

Mechanisms of c-Myc Dependent Genomic Instability

By

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Abstract

Cancer is a disease that involves genomic instability, to which c-Myc contributes during its initiation and progression. Over 70% of all human cancers show deregulated levels of c-Myc protein. The term genomic instability refers to genetic and/or epigenetic changes that alter the normal organization and function of genes and chromosomes. Genomic instability is a hallmark of cancer and often is associated with cancer. Deregulated c-Myc expression generates genomic instability by initiating intra- and extrachromosomally locus-specific gene amplification, gene rearrangements and karyotypic instability that includes translocations, fusions, insertions and deletions. Out of the several outlined pathways by which deregulated levels of c-Myc can lead to genomic instability, the work described in this thesis focuses on three with direct relevance to tumorigenesis; gene amplification (increase in gene copy number), remodeling of the chromosomal and telomeric structures in the interphase nucleus and comparing the effect of Myc to that of Epstein Bar virus (EBV) infection in remodeling the nuclear structure that may lead to genomic instability.

We show that amplification of the Myc target genes *dihydrofolate reductase (DHFR)* and *cyclin D2 (CCND2)* upon Myc deregulation in mammalian immortalized cells is replication driven. We also show evidence that Myc attenuates the dissociation of the replication initiation complex in those cells which may lead to rounds of rereplication and result in gene amplification after few cell divisions.

We also show that Myc deregulation induces the formation of telomeric aggregates which remodel the three dimensional (3D) structure of the interphase nucleus. The aggregates lead to bridge-breakage-fusion (B-B-F) cycles and the generation of

several forms of karyotypic abnormalities including fusions and unbalanced translocations. Remodeling of the nuclear organization through the formation of telomeric aggregates also increased the frequency of chromosomal translocation between specific pairs of chromosomes. This highlights that the telomeric aggregates remodeled the spatial distribution of chromosomes inside the nucleus.

Finally we describe distinct types of 3D nuclear telomeric signatures in EBV-carrying established lymphoblastoid cell lines, in *ex vivo* B-cells freshly infected with EBV, in EBV-positive Burkitt's lymphoma (BL) cell lines and following the conditional expression of the EBV transcription factor EBV- nuclear antigen 2 (EBNA2) or Myc. We conclude that EBV infection promotes both nuclear telomere remodeling and chromosomal instability.

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List of Abbreviations

2D	Two-dimensional
3D	Three-dimensional
4HT	Four hydroxytamoxifen
ALL	Acute lymphocytic leukemia
ANOVA	Analysis of variance
ARS	Autonomously replicating sequence
ATL	Adult T-cell leukemia
B-B-F	Bridge-breakage-fusion
bHLHZip	Basic/helix-loop helix/leucine zipper
BL	Burkitt's lymphoma
BSA	Bovine serum albumin
B&W	Black and white
CCD	Charge coupled device
<i>CCND2</i>	Cyclin D2
CD	Cluster of differentiation
Cdc	Cell cycle dependent cyclines
CDK	Cycline dependent kinase
cDNA	Complementary DNA
CK2	Casein kinase II
CycC	Cyclin C
DAPI	4',6-diamidino-2-phenylindole
dCTP	Deoxycytosine triphosphate
dATP	Deoxyadenosine triphosphate
<i>DHFR</i>	Dihydrofolate reductase
dic	Dicentric
DIG	Digoxigenin
DLCL	Diffuse large cell lymphoma
DM	Double minutes
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DSBs	Double-strand breaks
E	Estrogen
EBNA1	EBV nuclear antigen 1
EBNA2	EBV- nuclear antigen 2
EBV	Epstein Bar virus
EEs	Extrachromosomal elements
EGF	Eukaryotic growth factor
ER	Estrogen receptor
<i>F</i> values	Frequency values
FBS	Fetal bovine serum
<i>FISH</i>	Fluorescent in situ hybridization
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase
GLM	General linear Modeling
HAM	HTLV-I-associated myelopathy

HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
HHV-8	Human herpesvirus 8
HIV	Human immunodeficiency
HMGI(Y)	High mobility group protein I(Y)
HPV	Human papilloma virus
HR	Homologous recombination
HRP	Horse reddish peroxidase
hrs/h	Hours
HSV	Herpes simplex virus
HTLV-I	Human T-cell leukemia virus type I
IgG	Immunoglobulin G
IgH	Immunoglobulin H
IHC	Immunohistochemistry
IP	Immunoprecipitation
JCV	John Cunningham virus
Kb	Kilobases
kDa	Kilodalton
LCL	Lymphoblastoid
Lit/L	Liter
LMP	Latent membrane protein
MBI	Myc box I
MBII	Myc box II
MCM	Minichromosome maintenance
MHRC	Manitoba Health Research Council
Min	Minutes
MM	Multiple myeloma
mmol	Milli molar
MOPC	Mouse plasma cytoma
mRNA	Messenger RNA
msec	Milli seconds
NBL	Neuroblastoma
ng	Nanogram
NHEJ	Non homologous end joining
nM	Nanomolar
nm	Nanometer
NPC	Nasopharyngeal carcinoma
NPC	Nuclear pore complexes
ODC	Ornithine deoxycarboxylase
Orc	Origin recognition complex
<i>P</i> values	Probability values
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCL	primary plasma cell leukemia
Q-FISH	Quantitative FISH

R1	Ribonucleotide reductase 1
R2	Ribonucleotide reductase 2
Rb	Retinoblastoma
RNA	Ribonucleic acid
ROI	Region of interest
ROS	Reactive oxygen species
rpm	Rotation per minute
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SCC	Squamous cell carcinoma
SCLC	Small cell lung carcinoma
SDS	Sodium dodecyl sulphate
sec	Seconds
SKY	Spectral karyotyping
SSC	Saline-sodium citrate
SV40	Simian virus 40
TAs	Telomeric aggregates
TAD	Transactivation domain
TBE	Tris/borate/EDTA
TRF2	Telomere repeat binding protein
TSP	Tropical spastic paraparesis
U	Units
UV	Ultra violet
V	Volts
μ M	Micromolar

1.0 Introduction

1.1 History of Cancer

The origin of the word cancer is credited to the Greek physician Hippocrates (460-370 BC.), the "Father of Medicine." Hippocrates used the terms *carcinoma* and *carcinoma* to describe non-ulcer forming and ulcer-forming tumors. In Greek those two words refer to a crab, most likely applied to the disease because the finger-like spreading projections from cancer resembling the shape of a crab (Karpozilos, 2004).

Human cancer is probably as old as the human race. It is obvious that cancer did not suddenly start appearing after modernization or industrial revolution. Diseases that are thought to be rare today have had a long history. The oldest available specimen of a human cancer is found in the remains of skull of a female who lived during the Bronze Age (1900-1600 BC). The tumor in the women's skull was suggestive of head and neck cancer. The mummified skeletal remains of Peruvian Incas, dating back 2400 years ago, contained abnormalities suggestive of malignant melanoma. Cancer was also found in fossilized bones recovered from ancient Egyptians. Louis Leakey found the oldest possible hominid malignant tumor in 1932 from the remains of a body, which could be either that of *Homo erectus* or an *Australopithecus*. This tumor had features suggestive of a Burkitt's lymphoma (Kardinal and Yarbrow, 1979).

The oldest known description of human cancer is found in seven Egyptian papyri or writings that dated between 3000-1500 BC. Two of them are known as the "Edwin Smith" and "George Ebers" papyri. They contain details of conditions that are consistent with modern descriptions of cancer. The Edwin Smith papyrus, describes 8 cases of tumors or ulcers of the breast. The document acknowledged that there is no treatment for

this condition and recommended cauterization (the fire drill) as a palliative measure (Breasted, 1922).

The ancient Egyptian medicine typically mixed medicine and religion. Old Egyptian physicians probably treated patients for several forms of cancer. Although Hippocrates is credited for being the first to recognize the difference between benign and malignant tumors, Hieroglyphic inscriptions and papyri manuscripts suggested that these ancient physicians were able to distinguish between benign and malignant tumors. They described that the surface tumors may be removed surgically much similar to the current medical practice. Compounds of barley, pigs' ears and other indigenous materials were prescribed as treatment for cancer of the stomach and the uterus. Other commonly dispensed medications included ointments, enemas, castor oil, suppositories, poultices and animal parts (Gallucci, 2000).

Figure 1. 1 The Edwin Smith papyrus, the world's oldest surviving surgical document. Written in hieratic script in ancient Egypt around 1600 BC (Beasted JH, 1922).



After the above mentioned discoveries, thousands of years had past without documented discoveries in cancer specifically during the Renaissance. Between the 15th - 17th centuries European scientists were able to develop better understanding of the human body. Galileo and Newton began to develop and use scientific methods, which were later used to study disease. For example autopsies performed by Harvey (1628), allowed an understanding of the circulation of blood through the heart and the body that had been a mystery till then (Skinner, 2001).

In 1761, Giovanni Morgagni was the first to perform autopsies to relate the patient's illness to the pathologic findings after death. This laid the foundation for scientific oncology, the study of cancer. In the same century, the Scottish surgeon John Hunter (1728-1793) suggested that some cancers might be cured by surgery and described how the surgeon might decide which cancers to operate on. If the tumor had not invaded nearby tissue and was "movable," he considered that there is no harm in removing it. A century later the development of anesthesia allowed surgery to flourish and the classic cancer operations such as radical mastectomy were developed (Grange et al., 2002).

Several major discoveries followed in the 18th and 19th centuries including the Dutch Scientist Hermann Boerhave (1668-1738) who suggested that inflammation could lead to cancer, Later on towards the end of the same century the German scientist Rudolph Virchow, proposed that chronic inflammation – the site of a wound that never heals- was the cause of cancer. The French physician Le Dran (1685-1770) described that breast cancer cells could migrate to the lymph nodes and lead to poorer prognosis. However, in 1829, the French gynecologist Recamier was the first to use the term

metastasis in the 19th century. Also in 1845 the Scottish physician Bennett in Edinburgh described leukemia as an excessive proliferation of blood cells. Early in the 20th century, the distinguished German embryologist, Theodore Boveri (1914), suggested that chromosomal imbalance, what we call today “genomic instability” lead to the initiation of cancer. By the middle of the 20th century, scientists had in their hands the instruments needed to begin solving the complex problems of chemistry and biology presented by cancer. Understanding how genes worked and how they could be damaged by mutations made it possible to pinpoint the exact site of the damage to a specific gene (Harvey, 1974).

1.2 What is Cancer?

Cells are the smallest units in all living organisms, including the human body. Cell growth and cell division are essential events that are involved in natural process of replacing aging or apoptotic cells. Normal body cells do grow, divide, and die in a controlled fashion. Cellular growth, division and death are strictly regulated by cellular control mechanisms and checkpoints. During the process of cell division one cell may acquire some genetic mutations that would alter the cell division control mechanisms of that cell. In general cancer develops when the cellular growth control mechanisms are disrupted. When this happens cells begin to grow neglecting all growth control mechanisms resulting in cancer (Shimkin, 1976).

The human body includes many different cell types that behave differently from each other in their protein expression profile, life span and certainly function. Most cancer cases (90%-95%) are sporadic and occur by chance. It is likely that only 5%-10%

of the disease occurs because of an inherited predisposition or gene defaults. These inherited gene defaults, though comparatively rare give rise to a high lifetime risk of developing cancer. In families where there is an inherited predisposition, cancer tends to occur at a younger age. Moreover, the same type of cancer may affect several family members and in some cases there may be unusual cancers or two different cancers that affect a single individual. It is then thought that a further 10%-20% of breast, colorectal and ovarian cancers may be caused by other inherited "medium risk" gene defaults which can also give rise to significant increased risk (Wu et al., 2002).

As mentioned, normal cell division may result in a low level of spontaneous mutations during different cellular processes like DNA replication, also spontaneous DNA damage can result from cytosine deamination at physiological temperatures, from oxidative damage associated with normal cellular physiology or from low risk mutagens that are present in food, air, or water (Loeb, 1989).

1.3 Cancer is a multi-step process.

Many steps are required to convert a normal cell into a cancerous one. The cancer cell must be able to multiply under conditions that a normal cell would not and to be able invade surrounding tissue and spread throughout the body. Both genetic changes, such as activation of oncogenes or inactivation of tumor suppressor genes, and epigenetic changes that may induce stimulation of cell proliferation, contribute to the development of cancers (Bishop, 1987).

Chemical agents can increase the probability of malignant transformation by inducing mutations that can ultimately lead to tumor formation, by promoting the

development of tumors in cells with preexisting genetic damage or by increasing the rate of acquisition of malignant traits by benign tumors. Chemical carcinogens are structurally diverse, but all initiating agents are either already electrophiles or can be converted to electrophilic reactants through metabolic activation and hence may react with DNA forming adducts that can initiate cancer (Shelby, 1988; Loeb and Harris, 2008).

Genetic and environmental factors can alter an individual's ability to metabolize carcinogens, to repair DNA damage or to respond to mitogenic stimuli, all of which can alter susceptibility to chemical carcinogenesis. The incidence and time required for appearance of tumors appear to be dose-related, but the existence of no-effect doses of carcinogens remains controversial (Kato et al., 1990; Nielsen and Ovrebo, 2008).

Cancer is a major chronic health problem that may be associated with toxicological substances. The long latency period of cancer induction is a major problem in the evaluation of toxicological hazards and risk assessment. The underlying reasons for the time requirement in cancer initiation is now clear, at least in part. For a normal cell to evolve into a cancer cell, multiple transmissible changes within the individual cell are required. One of the underlying premises of most multistep models of carcinogenesis is that genetic and/or epigenetic alterations of multiple, independent genes are involved. The process of carcinogenesis is often separated operationally into three stages; initiation, promotion, and progression, but the number of genetic changes involved in each of these operationally defined stages has not yet been determined (Pitot, 1986; Rajaraman et al., 2006). Initiation involves the induction of an irreversibly altered cell and is frequently equated with a mutational event. The mechanisms of initiation may vary, however, in

different tissues or with different initiators in the same tissue (Quintanilla et al., 1986; Ashkenazi et al., 2008).

Promotion is the experimentally defined process by which the initiated cell clonally expands into a visible tumor, often a lesion such as a papilloma (Scheinfeld, 2006). This process undoubtedly involves epigenetic factors that selectively influence the proliferation of the initiated cell (Blanco et al., 2007). These cells must undergo one or more additional transmissible changes during the progression to a malignant neoplasm.

The number of genes involved in neoplastic development is not known with certainty. Estimates of as many as ten or more mutational changes have been proposed to occur in adult human cancers (Callahan, 1989; Pichiorri et al., 2008). These findings are consistent with “*multi-hit*” model developed on the basis of specific incidence rates of cancers increasing exponentially with the fifth to seventh power of age (Callahan, 1989; Pichiorri et al., 2008).

Analysis of multistep carcinogenesis at the molecular level, therefore, indicates that the process of neoplastic evolution is significantly more complicated than the relatively simple two stage (initiation and promotion) model of carcinogenesis or even a three-stage model of initiation, promotion, and progression. Thus, the progression phase of carcinogenesis represents multiple stages that might influence the neoplastic process (Vesselinovitch et al., 1979; Nam et al., 2005).

There are three general mechanisms by which a substance can influence the multistep carcinogenic process. 1. A substance can induce a heritable alteration in one or more critical genes. 2. The change caused by the substance may impact the epigenetic program of the cell. Although considerable insight into the mechanisms of genetic

changes by chemicals exists, little is known about the mechanisms of carcinogen-induced epigenetic, heritable changes. 3. A substance can influence multistep carcinogenesis is the facilitation of clonal expansion of an initiated or intermediate cell, which increases the probability of additional, spontaneous (mutational or epigenetic) transmissible changes (Blanco et al., 2007).

1.4 Genomic Instability and Cancer

The presence of an abnormal chromosomal content is probably the most universally conserved hallmark of cancer cells. Predicted at the beginning of the 20th century as the origin of tumors, and extensively documented thereafter, genomic instability lies at the core of neoplastic transformation (Boveri, 1912). Nowadays, genetic, or genomic, instability refers to a series of observed genetic changes occurring at an accelerated rate in cell populations derived from the same ancestral precursor (Hoeijmakers, 2001).

Theodor Boveri, a distinguished German embryologist, postulated more than 100 years ago that cancer cells may arise from a single multipolar cell division (Boveri, 1912). He proposed that genomic instability is present in all tumor cells and it could be the source of neoplastic transformation. In his publication, Boveri described genomic instability as an imbalance in the number of chromosomes during cell division due to a defect in the centromere (Manchester, 1995). This type of genetic instability is now known as aneuploidy (Hanks et al., 2004). Extensive research over the past few decades revealed that genomic instability is not limited to whole chromosomal gain or loss but

involves a variety of abnormalities including deletions, insertions, translocations, gene amplifications and even point mutations (Boehm and Hahn, 2005).

The majority of the genetic alterations contributing to the malignant transformation are seen in growth regulatory genes and in genes involved in cell cycle progression and arrest. Genomic instability in the form of alterations in the length of short repeat stretches of coding and non-coding DNA result in microsatellite instability. Tumors with such profiles are referred to as exhibiting a “*mutator*” phenotype, which is commonly a consequence of inactivating mutations that take place in DNA damage-repair genes (Bignold, 2004).

Neoplastic cells typically possess numerous genomic lesions which may include sequence alterations (point mutations, small deletions, and insertions) and/or gross structural abnormalities in one or more chromosomes (large-scale deletions, rearrangements, gene amplifications). Based upon this general observation cancer cells are genetically unstable and the acquisition of genomic instability represents an early step in the process of carcinogenesis and a general feature of many human tumors (Vineis et al., 2003; Jeggo, 2005).

Regardless of the form of genetic instability whether it is aneuploidy, chromosomal translocations, gene amplification, point mutations, gene deletion or silencing, unstable cells now have mutations that may render resistance. For example inhibition of BubR1 or overexpression of Aurora kinase A, the spindle checkpoint regulators implicated in chromosomal instability, promote resistance to treatment by microtubule inhibitors. More common in leukemias and small cell lung cancer, mutations in cellular apoptotic machinery especially p53 can render cells resistant against apoptosis

inducing drugs (Scarfi et al 2003). Furthermore, it has been shown in several cancers that the high rate of mutations taking place in genetically unstable cells lead to high levels of heterogeneity and explain the rapid appearance and disappearance of multidrug resistance in tumor cells (Scarfi et al., 2003; Stock and Bialy, 2003; Duesberg et al., 2004).

Therefore, if a mutation is selected, then genetic instability is selected too so one can say genetic instability provides a means for selection (Nowell, 1976; Rubin, 1999; Marx, 2002).

The increased gene dosage by itself in the absence of gene mutations is strongly associated with the formation of a number of cancers. Thus, the aneuploidy-driven theory of carcinogenesis suggests that disruption of gene dosage has a significant role in the initiation of cancer formation (Boland and Ricciardiello, 1999; Fodde and Smits, 2002; Albertson et al., 2003).

Another form of instability that may lead selection and clonal expansion is telomere length and telomerase activity that are important in maintaining chromosomal structure and in regulating the normal lifespan of a cell. It has been shown that telomeres may play a role in both suppressing and facilitating instability and malignant transformation (Desmaze et al., 2004; Cosme-Blanco and Chang, 2008). In addition to such direct sequence and structural changes, gene silencing through the hypermethylation of promoter regions, or increased gene expression through the hypomethylation of such regions form an alternative epigenetic mechanism also leading to instability (El-Osta, 2004; Jacinto et al., 2007). Carcinogenesis can also result from aberrations of genomic DNA methylation that include hypermethylation and hypomethylation of promoter or first exon of cancer-related genes. Changes in methylation of promoter or first exon may

mimic the effect of mutations of tumor suppressor genes by inactivating the gene (Jacinto et al., 2007).

Hypermethylation of promoter and first exon of various tumor suppressor genes causes their transcriptional silencing. However, hypomethylation of regulatory DNA sequences activates transcription of protooncogenes, retrotransposons, as well as genes encoding proteins involved in genomic instability and malignant cell metastasis. Tumor suppressor gene products are normally involved in holding cellular growth at the checkpoint and inhibit expression of the tumorigenic phenotype. Inactivation or loss of tumor suppressor gene products removes a barrier of normal proliferation, which may result in malignant transformation (El-Osta, 2004; Jacinto et al., 2007). For example silencing the *MLH1* gene expression by its promoter methylation stops the formation of MLH1 protein, and prevents the normal activation of the DNA mismatch repair gene. This is an important cause for genomic instability and cell proliferation to the point of colorectal cancer formation (Au, 1993). Moreover, genomic instability allows numerous genetic and epigenetic alterations to accumulate during carcinogenesis without markedly changing phenotype until they are qualitatively or quantitatively sufficient to be selectively advantageous in the tumor microenvironment (Au, 1993; Nakanishi et al., 2006; Laconi et al., 2008).

In any case, it is evident that genomic instability represents a general feature of neoplasia that can be acquired through a genetic process and is thus mechanistically related to neoplastic development (Drake and Baltz, 1976; Digweed, 1993; Laconi et al., 2008).

1.5 Tumor Viruses

Viruses are obligatory intracellular parasites and hence their life cycles are irrevocably coupled to that of their host cells. Due to the limited coding capacity of viral genomes that is imposed by packaging limits, viruses had to develop strategies to target host cellular regulatory mechanisms and reprogram them for their own benefit (Weinberg, 1997; Carbone et al., 2004).

It has been known for more than four decades that members of different virus families can induce chromosome damage in infected cells, and chromosome breakages have been observed in leukocytes isolated from patients experiencing systemic viral active infections (Nichols, 1970; Fortunato and Spector, 2003). In recent years evidence has accumulated indicating the ability of different viruses to induce abnormal mitosis and consequently genetic instability *in vitro* (Aliab'eva et al., 1970; Munger et al., 2006).

The concept of viral carcinogenesis was originally derived from studies with animal-viruses with infectious entities, many of which were later identified as retroviruses and were shown to cause formation of malignant tumors (Huang and Baltimore, 1970). Retrovirus may contribute to carcinogenesis by insertion mutagenesis, whereas integration of provirus in host cell genomes causes deregulated expression of a cellular protooncogene or disruption of tumor suppressor genes (Jolicoeur et al., 1978; Zimonjic et al., 2001; Sugimoto et al., 2004).

Integration of a provirus upstream of a protooncogene in some situations may produce a chimeric virus–cell transcript. Alternatively, recombination during replication could lead to incorporation of the cellular gene into the viral genome (Kung et al., 1991; Li et al., 1997). Typically, the inserted cellular sequences are copies of spliced transcripts,

containing no introns (cDNA). Since this process is generally associated with deletions in viral coding sequences, many oncogenic retroviruses are intrinsically defective for completing the infectious life cycle, and require normal “helper” viruses for replication (Fourel et al., 1990).

It is well known that viruses can transform non permissive cells and several human viruses cause tumors if introduced in experimental animals. However in human, virus associated tumors develop in permissive tissues. It was repeatedly shown that six viruses are consistently associated with the onset of tumors in human (zur Hausen, 2001b). Etiologically this accounts for up to 20% of all cancer cases worldwide. Furthermore, viruses have been shown to influence tumor sustainment and progression and induce escape pathways from apoptosis and immunosurveillance (Fortunato and Spector, 2003).

Tumor viruses were first detected early in the twentieth century, with the cell-free transmission of human warts by Ciuffo in 1907, of chicken leukemia by Ellermann and Bang in 1908 and of a chicken sarcoma by Rous in 1911. These results were initially judged to be irrelevant, because leukemia was not then considered to be a form of cancer, the chicken was viewed as too unrelated to humans to be meaningful and cancer in humans was not contagious, so transmissibility in chickens was not seen as applicable to human disease. Murine leukemia viruses were demonstrated to be transmissible to newborn animals by Gross in 1950 (Rothermell et al, 1997). The following two decades witnessed the isolation of several animal viruses, including many retroviruses having tumorigenic properties in animals. However, attempts to isolate similar viruses from humans was not successful, raising doubts at that time that human cancer viruses existed. During this time, the human herpesvirus Epstein–Barr virus (EBV) was observed by

electron microscopy in cultured cells from Burkitt's lymphoma (BL) in 1964 and the hepadnavirus hepatitis B virus (HBV) virion was visualized in human sera positive for hepatitis B surface antigen in 1970 (Epstein and Barr, 1964; Dane et al., 1970; Poiesz et al., 1980), while novel types of a genital human papilloma virus (HPV) were isolated from cervical cancer lesions around 1983 (Kitagawa et al., 1996).

EBV infects B lymphocytes and immortalizes them by amplification of CD4+ and CD8+ T-cells, thus displaying the ability to code for immortalizing functions. The virus may stay in latency for decades before any pathogenic implications take place. When EBV infects B lymphocytes, its linear genome circularizes to form an episome, or extrachromosomal element, in the nucleus of the cell (Swendeman et al., 1987). The result is a transformation of the infected B cells, which acquire the capacity to proliferate indefinitely. *in vitro*, these latently infected B cells express only 10 of the approximately 80 genes encoded by the virus. The state of latent infection is maintained by the Epstein-Barr virus nuclear antigen 1 (EBNA1) protein; it binds to a nucleotide sequence, termed *oriP*. The binding of EBNA1 to *oriP* allows the viral genome to be maintained in the nucleus of the B cell (Cohen et al., 1989).

Two of the EBV latent genes encode proteins that transactivate other viral genes. EBNA1 leads to transactivation of the EBNA proteins, whereas Epstein-Barr virus nuclear antigen 2 (EBNA-2) as the major EBV transcription factor transactivates the expression of two EBV latent membrane proteins, LMP-1 and LMP-2. EBNA2 also transactivates CD21, CD23. The secreted (truncated) form of CD23 may be a B-cell growth factor, but the full-length molecule may be a receptor, thus providing autocrine stimulation of EBV-infected B cells (Aubry et al., 1992). The latent gene product, LMP-

1, acts as a direct oncogene in transformation assays (Cohen et al., 1989). Expression of LMP-1 in epithelial cells transforms them morphologically, and in B-lymphoma cells, LMP-1 prevents programmed cell death, or apoptosis (Henderson et al., 1991). Genetic analyses using viral mutants indicate that EBNA-2 and LMP-1 are essential for EBV-induced transformation (Cohen et al., 1989).

Viruses are now recognized as typical members of the group of agents known to be cancer-causing in humans. Numerous viral isolates from primates and lower animals produce cancer in animal model systems, and these have been utilized to define mechanisms of viral carcinogenesis that form the basis of much of our understanding today (Parkin et al., 1999).

Viruses are associated with a variety of types of human malignancies. Some viruses are associated with a single tumor type (e.g., HBV), whereas others are linked to multiple tumor types (e.g., EBV); these differences reflect the tissue specificity of a given virus. A virus associated with human tumors may also produce non-neoplastic disease in some hosts. Human T-cell leukemia virus type I (HTLV-I) is the cause of HTLV-I-associated myelopathy or tropical spastic paraparesis (HAM/TSP), a neurological disease that develops even more rarely in infected persons than adult T-cell leukemia (ATL); HPVs cause a variety of benign hyperplasias; and both HBV and Hepatitis C virus (HCV) cause hepatitis. The frequency of disease development varies widely, reflecting the basic characteristics of the particular virus and features of the virus–host relationship, including the age at primary infection (Klein, 2002; Mossman et al., 2004).

Table 1.1 Retroviruses containing cellular oncogenes (adapted from Rosenberg & Jolicoeur 1997)

General class	Oncogene	Virus			Protein product
		Name	Abbreviation	Origin	
Non-receptor protein tyrosine kinase	<i>abl</i>	Abelson murine leukemia virus	Ab-MLV	Mouse	Tyrosine kinase
	<i>fes</i>	ST feline sarcoma virus	ST-FeSV	Cat	Tyrosine kinase
	<i>src</i>	Rous sarcoma virus	RSV	Chicken	Tyrosine kinase
Receptor protein tyrosine kinase	<i>erbB</i>	Avian erythroblastosis virus	AEV-ES4	Chicken	Epidermal growth factor receptor
	<i>kit</i>	Hardy-Zuckerman-4 feline sarcoma virus	HZ4-FeSV	Cat	Stem cell factor receptor
Serine/threonine protein kinase	<i>mil</i>	Avian myelocytoma virus	MH2	Chicken	Serine/threonine kinase
	<i>raf</i>	Murine sarcoma virus 3611	MSV3611	Mouse	Serine/threonine kinase
Growth factor	<i>sis</i>	Simian sarcoma virus	SSV	Monkey	Platelet-derived growth factor
G protein	<i>H-ras</i>	Harvey murine sarcoma virus	Ha-MSV	Rat	GDP/GTP binding
	<i>K-ras</i>	Kirsten murine sarcoma virus	Ki-MSV	Rat	GDP/GTP binding
Transcription factor	<i>erbA</i>	Avian erythroblastosis virus	AEV-ES4	Chicken	Transcription factor (thyroid hormone receptor)
	<i>fos</i>	FBJ osteosarcoma virus	FBJ-MSV	Mouse	Transcription factor (AP1 component)
	<i>jun</i>	Avian sarcoma virus-17	ASV-17	Chicken	Transcription factor (AP1 component)
	<i>myb</i>	Avian myeloblastosis virus	AMV	Chicken	Transcription factor
	<i>Myc</i>	MC29 myelocytoma virus	MC29	Chicken	Transcription factor

Adapted from Rosenberg and Jolicoeur (1997). This list is representative, not exhaustive.

In cancers that have a viral etiology, the virus appears to be necessary, but not sufficient, for tumor development. The interpretation is that viruses usually do not behave as complete carcinogens, but rather act as initiating or promoting factors.

Additional changes must accumulate to complement those mediated by viral functions in order to disable the multiple regulatory pathways and checkpoints in normal cells and to allow a cell to become completely transformed (Klein et al., 2002; Kelly et al., 2006).

Long latent periods are the norm between the time of initial virus infection and tumor appearance in normal individuals. Chinese with chronic HBV infections acquired as newborns usually develop hepatocellular carcinoma (HCC) beyond 50 years of age, there may be >30 years between EBV infection and development of nuclear pore complexes (NPC) or Hodgkin's disease, 3–4 decades typically elapse between HPV infection and development of cervical cancer and, although most HTLV-I infections are acquired in infancy, ATL usually arises in people in their forties and fifties (Stubenrauch and Laimins, 1999; Buti et al., 2005; Carbone et al., 2008).

Human tumor viruses display different mechanisms of cell transformation and fall into both direct- and indirect-acting categories. Direct-acting viruses carry one or more viral oncogenes for example HPV and human polyoma virus encode proteins that target and inactivate cellular tumor suppressor genes especially retinoblastoma (Rb) and p53. The indirect-acting viruses appear not to possess actual oncogenes, but their infection renders the host more vulnerable for acquiring additional infections with other direct acting viruses or to accumulate enough mutations to trigger transformation. For example

the detection of HCV following HBV infection is thought to take place through promoter insertion mechanism. Indirect acting virus generally lack the transforming activity in *in vitro* assays (DiMaio et al., 1998).

Another herpesvirus associated with human cancers that was discovered relatively not too long time ago is Human herpesvirus 8 (HHV-8). HHV-8 was discovered in 1994 and found to be associated with 2 rare B cell lymphomas, primary effusion lymphoma and multicentric Castleman's disease, in addition to Kaposi's sarcoma in which HHV-8 genomic sequences were initially identified (Ensoli et al., 2001). HHV-8-induced oncogenesis is a complex process that is likely to involve both paracrine-mediated promotion of cell proliferation and survival by viral lytic gene products and classical cellular transformation induced by the activity of latency proteins. The 3 malignancies associated with HHV-8 infection are likely to be the products of different pathogenic mechanisms, including differential involvement of individual HHV-8 latency and lytic genes (Guo et al., 2003).

The diversity of oncogenic mechanisms by human tumor viruses emphasizes that there is no single mode of transformation underlying viral carcinogenesis. As mentioned, even the direct-acting viruses are not complete carcinogens. The proven human cancer viruses are all replication competent and establish long-term persistent infections in various cell types; the occasional destructive outcome, cancer, is an accidental side-effect of viral replication strategies (Carbone et al., 2004).

Viruses that establish persistent infections must avoid detection and recognition by the immune system that would eliminate the infection. Different viral evasion strategies have been identified, including: (i) restricted expression of viral genes and

proteins that makes the infected cells nearly invisible to the host (e.g., EBV in B cells); (ii) infection of sites that seem relatively inaccessible to immune responses like John Cunningham virus (JCV) and herpes simplex virus (HSV) in the central nervous system, HPV in the epidermis and polyomaviruses and cytomegalovirus in the kidney; (iii) variation in viral antigens that allows escape from antibody and T-cell recognition (e.g., human immunodeficiency (HIV) and influenza virus). It is of interest that the majority of tumor viruses can infect lymphocytes and monocytes, although other cell types may be the primary targets for infection (Buti et al., 2005).

Despite those viral evasion mechanisms, the immune system frequently prevails. For example, the prevalence of HPV infections may be as high as 50% among young women, but declines with age. This suggests that the immune system renders most, but not all, infections transient. Perhaps the high-risk HPV types are relatively more efficient than other types at establishing persistent infections, contributing to their disease potential (Buti et al., 2005; Carbone et al., 2008).

1.6 Oncogenes and tumor suppressor genes

The recognition that viral-transmitted oncogenes are in fact no more than altered versions of cellular protooncogenes had a major impact on understanding the onset of carcinogenesis. For example, we now know that in human cancers, protooncogenes are initially cellular genes that can induce transformation under certain abnormal conditions, are frequently mutated and activated through cell intrinsic mechanisms, including point mutations, gene amplification, gene fusion, or alterations that lead to increased mRNA or protein stability (Parada et al., 1982; zur Hausen, 2001a).

Oncogenes of human tumor viruses are virally encoded genes that play integral roles for the viral life cycle. To fulfill their roles in the viral life cycle, human tumor virus oncogenes target critical cellular regulatory circuits, including cellular protooncogenes and tumor suppressor pathways, and cause their activation or inactivation, respectively (Munger et al., 2004). Some viral oncogenes also subvert cellular processes that are necessary for maintaining genomic integrity of the host cell. Hence, some human tumor viruses contribute to human carcinogenesis by facilitating the generation and accumulation of activating mutations of cellular oncogenes and/or inactivating mutations of tumor suppressors in the host genome. Alternatively, other protooncogenes like *myc* do not require a mutation to contribute to carcinogenesis and deregulation of their expression is sufficient to drive tumorigenesis. Such viruses contribute not only to initiation but also to the progression of human cancers (Kinzler and Vogelstein, 1997; Munger et al., 2004).

1.6.1 Oncogenes and Oncoproteins

All of the oncogenes carried by transforming retroviruses were subsequently shown to be derived from the cell. The progenitor cellular genes, referred to as protooncogenes, have been identified as classes of genes involved in mitogenic signaling and growth control, including protein kinases, growth factor receptors, growth factors, G proteins, transcription factors and adapter proteins (Klein and Klein, 1985). The observation that first linked an oncogene from a chicken sarcoma virus to a cellular transcription factor was quite unanticipated. More than 30 transduced oncogenes in

transforming retroviruses have been identified (Tabin et al., 1982; Delgado and Leon, 2006).

Conversion, or activation, of a protooncogene into an oncogene generally involves a mutation. At least three mechanisms can activate oncogenes as compared to their corresponding protooncogenes (Schafer et al., 2007). Point mutations in a protooncogene that result in a constitutively active protein product, localized gene amplification within a DNA segment that includes a protooncogene, leading to overexpression of the encoded protein and finally chromosomal translocation that brings a growth-regulatory gene under the control of a different promoter that causes inappropriate expression of the gene (Hunter, 1991; Jahner and Hunter, 1991; Rajalingam et al., 2007; Felsher, 2008).

Based on biological activity, oncogene products are classified into several groups that are analogous to components of the mitogenic pathway, such as growth factors, membrane receptors, signal transducing proteins and nuclear proteins (Bechade et al., 1985). Deregulation results in stimulation of growth or phenotypic change, or both. Protein products of some oncogenes stimulate excretion of growth factors. Cells transfected by some oncogenes (such as *ras*, *src*, *middle T*, *mos*, *fes*, *abl*, *fps*, *erb B*, *yes* and *mil/raf*) release growth stimulating factors (Downward et al., 1984; Scaltriti and Baselga, 2006). The growth factors are not encoded by the oncogenes themselves but by quite separate genes whose expression is indirectly stimulated by transfected oncogene. Some oncogenes, such as *c-sis*, encode growth stimulatory proteins, and if deregulated, may assume the status of active oncogenes (Wakelam et al., 1986). Irrespective of whether increased secretion of growth factors is due to stimulation by oncogene products

or by their direct transcription, cells must express the growth factors-receptors first before a positive feedback loop can be established. The establishment of these loops provides cells with a steady stream of growth stimulatory signals and frees them from dependence of growth factors imported from elsewhere (Alvarado and Giles, 2007).

Deregulation of the receptors of growth factors can also activate the mitogenic pathway. Here, the receptors themselves are changed in ways which continuously provide the cell with growth stimulatory signals, even in the absence of growth factors. In this scenario the growth factor receptor assumes the role of an oncogenic protein (Downward et al., 1984; Boulougouris and Elder, 2002; Zhang et al., 2007). For example, if the eukaryotic growth factor (EGF) receptor spontaneously in absence of EGF stimulates nucleotide binding by p21 Ras. Ras protein in turn stimulates the growth promoting effect of a variety of growth factors by stimulating inositol phospholipid metabolism, which participates in the signal transducing pathway. The growth promoting effect of EGF, however, which is independent of inositol phospholipid turnover, is also stimulated by Ras proteins. The exact mechanism of growth stimulation by mutated Ras protein is still obscure (Gaynor et al., 1984; Prenzel et al., 2001; Leicht et al., 2007).

The protein products of some protooncogene are located in the nucleus and these may have a role in growth control. The *ela* oncogene of human adenovirus is a regulator of transcription of viral and cellular genes (Gaynor et al., 1984; Kingston et al., 1984). Also Myc protein perturbs the activity of the cellular transcription machinery and mobilizes the expression of a number of cellular genes whose protein products are critical for growth and differentiation (Janz, 2006). The normal cell genome carries multiple protooncogenes whose products are nuclear (Myc, N-Myc, Myb, Fos, and Ski). Each of

the proteins encoded by these genes may activate a slightly different group of cellular genes (Cole and Nikiforov, 2006; Eilers and Eisenman, 2008).

1.6.2 Tumor suppressor genes

Tumor suppressor genes generally encode proteins that in one way or another inhibit cellular proliferation. Loss of one or more of those “brakes” contributes to the development of many cancers. Five broad classes of proteins are generally recognized as being encoded by tumor suppressor genes: Intracellular proteins, such as the p16 cyclin-kinase inhibitor, that regulate or inhibit progression through a specific stage of the cell cycle, receptors for secreted hormones (e.g., tumor derived growth factor β) that function to inhibit cell proliferation, checkpoint-control proteins that arrest the cell cycle if DNA is damaged or chromosomes are abnormal, proteins that promote apoptosis or enzymes that participate in DNA repair (Finlay et al., 1989; Neil et al., 1997; Colot-Teixeira et al., 2004; Stafford et al., 2008).

Tumor suppressor genes were initially discovered during studies involving viral oncogenes. Specifically the oncogenes of the small DNA tumor viruses (polyomavirus, papillomavirus and adenovirus) like E6, E7, E1A and E1B p55 were found to be essential for both viral replication and cell transformation (Lednický and Butel, 1999). The protein products of those oncogenes target and inactivate the tumor suppressor genes in the host cells. Studies of these DNA viruses led to the discovery of tumor suppressor genes, the second group of genes that are critically important in cancer development (Raja et al., 2002; Woo and Poon, 2004).

One important class of tumor suppressor genes is DNA repair enzymes. Although DNA-repair enzymes do not directly function to inhibit cell proliferation, cells that have lost the ability to repair errors, gaps, or broken ends in DNA accumulate mutations in many genes, including those that are critical in controlling cell growth and proliferation. The loss-of-function mutations in the genes encoding DNA-repair enzymes promote inactivation of other tumor suppressor genes as well as activation of oncogenes (Jack et al., 2004; Lavin et al., 2005; Martin et al., 2008).

Since generally one copy of a tumor-suppressor gene suffices to control cell proliferation, both alleles of a tumor-suppressor gene must be lost or inactivated in order to promote tumor development. Thus oncogenic loss-of-function mutations in tumor suppressor genes act recessively. Tumor suppressor genes in many cancers have deletions or point mutations that prevent production of any protein or lead to production of a nonfunctional protein (Wani and Nair, 2003). In traditional tumor suppressor genetics, inherited loss of one tumor suppressor allele leads to accelerated tumorigenesis due to the need to inactivate only one remaining allele. Haploinsufficient tumor suppressor genes also lead to accelerated tumorigenesis, however, without the requirement for inherited mutation of one allele. In the past decade several tumor suppressor genes including p53 and p27 were reported to be haploinsufficient for tumor suppression or at least to control tumor progression in dose-dependent manner (Yanagawa et al., 2008).

Out of various known tumor suppressor proteins, p53 and pRb were and are still extensively studied. The binding of viral oncoproteins to cellular tumor suppressor proteins p53 and pRb is fundamental to the effects of the small DNA tumor viruses on host cells (Garner and Raj, 2008). As several different DNA tumor viruses encode unique

oncoproteins that target pRb and p53, this emphasizes the central power these two proteins exert over cell growth-control and the viral imperative to circumvent that control (Lan et al., 2007; Verma et al., 2007). For example, Rb functions as a transcriptional repressor able to bind to the E2F family of transcription factors and regulate the expression of genes required for S-phase entry and progression. Upon phosphorylation, Rb releases E2F and allows transcription of proteins required to the progression of the cell cycle into the S-phase. Loss of Rb function due to structural mutation that will allow its continuous phosphorylation or as a result of haploinsufficiency or chromatin hypermethylation will allow the cell to escape this important checkpoint and may contribute to the initiation of cancer. Similarly, p53 controls the entry of cells into G₂M phase of the cell cycle. In case of anti-mitogenic signals due to DNA damage for example, p53 is phosphorylated and triggers a cascade of pathways that lead to cell cycle arrest and apoptosis. In case of p53 inactivation, cells that carry damaged DNA or abducted chromosomes will allow to divide and pass the abnormality to daughter cells (Braithwaite et al., 2006).

1.7 The oncoprotein Myc

c-myc protooncogene was identified as the cellular homolog of the viral oncogene *v-myc* encoded by the avian myelocytomatosis virus (Vennstrom et al., 1982). *c-Myc* (often termed “Myc”) is a transcription factor which specifically binds to so-called E-box sequence (CACGTG) and regulates expression of multiple genes involved in control of cell growth, proliferation, differentiation, apoptosis, angiogenesis, cellular adhesion, DNA metabolism and repair (Eisenman, 2001; Pelengaris et al., 2002a).

Deregulation of c-Myc expression is observed in many human cancers and has been implicated in a number of cellular processes associated with tumorigenesis such as reduction of growth-factor requirements, immortalization, resistance to anti-mitogenic signaling, increase of angiogenesis, changes in adhesion and genomic instability (Baudino et al., 2002; Pelengaris et al., 2002b). The ability of c-Myc to induce unrestrained and autonomous cell growth and proliferation seems to be particularly important for tumorigenesis.

c-Myc acts at different stages of cell cycle. It enforces transition through G₁/S and prolongs the G₂/M phase (Felsher and Bishop, 1999) and is able to overcome cell cycle arrest induced by DNA damage (Sheen and Dickson, 2002). The effects of c-Myc on the cell cycle are mediated by transcriptional activation or repression of genes encoding cell cycle regulators (Hermeking et al., 2000).

Genomic instability induced by c-Myc may involve a variety of different mechanisms including inappropriate cell cycle transition, perturbation of DNA replication, bypass of cellular check-points, suppression of DNA repair, induction of reactive oxygen species (ROS) production, chromosome and telomere remodeling (Chernova et al., 1998). c-Myc-induced genomic instability can be classified into two categories: abnormal chromosomal numbers (aneuploidy) and defects in chromosomal integrity including chromosomal breaks, fusions and translocations (Mai et al., 1996a; Felsher and Bishop, 1999; Li and Dang, 1999; Karlsson et al., 2003).

1.7.1 Myc structure and function

In humans, the c-Myc gene is located on chromosome 8q24 with the coding region consisting of three exons. Its transcription may be initiated at one of three promoters. Translation at the AUG start site in the second exon produces a major 439 amino acid, 64 kDa Myc protein. Alternative translational initiation start sites result in both longer and shorter forms of the protein, termed p67 Myc and MycS, respectively (Kato et al., 1990). The Myc protein is O-linked glycosylated and phosphorylated, and these modifications may alter the protein half-life. The Myc sequence contains several conserved domains at the N-terminal, termed Myc boxes, which are found in closely related proteins, N-Myc and L-Myc (Barrett et al., 1992; Crouch et al., 1993).

Myc is a member of the MycC/Max/Mad network and belong to the basic/helix-loop helix/leucine zipper (bHLHZip) family of transcription factors, where the basic region mediates DNA-binding and the helix-loop-helix and the leucine zipper mediate protein-protein interactions (Davis and Halazonetis, 1993). The Myc family members are short-lived phosphoproteins with a half life of 20-30 minutes. Myc dimerizes with the bHLHZip proteins Max or Mad. Dimerization of Myc with either Max or Mad takes place on a competitive bases. Dimerization of Myc with Max has proven necessary for Myc to bind DNA and exert its functions while dimerization of Myc with Mad inhibits Myc's binding to DNA and negatively regulates Myc's function (Amati et al., 1992). The Myc/Max complex bind E-boxes, regulatory recognition elements found in many promoters. The N-terminus of Myc harbors a transactivation domain (TAD) containing two conserved regions Myc box I (MBI) and Myc box II (MBII) roughly spanning amino acids 45-63 and amino acids 129-141, respectively (Figure 1. 2) (Blackwood and

Eisenman, 1991). Recent structure function studies have shown that MBII is necessary for Myc induced tumorigenesis (Fest et al., 2005).

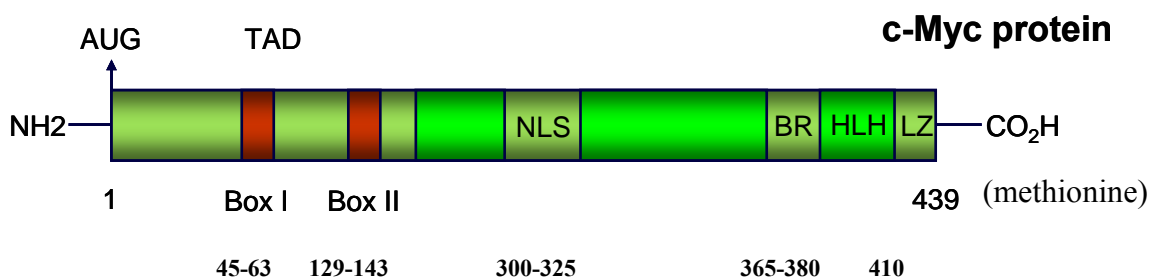
1.7.2 Myc Biology

In general, Myc proteins are phosphorylated at multiple sites distributed over the entire protein. The major sites were identified almost 20 years ago within two areas, the acidic domain and near the basic region. Phosphorylation sites in both areas are substrates of protein kinase casein kinase II (CK2), a kinase that is known to have implications in tumorigenesis (Beckmann et al., 1990; Fisher et al., 1991). Recent studies showed correlation between CK2 activity and Myc protein levels suggesting that CK2 stabilizes Myc (Gregor et al., 1990).

In addition to these two areas of phosphorylation, a third is located within the TAD of Myc (Figure 1.2) (Luscher et al., 1989; Lutterbach and Hann, 1994). Two sites, Thr-58 and Ser-62 within MBI, that are targeted by GSK3 and by proline-directed kinases, respectively (Henriksson et al., 1993). Those two sites were found to be frequently mutated in BL (Henriksson et al., 1993; Albert et al., 1994). The Thr-58 and Ser-62 sites are interdependent and the Ser-62 phosphorylation is a required for modification of Thr-58 (Bhatia et al., 1993). Phosphorylation at this site appears to affect the stability of Myc at the G₀ to G₁ transition and to regulate Myc-DNA binding. Because the location of the phosphorylation sites at Thr-58 and Ser-62 is within the TAD, it is believed that those two sites are involved in the regulation of gene transcription (Classon et al., 1993).

The phosphorylation sites at Thr-58 and Ser-62 within MBI play an important role in controlling Myc protein stability through regulating Myc's ubiquitination and degradation (Kohl and Ruley, 1987; Eisenman, 2001). As mentioned, phosphorylation of Ser-62 stabilizes Myc, whereas upon phosphorylation of Thr-58 a series of events take place and lead to Myc degradation (Mougneau et al., 1984; Eisenman, 2001). Once both sites are phosphorylated, the protein phosphatase 2A gains access to Myc and dephosphorylates Ser-62. The Thr-58 only phosphorylated form of Myc is recognized by ubiquitin-protein isopeptide ligase complex that stimulates polyubiquitinylation and subsequent proteasomal degradation of Myc. The relevance of the two phosphorylation sites in controlling Myc stability is supported by the fact that those sites are frequently mutated in Burkitt lymphoma and other Myc driven cancers (Karn et al., 1989; Eilers and Eisenman, 2008).

Figure 1.2: A cartoon showing the schematic structure of the c-Myc protein with its different domains. LZ: leucine zipper, HLH: helix loop helix, BR: basic region, NLS: nuclear localization signal, TAD: Transactivation domain, AUG: first translated codon



1.7.3 Mechanisms of c-Myc activation in cancer

Overexpression of *c-myc* has been found in up to 70% of all human cancers (Nesbit et al., 1999). Elevated c-Myc expression correlates with clinically aggressive tumors, which have a worse prognosis than tumors without Myc overexpression (Gamberi et al., 1998). The activation of *c-myc* occurs mainly through genomic and transcriptional alterations. One of the common genomic changes in hematopoietic malignancies as Burkitt's lymphoma are translocations of the *c-myc* gene, which is as mentioned located on chromosome 8q24, to the immunoglobulin μ heavy chain or the λ and κ light chain enhancers located on chromosome 2p12, 14q32 or 22q11, respectively (Dalla-Favera et al., 1982). Rearrangements of *c-myc* gene were also found in diffuse large cell lymphoma (DLCL), acute lymphocytic leukemia (ALL), multiple myeloma (MM), and primary plasma cell leukemia (PCL) (Nesbit et al., 1999). Rare cases of T-cell leukemia, in which the *c-myc* gene is translocated to T-cell receptor, have also been reported (Harrison, 2000).

The deregulated expression of *c-myc* may be activated by mutations in genes laying in pathways upstream of c-Myc, like mutations in the APC/ β -catenin pathway that lead to activation of c-Myc in colorectal cancer (He et al., 1998). Likewise, c-Myc deregulation in malignant melanoma might be *via* APC/ β -catenin pathway as well (Rubinfeld et al., 1997).

Another mechanism of c-Myc activation in solid tumors is gene amplification (Vita and Henriksson, 2006). In case of malignant melanoma, extra *c-myc* copies were found in 61% of nodular melanomas, in 28% of superficially spreading melanomas, and in 30% of metastatic tumors. Amplification of *c-myc* was detected in 40% of tumors with

overexpression of c-Myc protein in ovarian cancer (Treszl et al., 2004). Up to 23% of lung carcinoma samples displayed amplifications, which were also found in 10% of esophageal squamous cell carcinoma (SCC) patients treated by surgery and in 30% of patients subjected to multimodal treatment (Bitzer et al., 2003). All three transforming members of the Myc family have been shown to be amplified in small cell lung carcinoma (SCLC), with a frequency of 10% (*mycN*), 13% (*L-myc*), and 20% (*c-myc*) (Nesbit et al., 1999; Gugger et al., 2002) whereas 30% of all neuroblastoma (NBL) show specific amplification of *mycN* (Brodeur, 1995).

1.8 Mechanisms of c-Myc induced genomic instability

In solid tumors abnormal c-Myc expression correlates with genomic instability. *In vivo* and *in vitro* models of c-Myc-overexpression revealed induction of karyotypic changes, including alterations in copy number and chromosomal rearrangements (Mai and Mushinski, 2003; Felsher, 2008) or locus specific instability involving amplification of certain genes (Mai, 1994; Mai et al., 1996b; Kuschak et al., 1999; Mushinski et al., 1999) (*CCND2*, *ribonucleotide reductase2 (R2)*, *DHFR*).

After inactivation of Myc in conditional mice models most tumors undergo proliferative arrest, differentiation and apoptosis (Arvanitis and Felsher, 2006). However, some of tumors can become independent of Myc overexpression by acquiring additional genetic events such as chromosomal translocations (Felsher and Bishop, 1999; Mai and Garini, 2005). These observations suggest that c-Myc functions as dominant mutator gene by promoting chromosomal instability. Identical translocations were present in

multiple relapsed tumors arguing that these genomic events may contribute to the independence from c-Myc (Arvanitis and Felsher, 2006).

1.8.1 Myc promotes transition into S phase

c-Myc activation is sufficient and necessary for induction of G₁/S-transition (Trumpp et al., 2001). In *Drosophila* ectopic expression of dMyc increases both cell mass and cell number (Pierce et al., 2004). Targeted disruption of the *c-myc* gene by preventing DNA unfolding at Myc's promoter region in rat fibroblasts resulted in a significant lengthening of the G₁ and G₂ phases, whereas the duration of S-phase was not affected (Iritani and Eisenman, 1999; Trumpp et al., 2001). c-Myc induces transcription of several target genes involved in G₁/S transition such as *ODC*, *Cull*, *CDK4*, *Cdc25A* and *Id2* (Bello-Fernandez et al., 1993; Muller et al., 1997; O'Hagan et al., 2000).

Furthermore, c- Myc activation leads to down-regulation of inhibitors of cyclin/Cdk complexes (Blagosklonny and Pardee, 2002). As a result, c-Myc leads to activation of cyclin/Cdk complexes, phosphorylation of pRB and release of active E2F/DP transcription factors (Blagosklonny and Pardee, 2002). Under such conditions inappropriate expression of cyclin E can lead to genomic instability (Spruck et al., 1999). Also, excessive Cdk activity influences the fidelity of chromosome segregation, including licensing of replication origins to fire, which has been linked to instability (Walter et al., 1998). In addition, studies in yeast suggested that precocious Cdk activation may cause genomic instability via delayed firing of replication origins, leading to breaks during mitosis of incompletely replicated chromosomes (Lengronne and Schwob, 2002).

1.8.2 c-Myc abrogates G₂/M arrest

The error-free transmission of genomic information to the next generation of cells requires complete, damage-free DNA replication and faithful mitotic segregation of chromosomes into two daughter cells. Checkpoint mechanisms in G₂- and M-phase ensure the proper segregation of the duplicated chromosomes. Dysfunction of G₂ and/or mitotic checkpoints may result in karyotypic abnormalities and/or endoreduplication (Niculescu et al., 1998; Stewart et al., 1999).

Ectopic expression of c-Myc compromises a stable G₁-arrest and causes aneuploidy and endoreduplication (Andreassen and Margolis, 1994; Khan and Wahl, 1998; Li and Dang, 1999). c-Myc can compromise a G₁-like arrest of cells undergoing mitotic slippage caused by drug-induced microtubule perturbation or sequestration of E2F transcription factors and leads to reduplication. p27 suppresses c-Myc-induced endoreduplication at low, but not at high levels of c-Myc expression (Santoni-Rugiu et al., 2000; Deb-Basu et al., 2006).

In the latter case no influence on c-Myc dependent chromosomal breaks or fusion formation was detected. A possible explanation for this observation is that p27 suppresses mitotic division and endoreduplication or the ability of c-Myc to cause accelerated entry into the S phase. The DNA damage generated by c-Myc overexpression is sufficient to activate the G₂/M checkpoint and arrest cells with a 4N DNA content (Felsher et al., 2000). Nevertheless, c-Myc activation is able to enforce G₂ to S transition probably through the re-initiation of DNA synthesis or potential leakiness of the G₂/M checkpoint. This bypass contributes to an increase in ploidy. An explanation for such mechanism

could be the premature activation of cyclin/Cdk complexes or other factors involved in replication origin licensing and initiation of S-phase by c-Myc.

1.8.3 c-MYC modulates replication, DNA damage response and repair pathway

c-Myc expression was shown to influence processes which maintain the integrity of the genome such as DNA repair and the response to DNA damage. Perturbation or attenuation of these processes may contribute to genomic instability. c-Myc deregulation interferes with the repair of double-strand breaks (DSBs) and results in an increase in chromosomal breaks and translocations (Karlsson et al., 2003). It is not clear whether c-Myc is able to inhibit repair directly *via* modulation of DNA damage response i.e. repair genes or it is an indirect function of Myc. In this context c-Myc dependent induction of DNA repair genes (Grandori et al., 2003) might have a dual effect on c-Myc-driven tumorigenesis.

Another function of Myc was revealed from several gene expression studies which described that c-Myc can upregulate genes involved in DNA replication including: Mcm4, Mcm6, Mcm7, Cdt1, Cdc6 and TOP1 (O'Hagan et al., 2000; Watson et al., 2002; Fernandez et al., 2003). In a proteomic approach c-Myc was shown to directly interact with MCM7, RFC and others components of DNA replication machinery (Koch et al., 2007). Therefore, c-Myc activation interferes with or modulates DNA replication, which in the case of constitutively active, oncogenic c-Myc expression may lead to induction of genomic instability (Louis et al., 2006; Dominguez-Sola et al., 2007; Lebofsky and Walter, 2007).

On the one hand activation of repair genes might increase fidelity of DNA replication and facilitate resolution of breaks arising during replication and thus ensure

replication fork progression. On the other hand, aberrant activation of repair enzymes may cause unscheduled repair of replication intermediates and increase the probability of chromosomal aberrations (Schar, 2001). Further studies are needed to clarify the exact role of Myc in those two important cellular processes.

1.8.4 c-Myc increases reactive oxygen species (ROS)

c-Myc couples mitogenic signaling to transcriptional induction of genes which promote growth and proliferation. Furthermore, c-Myc induces numerous target genes involved in glycolytic, respiratory and biosynthetic pathways (Gomez-Roman et al., 2003; O'Connell et al., 2003). Rapid elevation of metabolism associated with transition from quiescence to S-phase could potentially lead to long term accumulation of ROS, which may cause modifications and breaks of genomic DNA. Several studies suggest that c-Myc induces ROS which generate DNA damage (Tanaka et al., 2002; Vafa et al., 2002).

1.8.5 Myc induces non random gene amplification

Replication occurs only once per cell cycle during S-phase and under the control of cyclin dependent kinases and cell cycle checkpoint proteins (Piatti et al., 1996). The expression of all these proteins is regulated by transcription factors. Thus, it is widely accepted that transcription factors indirectly control replication by controlling the expression of specific Cdks and cyclins.

Alternatively, in viral systems such as Simian virus 40 (SV40) or EBV, the auxiliary elements that are non essential components of the origin recognition sequence

are transcription factors binding sites (Kohzaki et al., 1999). In yeast, similarly, the nonessential B3-element in autonomously replicating sequence (ARS) is a transcription factor binding site (Marahrens and Stillman, 1992). The commonly known role for transcription factors in replication is the recruitment of chromatin remodelling factors to create either nucleosome-free regions or regions of specifically spaced nucleosomes (Collins et al., 2002). This results in the activation of specific origins, presumably by making the origin region accessible to replication complexes (Figure 1.3).

It was first reported in 1987 that Myc antibodies interfered with the replication of plasmids containing autonomously replicating sequence in mouse liver cells (Iguchi-Arigo et al., 1987). At the same time, it was also reported that Myc promotes replication of plasmids containing SV40 replicating *ori* in human Raji cells 100 times more than in cells lacking Myc expression. As early as 1987 it was proposed by Iguchi-Arigo *et al* that Myc binds the initiation site of replication (Classon et al., 1987; Iguchi-Arigo et al., 1987). However, this finding was not further investigated. Importantly, binding to *ori* elements does not necessarily confirm a promotion of replication through interactions with the initiation complexes. The role of Myc in promoting replication was further investigated by several groups and elucidated Myc contribution to the amplification of several proliferation promoting genes (Kuschak et al., 1999; Mai et al., 1999; Mai and Mushinski, 2003).

Subsequently, it was reported that SV40 DNA replication is dependent on the levels of Myc in human tumor cell lines and that N-Myc and L-Myc could substitute c-Myc to facilitate replication of SV40 DNA in Burkitt's lymphoma cells (Henriksson et al., 1988; Classon et al., 1990). Furthermore, it was described that Myc was the limiting

factor for ionizing radiation-induced SV40 gene-amplification in semi-permissive Chinese hamster embryo cells. It was shown that Myc strongly influences the binding of protein complexes at the minimal origin of SV40 (Lucke-Huhle et al., 1989; Classon et al., 1993). Later on it was shown that deregulation of Myc protein induces chromosomal instability and locus-specific gene amplification of several target genes including the *DHFR* locus in human, hamster, mouse and rat cell lines as well as in primary lymphocytes (Mai et al., 1996b, Taylor et al., 1997). Other targets are *CCND2* and *R2* (Kuschak et al., 1999; Mai et al., 1999; Mai and Mushinski, 2003).

The binding of Myc/Max heterodimer to the two adjacent 5' E-box sequences upstream the *DHFR* locus was found to be enhanced and correlated with cellular proliferation. Gel retardation assays using oligonucleotides representing the two adjacent E-boxes revealed the formation of several oligonucleotides-protein complexes. Later on the amplification of *DHFR* gene together with downstream genes sharing the same regulatory elements occurs within three cell divisions and reaches over four fold increase in gene copy number (Mai and Jalava, 1994). The enhanced binding of Myc to one E-box as compared to the second suggests a different role for each E-box sequence. Interestingly, the affinity of Myc/Max binding to the E-box was enhanced upon cell cycle progression from G₁- to S- phase (Mai et al., 1996b).

While transient overexpression of Myc induced a transient amplification of *DHFR*, constitutive overexpression of Myc in plasmacytoma cell lines showed ongoing amplification and rearrangements in the *DHFR* locus (Mai et al., 1996b). The rearrangements were observed both chromosomally and extrachromosomally. Fluorescent in situ hybridization (*FISH*) analysis using *DHFR* gene specific probe

showed amplicons that were present on several chromosomes. Moreover, extrachromosomal elements that carried the *DHFR* signal were observed and varied in size (Mai et al., 1996b). Southern blot analysis of Myc induced Chinese hamster ovary cells (CHO-9M) using probes specific to different exons of the *DHFR* gene suggested that *DHFR* gene undergoes internal rearrangements in their order but all exons in the gene are amplified. *DHFR* amplification upon Myc deregulation correlated with cellular increased resistance to methotrexate (Mai et al., 1996b). The amplification was shown to be locus specific since other loci that are Myc transcription targets such as *cyclin C* (*CycC*), *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *ribonucleotide reductase 1* (*R1*) were not affected (Mai and Jalava, 1994; Mai et al., 1996b).

Also upon Myc deregulation the *R2* gene was amplified within a similar time frame. However, unlike *DHFR* and *CCND2* amplification, the level of mRNA of *R2* was not elevated upon amplification (Kuschak et al., 1999; Kuschak et al., 2002; Smith et al., 2003). It was shown that Myc dependent amplification of the *R2* gene, interestingly, takes place through an alteration in the replication program of that gene. It is important to mention that no alteration in the replication pattern was detected at the locus of the control gene *CycC* (Kuschak et al., 2002).

Upon experimental Myc deregulation it was observed that four replication forks originate at the *R2* locus *versus* a single fork observed in cell with normal Myc expression. Multiple replication forks at the *R2* locus were detected by two-dimensional (2D) gels analysis. The multiple replication forks lead to the generation of multiple

copies of the DNA region starting from *R2* gene and other downstream genes sharing the same regulatory elements. This amplified region (amplicon) may be recognized as DNA damage site and may lead to further amplifications. Alternatively the amplicon may be recognized by the homologous and/or non homologous recombination machinery inside the cell. Consequently this amplicon will be either integrated chromosomally or will form circular double minute elements (extrachromosomal elements) (Kuschak et al., 2002).

Replication dependent mechanism is then suggested behind such amplification (Figure 1.3). Concomitant with what was observed at the *DHFR* locus, the binding of Myc/Max heterodimers to the E-boxes upstream *R2* gene was documented by gel retardation assays and was confirmed by super shifts assays using My and Max antibodies.

Simultaneously, Myc enhances replication of the chromosomal region that expands beyond the *R2* locus on mouse chromosome 12 and induces enhanced replication of additional regions downstream the *R2* gene (Kuschak et al., 2002).

Similarly, amplification of *CCND2* was observed within three days upon Myc experimental deregulation or in the mouse plasmacytoma cell line MOPC 460D constitutively expressing high levels of Myc. This tumor cell line frequently showed microscopic evidence of extrachromosomal elements (EEs) (Mai et al., 1999). Importantly, this study points out that the overexpression of *CCND2* upon Myc deregulation is not due to enhanced transcription of the normal number of copies of the

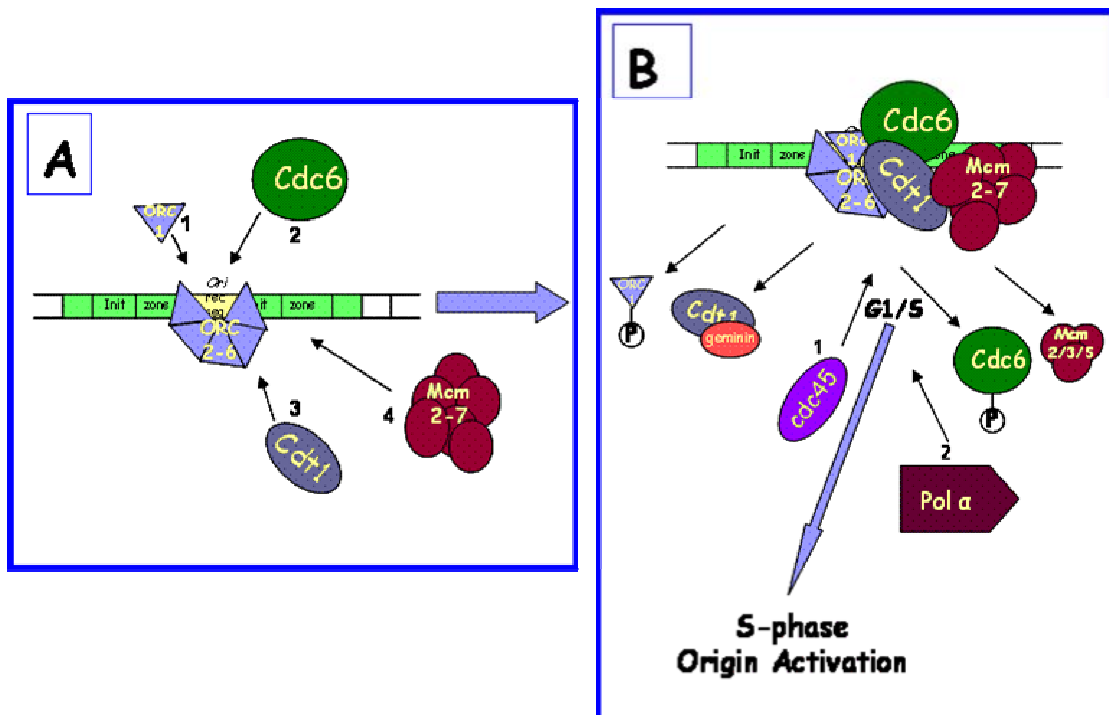
CCND2 gene but due to amplification of the gene. *CCND2* is one important protein expressed in G₁ phase of the cell cycle to facilitate the transition to S-phase.

The amplification of *CCND2* describes one mechanism by which Myc may accelerate the transition from G₁- to S-phase of the cell cycle. *CCND2* gene sequence contains four Myc/Max binding E-box motifs upstream exon one, which suggests that the amplification may take place with a similar mechanism to that described for *R2* gene (Mai et al., 1999; Kuschak et al., 2002).

Each Myc target gene described here is located on a different chromosome either in mouse or human genomes. This fact supports the concept that Myc dependent gene amplification is a non random process that takes place through a common mechanism upon Myc deregulation and not a process that accidentally takes place and leads to the amplification of a random genomic region (Mai et al., 1996a). Interestingly, the ODC gene which is located on the same chromosome as *R2* gene in the mouse genome is selectively amplified in certain cell lines but not others (Mai et al., 1996b; Smith et al., 2003).

The data that were discussed from the characterization of *R2* gene amplification suggest that the mechanism of Myc dependent gene amplification at specific loci involves the alteration in the replication pattern at those loci. Recently reports showed a non transcriptional role of Myc in DNA replication through the interaction of Myc with early replication proteins (Dominguez-Sola et al., 2007). Characterizing a common mechanism by which Myc contributes to the amplification of its target genes is one of the objectives of this thesis work.

Figure 1.3: A cartoon showing the assembly of the initiation complex at mammalian origins. **A:** In early G1 phase, Orc1 is recruited to the origin site where it binds the rest of the Orc subunits forming an active hexamer (1). The Orc1-6 complex then recruits Cdc6 (2), which in turn recruits Cdt1 (3). Cdt1 loads Mcm2-7 complex, and as a result, origin licensing takes place (4). At this point the complex is called pre-initiation complex. **B:** After Binding of the Mcm2-7 complex, a series of events is triggered, including loading of Cdc45 (1), removal of Cdt1 upon binding of geminin, phosphorylation (P) of Cdc6 and its removal from the origin site. Cdc45 recruits DNA polymerase alpha (2). The Mcm hexamer is then dissociated to generate a subcomplex Mcm4/6/7 that exhibits helicase activity. At this step, the complex is called initiation complex, and the origin is activated (adapted from Louis et al., 2006).



1.8.6 Myc dependent remodelling of nuclear organization

Chromosomes can easily be seen in cells during mitosis when they appear as distinct, highly condensed entities. However, their morphological structure during interphase remained unclear for many years. Advances in fluorescent microscopy allowed researchers to study the positions, structures and even volumes of chromosomes in the interphase nucleus. These techniques can identify the localization of any part of the genome in space (Croft et al., 1999). Chromosomes can be visualized directly by *in situ* hybridization using chromosomes-specific fluorescently labeled probes.

Based on fluorescent imaging studies, it is now accepted that the genetic material that constitutes a single chromosome is not randomly distributed all over the interphase nucleus. Instead, chromosomes represent genetic units and occupy well defined, exclusive spaces of the nuclear volume currently known as chromosomal territories (Cremer et al., 1982b).

Several models emerged speculating and interpreting data about the positions and the mobility of chromosomes in the interphase nucleus. Nevertheless, the issue remains controversial. One appreciated model is that each chromosome has a specific position inside the nucleus. This model was based on the analysis of gene density on each chromosome and implies a functional role for chromosomes positioning (Cremer et al., 1982a). An alternative model suggests that the arrangement of chromosomes is according to the preferential positioning of chromosomes relative to each other. This model

suggests that chromosomes occupy preferential positions within the nucleus depending on neighboring chromosomes (Gerlich et al., 2003; Thomson et al., 2004).

Chromosomal mobility during interphase is documented but the dynamics of such movement is believed to be limited (Gerlich et al., 2003). Mammalian gene loci and chromosomes undergo an increased mobility during early stages of G₁ phase of the cell cycle when nuclei reform (Thomson et al., 2004). Thus, in normal cells chromosome territories do not seem to significantly change position during most of the phases of the cell cycle (Parada and Misteli, 2002).

It was recently described that positioning of chromosomes within the nucleus is only partly conserved between normal and cancer cells (Parada et al., 2002; Cremer et al., 2003). However, in tumor cell lines or upon the experimental deregulation of oncoproteins like Myc, a deviation from the normal spatial organization of chromosomes may be observed *via* a telomere-dependent pathway (Parada et al., 2002; Louis et al., 2005; Mai and Garini, 2005).

Telomeres are repetitive DNA sequences located at the termini of linear chromosomes of most eukaryotic organisms, and a few prokaryotes. Telomeres compensate for incomplete semi-conservative DNA replication at chromosomal ends. The protection against homologous recombination (HR) and non-homologous end joining (NHEJ) constitutes the essential “capping” role of telomeres that distinguishes them from DSBs (Lundblad, 2000; Ferreira et al., 2004).

Beside the distinct structure of telomeres that enable capping of the chromosomal ends, telomeres are bound with a number of proteins comprising the telomeric capping complex shelterin. Failing of the capping process due to telomeric shortening or any manipulation of the capping complex may lead to cellular transformation often due to telomeric fusions (Denchi et al., 2007).

1.9 References

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Objectives

The objective of the work described in this thesis is to characterize three mechanisms by which the deregulation of Myc induces genomic instability in mammalian cells that may lead consequently to cancer development. The mechanisms of interest are:

1. Myc dependent gene amplification through induction of illegitimate replication.
2. Myc induced karyotypic instability via the alteration of the three-dimensional organization of telomeres in the interphase nucleus.
3. The mechanism by which Myc dependent Burkitt's lymphoma phenotype evolves in EBV positive B-cells and if EBV infection plays a role to promote the development of that cancer.

2.0 Myc dependent gene amplification takes place through a replication driven mechanism

Manuscript prepared for submission

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2.1 Abstract

Myc is a protooncogene that does not require a mutation to contribute to the initiation and development of cancer. The deregulation of Myc alone is sufficient to trigger several forms of genomic instability that may lead to cancer. Myc was found to be deregulated in 70% of human cancers. Gene amplification is one important form of genomic instability induced upon Myc deregulation. The transforming activity of Myc was generally thought to lie in its ability to modulate the expression of a series of genes, among them certain proliferation-promoting genes. However, recent studies showed that Myc is a multifunctional protein that is capable of affecting cellular functions in non-transcriptional dependent pathways. In this study we investigate the presence of a common non transcriptional mechanism by which deregulated Myc induces locus specific gene amplification in mammalian cells. We report that Myc induces gene amplification at the *DHFR* and *cyclin D2* loci through a replication-driven pathway in two immortalized but karyotypically stable mammalian cell lines. We show the initiation of replication intermediates suggesting refiring of the origins of replication at those loci multiple times in the same cell cycle. We also investigated Myc interactions with replication initiation proteins and we report specific interactions taking place between Myc and Orc 1, Cdc6 and Cdt1. We conclude that upon Myc deregulation, Myc stabilizes Cdt1 at the initiation site which leads to rereplication. Amplicons that result from the rereplication events lead to intra-and interchromosomal gene amplifications.

2.2 Summary

Throughout the past two decades it was shown that Myc contributes to cancer through the induction of genomic instability that consequently may lead to cancer (Chernov et al. 1998). Several forms of genomic instability were found to be induced after Myc deregulations and one of those is gene amplification (Mai et al. 1994). Gene amplification is a causal factor for many forms of cancer including breast, lung and a number of blood cancers (Vita and Henriksson 2006). In order to develop intervention methods to control Myc dependent gene amplification it is important to understand the molecular mechanism(s) by which the amplification takes place and that is the focus of this chapter in the thesis.

It has been shown that gene amplification may be triggered as a result of DNA damage especially in close proximity to fragile sites where DNA is more susceptible to damaging agents especially in the presence of defects in cellular DNA repair mechanisms (Debatisse and Malfoy 2005; Shimizu et al. 2005). However, evidence from studies investigating developmental stages in *Drosophila* suggested that gene amplification may also result due to special regulation or deregulation in the replication events that take place during normal cell cycle (Maines et al. 2004).

Previous studies conducted by our group looking at the amplification of the *R2* gene reported that the initiation of *R2* gene amplification takes place during S-phase (Kuschak et al. 2002). The report also documented the presence of four replication forks instead of one during the replication of that gene only after Myc deregulation (Kuschak et al. 2002). The cell line used in that study was mouse Pre B that is karyotypically stable diploid and has functional wild type p53 and DNA repair mechanisms.

The results obtained from studying *R2* gene amplification raised two questions that constitute the objective of this chapter of the thesis. First, is replication driven gene amplification a common cell type independent mechanism by which Myc induces the amplification of other target genes? Second, what is the molecular pathway by which Myc interferes in the replication process to allow the induction of multiple replication forks that lead to gene amplification?

To address the first question, we chose two other Myc amplification target genes; *DHFR* and *CCND2*. We also included in the study the *R2* gene that was previously characterized as a positive control for the initiation of replication driven gene amplification and *CycC* gene that is not amplified upon Myc deregulation as a negative control. We included two different cell lines in the study; the mouse Pre B line that was used in studying the *R2* gene and a rat fibroblast cell line. Both lines are stably transfected with constructs that spontaneously express Myc-estrogen receptor fusion protein. The fusion protein is kept outside the nucleus till the specific estrogen receptor-ligand is added to the media, then the fusion protein is activated and can translocate to the nucleus where Myc performs its biological functions.

We wanted to monitor if Myc deregulation will induce the generation of additional replication forks of our genes of interest (*CCND2* & *DHFR*) similar to what was observed previously with the *R2* gene also after Myc activation. We performed our experiments on cells just released from cell cycle arrest at G₁/S phase. Myc was activated at the point of releasing cells from the cell cycle arrest and this was considered time point zero. We considered in our experiments six time points: 1, 2, 3, 24, 48 and 72 hours. We analyzed the replication pattern by performing Southern blot using gene specific probes

after 2 dimensional agarose gel electrophoresis of genomic DNA isolated from both cell lines at the respective time points. We included mock activated Myc as control in all our experiments and we collected control cells from all respective time points. We observed additional replication forks at *DHFR*, *CCND2* and the positive control *R2* loci but not at the negative control *CycC* at time points 1, 2 and 24 hours. To investigate if the amplicons observed at *DHFR*, *CCND2* and *R2* loci included Myc binding sequence (E-box element), we performed Southern blot using oligonucleotides designed to include the E-box sequence in each of the three loci.

The second aim of this study was to characterize possible protein-protein interactions between Myc and the replication proteins that may allow Myc to interfere in the replication process. We chose to analyze Myc's interaction with early replication proteins for the reason that replication intermediates similar to what we observed in our 2D agarose gel electrophoresis experiments were observed by other groups performing structure/function studies on replication initiation proteins targeting to stabilize the replication initiation complex. These observations encouraged us to hypothesize that Myc interference in the replication process takes place by binding one or more of the members of the replication initiation complex and stabilizing the complex which allow refiring at those loci.

We included in our study the replication initiation proteins Orc1, Cdc6, Cdt1, and Mcm7, that is a member of the Mcm complex. We also included PCNA and Pol α that are mid and late replication proteins respectively as internal controls to our experiments. We observed strong interactions between Myc and each of Orc1, Cdc6 and Cdt1 at the time points when we observed the initiation of multiple replication intermediates. We also

observed relatively weak but consistent interaction between Myc and Mcm7 throughout all the time points of the experiment. Those results suggested to us that Myc interference in the replication initiation process takes place at a step prior to the recruitment of Mcm7.

Cdt1 is the last protein that joins the initiation complex prior to Mcm7 and in fact allows recruiting the Mcm complex to the replication initiation complex. After the origin firing, Cdt1 is removed from the initiation site and escorted outside the nucleus by the protein Geminin. The removal of Cdt1 from the origin site is crucial in dissociating the origin and preventing multiple firing. For this reason we included Geminin in our analysis to determine if Myc stabilizes the initiation complex by binding Cdt1 and preventing Geminin from escorting Cdt1 outside the nucleus. We analyzed the level of interaction that takes place between Geminin and Cdt1 at the respective time points where Myc is bound to the initiation complex.

2.3 Introduction

c-Myc is a DNA-binding protein of the helix-loop-helix family that must interact with Max to translocate into the nucleus and bind DNA (Blackwood and Eisenmann 191; Cole and Nikiforov, 2006). The structure of Myc includes a number of domains that are capable of undergoing a variety of protein-protein interactions. Although Myc is being extensively studied for the past few decades and the function of several domains have been explored including Myc box I, Myc box II and the transactivation domain, however, some domains in the basic region are still of unknown functions.

In its physiological role, Myc has been implicated in wide variety of cellular activities especially those that are of proliferative outcome. Most importantly Myc was shown to be essential in cell cycle progression (Lee and Dang 1997). Under cell-culture conditions, Myc is rapidly induced in response to various mitogenic stimuli, yet only few of the pathways that regulate Myc expression have been fully elucidated. Concomitant with promoting cell growth and proliferation, Myc inhibits terminal differentiation of most cell types (Mai and Mushinski, 2003; Eilers and Eisenman, 2008).

Myc is also broadly expressed during embryogenesis and in tissue compartments that possess high proliferative capacity in adult (such as skin epidermis and gut). It has been also shown that Myc plays essential roles in early developmental stages in several species including *Drosophila*, *Tetrahymena* and mouse embryos (Pierce et al., 2004).

Since the discovery of its viral homologue (v-Myc) in Simian virus 40 (SV40), Myc was thought to be only a transcription factor. However, reports within the last decade discriminated between Myc domains that are required for its transcriptional activity versus those involved in promoting cell proliferation (Dominguez-Sola et al.,

2007; Lebofsky and Walter, 2007). Those observations suggested that Myc is able to alter cellular activities in mechanisms different from its ability to manipulate gene expression. For example, in Fest T et al 2002, it was shown that the Delta 106 - mutant Myc with a deletion in Myc Box II, a domain essential for Myc's function as a transcription factor, still induced karyotypic instability when deregulated in Ba/F3 cells (Fest et al 2002; Fest et al., 2005; Guffei et al., 2007).

In different studies a deletion of the N-terminal Myc Box I within its transcriptional activation domain was reported as defective for cellular transformation *in vitro* but retained the transcriptional activity (Herbst et al., 2005). Those observations suggested that Myc may contribute to oncogenic transformation *via* non-transcriptional mechanisms.

As a proto-oncoprotein, Myc does not require a structural mutation to contribute to the initiation and development of cancer. In fact the expression of Myc was found to be deregulated in over 70% of human cancers (Nesbit et al., 1999). The deregulation of Myc expression alone is sufficient to induce various forms of genomic instability that lead to the formation of neoplastic lesions (Mai, 1994; Felsher and Bishop, 1999; Li and Dang, 1999). One form of genomic instability induced by Myc deregulation is gene amplification (Mai et al., 1996a).

Gene amplification has been implicated in several cancers including breast, lung and colon cancers (Vita and Henriksson, 2006). As one form of genomic instability, gene amplification may contribute to the initiation and development of cancer in two ways; one way if the amplicon includes "tumor promoting" oncogene(s) and the other way if the amplicon includes a set of target genes that might have high proliferative functions or

drug resistance functions (Debatisse and Malfoy, 2005). Two types of amplification events have been detected cytogenetically: intra- and interchromosomal amplicons (Stark et al., 1989; Windle and Wahl, 1992).

Intrachromosomal amplicons or double minutes (DM) may contain up to several hundred copies of a genomic segment and may form mini-chromosomes with inverted symmetry. Intrachromosomal amplicons, also described as abnormally banded or homogeneously staining regions, are head-to-tail or tail-to-tail repeats, which have ten or fewer copies at early stages of amplification. Moreover, some gene amplifications are accompanied by aneuploidy, deletions, or translocations (Albertson et al., 2003; Arvanitis and Felsher, 2005; Kuttler and Mai, 2007).

Several mechanisms have been proposed to identify the initiation event of gene amplification including double-strand breaks (DSB) dependent, breakage-bridge-fusion (B-B-F) or replication-driven (Debatisse and Malfoy, 2005). DSB-driven amplification is triggered under the influence of hyperactivity of nucleases or DNA-damaging agents including chemicals and ionizing radiation (Watanabe and Horiuchi, 2005). In addition, in human and rodent cells, chromosomal regions containing fragile sites, which are natural hot spots for breakage and recombination, are highly susceptible to amplification (Kobayashi et al., 2004; Debatisse and Malfoy, 2005; Shimizu et al., 2005).

B-B-F-dependent gene amplification can explain intrachromosomal amplification in cancers (McClintock, 1941; Shimizu et al., 2005). However, B-B-F model also depends on an initial DSB step; fusion of sister chromatids; formation of a bridge during anaphase; and asymmetrical breakage due to mechanical tension, which generates one

chromatid with an inverted repeat at the broken end and the second one with a deletion (Lobachev et al., 2002).

The third suggested mechanism is replication-driven. Replication dependent gene amplification takes place in some species during normal developmental stages (Maines et al., 2004). The theory postulates that rereplication of a gene occurs within a single cell cycle. This model allows for the formation of intrachromosomal amplification. It can also account for the formation of extrachromosomal DNA molecules that are released from their chromosomal locations by recombination (Kuschak et al., 2002; Debatisse and Malfoy, 2005). A well-known example is the amplification of chorion genes during *Drosophila* oogenesis (Spradling, 1981). Recent studies showed that dMyc (the *Drosophila* homolog of Myc) is essential for promoting the endoreplication process that takes place during the germ line development of *Drosophila* (Maines et al., 2004; Pierce et al., 2004).

Several proliferative genes were identified as targets genes for Myc dependent gene amplification including *dihydrofolate reductase (DHFR)*, *cyclin D2 (CCND2)*, *ribonucleotide reductase 2 (R2)*. In previous studies our group showed that the amplification of those genes *in vitro* takes place upon Myc deregulation (Mai et al., 1996b; Kuschak et al., 1999; Mai et al., 1999). Furthermore, in Kuschak et al 2002 our group showed that the amplification of the *R2* gene upon Myc deregulation occurred as a result of the initiation of multiple replication forks at the *R2* origin in a single cell cycle but in non transcriptional dependent fashion (Kuschak et al., 2002).

In this study, we investigate the presence of a common mechanism by which Myc dependent locus specific gene amplification takes place. We show in two mammalian

immortalized but karyotypically stable cell lines that c-Myc-dependent rereplication events take place during the replication of *CCND2* and *DHFR* loci. Consistent with this interpretation, we also show DNA structures suggestive of multiple firing events from the *DHFR* origin of replication. We also investigated a possible cross talk between Myc and early replication proteins. We report specific interactions between Myc and members of the replication initiation complex. We suggest that the ectopically induced deregulation of Myc stabilizes the replication initiation complex and leads to refiring of replication from origins at specific loci in a DNA damage independent mechanism.

2.4 Materials and Methods

2.4.1 Cell lines and culture conditions

Two cell lines were included in the study, mouse Pre B lymphocytic cell line and R1A rat fibroblasts (Littlewood et al., 1995; Mai et al., 1996a). Both cell lines are immortalized but karyotypically stable (Mai et al., 1996b; Louis et al., 2005). Pre B cell line are propagated in RPMI 1640 (Biofluids, Inc, Rockville, MD), supplemented with 10% heat-inactivated (30 minutes, 56°C) fetal bovine serum (FBS) (Gibco/BRL, Germantown, MD), 2 mmol/L glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin and 0.1% β-mercaptoethanol (Invitrogen/Gibco, Burlington, ON, Canada) at 37°C in a humidified atmosphere and 5% CO₂. The R1A cell line were grown in DMEM media (Gibco/BRL, Germantown, MD) supplemented with 10% heat-inactivated (30 minutes, 56°C) estrogen stripped fetal bovine serum (Gibco/BRL, Germantown, MD), 2 mmol/L glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a humidified atmosphere and 5% CO₂. Both cell lines carry an exogenous spontaneous MycERTM expression system. In Pre B cells ectopically expressed Myc requires the addition of four hydroxyl tamoxifen (4HT) to translocate into the nucleus because the estrogen receptor component of the chimera protein is modified to be sensitive only to 4HT (Littlewood et al 1995). 4HT (Sigma-Aldrich, Oakville, ON, Canada) is added in PreB cells to a final concentration of 100 nM in 10⁵ cells/ml. In R1A MycERTM is translocated to the nucleus after the addition of estrogen. R1A cells were induced by adding the estrogen homologue β-oestradiol (Sigma-Aldrich, Oakville, ON, Canada) to a final concentration of 50 nM in 10⁵ cells/ml.

2.4.2 Cell synchronization and cell cycle arrest

For G₁/S synchronization, PreB cells were incubated for 42 hours in RPMI 1640 minimal media that have been depleted of the amino acids methionine, cysteine, and L-glutamine (Gibco/BRL, Germantown, MD). After the 42 hours incubation, cells were returned to complete RPMI 1640 media (Gibco/BRL, Germantown, MD) supplemented with mimosine at a concentration of 0.4 µg/ml for 8 hours. Mimosine is an alkaloid isolated from *Mimosa pudica* and known to arrest cell cycle at G₁/S boundary by competing with the availability of cellular nucleotides. In contrast to the synchronization scheme employed for Pre B cells, R1A were synchronized at G₁/S transition without amino acids depletion. Cells were incubated in growth media supplemented with mimosine at a final concentration of 0.1 µg/ml for 8 hours.

2.4.3 Cell Cycle Profiles

Cell cycle profiles were studied for both cell lines. Cells were analyzed according to their DNA content for the determination of G₁- and/or G₂-phase by flow cytometry. Synchronized cells were washed twice with PBS followed by fixation on ice for 30 min using 70% ethanol in PBS or at 4°C for 30 min. Ethanol fixed cells were washed twice with PBS and incubated with 100 µg/mL RNase and 0.5 mg/mL propidium iodide at 4°C until just before analysis. Flow analysis was carried out using an EPICS Altra cytometer (Beckman-Coulter, Mississauga, ON, Canada).

2.4.4 Myc activation.

Myc activation in both cell lines were carried as described in 2.3.1 after releasing the cells from the cell cycle arrest by placing the cells in fresh growth media free of mimosine. The time when Myc inducing/activating agent was added to media was considered zero time point. Cells were harvested at zero, 1, 2, 3, 24, 48 and 72 hours.

2.4.5 Immunofluorescence assay

Pre B cells were resuspended in PBS buffer and deposited on slides whereas R1A cells were grown on slides. Cells were fixed in 3.7% formaldehyde in PBS/50mM MgCl₂. Cells were then permeabilized using 1% Triton for 10 minutes and then blocked using FBS. Cells were stained or co-stained with one or two polyclonal primary antibodies to final concentration of 25 ng/slide for 45 minutes (min) at room temperature (RT). Primary antibodies used in these experiments are against Myc, Orc1, Cdc6, Cdt1, Geminin, Mcm7, Pol α and PCNA were all purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Following the antibody incubation, cells were washed to remove unbound primary antibody, reblocked and incubated with 10 ng/slide of the appropriate secondary antibody for 30 minutes at RT. Slides were then washed and 20 μ l of vectashield was applied to minimize bleaching. Cells were visualized and imaged using a Zeiss Axiophot 2 microscope (Zeiss Ottawa, Ontario, Canada). Images were acquired with a Cooke CCD SensiCam Camera. Fluorescent signals were quantified using Northern eclipse software (EMPIX Imaging Inc Mississauga, ON, Canada). Intensity was expressed as arbitrary grey scale units. Statistical analyses were done using Microsoft Excel software.

2.4.6 Fluorescence in Situ Hybridization (*FISH*)

FISH was performed on fixed interphase cells either laid on the slides (PreB) using a cytocentrifuge or grown on slides (R1A). After acetic acid fixation cells were treated with 100 µg/ml Rnase for 1 hour (hr) at 37°C. Cells were washed in 2X SSC and incubated with 50 µg /ml pepsin for 10 min at 37°C. Slides and probes were then denatured and then incubated with 1ng of digoxigenin (DIG) labeled probes specific to either *DHFR* (1.5kb), *CCND2* (2.2 kb), *R2* (1.5kb) and *CycC* (400 & 500 bases) overnight. Slides were then washed and incubated with anti DIG antibody (Roche Diagnostics, Laval, Québec, Canada) labeled with either Texas red or FITC™ for visualization. Cells were visualized and signals were quantified as mentioned above.

2.3.6. Co-immunoprecipitation

Co-immunoprecipitation was performed according to the manufacturer suggested protocol. 50-100 µg of whole cell extracts from each time point were incubated for four hours or overnight at 4°C with 50 ng of one polyclonal antibody (same antibodies mentioned in 2.3.4 in addition to anti Max antibody (Santa Cruz biotechnology) which has been preabsorbed to protein G-bound to sepharose beads (Sigma-Aldrich, Oakville, ON, Canada). Following incubation beads were washed to remove excess unbound extracts, resuspended in 2X Laemmli sample buffer. Samples were then boiled and briefly centrifuged. Supernatant was subjected to SDS-PAGE and Western blot analysis.

2.3.7. SDS-PAGE and Western Blot analysis

Whole cell extracts were prepared at the mentioned time points by applying three freeze and thaw cycles. Western blots were then performed as described in Albihn et al., 2006 (Albihn et al., 2006). In brief, 10-15 µg of extracts were loaded on a 10% SDS–polyacrylamide gel electrophoresis (Invitrogen Corp., Carlsbad, CA, USA) and transferred to a nitrocellulose Hybond membrane N⁺ (Amersham, UK). The blots were blocked with 3% bovine serum albumin (BSA) (Roche Diagnostics, Laval, Québec, Canada) for 1 hr and probed with 4 ml of 25 ng/ml with one of the same primary antibodies mentioned in 2.3.5 for 1-4 hrs at RT. Blots were washed and reblocked in 1% BSA followed by incubation with 4 ml of 1/25000 anti rabbit/mouse/goat HRP secondary antibody (Amersham UK) for 1 hr at RT. Blots were visualized using Nitrocellulose, Hybond ECL kit (Amersham, UK) and analyzed using a Fuji LAS 1000 system (FujiFilm Medical Systems Inc., Stamford, CT, USA). Signals were quantified using a Fuji densitometer (FujiFilm Medical Systems Inc., Stamford, CT, USA).

2.3.8. Two dimensional neutral /neutral gel electrophoresis

Neutral-neutral 2-dimensional (2D) gel electrophoresis was performed as follows: Genomic DNA was isolated from synchronized Myc non-activated and activated cells by standard procedures. 150 µg genomic DNA of each sample was digested overnight at 37°C with *Hind*III (Roche Diagnostics, Laval, Québec, Canada). The samples were electrophoresed in two dimensions under neutral-neutral conditions (Kuschak et al., 2002). In brief, Restriction digests were separated in the first dimension on a 0.4% agarose gel that was run for 3 – 4 hrs at 50 to 70 Volts (V) in TBE (89 mM Tris-HCl [pH

8.0], 89 mM boric acid, 2 mM EDTA) at room temperature. The gel was stained with 0.3 µg/ml of ethidium bromide. Gels were briefly illuminated with UV light to enable individual lanes to be excised with a blade. The excised lanes were turned 90° and placed at the top of a 1% agarose gel made up in TBE. Separation in the second dimension was performed in the cold room for 4 - 6 h at 70 to 90 V. The samples were then transferred overnight to nitrocellulose Hybond C+ membrane (Amersham, UK). Blots were then washed and blotted.

The probes that were used in the *FISH* experiments were also used in the Southern blot analyses of the 2D gels (2.3.6). 50 ng of each probe was labeled with 50uCi ³²P-α-dCTP (Amersham, UK) by random priming using an Oligolabelling Kit (Amersham, UK). Signals were visualized using a Fuji LAS 1000 system (FujiFilm Medical Systems Inc., Stamford, CT, USA). In addition to genes specific probes, we used oligonucleotides designed from the sequence of each gene of interest (*DHFR*, *CCND2* & *R2*) that include one of Myc E-boxes sequence (underlined); A 24 mer scrambled oligonucleotide (tandom repeats of TTAAGG) was included in the study to determine binding specificity. Oligonucleotides were end-labeled with 50uCi ³²P-γ-dATP (Amersham, UK). The sequences and location of the oligonucleotide are as follows:

DHFR (DHFR I; -573 to -550): 5'-GGCGCGACACCCCACGTGCCCTCT-3';

CCND2 (E3; -955 to -930): 5'- ATGCTATCTGCCACGTGCCCAGCGTGGC-3'' and

R2 (R2-3; 4781 to 4805): 5'- GAGCCCCCACGGTGACCTTGAACG-3'

2.5 Results

2.5.1 Ectopic activation/expression of Myc in mouse Pre B and rat R1A cells

The level of expression of Myc in the two cell lines was measured after cells were activated by adding 4HT or β -oestradiol to Pre B and R1A cell lines respectively. Myc levels were measured at zero, 1, 2, 4, 6, 24, 48 and 72 hours after activation. Starting from 1 hour time points Myc was detected in the nucleus in both cell lines (Figure 2. 1 A ,B , C & D). The maximum presence of Myc in the nuclei took place at 5 or 6 hours time point in Pre B and R1A cell lines respectively and then started to decline. At 24 hours time point Myc level was still significantly higher than zero but lower than 1 hour time point (Figure 2. 1 A, B, C & D). Myc expression and translocation to the nucleus was confirmed by immunofluorescence each time. The experiments were repeated 3 times.

2.5.2 Cell synchronization at G₁/S phase

In order to assess the changes in the replication pattern upon Myc activation or induction, our experiments were designed to be performed on cells synchronized at G₁/S-phase boundary. Synchronization was achieved by either growing cells in minimal media followed by mimosine treatment in Pre B cells or mimosine treatment only in R1A cells. Only cells that had over 80% synchronization at G₁/S boundary were considered for further analysis (Figure 2. 1 E).

2.5.3 System validation: c-Myc-induced amplification of selected target genes

In our studies we analyzed the replication pattern of *DHFR* and *CCND2* genes after Myc deregulation. We also considered *R2* gene which was previously analyzed in Kuschak et al 2002 as a positive control. There are previous reports that all three genes are amplified upon Myc deregulation. We were interested to see the amplification of those genes in our experiments before moving for further analyses. We also considered *CycC* gene in the study as a negative control because *CycC* copy number is not affected by Myc deregulation. We analyzed gene amplification by *FISH* using specific probes for each gene at zero and 72 hours time points after Myc deregulation. In Figure 2 we show amplification of *DHFR*, *CCND2* & *R2* but not *CycC* at 72 hours time point.

Amplification was detected in both cell lines (Pre B & R1A) either by visual observation of multiple signals or by comparing intensity of signals between zero and 72 hours time points (Figure 2. 2 A, B & C).

Figure 2. 1 Activation of ectopic Myc in Pre B and R1A cell lines and cell cycle arrest of Pre B and R1A at G₁/S boundary. A: A representative image showing PreB cell with mock Myc activation (cells were mock treated with ethanol) (left panel) or cells activated with 4HT at six hours (right panel). **B:** A graph showing quantification of Myc signals in Pre B cells after Myc activation. Minimum 40 cells were analyzed per time point. Error bars represent the standard deviation.

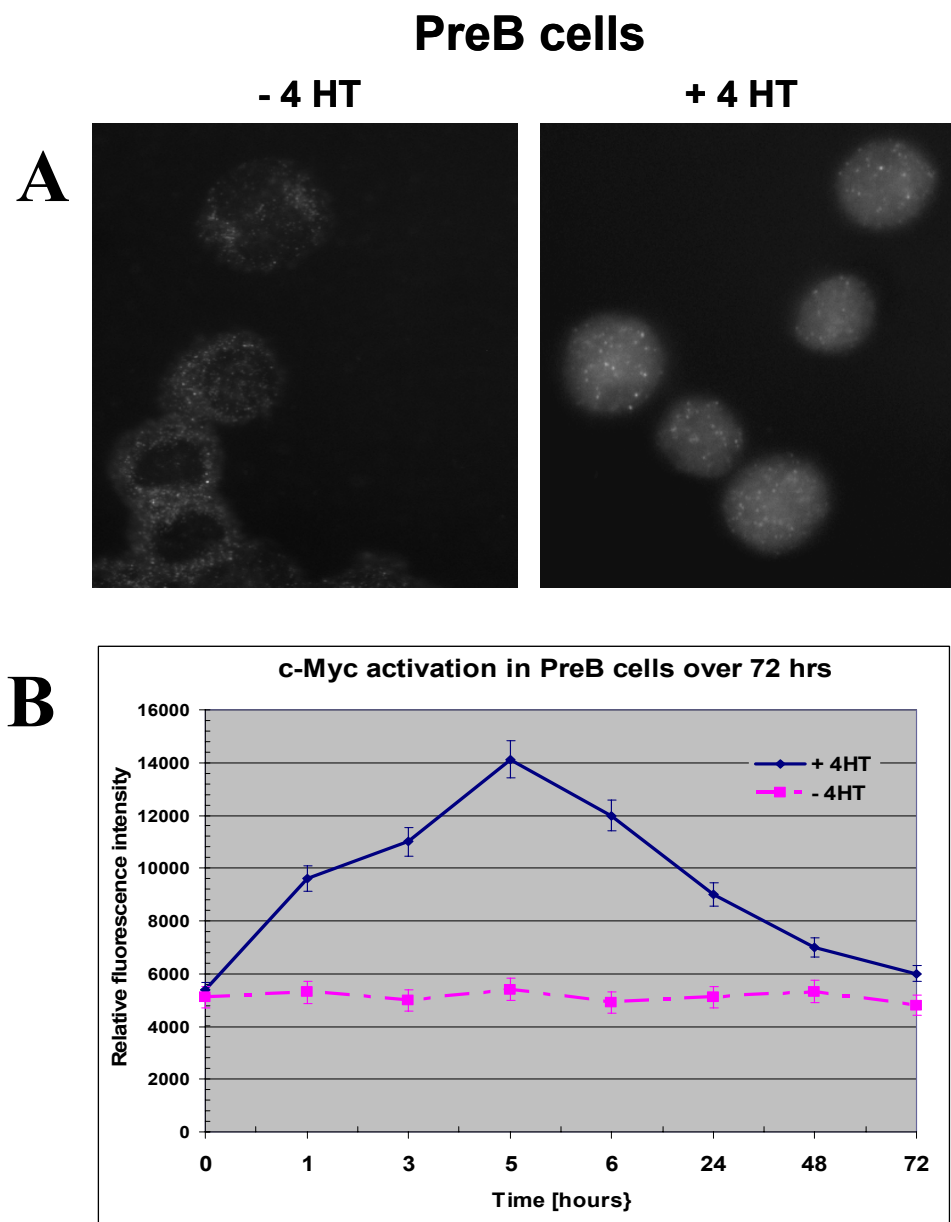
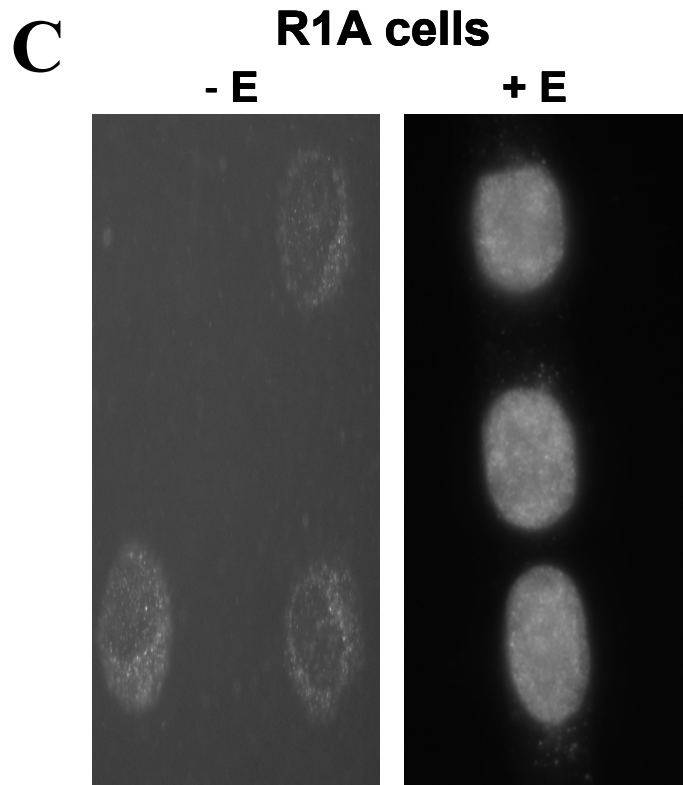


Figure 2. 1 (continue) C: A representative image showing R1A cells with mock Myc (-E) (cells were mock treated with ethanol, left panel) or cells activated with the estrogen homologue β -oestradiol (+E) at six hours (right panel). **B:** A graph showing quantification of Myc signals in R1A cells after Myc activation. Minimum 40 cells were analyzed per time point. Error bars represent the standard deviation.



D

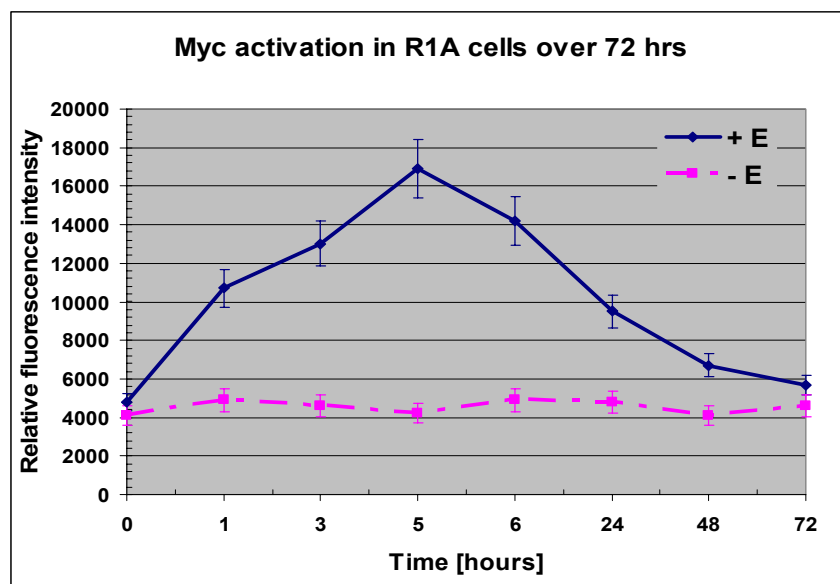


Figure 2.1. E A graph showing the flow cytometric cell cycle analysis of Pre B cells (top panel) or R1A cells (bottom panel) under normal growth conditions (left) or arrested at G₁/S transition (right).

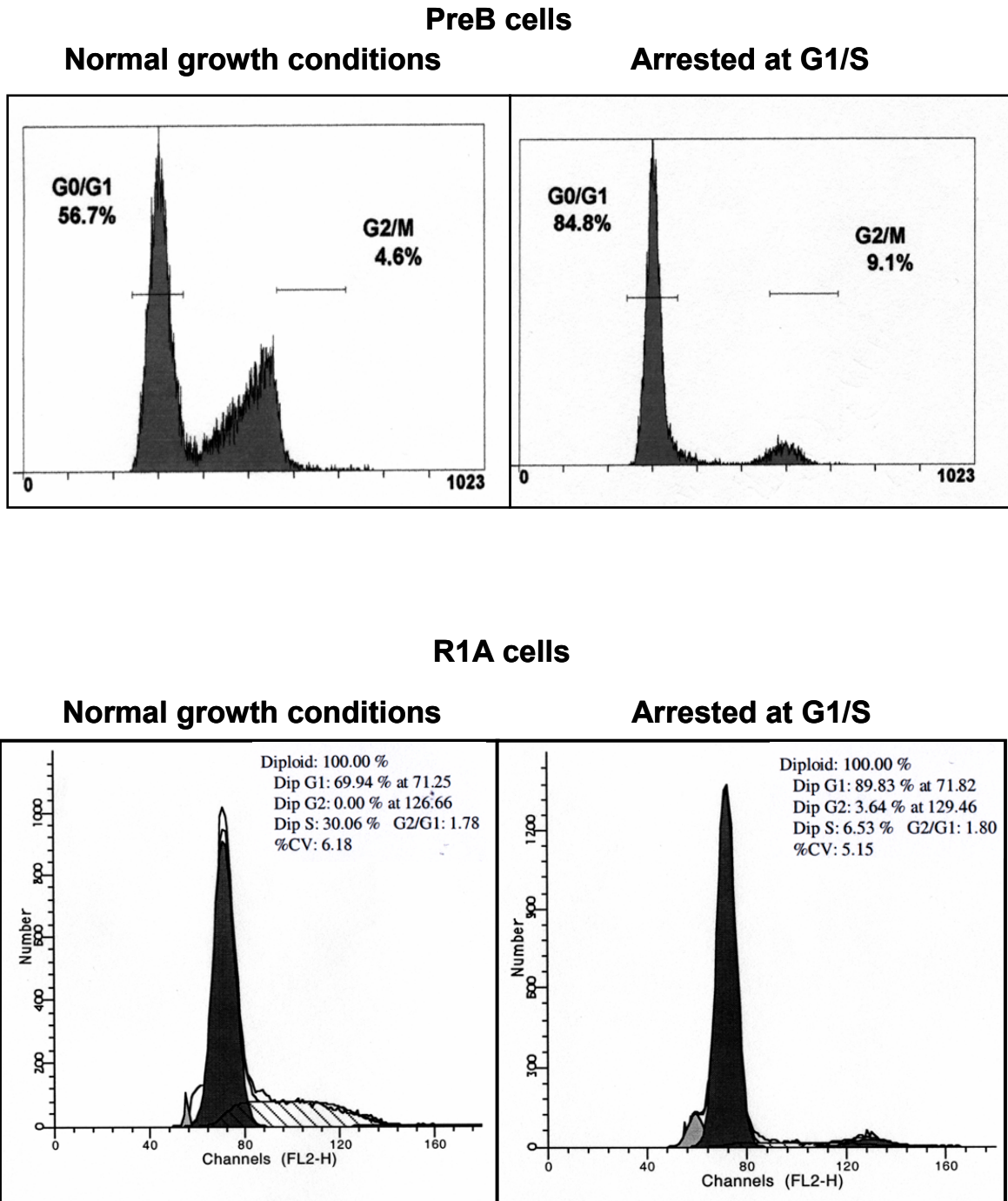


Figure 2.2 FISH Analysis of gene amplification at the *DHFR*, *CCND2*, *R2* and *CycC* loci in Pre B and R1A cells. A: Representative images showing *FISH* performed using gene specific probes on Pre B cells after Myc activation at time point zero or 72 hours (top hours). Bottom panel is a graph showing the quantification of the fluorescence intensity signals. Purple arrows point the *FISH* signals.

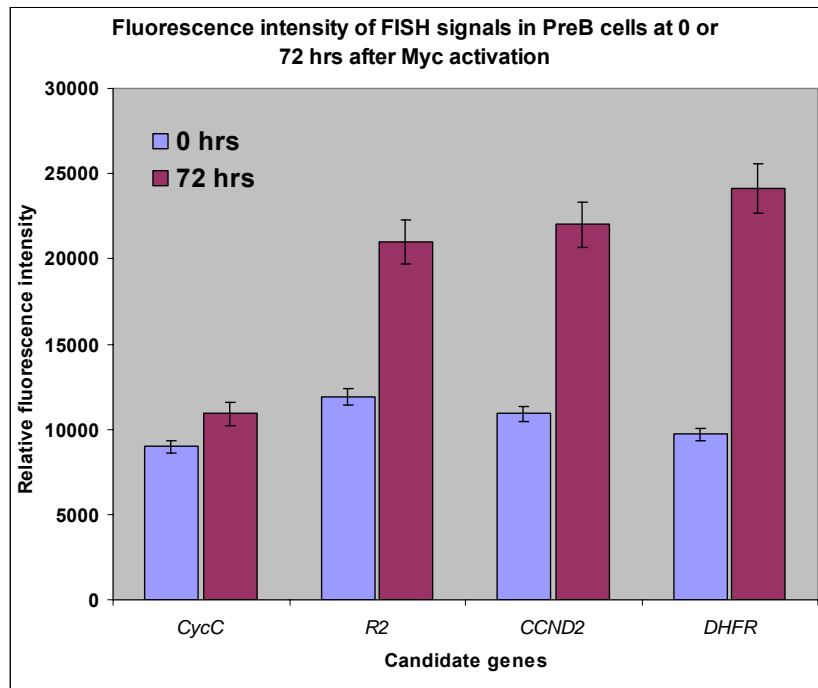
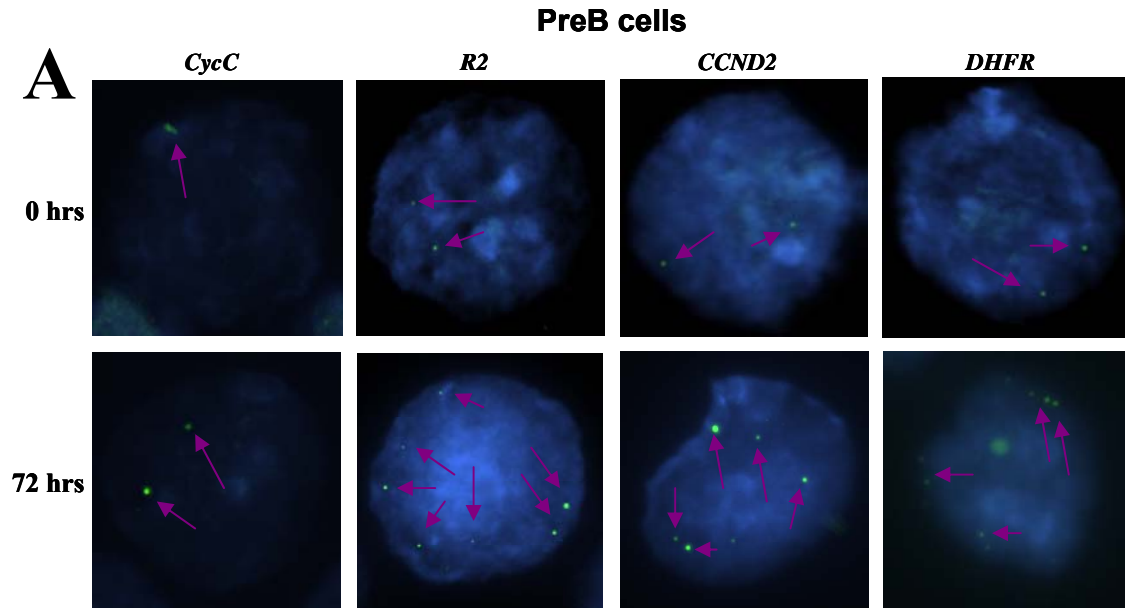


Figure 2.2 B: Representative images showing *FISH* performed using gene specific probes on R1A cells after Myc activation at time point zero or 72 hours. Bottom panel is a graph represents the quantification of the signals fluorescence intensity. Purple arrows point the *FISH* signals.

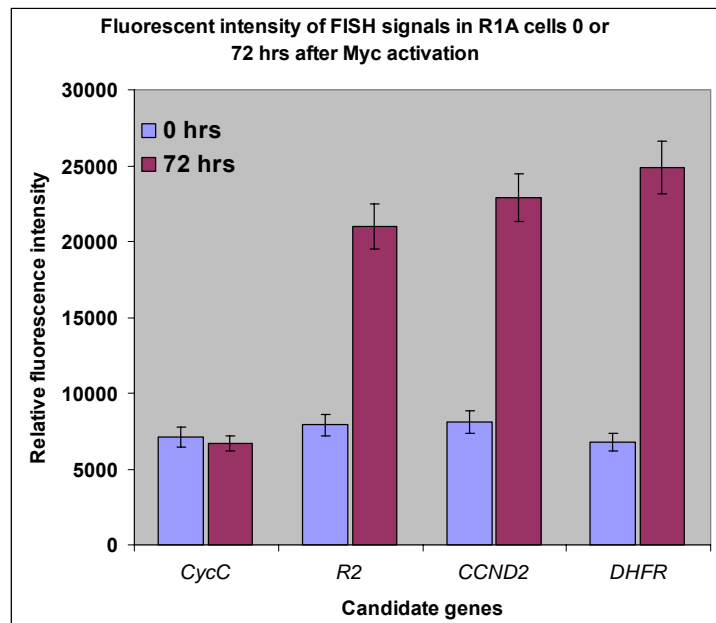
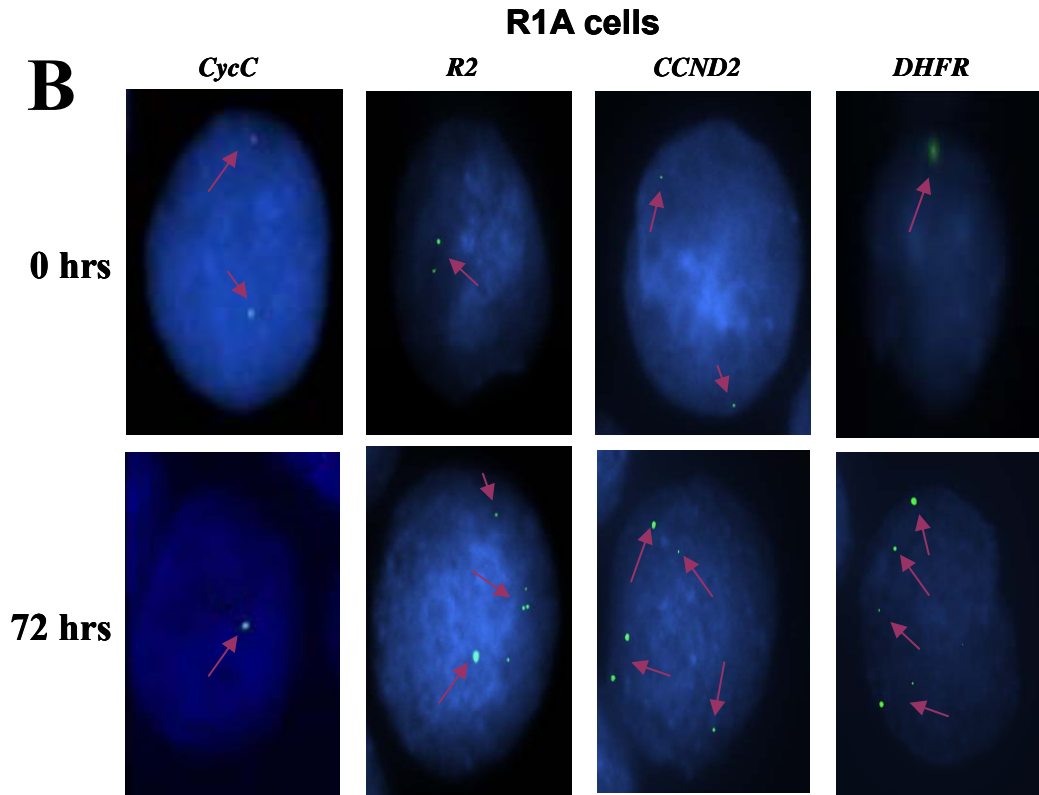
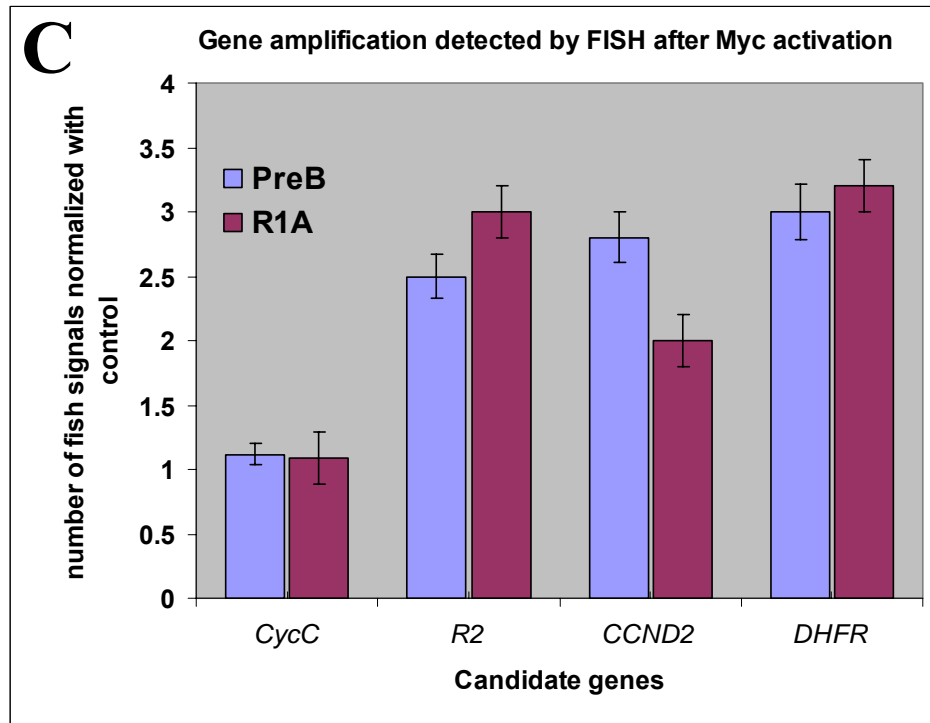


Figure 2.2 C: A graph showing the ratio of amplified *FISH* signals 72 hours after MycER™ activation normalized by the counts of signals at 0 time point. Blue represents PreB cells and red represents R1A cells respectively.



2.5.4 Analysis of the replication pattern of target genes

To investigate if the gene amplification that we observed takes place through a replication dependent mechanism, we analyzed the replication patterns at the four loci of interest using 2D neutral/neutral gel electrophoresis followed by Southern blot analysis. We analyzed time points zero, 1, 2, 3, 24 and 48 hours after the release from G₁/S arrest and Myc deregulation (Figure 2. 3). We also analyzed side by side cells with mock Myc activation from both cell lines at the respective time points.

Initially we probed the membranes with a probe specific for each gene in a sequential fashion. In both cell lines we observed the generation of several replication intermediates at time points 1, 2 and 24 hours at *DHFR*, *CCND2* and *R2* loci but not *CycC* after Myc deregulation (Figure 2. 3 B & C). Those replication intermediates resembled the simple Y-arc, commonly interpreted as to represent the propagation of a replication fork (Figure 2. 3 A). Normally a single Y-arc should be observed at each locus. Cells with mock Myc showed only one simple Y-arc (Figure 2. 3 B & C). The replication intermediates varied in number from one gene to the other and from one cell line to the other (black arrow on Figure 2. 3 B & C). In membranes blotted with *DHFR* probes, we also observed at 1 hour time point intermediates that have the shape of a bubble arc commonly interpreted to represent an origin of replication (Figure 2. 3 A). In cells with mock Myc activation we only observed one bubble arc in both cell lines but in cells with deregulated Myc we observed more than one bubble arc at the 1 hour time point (grey arrow on Figure 2. 3 B & C).

Figure 2.3 Analysis of the replication pattern at the *DHFR*, *CCND2*, *R2* and *CycC* loci in Pre B and R1A cells using 2D neutral/neutral gel electrophoresis.

A: A generated cartoon showing the patterns of typical replication intermediates separated by two-dimensional neutral/neutral gel electrophoresis. Cartoon represents the behavior of digested genomic DNA of mammalian species.

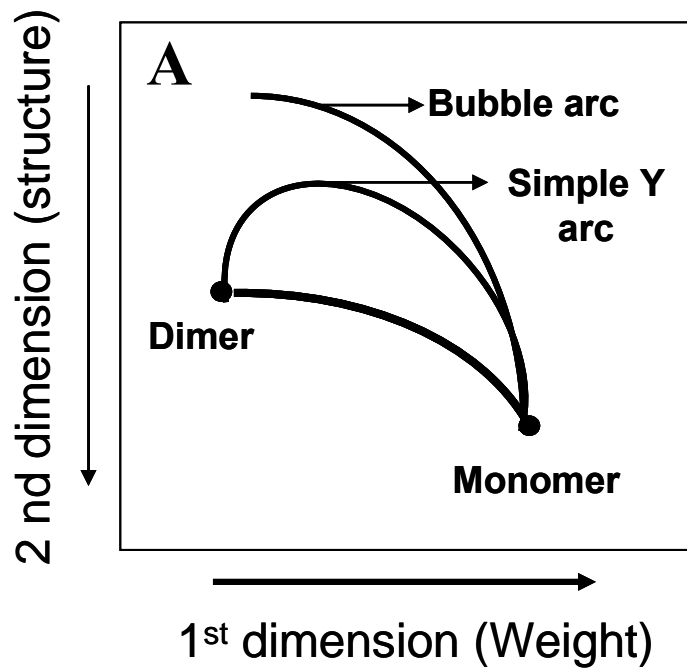


Figure 2. 3 B Analysis of the replication pattern in Pre B cells. The black arrows point the observed multiple Y- arcs and the grey arrows point the observed multiple bubble arcs.

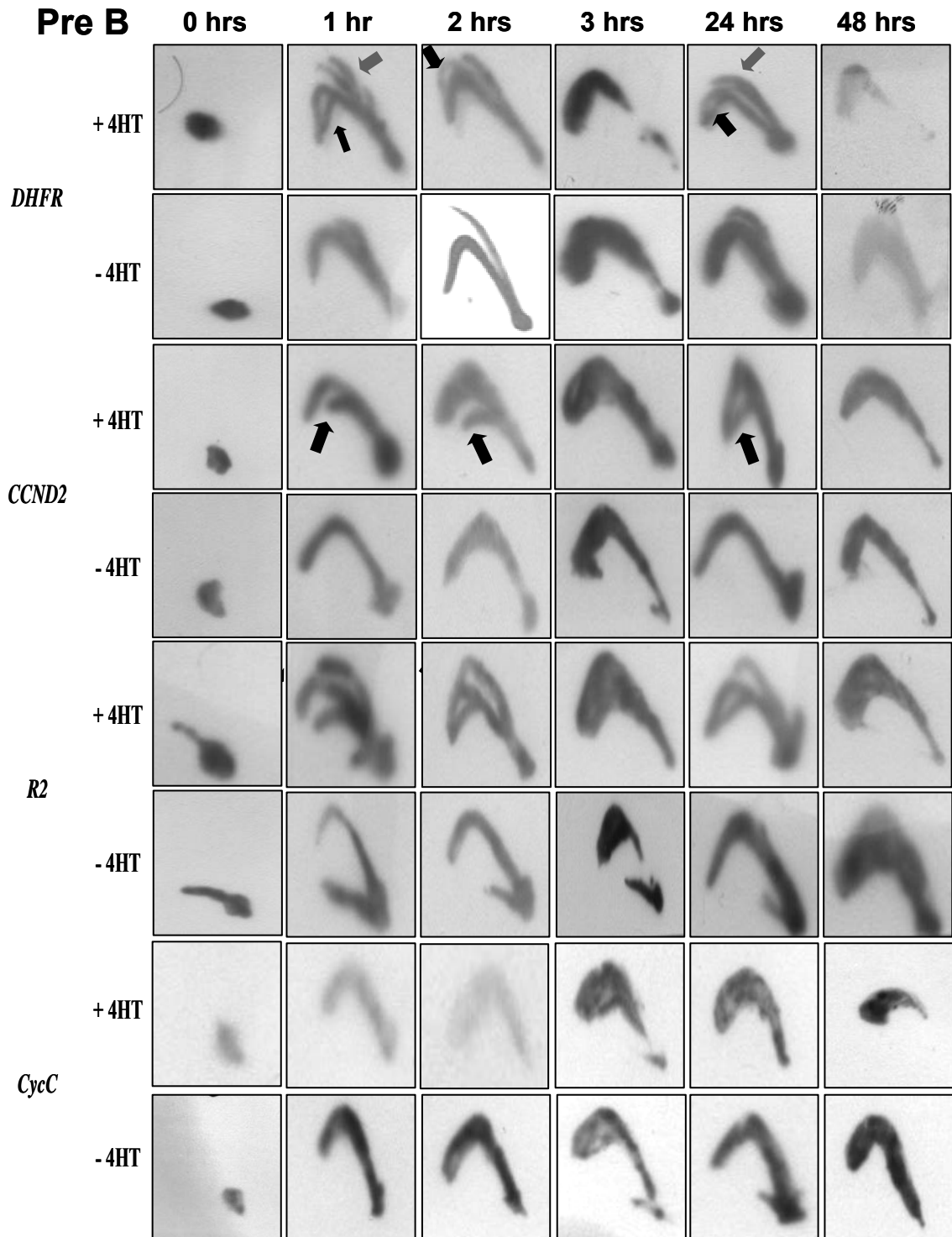


Figure 2. 3 C Analysis of the replication pattern in R1A cells. The black arrows point the observed multiple Y- arcs and the grey arrows point the observed multiple bubble arcs.

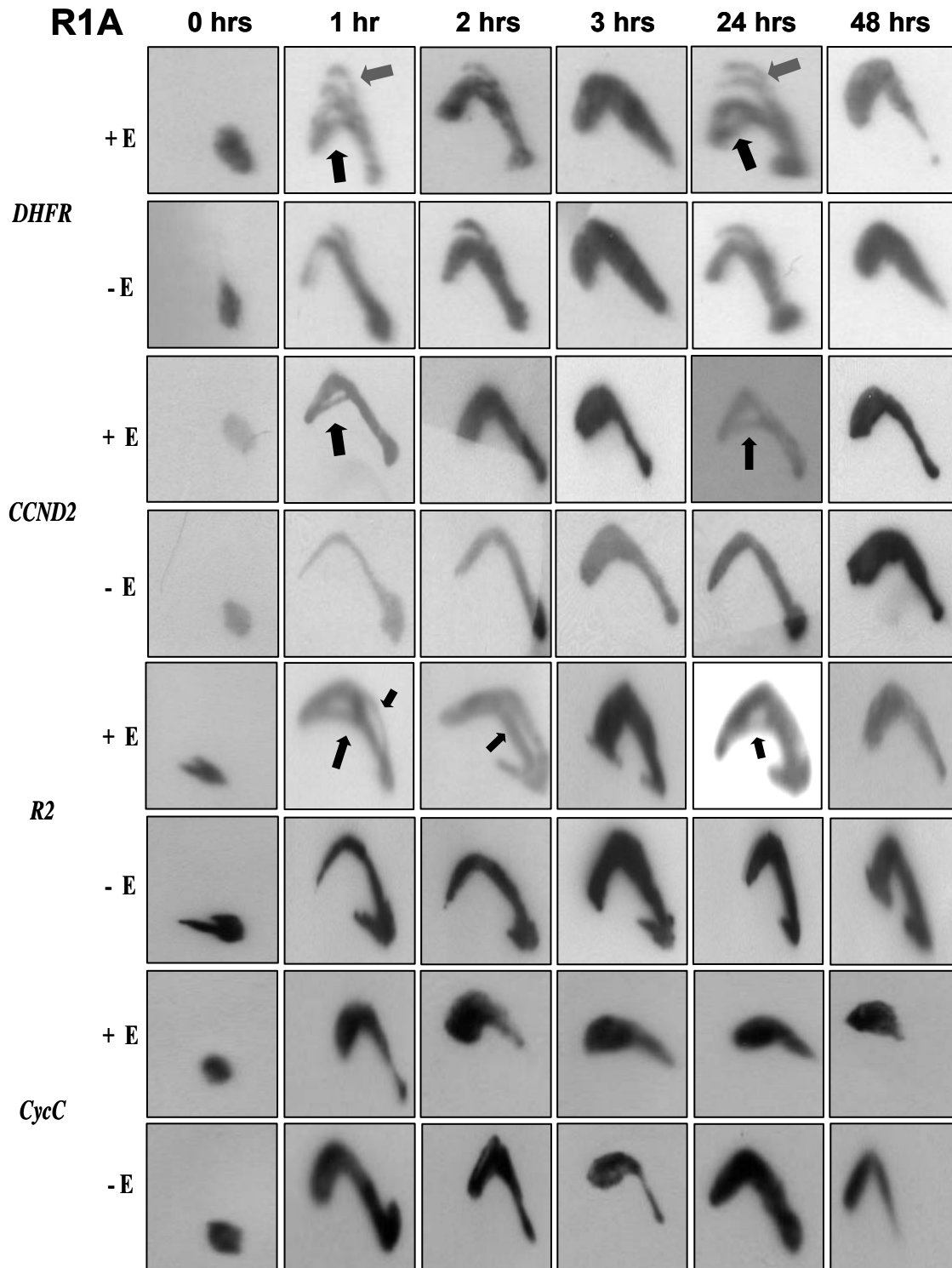


Figure 2. 3 D Analysis of the replication pattern in Pre B cells. Membranes were stripped and blotted with oligo nucleotides designed to include upstream sequence of each of the genes of interest that contains Myc E-Box binding elements. Bottom panel shows hybridization with scrambled sequence nucleotide.

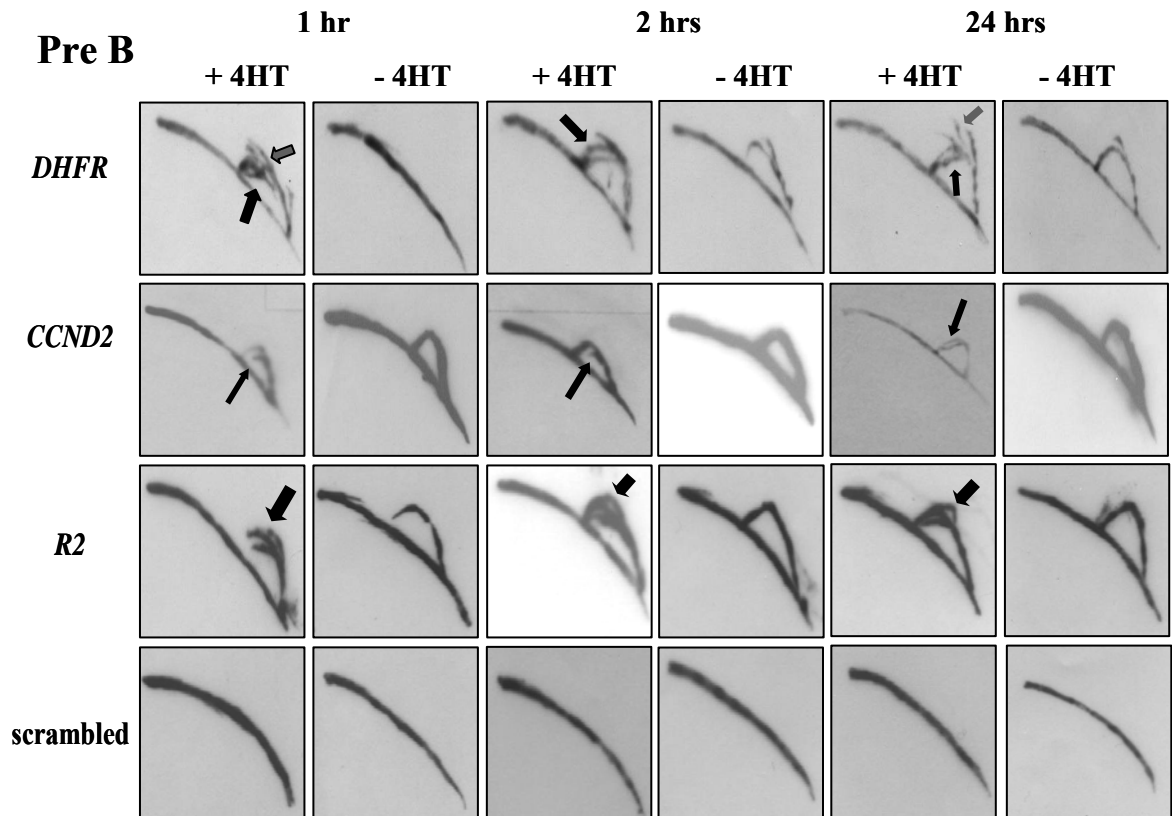
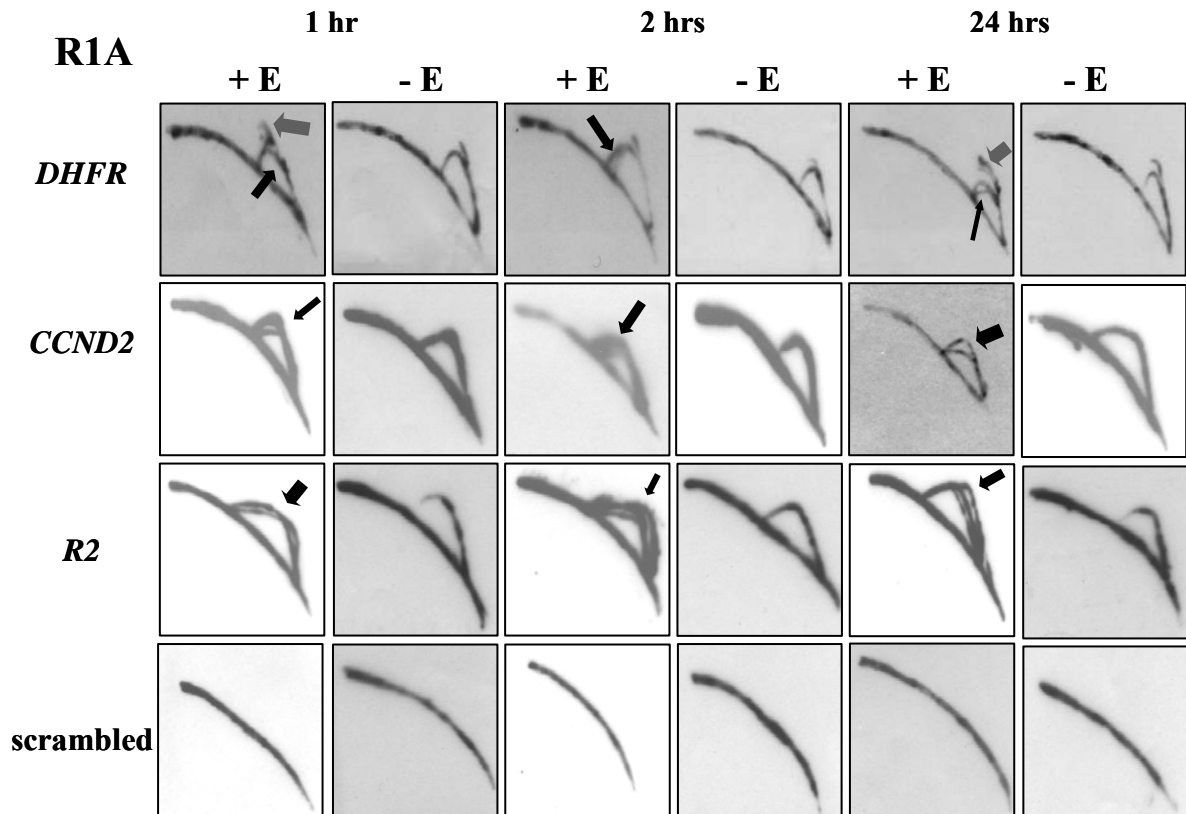


Figure 2.3 E Analysis of the replication pattern in R1A cells. Membranes were stripped and blotted with oligo nucleotides designed to include upstream sequence of each of the genes of interest that contains Myc E-Box binding elements. Bottom panel shows hybridization using scrambled sequence oligos.



2.5.5 Myc E boxes (binding elements) are present in the generated DNA intermediates

To determine if Myc E-box sequence is part of the observed DNA replication intermediates we hybridized the membranes with oligonucleotides carrying the upstream sequence of each gene including the E-Box sequence (Materials and methods **2.3.8.**) We also included oligonucleotides with scrambled sequence to confirm the specificity of the signals. We observed DNA structures similar to the ones generated upon using gene specific probes (Figure 2. 3 D & E) except for the scrambled oligonucleotides we only observed non specific hybridizations along the genomic DNA. *CycC* was not included in this analysis because no rereplication was observed when gene probe was used (Figure 2. 3 B & C)

2.5.6 Myc selectively interacts with members of replication initiation complex

To obtain evidence about Myc's role in initiating the replication arcs that we observed we investigated possible protein-protein interactions taking place between Myc and a number of proteins involved in DNA replication including those involved in the initiation and elongation processes. Our primary analysis performing co-immunofluorescence with Myc antibody together with antibody of one replication protein at a time indicated co-localization of Myc with Orc1 and Cdc6 but not with Mcm7, PCNA, Pol α (Figure 2. 4 A & B). To elucidate more details about such interactions we performed co-immunoprecipitation experiments. We used cell extracts obtained at the respective time points used in the 2D-gel analyses and under the same conditions. In addition to the antibodies used in the immunofluorescence experiments we included

antibodies against Cdt1, Geminin a protein that is not a member of the initiation complex but function to bind and escort Cdt1 away from the origin site once the origin fires to prevent rereplication. We also included antibody against Max the protein that binds Myc and enables the heterodimer to bind DNA. We started pulling down complexes using Myc antibody followed by Western blot using antibodies against the replication proteins. Myc was found to be in complexes with Max, Orc1, Cdc6, Cdt1 but not PCNA, Pol α or Geminin (Figure 2. 4 C; Figure 2. 5). Interestingly, at time points 1 and 24 hours the complexes were enriched with Orc1, Cdc6 and Cdt1 as compared to other time points (Figure 2. 4 C & Figure 2. 5). Mcm7 was found to be in the complex with Myc at low levels through out the time points of the experiment unlike Cdc6 or Orc1 (Figure 2. 4 C).

To confirm the data obtained from Myc IP we performed IP experiments using the respective replication proteins one at a time followed by Western blot analyses using antibodies against Myc, Max and the other replication proteins (Figure 2. 4 D). Orc1 and Cdc6 were indeed in a complex with Myc and Max and the complexes were also enriched at the identical time points when the complexes pulled by Myc were enriched (Figure 2. 4 D). Mcm7 also pulled Myc at low but consistent levels throughout all the time points of the experiment (Figure 2. 4 D).

Interestingly, the complexes precipitated by Myc or by Geminin antibodies showed a switch in the amounts of Cdt1 (Figure 2. 5 A & B). At the time points when complexes pulled by Myc antibody had high levels of Cdt1, complexes pulled by Geminin antibody showed low levels of Cdt1 (Figure 2. 5). This observation was also confirmed from analyzing the amounts of Myc and Geminin that were in complexes pulled by Cdt1 antibody at the respective time points.

Figure 2.4 Analysis of the interactions taking place between Myc and replication proteins in Pre B and R1A cells. A: co-immunofluorescence analysis showing colocalization of Myc and early replication proteins but not late replication or maintenance proteins in Pre B cells. Grey arrows point to co-localization of Myc and replication proteins.

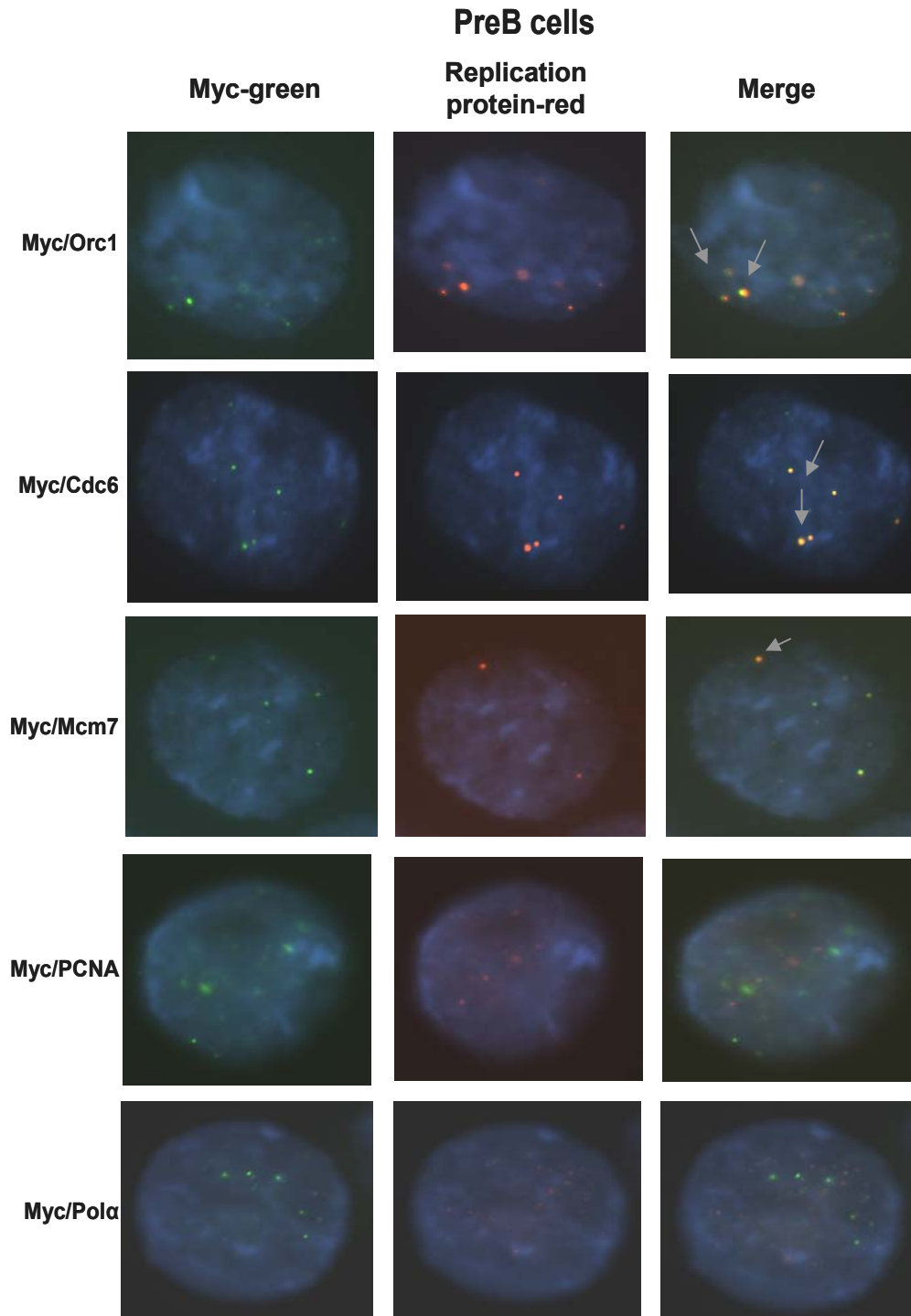


Figure 2. 4 B Co-immunofluorescence analysis showing colocalization of Myc and early replication proteins but not late replication or maintenance proteins in R1A cells. Grey arrows point the localization of Myc and replication proteins.

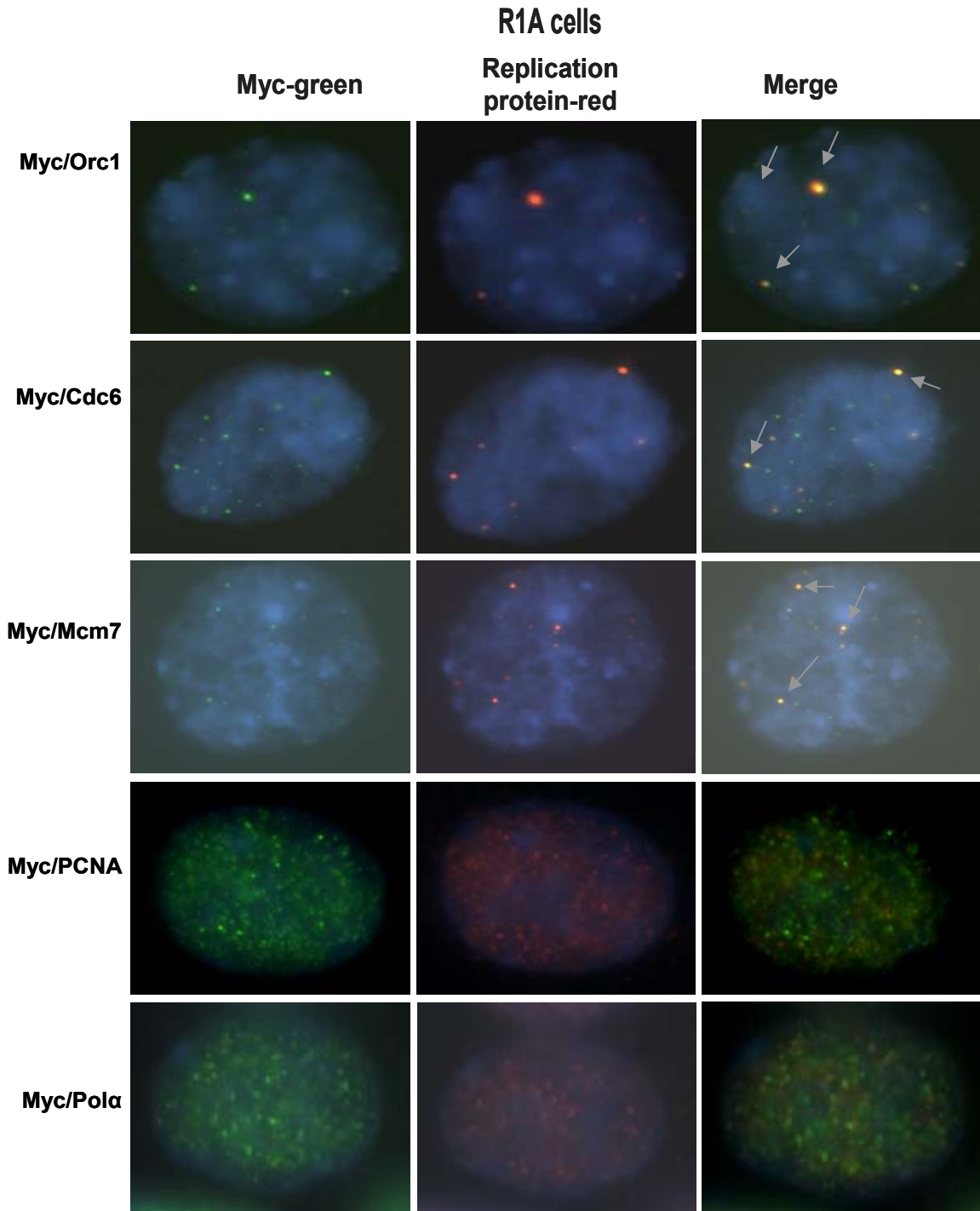


Figure 2. 4 C Co-immunoprecipitation analysis using Myc antibody (N262, Santa cruz) followed by Western blot analysis using antibodies against replication proteins. Top panel shows extracts from Pre B cells and right panel shows extracts from R1A cells. Both cell lines were synchronized. Time point zero represents release from G₁/S arrest and activation of MycERTM. 67KDa band of endogenous Myc is not shown.

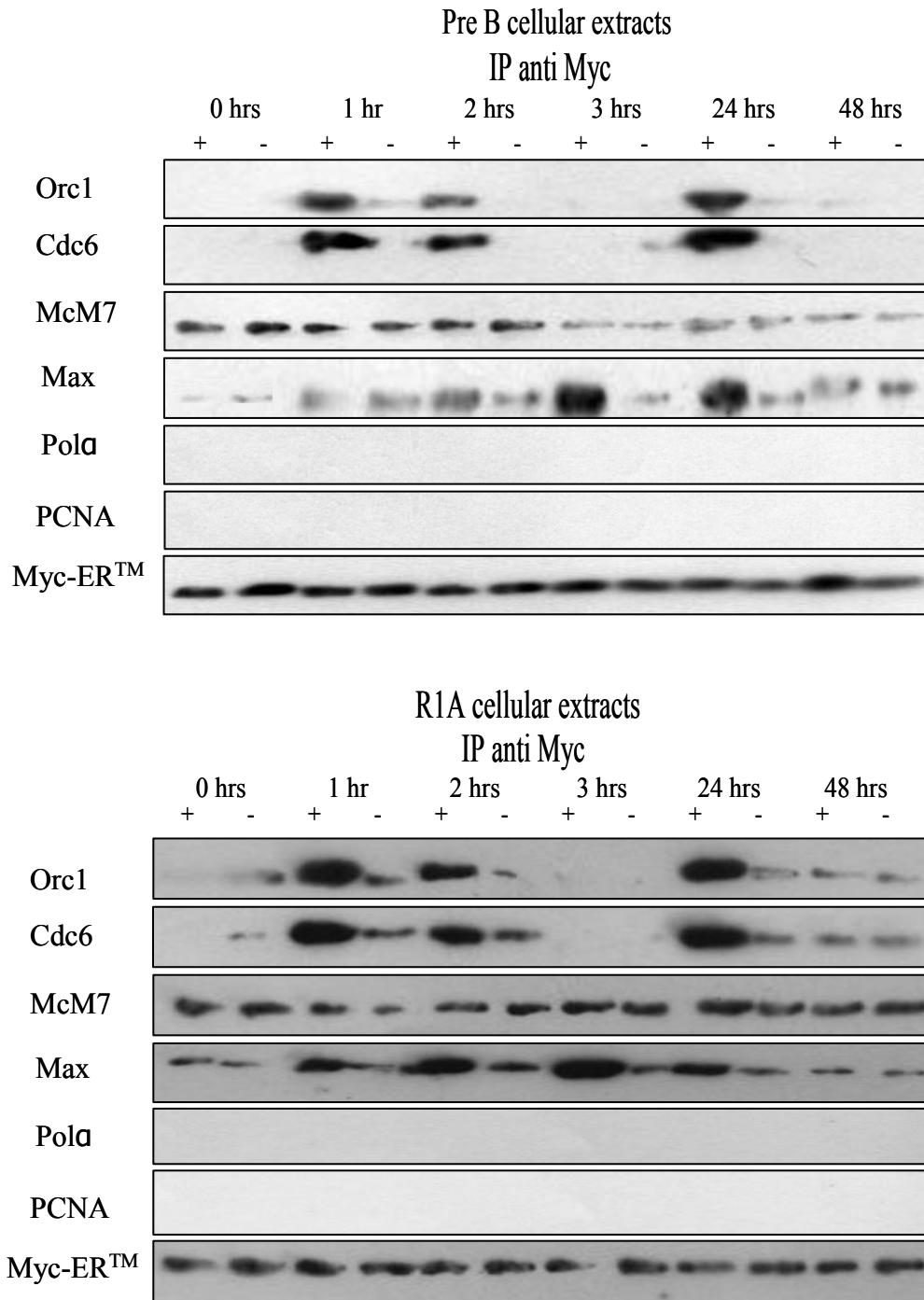


Figure 2. 4 D co-immunoprecipitation analysis using antibodies against replication proteins followed by Western Blot analysis using Myc, Max or other replication proteins antibodies. 67KDa band of endogenous Myc is not shown.

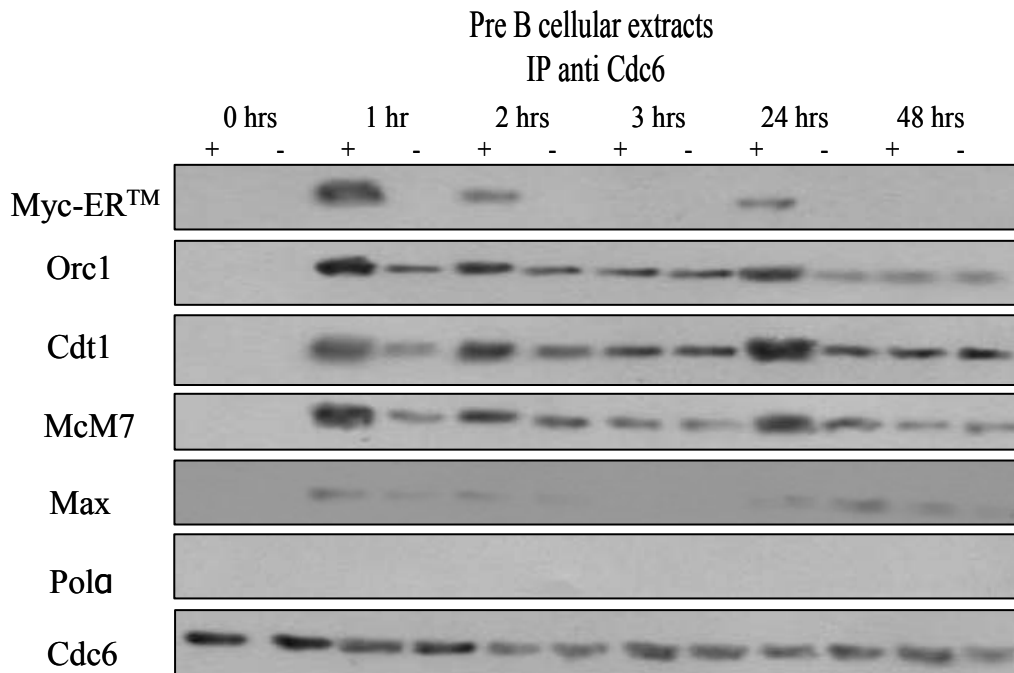
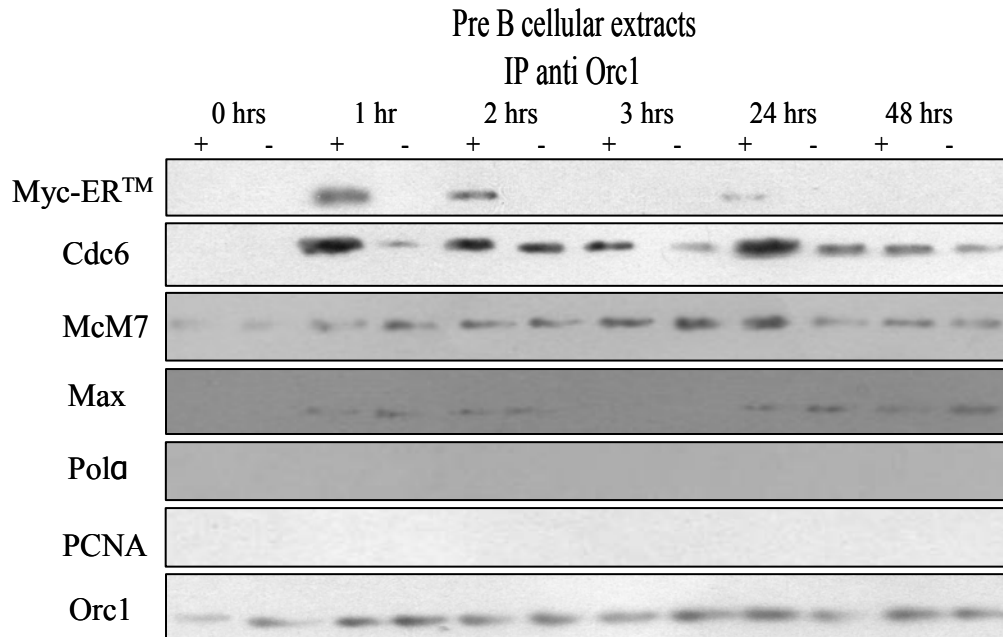


Figure 2. 4 D (continue): co-immunoprecipitation analysis using antibodies against replication proteins followed by Western Blot analysis using Myc, Max or other replication proteins antibodies. 67KDa band of endogenous Myc is not shown.

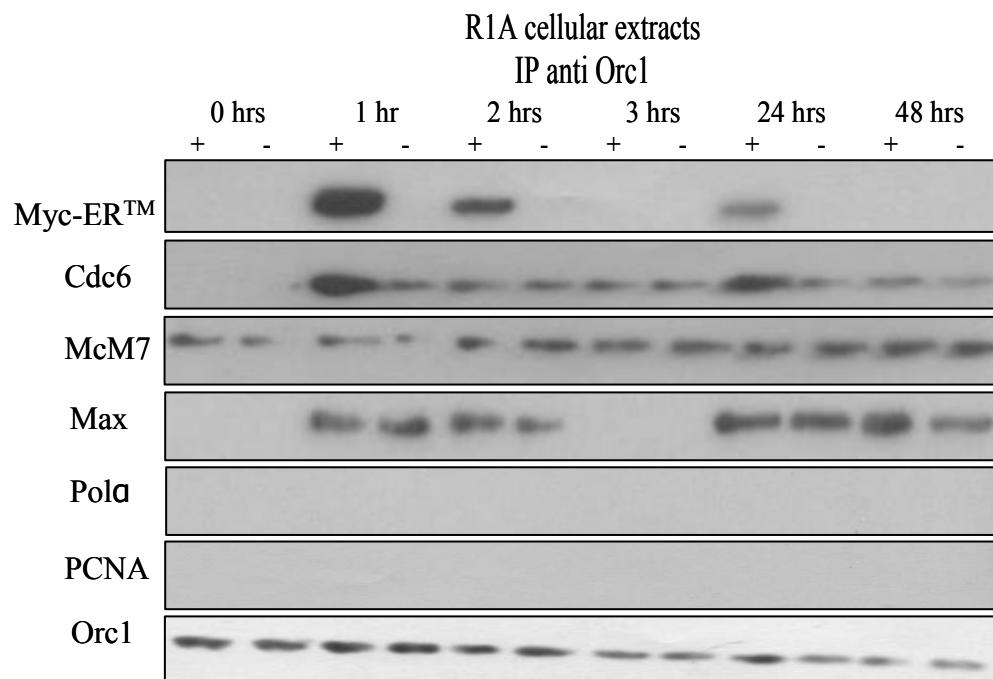
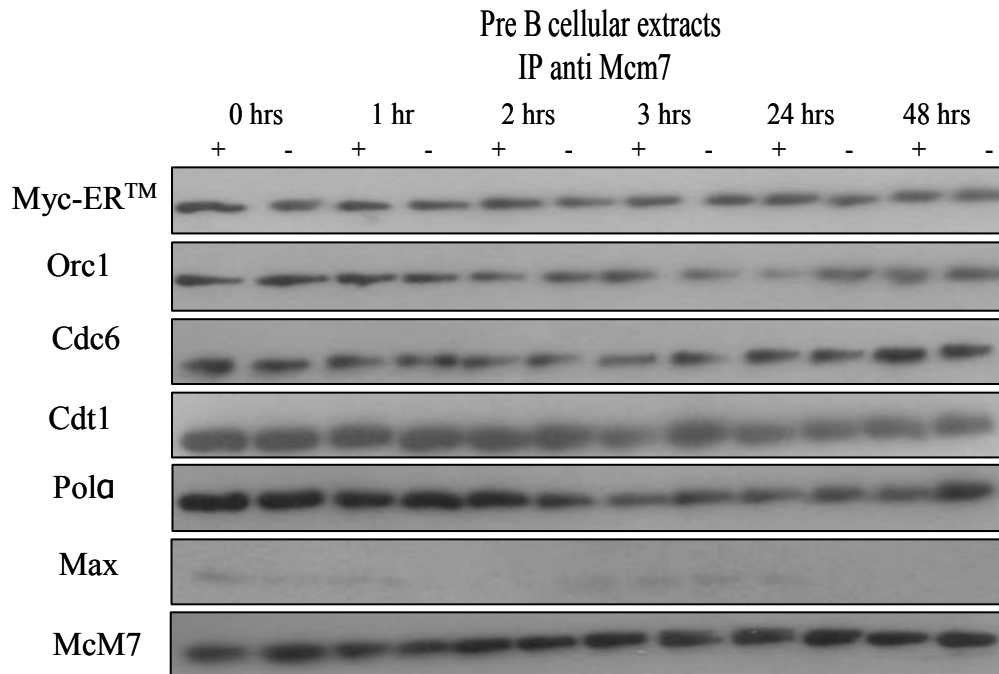


Figure 2. 4 D (continue) : co-immunoprecipitation analysis using antibodies against replication proteins followed by Western Blot analysis using Myc, Max or other replication proteins antibodies. 67KDa band of endogenous Myc is not shown.

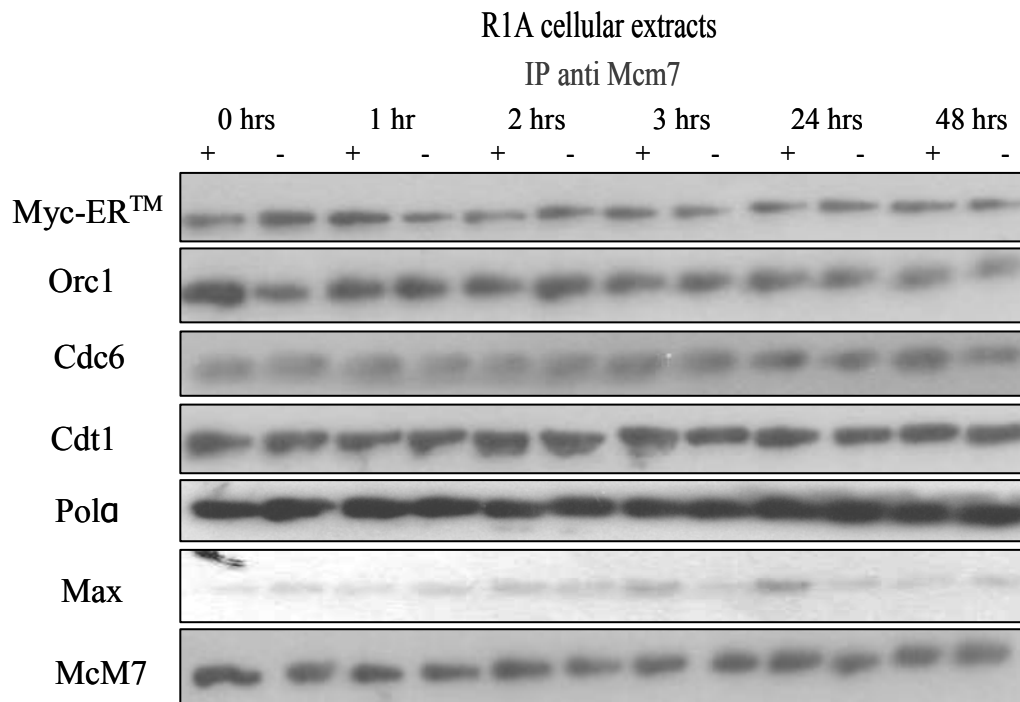
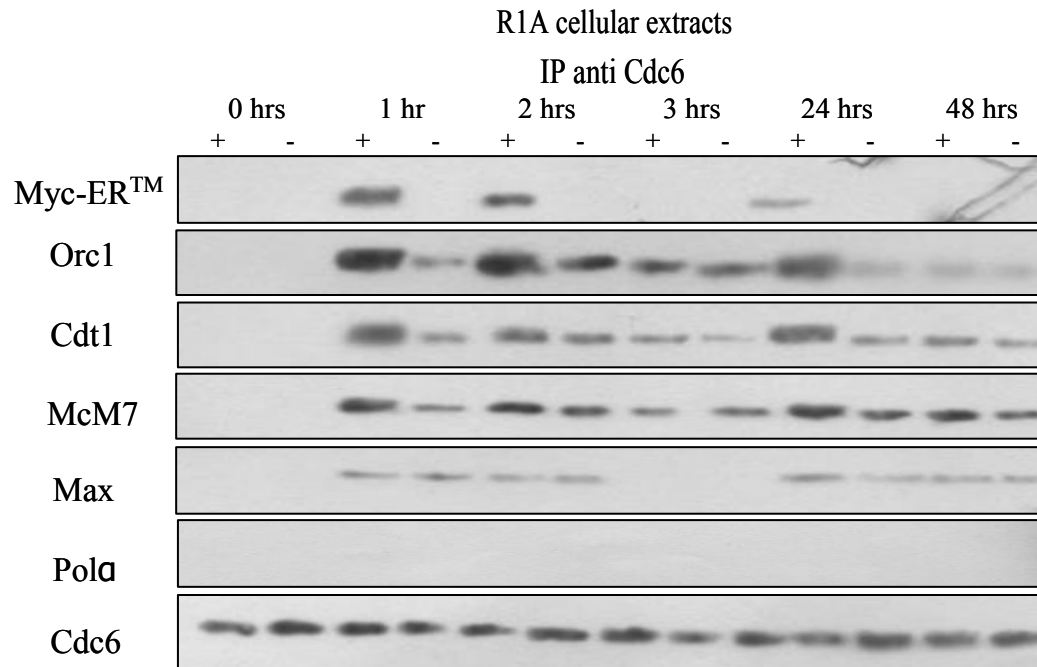


Figure 2.5 Interactions of Cdt1 with Myc and Geminin. A: Co-IP using Myc/Cdt1/Geminin antibodies performed on PreB cellular extracts followed by Western blot analysis. 67KDa band of endogenous Myc is not shown.

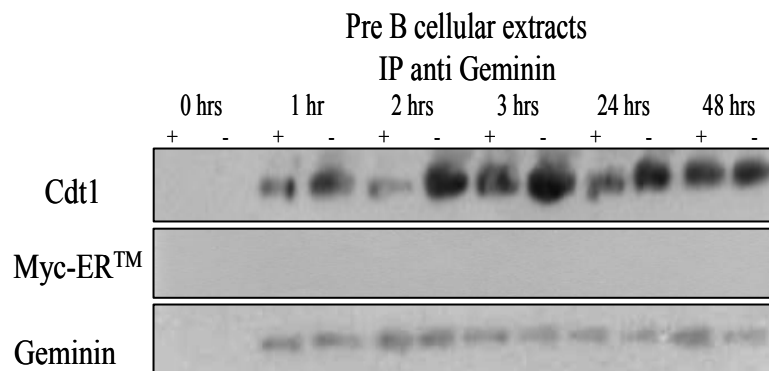
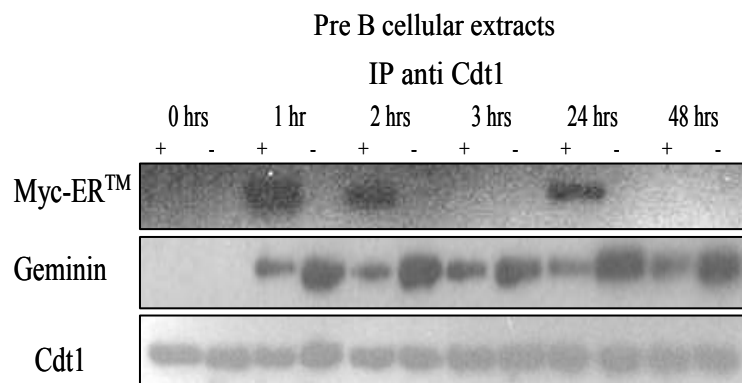
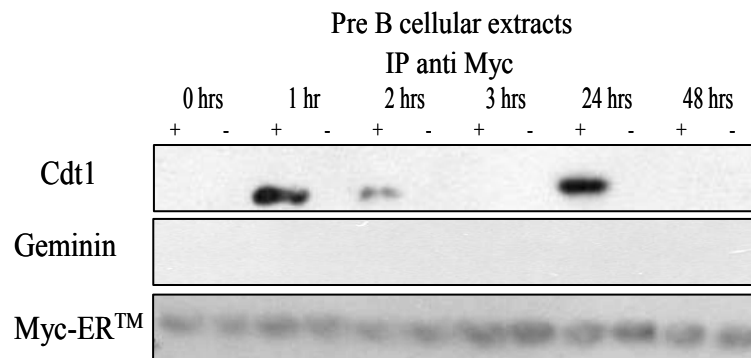
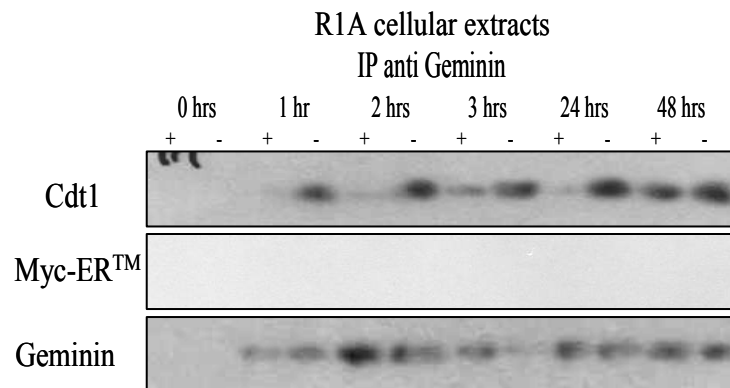
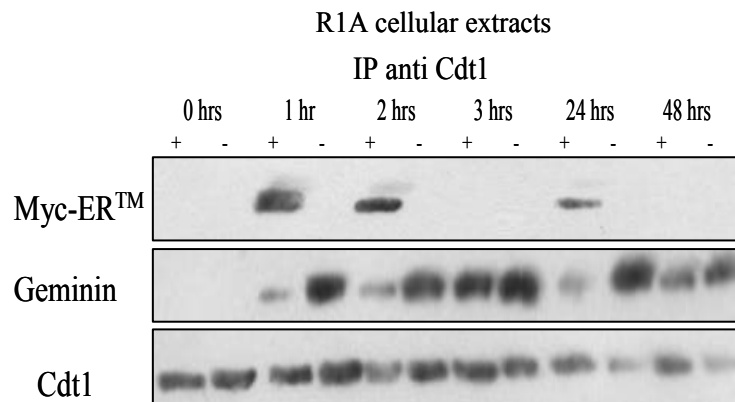
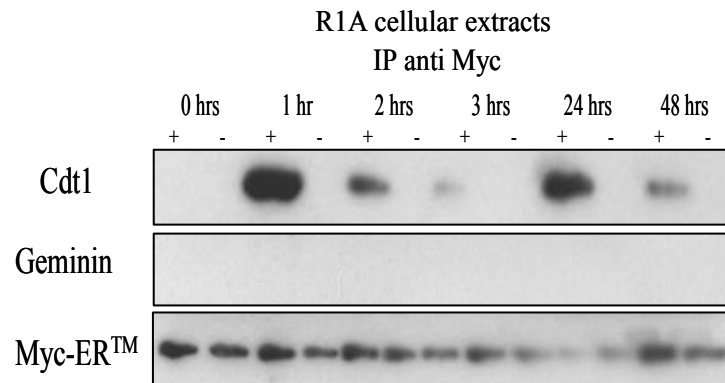


Figure 2. 5 B Co-IP using Geminin antibody followed by Western blot analysis using antibodies against Myc and Cdt1. Experiment performed on extracts from both Pre B and R1A. 67KDa band of endogenous Myc is not shown.



2.6 Discussion

Myc is multifunction protein that contributes to a number of cellular processes including proliferation. In normal cells Myc expression is tightly regulated. Deregulation of Myc level alone is enough to induce cellular transformation. Myc was found to be deregulated in 70% of human cancers (Nesbit et al., 1999). One way Myc contributes to cancer is through the induction of genomic instability. Gene amplification is an important form of genomic instability that is present and considered a causal factor for several human cancers (Jeggo, 2005) . Gene amplification was thought to develop through a DNA damage event that will be illegitimately followed by non homologous end joining recombination (NHEJ) or B-B-F cycles that may involve sister chromatid fusion followed by the formation of anaphase bridges and assymetric breakage of the two sister chromatids during cellular division (Debatisse and Malfoy, 2005).

Gene amplification in fact is a normal event in the development of several organisms the most studied of which is *Drosophila*. In the normal developmental stages of larvae in *Drosophila*'s, cycles of whole genome amplification take place without cellular division in a process known as endoreplication (Tsuyama et al., 2008). Alternatively specific genes may be selectively amplified like the chorion gene (Maines et al., 2004). Recent studies showed that Myc is essential for both processes. However, a unique mechanism by which Myc influence developmental gene amplification was not identified (Maines et al., 2004; Pierce et al., 2004).

A number of years ago our group reported that Myc deregulation lead to illegitimate replication at *R2* locus which later on leads to gene amplification. Here we asked the question if this is a common mechanism by which Myc deregulation will lead

to the amplification of additional Myc amplification target genes. We were also interested to know if this phenomenon was cell line dependant. We used two immortalized but karyotypically stable mammalian cell lines both are capable of ectopically expressing high levels of Myc. We focused our studies on the two genes *DHFR* and *CCND2* that are known to be amplified upon Myc deregulation. We also included *R2* studied in Kuschak T *et al.* (2002) as a positive control and *CycC* as a negative control.

Our data obtained after Myc activation in both cell lines showed the initiation of a number of replication intermediates with structures similar to the simple Y-arc that represents the replication fork at each of the *DHFR*, *CCND2* and *R2* loci. Under normal conditions only one replication fork (Y-arc) initiates per origin of replication per cell cycle and that was the case in our cell lines with mock Myc activation or at the *CycC* locus. The additional Y-arcs were observed at time points 1 and 24 hours and varied in number from one locus to the other and from one cell line to the other. Moreover, at the *DHFR* locus where well identified mammalian origins of replication are harbored we observed the initiation of an additional bubble arc suggesting more than one firing of that origin only upon Myc deregulation at time points 1 and 24 hours.

The observation of those *de novo* replication intermediates suggests that rereplication events are taking place upon Myc deregulation. Rereplication events are usually observed in structure function studies of the replication initiation complex were mutations are introduced to stabilize the pre-initiation complex at the origin of replication after the origin fires (Lutzmann *et al.*, 2006; Honey and Futcher, 2007).

To confirm that Myc is inducing rereplication events at the loci included in our study we probed the membranes obtained from the 2D replication gels with oligonucleotides each designed with specific sequence of one of the studied genes containing the E-box Myc binding site and we observed similar replication structures to those observed using gene probes at the respective time points.

We then analyzed Myc interactions with the replication initiation proteins in the sequence by which they are recruited at origins of replication. We also included maintenance and late replication proteins to ensure the specificity of our results. Our data, in agreement with other published studies, showed that Myc is in complex with replication initiation proteins but not with maintenance or late replication ones (Dominguez-Sola et al., 2007, Kim et al., 2008)

The control of origins of replication in mammalian cells to fire once and only once per cell cycle is not fully understood. Confirmed is that such control is primarily exerted by the assembly and disassembly of the preinitiation complex. Our data show enrichment of complexes including Myc and three members of the pre initiation complex (Orc1, Cdc6 and Cdt1) at the specific time points where the *de novo* replication intermediates are observed. Both findings suggest that Myc binds and stabilizes the replication initiation complex at time points 1 and 24 hours which in turn leads to re-firing of the origins at those loci.

More important, the switch in the level of enrichment of Cdt1 in complexes precipitated by either Myc or Geminin antibodies suggests a specific role that Myc plays to induce rereplication. In the assembly of the preinitiation complex, Orc1 binds the rest of the Orc complex at the origin site followed by Cdc6 then Cdt1. Cdt1 then recruits

Mcm7 and the rest of the Mcm complex that is known to have helicase activity. The origin is then said to be “licensed to fire” (Louis et al., 2006). Immediately after the firing of the origin Cdt1 exits the preinitiation complex and is escorted by Geminin to the cytosol where it is degraded. In our experiments Cdt1 was enriched in Myc complexes at time points where rereplication was observed while Mcm7 (the protein that binds the initiation complex after Cdt1) levels that are in complex with Myc are consistent throughout all the time points of the experiment. Those two observations suggest that the extended bioavailability of Myc due to its ectopic deregulation in our cell lines selectively bind and prevent Cdt1 from exiting the preinitiation complex. Consequently the preinitiation complex is stabilized at the origin site and that in turn leads to refiring of the origin. This conclusion is also supported by the fact that Cdt1 levels that are in complex with Geminin were decreased only at time points where they were enriched in Myc complexes.

The time points 1 and 24 hours, when we observed Myc inducing rereplication, coincide with the replication timing of early replicating genes. All known Myc gene-amplification targets are early replicating genes involved in cellular proliferation especially in the bioavailability of precursors (Robson et al., 2006).

According to our findings there are two events required to achieve rereplication. One is Myc binding Cdt1 and preventing it from exiting the preinitiation complex. The other event is Myc/Max complex binding Myc-specific E-box sequence withing the locus at a close proximity from the firing origin. Those two events may be taking place in a way that one event leads to the other.

Recent reports by Dominguez-Sola et al 2007 suggested a non transcriptional but global role for Myc in DNA replication. In contrast to our study, Dominguez-Sola et al 2007 examined origins that are not targets for Myc dependent gene amplification. Our data agrees with Dominguez-Sola *et al* 2007 findings concerning Myc's direct contribution to the initiation of replication through protein interactions with early replication proteins. However, we propose that two events are required to achieve rereplication at Myc amplification target genes. It appears that Myc interaction with the replication initiation complex is a global event that can take place in the assembly of any replication initiation complex. This event transiently stabilizes the preinitiation complex and allows a single firing at the origin, but then in absence of Myc/Max specific binding sequence (E-box sequence) the second event will not take place. Instead, Myc at its physiological low levels and its short half life will then be promptly removed from the origin site allowing Cdt1 to exit the origin of replication before refiring takes place. This proposed mechanism also explains the essential role of Myc in whole genome or specific genes amplification at early developmental stages in some organisms.

The *de novo* replication intermediates and rereplication were observed as early as 1 hour time point, why then we do not see confirmed gene amplification till 72 hours? Not every copy of the initiating replicons or rather amplicons will be tolerated and preserved by the cell. The amplified DNA may be sensed as DNA damage and the cell may be killed by cellular apoptotic mechanisms. Alternatively, the amplicons may be ligated with other nascent DNA strands and integrated chromosomally or extra-chromosomally to form extrachromosomal elements or double minute chromosomes,

some of which will be lost during subsequent cell divisions and some will be preserved and will accumulate to yield amplifications that are experimentally detectable.

The two time points when rereplication events were observed do not overlap with the time points when maximum nuclear availability of the ectopically expressed Myc is achieved in both cell lines. One possible explanation is that the level of nuclear Myc after 1 hour is sufficient to contribute to the observed results and the increase of Myc's level beyond the 2 hours may be beyond saturation for this function, a phenomenon that was also observed in another study (Bouchard et al., 2001). Another reason may be related to the replication timing of the genes included in our study.

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3.0 c-Myc induces chromosomal rearrangements through telomere and chromosome remodeling in the interphase nucleus

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Contribution:

SL participated in designing the experiments and coordinated the project between the different authors and collaborators. SL also performed the majority of the experimental work, participated in the imaging and analysis. Collected data and produced figures. SL also wrote materials and methods and the supplementary material. The estimated overall contribution of SL is 50%.

3.1 Abstract

In previous work, we showed that telomeres of normal cells are organized within the 3D space of the interphase nucleus in a non-overlapping and cell cycle-dependent manner. This order is distorted in tumor cell nuclei where telomeres are found in close association forming aggregates of various numbers and sizes. Here we show that c-Myc overexpression induces telomeric aggregations in the interphase nucleus. Directly proportional to the duration of c-Myc deregulation, we observe three or five cycles of telomeric aggregate formation in interphase nuclei. These cycles reflect the onset and propagation of breakage-bridge-fusion cycles that are initiated by end-to-end telomeric fusions of chromosomes. Subsequent to initial chromosomal breakages, new fusions follow and the breakage-bridge-fusion cycles continue. During this time, nonreciprocal translocations are generated. c-Myc-dependent remodeling of the organization of telomeres thus precedes the onset of genomic instability and subsequently leads to chromosomal rearrangements. Our findings reveal that c-Myc possesses the ability to structurally modify chromosomes through telomeric fusions, thereby reorganizing the genetic information.

3.2 Summary

Chromosomal instability is one major form of genomic instability induced upon Myc deregulation *in vitro* and *in vivo* (Mai and Mushinski 2003). However, the molecular mechanism for the initiation of such instability has not been described

The objective of this chapter of the thesis is to characterize one possible molecular pathway by which Myc dependent chromosomal instability initiates. The experiments described in this chapter were designed to answer questions based on observations that were previously published by our group reporting that the 3D volume and structure of telomeres in tumor tissue and cells are different than in normal ones (Chuang et al. 2004). The tumor tissues screened in that study were associated with high levels of Myc and included cells from one Burkitt's lymphoma cell line, primary mouse plasmacytoma and tissue from a primary human head and neck squamous cell carcinoma (stage IV) in addition to normal primary human lymphocytes, primary human fibroblasts and human epithelial tissue.

Based on Chuang et al 2004, we designed two specific aims. The first one is to investigate if the difference in telomeric volume and structure between normal and tumor cells and tissue is Myc dependent? If so we should be able to reproduce those structures upon up regulating Myc in non transformed cells in culture. Our other aim was to characterize the presence of a cause-effect relationship between the generation of the telomeric aggregates and the chromosomal instability.

To assess if the formation of the telomeric aggregates is Myc dependent we chose the mouse Pre B cell line as our model (described in the introductory section of Chapter 2). We designed several Myc activation schemes that varied in time and we collected

cells every six hours over a time period of five days. The initial 3D imaging and analysis counting the telomeric aggregates for each of Myc activation schemes revealed that the formation of the telomeric aggregates depended on Myc's availability. The formation of the aggregates was observed 18 hours after Myc activation and took place in cycles that lasted for 12 hours. Moreover, the formation and dissociation of the aggregates took place in cycles and the number of cycles depended on the duration of Myc's availability.

Those results allowed us to design experiments to address the second question investigating the presence of a relationship between the formation of the aggregates and the formation of chromosomal instability. We performed spectral karyotyping analysis (SKY) on time points where we observed the aggregates and subsequent time points immediately after the dissociation of the telomeric aggregates. Out of our SKY data we observed two different profiles of chromosomal abnormalities; the first was associated with time points when the aggregates are formed and included predominantly end to end fusion and telomeres-free chromosomal ends (broken chromosomes). The second profile of chromosomal abnormalities was associated with time points following the dissociation of the aggregates and included more complex karyotypes with multiple unbalanced translocations and more telomeres free chromosomal ends. The two karyotypic profiles alternated along the time points of the experiment and coincided with the formation and dissociation of the telomeric aggregates suggesting the dependency of the chromosomal profile on the telomeric status.

The results obtained from those experiments addressed our initial questions/objectives, yet they encouraged us to add more specific aims to this chapter.

The alternating chromosomal profile that we observed was reminiscent of the genomic instability process first described by Barbra McClintock early in the 20th century and known as breakage bridge fusion (B-B-F) (McClintock 1939). In addition to the chromosomal profiles, B-B-F cycles initiate due to chromosomal end to end fusions and the telomeric aggregates that we observed may be representing those end to end fusions. We then designed experiments to investigate if whether we are observing cycles of B-B-F in our cells. We performed telomeres *FISH* on metaphases from the respective time points to investigate the presence of interstitial telomeres that marks the presence of end to end fusions, which is the crucial step that leads to B-B-F cycles. We also screened for the presence of anaphase bridges that are also characteristic markers for B-B-F.

The last specific aim in this chapter was to investigate if the fusion of two chromosomes in B-B-F cycles will increase the frequency of balanced translocations between specific chromosomes. Our SKY analysis showed that at the latter time points of the experiment the frequency of balanced translocations especially between specific pairs of chromosomes is increased. To characterize the role of Myc activation and the generation of telomeric aggregates in those translocations, we decided to investigate the positions of each pair of those chromosomes in the interphase nucleus to find if Myc activation causes alteration in the positions of those chromosomes as a consequence of the formation of the telomeric aggregates. We performed chromosome-painting on specific pairs of chromosomes one pair at a time. We acquired 3D images at different time points and we were able to compare the 3D positions of each pair of chromosomes in Myc activated cells versus mock Myc activated cells over the time points of the experiments.

3.3 Introduction

Multiple alterations accompany tumor initiation and progression resulting in the modulation of gene expression and in genomic instability. These interconnected changes occur within nuclei that harbor an altered 3D organization (1–3). In agreement with this concept, recent reports suggest tumor-associated changes of chromosomal organization in an altered 3D nucleus (3–8). However, mechanisms leading to structural changes of telomeres and chromosomes remain elusive.

We recently reported that the normal interphase nucleus has a unique 3D telomeric organization that is cell cycle dependent (9, 10). Telomeres are organized in a non-overlapping manner and align into a central telomeric disk during the late G₂ phase of the cell cycle (9). In contrast, tumor cells display an aberrant organization of telomeres that can be objectively measured in nuclei showing telomeric aggregates of various complexity and sizes (9).

Constitutive expression of c-Myc due to chromosomal translocations, mutation, or amplification contributes to the development and progression of many cancers (11, 12). c-Myc deregulation directly promotes genomic instability (13), causing locus-specific and karyotypic instability (14–18). Additionally, c-Myc induces illegitimate replication initiation (19, 20), DNA breakage (21), alterations of DNA repair (22, 23), and a low level of point mutations (24, 25). Effects of c-Myc on genomic instability are reversible after a transient experimental activation of c-Myc (15). However, c-Myc continues to generate instability after constitutive deregulation (16). *in vivo*, c-Myc deregulation directly initiates and promotes tumorigenesis (26–30). When c-Myc deregulation is

abolished, *in vivo* tumorigenesis is reversible, provided that no additional mutations had occurred (29–34).

Prompted by the complexity of downstream genetic alterations that result from c-Myc deregulation, we investigated whether c-Myc affected the 3D organization of the mammalian interphase nucleus and whether this remodeling had an impact on genomic stability. We show that c-Myc deregulation causes remodeling of the 3D nuclear organization of telomeres and chromosomes, thus creating the topological conditions that initiate genomic instability.

3.4 Materials and Methods

3.4.1 Cells and Conditional Myc Activation

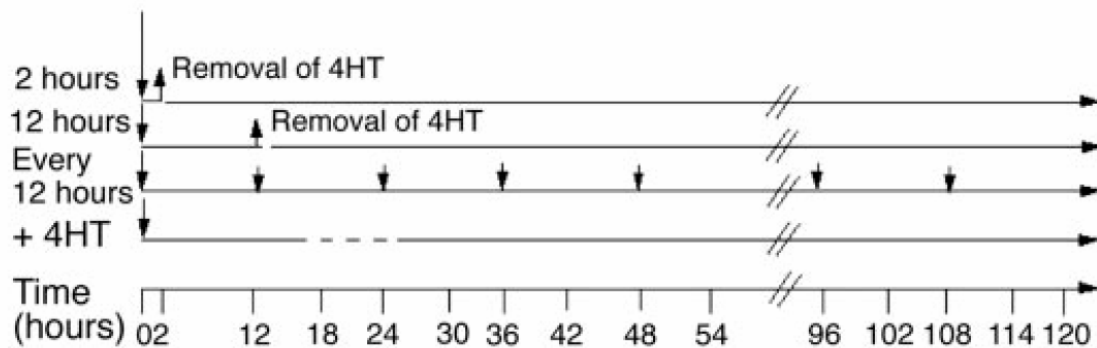
Culture conditions have been described for Ba/F3 (35) and PreB (36) cells. The plasmacytoma cell line MOPC460D was a gift of J. Mushinski (National Institutes of Health, Bethesda). Cell viability was determined by hemocytometer counts by using trypan blue. The primary mouse plasmacytoma DCPC21 was isolated from a BALB/c mouse (37). *v-abl/myc*-induced plasmacytomas (38) and primary lymphocytes were collected from BALB/c mice (Central Animal Care protocol 02-039).

To activate MycER (39) in Ba/F3 or PreB cells, 10^5 cells per ml were treated with 100 nM 4-hydroxytamoxifen (4HT). Cells were split 24 h before 4HT treatment. Non-4HT treated control cells were cultivated in ethanol, which is used to dissolve 4HT (25, 26, 39). Two different MycER™ activation schemes were performed. First, analyses of c-Myc-induced changes in 3D telomere organization were carried out after a single addition of 4HT that was left in the culture medium until its biological effects subsided (40–42). Nuclei were examined every 24 h over a 10-day period. A second time course was performed every 6 h for 120 h (Figure 3.1). To enable a time-dependent analysis of Myc activation, 4HT was given for 2 or 12 h and was removed. Alternatively, 4HT was added every 12 h or was given once but left in the culture. MycER activation was determined by fluorescent immunohistochemistry.

3.4.2 Immunocytochemistry (IHC)

Fluorescent IHC of Myc protein was performed as described in ref. (43) by using a polyclonal anti-c-Myc antibody (N262; Santa Cruz Biotechnology) and a goat anti-rabbit IgG FITC™ antibody, each at a dilution of 1:100. Analysis was performed by using a Zeiss Axiophot 2 microscope. Images were acquired with a Cooke CCD SensiCam Camera.

Figure. 3.1 MycER™ activation scheme. Time-dependent analysis of c-Myc deregulation. 4HT, added at various time points and durations, was administered for 2 or 12 hours and thereafter removed from the medium. In parallel, 4HT was added every 12 hours or just once and not removed. The effects of 4HT last 15-24 hours in cell lines (40-42) as indicated by dashed lines. Cells were harvested every 6 hours over a time period of 120 hours. Mock-treated control cells were processed in parallel.



3.4.3 Cell death

Apoptotic bodies for control and MycERTM-activated cells were assessed by two independent observers who scored 300 DAPI-stained nuclei per time point in the presence or absence of MycERTM activation.

3.4.4 Telomere fluorescent in situ hybridization (*FISH*)

Ba/F3, Pre-B and plasmacytoma cells were collected (200xg for 10 minutes) and resuspended in phosphate buffered saline containing 3.7% formaldehyde (Fluka, Basel, Switzerland) and incubated for 20 minutes. Thereafter, the telomere *FISH* protocol was performed (1, 44) using Cy3- or FITCTM labeled PNA probes (DAKO, Glostrup, Denmark). Three independent experiments were performed. The number of nuclei examined was >30 per experiment; the number of metaphases examined by telomere *FISH* was 20. 3D image acquisition of nuclei was performed as described below and in (1).

Imaging of metaphases after telomere *FISH* was performed using Axioplan 2 with a cooled AxioCam HR B&W, DAPI, Cy3TM or FITCTM filters in combination with Planapo 63x/1.4 oil (all Carl Zeiss Inc., Canada). Metaphase images were acquired using the Axiovision 3.1 software (Carl Zeiss Inc., Canada) in Multichannel mode. Twenty metaphases were examined per time point. Due to the presence of multiple variables, the General linear Modeling (GLM) procedure was used. To test average aggregates among different groups and two-way ANOVA test was performed for normality and robustness of the data (> 30 nuclei per time point). For details of all tests performed, see *Supplemental Information*

3.4.5 3D image acquisition

For each time point, at least thirty nuclei were analyzed by 3D imaging using Axioplan 2, a cooled AxioCam HR B&W. DAPI, FITC™ and Cy3™ filters were used in combination with Planapo 63x/1.4 oil (all Carl Zeiss Inc., Canada), giving a sampling distance of 107 nm in the XY-direction. Axiovision 3.1 software with deconvolution module and rendering module were used (Carl Zeiss Inc., Canada). For every fluorochrome the 3D image consists of a stack of 100 images with a sampling distance of 200 nm in the z-direction. The constrained iterative algorithm option was employed (45).

3.4.6 3D image analysis for telomeres

Telomere measurements were done with TeloView (1, 46). By choosing a simple threshold for the telomeres a binary image is found. Based on that, the center of gravity of intensities is calculated for every object resulting in a set of coordinates (x, y, z) denoted by crosses on the screen. The integrated intensity of each telomere is calculated since it is proportional to the telomere length (47). The integration region is determined by growing a sphere on top of the found coordinate. After every step of growth (iteration) the sum under this volume (the telomere) is subtracted by the sum just surrounding it (background level). When the process of the growth of the sphere does not contribute to an integrated intensity increase, the algorithm stops and the integrated intensity of the telomere with an automatic background correction is obtained.

3.4.7 Chromosome painting and measurements of chromosomal overlap(s) in interphase nuclei

Chromosome painting was carried out as described (48) using paints for mouse chromosomes 5 (Cy3TM), 13 (FITCTM), 7 (Cy3TM), 10 (FITCTM) and 17 (FITCTM) from Applied Spectral Imaging (Vista, CA, USA). 3D image acquisition of painted nuclei was performed as described above. Measurements of chromosomal overlaps were performed after 3D image acquisition and constrained iterative deconvolution as follows; (1) based on the DAPI counter-stain image, we determined the 3D boundary of the nuclear volume. Data outside that volume were ignored. (2) For each one of the chromosomes, we determined an intensity threshold and referred only to voxels that were above the threshold that belonged to the specific chromosomes. The total volume occupied by each one of the chromosome pairs is measured (V1 and V2). (3) The volume occupied by both chromosome pairs is measured, V₀. By dividing this value by V1 and by V2, the level of overlap relative to the total volume of each chromosome pair was measured, V₀/V1, V₀/V2. For more details, see *Supplemental Information*.

3.4.8 Spectral karyotyping (SKY)

Mouse SKY was done as described (37) by using a SKY system (Applied Spectral Imaging, USA). Twenty metaphases were examined per time point. Significant values for chromosomal rearrangements were determined after MycERTM activation. Mean total chromosomes and numbers of each chromosome observed for control and Myc-activated cells were compared over time by two-way analysis of variance. For other chromosomal aberrations the common (at least 10%) incidences in specific chromosomes

were also compared for both groups of cells over time with the Mantel-Haenzsel stratified analysis. In addition, statistical analyses were performed for the occurrence of translocations, breakages and fusions over the experimental period of 120 hours. p values of less than 0.05 were considered significant. Only the Frequency procedure was used followed by Fisher's exact test. The p value of the overall study was < 0.0001 .

3.5 Results

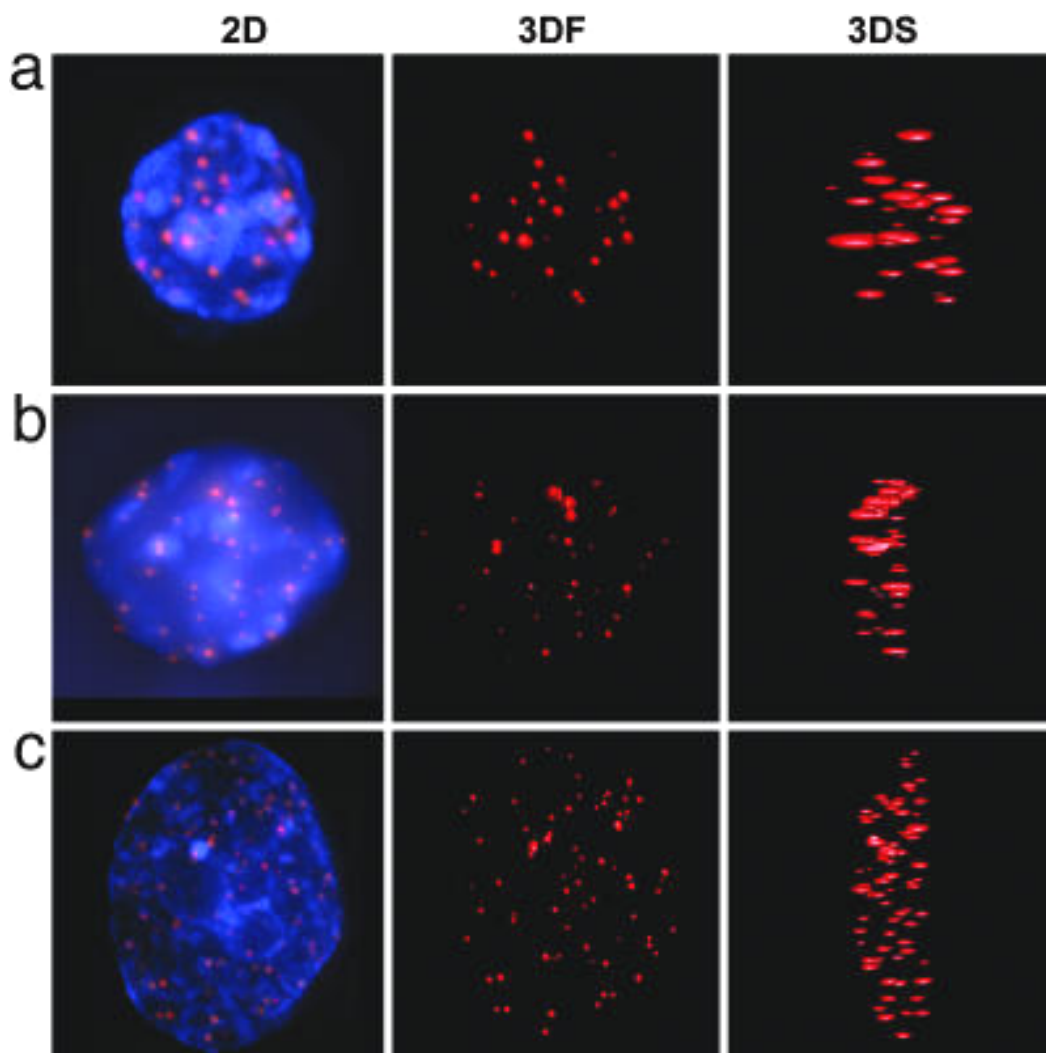
3.5.1 The 3D Organization of Telomeres Before c-Myc Activation

We examined whether c-Myc deregulation affected the 3D organization of telomeres in the interphase nucleus. To this end, we analyzed the effect of conditional c-Myc expression in two independent immortalized mouse B lymphocyte lines, Ba/F3 (35) and PreB (36), stably transfected with MycERTM (39). For both cell lines, we first evaluated the 3D organization of telomeres in nuclei of non-MycERTM-activated cells by using primary BALB/c B lymphocytes as a control. Consistent with our previous studies (9), telomeres of normal primary BALB/c B nuclei showed non overlapping telomere positions as determined by 3D imaging (Figure 3. 2*a*). Without MycERTM activation, both PreB and Ba/F3 interphase nuclei also displayed non overlapping telomere positions (Figures 3. 2 *b* and *c*, respectively). Therefore, the above cell lines were appropriate to study the effects of conditional c-Myc activation on the 3D telomeric organization.

3.5.2 c-Myc-Dependent Disruption of the 3D Telomeric Organization: Formation of Telomeric Aggregates (TAs) in Interphase Nuclei

We next analyzed the effect of conditional c-Myc expression on the 3D organization of telomeres. After a transient MycERTM activation with 4HT, nuclear c-Myc signals were observed in both PreB and Ba/F3 cells (Figures 3. 9 *b* and *d*). In non-4HT treated control cells, MycERTM was found in the cytoplasm (Figures. 3. 9 *a* and *c*; see also ref. 39).

Figure 3. 2 Telomere organization in nuclei of primary mouse B cells, in Pre B and Ba/F3 lymphocytes. Telomeric organization in interphase nuclei of primary and immortalized cells without overlap in telomere positions. **a:** Primary B cell nucleus; **b:** nucleus of near diploid Pre B cell; **c:** nucleus of tetraploid Ba/F3 cell. Telomeres (red), nuclei (blue). 2D: two-dimensional; 3D: three-dimensional; 3DF: three-dimensional front view; 3DS: three dimensional side view.



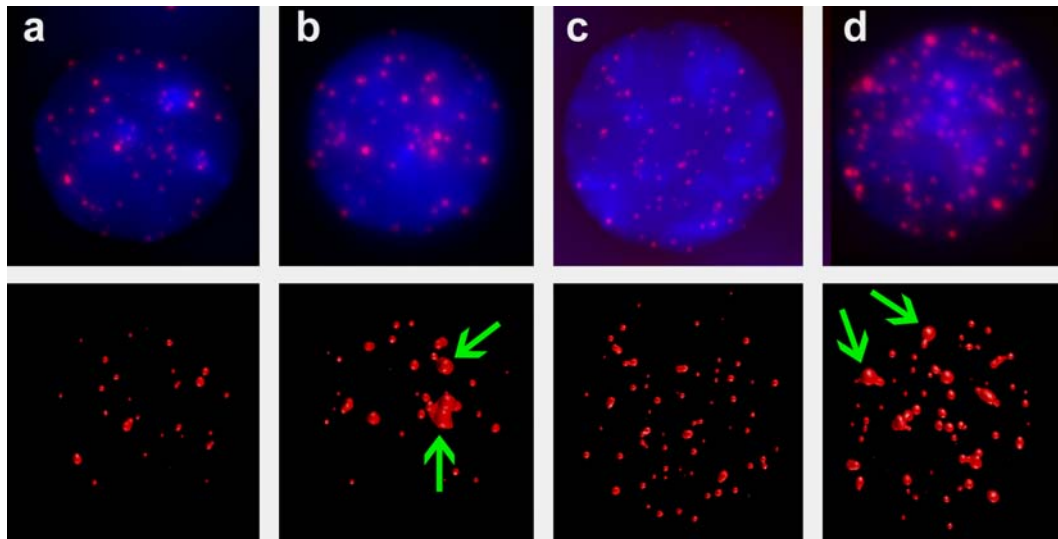
To determine whether c-Myc deregulation affected the 3D organization of telomeres, we performed time course experiments. In the first set of experiments, c-Myc deregulation and 3D telomeric organization were investigated in both PreB and Ba/F3 cells after a single 4HT treatment. Nuclei were analyzed after c-Myc deregulation at 0, 24, 48, 72, and 96 h and at 10 days and compared with nuclei from mock-treated control cells. In both cell lines, analyses of the 3D nuclear organization of telomeres revealed that c-Myc deregulation induced the formation of TAs. TAs are group(s) of telomeres that are found in clusters and, thus, in close association in the interphase nucleus. This 3D telomeric organization is distinct from the normal 3D organization of non-MycERTM-activated PreB, Ba/F3 cells and primary mouse lymphocytes (Figure 3. 2). Figure 3. 3 illustrates the presence of TAs in interphase nuclei of MycERTM-activated PreB and Ba/F3 cells (Figure 3. 3 b and d respectively). Although such TAs had been observed in tumor cell nuclei previously (9), their presence in conditional c-Myc expressing cells is a previously uncharacterized finding.

3.5.3 c-Myc Induces Cycles of TAs in Interphase Nuclei

In subsequent experiments, we investigated the time relationship between c-Myc deregulation and the formation of TAs more closely. To this end, cells were harvested every 6 h over a time period of 120 h. We also varied the duration of conditional c-Myc expression (Figure 3. 1), confirming nuclear c-Myc staining as above (Figure 3. 9 and 3. 11). Next, the 3D organization of telomeres was determined (Figure 3. 4). At this point, we focused on near diploid PreB cells only (49). Our positive controls were cells

constitutively overexpressing c-Myc [mouse plasmacytomas (27) and a plasmacytoma line (Figure 3. 4 Ae)]. Negative controls were mock-treated PreB cells (Figure 3. 4 Aa).

Figure 3.3 c-Myc deregulation induces TAs in interphase nuclei of PreB and Ba/F3 cells shown at 72 h after 4HT-treatment. (a) Mock-treated PreB cells show non overlapping telomeres (red). **(b)** MycER™-activated PreB cells with TAs (green arrow). **(c)** Mock-treated Ba/F3 cells show non overlapping telomeres. **(d)** MycER-activated Ba/F3 cells show the formation of TAs (green arrow).



This time course confirmed that c-Myc deregulation induced TAs. Representative images show that TAs varied in size and numbers per MycER™-activated PreB cell nucleus (Figure 3. 4A b–d, red arrows). High induction levels of TAs were observed at 30, 48, 72, and 96 h declining after 96 h (Figure 3. 4 B, arrows). The highest levels of TA formation will hereafter be referred to as peaks of TAs. The 6-h time course performed over 120 h indicated that TAs formed in a c-Myc-dependent manner and showed a cyclic appearance (Figure 3. 4 B). The number of TA cycles was directly linked to the duration of c-Myc deregulation. For example, 2 h of Myc activation induced three such cycles, whereas 12 h led to five cycles (Figure 3. 4 B, black and red lines, respectively). 4HT, left in the culture medium until its biological effects on our cells subsided (Figure 3. 1), also induced five TA cycles (Figure 3. 4 B, green line). In this context, repeated consecutive activations of MycER™ given every 12 h caused TAs in 96% of all nuclei. These cells died after 30 h (Figure 3. 4 B, blue line) because of repeated cycles of c-Myc deregulation and not due to toxicity exerted by 4HT (50). Thus, only a single TA cycle is observed in this experimental setting (Figure 3. 4 B, arrowhead). The increase in TAs and 3D volumes was significant (Table 3. 3).

3.5.4 The c-Myc-Induced TA Cycles Represent Breakage-Bridge-Fusion (BBF) Cycles and Chromosomal Rearrangements

The cycles of c-Myc induced TAs in PreB nuclei showed similar periodicity for all c-Myc activation periods (Figure 3. 4 B). We reasoned that these cycles might reflect both ongoing associations and dissociations of telomeres or B-B-F cycles. The B-B-F

cycle could be induced by the breakage of dicentric chromosomes during anaphase-inducing apoptosis of cells having multiple or large TAs per nucleus.

Figure 3. 4 c-Myc-induced telomeric aggregates appear in cycles. (A) Conditional c-Myc deregulation causes TA formation. (Aa) Negative control: non-Myc-deregulated PreB nucleus with non overlapping 3D telomeric nuclear positions. (Ab–Ad) TAs of various sizes and numbers are present after conditional c-Myc expression at any given time point of TA formation. Telomeres are shown in green; TAs by red arrows. (Ae) Positive control: plasmacytoma cell line, MOPC 460D, with constitutive c-Myc deregulation due to T12;15, shows TAs. Similar results were obtained with primary plasmacytoma cells (data not shown).

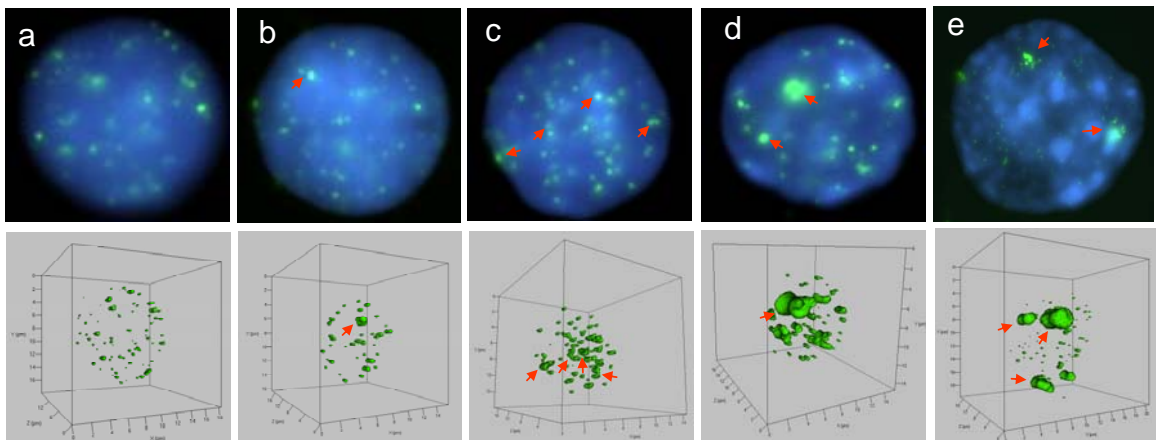
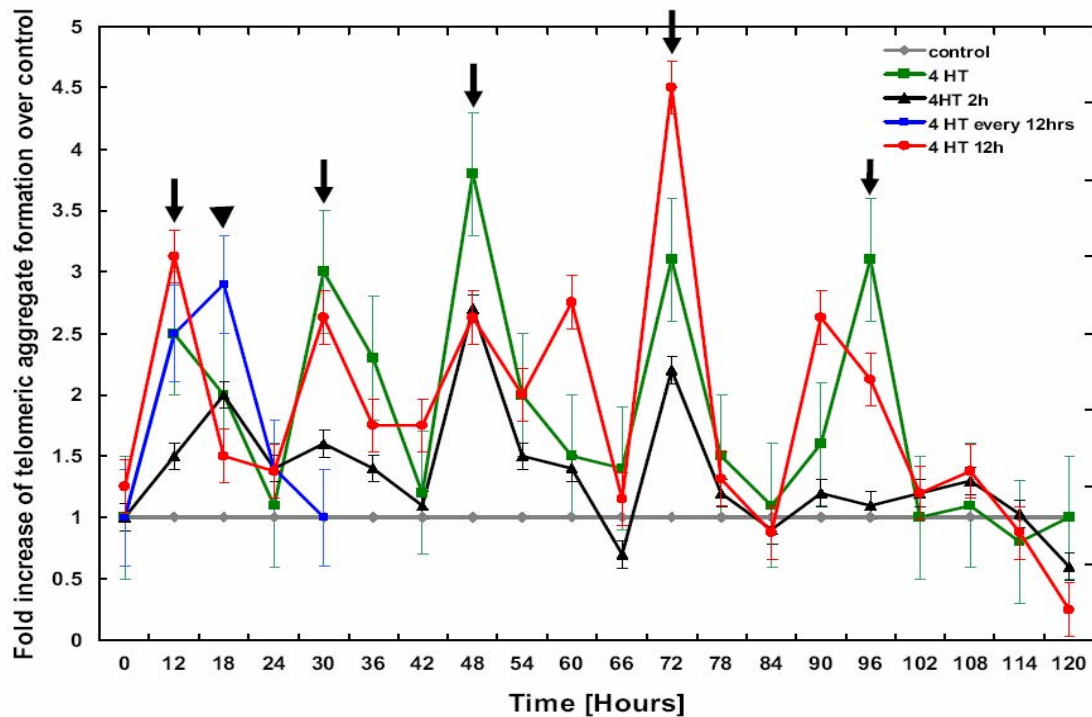


Figure 3. 4 B c-Myc induces cycles of TAs. Fold increase in TAs over control levels during a period of 120 h. During this period, c-Myc had been up-regulated for different lengths of time (see Figure 3.1). Black, 4HT given for 2 h and removed; red, 4HT administered for 12 h and removed; green, 4HT added once and not removed; blue, 4HT added at 0, 12, and 24 h; gray, control cells. The highest levels of TA formation and a single TA peak observed after consecutive activations of MycER™ are shown by arrows and an arrowhead, respectively. Error bars represent a 95% confidence interval of binomial distributions.



To address these possibilities, we first examined metaphase chromosomes at different times: prior, during, and after the peaks of TA formation for a 120-h period. We used both whole-genome analysis by mouse SKY and telomeric FISH of metaphase chromosomes. A significant level of dicentric chromosomes was noted (Figure 3. 5). Control cells had normal karyotypes . In MycERTM-activated PreB cells, however, fusions had occurred. We show as example fusions at the telomeric ends of chromosomes 18 and 4 (Figure 3. 5a), red and green arrows) and between two chromosomes 1 (Figure 3. 5a, green arrow). Chromosome 1 was probably broken in the previous anaphase (Figure 3.5a, green circle). An additional terminally deleted chromosome 1 is in the center of the same metaphase plate, and chromosomes 2, 3, and 7 reveal terminal deletions (Figure 3. 5a). Telomeric fusions involving both ends of chromosomes as well as sister chromatids were confirmed by telomeric FISH (Figure 3. 5b). Anaphase bridges and ring chromosomes were present (Figure 3. 5c).

The nature of c-Myc-induced 3D structural changes in interphase nuclei of conditionally Myc expressing cells was as follows: at peaks of TA formation and thereafter, a significant increase in end-to-end chromosomal fusions over control levels was observed. This result was followed by a significant increase in broken chromosomes and nonreciprocal translocations (Figures 3. 5d and 3. 6 and Table 3. 2). In conclusion, TA cycles unveil B-B-F cycles, namely the fusions of two chromosomes, consequently, the formation of dicentrics and their subsequent breakage in anaphase (Figure 3. 5). The cycles are induced by conditional Myc deregulation and lead to the onset of genomic instability, demonstrated by the chromosomal rearrangements resulting from these B-B-F cycles (Figures 3. 5 and 3. 6 and Table 3. 2).

Figure 3. 5 Molecular cytogenetic evidence of BBF cycles in MycER-activated PreB cells. (a) SKY analysis reveals telomeric fusions and chromosome breakage. (*a Upper*) Metaphase, raw image (*Left*); metaphase, classified image (*Center*); and metaphase, inverted DAPI image (*Right*). (*a Lower*) Spectral karyotype. End-to-end fusion of chromosomes 18 and 4 (red arrow) and fusion of chromosome 1 with a broken piece of chromosome 1 (green arrow) are shown. One broken chromosome 1 is circled. Note additional broken chromosomes 1, 2, 3, and 7.

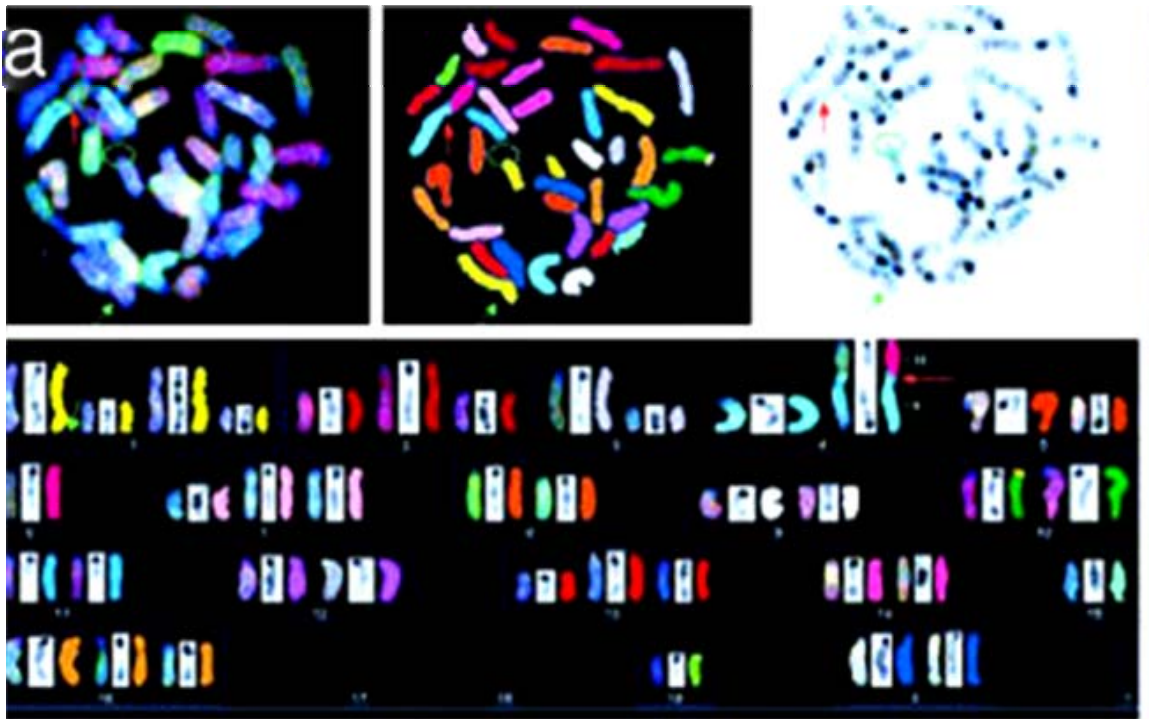


Figure 3. 5 b End-to-end fusions of chromosomes revealed by telomere FISH. (*Upper*) Centromeric fusion (see arrow and insert). (*Lower*) Telomeric fusion (see arrow and insert). **C:** Anaphase bridges. (*Upper*) Short exposure of DAPI stained nucleus (100 msec). (*Lower*) Longer exposure (500 msec) of same image makes anaphase bridge visible (white arrow) but overexposes nuclei.

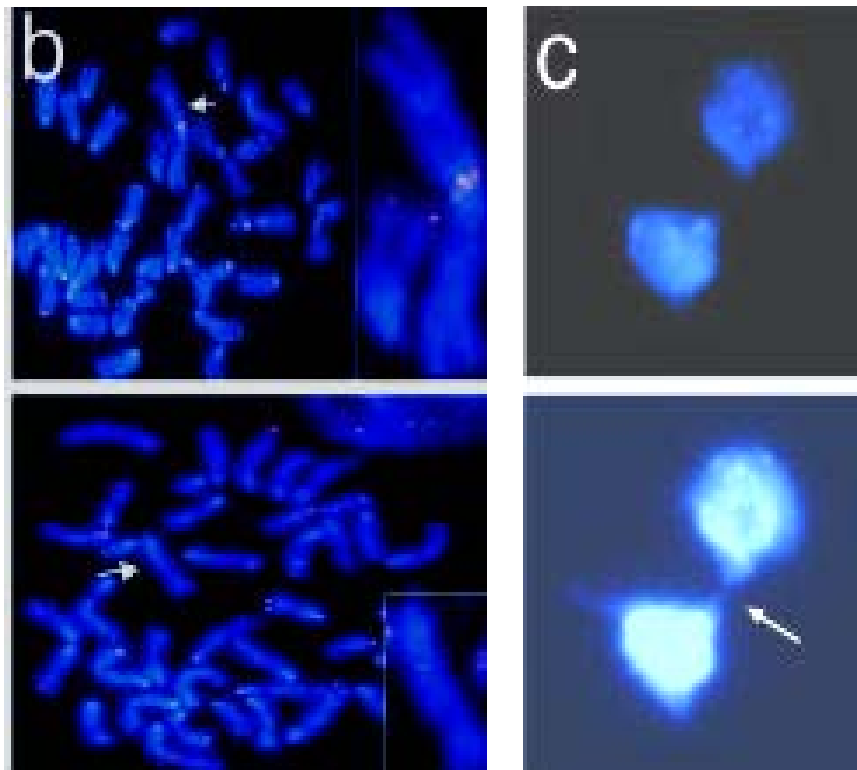
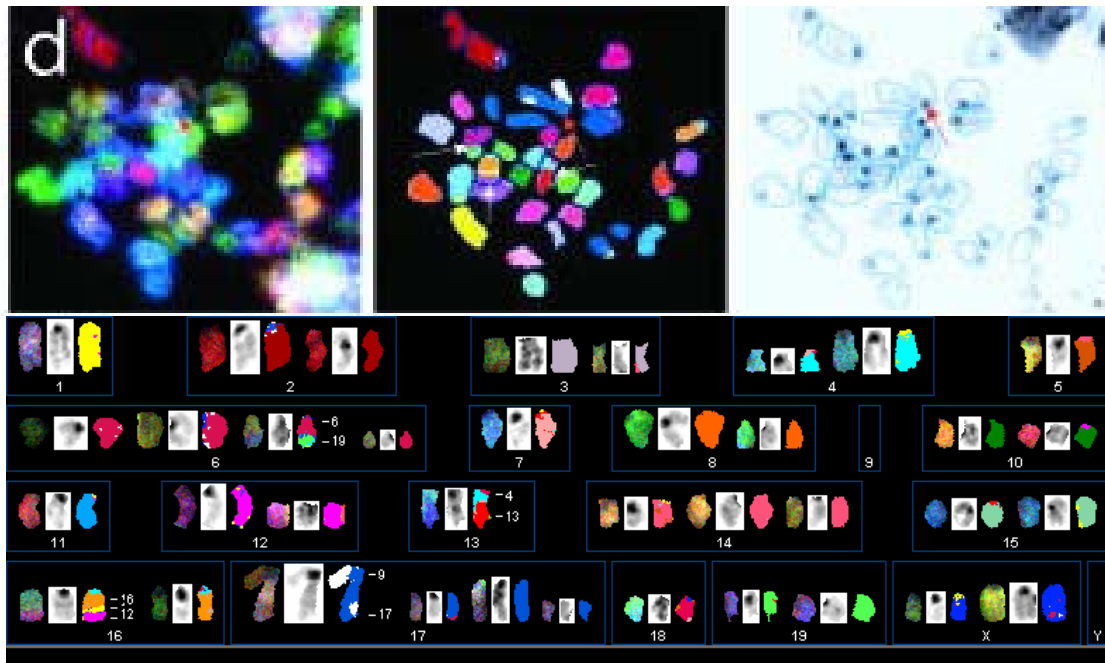


Figure 3. 5 D SKY illustrating chromosomal fusions (red arrow) and nonreciprocal translocations (white arrows). Broken chromosomes are also present (chromosomes 4, 6, 12, and 17).



Next, we investigated whether cells with TAs died during the course of the experiments. If this possibility was the case, we would expect a correlation of cell death in Myc-activated cells at the peak of TA formation or shortly thereafter. The level of apoptosis was ≈ 2 -fold higher in Myc-activated cells than in control cells (Table 3. 1). There was no preference in apoptotic cell death for any specific time point during the 120 h. We concluded that BBF cycles, not apoptosis, contributed to the cycles of TA formation.

3.5.5 3D Organization of Chromosomes in c-Myc Activated Interphase Nuclei.

TAs and the initiation of B-B-F cycles with subsequent chromosomal rearrangements prompted us to investigate whether chromosomes were affected in their 3D nuclear positions during MycERTM activation. To this end, we examined the overlap of specific chromosomes over the 120-h period. SKY of MycERTM-activated PreB cells suggested chromosomal rearrangements involving chromosomes 7, 13, and 17. Additional rearrangements were found but did not reach significant levels (data not shown). We examined three combinations of chromosomes over a 96-h period. This period covered all peaks of TA formation (Figure 3. 4 B). As shown in Figure 3.7, we observed a change in overlaps between chromosomes 5 (red) and 13 (green) over the time course (Figures 3. 7 A and B). Both chromosomes were found in closer vicinity as the cells entered into the first TA cycle. Chromosomes 10 (green) and 7 (red) also showed increases in the percentage of overlap (Fig 3.7 A and B), as did chromosomes 7 (red) and 17 (green) (Figure 3. 7 A and B).

Figure 3. 6 Chromosomal aberrations in MycER-activated PreB cells over a period of 120 h after a single administration of 4HT. End-to-end fusions (blue) increase to 40% in the first 12 h. Over time, the percentage of fusions decreases. Translocations (orange) appear at 12 h and reach a maximum of 35% at 42 h. Telomere-free chromosomal end(s) (green) increase over time peaking at 30 h with 75% of metaphases having at least one telomere-free chromosomal end. Subsequently, the percentage of telomere-free chromosomal end(s) decreases. Q-FISH experiments confirmed healing of telomeric ends at later time points. The error bars show the 95% confidence interval for binomial distributions (51). Because of a confidence interval, the error bars are larger than expected when a standard error would have been used, which was not applicable in this situation. For details on each time point and aberration, see Table 3. 2.

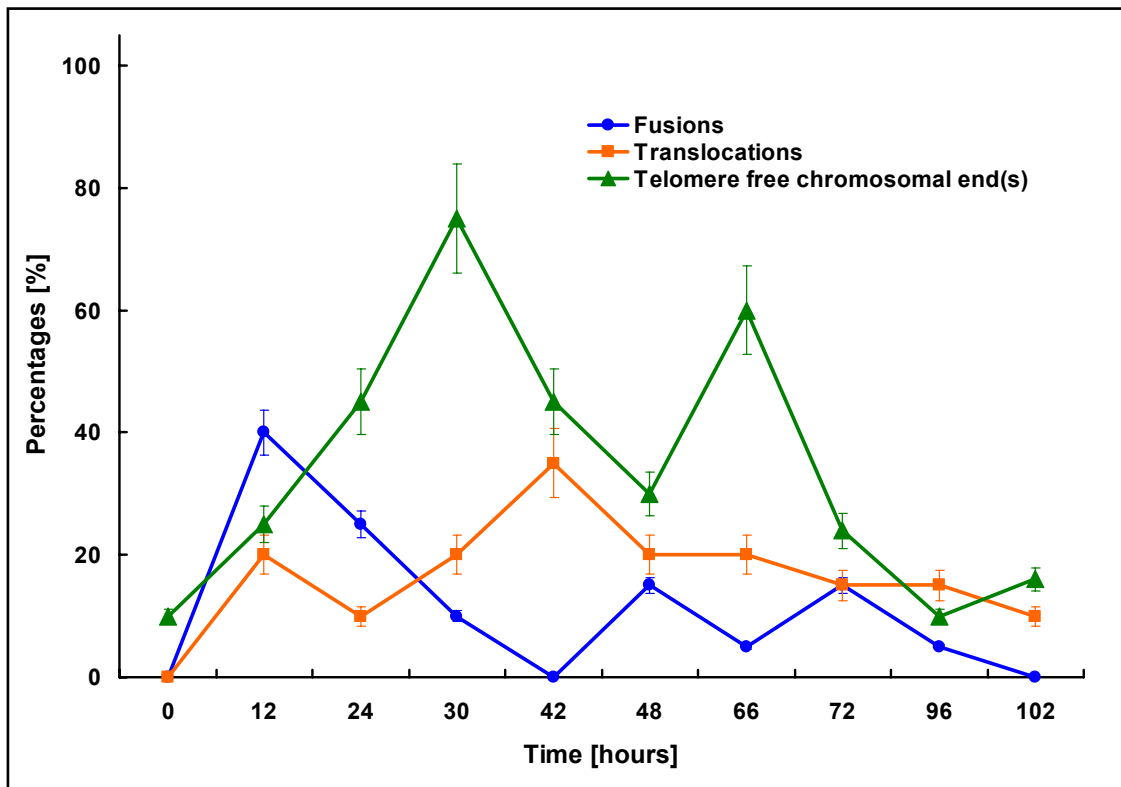


Figure 3. 7 Chromosome positions in Myc-activated nuclei. (A) Representative nuclei painted with chromosome paints over a period of 96 h after 4HT (Figure 3.1). (*Top*) Chromosomes 5 and 13. (*Middle*) Chromosomes 7 and 10. (*Bottom*) Chromosomes 7 and 17. (**B**) Measurements of chromosomal overlaps in nuclei of c-Myc deregulated cells for chromosomes 5 and 13 (*Left*), 7 and 10 (*Center*), and 7 and 17 (*Right*) over a 96-h period

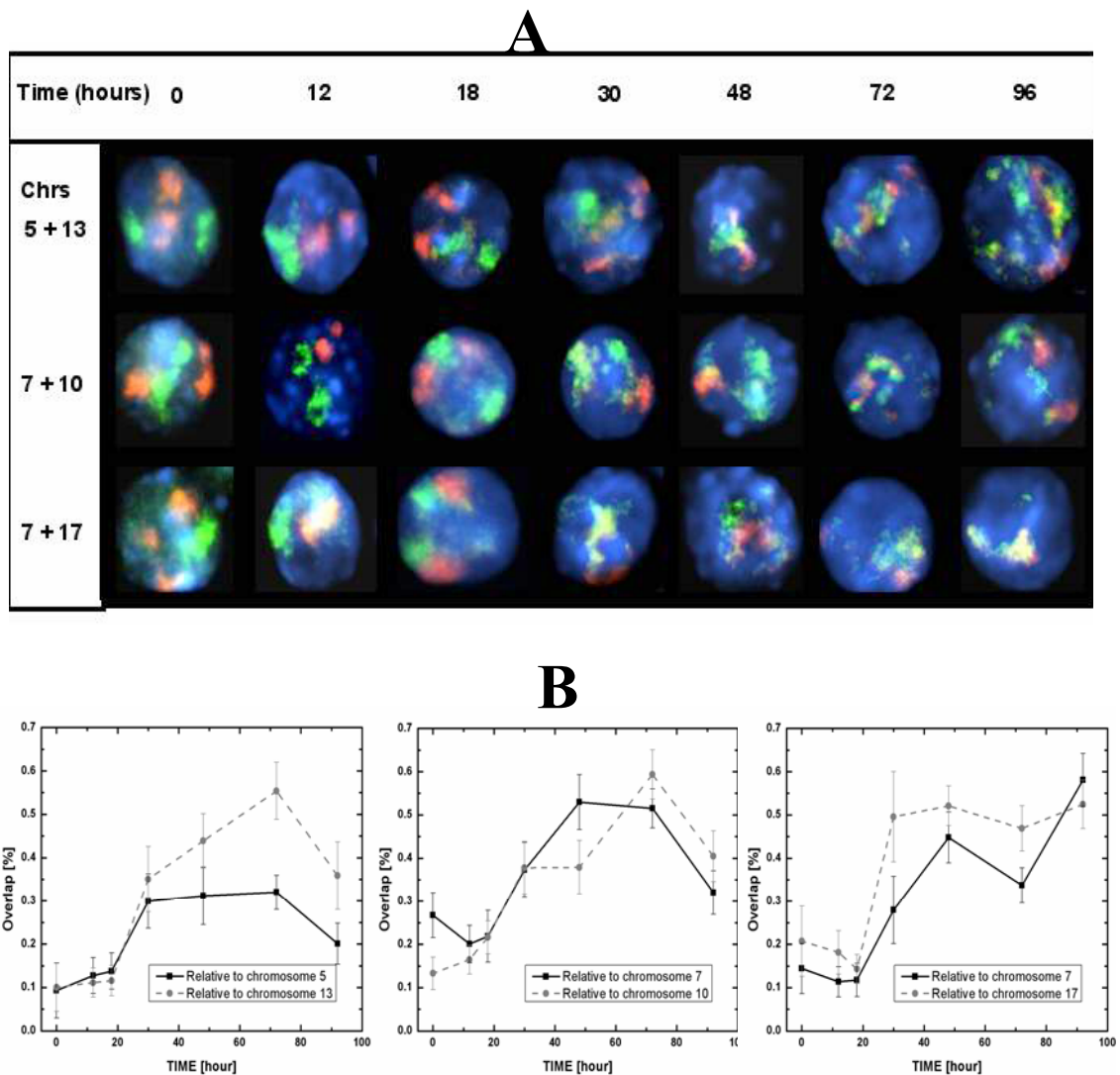


Table 3. 1 Apoptosis levels in non-MycER and MycER-activated PreB cells

% apoptosis			
Time, h	Controls	MycER-activated PreB cells	Fold increase
0	3	3	1
12	2	4	2
24	6	12	2
30	4	10	2.5
42	2	8	4
48	5	10	2
66	4	11	2.7
72	3	8	2.7
84	3	5	1.7
96	3	3	1.0
102	2	3	1.5

3.6 Discussion

3.6.1 c-Myc Induces Telomeric Aggregates, Fusions, and B-B-F Cycles.

Previous studies have shown that c-Myc triggers a complex network of genomic instability at the level of single genes (14, 15, 19) and whole chromosomes (16–18) (for review, see ref. 13). In addition, c-Myc induces illegitimate replication initiation (19, 20), chromosomal rearrangements (18), DNA breakage, alterations of DNA repair (21–23), and a low level of point mutations (24, 25). A previously uncharacterized mechanism underlying c-Myc-dependent genomic instability at the chromosomal level directly affects the integrity of the telomeres and was revealed in this study.

The clear periodicity of the TA cycles that was found with four different Myc-activating treatments suggested a biological relevant Myc-dependent process. Theoretically, cycles of Myc-induced TAs could reflect (*i*) nuclear remodeling with the transient association and subsequent dissociation of telomeres; (*ii*) end-to-end chromosomal fusions that initiate B-B-F cycles (52, 53); (*iii*) c-Myc induced cell death; and (*iv*) a combination of all of the above. Our data are consistent with B-B-F cycles and exclude apoptosis as a direct contributor to the TA cycles. Apoptosis occurred at equal levels throughout the study and consistently reached about twice the levels seen in the control cells. The loss of cells was compensated by a 2-fold increase in proliferation in MycERTM-activated PreB (19). These data also indicate that there is genetic separation of genomic instability and apoptosis as reported in ref. 54. Whether telomere associations and dissociations (55) contributed to the TA cycles is presently unknown.

Direct evidence of B-B-F cycles in the periodicity of TAs came from a detailed analysis of chromosomal fusions, breakage, and rearrangements observed over the time

course of five TA cycles. We demonstrated the occurrence of end-to-end fusions that generated dicentric chromosomes and breaks during anaphase, leaving one chromosome or chromatid with a piece from another chromosome or chromatid. The resulting telomere-free ends continue to undergo fusions with other chromosomes, a cycle of events termed B-B-F cycle (52, 53). Experimental data support these events from fusions to breakages and nonreciprocal translocations. The periodicity of the TA cycles is consistent with a ≈ 12 h population doubling time of the PreB cells (19). Each peak of TAs is consistent with the repeated formation of TAs. Time points after the peak are in agreement with the breakage of dicentric chromosomes. Telomere-free ends initiate new B-B-F cycle(s) until no more telomere-free chromosomal end(s) persist.

From Telomeres to Chromosomal Rearrangements: A New Pathway of c-Myc-Dependent Genomic Instability.

Muller (51) and McClintock (52) first described B-B-F cycles, a mechanism of chromosomal end-to-end fusion that contributes to the onset of genomic instability. B-B-F cycles contribute to deletions, gene amplification, nonreciprocal translocation, and overall genetic changes that are associated with tumorigenesis (56–63).

Our study showed that c-Myc is one key factor that initiates genomic instability through B-B-F cycles. Such B-B-F cycles in telomerase-positive immortalized mouse PreB cells (unpublished data) with long telomeres are distinct from B-B-F cycles reported for critically short telomeres (61, 64). Some TAs (but not necessarily all) represent fusions, as evident by the analysis of metaphase chromosomes. TAs and end-to-end fusions depended on time and levels of c-Myc activation. Analysis of frequencies of both

events showed that they are closely linked. As the fusions initiate B-B-F cycles, the frequencies of breakage and nonreciprocal translocations increase over time.

A previously uncharacterized pathway of c-Myc-dependent genomic instability thus starts at the telomeric ends of the chromosomes. Both TAs and B-B-F cycles are the manifestation of deregulated Myc expression, leading to chromosomal rearrangements and subsequently to genomic instability.

Local chromosome movement increases chromosomal overlap in the nucleus. This temporal change in local positioning may permit the direct contact of chromosomal ends and facilitate recombinations and/or fusions. Such movements were observed after c-Myc deregulation and suggested an impact of the oncoprotein on local nuclear positioning of chromosomes. Chromosome movements were previously studied and found by others as well (65–69).

Several regulatory pathways involving oncogene deregulation may affect the 3D nuclear organization. Oncoproteins, including c-Myc, can alter the 3D nuclear organization and the organization of chromatin (70–72). They also affect the nuclear matrix. High mobility group protein I(Y) (HMGI(Y)) is a c-Myc-dependent nuclear matrix protein (73) with increased expression during neoplasia (2). The analysis of *myc*-binding sites in the human genome suggests that c-Myc binds to genes encoding nucleoskeletal components (74). Furthermore, constitutive c-Myc expression was shown to be associated with down-regulation of the telomere repeat binding protein (TRF2) (10), a protein required for telomere capping and genome stability (75). Myc is also involved in the regulation of DNA repair (22, 23) and has been shown to induce DNA

breakage (21). Thus taken together, many different c-Myc-dependent mechanisms could potentially affect the nuclear organization and, as shown here, converge at the telomeres.

3.7 Supplementary information

3.7.1 Statistical Analyses of Telomeres.

Because of the presence of multiple variables, the general linear modeling (GLM) procedure was used. To test average aggregates among different groups, a two-way ANOVA test was performed for normality and robustness of the data (³ 30 nuclei per time point). The test was followed by least square means Bonferroni test for correction significance comparison. To ensure protection level, only probabilities associated with preplanned comparisons were used. To test the percentage of the occurrence of the aggregates, the frequency procedure was used. For statistic significance in GLM comparisons, the χ^2 value (probability value) was calculated. Comparisons with probability values (P values) of <0.01 were considered significantly different. For the frequency comparisons among different groups, the Breslow–Day test for homogeneity of the odds ratios was performed. Frequency values (F values) were calculated providing the P values. For the telomeric aggregates study, the P values of the overall frequency procedure and the GLM procedure were 0.0019 and <0.0001 , respectively.

Figure 3. 8 (Top) A chromosome pair. We clearly see that the larger part is background and the smaller parts are the chromosomes. We also see this in the histogram (*Middle*). The large peak is from the background (with the lower intensities), and the smaller peak is from the chromosomes (higher intensities). The threshold is chosen where the fitted Gaussian shaped curve of the background histogram and of the chromosome histogram intersect. When we do this for both channels (chromosome pair A and B), as illustrated in *Bottom*, we can calculate the volume occupied by chromosome pair A

(green plus yellow), chromosome pair B (red plus yellow) and the overlap (only yellow). A more detailed description of these steps is as follows: (i) The region of interest (ROI) is selected by using the DAPI channel. The grayscale image from this channel is thresholded by using an isodata algorithm (1). This threshold is the mean value of the object intensities plus the mean value of the background intensities divided by two. We fill the holes of the resulting binary image by binary propagation with the edge as seed and the inverted DAPI binary as a mask. Now we invert the resulting binary image, leaving us with the holes filled. (ii) Now that we have the ROI (i.e., the location of the DNA), we can use this finding to make a histogram in this ROI of the channel of interest (i.e., the red or green channel). We use an adaptation of minimum-error thresholding (2). Here, we estimate the background level from the histogram by fitting a Gaussian curve on the lower (large) part of the histogram, assuming the larger part of the image to be background. We use a least squares fit for this. After we subtract this fitted Gaussian curve, we end up with the histogram belonging to the signal, in our case, the chromosomes. After this step, we threshold where the signal histogram and the background fitted curve intersect. (iii) Performing this algorithm on both channels leaves us with two binary images. After a logical AND operation (3), where we input these binary images we end up with a binary image of the overlap. The sum of the voxels divided by the sum of the voxels of either the red or green binary image gives us a normalized parameter indicating the level of overlap.

1. Ridler, T. W. & Calvard, S. (1978) *IEEE Trans. Syst. Man Cybern.*, **8**, 630–632.
2. Rosenfield, A. & Kak, A. C. (1976) in *Digital Picture Processing*, Computer Science and Applied Mathematics (Academic, New York), pp. 269–275.
3. Young, I. T., Gerbrands, J. J. & van Vliet L. J. (1998) in *The Digital Signal Processing Handbook*, eds. Madisetti, V. K. & Williams, D. B. (CRC, Boca Raton, FL).

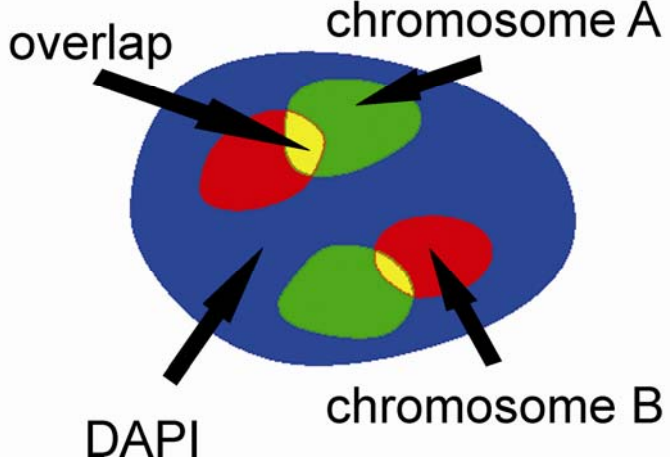
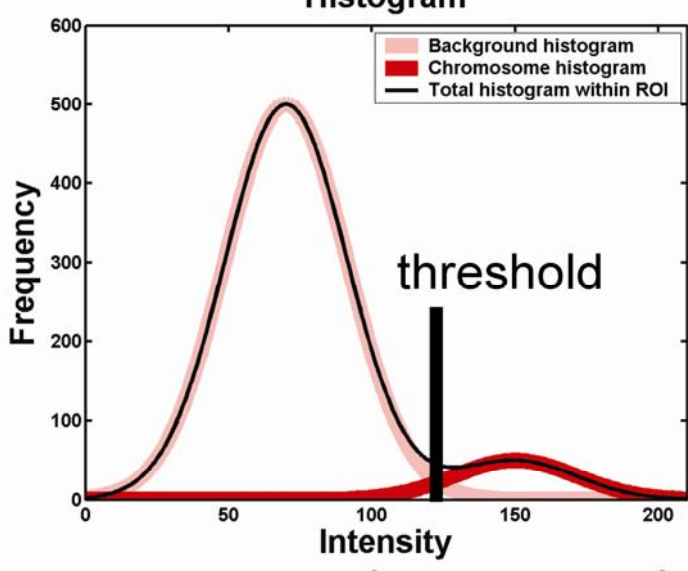
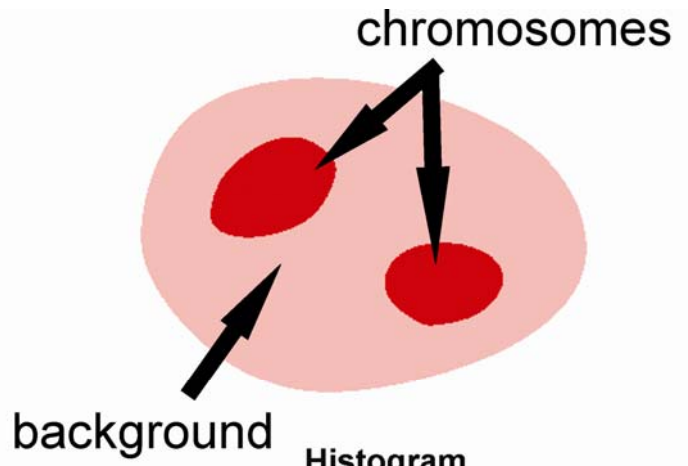


Figure 3. 9 Experimental activation of 4-hydroxytamoxifen (4HT) in PreB and Ba/F3 nuclei, respectively. Immunohistochemistry images at 0 and 4 h after MycER™ activation. (a) PreB cells without 4HT. (b) PreB cells with 4HT. (c) Ba/F3 cells without 4HT. (d) Ba/F3 cells with 4HT.

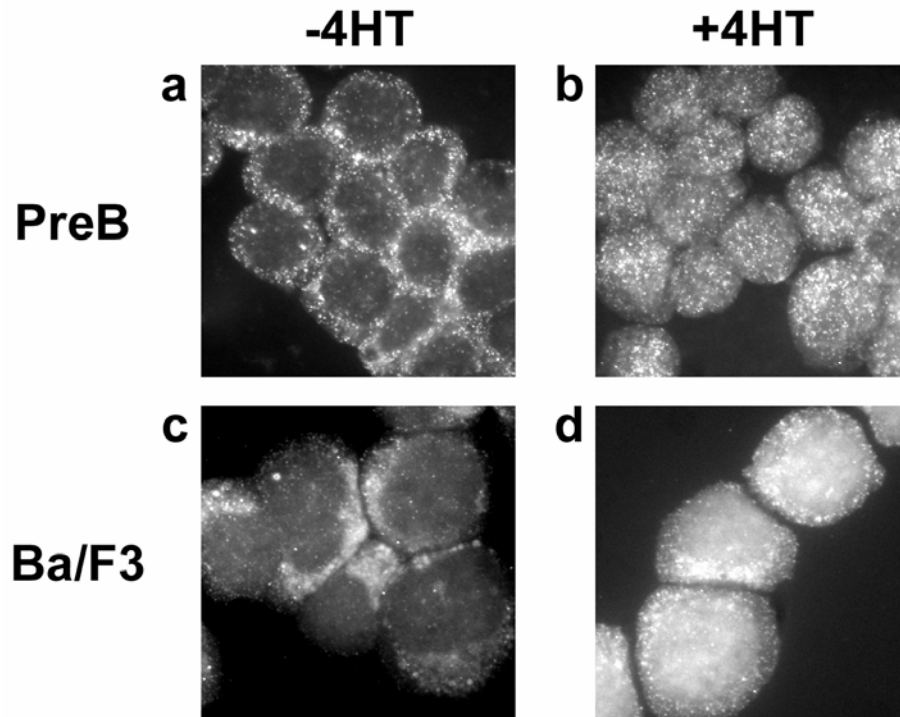


Figure 3.10 Shown is a comparison between the volume of telomeres of mock-treated PreB cells and cells treated with 4HT before, during, and after the first peak of telomeric aggregation.

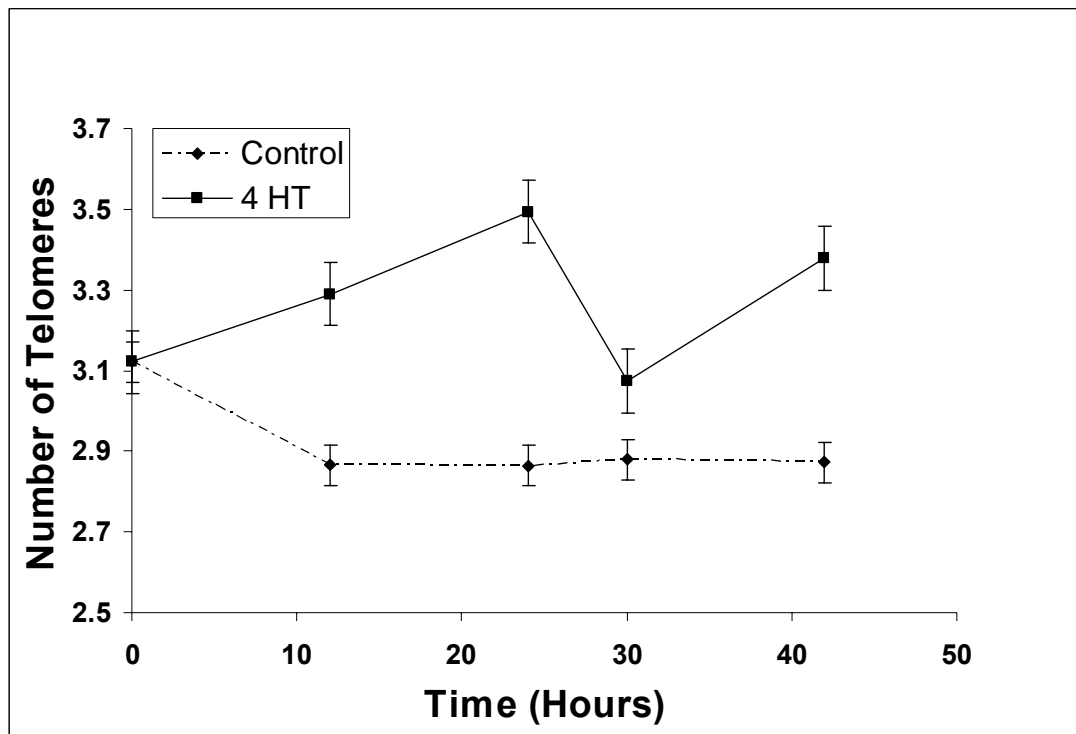


Figure 3. 11 Conditional c-Myc deregulation in PreB cells as examined by fluorescent immunohistochemistry. Relative fluorescent nuclear Myc staining is determined by immunohistochemistry. Different levels of nuclear c-Myc protein are observed as a result of different lengths of MycER™ activation. For details, see Fig. 1. Error bars show the standard deviation.

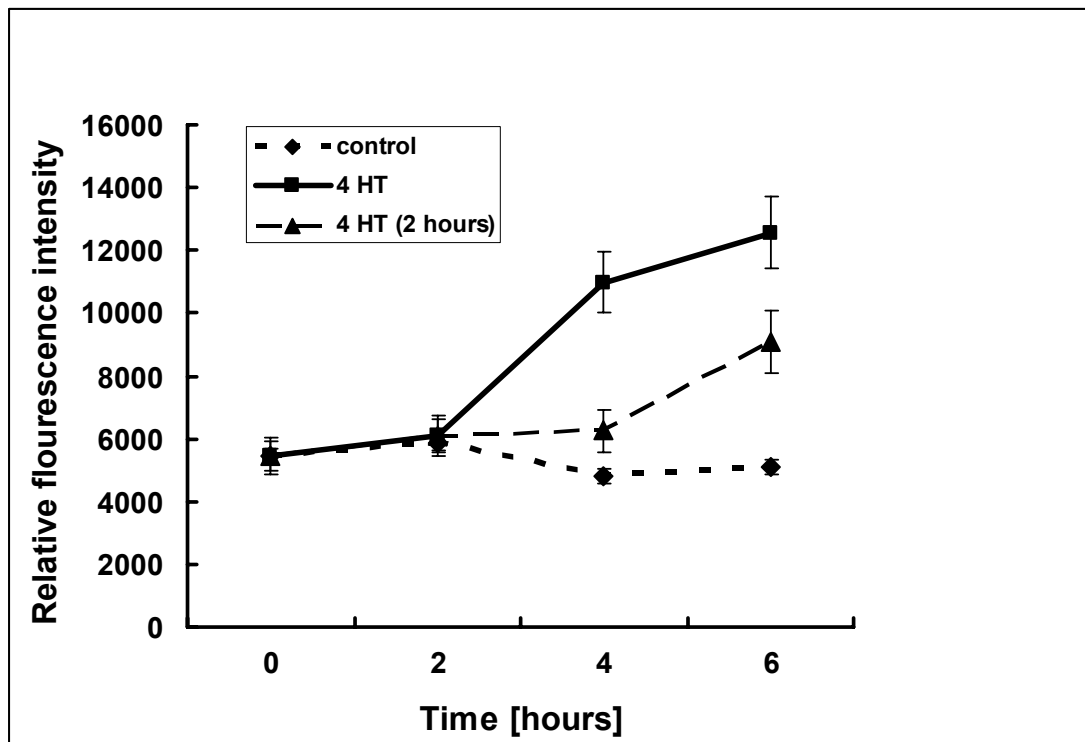


Figure 3. 12: Molecular cytogenetic evidence of breakage-bridge-fusion (B-B-F) cycles in MycER™-activated PreB cells is shown in Fig. 5. Shown here is the spectral karyotype for control preB cells. SKY was performed as described in *Materials and Methods*.



Table 3. 2 Significance of c-Myc-dependent formation of telomeric aggregates

Time [h]	% Telomeric aggregates/nucleus				Number of telomeric aggregates/ nucleus			
	4HT/ ctrl	12 h 4HT/ ctrl	2 h 4HT/ ctrl	Every 12 h 4HT/ctrl	4HT/ ctrl	12 h 4HT/ ctrl	2 h 4HT/ ctrl	Every 12 h 4HT/ctrl
0								
12	0.0014	0.0014	0.0095	0.0014	0.0001	0.0001	0.1502	0.0001
18	0.0112	0.5960	0.1896	<0.0001	0.0012	0.3661	0.0009	<0.0001
24	0.6768	0.6965	0.1448	0.1132	0.5511	0.4580	0.1328	0.0658
30	<0.0001	0.0107	0.1448	0.89	<0.0001	0.0017	0.0794	0.7691
36	0.0022	0.3324	0.3032		0.0002	0.3548	0.2785	
42	0.5580	0.2204	0.6980		0.6003	0.2355	0.6438	
48	0.0009	0.0863	0.0181		0.0004	0.0782	0.0115	
54	0.0900	0.2602	0.3251		0.0877	0.2841	0.2679	
60	0.1904	0.0055	0.3031		0.1268	0.0017	0.2234	
66	0.3031	0.8332	0.4178		0.3861	0.8130	0.3986	
72	0.0480	0.0128	0.2043		0.0388	0.0077	0.3599	
78	0.2973	0.9325	0.6072		0.3480	0.8874	0.6399	
84	0.8004	0.3453	0.8556		0.6853	0.3121	0.8281	
90	0.2797	0.0595	0.7238		0.2019	0.0567	0.6566	
96	0.0050	0.3209	0.9422		0.0008	0.3672	0.9951	
102	1.0	0.9375	0.6140		1.0	0.9845	0.6800	
108	0.8575	0.8598	0.5722		0.8738	0.7708	0.6128	
114	0.6233	0.2501	0.9336		0.7194	0.3015	0.9767	
120	1.0	0.0053	0.1914		0.8071	0.0105	0.1420	

Table 3.3 3D volumes of telomeres in the presence and absence of c-Myc deregulation.

Volumes of telomeres measured using TeloView™ software. Time points were chosen during and after the first peak of telomeric aggregate. Numbers represent arbitrary grey scale voxels to quantify 3D volumes of telomeres in the interphase nucleus.

0 hours	12 hours control	12 hours 4 HT	24 hours control	24 hours 4 HT	30 hours control	30 hours 4 HT	42 hours control	42 hours 4 HT
2.7480179	3.155118	3.2255818	2.9765258	3.0824138	2.4318691	3.9349915	2.2360609	3.1882868
3.4454789	2.4077906	3.2933468	2.6384064	2.9307845	2.7044453	3.3867977	2.8131164	2.5005747
2.785264	3.2405494	2.8523773	2.1905873	5.0193344	2.4706524	3.1371165	2.8124572	3.214996
2.7991088	2.832567	2.6886955	2.4466997	2.462482	2.8427024	2.8460764	3.2652854	4.091355
2.6946167	3.1279687	2.6804148	2.6078084	4.1385859	3.156248	3.2185313	2.5450675	3.5085828
3.01823	2.9748873	9.7853907	2.1347668	3.7675676	2.9989503	3.3201373	3.551176	4.8515327
3.165054	3.0696883	3.5526582	2.5988996	3.0408704	2.3935667	3.3037041	3.6279766	2.4653457
2.9904209	2.6450393	2.8253527	2.83635	4.5035069	2.8910234	2.4799326	2.7213938	3.5443861
2.9919276	2.9971305	2.6955836	3.4705191	4.248744	3.0045352	3.2831408	3.7790187	2.4848301
2.6610098	2.5518338	3.1429217	2.6428997	2.8447596	3.3133303	2.8017184	2.4112111	2.7095275
2.890081	3.5141001	2.7814745	3.7133303	3.896549	2.9299166	2.8391846	2.3847161	4.8238688
3.8733312	3.6647535	3.0174883	2.9066	2.512283	3.8507741	2.5826016	3.8871115	2.7186366
3.2712253	2.4579057	2.4096787	3.1469761	3.8031042	2.4921159	3.6471994	2.8038163	2.6715952
2.859023	2.5333973	3.5642962	2.8244377	3.1206752	2.7138448	2.9304941	2.3567523	2.6697354
3.3318544	2.5564695	2.8565793	2.1827726	4.3021358	2.3713578	2.8887614	2.5424156	2.6604817
4.0216141	2.8054766	3.1763448	4.2173983	3.3192559	2.9903568	2.6070964	3.3669119	6.9566695
4.345595	2.5119708	2.8907648	2.184112	3.2800808	2.5179793	3.2266854	2.5074678	3.3555232
2.7101006	3.0150602	2.4245282	4.5794647	3.4591871	3.0801492	2.4966972	2.5271163	3.4915949
2.7394983	2.3948586	2.6482404	2.6380369	2.8303021	2.703096	3.8250294	3.031217	2.8287691
3.0957992			2.3588044	3.2984892	3.720478	2.7233904	2.279984	2.8261393
3.1218625	2.866135	3.2900904	2.8647698	3.4930556	2.8788696	3.0739643	2.8725136	3.3781215
0.4746781	0.3745998	1.6069797	0.6723212	0.6940812	0.4125482	0.425611	0.5272924	1.1026799
0.2221563	0.1805515	0.77454	0.3146561	0.3248401	0.1930786	0.1991921	0.2467805	0.5160702

Table 3. 4 A summary for the data obtained by spectral karyotyping (SKY).

Metaphases were prepared and analyzed after a single administration of 4-hydroxytamoxifen (4HT) (see Fig. 1). A minimum of 20 metaphases was examined per time point. Q-FISH on additional 20 metaphases per time point confirmed the SKY data with respect to fusions and breakages. Translocations were only determined by SKY. End-to-end fusions predominate at early TA cycles (40% of the metaphases show fusions, $P < 0.0001$). Chromosomal breakages increase after the telomeric aggregate peaks. Q-FISH experiments confirmed that at later time points, most of chromosomal breakages were healing, leaving low levels of telomere-free chromosomal end(s) at 120 h. Nonreciprocal translocation were also found in the first TA (telomeric aggregate) cycle (in 20% of the metaphases) and reached significance, albeit at lower levels. As time progresses, fusions decreased while translocations remained steady. Fusions are shown in blue rows, and translocations and breakages are shown in yellow and green, respectively. The table shows the description of the aberrations found, their percentage of occurrence, and significance P values. M, metaphase. Numbers after M indicate metaphase numbers (i.e. M2: metaphase 2). Listing of aberrations followed (1).

1. Committee on Standardized Genetic Nomenclature for Mice (1969) *Mouse News Letter* **17**, 481–487.

Time point	Aberrations	%	p value
0 hrs	Fusions: none	-	-
	Translocations: none	-	-
	Breakages per metaphase: 2/20	10	0.1468
12hrs	Fusions: M2:5:16, M5:9:1:11, M6:6:16, M8:X:X, M10:12:6, M11:15:Y:12, M17:7:13, M18:17:14	40	<0.0001
	Translocations: M8:T3;15, T15;10, M10:TX;16, TX;5, M14:TX;1, M16:T1;5, T1;8.	20	0.0138
	Breakages per metaphase: 6/20	30	0.0101
24hrs	Fusions: M4:15:19, M7:14:X, M9:4:6, M10:8:3, M13:4:18	25	0.0168
	Translocations: M5:T6;19, T13;4, T16;1;12, M9:T11;1, T9;19	10	0.1468
	Breakages per metaphase: 9/20	45	0.0006
30hrs	Fusions: M10:15:10, M20:9:19	10	0.1468
	Translocations: M4:T18;4, M13:T16;3, M17:T2;17	15	0.035
	Breakages per metaphase: 15/20	75	<0.0001
42hrs	Fusions: none	-	-
	Translocations: M1:T11;3, T12;6, M4:T16;3, M10:T2;19, M15:T8;16, M17:T11;2, M18:T15;14, M20:T13;19	35	0.0036
	Breakages per metaphase: 9/20	45	0.0006
48hrs	Fusions: M16:10:X, M18:6:13, M19:14:3	15	0.0717
	Translocations: M2:T4;15, M3:T11;19, M4:T8;2, M7:T1;16	20	0.035
	Breakages per metaphase: 6/20	30	0.0101
66hrs	Fusions: M15:2:9, 3:16	5	0.3112
	Translocations: M1:T3;12, M15:T13;15, M7:T10;12, T2;12, M10:T5;X,	20	0.035
	Breakages per metaphase: 12/20	60	0.0003
72hrs	Fusions: M3:6:17,8:18, M4:7:4, M9:5:4	15	0.0717
	Translocations: M4:TY;15;4, M10:T15;4, M14:T19;8	15	0.0717
	Breakages per metaphase: 5/21	24	0.0314
96hrs	Fusions: M13:2:15	5	0.3112
	Translocations: M4:T15;6, M9:T14;5, M15:T4;8, T6;19, T16;4	15	0.0717
	Breakages per metaphase: 3/25	12	0.1101
102hrs	Fusions: none	-	-
	Translocations: M1:T16;3, M2:T15;11	10	0.1468
	Breakages per metaphase: 4/25	16	0.0619
120hrs	Fusions: none	-	-
	Translocations: none	-	-
	Breakages per metaphase: 3/22	13	0.0932

3.8 References

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4.0 Epstein-Barr virus associated remodeling of three-dimensional nuclear telomere signatures precedes chromosomal instability

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4.1 Abstract

Alterations in the three-dimensional (3D) nuclear organization of telomeres precede the onset of chromosomal rearrangements in cells exhibiting Myc protein deregulation. In the present study, we investigated whether Epstein-Barr (EBV) virus remodels the 3D nuclear organization of telomeres and whether alterations in 3D telomeric signatures are associated with chromosomal instability. We describe distinct types of 3D nuclear telomeric signatures in EBV-carrying established lymphoblastoid (LCL) cell lines, in *ex vivo* B-cells freshly infected with EBV, in EBV-positive Burkitt's lymphoma (BL) cell lines, and following the conditional expression of the EBV transcription factor, EBNA2 or Myc. Spectral karyotype analyses demonstrate that altered telomeric signatures precede chromosomal instability. The forms of chromosomal instability observed were both structural and numerical in nature and included unbalanced translocations, the formation of dicentric and Robertsonian chromosomes as well as mono- and trisomies and high occasional ploidies. Non-random chromosomal aberrations were found in BL lines. However, LCLs and *ex vivo* infected B cells only displayed random chromosomal instability. We conclude that EBV infection promotes both nuclear telomere remodeling and chromosomal instability.

4.2 Summary

Our recent findings described in Chapter 3 allowed us to discriminate between normal cells and transforming/cancer cells based on the 3D telomeric volume in the interphase nucleus (Louis et al. 2005). In Vermolen et al 2005, we developed the TeloViewTM software that can measure the 3D volume of each individual telomere in individual cells with resolution of 200 nm. Upon plotting data from statistically acceptable number of cells for a given cell/tissue type we can get a representation of the telomeric distribution of that cell type (Vermolen et al. 2005).

In the experiments described in this chapter of the thesis we had the objective of using TeloViewTM to shed light on the mechanism involved in the onset of Burkitt's lymphoma phenotype in EBV positive B-cells and also to investigate if EBV infection contributes to the development of that disease. BL is associated with and in fact characterized by the Myc/Igh translocation that leads to the expression of high levels of Myc and an aggressive malignant phenotype (Klein et al. 2007). To date there is no evidence that EBV infection induces or promotes the initiation of this translocation (Klein, et al. 2007).

EBV infects human B-cells and stay in latency for many years or even life time in over 90% of infected humans without progressing into any symptomatic disease (Thompson and Kurzrock 2004). However, recent reports have shown that EBV infection induces genomic instability and even may provide a surviving advantage for cells with Burkitt's lymphoma phenotype (Kamranvar et al. 2007).

The first aim for this objective was to use TeloViewTM to blindly categorize a number of established cell lines including BL-EBV positive cells, BL-EBV negative

cells, B-cell lymphoma and lymphoblastoid (LCL) lines (cell lines generated from B-cells by EBV infection but do not have a malignant phenotype). A total of nine cell lines were analyzed and except for one cell line they were all successfully categorized based on their telomeric distribution into either “transformed” or “non transformed” cells. The one lymphoblastoid cell line, IARC171 that was placed into transformed cells category based on the telomeric distribution turned to be donated by a donor who developed BL.

Those results encouraged us to add another aim to the study to assess the immediate changes in the telomeric distribution upon the switch between LCL to BL phenotypes. The proliferation of LCLs in culture is predominantly driven by the expression of the EBV transcription factor EBV-nuclear antigen 2 (EBNA2), while the proliferation in BL cells is driven by Myc. We then employed the p493 cell line that is an LCL line stably transfected with two expression constructs (Kempkes et al 1995). The cell line is kept in culture under one of two conditions, either conditionally expressing Myc conditionally expressing EBNA2. We analyzed the telomeric profile of those two forms of the cell line and also we switched the protein being conditionally expressed in each form and analyzed the telomeric profile on 24 hours bases for four days. The switch from EBNA2 driven proliferation to Myc driven proliferation, which artificially mimics the development of BL in EBV positive patients, shifted the telomeric distribution towards typical transformed cells profile. The experimental switch from Myc to EBNA2 driven proliferation did not return the telomeric distribution to the baseline telomeric profile of EBNA2 driven proliferation suggesting that EBNA2 can rescue the transformed phenotype.

To this point all the experiments described in this chapter/thesis objective were performed in cell lines that have been in culture for years. To avoid any bias in our data that may be caused by cell culture artifacts we added another special aim to our objective. We generated lymphoblastoids by infecting freshly donated *ex-vivo* B-cells with EBV and monitored the telomeric distribution of those cells on 24 hours bases for the first four days and then on monthly bases for seven months. We observed two changes in the telomeres distribution took place at 96 hours time point and at three months time point. The change in the telomeric distribution after 96 hours was considered due to the presence of a mixed population in the culture before an EBV immortalized colony be established.

To examine if the change in the telomeric distribution at three months time point induced any chromosomal instability we analyzed the cells using SKY. We observed several chromosomal abnormalities including high translocation frequency between chromosomes 8 and 14. To confirm if telomeric aggregates played a role in inducing such translocations we performed telomeres *FISH* on the same slides used for the SKY experiments and we observed the presence of interstitial telomeres marking end to end fusions and B-B-F cycles.

The last aim of this objective was to confirm that the chromosomal changes we observed in the pervious aim were due to EBV infection and not just forcing primary B-cells to enter the cell cycle and divide in culture. To address this question we put freshly donated B-cells in culture and we activated them to divide by adding mitogens known to blast activate primary B-cells. Cells did not survive longer than 30 days and no

significant telomeric or karyotypic changes were observed as compared to the EBV infected B-cells.

4.3 Introduction

It is not clear how the three-dimensional (3D) order of the nucleus is maintained. Boveri's early work suggested that chromosomes have their specific locations within the nucleus, which he termed chromosome regions and which were later coined 'chromosome territories' [Cremer and Cremer, 2001]. Boveri further deduced that an altered nuclear organization of chromosomes contributed to the development of malignant tumors [Boveri, 1902, 1914]. A century later, the identification of mechanisms leading to nuclear changes that contribute to the onset of tumor development remains elusive. While it has been reported by many groups that the nuclear organization is severely impeded in cancer cells [Pienta et al., 1989; Schmid et al., 2006; Zink et al., 2004], we do not understand how and in which order these changes take place. Are there step-wise events, that, similar to multi-step carcinogenesis, remodel and refine the nuclear micro and nano-environments to ultimately permit the formation of malignant clone(s)? Or is the altered nuclear organization already established in cancer stem cells? Are there reversible and irreversible alterations in the nuclear organization? And if so, when do such alterations become irreversible? When do such changes promote tumorigenicity?

Recent studies focusing on nuclear remodeling that is associated with malignant cellular transformation have shown that telomeres and centromeres assume altered 3D nuclear positions during cellular transformation [Chuang et al., 2004; Sarkar et al., 2007]. Oncogene activation, exemplified by the conditional deregulation of Myc, promoted the nuclear remodeling of telomeres, chromosomes and centromeres [Louis et al., 2005; Guffei et al., 2007]. Telomeres formed telomeric aggregates, chromosomes were found in

altered positions, and centromeres were observed at higher frequencies within the nuclear centre and away from the nuclear periphery. Importantly, the induction of these nuclear changes permitted the occurrence of specific types of genomic instability, such as the formation of dicentric and Robertsonian chromosomes, and unbalanced translocations. Breakage-bridge-fusion cycles were induced, leading to the formation of dicentric chromosomes and subsequent chromosome breakages in anaphase [Louis et al., 2005].

As we currently investigate induced nuclear remodeling, we focused on EBV. EBV is a ubiquitous human gamma herpes virus with dual tropism for human B lymphocytes and epithelial cells [Kempkes et al., 1995]. EBV contributes to several malignancies including Burkitt's lymphoma (BL), B cell lymphomas, Hodgkin's disease, and nasopharyngeal carcinoma (NPC) [Klein et al., 2007; Sugimoto et al., 2004; Thompson and Kurzrock, 2004]. Earlier studies by others had shown that EBV-infected human B cells display karyotypic changes in culture [Kataoka et al., 1997; Okubo et al., 2001; Kamranvar et al., 2007].

In the present study, we examined the role of EBV in nuclear telomere remodeling and the generation of chromosomal changes in human B cells. The 3D telomere organization was examined in p493 cells with conditional EBNA2 and Myc expression respectively, [Kempkes et al., 1995; Pajic et al., 2001; Schuhmacher et al., 1999], and in comparison with established EBV carrying B cell lymphoma and EBV-positive Burkitt lymphoma (BL) clones. Freshly EBV infected or mitogen-activated *ex vivo* B cells were tested as well. We report that the telomeric signatures of the LCLs differed from the BL cells. EBV infected but not mitogen-activated *ex vivo* B cells

showed two major shifts in telomeric signatures over the period of seven months.

Telomeric changes coincide with the onset of genomic instability.

4.4 Materials and Methods

4.4.1 Cell lines and conditional activation/expression of EBNA2 and Myc respectively

Nine cell lines were included in the study. Four BL cell lines, three of them are EBV-negative including BL41, Ramos and DG75 and the EBV- positive Raji. The EBV-negative Bjab cell line has been derived from B-cell lymphoma. The four LCLs IARC171, LCL 970402, LCL 980215 and LCL 910410 were derived from healthy donors. Conditional expression of Myc or of EBNA2 respectively was performed in p493 cells, an LCL carrying estrogen regulated EBNA-2 (EBNA2-ER) and a tetracycline regulated *myc* gene (*tet-myc*) [Kempkes et al., 1995; Pajic et al., 2000]. EBNA2 is activated in the presence of 2 μ M β -estradiol (Sigma) (+) while the *myc* gene can be repressed by 0.1 μ g/ml of the tetracycline analogue doxycycline (+). p493 +/+ cells were grown under the following two conditions in parallel; the presence of estrogen and doxycycline. Under this condition the cells express exogenous EBNA2 but not exogenous Myc. Those cells have a lymphoblastoid (LCL) phenotype. Alternatively, p493 -/- cells were also kept deprived of estrogen and doxycycline. In this case exogenous EBNA2 will not be expressed but the withdrawal of doxycycline lead to overexpression of exogenous Myc. Phenotypically, these were BL like. The switch of p493+/+ to -/- indicates that the cells have been switched from the estrogen driven state to the Myc driven state and vice-versa. Cells were harvested at time points zero, 24, 48, 72 and 144 hours after the switch. Three independent experiments were performed.

EBV infected *ex vivo* B-cells were prepared by isolating B-cells from freshly donated human blood sample from EBV negative donor. Cells were incubated with the

EBV strain B95-8 (batch 13-12-04) at a titer of $1\text{ml}/10^6$ cells for 1 hour at 37°C . Infected cells were plated in fresh media and incubated at 37°C in 5% CO_2 incubator. They were harvested after zero, 24, 48, 72, 96 hours and every month thereafter up to seven months.

Mitogen activated B-cells were prepared by isolating lymphocytes from freshly donated human blood sample from an EBV negative donor [O'neils et al., 2004]. B-cells were separated from T-cells by labeling cells with magnetic beads conjugated anti CD19 antibody (Miltenyi Biotec CA, USA) and passing them through AutoMax cell sorter (Miltenyi Biotec CA, USA). Cells were plated with 2 ng/ml CD40 (Sigma Aldrich, Oakville ON, Canada) and 0.2 mg/ml IL-4 (sigma Aldrich, Oakville ON, Canada).

4.4.2 SDS- PAGE and Western blot analysis

Whole cell extracts were prepared from p493 cells at the mentioned time points using 2X sodium dodecyl sulfate (SDS) sample buffer. Western blots were prepared to confirm the conditional expression or repression of Myc [Albihn et al., 2006]. In brief, 10-15 μg of extracts were loaded on a 10% SDS-polyacrylamide gel electrophoresis (Invitrogen Corp., Carlsbad, CA, USA) and transferred to a nitrocellulose Hybond N+ membrane (Amersham, UK). The blots were probed with antibodies to Myc (N262, Santa Cruz Biotechnologies Santa Cruz CA, USA) followed by anti rabbit HRP secondary antibody (Amersham UK). Blots were visualized using Fuji LAS 1000 system (FujiFilm Medical Systems Inc., Stamford, CT, USA).

4.4.3 Three dimensional (3D) telomere FISH

Cells were removed from the culture plate to appropriately sized falcon tubes and were centrifuged for 10 minutes at 800 *rpm*. They were 3D fixed by incubating for 10 min in 0.075M KCl hypotonic solution followed by overlay and slow mixing with 1ml of fresh 3:1 vol/vol methanol/ acetic acid fixative. Telomeres were stained using a telomere-specific CY³-labeled PNA probe (DAKO, Mississauga, ON, Canada) in FISH hybridization experiments as described in Chuang *et al* 2004 [Chuang et al., 2004]. In brief 3D cells were deposited onto a slide and fixed in 3.7% formaldehyde in 1X PBS buffer. Cells were then treated with pepsin for 10 min, re-fixed in 3.7% formaldehyde, dehydrated with successive dipping in ethanol 70%, 90% and 100% for 2 minutes each. 6 µl of probe were then applied to each slide, slides were sealed with rubber cement. Hybridization took place using a HybriteTM (Vysis, Chicago, IL, USA) where slides were first denatured for 3 minutes at 80°C and then hybridized for 2 hours at 37°C. Slides were then washed 2 X 15 min in 70% formamide/10mM Tris p H 7.4 followed by 5 minutes wash in 0.1% SSC at 55°C and 2 X 5 minutes in 4XSSC/0.01 Tween-20. Slides were then dehydrated with ethanol as mentioned above and counterstained with 0.1 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Oakville ON, Canada).

4.4.4 Three dimensional imaging and analysis

Slides were 3D imaged on a Zeiss Axioplan 2 microscope with Axiovision software v3.1, equipped with a Zeiss cooled AxioCam HR camera and a motorized stage. Images were acquired using DAPI or Cy3TM filters with Planapo 63x 1.4 oil objective lens (Zeiss, North York, ON, Canada). Each image consisted of 90 Z-stacks with a 200

nm step between each stack along the Z- axis. Images were deconvolved using a constrained iterative algorithm [Schaefer et al., 2001]. Telomere volume measurement and analysis was performed using TeloViewTM software [Vermolen et al., 2005]. 25-30 nuclei were examined per time point.

4.4.5 Telomere FISH on metaphase

Chromosomes were prepared from harvested cells according to Mai and Wiener 2002 [Mai and Wiener 2002]. Telomere FISH were performed as mentioned under 3D telomere FISH. Cells were imaged and analyzed on the ASI (Applied Spectral Imaging, Vista, CA, USA) station using the FISHVIEW 5.5 software.

4.4.6 Spectral Karyotyping (SKY)

SKY was performed as described in Schrock E et al 1997 by using the ASI (Applied Spectral Imaging, Vista, CA, USA) kit for human and the supplier's hybridization protocol [Schrock et al., 1997]. Slides were imaged using the Spectral Cube on a Carl Zeiss Axioplan 2 microscope and 63X oil objective. Images were acquired and analyzed using SKYVIEW 1.6.2 and 2.0 software for PC. 20 metaphases were examined per cell type.

4.4.7 Statistics analyses

Statistics were performed using Excel software. The *Chi*-square was calculated by comparing the variance of each data group from the mean. *p*-values > 0.05 were considered significant.

4.5 Results

4.5.1 Analysis of telomere volumes in LCL, BL and B-cell lymphoma cell lines

In this study nine cell lines were included four BL lines BL41, Raji, Ramos and DG75; one B-cell lymphoma Bjab and four LCLs IARC171, LCL 970402, LCL 980215 and LCL 910410. Cells were 3D fixed, hybridized with a peptide-nucleic acid (PNA) telomere probe labeled with a Cy3TM molecule, and then cells were 3D imaged and analyzed. We were able to distinguish the tumor derived cell lines from the LCLs based on the telomere 3D volume with the exception of IARC 171 LCL cell line that looked more like a BL rather than an LCL. In general, telomeres in BL lines were larger in size than in most LCL lines (Table 4. 1 and Figure 4. 1 A, Figure 4. 1 B, C and D). The analysis of the formation of telomeric aggregates revealed higher percentages of aggregates in tumor cell lines as compared to the LCLs (Figure 4. 2A, Table 4. 1). The analysis of the nine cell lines suggests that 3D telomere analysis may differentiate between cell lines derived from normal or tumor origins.

4.5.2 Analysis of telomere volumes in the conditional p493 cell line when driven by EBNA2 or by Myc, respectively

p493^{+/+} cells were subjected to estrogen and doxycycline deprivation to switch from EBNA2 driven phase to Myc driven phase and *vice-versa* for p493^{-/-} cells (Figure 4. 2 C) (see Materials and Methods). Cells were harvested and 3D fixed at time points; 0, 24, 48, 72, 144 hours. Individual nuclei were 3D imaged and deconvolved (Schaefer et al., 2001) and telomere volumes for each nucleus were assessed using TeloViewTM software (Figure 4. 2 A, B and D) [Vermolen et al., 2005].

Figure 4. 1 Three-dimensional (3D) telomere analysis of BL, B-cell lymphoma and LCLs. A: A graph showing percentages of formation of telomere aggregates in BL, B-cell lymphoma and LCL cell lines. Error bars present standard deviation between three independent hybridizations.

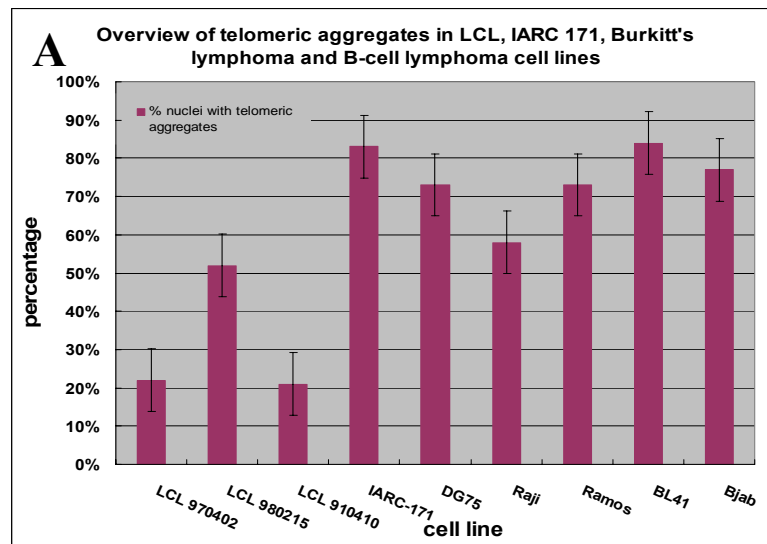


Figure 4. 1 B & C Representation of the distribution of telomere sizes in BL, B-cell lymphoma and LCL cell lines. Fluorescent intensities are proportional to size (Poon et al., 1999) and are illustrated by relative fluorescence intensity units. **B:** graph showing a comparison of BL cell lines DG75, Raji, Ramos and LCL910410. **C:** graph showing a comparison of BL cell line BL41, B-cell lymphoma cell line Bjab and LCL 910410.

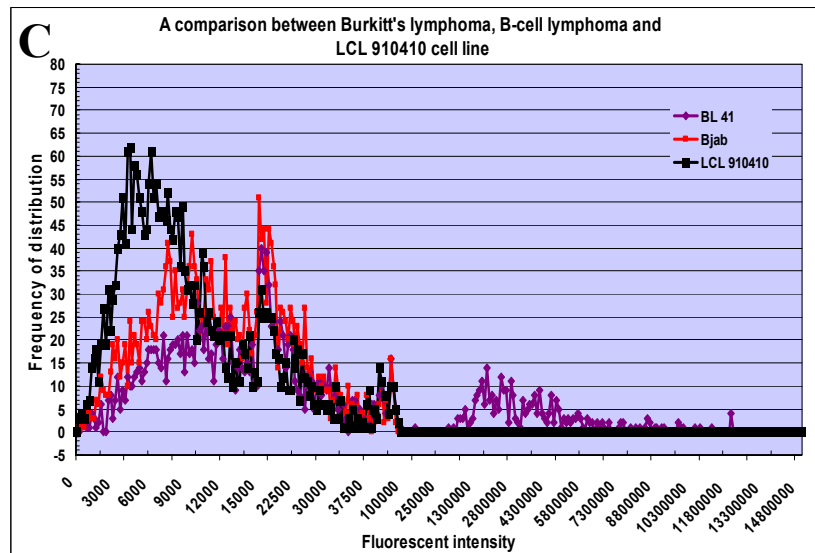
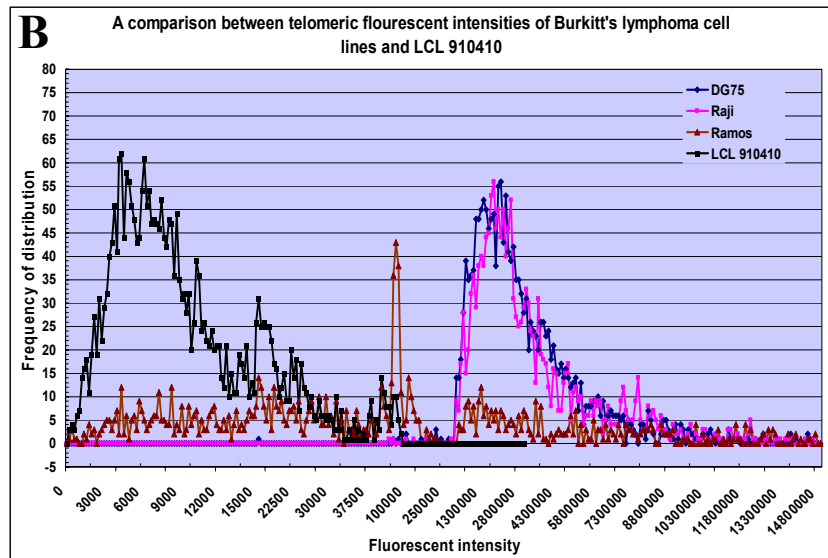
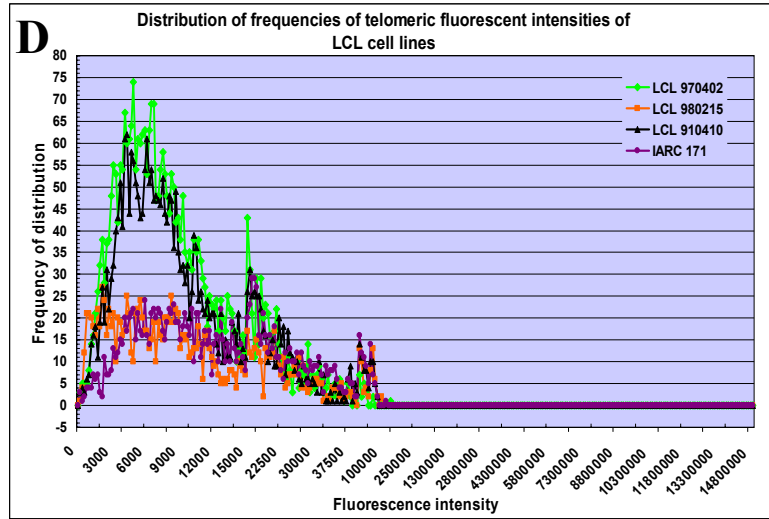


Figure 4. 1 D graph showing comparison between LCL cell lines 970402, 980215, 910410 and IARC 171.



The conditional expression of either of Myc and EBNA2 proteins in p493 cells affected the telomere distribution over time and favored the formation of telomere aggregates. Upon estrogen and doxycycline deprivation in p493^{+/+} cells (EBNA2 downregulated and Myc upregulated) more telomeric aggregates were observed over time. Conversely, upon estrogen and doxycycline administration in p493^{-/-} cells (EBNA2 upregulated and Myc downregulated) the telomeric aggregates decreased over time (Figure 4. 2D). These data suggest that the upregulation of both Myc and EBNA2 contributes to the alteration in the telomeric volume but only the upregulation of Myc leads to the generation of measurable telomeric aggregates.

4.5.3 Analysis of Freshly EBV-infected or mitogen activated *ex vivo* B-cells

In view of the fact that BL/LCL comparisons were done on long-propagated lines, it was deemed important to analyze freshly EBV infected B-cells. Mitogen activated B-cells were also included in the study. After infecting *ex vivo* B-cells with EBV virus, we analyzed the time points 0, 24, 48, 72, 144 hours and once every month till the seventh month thereafter. The telomeric distribution of EBV-infected *ex vivo* B cells starting from 24 hours after infections showed difference in their profile overtime. The distribution curve shifted towards smaller sized - telomeres compared to zero time point. However at time point 96 hours the shift towards smaller telomeres was more robust than the shift observed in 24, 48 and 72 hours. In later time points of one to three months, the graphs showed similar data to what was observed on 96 hours (Figure 4. 3 A, B and C). Starting at 4 months a significant shift in the distribution curve towards larger telomeres was observed. Similar curves were observed till 6 months (last time point analyzed) (Figure 4.

3 D). To investigate if EBV infection contributed to the observed changes in the telomeric profile overtime, we analyzed mitogen activated B-cells at zero and 30 days time point (Figure 4. 3 E). Earlier or later time points were not analyzed due to the limitation in the ability to prolong the life of mitogen activated B-cells in culture. A slight shortening in the relative telomeric size was observed between zero and one month, however in EBV-infected B-cells the change in the telomeric profile at the same time point was more robust (Figure 4. 3 E & F). This data suggest that EBV infection induces a fast but prolonged telomere lengthening in infected B-cells.

Table 4. 1 An overview of the formation of telomere aggregates in BL, B- cell lymphoma and LCL cell lines. Statistics data were obtained by performing *Chi*-square analysis for the variance from the means. *p*-values are the probabilities obtained based on the *Chi*-square analysis. *p*-values of <0.05 were considered significant. * represents statistical significance

Cell line	# of nuclei	% aggregates	<i>p</i> -value	#agg/# of nuclei
LCL 970402	27	22%	0.2	12/27
LCL 980215	25	52%	0.08	20/25
LCL 910410	27	21%	0.2	12/27
IARC-171	30	83%*	0.0003	50/30
Raji	26	58%	0.07	23/26
Ramos	26	73%*	0.004	50/26
BL41	26	84%*	0.0003	63/26
Bjab	30	77%*	0.001	51/30
DG75	26	73%*	0.004	48/26

Figure 4. 2 Telomeres analysis of p493^{+/+} and p493^{-/-} cell lines after estrogen/doxycyclin deprivation (p493^{+/+}) or vice versa (p493^{-/-}). **A:** Representative image of a nucleus of p493 ^{+/+} cell at zero hours time point showing normal telomere distribution. The Left panel shows 2D image, the right panel shows 3D image. **B:** Representative image of p493 ^{+/+} cells at 72 hours after estrogen/ doxycycline deplete on showing telomere aggregates. The Left panel shows 2D image, and the right panel shows 3D image. Black arrows point the telomere aggregates in the 3D.

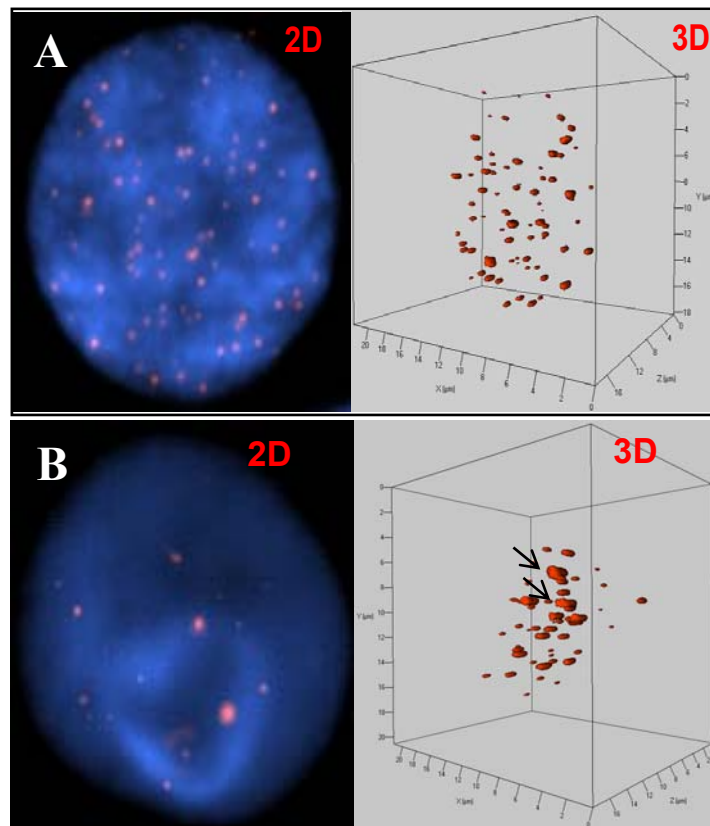


Figure 4. 2 C Western blot analysis showing the change in Myc expression in p493 cell lines after estrogen/doxycycline depletion in p493^{+/+} cells (left panel ^{+/+} to ^{-/-}, LCL phenotype to BL phenotype) or estrogen/doxycycline administration to p493^{-/-} (right panel ^{-/-} to ^{+/+}, BL phenotype to LCL phenotype) at time the points shown. **D:** A graph showing the change of percentages of the formation of telomere aggregates in p493 cell lines upon estrogen/doxycycline depletion or administration. Error bars present the standard deviation between three independent reverse induction experiments.

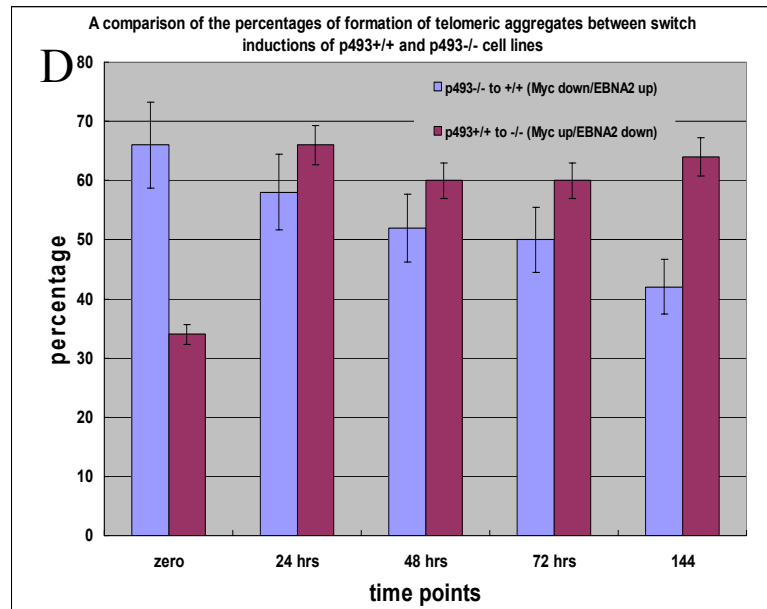
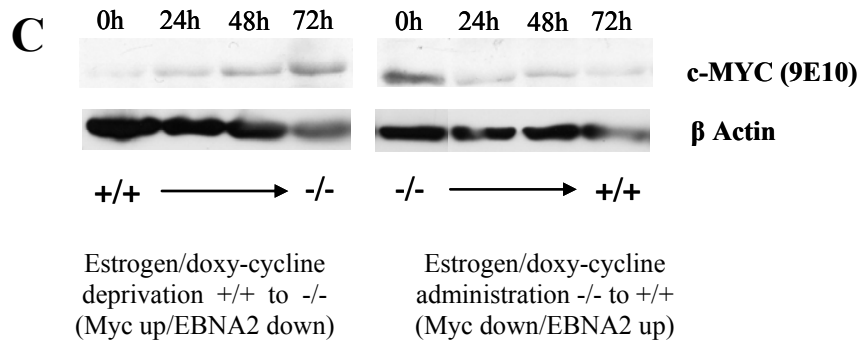


Figure 4. 3 3D telomere analysis of EBV infected *ex vivo* B-cells: A: A comparison between 96 hours, 3 and 4 months time points. **B:** A comparison between 96 hours and LCL lines.

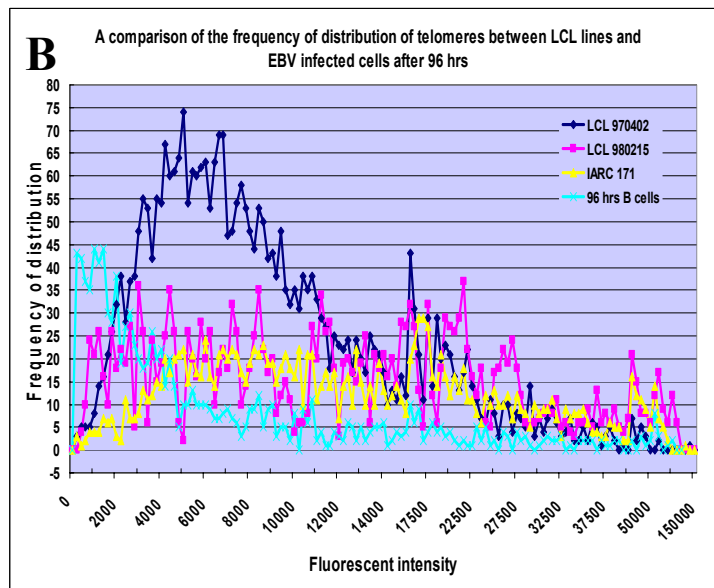
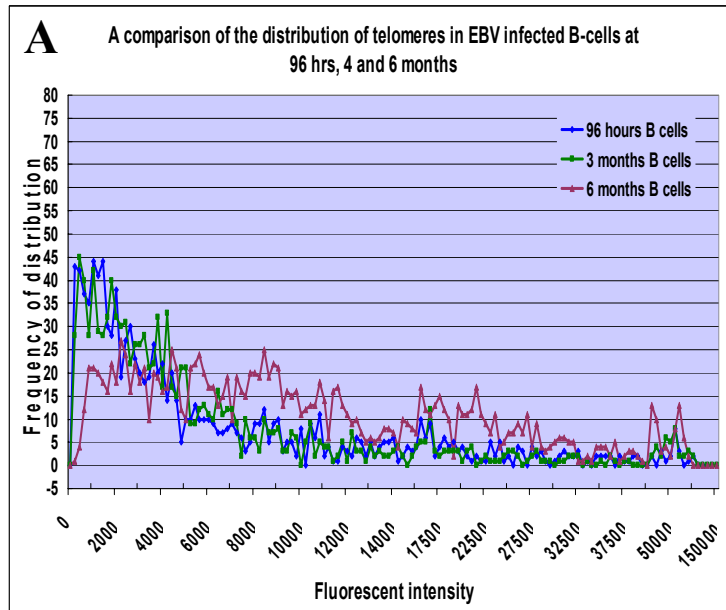


Figure 4.3 C A comparison between 3 months time point and LCL lines **D:** A comparison between 4 months time point and LCL lines. Note that 1 to 3 months had similar graphs (3 months only is shown) and 4 to 7 months (4 months only is shown) had similar graphs.

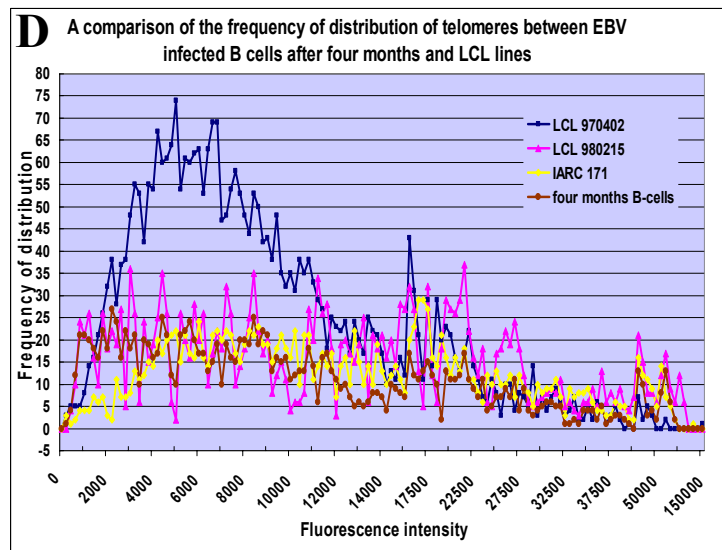
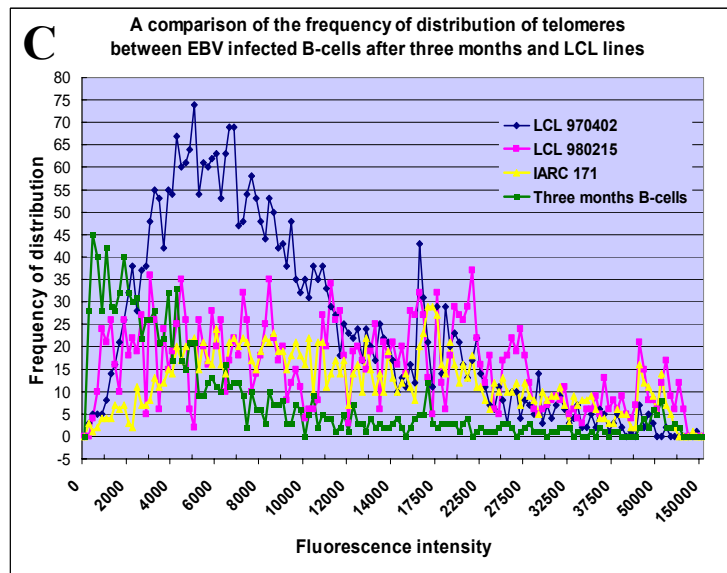
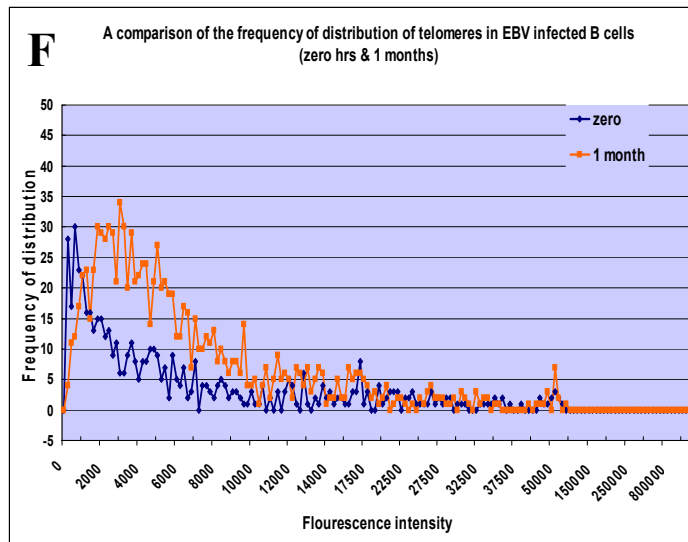
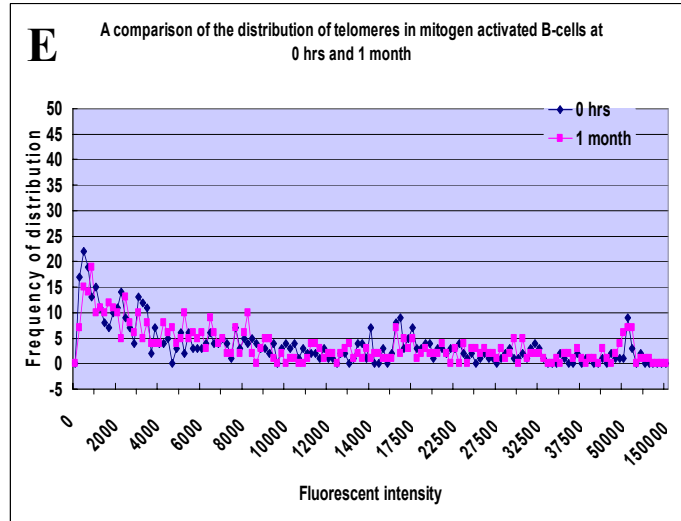


Figure 4.3 E A comparison between mitogen activated B cells at time point 0 hrs and 1 month. **F:** A comparison between EBV infected B-cells at time points 0 and 1 month



4.5.4 SKY analysis for BL and LCL cell lines

To investigate if the alterations in the telomere profiles are associated with the formation of karyotypic instability, we performed SKY analysis of BL41 (Figure 4. 4 A), IARC171, LCLs, and EBV-infected *ex vivo* B-cells at time points 1 and 6 months (Figure 4. 4 B and C respectively). SKY analysis revealed the following (Table 4. 2): In BL41, 18/22 metaphases had the translocation t (8;14). Two metaphases had monosomy 14 also other random numerical and structural aberrations were observed (Table 4. 2). Four LCL cell lines were analyzed including IARC 171, LCL 970402, LCL 980215 and LCL 910410. In IARC 171, low levels of random translocations were observed (Table 4. 2). However, several trisomies and an increase in certain ploidy levels of individual chromosomes were found including chromosomes 5, 9, 12 and 18. Moreover, broken chromosomes were also present in 18/ 20 metaphases; these included chromosomes 2, 8, 14, 3, 12, 21, 15 and Y. For LCL 910410, only a single translocation was observed in one metaphase T(14;12). Random numerical aberrations were found in the form of individual chromosomes ploidies especially chromosomes 3, 4, 9, 14 and 16. The SKY analysis of BL and LCL cell lines shows that cell lines derived from a tumor background had non random structural and random numerical aberrations while those driven from normal background had only random numerical aberrations.

4.5.5 Sky analysis for EBV infected and mitogen activated *ex vivo* B-cells

For the one month time point, 12/20 metaphases of the EBV infected B-cells showed normal karyotypes. The remaining eight metaphases had numerical aberrations, including monosomies and trisomies (Figure 4. 4 B, Table 4. 2). The karyotypic analysis

of mitogen activated B-cell after one month in culture also showed numerical aberrations in 2/20 metaphases. The aberrations included monosomies, trisomies and ploidies (Table 4. 2). Unfortunately mitogen activated B-cell did not survive in culture to allow analysis of time points beyond the 30 days.

At the 4 months time point all 20 metaphases analyzed from EBV infected B-cells had numerical aberrations and/ or broken chromosomes. Also a large number of random unbalanced translocations were observed as well as chromosomal fusions (Table 4. 2). For the 6 months time point, all 20 metaphases also had numerical and/or structural aberrations. 4/20 metaphases had T(8;14) translocation that appeared to be different from BL characteristic T(8:14) translocation (Figure 4. 4 A and C). It is worthy to mention that all four metaphases that showed this translocation had broken parts of chromosome 8. In Figure 4. 4 C the metaphase shown also had a trisomy 8. At this time point both Robertsonian (Rb) and dicentric (dic) fusions were observed in 8/20 metaphases including Rb(15.11), Rb(1.6), dic(5.13), dic(9.13), dic(3.4), dic(18.22) and dic(22.7) (Table 4. 2). The formation of random genomic aberrations overtime in EBV infected *ex-vivo* B-cells may suggest a role for EBV in priming cells for acquiring genomic aberrations.

Figure 4. 4 SKY analysis of BL41, EBV-infected ex-vivo B-cells at 1 months and 6 months .

A: BL41 BL cell line, Note that left top panels (A-C) represent from left to right, the raw spectral image, the inverted DAPI image, and the classified image. The bottom panel shows the SKY karyotypes. Red circles in A and C highlight the rearrangements involving chromosomes 8 and 14.

A

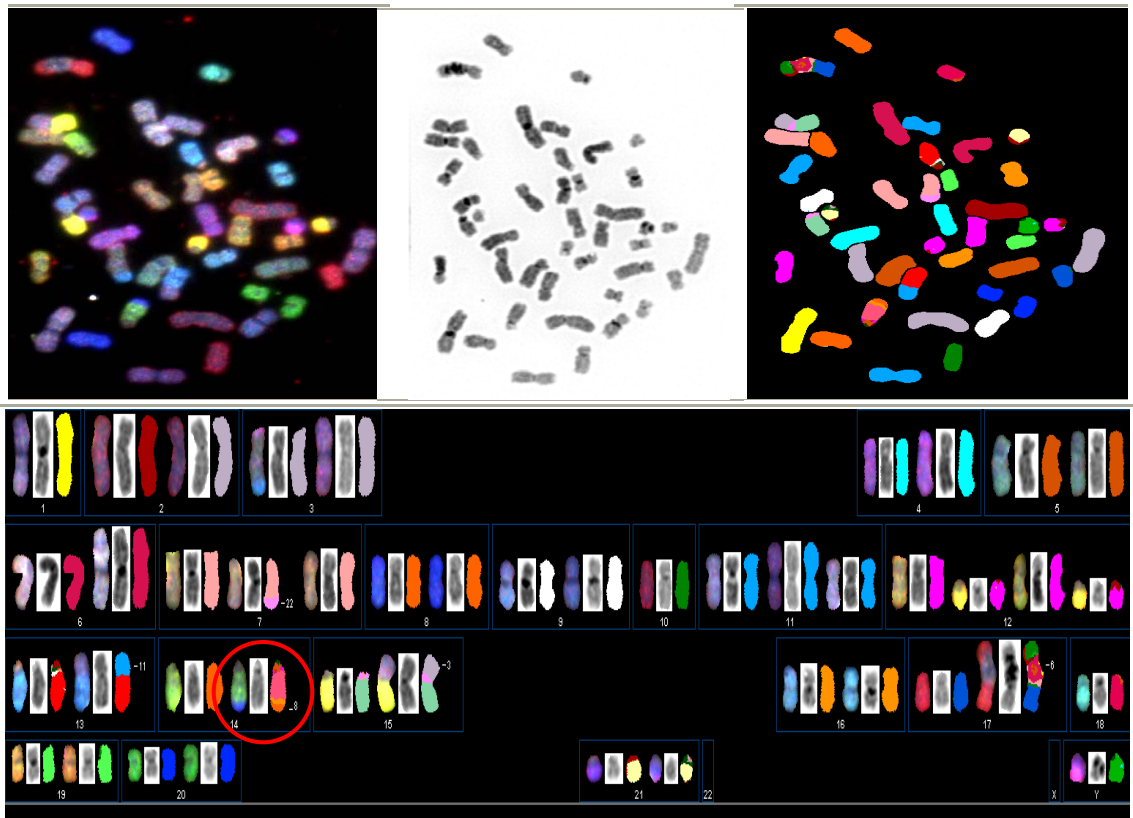


Figure 4. 4 B EBV- infected *ex vivo* B-cells at 1 month time point showing normal karyotype.

B

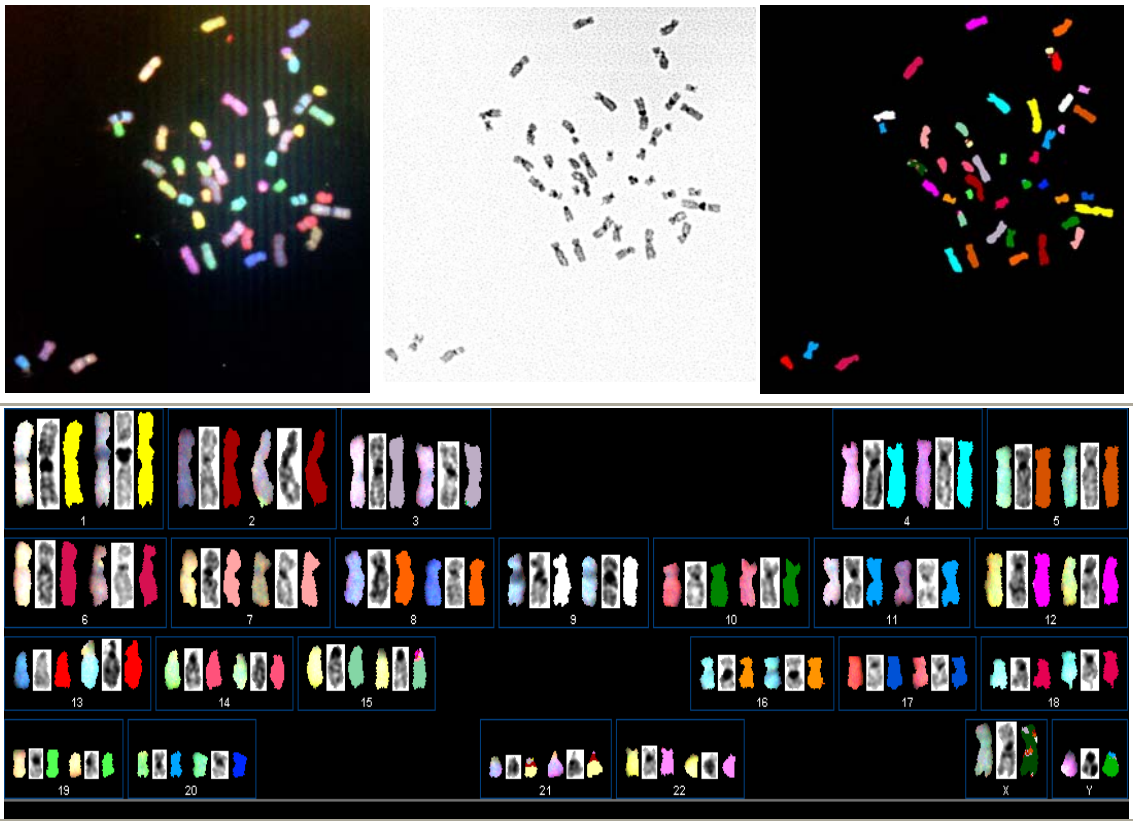


Figure 4. 4 C EBV- infected *ex vivo* B-cells after 6 months showing fusion/translocation involving chromosomes 8 and 14 (red circle).

C

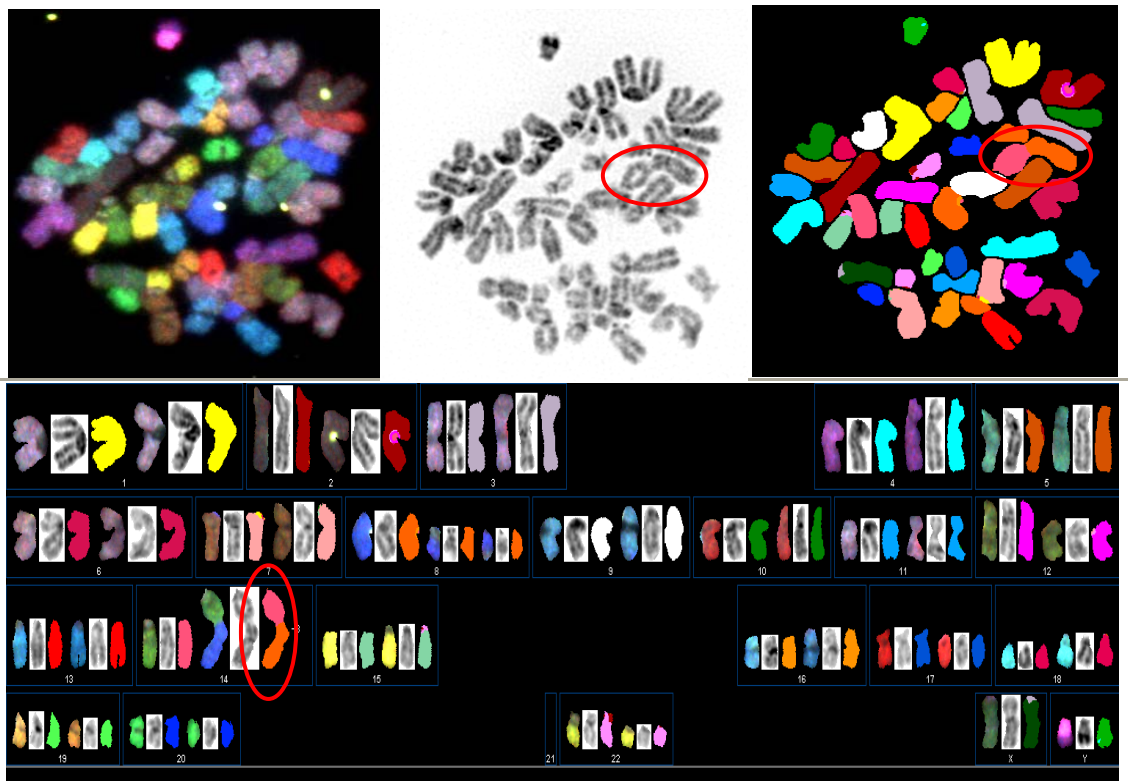


Table 4. 2 Summary of aberration found By SKY analysis

Cell type	Translocation		Fusions		Insertions		Telomere free chr. ends		Numerical Aberrations	
	Aberration	%	Aberration	%	Aberration	%	Aberration	%		
BL41 (22 Ms)	T(8;14)	82%	M1:[dic(10.17), Rb(15.3)]	5%	Is(10;6)	18%	8, 12	10%	All metaphases. (Monosomies, trisomies and individual chromosomes ploidies)	
	T15;3)	72%		5%	Is(10;1)	5%	2,3,10	20%		
	T(13;11)	68%	M15:dic(10.6)	5%	Marker Is(10;6;17)	18%	13	15%		
	T(10;17)	13%	M21:Rb(6.9)	5%	Marker Is(10;1;17)	27%	17,Y	5%		
	T(13;6)	9%								
	T(7;19)	9%								
	T(14;22)	9%								
IARC 171 (20 Ms)	T(12;Y)	5%	0%	0%	0%	0%	2,3,4,5,8	5%	All metaphases (Monosomies, trisomies and individual chromosomes ploidies)	
	T(13;9)	5%					9,11,12,15,16,17,18, 21,22, Y	10%		
	T(12;3)	5%								
LCL 910410 (20 Ms)	T(14;12)	5%	0%	0%	0%	0%	8	10%	All metaphases has individual chromosomes ploidies	
B-cells + EBV 1 months (20 Ms)			0%	0%	0%	0%	0%	0%	Monosomies	%
									2	5
									8	25
									14	10
									22	10
									Trisomies	%
									1	5
									3	15
		6	5							
		7	5							
B-Cells + EBV 4 months (20 Ms)	[T(18;17),T(5;4)],[T(18;3),T(13;3),T(17;1)] [T(15;2)]	5% each	M3: dic(5.13), M8: dic(21.13), M16: dic(3.2), M19: dic(10.1), M20: [dic(3.16), dic(8.22)]	5% each	0%	0%	8	15%	50% of metaphases (Monosomies, trisomies and individual chromosomes ploidies)	
	[T(14;12)]						12	15%		
	[T(18;4),T(18;9)]						3	10%		
	[(T8;4)] [T(1;22), T(4;3)]						Y	10%		
	[T(1;3),T(1;22)]						9, 18, 21, 22	5%		
B-Cells + EBV 6 months (20 Ms)	M1,4,15,20: T(8;14)	20%	M1: Rb(15.11)	5%	M14: Is(15;12)	5%	0%	0%	50% of metaphases (Monosomies, trisomies and individual chromosomes ploidies)	
	M5,6,12:T(14;12)	15%	M3,M6:dic(1.6)	10%	M14: Is(4;1)	5%				
	M1,20: T(11;13)	10%	M12: dic(5.13)	5%						
	M1:T(19;16)	5%	M13: dic(9.13)	5%						
	M6: T(1;6)	5%	M14: dic(3.4)	5%						
	M11: T(15;22)	5%	M15dic(18.22)	5%						
M15: T(9;1)	5%	M19: dic(22.7)	5%							

4.5.6 Telomere FISH on metaphase preparation

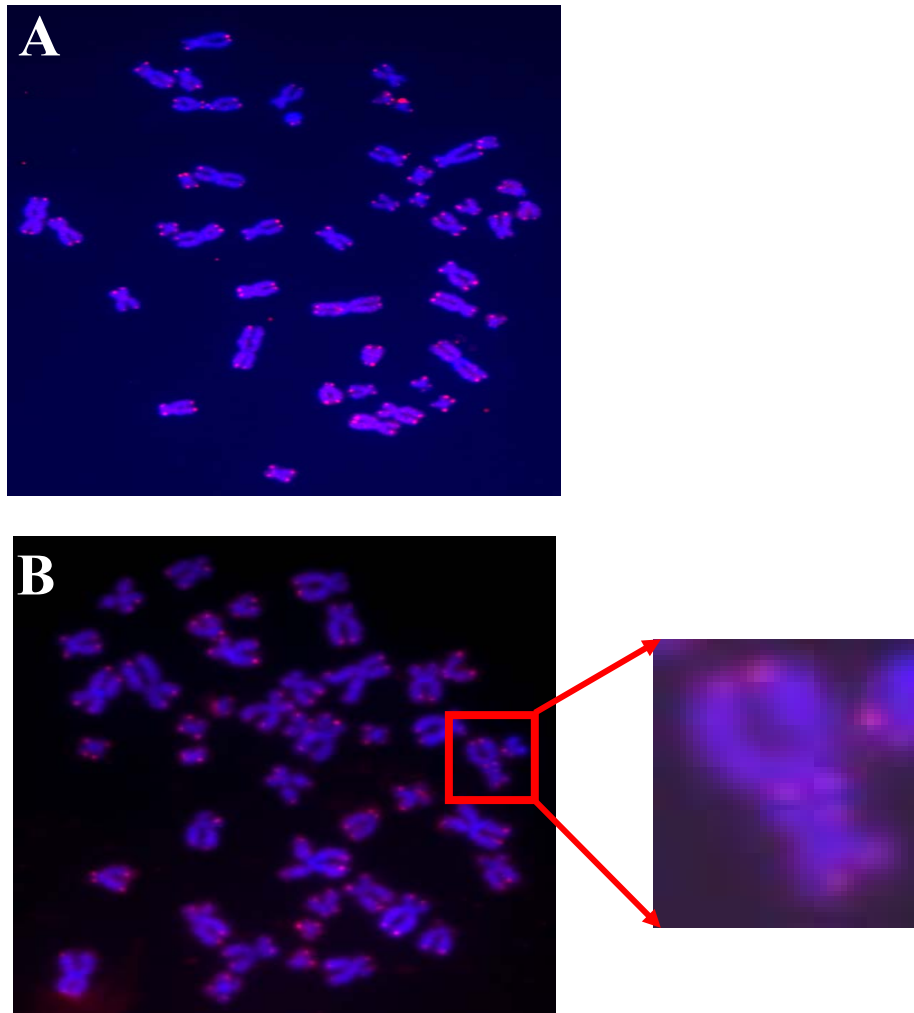
In order to verify if telomere fusions played a role in the translocations/fusions observed by SKY, we performed telomere FISH on metaphases prepared from BL41, LCLs, IARC171 cells, EBV-infected cells at time points 1, 4 and 6 months and mitogen activated B-cells at 1 month time point, to determine the presence of dicentric chromosomes or interstitial telomere signals. Dicentric fusions were observed and also interstitial telomeres were present at low percentages in the different cell types that we analyzed except for the EBV infected *ex vivo* B cells at the 4 and 6 months time points. BL 41, IARC 171 and LCL 910410 also showed low percentages of dicentric and Robertsonian fusions that ranged between 5-10 % per cell line (Table 4. 3), however, no interstitial telomeric signals were observed.

In one month time point of *ex vivo* B- cells, 5% of the metaphases had dicentric fusions but again no interstitial telomeric signals were observed. In four and six months time points the dicentric fusions were 15% and 25% respectively (Figure 4. 5, Table 4. 3) and included interstitial telomeric signals. The data of this set of experiments confirmed the fusions that were observed using SKY in the four and six months time points.

Table 4.3 Summary of telomere-FISH analysis. Telomere fusions/interstitial telomere signals were detected unless otherwise noted in the comments column. Fusions are shown as number of metaphases with fusions over total number of metaphases analyzed per group.

Cell type	Chr Fusions	%
BL41	2/22	9%
IARC 171	4/20	20%
LCL 910410	1/20	5%
B-Cell+EBV 1 month	1/22	5%
B-Cell+EBV 4 month	3/20	15%
B-Cell+EBV 6 month	5/20	25%

Figure 4.5 Telomere-FISH analysis on metaphases. Representative images showing chromosomal fusions and interstitial telomeres in EBV infected *ex vivo* B-cells. **A:** normal metaphase. **B:** image from 4 months time point showing dicentric chromosomal fusion.



4.6 Discussion

4.6.1 Unique telomere distribution for each of BL and LCLs

The analysis of BL, B-cell lymphoma and LCL lines showed the ability of the 3D telomere volume measurements to differentiate between cell lines of normal or tumor origins. Although minor overlaps existed, the discrimination between the two groups was significant except for IARC 171 that was established from a sample donated by the same patient who donated the BL41 cell line [Cohen et al., 1987].

Interestingly, p493 cells (LCLs stably transfected with EBNA2 activation/Myc expression constructs) developed increased percentages of telomere aggregates when proliferation was driven by Myc versus EBNA2. Once Myc takes over the proliferation driving function from EBNA2, several phenotypic changes take place including the separation of the clumping immunoblasts. The cells lose their villi, down-regulate their adhesion molecules, decrease their MHC I expression, lose activation markers and acquire some BL associated markers [Kataoka et al., 1997]. In this work we show a change in the telomeric signature favoring the formation of telomeric aggregates upon Myc upregulation.

The mentioned phenotypic changes are fully reversible by switching EBNA2 „on“ and Myc „off“. However, we show that the change in the telomeric signature was only partially reversible upon switching back proliferation to be EBNA2 driven.

These findings coincide with our data obtained from the analysis of the BL and LCL cell lines where BL cells, constitutively expressing high levels of Myc due to the Myc/Ig translocation, had the majority of telomeres with larger volumes when compared

with LCLs that lack the Myc/Ig translocation and express lower levels of Myc (Figure 4. 2).

4.6.2 Karyotypic instability via telomeric dependent mechanism

The telomere analysis of EBV-infected *ex vivo* B-cells revealed the time frame of two changes in the 3D telomere volume distribution that may contribute to EBV induced genomic instability and cellular transformation. The first change took place after 96 hours and the second was observed after four months. Starting from the four months time point the SKY analysis revealed the presence of large numbers of random non-reciprocal translocations, telomere-free chromosomal ends suggesting that the change in the telomeric distribution may be a cause for the karyotypic abnormalities.

At six months time point we observe that 20% of the analyzed metaphases had non BL T(8;14) translocations (Figure 4. 4 C). At the same time points telomere FISH on metaphases showed that 25% of the analyzed metaphases had dicentric chromosomes/ interstitial telomere signals and chromosomal breakages. The presence of a specific translocation in 20% of the metaphases suggests that this translocation may be selected and propagated through cellular division. Moreover, the fact that the majority of the structural abnormalities are dicentric chromosomes suggests a link between EBV infection, the change of the telomeric distribution and the generation of the dicentric fusions.

These findings agree with the recent published data, by Kamranvar *et al.* (2007), comparing EBV + and EBV – BL [Kamranvar *et al.*, 2007]. In Kamranvar *et al* it was demonstrated that EBV primes B cells for genomic instability. EBV + BL cell lines had

more abnormalities as compared to EBV- BL cell lines. They also showed that EBV+ cell lines have larger number of dicentric fusions as compared with EBV- cell lines [Kamranvar et al., 2007]. In our experiments we show that the telomeric distribution was altered twice after EBV infection and within a specific time frame followed by the generation of high levels of dicentric fusions. This suggests a telomere dependent mechanism that also involves the formation of telomere aggregates.

4.6.3 Types of EBV induced karyotypic instability in BL and LCLs

In our study we observed two groups of genomic instabilities; random and non random. The appearance of a large number of random non-reciprocal translocations starting from four months time point in *ex- vivo* B-cells after EBV infection supports the fact that EBV induces chromosomal fusions followed by chromosomal breakages during cell division. The observed high percentage of dicentric fusions and telomeric aggregates suggest that the chromosomal fusion takes place through telomeric fusions.

Chromosomal breakages from the one hand may lead to translocations, on the other hand may lead to further chromosomal fusions through the exposed telomere ends. EBV infection was reported to induce chromosomal breakage through various mechanisms including the induction of oxidative stress or even as a result of the integration of the viral genome into the host cell genome which creates fragile like sites sensitive to DNA damaging agents [Gualandi et al., 2001; Jox et al., 1997]. In our analysis we also observed numerical aberrations in all cell types that were included in the study (Table 4. 2) namely: BL, LCL and *ex-vivo* B-cells. The aberrations were mostly in the form of trisomies, monosomies or occasional increase in individual chromosomal number (>4). It

was previously reported that numerical aberrations occur in BL cell lines due to mitotic infidelity or centrosome duplication errors [Stavropoulou et al., 2005]. In our studies BL and IARC 171 cell lines both had numerical aberrations in all analyzed metaphases, however, *ex-vivo* B-cells numerical aberrations increased overtime between 1 to 6 months but did not reach the percentages observed in BL41 or IARC 171. Our data suggest that EBV may also contribute to the numerical aberrations observed in BL. However, it is note worthy that those cell lines have been in culture for long time and thus may have accumulated *in vitro* culture-dependent aberrations.

4.6.4 EBV contributes to remodeling of the nuclear structure

It was previously reported that EBV alters the chromosomal segregation in B-cell lymphoma and leads to the generation of micronuclei that contain mainly centromeres suggesting a mechanism that involve the nuclear structure of infected cells [Gualandi et al., 2001]. Here, we showed that the karyotypic instability was observed after the alteration of the telomeric distribution took place. We previously showed that telomeric aggregates lead to the formation of karyotypic instability after Myc deregulation [Caporali et al., 2007; Louis et al., 2005].

4.6.5 Nuclear organization and cancer

We have recently shown that genomic instability is not necessarily coupled to cellular transformation [Fest et al., 2005]. However, genomic instability is associated and often is a result of alteration in the nuclear structure which in turn may prime cells for

transformation. Alterations in the nuclear structure can take place in various forms either genetic or epigenetic, reversible or irreversible and finally spatial or temporal [Cremer and Cremer, 2001; Espino et al., 2005; Misteli, 2004]. Each form of alterations may lead to different forms of instability that may lead to the formation of a colony or may just terminate by the death of the cell that carried the alteration [Mitelman, 2000]. Massive structural modifications usually lead to cell death but small changes that involve the deletion of a tumor suppressor gene or amplification of a region that contains an oncogene may be propagated through cell division and lead to transformation [Rowley, 2000]. In many cases reversible changes lead to irreversible ones that will in turn promote tumorigenesis. For example alteration in chromatin remodeling in a certain region may lead to the overexpression of an oncogene which in turn will lead to cellular transformation [Espino et al., 2005].

In conclusion, our data highlight the difference in the telomeric signature between LCL cells when proliferation is Myc driven or EBNA2 driven. The study provides an effective tool employing 3D imaging and deconvolution techniques to discriminate between cells from normal origin from those from tumor background. We also showed the different cellular stages that take place after EBV infection based on the 3D telomeric signature.

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4.8 References

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5.0 Discussion and Future prospective

The protooncogene Myc is involved in regulating growth and proliferation and its deregulation contributes to the development and progression of multiple human cancers. Deregulated expression of Myc contributes to neoplastic transformation by altering cellular pathways and facilitating the induction of several forms of genomic instability including locus specific gene amplification, structural karyotypic instability, DNA damage, point mutations and several others. Myc-dependent genomic instability is being studied for several decades however the exact mechanisms by which this instability are initiated were not characterized.

The overall objective of this thesis work was to characterize molecular mechanisms by which three of Myc dependent pathways leading to genomic instability take place. Those mechanisms are 1. Myc dependent locus specific gene amplification; 2. Myc induced karyotypic instability and 3. The mechanism by which Myc dependent Burkitt's lymphoma phenotype evolves in EBV positive B-cells and if EBV infection plays a role to promote the development of that cancer.

The three chosen pathways though appear to be divergent but they all share high relevance in cancer promotion and to our hypothesis they may be all be triggered by non transcriptional role(s) for Myc upon its deregulation.

5.1 Myc dependent replication driven gene amplification

In Chapter 2 the objective was to investigate a replication driven mechanism for Myc dependent gene amplification. Myc was shown to be necessary for the endoreduplication cycles that take place during the developmental stages of several

organisms especially *Drosophila* (Pierce, Yost et al. 2004). In this example, Myc contributes to gene amplification specifically at the chorion loci in *Drosophila* oocytes. This role highlights that gene amplifications under certain conditions is legitimate cellular process and is regulated. Furthermore, from the amplification of chorion genes we learn that Myc expression and may be over expression is essential for the process and that this amplification takes place during normal genomic replication in the S-phase of the cell cycle but no molecular mechanism was suggested or characterized.

The observation that Myc dependent gene amplification that contributes to genomic instability and cancer is replication driven was not initially reported in this thesis work. It was reported in Kuschak et al in 2002. However, Kuschak et al described the amplification of one gene in one cell line and showed only one time point. The actual focus of the first objective in the thesis is to investigate and establish the concept that replication driven Myc dependent gene amplification is a common mechanism by which Myc amplifies several target genes in different cell types. That is why several genes were included in the study and two cell lines were considered.

In the experiments described in Chapter 2 we indeed show that Myc induces gene amplifications of target genes through a replication-dependent mechanism. We showed rereplication events taking place at specific loci and leading to the amplification of the *DHFR*, *CCND2* and *R2* loci. The data described allow us to make the argument that this is a common mechanism for Myc dependent gene amplifications and fulfill the first question raised in this objective. Furthermore, the data obtained from analyzing replication event at *CycC* locus in the two cell lines confirms that this process is not random and does not take place at all replicating loci.

The second question that we addressed in Chapter 2 was characterizing how Myc alters the replication event at specific loci. Using fluorescence and molecular techniques we were able to point out that Myc binds specific members of the replication initiation complex and interferes with the dissociation of the complex or rather stabilizing the complex after the origin firing. We also pointed that this takes place by preventing the protein Geminin from binding and escorting Cdt1 out of the nucleus after origin firing.

These data all together establish two concepts: 1. Myc is a replication factor and its role in DNA replication is independent on its function as a transcription factor. Here our data agrees with Dominguez-sola et al 2007 that also reported a role for Myc in DNA replication; 2. The increase in Myc's bioavailability upon its deregulation will allow rereplication at loci that include Myc/Max binding sites (E-boxes) and are in close proximity to replicating DNA origins. At those loci, Myc/Max binding to the E-box sequence will act as an enhancer to stabilize Myc/replication initiation complex and allow the multiple firing that we observed in our experiments.

The experiments described in the chapter 2 were all done in the two cell lines mouse Pre B and rat fibroblasts R1A. Both equipped with Myc activation systems that are sensitive to 4HT or E respectively. One important issue that needs to be addressed is whether the results described in that chapter are due to Myc activation or are cell culture artefacts arising under the influence of adding the activation agent (4HT or E) regardless of Myc's activity.

The mouse Pre B cell line was first reported by Littlewood et al in 1995. The chimera protein that is expressed by this cell line includes Myc and a modified ER that is only sensitive to 4HT and not estrogen. In that report Littlewood et al included several

controls and non of them showed any physiological changes based on the addition of 4HT (Littlewood, Hancock et al. 1995). For the R1A cell line, the construct is sensitive to estrogen and this cell line is cultured and maintained in estrogen free serum/phenol free media to avoid Myc activation. R1C cell line is the parental line for R1A and lacks Myc expression system. R1C cells are karyotypically stable and do not spontaneously develop genomic instability in spite of being maintained continuously in complete media and exposed to. This control cell line proves that the instability observed in R1A cells are due to Myc activation and not culture conditions.

5.1.1 Future directions

In the experiments described in Chapter 2 we showed the initiation of the newly synthesized amplicons; however we did not characterize how those amplicons will be processed and integrated chromosomally or form extra-chromosomal elements (double-minute chromosomes). The first detectable gene amplification is observed 72 hours after Myc deregulation. This fact highlights that there are other steps that take place over several cell divisions possibly involving recombination and non homologous end joining. Characterizing the molecular mechanism by which the synthesized amplicons being processed is an important future development for this project and will help developing intervention methods to control the integration of amplified genes in the host cell genome.

Our data and the data described in Dominguez-Sola et al characterizing Myc's interaction with the replication complex were obtained from immunoprecipitation experiments followed by Western blot analysis. Any data obtained from this type of analysis just prove that the two proteins being studied are in complex together but do not

prove that they are in direct interaction with each other or rule out the presence of other proteins that may be playing roles in this process. Further characterization for individual protein-protein interaction using advanced techniques like fluorescence resonance energy transfer (FRET) will provide concrete evidence on the exact sequence of events.

Finally our data not only characterize a replication driven mechanism for gene amplification upon Myc activation but the data also points out a possible role for Myc in DNA replication in normal mammalian cells. To date this role for Myc as a replication factor is hypothetical and need to be further investigated. In both our experiments and in Dominguez-Sola et al experiments only cell lines were used. Experiments using normal primary cells and *in vivo* studies are needed before Myc is confirmed as a replication factor in mammalian cells. One useful approach is to screen genes that are not Myc transcription targets but meanwhile have upstream the E-box element and examine if Myc at its normal physiological level is associated with the initiation complex during the replication of those loci.

5.2 Myc dependent karyotypic instability

The second objective of the thesis which is described in Chapter 3 characterizes a novel mechanism by which Myc induces chromosomal instability through remodelling of the interphase nucleus. Our first question was to clarify if the formation of telomeric aggregates is Myc dependent. Our data show not only that Myc deregulation induced cycles of telomeric aggregates but the number of cycles was also dependent on Myc's availability. The formation of TAs appeared as early as 12 hours after Myc deregulation

and preceded the formation of chromosomal abnormalities suggesting that TAs control or rather orchestrate the induction of the chromosomal instability that are usually observed in later time points after Myc deregulation.

Furthermore, the SKY analysis revealed high frequency of end to end fusions at the respective time points when the TAs were observed suggesting that TAs may be in fact telomeric fusions marking the whole end to end chromosomal fusions detected by SKY.

In the three dimensional interphase nucleus those fusions may bring specific pairs of chromosomes in closer proximity and allow recombination and the initiation of balanced translocations. Several cancers are driven each by a specific translocation. Here we suggest a novel mechanism by which those translocations may be taking place. We analyzed the positions of three pairs of chromosomes each with high frequency of balanced translocations. We showed alterations in the 3D positions of those pairs of chromosomes upon Myc deregulation. Further analyses characterizing specific cancer-causing translocations are needed to confirm this hypothesis.

The experiments described in this chapter were performed in the mouse Pre B cells used in chapter 2. As additional control we included the mouse plasmacytoma cell line MOPC 460D in which cells spontaneously express high levels of Myc without adding any reagents like 4HT. The results obtained confirm that the formation of TAs is due to Myc activation and not the effect of 4HT on the cells in culture. Later study published by our group using a mutant Myc activated by 4HT confirmed that only wild type Myc can induce TAs.

5.2.1 Future directions

These findings suggest the presence of a tight molecular pathway by which those aggregates are formed, and that has yet to be characterized. One can speculate that Myc is involved in the regulation of telomeres capping by interacting with one or more member(s) of the telomeres protection complex shelterin, preventing the complex from binding to the telomeres after replication and allowing unprotected telomeres to aggregate and fuse during G₂ phase. Alternatively, Myc binding to any member of the shelterin complex may prevent the formation of the T-loop structure allowing telomeres from different chromosomes to join.

Another process that needs to be further investigated is how Myc induces or affects the motility of chromosomes in the interphase nucleus. The data from our chromosome painting experiments show an alteration in the chromosomal positions upon Myc activation. It is known that Myc interacts with the nuclear skeleton proteins which control the positions and motility of chromosomes in the interphase nucleus especially the High mobility group protein I(Y) (HMGI(Y)) that is Myc dependent. It is possible that Myc dynamic association and dissociation with such proteins and complexes induce the observed alteration in the chromosomal motility. However, this pathway is hypothetical and need to be elucidated in the future.

Another future direction is to find which domain in Myc structure is responsible for each of the proposed pathways. It was recently published that mutant Myc lacking MBII does not induce TAs but still able to induce genomic instability (Caporali et al 2007, Fest et al 2005). Further mutation analyses are required to characterize the non-transcription role of each of Myc domains.

5.3 Myc's role in the development of Burkitt's lymphoma in EBV positive B-cells

In Chapter 4 our objective was to shed light on the mechanism by which Burkitt's lymphoma phenotype evolves in EBV positive B-cells and if EBV infection provides any survival advantage to Burkitt's lymphoma cells.

Our first set of experiments described in this chapter categorizing transformed and non transformed B-cell lines based on their telomeres distribution was successful. This assay was initially developed based on our findings that TAs are formed earlier before the chromosomal abnormalities are detectable but marks the onset of transformation. Our results analyzing 9 B-cell lines marked a non transformed line as transformed in spite that this line did not have BL translocation but was from a donor that developed BL.

Another question that we addressed in this chapter was to compare the remodeling of nuclear structure and the formation of telomeric aggregates and karyotypic instability when proliferation is driven by Myc or EBNA2 the EBV transcription factor. The results obtained showed that proliferation driven by EBNA2 can rescue the telomeric aggregates phenotype induced by Myc. These results supports and provide a mechanism for recent reports suggesting a survival advantage of EBV positive BL as compared to EBV negative BL.

In the last set of experiments described in Chapter 4 we monitored the telomeric distribution in freshly generated lymphoblastoids over seven month. We were investigating if the EBV infection will induce telomeric dependent genomic instability. Starting at three month time point we observed EBV infection significantly increased the frequency of formation of chromosomal translocation most interesting between

chromosomes 8 and 14 that carry the *myc* and *Igh* genes in human cells respectively. Although the t(8:14) translocations that we observed did not match the typical Myc/Igh translocation that characterizes BL phenotype, yet it proves that EBV infection at least increases the chance of translocations between those two chromosomes.

5.3.1 Future directions

Our findings agree with recent data suggesting high frequency of translocation between chromosomes that lies in close proximity in the special organization of the nucleus. Our data are consequently suggestive of the likelihood for EBV infected B-cells to accumulate the Myc/IgH translocation once the translocation is colonized and selected. However, further analysis including large number of lymphoblastoids freshly generated from different donors is required before this conclusion is validated.

5.4 Summary

One major focus of health research is to translate and evolve our knowledge about diseases from the level of observation to the level of characterization. Understanding the mechanism by which a disease develops is the only way to design effective diagnostic, treatment and even avoidance measures against that disease. Unfortunately our level of knowledge for several complex diseases is still at the level of observation. Extensive research is taking place all over the world to improve our knowledge to the level of characterization about those complex diseases including the projects described in this thesis.

Our work focused on understanding the sequence of events that take place in mammalian cells upon Myc deregulation. We used several mammalian cell systems including immortalized mouse lymphocytes, immortalized rat fibroblasts, human B-cell lines and human primary B-cells. The mechanisms that were investigated were diverse but all contributes to our understanding of the mechanisms of Myc dependent genomic instability which leads to cancer deposition. The future direction for our findings is to design effective tests for early diagnosis of several cancers.

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