THESIS

TWO MODEL SYSTEMS FOR STUDYING THE EFFECTS OF ACUTE RADIATION EXPOSURE ON GENE DELETIONS AND AMPLIFICATIONS

Submitted by

Rabab S Sharif

Department of Environmental and Radiological Health Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Fall 2014

Master's Committee:

Advisor: Michael Weil
Co-Advisor: Juan Lucas Argueso

Douglas Thamm

ABSTRACT

TWO MODEL SYSTEMS FOR STUDYING THE EFFECTS OF ACUTE RADIATION EXPOSURE ON GENE DELETIONS AND AMPLIFICATIONS

Ionizing radiation (IR) poses a severe threat to genome integrity, and is an important source of environmental damage, arising from naturally occurring sources (e.g. radon and cosmic radiation) and medical imaging and therapy. Radiation exposure can lead to somatic changes in chromosomal structure such as copy number alterations (CNAs) resulting in gain or loss in copies of sections of DNA. To study copy number alterations in the human genome resulting from gamma radiation, early passage cultures of normal human fibroblasts were exposed to a single acute 4 Gy dose of radiation. Irradiated cells were kept for 48 h to allow repair of initial DNA damage. Single cell cloning was done by serial dilution in 96 well plates. Standard PCR was performed using seven sequence tagged site (STS) markers (SY 83, SY86, SY88, SY1190, SY1191, SY1201, and SY1206) of the azoospermia (AZF) region in the Y chromosome to test for microdeletions, in irradiated and non-irradiated cells. The comprehensive analysis of the molecular mechanism of copy number changes, requires a more elaborate experimental system in a model organism. Hence, we also investigated copy number alterations in diploid budding yeast cells after exposing them to two acute gamma radiation doses and detecting CNAs via a unique selection system, that involves events at two chromosomes. The copy number selective system used in our yeast samples allowed us to select for copy number alterations (duplications and deletions) in all samples after exposure to radiation, which lead to nonreciprocal translocation events formed by nonallelic homologous recombination (NAHR) mechanism.

These results lead us to conclude that acute exposures to gamma radiation, induced deletions and amplifications as shown in both models. The experiments described in the thesis provide a platform for future work aimed at investigating the role low dose ionizing radiation on genome stability.

ACKNOWLEDGEMENTS

"The teacher who is indeed wise does not bid you to enter the house of his wisdom but rather leads you to the threshold of your mind" *Khalil Gibran*

There are many individuals whom I am grateful to, but first I would like to acknowledge and extend my heartfelt gratitude to Dr. Weil for his continued support and inspiration throughout my time here at Colorado State University. He has not only been my mentor along with Dr. Argueso but also my guide into the world of radiation cancer biology, with his vast knowledge and experience, I am grateful to have had such an opportunity to work under his supervision. As for Dr. Argueso, he has shown me that it is possible to bring my dreams to reality. Through his energy and commitment to teaching, he has enabled me the opportunity to bring the world of medicine and marry it with the world of research, allowing for an outstanding outlook on the possibilities of knowledge and technology. Thank you both!

I would also like to express my gratitude to Dr. Thamm for his enormous support. He has continuously offered his assistance in various ways, whether by providing his expertise, or by always expressing positive encouragement. I am grateful to Dr. Genik who always made herself available with her time and assistance, whether I had come to her for advice or to request help with a task. I would also like to acknowledge the Radiation Control Office: Jim, Joe, Ronda, Christy, Sue, your help was greatly valued. And to the staff of the ERHS department, especially Julie Asmus, Jeanne Brockway, Chris Loucks, for their help in navigating my academic pathway. Finally I would like to extend my gratitude to my fellow graduate students in both the Weil, and Argueso Lab for their assistance. And a special thanks to Brock, for his support, as a colleague and a friend his assistance in the lab was tremendous.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS

^{0}C	degree Celsius
%	percentage
bp	base pairs
Gy	Gray
Kb	kilobases
µl	microliter
min	minute
mM	millimolar
M	molar
mL	milliliters

LIST OF ACRONYMS

CAN	Copy Number Alteration
CNV	Copy Number Variation
DNA	Deoxyribose Nucleic Acid
DSB	Double-Strand Break
SSB	Single-Strand Break
HR	Homologous Recombination
NAHR	Nonallelic Homologous Recombination
IR	Ionizing Radiation
NHEJ	Nonhomologous End-Joining
PCR	Polymerase Chain Reaction
FBS	Fetal Bovine Serum
YPD	Yeast Peptone dextrose
AZFc	Azoospermia Factor-c
STS	Sequence Tagged-Site
FCR	Formaldehyde Copper Resistant
YNB	Yeast Nitrogen Base

CHAPTER ONE

INTRODUCTION

1.1. RADIATION AND COPY NUMBER ALTERATIONS

Contrary to general assumptions, it has been shown that DNA is not a very stable molecule. When it comes to cancer and genetic diseases, genomic instability is an essential factor, characterized by an increased rate of acquisition of alterations in the human genome [1]. Ionizing radiation (IR) is an important source of environmental damage, arising from cosmic radiation, other naturally occurring sources (e.g. radon) and intensive cancer therapy. Radiation exposure leads to somatic mutations such as copy number alterations (CNAs) which cause somatic changes to chromosomal structure that result in gain or loss in copies of sections of DNA and are prevalent in many types of cancer. This instability is observable in a large collection of endpoints that include amplification of genetic material, an increased rate of chromosomal rearrangements or aberrations, microsatellite instability, gene nucleotide sequence mutation and aneuploidy.

1.2. CHROMOSOMAL REARRANGEMENTS

Chromosome structural changes are among the major genetic instabilities induced by ionizing radiation, and can be responsible for inherited as well as sporadic traits [2]. Serving an evolutionary function similar to base pair changes, rearrangements introduce variation into the genome for natural selection to act upon. These rearrangements may cause Mendelian diseases, or represent benign polymorphic changes. While Watson–Crick DNA base pair changes are well-known as a mechanism for mutation, rearrangements of the human genome (including deletions, duplications or inversions) have only been considered more recently as a major source of genetic variation. Deletion and duplication mutations may vary in size - from thousands to

hundreds of thousands or millions of base pairs in length - and may require the use of specialized technologies to be visualized. The architecture of the human genome may result in region-specific sensitivity to rearrangements [3].

1.3. DEFINING DNA COPY NUMBER ALTERATIONS

Alterations in DNA copy number, whether confined to specific genes or affecting whole chromosomes have been identified as causes of diseases and developmental abnormalities. DNA copy number alterations are referred to by various terms, depending on their size. Variations smaller than 500 kbp are called submicroscopic alterations, and microscopic alterations when they're higher than 500 kbp Between 1 bp and 1 kbp in size, they are categorized into insertions or deletions, depending on whether DNA is gained or lost [4], [5]. Sub-microscopic DNA copy number alterations that are between 1 kbp and 1 Mbp in length are referred to as copy number variations (CNVs) [6].

1.4. COPY NUMBER VARIATIONS (CNVs)

Copy number variation (CNV) is a primary determinant of genetic variation among many (maybe all) living species, including humans. Normally, humans carry two copies of most genes, one from each parent. However, errors may occur during meiosis and mitosis, and result in deletions and duplications of genes, which can then lead to disorders. By definition, CNVs are deletions or duplications of 50 bp to over a mega-base [7], resulting in an abnormal number of copies of one or multiple regions of DNA. Copy number variant formation occurs by both replication-based and recombination-based mechanisms; de novo locus-specific mutation rates also appear to be much higher for CNVs than they do for nucleotide mutations. In 2004, thanks to the investigation of DNA content through genome-wide analysis tools, two studies [8],[9] revealed that CNVs are widespread in human genomes and serve as a powerful source of genetic

variation. Since then, with the help of genome-wide, high-resolution tools, including array comparative genomic hybridization (aCGH) and next generation whole genome sequencing platforms, over 38,000 CNVs (>100 bp in size) and a variety of structural variations, such as balanced inversions and translocations, have been characterized [10].

Moreover, thorough examination of the genomic content of certain CNVs revealed that these genomic regions feature many functional genes involved in regulating cell growth and metabolism, hence implicating CNVs in human diseases, traits, and evolution [11]. Therefore, a significant number of CNVs are associated with human diseases such as Charcot–Marie–Tooth syndrome, Williams syndrome, autosomal dominant leukodystrophy, autism-related and other neurodevelopmental disorders, as well as several cancer predispositions [12].

The most compelling evidence showing that CNVs are linked to cancer predisposition is the fact that a lot of the affected genes have been involved in the tumors themselves previously [13]. A good illustration is given by the study of Tang and colleagues [6], It focuses on a family with a history of melanoma, which entails a rare 4q13 duplication. Even though this duplication was only observed in this one family, it distinguishes melanoma in all three affected patients.

In another CNV case related to breast and colorectal cancer, two separate studies reported a similar 9p21.3 germline microdeletion affecting the *KIA1797* and *MIR491* genes, which confirms a pathologenic role in cancer predisposition. In addition, the burden of large (>500 kbp) CNVs is most prevalent among cases of intellectual disability [14], and decreases for schizophrenia, bipolar disorder and autism [15]. Certain conditions (e.g. dyslexia) show no indication of increased rates or burden of CNVs. It follows that for “less acute” adult phenotypes (e.g. bipolar disorder), de novo CNVs may be smaller in size, affecting fewer genes or manifesting as an excess of duplications. It is recognized that certain CNVs are much more diverse in their

outcome, having been associated with a wide range of phenotypes, and that the progression to intellectual disability among pediatric cases associates with a significant excess of additional CNVs, so-called “second hits” [7]. Hence, it is probable that a subset of schizophrenia and bipolar disorder are elements of a spectrum of neurodevelopmental diseasein which the effects of both de novo and inherited events are additive.

1.5. COPY NUMBER ALTERATIONS AND CANCER

Genome copy number alterations (CNAs) exist in nearly all tumor genomes[16]. Such alterations can indicate the genomic instability of a tumor and are the result of acquired somatic mutations in the evolution of the tumor cells from a normal state to a neoplastic state. The identification of somatic copy number alterations (SCNAs), whether it's observing the deletion of tumor suppressors or the amplification of oncogenesin tumors, has proved an invaluable tool in cancer research and has therefore prompted the development of several new methods for their analysis [17]. Shlien and collegues [18] reported excessive genomic CNAs in Li-Fraumeni syndrome (LFS), which is an autosomal dominant disorder characterized by increased risk of various cancers in individuals with germline TP53 mutations. In addition, the analysis of 371 lung adenocarcinoma samples through a 250,000 probe array [19] identified seven recurrent homozygous deletions and 24 recurrent amplifications. In addition, the identification of a subset of head and neck squamous cancers (HNSCC) that exhibited epidermal growth factor receptor (EGFR) copy number alterations, in the form of small deletion in EGFR exons 18 to 21 indicates a role of these events in HNSCC [20]. The most widespread type of structural variation in the genome has been linked to germ-line CNV, according to recent studies [21], [9]. In the case of CNVs entailing deletions, close to 30% of highly penetrant cancer-predisposing genes have been affected, including mismatch repair genes, as well as *APC, BRCA1, BRCA2, TP53 and SMAD4*

[22]. Other recent studies [23], [24] have highlighted the role of germ-line gains and losses of large DNA segments in the predisposition of humans to colorectal and prostate cancer, *BRCA1*-associated ovarian cancer as well as neuroblastoma. It is thought that the loss of homologous recombination by inactivation of *BRCA1* results in inappropriate repair of double-strand DNA break via nonhomologous end-joining and single strand annealing, which leads to genomic instability through increased deletions and translocations [25].

1.6. COPY NUMBER VARIATION: MECHANISMS OF FORMATION

Although their fundamental mechanisms of formation are not fully understood, duplications and deletions in genes frequently are associated with four major mechanisms: Nonallelic homologous recombination (NAHR), nonhomologous end-joining (NHEJ), fork stalling and template switching, and retro transposition. These mechanisms are responsible for structural variations in humans [26]. NAHR involves the alignment of and subsequent crossover between two nonallelic DNA sequence repeats sharing high similarity to each other which may occur in meiosis, and by the segregation of marker genotypes it arises in unequal crossing over, and induces constitutional genomic rearrangements that may be benign polymorphisms or manifest sporadic *de novo*, or inherited genomic disorders [27]. In mitosis, NAHR can result in mosaic populations of somatic cells carrying copy number alterations, such as cancer cells [28]. In addition, transposable element insertions that contain regions of dispersed highly repetitive DNA sequences, and other low copy repeats (LCRs) are extremely predisposed to frequent rearrangements caused by NAHR [29]. Nonhomologous end-joining is promoted by human cells in order to repair DNA double-strand breaks (DSBs) caused by ionizing radiation or reactive oxygen species and for physiological V(D)J recombination. In contrast to NAHR, NHEJ does not require substrates with extended homology [30]. Rearrangements induced by fork stalling

and template switching that are described as microhomology-mediated break-induced replication (FoSTeS/MMBIR) vary in complexity and size and result from errors during DNA replication [27]. Results from the 1000 Genomes Project have shown that 70 to 80% of deletions resulted from NHEJ or FoSTeS/MMBIR, while large deletions or duplications were induced by NAHR [29].

1.7. COPY NUMBER VARIATION AND MUTATION RATES

The estimates for CNV locus-specific mutation rates range from 1.7×10^{-6} to 1.0×10^{-4} per locus per generation [5]. Distinct from the relatively constant mutation rates of SNPs that initially arise via rare spontaneous mutations (approximately 10^{-8} per base pair per generation), mutation rates of CNVs may broadly vary at different loci, probably mirroring the differences in CNV formation mechanism and genomic architecture inciting genome instability.

1.8. COPY NUMBER VARIATION AND ENVIRONMENTAL EXPOSURES

1.8.1. Ionizing radiation

The rate of copy number variation formation increases in response to a wide range of DNA damaging agents and genotoxins. Although the mechanisms by which these agents act are not fully understood, DNA damage such as double strand breaks are initiators that trigger the mechanisms that can lead to chromosomal rearrangements involved in CNA formation. This damage can be the result of environmental exposures such as ionizing radiation, or intrinsic cellular factors. Previous studies [31] showed that at least two chemical agents that cause replication stress via impairment of replication fork progression, hydroxyurea (HU) and aphidicolin (APH), result in *de novo* CNVs by template switching and microhomology-mediated break-induced repair (MMBIR).

A previous study by [32], in which normal human fibroblasts irradiated with acute doses of 1.5-3 Gy, showed induced *de novo* CNVs via mechanisms similar to that in CNVs induced by APH and HU, and are often located within the same hotspot regions. IR might induce CNVs in at least two distinct ways. First, the DSBs mainly responsible for the cytotoxicity of IR might actually be the direct substrates of CNV formation via an end joining process. However, several factors seem to suggest this is not the most likely explanation for IR-induced CNVs. Additionally, if random DSBs were the key intermediate, it remains to be seen why they would lead to the identical nonrandom genomic distribution of CNVs as replication stress. Moreover, it is difficult to explain the prevalence of copy number gains with an end-joining repair mechanism. Therefore, classical NHEJ, which is expected to act in the repair of IR DSBs, has been excluded as a deciding factor when it comes to CNV formation after replication stress in mouse ES cells [33], even though alternate end-joining remains a conceivable mechanism of formation for at least the deletion CNVs. In contrast, IR might induce CNV formation via secondary effects, which result from the more abundant non-DSB type of lesions, including base lesions and SSBs, or from alterations in the cell state resulting in altered replication or repair function [32]. Previous conclusions regarding similarity between APH/HU-induced and IR-induced CNVs strongly implies that low-dose IR-induced CNVs result from IR-dependent replication stress and replication errors, in contrast with direct joining of IR-induced DSB ends.

1.8.2. Exposure to high natural background radiation

Throughout their lives, humans are exposed to varying doses of ionizing radiation, whether it's due to living in regions with high natural background radiation, occupationally or medically. Radiation comes from outer space in the form of galactic cosmic rays as well as from radioactive materials located in the earth’s crust which possess high levels of thorium. In some

areas, natural exposure is due to radon (^{222}Rn), a gas from the earth's crust. Additionally, the human body (bones, muscles and other tissues) contains trace amounts of naturally occurring radioactive isotopes, the most important being potassium (^{40}K). Besides radiation from natural sources, people are exposed to man-made sources of radiation, which mainly consist of medical radiation, but also includes commercial, and industrial activities that contribute to the total radiation exposure of humans. It is commonly accepted that radiation doses above natural background doses do cause increased health risks, notably an augmentation in the induction of cancers. One of the most studied areas with high levels of terrestrial radiation is Kerala, India, which is a densely populated, monazite-bearing region, where radiation exposure is mainly due to the presence of thorium and its decay products in the surface soil. On average, its 360,000 inhabitants receive annually external whole-body doses of about 4.5 mGy from gamma-rays, plus an internal dose of 2.4 mSv following exposure to radon. Attempts have been made to establish a correlation between natural background radiation (NBR) and copy number changes in the human genome, a previous study showed that exposure to natural background radiation correlated with several Y chromosome copy number alterations [34]. In this study, random microdeletions in the Azoospermia factor (*AZF*) a, b and c regions, and tandem duplications of 11 different Y-linked genes were observed in ~80% of males exposed to NBR. In another attempt to demonstrate a correlation between radiation exposure and copy number changes in the *AZF* regions, a study by Moghbeli-Nejad et al.,2012 [35], in which blood samples from normal, oligospermia, and azoospermia individuals were irradiated with 2 and 4 Gy doses of gamma rays, revealed that susceptibility to instability in the AZF region in lymphocytes of infertile men exposed to radiation was more prevalent than in normal males. The susceptibility of the AZF region to radiation exposure was suggested to be due to repeated DNA blocks called amplicons,

as well as to highly recombinogenic loci. Amplifications and deletions are one of the most common structural rearrangements in the AZF region [35]. Recombination is triggered by the formation of a DNA double strand break (DSB) within an amplicon. The biological effects in cells exposed to ionizing radiation include gene mutation, chromosomal rearrangements, and cell death [36].

In our study, we attempted to develop the technical capacity to identify chromosomal changes due to chronic low-level exposures to ionizing radiation, and provide the background for initiating these studies. Since the estimation of risk in humans exposed to low dose radiation is based on the extrapolation from documented effects observed at high doses (UNSCEAR 1993), we used early passage normal human fibroblasts and exposed them to a single dose of 4 Gy. Sequence tagging site (STS) markers and clonal genomic DNA were used to perform standard PCR and identify AZF region deletions in the Y chromosome. In addition to studying the more detailed mechanisms involved in radiation-induced copy number alterations will require a simpler system that was more amenable to experimental control. Therefore, we chose the budding yeast *Saccharomyces cerevisiae* and a unique copy number selective system to study these effects.

CHAPTER TWO

RADIATION INDUCED COPY NUMBER ALTERATION IN HUMANS

2.1. BACKGROUND

Although uneven geographically, natural background radiation (NBR) has always impacted all life forms. Prolonged exposure to ionizing radiation leaves an imprint on the genome that may be used as a biomarker, allowing us to track and study the progression of tumors, copy number variation (CNV), and other alterations of DNA. Attempts have been made to establish a correlation between background radiation and phenotypic changes in mice, Dubrova et al.,2002 [37] analyzed the effects of ionizing radiation on germline mutations, using hypervariable mouse expanded single tandem repeat (ESTR) loci (minisatellite) which are both highly sensitive to IR . These studies suggest that radiation may also have an indirect effect on somatic genome stability that is transmitted through the germline of irradiated parents. Another study in humans revealed an elevated minisatellite mutation rate in families in certain regions of Ukraine, which were heavily contaminated by radionuclides after the Chernobyl accident [38]. In contrast to man-made radiation sources, the coastal areas of Kerala in Southern India, provides a natural setting to study the radio-genomic effects of background radiation on humans. With a high percentage of thorium phosphate monazite covering a large range of doses, this densely populated area is an ideal candidate to study the consequences of low-level, chronic radiation exposure on humans. One such study investigated the relationship between natural background radiation and human genome stability [34]. It raised questions regarding the results of low dose and low dose rate ionizing radiation on the stability of the Y chromosome of individuals exposed to high natural background radiation exposure.

Among those effects are copy number variation (CNV) in the form of randomly scattered microdeletions in the AZF region of the Y chromosome, which are known to be an important factor inducing impaired spermatogenesis [39],[40]. Previous studies showed that after irradiation of blood samples from a group of healthy fertile men, no microdeletions were detected after an acute dose of 2 Gy radiation, but with the increase of radiation dose to 4 Gy the frequency of these deletions increased to 6.6% [35]. This inspired us to expose our samples to a 4 Gy dose in this study. We set out to determine whether these results could be induced by ionizing radiation in cultured normal human fibroblasts by picking a low passage human fibroblasts strain with regular radio-sensitivity, derived from a supposedly normal individual with no previous known genetic disorders. Despite the fact that the entire genome could be affected, the Y chromosome was chosen to study these effects, owing to its haploid status, clonal inheritance and absence of recombination. Hence, it is highly prone to genetic variation. It is only passed from father to son, therefore its DNA is often used for familial research. The DNA present in the Y chromosome is constituted of over 59 million base pairs. In humans, the *SRY* gene is contained in the Y chromosome, and generates testis development. The mentioned AZF regions were screened in human fibroblasts from both irradiated and unirradiated cultures for the presence or absence of STS markers. Details of the primers used are given in Table 1. In this study we used a standard PCR technique to detect copy number changes that might be induced by gamma radiation.

2.2. MATERIAL AND METHODS

2.2.1. Cell line and culture conditions

Normal human nonfetal foreskin fibroblast (BJ1 cells, obtained from the National institute on Aging [NIA] cell repository), were derived from an apparently normal individual

with no known genetic abnormality. Early passage BJ1 cells were maintained in a 4:1 mixture of D-MEM high glucose medium (Hyclone)/ M-199(Hyclone) supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum and 1% Glutamax (Hyclone). Cells were grown and routinely maintained by standard techniques in 75 cm^2 or 25 cm^2 polystyrene flasks at 37 C^o in a humidified atmosphere of 5% CO_2 and 95% air. For subculture, cells adherent to the culture flask were detached using 0.25% trypsin solution containing EDTA. Prior to initiating this study, sufficient amounts of cells from this human cell line were grown and stored as aliquots in liquid nitrogen in cryovials, each containing at least 1 X 10^6 cells suspended in in 1 mL of D-MEM medium with 10% dimethyl sulfide as a cryopreservant. When cells were needed for this study, a vial was removed from liquid nitrogen, thawed quickly at 37^{o}C and added to a 75 cm^2 flasks containing pre warmed, pH-adjusted medium. The cell culture medium was replaced the next day with 5 mL of fresh cell culture medium. Cells that attached to the bottom surface of the 75 cm^2 flask were trypsinized and 5 X10^5 cells were inoculated into each of the required number of 25 cm^2 flasks together with 4 mL of pre-warmed, pH-adjusted medium. The cells at passages 6-10 were seeded at 1 x 10^4 cells/cm^2, and media was changed on days 4, 7, and 9 and used for irradiation experiments on day 11. This procedure was followed to guarantee confluent density-inhibited cultures. To determine their density, the cells were counted at confluence using a Coulter counter.

2.2.2. Irradiation conditions

Cells were grown to confluence to minimize cell cycle distribution, and were exposed to an acute dose of 4 Gy at a dose rate of 2.030 Gy/min with rotation, 48 hours after the final medium change. ^{137}Cs γ-rays were delivered by a Mark-1 irradiator (J.L Shepherd) located at Colorado State University.

2.2.3. Post-irradiation recovery

It is known that trypsinizing and replating irradiated fibroblasts causes them to immediately resume replication and reenter the cell cycle [41], leading to IR damage-induced replication stress. So cells in this experiment were given a 12-24 hour post-irradiation recovery period to allow for potentially lethal damage repair to occur, before being trypsinized and plated for clone isolation.

2.2.4. Cell cloning

Single cell cloning was done by serial dilution in flat bottom 96 well microtiter plates. This procedure was used to ensure clonal isolation of BJ1 cells in cultures that were both exposed and unexposed to gamma radiation. We used filtered conditioned medium (medium in which cells have been previously grown for 24 hours) which was added to the wells, in an attempt to increase the success rate (cloning efficiency), which ultimately helped us determine cell survival. This procedure was performed as in (www.corning.com/lifesciences), starting with a cell suspension of $2x10^4$cells/ml of which 200 µL was then added to well A1, all other wells had 100 µL of media (except the wells in the last column, A12 through H12). Then by using a single channel pipettor 100 µL was transferred from the first well to well B1 and mixed by gently pipetting, using the same tip, these 1:2 dilutions were repeated down the entire column. Using an 8-channel micropipettor, an additional 100 µL of media was added to each well in column 1 (giving a final volume of cells and medium of 200 µL/well). Then using the same pipettor, l00 µL was transferred from the wells in the first column (Al through H1) to those in the second column (A2 through H2) and mixed by gently pipetting. Using the same tips, these 1:2 dilutions were repeated across the entire plate, so that all the wells ended up with 100 µL of cell suspension (Figure 1) .This brought the final volume of all the wells to 200 µL. Plates were

incubated at 37°C in a humidified CO_2 incubator undisturbed. In another attempt to detect genomic instability, the previous procedure was performed, but in this case each clone from the 96 well plates was further passaged into a single well in a 48 well plate or a 12 well plate. Clones were detectable by microscopy after 5 to 7 days and ready to score after 10 to 14 days. Only wells confirmed to contain a single cell by microscopic examination were used further. For every irradiated culture, parallel unirradiated cultures were also plated at the same time. This was to ensure that over a period of time cell viability was not changing for reasons not related to radiation exposure. Both controls and irradiated samples were kept in the same incubator.

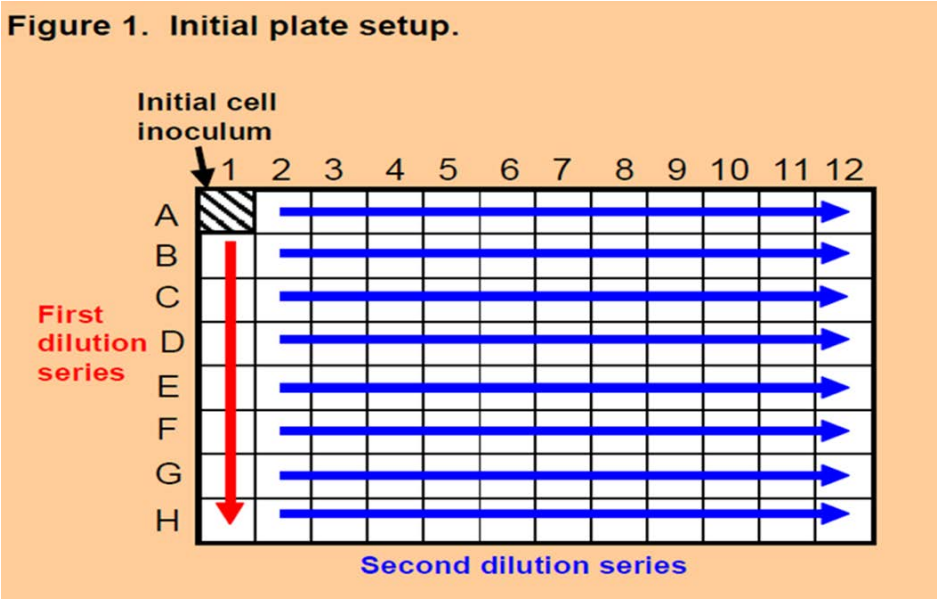

Figure 1: Cell cloning by serial dilution in 96 well plates (www.corning.com/lifesciences)

2.2.5. DNA extraction

50 clones from each condition (irradiated and unirradiated) were isolated and genomic DNA was prepared from each clone using a Blood and Cell Culture DNA Mini Kit (Qiagen).

2.2.6. Polymerase chain reaction (PCR)

DNA samples from both irradiated and nonirradiated clones were quantified and a concentration of 1.35 ng/µL was used. PCR was performed in 0.2 ml individual tubes containing 13µL of master mix consisting of 7.5 µL 2X Taq mix (Bioline), 1.2 µL of primer pairs at a concentration of 0.4 pmol/µL per reaction and 2µL of genomic DNA.

2.2.7. STS analysis of AZF region

Prior STS mapping by Premi et al., 2009 [34] indicated microdeletions randomly dispersed in AZF regions (a, b, and c) of the Y chromosome of the exposed males. However, the rate of microdeletions was highest in the AZFc region. Therefore, for the purpose of our study seven STS markers (Sy83, Sy86, Sy88, Sy1190, Sy1191, Sy1201, and Sy1206) were chosen to analyze the AZF region on the Y chromosome. PCR was done using a Bio-Rad thermal cycler. Following initial denaturation and *Taq* activation at 95^0C during 1 minute, samples were then subjected to PCR amplification using 35 cycles of 95^0C for 15 seconds, 60^0C for 18 seconds and 72^0C for 45 seconds, and a final elongation at 72^0C for 3 minutes, followed by a final hold at 4^0C. Control samples of unexposed bulk (nonclonal) cultures were included in all cases.

Table 1: The STS markers and primers used in standard PCR [34].

STS MARKERS	*PRIMER*	*5' to 3' SEQUENCE*	*AMPLIFICATION SIZE*
SY 83	A B	CTTGAATCAAAGAAGGCCCT CAATTTGGTTTGGCTGACAT	301bp
SY 86	A B	GTGACACACAGACTATGCTTC ACACACAGAGGGACAACCCT	306bp
SY 88	A B	CACCCAGCCATTTGTTTTAC CACCCAGCCATTTGTTTTAC	123bp
SY 1190	A B	TTGTGAGGTGGTGATGG CTGATTTGGAAACTCGTCCC	666bp
SY1191	A B	CCAGACGTTCTACCCTTTCG GAGCCGAGATCCAGTTACCA	385bp
SY 1201	A B	CCGACTTCCACAATGGCT GGGAGAAAAGTTCTGCAACG	677bp
SY 1206	A B	AGGAGGCATAGCTAGGAGGC TGCACTGCCTTTATGAGCTG	515bp

2.3. RESULTS

2.3.1. Copy number alteration analysis of STS markers

Our aim in this experiment was to detect copy number variation in the form of deletions in the *AZFc* region of the Y chromosome, and to determine whether copy number changes by radiation. Using *AZFc* specific STS analysis, we identified 3 clones from human fibroblasts that contained partial *AZFc* deletions among the irradiated samples (3/50, 6%), but no deletions were found among the nonirradiated human fibroblast clones which would be expected to accumulate some spontaneous chromosomal variations during proliferation from a single cell. As expected, all control bulk DNA cultures did not show deletions using all seven STS markers, and three irradiated clones (YCR-2, YCR-3 and YCR-13) revealed a negative signal for sY83, sY1191, and sY1201 indicating a deletion, but no detectable deletion for sY86, sY88, sY1190, sY1206 (an example is shown in Figure 2). The same results were obtained from clones that were passaged from 96 well plate to 24 or 12 well plates, with the addition that clone YCR-3`showed a detectable deletion for sY1206, indicating that genomic instability was manifested in the progeny of those cells surviving radiation.

2.3.2. Reduced plating efficiency (PE)

Our data in Table 2, shows that colonies surviving 4 Gy of gamma irradiation have depressed PEs (0.1 %) in comparison with the control population plating efficiency (0.5%).

Table 2: This table represents the plating efficiency for doses of 0 Gy and 4 Gy at 4 cells/well.

Dose Gy	Cell no. of PE	Negative wells	Total no. wells	P.E
4	**4**	**89**	**96**	**0.01893**
4	**4**	**93**	**96**	**0.00794**
4	**4**	**93**	**96**	**0.00794**
4	**4**	**91**	**96**	**0.01337**
4	**4**	**89**	**96**	**0.01893**
4	**4**	**86**	**96**	**0.0275**
4	**4**	**92**	**96**	**0.01064**
4	**4**	**85**	**96**	**0.03042**
0	**4**	**7**	**96**	**0.65461**
0	**4**	**10**	**96**	**0.56544**
0	**4**	**21**	**96**	**0.37996**
0	**4**	**8**	**96**	**0.62123**

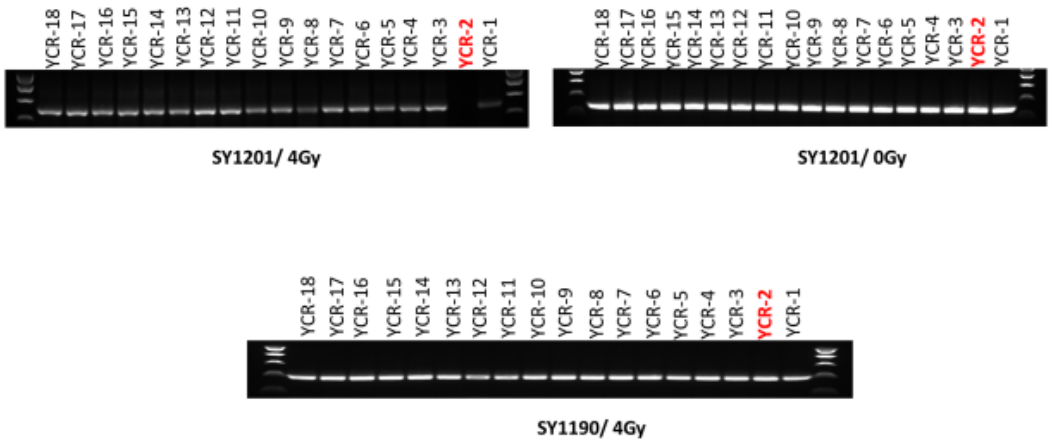

Figure 2: Microdeletions and STS markers. Detection of STS marker SY1201(upper images) and SY1190 (Lower image) in the progeny of 18 fibroblasts exposed to 4 Gy and 0 Gy. An absent band for clone YCR-2 indicating a deletion, using SY1201 marker of the AZF region. But when using marker SY 1190 for clone YCR-2 no deletion was detected.

CHAPTER THREE

COPY NUMBER ALTERATIONS IN YEAST

3.1. OBJECTIVES

The main purpose of the research described in this chapter was to use the yeast CNV assay to investigate the effects of ionizing radiation on the formation of *de novo* CNV events. *Saccharomyces cerevisiae* cells were exposed to two doses of ionizing radiation to characterize the mechanisms involved.

3.2. A YEAST MODEL SYSTEM TO INVESTIGATE MECHANISMS FOR MAINTANENCE OF GENOME STABILITY

In this study we used diploid cells of the budding yeast *Saccharomyces cerevisiae* which have 16 pairs of chromosomes, ranging from 0.2 to 2.5 Mb in size, to investigate CNV formation mechanisms. Although much smaller than the human genome, the yeast genome shares many of the basic biologic properties: conserved DNA replication and repair pathways, similar organization of the chromosomes and dispersed repetitive DNA sequences. In this model, a CNV event is a duplication or deletion that includes at least one gene (approximately 2 kb) but not more than one chromosome arm (approximately 400 kb). The rationale for using yeast in such model studies lies in the ease of handling, as well as the availability of advanced molecular genetic methods for further analysis. *S. cerevisiae* based genetic assays have been developed to evaluate genes and pathways that suppress genome instability, from point mutation to gross chromosomal rearrangements. Previous studies of unselected CNV in the yeast genome have shown that directly oriented retrotransposons are preferential sites for genomic rearrangements [42, 43]. In other studies, selective assays have been developed to study the genetic and environmental control of deletions in yeast. One such assay involving gross chromosomal

rearrangements (GCR) exhibited the simultaneous loss of two genes *CAN1* and *URA3*, located on the left end of chromosome 5 (Chr5). It revealed that the presence of dispersed repeats in this region increased the rate of NAHR-mediated deletions, while in regions without repeats infrequent deletions weregenerally due to NHEJ. In later implementations of the same assay, the insertion of a Ty1 element proximal to the *CAN1* and *URA3* genes augmented the rate of deletion events about 400-fold [44]. In yeast gene duplication, events involve more diverse types of mechanisms, which appear to be dependent on chromosome context and ploidy. These duplication events can involve an increase in copy number from a duplication of a region originally present in only one copy, or from a pre-existing duplication. An assay selected for amplification of a cassette containing *CUP1* and *SFA1* by use of medium that contained high levels of copper (Cu) and formaldehyde (FA) was developed [45] *SFA1* encodes formaldehyde dehydrogenase which acts to detoxify formaldehyde, and *CUP1* encodes metallothionein which has the capability to sequester copper and thus mediate resistance to high copper concentrations. In the previous study by Narayanan et al.,2006 [45], this reporter system was optimized to select for low-order amplification events (one extra copy) that were more similar to the duplication associated with human diseases. This system was used to examine gene duplications in both haploid and diploid yeast, as well as wild-type and mismatch-repair defective strains.
When examining this chromosomal context, most duplications reflected homologous recombination beween flanking Ty repeats in haploids, while diploids mostly showed nonreciprocal translocations between Ty elements on Chr5 and Ty elements on other chromosomes, notably Chr13 [46]. This cassette reporter system was the inspiration for the development of the assay detailed in this study.

3.3. MATERIALS AND METHODS

3.3.1. Yeast strains used in this study

All yeast strains used in the CNV assay were genetically identical to strain MS71, with the exception of locus-specific modifications introduced by transformation [47]. MS71 is essentially isogenic to the CG379 strain background [43],[48]The specific diploid strains that were used in this study are listed in Table 3. The CNV reporter system [45] (Figure 3) was incorporated into the yeast genome through PCR-mediated homologous recombination, while transformants were selected in plates containing geneticin. The *SFA1* gene in the reporter system contained a dominant mutation that encodes a Valine 208 to Isoleucine substitution *(SFA1-V208I*) which is expected to increase the activity of formaldehyde dehydrogenase, thus enabling higher tolerance to formaldehyde. The reporter system was integrated [46],[49] at two different positions (one after the other) in the haploid yeast genome first on Chr15 about 5 Kb distal to the *RPL20B* gene, between the *SFG1* and *COT1* genes, between SGD coordinates 905,937 and 906,020 (*SFG1::SFA1-V208I-CUP1-KanMX4*); then on Chr4 between the *PML2* and *SAM2* genes, at SGD coordinate 1,453,103. In order to form the diploid strains used in this study, each haploid that carried a specific reporter insertion was mated to a haploid of the opposite mating type, carrying a *TRP1* insertion at the same respective positions. The main advantage of our CNV assay system is that it uses diploid parent strains, while the majority of previous assays used to model genome stability in yeast were limited to haploid cells. Therefore, it isn't limited as far as CNV formation mechanisms, which means that all of the pathways outlined in the Introduction section can be successfully recovered, notably the events involving large genomic deletions, which are lethal in haploids. This resulted in an unbiased sampling of naturally occurring CNV events, providing a much more relevant model for the radiation-induced genomic

changes that take place in the diploid human genome. The CNV assay system was built on the phenotypic selection of yeast cells containing local genomic increases in gene dosage (Figure 4). A CNV reporter system containing the *SFA1* and *CUP1* genes (Figure 3), which confer gene dosage-dependent tolereance to formaldehyde (FA) and copper (Cu), was integrated to the diploid strains that were built and studied [45],[46]. The parent strains which contained a single copy of the CNV reporter system were able to grow in media that included low levels of FA and Cu, but not high levels. With selective conditions optimized at the inhibitor concentrations combination of 2.0 mM FA and 160 μM Cu, cells that had undergone genomic amplification events which resulted in the presence of two or more copies of the CNV reporter system were the only ones capable of growing. Two markers were included in the reporter cassette: the *KanMX4* marker [50] which was used to select for initial integration of the cassette into the genome, and the auxiliary marker gene *TRP1* [49],[46], which was inserted on the homologous chromosome at the allelic position. Essential for growth in media lacking tryptophan, this auxiliary marker further augmented the sensitivity of the CNV assay, allowing us to focus our study on the relatively rare nonallelic recombination events responsible for pathogenic CNV formation in humans. Plating cells in Trp dropout media suppressed the growth of clones that may otherwise have acquired a second copy of the CNV reporter by allelic mitotic recombination (Loss-of-Heterozygosity; LOH). This class of allelic double strand break repair mechanism does not generate chromosomal rearrangements and hence was not a target of this research project. Two parent diploid strains were built [46],[49] with the CNV reporter system inserted at different genomic sites: Chr15 (JAY685) and Chr4 (JAY654). Schematic representations of both strains chromosomal insertions are shown in Figure 3. The genomic context of the reporter insertion largely differed between both parent strains. Chr15 is a smaller chromosome (1,091 Kb) and the

reporter cassette was inserted far away from flanking repetitive elements, immediately distal to the *RPL20B* gene. This location was chosen to allow us to compare our results with those previously reported using selection for duplication of the *RPL20B* gene [51]. The other parent diploid strain, Chr4, is the second largest chromosome in the yeast genome (1532 Kb). The reporter system was inserted distal to the last annotated dispersed repeat, not flanked by any type of homology. The analysis of both host chromosomes sizes and reporter location provided a wide spectrum of the CNV mechanisms that are possible throughout the yeast genome.

Table 3: Diploid strains studied. These strains are part of the Argueso Laboratory collection.

Strain	Description	Genotype
JAY685	Diploid formed by crossing JAY681 and JAY657. Reporter on Chr15.	
JAY681	MAT a	*ade5-1 his7-2 leu2-3,112 LEU+ ura3-52 trp1-289 cup1Δ RSC30 sfa1Δ::hisG RPL20B::SFA1-V208I-CUP1-Kan 3'-5'*
JAY657	MAT alpha	*ade5-1 his7-2 leu2-3,112 LEU+ ura3-52 trp1-289 cup1Δ RSC30 sfa1Δ::hisG RPL20B::TRP1*
JAY654	Diploid formed by crossing JAY648 and JAY644. Reporter on Chr4.	
JAY648	MAT alpha	*ade5-1 his7-2 leu2-3,112 LEU+ ura3-52 trp1-289 cup1Δ RSC30 sfa1Δ::hisG PLM2::SFA1-V208I-CUP1-Kan*
JAY644	MAT a	*ade5-1 his7-2 leu2-3,112 LEU+ ura3-52 trp1-289 cup1Δ RSC30 sfa1Δ::hisG PLM2::TRP1*

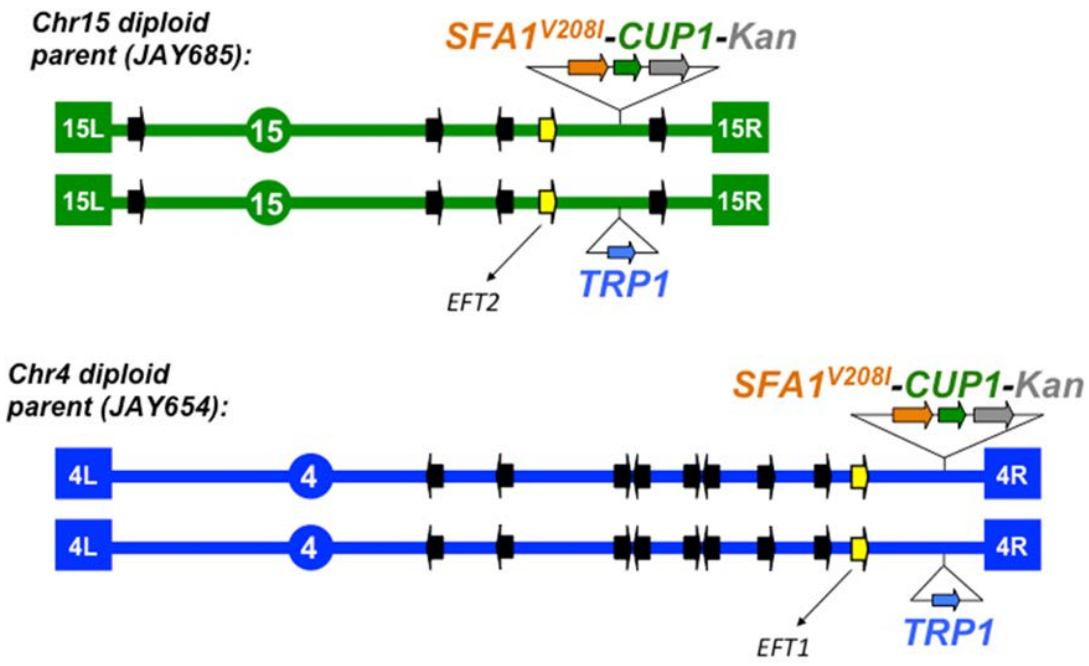

Figure 3: **Copy number reporter system.** Illustrates reporter system insertion sites on Chr15, and Chr4 diploid strains. Circles represent centromeres, and boxes "L" and "R" indicate left and right telomeres, respectively. The Ty retrotransposons elements are represented by black arrows.Yellow arrows represent the *EFT1* and *EFT2* genes [49],[46].

3.3.2. CNV assay and ionizing radiation exposure

The CNV reporter parent diploid strains individual colonies were isolated on YPD plates (rich media; 20 g glucose, 20 g peptone, 10 g yeast extract, and 10 g agar in 1 L of distilled water), and then incubated in 7 mL liquid YPD at 30^0C for 24 hours of growth (saturated cultures). 50 µL of the incubated cell cultures were reinoculated in 7mL of fresh prewarmed liquid YPD, and incubated at 30^0C for an extra 6 hours before irradiation. Using this procedure, the cells reached the irradiation point as an exponentially growing asynchronous cell population. Irradiation exposures were performed using a Mark-1 irradiator (J.L Shepherd) source of ^{137}Cs γ-rays, located at Colorado State University. The cells were exposed to acute doses of 200, 50, or 0 Gy at dose rate of 3.9 Gy/min at room temperature. After radiation exposure, cultures were returned to the incubator, incubated overnight at 30^0C until saturation, and plated the next day. 1 mL of each culture was centrifuged down to a pellet and the supernatant was discarded. The pellet was then washed twice using sterile distilled water. The washed pellets were resuspended in 1 mL of water, and the samples were serially diluted. 150 µL of the 10^{-4} dilution was plated on one permissive SC Trp drop-out plate and two 90 µL aliquots of the 10^{-1} dilution were plated on two selective, permissive synthetic media Trp dropout plates supplemented with 2.0 mM FA and 160 µM $CuSO_4$. The SC Trp dropout plates were prepared with 1.7 g Yeast nitrogen base (YNB) (with out amino acids, with out ammonium sulfate), 1.4 g Trp dropout mix, 5 g ammonium sulfate, 20 g bacteriological agar, 20 g glucose and 1L distilled water were mixed and autoclaved. Once the media cooled down to 70^0C, Cu and FA were added. Fresh 1 M dilutions from a methanol-stabilized stock were prepared for each new batch of selective media, since FA is not stable in water soution. Plates were always used within 24 hours of pouring.

Cells were grown for 5 days and colonies were counted after 3, 4 and 5 days of growth. To calculate the mutation rate using the Lea and Coulson method of the median, the total number of colonies that had formed on the two selective plates after 5 days were added and compared to the colonies formed on the permissive plate [52]. Only one colony from each culture was selected from a selective plate to be retested and later used in pulse field gel electrophoresis (PFGE) and array-CGH assays [43], to ensure the independence of the CNV events studied. The two diploid parent strains were used to investigate two fundamental guidelines of CNV formation presented below: (1) using the quantitative fluctuation mutation assays, we measured the rate at which gene amplification events occur; and (2) using a combination of pulse field Gel electrophoresis (PFGE) and microarray based-comparative genome hybridization (array-CGH), we characterized the qualitative nature of the genome rearrangements present in the selected CNV clones by molecular karyotyping. We first determined these parameters in yeast cultures under normal (unirradiated) conditions. Then we used the assay as a tool to investigate the effect of ionizing radiation exposure on CNV formation by exposing the yeast cultures to two doses of gamma radiation.

3.3.3. Phenotypic retests

A colony was selected from each culture, then streaked on low concentration 0.5 μM Cu and 1 mM FA plates to grow isolated colonies, and tested again to confirm the occurrence of the copy number amplification of the reporter [45]. This was achieved by plating dilutions of the medium that contained the experimental culture, which was inoculated for 24 to 48 hours on 4 different plates consisting of various combinations of copper and formaldehyde. One plate did not contain any copper or formaldehyde, to act as a positive viability reference for the cells plated. The three other plates contained 2.0 mM FA and 160 μM Cu (as did the selective plates

used in the CNV assay), 0 mM FA and 300 μM Cu, and 2.0 mM FA and 0 μM Cu. The plates that only contained either Cu or FA revealed information about the presence of the two individual genes inserted in the reporter cassette. For each plate, we compared the growth of the candidate CNV clones to the viability of the parent that contained one copy of the reporter system (shown in figure 4).

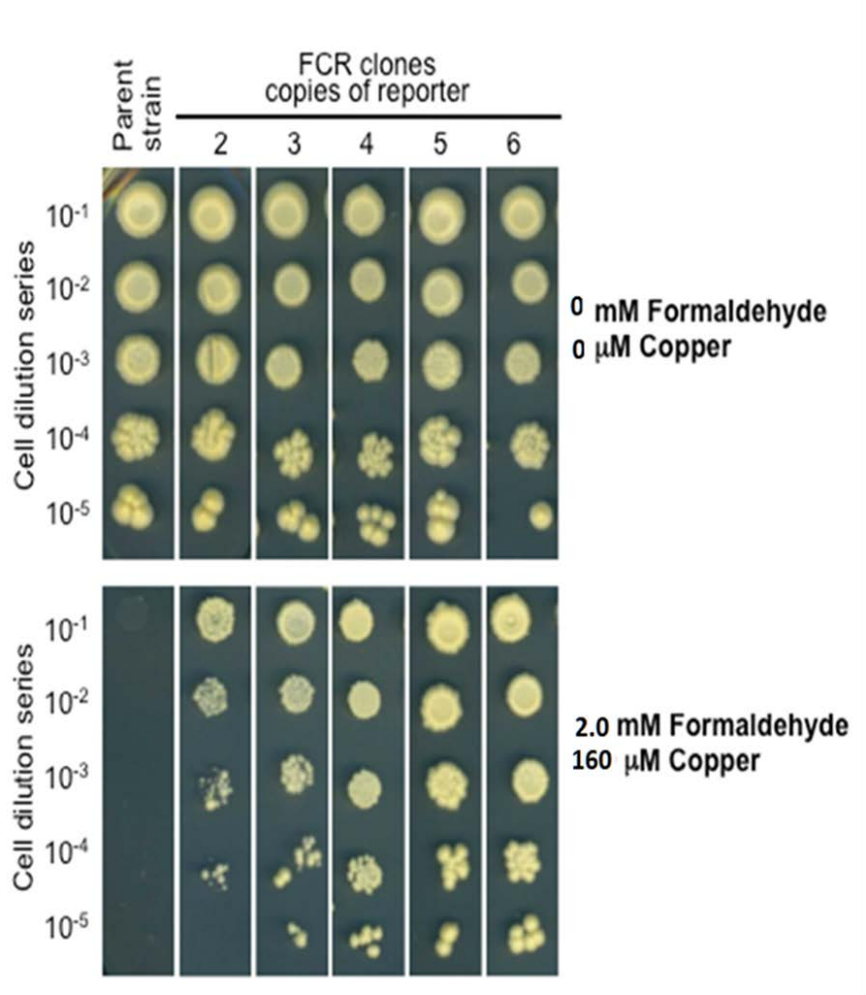

Figure 4. Phenotypic retests. The upper image represents the growth appearance of the parent strain and of Formaldehyde and Copper Resistant (FCR) clones on medium containing no copper nor formaldehyde. The same strains are plated on medium with FA and Cu, as depicted in the lower image, and the FCRs then manage to grow to full viability. The parent strain contains only one copy of the reporter and cannot grow on the plates that contain copper and formaldehyde [46],[49].

The next two procedures (PFGE, Array-CGH), were performed using the same protocols as those described by and Argueso et al.,2008 [43]and Zhang et al.,2013 [46].

3.3.4. Pulse field gel electrophoresis (PFGE)

Agarose-embedded full length chromosomal DNA was used from selected formaldehyde-copper resistant (FCR) clones to analyze CNV events using PFGE karyotyping. These samples, or plugs, were made in sufficient quantities, so that a single source of full-length chromosomal DNA could be analyzed repetitively, or studied in different assays without the need for secondary DNA preparations that might have led to variations in clones carrying unstable chromosomal rearrangements. The plugs consisted of cultures that were grown in 7 mL of YPD for 24 to 48 hours then centrifuged, the supernatant was discarded and the pellets were weighed. The proper amount of zymolyase and low-melting point agarose were mixed with the cells, then transferred to Bio-Rad plug molds. The plugs were incubated in the cold room for about 30 minutes, then put in a solution consisting of 500 mM EDTA, 10 mM Tris, pH 7.5 once they were solidified. Afterwards, they were incubated overnight at 37^0C. The next day, 400 µL of a solution containing 5% sarcosyl, 5 mg/ml proteinase K in 500 mM EDTA pH 7.5 was added and incubated at 50^0C for five hours, then plugs were washed 3 to 5 times in 1x TE (10 mM Tris PH 7.5 and 1 mM EDTA) solution. These plugs were trimmed and then loaded on a 180 mL gel that was prepared with 1% Bio-Rad Pulse Field Certified agarose, in 0.5% TBE buffer. We used the wide/long BioRad CHEF gel to cast the gel. The separation program was run at 5 volts and 14^0C, with an initial switch time of 47 seconds, a final switch time was of 170 seconds, and a run time ranging from 50 to 58 hours. With this technique, the voltage changes direction diagonally, resulting in a net forward movement of the DNA, contrary to a standard gel electrophoresis. The

different sized chromosomes react to this change of voltage at various rates, resulting in separation of large DNA molecules in the size ranges of *S.cerevisiae* chromosomes.

3.3.5. Array comparative genomic hybridization (array-CGH)

The CNV events were identified through the use of Array-CGH experiments. The DNA was first extracted from the agarose-embedded full length chromosomal DNA plugs, by using 4 plugs that were dried on a paper wipe and then stored in a tube for each sample. 250 μL of binding buffer was added from the GeneJET Extraction Kit (#K0702) and incubated at 37^0C, until the plugs dissolved. Every sample was then sonicated for 30 minutes with the Bioruptor UCD-200 by Diagenode. The manufacturer's recommended procedure for the GeneJET Gel extraction kit was followed using 15 μL of elution buffer. To determine the concentration of the extracted DNA, the Invitrogen Qubit fluorometer and the dsDNA broad range Quant-iT assay kit were used. An agarose gel was run to confirm the desired size range of the fragmented DNA (0.5 to 2.0 Kb). From every sample, an aliquot of 1.5 μg of genomic DNA was diluted with deionized water to a total volume of 7 μL. Afterwards, we added 6.7 μL of 2.5x random primers from the Invitrogen BioPrime Array CGH Genomic Labeling module kit to every sample. These samples were then vortexed and incubated for five minutes at 95^0C to denature the genomic DNA. During this time, the Cy3 labeling mix and Cy5 reaction labeling mix were prepared, using dUTP mix and Klenow Pol from the BioPrime Array CGH Genomic Labeling module kit as well as Cy5-dUTP and Cy3-dUTP. The whole Cy3 mix was then added to the parent strain sample, while the Cy5 mix was distributed equally among all of the experimental CNV clone samples. The samples were then incubated at 37^0C for 3 hours. Before the Cy3 mixture was evenly divided into each of the Cy5 reaction. The GeneJET PCR Purification Kit (#K0702) was employed to clean up the reaction, using 22 μL of elution buffer in order to recover the purified

DNA. A hybridization mixture consisting of salmon sperm DNA, Agilent 10x blocking agent, as well as Agilent 2x Hi-RPM buffer was prepared. 35 μL was added to 20 μL of each sample, which were then incubated for 30 minutes at 95^0C and 20 minutes at 37^0C. Next, the Cy-3 labeled parental DNA samples and the Cy5-labeled experimental DNA were loaded on Agilent Technologies oligonucleotide microarray slides and co-hybridized at 65^0C in a rotating oven for at least 14 hours overnight. The arrays contained approximatively 15,000 60-mer probes distributed equally across the whole yeast genome, with a median spacing of about 700 bp [46]. The following day, the slides were washed for five minutes with Agilent wash buffers 1 and 2 and scanned using the PerkinElmer Scan Array Express microarray scanner. Gene Pix Pro 6.1 was run in order to grid the arrays and extract the relative Cy5/Cy3 hybridization signals from the images. The GenePix data was then analyzed by the Nexus Copy Number software,allowing visualization of the results in the form of the graphical plots (Figures 5-11).

3.4. RESULTS

3.4.1. Phenotypic retests and amplification mutation rates

In order to study the molecular nature of the endpoint genome rearrangements and to describe the mechanism of exposure-dependent CNV mutations, experiments were performed for unirradiated cultures as well as for cultures exposed to 50 or 200 Gy of ionizing radiation. Determining the amplification mutation rate was achieved with the classic fluctuation analysis assays described by Lea et al., 1949 [52], by plating dilutions of various independent yeast cultures on permissive and nonpermissive FA/Cu media, before counting the resulting yeast colonies in an attempt to estimate the number of mutants per culture. The results of the mutation rate assays are summarized in Table 4, which include both the 95% confidence interval and the median rate.

Table 4. **Quantitative analysis of reporter amplification**. Shown below are the median rates of amplification per cell per cell division, as well as the relative rate in comparison with the unirradiated cultures. The 95% confidence intervals for the rates and the total amount of cultures for every strain are also displayed.

	Parent strain - diploid					
	Chr4			**Chr15**		
	0 Gy	**50 Gy**	**200 Gy**	**0 Gy**	**50 Gy**	**200 Gy**
Relative rate	1	2.2	1.9	1	1.8	2.7
Absolute rate (x10-6)	9.7	20.8	18	2.5	4.5	6.8
95% confidence (x10-6)	7-12.9	16-26	14.8-24	0.5-3.2	2.8-5	6-10.5
Number of cultures	25	23	24	13	20	19

The genomic background of the CNV reporter played an important role in the variation of mutation rate absolute values that are shown for each radiation dose. The rate measured for Chr15 was lower when compared to the mutation rates for Chr4 reporter. Cultures exposed to 50 Gy and 200 Gy showed ~ 2 fold elevation in CNV mutation rate. The other significant parameter that we examined was the number of positive phenotypic retests for every mutation rate assay. The CNV assay is built on scoring the progressive phenotypic enhancement associated with extra copies of the reporter cassette. We chose one individual colony selected on FA/Cu plates from each culture. This method was used to ensure that all growing phenotypic retests were true amplifications. Our double selection system was highly specific, because the majority of the clones retested retained resistance to FA/Cu.

Our phenotypic results showed that:

Chr 4 retests:

- 12 /14 colonies (exposed to 200 Gy) tested positive.

- 2/12 colonies tested negative.
- 9/10 colonies (exposed to 50 Gy) tested positive, and one negative.

Chr15 retests:

- 13/14 colonies (exposed to 200 Gy), tested positive and one tested negative.
- 11/14 colonies (exposed to 50 Gy) tested positive, and 3/14 tested negative.

Therefore, 14 true colonies from Chr4 and 16 true colonies from Chr15 were chosen as FCRs to perform PFGE, Array-CGH investigations.

3.4.2. Investigating the associated chromosomal rearrangements

Every individual FA/Cu resistant clone (FCR) was examined using PFGE karyotyping and array-CGH after being confirmed phenotypically to contain a copy number event, in an attempt to determine the molecular nature of their chromosomal rearrangements.

The microarrays that were chosen this study were specifically designed to contain a high number of probes within the *SFA1* and *CUP1* genes. For these high-density reporter probes, we calculated the FCR to parent ratio separately from the global copy number changes in the rest of the genome, in order to determine the copies of the reporter cassette in the mutated strains with a reliable method. Regardless of the position where the CNV reporter was inserted in the genome, the signal for the *SFA1* and *CUP1* genes was always displayed at their original positions on Chr4 and Chr8. Hence, the positive spike that appeared at these two locations on the array-CGH analysis was present in every FCR clone.

As can be seen in Table 5, the majority of the unirradiated FCR clones contained only one extra copy of the reporter, and this was also the case for the FCRs derived from radiation exposures. This observation substances that the FA/Cu resistance selection conditions were correctly

adjusted and that radiation exposures stimulated CNV formation that was not more complex in structure than spontaneous CNVs.

Table 5. Reporter copy number amplification rate

Data collected from array-CGH analysis, determined the number of copies of the reporter based on the comparative hybridization signal between parent strain and FCR clones, in regards to the high density hybridization probes located at *SFA1* and *CUP1* . The unirradiated samples were done by Ane Zeidler; previous Argueso lab member.

	Parent strain - diploid					
Reporter copy number	Chr4			Chr15		
	0 Gy	50 Gy	200 Gy	0 Gy	50 Gy	200 Gy
2	22	6	5	22	7	7
3	2	0	0	1	0	0
4	0	0	0	0	0	0
5+	0	0	0	1	0	0
Total FCR clones analyzed by array CGH	24	6	5	24	7	7

The two parental strains analyzed showed very similar CNV of reporter levels, shown in Table 5. In irradited and unirradiated cultures, the reporter copy number was mainly 2.
The structural properties of the genome rearrangements mainly showed that multiple chromosomes were involved (translocations), except for one aneuploidy event. The breakpoints were also scored to determine if recombination occurred at sites containing dispersed repetitive elements or at single copy sequences. As observed in Table 6, all the breakpoints in Chr4 (unirradiated and irradiated) were present at dispersed Ty repeats, in contrast to Chr15 where a majority of breakpoints were found at sites containing other forms of conserved sequences such as gene family members (e.g. *EFT1* and *EFT2).* They both share a 99.80% nucleotide sequence identity over a 2.5 kb region, and encode the translational elongation factor 2 [EF-2].

Table 6. Mechanisms and chromosomal rearrangement breakpoints associated with the reporter

CGH-array data of the CNV events associated with the reporter was analyzed to find out whether the regions of the breakpoints contained dispersed repetitive DNA elements.

		Parent strain - diploid							
		Chr4				Chr15			
		0 Gy	50 Gy	200 Gy	All IR	0 Gy	50 Gy	200 Gy	All IR
CNVs associated with the reporter	Aneuploidy	5	0	0	0	1	1	0	1
	Segmental duplication	0	0	0	0	1	0	0	0
	Translocation	21	6	5	11	23	7	7	14
Rearrangement breakpoints	At Ty or LTR repeats	42	6	5	11	31	1	5	6
	At other repeats	0	0	0	0	14	6	2	8
	At single copy sequences	0	0	0	0	3	0	0	0

We also detected chromosomal rearrangements that were not associated with the CNV reporter. These events are shown in Table 7, in which the majority of breakpoints were located at dispersed Ty repeats. Even though these events also included aneuploidies, segmental duplications and deletions, as well as nonreciprocal translocations, it did not directly affect the selected phenotype.

Table 7: Mechanisms and chromosomal rearrangement breakpoints not associated with the reporter

A couple of explanations for this high incidence include: (1) the presence of formaldehyde may have triggered these unassociated rearrangements; (2) these events may have been indirectly chosen in the FCR clones as second site suppressors, that compensated the negative effects of the gene dosage imbalances consecutive to the reporter associated rearrangements.

		Parent strain - diploid							
		Chr4				Chr15			
		0 Gy	50 Gy	200 Gy	All IR	0 Gy	50 Gy	200 Gy	All IR
CNVs not associated with the reporter	Aneuploidy	5	2	1	3	1	0	0	0
	Segmental duplication	1	0	0	0	2	0	0	0
	Segmental deletion	1	0	0	0	0	0	0	0
	Translocation	5	4	4	8	2	7	7	14
Rearrangement breakpoints	At Ty or LTR repeats	10	7	5	12	7	3	10	13
	At other repeats	0	0	0	0	0	5	2	7
	At single copy sequences	0	0	0	0	1	0	0	0

3.4.3. Events associated with chromosomal rearrangements

In this study, a total of 25 FCR clones carrying some form of genomic rearrangement were analyzed by molecular karyotyping. We will only describe some of the more commonly seen events.

3.4.4. Results of a radiation-induced Chr4 amplification event

FCR 348, as illustrated in Figure 5 and 6, was a clone induced by 50 Gy of ionizing radiation, and had a Chr7 deletion associated event. A new band around 1363 Kb and a faint

band for Chr8 appeared on the PFGE. The array-CGH data revealed an aneuploidy event on Chr8, and a 273 kb deletion on Chr7's right arm at *YGRWTy2-3*/*YGRCTy1-3*, and a 544 kb amplification on the right arm of Chr4 at *YDRWTy2-2*/ *YDRCTy1-3* was also detected. Hence, the karyotype changes for this FCR clone contained a Chr7/Chr4 NAHR terminal translocation and an aneuploidy event on Chr8. Another example of a radiation-induced Chr4 amplification event, is illustrated in Figure 7,8. An FCR 345 clone was induced by 200 Gy of ionizing radiation, a new band around 1470 kb was detected by PFGE. Array-CGH data analysis indicated a 654 kb amplification on the right arm of Chr4 at *YDRWTy2-2*/ *YDRCTy1-2*, and deletion on the right arm of Chr7 at *YGRWTy2-2*/ *YGRCTy1-3*. Thus, these karyotype changes for FCR 345 clone indicating a Ch7/ Chr4 NAHR terminal translocation.

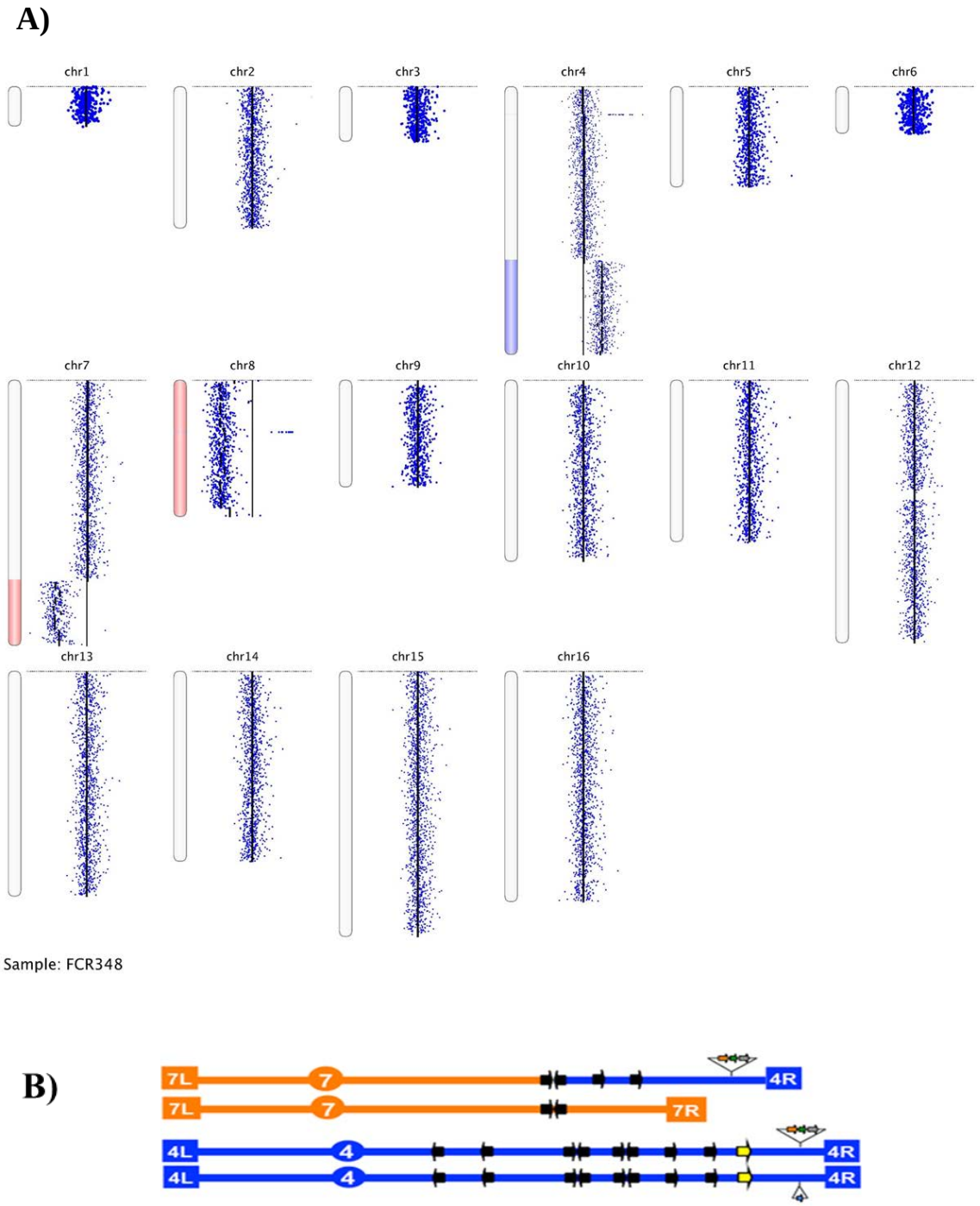

Figure 5: Qualitative analysis of CNV associated rearrangements in Chr4 diploid strain (FCR 348) exposed to 50 Gy. **(A)** Whole genome map for FCR348. And a diagram of these events is shown in **(B)** FCR 348 karyotype was a Chr7/Chr4 reporter associated translocation event occurring at Ty repetitive sequences.

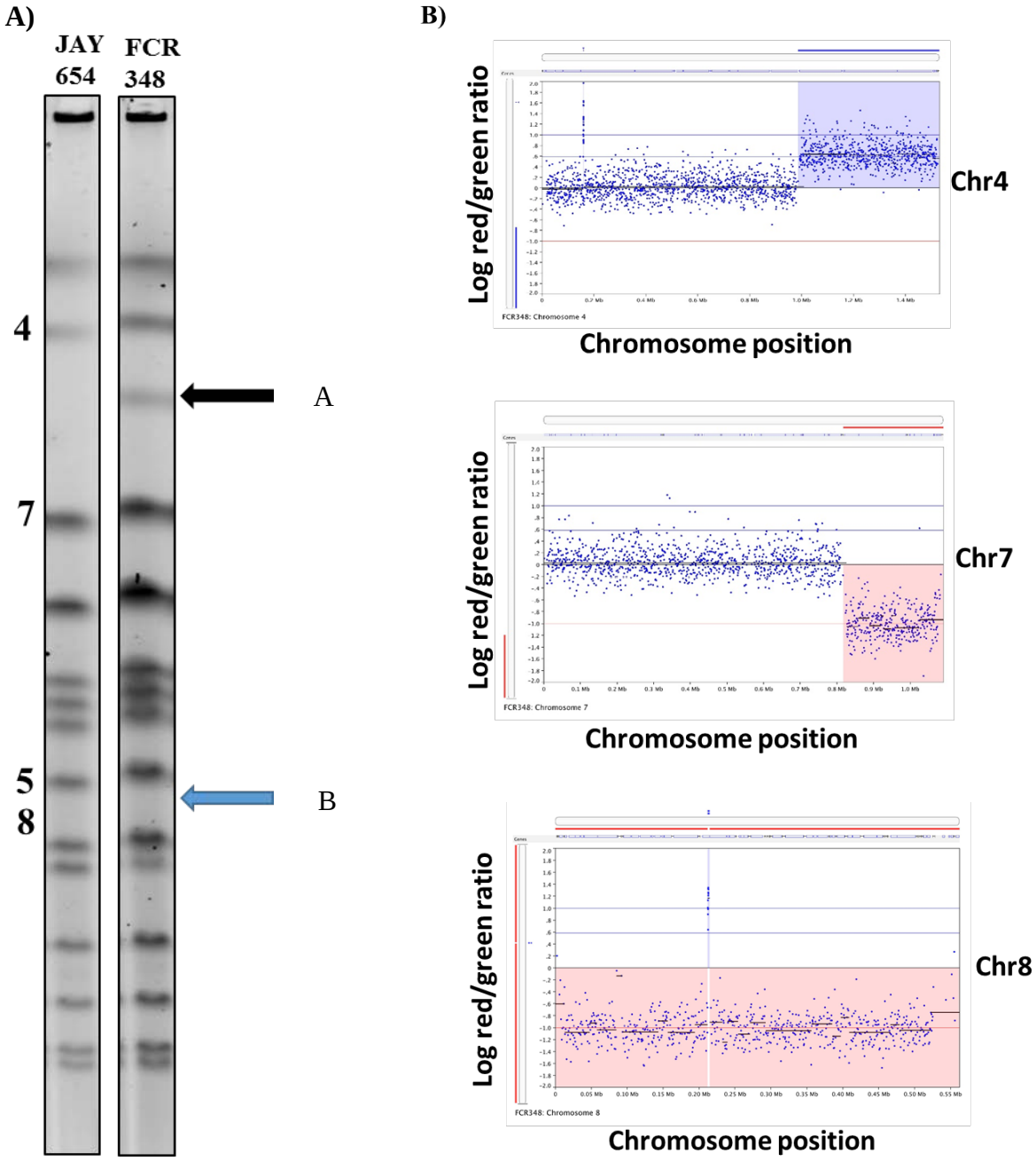

Figure 6: PFGE and array-CGH analysis of CNV associated rearrangements in Chr4 diploid strain (FCR 348) exposed to 50 Gy. PFGE (A) of the sample FCR348, and the parent (left) show two new events as marked by the arrow A and B. Comparing CGH-array data **(B)** for this sample with the PFGE allows for the interpretation of the mutation that occurred.

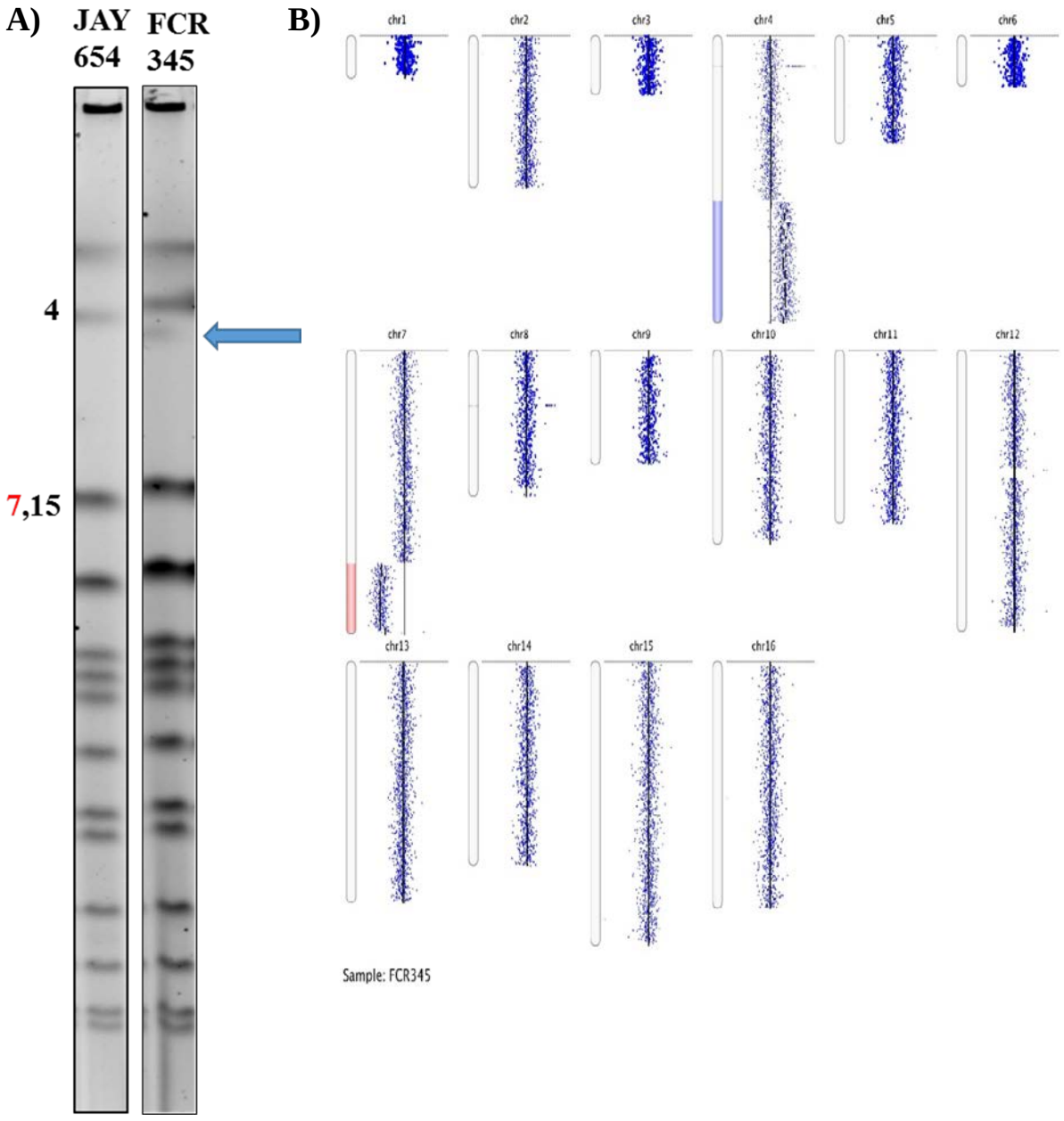

Figure 7: PFGE and array-CGH analysis of CNV associated rearrangements in Chr4 diploid strain (FCR 345) exposed to 200 Gy. PFGE **(A)** of the sample FCR345, and the parent (left) show new band as marked by the arrow. Whole genome CGH-array data **(B)** for this sample indicates two new events occurring on Chr4, Chr7.

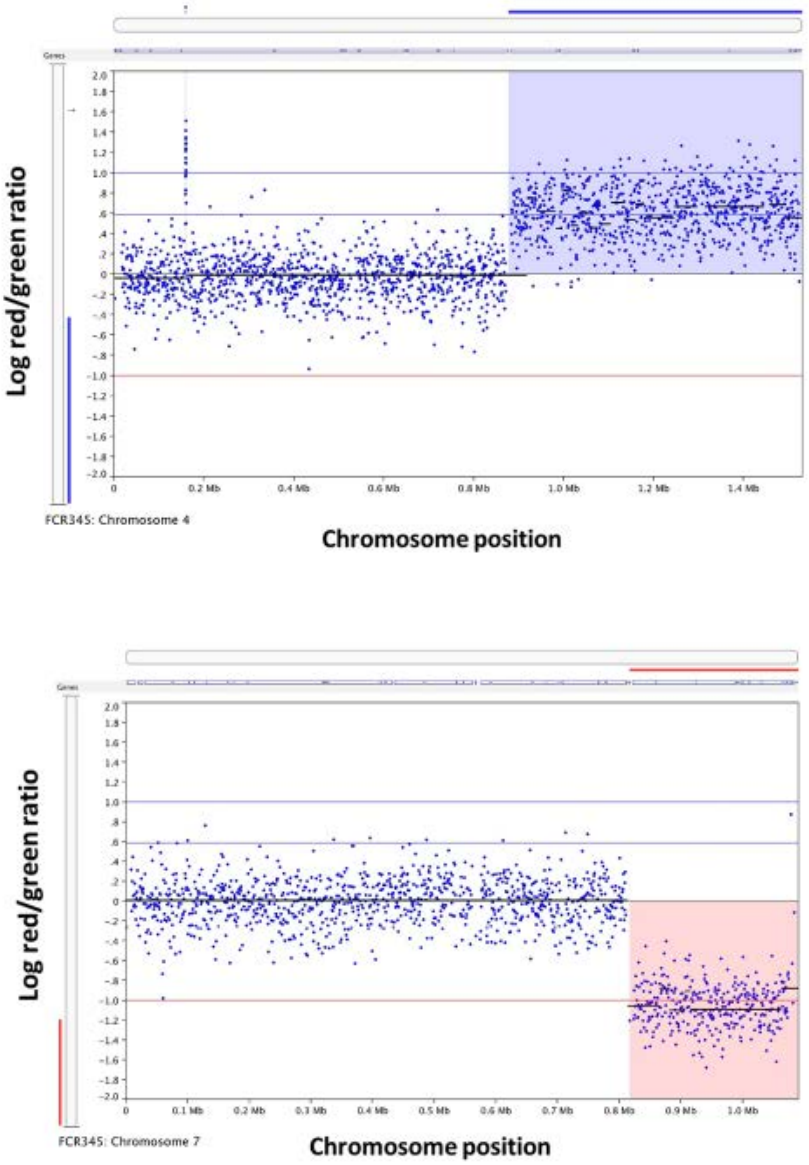

Figure 8: Array-CGH analysis of CNV associated rearrangements in Chr4 diploid strain (FCR345) exposed to 200 Gy. (A) Represents an amplification event on Chr4. **(B)** Represents a deletion event on Chr7.

3.4.5. Results of a radiation-induced Chr15 amplification event

This case shows that while the majority of events were mediated by recombination between Ty repeats, we also discovered cases of other classes of repeats (Figures 9,10) that are *EFT1*, *EFT2* duplicated genes.

FCR 337, as illustrated in Figures 9 and 10, was cloned from a culture that was previously exposed to 50 Gy of gamma rays. The PFGE for FCR 337 indicated a new band around 1700 kb. The array-CGH detected a deletion on the right arm of Chr4 from *EFT1* gene to TEL04R, and the amplification of the CHr15 containing the reporter from *EFT2* gene to TEL15R. The predicted size for the Chr4/Chr15 translocation at the *EFT* genes matched the size of the new band observed by PFGE. FCR 328, as illustrated in Figures 11 and 12, was cloned from a culture that was previously exposed to 200 Gy of gamma rays. The PFGE for FCR 328, indicated a new band around 1591kb. Analysis of the array-CGH indicated a 493 kb amplification on the right arm of Chr15 from *YDRWTy1-2* to *TEL15R*, and a 433 kb deletion on the right arm of Chr4 from *YDRWT1-4* to *TEL04R*. The new band on PFGE also matched the predicted size for the Chr4/Chr15 translocation but at a Ty breakpoint.

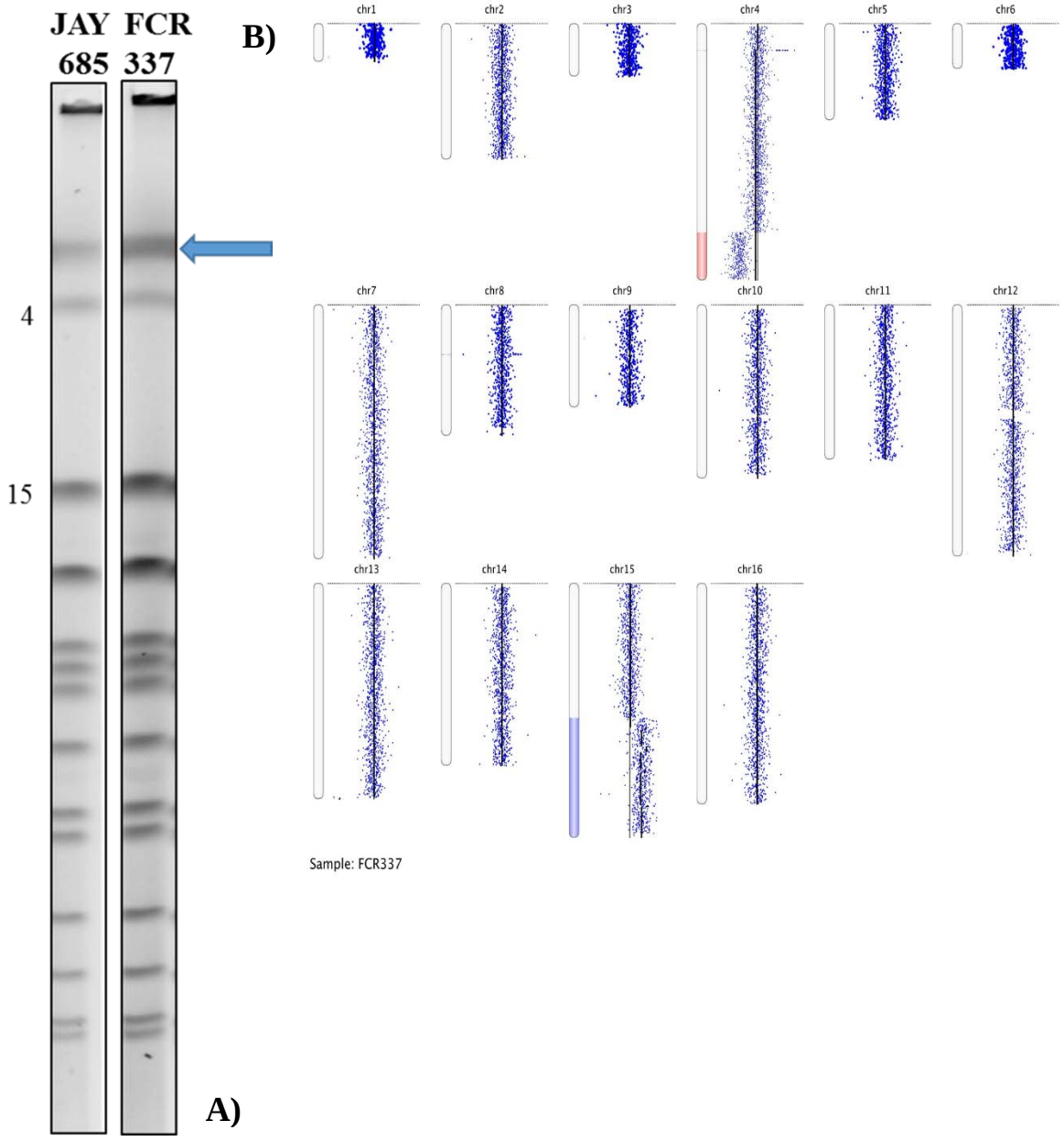

Figure 9. PFGE and array-CGH analysis of CNV-associated rearrangements in Chr15 diploid strain (FCR337) exposed to 50 Gy. PFGE **(A)** of the sample FCR 337, and the parent (left) show a new band as marked by an arrow. Whole genome CGH-array data **(B)** for this sample.

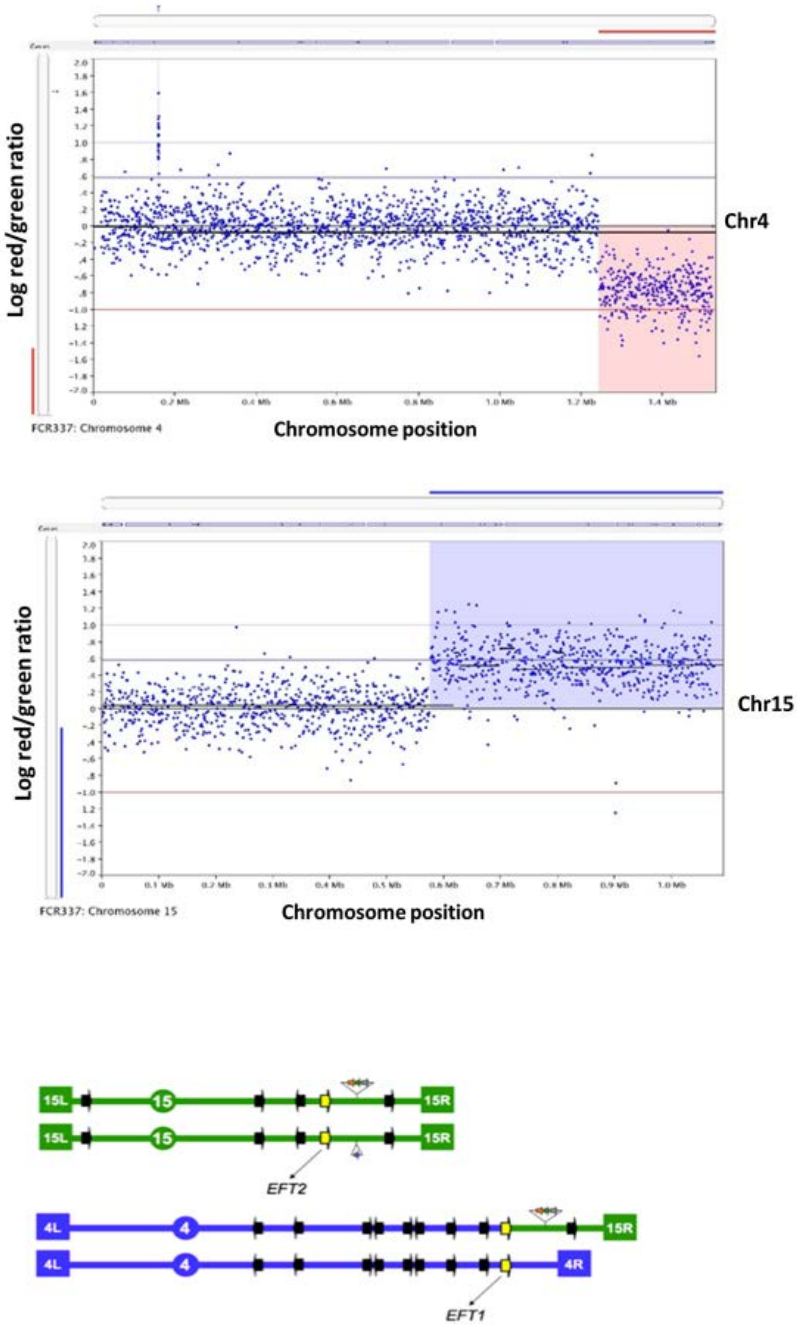

Figure 10. **Qualitative analysis of radiation-induced CNV events of Chr15 (FCR 337). (A)** Array-CGH plot for Chr15 diploid strain (FCR337). **(B)** Diagram showing the karyotype for FCR 337 was a Chr4/Chr15 reporter associated translocation event occurring at *EFT1/EFT2* sequences.

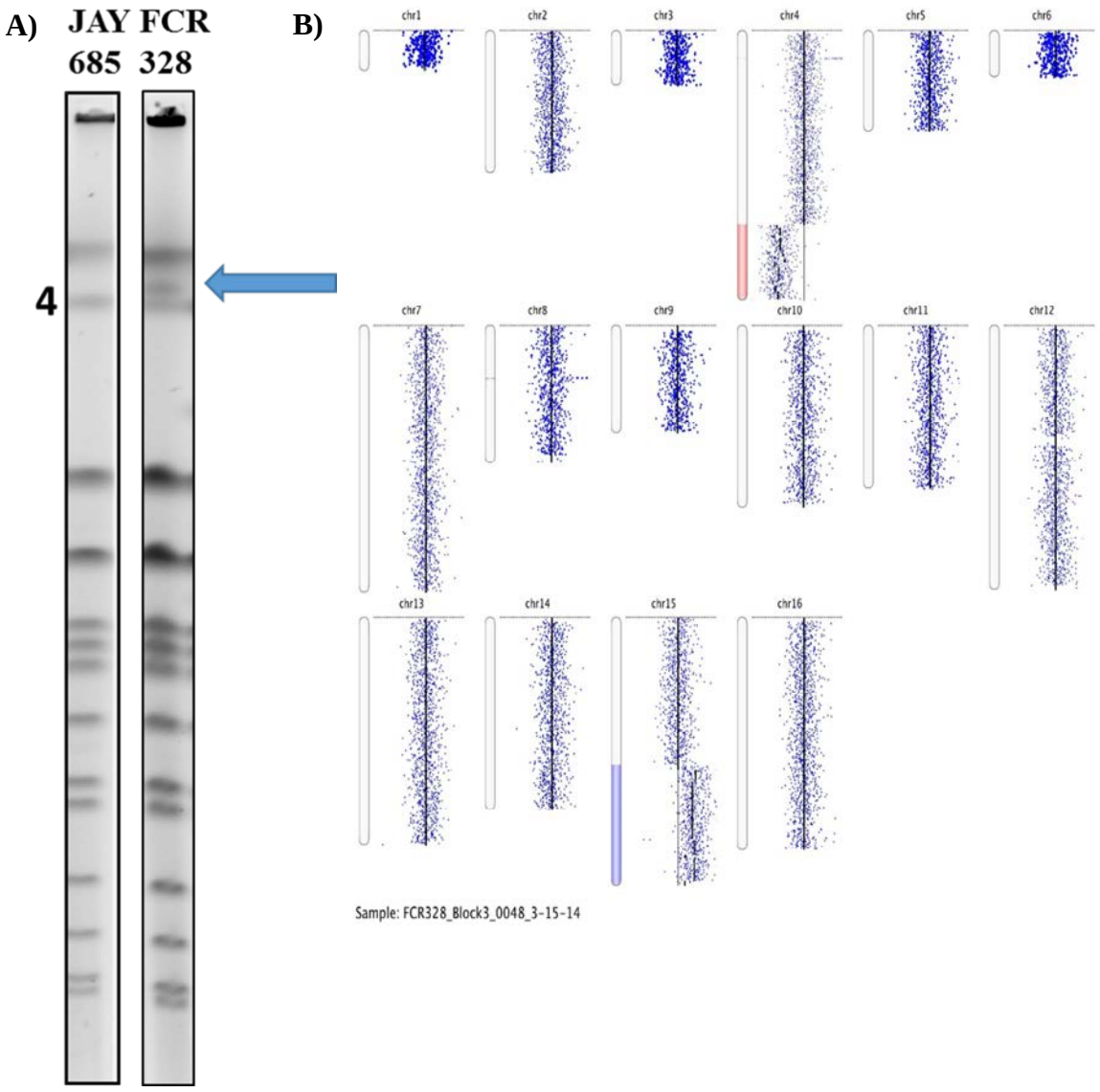

Figure 11: PFGE and array-CGH analysis of CNV associated rearrangements in Chr15 diploid strain (FCR328) exposed to 200 Gy. PFGE **(A)** of the sample FCR328, and the parent (left) show a new band as marked by the arrow. In **(B)** a whole genome CGH-array data for this sample.

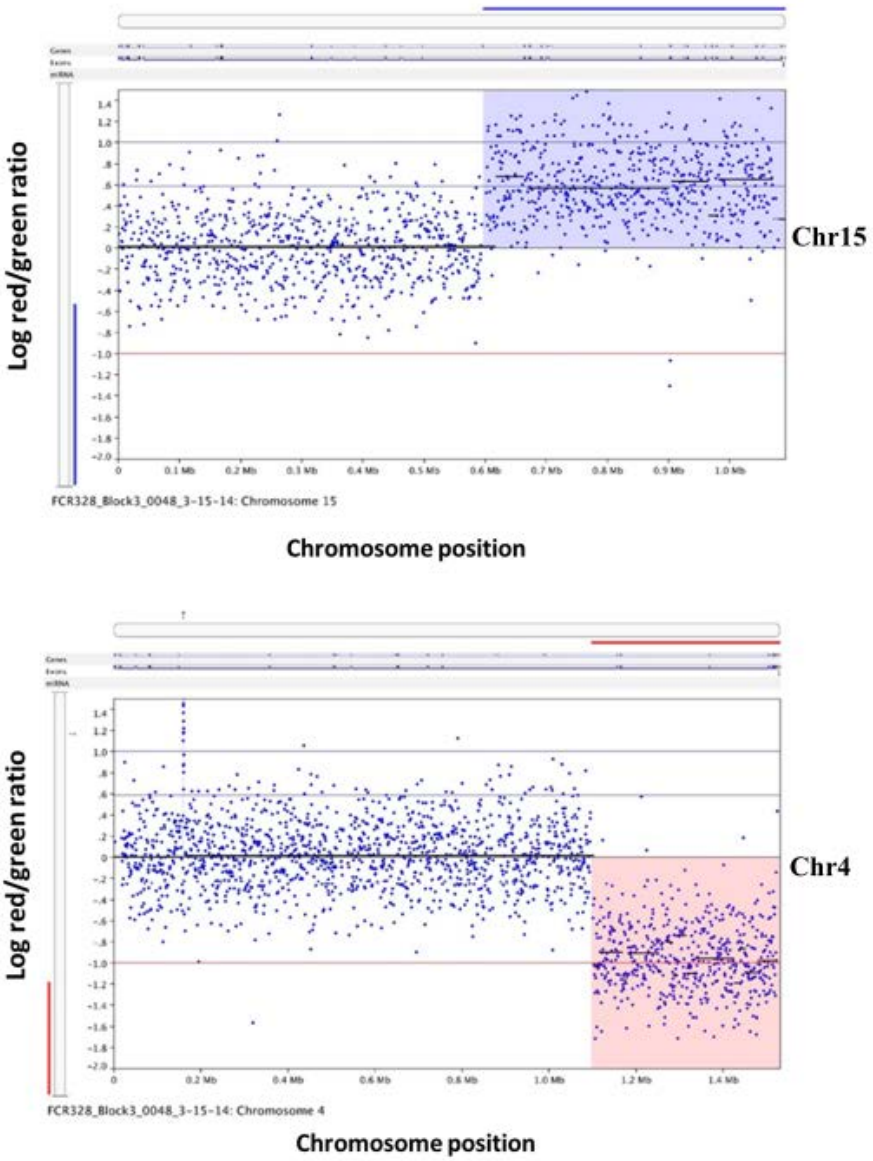

Figure 12: **Qualitative analysis of radiation-induced CNV events of Chr15 (FCR328)**. Array-CGH analysis of CNV associated rearrangements in Chr15 diploid strain (FCR328) exposed to 200 Gy. Showing two radiation-induced events on Chr15 and Chr4.

In conclusion, the primary outcome observed of gene amplification on both Chr4 and Chr15 selected FCRs resulted was unbalanced translocations involving the loss of a terminal segment of one chromosome and the gain of an extra copy of the chromosome arm which contains the reporter. One important goal of our study was to identify the recombination breakpoints associated with chromosomal rearrangements arising after exposure to an acute high dose of ionizing radiation. Since most CNV breakpoints contained repeated sequences such as Ty or conserved gene family members, the presumed molecular mechanism involved is homologous recombination, and not nonhomologous or micro-homology mediated pathways.

CHAPTER FOUR

CONCLUSIONS AND DISCUSSIONS

Ionizing radiation-induced DNA damage resulting in chromosomal rearrangements through single and double strand breaks (SSBs and DSBs, respectively) is a crucial component of cancer research. The availability of methods and assays that provide a quick and simple way to detect such alterations of the genome is invaluable. This present study showcases the development of two systems that were selected to evaluate the risk of chronic low dose ionizing radiation exposure to the human population. It is based on prior research focused on high dose exposure delivered in laboratory models. We looked for gene deletions and amplifications by selecting two research models: normal human fibroblasts, a radio-resistant cell line, and diploid cells of the budding yeast *Saccharomyces cerevisiae.* Our results from the first model, revealed instability of the Y chromosome, as three potentially radiogenic deletions were detected in proximal (SY83), and distal (SY 1191, SY1201) markers of the AZF region. Therefore a wide portion of the AZF region was affected by radiation, which is indicative of random intra chromosomal variations. These observations are in line with the suggestions made by [34], regarding random intrachromosomal breakage being a possible mechanism for radiation- induced microdeletions. We observed a difference between the frequency of copy number changes in fibroblast clones from irradiated samples, in the form of microdeletions, after an acute dose/dose rate of 4 Gy, delivered at2.030 Gy/min. No deletions were detected in the unirradiated clones. With the implementation of high-resolution genome-wide analyses, our yeast model helped us detect and evaluate copy number variations in the form of genome amplifications in normal diploid yeast cultures exposed to two acute high radiation doses. We took advantage of a previous system that used the *SFA1* and *CUP1* genes to detect amplification through their gene

dosage-dependent phenotypes [46], [49] After our study confirmed the resistance phenotype, we analyzed FCR clones for chromosome size changes using PFGE. In addition, we analyzed 25 FCR clones by array-CGH and detected both CNVs that were associated with the reporter and other CNVs that were not. Our results showed that the major mechanism associated with CNV in diploid cells is nonreciprocal translocations. This was true for both Chr4 and Chr15 when exposed to acute doses of 50 Gy or 200 Gy. As a result of high dose exposure to gamma radiation, we expected to see high mutation rates with high doses in comparison with unirradiated cultures. However, there was only a twofold increase in mutation rates following these high doses. We also detected multiple breakpoints throughout Chr4 and Chr15 used in this study, which were associated with chromosomal rearrangements mainly involving Ty repeats, with a few breakpoints occurring at other repetitive sequences (*EFT1*, *EFT2* genes). The results obtained in this study, for both unexposed and exposed cultures, confirmed that NAHR is the dominant DSB repair pathway responsible for the formation of copy number changes in yeast. In order to compare and validate systems to detect copy number variation in radiation exposed samples, we mentioned previous studies, which reported that ionizing radiation in the range of 1.5-3.0 Gy effectively induces de novo CNV mutations in cultured normal human fibroblasts [32]. In the same study, it was shown that acute IR induces de novo CNVs via mechanisms similar to that in CNVs induced by genotoxins like hydroxyurea (HU) and aphidicolin (APH). This was not expected since these studies focused on acute doses and effects, in contrast to the chronic dose effects of these genotoxins on the human genome. While comparing the two models used in this study, we observed that both systems responded to acute doses of ionizing radiation. Resulting in an increased number of events occurring in irradiated samples but not in unirradiated cultures.

These present findings in both normal human cell lines and budding yeast, have implications for assessing the genetic risk of acute radiation exposure on human genome stability, and its ability to promote the formation of copy number changes. This was performed in an attempt to build a reliable assay system for possible future studies to explore and estimate the risks related to humans exposed to chronic low-dose radiation, and the nature of the copy number alteration events to help determine the risk of radiation-induced cancer. In subsequent studies, it would be interesting to map the deletions resulting from acute radiation exposure of human fibroblasts to a dose of 4 Gy, and estimate the exact size and mechanism of these deletions. This study allowed us to determine acute-dose ionizing radiation-induced copy number alterations, in human fibroblasts in the form of deletions in the *AZF* region of the Y chromosome, as well as amplifications formed by NAHR mechanism in the yeast genome.

REFERENCES

1. Limoli, C.L., et al., *Genomic instability induced by high and low LET ionizing radiation.* Adv Space Res, 2000. **25**(10): p. 2107-17.

2. Bortoletto, E., et al., *Chromosome instability induced in the cell progeny of human T lymphocytes irradiated in G(0) with gamma-rays.* Mutagenesis, 2001. **16**(6): p. 529-37.

3. Lupski, J.R. and P. Stankiewicz, *Genomic disorders: molecular mechanisms for rearrangements and conveyed phenotypes.* PLoS Genet, 2005. **1**(6): p. e49.

4. Feuk, L., A.R. Carson, and S.W. Scherer, *Structural variation in the human genome.* Nat Rev Genet, 2006. **7**(2): p. 85-97.

5. Lupski, J.R., *Genomic rearrangements and sporadic disease.* Nat Genet, 2007. **39**(7 Suppl): p. S43-7.

6. Tang, Y.C. and A. Amon, *Gene copy-number alterations: a cost-benefit analysis.* Cell, 2013. **152**(3): p. 394-405.

7. Girirajan, S., C.D. Campbell, and E.E. Eichler, *Human copy number variation and complex genetic disease.* Annu Rev Genet, 2011. **45**: p. 203-26.

8. Iafrate, A.J., et al., *Detection of large-scale variation in the human genome.* Nat Genet, 2004. **36**(9): p. 949-51.

9. Sebat, J., et al., *Large-scale copy number polymorphism in the human genome.* Science, 2004. **305**(5683): p. 525-8.

10. Meyerson, M., S. Gabriel, and G. Getz, *Advances in understanding cancer genomes through second-generation sequencing.* Nat Rev Genet, 2010. **11**(10): p. 685-96.

11. Iskow, R.C., O. Gokcumen, and C. Lee, *Exploring the role of copy number variants in human adaptation.* Trends Genet, 2012. **28**(6): p. 245-57.

12. Abrahams, B.S. and D.H. Geschwind, *Advances in autism genetics: on the threshold of a new neurobiology.* Nat Rev Genet, 2008. **9**(5): p. 341-55.

13. Krepischi, A.C., P.L. Pearson, and C. Rosenberg, *Germline copy number variations and cancer predisposition.* Future Oncol, 2012. **8**(4): p. 441-50.

14. Girirajan, S., et al., *Relative burden of large CNVs on a range of neurodevelopmental phenotypes.* PLoS Genet, 2011. **7**(11): p. e1002334.

15. Zhang, J.P., et al., *Genetic variation in BDNF is associated with antipsychotic treatment resistance in patients with schizophrenia.* Schizophr Res, 2013. **146**(1-3): p. 285-8.

16. Hanahan, D. and R.A. Weinberg, *The hallmarks of cancer.* Cell, 2000. **100**(1): p. 57-70.

17. Lonigro, R.J., et al., *Detection of somatic copy number alterations in cancer using targeted exome capture sequencing.* Neoplasia, 2011. **13**(11): p. 1019-25.

18. Shlien, A. and D. Malkin, *Copy number variations and cancer.* Genome Med, 2009. **1**(6): p. 62.

19. Weir, B.A., et al., *Characterizing the cancer genome in lung adenocarcinoma.* Nature, 2007. **450**(7171): p. 893-8.

20. Temam, S., et al., *Epidermal growth factor receptor copy number alterations correlate with poor clinical outcome in patients with head and neck squamous cancer.* J Clin Oncol, 2007. **25**(16): p. 2164-70.

21. Conrad, D.F., et al., *Origins and functional impact of copy number variation in the human genome.* Nature, 2010. **464**(7289): p. 704-12.

22. Bunyan, D.J., et al., *Dosage analysis of cancer predisposition genes by multiplex ligation-dependent probe amplification.* Br J Cancer, 2004. **91**(6): p. 1155-9.

23. Yoshihara, K., et al., *Germline copy number variations in BRCA1-associated ovarian cancer patients.* Genes Chromosomes Cancer, 2011. **50**(3): p. 167-77.

24. Diskin, S.J., et al., *Copy number variation at 1q21.1 associated with neuroblastoma.* Nature, 2009. **459**(7249): p. 987-91.

25. Turner, N., A. Tutt, and A. Ashworth, *Hallmarks of 'BRCAness' in sporadic cancers.* Nat Rev Cancer, 2004. **4**(10): p. 814-819.

26. Zhang, F., et al., *Copy number variation in human health, disease, and evolution.* Annu Rev Genomics Hum Genet, 2009. **10**: p. 451-81.

27. Lee, J.A., C.M. Carvalho, and J.R. Lupski, *A DNA replication mechanism for generating nonrecurrent rearrangements associated with genomic disorders.* Cell, 2007. **131**(7): p. 1235-47.

28. Lam, K.W. and A.J. Jeffreys, *Processes of de novo duplication of human alpha-globin genes.* Proc Natl Acad Sci U S A, 2007. **104**(26): p. 10950-5.

29. Malhotra, D. and J. Sebat, *CNVs: harbingers of a rare variant revolution in psychiatric genetics.* Cell, 2012. **148**(6): p. 1223-41.

30. Yu, K. and M.R. Lieber, *Nucleic acid structures and enzymes in the immunoglobulin class switch recombination mechanism.* DNA Repair (Amst), 2003. **2**(11): p. 1163-74.

31. Arlt, M.F., et al., *Hydroxyurea induces de novo copy number variants in human cells.* Proc Natl Acad Sci U S A, 2011. **108**(42): p. 17360-5.

32. Arlt, M.F., et al., *Copy number variants are produced in response to low-dose ionizing radiation in cultured cells.* Environ Mol Mutagen, 2014. **55**(2): p. 103-13.

33. Arlt, M.F., et al., *De novo CNV formation in mouse embryonic stem cells occurs in the absence of Xrcc4-dependent nonhomologous end joining.* PLoS Genet, 2012. **8**(9): p. e1002981.

34. Premi, S., et al., *Unique signatures of natural background radiation on human Y chromosomes from Kerala, India.* PLoS One, 2009. **4**(2): p. e4541.

35. Moghbeli-Nejad, S., et al., *Genome instability in AZFc region on Y chromosome in leukocytes of fertile and infertile individuals following exposure to gamma radiation.* J Assist Reprod Genet, 2012. **29**(1): p. 53-61.

36. Eric J.Hall., G., Amato, *radiobiology for the Radiologist 7th Edition*. 2011.

37. Dubrova, Y.E. and M.A. Plumb, *Ionising radiation and mutation induction at mouse minisatellite loci. The story of the two generations.* Mutat Res, 2002. **499**(2): p. 143-50.

38. Dubrova, Y.E., et al., *Elevated minisatellite mutation rate in the post-chernobyl families from ukraine.* Am J Hum Genet, 2002. **71**(4): p. 801-9.

39. Goncalves, J. and J. Lavinha, *[Y chromosome and male infertility]*. Acta Med Port, 1998. **11**(4): p. 365-72.

40. Tiepolo, L. and O. Zuffardi, *Localization of factors controlling spermatogenesis in the nonfluorescent portion of the human Y chromosome long arm.* Hum Genet, 1976. **34**(2): p. 119-24.

41. Gadbois, D.M. and B.E. Lehnert, *Temporal position of G1 arrest in normal human fibroblasts after exposure to gamma-rays.* Exp Cell Res, 1997. **232**(1): p. 161-6.

42. McCulley, J.L. and T.D. Petes, *Chromosome rearrangements and aneuploidy in yeast strains lacking both Tel1p and Mec1p reflect deficiencies in two different mechanisms.* Proc Natl Acad Sci U S A, 2010. **107**(25): p. 11465-70.

43. Argueso, J.L., et al., *Double-strand breaks associated with repetitive DNA can reshape the genome.* Proceedings of the National Academy of Sciences, 2008. **105**(33): p. 11845-11850.

44. Chen, C. and R.D. Kolodner, *Gross chromosomal rearrangements in Saccharomyces cerevisiae replication and recombination defective mutants.* Nat Genet, 1999. **23**(1): p. 81-5.

45. Narayanan, V., et al., *The pattern of gene amplification is determined by the chromosomal location of hairpin-capped breaks.* Cell, 2006. **125**(7): p. 1283-96.

46. Zhang, H., et al., *Gene copy-number variation in haploid and diploid strains of the yeast Saccharomyces cerevisiae.* Genetics, 2013. **193**(3): p. 785-801.

47. Lemoine, F.J., et al., *Chromosomal translocations in yeast induced by low levels of DNA polymerase a model for chromosome fragile sites.* Cell, 2005. **120**(5): p. 587-98.

48. Morrison, A., et al., *Eukaryotic DNA polymerase amino acid sequence required for 3'---5' exonuclease activity.* Proc Natl Acad Sci U S A, 1991. **88**(21): p. 9473-7.

49. Stanton, J., *A Yeast-Based Assay System for the Study of Environmentally Induced Copy Number Variation*, in *Environmental and Radiological Health Sciences*. 2012, Colorado State University. p. 82.

50. Wach, A., et al., *New heterologous modules for classical or PCR-based gene disruptions in Saccharomyces cerevisiae.* Yeast, 1994. **10**(13): p. 1793-808.

51. Payen, C., et al., *Segmental duplications arise from Pol32-dependent repair of broken forks through two alternative replication-based mechanisms.* PLoS Genet, 2008. **4**(9): p. e1000175.

52. Lea, D.E. and C.A. Coulson, *The distribution of the numbers of mutants in bacterial populations.* J Genet, 1949. **49**(3): p. 264-85.