Chapter - 4

Characterization of Nkp2
4.1 Introduction

Kinetochores are macromolecular machines that are specialized protein structure assemblies on centromeric DNA and couple chromosomes to the dynamic spindle during cell division (Cheeseman and Desai, 2008; Santaguida and Musacchio, 2009). Transfer of genetic material to daughter cells during cell division requires the proper partitioning of chromosomes mediated by the kinetochore. Although kinetochore function is conserved from yeast to mammals, the size and sequence of the centromeric DNA are highly variable (Henikoff et al., 2001). In *Saccharomyces cerevisiae*, the centromeric sequences have been defined that are sufficient to mediate the kinetochore assembly but, in fission yeast and multicellular organisms, centromeres consist of megabases of DNA. Despite their variation in size and sequence composition, epigenetic aspects of centromeres are highly conserved and are essential for assembly of kinetochore (Buhler and Gasser, 2009; Mytreye and Bloom, 2003; Tanaka et al., 1999). Failure of kinetochore assembly on centromere leads to improper microtubule attachment and can result in chromosome loss. In higher organisms, during mitotic division, such failures can drive tumour formation and in meiosis affect the fertility of the organism (Weaver and Cleveland, 2007).

In *Saccharomyces cerevisiae*, kinetochore complex are composed of approximately 40 different proteins; these kinetochore proteins associate with each other and perform their function by connecting centromere and microtubules to segregate accurately the genetic material to the daughter cells during cell division (Cheeseman et al., 2002). Recently, the organization of yeast kinetochores has been better defined by biochemically purifying the subcomplex within the kinetochore (Akiyoshi et al., 2010; Cheeseman et al., 2001; Janke et al., 2001; Janke et al., 2002; Li et al., 2002). However, the physical interactions between these proteins are yet to be established and the organization of these subcomplexes also needs to be elucidated.

Previously, it was reported that, in *Schizosaccharomyces pombe* and multicellular organisms, centromere proximal regions of chromosomes are silenced. In contrast, the relation between heterochromatin proteins and kinetochore function in the budding yeast remains largely unexplored. The Sir1 heterochromatin protein is a
component of centromeric chromatin and contributes to the mitotic chromosome stability (Sharp et al., 2003). Sir1 binds to Cac1, a subunit of chromatin assembly factor-I (CAF-I) and helps to retain the Cac1 at the centromeres, thereby retaining Caf1 at the centromeric chromatin. This function is independent of other Sir proteins (Sharp et al., 2003). In order to find out if there were additional interactions between proteins that bind to the centromere and heterochromatin, we first tested if Nkp2 has any role in heterochromatin establishment at HMR and telomeres by generating null mutants of nkp2.

4.2 Results

4.2.1. Targeted silencing in nkp2Δ

In order to examine the role of Nkp2 on silencing, we constructed a nkp2 mutant (described in materials and methods). This strain was crossed to KRY28 (hmraeB::1XUASgURA3) to obtain KRY311 which contain (nkp2::TRP1 hmrΔaeb::1XUASgURA3). To check targeted silencing, we transformed the plasmids encoding Gbd, Gbd-Rap1, Gbd-Sir4 and Gbd-Yif1 in wild type and nkp2 mutant. Transformants were grown on histidine drop-out liquid broth overnight and 10 fold serial dilutions was spotted on uracil deficient agar plates to check the targeted silencing.

In wild type, Gbd-Sir4 plasmid establishes silencing at the modified hmr loci and no growth in uracil deficient agar plate was detected whereas, Gbd-Rap1 and Gbd-Yif1 show little effect on silencing (Figure 12). However in nkp2 mutant, we observed the opposite results; Gbd-Rap1 showed no growth in uracil deficient plate. These two results are inconsistent with each other and are unlikely to represent true loss in silencing. These results indicated that loss of growth on uracil deficient plates might mean there might be a spontaneous loss of the URA3 marker from HMR locus, which might indicate increased genome instability.
Panel A

Figure 12: Targeted silencing does not affected in \( nkp2 \). KRY28 (\( hmrAaeB::1XUASgURA3 \)) and KRY311 (\( nkp2 hmrAaeB::1XUASgURA3 \)) were transformed with Gbd, Gbd-Rap1, Gbd-Sir4 and Gbd-Yif1. Transformants were grown in SC-His broth and 5μl of 10-fold serial dilutions was spotted on SC-His (to select the plasmid) & SC-His Ura (to assay silencing).
4.2.2. Telomere silencing in \textit{nkp2}Δ

Simultaneously, we also constructed \textit{nkp2} mutant strains to assay TPE at \textit{URA3} locus on the left telomere of chromosome VII. Gene silencing at telomeres was measured by growth on 5-FOA plates. Six different \textit{nkp2} segregants were inoculated in synthetic complete broth and 10 fold serial dilutions were made and spotted on 5-FOA. Plates were incubated at 30ºC for 2 days. Among six \textit{nkp2} mutants, two of them showed more resistance than wild type strain. Again this was inconsistent, not all \textit{nkp2} mutants behaved identically (Figure 13).

![Figure 13: Telomere position effect in \textit{nkp2}Δ. WT (Tel VII-URA3) and \textit{nkp2} (\textit{nkp2}::his5+ Tel VII-URA3) were grown in SC-Leu broth and 5ul of 10 fold serial dilutions was spotted on SC, SC- Ura (control) and SC+5-FOA (to check loss in TPE).](image)

These results were puzzling and because not all \textit{nkp2} mutants behaved identically, it rules out a silencing defect. If the growth on uracil (Figure 12) or 5-FOA (Figure 13) is not due to silencing, then the other possibility is that they are losing the
URA3 gene. As these are haploid, it is not possible to lose the chromosome and survive. Therefore we reasoned that maybe these strains have uneven number of chromosomes.

In the process of construction of nkp2 mutant with defective hmr strain, we crossed wild type strain with the hmr::URA3 with nkp2 mutant and random sporulation was done to isolate the nkp2 mutant with defective hmr strain. It is possible we obtained some strains with extra chromosomes and were losing those at an elevated rate. For eg., it is possible that two chromosome VIIL may be present in the cell with one modified telomere and one wild type and the cell was losing one of them, hence we see either URA plus (retain modified chromosome) or URA minus (lose the modified chromosome).

4.2.3 Abnormal segregation of markers in nkp2 homozygous strain

To test this possibility more directly, we constructed 2 different nkp2 mutant strains with different auxotrophic marker KRY277 (nkp2::his5+) and KRY311 (nkp2::TRP1 hmr::lXUASgURA3). We crossed these strains and sporulated. We constructed a diploid nkp2 homozygous strain with one nkp2 replaced with his5+ and the other with TRP1. This was made by crossing nkp2::his5+(Mat a) with nkp2::TRP1 with URA3 reporter gene at the defective hmr loci (Mat a), diploids were selected on tryptophan and histidine dropout medium. Diploid cells were kept on non-fermentable carbon source i.e., potassium acetate for spore formation. After 3 days spores were checked under microscope and the formation of tetrads was similar to wild type diploid strain. Spores were digested with zymolyase and random spore analysis was performed to score the segregants with auxotrophic markers. We observed that ~25% of segregants (73 out of 300 segregants analyzed) grew on both tryptophan and histidine dropout medium and also showed a specific mating type, either Mat a or Mat a. This is unexpected, as a haploid can have only one of the two nkp2 chromosomes. We reasoned this could be due to improper segregation of chromosomes: these strains may contain uneven number of chromosomes and might carry additional chromosome XII, and just one of Chromosome III, therefore can mate. These experiments together suggested that
nkp2 mutants do not have any effect on silencing but might affect chromosome segregation.

4.2.4 Homozygous NKP2 deleted cells were able to mate with haploid strain.

The previous experiments show that there is some chromosome missegregation in nkp2 mutants. To test this further, we did southern blots to check the nkp2 locus status. We performed genomic southern to check the nkp2 loci in a strain with one copy of chromosome III i.e., either MAT a or MAT a locus. NKP2 gene is present on right

![Figure 14: Southern blot analysis of nkp2Δ with mating behavior in diploid cells.](image)

Southern blot was performed with homozygous nkp2 diploid strain and triploid strain. The genomic DNA isolated and digested with Xhol and SalI double digest transfer to nylon membrane and probe with NKP2. Wild type copy of NKP2 gives ~0.7 kb where as NKP2 gene replaced with TRP1 marker gives ~1.9 kb and his5+ gives about 2.2 kb fragments. From lane 4-9 are diploid cells which shows the mating type either Mat a or Mat α, these diploid cells were mated with wild type haploid to make triploid from lane 10-15.
arm of chromosome XII. Genomic DNA was digested with two enzymes Xhol and SalI, and probed with NKP2 gene fragment. Wildtype gives about 0.7kb fragment whereas NKP2 gene replaced with either TRP1 or his5+ gives approximately 1.9 kb or 2.2 kb respectively. We found that the nkp2::his5+/nkp2::TRP1 contained two copies of nkp2 locus (Figure 14, lane 4-9) and lost one of the chromosome III. Next we mated this strain with an authentic haploid strain. The resultant strain contains 3 nkp2 loci: 2 of the mutant marked loci from the diploid parent and one from the haploid carrying wild type NKP2 (Figure 14, lane 10-15).

The southern blot clearly indicates that these strains contain 2 nkp2 loci to begin with and then mate with wild type to produce a strain with 3 NKP2 loci. However, this does not say if the strain was a true diploid or was disomic for the chromosome number XII which contains NKP2. To answer this question, we performed a sporulation test. We sporulated the haploids that mate with haploid wild type strain (lanes 4-9 in Figure 14). Diploids with 2 nkp2 copies can give a large number of viable spores. Triploid on the other hand cannot (lanes 10-15). We dissected the nkp2 diploid and so called triploid spores and tested the viability, the viability from diploid cells was about 98% and segregated into a 2:2 his5+:TRP1 whereas so called triploid, the viability was decreased to below 50%.

Southern blot and tetrad dissection results indicated that the nkp2::his5+/nkp2::TRP1 is a diploid that can mate. This means that there is a high rate of loss of chromosome III in nkp2 mutant leading to the production of monosomy at chromosome III. We tested this more directly by performing specific chromosome loss assay for chromosome III.

We performed a plate based mating assay to test if diploids of nkp2 undergo mating. To this end, we grew cultures wild type and nkp2 mutant overnight, equal numbers of cells are mixed with fixed number of mating tester MAT a or MAT α. These cultures were grown overnight and 10 fold serial dilutions were made and cells were spotted on synthetic deficient media without amino acids. In the mating assays, nkp2 mutant cells show more growth on synthetic deficient medium; this is because nkp2
mutant loses chromosome III more frequently than wild type. All these results together suggest that, chromosome loss was prominent in nkp2 mutant as seen in Figure 15.

![Mating assay in nkp2 homozygous diploid cells.](image)

**Figure 15: Mating assay in nkp2 homozygous diploid cells.** Mating assays were performed with nkp2 homozygous diploid. Cells grown in YPD were mixed with either MAT a or MAT α and incubated for overnight. 5μl of 10-fold serial dilutions was spotted on YPD (to select the experimental strain and mating tester) & SD (to select those cells which had mated with the mating tester).

As the plate-based assay indicated that nkp2 mutants indeed tend to mate frequently, we quantified this phenotype to get a measure of the extent of loss of chromosome III. Diploid cells contain MAT a and MAT α information on two copies of chromosome III. Wild type and nkp2 mutant diploid cells were grown overnight and equal number of cells are mixed with the mating tester strain either MAT a or MAT α, and placed on nitrocellulose membrane for 4 hours on YPD at 30°C. These cells were washed in phosphate buffer and plated on synthetic deficient plates for growth. In this assay, diploid cells which have lost any one of the chromosome III, either MAT a or MAT α, can mate with the opposite mating tester strain (Figure 15). For example if
diploid cell loses *MAT a* marked chromosome III, it can mate with mating tester strain *MAT a* and vice versa (Table 7). *nkp2* homozygous mutant shows about four fold increase in loss of chromosome III compared to the wild type strain.

**Table 7: Quantitative mating assay in wild type and nkp2Δ**

<table>
<thead>
<tr>
<th>Name of the strain</th>
<th>Total number of colonies screened</th>
<th>Mated colonies</th>
<th>Total percentage of mated colonies (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>2x10^6</td>
<td>600</td>
<td>0.03</td>
</tr>
<tr>
<td>nkp2</td>
<td>2x10^6</td>
<td>2230</td>
<td>0.11</td>
</tr>
</tbody>
</table>

### 4.2.5. Chromosome loss upon overexpression of NKP2

All these results suggest that Nkp2 may have role in chromosome segregation but not in silencing. As Nkp2 is a kinetochore protein, we decided to test the role of Nkp2 in chromosome loss upon overexpression. We performed the colony colour assay to measure the chromosome instability. The wild type strain carrying a cloned ochre-suppressing form of a tRNA gene, *SUP11*, serves as a marker on artificial chromosome. To measure chromosome loss, strain carrying an artificial chromosome with a cloned ochre-suppressing form of a tRNA gene, *SUP11*, was used. Strain carrying artificial chromosome are *ADE2+* (due to the suppression of the non-sense mutation in the *ade2* gene) and white in colour whereas loss of the artificial chromosome causes colonies to appear red. To perform loss assays, we transformed the plasmid encoding multiple copies of *NKP2* and empty vector to check chromosome loss in wild type strain. Initially, transformants were selected on leucine, adenine deficient selective media to retain the *SUP11* and then plated on leucine drop out agar plates. To quantify the loss of chromosome, we initially plated approximately 3,500 cells, grew them for 3 days at...
30°C and stored at 4°C for 6 days to assess the colour. As shown in Figure 16, we found that a few colonies lost the SUP11 chromosome and became red in colour upon overexpression of NKP2. These results suggest that overexpression of NKP2 has small enhancing effect of SUP11 loss.

![Vector](image1) ![NKP2](image2)

**Figure 16: Artificial chromosome loss (SUP11) upon overexpression of NKP2.** Wild type cells with artificial chromosome SUP11 was transformed with either empty vector or NKP2 plasmids. Plate about 3,500 cells on Leucine dropout agar plates to access the colour.

### 4.2.6. Chromosome loss in nkp2Δ

The results presented earlier suggest that nkp2 mutants are defective in chromosome transmission. It has been shown that incorrect kinetochore assembly or kinetochore-microtubule attachment can result in loss or gain of chromosomes (Raghuraman et al., 2001). During mitosis, in the somatic cells of higher organisms, such failure can cause tumour formation and in meiosis, defective chromosome segregation leads to aneuploidy (Weaver et al., 2007). We tested the chromosome loss in nkp2 mutant more directly using an artificial chromosome loss.

We performed the colony colour assay described above in nkp2 mutants to measure chromosome instability. We constructed the nkp2 mutant strain carrying the SUP11 artificial chromosome by crossing nkp2 mutant with a wild type strain (kind gift.
of Marston). We used ctf3 mutant as positive control as Ctf3 is a kinetochore protein and is involved in correct segregation of chromosomes. Initially colonies were selected on adenine drop out broth overnight to select for SUP11 and approximately, 3,500 cells

![Image](Image1.png)

**Table 8: Artificial chromosome loss in Ctf19 kinetochore mutants**

<table>
<thead>
<tr>
<th>Name of the strain</th>
<th>SUP11 chromosome loss</th>
<th>Total percentage of SUP11 loss (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>WT</strong></td>
<td>15</td>
<td>0.4</td>
</tr>
<tr>
<td><strong>nkp2</strong></td>
<td>60</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>nkp1</strong></td>
<td>72</td>
<td>2.0</td>
</tr>
<tr>
<td><strong>ctf3</strong></td>
<td>164</td>
<td>4.7</td>
</tr>
</tbody>
</table>

**Figure 17: Artificial chromosome loss was elevated in nkp2Δ.** 3,500 wild type, nkp2, nkp1 and ctf3 cells with artificial chromosome SUP11 cells were plated on YPD agar plates to score the half sector colonies. Panel B shows graphical representation of artificial chromosome loss (SUP11) and Table 8 represents with numbers and percentages.
were plated on rich media agar plates, these agar plates were incubated at 30°C for 3 days and shifted to 4°C for one week. The percentage of sectored colonies, which were at least half red indicating loss in the first division after plating were scored. We found that \textit{nkp2} shows significant loss of \textit{SUP11} compared to wild type (Figure 17/Table 8). We also included \textit{nkp1} mutant; it’s a kinetochore protein that is thought to interact with Nkp2 protein. \textit{ctf3} mutants show more significant loss than the \textit{nkp2} mutant, whereas \textit{nkp1} is comparable to \textit{nkp2} (Figure 18). These results suggest that Ctf19 components are required for accurate chromosome transmission during mitosis.

4.2.7 Tetrad analysis shows gene conversion events

In order to study the segregation of chromosomes more systematically, we dissected the tetrads from \textit{nkp2} homozygous diploids and looked at each meiotic event

![Tetrad analysis image](image)

\textbf{Figure 18: Tetrad analysis shows gene conversion events in \textit{nkp2A} homozygous diploid.} Panel (A) is a pictorial representation of the meiotic segregation of wild type strain. Panel (B) shows the spores obtained from dissecting of \textit{nkp2} homozygous diploid.
for the segregation of \textit{nkp2} and \textit{MAT} locus. We dissected \textit{\textasciitilde}100 tetrads and each tetrad segregated 2:2 with respect to \textit{his5}+ and \textit{TRP1} cells and the \textit{MAT} locus (\textit{a} and \textit{\alpha}). Among the 100 tetrads, few of them showed 3 \textit{his5}+ and 1 \textit{TRP1} and in a few tetrads, all 4 were \textit{TRP1} or \textit{his5}+ (Figure 18 and Table 9). Both these patterns of segregation represent gene conversion events that took place post meiotically (3:1, a classic single event during meiosis II stage); or in case of 4:0, it could also be a mitotic gene conversion event that took place just prior to meiosis, leading to loss of heterozygosity.

4.3 Discussion

In our study, we observed that chromosome loss was seen rather than loss in silencing at the \textit{HMR} locus in \textit{nkp2} mutant. We observed slightly elevated levels of chromosome loss upon \textit{NKP2} overexpression. However, we saw an even more elevated chromosome loss in \textit{nkp2} deletion. These results are consistent with the idea that any defect in the kinetochore components is detrimental to proper chromosome segregation. These data also indicate that Nkp2 function is required for proper segregation of chromosomes.