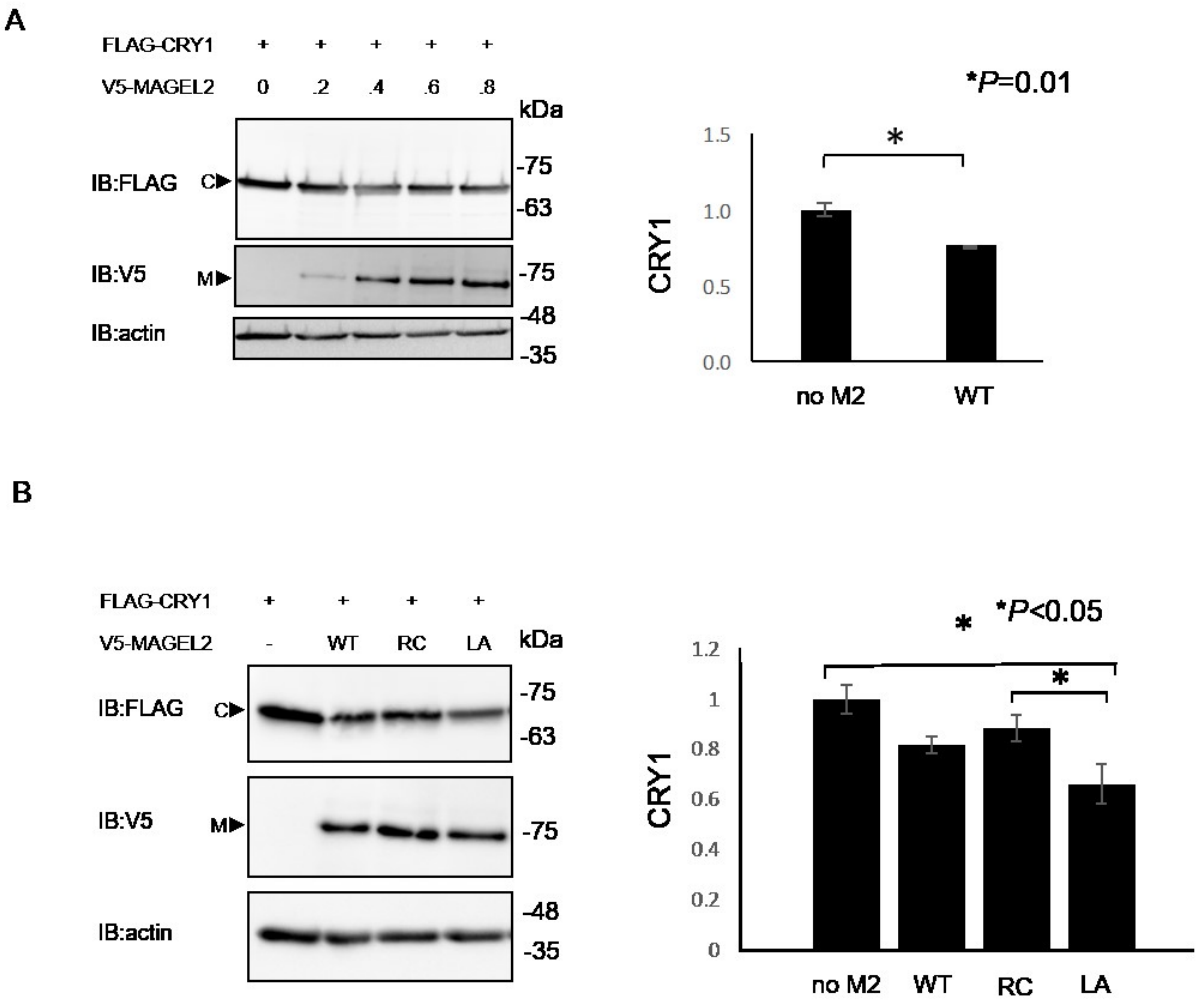


Figure 3.7 Co-expression of MAGEL2 reduces the abundance of CRY1. A) U2OS cells were transiently transfected with cDNA constructs encoding FLAG-CRY1 and varying amounts of V5-MAGEL2, and signal intensities were measured in cell lysates and normalized to signal intensity of actin (representative blot shown). Graph represents relative CRY1 signal intensity from a triplicate co-transfection at a 1:1 ratio of MAGEL2 (or empty vector) and FLAG-CRY1 (technical triplicate, blots not shown). **P*=0.01 comparing abundance of CRY1 with empty vector to co-expression of MAGEL2 by Student t-test. Abundance experiment was repeated three times. B) FLAG-CRY1 was co-transfected with V5-MAGEL2 wild-type (WT), V5-MAGEL2p.R1187C (RC), or V5- MAGEL2p.LL1031AA (LA) and the signal intensity of FLAG-CRY1 was measured

in cell lysates (technical triplicate, representative lanes shown). Graph represents relative CRY1 signal intensity from a triplicate co-transfection at a 1:1 ratio of MAGEL2 (WT, RC, or LA or empty vector) and FLAG-CRY1. *, $P<0.05$ comparing empty vector, co-expression of MAGEL2 WT, R>C, or LL>AA, using the One-Way analysis of variance (ANOVA) with Tukey's Honest Significant Difference POST Hoc test. Only a single representation of the triplicate experiment (with CRY1 alone and with wild-type and mutant MAGEL2 proteins) is shown. Experiment was repeated twice.

The stability of the CRY proteins is regulated by phosphorylation and ubiquitination, and CRY1 is deubiquitinated and stabilized by USP7 (Papp et al., 2015). U2OS cells were co-transfected with FLAG-CRY1 with or without V5-USP7. With V5-USP7, 2.6-fold more FLAG-CRY1 was detected in the cell lysates, confirming that USP7 stabilizes CRY1 (Figure 3.8A, n=4 technical replicates, p=0.001). To examine the mechanism by which these effects take place, we tested whether USP7 can deubiquitinate CRY1 in a ubiquitination assay. Co-transfection of FLAG-CRY1 and V5-USP7 together with HA-ubiquitin followed by immunoprecipitation with anti-FLAG antibodies and detection of ubiquitination of CRY1 demonstrated that CRY1 can be deubiquitinated by USP7 (Figure 3.8B, ubiquitin smear in lane 3 compared to lane 2). We previously showed that BirA-MAGEL2 can form a complex with USP7, using co-transfection and BioID (Wijesuriya et al., 2017). As well, endogenous USP7 was recovered in an immunoprecipitation of stably expressed TAP-tagged MAGEL2 in HEK 293T cells (Hao et al., 2015). We confirmed this interaction, performing the converse BioID experiment, in U2OS cells transiently co-transfected with FLAG-tagged BirA*-USP7 as bait and V5-tagged MAGEL2 as prey. V5-MAGEL2 was biotinylated by BirA*USP7, indicating an interaction (Figure 3.8C). Although an RBX1-containing complex is responsible for the ubiquitination of CRY1, MAGEL2 may modify CRY1 ubiquitination levels thereby affecting its stability. In a ubiquitination assay, co-transfection with MAGEL2 increased ubiquitination of CRY1 (Figure 3.8D, compare lane 3 to lane 2). In contrast, co-expression of V5-tagged MAGEL2 mutant constructs (MAGEL2R>C and MAGEL2LL>AA) did not increase CRY1 ubiquitination (Figure 3.8D, compare lane 3 to lanes 4 and 5).

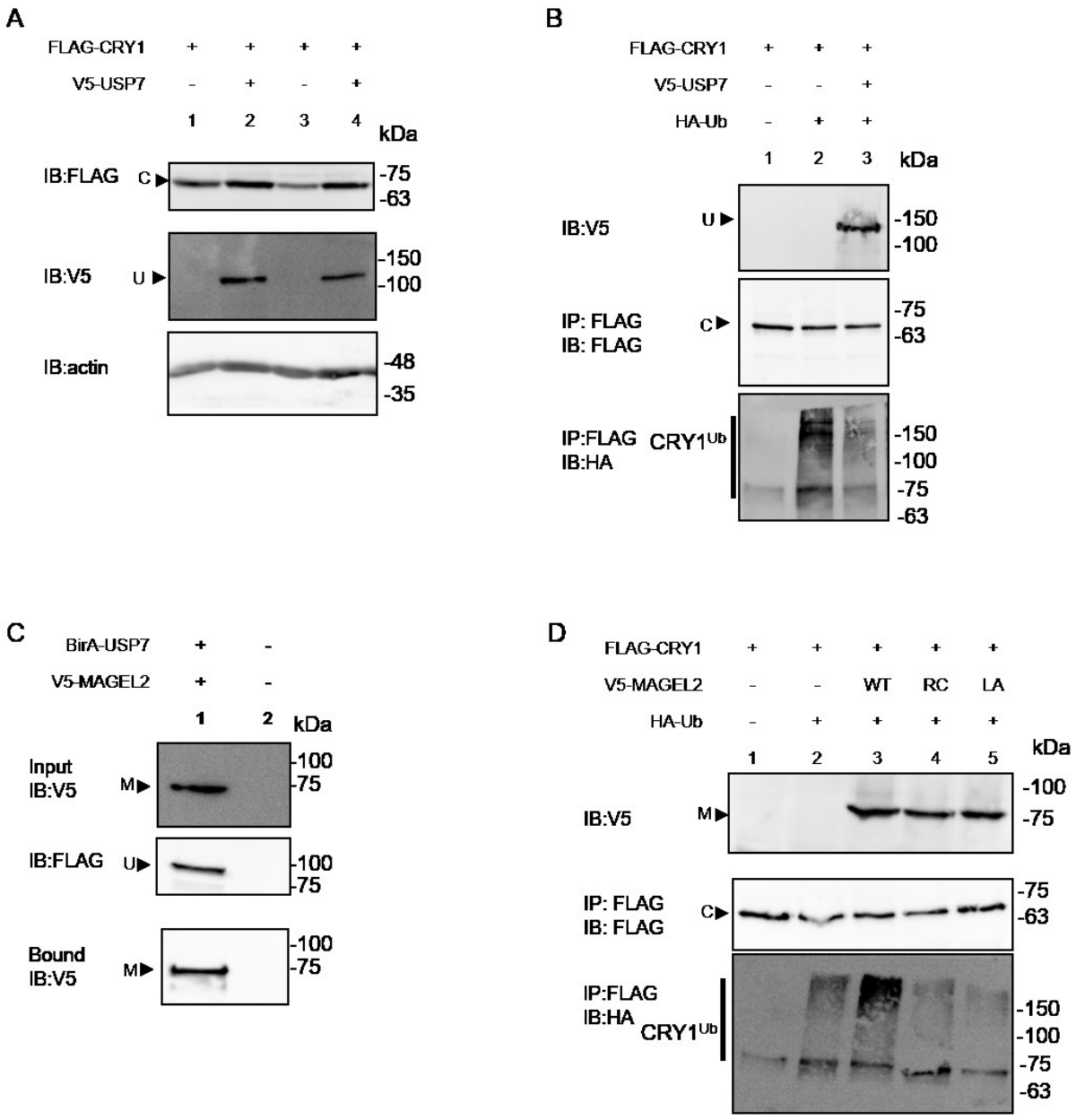


Figure 3.8 MAGEL2 and USP7 alter CRY1 stability through ubiquitination-related processes. A) USP7 stabilizes CRY1 when co-expressed in U2OS cells. U2OS cells were transiently transfected with cDNA constructs encoding V5-USP7 and FLAG-CRY1 in equal amounts in a technical quadruplicate. B) In a ubiquitination assay, HEK 293T cells were co-transfected with epitope-tagged constructs, and treated with MG132 and chloroquine. After 24 h,

cell lysates were collected, an aliquot retained as input and immunoblotted (IB) to confirm expression of V5-USP7. FLAG-CRY1 was immunoprecipitated (IP) using anti-FLAG beads from the remaining lysate. FLAG-CRY1 was detected in the immunoprecipitate, and ubiquitinated CRY1 ($Cry1^{Ub}$) was detected by probing the immunoprecipitate with anti-HA antibodies to detect HA-ubiquitin (HA-Ub, smear above molecular weight of CRY1). C) The deubiquitinase USP7 interacts with MAGEL2 as detected by BioID. U2OS cells were transiently transfected with constructs encoding epitope-tagged proteins, incubated with biotin, and collected 24 h after transfection. A portion of the cell lysate was removed and retained as input. Subsequently, streptavidin affinity purification captured V5-tagged MAGEL2 that was biotinylated by BirA*-USP7 (bound). D) Ubiquitination assay showing effect of co-expression of MAGEL2 (V5-MAGEL2 wild-type (WT), V5-MAGEL2p.R1187C (RC), or V5- MAGEL2p.LL1031AA (LA)) on the ubiquitination of CRY1 ($Cry1^{Ub}$) in transfected HEK 293T cells. Fig. 3A was performed once, while B and D repeated twice, and C was repeated three times.

3.3 Discussion

Protein truncating mutations in *MAGEL2* cause Schaaf-Yang syndrome, while loss of *MAGEL2* and other contiguous genes causes Prader-Willi syndrome. Children with PWS have circadian rhythm disruptions and sleeping problems such as night time wakening and excessive daytime sleepiness. To date, there are no studies on circadian rhythm and sleep disruptions in children with SYS due to small sample size and recent clinical discovery. Interestingly, *Magel2* mutant mice exhibit circadian rhythm defects having a reduced amplitude of activity, increased daytime activity, and fragmented night time activity as measured by recording running-wheel activity (Kozlov et al., 2007). In a *Magel2* mutant mouse (Magel2^{tm1Stw}) with lacZ transgene expression, *Magel2* mRNA localizes to neurons in the dorsal suprachiasmatic nucleus that express the neuropeptide vasopressin important for mediating circadian rhythm output signals (Kozlov et al., 2007; Silver & Schwartz, 2006). *Magel2* mRNA exhibits a circadian rhythm expression with highest expression in the light period (subjective night), specifically at the light to dark transition in wild-type mice (Figure 3.3, Kozlov et al., 2007). In wild-type mice, *Magel2* mRNA is highly expressed in a circadian fashion in the suprachiasmatic nucleus of the hypothalamus and its expression is abnormal in *Clock* mutant mice (Figure 3.3). In summary, *Magel2* is expressed in a circadian fashion, lack of Magel2 in mice (Magel2^{tm1Stw}) causes circadian rhythm defects, and children with loss of function of *MAGEL2* exhibit circadian rhythm defects. However, the role *MAGEL2* plays in regulating circadian rhythm at the cellular level remains to be fully elucidated.

The *CLOCK* gene along with *BMAL1* encodes bHLH-PAS proteins that heterodimerize to control expression of circadian rhythm proteins at the E-box elements in target gene promoters through their histone acetyl transferase domains (Gekakis et al., 1998). CLOCK:BMAL1 are known as the master clock regulators in the SCN that control approximately 10% of genes in the

mammalian genome to regulate circadian rhythm outputs (Panda et al., 2002). In turn, the transcription of CLOCK:BMAL1 is regulated by core clock controlled genes Period (*PER1, PER2, PER3*) and cryptochrome *(CRY1, CRY2)* creating a negative feedback loop (Gekakis et al., 1998; Kume et al., 1999). Period and cryptochrome proteins are then regulated by post-translational modifications such as ubiquitination to control protein levels and availability. *Magel2* dysregulation in *Clock* mutant mice indicate *Magel2* is a clock-controlled gene, which may in turn regulate expression of other important circadian rhythm proteins at the light to dark transition (subjective night to subjective day, respectively) to fine tune the circadian rhythm cycle.

Magel2 regulates cellular circadian rhythm (Devos et al., 2011). Magel2 interacts with circadian rhythm proteins Bmal1 and Per2 in a co-immunoprecipitation assay (Devos et al., 2011). Magel2 acts as a negative repressor of Clock:Bmal1 activity at the *Per2* promoter resulting in negative regulation of the circadian rhythm cycle (Devos et al., 2011). However, given the role MAGEL2 plays in ubiquitination, we wanted to determine if MAGEL2 also affects circadian rhythm protein ubiquitination and therefore circadian rhythm protein levels. The Skp1-Cullin-F box complex that encompasses the RING E3 ligase RBX1 targets CRY proteins for ubiquitination and subsequent degradation. Along with regulation of PER, CRY protein levels determine the period of the circadian clock (Maywood et al., 2011; Meng et al., 2008; Siepka et al., 2007; Yoo et al., 2013; Zheng et al., 2002). Double knockout Cry1/Cry2 mice had a complete loss of circadian rhythmicity, indicting the vital role CRY proteins have on maintaining circadian rhythm (Van der Horst et al., 1999). Fine-tuned CRY protein levels through ubiquitination mediated pathways are important in the regulation of circadian rhythm.

MAGE proteins are regulators of ubiquitination through their interaction with RING E3 ubiquitin ligases and deubiquitinases (Doyle et al., 2010). First, we wanted to identify an

interaction between MAGEL2 and RBX1. RBX1 interacted with wild-type MAGEL2, but not with MAGEL2 harboring mutations in the MAGE homology domain (p.R1187C and p.LL1031AA) (Figure 3.4). Mutation of the equivalent dileucine motif in the winged helix A motif of the MAGE homology domain of MAGEG1/NSMCE3 (NSMCE3p.LL96AA) disrupts its binding to the RING-containing protein NSE1 (Doyle et al., 2010). As well, MAGEC2 carrying an equivalent dileucine to dialanine mutation (MAGEC2p.LL152AA) can no longer bind to TRIM28 (Doyle et al., 2010). Furthermore, both these MAGEL2 mutations suppressed E3 ligase RNF41 stabilization and negated the ability of MAGEL2 to increase trafficking of leptin receptor to the cell surface (Wijesuriya et al., 2017). This result sheds further light on the effect of mutations in the MHD and how insults to the MHD could result in inability of MAGEL2 to bind its protein interactors.

MAGEL2 interacts with ubiquitinated proteins and modulates their ubiquitination levels. CRY proteins are ubiquitinated and regulate transcription factors CLOCK and BMAL1 (Hirano et al., 2016; Papp et al., 2015; Siepka et al., 2007; Yoo et al., 2013). We found that MAGEL2 interacts CRY1 and CRY2 proteins, with CRY1 interacting with MAGEL2 MAGE homology domain mutants as well (Figure 3.5). In summary, MAGEL2 interacts with both cryptochrome proteins and RBX1, the RING E3 ubiquitin ligase responsible for ubiquitination and stability of CRY1 and CRY2.

MAGEL2 has been shown to co-localize with various E3 ligase-deubiquitinase-substrate complexes in the cytoplasm to alter either protein localization or stability. We hypothesized that if MAGEL2 interacted with RBX1-SCF complex, that MAGEL2 and RBX1 proteins would also co-localize in the cytoplasm. Although MAGEL2 does not alter the subcellular localization of RBX1, it does have overlapping expression in the cytoplasm where it could enhance ubiquitination of target circadian rhythm proteins (Figure 3.6). We reasoned that MAGEL2 could play a role in

regulating circadian rhythm proteins by altering their subcellular localization. CRY proteins are localized to both the nucleus and cytoplasm. In the nucleus, CRY and PER protein function to inhibit CLOCK/BMAL1 transcription. In the cytoplasm, CRY proteins accumulate and bind to PER proteins that are then imported into the nucleus (Kume et al., 1999). Casein kinase 1 ε (CK1ε) phosphorylates Per and retains Per protein in the cytoplasm (Vielhaber, Eide, Rivers, & Gao, 2000). CRY1 binding of PER1 abolishes CK1ε cytoplasmic retention and CRY/PER can then be translocated into the nucleus to inhibit CLOCK/BMAL1 transcription (Eide, Vielhaber, Hinz, & Virshup, 2002; Kume et al., 1999; E. Lee & Kim, 2014). In our localization experiments, CRY1 expressed alone had a mostly cytoplasmic localization, while CRY1 co-transfected with MAGEL2 was mostly nuclear. We conclude that MAGEL2 may enhance CRY1 ubiquitination, targeting cytoplasmic CRY1 for proteasomal degradation and therefore we see a reduction of CRY1 protein in the cytoplasm in the presence of MAGEL2. CRY1 ubiquitination and subsequent degradation in the cytoplasm at the beginning of the subjective day (when CLOCK/BMAL1 transcription activation is required) is necessary to inhibit CRY/PER complexes from accumulating and translocating into the nucleus.

Other components of the SCF complex that bind to CRY proteins include F-box proteins FBXL21 and FBXL3 that differentially recognize CRY proteins in the nucleus and cytoplasm (Yoo et al., 2013). In the nucleus, FBXL21 binds to CRY to stabilize the protein and inhibit ubiquitin mediated degradation by RBX1 and exclusively nuclear protein FBXL3 (Yoo et al., 2013). However, in the cytoplasm FBXL21 is responsible for CRY degradation, where it promotes ubiquitination along with RBX1 (Yoo et al., 2013). Given the overlap in expression of MAGEL2 with RBX1 and CRY1 in the cytoplasm, MAGEL2 may interact with FBXL21 acting as a scaffold

bridging MAGEL2 to the SCF protein complex to destabilize CRY proteins. The relationship between MAGEL2 and FBXL21 warrants further investigation.

Next, we reasoned that if there is less CRY1 protein in the cytoplasm in the presence of MAGEL2, then MAGEL2 may affect CRY1 protein levels. Increasing amounts of *MAGEL2* resulted in a decrease in CRY1 protein levels (Figure 3.7A). However, when the experiment was repeated using wild-type MAGEL2 and MAGEL2 mutant constructs (MAGEL2p.R1187C and MAGEL2p.LLAA) only the MAGEL2p.LLAA significantly reduced CRY1 protein levels. In this experiment wild-type MAGEL2 did not significantly reduce CRY1 protein levels highlighting the variability that is possible in cell culture experiments involving transfections. Interestingly, wild-type MAGEL2 and MAGEL2p.LLAA were not significantly different from each other, indicating there is a decrease in CRY1 protein levels when co-expressed with wild-type MAGEL2 in this experiment, although not significant. MAGEL2 carrying a mutation at a highly conserved arginine residue (MAGEL2p.R1187C) had no effect on CRY1 protein levels. This indicates that different mutations do not affect CRY1 protein levels similarly. Different mutations to the MHD may disrupt particular MAGEL2 interactions and therefore result in lack of MAGEL2 modulation on substrate protein ubiquitination levels.

Through its interaction with RING E3 ligase TRIM27, MAGEL2 facilitates ubiquitination of the WASH complex important in endosomal F-actin nucleation required for retromer mediated transport (Hao et al., 2013). MAGEL2 stabilized USP8 protein levels by reducing RNF41 ubiquitination of USP8 (Wijesuriya et al., 2017). CRY proteins are deubiquitinated and stabilized by deubiquitinase USP7 (Papp et al., 2015). As expected, CRY1 ubiquitination levels were decreased in the presence of USP7, and CRY1 protein levels increased (Figure 3.8A). MAGEL2 interacts with CRY1 and both RBX1 and USP7. If CRY1 protein levels are decreased in the

presence of MAGEL2, we hypothesized that MAGEL2 may enhance ubiquitination of CRY1. We therefore tested if MAGEL2 was able to modify CRY1 ubiquitination levels in a ubiquitination assay. Here we found that wild-type MAGEL2 increased CRY1 ubiquitination levels, but MAGEL2 proteins with MAGE homology domain mutations (p.R1187C and p.LL1031AA) did not alter CRY1 ubiquitination (Figure 3.8D). An intact MAGE homology domain is thus required for MAGEL2 to facilitate the ubiquitination of and destabilize CRY1, likely through interactions between MAGEL2 and the RBX1-containing SCF complex.

MAGEL2 interacts with core circadian rhythm protein CRY1, RBX1 the E3 ligase responsible for CRY1 ubiquitination and degradation, and USP7 the deubiquitinase responsible for CRY1 deubiquitination and stabilization. MAGEL2 enhances CRY1 ubiquitination resulting in reduced CRY1 protein levels and less CRY protein in the cytoplasm, targeting CRY1 for proteasomal degradation. CRY1 protein is tagged with ubiquitin and sent to the proteasome for degradation at the beginning of the subjective day when CLOCK:BMAL1 repression is no longer needed. *Magel2* has highest expression at the light to dark transition (subjective night and day respectively) (Kozlov et al., 2007). At the transition from the subjective night to day *CLOCK:BMAL1* expression is at its highest in order to initiate transcription of clock controlled genes during the subjective day. At this time, repression of *CLOCK:BMAL1* transcription by CRY and PER proteins is halted through degradation of these proteins facilitated by phosphorylation and ubiquitination posttranslational modifications (Maywood et al., 2011; Meng et al., 2008; Siepka et al., 2007). MAGEL2 may be important in reducing CRY1 protein levels at the subjective night to day transition, facilitating positive regulation of the circadian rhythm cycle through its interactions with RBX1, or could inhibit USP7 deubiquitination (Figure 3.9). The next step would be to test the effect on co-expression of MAGEL2 and USP7 on CRY1 ubiquitination to determine

if MAGEL2 is inhibiting USP7 deubiquitination of CRY1. MAGEL2 may be acting as a further form of positive regulation on CLOCK:BMAL1 transcription and circadian rhythm gene expression. It would be interesting to investigate if there are higher levels of CRY protein in the subjective day (dark) in *Magel2* mutant mice. It would also be of interest to investigate the effect of mutations in MAGEL2-USP7-RBX1 complex on the regulation of CRY protein levels.

In summary, Magel2^{tm1Stw} mice show circadian rhythm defects and people with PWS and mutations in *MAGEL2* also exhibit circadian rhythm disruptions (Camfferman et al., 2008; Kozlov et al., 2007; Weselake & Wevrick, 2012). At the cellular level, MAGEL2 is responsible for fine-tuning core circadian rhythm protein levels such as CRY1, through the ubiquitin mediated proteasomal degradation pathway. Disruptions in the regulation of core circadian protein levels due to loss of MAGEL2 could result in the circadian rhythm and sleep disruptions seen in children with PWS and SYS.

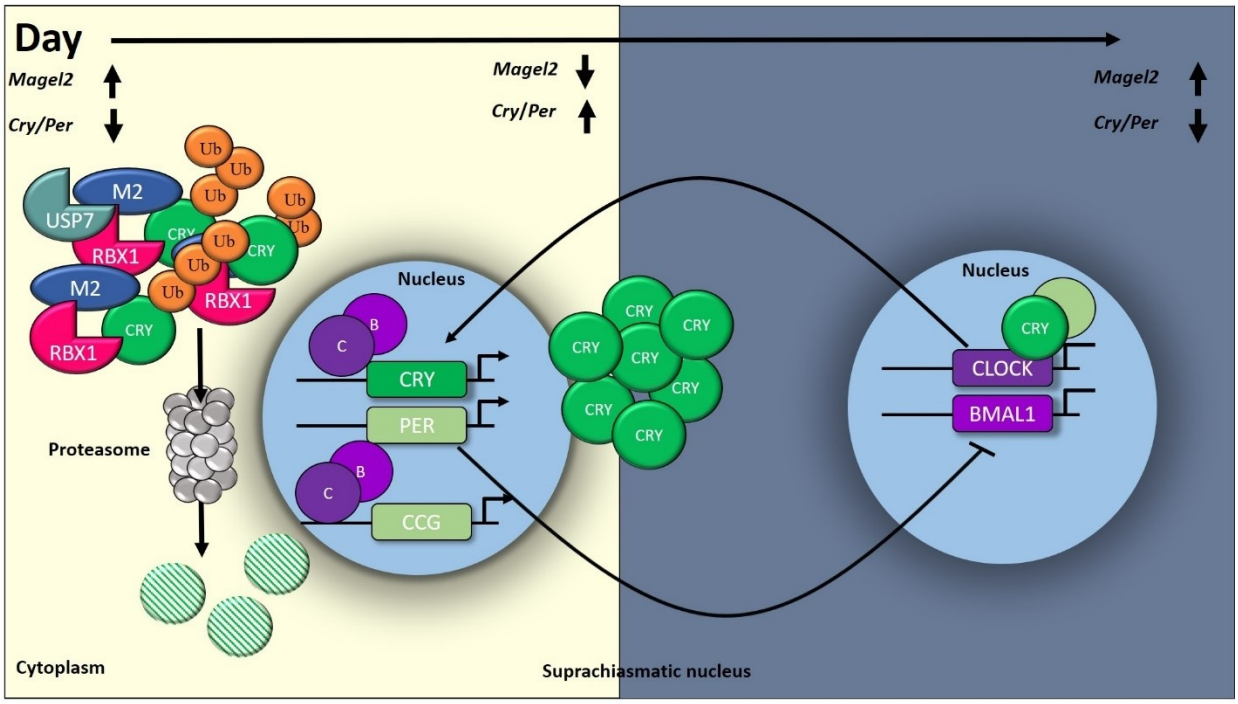


Figure 3.9 MAGEL2 modulates ubiquitination of CRY1, a core circadian rhythm protein, to fine-tune protein levels in the suprachiasmatic nucleus. MAGEL2 interacts with core circadian rhythm protein CRY1, its E3 ubiquitin ligase RBX1, and its deubiquitinase USP7. MAGEL2 increases the ubiquitination of CRY1 protein in the cytoplasm. MAGEL2 may be important in regulating CRY1 protein levels at the beginning of the subjective day to positively regulate CLOCK and BMAL1 transcription. Striped green circles indicated degraded CRY1 protein. C, CLOCK; B, BAML1; CCG, Clock-controlled genes; M2, MAGEL2; Ub, ubiquitin.

4 Chapter 4: MAGEL2 modulates the ubiquitination of BBS2 through its interaction with E3 ubiquitin ligase TRIM32, implicated in Bardet-Biedl syndrome

4.1 Introduction

Intracellular signaling is mediated by cell surface receptors that sense and respond to a variety of signals in the extracellular environment. One mechanism for the regulation of receptor activity involves trafficking of the receptor through the endosomal sorting process for either recycling back to the plasma membrane or to lysosomes for degradation. The endosomal sorting process involves endocytosis of molecules from the cell surface through vesicles. Vesicles and adapter proteins internalize molecules such as receptors into sorting/early endosomes (EE). Cargo is either trafficked to late endosomes/multivesicular bodies (MVBs) for transport to lysosomes for degradation , can be transported to the trans-Golgi through retromer transport for sorting, or finally they can be recycled back to the cell membrane through recycling endosomes (Figure 4.1Naslavsky & Caplan, 2018). Recycling of receptors back to the plasma membrane can occur through fast recycling directly through the recycling endosomes or slow recycling where cargo is first transported to the endocytic recycling compartment (ERC) before being recycled to the plasma membrane (Naslavsky & Caplan, 2018).

Ubiquitination is the major post translational modification that sends receptors into the endosomal sorting process, with the final stages of ubiquitination regulated by sets of proteins with antagonistic activities: E3 ubiquitin ligases, deubiquitinases (DUBs), and regulatory proteins such as melanoma antigen (MAGE) proteins. Although MAGE proteins do not have intrinsic enzymatic activity, they bind to and regulate the activity of E3 ubiquitin ligases and DUBs (Doyle et al., 2010; Feng et al., 2011; Lee & Potts, 2018). This modulation occurs through interactions between the ~200 amino acid conserved MAGE homology domain (MHD) of the MAGE protein and the

variable domains of the E3 ubiquitin ligases. Endosomal sorting complexes required for transport (ESCRT) are critical for the sorting of receptors and other proteins and are sensitive to the ubiquitination status of these proteins. Dysfunction in the endosomal sorting of cell surface receptors is associated with many human conditions and with the accumulation of cellular waste products, defective autophagy and degeneration.

TRIM (tripartite motif) proteins are E3 ubiquitin ligases that characteristically contain a N-terminal RING (Really Interesting New Gene) domain, one to two B-box domains and a Coiled-Coil region, followed by a variable C-terminal domain. TRIM27 (tripartite motif-containing 27, or Ret finger protein RFP) has roles in intracellular signaling, cell cycle, apoptosis and the regulation of transcription primarily through interactions between its C-terminal SPRY-domain and other proteins (Dho & Kwon, 2003; Shimono, Murakami, Hasegawa, & Takahashi, 2000; Tacer & Potts, 2018; Zaman et al., 2013). TRIM32 (tripartite motif-containing 32, BBS11) is similar in structure to TRIM27, but has six NHL repeats in its C-terminus. TRIM32 ubiquitinates a variety of proteins including Glis2, actin, dysbindin, and c-myc (Locke, Tinsley, Benson, & Blake, 2009; Ramachandran et al., 2014; Ryu et al., 2011; Shieh, Kudryashova, & Spencer, 2011). Both TRIM27 and TRIM32 associate with and antagonize the activity of the deubiquitinase USP7 (Nicklas et al., 2019; Zaman et al., 2013). USP7 has deubiquitinating activity towards many substrate proteins that function in a range of cellular processes (Kim & Sixma, 2017; Rawat, Starczynowski, & Ntziachristos, 2019). The USP7-TRIM27 complex can incorporate the MAGE protein MAGEL2, forming a complex that is necessary for the regulation of ubiquitination of the actin-nucleating protein WASH, facilitating endosomal actin assembly and protein trafficking (Hao et al., 2015). *MAGEL2* also regulates the endosomal trafficking of the leptin receptor together with another MAGE protein, necdin, and with the RING domain-containing E3 ubiquitin ligase

RNF41, the DUB USP8, and the ESCRT-0 component STAM (Wijesuriya et al., 2017). Yet another MAGE protein, MAGED1, binds to necdin and regulates the nerve growth factor-induced endosomal association of the p75 neurotrophin receptor (Bronfman, Tcherpakov, Jovin, & Fainzilber, 2003).

Primary cilia are organelles composed of microtubules, are non-motile and are responsible for sensing the extracellular matrix environment resulting in intracellular signalling cascades (Christensen, Morthorst, Mogensen, & Pedersen, 2017). Cilia protrude into the cytoplasm, known as the ciliary pocket, where they interact with endocytic structures and participate in endosomal trafficking (Clement et al., 2013; Molla-herman et al., 2009). Primary cilia-associated proteins mediate receptor signaling pathways by regulating endosomal trafficking: ALMS1, ARL13B, and BBS proteins (BBS1, BBS3 and BBS4) are all cilia-associated proteins with roles in the trafficking of receptors through endosomes (Barral et al., 2012; Collin et al., 2012; Leitch, Lodh, Prieto-echagu, Badano, & Zaghloul, 2014). Most BBS genes encode subunits or assembly factors for an octameric protein complex (the BBSome) required for the biogenesis and function of the primary cilium (Novas, Cardenas-rodriguez, Irigoín, & Badano, 2015).

The BBSome mediates protein trafficking and recycling of receptors to the plasma and ciliary membranes (Figure 4.1, Guo et al., 2016; Jin et al., 2010, Leitch et al., 2014; Seo et al., 2009; Starks, Beyer, Guo, Boland, & Zhang, 2015). The BBS protein BBS1 is required for proper trafficking of the leptin receptor back to the cell surface (Guo et al., 2016; Seo et al., 2009). BBS1 interacts with the LepR and knockdown of BBS1 or BBS2 resulted in a decrease of LepR abundance at the cell surface (Guo et al., 2016; Seo et al., 2009). Similarly, MAGEL2 interacts with the LepR and increases its abundance at the cell surface through interactions with the ubiquitination system (Wijesuriya et al., 2017). The LepR is recycled through clathrin dependent

endocytosis regulated by ubiquitination signals (Belouzard & Rouille, 2006). It has been suggested that TRIM32 may regulate the stability of the BBSome protein complex, possibly acting on BBS2, a core BBSome component that is susceptible to proteasomal degradation (Zhang, Yu, Seo, Stone, & Sheffield, 2012).

Consistent with a key role of endosomal trafficking in cellular homeostasis, many of the genes encoding the proteins described above are mutated in heritable genetic disorders. Interestingly, many of these disorders affect the development and function of the nervous system and muscle. Mutations in *TRIM32*, *ARL13B*, *BBS1*, *BBS3* and *BBS4* cause autosomal recessive disorder Bardet-Biedl syndrome (BBS, OMIM #209900, Table 1, Hamosh et al., 2005) and mutations in *ALMS1* cause the related autosomal recessive disorder Alström syndrome (OMIM #203800, Hamosh et al., 2005). Alström syndrome and BBS are complex genetic disorders affecting multiple body systems. While symptoms vary among individuals, Alström syndrome and BBS typically present with retinal degeneration, obesity, intellectual disability, delayed development, hypogonadism, and renal abnormalities (Forsythe & Beales, 2013; Marshall, Bronson, Collin, & Nordstrom, 2005; Vaisse, Reiter, & Berbari, 2018). A homozygous missense mutation (p.P130S) in the B-box of TRIM32 was identified in a consanguineous family with BBS (Chiang et al., 2006). Mutations in *TRIM32* also cause progressive muscle disease, either limb girdle muscular dystrophy (LGMD2H, OMIM #254110, Hamosh et al., 2005) or sarcotubular myopathy (Johnson et al., 2019; Lazzari & Meroni, 2016; Lazzari et al., 2019). Homozygosity for a frameshift mutation in the RING domain of TRIM32 caused both the progressive muscle weakness typical of LGMD2H and hypogonadism, hearing loss, and behavioral abnormalities seen in BBS in two other cases (Servian-Morilla et al., 2019).

Inactivating mutations in *MAGEL2* cause Schaaf-Yang syndrome (Schaaf et al., 2013a). Infants with SYS present with hypotonia, hypogonadism and developmental delays, and later develop intellectual disabilities, autism spectrum disorder, maladaptive behavior, endocrine dysfunction and excess fat mass (Mccarthy et al., 2018). *MAGEL2* is also one of a small set of genes inactivated in Prader Willi syndrome (PWS, OMIM 176270, Hamosh et al., 2005), a neurodevelopmental disorder that overlaps phenotypically with SYS. Mice carrying gene targeted mutation in either *Magel2* or BBS genes have excess fat mass, reduced sensitivity to leptin, and abnormal trafficking of the leptin receptor (Mercer et al., 2013; Nishimura et al., 2004; Seo et al., 2009; Wijesuriya et al., 2017). There were no morphological differences (length and shape of cilia) between cilia in the hypothalamus Magel2^{tm1Stw} mice compared to wild-type mice stained with primary cilia marker Adenylyl Cyclase type 3 (ACIII) (unpublished data from the Wevrick lab). Additionally, there were no differences in the amount of ACIII positive cilia in the hypothalamus of wild-type and Magel2^{tm1Stw} mice (unpublished data from the Wevrick lab). MAGEL2 and another PWS region protein Necdin interact with BBSome protein BBS4 (Lee et al., 2005). MAGEL2 may not have a role in cilia biogenesis but instead may be involved in the receptor trafficking function of cilia through its interactions with BBSome proteins. Other neurodevelopmental disorders are also caused by mutations in genes encoding components of the endosomal trafficking system (e.g. *WASHC4*, *VPS13B*, *VPS15*, *VPS51*, and *STAMBP*) (Hartwig et al., 2019; Patak, Zhang-James, & Faraone, 2017). Shared cell biological mechanisms may be responsible for the phenotypic overlaps in neurodevelopmental disorders caused by defects in ubiquitin-dependent endosomal trafficking of signaling receptors.

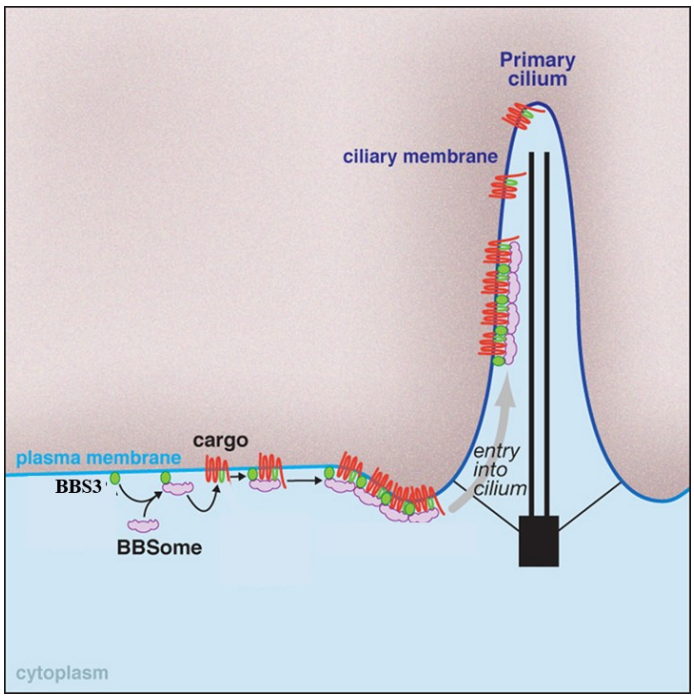


Figure 4.1 The BBSome mediates receptor and cargo trafficking to the plasma and ciliary membranes. The BBSome (made up of BBS1, BBS2, BBS4, BBS5, BBS7, BBS8, BBS9) interacts with BBS3 at the plasma membrane to facilitate trafficking of cargo to the ciliary and plasma membrane. BBS3 acts as an anchor at the plasma membrane to recruit the BBSome to the membrane. The BBSome can interact with receptors and cargo before being recruited to the plasma membrane by BBS3 or can facilitate the movement of cargo into the primary cilia, as shown in this figure. Figure modified from Jin et al., 2010.

4.1.1 Aims and Hypothesis

Given the phenotypic overlap in PWS, SYS, and BBS, we hypothesize that MAGEL2 may play a role in the ubiquitination of the BBSome important for receptor trafficking, specifically that of the leptin receptor. Firstly, we hypothesize that TRIM32, the proposed BBS2 E3 ubiquitin ligase in fact does ubiquitinate BBS2 protein. We also test the effect of pathogenic TRIM32 mutations P130S and D487N on BBS2 ubiquitination. We hypothesize that MAGEL2 could modulate BBS2 ubiquitination through interactions with the E3 ubiquitin ligases TRIM27 and TRIM32/BBS11.

4.2 Results

4.2.1 Interactions among MAGEL2, E3 ubiquitin ligases including TRIM32.

MAGEL2 interacts with TRIM27 through binding of the MAGE homology domain of MAGEL2 and the coiled-coil domain of TRIM27 (Hao 2013). Since MAGEL2 can affect E3 ligase activity, we wanted to determine if MAGEL2 also interacts with RING E3 ubiquitin ligase TRIM32. U2OS cells were transiently co-transfected with cDNAs encoding FLAG-tagged biotin ligase-fusion proteins (BirA*-MAGEL2 or BirA*-TRIM32) and epitope-tagged potentially interacting proteins. A proximity-dependent biotin identification assay (BioID) assay was performed to detect interactions between proteins. In short, streptavidin-affinity purification of biotinylated proteins from cell lysates of transiently transfected cells cultured in excess biotin was followed by immunoblotting to detect biotinylated proteins (Roux, Kim, & Burke, 2013; Wijesuriya et al., 2017). MAGEL2 interacted with both TRIM32 and TRIM27 by BioID (Figure 4.2A, lanes 2 and 6 and Figure 4.2B, lane 1), and TRIM32 and TRIM27 interacted with each other (Figure 4.2A, lane 7). MAGEL2 proteins carrying mutations in the MHD that impair some aspects of MAGEL2 function (p.R1187C and p.LL1031AA) nonetheless still interact with TRIM32 (Figure 4.2A, lanes 3 and 4) and TRIM27 (Figure 4.2B, lanes 2 and 3). To further explore this interaction, we performed another BioID experiment, this time using BirA*-TRIM32 as the bait. TRIM32 interacted with both MAGEL2 and TRIM27 in this experiment (Figure 4.2C, lanes 2 and 4).

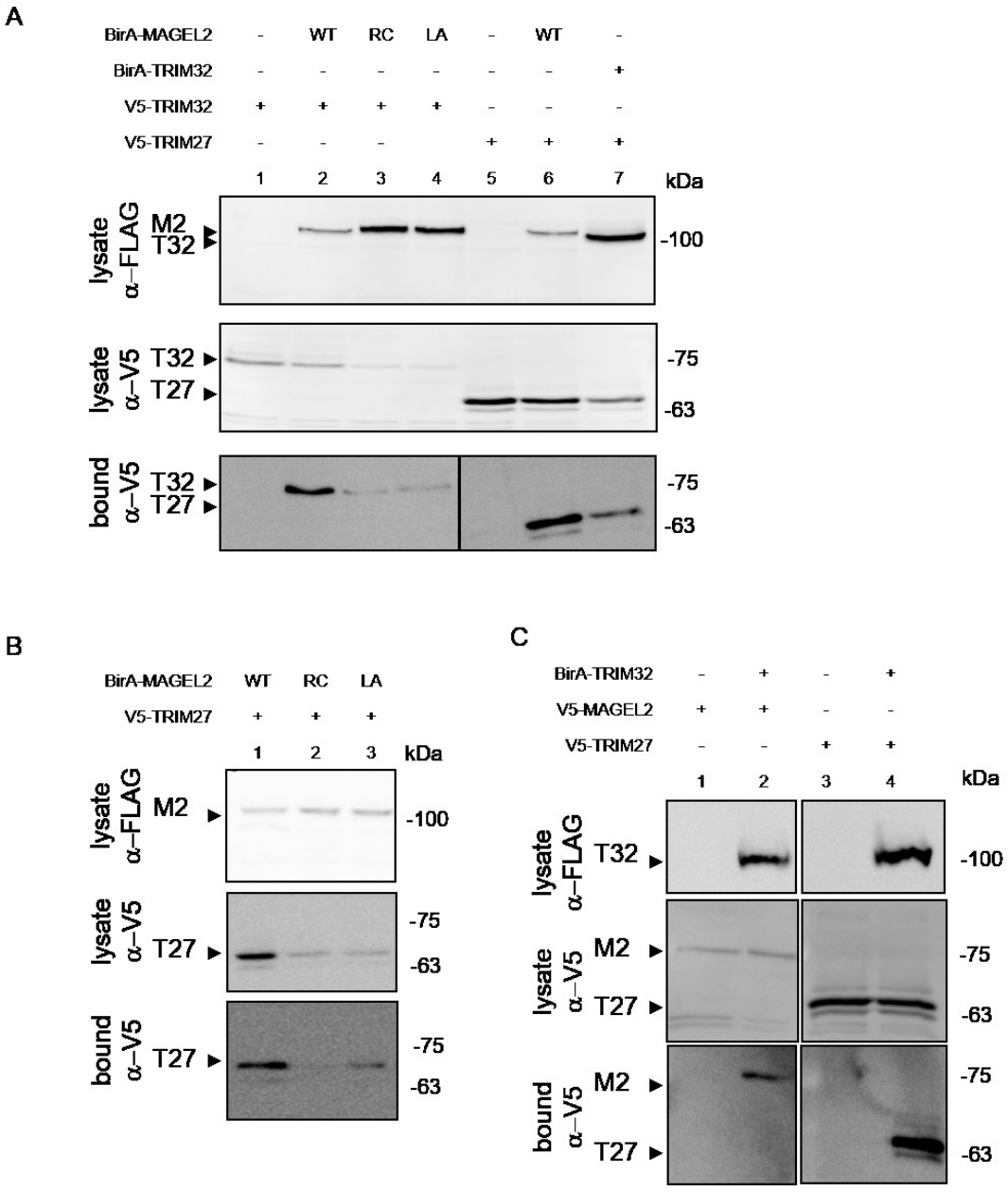


Figure 4.2 MAGEL2 interacts with E3 ubiquitin ligases TRIM32 and TRIM27, and TRIM32 interacts with TRIM27. U2OS cells were transiently transfected with combinations of epitope-tagged cDNA constructs. Cells were incubated with biotin for 24 h then processed for BioID. Cell lysates were collected and a fraction was retained (lysate). Biotinylated (bound) proteins were recovered from the remainder of the sample by streptavidin affinity purification, then detected by

immunoblotting. A) BirA*-FLAG-MAGEL2 wild-type (WT), BirA*-MAGEL2p.R1187C (RC), BirA*-MAGEL2p.LL1031AA (LA) (all indicated as M2) or BirA*-TRIM32 were co-transfected with V5-TRIM32 (T32) or V5-TRIM27 (T27) and processed for BioID. B) BirA*-FLAG-MAGEL2 (wild-type, WT), BirA*-MAGEL2p.R1187C (RC), BirA*-MAGEL2p.LL1031AA (LA) (all indicated as M2) were co-transfected with V5-TRIM27 (T27) and processed for BioID. C) BirA*-FLAG-TRIM32 was co-transfected with V5-MAGEL2 or V5-TRIM27 and processed for BioID. All interactions have been repeated at least three times.

4.2.2 MAGEL2 affects the abundance of E3 ubiquitin ligase proteins TRIM32 and TRIM27

MAGEL2 has been shown to affect the abundance of interacting E3 ubiquitin ligases (Wijesuriya et al., 2017). For example, co-expression of MAGEL2 increased the abundance of RNF41 expressed in co-transfected cells. Consistent with this result, levels of endogenous Rnf41 protein were lower in tissues from Magel2^{tm1Stw} mice carrying a gene-targeted *Magel2* allele (Wijesuriya et al., 2017). To investigate the extent to which the stability of TRIM32 and TRIM27 could be modulated by co-expression of MAGEL2, we measured the abundance of these E3 ubiquitin ligases in cultured cells that were co-transfected with cDNA constructs encoding epitope-tagged TRIM32 or TRIM27, with and without co-expression of MAGEL2 (Figure 4.3). More TRIM32 protein was detected in the presence of co-transfected MAGEL2 compared to when TRIM32 was transfected with empty vector control (performed in triplicate, Figure 4.3A). There was some variability in this experiment, with co-expression of MAGEL2 resulting in an increase in TRIM32 protein levels in two biological replicates (with 3 technical triplicate replicates each) and a decrease in TRIM32 protein levels in two biological replicates (with 3 technical triplicate replicates each). In contrast, less TRIM27 was detected in the presence of co-transfected MAGEL2 compared to when TRIM27 was transfected alone (Figure 4.3B). Interestingly, co-expression of mutant forms of MAGEL2 (p.R1187C and p.LL1031AA) resulted in a decrease in the abundance of recombinant TRIM32 compared to co-expression of WT-MAGEL2 or of TRIM32 alone, as detected on immunoblots of cell lysates (Figure 4.2A, lanes 3-4 compared to lanes 1-2, lysate immunoblotted with anti-V5). Co-expression of MAGEL2 MHD mutants resulted in a further decrease in the abundance of recombinant TRIM27 compared to co-expression of wild-type MAGEL2 (B, lanes 2-3 compared to lane 1).

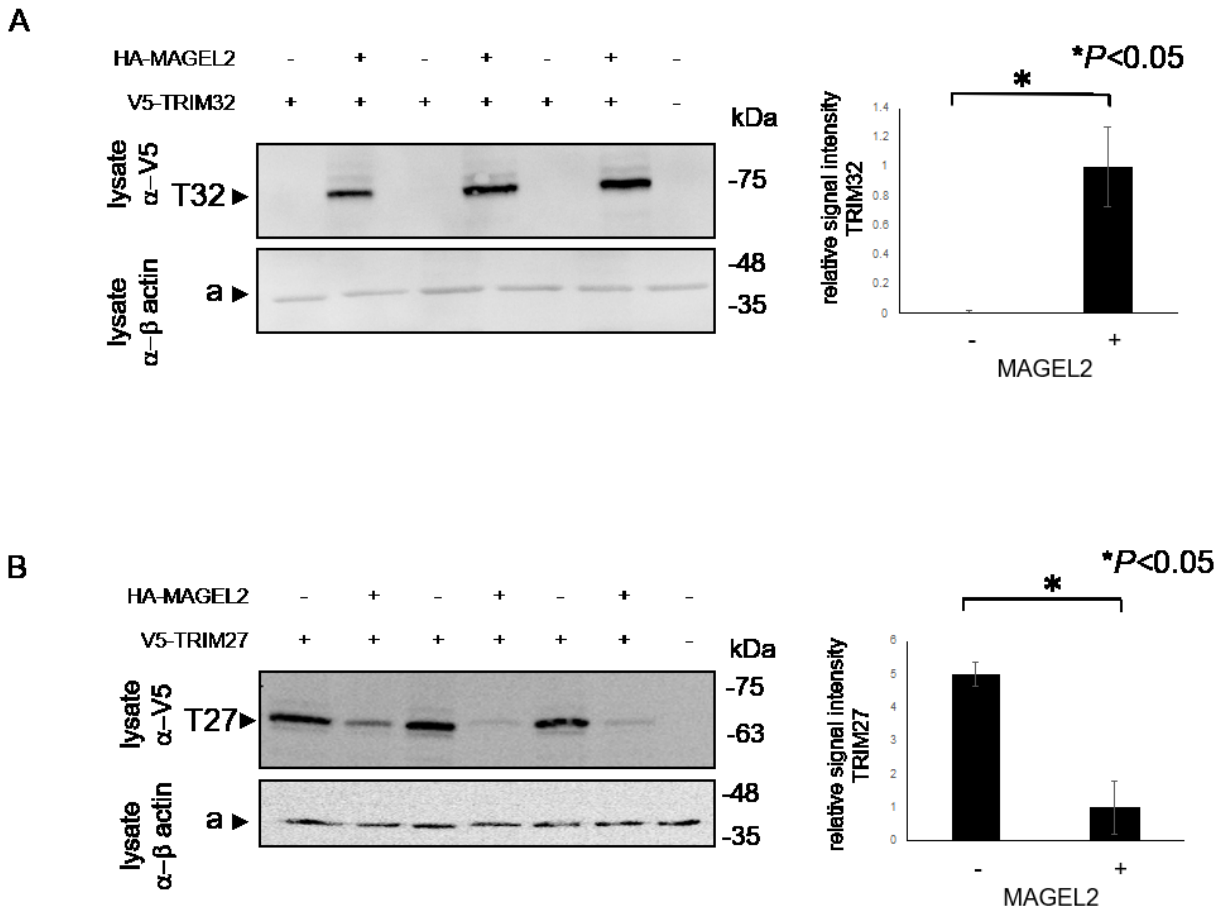


Figure 4.3 Co-expression of MAGEL2 increases the abundance of TRIM32 protein and decreases the abundance of TRIM27. U2OS cells were transiently transfected with combinations of epitope-tagged cDNA constructs encoding V5-TRIM32 (T32), V5-TRIM27 (T27) and HA-MAGEL2, then cell lysates were immunoblotted to detect recombinant proteins. A) Co-transfection of V5-TRIM32 and HA-MAGEL2. *, P<0.05 comparing TRIM32 abundance with co-expression of empty vector or MAGEL2 by Student t-test, technical triplicate. B) Co-transfection of V5-TRIM27 and HA-MAGEL2. Blots were re-probed with β-actin (a), used as a loading control. Abundance experiments have been repeated at least three times. *, P<0.05 comparing TRIM27 abundance with co-expression of empty vector or MAGEL2 by Student t-test, technical triplicate.

4.2.3 TRIM32 localizes similarly to MAGEL2 in the cytoplasm

We then tested whether MAGEL2 and TRIM32 proteins subcellular localization overlaps within the cell, by confocal microscopy. U2OS cells were transiently transfected with expression constructs encoding HA-tagged MAGEL2 or V5-tagged TRIM32, and recombinant proteins were detected using immunofluorescence microscopy. Nuclei were counterstained blue with Hoechst dye. MAGEL2 protein was diffusely localized to the cytoplasm, with some punctate expression of greater intensity (Figure 4.4A, single). TRIM32 was also largely localized to the cytoplasm, with distinct cytoplasmic speckles located around the nucleus (as in Lazzari et al., 2019; Locke et al., 2009). In doubly-transfected cells, MAGEL2 and TRIM32 were both localized to the perinuclear region of the cell (Figure 4.4A, double). Mutations in TRIM32 (p.P130S, located in the B-box, and p.D487N, located in the third NHL repeat) cause Bardet-Biedl syndrome and limb-girdle muscular dystrophy type 2H (LGMD), respectively (Chiang et al., 2006; Frosk et al., 2002). We next wanted to determine if TRIM32 mutations affected the subcellular localization of TRIM32 in the presence of co-expressed MAGEL2. In U2OS cells transfected with MAGEL2 and TRIM32 mutants followed by immunofluorescence, TRIM32p.P130S and TRIM32p.D487N localized similarly to MAGEL2 in the perinuclear region in a similar manner to wild-type TRIM32 (Figure 4.4B and C, respectively).

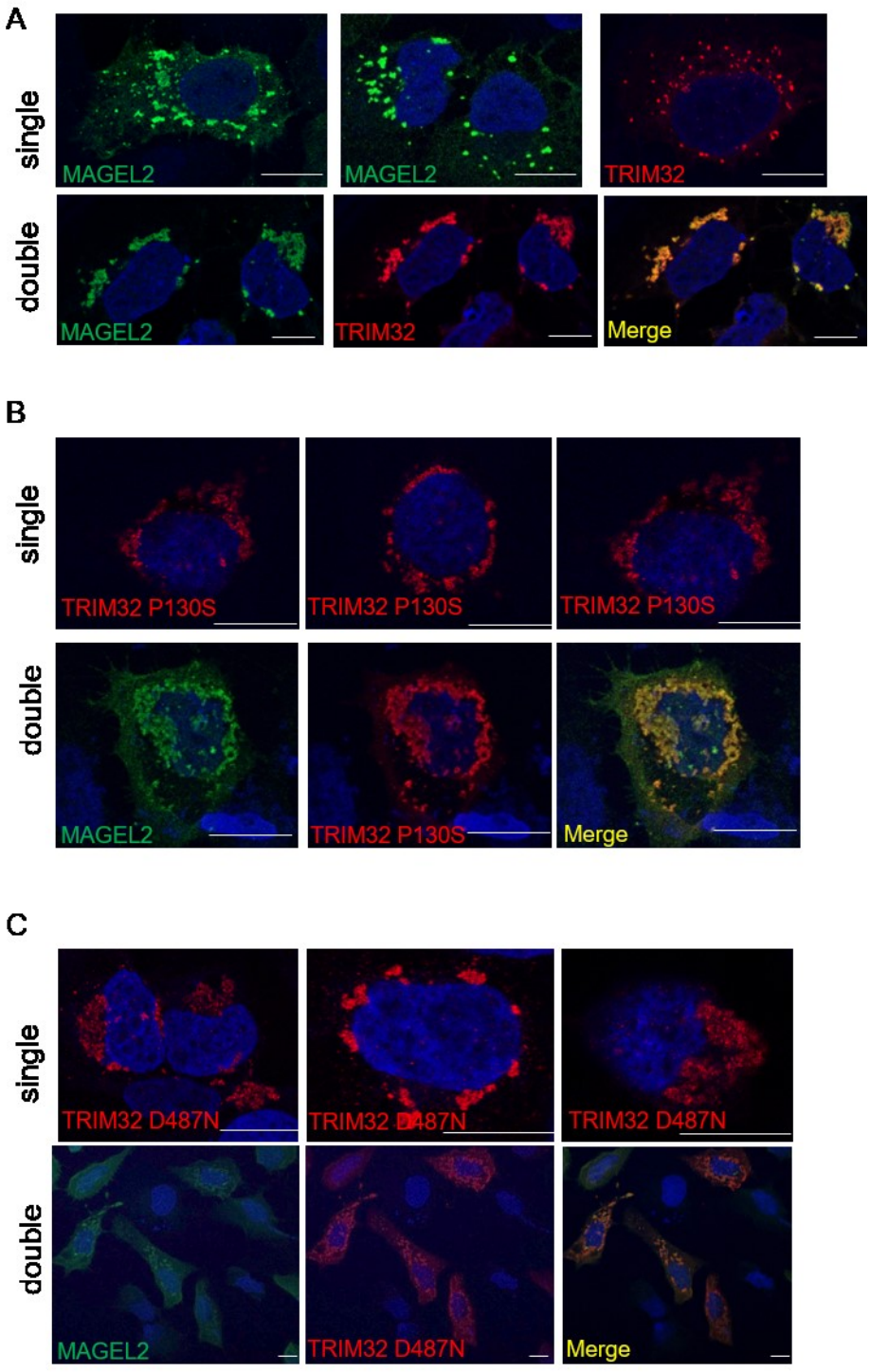


Figure 4.4 MAGEL2 expression overlaps with TRIM32 in the cytoplasm in transfected U2OS cells. A) Recombinant TRIM32 (red) and MAGEL2 (green) were detected in either singly transfected (single) or co-transfected (double) U2OS cells by immunofluorescence microscopy.

Yellow signal in merged image indicates where recombinant proteins overlap in the cell. B) Recombinant TRIM32 P130S (red) and MAGEL2 (green) were detected in transfected U2OS cells. C) Recombinant TRIM32 D487N (red) and MAGEL2 (green) were detected in transfected U2OS cells. Representative cells are shown. Nuclei were counterstained blue with Hoechst dye. Scale bars: 10 μm.

4.2.4 MAGEL2 does not affect ubiquitination of TRIM32

TRIM32 auto-ubiquitinates in the presence of specific E2 enzymes (Kudryashova et al., 2005). TRIM32 mono-ubiquitination regulates its sub-cellular localization, and some LGMD mutations may impair TRIM32 function by altering its ubiquitination (Lazzari 2016). We next investigated whether the co-expression of MAGEL2 affected the ubiquitination of TRIM32. HEK 293T cells were co-transfected with combinations of cDNAs encoding FLAG-TRIM32, V5-MAGEL2 and HA-ubiquitin. FLAG-TRIM32 was immunoprecipitated, and both TRIM32 and ubiquitinated TRIM32 were detected on immunoblots (Figure 4.5). Poly-ubiquitinated TRIM32 was evident as a smear above the expected molecular weight of TRIM32. More ubiquitinated TRIM32 was present when MAGEL2 was co-expressed (Figure 4.5, lane 4 compared to lane 3), but consistent with our co-expression studies (Figure 4.3A), there was also more TRIM32 protein in the presence of MAGEL2. Therefore, it was difficult to determine whether MAGEL2 alters the ubiquitination of TRIM32, although from the relative abundance of total and ubiquitinated TRIM32, it appears that MAGEL2 could increase the stability of TRIM32 by reducing the extent to which TRIM32 is ubiquitinated.

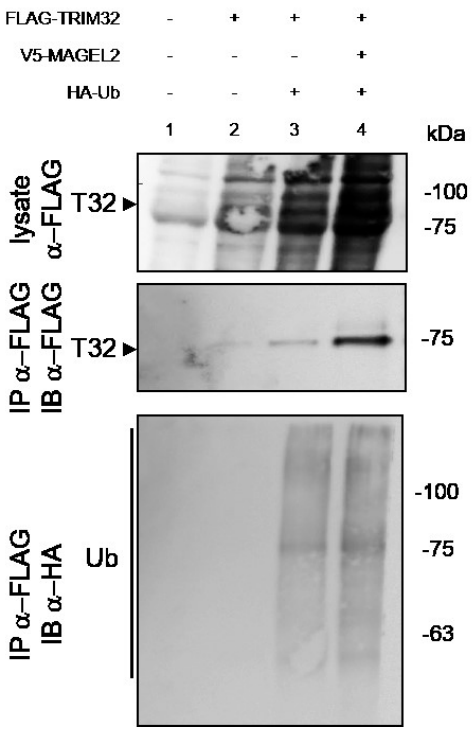


Figure 4.5 TRIM32 is polyubiquitinated but the effect of MAGEL2 on this ubiquitination is unclear. HEK 293T cells were transiently co-transfected with combinations of cDNA constructs encoding FLAG-TRIM32, V5-MAGEL2 and HA-Ubiquitin. Cells were treated with chloroquine and MG132 overnight, and a fraction of the collected cell lysate was retained (lysate). FLAG-TRIM32 was immunoprecipitated (IP) from remainder of lysate. Immunoprecipitated proteins were probed with anti-FLAG antibodies to detect FLAG-TRIM32 protein or with anti-HA antibodies to detect ubiquitinated FLAG- TRIM32 protein (appears as a smear above the expected size of TRIM32).

4.2.5 Interactions among MAGEL2, TRIM32 and BBS2

The BBS2 protein forms a multiprotein complex (the BBSome) with seven other BBS proteins, and mutations in the *BBS2* gene cause Bardet-Biedl syndrome. The stability of BBS2 is dependent on its interactions with other proteins, including BBS7 and BBS9 (Zhang et al., 2012). BBS2 is ubiquitinated and degraded through the proteosomal pathway, and TRIM32 has been proposed to regulate BBS2 stability through its E3 ubiquitin ligase activity. We had also previously shown that MAGEL2 co-localizes with BBSome components BBS4 and BBS8 (Lee et al., 2005). We therefore postulated that regulation by MAGEL2 could affect BBSome stability through its effects on TRIM32 and BBS2.

We first tested whether BBS2 interacts with MAGEL2 and TRIM32 in co-transfected cells, using BioID with combinations of BirA* tagged prey proteins (BirA*-TRIM32 or BirA*-MAGEL2) and bait proteins (BBS2 and TRIM32). BBS2 interacts with TRIM32 (Figure 4.6A) and with MAGEL2 by BioID (Figure 4.6B, TRIM32 as positive control). In addition, co-expression of MAGEL2 with BBS2 in U2OS cells increased the steady-state abundance of BBS2 (Figure 4.6C and D). We then detected endogenous BBS2 in HEK 293T cells that either expressed only residual levels of MAGEL2 (uninduced) or expressed recombinant MAGEL2 under the control of a tetracycline inducible promoter (+Tet: MAGEL2). However, we could not detect any difference in steady-state endogenous BBS2 protein levels between induced and uninduced cells (Figure 4.7).

Since TRIM32 is proposed to regulate BBS2 ubiquitination through its E3 ubiquitin ligase activity, and we determined that TRIM32 interacts with BBS2, we wanted to determine if TRIM32 and BBS2 co-localized in the cell. We transfected epitope-tagged BBS2 and TRIM32, either alone or together. When BBS2 was transfected alone in U2OS cells the protein had a diffuse cytoplasmic localization (Figure 4.8, single), while TRIM32 protein was present in a diffuse and punctate

pattern in singly transfected cells (Figure 4.8, single and Figure 4.4A). When BBS2 was co-transfected with TRIM32, a portion of the BBS2 protein expression overlapped with TRIM32 in a punctate fashion, while the remaining BBS2 protein retained its diffuse cytoplasmic pattern (Figure 4.8, double).

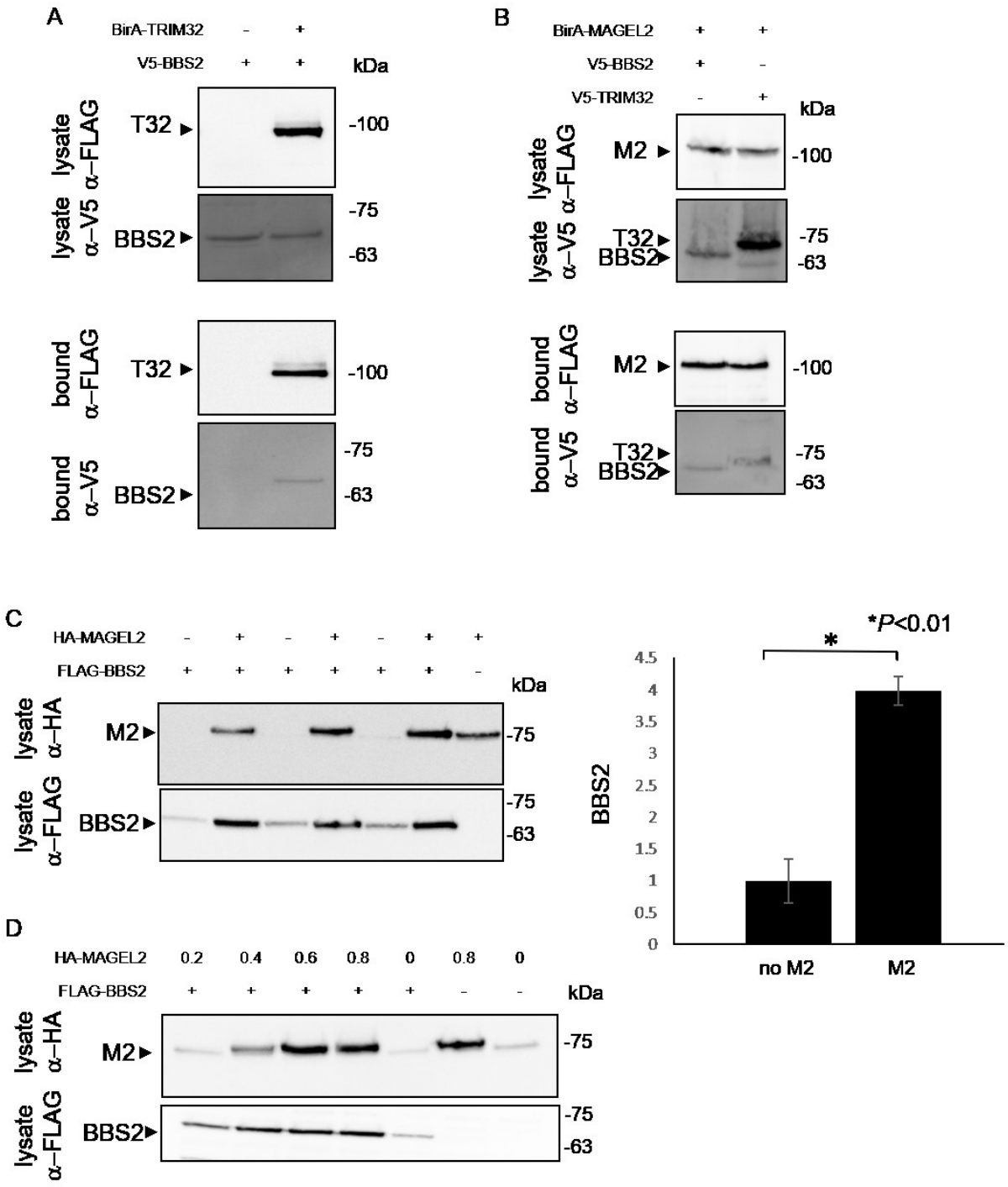


Figure 4.6 TRIM32 interacts with BBS2 and MAGEL2, while MAGEL2 increases BBS2 abundance. U2OS cells were transiently transfected with combinations of cDNA constructs encoding epitope tagged proteins. A) Cells were transfected with the indicated cDNA constructs

(BirA*-FLAG-tagged TRIM32 (T32) and V5-BBS2), incubated with biotin, then processed for BioID. B) Cells were transfected with the indicated cDNA constructs (BirA*-FLAG-MAGEL2 (M2), V5-BBS2, V5-TRIM32), incubated with biotin, then processed for BioID. C) U2OS cells were transfected with FLAG-BBS2 in the absence and presence of HA-MAGEL2 (M2), in technical replicates. Protein was quantified and equal amounts of protein were loaded into each lane. BBS2 was detected by immunoblot analysis of the cell lysates. *, $P<0.01$ comparing abundance of BBS2 with empty vector to co-expression of MAGEL2 by Student t-test, technical triplicate. D) Equal amounts of FLAG-BBS2 cDNA were transfected with increasing amounts of HA-MAGEL2 plasmid (0 to 0.8 μg) with empty vector used to equalize the total amount of plasmid transfected. Recombinant proteins were detected by immunoblotting. Results repeated at least three times.

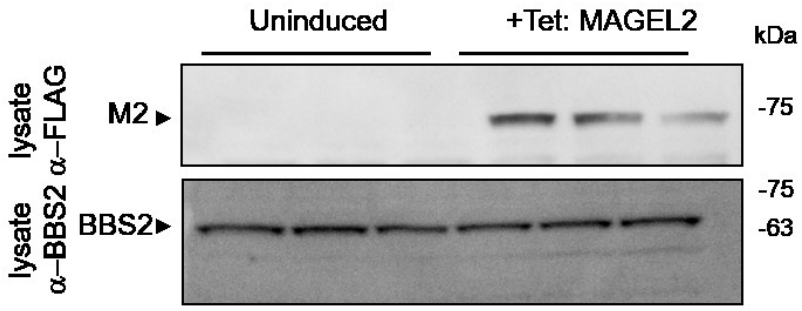


Figure 4.7 Endogenous BBS2 protein levels do not change in cells induced to express MAGEL2. Cells from a HEK 293T Flp-In stable cell line carrying a tetracycline inducible BirA*-FLAG-MAGEL2 cDNA were not treated (uninduced) or treated with tetracycline (+Tet), then cell lysates were collected after 24 h. Endogenous BBS2 protein was detected by immunoblotting against BBS2. Experiment was done as a technical triplicate.

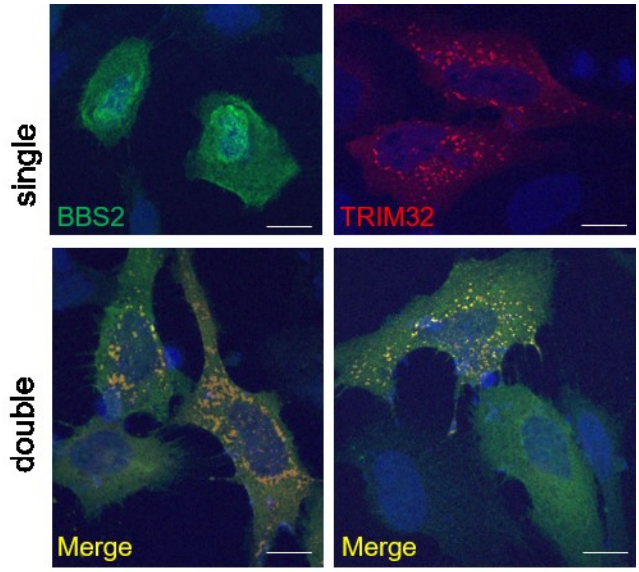


Figure 4.8 BBS2 localization changes in the presence of TRIM32. cDNAs encoding epitope-tagged BBS2 (green) or TRIM32 (red) were transfected alone (single) or together (double) in U2OS cells and recombinant proteins detected by immunofluorescence microscopy. Yellow signal in the merged images indicates where the proteins overlap in the cell. Scale bars: 10 μm.

4.2.6 MAGEL2 affects the ubiquitination of BBS2 in cooperation with TRIM32 and TRIM27

BBS2 is ubiquitinated and degraded through the ubiquitin-proteasomal pathway (Zhang et al., 2012). We determined that TRIM32 has similar cytoplasmic localization and interacts with BBS2, and MAGEL2 interacts with and increased the steady-state abundance of recombinant BBS2 protein. We therefore investigated whether MAGEL2 affects the ubiquitination of BBS2. Transfection of HA-ubiquitin with FLAG-BBS2 followed by immunoprecipitation with anti-FLAG beads demonstrated that BBS2 is ubiquitinated (Figure 4.9, lane 2). Ubiquitinated BBS2 was evident as a smear above the expected molecular weight of BBS2. The amount of ubiquitination of BBS2 was increased when either TRIM32 or TRIM27 was co-transfected (Figure 4.9, lanes 3 and 5). Co-transfection of MAGEL2 decreased the ubiquitination of BBS2 by either TRIM32 or TRIM27 (Figure 4.9, lanes 4 and 6).

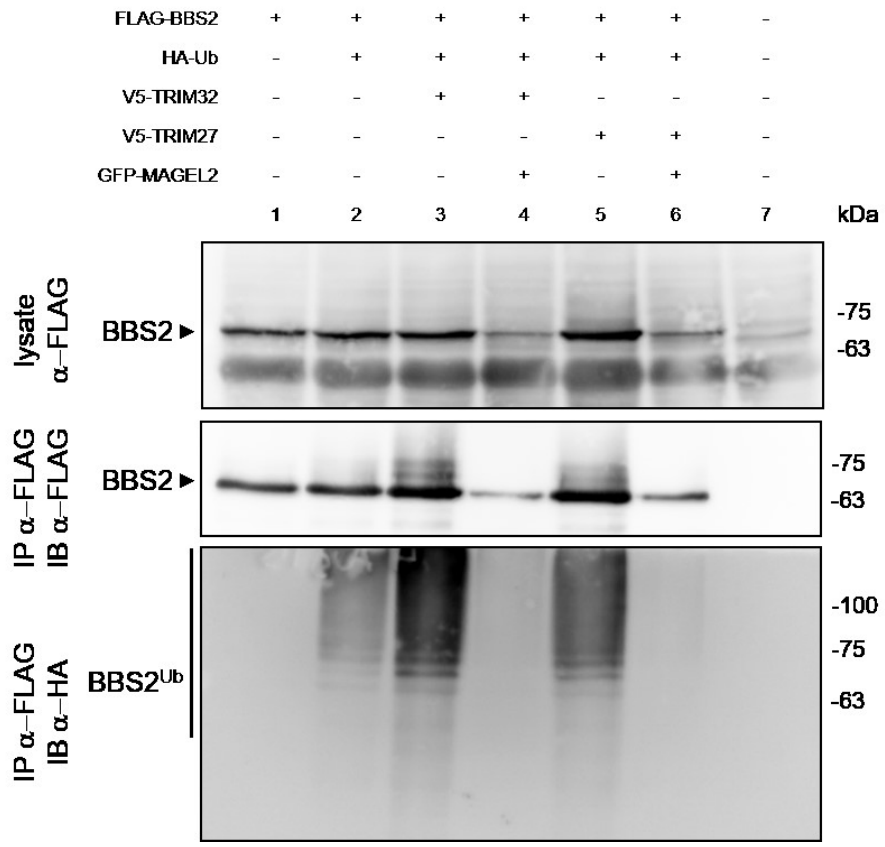


Figure 4.9 TRIM32 and TRIM27 increase polyubiquitination of BBS2, and MAGEL2 reduces this ubiquitination. A) HEK 293T cells were transiently co-transfected with combinations of cDNA constructs encoding FLAG-BBS2, V5-TRIM32 or V5-TRIM27, GFP-MAGEL2, and HA-ubiquitin. Cells were treated with chloroquine and MG132 overnight, and a fraction of the collected cell lysate was retained (lysate). FLAG-BBS2 was immunoprecipitated (IP) from remainder of lysate. Immunoprecipitated proteins were immunoblotted (IB) with anti-FLAG antibodies to detect FLAG-BBS2 protein or with anti-HA antibodies to detect ubiquitinated FLAG-BBS2 protein (appears as a smear above the expected size of BBS2). Repeated three times.

4.2.7 Mutations in TRIM32 alter ubiquitination of BBS2

We next tested whether mutations that impair the RING activity of TRIM32 affect the ubiquitination of BBS2. TRIM32iRING (inactive RING) is a mutant form in which Cys20, Cys39, and His 41 in the RING domain are replaced by alanines (C20A/C39A/H41A). Despite the mutations in the RING domain, co-expression of TRIM32iRING nonetheless increased the ubiquitination of BBS2 to an equivalent extent as WT-TRIM32 (Figure 4.10A, ubiquitinated BBS2 in lanes 3 and 5 compared to lane 2). Co-transfection with MAGEL2 counteracted the effect of TRIM32, so that there was less ubiquitination of BBS2 in the presence of MAGEL2 with either co-expression of TRIM32 or TRIM32iRING (Figure 4.10A, lanes 4 and 6).

We repeated this assay to investigate the effect of other TRIM32 mutants (p.P130S and p.D487N) on BBS2 ubiquitination. As in the previous experiment (Figure 4.10A), both wild-type and iRING forms of TRIM32 increased the ubiquitination of BBS2 (Fig. 9B, ubiquitinated BBS2 in lanes 3 and 9 compared to lane 2). In contrast, expression of TRIM32 p.P130S did not increase the ubiquitination of BBS2 (Figure 4.10B, ubiquitinated BBS2 in lane 5 compared to lane 2), while expression of TRIM32 p.D487N had a similar effect as wild-type TRIM32. Co-transfection with MAGEL2 counteracted the effect of wild type and p.D487N TRIM32 proteins on BBS2 ubiquitination, but had no effect on ubiquitination of BBS2 in the presence of TRIM32p.P130S (Figure 4.10B, lanes 4, 6 and 8). Thus, the P130S mutation in TRIM32, but not the iRING or D487N mutations, appears to inhibit the ability of TRIM32 to promote the ubiquitination of BBS2 and inactivates the ability of MAGEL2 to inhibit BBS2 ubiquitination.

Next, we investigated the type of polyubiquitin chains attached to BBS2 in the presence of E3 ubiquitin ligases. FLAG-BBS2 protein was immunoprecipitated and protein lysates were probed with lysine-48 (K48) and lysine-63 (K63) antibodies to identify chains that typically send

protein for proteasomal degradation or target protein for other processes respectively. TRIM32 can facilitate the polyubiquitination of BBS2 on both K48 and K63 (Figure 4.11, lane 3). The amount of ubiquitination of BBS2 on both K48 and K63 was also increased by co-expression of TRIM27 (Figure 4.11, lane 5). MAGEL2 co-transfection again counter-acted the increased ubiquitination of BBS2 by either TRIM32 or TRIM27 co-expression (Figure 4.11, lanes 4 and 6). In summary, TRIM27 and TRIM32 interact with each other, with MAGEL2, and with BBS2, and together these proteins regulate the ubiquitination and the abundance of the key BBSome component BBS2.

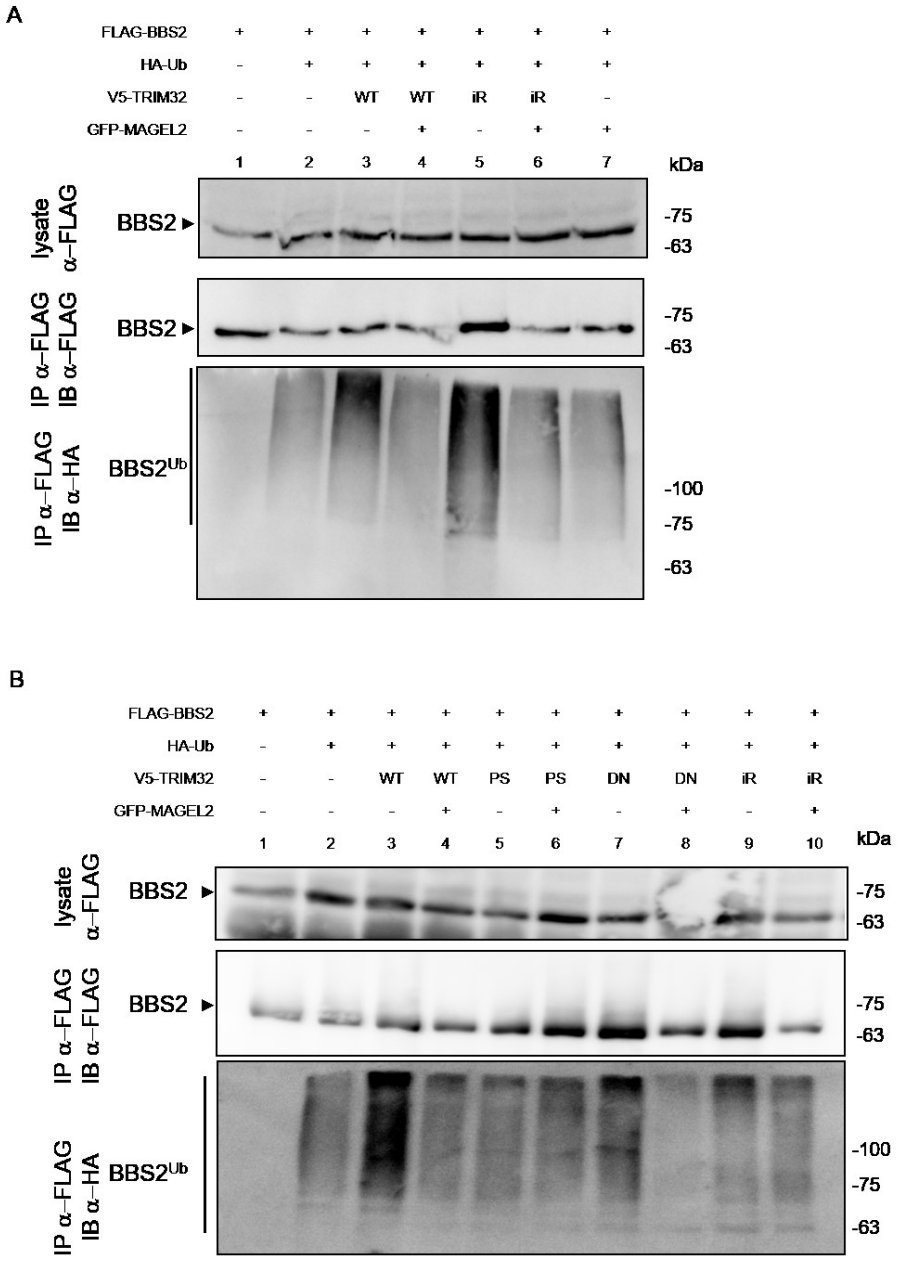


Figure 4.10 TRIM32 variant proteins and MAGEL2 variably affect the ubiquitination of BBS2. A) HEK 293T cells were transiently co-transfected with combinations of cDNA constructs encoding FLAG-BBS2, V5-TRIM32 (wild-type, WT) or V5-TRIM32 with mutations in the RING domain (iRING, iR), GFP-MAGEL2 and HA-ubiquitin. Cells were treated with chloroquine and MG132 overnight, and a fraction of the collected cell lysate was retained (lysate). FLAG-BBS2 was immunoprecipitated (IP) from remainder of lysate. Immunoprecipitated proteins were

immunoblotted (IB) with anti-FLAG antibodies to detect FLAG-BBS2 protein or with anti-HA antibodies to detect ubiquitinated FLAG-BBS2 protein (appears as a smear above the expected size of BBS2). B) HEK 293T cells were transiently co-transfected with combinations of cDNA constructs encoding FLAG-BBS2, V5-TRIM32 (wild-type, WT) or V5-TRIM32 with various mutations (P130S (PS), D487N (DN), RING domain (iR)), GFP-MAGEL2 and HA-ubiquitin. Cells were treated with chloroquine and MG132 overnight, and a fraction of the collected cell lysate was retained (lysate). FLAG-BBS2 was immunoprecipitated (IP) from remainder of lysate. Immunoprecipitated proteins were probed with anti-FLAG to detect FLAG-BBS2 protein or with anti-HA to detected ubiquitinated FLAG-BBS2 protein (appears as a smear above the expected size of BBS2). Results were repeated twice.

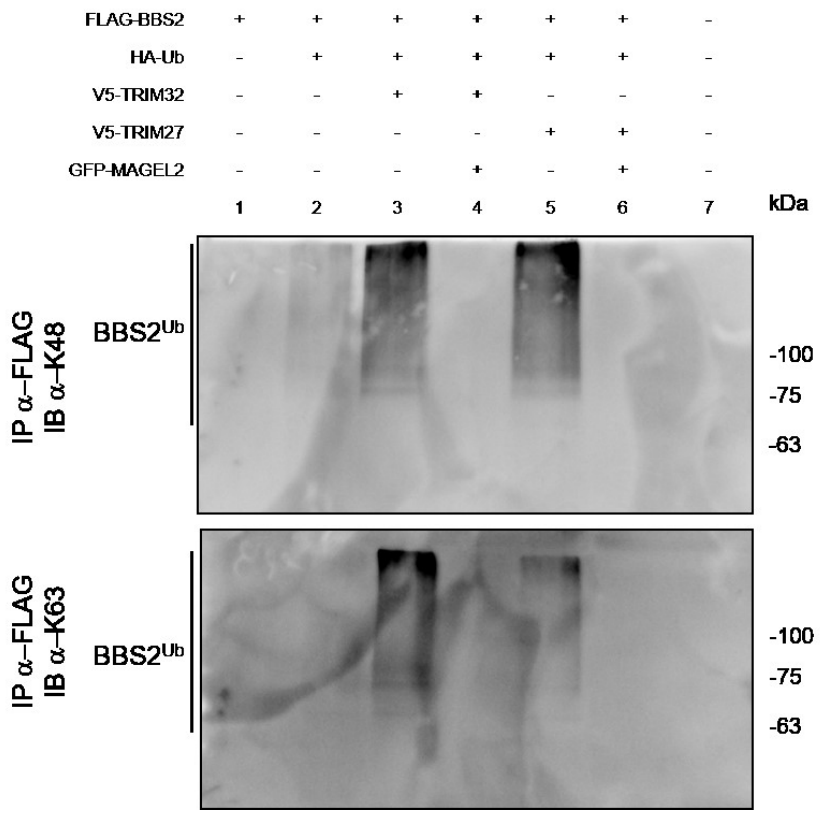


Figure 4.11 BBS2 is ubiquitinated on both lysine 48 and 63 by TRIM32 and TRIM27, with MAGEL2 reducing this ubiquitination. HEK 293T cells were transiently co-transfected with combinations of cDNA constructs encoding FLAG-BBS2, V5-TRIM32 or V5-TRIM27, GFP-MAGEL2 and HA-ubiquitin. Cells were treated with chloroquine and MG132 overnight, and a fraction of the collected cell lysate was retained (lysate). FLAG-BBS2 was immunoprecipitated (IP) from remainder of lysate. Immunoprecipitated proteins were immunoblotted (IB) with antibodies to detect lysine-48 (K48) and lysine-63 (K63) ubiquitin chains. (appears as a smear above the expected size of BBS2). Results repeated twice.

4.3 Discussion

Neurodevelopmental disorders with overlapping phenotypes could result from perturbations in common cellular pathways. Ubiquitination of proteins is vital for determining protein turnover rates and downstream protein functions. Mutations in genes involved in the ubiquitination of target proteins could result in similarities across different complex neurodevelopmental disorders. In this study, we wanted to determine the underpinning cellular process that could result in common phenotypes seen in people with PWS, SYS, and BBS. Specifically, with the overlapping cellular functions of genes implicated in PWS and BBS in endosomal trafficking, we wanted to determine if MAGEL2 was a modulator of ubiquitination of the BBSome complex. In this study we provide evidence that TRIM32 and TRIM27 ubiquitinate BBS2 and MAGEL2 is a modulator of this ubiquitination.

MAGE proteins interact with E3 ubiquitin ligases to modulate their activity. We found that MAGEL2 interacts with TRIM32 and confirmed the MAGEL2-TRIM27 interaction (Figure 4.2). However, MAGEL2 with mutations in the MHD still interacts with TRIM32. MAGEL2 interaction with TRIM32 could be facilitated by a different domain of the MAGEL2 protein, as these mutations have been shown to disrupt other MAGEL2-E3 ubiquitin ligase complexes (Wijesuriya et al., 2017). TRIM proteins form homodimers and heterodimers that could be responsible for substrate binding specificity (Hatakeyama, 2017; Reymond et al., 2001). We also found that TRIM27 and TRIM32 interact with each other, confirming a previously identified TRIM heterodimer interaction (Hatakeyama, 2017). These results suggest that MAGEL2, TRIM27, and TRIM32 can form a protein complex in cells, and that this complex can still form even when the MHD of MAGEL2 carries mutations that disrupt some MAGEL2 functions. MAGE proteins MAGEL2 and Necdin interact with the E3 ubiquitin ligase RNF41 and deubiquitinase

USP8 to modulate LepR levels at the cell surface through the endosomal protein trafficking pathway (Wijesuriya et al., 2017). BBS1, a component of the BBSome, interacts with LepR and mice lacking BBS1 or other BBS proteins were obese and had impaired LepR signaling (Guo et al., 2016; Seo et al., 2009). Given the role MAGEL2 and the BBSome play in LepR recycling through endosomal trafficking, we demonstrate a new MAGEL2-E3 ligase complex where MAGEL2 interacts with TRIM32 the E3 ubiquitin ligase proposed to be responsible for BBSome ubiquitination and subsequent degradation.

E3 ubiquitin ligases participate in auto-ubiquitination to regulate their own bioavailability (Bie & Ciechanover, 2011; Kudryashova, et al, 2005). Specifically, TRIM32 regulates its own mono-ubiquitination, which typically is responsible for targeting protein for proteasome independent processes (Locke et al., 2009). Here we show TRIM32 is also polyubiquitinated, although MAGEL2 has no effect on the level of TRIM32 poly-ubiquitination (Figure 4.5). However, MAGEL2 did affect TRIM32 protein levels with co-transfection of MAGEL2 resulting in more TRIM32 protein, with some variability in this result (Figure 4.3). Conversely, co-expression of MAGEL2 resulted in less TRIM27 protein. Co-expression of MAGEL2 mutants (p.R1187C and p.LL1031AA) resulted in a decrease of TRIM32 and TRIM27 protein levels when compared to co-expression with wild-type MAGEL2. Although the MAGEL2 mutants did not affect the ability of MAGEL2 to biotinylate TRIM32 in a BioID proximity assay, they do affect MAGEL2 ability to regulate TRIM32 protein levels. These results suggest that the MHD of MAGEL2 is important for the effects of MAGEL2 on the stability of E3 ubiquitin ligases with which it interacts, including RNF41 (Wijesuriya et al., 2017), TRIM27, and TRIM32. An alternative mechanism could be that MAGEL2 influences the deubiquitination rather than ubiquitination of TRIM32 through interactions with deubiquitinases, such as USP7 or USP8.

MAGE proteins modulate protein ubiquitination and can affect protein degradation levels, as well as subcellular localization (Wijesuriya et al., 2017). TRIM32 localizes in speckles in distinct cytoplasmic regions known as cytoplasmic bodies (Lazzari et al., 2019; Locke et al., 2009). TRIM32 alone had a punctate cytoplasmic localization (Figure 4.4). When co-transfected with MAGEL2, both MAGEL2 and TRIM32 had overlapping punctate perinuclear expression within the cell. Mutations in TRIM32 (P130S and D487N) cause BBS and LGMD2H respectively. Proteins carrying these mutations had similar punctate cytoplasmic expression to that of wild-type TRIM32 (Chiang et al., 2006; Frosk et al., 2002). MAGEL2 also had similar protein localization with TRIM32 mutants and did not affect this subcellular localization. Unlike previous findings that showed the two TRIM32 mutant proteins having a more diffuse cytoplasmic localization with some punctate expression when compared to wild-type TRIM32, we found the mutant proteins to have similar subcellular localization to wild-type (Lazzari et al., 2019; Locke et al., 2009). Differences between our findings and previous findings could be due to the different cell lines used to study TRIM32 localization. In our experiments we used U2OS cells, a human osteosarcoma cell line whereas Lazzari et al., 2019 used C2C12 myoblasts derived from mouse and Locke et al., 2009 used Cos-7 cells derived from African green monkey kidney. We show normal localization of mutant TRIM32 protein associated with BBS and LGMD2H type II and MAGEL2 has overlapping localization with these TRIM32 mutant proteins.

BBS2, a component of the BBSome complex, is ubiquitinated and targeted for proteasomal degradation (Zhang et al., 2012). TRIM32 was proposed as the E3 ligase that ubiquitinates BBS2 (Zhang et al., 2012) and MAGEL2 was previously identified to interact with other BBSome proteins (Lee et al., 2005). We showed MAGEL2 interacts with BBS2 and TRIM32, and TRIM32 also interacts with BBS2 (Figure 4.6). MAGEL2 interacts with the ubiquitin-protease system to

modulate substrate protein levels. As previously described, MAGEL2 affects TRIM32 protein levels and it could affect the protein levels of the TRIM32 substrate, BBS2. Co-expression of MAGEL2 and BBS2 results in increased BBS2 protein suggesting that MAGEL2 stabilizes BBS2 levels. However, in induced cells stably expressing MAGEL2, there was no difference in endogenous BBS2 protein levels (Figure 4.7). This discrepancy could be due to detection of endogenous BBS2 in HEK 293T cells which may not express BBS2 protein to the same extent to that of experiments where epitope-tagged BBS2 is transiently transfected. Therefore, there may not be enough BBS2 protein for stably transfected MAGEL2 to effect the protein levels within the cell. Alternatively, to investigate the functional significance of the role of MAGEL2 on BBS2 protein levels, we could look at changes in protein abundance of BBS2 in the hypothalamus of wild-type mice versus Magel2^{tm1Stw} mice.

We showed that TRIM32 interacts with BBS2 and they also localize similarly in the cell. Epitope-tagged BBS2 has a cytoplasmic distribution with some expression at the basal body of cilia (Berbari, Lewis, Bishop, Askwith, & Mykytyn, 2008). We showed that when BBS2 is expressed alone it has a diffuse cytoplasmic localization, however when co-expressed with TRIM32 it took on a more punctate localization similar to that of TRIM32 (Figure 4.8). Co-localization of BBS2 and TRIM32 to cytoplasmic bodies suggests TRIM32 complexes with BBS2 to re-localize the protein, possibly priming BBS2 for ubiquitination at these punctate regions. To further characterize the relationships between TRIM32 and BBS2, we showed BBS2 is ubiquitinated in the presence of TRIM32 (Figure 4.9). TRIM27 also increased BBS2 ubiquitination. Interestingly, MAGEL2 reduced the increase in BBS2 ubiquitination levels caused by TRIM32 and TRIM27. Reduced levels of BBS2 ubiquitination by co-expression of MAGEL2 could explain the increase in BBS2 protein levels observed in co-transfected cells (Figure 4.6).

Co-expression of MAGEL2 in the presence of either TRIM32 or TRIM27 reduced the abundance of BBS2, in contrast to the effect of MAGEL2 on BBS2 abundance in the absence of over-expressed E3 ubiquitin ligase. This suggests a complex interplay among the E3 ubiquitin ligases and the modulator protein MAGEL2 in the regulation of abundance of substrate proteins. TRIM32 mutant with an inactive RING domain (TRIM32iRING) was still able to ubiquitinate BBS2 similar to the extent of wild-type TRIM32. This phenomenon was previously observed for Gli2, a protein whose ubiquitination is increased by co-expression of either WT or RING mutant TRIM32 (Ramachandran et al., 2014). The TRIM32 ring mutant construct may still have residual ligase activity, and possibly using a TRIM32 truncated protein lacking the RING domain entirely may be a better option to study the ligase activity.

Mutations identified in TRIM32 have mostly been associated with limb girdle muscular dystrophy type 2H and are located in the NHL domain at the C-terminal (Lazzari & Meroni, 2016). However, a single mutation (TRIM32 p.P130S) found in a consanguineous family located in the B-box domain of TRIM32 associates with Bardet-Bield syndrome (Chiang et al., 2006). We found that TRIM32 LGMD2H mutation p.D487N could increase ubiquitination levels of substrate BBS2 similar to that of wild-type while the BBS mutation p.P130S did not increase BBS2 ubiquitination (Figure 4.10). Subsequently, MAGEL2 modulated wild-type and p.D487N ubiquitination of BBS2 but had no effect on p.P130S ubiquitination of BBS2. TRIM32 p.P130S mutation may inhibit MAGEL2 from modulating BBS2 protein since on its own p.P130S did not affect BBS2 ubiquitination levels. It is important to note however that MAGEL2 could not decrease BBS2 ubiquitination levels from that of baseline and therefore wild-type TRIM32 may be necessary for MAGEL2 to modulate BBS2 ubiquitination.

Proteins can either be mono- or poly-ubiquitinated with ubiquitin chains forming on seven lysine residues of the ubiquitin protein. Lysine-48 (K48) and lysine-63 (K63) ubiquitin chains are the most common (Li et al., 2008; Welchman et al., 2005). Polyubiquitination of K48 is responsible for targeting substrate proteins towards the proteasomal pathway while polyubiquitination of K63 modulates performs primarily non-proteolytic functions such as changes in protein trafficking, localization, or function (Jacobson et al., 2009; Li et al., 2008). TRIM32 and TRIM27 were able to enhance K48 and K63 polyubiquitination of BBS2, suggesting ubiquitination by these E3 ubiquitin ligases can mediate proteasomal degradation signals as well as further downstream changes to BBS2 function (Figure 4.11). MAGEL2 reduced the K48 and K63 polyubiquitination levels of BBS2 suggesting MAGEL2 modulates proteasomal dependent and independent ubiquitination of BBS2. MAGEL2-TRIM32 modulation of BBS2 protein ubiquitination provides further evidence of the interplay between MAGEL2-TRIM32 and BBS2 at the cellular level. Moreover, the pathogenicity of the Bardet-Biedl syndrome mutation p.P130S in TRIM32 may be related to its inability to promote the ubiquitination of BBS2, as well as to render BBS2 ubiquitination insensitive to regulation by MAGEL2.

The BBSome mediates leptin receptor trafficking to the cell membrane (Guo et al., 2016; Seo et al., 2009). BBS1 interacts with LepRb, the signaling isoform of the leptin receptor, and knockdown of BBS1 or BBS2 in cells results in reduced LepR at the cell surface (Guo et al., 2016; Seo et al., 2009). Mice with targeted BBSome protein (BBS1, BBS2, BBS4, or BBS6) had impaired LepR signaling and became obese (Guo et al., 2016; Seo et al., 2009). The BBSome is associated with cilia and basal bodies and regulates receptor trafficking at the cell membrane through the endosomal sorting process (Clement et al., 2013; Molla-herman et al., 2009). MAGEL2 forms a complex with E3 ubiquitin ligase RNF41 and DUB USP8 (Wijesuriya et al.,

2017). This protein complex interacts with Necdin (another MAGE protein), which binds to the LepR to regulate receptor levels at the cell surface (Wijesuriya et al., 2017). This complex interacts with the ESCRT-0 complex of endosomes to transport LepR through the endosomal trafficking pathway (Wijesuriya et al., 2017). Another identified function of MAGEL2 is the MAGEL2-TRIM27-USP7 complex that regulates the ubiquitination of the WASH complex that facilitates protein trafficking through the endosome (Hao et al., 2015). Here we show the interplay between the regulation of LepR in two different protein complexes with genes implicated in disorders (PWS, SYS, BBS) with overlapping phenotypes. Given the role MAGEL2 has been shown to play in the endosomal sorting process, our data integrates another E3 ubiquitin ligase, TRIM32 and substrate BBS2 into MAGEL2 function important for trafficking of receptors, specifically the leptin receptor. In our model, MAGEL2 interacts with TRIM32, TRIM27, and BBS2 to modulate BBS2 ubiquitination and degradation that could facilitate LepR receptor trafficking through the endosomal protein trafficking pathway (Figure 4.12). Degradation of BBS2 is responsible for BBSome bioavailability and the TRIM32-MAGEL2-TRIM27 complex could regulate these levels to ultimately influence LepR cell surface expression. In my model, I show TRIM32 and TRIM27 promoting degradation of BBS2 (and therefore BBSome) however an alternative possibility is that these E3 ligases are also responsible for targeting the BBSome to the plasma membrane. TRIM32 and TRIM27 facilitate K63 ubiquitin linkages of BBS2, and can therefore influence BBS2 localization and trafficking. MAGEL2 may fine tune both K48 and K63 linkages on BBS2 to modulate protein stability and localization.

Perturbations in LepR trafficking and signaling are a proposed mechanism for obesity in neurodevelopmental disorders. The impaired ubiquitination of BBS2 by TRIM32 p.P130S mutant implicated in BBS, could result in faulty LepR trafficking and ultimately the obesity phenotype

seen in patients with this mutation. MAGEL2 modulation of this ubiquitination could be important in fine tuning LepR levels and interplay between all the mentioned proteins could result in syndromic obesity seen in PWS and BBS. Evaluating changes in the BBSome complex function to traffic the leptin receptor in the presence of TRIM32 or MAGEL2 mutations or in knockdowns would be valuable to understand the overall role of the TRIM32-MAGEL2 complex on BBSomse regulation.

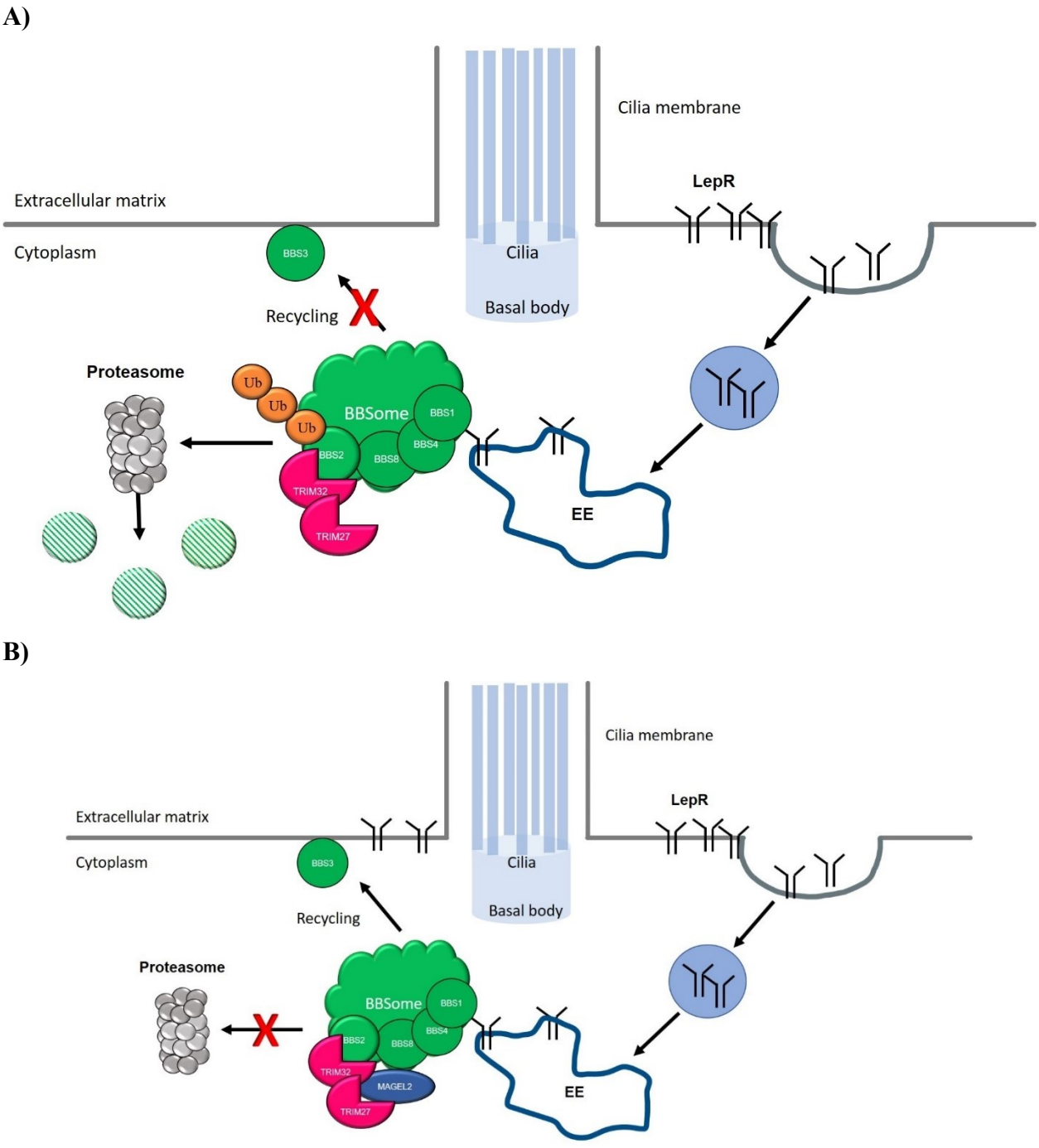


Figure 4.12 MAGEL2 regulates BBS2 ubiquitination through a ubiquitin dependent pathway. Through the endosome sorting process, LepR is internalized and taking to early endosomes. The BBSome interacts with LepR to recycle LepR back to the cell surface. **A)** We identified E3 ubiquitin ligases TRIM32 and TRIM27 as interactors of BBS2 that ubiquitinate

BBS2 protein with both lysine-48 and lysine-63 ubiquitin linkages. TRIM32 and TRIM27 target BBS2 towards proteasomal degradation. **B)** In this study, we found MAGEL2 interacts with E3 ubiquitin ligases TRIM32 and TRIM27 as well as substrate protein BBS2, the ubiquitinated BBSome complex protein. TRIM32 or TRIM27 enhance poly-ubiquitination of BBS2, and MAGEL2 decreases this increase. BBS2 protein levels increased with co-expression of MAGEL2 indicating MAGEL2 may stabilize BBS2 protein. MAGEL2 modulation of BBS2 may be important for BBSome bioavailability and therefore LepR trafficking to the cell surface. Striped green circles indicate degraded BBSome protein. EE, early endosomes; LepR, leptin receptor; ub, ubiquitin.

5 Chapter 5: Overall Discussion and Conclusions

Perturbations in MAGEL2, a gene implicated in Prader-Willi and Schaaf-Yang syndromes result in various symptoms that affect multiple body systems such as the nervous, endocrine, and musculoskeletal systems. The cellular role of MAGEL2 has not been fully elucidated, but in recent years MAGEL2 has been identified as a modulator of ubiquitination (Hao et al., 2013, 2015; Wijesuriya et al., 2017). Ubiquitination is required for almost all cellular functions, whether it is determining protein half-life and availability or tagging proteins for altered functions. There are many steps in the ubiquitination process where modulators or scaffold proteins play a critical role in fine tuning ubiquitinated protein levels and function. We sought to determine the role of MAGEL2 in ubiquitination of proteins involved in circadian rhythm and those involved in obesity caused by leptin receptor trafficking defects, similar to symptoms seen in Bardet-Biedl syndrome. We also show for the first time that MAGEL2 not only enhances ubiquitination levels of E3 ubiquitin substrates, as in the case of CRY1 and WASH1 (Hao et al. 2013), but can also reduce ubiquitination levels of substrate proteins, as in the case of BBS2.

Ubiquitination determines protein turnover important in regulating the circadian rhythm cycle. Defects in ubiquitination of core circadian rhythm proteins could result in sleep abnormalities and circadian rhythm defects in people with mutations in any component of the circadian rhythm system. Fine-tuned CRY protein levels throughout the circadian rhythm cycle are vital in determining circadian rhythm outputs and governing behaviour. We identified MAGEL2 as a modulator of CRY1 protein stability and ubiquitination. Through MAGEL2 interactions with E3 ubiquitin ligase RBX1 and deubiquitinase USP7, MAGEL2 modulates CRY1 protein levels. Defects in circadian rhythm seen in mice and people with mutations in MAGEL2 could be caused by dysregulated ubiquitination of CRY.

Neurodevelopmental disorders PWS and BBS have overlapping phenotypes such as abnormal body composition with increased fat mass and obesity. BBS is caused by mutations in any one of at least 21 genes, some of which are involved in the BBSome complex important in cilia biogenesis and protein trafficking (Priya, Nampoothiri, Sen, & Sripriya, 2016; Sattar & Gleeson, 2011). PWS and BBS present with overlapping phenotypes such as learning disabilities, hypogonadism, and obesity. Mice lacking either *Magel2* or BBS genes have abnormal leptin trafficking, reduced leptin sensitivity, and increased fat mass (Mercer et al., 2013; Nishimura et al., 2004; Seo et al., 2009; Wijesuriya et al., 2017). MAGEL2 and the related MAGE protein Necdin interact with BBS4, a component of the BBSome (Lee et al., 2005). The BBSome complex is regulated by ubiquitination of BBSome protein BBS2, although the E3 ubiquitin ligase responsible for ubiquitination has yet to be identified (Zhang et al., 2012). We identified TRIM32 and TRIM27 as E3 ubiquitin ligases responsible for BBS2 ubiquitination with MAGEL2 acting as a modifier of this ubiquitination. Given the interactions between MAGEL2, Necdin, BBS4, BBS8, TRIM32, and BBS2, and the modulation of ubiquitination of BBS2 by MAGEL2, it is reasonable to speculate that MAGEL2 functions in BBSome regulation. The function of MAGEL2 as a ubiquitination modulator of the BBSome could explain the overlapping phenotypes seen in PWS and BBS. It has been suggested that the interplay between different leptin signaling pathways could be required for coordinating leptin functions in the hypothalamus and the coordination of these different pathways could be important for proper LepR trafficking (Roujeau, Jockers, & Dam, 2019). Understanding the cellular roles of proteins involved in neurodevelopmental disorders with overlapping phenotypes could help in identifying perturbed pathways that cause these phenotypes.

A major limitation to the experiments performed in this thesis is the use of over-expression of exogenous genes through plasmid transfections. To study MAGEL2 function, proteins were

epitope-tagged in order to probe for MAGEL2, as endogenous MAGEL2 has not been successfully detected using antibody techniques. Collaborative efforts are being taken and development of a working antibody was deemed a priority at the latest PWS international conference. Transfections in cell lines result in over-expression of proteins that would normally not be present at that level in human cells. Although transfections are a widely used and accepted method to study protein function, the difference between endogenous gene expression and exogenous gene expression cannot be ignored. Constructs used in the experiments in this thesis were epitope tagged. These tags could influence protein interactions, function, or localization. We also used partial MAGEL2 in all experiments within this thesis, which harbors the MAGE homology domain but does not contain the full N-terminal proline rich region. The full length MAGEL2 protein proved difficult to immunoprecipitate and therefore the choice was made to use partial MAGEL2. Given the role other MAGE protein domains could play in protein-protein interactions it would be valuable to understand the functions and properties of the full length MAGEL2 protein. As more is understood about the MAGEL2 protein it would be important to investigate the mutations found in these domains in children with SYS. Another limitation when studying MAGEL2 protein function is the lack of reliable antibody towards MAGEL2.

It would be interesting to investigate changes in protein levels identified in these MAGEL2 complexes in Magel2^{tm1Stw}. I would expect there to be abnormal ubiquitination of the substrate proteins studied, resulting in changes in protein levels. It would also be vital to investigate the effect of overexpression or loss of *MAGEL2* on the other proteins in the protein complexes mentioned in the two systems studied. This would lend a more thorough understanding of how MAGEL2 is affecting complex ubiquitination and function. Regarding the circadian rhythm results, it would be interesting to study *MAGEL2* mutations, over expression, and silencing in NIH

3T3 cells, a fibroblast cell line exhibiting circadian oscillations. Since MAGEL2 is a modulator of BBS2 ubiquitination, and BBS2 is an important part of the BBSome core complex, it would be intriguing to look at the effects of BBSome complex assembly in the absence of MAGEL2. To better understand how different *MAGEL2* mutations could result in the clinical variability seen in SYS patients, it would be interesting to examine the effect of MAGEL2 on substrate ubiquitination levels using cell lines stably expressing different *MAGEL2* patient mutations. Of most interest to investigate would be the c.1996dupC *MAGEL2* mutation that showed the most severe clinical presentation among Schaaf-Yang syndrome patients (Mccarthy et al., 2018). This would also help elucidate the differences in function of complete loss of *MAGEL2* as seen in PWS, versus truncating mutations of MAGEL*2* identified in SYS. The different results observed for the effect of the MHD mutant proteins used in this study (MAGEL2 p.LL1301AA and MAGEL2 p.R1187C) on MAGEL2 binding and ubiquitination of substrate proteins indicates different mutations may affect MAGEL2-interacting proteins in different ways.

Results from this thesis, coupled with previous investigations into the role of MAGEL2 highlight the importance of MAGEL2 as a modulator of ubiquitination in the proteasomal dependent and independent pathways (Hao et al., 2013, 2015; Wijesuriya et al., 2017). The diverse phenotypes in PWS and SYS affecting multiple body systems could result from perturbed MAGEL2 functions in the ubiquitination of substrate proteins in various pathways (Figure 5.1). One example that warrants investigation of MAGEL2 function in ubiquitination pathways involves its possible role in muscle development and maintenance. *Magel2* is also expressed in developing muscle, developed skeletal muscle, bone marrow, and mesodermal cells involved in the formation of muscle, bone, and fat (Hao et al., 2015; Kamaludin et al., 2016; Kozlov et al., 2007). People with PWS have various musculoskeletal abnormalities resulting in reduced limb strength and

endurance with reduced energy expenditure, poor balance, and scoliosis (Capodaglio et al., 2011; Edouard et al., 2012; Reus et al., 2013; Weiss & Goodall, 2009). Children with PWS exhibit abnormal body composition with less lean muscle and increased fat mass, which is recapitulated in the Magel2^{tm1Stw} mice (Kamaludin et al., 2016; Reus et al., 2011). Magel2^{tm1Stw} mice recapitulated many of the musculoskeletal phenotypes observed in people with PWS (Kamaludin et al., 2016). Perturbations in ubiquitination have been linked to muscle phenotypes such as atrophy (Sandri, 2013). At the cellular level, MAGEL2 could modulate the ubiquitination and deubiquitination of muscle specific E3 ubiquitin ligases, deubiquitinases or their substrates. In addition to BBS2 investigated in this thesis, TRIM32 also has several muscle specific substrates such as N-myc downstream-regulated gene (NDRG), myosin, actin, dysbindin, and plakoglobin (Cohen, Lee, Zhai, Gygi, & Goldberg, 2014; Kudryashova et al., 2005; Locke et al., 2009; Mokhonova et al., 2015; Nicklas et al., 2012, 2019). Other possible interacting E3 ubiquitin ligases include the muscle-specific ring finger (Murf) proteins Murf1 (Trim63) and MURF2 (TRIM55/RNF29) that were abnormally expressed in adult Magel2^{tm1Stw} mice and in PWS fibroblasts, respectively (Horsthemke et al., 2003; Kamaludin et al., 2016). MAGEL2 could modulate ubiquitination of MURF substrates that include muscle proteins actin and myosin (Rubel et al., 2013).

Many MAGE proteins may function in modulating ubiquitination of substrates involved disease and development. MAGED1 enhances ubiquitination and proteasomal degradation of the serotonin transporter (SERT) (Mouri et al., 2012). SERT is responsible for the regulation and recycling of serotonin. Serotonin is a neurotransmitter responsible for regulating behaviours regarding social interactions, mood, and appetite. Maged1 knockout mice exhibit deficits in social interaction, increased anxiety, and symptoms of depression (Dombret et al., 2012; Mouri et al.,

2012). Perturbations of ubiquitination levels of SERT in the absence of Maged1 could result in deficits in neurobehaviours seen in mice lacking Maged1. MAGED1 has not yet been implicated in a neurodevelopmental disorder but given its role in serotonin regulation and the phenotypes seen in knockout mice, there could be unidentified pathological variants of MAGED1 in people with undiagnosed syndromes. Technological advances in diagnostics could unveil further MAGE proteins involved in neurodevelopmental genetic disorders and understanding MAGE protein function in these phenotypes could help guide therapeutics.

MAGE proteins are conserved in all eukaryotes with some organisms only having one MAGE protein. In mammals, the MAGE protein has seen rapid evolutionary expansion and the MAGE homology domain is highly conserved. Due to this conversation many MAGE proteins may have overlapping protein functions. Maged1 knockout mice recapitulate many of the phenotypes seen in both Magel2 mouse models (Magel2^{tm1Stw} and Magel2$^{tm1.1Mus}$) and people with PWS such as hyperphagia, impaired social interactions, and deficits in motor activity (Dombret et al., 2012; Mouri et al., 2012; Carias et al., 2019). Both Maged1 mice and Magel2 mice (Magel2^{tm1Stw} and Magel2$^{tm1.1Mus}$) had decreased oxytocin levels and OT treatment rescued phenotypes in all three mouse models (Carias et al., 2019; Dombret 2012; Schaller et al., 2010). However, certain MAGE proteins may be more important in regulating ubiquitination of specific substrates within certain tissues, making each MAGE protein similar in function yet unique in substrate specificity. Loss of different MAGE proteins would result in distinct phenotypes as seen in MAGEL2 in SYS versus MAGED2 in antenatal Bartter syndrome.

To truly understand how to tackle therapies and treatments for complex genetic disorders, we must first understand the gene's role at the most basic science level. Given that there are over 600 E3 ubiquitin ligases and over 100 DUBs, there are many MAGE-E3 ubiquitin ligase-DUB relationships that could play critical roles in fine-tuning protein stability and function, as well as

roles in disease. With emerging evidence for the role of the MAGE family of proteins in the modulation of ubiquitination, it will be critical to investigate unidentified MAGE protein targets and pathways. Further investigation into the role of MAGE proteins will elucidate the role of MAGEs in ubiquitination of substrate proteins and their role in disease through their interactions with the ubiquitin system (Table 2) and those that remained to be discovered. Overall MAGE proteins may cause disease through the fine tuning of substrate ubiquitination that may causes symptoms ranging from mild to severe depending on the effect of the MAGE protein on ubiquitination levels and overall protein abundance, localization, trafficking or function.

Various type II MAGE proteins are expressed in the brain, indicating this class of MAGE proteins could be responsible for neuronal development and/or function (Lee & Potts, 2017). Approximately 13% of E3 ligases are associated with over 70 different neurological disorders (George et al., 2018). Copy number variants in genes implicated in autism were found in genes encoding various E3 ubiquitin ligases (Glessner et al., 2009). In a co-expression analysis of 455 genes associated with autism, many of the identified candidate genes were enriched in the gene ontology category relating to functions in ubiquitination (Mahfouz et al., 2015). It is interesting to hypothesize that many MAGE proteins could play a role in neurodevelopment disorders through their interactions with E3 ligases. In PWS and SYS, ASD could be a result of perturbed ubiquitination of MAGEL2-E3 ubiquitin ligase complex on substrates involved in brain function. In a genome-wide association study, TRIM27 was identified as a strong candidate for social communication deficits associated with ASD (St Pourcain et al., 2013). TRIM27 is a MAGEL2 interacting protein, where perturbed ubiquitination of unidentified TRIM27 substrates involved in neuronal development or function could result in the ASD phenotypes. Dysbindin, a TRIM32 substrate is involved in endosomal-lysosomal trafficking and is associated with schizophrenia and

childhood onset psychosis (Benson, Sillitoe, & Blake, 2004; Gornick et al., 2005; Locke et al., 2009). We show TRIM32 interacts with MAGEL2 and modulates BBS2 substrate ubiquitination levels. MAGEL2 could also fine-tune ubiquitination important in Dysbindin protein stability, resulting in deficits in neurobehaviours. Different MAGE proteins could affect a variety of substrates to ultimately fine-tune neuronal development and function.

Overall the results of this thesis shed further light on the cellular role of MAGE proteins in ubiquitination, a critical post translational modification, and how perturbations in this family of proteins could result in genetic disorders. The role of MAGEL2 as a modulator of ubiquitination of various substrates could explain how the deletion of one gene could cause such variable symptoms affecting multiple body systems. Our results contribute to understanding the etiology of complex genetic disorders involving mutations in *MAGEL2.* Moving forward, fully characterizing the role of MAGEL2 in ubiquitination pathways of substrate proteins will guide the development of pharmaceutical interventions and gene therapies in PWS, SYS, and related neurodevelopmental disorders such as BBS.

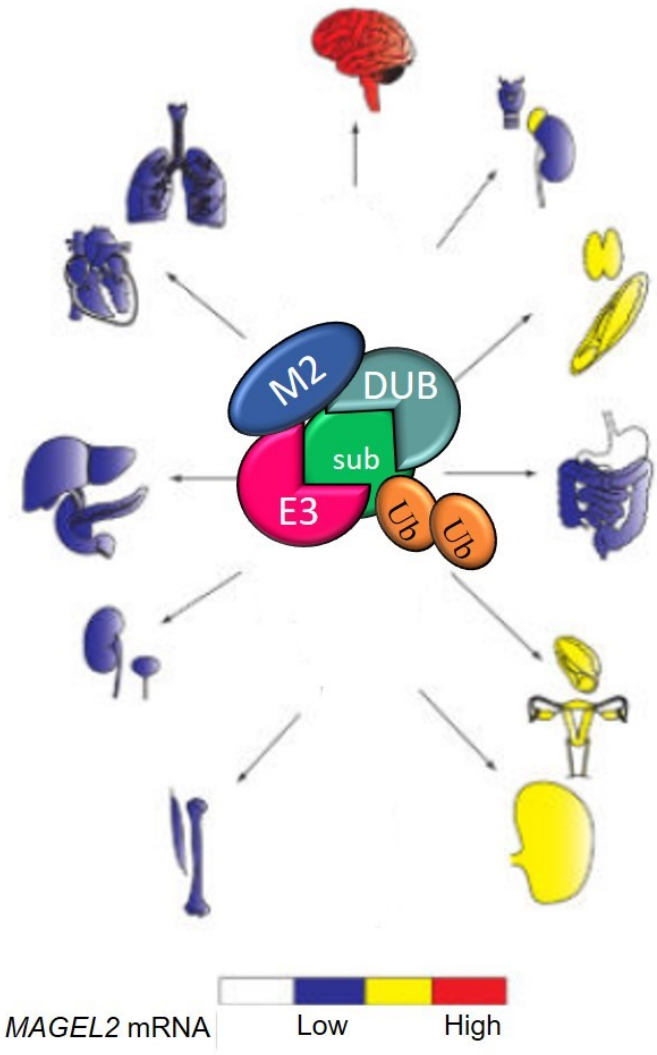


Figure 5.1 *MAGEL2* is expressed in multiple tissues of the human body and may modulate ubiquitination of tissue specific substrates. *MAGEL2* mRNA expression was identified in various tissues with highest expression in the brain, specifically the hypothalamus. MAGEL2 plays a role in modulation of ubiquitination. If loss of function of MAGEL2 occurs there may be perturbations in ubiquitination levels of substrate proteins resulting in phenotypes seen in *MAGEL2* related genetic disorders. Representative tissues are depicted with lowest *MAGEL2* mRNA expression in blue and highest expression in red. M2, MAGEL2; E3, E3 ubiquitin ligase; DUB, deubiquitinase; sub, substrate; ub, ubiquitin. Figure modified from (Tacer & Potts, 2018).

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