DEPARTAMENTO DE CIÊNCIAS DA VIDA
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Age-dependent sensitivity to anti-mitotics: the role of FoxM1 and its therapeutic potential

Sara Marisa Duarte Vaz

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica do Professor Doutora Elsa Logarinho (Aging and Aneuploidy, Instituto de Biologia Molecular e Celular da Universidade do Porto) e do Professor Doutor Carlos Duarte (Departamento de Ciências da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra).

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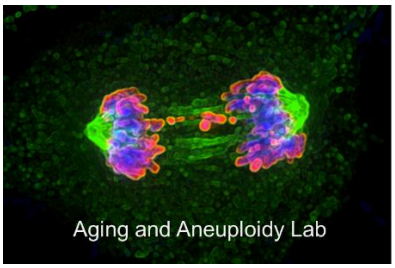

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“Do not regret growing older. It is a privilege denied to many.”

- Popular saying.

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Abstract

Oncogenic transcription factor FoxM1 is overexpressed in the majority of human cancers. Emerging data suggest that targeting FoxM1 in mono- or combination therapy may have promising therapeutic benefits for the treatment of cancer. FoxM1 expression is associated with the proliferative capacity of the cell, consistently with its role in primarily driving the expression of G2/M specific genes, with associated phenotypic expression of mitotic defects and chromosome aberrations when defective. Using high-resolution live cell imaging to observe individual cell behavior, we found that human elderly dermal fibroblasts reproduce the mitotic defects of FoxM1 repression. In agreement, expression of FoxM1 and its downstream targets decreases progressively with chronological ageing, suggesting it counters senescence. Importantly, we also perceived that old fibroblasts are sensitized to cell death when treated with anti-mitotic drugs commonly used in chemotherapy. Therefore, we not only depleted FoxM1 in young cells but also overexpressed a constitutively active form of FoxM1 in old cells, to follow their cell fate upon anti-mitotic drug treatment. Our results indicate that FoxM1 downregulation accounts for the increased sensitivity to anti-mitotics in aged cells. Therefore, a combinatorial treatment using FoxM1 inhibition plus microtubule poisons was tested and our data suggest a pro-apoptotic efficacy against tumor cells overexpressing FoxM1.

Keywords: FoxM1; ageing; anti-mitotic drugs; cancer treatment.

Resumo

O fator de transcrição oncogénico FoxM1 é sobre-expresso na maioria dos cancros humanos. Dados recentes sugerem que a inibição de FoxM1 em terapia mono- ou combinada pode ter benefícios terapêuticos promissores para o tratamento de cancro. A expressão de FoxM1 está associada com a capacidade proliferativa da célula, o que é consistente com a sua função principal de promover a expressão de genes G2/M. A repressão de FoxM1 induz uma manifestação fenotípica de defeitos mitóticos e aberrações cromossómicas. Usando microscopia de alta resolução para aquisição de imagens de células vivas e observação do comportamento individual de células, descobrimos que fibroblastos dérmicos humanos idosos reproduzem os defeitos mitóticos de repressão de FoxM1. Consistentemente, a expressão de FoxM1 e de genes alvo diminui progressivamente com a idade cronológica, sugerindo uma função do FoxM1 na prevenção da senescência celular. Relevantemente, observámos também que os fibroblastos de idade avançada são mais sensíveis à morte celular em mitose quando tratados com drogas anti-mitóticas comummente usadas em quimioterapia. Deste modo, diminuímos os níveis de FoxM1 em células jovens e sobre-expressámos uma forma constitutivamente ativa de FoxM1 em células velhas, e seguimos o seu destino celular após tratamento com drogas anti-mitóticas. Os nossos resultados indicam que a redução dos níveis de FoxM1 é responsável pelo aumento da sensibilidade aos anti-mitóticos em células idosas. Finalmente, testámos um tratamento combinado usando inibição de FoxM1 e drogas anti-mitóticas e os nossos dados sugerem uma eficácia pró-apoptótica contra células cancerosas que sobre-expressam FoxM1.

Palavras-chave: FoxM1; envelhecimento; drogas anti-mitóticas; tratamento de cancro.

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Abbreviations

AA: Antibiotic-Antimycotic

APC/C: Anaphase-Promoting Complex/Cyclosome

APS: Ammonium Persulfate

A.U.: Arbitrary Units

BP: Bipolar

C. elegans: Caenorhabditis elegans

CAK: Cdk-activating kinase

Cdk: Cyclin-dependent kinase

Cyc: Cyclin

ddH2O: Desionizated water

DMEM: Dulbecco's Modified Eagle Medium

DNA: Deoxyribonucleic acid

DOX: Doxycycline

EDTA: Ethylenediaminetetracetic acid

EGTA: Ethyleneglycoltetraacetic acid

FBS: Fetal Bovine Serum

g: Gram

G1: Gap phase 1

G2: Gap phase 2

GFP: Green Fluorescent Protein

GOI: Gene of interest

HDF: Human Dermal Fibroblast

hr: Hours

Kbp: Kilo base pairs

kDa: KiloDalton(s)

M phase: Mitosis

M: Molar

MCC: Mitotic Checkpoint Complex

MEF: Mouse Embryonic Fibroblast

MEM: Minimum Essential Media

min: Minutes

ml: Mililiter

mM: Milimolar

MP: Multipolar

mRNA: Messenger RNA

n: Number of samples in the study

NEB: Nuclear Envelope Breakdown

nM: NanoMolar

NOC: Nocodazole

ºC: Celsius degree

ON: Over Night

PBS: Phosphate-buffered saline

RNA: Ribonucleic acid

RNAi: RNA interference

ROS: Reactive oxygen species

rpm: Rotations per minute

RT: Room temperature

S phase: DNA synthesis phase

SAC: Spindle Assembly Checkpoint

SASP: Senescence-associated secretory phenotype

SCF: Skp1/Cullin/F-box protein

SD: Standard deviation

SDS: Sodium dodecyl sulphate

SDS-PAGE: Sodium dodecyl sulfate-Polyacrylamide Gel Electrophoresis

siRNA: Small interference RNA

STLC: S-trityl-L-cysteine

TBS: Tris-buffered saline

TBST: Tris-buffered saline with Tween 20

TEMED: Tetramethylethylenediamine

V: Volt

µg: Microgram

µl: Microliter

µM: MicroMolar

Chapter I - Introduction

I-1 Mitosis: cancer is the illness of the cell cycle

I-1.1 The cell cycle - overview

Omnis cellula et cellula. - Where a cell arises, there a cell must previously have existed. This is the vital cell reproduction dogma confirmed by Rudolf Virchow in 1855 (Rudolf Virchow work reviewed in Lindkvist, 1999). Cell division is fundamental to the development and function of all life. The sequence of events that leads to cell reproduction is known as the cell cycle, and they are highly regulated by a control system composed of a protein network that ultimately ensure the fidelity of the transmission of genetic information.

There are two major phases in the cell cycle: the S-phase, where DNA duplication occurs and the M-phase or Mitosis, where chromosome segregation and cell division follows. Partly to permit more time to grow and double their mass of protein and organelles, most cell cycles have extra gap phases: a G1-phase between M-phase and S-phase and a G2-phase between S-phase and Mitosis. Cells can also exit from cell cycle entering G0. This situation may occur due to adverse growth conditions, inhibitory signal from other cells or intracellular damage (Reviewed in detail in Morgan, 2007), figure 1.

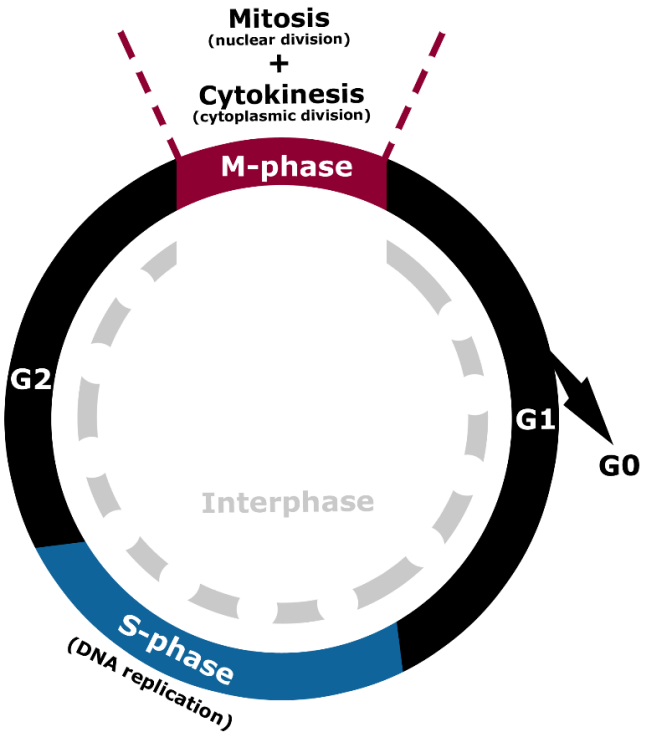

Figure 1 – The Cell Cycle. Representation of the eukaryotic cell cycle, composed of two main phases, the S-phase (for DNA replication) and the M-phase (for nuclear and cytoplasmic division).

In the human body, every tissue has optimized cell size, number and type. When cells no longer respond to inter- and intra-cellular control signals and develop an excessive proliferation rate, a tumor might arise. Therefore, it is crucial to understand the cell cycle, especially mitosis, in order to better understand cancer and develop strategies to prevent/reverse it.

I-1.2 The cell cycle control system

The fundamental components of the cell cycle control system are a family of enzymes called cyclin-dependent kinases (Cdks). Cdks are activated when bound to regulatory proteins called cyclins. These kinases activities rise and fall as the cell advances through the cycle, primary due to changes in cyclin levels. These oscillations result in cyclical alterations in the phosphorylation status of the components of the cell cycle machinery, triggering the initiation of events (Minshull et al., 1989).

There are several classes of cyclins each defined by the stage of the cell cycle at which they bind Cdks. The G1-cyclins (cyclin D in vertebrates), that help govern the activities of the G1/S-cyclins (cyclin E), which trigger progression through G1 to S-phase, ensuing a commitment to cell cycle entry. Their levels fall in S phase. The S-cyclins (cyclin A) that promote chromosome duplication. Their levels remain elevated until mitosis, contributing to the control of some early mitotic events. The M-cyclins (cyclin B) that fuel the entry into mitosis and which the levels suddenly decline before the end of the M-phase (Minshull et al., 1989; reviewed in Alberts et al., 2012).

The other central components of the cell cycle control system are the checkpoints. Checkpoints are constitutively active control pathways that have a role of checking if all the pre-requisites to progress the cell cycle have been properly satisfied before the division is allowed to continue (Hartwell and Weinert, 1989). The first checkpoint is called Start, at the late G1 and it checks if there are ideal conditions for cell proliferation, in the microenvironment and in the cell itself. The second checkpoint, at the G2/M transition, is the G2/M checkpoint that ensures that all DNA has been properly replicated and that mitosis is ready to advance. The third checkpoint, that will be described latter in this work, is the Spindle Assembly Checkpoint (SAC) that certifies that all the chromosomes are correctly aligned at the

metaphase plate before anaphase is triggered (reviewed in Hartwell and Weinert, 1989; Morgan, 2007; Alberts et al., 2012), figure 2.

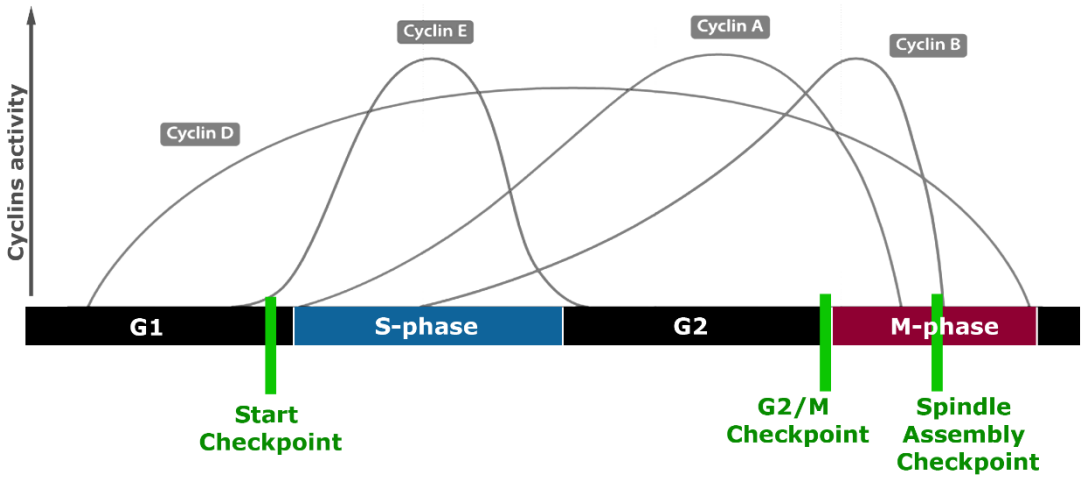

Figure 2 – The Cell Cycle Control System. Representation of the two main control systems of the cell cycle: cyclins activity and checkpoints. There are three checkpoints, the Start, G2/M and SAC, located at specific stages. There are several classes of cyclins each defined by the stage of the cell cycle at which they bind to Cdks. (Adapted from Morgan, 2007).

I-1.3 Mitosis

Based on the chromosome behavior observed through the microscope, mitosis is traditionally divided in five stages: prophase, prometaphase, metaphase, anaphase and telophase. Afterwards, a cell enters a stage of division into two halves, cytokinesis, figure 3. Prophase is categorized by the condensation of DNA into chromosomes, followed by separation of centrosomes that start to move to opposite poles of the cell and begin to assemble the mitotic spindle. Prometaphase begins upon nuclear envelope breakdown (NEB). Microtubules invade the nuclear space and start to interact with chromosomes at the kinetochore, a specialized structure that is assembled over the centromeric DNA. During this stage, the mitotic spindle becomes fully organized. At metaphase, the bi-oriented chromosomes are aligned at the equator of the spindle, so that each sister kinetochore is attached to microtubules emanating from opposite poles. At anaphase, sister chromatids synchronously separate to opposite spindle poles, as the kinetochore microtubules get shorter (anaphase A) and the poles move apart (anaphase B). Normally, mitotic duration is defined as the minutes from NEB to anaphase onset, since abscission (the moment when the two daughter cells completely separate from each other) may

already occur in G1 of the next cell cycle. Lastly, during telophase, the nuclear envelope reforms around each set of chromosomes and there is separation on the cell cytoplasm (cytokinesis) (reviewed in Mitchison and Salmon, 2001; Morgan, 2007; Alberts et al., 2012).

However, from a regulatory point of view, mitosis can be divided into two major stages: early mitosis and late mitosis.

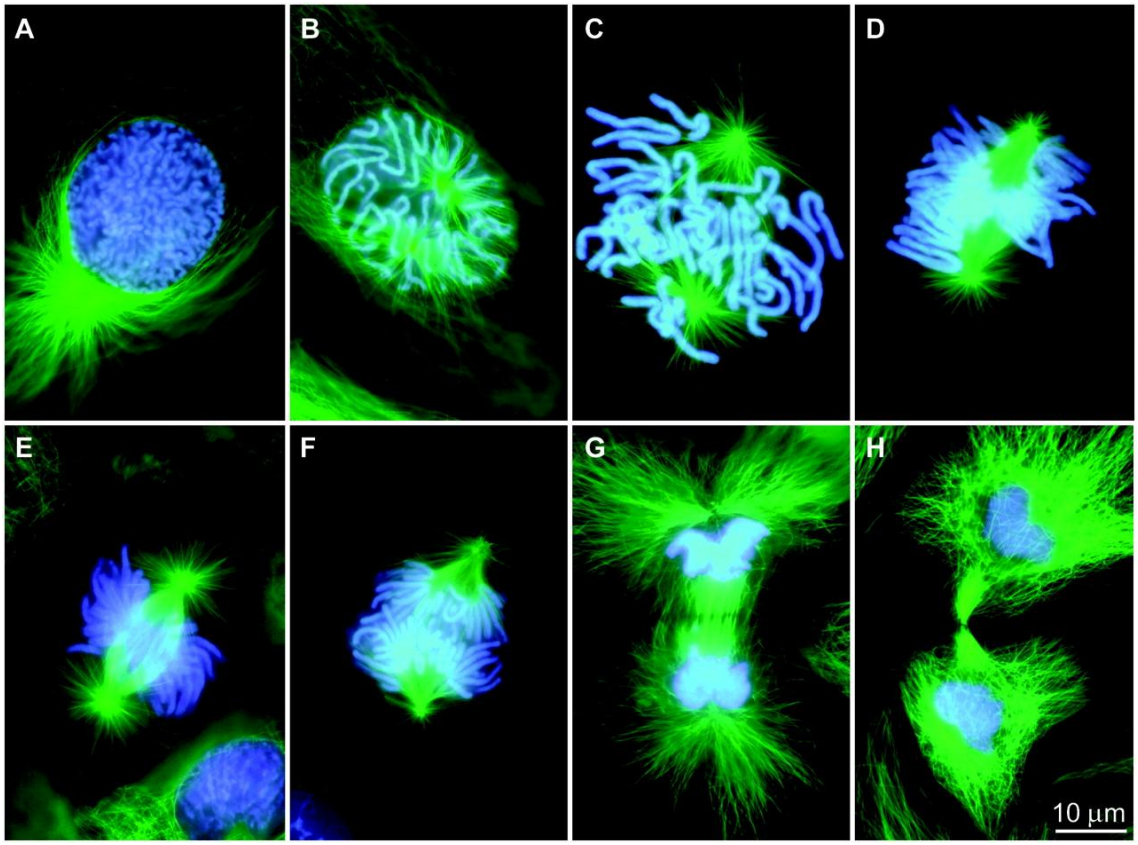

Figure 3 – Mitosis. Newt lung cells fixed and stained with DAPI (blue) and tubulin (green). Representative images of the different stages of mitosis. (A-B) Prophase. (C-D) Prometaphase. (E) Metaphase. (F) Anaphase. (G) Telophase. (H) Cytokinesis. (Rieder and Khodjakov, 2003)

I-1.3.1 Early mitosis

An abrupt increase in M-Cdk activity at the G2/M checkpoint triggers the events of early mitosis (prophase, prometaphase and metaphase). In vertebrates, Cdk1 and cyclin B1 (cycB1) are the M-Cdk complex and they are responsible for initiating chromosome condensation, NEB and the assembly of the mitotic spindle.

As the cell approaches mitosis, there is increased gene transcription of cyclin B1, leading to accumulation of Cdk1/CycB1 complexes. These complexes are phosphorylated at an activating site by Cdk-activating kinases (CAK), however,

Cdk1/CycB1 is kept inactive by phosphorylation of an inhibitory site by Wee1 and Myt1. The crucial event is the activation of the phosphatases Cdc25 that remove the inhibitory phosphate of Cdk1/CycB1. Cdc25B activity rises in late S-phase and in parallel with Cdk2/ Cyclin A, they activate a small amount of Cdk1/CycB1 that afterwards generates a positive feedback loop (Lindqvist et al., 2005). Active Cdk1/CycB1 activates Polo-like kinases (Plk) (Abrieu et al., 1998) that in one hand inhibit Myt1 and Wee1 and in the other hand activate CDC25A and –C that remove the inhibitory phosphates of Cdk1/CycB1 (Mailand et al., 2002, Morgan, 2007).

At the beginning of mitosis, Cdk1/CycB1 translocate to the nucleus, where it phosphorylates subunits of condensin I, stimulating the ability of condensin to supercoil DNA, and thus providing one mechanism by which M-Cdk initiates chromosome condensation (Kimura et al., 1998).

A fundamental early event of nuclear envelop breakdown seems to be the phosphorylation of some components of the nuclear pore complex, which cause its disassembly into smaller subcomplexes that disconnect from the envelope membranes. It is likely that Cdk1/CycB1 is responsible for this phosphorilations (Lénárt et al., 2003).

Although Cdks are master regulators of mitosis, they do not act alone. There are two major groups of mitotic kinases that are phosphorylated by Cdk1/CycB1 and promote mitotic progress: the Polo-like kinases and the Aurora kinases. Plks are involved in spindle assembly, kinetochore function and cytokinesis. Aurora A is implicated in spindle assembly and centrosome function, whereas Aurora B is involved in chromosome condensation, spindle assembly, attachment of kinetochores, sister-chromatid segregation and cytokinesis (reviewed in Morgan, 2007).

I-1.3.2 The Spindle Assembly Checkpoint

In most animal cells, after centrosome duplication and separation to opposite poles, rapidly growing and shrinking microtubules radiate from the centrosomes and search the space between the poles to bind to kinetochores, establishing the mitotic spindle (reviewed in Wittmann et al., 2001). Many sister-chromatid pairs are initially attached by this search-and-capture mechanism, however, anaphase must

wait until all chromosome are correctly bi-oriented in the spindle. This "wait anaphase signal" is executed by the SAC (Musacchio and Salmon, 2007).

The SAC principles are the following: in prometaphase, unattached kinetochores and Mps1 catalyze the assembly of the mitotic checkpoint complex (MCC), composed of BubR1, Bub3, Mad2 and Cdc20. The sequestering of Cdc20 inhibits the activation of the anaphase promoting complex, or cyclosome (APC/C), an E3 ubiquitin ligase that targets several proteins for proteolytic degradation. Once all chromosomes are aligned and their kinetochores attached to the spindle, the formation of the MCC stops and Cdc20 can activate the APC/C. This leads to ubiquitination and degradation of securin and cyclin B. Degradation of securin releases separase that in turn cleaves a subunit of the cohesin complex, a multimeric ring structure that encircles the replicated sister chromatids, allowing them to separate. In the meantime, degradation of cyclin B inactivates Cdk1 leading to mitotic exit (Musacchio and Salmon, 2007; Kops, 2008; Lara-Gonzalez et al., 2012).

Weak mitotic checkpoint activity is prevalent in human tumors (Weaver and Cleveland, 2006). Heterozygous mice for several mitotic checkpoint genes produce aneuploid cells with high frequency and they are more prone to tumor development as a consequence of chromosomal instability, particularly when mitotic checkpoint weakening is combined with frequent carcinogens or tumor suppressor gene mutations (reviewed in Baker et al., 2005; Kops et al., 2005).

I-1.3.3 Late mitosis

In contrast to the mechanisms of the mitotic entry, little is known about what controls the orderly exit from mitosis, especially in mammalian cells. However, inactivation of mitotic kinases, predominantly Cdk1/CycB1, due to cyclin B degradation and Cdk1 dephosphorilation, is required for spindle disassembly, daughter cell nuclei formation and cytokinesis (reviewed in Weiss, 2012). In budding yeast, mitotic exit control can be divided into four interconnected regulatory systems that are chronologically activated. The first to act is the APC/C – Cdc20 (Pines, 2011), which initiates degradation of the mitotic cyclins. The second and third regulatory systems to act are the Cdc14 early anaphase release (FEAR) pathway and the mitotic exit network (MEN). These pathways control Cdc14, which dephosphorylates mitotic Cdk substrates, and also directly regulate the actomyosin

ring contraction, which is essential for cytokinesis (Dumitrescu and Saunders, 2002). The last system, through the activation of the transcription factor Ace2, acts to promote expression of separation genes, the Ace2 and morphogenesis (RAM) network (Nelson et al., 2003).

I-2 Anti-mitotic Drugs

Nowadays, a major part of the chemotherapeutic anti-cancer drugs used in the clinics, either for younger or older patients, comprises agents that target the cell cycle in order to inhibit the excessive proliferation of cancer cells and to induce apoptosis or premature senescence (Lee and Schmitt, 2003). These anti-proliferative drugs can be sub classified into three groups: drugs that interfere with DNA synthesis; drugs that induce DNA damage; drugs that perturb the progression through mitosis. The last group have been recognized to be remarkably successful in the clinic (reviewed by Jordan and Wilson, 2004). Several therapeutic strategies that have been proposed for targeting mitosis in cancer treatment are described below.

I-2.1 Targeting the Spindle Assembly

I-2.1.1 Classical microtubule inhibitors

Microtubule inhibitors, also frequently called as classical spindle poisons, interfere with the microtubule dynamics by directly binding to tubulin subunits. The obstruction of this dynamics prevents the normal alignment of the chromosomes in the prometaphase/metaphase stage of mitosis, thus activating the SAC, that halts the cycle until all the chromosomes are correctly aligned in the spindle, which in turn mediates a mitotic arrest (Jordan and Wilson, 2004; Kaestner and Bastians, 2010). A prolonged mitotic arrest is, in the ideal circumstances, followed by the induction of apoptosis, as it will be discussed later.

Traditionally, spindle poisons are divided into two groups: agents that stabilize microtubules (such as taxanes and epothilones) and agents that destabilize microtubules (such as *Vinca* alkaloids and nocodazole).

Taxanes are drugs that bind to the taxane site in β-tubulin, but only when the monomer is integrated in a microtubule. This binding makes a conformational change on the structure of the polymer, which increases the affinity of the interaction among tubulin molecules, thereby reducing the depolymerisation (Nogales, 2000). Taxanes include different analogues of paclitaxel (Taxol ®) and docetacel (Taxotere ®). Paclitaxel was originally isolated from the bark of *Taxus brevifolia* (pacific yew tree), while docetacel was synthesized from a precursor isolated from the *Taxus baccata* (European yew tree) (Schmidt and Bastians, 2007).

Paclitaxel was first approved in 1992 for the treatment of ovarian cancer (Food and Drug Administration, 1992) and in 1994 was also approved for the treatment of breast cancer (Food and Drug Administration, 1994). Currently, paclitaxel is used at rather low concentrations as well for the treatment of non-small cell lung cancer, head and neck cancer, Kaposi's sarcoma and is in trial for numerous other tumors. Up to date, Taxol ® is the best-selling cancer drug ever manufactured (EORTC, 2014).

Epothilones and discodermolide also bind to the taxane site in β-tubulin and show microtubule stabilizing activity. Both are much more potent than taxanes, showing a strong antitumor activity *in vitro* and in clinical trials, having the advantage of still being active in taxane-resistant tumors (Larkin and Kaye, 2006). Epothilones were originally isolated from the myoxobacterium *Sorangium cellulosum* and discodermolide from the marine sponge *Discodermia dissolute* (Schmidt and Bastians, 2007).

Vinca alkaloids attach to the *Vinca* binding site, a region adjacent to the GTP-binding site of β-tubulin, at the plus-tip (Rai and Wolff, 1996). At a lower concentration, it inhibits microtubule dynamics without altering polymer levels, whereas at higher concentrations, it increases depolymerisation of the microtubule. In both circumstances, cells fail to complete a normal mitosis since the mitotic spindle formation is disturbed (Jordan et al., 1991). Various *Vinca* alkaloids were originally derived from the periwinkle plant *Vinca rosea*, also known as *Catharanthus roseus*, such as Vinblastine (Velban ®) and Vincristine (Oncovin ®), that were introduced to the clinic in the late 1950s for the treatment of leukemia's, lymphomas and Hodgkin's disease.

Nocodazole is a synthetic drug that binds at the interface between the α- and β-tubulin dimers. Similarly to Vinca alkaloids, the effect of nocodazole on microtubules is highly concentration-dependent. At high concentrations inhibits the polymerization, while low concentrations of inhibit microtubule dynamic instability, leading to mitotic arrest in both cases (De Brabander et al., 1976; Xu et al., 2002). This drug was originally identified in a chemical library screen for antitumor activity (reviewed by Park et al., 2012), but it is not currently used in the clinics due to its strong cytotoxic effects (National Cancer Institute, 2014).

Lastly, even though microtubule inhibitors have been recognized to be remarkably successful in the clinic, there are secondary side effects of the treatments. Microtubule poisons affect other cells as well, since resting and differentiated cells also need dynamic microtubules for the preservation of cytoskeletal functions and intracellular transport processes. This is the case of neuronal cells, where axonal transport of neurotransmitter vesicles is guided by microtubules. This neurotoxicity is displayed primarily by a motor and sensory polyneuropathy. Furthermore, myelosuppression is also observed, mainly neutropenia, the abnormally low number of neutrophils, because the proliferation rate of hematopoietic precursor cells is disturbed (Rowinsky et al., 1993). Myelosuppression is clinically manageable, but, by contrast, neurotoxicities can frequently cause permanent damage. In addition, patients treated with microtubule inhibitors frequently acquire resistance to it. Therefore, there is a need to develop new effective agents that should disrupt mitosis of abnormal cells, without interfering with microtubule dynamics in non-dividing cells, this way avoiding neuropathies (Gascoigne and Taylor, 2009).

I-2.1.2 New spindle targets

As described by Kaestner and Bastians, 2010, an ideal mitotic drug target should: (a) have a crucial role during mitosis and preferably no function in non-dividing cells; (b) its inhibition should rigorously abolish the progression through mitosis leading to the induction of apoptosis; (c) have a high specificity for cancer cells; (d) be druggable; and (e) offer robust diagnostic biomarkers (Kaestner and Bastians, 2010). Consequently, in recent years, many "next generation" spindle drug targets have risen to try to match some of these criteria, being the most studied kinesin Eg5, centromere-associated protein E (CENP-E), Aurora-A and -B kinases, and polo-like kinase 1 (Plk1).

Eg5 is a plus end-directed kinesin motor protein required for centrosome separation and assembly of a bipolar spindle (Wordeman, 2010). Inhibition of Eg5 activity produces monopolar spindles that leads to mitotic arrest, which terminates in apoptosis in a broad range of tumor cell lines, both *in vitro* and *in vivo* (Sakowicz et al., 2004; Tao et al., 2005). Also, cancer cells resistant to taxanes remain sensitive to Eg5 inhibition (Marcus et al., 2005). A number of Eg5 inhibitors have been

identified, and some of them have entered into clinical trials, such as Ispinesib, AZD4877, ARRY-520. Secondary side effects seem moderate, but the clinical efficacy of Eg5 inhibitors has been limited, with only few studies validating a partial response (Huszar et al., 2009).

Another plus end-directed kinesin motor protein that is a promising target is CENP-E, since no other role has been found for it outside of mitosis. CENP-E stabilizes the interactions between microtubules and kinetochores and regulates the activity of BubR1, a kinase involved in the SAC (Mao et al., 2003). Its complete inhibition leads to mitotic delay, associated with misaligned chromosomes and apoptosis (Wood et al., 2010). However, the main concern about the CENP-E is that there is some disagreement about the effects of only partial inhibition of this kinesin: some studies claim that partial inhibition has anti-tumor activity, while others say that it might bear the risk to induce aneuploidy, which in turn contributes to tumor growth (Weaver et al., 2007; Wood et al., 2010). Nevertheless, farnesyltransferase inhibitors that hamper CENP-E activity and specific small molecule inhibitors, such as GSK923295A, are currently in clinical trials (Huszar et al., 2009).

Mitotic kinases Aurora-A and -B are often overexpressed in human cancer and regularly correlate with poorer prognosis, making them attractive mitotic targets (Keen and Taylor, 2004). Inhibition of Aurora-A by siRNAs causes a mitotic arrest triggered by a monopolar spindle, that often results in the induction of cell death (Hata et al., 2005). Several compounds specific against these kinases have already entered clinical trials, such as MLN-8237 or MLN-8054 for Aurora A, and GSK1070916A or AZD1152 for Aurora B, presenting partial responses in specific tumors (de Castro et al., 2008).

Similar to Aurora-A, Plk1 is also involved in the mitotic entry, bipolar spindle assembly and mitotic exit, and is also frequently overexpressed in human cancers (Lens et al., 2010). Inhibition of Plk1 activity induces a SAC-dependent mitotic arrest as a result of monopolar spindle formation, followed by cell death in multiple cancer cell lines (Degenhardt and Lampkin, 2010). Inhibitors of Plk1 have already entered clinical trials, such as GSK461364, HMN-214 or ON01910 (de Castro et al. , 2008).

In conclusion, the list of potential proteins for mitotic targeting is far from being complete. Further efforts are now devoted to understanding the mechanisms

behind these inhibitions and therefore, its consequences on transformed and non-transformed cells.

I-2.2 Targeting other mitotic processes

The spindle assembly checkpoint is a crucial surveillance pathway and might represent a novel therapeutic target for cancer treatment. A partial downregulation of spindle checkpoint genes in human tumor cell lines causes chromosome missegregation leading to aneuploidy and drug resistance, but, a much more severe repression of it causes a massive missegregation that is associated with apoptosis. This is, the goal of this strategy is targeting the SAC to a level that is no longer compatible with cell survival (Kops et al., 2005). In line with this, genetic elimination of Mad2 or BubR1, kinases known to function in the SAC, outcomes in extensive chromosome missegregation that triggers programmed cell death (Kops et al., 2004). Also, chemical inhibition of Mps1 by a selective bioavailable MPS1 small-molecule inhibitor, has been evaluated as a good antitumor strategy in xenografted human tumors, reducing cancer cells proliferation, leaving normal cells almost unaffected (Colombo et al., 2010). Remarkably, cancer cell lines are more prone to suffer cell death in response to checkpoint disruption when compared with non-transformed cells. It is not clearly understood why, but it has been hypothesized that that predisposition might be a consequence of the extra chromosomes and cancer cells might need more time to correctly align them at mitosis, being thereby, more sensitive to die because of being more dependent on SAC activity (Manchado et al., 2012). However, there are major difficulties in reaching a complete inhibition *in vivo* of the SAC, and a drug combination approach could be the best therapeutic strategy, as it will be discussed later.

Cdc20 knockdown slowed cyclin B degradation, allowing more time for death initiation, and that was sufficient for inducing apoptosis in human cancer cells. Killing by Cdc20 knockdown did not demand checkpoint activity (Huang et al., 2009). Similarly, *in vivo* studies with genetically engineered mice, showed that deletion of Cdc20 allows efficient regression of aggressive tumors, while current anti-mitotic drugs exhibit partial effects (Manchado et al., 2010). But, with such promising results, the question of the selectivity of this strategy to distinguish between tumor and normal proliferating cells was raised. One possibility is that

APC/C–Cdc20 inhibitors could be beneficial in combination with other anti-mitotic drugs (Rieder and Medema, 2009). Further investigation will be necessary to identify compounds that could validate this therapeutic strategy in specific tumors *in vivo*/clinical trials.

Moreover, most non-transformed cells have two centrosomes that form bipolar spindles in mitosis, whereas cancer cells frequently have more than two, or "supernumerary" centrosomes. Such cancer cells accomplish bipolar division by clustering their centrosomes into two functional poles (Ganem et al., 2009). Inhibiting this process would lead to induction of multipolar spindles, thus massive chromosome segregation defects and a cancer-specific cell death. Such strategy would theoretically spare healthy cells, would specifically target cancer cells and overcome one limitation of current cancer treatments – selectivity (Kwon et al., 2008; Kawamura et al., 2013). One of the first targets identified as required for centrosome clustering was the mitotic kinesin KIFC1/HSET. Ablation of HSET induced multipolar spindles and cell death specifically in cells with supernumerary centrosomes (Kwon et al., 2008). Other novel small molecule inhibitors of centrosome clustering were identified and remain to be studied as efficient compounds *in vivo* (Kawamura et al., 2013).

I-3 Molecular determinants of cellular response to anti-mitotics

Although spindle poisons have been used in the clinics for many years and despite extensive research, the mechanisms by which these drugs lead to cell death are still not fully understood (reviewed by Rieder and Maiato, 2004; Gascoigne and Taylor, 2009; Janssen and Medema, 2011). As it has been mentioned, disturbance in the mitotic spindle caused by impaired microtubule dynamics, activates the SAC, inducing a prolonged mitotic arrest (Musacchio and Salmon, 2007), and ultimately a number of outcomes have been described to follow this chronic arrest (Rieder and Maiato, 2004, Weaver and Cleveland, 2005). It is crucial to understand the factors that dictate the different outcomes, or different fates, as it will be termed is this work, in order to predict which tumors would be likely to respond to this type of drugs and to increase their efficiency and decrease their side effects. Figure 4 illustrates the possible cell fates that a cell can undergo after a mitotic arrest.

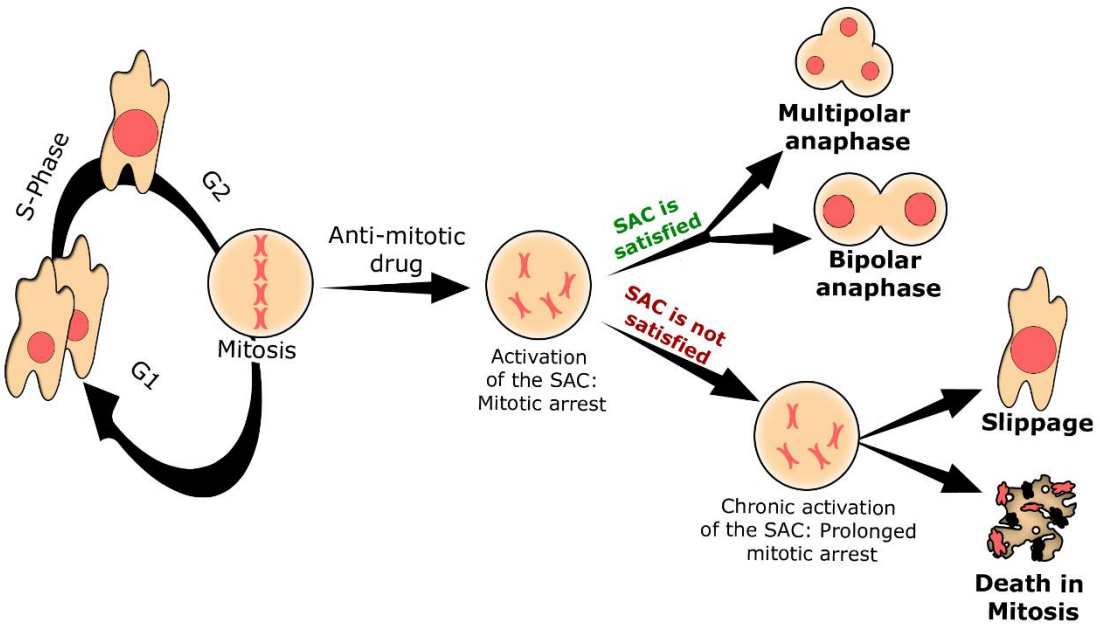

Figure 4 – Possible cell fates in response to anti-mitotics. When cells are in a state called mitotic arrest caused by impaired microtubule dynamics and SAC activation, they can undergo one of several fates. If cells are able to satisfy the SAC, the APC/C will degrade cyclin B1 and securin and a bipolar or multipolar anaphase will occur. If cells are not able to satisfy the SAC after several hours, they are in a state called prolong mitotic arrest that ultimately will lead to death in mitosis or mitotic slippage (an exit from mitosis without undergoing genome division).

If a mitotic cell is exposed to anti-mitotics that stabilize its microtubules, as for instance, low nanomolar concentrations of taxol, the SAC will prevent anaphase, as some chromosomes cannot align in the spindle correctly. However cells ultimately satisfy the SAC (Brito and Rieder, 2009). The APC/C will degrade cyclin B1 and securin and anaphase will occur. Although cells often initially assembled a bipolar spindle with low concentrations of taxol, extra spindle poles recurrently develop before anaphase onset, generating aneuploid daughter cells (Zasadil et al., 2014).

With high concentrations of anti-mitotics, that completely depolymerize all microtubules, (≥100 nM taxol, for example) mitotic cells are not able to satisfy the SAC after several hours and they are in a state called prolong mitotic arrest. This will ultimately lead to death in mitosis or mitotic slippage (an exit from mitosis without undergoing genome division) (Rieder and Maiato, 2004, Weaver and Cleveland, 2005).

Using a time-lapse light microscopy approach and 15 different cell lines, Gascoigne and Taylor (2009) developed a model to explain the molecular mechanisms that are responsible for the different cell behaviors in response to anti-mitotics. It seems that the decision to die in mitosis or to do a mitotic slippage is dictated by two independent processes during the arrest (figure 5): a slow, but progressive loss of cyclin B1 and a slow, but steady rise in cell death proteins activity. The fate of the cell is decided by which threshold is breached first. This is, if cyclin B1 levels fall below the mitotic-exit threshold first, slippage occurs. If the death threshold is breached first, then the cell dies in mitosis. Moreover, if a certain cell line, under specific concentrations of a specific anti-mitotic, is slippage-prone (this is, the predominant fate of that cell line is slippage), delaying mitotic slippage, by overexpressing cyclin B1, shifts the fate profile to death in mitosis. The other way around is also observed: if in a death-prone cell line death is delayed, by inhibiting caspase activation, as death in mitosis is caspase-dependent, the fate profile will shift to slippage. Also, it seems that cell fate is not necessarily genetically predetermined and the duration of the mitotic arrest does not dictate fate (Gascoigne and Taylor, 2009).

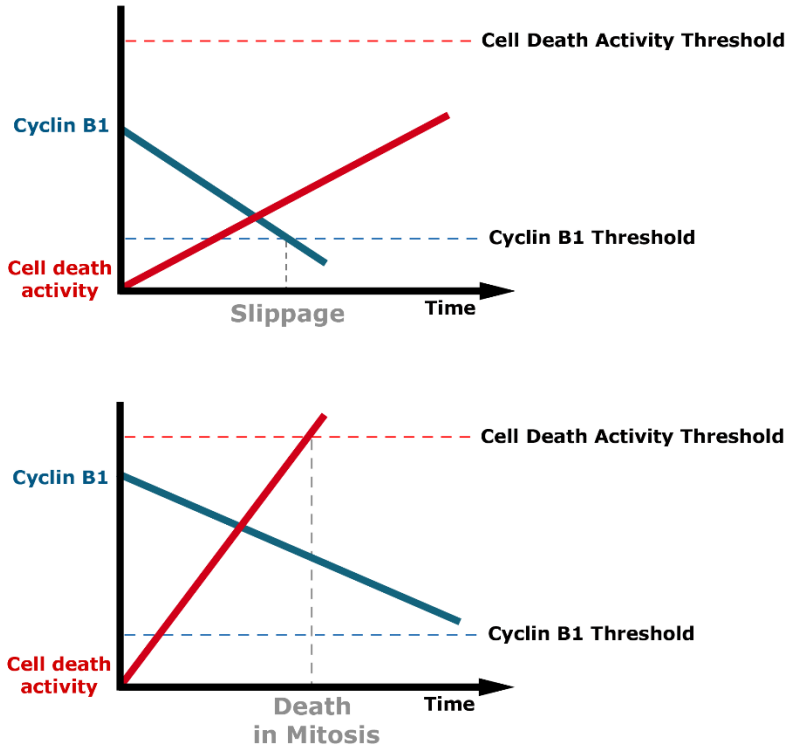

Figure 5 – The two competing networks model. After a chronic activation of the SAC and a prolonged mitotic arrest, the decision to die in mitosis or to slip mitosis is dictated by two independent processes during the arrest: a slow, but progressive loss of cyclin B1 and a slow, but steady rise in cell death proteins activity. The fate of the cell is decided by which threshold is breached first (Adapted from Gascoigne and Taylor, 2009).

However, this model does not account for recent evidence on the action of Cdk1/cyclin B1 on some members of protein families that directly control apoptosis, suggesting that the levels of cyclin B1 and the generation of mitotic death signals are not independent, but rather, are interdependent, figure 6 (Harley et al., 2010; Wertz et al., 2011; Sakurikar et al., 2012).

This key role of Cdk1/cyclin B1 appears at many levels. On one hand, phosphorylation of the anti-apoptotic protein Mcl-1 by Cdk1/cyclin B1 initiate its destruction by the proteasome after a mitotic delay, which in turn is necessary for mitochondrial apoptosis and caspase activation (Harley et al., 2010; Wertz et al., 2011). Moreover, it was showed that several death-prone cancer cell lines exhibited a robust Cdk1 activity and extensive Mcl-1 phosphorylation/degradation, while slippage-prone cell lines displayed weak Cdk1 activity and partial/transient Mcl-1 phosphorylation. Additionally, these events are linked with mitotic death and they are not detected in the circumstance of non-mitotic death, suggesting that this might be a "mitotic death signature" in cancer cell lines (Sakurikar et al., 2014).

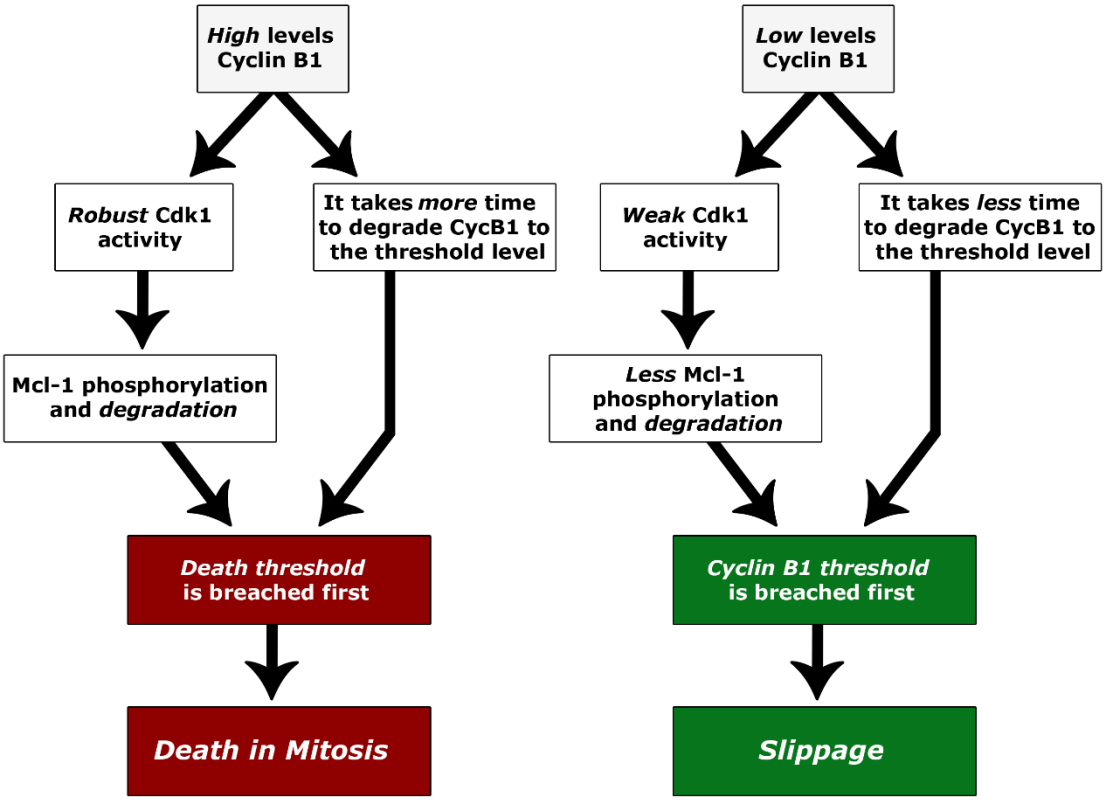

Figure 6 – The model for a dual role of Cdk1/cyclin B1 in the fate decision. After a chronic activation of the SAC and a prolonged mitotic arrest, the fate decision is influenced by the cyclin B1 levels. If there is high abundance of cycB1, it will take longer to degrade it to the threshold level and at the same time, there will be a robust Cdk1/cycB1 activity that will phosphorylate Mcl-1, marking it for ubiquitination and proteasomal degradation. Without this anti-apoptotic protein, pro-apoptotic signals will accumulate and the death threshold we be breached, causing death in mitosis. If there are low levels of cycB1, the opposite will happen, the cyclin B1 threshold will be breached first and mitotic slippage will follow (Adapted from Sakurikar et al., 2012).

On the other hand, during a normal mitosis, phosphorylation of caspase-9 by Cdk1/cyclin B1 restrains apoptosis (Allan and Clarke, 2007). Eventually, during a prolonged mitotic arrest, as cyclin B1 levels fall, caspase-9 phosphorylation rate decreases and it permit its activation.

It was suggested that there is a temporal series of events that chronologically eliminates the brakes on the apoptotic pathway throughout a prolonged mitotic arrest, (Harley et al., 2010), however, there is still a long way to go in understanding how the anti- and pro-apoptotic functions of Cdk1 signaling are combined and integrated in the fate decision after a mitotic arrest (Sakurikar et al., 2012). The existence of substantial crosstalk between the two pathways of slippage and death in mitosis raises the possibility that the fate decision may depend not only in which threshold is breached first, but also in the balance between both of them.

Concerning post-mitotic response, this is, the outcome of a 4N interphase cell that slipped mitosis, our understanding is much more obscure. There seems to be a complex system that is cell type-, anti-mitotic type- and time-dependent that regulates the fate of these tetraploid cells (Sakurikar et al., 2012). Nevertheless, these cells can die in interphase, arrest in interphase or continue with the cell cycle progression (Rieder and Maiato, 2004, Weaver and Cleveland, 2005). It remains to be clarified if the apoptosis is a result from the pro-apoptotic pathways activated during the mitotic arrest, or if it is a *de novo* signaling due to some impairment, as possibly DNA damage (Gascoigne and Taylor, 2009; Sakurikar et al., 2014). The cell cycle arrest it's also not fully understood, but it seems that it's p53-dependent (Demidenko et al., 2008). This interphase arrest might also lead to senescence, and senescence is strongly associated with ageing and cancer, as it will be addressed in the next topic.

The death of a cancer cell is the ideal fate of chemotherapy with anti-mitotics. However, recently, some researchers have been raising the question on if the mitotic death after anti-mitotic drug treatment is a clinically relevant mechanism. Zasadil and colleagues demonstrated that in primary breast tumors, taxol levels are at low concentration and in this condition, cells cannot sustain a long mitotic arrest and they proceed through mitosis and divide their chromosomes on multipolar spindles. This is, mitotic arrest is unessential for tumor regression in patients and cell death occurs in interphase due to massive chromosome missegregation not compatible with cell survival (Zasadil et al., 2014). Furthermore, Komlodi-Pasztor and colleagues pointed that human tumors have a slow doubling time, low mitotic index and that new generation of agents that specifically target mitosis have poor efficacy in clinical trials when compared to classical microtubule inhibitors. This suggests that the toxicity in the clinics of microtubule inhibitors such as taxanes might arise from their ability to inhibit interphase rather than mitotic microtubule function (Komlodi-Pasztor et al., 2012). Therefore, there is an imperative need of basic research to clarify the molecular determinants of cellular response to anti-mitotics and their relevance in the patient clinical context.

I-4 Ageing and Cancer

Ageing is a virtually universal characteristic of all biological organisms. Amongst multicellular organisms, ageing is an ongoing degeneration that arises at the molecular, cellular, tissue, and organismal levels. On the other hand, in organisms with renewable tissues, ageing might also be evidenced by gain-of-function alterations that allow cells to acquire an improperly high rate of proliferation, a hyperplasia, the most serious of which are cancers. Since, more than 80% of all human cancers are diagnosed after the age of 50 (Henry et al., 2011), ageing represents the single most important prognostic risk factor for many cancers (Campisi, 2013).

Therefore, there is this apparent paradox of how can ageing, characterized by reduced cell proliferation, can promote late-life cancer, characterized by uncontrolled cell proliferation.

I-4.1 Cellular senescence and the ageing process

Mounting evidence proclaims that ageing, and multiple pathologies associated with it, are at least partly linked to a common cellular stress response known as cellular senescence. Cellular senescence is the irreversible arrest of cell proliferation that happens when cells experience potentially oncogenic stress, such as dysfunctional telomeres, non-telomeric DNA damage, excessive mitogenic signals and non-genotoxic stress as perturbations in chromatin organization (Campisi and Fagagna, 2007).

There are two main potential pathways in which cellular senescence may contribute to the ageing process: (1) senescence decreases the number of self-renewing cells, thus compromising tissue regeneration; (2) through the powerful paracrine activities of senescence-associated secretory phenotype (SASP), cellular senescence might disrupt tissue organization, local inflammation and a tolerant microenvironment for the growth of a hyperplasia. Compromised tissue homeostasis and function derived from both pathways can ultimately lead to the ageing process (Chen et al, 2007).

This irreversible growth arrest evokes the idea that the senescence response developed at least in part to suppress the development of cancer (Sager, 1991). Besides the arrested growth, senescent cells display modifications in chromatin

organization and gene expression that leads to the SASP. The SASP include the secretion of numerous pro-inflammatory cytokines, chemokines, growth factors, and proteases (Chen et al, 2007). SASP likely evolved to stimulate tissue repair or regeneration in the case of injury, however, it can be either beneficial or deleterious, depending on the physiological context. It can create a permissive microenvironment for neoplastic growth, with factors for promoting angiogenesis, cell proliferation, chemotherapy resistance, epithelial-to-mesenchymal transition, and inflammation (Campisi, 2013). The findings pointing that senescent cells can fuel tumor growth are very paradoxal since, after all, cells enter the senescent state to avoid the division of damaged cells, which is a key risk factor for cancer development (Campisi, 2013). One possible explanation is that senescent cells are advantageous when present only transiently. For example, in the skin, temporary senescent cells stimulate ideal wound healing (Jun and Lau, 2010) but, when chronically present, they may induce phenotypes related with skin ageing (Velarde et al., 2012). Further research is required to outline the role of the chronological time and tissue microenvironment in the functions of senescent cells.

I-4.2 Models linking ageing and tumorigenesis

Regarding the question of why cancer occurs more regularly with advancing age, the most recognized justification is that accumulation of oncogenic mutations with age should facilitate cancer evolution (Peto, 2001). However, a rising amount of evidence suggests that the fitness effects of oncogenic alterations are highly context dependent and that Darwinian evolution selection is a more correct representation of somatic evolution of cancers rather than linear step accumulation models (Greaves, 2010).

Henry et al., 2011, proposed an Adaptive Oncogenesis Hypothesis, where healthy populations of young cells hold inherent high fitness that allows its adaptive selection. But, with ageing, cellular fitness declines due to age-related alterations in the microenvironment, such as the SASP, leading to an increased selection for certain mutations/epimutations that are adaptive, this is, that improve fitness in that context allowing that cell's a higher proliferation (figure 7). For example, the gain-of-function of certain oncogenes in an aged background would be adaptive by either improving or avoiding deficiencies in old cells. Also, any oncogene mutation

that would turn an old cell more resistant to ageing-associated insults would be selected in this context.

A more complete comprehension of how age-associated alterations in cells and tissues induce on carcinogenesis is needed since it will contribute to the improvement of more valuable therapeutic and prevention approaches for cancer (Henry et al., 2011).

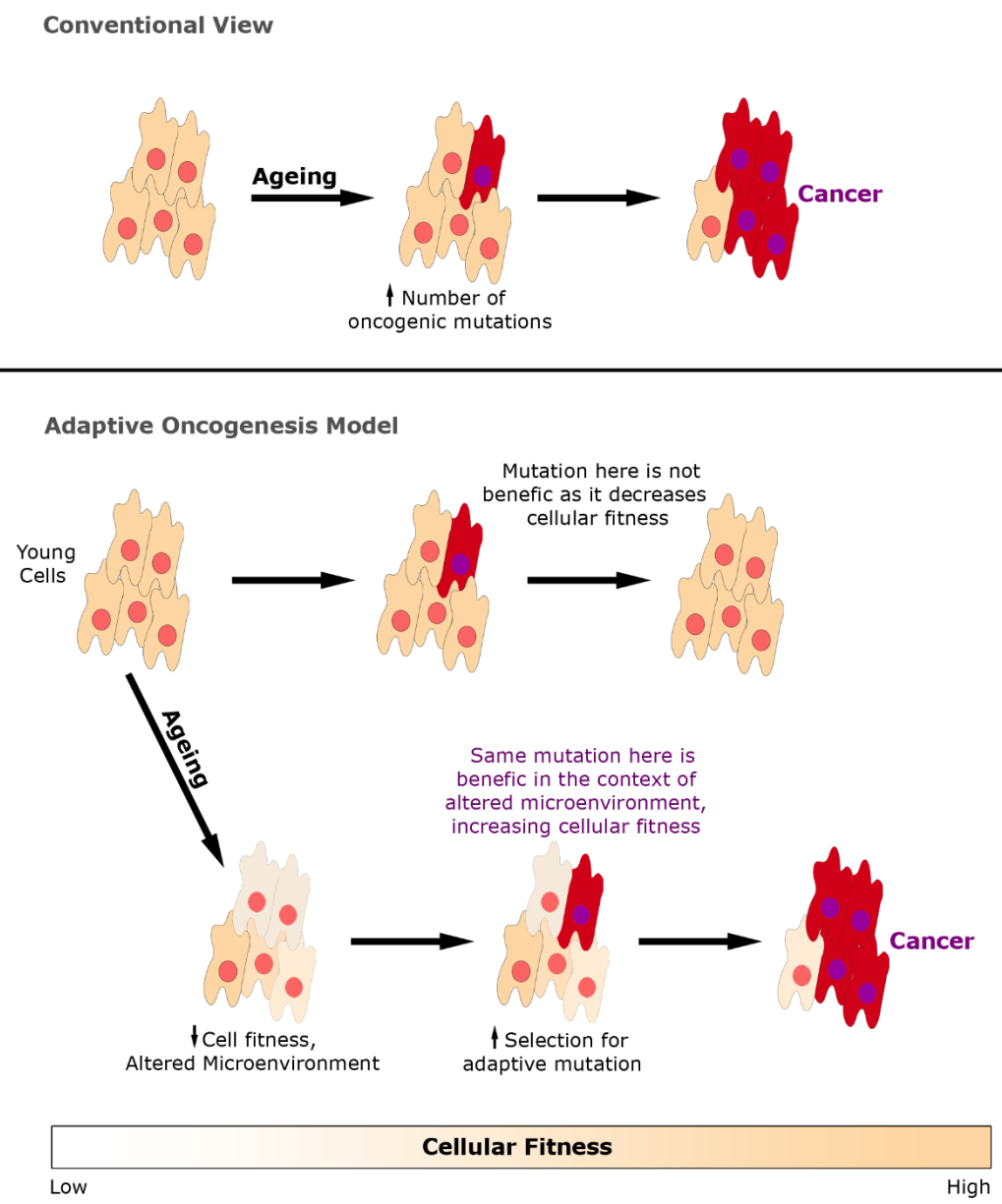

Figure 7 - Conventional and Adaptive Oncogenesis Models for Tumorigenesis. Healthy populations of young cells hold inherent high fitness that allows its adaptive selection. On the other hand, with ageing, cellular fitness drops due to alterations in the microenvironment, leading to an increased selection for certain mutations/epimutations that are adaptive (adapted from Henry et al., 2011).

I-4.3 Downregulation of cell cycle genes during ageing

It seems that neither in chemotherapy strategies nor in basic mechanism research, the cellular chronological age and its implications are not fully being taken in account. As described, ageing is characterized by a progressive loss of cell proliferation. In 1966, Jacobs and Brown linked ageing to aneuploidy (Jacobs and Brown, 1966). Studies with fibroblasts isolated from young, middle-age, and old-age humans and humans with progeria (a syndrome of premature ageing), showed that several mitotic genes were downregulated in human aged cells, such as cyclins –A and -B, Plk1, Cdc20, CENP-A, CENP-F, MKLP1, Eg5 and MCAK (Ly et al., 2000). Therefore, this downregulation might result in the induction of different cell fates in response to anti-mitotics, as it will be addressed in this project. Interestingly, many of these mitotic genes were shown to be direct downstream targets of a transcription factor, Forkhead Box M1 or FoxM1 (Laoukili et al., 2005). FoxM1 has also been recognized as one of the most upregulated genes in tumors (Pilarsky et al., 2004).

Both ageing and cancer are greatly complex and their causes are unquestionably multifactorial, so there is no surprise that the underlying links between them will also need to be multifaceted. However, it seems that FoxM1 fits this complex duality, making it a good candidate gene for the study of ageing, cancer and mitosis, as it will be approached in this work.

I-5 FoxM1

Transcription factor Forkhead Box M1 (FoxM1) belongs to the wide-ranging family of Forkhead transcription factors, whose members are characterized by an evolutionarily conserved DNA binding domain called Forkhead or winged-helix domain (reviewed by Laoukili et al., 2007). Forkhead family members are tangled in an extensive range of biological processes comprising embryogenesis, proliferation, differentiation, apoptosis, tumorigenesis, longevity and metabolic homeostasis (Kalin et al., 2011).

The human FoxM1 is a 10-exon structure covering approximately 25 kb on the 12p13.3. Human FoxM1 is alternatively spliced in three different variants, which arise from the same gene through differential splicing of the two facultative exons A1 and A2. FoxM1a comprises exon A1 and exon A2; FoxM1b lacks exon A1 and exon A2; and FOXM1c contains exon A1, but lacks exon A2. Both FoxM1b and -c are transcriptionally active, while FoxM1a is inactive, due to having exon A2 in the C-terminal transactivation domain (Yao et al., 1997). FoxM1b appears to display a higher DNA-binding affinity than FoxM1c (Hegde et al., 2011). Since FoxM1a can act as a dominant-negative variant, the alternative splicing may consequently act to modulate the overall FoxM1 activity in certain situations, most likely due to competition between the splice variants for binding to the FoxM1-responsive elements (Laoukili et al., 2007).

I-5.1 Cellular functions of FoxM1

I-5.1.1 FoxM1 is a key cell cycle regulator

FoxM1 expression correlates with the proliferative state of the cell. It is expressed in all embryonic tissues, principally in proliferating epithelial and mesenchymal cells. In adult tissues, FoxM1 expression is narrowed to actively dividing cells. On the other hand, its expression is abolished in resting or terminally differentiated cells. Also, FoxM1 mRNA and protein levels are hardly detectable in quiescent cells, but both are upregulated in the late G1-phase of the cell cycle and continue during the G2 and M phases (Korver et al., 1997; Laoukili et al., 2007).

FoxM1 transcriptional activity is kept low during G1/S through the action of its N-terminal autoinhibitory domain (Laoukili et al., 2008). Afterwards, the transcriptional activity positively correlates with its phosphorylation level. Both

progressively rise as cells advance through the cell cycle achieving maximum levels at the G2/M transition. FoxM1 is firstly phosphorylated in late G1 and then further phosphorylated in a sequential order by multiple protein kinases, such as Cdk2, Cdk1, MAPK and Plk-1 (Laoukili et al., 2007; Chen et al., 2009). In the C-terminal region of the FoxM1 protein there is a LXL docking sequence for the cyclins binding, that permits efficient phosphorylation by the Cdk's. This type of phosphorylation seems to be essential to the recruitment of a transcriptional co-activator, the histone deacetylase p300/CREB binding protein (p300/CBR) (Major et al., 2004). The MAPK pathway appears to play an important role in the link between mitogenic signals and FoxM1 activation. This pathway mediates FoxM1 phosphorylation and nuclear translocation, mediating this way its transcription activation. Inhibition of the MAPK pathway caused a solid decline in the transcription of the genes dependent in FoxM1 (Ma et al., 2005). Moreover, the Cdk inhibitor $p27^{Kip1}$ and $p19^{ARF}$ can both, individually, associate with FoxM1 and inhibit its activity (Laoukili et al., 2007).

Additionally, FoxM1 is involved in a positive auto-regulatory loop, where FoxM1 activation promotes its own transcription by binding to its own promoter (Halasi and Gartel, 2009).

Concerning, FoxM1 downstream targets, this protein regulates the expression of over 220 genes, in which 80 genes are direct target genes (Wierstra, 2013). Consistent with the fact that FoxM1 expression correlates with the proliferative state of the cell, it was uncovered a network of genes intimately involved in cell cycle progression that are under the direct transcriptional regulation of FoxM1 (Laoukili et al., 2005). In overview, FoxM1 induces expression of cyclin A2, JNK1, ATF2 and Cdc25A phosphatase, all of which are crucial for G1/S transition and DNA replication. It also induces transcription of Skp2 and Cks1 which encode subunits of the Skp/Cullin-1/F-box (SCF) ubiquitin ligase complex, which is necessary for proteolysis of Cdk2 inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ through the G1 phase. Mitotic entry demands activation of Cdk1 through the elimination of inhibitory phosphates by the Cdc25B and Cdc25C phosphatases. Both Cdc25B and cyclin B1 are direct transcriptional targets of FoxM1. In addition, FoxM1 is a transcriptional activator of various genes critical for chromosome segregation and cytokinesis, such as Aurora B, Plk-1, Survivin, CENP-A, -B and -F isoforms (Kalin et al., 2011).

This crucial cell cycle control function of FoxM1 is confirmed by depletion studies with FoxM1-deficient MEFs (mouse embryonic fibroblasts) as well as in FoxM1-depleted human cancer cells with RNA interference (RNAi). The inhibition of FoxM1-dependent gene expression caused a large diversity of cell cycle defects, associated with premature cellular senescence and/or cell death, as severe delay in mitotic entry, chromosome missegregation, aneuploidy (Laoukili et al., 2005), mitotic spindle aberrations, centrosome amplification (Schüller et al., 2007) and endoreduplication (Korver et al., 1997) .

Therefore, it became clear that FoxM1 plays a key role in leading cell cycle progression and genomic integrity, being irrevocably related to its function as a transcriptional regulator of the mammalian G2/M-specific gene cluster (Laoukili et al., 2007).

I-5.1.2 FoxM1 and organogenesis/tissue regeneration

Complete deletion of FoxM1 is embryonic lethal due to defects in the organogenesis of major organs, while heterozygous appear to be normal (Korver et al., 1998). Histological analysis of the FoxM1−/− mouse embryos showed abnormalities in the heart and liver, with 50-fold and 6-fold increased polyploidy in cardiomyocytes and hepatocytes, respectively, suggesting that FoxM1 is required to prevent premature endoreplication *in vivo*, coordinating the S- and M-phase (Korver et al., 1998; Krupczak-Hollis et al., 2004). Similarly, FoxM1 knockout mouse embryos also displayed reduced hepatoblast proliferation, decreased liver perfusion and a lack of intrahepatic bile ducts, proposing that FoxM1 is crucial for hepatoblast proliferation and differentiation toward biliary epithelial cell lineages (Krupczak-Hollis et al., 2004). Pulmonary defects in these FoxM1−/− mice were also reported which are likely due to lack of proliferation of cells of the embryonic pulmonary mesenchyme origin (Kim et al., 2005). Equally, FoxM1 expression pattern in mouse embryos seems to be much broader. FoxM1 staining was also detected in hair follicles, follicles of vibrissae and keratinocytes of basal layers, developing kidney, cartilage, epithelial and mesenchymal cells of pancreas and stomach, nasal cavity, tongue, tooth primordium, salivary glands, muscles and the Rathke's pouch (Kalin et al., 2011). These data implies that FoxM1 plays a role in proliferation and differentiation of progenitor cells in several tissues/cell types.

In line with this, FoxM1 expression is re-activated after organ injury for tissue regeneration, in some tissue types. In induced lung injury, FoxM1 protein was found in pulmonary epithelial, endothelial and smooth muscle cells (Kalinichenko et al., 2003). Following partial hepatectomy or pancreatectomy, FoxM1 expression was induced in hepatocytes and pancreatic endocrine cells, respectively (Ye et al., 1999; Misfeldt et al., 2008). However, in non-regenerating hepatocytes, induced expression of a FoxM1 transgene does not cause abnormal proliferation, implying that FoxM1 expression is important for tissue regeneration and repair, but it need to be properly activated by mitogenic cues (Laoukili et al., 2007).

I-5.2 FoxM1 and ageing

Elderly transgenic mice with induced overexpression of FoxM1 showed an improved hepatocyte proliferation rate after partial hepatectomy, with similar results to young mice controls (Wang et al., 2001). Consistently with this, a conditional FoxM1 knock-out in the liver of adult mice led to a significant reduction in hepatocyte proliferation after partial hepatectomy (Wang et al., 2002).

Moreover, two major cell cycle inhibitors, p16Ink4a and p19Arf, which are both encoded at the Ink4/Arf locus, were reported to be the main mechanism to induce cellular senescence, through pRb and p53, respectively (Serrano et al. , 1997). Their relevance in ageing is even more remarkable, as the levels of p16Ink4a and p19Arf strongly correlate with the chronological age of all tissues analyzed both in mice and humans, being the most robust biomarker of mammalian aging up to date (Krishnamurthy et al., 2004). Interestingly, FoxM1 is an inhibitory target of p19Arf (Kalinichenko et al., 2004), pRB (Wierstra and Alves, 2006) and p53 (Pandit et al., 2009) tumor suppressors.

Furthermore, in *C. elegans*, the MAPK signaling pathway was significantly down-regulated from mid-to-late adulthood stages (He et al., 2014). Concordantly, MAPK pathway employs its G2/M regulatory effect via FoxM1 (Ma et al., 2005).

It was also been described that SUMOylation actively contributes to the execution of the senescence program (Bischof and Dejean, 2007) and experiments with rodent spleen tissue have shown that there is an increase of the general amount of SUMOylated proteins (Zhang et al., 2007). Recently, it was shown that in response

to some cytotoxic drugs, SUMOylation attenuates FoxM1 activity and causes mitotic delay (Myatt et al., 2014).

Much more research is needed since we are only starting to understand the importance of FoxM1 in ageing. It is true that induced expression of FoxM1 can revert some of the proliferative deficiencies that come with age, but it remains to be seen if the overall age can be affected (Laoukili et al., 2007).

I-5.3 FoxM1 and cancer

Genome-wide gene expression profiling of cancers has consistently recognized FoxM1 as one of the most upregulated genes in tumors (Pilarsky et al., 2004). Commonly, increased expression of FoxM1 in tumors strongly correlates with poor prognosis, suggesting that FoxM1 could be a novel prognostic marker (Liu et al., 2006; Bektas et al., 2008).

Studies with xenograft models have provided valuable information about the role of FoxM1 in cancer. Overexpression of FoxM1 in human cancer cell lines improved their tumorigenicity in xenograft models. Additionally, FoxM1 suppression inhibited xenograft tumor development of human cancer cells in nude mice (Li et al., 2009). Equally, *in vitro* knockdown with RNAi in human cancer cells declined proliferation, migration, invasion, angiogenic capacity, and anchorage independent growth (Dai et al., 2007; Zhang et al., 2008), suggesting that FoxM1 is involved in several steps of tumorigenesis, such us tumor initiation, progression and metastasis. However, it still remains to be addressed if FoxM1 is universally required for oncogenesis (Halasi and Gartel, 2013).

Regarding the increased expression and activity of FoxM1 in cancer, the following mechanisms are currently proposed: (a) amplification of the FoxM1 locus (Korver et al., 1997); (b) increased stability or activity of FoxM1 via the interaction with different proteins or by different modifications; (c) higher transcription of the FoxM1 gene by other transcription factors that directly bind to the FoxM1 promoter and stimulate its expression, such as E2F (Julie Millour et al., 2011) and c-Myc (Blanco-Bose et al., 2008); (d) mutations on tumor suppressor as p53, pRB and $p19^{ARF}$ that negatively regulate FoxM1 expression (Laoukili et al., 2007; Pandit et al., 2009); (e) activation of FoxM1 by oncogenic signaling pathways, mainly PI3/Akt and Raf/MEK/MAPK pathways (Wang et al., 2010).

I-5.3.1 FoxM1 contributes to multiple hallmarks of cancer

In 2000, Hanahan and Weinberg proposed the hallmarks of cancer, which comprise six biological capabilities acquired during the multistep development of human tumors. They include sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis (Hanahan and Weinberg, 2000). Eleven years later, both authors published an update to this hallmarks of cancer, proposing two additional hallmarks (deregulating energy metabolism and evading immune destruction) and also clarifying that there are enabling characteristics underlying the hallmarks, as genomic instability and inflammation (Hanahan and Weinberg, 2011). In recent years, experimental data has emphasized the relevance of FoxM1 in diverse cellular processes and demonstrated that it is involved in multiple hallmarks of cancer (figure 8). In the context of this work, three hallmarks will be specially addressed: excessive proliferation, apoptosis resistance, replicative immortality and genomic instability.

The most basic characteristic of cancer cells is their unlimited ability to proliferate, therefore since FoxM1 is a key cell cycle regulator, there is no surprise that this oncogenic transcription factor is intimately related to the sustaining proliferative signaling of cancer cells. Besides the role of FoxM1 in cell cycle progression that were already discussed in the subsection I-5.1.1, there are several other targets in which it interferes. FoxM1 directly binds to the promoter and stimulates the transcription of several membrane receptors that promote cell proliferation, such as ERα (estrogen receptor α) (Millour et al., 2010) and HER2 (human epidermal growth factor receptor-2) (Francis et al., 2009). FoxM1 was also found to be a downstream target of these receptors, and so, the positive feedback loop is expected to have a crucial role in breast cancer expansion, since it increases the mitogenic effects of growth factors. Furthermore, overexpression of FoxM1 antagonizes the anti-proliferative effects of ERβ1 (Horimoto et al., 2011). TOPO-2α (topoisomerase-2α) is another direct target of FoxM1 and its increased expression correlates with overexpression of FoxM1 in lung tumors, thus contributing to proliferation of cancer cells (Wang et al., 2009).

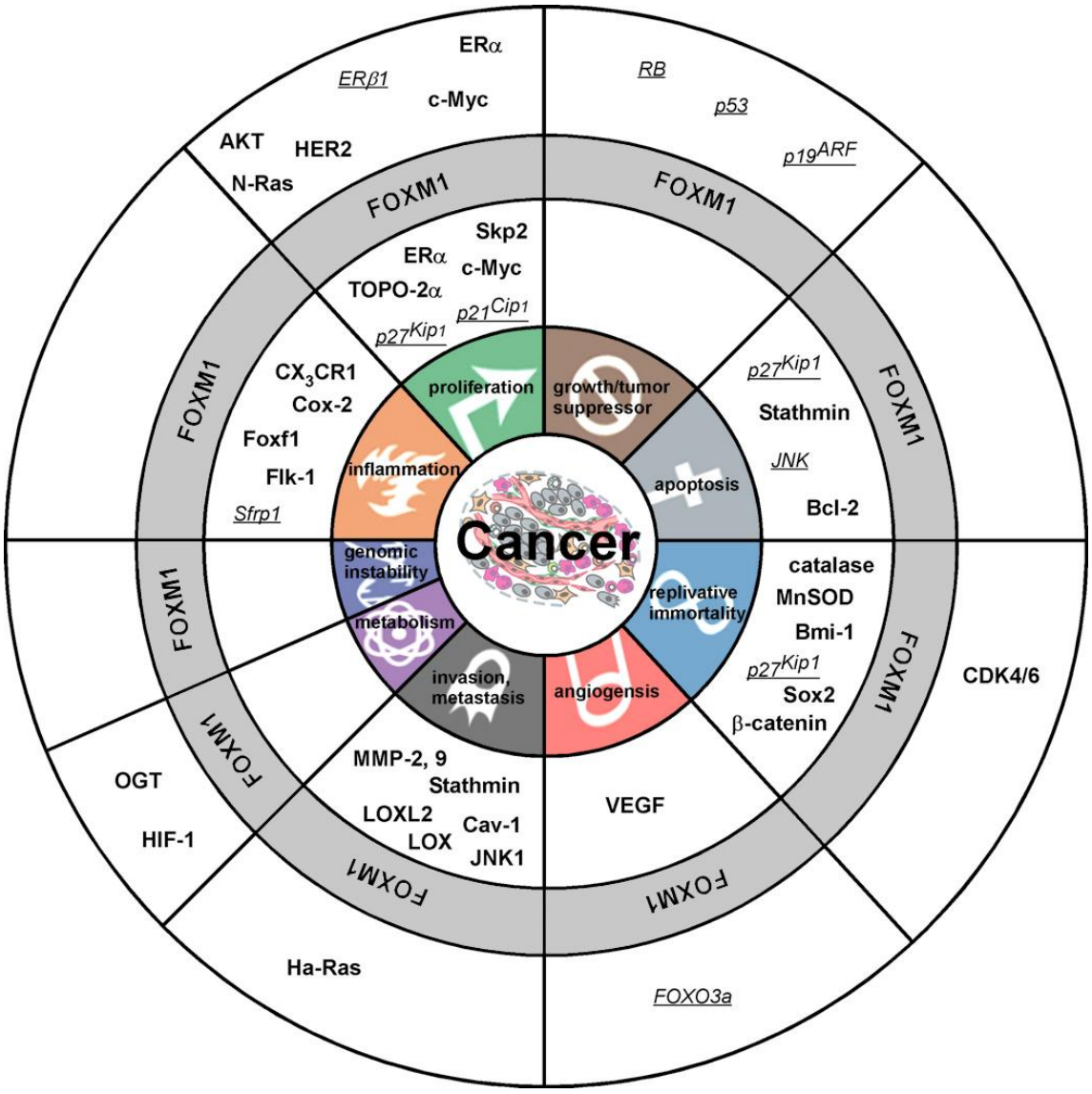

Figure 8 - FoxM1 is involved in multiple hallmarks of cancer. The scheme illustrates the involvement of FoxM1 in multiple hallmarks of cancer. Negative (in italics and underlined) and positive (in bold) downstream targets (inner circle) or upstream regulators (outer circle) of FoxM1 (Adapted from Hanahan and Weinberg, 2011 and Halasi and Gartel, 2013).

Interestingly, c-Myc, that itself is a regulator of proliferation, differentiation and cell death, was found to be both a direct transcriptional target and an upstream regulator of FoxM1 which suggest that there is a positive feedback loop that possibly strengthens and maintains the expression of c-Myc and FoxM1 target genes in order to stimulate constant proliferation (Blanco-Bose et al., 2008). In addition, increased activity of the kinases AKT and N-Ras in the mouse liver radically enhanced the development of liver tumors due to elevated proliferation and angiogenesis, mainly through a FoxM1-dependent pathway (Ho et al., 2012). Also, MAPK pathway, which

is commonly deregulated in cancer cells, exerts its G2/M regulatory effect via FoxM1 (Ma et al., 2005).

Another characteristic of FoxM1 is that it increases resistance of cancer cells to apoptosis, however the downstream effectors that mediate that mechanism have just started being uncovered. Nevertheless, studies in breast cancer cells overexpressing FoxM1 point to a role in $p27^{Kip1}$ and JNK downregulation and Stathmin and Bcl-2 upregulation as involved in potential underlying mechanisms (Carr et al., 2010; Halasi and Gartel, 2012). Recently, Mcl-1 was also proven to be a direct target of FoxM1 (Hu et al., 2015).

Avoiding cellular senescence could promote infinite replicative capacity leading to cancer expansion. FoxM1-deficient MEF's displayed some characteristics of premature senescence, such as high levels of β-galactosidase, high nuclear levels of $p19^{ARF}$ and $p16^{INK4A}$, suggesting that FoxM1 might protect against senescence (Wang et al., 2005). Moreover, FoxM1 knockdown in gastric cancer cells increased senescence, partially facilitated by $p27^{Kip1}$ (Zeng et al., 2009). Via direct activation of antioxidant enzymes transcription, catalase and MnSOD, FoxM1 protected cancer cells from oxidative stress-induced premature senescence by dropping ROS levels (Park et al., 2009). Recently, FoxM1 has also been pointed out to promote stem cell-like properties (constant proliferation, immortality and the capacity for self-renewal) through the direct interaction with β-catenin (Zhang et al., 2011) and direct regulation of the expression of pluripotency gene Sox2 (Wang et al., 2011).

Genomic instability is the driving force that fuels cancer development, since it allows cancer cells to gain advantageous genetic alterations that facilitate tumor progression. As discussed before, inhibition of FoxM1-dependent gene expression was proven crucial for an appropriate chromosomal segregation and genomic stability (Laoukili et al., 2005; Kalin et al., 2011). However, in most cancers, FoxM1 is upregulated. To explain this apparent paradox it was proposed that even though FoxM1 is involved in DNA repair via direct transcriptional regulation of XRCC1 and BRCA2 DNA repair genes, it seems that its anomalous upregulation expression rather assistances the acquirement of genomic instability than increases DNA repair (The et al., 2010).

I-5.3.2 Targeting FoxM1

Without doubt, the significant and diverse role of FoxM1 in oncogenesis validates its importance for a mono- or combination therapeutic intervention (Halasi and Gartel, 2013b).

Ablation of FoxM1 by RNAi decreased proliferation of many cancer cell lines. Yet, *in vivo*, there are major difficulties in the delivery of anti-FoxM1 RNAi. Some strategies arise to overcome this issue, such us direct intratumoral injection of PEI-encapsulated FoxM1-specific small interference RNA (siRNA) or intravenous injection of nanoparticle-encapsulated anti-FoxM1 siRNA (Halasi and Gartel, 2013b).

Proteasome inhibitors and thiazole antibiotics, such as Siomycin A and Thiostrepton are potent FoxM1 inhibitors, not only inhibiting the transcriptional activity, but also downregulating the mRNA and protein FoxM1 levels, because of the positive autoregulatory loop of this transcription factor (Bhat et al., 2009). Both drugs also revealed encouraging anti-cancer properties since they showed specificity toward transformed cells compared to non-transformed cells (Radhakrishnan et al., 2006). Other proteasome inhibitors, as Bortezomib or MG132 or MG115, also mediated apoptosis associated with the suppression of FoxM1 (Pandit and Gartel, 2010). Bortezomib is in clinical practice and several new proteasome inhibitors are in clinical trials (Kisselev et al., 2012).

Nevertheless, emerging data proposes that targeting cancer by combination treatment offers a more effective therapeutic approach, as also discussed for the combinatorial therapy with anti-mitotic drugs. Combination therapy can improve treatment efficiency, by reducing the adverse side effects of chemotherapy and overcoming possible drug resistance by merging agents with distinct mechanisms of action (Halasi and Gartel, 2013b). For example, FoxM1 suppression by RNAi sensitized human cancer cells to apoptotic cell death induced by proteasome inhibitors, including Thiostrepton, MG132 and Bortezomib (Pandit and Gartel, 2011). Also, Curcumin in combination with Bortezomib synergistically induced apoptosis in cancer cells, suggesting that a combination of proteasome inhibitors could lead to stronger apoptotic effects (Park et al., 2008). Furthermore, FoxM1 proteasome inhibitors in combination with DNA-damaging agents powerfully induced apoptosis in pancreatic and breast cancer cells (Halasi and Gartel, 2012).

All the research outcomes point to the fact that this single oncogene might hold the key to a substantial progress in cancer treatment, either alone or in combination strategy.

I-5.3.3 Drug combination approaches

Although single-agent therapy may play a role in a few selected cases, most current cancer treatments are combinations of chemotherapeutic agents. It is expected that future strategies should comprise combination treatments designed to deal with the crucial issue of drug resistance, together with rational approaches to patient selection. Moreover, the potential for combination with other drugs may enhance their cytotoxicity towards cancer cells while reducing secondary effects (Jackson et al., 2007).

These two are the main objectives of combinatorial therapy, avoiding resistance and the secondary side effects, but there are other challenges, like achieving the *in vitro* results *in vivo*. For instance, there are major difficulties in reaching a complete inhibition *in vivo* of the SAC, but a partial reduction of essential mitotic checkpoint components in combination with sub-lethal doses of microtubule poisons, such as taxol, boosted the amount and severity of chromosome segregation errors, causing selective cell death of tumor cells. That could be an effective anti-tumor approach in clinical settings (Janssen et al., 2009). Similarly, drugs that prevent mitotic slippage may well prove superior to other drug-based strategies (Huang et al., 2009), but alone, they might not be selective for cancer cells. Since compared to non-transformed cells, tumor cell lines seem more predisposed to die in mitosis in response to spindle poisons, a combination of spindle poisons with drugs that block mitotic exit could theoretically offer appropriate tumor selectivity (Rieder and Medema, 2009).

Moreover, combination of anti-mitotic drugs with other drugs that restore the activity of tumor suppressors or inhibit oncogenes might also provide a capable strategy for combating relapse and metastasis of cancer (Louwen and Yuan, 2013).

Recently, FoxM1 depletion was shown to sensitize breast cancer cells and mouse embryonic fibroblasts into entering taxol-induced senescence, with the loss of clonogenic ability, and the induction of senescence-associated β-galactosidase activity (Khongkow et al., 2015).

It is clear that we are arriving a new era in anti-mitotic therapy with the identification and clinical translation of new targets in mitosis, as FoxM1. There is the major necessity of refining our knowledge of the main links between disrupting key components of the mitotic machinery and the apoptotic pathway. Understanding what underlies beneath this complexity will ultimately boost the advancement of cancer treatment (Jackson et al., 2007).

I-6 Project outline

Our work stands on the idea that cellular chronological ageing research might be helpful in the improvement of chemotherapy with anti-mitotics.

We observed that FoxM1 and SAC genes are downregulated in mitotic old cells (our unpublished data), raising the question whether the robustness of the spindle assembly checkpoint function is compromised and changes the cell fate in response to anti-mitotics. To address this problem, we used long-term phase-contrast live cell imaging to observe individual cell behavior in response to anti-mitotic drugs. Preliminary data in our lab showed that independently of the type of anti-mitotic drug used, old cells are more prone to die in mitosis than young cells, suggesting that there is an increased sensibility to anti-mitotics in aged cells.

Consequently, the first question to address in this project was whether FoxM1 downregulation accounts for the increased sensibility to anti-mitotics in old cells. To answer this question, we used time-lapse microscopy to access the cell fate of young (10 years) and old (87 years) fibroblasts following drug treatment. Then, using siRNA, we knocked-down FoxM1 in young cells and examined whether these cells become more sensitive to spindle poisons. On the other way round, we expressed a constitutive active form of FoxM1 in old cells and determined whether these cells become less sensitive to spindle poisons.

Moreover, the significant and diverse role of FoxM1 in oncogenesis validating its relevance for chemotherapeutic intervention, led us to the second question of this project: can induced downregulation of FoxM1 in combination with anti-mitotics act as an efficient combined chemotherapy to kill cancer cells? To address this, we again used long-term phase-contrast live cell imaging to access the cell fate in response to anti-mitotics of a tumor cell line that overexpresses FoxM1. Then, we knocked-down FoxM1 in this tumor cell line and tested whether they become more sensitive to anti-mitotics. We found evidence for an increased pro-apoptotic efficacy of anti-mitotics following FoxM1 downregulation that might be explored in the future as a chemotherapeutic strategy.

Chapter II - Material and Methods

II-1 Cell Culture

Human Dermal Fibroblasts (HDFs) of neonatal (DFM021711A, ZenBio, USA), 10 years-old (GM03348, Coriell Cell Repository, USA), 22 years-old (DFME102710, ZenBio, USA), 52 years-old (GM28967, Coriell Cell Repository, USA) and 87 years-old (AG10884, Coriell Cell Repository, USA) Caucasian males, were cultured in MEM (Gibco®) supplemented with 15% Fetal Bovine Serum (FBS) (Gibco®), Antibiotic-Antimycotic (AAs) (1:100) (Gibco®) and L-Glutamine (1:100) (Gibco®). Fibrosarcoma cell line HT-1080 (ATCC®) was cultured in MEM (Gibco®) supplemented with 10% FBS (Gibco®), AAs (1:100) and L-Glutamine (1:100). Cells were cultured under optimal conditions of temperature 37°C and humidified atmosphere with 5% CO2, using ventilated T25 and T75 flasks (SARSTEDT®). Three anti-mitotic drugs were used: Nocodazole (Sigma-Aldrich®) (3,3 μM), S-trityl-L-cysteine (STLC) (Biogen®) (5 μM), Taxol (Sigma-Aldrich®) (5 nM and 500 nM).

II-2 Cell transfection with siRNA

For primary cell transfection, 1,5 x10^5 cells were platted per well in a 6-well plate in 1,5ml of MEM without FBS. For FoxM1 siRNA transfection (75 nM) two tubes were prepared: Tube 1 with 250 μL Opti-MEM (Gibco®) + 7,5 μL FoxM1 siRNA (Sigma®), target sequence (5'-3') (CAACUCUUCUCCCUCAGAU[dT][dT]); Tube 2 with 250 μL Opti-MEM (Gibco®) + 5 μL Lipofectamine RNAiMax (Invitrogen®). After 5 min, tube 2 was added to tube 1 and the solution rested 1 hour at room temperature (RT). Afterwards, the mix was added carefully to the cells, drop-by-drop, and 500 μL of Opti-MEM + 5 μL Lipofectamine RNAiMax was added to the mock control. After 16 hours, medium was replaced by fresh MEM with 15% FBS. Cells were analyzed 72 hours after the initial transfection with FoxM1 siRNA. .

For HT-1080 cell line transfection, 1,5 x10^5 cells were platted per well in a 6-well plate in 1.5 ml of MEM with 5% FBS. FoxM1 siRNA oligonucleotide was used at 20 nM final concentration. 5 hours after transfection, MEM with 10% FBS was added. Cells were analyzed 72 hours after the initial transfection with FoxM1 siRNA.

II-3 Viral production and cell infection

FoxM1 ΔNΔKEN plasmid construct was generously provided by René Medema (NKI, Amsterdam). FoxM1 ΔNΔKEN coding sequence was subcloned into the lentiviral vector pLVX-Tight-Puro Vector (Clontech®, CA, USA) by Joana Macedo (IBMC, Porto).

Lentivirus production was performed with the Lenti-X™ Tet-On® Advanced Inducible Expression System (Clontech®, CA, USA). This system allows the control of the expression level of your gene of interest (GOI) by regulating the concentration of the system's inducer, doxycycline. There are two main elements of the Tet-On Advanced System: the Regulator Vector, pLVX-Tet-On Advanced, which constitutively expresses the tetracycline-controlled transactivator rtTA-Advanced, and the Response Vector, pLVX-Tight-Puro, which contains PTight, the inducible promoter that controls expression of your GOI. Upon induction with doxycycline, rtTA-Advanced binds to the PTight promoter on the response vector, thereby activating transcription of the downstream gene.

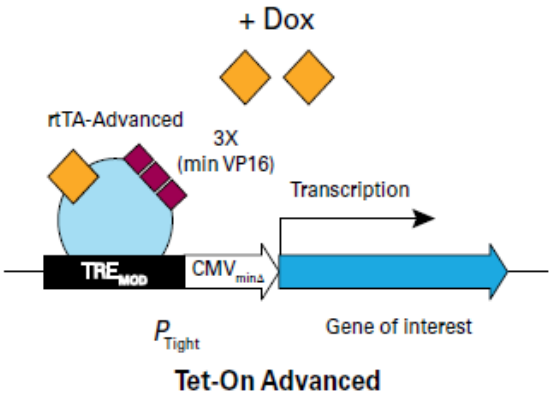

Figure 9 - Gene induction in the Tet-On® Advanced Systems. The Tet-controlled transactivator rtTA-Advanced is a fusion protein derived a mutant version of the E. coli Tet repressor protein. When the transactivator is bound to the tetracycline response element (TRE) in PTight, the Tet-On Advanced Systems is inactive. However, it's activated in the presence of doxycycline (Dox), triggering transcription of the downstream gene (Adapted from Clontech Laboratories, 2010).

5-6x10^6 Hek 293 T cells were plated in DMEM+10% FBS tetracycline free per 100 mm plate. After 24h, the culture medium was changed to DMEM only. As described in the kit, two mixes were prepared: Plasmid DNA Mix (575 µl xfect reaction buffer, 36 µl packaging Mix and 7 µl Lenti vector DNA (1 µg/µl)) and Polymer Mix (592.5 µl xfect reaction buffer and 7.5 µl Xfect Polymer). Polymer Mix was added to the Plasmid DNA Mix and incubated for 10 min at room temperature

to allow nanoparticle complexes to form. The solution was added carefully to the cells, drop by drop. Cells were incubated at 37ºC overnight. Afterwards, the transfection medium was replaced with fresh DMEM + 10% of Tet System Approved FBS and cell were incubated at 37 ^{0}C for 24-48-72 hours. At those time points, the supernatants containing infectious lentivirus were collected, centrifuged at 1200 rpm for 10 min, and filtered through 0.45 μm (cellulose acetate) to eliminate cell debris. For viral concentration, the resulting supernatant was centrifuged at 4000xg for 20min through an Amicon® Ultra-15 Centrifugal Filter Device (Millipore®). Virus particles were aliquoted and stored at -80ºC until further use.

For primary cell lentiviral infection, 1.5x10^{5} cells were platted per well in a 6-well plate in 1.2 ml of MEM without FBS. 5 μl of FoxM1 ΔNΔKEN viral particles, 2.5 μl of rTTA and 0.3 μl of polybrene were added to each well. After 12 hours of infection, the medium was replaced with complete medium and cells allowed to recover for one day before the start of transduction with doxycycline (1:1333).

II-4 Western Blotting

Cells were detached from the culture flasks with Trypsin (Gibco®). The cell suspension was centrifuged at 1200 rpm for 5 min at room temperature (RT). The supernatant was discarded and the pellet washed with PBS 1x (Gibco®), and another centrifugation step was performed. The pellet was suspended with lysis buffer (150 nM NaCl + 10 nM Tris.HCl pH 7.4 + 1 nM EDTA + 1 nM EGTA + 0,5% Isgepal + autoclaved water) plus protease inhibitors (1:50). The samples were frozen in liquid nitrogen. The extracts were then defrosted on ice and centrifuged at 13300 rpm (maximum speed) for 20 min at 4ºC. The supernatant was transferred to a new eppendorf. Protein quantification was performed with the DC™ Protein Assay (Lowry method) (BioRad®). Samples were kept at -20^{0}C until required.

20 μg of total extract was used for analysis plus 5x Laemmli buffer (250 nM Tris.Cl, 10% SDS, 0.5% bromophenol blue, 50% Glycerol and 500 mM β-mercaptoethanol). The Mini-PROTEAN® Tetra Handcast Systems (BioRad®) was assembled. After boiling samples for 5 minutes at 95ºC, the extracts were loaded in the acrylamide gel, with the PageRuler Prestained Protein Ladder (Thermo Scientific®). The gels recipe is described in Table 1. The electrophoresis ran with

running buffer (387.3 mM Glycine e 50.2 mM Trizma base + 1% SDS, pH 8.3) until the desired resolution of bands was achieved.

Table *1* - SDS-PAGE gels recipe (BioRad®).

	4% Stacking Gel	7% Resolving Gel	10% Resolving Gel
40% Acrylamide/Bis-acrylamide (29:1)	300 µL	875 µL	1.25 mL
Stacking Buffer	756 µL	-	-
Resolving Buffer	-	1.25 mL	1.25 mL
10% SDS	30 µL	50 µL	50 µL
ddH_2O	1.908 mL	2.8 mL	2.425 mL
TEMED	3 µL	2.5 µL	2.5 µL
10% APS	15 µL	50 µL	50 µL
Total Volume	3 mL	5 mL	5 mL

The gel was transferred to a nitrocellulose membrane (Whatman) using a wet system (BioRad®) with transfer buffer (39 mM Glycine, 48 mM Trizma base and 20% Methanol), overnight (ON) at 30V, in a cold chamber at 4ºC or for 2 hours at 100V at room temperature (RT). To analyze transfer efficiency, the membrane was incubated for 1 min with Ponceau S (0.1% Ponceau S and 5% acetic acid).

Nitrocellulose membrane was blocked for 1 hour at RT with 5% non-fat milk in 1x TBS (500 mM Tris-Cl, 150 mM NaCl) + 0.05% Tween20 (TBST). Primary antibodies were diluted in 2% non-fat milk in TBST and incubation was done for 2 hours at RT or overnight at 4ºC. Primary antibodies were diluted according to Table 2:

Table 2 - Primary antibodies dilutions.

	Animal	Dilution	Purchase from
Actin	Mouse	1:50000	Sigma-Aldrich®
CENP-F	Sheep	1:1000	Upstate Biotechnology®
Cyclin B1	Mouse	1:1500	Cell Signaling Technology®
FoxM1	Rabbit	1:1000	Proteintech Group®
GAPDH	Mouse	1:30000	Proteintech Group®
Plk1	Mouse	1:1000	Santa Cruz Biotechnology®
Tubulin	Mouse	1:100000	Sigma-Aldrich®

After primary antibody incubation, membranes were washed (2x) for 15 minutes with 2% milk solution in TBST, and incubated for 1 hour with secondary

antibody [goat anti-rabbit IgG-HRP (SC-2004), goat anti-mouse IgG-HRP (SC-2005), donkey anti-sheep IgG-HRP (SC- 2473), from Santa Cruz Biotechnology]. All secondary antibodies were diluted in 1:3000. After wash (2x) for 20 minutes with TBST and (1x) for 20 minutes with TBS, the membranes were processed with Clarity™ Western ECL Blotting Substrate (BioRad®).

For band quantification, it was used the Molecular Imager GS800 Calibrated Densitometer (BioRad®), with Quantity one I-D Analysis Software version 4.6 (BioRad®).

II-5 Time-lapse Live Cell Imaging

Cells were plated in glass bottom 35 mm μ-dishes (Ibidi®) or 2-well μ-slide (Ibidi®) coated with fibronectin (Sigma-Aldrich®). Images were acquired on a Zeiss Axiovert 200M inverted microscope (Carl Zeiss®, Germany) equipped with a CoolSnap HQ camera (Roper), XY motorized stage and NanoPiezo Z stage, controlled temperature (37^{o}C), atmosphere (CO2 5%) and humidity (99%), and operated by Micro-Manager 1.4 software. Fields were imaged every 10 minutes for 72 hours using a 20x A-Plan NA 0.30 objective. Grids of neighboring fields were generated using the plugin Stitch Grid (Stephan Preibisch) from open source Fiji/ImageJ (http://rbs.info.nih.gov/ij/).

II-6 Statistical Analysis

All statistical analyses were performed with Prism version 6.00 for Windows, GraphPad Software®, La Jolla California USA, www.graphpad.com.

Chapter III - Results

III-1 FoxM1 downregulation accounts for increased sensitivity to anti-mitotics in human aged cells

III-1.1 FoxM1 downregulation in human aged cells

We analysed FoxM1 expression levels in total cell extracts of asynchronous cell cultures of human dermal fibroblasts (HDFs) of different ages (neonatal, 10, 22, 52, 87 years old Caucasian males). We observed that FoxM1 expression levels decrease progressively with advancing age (Figure 10).

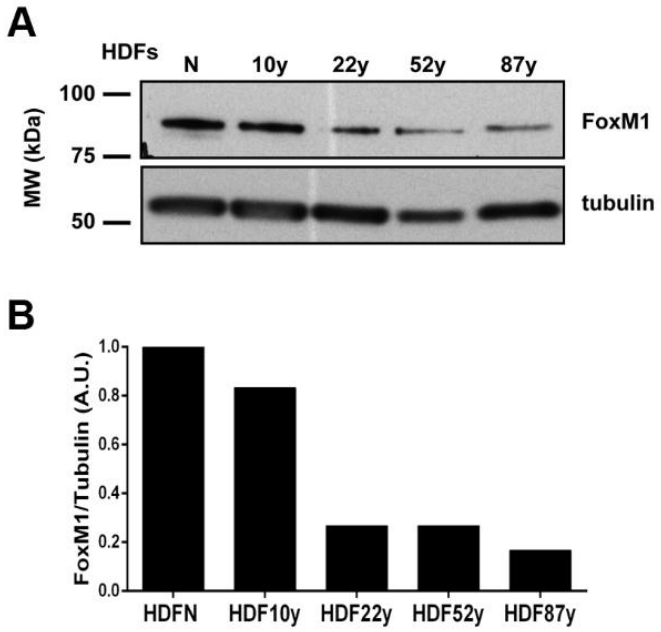

Figure 10 - FoxM1 downregulation during human chronological ageing. (A) FoxM1 expression levels in asynchronous cell culture extracts of human dermal fibroblasts (HDFs) of different ages (N-neonatal, 10, 22, 52, 87 years old Caucasian males). (B) Protein levels were normalized against tubulin levels used as loading control.

FoxM1 is responsible for the transcriptional activation of the G2/M-specific gene cluster in mammalian cells. Consistently, Ly et al., 2000, found several mitotic genes (such as cyclins –A and -B, Plk1, Cdc20, CENP-A, CENP-F, MKLP1, Eg5 and MCAK) to be downregulated with advancing age in fibroblasts isolated from young, middle-age, and old-age humans. However, these studies were performed with asynchronous cell cultures. Since FoxM1 levels correlate with the cellular proliferative state, we asked if there was indeed intrinsic cellular downregulation of

FoxM1 due to chronological ageing or if this was just a consequence of reduced cell proliferation and mitotic index in an asynchronous population. Therefore, we synchronized cell cultures in mitosis with the kinesin-5 small molecule inhibitor STLC, which prevents formation of bipolar spindles. Following 15 hours treatment and mitotic cell shake-off, we prepared mitotic cell extracts for western blot analysis. Again, we found FoxM1 to be downregulated in mitotic cells from elderly cultures (Figure 11). To further validate this, we also checked the protein levels of known direct targets of FoxM1. CENP-F, cyclin B and Plk-1 were downregulated in mitotic cells with advancing age, pointing indeed to a cell intrinsic FoxM1 downregulation during human physiological ageing that in turn represses many mitotic genes.

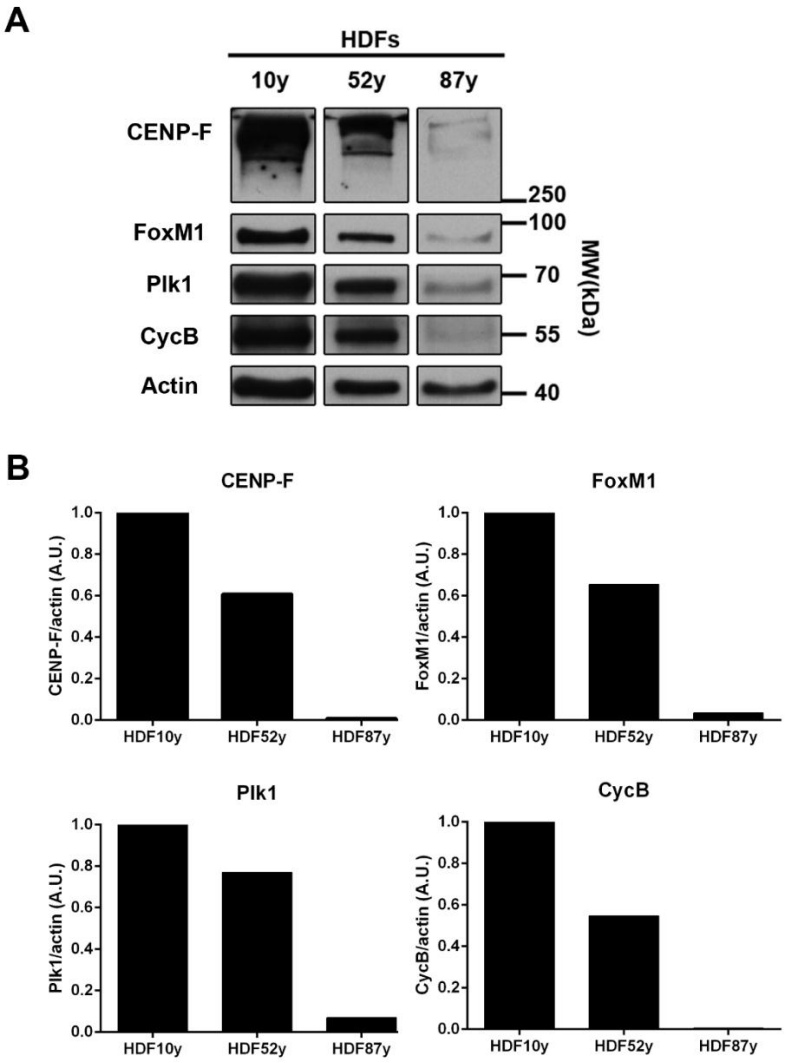

Figure 11 - FoxM1 downregulation in mitosis during human chronological ageing. (A) CENP-F, FoxM1, Plk1 and CycB expression levels in mitotic cell culture extracts of human dermal fibroblasts (HDFs) of different ages (10, 52 and 87 years old Caucasian males), synchronized with STLC (5μM) during 15 hours and mitotic shake-off. (B) Protein levels were normalized against actin levels used as loading control.

III-1.2 Old cells display an increased sensitivity to anti-mitotics

Two independent sets of microarray data showed that there are several SAC genes downregulated with human chronological ageing (Ly et al., 2000; Geigl et al., 2004). Therefore, we hypothesized that old cells might have a weakened spindle assembly checkpoint function. Also, as mentioned before, upon exposure to anti-mitotic drugs, the decision to die in mitosis or to slip mitosis is dictated by two independent processes during the arrest: a slow, but progressive loss of cyclin B1 and a slow, but steady rise in cell death proteins activity. If cyclin B1 levels fall below the mitotic-exit threshold first, slippage occurs and if the death threshold is breached first, then the cell dies in mitosis (Gascoigne and Taylor, 2008). Besides downregulation of SAC genes, old cells exhibit decreased cyclin B1 levels, suggesting that they likely breach the mitotic-exit threshold first and are more slippage-prone than young cells in response to anti-mitotics.

To address this issue, we used an inverted microscope, Zeiss Axiovert 200M (Carl Zeiss®, Germany), under controlled temperature (37^{o}C), atmosphere (CO_2 5%), humidity (99%) and only 5 volts of luminosity to do long-term phase-contrast live cell imaging over 72 hours and record individual cell behavior every 10 minutes.

Under optimal conditions, HDFs divide with a normal bipolar anaphase (Figure 12A). However, young cells (HDF 10 years) do mitosis faster than old cells (HDF 87 years), taking an average of 23,4±9,4 minutes and 32,3±11,4 minutes, respectively, from NEB to anaphase onset (Figure 12B).

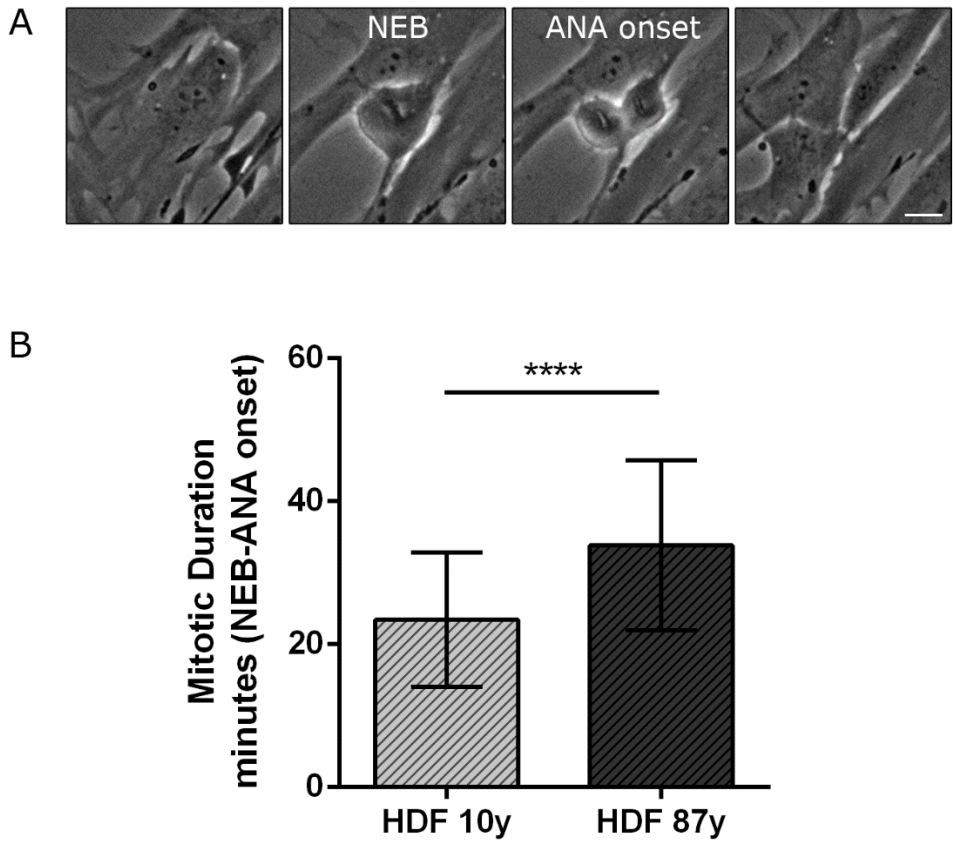

Figure 12 - Mitotic duration in young cells *versus* old cells. (A) Example of time-lapse sequence illustrating a normal bipolar division of human dermal fibroblasts (HDFs). Mitotic duration was defined as the time from the nuclear envelope breakdown (NEB) to anaphase onset. Scale bar = 10 μm. (B) Mean with standard deviation of the mitotic duration of HDF 10y (23,4±9,4 min) and HDF 87y (32,3±11,4 min). Statistical analysis performed with Mann-Whitney test of three independent experiments with n>200 cells in each condition, ****p≤0,0001.

To access the robustness of SAC activity in old cells, we challenged the SAC with three different anti-mitotic drugs: Nocodazole (NOC) (3,3μM), that promotes the despolymerization of microtubules, S-trityl-L-cysteine (STLC) (5μM), an inhibitor of Eg5/kinesin-5 that provokes monopolar spindles, Taxol (500nM), that stabilizes microtubules, and Taxol (5nM), at a low but clinically relevant dose that perturbs the microtubule dynamics. We followed individual cell behavior in response to the drug treatments for 72h under phase-contrast microscopy and scored the number of mitotic cells exhibiting each one of the following possible cell fates: bipolar division, multipolar division, slippage or death in mitosis (Figure 13).

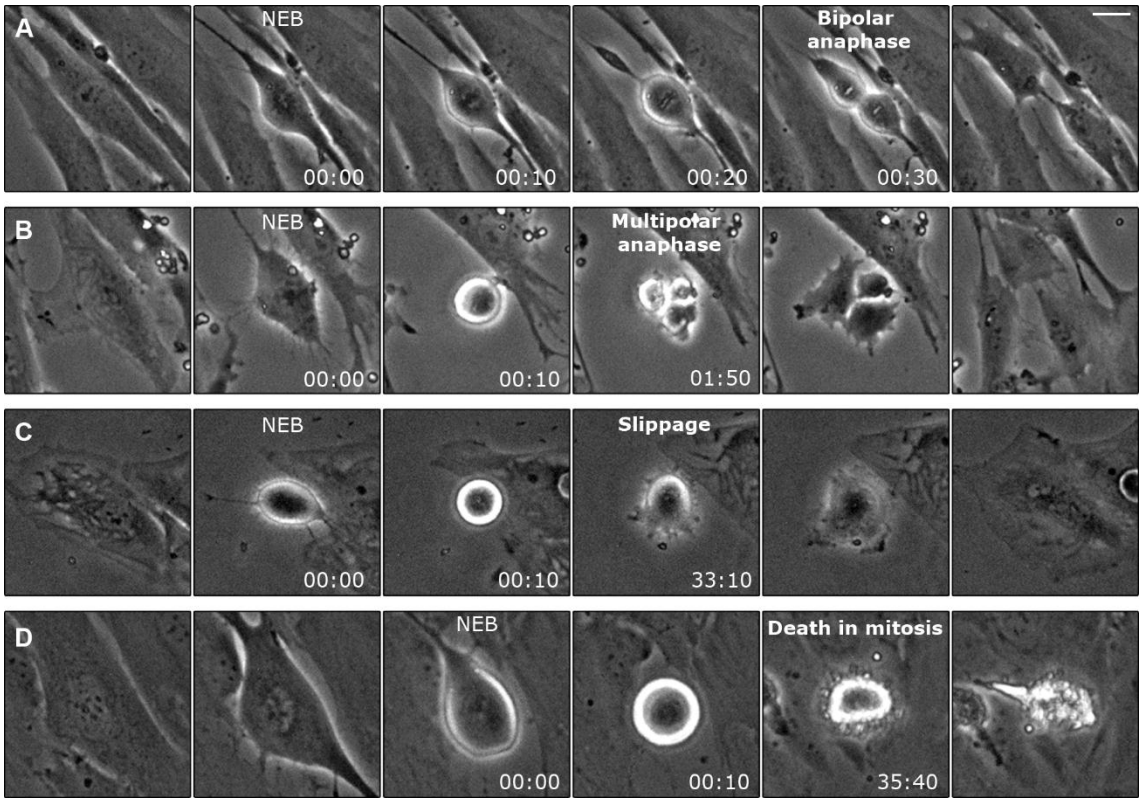

Figure 13 – Cell fates of human dermal fibroblasts in response to anti-mitotics. Examples of time-lapse sequences illustrating the fates exhibited by HDFs following prolonged exposure to anti-mitotic drugs. Mitotic duration in hr:min from the NEB to the respective fate. Scale bar = 10 μm. (A) Bipolar anaphase. (B) Multipolar anaphase. (C) Slippage. (D) Death in mitosis.

III-1.2.1 Drugs that cause a chronic activation of the SAC

Both young and old cells when exposed to high concentrations of anti-mitotics that cause a chronic activation of the checkpoint exhibited one the following fates, slippage or death in mitosis.

When treated with nocodazole, HDFs 10y exhibited 65,4% of death in mitosis *versus* 34,6% of slippage, while HDFs 87y exhibited 95,5% of death in mitosis versus 3,5% of slippage. With high concentrations of Taxol, 55,4% of young cells died in mitosis whereas 68,3% of the old cells had mitotic death. With STLC, only 19,5% of HDFs 10y died while HDFs 87y had 58,5% of mitotic death (Figure 14). So, mitotic death following chronic activation of the SAC by any of the drug treatments increases significantly in elderly cells. This is contradictory to our initial idea, where we would expect old cells to be more slippage-prone than young cells.

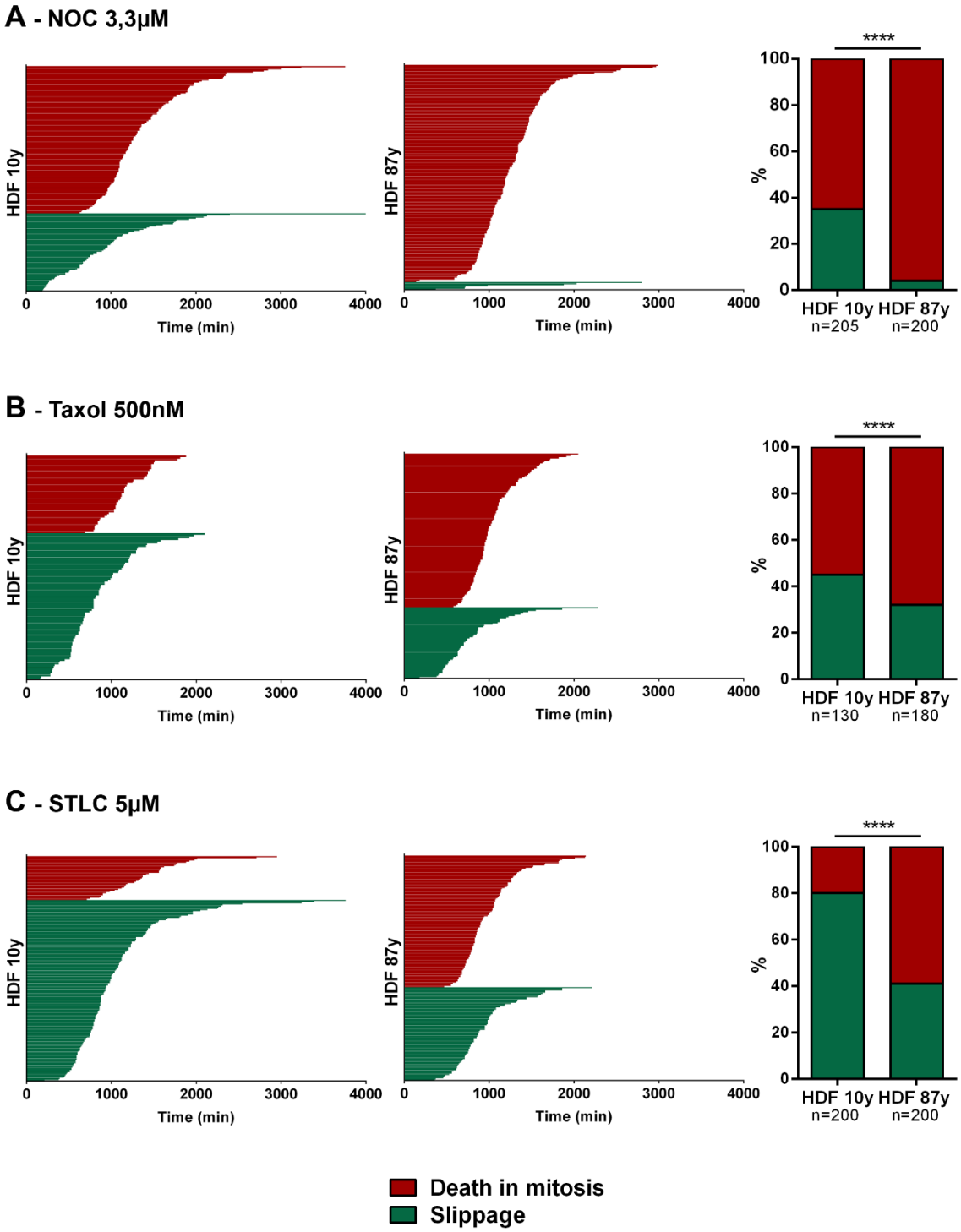

Figure 14- Mitotic cell fate of human dermal fibroblasts in response to anti-mitotic agents. Fate profiles of HDF 10y and HDF 87y exposed to (A) nocodazole (NOC) 3,3µM, (B) taxol 500nM and (C) S-trityl-L-cysteine (STLC) 5µM. Each individual bar in the graphs represents one individual cell analyzed by long-term phase-contrast time-lapse microscopy. The duration of the mitotic arrest was measured as the time from the NEB to the respective fate. Statistical analysis performed with Fisher's exact test of three independent experiments with n≥130 cells in each condition, ****p≤0,0001.

We asked whether the duration of the mitotic arrest induced by the anti-mitotics could account for the different cell fate profiles found between young and

old fibroblasts. The time the cells take to die in mitosis or to slip mitosis under the different drug treatments is illustrated in Figure 15. Older cells die faster in response to taxol 500nM and STLC 5uM, but not in response to nocodazole 3.3uM. However, we acknowledge that changes in cellular morphology determined under phase-contrast microscopy are not an accurate methodology to measure apoptosis onset (some cells suddenly blast disassembling into apoptotic bodies, whereas others slowly shrink overtime). Concerning slippage, time to slip mitosis was measured as the interval between nuclear envelope breakdown and cell adhesion. Independently of the anti-mitotic drug used, there was no significant difference in the time of slippage between the young and old HDFs. Altogether, the data suggest that older cells preferentially follow to mitotic death fate in response to anti-mitotics therefore reaching the apoptosis threshold faster accordingly to the model of (Gascoigne and Taylor, 2008).

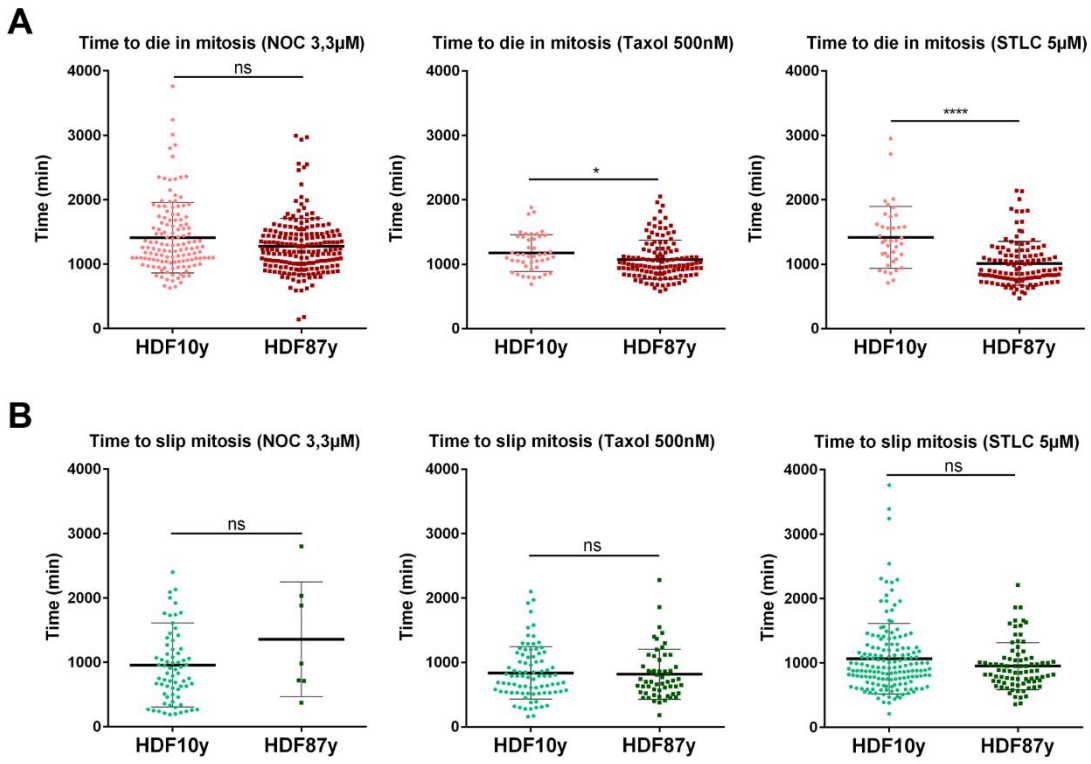

Figure 15- Duration of the mitotic arrest until cell fate decision, death or slippage, of human dermal fibroblasts treated with different anti-mitotic agents. Each dot represents one cell. (A) Time to die in mitosis, measured as the interval between NEB and first morphological evidence of apoptosis. (B) Time to slip mitosis, measured as the interval between NEB and cell adhesion. Statistical analysis performed with Mann-Whitney test for 3 independent experiments. ns=no significance=p>0.05, *=p≤0.05, ****=p≤0.0001.

Concerning the post-mitotic response, 95% of the cells that slipped mitosis entered an interphase arrest (≥24h), during at least the duration of our experiment, while 5% of them died in interphase. There was no significant difference between HDF 10y and HDF 87y.

III-1.2.2 A clinically relevant dose of Taxol

A recent study points to low concentrations of anti-mitotics as having greater clinical importance (Zasadil et al., 2014). We therefore challenged our cells with 5nM of taxol, described to be the concentration that indeed reaches the tumor's core of breast cancer cells (Zasadil et al., 2014). Under this concentration, cells can ultimately satisfy the SAC and exit mitosis in a bipolar or multipolar anaphase. We found that old cells most often display multipolar anaphases than young cells (88,9% vs 44,9%, respectively) (Figure 16).

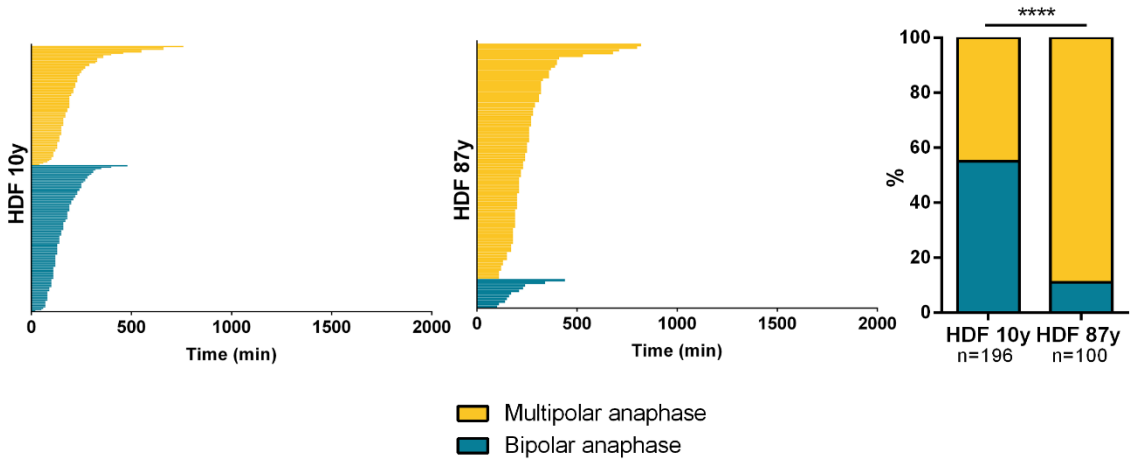

Figure 16- Mitotic cell fate of human dermal fibroblasts in response to 5nM of Taxol. Each individual bar in the graphs represents one individual cell analyzed by long-term phase-contrast time-lapse microscopy. The duration of the mitotic arrest was measured as the interval between NEB and anaphase onset. Statistical analysis performed with Fisher's exact test, of three independent experiments, n≥100cells in each condition. ****p ≤0,0001.

Regarding how long cells were arrested in mitosis before they proceeded to anaphase, we found old cells to arrest longer before ensuing to multipolar anaphase (Figure 17), which is contradictory to our initial assumption. However, the average time to do a bipolar anaphase was not significantly different between young and older cells, even though the number of old cells doing bipolar anaphases might be poorly representative.

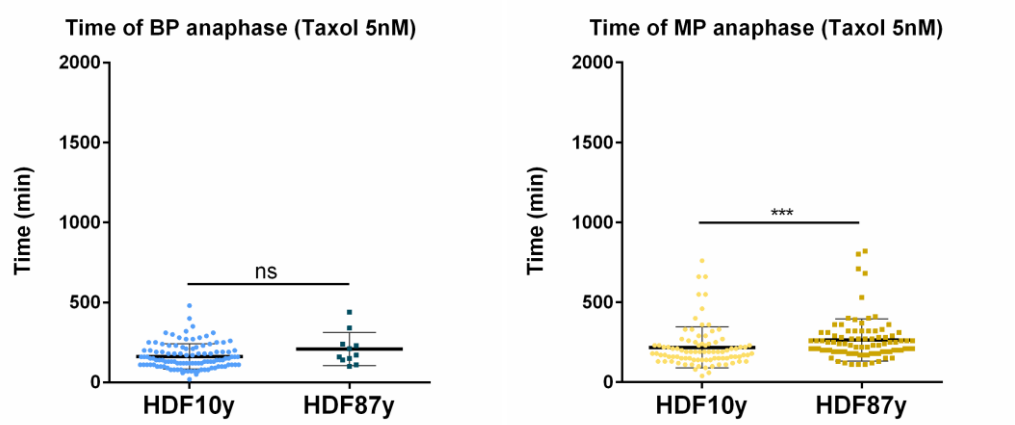

Figure 17- Duration of the mitotic arrest in human dermal fibroblasts in response to 5nM of Taxol. Each dot represents one cell. Time was measured from NEB to anaphase onset. Statistical analysis performed with Mann-Whitney test. ns=no significance=p>0.05, ***p<0,001.

The post-mitotic response of cells after treatment with 5nM of taxol was also analyzed, and the results are illustrated in table 3 and table 4. In HDFs 10y, we observed an increase of cell death in the case of cells that derived from a multipolar anaphase, consistently to the fact that massive chromosome mis-segregation compromises cell survival. There was also a decrease of cell cycle progression, which is in line with the fact that interphase checkpoints are halting the cell cycle due to DNA imbalances. Therefore, 5nM of taxol exerts its toxicity towards cells in the post-mitotic response. In HDFs 87y, we only quantified the post-mitotic cell fate of n=50 cells because the cell confluence in two out of three experiments was too high complicating cell tracking, and many cells only entered mitosis on the second and third days of live cell records leaving short time interval to determine their post-mitotic fate. Nevertheless, our data show that HDFs 87y post-mitotic cells typically arrest in interphase independently of arising from a bipolar or multipolar anaphase, and most importantly, no cells further progressed in the cell cycle. Therefore, resistance to taxol is unlikely to arise in the old cell population.

Table 3- Post-mitotic response of HDFs 10y after treatment with 5nM of Taxol. Data from three independent experiments with n=196.

Bipolar Anaphase			Multipolar Anaphase		
55%			45%		
Cell Cycle Progression	Interphase Arrest	Death in Interphase	Cell Cycle Progression	Interphase Arrest	Death in Interphase
6,3%	42,4%	6,3%	1,5%	24,6%	18,9%

Table 4- Post-mitotic response of HDFs 87y after treatment with 5nM of Taxol. Data from one experiment with n=50.

Bipolar Anaphase			Multipolar Anaphase		
10%			90%		
Cell Cycle Progression	Interphase Arrest	Death in Interphase	Cell Cycle Progression	Interphase Arrest	Death in Interphase
0%	8%	2%	0%	86,7%	3,3%

We conclude that the pro-apoptotic efficiency of anti-mitotics is increased in old cells, suggesting that cellular senescence is determinant on cell fate decision following chronic activation of the SAC. Because old cells take as long to slip out of mitosis as young cells, this suggests also that SAC activity is not compromised. Therefore, we hypothesize that cellular ageing is alternatively impacting on the molecular threshold for mitotic death, possibly by leading to either increased levels of pro-apoptotic proteins or decreased levels of anti-apoptotic proteins.

III-1.3 – FoxM1 accounts for increased sensitivity of human aged cells to anti-mitotic chemotherapy

III-1.3.1 FoxM1 silencing in young HDFs

Being FoxM1 the major transcription factor in G2/M transcription, it is likely that it regulates the expression levels of the molecular determinants of cell fate decision upon prolonged mitotic arrest. Therefore, we asked whether FoxM1 downregulation in elderly cells was responsible for the distinct mitotic fate profiles and increased sensitivity to anti-mitotics. For that, we developed an RNAi transfection protocol for our primary cells. We silenced FoxM1 in HDFs 10y (Figure 18A) to levels similar to those in HDFs 87y and accessed their individual cell behavior under long-term phase-contrast live cell microscopy.

Under normal conditions, FoxM1 siRNA-depleted HDF 10y have a mitotic duration of 31,3±8,6 min, not significantly different from HDF 87y (Figure 18B).

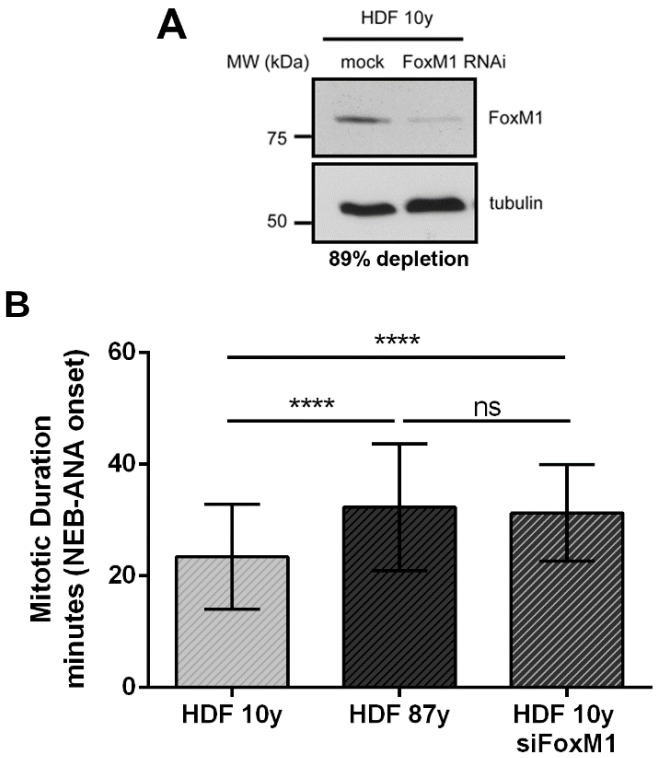

Figure 18- FoxM1 RNAi silencing in HDF 10y. (A) FoxM1 protein levels following RNAi silencing in 10 years old fibroblasts. Depletion levels were quantified against the levels of tubulin used as loading control. An average of 89± % of depletion in three independent experiments was obtained. (B) Mean with standard deviation of the mitotic duration of HDF 10y (23,4±9,4 min), HDF 87y (32,3±11,4 min) and HDF 10y siFoxM1 (31,3±8,6 min). Statistical analysis performed with Mann-Whitney test of three independent experiments with n>100 cells in each condition. ns=no significance=p>0.05, ****p≤0,0001.

We challenged HDF 10y siFoxM1 with the same anti-mitotic drugs and observed that these cells display a mitotic fate profile more similar to HDF 87y (Figure 19). In nocodazole treatment, 87,8% of cells die in mitosis, which is more statistically different from HDF 10y ($p \leq 0.0001$) than from HDF 87y ($p \leq 0.001$) . Additionally, in the Taxol 500nM and STLC 5µM treatments, the death percentages in HDF 10y siFoxM1 were 65,5% and 62,3% respectively, and non-significantly different from HDF 87y.

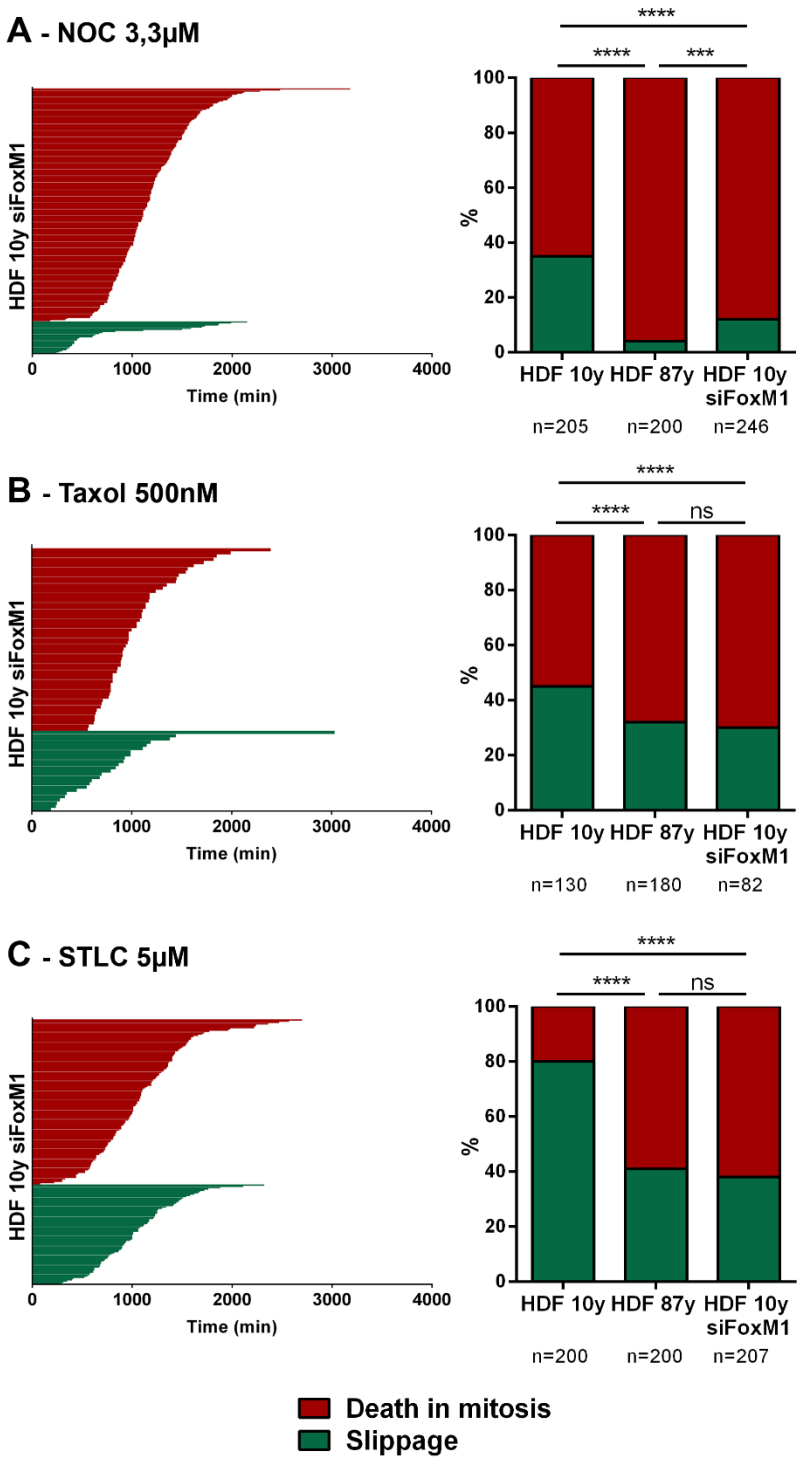

Figure 19- Mitotic cell fate of FoxM1 RNAi-depleted 10 years old fibroblasts in response to anti-mitotic agents. Fate profiles of HDF 10y siFoxM1 exposed to (A) nocodazole (NOC) 3,3µM, (B) taxol 500nM and (C) S-trityl-L-cysteine (STLC) 5µM. Each individual bar in the graphs represents one individual cell analyzed by long-term phase-contrast time-lapse microscopy. The duration of the mitotic arrest was measured as the time from the NEB to the respective fate. Statistical analysis performed with Fisher's exact test of three independent experiments with n≥82 cells in each condition. ns=no significance=p>0.05, ***=p≤0.001, ****=p≤0.0001.

Concerning the duration of the mitotic arrest, we found that FoxM1 RNAi-depleted cells die faster than HDF 10y, with the exception of taxol 500nM treatment.

Again, time to slip out of mitosis consistently showed no significant difference between the different conditions (Figure 20).

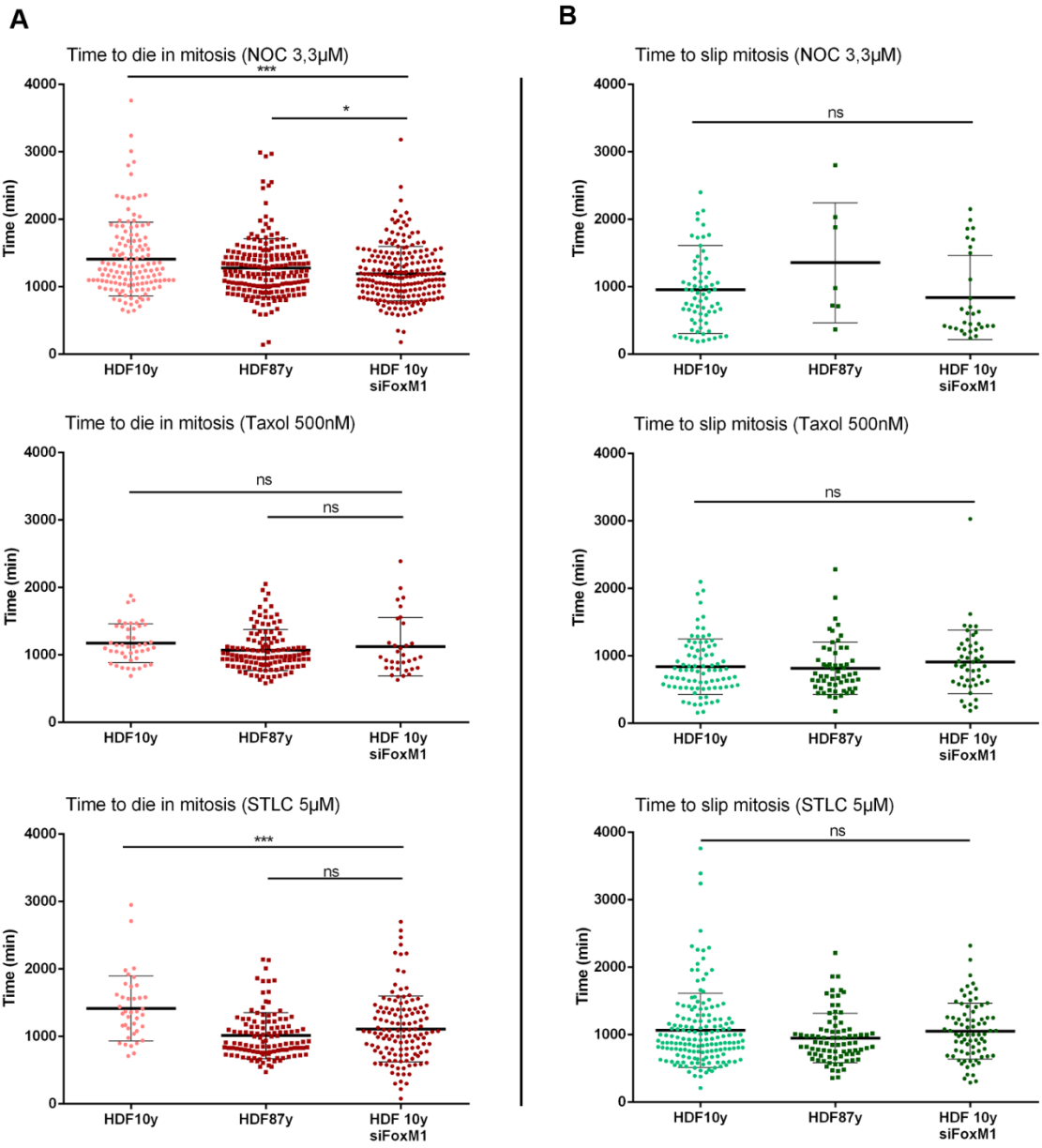

Figure 20- Duration of the mitotic arrest until cell fate decision (death or slippage) of young, elderly and FoxM1-depleted young fibroblasts treated with anti-mitotic drugs. Each dot represents one cell. (A) Time to die in mitosis, measured from the frame that showed NEB to the first frame that presented a morphological evidence of apoptosis. (B) Time to slip mitosis, measured from NEB to the first frame where the cell started to adhere. Statistical analysis performed with Mann-Whitney test of three independent experiments.ns=no significance=p>0.05, *=p≤0.05, ***=p≤0.001.

Regarding the 5nM Taxol treatment, we found FoxM1 RNAi-depleted cells to exhibit a fate profile non-significantly different from elderly cells, with 88,6% of cells doing a multipolar anaphase (Figure 21).

Concerning how long cells are arrested in mitosis before doing anaphase, we observed that cells with low levels of FoxM1 delay in anaphase onset (Figure 22). The average time to do both bipolar and multipolar anaphases increases in HDF 10y siFoxM1 in comparison to HDF 10y, and is not significantly different from HDF 87y.

Altogether, the data suggest that FoxM1 is a molecular determinant of cell fate decision upon exposure to anti-mitotic drugs.

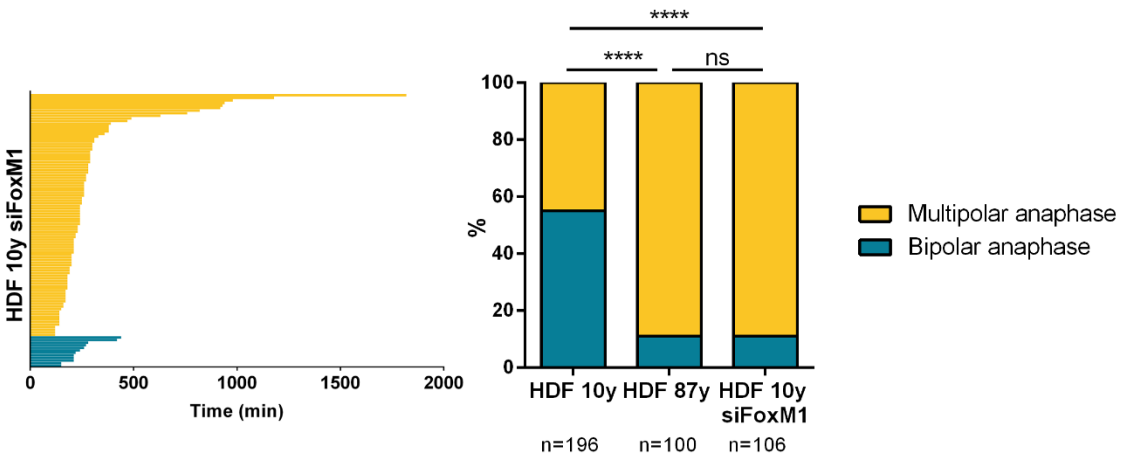

Figure 21 - Mitotic cell fate of HDF 10y siFoxM1 in response to 5nM of Taxol. Each individual bar in the graph on left represents one individual cell analyzed by long-term phase-contrast time-lapse microscopy. The duration of the mitotic arrest was measured as the interval between NEB and anaphase onset. Fisher's exact test was used for statistical analysis of three independent experiments (n≥100 cells). ns=no significance=p>0.05, ****p ≤0,0001.

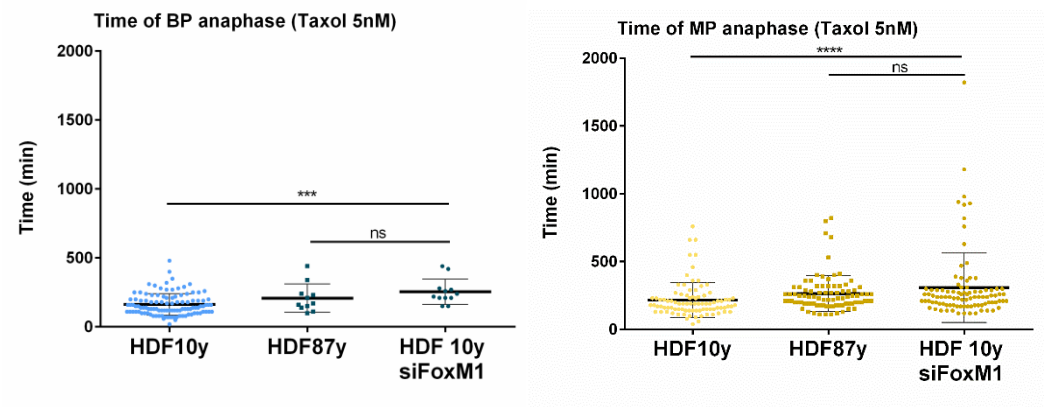

Figure 22- Duration of the mitotic arrest of HDFs 10y, 87y and 10y siFoxM1 in response to 5nM of Taxol. Each dot represents one individual cell analyzed by long-term phase-contrast time-lapse microscopy. The duration of the mitotic arrest was measured as the interval from NEB to anaphase onset. Mann-Whitney test was used for statistical analysis of three independent experiments . ns=no significance= p>0.05, ***=p≤0.001, ****p ≤0,0001.

III-1.3.2 FoxM1 overexpression in old HDFs

To further validate our hypothesis, we needed to do the reverse experiment, this is, to increase the levels of FoxM1 in old cells and see if the cell fate profiling becomes similar to that of young cells. We overexpressed a N-terminal truncated form of FoxM1, also deleted for the KEN-box destruction signal (FoxM1 ΔNΔKEN), to ensure constitutive transcriptional activity of FoxM1 in old cells. Deletion of the N-terminal prevents FoxM1 auto-inhibition, and deletion of the KEN-box impairs FoxM1 degradation by the proteasome.

Figure 23A shows the expression of FoxM1 ΔNΔKEN in HDF 87y following lentiviral infection. Using time-lapse microscopy to access individual cell behavior, we found HDF 87y ΔNΔKEN to have an average mitotic duration of 24,4±8,5 min, not significantly different from HDF 10y (Figure 23B).

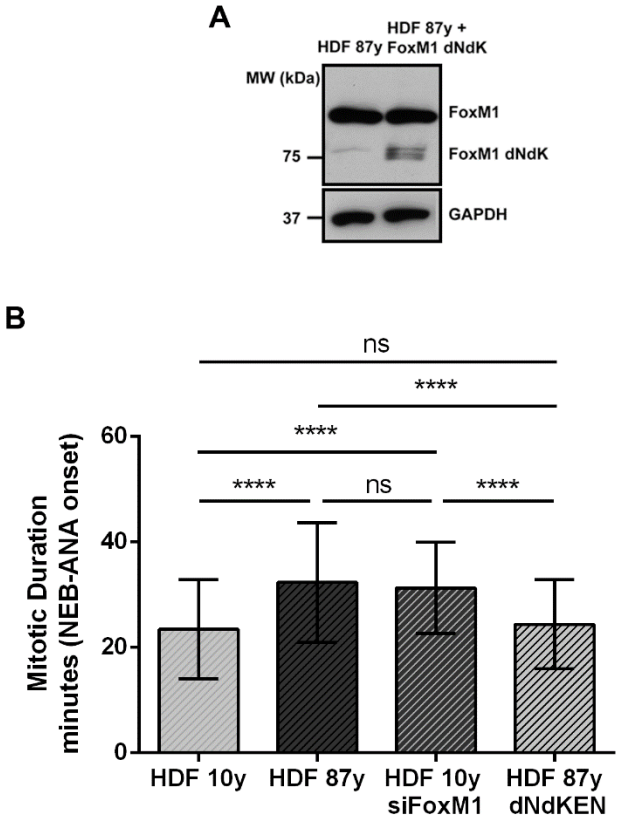

Figure 23 - Overexpression of constitutively active FoxM1 (FoxM1 ΔNΔKEN) in HDF 87y. (A) Expression of FoxM1 ΔNΔKEN in HDF 87y following lentiviral infection as determined by western blot detection in total cell extracts. GAPDH was used as loading control. (B) Mean with standard deviation of the mitotic duration of HDF 10y (23,4±9,4 min), HDF 87y (32,3±11,4 min), HDF 10y siFoxM1 (31,3±8,6 min) and HDF 87y ΔNΔKEN (24,4±8,5 min). Mann-Whitney test was used for statistical analysis of three independent experiments with n>100 cells in each condition. ns=no significance=p>0.05, ****p≤0,0001.

When exposed to anti-mitotic drugs, HDF 87y ΔNΔKEN exhibited a cell fate profile more similar to HDF 10y (Figure 24). All drug treatments significantly decreased the percentages of old cells dying in mitosis ($p \leq 0{,}0001$). However, the cell fate profile only reverted to similar percentages as those in HDF 10y in the case of taxol 500nM treatment, suggesting that FoxM1 more tightly regulates molecular determinants of cell response to taxol.

Regarding the time to die in mitosis, we found HDF 87y ΔNΔKEN cells to still die faster in response to nocodazole and STLC in comparison to HDF 10y, but not to differ in the case of taxol. This is in agreement with the fact that we found partial revertion of the mitotic death phenotype in nocodazole and STLC treatments, but a complete rescue to HDF 10y percentage in taxol condition (Figure 25A). Interestingly, concerning the time to slip out of mitosis (Figure 25B), HDF 87y ΔNΔKEN cells became faster in all conditions in comparison to HDF 10y. This might account for the increased percentage of slippage when comparing to HDF 87y.

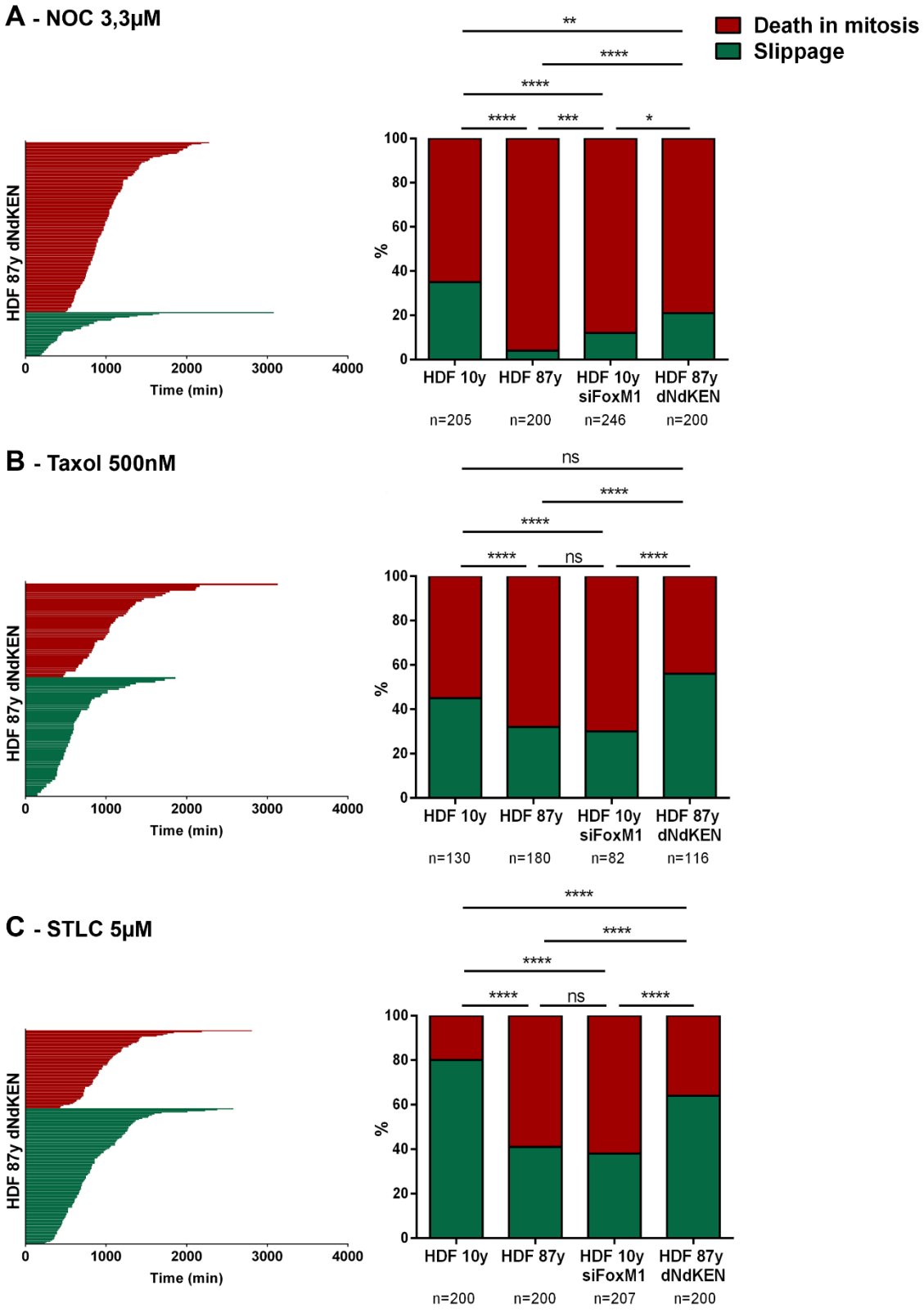

Figure 24- Mitotic cell fate of FoxM1 overexpressing HDFs (HDFs 87y ΔNΔKEN) in response to anti-mitotic agents. Fate profiles of HDFs 87y ΔNΔKEN exposed to (A) nocodazole (NOC) 3,3µM, (B) taxol 500nM and (C) S-trityl-L-cysteine (STLC) 5µM. Each individual bar in graphs on left represents one individual cell analyzed by long-term phase-contrast time-lapse microscopy. The duration of the mitotic arrest was measured as the time from NEB to the respective fate. Fisher's exact test was used for statistical analysis of three independent experiments with n ≥82 cells. ns=no significance=p>0.05, *=p≤0.05, ***=p≤0.001, ****=p≤0.0001.

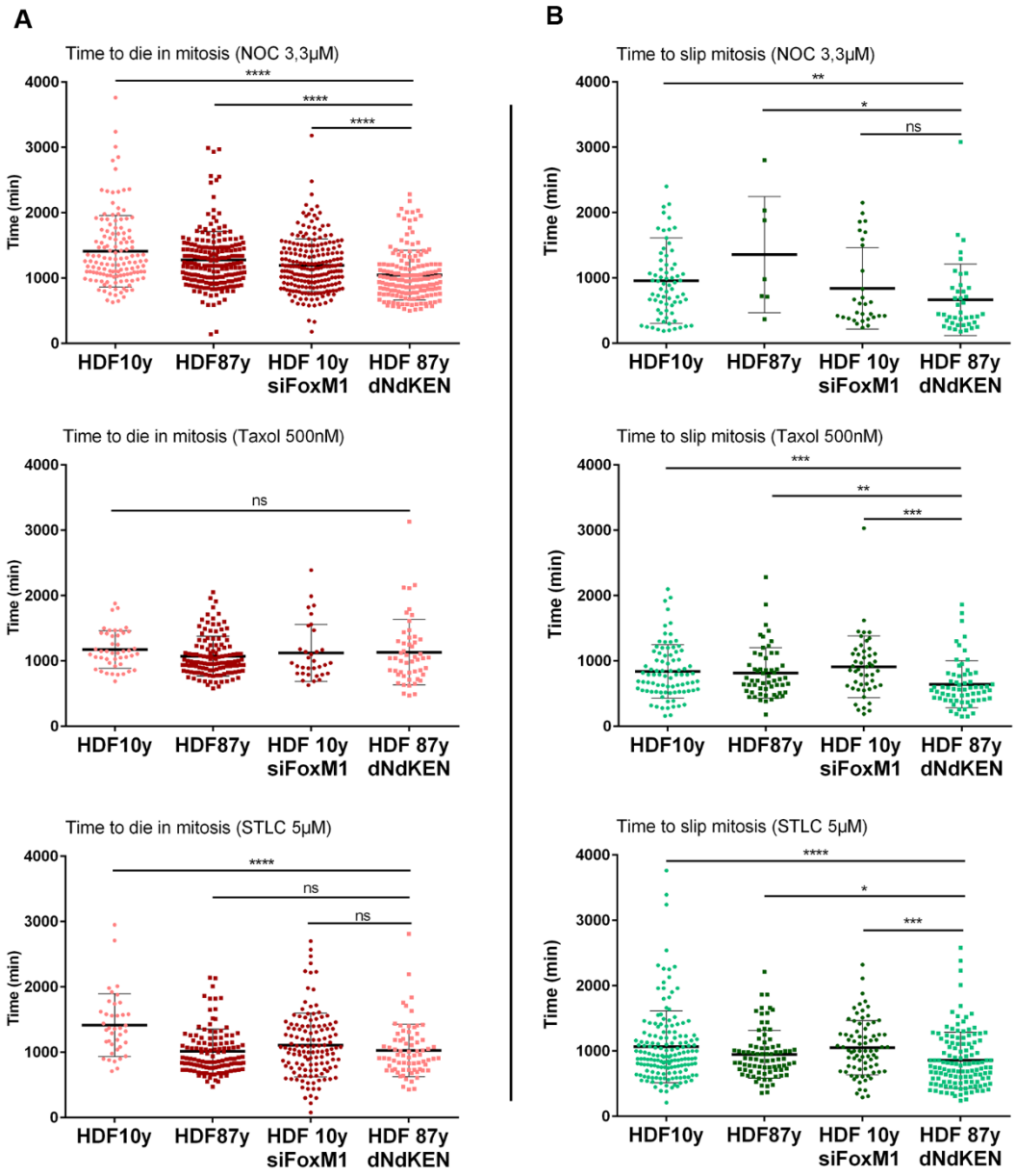

Figure 25- Duration of the mitotic arrest in FoxM1 overexpressing HDFs (HDFs 87y ΔNΔKEN) upon exposure to anti-mitotic drugs. Each dot represents one cell. (A) Time to die in mitosis, measured as the interval from NEB to the first morphological evidence of apoptosis. (B) Time to slip mitosis, measured as the interval from NEB to cell adhesion. Mann-Whitney test was used for statistical analysis of three independent experiments. ns=no significance= p>0.05, *=p≤0.05, ***=p≤0.001, ****=p≤0.0001.

In terms of HDFs 87y ΔNΔKEN response to low dose taxol, we observed that these cells exhibit a cell fate profile similar to young cells (Figure 26).

As for chronic activation of the SAC (Figure 26), in the non-chronic activation with low dose taxol, HDFs 87y ΔNΔKEN resolve mitosis faster (Figure 27).

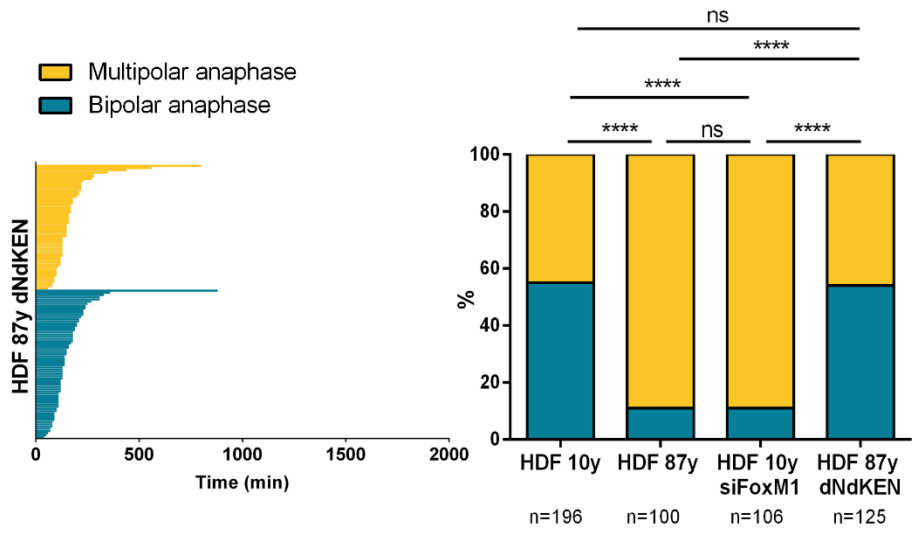

Figure 26 - Mitotic cell fate of HDFs 87y ΔNΔKEN in response to 5nM of Taxol. Each individual bar represents one individual cell analyzed by long-term phase-contrast time-lapse microscopy. The duration of the mitotic arrest was measured as the time from the NEB to anaphase onset. Statistical analysis performed with Fisher's exact test, of two independent experiments, n≥100, ns=no significance=p>0.05,****p ≤0,0001.

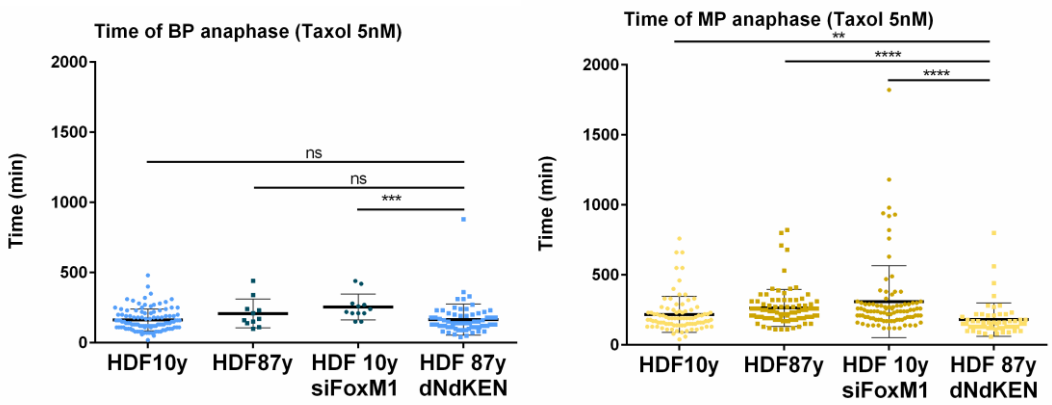

Figure 27- Duration of the mitotic arrest of HDFs versus HDFs 87y ΔNΔKEN in response to 5nM of Taxol. Each individual bar represents one individual cell analyzed by long-term phase-contrast time-lapse microscopy. The duration of the mitotic arrest was measured as the time from the NEB to anaphase onset. Statistical analysis performed with Mann-Whitney testof two independent experiments, ns=no significance=p>0.05, **=p≤0.01, ***=p≤0.001, ****=p≤0.0001.

In conclusion, we have shown that FoxM1 downregulation in mitotic old cells determines their cell fate in response to anti-mitotic drugs, turning mitotic death the major outcome.

III-2 – FoxM1 repression increases the pro-apoptotic efficacy of anti-mitotics in a fibrosarcoma cell line

Taking into account that FoxM1 came out as a molecular determinant of the mitotic cell fate decision in the presence of anti-mitotic drugs, and given its significant role in oncogenesis, we asked if induced downregulation of FoxM1 in combination with anti-mitotics could act as an efficient therapy to kill cancer cells.

To address this, we used long-term phase-contrast live cell imaging to examine the cell fate in response to anti-mitotics of a fibrosarcoma cell line (HT-1080) that overexpresses FoxM1 (Figure 28).

We knocked-down FoxM1 in this tumor cell line (Figure 29A) and we observed that under optimal conditions, there is a significant increase of the mitotic duration when FoxM1 is downregulated (Figure 29B), which corroborates with our findings in primary human dermal fibroblasts.

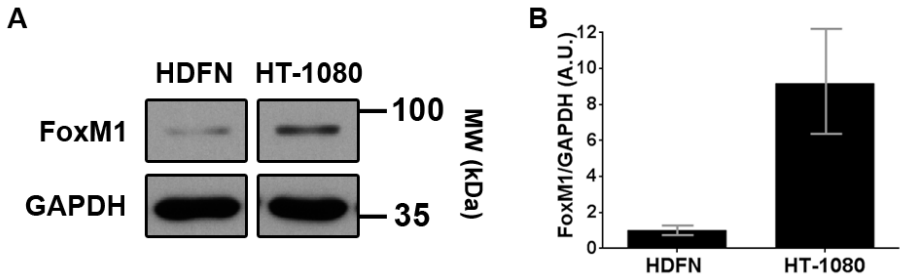

Figure 28 - FoxM1 overexpression in the fibrosarcoma cell line HT-1080. (A) FoxM1 expression levels in total cell extracts of asynchronous cell cultures of HDFN and HT-1080. (B) Protein levels were normalized against GAPDH levels used as loading control. In three independent experiments, there was an average of 9-fold increased expression of FoxM1 in HT-1080 when comparing with HDFN.

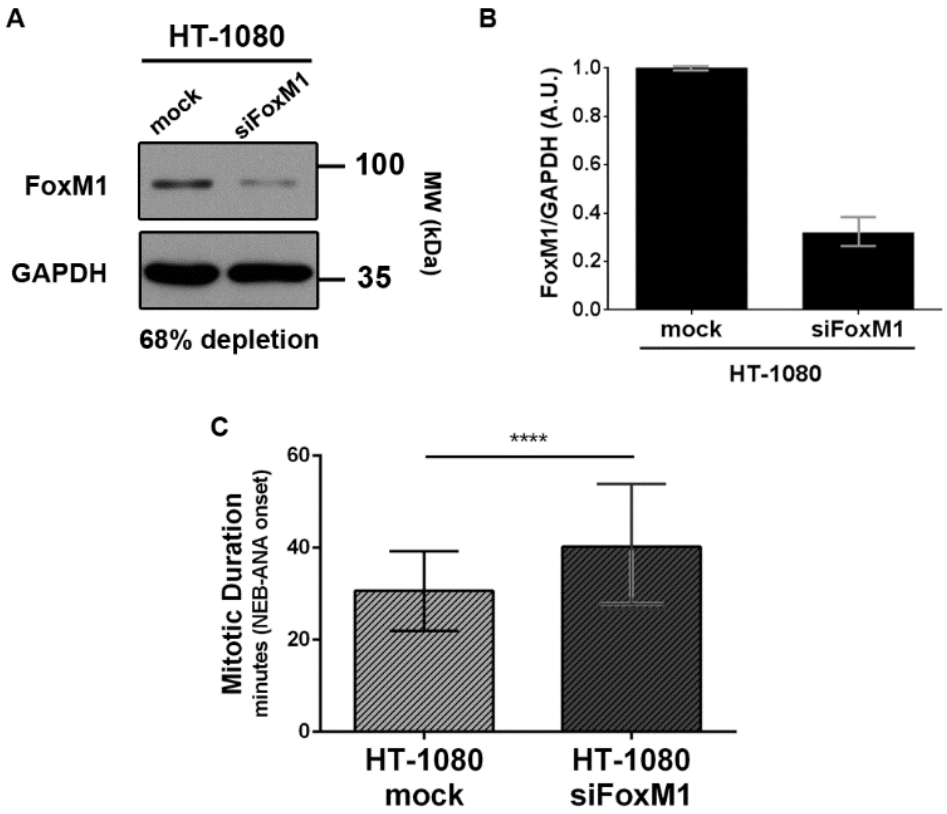

Figure 29- FoxM1 RNAi silencing in HT-1080. (A) FoxM1 protein levels following RNAi silencing in HT-1080. An average of 68% of depletion in three independent experiments was obtained. (B) Depletion levels were quantified against the levels of GAPDH used as loading control. (C) Mean with standard deviation of the mitotic duration of HT-1080 (30,6±9,6 minutes) and HT-1080 siFoxM1 (40,2±12,1 minutes). Statistical analysis performed with Mann-Whitney test of three independent experiments, with n≥100 cells for each condition, ****p≤0,0001.

We then treated the fibrosarcoma cell line with three different anti-mitotic drugs (nocodazole (3,3μM), Taxol (500nM) and STLC (5μM)) and found evidence for a pro-apoptotic efficacy of FoxM1 downregulation in combination with anti-mitotics (Figure 30). This is, independently of the anti-mitotic drug used, downregulating FoxM1 in combination increases the percentage of fate decision to 98-99% of death in mitosis.

Again we measured the duration of the mitotic arrest from NEB to mitotic death in the different conditions (Figure 30). We found that FoxM1 repression in HT-1080 decreases the time cells take to die (Figure 31). Time to slip out of mitosis is not shown, as the number of FoxM1 RNAi-depleted cells that chose slippage was too small.

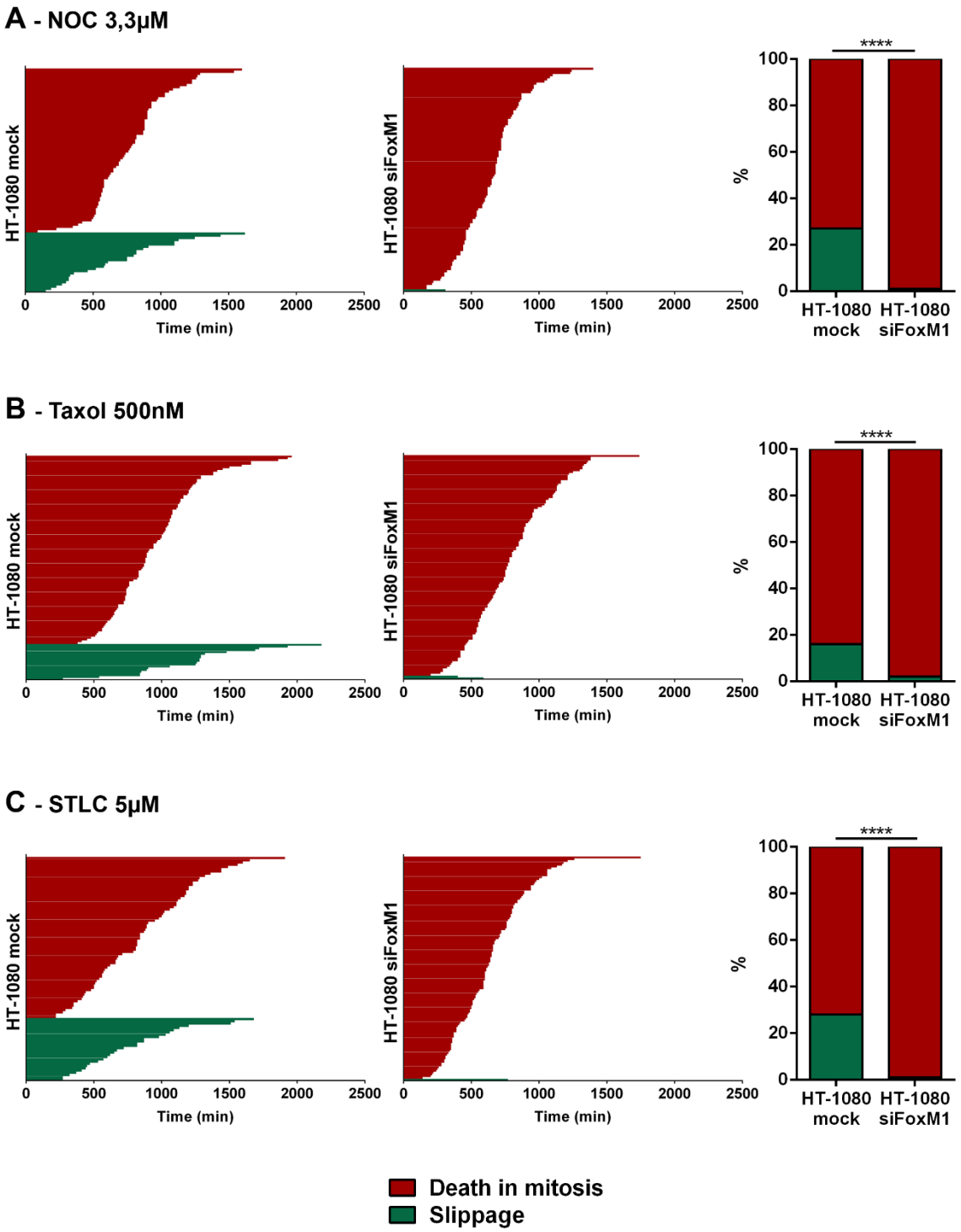

Figure 30 - Mitotic cell fate of HT-1080 fibrosarcoma cells in response to anti-mitotic agents and FoxM1 depletion. Fate profiles of mock- and FoxM1 siRNA-treated HT-1080 cells exposed to (A) nocodazole (NOC) 3,3µM, (B) taxol 500nM and (C) S-trityl-L-cysteine (STLC) 5µM. Each individual bar in the graphs represents one individual cell analyzed by long-term phase-contrast time-lapse microscopy. The duration of the mitotic arrest was measured as the time from the NEB to the respective fate. n≥100 cells in each condition. Statistical analysis performed with Fisher's exact test of three independent experiments, ****=p≤0.0001.

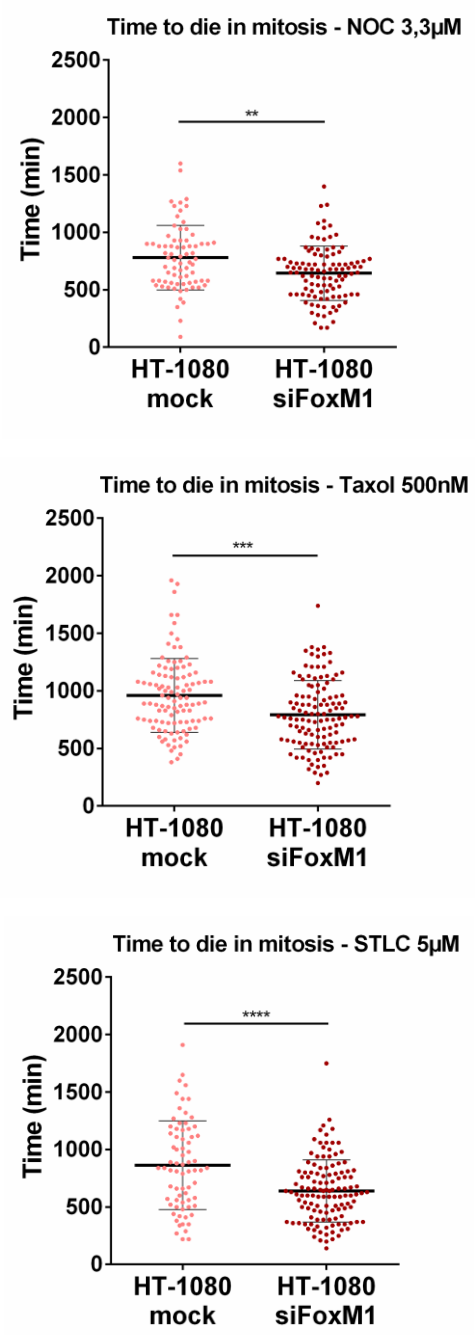

Figure 31- Duration of the mitotic arrest from NEB to cell death in mock- and FoxM1 siRNA-depleted HT-1080 cells upon exposure to anti-mitotic drugs. Each dot represents one cell. Time to die in mitosis, measured from the frame that showed NEB to the first frame that presented a morphological evidence of apoptosis. Statistical analysis performed with Mann-Whitney test of three independent experiments, **=p≤0.01, ***=p≤0.001, ****=p≤0.0001.

In sum, our data show that FoxM1 repression increases the pro-apoptotic efficacy of anti-mitotics against the HT-1080 fibrosarcoma cell line, and support the therapeutic potential of this combined strategy to kill cancer cells that overexpress FoxM1.

Chapter IV - Discussion

FoxM1 is a determinant of mitotic cell fate decision in response to anti-mitotics

In this project, we aimed to assess the fate profile of young versus old cells in response to anti-mitotic drug treatment and if it was dependent on FoxM1 expression levels.

We initially observed FoxM1 downregulation in asynchronous extracts of primary human dermal fibroblasts from individuals with advancing age, in agreement with previous findings showing that several mitotic genes, many of them direct targets of FoxM1, were gradually downregulated in fibroblasts isolated from young, middle-age, and old-age humans showed (Ly et al., 2000). However, being FoxM1 responsible for the transcriptional activation of the G2/M-specific gene cluster in mammalian cells and its levels correlating with the cellular proliferative state, we asked if there was indeed an intrinsic downregulation of FoxM1 in old mitotic cells or if this was just a consequence of the lower mitotic index of elderly cell populations. Using mitotic protein extracts from young, middle and old age fibroblasts, we demonstrated that there is an intrinsic cellular downregulation of FoxM1 due to chronological ageing, as well as of its well characterized direct targets, CENP-F, cyclin B and Plk-1.

Because protein levels of several mitotic regulators were reduced in old cells, we asked if SAC robustness was weakened in those cells. Therefore, we challenged the SAC with different anti-mitotic drugs and we tracked individual cell behavior using long-term phase-contrast live cell microscopy. This methodology circumvents the confusing interpretations of population-based assays. We found that independently of the anti-mitotic drug used, old cells display an increased sensitivity, i.e. higher percentage of mitotic cell death. When we downregulated FoxM1 in young cells, the percentage of cells dying in mitosis increased significantly. Equally, when we overexpressed FoxM1 in old cells they became less prone to mitotic death. Overall, this validates the role of FoxM1 in the mitotic cell fate decision in response to anti-mitotics, as low FoxM1 levels correlate with a higher sensitivity to these drugs (Figure 32). This corroborates with the fact that in tumors, high expression of FoxM1 correlates with poor prognosis (Gentles et al., 2015).

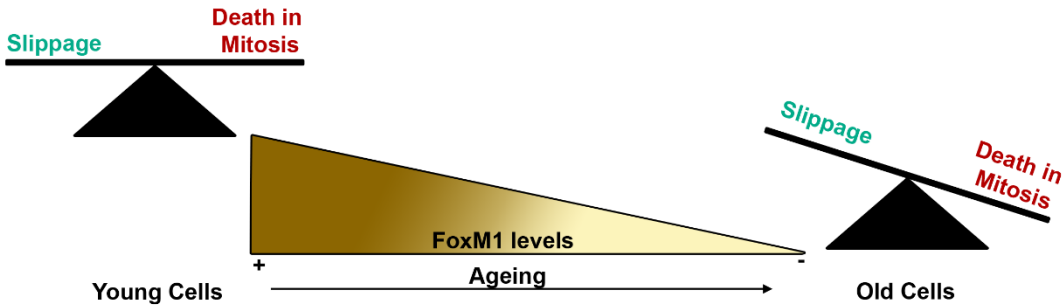

Figure 32 – The role of FoxM1 in the mitotic cell fate decision in response to anti-mitotics during human chronological ageing. As we get older, low expression of FoxM1 correlates with a higher sensitivity to these drugs.

In this study, human aged fibroblasts were exposed to anti-mitotics and imaged by long-term phase contrast microscopy for the first time. Comparing with RPE-1, a human immortalized retinal pigment epithelial cell line normally used as an untransformed control in cell line studies, HDFs exhibited very similar durations of the mitotic arrest imposed by the chronic activation of the SAC with spindle poisons. However, HDFs were more susceptible to die in mitosis than RPE-1 cells (Brito and Rieder, 2009). This supports the idea that the relationship between the duration of a mitotic arrest and survival is drug and cell type specific.

Based on the idea that in the complete absence of microtubules (NOC 3,3μM condition) the duration of a mitotic arrest reflects the "strength" of the SAC (Weaver and Cleveland, 2005), we observed that even though several mitotic and SAC genes were downregulated in old cells, their SAC is not weakened, as the duration of the mitotic arrest is not significantly different from young cells, or even higher, as in the case of Taxol 5nM treatment. This is in line with the idea that partial inactivation of the SAC generates a feedback-signaling loop that leads to SAC activation (Jeganathan et al., 2007; Logarinho and Bousbaa, 2008). Therefore, we hypothesize that even though old cells have an overall decrease in the quantity of SAC proteins, they have more activation of this checkpoint due to the feedback-signaling loop. Considering the model currently proposed in the literature for the fate decision in response to anti-mitotic drugs (Gascoigne and Taylor, 2008), we were initially expecting that old cells, because they have less cyclin B1, would breach the mitotic-exit threshold first and they would be more slippage-prone than young cells. However, we found old cells to be much more susceptible to die in mitosis than young cells, and this cell fate decision to be determined by FoxM1 levels.

Confronted with this data, we thought of three possibilities that could be occurring: (1) old cells have less cyclin B1, however, they are degrading it slower during the mitotic arrest; (2) the levels of the mitotic-exit threshold and/or the death threshold are different between young and old cells; (3) old cells might have more pro-apoptotic (or less anti-anti-apoptotic proteins) in their basal conditions, and therefore breach the death threshold easily during an extended mitotic arrest. Of course, more than one possibility might be true. We observed that within the cells that slip out of mitosis, there is no significant difference between the time to slippage of HDFs 10y, HDFs 87y and HDFs 10y siFoxM1, this is, the mitotic-exit threshold is being breached at the same time. Also, being Cdc20 (one activator of APC/C) a FoxM1 target (Wang et al., 2002) and one of the mitotic genes downregulated with ageing (Ly et al., 2000), possibility number 1 is compatible with this data. Moreover, in a *C. elegans* model it seems that in a normal mitosis, cyclin B1 is degraded slower in adult mitotic germ cells than in the same germ cells derived from an early stage of development (Gerhold et al., 2015). We cannot dismiss possibility number 2, as it is completely unknown in the literature, however, it seems unlikely, since we are working with primary cells from the same cell type. Ideally, as future work, in our ageing model we would like to replace one allele of cyclin B1 with a cyclin B1-GFP and observe the degradation kinetics during a mitotic arrest caused by anti-mitotic drugs. Regarding possibility number 3, we would like to see if there is a downregulation of the anti-apoptotic protein Mcl-1, a direct target of FoxM1 (Hu et al., 2015), with human chronological ageing. Many studies claim that Mcl-1 is one of the major proteins involved in the decision to die in mitosis or to slip mitosis (Harley et al., 2010; Wertz et al., 2011). Therefore, in an aged cell, less FoxM1 would lead to less Mcl-1, the cell death activity threshold would be breached first, and death in mitosis would occur.

Our data suggest that old cells die faster in mitosis than young cells. We acknowledge that phase-contrast microscopy analysis does not provide a robust marker for the initiation of apoptosis. In this cell type, some cells suddenly blast, disassembling into apoptotic bodies, while others slowly shrink overtime and it's impossible to pinpoint the exact time when that phenomenon started to occur. So, in old cells, time to slip out of mitosis and the overestimated time to die in mitosis was similar, which means that indeed the real time to die is mitosis is faster than the

time to slip mitosis. Again, as for cyclin B1, we would like to replace an endogenous apoptotic protein marker for a fluorescence one and observe it's kinetics during a mitotic arrest caused by anti-mitotic drugs. However, we must keep in mind that our ageing model are primary cells, and in these cells it is extremely hard to do any type of genome editing, as well as they are very sensitive to phototoxicity.

Taking all these data together, we have developed a model to try to explain the age-dependent response to anti-mitotic drugs (Figure 33). The decision is dictated by two independent processes during the arrest: a slow, but progressive loss of cyclin B1 and a slow, but progressive loss of anti-apoptotic proteins. We believe that it is a loss of anti-apoptotic proteins instead of a gain of pro-apoptotic proteins since the fate profile is FoxM1-dependent and downregulation of transcriptor factor during chronological ageing will decrease the levels of anti-apoptotic target genes. Then, there are two thresholds, which were represented at the same level just for schematic simplicity. If cyclin B1 levels fall below the mitotic-exit threshold first, slippage occurs or if the anti-apoptotic proteins levels fall below the necessary ones to prevent apoptosis, then the cell dies in mitosis. Comparing young and old cells, we know that young cells have a higher amount of cyclin B1, however, the time to slip mitosis is occurring at the same time (around 1000min), which means that the velocity of cyclin B1 degradation is slower in old cells. Therefore, if the process of cyclin B1 degradation isn't a decisive variation factor in our model, it must be the loss of anti-apoptotic protein functions that is switching the fate profile of old cells to death in mitosis. We propose that old cells have less anti-apoptotic proteins in their basal conditions, and once they enter mitosis and are in the condition of a prolonged mitotic arrest, they reach the death threshold before the cyclin B1 threshold and therefore they die in mitosis.

As described for Cdk1 (Sakurikar et al., 2012), we found a molecular determinant that also controls both processes of the competing model. Depending on the cell type, it is crucial understanding how the anti- and pro-apoptotic functions of FoxM1 signaling are combined and integrated in the fate decision after a mitotic arrest.

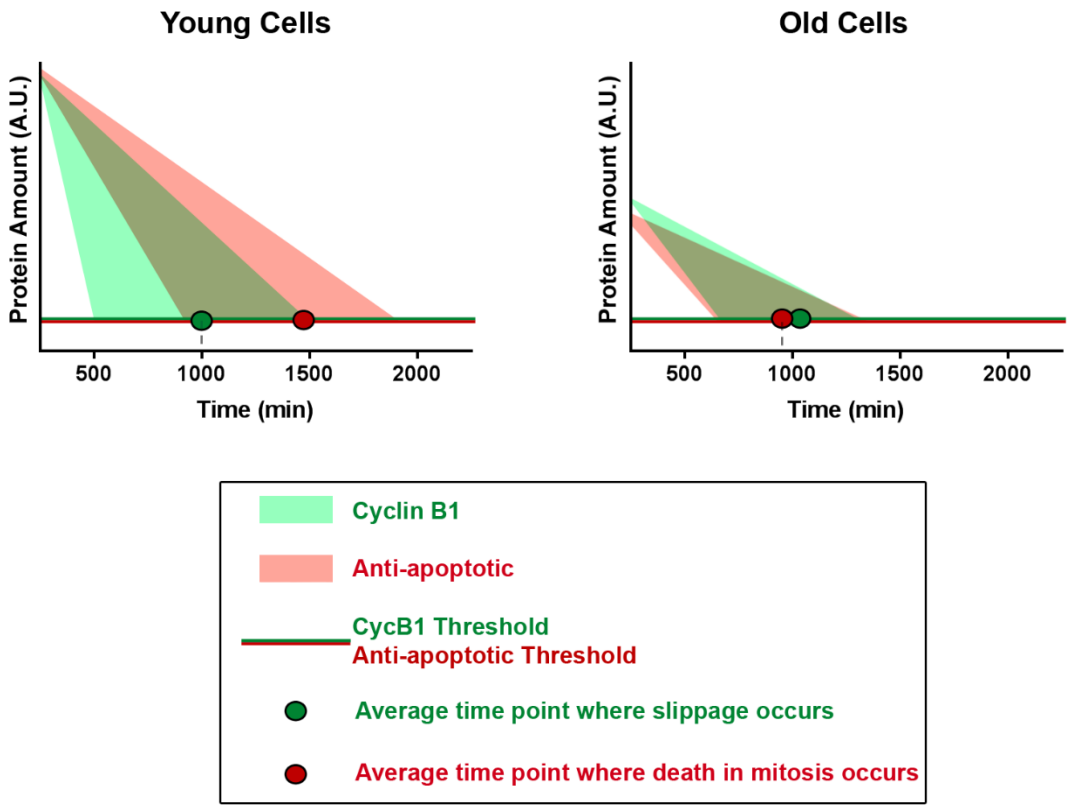

Figure 33 – Proposed Model for the response to anti-mitotic drugs in young and old cells. The decision is dictated by two independent processes during the arrest: a slow, but progressive loss of cyclin B1 and a slow, but progressive loss of anti-apoptotic proteins. Then, there are two thresholds, which were represented at the same level just for schematic simplicity. If cyclin B1 levels fall below the mitotic-exit threshold first, slippage occurs (young cells) or if the anti-apoptotic proteins levels fall below the necessary ones to prevent apoptosis, then the cell dies in mitosis (old cells).

Ultimately, there are some challenges in this research field. Firstly, it would be ideal to monitor molecular events in living cells, however, currently there are just a few bioprobes. Secondly, the ideal experiment that we could do to answer our questions, would be to expose a cell to anti-mitotics and observe its fate while simultaneously quantifying bioprobes of the molecular determinants of cell fate, however that is very demanding in primary cells.

Nevertheless, this work has provided a new molecular determinant of cellular response to anti-mitotics, FoxM1, which seems to be upstream of all the players described up to date. Furthermore, it has also been shown that cellular chronological age has implications in the response to spindle poisons, and therefore, the clinics should be aware of this implication in chemotherapy dosage.

Anti-mitotic drug treatment in combination with FoxM1 repression as an efficient chemotherapeutic strategy to kill cancer cells

In this project, we aimed to determine if induced downregulation of FoxM1 in combination with anti-mitotics could work as an efficient combined chemotherapy to kill cancer cells. We used the fibrosarcoma cell line HT-1080 i) to allow comparative analysis with the nontransformed fibroblasts counterparts, and ii) because we found this cell line to overexpress FoxM1.

Despite of overexpressing FoxM1 nine fold more than young fibroblasts, the HT-1080 cells were found to be more sensitive to nocodazole, taxol and STLC treatments (higher percentage of mitotic death) in comparison to the young fibroblasts. This was in contrast with what we expected considering our results from nontransformed fibroblasts showing that FoxM1 overexpression decreases mitotic death. However, previous studies have shown that cancer cells are less likely to survive mitosis upon exposure to anti-mitotics than nontransformed cells (Brito and Rieder, 2009). Also, we were expecting HT-1080 to divide faster than the control fibroblasts considering their higher FoxM1 levels. Again this was not the case, but actually is in agreement with prior reports describing that the average duration of a normal mitosis is prolonged in most cancer cell lines, when compared with nontransformed cell lines (Brito and Rieder, 2009).

However, when using a combination of FoxM1 downregulation with anti-mitotic drug treatment, independently on the drug, we increased the percentage of mitotic cell death to 98%-99%. Moreover, we have evidence that in HT-1080 siFoxM1, besides the increased the percentage of mitotic cell death, we also have a decrease in the time of the mitotic duration. This is, cells die more and faster, which is ideal in a chemotherapeutic context. We still need to observe the response of HT-1080 and HT-1080 siFoxM1 to taxol 5nM, as it is described a more realistic concentration in patients (Zasadil et al., 2014).

As a future perspective, we also want to address this drug combination approach in other cancer cell lines and afterwards, in a xenograft model too. It is of major importance to emphasize that our strategy is based on the circumstance in which we don't need a complete inhibition of FoxM1, as long as we combine it with anti-mitotics.

Ultimately, our results provide data evidencing a pro-apoptotic efficacy against this cancer cell lines overexpressing FoxM1, and reinforce the value of this type of combined chemotherapeutic strategy in the future in cancer cells that overexpress FoxM1.

Chapter V - Concluding Remarks

Using long-term phase-contrast live cell microscopy to track individual cell behavior, we came out with a model trying to explain the age-dependent response to anti-mitotic drugs. Old cells are more sensitive to this type of drugs than young cells and the fate decision is likely dictated by two processes during the arrest: a slow, but progressive loss of cyclin B1 and a gradual loss anti-apoptotic proteins. Comparing young and old cells, we know that young cells have a higher amount of cyclin B1, however slippage occurs at the same time, which means that the velocity of cyclin B1 degradation is slower in old cells. Therefore, if the process of cyclin B1 degradation isn't a decisive variation factor in our model, it must be the loss of anti-apoptotic protein functions that is switching the fate profile of old cells to death in mitosis. We propose that old cells have less anti-apoptotic proteins in their basal conditions, and once they enter mitosis and are in the condition of a prolonged mitotic arrest, they reach the death threshold before the cyclin B1 threshold and therefore they die in mitosis. Our working model brings up a new molecular determinant of cell response to anti-mitotics, FoxM1, which seems to be upstream of all the players described up to date and makes the cross-talk between the slippage and death processes.

Furthermore, we show evidence that the cellular chronological age has implications in the response to spindle poisons, and therefore, the clinics should consider the patient's age for chemotherapy dosage. Most importantly, our data opens a new window for therapeutic intervention. Induction of cellular senescence in cancer cells, by FoxM1 repression or any other strategy, is a way to increase the pro-apoptotic efficacy of anti-mitotics, and a combined therapy might add significant value particularly in the treatment of FoxM1 overexpressing tumors.

Chapter VI - References

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