Investigating the Magnesium-Dependent Post-transcriptional Regulation of Phosphatases of Regenerating Liver

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List of Abbreviations

ADP adenosine diphosphate

Akt Protein Kinase B

AMP adenosine monophosphate

AMPK AMP-activated protein kinase

ATP adenosine triphosphate

BSA Bovine rerum albumin

cDNA Complementary deoxyribonucleic acid

CNNM cyclin-M

DNA deoxyribonucleic acid

Dox doxycycline

DUSP dual specificity phosphatases

EDTA ethylenediaminetetraacetic

FBS fetal bovine serum

HA hemagglutinin

KO knockout

MEF2C transcription factor myocyte enhancer factor 2C

mRNA messenger ribonucleic acid

mTOR mechanistic target of rapamycin

PRL-1 or *PTP4A1* Phosphatase of Regenerating Liver 1

PRL-2 or *PTP4A2* Phosphatase of Regenerating Liver 2

PRL-3 or *PTP4A3* Phosphatase of Regenerating Liver 3

pSer phosphoserine

PTEN phosphatase and tensin homologue

pThr phosphothreonine

PTP Protein Tyrosine Phosphatase

pTyr phosphotyrosine

qPCR quantitative polymerase chain reaction

RT-PCR reverse transcription polymerase chain reaction

SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis

STAT3 signal transducer and activator of transcription 3

TRPM7 Transient Receptor Potential Cation Channel Subfamily M Member 7

uAUG upstream AUG

uORF upstream open reading frame

uORF upstream open reading frame

UTR untranslated region

VEGF vascular endothelial growth factor

WT wild type

Abstract

The three-membered **P**hosphatases of the **R**egenerating **L**iver (PRL-1, -2, -3) are a subfamily of protein tyrosine phosphatases. They have been found to be highly expressed in a majority of tumors and to be associated with cancer metastasis. Recently, PRLs have been implicated in magnesium homeostasis by forming a complex with the cyclin-M (CNNM) magnesium transporter family. Under magnesium-depleted conditions, PRL-1/-2 levels have been shown to be upregulated. We confirmed this magnesium-dependent regulation by modulating the expression of the magnesium transporter TRPM7. We report here that this magnesium-dependent regulation is post-transcriptional, specifically related to the 5'untranslated region (5'UTR) of the PRL mRNA. In the overexpression system, we observed that the presence of the 5'UTR inhibited PRL-2 expression and conveyed magnesium sensitivity. Furthermore, through ribosome-profiling data, we identified a conserved upstream open reading frame (uORF) in PRL-2 mRNA which appeared to stall ribosomes under standard magnesium conditions. Targeting of the PRL-2 uORF region via CRISPR-Cas9 system led to an increase in endogenous PRL-2 expression and reduced its magnesium sensitivity. The same observation was seen in PRL-1 uORF-targeted cells. In parallel, we observed that knocking out AMPK and inhibiting mTORC2 activity, respectively, reduced the magnesium-dependent upregulation of PRL-1/-2, implicating these as upstream modulators. Defining this magnesium-dependent regulation provides not only insight into understanding the physiological processes governed by PRLs, but also their implication in progression of cancer with more aggressive phenotypes.

Résumé

Les trois **P**hosphatases of **R**egenerating **L**iver (PRL-1, -2, -3) forment une sous-famille de protéines tyrosine phosphatases. Ils ont été trouvés surexprimé dans une majorité de tumeurs et sont impliquées dans les métastases cancéreuses. Notre groupe a trouvé que les PRLs jouent un rôle dans l'homéostasie du magnésium en formant un complexe avec les cyclin-M (CNNM) transporteurs de magnésium. Dans les conditions d'appauvrissement en magnésium, les niveaux de PRL-1/-2 sont surexprimés. Nous avons confirmé cette régulation dépendant du magnésium en modulant les niveaux de transporteur de magnésium TRPM7. Nous rapportons ici que cette régulation dépendant du magnésium est post-transcriptionnelle, spécifiquement liée à la région 5' non traduite (5'UTR) de l'ARNm de PRL. Dans le système de surexpression, nous avons observé que la présence de 5'UTR inhibe l'expression de PRL-2 et transmet la sensibilité au magnésium. De plus, grâce à des données du profilage ribosomique, nous avons identifié un cadre de lecture ouvert (uORF) conservé dans l'ARNm de PRL-2 qui semble bloquer la progression des ribosomes dans des conditions de magnésium standard. Le ciblage de la région uORF de PRL-2 via le système CRISPR-Cas9 a conduit à une augmentation de l'expression de PRL-2 endogène et a réduit sa sensibilité au magnésium. La même observation a été observée dans les cellules où PRL-1 uORF était ciblé. Parallèlement, nous avons observé que l'inhibition de l'AMPK et mTORC2 réduisaient respectivement la régulation de PRL-1/-2 dépendant du magnésium, ce qui les impliquait comme facteurs dans cette régulation. Déterminer cette régulation dépendante du magnésium fournit non seulement un aperçu de la compréhension des processus physiologiques régis par les PRL, mais aussi leur implication dans la progression du cancer avec des phénotypes plus agressifs.

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Chapter 1: Introduction

Protein Tyrosine Phosphatases

Protein phosphorylation is an important post-translational modification that provides a rapid response to internal and external cues.[1] More than 35 000 proteins have been reported to have at least one phosphorylation site in the PhosphoSitePlus[2] database. In eukaryotes, protein phosphorylation typically occurs on serine, threonine, and tyrosine residues.[3] The balance of phosphorylation is maintained through protein kinases that add phosphate groups and protein phosphatases that remove them.

There are in total 107 genes identified in the family of protein tyrosine phosphatase (PTPs).[1] They play a regulatory role in various biological processes that are directly relevant to cancer, such as proliferation, differentiation, migration, and metabolism.[4] Since PTPs can both activate kinases or counteract their activity by dephosphorylating the kinase itself or its downstream target, they can either positively or negatively regulate different signaling pathways.[4, 5] The PTP superfamily is defined by an active-site signature motif HCX_5R[6] and is broken into classical phosphotyrosine (pTyr)-specific phosphatases and dual specificity phosphatases (DSP).[7, 8]

The classical PTPs are further categorized as receptor-like or non-transmembrane cytosolic proteins, and additional diversity is introduced through the use of alternative promoters and alternative mRNA splicing.[6] On the other hand, the DSPs form a more heterogeneous group of phosphatases.[6] They are more structurally diverse and possess a shallower catalytic domain than classical PTPs.[6] Their active sites are able to accommodate phosphoserine (pSer), phosphothreonine (pThr), in addition to pTyr residues.[6]

Phosphatases of Regenerating Liver

The three members of the subfamily of **P**hosphatases of **R**egenerating **L**iver (PRL-1,-2,-3 or *PTP4A1, 2, 3*) are about 20kDa with at least 75% amino acid sequence identity.[9] Their sequences are presented in Figure 1. Unlike other PTPs, the PRL family members are unique in possessing a CAAX prenylation motif in the C-terminal region.[10] The prenylation of PRLs is essential for their localization to endosomal compartments and to the plasma membrane.[11, 12] Deletion of the CAAX motif redirects the PRLs into the nucleus.[11, 13, 14] While PRL-1 and PRL-2 are expressed ubiquitously, PRL-3 is mainly expressed in the heart, skeletal muscle, and prostate.[9] PRL-3 has very low expression in all other tissues.[9]

PRLs are categorized as DSPs.[4] As for other PTPs, the catalytic cysteine of the $C(X)_5R$ motif initiates the nucleophilic attack to remove the phosphate from the substrate while the conserved arginine of the WPD loop acts as a general acid/base catalyst for the dephosphorylation reaction.[16] Following a similar mechanism as other DSPs, including PTEN, PRLs are subject to redox-regulation.[15] The cysteine of the active site can form a disulfide bond with a spatially proximal cysteine residue.[16, 17] Disulfide bond formation is reversible and enzymatic function is restored via reduction.[16-19] Interestingly, PRLs contain an alanine residue instead of the conserved ser/thr residue in its active site p-loop. [16, 17] It was shown that the presence of this alanine results in low *in vitro* activity of the enzymes.[16, 17],

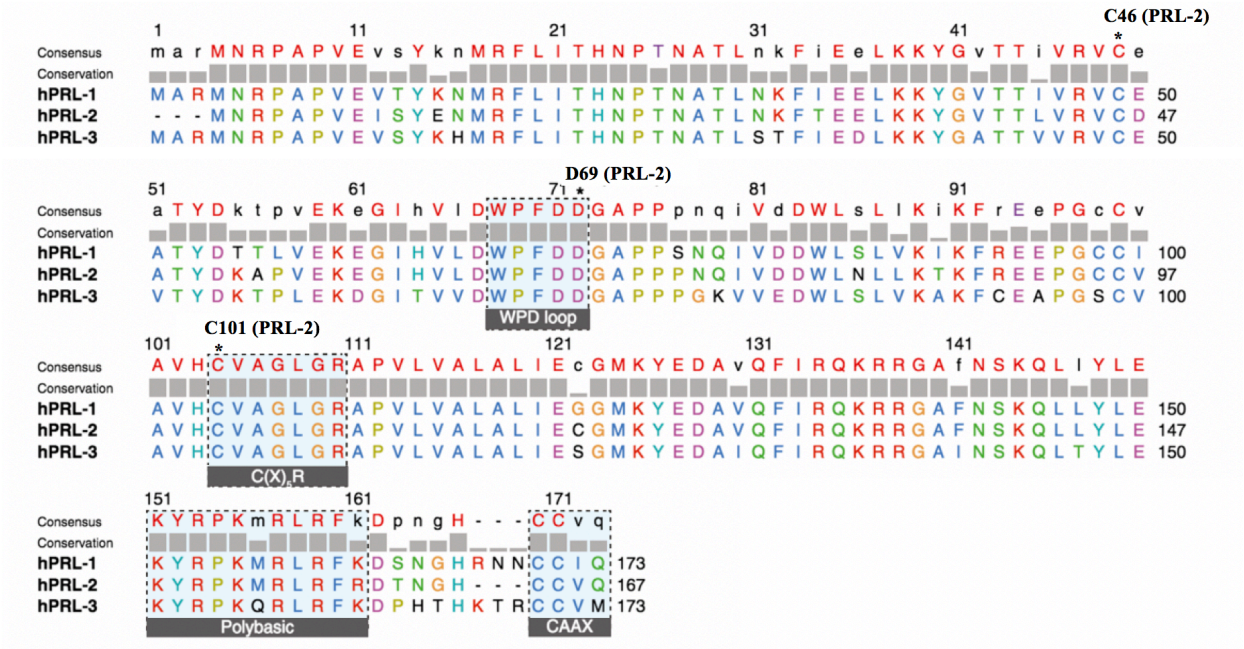

Figure 1. Sequence alignment of members of the PRL family.

The three PRLs share over 75% amino acid sequence identity. The $C(X)_5R$ and WPD loop are characteristics of protein tyrosine phosphatases. The polybasic domain and prenylation motif are critical for PRL localization. Figure adapted from Hardy *et al.*[9]

Role of PRLs in Cancer

The PRL subfamily gained attention over a decade ago when the Vogelstein group identified PRL-3 as the only gene consistently expressed at a higher level among 144 upregulated genes in all 18 metastatic colorectal liver samples analyzed.[20] Since then, the PRL family members have been found to be highly expressed in a majority of human solid tumors and shown to correlate strongly with cancer progression.[21] Both their catalytic activity and CAAX motif are necessary for tumor- and metastasis-related phenotypes.[1-4]

Our group has shown that PRL-2 mRNA is elevated in primary breast tumors relative to matched normal tissue, and is also further increased in lymph node metastatic tissue compared to primary

tissue.[5] In a breast cancer transgenic mouse model, mammary tumors were observed to form earlier when PRL-2 was overexpressed.[5] While the literature has linked PRLs to major oncogenic signalling pathways involved in cell proliferation, survival, migration and adhesion including Rho-family GTPase, PI3K-Akt, STATs, and Ras-MAPK pathways, to date no substrate has been clearly identified for the PRLs. [6, 7]

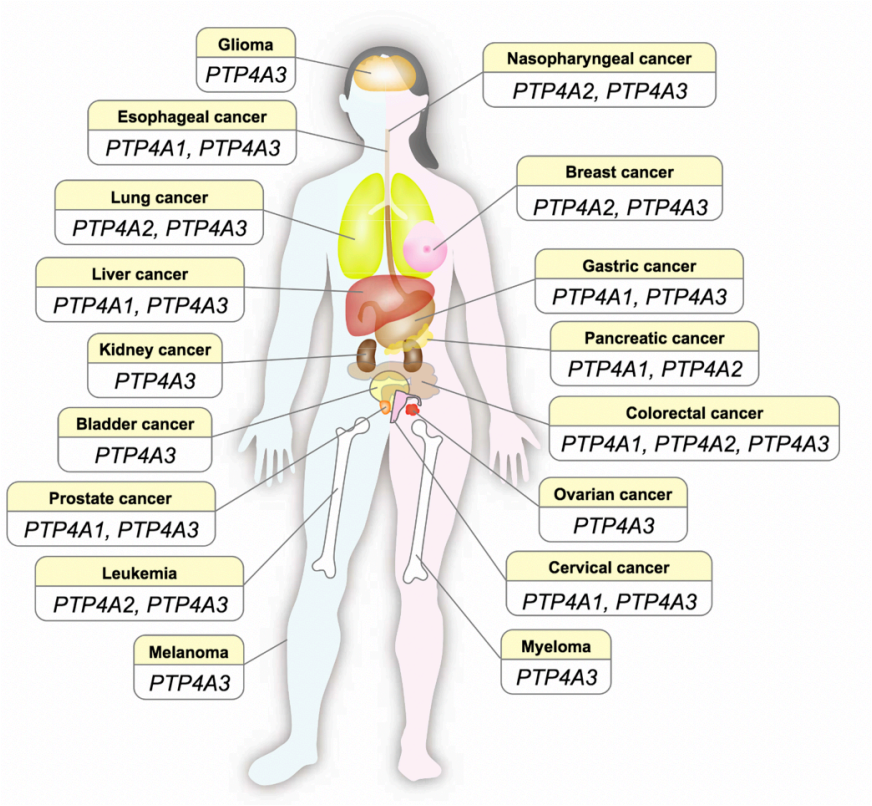

Figure 2. Overexpression of PRLs in different type of cancers.

Overexpression of the PRLs (*PTP4A*s) at either mRNA or protein levels by different types of cancer. Figure adapted from Hardy *et al*.[8]

Regulation of PRL Expression

Protein expression is tightly regulated spatiotemporally. Regulation is especially critical during development, in response to environmental stimulation, and for reestablishment of homeostasis.[9]

Protein expression can be modulated at different stages, from transcriptional initiation, RNA processing, translation to post-translational modification[9].

Regulation at the level of transcription is typically the slowest, requiring the rewiring of different transcription factors and coregulators to modulate the level of mRNA transcription.[10, 11] Regulation at the level of RNA processing involves capping, splicing, and addition of a polyA tail.[12-14] Along with small RNAs[15], these are all critical for modulating the rate of translation and mRNA stability. Protein expression is a balance between the rate of translation and protein degradation. Lastly, post-translational modification can alter protein activity and location. Post-translational modification, such as phosphorylation, enables fast modulation[16], while transcriptional reprogramming exerts broader and longer-lasting effects.[17] These different modes of regulation are necessary for increasing the versatility and adaptability of an organism[9].

PRL expression is regulated at the level of transcription, translation, and protein stability. Many of the studies have however focused their investigation on PRL-3 only. Transcriptional regulation of PRLs has been associated to tumor suppressor p53.[18] While two PRL-3 introns contain a p53 consensus sequence, p53-mediated regulation appears to be cell-type dependent.[18] Transcription of PRL-3 is also suggested to be controlled by tissue-specific transcription factors. For instance, PRL-3 is activated by VEGF through transcription factor MEF2C in human umbilical vein endothelial cells.[19] Others factors implicated in PRL-3 transcription regulation include STAT3[20], snail[21], and TGFß[22] among others.

At the level of translation, polyC-RNA-binding protein 1 (PCBP1) has been reported to regulate PRL-3 expression.[23] PCBP1 binds to GC-rich motifs found in the 5' and 3' untranslated region of the mRNA to retard its incorporation into polyribosomes.[23] Lastly, deubiquitinating enzyme

ubiquitin specific protease 4 (USP4) has also been reported to interact and stabilize PRL-3 via deubiquitination.[24]

Magnesium and PRLs: The PRL/CNNM Complex

Magnesium is the second most abundant intracellular cation, after potassium.[25] It is an essential intracellular cation involved in over 600 enzymatic reactions including energy metabolism and protein synthesis.[25] Intracellular concentration of magnesium falls between 10 to 30 mM.[25] Since most of this magnesium is bound to ribosomes, nucleotides and ATP, the concentration of "free" magnesium falls within 0.5 to 1.2 mM, which is comparable to the extracellular concentration.[26] Magnesium homeostasis is important for cell survival, and its levels have shown to be closely related to ATP levels.[27] In human lens epithelial cells, incubation in magnesium-deficient medium has been shown to decrease ATP levels compared to incubation in standard magnesium medium.[27] An imbalance of intracellular magnesium levels is also observed in transformed cells and is linked to alterations of several hallmarks of cancer.[28] The balance of intracellular magnesium is regulated by different magnesium transporters as shown in Figure 3.[25] TRPM7, a ubiquitously expressed divalent cation channel[41], is a major channel responsible for maintaining intracellular magnesium flux and has been reported to be an essential gene for cell survival.[29] Some of the other magnesium transporters include TRPM6, MagT1, SLC41A1-2, and CNNMs.[25]

Hardy *et al.* first identified an interaction between the PRLs and the magnesium transporter CNNMs.[30] PRL-2 was found to form, via its catalytic domain, a heterodimer with CNNM3.[30] CNNM3 has a unique conserved elongated loop located in the Bateman domain which interacts with PRL-2 catalytic domain.[30] Interestingly, in a phylogenetic analysis of both PRL and CNNM enzymes in 150 genomes across all kingdoms, the unique loop on CNNM was conserved only when a PRL ortholog was also present in the same organism.[30] Furthermore, when this complex

formation was disrupted via a single point mutation (D426A) in the CNNM3 loop region, tumor growth and cell proliferation were shown to decrease.[31] This PRL/CNNM interaction was also reported independently by another group [32] and validated by structural studies[33-35]. The structure of the complex showed that the binding of PRLs to CNNMs favored a twisted to flat conformational change, which is believed to affect the transmembrane domains of CNNMs and to modulate magnesium transport.[35, 36] The PRL/CNNM complex thus provides a novel mechanism of controlling magnesium homeostasis.

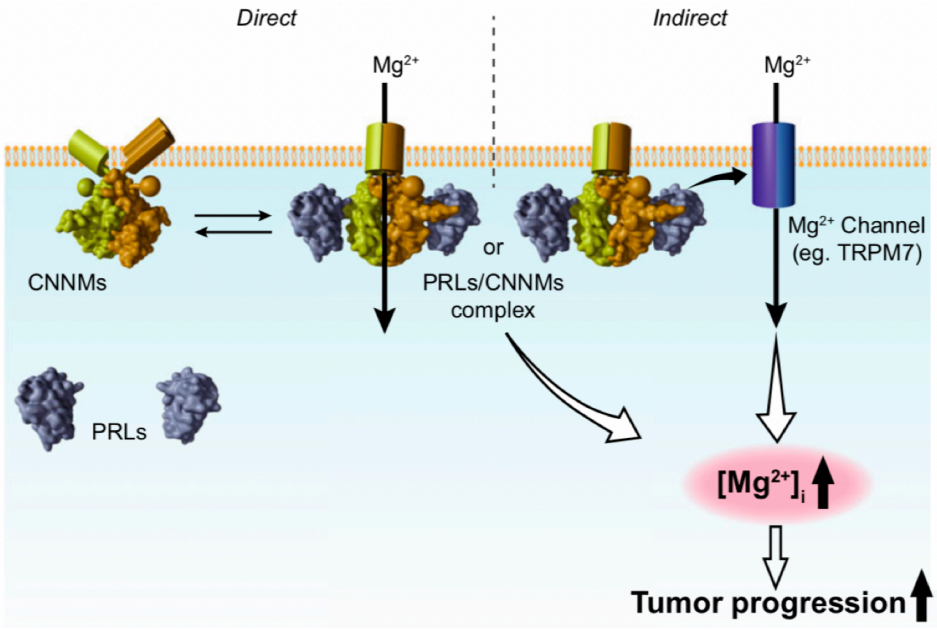

Figure 3. Regulation of magnesium homeostasis by the CNNM/PRL Protein Complex.

When intracellular magnesium becomes limiting, PRL is recruited to the CNNM/PRL complex to either directly or indirectly increase intracellular magnesium concentration to promote cancer progression. Figure adapted from Hardy *et al*.[8]

Magnesium-Dependent Regulation of Protein Expression

Since the balance of intracellular magnesium is essential for the organism's survival, there must exist an exquisite regulation to maintain magnesium homeostasis. AMPK is a sensor of cellular energy; it is activated via phosphorylation of threonine-172 in the alpha catalytic subunit under condition of energy stress.[37] A well-characterized downstream effector of AMPK activation is the rapamycin-sensitive mammalian target of rapamycin complex (mTORC1) pathway. AMPK inhibits this pathway via both the direct phosphorylation of the critical mTOR binding partner raptor[38] and the indirect phosphorylation of the critical upstream mTORC1 complex inhibitor tuberous sclerosis complex (TSC)[39]. The mTORC1 complex is an intracellular sensor, sensitive to nutrient levels (glucose and amino acids) and growth factors.[40] Its inhibition suppresses cell growth and biosynthetic processes.[38] Via its downstream targets 4E binding protein 1 (4EBP1) and ribosomal S6 kinase (S6K), mTORC1 also acts as a major regulator of protein synthesis.[37]

Under conditions of magnesium deprivation, the essential magnesium channel TRPM7 protein expression is shown to be upregulated.[41] Its regulation by magnesium has been proposed to be controlled at the level of translation by an upstream open reading frame (uORF).[41] In TRPM7, it has been proposed that the first uORF inhibits the overall translation of the main coding sequence while the second uORF confers regulation in response to changes in magnesium.[41]

More traditionally, uORFs have often been described as a constitutive repressor of translation at the main coding sequence (CDS).[42] This is achieved in several ways: direct ribosome elongation stalling during translation of the uORF could occur due to codon usage bias, polypeptide sequences, or interaction with trans-acting factors.[43-45] Another form of repression by uORF is the promotion of ribosome dissociation from the mRNA and subsequent decreased translation at the main CDS. [46-48] Another interesting phenomenon associated with uORF is ribosome bypass. Bypass of an

uORF is dictated either constitutively by a poor Kozak consensus context surrounding the uORF or by induction of physiological signals.[48-50]

In prokaryote, the presence of riboswitch allows for RNA to undergo conformational change in response to metabolic or stress cues to control gene expression.[51] In *B. subtilis,* magnesium-specific regulation of the mgtE magnesium channel is controlled by a riboswitch found in the 5'untranslated region.[52] Similarly, protein expression of the MgtA magnesium channel is regulated by a riboswitch mechanism.[53] The sensitivity to magnesium is thus built into the mRNA transcripts.[52, 53]

Interestingly, in the PRL/CNNM complex, PRL-1/-2 but not CNNMs were shown to be regulated by magnesium levels.[30] In various cell lines, it was shown that upon Mg^{2+} depletion, PRL-1/-2 protein levels were upregulated.[30] This leads to improved association with the CNNMs and increased intracellular magnesium levels as shown in Figure 4.[30] Since PRLs could play a pivotal role in the response to decreased magnesium availability, such as in cancer progression, it is of interest to study its magnesium-dependent regulation.

Objectives of this study

The PRLs have been established to promote cancer progression. Recently, our group have identified the magnesium transporter CNNMs as binding partners of PRLs. Interestingly, we have also uncovered that the protein expression of PRLs is increased under hypomagnesaemic conditions. The study's overall aim is thus to investigate the mechanism behind this magnesium-dependent upregulation. Initial studies in our lab linked this regulation to the 5' untranslated region of the mRNA and this study will seek to further characterize this regulation. In parallel, we are also interested in uncovering upstream factors regulating the magnesium-dependent PRL expression. Taken together, we believe that understanding the magnesium-dependent expression of PRL will provide insight into its role in normal physiology and cancer progression.

Chapter 2: Materials and Methods

Cell culture

HeLa, MDA-MB-231, MCF-7, BT474 and HEK293 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, HyClone) supplemented with 10% fetal bovine serum (FBS, Gibco), 20 mM HEPES (Multicell), and 50 ug/mL gentamicin sulfate (Multicell) and cultured in a controlled humid environment (37℃, 5% CO_2). For magnesium deprivation experiments, cells were growth in magnesium-free DMEM (HyClone) supplemented with dialyzed FBS (Gibco); and when standard magnesium concentration was required, magnesium was supplemented to 1mM using $MgSO_4$. Unless otherwise specified, magnesium treatment is 4 hours in HeLa cells and 24 hours in breast cancer cell lines.

For inhibitor treatments, cells were seeded to achieve 70-80% confluency. The inhibitors were used at the following concentrations: actinomycin D (50 mM), cycloheximide (50 ug/mL), MG132 (5 uM), PP242 (2.5 uM), Torin 1(100 nM), and rapamycin (50 mM).

Overexpression of Flag-PRL-2 Constructs

Different PRL-2 constructs were cloned into pcDNA™3.1/Zeo (+) plasmids. Transfections into HeLa cells were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. After an overnight transfection, cells were subjected to magnesium treatment.

Lentivirus Production and Infection of CRISPR/Cas9 constructs for genome-editing

Single guide RNA (sgRNA) were generated by using the online tool (http://crispr.mit.edu) and are listed in Table 1. TRPM7 sgRNA were cloned into the SFFV construct and transfected into Hek 293 T17 cells for viral production using Lipofectamine 2000 (Invitrogen). Following 48 hours

incubation, lentivirus-containing supernatants were s harvested, filtered (0.45 um), and used for infection of Dox-inducible HA-TRPM7 HEK293 cells (gift from Drs. Carsten Schmitz and Anne-Laure Perraud) in the presence of 5 ug/mL polybrene (Sigma-Aldrich) to generate TRPM7 KO cells.

sgRNA targeting PRL-1 and PRL-2 uORF region were cloned into the LentiCRISPR-V2 and LentiCRISPR-V2-GFP, respectively. Lentiviral particles were produced and used to infect MDA-MB-231 and MCF-7 in the presence of 5 ug/mL polybrene (Sigma-Aldrich) to generate PRL-1 and PRL-2 uORF targeted cells.

sgRNA targeting the AMPK alpha subunit were cloned into the LentiCRISPR-V2. Lentiviral particles were produced and used to infect MDA-MB-231, MCF-7, and BT474 in the presence of 5 ug/mL polybrene (Sigma-Aldrich) to generate AMPK KO cells.

A LacZ sgRNA control was cloned in all of these lentiviral vectors.

Oligonucleotide	Sequence
TRPM7 sgRNA #1	F: CACCGCATCCTGGAAGGCATCTGTG

	R: AAACCACAGATGCCTTCCAGGATGC
TRPM7 sgRNA #2	F: CACCGGAGTCATAAATTTTCAAGG R: AAACCCTTGAAAATTTATGACTCC
TRPM7 sgRNA #3	F: CACCGAGAAAGCACTTTGACCAAGA R: AAACTCTTGGTCAAAGTGCTTTCTC
TRPM7 sgRNA #4	F: CACCGAAATTTGTCAGCAACTCGTC R: AAACGACGAGTTGCTGACAAATTTC
PRL2 sgRNA	F: CACCGAGTGCATTGTGTTGCAGGAT R: AAACATCCTGCAACACAATGCACTC
PRL-1 uORF #1	F: CACCGCATAGAGGTCGTGCTGTGCC R: AAACGGCACAGCACGACCTCTATGC
PRL-1 uORF #3	F:CACCGGGCAGTGGAGATTACTGCC R: AAACGGCAGTAATCTCCACTGCCCC
PRL-2 uORF #2	F: CACCGTTATGGCGATTCTGAGTGTG R: AAACCACACTCAGAATCGCCATAAC
PRL-2 uORF #3	F: CACCGTGTGAGGGCAGACTTCTGCC R: AAACGGCAGAAGTCTGCCCTCACAC
AMPK alpha subunit #1	F: CACCGCACGACGGGCGGGTGAAGAT R: AAACATCTTCACCCGCCCGTCGTGC
AMPK alpha subunit #2	F: AAACATCTTCACCCGCCCGTCGTGC R: AAACGCCCGTCGTGTTTCTGCTTCC
Vector	Addgene plasmid number
pcDNA™3.1/Zeo (+) plasmids	V86020
LentiCRISPR v2	52961
LentiCRISPR v2 GFP	82416
pL-CRISPR.SFFV.GFP	57827

Table 1. Sequences for CRISPR sgRNA and Addgene plasmid number for different vectors.

Immunoblotting

Cells were washed with phosphate buffered solution (PBS), and lysed on ice in RIPA buffer [150 mM NaCl, 50 mM Tris (pH 7.4), 0.1% SDS, 1% NP40, 0.5% sodium deoxycholate] supplemented with inhibitors [50 mM NaF, 1 mM Na_3VO_4, and Complete protease inhibitors (Roche)]. Using the bicinchoninic acid (BCA) assay (Thermo Scientific) and a 96-well plate reader (Varioskan), the protein concentrations of lysed samples were quantified. Denatured proteins were then separated on SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The membranes were blotted using antibodies specific for PRL-1/-2

(Millipore), p-p53 (Cell Signaling), actin (Sigma), CNNM3 (Proteintech Group), p-S6 (Cell Signaling), S6 (Cell Signaling), p-AMPK (Cell Signaling), AMPK (Cell Signaling), Flag (Sigma) according to the manufacturer's instructions. All immunoblots were visualized on a BioRad Imager (LI-COR Biosciences). When required, blots were stripped [62.5 mM Tris-HCl (pH 6.8), 2% SDS] and reblotted according to the instructions from the manufacturer. Immunoblots were visualized and band density quantified on a Bio-Rad Image Lab (BioRad) and using ImageJ software.

RNA isolation and RT-PCR

Total RNA was extracted from cells using TRIzol (Invitrogen) according to manufacturer's instructions. TRIzol and chloroform were used to extract total RNA. RNA was precipitated with the addition of isopropanol. The pellets were washed with cold ethanol. Contaminated genomic and plasmid DNA were removed with DNAseI treatment at 37 °C for 30 minutes. DNAseI was inactivated by adding DNase I inhibition beads for 2 minutes at room temperature. 1 ug of extracted RNA was used for synthesizing cDNA with SuperScript III reverse transcriptase (Invitrogen) according to manufacturer's instructions. After the addition of random primers and dNTP, the mixture was heated at 65°C for 5 minutes followed by an incubation on ice. First-Strand buffer, DTT and reverse transcriptase were added and cDNA was transcribed. The cDNA was treated with E. Coli RNase H at 37°C for 20 minutes.

qPCR reaction was performed using the following primers.

PTP4A1F: (5'TGCTGTTCATTGCGTTGCAG3')

PTP4A1R: (5'CCACGCCGCTTTTGTCTTATG3')

PTP4A2F: (5' GGAATCCACGTTCTAGATTGGC3')

PTP4A2R: (5': AACACAGCAACCTGGCTCTT3')

RPLP0F: (5'CGTCCTCGTGGAAGTGACAT3')

RPLP0R: (5'ATCTGCTGCATCTGCTTGGA3')

The *PTP4A1* primers amplify a region between exon 3 and 4 while the *PTP4A2* primers amplifies a region between exon 2 and 3 of *PTP4A2*. *RPLP0* was used for normalization of PRL-1 and PRL-2 mRNA expression. qPCR was performed with 2X SYBR master mix (Bio-Rad), and a 3-step amplification repeated for 45 cycles on LightCycler480 (Roche). The mean ± SD was determined from three independent experiments.

IncuCyte

MDA-MB-231 were plated at a density of 3000 cells per well in a cell culture treated flat bottom 96-wells plate. Cells were left to grow over 96 hours in IncuCyte ZOOM system (Essen Bioscience). Pictures of the cell confluency were taken every 4 hours. Cell confluency was analyzed using IncuCyte ZOOM integrated software. The slope of the proliferation curve was quantified by fitting an exponential equation onto the curve with the PRISM software. IncuCyte data was reported based on three to five replicates.

ICP-OES

For ICP-OES analyses, confluent 10-cm plates were scraped and digested in 0.5 ml nitric acid (Macron Fine Chemicals) at 100°C for 1 hour followed by digestion in 0.5 ml of 30% hydrogen peroxide (BioShop) at 100°C for 30 minutes. Samples were then diluted to a final concentration of 5% nitric acid and analyzed with ICP-OES (Thermo Fisher Scientific iCAP™ 6000). Intracellular magnesium levels were normalized to total protein content. ICP-OES data reported is based on four or more replicate.

Analysis of publicly available data

For the analysis of publicly available data the GWIPS-viz browser (https://gwips.ucc.ie) [54, 55] was used. For the analysis of nucleotide conservation, 100-way vertebrate alignment was explored with phyloP[56] and CodAlignView (I. Jungreis, M. Lin and M. Kellis, manuscript in preparation).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism Version 7.0. One-way ANOVA statistical analysis was performed where applicable.

Chapter 3: Results

Magnesium-Dependent Regulation of PRL-1/-2 is Post-transcriptional

Previously, levels of PRL-1/-2 have shown to be modulated through changes in magnesium levels in the culture media. To take it a step further, we wanted to modulate intracellular magnesium by modulating the expression of the magnesium transporter TRPM7, which is an essential magnesium transporter that maintains intracellular magnesium homeostasis.[29] To this end, we over-expressed the TRPM7 magnesium transporter using the well-characterized HEK293 doxycycline-inducible system described to trigger an increase in intracellular magnesium levels.[29] We observed a decrease in PRL-1/2 expression following doxycycline-induced TRPM7 expression (Figure 4A). On the other hand, when we knocked out TRPM7 using four different sgRNA using the CRISPR-Cas 9 system in this overexpression system, we saw an increase in PRL-1/-2 expression (Figure 4B). Combined with previous results, these observations reinforce that PRL-1/-2 expression is modulated by intracellular magnesium levels.

To further assess this magnesium-dependent regulation, we first assessed whether the regulation was taking place at the transcriptional level. Following treatment with the transcriptional inhibitor Actinomycin D for four hours in HeLa cells, we did not detect changes in the magnesium-dependent increase of PRL-1/-2 levels (Figure 5A), suggesting that this upregulation is post-transcriptional. Of note, when we look at the half-life of PRL-1/-2 protein by performing a cycloheximide-chase experiment, we observed a decrease starting around eight hours (Figure 5B).

To assess the role of proteasome degradation in the regulation of PRL-1/-2, we then treated cells with the proteasome inhibitor MG132. We observed no difference in PRL-1/-2 protein levels under standard and magnesium-depleted conditions compared to control treatment (Figure 5C). This suggested that the upregulation of PRL-1/-2 under magnesium removal is independent of the proteasomal degradation of the protein. Treatment of cells with the translation elongation inhibitor cycloheximide on the other hand was able to abolish the upregulation (Figure 5C). Taken together, it would appear that the magnesium-dependent regulation of PRL-1/-2 occurs post-transcriptionally and is independent to proteasomal-regulated degradation.

Expression of 5'UTR regulates PRL-2 protein levels in standard and magnesium-depleted conditions.

Upon examining the mRNA of PRL-2, we observed that it has a very long 5' untranslated region (5'UTR), a characteristic shared by many translationally-regulated mRNA[57]. To assess the role of the 5'UTR, we cloned PRL-2 with and without its 5'UTR for overexpression in HeLa cells (Figure 6A). Cells transfected with the PRL-2 only construct had higher expression than those transfected with the 5'UTR-PRL-2 (Figure 6B), indicating a strong regulation by the presence of this region. Still, PRL-2 appeared to require the 5'UTR to respond to the condition of magnesium depletion (Figure 6B). We also included a PRL-2 C101S mutant that is unable to bind to CNNMs[5] and their expressions are similar to corresponding the PRL-2 WT (Figure 6B), suggesting that the effect of the UTR is independent of PRL-2 interaction with the magnesium transporter. When we assessed the levels of intracellular magnesium in these cells by ICP-OES, we observe a trend of decreased intracellular magnesium with magnesium-depletion treatment (Figure 6C). However, there is no significance difference between the different constructs in both standard and magnesium-depleted conditions (Figure 6C).

Identification of uORF in 5' Untranslated Region Controlling PRL-1/-2 Regulation

Upon further examination into the 5'UTR of PRL-2, we uncovered the presence of several upstream canonical-AUG codons which encode putative upstream open reading frames (uORFs). Ribosome-profiling data obtained from the publicly available GWIPS-viz Riboseq database (https://gwips.ucc.ie)[54, 55] revealed strong signals for ribosome protected fragments in the 5'UTR of PRL-2 downstream of an uAUG, specifically towards the end of the putative uORF (Figure S1A). This strongly suggested that ribosomes are potentially being stalled in this region in presence of normal magnesium condition. The uORF of our interest is about 60 nucleotides in length and nucleotide conservation of a 100-way vertebrate alignment analysis using phyloP[56] and CodAlignView (I. Jungreis, M. Lin and M. Kellis, manuscript in preparation) revealed that the region is very well conserved (Figure S1A).

Then, we wanted to assess the function of this uORF in PRL-2. We started by targeting this uORF region using the CRISPR-Cas9 system in the MDA-MD-231 breast cancer cell line. We then examined PRL-1/-2 expression in these PRL-2 uORF targeted cells at standard and magnesium-depleted conditions. These cells had higher PRL-2 protein expression at standard magnesium conditions and showed a reduced response to magnesium depletion (Figure 7A-B). PRL-1 on the other hand was not affected and was responsive to magnesium depletion. To confirm that the uORF-targeted effect was not cell-line dependent, we also targeted the PRL-2 uORF region in the MCF-7 breast cancer line. Similarly, PRL-2 protein expression under standard magnesium conditions was increased and there was a reduced response to magnesium-dependent regulation of PRL-2 (Figure 7C-D). Again, PRL-1 protein levels and response were not affected.

When looking at the conservation of this regulation in the other PRL members, we determined that the uORF in PRL-2 was conserved in PRL-1 using ClustalW2[68] (Figure S1C) and ribosomes were

also stalled at the PRL-1 uORF according to GWIPS-viz Riboseq data[54, 55] (Figure S1B). Again, a CRISPR sgRNA was designed against the region to assess the role of the uORF on PRL-1 expression. Similar to PRL-2 uORF-targeted cells, PRL-1 uORF-targeted cells had an increase in PRL-1 expression under standard magnesium concentration and were less responsive to magnesium-dependent upregulation in both MDA-MB-231 (Figure 8A-B) and MCF-7 (Figure 8C-D) breast cancer cell lines. PRL-2 expression and response to magnesium levels were unaffected.

Importantly, these uORF-targeted cells also did not show significant differences in *PTP4A2* mRNA or *PTP4A1* mRNA respectively in PRL-2 uORF-targeted (Figure 9A) and PRL-1 uORF-targeted MDA-MB-231 cells, respectively (Figure 9B). These results further reinforced that the increase in PRL-1/-2 expression is regulated post-transcriptionally.

Still, in MDA-MB-231 PRL-2 uORF-targeted cells, we observed no conclusive changes in 2D proliferation compared to the control cells using IncuCyte (Figure 10).

AMPK/mTOR2 Involved in the Regulation of Magnesium-Dependent Expression of PRL-1/-2

In parallel, we were also interested in uncovering the upstream signaling pathway regulating magnesium-dependent regulation of PRL-1/-2. We first observed that upon magnesium-depletion treatment, there is an increased phosphorylation of AMPK, suggesting an increase in AMPK activity (Figure 11A). Since AMPK is a well-known sensor of energy, we were interested to test whether AMPK is involved upstream of PRL-1/-2 magnesium-dependent regulation. Using the CRISPR-Cas9 system, we targeted the catalytic alpha subunit of AMPK using two different sgRNAs across three breast cancer lines, MDA-MB-231, MCF-7, and BT474. In these KO cells, we observed that there was a reduced upregulation of PRL-1/-2 expression to magnesium-

depletion (Figure 11B-D). This indicated that AMPK is an upstream regulator of PRL-1/-2 protein expression in hypomagnesaemic condition.

Since a well-known downstream target of AMPK is the mammalian target-of-rapamycin (mTOR), we wanted to assess whether either mTORC1 or mTORC2 could be involved in the magnesium-dependent regulation of PRL-1/-2. We cultured the MCF-7 cell line with either mTORC1/2 inhibitor PP242 or mTORC1/2 inhibitor Torin1 or mTORC1-specific inhibitor rapamycin for 24 hours under standard or magnesium-depleted conditions. We observed that only PP242 and Torin1 were able to abolish the magnesium-dependent upregulation of PRL-1/-2 (Figure 12A). Furthermore, we showed that PP242 treatment was also able to reduce PRL-1/-2 upregulation in breast cancer cell lines BT474 and MDA-MB-231 (Figure 12B). Taken together, it appeared that AMPK and mTORC2 might be mechanistically linked to the magnesium-dependent upregulation of PRL-1/-2.

Chapter 4: Discussion

Homeostasis of intracellular magnesium is achieved by regulating expression of various proteins, including magnesium transporters. The PRLs subfamily of protein phosphatases has previously been shown to respond to changes in extracellular magnesium levels.[30] In this study, we modulated intracellular magnesium levels by overexpressing and knocking out the essential magnesium channel TRPM7. Overexpression of TRPM7 led to decreased expression of PRL-1/-2 while knockout of the channel led to increased expression of PRL-1/-2. This confirmed that PRL-1/-2 expression is regulated in a magnesium-dependent manner.

Interestingly, there was a recent report of magnesium-dependent regulation of PRL-1/-2 by STAT1 at the transcriptional level in HeLa cells [58]. In our hands however, treatment with transcriptional inhibitor actinomycin D was unable to abolish this magnesium-dependent upregulation, suggesting that the regulation is post-transcriptional. Furthermore, we used the STAT1 inhibitor described in the discussed paper. While we observed a reduction in the magnesium-dependent regulation of PRL-1/-2 at 24 hours, we did not see any differences at the four-hours timepoint where PRL-1/-2 protein expression was clearly seen to be upregulated (Hardy S. and Tremblay ML., unpublished results). Hence, it seemed that the acute response of PRL-1/-2 upregulation by magnesium is post-transcriptional, and that there could be an activation of transcriptional reprogramming at later timepoints.

It was also reported that PRL-3 protein stability is regulated by the ubiquitin-proteasome system.[59] When we tested the effect of the proteasome inhibitor MG132 under standard and magnesium-depleted conditions to assess whether magnesium affected the proteasomal degradation of PRL-1/-2, we observed that PRL degradation did not play a major role in the magnesium-dependent

regulation. Taken together, our data strongly supported that the acute magnesium-dependent regulation of PRL-1/-2 is post-transcriptional and is independent of proteasomal degradation.

Like many other translationally-regulated proteins, PRL-1/-2 have a very long 5'UTR. PCBP1 was reported to bind to GC-rich motifs found in the UTR to retard PRL-3 mRNA transcript incorporation into polyribosomes under standard magnesium conditions.[23] Initial data from an *in vitro* luciferase assay suggested that the 5' untranslated region was associated with the magnesium-dependent regulation of PRL-2; the 3'UTR, on the other hand, did not seem to be involved in this regulation (Hardy S. and Tremblay ML., unpublished results). When we overexpressed PRL constructs with and without the 5'UTR, we observed that the presence of the 5'UTR decreased the expression of PRL-2 but allowed the latter to respond to magnesium levels. This confirmed that the presence of the 5'UTR represses the translation at the main coding sequence but has a pivotal role for the "sensing" of magnesium. In addition, since the PRL-2 (C101S) mutant construct, which is unable to bind CNNM[5], behave similarly, it also suggested that the binding to CNNM did not affect the ability of PRL-2 to respond to magnesium levels. In line with this, when we measured the total intracellular magnesium content in these cells, we were unable to see significant differences. Still, we cannot exclude that free intracellular magnesium was not affected.

We identified an upstream AUG in the 5'UTR of PRL-1/-2 which is responsible for magnesium-dependent regulation of PRL-1/-2. Ribosome profiling data under standard conditions revealed an accumulation of ribosomes towards the end of this uAUG, suggesting that the latter encode a upstream open reading (uORF).[60] Furthermore, this uORF is highly conserved across different organisms and between PRL-1 and PRL-2. When this region was targeted by CRISPR, there was increased PRL expression and reduced magnesium-dependent upregulation. We speculate that under standard conditions, ribosomes likely initiate at this first uORF and become stalled by some

unknown mechanism, leading to a decreased availability of ribosomes to initiate at the main PRL coding sequence. When the uORF region is targeted by CRISPR, ribosomes are no longer able to initiate at the uORF, and instead more ribosomes are available for initiation at the main coding sequence. It is clear then that the uORF has a strong inhibitory effect on PRL expression, as has shown for many other proteins.[61]

The importance of the uORF in the response of PRL-1/-2 to magnesium modulation is perhaps harder to explain. There is the possibility that the disruption of the uORF simply confers sufficient PRL levels to sustain an acute response to magnesium deprivation. However, given that PRL-1 still responds to magnesium upregulation in PRL-2 uORF targeted cells and that PRL-2 still responds in PRL-1 uORF targeted cells, it suggested that the uORF might play a more direct role in the sensing of magnesium concentration. First, it is possible that magnesium promotes the skipping of uORF initiation, thus allowing more ribosomes to initiate at the main PRL-coding sequence. For instance, the uORF in PRL-1/-2 could play a similar role to the two uORFs found in TRPM7 where it has been proposed that the first uORF inhibited the overall translation of the main coding sequence while the second uORF conferred regulation in response to changes in magnesium levels.[41] Another possibility is that under magnesium deprivation, instead of being stalled, the ribosome is able to complete the translation of the uORF and will release a regulatory short peptide into the cellular environment. Ribosomes will then continue to scan and reinitiate at the main PRL-coding sequence. Given the high sequence conservation of this uORF across different species, it would be possible to imagine that the uORF not only plays an inhibitory role on PRL expression, but also plays a role as a cis-regulatory element. In *Salmonella*, a magnesium regulatory peptide MgtR has been described to interact and modulate the protein expression of the MgtA transporter.[62] It would be interesting to study whether the PRL-1/-2 uORF peptide could exist to modulate magnesium homeostasis potentially via the PRL/CNNM complex.

So far, we have provided strong evidence that the magnesium-dependent upregulation of PRL-1/-2 is regulated at the level of mRNA translation, specifically linked to the 5'UTR. It was then interesting to investigate the upstream signalling cascade regulating this magnesium response. Giving the critical role of magnesium in various biosynthetic processes, we were able to identify AMPK and mTORC2 as upstream factors. We thus propose the following mechanism for the upregulation of PRL-1/-2 in response to magnesium depletion (Figure 13). AMPK is an energy sensor which is activated via phosphorylation when the ratio of AMP or ADP to ATP is high.[63] In the cell, ATP is bound to magnesium and the cation stabilizes the negative charges of the phosphate moiety of ATP.[25] It has been shown that incubation in magnesium-depleted medium leads to decreased ATP levels.[27] Hence, we believe that upon magnesium depletion, there is a decrease in magnesium-ATP. Subsequently, this leads to higher levels of AMP and activation of AMPK via phosphorylation, which we observed. A well-known target of AMPK is the mTOR pathway. mTORC1 has been well described to be inhibited by AMPK activation and leads to lower phosphorylation of its downstream target S6 ribosomal protein. The link between AMPK and mTORC2 is less clear. One report suggested that AMPK can upregulate mTORC2 activity.[64] There is also a more complex regulation involving Akt, which is both a downstream target of mTORC2 and is an upstream regulator of mTORC1 activity[65, 66]. Thus, we believe that AMPK activation leads to mTORC2 activation, either directly by AMPK or by a feedback mechanism through the indirect inhibition of mTORC1, which leads to PRL-1/-2 upregulation in condition of magnesium deprivation.

Overall, we showed that the control of PRL expression involves a regulatory uORF. Furthermore, this cis-regulatory element is involved in the sensing of magnesium levels in which participate an AMPK/mTORC2 dependent pathway to upregulate PRL-1/-2 protein levels under hypomagnesaemic condition. We have previously characterized the role of PRL-1/-2 in

proliferation[5] and in metabolic reprogramming[67]. Acute regulation of PRL-1/-2 expression at the translational level is a cellular response to re-establish magnesium homeostasis. Defining this magnesium-dependent regulation thus provides insight in understanding the physiological role of PRLs and their implication in progression of cancer with more aggressive phenotype.

Chapter 5: Conclusion

In our study, we investigated the mechanism behind the regulation of PRLs under hypomagnesaemic conditions. We demonstrate that intracellular magnesium level is capable of modulating PRL-1/-2 protein expression and that this magnesium-dependent regulation is post-transcriptional. We identified a conserved uORF in the 5'untranslated region of PRL-1/-2 which mediates the response of PRL to magnesium-dependent regulation. In cells with CRISPR-Cas9 targeting of uORF, there is higher expression of PRLs and decreased response to magnesium-dependent regulation. It also appears that AMPK and mTORC2 are implicated upstream of this regulation. We believe that this study provides a first insight into how the upregulation of PRLs promote cancer progression by providing a survival advantage in stringent magnesium-deprived growth environment.

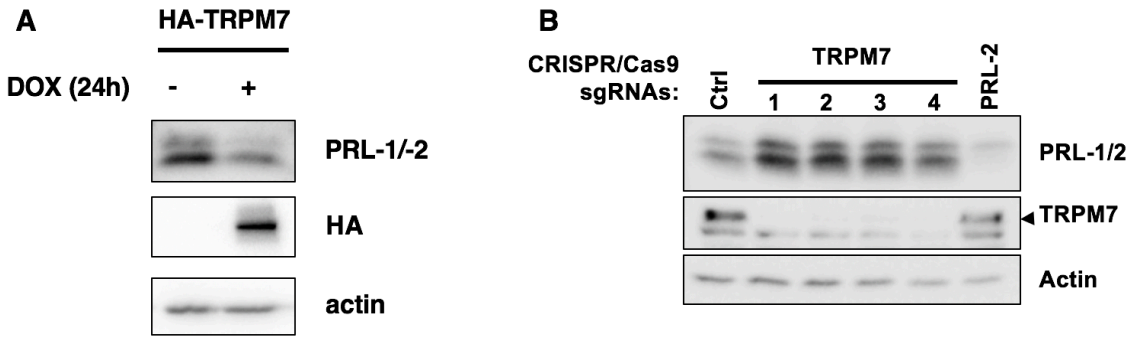

Figure 4. PRL-1/-2 expression is regulated by the magnesium channel TRPM7.

(A) Immunoblot analysis of dox-inducible HA-TRPM7 HEK293 cells with antibodies against PRL-1/-2, HA, and actin. Addition of dox induced the overexpression of HA-TRPM7 and led to decreased PRL-1/-2 expression. (B) Immunoblot analysis of TRPM7 knockout dox-inducible HA-TRPM7 HEK293 cells with antibodies against PRL-1/-2, TRPM7, and actin. HA-TRPM7 were first infected with either TRPM7 or PRL-2 sgRNA. Then, dox was added to induce HA-TRPM7 overexpression. Cells expressing TRPM7 sgRNA have increased PRL-1/-2 protein levels.

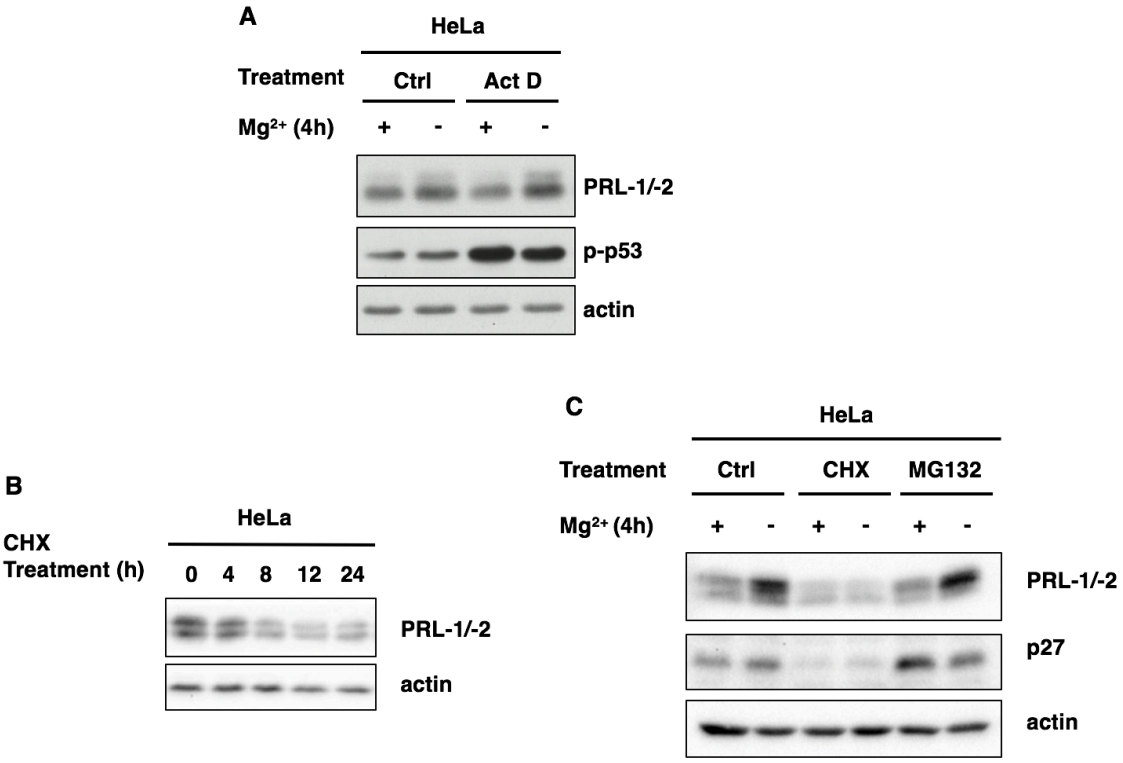

Figure 5. Magnesium-dependent upregulation of PRL-1/-2 is post-transcriptional.

(A) Immunoblot analysis of HeLa cells treated with transcriptional inhibitor Actinomycin D (50 mM) or DMSO under standard and magnesium-depleted conditions with antibodies against PRL-1/-2, p-p53, and actin. Actinomycin D treatment did not abolish the magnesium-dependent upregulation of PRL-1/-2. (B) Cycloheximide pulse experiment of PRL-1/-2 protein stability over 24 hours. PRL-1/-2 protein expression decreased starting at 8 hours. (C) Immunoblot analysis of HeLa cells treated with translational inhibitor cycloheximide (50 ug/mL) and proteasome inhibitor MG132 (5uM) under standard and magnesium-depleted conditions with antibodies against PRL-1/-2, p27, and actin. MG132 treatment did not affect the level of PRL-1/-2 under standard or magnesium-depleted conditions.

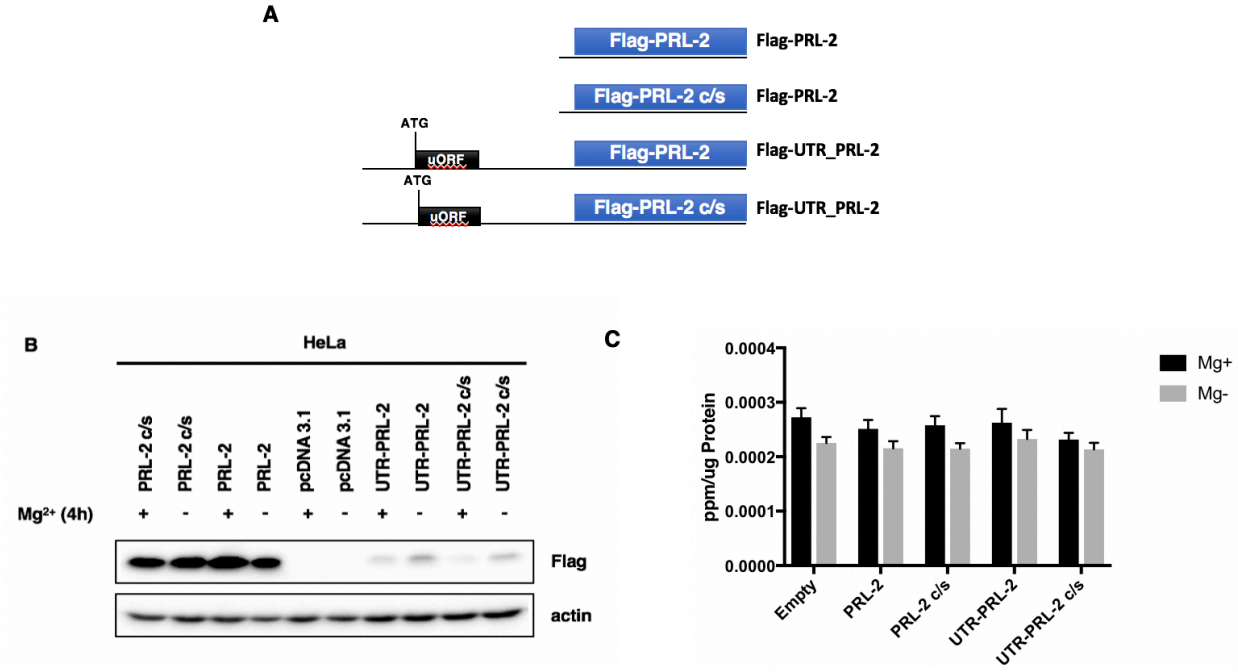

Figure 6. Presence of 5'UTR regulates PRL-2 expression in a magnesium-dependant manner.

(A) Different Flag-PRL-2 constructs were transiently transfected in HeLa cells followed by magnesium treatment for 6 hours. (B) Immunoblot analysis of Flag-PRL-2 expression. Flag-PRL-2 protein levels was lower in constructs with the UTR. Only constructs with the UTR responded to conditions of magnesium depletion. (C) Total intracellular magnesium concentration of transiently transfected HeLa cells. Samples were digested in 0.5 ml nitric acid at 100°C for 1 hours followed by digestion in 0.5 ml of 30% hydrogen peroxide at 100°C for 30 minutes. Samples were then diluted to a final concentration of 5% nitric acid and analyzed with ICP-OES. Magnesium concentration was normalized to total protein concentration. No significant differences are observed.

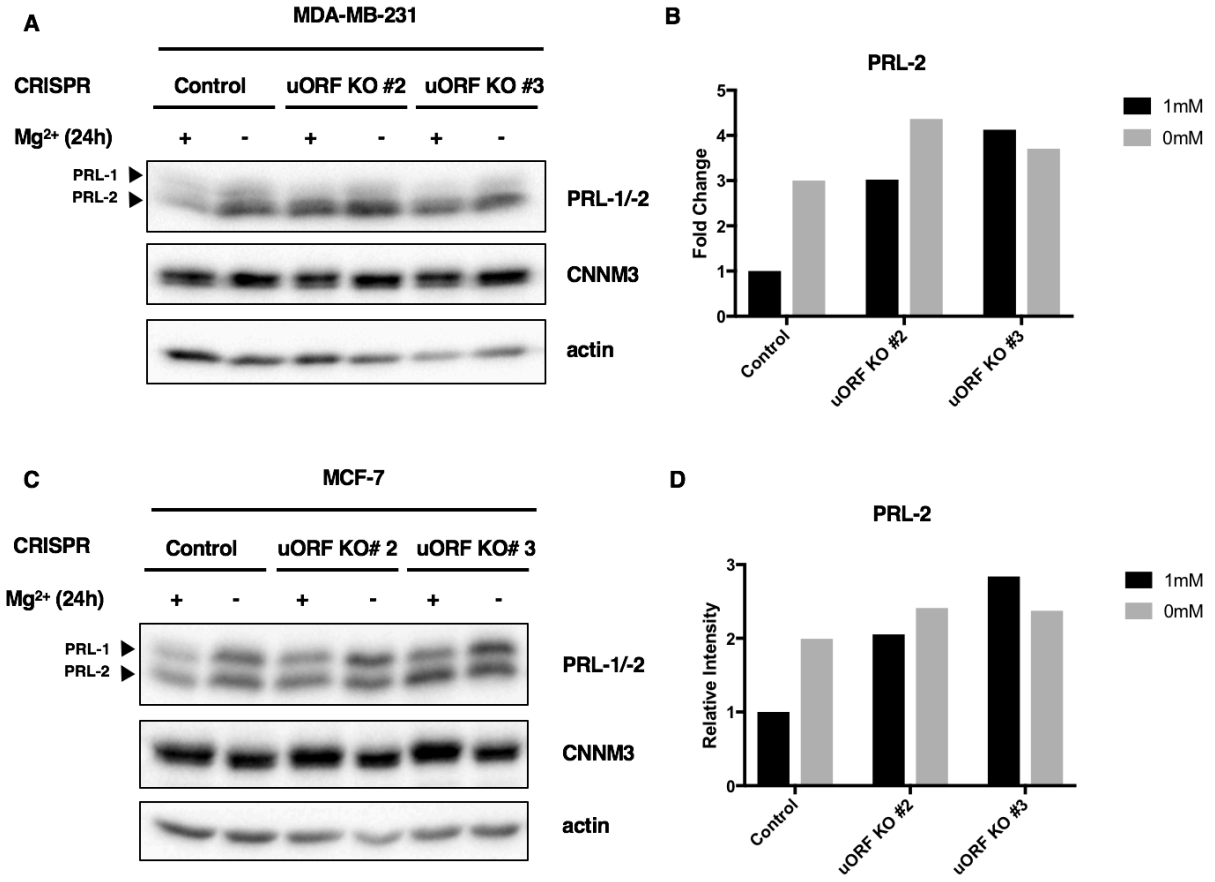

Figure 7. Effect of PRL-2 uORF targeting by CRISPR/Cas9 on PRL-2 protein levels.

From ribosome-sequencing and conservation data, two sgRNA against the uORF region were designed. sgRNA were infected in MDA-MB-231 and MCF-7 cells and these cells were treated under standard or magnesium-depletion conditions for 24 hours. (A) Immunoblot analysis of MDA-MB-231 PRL-2 uORF targeted cells with antibodies against PRL-1/-2, CNNM3, and actin. uORF-targeted cells showed increased PRL-2 expression and reduced magnesium-dependent upregulation. (B) Quantification of immunoblot normalized to actin loading control and control expression. (C) Immunoblot analysis of MCF-7 PRL-2 uORF targeted cells with antibodies against PRL-1/-2, CNNM3, and actin. (D) Quantification of immunoblot normalized to actin loading control and control expression. Similar results were observed in MCF-7.

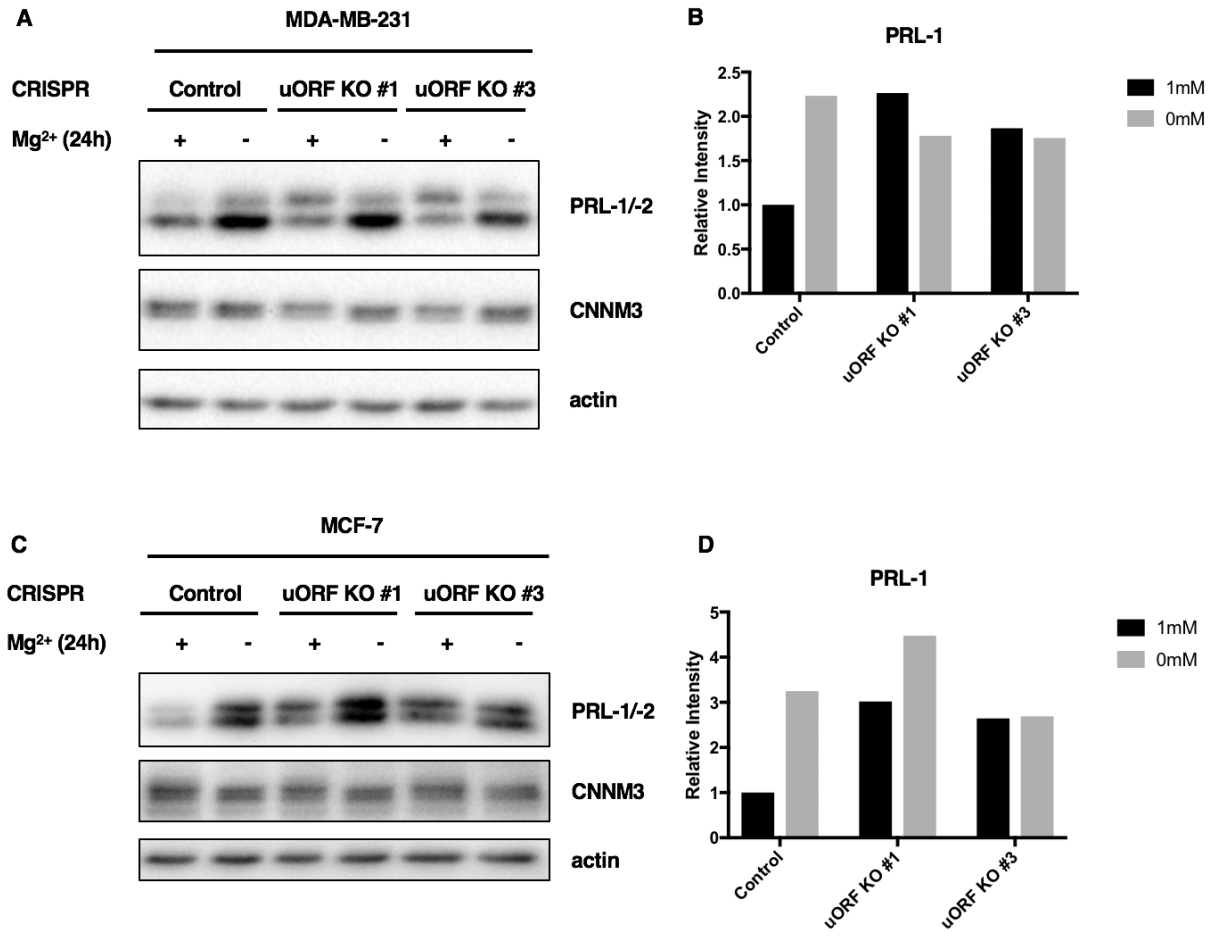

Figure 8. Effect of PRL-1 uORF targeting by CRISPR/Cas9 on PRL-1 protein levels.

From ribosome-sequencing and conservation data, two sgRNA against the uORF region were designed. sgRNA were infected in MDA-MB-231 and MCF-7 cells and these cells were treated under standard or magnesium-depletion conditions for 24 hours. (A) Immunoblot analysis of MDA-MB-231 PRL-2 uORF targeted cells with antibodies against PRL-1/-2, CNNM3, and actin. uORF-targeted cells show increased PRL-1 expression and reduced magnesium-dependent upregulation. (B) Quantification of immunoblot normalized to actin loading control and control expression. (C) Immunoblot analysis of MCF-7 PRL-2 uORF targeted cells with antibodies against PRL-1/-2, CNNM3, and actin. (D) Quantification of immunoblot normalized to actin loading control and control expression. Similar results were observed in MCF-7.

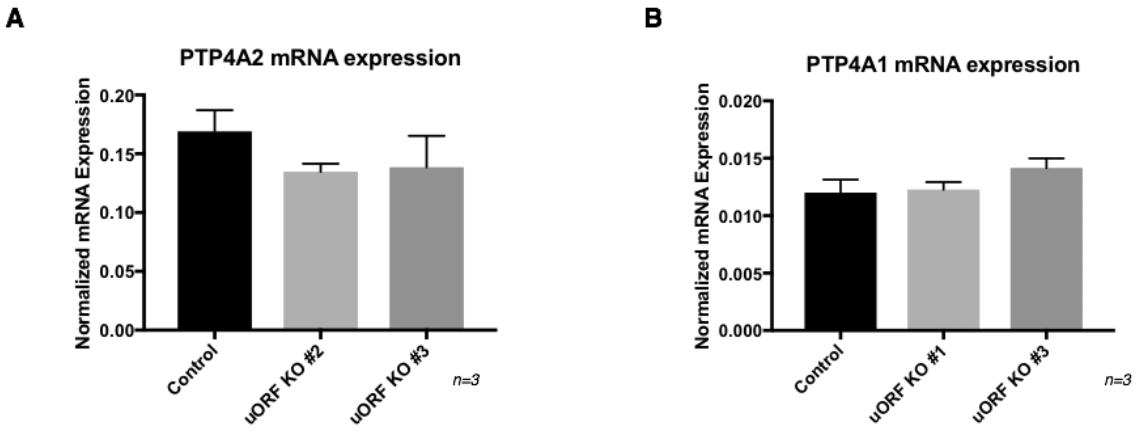

Figure 9. Effect of PRL-1/-2 uORF targeting by CRISPR/cas9 on PRL-1/-2 mRNA expression.

(A) *PTP4A2* mRNA expression of MDA-MB-231 PRL-2 uORF targeted cells under standard condition from three independent experiments. (B) *PTP4A1* mRNA expression of MDA-MB-231 PRL-1 uORF target cells under standard condition from three independent experiments. No significant differences in mRNA levels were observed.

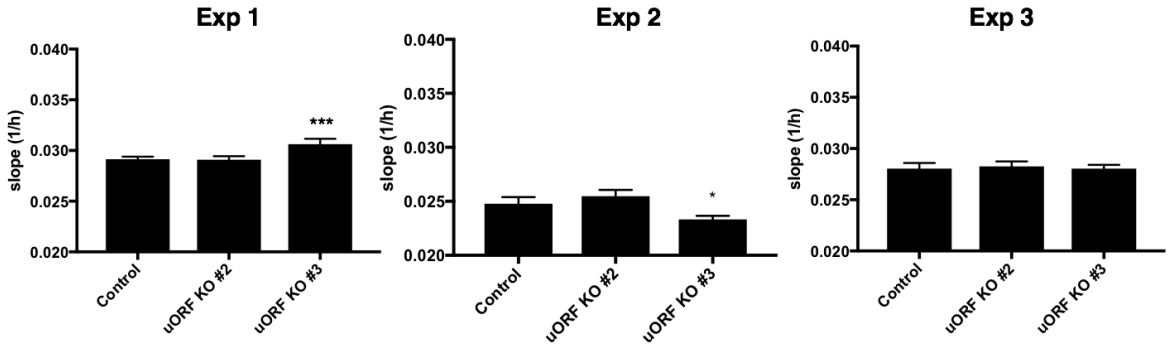

Figure 10. uORF targeted cells shows no 2D proliferative advantage.

Slope of the proliferative curve analyzed using IncuCyte ZOOM integrated software showed no conclusive differences across three independent experiments. One-way ANOVA statistical analysis was performed. *$p<0.05$, *** $p<0.0001$

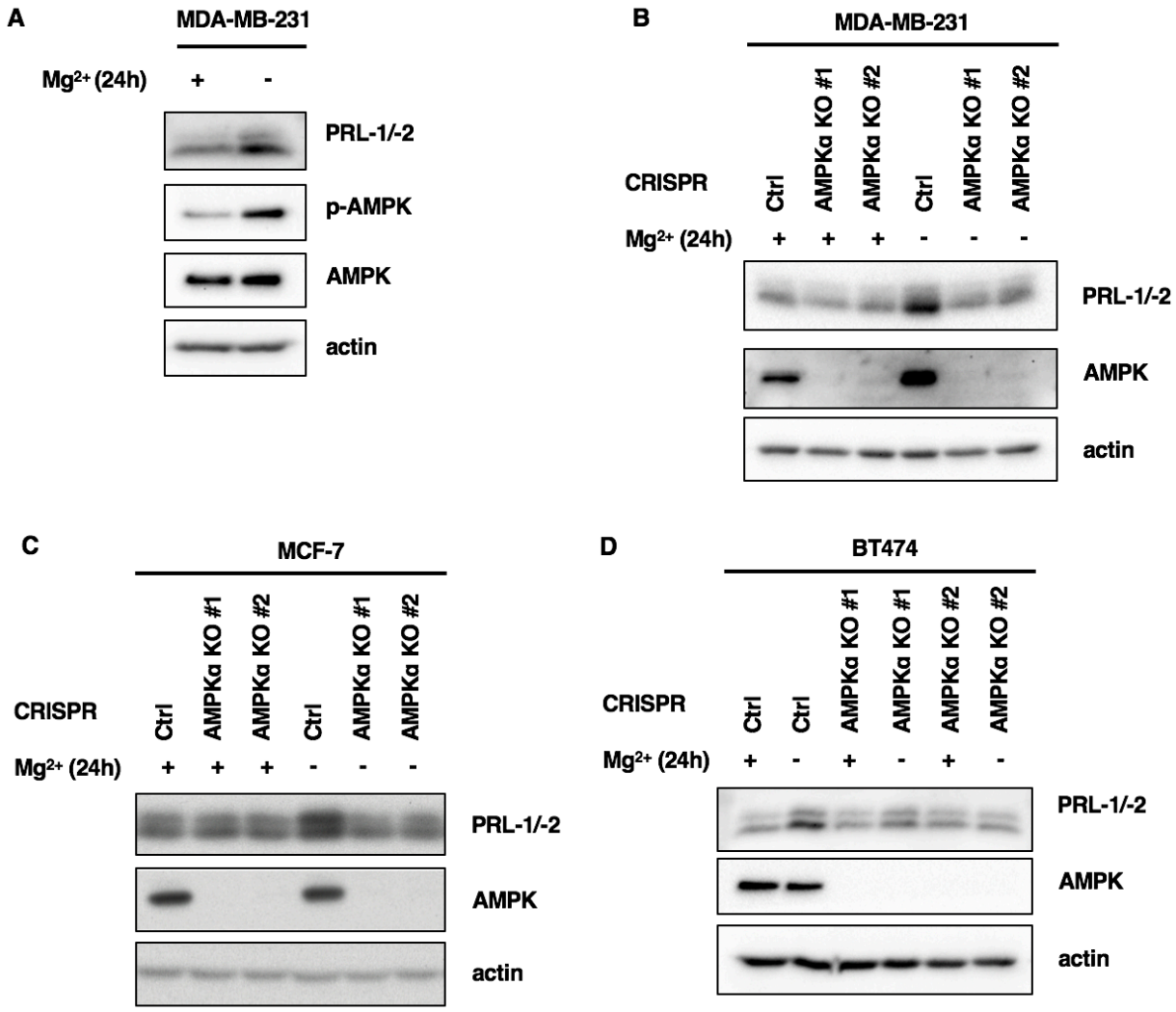

Figure 11. AMPK is implicated in the magnesium-dependent regulation of PRL-1/-2.

(A) Immunoblot of MDA-MB-231 cells treated with magnesium-depletion for 24 hours with antibodies against PRL-1/-2, p-AMPK, AMPK, and actin. Magnesium depletion resulted in increased phosphorylation of AMPK. Breast cancer cell lines MDA-MB-231 (B), MCF-7 (C), and BT474 (D) were infected with sgRNA against the catalytic subunit of AMPK and treated with standard or magnesium-depleted conditions for 24 hours. KO cells had a reduced PRL-1/-2 upregulation response to magnesium depletion.

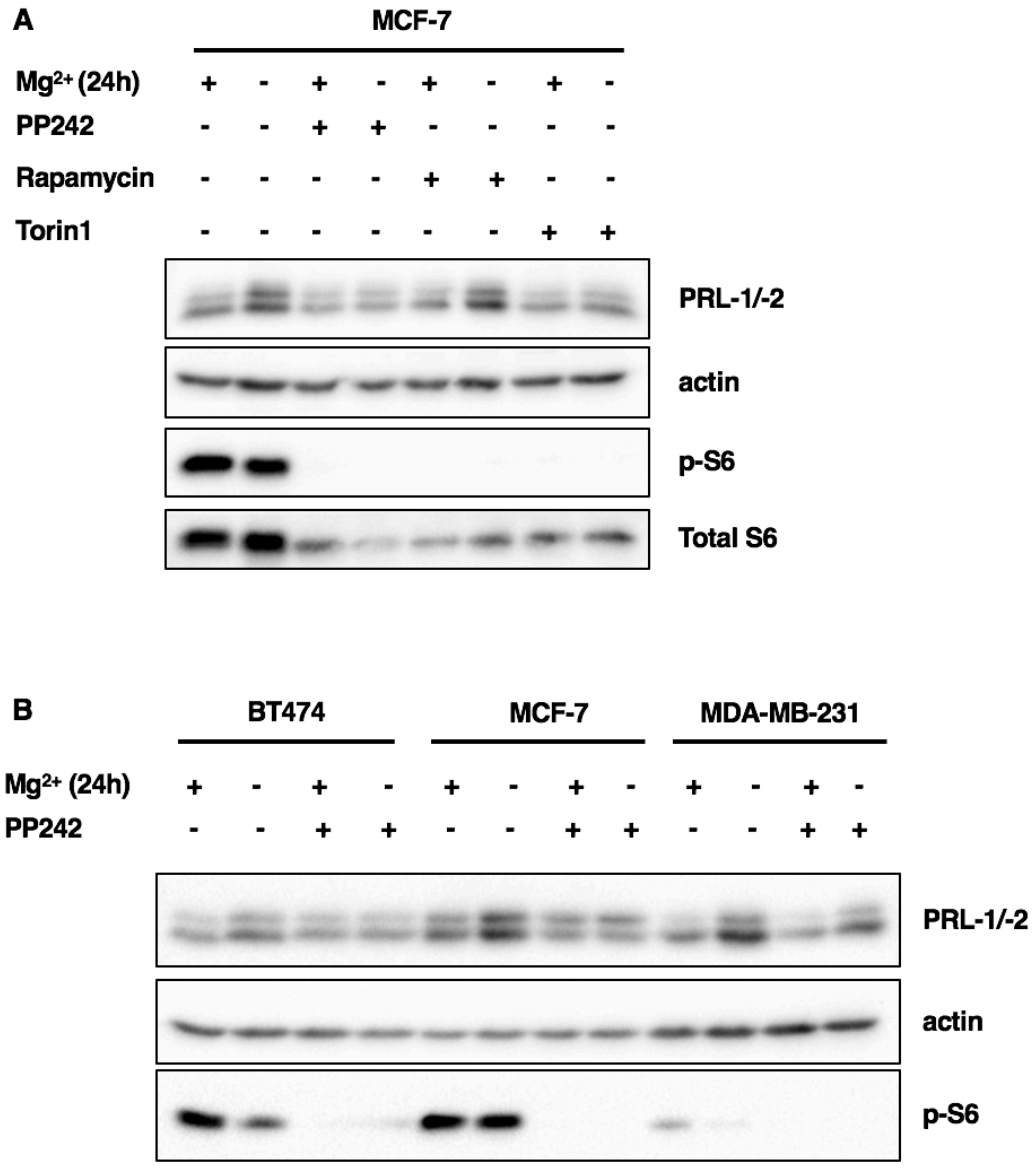

Figure 12. mTORC2 inhibition reduces the magnesium-dependent upregulation of PRL-1/-2.

(A) Immunoblot analysis of MCF-7 treated with mTORC1/2 inhibitor PP242 or Torin1, and mTORC1-selective inhibitor rapamycin for 24 hours under standard and magnesium-depleted conditions with antibodies against PRL-1/-2, p-S6, S6, and actin. Only PP242 and Torin1 were able to abolish the magnesium-dependent upregulation. (B) Immunoblot analysis of BT474, MCF-7 and MDA-MB-231 with showed antibodies against PRL-1/-2, p-S6, and actin. PP242 treatment was able to reduce magnesium-dependent upregulation of PRL-1/-2 in all three different breast cancer cell lines.

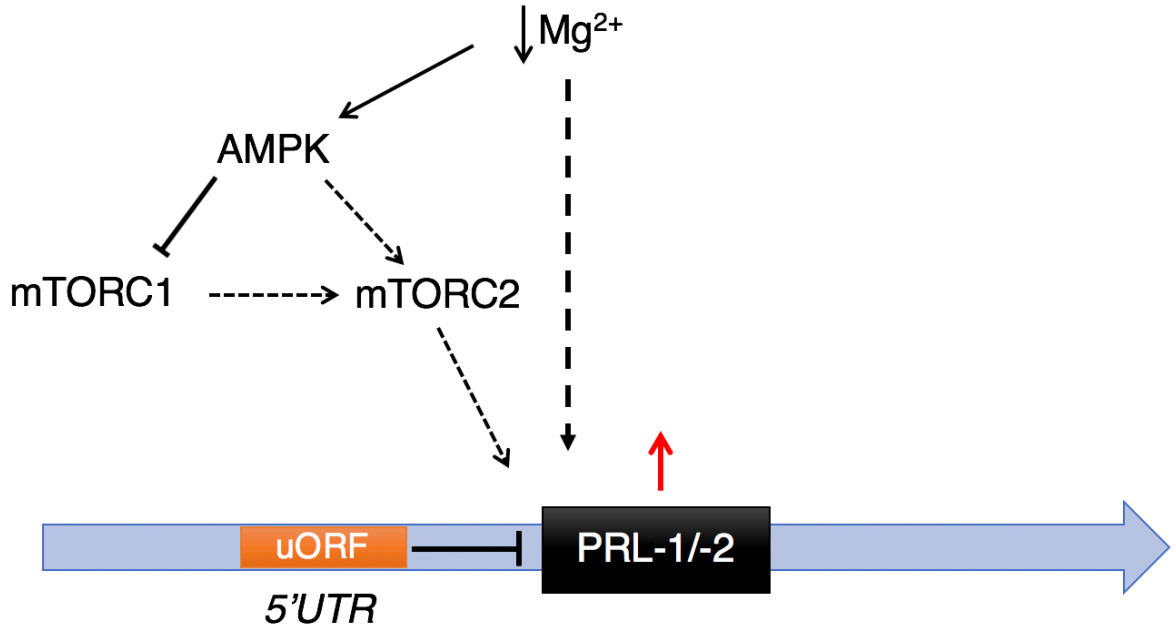

Figure 13. Proposed mechanism of magnesium-dependent upregulation of PRL-1/-2.
When intracellular magnesium decreases, AMPK is activated leading to inhibition of mTORC1. AMPK also leads to induction of mTORC2 activity which results an increase in PRL-1/-2 protein expression. PRL-1/-2 protein expression has also shown to be related to an upstream open reading frame (uORF) in the 5'untranstrated region (5'UTR) which has both an inhibitory effect on PRL expression and appears to convey magnesium sensitivity.

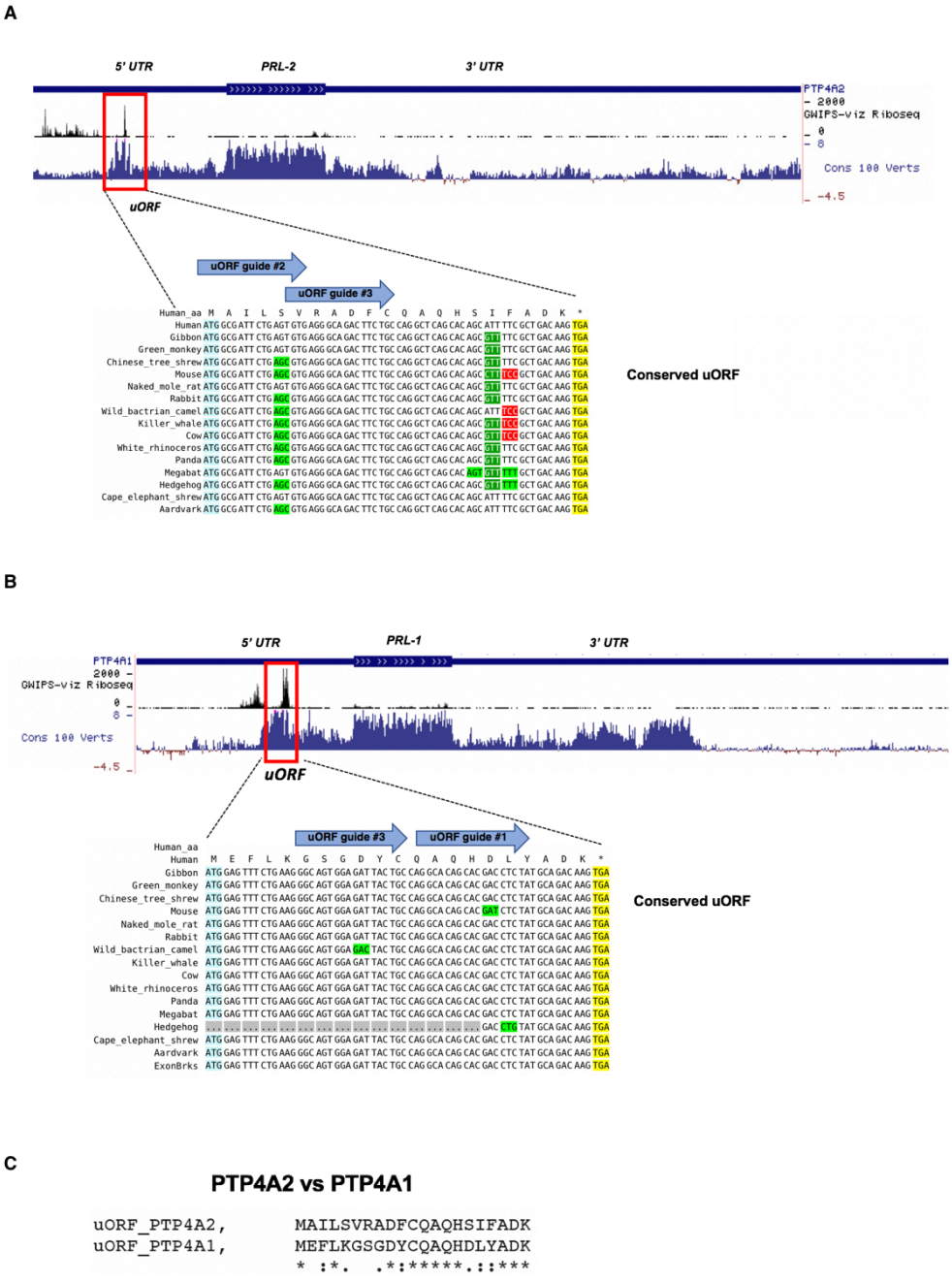

Figure S1. Ribosomes are stalled at the conserved uORF in PRL-1 and PRL-2.

Ribosome-sequencing data were extracted from publicly available data on the GWIPS-viz browser[54, 55] and nucleotide conservation was explored visually using a 100-way vertebrate alignment using phyloP[56] and CodAlignView (I. Jungreis, M. Lin and M. Kellis, manuscript in preparation) for (A) *PTP4A2* and (B) *PTP4A1* (C) Conservation of the putative peptide encoded by the uORFs was performed in ClustalW2[68].

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