The location of the Fun26 transporter in H. polymorpha

Research Project Molecular Life Sciences

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Contents

Abstract

In this research bachelor project, we investigated if the Fun26 transporter is also located in the peroxisomal membrane. Originally is the Fun26 transporter a vacuolar membrane transporter. A mass spectrometry study was done on protein transcription levels and the abundance of proteins in a WT *baker's yeast* strain and a *pex3 baker's yeast* strain. The protein transcription levels and protein abundance levels are compared to each other for both strains. The *pex3* strain has no functional peroxisomes present in the cells and has a reduction in the protein abundance level of the Fun26 transporter compared to the WT strain whereas the transcription levels of the Fun26 protein were roughly the same for both strains. Fun26 is a transporter of nucleosides and nucleobases which are used in peroxisomes as substrate by peroxisomal enzymes. The *MEGFP-FUN26* gene (N-terminal fusion) and the *FUN26-MEGFP* gene (C-terminal fusion) were transformed into the genome of *H. polymorpha ku80 PEX3-MKATE2.* Fluorescence microscopy was done to colocalize the meGFP fusion proteins with Fun26 and Pex3-mKate2 (if this is the case). Western blot was also done to see if the meGFP fusion proteins with Fun26 are present in the strains. There is no colocalization between meGFP and mKate2 so the Fun26 transporter is not localized in the peroxisomal membrane. Observation of meGFP fusion proteins with Fun26 is really difficult to prove and therefore it is difficult to draw conclusions on the location of the Fun26 transporter so further research is needed.

Introduction

Peroxisomes are conserved, intracellular organelles which are present in eukaryotic cells. β-oxidation of fatty acids and the detoxification of hydrogen peroxide, which is produced during fatty acid oxidation by catalase are some of the multiple metabolic pathways housed in peroxisomes. (Farré et al., 2019) Peroxisomes arise from pre-existing peroxisomes, after acquiring the peroxisomal membrane proteins and peroxisomal matrix proteins from the cytosol. The capacity of peroxisomes to import folded proteins into the peroxisomal lumen is crucial to the existence of the peroxisomal organelle. (Plett et al., 2020) Peroxins play an important role in the biogenesis of peroxisomes and also in peroxisome proliferation. Peroxins are also required for import of peroxisomal matrix proteins and for targeting of peroxisomal membrane proteins (PMPs) to the peroxisome membrane. (Farré et al., 2019) Peroxin 3 (Pex3) is a crucial peroxin and also a peroxisomal membrane protein (PMP)

related to locating other peroxisomal membrane proteins in the membrane of peroxisomes. (Yamashita et al., 2014)

Peroxisomes lack protein synthesis machinery and therefore peroxisomal transporters are very important to the functions of peroxisomes. (Plett et al., 2020) Many biochemical reactions are housed in peroxisomes and therefore peroxisomes are dependent on a wide range of cofactors, like ATP, CoA and NAD. (Plett et al., 2020) A large number of chemically diverse compounds must enter the peroxisome and products formed must be transported out of the peroxisome. (Rottensteiner et al., 2006) Membrane contact sites (MCSs) between peroxisomes and other organelles is a way of transport used for non-vesicular transport. (Rottensteiner et al., 2006) Another way of transport is via nonspecific porin-like channels that allows free diffusion of low-molecular weight compounds with a broad substrate specificity (Plett et al., 2020). Transporter members of the ABC transporter superfamily are involved in peroxisomal import of β-oxidation substrate, Pxa1 and Pxa2 being the most important in this process. (Rottensteiner et al., 2006) Adenine nucleotide transporter (Ant1) is the best characterized peroxisomal transporter in *S. cerevisiae*, this transporter is required for activation of medium chain fatty acids. Cross-membrane traffic of adenine nucleotides (ATP in, AMP out) is catalyzed by Ant1, which is a member of the mitochondrial carrier family. (Rottensteiner et al., 2006)

A study was done on the influence of the absence of functional peroxisomes in *S. cerevisiae* yeast cells. (Kosir et al., 2024) Pex3 is important to form peroxisomes since it is necessary for localizing membrane bound peroxins and PMPs. (Yamashita et al., 2014) The study revealed that absence of the *PEX3* gene results in no functional peroxisomes. (Kosir et al., 2024) Pex3 is crucial for targeting peroxisomal membrane proteins into the membrane and absence of this gene results in no target for these membrane proteins and therefore the proteins will not reach and form the peroxisomal membrane. (Kosir et al., 2024) A wild-type *S. cerevisiae* strain and a peroxisome-deficient *S. cerevisiae pex3* strain were compared to each other based on cellular proteins levels; protein levels are obtained with mass spectrometry. In *pex3* cells were several non-peroxisomal transcripts and proteins with a change in abundance, also the Fun26. (Kosir et al., 2024) The protein level of Fun26 is significantly lower in the *pex3* strain compared to the wild-type strain, whereas the transcription levels were in both strains the same. (Kosir et al., 2024). This is an indication for degradation of the Fun26 transporter. This is interesting since the absence of functional peroxisomes goes together with the drop in protein levels of the Fun26 transporter. (Kosir et al., 2024)

Fun26 is a nucleoside and nucleobase transporter with a broad selectivity and high affinity normally located in yeast vacuolar membranes. Fun26 is also a member of the equilibrative nucleoside transporter (ENT) family, these are polytopic integral membrane proteins that transport nucleobases and nucleosides across membranes. (Boswell-Casteel et al., 2014) Interestingly, the substrates transported by the Fun26 are also compounds which are utilized by two peroxisomal enzymes: ScNPY1 is a peroxisomal NADH pyrophosphatase which converts NAD(H) to AMP and nicotinamide mononucleotide. (AbdelRaheim et al., 2001) The mechanism to generate a NAD pool inside a peroxisome is also still unknown, making Fun26 an interesting possibility for this mechanism (Plett et al., 2020). The second peroxisomal enzyme is ScPnc1, this is a peroxisomal nicotinamidase. (Effelsberg et al., 2015)

In this project we will investigate if the Fun26 transporter is also present in the peroxisomal membrane. We will do this by making fusion proteins with meGFP fused to the N- and C-terminal of the Fun26 transporter. The used promoter for this fusion gene is the Padh1 promoter which is a strong promoter. A strong promoter is used to see really overexpression of the fusion protein gene, this was done to really see the fusion protein with fluorescence microscopy and the Western blot. The genes for these fusion proteins are transformed to the genome of *ku80 PEX3-MKATE2* yeast strains to see colocalization between Pex3 and Fun26 when using fluorescence microscopy (when

present). This particular strain is used because the transformed DNA of interest is now inserted in the right place in the genome. Western blot is also done to see the size in kDa's of the meGFP fusion proteins present in the created strains. It turns out there is no colocalization between the meGFP (Fun26) and mKate2 (Pex3) so there are no Fun26 transporters present in the peroxisomal membrane based on the finding of this project.

Materials and methods

Cloning of the *MEGFP* fusion gene using the *E. coli DH5α* strain

The GeneJET Plasmid Miniprep Kit was performed to prepare high quality plasmid DNA from *E. coli DH5α* strain with the plasmid of interest. The plasmids of interest have a Padh1 promoter which is a strong promoter resulting in overexpression of meGFP-W1Q7X1 or W1Q7X1-meGFP depending on which plasmid is present in the *E. coli DH5α* strain. pHIPZ18 *MEGFP-W1Q7X1* and pHIPZ18 *W1Q7XI-MEGFP* are the two plasmids of interest, also both plasmids have a gene for ampicillin and zeocin resistance. The protocol can be found on the Thermo Fisher Scientific site (Thermo Fisher Scientific, 2015). Nanodrop was done afterwards to check the concentrations of the plasmid DNA in ng/µL, based on the concentrations of the plasmid DNA, a restriction reaction was done to check if the pHIPZ18 *MEGFP-W1Q7X1* and pHIPZ18 *W1Q7XI-MEGFP* plasmids were really present. For the sample with the pHIPZ18 *MEGFP-W1Q7X1* vector, 2 µL of the vector was added to 14 µL Milli Q water, 2 µL buffer (10X) and 2 µL of the restriction enzyme duo (1 µL of both). For the sample with the pHIPZ18 *W1Q7XI-MEGFP* vector, 3 µL of the vector was added to 13 µL Milli Q water, 2 µL buffer (10X) and 2 µL of the restriction enzyme duo (1 µL of both restriction enzymes). Restriction enzyme duo 1 is: BamHI and HindIII and restriction enzyme duo 2 is: BglII and PstI, both restriction enzyme duo's were added to both plasmids so a total of 4 samples. These four restriction enzymes are all FastDigest and from Thermo Scientific. Restriction of the 4 samples was done for 1,5 hours at 37 degrees. Agarose gel for electrophoresis was prepared to check if the restriction fragments had the same size like we expected. 1000 milliliter 1X TAE was prepared by adding 100 milliliter 10X TAE to 900 milliliter demiwater. To 500 milliliter 1X TAE 5 grams of agarose was added (1% gel) and 4 µL of DNA stain G. The rest of the agarose gel electrophoresis protocol was done according to protocol on Addgene (Agarose Gel Electrophoresis, 2018). PCR was done according to '01-PCR of fragments for cloning' which can be found in the protocol book for Molecular Cell Biology. Genomic DNA of the *H. polymorpha ku80* was used as template DNA to get the *FUN26* gene as PCR fragment. The primer duo's used are: FUN26-C-Fw primer + FUN26-C-rev primers and the FUN26-N-Fw + FUN26-N-rev primers because we have N-terminal and C-terminal fusion of *MEGFP* to the *FUN26* gene. More information on the primers can be found in the Appendix section. Afterwards agarose gel electrophoresis was done to check if the *FUN26* gene was present in the PCR reaction samples. *FUN26* was present and PCR purification was done to get the pure *FUN26* PCR fragments. PCR purification was done according to: 'GeneJET PCR purification' which can be found on the Thermo Scientific (Thermo Fisher Scientific, 2015). Medium was made to grow *E. coli DH5α* strains on agar plates according to 'I A-2 Media for growing *E. coli*' which can be found in the protocol book of Molecular Cell Biology. For pouring the agar plates, 3,75 grams of agar and 250 µL ampicillin was added to 250 milliliter LB medium, when this liquid got to a lower temperature, the liquid got to a more like gel phase. An overnight *E. coli DH5α* strain was grown in a sterile flask with 25 milliliter of LB medium, a WT *E. coli DH5α* colony was picked from an agar plate and put in the flask with 25 milliliter of LB medium. Restriction of fragments for ligation was done according to '02 Restriction of fragment for ligation' which can be found in the protocol book of Molecular Cell Biology. For the V1 (pHIPZ18 *MEGFP-W1Q7X1*) restriction reaction: 78 µL milli Q water, 10 µL buffer (10X), 8 µL V1 (2

nanogram), 2 µL of BamHI enzyme and 2 µL of XhoI enzyme. For the V2 (pHIPZ18 *W1Q7XI-MEGFP*) restriction reaction: 76 µL milli Q water, 10 µL buffer (10X), 10 µL V2 (2 nanogram), 2 µL of HindIII enzyme and 2 µL of BglII enzyme. For the PCR1 (*MEGFP* fusion to the N-terminal of *FUN26*) restriction reaction: 66 µL milli Q water, 10 µL buffer (10X), 20 µL PCR product (cleaned by PCR clean-up), 2 µL BamHI enzyme and 2 µL SalI enzyme. For the PCR2 (*MEGFP* fusion to the C-terminal of *FUN26*) restriction reaction: 66 µL milli Q water, 10 µL buffer (10X), 20 µL PCR product (cleaned by PCR clean-up), 2 µL BamHI enzyme and 2 µL SalI enzyme. The used restriction enzymes are FastDigest and from Thermo Scientific. Agarose gel electrophoresis was done to check the lengths of the fragments present in the V1, V2, PCR1 and PCR2 restriction samples. When the fragments with the right sizes werd obtained, purification of the V1, V2, PCR1 and PCR2 restriction fragments was done. The 'GeneJET PCR purification' protocol which can be found on the Thermo Scientific site (Thermo Fisher Scientific, 2015) was used. Ligation of V1 with PCR1 was done and ligation of V2 with PCR2 was done with the 'rapid DNA ligation kit' by Thermo Fisher (Thermo Fisher Scientific, 2015). The '03-ligation' protocol was used and can be found in the protocol book of Molecular Cell Biology. Competent *E. coli DH5α* cells (with ligated plasmid of interest) were made according to 'B.1 E. coli transformation: CaCl2 competent' which can be found in the protocol book of Molecular Cell Biology. YPD was made according to 'B. 4 Electrotransformation of *Hansenula Polymorpha*' YPD plates were also made with agar and zeocin. 3,75 gram of agar and 250 µL zeocin were added to 250 milliliter YPD. Also STM and TE(D) were prepared according to 'B. 4 Electrotransformation of *Hansenula Polymorpha'* which can be found in the protocol book of Molecular Cell Biology. The agar plates with competent *E. coli DH5α* colonies were used to pick single colonies which were used for colony PCR to confirm that the *FUN26* gene was present in the plasmids. 2 master mixes were made to perform the 'DreamTaq DNA Polymerase' which can be found on (Thermo Fisher Scientific, 2015). We need 1 master mix for 5 single meGFP-Fun26 colonies (N-terminal fusion) and 1 master mix for 5 single Fun26-meGFP colonies (C-terminal fusion). 1 master mix had volume for 6 reactions and has a total volume of 150 µL (25 µL per reaction). 1 master mix contains: 15 µL 10X DreamTaq buffer, 3 µL dNTP Mix 10mM, 1 µL Fw. primer, 1 µL Rev. primer, template DNA (single colony picked from agar plates with competent *E. coli* colony), 1 µL DreamTaq DNA polymerase and 129 µL water (nuclease-free). The master mix for the N-terminal fusion colonies contained the FUN26-N-Fw primer and the FUN26-N-rev primer whereas the master mix for the C-terminal fusion colonies contained the FUN26-C-Fw primer and the FUN26-C-rev primer. Agarose gel electrophoresis was done with the DreamTaq DNA polymerase samples after colony PCR. Based on the results of the agarose gel electrophoresis, colonies with the insert of interest (*FUN26*) were picked to grow overnight in LB medium + ampicillin in a shaker at 37 degrees in a tube. The 'GeneJET Plasmid Miniprep Kit' was performed according to the protocol which can be found on the Thermo Scientific site (Thermo Fisher Scientific, 2015). Pellet of the N-terminal fusion colony N2 and N3 were used for the plasmid miniprep and pellet of the C-terminal fusion colony C1 and C5 were also used. Nanodrop was done to check the DNA concentrations of N2, N3, C1 and C5. FastDigest of the 4 colonies with different duo's of restriction enzymes was done according to the protocol 'Fast digest protocol for vectors'. One fast digest sample contains 2 µL vector DNA (when the DNA concentration is ~300 ng/ µL), 2 µL 10X buffer, 14 µL milli Q water and 2 µL of the restriction enzyme duo (1 µL of each restriction enzyme). The N-fusion colonies had 2 duo's: BamHI + XhoI and HindIII + ApaI. The C-fusion colonies had 2 duo's: HindIII + BglI and HindIII + ApaI. Used enzymes were FastDigest restriction enzymes and are from Thermo Scientific. This resulted in 8 FastDigest samples in total which can be stored at a heath block of 37 degrees for 5 minutes. Agarose gel electrophoresis was done to check if the fragments were in line with the size of the fragments which were expected. The N2 and C1 gave the best results and were used for sequencing of the *FUN26* gene. The sequencing samples for N2 were prepared in the following way: 1.25 µL of concentrated plasmid DNA, 3.75 µL milli Q water and 5 µL of one primer.

SeqGFP Fw. and Tamo rev. were the two primers, so this resulted in 2 sequencing samples. The sequencing samples for C1 were prepared in the following way: 1.5 µL of concentrated plasmid DNA, 3.75 µL milli Q water and 5 µL primer. pHIPN18 Fw. and mGFP rev check were the two primers, so this resulted in 2 sequencing samples. All the primers can be found in the appendix section.

Cloning using *H. polymorpha* strains

Linearization of the plasmid with the right insert (*FUN26*) was done according to the '02-Restriction of fragment for ligation' protocol with the BshTI restriction enzyme (not FastDigest, Thermo Scientific), the protocol can be found in the protocol book for Molecular Cell Biology. The BshTI restriction site is present in the promoter so this makes sure that the fusion proteins of the meGFP to the Fun26 protein were expressed. Agarose gel electrophoresis was done with the linearized fragments to see if the fragments were linear, when this is the case, the DNA was purified after to get purified linear fragments which can be used for insertion in the genome of *H. polymorpha ku80 PEX3-MKATE2*. Purification of the linearized plasmid of the N2 and C1 colony was done according to the 'GeneJET PCR Purification Kit' protocol which can be found on the Thermo Scientific site (Thermo Fisher Scientific, 2015). Instead of 50 µL elution buffer, we used 30 µL to get a higher concentration of the linearized plasmid DNA. Afterwards a nanodrop was done to check the concentrations of the purified linearized plasmid DNA of colony N2 and C1. Growing of *H. polymorpha ku80 PEX3-MKATE2* cells was done to create in the end competent cells according to the protocol for competent cells 'B.4 Electrotransformation of *Hansenula Polymorpha*' which can be found in the protocol book for Molecular Cell Biology. The transformation step of the linearized plasmid DNA into the genomic DNA of *H. polymorpha ku80 PEX3-MKATE2* was done according to the transformation protocol 'B.4 Electrotransformation of *Hansenula Polymorpha*' which can be found in the protocol book of Molecular Cell Biology. The *Hansenula Polymorpha* YPD antibiotics agar plates were plated with competent *H. polymorpha ku80 PEX3-MKATE2* with *MEGFP-FUN26/FUN26-MEGFP*. according to the transformation protocol 'B.4 Electrotransformation of *Hansenula Polymorpha*' which can be found in the protocol book of Molecular Cell Biology. A colony PCR protocol was tested with WT *H. polymorpha ku80* cells according to 'Tested colony PCR protocol for yeast cells' which can be found in the 'Materials and methods' section of this report. Colony PCR was done with our plated *H. polymorpha ku80 PEX3-MKATE2* with *MEGFP-FUN26/FUN26-MEGFP* colonies to check which single yeast colony had the linearized plasmid DNA of interest inserted (the *FUN26* and *MEGFP* fusion gene) into the genome, the 'Tested colony PCR protocol for yeast cells' which can be found in the 'Materials and methods' section of this report was used. The extension step was set at 1 minute. 5 single N-colonies and 5 single C-colonies were picked from the YPD agar plates and used for PCR and streaked out on new YPD agar plates. 2 master mixes of each 149 µL were prepared for the colony PCR, one for the 5 N-fusion colonies and one for 5 C-fusion colonies. The master mix consisted of: 128 µL water (nuclease free), 15 µL DreamTaq buffer, 3 µL dNTP mix 10 mM, 1 µL cPCR Padh-fw primer, 1 µL mGFP rev. check primer, 1 µL DreamTaq DNA polymerase. 1 µL DNA from N- or C-fusion colony resuspended in 50 µL milli Q water was added last to the PCR tube with 24 µL master mix in it. Primers can be found in the Appendix section. Agarose gel electrophoresis was done to check the colony PCR and to see if PCR products with expected sizes were present. Positive N-colony N2, C-colonies C1 (with the linearized plasmid of interest inserted in the genome) and WT *H. polymorpha ku80* were grown overnight in flasks with glucose mineral medium for fluorescence microscopy and preparing TCA samples the next day. The next morning were the cultures diluted to an O.D. of 0.1. TCA samples were prepared with 2 milliliter of the cultures with an O.D. of ~1.5 according to the 'C.10 preparing crude extract of yeast cells in presence of TCA' protocol which can be found in the protocol book Molecular Cell Biology. 1% SDS in 0.1 M NaOH was freshly made by adding 1 milliliter of SDS 10% and 1 milliliter 1 M NaOH to 8 milliliter demiwater. Fluorescence microscopy was done

when the diluted cultures reach an O.D. of ~1 with the confocal (LSM800) microscope according to 'Fluorescence microscopy' which can be found in the 'Materials and methods' section of this report. N2, C1 and WT *H. polymorpha ku80* strains were grown overnight in flasks filled with 20 milliliter of YPD medium (37 degrees, in a shaker). The next days were the overnight cultures diluted to an O.D. of 0.1 in flasks with 20 milliliter methanol mineral medium, the yeast cultures grew slower on methanol mineral medium compared to glucose mineral medium so the O.D. of ~1 was not reached this day, this O.D. indicates exponentially growth of the yeast cells. After 5 hours the cultures only had an O.D. of ~0.2 so another way to get cell pellet was performed. 10 milliliter of a culture was put in a glass tube and centrifuged for 2 minute at 3220 rcf. ~1.5 milliliter of the 10 milliliter supernatant in the glass tube remained and the other ~8.5 milliliter supernatant was removed. The remaining 1.5 milliliter was resuspended by pipetting up and down and added to an Eppendorf tube which was centrifuged for 1 minute 3381 rcf. Now a pellet could be observed and this could be used for the preparation of fluorescence microscopy samples according to 'Fluorescence microscopy' which can be found in the 'Materials and methods' section. The confocal (LSM800) microscope was used for the fluorescence microscopy.

DTT was added to TE(D) resulting in the formation of TED, 100 milligram DTT per 25 milliliter TE(D). Mineral medium was made for the *H. Polymorpha* strains according to 'I E.1 Media for growing yeast H. Polymorpha', this protocol can be found in the protocol book for Molecular Cell Biology. The total amount was divided over smaller bottles of 50 or 100 milliliter for example. To 100 milliliter of mineral medium for H. Polymorpha was 1 milliliter Leucine (100X) added, 2 milliliter glucose (25% to 0.5%) or 1 milliliter methanol (50% to 0.5%) depending on if glucose mineral medium was prepared or methanol mineral medium, and at least 2 drops of vitamin. 10 milliliter of demiwater was added to 10 milliliter of methanol to get to the 50% methanol stock.

Fluorescence microscopy

Strains of *H. polymorpha* were grown overnight on mineral medium (glucose/methanol). The overnight cultures were diluted the next morning in new flasks with mineral medium (Leucine, glucose/methanol and vitamin drops were added) to an O.D. of 0.2 (around 11:00), you want the cultures around an O.D. of 1 for fluorescence microscopy. When an O.D. of ~1 was reached, 1 milliliter of the flask with the culture was put in an Eppendorf tube and centrifuged for 1 minute at 3381 rcf (25 degrees). A pellet was formed and ~970/980 µL was taken out the Eppendorf tube by pipetting and the remaining medium was used to resuspend the pellet in the remaining medium by pipetting up and down. 1 µL of the resuspended pellet was put on a glass plate and covered by a small cover glass, now fluorescence microscopy could be done with the prepared sample.

All images were taken at room temperature using a 100x 1.30 NA Plan Neofluar objective. Wide-field images were taken using a Zeiss Axioscope A1 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Images were taken using a Coolsnap HQ2 digital camera and MICRO MANAGER software. A 470/40 nm bandpass excitation filter, a 495 nm dichromatic mirror and a 525/50 nm bandpass emission filter were used to visualize the meGFP signal. The mKate2 fluorescence were visualized with a 587/25 nm band pass excitation filter, a 605 nm dichromatic mirror and a 647/70 nm band pass emission filter (Wu, 2020). CMAC/DAPI was visualized by excitation with a 405 nm laser (Krikken, et al., 2020). (Thomas et al., 2018)

Airyscan images were captured with a confocal laser scanning microscope (LSM800, Zeiss) equipped with a 32-channel gallium arsenide phosphide photomultiplier tube (GaAsP-PMT) and a 63× 1.4 NA oil immersion objective, in Airyscan mode. Images were analysed in Fiji software (ImageJ 2.14.0) (Jansen et al., 2024)

Western Blot

The 'C.60 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) using mini-gel system' was performed to make mini gels (10%). The protocol can be found in the protocol book of Molecular Cell Biology. The protein samples were prepared according to 'C.10 preparing crude extract of yeast cells in presence of TCA' and the gel was loaded and run according to 'C.60 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE) using mini-gel system' which can both be found in the protocol book of Molecular Cell Biology. Western blotting was done with the mini-gels according to the 'C.68 Western blotting; Semi-dry' protocol which can be found in the protocol book of Molecular Cell Biology. The 'C.66 Ponceau S staining for nitrocellulose blots' protocol was performed which can be found in the protocol book of Molecular Cell Biology. The primary antibody which was used is the mouse monoclonal α-GFP (Thermo Fisher Scientific); the secondary antibody used for detecting the Western blot with HRP-substrate was the Goat anti-mouse IgG-HRP (Thermo Fisher Scientific); the secondary antibody used for detecting the Western blot with AP-substrate is AP-conjugate antibody Goat anti-mouse IgG AP. 1X TBS was made (400 milliliter + 100 milliliter 5X TBS) and also a blocking buffer was prepared: a 50 milliliter bottle was filled with 1 gram milk powder (2%) and 50 milliliter 1X TBS. The nitrocellulose blots were put in 25 milliliter blocking buffer and put at a temperature of 4 degrees to shake overnight according to 'C.70 or C71' which can be found in the protocol book of Molecular Cell Biology. The protocols 'C.70 Detection of proteins on Western blots using an AP-conjugate' and 'C.71 Detection of proteins on Western blot using an HRP-conjugate' were performed and can be found in the protocol book of Molecular Cell Biology.

DAPI/CMAC staining

The N2 and C1 colonies were grown overnight on glucose mineral medium in order to do staining with DAPI and CMAC the next day. The next morning are the cultures diluted to an O.D. of 0.1 in flasks with 20 milliliter glucose mineral medium. When the diluted cultures of N2 and C1 reach an O.D. of ~1, DAPI and CMAC staining is done to 1 milliliter of both N2 and C1 culture so a total of 4 Eppendorf tubes are incubated after adding the DAPI and CMAC stain according to the 'DAPI staining' and 'D.7 Fluorescent staining of vacuolar lumen using CMAC cell tracker blue', both protocols can be found in the protocol book for Molecular Cell Biology. After both protocols are performed fluorescence samples can be made according to 'Fluorescence microscopy' which can be found in the 'Materials and methods' section of this report.

Results

Cloning of the *MEGFP* fusion gene using the *E. coli DH5α* strain

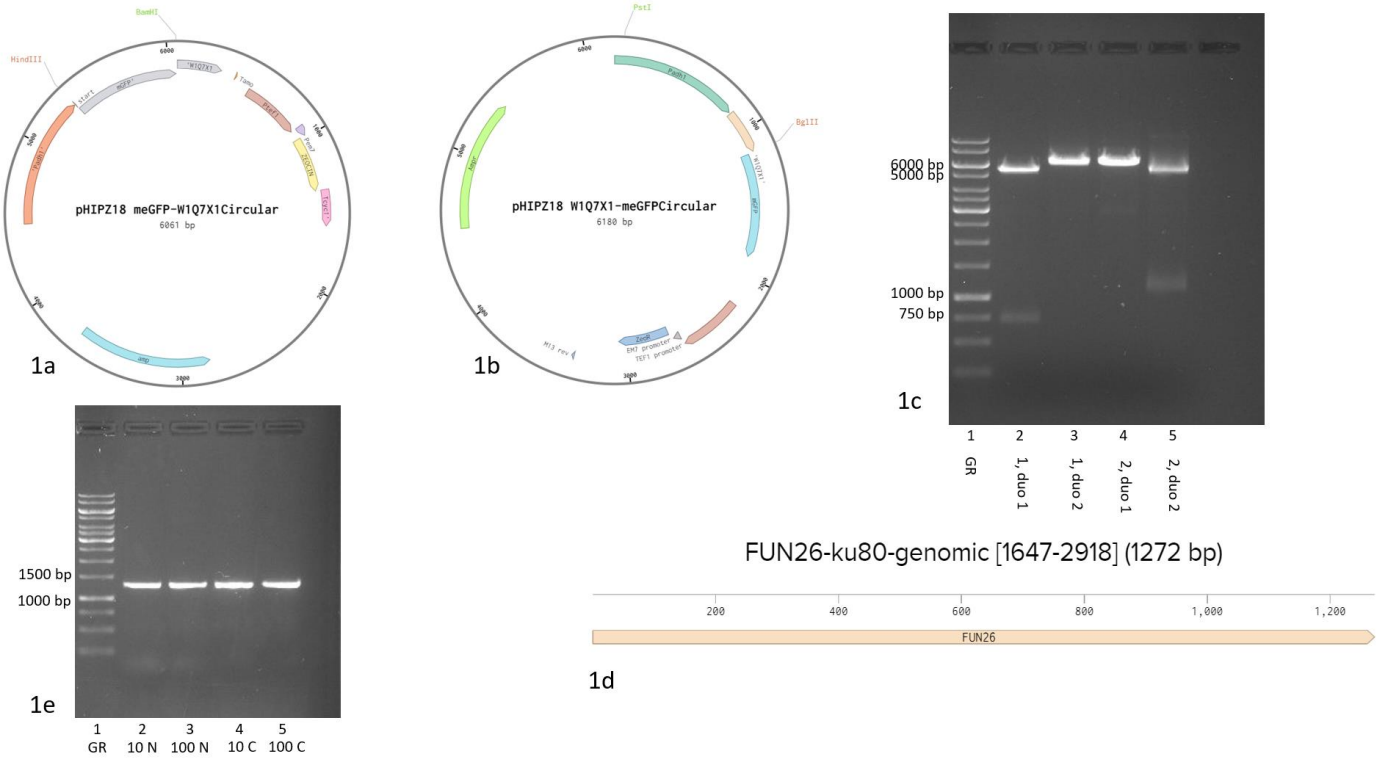


***Figure 1**. In **figure 1a** can the pHIPZ18 MEGFP-W1Q7X1 plasmid be observed. 2 restriction sites are present: the HindIII and the BamHI site. The fragment between the two cutting sites is 723 base pairs. In **figure 1b** can the pHIPZ18 W1Q7X1-MEGFP plasmid be observed. 2 restriction sites are present: the PstI and the BglII site. The fragment between the two cutting sites is 1092 base pairs. In **figure 1c** is the agarose gel electrophoresis of the plasmid miniprep check by restriction presented. Well 1 contains the gene ruler; well 2 contains plasmid 1 with restriction enzyme duo 1; well 3 contains plasmid 1 with restriction enzyme duo 2; well 4 contains plasmid 2 with restriction enzyme duo 1; well 5 contains plasmid 2 with restriction enzyme duo 2. Some relevant sizes of fragments present in the gene ruler are indicated. Plasmid 1, plasmid 2, restriction enzyme duo 1 and 2 can be found in the results section. In **figure 1d** can the FUN26 gene be observed which is present in the genomic DNA of the H. polymorpha ku80, this gene will be inserted in plasmid 1 and 2 and replaces the W1Q7X1 gene. In **figure 1e** is the agarose gel electrophoresis of the FUN26 PCR products presented. Well 1 contains the gene ruler with some labelled fragments in size which are relevant for this agarose gel electrophoresis. Well 2 contains the PCR product of FUN26 with primers for N-terminal fusion of the MEGFP to the FUN26. The template DNA used for the PCR is 10x diluted genomic DNA of the H. polymorpha ku80 strain. Well 3 contains the PCR product of FUN26 with primers for N-terminal fusion of the MEGFP to the FUN26. The template DNA used for the PCR is 100x diluted genomic DNA of the H. polymorpha ku80 strain. Well 4 contains the PCR product of FUN26 with primers for C-terminal fusion of the MEGFP to the FUN26. The template DNA used for the PCR is 10x diluted genomic DNA of the H. polymorpha ku80 strain. Well 5 contains the PCR product of FUN26 with primers for C-terminal fusion of the MEGFP to the FUN26. The template DNA is 100x diluted genomic DNA of the H. polymorpha ku80 strain.*

Both of the DNA plasmids miniprepped from the *E. coli* strain can be observed in figure 1a and 1b. In **figure 1a**, the pHIPZ18 *MEGFP-W1Q7X1* plasmid is present which is used for the N-terminal fusion of *MEGFP* to *FUN26* gene and in **figure 1b** the pHIPZ18 *W1Q7XI-MEGFP* is present which is used for the C-terminal fusion of *MEGFP* to the *FUN26* gene. Both plasmids also have ampicillin resistance and zeocin resistance, this enables selecting competent *H. polymorpha ku80* and *E. coli DH5α* colonies after the cells are plated on agarose plates with either ampicillin (for selecting *E. coli* colonies) or zeocin (for selecting *H. polymorpha* colonies) on them. **figure 1c** shows an agarose gel electrophoresis which was done to check if the pHIPZ18 *MEGFP-W1Q7X1* (plasmid 1) and pHIPZ18 *W1Q7XI-MEGFP* (plasmid 2) plasmids were present after the miniprep and not some other plasmid, restriction enzyme duo's were added to the two plasmids and certain fragment sizes were expected (Benchling, 2024). Restriction enzyme duo 1 contained: BamHI and HindIII; restriction enzyme duo 2 contained: BglII and PstI. The sample in well 2 of the agarose gel contained plasmid 1 and restriction

enzyme duo 1, a DNA fragment of 5338 base pairs was expected and a DNA fragment of 723 base pairs was expected. A DNA fragment of ~5500 base pairs was observed and a fragment of ~750 base pairs was observed, just like expected. The sample in well 3 of the agarose gel contained plasmid 1 and restriction enzyme duo 2, a DNA fragment of 6061 base pairs was expected (linear, the BglI restriction enzyme has no cut site in this plasmid). A DNA fragment of ~6100 base pairs was observed. The sample in well 4 of the agarose gel contained plasmid 2 and restriction enzyme duo 1, a DNA fragment of 6180 base pairs was expected (linear, the BamHI restriction enzyme has no cut site in this plasmid). A DNA fragment of ~6100 base pairs was observed. The sample in well 5 of the agarose gel contained plasmid 2 and restriction enzyme duo 2, a DNA fragment of 5088 base pairs was expected and a DNA fragment of 1092 base pairs was expected. A DNA fragment of ~5200 base pairs was observed and a fragment of ~1200 base pairs was observed, just like expected. The *FUN26* gene which replaced the *W1Q7X1* gene in plasmid 1 and 2 after restriction for ligation and ligation itself can be observed in **figure 1d**. PCR for cloning was done with the *H. polymorpha ku80* genome as template DNA. The primer duo decide which PCR product of *FUN26* was produced, either the PCR fragment for fusion of *MEGFP* at the N-terminal of *FUN26* (N-terminal fusion) or the PCR fragment for fusion of *MEGFP* at the C-terminal of *FUN26* (C-terminal fusion). The PCR product for the N-terminal fusion had the FUN26-N-fw primer with a BamHI restriction site and the FUN26-N-rev primer with the SalI restriction site. The PCR product for the C-terminal fusion had the FUN26-C-fw primer with a HindIII restriction site and the FUN26-C-rev primer with the BglII restriction site. The PCR product for the N-terminal fusion had 1290 base pairs and the PCR product for the C-terminal fusion has 1287 base pairs (Benchling, 2024). In **figure 1e**, the agarose gel electrophoresis of the *FUN26* PCR products with primers can be observed. Well 2 and 3 contained the PCR product for N-terminal fusion, a PCR fragment of ~1300 base pairs could be observed. Well 4 and 5 contained the PCR product for C-terminal fusion, a PCR fragment of ~1300 base pairs could be observed.

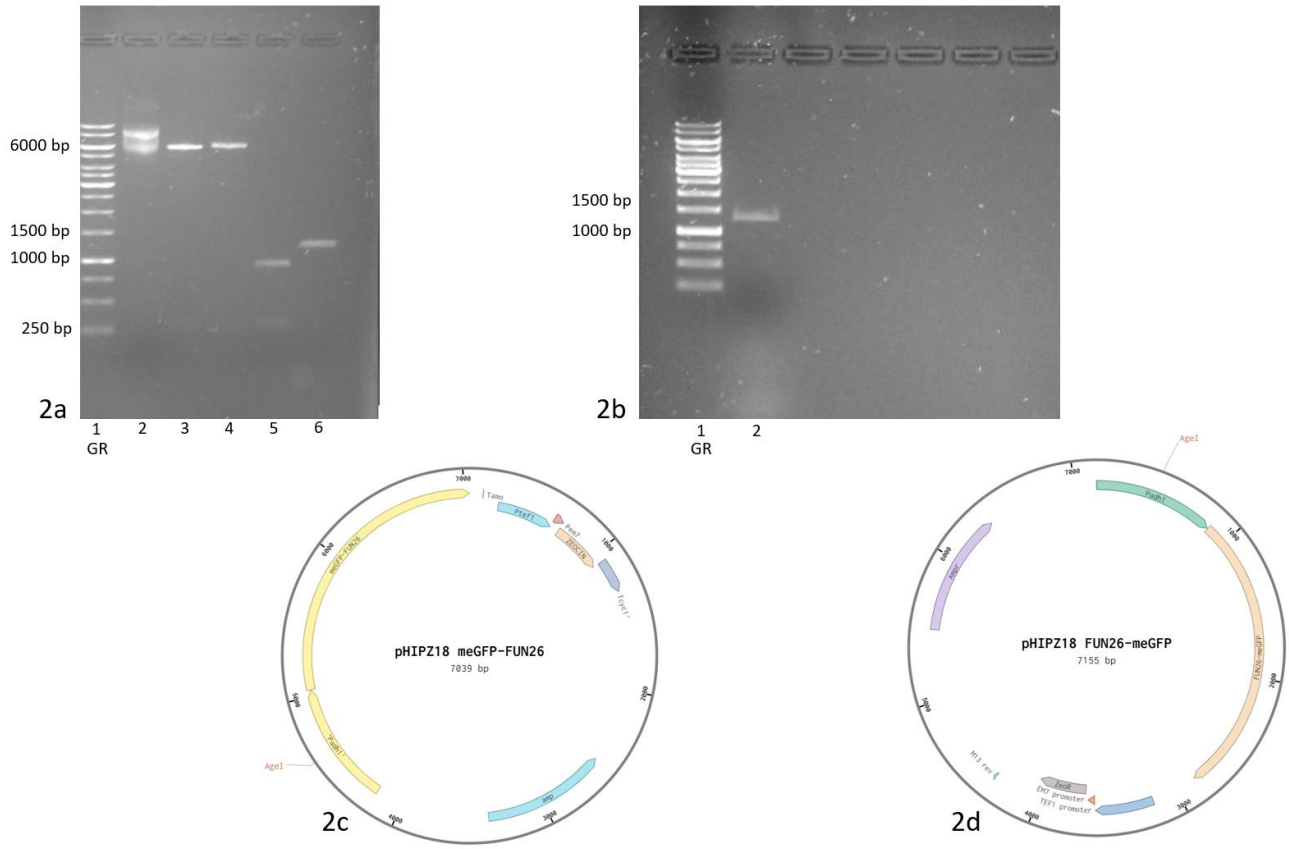


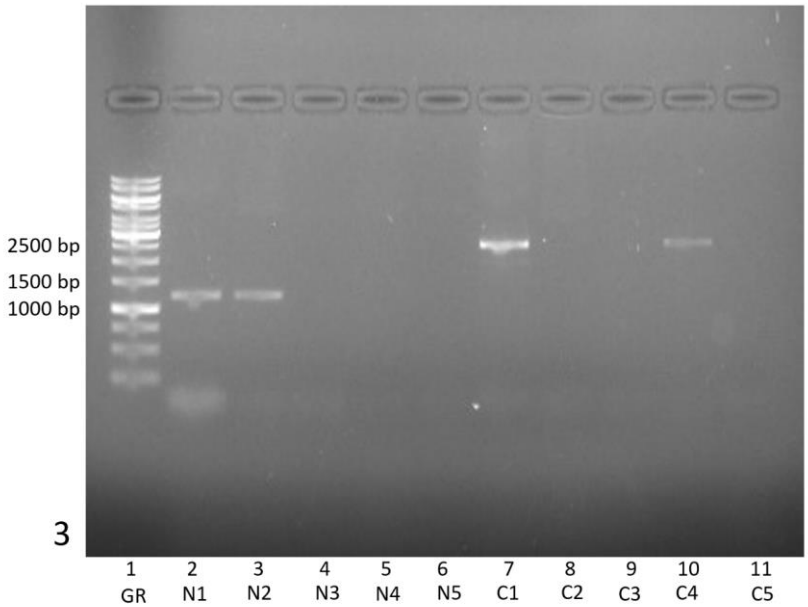
***Figure 2**. In **figure 2a** can the agarose gel electrophoresis performed with restriction fragments for ligation be observed. Well 1 contains the gene ruler and some relevant fragment sizes are indicated; well 2 contains plasmid 1 (not restricted); well 3 contains plasmid 1 cut with restriction enzymes BamHI and XhoI; well 4 contains plasmid 2 cut with restriction enzymes HindIII and BglII; well 5 contains PCR product N-terminal fusion cut with restriction enzymes BamHI and XhoI; well 6 contains PCR product C-terminal fusion cut with restriction enzymes HindIII and BglII. In **figure 2b** is the agarose gel electrophoresis performed again for the PCR product for N-terminal fusion but this time with the BamHI and SalI restriction enzymes. In **figure 2c** can the resulting pHIPZ18 MEGFP-FUN26 be observed, the FUN26 gene replaces the W1Q7X1 gene and the MEGFP is fused to the N-terminal of FUN26. In **figure 2d** can the resulting pHIPZ18 FUN26-MEGFP be observed, the FUN26 gene replaces the W1Q7X1 gene and the MEGFP is fused to the C-terminal of FUN26.*

After restriction the of PCR products and vectors, the ligation was done. PCR product for N-terminal fusion has the BamHI and SalI restriction sites present, in both primers one of the two restriction sites. plasmid 1 has the BamHI and XhoI restriction sites at both ends of the *W1Q7X1* gene one. PCR product for N-terminal fusion was inserted in plasmid 1 since the PCR product for N-terminal fusion was added in excess to the ligation reaction and the BamHI site present in PCR product and plasmid had compatible ends, the SalI site in the PCR product had compatible ends with the XhoI site in plasmid 1. The primer FUN26-N-rev had no XhoI restriction site since there was a XhoI restriction site present in the *FUN26* gene. PCR product for C-terminal fusion had the HindIII and BglII restriction sites present in both primers one. plasmid 2 had the HindIII and BglII restriction sites at both ends of the *W1Q7X1* gene one. PCR product for C-terminal fusion was inserted in plasmid 2 since the PCR product for C-terminal fusion was added in excess to the ligation reaction and the HindIII and BglII site was present in both PCR product and plasmid. The HindIII site in the PCR product had compatible ends with the HindIII site in plasmid 2. The same was true for the BglII restriction sites in both PCR product and plasmid. An agarose gel electrophoresis was done on the restriction fragments for ligation, this can be observed in **figure 2a and 2b.** After restriction of PCR product for N-terminal fusion with BamHI and SalI a fragment of 1278 base pairs was expected and after restriction of PCR product for C-terminal fusion with HindIII and BglII a fragment of 1275 base pairs was expected. After

restriction of plasmid 1 with BamHI and XhoI a fragment of 5770 base pairs and 291 base pairs was expected and after restriction of plasmid 2 with HindIII and BglII a fragment of 5892 base pairs and 288 base pairs was expected. In **figure 2a**, we can see for well 3 (plasmid 1 restricted) and 4 (plasmid 2 restricted) a fragment around 5800 base pairs like expected, the smaller *W1Q7XI* fragment travelled probably too far through the gel to be observed on the gel. We can also see that the fragment present for plasmid 2 in well 4 is slightly bigger in size than the fragment for plasmid 1 in well 3. In well 6 (PCR product C-terminal fusion) we see a band around 1300 base pairs like expected, in well 5 (PCR product N-terminal fusion) we observe a band around 900 base pairs and a band around 350 base pairs (not like expected). For well 5 I used the BamHI and XhoI restriction enzymes instead of the BamHI and SalI, *FUN26* has a XhoI restriction site in the gene, this explains the two different fragments. In **figure 2b** was a fragment around 1300 base pairs (like expected) observed in well 2, this was the PCR product N-terminal restricted with BamHI and SalI.

Cloning using *H. polymorpha* strains

In **picture 2c,** the ligated pHIPZ18 *MEGFP-FUN26* plasmid can be observed which was later on linearized with the AgeI enzyme which cut in the Padh1 promoter. The linearized plasmid was inserted in the genome of the *H. polymorpha ku80 PEX3-MKATE2*. In **figure 2d** the ligated pHIPZ18 *FUN26-MEGFP* plasmid can be observed which was later on linearized with the AgeI enzyme which cut in the Padh1 promoter. The linearized plasmid was inserted in the genome of the *H. polymorpha ku80 PEX3-MKATE2*.

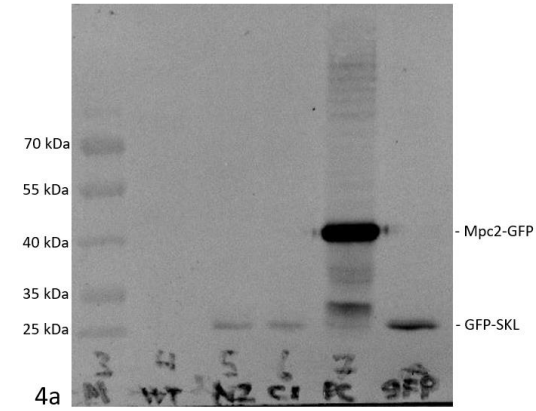


*In **figure 3** can the agarose gel electrophoresis which is performed to check for competent colonies of H. polymorpha ku80 PEX3-MKATE2 with the MEGFP-FUN26/FUN26-MEGFP gene. after colony PCR. Well 1 contains the gene ruler and some relevant fragment sizes are also indicated. Well 2 contains the sample with the N-terminal fusion of MEGFP to FUN26 genome from colony 1 as template DNA for colony PCR. This is colony N1 from the YPD zeocin agar plate. Well 3 contains the sample with the N-terminal fusion of MEGFP to FUN26 genome from colony 2 as template DNA for colony PCR. This is colony N2 from the YPD agar plate. Well 4 contains the sample with the N-terminal fusion of MEGFP to FUN26 genome from colony 3 as template DNA for colony PCR. This is colony N3 from the YPD zeocin agar plate. Well 5 contains the sample with the N-terminal fusion of MEGFP to FUN26 genome from colony 4 as template DNA for colony PCR. This is colony N4 from the YPD agar plate. Well 6 contains the sample with the N-terminal fusion of MEGFP to FUN26 genome from colony 5 as template DNA for colony PCR. This is colony N5 from the YPD zeocin agar plate. Well 7 contains the sample with the C-*

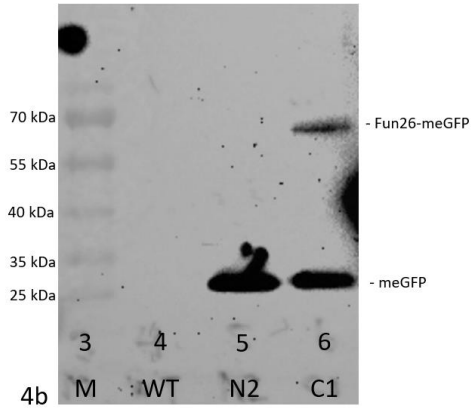
terminal fusion of MEGFP to FUN26 genome from colony 1 as template DNA for colony PCR. This is colony C1 from the YPD zeocin agar plate. Well 8 contains the sample with the C-terminal fusion of MEGFP to FUN26 genome from colony 2 as template DNA for colony PCR. This is colony C2 from the YPD zeocin agar plate. Well 9 contains the sample with the C-terminal fusion of MEGFP to FUN26 genome from colony 3 as template DNA for colony PCR. This is colony C3 from the YPD zeocin agar plate. Well 10 contains the sample with the C-terminal fusion of MEGFP to FUN26 genome from colony 4 as template DNA for colony PCR. This is colony C4 from the YPD zeocin agar plate. Well 11 contains the sample with the C-terminal fusion of MEGFP to FUN26 genome from colony 5 as template DNA for colony PCR. This is colony C5 from the YPD zeocin agar plate.

Cloning of the gene of interest fused to *MEGFP* into the genome of the *H. polymorpha ku80 PEX3-MKATE2* done by transformation leads to two different genomes for the *H. polymorpha ku80 PEX3-MKATE2* strains. We had a strain with the *MEGFP-FUN26* plasmid inserted in the genome of the *H. polymorpha ku80 PEX3-MKATE2* (positive N-terminal fusion strains) and we had a strain with the *FUN26-MEGFP* plasmid inserted in the genome of the *H. polymorpha ku80 PEX3-MKATE2* (positive C-terminal fusion strain). Colony PCR was done to check if transformation succeeded and if the *H. polymorpha ku80 PEX3-MKATE2* strains really have the *FUN26-MEGFP*/*MEGFP-FUN26* gene. For the genome with the *MEGFP-FUN26* gene inserted were the cPCR Padh-fw and mGFP rev check primers used for the colony PCR this resulted in a PCR product of 1234 base pairs in size. For the genome with the *FUN26-MEGFP* gene inserted were the cPCR Padh-fw and mGFP rev check primers used for the colony PCR this resulted in a PCR product of 2506 base pairs in size. The primers can be found in the Appendix section. An agarose gel electrophoresis was done to check the colony PCR, in **picture 3** this can be observed. For the N-fusion (*MEGFP*) positive colonies, N1 (well 2) and N2 (well 3), PCR fragments around 1234 base pairs could be found like expected. For the C-fusion (*MEGFP*) positive colonies, C1 (well 7) and C4 (well 10), PCR fragments around 2506 base pairs could be found like expected. The cPCR Padh-fw primer was located before the Padh1 promoter and only gives PCR fragments of these sizes when the plasmids of interest were inserted at the correct sport in the *H. polymorpha ku80 PEX3-MKATE2* genomic DNA. The N-fusion positive colony N2 and the C-fusion positive colony C1 was used for fluorescence microscopy and also for the Western blotting.

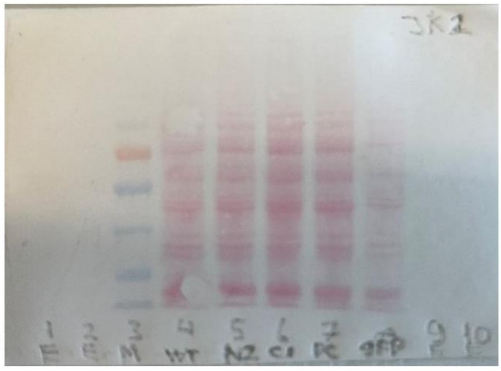
Western blotting



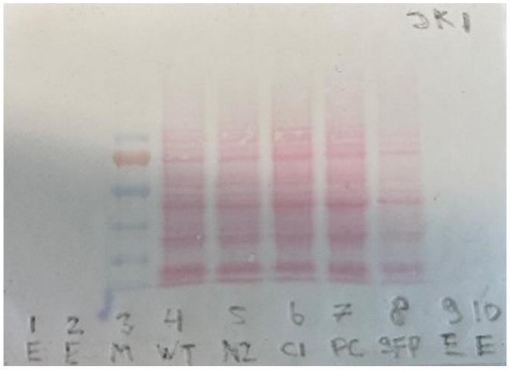
4a



4b



4c



4d

Figure 4*. In* ***figure 4a*** *can the Western blot performed with AP-conjugate be observed. Well 3 has the marker (M) which indicates protein sizes of interest. TCA samples of proteins are prepared and loaded in the wells of the mini gel. Well 4 contains the TCA sample of WT proteins; well 5 contains the TCA sample of proteins in colony N2, the meGFP-Fun26 should be present; well 6 contains the TCA sample of proteins in colony C1, the Fun26-meGFP should be present; well 7 contains the TCA sample of proteins in the positive control, the positive control is the Mpc2-GFP protein. well 8 contains the TCA sample of the GFP-SKL protein. In* ***figure 4b*** *can the Western blot performed with HRP-conjugate be observed, a part of the nitrocellulose is cut off because of the brightness of the band of the PC. Well 3 has the marker (M) which indicates protein sizes of interest; Well 4 contains the TCA sample of WT proteins; well 5 contains the TCA sample of proteins in colony N2, the meGFP-Fun26 should be present; well 6 contains the TCA sample of proteins in colony C1, the Fun26-meGFP should be present. In* ***figure 4c*** *can the Ponceau S staining of the nitrocellulose membrane before performing Western blot with AP-conjugate be observed. In* ***figure 4d*** *can the Ponceau S staining of the nitrocellulose membrane before performing Western blot with HRP-conjugate.*

Detection of proteins on Western blots was also performed using both an AP-conjugate and an HRP-conjugate. We want to check if the meGFP-Fun26 fusion protein was present in the TCA samples of the N2 cells. The same was checked for the Fun26-meGFP fusion protein in the TCA samples of the C1 cells. In **picture 4a,** the Western blot performed with an AP-conjugate can be observed, as we can see are for both well 5 (N2 colony) and well 6 (C1 colony) one band around ~27 kDa is observed. This is free meGFP since meGFP has this size as a protein and the whole point of this Western blot is the observation of meGFP and if this is fused to a different protein, the size of the whole fusion protein can be confirmed, but this is only because it is labeled to the meGFP. The chosen antibodies enable the observation of meGFP on the Western blot. No fusion proteins with meGFP were observed for the N2 and C1 colonies on the Western blot performed with an AP-conjugate. For well 7 (the positive control), multiple bands were present, especially one bright band around 40 kDa and a band around 30 kDa which was a little bit less bright. This was the Mpc2-GFP protein and was probably broken down in smaller pieces to which the GFP protein was still attached. In well 8 we see the meGFP, this

GFP also had a band around ~27 kDa like in well 5 and 6 and served as a control to see if the meGFP in the N2 and C1 wells were free or fused to another protein. In **figure 4b,** the Western blot performed with HRP-conjugate can be observed. A part of the nitrocellulose blot was cut of the picture otherwise the important bands of low intensity could not be observed because of the brightness of the PC bands. We can observe some very darkish dots on the blot which was just dirt, we can observe the free meGFP bands again for well 5 (N2) and well 6 (C1) just like on the blot performed with AP-conjugate. On this blot a new band was present in the C1 well, a band around the 70 kDa can be observed, this was probably the Fun26-meGFP fusion protein since this fusion protein had a size around the 74 kDa. In the C1 colony was some meGFP detached from Fun26 and in the N2 colony it seems like all the meGFP was detached from Fun26 and was just free meGFP. In **figure 4c and 4d** can both nitrocellulose membrane 2 and 1 be observed after Ponceau S staining. Nitrocellulose membrane 2 (**figure 4c**) was later on used for Western blotting with AP-conjugate, we can see that some air bubbles were present on the membrane in the WT region. Furthermore, we can see that in the TCA sample of C1, similar amount of proteins were present compared to the rest of the TCA samples. In the TCA samples for the PC and N2 were a little bit less proteins present compared to the C1 TCA sample. The WT and GFP-SKL TCA samples contained the least amount of proteins. Nitrocellulose membrane 1 (**figure 4d**) is later on used for Western blotting with HRP-conjugate. We can see that in the TCA samples of C1 and the PC, most proteins were present compared to the rest of the TCA samples. In the TCA samples for the N2 and the WT were a little bit less proteins present compared to the C1 and PC TCA samples. The GFP-SKL TCA sample contained the least amount of proteins.

Fluorescence microscopy

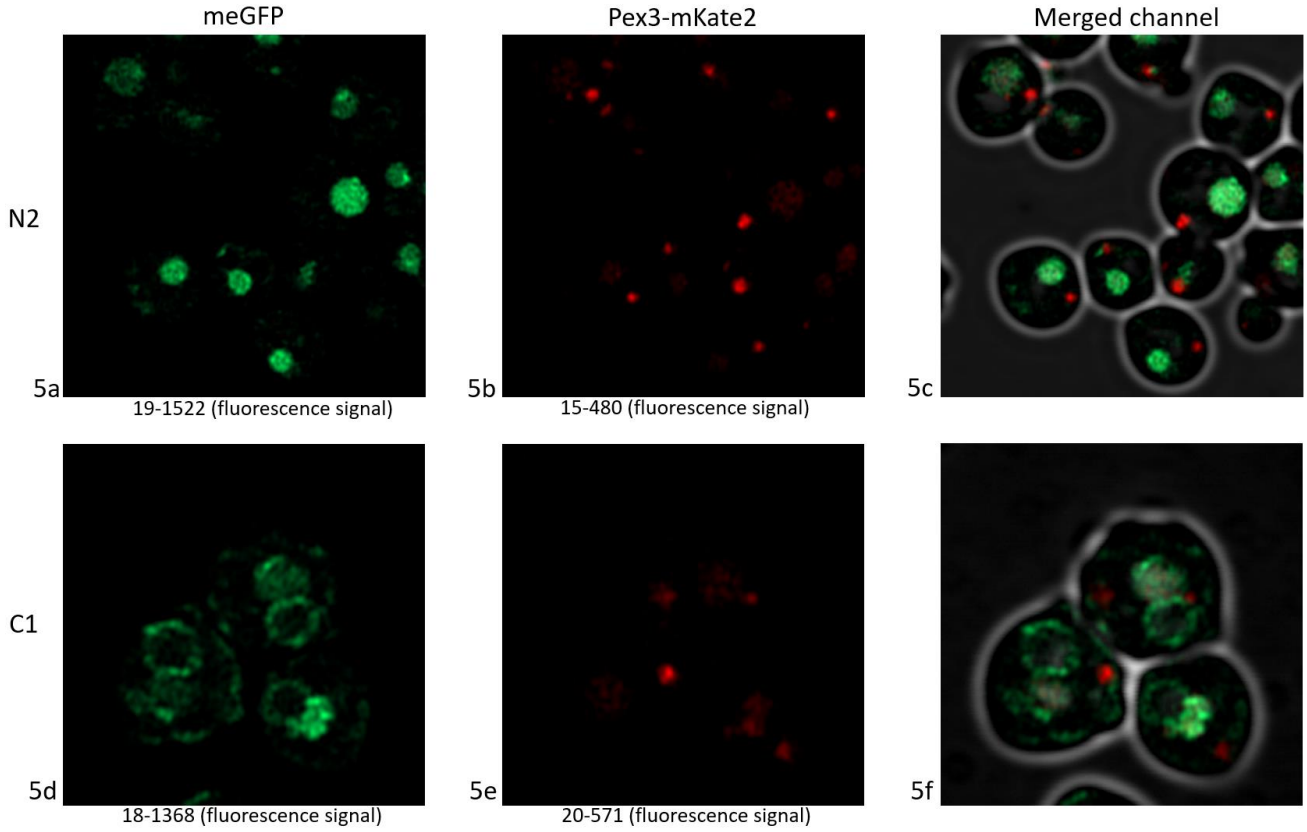


*Figure 5. In **figure 5a** can positive N-terminal fusion colony N2, which is a ku80 yeast with the MEGFP-FUN26 gene in the genome, be observed. The green we observe is the meGFP. In **figure 5b**, we still look at the N2 colony. The red we observe is the mKate2 fluorescent protein labeled to Pex3 which is located in the membrane of the peroxisome. In **figure 5c**, we still look at the N2 colony. We now observe a merged channel, this means that mKate2 and meGFP can be observed in the same figure. In **figure 5d** can positive C-terminal fusion colony C1, which is a ku80 yeast with the FUN26-MEGFP gene in the genome, be observed. The green we observe is the meGFP. In **figure 5e**, we still look at the C1 colony. The red we observe is the mKate2 fluorescent protein labeled to Pex3 which is located in the membrane of the peroxisome. In **figure 5f**, we still look at the C1 colony. We now observe a merged channel, this means that mKate2 and meGFP can be observed in the same figure. The N2 and C1 colonies are grown on glucose mineral medium. The fluorescence signal (measured with ImageJ) can also be found for the meGFP and mKate2 under the figure for either the N2 or C1 colony. The figures are made with the confocal (LSM800) microscope.*

In **figure 5 a,b,c** can the fluorescent microscopy of positive N-terminal fusion (*MEGFP-FUN26*) colony N2 be observed and in **figure 5 d,e,f** can the fluorescent microscopy of positive C-terminal fusion (*FUN26-MEGFP*) colony C1 be observed. Pex3 is labelled with mKate2 which is present in the membrane of peroxisomes. This is the way to label peroxisomes and this enables to visualize colocalization (if this is the case) with the meGFP which is linked to the Fun26 protein. All these figures are *ku80* yeast cells grown on glucose mineral medium. In **figure 5a** we can nicely see the meGFP in a circle of which I suspect it to be the lumen of the vacuole. In **figure 5b** we can see the red of the mKate2 labelled to the peroxisomal membrane. In **figure 5c** we can both see the green (meGFP) and the red (mKate2), we can also see that there is no colocalization between the two. Thus, we can conclude that the Fun26 transporter protein is not present in the peroxisomal membrane for the Fun26 protein with N-terminal fusion of meGFP. In **figure 5d** we can see the green fluorescence of the meGFP but it is not localized in a circle like for the N2 colony, it seems like the meGFP is mostly located outside the vacuolar lumen and also outside the vacuolar membrane, in a ring shape structure. In **figure 5e** we can see the red of the mKate2 labelled to the peroxisomal membrane. In **figure 5f** we can both see the green (meGFP) and the red (mKate2), we can also see

that there is no colocalization between the two. Thus, we can conclude that under the present growth medium (glucose), the Fun26 transporter protein is not present in the peroxisomal membrane for the Fun26 protein with C-terminal fusion of meGFP.

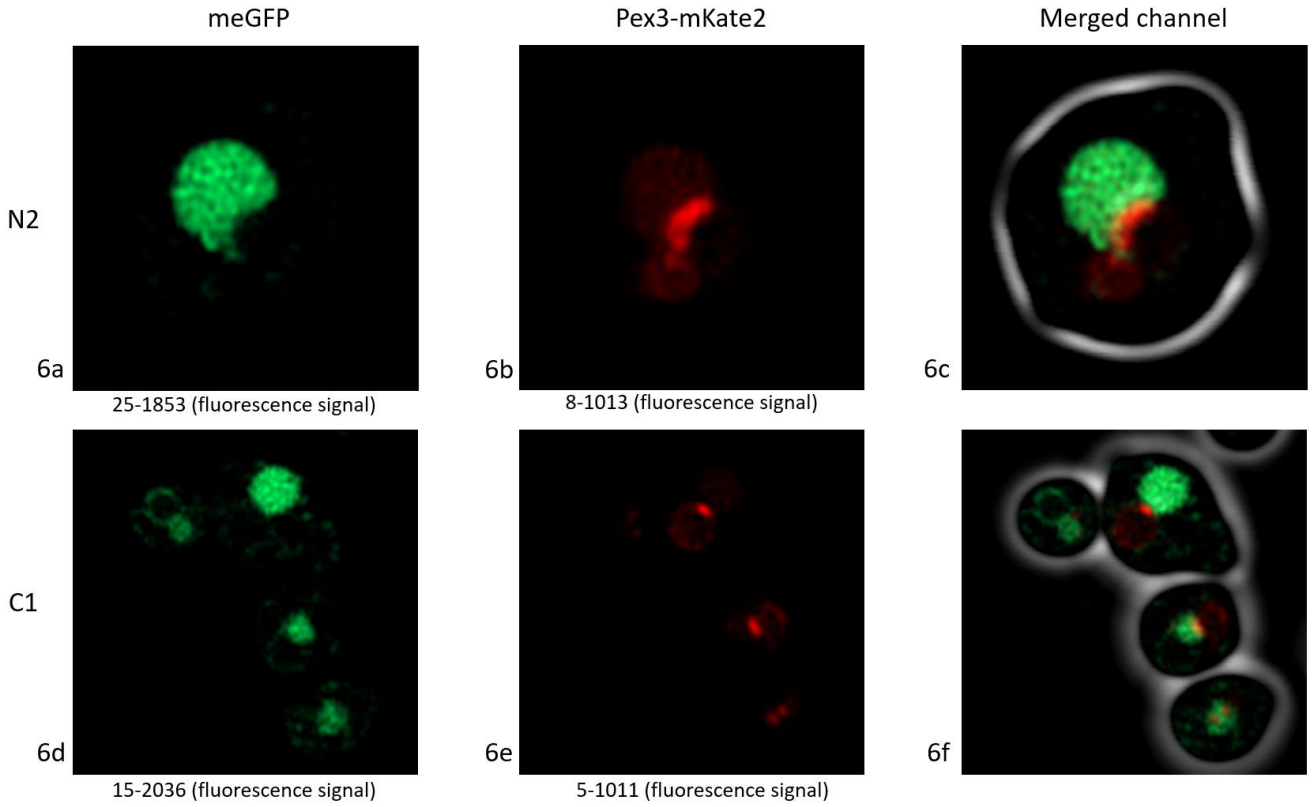


*Figure 6. In **figure 6a** can positive N-terminal fusion colony N2, which is a ku80 yeast with the MEGFP-FUN26 gene in the genome, be observed. The green we observe is the meGFP. In **figure 6b**, we still look at the N2 colony. The red we observe is the mKate2 fluorescent protein labeled to Pex3 which is located in the membrane of the peroxisome. The peroxisomes appear to be bigger, we can really see the membrane of the peroxisomes (the size is like a ring). This is because the yeast colonies N2 and C1 are grown first on YPD medium and afterwards on methanol mineral medium. This results in growth in size of peroxisomes (van Zutphen, 2008) In **figure 6c**, we still look at the N2 colony. We now observe a merged channel, this means that mKate2 and meGFP can be observed in the same figure. In **figure 6d** can positive C-terminal fusion colony C1, which is a ku80 yeast with the FUN26-MEGFP gene in the genome, be observed. The green we observe is the meGFP. In **figure 6e**, we still look at the C1 colony. The red we observe is the mKate2 fluorescent protein labeled to Pex3 which is located in the membrane of the peroxisome, explanation on peroxisomes can be found in the explanation for **figure 6b** of this legenda. In **figure 6f**, we still look at the C1 colony. We now observe a merged channel, this means that mKate2 and meGFP can be observed in the same figure. N2 and C1 colony are first grown on YPD and afterwards on methanol mineral medium. The fluorescence signal (measured with ImageJ) can also be found for the meGFP and mKate2 under the figure for either the N2 or C1 colony. The figures are made with the confocal (LSM800) microscope.*

We also checked the colocalization of the fusion protein using medium with methanol, which results in peroxisome induction (van Zutphen, 2008). In **figure 6 a,b,c** the fluorescent microscopy of positive N-terminal fusion (*MEGFP-FUN26*) colony N2 can be observed and in **figure 6 d,e,f** can the fluorescent microscopy of positive C-terminal fusion (*FUN26-MEGFP*) colony C1 be observed. Pex3 was labelled with mKate2 which is present in the membrane of peroxisomes. All these figures are cells grown first on YPD and afterwards on methanol mineral medium. Growth on these medium results in bigger peroxisomes and this should make colocalization between meGFP fused to Fun26 and mKate2 fused to Pex3 easier to see when colocalization is present. In **figure 6a** we can nicely see the meGFP in a circle of which I suspect it to be the lumen of the vacuole. In **figure 6b** we can see the red fluorescence of the mKate2 fused to the peroxisomal membrane (Pex3). In **figure 6c** we can both

see the green (meGFP) and the red (mKate2), we can also see that there is no colocalization between the two. Thus, we can conclude that the Fun26 transporter protein is not present in the peroxisomal membrane for the Fun26 protein with N-terminal fusion of meGFP (like expected according to **figure 5**). In **figure 6d** we can see the green fluorescence of the meGFP but it seems to be located a little bit more in the vacuolar lumen but also outside the vacuolar lumen and vacuolar membrane, in a ring shape structure. In **figure 6e** we can see the red of the mKate2 labelled to the peroxisomal membrane, we can see nicely the membrane of the peroxisomes. In **figure 6f** we can both see the green (meGFP) and the red (mKate2), we can also see that there is no colocalization between the two. This concludes that the Fun26 transporter protein is not present in the peroxisomal membrane for the Fun26 protein with C-terminal fusion of meGFP (like expected based **figure 5**).

Staining of the vacuolar lumen with CMAC was done to see the colocalization (if present) of meGFP and CMAC, colony N2 and C1 are grown on glucose mineral medium for this staining. This can be observed in **figure 7**. After this staining better conclusions on where meGFP is located can be drawn.

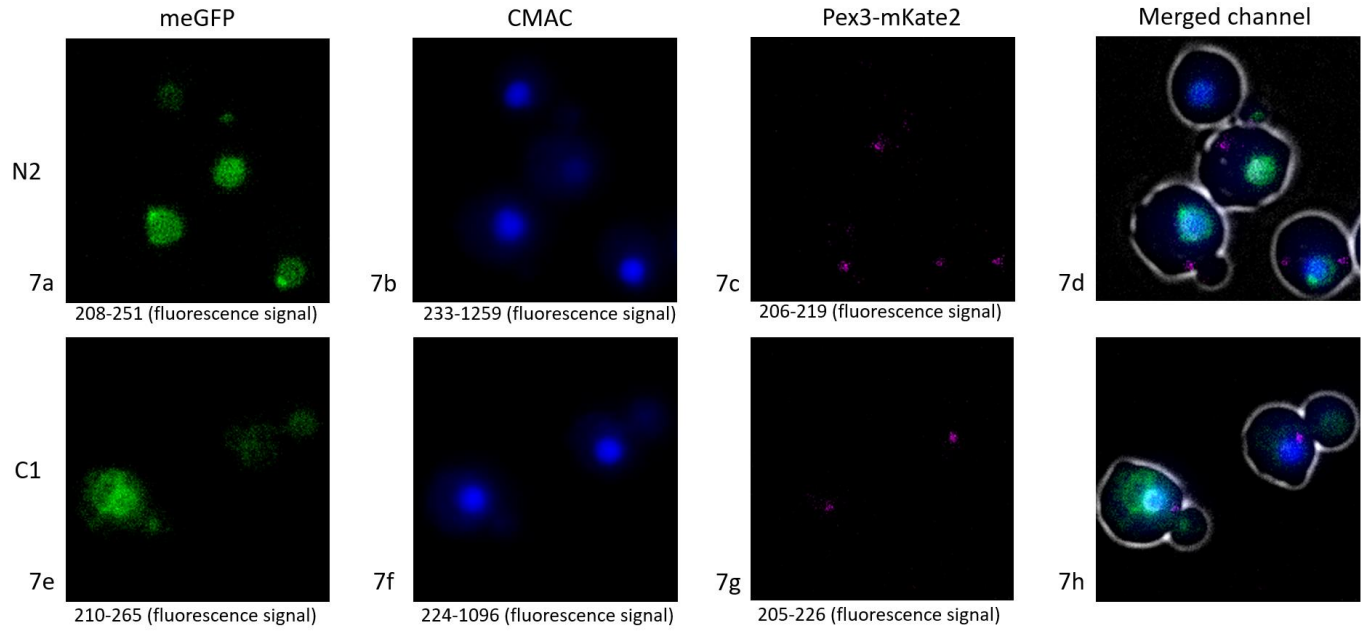


Figure 7. The N2 and C1 ku80 yeast colonies are grown on glucose mineral medium for this staining procedure. ***Figure 7 a,b,c,d*** *are fluorescence microscopy figures from the N2 colony.* ***Figure 7 e,f,g,h*** *are fluorescence microscopy figures from the C1 colony. In* ***figure 7a*** *can the green fluorescence of meGFP be observed in the N2 colony. In* ***figure 7b*** *can the fluorescence of the CMAC stain be observed, the vacuolar lumen is stained blue. In* ***figure 7c*** *can a few magenta fluorescence dots be observed which is the Pex3-mKate2 in the N2 colony. In* ***figure 7d*** *we have the merged channel of the meGFP, CMAC and mKate2 for the N2 colony. In* ***figure 7e*** *can the green fluorescence of meGFP be observed for the C1 colony. In* ***figure 7f*** *can the fluorescence of the CMAC stain be observed, the vacuolar lumen is stained blue. In* ***figure 7g*** *can a few magenta fluorescence dots be observed which is the Pex3-mKate2 in the C1 colony. In* ***figure 7h*** *we have the merged channel of the meGFP, CMAC and mKate2 for the C1 colony. The fluorescence signal (measured with ImageJ) can also be found for the meGFP, mKate2 and CMAC under the figure for either the N2 or C1 colony. The figures are made with the Axioscope (upright) microscope.*

The staining of the vacuolar lumen with the blue fluorescent CMAC is done to see where the meGFP is located compared to the vacuole for the N2 and the C1 colony. In **figure 7b** we can nicely see the blue vacuolar lumen for the N2 colony and in **figure 7f** we can nicely see the blue vacuolar lumen for the C1 colony. In **figure 7a** we can nicely see the green fluorescent meGFP for the N2 colony and in

figure 7e we can nicely see the green fluorescent meGFP for the C1 colony. We can indeed see in **figure 7d** that there is colocalization between the meGFP and the vacuolar lumen, it seems like the meGFP is inside the vacuolar lumen and not on the membrane of the vacuole. For **figure 7h** this is slightly different and more complicated, it seems like there is a little bit colocalization between the green fluorescent meGFP and the blue fluorescent CMAC (vacuolar lumen) but most of the meGFP seems to be located outside the vacuole.

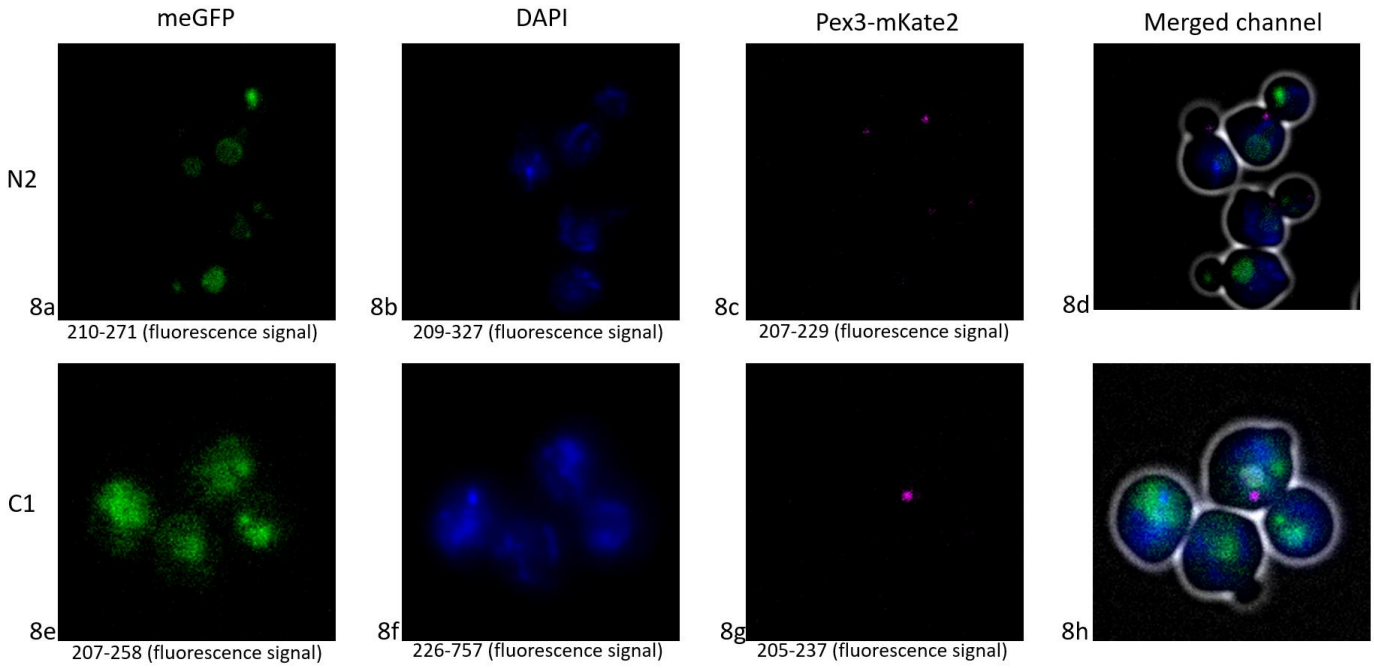


Figure 8. The N2 and C1 ku80 yeast colonies are grown on glucose mineral medium for the DAPI staining procedure. ***Figure 8 a,b,c,d*** *are fluorescence microscopy figures from the N2 colony.* ***Figure 8 e,f,g,h*** *are fluorescence microscopy figures from the C1 colony. In* ***figure 8a*** *can the green fluorescence of meGFP be observed in the N2 colony. In* ***figure 8b*** *can the fluorescence of the DAPI stain be observed, the dsDNA in the yeast cells is stained blue. In* ***figure 8c*** *can a few magenta fluorescence dots be observed which is the Pex3-mKate2 in the N2 colony. In* ***figure 8d*** *we have the merged channel of the meGFP, DAPI and mKate2 for the N2 colony. In* ***figure 8e*** *can the green fluorescence of meGFP be observed for the C1 colony. In* ***figure 8f*** *can the fluorescence of the DAPI stain be observed, the dsDNA in the yeast cells is stained blue. In* ***figure 8g*** *can a few magenta fluorescence dots be observed which is the Pex3-mKate2 in the C1 colony. In* ***figure 8h*** *we have the merged channel of the meGFP, DAPI and mKate2 for the C1 colony. The fluorescence signal (measured with ImageJ) can also be found for the meGFP, mKate2 and DAPI under the figure for either the N2 or C1 colony. The figures are made with the Axioscope (upright) microscope.*

The staining of the dsDNA with the blue fluorescent DAPI was done to see where the meGFP is located compared to nucleus and mitochondria in the yeast strains and in particular for the C1 colony because it seems that the meGFP for this colony is neither located in the vacuolar lumen nor on the membrane of the vacuole. For the N2 colony we can really see the meGFP inside the vacuolar lumen. In **figure 8b** we can nicely see blue stained dsDNA for the N2 colony and in **figure 8f** we can nicely see the blue stained dsDNA for the C1 colony. In **figure 8a** we can nicely see the green fluorescent meGFP for the N2 colony and in **figure 8e** we can nicely see the green fluorescent meGFP for the C1 colony. We can indeed see in **figure 8d** that there is no colocalization between the meGFP and the nucleus and mitochondria (dsDNA), like expected since the meGFP is located inside the vacuole. For **figure 8h** this is slightly different and more complicated, it seems like there is a little bit colocalization between the green fluorescent meGFP and the blue fluorescent DAPI. This indicates that the meGFP

is most likely to be present on membrane of the nucleus and therefore also in the ER (de Haan, et al., 2006) than in the vacuole.

Discussion

The Fun26 transporter protein is known to be located in the vacuolar membrane (Boswell-Casteel et al., 2014). A mass spectrometry study was done to analyze the transcription levels of proteins and the protein abundance in a wild-type *S. cerevisiae* strain and a peroxisome-deficient *S. cerevisiae pex3* strain. The protein transcription and protein abundance are compared to each other for these two strains (Kosir et al., 2024). According to the study it turned out that for both the strains the transcription levels of Fun26 is roughly the same but the protein abundance in the *S. cerevisiae pex3* strain is reduced compared to the wild-type *S. cerevisiae* (Kosir et al., 2024). The *S. cerevisiae pex3* strain has no functional peroxisomes anymore and the protein level of Fun26 is also decreased, maybe there is a link here. Furthermore, is the Fun26 protein a transporter of nucleosides and nucleobases which serve as substrate for the ScNPY1 and ScPnc1 enzymes inside the peroxisome (Effelsberg et al., 2015) (AbdelRaheim et al., 2001). Hypothetically, it is a possibility that the Fun26 transporter is also located in the peroxisomal membrane based on these two findings.

The Fun26 of *H. polymorpha ku80* is an integral membrane protein of 424 amino acids (Benchling, 2024) with 11 transmembrane segments (Health tech DTU, 2024). Health tech DTU predicts the translocation of the Fun26 membrane based on amino acid sequence, the N-terminal of the Fun26 is located in the lumen of the vacuole whereas the C-terminal of the Fun26 protein is located in the cytosol based on these predictions.

N2 analysis

For the fluorescence microscopy on the N2 colony (*H. polymorpha ku80 PEX3-MKATE2 MEGFP-FUN26* Colony 2) we observe a green fluorescence circle in the lumen of the vacuole, this could be cleaved off meGFP which was fused to the N-terminal of the in the vacuolar membrane located Fun26 transporter. Another possibility is that the meGFP-Fun26 protein is mislocated and transported to the vacuole and downgraded in the lumen of the vacuole. The Western blot performed with HRP-conjugate (**figure 4b**) confirms that only free meGFP is present for the N-terminal meGFP fusion to Fun26, this makes sense because in the vacuole can the meGFP be cleaved off the N-terminal of the Fun26 protein, because of the harsh environment of the lumen of the vacuole (Thumm, 2000). Another possibility is that the meGFP-Fun26 protein is mislocated and transported to the vacuole and downgraded in the lumen of the vacuole, the meGFP is a stable protein and is not downgraded in the lumen yet at the time of fluorescence microscopy, that is also a possibility why we see the meGFP in the lumen of the vacuole (Monosov, 1996). The meGFP is not localized on the membrane of the vacuole since we observe a more like circle structure and not a ring (**figure 5/6**). Because of this it is also difficult to draw conclusions on the location of the Fun26 since we cannot really observe a fusion protein. When there was a ring like structure on the vacuole we could say something about the location of the meGFP-Fun26 protein. Based on the staining of the vacuolar lumen done with the blue CMAC stain, we can conclude that the meGFP is located in the vacuolar lumen since the CMAC and the meGFP colocalize (**figure 7**). If we look at the Western blot performed with the HRP-conjugate (**figure 4b**), we can see that there are no meGFP fusion proteins present in the N2 cells TCA sample. A band around 27 kDa is present, this is free meGFP since it has a protein size of 27 kDa. We can conclude that the N-terminal of Fun26 is located inside the lumen of the vacuole or that because of overexpression and mislocation of the meGFP-Fun26 protein,

transport to the vacuole for degradation of the fusion protein happens. Due to the environment (acidic) and proteases inside the lumen of the vacuole is the meGFP cleaved off and/or the fusion protein degraded, this is the reason for all the free meGFP (Thumm, 2000). We can still observe the green of the fluorescent protein since the meGFP is a stable protein which is not immediately degraded by the vacuole (Monosov, 1996).

C1 analysis

If we look at the fluorescence microscopy for the C1 colony (*H. polymorpha ku80 PEX3-MKATE2 FUN26-MEGFP* Colony 1) we can see that most of the meGFP seems to be located outside the vacuole. In **figure 5d** we see a more like ring structure outside of what I suspect to be the vacuole. In **figure 6d and 6f** it seems that the meGFP is still partly located outside the vacuole but it also seems that some of the meGFP is located in what I suspect to be the vacuolar lumen. In **figure 6** are the cells grown on methanol which makes the peroxisomes increase in size (Van Zutphen, 2008), we can see for **figure 6d and 6f** that the Pex3-mKate2 has a contact site with an organelle, this is the vacuole since peroxisomes (especially Pex3) and the vacuole have contact sites with each other (Wu, 2018) and exactly in the lumen of this organelle is also green fluorescence of meGFP present. The C1 colony is also stained with CMAC which stains the lumen of the vacuole blue. In **figure 7e** we can still observe green fluorescence of the meGFP outside the vacuole, in **figure 7h** we can observe the merged channel of the meGFP, CMAC and mKate2; some colocalization between the meGFP and the CMAC stain can be observed at the membrane of the vacuole and also some meGFP located inside the lumen of the vacuole can be observed. The C1 colony is also stained with DAPI which stains the dsDNA in the nucleus and also in mitochondria. In **figure 8e** we can see the green fluorescence of the meGFP for the C1 colony, the meGFP is not concentrated in a nice circle but more spread over the entire cell. In **figure 8f** we can observe the C1 colony with the blue DAPI stain, also the DAPI stain is spread over the entire cell, this is the dsDNA in mitochondria. In **figure 8h** there is only very little colocalization between the meGFP and the DAPI stain so I suspect that the meGFP is localized in the ER.

The Western blot performed with HRP-conjugate (**figure 4b**) shows us a band of free meGFP (a band around 27 kDa) and a band of the Fun26-meGFP (around 70 kDa) can be observed. The Fun26-meGFP has a protein size of 73.7 kDa so this seems about right (Benchling, 2023) (DNA to Protein, 2024). So, in the C1 colony is most of the meGFP cleaved off the Fun26 protein but there also still some intact Fun26-meGFP proteins present.

It is a possibility that part of the Fun26-meGFP is located in the ER because apparently the ER-membrane is also a location of the Fun26 transporter. Another possibility is that because of the C-terminal fusion of meGFP to Fun26, the transport of the Fun26 protein to the vacuole is slowed down and because the pathway of the transport to the vacuole is via the ER, we can still see some Fun26-meGFP in the ER (Klionsky, 1998). It is also a possibility that because of overexpression of the Fun26-meGFP, some of the fusion proteins are mislocated and transported via the ER to the vacuole to be degraded and that is why we see some intact fusion proteins located on the ER and also some meGFP in the vacuolar lumen. It is also a possibility that the C-terminal is located inside the lumen of the vacuole and that is the reason of the meGFP in the lumen of the vacuole after it is cleaved off the C-terminal of Fun26. I suspect that the intact Fun26-meGFP proteins are located in the ER, whether it is their location in the ER-membrane or the fusion protein is present there because of transport pathways via the ER, both options are both possible (Klionsky, 1998). In **figure 7h** it seems like there is some colocalization between the meGFP and the vacuolar membrane but there is also meGFP located inside the lumen of the vacuole. It is difficult to draw a conclusion on wheter the C-terminal

of the Fun26 is located inside or outside the lumen of the vacuole and that is why future research is needed.

In the end it is also difficult to draw a conclusion on the location of the Fun26 protein since we are not sure if there is even a fusion protein of meGFP to Fun26 observed for both the N2 and C1 colonies. Maybe on the ER-membrane and in **figure 7h** for the C1 colony but for the N2 colony it is really difficult to prove that a fusion protein is observed since the meGFP seems like free meGFP, this is also what the Western blot performed with HRP-conjugate (**figure 4b**) confirms. But one thing we know for sure and that is that the Fun26 transporter is not localized on the peroxisome.

PMSF is also added to the N2 and C1 colony since PMSF is an inhibitor of some proteases in the vacuole (Zieske, 1991), we unfortunately did not find other/new results compared to **figure 5**, the location and fluorescence signal of meGFP was roughly the same. A suggestion for future research is using a *H. polymorpha ku80 PEX3-MKATE2* strain in which the *PEP4* gene is knocked out and the *MEGFP* gene fused to *FUN26* (C- or N-terminal) is also transformed in this strain. Yeasts are easy to use for genome editing like knock out of certain genes (Fraczek, 2018). Pep4 is required for post-translational precursor maturation of vacuolar membranes (PEP4 / YPL154C Overview, 2024). Maybe because of the knock out of this gene, the meGFP fused to the Fun26 protein can be observed in the membrane of the vacuole since the meGFP is not cleaved of. Another suggestion for future research is using an inducible system with a weak promoter for the transcription of the fusion gene so we do not get overexpression of the fusion protein but now we really can see the location and the fluorescence signal of the meGFP at different time points by fluorescence microscopy.

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Appendix

Plasmids:	**Description**
pHIPZ18 *MEGFP-W1Q7X1*	Padh1 promoter, zeocin resistance, ampicillin resistance, N-terminal fusion of meGFP to Fun26 (*W1Q7X1* replaced)
pHIPZ18 *W1Q7XI-MEGFP*	Padh1 promoter, zeocin resistance, ampicillin resistance, C-terminal fusion of meGFP to Fun26 (*W1Q7X1* replaced)
pHIPZ18 *MEGFP-FUN26*	*H. polymorpha ku80 PEX3-MKATE2* integrating plasmid, Padh1 promoter, zeocin resistance, ampicillin resistance, *MEGFP-FUN26*
pHIPZ18 *FUN26-MEGFP*	*H. polymorpha ku80 PEX3-MKATE2* integrating plasmid, Padh1 promoter, zeocin resistance, ampicillin resistance, *FUN26-MEGFP*

Strains:	**Description:**
E. coli DH5α	F– ϕ80lacZΔM15 Δ(lacZYA-argF)U169 recA1 endA1 hsdR17(rK–, mK+) phoA supE44 λ–thi-1 gyrA96 relA1
H. polymorpha ku80	Leu 1.1
H. polymorpha ku80 PEX3-MKATE2	Leu 1.1, Natr
H. polymorpha ku80 PEX3-MKATE2 MEGFP-FUN26	Leu 1.1, Natr, ZeoR
H. polymorpha ku80 PEX3-MKATE2 FUN26-MEGFP	Leu 1.1, Natr, ZeoR

Primers:	**Sequence:**
cPCR Padh1-fw	5’-CACGGCAGCAGAATTGGAATTG-3’
FUN26-C-Fw	5’-CCCAAGCTTATGTCTGCTAGTAATACGTC-3’
FUN26-C-Rev	5’-CGCAGATCTTGCACTCACCAGATACACAA-3’
FUN26-N-Fw	5’-CGCGGATCCATGTCTGCTAGTAATACGTCC-3’
FUN26-N-Rev	5’-CCGGTCGACCTATGCACTCACCAGATACAC-3’
mGFP rev check	5’-AAGTCGTGCTGCTTCATGTG-3’
Tamo-rev	5’-TGATGCCTACAGACCAGATG-3’
Seq GFP Fw	5’-TGCCCGACAACCACTACCTG-3’
pHIPN18 Fw	5’-CCCACTCGGAGTAAAGCAA-3’