CHARACTERIZATION OF MAM301 IN FISSION YEAST

Bachelor thesis Medical Laboratory Science

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Preface

This thesis was written as the final part of my bachelor's degree in Biomedical laboratory science at NTNU Trondheim, Faculty of Natural Sciences (NV), in the period between March 11th and June 11th in 2022. The project was executed in Dr. Amanda Bird's lab at the Ohio State University, departments of Human Nutrition and Molecular Genetics. The Bird Lab's primary focus is gaining a better understanding of the mechanisms that maintain metal homeostasis.

I want to give a huge thanks to my supervisor at Ohio State University, Dr. Amanda Bird, both for taking me in, and for being an amazing supervisor and teacher. Thank you for your outstanding patience both with answering all my questions even if I asked several times, and whenever I did something the wrong way. I also want to give my thanks to the students in the lab; Andy, Derek, Sarah, and Michele for helping me out whenever I needed it and for great company both in and outside of the lab.

I also want to give my thanks to Liv Thommesen as my supervisor from NTNU back in Norway, who has been a part of the writing process, for keeping me in line and reeling me in. Another thanks to Kristin Solum Steinsbekk and Patrice Hamel for making it possible to go overseas to do this project. None of this would be possible without you, and I hope you will keep up the good work so more students get the chance.

A huge thanks to all of you, and everyone else I've met here, for making my time at OSU, and in Columbus, one of the highlights of my life!

June 10th 2022, Columbus Ohio

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Abstract

Background: Magnesium is an essential ion that is a cofactor for many important proteins and enzymes in the body. It also functions as a counterion for negative ions and thus helps stabilize many important molecules. Although magnesium is vital for life, in excess, magnesium can be toxic and can lead to muscular paralysis and respiratory failure. In humans, genetic mutations in CNNM2 and CNNM4 interfere with magnesium homeostasis and cause the genetic diseases familial hypomagnesemia and Jalili syndrome. It is currently not clear if this protein transport or sense magnesium. The objective of this study was to gain new insight into the function of these proteins by characterizing the homologous gene Mam301 in *Schizosaccharomyces pombe.*

Methods: To investigate the function of Mam301 from fission yeast*,* the Mam301 protein was tagged with GFP (green fluorescent protein) to visualize the protein by microscopy and western blot analysis. To determine if Mam301 is important for growth in high or low magnesium, a series of drop tests were performed with wild-type and *mam301* deletion cells. Western blot analysis was used to determine if Mam301 protein levels were dependent upon magnesium ion concentration. Additional experiments also examined the growth and regulatory function of Cis4 and Zrg17, two zinc transporters that are required for growth in high magnesium.

Results: The drop tests showed that Mam301 protects cells from high Mg^{2+} toxicity, and that *cis4* and *zrg17* deletions have higher sensitivity to high magnesium than a *mam301* deletion. The western blot analyses show that Mam301 expression is dependent upon magnesium, and that the *mam301* regulation is impaired in cells containing *cis4* and *zrg17* deletions.

Conclusion: The *mam301* deletion showed growth inhibition with high magnesium, suggesting that Mam301 is important in protecting cells from high Mg^{2+}. Further it was shown that Mam301 levels are regulated by magnesium levels up to 100 mM MgCl2, and that the function of Mam301 is impaired in the *cis4* deletion strain. The latter strengthens the hypothesis that *cis4* and probably *zrg17* have an important regulatory role.

Table of Contents

Preface I

Abstract II

Genes mentioned in this thesis 0

1.0 Introduction 1

1.1 Metal ion homeostasis 1

1.2 Magnesium 2

1.3 Use of fission yeast as a model system 5

1.4 Aims of the study 8

2.0 Material and methods 9

2.1 Model system 9

2.2 Equipment, reagents and primers 9

2.3 GFP tagging the Mam301 gene 9

2.4 Yeast transformations 10

2.5 Drop tests 12

2.6 Crossing Mam301GFP to Δzrg17 and Δcis4 13

2.7 Western blots 15

3.0 Results 17

3.1 Δmam301 cells display growth defect in high magnesium concentration 17

3.2 Generation of a yeast strain expressing Mam301-GFP 18

3.3 The Mam301-GFP is a functional protein 19

3.4 Mam301 levels are regulated by magnesium 20

3.5 Deletion of cis4 and zrg17 leads to growth defect with high magnesium concentrations 22

3.6 Genetic crosses of Mam301-GFP to Δcis4, and Mam301-GFP to Δzrg17 23

3.7 Cis4 is required for magnesium dependent regulation of Mam301 25

4.0 Discussion 26

5.0 Conclusion 30

6.0 References 31

7.0 Appendix 33

Table 7: Strains used in this study 33

Table 8: Primers used in PCR reactions 34

Table 9: PCR programs used in this study 35

Table 10: Reagents used in the study and how they were made 36

Genes mentioned in this thesis

Table 1: An overview of terms for important genes mentioned in this thesis. All S. pombe gene information was found at www.pombase.org, a scientific resource for fission yeast. Information about human genes were found at https://www.uniprot.org/.

Protein name	**Gene name**	**Organism**	**Protein function**
Mam301	*SPCC4B3.03c*	*S. pombe*	Predicted to be important for magnesium homeostasis
Mnr2	*SPAC17A2.14*	*S. pombe*	Vacuolar magnesium transporter, CorA family
Cis4	*SPAC17D4.03c*	*S. pombe*	Golgi zinc importer, CDF family
Zrg17	*SPBC16E9.14c*	*S. pombe*	Golgi zinc importer, CDF family
CNNM4	*KIAA1592*	*Homo sapiens*	Probable magnesium transporter
ZnT5	*SLC30A5*	*Homo sapiens*	Golgi zinc importer, CDF family
ZnT6	*SLC30A6*	*Homo sapiens*	Golgi zinc importer, CDF family

1.0 Introduction

1.1 Metal ion homeostasis

Metal ion homeostasis is defined as a process involving the maintenance of the internal steady state of metal ions within a cell or an organism (1). Essential metal ions including magnesium, zinc, manganese, and iron are necessary for many vital functions in the human body and inside of cells. At the same time, they are toxic in excess (2). This essential but toxic nature of metals is the reason why regulation of ions is important. When metal ions are bound to protein, they can facilitate catalytic reactions and stabilize structural domains by for example neutralizing charge (3). Metal ions can also have specialized functions, such as being modulators of synaptic transmissions and functioning as intracellular secondary messengers (4,5).

Different type of metal ions have different functions in the organism, but one feature they all have in common is that they cannot move across cellular membranes without a specialized transporter or channel. The uptake and distribution of metal ions in the cell is often regulated by controlling the expression of these transporters and other genes important for storage and compartmentalization. Depending on the metal and the organism, gene expression is often controlled by metal-responsive factors which play an important role in maintaining homeostasis by controlling protein activity, transcription, alternative splicing, mRNA- and protein stability (4).

Genetic mutations resulting in defective metal transporters affect the distribution and level of metal ions in a way that can lead to disease (4). High metal levels are toxic for the cells, and too low concentrations will inhibit important cell functions that depends on metal ions (6). One example is excess magnesium concentration in blood, over 15 mg/dL, which may cause muscular paralysis and cardiac or respiratory failure (2). Excess of specific metals may lead to binding of the incorrect metal to proteins making them inactive (4). All of these facts highlight the importance of strictly regulating metal ions levels in the organism.

1.2 Magnesium

Magnesium is the fourth most abundant cation in the body, and intracellularly the concentration ranks second to potassium. Magnesium, which is a divalent cation, has a lot of important functions in the body, both as a cofactor in a lot of proteins and enzymes and as a counter ion for negative charged ions, which helps stabilize many important molecules. This can for an example be seen in how magnesium is helpful in stabilizing ATP (2).

The recommended daily intake of magnesium is 400-420mg for men and 310-320mg for women, with an additional 40mg pr day recommended during pregnancy (7). Good sources for magnesium include green leafy vegetables as spinach and legumes, as well as nuts, seed, whole grain, and potatoes. A noticeable amount is also found in unfiltered tap water and in meats such as salmon, beef, and chicken (2,7).

Most of the magnesium in the body (55-60%) is found in the bone tissue. In bone, magnesium ions are located with the crystal lattice. They are also located on the surface in an amorphous form. The magnesium found on the surface is thought to be extendable magnesium, which can be used if there is lack of magnesium in other places in the body (2,8). Magnesium is also found in extracellular fluids, soft tissues, muscle and in organs such as the kidney and liver. Some magnesium is found in plasma, and normal plasma concentrations are between 0.65-1.05mmol/L (2).

Magnesium is absorbed mostly through the small intestine, though the colon has been found to absorb magnesium in diseases that interferes with uptake in the small intestine (2,9). There are two transport systems for magnesium uptake in the small intestine, one which is an active, tightly regulated transporter that transports the ions through the epithelial cells, and simple diffusion where the ions pass through the small spaces between the cells (2,9).

Paracellular transport, which is the passage of molecules between epithelial cells, is responsible for most of the intestinal magnesium uptake, about 80-90%, and is driven by a high Mg^{2+} concentration in the lumen (9,10). The active transport is connected to Transient Receptor Potential cation channels 6 and 7 (TRPM6 and TRPM7), which are found on the brush border membrane of small intestinal cells. The function of these channels, and therefore magnesium uptake, is inhibited by high intracellular magnesium concentrations

(2,9). The TRPM6 and TRPM7 channels allows Mg^{2+} to enter the cell, and even though the basolateral mechanism is still undiscovered, evidence suggests that it may be dependent of the Na^+ gradient mediated by Na^+/K^+/ATPase (9).

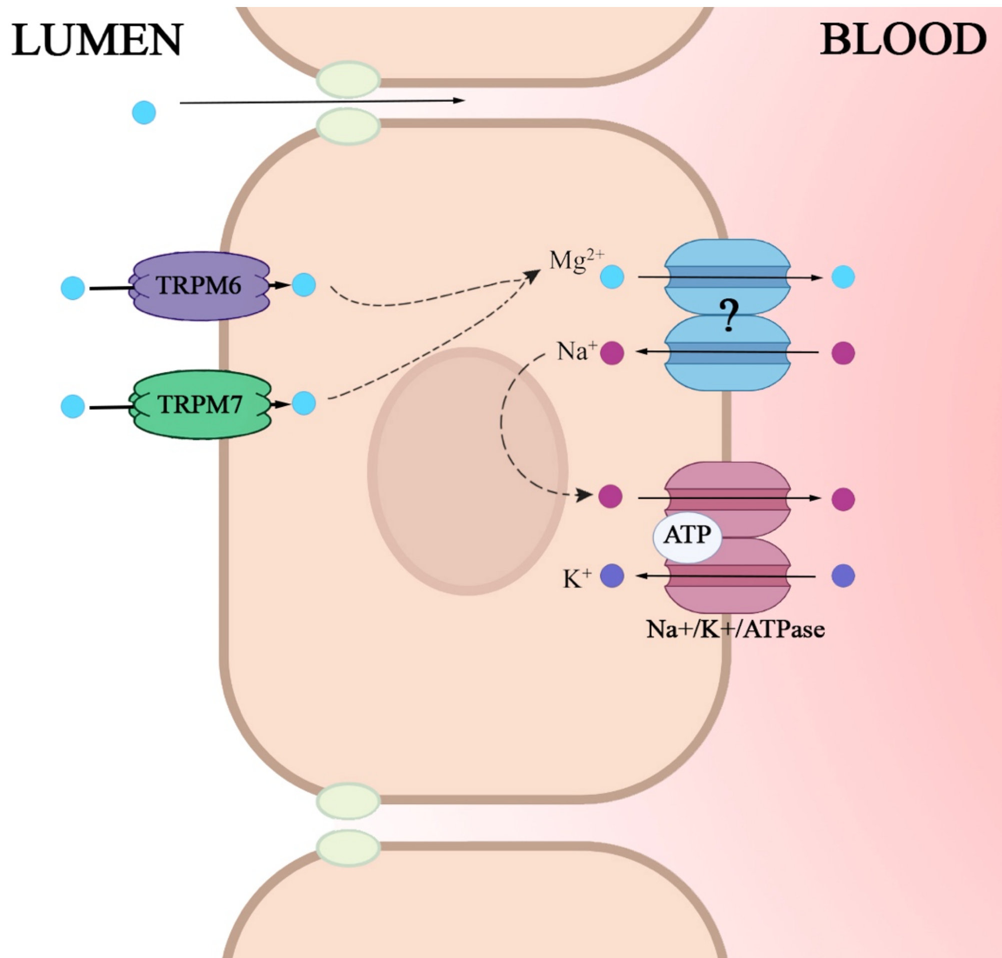

Figure 1: Paracellular and active transport through the intestine. Though the basolateral mechanism is unknown, evidence suggests the Na^+ gradient mediated by Na^+/K^+/ATPase is important (9).

The elimination of magnesium from the body occurs through renal excretion, where approx. 2400mg of magnesium ions are filtered in the glomeruli, and 90-95% are retrieved (9). Most of the reabsorption happens in the loop of Henle, as well as some by paracellular passive transport in the thick ascending limb. The fine tuning of the reabsorption happens in the distal convoluted tubule (9).

In cells magnesium can be found as a part of the cell membrane bound to phospholipids which is thought to be helpful in membrane stabilization (2). It has been noticed that different types of cells handle magnesium differently, and cell types like skeletal muscle, fat tissue, brain tissue and lymphocytes have different rates for the intra- and extracellular exchange of

magnesium (11). Still little is known about cellular magnesium homeostasis, although magnesium transporters belonging to the ancient conserved domain family are thought to have an important role (12,13). Proteins belonging to this family include Mam301 in fission yeast and *CNNM1-4* in humans. Not much is known about the *CNNM1-4* genes, however mutations in *CNNM2* lead to renal hypomagnesemia and mutations in *CNNM4* lead to Jalili syndrome and cone rod dystrophy (14–16).

Mam301, which in the budding yeast *Saccharomyces cerevisiae* is called Mam3, is a magnesium transporter in the vacuole wall (17), and the deletion of this gene (*Δmam3*) leads to a growth defect in high magnesium concentration (17). In fission yeast, there is still much unknown when it comes to Mam301, but deletion of *cis4* and *zrg17* in *S. pombe*, genes encoding zinc transporters that function as a heterodimer (18), leads to increased sensitivity to high magnesium concentrations (19). Interestingly, this may indicate that Cis4 and Zrg17 are important also in magnesium homeostasis.

1.3 Use of fission yeast as a model system

Schizosaccharomyces pombe (S. pombe) is a type of fission yeast that has been widely used in biology laboratories around the world. The name *pombe* comes from the Swahili word for beer, which is a nod to the millet beer from where the yeast was originally isolated (20). *S. pombe* is a rapidly growing eukaryote yeast that is easy to manipulate, making it a valuable model to understand biological processes like for example the function of transporters (20,21).

Although *S. pombe* can be found as either a haploid or diploid it is typically a haploid. The haploid yeast cell is about 4 µm in diameter with a length from 7-14 µm. At 14 µm length the cell enter mitosis and split into two daughter cells at 7 µm each (22). The *S. pombe* genome is 13.8 Mb divided on 3 relatively large chromosomes, with 5054 protein encoding genes (23,24).

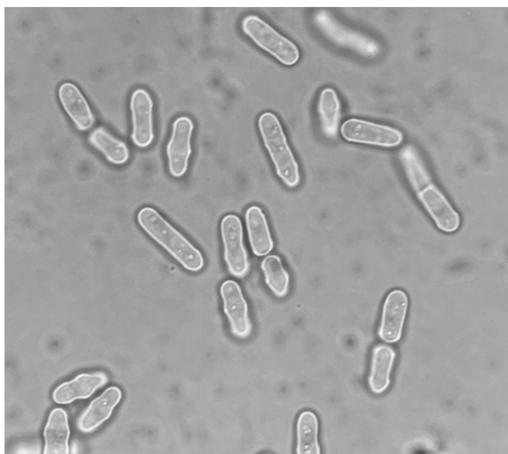

Figure 2: S. pombe yeast cells (100x)

The fact that *S. pombe* usually is found as a haploid makes it easy to cross strains to make strains with new properties. For a genetic cross to be successful two strains of the opposite mating type need to be crossed together. Fission yeast has the mating types h+ and h-, so all crosses include one h- strain and one h+ strain. Most lab strains of fission yeast are haploid cells which have complementing *ade6* alleles, which can be either *ade6-M210* or *ade6-M216*. Haploid cells containing either of these alleles are not able to grow without an adenine supplement. However, when these alleles are combined in a diploid cell, they complement the *ade6* mutation and provide an ade plus phenotype which can be seen by the colonies turning white (23). These alleles can therefore be used to isolate diploids. In an adenine

limiting environment cells without the ade plus will turn bright pink on an adenine negative (-ade) plate.

S. pombe has five known magnesium transporters, three of which have human homologs. An overview of magnesium transporters in *S. pombe* can be seen below in table 2.

Table 2: Known magnesium transporters in S. pombe and their human homologs, family, and function. All gene information for S. pombe genes are found at www.pombase.org.

Protein name	**Gene name**	**Human homolog**	**Protein family**	**Function**
Mam301	SPCC4B3.03c	CNNM4	Ancient conserved domain protein	Implicated in magnesium homeostasis
Mme1	SPCC1442.03	-	Mitochondrial carrier domain superfamily	Mitochondrial carrier
Ost3	SPAPB17E12.11	MAGT1	OST3 / OST6 family	Part of the pathway protein glycosylation
Mnr2	SPAC17A2.14	-	CorA	Transmembrane transporter
	SPBC27B12.12c	-	CorA	Transmembrane transporter
Mrs2	SPBC25H2.08c	MRS2	CorA	Mitochondrial inner membrane transmembrane transporter

In this thesis the focus has been on Mam301 which is predicted to play an important role in magnesium homeostasis (13). In addition, deletion of *cis4/zrg17* leads to high sensitivity to magnesium. Cis4 and Zrg17, and their human homolog ZnT5 and Znt6, are members of the cation diffusion facilitator (CDF) family that form a heteromeric complex that transports zinc ions into the cis-Golgi (18,19). As Cis4 and Zrg17 are required for growth in high magnesium, here hypothesize that Cis4 and Zrg17 also have an important role in magnesium homeostasis.

1.4 Aims of the study

As little is known about cellular magnesium homeostasis, including why mutations in the *CNNM4* gene are linked to several diseases including Jalili syndrome and cone rod dystrophy (14,15). The goal of this thesis was to study the *S. pombe* homolog of the *CNNM4*, which is called Mam301. Given the level of conservation between *CNNM4* and Mam301, we predict that information about Mam301 function in fission yeast will provide insight into *CNNM4* function in humans.

To gain a better understanding of Mam301 function in fission yeast, we followed these aims:

Aim 1: Determine whether Mam301 protects cells from high Mg^{2+} toxicity
Aim 2: Determine if Mam301 expression is dependent upon magnesium
Aim 3: Determine if Mam301 function is impaired in cells containing deletions in the *cis4* and *zrg17* genes

2.0 Material and methods

2.1 Model system

The fission yeast *Schizosaccharomyces pombe* (*S. pombe)* was used as a model system. An overview of the strains used in this study can be found in the appendix (Table 7).

2.2 Equipment, reagents and primers

Reagents and buffers used is shown in the appendix (Table 10). Primers were purchased from Sigma Aldrich (St Louis, Missouri, USA) and the sequences are shown in table 8 in the appendix. Buffers, dNTP's and Taq polymerase used in PCR reactions were purchased from New England biolabs (https://www.neb.com/) and Bio-Rad (https://www.bio-rad.com/).

2.3 GFP tagging the Mam301 gene

To detect the Mam301 protein, a fusion between the Mam301 and GFP (green fluorescence protein) was made. This involved generating a gene fusion where the coding sequence of GFP was fused to the 3' end of the *mam301* open reading frame.

The gene fusion was generated using a PCR (Polymerase chain reaction), where a specific gene sequence is amplified using sequence-specific primers and the enzyme Taq polymerase. The first step was to amplify the genes needed for the fusion, which was done in 2 separate PCR reactions, where PCR A amplified the kanMX6-GFP cassette which contains the GFP and a kanamycin resistant gene, and PCR B amplified the *mam301* 3' UTR. The template for PCR A was the plasmid pFA6aMX6-GFP (25), and the template for reaction B was genomic DNA. When a plasmid template was used it was pipetted into the master mix. When genomic DNA was used it was pipetted into each PCR tube after the master mix had been split. Set up for PCR A and B can be seen below in table 3, the programs can be found in the appendix (table 9).

To purify the products as well as confirm that the correct product the PCR products were loaded into an 1% agarose TAE gel and run at 100V for 25 minutes. The bands with the correct size were then cut out from the gel and purified from the gel using QIAquick PCR Purification Kit following the manufacturer's instructions (https://www.qiagen.com/us/).

The products from PCR A and PCR B were then used to make the overlap PCR AB. As this PCR product was larger in size, a different high fidelity Taq polymerase (iProof from BIORAD). The set up for PCR AB can be seen in table 3, while the PCR program utilized is shown in the appendix (table 9).

Table 3: PCR set up for A, B, and the overlap AB, about Primers can be found in the appendix (table 8).

PCR A	**PCR B**	**PCR AB**
1 µL pFabakanMX6 (10 µM)	1 µL genomic DNA template of *Mam301*	1.25 µL PCR A product 1.25 µL PCR B product
Forward primer: 1 µL Mam3GFPF (10 µM)	Forward primer: 1 µL Mam301UTRF (10 µM)	Forward primer: 1 µL Mam3GFPF
Reverse primer: 1 µL Mam3GFPR (10 µM)	Reverse primer: 1 µL Mam301UTRR (10 µM)	Reverse primer: 1 µL Mam3UTRR
1 µL dNTPs	1 µL dNTPs	1 µL dNTPs
5 µL 10x Thermopol buffer	5 µL 10x Thermopol buffer	5 µL iProof buffer
0.5 µL Taq polymerase	0.5 µL Taq polymerase	0.5 µL iProof Taq polymerase
40.5 µL dH_2O	40.5 µL dH_2O	0.5 µL $MgCl_2$
		34.5 µL dH_2O

The PCR product AB was loaded into a gel and run at 100V for 25 minutes.

2.4 Yeast transformations

The yeast transformation was done by adding 30mL fresh Yeast extract containing 3% glucose and supplements leucine, uracil, and adenine (YE LUA), to a sterile flask along with the wild type (WT) yeast. The cells were grown in a 31°C incubator with shaking at 200rpm for 4-6 hours. The cells were transferred to a sterile 50mL Falcon tube and spun down at 3500rpm for 2 minutes before discarding the supernatant. The cells were accordingly washed with 10mL Lithium Acetate (LiAc) solution, vortexed and spun down again. After the supernatant was discarded the cells were resuspended in the remaining solution. Subsequently 10 µL of the DNA was pipetted into a 1.5mL tube along 60mL of cells before placed in a 31°C incubator.

Sheared salmon sperm DNA (10mg/mL) was boiled for 5 minutes and cooled on ice for 2 minutes before adding 7 µL of the sperm and 350 µL LiAc with 40% polyethylene glycol (PEG) to the sample. The cell suspension was incubated at 31°C for 25 minutes before they were heat shocked at 42°C in a water bath for 25min. Afterwards cells were spun at 4000rpm for two minutes. The supernatant was discarded, and cells resuspended in 400 µL YE LUA. 150 µL of cells were then plated on a YE LUA plate, which was placed in the 31°C incubator overnight.

When the transformed cells had grown for 1-2 days, they were replica plated onto a selective YE G418 agar, including the antibiotic geneticin/G418 (200 mg/ml) which will kill off everything except cells with the kanMX6 cassette, which is a plasmid module which allows for selection of G418 resistant cells (25).

After colonies were formed, they were spread onto a new YE G418 plate by picking different colonies from the original plate and spreading them on the new plate. This was done to keep the cells growing, and to isolate individual G418 resistant colonies.

Some cells from the new plate were added to a microscope slide to check if the transformation was successful and whether the mam301-GFP fusion was localized to the vacuolar membrane. This was done with an Olympus IX71 fluorescence microscope.

The next step was to check if the protein is still functionable, which was done by setting up a drop test with the strains expressing Mam301-GFP and wildtype (JW81) yeast (positive control) and Δ*mam301* (negative control). The ability of JW81, Δ*mam301* and strains expressing Mam301-GFP to grow in high magnesium was tested by plating 10-fold serial dilution onto different places on the plate. All strains were plated on a normal YE LUA plate as well as a YE LUA plate with 200mM $MgCl_2$.

2.5 Drop tests

Drop tests were used for confirming that *Δmam301* was sensitive for high magnesium, to check if the *mam301-GFP* strain was able to grow in high magnesium and to determine whether a deletion of *cis4* and *zrg17* had the same growth defect on high magnesium as deletion of *mam301*.

Drop tests were done by growing up strains in 5 mL media overnight, either YE LUA or Edinburgh Minimal Media (EMM) depending on the conditions the strains should be tested. The day after the cells were diluted; 1:20 with YE LUA or 1:10 with EMM and OD (600nm) assessed with an Agilent 8453 spectrophotometer. Afterwards cells were diluted to an OD_{600} = 1.0 stock, before four sequential tenfold series dilution were made by pipetting 100 µL cells into 900 µL media.

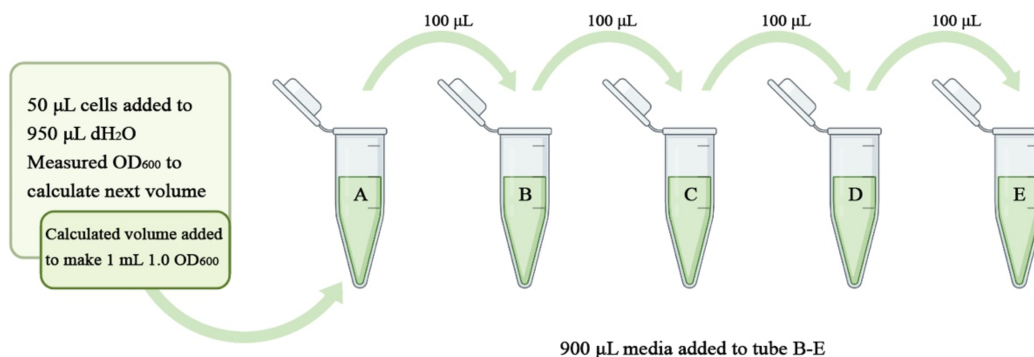

Figure 3: Dilutions for drop tests

The dilutions were then pipetted into plates in small 5 µL drops. After pipetting the plates was left in room temperature for 10-20 minutes to allow the drops to set into the medium before they got placed in a 31°C incubator for 2-4 days.

2.6 Crossing Mam301GFP to Δzrg17 and Δcis4

The crossing of two yeast colonies was done on a nutrient poor plate with malt extract (ME) that makes the two haploids join as a diploid to better survive (23). The crossing was done by spreading a couple colonies of strain 1 in a small circle on the ME plate, then adding the same amount of strain 2 before mixing well with 5 µL dH_2O. The plate was left at room temperature overnight, before streaked to single colonies on an EMM -ade (adenine negative) plate to select for diploids.

The -ade plate was left to grow in the incubator for 3-5 days before a couple white colonies from the -ade plate were suspended in 100 µL dH_2O and 5 µL of the suspension pipetted and streaked onto YE LUA plates. For tetrad dissection analysis four new haploids from the split sporulated diploid were moved to specific marked places on the plate. After 1-3 days in the 31°C incubator, the tetrads were moved over to a new YE LUA as a master plate as well as streaked out to single colonies on a G418 plate and a YE -ade plate to check for resistance and allele type. The G418 plate was utilized to examine if the kanMX6 cassette was present in the colonies. The -ade plate was chosen to select for the *ade6* allele, where their color would reveal their *ade6* allele. When grown on an YE-ade plate *ade6-M210* strains should be light red and *ade6-M216* dark red (23).

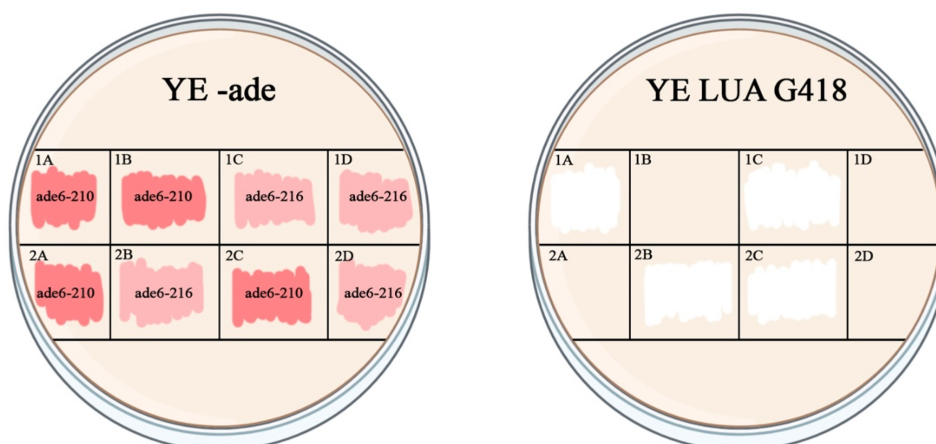

Figure 4: Genetic crosses streaked on YE -ade and G418 plates for selection of allele type and G418 resistance.

To check if the crosses were successful, a diagnostic PCR was performed on genomic DNA isolated from tetrads that looked promising. To extract genomic DNA from selected tetrads, cells were grown overnight in YE LUA before transferring to a 50 mL Falcon tube and spun down for 2 min at 3500rpm. Discarding the supernatant, the cells were transferred to a new 1.5 mL tube before a short spin to remove residual supernatant. The cells were washed with 500 µL dH_2O before a 30 second spin at 12000rpm. After removing 400 µL of dH_2O, the remaining cells were resuspended in 300 µL Blue buffer and 0.3g micro glass beads were added along with 300 µL Phenol/Chloroform (pH 6.5) before the tube was vortexed for 3-5 min. The mixture was then spun down at 12000rpm for 5 minutes and 150 µL of the upper layer was transferred to a new 1.5 mL tube. DNA was precipitated by adding 1 mL of cold ethanol (-20°C) and centrifugation at 12000 rpm for 6 minutes. The supernatant was then discarded, and the tubes pulsed before removing residual supernatant. The pellet was left to dry in an open tube at 37°C for 10-20 minutes. When the pellet was completely dry it was resuspended in 100 µL EB buffer (10mM TRIS pH 8.0). The concentration of DNA in the tube was then determined by measured absorbance at 260/280 nm using a NanoDrop ND-1000 spectrophotometer. Genomic DNA was diluted with dH_2O to a concentration of approximately 1 µg/µl.

Diagnostic PCRs were used to check for the genetic combinations we were searching for. For example, when *mam301-GFP* was crossed to Δ*cis4,* the goal was to isolate a double mutant containing Mam301-GFP and Δ*cis4*. One set of diagnostic PCRs checked for the presence of the Δ*cis4::kanMX6* allele while another checked for the *mam301-GFP* allele. The same method was used for screening tetrad from the *Δzrg17* Mam301-GFP cross. Setup for these diagnostic PCRs (PCR C) is shown in table 4, with the PCR program in the appendix (table 9).

Table 4: PCR setup for identification of successful crosses from the tetrad analysis.

PCR C
1 µL genomic DNA
0.5 µL Forward primer (10 µM)
0.5 µL Reverse primer (10 µM)
0.5 µL dNTPs
2.5 µL 10x Thermopol buffer
0.25 µL Taq polymerase
20 µL dH_2O

2.7 Western blots

To generate protein extracts for western analysis, cells were grown overnight in YE LUA, before 500 µL was inoculated into plastic metal-free tubes containing 5 ml of fresh YE LUA with different magnesium concentrations. These cells were left to grow for 4 hours at 31°C with shaking before cells were harvested by centrifugation at 3500rpm for 2 minutes. The supernatant was then removed, and the cells transferred to a 2 mL tube and kept on ice. A urea protein prep that specifically enriches for membrane proteins (26) was chosen on the on the background that Mam301 is a transmembrane protein (13). The cells were pulsed in the centrifuge to remove any residual supernatant before resuspension in membrane buffer. Further, 0.3g of zirconia beads was added to each tube, and cells disrupted by vortexing for 1x7 minutes with a minimum of 2 minutes on ice between. The supernatant was then transferred to a 1.5 mL tube and left on ice for 2 hours to precipitate membrane proteins.

After two hours the tubes were spun down at 16000g for 10 minutes and the supernatant removed. The pellets were washed with 1 mL of cold 1M NaCl before a 1-minute spin to remove supernatant. To remove all remaining NaCl, tubes were spun briefly in the centrifuge before the remaining supernatant was removed by pipetting. Pellets were resuspended in 100-150 µL of urea buffer with 1% β -Mercaptoethanol. The tubes were mixed for 10 minutes and spun down at 16000g for 1 minute before loading 15 µL of the supernatant onto a 10% SDS_PAGE gel. The gel was run at 170V for 1 hour 5 minutes, before transferring the proteins from the gel to a nitrocellulose membrane (90V for 1 hour 15 minutes).

The nitrocellulose membrane was subsequently incubated with the primary antibody (anti-GFP, 1:1000 dilution in TBST 5% skimmed milk), for either 1 hour at room temperature or overnight in a cold room on a rocking platform. The membrane was washed with TBST 3 times for 5-10 minutes before a 1-hour incubation at room temperature with the secondary antibody (IRDye 680RD Goat anti-rabbit, 1:10000 dilution in TBST 5% skimmed milk). The membrane was washed twice with TBST and once with TBS before scanning with Odyssey Infrared Imaging System (LICOR).

3.0 Results

3.1 *Δmam301* cells display growth defect in high magnesium concentration

To determine whether Mam301 protects cells from high Mg^{2+} a drop test was performed with JW81 (WT), *Δmam301*, and *Δmnr2* cells. Mnr2 is a membrane protein that transports magnesium out of the vacuole and is predicted to release magnesium from vacuolar stores (17). The drop tests were used to examine cell growth with different magnesium conditions. The results are shown in figure 5. When compared to the wildtype JW81 and the *Δmnr2*, the *Δmam301* shows a severe growth defect on high magnesium plates. This result confirms the hypothesis that Mam301 protects cells from high magnesium environments.

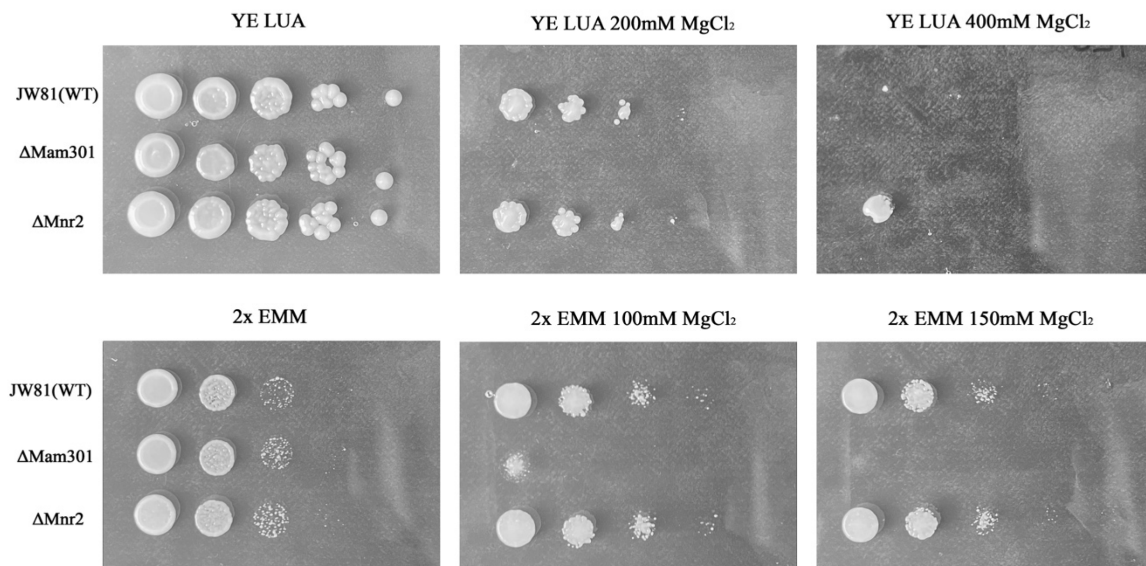

Figure 5: Drop tests performed with ΔMam301 in various Mg concentrations. JW81 and ΔMnr2 was used as controls.

3.2 Generation of a yeast strain expressing Mam301-GFP

To be able to visualize the Mam301 protein a strain was generated expressing Mam301 fused to GFP. This strain was generated by initially using overlap PCR to generate a GFP-kanMX6 cassette flanked by *mam301* gene sequences. The Mam301-GFP-KanMX6 cassette was then introduced into a wild-type yeast by transformation. After isolating G418-resistant colonies, the PCR reaction was performed to identify strains where the Mam301-GFP-KanMX6 cassette had correctly integrated downstream of the *mam301* open reading frame. The PCR reactions used primers that bound to *mam301* gene outside of the Mam301-GFP cassette, and to the GFP open reading frame, which would result in the generation of a 1kb diagnostic PCR product. Figure 6 shows a 1 kb band in 4 different transformants, indicating that these strains all contain a successful integration with the coding sequence for GFP immediately downstream of the *mam301* gene.

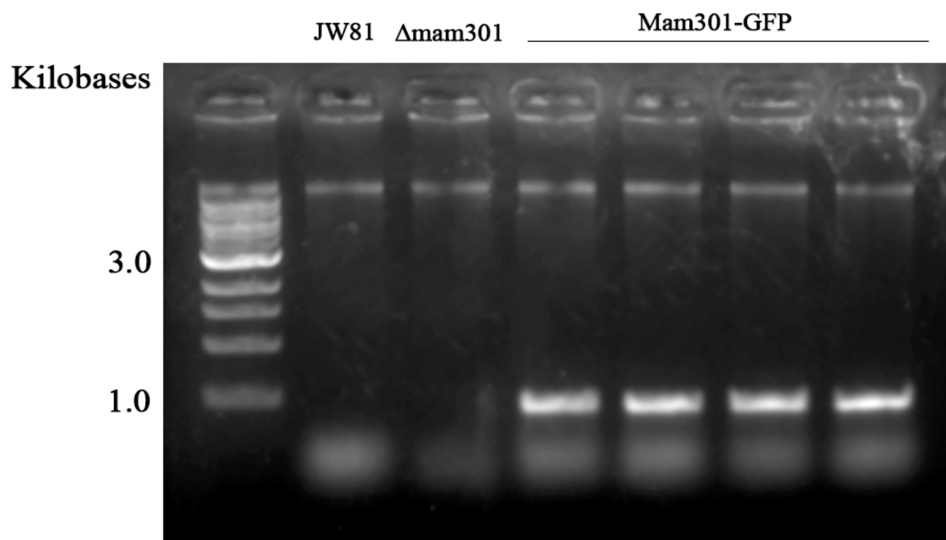

Figure 6: Agarose gel showing four bands of the successful Mam301-GFP fusion (1 kb). PCR reactions using genomic DNA purified from JW81 and Δmam301 were included as negative controls. Bands visualized with ethidium bromide.

The cells were also looked under Olympus IX71 fluorescence microscope which showed the tagged Mam301, this can be seen in figure 7. The figure also confirms the localization of the Mam301 in the vacuole membrane.

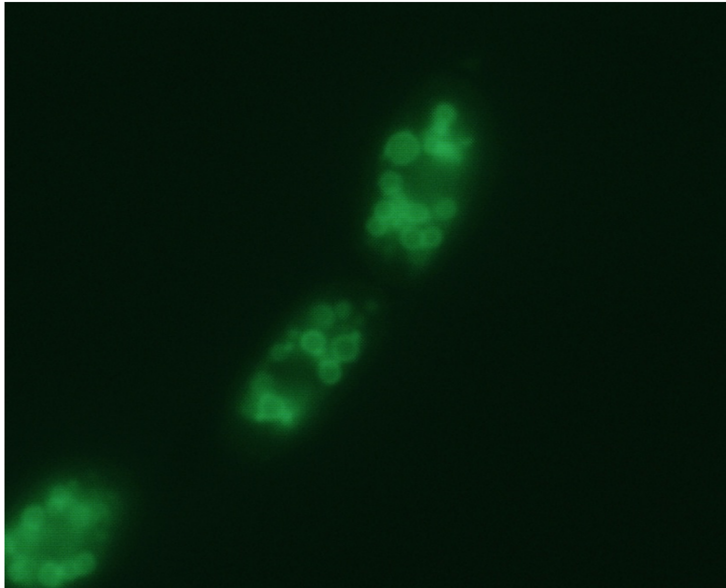

Figure 7: Picture from Olympus IX71 fluorescence microscope showing the tagged Mam301 and confirming Mam301's localization to the vacuole membrane.

3.3 The Mam301-GFP is a functional protein

To determine whether the Mam301-GFP fusion was a functional protein, additional drop tests were performed involving Mam301-GFP and the controls JW81 (WT) and Δ*mam301.* The drop tests were used to examine cell growth from the three strains on YE LUA and YE LUA with 200mM $MgCl_2$. The results are shown in figure 8. JW81 and the Mam301-GFP grows well on both plates, while the *mam301* deletion is struggling on the high magnesium plate. When comparing to Δ*mam301* on the magnesium plate we found that the Mam301-GFP grows as well as the wild-type JW81. This result confirms that the Mam301-GFP protein is functional and can rescue cells on high magnesium.

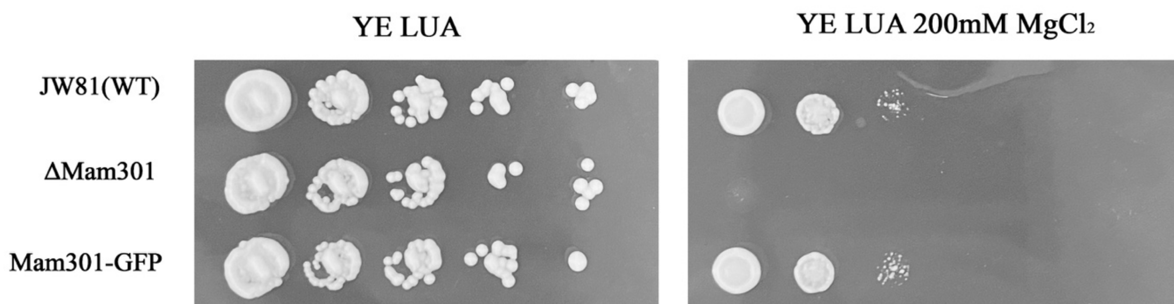

Figure 8: Drop tests performed with Mam301-GFP on two magnesium concentrations. Wild-type strain JW81 and ΔMam301 were used as controls.

3.4 Mam301 levels are regulated by magnesium

To determine whether Mam301 levels were regulated by magnesium, Mam301-GFP cells were grown in increasing magnesium concentration for four hours before proteins were isolated for western analysis. The western blot in figure 9 shows that Mam301 expression is higher with high magnesium concentration, which confirms that Mam301 expression is dependent upon magnesium.

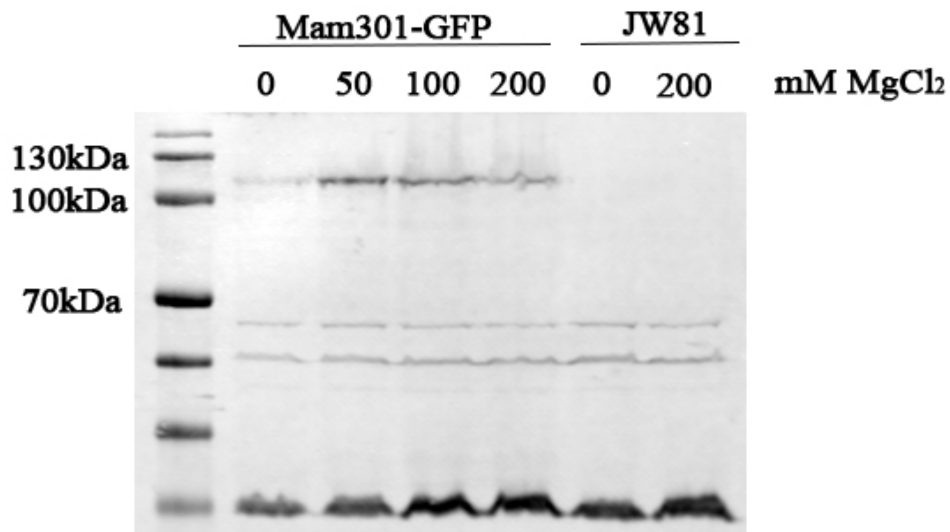

Figure 9: Western blot showing regulation of Mam301 with increasing magnesium concentrations.JW81 is used as control.

While Mam301-GFP abundance increased between 0 and 50, it levels were modestly decreased at 100mM $MgCl_2$, and strongly decreased at 200mM $MgCl_2$. The fact that expression of the Mam301 seems to decrease when it reaches a 100mM $MgCl_2$ and is further decreased at 200mM $MgCl_2$ may indicate that cells are reaching toxic levels of $MgCl_2$. To test this hypothesis an additional western blot analysis was carried out. The result is shown in figure 10, which does not show as clear increasing as figure 9, there is still evident that there is regulation in the increasing magnesium concentration, and expression seems to hit its peak at 40mM $MgCl_2$.

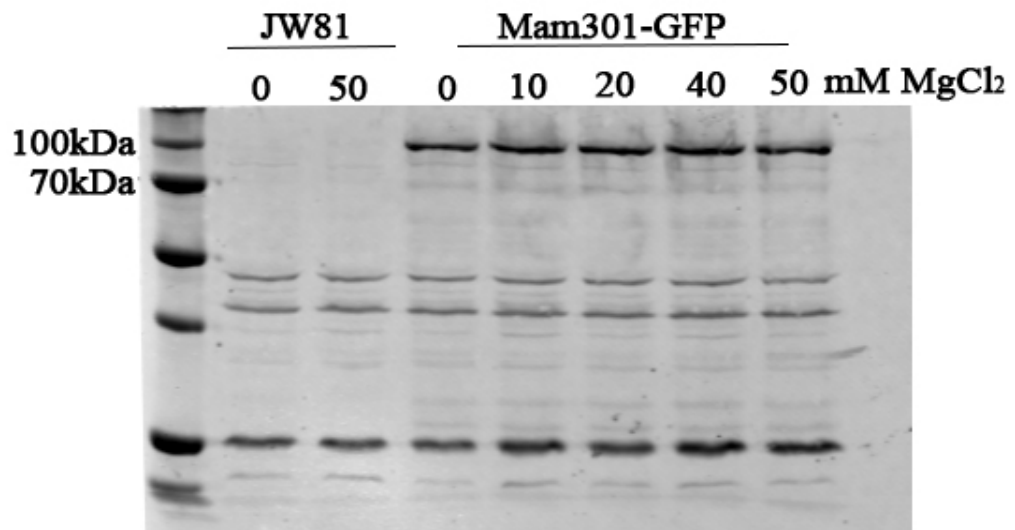

Figure 10: Western blot showing regulation of Mam301 with increasing magnesium concentrations at a lower curve. JW81 is used as control.

3.5 Deletion of cis4 and zrg17 leads to growth defect with high magnesium concentrations

As Cis4 and Zrg17 are also important for survival on high magnesium, we hypothesized that they supply zinc ions to a protein that is important for Mam301 function. To test this hypothesis, another set of drop tests were performed with deletions of *mam301, cis4, zrg17* and a double deletion of *cis4* and *zrg17*. These were performed on plates with different magnesium concentrations and with JW81 as a control. The results show that all deletions have a growth defect on high magnesium concentrations (figure 11). When compared to Δ*mam301* both Δ*cis4* and Δ*zrg17,* and the Δ*cis4* Δ*zrg17* double mutant, show a more severe growth defect on higher magnesium concentrations. The stronger growth inhibition of Δ*cis4* and Δ*zrg17* indicate that these genes are important for magnesium homeostasis. Potential explanations for the stronger growth defect include that they participate in a different pathway that protects cells from high magnesium, or that they have other regulative functions that protect cells from excess magnesium.

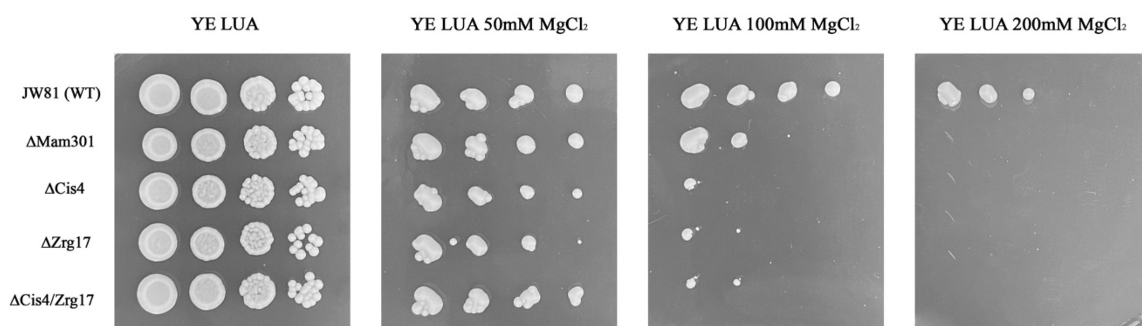

Figure 11: Drop tests performed with deletion strains as indicated. JW81(WT) was used as control.

3.6 Genetic crosses of Mam301-GFP to Δcis4, and Mam301-GFP to Δzrg17

To be able to determine if Cis4 and Zrg17 are required for magnesium-dependent regulation of Mam301, the *mam301-GFP* was crossed with the Δ*cis4* strain, and in another cross to a Δ*zrg17* strain. This was done through tetrad dissection analysis. Resulting tetrad colonies can be seen below in table 5 for the cross to Δ*cis4*, and table 6 for the cross to Δ*zrg17*.

Table 5: Genetic cross of Mam301-GFP and Δcis4. The successful double mutants are marked with bold.

Colony	**G418**	**-ade color**	**Δcis4**	**Mam301-GFP**	**Strain information**
1A	Growth	Dark			
1B	Growth	Light			
1C	Growth	Dark			
1D	Growth	Light			
2A	Growth	Dark			
2B	Growth	Light			
2C	Growth	Light			
2D	Growth	Dark			
3A	Growth	Dark	x	✓	Mam301-GFP ade6-210
3B	**Growth**	**Dark**	✓	✓	**Δcis4 Mam301-GFP ade6-210**
3C	-	-			
3D	Growth	Light	✓	x	Δcis4 ade6-216
4A	-	Dark	x	x	
4B	-	Light	x	x	
4C	**Growth**	**Light**	✓	✓	**Δcis4 Mam301-GFP ade6-216**
4D	Growth	Dark			

Two hybrid strains that contained Δ*cis4* and *mam301-GFP* (3B and 4C) were isolated from the cross of Δ*cis4* to *mam301-GFP*. Both strains were frozen, and stored at -80°C. Colony 4C was also added to the labs biobank and was used in all further analyses.

Table 6: Genetic cross of Mam301-GFP and Δzrg17. The successful double mutants are marked with bold.

Colony	**G418**	**-ade color**	**Δzrg17**	**Mam301-GFP**	**Strain information**
1A	Growth	Dark	x	✓	Mam301-GFP ade6-210
1B	-	-			
1C	Growth	Light	✓	x	Δzrg17 ade6-216
1D	Growth	Light	✓	x	Δzrg17 ade6-216
2A	Growth	Dark	x	✓	Mam301-GFP ade6-210
2B	Growth	Light	✓	x	Δzrg17 ade6-216
2C	Growth	Light	✓	x	Δzrg17 ade6-216
2D	-	-			
3A	Growth	Dark	x	✓	Mam301-GFP ade6-210
3B	Growth	Light	✓	x	Δzrg17 ade6-216
3C	-	Dark	✓	x	Δzrg17 ade6-210
3D	Growth	Light	x	✓	Mam301-GFP ade6-216
4A	-	Light	x	x	
4B	**Growth**	**Dark**	✓	✓	**Δzrg17 Mam301-GFP ade6-M210**
4C	**Growth**	**Dark**	✓	✓	**Δzrg17 Mam301-GFP ade6-M210**
4D	-	Light	x	x	

The Δ*zrg17 mam301-GFP* also gave two successful crosses, 4B and 4C. Both strains were frozen down and 4C was added to the laboratory's biobank and used in further work.

3.7 Cis4 is required for magnesium dependent regulation of Mam301

Earlier studies have shown that a deletion of *cis4* and *zrg17* leads to a sensitivity against magnesium. To determine whether Cis4 and Zrg17 are required for the magnesium-dependent regulation of Mam301, the *mam301-GFP*, Δ*cis4 mam301-GFP* and Δ*zrg17 mam301*-GFP strains were grown in high and low magnesium. Proteins extracts were then isolated using a urea protein prep for western analysis. JW81 (WT) was included as a control. The western blot in figure 12 shows that the 0 mM $MgCl_2$ for the *cis4* deletion has the same brightness as the 50 mM $MgCl_2$ of the tagged wildtype. This supports that Cis4 is required for magnesium-dependent regulation of Mam301. The 50 mM $MgCl_2$ band for the *cis4* deletion appears to be slightly decreased, although it is unsure if the cause is a downgrade in regulation or because the concentration is reaching toxic levels for the mutant.

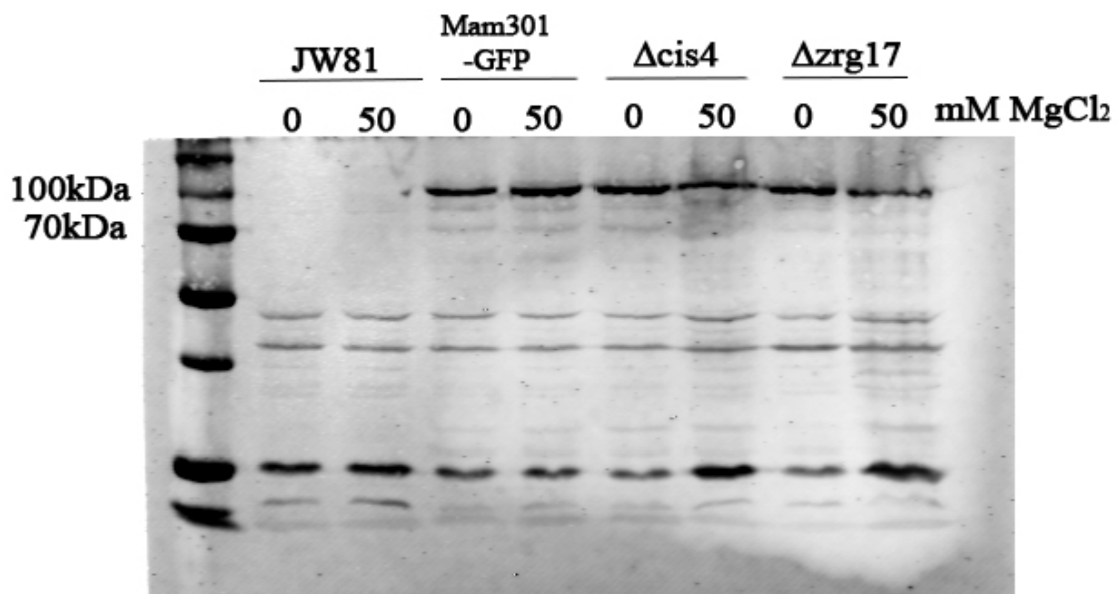

Figure 12: Western blot showing regulation of Mam301 in the tagged wildtype and in deletions of cis4 and zrg17. JW81 is used as control.

When cells were grown for the above experiments, the *zrg17* deletion has a severe growth defect in the presence of 50mM $MgCl_2$. Under this growth condition cells started dying, and the band at 50mM $MgCl_2$ cannot be used to determine if regulation is changed or not. The band at 0mM $MgCl_2$ does not appear to be brighter than 0 for the Mam301-GFP WT, which suggests that Zrg17 is not directly regulating Mam301 expression.

4.0 Discussion

The goal of the study was to characterize *mam301* gene from *S. pombe*, which is predicted to be important in magnesium homeostasis. A set of drop tests was utilized to determine whether Mam301 protects cells from high magnesium concentration. These drop tests show a clear difference between the WT yeast and Δ*mnr2*, and the deletion of *mam301*. Both the WT and Δ*mnr2 strains* grew well on low and high magnesium concentrations, while Δ*mam301* shows a clear growth inhibition on plates with high magnesium concentrations. These results demonstrate that Mam301 is important for cells to survive in high magnesium environments. These findings are similar to those obtained with the *Saccharomyces cerevisiae* homolog *mam3* (17), indicating that proteins belonging to the ancient domain family (Mam301 and Mam3) have a conserved role in protecting yeast from magnesium toxicity.

We confirmed that the Mam301 is localized in the vacuole membrane. This finding supports the hypothesis that Mam301 is a transporter that transports excess magnesium into the vacuole for storage and that its function is the opposite of Mnr2 that exports magnesium out of the vacuole. This correlates with what was found in the budding yeast, *S. cerevisiae*, homolog Mam3 (17).

Membrane proteins are often more difficult to isolate than cytosolic proteins. During this research I found that isolating Mam301, a transmembrane protein (13), for western blots proved to be a challenge. A lot of time was spent on optimizing the protein prep to best visualize mam301 on the membrane. The urea prep was chosen because it specifically enriches for membrane proteins (26), and although Mam301-GFP was clearly detected on the first membrane protein preparation I was not able to detect any protein on the following runs. After troubleshooting the experiment, and making changes, in the end it was found that the time taken to resuspend the Mam301 protein in the urea buffer on the twirler was important, and the optimal timing was about 10 minutes.

The results showing that Mam301 levels are increased by magnesium concentrations suggests that these magnesium levels somehow regulate Mam301 levels. The mechanisms underlying this regulation is still unknown, but the fact that a deletion of the zinc transporters Cis4 and Zrg17 leads to a sensitivity to magnesium is interesting, and we hypothesized that Cis4 and

Zrg17 could be involved in regulation of Mam301 expression or some other unknown mechanism in magnesium homeostasis.

It was not known beforehand which levels of magnesium concentrations would be toxic to the cells, so we had to try out different concentrations. The results suggests that concentrations over 100mM $MgCl_2$ could be reaching toxic levels, which can be seen in figure 9. The curve that is seen in figure 9 was the first experiment that was performed. At this time it was not clear what levels of $MgCl_2$ would be toxic to cell growth. The results from this western blot inspired a new experiment with lower magnesium levels to see how the regulation changed between 0 and 50mM $MgCl_2$, which is where we could see the biggest change. The grading of the 0 through 50 curve suggests that these concentrations is where regulation is most effective.

One thing worth noticing is that the YE LUA growth medium used in these studies has an adequate amount of magnesium, and this opens for the possibility that what is seen in these figures is the upper region of the regulation, and that there could be lower levels of Mam301 expressed if cells were to grow in low magnesium environments. This theory was not tested within the timeframe of this project. This is still an interesting research question which would be essential to follow up with additional experiments.

The drop tests showing that deletion of *cis4, zrg17* and the double deletion of *cis4* and *zrg17* had a stronger growth defect on high magnesium concentrations suggests that Cis4 and Zrg17 are in a different pathway than Mam301, but that both of these pathways are important for magnesium homeostasis. This finding is also consistent with Cis4 and Zrg17 having a general regulatory role, which is something that has been a thought in the Bird Lab. What this regulatory role, and all it regulates, is still unknown, but the fact that *cis4* and *zrg17* deletions show a higher magnesium sensitivity than *mam301* deletions suggest that Cis4 and Zrg17 regulate the expression of Mam301 and additional genes important for magnesium homeostasis.

The *zrg17* deletion had a more severe growth defect in high magnesium in liquid cultures when compared to the *cis4* deletion. This result suggests that Zrg17 may be even more important for magnesium homeostasis than Cis4. These results were found during the last weeks of the project, and there was not enough time to go lower on concentrations to find

where the cutoff level seems to be for the *zrg17* deletion, though this would be interesting to find out. The *cis4* deletion shows a loss of regulation of Mam301, which could indicate that Cis4 has an important regulatory role for Mam301. Though it is not clear from figure 12 if the decreased concentration of Mam301 in the 50 mM $MgCl_2$ is because of downgrade of regulation or because the concentration is reaching toxicity, the hypothesis is that the latter is more likely. In the early stages when the cells were looked at under fluorescence microscope it appeared to be a clear loss of regulation in the *cis4* deletion, with the same brightness in all concentrations. What was surprising was that the *zrg17* deletion did not show the same loss of regulation of Mam301, and this suggests that Zrg17 and Cis4 could have different roles in magnesium homeostasis.

Some time was spent finding out the high and low concentration of magnesium to grow cells in. As can be seen in figure 9 100mM $MgCl_2$ and higher seems to be closing on toxic levels of magnesium, and though the tagged wildtype was able to grow some, the deletions were having trouble. The choice was first made to decrease concentration from 200mM $MgCl_2$ to 100mM $MgCl_2$, but when even this proved to be too tough on the deletion strains the concentration was lowered to 50mM $MgCl_2$. As a point made above, the *zrg17* deletion was still having a lot of trouble, but because the project nearing the end the choice was made to let it go and settle for 50mM $MgCl_2$.

What was interesting was the *cis4* deletion showing loss of regulation on the western blot, which gives a higher expression of Mam301. Why would this phenotype then show a lower tolerance for magnesium? One possible reason could be that Cis4 and probably Zrg17 are important in sensing magnesium going into the cell, and when these are deleted the cell loses this regulation and the uptake of magnesium into the cytosol is increased. This would in turn lead to an increased expression of Mam301 to remove excess magnesium into the vacuole. This is just a hypothesis, and this needs to be probed further to find out more. One way to test whether deletion of *cis4* and *zrg17* affects magnesium levels would be to use magnesium specific dyes (e.g. Mag-Fura-2 from Thermofisher (27), that detect the level of free magnesium ions inside of cells. The Bird lab are planning on finding out more about this and is currently in the work of researching other proteins that are predicted to be linked to magnesium homeostasis.

Another way to further research this is to examine changes in the entire proteome in *cis4* and *zrg17* deletions in high magnesium. This potentially could reveal other proteins that are regulated by magnesium in a manner that is dependent on Cis4 and Zrg17. This approach could give a series of proteins that could be interesting to study further to see if they also have important roles in maintaining magnesium homeostasis.

5.0 Conclusion

In this study I have characterized several things about the Mam301 protein in *S. pombe* to gain insight about how this protein family function in humans. Mam301 was predicted to have a role in magnesium homeostasis.

My work revealed that Mam301 is important in protecting cells from high Mg^{2+} environments (Aim 1), and evidence suggests that it could be a magnesium transporter that transports Mg^{2+} into the vacuole for storing. Through western blot analysis I was able to confirm that Mam301 expression is regulated by magnesium levels, and that this regulation goes up to around 100 mM $MgCl_2$ (Aim 2). After reaching 100 mM $MgCl_2$ regulation gets lost, which suggests that levels higher than 100 mM $MgCl_2$ are toxic for the cell.

On the background of earlier studies showing that deletions of *cis4* and *zrg17* leads to a sensitivity to magnesium, I tested to see if they have anything to do with Mam301's function. It was found that *cis4* and *zrg17* deletions are more sensitive to magnesium than a *mam301* deletion, which suggests that Cis4 and Zrg17 are in a different pathway and is consistent with them having a general regulatory role. I was also able to show that Mam301 regulation is lost in cis4 deletions, which in turn strengthens the suggestion that Cis4 has an important regulatory role for Mam301 (Aim 3). Deletion of zrg17 does not show loss of regulation, but is highly sensitive to high magnesium concentrations, which suggests that Zrg17 has an important role in magnesium homeostasis even though exactly how is still unclear.

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7.0 Appendix

Table 7: Strains used in this study

Strain	Genotype	Reference
JW81	ade6-M210 leu1-32 ura4-D18	Wu et al, 2003
ΔMam301	ade6-M216 leu1-32 ura4-D18 mam301::KANMX4	Bioneer
ΔMnr2	ade6-M216 leu1-32 ura4-D18 mnr2::KANMX4	Bioneer
Δcis4	ade6-M216 ura4-D18 Δcis4::KANMX4	Andy Weeks
Δzrg17	ade6-M216 leu1-32 ura4-D18 Δzrg17::KANMX4	Derek Boehm
Mam301-GFP	ade6-M210 leu1-32 ura4-D18 mam301-GFP::KANMX6	Made in this study
Δcis4 Mam301-GFP	ade6-M216 leu1-32 ura4-D18 Δcis4 mam301-GFP::KANMX6	Made in this study
Δzrg17 Mam301-GFP	ade6-M210 leu1-32 ura4-D18 Δzrg17 mam301-GFP::KANMX6	Made in this study

Table 8: Primers used in PCR reactions

Primer name	**Function**	**Primer sequence (5’-3’)**
KanB	KanMX6 reverse primer	5’ – CTGCAGCGAGGAGCCGTAAT – 3’
Mam301GFPF	Mam301-GFP forward primer	5’- CGTCATCTTCAAAGGTACCTCGAAA TAAGCGGAAACGAAGAAAGGTAAAAG CAAGAAACGGATCCCCGGGTTAATTAA - 3’
Mam301GFPR	Mam301-GFP reverse primer	5’ – CAGTACTAATTCAATAATCCA GCCGTGAATTCGAGCTCGTTTAAAC – 3’
Mam301UTRF	Mam301 UTR forward primer	5’ - GTTTAAACGAGCTCGAATTCACGGCTGGAT TATTGAATTAGTACTG – 3’
Mam301UTRR	Mam301 UTR reverse primer	5’ – GCTTCAACTGAACCTCGTCGTCCC - 3’
GFPR	Mam301-GFP confirmation	5’ – GACAAGTGTTGGCCATGGAAC – 3’
Mam301F	Mam301-GFP confirmation	5’ –GAGAGTAATGTTGTATCACCCG– 3’
Mam301prom	Deletion confirmation	5’ – ACATCCAAGCGTCATCCCAA – 3’
Cis4-Prom	Deletion confirmation	5’- ACCACAGCTTTTGGGTGTTCCTGACGAC – 3’
Zrg17-prom	Deletion confirmation	5’ – TACCCATCCACTCCACCAAACG – 3’

Table 9: PCR programs used in this study

Step	Temperature	Duration	Cycles
		PCR A and B	
	94°C	3 min	1
Denaturation	94°C	30 s	28
Annealing	58°C	30 s	
Extension	72°C	3 min	
	72°C	6 min	1
		PCR AB	
	95°C	3 min	1
Denaturation	98°C	10 s	24
Annealing	58°C	30 s	
Extension	72°C	4 min	
	72°C	6 min	1
		PCR C	
	94°C	3 min	1
Denaturation	94°C	30 s	32
Annealing	58°C	30 s	
Extension	72°C	1 min	
	72°C	2 min	1

Table 10: Reagents used in the study and how they were made

Reagent	Ingredients	Directions
2x YE	2.5 g Yeast extract	Made up to 250 mL with dH_2O Autoclaved
2x EMM	1.5 g KH phthalate 2.07 g Na_2HPO_4 $6H_2O$ 2.5 g NH_4Cl	Made up to 250 mL with dH_2O with pH 5.6 Autoclaved
1x YE LUA	250 mL 2x YE 37.5 mL 40% Glucose 5 mL 100x Leucine 10 mL 50x Uracil 15 mL 50x Adenine dH_2O	Made up to 500 mL with dH_2O
1x EMM LUA	250 mL 2x EMM 25 mL 40% Glucose 5 mL 100x Leucine 10 mL 50x Uracil 15 mL 50x Adenine 10 mL 50x Salt stock 500 µL 1000x Vitamin stock 500 µL Na_2SO_4 50 µL 10000x Mineral stock dH_2O	Made up to 500 mL with dH_2O
100x Leucine	0.75 g Leucine dH_2O	Made up to 100 mL with dH_2O Autoclaved
50x Uracil	0.75 g Uracil dH_2O	Made up to 200 mL with dH_2O Autoclaved
50x Adenine	0.75 g Adenine dH_2O	Made up to 200 mL with dH_2O

Reagent	Ingredients	Directions
50x Salt stock	26.65 g $MgCl_2$ x $6H_2O$ 0.368 g $CaCl_2$ x $2H_2O$ 25 g KCl 1 g 1000x Na_2SO_4 dH_2O	Made up to 500 mL with dH_2O
10000x Mineral stock	1.25 g H_3BO_3 5.2 mM $MnSO_4$ H_2O 1 g $ZnSO_4$ $7H_2O$ 0.5 g $FeCl_3$ $6H_2O$ 0.4 g MoO_4 $2H_2O$ 0.25 g KI 0.1 g $CuSO_4$ $5H_2O$ 2.5 g Citric acid	Made up to 250 mL with dH_2O Filter sterilized
1000x Vitamin stock	1 g Nicotinic acid 1 g Inositol 1 mg Biotin 200 mg D-Calcium Pantothenate	Made up to 100 mL with dH_2O Filter sterilized
1x TAE buffer	40 mL TAE 50M stock dH_2O	Made up to 1 L with dH_2O
Primary antibody	10 mL TBST 5% skimmed milk 10 µL Anti-GFP from Rabbit	
Secondary antibody	10 mL TBST 5% skimmed milk 1 µL IRDye 680RD Goat anti-rabbit 926-68071 from Li-Cor Lot nr: C60920-06	
SBS PAGE 10% separating gel	1.9 mL dH_2O 1.3 mL 1,5M Tris pH 8,8 1.7 mL 30% Acrylamide 50 µL 10% SDS 50 µL 10% Ammonium Persulfate 2 µL TEMED	Add into beaker in listed order, stir and load into plates

Reagent	Ingredients	Directions
SDS PAGE 5% Stacking gel	1.4 mL dH_2O 250 µL 1,5M Tris pH 8,8 330 µL 30% Acrylamide 20 µL 10% SDS 20 µL 10% APS 2 µL TEMED	Add into beaker in listed order, stir and load on top of separating gel
Western transfer buffer	29.4 g Glycine 6 g TRIS base 100-200 mL Methanol dH_2O	Made up to 2 L with dH_2O
TBS(T) buffer	20 mL 1M TRIS-HCl pH 7.5 30 mL 5M NaCl (1 mL Tween) dH_2O	Made up to 1 L with dH_2O
Malt extract agar	3 g Fisher scientific malt extract 2 g Fisher scientific agar dH2O	Adjusted pH to 5.5 with NaOH and made up to 100 mL with dH_2O
Blue buffer	2% Triton X-100 1% SDS 100mM NaCl 10 mM TRIS-Cl pH 8.0 1 mM Na_2EDTA	
Cold Membrane Buffer	10 mL 5M Ammonium Acetate 1.5 mL 5M NaCl 1.5 mL 1M TRIS pH 7.4 500 µL 0.5M EDTA dH_2O	Made up to 50 mL with dH_2O