Identification of Genes Which Affect Chromosomal Instability (CIN) in a Dosage-Sensitive Manner in *Saccharomyces cerevisiae*

Thesis

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“Identification of genes which affect chromosomal instability (CIN) in a dosage-sensitive manner in *Saccharomyces cerevisiae*”

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A thesis submitted in fulfillment of the requirements of the Open University for the Degree of Master of Philosophy

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an Affiliated Research Center of
the Open University, United Kingdom

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<table>
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<tr>
<td>ALF</td>
<td>A-like faker</td>
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<tr>
<td>AMKL</td>
<td>Acute megakaryoblastic leukemia</td>
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<tr>
<td>AML</td>
<td>Acute myeloid leukemia</td>
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<tr>
<td>BP</td>
<td>Base pairs</td>
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<td>CIN</td>
<td>Chromosome instability</td>
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<tr>
<td>CNV</td>
<td>Copy number variation</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>ESR</td>
<td>Environmental stress response</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorting</td>
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<tr>
<td>GCN</td>
<td>Gene copy number</td>
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<tr>
<td>GCR</td>
<td>Gross-chromosomal rearrangement</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>GO</td>
<td>Gene ontology</td>
</tr>
<tr>
<td>HI</td>
<td>Haploid-insufficient</td>
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<tr>
<td>HT</td>
<td>High-throughput</td>
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<tr>
<td>iCTF</td>
<td>Improved GFP-based chromosome transmission fidelity</td>
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<tr>
<td>K-MT</td>
<td>Kinetochore-Microtubule</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
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<tr>
<td>MoBY-ORF</td>
<td>Molecular barcoded yeast open reading frame</td>
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<tr>
<td>MudPIT</td>
<td>Multidimensional protein identification technology</td>
</tr>
<tr>
<td>MVA</td>
<td>Mosaic variegated aneuploidy</td>
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<tr>
<td>O/N</td>
<td>Over night</td>
</tr>
<tr>
<td>OD</td>
<td>Over-dosage</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
</tr>
<tr>
<td>RPM</td>
<td>Rounds per minute</td>
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<tr>
<td>RT</td>
<td>Room temperature</td>
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<tr>
<td>SAC</td>
<td>Spindle Assembly Checkpoint</td>
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<td>SGD</td>
<td>Saccharomyces genome database</td>
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<tr>
<td>SILAC</td>
<td>Stable isotope labeling with amino acids in cell culture</td>
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<tr>
<td>WT</td>
<td>Wild type</td>
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<tr>
<td>YAC</td>
<td>Yeast artificial chromosome</td>
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<tr>
<td>XCI</td>
<td>X chromosome inactivation</td>
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First and Foremost, I would like to thank Dr. Rong Li for her advice and guidance during my time in her lab. She gave me the opportunity to be part of a great team of talented researchers and was very supportive and understanding from the very first day. I would say nearly equally as important was the support, mentorship and friendship of Dr. Jin Zhu, who was gracious enough to allow me to work with him and who showed me the wonderful world of yeast. I have never encountered someone as enthusiastic about science and I know for sure that he will go places.

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At last, I would like to thank my family and friends which were always very supportive of my decisions, even though they resulted in me moving far away from them.
Dedication

“I would like to dedicate this thesis to my girlfriend Nina K., whose love and support matters to me the most and who never ceases to amaze me.”

“Knowledge is in the end based on acknowledgement”

- Ludwig Wittgenstein, Ph.D. -
Abstract

Chromosomal instability (CIN) refers to circumstances which can alter the chromosomal content of a cell during its division. Aneuploidy is known to be a direct result of CIN but importantly has recently been shown to affect CIN itself. A possible reason as to why aneuploidy could influence CIN is by gene copy-number-variation (CNV) of dosage sensitive genes that are present on the chromosome which was lost or gained by the aneuploid cell. To test this hypothesis, our lab developed a novel form of CIN assay in budding yeast, termed Improved GFP-based Chromosome Transmission Fidelity (iCTF) assay, which allows us to determine the effects that slight copy number changes of individual genes have on CIN in a high-throughput manner. We utilized this assay to systematically screen for genes which can affect the loss rate of a yeast artificial chromosome (YAC) when (1) their copy number was increased by a gene containing plasmid (Over-Dosage CIN) or (2) decreased due to haploid insufficiency (HI-CIN).

We identified and validated 36 CIN genes in the Over-Dosage CIN screen as well as 139 CIN genes in the HI-CIN screen. From these 175 CIN genes, in total, only 25 known CIN genes were identified by previous screens, which leave 150 novel CIN genes. Most interestingly, 9 out of 175 CIN gene candidates decrease CIN. To our knowledge this is the first reported case of this phenotype.

CIN and aneuploidy are widely known to frequently co-exist in tumorigenic tissues and that they can be caused by loss or gain of certain genes, often involved in maintenance of genomic integrity. The spectrum of such genes is only partially known and it is so far impossible to predict the effects that individual mutations could have on chromosomal instability, especially in such a complex and diverse background as cancer cells. To address this issue we present here a fast and reliable method to determine the effects of single copy number variations in CIN in a quantitative manner.
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1. Introduction

The genetic information which defines an organism is stored in the form of deoxyribonucleic acid (DNA) on a species specific number of chromosomes, collectively called a genome. The so called karyotype of each organism describes the total sum of individual attributes of its corresponding genome, e.g. the total number of chromosomes and chromosome duplicates as well as the size and content of every individual chromosome. Karyotypes therefore genetically define an organism and any changes might drastically affect its characteristic properties and functions on a cellular level (Heng et al., 2013).

Genetic changes can happen due to several means and lead to various more or less severe variations from the as standard considered wild type (WT) karyotype. Deviations in DNA content are categorized by the affected DNA quantity which can be utilized to detect genomic alterations. Smaller, more constricted chromosomal changes can be classified as point mutations, which affect only a few base pairs (bp), or larger copy number variations (CNV), that can affect long stretches of DNA sequence (Tang and Amon, 2013). Both of these alterations are caused by DNA replication errors that can lead to the gain or loss of functional genes and can only be detected by sequencing the affected chromosomal regions. In comparison to these smaller alterations, are the more severe changes of a cell’s karyotype, namely polyploidy, aneuploidy or segmental aneuploidy, which describe the gain or loss of a whole chromosome set, individual chromosomes or sub-chromosomal regions, respectively. These, much larger alterations are caused by the unequal segregation of a cell’s chromosomal content during cell division and are often large enough to be observed using a light microscope (Torres et al., 2008).

Chromosomal instability (CIN) describes a cell’s frequency of chromosome missegregation during mitosis, causing the gain or loss of partial or entire chromosomes, which in turn determines the cell’s ability to maintain a stable karyotype. The resulting cellular state of having an abnormal number of chromosomes, termed aneuploidy, has long
been observed to exist in a multitude of diverse organisms as well as varying tissues and has been identified to be mainly a direct result of CIN. In addition, cells with a high degree of aneuploidy are frequently observed in many types of cancer cells, causing a high level of genetic diversity among individual tumors.

Several studies have shown that the copy number of genes directly corresponds to the level of gene expression in aneuploid cells (Oromendia and Amon, 2014). Due to this can any genetic change, which leads to the presence or absence of functional genes, directly affect the amount of the corresponding protein product. Aneuploidy has, due to its large deviation in chromosomal content, a high impact on gene expression levels and is able to alter the balance of stoichiometrically sensitive pathways and protein complexes which can cause a disruption of their cellular functions. The phenotypic consequences for each specific aneuploidy will rely on the genes which are present on the imbalanced chromosomes as well as on their interactions with other genes, which might have been affected by aneuploidy themselves (Girirajan et al., 2011).

The mechanisms by which aneuploidy could specifically influence chromosomal instability might depend on two connected mechanisms. First, it could happen due to a stoichiometric imbalance between individual proteins, which are involved in chromosome segregation. Second, it could be due to a lack of the overall scaling between the increased chromosome load that has to be segregated and the capacity of the chromosome segregation machinery, which could again be affected by specific stoichiometric imbalances between proteins located on imbalanced chromosomes (Potapova et al., 2013).

Mutations of several individual genes such as components of the mitotic spindle or cell cycle checkpoints, are well known to cause an increase in CIN and therefore raise a cell's chance to become aneuploid (Aguilera and Gomez-Gonzalez, 2008). According to this model it is important for a mutation to occur in a gene which is directly relevant for chromosomal segregation, to lead to aneuploidy by elevation of CIN. This starting mutation is considered the driving force of the initial, as well as all following,
chromosome missegregation (Hanahan and Weinberg, 2011). In contrast, does the great genomic diversity observed in cancer support a more genome-centric perspective. Accordingly, aneuploidy can lead to changes in CIN as well as vice versa, increasing thereby the cancer cell’s adaptability to non-beneficial conditions (Heng, 2009). Recent studies in our lab have shown that aneuploidy itself can be a driving force for further CIN instability, most likely due to scaling differences of the chromosome segregation machinery. More importantly, the same study showed a direct correlation between CIN and the stoichiometric imbalance of certain chromosome pairs which harbor cell cycle checkpoint proteins which rely on direct interaction with each other to fulfill their functions (Zhu et al., 2012).

Because of this, we propose that an increase or decrease in the copy number of certain genes can cause a stoichiometric imbalance between the affected gene and its interacting partners. If said imbalance happens to a protein which is involved in chromosome segregation, the subtle change could be amplified by reducing the entire machinery’s functionality and therefore severely influence a cell’s chromosomal loss and gain rate.

To identify these specific genes which demonstrate a high impact on CIN due to copy number variation we designed a novel assay to measure the chromosome loss rate in budding yeast in a sensitive but high-throughput manner.

1.1 Chromosomal segregation defects as a pathway to aneuploidy

Chromosome segregation, in eukaryotic cells, is facilitated by the mitotic spindle apparatus which is a bipolar subcellular structure composed of several different proteins, most importantly the tubular polymers known as microtubules. To guarantee the accurate segregation of chromosomes, cells use a complex safeguarding mechanism known as spindle assembly checkpoint (SAC). In budding yeast, the genes that belong to the SAC signaling pathway were identified in two separate screens looking for mutants capable of
cell cycle progression even in the presence of drugs known to damage mitotic spindle components (Hoyt et al., 1991; Li and Murray, 1991). The identified conserved proteins, i.e. MAD1, MAD2, MAD3, BUB1 and BUB3, allow the cell to detect if the correct and stable attachment between the mitotic spindle structure and the chromosomes kinetochore region has been established before allowing the cell to progress through anaphase (Musacchio and Salmon, 2007).

Despite all the safety precautions that have evolved to increase chromosome transmission fidelity, it is still possible for errors to occur during chromosome segregations, even without the presence of any pre-existing defects. The chromosome loss rate of budding yeast in a diploid WT background lies between $10^{-7} - 10^{-5}$ per division, depending on the length of the chromosome which is lost, i.e. a longer chromosome has a smaller loss rate (Kumaran et al., 2013). Studies in human cell culture have shown that missegregation of chromosome VII occurs with a rate of $7 \times 10^{-3}$ (Cimini et al., 1999) while HeLaS3 cell lines have a chromosome loss rate ranging from $2 \times 10^{-4} - 4 \times 10^{-3}$ per division (Burns et al., 1999). Recent studies also identified the presence of chromosome missegregation in vivo and found that aneuploid cells can be a very common occurrence in certain tissues, as for example human hepatocytes that showed to have an aneuploid population ranging from 25-50% (Duncan et al., 2012).

Single gene defects affecting cellular mechanisms and thereby causing an increase in CIN have been identified using gene deletions as well as conditional mutations in budding yeast (Stirling et al., 2011). Current studies suggest that 692 of such "CIN genes" exist in the budding yeast genome of which about 40% function in expected pathways like mitosis, DNA replication and repair, while additional 20% were already known to be able to indirectly affect genome integrity i.e. transcription, nuclear transport and RNA processing (Stirling et al., 2011). The remaining 40% of genes are distributed throughout various cellular pathways of which some only recently have been linked to CIN e.g. iron-sulfur cluster defects (Veatch et al., 2009). CIN genes directly related to chromosome
misseggregation and the underlying mechanisms have been reviewed in great detail (Compton, 2011; Orr and Compton, 2013; Thompson et al., 2010). Most commonly, these defects interfere with (1) spindle assembly checkpoints, (2) cohesion complex formation and stability, (3) kinetochore–microtubule attachment (Thompson et al., 2010). Figure 1 as well as the following subchapters will illustrate and discuss these specific defects in chromosome segregation in more detail.

Figure 1: Pathways illustrating the involvement of chromosome misseggregation as a cause for a cell to enter an aneuploid state.

(a) Spindle assembly checkpoint defect, a compromised checkpoint signaling enables cells to enter anaphase even though not all kinetochores have been connected to microtubules. This may result in both copies of one chromosome being segregated into the same daughter cell. (b) Cohesin
complex defect, a premature loss of cohesin between sister chromatids can cause missegregation when the cell finally enters anaphase. (c) Incorrect spindle attachments, most commonly known as merotelic attachment, are attachments between one kinetochore and two microtubules from the opposite spindle poles. During anaphase these defects result in lagging chromosomes which can lead to chromosome missegregation or complete chromosome loss.

1.1.1 Mitotic checkpoint defects

The complete loss of the spindle assembly checkpoint is lethal for unicellular as well as multicellular organisms (Schvartzman et al., 2010) and therefore cannot cause the generation of aneuploid cells. On the other hand, not all the individual SAC genes have been found to be essential in budding yeast and a loss of one or two of them is very likely to have a negative impact on SAC signaling (Schvartzman et al., 2010). The loss of SAC genes results in a defective spindle apparatus which is prone to prematurely segregate chromosomes causing aneuploidy. Several studies have been done which confirm this effect for MAD1 (Iwanaga et al., 2007), BUB1 (Jeganathan et al., 2007), BUBR1 (Baker et al., 2004; Dai et al., 2004) and BUB3 (Kalitsis et al., 2000). Interestingly, the overexpression of the essential MAD2 gene did seem to delay the exit from mitosis, which caused the generation of lagging chromosomes in mouse embryonic fibroblasts and thereby the generation of aneuploid and tetraploid cells (Sotillo et al., 2007).

Further, MAD1 overexpression has been shown to cause aneuploidy by miss-localization of its binding partner MAD2, which was shown to weaken SAC signaling in various cell lines (Ryan et al., 2012). In addition, mutations in BUBR1 have been commonly identified in patients with mosaic variegated aneuploidy (MVA) syndrome (Suijkerbuijk et al., 2010) which can cause up to 25% of a cellular population to become aneuploid (Hanks et al., 2004).
1.1.2 Cohesion complex defects

The main role of the cohesin complex is to keep sister chromatids associated before anaphase, but in addition has been proven to be involved in gene transcription regulation as well as the DNA damage response (Nasmyth and Haering, 2009). A screen conducted in budding yeast, using conditional mutations, identified all of the cohesin core components, namely SMC1, SMC3, IRR1 and MCD1, as well as the related regulatory genes, SCC2, SCC4 and SMC4, all of which were also shown to increase chromosomal instability (Stirling et al., 2011). Further studies looking at the homologs of yeast CIN genes showed that mutations in colorectal cancer cells had an enrichment for genes implicated to play a role in sister chromatid cohesion, such as CSPG6, SMC1L1, NIPBL and STAG2 (Barber et al., 2008). Additional studies looking at STAG2 mutations, homolog of SCC3, showed that they occur in a wider range of tumors (Solomon et al., 2011) and that STAG2 inactivation can lead to defects in cohesin complex formation and aneuploidy (Xu et al., 2011). RAD21 mutations, homolog of MCD1, was also frequently found in different breast cancers and related to increased adaptability of cancer cells (Xu et al., 2011). Importantly, cohesin complex stability is essential for the prolonged arrest of oocytes in prophase I, a state which lasts several years. Multiple studies have shown that the increased age of oocytes corresponds to a continuous destabilization of the cohesin complex which results in a higher risk for chromosome missegregation, which would further be increased if the cohesin complex was defective from the beginning (Chiang et al., 2010; Lister et al., 2010; Revenkova et al., 2010).

1.1.3 Incorrect spindle attachments

A high risk factor for chromosome missegregation is the incorrect assembly of kinetochore-microtubule (K-MT) attachments. One prominent example are merotelic attachments, meaning that the same kinetochore is attached to two spindle microtubules from opposite poles, which has been recognized as a major contributor to aneuploidy
using mammalian cell culture (Cimini et al., 2001). Merotelic attachments cannot be recognized by the spindle assembly checkpoint (SAC) as they are only able to detect unconnected kinetochores. Therefore mitosis could still progress and likely result in missegregation due to lagging chromosomes (Crasta et al., 2012). In comparison, budding yeast does not show merotelic attachments, because only one microtubule attaches to one kinetochore, while in mammalian cells ~25 microtubules can attach to one kinetochore (Bouck et al., 2008). Correction of merotelic attachments was shown to be possible by the use of microtubule depolymerizing agents i.e. microtubule severing kinesin MCAK and Aurora B kinase (Cimini et al., 2003; Gregan et al., 2011). Stabilization of microtubules could therefore lead to an increase in merotelic attachments which was shown by RNAi knock-down of MCAK and the microtubule-depolymerizing Kif2b (Bakhoum et al., 2009). Centromere over-amplification is another common source for merotelic attachments as it increases the chance that microtubules from different spindle poles attach to a centromere located on the same chromosome (Ganem et al., 2009).

1.2 Cellular consequences caused by gene imbalance due to aneuploidy

While most commonly studied model organisms, such as human and mouse, are capable of regulating gene expression on sex chromosomes, their cells lack a mechanism of dosage compensation for genes located on autosomes (Nguyen and Disteche, 2006). Therefore, they are unable to react to sudden changes in gene copy number (GCN), present due to CIN, and accordingly cannot normalize gene expression levels. It has already been shown by us, as well as others, that after the gain or loss of individual or multiple genes the corresponding mRNA and protein levels reflect the changes in gene copy number. This is the case for example in S. cerevisiae (Pavelka et al., 2010; Torres et al., 2007), mouse cell lines (Williams et al., 2008) and human cell lines (Stingele et al., 2012).
Gene copy number alterations and the related gain or loss of proteins, independent of their size, are in general known to be detrimental for organisms which harbor these kind of deviations (Torres et al., 2008). The origins of several very unique diseases are known to be caused by copy number changes in certain individual genes (Girirajan et al., 2011). In addition, the loss or gain of so called oncogenes can lead to the formation of different types of cancer, influenced by the affected oncogene (Shlien and Malkin, 2009). In contrast, non-specific variations of larger chromosomal regions have been shown to be responsible for general phenotypes which are thought to be a consequence of the sum of multiple gene copy number changes. The phenotypes often associated with chromosomal copy number alterations are (1) cellular proliferation defects, (2) metabolic stress, (3) proteotoxic stress, (4) environmental stress response (ESR) such as gene expression, (5) and increase in the probability of further missegregation. Compared to these large scale changes which introduce dramatic differences in the karyotype and lead to the mentioned general phenotypes, is it very difficult to look at small individual changes of one gene and what such a change in their dosage could affect. To understand by which specific means aneuploidy causes such diverse cellular consequences, its effects on transactional and translational regulation and the subsequent changes in phenotype need to be studied in greater detail.

1.2.1 Transcriptional and translational changes caused by aneuploidy

The capability of aneuploidy to cause large scale consequences is mainly due to the direct correlation between gene dosage and the caused increase in its functional products, mRNA and protein. This concept has been known for years and states that the concentration of gene products directly correlate with the number of gene copies inside a cell (Epstein, 1986). Cells in a monosomic or trisomic state transcribe and translate each present gene copy with the same efficiency and therefore have 50% less or 50% more proteins, respectively, compared to the diploid state.
Many studies in budding yeast have concluded by using microarray that severe
gene dosage changes are often found in cells which became aneuploid due to the gain of
one or multiple chromosome copies (Pavelka et al., 2010; Rancati et al., 2008; Torres et
al., 2007). These studies did not only show that gene copy number predicts gene
expression levels but also that gene expression profiles could be used to determine the
karyotype of aneuploid strains. A previously performed large scale analysis of ~300
budding yeast deletion mutants had already shown that ~8% of the mutant strains became
aneuploid (Hughes et al., 2000). Studies performed using human cell culture showed
comparable results when examining aneuploidy and gene expression in colon- and uveal
cancer or human induced pluripotent stem cells (Hertzberg et al., 2007; Mayshar et al.,
2010). Furthermore, a study conducted in mouse embryonic fibroblasts, which had gained
a copy of chromosome 16, showed a 152% increase in expression of genes on that
particular chromosome (Williams et al., 2008).

An upstream event of this gene dosage concept is the increase in the expressions of
genes which are not located on a chromosome that was gained or lost in the aneuploid
state, but which is affected indirectly. Investigations into complex aneuploid patters in
budding yeast discovered that genes which underwent changes in gene expression were
not only on aneuploid chromosomes but were equally often found to be present in
chromosome in a euploid state (Rancati et al., 2008). A closer look revealed that up to
78% of these particular genes were the downstream targets of genes coding for
transcription factors that were present on aneuploid chromosomes. One very important
example in humans are trisomy 21 cells which show a two-fold overexpression of genes
not located on chromosome XXI but on chromosomes III, X and XVII (Guo et al., 2007).

The extent by which gene copy number alteration and corresponding change in
gene expression levels influence an organism, is highly dependent on the affected gene’s
location inside the genome, (e.g. on an autosome or sex chromosome) as well as possible
regulatory mechanisms which the organism has developed. In Drosophila cell culture, it
was found using DNA-Seq and RNA-Seq that 42% of autosomes and 17% sex chromosomes are considered segmental aneuploid (Zhang et al., 2010). Further, while gene regulation on autosomes was shown to be normalized by gene silencing in *Drosophila*, the silencing mechanism was not sufficient to fully compensate for the gene dosage defects.

While gene expression was shown to be directly correlated to gene copy variation in aneuploid cells, it was still unclear if the same correlation could be found when analyzing the concentration of the corresponding proteins. Using different approaches, recent studies have reported proteome changes in aneuploid budding yeast as well as human cell culture. Experiments in budding yeast using stable isotope labeling with amino acids in cell culture (SILAC) – mass spectrometry showed that proteins of genes located on the gained chromosomes V and XIII were enriched 1.8 - 1.9 fold (Torres et al., 2010). For strains with more complex karyotypes, multidimensional protein identification technology (MudPIT) was used, which detected an overall proportional increase of gene products present on the gained chromosomes (Pavelka et al., 2010). Additional analysis of both independent studies showed the indirect effect of aneuploidy on the protein concentration but only demonstrated a very small overlap when looking at the proteins found to be affected by copy number variation. This might be due to the different methods employed by the two different labs, since protein levels were measured by very different means, which may lead to the small overlap of results.

Human cell lines show a similar correlation between gene copy number and protein concentration while being less extreme as compared to RNA level changes in yeast. Using the SILAC assay in cells tetrasomic for chromosome V it was determined that the corresponding proteins increased by only ~1.6 fold (Stingele et al., 2012). The same results were observed for a haploid leukemia cell line, disomic for chromosome VIII (Burckstummer et al., 2013). This reduced correlation of gene copy number and protein levels in mammalian cells could mean that protein translation loses efficiency or that
extensive protein levels could degrade more rapidly under aneuploid conditions. An overview of how changing protein levels could affect cellular functions is shown in Figure 2.

**Figure 2:** Different mechanisms by which gene copy number variation (CNV) of certain genes can influence directly related or unrelated cellular functions.

(a) Variation in catalytic genes, changes in rate limiting enzymes (B) can increase or decrease the transformation from educt (A) to product (C). 

(b) Variation in structural genes, changes in structural genes of which the proteins (A&B) are required for the assembly of protein complex (C) can not only increase or decrease the amount of protein complex (C) but also lead to an increase in unused subunit proteins (B) which could act inhibitory on the overall complex assembly process.

(c) Variation leading to Off-target effects, increase in educt (A) can affect indirectly related pathways (D) unrelated to the transformation into the product (C).
1.2.2 Correlation between Gene Dosage-Sensitivity and Protein complex formation

Evidence supports a direct correlation between protein dosage imbalances and its influence on cellular fitness, shown in yeast by the direct connection of gene deletions and the involvement of the corresponding protein in macromolecular complex formation (Papp et al., 2003). According to this data, it is hypothesized that the overexpression of genes, through e.g. gene duplications, of certain subunits can lead to similar effects. It was also shown that gene pairs which encode interacting protein subunits have the same number of paralogs to avoid imbalance, and that larger gene families are less likely to code for subunits of macro complexes. The same was confirmed in a study conducted in human (Yang et al., 2003), which also found that the higher the number of subunits is in a complex the more likely it is that the subunits are encoded by a non-duplicated gene.

Examples supporting this hypothesis are provided by the yeast genes GPA1, STE4 and STE18 which encode the homologs of the mammalian G-protein α, β and γ subunits, respectively (Cole et al., 1990; Hamm, 2001). These three proteins form a heterotrimer of the X-Y-Z-type which is essential for the transduction of the mating pheromone signal in haploid cells. Overexpression of the bridge subunit STE4 (Y) did lead to an abnormal increase in pheromone signal transduction, caused by excessive amounts of the STE4-STE18 (Y-Z) subunit dimer, while an overexpression of STE18 (Z) had no such effect (Cole et al., 1990). Another potential example is the SNF/AMP-activated protein kinase which is involved in cellular stress response of budding yeast. Snf1 complexes consist of Snf1 as catalytic X-subunit, one of the three related Y-subunits GAL83, SIP1or SIP2 as bridge and SNF4 as the Z-subunit. Overexpression of any of the three bridge subunits showed not only a decrease in SNF1 activity but also an alteration of its subcellular localization (Hamm, 2001).

Thus, it is expected that over-expression of gene products cause disadvantages if the affected gene product is a subunit which occupies a specific position inside a macromolecular complex. The described mechanisms for the stochiometric imbalance of
protein subunits could therefore be explanatory for the effects caused by the gain or loss of gene copies for the more dosage-sensitive genes. An explanation of the significance of the position that the imbalanced protein occupies inside a multi-subunit complex is visualized in Figure 3.

**Figure 3: Overview of the effect that the concentration increase of a dosage-sensitive internal subunit has on the assembly of a multi-subunit complex.**

(a) The two-step assembly process of a three subunit complex (X-Y-Z) in the presence of equal levels of each individual subunit and under irreversible conditions. Due to the balance between all subunits the incompletely assembled complexes can react further with still present individual subunits to form the maximum number of completely assembled complexes. (b) Two-step assembly process of a three subunit complex (X-Y-Z) with a 50% increase in internal subunit Y levels, under irreversible conditions. The increase in the internal subunit Y increases the assembly of intermediate complexes with insufficient amounts of remaining complementary single subunits. Due to the irreversible nature of both reactions, the intermediate complexes cannot react further with each other and the level of fully assembled complexes will be reduced.
1.2.3 Phenotypic changes caused by aneuploidy

While aneuploidy gives rise to large scale transcriptional and translational changes, which can lead to extensive variations of a cell's transcriptome and proteome, it also leads to diverse phenotypes distinguishable by various cellular attributes.

Multiple studies have been conducted which focused mainly on proliferative defects in aneuploid cells under standard or unfavorable growth conditions. In induced pluripotent stem (IPS) cells as well as human embryonic stem (hES) cells, it was shown that an enrichment of cells with trisomy I, XII, XVII, and/or XX takes place (Mayshar et al., 2010; Olariu et al., 2010; The International Stem Cell, 2011), which is thought to be due to an increase in growth proliferating genes present on these four chromosomes. The same holds true for euploid mouse cells which develop complex cases of aneuploidy (Longo et al., 1997). Especially trisomy VIII cells were shown to proliferate faster in comparison to euploid cells (Liu et al., 1997). Human trisomy XI IPS cells on the other hand were observed to proliferate slower than euploid cells (Li et al., 2012). Similar observations were made in mouse embryonic fibroblast where trisomy I, XIII, XVI and XIX clearly showed to decrease proliferation efficiency of cells when compared to their original euploid background cells (Williams et al., 2008). These results indicate that proliferation efficiency is dependent on karyotype, species, growth conditions as well as the tissue type.

Another important discovery was made while observing the effects that aneuploidy has on cell proliferation in budding- or fission yeast (Niwa et al., 2006; Pavelka et al., 2010; Torres et al., 2007). Under normal growth conditions it was demonstrated that aneuploidy, in the majority of cases, provides a proliferative disadvantage while under unfavorable conditions caused by the presence of certain drugs, gave rise to some aneuploid karyotypes that are capable to outcompete euploid cells (Pavelka et al., 2010). Additionally, yeast trisomy XIII cells demonstrated an increase in the resistance against the mutagenic reagent 4-nitroquinoline-N-oxide (4-NQO). This resistance is very likely due to an increase in the transporter protein- ATR1, which can be also observed in the
pathogenic yeast species *Cryptococcus neoformans* (Ni *et al.*, 2013). Furthermore, this particular yeast karyotype can develop a resistance to fluconazole which is linked to gain of chromosome X (Sionov *et al.*, 2010).

Aneuploidy studies on an organismal level focus on differences in intelligence and lifespan between euploid and aneuploid organisms. In addition, comparisons of aneuploidies in human have to distinguish between aneuploidies affecting autosomes or sex chromosomes. Regarding this it has been shown that women with triple XXX (47, XXX) syndrome (Otter *et al.*, 2009) are nearly indistinguishable from euploid females except for a small decrease in intelligence when compared to individuals with down syndrome (trisomy 21), which have been shown to have severe defects in mental capacity (Dykens and Kasari, 1997). It has been shown that the life spans triple X females (Turner syndrome) show no significant difference compared to the euploid human population while people with trisomy XI show a decreased average life span of ~60 years in developed countries (Bittles *et al.*, 2007). The silencing capability of the X chromosomes (Ahn and Lee, 2008), called X chromosome inactivation (XCI) have been identified to be responsible for this difference in life span. This form of inactivation allows the cells to silence two of the three present X chromosomes while the imperfection of the silencing leads to the less severe phenotypes (Brown *et al.*, 1991). In euploid females several different forms of silencing mechanisms have evolved to compensate for gender specific diversity between chromosome numbers (Chaumeil *et al.*, 2011). This is thought to be the reason why sex chromosome aneuploidies are more easily tolerated.

Most importantly, it has to be mentioned that in multicellular organisms many karyotypes, including every loss of an autosome, leads to the death of the developing organism by spontaneous abortion (Nagaoka *et al.*, 2012). In humans only the trisomies of chromosome XIII, XVIII and XXI are viable but also cause an increase in the risk of pregnancy abortion. In comparison, unicellular organisms show a wider diversity of viable karyotypes. Especially budding yeast has been shown to not only tolerate but even benefit
from certain types of aneuploidy under various conditions (Zhu et al., 2012). Therefore, budding yeast is considered a very promising organism to study the effects of individual genes on chromosomal instability.

1.3 Screening methods to detect variation in chromosomal instability (CIN)

1.3.1 Previous CIN screens and identified CIN genes

For the past two decades several CIN assays employing different principles have been developed to measure chromosome instability in budding yeast. All of the previous CIN assays are based on certain morphological changes in yeast colonies which are triggered by the loss of certain markers unique in between different CIN assay methods (Stirling et al., 2011).

Three CIN assays are most common, namely (1) the chromosome transmission fidelity (CTF) assay which measures the loss of whole chromosomes, (2) the A-like faker (ALF) assay which also detects chromosome loss as well as rearrangements and gene conversion and (3) the gross-chromosomal rearrangements (GCR) assay which only detects terminal chromosomal deletions (Kanellis et al., 2007; Smith et al., 2004; Spencer et al., 1990; Yuen et al., 2007). With the help of these assays in combination with gene deletion or conditional gene deletion libraries 692 CIN genes have been identified so far (Stirling et al., 2011). Due to several restrictions and the very nature of the mechanisms behind the assays it has been impossible so far to conduct a quantitative and accurate CIN screen in a high-throughput format. These traditional CIN assays also cannot detect the effect that slight changes like a minimal gene copy number increase or decrease would have on a chromosomal loss rate. In case the traditional CIN assay were able to detect these changes it would still be a very labor intensive and time consuming approach to conduct a high-throughput screen with several hundred samples, not including replicates. Figure 4 provides a quick schematic to explain the mechanism behind the discussed traditional CIN assays.
Figure 4: Mechanisms behind three traditional chromosomal instability assays.

(a) Chromosome Transmission Fidelity (CTF), loss of SUP11, present on a chromosome 3 fragment, leads to the accumulation of red pigments due to a block in adenine production (ade2-101), after the loss of the mini-chromosome.

(b) A-like Faker (ALF), loss or deletion of the MATa locus, present on Chromosome 3, leads to differentiation into a MATa mating type which is able to mate and form colonies on selective medium.

(c) Gross Chromosomal Rearrangements (GCR), URA3 and CAN1 are inserted into a distal and non-essential part of chromosome 5, for which the loss of can then be determined by plating the cells on selective medium containing canavanine sulfate (CAN) and 5-fluoroorotic Acid (5'FOA).
1.3.2 High-throughput screen to identify Dosage-Sensitive Genes which affect CIN

Our lab has developed a novel screening method in *S. cerevisiae* by which we aim to identify novel gene candidates which when they experience a 1-fold copy number increase, show a significant increase or decrease in CIN.

The screen combines the natural mating type switch system of budding yeast with a fluorescent reporter system to create a natural and highly reliable system which indicates the loss of an introduced yeast artificial chromosome (YAC). In brief, an increase in the copy number of one individual gene by one would lead to an equal increase in the level of its corresponding protein. While the YAC, containing the Mat-α locus is present, the cell will act like an a/a cell and not express the a-type specific MFA1-3xGFP fusion-protein. If during cell division the YAC is lost, the cell will only have the Mat-α locus and it starts to express the a-factor MFA1-3xGFP which would result in a detectable fluorescent signal. We utilize flow-cytometry to detect the number of individual fluorescent cells in large cellular populations for a high-throughput screen.

Due to the specific and subtle nature of the introduced alterations we aim to gain information about subunits of protein complexes and corresponding cellular pathways which have a great impact on the cell’s chromosome loss rate.

1.4 Aims and Significance

Mechanisms to ensure genome stability, particularly ensuring the accurate segregation of a cell’s genomic content during mitosis, are absolutely vital for a cell’s long term persistence. Correct segregation of the chromosomes guarantees the inheritance of the cell’s individual karyotype and diminishes the appearance of possibly detrimental phenotypes. One such detrimental phenotype, important for multicellular organisms would be the emergence of cancerous cells which has been shown to frequently co-exist with aneuploidy, as a direct result of CIN. Many cancer cells have hallmark mutations which can be deletions or amplifications of genes which then result in the gain of cancerous
properties, including CIN. Considering the impact that under- or overexpression of one particular protein can have on a cell’s functionality, here with focus on CIN, it is of vital importance to identify and characterize the proteins which, when they experience such subtle changes, can result in severe phenotypic differences.

Aneuploidy, a product of CIN, itself has recently been shown to be able to affect CIN but the mechanism by which this happens is still not fully determined. One hypothesis is that aneuploidy can lead to copy number variation (CNV) of dosage-sensitive genes which are involved in chromosome segregation. For testing this hypothesis we established the protocol for a novel CIN assay in budding yeast, which allowed us to detect the effects of CNV for individual genes in a high-throughput manner. After establishing the new CIN assay we used it to conduct two different screens, both based on minimal copy number variations, to ask two different questions.

First, which are the genes that affect CIN when their dosage is increased? Using the molecular barcoded yeast open reading frame (MoBY-ORF) library, which includes each gene with its native promoter and terminator, we transformed the centromeric plasmids individually into our reporter strain. We then observed its effect on the missegregation rate of the strain specific mini-chromosome.

Second, which are the haploid-insufficient genes that affect CIN? For this we combined our mini-chromosome reporter strain with a collection of strains which are heterozygous for non-essential genes in budding yeast. We will again observe its effect on the missegregation rate of the strain specific mini-chromosome.

Our results will include genes which by an increase or by a decrease in copy number affect CIN. Thereby identified gene candidates can then be further analyzed and compared to the known CIN genes from previous screens.
2. Results

2.1 Development of the novel high-throughput iCTF assay in budding yeast

The development of the improved GFP-based chromosome transmission fidelity (iCTF) assay allowed us to bypass the many limitations that traditional CTF assays have. Specifically, the mechanisms employed by the iCTF assay allow for a more reliable, sensitive and, very importantly, a high-throughput compatible way to conduct our screen. To have a reliable system we utilized the budding yeast’s own two haploid mating types, MATa and MATα. Both mating types are defined by the presence of the MATa or MATα locus, respectively, which enables the expression of certain mating type specific genes, while at the same time inhibits the expression of genes specific for the opposite mating type. In addition a diploid cell with both mating types switches off the expression of MATa and MATα specific genes and starts to express MATa/α specific genes. In our system we added the MATα locus into the right arm of an artificial mini-chromosome which we then transformed into a haploid MATa strain. The strain was therefore haploid but had both the MATa and MATα present and therefore did not express MATa and MATα specific genes. Inside the haploid strain we modified the MATa specific MFA1 gene to contain a 3xGFP at the c-terminal end. MFA1 was used as the MFA1p shows the highest protein concentration in comparison with all the MATa specific genes (Breker et al., 2013). In the presence of the mini-chromosome, expression of the MFA1-3xGFP fusion protein would, like the other MATa specific genes, not be possible as the MATα locus suppresses all MATa gene expression. If the mini-chromosome, and thereby the MATα locus, are lost due to a chromosome missegregation event, the MFA1-3xGFP gene would be transcribed leading to the presence of MFA1-3xGFP fusion protein and the corresponding bright fluorescent signal. The mechanism is illustrated in Figure 5a.

While in theory, other genetic changes, different from the loss of the mini-chromosome, could result in a defect of the reporter system this is very unlikely due to the specific structure of the mini-chromosome. The mini-chromosome consists of the original
chromosome III short left arm and the only the MATα locus with the selective marker as the right arm (Spencer et al., 1990) making it highly telocentric. In addition, the mini-chromosome’s loss rate is $2 \times 10^{-4}$ per cell division (Chen et al., 2012), which is higher as the estimated $3 \times 10^{-6}$ per cell division chance for a mutation to occur inside the reporter genes. Furthermore, it was also shown that the presence of the mini-chromosome does not affect the proliferation efficiency for several tested strains (Spencer et al., 1990a) which allowed us to give an accurate quantitative measurement when considering the presence of essentially two different cell populations, with and without the mini-chromosome.

The sensitivity of the system is due to that fact that the phenotypic switch of budding yeast from MATa/a to MATa happens in less than one cell cycle phase, which is in average 90 - 120 min (Evdokimov et al., 2006) depending on the growth conditions. MATa specific proteins where shown to be rapidly degraded by the proteasome shortly after the MATa locus was lost (Laney et al., 2006). On the other hand, the generation of MATa specific proteins, was quickly facilitated as well as the generation of the 3xGFP fluorescent reporter gene which takes about ~60 min (Evdokimov et al., 2006). Therefore, the switch between mating types and the generation of the fluorescent reporter protein is very unlikely to suffer from phenotypic lagging and sensitive enough to be quickly detectable by flow-cytometry in a quantitative fashion. The green fluorescent cell population as shown by flow-cytometry with selection for the mini-chromosome (A) and without the selection of the mini-chromosome (B) is shown in Figure 5b.

To calculate the mini-chromosome loss rate, also referred to as CIN rate, we measured the OD$_{600}$ to determine the cell number and the percentage of GFP-positive cells inside a larger cell population at two subsequent time points. Using this information as well as the basal level derived from the control strain we used the function shown in Figure 5c to calculate the CIN rate.

For validation of the iCTF assay we first measured the CIN rate of a wild type strain with or without centromeric empty MoBY-ORF plasmid. The calculated basal loss
rate without the blank plasmid was $1.96 \pm 0.06 \times 10^{-4}$ per cell division, which was very
similar with our previous result of $2 \times 10^{-4}$ per cell division when using a traditional colony
based assay (Chen et al., 2012). For the the control strain with the empty plasmid we
observed a 1.5 fold increase in CIN rate, seen in Figure 5d, which demonstrates first, that
even one additional centromere harbored inside the plasmid can affect CIN and second
that the iCTF assay is sensitive enough to detect these subtle changes. The fact that the
presence of one additional centromere increases the CIN in such a dramatic way also shows
that the chromosome segregation machinery has, without any additional influence, a
limitation when it comes to the chromosome workload that it can successfully segregate (Zhu
et al., 2012).
Figure 5: Basic concept behind the modified yeast strain and the evaluation of the improved GFP-based chromosome transmission fidelity (iCTF) assay.

(a) Natural MATα yeast strain with indicated modifications, (1) MFA1-3xGFP fusion-protein integrated in endogenous Chr. IV, and (2) yeast artificial chromosome (YAC) containing the Mat-α locus which suppresses the expression of MFA1-3xGFP and the LEU-marker for selection. Loss of the YAC results in the accumulation of MFA1-3xGFP inside the cell which in turn makes the cell detectable using flow-cytometry. (b) Example of flow-cytometry results showing an increase in GFP+ cell populations between a cell population grown under selective conditions for the mini-chromosome (A, 0.046% GFP+) and without selective condition for the mini-chromosome (B, 0.247%). (c) Formula used to calculate the chromosomal loss rate using the difference between both cell populations and the number of generations. (d) Comparison between mini-chromosome loss rate for only the background strain and the background strain containing the empty MoBY-ORF plasmid. Mean ± SEM, Unpaired Student's t-Test, p<0.0001, n=7.
2.2. Identification of genes that affect CIN in an over-dosage dependent manner

To determine the effect that genes have when increased in copy number in our over-dosage (OD)-CIN screen we utilized the MoBY-ORF library which contains ~4956 ORFs with their natural promoter and terminator. This covers about ~90% of all the Open reading frames (ORFs) currently annotated in the Saccharomyces Genome Database (SGD) when it was established (Ho et al., 2009). Due to the fact that the MoBY-ORF plasmid is a centromeric plasmid it would cause an increase in gene copy number by 1-3 fold, depending on the number of plasmids inside the cells. The individual centromeric MoBY-ORF plasmids, each containing a different ORF, were transformed in a high throughput manner into our modified haploid yeast strain with an efficiency of 88%, resulting in 4389 genes. The modified strain for the OD-CIN screen and the process are shown in Figure 6a. The CIN rate distribution of all genes showed a narrow peak located around the CIN rate of the empty control plasmid which confirmed that the majority of genes, when increased, have no significant effect on CIN, shown in Figure 6b. It was also observed that outlier genes with a large difference to the control plasmid were more commonly detected and successfully validated when showing an increase in CIN compared to a CIN decrease. The same holds true when comparing the distribution pattern of the various MoBY-ORF plasmids to the distribution of 60 control plasmid replicates, as seen in Figure 6c. The control strain samples were present inside each 96-well block and taken into account when calculating the chromosome loss rate.
Figure 6: Modified yeast strain, experimental procedure and results of the Overdosage chromosomal instability (OD-CIN) screen in *S. cerevisiae*.

(a) Haploid a/α yeast strain with indicated modifications, importantly (1) MoBY-ORF plasmid containing one additional gene copy with natural promoter, (2) MFA1-3xGFP a-factor fusion-protein integrated in the endogenous Chr. IV, and (3) the mini-chromosome III containing the Matα locus which suppresses the expression of MFA1-3xGFP. For the screening process the modified strain with a specific MoBY-ORF plasmid undergoes two subsequent growth phases in different selective media. This generates two separate cell populations representing (1st) the basal level in
selective- and (2nd) the level of cells that lost the mini-chromosome without selection, to calculate the individual chromosomal loss rate. (b) Diagram showing the distribution pattern of all 4389 genes (n=1 per gene) with the majority of genes showing to be distributed around the loss rate of the empty control plasmid (~0.00025). Dashed line indicates position of control plasmid Jz666. (c) Diagram showing the comparison between the loss rate of the empty control plasmid and the loss rate distribution pattern of all 4389 genes (n=1 per gene) from the MoBY-ORF library compared to n=60 replicates for the control plasmid Jz666, Mean ± SEM. (d) Chromosomal instability rate of the 36 validated CIN gene candidates and the MoBY-ORF control plasmid 666 arranged from low to high chromosomal instability rate. Mean ± SEM, n=11 or 12 for the MoBY-ORF plasmids and n=24 for the control plasmid Jz666. Unpaired Student's t-Test, p<0.0001 Arrow indicates location of control plasmid Jz666. The known CIN gene MAD1 is the highest hit in the over-dosage CIN screen.

After the primary screen of the whole yeast genome was conducted we picked the genes with the top100 highest and the top100 lowest chromosomal loss rates based on one experimental sample. Thus, we continued with 200 genes which we validated by two subsequent validation screens, the first with eight and the second with 12 biological replicates, respectively. After both validation screens had been finished we were left with 33 high confidence gene candidates. The actual final distribution pattern for the validated gene candidates is presented in Figure 6d, which gives the actual ranking, taking each gene’s averaged chromosomal loss rate into account.

One of the major findings is that the iCTF due to its quantitative nature and high accuracy can detect genes which decrease CIN. Out of 100 low CIN genes that we picked for validation we confirmed that 3 of them actually have the capability of leading to a significant decrease in CIN with an increase in copy number. This seems to be the first study which is able to detect this particular phenotype and we decided that it is of high importance to follow up on these particular genes as well as performing the CIN screen for genes under haploid insufficient conditions.
2.3 Identification of haploid insufficient genes that affect CIN

To determine the effect that genes have when decreased in copy number by our haploid-insufficient (HI)-CIN screen we utilized a yeast MATα Knock-Out gene library containing ~4786 ORFs for non-essential genes. Our CIN yeast strain was mated with the library collection to produce the diploid CIN strain containing the deletion as well as the mini-chromosome. The HI-CIN screen took place utilizing the same experimental procedure as the previous OD-CIN screen but it will show the effect a copy number decrease can have in a diploid background with only one functional gene copy remaining. After the primary screen of the whole yeast genome was conducted we again picked the genes with the top 100 highest and the top 100 lowest chromosomal loss rates based on one experimental sample. Thus, we continued with 200 genes which we validated by only one validation screen, with eight biological replicates each. After the validation screen had been finished 154 high confidence gene candidates remained.

The results indicated that the loss of one copy of certain genes can reduce CIN, similar to the results observed in the OD-CIN screen. Out of the 100 low CIN genes that we picked for validation we confirmed that three of them actually have the capability of leading to a significant decrease in CIN when increased in copy number. The binned distribution pattern for the validated gene candidates is presented in Figure 7b, which gives the range of CIN for the validated genes, taking into account the average from the biological replicates. We did a ploidy analysis for all the validated hits to detect if some of them had entered an aneuploid state. Aneuploid strains would obviously have to be excluded from our gene candidate list since due to the huge genomic variation beside our specific gene deletion. Out of the 154 strains we found 15 strains to contain various aneuploidies which were excluded. Therefore, only 139 gene candidates remained. The gene deletion inside the aneuploid strains and their ploidy level are shown in Figure 7c.

To identify possible overlaps between our gene candidates as well as previously known CIN genes from gene deletion screens we created a Venn diagram including these
three populations, shown in Figure 7d. The diagram indicates a very small overlap between all three groups. This shows that our CIN gene candidates haven’t been detected by previous CIN screens and are so far unknown to have an influence on CIN.

Figure 7: Schematic of the modified yeast strain, the experimental procedure and results of the HI-CIN screen as well the ploidy analysis of all HI strains and the a visualization of overlapping hits between our screens and the known deletion screen.
(a) Diploid α/α yeast strain with indicated modifications, importantly (1) Gene deletion of one of the two present gene copies, (2) MFA1-3xGFP α-factor fusion-protein integrated in the endogenous Chr. IV, and (3) the mini-chromosome III containing the Mat-α locus which suppresses the expression of MFA1-3xGFP. For the screening process the modified strain with a specific gene deletion undergoes two subsequent growth phases in different selective media. This generates two separate cell populations representing (1) the basal level in selective- and (2) the level of cells that lost the mini-chromosome without selection, to calculate the individual chromosomal loss rate. (b) Binned chromosomal instability rate of the 140 validated CIN gene candidates and the control strain arranged from low to high chromosomal instability rate. Arrow indicates the location of the background strain used as control (~0.000075). n=7 or 8 for the haploid-insufficient genes and control background strain. (c) Excluded strains with gene deletions, found to differ in ploidy in relation to their CIN rate distribution. Yellow bars indicate the CIN rate and blue dots indicate the ploidy. (d) Venn diagram showing the overlaps between our two performed screens (1) over-dosage CIN in blue and (2) haploid-insufficient CIN in yellow with the previously performed Deletion-CIN screen in green, performed with traditional CIN assays by the Hieter lab (Stirling et al., 2012). MAD1 is the only gene present in all three screens.

2.4 GO-Term network analysis for biological processes of gene candidates

After conducting both the OD-CIN and HI-CIN screens and validating the potential hits we combined both gene candidate populations to detect if certain types of genes are shown to be enriched. We therefore analyzed the 175 gene candidates using a Gene Ontology (GO) -term network analysis program which plots the enriched GO-terms for cellular processes in a network pattern while also indicating the most significantly enriched Go-terms. Using this method we identified three different enriched GO-term groups in our overall population of gene candidates. The three populations shown to be slightly enriched consist of GO-terms well known to be connected to chromosomal instability.

The first and largest group shows general GO-terms indicating the involvement of the gene candidates during mitosis and cell division. The second group is already greatly diminished in size but indicates the enrichment of genes acting during cell component- and spindle pole organization. The third and last enriched GO-term is showing an involvement of genes influencing the Cyclin-dependent kinase activity during cell cycle. All these three
groups consist of GO-terms that are highly expected to have a connection to chromosome segregation and subsequently could cause defects in chromosomal instability when affected by copy number alteration. While this does not give the involvement of a very specific or unexpected process it shows that our screen succeeds in its main function which is to detect dosage-sensitive genes which affect CIN.

**GO-Term Analysis for Biological Processes**

![GO-Term Network Analysis](image)

Figure 8: GO-Term network analysis of 175 gene candidates identified in the over-dosage and haploid-insufficient CIN screens to define enriched biological processes. The GO-Term network analysis shows which biological processes are enriched in the validated gene candidate populations which were confirmed to significantly affect CIN in the OD-CIN or HI-CIN screen. The color scale indicates the significance of the GO-terms with yellow nodes being of less significance and orange nodes being of higher significance. Three distinct populations were magnified and the GO-terms added. These three populations contain GO-terms
which indicate that the genes are majorly involved in (I.) Mitosis, including Cell cycle phases and Nuclear Division, (II.) the organization of cellular components, with additional emphasis on the spindle pole body and (III.) Cyclin-dependent protein kinase activity. The GO-term analysis which is shown was performed using the free software Cytoscape Version 3.0.1. in combination with the free Application BINGO Version 2.4.4 (Maere et al., 2005).

2.5 Testing dosage dependent effect of gene candidates shown to decrease CIN

We followed up on two validated CIN gene candidates, NPL3 and MCD1 from the OD-CIN screen, which surprisingly showed to decrease CIN.

NPL3 and MCD1 were shown to effect CIN in a dosage-sensitive manner when one additional gene copy was present, but it had to be determined if the observed phenotype still persists when several additional gene copies are added. To obtain more information about the effect that an increase in the copy number of these two genes has on CIN we inserted different copy numbers of NPL3 or MCD1 directly into the CIN strains genome at the original gene position. After identifying strains with increases in copy numbers by qPCR we repeated the iCTF assay comparing these strains to the WT strain. The increase in copy number of NPL3 or MCD1 caused two different types of behavior, shown in Figure 9a. For NPL3, we could see a further decrease in CIN when three NPL3 copies were present. After this every further copy kept increasing the CIN but our highest copy number of five NPL3 genes still would not show a recovery of the CIN rate to the basal level. For MCD1, we observed a strong initial decrease when two copies of MCD1 are present which stays relatively constant with up to six MCD1 copies. Interestingly, the strain with our highest MCD1 copy number of ten shows a very strong decline in CIN which is the lowest chromosomal loss rate that we detected.

To further dissect possible mechanisms that lead to a decrease in CIN we utilized two different approaches to specifically look at NPL3 and MCD1.

NPL3 is an RNA-binding protein which carries poly(A)-mRNA from nucleus to cytoplasm, promotes elongation, and regulates transcription termination (Kress et al.,
2008). To determine if NPL3’s interaction with RNA plays a role in decreasing CIN we used mutants, obtained from Pamela A. Silvers lab (Lee et al., 1996), which had different kinds of point mutations in NPL3’s two mRNA binding domains. Comparing the chromosomal loss rate we could identify two mutants, NPL3-1 and NPL3-48, leading to an increase in CIN, shown in Figure 9b. Both of these mutants have point mutations in NPL3’s RNA-binding domain 2 and show miss-localization of NPL3. This could indicate that the RNA-binding domain 2 in NPL3 is necessary for decreasing CIN.

MCD1 is a main subunit of the cohesin complex, which holds together sister chromatids and has to be cleaved for sister chromatid separation during mitosis and meiosis. The increase in MCD1 could lead to a more stable cohesion complex and stability between sister chromatids which could decrease CIN. To see if MCD1’s function in cohesin complex formation is responsible for decreasing CIN, we increased the copy number of separase ESP1 in a yeast strain. ESP1 is the enzyme which cleaves the cohesin complex of which MCD1 is a subunit. Our results show that independent of the presence of additional MCD1, an increase in the separase copy number causes a tremendous increase in CIN bringing it up to the same loss rate as without additional MCD1. Due to the strong effect of additional separase we are unable to determine by this experiment if additional MCD1 decreases CIN by an increase in the formation or the stability of the cohesin complex.
Figure 9: Changes in chromosomal instability caused either by an increase in the gene copy number, the introduction of mutated genes (for NPL3) or the introduction of antagonistic genes (for MCD1).

(a) Changes in chromosomal instability rate after integrating multiple copies of NPL3 (blue; left side) and MCD1 (red; right) into the genomic DNA. The total copy number of the corresponding gene is given at the end of sample name indicated by X for times. (b) Changes in chromosomal instability after adding a URA-plasmid containing different mutated copies of the NPL3 gene in addition to the WT NPL3 copy. (c) Changes in chromosomal instability due to the addition of two different plasmids, containing either the MCD1 gene candidate, the ESP1 gene coding for the separase enzyme or as being empty and acting as a control plasmid.
3. Discussion

In this study, we demonstrated the developmental path and performance of a novel method, named improved GFP-based chromosome transmission fidelity (iCTF) assay, which allowed us to observe the loss rate of an artificial mini-chromosome in budding yeast. It does so by detecting GFP-expression, induced by loss of the mini-chromosome, of single cells that can be counted using a flow-cytometer. This allowed us the analysis of large cell populations (~200-300k) of several hundred different samples (~5k) in a high-throughput manner. The mechanism behind this new method, in comparison to older more conventional CTF assays, made it possible to perform two yeast whole-genome screens to identify individual genes which affect chromosomal instability (CIN) when their copy number is increased or decreased. The iCTF assay is sensitive enough to detect the impact that a small change in gene copy number can have on CIN, not only the increase in CIN but also a decrease in CIN. Furthermore, the iCTF assay is sensitive enough to detect the CIN increase caused by the presence of one additional centromere located on a plasmid. This data further proves the earlier results from our lab (Zhu et al., 2012), which showed that CIN can be influenced by scaling differences between the chromosome segregation machinery and the load of centromeres which have to be separated.

Due to the mentioned points we are confident that the iCTF assay is superior to previously applied assays that detect CIN. Further, the iCTF can be adapted for detecting drugs which affect CIN, not only in a negative but potential positive manner as well as screen through environmental samples to detect substances able to induce aneuploidy. Additionally, the iCTF can be modified to investigate not only for the loss rate of an artificial chromosome but to detect the loss rate of endogenous chromosomes, which would give great insight into the relation between chromosomal properties and the chromosome missegregation rate.

Analyzing the results obtained by both our screens, we make two observations. First, there is only a very slight overlap in hits between our assay as compared to
previously performed CIN assays with deletion strains. Second, our validated hits are enriched for cellular processes known to directly affect CIN. With regards to the small overlap between the hits from this screen and previously performed screens, we must take into consideration the different natures of the screens as well as their limitations. The previously performed deletion CIN screen was performed using traditional methods and used gene deletions and temperature sensitive mutants by which the gene and its products were completely removed from the system (Stirling et al., 2011). Thereby, the screen was not aimed to find dosage-sensitive genes but genes whose complete absence would increase the CIN rate. The complete deletion of genes is more likely to cause detrimental effects as cells might be able to handle an increase of gene products but are unable compensate for the complete absence of certain important genes. This could lead to the cell’s death or make them sick which would exclude them from the list of possible hits that can be picked up by the screen. In contrast, our screen observes the effects of gene copy number changes which are less likely to cause these severe defects and is also relevant in the light of cancer cells which are still able to survive and proliferate with gene copy number imbalances.

For our two screens, we used two different libraries which did not contain every ORF present in the yeast genome. For example the gene MAD2 is absent from the MoBY-ORF library, MAD2 interacts with our top hit MAD1 and is known to lead to CIN. Furthermore, additional genes were lost during the set-up of our CIN screen due to unsuccessful transformations or mating. For both screening procedures one of the main disadvantages is the limited number of replicates of each sample for the primary whole genome screen. This means that each gene is initially only tested once for its CIN rate. If the first sample does not show a significant change in CIN during the screening process or the strain preparation, it will be neglected and won’t be repeated in the follow up screen. Therefore, some hits could be lost during the screen. Any of these reasons listed above which account for differences between our set and previously identified CIN genes could
also be applied to explain the reason why our GO-term analysis showed enrichment in cellular processes that were also shown in previous CIN gene studies. One has to consider that while both screens are testing for genes affecting CIN they are looking for two different mechanisms by which this effect takes place. The known CIN genes are mostly genes which require deletion to influence CIN, while in our screen a gene only requires a copy number increase or decrease to reach the same effect. While genes can be present in both populations they don't have to be but can still act in the same cellular pathway or even be present as a subunit in the same macromolecular complex. It has to be considered that so far we have only performed the iCTF assay relying on a yeast artificial chromosome (YAC). For the future it would be an essential step to validate our candidate genes using the loss of different endogenous natural chromosome or maybe even repeating the whole genome screen.

After developing and validating the iCTF assay, our lab was able to perform a screen which, for the first time, looked at the effect that an individual increase in gene copy number can have on CIN. Thereby we identified 33 genes which caused an increase in CIN of which 6 genes, namely MAD1, VIK1, CLB3, RFA1, TAF1 and GLC7, were known from previous screens to have the same effect when deleted. This gave us confidence that our screen fulfilled its role while at the same time brings up the questions about specific mechanisms which could be affected in a dose-dependent manner by the gain or loss of these proteins. Even more importantly, the first screen resulted in the identification of 3 proteins, NPL3, MCD1 and STE4, which actually decrease CIN when their copy number is increased. We followed up on two of these genes, NPL3 and MCD1, which we expected to have different mechanisms by which they are able to decrease CIN.

First, NPL3 codes for an RNA binding protein which is involved in pre-mRNA splicing and the transport of poly-A mRNA from the nucleus into the cytoplasm (Bossie et al., 1992; Kress et al., 2008; Lee et al., 1996). It has been previously shown that its deletion decreases yeast artificial chromosome (YAC) stability (Wahba et al., 2011) with
the hypothesis that without it, the concentration of RNA in the nucleus might increase which might also increase the formation of DNA:RNA hybrids. These DNA:RNA structures, called R-loops, have been shown to lead to DNA damage which is known to be a source for genomic instability (Aguilera and García-Muse, 2012). Recently it has been shown that it is the direct involvement of the NPL3 heterogeneous ribonucleoprotein particle (hnRNP) complex that inhibits R-loop stabilization and thereby decreases genome instability (Santos-Pereira et al., 2013).

Second, MCD1 codes for the essential alpha-kleisin subunit of the cohesin complex which facilitates sister chromatid condensation (Guacci et al., 1997). For meta- to anaphase transition the cohesion complex must be cleaved (Xiong and Gerton, 2010) which requires the proteolysis of MCD1 by the separase ESP1 (Ciosk et al., 1998). Presumably, the increased expression of MCD1 could extend the time needed by ESP1 to completely cleave MCD1 and therefore increase the time between meta- to anaphase transition, which could improve accurate chromosome segregation.

Both NPL3 and MCD1 are very different from each other when comparing their cellular functions. To get an idea about the mechanism by which they actually affect CIN, we introduced multiple gene copies into the genome and repeated the iCTF assay to see how the increased dosage further affects CIN. With NPL3 we observed further a steady decrease in CIN even after adding two additional gene copies, three copies in total. Everything exceeding three gene copies reversed the phenotype and lead to an increase in CIN, which reached the basal CIN level with five copies of NPL3 present in total. Considering that NPL3 hnRNP binds RNA and stabilizes it before is exported out of the nucleus we assumed that its interaction and binding to RNA is involved in its mode of action by which it affects CIN. To test this we used multiple NPL3 mutants with point mutations inside its RNA binding domains. We cloned these mutant copies into a plasmid and used the iCTF assay to determine whether any of the mutants no longer show any decrease in CIN. We would thereby not only test our hypothesis but also possibly identify
the specific binding site which is essential for decreasing CIN. Two mutations increased the CIN in contrast to the WT NPL3 decrease in CIN. The point mutations in these two NPL3 copies are located in the same mRNA binding site and have also been shown to lead to NPL3 protein miss-localization and enrichment in the cytoplasm (Lee et al., 1996). To determine if both the mutations in the mRNA binding domain as well as the NPL3 miss localization are connected and how they lead to a sudden increase in CIN has to be determined by future studies.

For MCD1 we made the surprising observation that with each additional copy inside the genome we detected a steady decrease in CIN. Even with a very high number of ten MCD1 copies present inside the genome we detected a CIN decrease. Considering our previous hypothesis about the increased time it would take ESP1 to cleave the additional MCD1 it would be logical to assume that an increase in ESP1 would decrease the MCD1 phenotype and lead to an increase in CIN in comparison. To test this we inserted additional copies of ESP1 into the genome to see if there is a correlation between the ratio between MCD1 and ESP1 copies and in which way it will affect CIN. The addition of only one additional copy of Esp1 proved to tremendously increase the CIN and we were therefore unable to specifically determine if the ratio between MCD1 and ESP1 is important. It could still be that the ratio between both genes is not linear but that multiple copies of MCD1 could counteract the single copy number increase of ESP1.

4. Summary and Conclusion

Our results clearly demonstrate that the novel improved GFP-based chromosome transmission fidelity (iCTF) assay developed by our lab not only fulfills its intended purpose but in addition surpasses our expectations with regards to speed and sensitivity.

We performed two different whole genome screens in yeast followed by validation screens with biological replicates. The whole screening procedure only took ~6 months and the
workload could be managed by one to two researchers. With regards to sensitivity, the iCTF assay is the only assay so far to be able to detect a decrease in chromosomal instability. Furthermore, were we able to detect an increase in CIN rate caused by the presence of a single centromeric plasmid. The identified and validated gene candidates were shown to be enriched in cellular processes which are known to be directly related to changes in CIN. While we did not identify a link between a cellular process unknown to affect CIN, we can say that this is a further indicator that our screen successfully performed its given function. In addition, this shows that processes known to have the most severe impact on CIN are dosage sensitive and a slight change in copy number can significantly affect the chromosome segregation machinery and thereby CIN. Following up on two candidate genes NPL3 and MCD1, which were identified to decrease CIN, we confirmed the observed phenotype occurs when gene copy number alterations were located inside endogenous chromosomes. Additional experiments, conducted to dissect the specific mechanisms by which NPL3 and MCD1 decrease CIN have so far not yielded definite results but we will continue to test different possibilities.

This study shows that slight CNVs of certain genes, are capable of not only increasing but also decreasing CIN. This does support our hypothesis that the imbalance for certain dosage sensitive-genes can in fact have very severe consequences for cellular functions, such as CIN. It is therefore possible to assume that the same is true if these kind of CNVs are due to chromosomal imbalances in case of aneuploid cells. Therefore, aneuploidy should not only be considered a direct result of CIN but recognized as an important source for CIN itself. With the results shown here we identified an important mechanism by which aneuploidy can give rise to further genomic variation and lead to an increase in cellular diversity, especially important with regards to aneuploid cancer cells.
5. Materials & Methods

5.1 Construction of plasmids and strains for High-Throughput CIN screen

The multiple plasmids and strains that were facilitated during both CIN screens, gene-copy-number-increase and the haploid-insufficient, were constructed as follows.

5.1.1 Construction of the yeast reporter strains JZY569 and JZY657

The MATα locus was inserted into the pRS305 vector, containing a LEU2 marker. Together the MATα locus and LEU2 marker sequence were amplified using PCR and transformed into YPH278 (Spencer et al., 1990) to replace the SUP11 and URA3 sequence on the present mini-chromosome. The resulting strain was then mated with the RLY2626 strain, which is modified to have the MATα specific MFA1 gene tagged with 3XGFP-HIS5, a strong fluorescent reporter. After inducing sporulation, the meiotic progeny were screened for showing the specific HIS+, LEU+ and ADE2 growth phenotype, resulting in the desired JZY569 strain. To further generate the JZY657 strain the MATα locus of the JZY569 strain was deleted using the natMX module contained in the pFA6a-natMX plasmid (Longtine et al., 1998).

5.1.2 Construction of MoBY-ORF Control plasmid JZP666

The MoBY-ORF control plasmid JZP666 was constructed as described previously (Ho et al., 2009). I short, the KanMX4 module was PCR amplified from the pFA6a-KanMX4 plasmid and subsequently co-transformed into the RLY2626 WT strain together with the Xhol-linearized p5472 plasmid as backbone. Yeast colonies growing on YPD-G418 media plates were used to inoculate SC-complete + G418 liquid medium from which the plasmid was recovered using a MasterPure Yeast DNA Purification Kit (tebu-bio, MN: MPY80200). The isolated plasmid was then transformed into the bacterial host strain BUN20, resulting in the bacterial strain JZB666. For confirmation the JZP666 plasmid was isolated from the bacterial JZB666 strain using the GenElute™ Plasmid
Miniprep kit (Sigma Life Science, MN: PLN350-1KT) and subsequently sequenced to confirm the presence of the UPTAG- (TATTTACGCGGGAGACTCGT) and DOWNTAG- (ATACACGTCGAAGGAGTGCC) barcode.

5.1.3 Construction of strains for the dosage-increase (OD)-CIN screen

The MoBY-ORF library collection was transformed in a high-throughput manner into the previously constructed JZY569 yeast strain in a 96-well format using the BioMek FX Laboratory Automation Workstation/Biomek Software (Beckman Coulter, MN: 717013). Briefly, JZY569 strain was inoculated into 200ml YPD liquid medium and incubated overnight (O/N) at 30°C/250rpm in a Multitron Infors Shaking Incubator (ATR Biotech, MN: 80120805BC2). After ~12h the OD\textsubscript{600} was measured using an Ultrospec 3100 pro spectrophotometer (Amersham Biosciences, MN: 80-2112-31) and diluted to an OD\textsubscript{600} of 0.1 using fresh YPD liquid medium. The cell culture was further incubated at 30°C/250rpm for 3h after which the cell culture was transferred into 50ml Centrifuge Tubes with screw caps (VWR, MN. 21008-178) and spun down at 3000rpm for 3min using an Allegra X-22R centrifuge. Supernatant was aspirated, the pellet was washed twice with 5ml ddH\textsubscript{2}O, once with 20ml of 0.1M Lithium acetate (LiOAc) and then resuspended in 1.25ml 0.1M LiOAc. The prepared yeast cell suspension was then added to 1.875ml 1M LiOAc, 0.625ml ddH\textsubscript{2}O, 2.5ml of 2mg/ml denatured/sheared salmon sperm DNA, and 12.5ml 50% polyethylene glycol (PEG) 3350. For each well 100µl yeast transformation mix was distributed into a 96-well half skirt PCR plate (Phenix Research Products, MN: MPX-96M2), after which 10µl of MoBY-ORF plasmid was added and repeatedly mixed using the BioMek FX Laboratory Automation Workstation/Biomek Software. The 96-well half skirt PCR plate containing the transformation mix was then covered with breathable Airpore Tape Sheets (Qiagen, MN: 19571) and heat-shocked at 42°C/220rpm for 1h using a Multitron Infors Incubator. After heat shock the PCR plate was spun down at 2500rpm for 5min using an Allegra 25R Centrifuge (Beckman Coulter,
MN: 425752) and the supernatant was aspirated. Pellets were resuspended in 15μl ddH₂O, re-sealed with breathable Airpore Tape Sheets and shaken for 5min at 1500rpm using an Eppendorf Mixmate (Eppendorf, MN: PCB-11). From the resuspended wells 7μl were spotted onto SD-URA agar plates. SD-URA agar plates were grown at 30°C in a low temperature 815 Precision Incubator (Thermo Electronic Corporation, MN: 3721) for 1 day. Transformants were picked with a 96 Solid Pin Multi-Blot Replicator (V&P scientific MN: VP407) and used to inoculate 100μl SD-URA liquid medium in an untreated 96-well flat bottom with lids microplate (Evergreen Scientific, MN: 222-8030-F1K). The cell culture was incubated overnight at 30°C/220rpm using a Multitron Infors Incubator and then mixed with 100μl of 50% glycerol to make a saturated library stock. Prepared untreated 96-well flat bottom w/lids microplates were then sealed using Seal & Sample Aluminum Foil Lids (Beckman Coulter, MN:536619) and stored until use in a Revco Ultima II -80°C Freezer (Kendro Laboratory Products, MN: Ult2586-9-A38).

5.1.4 Construction of strains for haploid insufficient (HI)-CIN screen

JZY657 cells were pinned in quadruplicates from 96-well plate glycerol stocks onto SC-Leu + CloNat (100μg/ml) agar plates using the Singer RoToR HDA Robot (Singer Instruments, MN: RO7026-100Y) with 96 long Pin Repads (Singer Scientific, MN: RP-MP-2L). The MATa haploid yeast Knockout (KO) collection was pinned from 384-well plate glycerol stocks onto YPD+ with G418 (200μg/ml) agar plates using the Singer RoToR HDA Robot with 384 long Pin Repads (Singer Scientific, MN: RP-MP-3L). Both strains were then incubated for 2-3 days at 30°C in a low temperature 815 Precision Incubator (Thermo Electronic Corporation, MN: 3721). When the colonies reached a sufficient size, JZY657 cells and MATa haploid KO cells were combined on YPD+ agar plates using the Singer RoToR HDA Robot with 384 short Pin Repads (Singer Scientific, RP-MP-384). The cells from the JZY657 strain were pinned onto the YPD+ plates first followed by the cells of MATa KO strain on top. The cells were then incubated
at 30°C for overnight (O/N) in a low temperature 815 Precision Incubator. Following incubation, diploids were selected for by pinning from the YPD+ plates onto SC-Leu + CloNat (100μg/ml) + G418 (200μg/ml) agar plates using the Singer RoToR HDA Robot with 384 short Pin Repads. The cells were then incubated at 30°C for 1-2 days. After incubation, the cells were transferred into an untreated 96-well flat bottom with lids microplate (Evergreen Scientific, MN: 222-8030-F1K) containing SC-Leu + CloNat (100μg/ml) + G418 (200μg/ml) liquid medium using the Singer RoToR HDA Robot with 96 long Pin Repads. The 96-well microtiter plates were sealed with breathable Airpore Tape Sheets (Qiagen, MN: 19571) and allowed to grow for 1-2 days at 30°C/220rpm in a Multitron Infors Shaking Incubator (ATR Biotech, MN: 80120805BC2). From the resulting cell suspension were 100μl transferred into 100μl 50% Glycerol (1:2 Dilution) inside a new 96-well microtiter plate. Prepared untreated 96-well flat bottom w/lids microplates were then sealed using Seal & Sample Aluminum Foil Lids (Beckman Coulter, MN:536619) and stored until use in a Revco Ultima II -80°C Freezer (Kendro Laboratory Products, MN: Ult2586-9-A38).

5.2 High-throughput chromosomal instability (CIN) screening procedure in yeast

The procedure for both the OD-CIN and HI-CIN screen facilitate the same mechanism with small adjustment that will be indicated at the end of the description.

5.2.1 Over-dosage (OD) -CIN and haploid-insufficient (HI) -CIN screening process

For the Over-dosage (OD)-CIN screen, 30μl of glycerol stock were used to inoculate 1.5ml of SD-Leu-Ura medium (1:50 Dilution) in a 2.2ml Deep Well Plate, Sterile PP Wells W/Conical Bottom (PHENIX Research Products, MN: M1810S) using a Biomek FX Laboratory Automation Workstation/Biomek Software (Beckman Coulter, MN: 717013). The 30μl of control strain JZY666, containing the empty MoBY-ORF plasmid, were manually pipetted into a well which was empty due to unsuccessful
transformation. Subsequently the 2.2ml Deep Well Plate containing the cell suspension was sealed with AeraSeal Sterile Microporous Sealing Film (PHENIX Research Products, MN: LMT-AERAS-EX) and incubated for 24h, attached to a TC-7 Rotor (New Brunswick Scientific, MN: 500484410) set to speed level 10, inside a low temperature 815 Precision Incubator (Thermo Electronic Corporation, MN: 3721) at 30°C. After the 1st growth phase, the block was mixed for 5min using an Eppendorf Mixmate (Eppendorf, MN: PCB-11) set to 1400rpm and 23μl of cell suspension was diluted in 207μl SD-Leu-Ura medium inside a 96-well Non-Tissue Culture plate with Flat-bottom and Low-Evaporation Lid (BD Falcon®, Model-No. 351172). The 96-well plate is shaken for 3min at 1000rpm using an Eppendorf Mixmate and the OD_{600} of each strain was measured on a SpectraMax M2 Multimode Microplate reader (Molecular Devices, MN: DE05224). After this, 30μl of the cell suspension was diluted into 1.5ml SC-Ura medium (1:50 Dilution) inside a new 2.2ml Deep Well Plate using the Biomek FX Laboratory Automation Workstation. The remaining 200μl of cell suspension inside the 96-well plate were spun down using an Allegra 25R Centrifuge (Beckman Coulter, MN: 425752) set to 3000rpm for 3min and the supernatant aspirated by the Biomek FX Laboratory Automation Workstation. After that the cell pellets were resuspended in 200μl 4% paraformaldehyde (PFA), shaken for 3min at 1000rpm using a Eppendorf Mixmate and fixed for 15min at room temperature (RT). The fixed cells were then washed twice by centrifugation using the Allegra 25R Centrifuge set to 3000rpm for 3min, aspirating the supernatant with the Biomek FX Laboratory Automation Workstation and resuspending them in 200μl PBS pH7.4. For the last wash the fixed cells were spun down using the Allegra 25R Centrifuge set to 3000rpm for 3min, resuspended in 250μl PBS pH7.4 and shaken on the Eppendorf Mixmate for 3min at 1000rpm. The plates containing the fixed and washed cells were then stored at 4°C in the dark for flow-cytometry analysis. The new 2.2ml Deep Well Plate with the inoculated the SC-URA was again sealed with AeraSeal Sterile Microporous Sealing Film.
and incubated for 24h, attached to a TC-7 Rotor set to speed level 10, inside a low temperature 815 Precision Incubator at 30°C.

After the 2\textsuperscript{nd} growth phase, the previously described screening process was repeated with the only exception being that no new 2.2ml Deep Well Plate was inoculated and the current 2.2ml Deep Well Plate was stored in a Revco Ultima II -80°C Freezer (Kendro Laboratory Products, MN: Ult2586-9-A38) for later sequencing confirmation.

For the Haploid Insufficient (HI)-CIN screen, the same process was followed except for the fact that the cells were first grown in SC-LEU liquid medium and then in SC-Complete liquid medium.

5.2.2 Flow-cytometric analysis of screening samples

Fixed cells contained inside the 96-well Non-Tissue Culture plate with flat-bottom and low-evaporation lid (BD Falcon®, MN: 351172) were transferred into a 96-well MatriTube\textsuperscript{TM} Storage Plate with clear tubes (Matrical Bioscience, MN: MSP096-A) and covered with a SonicMan\textsuperscript{TM} Pinned Lids (Matrical Bioscience, MN: SL0096-P19-SS). The 96-well MatriTube\textsuperscript{TM} Storage Plate was then sonicated for 20secs at 50% power using a SonicMan HT-sonication instrument (Matrical Bioscience, MN: SCM1000). Sonicated cells were then transferred back into an untreated 96-well flat bottom with lids microplate (Evergreen Scientific, MN: 222-8030-F1K) and placed on the MACSQuant Analyzer (Miltenyi Biotec, MN: 130-092-197) to count the number of GFP-positive cells in the overall cell population. For both screens 125\mu l of mixed cell suspension was ran per sample with the difference that for the OD-CIN screen we counted 0.2mio events while for the HI-CIN screen we counted up to 0.3mio events in total. Data obtained from the MACSQuant Analyzer was then analyzed using FlowJo software 7.6.5 (Tree Star) to calculate the percentage of GFP-positive cells. The previous OD\textsubscript{600} readings combined with the difference in GFP-positive cell population was entered into Excel2010 (Microsoft Office, Vers. 14.0.7106.5003) to calculate the chromosomal instability rate.
5.2.3 Statistical evaluation and exclusion of candidate CIN genes

After both primary whole genome CIN screens, including the MoBY-ORF Library for the over-dosage CIN screen and the deletion library MATa haploid yeast Knockout (KO) for the haploid-insufficient CIN screen, the 100 genes with the highest- and the 100 genes with the lowest chromosomal loss rate were picked from each screen for validation. The subsequent validation screens included 8 biological replicates for the first and 12 biological replicates for the second validation screen. Candidate genes were excluded when the unpaired Student's t-test indicated no significant difference (p>0.05) between the compared data sets. The final data is presented as column diagrams with the corresponding Mean±SEM for the sample populations, the sample size n, and the determined p-value.

5.2.4 Isolation and sequence confirmation of MoBY-ORF Plasmids

2.2ml Deep Well Plates, sterile PP wells with conical bottom (PHENIX Research Products, MN: M1810S) were thawed at room temperature (RT). The complete 1.5ml contained in the well was transferred to a 2ml Eppendorf Safe-Lock Tubes™ (Eppendorf, CN: 0030 120.094). Plasmid isolation was performed according to the standard protocol for liquid culture of the Zymoprep™ Yeast Plasmid Miniprep II kit (Zymo Research, CN: D2004) with two important changes. First, the centrifugation of the culture was done for 3min at 13200rpm in a Eppendorf Centrifuge 5415D (Eppendorf, CN: 022621408) and second, instead of the zylomylase incubation at 37°C the cell pellet resuspended in Solution 1 was bead beaten for 10min using 50μl of 0.6mm acid washed glass beads (Sigma-Aldrich, CN: G8772-10G) on a Fisher Vortex Genie 2 (Fisher Scientific, CN: 12-812). The ORF region of the isolated plasmid was sequenced using the standard MoBY-ORF primers for the 5' (ACGTTTCAGACGTATCATCAGTACATCACGAGACTACTA) and 3' (ATGTTACTTACCACATCACGATAGGTCTCAGATC) position.

5.3 Manual transformations of individual plasmids into *S. cerevisiae*
To transform plasmids into budding yeast we inoculated 5ml of YPD liquid medium with a single yeast colony and incubated it O/N at 30°C/230rpm in a Multitron Infors Shaking Incubator (ATR Biotech, MN: 80120805BC2). The culture was then diluted up to 50ml (1:10 Dilution) with fresh YPD liquid medium and incubated for 4h at 30°C/230rpm. Salmon sperm DNA was boiled for 10min in boiling water and then put on ice for 5min. Cells were centrifuged for 3min/3000rpm. The cell pellet was washed with 1ml of 1M Lithium Acetate and transferred into a 1.5ml tube. Cells were centrifuged for 3min/3000rpm. The cell pellet was resuspended in 1 volume of 1M Lithium Acetate. Transformation vials were prepared mixing, (1) 12ul cell suspension, (2) 5ul boiled salmon sperm DNA, (3) ~200-500ng plasmid DNA and (4) 45ul PEG50%. Samples were incubated for 1h at RT, after which 6ul 60% Glycerol was added followed by 1h additional incubation at RT. Cells were heat shocked at 45°C for 10min in a water bath. The cells were then transferred to selective agar plates and incubated at 30°C for 24-48h.

5.4 Increasing the gene copy number inside the genome with qPCR confirmation

To integrate additional copies of candidate genes into the genome we cloned the corresponding gene from our MoBY-ORF plasmid by PCR amplification into pRS306, an integrating plasmid with a -URA3 marker. The resulting plasmid was transformed into home-made Top10 bacterial cells, of which colonies were picked and amplified in 2xYT + Amp liquid medium. Plasmid isolation was performed using the GenElute™ Plasmid Miniprep kit (Sigma Life Science, MN: PLN350-1KT) followed by sequencing with the standard T3 (GCAATTAACCCTCACTAAAGG) and T7 (TAATACGACTCACTATAGGG) primers to determine the presence of the inserted gene. The plasmid was then digested using a restriction enzyme which specifically only cuts once inside the middle of the gene sequence (XhoI or SacI in this case) and transformed into the JZY659 yeast strain, containing the mini-chromosome. The transformed cells were plated on SD-LEU-URA plates and grown at 30°C in a low
temperature 815 Precision Incubator (Thermo Electronic Corporation, MN: 3721) for 2 days. After incubation, 18 individual colonies were picked and amplified in SD-LEU-URA liquid medium from which the genomic DNA (gDNA) was extracted using the standard protocol of the Masterpure™ Yeast DNA Purification Kit (epicenter, MN: MPY80200). The qPCR analysis, to determine gene copy number variation, was done in two steps. First, 3 different primer pairs for the gene of interest were validated for their specificity using haploid wild type (WT) gDNA and including ACT1 as endogenous control. Haploid WT gDNA was diluted in series (1:5 Dilution) ranging from 100ng/μl to 0.0064 ng/μl and mixed with 5μM Forward + Reverse primer mix inside a Microamp® Optical 384-well Reaction plate with barcode (Applied Biosystems, MN: 4309849) using a CAS-4200 Liquid Handling System (Corbett Robotics, CAS4200). The prepared Microamp® Optical 384-well Reaction plate with barcode was sealed with MicroAmp Optical Adhesive Films (Applied Biosystems, MN: 4311971), spun down for 15sec and analyzed with 7900HT Fast Real-Time PCR system (Applied Biosystems, MN4329002).

Second, the primer pair and gDNA concentration giving the best peak specificity was chosen for the actual copy number determination. gDNA from each of the 18 colonies and one haploid WT was combined with the previously determined most specific primer pair as well as 4 endogenous controls, namely ACT1, TUB1, CDC28 and ZWF1. The Microamp® Optical 384-well Reaction plate with barcode, containing 4 replicates for gDNA with each primer combination, was prepared with the CAS-4200 Liquid Handling System. The prepared Microamp® Optical 384-well Reaction plate with barcode was sealed with MicroAmp Optical Adhesive Films, spun down for 15sec and analyzed with 7900HT Fast Real-Time PCR system. The analysis of the gene copy number was done using the Biogazelle qbase PLUS 2.6 system.
5.5 GO-Term network analysis of validated candidate genes

For the GO-term network analysis we used the free software Cytoscape (Version 3.0.1.) in combination with the free Application BINGO (Version 2.4.4.). In brief, the cytoscape program was opened in new network setting and BINGO was selected from the application section. Validated candidate gene candidates, from both screens, were imported using the “Paste genes from text option” and “Overrepresentation” and “Visualization” was selected as output settings. The following standard settings of the program were selected for the analysis, (1) Binomial statistical test, (2) Bonferroni Family-Wise Error Rate (FWER) correction, (3) 0.05 Significance level, (4) Overrepresented categories after correction, (5) Use whole annotations as reference set, (6) GO_Biological_Process, and (7) Saccharomyces cerevisiae. Node module and p-value significance were visualized using a yellow (5.00E-2) to red (5.00E-7) scale bar. The node labels were removed for non-significantly enriched terms and the whole network was exported as a .png file (Maere et al., 2005).
6. Contributions

The contribution of individual researchers towards this project is detailed below. The order of contributions follows the order of the materials and methods section.

The construction of the reporter and control strains JZY569, JZY657, and JZP666 was performed by Dr. Jin Zhu. The formula used for calculating the chromosomal loss rate was derived and implemented by Dr. Boris Rubinstein. The high-throughput transformation of the MoBY-ORF plasmids and high-throughput mating with the Knockout collection were performed by Dan Bradford and Scott McCroskey. The parameters used for GFP detection in yeast were configured by Jeff Haug and Andrew Box. The primary CIN screen for the over-dosage genes, including the evaluation by flow-cytometry, was performed by Dr. Jin Zhu and Wahid Mullah. The first and second confirmation screen for the over-dosage genes as well as the primary and confirmation screen for the haploid-insufficient genes, including the evaluation by flow-cytometry, was performed by Dominic Heinecke. The individual isolation, sequencing, and transformation of the MoBY-ORF plasmids, was performed by Dominic Heinecke. The increase of gene copy number, involving the transformation and qPCR analysis, was performed by Dominic Heinecke. The GO-term network analysis, using the programs Cytoscape and Bingo, was performed by Dominic Heinecke.
7. References


correction is achieved by two different mechanisms. Journal of Cell Science 116, 4213-4225.


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