Subcellular localization of Mtm1 in *Hansenula Polymorpha*

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Abstract

Peroxisomes are organelles found in eukaryotic cells. Transporter proteins are needed to transport solutes across the peroxisomal membrane. There is still a lot unknown about the metabolite transport. In this research the localization of the pyridoxal 5'phosphate transporter protein Mtm1 was studied using fluorescence microscopy. To study this plasmids encoding for a N-terminal and encoding for a C-terminal fusion of Mtm1 to meGFP was made. This plasmid was transformed into *Hansenula polymorpha* Δ*ku80 with* a peroxisomal marker Pex3-mKate2. These transformants were then used for a western blot and fluorescence microscopy. The western blot was detected using and AP-conjugate. The cells for fluorescence microscopy were grown on glucose, methanol and on glucose after which they were stained with MitoTracker Red. The observed location of the GFP was not colocalized with the mKate2 location. The western blot showed the presence of the fusion protein for both the N- and C-terminal fusion. From the obtained data it was concluded that there was no colocalization between the fusion protein and the peroxisomes. It was also found that the Mtm1 is located at the mitochondria for the cells grown on glucose.

Introduction

Peroxisomes are conserved organelles that are found in eukaryotic cells. The functions of peroxisomes, as well as their morphology, are remarkably diverse and flexible. Not only can these change from organism to organism, but they can even differ based on environmental conditions (Lazarow, 2023). Peroxisomes are unable to synthesize proteins since they do not contain DNA. This means all peroxisomal proteins come from the cytosol, and need to be transported into to the peroxisome. At this time it is thought that transport proteins are responsible for the transport of solutes in and out of the peroxisomes (Plett et al., 2020), but there is still a lot unknown about metabolite transport (Rottensteiner & Theodoulou, 2006).

There is also still a lot to be discovered about the proteome of the peroxisome. Knowledge on the peroxisome is essential for many different reasons; Not only is it important to better understand peroxisomal diseases, but also other health related topics like immunity, viral infection, neurogenerative diseases, aging and malignant transformation (Yifrach et al., 2022). This makes the proteome of the peroxisome an important field of research.

In a recent study it was found by Mass spec analysis of *Saccharomyces cerevisiae* proteins that certain proteins have a deviant level in *pex3* strains compared to their wild type (Kosir et al., preprint 2024). This study used transcriptomics and quantitative proteomics to study these strains. The *pex3* strain has no functional peroxisomes (Erdmann et al., 1989), and also no peroxisomal membrane. The membrane proteins of peroxisomes could therefore show a difference in membrane protein levels for the *pex3* mutant, since synthesized peroxisomal membrane proteins without a target are relocated or degraded (Cooper GM, 2000), where degradation is based on the half-life of the protein. In the study from Kosir et al (preprint 2024) it was found that the transporter protein Mtm1 had the same transcription level for the *pex3* and the wild type, however, its protein level was lower for the *pex3* mutant. This suggests the protein might also be located in the membrane of the peroxisome in the wild type.

Mtm1 is part of the mitochondrial carrier family. This family has transporters which have both the N- and C-terminus in the inner membrane space, due to having six membrane spans in *S. cerevisiae* (Mentel et al., 2021). Mtm1 is a mitochondrial transporter protein, and supposedly transports the co-factor pyridoxal 5'-phosphate (PLP) in *S. cerevisiae* (Whittaker et al., 2015). Earlier research has

already shown that proteins can be dually localized to both the mitochondria and the peroxisome (Yifrach et al., 2022). For the synthesis of PLP Bud16 is a key enzyme. Bud 16 is a pyridoxal kinase that localizes not only to the cytosol but also to the peroxisome (Yifrach et al., 2022).

Furthermore, the aspartate aminotransferase called Aat2 is found to be localized in the peroxisomes of *S. cerevisiae* cells grown on oleate (Verleur et al., 1997), as well as in the peroxisomes of *Hansenula polymorpha* (Thomas et al., 2018). The Aat2 enzyme contains PLP as a co-factor, suggesting that PLP needs to be transported into the peroxisomes. The peroxisome is not freely permeable and might therefore be in need of a PLP transporter. However, one might argue that it is also possible PLP is transported into the peroxisome together with the Aat2, however, there is currently no data supporting this hypothesis.

Taken together this knowledge about Mtm1 and the results form Kosir et al. (preprint 2024), it was hypothesized that the mitochondrial transporter Mtm1 might also be located in the peroxisome. This led to the research question: does the Mtm1 transporter protein in *H. polymorpha* localize not only to the mitochondria membrane but also to that of the peroxisome?

To answer this question a 5' and 3' fusion of *meGFP* to the *MTM1* gene was made. These fusion genes were made in two different *H. polymorpha* plasmids (Table S2) which contain the strong promoter P_{ADH1}, resistance markers for ampicillin and zeocin and integrated a stuffer gene at the 5' or 3' to the *meGFP* gene. These two different plasmids were fused into the genome of the methanotrophic *H. polymorpha*, containing Pex3-mKate2 as peroxisomal marker. *H. polymorpha* was chosen due to its characteristic that it will make very large, and even multiple peroxisomes when grown on methanol. This has the advantage that visualization with the fluorescence microscope of the peroxisome is clearer. The *H. polymorpha* cells with the integrated plasmid were used for fluorescence microscopy and western blot analysis.

It was observed that neither the Mtm1 with the N- nor the C-terminally fused meGFP colocalized with the Pex3-mKate2. The western blot did show that the Mtm1 fused to meGFP was present for both the cells with the N- and C-terminally fused meGFP to Mtm1. The results from the MitoTracker Red CMXRos, from Life Technologies, showed that the meGFP localizes to the mitochondria. This led to the conclusion that Mtm1 is a mitochondrial transporter protein which does not also localize to the peroxisome in *H. polymorpha*.

Material and methods

The standard protocols used for this study came from the protocol book from Molecular Cell Biology (MCB, 2024).

Construction of plasmids for cloning

Construction of two plasmids was done, one encoding a N-terminal fusion and one encoding a C-terminal fusion of meGFP to Mtm1, resulting in the plasmids pHIPZ18 *meGFP-MTM1* and pHIPZ18 *MTM1-meGFP*. The plasmid details can be found in Table S2. The promoter P_{ADH1} is a strong promoter causing overexpression of the *MTM1* gene.

To obtain the N-terminal meGFP fusion the following methods procedures were followed. To obtain the gene of interest *MTM1,* Phusion PCR, using primers *MTM1*-N-fw and *MTM1*-N-rev (Table S3), was performed on chromosomal DNA from *H. polymorpha* Δ*ku80 (leu1.1)*. For this PCR the Phusion High-Fidelity PCR Kit was used. The PCR was done as suggested by the manual from the supplier Thermo

Scientific. The following settings were used: 98°C for 2 minutes, then 35x 98°C for 10s, 60°C for 10s and 72°C for 1 min in cycles and lastly 72°C for 5 minutes. This resulted in a PCR product which was restricted with BamHI and XhoI, resulting in a fragment of 1101bp. From *Escherichia coli* DH5α plasmid pHIPZ18 *meGFP-W1Q7X1* was obtained by using the Thermo Scientific GeneJet Plasmid Miniprep kit. From this plasmid the *W1Q7X1* gene was removed by restriction with BamHI and XhoI. The plasmid should have a concentration of about 200ng/µL, which was measured using a nanodrop. The PCR product was purified using the Thermo Scientific GeneJet PCR Purification kit.

To obtain the plasmid with the C-terminal fusion, pHIPZ18 *MTM1-meGFP*, the same principle was used. Here however the primers used for the first PCR to obtain the *MTM1* gene were primers *MTM1*-C-fw and *MTM1*-C-rev (Table S3). The resulting PCR product was restricted with HindIII and BamHI, resulting in a fragment of 1098bp. From the plasmid pHIPZ18 *W1Q7X1-meGFP* the *W1Q7X1* gene was removed by restriction with HindIII and BglII.

The restricted PCR fragment and the cut plasmids were ligated according to the manual of the Rapid DNA ligation kit from Thermo Fisher. Ligation resulted in the plasmids pHIPZ18 *meGFP-MTM1* and pHIPZ18 *MTM1-meGFP*. Most restriction enzymes used in this study were Fast digest enzymes. The enzymes came from the company Thermo Fisher. The only regular digest enzyme used is BshTI (AgeI), which needs to restrict for 1.5h.

Transformation

The constructed plasmids were transformed into *E. coli* DH5α according to protocol B.1 *E. coli* transformation: $CaCl_2$ competent, however sterile demi water was used instead of sterile MilliQ. These cells were plated on LB agar ampicillin 100µg/mL plates in concentrations of 0.1%, 10% and 89.9%. Colony PCR was used to check for correctly integrated plasmids in the *E. coli* colonies. For the colony PCR Thermo Scientific DreamTaq DNA Polymerase protocol was used, with the primers *MTM1*-N-fw and *MTM1*-N-rev for the N-terminal colonies and *MTM1*-C-fw and *MTM1*-C-rev for the C-terminal colonies (Table S3). Correct colonies were incubated in 5mL LB ampicillin 100µg/mL overnight. Thermo scientific GeneJet plasmid miniprep kit was used to obtain the plasmids. These plasmids were checked by a 1% agarose gel electrophoresis with different restriction enzyme combinations and by sequencing before transformation into *H. polymorpha*.

The integration test with restriction enzymes of pHIPZ18 *meGFP-MTM1* was done with the restriction enzyme combination HindIII + BglII and combination BamHI + XhoI. The integration test of pHIPZ18 *MTM1-meGFP* was done with the restriction enzyme combination HindIII + BglII and combination HindIII + SalI. After this, the sequencing of plasmids, which had the expected restriction pattern, was done by Eurofins Genomics. For the sequencing of the pHIPZ18 *meGFP-MTM1* the primers Seq GFP Fw and Tamo rev were used. For the sequencing of the pHIPZ18 *MTM1-meGFP* the primers pHIPN18 Fw and mGFP rev check were used (Table S3).

The plasmids were linearized using restriction enzyme BshTI (AgeI). The linearization was checked by 1% agarose gel electrophoresis. Electro transformation of *H. polymorpha yku80* + *PEX3-mKATE2* (WT) (Table S1) with the two different linearized plasmids pHIPZ18 *MTM1-meGFP* and pHIPZ18 *meGFP-MTM1* was done according to protocol B.4 Electrotransformation of *Hansenula polymorpha*.

The transformed yeast cells were plated on YPD agar zeocin 100µg/mL plates in concentrations of 1%, 10% and 89% and incubated for two days in a 37°C incubator. The transformants from the 1% plates for both fusions were numbered and replated on fresh YPD agar zeocin plates and again incubated at 37°C for one day. Colony PCR was done to check for correct integration. The colony PCR was done according to the Thermo Scientific DreamTaq DNA Polymerase protocol, using primer:

cPCR Padh-fw and primer: mGFP rev check (Table S3). The cells from the numbered colonies plates were resuspended in 50µL Milli-Q water, and used as template DNA. A total volume of 25µL, instead of 50µL, was used for each PCR tube. The dNTP mix used had a molarity of 10mM instead of 2mM.

Cultivation conditions

Both *E. coli* and *H. polymorpha* were grown in liquid media at 37°C while shaking at 200rpm in the incubator. Plated cells were grown in the 37°C incubator.

LB medium was prepared according to protocol I A.2. LB was used for both overnight cultures of *E. coli* as for LB agar ampicillin plates. The ampicillin was added to a concentration of 100µg/mL, to select for resistant strains.

Mineral medium for *H. polymorpha* was prepared according to protocol I E.1. As additional constituent l-Leucine was added according to Table 6 in the protocol. Two different types of mineral media were made with different carbon sources. The first used as carbon source 0.5% glucose, and the second 0.5% methanol.

YPD was also prepared according to protocol I E.1. YPD was used for both overnight cultures of *H. polymorpha* and for YPD agar zeocin plates. The zeocin was added to a concentration of 100µg/mL, to select for resistant strains.

Protein detection using western blot

Extracts from WT, WT + *meGFP-MTM1* and WT + *MTM1-meGFP* cultures (Table S1) were prepared for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis by following protocol C.10 Preparing crude extract of yeast cells in presence of Trichloroacetic acid (TCA). Protocol C.60 SDS-PAGE using mini-gel system, was followed to run two SDS-PAGE gels of the WT, as negative control, WT + *meGFP-MTM1* and WT + *MTM1-meGFP*, a pre-stained marker and two positive controls. The percentage of acrylamide in the gel was 10%, and the gel ran for 1h. The first positive control was the fusion protein Mpc2-GFP which has a size of 41 kDa, and the second GFP-SKL which has a size of 27 kDa. Protocol C.68 Western blotting; Semi-dry, was followed to make two western blots from the SDS-PAGE gels. Protocol C.66 Ponceau S staining for nitrocellulose blots was followed to visualize the protein transferred from the gels to the blots. The incubation of the dye was approximately 1 minute.

For the first blot protocol C.70 Detection of proteins on western blots using an AP-conjugate was followed. The primary antibody used was αGFP mouse monoclonal anti-GFP. The secondary antibody was Goat anti-Mouse IgG AP-conjugate. For the second blot protocol C.71 Detection of proteins on western blots using an HRP-conjugate was followed. The primary antibody used was also mouse monoclonal αGFP. The secondary antibody used was Goat anti-Mouse HRP-conjugate. The blot was exposed from 1 second – 188.6 seconds, with images taken about every 6 seconds. This was done using the Bio-Rad molecular imager ChemiDoc RXS+ imaging system.

Fluorescence microscopy

All fluorescence imaging was done at room temperature. Images of the WT + pHIPZ18 *meGFP-MTM1* and WT + pHIPZ18 *MTM1-meGFP* were made under different conditions. Firstly cells were grown on mineral media with glucose, secondly cells were grown on mineral media with methanol and lastly glucose grown cells were stained with MitoTracker Red CMXRos from the company Life technologies. All images were processed using Fiji software, ImageJ 2.14.0.

Samples of the cells grown on glucose were prepared by growing an overnight culture in mineral media with glucose. The overnight culture was diluted to an OD_{660} of 0.1 and grown for

approximately 6h until a reached OD_{660} of 0.8 – 1.5. From the culture 1mL was pipetted into a 1.5mL Eppendorf tube. The cells were pelleted with an Eppendorf centrifuge for 1 minute on 3381 rcf. 950µL of the supernatant was removed and the pellet was resuspended in the remaining supernatant. 1µL of the resuspended cells was pipetted on an object slide and covered with a cover glass.

For the samples of cells grown on methanol the culture was grown overnight on YPD. The overnight culture was diluted to an OD_{660} of 0.1 in mineral media with methanol as its carbon source. Approximately 6h later 10mL of the culture was centrifuged for 2 minutes at 3220 rcf. Most of the supernatant was removed, and the pellet was resuspended in approximately 1mL of supernatant. The resuspended cells were again centrifuged for 1 minute at 3381 rcf. 950µL of the supernatant was removed and the pellet was resuspended in the remaining supernatant. 1µL of the resuspended cells was placed on an object slide and covered with a cover glass.

The samples for fluorescence microscopy of the cells grown on glucose with MitoTracker Red CMXRos was done according to protocol D.3 fluorescent staining of yeast mitochondria. The agarose pad object slides were made by first preparing 1% agarose in 10mL mineral media with glucose. This mixture was microwaved until a clear solution was obtained. Warm agarose solution was pipetted on an object slide and covered with another object slide, and let to solidify at room temperature. The slides were stored in a closed Petri dish for approximately 1-1.5h before imaging. Right before imaging the objective slides were removed from one another by sliding one away. The agar pad was on one of the object slides, which was used for microscopy. 2µL of the cell suspension from protocol D.3 was applied to the agarose pad and covered with an object slide.

Two different microscopes were used for imaging. The first being the confocal microscope LSM800 Airyscan from Zeiss microscopy. This microscope was equipped with a 32-channel gallium arsenide phosphide photomultiplier tube (GaAsP-PMT). The objective used was a 63x 1.4 NA oil immersion objective. This objective was set to Airyscan mode. This microscope was used to make the images of the glucose and methanol grown cells.

The second microscope used is the Zeiss Axioscope A1 fluorescence microscope form the company Carl Zeiss microscopy. A Coolsnap HQ2 digital camera was used together with µManager software to make the images. To visualize the meGFP signal a bandpass (BP) 470/40 nm excitation filter and a BP 525/50 nm emission filter were used together with a 495 nm dichromatic mirror. To visualize the MitoTracker Red CMXRos signal a BP 587/25 nm (High efficiency) excitation filter and a BP 647/70 nm (High efficiency) emission filter were used together with a 605 nm dichromatic mirror (High efficiency). This microscope was only used for images of the cells stained with MitoTracker Red CMXRos.

Results

Cloning

The goal of the cloning steps was to obtain one *H. polymorpha* strain with its genome containing an integrated plasmid encoding a N-terminal fusion of meGFP to Mtm1, and one with its genome containing an integrated plasmid encoding a C-terminal fusion of meGFP to Mtm1. Insertion of the gene *MTM1* into the pHIPZ18 *meGFP-W1Q7X1* and pHIPZ18 *W1Q7X1-meGFP* (Table S2) using restriction enzymes was done. Ligation of the PCR product of the *MTM1* gene and the restricted plasmid was done to obtain the plasmids pHIPZ18 *meGFP-MTM1* and pHIPZ18 *MTM1-meGFP*. Figure

1A shows the plasmids, with its used restriction sites. As well as its important promoters and genes (Table S2). These plasmids were integrated into the genome of the WT (Table S1), resulting in the strains WT + *meGFP-MTM1* and WT + *MTM1-meGFP.*

The primers *MTM1*-N-fw, *MTM1*-N-rev, *MTM1*-C-fw and *MTM1*-C-rev (Table S3) were used to obtain the PCR product of the N-terminus and C-terminus, respectively. The N-terminal PCR product was restricted with BamHI and XhoI, resulting in a fragment of 1101bp (Figure 1B). The C-terminal PCR product was restricted with HindIII and BamHI, resulting in a fragment of 1098 (Figure 1B). The 1% agarose gel electrophoresis results seen in Figure 1B show clear clean bands at the expected sizes of the PCR products.

The plasmid pHIPZ18 *meGFP-W1Q7X1* was restricted with BamHI and XhoI resulting in two fragments: 300bp (*W1Q7X1* gene) and 5761bp (remaining plasmid). The plasmid pHIPZ18 *W1Q7X1-meGFP* was restricted with HindIII and BglII also resulting in two fragments: 300bp (*W1Q7X1* gene) and 5880bp (remaining plasmid). The remaining plasmids were now linearized. In these linearized plasmids the PCR fragments were inserted resulting in plasmids in Figure 1A.

Two clear bands can be observed in Figure 1B for both plasmids, one faint band at 300bp and one strong band at approximately 6kbp, with the band of plasmid pHIPZ18 *meGFP-W1Q7X1* being a bit smaller than the band for pHIPZ18 *W1Q7X1-meGFP*. These band correspond to the correctly restricted plasmid.

The integration of the plasmid pHIPZ18 *meGFP-MTM1* was tested with the restriction enzyme combinations HindIII + BglII and BamHI + XhoI. The correctly integrated plasmid should give two fragments (5692+1170bp) instead of a linear fragment (6061bp) for the first combination and two fragments (5761+1101bp) instead of two fragments (5761+300bp) for the second combination. For plasmid pHIPZ18 *MTM1-meGFP* the restriction enzyme combinations HindIII + BglII and HindIII + SalI were used. Here, the correctly integrated plasmid should give two fragments (6531+447bp) instead of two fragments (5880+300bp) for the first combination and two fragments (5143+1836bp) instead of two fragments (5143+1037bp) for the second combination (Figure 1C).

It can be observed in Figure 1C that the two N-terminal plasmids had the expected restriction pattern however, there are some additional bands for the first plasmid restricted with the first restriction enzyme combination. These bands are above the expected size. The two C-terminal plasmids had the expected restriction pattern, however, they also both showed an additional band above the expected size for the first restriction enzyme combination. The additional bands are likely due to partially DNA.

These results led to the decision to sequence the second N-terminal plasmid and the second C-terminal plasmid. The sequence data can be found in the appendix (Figure S4). The sequencing data showed that the sequences for both the N- and C-terminal plasmid were correct.

The resulting plasmids were now linearized for transformation into *H. polymorpha* (WT) cells (Table S1). The linearization was done by restriction with BshTI (AgeI) of the plasmids. This restriction enzyme cuts in the P_{ADH1} promoter which is both in the genome of the WT and in the plasmids pHIPZ18 *meGFP-MTM1* and pHIPZ18 *MTM1-meGFP* (Figure 1A). Bands at the expected size of 6978 bp for pHIPZ18 *meGFP-MTM1* and 6862 bp for pHIPZ18 *MTM1-meGFP* can be seen in the gel (Figure 1D). This means the linearization was done correctly, and the linear plasmids can be transformed into the genome of the WT.

The transformation led to WT + *meGFP-MTM1* and WT + *MTM1-meGFP* transformants. These colonies were used for colony PCR. In Figure 1E the results can be observed of the colony PCR. The

first gel shows the results of the N-terminal colonies, 4/5 were positive. The second gel shows the results of the C-terminal colonies. Here 4/10 colonies were positive (Figure 1E). The positive N-terminal colonies show bands at the expected size of 1234bp. The positive C-terminal colonies show bands at the expected size of 2329bp. These positive colonies could now be used for protein detection using western blot and for fluorescent microscopy. For both the N- and the C-terminal fusion one colony was chosen for further analysis. For the N-terminal fusion the chosen colony was colony #3 and for the C-terminal fusion the chosen colony was colony #14. From here on colony #3 will be called colony N and colony #14 will be called colony C.

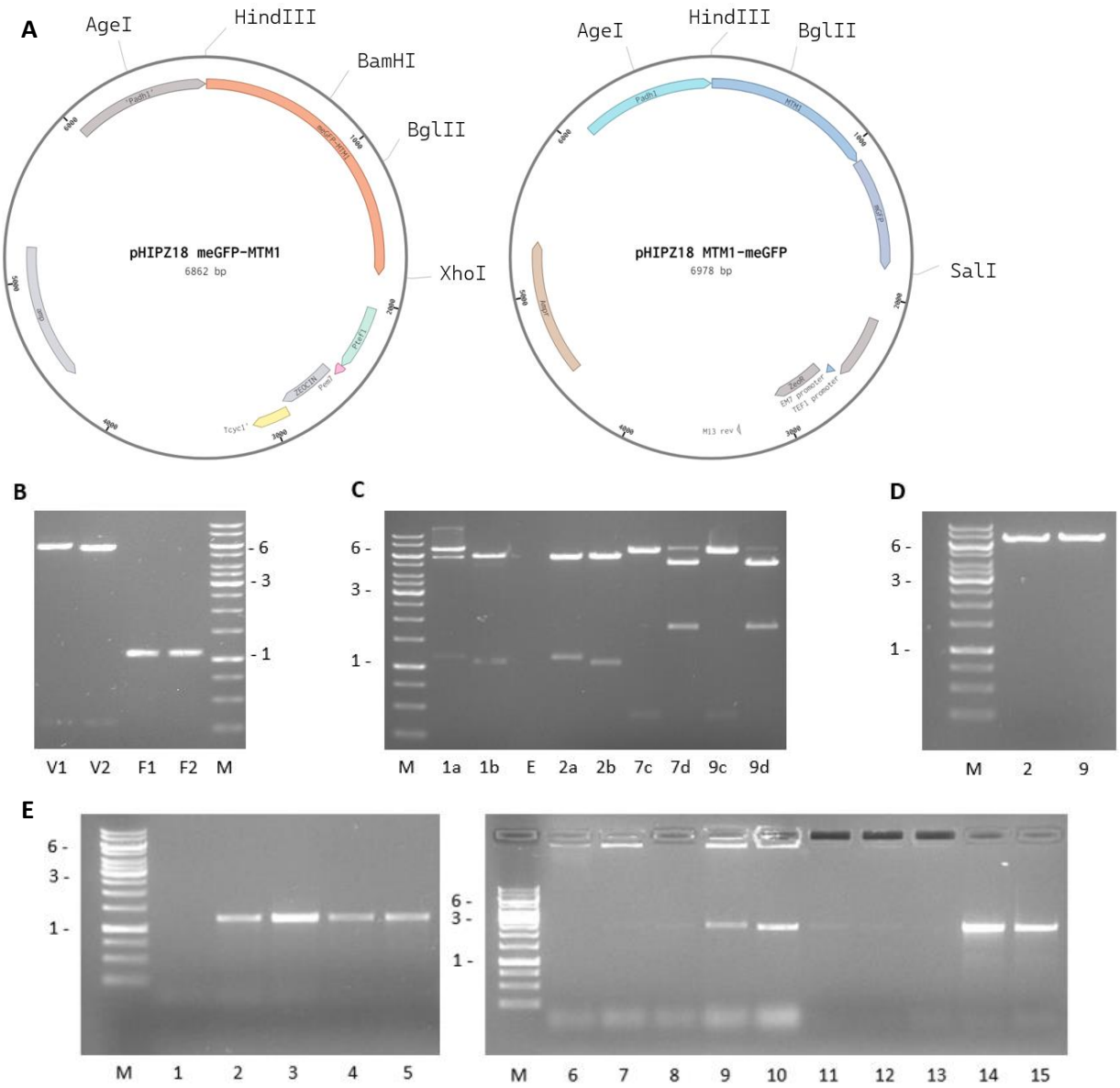

Figure 1: Schematic plasmid map of the expected plasmids and agarose gel electrophoresis tests. For all gels the M corresponds to the Thermo Scientific O'GeneRuler 1 kb DNA Ladder, Ready-to-Use- 250-10,000 bp. The numbers on the marker site of the gels correspond to the size in kbp. (A) The plasmid construction of plasmid pHIPZ18 *meGFP-MTM1* and pHIPZ18 *MTM1-meGFP*. The used cut sites are shown on the plasmids. The genes for ampicillin and zeocin resistance, the promoter P_{ADH1} and the *mtm1* fusion to GFP are annotated. (B) The 1% agarose gel electrophoresis gel of the PCR fragments of *mtm1* and the restricted plasmids pHIPZ18 *meGFP-W1Q7X1* and pHIPZ18 *W1Q7X1-meGFP.* The abbreviations under the gel: V1 corresponds to the restricted plasmid with the C-terminal meGFP, V2 to the restricted plasmid with the N-terminal meGFP. F1 corresponds to the C-terminal PCR fragment, and F2 corresponds to the N-terminal PCR fragment. The expected sizes for V1 is 300bp + 5880bp, for V2 is 300bp + 5761bp, for F1 is 1098 and for F2 1101bp. (C) The 1% agarose gel electrophoresis gel of the plasmid integration test. The abbreviations: numbers 1 and 2 correspond to the constructed N-terminal plasmids, 7 and 9 correspond to the constructed C-terminal plasmids. The letters a, b, c and d indicate the restriction enzyme combinations. Combination a is HindIII + BglII, b is BamHI + XhoI, c is HindIII + BglII and d is HindIII + SalI. The expected sizes are for 1a and 2a 5692bp + 1170bp, for 1b and 2b 5761+1101bp, for 7a and 9a 6531bp + 447bp, and for 7b and 9b 5143bp + 1836bp. (D) the 1% agarose gel electrophoresis gel of the linearization control. Number 2 corresponds to the N-terminal plasmid, and number 9 tot the C-terminal plasmid. The expected sizes are 6978 for 2 and 6862 for 9. (E) The gels on the left corresponds to the colony PCR of colonies 1-15. Colonies 1-5 were N-terminal colonies, 6-15 the C-terminal colonies. For the colony PCR colonies of the strains WT + *meGFP-MTM1* for the N-terminal colonies and WT+ *MTM1-meGFP* for the C-terminal colonies were used (Table S1). The primers used are cPCR Padh-fw and primer: mGFP rev check (Table S3). The expected sizes are 1234bp for the N-terminal colonies and 2329bp for the C-terminal colonies.

Western blot

Ponceau S staining was done to visualize how much protein was loaded in the different lanes by. Western blot was detected to visualize the Mtm1 protein fused to meGFP. This detection was done using AP-conjugate. To obtain the western blot first a SDS-PAGE was run. The gel was blotted on the nitrocellulose membrane, which was then used for the staining and detection.

The Ponceau S staining showed clear separate lanes on the blot (Figure 2A). There were some bubbles present in the WT and N lanes, however these were relatively small and did not obstruct an entire lane. The intensity of most of the different loaded samples was similar, except for the GFP-SKL, which was a lot fainter. These results led to the conclusion that the amount of protein loaded was the same for both colonies.

The western blot detection using an AP-conjugate resulted in clear GFP-specific bands for the N and C colonies. These colonies correspond to the strains WT + *meGFP-MTM1* and WT + *MTM1-meGFP* from Table S1. The N colony shows two small bands right above one another. These bands are located at approximately at the expected size of 67.89 kDa. Colony C shows a thick band and a smaller band right below it. These bands are approximately located at the expected size of 67.75 kDa. A extremely faint band of free GFP can also be observed for the colony. The positive control shows a thick band at the expected size of 41 kDa. GFP-SKL shows a very thin band at the expected size of 27 kDa. This led to the conclusion that the N and C colonies indeed contained a fusion protein with meGFP, and that the amount of it is higher in the C colony.

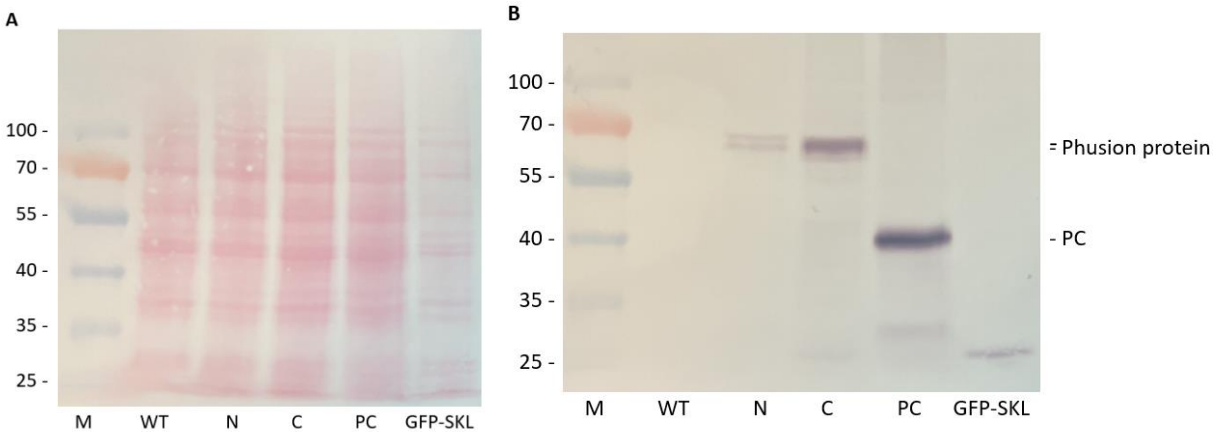

Figure 2: Ponceau S staining and western blot AP detection. The western blot was developed using primary antibody αGFP mouse mitochondrial anti-GFP and secondary Goat anti-Mouse AP-conjugated antibody. Abbreviations: M is the PageRuler Plus Prestained Protein Ladder from Thermo Fisher Scientific, WT corresponds to the WT from Table S1, N and C are WT + *meGFP-MTM1* and WT + *MTM1-meGFP* (Table S1) and PC is the positive control MPC2-GFP. The protein sizes on the left side of the blots are given in kDa. (A) The staining of Ponceau S stain of the western blot. The application was performed for approximately 1 minute. (B) AP detection of the western blot.

Fluorescence microscopy

The goal of fluorescence microscopy was to visualize the location of the fluorescent proteins in the cells of strain WT + *meGFP-MTM1* and WT + *MTM1-meGFP*. The location of the meGFP signal gives information on the location of the Mtm1 protein. The location of the mKate2 signal gives information on the location of the peroxisome.

For the images of cells grown on glucose and methanol the microscopes LSM800 Airyscan confocal microscope was used. For the cells with mitochondria stained with MitoTracker Red CMXRos the Axioscope A1 were used. All images were processed using Fiji software ImageJ 2.14.0. For all three different conditions the intensity of the fluorescence was set to the same intensity for both cells. The cells grown on glucose show red dots from the Pex3-mKate2 (Figure 3A). These dots are the locations of the peroxisomes. The meGFP signal intensity from the N-terminal fusion is very similar to the C-terminal fusion. The location of the meGFP is the same for both fusions. There is no overlay of the meGFP and the mKate2 signals. This led to the conclusion that on glucose the Mtm1 and Pex3 proteins do not colocalize.

The cells grown on methanol show a red ring from the Pex3-mKate2 (Figure 3B), since the peroxisomes are larger when the cells are grown on methanol. The meGFP signal intensity is higher for the C-terminal fusion than the N-terminal fusion. The meGFP for the N-terminal fusion is located again at the mitochondria. There is no ring visible that overlays with the ring from the peroxisome. The C-terminal fusion is located in bright green spots. These spots do not overlay with the Pex3-mKate2 signal. This led to the conclusion that also the cells grown on methanol show no colocalization of the Mtm1 and Pex3 proteins.

The meGFP for both the N- and C-terminal fusion grown on glucose show colocalization with the MitoTracker Red CMXRos signal (Figure 3C). The fluorescence signal of meGFP is higher for the C-terminal fusion of meGFP. The results show exact overlap of Mtm1 and the mitochondria.

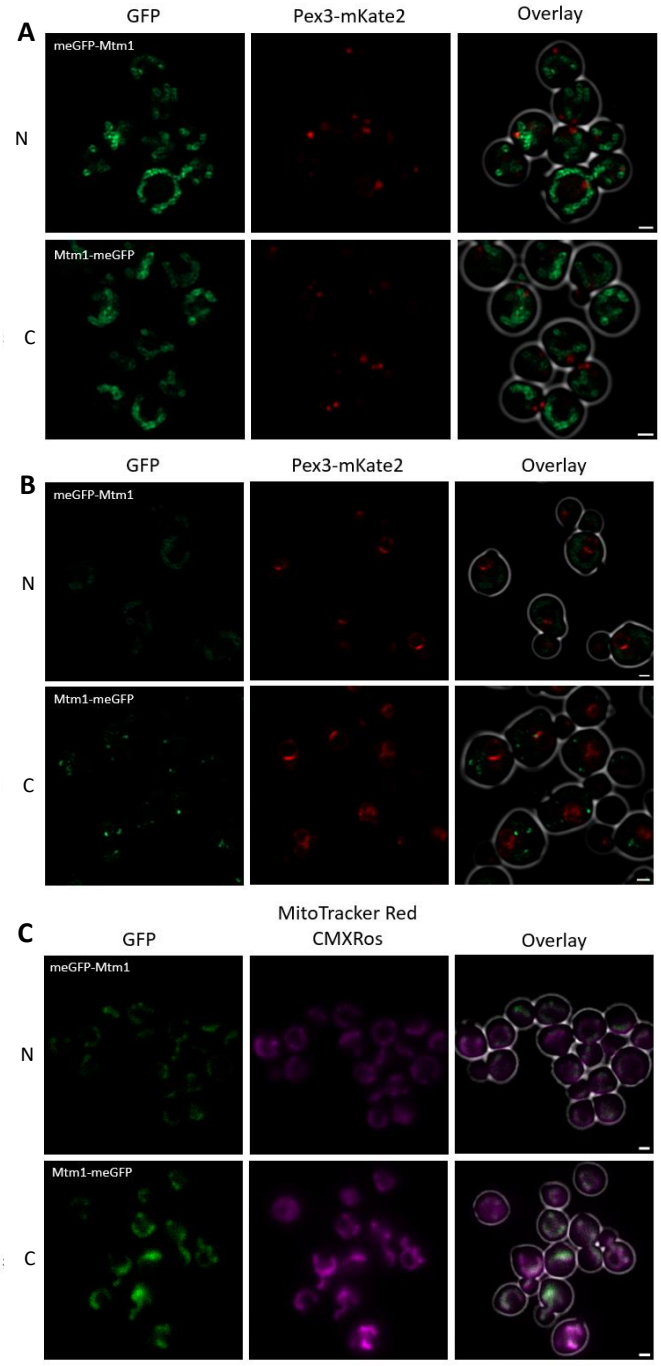

Figure 3: Fluorescence microscopy images. (A) Fluorescence microscopy images made with the LSM800 confocal microscope of cells grown on glucose. N is the N-terminal colony, and C the C-terminal colony. The scalebar represents 1µm. (B) Fluorescence microscopy images made with the LSM800 confocal microscope of cells grown on methanol. N is the N-terminal colony, and C the C-terminal colony. Scalebar represents 1µm. (C) Fluorescence microscopy images of cells stained with MitoTracker Red CMXRos. N is the N-terminal colony, and C the C-terminal colony. Images were taken with the Zeiss Axioscope A1, using a Coolsnap HQ2 digital camera. The GFP signal was visualized using a (BP) 470/40 nm excitation filter, a BP 525/50 nm emission filter and a 495 nm dichromatic mirror. The intensities of the fluorescent signal is set the same for both colonies. Scale bar represents 1µm.

Discussion

This study was set out to find novel peroxisomal proteins, specifically investigating the membrane protein Mtm1, by using fluorescence microscopy. The hypothesis was that the mitochondrial PLP transporter Mtm1 might also be located at in peroxisomal membrane. This led to the research question: does the Mtm1 transporter protein in *H. polymorpha* localize not only to the mitochondria membrane but also to that of the peroxisome? This was answered by creating a fusion of meGFP to Mtm1 at the N- and the C-terminus, and analyzing its location using a fluorescence microscope. It was found that both the N- and the C-terminal fusion of meGFP do not localize to the peroxisome when the cells are grown on methanol or glucose. Furthermore it was found that both the N- and the C-terminal fusion of meGFP localize to the mitochondria when the cells are grown on glucose. Using the stain MitoTracker Red CMXRos the location of the mitochondria was confirmed for the cells grown on glucose.

Contrary to the hypothesis it was found that the Mtm1 fused to meGFP does not localize to the peroxisome in *H. polymorpha* grown on glucose and methanol. The fluorescence results show no colocalization between the meGFP signal and the mKate2 signal, resulting in the conclusion that Mtm1 and Pex3 do not colocalize. The findings are consistent with the results from Whittaker et al. (2015), who showed that the Mtm1 is located in the mitochondria of *S. cerevisiae*. This illustrates that the localization of Mtm1 to the mitochondria is conserved for *H. polymorpha* when the cells are grown on glucose.

The results from the colony PCR must be taken with caution due to the following reasons. During the first colony PCR there was approximately half an hour to 45 minutes between the preparation of the mixture and the start of the PCR. This might have led to poor results. This is also the reason why a new colony PCR was done for the colonies 6-10 together with an additional 5 colonies with a C-terminal fusion. The second gel showed positive colonies in the 6-10 colonies which showed a negative result in the first gel. This means there were false negatives for the colony PCR. Another reason why these false negatives might have occurred is due to the fact that the cells were only resuspended in water and then used as template DNA for the PCR. Normally protocol B.31 Colony PCR of yeast cells is followed to make spheroplasted cells for colony PCR. This was not done for this study. It might therefore be the case that the denaturation step did not work properly during the PCR, resulting in false negatives.

In the western blot a faint line can be observed right below the expected sizes of the bands for both the N and C. These lines might be due to post translational modifications (PTM), like phosphorylation. Protein phosphorylation result in a band with a slightly higher molecular weight than the expected band, which is exactly what is observed in Figure 2B. It was found by Lanz et al. (2021) that Mtm1 has a phosphorylation site in *S. cerevisiae*. At S7, a phosphorylated residue was found (Lanz et al., 2021). This phosphorylation site is not exactly conserved in *H. polymorpha*, however around the same region other serines can be found (Figure S2). Prediction of serine phosphorylation sites was done (Figure S3), which showed possible serine phosphorylation sites around the same region at the N-terminal site of Mtm1. To further investigate if this double band is due to phosphorylation additional research must be done, due to the lack of information on post translational modifications in *H. polymorpha*. A way to determine if Mtm1 is phosphorylated is by tandem mass spectrometry (MS/MS). This allows identification of specific phosphorylation sites (Taracha et al., 2017).

The western blot detection using HRP showed the same results for N, however, it was unable to show the faint band for C. This is due to the thicker band being too intense to visualize the faint band

even at a very low exposure time. Furthermore, the positive control MPC2 had a very high intensity. This made the visualization using HRP very difficult. With a low exposure time the PC already showed a thick band. Due to this reason too long exposure was impossible. This made it very difficult to detect the blot in this way, and it is therefore not used to draw conclusions. The HRP detected western blot results, and its Ponceau S staining results, can be found in the Appendix (Figure S1).

The location of the Mtm1 fused to meGFP is not the same for the N- and C-terminal fusion for the cells grown on methanol. The bright spots of the C-terminal fusion of meGFP to the Mtm1 are not located at the characteristic location of the mitochondria. The N-terminal fusion of meGFP to Mtm1 does show this mitochondrial pattern, which is also observed in the cells stained with the MitoTracker. To find out where the meGFP is located when the cells are grown on methanol further research needs to be done. To find out if the localization is still on the mitochondria, the MitoTracker Red CMXRos could be used again, this time growing the cells on methanol. The meGFP however, still does not colocalize with the Pex3-mKate2, meaning the Mtm1-meGFP does not localize to the peroxisomes.

The similar meGFP signal intensity for the N and C colony grown on glucose does not correspond to the observed intensity difference for the cells grown on methanol and the cells grown on glucose stained with the Mitotracker Red. The imaging of the glucose grown cells should be duplicated to see if the difference in signal can be seen.

Differences between the N- and C-terminal fusion could be due to potentially masking a N-terminal signal sequence (Palmer & Freeman, 2004). Furthermore, the fusion with meGFP can interfere with the folding of the amino acid chain. This could result in the protein no longer fitting in the membrane, changing its native location (Palmer & Freeman, 2004).

Due to the limited options of mitotrackers it was not possible to visualize both the Pex3-mKate2 and the Mitotracker in the same picture. The fluorescence signals of the fluorophores are too close together to be visualized individually. The intensity of the MitoTracker Red CMXRos was way higher than the Pex3-mKate2 signal. This resulted in pictures only showing the MitoTracker and not the peroxisomes.

A major strength of this study is the fact that both a fusion of meGFP at the N-terminal site of Mtm1 as well as one at the C-terminal site of Mtm1 was constructed. By doing this it was possible to draw conclusions based on both images. It is possible the fusion of meGFP obstructs the native localization of a protein. This effect can change regarding the location of the fusion (Palmer & Freeman, 2004). This is why it is important to look at both options. Additionally, both fusions were imaged the confocal LSM800 Airyscan confocal microscope. This microscope makes high resolution images of the fluorescently labeled cells.

The fluorescence microscopy results do need to be taken with caution due to the use of a strong promoter resulting in overexpression of the gene. Since Mtm1 is a mitochondrial membrane protein, overexpression might lead to morphological changes of the mitochondria. A strong promoter also results in a higher fluorescence intensity, which might overshadow any weaker fluorescence signal present in the cell. To further investigate if the Mtm1 might still be located on the peroxisome, its own promoter might be used to tackle the overexpression. This might however result in the lack of any fluorescence.

Further investigation into the transport of PLP to the peroxisome is needed, since the Mtm1 transporter did not prove to be in the peroxisome and it can therefore not be responsible for the transport. The main lesson is that intracellular trafficking of PLP is still poorly understood and that

Mtm1 is not the answer to this question. It is still even unclear whether free PLP is imported into peroxisomes (Chornyi et al., 2021). As mentioned before there are some arguments suggesting PLP might be transported into the peroxisome. These being Bud16, a key enzyme for PLP synthesis being located peroxisome (Yifrach et al., 2022), and enzyme Aat2, also located at the peroxisome (Thomas et al., 2018) which has PLP as cofactor. This highly suggests there must be PLP transport into the peroxisome. Further work on the transport of PLP into the peroxisome might lead to better understanding transport in and out of the peroxisome in general, and might lead to the discovery of novel peroxisomal transporter proteins, achieving this studies goal, even if it is not Mtm1.

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Appendix

Table S1*: E. coli* and *H. polymorpha* strains used in this study.

Strains	**Characteristics**
E. coli DH5α	^{F}-φ80*lac*ZΔM15 Δ(*lacZYA-argF*)U169 *rec*A1 *end*A1 *hsd*R17(rK–, mK+) *pho*A *sup*E44 λ–*thi*-1 *gyr*A96 *rel*A1
H. polymorpha Δ*ku80* (leu1.1)	NCYC495, *leu1.1 YKU80::URA3*
H. polymorpha yku80 + *PEX3-mKATE2* (WT)	NCYC495, *leu1.1 YKU80*::*URA3* + *PEX3-mKATE2*
WT + *meGFP- MTM1*	*YKU80::URA3* + *PEX3-mKATE2* + pHIPZ18 *meGFP- MTM1*
WT + *MTM1-meGFP*	*YKU80::URA3* + *PEX3-mKATE2* + pHIPZ18 *MTM1-meGFP*

Table S2: Plasmids used in this study.

Plasmid name	**Description**
pHIPZ18 *meGFP-W1Q7X1*	pHIP vector, *W1Q7X1* expressed from the P_{ADH1} promoter with N-terminal meGFP fusion, containing Amp^{R} , Zeo^{R},

pHIPZ18 *W1Q7X1-meGFP*	pHIP vector, *W1Q7X1* expressed from the P_{ADH1} promoter with C-terminal meGFP fusion, containing Amp^R , Zeo^R,
pHIPZ18 *meGFP-MTM1*	pHIP vector, *MTM1* expressed from the P_{ADH1} promoter with N-terminal meGFP fusion, containing Amp^R , Zeo^R,
pHIPZ18 *MTM1-meGFP*	pHIP vector, *MTM1* expressed from the P_{ADH1} promoter with C-terminal meGFP fusion, containing Amp^R , Zeo^R,

Table S3: Primers used in this study. The primers came from the company Biolegio.

Primer	Sequence 5' – 3'
MTM1-N-fw	CGCGGATCCATGTCGCAAATAGAACTGATAG
MTM1-N-rev	CCGCTCGAGTTATAACATGTCTGCAAATAATTTTTTGC
MTM1-C-fw	CCCAAGCTTATGTCGCAAATAGAACTGATAG
MTM1-C-rev	CGCGGATCCTAACATGTCTGCAAATAATTTTTTGC
cPCR Padh-fw	CACGGCAGCAGAATTGGAATTG
mGFP rev check	AAGTCGTGCTGCTTCATGTG
primer Seq GFP Fw	TGCCCGACAACCACTACCTG
primer Tamo rev	TGATGCCTACAGACCAGATG
pHIPN18 Fw	CCCACTCGGAGTAAAGCAA

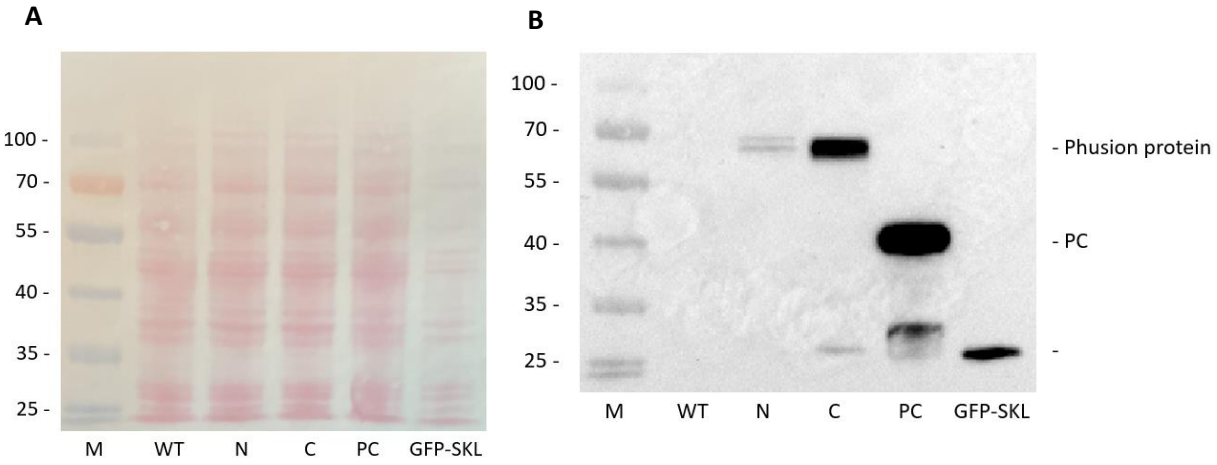

Figure S1: The Ponceau S stain and HRP detection of the second western blot. Abbreviations: M is the PageRuler Plus Prestained Protein Ladder from Thermo Fisher Scientific, WT corresponds to the WT from Table S1, N and C are WT + *meGFP-MTM1* and WT + *MTM1-meGFP* (Table S1) and PC is the positive control MPC2-GFP. The protein sizes on the left side of the blots are given in kDa. (A) The staining with Ponceau S stain of the western blot. (B) The HRP detection of the western blot. The western blot was developed using primary antibody αGFP mouse mitochondrial anti-GFP and the secondary antibody Goat anti-Mouse HRP-conjugate. The exposure time was 61.5 seconds merged with the white image of the blot. The image was made using the Bio-Rad molecular imager ChemiDoc RXS⁺ imaging system.

```
S.cerevisiae      ---------------MSDRNTSNSLTLKERMLSAGAGSVLTSLILTPMDVVRIRLQQQQM
H.polymorpha      MSQIELIEDATLPRLESRPQPGSDITITQRMLSACTGSLLTSLVVTPFDVVRIRLQQQQL
                                 *  :....:*:.:***** :**:****::**:***********:

S.cerevisiae      IPDCSCDGAAEVPNAVSSGSKMKTFTNVGGQNLNNAKIFWESACFQELHCKNSSLKFNGT
H.polymorpha      LFPPHFRQTATCCKKVFWEDATRPSK----------------DYFCSSNACAQELKINGT
                  :       :*   : *   .  :. .                  * . :.  ..**:***
```

```
S.cerevisiae       LEAFTKIASVEGITSLWRGISLTLLMAIPANMVYFSGYEYIRDVSPIASTYPTLNPLFCG
H.polymorpha       FSGLSKIAVNEGVFTLYRGLSLMLIMAVPSNMVYFSGYEYLRDRSPLKNQFPIFNPLLCG
                   :..::***  **: :*:**:** *:**:*:**********:** **: . :* :***:**

S.cerevisiae       AIARVFAATSIAPLELVKTKLQSIPRSSKSTKTWMMVKDLLNETRQEMKMVGPSRALFKG
H.polymorpha       SFARIMAATVVAPLELIKTRLQAVPTSSSTSSEIMKMVVTNSFKEVQNKGFFS---LFKG
                   ::**::*** :*****:**:**::* **.::.  * :    . .. : * . .   ****

S.cerevisiae       LEITLWRDVPFSAIYWSSYELCKERLWLDSTRFASKDANWVHFINSFASGCISGMIAAIC
H.polymorpha       LQLTLWRDVPFSGIYWSSYEYLNDRLQRLQIFSSPDHQHAEIFARSFISGSLSGVLAAIF
                   *::*********.*******  ::**   .   :... :   * .** **.:**::***

S.cerevisiae       THPFDVGKTRWQISMMNNSDPKGGNRSR----NMFKFLETIWRTEGLAALYTGLAARVIK
H.polymorpha       TNPFDVGKTRLQVSLEDAGSLNKQVNSKSTKESMFKSLHTIYKNEGMSSLFVGLAPRCLK
                   *:******** *:*: : .. :   .*:    .*** *.**::.**:::*:.***.* :*

S.cerevisiae       IRPSCAIMISSYEISKKVFGNKLHQ
H.polymorpha       IAPSCAIMISTYEISKKLFADML--
                   * ********:******:*.: *
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Figure S2: alignment of the sequence of Mtm1 in *S. cerevisiae* and the sequence of Mtm1 in *H. polymorpha.* The highlighted S is the phosphorylated residue (Lanz et al., 2021). Alignment was made using Multiple Sequence Alignment by CLUSTALW. The sequence of *H. polymorpha* was taken from the genomic *MTM1* gene. The *S. cerevisiae* sequence came from Saccharomyces Genome Database (SGD, http://www.yeastgenome.org/).

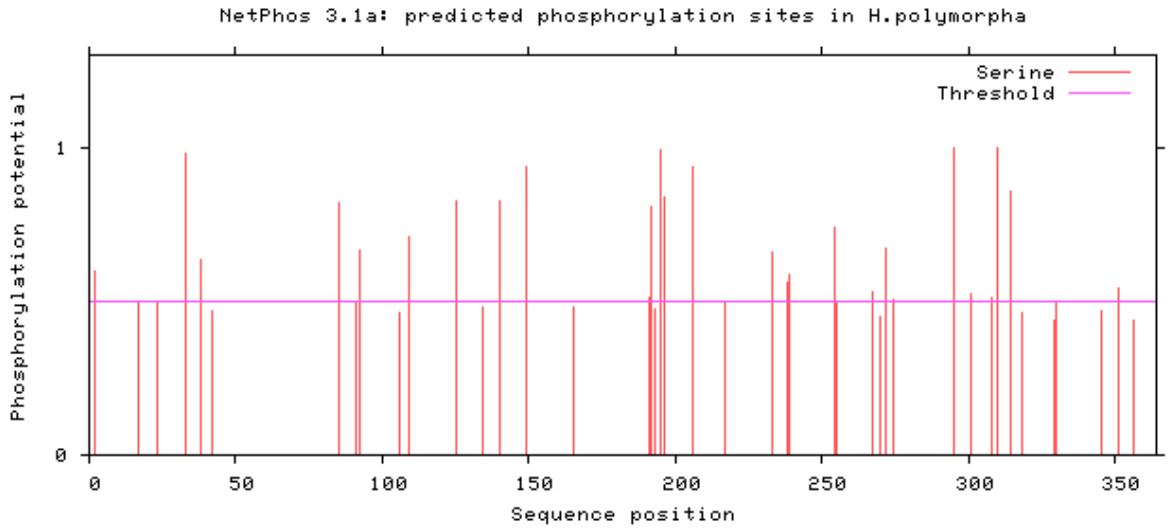
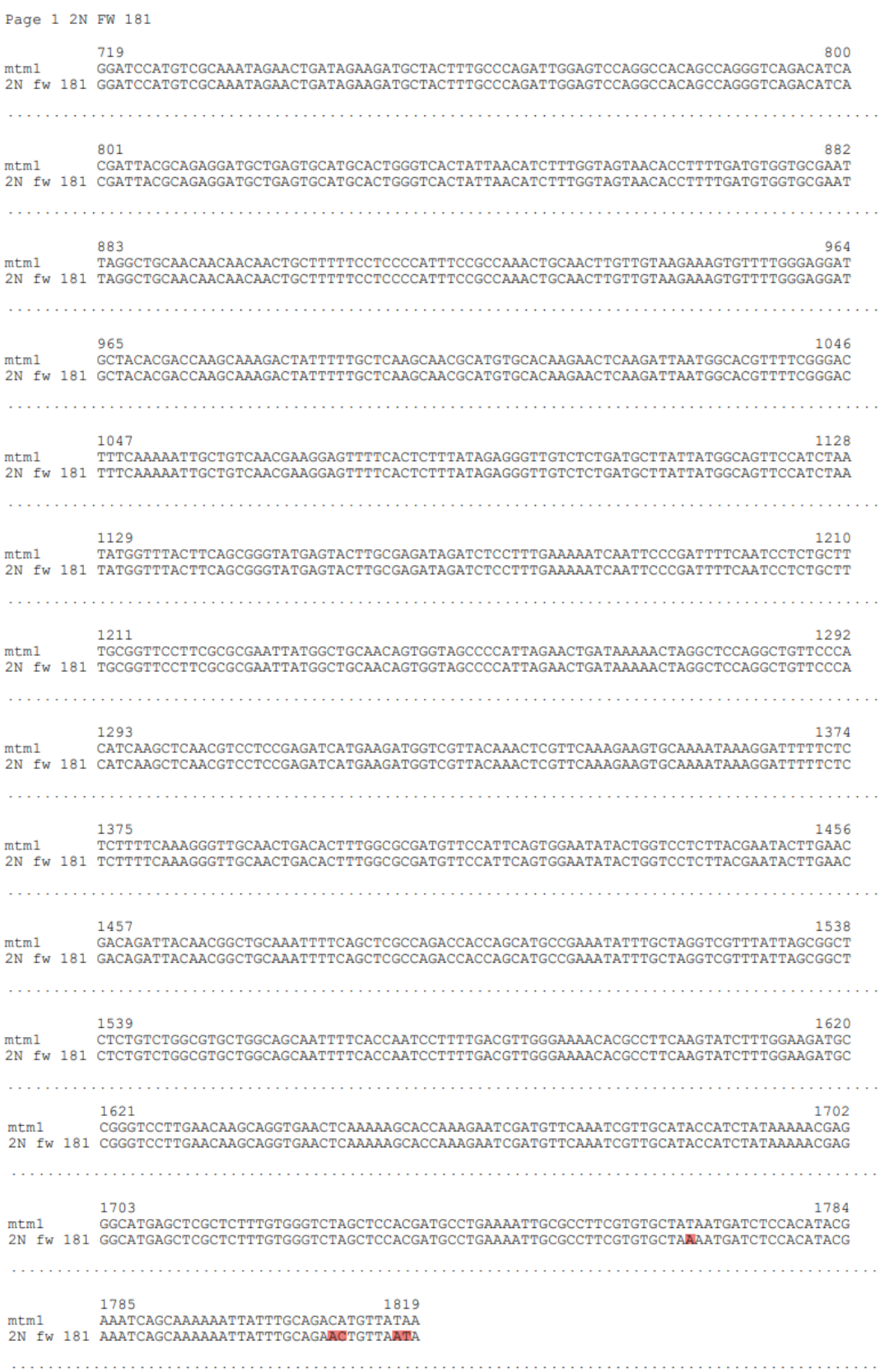


Figure S3: Predicted Serine phosphorylation sites in *H. polymorpha MTM1.* These predictions were made using the website from DTU Health Tech (Blom et al., 1999), with the same data as Figure S3.


Page 1 2N FW 181
719 800
mtm1 GGATCCATGTCGCAAATAGAACTGATAGAAGATGCTACTTTGCCCAGATTGGAGTCCAGGCCACAGCCAGGGTCAGACATCA
2N fw 181 GGATCCATGTCGCAAATAGAACTGATAGAAGATGCTACTTTGCCCAGATTGGAGTCCAGGCCACAGCCAGGGTCAGACATCA
801 882
mtm1 CGATTACGCAGAGGATGCTGAGTGCATGCACTGGGTCACTATTAACATCTTTGGTAGTAACACCTTTTGATGTGGTGCGAAT
2N fw 181 CGATTACGCAGAGGATGCTGAGTGCATGCACTGGGTCACTATTAACATCTTTGGTAGTAACACCTTTTGATGTGGTGCGAAT
883 964
mtm1 TAGGCTGCAACAACAACAACTGCTTTTTCCTCCCCATTTCCGCCAAACTGCAACTTGTTGTAAGAAAGTGTTTTGGGAGGAT
2N fw 181 TAGGCTGCAACAACAACAACTGCTTTTTCCTCCCCATTTCCGCCAAACTGCAACTTGTTGTAAGAAAGTGTTTTGGGAGGAT
965 1046
mtm1 GCTACACGACCAAGCAAAGACTATTTTTGCTCAAGCAACGCATGTGCACAAGAACTCAAGATTAATGGCACGTTTTCGGGAC
2N fw 181 GCTACACGACCAAGCAAAGACTATTTTTGCTCAAGCAACGCATGTGCACAAGAACTCAAGATTAATGGCACGTTTTCGGGAC
1047 1128
mtm1 TTTCAAAAATTGCTGTCAACGAAGGAGTTTTCACTCTTTATAGAGGGTTGTCTCTGATGCTTATTATGGCAGTTCCATCTAA
2N fw 181 TTTCAAAAATTGCTGTCAACGAAGGAGTTTTCACTCTTTATAGAGGGTTGTCTCTGATGCTTATTATGGCAGTTCCATCTAA
1129 1210
mtm1 TATGGTTTACTTCAGCGGGTATGAGTACTTGCGAGATAGATCTCCTTTGAAAAATCAATTCCCGATTTTCAATCCTCTGCTT
2N fw 181 TATGGTTTACTTCAGCGGGTATGAGTACTTGCGAGATAGATCTCCTTTGAAAAATCAATTCCCGATTTTCAATCCTCTGCTT
1211 1292
mtm1 TGCGGTTCCTTCGCGCGAATTATGGCTGCAACAGTGGTAGCCCCATTAGAACTGATAAAAACTAGGCTCCAGGCTGTTCCCA
2N fw 181 TGCGGTTCCTTCGCGCGAATTATGGCTGCAACAGTGGTAGCCCCATTAGAACTGATAAAAACTAGGCTCCAGGCTGTTCCCA
1293 1374
mtm1 CATCAAGCTCAACGTCCTCCGAGATCATGAAGATGGTCGTTACAAACTCGTTCAAAGAAGTGCAAAATAAAGGATTTTTCTC
2N fw 181 CATCAAGCTCAACGTCCTCCGAGATCATGAAGATGGTCGTTACAAACTCGTTCAAAGAAGTGCAAAATAAAGGATTTTTCTC
1375 1456
mtm1 TCTTTTCAAAGGGTTGCAACTGACACTTTGGCGCGATGTTCCATTCAGTGGAATATACTGGTCCTCTTACGAATACTTGAAC
2N fw 181 TCTTTTCAAAGGGTTGCAACTGACACTTTGGCGCGATGTTCCATTCAGTGGAATATACTGGTCCTCTTACGAATACTTGAAC
1457 1538
mtm1 GACAGATTACAACGGCTGCAAATTTTCAGCTCGCCAGACCACCAGCATGCCGAAATATTTGCTAGGTCGTTTATTAGCGGCT
2N fw 181 GACAGATTACAACGGCTGCAAATTTTCAGCTCGCCAGACCACCAGCATGCCGAAATATTTGCTAGGTCGTTTATTAGCGGCT
1539 1620
mtm1 CTCTGTCTGGCGTGCTGGCAGCAATTTTCACCAATCCTTTTGACGTTGGGAAAACACGCCTTCAAGTATCTTTGGAAGATGC
2N fw 181 CTCTGTCTGGCGTGCTGGCAGCAATTTTCACCAATCCTTTTGACGTTGGGAAAACACGCCTTCAAGTATCTTTGGAAGATGC
1621 1702
mtm1 CGGGTCCTTGAACAAGCAGGTGAACTCAAAAAGCACCAAAGAATCGATGTTCAAATCGTTGCATACCATCTATAAAAACGAG
2N fw 181 CGGGTCCTTGAACAAGCAGGTGAACTCAAAAAGCACCAAAGAATCGATGTTCAAATCGTTGCATACCATCTATAAAAACGAG
1703 1784
mtm1 GGCATGAGCTCGCTCTTTGTGGGTCTAGCTCCACGATGCCTGAAAATTGCGCCTTCGTGTGCTATAATGATCTCCACATACG
2N fw 181 GGCATGAGCTCGCTCTTTGTGGGTCTAGCTCCACGATGCCTGAAAATTGCGCCTTCGTGTGCTAAAATGATCTCCACATACG
1785 1819
mtm1 AAATCAGCAAAAAATTATTTGCAGACATGTTATAA
2N fw 181 AAATCAGCAAAAAATTATTTGCAGAACTGTTAATA

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           332                                                                 413
mtm1       GGATCCATGTCGCAAATAGAACTGATAGAAGATGCTACTTTGCCCAGATTGGAGTCCAGGCCACAGCCAGGGTCAGACATCA
2N rev 182 GGATCCATWTCGCAAATAGAACTGATAGAAGATGCTACTTTGCCCAGATTGGAGTCCAGGCCACAGCCAGGGTCAGACATCA

           414                                                                 495
mtm1       CGATTACGCAGAGGATGCTGAGTGCATGCACTGGGTCACTATTAACATCTTTGGTAGTAACACCTTTTGATGTGGTGCGAAT
2N rev 182 CGATTACGCAGAGGATGCTGAGTGCATGCACTGGGTCACTATTAACATCTTTGGTAGTAACACCTTTTGATGTGGTGCGAAT

           496                                                                 577
mtm1       TAGGCTGCAACAACAACAACTGCTTTTTCCTCCCCATTTCCGCCAAACTGCAACTTGTTGTAAGAAAGTGTTTTGGGAGGAT
2N rev 182 TAGGCTGCAACAACAACAACTGCTTTTTCCTCCCCATTTCCGCCAAACTGCAACTTGTTGTAAGAAAGTGTTTTGGGAGGAT

           578                                                                 659
mtm1       GCTACACGACCAAGCAAAGACTATTTTTGCTCAAGCAACGCATGTGCACAAGAACTCAAGATTAATGGCACGTTTTCGGGAC
2N rev 182 GCTACACGACCAAGCAAAGACTATTTTTGCTCAAGCAACGCATGTGCACAAGAACTCAAGATTAATGGCACGTTTTCGGGAC

           660                                                                 741
mtm1       TTTCAAAAATTGCTGTCAACGAAGGAGTTTTCACTCTTTATAGAGGGTTGTCTCTGATGCTTATTATGGCAGTTCCATCTAA
2N rev 182 TTTCAAAAATTGCTGTCAACGAAGGAGTTTTCACTCTTTATAGAGGGTTGTCTCTGATGCTTATTATGGCAGTTCCATCTAA

           742                                                                 823
mtm1       TATGGTTTACTTCAGCGGGTATGAGTACTTGCGAGATAGATCTCCTTTGAAAAATCAATTCCCGATTTTCAATCCTCTGCTT
2N rev 182 TATGGTTTACTTCAGCGGGTATGAGTACTTGCGAGATAGATCTCCTTTGAAAAATCAATTCCCGATTTTCAATCCTCTGCTT

           824                                                                 905
mtm1       TGCGGTTCCTTCGCGCGAATTATGGCTGCAACAGTGGTAGCCCCATTAGAACTGATAAAAACTAGGCTCCAGGCTGTTCCCA
2N rev 182 TGCGGTTCCTTCGCGCGAATTATGGCTGCAACAGTGGTAGCCCCATTAGAACTGATAAAAACTAGGCTCCAGGCTGTTCCCA

           906                                                                 987
mtm1       CATCAAGCTCAACGTCCTCCGAGATCATGAAGATGGTCGTTACAAACTCGTTCAAAGAAGTGCAAAATAAAGGATTTTTCTC
2N rev 182 CATCAAGCTCAACGTCCTCCGAGATCATGAAGATGGTCGTTACAAACTCGTTCAAAGAAGTGCAAAATAAAGGATTTTTCTC

           988                                                                1069
mtm1       TCTTTTCAAAGGGTTGCAACTGACACTTTGGCGCGATGTTCCATTCAGTGGAATATACTGGTCCTCTTACGAATACTTGAAC
2N rev 182 TCTTTTCAAAGGGTTGCAACTGACACTTTGGCGCGATGTTCCATTCAGTGGAATATACTGGTCCTCTTACGAATACTTGAAC

           1070                                                               1151
mtm1       GACAGATTACAACGGCTGCAAATTTTCAGCTCGCCAGACCACCAGCATGCCGAAATATTTGCTAGGTCGTTTATTAGCGGCT
2N rev 182 GACAGATTACAACGGCTGCAAATTTTCAGCTCGCCAGACCACCAGCATGCCGAAATATTTGCTAGGTCGTTTATTAGCGGCT

           1152                                                               1233
mtm1       CTCTGTCTGGCGTGCTGGCAGCAATTTTCACCAATCCTTTTGACGTTGGGAAAACACGCCTTCAAGTATCTTTGGAAGATGC
2N rev 182 CTCTGTCTGGCGTGCTGGCAGCAATTTTCACCAATCCTTTTGACGTTGGGAAAACACGCCTTCAAGTATCTTTGGAAGATGC

           1234                                                               1315
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2N rev 182 CGGGTCCTTGAACAAGCAGGTGAACTCAAAAAGCACCAAAGAATCGATGTTCAAATCGTTGCATACCATCTATAAAAACGAG

           1316                                                               1397
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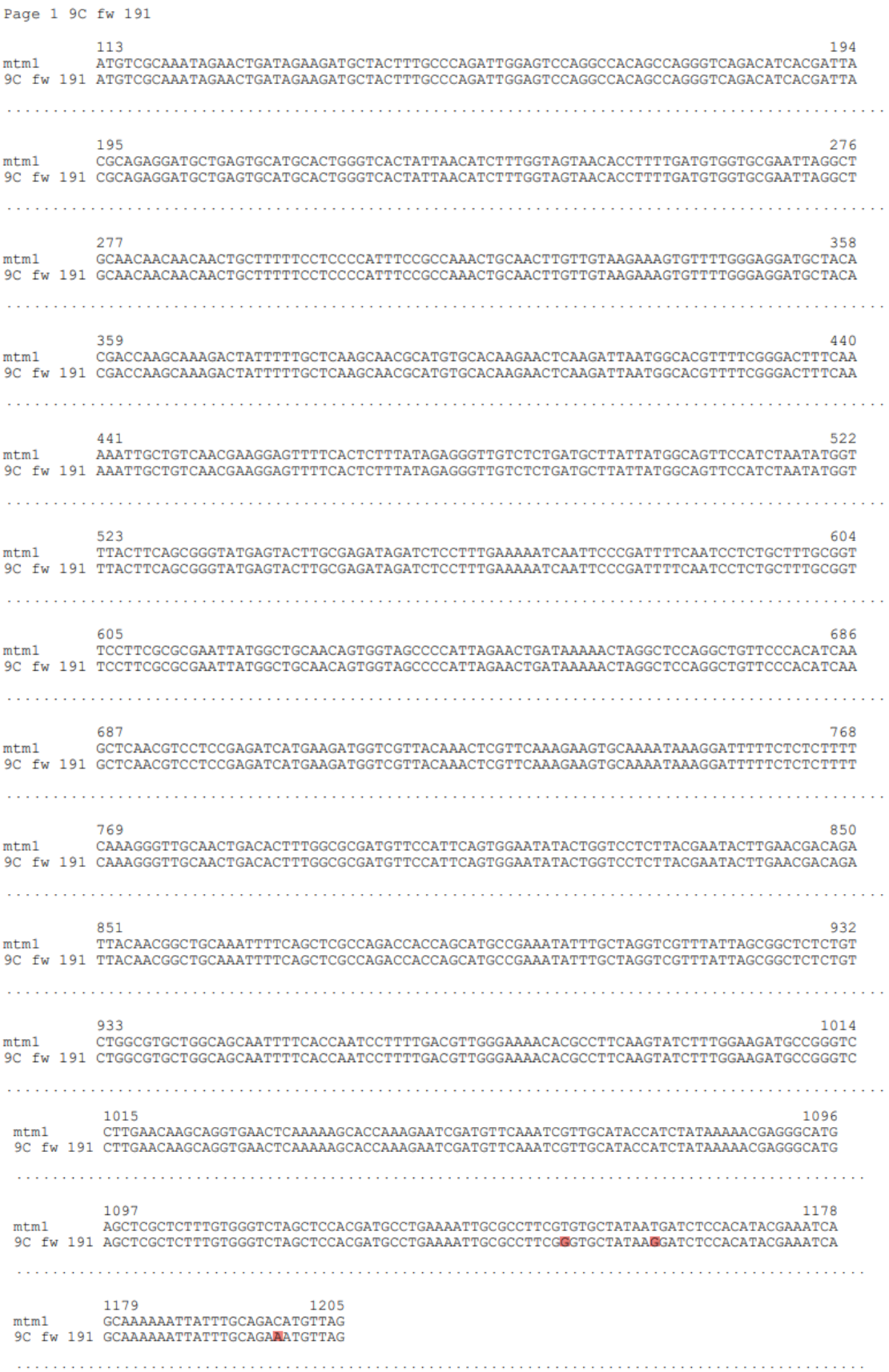
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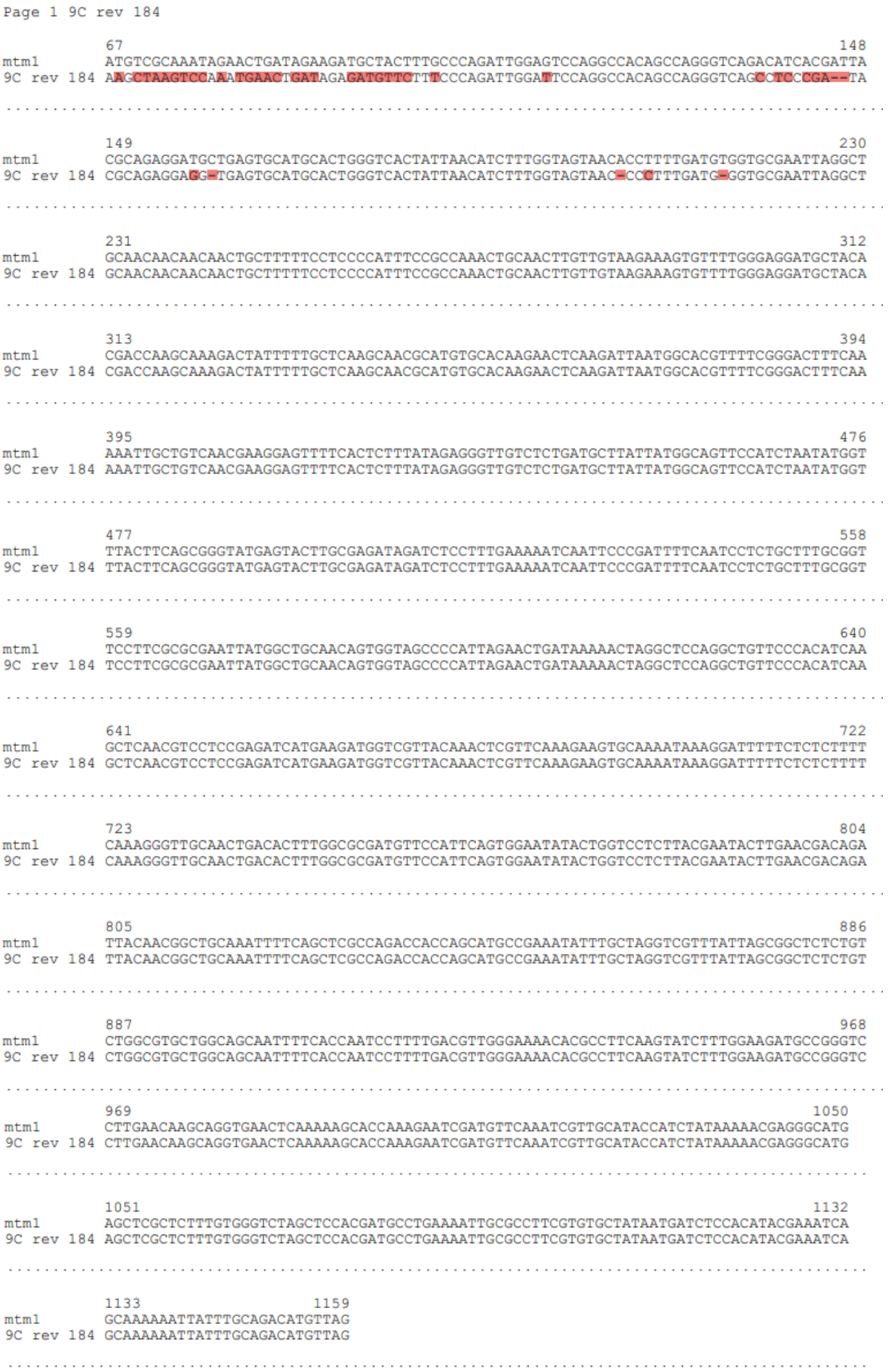

Figure S4: Sequencing data alignment with the *MTM1* gene. 2N forward, 2N reverse 9C forward and 9C reverse can be found below. Highlighted bases are mismatches.