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"ALEXANDER FLEMING"
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"Escape" from oncogene induced senescence

Master's Thesis
M.Sc in Molecular Biomedicine

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Place of research

The current study was performed in collaboration of the National and Kapodistrian University of Athens in Greece with the University Medical Center of Göttingen in Germany. A large part of my thesis took place in Professor Papantonis' laboratory at Translational Epigenetics Group of the Institute of Pathology in the University Medical Center of Göttingen in Germany.

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Thank you,
Andriani Angelopoulou

Contents

ABSTRACT

The reversibility of senescence has been an issue highly disputable until recently, thus the factors driving this event remain still elusive. Using a human epithelial model, we recapitulate the whole spectrum of lung carcinogenesis, promoted by CDC6 overexpression, including the precancerous (senescent) state and the escape from senescence providing clear evidence about the increased proliferative capacity and aggressiveness acquired by the escaped clones. We also focus on the genetic and epigenetic determinants driving and/or contributing to the phenomenon of the oncogene induced escape from senescence. BHLHE40 was identified as a crucial gene, since an inversion in the region encoding this locus promoted the altered expression of numerous genes while it was found essential for the survival of the escapees. Apart from the genetic alterations, the aberrant expression of histone marks, such as H4K16ac, may facilitate the phenomenon by affecting the expression of genes related to senescence and the evasion from it. Further research is required to delineate the genetic and epigenetic contribution to the escape from oncogene induced senescence.

1. INTRODUCTION

1.1 Replication

DNA replication in eukaryotic cells is an elaborate and finely tuned set of control processes which assure the precise and timely DNA duplication and its coordination with other events of cell cycle, so as to avoid the establishment of DNA lesions and the accumulation of mutations to the next generations. Thus, the "replication machinery" has evolved to respond to numerous signals ensuring replication fidelity and maintaining genomic integrity (Bell and Dutta 2002; Sancar et al. 2004).

DNA replication takes place in two steps, the licensing and the firing of particular genomic sites, known as replication origins where replication initiates. The origins are recognized by Origin Recognition Complex (ORC), which functions as a platform to recruit licensing factors and Minichromosome maintainance protein complex helicase (MCM), forming the pre-replication complex (pre-RC) **(Figure 1.1)**. MCM loading functions as DNA replication licensing during G1 phase of every cycle (DePamphilis 2003; Fragkos et al. 2015). The pre-replication complex leads to the assembly of other proteins which activate MCMs.

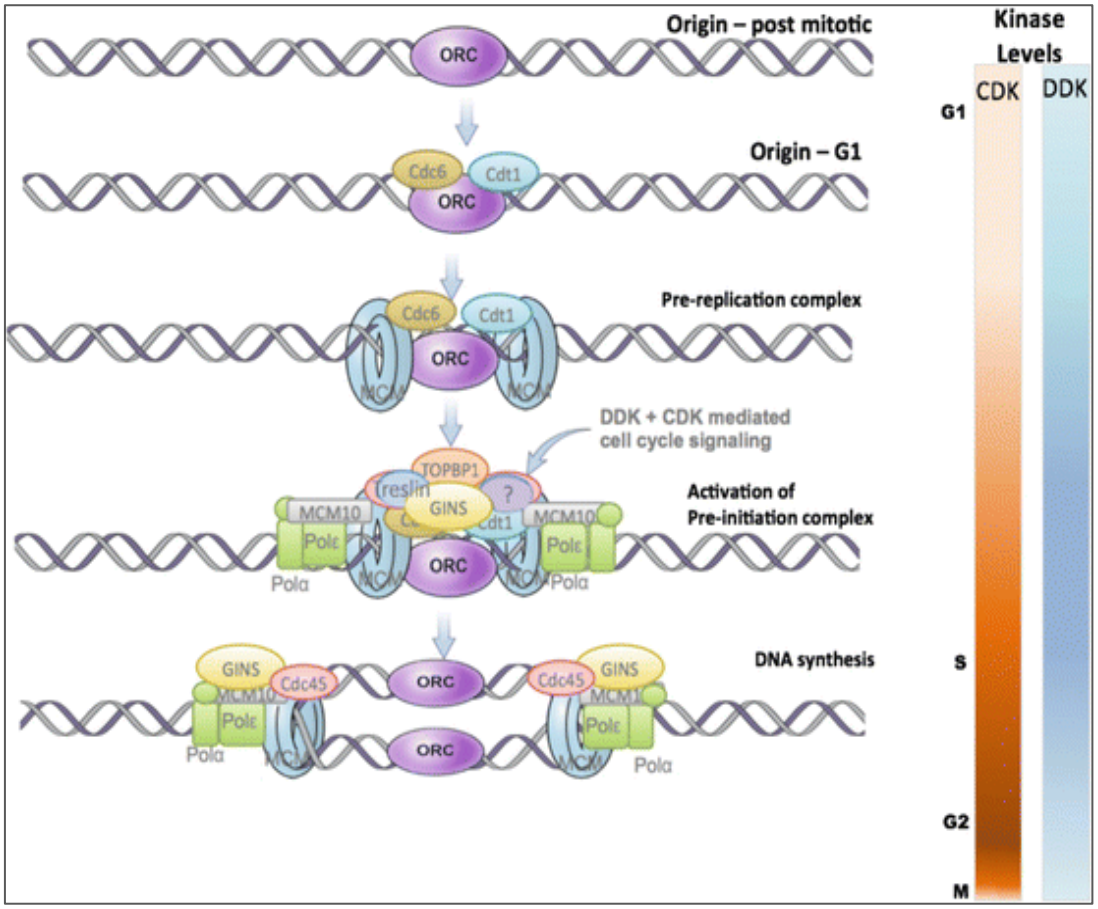


Figure 1.1: The DNA replication machinery. The DNA replication requires the "licensing" of replication origins and the origin firing. (Marks et al., 2017)

triggering origin firing and DNA synthesis during S phase (Tanaka and Araki 2013).

In more detail while the cell is exiting mitosis (late M to G1), ORC binds to DNA. Then, the licensing factors Cell division cycle 6 (CDC6) and CDC10-dependent transcript 1 (CDT1) bind ORC, allowing the recruitment of inactive helicase MCM2-7 onto origins, including the dormant ones (Evrin et al. 2009; Speck et al. 2005). 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 (Randell et al. 2006). Despite the fact that two MCM2-7 hexamers are required on each replication origin, an excess of MCMs bind DNA probably in an attempt to ensure that the cell will be replicated properly in case of replicative stress (Ge, Jackson, and Blow 2007). It has been shown that MCMs are also loaded in regions distant to ORC binding sites. These MCMs can potentially act as origins.

Origin firing 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. The formation of the pre-IC is promoted by DBF4-dependent kinase (DDK) and cyclin-dependent kinases (CDKs) at the G1–S phase transition, while its transformation into a functional replisome takes place during 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) leading to their recruitment onto the origins.

Entering S phase, CDC45, GINS and other proteins loading occurs via the activity of CDK and CDC7 kinases, which are activated in this phase, promoting in this way the helicase activity of MCM (Bleichert, Botchan, and Berger 2017; Heller et al. 2011). It leads to the unwinding of DNA and the subsequent loading of proteins including replication factor C (RFC), proliferating cell nuclear antigen (PCNA), replication protein A (RPA) and other DNA polymerases that start DNA replication.

In order to avoid MCM re-loading during S phase of the same cycle, it is essential to negatively regulate the MCM loaders. This can be achieved via regulation of Cdt1, which is mediated by ubiquitin-based degradation and its inhibitor, known as geminin. In addition, when CDK phosphorylates CDC6 during S the relicensing is suppressed via CDC6 nuclear export. As for the ORC1, it is ubiquitinated and degraded after phosphorylation by CDK (Marks, Fu, and Aladjem 2017)

CDC45, MCM hexamer and GINS are known as the CMG complex (Heller et al. 2011; Kang et al. 2014) and is activated by the MCM10. Next, DNA polymerase α-primase (Pol α) primes DNA synthesis via the DNA polymerases, Pol δ synthesizes from the lagging strand, while Pol ε synthesizes from the leading strand (Stillman 2015). The lagging strand is synthesized discontinuously via Okazaki fragments, while the leading strand is polymerized continuously. Okazaki fragments are joined

together by DNA ligase. In eukaryotes, replication ends when opposing forks coming from adjacent origins meet together, leading to the ubiquitin-dependent removal of the CMG from chromatin (Bell 2017)

Of note, only 33% of the origins are fired in each replication unit, indicating that the rest is not activated although the licensing process has been performed. Furthermore, the activation of origins may differ among cell types or even among cells of the same cell population (Fragkos et al. 2015).

1.2 Cell cycle

1.2.1 Cell cycle regulation

The cell cycle can be separated in 2 parts: the interphase, including G1, S (Synthesis) and G2 phases, and the M (mitotic) phase consisting of prophase, prometaphase, metaphase, anaphase, telophase and cytokinesis. G1 and G2 stand for the "gaps" required for the preparation for DNA synthesis and division, respectively (Vermeulen, Van Bockstaele, and Berneman 2003). More specifically, the cell cycle follows a progression of events that lead cells from a resting state (G0), growth to the suitable size (G1), DNA replication (S phase), preparation for segregation and chromosomes integrity (G2) and their accurate segregation during mitosis (M phase). **(Figure 1.2)**

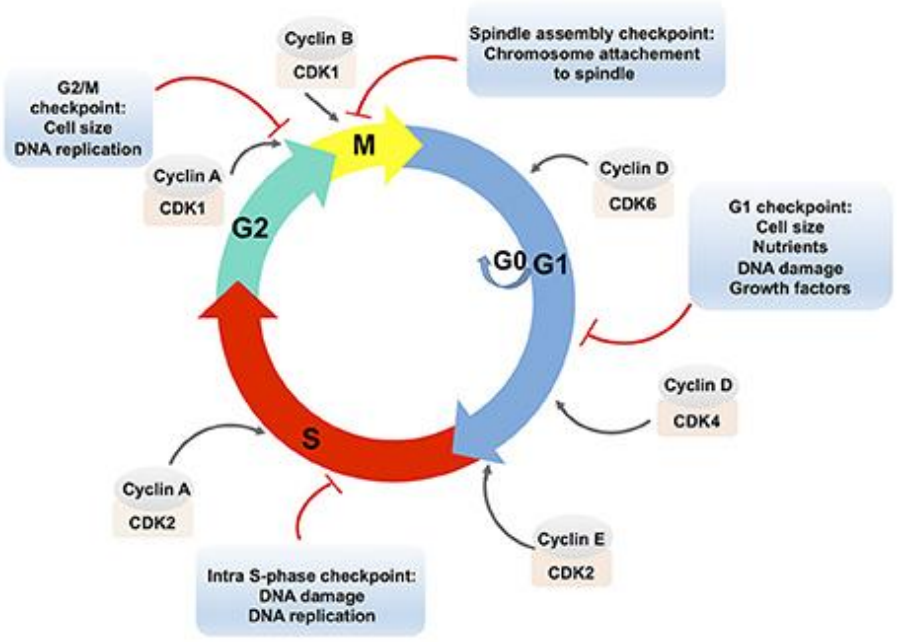


Figure 1.2 : Eukaryotic cell cycle and its regulation. (Front. Cell. Infect. Microbiol., 2017)

It is known that our genome is constantly in danger by numerous endogenous and exogenous agents, which could dramatically affect the integrity of the cell and potentially lead to carcinogenesis. To ensure the normal regulation and completion of

each phase, taking into account the order, the integrity and the fidelity, cell cycle checkpoints have evolved and have been conserved (Hartwell and Weinert 1989). This control is essential so that the cell cycle can be arrested until DNA damage is repaired or in case of extended damage to promote cell death. This may explain why malignant cells are often defective in factors involved in these checkpoints (Curtin 2012).

The progression of the cell cycle is mediated by Cyclin-Dependent Kinases (CDKs), which 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, able to phosphorylate key targets due to the serine-threonine kinase activity (Malumbres and Barbacid 2005). The activity of CDKs depends on the binding of cyclins to their catalytic partner offering an altered conformation to the kinase, while the levels of the cyclins are strictly regulated during the cell cycle through synthesis and ubiquitin based degradation.
CDKs activity can be also negatively regulated via the interaction with Cdk inhibitors (CKIs), which block their activity, controlling cell cycle progression. Two categories of CKIs have been identified based on their structural homology, amino acid similarity and CDK specificity: the INK4 and the CIP/KIP family. The INK4 family includes the $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ και $p19^{INK4d}$ inhibitors which are specific for the CDK4 and CDK6 and block their connection with the cyclin-D. As for the CIP/KIP family, it includes the p21 $^{Cip1/Waf1/Sdi1}$, $p27^{Kip1}$ and $p57^{Kip2}$ inhibitors which have the same region of homology that facilitates their binding with the CDKs. There is also evidence 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 (Besson, Dowdy, & Roberts, 2008). To sum-up, Cdks are perceived as the machines promoting cell cycle progression while cyclins are considered to be the factors that aid the transition from one phase to the other.

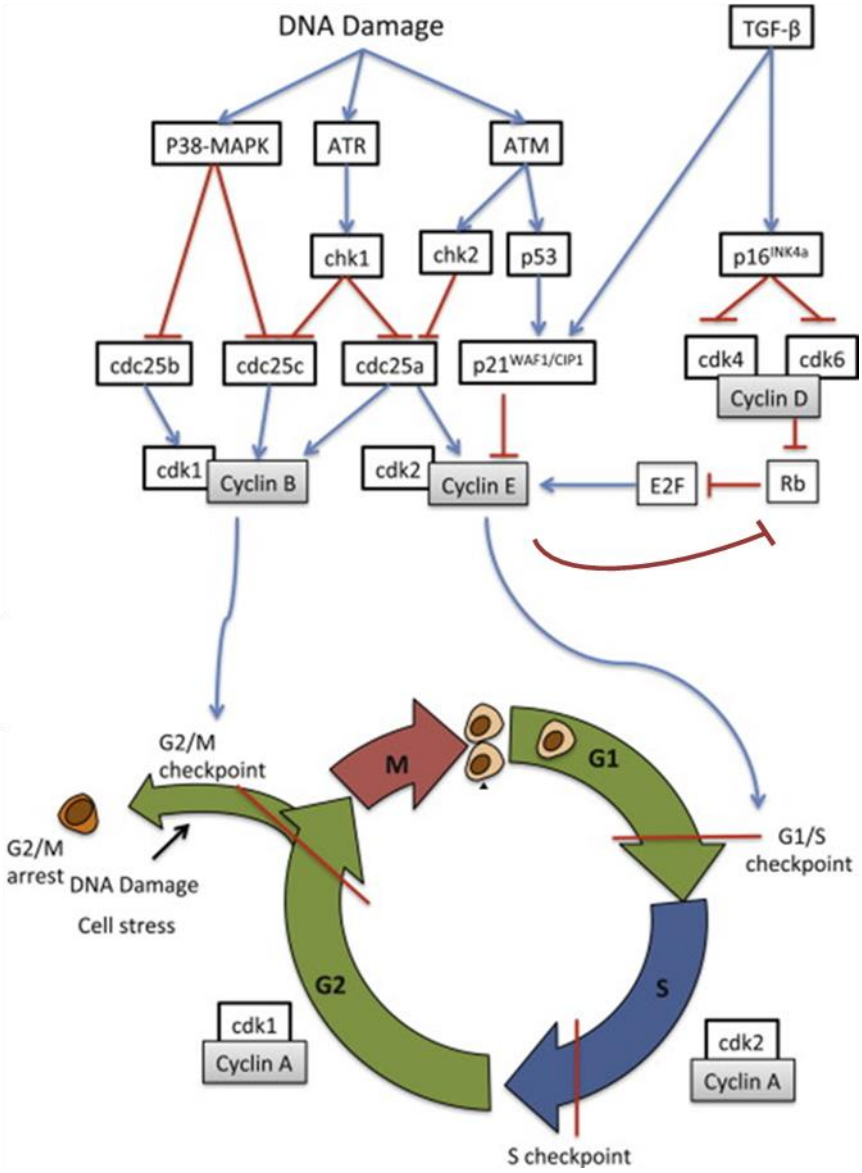


Figure 1.3 : The DNA-damage response pathway. Chk1 and Chk2 are activated upon DNA damage through phosphorylation by ATR and ATM, respectively. Downstream phosphorylation events result in G2/M- and S-phase and G1 cell cycle arrest. P16 may also promote G1 arrest as a response to replicative senescence and transforming growth factor (TGF)-β. (Ferenbach & Bonventre, 2016)

1.2.2 DNA damage checkpoints

As mentioned before, the existence of checkpoints, which can be activated when it is necessary, and block the transition to the next phase protects the cell from replicating and dividing under non favorable conditions. More precisely, upon DNA damage, cycle checkpoints can be activated in G1 phase, in S phase and at the G2/M transition.(Bartek and Lukas 2007; Ferenbach and Bonventre 2016; Kastan and Bartek 2004)

Regarding the G1 phase arrest, it may be promoted by two pathways. One involves activation of $p16^{INK4a}$, as takes place in replicative senescence and in response to

transforming growth factor (TGF)-β in vivo. The production of p16INK4a leads to its binding with Cdk4/6, preventing cyclin D binding to the kinase and phosphorylation of Rb (Russo et al. 1998). In its un-phosphorylated form, Rb binds E2F. Rb acts as a repressor, so in complex with E2F it prevents expression of E2F-regulated genes, which are related to proliferation and this inhibits cells from progressing through G1. More specifically, active cyclin D/Cdk4 and Cdk6 inhibit Rb by partial phosphorylation, reducing its binding to E2F and thereby allowing E2F-mediated activation of the transcription of the cyclin E gene and the cell progresses towards S-phase. Subsequently, cyclin E/Cdk2 fully phosphorylates Rb and completes its inactivation (Musgrove, Lee, Buckley, & Sutherland, 1994).
The other pathway is promoted by DNA double strand breaks which activate Ataxia Telangiectasia Mutated (ATM) kinase and promote G1 arrest via phosphorylation and activation of Checkpoint Kinase 2 (Chk2) (Matsuoka, Huang, and Elledge 1998).
Chk2 inhibits Cdc25A, a phosphatase that removes inhibitory phosphorylation of the cyclin E/Cdk2 (and cyclin A/Cdk2) complexes, preventing cells from proceeding into S phase (Falck et al. 2001). The G1 checkpoint is also dependent on p53. In addition, ATM triggers phosphorylation of p53, reducing its affinity for the negative regulator, the ubiquitin ligase Mdm2, leading to p53 stabilization (Banin et al. 1998; Canman et al. 1998; Shieh et al. 1997). Stabilized p53 induces $p21^{Cip1/Waf1/Sdi1}$, that binds and further inhibits cyclin A/Cdk2 (and cyclin E/Cdk2) complexes, DNA repair proteins and, upon protracted checkpoint activation, apoptotic cell death promoters (Vousden and Lu 2002). **(Figure 1.3)**

Upon DNA damage occurring in S phase, for instance due to replication stress and stalled forks or double strand breaks, the S phase checkpoint is activated to ensure that the replication will not proceed. The sensor of DNA damage is Ataxia Telangiectasia and Rad3-related (ATR) kinase that activates Checkpoint Kinase 1 (Chk1), induces Cdc25A proteosomal degradation, inhibition of cyclin-dependent kinase 2 (Cdk2)/cyclin A complex, preventing in this way the cell from further progression through S phase. (Mailand et al. 2000; Xiao et al. 2003).
ATR and Chk1 also trigger the G2/M checkpoint, which prevents cells with damaged DNA from entering mitosis. Mitosis onset requires activity of the mitotic kinase cyclin B-dependent kinase 1 (Cdk1) (Nigg 2001). Cdk1 catalytic activity is inhibited during the S and G2 phases through the phosphorylation on T14 and Y15 induced by the kinases Wee1 and Myt1. These phosphorylations are removed at the G2/M transition by the Cdc25C phosphatase. To prevent cells with damaged DNA from entering mitosis, ATR inhibits cyclin B/Cdk1 activation by stimulating the Cdk1 inhibitory kinase Wee1 and inhibiting Cdc25C via Chk1 (O'Connell et al. 1997; Sanchez et al. 1997). **(Figure 1.3)**
In response to DNA damage ATM and ATR not only stop cell cycle progression but also initiate DNA repair by phosphorylating several other substrates. If damage cannot be repaired, the cell destiny might be death (apoptosis) or permanent growth arrest (senescence) (Cimprich and Cortez 2008; Shiloh and Ziv 2013).

1.3 Senescence

Senescence was first reported as the limited proliferation capacity of human diploid fibroblasts after a particular number of cellular divisions in vitro due to telomere shortening (Hayflick and Moorhead 1961). However, we know now that senescence is considered the permanent growth arrest activated by various stimuli as a response to stress. There is bulk of evidence showing the role of senescence in embryonic development, wound healing, its tumor suppressive aspect and the association with organismal ageing as senescent cells tend to accumulate with age in mammals. (Dimri et al. 1995).

There are two types of senescence: the replicative (RS) and the stress induced premature senescence (SIPS). The replicative senescence is promoted by telomere shortening and takes place in all somatic cells apart from some stem cells and cancer cells. (Campisi 1997). On the other hand, several stressful stimuli are able to accelerate or trigger premature senescence independently of telomere attrition. Oncogene overexpression, ROS-mediated DNA damage, mitochondrial dysfunction and inflammation are some of the factors capable of promoting senescence.

1.3.1 The molecular mechanisms of senescence

Despite the fact that a variety of stressful stimuli may promote senescence through different pathways based on the cell type, it is known now that the majority of them converge on p53 and p16INK4^{a} -Rb pathways, while the inactivation of p53 or Rb is adequate to block senescence (Shay, Pereira-Smith, & Wright, 1991; Wei, Herbig, Wei, Dutriaux, & Sedivy, 2003).

DNA damage, similar to that promoted by exogenous agents including ionizing radiation and chemotherapeutics, may be triggered by telomere shortening. DNA damage is sensed by ATM/ATR that phosphorylate Chk1/Chk2, which in turn phosphorylate p53 stabilizing it (Ruiz et al., 2008; Serrano, Lin, McCurrach, Beach, & Lowe, 1997). **(Figure 1.4)**

Senescence is also associated with INK4A/ARF locus (CDKN2A) encoding three tumor suppressors (p15^{INK4b}, p16^{INK4a}, ARF). P16^{INK4a} inhibits CDK4 and CDK6, while ARF affects p53 stability by suppressing Mdm2, an ubiquitin ligase that targets p53 for degradation (Stott et al. 1998). The target of p53, p21$^{(CIP1/WAF1)}$ binds to and inhibits some CDK–cyclin complexes, particularly those involving CDK2, and subsequently inhibit the pRB phosphorylation. The pRB pathway prevents cellular proliferation through downstream effectors. For instance, pRB inhibits the E2F family of transcription factors, whose target genes are necessary for progression through S-

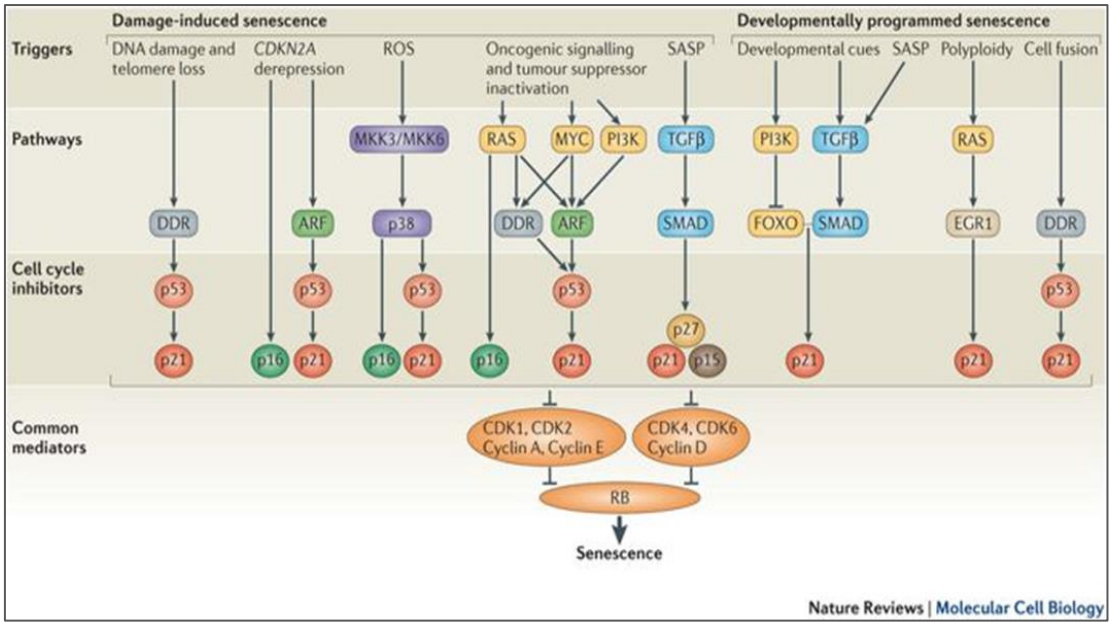


Figure 1.4: Molecular mechanisms of senescence. Several stressful stimuli, including the DNA damage response pathway (DDR), the derepression of CDKN2A locus, reactive oxygen species (ROS), activation of oncogenes and SASP can promote the induction of senescence converging on the inactivation of tumor suppressor Rb.

phase (Nevins 2001). Both $p16^{INK4a}$ and $p21^{Cip1/Waf1/Sdi1}$ activation leads to G1 cell cycle arrest. As for the mechanisms that regulate INK4/ARF expression, leading to its derepression and subsequently to senescence, they have not been fully clarified. Until now, it is known that INK4/ARF expression is affected by the loss of Polycomb repressive complexes (Bracken et al. 2007), while DNA damage is able to reduce ARF levels by promoting its degradation (Velimezi et al. 2013).

Senescence can be triggered by chemotherapeutics, telomeres erosion, DNA damage and oncogene activation due to high levels of Reactive Oxygen Species (ROS) (Passos et al. 2009). There is evidence that increased levels of ROS via the RAS–RAF–MEK–ERK cascade activate the p38 MAPK. The substrate of p38 MAPK becomes activated by the kinase and in turn phosphorylates and activates p53 promoting senescence in this way (Sun et al. 2007).
As for the oncogene induced senescence (OIS), it may occur due to the loss of tumor suppressors or oncogenes overexpression and is often mediated by derepression of INK4/ARF locus (W. Y. Kim and Sharpless 2006). Furthermore, OIS may result in DNA damage response pathway because of abnormal replication (Bartkova et al. 2006; Di Micco et al. 2006) or ROS (Debacq-Chainiaux et al. 2010). **(Figure 1.4)**

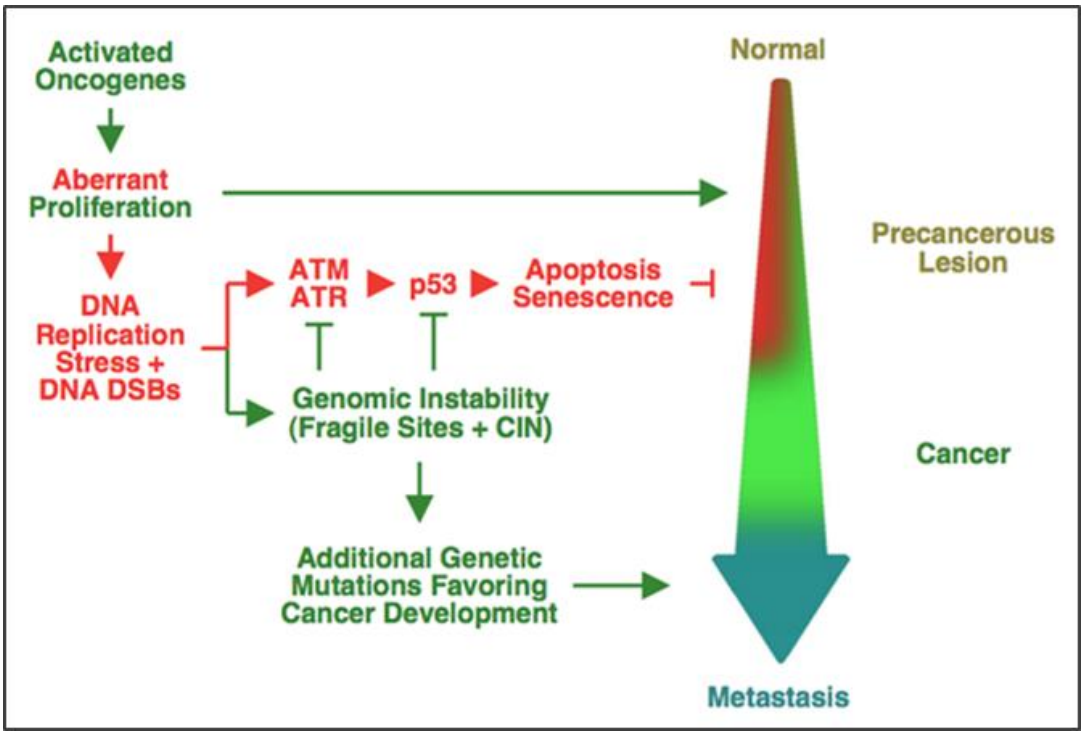


Figure 1.5: A model about the evolution of cancer promoted by oncogene activation. DNA damage, tumor suppressing mechanisms and genetic alterations are present in premalignant lesions before the emergence of the tumor. (Halazonetis et al., 2008

1.3.2 The role of senescence in tumorigenesis

The main function of cell cycle arrest during the senescent state is to prevent dysfunctional cells from propagating, whether it is prompted by telomere erosion (Campisi 1997) or by other factors including oncogene activation (Bartkova et al. 2006) . However, there is strong evidence that chronic maintenance of senescence may have deleterious effects especially in the context of tissue environment and probably contributes to cancer progression. The tumorigenic aspect of senescence could be partially attributed to the fact that the senescent cells are metabolically active and their ability to communicate with the surrounding cells through Senescence Associated Secretory Phenotype (SASP) including the release of cytokines and chemokines (Coppe et al. 2008; Krtolica et al. 2001; Kuilman et al. 2008). Apart from the SASP effect, the genetic changes identified in malignant cells may also contribute to the evasion from preventive mechanisms and the development of tumor (Coppé et al. 2010), while they could be acquired during the long term presence of senescent cells.

Several studies have reported the existence of activated oncogenes at premalignant lesions and tumors which promote DNA fork stalling and collapse due to aberrant

replication resulting in DNA double strand breaks formation. This, in turn, contributes to the emergence of genomic instability, a feature shared by many malignancies. So, the DNA damage response pathway, and subsequently senescence or apoptosis are activated in precancerous lesions, while genomic instability, gradually acquired by the cells, probably provides the cells with the ability to overcome these barriers favoring the development of cancer overtime. (Halazonetis, Gorgoulis, and Bartek 2008) **(Figure 1.5)**

1.3.3 Chromatin re-organization during senescence

Senescence is also accompanied by significant alterations in the chromatin structure which affect the accessibility and the transcriptional program of numerous genes (Criscione, Teo, and Neretti 2016). The chromatin changes might significantly differ in a cell type and stimulus dependent manner.

Common feature of OIS is the presence of Senescence Associated Heterochromatin Foci (SAHF). SAHF are chromatin foci visible by staining with DAPI and consist of the High-Mobility Group A (HMGA1 and HMGA2) proteins, the histone variant mH2A, and HP1 Protein (Huang et al. 2005; Narita et al. 2006). They are enriched for heterochromatic marks including H3K9me3 and H4K20me3 (Nelson et al. 2016), with the facultative heterochromatin mark H3K27me3 located at the periphery (Chandra et al. 2012). Although this phenotype has been described a lot as an OIS feature, it should not be considered a universal characteristic of all types of senescent cells (Parry and Narita 2016). More specifically, it has been reported that SAHF formation requires an intact $p16^{INK4a}$-pRB pathway and they are based on the type of stimulus promoting the senescent state and the cell type, as well (Kosar et al. 2011; Narita et al. 2003).

There have also been reported changes in the lamina of senescent cells. The lamina is located in the interior of the nuclear membrane, consists of lamins A and B and comes in contact with gene poor regions of heterochromatin known as lamina-associated domains (LADs) (Guelen et al. 2008). In premature senescence, LaminB1 (LMNB1) is downregulated, while LMNB1 depletion at LADs results in spatial redistribution of heterochromatic regions marked by H3K9me3, which is crucial for the formation of SAHF (Sadaie et al. 2013).

Post translational modifications of histone tails also play an important role in dynamic processes, such as senescence. Histone modifications are able to affect the affinity of the tails with DNA and interacting proteins contributing to the formation of active or repressive chromatin states and affecting transcriptome in this way. The regulation of transcription can also be affected by possible three-dimensional spatial alterations that occur during senescence (Yang and Sen 2018).

1.4 Carcinogenesis

Cancer is the second cause of death globally after cardiovascular diseases. Despite the wide spectrum of cancer types, they share a common characteristic: all of them derive from cells of a normal tissue. Thus, a gradual process always mediates the transition from the normal to the cancerous state, known as carcinogenesis. It is a multi-step process including numerous genetic and epigenetic alterations eventually leading to the formation of cancer.

There are several types of cancer (malignant neoplasias) based on their origin. They are known as carcinomas if they have epithelial origin and sarcomas in case that they come from connective tissue. Other types are leukemias, referring to the blood cancers, and the cancers of nervous system. Strikingly, 90% of human cancers are of epithelial origin.

The pathogenesis of cancer starts with the uncontrollable cell proliferation, which is known as hyperplasia, remains localized and is not dangerous for the organism, while the cells are absolutely normal and maybe never drive cancer formation. The next stage towards cancer formation is dysplasia, which can be separated to low grade and high grade, with the latter one to be closest to the cancer. They are still restricted to the basal membrane, but they are considered pre-cancerous and look abnormal under the microscope until the point that the cells acquire the capability of invading the basement membrane and metastasize to distal tissues/organs (malignant neolpasia). (Halazonetis, Gorgoulis, and Bartek 2008)

1.5 CDC6

1.5.1 Structure and Function

CDC6 was initially identified in yeast and we know now that it is highly conserved, as it is found in all eukaryotes, while a homolog has been also identified in Archaea. Cdc6 is a 60kDa protein which belongs to the AAA^+ family of ATPases (Neuwald et al. 1999) and is essential for the replication licensing. The human Cdc6 is located at chromosome 17q21.3 and it is regulated by E2F/ retinoblastoma transcription factors (Hateboer et al., 1998). As all proteins of the AAA^+ family, Cdc6 consists of Walker A and Walker B motifs, which are responsible for the ATP binding and ATP hydrolysis, respectively (D. G. Lee et al. 2000). Cdc6 consists of a Winged Helix Domain (WHD) mediating the Cdc6 binding on the DNA, a Nuclear Location Sequence (NLS) and 2 Nuclear Export Signals (NES) allowing its cytoplasmic translocation during S phase after phosphorylation at S74 and S54 by cyclin A/Cdk2 (Hwang et al. 2014). Cdc6 also consists of PCNA interacting protein (PIP) box to the N-terminal facilitating the binding of the DNA-clamp proliferating cell nuclear antigen (PCNA), the loading of which is necessary for the recruitment of DNA polymerases (Masai et al. 2010).Apart from that the PIP box may

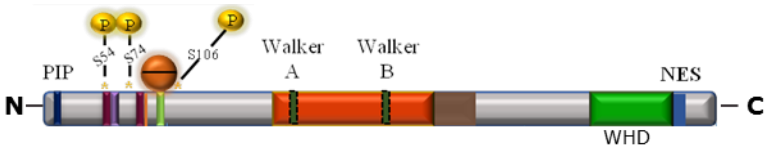


Figure 1.6: The conserved domains of Cdc6.

facilitate the degradation of Cdc6 in order to avoid the nuclear re-accumulation of it at S phase (Clijsters and Wolthuis 2014).

Cdc6 can be phosphorylated in 3 different sites and translocated from the nucleus to the cytoplasm during S phase, probably due to the unmasking of NES via conformation changes mediated by the CDK phosphorylation in order to prevent re-replication after origin firing (Saha et al. 1998). However, although the exogenous Cdc6 is exported to the cytoplasm, the endogenous Cdc6 is located in the nucleus bound to the chromatin even in S phase, while only a small portion of it is translocated to the cytoplasm (Alexandrow and Hamlin 2004). In higher eukaryotes, another mechanism regarding Cdc6 degradation during the S phase was recently reported and it probably functions as an additional way of replication control. More specifically, during S phase Cdc6 is targeted for degradation, mediated by proteasome, which is triggered by the $SCF^{Cyclin\ F}$ ubiquitin ligase complex. It is achieved via interaction between CDC6 and Cyclin F at particular motifs which promote the ubiquitylation and degradation of Cdc6. (Walter et al. 2016)
Independent of its ATPase activity, Cdc6 is essential to recruit Cdt1 and MCM complex to the origin, as ORC alone is not able to do that (Randell et al. 2006). Based on its ATPase activity CDC6 has two functions which are crucial for DNA replication. The first one, which has been shown only in yeast so far, is the ability to affect the specificity of ORC binding DNA and target in this way the origin sequence (Mizushima, Takahashi, and Stillman 2000). Despite the fact that ORC binds origins of replication, it is not the sole factor that determines their location. ORC requires Cdc6 to target the initiation at particular regions and Cdc6 acts together with Cdt1 to promote the MCM helicase loading onto DNA (Speck and Stillman 2007). ATPase activity of Cdc6 is affected by the DNA sequence where it is bound. In case that the sequence is not origin DNA, Cdc6 and ORC - due to Cdc6 ATPase activity- are able to dissociate, preventing the initiation of replication, while origin DNA negatively regulates the ATPase activity stabilizing the ORC-Cdc6-DNA complex.
The second function based on Cdc6 ATPase activity, concerns the loading of the MCM ring on DNA. Although the MCM complex is recruited to the origin by ORC-

Cdc6, it cannot be engaged to the DNA without ATP hydrolysis. In more detail, inhibition of Cdc6 ATP hydrolysis prevents the formation of pre-RC complex and traps Cdt1 to the origin, a factor necessary for MCM recruitment and through its release seems to facilitate the loading of MCMs, which could probably take place to prevent the repeated MCM loading. There is also evidence that in yeast apart from Cdt1 dissociation ATP hydrolysis by Cdc6 promotes Cdc6 disengagement from pre-RC after MCM loading. (Randell et al. 2006).

Apart from its role in replication, it has been reported that Cdc6 plays important role in the activation of checkpoint mechanisms which control the progression to the M phase (Borlado and Méndez 2008). There is evidence that Cdc6 may prevent early entry into mitosis (before DNA replication has been completed), independently from its replication licensing ability, maybe due to direct activation of Chk1 (Clay-Farrace et al. 2003). In addition, several studies have shown the oncogenic potential of Cdc6 that will be discussed later.

1.5.2 Cdc6 in human cancer

The expression of CDC6 is deregulated in many malignancies. High levels of Cdc6 (more than fourfold change) have been detected in 50% of Non-Small-Cell Lung Carcinomas (NSCLC), which is the most common lung malignancy (Karakaidos et al. 2004), while the increased levels of Cdc6 (and Cdt1) are obvious from the stage of dysplasia. More specifically, different stages of cancer progression, including hyperplasia, dysplasia and full malignancy, have been investigated in lung, larynx and colon and the results reveal that CDC6 overexpression is an event occurring early during cancer evolution (**Figure 1.7**) and it is not a result of increased proliferation, as there is no correlation between the replication licensing factors and the levels of the proliferative marker Ki-67. (Liontos et al., 2007)

Overexpression of CDC6 has been also associated with cervical cancer and may be exploited as a marker of advanced cervical cancer lesions, since increasing levels of Cdc6 have been highly correlated with the severity of dysplasia. (Murphy et al. 2005). Furthermore, CDC6 expression can be used as a marker of proliferative capacity in brain tumors, while 55% of them overexpress Cdc6 (Ohta et al., 2001).

Interestingly, apart from Cdc6 overexpression, it has been reported its down-regulation in aggressive prostate cancer (Robles et al. 2002), indicating together with the above-mentioned results that the deregulation of this factor affects the cellular proliferation and has severe impact on cell integrity and subsequently contributes to the development of cancer, revealing in this way its oncogenic function.

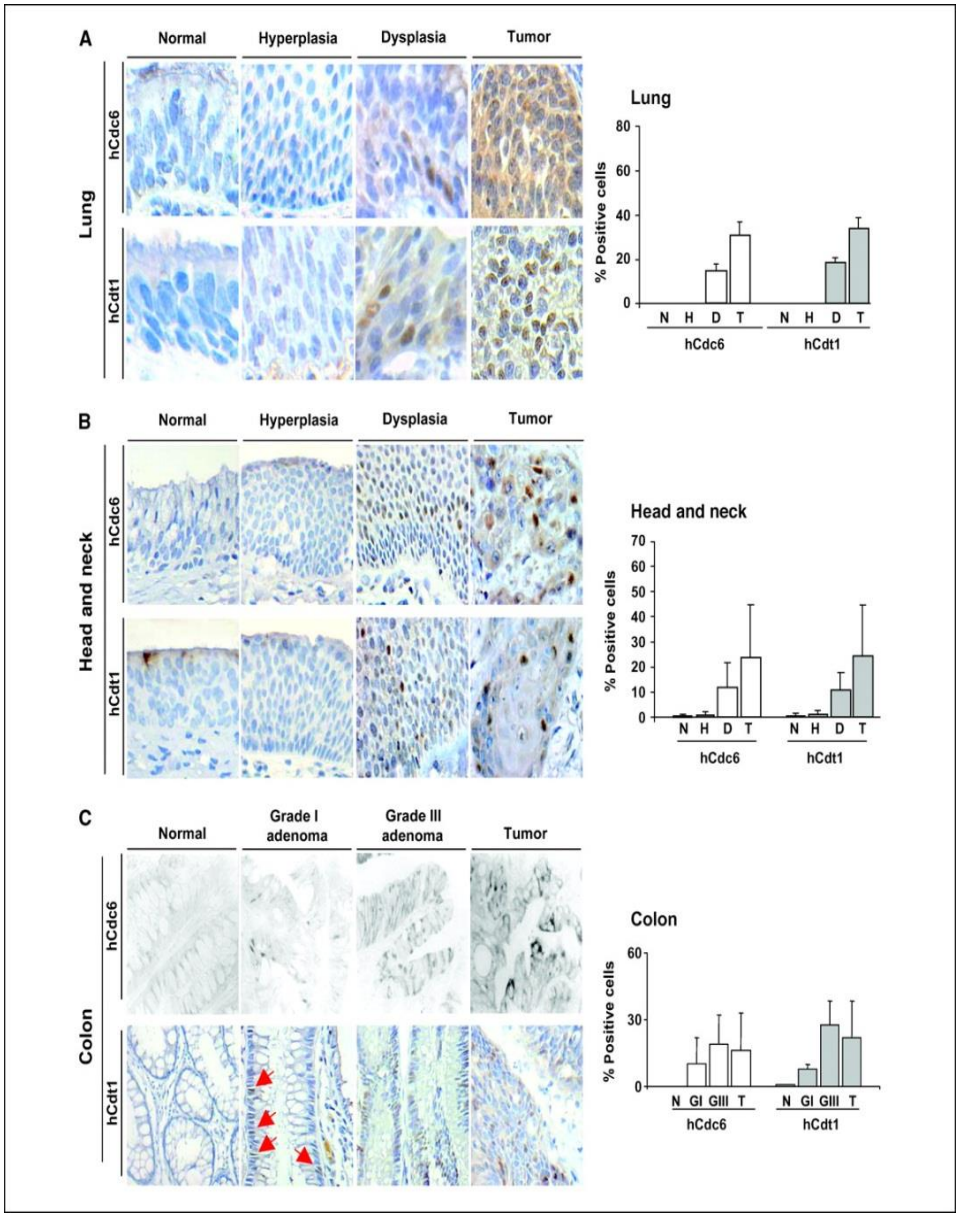


Figure 1.7: Cdc6 and Cdt1 levels during cancer development. Immunohistochemistry analysis performed in 3 different tissues (lung, larynx, colon) and 4 grades of malignancy indicated that high levels of replication licensing factors are detected from the early stages of malignancies (Liontos et al., 2007)

1.5.3 Oncogenic potential of Cdc6

The way that Cdc6 is associated with the tumorigenesis remains still unclear. There is evidence that Cdc6, when overexpressed, may act as a transcription factor. It is achieved by binding to the E-box of CDH1 and displacing CTCF and histone H2A.Z which leads to the repression of the CDH1 through heterochromatinization of the CDH1 promoter It also activates origins of replication proximal to the particular promoter **(Figure 1.8)** (Sideridou et al. 2011). CDH1 is coding for E-cadherin which is crucial for cell-cell adhesion in epithelial tissues. Inactivation of E-cadherin may be

associated with Epithelial to Mesenchymal Transition (EMT) that takes place in cancer and through which the epithelial cells lose their epithelial characteristics and acquire migratory and invasive properties of mesenchymal cells (Kalluri and Weinberg 2009). In addition, Cdc6 overexpression in murine, premalignant epithelial cells promotes a mesenchymal transition, revealing the EMT phenomenon due to Cdc6 stimulus. (Liontos et al. 2007)

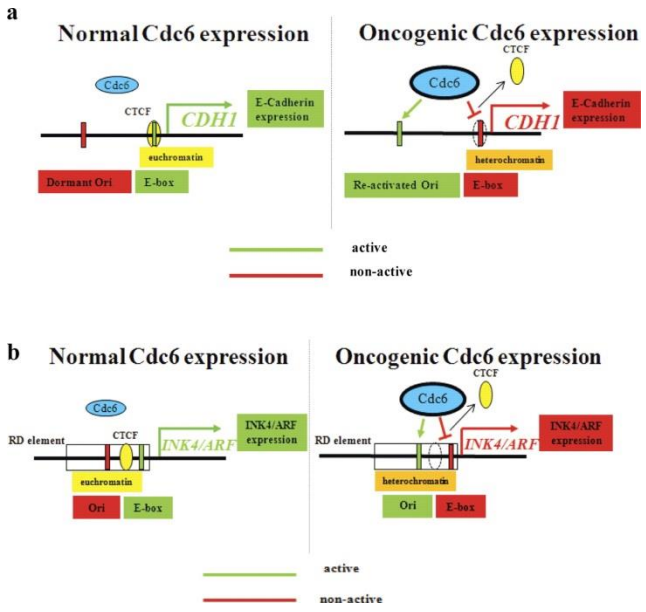


Figure 1.8: The ability of oncogenic Cdc6 to function as a transcription factor (a) atCDH1 promoter and (b) at INK4/ARF locus.

To understand in depth the Cdc6 oncogenic potential, we should take into account that it is possibly close to the mechanism by which the oncogenes -when deregulated- are able to promote cancer progression. It is known now that this process is mainly dependent on genomic instability, a hallmark of cancer, which is usually prompted by re-replication (Blow and Gillespie 2008; Halazonetis, Gorgoulis, and Bartek 2008). In more detail, precancerous cells usually are unable to control replication, due to the overexpression of oncogenes/licensing factors that probably lead to abnormal replication promoting replication stress. Replication stress is used to describe every condition that may interfere with DNA replication and hinder its progression. So, the replication stress results in replication fork stalling and possibly in fork collapse. Double strand breaks from the collapsed forks in turn activate the DNA damage response pathway and transient cell cycle arrest occurs until the damage is repaired. However, if the harmful stimulus is induced continuously, as in the case of Cdc6 overexpression, DNA damage is extended and is likely to lead to senescence or apoptosis. These are considered to be the main tumor suppressor responses against the propagation of damaged cells, but long-term retain of senescence apart from the fact that it is a metabolically active state and may affect the surrounding cells through SASP, it can also allow genomic re-arrangements to occur during the damage repair and this will eventually lead to genomic instability giving rise to cancerous cells.

(Halazonetis, Gorgoulis, and Bartek 2008). The genomic alterations taking place during the senescence state could be accompanied by epigenetic changes that potentially contribute, if not drive, this event.

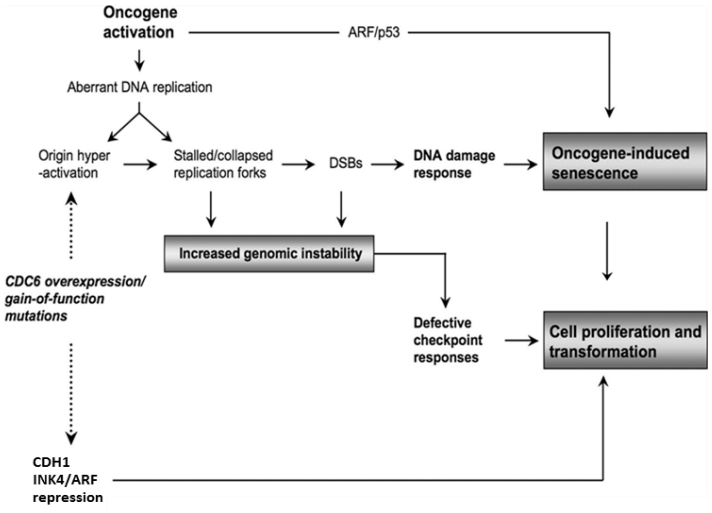


Figure 1.9: Suggested model for cellular response to Cdc6 hyper-activation. It includes a burst of proliferation accompanied by aberrant DNA replication leading to double strand breaks and activation of DNA damage response increasing genomic instability and potentially promoting senescence (Borlado & Méndez, 2008).

1.6 Epithelial model recapitulating lung cancer progression

The fact that the majority of the models employed so far for the -in depth- understanding of carcinogenesis are mesenchymal, whereas most of malignancies are of epithelial origin prompted our group to create a non- malignant epithelial model recapitulating the spectrum of cancer evolution under in vitro conditions. Thus, Human Bronchial Epithelial Cells (HBECs) were employed by constantly over-expressing Cdc6 in an inducible manner.

First, HBECs immortalization was performed through overexpression of human telomerase reverse transcriptase (hTERT) and cyclin dependent kinase 4 (Cdk4). hTERT is essential to be over-expressed in order to avoid telomere shortening and subsequently replicative senescence. In addition, it has been reported that epidermal keratinocytes and human mammary epithelial cells after a period of propagation are arrested, mediated by up-regulation of the cyclin-dependent kinase (Cdk) inhibitor, $p16^{INK4a}$, probably due to cell culture stress. So, over-expression of CDK4 was performed in order to avoid the premature growth arrest (Ramirez et al. 2004).

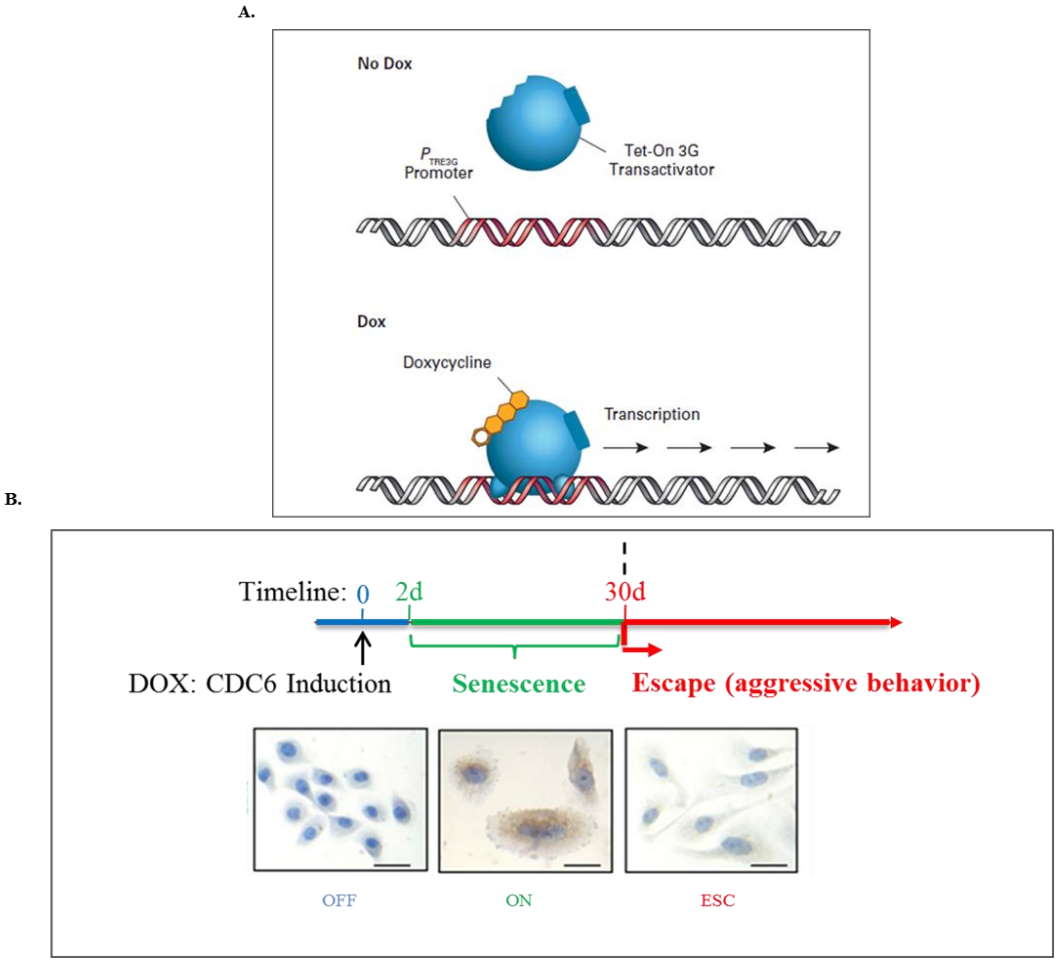


Figure 1.10 : A) The Tet-On 3G system allows inducible gene expression in the presence of doxycycline. When Dox binds, the transactivator undergoes a conformational change allowing it to bind tet operator (tetO) repeats within the TREG Promoter (PTRE3G). The transactivator activates expression through transcription activation domain repeats. B) A non -malignant epithelial model recapitulating all the stages of lung carcinogenesis, including the normal epithelium, the precancerous (stained with SenTraGor (GL13 compound) binding to the metabolic bio-product of senescent cells, known as lipofuscin) and the cancerous cells. (Modified figure from Komseli et al. 2018)

The Lenti-X™ Tet-On® 3G Inducible Expression System was used to create the CDC6 inducible over-expression model. The immortalized HBECs were transfected with the plasmids carrying PLVX-TET3G and PLVX3G-TRE-CDC6 by employing lentiviruses. So, the HBECs expressing a) the Tet-On 3G trans-activator protein and b) CDC6 gene (including Myc tag at the N- terminus of Cdc6) under the control of a TRE3G (Tetracycline Response Element) promoter (*P*TRE3G) will express high levels of CDC6, only in the presence of doxycycline (Dox), a tetracycline analog. When bound by Dox, the Tet-On 3G protein undergoes a conformational change that allows it to bind to tet operator *(tetO)* sequences located in *P*TRE3G **(Figure 1.10).**

Characteristic features of the system upon induction of Cdc6 include an initial reduction in the proliferative rate, followed by a gradually imposition of the senescent phenotype which is totally manifested within 6 days. Interestingly, at least 40 days are required after the Cdc6 overexpression, so that a subset of the previously senescent cells re-enters proliferation adopting a mesenchymal morphology, which in combination with the existence of respective cellular markers reveal that these cells escape from senescence and manifest EMT (Komseli et al. 2018).

2. AIMS

The senescence promoted by oncogene overexpression is considered to be one of the major defense mechanisms against cancer. However, there is strong evidence regarding the dynamic role of senescence in cancer and its ability to gradually contribute to the development of it, in case it is not cleared by the immune system in due time (Medema 2018; Saleh et al. 2019).

The mechanism through which the phenomenon of escape from senescence takes place remains still unclear. Having established a human epithelial cellular system representing all the spectrum of lung cancer progression in a relatively short period of time, including the senescent state (precancerous phase) following by the re-entry of a subset of cells into the cell cycle and the emergence of more aggressive clones (cancerous phase), we were prompted to have an insight into the mechanistic aspect of the oncogene- induced- escape from senescence.

First, we investigated the aggressive characteristics acquired by the cells escaped from senescence, while we tried to delineate the mechanism through which the DNA damage response pathway leads to senescence.

Apart from that, we were interested in identifying the molecular events taking place that provide the cells with the ability to escape from senescence. Taking into account that the DNA damage response pathway is activated from the early stages of carcinogenesis in combination with the genomic instability found in the majority of malignancies, it is likely that the escape from senescence is mediated by the accumulation of genetic alterations. They could potentially "exert" a selective pressure on the arrested cells favoring in this way the survival of more aggressive clones. In addition, recent studies link the epigenetic determinant to cancer development. It has been reported that epigenetic alterations (such as DNA methylation) occurring during senescence could provide the cells with tumor promoting abilities (Xie et al. 2018).

The above-mentioned evidence in conjunction with the altered transcriptome promoted by the aberrant Cdc6 expression (Komseli et al. 2018) urged us to study the genetic and epigenetic changes taking place during the escape from the oncogene induced senescence. The goal is to delineate if the evasion from senescence occurs exclusively due to DNA damage promoting genomic instability, and/or epigenetic alterations may also contribute to the phenomenon.

3. MATERIALS AND METHODS

3.1 Cell culture

HBECs

- Keratinocyte Serum-Free Medium (#17005-075, Invitrogen)
- 50 μg/ml bovine extracts
- 5 ng/ml hEGF (# 17005-075, Invitrogen)
- Trypsin neutralizer
- 1 μg / ml doxycycline (Sigma)
- PBS

Human Bronchial Epithelial cells are vulnerable to culture stress, thus special handling is required. Serum free culture media (Keratinocyte) and supplements, including Epidermal Growth Factor and bovine extracts, are used. Special neutralizer is also preferred to deactivate the trypsin. The cells have been immortalized and the over-expression of Cdc6 is maintained by adding doxycyclin every second day. The cells are incubated at 37°C και 5% CO_2.

3.2 Immunofluorescence

- Apply 0,3% Triton-X-100 for 5minutes
- 3 washes with PBS
- Blocking with 3% BSA, 10% FBS in PBS for 1 hour RT
- 3 washes with PBS
- Add primary antibody in recommended dilution and incubate overnight at 4°C or 1hour RT.
- 3 washes with PBS
- Incubation with secondary fluorescent antibody (diluted in Blocking solution) for 1 hour RT in the dark
- 3 washes with PBS
- Apply DAPI for 5min in the dark (1:10000)
- Rinse with PBS
- Rinse with dH_20
- Mount slides with coverslips using DPX mountant

3.3 Detection of senescence with GL13 (SenTraGor)

Materials

- Compound GL13 (SenTraGor)
- Ethanol 99.9%, 70%, 50%
- 1ml syringe
- Filter 0.22μm

- Coverglass
- 0.5% TritonX-100/TBS
- Anti-biotin antibody (ab201341, Abcam)
- Immunohistochemistry kit UltraVisionQuanto Detection system (Thermo Scientific)
- 3,3'Diaminobenzidine (DAB) chromogen
- Glycerol(Sigma-Aldrich)
- Tris Buffered Saline (TBS 10x): 1.5 M NaCl, 0.1 M Tris–HCl, pH 7.4
- Light microscope

Preparation of SenTraGor:
Dilute 40mg of SenTraGor in 6.660µl Ethanol 99.9%.
Incubate at 56°C in a waterbath for 2 hours

- Place slides in 3% hydrogen peroxide for 13 minutes
- 3 washes with PBS
- Incubate the samples in ethanol 50% for 5 min at RT
- Incubate the samples in ethanol 70% for 5 min at RT
- Drops of GL13 (SenTraGor) are placed on tissue section (cover glass is used to avoid evaporation)
- Monitor reaction under light microscope for 10 min
- Remove gently the cover glass
- Wash in ethanol 50%
- 3 washes in PBS
- Place slides in 0.3% Triton-X-100 for 3 min
- 3 washes in PBS
- Incubate slides with anti-biotin antibody for 1.5 hour at 37°C or overnight at 4°C
- 3 washes in PBS
- Incubate with secondary antibody for 10 min at RT
- 3 washes with PBS
- Incubate with HRP polymer for 13 min in dark
- 3 washes in PBS
- Apply DAB (3,3'Diaminobenzidine) chromogen (1 DAB:200 DAB substrate) and monitor closely under the microscope
- Rinse with tap water in coverslips
- Counterstain sections with 1/4 diluted hematoxylin
- Mount slides with coverslips using glycerol

3.4 Immunocytochemistry

- Inactivation of endogenous peroxidase activity (to avoid non-specific staining)
  Place slides in 3% hydrogen peroxide for 13 minutes
- 3 washes with PBS

- Apply 0,3% Triton-X-100 for 5minutes
- 3 washes with PBS
- Blocking of non-specific signal
  Ultra V block (Thermo Scientific Lab Vision Ultra V Block kit) for 7minutes
- 3 washes with PBS
- Add primary antibody in recommended dilution and incubate overnight at 4°C or 1hour RT.
- 3 washes with PBS
- Incubation with secondary antibody for 10 minutes
- 3 washes with PBS
- Incubation with HRP polymer for 13 minutes
- 3 washes with PBS
- Apply DAB (3,3'Diaminobenzidine) chromogen (1 DAB:100 DAB substrate) and monitor closely under the microscope
- Rinse with H_20 in coverslips
- Counterstain sections with 1/2 diluted hematoxylin that stains the nuclei blue
- Mount slides with coverslips using DPX mountant

3.5 Flow cytometry

- Harvest of cells with trypsinization
- Centrifugation at 1700 rpm for 7 min at 20°C
- Resuspension of the pellets in 800μl ice-cold PBS and centrifugation at 1700 rpm for 3 min at 4°C. Supernatant is discarded
- Resuspension of the pellets in 100μl PBS-0,1% glucose and 1ml 70% cold ethanol with the use of vortex.The samples are stored overnight at -20°C
- Centrifugation of the samples at 1700 rpm for 5 min at 4°C.
- Resuspension of the pellets in 1ml ice cold PBS
- Centrifugation at 1700 rpm for 20 min at 4°C. The supernatant is removed.
- Staining of the cells 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)
- DNA content is assessed on a flow cytometer (FACS Canto II of Becton Dickinson (BD).

3.6 Protein extraction

In order to isolate proteins from cells, the particular cells are trypsinized and centrifuged for 5 minutes. The incubation of cells with RIPA buffer in the presence of protease and phosphatase inhibitors for 1 hour enables cell lysis allowing the extraction of cytoplasmic, membrane and soluble proteins after centrifugation for

15minutes at 4°C at 13.400 rpm. The quantification of the proteins is performed with Bradford assay, allowing the measurement of protein concentration contained in the samples.

3.7 Western Blot

Gel Preparation

Polyacrylamide gel electrophoresis (PAGE) is used to separate proteins based on their molecular weight. To address this aim 2 gels are required; the stacking gel the pores of which are quite large enabling the simultaneous alignment of proteins and a small pore gel, the separating gel, which allows the separation of the proteins. The stacking gel is placed on the top of the other one. The determinant factor for the concentration of the separating gel is the molecular weight of the desired protein. The higher the molecular weight of the protein, the smaller the concentration of the gel.

Separating gel	Stacking Gel
• Distilled water	Distilled water
• Acrylamide/bis 37:1	Acrylamide/bis 37:1
• 1.5 M Tris (pH 8.8)	1.5M Tris (pH 6.8)
• 10% SDS	10% SDS
• 10% Ammonium persulfate	10% Ammonium persulfate
• TEMED	TEMED

Protein Loading

The amount of the protein loading in the gel depends on the purity of the protein. Since our proteins are not purified, the preferable amount of protein is 30μg per well. Gel loading buffer (Laemmli sample buffer 2x) is added to equal amount to the protein volume so as to ensure the dissociation and the denaturation of the proteins and increase the density of the samples so that they can be loaded properly into the well. Then, the samples are heated for 8minutes at 95°C- in order to denaturate the proteins.

Laemmli sample buffer (2x)

- 65.8 mMTris-HCl, pH 6.8
- 26.3% (w/v) glycerol
- 2.1% SDS
- 0.01% bromophenol blue

Gel Electrophoresis

The polyacrylamide gel is placed into the special chamber filled with Running Buffer (1x) that facilitates the electrophoresis. Until the proteins reach the separating gel, the gel is typically run at a voltage of 100V. Next, the voltage can be increased at 120V. The duration of electrophoresis may vary from 1 to 3 hours according to the proteins

of our interest, while electrophoresis progress is monitored using particular ladder (BenchMark Pre-stained Protein Ladder), comprising proteins of various molecular weights.

Transfer / Blotting Gel

The electrophoresis is followed by the transfer of the proteins from the gel to a special matrix, such as polyvinylidenedifluoride (PVDF) membrane. PVDF is a hydrophobic surface with high affinity for proteins (and nucleic acids) that has to be incubated in methanol and dH2O (1 minute each) before the submersion to the transfer buffer. This buffer facilitates the transfer process and the methanol required during its preparation contributes to the removal of SDS from the proteins enhancing their capacity to bind to the membrane. The transfer is performed at 200mA (100 V) for 60 minutes at 4°C. The duration may increase if the molecular weight of the protein is large.

Transfer Buffer (1X)

- 10% transfer buffer
- 20% methanol
- dH20

Membrane blocking

The membrane has to be blocked so as to avoid background noise due to the non-specific binding of the antibodies. The blocking is performed with 5% non- fatty milk or 5% Bovine Serum Albumin (BSA) when it comes to phospho-proteins. The incubation lasts for 1 hour at RT.

Primary antibody

The primary antibody is diluted in 0.5% non-fatty milk or BSA (diluted in TBS-Tween) in the concentration suggested by the company. The membrane is rotated overnight at 4°C.

Secondary antibody

The membrane is washed 3 times (5 minutes each) with TBS-T. Then, the membrane is incubated for 1 hour at RT with a secondary antibody conjugated with HRP (Horsheradish Peroxidase) which is diluted in 0.5% milk.

Chemiluminescent Blotting

Chemiluminescence is the emission of light as a result of a chemical reaction. In the presence of horseradish peroxidase (HRP) and peroxide buffer, luminol oxidizes and forms an excited state product that emits light as it decays to the ground state. Thus, equal volumes of peroxide solution and an enhanced luminol solution are placed on the membrane. After 5 minutes of incubation, the light emitted from the reaction can be detected with x-ray film or CCD camera imaging devices.

3.8 Gene silencing via RNA interference (RNAi)

It provides sequence-specific regulation of gene expression triggered by double-stranded RNA (dsRNA).250μl Optimem is added in two separate eppendorfs. In one

of the two 5μL si-RNA is added, while in the other receives 5μL lipofectamin. The content of the eppendorf with the si-RNA is added to the eppendorf which contains lipofectamin. The mixture is incubated for 15 minutes at room temperature before applying for cell treatment.Before cell treatment, DMEM medium is removed and culture plates are washed with PBS. 1,5 ml (serum-free) Optimem medium is added in each plate and the mixture of si-RNA and lipofectamin is added in the medium. The medium is removed after 6-7 hours. The process is repeated every second day till the last timepoint.

4. RESULTS

4.1 Cdc6 and Cdt1 levels in HBEC CDC6 Tet-ON system

Antibiotic selection with 3 μg/ml blastisidin and 12.5 μg/ml zeocin led to the isolation of a mixed population of HBEC overexpressing CDC6 raising the necessity to check the overexpression of CDC6. Thus, immunofluorescence experiments were performed to test CDC6 and Myc-tag levels after the induction with 1mg/ml Doxycycline. As expected, Cdc6 is not

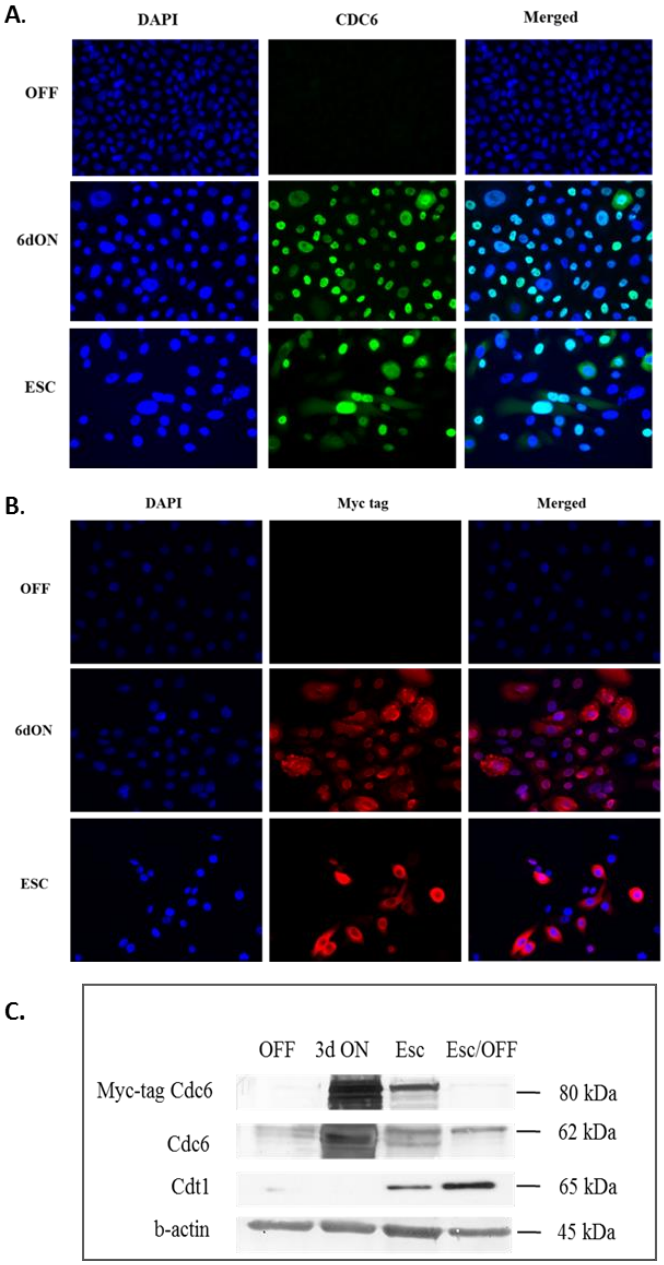


Figure 2.1: Cdc6 and Cdt1 levels in HBEC CDC6 Tet-ON system . A),B) Immunofluorescence for Cdc6 and Myc-tag, respectively in untreated (OFF), 6 days after the CDC6 induction (6dON) and escaped cells (ESC). C) Western Blot about Myc-tag, Cdc6 and Cdt1 in untreated cells, 3 days after CDC6 overexpression, escaped and escaped cells after interruption of CDC6 induction (Esc/OFF).

detected in untreated (OFF) cells because it is normally degraded during S phase after origin firing to prevent the re-licensing of DNA replication. Six days after the induction of CDC6, all the cells overexpress CDC6 (endogenous and exogenous) and Myc-tag (exogenous) (Figure 4.1A,B), while the escapees, cultured with Dox more than 40 days, they express slightly decreased levels of CDC6 and Myc-tag **(Figure 4.1A,B,C)**, while Cdt1 levels have been increased. Interestingly, when we stopped CDC6 over-expression (by non-addition of Doxycycline in the culture medium) in escaped cells we observed that the endogenous Cdc6 levels remained high although exogenous expression had ceased **(Figure 4.1C)**.

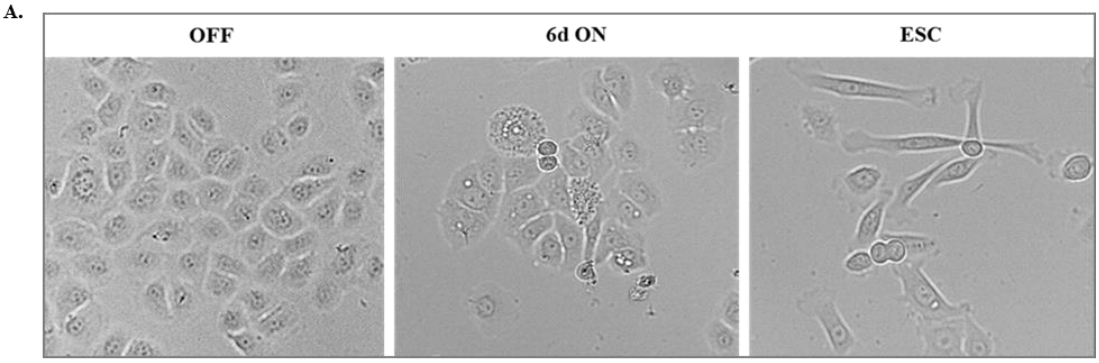
These results together imply that the "steering force" of CDC6 overexpression probably leads to a dynamic regulation of the expression levels of the licensing factors Cdc6 and Cdt1 in the escaped cells, maybe in an attempt to balance their expression to levels viable for the cancer cell.

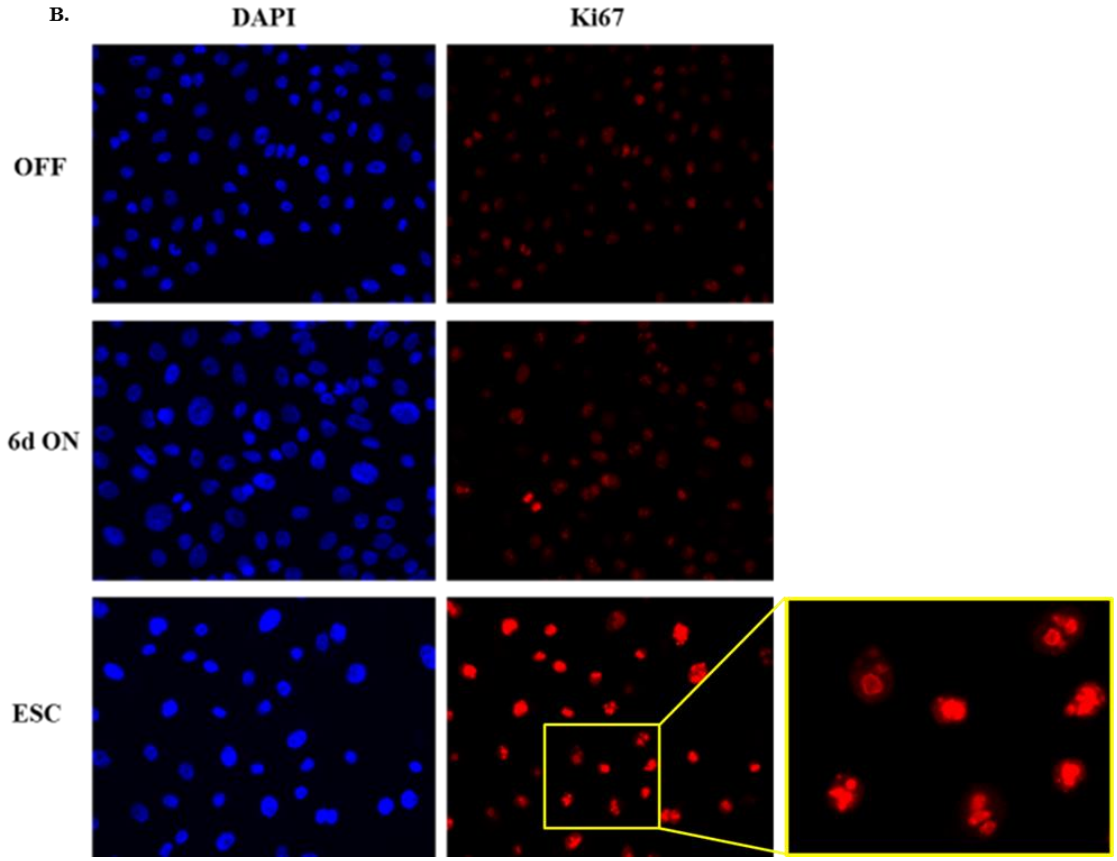
4.2 The increased proliferative capacity and aggressiveness of escaped cells have been established.

The HBEC system reflects the spectrum of lung cancer progression, from the normal epithelium to the senescent state promoted by Cdc6 overexpression and finally the escape from senescence. Regarding the characteristics of the cells escaped from senescence, Ki-67 staining and growth curve analysis showed their -significantly increased- ability to proliferate compared to the basic levels of the untreated cells, which is in agreement with the uncontrollable proliferative capacity of cancerous cells **(Figure 4.2 B,C)**. Ki-67 is a nuclear protein binding to DNA, the expression of which provides an estimation of the proliferative ability and is associated with tumor aggressiveness or progression in many cancers (Chirieac 2016; Gerdes 1990).

Apart from hyper-proliferation, the aggressive cancers are characterized by their ability to move beyond the basal membrane and invade to distal organs and tissues due to acquisition of a mesenchymal morphology, as they undergo EMT. Strikingly, the phenotypic transition from the epithelial to a mesenchymal-like morphology is obvious in our model, as the escaped cells manifest a mesenchymal phenotype **(Figure 4.2A)**. This observation in combination with the invasion assays performed in our system suggests that the cells that escaped from senescence have acquired an invasive behavior, as expected by cells able to promote cancer. Of note, these cells maintain the mesenchymal morphology and the aggressive characteristics even in the absence of the stimulus derived from Cdc6 induced overexpression **(Figure 4.2C,D)**.

In conclusion, the escaped -from senescence- cells have transformed to mesenchymal-like cells acquiring a different cellular identity and more aggressive features, which seem to have been established as they are independent of the exogenous Cdc6 stimulus.


A.
OFF
6d ON
ESC


B.
DAPI
Ki67
OFF
6d ON
ESC

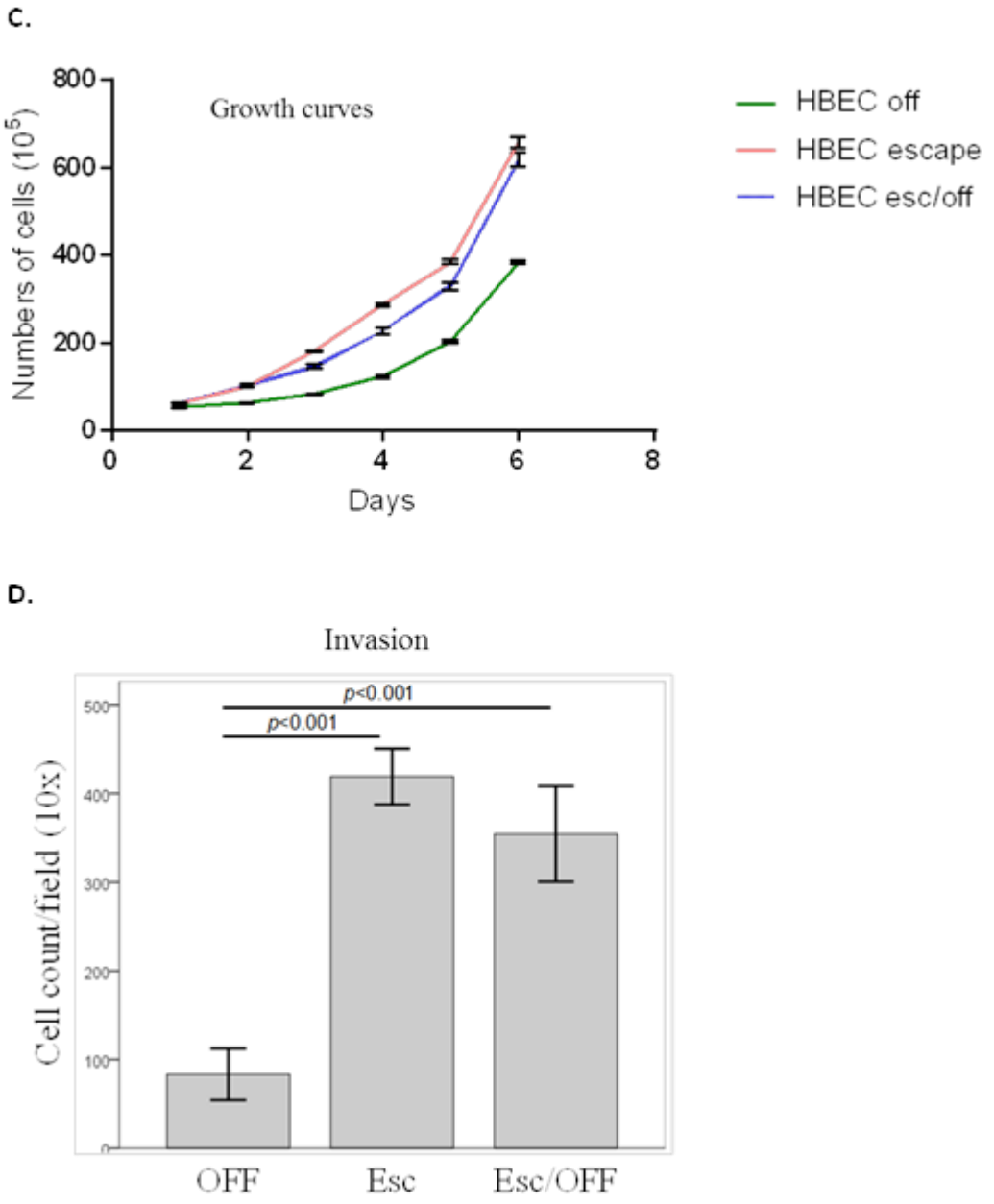


Figure 4.2 : The proliferative capacity and aggressiveness of escaped cells. A) Epithelial to Mesenchymal transition (EMT) during the escape from senescence. B) Immunofluorescence for Ki-67 reveals the increased proliferation of escapees, C) Growth curves and D) invasion assay of escaped cells upon exogenous expression of Cdc6 (Esc) and without (Esc/OFF).

4.3 Activation of p53/p21$^{Cip1/Waf1}$ pathway during CDC6 induced senescence in HBECs

Next, we wanted to delineate the pathway through which the oncogene induced senescence is activated in our model. Since, the CDK4 is overexpressed in immortalized HBECs (to prevent the premature arrest) and subsequently the p16^{INK4a} is not able to promote senescence due to constant phosphorylation of Rb, we investigated the p53 pathway. Indeed, p21$^{Cip1/Waf1}$ levels were significantly increased 6 days after the induction of Cdc6, while the escaped cells manifested a subtle detection

of it, suggesting that the activation p53/ p21 pathway possibly mediates the senescent phenotype in HBEC- CDC6 -TetON system. Immunoblot analysis also indicated that the activation of p53 and its downstream effector $p21^{Cip1/Waf1}$ are detected 3 days after the induction of CDC6, which is probably attributed to the replication stress promoted by CDC6 overexpression and finally leads to cell cycle arrest, known as oncogene induced senescence. As expected, these proteins have been almost eliminated in the escaped cells, since they have evaded the senescent state and they are able to proliferate again **(Figure 4.3A,B).**

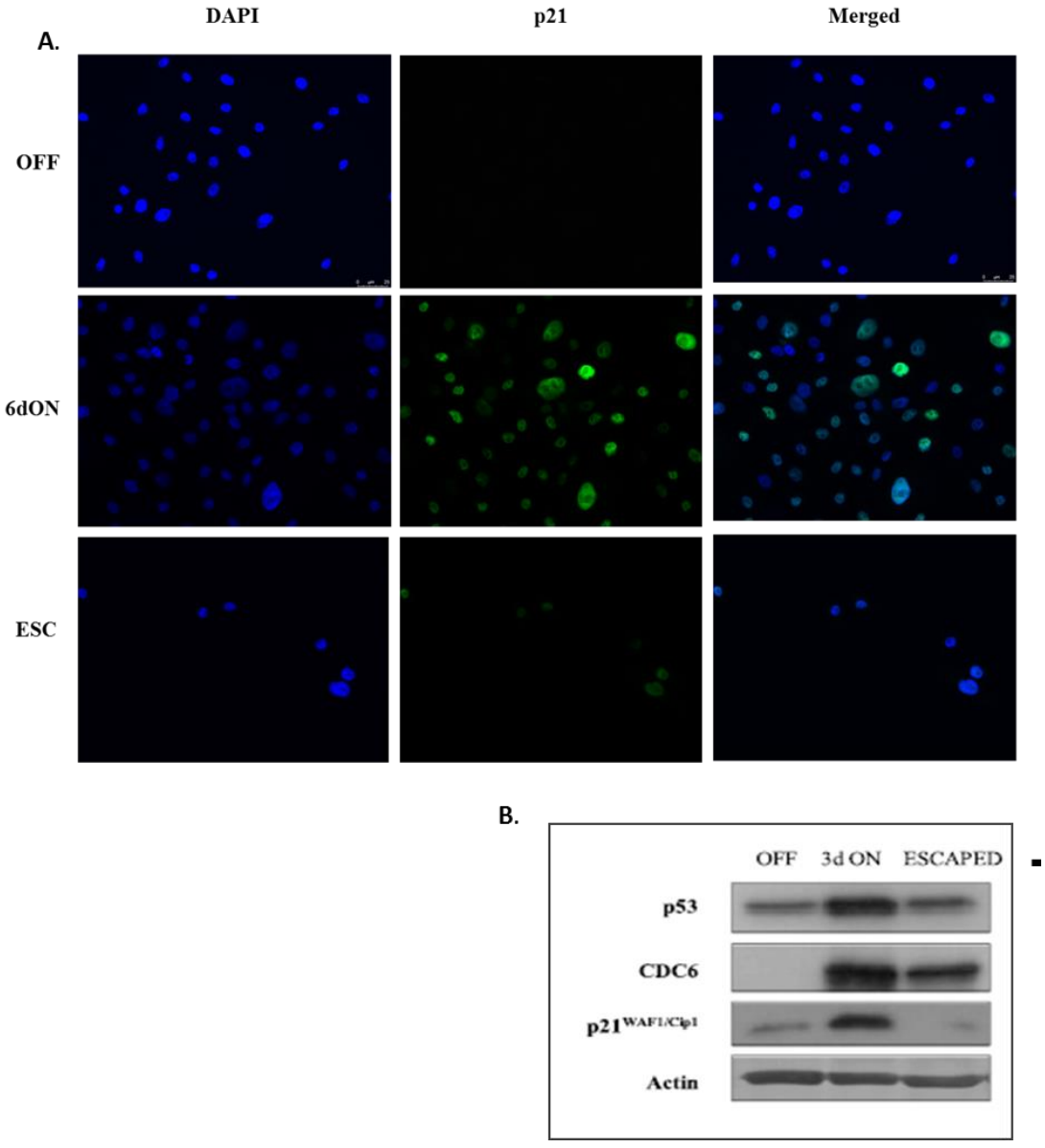


Figure 4.3: The senescence in HBEC CDC6 TetON system is mediated by p53/p21 pathway. A) Immunofluorescence for p21 in untreated (OFF), 6days after CDC6 overexpression (6dON) and escaped cells and B)Western Blot about p53 and p21 performed in untreated cells, 3 days after CDC6 overexpression (3d ON), escaped indicated the activation of p53/p21 pathway.

4.4 Absence of SAHF and intact nuclear lamina in HBEC CDC6 TetON system

As mentioned before, some of the common features of oncogene-induced senescence is the emergence of SAHF, which include the dissociation of heterochromatin from the nuclear lamina to the interior of the nucleus, and the down regulation of LMNB1(coding for Lamin B1). Thus, we decided to investigate these factors in our system and observed that SAHF are not formed in HBEC system, while the laminb1 levels remain the same during senescence indicating that the nuclear lamina is intact in our model. The absence of SAHF could be potentially attributed to the fact that CDK4 is mutated (overexpressed) in HBEC system so that the cells can be immortalized avoiding the premature growth arrest due to p16 activation, while the SAHF formation requires the integrity of Rb pathway. In addition, SAHF are linked to Lamin-Associated Domains (LAD) re-organization and there is evidence that deregulation of LMNB1 might be fostering a pre-SAHF nuclear landscape (Lochs, Kefalopoulou, and Kind 2019). This could be a possible explanation of the intact laminb1 levels in our system **(Figure 4.4).**

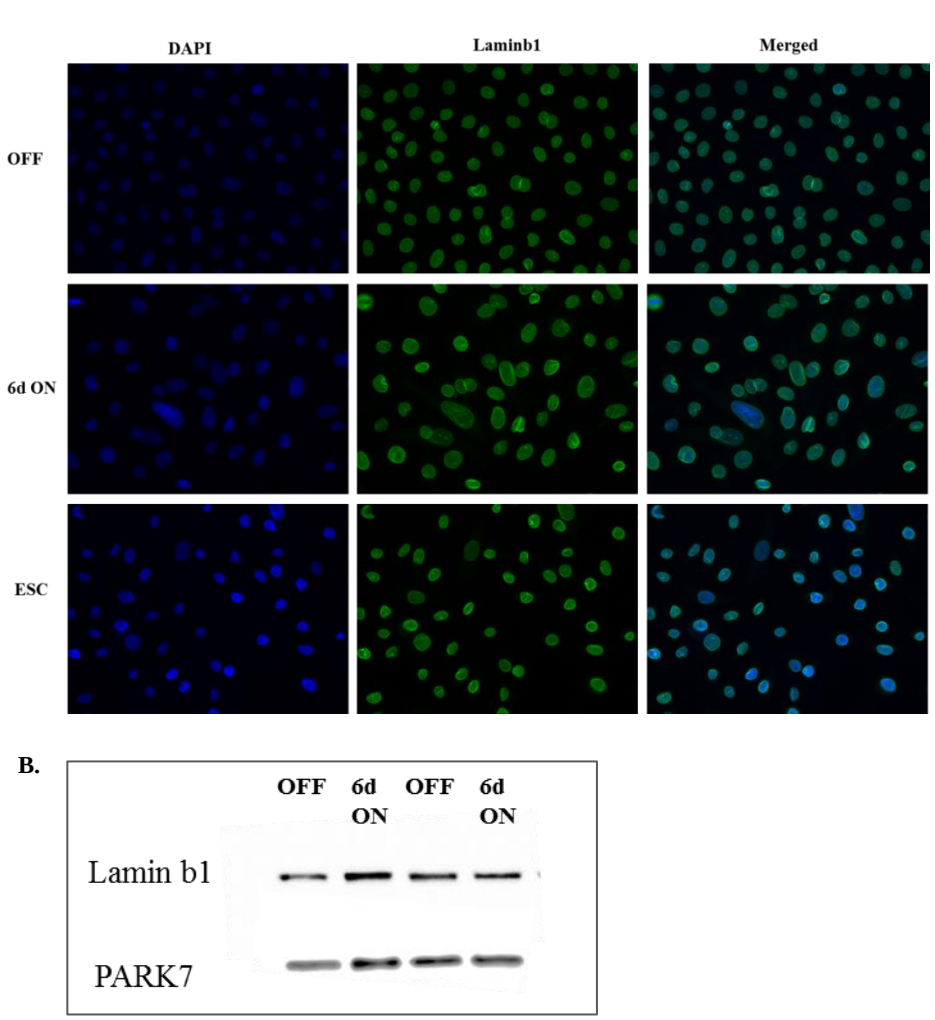


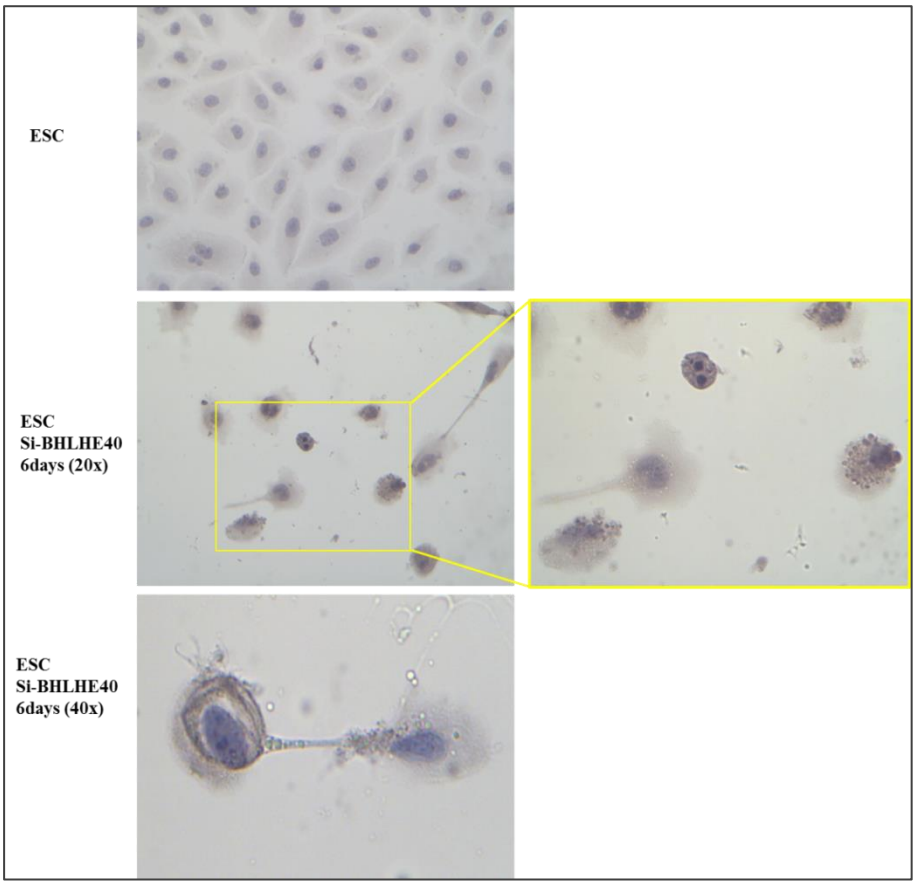
Figure 4.4 : LaminB1 levels indicated by A) Immunofluorescence in untreated cells (OFF), 6 days after CDC6 overexpression (6dON) and escaped cells and B) Western Blot in untreated cells (OFF), 6 days induced after CDC6 overexpression (6dON) performed in 2 biological replicates.

4.5 BHLHE40 is essential for the survival of escaped cells

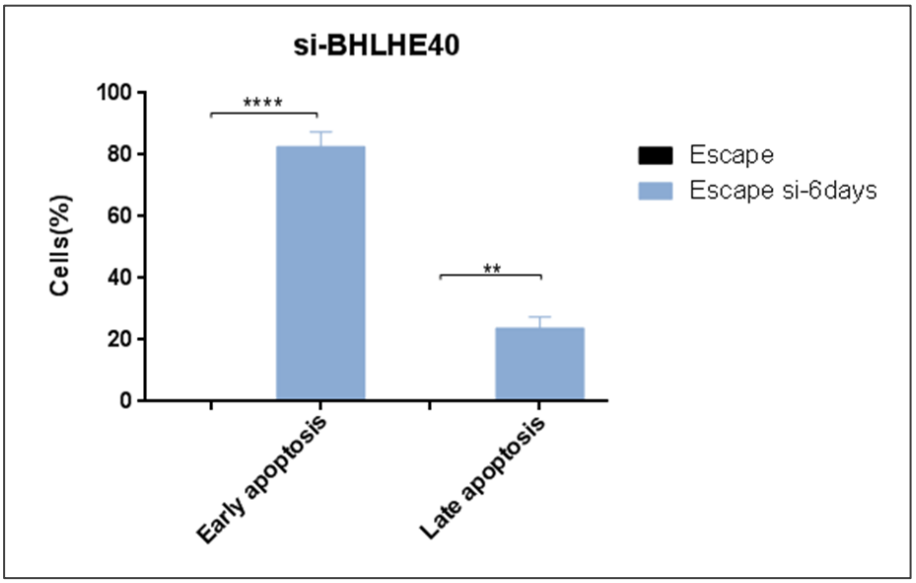
Having as a goal to identify genetic changes taking place during the oncogene induced escape from senescence, Whole Genome Sequencing (WGS) was performed in untreated and escaped cells. Among many Single Nucleotide Polymorfisms (SNPs) and Copy Number Variations (CNVs) found in escaped cells that potentially contribute to the phenomenon of evasion from senescence, we identified an inversion (> 3.7 Mb) on chromosome 3, where the Basic Helix-Loop-Helix Family Member 40 (BHLHE40) gene is located. BHLHE40 is a transcription factor with multiple roles including circadian rhythm regulation, differentiation, proliferation, apoptosis, and metabolism. High levels of BHLHE40 have been reported in many malignancies, including NSCLC (Chakrabarti et al. 2004; Giatromanolaki et al. 2003). There is also evidence connecting BHLHE40 with EMT, while the exact mechanism has not been clarified yet (Wu et al. 2012).

These studies in combination with the inversion observed in our system and the high expression levels of BHLHE40 in the escaped cells, prompted us to silence the particular gene in the escapees. Interestingly, there was a significant change in their morphology implying a possible activation of the apoptotic mechanism. To prove this, the presence of Caspase-3 was first tested in escaped cells before and after the silencing of BHLHE40 by performing immunocytochemistry. Indeed, almost all the escaped cells were stained with Caspase-3 6 days after the silencing of BHLHE40, while there was also a clear transportation of extracellular vesicles between (pre)apoptotic cells **(Figure 4.5A)**. However, the morphology of the stained cells was not homogeneous among the cells. Thus, the early apoptotic cells (retainingmembrane integrity) and late apoptotic cells (fragmented cells forming apoptotic bodies) were quantified and compared to the untreated escaped cells. The cells detected at the phase of early apoptosis were approximately 82%, while 23% of the cells were found in the late apoptosis **(Figure 4.5B)**. In addition, FACS analysis in escaped control and escaped si-BHLHE40 confirmed the immunocytochemistry results, indicating that 21,61% of the cells had been apoptotic 6 days after gene silencing (20 fold change) **(Figure 4.5C)**. These results suggest that BHLHE40 dramatically affects the cell cycle progression and is essential for the survival of escaped cells.

A.


ESC
ESC
Si-BHLHE40
6days (20x)
ESC
Si-BHLHE40
6days (40x)

B.


si-BHLHE40
Cells(%)
100
80
60
40
20
0
****
**
Escape
Escape si-6days
Early apoptosis
Late apoptosis

C.

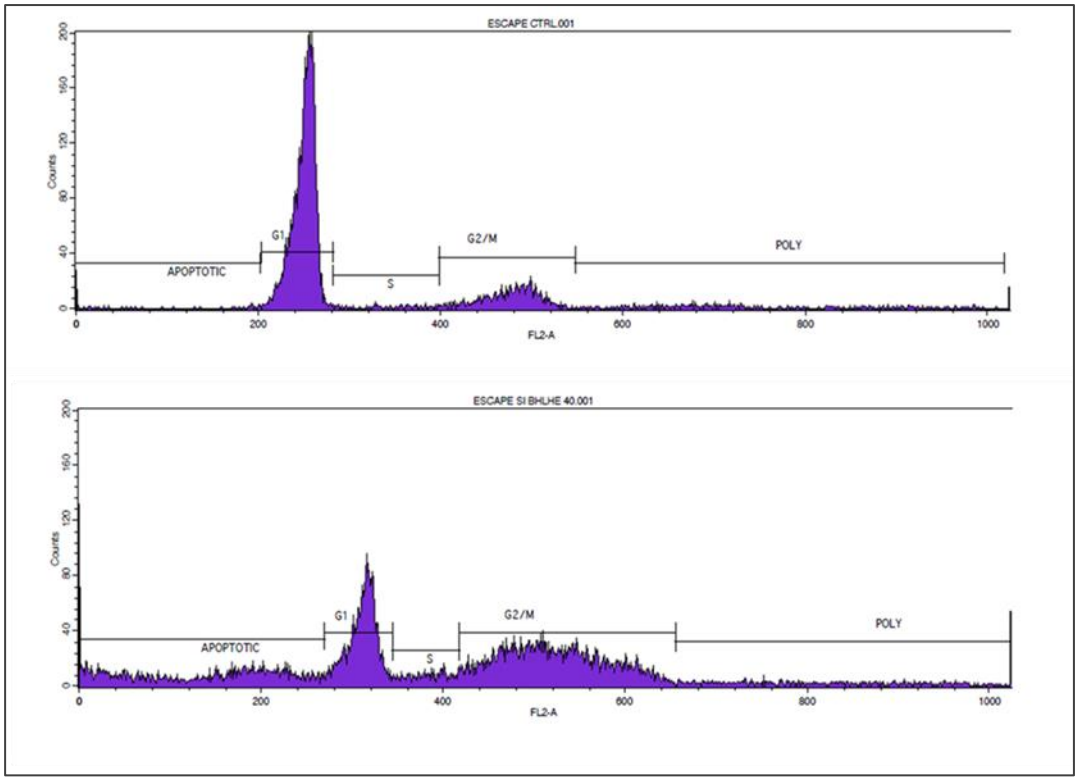


Figure 4.5: BHLHE40 silencing mediated apoptosis in escaped cells. A) Immunocytochemistry assay for Caspase-3 indicating apoptosis in escaped cells 6 days after BHLHE40 silencing. Extracellular vesicles are transferred between cells (ESC Si-BHLHE40 6days (40x)), B) Quantification of early and late apoptotic cells in escape cells before and after si-BHLHE40. C) FACS analysis indicating apoptosis promoted by silencing BHLHE40 gene.

4.6 The levels of H4K16ac fluctuate in HBEC CDC6 TetON system

The acetylation on lysine 16 of histone 4 is associated with gene activation and DNA damage repair (Verdone, Caserta, and Mauro 2005). Immunofluorescence analysis of H4K16ac revealed a significant increase of H4K16ac levels in escaped cells, which was probably accompanied by slight reduction in the senescent cells (6 days after CDC6 overexpression). Immunoblot analysis at the same timepoints confirmed these results, indicating a fluctuation at H4K16ac levels in our model **(Figure 4.6A, B)**.

These results possibly strengthen the notion regarding the role of hypoacetylation of H4K16 in senescence, revealing also its contribution in the oncogene-induced senescence. Apart from that, the enrichment of H4K16ac in escaped cells could be associated with the activation of genes driving or contributing to the escape from senescence, as this histone mark regulates the accessibility of the chromatin affecting the interaction between nucleosomes and subsequently the expression levels of genes (Zhang, Erler, and Langowski 2017).

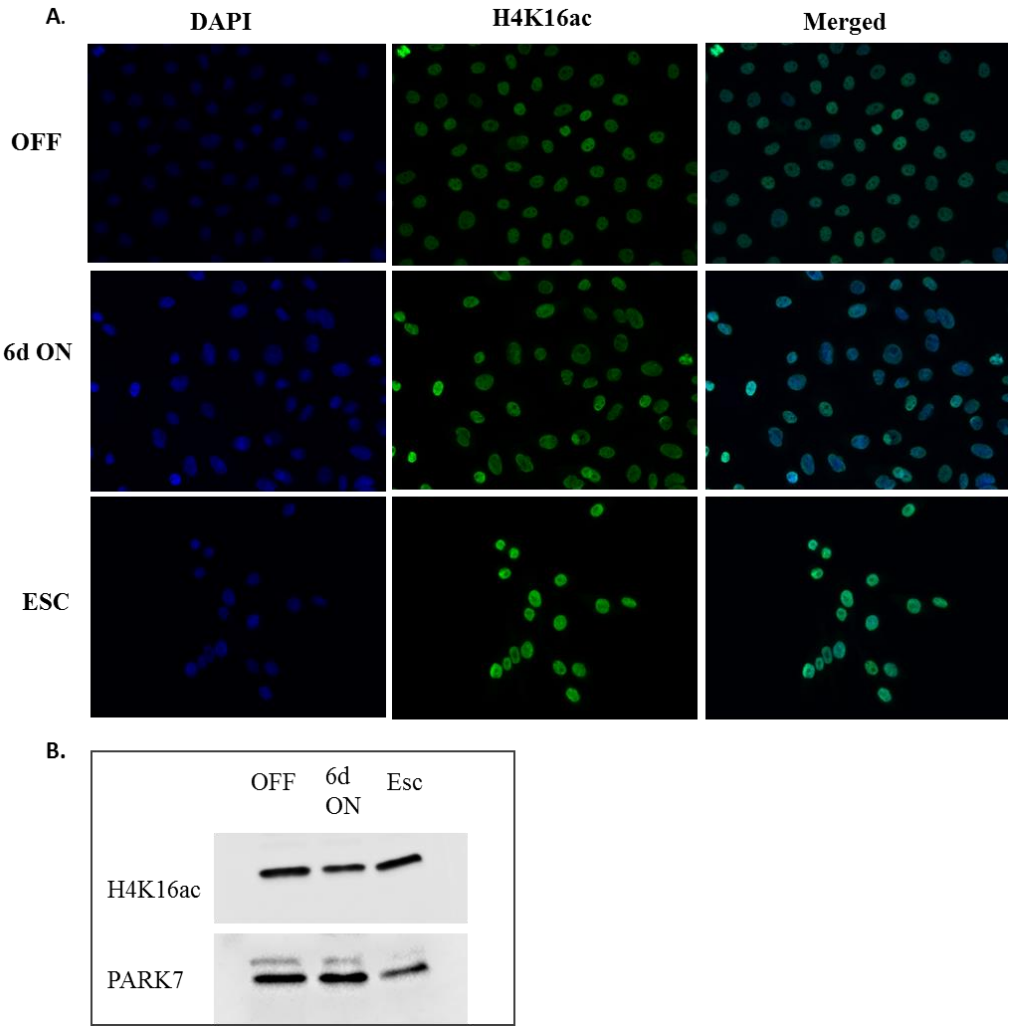


Figure 4.6: H4K16ac levels in HBEC CDC6 TET-ON system indicated by A) Immunofluorescence assay and B) Western Blot in untreated cells (OFF), 6 days after CDC6 overexpression (6dON) and escaped cells.

5. DISCUSSION

Cancer refers to a group of diseases manifesting uncontrollable cell proliferation, invasion into the surrounding tissues and metastasis to distant organs via lymphoid system or blood circulation. It may arise anywhere in the body and it requires a long term process, known as carcinogenesis. Numerous are the factors that may contribute to this process, but carcinogenesis usually starts with genetic mutations or chromosomal re-arrangements affecting crucial genes (oncogenes, tumor-suppressors) providing the cell with the capability of bypassing the checkpoint mechanisms that control cell cycle and proliferation.

The fact that 90% of human cancers arise in epithelial cells, in combination with the existence of many mesenchymal models, prompted us to create a unique epithelial model to study the lung cancer initiation and progression. So, in this study, we used an inducible cellular system over-expressing the replication licensing factor CDC6 which is deregulated from the early stages of carcinogenesis in several cancers (Karakaidos et al. 2004; Liontos et al. 2007). In addition, when CDC6 is over-expressed, it is able to function as an oncogene mainly by promoting genomic instability due to re-replication (Bartkova et al. 2006; Komseli et al. 2018). It may also regulate transcription (upon its overexpression) through its binding to particular regions (such as the promoter of CDH1 locus), leading to the displacement of CTCF and the repression of particular genes (Sideridou et al. 2011).
Human bronchial epithelial cells (HBECs) immortalized by over-expressing the human telomerase (hTERT) to prevent the replicative senescence and CDK4 to avoid the cell cycle arrest due to activation of the signaling pathway p16INK4^{a} have been employed as a non-malignant model to study the different phases of tumorigenesis and mainly to investigate the "driver" events that led to the emergence of cells with aggressive behavior (Komseli et al. 2018; Ramirez et al. 2004).

In the current thesis, first the CDC6 overexpression was confirmed in HBECs, validating the induction of senescence in almost all the cells at 6 days after the induction with Doxycycline. The senescent state of the cells was visible morphologically, as the cells manifested a flattened morphology with enlarged nuclei, while GL13 (SenTraGor) staining that detects the lipofuscin (a non-degradable metabolic bioproduct accumulated in senescent cells) also supported this notion (Evangelou et al. 2017; Komseli et al. 2018).
In parallel with the exogenous expression of Cdc6, its endogenous levels seem to significantly increase in the senescence and escaped from senescence cells, suggesting that Cdc6 is probably able to be self-regulated maybe due to direct binding on CDC6 promoter, which is not surprising if we take into account that Cdc6 acts as a transcriptional regulator upon overexpression. In addition, the increased levels of the licensing factor Cdt1 in the escaped cells imply that the pressure from the constant CDC6 stimulus dynamically regulated the expression levels of both licensing factors Cdc6 and Cdt1 in the escaped cells, while the maintenance of the expression levels

even in the absence of exogenous Cdc6 confirm that these changes have been established and are not dependent on the "driving" force of the exogenous Cdc6 expression any longer.

Investigating the proliferative capacity of the cells that escaped from senescence, the significantly increased levels of Ki67, a marker highly associated with poor prognosis in NSCLC (Martin et al. 2004), also supported by growth curve analysis revealed that the escapees are able to proliferate extremely fast as expected by cells evadingsenescence. In addition, these cells have changed cellular identity, as they have acquired a mesenchymal-like morphology which is in agreement with the invasion assays, strengthening the notion that the cells managed to bypass the anti-tumor barriers have acquired more aggressive characteristics shared among cancerous cells. Therefore, the HBEC CDC6 Tet-ON system is a model that properly recapitulates all the phases of carcinogenesis manifesting a total change of cellular identity via epithelial to mesenchymal transformation and gaining invasive capabilities, which have been established and are independent of the CDC6 exogenous expression.

Regarding the molecular basis of Cdc6 induced senescence in the HBEC system, we observed the activation of p53/p21 pathway in senescent cells, which is reasonable since Cdc6 overexpression promotes re-replication, and subsequently DNA damage response pathway due to the emergence of double strand breaks. More specifically, ATM phosphorylates and stabilizes p53, as it is not degraded by Mdm2, and promotes p21 activation. Normally, p21 inhibits Cyclin E/Cdk2 activating (hypophosphorylating) in this way the Rb protein, which binds to the transcription factor E2F preventing the cell cycle progression. However, in HBEC system the CDK4 overexpression required for the immortalization of the cells in combination with the repression of the INK4/ARF locus by Cdc6 overexpression leads to the constant phosphorylation of Rb, maintaining its inactive form and allowing the expression of genes regulated by E2F, such as Cyclin E, and promoting proliferation. Therefore, the cell in an attempt to be protected by the extended DNA damage promoted by CDC6 overexpression possibly induces senescence through the activation of an alternative pathway including p53/p21/Cdk1. There is evidence that p21 is able to inhibit Cdk1 and promote G1/S arrest (Satyanarayana, Hilton, and Kaldis 2008). This notion could potentially apply in our system because CDK1 is down-regulated in senescent cells, as indicated by RNA sequencing analysis (Komseli et al. 2018).

Taking into account the dynamic nature of senescence in combination with the necessity of the senescence induction in order to protect the cell upon the continuous stressful stimulus of Cdc6 overexpression, the above cellular pathway could act synergistically with other pathways, which still have to be investigated. For instance, the fact that TP73 is upregulated during senescence in our system (3.98 fold change compared to the untreated) (Komseli et al. 2018) could imply that p21 can be also activated by this gene. So, in case that p53 is deficient or depleted, the induction of senescence could occur through TP73. Apart from that, there are studies showing the ability of IGF binding protein-5 (IGFBP-5) to induce premature (and replicative)

senescence mediated by p53 activation through the activation of inflammatory pathways (K. S. Kim et al. 2007; Sanada et al. 2018). Actually the suggested molecular pathway includes the induction of IL-6 activating STAT3 which in turn activates IGFBP-5 leading to DNA damage through ROS and subsequently p53 induction. In the HBEC cellular system, IL-6R, STAT3 and IGFBP-5 are upregulated in the senescent state induced by CDC6 overexpression and are estimated to a 2.96, 1.68 and 23.74 fold change, respectively (compared to the cells before the CDC6 overexpression). This pathway could potentially function as supplementary, since the senescent cells tend to communicate through SASP which could trigger this pathway reinforcing senescence.

Of note, CDC6 induced senescence does not include the SAHF formation or lamina dissociation, which often occur in the oncogene induced senescence (OIS). However, these characteristics should not be considered hallmarks of OIS since there are studies supporting that SAHF require the integrity of the $p16^{INK4a}$-pRB pathway in order to be formed, which is not the case in our model, while they are also dependent of the type of stimulus promoting the senescent state and the cell type (Kosar et al. 2011; Narita et al. 2003; Parry and Narita 2016). As for the intact lamina during senescence, it could be attributed to the fact that Lamin-Associated Domains (LAD) are re-organized and the lamina is dissociated only in view of SAHF formation, as changes in the heterochromatin landscape are essential for the emergence of these structures (Lochs, Kefalopoulou, and Kind 2019).

Taken together, these results prompt us to the assumption that the type of senescence induced by Cdc6 overexpression in HBEC system differs to an extent from the "typical" oncogene-induced senescent models described so far. This could mainly attributed to the abrogation of p16/Rb pathway in HBECs, which could potentially lead to a "lighter" state of senescence than the "deep" senescence promoted when this pathway is intact (S. Lee and Schmitt 2019). If this hypothesis is true, the abrogation of this pathway could be employed as a prognostic factor as it may potentially facilitate the escape from oncogene (and therapy) induced senescence, and maybe the fact that Cdc6 abrogates this pathway through INK4/ARF locus repression is one of the reasons that its overexpression is highly associated with poor prognosis in many malignancies.

Apart from the characterization of CDC6 induced senescent and escaped cells in the HBEC system, we tried to identify the genetic and epigenetic changes taking place and potentially drive or at least contribute to the phenomenon of escape from senescence. Among numerous genetic changes identified in escaped cells, we considered of great importance the inversion on BHLHE40 gene, as it affects a large number of genes which are differentially expressed in our system (Komseli et al. 2018). The silencing of this BHLHE40 in escaped cells revealed that it is essential for the survival of the cells and the maintenance of the escape phenotype. Apart from that, the fact that an inversion of size bigger than 3.7 Mb leads to the overexpression of the gene, instead of its inactivation, maybe implies that the inversion affected the expression of the gene through epigenetic mechanisms. More specifically, this

genomic re-arrangement could bring the promoter of the gene in close proximity to enhancers or even disrupt the existing physical barriers, mediated by chromatin insulators such as CTCF, facilitating the altered expression of BHLHE40. These assumptions should be further investigated with supplemental experiments.

Last but not least, regarding the epigenetic aspect of the phenomenon we observed that H4K16 is hypoacetylated during CDC6 induced senescence in HBECs, which is consistent with another study mentioning that H4K16 hypoacetylation is able to promote early cellular senescence (Krishnan et al. 2011). In addition, the increased levels of H4K16 acetylation in the cells that escaped from senescence implies the activation of genes that are possibly related to the manifestation of EMT and cancer progression and contributed to the evasion from senescence.

To conclude, in this study we attempted to have a versatile approach to the phenomenon of escape from senescence in a human epithelial cellular system, investigating the molecular pathways facilitating this phenomenon, which has been disputable until recently, as senescence was considered to be an irreversible state. By delineating the molecular events driving this phenomenon, we could comprehend the mechanisms of lung carcinogenesis in depth and potentially lead the way for new therapeutic approaches.

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