UPPSALA
UNIVERSITET

The Role and Function of Clr2 in *Schizosaccharomyces pombe*

Gordon Henry Virgo

Degree project in biology, Master of science (2 years), 2012
Examensarbete i biologi 30 hp till masterexamen, 2012
Biology Education Centre and IMBIM - Department of Medical Biochemistry and Microbiology, Uppsala University
Supervisor: Pernilla Bjerling

Table of Contents

1 Introduction

1.1 Background

Epigenetics is defined as the study of changes in gene expression that are not due to changes in the DNA sequence. These epigenetic changes can be heritable and can involve for example changes in DNA methylation and histone modification. Shifts in the epigenome are important during development processes and in diseases, such as cancer. Improper silencing of genes or inappropriate expression can lead to severe conditions and epigenetics is therefore an important research area in order to learn more about gene regulation and different diseases and possible drug targets (Portela and Esteller, 2010).

In eukaryotic organisms there are two types of chromatin, euchromatin and heterochromatin. Euchromatin is the transcriptionally active form whereas heterochromatin is more densely packed and is transcriptionally repressed. Nucleosomes, which DNA is wrapped around, consist of eight histone proteins and at the N-terminal of these histones different modifications are established for the two types of chromatin. Euchromatin has methylation of lysine 4 (H3K4me) and acetylation of lysine 9 (H3K9ac) on histone 3. In heterochromatin it is the other way around with methylation of lysine 9 (H3K9me) that enables interaction with heterochromatin protein 1 (HP1) (Goto and Nakayama, 2011). In this project the yeast *Schizosaccharomyces pombe* have been used to study epigenetically silent heterochromatin. In *S. pombe* there are heterochromatic regions at the centromere, telomeres and at the mating type region (Olsson and Bjerling, 2011). Heterochromatin formation in *S. pombe* involves different complexes that act in different ways. There are complexes that interact directly with the chromatin and also RNA interference (RNAi) mediated complexes (Goto and Nakayama, 2011). The RNA-induced complex (RITS) consists of a group of proteins, which together with the enzyme Dicer generate siRNAs. The RITS complex then recruits the Clr4 methyltransferase complex (ClrC complex) that contains Clr4 that methylates H3K9 (Cam *et al*, 2009). Another important complex that has a role in establishment of heterochromatin is the multieffector complex named SHREC complex. This complex accommodates Clr3,

which is a histone deacetylase, and generates the closed structure of heterochromatin (Cam *et al*, 2009). In this project the focus is to study one of the members in the SHREC complex, namely Clr2.

The SHREC consist of four proteins, Clr1, Clr2, Clr3 and Mit1. Together the SHREC complex achieves transcription gene silencing by restricting the occupancy of RNA pol II at heterochromatic regions (Sugiyama *et al*, 2007). Clr3 contains HDAC activity and the Mit1 protein is a chromatin remodelling protein. Very little is known about the two other components. Clr2 was first characterized as a factor involved in silencing of the mating type region but was later shown to be involved in silencing at all major heterochromatic regions in *S. pombe*. Clr2 is needed for hypoacetylation of histone N-terminal tails by Clr3 (Bjerling *et al*, 2004). Clr2 are expressed at low levels in wild type and over expression of the protein do not result in changes in silencing (Bjerling *et al*, 2004). No other information about Clr2 has been found and no homologs have been present until recently when newly sequenced fungi genomes have been published. From this data a Clr2 region has been established (Our unpublished data). In this region three conserved motifs were found with some amino acids being highly conserved throughout the motifs (see Appendix).

1. 2 *Schizosaccharomyces pombe* as a model organism

The yeast *Schizosaccharomyces pombe* is a unicellular eukaryote and is often called fission yeast because it divides by medial division instead of budding like the baker's yeast *Saccharomyces cerevisiae*. The cells are usually about 3 μm in diameter and 7 μm in length and they elongate from both ends until the reach approximately the double size. Then the cells separate at the medial division septum and result in two daughter cells (Egel, 2004). The fission yeast grows rapidly and has a division time of about 2 hours in rich medium. It can be in both haploid and diploid state but prefers the haploid state. During nitrogen starvation, haploid yeast cells of opposite mating type can fuse and form a diploid cell called zygote. During meiosis this zygote will sporulate and form an ascus with four viable spores (Egel, 2004). The mating type region on chromosome II determines the mating type of the yeast cell. The mating type region has three different loci, *mat1*, *mat2-P* and *mat3-M* but only *mat1* is

expressed. The non-expressed loci are in a heterochromatic region but material from *mat2-P* and *mat3-M* can be transferred to the active locus and by this mechanism the yeast cells can switch mating type. This occurs in a homothallic, h^{90} strain. Rearrangements of the mating-type region can occur, resulting in heterothallic strains with more or less stable mating types, h^{+} mating as P and h^{-}, mating as M. The regions between the three loci are called L and K region. The L region contains a few genes whereas the K region contains a *cenH* element which are similar to centromeric dg/dh repeats, together with *mat3-M* and *mat2-P* this make up the transcriptional repressed heterochromatic area (Arcangioli *et al,* 2007).

Schizosaccharomyces pombe was the sixth eukaryotic organism to be sequenced and the complete genome was published in 2002 (Wood *et al*, 2002). The genome with size of 15 Mb is spread on three chromosomes and approximately 5000 open reading frames have been found. *S. pombe* shares many features with higher eukaryotes, for example the centromere structure is similar to centromeres found in human, histone modifications are present and RNAi is found in *S. pombe* (Wood *et a*l, 2002; Goto and Nakayama, 2011). Since the sequencing of the genome of this organism it has gained popularity for studying particularly the cell cycle and chromatin. Moreover, efficient homologus recombination enables targeted integration into the genome, a feature that has been exploited in this study.

1.3 Aim of the project

In order to find out more about Clr2, protein engineering of the Clr2 has been undertaken. Previously, conserved regions of Clr2 have been identified among newly sequenced fungi genomes. From this data three motifs have been established and conserved amino acids in these motifs have been found. In this project point mutations in these conserved amino acids have been preformed that is expected to give clues about the protein function. The well-defined mating type region in *S. pombe*, which contains a heterochromatic region, has been used to examine silencing. In this region a reporter gene has previously been inserted and silencing of this reporter gene is examined after changes in Clr2.

2 Materials and Methods

2.1 Media

In this project five different media were used for yeast and two for bacteria. All recipes can be found in the Appendix. Particularly YEA medium have been used for taking up strains from the – 80 °C freezer, but also for storage and control plates. Minimal media was used for selection of transformants and examination of silencing of the reporter gene *ade6*$^{+}$. For yeast transformations Tom's FOA medium were used. This media contains Fluorootic acid monohydrate (FOA), which represses growth of Ura+ strains. The other minimal media used were AA drop out media which contains a mix of amino acids. In AA Total, all amino acids are included, whereas in AA -Ade, adenine has been left out. SOB Media was used for growing *Escherichia coli* cells when making competent bacterial cells and also LB medium were used for transformation and growth of *E. coli*.

2.2 Strains

The *S. pombe* strains used for the experiments are listed in table 1. The strain PJ1085 was used for transformation of different constructs of Clr2. In PJ1085, wt *clr2* is deleted which has to be done in a h^{90} strain otherwise the yeast will sporulate and form deformed spore ascus because the deletion of *clr2* lead to disrupt silencing of *mat2-P* and *mat3-M* (Bjerling *et al,* 2004). This strain contains the gene *ura4*$^{+}$, which during successful transformation is replaced by the construct. In this strain there is also an *ad6*$^{+}$ reporter gene inserted in the mating type region. The other strain PJ121 was mainly used as a control since it has the wild type form of Clr2.

Table 1. *Schizosaccharomyces pombe strains used in the project*

Strains	Genotype
PJ1085	h^{90} *mat3-M:ade6*$^{+}$ *leu1-32 ura4-D18 ade6-DN/N clr2::ura4*$^{+}$
PJ121	h^{+} *ura4-D18 leu1-32 ade6-M216*

Apart from the two yeast strains, the bacterial strain *E. coli* DH5α was used for making competent *E. coli* cells.

2.3 Site directed mutagenesis

Different point mutations were introduced in Clr2 by a Polymerase chain reaction (PCR) based method (Qi and Scholthof, 2008). The principle is to have the gene of interest in a plasmid, in this case in pCR®2.1-TOPO® (Invitrogen). Overlapping primers with the mutation in the middle anneal to the gene and the polymerase start the amplification and plasmids with the desired mutation are generated. Primers were designed based on information from Qi and Scholthof (2008), Stratagene and Matt Lewis (University of Liverpool). The general recommendations were to have a T_m above 78 °C in order to avoid self-annealing. Also the primers must be PAGE purified to minimize the risk of introducing unintended mutations. The PAGE purification get rid of primers with shorter bp which otherwise could compete with full length oligonucleotides. Regarding the length the primers, they should be between 25 and 45 bases and the mutation should be in the middle of the primers. All primers were designed to start and end with either a G or C. The primer manufacturer (Thermo Fisher Scientific) only PAGE purify primers that are at least 40 bases in length. The primers listed in the appendix were used for introducing 9 single mutations and 6 double mutations. The same references used for primer design was also used together with discussion from colleagues for establishing a protocol for PCR mutagenesis. The PCR program started with a 2 min denaturation step at 95 °C followed by 18 cycles of 95 °C for 45 s, 58 °C for 45 s for annealing of primers, extension at 72 °C for 1 min and then final extension at 72 °C for 10 min. The programme was changed for some primers that did not work with the initial programme. In these cases problems were overcome by running gradient PCR with annealing temperatures from 54 – 70 °C and also increasing the number of cycles. High-fidelity DNA polymerase Phusion (Finnzymes) was used for the amplification. Other reagents as buffer and DMSO were also from Finnzymes. Full protocol is shown in the appendix. When having trouble with some primer pairs, then concentrations of 3% or 5% of DMSO were used to avoid secondary structure formation and decrease the annealing temperature. The PCR products were analysed on a 1% agarose gel and for samples were a band was observed the tubes were treated with 0.5 µl *Dpn*I and incubated at 37 °C for 2 hours. The template is methylated by the restriction modification system and the enzyme *Dpn*I recognize and cleave

methylated DNA. The template is eliminated by this way and only the plasmids with the mutation are left and can be the transformed into competent cells.

2.3 Bacterial transformation

Competent cells were prepared from a DH5α strain, that was streaked on a LB plate from a stock kept at – 80 °C and incubated overnight at 37 °C. Colonies from the fresh LB plate were inoculated in 250 ml SOB medium and grown at 18 °C at 200 rpm until OD of A_{600} = 0.6. The culture was then put on ice for 10 min and the cells were harvested by spinning at 4500 rcf in a centrifuge (Heraeus Labofuge 400R) for 10 min at 4 °C. The pellets were resuspended in 40 ml ice cold TB (recipe in appendix) and incubated on ice for 10 min. The cells were harvested again by centrifugation and resuspended in 20 ml TB together with 7 % DMSO by swirling and then incubated on ice for 10 min. The cells were divided into aliquots of 50 µl in prechilled Eppendorf tubes and then frozen quickly in dry ice by applying ethanol. The cells were then kept at –80 °C prior to transformation. The transformation efficiency was measured by transforming a plasmid with known concentration. Also, two tubes of competent cells with higher transformation efficiency were a gift from Andreas Karlsson (Uppsala University). These cells were used for two mutations were only weak products were generated by PCR. The transformations were carried out by thawing the competent cells on ice and then mix with 5 µl of sample to be transformed. The samples were incubated on ice for 15 min and then heat shocked in a water bath at 42 °C for 45 s and the returned to ice for 2 min. Each sample was then mix with 300 µl of LB + amp and incubated at 37 °C for 20 min. The whole volumes were then plated on LA + amp and transformants appeared after 24 hours. The transformants were then streaked for single colonies on new LA + amp plates and plasmids were harvested by growing single cell cultures and using E.Z.N.A® Plasmid Mini Kit I (Omega Bio-tek) according to the manufactures instruction. The plasmids were then analysed on a 1 % agarose gel by running 1 µl of plasmid and 5 µl 5X TE. Samples were prepared for sequencing in order to confirm the mutations.

2.4 Cloning into Yeast vector

The pCR®2.1-TOPO® (Invitrogen) plasmids containing the mutated versions of Clr2 were cut with *Bam*HI (Fermentas) for 2 hours at 37 °C according to the manufacturer's instructions. For the different mutations, four reactions of 20 µl for each mutation were set up. Also cuttings for the vector pREP41PkN (V5) were set up. The cuttings were run on 1% agarose gel with big wells and the fragments containing the Clr2 gene were excised by scalpel under UV illumination. As much agarose as possible were removed from the bands and then purified using QIAquick® Gel extraction kit (Qiagen). The purified constructs and empty vector were analysed on a 1 % agarose gel in other to measure sizes of vector and insert and amounts of DNA. Based on the gel picture different ligation reactions were set up using T4 ligation kit (Fermentas) according to their instructions. The ratios used, vector:insert, were 1:2 and 1:5. Also different amounts of T4 ligase were used both 1 unit and 5 units. Before the ligation the empty vector were dephosphorylated by FastAp phosphatase (Fermentas). Phosphate treated vector and untreated vector were used as control. The ligation reactions were incubated in a water bath at 16 °C overnight and the next day 5 µl of reaction were transformed into competent DH5α cells and plasmid was harvest by the E.Z.N.A® Plasmid Mini Kit I (Omega Bio-Tek). The *S. pombe* vector used for cloning was pREP41PkN (Craven *et al.* 1998), which have a V5 tag in the N-terminal, GKPIPNPLLGLDST, which can be detected using V5 antibodies. Successful ligations were then checked to have the right orientation of the gene in the vector. This was done by cutting the vector with *Sal*I (Fermentas) and checking the size of fragments on a 1% agarose gel. Correct orientation of the gene in the plasmid will after *Sal*I digestion generate a fragment of approximately 500 bp whereas a plasmid with the insert at wrong rotation would give product of approximately 1000 bp.

2.4 Yeast transformation

The constructs to be integrated into the strain PJ1085 were all PCR products of the pREP41PkN (V5) vector with different versions of Clr2, generated by the primers D80 and D81. All yeast transformations in this project have been carried out by electroporation by using Bio-Rad gene pulser (Bio-Rad) whereas for bacterial

transformation chemical transformations have been used. The strain PJ1085 was grown in a 200 ml culture until log phase with density of 1 x 10^7 cells ml^{-1} by counting cells in a Bürker chamber under light microscope. The cells were harvested in a centrifuge (Heraeus Labofuge 400R) at 4500 rcf for 10 min at 4 °C. The cells were then re-suspended in filter sterilized 1.2 M ice-cold sorbitol and transferred to a new 50 ml falcon tube. Subsequently the cells were washed 3 times with 1.2 M sorbitol. The next step was to dilute the pellet in 1.2 M ice-cold sorbitol to the concentration 1 x 10^9 cells ml^{-1}. The cells were divided into aliquots of 100 µl and mixed with DNA just prior to adding the solutions in electroporation cuvettes (Merck). The cuvettes were put in to the Bio-Rad gene pulser (Bio-Rad) and pulse was applied by the settings of 2.25 kV, 200 Ω and 25 mF. After each pulse a shot of 0.7 ml ice-cold sorbitol was added and the cells were plated on YEA plates. After approximately 24 hours the plates were replica plated to Tom's FOA plates. These plates contain Fluoroorotic acid monohydrate (FOA) that generates a toxic metabolite in Ura^+ strains. The strain PJ1085 was used because *clr2* have been replaced with an *ura4*$^+$ gene therefore only colonies were *ura4*$^+$ have been replaced by *clr2* will be able to grow. The growth on these plates is very slow and colonies appeared after 5 – 12 days. Transformants were then streaked for single colonies on Tom's FOA plates and all candidates were checked for the loss of *ura4*$^+$ with primers A6 + A7 and integration of *V5-clr2* with primers F18 + F19 by colony PCR using the JTAG-program (see appendix). The candidates for wild type integration were also checked on AA-ade plates since the strain PJ1085 have an *ade6*$^+$ reporter gene in the mating type region.

Yeast transformations were also made based on information from Suga and Hatakeyama (2001, 2003). In this second method the cells were grown in minimal media, SD + leu, instead of rich media. The cells were also treated with DTT-buffer (25 mM dithiothreitol, 0.6 M sorbitol, 20 mM Hepes, pH7.5). This treatment is expected to lead to increased transformation efficiency. The washing procedure with sorbitol was the same as previously described and the cells were aliquoted into Eppendorf tubes with a volume of 50 µl and with final density of approximately 10^9 cells/ml. These cells had an advantage of being able to be stored at –80 °C for 6 months without loss in transformation efficiency according to the authors.

2.5 DNA precipitation

The PCR products of *V5-clr2* were low in DNA concentration for sequencing, so it had to be concentrated. This was done by mixing the PCR products and adding up with dH_2O to 500 µl in a 1.5 ml Eppendorf tube. Then 100 µl of 10 M Ammonium acetate was added and also 1250 µl of 95% ethanol. The solution were mixed vigorously by vortex and incubated at –20 °C over night. The next the day the samples were centrifuged in a bench top centrifuge (Eppendorf 5424 R) at 13000 rpm for 20 min 4 °C. The pellet was then washed by adding 300 µl of ice-cold 70 % ethanol and then centrifuged again for 10 min at 4 °C. As much as possible of the ethanol were removed by pipetting. The Eppendorf tubes were left with the lids open for drying. Eventually when the pellet were completely dry then 30 µl of sterile water was added and the concentration were examined on a 1% agarose gel by electrophoresis. The DNA used in transformation into yeast were desalted on 0.025 um VSWP filters (Millipore). Filters were floating in sterile water in a petri dish and the DNA was incubated on the filters for approximately 15 min.

2.4 Sequencing

All samples that were sequenced were sent to Eurofins mwg operon (Edsberg, Germany). The DNA was sent in a final volume of 15 µl and with concentrations of 50 – 100 ng / µl for plasmid DNA and approximately 2 ng / µl for PCR products. The pCR®2.1-TOPO® (Invitrogen) plasmid were sequenced with M13 Forward (-21) and as reverse primer M13 Reverse (-49) or M13 Reverse (-29) were used. PCR products of *V5-clr2*$^+$ with primers A7 as forward and A6 as reverse (see appendix). All sequences were analysed in the software CLC Main workbench 6.

2.5 Spotted dilution series

To investigate the effects of the two point mutations, P137 → G137 and R170 → G170, strains containing the mutations as well as control strains were grown in YEA media and then serial diluted. The cells were grown to early log phase (5×10^6 cells / ml) and the density was determined by counting cells in a Bürker chamber. For each

strain, six spots were made on YEA plates, AA Total plates and AA -Ade plates. Dilutions were made so the first ring contained 3125 cells and then five-fold dilutions were made for the other five spots. For each dilution, 5 µl was spotted onto the different plates.

3 Results

1.1 Site-directed mutagenesis

The primer pairs F27 + F28 and F25 + F26 were tested for optimum annealing temperature by running gradient PCR. Both primer pairs resulted in more PCR products with annealing temperatures between 58 – 59.7 °C, which can be seen in Figure 1. These two were tested to evaluate the primer design before ordering more primers. Unfortunately not all primer could be designed based on this information because the need of having the mutation in the middle and the AT-rich genome of *Schizosaccharomyces pombe*. For some primer pairs, generally those with higher T_m, no PCR product could be observed within the temperature interval 58 – 59.7 °C. PCR products were generated from these primers by increasing annealing temperature, number of cycles and addition of DMSO.

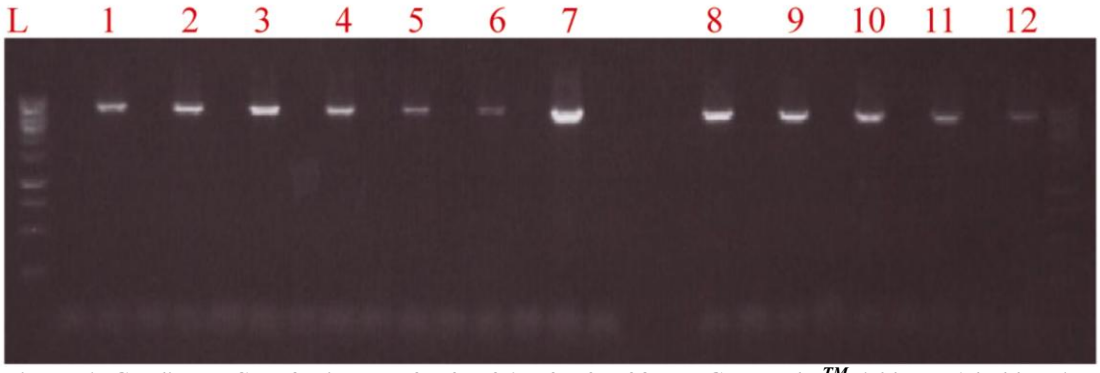


Figure 1. Gradient PCR of primers F25 & F26, F27 & F28. L= GeneRuler™ 1 kb DNA ladder, 1 - 6 are PCR products of primers F25 & F26 and 7 – 8 are PCR products of F27 & F28. The gradient started with 58 ºC and then continued with 58.5 ºC, 59.7 ºC, 61.3 ºC, 62.9 ºC and with final temperature of 64 ºC. The six different temperatures follow the order of the samples 1 – 6 and from 7 – 12. Between each samples are the negative controls with exception for two empty wells on the left of sample 8.

All PCR products were succesfully transformed into competent *E. coli* with transformation efficiency of 1.4×10^6 cfu (µg / DNA)$^{-1}$, except for mutation M13 (Table 3) which was transformed into competent cells with higher transformation efficiency (1×10^8 cfu (µg / DNA)$^{-1}$). In Figure 2, an example is shown how the mutations were analysed in CLC main work bench 6. The sequence for wt *clr2*$^+$ was from Pombase and the sequence was aligned with sequence obtaining the mutation. Figure 2 shows successful change of P137 to G137 by point mutation of CC to GG.

All mutations listed in Table 2 and Table 3 were successfully generated and confirmed by sequencing.

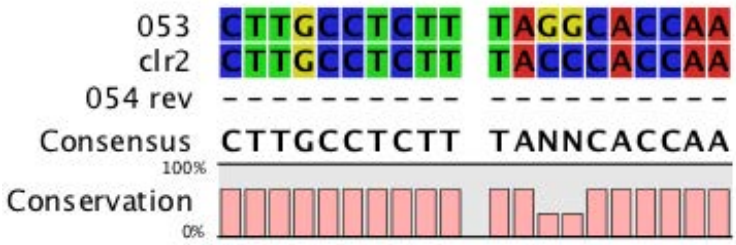


Figure 2. Sequence example from mutation P137 → G137 in motif 1. The sequence in the example was analyzed in the software CLC main work bench 6.

Table 2. Single mutations in conserved amino acids of Clr2

Name of mutation	Change in amino acid sequence	Primers used	Sequence analysis
M1	R170 → G170	F27 + F28	Ok
M2	H178 → G178	F29 + F30	"
M3	F175 → G175	F42 + F49	"
M4	L182 → G182	F43 + F44	"
M5	A385 → G385	F45 + F46	"
M6	E386 → G386	F47 + F48	"
M7	P137 → G137	F25 + F26	"
M8	Y140 → G140	F38 + F39	"
M9	L142 → G142	F40 + F41	"

Table 3. Double mutations in conserved amino acids of Clr2

Name of mutation	Change in amino acid sequence	Primers used	Sequence analysis
M10	R170 + H178 → G170 + G178	F27 + F28, F29 + F30	Ok
M11	R170 + L182 → G170 + G182	F27 + F28, F43 + F44	"
M12	F175 + L182 → G175 + G182	F42 + F49, F43 + F44	"
M13	A385 + E386 → G385 + G386	F47 + F48, F31 + F32	"
M14	P137 + L142 → G137 + G142	F27 + F28, F40 + F41	"
M15	P137 + Y140 → G137 + G140	F25 + F26, F35 + F36	"

Table 4. Point mutation overview of Clr2

Motif 1	Motif 2	Motif 3
M7	M1	M5
M8	M2	M6
M9	M3	M3
M14	M4	
M15	M10	
	M11	
	M12	

1.2 Spotted dilution series

The spotted dilution series in Figure 3 show similar growth for the first three spots on YEA plates and on AA Total plates for all strains, Although there are different number of cells for the other three spots. The YEA plates and AA Total plates are used as control plates and the AA –Ade plates show repression of the reporter gene. On AA -Ade plates there is no growth for PJ121 with wt *clr2*$^{+}$ and also for PJ1335 with *V5-clr2*$^{+}$. The three strains with point mutations grows better as compared to the wild type with *clr2*$^{+}$, these strains are less repressed but still partly repressed compared to PJ1085 which lacks Clr2. In Figure 4, streaks of the same strains are compared on AA Total and AA -Ade plates. This figure shows that strains with mutated Clr2 grow as well as the strain with Clr2 knockout (PJ1085). These results confirm the loss in repression of strains that contain the point mutations.

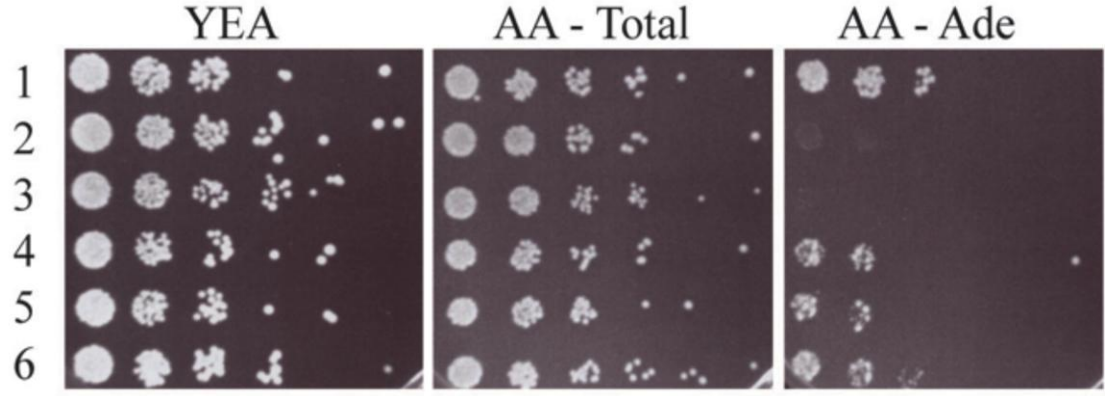


Figure 3. Spotted dilution series of 1 = PJ1085 (ΔClr2), 2 = PJ121 (Clr2), 3 = PJ1335 (V5-Clr2), 4 & 5 are both point mutation R170 → G170 in motif 2 and 6 = point mutation P137 → G137 in motif 1.

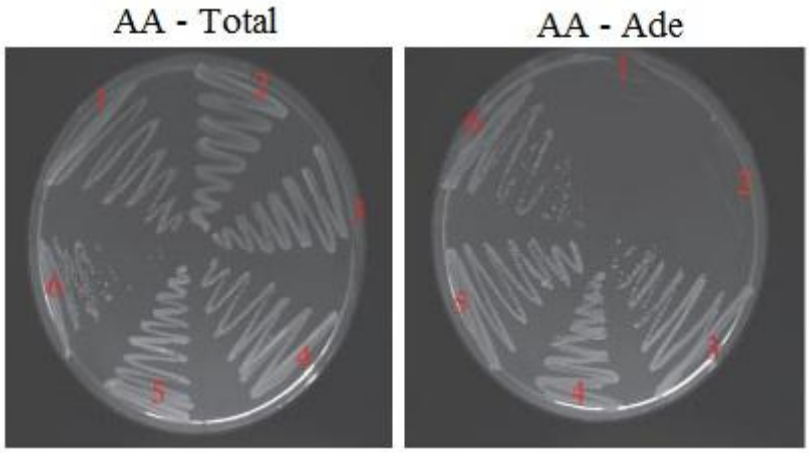


Figure 4. Streaks of 1 = PJ1335 (V5-Clr2), 2 = PJ121 (Clr2), 3 = PJ1085 (ΔClr2), 4 = Point mutation P137 → G137 in motif 1 and 5 & 6 are both point mutation R170 → G170 in motif 2.

4 Discussion

4.1 Point mutations

The protocol developed for point mutations (see Appendix) was very efficient for most primers. It generated the desired mutations efficiently with very low error-rate. Primers with T_m about 71 – 75 °C (using Finnzymes calculator) were successful with this protocol. Melting temperatures within this interval should be the aim when designing primers that will be used with this protocol. However, this is not always possible because there could be small margins when designing primers for this purpose. The *S. pombe* genome is very A-T rich and the mutations should be in the middle of primer that are approximately 40 nucleotides long. Problems with primers with high T_m were overcome by increasing annealing temperatures and with additions of DMSO, which probably prevent secondary structure formation and decrease annealing temperature.

4.2 Transformation

The constructs with wild type *V5-clr2*$^+$ were successfully integrated into PJ1085. However, later when all constructs with point mutations were to be integrated a lot of problems occurred. All colonies that appeared where false positives and many attempts of transformation resulted in no colonies on FOA plates. It could be possible that the transformation worked but the selection process was the problem. There were a lot of problem with background-growth on these plates that might inhibits growth of real transformants. The toxicity of FOA and the slow growth on these plates could accumulate other point mutations in the *ura4*$^+$ gene, which makes it possible for them to grow and therefore result in many false positives. To improve transformation efficiency, another transformation protocol developed by Suga and Hatakeyama (2003) was used and showed more promising results. The difference in this protocol is that the cells are grown in minimal media and treated with buffers containing DTT. So far have only two out of 16 mutations been integrated but with this new protocol all the other mutations will hopefully be integrated in the near future.

4.3 Results of spotted dilution series

The spotted dilution series show that the amino acids P137 in motif 1 and R170 in motif 2, are important for the function of Clr2. These two point mutations result in partial derepression of the *ade6*$^{+}$ reporter gene. The reporter gene is still partly repressed because the strains with point mutations do not grow as well as the control strain PJ1085. Also, the colonies of the strains with point mutation have a slightly red pigment that indicates that repression still occur. The dilutions for the spotted dilution series are a bit uneven and the control strain PJ1085 does not grow fully on AA- Ade plates as expected. This could be that the strain is too old and therefore the spotted dilution series should be repeated with a fresh isolate of PJ1085 from the -80 °C frezzer. The spotted dilution series therefore need to be repeated to fully examine the de-repression compared to the control strain. Although the dilutions are slightly uneven, this clearly shows that the point mutations result in a partially dysfunctional Clr2 protein. Also, the streaks on AA-total and AA- Ade plates further back up these results. It is interesting that the two point mutations, which occur in different motifs, show almost the same phenotype. This indicates that these two motifs are very important for the Clr2 protein. Perhaps the point mutations change the properties of the protein, which could result in less efficient binding to other SHREC components. It will be interesting to examine if the double mutations in these two motifs leads to greater loss of repression of the *ade6*$^{+}$ gene. The conserved amino acids P137 and R170 are important for the function of the Clr2 but more work need to be done to understand how they affect properties of Clr2.

4.4 Further research

The other fourteen mutations will be integrated into the same strain PJ1085 and spotted dilutions will be preformed. Hopefully this will lead to more clues about the importance of the different motifs. It would also be interesting to examine if more point mutations of the highly conserved amino acids within the same motif will result in more de-repressed phenotype. It would be interesting to examine if the changes in Clr2 effect silencing of other regions than the mating type region. The strains with the different point mutations should therefore be crossed with strains that have reporter

genes in other heterochromatic regions such as the inner repeats of centromere, central core of centromere and the rDNA. All strains needs to be sequenced to confirm that no other point mutations have occurred during the integrations. So far have only the strain PJ1335 with *V5-clr2*$^{+}$ has been confirmed by sequencing. Since the two point mutations show partly repressed phenotype it would be interesting the compare the amount of *ade6*$^{+}$ transcript to the wild type *V5-clr2*. Also, western blots should be carried out to detect the engineered forms of Clr2. The detection of the proteins is important so that the phenotype cannot simply be explained by lack of the protein due to degradation. Hopefully, further studies with these constructed strains will reveal importance of the three motifs for the protein, and generate more information on how Clr2 functions, for example how Clr2 interact with other SHREC components.

5 Acknowledgements

Thanks to Pernilla Bjerling for great project and supervision. Also thanks to former PhD-student Carolina Kristell for previous work on Clr2. Thanks to new members of the Bjerling group for making the group cheerful and good luck with your projects.

6 References

Arcangioli B, Roseaulin L, Holmes A. 2007. Mating-type switching in *S.pombe*. Current genetics. 17: 251-283.

Bjerling P, Ekwall K, Egel R, Thon G. 2004. A novel type of silencing factor, Clr2, is necessary for transcriptional silencing at various chromosomal locations in the fission yeast *Schizosaccharomyces pombe*. Nucleic acids research. 32: 4421-4428.

Craven RA, Griffiths DJF, Sheldrick KS, Randall RE, Hagan I, Carr AM. 1998. Vectors for the expression of tagged proteins in *Schizosaccharomyces pombe*. Gene 221: 59-68.

Cam HP, Chen ES, Grewall S. 2009. Transcriptional scaffolds for heterochromatin Assembly. Cell 136: 610-614.

Egel R. 2004. Fission yeast in general genetics. In: Egel R (ed). The molecular biology of *Schizosaccharomyces pombe*: genetics, genomics and beyond, pp. 1- 10. Springer, Copenhagen.

Egger G, Liang G, Aparicio A, Jones PA. 2004. Epigenetics in human disease and prospects for epigenetic therapy. Nature 429: 457-463.

Goto DB and Nakayama. 2011. RNA and epigenetic silencing: Insight from fission yeast. Development, growth & differentiation. 54: 129-141.

Olsson I, Bjerling P. 2011. Advancing our understanding of functional genome organisation through studies in the fission yeast. Current genetics. 57: 1-12.

Portela A, Esteller M. 2010. Epigenetic modifications and human disease. Nature Biotechnology 10: 1057-1068.

Qi D, Scholthof KBG. 2008. A one-step PCR-based method for rapid and efficient site-directed fragment deletion, insertion, and substitution mutagenesis. Journal of Virological Methods 149: 85-90.

Suga M, Hatakeyama. 2003. High-efficiency electroporation by freezing intact yeast cells with addition of calcium. Current genetics. 43: 206-211.

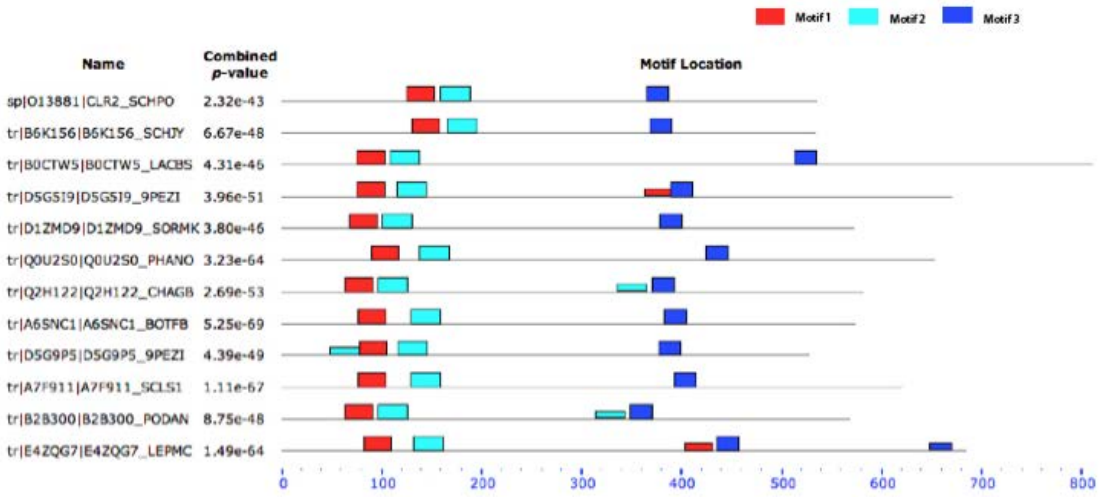
Suga M, Hatakeyama. 2001. High efficiency transformation of *Schizosaccharomyces pombe* pretreated with thiol compounds by electroporation. Yeast. 18: 1015-1021.

Sugiyama T, Cam HP, Sugiyama R, Noma K, Zofall M, Kobayashi R, Grewal S. . SHREC, an effector complex for heterochromatic transcriptional silencing. Cell 128: 491-504.

Wood V, Gwilliam R, Rajandrem MA, Lyne M, Lyne R, Stewart S, Sgouros J, Peat N, Hayles J et al. 2002. The genome sequence of *Schizosaccharomyces pombe*. Nature. 415: 871-880.

7 Appendix

7.1 Motifs



Motif 1

Motif 2

Motif 3

7.2 Media

YEA (1 litre)

5 g Yeast extract
2 g Casamino acids
0.1 g Adenine
0.1 g Uracil
20 g Agar
150 ml 20 % Glucose

The Glucose and the media were autoclaved separately and then mixed.

Tom's FOA plates (1 litre)

1.2 g FOA
50 mg Uracil
2 g AA-uracil
1.7 g Difico Yeast Nitrogen Base (w/o amino acids and ammonium sulphate)
5 g ammonium sulphate
50 ml 40% glucose
20g agar

The agar and glucose were autoclaved separately. The FOA was dissolved in 200 ml dH_20 at 55 °C. The media was filtered through a 45 μm (Sarstedt) and then mixed with autoclaved agar and glucose.

AA drop out media (1 litre)

<u>Solution 2</u>
1.7 g Difico Yeast Nitrogen Base w/o amino acids and ammonium sulphate)
5 g Ammonium sulphate
2 g Drop out mix (Formedium)
450 H_20

<u>Solution 1</u>
20 g Agar
450 ml H_20

Both solutions were autoclaved in separate bottles and then mixed together with 100 ml 20 % glucose.

SOB medium (1litre)

20 g Bacto tryptone
5 g Bacto yeast extract
5.84 g NaCl
0.1864 g KCl

Ingredients were mixed and autoclaved. Prior to use a 2 M Mg^{2+} solution was added.

2 M Mg^{2+} solution (50 ml)

10.165 g $MgCl_2$
12.32 g $MgSO_4$

LB/LA (1 litre)

10 g NaCl
5 g Yeast extract
10 g Tryptone

(20 g agar)

The media were sterilized by autoclaving and for some batches ampicillin was added to a concentration of 100 µg / ml.

7.3 Solutions

TB (250 ml)

0.756 g PIPES (10 mM)
0.416 g $CaCl_2$ (15 mM)
3.73 g KCl (250 mM)
2.72 g $MnCl_2$ (15 mM)

The solution were sterilised by filtration (Sarstedt) and kept at 4 °C.

DTT buffer (40 ml)

1 ml DTT (25 mM)
4.37 g Sorbitol (0.6 M)
0.19 g Hepes (20 mM)

The solution were sterilised by filtration (Sarstedt) and kept at 4 °C.

7.4 Primers

Primers for point mutations were labelled from F25 to F48

Table 5. Primers used for introducing point mutations

Primer	
F25 (for)	5'-GAAAATTATTATCTTGCCTCTTTAGGCACCAATTACCAG-3'
F26 (rev)	5'-CTGGTAATTGGTGCCTAAAGAGGCAAGATAATAATTTTC-3'
F27 (for)	5'-GTGGTCGACCATTTGGATCAGTTAATGATTTC-3'
F28 (rev)	5'-GAAATCATTAACTGATCGAAATGGTCGACCAC-3'
F29(for)	5'-GTTAATGATTTCCTTCATGGTTTGTATTGGCTTATATC-3'
F30 (rev)	5'-GATATAAGCCAATACAAACCATGAAGGAAATCATTAAC-3'
F31(for)	5'-CATTTTTACGGAATTTTCCGTGGAGGCGGAAAACTATGGATTAATG-3
F32 (rev)	5'-CATTAATCCATAGTTTTCCGCCTCCACGGAAAATTCCGTAAAAATG-3'
F35 (for)	5'-CTTGCCTCTTTAGGCACCAATGGCCAGCTATATCAGCGTGATTC-3'
F36 (rev)	5'-GAATCACGCTGATATAGCTGGCCATTGGTGCCTAAAGAGGCAAG-3'
F38 (for)	5'-CTTGCCTCTTTACCCACCAATGGCCAGCTATATCAGCGTG-3'
F39 (rev)	5'-CACGCTGATATAGCTGGCCATTGGTGGGTAAAGAGGCAAG-3'
F40 (for)	5'-CTTTACCCACCAATTACCAGGGATATCAGCGTGATTCAAAC-3'
F41 (rev)	5'-GTTTGAATCACGCTGATATCCCTGGTAATTGGTGGGTAAAG-3'
F42 (for)	5'-CATTTCGATCAGTTAATGATGGCCTTCATCATTTGTATTG-3'
F49 (rev)	5'-CAATACAAATGATGAAGGCCATCATTAACTGATCGAAATG-3'
F43 (for)	5'-CATCATTTGTATTGGGGTATATCAGATTTGACCCGTAAC-3'
F44 (rev)	5'-GTTACGGGTCAAATCTGATATACCCCAATACAAATGATG-3'
F45 (for)	5'-GAATTTTCCGTGGAGGCGAAAAACTATGGATTAATGATTTATGTG-3'
F46 (rev)	5'-CACATAAATCATTAATCCATAGTTTTTCGCCTCCACGGAAAATTC-3'
F47 (for)	5'-GAATTTTCCGTGGAGCCGGAAAACTATGGATTAATGATTTATGTG-3'
F48 (rev)	5'-CACATAAATCATTAATCCATAGTTTTCCGGCTCCACGGAAAATTC-3'

Table 6. Primer design of point mutation primers

Primer	Length (bases)	GC %	T_m °C (Finnzymes T_m calculator)
F25 (for)	39	35.9	73.29
F26 (rev)	39	35.9	73.29
F27 (for)	32	40.6	73.5
F28 (rev)	32	40.6	73.5
F29(for)	38	31.6	71.1
F30 (rev)	38	31.7	71.1
F31(for)	46	39.1	81.1
F32 (rev)	46	39.1	81.1
F35 (for)	44	50	83.6
F36 (rev)	44	50	83.6
F38 (for)	40	52.5	82.9
F39 (rev)	40	52.5	82.9
F40 (for)	41	43.9	79.2

F41 (rev)	41	43.9	79.2
F42 (for)	40	35	76.9
F49 (rev)	40	35	76.9
F43 (for)	39	38.5	75
F44 (rev)	39	38.5	75
F45 (for)	45	35.6	78.6
F46 (rev)	45	53.6	78.6
F47 (for)	45	37.8	79.5
F48 (rev)	45	37.8	79.5

Table 7. Primers used for sequencing, transformation & colony screening

Primer	
D80 (for)	5'-TCAATTTTCCGCTACACAAAATAATTTGCATACTTCTTTTGTTCCGTATA TTCAGCTATTGTCATTTTTAGTA-3'
D81(rev)	5'-ATATTATTTACATTATAAATAATGACCGTCACTGATCAATAAGTAGACA TCTATATCTAATTTTATCTGCCATA-3'
A6 (rev)	5'-TCACTGATCAATAAGTAGACAC -3'
A7 (for)	5'-GCGTCAATTTTCCGCTACAC -3'
A2 (for)	5'-GAGGGGATGAAAATTCCCA-3'
A3 (rev)	5'-TTCGACAACAGGATTACGAC-3'
F18 (for)	5'-CTGCTATTACTTGTGTTTGG -3'
F19 (rev)	5'-CAACTGCTGACACCATATC-3'
M13 (21) (for)	5'-TGACCGGCAGCAAAATGT-3'
M13 (29) (rev)	5'-CAGGAAACAGCTATGACC-3'
M13 (49) (for)	5'-GAGCGGATAACAATTTCACACAGG -3'

7.5 PCR programs

Colony PCR (JTAG)

5 µl Template (cell solution)
10 µl Buffer 5X
1 µl dNTP (10mM total)
0.3 µl Forward primer (100 µM)
0.3 µl Reverse primer (100 µM)
1 µl Phire polymerase
33.1 µl dH_20
---
50 µl

94 °C	2 min		
94 °C	15 s	}	X10
47 °C	30 s		
68 °C	4 min		
94 °C	15 s	}	X20
47 °C	30 s		
68 °C	4 min dt 15 s		(This means 15 s are added to each cycle)
4 °C			

Clr2i

1 µl Template
7 µl Buffer 5X HF
1.75 µl $MgCl_2$ (25 mM)
0.6 µl dNTP
0.3 µl D80 (100 µM)
0.3 µl D81 (100 µM)
0.3 µl Phusion
23.75 µl dH_20
---
35 µl

95 °C	2 min		
94 °C	50 s	}	X25
56 °C	50 s		
72 °C	2 min		
72 °C	10 min		

Point mutation protocol

1 µl Template (~ 10 ng)
1 µl Forward primer (20 µM)
1 µl Reverse primer (20 µM)
1 µl dNTP
1 µl Phusion polymerase
10 µl 5 X buffer (High-fidelity buffer)
0.5 µl DMSO
34.5 µl dH_20
---
50 µl

95 °C 2 min

95 °C 45 s
58 °C 45 s
72 °C 60 s / kb

X 18

72 °C 10 min

"Fake" hot start. Run 10 µl PCR-product on a gel and incubate remaining product with 0.5 µl DpnI at 37 °C for 1-3 hours.

Use 5 µl for transformation. Mix product and 50 µl competent cells on ice for 15 – 30 min. Heat shock the cells at 42 °C for 45 s and return to ice for 2 min. Recover cells in LB+amp broth for 15 – 30 min at 37 °C. Then Plate on LA+amp.

Primer design for point mutations

Try to make primers about 40 bp or shorter. Primers, 41 – 46, bp are more troublesome to work with. Use Finnzymes T_m calculator online http://www.finnzymes.fi/tm_determination.html which is based on Breslaure's thermodynamics. Aim for T_m 71 - 74 °C. Primers within this T_m –range worked perfect with this protocol whereas longer primers with higher T_m were difficult to work with. Problems with these primers could be solved by increasing annealing temperature, number of cycles and also with addition of 3 – 5 % DMSO. Particularly the addition of DMSO can be very effective.

Table 7. Primer design of point mutation primers and sucess of primers

Primer	Length (bases)	GC %	T_m °C (Finnzymmess calculator)	Product
F25 (for)	39	35.9	73.29	58 °C
F26 (rev)	39	35.9	73.29	-II-
F27 (for)	32	40.6	73.5	58 °C
F28 (rev)	32	40.6	73.5	-II-
F29(for)	38	31.6	71.1	58 °C
F30 (rev)	38	31.7	71.1	-II-
F31(for)	46	39.1	81.1	Failed
F32 (rev)	46	39.1	81.1	-II-
F35 (for)	44	50	83.6	5 % DMSO, 65 °C
F36 (rev)	44	50	83.6	-II-
F38 (for)	40	52.5	82.9	3 % DMSO
F39 (rev)	40	52.5	82.9	-II-
F40 (for)	41	43.9	79.2	3 % DMSO, 62 - 65 °C
F41 (rev)	41	43.9	79.2	-II-
F42 (for)	40	35	76.9	58 °C
F49 (rev)	40	35	76.9	-II-
F43 (for)	39	38.5	75	58 °C
F44 (rev)	39	38.5	75	-II-
F45 (for)	45	53.6	78.6	5 % DMSO, 58 °C
F46 (rev)	45	53.6	78.6	-II-
F47 (for)	45	37.8	79.5	61 – 64 °C, 30 cycles
F48 (rev)	45	37.8	79.5	-II-