Scuola Internazionale Superiore di Studi Avanzati - Trieste

Chromosomal regulation of aneuploidy in the yeast *Saccharomyces cerevisiae*

SANJEEV K. WAGHMARE
International Centre for Genetic Engineering and Biotechnology, Trieste, Italy

Thesis Submitted to ISAS / SISSA for the degree of

Doctor Philosophiae in Molecular Genetics

Candidate:
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Supervisor:
Prof. C.V. Bruschi

Academic Year 2003/2004

SISSA – Via Beirut 2-4 – 34014 TRIESTE – ITALY

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Abbreviations

BFP	Blue fluorescent protein
DsRed / RFP	Red fluorescent protein
FLP	2μ site-specific recombinase
FRT	Flp recognition target
GFP	Green fluorescent protein
IR	Inverted repeat
LOH	Loss of heterozygosity
NLS	Nuclear localization signal
STIK	Specific targeted integration of kanamycin resistance-associated non-selectable DNA
tetR	Tetracycline repressor

CHAPTER 1

Introduction

1 Introduction

1.1 *Saccharomyces cerevisiae*, a favoured model eukaryote

Saccharomyces cerevisiae is a fungus commonly known as baker's or brewer's yeast. It's a unicellular eukaryotic microorganism, and was the first eukaryotic model, the genomic DNA of which was fully sequenced (Mewes *et al.,* 1997). It can either grow aerobically and anaerobically, and utilizes glucose as a prefered carbon source. It exists as both stable haploid and diploid, its generation time is rapid and it is easy to handle in the laboratory. Also, genetic, biochemical and molecular biology studies can be carried out at ease in this microorganism.

In these years, research in *S. cerevisiae* has provided enormous knowledge in the area of cell cycle regulation, replication and repair etc. Moreover, yeast genes show high degree of homology as compared to humans. Understanding various mechanisms and extrapolating further into higher organisms will provide a way to know their likely role in higher system, and will greatly benefit for the cure of various human diseases. Below are discussed some of the structural elements of yeast that are related to our research work; and the molecular mechanism, the defect in which lead to chromosomal abnormalities in both yeast and human cells. In our research studies, we made an attempt to understand the regulation of chromosomal ploidy control in yeast. Our finding will contribute further understanding of the correlation between chromosomal aneuploidy and cancer in human cells.

1.2 2-μm circle

In the yeast *Saccharomyces cerevisiae*, there exist a 2-μm molecule, an extrachromosomal element having the capability to replicate on its own. It is present in the nucleus at a copy number of 60-100 per haploid cell and the number increases linearly with the ploidy (Clark-Walker and Miklos, 1974; Futcher and Cox, 1983). Since it is entirely devoted to its own survival, 2-μm is regarded to be a selfish molecule (Mead *et al.,* 1986). The 2-μm circle is a 6318 bp circular double-stranded DNA molecule containing two inverted repeat (IR) regions of 599 bp, which divide the circle into a large and a small unique region, comprising of 2774 and 2346 bp regions respectively. The 2-μm contains several genes, namely: *FLP*, *REP1*, *REP2*, *STB* (*REP3*) and *RAF1*. *FLP* encodes a protein that catalyzes site-specific recombination between the Inverted repeats. *REP1* and *REP2* codes for proteins required for the regulation of gene expression and plasmid segregation. The junction between the large unique region and one IR, contains a single Ori of replication, which is useful for its high copy number and stability in the cell. In the large unique region, there is the *STB* locus, which is considered to be a centromere of 2-μm, involved in proper plasmid segregation (Jayaram *et al.,* 1983; Jayaram *et al.,* 1985). *RAF1*, codes for a protein, which is involved in plasmid gene expression. *FLP* encodes a site-specific recombinase acting on two specific sites, termed as Flp recognition target (*FRT*), located at the center of the 599 bp inverted repeats (Senecoff and Cox, 1986; Broach and Volkert, 1991). Flp protein interacts specifically with a 50-bp DNA sequence that includes three 13 bp repeats; the third of which is inverted with respect to the other two, separated from them by an 8-bp core sequence (Andrews *et al.,* 1985; Senecoff *et al.,* 1988). Mutation in one *FRT* core sequence prevents recombination

with other wild-type *FRT*; however, identical mutations in the two *FRT's* do not affect site-specific recombination (Dixon and Sadowski, 1994; Storici *et al.,* 1999; Storici and Bruschi, 2000). Taking this into account, a series of vectors were constructed bearing different mutations in the *FRT's*. These vectors could be used to perform mutiple targeted disruption and integration; however, to date it was impossible to integrate any non-selectable DNA sequence. Recently, a modified version of the above mentioned vectors were constructed, which is termed as STIK system (Waghmare *et al.,* 2003). These vectors enable to perform integration, disruption and tagging of any non-selectable DNA in to any region in the genome.

1.3 Centromere

A centromere is a crucial component of the chromosome that aids in proper chromosome segregation during mitosis and meiosis. Its function is to hold sister chromatids together and, through its centromere DNA–protein complex, known as the kinetochore, binds spindle microtubules to bring about accurate chromosome segregation, thereby, maintaining the integrity of the cell. Centromeric DNA sequence shows variability when compared from yeast to mammals; however, proteins assembled at the kinetochore complex are highly conserved.

In yeast, average size of a centromere is around 125 bp, while *Schizosaccharomyces pombe* centromere size ranges from 40-100 KB, *Drosophila* has a size around 400 KB and human centromere have sizes approaching 1-4 MB.

In *S. cerevisiae*, centromere consists of three elements, namely: CDEI, CDEII and CDEIII. CDEI is an 8-bp sequence. CDEII is a region of 80 bp having high AT

nucleotide content and CDEIII is a 26 bp region. Mutation in the CDEI affects the function in mitosis, but not significantly in meiosis. Deletion or insertion in CDEII region that varies the length of AT content affects centromere function in both mitosis and meiosis. Moreover, CDEIII is an essential domain and any change in this region can severely affect or completely abolish the centromere function (Murphy *et al.,* 1991; Fleig *et al.,* 1995). In *Saccharomyces cerevisiae*, there are specific proteins (Choo, 2000; Cleveland *et al.,* 2003) that interact with the centromeric DNA sequence to form a centromere-kinetochore complex (**Figure 1**).

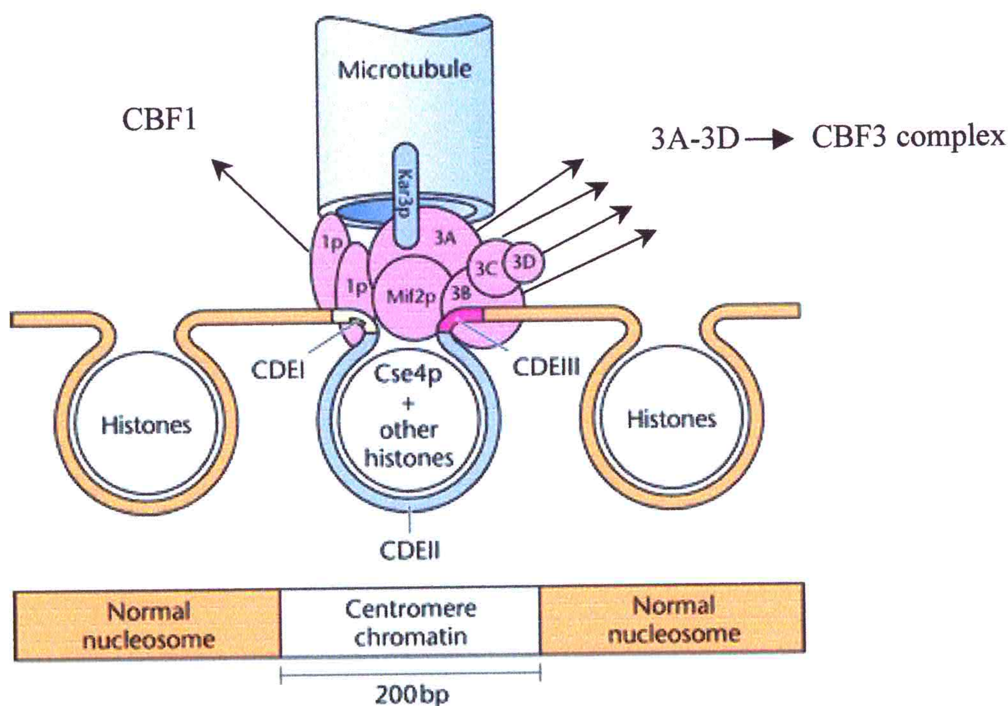

Figure 1 Organization of *Saccharomyces cerevisiae* centromere kinetochore chromatin complex, showing nucleosome formation and DNAprotein and protein-protein interactions. 1p, 3A-3D denote the centromere proteins CBF1 and CBF3A-D, respectively.

From Choo (2001) Enclyclopedia of Life Sciences, *Nature Publishing Group*, 1-7, modified

Cse4p, centromere-specific, histone H3-related protein is present at the kinetochore complex, which binds to CDEII element (Stoler *et al.,* 1995) and interacts with histone H4 and possibly with other histones. This in turn, allows the centromeric DNA to wrap around it to form a unique nucleosome structure. This protein shares a homology with the mammalian CENP-A protein. A multi-subunit protein complex CBF3 binds to the CDEIII region (Gardner *et al.,* 2001). This complex is composed of Ndc10p (3A), Cep3p (3B), Ctf13p (3C) and Skp1p (3D). The CDEIII-CBF3 complex performs multiple functions such as centromere targeting, association with mitotic spindle and regulation of cell cycle progression. Kar3p binds to the CBF3 complex, and a second CDEII interacting protein, Mif2p (Meluh and Koshland, 1995) is the homolog of a mammalian kinetochore chromatin binding protein CENP-C. To date, how these different trans-acting proteins and complexes attach and move the centromere-kinetochore along spindle microtubule is still yet to be understood.

1.4 Maintenance of genomic stability

Most human cancer shows some form of genomic instability, which occurs due to gross chromosomal rearrangements and alterations in the chromosome numbers. A critical step involved in the cell survival and normal cell growth is to prevent oncogenesis. The genetic instability is fuelled by the DNA damage and errors made by the DNA machinery. Double strand breaks also pose problems during mitosis, as intact chromosomes are pre-requisite for proper chromosome segregation during cell division. Therefore, these DNA lesions frequently cause chromosomal abberations including aneuploidy and translocation that are eventually associated with carcinogenesis.

A checkpoint pathway (**Figure 2,** Kolodner *et al.,* 2002) in cell promotes cell cycle delay in response to DNA damage, thereby giving the cell time to repair the damage, and therefore, checkpoint suppresses genome instability. In *S. cerevisiae*, mutations that disrupt the replication checkpoint such as *rfc5-1*, *dpb11-1*, *mec1*, *ddc2* and *dun1* shows increased rate of genome rearrangements (Myung *et al.,* 2001). Replication errors lead to genome rearrangements, and the function of replication check-point is to regulate cell cycle progression in response to replication errors. It is achieved by reducing the rates of DNA replication, to slower cell cycle progression and finally to restart DNA synthesis. There are two branches of intra-S checkpoint that includes: *RAD17* and *RAD24* and *SGS1*. Both these intra-S checkpoint are interfaced with the same downstream signal transduction and effector functions as shown in the **Figure 2**. It was shown that the checkpoint defects and recombination defects independently, increase chromosome loss in diploid cells, and the combination of these two causes a pronounced increase in the rate of chromosome loss (Klein, 2001). In yeast *Saccharomyces cerevisiae*, mutation in the *mad1*, *mad3*, *pds1* showed chromosome loss (Li and Murray, 1991; Yamamoto *et al*., 1996; Alexandru *et al*., 1999) but the frequency of chromosome loss was more in the *mad2* mutants (Li and Murray, 1991; Zang *et al*., 2002). In *S. cerevisiae*, mutation and combination of mutation in genes involved in homologous recombination pathway, such as *rad52, rad55 (23C only)*, *rad57 (at 23C only)*, *rad51 rad59* and *rad54 rdh54* showed more genomic rearrangements. Mutation in *mre11*, *rad50* and *xrs2* also causes genomic stability (Bressan *et al*., 1999; Myung and Kolodner, 2002), which are functional homologs of the mammalian *MRE11 /RAD50 / NBS1* respectively as seen in the **Figure 2**. Moreover, in the absence of Rad54p, homologous recombination is initiated normally

but not terminated and as a consequence leads to misrepair and chromosome loss (Schmuckli-Maurer *et al.*, 2003). Recently, it was shown that deletion of *Elg1* showed increased levels of recombination, chromosome loss (Ben-Aroya *et al.*, 2003). Moreover, Elgp shows homology with Rfc1p, Ctf18p and Rad24p; however, does not co-immunoprecipitate with Elgp (Ben-Aroya *et al.*, 2003). It was reported to form an alternative replication factor (RFC) complex, required for DNA replication and integrity (Ben-Aroya *et al.*, 2003; Bellaoui *et al.*, 2003; Kanellis *et al.*, 2003) and also, *ELG1* has a role in telomere length regulation (Smolikov *et al.*, 2004).

Therefore, multiple pathways are involved that control the genome rearrangement. The molecular mechanism that generates this instability that cause genome rearrangements or aneuploidy is still obscure.

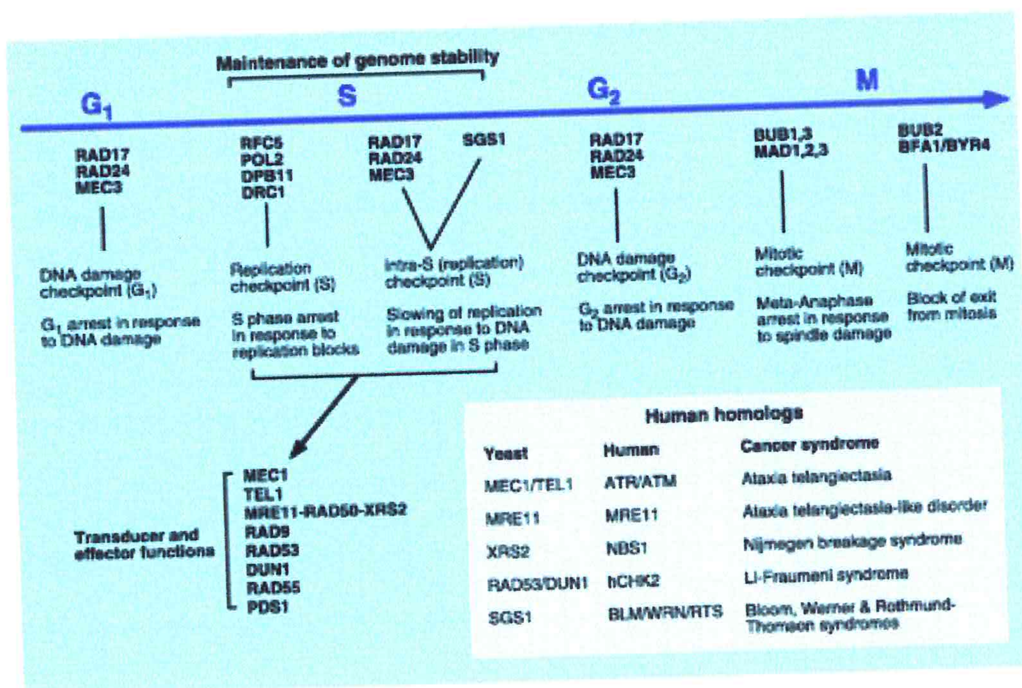

Figure 2 Summary of *S. cerevisiae* DNA damage, replication, and mitotic checkpoints.

The different stages of the cell cycle are indicated above the horizontal line. Below the line are listed a subset of the proteins that function in the indicated checkpoint branches. These proteins are thought to

detect the "damage" that triggers each checkpoint. The primary effect of activating each checkpoint is shown below the proteins. Listed below the three checkpoint branches that function in S-phase are many of the known proteins that function in the downstream signal transduction cascade or are targeted by this cascade. The box at the right lists human homologs of yeast checkpoint genes that are mutated in human cancer susceptibility syndromes.

From Kolodner *et al.* (2002), Science (297) 552-557

1.5 Loss of heterozygosity (LOH)

A genetic alteration that leads to phenotypic changes in diploid cell is more complex than those occurring in haploid cell. The process of loss of heterozygosity is due to chromosome loss, reduplication, gross rearrangement of chromosomes, and mitotic recombination between alleles (Tischfield, 1997; Lengauer *et al.*, 1998). In mammalian cells, LOH is one of the events responsible for the emergence of recessive oncogenic mutations resulting in the insurgence of cancer. The loss of heterozygosity of tumor suppressor genes in tumor cells is mainly associated to chromosomal alterations. Furthermore, an attempt was done to study spontaneous LOH, which has been found associated with aberrant chromosomes in human T cells and mouse primary fibroblasts cells (Gupta *et al.*, 1997; Tischfield, 1997), but the breakpoints of chromosomal rearrangements were difficult to determine. Therefore, the structural factors that mediate chromosomal aberrations are not well defined in mammalian cells. Since somatic cells are diploid, the mechanism of LOH could be easily studied in the yeast *Saccharomyces cerevisiae*. In addition, it may be relatively easy to examine the LOH mechanism, due to availability of genetic techniques. To this end, mitotic segregation of a given marker was examined in diploid and in hyperploid strain disomic for a particular chromosome (Campbell *et al.*, 1975; Esposito *et al.*, 1982; Bruschi *et al.*, 1995), which showed a LOH

mainly due to mitotic recombination between alleles. This suggested that in yeast, generally LOH could be mainly due to recombination between the alleles. Recently, LOH was observed, in which a genetic alterations that functionally inactivated a *URA3* marker hemizygously or heterozygously, located either on chromosome III and chromosome V. Further analysis showed that chromosome loss, chromosome aberrations and allelic recombination were the major events causing LOH events (Hiraoka *et al.*, 2000). Moreover, the frequency of these above phenomena was affected if the markers were displaced on different chromosomes. The result suggested that homologous recombination contributed to at least half of these alterations. Similar studies were performed in the *sgs1* mutant background, which is a member of the RecQ helicase family, which includes the human *BLM*, *WRN* and *RECQL4* genes responsible for Bloom and Werner's syndrome and Rothmund-Thomson syndrome, respectively. In *sgs1* mutant background, ectopic recombination between chromosomes was increased, however, intrachromosomal deletions between *MAT* and *HMR* was increased only slightly in the *sgs1* mutant (Ajima *et al.*, 2002). These results clearly indicate that *sgs1* mutant leads to an elevated incidence of LOH including chromosome loss and interchromosomal rearrangements, but not intrachromosomal deletion. Later, break point of the aberrant chromosomes in LOH clones was further analysed by sequencing the junctions, which revealed that most of the breakpoint occurred within the repeated sequence. This indicates that, in yeast, Ty1 element play crucial roles in chromosomal rearrangement (Umezu *et al.*, 2002). Moreover, to study the role of homologous recombination in detail, LOH was analyzed in *rad50Δ*, *rad51Δ*, *rad52Δ* deletants, as well as in *rad50Δ rad52Δ* and *rad51Δ rad52Δ* homozygous diploids (Yoshida *et al.*, 2003). In all the above strains, frequency of chromosome loss and

LOH events was increased. Therefore, homologous recombination plays significant roles in cellular processes leading to loss of heterozygosity. Furthermore, it was shown that deletion of some DNA check-point genes cause an increase in chromosomal rearrangement. Thus, DNA damage check-points are essential for preserving the normal chromosome number and act in concert with homologous recombination machinery. Failure of either of these processes causes increase in LOH (Klein, 2001). A specific induced chromosome V loss in a diploid resulted in restitution of diploidy with homozygosity for genetic markers, showing loss of heterozygosity (Zang *et al.*, 2002)

Therefore, determining the mechanisms leading to loss of heterozygosity in lower system such as yeast should be studied. This will provide an insight into the regulation and will allow in understanding the generation and progression of cancer in higher system.

1.6 Haploinsufficiency

In diploid organism, ideally two copies of a gene are adequate to perform normal function, however, sometimes one copy of a gene function well. Moreover, presence of one copy of a gene is not sufficient and therefore causes abnormality. This phenomenon is called as haploinsufficiency, which is due to an insufficient gene product in the cell to perform normal function. For instance, in humans, a few genes have been identified as haploinsufficient. These genes were identified because they resulted in abnormalities (Fischer and Scambler, 1994). To study haploinsufficiency, *Saccharomyces cerevisiae* is an ideal model system due to its ease of genetic manipulation. Moreover, it allows for the construction of heterozygous deletion strains in both essential and non-essential gene. It was shown that drug induced sensitivity occurs in diploid yeast, when gene dosage was lowered from two copies to one copy (Giaever *et al.*, 1999). These heterozygotes showed

increased drug sensitivity as compared with the wild-type strain. Moreover, a microarray screen was done in 233 bar coded heterozygous yeast strain, which were grown competitatively in the presence of a drug, whose target in yeast cell was known (Giaever *et al.*, 1999). In human cells, *RAD17*, a component of the checkpoint clamp loader complex is required for the response to DNA damage and replication stress. The yeast cells lacking *RAD17* exhibited endoreduplication at a higher rate, and thus it is linked to ploidy control (Wang *et al.*,. 2003). Haploinsufficiency of *NDC1* in a diploid cell leads to aneuploidy and inviability (Chial *et al.*, 1999). A loss of functional mutation of *BubR1*, a crucial component of the spindle check-point, has been detected in human cancer. *BubR1* +/- mouse embryonic fibroblasts are defective in spindle-check-point activation, and exhibit a greater level of micronuclei than wild-type (Dai *et al.*, 2004). Thus, BubR1 haploinsufficiency in mice showed rapid development of lung and intestinal carcinomas in response to carcinogen. Recently, in *Saccharomyces cerevisiae*, a genome-wide haploinsuficiency screen was performed to identify drug mode of action (Baetz et al. 2004). In this screen, a drug called as dihydromotuporamine C (dh MotC), was used that belongs to a member of the motuporamine family. This assay identified two members of sphingolipid biosynthesis pathway, *lcb1Δ / + and tsc10Δ /* +, which are sensitive to this drug. Thus, dhMotC targets sphingolipid metabolism, resulting in a decrease of ceramide levels in yeast. By adding extracellular ceramide in yeast culture showed protection against the drug. The budding yeast shows < 40% of yeast proteins share conserved sequence with at least one known or predicted human protein. Therefore, this result provides a broad horizon that drug-induced haploinsuficiency in yeast can reveal pathways and this knowledge can be extrapolated into human cells.

1.7 Chromosomal aneuploidy and cancer

Every living cell faithfully divides it genetic material at high rate of fidelity. Failure of this event either results in the death of the cell or disease in the organism. A number of human diseases are reported, and a prime example of aneuploidy is the trisomy of chromosome 21. The chromosomal non-disjunction occurs during the meiotic II division of anaphase. For instance, human cancer involves both chromosome number as well as structure causing genetic instability, and in most if not in all the tumors there is occurrence of gross chromosomal rearrangements, chromosomal aneuploidy and gene amplification etc. (Friedberg *et al.*, 1995; Lengauer *et al.*, 1998; Schar *et al.*, 2001). Chromosomal instability leading to an aberrant chromosome number and missegregation is a hallmark of cancers. The chromosomal abnormalities could be categorized into those that affect chromosome number and/or structure. Chromosome number instabilities are seen in most of human cancer, which likely reflect the malfunction of chromosome segregation (Lengauer *et al.*, 1998). The numerical abnormalities have changes in the chromosome number such as increase or decrease of a copy number. The normal human complement is 46 chromosomes (diploid). Aneuploidy refers to the presence of an extra copy of chromosome, i.e., trisomy as seen in Klinefelter (XXY) syndrome, or to absence of a single chromosome is monosomy- Turners (XO) syndrome. However, little is know about the genetic and epigenetic factors that affect the chromosomal segregation process. Moreover, changes in the chromosome structure are frequent and these usually are chromosomal aberrations. It has been established that treatment of cells with the agents that induce double strand breaks leads to recombinational repair and also give rise to chromosomal rearrangements (Friedberg *et al.*, 1995). Structural rearrangements involve

chromosome breakage and reunion within a single chromosome or between two or more chromosomes. If a gain is of some fragment of the chromosome then it is partial trisomy or a loss is called as partial monosomy.

The defects in replication-associated Sgs1p, which is homologous to BLM of human cell cause increased mitotic recombination and chromosomal instability (Frei and Gasser, 2001). This suggests that irregularities during DNA replication can generate substrates for recombination and give rise to gross chromosomal rearrangements. Furthermore, defects in the cell cycle surveillance mechanism called as the spindle check-point contribute to chromosome instability and aneuploidy (Klein, 2001; Cahill *et al.*, 1998). Many cancers are associated with mutation in the DNA repair genes, e.g. *Xeroderma pigmentosum*, which is associated with mutations in the nucleotide excision repair pathway (Wood *et al.*, 1997). Also, specific forms of human colorectal carcinomas are associated with the mutations in the DNA mismatch repair genes-*MSH2* and *MLH1* (Kinzler and Vogelstein, 1996; Peltomaki, 2001). Mutations in the tumor suppressor genes such as *BRCA1*, *BRCA2* and *TP53* have been associated with aneuploidy, centrosome duplication and chromosomal instability (Fukasawa *et al.*, 1996; Tutt *et al.*, 1999; Xu *et al.*, 1999). However, relationship between the defect in repair pathway and the progression to aneuploidy is still unclear. In yeast, it was shown that nucleotide excision repair (NER) pathway is required for accurate chromosome segregation, and in the absence of *RAD1*, *RAD2* or *RAD4* resulted in non-disjunction and spontaneous levels of chromosome XV gain was seen in both diploid and haploid yeast strains (Howlett and Schiestl, 2004). A microarray analysis was used to study the expression profiles in 300 homozygous deletion mutants of *S. cerevisiae*, and reported that 8% of the strain showed

some degree of aneuploidy. Moreover, this screen revealed genes present on some chromosomes, which regulate the copy number of other chromosomes, i.e., if a gene present on one chromosome is deleted, it gives rise to disomy or trisomy of another chromosome (Hughes *et al.*, 2000). Further studies in detail would reveal the genetic control of individual chromosome, which will assist in understanding the machinery regulating the chromosome segregation.

1.8 Aim and outline of this study

In *Saccharomyces cerevisiae*, the aneuploid cell is fairly stable; however, the regulatory mechanism involved in controlling/tolerating the stability is far from being discovered. To gain insight about this regulation, we proposed to study the behaviour of aneuploid and wild-type strains of yeast. To this end, we asked two questions: 1) if a chromosome loss is induced in aneuploid strain- will the cell favor endo-reduplication of the remaining chromosome or the cells prefer to retain the aneuploid status? 2) Is there a chromosome counter in the cell, which keeps a vigil on chromosome number to maintain chromosomal integrity?

To address these questions, in our study, we used the aneuploid strains such as: disomic haploid and trisomic diploid as well as the control wild-type haploid and diploid strains. All these strains were tagged with fluorescent markers at *thr1* locus on chromosome VIII. A loss of chromosome VIII was induced by deleting the centromere, using the standard knock-out strategy used previously in our laboratory (Zang *et al.*, 2002). The fluorescent markers tagged on chromosome VIII facilitated in screening for the event of chromosome loss. In addition, a loss of chromosome V was induced in a trisomic diploid for chromosome VIII.

A physical loss of a chromosome was assessed by PCR and phenotypic analysis. Furthermore, Quantitative PCR and Southern analysis were performed to ascertain the presence of a copy number of chromosomes.

CHAPTER 2

Specific targeted integration of kanamycin resistance-associated non-selectable DNA in the genome of the yeast *Saccharomyces cerevisiae*

Sanjeev K. Waghmare, Valentina Caputo, Slobodanka Radovic and Carlo V. Bruschi*

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2.1 Abstract

Sophisticated genome manipulation requires the possibility to modify any inter- or intragenic DNA sequence at will, without leaving large amounts of undesired vector DNA at the site of alteration. To this end, a series of vectors was developed from a previous gene knockout plasmid system, to integrate non-selectable foreign DNA at any desired genomic location in yeast, with a minimum amount of residual plasmid DNA left. These vectors have two mutated *Flp* recognition targets (*FRT*) sequences flanking the *KanMX4* gene, and multiple sites for subcloning the DNA fragment to be integrated. The selectable marker can be recycled by Flp site-specific excision between the identical *FRT*s, thereby allowing the integration of further DNA fragments. With this system, the *NLS-tetR-GFP* and *DsRed* genes were successfully integrated at the *thr1* locus and the *RVB1* gene was tagged at the C-terminus with the V5-epitope-6-histidine tag. This plasmid system provides for a new molecular tool to integrate any DNA fragment at any genome location in *[cir$^+$]* yeast strains. Moreover, the system can be extrapolated to other eukaryotic cells, in which the *FLP/FRT* system functions efficiently.

2.2 Introduction

In the yeast *Saccharomyces cerevisiae*, the 2µm FLP encodes a site-specific recombinase acting on two specific sites, termed as Flp recognition target (*FRT*), that are located at the center of the inverted repeats (Broach and Volkert, 1991; Senecoff *et al.*, 1986). It had been shown that the Flp protein interacts specifically with a 50-bp DNA sequence that includes three 13 bp repeats; the third of which is inverted with respect to the other two and separated from them by an 8-bp core sequence (Andrews *et al.*, 1985; Senecoff *et al.*, 1988). Mutation in the *FRT* core sequence prevents recombination; however, identical mutations are well tolerated and do not affect site-specific recombination (Dixon and Sadowski, 1994; Storici *et al.*, 1999; Storici and Bruschi, 2000). Flp can also mediate intermolecular recombination between the two *FRT*s located on different plasmids (Bruschi and Howe, 1988; Bruschi and Ludwig, 1989) and it is known that DNA heterology between the *FRT* core region of the endogenous 2µm and of a foreign plasmid does not permit site-specific recombination between them. The *FLP/FRT* system carrying different mutations in the *FRT* core region has been employed for the construction of recyclable vectors for multiple gene disruption in yeast (Storici *et al.*, 1999; Storici and Bruschi, 1997). Within the recyclable marker methodology, several other systems are available to date for gene disruption and replacement (Akada *et al.*, 2002; Reid *et al.*, 2002; Toh-e, 1985; Wach et al. 1994) as well as for epitope tagging of chromosomal genes (De Antoni and Gallwitz, 2000; Knop and Schiebel, 1997), but none is capable of integrating at a specific locus any desired DNA sequence having no directly detectable phenotype. The *FLP/FRT* system has now been improved to implement a new, advanced strategy for *in vivo*

genomic DNA alterations. The new system, called specific targeted integration of kanamycin resistance-associated non-selectable DNA (STIK) allows the integration, at any genomic location of DNA sequences that express no directly selectable phenotype, such as spacers, tags, nuclear localization signal sequence and any intergenic or otherwise heterologous sequence. The STIK system accomplishes this task by exploiting the temporary integration of the recyclable, positively selectable *KanMX4* marker. To demonstrate the usefulness of this system, the two new plasmids pGKGE and pXKXE were used to perform gene disruption and replacement of the *THR1* gene with the *NLS-tetR-GFP* and *DsRed* fluorescent markers, respectively, while the new plasmid pHKHE was used for tagging the *RVB1* gene. After these alterations, the selectable marker could be excised and thus, similarly, other DNA sequences could be integrated in the same strain.

2.3 Materials and Methods

Strains, Media and Culture Conditions

The *S. cerevisiae* strain used in this study is the disomic strain for chromosome VIII, Z140-51D: a (*thr1 / +, CUP1 / +, arg4-2 / + , arg4-17 / +) his5 ade2 trp1 trp5 leu1 [cir*$^+$*]*, and its derivatives, in which one or both the *thr1* alleles were replaced with the fluorescent marker gene *GFP*: Z140-51G, a (*thr1 / THR1:: FRTG-NLS-tetR-GFP, CUP1 / +, arg4-2 / + , arg4-17 / +) his5 ade2 trp1 trp5 leu1 [cir*$^+$*]*, and GFP plus *DsRed:* Z140-51GR, a (*thr1:: RFP-FRTH /THR1:: FRTG-NLS-GFP, CUP1 / +, arg4-2 / + , arg4-17 / +) his5 ade2 trp1 trp5 leu1 [cir*$^+$*]*, respectively. Diploid strain DUPOT-SL: *gal2/+, leu2/leu2::FRTX, arg10/+, ade2/+, ura3-52/ura3-5 [cir*$^+$*]*, and haploid strain YPH250: a *ade2 leu2 lys2 his3 trp1 ura3 [cir*$^+$*]* were also used. Yeast peptone dextrose (YPD) and kanamycin-containing media were prepared as described (Sambrook *et al.*, 1989). The yeast transformation protocol was used as described in the EUROFAN protocol(http://www.mips.biochem.mpg.de/proj/eurofan/eurofan_1/b0/home_requisites/guideline/exp-transformation.html). *E. coli* strain *DH5*α was used for plasmid propagation. *E. coli* cultures were grown in LB broth supplemented ampicillin as described (Sambrook *et al.*, 1989).

Plasmids

YIplac128 vector, harboring the *NLS-tetR-GFP* chimeric DNA fragment under the yeast *URA3* promoter and followed by the *ADH1* terminator, was obtained from Kim Nasmyth's laboratory. The Living Colors^{R} pDsRed1-N1 vector was obtained from BD Biosciences Clontech (Palo Alto, CA, USA). In the *DsRed* gene construct, the cytomegalovirus (*CMV)* promoter was replaced with the yeast *LEU2* promoter. Plasmid

pTOPO-RUVB containing the *Bacillus subtilis RuvB* gene fused in frame with the V5His6x epitope was constructed in our laboratory using the pYES2.1 TOPO TA™ Cloning Kit (Invitrogen Technologies, Carlsbad, CA, USA). Plasmids: pGKG, pXKX and pHKH, having different mutations in the central core region of the *FRT* sequence and pWKW, having wild-type *FRT*s, were constructed in our laboratory (Storici *et al.*, 1999). From these plasmids the new series of vectors was obtained as described in the Results section, an example of which is shown in Figure 1A.

Primers

Table 1 lists the primers used in this study, and their details are described as follows. THR1ΔF1: chimeric forward primer, having a 40 bp homologous sequence from the ATG start codon of *THR1* gene and 20 bp homologous upstream of the 5' *FRT*; THR1ΔR1: chimeric reverse primer, having 40 bp homologous to the downstream sequence of *THR1* locus and 20 bp homologous downstream of the 3' *FRT*; THR1ΔR2: chimeric reverse primer, having 40 bp homologous to the sequence of *THR1* locus, further upstream of THR1ΔR1 primer and 20 bp homologous downstream of the *FRT*; THR1F1: forward primer homologous to 100 bp upstream of the ATG of the *THR1* gene; THR1R1: reverse primer homologous to 300 bp downstream of the ATG of the *THR1* gene; 1-V5 F: chimeric forward primer, having 40 bp homologous to the sequence immediately upstream of the *RVB1* STOP codon and 20 bp homologous to the sequence upstream of the V5 epitope in pH-RuvBV5H6; 1-V5 R: chimeric reverse primer, having 40 bp homologous to the sequence immediately downstream of the *RVB1* STOP codon and 20 bp homologous to the sequence downstream of the *FRT* ; RVB1F2: forward primer homologous to the sequence 533 bp upstream of the STOP codon of the *RVB1*

gene; RVB1R2: reverse primer homologous to the sequence 364 bp downstream of the STOP codon of the *RVB1* gene; K1: reverse primer homologous to a 500-bp sequence downstream of the ATG of the *KanMX4* gene; K2: forward primer homologous to the sequence 898bp upstream of the STOP of the *KanMX4* gene.

Standard molecular biology techniques

Plasmid DNA was extracted from *E.coli* using the Wizard^{R} Plus Promega mini-preparation kit (Promega, Madison, WI, USA). Restriction enzymes and DNA polymerase I Klenow fragment were obtained from New England Biolabs (NEB, Beverly, MA, USA) and used according to the manufacturer recommendation. Restriction fragments were separated by gel-electrophoresis and purified using the QIAquick^{R} gel extraction kit (Quiagen, Inc., Valencia, CA, USA) as specified by the manufacturer.

Fluorescent microscopy

The yeast strain was grown on G418 plates. Cells were harvested and washed once with water. Approximately, 10^7 cells were mounted on a glass slide and air-dried. Subsequently, 5µl of mounting medium (Vector Laboratories, Inc., Burlinghame, CA, USA) were added over the dried culture. The green fluorescence was visualized using a Zeiss Axiovert^{R} 100M confocal microscope (Carl Zeiss Jena, Germany).

PCR analysis

Yeast colony PCR: Yeast colonies picked from the plate were suspended in 50 µl of sterile water, and incubated with 5µl of Lyticase™ (Sigma Aldrich, St. Louis, MO, USA) at 40 U/µl for 15 min at room temperature. The suspension was centrifuged at 400X g for 1 min., harvested, and the supernatant discarded. Cells were denatured at 100°C for 10

min and placed on ice for 5 min. The pellet was then suspended in 40 µl of sterile water and 10 µl were used as template for PCR. The reaction was performed following the standard EUROFAN B0 Program protocol. PCR programme for the amplification of replacement cassettes consists of one cycle of 2 min at 94°C followed by 20 cycles at 94°C for 30 sec, 54°C for 30sec and 68°C for 4 min. Subsequently, 10 cycles at 94°C for 30 sec, 59°C for 30sec and 68°C for 4 min. followed by one cycle of 68°C for 5 min. A mixture of Taq DNA polymerase and Pfu DNA polymerase (Promega) in ratio 1:1 was used for amplification of the replacing cassettes. After amplification, 10µl of the samples were run on a standard 1.2% agarose gel for size analysis.

Subcloning the *NLS-tetR-GFP, DsRed* and *RUVB-V5His6x* DNA fragment in the pGKGE, pXKXE and pHKHE vectors, respectively

To sub-clone *NLS-tetR-GFP* in the pGKGE vector, this was cut with the SnaBI restriction enzyme to create blunt ends. The 2.2 kb region bearing the *NLS-tetR-GFP* fragment was excised from the vector YIplac128 by double digestion with restriction enzymes EcoRI and BamHI. The recessed 5' ends of this fragment were gap-filled using DNA polymerase I Klenow fragment. Lastly, this 2.2 kb fragment was further subcloned into the pGKGE vector (Figure 1C, I). Similarly, the *DsRed* fragment was subcloned into the pXKXE vector (Figure 1C, II).

To subclone *RuvB-V5His6x* in the pHKHE vector, pTOPO-RuvB was cut by double digestion with *PvuII* and *XbaI* restriction enzymes. The recessed 5' end of this fragment was gap-filled using the DNA polymerase I Klenow fragment. Finally, the 1.5 kb DNA fragment was subcloned into pHKHE SnaBI site (Figure 1C III), which gave rise to plasmid pH-RuvBV5His6x.

Immunochemical Techniques

The V5 epitope-tagged protein was detected using the mouse monoclonal Anti-V5 Antibody (Invitrogen). Whole protein extracts were prepared following the protocol available at the URL http://www.pmci.unimelb.edu.au/core_facilites/manual/mb460.asp. Western blotting was performed as previously described (Sambrook *et al.*, 1989). The proteins were visualized on the blot membrane after Western blot hybridization by staining with a solution of 0.1% Ponceau-S Red stain and 1% acetic acid for 1 minute.

Table 1. Primers used in this work

THR1ΔF1: 5'ATGGTTCGTGCCTTCAAAATTAAAGTTCCAGCTTCTTCCGAA AAATAGGCGTATCACGAG3'

THR1ΔR1: 5'GCTGTTCGACGCTAGCACCATCGTATGGCAGGCTCAGTAGTC GATGATAAGCTGTCAAAC3'

THR1ΔR2: 5'CGAACCTGTTGATAATTTCTTGAGAGATTTCTTCGAATTCTTA ACTGTGCCCTCCATGG3'

THR1F1: 5'GAGTCATCATCTCGAAAAG3'

THR1R1: 5'TTAGCATCAGAACGCAATGG3'

1-V5F: 5'AAGGTCAACAAAGATTTTAGAAACTTCCGCAAATTATTTG AAGGGCGAGCTTCGAGGTCAC3'

1-V5R: 5'TATTTTTATTTATGAAATGTGCTTTAGGCTTTCTTCACTGTCG ATGATAAGCTGTCAAAC3'

RVB1F2: 5'CAAGATGTTACCTTGCACGA3'

RVB1R2: 5'CAAGTAATAGCAGCAACAAC3'

K1: 5'CAATCGATAGATTGTCGCAC3'

K2: 5'TTATGCCTCTTCCGACCATC3'

Polylinker: 5'TACGTACGTACGCCGCGGCCGCGAATTC3'

2.4 RESULTS

2.4.1 Construction of New Recyclable STIK vectors for Genomic Integration of Nonselectable DNA fragments

A series of vectors carrying the selectable marker *KanMX4* flanked by different FRT sequences such as pWKW, pGKG, pHKH, and pXKX were previously constructed in our laboratory (Figure 1A) (Storici *et al.*, 1999; Storici and Bruschi, 1997). The vector pWKW has wild-type *FRT* sequences, whereas the other three vectors have different mutations in the *FRT* core region. Figure 1B shows the sequences of the wild-type and mutated *FRT* are reported. These vectors were further modified by deleting one of the two EcoRI sites flanking the *FRT's* and subcloning a multiple-cloning site (MCS) (SnaB1, Spl1, SacII, Not I and EcoRI) at the remaining EcoRI site. The position and orientation of the MCS were determined by DNA sequencing (CRIBI, University of Padova) (Figure 1C). These modified vectors were designated as pGKGE, pXKXE, pHKHE and pWKWE according to the core *FRT* sequence present and the EcoRI restriction site that was used for cloning (Figure 1C I-IV, respectively). The MCS enables sub-cloning of the desired sequence in the plasmid to be used as template for PCR amplification of the integration cassette. After integration of the cassette, Flp-induced site-specific recombination occurs only between identical *FRT* sequences, thus allowing excision of the selectable marker, while leaving one *FRT* scare sequence integrated in the DNA, together with the sequence of interest (Figure 2).

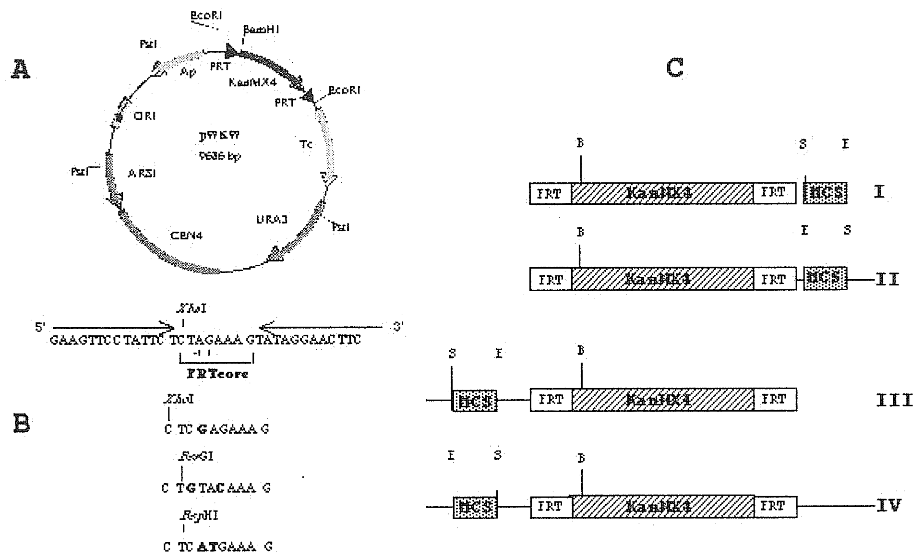

Figure 1. Schematic representation of the wild-type pWKW vector. Panel A shows the major plasmid elements and restriction sites. The different sequences of the FRT core present on the various vectors are indicated in panel **B**. The wild-type FRT sequence with restriction site XbaI, the FRT sequence with the mutation generating the XhoI site, that generating the BsrGI site, and that generating the BspHI site are present on the plasmids pWKW, pXKX, pGKG, and pHKH, respectively. In this vectors, the MCS has been cloned in both orientations, in either EcoRI restriction site giving rise to the four types of vectors (I: pHKGE, II:pXKXE, III:pHKHE, IV:pWKWE) represented in panel **C**.

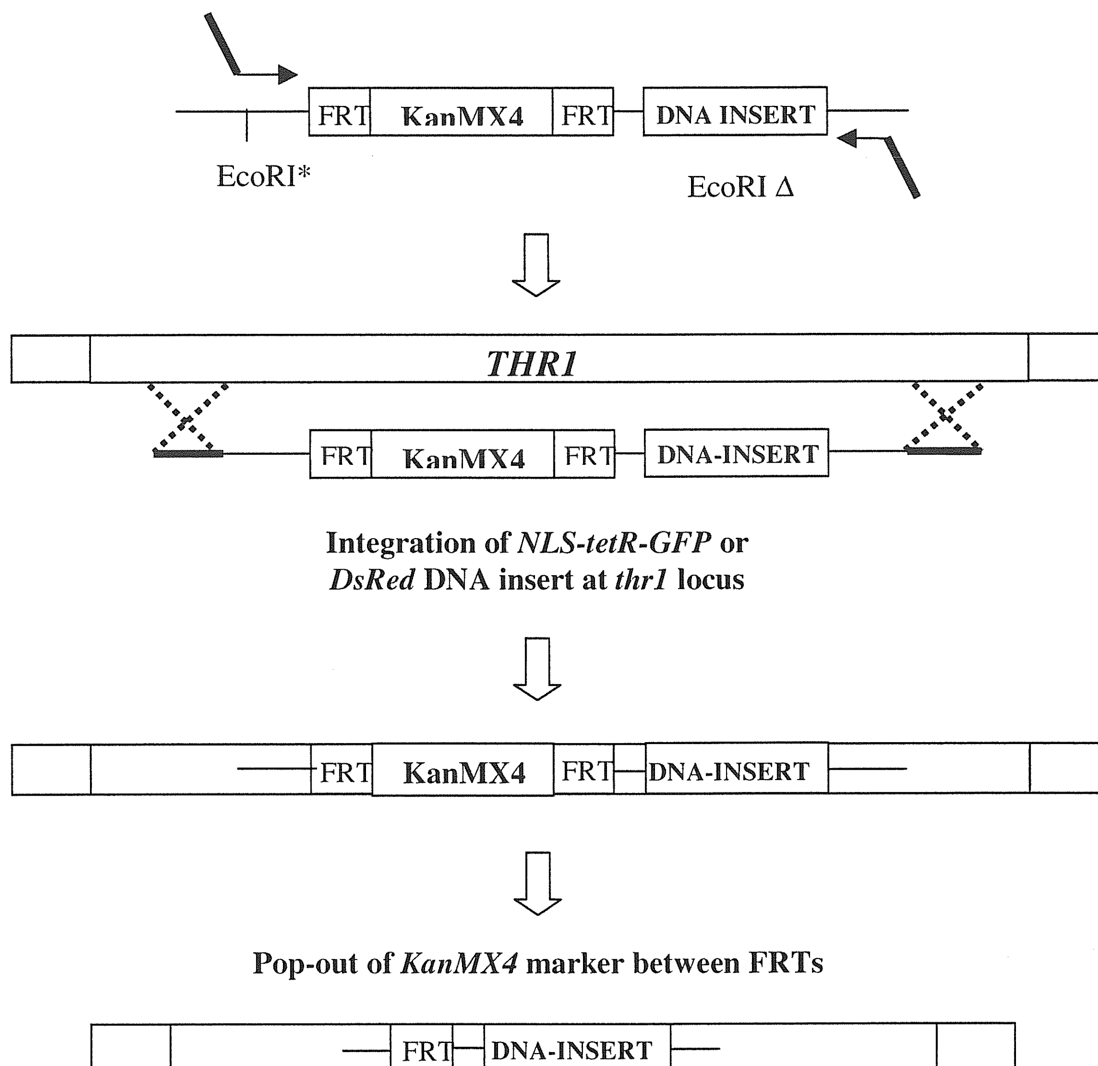

Figure 2.

Schematic representation of the STIK-based integration of a *NLS-tetR-GFP* or *DsRed* fluorescent marker at the *thr1* locus on chromosome VIII. After integration of the fluorescent marker and pop-out of *KanMX4*, the second fluorescent marker has been integrated in the same way into the second allele. EcoRIΔ and EcoRI*, remaining and deleted restriction site, respectively.

2.4.2 Integration of *NLS-tetR-GFP* and *DsRed* sequence at the *THR1* loci

To demonstrate that our system could be used for repeated targeted integration we replaced both threonine (*thr1)* loci on chromosome VIII in *Saccharomyces cerevisiae* strain Z140-51D disomic for that chromosome. Each *THR1* and *thr1* threonine allele was subsequently replaced with *NLS-tetR-GFP* and *DsRed* constructs, respectively. The *tetR* gene fused in frame with the (SV40) NLS sequence at its 5' end and *GFP* at its 3' end was subcloned into pGKGE plasmid (Figure 1C, I). This plasmid was then used as a template for the amplification of the replacement cassette using primers THR1ΔF1 and THR1ΔR1. Both the forward and reverse THR1ΔF1 and THR1ΔR1 chimeric primers (60 bp) were designed in such a way that 40 bp are homologous to the sequences upstream and downstream of *THR1* respectively. The remaining 20 bp are homologous to the sequence of the vector upstream of the 5' *FRT* and to the sub-cloned *NLS-tetR-GFP* fragment respectively (Figure 2). Yeast strain ZI40-51D was transformed with the PCR-amplified, linear 3.6 kb *NLS-tetR-GFP* cassette, and the transformants were selected on YPD plates supplemented with geneticin (G418). Eventually, the *THR1* gene was substituted with the *NLS-tetR-GFP* and two *FRT*s flanking the *KanMX4* gene. To confirm that the integration event occurred at the *THR1* locus, geneticin-resistant transformants were further analyzed by yeast colony PCR using primers THRF1, THRR1 and K1 (data not shown). Moreover, the new strain, designated as Z140-51G, harboring the substitution of *THR1* with the *NLS-tetR-GFP* cassette, fails to grow on minimal medium without threonine, as expected. The *KanMX4* marker was excised spontaneously by growing the G418-resistant, Z140-51G strain in the non-selective liquid medium (YPD) for several generations, to relieve the selective pressure for the marker. Approximately,

one hundred cells per plate were plated onto solid YPD. After incubation, the colonies obtained were further replica-plated onto YPD containing G418 to screen for G418-sensitive colonies. The frequency of the G418-sensitive colonies was found to be of the order of 10^{-3}, which is similar to that reported for the former set of knockout plasmid vectors (Storici *et al.*, 1999). The loss of the marker was further confirmed by yeast colony PCR analysis using primers, THR1F1, THRR1 and K1. At this stage, the remaining *thr1* locus was replaced similarly with the *DsRed* sequence encoding the red protein. This was initially subcloned into the pXKXE vector (Figure 1C, II) that served as a template for the PCR amplification of the *DsRed* replacement cassette using primers THR1ΔF1 and THR1ΔR2. The cassette was then integrated at the remaining threonine locus, which created a new G418-resistant strain Z140-51GR. The integration event was again confirmed by PCR analysis using primers THR1F1, THRR1 and K1 (data not shown).

The green and red fluorescence thus expressed by the integrated *NLS-tetR-GFP* and *DsRed* genes was visualized using confocal microscopy (Figure 3). From the picture, one can see that the fluorescence is present in almost all of cells. The green fluorescence is localized within the nucleus, due to the presence of the SV40 virus NLS sequence in the tetR-GFP construct, while the DsRed protein stains the entire cell due to its absence. Thus, the *NLS-tetR-GFP* and *DsRed* genes were successfully integrated at both *thr1* loci.

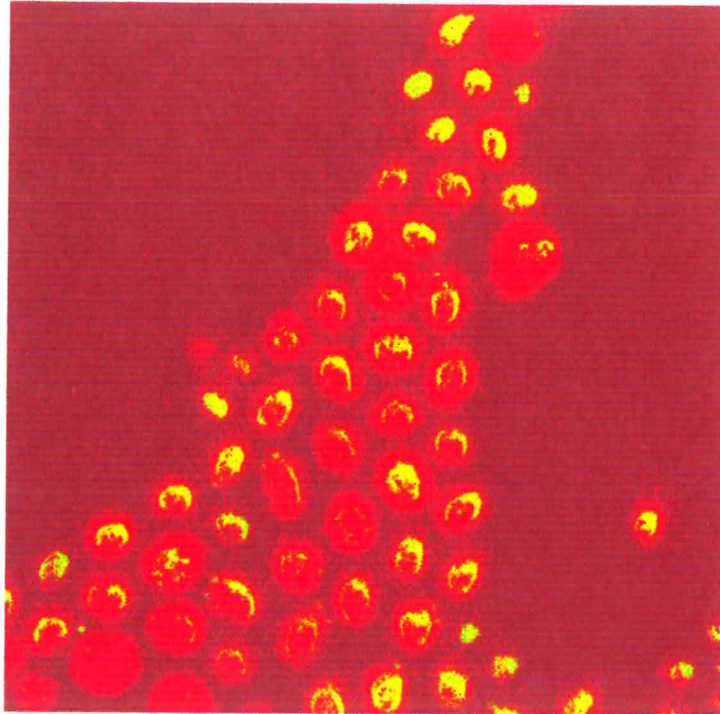

Figure 3.

Confocal fluorescence microscopy of disomic cells (1n+1) of the yeast *Saccharomyces cerevisiae* harboring *NLS-tetR-GFP* and *DsRed* fluorescent marker genes integrated each at the *thr1* locus on the two copies of chromosomes VIII. The green fluorescence is visible within the nucleus of the cells, while the red is present in the cytoplasm. Cells having both disomic chromosomes exhibit both red and green fluorescence. Some cells have lost either the green or red fluorescence, indicating the loss of the related copy of the chromosome.

2.4.3 Integration of the PCR-Tagging Cassette

To demonstrate the versatility of the STIK system for tagging chromosomal genes, we subcloned a DNA fragment encoding the V5His6x epitope into plasmid pHKHE (Figure 1C III) to create plasmid pH-V5His6x that was used as template for the construction of the tagging cassette. The 65 bp forward (1-V5F) and reverse (1-V5R) chimeric primers required for the construction of the *RVB1* locus tagging cassette are reported in Table 1 and their position relative to target genomic sequence is shown below in Figure 4.

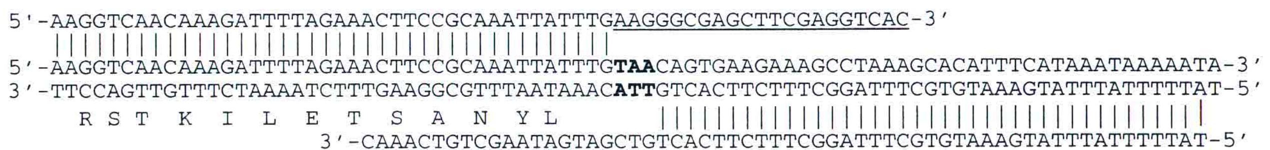

Figure 4. Position of the chimeric primers 1-V5F and 1-V5R used for the in-frame cloning of the V5His6x epitope at the 3'end of RVB1. Forty base pairs of the forward and reverse primers (vertical) are homologous to the *RVB1* ORF sequence upstream, and within its terminator downstream, of the STOP (TAA) codon (in bold), respectively Additional 20 bases complementary to the subcloned V5His6x DNA fragment and to the sequence outside the *FRT*s (corresponding to the vector) are underlined.

Both, haploid YPH250 and diploid DUPOT-SL cells were transformed with the 1.75 kb PCR-amplified linear tagging cassette and transformants were selected on YPD plates supplemented with geneticin (G418). Cassette integration was confirmed by yeast colony

PCR analysis using primers RVB1F2, RVB1R2 and K2 (not shown). No further excision of the *KanMX4* marker was applied in this case. Whole cell proteins were visualized by staining the membrane with Ponceau-S Red stain (Figure 5 left panel). The transformants were assayed for correct expression of the desired Rvb1p-tagged variant by Western blot analysis (Figure 5 right panel). As it can be seen overall from Figure 4, the insertion of the DNA sequence tag was successful and did not alter the expression or the structure of the Rvb1 protein.

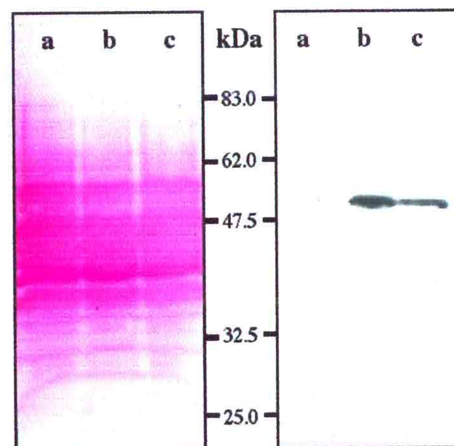

Figure 5.

Whole cell proteins visualized by Ponceau-S Red staining (left panel) and Western blot analysis (right panel) from yeast strains: wild-type, lane a; *RVB1*-tagged YPH250, lane b, *RVB1*-tagged DUPOT-SL, lane c, RVB1-tagged DUPOT-SL

2.5 DISCUSSION

The objective of this work was to construct a series of plasmid vectors that can be used to integrate any DNA sequence of interest, including those non-selectable phenotypically, to a desired coding or non-coding genomic target location, using the *FLP/FRT*-mediated recombination system and the *KanMX4* as a selectable marker in *S. cerevisiae*. A series of PCR-template vectors bearing different mutations in the core region of the *FRT*s was previously reported from our laboratory (Storici *et al.*, 1999; Storici and Bruschi, 1997). These vectors enable to perform multiple gene disruption and knockout using a recyclable selectable marker. In our new STIK system, the PCR amplified *FRT - KanMX4 - FRT* DNA fragment directs the integration to the genomic location of homology, allows its own positive selection and then it is lost by *FLP/FRT* mediated site-specific excision. This leaves the DNA sequence of interest integrated at the selected chromosomal location, together with a 54 bp DNA "scar" consisting of 20 bp homologous to the plasmid template, which can be either: 5'TCGATGATAAGCTGTCAAAC3' or 5'AAAAATAGGCGTATCACGAG3', depending on the EcoRI site that was used for polylinker insertion, and 34 bp of the mutated FRT employed (Storici *et al.*, 1999). Recombination between wild-type and mutated scars, as well as between different FRT scars is not favored; therefore their presence is not inducing any genomic instability due to internal recombination. The most salient feature of our work is that our newly constructed series of PCR-template vectors, pGKGE, pHKHE, pXKXE and pWKWE overcomes the limitation laid down by the impossibility of performing the targeted integration of DNA fragments for which there is no direct selection possible. Therefore, this system provides a broad horizon of

possibilities to integrate any non-homologous DNA sequence at the desired chromosomal location, without leaving relevant exogenous vector sequences that could interfere with the experiment. All the above vectors can be used in *[cir⁺]* strains; however, pWKWE can only be used in *[cir°]* strains due to the presence of wild-type *FRT*s in this vector that could recombine with the *FRT*s of the endogenous 2μm circle. The extrapolation of this system to *[cir°]* strains involves the presence of the endogenous *FLP* site-specific recombinase gene in the vectors themselves. The existing plasmids pGFKF, pHFKH, pXFKX, from our previous work (Sambrook *et al.*, 1989), carrying the endogenous *FLP* gene, can be used as substrate for the construction of additional vectors to be employed in *[cir°]* strains.

To validate our work, the *NLS-tetR-GFP* and *DsRed* DNA fragments were sub-cloned in the pGKGE and pXKXE vectors, respectively, and the entire region was PCR-amplified along with the two *FRT*s and *KanMX4*. These cassettes were integrated in the ZI40-51D strain at the *thr1* locus on chromosome VIII and the phenotypic expression of the fluorescent markers was observed by confocal microscopy (Figure 3). Moreover, to demonstrate the usefulness of this system for protein tagging, we constructed a module that contains the V5 epitope in combination with a 6His-tag. This module allows for the expression of tagged proteins under their own regulatory elements and, subsequently their immediate use for a large set of biochemical and cell biology tests (Wach *et al.*, 1994). We tagged the essential *RVB1* gene in haploid and diploid genetic background using YPH250 and DUPOT-SL strain, respectively. Both strains correctly expressed the C-terminal tagged Rvb1 protein, as demonstrated by Western blot analysis (Figure 5), although in this experiment the selectable *KanMX4* marker was not popped out. This

confirms also that the presence of the 3' *FRT* in the construct can act as an effective transcription terminator as previously reported (Storici and Bruschi, 2000) because the original terminator sequence was disrupted in the tagging process.

Our STIK plasmid system can be used for the construction of multiple deletions, replacement of endogenous regulatory elements and tagging of gene products with high efficiency, using the common *KanMX4* as recyclable selectable marker. Furthermore, this plasmid system can be easily extrapolated to other eukaryotic cells like *Schizosaccharomyces pombe*, *Drosophila melanogaster,* maize, *Xenopus laevis* embryos, and human cells, in which the FLP/*FRT* site-specific recombination has been demonstrated to function effectively (Knop and Schiebel, 1997; Golic *et al.*, 1997; Lyznik *et al.*, 1996; Ng-P Cummings *et al.*, 2000; Werdien *et al.*, 2001). In these cases, an inducible FLP recombinase gene should be co-transfected together with the new vector.

This new STIK system will greatly benefit, in particular, those experiments of cell biology, in which fewer markers genes are available for DNA sequence integration.

To this end, we have made constructions of the plasmid series containing the GFP variant blue fluorescent protein (BFP) and the herpes simplex virus (HSV) epitope tag. We will also be available to insert other commonly used tags, such as GST, if requested.

ACKNOWLEDGEMENTS

We would like to thank V. Tosato and K. Nasmyth for providing us with the *RuvB* and the *NLS-tetR-GFP* gene, respectively, and V. Rapisarda for technical help.

2.6..Implementation of DNA manipulation technology to study chromosomal ploidy

To study the behaviour of chromosomal aneuploidy for chromosome VIII by induced chromosome knock-out, our experimental approach was to construct strains such as disomic haploid, trisomic diploid and a wild-type diploid. In order to examine the event of chromosome loss in these strains, the screening required markers located on chromosome VIII because fewer markers are present on chromosome VIII. Therefore, there was a need to tagged markers, which would facilitate the experimental work.

In this context, firstly a *FLP/FRT* system was modified as mentioned in Chapter 2, which is now designated as STIK system (Specific targeted integration of kanamycin resistance-associated non-selectable DNA) to perform multiple disruption and integration of any non-selectable sequence, as well as epitope tagging at any desired location in the genome. We exploited this nature of STIK system as a genomic tool to overcome the limitation of markers located on chromosome VIII in the strains. Initially, fluorescent markers were individually sub-cloned into the STIK plasmids. Subsequently, these plasmids were used as a template for the amplification of a linear DNA cassette containing, the *FRT's*, *KanMX4* marker and the fluorescent marker, which was integrated at the desired *THR1* locus on chromosome VIII. In summary, the STIK system was used to tagged the chromosome VIII with fluorescent markers such as *GFP*, *RFP* and *BFP* in the aneuploid and wild-type strains. This in turn greatly benefited to address our questions that are mentioned in the Aim and Outline of this study. The results are shown and discussed in Chapter 3.

CHAPTER 3

Differential chromosome control of ploidy in the yeast *Saccharomyces cerevisiae*

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To be Submitted to Yeast Journal

3.1 Abstract

In the yeast *Saccharomyces cerevisiae*, aneuploidy is physiologically well tolerated and usually stable. To study the control of the chromosome complement in aneuploid cells, we analyzed whether the induced loss of a disomic chromosome favors endo-reduplication of the remaining chromosome or whether the cells prefer to retain the normal euploidy acquired by the induced loss. Strains disomic and trisomic for chromosome VIII were tagged with markers such as *GFP* (encoding Green fluorescent protein), *RFP/DsRed* (encoding Red fluorescent protein) and *BFP* (encoding Blue fluorescent protein) integrated at the *thr1* locus using our newly designed STIK (Specific Targeted Integration of Kanamycin resistance-associated, non-selectable DNA) plasmid system. A centromere knockout cassette for *CEN8* (centromere 8) was constructed with the hygromycin-B marker, which was transformed into the above mentioned strains. The transformants lost sensitivity to hygromycin, thereby indicating the event of centromere replacement. PCR and Southern analysis demonstrated the subsequent physical loss of a chromosome. Moreover, Quantitative PCR and Southern analysis were performed for chromosome VIII copy number determination by probing the markers located on both the right (*GFP*, *RFP* and *BFP* inserted at the *thr1* locus) and left arm (*GUT1*), whereas, for chromosome V, markers such as *HIS1,* located on right arm, and *URA3* (on left arm) were used.

The event of an extranumerary chromosome VIII loss in a disomic haploid (1n+1) and trisomic diploid (2n+1) leads to stable, normal euploidy. Furthermore, in a wild-type diploid (2n), deletion of a copy of chromosome VIII, leads to monosomy (2n-1), and restoration of normal euploidy after 20-21 generations, by reduplication of chromosome VIII, and consequent LOH (loss of heterozygosity). However, chromosome V knockouts in

a strain background trisomic for chromosome VIII, still showed LOH and duplication of chromosome V, with return to the aneuploid condition. These results suggest the possibility that yeast cells control the integrity of their genetic complement by a mechanism that acts not at the whole-genome level, but at that of the individual chromosomes.

Keywords: Aneuploidy, chromosome loss, disome, LOH, monosome, ploidy, trisome, yeast

3.2 Introduction

Every living organism faithfully divides its genetic material from a cell to its daughters during cell division. Any change or variability in this phenomenon is bound to generate abnormalities that often lead to cell death. The control mechanisms may be categorized into those that affect chromosome number and/or structure. Abnormalities in chromosome number are seen in human genetic disorders, like Klinefelter syndrome (XXY), Down syndrome (trisomy 21) etc., where (in most of the cases) non-disjunction occurs during the anaphase of the meiotic II division (Luthardt *et al.*, 2001). For instance, human cancer involves changes in both chromosome number as well as structure causing genetic instability and in most, if not in all tumors, there is an occurrence of gross chromosomal rearrangements, chromosomal aneuploidy and gene amplification etc (Lengauer *et al.*, 1998; Friedberg *et al.*, 1995; Schar *et al.*, 2001). However, the underlying mechanism has yet to be elucidated, in particular with respect to its cause-effect relationships.

In eukaryotic cells, the mitotic chromosome segregation error rate is 10^{-5} per generation (Brown *et al.*, 1991). In mitosis, during anaphase, the segregation of chromosomes to the opposite poles is guaranteed by the attachment of the centromeres to the microtubules that extends from opposite spindle poles (Spencer and Hieter, 1992). In the yeast, *Saccharomyces cerevisiae*, an average size of a centromere is around 125 bp, and this is where the kinetochore proteins bind. These centromeres have three well-defined regions comprised of CDEI, CDEII and CDEIII that are highly conserved, being essential for centromere function (Fleig *et al.*, 1995; Murphy *et al.*, 1991; Choo, 2001), and mutations in these element affect chromosome segregation. Moreover, it was

demonstrated that inducing a centromere knockout for chromosome V in a diploid strain leads to chromosome loss during the successive rounds of cell division followed by its endoreduplication (Zang *et al.*, 2002). To date, however, very little information is available on the maintenance of chromosomes present in aneuploid conditions.

Here, we report the behavior of disomic (n+1), trisomic (2n+1) and diploid (2n) yeast strains challenged by the induced loss of a copy of chromosome VIII. This was achieved by deleting one of their centromeric sequence and replacing it with a selectable marker, using a standard knockout strategy. Quantitative PCR and Southern analysis were performed in order to verify the actual copy number of the chromosome. Our data confirm the restitution of euploidy for chromosome VIII in a disomic and trisomic strain, which implies that the cell favors the loss of an extra copy of a chromosome, rather than maintaining its original aneuploid status. However, in a diploid strain, chromosome VIII loss by centromere knockout showed no immediate reduplication of that chromosome, leading to its monosomy, but eventually (after 20-21 generations) restoration of normal diploidy occurred. Moreover, when a copy of chromosome V was deleted in a diploid that was trisomic for chromosome VIII, we noticed the endoreduplication of chromosome V leading to LOH of its markers in spite of the return to the aneuploid condition.

Our investigative approach possibly could be applicable to higher systems, if a similar technique, or modification of the same, could be used to specifically eliminate an extra copy of a chromosome.

3.3 Materials and methods

Strains, media and culture conditions

Saccharomyces cerevisiae strains used in this study are listed in the **Table 1.** YPD and kanamycin-containing media were prepared as described (Sambrook *et al.*, 1989). YPD medium containing hygromycin was prepared as described in Goldstein *et al.* (1999). Yeast transformation protocol was used as described in the EUROFAN programme(http://www.mips.biochem.mpg.de/proj/eurofan/eurofan_1/b0/home_requisites/guideline/exp-transformation.html). *E. coli* strain DH5α was used for plasmid propagation. *E. coli* cultures were grown in LB broth supplemented with ampicillin as described (Sambrook *et al.*, 1989).

Plasmids

Plasmids: pGKGE, pXKXE and pHKHE, bearing different mutations in the central core region of the *FRT* sequence and pWKWE, having wild-type *FRT*s, were constructed in our laboratory (Storici *et al.*, 1999). YIplac128 vector, harboring the *NLS-tetR-GFP* chimerical DNA fragment under the yeast *URA3* promoter and followed by the *ADH1* terminator, was obtained from Kim Nasmyth's laboratory. (Michaelis *et al.*,1997). The *DsRed* vector was obtained from Clontech (BD Biosciences Clontech Laboratories, Inc., Palo Alto, CA, USA). In the *DsRed* gene construct, the *CMV* promoter (*Cytomegalovirus* promoter) was replaced with the yeast *LEU2* promoter. Further, it was sub-cloned into the pXKXE. Initially, the *URA3* promoter plus *NLS-tetR* sequence was sub-cloned in pQBI50 (Amersham Biosciences, Uppsala, Sweden) . Subsequently, the fragment containing the *URA3* promoter-*tetR-BFP* was excised and sub-cloned in to pHKHE vector. *GFP*, *RFP* and *BFP* fluorescent markers were sub-cloned into pGKGE, pXKXE and pHKHE

respectively. Plasmid pAG32 (Goldstein *et al.*, 1999), containing the hygromycin resistance gene *hphMX4* was kindly provided by John McCusker from Duke University Medical Center. The construction of the aforementioned plasmids are reported in Waghmare *et al.* (2003).

Primers

The primers used in this study are listed in **Table 2** and their details are described herewith. THR1ΔF1: chimeric forward primer, having a 40 bp homologous sequence from the ATG start codon of *THR1* gene and 20 bp homologous upstream of the 5' *FRT*; THR1ΔR1: chimeric reverse primer, having 40 bp homologous to the downstream sequence of *THR1* locus and 20 bp homologous downstream of the 3' *FRT*; THR1ΔR2: chimeric reverse primer, having 40 bp homologous to the sequence of *THR1* locus, further upstream of THR1ΔR1 primer and 20 bp homologous downstream of the *FRT*; THR1F1: forward primer homologous to 100 bp upstream of the ATG of the *THR1* gene; THR1R1: reverse primer homologous to 218 bp downstream of the ATG of the *THR1* gene; THR1R3: another reverse primer homologous to 106 bp downstream of stop codon of *THR1* gene ;K1: reverse primer homologous to a 500-bp sequence downstream of the ATG of the *KanMX4* gene; K2: forward primer homologous to the sequence 898bp upstream of the STOP of the *KanMX4* gene. ARG4ΔF: chimeric forward primer, having a 40 bp homologous sequence upstream of ATG start codon of *ARG4* gene and 20 bp homologous upstream of the 5' *FRT*; ARG4ΔR: chimeric reverse primer, having 40 bp homologous to the downstream sequence of *ARG4* locus and 20 bp homologous downstream of the 3' *FRT*; ARG4F1: forward primer homologous to 165bp upstream of the ATG of the *ARG4* gene; ARG4R1: reverse primer homologous to 322 bp downstream of the ATG of the

ARG4R gene; ARG4R2: another reverse primer homologous to 68 bp downstream of the stop codon of *ARG4* locus.

CEN8F: chimeric forward primer, having 40 bp homologous sequence 284 bp upstream of the *CEN8* sequence and 20 bp homologous upstream of hygromycin-B gene in pAG32.

CEN8R: chimeric forward primer, having 40 bp homologous sequence upstream 288 bp 3' of CEN8 sequence and 20 bp homologous downstream of hygromycin-B gene in pAG32.

CEN5F: chimeric forward primer, having 40 bp homologous sequence 1022 bp upstream of CEN5 sequence and 20 bp homologous upstream of the hygromycinB gene in pAG32.

CEN5R: chimeric forward primer, having 40 bp homologous sequence upstream 1068 bp 3' of CEN5 sequence and 20 bp homologous downstream of hygromycin-B gene in pAG32.

Standard molecular biology techniques

Plasmid DNA was extracted from *E.coli* using the Promega (Promega, Madison, WI, USA) mini-preparation kit. Restriction enzymes and DNA polymerase I Klenow fragment were obtained from New England Biolabs (NEB, Beverly, MA, USA). Both of them were used according to the manufacturer's recommendation. Restriction fragments were separated by gel electrophoresis and purified using the Qiagen gel extraction kit (Quiagen, Inc., Valencia, CA, USA) as specified by the manufacturer.

Fluorescent microscopy

The yeast strains were grown on G418 plates. Cells were harvested and washed once with water. Approximately, 10^7 cells were mounted on a glass slide and air-dried. Subsequently, 5μl of mounting medium (Vector Laboratories, Inc., Burlinghame, CA, USA) were added over the dried culture. The green and red fluorescence was visualized using the Zeiss

Axiovert 100M confocal microscope equipped with an argon laser (Carl Zeiss Jena, Germany). The blue fluorescence was visualized with a Leica DMLB photomicroscope equipped with a CCD computer-driven camera.

PCR analysis

Yeast colony PCR: Yeast colonies picked from the plate were suspended in 50 μl of sterile water and incubated with 5μl of Lyticase™ (Sigma Aldrich, St. Louis, MO, USA) at 40 U/μl for 15 min at RT. The suspension was centrifuged at 2,000 rpm for 1 min., harvested, and the supernatant discarded. Cells were denatured at 100°C for 10 min and placed on ice for 5 min. The pellet was then suspended in 40 μl of sterile water and 10 μl of it were used as template for PCR. The reaction was performed following the standard EUROFAN B0 Program protocol. The PCR programme for the amplification of replacement cassettes consists of one cycle of 2 min at 94°C followed by 20 cycles at 94°C for 30 sec, 54°C for 30sec and 68°C for 4 min. Subsequently, 10 cycles at 94°C for 30 sec, 59°C for 30sec and 68°C for 4 min. followed by one cycle of 68°C for 5 min. A mixture of *Taq* DNA polymerase and *Pfu* DNA polymerase (Promega) in ratio 1:1 was used for amplification of the replacing cassettes. After amplification, 10μl of the samples were run on a standard 1.2% agarose gel for size analysis.

Quantitative PCR analysis

Genomic DNA was isolated using the "Yeast genomic DNA extraction Kit" (Pierce). In each reaction (100 μl), approximately 2x 10^6 copies of purified genomic DNA, were used as a template. All the primers were used to a final concentration of 2μM/ml each. The cycle number were 24, 26 and 28 using a standard PCR programme, consisting of 2 min at

94°C followed by 24/26/28 cycles at 94°C for 30 sec, 54°C for 30sec and 72°C for 2 min. Subsequently, followed by one cycle of 72°C for 3 min.

After PCR amplification, the samples were run on 1% agarose gel for size determination. The photograph of PCR was taken using Polaroid 545 Pro camera, having both positive and negative film. Later, the negative film was scanned and the intensity of the bands in each lane, expressed in O.D., was quantified by computerized laser-scanning densitometry at 633 nm wavelength with an UltroScan XL (Pharmacia LKB, Uppsala, Sweden). Each ratio between control and sample was calculated and plotted inclusive of standard error.

Chromosomal DNA preparation for CHEF

Yeast strains were grown at 30°C to stationery phase for 24 hrs. The cells were collected and washed once with water and then with 50 mM EDTA pH 7.5. Resuspended in 150 μl of CPES buffer (40mM citric acid, 120 mM Na_2HPO_4, 1.2 M sorbitol, O.5 M EDTA, pH 7.5). The cell suspension was mixed with 50 μl of Zymolase (20mg/ml), and 250 μl of 1.3% low-melting agarose kept at 50°C. 100 μl were allocated into block formers (Bio-Rad, Richmond, CA) and kept at 4°C. After solidification, the blocks were transferred to CPE buffer (CPES without sorbitol) and incubated at 37°C overnight, shaking at low speed. Blocks were then washed thrice with 50 mM EDTA pH 9.0 at room temperature and treated with Proteinase K overnight or more till they appeared transparent. Afterwards, the blocks were washed twice in 50 mM EDTA pH 9.0 every hour at room temperature. Finally, they were suspended in 0.5 M EDTA and stored at 4°C for future use.

Yeast chromosomal DNA separation by CHEF and Southern blot

Intact yeast chromosomes were separated using the Bio-RAD, CHEF-DRII (clamped homogenous electric field) system. 1% agarose gel was poured and pre-made blocks were

cut, put into the slots, then sealed on top with 0.5% low melting agarose. The gel was run in 0.5XTBE buffer at pulse time between 50 seconds to 100 seconds for 24 hrs. chromosome bands were stained with 1μg/ml ethidium bromide for 30' and transferred onto a nylon membrane (Amersham, USA) according to manufacturer recommendation. Before the transfer, the gel was pre-treated, twice with 0.25M HCl for 15 min., then washed with sterile distilled water. Further, the gel was equilibrated with 0.5N NaOH for 30 mins and transferred onto a nylon membrane by capillary blotting for 12-24 hrs. The membrane was neutralized twice with 1M Tris-HCL pH 8.0 for 30 min. and UV cross-linked, using the Stratagene cross linker.

Quantitative Southern analysis

For Southern hybridization, the membrane was treated as described in Sambrook *et al.* (1989) using PCR-amplified, DIG-labeled probes. After hybridization, the membrane was incubated with anti-DIG-AP chemiluminescent substrate CDP-Star (Roche) and bands were visualized by exposing the membrane to X-film (Kodak, Rochester, NY). After developing, the intensity of the bands was scanned with an UltroScan, as previously described.

Tetrad analysis

Yeast cells were grown on YPD plates, and then were streaked, onto sporulation medium (1% potassium acetate, 0.03% yeast extract), and incubated for 5-6 days at 30°C. Before dissection, the sporulated cells were treated with lyticase at room temperature for 10 min. Well-digested four-spore asci were dissected on thin YPD dissection plates using a Leitz twin-joystick electric micromanipulator with fibrecore dissection needles (Singer Instruments Co. Ltd., Watchet, UK) mounted on a FLUOVERT SF microscope (Leitz

Wetzlar, Germany). The plates were then incubated at 30°C to form colonies and were further analysed.

Table 1

List of strains

Z140-51D ***MAT*** **a** (*thr1 / +, CUP1 / +, arg4-2 / + , arg4-17 / +) his5 ade2 trp1 trp5 leu1 [cir+]*

Z140-51G ***MAT*a** (*thr1/THR1::FRTG-NLS-tetR-GFP, CUP1 / +, arg4-2 / + , arg4-17 / +) his5 ade2 trp1 trp5 leu1 [cir+]*

Z140-51GR ***MAT*a** (*thr1::FRTX-RFP / THR1::FRTG-NLS-tetR-GFP, CUP1 / +, arg4-2 / +, arg4-17 / +) his5 ade2 trp1 trp5 leu1 [cir+]*

FAS20 ***MAT*α** *ade1 ade2 ade8 ura3-52, leu2-3, trp1-289, can1[cir+]*

YPH250 ***MAT*a** *ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2Δ1[cir+]*

YPH250G ***MAT*a** *ura3-52, lys2-801, ade2-101, trp1-Δ1, his3-Δ200, leu2Δ1, ARG4::FRTG-NLS-tetR-GFP [cir+]*

FAS20R ***MAT*α** *ade1 ade2 ade8 ura3-52, leu2-3, trp1-289, can1, ARG4::FRTG-RFP [cir+]*

SAN1 ***MAT*a/α** *ura3-52/ ura3-52, lys2-801/ +, ade1 / +, ade2-101 / ade2, ade8 / +, trp1-Δ1 / trp1-289, his3-Δ200 / +, leu2Δ1 / leu2, can1 / +, ARG4::FRTG-NLS-tetR-GFP/ARG4::FRTG-RFP*

TRISOME ***MAT*a/α** (*thr1::FRTX-RFP/ THR1::FRTG-NLS-tetR-GFP/THR1::FRTH-BFP, CUP1 / + / +, arg4-2 / + /+ , arg4-17 / +) his5 / +,ade1 / +, ade2 / ade2, ade4 / +, trp1 / trp1-289, trp5 / +, leu1 / +, + / leu2-3, + / ura3-52, + / lys2-80, can1 / + [cir+]*

Table 2. Primers used in this work

THR1ΔF1: 5'ATGGTTCGTGCCTTCAAAATTAAAGTTCCAGCTTCTTCCGAA
AAATAGGCGTATCACGAG3'
THR1ΔR1: 5'GCTGTTCGACGCTAGCACCATCGTATGGCAGGCTCAGTAGTC
GATGATAAGCTGTCAAAC3'
THR1ΔR2: 5'CGAACCTGTTGATAATTTCTTGAGAGATTTCTTCGAATTCTTAAT
GTGCCCTCCATGG3'
ARG4ΔF: 5'TACCTTTCTTTAGCTAGGGGAGAATATTCGCAATTGAAGAAAAA
ATAGGCGTATCACGAG3'
ARG4ΔR: 5'CTAATTTAATTGGGATTTCAAATTATCCAATTGCTTCAATCTTAA
CTGTGCCCTCCATGG
CEN8F: 5'GGTTAGTTTCCTATTTCTAAATGAAAGAAGGAGGAATCTTTCCA
CATACGATTTAGGTGACAC3'
CEN8R: 5'TTCTTTGCGGCTCCATTATAAATACACTTTACCATGAGAGAATA
CGACTCACTATAGGGAG3'
CEN5F: 5'GACATTGTCGCTATTATTAGCTGATATGGCAGCACAGAAGCAC
ATACGATTTAGGTGACAC3'
CEN5R: 5'CGAGAGCGTGTCTAAAGATAAGCCGATAGTTTGTATCCTAA
ACTCACTATAGGGAG3'
HIS1-INF: 5'ACCTGTAGCGTTGGTCTTTC3'
HIS1-INF: 5'GAAATGGTTGGTGCTCTACG3'
ARG4F1: 5'GGATAAGCTGGCGCGCAAATTG3'
ARG4R1: 5'CTTTACCAGCAATATCGCGG3'
ADE2F2: 5'GCCCCAAGGCCTCACAACTC3'
ADE2R2: 5'AAGGACACCTGTAAGCGTTG3'
DsRedF1: 5'GAAGGGCGAGATCCACAAGG
DsRedR1: 5' CTCCATGCGCACCTTGAAGC3'
TETR1 : 5' ATATACCCCCCGCACATTGTACTGAGAGTGC3'
TETF1 : 5'ATATACCGCGGGCAGATTGTACTGAGAGTGC3'
TETR-F1: 5'GCAGATTGTACTGAGAGTGC3'
TETF2: 5'ACTTTTGCCCTTTAGAAGGG3'
TETR2: 5'GTTAAACCTTCGATTCCGAC3'
THR1F1: 5'GAGTCATCATCTCGAAAAG3'
THR1F2: 5'TGCCCTGAAACACCTTTCTTC3'
THR1R1: 5'TTAGCATCAGAACGCAATGG3'
THR1R3: 5'ACGGGTAACGGAAGAAGACTC3'
BFPR2: 5"GGCTGGATCGGTCCCGGTG3'
K1: 5'CAATCGATAGATTGTCGCAC3'
K2: 5'TTATGCCTCTTCCGACCATC3'
GUT1F1: 5'TCGATGCTGAAGTTGAAT3'3'
GUT1R1: 5'AGGGTGTGGAGTAGCATAGTG3'
URA3F1: 5'ACCAGAGTCAAACGACGTTG3'
URA3R1: 5'CCAATCTAAGTCTGTGCTCC3'

Underlined sequences of primer CEN5F and CEN5R are the regions for homologous recombination flanking centromere V. Coordinates: CEN5F (150888-150927); CEN5R (153182-153221).Underlined sequences of primer CEN8F and CEN8R are the regions for homologous recombination flanking centromere VIII. Coordinates: CEN8R (105259-105297); CEN8F (105986-106030).

3.4 Results

3.4.1 Construction of disomic, trisomic and diploid strains

Strain, Z140-51D, disomic for chromosome VIII, had its *THR1* and *thr1* threonine alleles replaced with *NLS-tetR-GFP* and *DsRed* constructs, respectively. The *tetR* gene (tetracycline repressor) fused in-frame with the SV40 virus nuclear localization signal (NLS) sequence at its 5' end and *GFP* at its 3' end, was sub-cloned into pGKGE. This plasmid was then used as a template for the amplification of the replacement cassette using primers THR1ΔF1 and THR1ΔR1. Both the forward and reverse THR1ΔF1 and THR1ΔR1 chimeric primers (60 bp) were designed in such a way that 40 bp are homologous to the sequences upstream and downstream of *THR1* respectively. The remaining 20 bp are homologous to the sequence of the vector upstream of the 5' *FRT* and to the sub-cloned *NLS-tetR-GFP* fragment. ZI40-51D strain was transformed with the PCR-amplified, linear 3.6 kb *NLS-tetR-GFP* cassette, and the transformants were selected on YPD plates supplemented with geneticin (G418). Eventually, the *THR1* gene was substituted with the *NLS-tetR-GFP* and two *FRT*s flanking the *KanMX4* gene. To confirm that the integration event occurred at the *THR1* locus, geneticin-resistant (G418) transformants were further analyzed by yeast colony PCR using primers THRF1, THRR1 and K1 (data not shown). Moreover, the new strain, designated Z140-51G, harboring the substitution of *THR1* with the *NLS-tetR-GFP* cassette, fails to grow on minimal medium without threonine, as expected. The *KanMX4* marker was excised spontaneously by growing the G418-resistant, Z140-51G strain in the non-selective liquid medium (YPD) for several generations to relieve the selective pressure for the marker, according to the procedure developed in our laboratory (Storici *et al.*, 1999). Approximately, one hundred cells per plate were plated on

YPD. After incubation, the colonies obtained were further replica-plated onto YPD containing G418 to screen for G418-sensitive colonies. The frequency of the G418-sensitive colonies was found to be of the order of 10^{-3}, similar to that reported for the former set of knockout plasmid vectors (Storici *et al.*, 1999). The loss of the *G418* marker was further confirmed by yeast colony PCR analysis using primers, THR1F1, THRR1 and K1. At this stage, the remaining *thr1* locus was replaced similarly with the *DsRed* sequence encoding the red protein. This was initially sub-cloned into the pXKXE vector that served as a template for the PCR amplification of the *DsRed* replacement cassette using primers THR1ΔF1 and THR1ΔR2. The cassette was then integrated at the remaining *thr1* locus, creating a new G418-resistant strain Z140-51GR in which both the *GFP* and *RFP* fluorescent markers were inserted. The integration event was again confirmed by PCR analysis using primers THR1F1, THRR1 and K1 (data not shown).

A trisomic strain was constructed by crossing the disomic strain Z140-51GR with FAS20, to obtain a diploid trisomic (2n+1) for chromosome VIII. To integrate BFP into the third chromosome VIII, *URA3* promoter plus *NLS-tetR* sequence was sub-cloned in pQBI50 obtained from Dr. Giacca's laboratory, ICGEB, Trieste. Subsequently, the fragment containing this construct was excised out and sub-cloned into pHKHE vector. This was used as template for PCR amplification of the BFP cassette using primers THR1ΔF1 and THR1ΔR1, which was integrated into the trisomic strain at the *THR1* locus, confirmed by PCR (data not shown). In a diploid *SAN1* strain, *NLS-tetR-GFP* and *DsRed* marker were integrated at the *ARG4* locus. Both the forward and reverse ARG4ΔF and ARG4ΔR chimeric primers (60 bp) were designed in a such way that 40 bp are homologous to the sequences upstream and downstream of *ARG4* locus, respectively. The

remaining 20 bp are homologous to the sequence of the vector upstream of the 5' *FRT* and to the sub-cloned *NLS-tetR-GFP and* DsRed fragment. Initially, *tetR-GFP* was integrated at the *ARG4* locus using primers ARG4ΔF and ARG4ΔR in YPH250G haploid strain, and *DsRed* marker was integrated at the *ARG4* locus in FAS20R strain. To confirm that the integration event occurred at the *ARG4* locus, geneticin-resistant transformants were further analyzed by yeast colony PCR using primers ARG4F1, ARG4R1 and K1 (data not shown). These two strains: YPH250G and FAS20R were crossed, resulting in the diploid SAN1 strain harboring green and red fluorescent markers (data not shown).

3.4.2 Induction of a chromosome loss in disomic haploid and trisomic diploid strains

A disomic strain for chromosome VIII was labeled with fluorescent markers such as GFP and DsRed as described earlier. The amplification of a 2 kb cassette to delete the *CEN8* sequence was performed using primers CEN8F and CEN8R and plasmid pAG32, bearing the hygromycin-B resistance gene flanked on either side by a 40 bp sequence homologous to *CEN8*, as template. 10^8 cells were transformed with the cassette and plated onto 4-5 plates containing YPD plus hygromycin. Typically, the transformant yield was 1-2 colonies per plate, with a total of approximately 10 colonies per transformation. These colonies were then re-streaked on a YPD permissive medium to relieve the selective pressure, thus allowing the cells to lose one of the chromosomes. Subsequently, after the initial rounds of cell division, the acentric chromosome is lost. Similarly, an induced chromosome loss was performed in a trisomic diploid (2n+1) for chromosome VIII. The same *CEN8* knockout cassette, used for the disomic strain was transformed into the trisomic diploid, and the transformants were selected on YPD medium containing hygromycin. 10^8 cells were plated and the yield of the transformants was 1-2 colonies per

plate, similar to that obtained for the disomic strain. The transformants that were bigger in size were picked and then PCR analysis was performed to determine the chromosome loss event. Out of the picked transformants, 30-40% of them showed false positives after PCR analysis. Furthermore, we used the *CEN8* knock-out cassette with DNA end sequences homologous to the peri-centromeric region for chromosome VIII and V. This prevented misintegration into other chromosomes.

3.4.3 Investigation of chromosome loss

The event of a chromosome VIII loss was assessed by colony PCR analysis, using primers THR1F1/TETR-R2, THR1F1/DSRedR1 and THR1F2/BFPR2 upstream, downstream and inside of the *GFP*, *DsRed* and *BFP* genes, respectively (data not shown). In a disomic strain, either one of the two copies of chromosome VIII is lost, i.e., the *GFP*-bearing chromosome or the *DsRed*-bearing chromosome, as compared to the control strain. Furthermore, in the trisomic diploid strain, chromosome loss was also verified by yeast colony and genomic DNA-PCR analysis, and the result showed the loss of either of the chromosomes as compared with the control trisomic diploid strain (data not shown).

3.4.4 Quantitative PCR analysis

Quantitative PCR analysis was performed in order to ascertain the chromosomal copy number after the event of a chromosome loss in both disomic and trisomic strain. Yeast genomic DNA was extracted from the transformants showing chromosome loss and from its respective parental strain. Primers ADE2F2 and ADE2R2 were used to amplify a 1030 bp of *ADE2* fragment located on chromosome XV, which was used as a control chromosome having normal ploidy. Primers ARG4F1 and ARG4F2, amplify a 487 bp

DNA regions located on the right arm of chromosome VIII, 34.3 kb from its centromere, which was used to determine the chromosome VIII number. PCR analysis (Figure 1A, I) showed lower intensity bands in the knockout as compared to the original disomic strain. These data were quantitatively analyzed by laser-scanner densitometry, which shows a band ratio for the knockout strains (lanes, 1-4) approximately half that of the disomic control (lane 5) and the same of the haploid control (lane 6) (Figure 1A, II). Similarly, in the trisomic knockout strain (Figure 2A, I and II), the ratio of chromosome VIII / XV in trisome-knock out strain (lane 1) is similar as compared to diploid strain (lane 2) having two copies of chromosomes VIII and half as compared to haploid strain (lane 3). Moreover, to demonstrate that indeed both arms of the chromosome had been lost in disomic and trisomic strain, quantitative PCR analysis was performed by amplifying the *ADE2* and the *GUT1* DNA fragment, using primers GUT1F and GUT1R. These amplify a 450 bp fragment located on the left arm of chromosome VIII, 69.2 kb from its centromere. PCR data were then analyzed by laser-scanner densitometry which shows indeed that the left arm of chromosome VIII has been lost in the disome (Figure 1B, I and II). The ratio of chromosome VIII/XV in the disome knock-out (lane 1) is half as compared to the disome having two copies of chromosome VIII (lane 2) and haploid (lane 3) and shows similar ratio to the knock-out. Similarly in the trisome, the left arm of chromosome VIII was assessed (Figure 2B, I and II). The ratio of chromosome VIII/XV in trisome knock-out (lane 1) and diploid (lane 2) is half as compared to trisome (lane 3), having three copies of chromosome VIII.

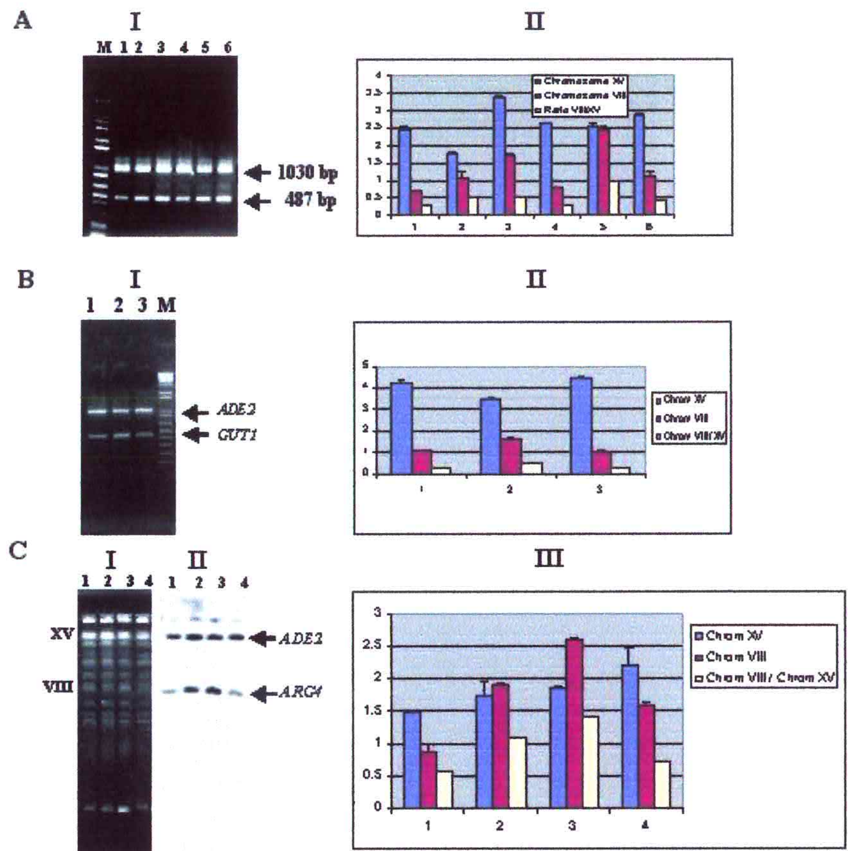

Figure 1: Quantitative PCR and Southern analysis in disomic strain

A(I) Quantitative PCR with probes *ADE2* and *ARG4*. ADE2F2 and ADE2R2 primers were used to amplify *ADE2* fragment located on chromosome XV, which was used as a control chromosome having normal ploidy._Lanes1-4 are Z140-51GR knockout strains; lane 5 is Z140-51GR disomic strain and lane 6 is the YPH250G control haploid strain. B(I) Quantitative PCR with probes *ADE2* and *GUT1*. Lane 1, Z140-51GR knockout strain; lane 2, Z140-51GR disomic strain and Lane 3, FAS20 control haploid strain. C(I) Ethidium bromide staining of pulsed-field electrophoresis gel. (II) Southern hybridization with DIG-labeled *ADE2* (chromosome XV) and *ARG4* (chromosome VIII) DNA fragments as probes. Lane 1 and 2: Z140-51GR-knockout strains; lanes 3 and 4: Z140-51GR disomic strain and YPH250G control haploid, respectively. A (II) B (II)and C (III) right panels show laser-scanner densitometry quantitation of the PCR and hybridization bands from their corresponding left panels. In A(I) and B(I), lane M represents 1kb plus DNA ladder.

3.4.5 Restoration of euploidy in disomic and trisomic strains

To verify further the results of PCR analysis, Southern hybridization analysis was performed. Yeast chromosomal DNA was extracted and separated by CHEF (Contour-clamped homogenous electric field) followed by Southern hybridization. Two DIG-labeled probes were used. *ADE2* (chromosome XV) was amplified from the genomic DNA as internal control probe, using primers ADE2F2 and ADE2R2. An *ARG4* DNA fragment was amplified using ARG4F1 and ARG4R1 primers to hybridize with chromosome VIII. After hybridization and exposure to X-ray film the intensity of the band was quantified by using laser-scanner densitometry, and the corresponding ratio was calculated. As far as quantitative Southern analysis in disome is concerned (Figure 1C, I, II and III), the ratio of chromosome VIII/XV in the disome knock-out (lanes1 and 2) is lower as compared to disome having two copies of chromosome VIII (lane 3) and haploid strain having one copy (lane 4).

Similarly, quantitative Southern analysis in trisome (Figure 2C, I, II and III) shows that the ratio of chromosome VIII/XV in a trisome knock-out (lanes1 and 2) is half as compared with trisome having three copies of chromosome VIII (lane 3) and the diploid strain shows a similar ratio as the trisome knockout having two copies (lane 4). These results demonstrate that indeed the cell favors normal euploidy rather than aneuploidy, in both the disomic haploid and trisomic diploid strains.

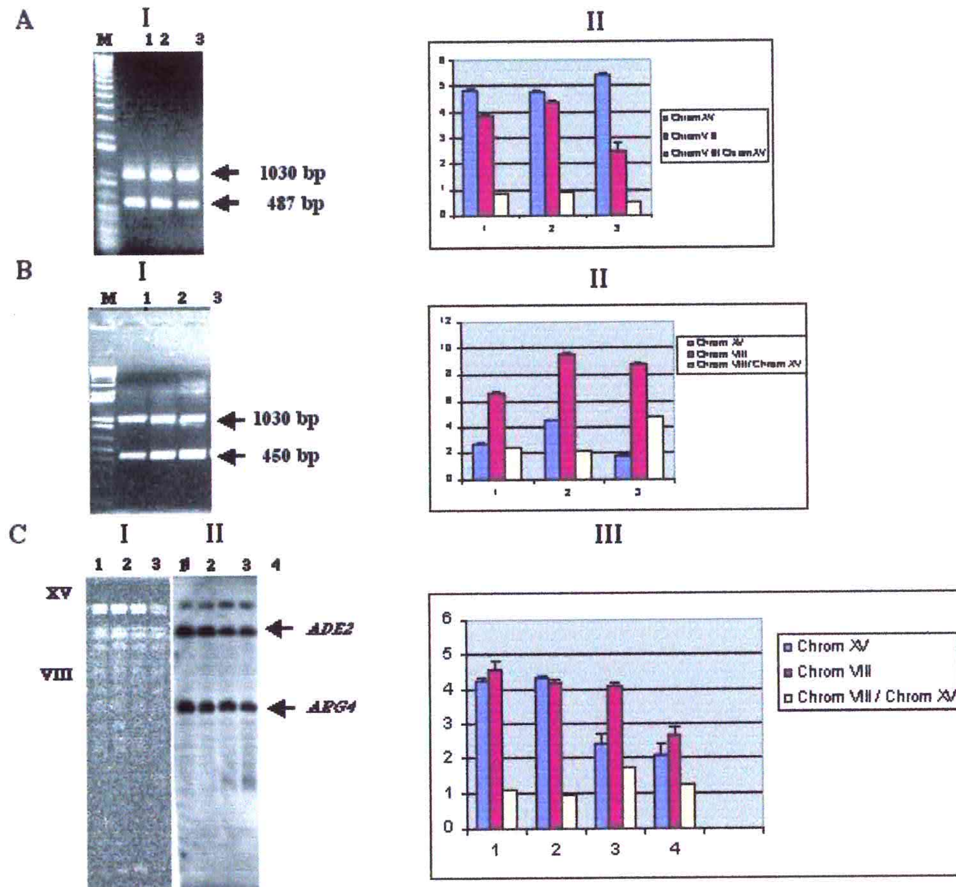

Figure 2: Quantitative PCR and Southern analysis in trisomic strain

A(I) Quantitative PCR with probes *ADE2* and *ARG4*. Lane 1, trisome-knockout strain; lane 2, SAN1 control diploid strain and Lane 3, YPH250G control haploid strain. B(I) Quantitative PCR with probes *ADE2* and *GUT1*. Lane 1, trisome-knockout strain; lane 2, SAN1 control diploid strain and Lane 3, trisomic strain. C(I) Ethidium bromide staining of pulsed-field electrophoresis gel. C(II) Southern hybridization with DIG-labeled *ADE2* (chromosome XV) and *ARG4* (chromosome VIII) . Lanes 1-2: trisome knockout strains; lane3: trisomic strain, lane 4: SAN1 diploid strain. A(II) and B (II) and C (III) right panels show laser-scanning densitometry quantitation of the hybridization bands from their corresponding left panels. In A(I) and B(I), lane M represents 1kb plus DNA ladder.

3.4.6 Restoration of diploidy for chromosome VIII

A diploid strain was constructed labeling chromosome VIII with the fluorescent markers, *GFP* and *RFP*. Chromosome VIII, loss was induced using the identical *CEN8* knockout cassette, which was used for disomic and trisomic strains. The transformants were selected on a YPD containing hygromycin. The loss was assessed by colony and genomic DNA PCR analysis using the primers inside, upstream and downstream of the *GFP* and *DsRed* gene (data not shown). Quantitative PCR and Southern hybridization were further analyzed in the transformants, using the ADE2 probe (chromosome XV) as an internal control, and primers THR1F1 plus THR1R1 for the *THR1* locus (chromosome VIII), which amplify a 318 bp fragment located on the right arm of chromosome VIII, 53.7 kb from its centromere. For Southern hybridization, DIG labeled *ADE2* and *THR1* probes were used. Quantitative PCR and Southern analysis are shown in Figure 3A, I and II and Figure 3B, I, II and III, respectively. The ratio of chromosome VIII/XV in the diploid knock-out strains (lanes 1 and 2) are half as compared with diploid having two copies of chromosome VIII (lane 3) and a haploid, bearing one copy (lane 4), shows similar ratio as compared to diploid knock-outs. Both results are in agreement that induced chromosome loss in a diploid promotes monosomy for chromosome VIII.. Moreover, to demonstrate that indeed both arms of the chromosome have been lost, quantitative PCR analysis was performed using the *ADE2* and the *GUT1* probes, as mentioned above, with primers GUT1F and GUT1R to amplify a 450 bp DNA fragment located on the left arm of chromosome VIII. PCR data were then analyzed by laser-scanning densitometry, which shows indeed that both the left and right arm of chromosome VIII have been lost (Figure 3C, I and II). The ratio of chromosome VIII/XV in the diploid knock-out strain (lane 1) is

lower as compared with the diploid having two copies of chromosome VIII (lane 2). Lane 3 shows the ratio between the two chromosomes in the haploid.

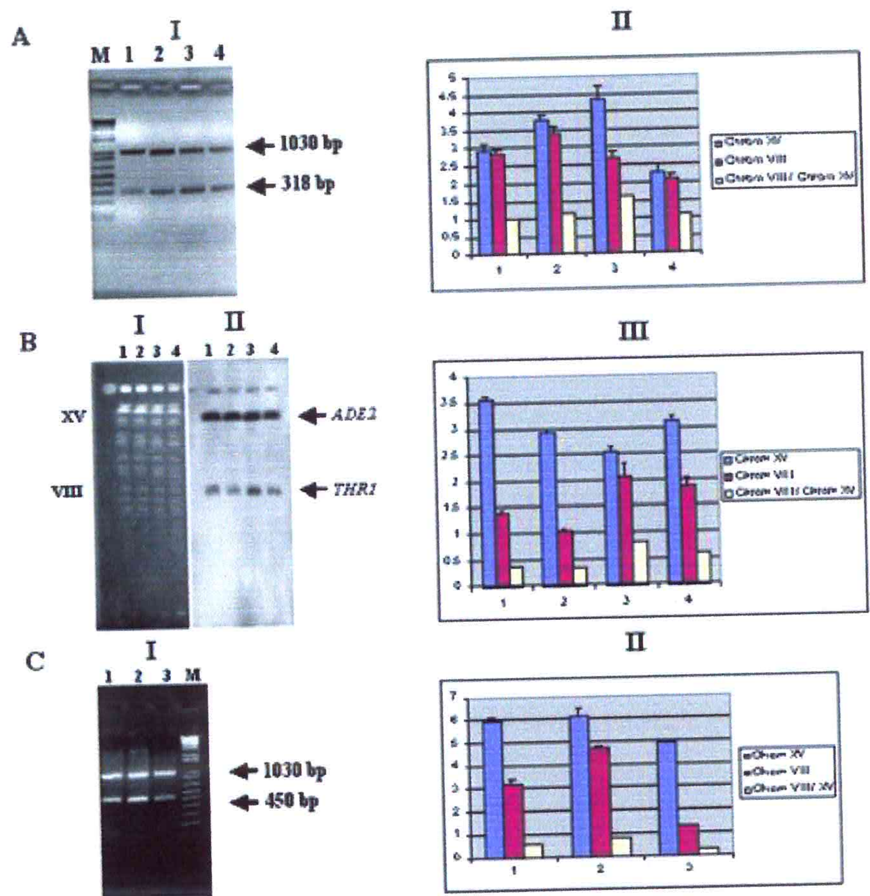

Figure 3: Quantitative PCR and Southern analysis in diploid strain

A(I) Quantitative PCR with probes *ADE2* and *THR1*. Lanes 1-2: SAN1 knockout strain; lane 3: SAN1 control diploid strain; lane 4: FAS20 control haploid strain. B(I) Ethidium bromide staining of pulsed-field electrophoresis gel. (II) Southern hybridization with DIG-labeled *ADE2* (chromosome XV) and *THR1* (chromosome VIII) DNA fragments as probes. Lanes 1-4 are the same as mentioned in A(I). C(I) Quantitative PCR with probes *ADE2* and *GUT1*. Lane1: diploid-knock out strain; lane 2: SAN1 diploid strain and lane 3: FAS20 control haploid strain. A(II), B(III) and C(II) right panels show laser-scanner densitometry quantitation of the PCR and hybridization bands from their corresponding left panels. In A(I) and C(I), lane M represents 1kb plus DNA ladder.

Furthermore, it was found that the monosome for chromosome VIII does not sporulate, as was expected. In order to assess the stability of its aneuploid status, the cells were grown on YPD medium for several generations and checked for the ability to sporulate at different times. Surprisingly, the result showed sporulation after 20-21 generations with 4-spored mature asci.

Tetrad analysis of 10 random asci yielded a 4:0 viability pattern, indicating restoration of euploidy, i.e. reduplication of a remaining chromosome VIII. This was indeed confirmed by PCR and phenotype analysis of the tetrads showing reduplication of chromosome VIII, leading to LOH (data not shown).

3.4.7 Restoration of diploidy for chromosome V in a chromosome VIII trisome suggests that yeast cells do not count chromosomes

In a trisomic diploid heterozygous for the *URA3* marker, chromosome V loss was induced using the knockout cassette consisting of 40 bp homologous to the pericentromeric region flanking, on either side, the hygromycin-resistance marker, by using the primers CEN5F and CEN5R. Transformants were initially selected on YPD-containing hygromycin plates and those that lost the wild-type *URA3* gene were selected on synthetic complete plates containing 5-FOA (data not shown). The 5-FOA-positive transformants were further subjected to quantitative PCR analysis. Primers ADE2F2 and ADE2R2 were used to amplify the *ADE2* fragment on chromosome XV, whereas, HIS1-INF and HIS1NR were used to amplify the 609 bp *HIS1* internal coding region located on the right arm of chromosome V, 112 kb from its centromere. Quantitative PCR and Southern analysis are shown in Figure 4A, I and II and Figure 4B, I, II and III,

respectively. The ratio of chromosome V /XV in the trisome knock-out strains (lane 1) is similar as compared with trisome having two copies of chromosome V (lane 2), whereas the haploid, bearing one copy (lane 3), shows a lower ratio as compared with both the trisome and the trisome knock-out.

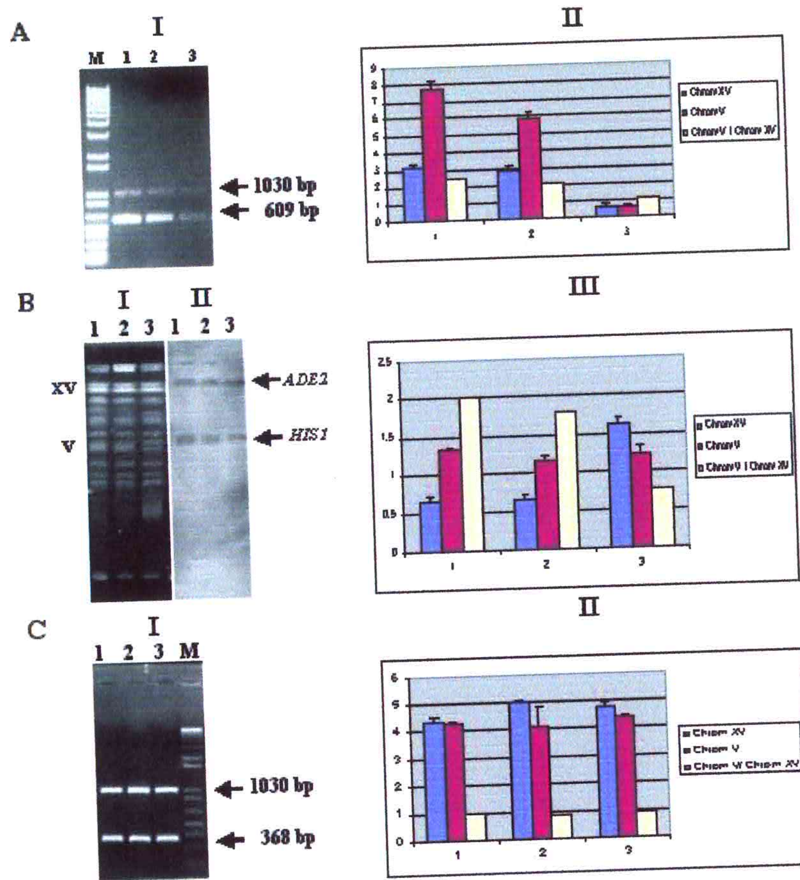

Figure 4: Quantitative PCR and Southern analysis for chromosome V LOH in trisome strain

A(I) Quantitative PCR with probes *ADE2* and *HIS1*. Lane 1: chromosome V knockout trisome strain; lane 2: trisome control strain; lane 3: FAS20 control haploid strain. B(I) Ethidium bromide staining of pulsed-field electrophoresis gel. (II) Southern hybridization with DIG-labeled *ADE2* (chromosome XV) and *HIS1* (chromosome V) DNA fragments as probes. Lanes 1-3 are the same as mentioned in A(I). C(I) Quantitative PCR with probes *ADE2* and *URA3*. Lane 1, chromosome V knockout trisome strain; lane 2: trisome control strain; lane 3: SAN1 diploid control. A(II), B(III) and C(II) right panels show laser-scanner densitometry

quantitation of the PCR and hybridization bands from their corresponding left panels. In A(I) and C(I) lane M represents 1kb plus DNA ladder.

Furthermore, to demonstrate that indeed both arms of the chromosome have been lost, quantitative PCR analysis was performed using the *ADE2* probe and the *URA3* probe obtained with primers URA3F1 and URA3R1 to amplify 368 bp DNA fragment located on the left arm of chromosome V, 34.9 kb from its centromere. PCR data were then analyzed by laser-scanning densitometry, which shows indeed that the left arm of chromosome V has also been lost (Figure 4C, I and II). The ratio of chromosome V/XV in the trisome knock-out strains (lane 1) is similar as compared with both the trisome having two copies of chromosome V (lane 2) and diploid bearing two copies (lane 3). These results clearly demonstrate that indeed there is a duplication of chromosome V, with an LOH phenotype, in a diploid background trisomic for chromosome VIII

3.5 Discussion

The aim of this work was to explore the existence of a cellular surveillance system that controls the chromosome complement in the yeast *Saccharomyces cerevisiae*, thereby maintaining its genomic stability. There are several reports of induced chromosome loss achieved by treating yeast cells with chemical and physical agents. However, the loss is random having additional cellular effects (Hartwell and Smith, 1985; Esposito *et al.*, 1982; Howlett and Schiestl, 2000). Mitotic segregation of genetic markers was examined in diploid and disomic strains for a particular chromosome (Campbell *et al.*, 1975; Esposito *et al.*, 1982; Bruschi *et al.*, 1995), which showed a LOH mainly due to mitotic recombination between alleles. More recently, a major cause of LOH was found to be chromosome loss and chromosome aberration (Hiraoka *et al.*, 2000). Moreover, the frequency of these two phenomena was affected if the markers were displaced on different chromosomes. It was also documented that deletion of some DNA check-point genes causes an increase in chromosomal rearrangement (Klein, 2001).

A novel approach to obtain specific chromosome loss by its centromere knockout was reported, wherein one copy of chromosome V was deleted in a diploid resulting in restitution of diploidy with homozygosity for genetic markers (Zang *et al.*, 2002). To better investigate the phenomenon of restitution of diploidy, we addressed a further question: how would an aneuploid strain would behave if its extra copy of a chromosome was deleted? In this context, a disomic haploid strain (1n+1) and trisomic diploid (2n+1) strain for chromosome VIII were constructed. In order to facilitate the screening for the event of chromosome loss, fluorescent markers such as *GFP*, *DsRed* (Waghmare *et. al.*, 2003) and *BFP* were integrated on chromosome VIII. However, these markers were not used for their

fluorescence as such, since this, or the lack of it, would not be directly diagnostic of the presence or absence of a particular chromosome at its DNA level. On the contrary, the prolonged half-life of the fluorescent proteins would hinder the precise scoring of the chromosome number. Therefore, the physical presence of the DNA sequence of the markers, detected by PCR, was used instead as proof of the presence of the chromosome bearing it.

The centromere knockout transformants were selected on YPD plates containing hygromycin in order to select for those that have lost the chromosome. This screening, therefore, eliminates the possibility of selecting spontaneous chromosome loss colonies. In disomic and trisomic strains, in order to detect the physical loss of a chromosome, PCR analysis was carried out on *GFP*, *RFP* and *BFP* exogenous markers inserted at the *THR1* locus, on the right arm of chromosome VIII. Moreover, to ascertain the event of an entire chromosome loss, a *GUT1* marker located on the left arm of the chromosome was also used to verify the loss. In the knockout studies, it was observed that the CEN8 knockout cassette had an equal possibility to recombine, in the disome, either with *GFP* or *RFP*-bearing chromosomes, or in the trisome with the *GFP*, *RFP* and *BFP*-bearing chromosomes. Quantitative PCR and Southern hybridization analyses in these transformants showed the physical loss of one copy, and no duplication of the remaining copy, of chromosome VIII in both disomic and trisomic strains. Furthermore, in a diploid strain, an induced chromosome VIII knockout was performed and the transformants were selected for resistance to hygromycin. Diagnostic PCR was performed on *GFP* and *RFP* markers located on the same chromosome and quantitative PCR and Southern analysis was carried out using the *ARG4* and *GUT1* probes located on either arm of the

chromosome. These results showed that cells tolerate monosomy for chromosome VIII, but only for several generations, after which they undergo restoration of euploidy, i.e reduplication of the chromosome leading to LOH, in agreement with the restoration of diploidy found for chromosome V (Zang *et al.*, 2002). This indicates that the cell favors restoration of euploidy for chromosome VIII, perhaps because this is a status in which the quantitative interaction among gene products acting stoichiometrically reaches its optimum. This is possibly due also to the fact that the cell is less energetically stressed losing an extra copy of a chromosome than maintaining an altered chromosome complement, which is not required for its optimal viability.

At this point, in order to understand more details of how the cell controls the chromosome number, a chromosome V was deleted in a trisome for chromosome VIII. The idea was that, if a chromosomal counter system exists, then by deleting any chromosome, other than one of the extranumerary copies of chromosome VIII, the cell will not need to duplicate the lost chromosome, having reached a chromosome number identical to the normal euploid (32). In our experiments, loss of chromosome V was induced using the knockout cassette for *CEN5*. After the knockout, the trisomic strain was replica-plated onto 5-FOA plates, to counterselect against Ura^+ prototrophic cells in which we could not discriminate whether the induced chromosome loss had occurred or not. Quantitative PCR and Southern analysis was carried out using the *HIS1* marker located on the right chromosomal arm of chromosome V. The result showed the physical loss and then duplication of one chromosome V, consistent with loss of heterozygosity (LOH). These results demonstrate that there could be a differential control over individual chromosomes,

i.e. control could be ploidy-specific for each individual chromosome rather than pertaining to a general genomic control over the total number of chromosomes.

The duplication event of chromosome V in a trisomic background of chromosome VIII may be mainly dependent upon the presence of genes having a haploinsufficiency phenotype. For example, *RAD17*, which encodes a component of the checkpoint clamp loader complex, is required for the response to DNA damage and replication stress. It was shown that cells lacking *RAD17* exhibited endoreduplication at a higher rate, and thus it is linked to ploidy control (Wang *et al.*, 2003). Furthermore, in a diploid strain, an induced chromosome VIII knockout was performed and the transformants were selected for resistance to hygromycin. Diagnostic PCR was performed on *GFP* and *RFP* markers located on the same chromosome and quantitative PCR and Southern analysis was carried out using the *ARG4* and *GUT1* probes located on either arms of the chromosome. These results showed that a cell tolerates monosomy for chromosome VIII for some time, after which it restores diploidy. This is in agreement with the immediate restoration of diploidy found for chromosome V (Zang *et al.*, 2002). These observations lead us to hypothesize a differential genome homeostasis model. According to this model, the equilibrium between control and tolerance of aneuploidy does not occur at the whole-genome level but, rather, at the of individual chromosomes, perhaps through the action of DNA *cis*-acting elements. This hypothesis is supported by recent evidence for the existence of *cis*-acting genomic sites that constitute chromosome integrity determinants (CIDs) that contributing to the control of the integrity of the cell's genome complement (Huang and Koshland, 2003). Quantitative gene product network interaction, in particular for those genes that are known to have an haploinsufficiency phenotype, could trigger endoreduplication of the linkage

group that carries them. In contrast, haploinsufficiency of *NDC1* in a diploid cell leads to aneuploidy and inviability (Chial *et al.*, 1999) while it can result in drug sensitivity as reported by Giaever *et al.* (1999). On the other hand, it was earlier shown by microarray analysis that homozygous deletion mutants of *S. cerevisiae* give rise to disomy or trisomy. Moreover, this screen revealed genes present on some chromosomes, which regulate the copy number of other chromosomes (Hughes *et al.*, 2000).

Hitherto, little information has been gathered on the dynamics of yeast's karyotype, a topic that warrants further investigation. Indeed, a deeper insight into the molecular dynamics underlying karyotype homeostasis in yeast will foster a coherent understanding of eukaryotic evolution, while its extrapolation to higher cellular systems will ultimately benefit the prognosis of genetic disorders.

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Chapter 4

Summary and Conclusions

4 Summary and Conclusions

The objective of this work was to explore the surveillance mechanism that controls the tight regulation of chromosome copy number in *Saccharomyces cerevisiae*.

Initially, a series of plasmid vectors, namely, pGKGE, pXKXE, pHKHE and pWKWE were constructed called as STIK system. These plasmids enabled us to integrate any DNA sequence of interest, including those non-selectable phenotypically, to a desired coding or non-coding genomic target location, using the *FLP/FRT*-mediated recombination system and the *KanMX4* as a selectable marker in *S. cerevisiae.* This STIK system was further exploited in our studies for genome manipulation. To this end, the fluorescent markers such as *GFP*, *RFP* and *BFP* were subcloned into pGKGE, pXKXE and pHKHE respectively. Subsequently, these markers were integrated on a chromosome VIII in a disomic haploid strain (1n+1) and trisomic diploid (2n+1) and wild-type diploid strains.

Chromosome loss was induced by centromere knock-out in the aforementioned strains, and the transformants were selected on YPD plates containing hygromycin in order to chose for those that have lost the chromosome. In disomic and trisomic strains, in order to detect the physical loss of a chromosome, PCR analysis was carried out on *GFP*, *RFP* and *BFP* exogenous markers inserted at the *THR1* locus, on the right arm of chromosome VIII. In the knockout studies, it was observed that the *CEN8* knockout cassette had an equal possibility to recombine, in the disome, either with *GFP* or *RFP*-bearing chromosomes, or in the trisome with the *GFP*, *RFP* and *BFP*-bearing chromosomes. Moreover, to ascertain the event of an entire chromosome loss, a *GUT1* marker located on the left arm of the chromosome was also used to verify the loss. Quantitative PCR and

Southern hybridization analysis in these transformants showed the physical loss and no duplication of chromosome VIII in both disomic and trisomic strains.

In a diploid strain, when an induced chromosome VIII knockout was performed, the cell sustained monosomy for chromosome VIII, however, it showed restoration of diploidy for chromosome VIII after 20-21 generations, leading to the loss of heterozygosity.

Moreover, when chromosome V was deleted in a trisome for chromosome VIII, loss of chromosome V was induced using the knockout cassette for *CEN5*. Quantitative PCR and Southern analysis was carried out using the *HIS1* and *URA3* markers located on the right and left chromosome arms, respectively, on chromosome V. The result showed the physical loss and then duplication of one copy chromosome V, consistent with loss of heterozygosity (LOH) of the genetic markers.

The latter suggests that the reduplication event can be induced by the haploinsufficiency of gene products, which are essential for the viability of the cell. Moreover, *cis*-acting genomic sites –chromosome integrity determinants (CIDs) may be contributing to control the integrity of genome complement (Huang and Koshland, 2003). There could be a possibility of unique determinants present on the chromosomes, which allow discrimination among them. For instance, chromosome V loss in a trisomic diploid or wild-type diploid leads to immediate reduplication of that chromosome, whereas, deletion of chromosome VIII in a wild-type diploid didn't show duplication until 20-21 generations later. Therefore, depending on the necessity of the gene products that are lost, the cell in turn triggers the chromosomal duplication event, to maintain homeostasis. This phenomenon could be due to a differential control over individual chromosomes, i.e.,

control could be ploidy-specific for each individual chromosome, rather than pertaining to a general genomic control over the total number of chromosomes.

In summary, these results demonstrate that cell favors euploidy regardless of its initial ploidy status. If an extra copy of chromosome VIII is deleted in disomic and trisomic strains, then the cell responds by restoring haploidy and diploidy, respectively. If a copy of a chromosome is deleted in a diploid, it responds by restoring the normal diploidy through endoreduplication, although with different kinetics.

A deeper insight into the molecular mechanism will elucidate the determinants controlling the chromosomal complement in the yeast *Saccharomyces cerevisiae*. The extrapolation of these results to the mammalian karyotype will help settle the controversy over the hypothesis of the chromosomal origin of cancer.

Chapter 5

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