HELLENIC REPUBLIC
National and Kapodistrian University of Athens

Medical School of Athens

Master Thesis

MSc in Molecular Biomedicine

Assessment of DNA damage and DNA damage response pathway upon CDC6 induction in Human Bronchial Epithelial Cells

Eleni Kardala

Supervisor: Professor Vassilis Gorgoulis

Athens, July 2019

"ALEXANDER FLEMING"
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Place of research

The presented study was performed in National and Kapodistrian University of Athens, Medical School, Laboratory of Molecular Carcinogenesis Group.

A part of the research, also, took place in Reza Mirzazadeh Lab, Science for Life Laboratory, Division of Translational Medicine and Chemical Biology, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden

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Athens, July 2019

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Abstract

Oncogene-induced senescence (OIS) is a type of stress-induced premature senescence (SIPS), which acts as an anti-tumor barrier and must be bypassed for tumor progression. Senescence is a stress response and the outcome of a protracted DNA Damage Response (DDR) (D'Adda Di Fagagna, 2008).

This thesis aims to provide more detailed insights in the DNA damage and repair process mechanisms leading to oncogene senescence. For this purpose, we applied an epithelial cellular model in which over-expression of an oncogene was achieved in an inducible way through a doxycycline-inducible promoter. Immortalized human bronchial epithelial cells (HBECs) (hTERT/CDK4) were used for that purpose and the oncogene that has been chosen is the Cell division cycle 6 (CDC6).

Cell division cycle 6 (CDC6) is a 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. Importantly, considering that cancers are of epithelial origin, this system focuses on epithelial cancer development and particularly on the transition from the the non-malignant stage to senescence and then to complete transformation of the normal cells into a mesenchymal - cancerous state.

1 INTRODUCTION

1.1 Cell cycle

The cell cycle of a eukaryotic cell is a process that drives the cell in mitosis through a specific sequence of events. The cell cycle is divided in two phases: the interphase which consists of G1, S and G2 phases and the M (mitotic) phase which includes prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis (Schafer, 1998).

In G1 phase the cell prepares itself through active transcription for entering S phase, where DNA replication takes place. S phase is followed by G2 where the integrity of DNA is checked and the cell prepares for mitosis. There is, also, a subtype of G1 phase, named G0 in which cells arrest their cycle. G0 phase is a state where back do not proliferate and can remain for a prolonged time before turning again into proliferation depending on the cell type and on the signals received from microenvironment (B Alberts, A Johnson, J Lewis, M. Raff, K. Roberts, 2015; Schafer, 1998)

During M phase, nuclear division (mitosis) is followed by cell division (cytokinesis) generating two daughter cells identical with the parent cell. Afterwards, the two new cells enter G1 stage of interphase (B Alberts, A Johnson, J Lewis, M. Raff, K. Roberts, 2015; Schafer, 1998).

1.2 Cell cycle control system

The eukaryotic cell has developed mechanisms of cell cycle regulation, ensuring the correct sequence of the cell cycle phases and that each DNA segment is replicated completely, and only once per cell cycle, with no sections left unreplicated, or re-replicated. Thus, fidelity in DNA replication is achieved and the integrity of genome is protected (de Pamphilis, de Renty, Ullah, & Lee, 2012; Hartwell & Weinert, 1989). Disorders in cell cycle control lead to deregulated cell function e.g. cancer (Barnum & O'Connell, 2014).

Cell cycle checkpoints are the mechanisms which control the order and fidelity of the events of the cell cycle. There are three checkpoints identified: at the G1/S boundary, at the G2/M transition and at the metaphase/anaphase boundary of the M phase. The main molecular regulators of the cell cycle progression are cyclin/cyclin-dependent kinases (cyclin/CDKs) complexes (Barnum & O'Connell, 2014).

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, which interact with the CDKs as complexes and activate them. CDKs are a family of kinases with regulatory effect on the cell cycle. Each CDK possesses an ATP-binding domain, a cyclin-binding domain known as a PSTAIRE helix, and a T-loop domain M (Lim & Kaldis, 2013; Morgan, 2002). Those motifs contribute in the activation of the

CDKs by the cyclins via the PSTAIRE cyclin-binding domain. Once activated, a CDK-cyclin complex phosphorylates and activates other regulatory factors. Activation of those substrates results in the transition into the next phase of the cell cycle. There is specificity in cell cycle regulation by CDKs-cyclins (Lim & Kaldis, 2013).

Activity of CDKs can be affected by CDK inhibitors molecules (CKIs). Based on CDK specificity, CKIs are categorized in the INK4 and the CIP/KIP families (Hunter & Pines, 1994). The INK4 family consists of the p16INK4a, p15INK4b, p18INK4c και p19 INK4d inhibitors which inhibit the complex formation between CDK4 or CDK6 and cyclin-D. On the other hand, the CIP/KIP family includes the p21Cip1/Waf1/Sdi1, p27Kip1 and p57Kip2 inhibitors that block formation of cyclin D-, E-, A- and B-dependent kinase complexes (Besson, Dowdy, & Roberts, 2008). Alterations in the aforementioned processes could lead to cell cycle arrest, apoptosis or cancer (Johansson & Persson, 2012).

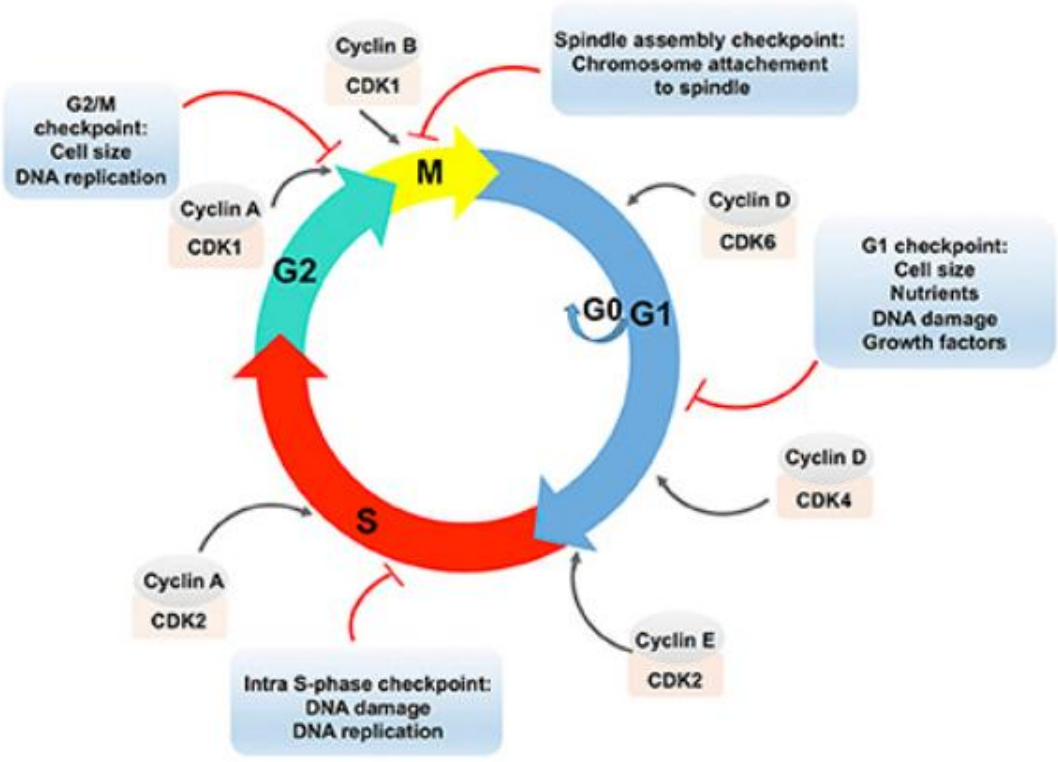

Figure 1.1: Schematic presentation of the eukaryotic cell cycle and its regulation (El-Aouar Filho et al., 2018).

1.3 Replication

Replication origins are the specific genomic sites, where replication starts (DePamphilis, 2003). In eukaryotic cells two processes determine the initiation of replication at origin sites: origin 'licensing' which takes place in the G1 phase of cell cycle, and origin 'firing' during the S phase. These processes contribute in maintenance of genome integrity (Fragkos, Ganier, Coulombe, & Méchali, 2015).

During replication licensing, replication origins are identified by the origin recognition complex (ORC), which binds the DNA and recruits other protein factors to establish the pre-replication complex (pre-RC) (DePamphilis, 2003). ORC is a multi-subunit DNA binding complex, which consists of 6 subunits, remains attached to DNA during the whole cell cycle and at the late M/early G1 phase activates the Cell division cycle 6 (CDC6) protein which, then, recruits the CDC10-dependent transcript 1 (DNA replication factor Cdt1). The last step of licensing is the recruitment of the mini-chromosome maintenance (MCM) helicase complex MCM2–MCM7 (Evrin et al., 2009). It is essential for cells to prevent re-licensing in the S phase in order for the whole genome to be replicated only once per cell cycle. This is achieved through i) the inhibition of Cdt1 by Gemini or ii) via Cdt1 ubiquitylation and degradation during the S phase (Blow & Gillespie, 2008).

In the G1-S transition, origin activation takes place with the establishment of the pre-initiation complex. Origin activation requires high levels of CDKs. Pre-IC assembly is triggered by DBF4-dependent kinase (DDK) and cyclin-dependent kinases (CDKs), although pre-RC assembly is independent of CDK activity (Fragkos et al., 2015). DDK and CDKs phosphorylate and load 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). Also, DDK and CDKs phosphorylate specific residues within the MCM2–MCM7 complex, leading to helicase activation and DNA unwinding. Then, other proteins such as replication factor C (RFC), proliferating cell nuclear antigen (PCNA), replication protein A (RPA) and other DNA polymerases are loaded and the pre-IC becomes 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 consists of CDC45, the MCM hexamer and the GINS complex (Kang, Warner, & Bell, 2014). The CMG complex is activated by the MCM10 and then DNA polymerase α-primase (Pol α) initiates DNA synthesis via the DNA polymerases Pol δ and Pol ε (Kunkel & Burgers, 2008).The lagging strand can be synthesized discontinuously in the form of short Okazaki fragments, which are joined by DNA ligase, while the leading strand is synthesized continuously. Replication ends, when two opposing forks coming from adjacent replication origins meet together, lead to the ubiquitin-dependent removal of the CMG from chromatin (Bell & Labib, 2016).

Disorders in genome replication can accumulated over the cell cycles and have been shown to be linked with a variety of human diseases, including many types of cancer. Therefore, replication is determined by coordinated activity of several proteins and enzymes, in order to avoid the generation and the transmission of mutations to daughter cells and to sustain replication fidelity and genome stability (Fragkos et al., 2015).

1.4 Replication stress

Replication stress is defined as the slowing or stalling of replication fork progression and/or DNA synthesis. This does not necessarily refer to all replication defects, such as re-replication or reduced numbers of origins, although these conditions may sensitize the cell to many of the sources of replication stress. Replication stress also does not refer to a physical structure, such as double-strand breaks (DSBs) associated with collapsed forks. Sources of replication stress are chromatin inaccessibility, oncogene-induced replication stress, DNA lesions, misincorporation of ribonucleotides, formation of unusual DNA structures, conflicts between replication and transcription and limitation of essential replication factors. Replication stress can be generated by a wide range of physical obstacles, and usually results in physical structures, namely stretches of single-stranded DNA (ssDNA). The persistence of ssDNA, bound by replication protein A (RPA), and adjacent to the stalled newly replicated double-stranded DNA, generates a signal for activation of the replication stress response: a primer-template junction. This structure serves as a signaling platform to recruit a number of replication stress response proteins, including the protein kinase ATM- and Rad3-related (ATR). ATR is one of the central replication stress response kinases, and once activated through co-localization with other factors that are recruited to these structures, it phosphorylates substrates which help the cell to survive and faithfully complete DNA replication in the face of the stress. Many of the common markers used to detect replication stress reflect activation of the ATR pathway, including phosphorylation of the histone variant H2AX (γH2AX). However, γH2AX can be activated by several kinases, which detect different types of DNA damage throughout the cell cycle. Thus, it is not a specific marker of replication stress (Zeman & Cimprich, 2014).

1.5 CDC6 role in cancer

CDC6 is a 60-kD protein and a member of AAA+ ATPases superfamily (Neuwald, Aravind, Spouge, & Koonin, 1999). The human CDC6 gene is located at chromosome 17q21.3. Walker A/B motifs and winged-helix fold domain play a role in CDC6 function, as mutations in Walker A/Walker B block its ATPase activity, which is necessary for pre-RC assembly (Liu et al., 2009; Randell, Bowers, Rodríguez, & Bell, 2006; Z. Yan et al., 1998).

In most cancer types, E2F/retinoblastoma transcription factors complexes, which control CDC6 expression, are frequently deregulated. In turn, CDC6 overexpression which has been observed in many tumor cells such as in various brain cancers, non-small cell lung carcinomas and mantle cell lymphoma (Borlado & Méndez, 2008; Karakaidos et al., 2004). Finally, CDC6 can be used as a marker for detecting early malignancy because of its absence in non-dividing differentiated and quiescent cells (Borlado & Méndez, 2008)

Deregulation of the replication licensing process promotes genomic instability that most of the times leads to carcinogenesis, due to DNA re-replication (Blow & Gillespie, 2008; Halazonetis, Gorgoulis, & Bartek, 2008; Negrini, Gorgoulis, & Halazonetis, 2010). Re-replication is a type of replication stress that contributes to replication fork stalling, DNA damage and finally genomic instability (Blow & Gillespie, 2008; Theodoros G. Petrakis et al., 2016). This phenomenon contributes to re-firing of the replication origins and inefficient fork progression (Di Micco et al., 2006). Stalled or collapsed replication forks 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. Permanent DNA damage (DSB) and subsequent DNA damage response (DDR) always lead to cell senescence (Bielak-Zmijewska, Mosieniak, & Sikora, 2018).

As CDC6 plays an important role in DNA replication licesing, its deregulation is expected to exert a negative impact on genome stability. Indeed, according to (Borlado & Méndez, 2008), the oncogenic activity of CDC6 may origin from the genomic instability that is an outcome of aberrant DNA replication. Proteasomal degradation of CDC6 occurs in G2/M phase in a Cyclin F-dependent manner, preventing re-replication and protecting genome integrity (Walter et al., 2016). Experimental data, also, has demonstrated that ectopic expression of CDC6 and Cdt1 causes DNA re-replication in tumor cells (C. et al., 2003).

Further evidence on 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 – following an epithelial to mesenchymal transition process (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. EMT is a characteristic of cancer and is associated with loss of E-cadherin which is a tumor suppressor (Thiery, Acloque, Huang, & Nieto, 2009). E-cadherin is encoded by the *CDH1* gene and plays a key role in cell-cell adhesion in epithelial tissues. 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). 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).

1.6 DNA damage response (DDR)

As DNA is the repository of genetic information, the ultimate goal of the DDR network is to preserve its integrity. However, because of the large number of DNA lesions induced in a cell every day, this task is not always achieved without cost. DNA lesions can be divided in two broad categories: S lesions occurring on one strand of the double helix such as modified bases, abasic sites, helix distorting base lesions, and single strand breaks and D lesions involving both strands which are interstrand crosslinks and double strand breaks (DSBs). The type of DNA lesion and the cell cycle phase largely dictate the repair programme to be engaged. Most types of damage are repaired by a series of catalytic events entailing multiple proteins and generally including two steps: (1) damage recognition by sensors; and (2) processing and repair of the lesions (Gorgoulis, Pefani, Pateras, & Trougakos, 2018).

1.6.1 Repair of S lesions

For category S lesions, subsequent to recognition, the following steps are required for processing and repair: (1) incisions flanking the damage; (2) excision of the damaged area; (3) filling of the gap by nucleotide polymerization; and (4) 'sealing' the gap with ligation. More specifically, the high fidelity (error free) pathways base excision repair (BER) and nucleotide excision repair (NER) deal with single base DNA defects and helix distorting base lesions, respectively, whereas repair of nucleotide misincorporation is mediated by mismatch repair (MMR). However, if BER and NER malfunction or are overloaded by 'fixing' demands, then the translesion synthesis (TLS) pathway, which is a low fidelity repair module (error prone) pathway known as DNA damage tolerance (DDT), takes over. To avoid replication of damaged DNA that could lead to fork collapse, DSB production, and genome destabilization, cells opt to recruit TLS/DDT to bypass encountered lesions and repair them at a later time. Thus, TLS/DDT is considered to be responsible for the majority of mutagenic events, playing a central role in carcinogenesis. Although the latter is an undesired event, from a broader perspective it is a 'cost' that the cell has to pay to avoid DSBs, thus preserving double helix continuity. In line with this, inhibition of factors involved in category S defect repair processes has the potential to induce DSB formation during replication, triggering replication stress and death if the cell is also deficient in components implicated in DSB repair (Gorgoulis et al., 2018).

1.6.2 Repair of D lesions

The second type of category D defects comprises DNA interstrand crosslinks (ICLs) that are generated by a class of agents such as mitomycin C (MMC), or circulating metabolites such as formaldehyde. ICLs are toxic, because the covalent links that they form prevent DNA from unwinding, thereby blocking replication and transcription, causing replication and transcription stress, respectively. These lesions are fixed by the Fanconi anaemia (FA)

pathway, which is a replication dependent repair mechanism that appeared relatively late in evolution. It is considered to be the most sophisticated repair route, enlisting modules of three classic repair pathways, i.e. TLS, NER, and HRR (Gorgoulis et al., 2018).

When DNA double-strand breaks occur, the cell cycle stage has a major influence on the choice of the repair pathway employed. DNA double-strand breaks (DSBs) are exceedingly dangerous chromosomal lesions. Failure to accurately repair DSBs can lead to gross chromosome rearangements or mutations at the break site, which can cause cell death, cell transformation, and tumorigenesis (Daley & Sung, 2014).

With the exception of immune receptor diversity V(D)J and class switch (CS) recombination and chromosomal crossover during meiosis II of gamete production, in which DSBs form part of physiological programmes, the cellular reaction to DSBs epitomizes an integrated cellular stress response to 'imminent danger'. Two classes of DDR proteins are recruited at damaged sites: those that present directly at DSBs (called sensors and mediators), and those associated with the DSB flanking chromatin, altogether constituting so called DDR foci. Over time, the DDR foci spread away from the DSB to distances up to megabases in mammals, forming an amplification mechanism recruiting signal transduction factors that further amplify the signal with effectors that set the cell in an 'alarm' state. This mechanism has, on the one hand, a local effect by relaxing the chromatin and increasing the concentration of repair factors at the damaged site, and, on the other hand, a systemic effect, termed checkpoint activation, that reduces the activity of CDKs. Notably, in certain cases and depending on the cellular context, checkpoint activation, apart from the DDR signalling cascade, also involves the parallel action of other stress response signalling routes, like the p38 mitogen activated protein kinase (p38MAPK) pathway, which coordinates several cellular functions. The endpoint of the stress response signalling cascade is always the cyclin–CDK complex. The cyclin–CDK complexes represent drivers of the cell cycle and, when they are suppressed, the cell enters a state of arrest, providing time for repair (Gorgoulis et al., 2018).

One of the earliest features that mark these DDR foci is histone variant H2AX phosphorylation at serine 139, (also referred to as γH2AX), by ataxia telangiectasia mutated (ATM) backed up by ataxia telangiectasia and Rad3 related (ATR) and DNA dependent protein kinase, catalytic subunit. All three kinases are members of the phosphatidylinositol 3 kinase (PI3K) family and key DDR signalling components (transducers) One of the earliest features that mark these DDR foci is histone variant H2AX phosphorylation at serine 139, (also referred to as γH2AX), by ataxia telangiectasia mutated (ATM) backed up by ataxia telangiectasia and Rad3 related (ATR) and DNA dependent protein kinase, catalytic subunit. All three kinases are members of the phosphatidylinositol 3 kinase (PI3K) family and key DDR signalling components (transducers) (Gorgoulis et al., 2018).The generation of DSBs

triggers the relocalization of many DNA damage response (DDR) proteins such as MRE11/NBS1/RAD50, MDC1, 53BP1, and BRCA1 to nuclear foci where these proteins colocalize and interact with γH2AX. Presumably, γH2AX foci specifically attract repair factors, leading to higher concentration of repair proteins surrounding a DSB site. Specific recognition of γH2AX by these repair factors requires the presence of protein domains, which bind to the phosphorylated carboxy terminus of γH2AX. Several lines of evidence suggest the critical role of H2AX phosphorylation at DSB sites for nuclear foci formation and induction of DSB repair. (i) H2AX-knockout cells manifested impaired recruitment of NBS1, 53BP1, and BRCA1 to irradiation induced foci. (ii) Both H2AX+/− and H2AX−/− mouse thymocytes show an increase in chromosomal aberrations. (iii) Mouse embryonic stem (ES) cells deficient in H2AX phosphorylation have alterations in efficiency of DNA repair by NHEJ or HR. As a result of these defects in DNA damage repair, such cells have increased sensitivity to DNA damage. All these facts suggest that γH2AX might serve as a docking site for DNA damage/repair proteins and functions to promote DSB repair and genome stability. (Podhorecka, Skladanowski, & Bozko, 2010)

Subsequently, the DNA damage mediator called mediator of DNA damage checkpoint 1 (MDC1) attaches to γH2AX, acting as a platform for the meiotic recombination 11 (MRE11)–Rad50–Nijmegen breakage syndrome 1 (NBS1) (MRN) sensor complex that activates ATM, thus forming an amplification loop. Concurrently, MRN complexes bind the DSB avidly, playing a pivotal role in the initial processing of the break, generating single strand (ssDNA) DNA 3′ overhangs that are recognized by replication protein A (RPA). This event brings into play the ATR transducer kinase, which, in cooperation with ATM, turns on the downstream transducer kinases checkpoint kinase 1 (Chk1) and checkpoint kinase 2 (Chk2). In concert, these kinases activate a key effector of the DDR pathway, namely p53. p53 is a transcription factor that governs a complex stress response programme covering a bewildering range of biological functions, explaining why p53 is frequently mutated in cancer. Activation of p53, mainly via PTMs, induces the expression of numerous downstream effectors, including the universal CDK inhibitor p21WAF1/Cip1, leading to cell cycle arrest. Concurrently, ATM imposes a transcriptional silencing programme by shutting down both RNA polymerase II and RNA polymerase I, thus saving the energy that transcription demands and preventing collision between transcription and repair. Concomitantly with these global effects, repair is facilitated by extensive chromatin modifications and remodelling at the site of the DSB. In brief, SWI/SNF dependent histone H2A.Z exchange for histone H2A destabilizes the nucleosomes surrounding the DSB. This nucleosome remodelling event exposes the N terminal tail of histone H4, which, in turn, is acetylated by TIP60 histone acetyltransferase,

further relaxing chromatin and enabling access to downstream repair factors (Gorgoulis et al., 2018).

Two mechanistically distinct pathways have evolved to eliminate DSBs from the genome: nonhomologous DNA end joining (NHEJ) and homologous recombination (HR) (Daley & Sung, 2014). HRR is considered to be an error free repair system occurring during the S and G2 cell cycle phases, whereas NHEJ is an error prone repair pathway dealing mainly with nonreplication associated DSBs, and represents the predominant repair route that functions irrespective of the cell cycle (Gorgoulis et al., 2018). 53BP1, first identified as a DNA damage checkpoint protein, and BRCA1, a well-known breast cancer tumor suppressor, are at the center of the choice between NHEJ and HR (Daley & Sung, 2014).

53BP1 promotes NHEJ repair and inhibits HR by antagonizing BRCA1 (Guo et al., 2018). In HRR, the intact sister chromatid is most often engaged as the information donor. This process is normally accurate but requires that cells be in the S or G2 phase of the cell cycle, when DNA replication generates the sister chromatid to direct the repair process (Daley & Sung, 2014). HRR is initiated by the binding of BRCA1 to the ubiquitin chain added by the E3 ligases ring finger protein 8 (RNF8) and ring finger protein 168 (RNF168) to the remodelled nucleosome. In this way the BASC connects sensing and signalling with the repair component BRCA2, which controls the Rad51 recombinase that replaces RPA. The BRCA2–Rad51 complex then invades the homologous template and primes DNA synthesis, copying and restoring the genetic information. When the homologue donor strand is the sister chromatid, HRR is accurate. However, recombination may take place across different genome regions, challenging previous notions concerning the error- free nature of HRR. Hence, to secure sealing of DSBs, various routes of HRR exist that may favour inappropriate pairing. Alternatively, three critical histone modifications, namely histone H4 lysine 20 dimethylation (H4K20me2) [catalysed sequentially by methyltransferases SU(VAR)39H1 and SETD8], ubiquitylation of histone H2A on lysine 15 (H2AK15ub) (induced by the E3 ligase RNF168), and histone H3 lysine 79 methylation (H3K79me), are recognized by the signalling mediator p53 binding protein 1 (53BP1) at DSBs, promoting NHEJ. Importantly, as aforementioned, 53BP1 accumulation antagonizes BRCA1- mediated HRR in favour of NHEJ (Gorgoulis et al., 2018).

NHEJ entails the tethering of the broken DNA ends and their ligation. NHEJ is active throughout the cell cycle. While NHEJ accurately repairs “clean” DSBs whose ends are compatible and harbor undamaged terminal nucleotides, it is also capable of joining mismatched termini or termini that harbor damaged. In the latter case, joining is associated with DNA sequence loss. Moreover, when ends from two different chromosomes are joined, a chromosomal translocation ensues (Daley & Sung, 2014). In NHEJ, the DSB is sensed by the

lupus Ku autoantigen protein p80 (Ku80)–lupus Ku autoantigen protein p70 (Ku70) heterodimer, which recruits and assembles the DNA–PK complex, which, in turn, processes the DNA ends and increases the recruitment of ligase IV/Xray repair complementing defective repair in Chinese hamster cells 4 (XRCC4), which carries out the rejoining reaction. Although HRR is the favoured pathway to deal with a DSB, ensuring DNA sequence fidelity, in the event that HRR is nonfunctional the cell 'prefers' to follow inappropriate repair routes to secure cell viability. In this case, the faster kinetics of the Ku heterodimer for DSBs compared to those of the HRR factors make the error prone NHEJ (the repair pathway of choice) operate even during S phase, with potential unfavourable effects for the cell (Gorgoulis et al., 2018).

A series of studies have implicated 53BP1 and the tumor suppressor BRCA1 in DNA end resection control. 53BP1 was shown to negatively regulate resection in G1 phase. Importantly, BRCA1 promotes the removal of 53BP1 in S phase to allow resection. Consequently, in cells lacking BRCA1, resection is not upregulated in S phase and inappropriate NHEJ occurs at replication-associated DSBs, leading to gross chromosomal rearrangements. In mice, deletion of 53BP1 suppresses the embryonic lethality and prevents the chromosomal rearrangements seen in BRCA1−/− animals, emphasizing the importance of BRCA1-dependent removal of 53BP1 to facilitate the transition from NHEJ to HR (Panier & Boulton, 2014).

1.7 Senescence

Senescence was firstly used five decades ago to describe the state of normal cells in the culture having a limited ability to proliferate (Hayflick & Moorhead, 1961). Cellular senescence is a condition in which cells, despite being alive, are unable to proliferate further. This is a stress response, and therefore is different from quiescence or terminal differentiation. Evidence indicates that senescence, triggered by different stimuli, is the outcome of a protracted DNA Damage Response (DDR) (D'Adda Di Fagagna, 2008). Senescence is classified in two types. The first one is replicative senescence (RS) caused by telomere shortening when short telomeres are recognized as DNA damage and DDR is triggered and the second one is stress-induced premature senescence (SIPS), which is determined as a response to various stimuli before telomere erosion occurs (D'Adda Di Fagagna, 2008; Sikora, Arendt, Bennett, & Narita, 2011). It is worth mentioning that even though induction of senescence is considered as a consequence of DNA damage and especially double strand DNA damage followed by DDR, there are a number of studies proving that cells can undergo senescence without accumulation of irreparable DNA damage and majority of them refer to senescence as a tumor suppressor mechanism. In other words, permanent DNA damage (DSB) and subsequent DNA damage response (DDR) always lead to cell senescence, but

senescence does not always depends on DNA damage and DDR (Bielak-Zmijewska et al., 2018).

The senescent phenotype is characterized by some features regarding cell function and structure. Senescent cells display a flat, enlarged, often multinucleated morphology, are characterized by a durable growth arrest, expression of anti-proliferative molecules (e.g. p16INK4a), activation of damage sensing signaling pathways (e.g. p38MAPK and NF-kB) and resistance to apoptosis. Growth arrest is often triggered by persistent DNA damage response (DDR) or stress signaling, and mechanistically executed by constitutive activation of the p16INK4a-RB and/or p53 pathways (Campisi, 2012; Campisi & D'Adda Di Fagagna, 2007; Childs, Durik, Baker, & Van Deursen, 2015; Muñoz-Espín & Serrano, 2014). Even though the mechanism underlying resistance to apoptosis is still indefinite, alterations in expression pattern of proteins controlling proliferation and apoptosis are being hypothesized as possible causes (Campisi & D'Adda Di Fagagna, 2007; Marcotte, Lacelle, & Wang, 2004).

Moreover, senescent cells demonstrate increased expression of lysosomal β-galactosidase and accumulation of lipofuscin which are both markers for senescence, but they lack proliferation markers such as Ki67 or PCNA (Campisi & D'Adda Di Fagagna, 2007; Dimri et al., 1995; Konstantinos Evangelou et al., 2017; Georgakopoulou et al., 2013). Also, except for short telomeres and activation of DNA damage response (DDR) signaling pathways other features of senescence are the expression or appearance of senescence-associated heterochromatin foci (SAHFs), and an increase in DNA-SCARS (DNA segments with chromatin alterations reinforcing senescence) (He & Sharpless, 2017; Rodier et al., 2011).

In addition, despite being hyporeplicative, senescent cells maintain their metabolic activity (Dörr et al., 2013). They secrete Senescence-Associated Secretory Phenotype-SASP factors which consist of soluble signaling factors (interleukins, chemokines, and growth factors), proteases and insoluble proteins/extracellular matrix (ECM) components. These factors contribute in modifying the tissue microenvironment of senescent cells and in facilitating cancer progression through paracrine effects on nearby cells (Coppé, Desprez, Krtolica, & Campisi, 2010).

Senescence can be caused by various factors: following many cell divisions, shorterning of human telomeres triggers senescence (Greider, Hemann, Strong, & Hao, 2001; Martens, Chavez, Poon, Schmoor, & Lansdorp, 2000). Furthermore, DNA damage, and especially double strand breaks (DSBs), play a significant role in transient or prolonged cell cycle arrest by activating p53 signaling pathway (Di Leonardo, Linke, Clarkin, & Wahl, 1994; Parrinello et al., 2003). Additionally, intracellular oxygen species or cytokines signaling (such as interferon-β) can trigger senescence (Campisi, 2012; Campisi & D'Adda Di Fagagna, 2007). Also, overexpression of oncogenes triggers senescence known as oncogene-induced

senescence-OIS. Indeed, oncogenic expression of RAS has been observed to transform normal cells to senescent (Dimri, Itahana, Acosta, & Campisi, 2009; Serrano, Lin, McCurrach, Beach, & Lowe, 1997). Oncogene activation also causes DDR activation and cellular senescence. Oncogene-induced DNA damage is caused by altered DNA replication, and oncogene-induced senescence is a barrier to cancer. Senescent cells can be observed *in vivo* in preneoplastic lesions (D'Adda Di Fagagna, 2008).

1.8 Oncogene-induced senescence and cancer development

According to the oncogene-induced DNA damage model for cancer development: i) oncogenes cause replication stress and eventually DNA damage, ii) DNA damage activates the DNA Damage Response (DDR) pathway, iii) depending on the amount and type of damage DDR triggers the anti-tumor barriers of apoptosis or senescence, iv) as DNA damage accumulates the DDR pathway and the error-free repair pathways are overwhelmed leading, due to selective pressure, to inactivation or exhaustion of vital DDR/R (DDR and Repair) components shifting v) to error-prone repair fueling genomic instability breaching the anti-tumor barriers and thus vi) creating a permissive environment for cancer initiation and progression (Gorgoulis et al., 2005, Gorgoulis et al., 2018, Halazonetis et al., 2008, Bartkova et al., 2006, Negrini et al., 2010)

1.9 Description of the epithelial cancer evolution experiment (ECEE)

An oncogene doxycycline-inducible (Tet-ON) non-malignant cellular system was generated by (Komseli et al., 2018) employing Human bronchial epithelial cells (HBECs) as a platform (Halazonetis et al., 2008**).** Immortalization with combined expression of hTERT and ectopic mutant cyclin-dependent kinase 4 (CDK4) was performed in order to bypass p16INK4A-induced premature growth arrest, maintaining the epithelial phenotype of the cells (Ramirez et al., 2004**,** K. Evangelou et al., 2013**,** Velimezi et al., 2013**).** Further genetic manipulations to develop the inducible system also did not affect the epithelial characteristics of this model (Komseli et al., 2018).

CDC6 was chosen as an inducible oncogene for the following reasons: i) it is a key component of the replication licensing machinery, found to be frequently deregulated in cancer from its earliest stages (Liontos et al., 2007**,** Karakaidos et al., 2004**,** Theodoros G. Petrakis et al., 2016) and ii) when over-produced it displays a multi-functional facet by compromising the replication process (re-replication: a form of replication stress) triggering genomic instability (Liontos et al., 2007)**,** Bartkova et al., 2006**,** Sideridou et al., 2011**,** (Galanos et al., 2016) and acquiring properties of a transcriptional regulator affecting: a) negatively the expression of the nodal tumor suppressors loci, *INK4/ARF* (encoding p16INK4A, ARF and p15INK4B) and *CDH1* (encoding E-cadherin) (Theodoros G. Petrakis

et al., 2016**,** Thodoris G. Petrakis, Vougas, & Gorgoulis, 2012)**,** Sideridou et al., 2011) and b) positively that of rDNA (ribosomal DNA), most probably impinging on RNA dynamics (Huang et al., 2016).

In the HBEC CDC6 Tet-ON system, CDC6 is expressed constitutively at levels relevant to those of tumor samples. Stimulation of CDC6 results in a progressive decrease of cell proliferation, as confirmed by reduced BrdU (bromodeoxyuridine) incorporation that ceases after 6 days of induction. As proliferation diminishes, the cells after a 3-day induction gradually acquire a senescent phenotype, as confirmed by GL13 staining that peaks at day 6. GL13 is a reagent which is applied in a non-enzymatic assay to detect lipofuscin, a non-degradable metabolic by-product that is considered a "hallmark" of senescence (Konstantinos Evangelou et al., 2017**,** Georgakopoulou et al., 2013)**.** A report showing CDC6-mediated inhibition of apoptosome formation through its binding onto cytochrome c activated Apaf-1 explains why senescence emerges as the only tumor suppressor mechanism in this system (Niimi et al., 2012)**.**

From a morphological point of view the microscopical analysis demonstrates senescent cells as enlarged and flattened cellular shapes, occasionally multinucleated, without the appearance of senescence associated heterochromatin foci (SAHF). Notably, this feature is observed during irreversible senescence in cells with an intact p16INK4A/Rb pathway, justifying its absence in the system (Narita et al., 2003)

After a protracted stalled growth phase (around a month after the first CDC6 induction), when all cells are senescent and uniformly express CDC6, a fraction of proliferating cells emerges with distinct morphological features compared to those of the "OFF" state. These cells, from now on termed "escaped", show traces of lipofuscin during the first cell divisions, proving that they came from senescent cells, while they are negative for GL13 after several passages and serial dilutions of the non-degradable metabolic by-product. They attain a spindle morphology resembling that of mesenchymal cells, insinuating an EMT, an embryonic program implicated in cancer invasion and progression (Nieto, Huang, Jackson, & Thiery, 2016)**.**

In accordance, E-cadherin, a fundamental adhesion molecule of epithelial tissues, is lost in the "escaped" cells, identifying a cardinal feature of the EMT program, whereas vimentin, a mesenchymal marker, is increased (Thiery et al., 2009).

1.10 Mechanistic insights into the senescent phase of the ECEE

CDC6 overexpression results in re-replication, a form of replication stress, that leads to replication fork stalling, collapse, DNA damage and DDR activation (Bartkova et al., 2006**,** Liontos et al., 2007**,** Walter et al., 2016)**.** This is, also, found in the HBEC CDC6 Tet-ON

system, since during the initial stages of senescence (3 and 6 days after the first induction) CDC6 overexpression results in DNA damage documented by alkaline comet assay and DDR stimulation confirmed by 53BP1 foci formation and induction of the p53 pathway. Replication stress caused by CDC6 overexpression leads, also, 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 (Bartkova et al., 2006; Di Micco et al., 2006). In line with the HBEC CDC6 Tet-ON system, overexpression of cyclin-dependent kinase inhibitor p21(WAF1/CIP1) (p21) in p53-null, cancerous and near-normal cellular models resulted in a senescence-like phase, followed by emergence of escaped p21-expressing proliferating cells, featuring increased genomic instability and aggressiveness. p21 (WAF1/CIP1) expression caused DNA damage, in the form of double strand breaks (DSBs). At the mechanistic level DSBs were generated: i) by deregulating the replication licensing machinery triggering re-replication and replication fork collapse and ii) by suppressing translesion synthesis and repair (TLS) converting single stand defects to double strand breaks (Galanos et al., 2016)**.** As DSBs accumulated, the DNA repair pathways engaged demonstrated a shift from Rad51-dependent high-fidelity (error-free) towards Rad52-dependent low-fidelity (error-prone) repair, promoting genomic instability (Galanos et al., 2016, Galanos et al., 2018)**.** Within the same vein, DNA damage and DDR followed CDC6 induction in HBEC CDC6 Tet-ON system and were attenuated in "escaped" cells, implying that a repair process takes place (Komseli et al., 2018).

An additional source of genomic instability is possibly R loop formation. R loops are three-stranded nucleic acid structures that encompass nascent RNA hybridized with DNA template, leaving single stranded the non-template DNA (ssDNA**)** (Skourti-Stathaki & Proudfoot, 2014)**.** They are also frequently produced at CFS, regions of the genome prone to replication stress, located in long human genes (≥ 800 kb); thus increasing the possibility of replication-transcription collision and genomic instability (Georgakilas et al., 2014). The fact that R loops are reported *in vivo* at origins of replication (Xu & Clayton, 1996, Baker & Kornberg, 1988, Carles-Kinch, 1997, Masukata & Tomizawa, 1984) and rDNA loci (Y.A. et al., 2014, El Hage, Webb, Kerr, & Tollervey, 2014) increases the probability of their formation by deregulated expression of the replication licensing factor, CDC6.

1.11 Aim

Aim of this thesis is: 1) to describe a "timeline" of senescence induction, 2) to provide more detailed insights in the DNA damage and repair process mechanisms leading to senescence, and 3) to define and analyse the cycle phase, during which DNA damage occurs upon CDC6 induction. Also, this thesis targets to determine the sources of DNA Damage, to evaluate the

risk of replication-transcription collision and to investigate the alterations in replication dynamics as well as the forms of replication stress triggered by CDC6 which probably contribute in genome instability.

2 MATERIALS-METHODS

2.1 Description of HBEC CDC6 –Tet-ON system

In this thesis, immortalized Human Bronchial Epithelial Cells (HBECs) were employed. Immortalization had already been performed with combined expression of hTERT and ectopic mutant cyclin-dependent kinase 4 (CDK4) in order to avoid p16INK4A-induced premature growth arrest and to maintain the epithelial phenotype (Ramirez et al., 2004, K. 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., 2018) in order to generate an inducible-expression cellular model in the immortalized Human Bronchial Epithelial Cells. Overexpression of CDC6 is achieved through treatment with doxocycline (at a concentration of 1μg/mL).

2.2 Cell culture

Immortalized HBECs (OFF cells) and HBEC CDC6 Tet-ON cells were cultivated in Keratinocyte-Serum-Free Medium (17005-075, Invitrogen) supplemented with 50μg/ml Bovine Pituitary Extract and 5ng/ml hEGF (#17005-075, Invitrogen) at 37^{o}C and 5% CO_2. CDC6 induction was conducted by treatment of the cell culture with 1 μg/ml doxocycline (DOX) (Applichem). (Komseli et al., 2018; Ramirez et al., 2004).

2.3 Cell splitting

For cell splitting the medium was removed and cells were washed with PBS 1X. An appropriate volume of Trypsin-EDTA (Thermo Scientific) was added in the plate or flask depending on the surface to be covered. Cells were incubated with Trypsin for 5 min at 37°C (5% CO_2). Trypsin neutralizer solution (0,5% FBS in PBS 1X) was added in the same volume as trypsin for inactivation of Trypsin. Cells were collected for centrifugation at 1700 rpm for 5 min at room temperature. The pellet was resuspended in the appropriate volume of Keratinocyte Serum-Free Medium (17005–075, Invitrogen) depending on the number of the plates and flasks required for cell distribution.

2.4 Freezing cells

The same method used for splitting cells was followed, except for the resuspension step, where pellet was resuspended in freezing medium consisting of Keratinocyte Serum-Free Medium 10% DMSO. 1,5 mL of that was transferred in cryovials which were placed at -20°C for 20 min and then stored at -80°C.

2.5 Defreezing cells

The content of a cryovial was transferred in a 15 ml falcon. 7 ml PBS 1X was added and the cells were centrifuged at 1700 rpm for 5 min at 20°C. The pellet was resuspended in 4-5ml Keratinocyte Serum-Free Medium (17005–075, Invitrogen) and was transferred in a plate or a flask.

2.6 Immunofluorescence

For indirect IF analysis HBECs were cultured on coverslips and CDC6 was induced using doxocycline. 3 days ON and 6 days ON HBECs were treated with DRB for 3 hours and incorporated with 5-EU for 1 hour. Cells were fixed with 4% paraformaldehyde for 10 min at room temperature and were stored at 4 °C until staining was performed. Following, cells were permeabilized with 0,3% Triton X-100 in PBS for 5 min at room temperature. A 10% fetal bovine serum and 3% bovine serum albumin in PBS solution was used as a blocking buffer for 1 h at room temperature. 53BP1 (Abcam #21083, 1:250) primary antibody was used, diluted in blocking buffer and incubated overnight at 4 °C. Secondary antibodies were goat anti-rabbit or goat anti-mouse, Alexa Fluor® 488 or Alexa Fluor® 568 (Invitrogen) diluted 1:500 in blocking buffer. Counterstaining was performed with 100 ng/ml DAPI (Sigma-Aldrich) and 5-EU. Image acquisition from multiple random fields was automatically obtained on a ScanR screening station (Olympus, Germany) and analyzed with ScanR (Olympus, Germany) software, or a Zeiss Axiolab fluorescence microscope equipped with a Zeiss Axiocam MRm camera and Achroplan objectives, while image acquisition was performed with AxioVision software 4.7.1.

2.7 Isolation of proteins from cells

Cells were 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. Cell pellets were resuspended in 1mL PBS 1X and centrifuged at 1500 rpm for 10 minutes. Pellets were dissolved in 50 μl RIPA buffer, consisting of protease inhibitor and phosphatase inhibitor at a concentration of 1:100, rotated for 1 hour in the cold room and then centrifuged at 13.400 rpm for 15 minutes. Proteins were obtained in the supernatant which was stored at -80^{o} C.

2.8 Preparing HBECs for BLISS

Cells were spotted onto coverslips pre-coated with poly-L-lysine (P8920-100ML, Sigma). Afterwards, 100 μl of paraformaldehyde 8% in 1 × PBS were gently added and incubated for

10 min at room temperature, followed by two washes in 1 × PBS at room temperature. The samples were stored in 1 × PBS at 4 °C up to 1 month before performing BLISS.

2.9 BLISS (Breaks Labeling In Situ and Sequencing)

The procedure was undertaken by Reza Lab. It starts by attaching cells fixed with formaldehyde onto a microscope slide or coverglass, which enables all the subsequent in situ reactions to be performed without centrifugations, thus minimizing the risk of introducing artificial DNA breaks and sample loss. DSBs are in situ blunted and then ligated with a double-stranded DNA oligonucleotide adapter containing the T7 promoter sequence, the RA5 Illumina sequencing adapter, a random stretch of 8–12 nucleotides (nt) that serves as unique molecular identifier (UMI) and a sample barcode suitable for multiplexing. Following genomic DNA (gDNA) extraction, the sequence immediately downstream to the tagged DSBs is linearly amplified via T7-mediated in vitro transcription, which has been shown to introduce fewer biases compared with exponential amplification by PCR when amplifying complementary DNA from low-input samples including single cells (W. X. Yan et al., 2017).

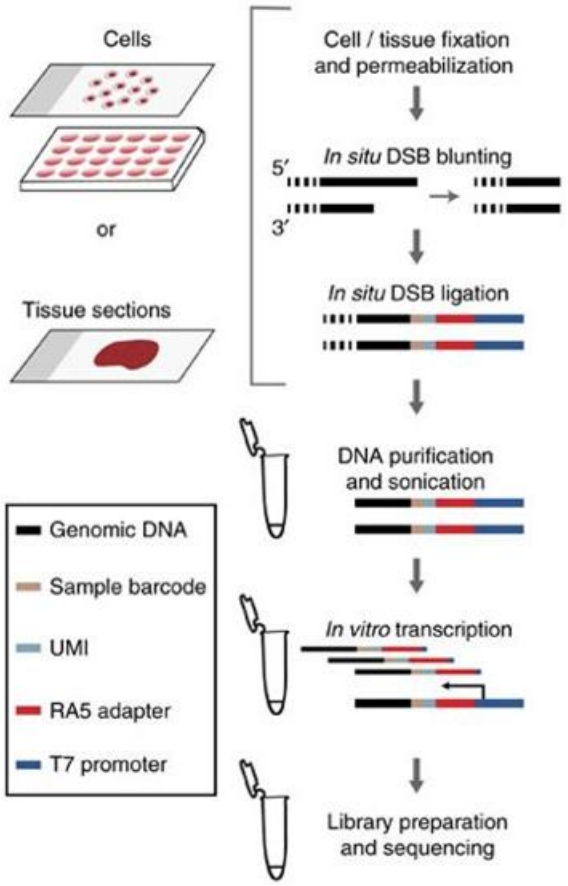

Figure 2.1: Schematic of BLISS.. The workflow starts by either fixing cells onto a microscope slide or in a multi-well plate, or by immobilizing already fixed tissue sections onto a slide. DSB ends are then in situ blunted and tagged with dsDNA adapters containing components

described in the boxed legend. Tagged DSB ends are linearly amplified using in vitro transcription and the resulting RNA is used for Illumina library preparation and sequencing (W. X. Yan et al., 2017).

2.10 Illumina library preparation and sequencing.

The resulting RNA was used for Illumina library preparation and sequencing as described by (W. X. Yan et al., 2017).

2.11 Estimation of DSBs per cell

The estimation of DSBs per cell and normalization was subsequently performed. For each sample, the number of DSBs per cell was estimated by counting the number of sequenced reads with correct prefix mapped to a unique genomic location and tagged by a unique UMI and assuming that on average one DSB produces two unique reads. Then, the data was fitted to the model $\mathrm{DSB} = \frac{r\mathrm{DSB}_{\max}}{r+k}$, where DSBmax is the number of DSB events per cell at saturation, r is the number of total reads and k is a constant. At saturation, the model estimated DSBmax=94 breaks per cell (95% confidence interval: 93.10–95.07) (W. X. Yan et al., 2017).

2.12 DNA combing assay

Molecular combing is a process whereby single DNA molecules (hundreds of Kbs) are stretched on a silanized glass surface (Bensimon et al., 1994). (Herrick & Bensimon, 1999). Briefly, HBEC-CDC6 Tet-ON cells were grown in the presence or absence of doxycyclin for the indicated time points and then pulsed-labeled with 25μM CldU for 20min, and then labelled with 250μM IdU for 20min. Cells were then harvested and lysed on glass slides in spreading buffer. The DNA was denatured and stained with rat anti-BrdU/CldU (1:1000, OBT0030F, Immunologicals Direct) and mouse anti-IdU/BrdU (1:500, clone B44, Becton Dickinson) primary antibodies. The length of the replication signals and the fork distances were measured in micrometers and converted to kilo bases according to a constant and sequence independent stretching factor (1μm = 2kb) (Herrick & Bensimon, 1999). Images were analyzed double blindly using Fiji (Schindelin et al., 2012).

2.13 Quantitative image-based microscopy (QIBC)

Protein accumulation on chromatin has traditionally been studied using immunofluorescence microscopy or biochemical cellular fractionation followed by western immunoblot analysis. As a way to improve the reproducibility of this kind of analysis, make it easier to quantify and allow a stream-lined application in high-throughput screens, we recently combined a classical immunofluorescence microscopy detection technique with flow cytometry. In addition to the

features described above, and by combining it with detection of both DNA content and DNA replication, this method allows unequivocal and direct assignment of cell-cycle distribution of protein association to chromatin without the need for cell culture synchronization. Furthermore, it is relatively quick (no more than a working day from sample collection to quantification), requires less starting material compared to standard biochemical fractionation methods and overcomes the need for flat, adherent cell types that are required for immunofluorescence microscopy (Forment & Jackson, 2015).

For immunostaining, cells growing on 12mm coverslips were fixed in formaldehyde 4% (VWR) for 15 min at room temperature. Primary antibodies were diluted in filtered DMEM containing 10% FBS and 0.05% Sodium Azide. Incubations with the primary antibodies were performed at room temperature for 1 to 3 hr. Coverslips were washed twice with PBS-Tween20 (0.01%) and incubated in DMEM/FBS/SA containing secondary fluorescently labeled antibodies (Alexa fluorophores, Life Technologies) for 45 min. PBS-T containing 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI, 0.5 mg/ml) was applied for 5 min at room temperature to stain DNA. After two more washes in PBS-T, coverslips were dipped in distilled water, placed on 3MM paper to dry, and mounted on 10 ml Mowiol-based mounting media: Mowiol 4.88 (Calbiochem)/Glycerol/TRIS.

Images were acquired with a ScanR inverted microscope High-content Screening Station (Olympus) equipped with wide-field optics, a 20×, 0.75-NA (UPLSAPO 20×) dry objective, fast excitation and emission filter-wheel devices for DAPI, FITC, Cy3, and Cy5 wavelengths, an MT20 illumination system, and a digital monochrome Hamamatsu ORCA-R2 CCD camera (yielding a spatial resolution of 320 nm per pixel at 20× and binning of 1). Images were acquired in an automated fashion with the ScanR acquisition software (Olympus, 2.6.1). Images were acquired, depending on cell confluency, containing at least 1,000 cells per condition. Acquisition times for the different channels were adjusted for nonsaturated conditions in 12-bit dynamic range, and identical settings were applied to all the samples within one experiment. Images were processed and analyzed with ScanR analysis software. TIBCO Software, version 5.0.0. software was used to quantify absolute, median, and average values in cell populations and to generate all color-coded scatter plots. (Ochs et al., 2016)

Secondary antibodies used were Alexa Fluor 647 (ab150115), while primary antibodies utilized were: 53BP1 (Abcam, ab3682) and gH2AX (Millipore, 05-636).

3 RESULTS

3.1 QIBC (Quantitative image-based microscopy)

In the present work we aim to define the DNA damage and the activation of the DDR signaling during the early phases of CDC6 induction in the HBEC-CDC6 Tet-ON system. As we have previously shown CDC6 induction results in a robust establishment of senescence that culminates at day 6 (Komseli et al, 2018). To further monitor the phenotypic response we employed the QIBC method, which is a combination of the classical immunofluorescence microscopy detection technique with flow cytometry. This method allows direct assignment of cell-cycle distribution of protein association to chromatin without the need for cell culture synchronization and is relatively quick (Forment & Jackson, 2015).

Following the kinetics of CDC6 induction FACs plots (Figures 3.1, 3.6) depicted that after 12 hours, S phase starts to gradually decrease, as replication stops and cells became arrested in S phase, entering senescence. The increase in 53BP1 foci and gH2AX during early CDC6 overexpression are signs of very early DNA Damage (Figures 3.3, 3.7). There are, also, marks of re-replication, (counts corresponding to DAPI intensity>500000) in 53BP1 QIBC FACs plot (Figure 3.6) observed, which is a potential factor of DNA damage and DNA damage response, leading cells to senescence. Moreover, in the QIBC quantification plots 53BP1 and gH2AX counts appear increased 12 hours after induction of CDC6 expression, although there is a decline in counts for both 53BP1 and gH2AX 48 hours after CDC6 induction (Figures 3.3, 3.7). On the other hand, the intensities of both 53BP1 and gH2AX signal show a gradual decrease from 12 hours to 24 hours but peak at 48 hours following CDC6 induction (Figures 3.4, 3.8).. Also, high 53BP1 and gH2AX intensity and DDR response is found in rereplicating cells. As previously mentioned, 53BP1 promotes NHEJ repair of replication induced DNA breaks and inhibits Homologous Recombination, therefore an increase in 53BP1 is indicative of increased error-prone NHEJ activity.

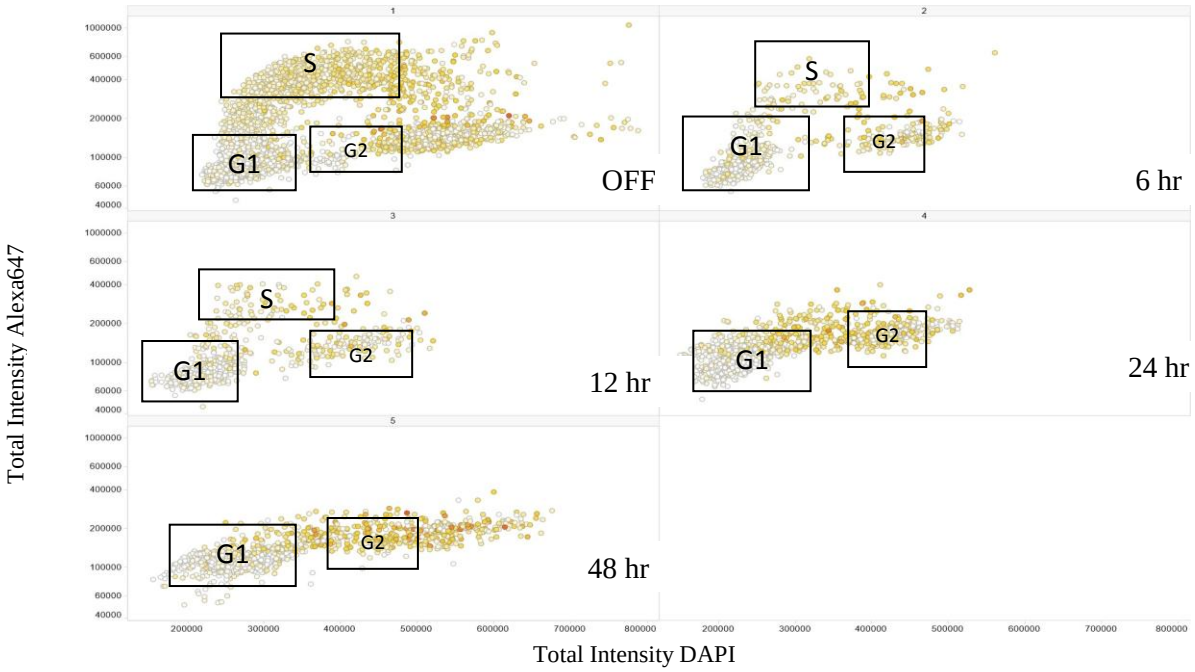

Figure 3.1: QIBC gH2AX counts FACs plot. QIBC of gH2AX counts per cell in HBECs at specific timepoints after CDC6 induction which were stained for nuclei (DAPI) and gH2AX antibody. Red corresponds to 35 counts, yellow corresponds to 10 counts, while white depicts the minimum limit. Counts corresponding to intensity DAPI>500000 represent rereplication. After 12 hours of CDC6 induction, S phase starts to gradually decrease.

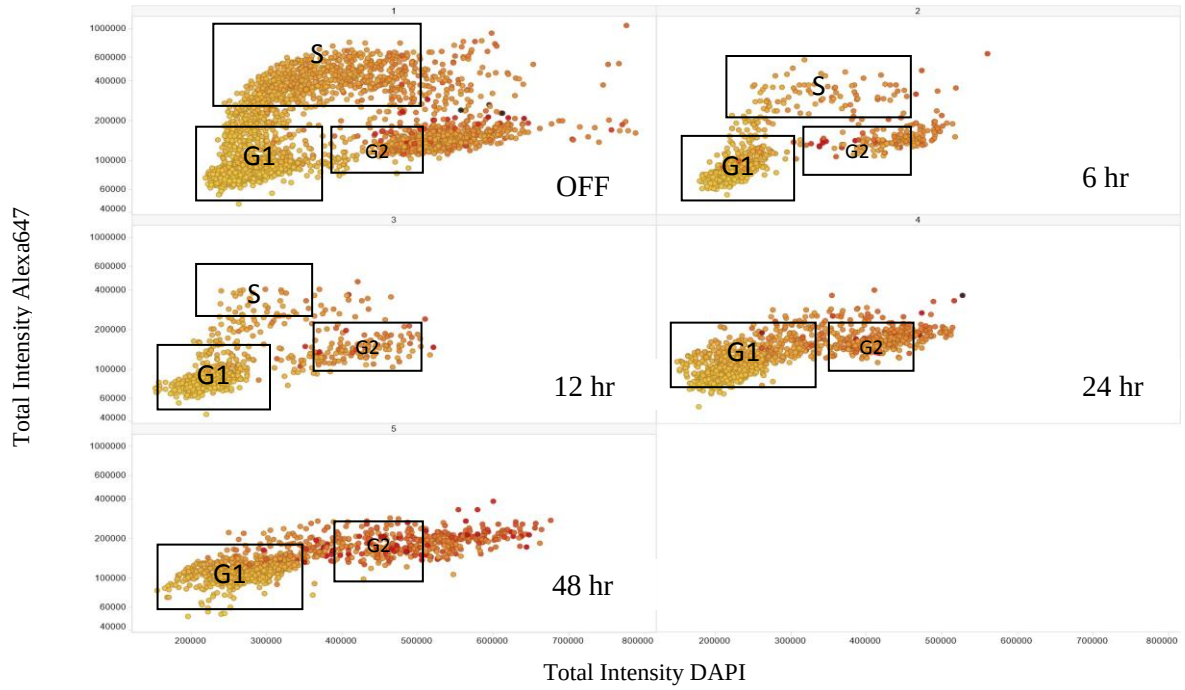

Figure 3.2: QIBC gH2AX intensity FACs plot. QIBC of gH2AX intensity in HBECs at specific timepoints after CDC6 induction which were stained for nuclei (DAPI) and gH2AX antibody. Red corresponds to intensity 2000000, yellow corresponds to 500000 counts, while white depicts 5000. Counts corresponding to intensity DAPI>500000 represent rereplication. After

12 hours of CDC6 induction, S phase starts to gradually decrease. Intensity of gH2AX demonstrates a significant increase at 48 hours after CDC6 induction especially in G2 phase and rereplication.

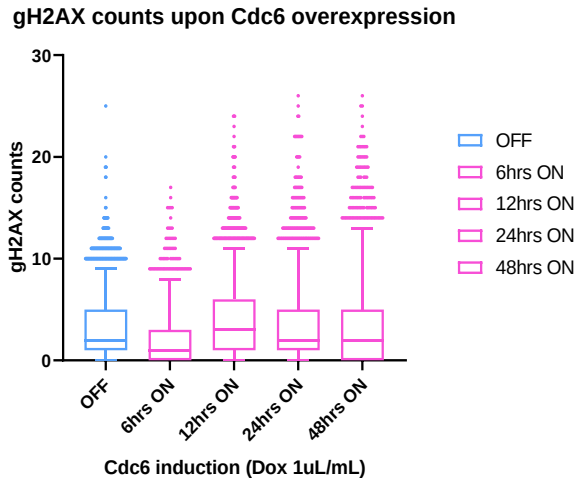

Figure 3.3: QIBC gH2AX counts quantification plot. Quantification of the gH2AX foci counts derived from the QIBC analysis. gH2AX counts are shown increased 12 hours after induction of CDC6 expression.

Figure 3.4: QIBC gH2AX intensity quantification plot. Quantification of the sum of the intensities of gH2AX foci per nucleus, derived from the QIBC analysis. Intensity of gH2AX signal peaks at 48 hours.

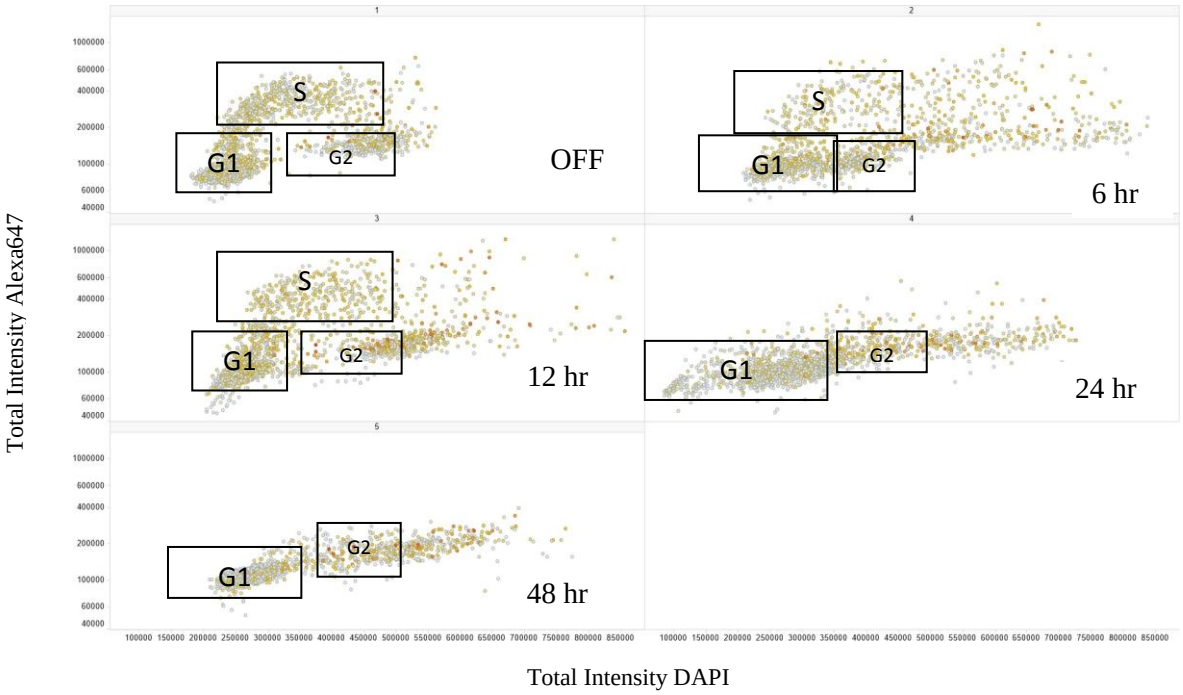

Figure 3.5: QIBC 53BP1 counts FACs plot. QIBC of 53BP1 counts per cell in HBECs at specific timepoints after CDC6 induction which were stained for nuclei (DAPI) and 53BP1 antibody. Red corresponds to 25 counts, yellow corresponds to 5 counts, while white depicts the minimum limit. Counts corresponding to intensity DAPI>500000 represent rereplication. After 12 hours of CDC6 induction, S phase starts to gradually decrease.

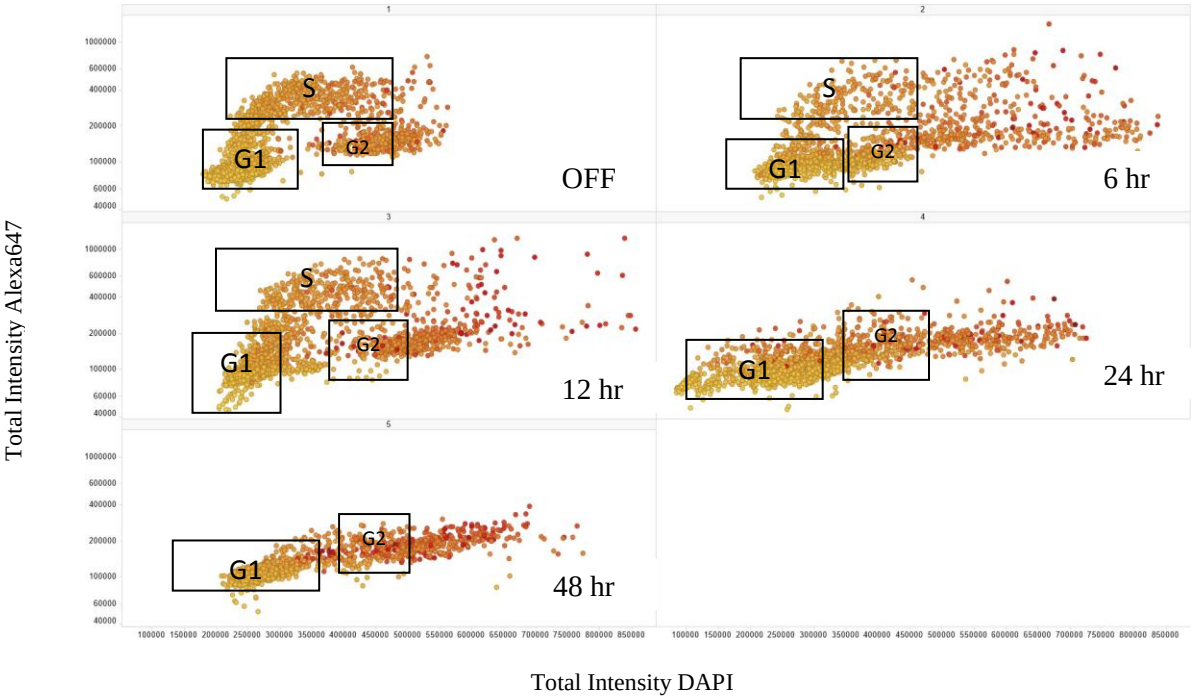

Figure 3.6: QIBC 53BP1 intensity FACs plot. QIBC of 53BP1 intensity in HBECs at specific timepoints after CDC6 induction which were stained for nuclei (DAPI) and 53BP1 antibody. Red corresponds to intensity 2000000, yellow corresponds to intensity 500000 counts, while

white depicts intensity 45000. Counts corresponding to intensity DAPI>500000 represent rereplication. After 12 hours of CDC6 induction, S phase starts to gradually decrease. Intensity of 53BP demonstrates a significant increase at 48 hours after CDC6 induction especially in G2 phase and rereplication.

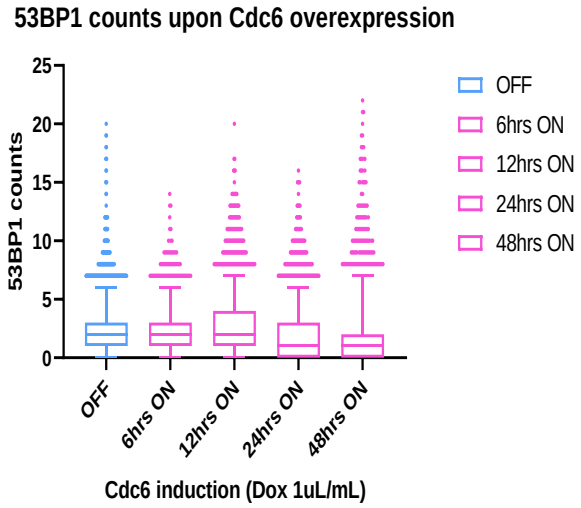

Figure 3.7: QIBC 53BP1 counts quantification plot. Quantification of the 53BP1 foci counts derived from the QIBC analysis. 53BP1 counts are shown increased 12 hours after induction of CDC6 expression.

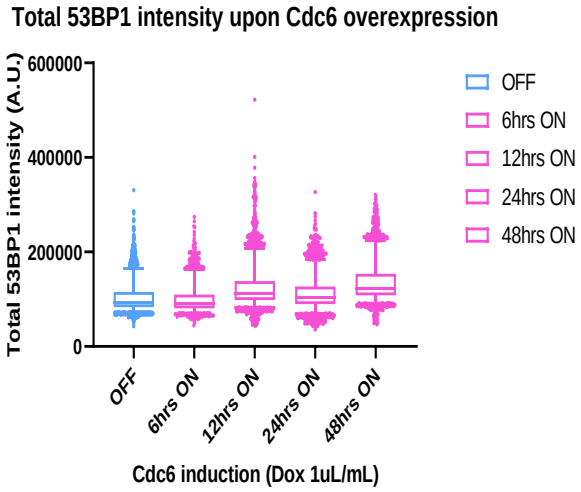

Figure 3.8: QIBC 53BP1 intensity quantification plot. Quantification of the sum of the intensities of 53BP1 foci per nucleus, derived from the QIBC analysis. Intensity of 53BP1 signal peaks at 48 hours.

3.2 Transcription-replication collision

As aforementioned, in the HBEC system activation of CDC6 is followed by DNA damage, an intense DDR and an increase formation of R-loops (Komseli et al, 2018), three-stranded RNA:DNA hybrid structures that favor replication-transcription collision (Skourti-Stathaki &

Proudfoot, 2014). The ectopic origin firing within active gene bodies triggering replication-transcription collision (Macheret & Halazonetis, 2018) in combination with re-replication due to overexpression of CDC6 (Komseli et al., 2018; Liontos et al., 2007; Theodoros G. Petrakis et al., 2016; Thodoris G. Petrakis et al., 2012) lead to aberrant replication program, replication stress and promote cancer development. In order to examine the scenario of replication-transcription collision, we attempted to inhibit global transcription with DRB (5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole). More specifically, the experiment we conducted includes treatment of OFF, 3 days and 6 days induced cells, termed ON, with DRB, a transcription inhibitor, for 4 hours, as well as incorporation of 5-EU for 1 hour, in order to assess total transcription levels. DRB was substituted with DMSO for treatment of control cells.

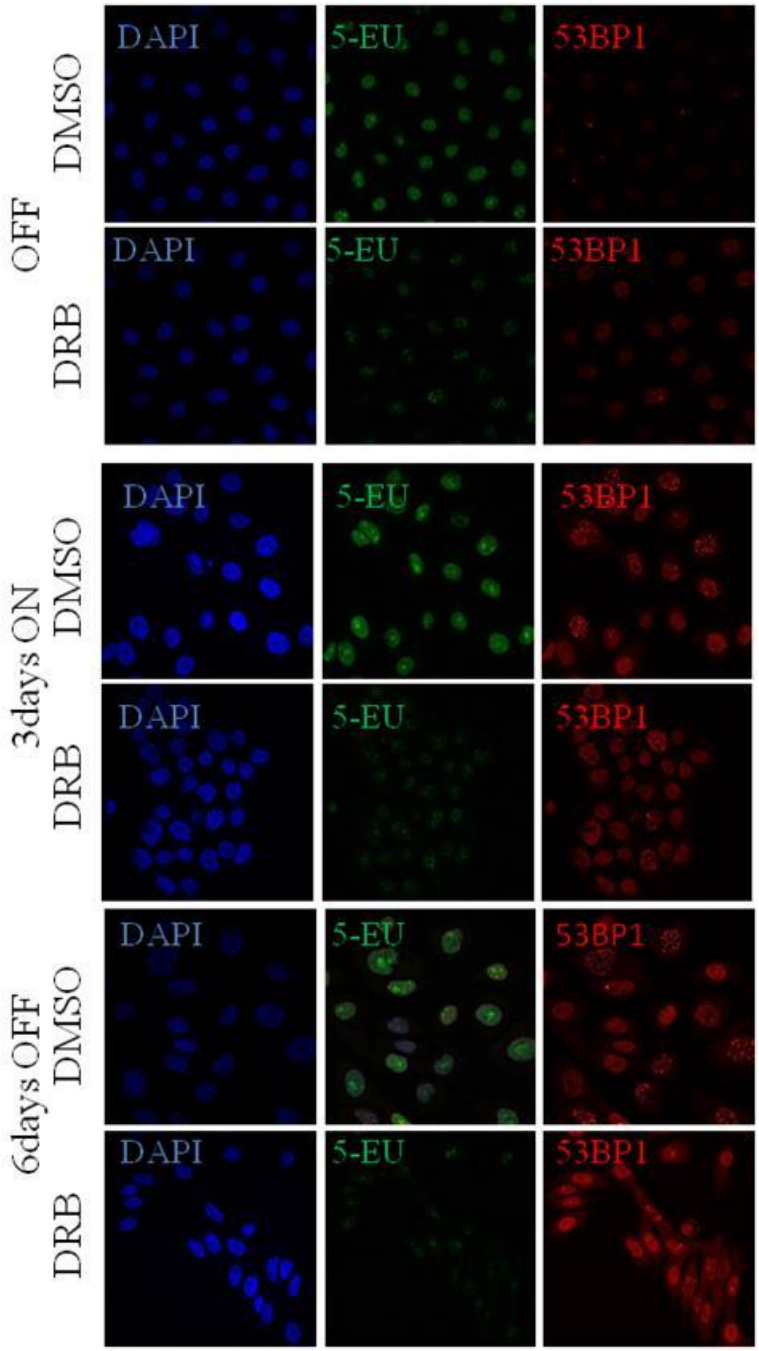

Figure 3.9: IF pictures. IF for 53BP1, 5-EU and DAPI in OFF, 3 days ON and 6 days ON HBECs treated with DRB (or DMSO) and incorporated with 5-EU, reflecting existence of replication-transcription collision.

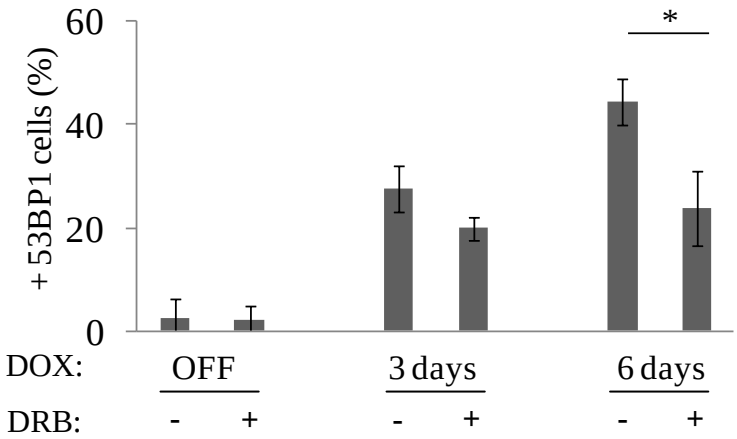

Figure 3.10: IF quantification plot. Percentage of OFF, 3 days and 6 days ON HBECs showing 53BP1 foci following treatment with DRB (or DMSO) and 5-EU incorporation.

Figures 3.9 and 3.10 depict that DRB treatment decreases 53BP1 foci formation and reduces DNA Damage Response in 3 days and 6 days ON HBECs compared to the 3 days and 6 days ON HBECs treated with DMSO (control ones), supporting the proposed scenario that replication transcription collision occurs and stands as a replication stress factor.

3.3 Alterations in DNA replication dynamics

As noted before, CDC6, as a replication licesing factor, when overexpressed, results in re-replication that leads to replication fork stalling DNA damage and DDR activation (Bartkova et al., 2006)**,** (Liontos et al., 2007)**,** (Walter et al., 2016)**.** Investigating aberrant replication dynamics can unveil essential mechanistic aspects of CDC6-driven replication stress and senescence. Therefore, we studied the effect of CDC6 overexpression on replication dynamics, by applying DNA fiber spreading (DNA combing) in order to examine fork speed and to measure fork distance in OFF HBECs and in ON HBECs at 6, 12, 15, 18 hours timepoints after CDC6 induction.

DNA fiber fluorography (combing assay) demonstrated accelerated fork progression and reduced origin firing, as depicted by the increased mean fork rate and origin distance, respectively (Figure 3.11, 3.12 and Tables 3.1. 3.2).

Table 3.1: Mean replication fork rate for each timepoint after CDC6 induction measured by DNA combing assay.

HBEC CDC6 –Tet-ON	**OFF**	**6hr**	**12hr**	**15hr**	**18h**
Mean replication fork rate (kb/min)	0,97	0,86	1,25	1,26	**1,47**

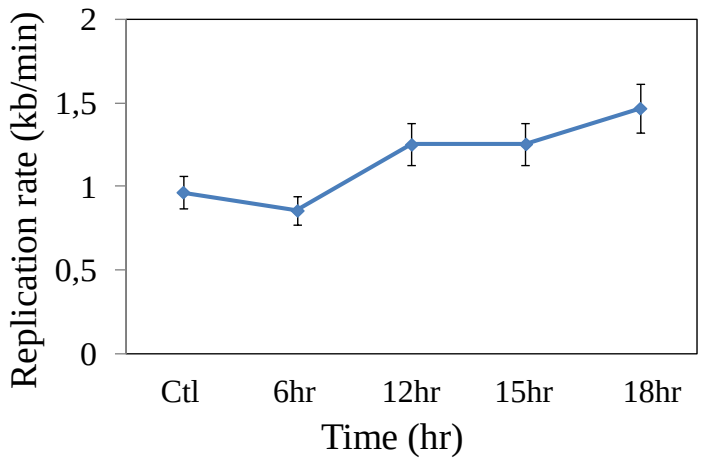

Figure 3.11: DNA Combing. Replication fork rate (kb/min) following CDC6 induction estimated by DNA combing assay

Table 3.2: Fork distance for each timepoint after CDC6 induction measured by DNA combing assay

HBEC CDC6 –Tet-ON	**OFF**	**6hr**	**12hr**	**15hr**	**18h**
Fork distance (kb)	89,76	85,79	127,38	130,73	**134,28**

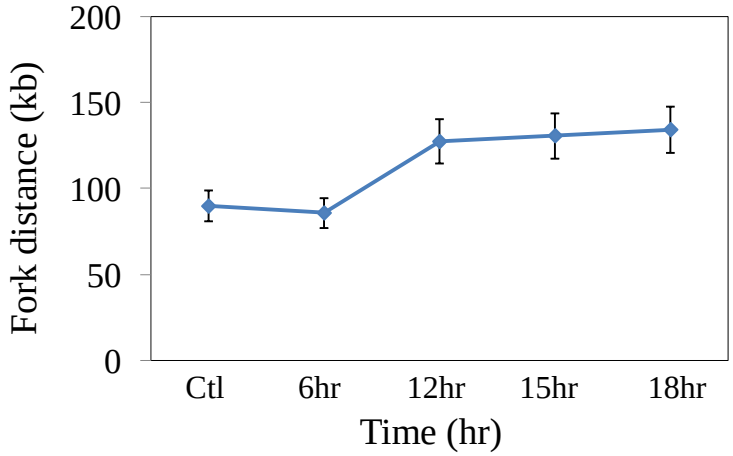

Figure 3.12: DNA Combing. Fork distance (kb) following CDC6 induction estimated by DNA combing assay

A similar pattern of replication dynamics is observed when the abundance of proteins involved in origin firing is altered (Maya-Mendoza et al., 2018; Zhong et al., 2013). Markedly, the fork velocity exceeded the reported tolerated threshold of 40% (OFF: 0,97 Kb/min vs 18h ON: 1,47), above which DNA damage and DDR occur (Maya-Mendoza et al., 2018).

3.4 BLISS analysis

The accumulation of DNA DSBs activates the DNA damage response pathways and contributes to genomic instability with final outcome the transformation of the cells into a senescent state (Bartkova et al., 2006; Di Micco et al., 2006). As aforementioned, overexpression of p21 in p53-null, cancerous and near-normal cells resulted in their entering senescence and caused DNA damage in the form of double strand breaks (DSBs), which were mechanistically generated by deregulation of the replication licensing machinery, re-replication and replication fork collapse (Galanos et al., 2016). In line with HBEC-CDC6 TET-ON system, we found increased replication fork rate and fork distance during early stages of CDC6 induction as assessed by DNA Combing assay. Thus, we examined whether deregulation of replication dynamics triggered formation of DNA DSBs, which we estimated performing a BLISS analysis in 3 days and 6 days ON HBECs. As shown below (Figure 3.13), DSBs appear increased in 3 days and 6 days ON HBECs compared to OFF HBECs, but are reduced in 6 days ON HBECs compared to 3 days ON HBECs, which implies that a DNA repair process took place during early senescent phase. As noticed by Komseli et al., 2018 the increase in 53BP1 foci in 3 days ON HBECs is indicative of error-prone NHEJ activity. This error prone DNA repair mechanism possibly fuels genomic instability and creates a permissive environment for breaching the anti-tumor barriers of senescence and initiating cancer (Gorgoulis et al., 2005).

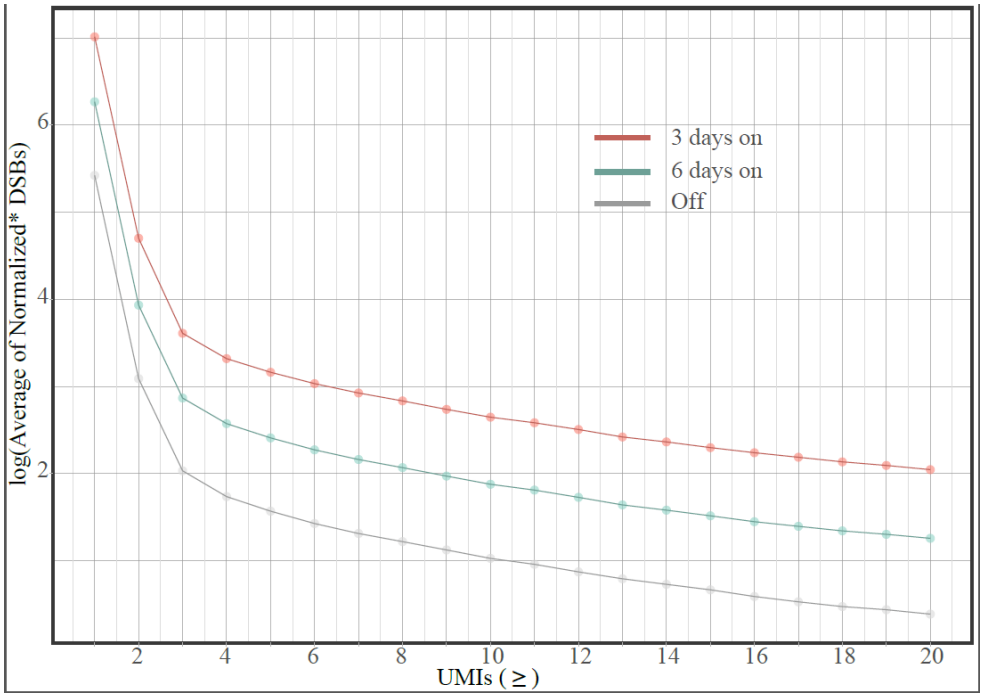

Figure 3.13: BLISS analysis plot. DSBs are increased in ON HBECs, but appear to decrease in 6 days ON HBECs.

4 DISCUSSION

In this thesis, we investigated mechanisms of oncogene-induced senescence (OIS), a type of stress-induced premature senescence (SIPS), which acts as an anti-tumor barrier and must be bypassed for tumor progression (Bartkova et al., 2006; Di Micco et al., 2006; Halazonetis et al., 2008; Komseli et al., 2018) and we studied the precancerous phase of epithelial tumorigenesis. We used the Immortalized Human bronchial epithelial cells (HBECs) with 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 prevent possible cycle arrest due to activation of the signaling pathway p16INK4) as a non-malignant model (K. Evangelou et al., 2013; Komseli et al., 2018). CDC6 was selected as an oncogene not only because of its important role as a replication licensing factor but also due to its deregulation in a variety of cancers (Karakaidos et al., 2004; Liontos et al., 2007). In the HBEC CDC6 Tet-ON system, CDC6 induction with doxocyxline results in a 3-day induction senescent phenotype, as shown by GL13 staining that peaks at day 6 and around a month after the first CDC6 induction, a fraction of cells escapes from senescence, acquires spindle morphology and starts proliferation (Komseli et al., 2018).

In this thesis we study the DNA damage and the DDR signaling during the early phases of CDC6 induction in the HBEC-CDC6 Tet-ON system. More specifically, CDC6 induction has been proved to result in a robust senescent phenotype that peaks at day 6 (Komseli et al., 2018). In order to investigate the response of HBEC-CDC6 Tet-ON during very early stages of CDC6 overexpression (hours following CDC6 induction), and to determine DNA damage and DDR throughout the cell cycle for each timepoint after CDC6 overexpression, we applied the QIBC method, a combination of immunofluorescence with flow cytometry, estimating intensity and counts of gH2AX and 53BP1 (per cell), markers of DDR. We concluded that DNA damage occurs very early, already at 12 hours after CDC6 overexpression, while simultaneously ON HBECs start entering the senescent stage and getting arrested at the S phase, as confirmed by the reduction of S phase at 12 hours, which disappears completely later. Marks of re-replication are also obvious, corresponding to counts with DAPI intensity >500000, indicating re-replication as potential factor fueling genomic instability.

Also, as CDC6 is a replication licensing factor, it is expected that its deregulation could cause dysfunction in replication dynamics. Moreover, it has been reported that CDC6 over-expression leads to replication fork stalling, collapse, DNA damage and DDR activation through triggering re-replication (Bartkova et al., 2006, Liontos et al., 2007, Walter et al., 2016), the presence of which was confirmed with QIBC. This motivated us to examine the alterations in replication dynamics following first stages of CDC6 induction. For this purpose, we applied DNA fiber spreading (DNA combing) in order to calculate fork speed and

measure fork distance in OFF HBECs and ON HBECs at 6, 12, 15, 18 hours following CDC6 induction. Our findings demonstrated that replication fork rate was increased, while the origin firing was reduced, as reflected by the increase in the distance between the replication forks (Figures 11, 12). Noticeably, the fork velocity exceeded the reported threshold of 40% (OFF: 0,97 Kb/min vs 18h ON: 1,47), above which DNA damage and DDR are caused (Maya-Mendoza et al., 2018).

As aforementioned, in line with our HBEC CDC6-Tet-ON system, induction of p21 in p53-null, cancerous and near-normal cells triggered DNA damage in the form of double strand breaks (DSBs), and resulted in their transformation in a senescent state. These DSBs were reported to arise mechanistically from deregulation of the replication licensing machinery, re-replication and replication fork collapse (Galanos et al., 2016)**.** As we proved not only the phenomenon of re-replication with QIBC and found deregulated replication dynamics with DNA fiber assay during first hours after CDC6 induction in our system, we decided to examine the existence of DSBs as a result of deregulated replication and re-replication. Thus, we ran a BLISS analysis in 3 days and 6 days ON senescent HBECs and found that DSBs are increased in senescent HBECs but appear to be decreased in 6 days ON compared to 3 days ON HBECs. This leads to the conclusion that a DSBs where repaired from 3 days ON to 6 days ON HBECs. Komseli et al., 2018 stated an increase in 53BP1 foci in 3 days ON HBECs and as 53BP1 activates the error prone NHEJ repair mechanism in favor of the error free Homologous Recombination (HR) (Guo et al., 2018), the activity of the error prone NHEJ repair mechanism could possibly fuel genomic instability and create a permissive environment for breaching the anti-tumor barriers of senescence and initiating cancer (Gorgoulis et al., 2005).

Furthermore, Komseli et al., 2018 stated R loop formation as another source of genomic instability, which raises the possibility of replication-transcription collision. Therefore, we finally investigated the scenario of the replication by inhibiting global transcription with DRB (5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole) in 3 days ON and 6 days ON HBECs and found that DRB resulted in reduction of 53BP1 foci, a finding which indicates reduced DNA Damage Response. So, the proposed scenario of replication transcription collision was confirmed, with replication transcription collision being the last factor proved in this thesis to cause DNA damage and lead to senescence.

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