HELLENIC REPUBLIC
National and Kapodistrian University of Athens

Cellular and molecular effects of CDC6-induced senescence in Human Bronchial Epithelial Cells

Foteini Karousi

Biologist

MSc in Molecular Biomedicine

Medical School of Athens

Supervisor: Professor Vassilis *G.* Gorgoulis

"ALEXANDER FLEMING"
Biomedical Sciences Research Center

ATHENS 2018

HELLENIC REPUBLIC
National and Kapodistrian University of Athens

Cellular and molecular effects of CDC6-induced senescence in Human Bronchial Epithelial Cells

Foteini Karousi

Biologist

MSc in Molecular Biomedicine

Medical School of Athens

Supervisor: Professor Vassilis *G.* Gorgoulis

"ALEXANDER FLEMING"
Biomedical Sciences Research Center

ATHENS 2018

Three-member Advisory and Examination Committee:

1. Prof. Vassilis G. GORGOULIS, MD, Head of the Department of Histology-Embryology, Medical School of Athens (supervisor).

2. Assistant Prof. Athanassios KOTSINAS, PhD, Department of Histology-Embryology, Medical School of Athens.

3. Assistant Prof. Sofia HAVAKI, PhD, Department of Histology- Embryology, Medical School of Athens.

Place of research

The presented study was collaboration between the National and Kapodistrian University of Athens, Greece and the Biomedical Sciences Research Center "Alexander Fleming" (BSRC Alexander Fleming), Greece.

A large part of the research took place in Dr. Fousteri's laboratory, Division of Molecular Biology and Genetics at the Biomedical Sciences Research Center "Alexander Fleming" (BSRC Alexander Fleming), Greece.

.

ACKNOWLEDGEMENTS

I would first like to thank my thesis advisor Professor Vassilis G.Gorgoulis of the Medical School of the National and Kapodistrian University of Athens. The door to Prof. Gorgoulis office was always open whenever I ran into a trouble spot or had a question about my research or writing.

I would also like to thank the experts who were involved in my three-member advisory and examination committee Dr. Sofia Havaki and especially Dr. Athanassios Kotsinas who was always really helpful and was there every time I asked for.

In addition I would like to thank Dr. Maria Fousteri who gave me the opportunity and trusted me to work in her laboratory. I also would like to thank Dr. Mat Lavigne for his guidance and support throughout my MSc research project.

Moreover, this thesis would not have been possible without the valuable help of Christos Zampetidis, Eirini Komseli, Ioanna Mourkioti and Sophia Rizou from Gorgoulis Lab. Furthermore, I would like to thank Tasos Liakos, Dimitris Konstantopoulos, Smaragda Fanourgakis and George Stefos and all the members of Fousteri lab. I am grateful to them for their help at several moments during this project.

Finally, I must express my very profound gratitude to my parents and to my friends for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them.

Thank you.

Fotini Karousi

Contents

ABSTRACT

The human body is a complex and wondrous living machine. Like any machine, our bodies are composed by smaller parts that work together – the organs. Organs consist of million cells that cooperate with each other to perform a specialized task. Cancer is the result of a long time process that typically happens when the cells of the human body present 'an antisocial behavior' and cannot work with other cells in harmony. Recently, it has been revealed that a type of cellular senescence, the Oncogene-Induced Senescence (OIS), may act as an anti-tumor barrier.

Cell division cycle 6 (CDC6) is an essential protein for cell's fate as it acts as replication licensing factor and prevents the cell from re-replication and genomic instability. Its over-expression has been associated with aberrant DNA replication and its deregulation has been linked with several types of cancer. Recent data have revealed a new oncogenic role of CDC6 and its newly identified participation in transcription regulation.

In this thesis, an epithelial cellular model in which over-expression of CDC6 was achieved in an inducible way through a doxycycline-inducible promoter is studied. Immortalized human bronchial epithelial cells (HBECs) (hTERT/CDK4) are used for that purpose. Most of the cancers are of epithelial origin and the above cell system simulates the whole spectrum of epithelial cancer development from the non-malignant stage to complete transformation of the normal cells into a mesenchymal - cancerous state. Importantly, in this thesis the part of carcinogenesis that we focus on is the cellular and molecular mechanisms controlling anti-tumor barrier-senescence.

The aim of this project was to study the role of CDC6 in cancer initiation and development. To facilitate our study, we examined how HBEC cells can be synchronized in the cell cycle. We confirmed by phase contrast microscopy and Flow Cytometry that simple deprivation of supplements was sufficient to reversibly block a large majority of the treated cells in G0/G1 phase. These experimental conditions thus enabled us to define a framework for addressing the second aim of this study: to monitor accurately the changes driven by CDC6 induction and the consequences on cell cycle dynamics and gene expression changes. We discovered a premature and accelerated entry of the cells in S phase. In addition, cells overexpressing CDC6 showed difficulties to carry on normal S phase and we suggest that replication may be blocked before the completion of the S phase. A third aim consisted in identifying the functional consequences of CDC6 induction at the chromatin structure and gene expression levels. In particular, we performed Chip-seq experiments to gain insights in the changes observed in the genome-wide binding of CTCF between HBEC OFF-control and HBEC cells that expressed CDC6 for 3 days (HBEC 3d Tet-ON). In HBEC 3d Tet-ON cells, we discover that the binding of CTCF on TSS is decreased at a subset of Transcription start sites (TSS). We propose that such structural changes may affect the gene expression program of the CDC6 overexpressing cells. To validate this hypothesis, we analyzed ATAC-seq data that were generated in the lab in

order to understand changes in chromatin accessibility upon CDC6 induction and we monitored the number and localization of Differentially Accessible Regions (DAR) as well as their correlated effect on gene expression.

1. INTRODUCTION
1.1: CHROMATIN STRUCTURE

The development of higher organisms requires the distinct specification of various cellular types. Although cells have exactly the identical genetic material in their nuclei, different cell types present different gene expression profiles (Margueron *et al.,* 2010). DNA is found as part of a nucleoprotein complex named chromatin.

Chromatin consists of DNA, RNA, histones and non-histones. More specifically, 147 bp of DNA are wrapped around 2*4 highly conserved core histones - two copies each of the four histones H2A, H2B, H3 and H4 - in order to compose the basic unit of chromatin, the nucleosome. Linker DNA is located between the nucleosomes. The role of chromatin is multiple: i) to pack DNA into a smaller volume in order to fit in the nucleus of the cell and ii) to protect its structure and sequence (Richmond *et al.,* 1984; Luger *et al.,* 1997; White *et al.,* 2001; Hayes *et al.,* 2001; Cutter *et al.,* 2015), and iii) to compartmentalize and regulate gene expression (Hübner *et al.,* 2013) as well as replication and DNA repair. There are two forms of chromatin: euchromatin and heterochromatin. The first one refers to chromosomal regions that contain transcriptional active or potentially active genes. The chromatin is decondensed and the genome regions are accessible to nucleases. Euchromatin is characterized by H3 and H4 hyperacetylation on their N-terminal lysine residues. On the other hand, heterochromatin refers to genome regions that stay highly condensed during the cell cycle. Heterochromatin contains inactive genes and lot of repetitive sequences. Unlike euchromatin, it is inaccessible to nucleases and it is characterized by histones hypoacetylation (Kosak *et al.,* 2004; Arney *et al.,* 2004; Quina *et al.,* 2006; Margueron *et al.,* 2010). ATAC-seq is the most optimal and widely accepted method for detecting accessible regions of chromatin (Buenrostro *et al.,* 2013; Tsompana *et al.,* 2014).

The modulation of the chromatin structure is of pivotal importance for the cell, as it regulates the chromatin accessibility and defines the position that the regulatory factors will be placed on the DNA. Frequently, the structure of the chromatin is found changed in its constituents because of different transcriptional conditions such as the presence of activators, repressors, chromatin remodelling complexes and histone modifications on its residues (Quina *et al.,* 2006). The amino-terminal ‘tail’ domain of each histone that protrudes from the nucleosome is prone to post transcriptional modifications (PTMs) such as phosphorylation, methylation, acetylation and monoubiquitylation (Fletcher *et al.,* 1996; Luger *et al.,* 1997). In addition to the above, it has been reported that specific PTMs are connected with specific cellular functions and are playing a role in gene regulation (Campos *et al.,* 2009).

Acetylation of histone tails facilitates the transcription machinery to access the promoters and hence initiates transcription (Studitsky *et al.,* 1997). The amino-terminal domains of the H3 and H4 histones are two of the most highly conserved sequences in eukaryotes and are subjected to acetylation. It has been reported that

acetyltransferases (HATs) are responsible for the acetylation of the histones N-termini and the activation of gene expression whereas histone deacetylases (HDACs) can reverse the modification (Sterner *et al.,* 2000; Chen *et al.,* 2001; Khochbin *et al.,* 2001). Thus, the cooperation and balance between HATs and HDACs is really important for gene expression output and plays a key role in alterations in chromatin structure and between transcription activity states. Their deregulation often leads to cancers and other human disorders (Timmermann *et al.,* 2001; Eberharter *et al.,* 2002). In particular, acetylation of the 27th lysine residue of H3 histone (H3K27ac) can be used as a marker for active enhancers and promoters (Wang *et al.,* 2009; Heintzman *et al.,* 2009; Creyghton *et al.,* 2010; Zhang *et al.,* 2013).

An upper level of organization of chromatin beyond the nucleosomes involves large-scale chromosomal regions that constitute specific territories in the nuclei of the cells (Van Bortle *et al.,* 2012). Within these territories chromatin alternates between heterochromatin and euchromatin in a way that is connected with transcription activity. Chromatin Conformation Analysis (3C) was used to study the 3D chromatin structure which has been considered as an important transcription regulator, and to capture physical interactions between chromatin segments (Crutchley *et al.,* 2010; De Laat *et al.,* 2012). Chromatin configuration is found to have an effect on the folding of genome due to long-range interactions- distantly genomic regions on the same or different chromosomes interact with each other. Long- range interactions have been revealed to affect the transcription. More specifically, physical interactions between regulatory DNA elements and gene-targets control the non-basal transcription (Vakoc *et al.,* 2005; Spilianakis *et al.,* 2005; Kagey *et al.,* 2010; Ferraiuolo *et al.,* 2012; Dekker *et al.,* 2015). The non-histone binding proteins CCCTC-binding factor (CTCF) and cohesin that play a key role in genome organization and gene expression profiles are considered to guide long-range interactions (Phillips *et al*., 2009; Hadjur *et al.,* 2009; Schmidt *et al.,* 2010; Merkenschlager, 2010).

CTCF is an eleven zinc finger DNA-binding protein which is highly conserved among higher species. Since the 3D structure of DNA influences the regulation of genes, CTCF's activity has an impact on the expression of genes. It acts as a multifunctional protein as it is considered to be not only a transcription factor, but also an insulator and a transcription repressor or activator (Lobanenkov *et al.,* 1990; Klenova *et al.,* 1993; Filippova *et al.,* 1996; Ohlsson *et al.,* 2001). There are several thousands of binding sites CTCF across the genome, often proximal to transcription start sites (TSS) (Kim *et al.,* 2007). Moreover, it was one of the first proteins that were found to change the chromatin structure, creating chromatin loops (Topologically Associated Domains –TADs) between its binding sites (Splinter *et al.,* 2006; Handoko *et al.,* 2011; Dixon *et al.,* 2012; Nora *et al.,* 2012). Within TADs enhancer-gene interactions are mediated (Dixon *et al.,* 2012, 2015). More specifically, TADs are defined by two CTCF molecules and this structure facilitates the interaction of enhancers and genes within the loop compared to the enhancers and genes outside the loop (Kim *et al.,* 2007; Barski *et al.,* 2007; Holwerda *et al.,* 2013; Hnisz *et al.,*

2016). The above structure which is connected to a cohesin complex and contains at least one gene has been defined as a ″insulated neighborhood″ (Dowen *et al.*, 2014; Ji *et al.*, 2016; Hnisz *et al.*, 2016) (see figure 8). This insulating function is pivotal for normal gene activation and repression (Hnisz *et al.*, 2016). In addition to the above, alterations on the CTCF binding sites in the chromatin loops have been observed in cancer cells (Ji *et al.*, 2016). Last but not least, replication of the genome starts within the TADs (Pope *et al.*, 2014).

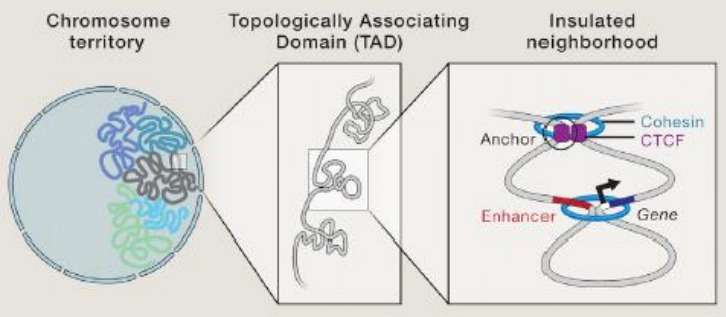


Figure 1.1: Organization of chromatin structure (figure from Hnisz *et al.*, 2016). Chromatin is organized in large-scale chromosomal regions that constitute specific territories in the nuclei of the cells. Topologically associating domains (TADs) consist the next level of chromatin organization. The insulated neighborhoods are the basic of TADs. Anchor refers to the binding of the cohesion ring with two CTCF molecules in order a insulated neighborhood to be formed.

1.2: REPLICATION

The procedure of DNA replication that occurs in every cell cycle has always been thought as a topic of 'basic research'. On the other hand, defects in genome replication which can be amplified and accumulated over the cell cycles, have been shown to be linked with a variety of human diseases, including many types of cancer and possibly underlie the process of ageing (DePamphilis, 2006; Borlado *et al.*, 2007; Fragkos *et al.*, 2015).

For that reason, it is extremely important for the process of DNA duplication to be kept regulated precisely. Importantly, the whole replication process needs to be strictly accurate in order to inhibit the generation and the transfer of unexpected mutations to daughter cells. For that reason, the coordinated action of several proteins and enzymes, acting either separately or in complexes is required. Consequently, cells have developed a so called "replication machinery", which is able to respond to a variety of signals and functions to preserve genome stability and to ensure DNA replication fidelity at replication forks (Denhardt, 1999; Bell *et al.*, 2002; Sancar *et al.*, 2004; Masai *et al.*, 2005).

DNA replication starts at specific genomic regions called replication origins. Replication origins are dispersed among tens of thousands of loci across the genome of eukaryotic cells. The replication region is initially recognized by specific proteins that form the origin recognition complex (ORC) which binds the DNA and recruits other protein factors to establish the pre- replication complex (pre-RC) (DePamphilis,

2003; Kawakami *et al.,* 2010; Chang *et al.,* 2011) . Initiation of Replication at origin sites in eukaryotic cells are determined by two steps: the recognition of the pre‑RC site,· a process known as replication origin 'licensing' which is restricted to the G1 phase of cell cycle, and the activation of DNA synthesis which is called origin 'firing' during the S phase. This two-step mechanism is essential for the inhibition of re‑replication within the same cell cycle and the prevention of genome instability (Abbas *et al.,* 2013; Fragkos *et al.,* 2015; Prioleau *et al.,* 2016). In addition to the above, it is important to note that the subset of the potential origins which are activated varies from cell to cell (Fragkos *et al.,* 2015).

More specifically, the process of 'licensing' of replication starts when the replication origins are first identified by the conserved 6-subunits (ORC1-6) origin recognition complex (ORC). ORC is bound to DNA during the whole cell cycle and at the late M/early G1 phase, recruits the Cell division cycle 6 (CDC6) protein which recruits the CDC10-dependent transcript 1 (also known as DNA replication factor CDT1). Additionally, the recruitment of the mini-chromosome maintenance (MCM) helicase complex-MCM2–7- is the last step of the what is called licensing process (Diffley *et al.,* 1994; Speck *et al.,* 2005; Chen *et al.,* 2007; Evrin *et al.,* 2009). Actually, within the nucleus of a eukaryotic cell, Cdt1 associates with the MCM2-7 complex and recruits it to the ORC-Cdc6-DNA complex via an interaction with Orc6 protein (Tanaka *et al.,* 2002; Evrin *et al.,* 2009). Although 2 MCM2-7 hexamers are required for the initiation and progression of replication in bidirectional replication forks, more hexamers are loaded onto each replication origin in order to protect the cells in case of replicative stress (Laskey *et al.,* 2003; Ge *et al.,* 2007).It is worth mentioning that Cdc6 ATP hydrolysis is required for MCM2-7 loading while Orc1 ATP hydrolysis facilitates the release of the MCM2-7 complex from ORC, enabling the process of pre-RC formation and DNA licensing to be completed (Bowers *et al.,* 2004; Randell *et al.,* 2006). The MCM2-7 complex remains inactive until the S phase (Evrin *et al.,* 2009) and is needed for the unwinding of DNA (Masai *et al.,* 2010). Once the replication origins are licensed, cells must prevent re‑licensing during the S phase to ensure that the whole genome is replicated only once per cell cycle. This checkpoint is achieved through i) the interaction of Cdt1 with its inhibitor· Geminin or ii) via Cdt1 ubiquitylation and degradation during the S phase, which is accompanied by the phosphorylation of several initiation factors (Blow *et al.,* 2008; Siddiqui *et al.,* 2013).

Origin activation requires the formation of a pre-initiation complex (pre-IC) and activation of the MCM helicase complex and takes place at the G1–S phase transition. Compared to the pre-RC assembly at origins, that requires no cyclin-dependent kinase (CDK) activity, origin activation requires high levels of CDKs (Takayama *et al.,* 2003). Assembly of the pre-IC is triggered by DBF4-dependent kinase (DDK) and cyclin-dependent kinases (CDKs) (which are Ser/Thr protein kinases) at the G1–S phase transition, and its transformation into a functional replisome occurs in the S phase (Fragkos *et al.,* 2015). DDK and CDKs phosphorylate the replication factors

MCM10, CDC45, ATP-dependent DNA helicase Q4 (RECQL4), treslin, GINS, DNA topoisomerase 2-binding protein 1 (TOPBP1) and DNA polymerase ε (Pol ε) to promote their recruitment onto origins. In addition to the above, DDK and CDKs phosphorylate specific residues within the MCM2–7 complex, leading to helicase activation and DNA unwinding. Helicase activation induces the loading of other proteins such as replication factor C (RFC), proliferating cell nuclear antigen (PCNA), replication protein A (RPA) and other DNA polymerases that transform the pre-IC into two functional replication forks that proceed in opposite directions from the activated origin, with the replisome at each fork. The functional helicase at the forks is considered to be the CMG complex which is composed of CDC45, the MCM hexamer and the GINS complex (Kang *et al.,* 2014; Heller *et al.,* 2011; Tanaka *et al.,* 2007; Masumoto *et al.,* 2002; Zegerman *et al.,* 2007; Ilves *et al.,* 2010; Kumagai *et al.,* 2010; Kumagai *et al.,* 2011; Thu *et al.,* 2013; Im *et al.,* 2009). The above mentioned CMG complex is activated by the MCM10 and then DNA polymerase α-primase (Pol α) primes DNA synthesis via the DNA polymerases Pol δ and Pol ε (Kunkel *et al.,* 2008). According to the prevailing view, Pol ε synthesizes from the leading strand while Pol δ synthesizes from the lagging strand (Stillman, 2015). The lagging strand can be synthesized discontinuously in the form of short Okazaki fragments, while the leading strand is polymerized continuously. Okazaki fragments are joined together by DNA ligase (Devbhandari *et al.,* 2017). Eukaryotic genome replication terminates when two opposing forks coming from adjacent replication origins meet together, leading to the ubiquitin-dependent removal of the CMG from chromatin (Bell et al., 2016). Noteworthy, in each replication unit only 33% of the origins are activated, implying that 66% remain inactivated, although they have been licensed. Therefore, a replisome is only formed in the activated origin. Moreover, in different cell types or even in the same cell population, different origins can be used in individual cells. In other words, a cell population includes a range of flexible origins. Inhibition of nearby origins within a replication unit is under the regulation of the checkpoint kinases ATR and ATM that activate checkpoint kinase 1 (CHK1) and 2 (CHK2) (Fragkos *et al.,* 2015).

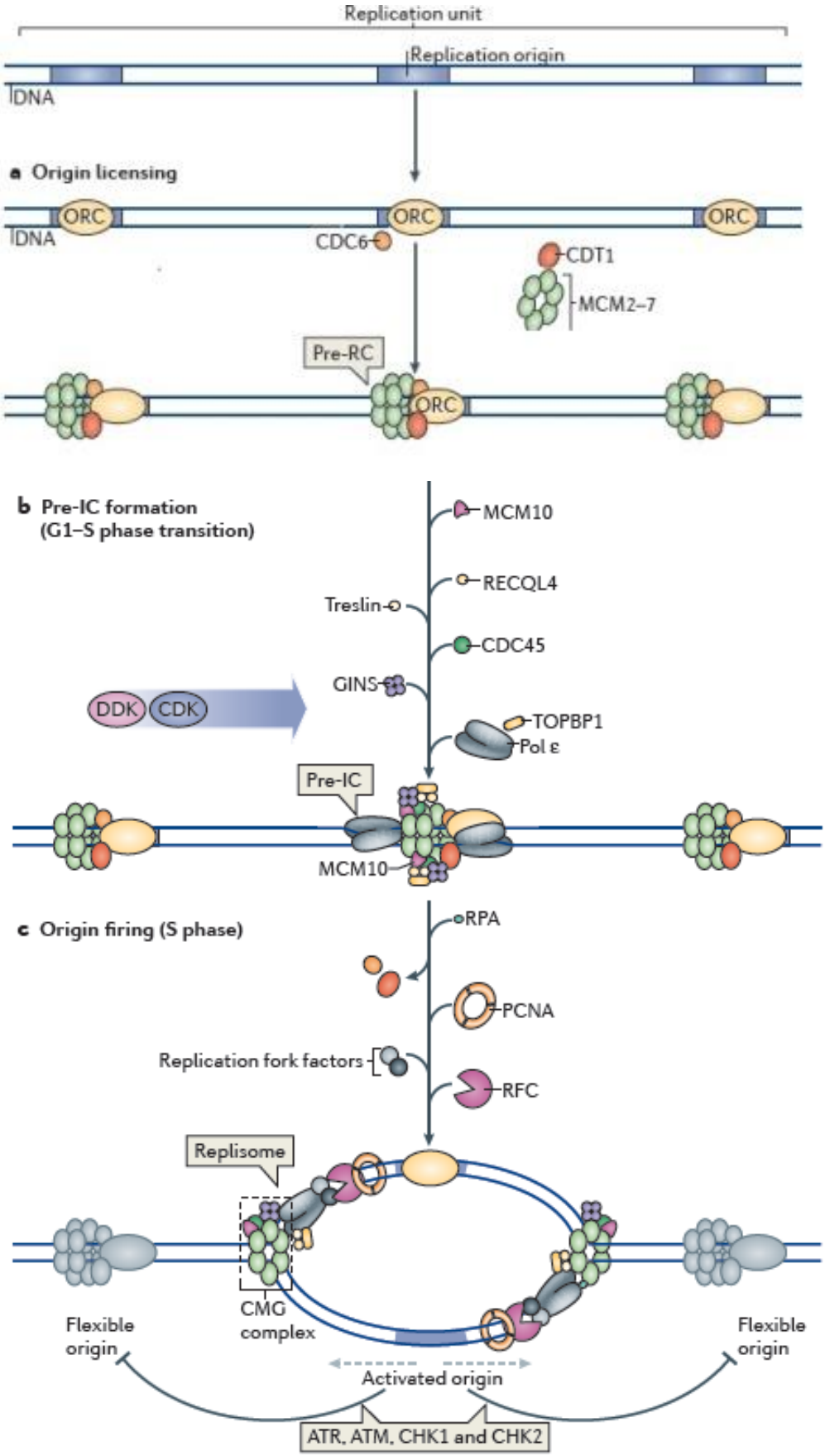


Figure 2.2: Licensing and activation of replication origins (modified figure from Fragkos *et al.*, 2015). a)Licensing of replication origins is restricted to the late M/early G1 phase of the cell cycle and results from the sequential loading of pre-replication complex (pre-RC) proteins on all potential origins in the genome.

b,c)Activation of replication origins requires the recruitment of the pre-initiation complex (pre-IC) onto the DNA and the activation of the MCM complex is essential for the unwinding of DNA.

1.3: CELL CYCLE

Cell division is required for an organism to be developed, to mature and to hold tissues. The cell cycle of a eukaryotic cell is a complicated procedure that drives the cell through a specific sequence of events in mitosis and the generation of two cells identical to the initial-parental one (daughter cells). The cell cycle can be subdivided in two phases: into interphase which consists of G1, S and G2 phases and into M (mitotic) phase which include prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. G1 and G2 symbolizes the time spent by a cell between the two landmarks· DNA synthesis and mitosis (Schafer, 1998; Vermeulen *et al.,* 2003).

G1 phase is especially important for the cell fate. If the external conditions and extracellular signals from other cells are suitable, the cell is actively transcribed and prepares its self for DNA replication which occurs in the S phase. DNA synthesis is followed by G2 where the integrity of DNA is checked and the cell is prepared for mitosis. Although the traditional phases of cell cycle are the above mentioned ones, there are cells that enter in a subtype of G1 phase, the phase G0 in which they arrest their cycle. G0 phase is a resting state where the cells do not grow in size and do not proliferate and they can remain for days to weeks, or even years before turning again into proliferation depending on the cell type and on the signals the cells accept from their microenvironment (Schafer, 1998; Alberts *et al.,* 2002).

During the mitotic phase (M phase), nuclear division (mitosis) is followed by cell division (cytokinesis) generating two daughter cells with the same genetic components as the parent cell. After the split, the two new cells enter G1 stage of interphase and are ready to begin their growth (Schafer, 1998; Alberts *et al.,* 2002; Vermeulen *et al.,* 2003). It is essential for the cells to replicate their DNA with maximum fidelity, which guarantees the maintenance of genomic stability. This is why the cells have developed several control mechanisms ensuring that each DNA segment is replicated completely and only once *per* cell cycle (De Pamphilis, 2006)

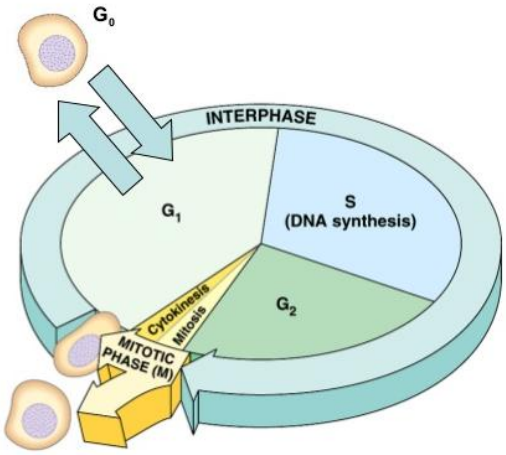


Figure1.3: The cell cycle of a typical eukaryotic cell (figure from Fletcher, 2014). Actively dividing eukaryote cells pass through a series of events known collectively as the cell cycle which is divided in two phases: the interphase and the mitotic phase (M). The interphase consists of the phase G1, the S phase in which the genetic material is duplicated and the G2 phase. The M phase is the last stage of the cell cycle in which mitosis partitions the duplicated genetic material as the cell divides (cytokinesis). In G0 phase· a subtype of G1 phase, the cells are not actively cycling anymore. They remain in G0 for days, weeks, or even years before resuming proliferation.

1.4: CELL CYCLE REGULATION

All multicellular living organisms are products of repeated rounds of cell growth and division. This is why the genomic material of each cell must be replicated precisely only once per cell cycle. The cell cycle is strictly regulated and the eukaryotic cell has developed a network of regulatory factors, the cell cycle control system, in order to be ensured for the replication fidelity of the chromosomal DNA with no sections left unreplicated, and no sections re-replicated (Bruce *et al.*, 2007). In most organisms there is a relationship of interdependence between the events of the cell cycle. Entry into one phase can only occur if the previous stage has been totally completed (Hartwell *et al.*, 1989). In other words, the correct sequence of the events is a robust characteristic of the cell cycle.

Control of the cell cycle is necessary and many disorder leads to deregulation of the cellular function as for instance in cancer. Checkpoints of the cell cycle usually are established at the transition between the cellular phases. By definition, 'cell cycle checkpoints are surveillance mechanisms that monitor the order, integrity, and fidelity of the major events of the cell cycle' (Barnum *et al.*, 2014). Three checkpoints have been identified· at the G1/S boundary, at the G2/M transition and at the metaphase/anaphase boundary of the M phase. It has been shown that the central molecular machines that control the progression of the cell cycle are the cyclin/cyclin-dependent kinases (cyclin/CDKs) (Tannoch *et al.*, 2002).

Cyclins are a family of several proteins·including cyclins A(1,2) , B(1,2,3), C, D(1,2,3), E(1,2) and F. They have been identified in higher organisms and are named cyclins because their concentrations are found to differ in each phase of the cell cycle. In addition to the above, they interact with the CDKs as complexes and activate them (Morgan *et al.*, 1997; Satyanarayana *et al.*, 2009; Lim *et al.*, 2013). Importantly, the

existence of a cyclin box in cyclins facilitates the binding with the CDKs (Gopinathan *et al.*, 2011).Upon dimerization, cyclins control the kinase activity of CDKs (Morgan *et al.*, 1997).

CDKs are a family of sugar kinases with evolutionary conserved regulatory function in the cell cycle. Each CDK consist of an ATP-binding domain, a cyclin-binding domain known as a PSTAIRE helix, and a T-loop domain (Pavletich *et al,* 1999; Lim *et al.*, 2013). Those motifs contribute in the activation of the CDK by the cyclins via the PSTAIRE-like cyclin-binding domain. When the cyclin binds to the PSTAIRE helix, a complex between CDK-cyclin is formed and the CDK is activated and ready to phosphorylate other substrates with regulatory action. Phosphorylation of those factors leads to the exit of the cell from the ongoing phase and to the transition into the next phase of the cell cycle. Furthermore, it has been reported that in different phases of the cell cycle, different complexes of CDKs-cyclins are activated (Nigg *et al.;* 1995; Lim *et al.*, 2013). Low activity of CDKs at the end of M phase and high activity at the late G1/early S phase ensures that the replication origins are licensed and activated only once per cell cycle (De Pamphilis 2006). Importantly, changes in the function of the above complexes may lead to cell cycle arrest or cell apoptosis or even to carcinogenesis. It is worth mentioning that in most cases of human cancer, mutations in the genes that encode the CDKs, their regulators and their substrates have been identified (Johansson *et al.*, 2008).

Importantly, CDKs activity can be modulated via the interaction with Cdk inhibitors molecules (CKIs)(ref), which block their activity, negatively controlling cell cycle progression. Studies conducted in metazoans have identified two categories of CKIs based on their structural homology, amino acid similarity and CDK specificity:· the INK4 and the CIP/KIP family (Hunter *et al.*, 1994; Quesenberry, 1998). The INK4 family which includes the $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ και $p19^{INK4d}$ inhibitors are specific for the CDK4 and CDK6 and block their connection with the cyclin-D. Differently, the CIP/KIP family which includes the $p21^{Cip1/Waf1/Sdi1}$, $p27^{Kip1}$ and $p57^{Kip2}$ inhibitors has the same region of homology that facilitates their binding with the CDKs. In addition to the above, it has been reported that the members of the CIP/KIP family interact not only with the CDKs but also with the cyclins and interfere with the activities of cyclin D-, E-, A- and B-dependent kinase complexes (Polyak *et al.*, 1994; Lee *et al.*, 1995; Sherr *et al.*, 1999; Besson *et al.*, 2008).

To sum-up, Cdks are considered as the engine that drives cell cycle progression whereas cyclins are perceived to be the mechanisms that aid the transition between the phases of the cell cycle. The kinase activity of Cdk/cyclin complexes is strictly controlled by a variety of Cdk inhibitors (CKIs), which serve as ″brakes″ to stop cell cycle progression if the cells appear under unsuitable conditions (Lim *et al.*, 2013)

1.5: SENESCENCE

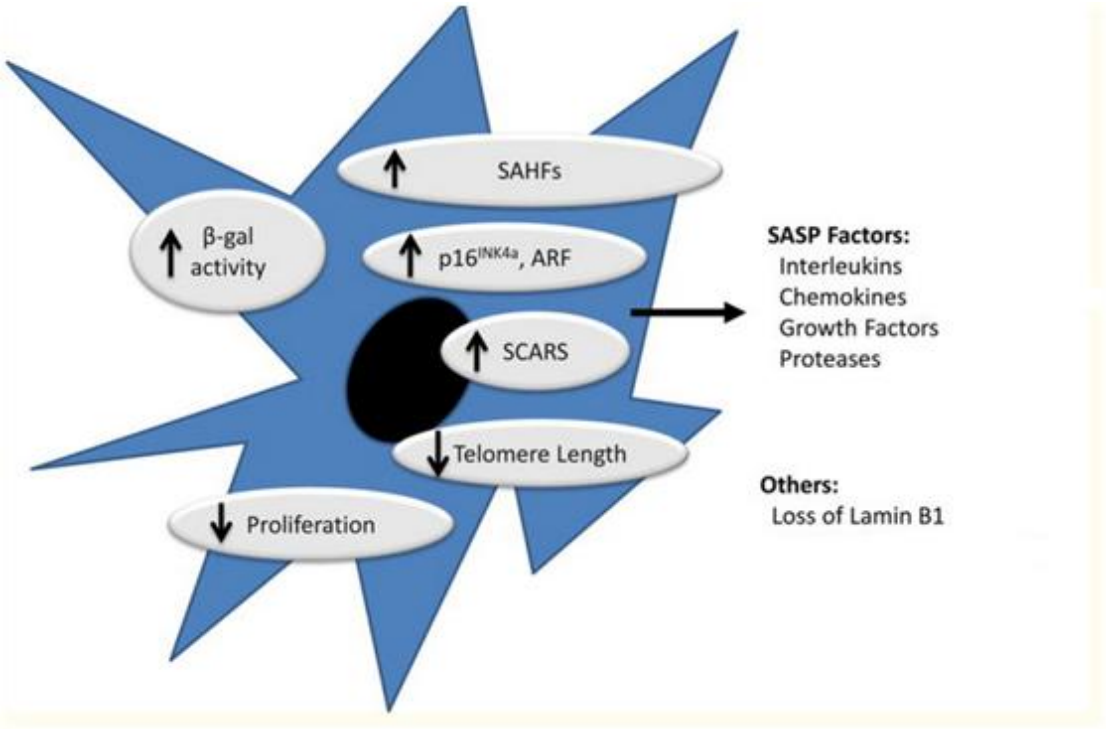
Cellular senescence is a stress-induce state, causing cell cycle arrest to cells normally proliferating, (He et al., 2017). The term was firstly reported five decades ago to describe that normal cells in the culture have a limited ability to proliferate (Hayflick *et al.,* 1961). Cells with characteristics of senescence were identified *in vivo,* with the number of those cells increasing with age in mammals, including humans (Dimri *et al.,* 1995; Krishnamurthy *et al.,* 2004; Melk *et al.,* 2004; Liu *et al.,* 2009). There are two hypotheses regarding how/why cells trigger senescence. The first one has to do with the perspective that senescence forms an anti-cancer or a tumor-suppressive mechanism. The second one suggests that cellular senescence is connected with ageing and, as time passes cells acquire an inability to regenerate. (Campisi *et al.,* 2007).

In mammals two types of senescence have been identified. The first one is replicative senescence (RS) that occurs because of telomere erosion and the second one is stress-induced premature senescence (SIPS) (Sikora *et al.,* 2011; Komseli *et al.,* 2018). SIPS does not entail telomere shortening and constitutes an acute response to a variety of stressful stimuli before telomere attrition shows up (Sikora *et al.,* 2011). As it will be mentioned below, oncogenes cause SIPS and this response represents a possible anti-tumor barrier (Braig *et al.,* 2005; Chen *et al.,* 2005; Collado *et al.,* 2005; Denchi *et al.,* 2005; Michaloglou *et al.,* 2005; Bartkova *et al.,* 2006; Di Micco *et al.,* 2006; Komseli *et al.,* 2018). Therefore, this type of senescence is called oncogene-induced senescence (OIS) and according to 'the oncogene-induced DNA damage model for cancer development, OIS must be bypassed for tumor progression' (Serrano *et al.,* 1997; Halazonetis *et al.,* 2008; Komseli *et al.,* 2018).

A myriad of stimuli can induce senescence: Firstly, after many cell divisions, human telomeres, become shorter and dysfunctional, thus triggering senescence (Martens *et al.,* 2000; Hemann *et al.,* 2001; Campisi *et al.,* 2007). Next, DNA damage, especially double strand breaks (DSBs), activates p53 signaling pathway, which arrest the cell cycle in a transient or prolonged manner. The latter can lead to senescence (Di Leonardo *et al.,* 1994; Parrinello *et al.,* 2003). Histone modifications play a role in triggering and establishing senescence. More specifically, chemical inhibition of histone deacetylase (HDACi) limits accessibility to chromatin and also triggers senescence (Ogryzko *et al.,* 1996; Munro *et al.,* 2004). Stress induced by intracellular oxygen radicals or the prolonged signaling by cytokines such as interferon-β or TGF-β trigger senescence (Campisi *et al.,* 2007; Campisi *et al.,* 2014). Additionally, as it has been referred above, oncogenes cause senescence (oncogene-induced senescence-OIS). An oncogenic form of RAS, which is involved in cellular signal transduction, and other targets of the downstream signaling pathway such as RAF and BRAF, as well as pro-proliferative nuclear proteins have been observed to transform normal cells to senescent when they are overexpressed or expressed as

oncogenic versions (Zhu *et al.*, 1998; Lin *et al.*, 1998; Dimri *et al.*, 2000; Michaloglou *et al.*, 2005; Serrano *et al.*, 2009).

The senescence response causes important changes in cellular phenotype. Changes in cell behavior, structure and function are characteristics of the senescent phenotype. The common features among the senescent cells are: the flat, enlarged, and often multinucleated morphology, the extended growth arrest that is possible induced because of the increased expression of the products- $p16^{INK4a}$ and ARF - of the *CDKN2a* locus, the secretion of anti-proliferative factors, the activation of the DNA damage sensing signaling pathways $p38^{MAPK}$ and NF-kB and of course development of resistance to apoptosis (Campisi *et al.*, 2007; Salminen *et al.*, 2012; Campisi,, 2013; Muñoz-Espín *et al.*, 2014; Childs *et al.*, 2015). Although, the mechanism that is used for apoptosis resistance remains still unclear, there are some hypothesis that this happens because of changes in expression pattern of the cell, concerning proteins that control proliferation or apoptosis (Marcotte *et al.*, 2004; Campisi *et al.*, 2007). Furthermore, senescent cells stay metabolically active (Dörr *et al.*, 2013). They secrete inflammatory mediators such as interleukins, numerous growth factors, chemokines and proteases, contributing to the senescence-associated secretory phenotype (SASP) (Coppé *et al.*, 2008). In addition to the above, senescent cells present increased expression of lysosomal β-galactosidase and accululation of lypofuscin which are both markers for senescence and they lack of proliferation markers such as KI67 or PCNA (Dimri *et al.*, 1995; Campisi *et al.*, 2007; Georgakopoulou *et al.*, 2013; Evangelou *et al.*, 2017). Moreover, other characteristics of senescence are short telomeres, activation of DNA damage response (DDR) signaling pathways, the expression or appearance of senescence-associated heterochromatin foci (SAHFs), and an increase in DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence) (Rodier et al., 2010; He *et al.*, 2017). Finally, a new marker for senescence is being investigated: the loss of Lamin B1, a highly conserved protein that is involved in nuclear stability, chromatin structure and gene expression (He *et al.*, 2017; Izdebska *et al.*, 2018).



Figoure 1. 4: The senescent phenotype (modified figure from He *et al.*, 2017). Senescent cells usually present flat, enlarged, and often multinucleated morphology, permanent arrest of cell proliferation, resistance to apoptotic signals and altered gene expression. In addition to the above, there is high SA β-gal activity and loss of Lamin B1 and other proliferation markers.. It has been observed an increase in SAHFs, SCARCs and in NF-kB signaling while senescent cells have short telomeres. Moreover, the senescent phenotype is accompanied by the secretion of inflammatory mediators (SASP).

1.6: CDC6

CDC6 is a 60-kD protein that belongs to the AAA^+ superfamily of ATPases (Neuwald *et al.*, 1999). CDC6-related genes have been identified in Archaea suggesting that is highly conserved in eukaryotic to prokaryotic organisms (Barry *et al.*, 2006). The human CDC6 gene is located at chromosome 17q21.3 and is regulated by the E2F/retinoblastoma transcription factors (Hateboer *et al.*, 1998; Ohtani *et al.*, 1998; Yan *et al.*, 1998). Mutations in Walker A/B motifs of the CDC6 protein block its ATPase activity (Herbig *et al.*, 1999). The only available crystallographic structure of CDC6 is of a fission yeast CDC18 homolog. It has been observed that there is also another interesting structural motif, the winged-helix fold domain which can be found in several DNA-binding proteins. Mutations in that site affect the function of the protein (Liu *et al.*, 2009). In addition to the above, there are three different positions on the CDC6 protein that can be phosphorylated by cyclin/CDKs, including those containing cyclin E (Mailand *et al.*, 2005). The loading of MCM complex, which enables pre-RC assembly, depends on CDC6 ATPase activity. More specifically, ATP hydrolysis by CDC6 facilitates the binding of the MCM ring with the DNA, while CDT1 is released (Bowers *et al.*, 2004; Randell *et al.*, 2006).

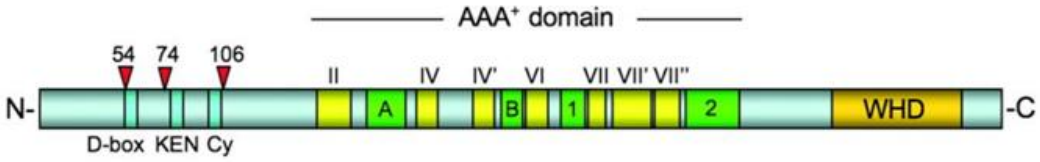


Figure1.5: Conserved motifs located in human CDC6 protein (figure from Borlado *et al.*, 2007). The red arrows represent the three positions of serine residues which can be phosphorylated by CDKs. D-box and KEN present 'protein degradation' sites whereas Cy indicates a cyclin-binding box. The different conserved AAA^+ boxes are represented with yellow, except Walker A, Walker B, Sensor 1 and Sensor 2 that are represented with green. WHD indicates the winged-helix fold domain of CDC6 protein.

Human CDC6 is destabilized in the G1 phase of the cell cycle by the action of the ubiquitin ligase APC^{Cdh1} and the proteasome (Méndez *et al.*, 2000; Petersen *et al.*, 2000) and its levels rise again before the entry into S phase. Although studies suggested that it remains stable until M phase (Williams *et al.*, 1997), recent studies indicate that proteasomal degradation of CDC6 in G2/M phase of cells occurs in a Cyclin F-dependent manner by forming an active SCF ubiquitin ligase complex- $SCF^{CyclinF}$. More specifically, CDC6 interacts directly with Cyclin F through the Cy-motif of CDC6 and the Cyclin box domain of Cyclin F (Walter *et al.*, 2016). In addition to the above, it has been reported that Cyclin F and the CDT1 inhibitor Geminin cooperate in maintaining genome integrity, via the suppression of the major licensing factors CDC6 and CDT1. Taking these together, it is proposed that Cyclin F prevents re-replication and maintains genome stability by targeting CDC6 for decomposition (Tada *et al.*, 2001; Zhu *et al.*, 2004; Melixetian *et al.*, 2004; Klotz-Noack *et al.*, 2012; Walter *et al.*, 2016).

1.7: CDC6 IN HUMAN CANCER

It is known that deregulation of the replication licensing process promotes genomic instability that most of the times leads to carcinogenesis, because of unexpected DNA re-replication (Blow *et al.*, 2008; Negrini *et al.*, 2010; Halazonetis *et al.*, 2008). Re-replication is a type of replication stress that contributes to replication fork stalling, DNA damage and finally leads to genomic instability (Blow *et al.*, 2008; Petrakis *et al.*, 2016). Considering the important role of *CDC6* in DNA replication, its deregulation is expected to have a negative impact in genome stability. Experimental data suggest that *CDC6* presents oncogenic characteristics. In addition, ectopic expression of CDC6 and CDT1 causes DNA re-replication in tumor cells (Stoeber *et al.*, 1998; Cook *et al.*, 2002; Vaziri *et al.*, 2003).

In most cancer types, E2F/retinoblastoma transcription factors, which control the CDC6 expression, are frequently deregulated. In turn, CDC6 overexpression which has been observed in many tumor cells such as in various brain cancers, in non-small cell lung carcinomas and in mantle cell lymphoma (Ohtae *et al.*, 2001; Karakaidos *et al.*, 2004; Pinyol *et al.*, 2006; Borlado *et al.*, 2007). Finally, CDC6 can be used as a marker for detecting early malignancy because of its absence in non-dividing differentiated and quiescent cells (Borlado *et al.*, 2007).

1.8: ONCOGENIC ACTIVITY OF CDC6

The perception that the aberrant DNA replication leads to genomic instability is suggested in several studies and it is proposed that abnormal formation of pre-RC and recruitment onto the DNA leads to inefficient S phase, contributing to chromosomal rearrangements (Lengronne *et al.*, 2002; Tanaka *et al.*, 2002; Sidorova *et al.*, 2003; Ekholm-Reed *et al.*, 2004). Consequently, the oncogenic activity of CDC6 may origin from the genomic instability that comes from the aberrant DNA replication (Borlado *et al.*, 2007).

Precancerous cells lose control of DNA replication. This phenomenom contributes to re-firing of the replication origins and inefficient fork progression (Di Micco *et al.*, 2006). Stalled or collapsed replication forks, as it mentioned above, usually cause double-strand breaks (DSB). When DSBs occur, the DNA damage response (DDR) pathway is activated and is responsible to eliminate the genomic threat. DDR is an early inducible barrier in carcinogenesis and usually leads the cells into a senescence state (Di Micco *et al.*, 2006; Bartkova *et al.*, 2006). In addition to the above, DDR seems to activate the signaling kinases ataxia-telangiectasia mutated (ATM) and the effector kinase Chk2 (Gorgoulis *et al.*, 2005; Bartkova *et al.*, 2006; Liontos *et al.*, 2007).

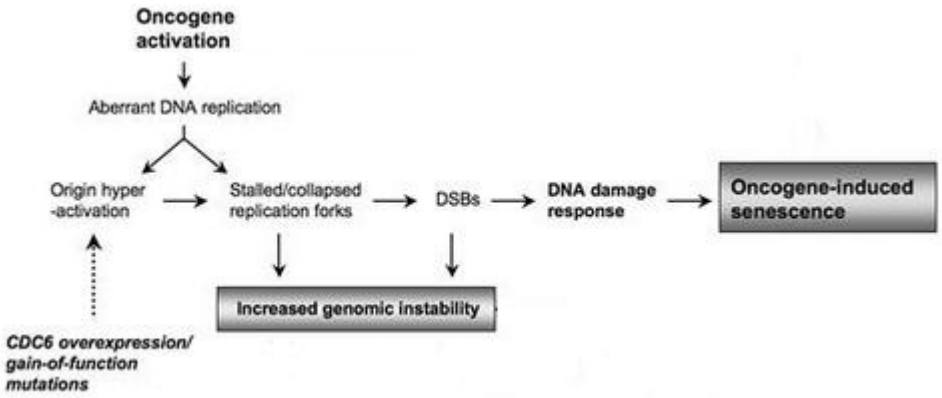


Figure 1.6: Oncogenic properties of CDC6 via replication stress (modified figure from Borlado *et al.*, 2007). Early cellular response to oncogene activation is characterized by abrnornal DNA replication which finally leads to DSBs. The accumulation of DNA double strand breaks (DSBs) activates the DDR but also contributes to genomic instability. The final outcome is the transforming of the cells into a senescent state.

A study has revealed that deregulation of CDC6 possibly affects the expression of INK4/ARF locus (Gonzalez *et al.*, 2006). The INK4/ARF locus which has been found deregulated in many cancers, encodes the $p16^{INK4a}$, $p15^{INK4b}$ and ARF genes, which are all tumor suppressors. The first two activate the retinoblastoma pathway and the last activates the p53 (Kim *et al.*, 2006). Overexpression of CDC6 in cultured cells has been found to impede the expression of INK4/ARF genes by blocking the action of CTCF (Gonzalez *et al.*, 2006; Sideridou *et al.*, 2011) (see CTCF section). Moreover, the mechanism by which, Cdc6 represses the INK4/ARF locus, involves induction of histone de-acetylases and heterochromatinization of the area (Petrakis *et al.*, 2012).

Further evidence of the oncogenic role of Cdc6 was demonstrated by the interesting observation that Cdc6 overexpression in murine, premalignant epithelial cells drives them into a mesenchymal state – epithelial to mesenchymal transition (EMT) (Liontos *et al.*, 2007; Sideridou *et al.*, 2011). EMT is a biological process by which the epithelial cells lose their epithelial characteristics and acquire migratory and invasive properties of mesenchymal cells (Kalluri *et al.*, 2003; Kalluri *et al.*, 2009). EMT is a characteristic of cancer and is associated with loss of E-cadherin which is a tumor suppressor (Hirohashi, 1998; Thiery *et al.*, 2009). E-cadherin is encoded by the CDH1 gene and plays a key role in cell-cell adhesion in epithelial tissues (Pećina-Šlaus, 2003). As CDC6 is overexpressed it binds to the E-boxes of the promoter of CDH1. and removes the chromosomal insulator CTCF and the histone H2A.Z from the area of the promoter. This represses the expression of E-cadherin and induces local heterochromatization. It also stimulates the replication origins near the CDH1 promoter (Sideridou *et al.*, 2011). Finally, high levels of CDC6 in mouse (P1mouse papilloma) and human (A549 lung cancer) cells results in significant increase of $CD24^{low}/CD44^{high}$ antigen phenotype, which is connected with stem-like features and is associated with EMT and the gain of stem cell properties (Hanahan *et al.*, 2011).

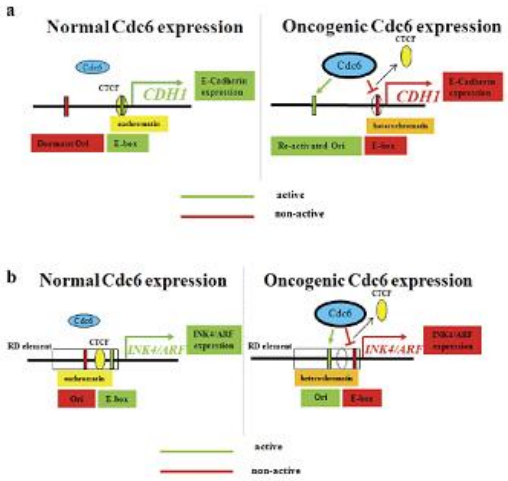


Figure 1.7: Oncogenic properties of CDC6 via transcription regulation (figure form Sideridou *et al.*, 2011). CDC6 overexpression represses transcription of the genetic locus CDH1 and INK4/ARF by removing CTC, heterochromatinizing the promoters and activating proximal replication origins.

The involvement of CDC6 in transcription is not only associated with repression of gene expression. Recently, it has been revealed that CDC6 acts as a transcription initiator, as it binds to the promoter of coding regions of rRNA genes and stimulates rDNA transcription in the nucleolus after mitosis/G1 phase (Huang *et al.*, 2016). More specifically, it is suggested that CDC6 localizes in the nucleolus thanks to its ATP-binding site, and is connected with B23-also known as nucleophosmin (NPM). B23 is connected with UBF (upstream binding factor) which binds to an upstream control element and the core promoter of the rRNA genes (Bell *et al.*, 1988; Bazett-Jones *et al.*, 1994; Bell *et al.*, 2002; Huang *et al.*, 2016). CDC6 facilitates the RRN3-mediated recruitment of Pol I onto the rDNA promoter site to start rDNA

transcription. Overexpression of CDC6 increases rDNA transcription, (Huang *et al.*, 2016).

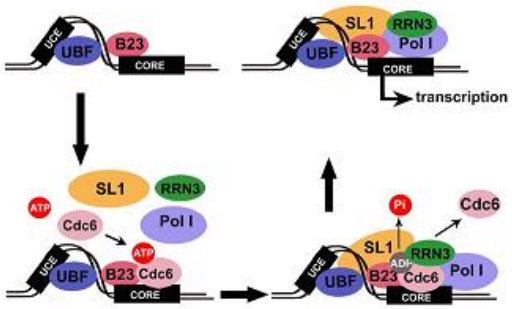


Figure 1.8: A suggested model of CDC6 as a transcription initiator (modified figure from Huang *et al.*, 2016). Overexpression of CDC6 increases rDNA transcription through the process that is described above.

2. AIMS

Cell division cycle 6 (CDC6) is a fundamental molecule that contributes to the normal progress of the cell cycle. More specifically, it plays an essential role in the 'licensing' of DNA replication as it interacts with the CDC10-dependent transcript 1 (also known as DNA replication factor CDT1) and facilitates the recruitment of the mini-chromosome maintenance (MCM) helicase complex MCM2-7 into the DNA at the G1 phase of the cell cycle (Diffley *et al.,* 1994; Speck *et al.,* 2005; Chen *et al.,* 2007; Evrin *et al.,* 2009).

Taking into consideration the importance of CDC6 in DNA replication, its deregulation is expected to have a negative impact in genome stability. Several studies support that deregulated expression of CDC6 exerts oncogenic properties and is associated with cancer (Ohtae *et al.,* 2001; Karakaidos *et al.,* 2004; Pinyol *et al.,* 2006; Borlado *et al.,* 2007, Sideridou *et al.,* 2011).

CDC6 overexpression is connected with abnormal DNA replication which finally leads to double strand breaks (DSBs). The accumulation of DNA DSBs activates the DNA damage response pathways but also contributes to genomic instability. The final outcome is the transformation of the cells into a senescent state (Di Micco *et al.,* 2006; Bartkova *et al.,* 2006). In addition, overexpression of CDC6 in tissue cultured cells has been found to impede the expression of the INK4/ARF locus, which encodes three important tumor suppressors, by blocking the action of CTCF (Gonzalez *et al.,* 2006). It is worth mentioning that CDC6 overexpression in murine, premalignant epithelial cells drives them into a mesenchymal state – epithelial to mesenchymal transition (EMT) (Liontos *et al.,* 2007; Sideridou *et al.,* 2011) which is followed by repression of CDH1 locus, which encodes E-cadherin (Hirohashi, 1998; Thiery *et al.,* 2009). However, the involvement of CDC6 in transcription is not only associated with repression of gene expression. Recent studies indicate that overexpression of Cdc6 increases rDNA transcription, probably due to increased cancer cell needs for protein synthesis (Huang *et al.,* 2016).

Cellular senescence is a stressed-induce state, where proliferating normally cycling cells are driven to arrest their cell cycle (He et al., 2017). Senescence is believed to constitute an anti-cancer or a tumor-suppressive mechanism. It is also said that cellular senescence is connected with ageing and the inability of cells to regenerate as the time passes *in vivo* (Campisi *et al.,* 2007). In mammals two types of senescence have been identified. The first one is replicative senescence and the second one is stress-induced premature senescence (SIPS) (Sikora *et al.,* 2011; Komseli *et al.,* 2018). SIPS constitutes an acute response to a variety of stressful stimuli before telomere attrition shows up (Sikora *et al.,* 2011). While oncogenes expression is associated with induced proliferative signals triggering uncontrolled cell multiplication, they also cause SIPS, a response aiming to counteract the proliferative effects and thus represents a possible anti-tumor barrier (Braig *et al.,* 2005; Chen *et al.,* 2005; Collado *et al.,* 2005; Denchi *et al.,* 2005; Michaloglou *et al.,* 2005;

Bartkova *et al.,* 2006; Di Micco *et al.,* 2006; Komseli *et al.,* 2018). This type of senescence is called oncogene-induced senescence (OIS) and according to 'the oncogene-induced DNA damage model for cancer development, OIS must be bypassed for tumor progression' (Serrano *et al.,* 1997; Halazonetis *et al.,* 2008; Komseli *et al.,* 2018).

In this thesis, an epithelial cellular model in which over-expression of the replication licensing factor CDC6 is achieved in an inducible manner via a doxycycline-inducible promoter is studied. More specifically, immortalized human bronchial epithelial cells (HBECs) (hTERT/CDK4) (Ramirez *et al.,* 2004) are used. Most of the cancers are of epithelial origin and the above cell system simulates 'the whole spectrum of epithelial carcinogenesis from the non-malignant stage to oncogene-mediated activation of the anti-tumor barrier of senescence, followed by the complete transformation of epithelial cells into a mesenchymal state (Komseli *et al.,* 2018). Importantly, in this thesis the part of carcinogenesis that we focus on is the cellular and molecular mechanisms controlling the anti-tumor of barrier-senescence. Therefore, the aim of this thesis is to better understand the exact role of CDC6 in cancer initiation and development. A number of experiments have been designed in order to understand what exactly happens during the cell cycle upon CDC6 overexpression. We first set to determine whether HBEC cells could be synchronized in the cell cycle to facilitate our study. We confirmed by phase contrast microscopy and Flow Cytometry that simple deprivation of supplements was sufficient to reversibly block a large majority of the treated cells in G0/G1 phase. These experimental conditions also enabled us to define a framework for addressing the second aim of this study: monitoring accurately the changes driven by CDC6 induction and the consequences on cell cycle dynamics. A third aim implied the study of CDC6 induction consequences at the chromatin structure and gene expression levels. As mentioned above, CDC6 overexpression blocks the action of CTCF- an important insulator that affects 3D chromatin structure and gene expression profile (Lobanenkov *et al.,* 1990; Klenova *et al.,* 1993; Filippova *et al.,* 1996; Ohlsson *et al.,* 2001). We therefore performed Chip-seq experiments to gain insights in the changes in genome-wide binding of CTCF between HBEC OFF-control and HBEC 3D Tet-ON cells. Finally, we analyzed ATAC-seq data generated in the lab in order to also understand the changes in open chromatin regions and the direct effect on gene expression.

To sum up, this thesis helps to clarify the mechanism driving HBEC cells entry into senescence and reveals how CDC6 overexpression can drive changes in chromatin and can affect both transcription and cell cycling.

3. MATERIAS & METHODS

3.1: Cell Culture and HBEC CDC6 Tet-ON system

Immortalized Human Bronchial Epithelial Cells (HBECs) were used in this thesis. Immortalization had already been done with combined expression of hTERT and ectopic mutant cyclin-dependent kinase 4 (CDK4) in order to overcome the problem of $p16^{INK4A}$-induced premature growth arrest and to keep the epithelial phenotype (Ramirez *et al.*, 2004; Evangelou *et al.*, 2013; Komseli *et al.*, 2018).

The Lenti-X™ Tet-On® 3G Inducible Expression System (Clontech Laboratories) was used by Komseli et al. to establish a CDC6 inducible-expression cellular model in immortalized Human Bronchial Epithelial Cells through doxycycline induction (1μg/ml). HBEC CDC6 Tet-ON overexpress CDC6 (Komseli *et al.*, 2018).

Immortalized HBECs and HBEC CDC6 Tet-ON cells were maintained in Keratinocyte Serum-Free Medium (#17005–075, Invitrogen) supplemented with 5 ng/ml hEGF, 50 μg/ml Bovine Pituitary Extract (#17005–075, Invitrogen), 50ug/ml Gentamycin (#22185.02,Serva) and Amphotericin-B (#15290026, Gibco) at 37 °C and 5% CO_2 (Ramirez *et al.*, 2004; Stewart *et al.*, 2012; Komseli *et al.*, 2018).

3.2: Cell Passage

The medium is removed and the cells are washed with 5ml PBS 1X. An appropriate volume of Trypsin-EDTA (Thermo Scientific) is added depending on the surface to be treated. Usually, we use 3ml for a T175, 2ml for a T75, 1ml for a T25 and 300μl for a well of a 6-well plate. Cells are incubated for 5min at 37°C (5% CO_2). Trypsin neutralizer solution (0,5% FBS in PBS) is added in the same volume as trypsin in order to inactivate it. Cells are collected by centrifugation at 1700 rpm for 7 min at 20°C. The pellet is resuspended in 4-5ml Keratinocyte Serum-Free Medium (#17005–075, Invitrogen) and cells are distributed in plates or flasks as required for the specific experiment (usually in dilution 1:2).

3.3: Freezing cells

The same protocol that is used for splitting cells but here the pellet is resuspend in freezing medium (Keratinocyte Serum-Free Medium 10% DMSO). 1,5-1,8 ml of that is transferred in cryovials and they are placed at 4°C for 15 min, at -20°C for 20 min and then at -80°C. The cry0vials must be transferred in liquid nitrogen for further storage.

3.4: Thawing Frozen Cells

The cells are thawed rapidly, less than 1 min in a 37°C water bath. The cells of the cryovial are transferred in a 15 ml falcon. 7 ml PBS 1X is added and the cells are

centrifuged at 1700rpm for 5min at 20°C. The pellet is resuspended in 4-5ml Keratinocyte Serum-Free Medium (#17005–075, Invitrogen) and is transferred in a plate or a flask.

3.5: Counting Cells with the use of Hematocytometer Neubauer

The initial steps are the same with the protocol of Cell Passage. After the centrifugation, the supernatant is removed and the pellets are resuspended in 5ml medium. 10μl of the supernatant are transferred to a new tube, mixed gently with 10 μl of 0,4% Trypan Blue **(**#T8154, Sigma) which is a dye for detecting dead cells (Crowley *et al.,* 2016). 20 μl of the solution are applied to the hemocytometer (BlauBrand) and unstained cells (live cells do not take up Trypan Blue) are counted in one set of 16 squares. The hemocytometer is moved to the next set of 16 corner squares and cells must be counted in all 4 sets of 16 corners. The average cell count from each of the sets of 16 corner square is multiplied by 10,000 (10^4) and by 2 to correct for the 1:2 dilution from the Trypan Blue addition. The final value is the number of viable cells/mL in the original cell suspension.

3.6: Total protein extraction and western blot analysis

Total protein extracts are obtained by homogenization in trypsin-EDTA (Thermo Scientific) and trypsin neutralizer (0,5% Fetal Bovine Serum-FBS in PBS 1X). The homogenate was centrifuged at 1700 rpm at 20 °C for 7 min. The pellet is resuspended in 10μl PBS 1X and 10μl Loading Buffer with DDT and the samples are stored in -20°C. Subsequently, the samples are incubated in 95°C for 15min. The samples are loaded on acrylamide/bis-acrylamide gels which consist of 2 different gels· the 5% stacking gel which contributes to the entry of the proteins in the separating gel and the 12% separating gel where the proteins are separated based on their molecular weight.

Ingredients	10ml stacking gel (5%)	10ml separating gel (12%)
H_2O	7.225ml	4.3ml
40% acrylamide	1.275ml	3ml
1M Tris (PH 6.8)	1.25	-
1.5M Tris (PH 8.8)	-	2.5ml
10% SDS	0.1ml	0.1ml
10% APS (Ammonium persulfate)	0.1ml	0.1ml
TEMED	0.01ml	0.004ml

Table 3.1: The ingredients of stacking and separating gels

The next step is the transfer of the proteins from the acrylamide gel to the PVDF membrane (Millipore, Cat No. IPFL00010). Methanol transfer buffer (200 ml

methanol, 700ml ddH20, 100 ml 10X Running Buffer) is used because the protein of interest has molecular weight < 120 kDa. The transfer is held for 1,5h at 4°C at 130V. The membrane is incubated with blocking buffer (PBS 1X: Licor, 1:1 (Odyssey Blocking Buffer, Cat No. 927-40000) for 1h in order to be covered all possible non-specific sites. Subsequently, the membrane is incubated with the primary antibody diluted in PBS 1x: Licor: 0.1% Tween 20 overnight (o/n) at 4°C. 4 washes with PBS 1X:Tween 20 0.1%, 5 min, RT each, are conducted and then the membrane is incubated with the secondary antibody (1:10000) diluted in the same buffer as the primary for 1 h, RT. 4 washes with PBS 1X:Tween 20 0.1%, 5 min, RT and one more with PBS 1X for 5 min are conducted. Then the membrane is transferred to the Odyssey CLX Imaging System for the visualization of the signal of the immunoblot.

Primary antibodies are used at the following dilutions: CDC6 (#9964, Santa Cruz) 1:500, actin (#1615, Santa Cruz) 1:2000. Goat Anti-mouse (#926-32210, LiCor Biosciences) and donkey anti-goat (#926-68074, LiCor Biosciences) secondary antibodies diluted at 1:1000 are used.

3.7: ChIP-seq assay

ChIP assay is performed in HBEC OFF and HBEC CDC6 3 days Tet-ON cells grown in 150 mm plates and induced with doxycycline for 2 days. Cells are cross-linked with 1% formaldehyde for 12 min at 4°C. Cross-linking is stopped by the addition of glycine to a final concentration of 125 mM for 6-7 min at 4°C on a rocking platform. Cross-linked cells are washed twice with ice cold PBS 1X, collected by scraping in PBS 1X, 1mM EDTA, 0.5mM EGTA, 1mM PMSF buffer and centrifuged at 2100 rpm for 10 min at 4°C. Pellets are resuspended again in the same buffer and centrifuged at 2800 rpm for 10 min at 4°C. Supernatant is removed and pellets are stored at -20°C. Approximately 2×10^6 cells which have been counted with the use of hemocytometer (BlauBrand**)** are resuspended in 1,5 ml Chro-lysis buffer (50mM Hepes-KOH pH 8.0, 1mM EDTA, 0.5mM EGTA, 140mM NaCl, 10% glycerol, 0.5% IGEPAL, 0.25% Triton X-100, 1mM PMSF, protease inhibitors) incubated for 10 min at 4°C on a rotator and centrifuged at 2800 rpm for 10 min at 4°C. Pellets are resuspended in 1,5 ml Wash Buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, 0.5 mM EGTA, 200mM NaCl, 1mM PMSF, 10mM NaPy, protease inhibitors) incubated for 10 min at 4°C on a rotator and centrifuged at 2800 rpm for 10 min at 4°C. Pellets are resuspended in 700ml RIPA buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 0.5mM EGTA, 140mM NaCl, 1% Triton X-100, 0.1% Na-Deoxycholate, 0.1% SDS, 1mM PMSF, 10mM NaPy, protease inhibitors) and incubated for 10 min at 4°C. Cells are sonicated with the Bioruptor Sonicator (Diagenode) for 27,5 min (12,5 min x 3) using ice. The Bioruptor Sonicator is active (ON) for 30 sec and then inactive (OFF) for 30 sec. Subsequently, the samples are centrifuged at 10000 rpm for 10 min at 4°C. The supernatant is stored as a soluble chromatin fraction (INPUT).

Immunoprecipitation is performed at 4°C overnight (o / n) incubating equal amounts of Chromatin Input per experimental condition with the desired antibody. CTCF (#70303, abcam) is used in my thesis. 16h later chromatin-antibody complexes are selected via the use of magnetic beads coated with protein A (Dynabeads Protein A for Immunoprecipitation, Thermo Scientific). Incubation of beads with antibody-chromatin complexes is performed at 4 ° C for 3h with rotation. Beads are washed twice with 300μl RIPA buffer, 3 times with 300 μl of RIPA solution containing 0.3 M NaCl, one time with 400 μl LiCl buffer (10mM Tris-HCl PH 8.0, 1mM EDTA, 0.5Mm EGTA, 0.25M LiCl, 0.5% Triton X-100, 0.5% Sodium Deoxycolate, 1mM PMSF, 10mM NaPy) and twice with 500 μl of TE solution (10 mM Tris, 1 mM EDTA). Afterwards, DNA-protein complexes are selected in 0.1M $NaHCO_3$, 1% SDS (2 cycles of incubation 20 min each at 65°C). Supernatant is collected and placed in 65°C overnight (o/n) –reverse cross-linking. Incubation with proteinase K 0.1 μg/μl for 1 h at 55°C is performed and DNA purification is performed using Agentcourt AMPURE XP beads (Beckman Coulter Life Sciences, Cat No. A63881). The DNA was stored at -20°C. It is worth mentioning that final ChIP DNA is quantified on a Qubit 2.0 Fluorometer (dsDNA HS Assay Kit, Thermo Scientific) and ChIP specificity is examined by qPCR analyses performed with 10–100 pg of ChIP and Input DNA in duplicate reactions with qPCRBIO SyGreen mix (PCR Biosystems) on Roche Light Cycler 96 instrument. If ChIPs showed enrichment in expected genomic regions, ChIP and Input DNA were then subjected to library preparation for high-throughput sequencing.

3.8: Illumina sequencing and library generation

Library generation was performed by Tasos Liakos as described before, with minor modifications. (Lavigne *et al.*,2015). Libraries are assayed on a BioAnalyzer (High Sensitivity DNA kit, Agilent) and next-generation sequencing is performed at Genecore-EMBL, using the Illumina HiSeq 2000 for single end reads.

3.9: Reads alignment and normalisation

Reads alignment and normalization were performed by Dimitris Konstantopoulos (DK) as described before (Lavigne *et al.,* 2017).

3.10: Flow cytometry

Cells are harvested with trypsinization and centrifuged at 1700 rpm for 7 min at 20°C. The pellets are resuspended in 800μl ice-cold PBS and centrifuged at 1700 rpm for 3 min at 4°C. Supernatant is discarded and pellets are resuspeded in 100μl PBS-0,1% glucose and 1ml 70% cold ethanol with the use of vortex. Afterwards, the samples are stored in -20°C. The next day the samples are centrifuged at 1700 rpm for 5 min at 4°C. The pellets are resuspended in 1ml ice cold PBS and centrifuged at 1700 rpm for

20 min at 4°C. The supernatant is removed and the cells are stained with 50 μg/ml propidium iodide in the presence of 100μg/ml RNase A. The samples are placed on a rocking platform for 30-40 min covered with aluminum foil and DNA content was assessed on a flow cytometer (FACS Canto II of Becton Dickinson (BD).

3.11: Senescence/GL13 staining

Cells developed in small round cover glasses (13mm) (VWR) are washed 3 times with PBS 1X, fixed with Paraformaldehyde (PFA) 4% (#P6148-500G, Sigma-Aldrich) and washed again 3 times with PBS 1X. Cover glasses should be hydrated and PBS 1X is added for that purpose. They are stock at 4°C. GL13 staining is performed in as described before (Evangelou *et al.*, 2013). GL13 compound is commercially available as SenTraGor™ from Arriani Pharmaceuticals (Cat no: AR8850040).

3.12: Starving assay

After passage, cells are plated in 6-well plates and keratinocyte with supplements-growth factors is added. Whenever the cells have the appropriate confluence, less than 60%, the cells are washed with PBS 1X and keratinocyte without supplements-growth factors is added for 48 and 72 h.

3.13: Phase Contrast Microscopy

Through the whole thesis, cells are microscopically observed in the phase contrast optical microscope (Leica DMI 3000 B). Images are taken using the above microscope which is connected to the camera (Infinity1-3C, Luminara).

3.14: USCS Genome Browser on Human (GRCh37/hg19)

The UCSC Human Genome Browser hosted by UC Santa Cruz, presents a variety of annotation datasets which are called tracks and they are presented graphically. Tracks of interest can be loaded in the browser. In this thesis, member of Fousteri Lab have loaded tracks about the accessibility of chromatin in HBEC OFF and HBEC 3d ON cells (ATAC-seq data) and the CTCF binding in the above two conditions (CHIP-seq data).

3.15: Bioinformatic analysis

The data analysis of the CHIP-seq experiment was conducted by Dimitris Konstantopoulos (DK). K-means clustering analysis (N=5 clusters) is used in order to see if there are different binding patterns of CTCF in the two examined conditions (OFF- HBEC 3D ON).

The primary data analysis of RNA-seq which has been obtained from Komseli *et al.*, 2018, and of ATAC-seq which has been conducted by Tasos Liakos, has been

performed by DK. We performed correlation analyses and identified common genes between the genes that are up/down-regulated and differentially accessible in HBEC 3d ON compared to HBEC OFF (gain or loss of differentially accessible regions-DARs) by using Venny 2.0 (http://bioinfogp.cnb.csic.es/tools/venny/index2.0.2.html). In addition, list of genes obtained from the meta-analysis of the RNA-seq analysis from Komseli *et al.,* 2018 performed by DK has been used in order to identify cluster of genes that are over-represented (enriched) in a previously published gene sets characterizing specific biological processes, looking for interesting signatures using Gene set enrichment analysis (GSEA) tools. Last but not least, *cis*-regulatory element annotation system (CEAS) has been used for finding out where the DARs are located across the genome.

4. RESULTS

4.1: The cellular system HBEC as a non-malignant human epithelial cancer model to study CDC6-induced senescence.

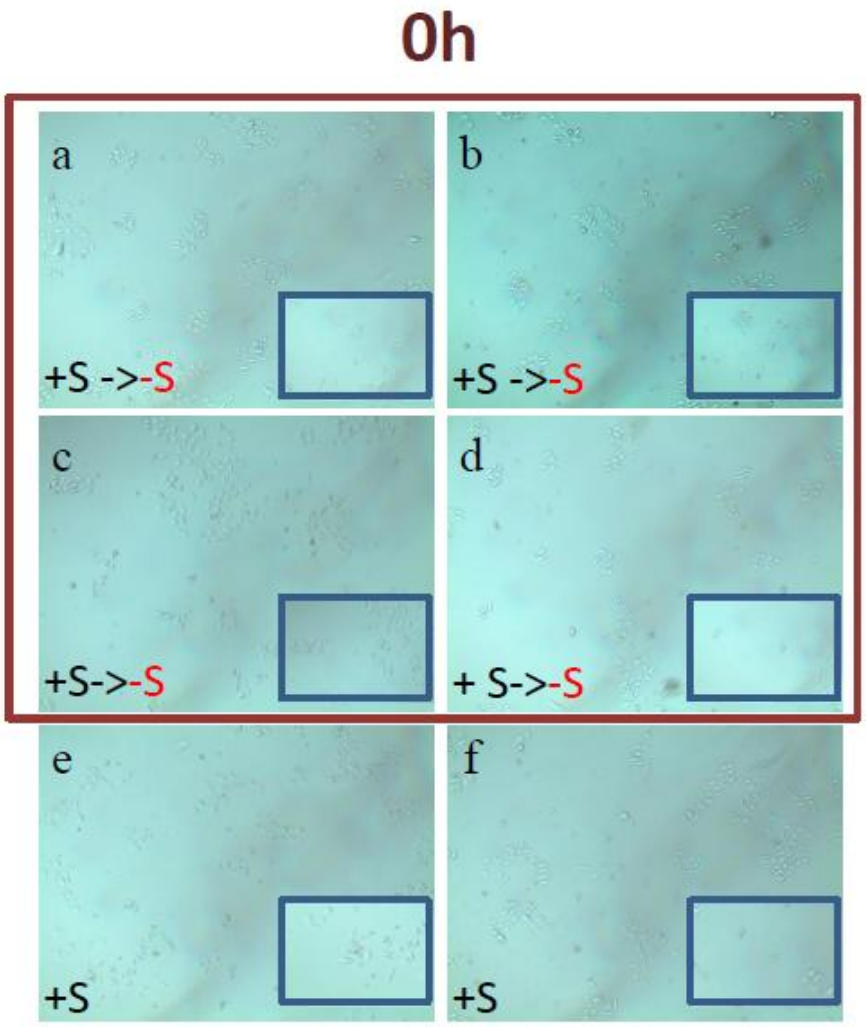
Human Bronchial Epithelial Cells (HBECs) are used as a tool in order to model the epithelial origin of cancer and its evolution. The immortalized HBECs can maintain their epithelial characteristics via the expression of mutant cyclin-dependent kinase 4 (CDK4) and hTERT (Ramirez *et al.,* 2004; Evangelou *et al.,* 2013; Komseli *et al.,* 2018). This model takes advantage of the genetic manipulations which were carried out by Dr Komseli in Prof. Gorgoulis lab in order to develop a doxycycline inducible system that over-expresses CDC6 (Komseli *et al.,* 2018). In order to confirm that the system is operational in my hands, I verified the efficiency of CDC6 induction by western blot and confirmed that upon dox treatment, the cells overexpress CDC6 at 3d and 6d post-induction (data not shown).

4.2: HBECs restore their proliferative ability after starving.

After passage, cells are plated in a 6-well-plate with Keratinocyte Serum-Free Medium with supplements. When the cells reach ≈50-60% confluence we record their initial state by phase contrast microscopy (Fig. 4.1, 0h). In four out of the 6 wells-Keratinocyte Serum-Free Medium without the supplements is added (red frame for a,b,c,d wells, Fig. 4.1). We monitored in a time-course the phenotypic changes observed upon starving (-S), release from starving (+S) and incuction of cdc6 (+dox) , as such: 48h after starving we verified that the cells survived in supplement-free medium and noticed that the growth was impaired in comparison to non-starved cells (arrows denote the areas where cells are missing, compare at 48h a-d with e-f, fig 4.1) . We assessed whether cells could be released from starving and multiply again by adding medium with supplements in well c at 48 h (Fig 4.1). At 72h we observed that for this well, cells started to multiply again as opposed to the wells a,b and, d, which were still maintained in starving medium. We then tested the effect of dox addition in well a (cdc6 induction at 72h) after adding supplements in wells a and d at the same time point. We checked after 1 and 3 days the effect of this treatment. At 96h and 144h we discover that the cells simultaneously induced by dox and supplements failed to recover their ability to proliferate as opposed to the cells that were supplied with supplements only (compare a and d at 96h and 144h, Fig 4.1). Interestingly, the cells that remained starved for as long as 144h (well b, Fig 4.1) did not multiply and survived the stress.

Therefore, this experiment allows us to conclude that the cells can survive the stress caused by prolonged starvation. Our results demonstrate how cells behave during starving and release treatment. Next, it would be interesting to test whether the cells that are subjected to these conditions are synchronized in G0/G1 phase of the cell cycle during starving. We set to study this phenomenon further by Flow Cytometry

(see below), as such experiment should also enable us to confirm the important role of CDC6 (when over-expressed) that appears to affect the proliferation rate and change how the cells resume their cell cycle during starvation-release.



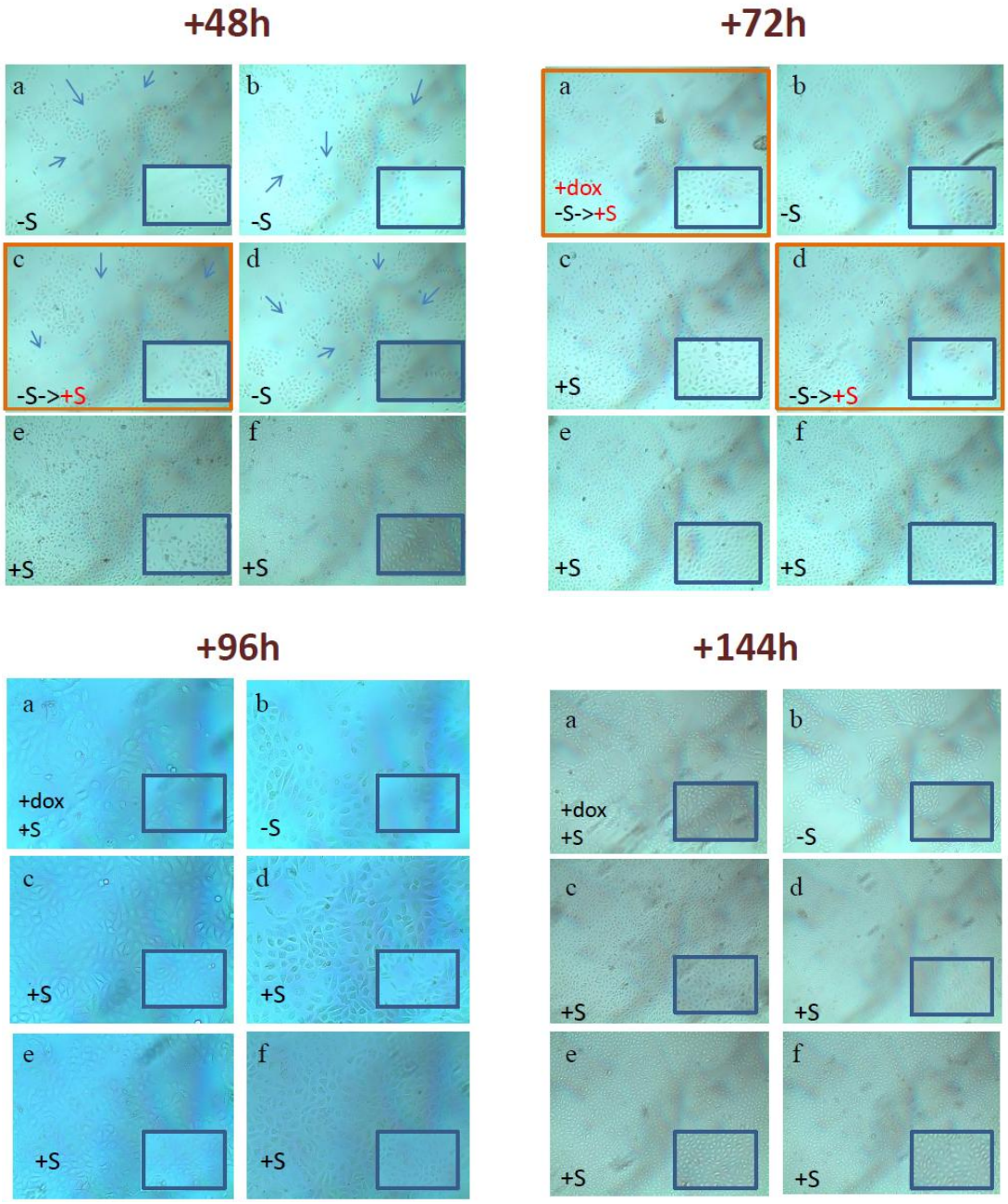


Figure 4.1: The starving experiment. A 6-well-plate is represented. The +S represents the keratinocyte with the supplements hEGF and BPE whereas the -S represents the keratinocyte without supplements. Black color represents the initial state of the cells in the well whereas red color represents the final. 0h represents the cells exactly before the experiment is conducted. In the four wells which are surrounded by the red frame (a,b,c,d wells), medium without supplements is added. 48h later in the well c, complete medium is added (orange frame) and 24h later in wells a and d, complete medim is added (orange frame) whereas doxycycline is added only in the well a.. The arrows in a,b,c,d wells show the areas where cells are missing compared to control cells in wells e and f. Different scale has been used in +96h compared to the onther time points. The blue frame represents a zoomed area of each well.

4.3: CDC6 overexpression leads to acceleration in the G1/S transition of the cell cycle.

After passage, cells are plated in two 6-well-plates (A and B) with Keratinocyte Serum-Free Medium with supplements. When the cells reach ≈50-60% confluence, induction of CDC6 expression is triggered by doxycycline addition in A3 and A6

wells. Cells of A3 well are harvested 2h later, whereas cells in A6 and A2 (cells with complete medium only-control) wells are harvested 4h after the dox induction in A6. Keratinocyte Serum-Free Medium without the supplements is added in A1 and A4 wells and in the B 6-well plate. After 72h of starving, complete medium is added in B3 and B6 wells, whereas doxycycline is added only in B3 well. After 8, the cells are harvested. The same process is followed and the cells are harvested after 14h, 15h, 16h and 24h after release of the cells into complete medium and dox induction. Furthermore, doxycycline is induced in A1 well and 3h later, cells of A1 and A4 wells are harvested, too. Flow Cytometry is followed. For the whole process, see the chapter Materials and Methods. The purpose of this experiment is to examine if starving cells can be synchronized in the G0/G1 phase and to study the impact of CDC6 over-expression via doxycycline induction on the cell cycle.

The not starved cells with and without doxycyxline have normal cell cycle. The pattern that is presented by the cells with doxycycline is approximately the same with the pattern of the not starved cells without doxycycline (Fig 4.2). In this case, we assume that CDC6-over-expression caused by dox induction, which lasts only for 4h, does not affect the cell cycle. The not released cells (for ever starved cells) with and without dox are synchronized in the C0/G1 (Fig 4.2). The cells arrest their cycle due to the depletion of growth factors. Nor in this case, CDC6 over-expression seems to affect the cell cycle.

The cells which have been starved and then released for specific time points into complete medium present some important differences in the pattern of the cell cycle in the two examined conditions (with and without dox addition).The most interesting part of the results is that in the five cases of release where dox is added, there is a shift in the start of the S phase. We clearly noticed a delay in the entry of the S phase (on the left side of the S phase, Fig 4.2) and we call it short fragment of replicated DNA (blue arrows represent the short fragment of replicated DNA, Fig. 4.2). We assume that the short fragment of replicated DNA occurs because the replication is halted and the cell cannot undergo efficient replication. The dox induced cells maybe have replicated small parts of DNA and they cannot replicate the larger ones, which are detected more on the right side of the histogram of this type of Flow Cytometry plot. This is compatible with the idea of replication-transcription conflict. In addition to the above, re-replication of DNA is possible to happen in the +dox induced cells probably due to the re-firing of ORCs because of CDC6 over-expression. However re-replication of the same short fragments could occur without the whole genome to be possibly replicated due to impeding by transcription-replication blocks and possible formation of double strand breaks. Furthermore, it is important to mention that in the most cases of the +dox induced cells, the cells that are in G2/M phase of the cell cycle are less than the non-inducted cells. This is probably happens due to the insufficient DNA replication.

Taking everything together, we reveal that CDC6 over-expression plays an essential role in the progress of the cell cycle and it drives the cells more quickly in the S phase

but is responsible for making short fragments of DNA that we cannot detect in the pattern of the cell cycle of the non-inducted with dox cells –control cells.

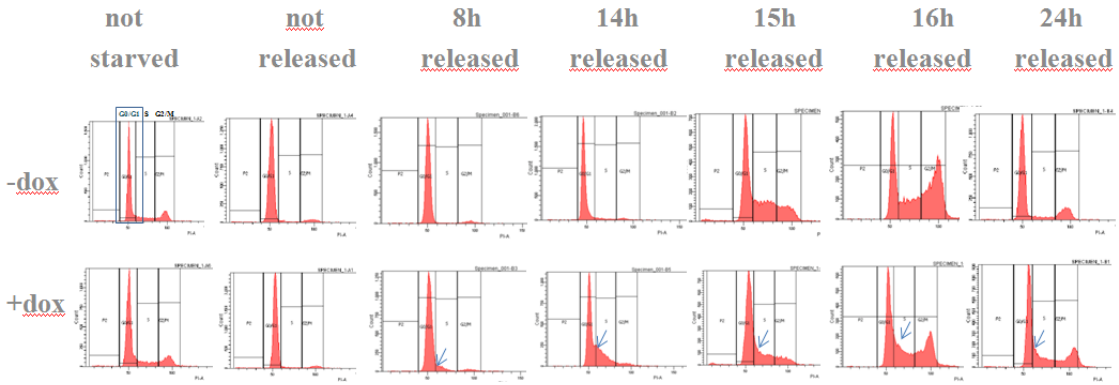


Figure 4.2: The FACS data. This figure reveals what happens in the cell cycle with and without doxycycline (CDC6 over-expression) induction in each experimental condition. The arrows represent the short fragment of replicated DNA (see text above).

	Not starved			Not released			8h released			14h released			15h released			16h released			24h released		
%	GO/G1	S	G2/M	GO/G1	S	G2/M	GO/G1	S	G2/M	GO/G1	S	G2/M	GO/G1	S	G2/M	GO/G1	S	G2/M	GO/G1	S	G2/M
w/o dox	66,9	12,85	17,7	91,6	2,3	4,9	94,8	0,7	4	90,9	5,3	3,4	47,3	30,5	19,1	30	21,4	45,5	85,3	1,9	12,1
with dox	68,8	12,3	16,8	91,8	2,5	4,2	86,8	8,8	3,7	62,4	30,7	6,1	59,6	24,7	12,2	39,5	26	31,4	73,9	12,4	12,9

Table 4.1: The percentages (%) of the cells that are in GI/G0, S and G2/M phase of the cell cycle, according to Flow Cytometry analysis.

4.4: Insights into the differentially expressed (DE) genes of the HBECs through the Gene Set Enrichment Analysis (GSEA) analysis.

We obtained and further analyzed the RNA-seq results from Komseli *et al.* 2018, that established a list of differentially expressed (DE) genes upon induction of CDC6 (3d ON vs OFF) in HBEC cells. In order to gain insights into the function of the DE genes, we performed a Gene Set Enrichment Analysis (GSEA) analysis. We first sorted DE genes by FC (normalized ratio of reads detected by RNA-seq between 3d ON and OFF conditions)-rank metric score from the most up-regulated to the most down-regulated. We ran the test for all the gene sets of GSEA database and summarize our main findings. One of the most interesting observation that derived from our results was the very significant positive correlation (p-val=0.0, NES= 3.3, ES=0.6) of the examined genes with genes that are found up-regulated in KRAS-dependent Lung-Breast cancer (a, Fig 4.3). In other words, most of the up-regulated genes in our case are also found to be up-regulated in Lung cancer tissues in comparison to normal samples. Some of those genes are the BBOX1, the GAS1, the CD36 and 32 more (data not shown).This result indicates that common genes are activated in our cell system and in cells undergoing KRAS-dependent transformation.

Therefore, CDC6 expression is sufficient to trigger, at least indirectly, the activation of key genes that participate in establishing cancer cells.

Furthermore, we noticed that the examined DE genes are significantly negative correlated with the set of genes that are found up-regulated during the Cell Cycle of normal samples (p-val=0.0, NES= -2.6, ES=-5.8 (b, figure 4.6). That means that there is a list of common genes that are down-regulated in the HBECs 3d ON and at the same time they are found up-regulated during the cell cycle of normal cells. Some of the most important genes are the CDK1 and the CDC25C from a list of 14 common genes in total (data not shown). This result suggests that CDC6 over-production affects the expression of several genes associated with the cell cycle and not to forget that the HBECs 3d ON according to Komseli *et al.*, 2008 are senescent cells meaning that they may have arrested their cycle.

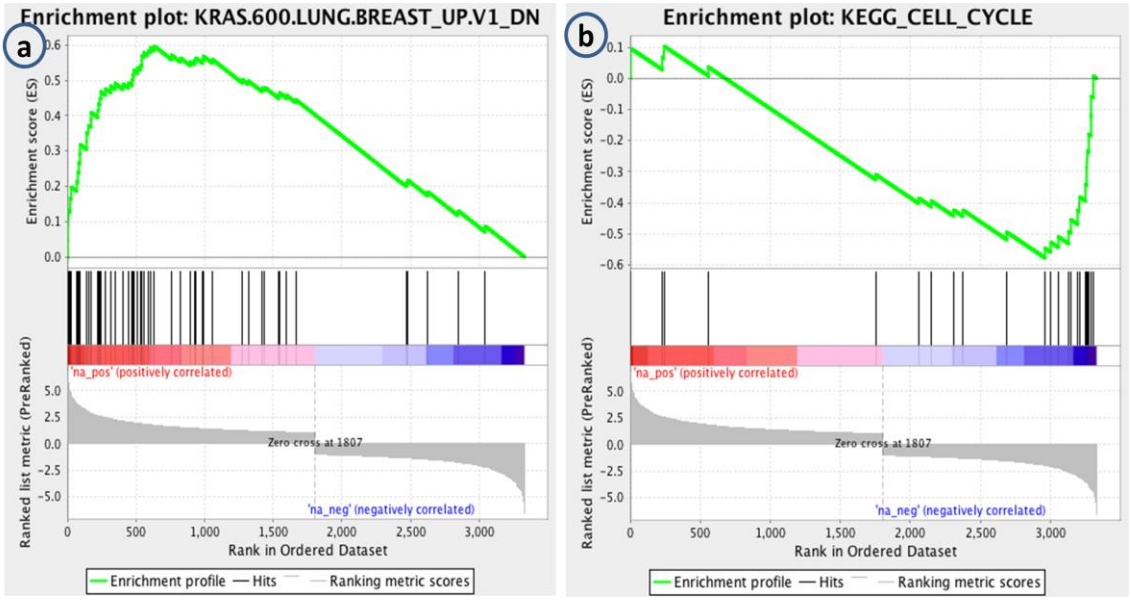


Figure 4.3: Enrichment plot of the DE of HBECs (3d ON/OFF) and the KRAS-dependent genes up-regulated in Lung cancer (a) and the down-regulated genes during the Cell Cycle (b).

4.5: Insights into the senescent state of the HBECs induced by CDC6 over-expression.

Lipofuscin is a marker of senescence which accumulates in the cells during the senescence process (Georgakopoulou *et al.*, 2013; Galanos *et al.*, 2016; Liakou *et al.*, 2016; Petrakis *et al.*, 2016; Evangelou *et al.*, 2016). Our lab synthesized GL13, a Sudan Black B (SSB) analogue which is connected with biotin, an easy detectable molecule by several immunohistochemical techniques (Evangelou *et al.*, 2016). GL13 is used for the detection of lipofuscin in the senescent cells and when 3,3'-Diaminobenzidine (DAB) is added to the sample according to the protocol described

in Material and Methods, the senescent cells are colored in brown whereas the normal cells are not (Evangelou *et al.*, 2016). In this study, HBEC cells induced with doxycycline for 6 days (dox is added per 2 days) and HBEC OFF cells (control) are stained for GL13.

We also recorded the phenotype before and after the cells are fixed with 4% PFA. PBS 1X washes are followed and then the staining as it mentioned above. After the staining, we show that the control cells are 100% confluent, which contrasts the status of HBEC 6d ON cells. In addition, the phenotype of 6d on cells comes in accordance with the theory of the senescent cells about the size, shape and structure. Debris are noticed in the two group of cells but are much increased in HBEC 6d ON, probably due to CDC6 overexpression and potential cell death. Concerning the GL13 staining, in 6d ON cells 'brown' cells – senescent cells are detectable (red arrows) whereas in OFF cells there are not such cells- see figure below.

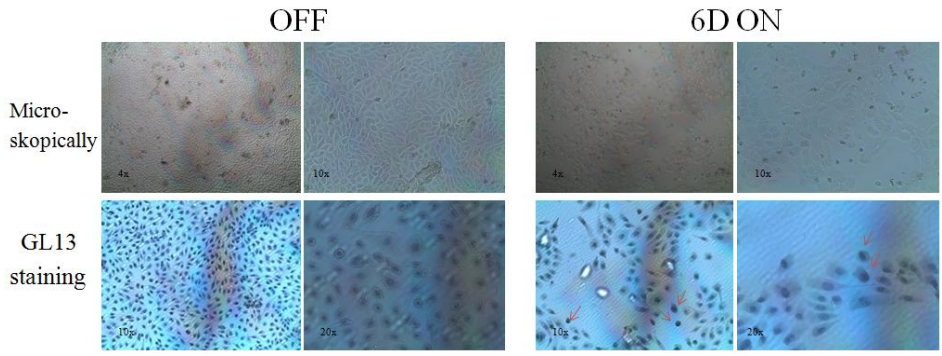


Figure 4.4: GL13 staining. The brown cells that are depicted in the figure are the senescent cells (red arrows).

4.6: CTCF binding is lost when CDC6 is over-expressed.

Chromatin immunoprecipitation (ChIP) followed by high-throughput sequencing (ChIP-Seq) is a new tool for studying chromatin binding states across the genome (Xu *et al.*, 2014). More specifically, ChIP assay is performed in HBEC OFF and HBEC CDC6 3 days ON cells in order to reveal the binding sites of CTCF across the genome and to find out the possible different binding patterns between these two different conditions (control vsCDC6 over-expression).

30.473 protein coding genes taken from the RefSeq database are studied at first level. Transcription start sites (TSS) and 2kb from both sides of TSS are studied extensively. K-means clustering (N=5) is conducted in order to detect probable differences in the binding sites of CTCF when CDC6 is over-expressed and when it is not. The below heat map presents 5 clusters where more intense CTCF binding is depicted with yellow color. Each horizontal line represents a gene fromTSS to 2kb from both sides of TSS. In the first three clusters the signal and the pattern of binding is similar in both conditions. However, in the fourth cluster where 7112 genes are

shown, the signal of CTCF binding is really strong in the HBEC OFF cells whereas in the HBEC CDC6 3 days ON cells the signal is significantly decreased. In other words, the signal of CTCF binding seems to be lost when there is a CDC6 over-expression for 3 days. In addition to the above, CTCF binding is restricted around TSS as it seems from the heat map. In the fifth cluster, CTCF is inactive in both conditions because there is no signal of CTCF binding.

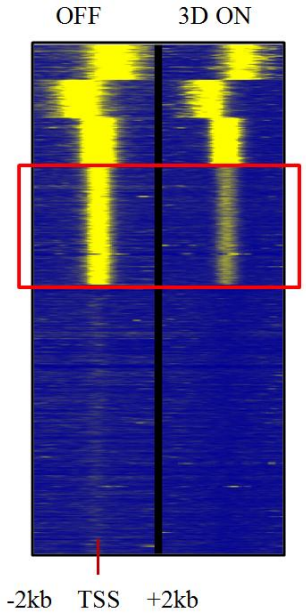


Figure 4.5: The binding sites of CTCF. Each horizontal line presents a TSS and 2kb from both sides of TSS. In the fourth cluster (red frame) a decrease in CTCF binding is observed in HBEC 3d on cells compared to the HBEC OFF (the control). In the other four clusters that are represented in the heatmap the CTCF binding pattern is similar in both conditions.

In the following figure, the average profile for 2858 coding genes of the third and 7112 genes of the fourth cluster of the above heat map is depicted. Importantly, the two samples (HBEC OFF and HBEC 3d on) have been normalized for the same number of reads sequenced. Strikingly, we confirm quantitatively that there is an important drop in CTCF binding in HBEC 3d ON cells compared to control cells for the 4^{th} cluster. Blue color is used for the control cells whereas pink for the HBEC 3d ON cells. This result suggests that the overexpression of CDC6 destabilizes the binding of CTCF to these loci and this may impact on the relative gene expression levels.

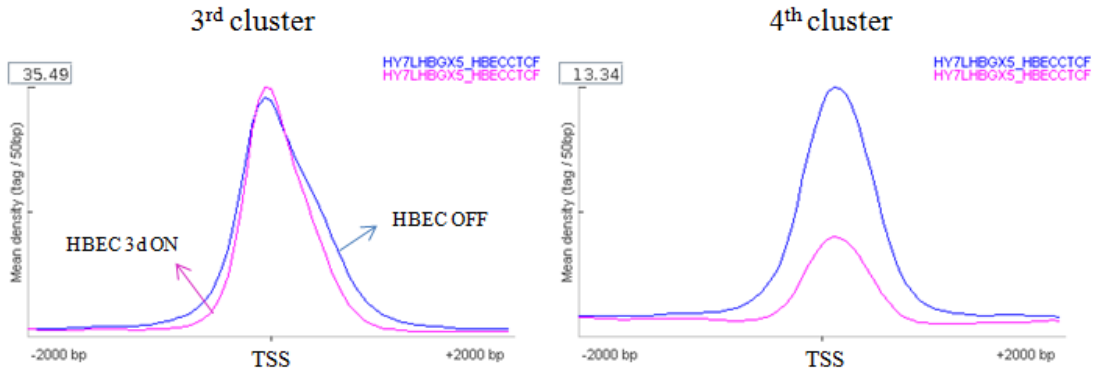


Figure 4.6: The average plot of CTCF binding on TSS and on 2kb from both sides of TSS of the 3rd and the 4th clusters of the heat map. It is clear that there is a drop of CTCF binding on TSS in the HBEC 3d ON cells.

4.7: DARs are annotated at important gene regulatory regions in HBECs 3d ON.

ATAC-seq data and RNA-seq data were analyzed to understand which genes are differentially regulated and differentially accessible in HBECs 3d ON compared to HBECs OFF (gain or loss of differentially accessible regions-DARs vs Up and Down regulation of the corresponding gene). The location of DARS in the genome can be annotated by the use of CEAS software.

We found that DARs that show gain of open chromatin sites (DAR^+) in HBECs 3d ON compared to controls, are mostly located throughout the genome with 49,1% in introns and 38,5% in distal intergenic sites (>10 kb from a gene). Interestingly 4, 4% of $DARs^+$ are annotated to promoters and 8% are located directly downstream of genes (Fig. 4.7). These regions are known to represent good candidate loci for gene regulation. Similarly, as for the loss of accessible regions (DAR^-) in HBECs 3d ON versus HBECs OFF, we find 54.5% of DARs at distal intergenic sites and 34, 7% in introns. 5% of DARs are located in promoters and 4, 9% downstream of promoters (Fig. 4.7).

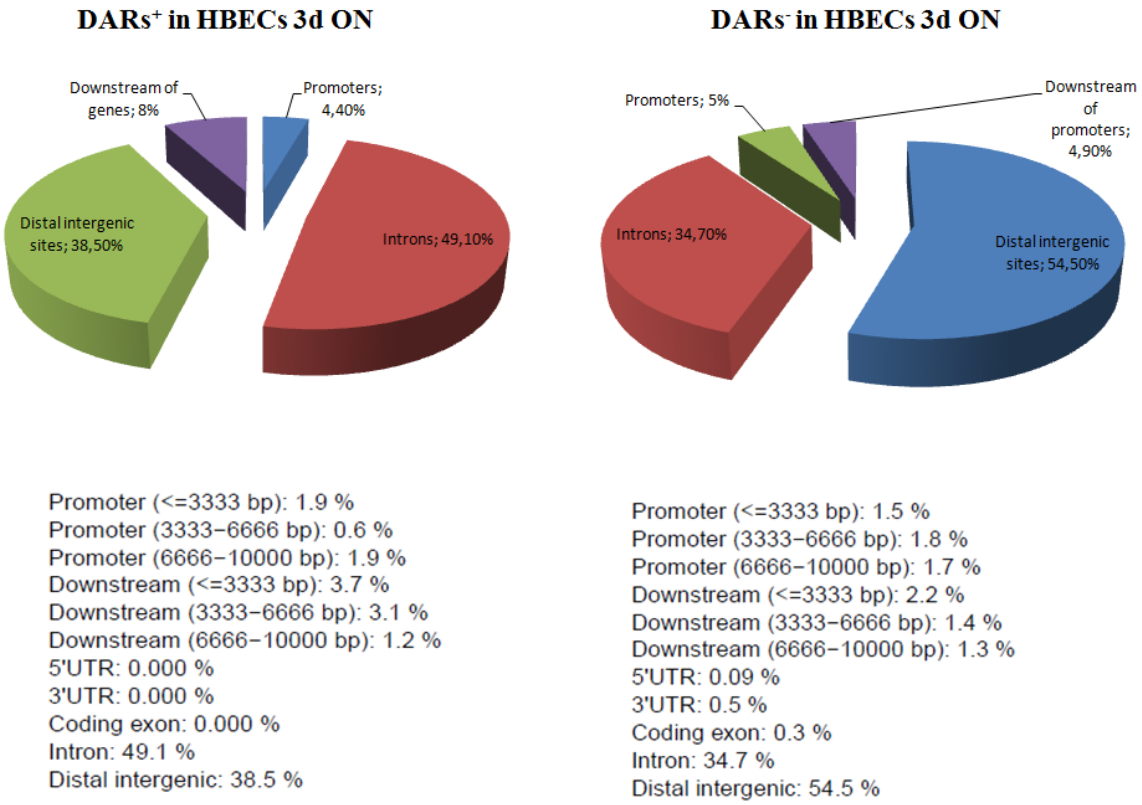


Figure 4.7: DARs annotation in the genome using CEASs software and representation of them using Venny.

.

4.8: An overall estimation of the results presented in this thesis using the UCSC Human Genome Browser (GRCh37/hg19).

We queried if the genes for which we find DARs also showed significant changes in gene expression by comparing gene lists in Venny and we show two examples of interesting cases.

In the first case, we show IP6K3 locus, for which we find that the gene is up-regulated in HBECs 3d ON (green bar, figure 4.8) as compared to control condition (HBEC OFF). According the ATAC-seq data, there are two DAR^+ (black boxes on the "ATAC ON gain" track) which mark a gain of accessibility in HBECs 3d ON compared to HBECs OFF (Note that the peaks height is different for the highlighted loci). We correlate the gain of ATAC-seq signal in HBECs 3d ON, (red frame, figure 4.8) to the possible recruitment of activating transcription factors and to the significant up-regulation of this gene as demonstrated by the presence of a green bar in the "Polyzos_ON_3d_Up" track. Importantly these $DARs^+$ are located near the promoter of the gene as expected from our CEAS results categorizing the locations of the analyzed DARs (figure 4.8). This type of figure also represents a good recapitulation of all the next generation sequencing (NGS) experiments presented in this thesis. Indeed, we observed that there is a drop of CTCF binding in HBECs 3d ON at a locus located nearby the IP6K3 gene, meaning that the TADs structure of this

locus may have been disturbed. We hypothesize that the enhancer and the promoter of the neighbor genes are differently associated after CDC6 induction and there is change in the expression and in the accessible chromatin profile.

Similarly, in the figure 4.9, the SQRDL gene locus is presented. This gene is down-regulated in the HBECs 3d ON compared to control cells (red bar) and corroboratively, we find that the HBEC OFF state is more accessible than the HBEC 3d ON (black frame) state (black bar above the ATAC-seq data). The loss of accessibility could be linked to the loss of binding of transcription activators, and the location of the DAR^{-} is again close to the gene promoter as predicted by our CEAS analysis. Finally, the binding of CTCF drops in HBEC 3d ON cells in contrast with the control cells, suggesting a correlation between TAD border location and cis-regulation of gene expression.

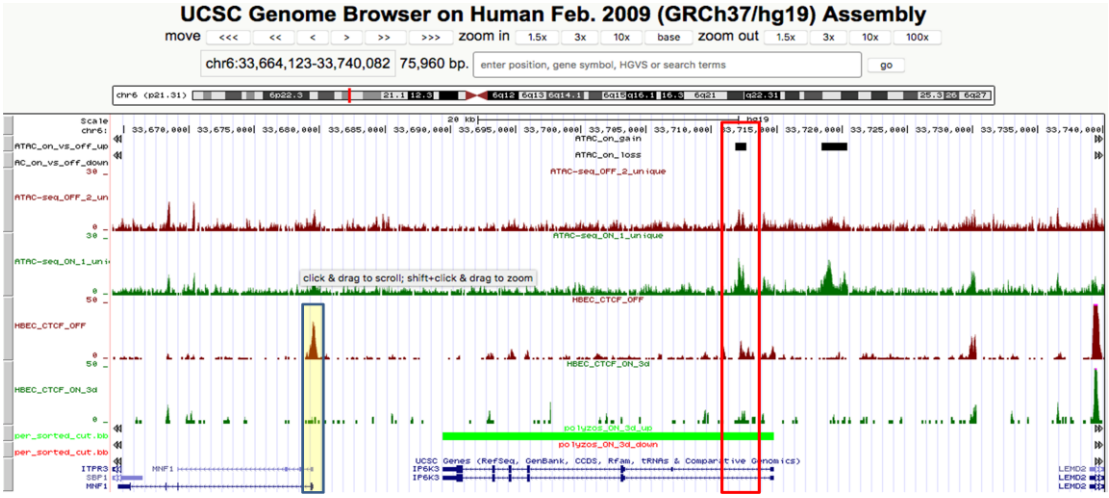


Figure 4.8: A chromosomal region of 75.960 bp is redicted. ATAC-seq, Chip-seq and CEAS data are presented. The gene IP6K3 is up-regulated in HBECs 3d ON.

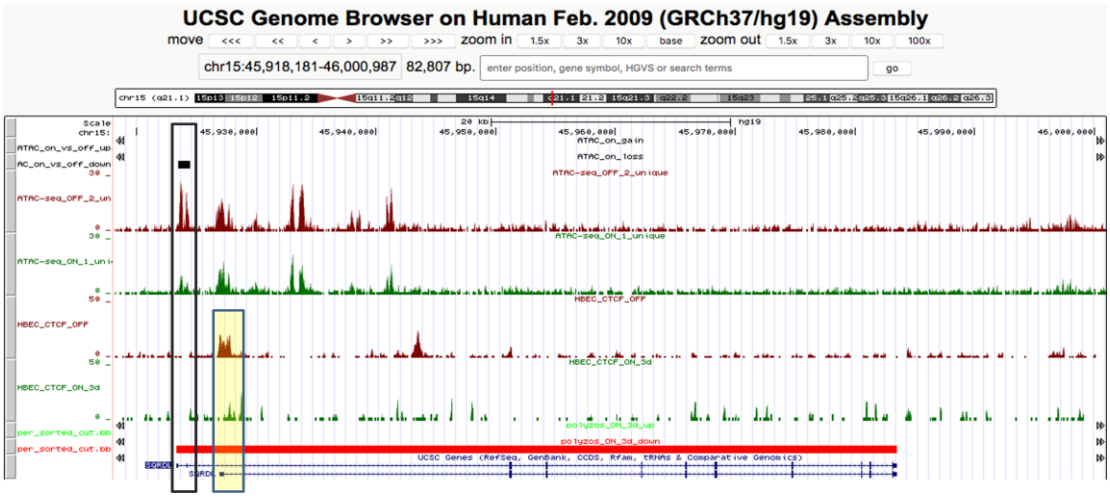


Figure 4.9: A chromosomal region of 75.960 bp is redicted. ATAC-seq, Chip-seq and CEAS data are presented. The gene SQRDL is down-regulated in HBECs 3d ON.

5. DISCUSSION

Cancer has been around since humanity began. It is a word given to a variety of diseases involving aberrant cellular growth that can potentially spread and threaten other tissues. It is the result of a long time process that typically happens when the cells of the human body cannot cooperate with other cells in harmony. Mutations accumulate in cells and transform them to cancerous. The fact that most cancers are of epithelial origin and the lack of cancerous epithelial models triggered our interest. The need of better understanding cancer initiation and development made us use an inducible cell system that over-expresses the replication licensing factor CDC6 which has been deregulated in several cancer types at the earliest stages of their development (Karakaidos *et al.,* 2004; Liontos *et al.,* 2007).

In this study a unique cellular tool to study carcinogenesis has been used. Immortalized Human bronchial epithelial cells (HBECs) with the ectopic expression of human telomerase (hTERT) (in order to prevent the replicative senescence (RS) that occurs because of telomere erosion) and of CDK4 (in order to overcome the problem of cell cycle arrest due to activation of the signaling pathway $p16^{INK4}$) have been used as a non-malignant model to study mostly the precancerous phase of epithelial tumorigenesis (Ramirez *et al.,* 2004; Evangelou *et al.,* 2013; Komseli *et al.,* 2018).

Oncogene-induced senescence (OIS) is a type of stress-induced premature senescence (SIPS) that has been examined in this thesis and according to theory; it acts as an anti-tumor barrier and must be bypassed for tumor progression (Serrano *et al.,* 1997; Braig *et al.,* 2005; Chen *et al.,* 2005; Collado *et al.,* 2005; Denchi *et al.,* 2005; Michaloglou *et al.,* 2005; Bartkova *et al.,* 2006; Di Micco *et al.,* 2006; Halazonetis *et al.,* 2008; Komseli *et al.,* 2018).

CDC6 is selected not only because of its important role as a replication licensing factor but also due to its deregulation in a variety of cancers. These observations triggered the interest of our lab for this molecule's role in preventing cancer initiation (Karakaidos *et al.,* 2004; Liontos *et al.,* 2007). Indeed, when CDC6 is over-expressed, it can cause re-replication and genomic instability (Bartkova *et al.,* 2006; Liontos *et al.,* 2007; Sideridou *et al.,* 2011; Galanos *et al.,* 2016; Komseli *et al.,* 2018). It can act as an oncogene but also as an expression regulator as it negatively controls the transcription of INK4/ARF which codes three important tumor suppressors by blocking the action of CTCF and it also represses the CDH1 locus which encodes E-cadherin (Hirohashi, 1998; Gonzalez *et al.,* 2006; Thiery *et al.,* 2009). Last but not least, CDC6 over-expression has been connected with the increase of rDNA transcription, probably due to increased cancer cell needs for protein synthesis (Huang *et al.,* 2016).

Indeed, in this thesis, we confirm that overexpression of CDC6 in a short period of time leads to senescence which is maintained for a prolonged period (precancerous phase). The cells are examined microscopically and changes in cell behavior and structure are observed in senescent cells. They become flat, enlarged, and often acquire multinucleated morphology. They cannot proliferate in the rate that control cells do, probably because of the extended growth arrest that characterize them. Interestingly, debris of cells are observed in the culture of HBEC 6d ON cells compared to HBEC OFF. This effect could be due to cell death although this hypothesis points against the claims that senescence brings resistance to apoptosis (Campisi *et al.,* 2007; Salminen *et al.,* 2012; Campisi,, 2013; Muñoz-Espín *et al.,* 2014; Childs *et al.,* 2015). Further experiments should be conducted to decipher if the cells that become senescent are only a proportion of the induced cells and if the rest of the cells that fail to senesce may favor the path of programmed cell death. Whether this path is apoptosis or is it necrosis, could be investigated as it is knowm that activation of Caspase-3 leads to apoptosis and it can be used as an important marker in order to clarify what really happens in the cell (Elmore, 2007).

GL13 is a biotin-connected Sudan Black B (SBB) analogue that detects lipofuscin which is present in senescent cells and is invented by our lab (Georgakopoulou *et al.,* 2013; Galanos *et al.,* 2016; Liakou *et al.,* 2016; Petrakis *et al.,* 2016; Evangelou *et al.,* 2016). After the Gl13 staining, the cytoplasm of the senescent cells is presented with brown color compared to the cytoplasm of control cells which is white (Evangelou *et al.,* 2016). HBEC OFF and HBEC 6d ON cells are used for the staining. It is known from previous work of our lab, that senescence fully appears (100% $GL13^+$ cells) 6 days after the first doxycycline induction in the HBEC cellular system (Komseli *et al.,* 2018). However, our data have shown that not 100% of the cells were detected as $GL13^+$. This could have happened because of the cellular system HBEC tet ON that used at the time of the experiments, was not optimal. Along this line of thinking, we discovered recently that the cells cannot homogeneously express CDC6 throughout the entire cell population upon doxycycline addition (data not show).

We successfully conducted a starving-release experiment for the first time using the HBEC system. One would have thought that, that kind of cells which are really sensitive, difficult to handle and to maintain in culture, should not survive the lack of supplements in the first hours after adding the starving medium (without the supplements-growth factors). To our surprise, we observed that cells can survive the starvation. In fact they can even proliferate to some extent until the growth factors that they have already up-taken run out. Of course as the time passes, the cells that are maintained in the starving medium cannot proliferate anymore. Furthermore, it is known that this type of handling causes stress to the cells but it is revealed from our data that when complete medium is added again, the cells can fully restore their proliferation. Most importantly, when complete medium is added again in the

presence of doxycycline, they cannot proliferate at the maximum rate, which is observed for the cells where only complete medium was added.

One of our major interests is to find out if the role of CDC6 as a transcription regulator affects only the expression of INK4/ARF, CDH1 and rDNA genes or CDC6 controls a global transcription program (Hirohashi, 1998; Gonzalez *et al.,* 2006; Thiery *et al.,* 2009; Sideridou *et al.,* 2011; Huang *et al.,* 2016). If so, does the mechanism involves the removal of CTCF as it happens in the case of INK4 / ARF and CDH1 loci or does not? Our first preliminary experiment in order to clarify this, is the CHIP-seq experiment for the CTCF which is conducted in HBEC OFF and HBEC 3D ON cells. More specifically, 30.473 protein coding genes taken from the RefSeq database have been examined at first level. We focus our study on the transcription start sites (TSS) and on 2kb from both sides of TSS. K-means clustering (N=5) is performed and although the binding of CTCF in both conditions seems to be about the same, there is one cluster (4^{th} cluster-7112 examined genes) that shows that the signal of CTCF is weak in the HBEC CDC6 3 days ON cells compared to HBEC OFF. Our data come in an agreement with the theory. When there is a CDC6 over-expression the binding of CTCF is lost exactly as it happens in the INK4 / ARF and CDH1 loci. However, further experiments should be performed in combination with CHIP-seq for the CDC6 and with ATAC-seq in both conditions (control and CDC6 overexpression) in order to study the accessibility of the chromatin and to deepen our knowledge about this particular multifactorial protein called CDC6.

The Flow Cytometry experiment reveled that CDC6 over-expression (via dox induction) does indeed have an impact on the progress of the cell cycle. CDC6 when is over-produced, is the driving force that leads cells into the S phase more quickly than the cells that express CDC6 in normal levels. Although, CDC6 facilitates the entry to S phase, it seems to make short fragments of DNA and the replication cannot be completed as it should be. The control cells-non dox inducted, do not present this shift in the start of their S phase. The above data agree with the theory of replication-transcription conflict due to the re-firing of the ORCs because of aberrant CDC6 production.

GSEA analysis has shown that there is a positive or a negative correlation of the genes found in HBECs 3d ON cells with the genes examined from the GSEA database each time. Regarding the ATAC-analysis, it has been shown that there are differentially accessible regions between the control and the cells that overexpress CDC6, meaning that the two group of cells present different expression profiles.

To sum up, CDC6 is an essential factor in a cell's life and its deregulation often leads to cancer. The experiments described above are preliminary and in the future we aim to deepen our knowledge about this multifactorial molecule. How CDC6 overexpression influences the cell cycle, what happens to the chromatin structure and how CTCF is affected by the CDC6 over-production and which molecular pathways

are activated during the CDC6-induced senescence are some of the questions we are going to answer.

6. REFERENCES

Abbas, T., Keaton, M. A., & Dutta, A. (2013). Genomic instability in cancer. *Cold Spring Harbor perspectives in biology*, *5*(3), a012914.

Alberts, B., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2002). Molecular Biology of the Cell, (Garland Science, New York, 2008). *Google Scholar*, 652.

Alexandrow, M. G., & Hamlin, J. L. (2004). Cdc6 chromatin affinity is unaffected by serine-54 phosphorylation, S-phase progression, and overexpression of cyclin A. *Molecular and cellular biology*, *24*(4), 1614-1627.

Arney, K. L., & Fisher, A. G. (2004). Epigenetic aspects of differentiation. *Journal of Cell Science*, *117*(19), 4355-4363

Barnum, K. J., & O'Connell, M. J. (2014). Cell cycle regulation by checkpoints. In *Cell Cycle Control* (pp. 29-40). Humana Press, New York, NY.

Barry, E. R., & Bell, S. D. (2006). DNA replication in the archaea. *Microbiology and Molecular Biology Reviews*, *70*(4), 876-887.

Bartkova, J., Rezaei, N., Liontos, M., Karakaidos, P., Kletsas, D., Issaeva, N., ... & Takaoka, M. (2006). Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*, *444*(7119), 633.

Barski, A., Cuddapah, S., Cui, K., Roh, T. Y., Schones, D. E., Wang, Z., ... & Zhao, K. (2007). High-resolution profiling of histone methylations in the human genome. *Cell*, *129*(4), 823-837.

Bazett-Jones, D. P., Leblanc, B., Herfort, M., & Moss, T. (1994). Short-range DNA looping by the Xenopus HMG-box transcription factor, xUBF. *Science*, *264*(5162), 1134-1137.

Bell, S. P., Learned, R. M., Jantzen, H. M., & Tjian, R. (1988). Functional cooperativity between transcription factors UBF1 and SL1 mediates human ribosomal RNA synthesis. *Science*, *241*(4870), 1192-1197.

Bell, S. P., & Dutta, A. (2002). DNA replication in eukaryotic cells. *Annual review of biochemistry*, *71*(1), 333-374.

Bell, S. P., & Labib, K. (2016). Chromosome duplication in Saccharomyces cerevisiae. *Genetics*, *203*(3), 1027-1067.

Bermejo, R., Vilaboa, N., & Calés, C. (2002). Regulation of CDC6, geminin, and CDT1 in human cells that undergo polyploidization. *Molecular biology of the cell*, *13*(11), 3989-4000.

Besson, A., Dowdy, S. F., & Roberts, J. M. (2008). CDK inhibitors: cell cycle regulators and beyond. *Developmental cell*, *14*(2), 159-169.

Blanton, J., Gaszner, M., & Schedl, P. (2003). Protein: protein interactions and the pairing of boundary elements in vivo. *Genes & development*, *17*(5), 664-675.

Blow, J. J., & Gillespie, P. J. (2008). Replication licensing and cancer—a fatal entanglement?. *Nature reviews Cancer*, *8*(10), 799.

Borlado, L. R., & Méndez, J. (2007). CDC6: from DNA replication to cell cycle checkpoints and oncogenesis. *Carcinogenesis*, *29*(2), 237-243.

Bowers, J. L., Randell, J. C., Chen, S., & Bell, S. P. (2004). ATP hydrolysis by ORC catalyzes reiterative Mcm2-7 assembly at a defined origin of replication. *Molecular cell*, *16*(6), 967-978.

Braig, M., Lee, S., Loddenkemper, C., Rudolph, C., Peters, A. H., Schlegelberger, B., ... & Schmitt, C. A. (2005). Oncogene-induced senescence as an initial barrier in lymphoma development. *Nature*, *436*(7051), 660.

Bruce, A., Johnson, A., Lewis, J., Raff, M., Roberts, K., & Walter, P. (2007). Molecular Biology of the Cell 5th edn (New York: Garland Science).

Buenrostro, J. D., Giresi, P. G., Zaba, L. C., Chang, H. Y., & Greenleaf, W. J. (2013). Transposition of native chromatin for fast and sensitive epigenomic profiling of open chromatin, DNA-binding proteins and nucleosome position. *Nature methods*, *10*(12), 1213.

Campisi, J., & di Fagagna, F. D. A. (2007). Cellular senescence: when bad things happen to good cells. *Nature reviews Molecular cell biology*, *8*(9), 729.

Campisi, J. (2013). Aging, cellular senescence, and cancer. *Annual review of physiology*, *75*, 685-705.

Campisi, J., & Robert, L. (2014). Cell senescence: role in aging and age-related diseases. In *Aging* (Vol. 39, pp. 45-61). Karger Publishers.

Campos, E. I., & Reinberg, D. (2009). Histones: annotating chromatin. *Annual review of genetics*, *43*.

Chang, F., May, C. D., Hoggard, T., Miller, J., Fox, C. A., & Weinreich, M. (2011). High-resolution analysis of four efficient yeast replication origins reveals new insights into the ORC and putative MCM binding elements. *Nucleic acids research*, *39*(15), 6523-6535.

Chen, H., Tini, M., & Evans, R. M. (2001). HATs on and beyond chromatin. *Current opinion in cell biology*, *13*(2), 218-224.

Chen, Z., Trotman, L. C., Shaffer, D., Lin, H. K., Dotan, Z. A., Niki, M., ... & Cordon-Cardo, C. (2005). Crucial role of p53-dependent cellular senescence in suppression of Pten-deficient tumorigenesis. *Nature*, *436*(7051), 725.

Chen, S., de Vries, M. A., & Bell, S. P. (2007). Orc6 is required for dynamic recruitment of Cdt1 during repeated Mcm2–7 loading. *Genes & development*, *21*(22), 2897-2907.

Childs, B. G., Durik, M., Baker, D. J., & Van Deursen, J. M. (2015). Cellular senescence in aging and age-related disease: from mechanisms to therapy. *Nature medicine*, *21*(12), 1424.

Cook, J. G., Park, C. H., Burke, T. W., Leone, G., DeGregori, J., Engel, A., & Nevins, J. R. (2002). Analysis of Cdc6 function in the assembly of mammalian prereplication complexes. *Proceedings of the National Academy of Sciences*, *99*(3), 1347-1352.

Collado, M., Gil, J., Efeyan, A., Guerra, C. J. S. A., Barradas, M., Benguria, A., ... & Serrano, M. (2005). Identification of senescent cells in premalignant tumours. *Nature*, *436*, 642.

Coppé, J. P., Patil, C. K., Rodier, F., Sun, Y., Muñoz, D. P., Goldstein, J., ... & Campisi, J. (2008). Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. *PLoS biology*, *6*(12), e301.

Creyghton, M. P., Cheng, A. W., Welstead, G. G., Kooistra, T., Carey, B. W., Steine, E. J., ... & Boyer, L. A. (2010). Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proceedings of the National Academy of Sciences*, *107*(50), 21931-21936.

Crowley, L. C., Marfell, B. J., Christensen, M. E., & Waterhouse, N. J. (2016). Measuring cell death by trypan blue uptake and light microscopy. *Cold Spring Harbor Protocols*, *2016*(7), pdb-prot087155.

Crutchley, J. L., Wang, X. Q. D., Ferraiuolo, M. A., & Dostie, J. (2010). Chromatin conformation signatures: ideal human disease biomarkers?. *Biomarkers in medicine*, *4*(4), 611-629.

Cutter, A. R., & Hayes, J. J. (2015). A brief review of nucleosome structure. *FEBS letters*, *589*(20), 2914-2922.

Dekker, J., & Misteli, T. (2015). Long-range chromatin interactions. *Cold Spring Harbor perspectives in biology*, *7*(10), a019356.

De Laat, W., & Dekker, J. (2012). 3C-based technologies to study the shape of the genome. *Methods (San Diego, Calif.)*, *58*(3).

Denchi, E. L., Attwooll, C., Pasini, D., & Helin, K. (2005). Deregulated E2F activity induces hyperplasia and senescence-like features in the mouse pituitary gland. *Molecular and cellular biology*, *25*(7), 2660-2672.

Denhardt, D. T. (1999). Signal transduction pathways and regulation of the mammalian cell cycle: cell type-dependent integration of external signals. *The Molecular Basis of Cell Cycle and Growth Control*, 225-304.

De Pamphilis, M. L. (2003). The 'ORC cycle': a novel pathway for regulating eukaryotic DNA replication. *Gene*, *310*, 1-15.

De Pamphilis Melvin L. (Ed.). (2006). *DNA replication and human disease*. CSHL Press.

Devbhandari, S., Jiang, J., Kumar, C., Whitehouse, I., & Remus, D. (2017). Chromatin constrains the initiation and elongation of DNA replication. *Molecular cell*, *65*(1), 131-141.

Diffley, J. F., Cocker, J. H., Dowell, S. J., & Rowley, A. (1994). Two steps in the assembly of complexes at yeast replication origins in vivo. *Cell*, *78*(2), 303-316.

Di Leonardo, A., Linke, S. P., Clarkin, K., & Wahl, G. M. (1994). DNA damage triggers a prolonged p53-dependent G1 arrest and long-term induction of Cip1 in normal human fibroblasts. *Genes & development*, *8*(21), 2540-2551.

Di Micco, R., Fumagalli, M., Cicalese, A., Piccinin, S., Gasparini, P., Luise, C., ... & Pelicci, P. G. (2006). Oncogene-induced senescence is a DNA damage response triggered by DNA hyper-replication. *Nature*, *444*(7119), 638.

Dimri, G. P., Lee, X., Basile, G., Acosta, M., Scott, G., Roskelley, C., ... & Pereira-Smith, O. (1995). A biomarker that identifies senescent human cells in culture and in aging skin in vivo. *Proceedings of the National Academy of Sciences*, *92*(20), 9363-9367.

Dimri, G. P., Itahana, K., Acosta, M., & Campisi, J. (2000). Regulation of a senescence checkpoint response by the E2F1 transcription factor and p14ARF tumor suppressor. *Molecular and cellular biology*, *20*(1), 273-285.

Dixon, J. R., Selvaraj, S., Yue, F., Kim, A., Li, Y., Shen, Y., ... & Ren, B. (2012). Topological domains in mammalian genomes identified by analysis of chromatin interactions. *Nature*, *485*(7398), 376.

Dixon, J. R., Jung, I., Selvaraj, S., Shen, Y., Antosiewicz-Bourget, J. E., Lee, A. Y., ... & Diao, Y. (2015). Chromatin architecture reorganization during stem cell differentiation. *Nature*, *518*(7539), 331.

Dörr, J. R., Yu, Y., Milanovic, M., Beuster, G., Zasada, C., Däbritz, J. H. M., ... & Kratzat, S. (2013). Synthetic lethal metabolic targeting of cellular senescence in cancer therapy. *Nature*, *501*(7467), 421.

Dowen, J. M., Fan, Z. P., Hnisz, D., Ren, G., Abraham, B. J., Zhang, L. N., ... & Young, R. A. (2014). Control of cell identity genes occurs in insulated neighborhoods in mammalian chromosomes. *Cell*, *159*(2), 374-387.

Eberharter, A., & Becker, P. B. (2002). Histone acetylation: a switch between repressive and permissive chromatin: Second in review series on chromatin dynamics. *EMBO reports*, *3*(3), 224-229.

Ekholm-Reed, S., Méndez, J., Tedesco, D., Zetterberg, A., Stillman, B., & Reed, S. I. (2004). Deregulation of cyclin E in human cells interferes with prereplication complex assembly. *The Journal of cell biology*, *165*(6), 789-800.

Elmore, S. (2007). Apoptosis: a review of programmed cell death. *Toxicologic pathology*, *35*(4), 495-516.

Evangelou, K., Bartkova, J., Kotsinas, A., Pateras, I. S., Liontos, M., Velimezi, G., ... & Andersen, C. L. (2013). The DNA damage checkpoint precedes activation of ARF in response to escalating oncogenic stress during tumorigenesis. *Cell death and differentiation*, *20*(11), 1485.

Evrin, C., Clarke, P., Zech, J., Lurz, R., Sun, J., Uhle, S., ... & Speck, C. (2009). A double-hexameric MCM2-7 complex is loaded onto origin DNA during licensing of eukaryotic DNA replication. *Proceedings of the National Academy of Sciences*, *106*(48), 20240-20245.

Ferraiuolo, M. A., Sanyal, A., Naumova, N., Dekker, J., & Dostie, J. (2012). Mapping chromatin interactions with 5C technology: 5C; a quantitative approach to capturing chromatin conformation over large genomic distances. *Methods (San Diego, Calif.)*, *58*(3).

Filippova, G. N., Fagerlie, S., Klenova, E. M., Myers, C., Dehner, Y., Goodwin, G., ... & Lobanenkov, V. V. (1996). An exceptionally conserved transcriptional repressor, CTCF, employs different combinations of zinc fingers to bind diverged promoter sequences of avian and mammalian c-myc oncogenes. *Molecular and cellular biology*, *16*(6), 2802-2813.

Fletcher, T. M., & Hansen, J. C. (1996). The nucleosomal array: structure/function relationships. *Critical Reviews™ in Eukaryotic Gene Expression*, *6*(2-3).

Fragkos, M., Ganier, O., Coulombe, P., & Méchali, M. (2015). DNA replication origin activation in space and time. *Nature Reviews Molecular Cell Biology*, *16*(6), 360.

Galanos, P., Vougas, K., Walter, D., Polyzos, A., Maya-Mendoza, A., Haagensen, E. J., ... & Canovas, B. (2016). Chronic p53-independent p21 expression causes genomic instability by deregulating replication licensing. *Nature cell biology*, *18*(7), 777.

Ge, X. Q., Jackson, D. A., & Blow, J. J. (2007). Dormant origins licensed by excess Mcm2–7 are required for human cells to survive replicative stress. *Genes & development*, *21*(24), 3331-3341.

Georgakopoulou, E. A., Tsimaratou, K., Evangelou, K., Fernandez, M. P., Zoumpourlis, V., Trougakos, I. P., ... & Gorgoulis, V. G. (2013). Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A method applicable in cryo-preserved and archival tissues. *Aging (Albany NY)*, *5*(1), 37.

Gonzalez, S., Klatt, P., Delgado, S., Conde, E., Lopez-Rios, F., Sanchez-Cespedes, M., ... & Serrano, M. (2006). Oncogenic activity of Cdc6 through repression of the INK4/ARF locus. *Nature*, *440*(7084), 702.

Gopinathan, L., Ratnacaram, C. K., & Kaldis, P. (2011). Established and novel Cdk/cyclin complexes regulating the cell cycle and development. In *Cell Cycle in Development* (pp. 365-389). Springer, Berlin, Heidelberg.

Gorgoulis, V. G., Vassiliou, L. V. F., Karakaidos, P., Zacharatos, P., Kotsinas, A., Liloglou, T., ... & Kletsas, D. (2005). Activation of the DNA damage checkpoint and genomic instability in human precancerous lesions. *Nature*, *434*(7035), 907.

Hadjur, S., Williams, L. M., Ryan, N. K., Cobb, B. S., Sexton, T., Fraser, P., ... & Merkenschlager, M. (2009). Cohesins form chromosomal cis-interactions at the developmentally regulated IFNG locus. *Nature*, *460*(7253), 410.

Halazonetis, T. D., Gorgoulis, V. G., & Bartek, J. (2008). An oncogene-induced DNA damage model for cancer development. *science*, *319*(5868), 1352-1355.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *cell*, *144*(5), 646-674.

Handoko, L., Xu, H., Li, G., Ngan, C. Y., Chew, E., Schnapp, M., ... & Wong, E. (2011). CTCF-mediated functional chromatin interactome in pluripotent cells. *Nature genetics*, *43*(7), 630.

Hartwell, L. H., & Weinert, T. A. (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science*, *246*(4930), 629-634.

Hateboer, G., Wobst, A., Petersen, B. O., Le Cam, L., Vigo, E., Sardet, C., & Helin, K. (1998). Cell Cycle-Regulated Expression of MammalianCDC6 Is Dependent on E2F. *Molecular and cellular biology*, *18*(11), 6679-6697.

Hayes, J. J., & Hansen, J. C. (2001). Nucleosomes and the chromatin fiber. *Current opinion in genetics & development*, *11*(2), 124-129.

Hayflick, L., & Moorhead, P. S. (1961). The serial cultivation of human diploid cell strains. *Experimental cell research*, *25*(3), 585-621.

He, S., & Sharpless, N. E. (2017). Senescence in Health and Disease. *Cell*, *169*(6), 1000–1011.

Heintzman, N. D., Hon, G. C., Hawkins, R. D., Kheradpour, P., Stark, A., Harp, L. F., ... & Ching, K. A. (2009). Histone modifications at human enhancers reflect global cell-type-specific gene expression. *Nature*, *459*(7243), 108.

Heller, R. C., Kang, S., Lam, W. M., Chen, S., Chan, C. S., & Bell, S. P. (2011). Eukaryotic origin-dependent DNA replication in vitro reveals sequential action of DDK and S-CDK kinases. *Cell*, *146*(1), 80-91.

Hemann, M. T., Strong, M. A., Hao, L. Y., & Greider, C. W. (2001). The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell*, *107*(1), 67-77.

Herbig, U., Marlar, C. A., & Fanning, E. (1999). The Cdc6 Nucleotide–Binding Site Regulates Its Activity in DNA Replication in Human Cells. *Molecular biology of the cell*, *10*(8), 2631-2645.

Hirohashi, S. (1998). Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *The American journal of pathology*, *153*(2), 333-339.

Hnisz, D., Day, D. S., & Young, R. A. (2016). Insulated neighborhoods: structural and functional units of mammalian gene control. *Cell*, *167*(5), 1188-1200.

Holwerda, S. J. B., & de Laat, W. (2013). CTCF: the protein, the binding partners, the binding sites and their chromatin loops. *Phil. Trans. R. Soc. B*, *368*(1620), 20120369.

Huang, S., Xu, X., Wang, G., Lu, G., Xie, W., Tao, W., ... & Zhang, C. (2016). DNA replication initiator Cdc6 also regulates ribosomal DNA transcription initiation. *J Cell Sci*, jcs-178723.

Hunter, T., & Pines, J. (1994). Cyclins and cancer II: cyclin D and CDK inhibitors come of age. *Cell*, *79*(4), 573-582.

Ilves, I., Petojevic, T., Pesavento, J. J., & Botchan, M. R. (2010). Activation of the MCM2-7 helicase by association with Cdc45 and GINS proteins. *Molecular cell*, *37*(2), 247-258.

Im, J. S., Ki, S. H., Farina, A., Jung, D. S., Hurwitz, J., & Lee, J. K. (2009). Assembly of the Cdc45-Mcm2–7-GINS complex in human cells requires the Ctf4/And-1, RecQL4, and Mcm10 proteins. *Proceedings of the National Academy of Sciences*, *106*(37), 15628-15632.

Izdebska, M., Gagat, M., & Grzanka, A. (2018). Overexpression of lamin B1 induces mitotic catastrophe in colon cancer LoVo cells and is associated with worse clinical outcomes. *International journal of oncology*, *52*(1), 89-102.

Ji, X., Dadon, D. B., Powell, B. E., Fan, Z. P., Borges-Rivera, D., Shachar, S., ... & Misteli, T. (2016). 3D chromosome regulatory landscape of human pluripotent cells. *Cell stem cell*, *18*(2), 262-275.

Johansson, M., & Persson, J. L. (2008). Cancer therapy: targeting cell cycle regulators. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, *8*(7), 723-731.

Kagey, M. H., Newman, J. J., Bilodeau, S., Zhan, Y., Orlando, D. A., van Berkum, N. L., ... & Taatjes, D. J. (2010). Mediator and cohesin connect gene expression and chromatin architecture. *Nature*, *467*(7314), 430.

Kalluri, R., & Neilson, E. G. (2003). Epithelial-mesenchymal transition and its implications for fibrosis. *The Journal of clinical investigation*, *112*(12), 1776-1784.

Kalluri, R., & Weinberg, R. A. (2009). The basics of epithelial-mesenchymal transition. *The Journal of clinical investigation*, *119*(6), 1420-1428.

Kang, S., Warner, M. D., & Bell, S. P. (2014). Multiple functions for Mcm2–7 ATPase motifs during replication initiation. *Molecular cell*, *55*(5), 655-665

Karakaidos, P., Taraviras, S., Vassiliou, L. V., Zacharatos, P., Kastrinakis, N. G., Kougiou, D., ... & Gorgoulis, V. G. (2004). Overexpression of the replication licensing regulators hCdt1 and hCdc6 characterizes a subset of non-small-cell lung carcinomas: synergistic effect with mutant p53 on tumor growth and chromosomal instability—evidence of E2F-1 transcriptional control over hCdt1. *The American journal of pathology*, *165*(4), 1351-1365.

Kawakami, H., & Katayama, T. (2010). DnaA, ORC, and Cdc6: similarity beyond the domains of life and diversity. *Biochemistry and cell biology*, *88*(1), 49-62.

Klenova, E. M., Nicolas, R. H., Paterson, H. F., Carne, A. F., Heath, C. M., Goodwin, G. H., ... & Lobanenkov, V. V. (1993). CTCF, a conserved nuclear factor required for optimal transcriptional activity of the chicken c-myc gene, is an 11-Zn-finger protein differentially expressed in multiple forms. *Molecular and cellular biology*, *13*(12), 7612-7624.

Khochbin, S., Verdel, A., Lemercier, C., & Seigneurin-Berny, D. (2001). Functional significance of histone deacetylase diversity. *Current opinion in genetics & development*, *11*(2), 162-166.

Kim, W. Y., & Sharpless, N. E. (2006). The regulation of INK4/ARF in cancer and aging. *Cell*, *127*(2), 265-275.

Kim, T. H., Abdullaev, Z. K., Smith, A. D., Ching, K. A., Loukinov, D. I., Green, R. D., ... & Ren, B. (2007). Analysis of the vertebrate insulator protein CTCF-binding sites in the human genome. *Cell*, *128*(6), 1231-1245.

Klotz-Noack, K., McIntosh, D., Schurch, N., Pratt, N., & Blow, J. J. (2012). Re-replication induced by geminin depletion occurs from G2 and is enhanced by checkpoint activation. *J Cell Sci*, jcs-100883.

Komseli, E. S., Pateras, I. S., Krejsgaard, T., Stawiski, K., Rizou, S. V., Polyzos, A., ... & Zampetidis, C. P. (2018). A prototypical non-malignant epithelial model to study genome dynamics and concurrently monitor micro-RNAs and proteins in situ during oncogene-induced senescence. *BMC genomics*, *19*(1), 37.

Kosak, S. T., & Groudine, M. (2004). Form follows function: the genomic organization of cellular differentiation. *Genes & development*, *18*(12), 1371-1384.

Krishnamurthy, J., Torrice, C., Ramsey, M. R., Kovalev, G. I., Al-Regaiey, K., Su, L., & Sharpless, N. E. (2004). Ink4a/Arf expression is a biomarker of aging. *The Journal of clinical investigation*, *114*(9), 1299-1307.

Kumagai, A., Shevchenko, A., Shevchenko, A., & Dunphy, W. G. (2010). Treslin collaborates with TopBP1 in triggering the initiation of DNA replication. *Cell*, *140*(3), 349-359 .

Kumagai, A., Shevchenko, A., Shevchenko, A., & Dunphy, W. G. (2011). Direct regulation of Treslin by cyclin-dependent kinase is essential for the onset of DNA replication. *The Journal of cell biology*, jcb-201102003.

Kunkel, T. A., & Burgers, P. M. (2008). Dividing the workload at a eukaryotic replication fork. *Trends in cell biology*, *18*(11), 521-527.

Laskey, R. A., & Madine, M. A. (2003). A rotary pumping model for helicase function of MCM proteins at a distance from replication forks. *EMBO reports*, *4*(1), 26-30.

Lavigne, M. D., Vatsellas, G., Polyzos, A., Mantouvalou, E., Sianidis, G., Maraziotis, I., ... & Thanos, D. (2015). Composite macroH2A/NRF-1 nucleosomes suppress noise and generate robustness in gene expression. *Cell reports*, *11*(7), 1090-1101.

Lavigne, M. D., Konstantopoulos, D., Ntakou-Zamplara, K. Z., Liakos, A., & Fousteri, M. (2017). Global unleashing of transcription elongation waves in response to genotoxic stress restricts somatic mutation rate. *Nature communications*, *8*(1), 2076.

Lee, M. H., Reynisdottir, I., & Massague, J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes & development*, *9*(6), 639-649.

Lengronne, A., & Schwob, E. (2002). The yeast CDK inhibitor Sic1 prevents genomic instability by promoting replication origin licensing in late G 1. *Molecular cell*, *9*(5), 1067-1078.

Liakou, E., Mavrogonatou, E., Pratsinis, H., Rizou, S., Evangelou, K., Panagiotou, P. N., ... & Kletsas, D. (2016). Ionizing radiation-mediated premature senescence and paracrine interactions with cancer cells enhance the expression of syndecan 1 in human breast stromal fibroblasts: the role of TGF-β. *Aging (Albany NY)*, *8*(8), 1650.

Lim, S., & Kaldis, P. (2013). Cdks, cyclins and CKIs: roles beyond cell cycle regulation. *Development*, *140*(15), 3079-3093.

Lin, A. W., Barradas, M., Stone, J. C., van Aelst, L., Serrano, M., & Lowe, S. W. (1998). Premature senescence involving p53 and p16 is activated in response to constitutive MEK/MAPK mitogenic signaling. *Genes & development*, *12*(19), 3008-3019.

Liontos, M., Koutsami, M., Sideridou, M., Evangelou, K., Kletsas, D., Levy, B., ... & Lygerou, Z. (2007). Deregulated overexpression of hCdt1 and hCdc6 promotes malignant behavior. *Cancer research*, *67*(22), 10899-10909.

Liu, J., Smith, C. L., DeRyckere, D., DeAngelis, K., Martin, G. S., & Berger, J. M. (2000). Structure and function of Cdc6/Cdc18: implications for origin recognition and checkpoint control. *Molecular cell*, *6*(3), 637-648.

Liu, Y., Sanoff, H. K., Cho, H., Burd, C. E., Torrice, C., Ibrahim, J. G., ... & Sharpless, N. E. (2009). Expression of p16INK4a in peripheral blood T-cells is a biomarker of human aging. *Aging cell*, *8*(4), 439-448.

Lobanenkov, V. V., Nicolas, R. H., Adler, V. V., Paterson, H., Klenova, E. M., Polotskaja, A. V., & Goodwin, G. H. (1990). A novel sequence-specific DNA binding protein which interacts with three regularly spaced direct repeats of the CCCTC-motif in the 5′-flanking sequence of the chicken c-myc gene. *Oncogene*, *5*(12), 1743-1753.

Luger, K., Mäder, A. W., Richmond, R. K., Sargent, D. F., & Richmond, T. J. (1997). Crystal structure of the nucleosome core particle at 2.8 Å resolution. *Nature*, *389*(6648), 251.

Mailand, N., & Diffley, J. F. (2005). CDKs promote DNA replication origin licensing in human cells by protecting Cdc6 from APC/C-dependent proteolysis. *Cell*, *122*(6), 915-926.

Marcotte, R., Lacelle, C., & Wang, E. (2004). Senescent fibroblasts resist apoptosis by downregulating caspase-3. *Mechanisms of ageing and development*, *125*(10-11), 777-783.

Margueron, R., & Reinberg, D. (2010). Chromatin structure and the inheritance of epigenetic information. *Nature Reviews Genetics*, *11*(4), 285.

Martens, U. M., Chavez, E. A., Poon, S. S., Schmoor, C., & Lansdorp, P. M. (2000). Accumulation of short telomeres in human fibroblasts prior to replicative senescence. *Experimental cell research*, *256*(1), 291-299.

Masai, H., You, Z., & Arai, K. I. (2005). Control of DNA replication: regulation and activation of eukaryotic replicative helicase, MCM. *IuBMB Life*, *57*(4-5), 323-335.

Masai, H., Matsumoto, S., You, Z., Yoshizawa-Sugata, N., & Oda, M. (2010). Eukaryotic chromosome DNA replication: where, when, and how?. *Annual review of biochemistry*, *79*.

Masumoto, H., Muramatsu, S., Kamimura, Y., & Araki, H. (2002). S-Cdk-dependent phosphorylation of Sld2 essential for chromosomal DNA replication in budding yeast. *Nature*, *415*(6872), 651.

Melk, A., Schmidt, B. M., Takeuchi, O., Sawitzki, B., Rayner, D. C., & Halloran, P. F. (2004). Expression of p16INK4a and other cell cycle regulator and senescence associated genes in aging human kidney. *Kidney international*, *65*(2), 510-520.

Melixetian, M., Ballabeni, A., Masiero, L., Gasparini, P., Zamponi, R., Bartek, J., ... & Helin, K. (2004). Loss of Geminin induces rereplication in the presence of functional p53. *J Cell Biol*, *165*(4), 473-482.

Méndez, J., & Stillman, B. (2000). Chromatin association of human origin recognition complex, cdc6, and minichromosome maintenance proteins during the cell cycle: assembly of prereplication complexes in late mitosis. *Molecular and cellular biology*, *20*(22), 8602-8612.

Merkenschlager, M. (2010). Cohesin: a global player in chromosome biology with local ties to gene regulation. *Current opinion in genetics & development*, *20*(5), 555-561.

Michaloglou, C., Vredeveld, L. C., Soengas, M. S., Denoyelle, C., Kuilman, T., Van Der Horst, C. M., ... & Peeper, D. S. (2005). BRAF E600-associated senescence-like cell cycle arrest of human naevi. *Nature*, *436*(7051), 720.

Morgan, D. O. (1997). Cyclin-dependent kinases: engines, clocks, and microprocessors. *Annual review of cell and developmental biology*, *13*(1), 261-291.

Muñoz-Espín, D., & Serrano, M. (2014). Cellular senescence: from physiology to pathology. *Nature reviews Molecular cell biology*, *15*(7), 482.

Munro, J., Barr, N. I., Ireland, H., Morrison, V., & Parkinson, E. K. (2004). Histone deacetylase inhibitors induce a senescence-like state in human cells by a p16-dependent mechanism that is independent of a mitotic clock. *Experimental cell research*, *295*(2), 525-538.

Negrini, S., Gorgoulis, V. G., & Halazonetis, T. D. (2010). Genomic instability—an evolving hallmark of cancer. *Nature reviews Molecular cell biology*, *11*(3), 220.

Neuwald, A. F., Aravind, L., Spouge, J. L., & Koonin, E. V. (1999). AAA+: A class of chaperone-like ATPases associated with the assembly, operation, and disassembly of protein complexes. *Genome research*, *9*(1), 27-43.

Nigg, E. A. (1995). Cyclin-dependent protein kinases: key regulators of the eukaryotic cell cycle. *Bioessays*, *17*(6), 471-480.

Nora, E. P., Lajoie, B. R., Schulz, E. G., Giorgetti, L., Okamoto, I., Servant, N., ... & Gribnau, J. (2012). Spatial partitioning of the regulatory landscape of the X-inactivation centre. *Nature*, *485*(7398), 381.

Ogryzko, V. V., Hirai, T. H., Russanova, V. R., Barbie, D. A., & Howard, B. H. (1996). Human fibroblast commitment to a senescence-like state in response to histone deacetylase inhibitors is cell cycle dependent. *Molecular and cellular biology*, *16*(9), 5210-5218.

Ohlsson, R., Renkawitz, R., & Lobanenkov, V. (2001). CTCF is a uniquely versatile transcription regulator linked to epigenetics and disease. *TRENDS in Genetics*, *17*(9), 520-527.

Ohta, S., Koide, M., Tokuyama, T., Yokota, N., Nishizawa, S., & Namba, H. (2001). Cdc6 expression as a marker of proliferative activity in brain tumors. *Oncology reports*, *8*(5), 1063-1066.

Ohtani, K., Tsujimoto, A., Ikeda, M. A., & Nakamura, M. (1998). Regulation of cell growth-dependent expression of mammalian CDC6 gene by the cell cycle transcription factor E2F. *Oncogene*, *17*(14), 1777.

Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., & Campisi, J. (2003). Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nature cell biology*, *5*(8), 741.

Pavletich, N. P. (1999). Mechanisms of cyclin-dependent kinase regulation: structures of cdks, their cyclin activators, and cip and INK4 inhibitors1, 2. *Journal of molecular biology*, *287*(5), 821-828.

Pećina-Šlaus, N. (2003). Tumor suppressor gene E-cadherin and its role in normal and malignant cells. *Cancer cell international*, *3*(1), 17.

Petersen, B. O., Wagener, C., Marinoni, F., Kramer, E. R., Melixetian, M., Denchi, E. L., ... & Helin, K. (2000). Cell cycle–and cell growth–regulated proteolysis of mammalian CDC6 is dependent on APC–CDH1. *Genes & development*, *14*(18), 2330-2343.

Petrakis, T. G., Vougas, K., & Gorgoulis, V. G. (2012). Cdc6: a multi-functional molecular switch with critical role in carcinogenesis. *Transcription*, *3*(3), 124-129.

Petrakis, T. G., Komseli, E. S., Papaioannou, M., Vougas, K., Polyzos, A., Myrianthopoulos, V., ... & Townsend, P. (2016, June). Exploring and exploiting the systemic effects of deregulated replication licensing. In *Seminars in cancer biology* (Vol. 37, pp. 3-15). Academic Press.

Phillips, J. E., & Corces, V. G. (2009). CTCF: master weaver of the genome. *Cell*, *137*(7), 1194-1211.

Pinyol, M., Salaverria, I., Bea, S., Fernández, V., Colomo, L., Campo, E., & Jares, P. (2006). Unbalanced expression of licensing DNA replication factors occurs in a subset of mantle cell lymphomas with genomic instability. *International journal of cancer*, *119*(12), 2768-2774.

Polyak, K., Kato, J. Y., Solomon, M. J., Sherr, C. J., Massague, J., Roberts, J. M., & Koff, A. (1994). p27Kip1, a cyclin-Cdk inhibitor, links transforming growth factor-beta and contact inhibition to cell cycle arrest. *Genes & development*, *8*(1), 9-22.

Pope, B. D., Ryba, T., Dileep, V., Yue, F., Wu, W., Denas, O., ... & Thurman, R. E. (2014). Topologically associating domains are stable units of replication-timing regulation. *Nature*, *515*(7527), 402.

Prioleau, M. N., & MacAlpine, D. M. (2016). DNA replication origins—where do we begin?. *Genes & development*, *30*(15), 1683-1697.

Quesenberry, P. J. (Ed.). (1998). *Stem cell biology and gene therapy*. John Wiley & Sons.

Quina, A. S., Buschbeck, M., & Di Croce, L. (2006). Chromatin structure and epigenetics. *Biochemical pharmacology*, *72*(11), 1563-1569.

Ramirez, R. D., Sheridan, S., Girard, L., Sato, M., Kim, Y., Pollack, J., ... & Milchgrub, S. (2004). Immortalization of human bronchial epithelial cells in the absence of viral oncoproteins. *Cancer research*, *64*(24), 9027-9034.

Randell, J. C., Bowers, J. L., Rodríguez, H. K., & Bell, S. P. (2006). Sequential ATP hydrolysis by Cdc6 and ORC directs loading of the Mcm2-7 helicase. *Molecular cell*, *21*(1), 29-39.

Richmond, T. J., Finch, J. T., Rushton, B., Rhodes, D., & Klug, A. (1984). Structure of the nucleosome core particle at 7 Å resolution. *Nature*, *311*(5986), 532.

Rodier, F., Muñoz, D. P., Teachenor, R., Chu, V., Le, O., Bhaumik, D., ... & Davalos, A. R. (2010). DNA-SCARS: distinct nuclear structures that sustain damage-induced senescence growth arrest and inflammatory cytokine secretion. *Journal of cell science*, jcs-071340.

Salminen, A., Kauppinen, A., & Kaarniranta, K. (2012). Emerging role of NF-κB signaling in the induction of senescence-associated secretory phenotype (SASP). *Cellular signalling*, *24*(4), 835-845.

Sancar, A., Lindsey-Boltz, L. A., Ünsal-Kaçmaz, K., & Linn, S. (2004). Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. *Annual review of biochemistry*, *73*(1), 39-85.

Satyanarayana, A., & Kaldis, P. (2009). Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. *Oncogene*, *28*(33), 2925.

Schafer, K. A. (1998). The cell cycle: a review. *Veterinary pathology*, *35*(6), 461-478.

Schmidt, D., Schwalie, P. C., Ross-Innes, C. S., Hurtado, A., Brown, G. D., Carroll, J. S., ... & Odom, D. T. (2010). A CTCF-independent role for cohesin in tissue-specific transcription. *Genome research*, *20*(5), 578-588.

Serrano, M., Lin, A. W., McCurrach, M. E., Beach, D., & Lowe, S. W. (1997). Oncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a. *Cell*, *88*(5), 593-602.

Siddiqui, K., On, K. F., & Diffley, J. F. (2013). Regulating DNA replication in eukarya. *Cold Spring Harbor perspectives in biology*, *5*(9), a012930.

Sideridou, M., Zakopoulou, R., Evangelou, K., Liontos, M., Kotsinas, A., Rampakakis, E., ... & Trougakos, I. P. (2011). Cdc6 expression represses E-cadherin transcription and activates adjacent replication origins. *J Cell Biol*, *195*(7), 1123-1140.

Sidorova, J. M., & Breeden, L. L. (2003). Precocious G1/S transitions and genomic instability: the origin connection. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *532*(1), 5-19.

Sikora, E., Arendt, T., Bennett, M., & Narita, M. (2011). Impact of cellular senescence signature on ageing research. *Ageing research reviews*, *10*(1), 146-152.

Sherr, C. J., & Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes & development*, *13*(12), 1501-1512.

Speck, C., Chen, Z., Li, H., & Stillman, B. (2005). ATPase-dependent cooperative binding of ORC and Cdc6 to origin DNA. *Nature Structural and Molecular Biology*, *12*(11), 965.

Spilianakis, C. G., Lalioti, M. D., Town, T., Lee, G. R., & Flavell, R. A. (2005). Interchromosomal associations between alternatively expressed loci. *Nature*, *435*(7042), 637.

Splinter, E., Heath, H., Kooren, J., Palstra, R. J., Klous, P., Grosveld, F., ... & de Laat, W. (2006). CTCF mediates long-range chromatin looping and local histone modification in the β-globin locus. *Genes & development*, *20*(17), 2349-2354.

Sterner, D. E., & Berger, S. L. (2000). Acetylation of histones and transcription-related factors. *Microbiology and Molecular Biology Reviews*, *64*(2), 435-459.

Stillman, B. (2015). Reconsidering DNA polymerases at the replication fork in eukaryotes. *Molecular cell*, *59*(2), 139-141.

Stewart, C. E., Torr, E. E., Jamili, M., Nur, H., Bosquillon, C., & Sayers, I. (2012). Evaluation of differentiated human bronchial epithelial cell culture systems for asthma research. *Journal of allergy*, *2012*.

Stoeber, K., Mills, A. D., Kubota, Y., Krude, T., Romanowski, P., Marheineke, K., ... & Williams, G. H. (1998). Cdc6 protein causes premature entry into S phase in a mammalian cell-free system. *The EMBO Journal*, *17*(24), 7219-7229.

Studitsky, V. M., Kassavetis, G. A., Geiduschek, E. P., & Felsenfeld, G. (1997). Mechanism of transcription through the nucleosome by eukaryotic RNA polymerase. *Science*, *278*(5345), 1960-1963.

Suzuki, T., Fujikura, K., Higashiyama, T., & Takata, K. (1997). DNA staining for fluorescence and laser confocal microscopy. *Journal of Histochemistry & Cytochemistry*, *45*(1), 49-53.

Tada, S., Li, A., Maiorano, D., Méchali, M., & Blow, J. J. (2001). Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. *Nature cell biology*, *3*(2), 107.

Takayama, Y., Kamimura, Y., Okawa, M., Muramatsu, S., Sugino, A., & Araki, H. (2003). GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes & development*, *17*(9), 1153-1165.

Tanaka, S., & Diffley, J. F. (2002). Deregulated G1-cyclin expression induces genomic instability by preventing efficient pre-RC formation. *Genes & Development*, *16*(20), 2639-2649.

Tanaka, S., & Diffley, J. F. (2002). Interdependent nuclear accumulation of budding yeast Cdt1 and Mcm2–7 during G1 phase. *Nature cell biology*, *4*(3), 198.

Tanaka, S., Umemori, T., Hirai, K., Muramatsu, S., Kamimura, Y., & Araki, H. (2007). CDK-dependent phosphorylation of Sld2 and Sld3 initiates DNA replication in budding yeast. *Nature*, *445*(7125), 328.

Tannoch, V. J., Hinds, P. W., & Tsai, L. H. (2002). Cell cycle control. In *Cancer Gene Therapy* (pp. 127-140). Springer, Boston, MA.

Thiery, J. P., Acloque, H., Huang, R. Y., & Nieto, M. A. (2009). Epithelial-mesenchymal transitions in development and disease. *cell*, *139*(5), 871-890

Thu, Y. M., & Bielinsky, A. K. (2013). Enigmatic roles of Mcm10 in DNA replication. *Trends in biochemical sciences*, *38*(4), 184-194.

Timmermann, S., Lehrmann, H., Polesskaya, A., & Harel-Bellan, A. (2001). Histone acetylation and disease. *Cellular and Molecular Life Sciences CMLS*, *58*(5-6), 728-736.

Tsompana, M., & Buck, M. J. (2014). Chromatin accessibility: a window into the genome. *Epigenetics & chromatin*, *7*(1), 33.

Vakoc, C. R., Letting, D. L., Gheldof, N., Sawado, T., Bender, M. A., Groudine, M., ... & Blobel, G. A. (2005). Proximity among distant regulatory elements at the β-globin locus requires GATA-1 and FOG-1. *Molecular cell*, *17*(3), 453-462.

Van Bortle, K., & Corces, V. G. (2012). Nuclear organization and genome function. *Annual review of cell and developmental biology*, *28*, 163-187.

Vaziri, C., Saxena, S., Jeon, Y., Lee, C., Murata, K., Machida, Y., ... & Dutta, A. (2003). A p53-dependent checkpoint pathway prevents rereplication. *Molecular cell*, *11*(4), 997-1008.

Vermeulen, K., Van Bockstaele, D. R., & Berneman, Z. N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. *Cell proliferation*, *36*(3), 131-149.

Walter, D., Hoffmann, S., Komseli, E. S., Rappsilber, J., Gorgoulis, V., & Sørensen, C. S. (2016). SCF Cyclin F-dependent degradation of CDC6 suppresses DNA re-replication. *Nature communications*, *7*, 10530.

Wang, Z., Zang, C., Cui, K., Schones, D. E., Barski, A., Peng, W., & Zhao, K. (2009). Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes. *Cell*, *138*(5), 1019-1031.

White, C. L., Suto, R. K., & Luger, K. (2001). Structure of the yeast nucleosome core particle reveals fundamental changes in internucleosome interactions. *The EMBO journal*, *20*(18), 5207-5218.

Williams, R. S., Shohet, R. V., & Stillman, B. (1997). A human protein related to yeast Cdc6p. *Proceedings of the National Academy of Sciences*, *94*(1), 142-147.

Wong, D. J., Liu, H., Ridky, T. W., Cassarino, D., Segal, E., & Chang, H. Y. (2008). Module map of stem cell genes guides creation of epithelial cancer stem cells. *Cell stem cell*, *2*(4), 333-344.

Xu, S., Grullon, S., Ge, K., & Peng, W. (2014). Spatial clustering for identification of ChIP-enriched regions (SICER) to map regions of histone methylation patterns in embryonic stem cells. In *Stem Cell Transcriptional Networks* (pp. 97-111). Humana Press, New York, NY.

Yan, Z., DeGregori, J., Shohet, R., Leone, G., Stillman, B., Nevins, J. R., & Williams, R. S. (1998). Cdc6 is regulated by E2F and is essential for DNA replication in mammalian cells. *Proceedings of the National Academy of Sciences*, *95*(7), 3603-3608.

Zegerman, P., & Diffley, J. F. (2007). Phosphorylation of Sld2 and Sld3 by cyclin-dependent kinases promotes DNA replication in budding yeast. *Nature*, *445*(7125), 281.

Zhang, B., Day, D. S., Ho, J. W., Song, L., Cao, J., Christodoulou, D., ... & Pu, W. T. (2013). A dynamic H3K27ac signature identifies VEGFA-stimulated endothelial enhancers and requires EP300 activity. *Genome research*, gr-149674.

Zhu, J., Woods, D., McMahon, M., & Bishop, J. M. (1998). Senescence of human fibroblasts induced by oncogenic Raf. *Genes & development*, *12*(19), 2997-3007.

Zhu, W., Chen, Y., & Dutta, A. (2004). Rereplication by depletion of geminin is seen regardless of p53 status and activates a G2/M checkpoint. *Molecular and Cellular Biology*, *24*(16), 7140-7150.