# PTP4A1 and PTP4A2 protein tyrosine phosphatase mechanism of action in magnesium controls murine organoid development

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*I dedicate this thesis to my girlfriend, Juliette.*

*For supporting me on my never-ending journey of personal growth.*

*I love you.*

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## Contribution of authors

Sanger sequencing was conducted by the Centre d'expertise et de services Génome Québec. The successful execution of the Western blotting experiments within this thesis can be attributed to the direction of Dr. Serge Hardy. The CNNM2-SpyTag project was directed by Noriko Uetani and the CRISPR guide RNA sequences, and template strand was designed by Dr. Serge Hardy. The mouse embryonic stem cells were genetically modified and validated by Jacinthe Sirois.

# List of Abbreviations

| | |
|---|---|
| ARL15 | ADP-ribosylation factor-like 15 |
| ATP | adenosine triphosphate |
| CBS | cystathionine β-synthase |
| CNNM | cyclin and CBS domain divalent metal cation transport mediator |
| CRISPR | clustered regularly interspaced short palindromic repeats |
| DMEM | Dulbecco's modified eagle medium |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| FBS | Fetal Bovine Serum |
| GFP | green fluorescent protein |
| IP | immunoprecipitation |
| KO | knockout |
| LIF | leukemia inhibitory factor |
| mESC | mouse embryonic stem cell |
| $[Mg^{2+}]$ | magnesium concentration |
| mTOR | mammalian target of rapamycin |
| NEAAs | non-essential amino acids |
| ORF | open reading frame |
| PRL | phosphatase of regenerating liver |
| PTP | protein tyrosine phosphatase |
| PVDF | polyvinylidene difluoride |
| RNA | ribonucleic acid |
| siRNA | small interfering RNA |
| TRPM | transient receptor potential cation channel subfamily M |

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# Abstract

Phosphatases of regenerating liver (PRL1, PRL2, and PRL3) are a family of dual-specificity phosphatases that regulate cell growth, differentiation, and proliferation. The PRLs are highly expressed in most cancers, and high expression of PRLs often correlates with poor patient prognosis. To elucidate the oncogenic role of PRLs, mouse embryonic stem cells with a deletion of PRL-2 were used to generate three-dimensional organoids. It was found that organoids with a PRL2 deletion had a significant disadvantage in growth, suggesting the importance of PRLs in neoplasia. Additionally, we found evidence suggesting that PRL1 and PRL2 have compensatory functions. Furthermore, the interaction between Cyclin M Metal Transporter (CNNM) proteins and PRLs has been found to play a role in regulating intracellular magnesium levels, which has been linked to increased tumour progression. CRISPR gene-editing technology was used to insert the SpyTag within the C-terminus of the murine CNNM2 gene to investigate this interaction. Overall, we found that PRL2 impacts the expression of several magnesium-regulatory proteins suggesting it’s crucial involvement in intracellular magnesium homeostasis.

# Résumé

Les phosphatases du foie régénérant (PRL1, PRL2 et PRL3) sont une famille de phosphatases à double spécificité impliquées dans la régulation de la croissance, de la différenciation et de la prolifération cellulaire. Les PRL sont fortement exprimées dans la majorité des cancers, et une forte expression des PRL est souvent corrélée à un mauvais pronostic pour les patients. Pour élucider le rôle oncogène des PRL, des cellules souches embryonnaires murine avec une suppression de PRL-2 ont été utilisées pour générer des organoïdes tridimensionnels. Il a été constaté que les organoïdes avec une suppression de PRL2 avaient un désavantage significatif de croissance, suggérant l'importance des PRL dans la néoplasie. De plus, nous avons trouvé des preuves suggérant que PRL1 et PRL2 ont des fonctions compensatoires. En outre, l'interaction entre les protéines Cyclin M Transporter de métaux (CNNM) et les PRL a été trouvée pour jouer un rôle dans la régulation des niveaux intracellulaires de magnésium, qui a été liée à une progression tumorale accrue. Pour étudier cette interaction, la technologie de l'édition de gènes CRISPR a été utilisée pour insérer la SpyTag dans le C-terminus du gène murin CNNM2. Dans l'ensemble, nous avons constaté que PRL2 a un impact sur l'expression de plusieurs protéines régulatrices de magnésium, suggérant son implication cruciale dans l'homéostasie intracellulaire du magnésium.

# Introduction

## Magnesium

Magnesium is an essential mineral that plays a vital role in the body's various biological and biochemical processes. It is involved in more than 300 enzymatic reactions, including those responsible for energy production, DNA synthesis, and protein synthesis[1]. Studies have shown that magnesium deficiency may be a risk factor for developing certain types of cancer, such as colorectal cancer, pancreatic cancer, and ovarian cancer[2].

One mechanism by which magnesium may influence cancer development is through its role in DNA repair. Magnesium is essential for the function of DNA polymerase, an enzyme involved in DNA replication and repair. Studies have shown that magnesium deficiency can lead to DNA damage and mutations, increasing the risk of cancer[3]. Another mechanism by which magnesium may affect cancer development is through its role in inflammation. The development of different types of cancer has been associated with chronic inflammation. Magnesium has anti-inflammatory properties, and studies have shown that magnesium supplementation may reduce inflammation markers in the body[4].

The numerous implications of magnesium dysregulation have made it an area of significant interest for researchers who are seeking to develop therapeutic treatments for magnesium-related diseases such as cancer. By better understanding the mechanisms behind magnesium dysregulation and its role in oncogenesis, we hope to identify new targets for therapeutic intervention and ultimately develop more effective treatments for these diseases.

## Protein Tyrosine Phosphatases

Protein tyrosine phosphatases (PTPs) are a class of enzymes that catalyze the dephosphorylation of tyrosine residues on proteins. They play important roles in regulating various cellular processes, including growth, differentiation, and signal transduction[5]. Research has demonstrated that the substrate specificity of PTPs is influenced by both the catalytic and noncatalytic segments of the enzyme in vivo. Noncatalytic domains can direct PTPs to specific intracellular compartments with high substrate concentrations, while the PTP catalytic domains enable site-specific protein dephosphorylation by recognizing the phosphotyrosine residue to be dephosphorylated, as well as its surrounding amino acids. Structural studies, kinetic analyses of PTP domains, substrate-trapping mutant studies, and PTP chimera studies have provided convincing evidence that isolated PTP domains can exhibit remarkable substrate selectivity[6].

Protein tyrosine phosphatases (PTPs) are marked by a critical attribute that sets them apart: their SH2 domain. This essential element enables PTPs to recognize and selectively bind to phosphorylated tyrosine residues, thereby unleashing a cascade of downstream signaling events that regulate key cellular processes. In other words, the SH2 domain of PTPs is a defining feature that underpins their vital role in cellular signaling pathways[7]. Research has shown that SH2 domains possess significant flexibility and versatility in their binding mechanisms with their ligands. This is supported by various examples such as the SH2-containing proteins from the SAP, Cbl, and SOCS families. These proteins are essential in immune responses, termination of protein tyrosine kinase (PTK) signaling, and cytokine response[8].

Dysregulation of PTPs has been associated with various diseases, including cancer. In cancer, alterations in PTP expression, activity, or localization can lead to aberrant signaling events that promote tumour growth and metastasis[9]. One PTP that has been extensively studied in this context is SHP2, which has been shown to have oncogenic activity when specific mutations activate it. The activation of SHP2 has been linked to increased cell proliferation, survival, migration, and resistance to chemotherapy and other treatments[10].

**Phosphatases of Regenerating Liver**

Phosphatases of the regenerating liver (PRL-1, PRL-2, and PRL-3 / PTP4A1, 4A2, 4A3) are a family of PTPs involved in regulating cell growth, differentiation, and proliferation[11]. PRLs were initially identified playing a critical role in liver regeneration after injury. They are also expressed in other tissues and are involved in various cellular processes, including the immune response, stress signaling, and cell cycle regulation[12].

The importance of PRLs lies in their ability to regulate key signaling pathways that are dysregulated in many diseases, including cancer. PRLs have been shown to regulate the MAPK and PI3K/AKT signaling pathways, which are frequently activated in cancer cells[13]. In addition, PRLs are involved in the regulation of cell cycle progression and apoptosis, which are critical processes in cancer development and progression[14].

All three PRLs share several conserved domains that are important for their enzymatic activity and cellular function[15].Understanding the structure and function of these domains is critical for the development of drugs and therapies that target PRLs and their associated diseases. Several PRL inhibitors have been developed as potential

cancer therapies. These inhibitors have shown promising results in preclinical studies and may represent a new approach for the treatment of cancer and other diseases associated with dysregulated PRL activity[16].

Studies have found that the overexpression of PRLs leads to increased intracellular magnesium levels, leading to increased tumour progression. The mechanism behind this effect is thought to be related to the role of magnesium in energy metabolism. Magnesium is required for the synthesis and hydrolysis of ATP, the main energy currency of the cell. Increased levels of intracellular magnesium can lead to increased production of ATP, which can provide the energy required for cellular proliferation and tumour growth[17].

**CNNMs**

CBS Domain Divalent Metal Cation Transport Mediators (CNNMs) proteins are a family of evolutionary conserved proteins that play a crucial role in maintaining intracellular magnesium homeostasis. They are involved in a wide range of physiological processes such as cell proliferation, neuronal development, bone formation, and insulin secretion. Mutations in the CNNM genes have been linked to a variety of diseases including hypomagnesemia, renal failure, and seizures[18]. The CNNMs include a transmembrane domain known as DUF21 with an unspecified function, a tandem domain of cystathionine-beta-synthase (CBS) also known as the Bateman domain, a cyclin-box domain, and a cyclic nucleotide-monophosphate-binding (cNMP) domain[19] (Fig. 1A). The crystallized structure of the CNNM transmembrane domain does not exist at the moment although a structure of the bacterial CNNM/CorC family $Mg^{2+}$ transporter transmembrane domain does[46]. It has been found that PRL-2 forms a functional heterodimer with CNNM3

through its CBS pair domain (Fig. 1C). Based on this data, we hypothesize that PRL-2 binds in close proximity to the cellular membrane to regulate intracellular magnesium homeostasis, likely through modulation of CNNM structure upon PRL binding (Fig. 1D)[21].

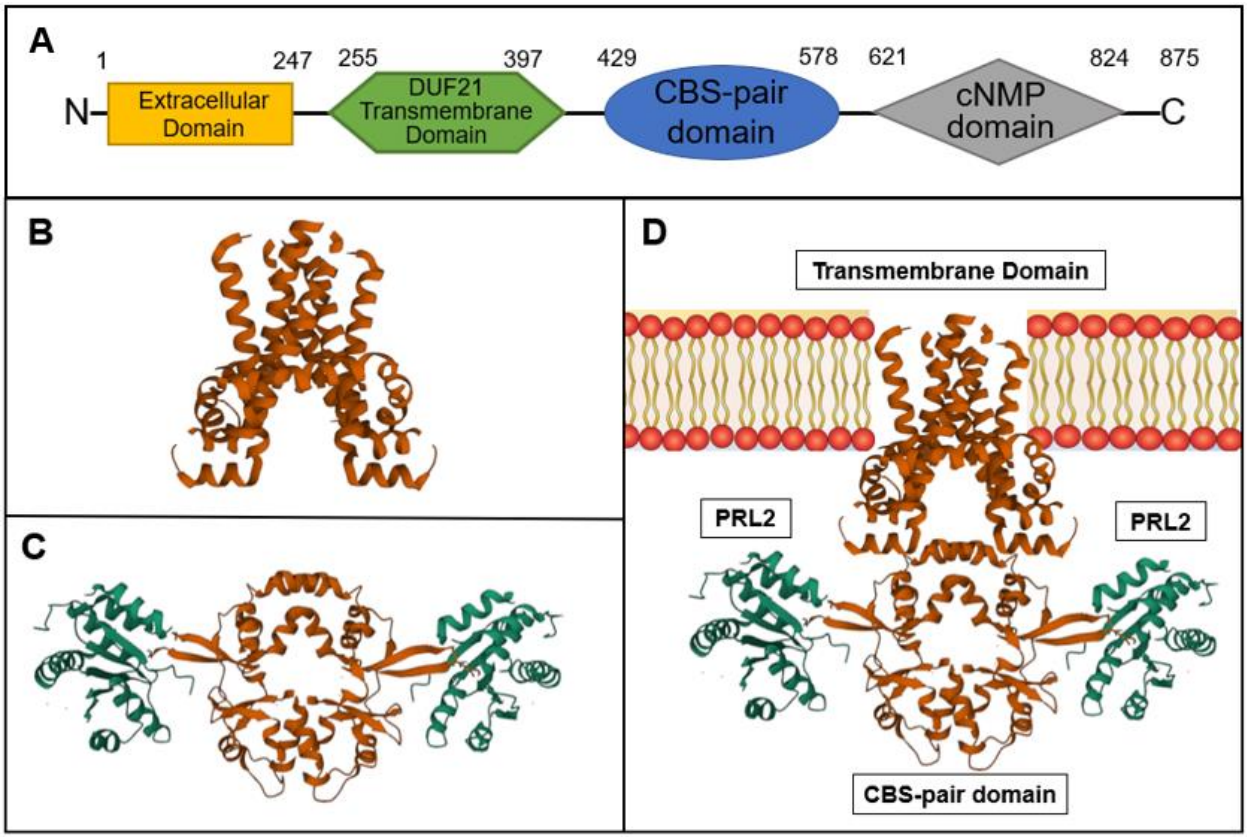


**Figure 1: CNNM binding domains (a)** The four domains of CNNMs: the extracellular domain (1-247), DUF21 Transmembrane Domain (255-397), CBS-pair domain (429-579), cNMP domain (621-824) (b) Structure of bacterial CNNM transmembrane domain (PDB: 7CFG) **(c)** PRL · CNNM CBS-pair domain complex (PDB ID: 5K24). (d) Hypothetical PRL · CNNM structure including CBS-pair domain and transmembrane domain. Generated and visualized using PDB Mol* Viewer

PRLs have been shown to modulate the magnesium regulatory activity of CNNMs through their phosphatase activity. This appears to play a role in regulating intracellular magnesium levels, which has been linked to increased tumour progression[20]. The protein-protein complex is dependent on the formation of an interaction between CNNM3 D426 and PRL2 C101 (Fig. 2) [20].

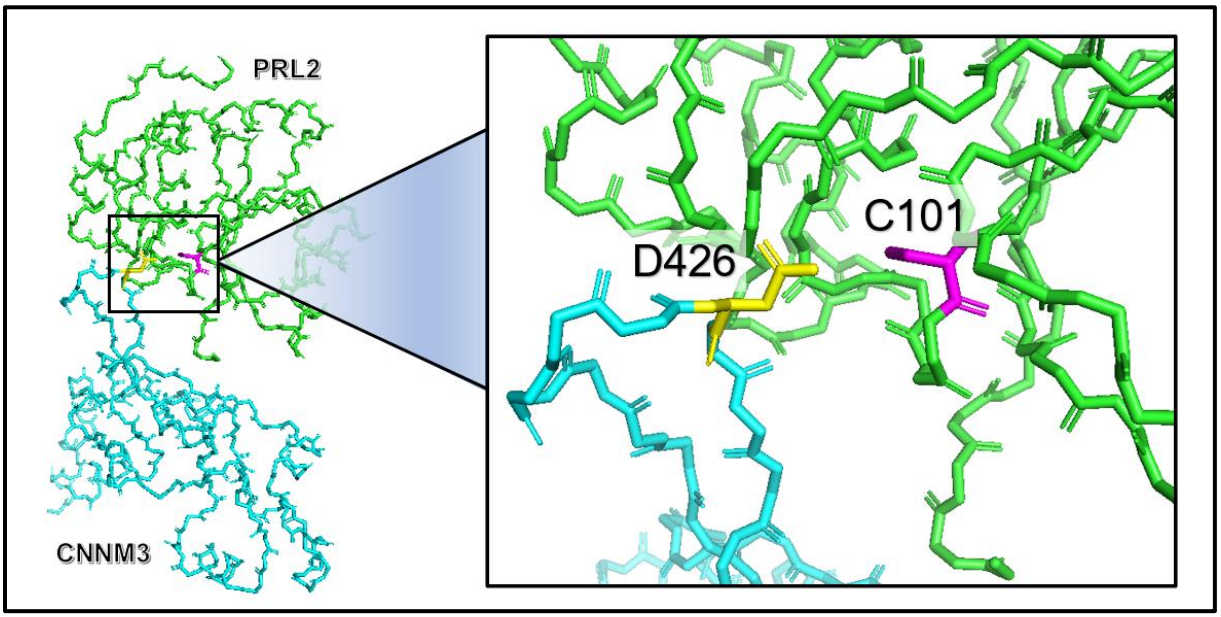


**Figure 2: Interface residues of the CNNM3 · PRL-2 complex.** Aspartic acid at position 426 of CNNM3 is essential for the interaction. The cysteine at position 101 of PRL-2 is essential for the interaction. Modelled using PyMOL.

The interaction between CNNMs and PRLs provides a potential target for the development of cancer therapies. Inhibiting the activity of PRLs or CNNMs could reduce intracellular magnesium levels and slow the growth of tumours. Further research is needed to better understand the role of magnesium in tumour progression and to identify specific targets for therapeutic intervention. Recent work in press (Hardy *et al.* PNAS 2023), the Tremblay lab investigates the role of PRLs in regulating the TRPM7-mediated magnesium influx. The paper proposes a model highlighting the significance of upregulating magnesium influx by outcompeting ARL15 in binding to CNNM3[21]. This

mechanism offers valuable insights into how PRLs perform their regulatory function and could contribute to their oncogenic effect if their expression becomes dysregulated.

**ADP-ribosylation factor-like GTPase 15**

ADP-ribosylation factor-like GTPase 15 (ARL15) is a small GTP binding protein with structural similarity to Ras-related GTP-binding proteins[22]. A recent genome-wide association study (GWAS) discovered that the ARL15 locus is linked to the excretion of magnesium in urine[23]. Recent studies in our lab reveal that CNNMs are direct partners of ARL15 (ADP-ribosylation factor-like GTPase 15) through the binding of the CBS1 domain of CNNM2. The study also demonstrates that ARL15 plays a crucial role in regulating the transport of magnesium ions within cells, by negatively influencing the N-glycosylation of CNNMs. Additionally, the expression of ARL15 and levels of intracellular magnesium ions were found to be inversely correlated[21].

Given the importance of this relationship, we became interested in understanding how it may be affected by other proteins that also interact with CNNMs. PRL proteins are one such group of proteins that have been shown to have an affinity towards CNNMs. Therefore, we decided to investigate how the relationship between ARL15 and magnesium homeostasis may be affected by PRL proteins, as this could shed light on the molecular mechanisms underlying magnesium regulation in cells. By examining the interactions between these proteins, we hope to gain a better understanding of the role that ARL15 and PRL proteins play in regulating cellular magnesium levels, which could have important implications for the development of new treatments for conditions associated with magnesium dysregulation.

**TRPM7**

TRPM7 (Transient Receptor Potential Cation Channel, sub family M, Member 7) is a widely distributed channel enzyme which has an ion channel that allows for the passage of divalent cations such as $Mg^{2+}$, $Ca^{2+}$, and $Zn^{2+}$, as well as an α-kinase that phosphorylates targets downstream. TRPM7 plays a key role in regulating many cellular processes, including cell proliferation, differentiation, and survival, as well as ion homeostasis and magnesium uptake. It is also involved in the regulation of gene expression, cell migration, and the response to oxidative stress[24]. Studies have shown that TRPM7 plays an important role in regulating $Mg^{2+}$ homeostasis in cells. TRPM7-mediated $Mg^{2+}$ uptake is necessary for cell survival, and TRPM7-deficient cells exhibit decreased levels of intracellular $Mg^{2+}$. This suggests that TRPM7 plays a crucial role in maintaining intracellular $Mg^{2+}$ concentrations.

Additionally, it has been reported that members of the CNNM family selectively bind to the TRPM7 channel to inhibit the entry of divalent cations into cells. It was found that the simultaneous overexpression of the CNNMs with the TRPM7 channel resulted in a significant increase in divalent cation uptake, which was rescued through an inactivating mutation in the TRPM7 channel pore. Furthermore, the study found that the overexpression of the PRLs stimulated the TRPM7-dependent divalent cation entry and that CNNMs were also necessary for this effect[25].

TRPM7 has been found to be an essential component in embryogenesis. The disruption of TRPM7 resulted in embryonic lethality before day 7 of development. The disruption of TRPM7 in induced pluripotent stem cells prevented the formation of neural stem cell monolayer. These findings suggest that the TRPM7-mediated intracellular

cation levels are essential towards the successful differentiation of pluripotent stem cells. This phenomenon is likely highly regulated by binding to TRPM7 towards CNNM3, and consequently the PRLs[26].

**Embryonic Stem Cells**

Embryonic stem cells are unique because they have the ability to develop into any type of cell in the body, a property known as pluripotency. During embryonic development, three germ layers form the basis for all the organs and tissues in the body. The three primary germ layers of an early embryo are the ectoderm, mesoderm, and endoderm. ESCs can differentiate into all three germ layers in vitro, providing a valuable tool for studying early development and cell differentiation[27].

Embryonic stem cells express multiple differentiation markers on their surface that allow to determine their level of pluripotency and how far along they are in the process of differentiating into specific cell types. For example, the stage-specific embryonic antigen (SSEA) and tumor-related antigen (TRA) are markers that are present on undifferentiated embryonic stem cells, but not on more mature or differentiated cells. Additionally, specific transcription factors such as Oct4, Sox2, and Nanog are expressed in undifferentiated embryonic stem cells and are downregulated as the cells differentiate into specific cell types. By analyzing the expression of these markers, researchers can gain insight into the state of embryonic stem cells and use this information to guide their experiments to direct the cells toward desired cell types[28].

Embryonic stem cells are an essential tool for studying cancer as they can be used to model the early stages of carcinogenesis. This provides a platform to investigate the

genetic and epigenetic changes that lead to cancer development and identify potential targets for cancer therapy.

**Embryoid Bodies**

Embryoid bodies (EBs) are three-dimensional aggregates of embryonic stem cells (ESCs) that resemble the early stage of embryonic development. They are formed when embryonic stem cells are cultured in suspension and have been found to mimic the 3D architecture of developing organs and tumors, making them an ideal model for cancer research. One of the most significant advantages of using EBs in cancer research is that they can be easily manipulated in vitro to mimic different stages of tumor development. By varying the culture conditions and growth factors, researchers can create EBs that closely resemble the early stages of tumor development, allowing them to study the mechanisms involved in tumor initiation and progression[29].

Studying mutations that impair the growth of embryonic bodies can provide valuable insights into the processes of early embryogenesis, development, and differentiation. Mutations that affect the growth and differentiation of embryoid bodies indicate disruptions in the normal developmental processes that occur during early embryogenesis. By studying these mutations, researchers can better understand the molecular and cellular mechanisms that control embryonic development and differentiation. Additionally, embryoid bodies are useful tools for modeling diseases and conditions that affect early embryonic development, such as birth defects, and can be used to test new treatments and therapies. Therefore, studying mutations that impair the growth of embryonic bodies can not only provide insights into fundamental aspects of

embryonic development, but also have important implications for the diagnosis and treatment of a wide range of diseases and conditions[30].

**SpyTag/SpyCatcher**

The SpyTag/SpyCatcher system is a molecular tool developed for protein labeling and protein-protein interactions. It is based on two small protein domains, called SpyTag and SpyCatcher, which can spontaneously react with each other to form a covalent bond. This reaction is fast, irreversible, and occurs under mild conditions, making it ideal for a wide range of biological applications[31].

SpyTag is a peptide composed of 13 amino acids that can be incorporated into the genetic sequence of the protein of interest through genetic fusion. SpyCatcher is a larger protein (approximately 12 kDa) that is expressed separately and purified as a recombinant protein. SpyCatcher contains an exposed lysine residue that reacts with the reactive peptide bond in SpyTag, forming a stable covalent bond between the two proteins. The SpyTag/SpyCatcher system can be used for a variety of applications, including protein labeling, protein immobilization, and protein-protein interactions. For example, a protein of interest can be labeled with a fluorescent or biotinylated SpyTag, and then captured on a surface coated with SpyCatcher. Similarly, two proteins of interest can be fused to SpyTag and SpyCatcher, respectively, and then brought together to form a stable complex (Fig 3).

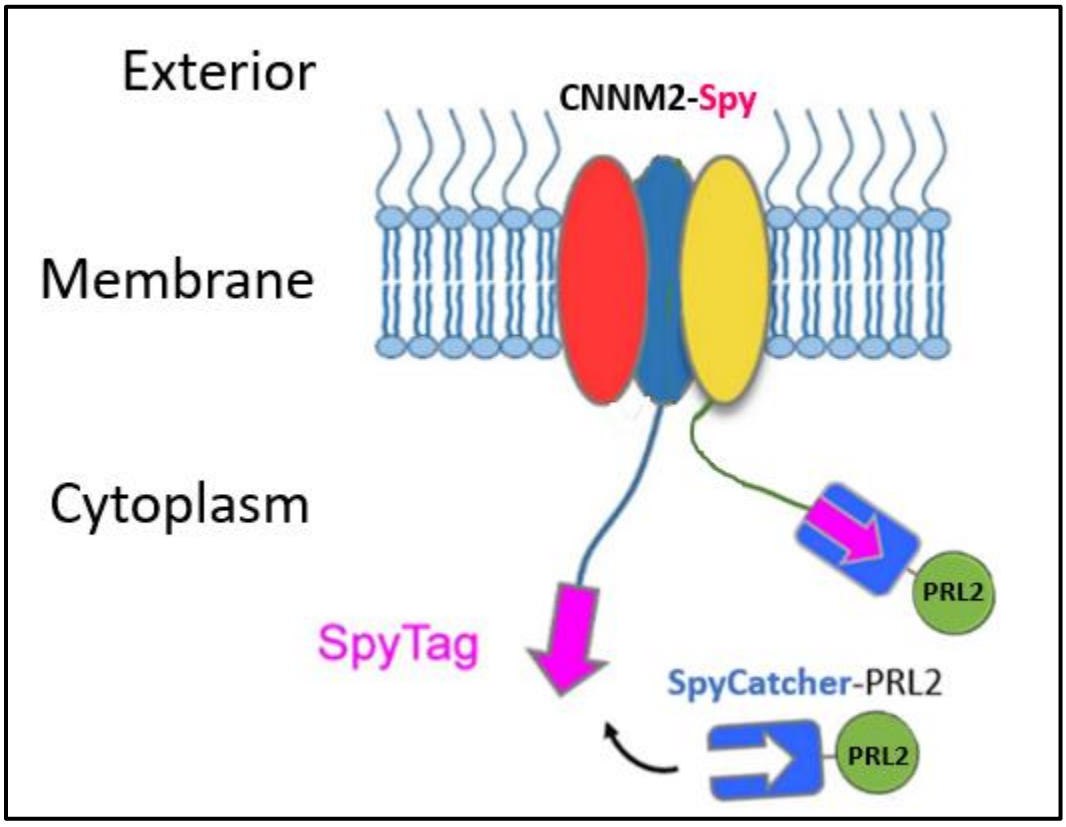


**Fig 3. The SpyCatcher-SpyTag system.** CNNM2-SpyTag and SpyCatcher-PRL2 being fused together to form a stable complex.

# Methods

## DNA Constructs

Human C-terminal V5-tagged ARL15 was obtained from the McGill Platform for Cellular Perturbation (MPCP) cloned into pLX317 plasmid. Human C-terminal FLAG-tagged CNNM3 was cloned into pDEST26 plasmid. Human C-terminal GST-tagged PRL2 was cloned into pDEST27 plasmid.

## HEK293 cell culture and transfection

HEK293 cells were grown in high glucose DMEM (Fisher Scientific, #SH30081.01) supplemented with 2 mM GlutaMAX (Life Technologies, #35050061), 1% gentamicin (Wisent Bioproducts, #450-135-XL) and 10% FBS Life Technologies, #12483020) at 37 ℃ in a humidified incubator with 5% (v/v) $CO_2$. Transfection was performed using Lipofectamine 2000 (Invitrogen #11668019) for protein overexpression at 1 µg DNA: 2 µL Lipofectamine or Lipofectamine RNAiMAX (Fisher Scientific #13-778- 150) for siRNA at 4 nM siRNA: 1 µl Lipofectamine.

HEK293 cells with doxycycline-inducible N-terminal HA-tagged TRPM7 expression and HEK293 cells with doxycycline-inducible CNNM3 expression were a gift from Drs. Carsten Schmitz and Anne-Laure Perraud (Integrated Department of Immunology, National Jewish Health and University of Colorado) TRPM7 expression was induced with 1 – 5 µg/mL doxycycline (Sigma-Aldrich, #D3072).

**Mouse embryonic stem cell culture**

ES cells were grown in KO DMEM (Invitrogen, #10829-017) supplemented with 1X GlutaMAX (Invitrogen, #35050061), 1X NEAAs (Invitrogen, 11140-050**)**, 55 μM β-mercaptoethanol (Invitrogen, #21985-023**),** 15% ES FBS (Invitrogen, #16141061) and 1X Penicillin-Streptomycin (Invitrogen, #10378-016**).** To maintain cells in an undifferentiated state, 1000 U/mL leukemia inhibitory factor (LIF) was supplemented. Cells were seeded in 0.1% gelatin-coated plates (Sigma-Aldrich, #ES-006-B**)**. The medium was replaced daily and cells were passaged every two days.

**Mouse embryonic stem cell transfection**

ON-TARGETplus™ SMARTpool PTP4A1 siRNA was purchased from Horizon for knockdown of PRL-1 and non-targeting control (D-001810-10-05). 1 x $10^5$ ES cells were seeded in one well of a 12-plate. 24 hours post-seeding, 12.5 nM siRNA was transfected using RNAiMAX protocol with 1:1 ratio of diluted Lipofectamine® RNAiMAX to siRNA. 24 hours post-transfection, cells were harvested and processed for western blot to detect knockdown of PRL1 protein.

**Cell proliferation assay**

mESC cells (1.5 x $10^4$) were seeded in one well of a 24-well plate (Corning) with ES culture medium supplemented with LIF. Medium was replaced daily. Cells were trypsinized and counted using a TC20 Automated Cell Counter (Bio-Rad, #1450102). Experiment was performed in biological triplicate and technical triplicate.

**Embryoid body formation assay**

To activate differentiation, ES cells were trypsinized and resuspended in ES cell medium without LIF. 500 ES cells were seeded in 20 uL droplets on the lid of a 15 cm Petri dish **(**Corning, #07-202-010) and incubated upside-down for 48 hours before transferring into low-adherence 6-well cell culture plates (Sarstedt, #83.3920.500**)** and maintained on a shaker at 65 RPM. Medium was replaced every three days. 15 mL PBS was added to the Petri dish to maintain humidity and prevent evaporation. Formation of embryoid bodies (EBs) was monitored and photographed on Days 2,4,6, 8. EBs were harvested on Day 8.

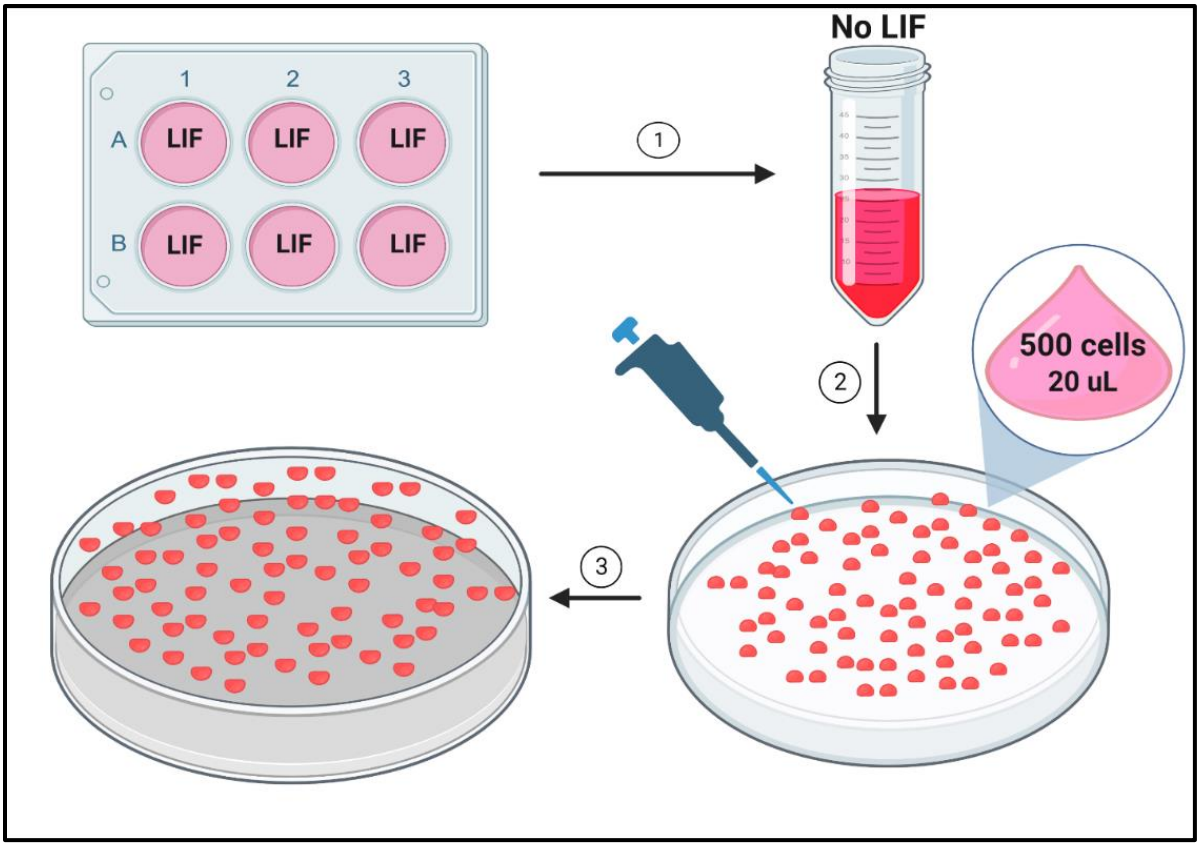


**Figure 4: Embryonic body formation assay workflow**. Generated using BioRender.

**Western blot**

Cells were lysed with lysis buffer and 1X complete protease inhibitor cocktail (Roche, #04693116001). The lysis buffer contained 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, 1% (v/v) Triton X-100. Protein concentrations were measured using a Bicinchoninic Acid protein assay (BCA) (Fisher Scientific, Hampton, NH, USA) and 10 µg protein was used for western blot. Gels were then transferred to PVDF membrane using the Trans-Blot Turbo Transfer System (Bio-Rad, #1704150). PVDF membrane was blocked for one hour with 5% (w/v) milk powder in PBS-T. Blots were then incubated in primary antibody overnight shaking at 4ºC. Primary antibodies were diluted in 5% BSA (w/v) diluted in PBS-T as follows: anti-FLAG (Sigma-Aldrich, #SAB4301135) diluted 1:10,000, anti-HA (Cell Signaling Technology, #3724) diluted 1:10,000, anti-β-actin (Sigma-Aldrich, #A2066) diluted 1:10,000, anti-ARL15 (ThermoFisher, #PA5-2077) diluted 1:10,000, anti-GST (Cell Signaling Technology, #2622) diluted 1:10,000, anti-PRL-2 (Sigma-Aldrich, #05-1583) diluted 1:2,000, phospho-S6 (S235/236) (Cell Signaling Technology, #4858) diluted 1:10,000, and anti-CNNM3 (ThermoFisher, #13976-1) diluted 1:10,000. Anti-PRL2 was raised in mouse, all others were raised in rabbit. After washing with PBS-T, blots were incubated for one hour at RT on a shaker in secondary antibody, either anti-mouse antibody raised in sheep (Sigma-Aldrich, #A9044) or anti-rabbit antibody raised in mouse (Sigma-Aldrich, #A0545). Proteins were ChemiDoc XRS+ (Bio-Rad Laboratories Inc). Image analysis was performed using Image Lab software.

**Co-immunoprecipitation**

For FLAG-tagged protein immunoprecipitation, 1 x $10^6$ HEK293 cells were seeded and transfected in 6-well TC-treated dishes (Corning, #3516). 48 hours after transfection, cells were lysed with lysis buffer. BCA assay was performed to measure the lysate protein concentrations. 300 ug protein of total cell lysate (TCL) were diluted in lysis buffer with 20 µg Anti-FLAG M2 Magnetic Beads (Millipore, #M8823) per sample and incubated overnight, rotated at 4$^o$C. Beads were washed with lysis buffer and 2X Laemmli sample buffer was added to collect bound proteins from beads. Western blot was performed.

**RNA isolation and quantitative reverse transcriptase-PCR**

For RNA isolation of ES cells, cells were seeded in 12-well plates at a concentration of 1 x $10^5$ cells/mL. 48 hours post-seeding, the cells were washed with PBS and RNA was isolated using RNeasy RNA isolation kit (Qiagen, #74004).

For RNA isolation of embryonic bodies, EBs were harvested 8 days post-seeding. EBs were washed with PBS and RNA was isolated using RNeasy RNA isolation kit (Qiagen, #74004).

Reverse transcription was performed using 1 µg RNA per sample with SuperScript™ IV Reverse Transcriptase (ThermoFisher, #18091050). Quantitative RT-PCR was performed (BioRad CFX Connect Real Time System) using SYBR® Green (Rocher). Relative mRNA expression levels were normalized to the housekeeping gene *Actin-β*. The runs were performed in technical triplicate and biological triplicate. Primers are presented in Table 1.

**Table 1. Mouse embryonic stem cell qPCR primers**

| Gene | Sequence |
|---|---|
| Actin_Forward | 5’- CGTGCGTGACATCAAAGAGAAG-3’ |
| Actin_Reverse | 5’- TGGATGCCACAGGATTCCATAC-3’ |
| PTP4A1_Forward | 5’- ATGCTGGGCGTGGTGGTGTG-3’ |
| PTP4A1_Reverse | 5’- TCGGAGCGCGCCAGGCTT-3’ |
| PTP4A2_Forward | 5’- TCGGGTGCTCTGTGACTTTA-3’ |
| PTP4A2_Reverse | 5’- CTGGCCAGTGTTGCTTTTCT-3’ |
| POU5F1_Forward (Oct4) | 5’- GTGGAGGAAGCTGCAAACTT-3’ |
| POU5F1_Reverse (Oct4) | 5’- CTCCAGGTTGGCTTTATGGT-3’ |
| Nanog_Forward | 5’- GACAGGGAGGAGAGGGTTTC-3’ |
| Nanog_Reverse | 5’- TGGTGCTGGAGTAGTTCTGG-3’ |
| Sox2_Forward | 5’- GCTGGAGTGGGAAACTGTGT-3’ |
| Sox2_Reverse | 5’- CCAGGTAGTGGATCGAGGTC-3’ |

## Statistical analysis

GraphPad Prism 9.5.0 was used for statistical analysis. Results are expressed as mean ± standard error of the mean. Unpaired T-test was used assuming a Gaussian distribution and assuming both populations have the same SD. At least 3 replicates were used for analysis. P-values reported using ns ($p > 0.05$), * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$), **** ($p \leq 0.0001$)

# Results

## 1. TRPM7 regulates PRL-1 and PRL-2 expression leading to activation of mTOR signaling pathway

HA-tagged TRPM7 was induced using a Tet-on doxycycline-inducible system in HEK293 cells. As TRPM7 expression increased, the expression of PRL-1 and PRL-2 gradually decreased, indicating that the reduced expression is proportional to the amount of TRPM7 being expressed (Fig. 5A). This suggests that the higher expression of TRPM7 cation channel results in increased intracellular magnesium levels leading to reduced expression of PRL-1 and PRL-2, further supporting that magnesium is an important regulator of PRL-2 expression.

To investigate the inverse relationship between TRPM7 and the PRLs, we looked into its role in regulating the oncogenic mTOR pathway (Fig. 5B). We found that as TRPM7 expression increased, the phosphorylation of serine 235 on the S6 ribosomal protein increases, suggesting that increased intracellular magnesium levels activate the mTOR signaling pathway. Additionally, we found that PRL2 likely plays a role in this activation by reducing its expression to decrease the cell's magnesium influx.

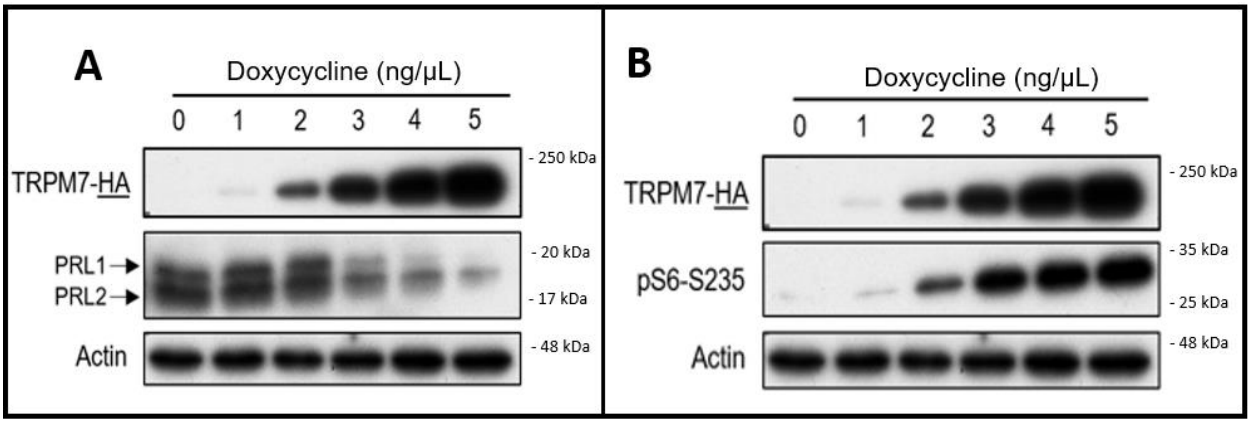


**Figure 5: TRPM7 regulates PRL-1 and PRL-2 expression leading to activation of mTOR signaling pathway**

**(a**) Different concentrations of doxycycline were used to induce HA-TRPM7 in HEK293 cells with doxycycline-inducible TRPM7 expression and Western blotting was performed. **(b)** Same as 5A.

## 2. PRL-2 outcompetes ARL15 for binding to CNNM3.

To determine the effect of CNNM3 on PRL expression, CNNM3 was induced using a Tet-on doxycycline-inducible system in HEK293 cells. As CNNM3 expression increased, the expression of PRL-1 and PRL-2 gradually increased, indicating that the increase in PRLs expression was proportional to the amount of CNNM3 being expressed (Fig. 6A). This suggests that CNNM3 reduces intracellular magnesium levels either through inhibition of magnesium influx or activation of magnesium efflux which leads to upregulation of its binding partner PRLs through post-transcriptional magnesium-dependent regulation of PRL expression. The data indicates that PRLs are upregulated in response to the reduction in intracellular magnesium which is mediated by the increased expression of CNNM3. This leads to a possible mechanism by which the interaction between PRL-2 and CNNM3 can enable cells to rescue their dysregulated magnesium levels. Thus, the formation of the complex seems to inhibit the magnesium-regulating function of CNNM3.

To gain further insight into the dynamics of protein-protein interactions, we conducted a rigorous investigation by introducing incremental amounts of PRL2-GST into HEK293 cells, while overexpressing a constant level of ARL15-V5 and CNNM3-FLAG (Fig. 6B). Our experimental findings proved to be intriguing, as we observed that an increase in PRL2 expression correlated with a concomitant decrease in both ARL15 and CNNM3 expression, thereby suggesting that PRL2 plays a significant role in regulating the stability of these two proteins.

To delve deeper into the intricate protein-protein interactions and stability of the CNNM/PRL/ARL15 trio, we proceeded to investigate the physical binding partners of

CNNM3 through co-immunoprecipitation with Flag antibody (Fig. 6C). Our co-IP results unequivocally demonstrated that PRL2 has a higher binding affinity for CNNM3 compared to ARL15. This finding leads us to hypothesize that ARL15 stability may be negatively impacted due to its inability to bind to CNNM3.

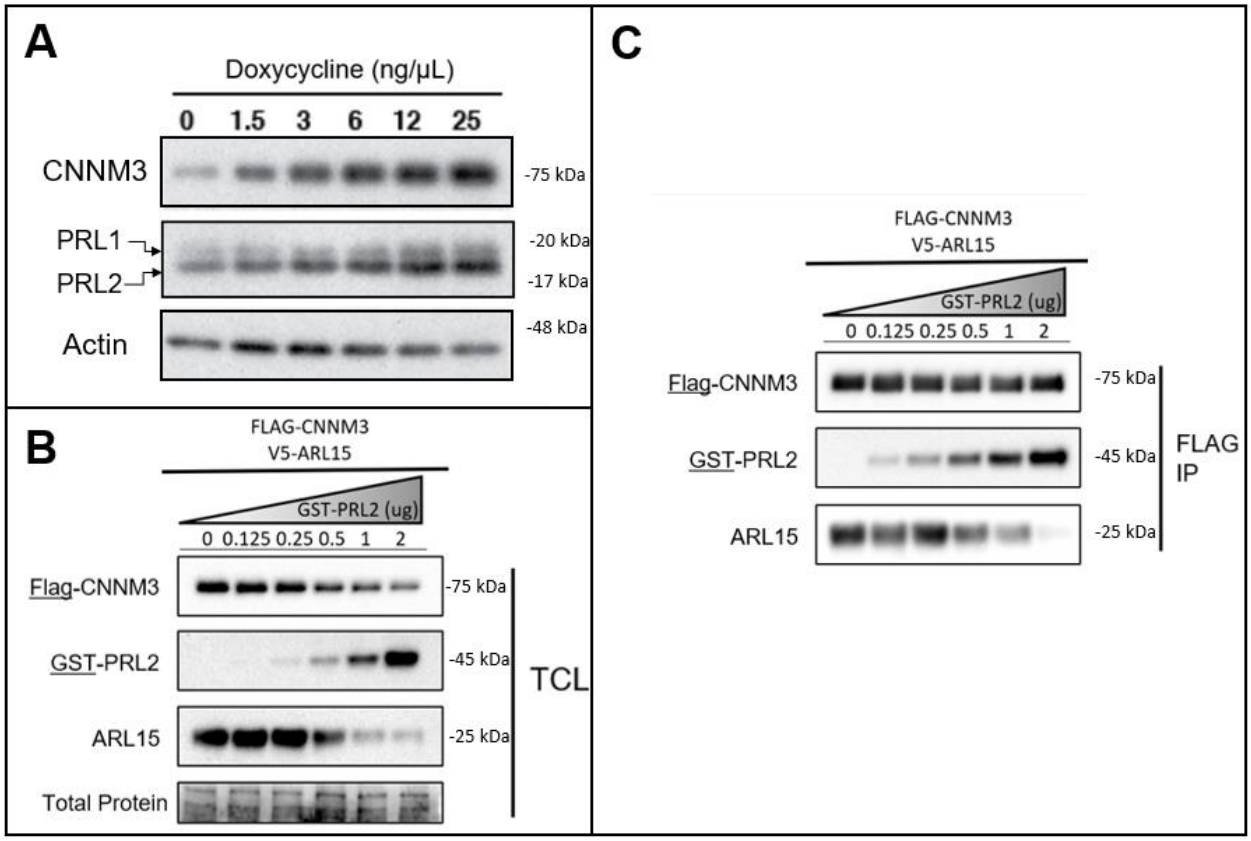


**Figure 6. PRL2 outcompetes ARL15 for binding to CNNM3. (a)** Increasing concentrations of doxycycline were used to induce CNNM3 in HEK293 cells with doxycycline inducible CNNM3 expression and Western blotting was performed. **(b)** Increasing concentration of GST-PRL2 were transfected in presence of 0.5 μg pLX317 plasmid encoding V5-ARL15 and 0.5 μg pDEST26 plasmid encoding FLAG-CNNM3. Western blotting was performed on total cell lysate. **(c)** Same as B. FLAG tag was immunoprecipitated and Western blotting was performed.

### 3. PRL-1 is upregulated in response to PRL-2 deletion

Given the remarkable role of PRL-2 in the modulation of critical magnesium regulatory proteins, we embarked on a study to investigate the function of this protein tyrosine phosphatase within a mouse embryonic stem cell (mESC) model. Utilizing CRISPR gene deletion, we successfully performed PRL-2 gene knockout, which elicited a notable compensatory response from PRL-1, further highlighting the significance of these proteins in maintaining cellular magnesium homeostasis (Fig. 7).

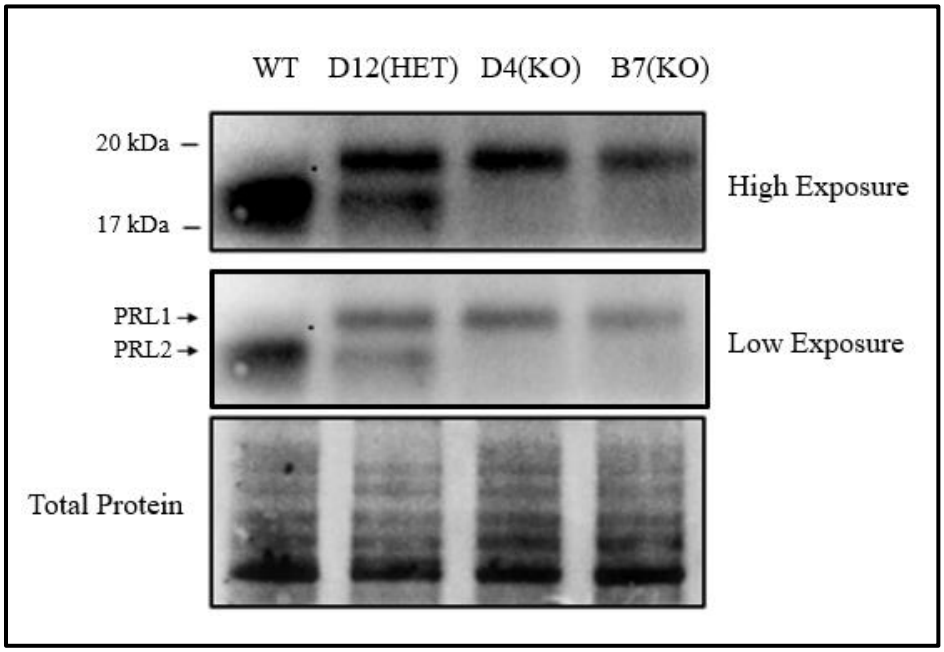


**Figure 7. PRL-1 is upregulated in response to PRL-2 deletion.** WT, Het, and KO mESC cells were seeded at 1 x $10^5$ cells per well of a six-well plate and harvested at 80% confluency. Western blotting was performed.

### 4. PRL-2 does not affect the rate of embryonic stem cell proliferation

To investigate the effect of PRL-2 deletion on mouse embryonic stem cells, we performed a cellular proliferation assay to determine if PRL-2 affected the growth rate in the cells. After seeding the mESCs, we measured cell count numbers on Days 2,3 and 5. Our results demonstrate that the rate of mESC proliferation is not affected by the deletion of the PRL-2 gene as there was no significant difference in the cell count numbers between the wild-type cells and the knockout cells (Fig. 8).

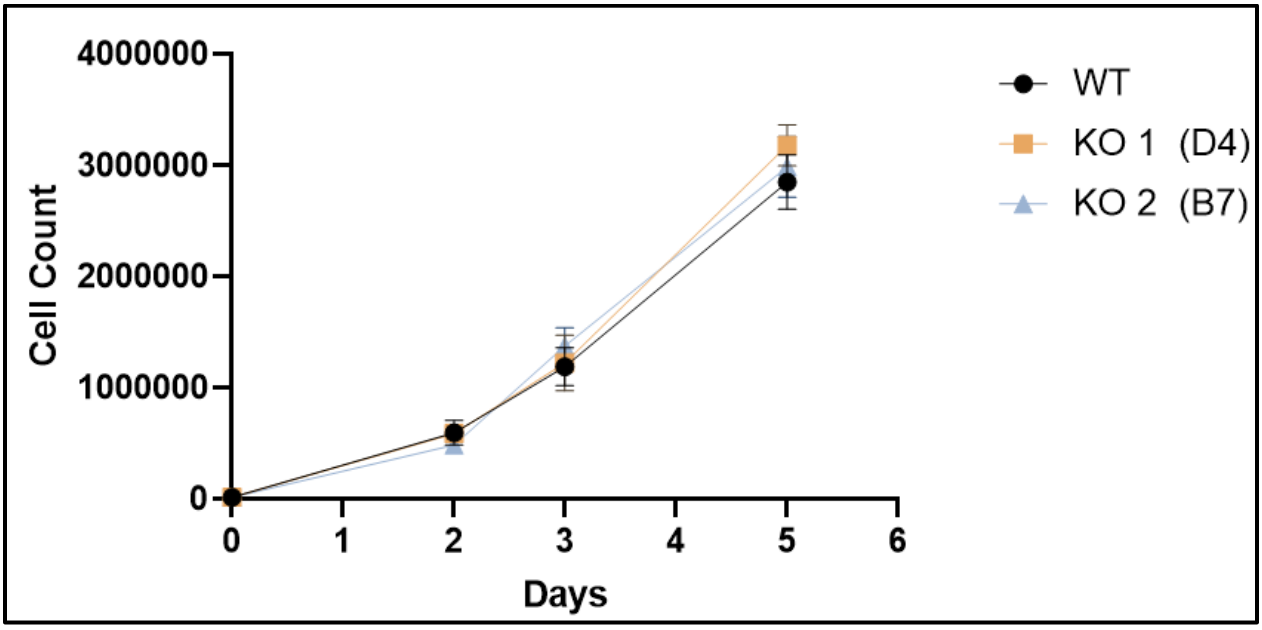


**Figure 8. PRL2 does not affect rate of cellular proliferation.** 2.5 x $10^4$ cells per well were seeded in biological triplicates in a 24-well plate. Cells were harvested at Days 2,3, and 5 and counted on the TC20 Automated Cell Counter.

### 5. PRL-2 does not affect embryoid body development size

To study the effect of PRL-2 disruption on a 3-D in-vitro cell model, we performed embryoid body formation assays to investigate the effect of PRL2 deficiency in the formation of early embryoid bodies. Upon initial investigation, we discovered that PRL2 knockout cells form embryoid bodies at a significantly similar size compared to wild-type cells (Fig. 9A, 9B).

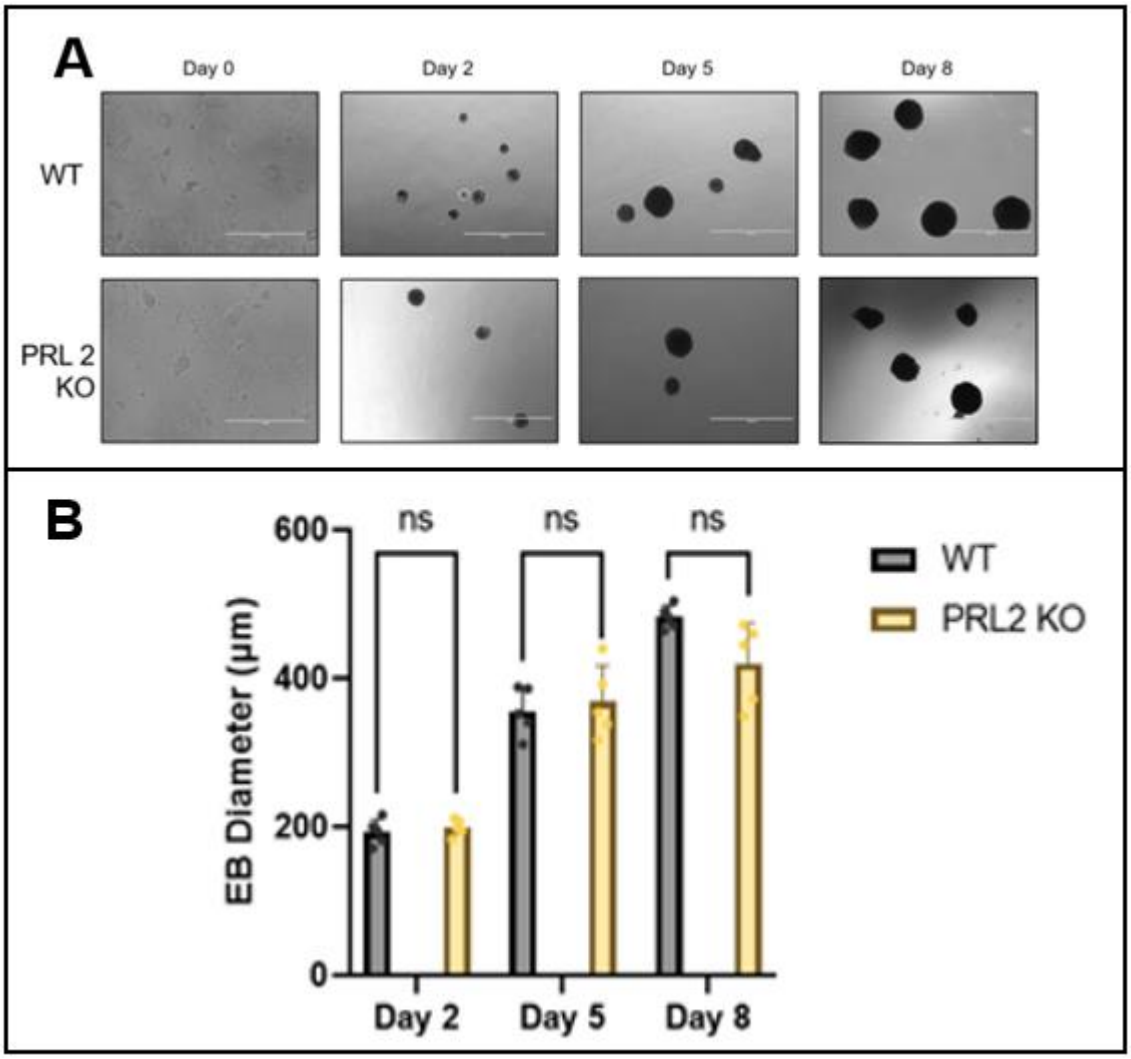


**Figure 9. PRL-2 does not affect embryoid body development size (a)** Undifferentiated ES cells and embryoid bodies formed at Days 2, 5, and 8 taken with EVOS M5000 Imaging System at 4X magnification, Scale Bar = 1000 µm. **(b)** Embryoid body diameters at Days 2,5,8. Measured using ImageJ software.

## 6. PRL-2 does not affect pluripotency marker expression

To investigate the effect of PRL-2 on the pluripotency marker expression of embryonic stem cells, we performed RT-qPCR on embryoid bodies to determine if there is a change in pluripotency marker expression among PRL2 KO embryoid bodies. We found that although both wild-type and PRL2 knockout cells downregulate their pluripotency markers as the embryoid bodies develop, there appeared to be no significant difference in the levels of expression in the PRL2 knockout cells compared to wild-type mouse embryonic stem cells (Fig. 10A, 10B).

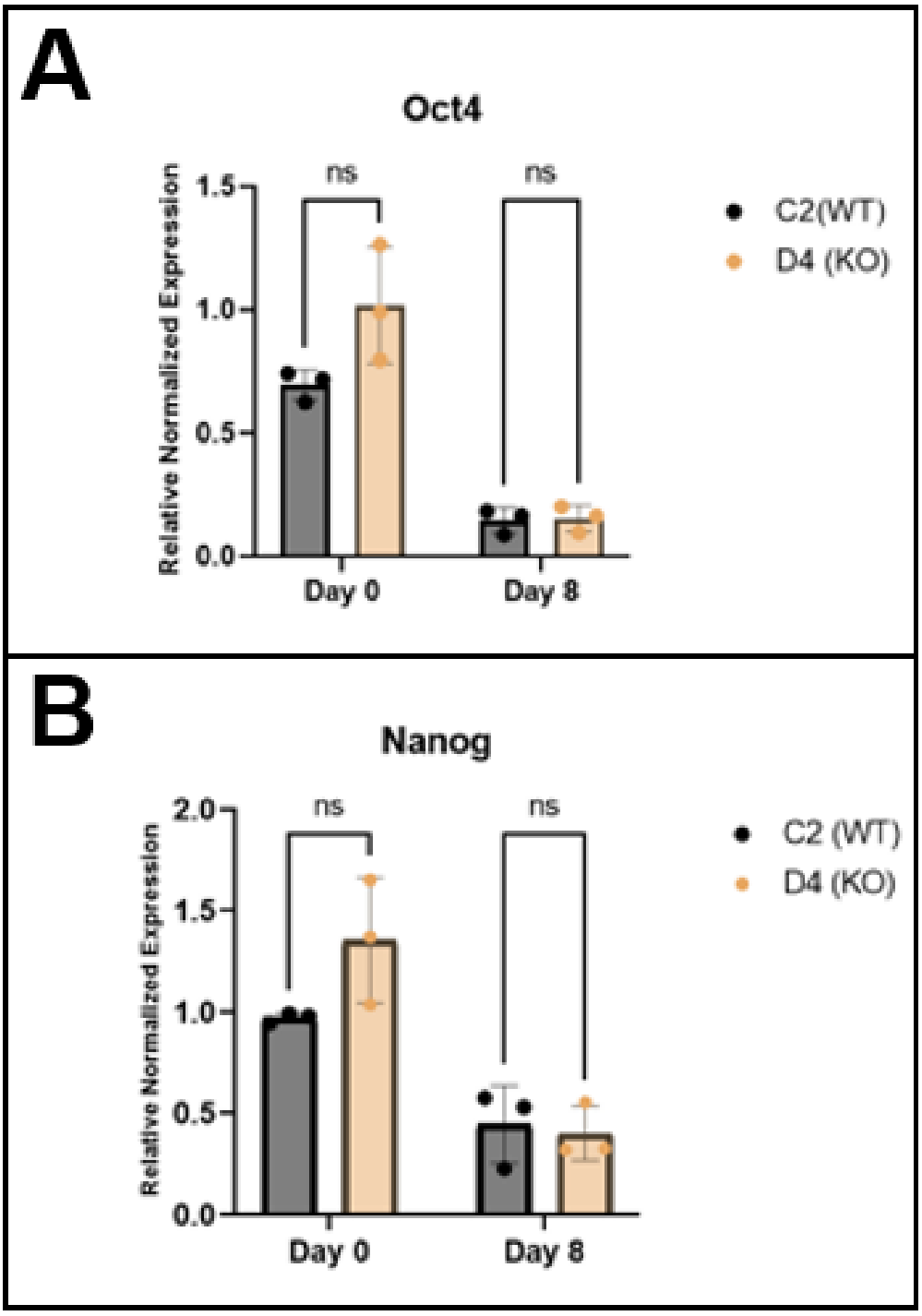


**Figure 10. PRL-2 does not affect pluripotency marker expression. (a)** Oct4 mRNA relative normalized expression to Actin-β at Day 0 and Day 8 of embryoid body formation assay. C2 (wild-type) cells compared against D4 (PRL2 knockout) cells, n = 3. **(b)** Nanog mRNA relative normalized expression to Actin-β.

## 7. PRL-2 is upregulated during embryoid body development

To study the expression levels of PRL2 during the process of embryoid body formation and development, we measured PTP4A2 mRNA transcript levels using RT-qPCR in wild-type mouse embryonic stem cells. We discovered that PTP4A2 mRNA transcript expression increases as the embryoid body develops, indicating that the protein has a higher level of expression among differentiated cell types in compared to pluripotent stem cells (Fig. 11).

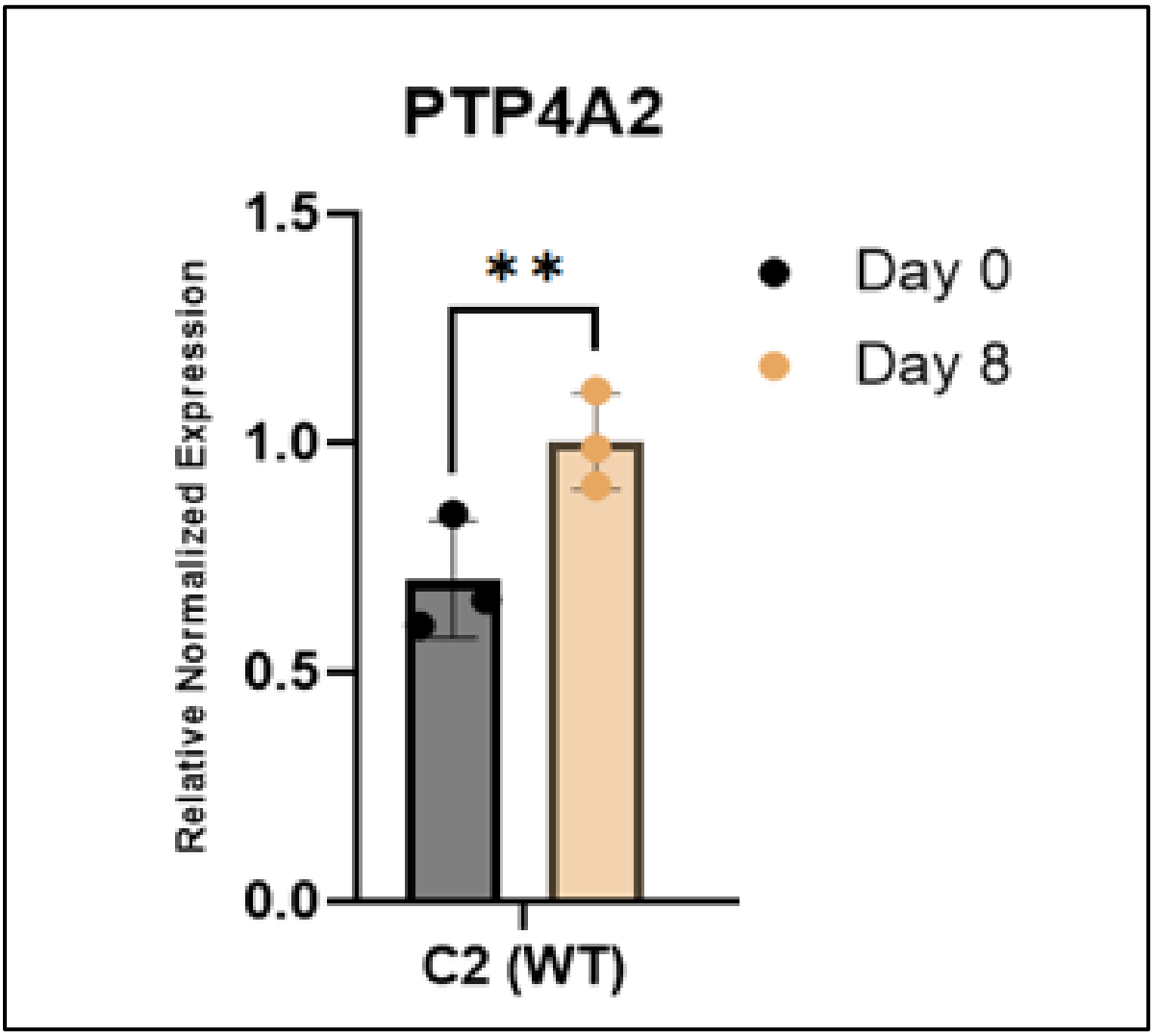


**Figure 11. PRL-2 is upregulated during embryoid body development.** Wild-type mESC PTP4A2 mRNA expression at Day 0 and Day 9 of embryoid body formation assay, n = 3. mRNA measured as relative normalized expression to Actin-β.

## 8. PRL2 knockout does not affect PRL-1 mRNA expression during embryoid body development

After discovering that PRL2 is upregulated in the process of embryoid body development, we expected to observe a similar phenomenon with respect to PRL1 mRNA expression. Unexpectedly, we did not detect any noteworthy rise in PRL1 mRNA expression throughout the progression of embryoid body development. (Fig. 12).

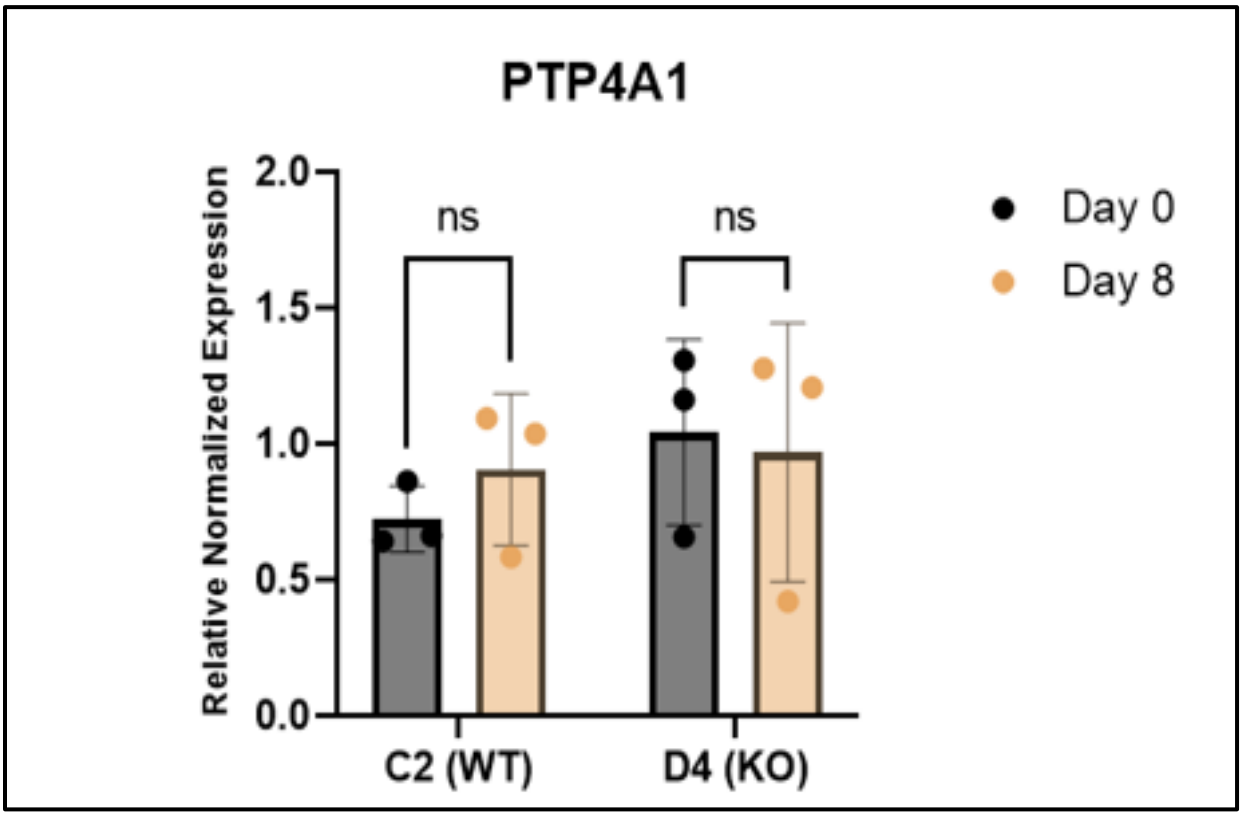


**Figure 12. PRL2 knockout does not affect PRL1 mRNA expression during embryoid body development.** Wild-type mESC PTP4A1 mRNA expression at Day 0 and Day 9 of embryoid body formation assay, n=3. mRNA measured as relative normalized expression to Actin-β.

## 9. Synthetic lethality between PRL-1 and PRL-2 dysregulates embryoid body development

To investigate this compensatory role of PRL-1, we treated mESCs with PTP4A1 siRNA which was determined to have a knockdown efficiency of approximately 50% (Fig. 13A, 13B). We found that the knockdown of PRL1 has a statistically significant reduction in embryoid body size (Fig. 13C). As expected, the deficiency of both genes (PRL-1/2) leads to a significantly greater reduction in embryoid body size (Fig. 13D). The observation that knockdown of PRL-1 leads to a significant reduction in embryoid body size suggests that PRL-1 plays a critical role in regulating embryonic stem cell differentiation. These findings are consistent with previous reports that implicate PRL-1 in cell proliferation, migration, and adhesion (Diamond, 1994). Together, these results suggest that PRL-1 and PRL-2 are essential components of the cellular machinery that regulates optimal embryonic development, and their precise functions need to be elucidated for a better understanding of these processes.

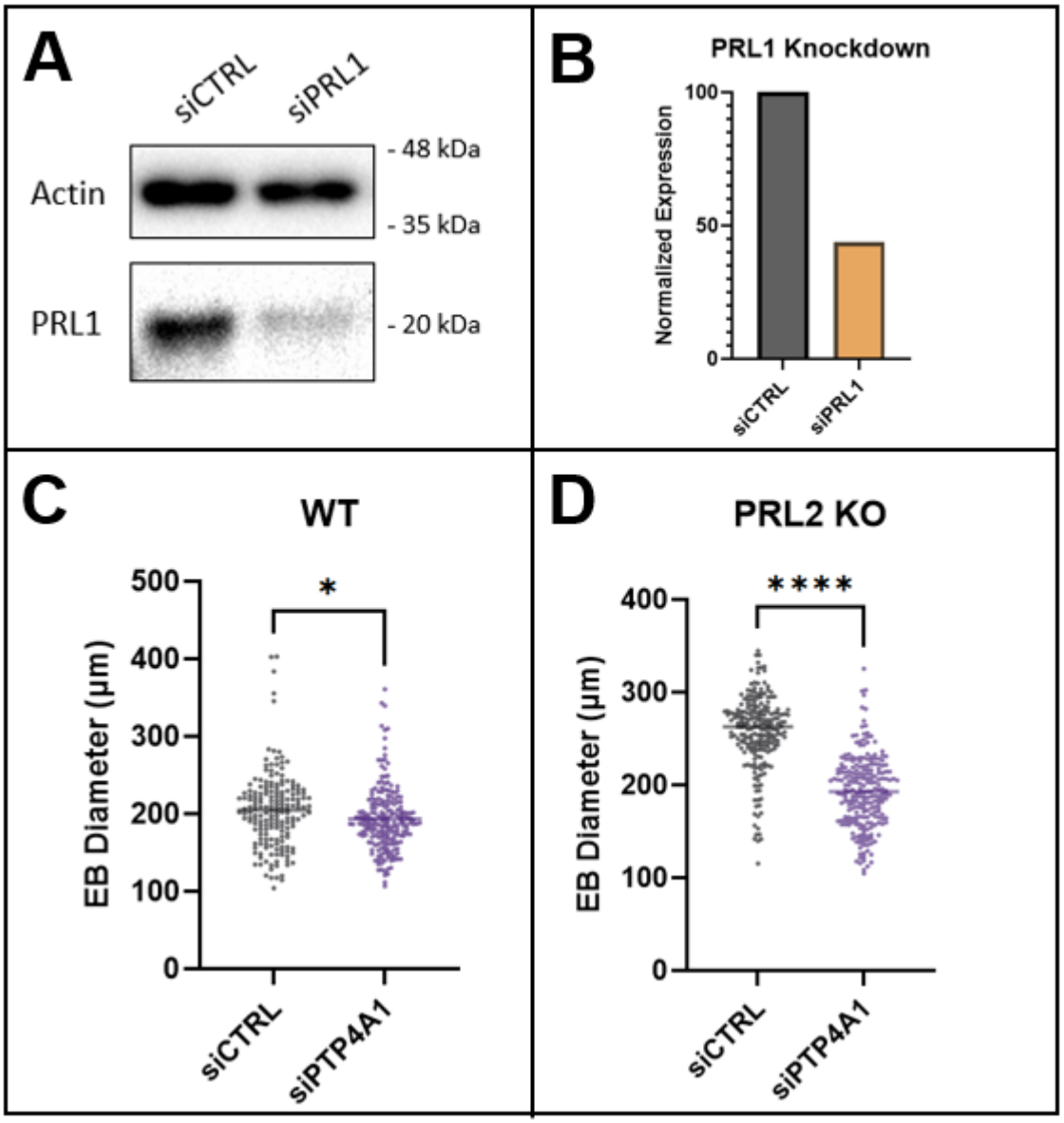


**Figure 13. Synthetic lethality between PRL1 and PRL2 dysregulates embryoid body development. (a)** Cells were seeded and treated with 12.5 nM siRNA 24 hours post-seeding and harvested 24 hours post-transfection. Western blotting was performed. **(b)** PTP4A1 siRNA knockdown efficiency was calculated using the normalized expression of PRL1 against Actin-β. **(c)** Wild-type mESC **e**mbryoid body diameters at 96H time point of embryoid body formation assay, n = 200. mESCs treated with PRL-1 siRNA at 0H (d) PRL-2 knockout mESC embryoid body diameters at 96h time point of embryoid body formation assay, n = 230. mESCs treated with PRL-1 siRNA at 0H. All EB diameters measured using ImageJ software.

## 10. CNNM2-SpyTag Mutant Generation

To validate the tag insertion, a PCR analysis was performed to detect a 50 base pair band shift in the PCR product of exon 8 of the CNNM2 gene (Fig. 14A). As the primers were designed to anneal outside of the target exon, the band shift would represent the insertion of the Spy tag in the CNNM2 gene. Mouse tissue samples which presented a band shift indicating a potentially successful insertion were selected for Sanger sequencing to determine if the tag insertion was in-frame with the coding sequence of CNNM2 (Fig. 14B). The analysis was performed on heterozygote mice, meaning we expected to see overlapping bands in the Sanger sequencing chromatogram representing the wild-type CNNM2 allele and CNNM2-spy allele. By aligning the insertion sequence with the chromatogram, we confirmed the presence of SpyTag directly after the final amino acid of CNNM2. The remaining protein characterization of the mouse is currently ongoing by other members of the Tremblay laboratory.

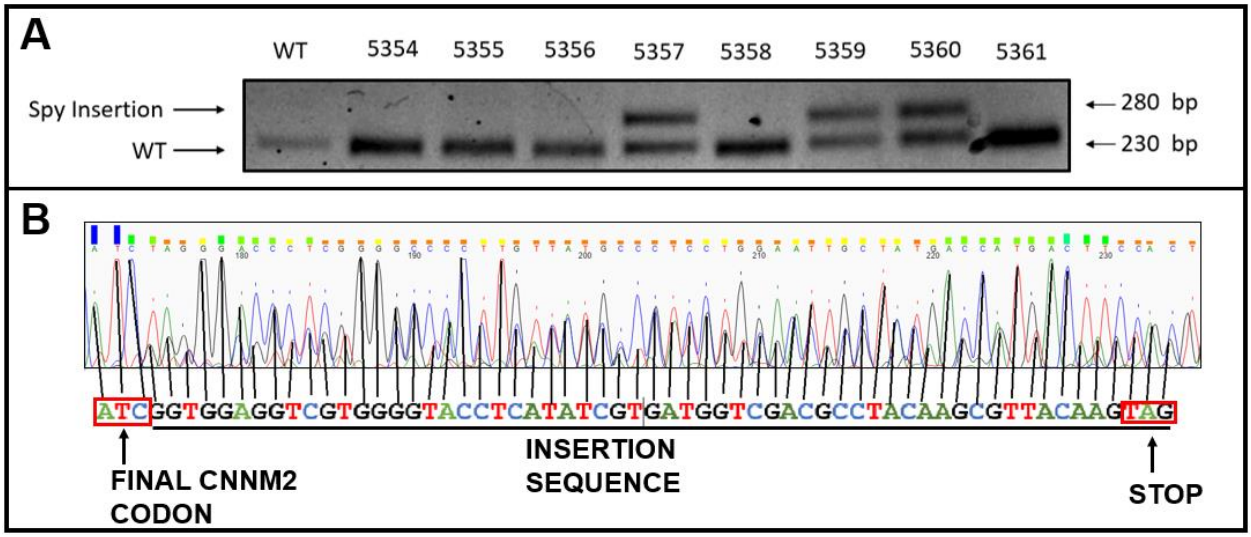


**Figure 14. Spy Tag Insertion Validation (a)** PCR analysis of CNNM2-Spy CRISPR insertion: detection of 50 bp insertion confirming successful gene editing **(b)** Sanger sequencing chromatogram analysis of Sample 5360 to confirm in-frame insertion

# Discussion

It has been found that PRL2 knockout hematopoietic stem cells show reduced activation of AKT signaling[32]. Cancer cells often hyperactivate the AKT-mTOR signaling pathway, increasing cell growth, proliferation, and survival. Various genetic and epigenetic alterations can cause activation of AKT and MTOR, such as mutations or amplification of genes encoding growth factor receptors, PI3K (phosphatidylinositol 3-kinase), AKT, or mTOR, or loss of expression of negative regulators such as PTEN.

The phosphorylation of S6 protein at serine 235 (p-S6K1/2-S235) is a downstream target of mTORC1 and is often used as a biomarker for activation of the mTOR signaling pathway[33]. When mTORC1 is active, it phosphorylates and activates its downstream target S6 kinase 1 (S6K1), which in turn phosphorylates S6 protein at several sites, including serine 235[34]. TRPM7 is essential for the control of cellular magnesium homeostasis[35]. Intracellular magnesium levels regulate the expression of PRL-1 and PRL-2 through a post-transcriptional feedback mechanism dependent on the 5'UTR-located upstream ORF of PTP4A2 mRNA[36].

To investigate the inverse relationship between TRPM7 and the PRLs, we looked into its role in regulating the oncogenic mTOR pathway. We found that as TRPM7 expression increased, the phosphorylation of serine 235 on the S6 ribosomal protein increases, suggesting that increased intracellular magnesium levels activate the mTOR signaling pathway. Additionally, we found that PRL2 likely plays a role in this activation by reducing its expression to decrease the cell's magnesium influx.

The CNNM3-PRL2 complex is believed to facilitate the transport of magnesium ions across the cell membrane by regulating the activity of CNNM3. Through this interaction, the complex has been found to promote oncogenesis[20]. Specifically, by utilizing xenograft tumor assays, our lab was able to establish that CNNM3 has inherent pro-oncogenic properties, and that the interaction between PRL-2 and CNNM3 is crucial in enabling tumor transformation. Additionally, our observations of human breast cancer tissues provide further evidence for this association, as we found that CNNM3 expression positively correlates with both PRL-2 expression and tumor proliferative index. The study demonstrates that oncogenic PRL-2 regulates tumor growth by influencing intracellular magnesium levels through binding with the CNNM3 magnesium transporter. Additionally, it has been determined that inhibition of the complex formation decreases breast cancer proliferation and tumor growth[37].

To build on the previous findings, our study provides compelling evidence of the intricate regulation of CNNM3 activity and expression through the differential expression of its binding partners, PRL2 and ARL15. Both proteins serve as pivotal regulators of CNNM3, and this competition interaction mechanism appears to have significant implications in the overall function of CNNM3, particularly in the regulation of magnesium homeostasis within the cell.

Interestingly, we found that the upregulation of PRL-1 in response to a heterozygous deletion of PRL-2 is similar, if not identical, to the homozygous deletion of PRL-2. This may indicate that PRL1 and PRL2 have similar functions and roles in the regulation of magnesium homeostasis. Alternatively, it could suggest that the upregulation of PRL1 is a compensatory response to the loss of PRL2, and that a certain

threshold of PRL2 expression is required to maintain normal levels of PRL1, in order to maintain healthy cell function. Further investigation would be necessary to determine the exact mechanism and implications of these findings. Regardless, the data suggests that to achieve a phenotype due to the loss of PRL functionality, the silencing of both proteins (PRL-1/2) is required.

Recent studies have determined that PRL-2 enhances hematopoietic stem and progenitor cell (HSPC) proliferation through its phosphatase activity[32]. We found that the rate of mESC proliferation was not affected by the deletion of the PRL-2 gene. This may be related to the compensatory upregulation of PRL-1 in response to PRL-2 gene knockout.

Stem cells are characterized by their ability to self-renew, which is the process by which they generate daughter cells that are identical to themselves. Self-renewal is crucial for maintaining the stem cell population and for enabling their differentiation into specialized cell types. Pluripotency, on the other hand, refers to the ability of stem cells to differentiate into any cell type in the body. Pluripotent stem cells express a set of markers, such as Oct4, Sox2, and Nanog, which are indicative of their pluripotency[38]. Maintaining the expression of these markers is critical for maintaining stem cell self-renewal and pluripotency. Thus, stem cell self-renewal and pluripotency marker expression are closely intertwined and mutually reinforcing processes. It has been found that PRL2 deficiency impairs hematopoietic stem cell self-renewal[32] and that PRL-2 deficiency leads to impaired placental development[39].

To investigate this phenomenon, we performed an embryoid body formation assay with PRL2 knockout embryonic stem cells. By inducing embryoid body formation from

pluripotent stem cells, we could mimic the early stages of embryonic development and investigate the molecular and cellular mechanisms that regulate placental formation. This technique enables the study of placental development under controlled conditions, allowing us to manipulate PRL2 expression to better understand its role in placental formation. Interestingly, we noticed that PRL2 is upregulated during the development of embryoid bodies. This suggests a potential role for the protein in regulating early stages of development. It is known that PRL2 is involved in regulating various cellular processes, including cell division and differentiation[40]. The upregulation of PRL2 in embryoid bodies could indicate its involvement in the differentiation of stem cells into specific cell types, such as trophoblasts, which give rise to placenta.

Further research is required to determine the specific function of PRL2 in embryonic development, but its upregulation in embryoid bodies provides a starting point for investigating its potential role in this process. Overall, this data suggests that PRL2 may have a more important role for differentiated cells. This is because the upregulation of PRL2 may be necessary to facilitate the differentiation process, indicating that the protein may play a critical role in driving the changes that occur as cells transition from a pluripotent to a more differentiated state.

The phosphatase of regenerating liver (PRL) family members, PRL-1 and PRL-2, are known to play crucial roles in cellular proliferation and differentiation. Surprisingly, we observed no significant increases in PRL-1 expression during embryoid body development. This information suggests that PRL-2 plays a more critical role during the differentiation process.

PRL2 is known to be the most ubiquitously expressed phosphatase of the PRLs family[12]. Due to this knowledge, we expected to see a larger change in expression of this protein compared to PRL1. Although knockout of PRL2 alone does not significantly affect embryoid body development, we did notice that its downregulation was complemented with the upregulation of PRL-1. The observed compensatory relationship between PRL-1 and PRL-2 underscores their importance in these processes. Due to their similar sequence homology, the proteins likely have redundant functions that are essential to cellular function. This is further supported by the fact that we were unable to generate a double knockout mutant through CRISPR-Cas9, suggesting that at least one of the proteins is required for cellular survival. The fact that deletion of either gene alone does not result in a significant developmental phenotype, but double knockout is lethal, highlights their redundant functions in cellular physiology.

We found that the knockdown of PRL1 has a statistically significant reduction in embryoid body size. As expected, the deficiency of both genes (PRL-1/2) leads to a significantly greater decrease in embryoid body size. The observation that knockdown of PRL-1 leads to a significant decrease in embryoid body size suggests that PRL-1 plays a critical role in regulating embryonic stem cell differentiation. These findings are consistent with previous reports implicating PRL-1 in cell proliferation, migration, and adhesion[41]. Together, these results suggest that PRL-1 and PRL-2 are essential components of the cellular machinery that regulates embryonic development. Their precise functions need to be elucidated further to understand these processes better.

Based on our discovery of the synthetic lethality interaction between PRL-1 and PRL-2, we posit that deleting both proteins in a cell population will lead to cellular

apoptosis. This hypothesis is supported by evidence indicating that embryonic bodies with reduced expression of both proteins were significantly smaller than those with only one deleted protein. To investigate further, we have developed CRISPR guide RNAs (gRNAs) targeting the start and end of exon 4 for both proteins, which we will introduce using an Amaxa nucleofector. Specifically, we plan to nucleofect PRL1-targeted gRNAs into PRL2 knockout mouse embryonic stem cells and PRL2 gRNAs into PRL1 knockout mouse embryonic stem cells. We anticipate observing significant cell death resulting from the knockout of both proteins in the majority of the cell population due to the high efficiency of RNA nucleofection with the Amaxa nucleofector.

To quantify the efficiency of nucleofection and CRISPR knockout, we will perform a time-course PCR experiment on the PRL-1/2 cell populations to monitor the change in signal strength of the targeted gene allele. PCR primers have been designed to obtain a signal dependent on exon 4 deletion. Once we confirm the dependence of mouse embryonic stem cells on at least one of the two proteins, we will investigate the differentiation process using the same approach. Additionally, we plan to investigate embryonic body formation failure resulting from simultaneous knockout of both PRLs.

Once the synthetic lethality interaction between PRL-1 and PRL-2 has been confirmed, the next step would be to investigate the underlying mechanism. It has been previously suggested that these proteins interact with CNNMs to promote oncogenesis, and that they are essential to the proper regulation of intracellular magnesium levels. As such, we plan on studying the changes in magnesium levels within the cells, as well as how the expression of CNNM and its competitive binder, ARL15, is affected. By examining these factors, we hope to gain a better understanding of how PRL-1 and PRL-

2 contribute to cellular processes such as cell signaling and gene expression regulation. Furthermore, identifying the role of these proteins in magnesium homeostasis could have important implications for conditions such as hypertension and diabetes, which are associated with alterations in magnesium levels. Overall, the investigation of the underlying mechanism behind the synthetic lethality interaction could provide valuable insights into the function of these proteins and their potential implications for human health.

The confirmation of synthetic lethality interaction between PRL-1 and PRL-2 genes in embryonic stem cells could have significant implications for the medical field. Understanding the role of these genes in development and differentiation could contribute to a better understanding of embryonic development and stem cell biology, which could lead to improved therapies for developmental disorders and regenerative medicine applications.

Moreover, the identification of synthetic lethal interactions is important in cancer research, as targeting synthetic lethal partners of cancer driver genes has been proposed as a promising therapeutic strategy. Therefore, identifying the synthetic lethality interaction between PRL-1 and PRL-2 could provide new insights into the mechanisms underlying oncogenesis and identify potential therapeutic targets for cancer treatment. Overall, the confirmation of this synthetic lethality interaction could pave the way for the development of novel therapeutic approaches in both developmental disorders and cancer research.

To finalize this thesis, we utilized CRISPR gene editing technology to insert a Spy-tag into the C-terminus of the mouse CNNM2 gene. CNNM2 is a gene that is expressed

in various tissues, including the brain, kidney, and liver, and has been shown to play a role in the regulation of intracellular magnesium levels. Similarly to CNNM3, CNNM2 functions as a magnesium transporter, facilitating the transport of magnesium ions across the cell membrane and into the cytoplasm. The protein has also been implicated in the development of some forms of hypertension, as well as in the regulation of bone mineralization. More recently, there has been interest in the potential role of CNNM2 in cancer, as it has been shown to interact with other proteins involved in oncogenesis, such as PRL-1 and PRL-2[21]. Overall, the CNNM2 gene and its protein product are important targets for research into various physiological and pathological processes, including magnesium homeostasis, hypertension, bone development, and cancer.

The SpyTag/SpyCatcher system is a powerful tool for studying protein-protein interactions, and the insertion of the SpyTag in the C-terminus of the CNNM2 gene could have several applications in this context. First and foremost, it would allow for the study of interactions between CNNM2 and other proteins of interest such as PRL-1/2, by fusing the SpyCatcher to the protein in question and observing its interaction with the CNNM2-SpyTag fusion protein. This could be particularly useful for studying proteins that are difficult to purify or that have low expression levels, as it would allow for the detection of weak or transient interactions that might otherwise go undetected[42]. The implications of this study extend beyond the specific interaction between PRL-2 and CNNM, as the methodology developed here can be applied to a wide range of protein interactions, providing valuable insights into the workings of cells and the mechanisms of disease.

In addition to studying protein-protein interactions, the SpyTag/SpyCatcher system can also be used to study protein localization and trafficking, by fusing the SpyCatcher to

a fluorescent protein and observing its localization in live cells[43]. Furthermore, the SpyTag/SpyCatcher system has been used for a variety of applications beyond protein-protein interactions, including the conjugation of enzymes and the creation of protein-based materials[44]. Overall, the insertion of the SpyTag in the C-terminus of the CNNM2 gene could open up a wide range of possibilities for studying protein interactions and function, and could have implications for a variety of fields including biotechnology, biochemistry, and cell biology. By studying the interaction between the PRLs and CNNM2, we aim to elucidate where in the cell this interaction occurs and how it may modulate magnesium homeostasis.

# Conclusion

The findings presented in this thesis demonstrate the crucial compensatory relationship between PRL-1 and PRL-2, underscoring their significance in embryonic differentiation and cellular proliferation. We propose a potential mechanism by which the dysregulation of PRLs could perturb cellular function by interfering with the interaction between CNNMs, ARL15, and PRLs. Additionally, we provide evidence that the dysregulation of intracellular magnesium levels may induce an oncogenic phenotype by activating the mTOR signaling pathway. Finally, this thesis concludes with the development of a genetically encoded SpyTag at the C-terminus of the mouse CNNM2 gene, enabling the investigation not only of the function and localization of the CNNM2 protein, a pivotal regulator of intracellular magnesium levels, but also of the localization and functional properties of the PRL-CNNM complex interaction.

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