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Chromatin dynamics during nitrogen depletion in fission yeast, *Schizosaccharomyces pombe*

Dennis Larsson

Project work in biology 45 hp, 2013
IMBIM
Supervisor: Pernilla Bjerling

Abstract
The fission yeast *Schizosaccharomyces pombe* is an excellent eukaryotic model organism for chromatin studies such as H3K9 (Histone 3 Lysine 9) methylation and heterochromatin formation. In a previous study regarding starvation responses, it was shown that the gene cluster Chr1, located in the middle of chromosome I, contains several genes up regulated by nitrogen depletion. It was also presented that this Chr1 region relocated out from the nuclear membrane towards the centre of the nucleus during early nitrogen starvation. This has given rise to the hypothesis that this relocalization is important for proper gene expression during early nitrogen depletion. In order to study this, the protein Gal4DBD-CCVC (Gal4 DNA Binding Domain-Cystein-Cystein-Valine-Cystein) was incorporated in a *S. pombe* strain. Gal4DBD-CCVC will then be targeted by farnesyltransferases, which adds a farnesol molecule to the first cystein. This gives the protein a hydrophobic tail, which attracts it to other hydrophobic structures, in this case the nuclear membrane, thus inhibiting the relocalization. A plasmid containing Gal4DBD-CCVC with an inducible *nmt1*-promoter (no message thiamine) was assembled and used for transformation into the genome. Strains containing fluorescent tags at Chr1, the nuclear membrane, and the spindle pole body were created by crossing various strains and used to study the Chr1 relocalization using LiveCell fluorescent microscopy. Together with Gal4DBD-CCVC, the effects of nitrogen starvation on Chr1 were studied.

List of abbreviations

Chr1: Chromatic region on chromosome 1 containing 7 nitrogen repressed genes.
DsRed: Red Fluorescent Protein.
E. coli: *Escherichia coli*.
EMM-N: Edinburgh Minimal Medium lacking nitrogen.
EMM: Edinburgh Minimal Medium.
FastAP: Fast Alkaline Phosphatase.
FD: Fast Digest.
FTases: Farnesyltransferases.
Gal4DBD-CCVC: Gal4 DNA Binding Domain-Cystein-Cystein-Valine-Cystein.
GFP: Green Fluorescent Protein.
H3K9: Histone 3 Lysine 9.
HP1: Heterochromatin Protein 1.
LA: Lysogeny Agar.
LB: Lysogeny Broth.
nmt1: No Message Thiamine.
PMG_{TOT}: Pombe Minimal Glutamate (Total).
RITS-complex: RNA-Induced Transcriptional Silencing Complex.
RNAi: RNA Interference.
RT-PCR: Reverse Transcriptase Polymerase Chain Reaction.
S. cerevisiae: *Saccharomyces cerevisiae*.
S. pombe: *Schizosaccharomyces pombe*.
TdTomato: Red Fluorescent Protein.
YEA: Yeast Extract Adenine.

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1. Introduction

1.1. Schizosaccharomyces pombe as a model organism for chromatin studies

The research field of epigenetics has been steadily growing during recent years. Epigenetics is defined as heritable changes in gene expression and phenotype that do not depend on genotypic change. This increased interest in epigenetics could be attributed to the links between epigenetic changes and a number of common conditions such as cancer (Esteller M 2007, Portela & Esteller 2012), autoimmune diseases (Javierre *et al.* 2009), and neurological diseases (Jakovcevski & Abkarian 2012) to name a few. Therefor, it is of great importance to understand the mechanisms of epigenetic changes. An organism that is especially useful in chromatin modification studies is the unicellular fission yeast, *Schizosaccharomyces pombe*. Compared to the yeast model organism *Saccharomyces cerevisiae*, it is more suitable for chromatin studies as it has fewer chromosomes (3 compared to 16) and because it has chromatin modifications similar to those of animals. Also, heterochromatin is highly regulated by H3K9 (Histone 3 Lysine 9) methylation, which is induced by the HP1 proteins Swi6/Chp2 (Al-Sady *et al.* 2013). This methylation of H3K9 is conserved from yeast to humans, making *S. pombe* ideal for studying basic chromatin mechanisms in. Finally, *Schizosaccharomyces pombe* have RNA interference (RNAi) that has effects on heterochromatin initiation through the recruitment of the RITS-complex (RNA-induced transcriptional silencing) (Marina *et al.* 2013), a system that *S. cerevisiae* lacks.

In the cell nucleus, there are roughly two different types of chromatin. Euchromatin, a lightly packed form associated with gene expression and heterochromatin, which is more tightly packed and associated with gene silencing and is often found in highly regulated genomic regions such as telomeres and the centromere.

1.2. Chromatin dynamics and the gene cluster Chr1

When *S. pombe* cells are starved of nutrients, primarily nitrogen, a response is triggered causing the cells to mate and initiate meiosis, leading to the formation of four haploid spores in a spore ascus. During early nitrogen starvation, numerous genes show various levels of up-regulation (Mata *et al.* 2002) and additionally, seven of these nitrogen regulated genes are located together in a cluster in a central region of chromosome I, called Chr1 (Alfredsson-Timmins *et al.* 2009). The studies of Chr1 and the chromatin dynamics during nitrogen starvation showed that the chromatin around Chr1 relocated after brief nitrogen depletion (Alfredsson-Timmins *et al.* 2009). In addition to this, there are also changes in nucleosome density and histone modifications, such as acetylation (Kristell *et al.* 2010). This has given rise to the hypothesis that relocalization of Chr1 is of great importance for full gene expression of the genes located in this region during early nitrogen starvation.

1.3. Inhibition of Chr1 relocalization

In order to investigate what effect the Chr1 relocalization has on gene expression, a method for inhibiting this movement had to be devised. Farnesyltransferases (FTases) are enzymes that attach a farnesol molecule to CaaX-motifs, a small chain of amino acids containing cysteine, two aliphatic amino acids, and an additional amino acid (most commonly alanine, glutamine, methionine, and serine), on proteins (Sousa *et al.* 2013). This molecule is highly hydrophobic and therefore is attracted to other hydrophobic regions, like the lipids in peroxisomes or the nuclear membrane. Since *S.*

pombe lacks peroxisomes, the most likely target would be the nuclear membrane. Gal4DBD-CCVC is a protein containing a Gal4 DNA Binding Domain with a cysteine-cysteine-valine-cysteine tail, a CaaX-motif, which will be used as a substrate for FTases and be farnesylated. The Gal4 DNA binding domain will bind to the Gal4 gene containing a Gal4DBD binding site that is located close to the Chr1 cluster. This combined will in theory cause farnesylated Gal4DBD-CCVC to bind to Gal4DBD binding sites and attract the DNA to the nuclear membrane, tethering the Chr1 and preventing relocalization during nitrogen depletion. It is desirable to be able to control the expression of Gal4DBD-CCVC because a high level of the protein is lethal to the cell. This means an inducible promoter such as the *nmt1*-promoter is suitable. The *nmt1*-promoter (no message in thiamine) is a thiamine (Vitamin B_1) repressed promoter and is a common inducible promoter used in *S. pombe* (Maundrell K, 1990). There are three different versions of this promoter, each allowing for various expression levels of the gene it precedes. These can be classified as weak (7x induction), medium (25x induction), and strong (300x induction) in concerns regarding gene expression (Maundrell *et al.* 1993) (Kumar & Singh 2006). In a previous study a strain containing the strong *nmt1*-promoter were used together with Gal4DBD-CCVC. However, this caused the cells to die (Engels M 2009). Therefore, a new strain with the weak *nmt1*-promoter was created (Larsson 2013) which did not affect cell viability. Early studies showed that it might give too low expression of Gal4DBD-CCVC for a significant inhibition of Chr1 relocalization. This gave rise to the need for a strain with the medium *nmt1*-promoter coupled to Gal4DBD-CCVC incorporated.

1.4. Fluorescent signals and the LacO/LacR-GFP system

In a previous study where the Chr1 relocalization during early nitrogen depletion was presented, Chr1 was tagged with a *LacO*/*LacR*-GFP system (Alfredsson-Timmins *et al.* 2009). The LacO/LacR-GFP system is an array of *LacO* introduced to the Chr1 region at the *ura4+* gene through homologous recombination. In conjunction with this, the LacR (LacI) protein is fused to GFP (Nabeshima *et al.* 1998). These will bind to all the *LacO* inserted giving a bright GFP signal in the Chr1 region when viewed under a fluorescent microscope. Together with this, additional structures of the nucleus had to get fluorescent signals in order to allow measurements of the Chr1 relocalization.

1.5. Aims of the study

The aim of the project was to investigate the circumstances around Chr1 relocalization and its gene regulation during nitrogen starvation in *S. pombe*. In order for this to be studied, several strains had to be generated with fluorescent tags at nuclear structures by crossing. Together with the fluorescent tags, a strain containing Gal4DBD-CCVC with a medium *nmt1*-promoter had to be created. In order for this to be achieved a plasmid had to be assembled by sub-cloning of Gal4DBD-CCVC from the expression vector pPB76 in to the empty expression vector paR41X containing the medium *nmt1*-promoter and then transforming a *S. pombe* strain with the newly created plasmid by electroporation. Using these strains together with a strain containing Gal4DBD-CCVC coupled to a weak *nmt1*-promoter constructed in a previous experiment (Larsson 2013), several experiments can be made. Firstly a RT-PCR expression analysis of the nitrogen repressed genes *urg1+* and *urg2+* was made in order to assure proper nitrogen starvation and then statistical analysis of Chr1 relocalization during nitrogen starvation using LiveCell fluorescent microscopy. Combining these results, performing the same experiments again with the expression of Gal4DBD-CCVC to inhibit the chromatin movement of the Chr1 region should give sufficient information

about the importance of this relocalization for proper gene expression of Chr1 during early nitrogen starvation.

2. Materials and methods

2.1. Crossing of strains

To create strains with genotypes necessary for the experiments, strains had to be crossed with each other to generate desired final strains (Table 1). Crossing of *S. pombe* is simply done by streaking desired strains of opposite mating type together on a nitrogen- and nutritional lacking medium plates (PMG_{TOT}) and growing them at 30°C over 2-3 days. These conditions will trigger cells of opposite mating type (h^+/h^-) to mate and generate spore asci. Once spore asci have formed (detectable under a light microscope), the cell and spore asci mixture is treated with the enzyme glusulase. This enzyme will digest the cell walls, which lyses the cells and the ascus but leaving the spores unharmed, thus leaving only spores and cellular debris. For the glusulase treatment, an inoculation loop containing cells and spores were suspended in filter sterilized 1 ml deionized and sterilized water mixed with 7.5 µl glusulase. This glusulase and spore mixture is then incubated at 30°C for over night. The free spores are then observed and counted using a light microscope and a Bürker chamber. A reasonable amount of spores (50-150) are then plated on rich medium plates (Yeast extract adenine, YEA) and then grown in 30°C over three days. When colonies were clear and visible, the plates are then used for replica plating (Figure 1) to select for desired genotype.

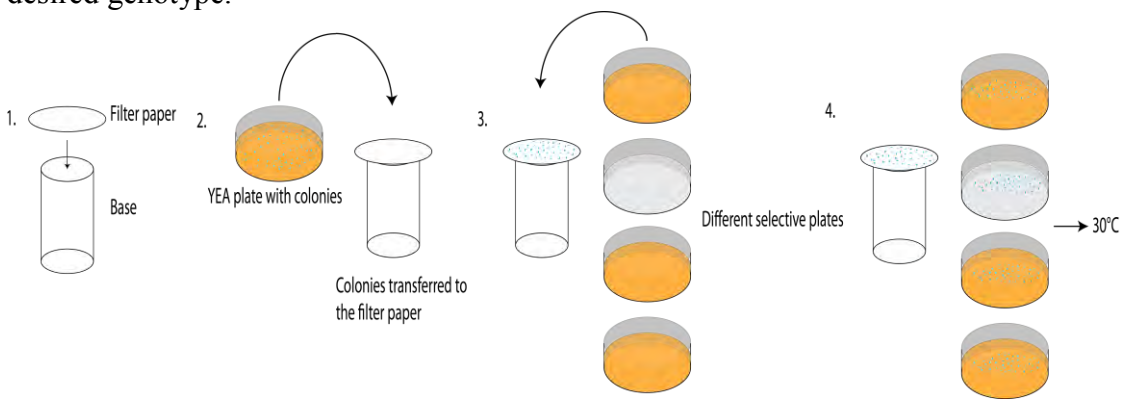

Figure 1: Procedure of replica plating. 1. Filter paper (Munktell Qualitative Filter Paper) was placed on a solid base structure. 2. The plate containing colonies was pressed on the filter paper, transferring the colonies. 3. Empty selective plates were gently pressed against the colonies on the filter paper transferring cells to the new plates without pulling off the colonies from the filter paper. 4. The selective plates containing transferred colonies were incubated at 30°C for three days. Colonies are coloured blue in this diagram for convenience.

Using replica plating with selective plates, colonies with the desired genotype were identified. These colonies were then re-streaked on YEA plates and grown in 30°C for 3 days and then subjected to another round of replica plating, using the same selective plates, to confirm the genotype. If the strains have genes for fluorescent proteins used for tagging certain structures (Chr1::GFP, spindle pole body::TdTomato, nuclear membrane::DsRed), they had to be grown and observed under a fluorescent microscope to verify the quality of the fluorescent signals. Once the signal and the genotype is confirmed, the strains were stocked in 25% glycerol at -80°C until further experiments.

Table 1. Crossing of strains to generate desired genotype.

Strains crossed	Desired genotype
PJ522×PJ1491	*h*$^+$ *his7*$^+$::*lacI*-GFP *tel1*::(*ura4*$^+$ *hphMX6 lacO*) *pom152-DsRed*::*natMX6 cut12-tdTomato*::*kanMX6 leu1-32 ura4-D18 ade6-*
PJ525×PJ1491	*h*$^+$ *his7*$^+$::*lacI*-GFP *chr1*::(*ura4+ hphMX6 lacO*) *pom152-DsRed*::*natMX6 cut12-tdTomato*::*kanMX6 leu1-32 ura4-D18 ade6-*
PJ1107×PJ1495	*h*$^+$ *his7*$^+$::*lacI*-GFP *chr1*::(*ade6*$^+$ *Gal4bindingsite hphMX6 lacO*) *pom152-DsRed*::*natMX6 cut12-tdTomato*::*kanMX6 hip1*::*kanMX6 leu1-32 ura4-D18 ade6-M210*
PJ1151×PJ1491	*h*$^+$ *his7*$^+$::*lacI*-GFP *chr1*::(*ade6*$^+$ *Gal4bindingsite hphMX6 lacO*) *pom152-DsRed*::*natMX6 cut12-tdTomato*::*kanMX6 arg3- leu1-32 ura4-D18 ade6-*
PJ1411×PJ1491	*h*$^+$ *his7*$^+$::*lacI*-GFP *chr1*::(*ade6*$^+$ *Gal4bindingsite hphMX6 lacO*) *nmt1p-gal4DBD-CCVC pom152-DsRed*::*natMX6 cut12-tdTomato*::*kanMX6 arg3*$^+$ *leu1-32 ura4- ade6-DN/N*

2.2. Creation of plasmid containing medium nmt1-promoter

In order to study the effects of the Chr1 re-localization, a strain with Gal4DBD-CCVC incorporated into its genome was created. The expression of Gal4DBD-CCVC had to be at a reasonable level in order to avoid killing the cells. Therefore, a promoter allowing medium expression was optimal. The first step involved in generating such a plasmid was growing *E. coli* containing the plasmid pPB76 (pREP3 plasmid containing strong *nmt1*-promoter and Gal4DBD-CCVC) and *E. coli* containing the plasmid paR41X and then extracting the plasmids. Strains of *E. coli* with pPB76 and paR41X were taken up from -80°C stock and subsequently plated on Lysogeny broth (LB) plates containing ampicillin (150 mg/L) and grown overnight at 37°C. The grown cells were then used to inoculate 3ml liquid LB containing ampicillin (150 mg/L), which were grown overnight at 37°C on a shaker. These cultures were then used for plasmid extraction using a GeneJet Plasmid Mini Kit (Thermo Scientific, #K0502, #K0503) with provided materials. The cells were transferred to 1.5 ml Eppendorf tubes, which were harvested at 8000 rpm and the supernatant was discarded by decantation. Acquired pellets were then re-suspended in 250 µl Re-suspension Solution. Once re-suspended, 250 µl Lysis Solution was added followed by brief mixing of the sample by inverting the tube and then 250 µl Neutralization Solution was added and mixed using inversion. With all these solutions added, the samples were centrifuged at 13200 rpm for 5 minutes in order to pellet unwanted material. The supernatant was then transferred to a provided GeneJET™ Spin Column and centrifuged at 13200 rpm for 1 minute and the flow-through was discarded. 500 µl Wash Solution was then added to the column and spun in the centrifuge at 13200 rpm for 1 minute. This step was repeated one additional time. Once the flow-through was discarded, the columns were centrifuged an extra time at 13200 rpm to dry the column of superfluous Wash Solution. The columns were then placed in new sterile 1.5 ml Eppendorf tubes and 25 µl Elution Buffer was added to the centre of the membrane in the column, incubated at room temperature for 2 minutes and then centrifuged at 13200 rpm for 2 minutes. The eluted plasmids was then placed on the membrane again and spun in the centrifuge as in previous elution step. Extracted plasmids were then separated on a 1% agarose gel using gel electrophoresis (120V) to control the purity and see the levels of extracted plasmid. After the purity control, the extracted plasmids were treated with the restriction enzyme Fast Digest (FD) *BamHI* according to

following protocol: **pPB76 (1)**: 2 µl FD BamHI, 5 µl 10X FD Buffer Green, 11 µl plasmid (~1 µg plasmid), 32 µl H_2O. **pPB76 (2)**: 2 µl FD BamHI, 5 µl 10X FD Buffer Green, 11 µl plasmid (~1 µg plasmid), 32 µl H_2O. **pPB76 (3)**: 2 µl FD BamHI, 5 µl 10X FD Buffer Green, 15 µl plasmid (~1 µg plasmid), 28 µl H_2O. **paR41X (1)**: 2 µl FD BamHI, 5 µl 10X FD Buffer Green, 2.3 µl plasmid (~1 µg plasmid), 40.7 µl H_2O. **paR41X (2)**: 2 µl FD BamHI, 5 µl 10X FD Buffer Green, 2.3 µl plasmid (~1 µg plasmid), 40.7 µl H_2O **paR41X (3)**: 2 µl FD BamHI, 5 µl 10X FD Buffer Green, 2.3 µl plasmid (~1 µg plasmid), 40.7 µl H_2O. The samples were treated with the restriction enzyme for 30 minutes at 37°C and thereafter treated with 1 µl FastAP, to prevent self-ligation of the plasmids by removing the phosphate at the 5' end disabling the 5'-3' connection, for 30 minutes in room temperature. After the FastAP treatment, the plasmids were separated on a 1% agarose gel using gel electrophoresis (120V). This separated the Gal4DBD-CCVC gene (insert) from pPB76 and the linearized paR41X plasmid (open vector). The insert and open vector were cut out of the agarose gel placed in a 2 ml Eppendorf tube and the genetic material was extracted through gel purification with QIAquick Gel Extraction Kit (QIAGEN, #28704, #28706) with the provided materials and following the manufacturer's protocol. First, gel slices were weighed and then dissolved in 3:1 volume to weight ratio (300 µl:100 mg) of Buffer QG by incubating at 50°C while on a shaker until gel slices were completely dissolved. Once dissolved, 1:1 volume to weight ratio of isopropanol was added to the sample and mixed by vortexing. The samples were then placed in a QIAquick column and centrifuged at 13200 rpm in order to bind the DNA to the membrane of the column. After centrifugation, flow-through was discarded and 500 µl of Buffer QG was added to the column and then centrifuged again in the same conditions in order to remove residual agarose. The flow-through was removed and 750 µl of Buffer PE was added to the column and incubated at room temperature for 5 minutes before centrifugation, as in previous steps. Yet again, the flow-through was removed and the column was subjected to centrifugation as in previous conditions to remove residual ethanol from Buffer PE. Once centrifuged, the columns were placed in new 1.5 ml Eppendorf tubes and 30 µl of Buffer EB was added and then incubated at room temperature for 1 minute after which the columns were centrifuged in order to elute the genetic material. The eluted DNA was then placed in the column yet again and the last step was repeated. The gel purified fragments and vectors were then subjected to various ligation setups with T4 DNA Ligase (Table 2) and incubated over night at room temperature.

Table 2. T4 DNA Ligase setup for ligation of plasmid with plasmid plus controls.

Setup	**Ligation mixture**
No DNA	2 µl 10X T4 DNA Ligase Buffer 0.75 µl 10 mM ATP 0.5 µl T4 DNA Ligase 16.75 µl H_2O
+K	2 µl 10X T4 DNA Ligase Buffer 0.75 µl 10 mM ATP 0.5 µl T4 DNA Ligase 0.89 µl paR41X (80 ng, un-cleaved plasmid) 15.86 µl H_2O
Cut plasmid	2 µl 10X T4 DNA Ligase Buffer 0.75 µl 10 mM ATP 1 µl paR41X (~80 ng FD BamHI-cleaved plasmid)

	16.25 µl H_2O
Cut plasmid ligated	2 µl 10X T4 DNA Ligase Buffer 0.75 µl 10 mM ATP 0.5 µl T4 DNA Ligase 3 µl paR41X (80 ng FD BamHI-cleaved plasmid) 12.75 µl H_2O
Cut FastAP treated ligated plasmid	2 µl 10X T4 DNA Ligase Buffer 0.75 µl 10 mM ATP 0.5 µl T4 DNA Ligase 4 µl paR41X (~80 ng FD BamHI-cleaved FastAP treated plasmid) 12.75 µl H_2O
Insert	2 µl 10X T4 DNA Ligase Buffer 0.75 µl 10 mM ATP 0.5 µl T4 DNA Ligase 5 µl Gal4DBD-CCVC (~30 ng FD BamHI-cleaved insert) 11.75 µl H_2O
Cut FastAP treated plasmid with insert	2 µl 10X T4 DNA Ligase Buffer 0.75 µl 10 mM ATP 0.5 µl T4 DNA Ligase 4 µl paR41X (~80 ng FD BamHI-cleaved FastAP treated plasmid) 12 µl Gal4DBD-CCVC (~20-30 ng FD BamHI-cleaved insert) 0.75 µl H_2O

After the incubation the newly created plasmids were used for cloning into competent *E. coli* $DH5_\alpha$ cells. These cells were thawed slowly on ice. Once thawed, 4 µl of each ligation sample (Table 2) were mixed with the cells (100 µl, 2.5×10^8 cells/ml) and let incubating on ice for an additional four minutes. Following the incubation the cells and ligation mixture were subjected to heat shocking at 42°C for one minute. Immediately after the heat shocking, the mixtures were put on ice to cool down for five minutes. After the cooling period, the cells were streaked on LA plates with ampicillin (150 mg/L) and incubated at 37°C over night. To control that the $DH5_\alpha$ cells had taken up the desired plasmid and that the plasmid had been assembled correctly, colonies appearing the day after were taken up and used to inoculate 3 ml LA medium with ampicillin (150 mg/L) and also re-streaked on new LA plates with ampicillin (150 mg/L). Cultures and plates were all incubated at 37°C over night, the cultures on a shaker. The next day the overnight cultures were used for plasmid extraction using the GeneJET Plasmid Mini Kit (Thermo Scientific, #K0502, #K0503) as described in section *2.2* and then cleaved with FD BamHI according to following protocol: **FD BamHI**: 2 µl 10X FD Green Buffer, 1 µl FD BamHI, 2 µl plasmid, 15 µl H_2O. The restriction enzyme cleaving was carried out over 50 minutes at 37°C. This should cleave out the inserted Gal4DBD-CCVC gene, which is then detected using gel electrophoresis to separate the inserted gene from the vector. This separation was performed on a 1% agarose gel (100V) until good separation could be observed. It is of great importance that the insert had been inserted in the right orientation. Therefore, the next step was to cleave the plasmids with insert using the restriction enzymes KspAI and SmaI that will generate two bands of different size depending on what orientation the insert has. This was carried out using the following protocol: **SmaI**: 2

µl 10X FD Green Buffer, 1 µl FD SmaI, 2 µl plasmid, 15 µl H_2O. **KspAI**: 2 µl 10X FD Green Buffer, 1 µl FD KspAI, 2 µl plasmid, 15 µl H_2O. **SmaI+KspAI**: 2 µl FD Green Buffer, 1 µl SmaI, 1 µl KspAI, 2 µl plasmid, 14 µl H_2O. **Positive control (pPB76) SmaI**: 2 µl 10X FD Green Buffer, 1 µl SmaI, 2 µl pPB76 plasmid, 15 µl H_2O. **Positive control (pPB76) KspAI**: 2 µl 10X FD Green Buffer, 1 µl FD KspAI, 2 µl pPB76 plasmid, 15 µl H_2O. **Positive control (pPB76) SmaI+KspAI**: 2 µl 10X FD Green Buffer, 1 µl FD SmaI, 1 µl FD KspAI, 2 µl pPB76 plasmid, 14 µl H_2O. The digestion was carried out at 37°C over 20 minutes. After the restriction enzyme digestion, the samples were separated on a 1% agarose gel using gel electrophoresis. The colonies corresponding to the plasmids with correct orientation was then re-streaked on LA plates with ampicillin (150 mg/L) to use for stocking. Once sufficient cells had grown they were put in 0.75 ml LA with ampicillin (150 mg/L) and 0.75 ml 50% glycerol and stocked at -80°C.

2.3. Transformation of S. pombe with constructed plasmid.

The newly constructed plasmids containing the Gal4DBD-CCVC with the medium *nmt1*-promoter had to be transformed into *S. pombe* using electroporation. In order for homologous recombination to occur, the plasmids need to be linearized before transformation. The linearization was performed by digesting the plasmids with Bsp681 according to following protocol: **Bsp681**: 2 µl Buffer O, 1 µl Bsp681, 15 µl plasmid, 2 µl H_2O. The samples were incubated at 37°C for one hour. The strain PJ1495 was then used to inoculate 100 ml YEA and grown over night at 30°C. When the cells reached log-phase and had a concentration of less than 2×10^7 cells/ml they were split up in two 50 ml test tubes. The samples were harvested in an ultra centrifuge, at 5000 rpm and 4°C and the supernatant was carefully discarded and the cell pellet was re-suspended in 20 ml ice-cold 1.2M sorbitol solution. After the addition of sorbitol, the cells were spun at 2500×g for five minutes at 4°C. The supernatant was removed yet again and the pellet was re-suspended in 10 ml ice-cold 1.2M sorbitol and was then spun in the same conditions as previous harvesting cycle. This was repeated two additional times to assure that there were no residual media left. When the final cycle had been performed, the cells were re-suspended in 600 µl 1.2M sorbitol and divided in to three aliquots with 200 µl in each. Each 200 µl aliquot was placed in a GenePulse cuvette put on ice. To avoid complications with the GenePulser, the plasmids had to be desalinized before use by placing the sample on Millipore Membrane Filters floating on deionized water over 10 minutes at room temperature and then relocated to a sterile 1.5 ml test tube. To five of the aliquots 5 µl of plasmid was added and one aliquot was used as a negative control with deionized water instead. Immediately after the plasmid was added to the cells, they were subjected to an electrical pulse using a BioRad Gene Pulser II (2.25 kV, 200Ω, 25 mF). As soon as the pulse has gone through the sample, 500 µl ice-cold 1.2M sorbitol was added and the sample was streaked out on a selective plate (PMG_{TOT}) and left to dry for a few minutes. Once the plates were dry enough they were incubated at 30°C over a number of days (3-5). When colonies could be detected on the plates, they were picked up and re-streaked on non-selective plates (YEA) and grown over night in 30°C. This was repeated two more times. After the last re-streak, the transformants were yet again re-streaked on non-selective plates although this time for single colonies. These single colonies were used for replica plating using PMG_{TOT} and grown over two days at 30°C. If stable transformants could be observed, they would need to be confirmed to contain the correct inserted gene PCR. The stable transformants were then used to inoculate 10 ml of YEA medium and grown in 30°C under constant aeration. Once the cells had reached log-phase and had a concentration of less than 2×10^7 cells/ml, the samples were used for genomic DNA extraction with a Yeast DNA Extraction Kit

(Thermo Scientific, #78870) using the manufacturer's protocol and the provided materials. First, cells were relocated to new 1.5 ml test tubes and then harvested at 3000×g at room temperature. The supernatant was discarded without disrupting the pellet and the pellet was then weighed. Depending on the weight of the pellet, different amounts of Y-PER Reagent were used (8 µl/1 mg). Once the correct amount of Y-PER Reagent was added, the samples were mixed by vortexing until the mixture is homogenized. When the samples was properly mixed, they were incubated at 65°C for 10 minutes to lyse the cells. After the incubation, the samples were centrifuged at 13200 rpm for 5 minutes and subsequently had the supernatant removed. The pellet was then re-suspended in 400 µl DNA of Releasing Agent A and 400 µl of DNA Releasing Agent B. Yet again, the samples were mixed by vortexing until a homogenized mixture was acquired and then incubated at 65°C for 10 minutes. The samples were then treated with 200 µl Protein Removal Reagent and mixed by inverting the tubes and then followed by centrifugation at 13200 rpm for 5 minutes. The supernatant was removed and placed in a new 1.5 ml test tube and to it 600 µl of isopropyl was added. When added, the samples were gently mixed by inversion and then centrifuged at 13200 rpm for 10 minutes to precipitate the genomic DNA. Without disturbing the pellet, the supernatant was removed. Once removed, 1.5 ml 70% ethanol was added in which the pellet was re-suspended by inversion. The re-suspended sample was then centrifuged at 13200 rpm for 1 minute to wash off residual buffers from sample. After the washing step, the ethanol was removed and the pellet was left to dry at room temperature. Once all the ethanol had evaporated, the pellet was re-suspended in 50 µl TE buffer. The extracted DNA was then treated with RNase to degrade RNA traces by adding 20 µl RNase A (Roche, #10109142001) to the samples and then incubating them at 37°C for approximately an hour. The treated genomic DNA was used in a PCR (Phire)(Tables 5, 6) with primers flanking the Gal4DBD-CCVC gene in order to detect the presence of incorporated Gal4DBD-CCVC.

2.4. RNA extraction and RT-PCR expression analysis

To make sure that the nitrogen starvation protocol was efficient and properly carried out and if the Chr1 tethering had an effect on gene expression, an expression analysis of mRNA using RT-PCR was performed. Firstly, cells of the strain PJ1411 and PJ1493 were grown and harvested in identical conditions as for the fluorescent microscopy study. As soon as the final incubation period was over, the cells were used for complete RNA extraction using an RNeasy Mini Kit (QIAGEN, #74104, #74106) using following protocol and provided reagents. The cells were harvested at 5000 rpm at 4°C for 5 minutes after which the supernatant was carefully removed by pipetting. The pellet was then re-suspended in 100 µl freshly prepared buffer Y1 with zymolase (2.5 mg/100 µl). This cell suspension was incubated at 37°C for 30 minutes on a shaker at low speed to generate spheroblasts. Once the incubation period was over, 350 µl of Buffer RLT was added and the samples were vortexed to lyse the spheroplasts. With the spheroblasts properly lysed, 250 µl of absolute ethanol (99.7%) was added. The samples were then transferred to RNeasy spin columns and centrifuged at 13200 rpm for 15 seconds. After the centrifugation, the flow-through was discarded and 700 µl of Buffer RW1 was added to the columns and they were subsequently centrifuged in the same conditions as earlier described and the flow-through was discarded. For the next step, 500 µl of Buffer RPE was added to the column and the samples were centrifuged as in previous steps. This step was performed twice in order to properly wash the membrane with flow-through being removed after each centrifugation. For the last washing step, the duration of the

centrifugation was increased to 2 minutes. Once the washing step was performed, the columns were placed in new 1.5 ml test tubes for elution. To elute the RNA, 30 µl of RNase-free water was added directly to the membranes and the columns were centrifuged 13200 rpm for 1 minute. The eluate was then placed back on the membrane and centrifuged again as in previous step in order to achieve higher RNA concentrations in the samples. The extracted RNA was then separated on a 1% agarose gel using gel electrophoresis in order to assure no RNase contamination. This RNA could then be used for RT-PCR analysis using the OneStep RT-PCR Kit (QIAGEN, #210210)(Supplementary data, Table 7) and the samples were then put in a thermal cycler for the RT-PCR (Table 8). Three genes were investigated, one control gene showing minimum difference before and after nitrogen starvation, *dis2+*, and two genes of interest showing high up-regulation during early nitrogen starvation, *urg1+* (26 times increase) and *urg2+* (90 times increase) (Kristell *et al.* 2010). After the RT-PCR, the generated cDNA was separated on a 1% agarose gel using gel electrophoresis.

2.5. Live cell fluorescence microscopy and cell counting

In order to observe the effect of farnesylation of Gal4DBD-CCVC on Chr1 re-localization, the cells were viewed under a fluorescent microscope before and after nitrogen starvation. The strain PJ1411 was grown in 3 ml EMM medium in 30°C over a period of three days, with and without thiamine. When the cells reached log-phase and had a concentration of less than 2×10^7 cells/ml, they were split to avoid the stationary phase, which could diminish the fluorescent signals. After three days of growth, the cells were harvested at room temperature at low force (~800 g) for two minutes and the supernatant was discarded carefully not to disturb the pellet. If the cells were to be starved of nitrogen, they were then washed with EMM-N (PMG_{TOT}) by re-suspending and harvesting for three cycles. After the last washing cycle, the cells were incubated in EMM-N (PMG_{TOT}) for 10 minutes in 30°C while shaking. When the incubation period was over, the cells were harvested under the same conditions as previously described and the supernatant was discarded, leaving a low amount of medium to re-suspend the pellet in order to get medium with a high cell concentration. 5 µl of this cell suspension was placed on a microscope cover slide together with 5 µl of lectin (1 mg/ml) and mixed in order to immobilize the cells, increasing the image quality. The cover slide was gently placed over an objective glass with 5 µl of EMM/EMM-N (PMG_{TOT}), for normal a condition and nitrogen depleted condition respectively, and then sealed with Dow Corning® High Vacuum Grease to create a air tight chamber. These slides were then observed under a fluorescent microscope.

For statistical analysis, each cell containing adequate GFP, DsRed, and TdTomato signals on the image from the fluorescent microscope were counted. Once a large enough sample size had been reached (60 cells from each condition), the distance that Chr1 was away from the nuclear membrane and to the spindle pole body was measured.

3. Results

3.1. Strain crossings

The crossed strains needed for these experiments were screened for desired genotype with replica plating on selective plates. These strains were then observed under a fluorescent microscope in order to detect fluorescent signals. Samples were prepared as presented in Materials and Methods, minus the nitrogen starvation step. All but one

strain showed positive fluorescent signals (PJ1107×PJ1495). The strains PJ1411 and PJ1491 mated properly but failed to provide colonies showing the desired genotype.

3.2. Construction of plasmid containing Gal4DBD-CCVC with medium nmt-promoter

Plasmids were extracted from cells containing pPB76 and paR41X with a concentration of 70-135 ng/µl and 90-440 ng/µl respectively. Cleaving these plasmids with BamHI generated Gal4DBD-CCVC bands on the agarose gel together with linearized paR41X (Figure 2) that could be cut out for gel extraction, yielding a concentration of 4-7 ng/µl of Gal4DBD-CCVC and 20-25 ng/µl of paR41X. Using these for ligation and subsequently cloning generated 9 successful integrations (Figure 3). These 9 candidates were tested for correct orientation using double restriction enzyme cleaving, identifying 5 Gal4DBD-CCVC genes inserted in the correct way (Figure 4).

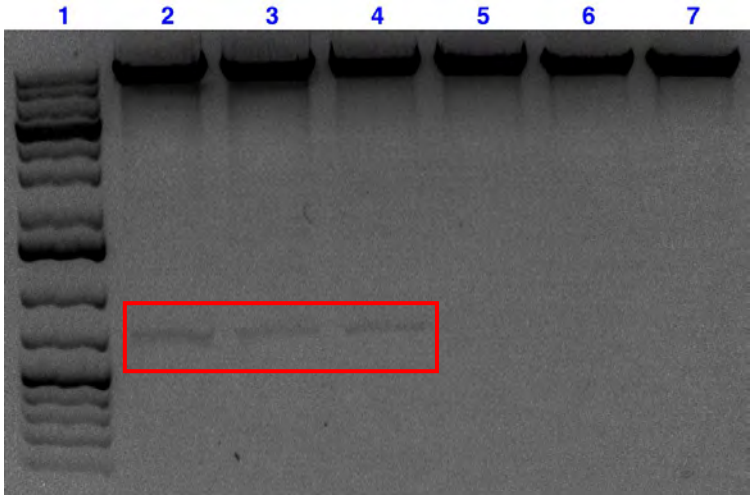

Figure 2. Gel showing Gal4DBD-CCVC cleaved out of pPB76 using BamHI. 1) GeneLadder 1 kb Plus. 2-4) pPB76 cleaved with BamHI showing Gal4DBD-CCVC (500 bp). 5-7) paR41X cleaved with BamHI.

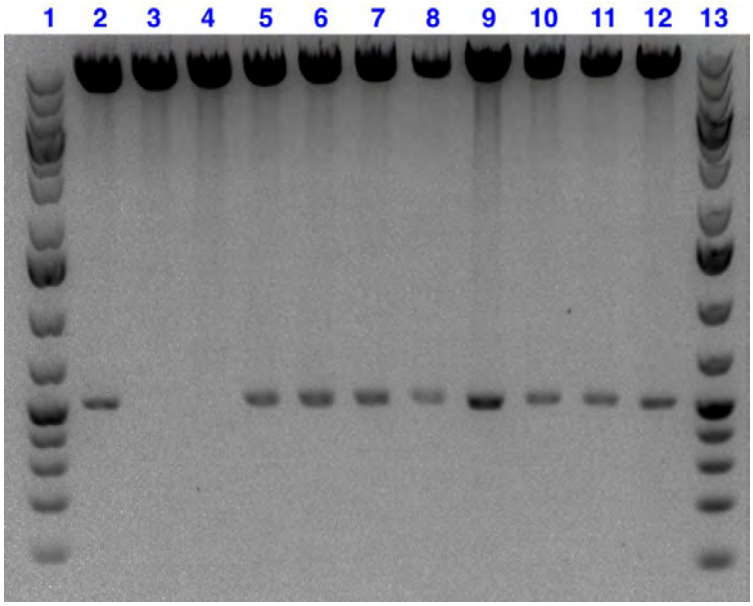

Figure 3. Constructed plasmid extracted from cloned DH5α-cells. 1&13) Gene Ladder 1 kp Plus. 2-12) paR41X ligated together with Gal4DBD-CCVC. Successful ligation and cloning in strains 2,5-12.

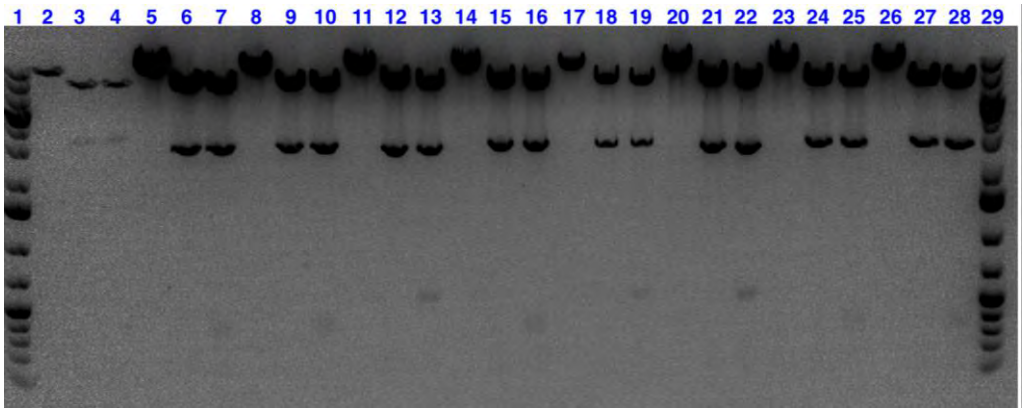

Figure 4. Gal4DBD-CCVC orientation analysis using restriction enzymes SmaI and KspAI (HpaI). 1&29) GeneLadder 1 kb Plus. 2) (SmaI) Positive control pPB76. 3) (KspAI) Positive control pPB76. 4) SmaI+KspAI) Positive Control pPB76. 5, 8, 11, 14, 17, 20, 23, 26) (SmaI) Investigated plasmid. 6, 9, 12, 15, 18, 21, 24, 27) (KspAI) Investigated plasmid. 7, 10, 13, 16, 19, 22, 25, 28) (SmaI+KspAI) Investigated plasmid. Lanes 7, 10, 16, 25 and 28 shows Gal4DBD-CCVC in the correct orientation.

3.3. Transformation of S. pombe using constructed plasmid

Several transformation experiments using electroporation were performed using this newly constructed plasmid. Despite this, no stable colonies or transformants could be detected either on PMG_{TOT}-plates or using PCR (Phire).

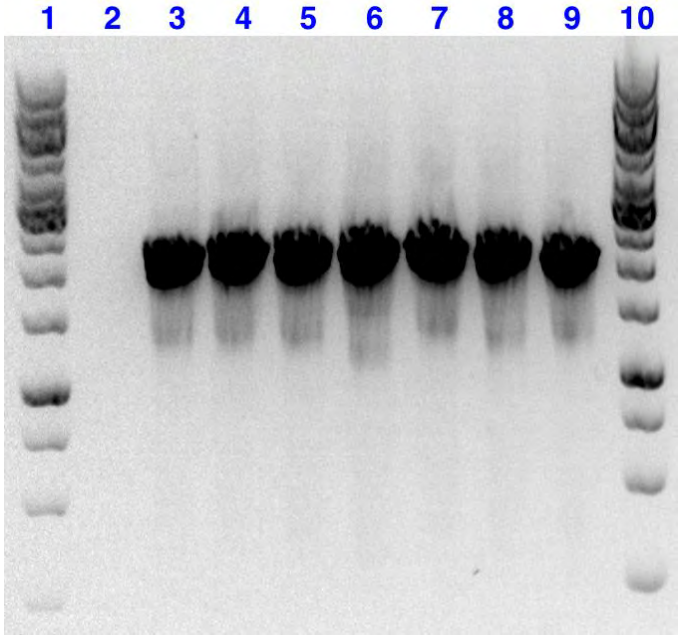

Figure 5. Detection of integrated plasmid using PCR (Phire). 1&10) GeneRuler 1 kb Plus. 2) Negative control. 3-9) Potential transformants. 3 kb bands showing $arg3^-$ gene without recombinated plasmid.

3.4. RT-PCR expression analysis

An RT-PCR expression analysis was performed in order to assure that the nitrogen starvation protocol is efficient. Two strains (PJ1411 and PJ1151) were grown in various conditions from which mRNA was extracted and used in an RT-PCR either with or without an RT-step (Figure 6) for the nitrogen repressed genes *urg1+* and *urg2+* and a gene not effected by nitrogen depletion, *dis2+*, as a negative control. This experiment indicates that proper nitrogen starvation is carried out and thus validating the nitrogen starvation protocol.

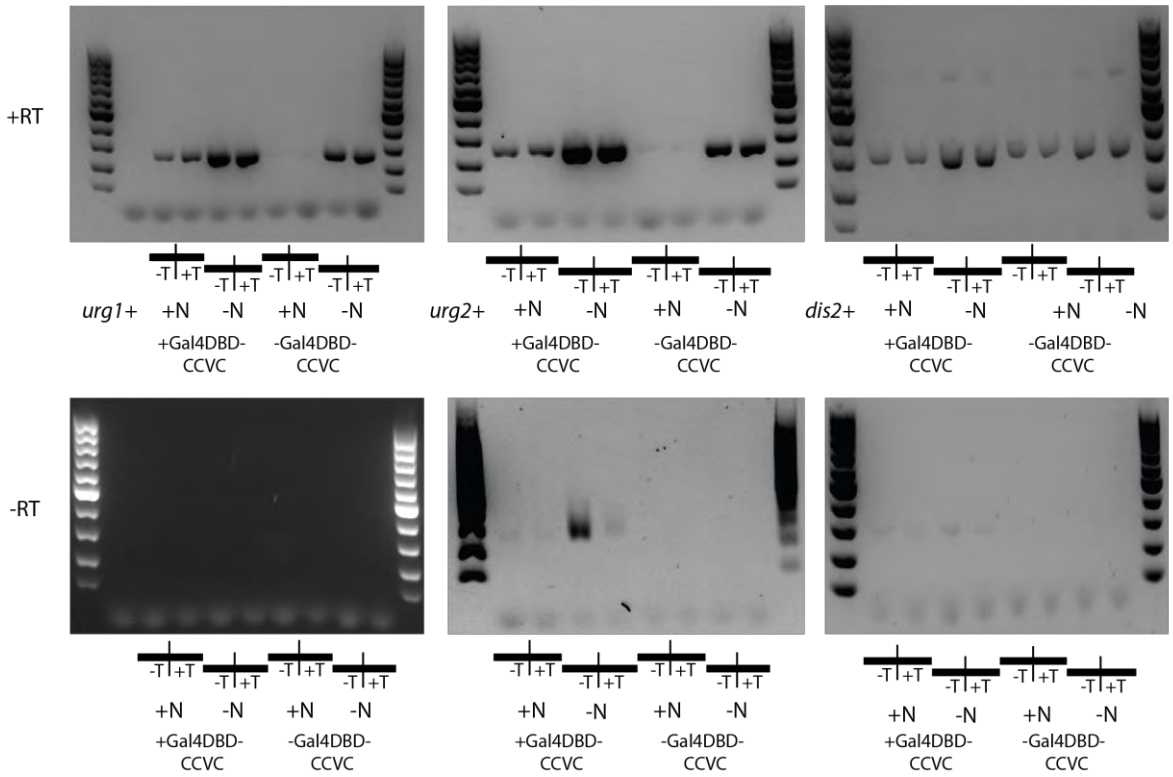

Figure 6. RT-PCR expression analysis used to ensure proper nitrogen starvation. +RT: Samples with cDNA created with reverse transcriptase. –RT: Samples lacking cDNA, checking DNA contamination. –T: Samples grown without the presence of thiamine. +T: Samples grown with the presence of thiamine. +N: Samples collected from strains grown in nitrogen rich medium (EMM). –N: Samples collected from strains grown in nitrogen deficient medium (EMM-N/PMG_{TOT}). +Gal4DBD-CCVC: Samples collected from strains containing Gal4DBD-CCVC. –Gal4DBD-CCVC: Samples collected from strains lacking Gal4DBD-CCVC. –RT, urg2+, +Gal4DBD-CCVC, –N, –T shows signs of DNA contamination.

3.5. Fluorescent microscopy and statistical analysis

With the aim of understanding the importance of the Chr1 relocalization, it was necessary to primarily reproduce the nitrogen starvation triggered chromatin movement presented in Alfredsson-Timmins *et al.* 2009. Therefor, the strain PJ1495 was subjected to the same nitrogen starvation conditions as the study and then observed under a fluorescent microscope. The cells containing adequate signal from Chr1 (GFP), the nuclear membrane (DsRed), and the spindle pole body (TdTomato) were used for the relocalization study. In Alfredsson-Timmins *et al.* 2009, three zones where Chr1 can be located were designated. The same zones were used in this study (Figure 7A). In the selected cells, the amount of Chr1 signals detected in various locations of the nucleus was counted (Figure 7B, 7C, 7D) using the image analysis software ImageJ with the plug-in programme ND2Reader. Each cell was magnified to a pixelated state (One pixel=0.217μm) and the distance of Chr1 to the nuclear membrane and the spindle pole body was determined. A minimum distance was set to one pixel for this analysis. This information was then used for statistical analysis of the location of Chr1 (Figure 8).

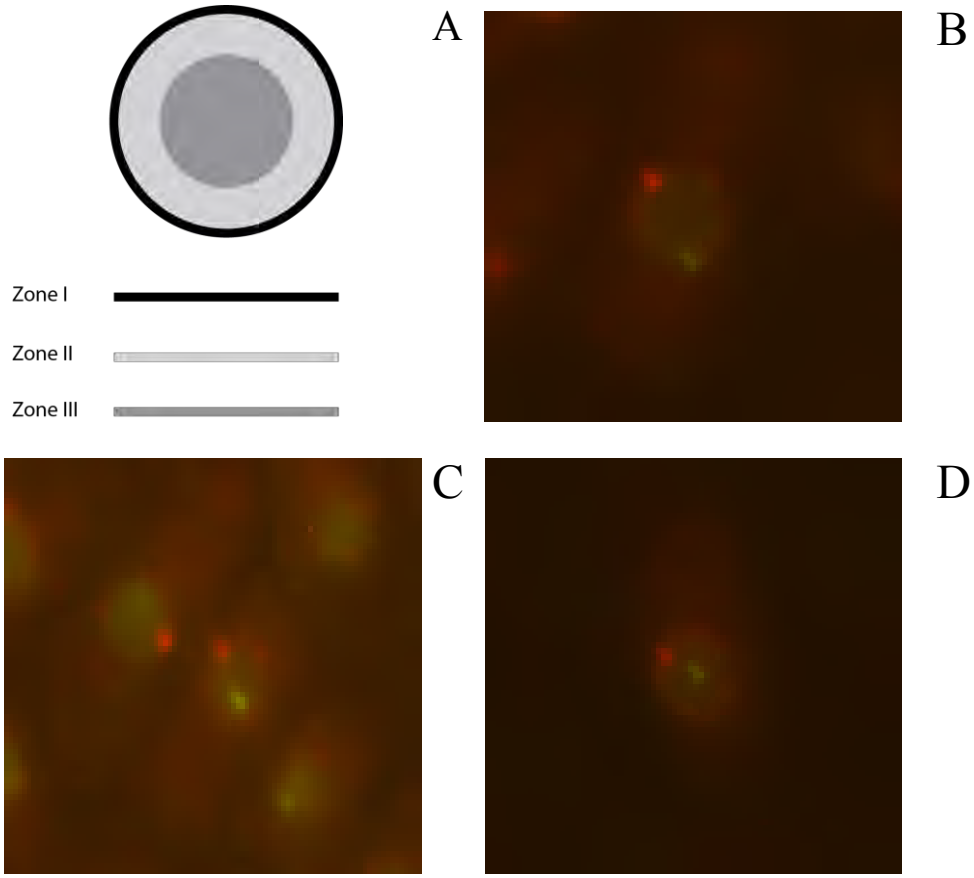

Figure 7. A) Designated zones where Chr1 can be localized. Zone I: 0-0.22 µm. Zone II: 0.23-0.51 µm. Zone III: 0.52-1.20 µm. Modified from Alfredsson-Timmins *et al.* 2009, Fig.3a. B) Image showing Chr1 signal in zone I. C) Image showing Chr1 signal in zone II. D) Image showing Chr1 signal in zone III.

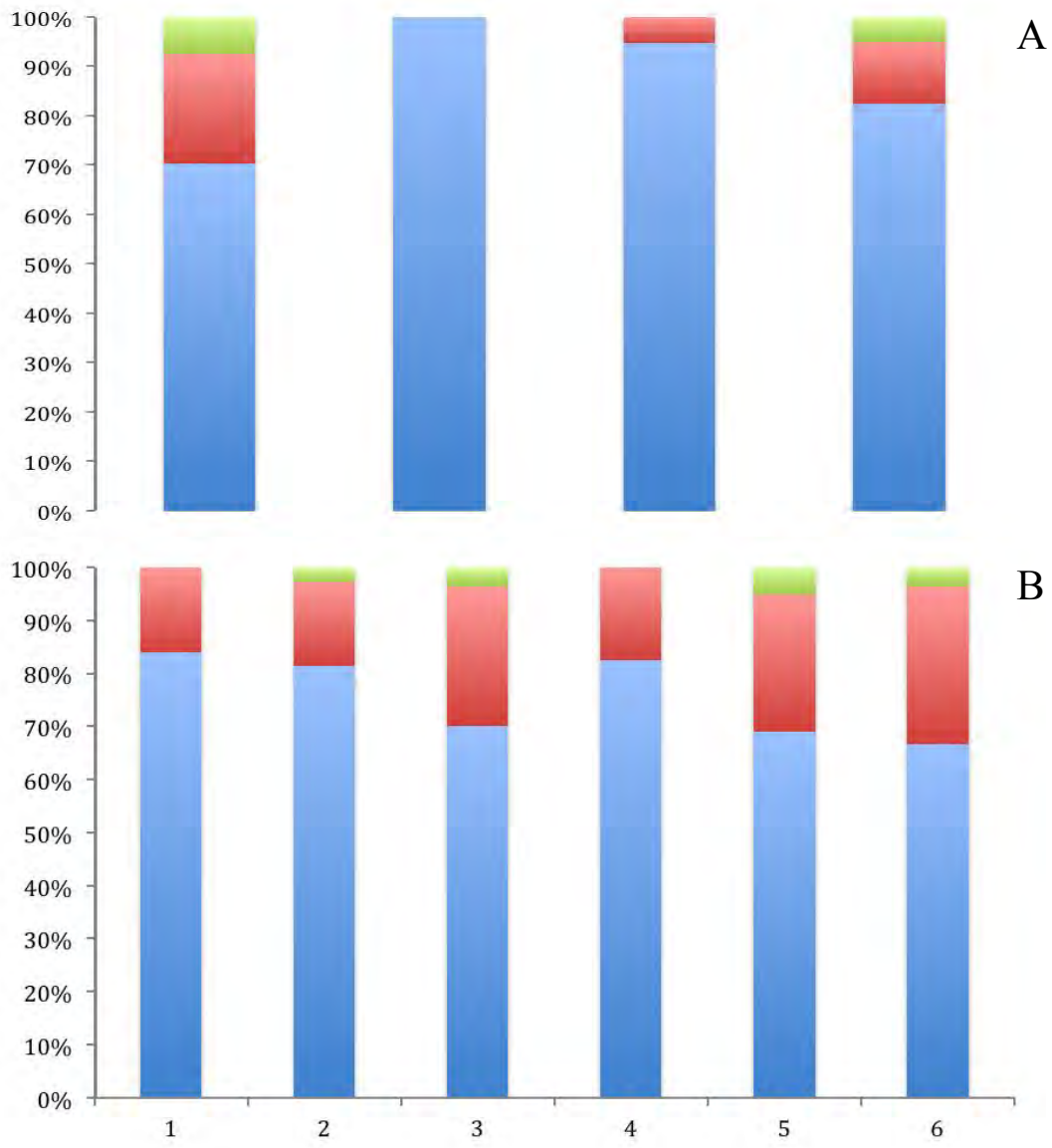

Figure 8. Statistical analysis of the position of Chr1before or after nitrogen starvation. A. Distribution of Chr1 signal in % during nitrogen rich conditions. B. Distribution of Chr1 signal in % during nitrogen deficient conditions. Blue: Zone I, Red: Zone II, Green: Zone III. Zone distribution assigned as in Figure 7.

4. Discussion

The objective for this study was to investigate whether Chr1 relocalization during early nitrogen starvation is important for gene regulation. Since new strains of *S. pombe* were used for these experiments, the first priority was to ensure that the results presented in Alfredsson-Timmins *et al.* 2009 could be replicated. Results from RT-PCR analysis to measure gene expression levels through mRNA extraction indicate that a proper nitrogen starvation protocol was performed due to an increase in *urg1+* and *urg2+* expression resembling results presented in Alfredsson-Timmins *et al.* 2009 and Kristell *et al.* 2010, which validates its use for fluorescent microscopy. Multiple fluorescent microscopy imaging sessions were carried out, yielding various results. The fluorescent signals bleached out quickly, which led to some signals becoming faint and hard to detect. Despite failed sessions, enough cells with good signals and visible Chr1 relocalization were available for a statistical analysis. However, strains carrying Gal4DBD-CCVC with a weak *nmt1*-promoter did not show a difference

before or after nitrogen starvation with or without thiamine regarding Chr1 relocalization and expression of Gal4DBD-CCVC nor any clear signs that the farnesylation of Gal4DBD-CCVC had any effect on the chromatin movement. There are a few reasons for why this did not work as expected. One reason could be that the Gal4DBD-CCVC expression is too weak, which is unlikely but plausible. Another explanation could be that the farnesylation was not enough to tether Chr1 to the nuclear membrane. If this were the case, a way to counteract this would be to incorporate more Gal4 binding sites in the Chr1 region. This should increase the amount of farnesylated proteins bound and potentially result in proper tethering. Human errors could also account for improper nitrogen starvation, but since there was minimum difference in media or protocol between the RT-PCR analysis and the fluorescent microscopy it is strange if that would be the case although one cannot rule out this as a potential cause.

During previous studies, the transformation by electroporation of *S. pombe* with the plasmid paR81X (weak *nmt1*-promoter) containing Gal4DBD-CCVC was successful (D. Larsson, unpublished data). Therefor, it is uncertain why it would not work with the newly constructed plasmid paR41X (medium *nmt1*-promoter). The transformation efficiency and homologous recombination of *S. pombe* is low which is a probable answer to why there were no stable transformants. It would still be valuable to try the transformation additional times, as it could just have been chance that no stable transformants could be detected.

For future studies, there are several potential experiments to run. Firstly, it would be preferable to have a *S. pombe* strain with integrated medium *nmt*-promoter Gal4DBD-CCVC gene in order to evaluate the effect of farnesylation on Chr1 relocalization. Once such a strain has been obtained, redoing the nitrogen starvation experiments with fluorescent microscopy could be most enlightening. If evidence of Chr1 relocalization inhibition can be observed, doing an RT-PCR expression analysis would tell if this relocalization is important for gene expression. Together with these experiments, similar setups could be used to evaluate the chromatin relocalization of Tel1, which has shown similar behaviour as Chr1, and its gene expression regulation. These experiments combined could provide clues on how chromatin movement affects gene regulation across the chromosomes.

5. Acknowledgements

First, I would like to give my deepest thanks to Pernilla Bjerling and Alejandro Rodriguez for their massive support, assistance, and constructive critique during my stay. I would also like to thank, in no particular order, Marcus Wäneskog, Daniel Steinhauf, Sanket Gaikwad, Jesper Boberg, Vladimir Maksimov, and Jacob Miro for creating a pleasant lab environment. Finally, I would like to thank Benjamin Holmgren for his assistance with the fluorescent image analysis.

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7. Supplementary data

7.1. Strains

Table 3. Yeast strains used for crossings.

Strains	Genotype
PJ522	*h*$^{+}$ *his7*$^{+}$::*lacI*-GFP *tel1*::(*ura4*$^{+}$ *hphMX6 lacO*) *ura4-D18 leu1-32 ade6-M210*
PJ525	*h*$^{+}$ *his7*$^{+}$::*lacI*-GFP *chr1*::(*ura4+ hphMX6 lacO*) *ura4-D18 leu1-32 ade6-M210*
PJ1107	*h*$^{-}$ *hip1*::*kanMX6 his7-366 leu1-32 ura4-D18 ade6-M210*
PJ1151	*h*$^{+}$ *his7*$^{+}$::*lacI*-GFP *chr1*::(*ade6*$^{+}$ *Gal4bindingsite hphMX6 lacO*) *arg3*$^{-}$ *leu1-32 ade6-DN/N ura4-*
PJ1411	*h*$^{+}$ *his7*$^{+}$::*lacI*-GFP *chr1*::(*ade6*$^{+}$ *Gal4bindingsite hphMX6 lacO*) *nmt1p-gal4DBD-CCVC arg3*$^{+}$ *leu1-32 ura4- ade6-DN/N*
PJ1491	*h*$^{-}$ *his7-366 pom152-DsRed*::*natMX6 cut12-tdTomato*::*kanMX6 leu1-32 ura4-D18 ade6-*
PJ1495	*h*$^{+}$ *his7*$^{+}$::*lacI*-GFP *chr1*::(*ade6*$^{+}$ *Gal4bindingsite hphMX6 lacO*) *pom152-DsRed*::*natMX6 cut12-tdTomato*::*kanMX6 arg3- leu1-32 ura4-D18 ade6-*

7.2. Primers

Table 4. Primers used for PCR

Name	Sequence
D1 (*dis2*+ Forward)	GAT GTG GAT TTG GAT TCC AT
D2 (*dis2*+ Reverse)	GCG AGC AAA AGA CAA ATT AC
D44 (*urg1*+ Forward)	TGT CGA TGA AGT TAC TCT TCG
D45 (*urg1*+ Reverse)	CTT GGC AGC TTC CTC AAT AC
D46 (*urg2*+ Forward)	GTG TGG AAG TAC ACT GAT GG
D47 (*urg2*+ Reverse)	GAA GCT CAG ACC AGA ATT CC
DL_F (*Gal4DBD-CCVC* Forward)	CAT GGT AGT TGT TGA TTT C
DL_R (*Gal4DBD-CCVC* Reverse)	GAT TCC AAG TCA CCG GAG

7.3. PCR protocol

Table 5. PCR (Phire) mixture

Ingredients	Volume (µl)
H_2O	9.96
5X Reaction buffer	4.0
10 mM dNTP	0.4
Forward Primer (DL_F)	0.12
Reverse Primer (DL_R)	0.12
Template (Genomic DNA)	5.0
Phire HotStart II DNA Polymerase	0.4

Table 6. PCR (Phire) thermal cycler protocol

Temperature	Time	Cycles
98°C	30 s	
98°C 56.3°C 72°C	5 s 5 s 2 min	30 cycles
72°C	1 min	
4°C	∞	

Table 7. RT-PCR mixture

Ingredients	Volume (µl)
H_2O	16.9
5X OneStep RT-PCR buffer	5.0
10 mM dNTP	1.0
Forward Primer (D1/D44/D46)	0.12
Reverse Primer (D2/D45/D47)	0.12
Template (Genomic DNA)	5.0
Phire HotStart II DNA Polymerase	0.4

Table 8. RT-PCR thermal cycler protocol

Temperature	Time	Cycles
50°C	30 m	
95°C	15 m	
94°C 59°C 72°C	30 s 30 s 1 min	25 cycles
72°C	10 min	
4°C	∞	