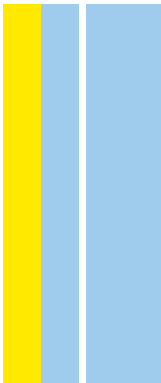


DOUTORAMENTO
BIOLOGIA MOLECULAR E CELULAR

Age-dependent sensitivity to antimetotics:
the role of FOXM1 and its therapeutic potential
Sara Marisa Duarte Vaz

D
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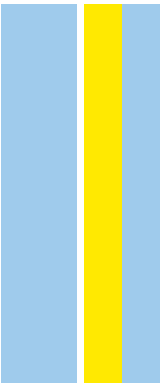


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Sara Marisa Duarte Vaz



SARA MARISA DUARTE VAZ

AGE-DEPENDENT SENSITIVITY TO ANTIMITOTICS: THE ROLE OF FOX M1 AND ITS THERAPEUTIC POTENTIAL

Tese de Candidatura ao grau de Doutor em
Biologia Molecular e Celular

Programa Doutoral da Universidade do Porto
(Instituto de Ciências Biomédicas Abel Salazar e
Faculdade de Ciências)

Orientadora - Doutora Elsa Clara Carvalho
Logarinho Santos

Categoria - Investigadora Principal

Afiliação - Instituto de Biologia Molecular e Celular
(IBMC), Instituto de Investigação e Inovação em
Saúde (i3S) da Universidade do Porto

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

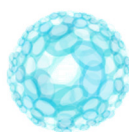
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***equal contribution**

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Abstract

Age-related molecular changes lead to a progressive failure of multiple organ systems and an accumulated incidence of chronic diseases, such as cancer. In the next decades, the European health care systems will be overwhelmed by the continuously increasing elderly population. Therefore, research focusing on the fundamental aspects of healthy ageing biology is at top priority. In this work, we bring mechanistic insight to FOXM1 proliferation-associated transcriptional activity in cellular ageing and cell response to antimitotic chemotherapy. Specifically, we show that along ageing, there is a gradual repression of FOXM1, that leads to mitotic dysfunction, generation of aneuploid cells and senescence. Re-establishment of FOXM1 expression in elderly cells ameliorates cellular ageing phenotypes. Moreover, we found FOXM1 levels to determine cell fate decision in response to antimitotic chemotherapeutic drugs. FOXM1 repression increases death in mitosis due to upregulation of the pro-apoptotic gene BMF. Interestingly, this regulatory axis is partly disrupted in chemotherapy-resistant tumour cells, thus providing a potential stratification biomarker for antimitotic chemotherapy response. Overall, our data disclose FOXM1 modulation as valuable therapeutic window against ageing and cancer.

Resumo

A acumulação de alterações moleculares durante o envelhecimento leva à disfunção progressiva de vários sistemas e órgãos, e a uma maior incidência de doenças crónicas, como o cancro. Nas próximas décadas, a alteração demográfica caracterizada pelo aumento da população idosa irá sobrecarregar os sistemas de saúde. Daí que a investigação científica focada nos aspetos fundamentais da biologia do envelhecimento seja prioritária. Neste trabalho, identificámos mecanismos moleculares da atividade transcricional do fator de proliferação FOXM1, que determinam tanto o envelhecimento celular e senescência, bem como a resposta celular a fármacos anti-mitóticos usados em quimioterapia. Mais especificamente, mostrámos que ao longo do envelhecimento ocorre uma repressão gradual do fator FOXM1, que induz disfunção mitótica, aneuploidia e senescência. O restabelecimento dos níveis de FOXM1 em células de dadores idosos conduz a uma melhoria dos fenótipos de envelhecimento celular. Adicionalmente, também demonstrámos que FOXM1 é um determinante molecular da resposta/sensibilidade celular a fármacos anti-mitóticos. A repressão de FOXM1 aumenta a morte em mitose devido à sobre-expressão do gene pró-apoptótico BMF. No entanto, este eixo de regulação está perturbado em células tumorais resistentes à quimioterapia, pelo que representa um potencial biomarcador de estratificação de pacientes que poderão beneficiar de tratamento com anti-mitóticos. No geral, os dados suportam a modulação da atividade FOXM1 como uma potencial estratégia terapêutica em envelhecimento e cancro.

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Abbreviations

APC/C - Anaphase promoting complex/cyclosome

BN - Binucleated

CCLE - Cancer Cell Line Encyclopedia

ChIP-seq - Chromatin immunoprecipitation sequencing

CHR - Cell cycle genes homology region

CIN - Chromosome instability

CRE - *Cis*-regulatory element

CTD2 - Cancer Target Discovery and Development

CytoD - Cytochalasin D

DBD - DNA-binding domain

DiM - Death in mitosis

DMEM - Dulbecco's modified Eagle's medium

DMSO - Dimethyl Sulfoxide

DOX - Doxycycline

DREAM - Dimerization partner, RB-like, E2F and MuvB

FACS - Fluorescence-activated cell sorting

FBS - Fetal bovine serum

FDR - False discovery rate

FISH - Fluorescence in situ hybridization

FOX - Forkhead Box

FOXM1-dNdK - Constitutively active truncated form of FOXM1

FPM - Fragments per million

FSC-A - Forward scatter area

GFP - Green fluorescent protein

GO - Gene Ontology

HDFs - Human dermal fibroblasts

HGPS - Hutchinson-Gilford Progeria Syndrome

IP - Intraperitoneally

KO - Knock-out

MAFs - Murine adult fibroblasts

MCC - Mitotic checkpoint complex

MEFs - Murine embryonic fibroblasts

MEM - Minimal essential medium Eagle–Earle

MI - Mitotic indexes

MMB - MYB-MuvB complex

MOMP - permeabilisation of the outer mitochondrial membrane

MT - Microtubule

MTA - Microtubule targeting agents

NA - Numerical aperture

NEB - Nuclear envelope breakdown

NRD - N-terminal repressor domain

OIS - Oncogene-induced senescence

OX - Overexpression

poly-HEMA - Poly-2-hydroxyethyl methacrylate

pS10-H3 - Phospho-Histone H3 (Ser10)

PTM - Post-translational modifications

RCM-1 - Robert Costa Memorial drug-1

RNAi - RNA interference

RNA-seq - RNA-sequencing

RT-PCR, qPCR - Real-time quantitative PCR,

SA - Senescence-associated

SAC - Spindle assembly checkpoint

SASP - Senescence-associated secretory phenotype

SA- β -gal - Senescence-associated- β -galactosidase

sgRNAs - Single guide RNAs

siRNA – Small interfering RNA

STLC - S-Trityl-L-cysteine

TAD - Transactivation domain

TIS - Therapy-induced senescence

TX - Paclitaxel

WO - Washout

C H A P T E R

I

General Introduction

The tight balance between cell division, senescence and death

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***equal contribution**

1. Ageing and cancer: two sides of the same coin

1.1 Overview

From a biochemical point of view, ageing is characterized by the increase of age-related molecular changes. Physiologically speaking, ageing is a virtually universal process of progressive failure of multiple organ systems and an accumulated incidence of chronic diseases, such as cancer. Indeed, cancer is an age-associated disease, with more than 80% of all malignancies being diagnosed after the age of 50 and 50% occurring in patients aged over 70 years (Roser & Ritchie, 2015). Consequently, there is a robust biological association among the mechanisms of ageing and carcinogenesis. In fact, there are several manipulations that simultaneously increase longevity and cancer protection (Serrano & Blasco, 2007), which points to ageing and cancer as being causally linked. Ageing arises due to accumulation of cellular damage and inefficient clearance of damaged cells. Cancer arises when damaged cells acquire uncontrolled proliferative advantage and cell death resistance. Consequently, the identification of genes involved in longevity pathways is a valuable strategy to unveil new and more efficient cancer therapies.

1.2 Cellular senescence and the ageing process

Several theories have been proposed to explain the accumulation of damaged cells during ageing and its impact in cancer development. Very briefly, they range from the model claiming that mutated genes accumulate throughout time (Medawar, 1952), to the theory stating that reproduction genes are naturally selected, becoming linked to disadvantages later in life (G. C. Williams, 1957). Others also suggest that in somatic (vs. germ line) cells there is reduced accuracy in energy saving and reproduction biological mechanisms, with the ultimate cost of deterioration and death (Kirkwood & Holliday, 1979). Some authors rely in the telomere shortening during each cell division (Lindsey et al., 1991) to explain cellular damage, ageing and cancer. Although none of these theories are universally accepted, they share a common denominator, the accumulation of cellular damage, namely, senescence.

Senescent cells are arrested in the cell cycle by the P16/RB or P53/P21 pathways (Childs et al., 2015), characterized by a number of nonexclusive markers such as the incapability to express proliferation genes (G. P. Dimri & Campisi, 1994; Goberdhan P. Dimri et al., 1996), high activity of senescence-associated- β -galactosidase (SA- β -gal) (Kurz et al., 2000) and an enlarged and flatter cell morphology (Druelle et al., 2016). Other markers include increased

levels of cell cycle inhibitors, such as P16INK4a, P21CIP1, P27, P19ARF, P53 and PAI-1 (Hernandez-Segura et al., 2018), increased DNA damage foci (Rodier et al., 2009), and loss of Lamin B1 (Hernandez-Segura et al., 2017). A major characteristic of senescent cells is the capacity of these cells to secrete a large number of factors, comprising growth factors, cytokines, chemokines, and proteases, known as the senescence-associated secretory phenotype (SASP) (Juan Carlos Acosta et al., 2013). Through their secretome, transient accumulation of senescent cells is crucial for beneficial functions such as limb patterning, wound healing, reprogramming and fibrotic resolution (Calcinotto et al., 2019). However, many markers of senescence persistently accumulate in aged human tissues (Chkhotua et al., 2003; G P Dimri et al., 1995; Y. Liu et al., 2009; Melk et al., 2004; Ressler et al., 2006). *In vivo* elimination of senescent cells (P16 positive cells) in naturally aged mice delayed the age-associated diseases in later life, while increasing median and maximum lifespans (Baker et al., 2016), supporting that chronic accumulation of senescent cells limits longevity. Overall, there are two major potential pathways in which cellular senescence may contribute to the ageing process: (1) senescence decreases the number of self-renewing cells; (2) through the SASP, cellular senescence can paracrinally disrupt tissue microenvironment. These lead to compromised tissue regeneration (J. H. Chen et al., 2007).

1.3 Senescence as an anti- and pro-cancer process

The permanent cell cycle arrest evokes the idea that senescence evolved at least in part to suppress the proliferation of damaged cells and tumorigenesis (Sager, 1991). Indeed, oncogene activation (such as mutated RAS, BRAF, HER2, EGFR, among others) in mammalian cells results in a permanent cell cycle arrest (Angelini et al., 2013; Braig et al., 2005; Courtois-Cox et al., 2008; Garbers et al., 2013; Serrano et al., 1997; Wajapeyee et al., 2008), a process called oncogene-induced senescence (OIS). Moreover, senescent cells are found in pre-neoplastic lesions, suggesting that OIS represents an initial preventive mechanism against excessive proliferation of oncogene-expressing cells (Collado et al., 2005; Collado & Serrano, 2010). It is known, that via the SASP, senescent cells can establish and maintain OIS of neighbouring cells, as inhibition of SASP factors such as IL-1, IL-6, IL-8 can promote OIS bypass and a more aggressive cancer cell phenotype (Juan C. Acosta et al., 2008; Di Mitri et al., 2014; Kuilman et al., 2008). Additionally, senescent cells can also facilitate cancer cell clearance through the recruitment of immune cells (Kang et al., 2011; Xue et al., 2007).

However, in recent years, increasing evidence has shown that the non-cell-autonomous effects of senescent cells through the SASP can also promote tumour progression (Coppe et al., 2006;

Kuilman et al., 2008; Laberge et al., 2015; Rodier et al., 2009). Several cancer chemotherapeutic drugs induce therapy-induced senescence (TIS) (Ewald et al., 2010). Likewise, patient treated with high-dose chemotherapy have been reported to have premature ageing features (Marcoux et al., 2013). While TIS is initially beneficial for cancer regression, it can lead to the chronic accumulation of senescent cells and tumour relapse, via increased proliferation of premalignant and malignant cells (Krtolica et al., 2001; Le Blanc et al., 2004), increased tumour vascularization (Coppe et al., 2006), the promotion of cancer stemness (Milanovic et al., 2018) and altered immunosuppression response (Di Mitri et al., 2014; Jackson et al., 2012). In fact, elimination of TIS cells in doxorubicin-treated mice decreased tumour recurrence and metastasis, as well as it decreased doxorubicin side-effects, such as bone marrow suppression, cardiac dysfunction, and fatigue (Demaria et al., 2017). This provided crucial evidence for the pro-cancer role of senescent cells and highlighted the elimination of senescent cells as an efficient strategy to avoid tumour relapse in cancer patients.

1.4 Apoptotic dysfunction as a hallmark of ageing and cancer

In 2000, Hanahan and Weinberg published the first hallmarks of cancer and described six barriers that cells need to overcome to become tumorigenic. One of them was acquiring the ability to evade cell death (Hanahan & Weinberg, 2000). More than 10 years later, senescence was fully accepted as a hallmark of ageing (López-Otín et al., 2013), and recently, apoptosis resistance was recognized as a hallmark of senescence (Hernandez-Segura et al., 2018). Therefore, damaged cells accumulated in cancer and ageing evolved mechanisms to circumvent apoptotic regulation.

In short, apoptosis comprises two pathways (extrinsic and intrinsic) that execute a highly regulated programmed cell death. When plasma membrane cell death receptors are activated, a signalling process activates Caspase-8 that, in turn, leads to the protease caspase cascade and the extrinsic apoptosis. The intrinsic or mitochondrial apoptosis is prompted by different damage stimuli that alter the tightly controlled balance of the pro- and anti-apoptotic proteins of the Bcl-2 protein family. When anti-apoptotic proteins are largely inactivated, BAX and/or BAK form permeabilizing pores at the outer mitochondrial membrane (MOMP) causing the release of Cytochrome c and other pro-apoptotic factors to the cytosol. These activate Caspase-9, that initiates the caspase cascade and programmed cell death (Green & Llambi, 2015).

It is well established that increased levels of anti-apoptotic Bcl-2 family members (BCL-2, BCL-XL, MCL-1, etc) are often found in cancer cells (Delbridge & Strasser, 2015; J. Y. Zhang, 2002), whereas pro-apoptotic proteins are often inhibited (Mi et al., 2000; Soengas et al., 2001). However, during ageing, the apoptotic dysfunction seems to be cell/tissue type specific, as some studies show increased apoptosis during ageing, while others describe apoptotic resistance (Cerella et al., 2016). For example, aged hematopoietic stem cells have an increased apoptosis rate and this is thought to contribute to the limited regenerative capacity of stem cells during ageing and reduced lifespan (J. Wang et al., 2014). On the other hand, a gradual age-dependent decline in the levels of several circulating pro-apoptotic serum markers (Cytochrome c, FASLG, among others) was found in healthy aged subjects (Kavathia et al., 2009). These contradictory results can be interpreted as an overall apoptotic deregulation during ageing that affects different cells, in different cell stages, with both up- and downregulation of apoptosis impacting on tissue integrity. Cells with accumulated irreversible damage should be able to execute programmed cell death as a tumour suppression mechanism. Therefore, damage accumulation during aging should lead to increased apoptosis. However, senescence can be an alternative cell fate (Childs et al., 2014). Senescent cells have been reported to be resistant to apoptosis (Hernandez-Segura et al., 2018; Seluanov et al., 2001). Thus, chronic accumulation of senescent cells during ageing reduces the apoptotic response in certain tissues, whereas non-senescent damaged cells increase apoptosis in other tissues. More studies will be needed to understand this duality.

1.5 Cell division deregulation in ageing and cancer

As for apoptosis, it is known that chromosomal instability (CIN), a mitotic dysfunction phenotype characterized by a high recurrent chromosome mis-segregation rate and the generation of aneuploid daughter cells (cells with a chromosome content that is not an exact multiple of the haploid complement), increases genetic heterogeneity and fuels tumour progression (Ben-David & Amon, 2020). Indeed, approximately 90% of solid tumours are aneuploid (Taylor et al., 2018). Although first arising at different stages in distinct cancer types, generally aneuploidy increases with tumour progression (Ben-David & Amon, 2020; Gao et al., 2016; Laubert et al., 2015; Ross-Innes et al., 2015; Teixeira et al., 2019).

Ageing has also been linked to increased aneuploidy for the past decades (Jacobs & Court Brown, 1966). This association has been well documented for oocytes and is considered to be the main cause of female reproductive infertility as well as of miscarriage and birth defects in humans (Nagaoka et al., 2012). Additionally, men are for long known to be subject to age-related loss of the Y chromosome (Jacobs et al., 1963; Pierre & Hoagland, 1972; Stone &

Sandberg, 1995). Also, loss of an X chromosome with advancing age has been reported for females (Fitzgerald & McEwan, 1977; Guttenbach et al., 1994), though the physiological consequence of this phenomenon remains unclear. Similarly, to sexual chromosomes, an increase in autosomal aneuploidy has been observed in elderly peripheral blood lymphocytes, bone marrow cells, myeloid cells and fibroblasts (Mukherjee et al., 1996; Mukherjee & Thomas, 1997; Nakagome et al., 1984). The relative high frequencies of multiple mis-segregation events (involving more than one chromosome) have raised the question whether there is a general dysfunction of the mitotic apparatus in aged cells (Carere et al., 1999). Moreover, oligonucleotide microarrays with fibroblasts isolated from young, middle-age, old-age humans and humans with progeria (a syndrome of premature ageing), showed that ageing is accompanied by decreased cell proliferation and an accumulation of mitotic defects, as several mitotic genes are downregulated, such as CCNA1, CCNB1, PLK1, CDC20, CENPA, CENPF, MKLP1, KIF11, KIF2C and FOXM1 (Ly et al., 2000). Taken together, the analysis of the mitotic process in ageing models, even though narrow, has often revealed the presence of dysfunctional mitotic phenotypes. However, functional analyses are limited, even though valuable, for future strategies to treat age-related diseases such as cancer.

1.6 The interconnected triad that governs ageing and cancer

Both ageing and cancer are impressively complex and their causes are incontestably multifactorial, so it is foreseeable that the underlying links between them will also be multidimensional. In this section, we have briefly discussed the importance of senescence, cell death and mitosis. Summarizing, as we age, we chronically accumulate senescent cells that transform the cell microenvironment and decrease cell/tissue fitness mainly through their secretome. Moreover, aged cells may also acquire resistance to apoptosis, while generating aneuploidy through general dysfunction of the mitotic apparatus, **Figure 1.1**. How mitotic dysfunction leads to generation of senescent cells during ageing will be discussed in **Chapter II**. Further studies are needed to understand the feedback loops between these three biological pathways.

In the ageing context, mutations and alterations that increase fitness, such as an uncontrolled proliferative advantage, will be naturally selected and increase the risk to develop cancer. This is termed the Adaptive Oncogenesis Model (Henry et al., 2011), **Figure 1.2**. After the selection of adaptive alterations, increased SASP, decreased mitotic fidelity (causing chromosome instability and aneuploidy) and resistance to apoptosis will further fuel tumour progression.

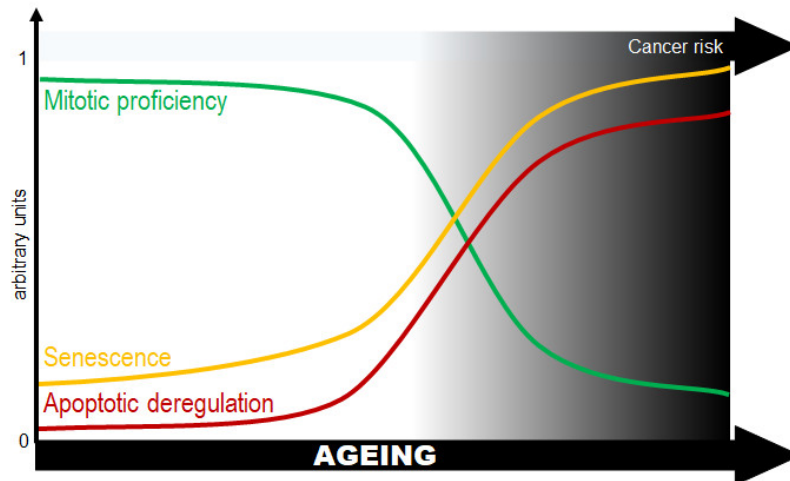


Figure 1.1. Senescence, mitosis and apoptosis during ageing. There is increasing evidence that as we age, we accumulate senescent cells, we deregulate the apoptotic machinery and we decrease mitotic proficiency. All these altered biological pathways contribute to an increased risk for cancer development.

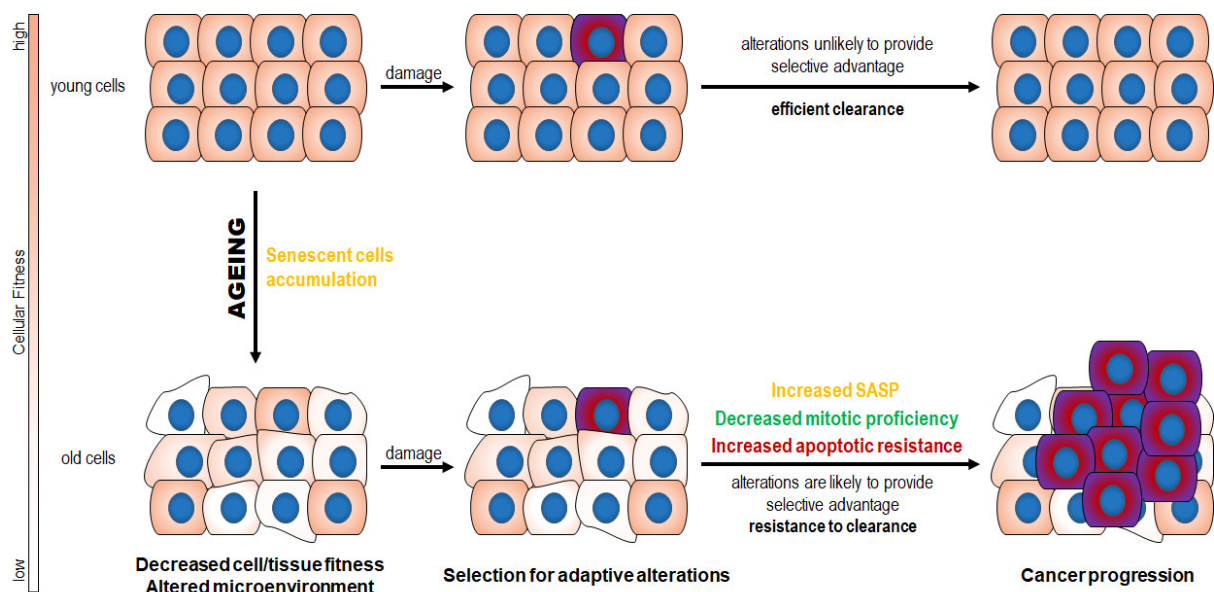


Figure 1.2 - Adaptive Oncogenesis Model. Healthy populations of young cells hold inherent high fitness that ensures their adaptive selection. Contrarily, with ageing, cellular fitness drops leading to an increased selection for certain alterations that are adaptive and increase cancer development risk.

A more comprehensive understanding of how age-associated alterations in cells and tissues induce and/or fuel carcinogenesis is in need, since it will contribute to the improvement of therapeutic approaches. Importantly, the impact of these alterations in the cellular response to antimetabolic drugs, widely used in mainstream chemotherapeutic strategies, remains unexplored. In the next sections, the current understanding of the mechanism of action of antimetabolites will be revised.

2. Cellular response to antimitotic chemotherapy

2.1 Antimitotics as cancer chemotherapeutic drugs

Targeting mitosis for cancer treatment is an age-old concept that has been around in the clinics for more than half a century. These anti-proliferative drugs, often called antimitotics, mitotic blockers, microtubule (MT) poisons or microtubule targeting agents (MTA), have been remarkably successful in treating a broad range of different malignancies as they target the persistent growth phenotype exhibited by cancer cells (Jordan & Wilson, 2004). Notwithstanding substantial research elucidating their mechanism of action, how clinical response is achieved remains elusive. The use of antimitotics is limited by their side effects in normal tissues and the development of drug resistance (McGuire et al., 1989).

2.2 Classical and new-generation MTAs

MTAs can be classified into classical spindle poisons, such as paclitaxel, a drug that binds to the taxane site in polymerized β -tubulin at the luminal side of microtubules. This binding generates a conformational change on polymer structure, which increases the affinity of the interaction among tubulin molecules, thereby increasing stability and reducing the depolymerisation rate (Derry et al., 1995; Nogales et al., 1998). Paclitaxel was approved by the Food and Drug Administration in 1992 for the treatment of ovarian cancer, and it is currently used also in breast cancer, non-small cell lung cancer and Kaposi's sarcoma, while docetaxel, its semi-synthetic analogue, is indicated for breast, gastric, head and neck, and prostate cancers (Mukhtar et al., 2014). Classical microtubule inhibitors have been successful in the clinics, but resistance to the treatment frequently arises. Although many molecular players have been shown to attenuate MTA response, antimitotic drug resistance is classified into three major pathways: 1) induction of multi-drug resistance efflux transporters, that pump taxanes out of the cell (Barbuti & Chen, 2015); 2) point mutations and alterations in tubulin isotypes, preventing MTA binding (Orr et al., 2003); 3) suppression of the apoptotic response via the Bcl-2 family (Whitaker & Placzek, 2019). Additionally, patients exhibit severe secondary side effects upon treatment. MTAs affect healthy proliferative cells, as well as resting and differentiated cells that also rely on dynamic microtubules for cytoskeletal functions and intracellular transport processes. Paclitaxel, given as a single agent or in combination with cisplatin, causes peripheral neurosensory manifestations, with some patients developing a severe sensory-motor polyneuropathy. Myelosuppression is also witnessed, mainly

neutropenia, the abnormally low number of neutrophils. While myelosuppression is clinically manageable, neurotoxicity can cause long-lasting damage (Rowinsky et al., 1993). Therefore, new generation antimetotics have been developed to circumvent resistance and secondary side effects. One example is the small-molecule inhibitor of kinesin 11 (KIF11/Eg5), a plus end-directed kinesin motor protein essential for centrosome separation, assembly of a bipolar spindle and therefore, correct chromosome segregation (Slangy et al., 1995). Inhibition of Eg5 activity produces monopolar spindles that lead to mitotic arrest, which terminates in cancer cell apoptosis (Mayer et al., 1999; Sakowicz et al., 2004). Despite decreasing the side effects, Eg5 inhibitors (Ispinesib, EMD534085 and Filanesib) failed to generate effective responses as a monotherapy in clinical trials (Gomez et al., 2012; Hollebecque et al., 2013; Lorusso et al., 2015). But interestingly, paclitaxel resistant cancer cells remain sensitive to Eg5 inhibition (Marcus et al., 2005), which opens a chemotherapeutic window for clinical trials in paclitaxel resistant tumours.

2.3 Mechanism of action of antimetotic drugs

Time-lapse live cell imaging revolutionized the understanding of MTA response, as single cell analysis circumvented the need for synchronization protocols and confusing interpretations of population-based assays. Due to impaired microtubule dynamics, MTAs activate the spindle assembly checkpoint (SAC), a control mechanism that prevents mitotic progression in the presence of improperly attached chromosomes to the spindle (Jordan & Wilson, 2004). This induces a prolonged mitotic arrest, as unattached kinetochores catalyse the formation of the mitotic checkpoint complex (MCC) complex that inhibits APC/C-CDC20 activity, thereby sustaining considerable levels of Securin, Cyclin B1, and CDK1 activity (Musacchio & Salmon, 2007). However, mitotic cells cannot maintain this status indefinitely due to slow erosion of Cyclin B1 levels, independent of active SAC signalling (Brito & Rieder, 2006). Ultimately, two major outcomes have been described to follow this chronic arrest (Rieder & Maiato, 2004; Weaver et al., 2007): the cell can die in mitosis or the cell can exit mitosis without dividing its genome. The currently accepted model for the mitotic cell fate decision proposes that during the chronic arrest, two competing pathways are in motion and the fate will be determined by the pathway that reaches its activity threshold first. The first network is the leaky and progressive loss of Cyclin B1. If the Cyclin B1 levels drop below the threshold necessary to maintain CDK1 activity and the mitotic state, the cell will exit mitosis as a 4N entity, a process also called mitotic slippage. The second network concerns the steady rise in cell death signalling up to the point-of-no-return from intrinsic apoptosis, and the cell will die in mitosis (Gascoigne & Taylor, 2008), **Fig. 1.3**. In support of the competing networks model, delaying

mitotic slippage by either Cyclin B1 (non-degradable) overexpression, APC/C pharmacologic inhibition, or CDC20 depletion shifts the fate profile to death in mitosis (DiM), whereas inhibiting caspase activation lays cells more prone to mitotic exit (Gascoigne & Taylor, 2008; Sloss et al., 2016a; Zeng et al., 2010). While the Cyclin B1 network is well understood, the intricate signalling network dictating DiM remains elusive (Ruan et al., 2019). Death in mitosis is controlled primarily by the Bcl-2 family, via the activation/oligomerization of the pore-former proteins at the mitochondria, as depletion of BAX and BAK1 blocks mitotic cell death and promotes mitotic exit (Haschka et al., 2015). However, the impact of different BCL-2 proteins in the context of MTA is still unclear, as well as the crosstalk between the competing networks

Fig. 1.4.



Figure 1.3. The competing networks model. After a prolonged mitotic arrest due to MTA exposure, the decision to slip out of mitosis (left) or to die in mitosis (right) is dictated by two processes during the arrest: a slow, but progressive loss of cyclin B1 and a slow, but steady rise in cell death activity. Mitotic cell fate is dictated by which threshold is breached first (Gascoigne & Taylor, 2008).

Among the anti-apoptotic proteins, MCL-1 has been reported as a main determinant of DiM. Its continued inhibitory phosphorylation during the chronic arrest operates as a clock (or mitotic death timer), leading to apoptosis. Therefore, upon exposure to antimetabolites, if MCL-1 levels are low, the arrested cell is tipped into the apoptotic cascade, whereas if MCL-1 levels are high, it will allow time for leaky Cyclin B1 degradation and slippage (Harley et al., 2010; Haschka et al., 2015; Sloss et al., 2016a; Tunquist et al., 2010; Wertz et al., 2011). Nevertheless, on its own, the loss of MCL-1 cannot explain the inter- and intra-line variation of cancer cells to antimetabolites. RNAi depletion or chemical inhibition of BCL-2 failed to lead to apoptosis (Bah et al., 2014; S. Huang et al., 2016; Shi et al., 2011), but BCL-2 overexpression can delay DiM long enough for Cyclin B1 levels to drop below the CDK1 activity threshold (Gascoigne & Taylor, 2008; Haschka et al., 2015; Topham et al., 2015). Another anti-apoptotic protein, BCL-W, has also been shown to prevent mitotic cell death (S. Huang et al., 2016). Lastly, BCL-XL has been described to act redundantly with MCL-1 as major negative regulators of DiM during the mitotic arrest. In cells with low BCL-XL levels, the abrogation of

MCL-1 alone is sufficient to promote apoptosis. However, MCL-1 loss is insufficient to promote DiM in cancer cells with high levels of BCL-XL, due to substantial BAX/BAK sequestration by BCL-XL (Bennett et al., 2016; Shi et al., 2011; Topham et al., 2015). Dual inhibition of MCL-1 and BCL-XL prompts substantial cell death in mitosis, but not in interphase (Shi et al., 2011), indicating that mitosis-specific pro-apoptotic signals contribute to the mitochondrial outer membrane permeabilization and caspase cascade activation in a prolonged mitotic arrest. These signals can be dictated by a set of pro-apoptotic BH3-only antagonists expressed or activated under MTA exposure, such as NOXA and BIM (Díaz-Martínez et al., 2014; Haschka et al., 2015; Moustafa-Kamal et al., 2013). NOXA acts mainly by mediating the degradation of MCL-1, that in turn releases BIM from MCL-1 sequestration, thus promoting DiM (Haschka et al., 2015). The influence of other pro-apoptotic BH3-only proteins to mitotic death upon antimitotic drug treatment is less understood (Erlacher et al., 2006; Labi et al., 2014; P. Wang et al., 2014). Interestingly, CDK1, the major kinase responsible for inducing and maintaining the mitotic state, has also been described to have opposite functions in the execution of DiM. In one hand, CDK1/Cyclin B1 can cause an inhibitory phosphorylation on Caspase-9, which protects cells from apoptosis during mitosis (Allan & Clarke, 2007). In the other hand, this kinase is also responsible for the previously mentioned inhibitory MCL-1 phosphorylation, that targets its proteasomal degradation (Harley et al., 2010), as well as inhibitory phosphorylation of other anti-apoptotic proteins, BCL-2 and BCL-XL (Eichhorn et al., 2013; Han et al., 2014; Sakurikar et al., 2012; Terrano et al., 2010), all favouring DiM. These opposing functions of CDK1 might be interpreted as a mechanism that ensures that the dividing cell does not die in a normal mitosis, due to inhibition of Caspase-9, whereas under chronic activation of the SAC, it starts inhibiting anti-apoptotic proteins and triggering DiM to prevent the generation of a polyploid cell. Moreover, other players have been described as major upstream determinants of mitotic cell fate decision. Of special relevance, c-MYC expression induces upregulation of BIM, BAD and NOXA, while repressing BCL-XL, sensitizing cells to antimitotic drugs (Topham et al., 2015). Another player is cGAS, that during the mitotic arrest, promotes a slow build-up of IRF3 phosphorylation that promotes DiM in a BCL-XL-dependent manner (Zierhut et al., 2019). DNA damage has also been proposed to play a role, as BID is phosphorylated by ATM/ATR, sensitizing paclitaxel-treated cells to DiM (P. Wang et al., 2014).

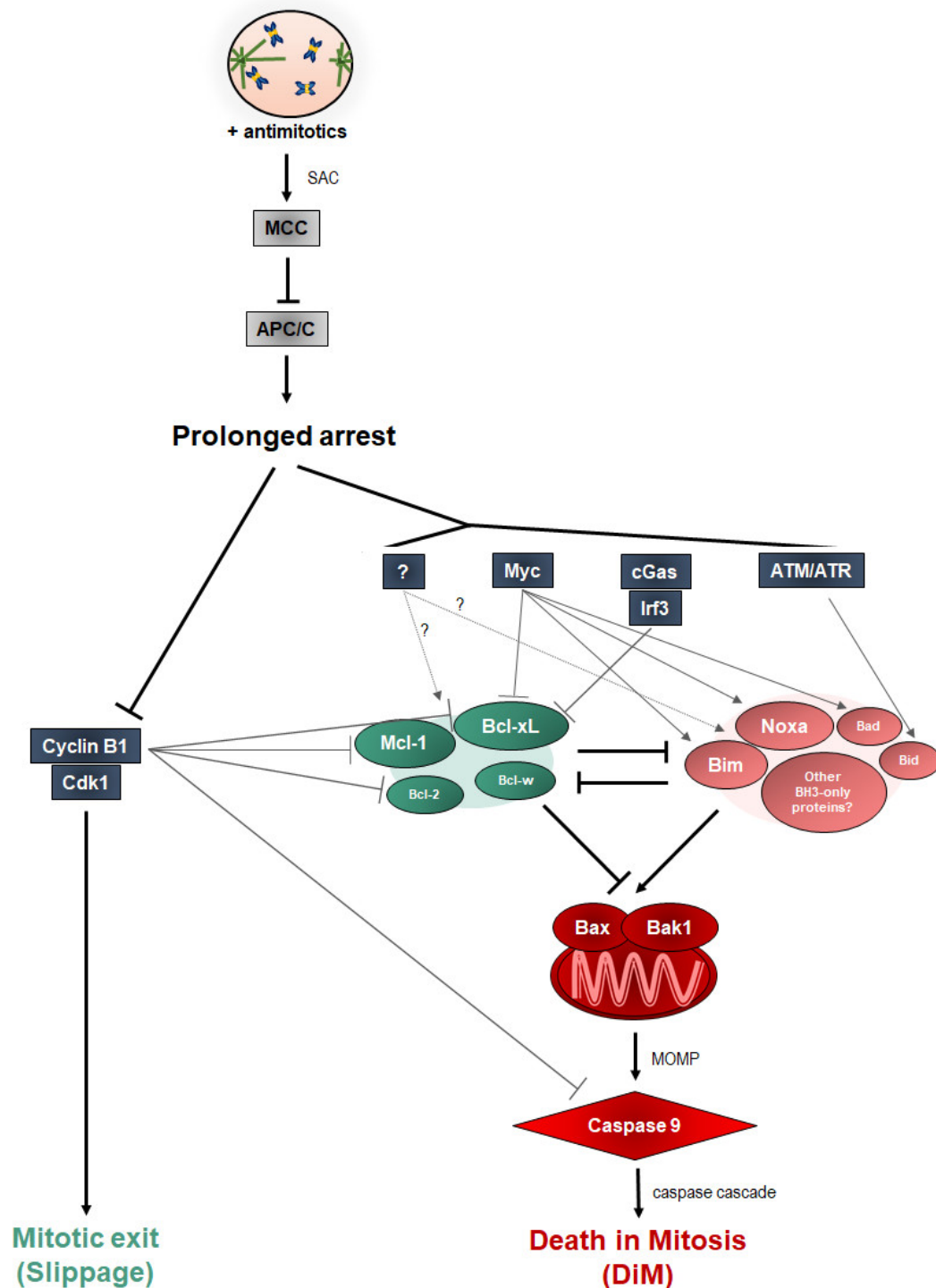


Figure 1.4. Molecular determinants of the mitotic cell fate decision. High concentrations of antimitotics chronically activate the spindle assembly checkpoint (SAC), causing a prolonged mitotic arrest. The fate will be dictated by the kinetics of gradual loss of function of CDK1 due to Cyclin B1 degradation or the caspase cascade activation. The presented model represents the main players already described to play a role in the competing networks.

Regarding the post-mitotic response, i. e., the fate of 4N interphase cells that slipped mitosis, the underlying mechanisms are incompletely understood. A cell type-, antimitotic type- and time-dependent complex system seems to regulate the outcome of these tetraploid cells

(Sakurikar et al., 2012). Nonetheless, these cells can die in interphase, arrest in interphase or continue cycling (Rieder & Maiato, 2004; Weaver & Cleveland, 2005). It remains to be elucidated if apoptosis results from the activation of pro-apoptotic pathways during the mitotic arrest, or from *de novo* signalling due to DNA damage (Gascoigne & Taylor, 2008; Sakurikar et al., 2012). The cell cycle arrest appears to be p53-dependent (Demidenko et al., 2008). Additionally, mitotic slippage after spindle poisons treatment is associated with genomic instability, senescence and the fueling of tumor progression. Elimination of these cells has been shown to be beneficial for chemotherapeutic response (Demaria et al., 2017). However, preventing slippage, and subsequent polyploidy, in the first place could prove to be a more advantageous strategy. For that, a better understanding of the molecular players that orchestrate the switch from mitotic slippage to mitotic cell death is crucial.

As already discussed in the previous section, an appealing approach is to study genes and pathways that modulate the interconnected triad of cell division, senescence and death. FOXM1 is a master regulator of cell division, but it is also known to regulate cell death and senescence. Thus, its role as molecular determinant of cell fate decision to MTA should be investigated.

3. FOXM1: a master regulator of cell division, death and senescence

3.1 The transcription factor FOXM1

Forkhead box M1, FOXM1, formerly called MPP2, FKHL16, HFH-11, INS-1 MPHOSPH2 or Trident, is a transcription factor of the Forkhead box protein superfamily which is characterized by an evolutionary conserved winged helix DNA-binding domain (Clark et al., 1993). FOXM1 primarily regulates the proliferation-associated gene cluster critical to G2/M transition, mitotic spindle assembly and chromosome segregation (Fischer & Müller, 2017; Laoukili et al., 2005).

The human FOXM1 gene is a 10-exon structure covering approximately 25 kb on the 12p13.3, in which exons Va and VIIa can be alternatively spliced (Korver, Roose, Heinen, et al., 1997). There are four isoforms identified to date. FOXM1a comprises both exons Va and VIIa and lacks transcriptional activity. The other three isoforms, FOXM1b (without exons Va and VIIa), FOXM1c (without exon VIIa) and FOXM1d (without exon Va) are transcriptionally active (Liao et al., 2018). The human FOXM1 protein has a conserved forkhead DNA-binding domain (DBD), an N-terminal repressor domain (NRD), and a C-terminal transactivation domain (TAD) (Anders et al., 2011; H. J. Park et al., 2008). FOXM1 binds both to chromatin regions containing a canonical consensus FKH motif (5'-RYAAAYA-3) and to non-consensus regions as a co-factor of large protein complexes (Sanders et al., 2015).

In the mouse embryo, the gene is expressed in virtually all tissues (Korver, Roose, & Clevers, 1997), while FOXM1 expression in adult mice is only easily detected in the proliferative intestinal crypts, thymus and testes (Kalin et al., 2008; Korver, Roose, & Clevers, 1997; H Ye et al., 1997). FOXM1 knock-out (KO) in mice is embryonic lethal due to defects in the organogenesis of the heart, lung and liver, whereas heterozygous animals seem to be normal. Histological analysis of FOXM1 KO embryos showed 50-fold and 6-fold increased polyploidy in cardiomyocytes and hepatocytes, respectively, suggesting that FOXM1 is necessary to prevent premature endoreplication *in vivo*, coordinating the S- and M-phase (Korver et al., 1998; Krupczak-Hollis et al., 2004). Pulmonary defects are likely due to lack of proliferation of the embryonic mesenchyme pulmonary cells (Kim et al., 2005). Moreover, FOXM1 expression is re-activated after induced lung, liver and pancreas injury, implying that FOXM1 also plays a role in tissue regeneration (Kalinichenko et al., 2003; Misfeldt et al., 2008; Honggang Ye et al., 1999).

Extensive experimental data has emphasized the relevance of FOXM1 in diverse cellular processes beside cell division. Indeed, FOXM1 has been directly or indirectly involved in all hallmarks of cancer (Halasi & Gartel, 2013). This is in line with the fact that FOXM1 is overexpressed in most solid tumours (Pilarsky et al., 2004) and its regulatory network is a major predictor of adverse outcomes across 39 human malignancies (Gentles et al., 2015).

Next, a more comprehensive view on the FOXM1 signalling pathways will be accessed, with particular focus on its functions in mitosis, senescence and apoptosis, the previously mentioned biological processes that govern ageing and cancer (**Figure 1.5**).

3.2 FOXM1 regulation

FOXM1 mRNA and protein levels are cell cycle-regulated at the G1/S and G2/M transitions. Its transcriptional activity rises at early S phase, peaks during late G2 /early M phases, and is abolished throughout mitotic exit (Laoukili, Alvarez-Fernandez, et al., 2008; H. J. Park et al., 2008).

At the transcriptional level, several factors have been described to bind directly to the FOXM1 promotor and to regulate its levels. c-MYC, HIF-1 α , ER α , E2F, STAT3, CREB, HSF1, TWIST1, GLI1, GLI2, FOXM1, CTCF and SP1 are among the ones that activate its expression, whereas MXI1-SR α , ER β , LXRA, SPDEF and P53 are known to repress it (Liao et al., 2018).

Nevertheless, cell cycle progression is vastly orchestrated via intricate networks of reversible post-translational modifications (PTM), and therefore, PTMs play a prominent role in the cellular localization, protein stabilization, and transcriptional activity of FOXM1. At G1 phase, FOXM1 protein displays low transcriptional activity due to cytoplasmic localization and N-terminal domain auto-inhibition of the C-terminal transactivation domain, in an RB-dependent manner (Ma et al., 2010; Wierstra & Alves, 2006b). Tumour suppressor P19ARF can also interact directly with the FOXM1 TAD, decreasing its transcriptional activity (Kalinichenko et al., 2004). At G1/S transition, active Cyclin D-CDK4/6 complexes initiate both the phosphorylation of multiple sites of FOXM1 and also remove the RB interaction, releasing the TAD from its repression (Anders et al., 2011; Wierstra & Alves, 2006b). This makes FOXM1 more stable, triggering an increase in FOXM1 transcriptional activity. At this stage, Cyclin E/A-CDK2 complex can already induce FOXM1 phosphorylation (Lüscher-Firzlaff et al., 2006), while B55 α (a subunit of protein phosphatase 2A) counteracts Cyclin A-CDK2, avoiding premature activation of FOXM1 (Alvarez-Fernández et al., 2011; Anders et al., 2011). In S phase, RAF/MEK/MAPK pathway phosphorylates FOXM1 in two ERK1/2 target sites, stimulating its nuclear translocation, thus promoting transcriptional activation (Ma et al., 2010).

Through the G2 phase, Cyclin A-CDK2 reaches its maximum activity, continuing to phosphorylate FOXM1, further relieving the repression of the TAD by NRD (Laoukili, Alvarez, et al., 2008; Lüscher-Firzlaff et al., 2006) and enabling Cyclin B1-CDK1-dependent phosphorylation of FOXM1 (Y. J. Chen et al., 2009). This Cyclin B1-CDK1 phosphorylation allows both the recruitment of PLK1 to also directly phosphorylate FOXM1 and the recruitment of P300/CREB binding protein to improve FOXM1 DNA binding affinity, protein stability, and phosphorylation sensitivity (Lv et al., 2016; Major et al., 2004). In addition, CDC25A interacts with the TAD and boosts CDK1-dependent FOXM1 phosphorylation (Sullivan et al., 2012). As Cyclin B1, CDC25A, and PLK1 are target genes of FOXM1, this creates a positive feedback loop, leading to a further increase in FOXM1 transcriptional activity (Fu et al., 2008; Ma et al., 2010; Major et al., 2004; Sullivan et al., 2012). The N-terminus of the FOXM1 protein contains a KEN box, which is an anaphase promoting complex/cyclosome (APC/C) recognition signal (Pfleger & Kirschner, 2000), that is responsible for the CDH1-APC/C-mediated ubiquitination, in late mitosis and early G1, which inhibits cell cycle progression (Laoukili, Alvarez-Fernandez, et al., 2008; Hyun Jung Park et al., 2008).

Full activation and degradation of FOXM1 safeguards the synchronized expression of the transcriptional network that is crucial for a timely and correct entry and exit from mitosis, respectively.

3.3 FOXM1 binding at transcriptional targets

Transcription factors are conventionally recruited to genomic regions based on a model of direct binding to high affinity consensus motif sequences. Yet, transcription factors often exhibit non-canonical modes of binding. These include recruitment by other protein complexes previously assembled at the DNA target site and/or binding to lower affinity non-consensus regions. Such mechanisms have been described for FOXM1 recruitment and may reflect increase regulatory flexibility for alternative cellular pathways (Sanders et al., 2015), **Figure 1.3**. As previously mentioned, FOXM1 has been shown to bind to canonical consensus FKH motif (5'-RYAAAYA-3') (Major et al., 2004; Sanders et al., 2015; H Ye et al., 1997). However, other studies have revealed FOXM1 enriched in many non-consensus motif sequences, such as CHR, ER α , SP2, NFYB, CTCF, ELK4, FOS, GCN4, AP1, NFYA, ARG81, amongst other motifs (Sanders et al., 2013, 2015). Indeed, it appears that less than 14% of FOXM1 chromatin immunoprecipitation sequencing (ChIP-Seq) peaks contain a FKH motif (X. Chen et al., 2013; Sanders et al., 2015). That suggests that an assisted recruitment of FOXM1 by other protein complexes is a more common mechanism of this transcription factor activity.

The major protein complex responsible for FOXM1 recruitment to the chromatin is the cell cycle control system MuvB complex. In brief, the mammalian MuvB core consists of LIN9, LIN37, LIN52, LIN54 and RBBP4 and binds to the cell cycle genes homology region (CHR) (5'-TTTGAA-3') motifs in the genome. During G0 and early G1, the MuvB core interacts with E2F4/5, DP1/2 and p130/p107 to form the DREAM (dimerization partner, RB-like, E2F and MuvB) repression complex of G1/S and G2/M gene expression. At G1/S, DREAM-specific proteins detach from the MuvB core, allowing the recruitment of B-MYB and assembly of the B-MYB-MuvB complex (MMB) that triggers the expression of S phase genes. In late G2, the MMB and active FOXM1-MuvB complexes orchestrate the expression of mitotic genes (Fischer & Müller, 2017). Three models have been proposed for the assembly of the FOXM1-MuvB complexes in the chromatin: (I) FKH and CHR motifs are in close proximity and allow the binding of FOXM1 and MuvB, respectively, forming the protein complex (Sadasivam et al., 2012); (II) FOXM1 is recruited by the MuvB complex at CHR sites, without the need of interaction between FOXM1 DBD and the DNA (X. Chen et al., 2013); and (III) FOXM1 binds with low affinity at non-canonical sites assisted by the MuvB protein-protein interactions. This mixed model accounts for the lack of FKH motifs in the promotor of many FOXM1 target genes, while it supports the controversial data showing that FOXM1 DBD mutations diminish genome-wide binding, even though they don't disturb protein-protein interactions (Sanders et al., 2015). Nevertheless, these models are not mutually exclusive.

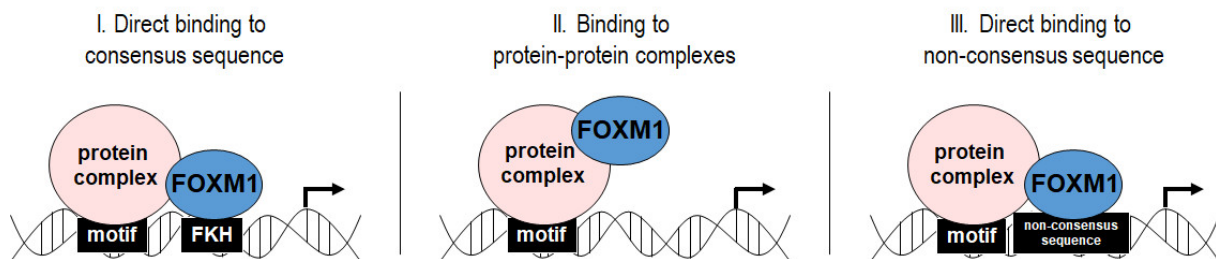


Figure 1.5. Alternative models of FOXM1 recruitment to the chromatin. FOXM1 can bind both to consensus and non-consensus sequences in the chromatin. Additionally, it has been reported that FOXM1 can drive gene expression without binding directly to the DNA, but as a co-factor of protein complexes, such as the MuvB.

3.4 FOXM1 as a major mitotic regulator

As mentioned above, the FOXM1-MuvB complex primarily drives the expression of G2/M genes. Virtually all mitotic genes are FOXM1 targets (Fischer, Grossmann, et al., 2016; Sadasivam et al., 2012; Wierstra, 2013), although functional promoter luciferase assay validation still lack for many of them. Consistently, studies using FOXM1 repression reinforced its role as a crucial regulator of cell proliferation and cell cycle progression. FOXM1 genetic

deletion causes a pleiotropy of cell cycle and mitotic defects, such as diminished mitotic progression, accumulation of polyploid cells, mitotic delay, spindle abnormalities, centrosomal amplification, chromosome misalignment and chromosome mis-segregation (Khongkow et al., 2016; Laoukili et al., 2005; I.-C. Wang et al., 2005; Wonsey & Follettie, 2005). These defects have been attributed to the downregulation of FOXM1 target genes with specific mitotic functions, such as CCNB1, CENPF, KIF20A, AURKA, PLK1, NEK2, SKP2, CKS1, CDC25B, CENPE, CENPA, CENPB, AURKA, BIRC5, all known for their importance in mitotic progression (Kalinichenko et al., 2004; Khongkow et al., 2016; Krupczak-Hollis et al., 2004; Laoukili et al., 2005; I.-C. Wang et al., 2005; Wonsey & Follettie, 2005).

3.5 FOXM1 as a suppressor of senescence

Interestingly, earliest FOXM1 depletion studies in mouse embryonic fibroblasts (MEFs) also described senescence as phenotypic outcome besides the mitotic defects (I.-C. Wang et al., 2005), later on validated also in human cells (Khongkow et al., 2016; O. H. Lee et al., 2020). Consistently, human fibroblasts undergoing senescence upon activation of the P16-RB and P53-P21 pathways exhibited FOXM1 downregulation, whereas FOXM1 overexpression was able to bypass senescence in this model (Rovillain et al., 2011). Additionally, FOXM1 overexpression was shown to avoid oxidative stress-induced senescence through the induction of BMI1, a negative regulator of P16 (Li et al., 2008), and/or through the induction of anti-oxidant genes, such as Catalase and MnSOD (Hyun Jung Park et al., 2009). DNA damage-induced senescence (e.g. ionizing radiation or epirubicin) was also reported to downregulate FOXM1 and to be rescued by ectopic expression of FOXM1 (Francica et al., 2016), likely through the transcriptional target NBS1, crucial for DNA repair (Khongkow et al., 2014).

3.6 FOXM1 and the Bcl-2 family

As previously mentioned, the Bcl-2 protein family governs the execution of intrinsic apoptosis, with the balance between anti- and pro-apoptotic Bcl-2 proteins determining the release of Cytochrome c and other factors from the mitochondria (Green & Llambi, 2015). In RNA interference (RNAi) studies, FOXM1 was first describe to impact positively the levels of BCL-2 (Halasi & Gartel, 2012), and more recently, to impact negatively the levels of BAX (L. Chen et al., 2020). In the case of MCL-1 (Hu et al., 2015) and PUMA (F. X. Chen et al., 2020), FOXM1 was shown to bind their promoters and trigger gene expression. These findings suggest a FOXM1 protective role against apoptotic induction, as it transcriptionally activates

BCL-2 and MCL-1 anti-apoptotic genes (Halasi & Gartel, 2012; Hu et al., 2015). However, regarding PUMA, this appears not to be the case (F. X. Chen et al., 2020). Concerning the many other Bcl-2 family members, still no report has proven their modulation by FOXM1. Nonetheless, based on increasing evidence that FOXM1 downregulation can sensitise a broad range of cells to apoptosis, as it will be discussed below, most likely FOXM1 controls many other apoptotic regulators.

3.7 Therapeutic opportunities with FOXM1

A multitude of studies have shown FOXM1 unequivocally overexpressed in most solid tumours (Nandi et al., 2018), and its higher levels to correlate with adverse prognosis outcomes (Gentles et al., 2015). Neoplastic transformation prompted by aberrant FOXM1 is through transcriptional activation of an extensive number of genes that are implicated in distinct phases of cancer development (Halasi & Gartel, 2013). Besides the genes covered above, FOXM1 induces critical cancer cell proliferation pathways, such as the ER α (Madureira et al., 2006), c-MYC (Wierstra & Alves, 2006a), and HER2 (Francis et al., 2009). Furthermore, FOXM1 is known to fuel invasion (Dai et al., 2007), angiogenesis (Y. Zhang et al., 2008) and anchorage-independent growth of cancer cells (Kalinichenko et al., 2004; M. Liu et al., 2006). The glucose metabolism reprogramming in cancer cells can also be induced by FOXM1 (Caldwell et al., 2010; Jiujie Cui et al., 2014; Y. Wang et al., 2016). Additionally, resistance to common chemotherapeutic drugs, such as tamoxifen, paclitaxel, cisplatin, epirubicin, temozolomide, gefitinib, and docetaxel, has been correlated with aberrant FOXM1 overexpression and of its target genes in different cancer types (breast, ovarian, cervical, glioma, pancreatic, prostate, lung, colorectal, hepatocellular and gastric) (Nandi et al., 2018). Thus, FOXM1 is a potential valuable predictive biomarker for chemotherapeutic outcome.

The significant and diverse role of FOXM1 in oncogenesis validates its promise for mono- or combination therapeutic interventions. Multiple knockdown studies have been done out to demonstrate this. Indeed, FOXM1 depletion reduced tumour burden in mouse models of hepatocellular carcinoma (T. Chen et al., 2014), breast cancer (C. Yang et al., 2013), lung adenocarcinoma (Wei et al., 2015) and nasopharyngeal carcinoma (Yu et al., 2015).

Thus, several drugs targeting directly or indirectly FOXM1 have been developed, trying to overcome the *in vivo* difficulties of RNAi delivery or gene editing. In brief, thiazolidinediones (Petrovic et al., 2010) and diarylheptanoids (Dong et al., 2017) inhibit FOXM1 mRNA expression; RCM-1 (Sun et al., 2017) prevents its nuclear translocation, increasing its protein degradation; thiostrepton (Hegde et al., 2011), siomycin A (Bhat et al., 2009) and honokiol

(Halasi et al., 2018) inhibit the binding of FOXM1 to target sites; FDI-6 (Gormally et al., 2014), FOXM1 Apt (Xiang et al., 2017) and peptide 9R-P201 (Bi et al., 2017) target the FOXM1 DBD, inhibiting therefore the expression of its target genes. Still, despite this extensive research, we still need to understand in more detail possible off-target effects of these drugs, to then safely validate them in clinical trials as single agents or in combination with low-dose chemotherapy for cancer treatment.

It is clear that we are reaching in a new era of treatment strategies against ageing and age-related diseases such as cancer, as we improve our knowledge of the links between key components of the mitotic machinery, the senescence state and the apoptotic pathway. FOXM1 provides a link between these biological processes. The systematic molecular dissection of this network will ultimately boost the design and clinical translation of strategies that target FOXM1.

4. Research aims

The major goal of this study was to molecularly understand the role of FOXM1 in the mitotic, senescence and apoptotic pathways, perceiving if FOXM1 modulation could be used as a future anti-ageing and/or anti-cancer therapeutic approach. For this purpose, the specific aims were:

- i) Dissect the link between ageing and mitotic proficiency decline and understand the role of FOXM1 in age-associated aneuploidization and cellular senescence (**Chapter II**).
- ii) Ascertain the impact of a novel FOXM1 small-molecule inhibitor in tumour progression (**Chapter III**).
- iii) Explore a combination treatment of FOXM1 inhibition plus antimitotic drugs as a means to induce apoptosis in cancer cells (**Chapter IV**).

C H A P T E R

I I

FOXM1 repression during human ageing leads to mitotic
decline and aneuploidy-driven full senescence

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2.1 Introduction

Numerous studies over the past decades have supported a link between ageing and aneuploidy (cells with a chromosome content that is not an exact multiple of the haploid complement, generated by a mitotic dysfunction phenotype characterized by mis-segregation of one or more chromosomes) (Jacobs & Court Brown, 1966). This association has been well documented for oocytes and is considered to be the main cause of miscarriage and birth defects in humans (Nagaoka et al., 2012). However, aneuploidy can also arise in somatic cells, and a number of studies have reported age-dependent increases in aneuploidy (Dumanski et al., 2015; Guttenbach et al., 1994; Mukherjee et al., 1996; Mukherjee & Thomas, 1997; Stone & Sandberg, 1995). These studies have shown that ageing is positively correlated with the incidence of chromosome mis-segregation, raising the question whether there is a general dysfunction of the mitotic apparatus in aged cells (Carere et al., 1999). Transcriptome analyses of a panel of fibroblast and lymphocyte cultures from young and old individuals revealed changes in the expression of genes controlling the mitotic machinery, including FOXM1, the major G2/M transition transcription factor (Geigl et al., 2004; Ly et al., 2000). However, the use of mixed cell populations in different stages of the cell cycle and the lower mitotic indexes (MIs) of elderly cell cultures has limited these studies from clearly demonstrating whether mitotic genes are repressed intrinsically in old dividing cells. Moreover, analysis of the mitotic process in ageing models and diseases has been scarce and a comprehensive analysis of cell division efficiency in naturally aged cells is largely missing.

More recently, aneuploidy has been also linked to ageing. Mutant mice with low levels of the spindle assembly checkpoint (SAC) protein BUB1B (crucial for correct chromosome segregation) were found to develop progressive aneuploidy along with a variety of progeroid features, including short lifespan, growth retardation, sarcopenia, cataracts, loss of subcutaneous fat, and impaired wound healing (Baker et al., 2004, 2013; Ricke & van Deursen, 2013; Wijshake et al., 2012). In addition, systematic analyses of disomic yeast, trisomic mouse, and human cells, all cells with an extra chromosome, have elucidated the impact of aneuploidy in cellular fitness (Y. C. Tang & Amon, 2013). The cumulative effect of copy number changes of many genes induces the so called pan-aneuploidy phenotypes, namely proliferation defects (Thorburn et al., 2013; B. R. Williams et al., 2008), gene signature of environmental stress response (Sheltzer et al., 2012; Torres et al., 2007), multiple forms of genomic instability (Blank et al., 2015; Sheltzer et al., 2011; Zhu et al., 2012), and proteotoxicity (Donnelly et al., 2014; Santaguida et al., 2015; Stinglele et al., 2013), which, interestingly, are all hallmarks of cellular ageing (Macedo et al., 2017).

Together, these observations suggest that there is a positive correlation between ageing and aneuploidy, but evidence for mitotic decline in elderly dividing cells and for aneuploidy-driven permanent loss of proliferation capacity (full senescence) is limited. Here, we used live-cell time-lapse imaging to investigate the mitotic behaviour of human dermal fibroblasts (HDFs) collected from healthy Caucasian males with ages ranging from neonatal to octogenarian and cultured under low passage number. We show that mitotic duration increases with advancing age, concurrent with a higher rate of mitotic defects. We demonstrate this mitotic decline to arise from a transcriptional repression of mitotic genes in pre-senescent dividing cells exhibiting senescence-associated secretory phenotype. We show short-term induction of FOXM1 transcriptional activity to improve mitotic fitness and ameliorate senescence phenotypes in elderly cells. Finally, we show aneuploidy to induce full senescence in naturally aged cells, which suggests that mitotic fitness enhancement may be a potential anti-ageing strategy.

2.2 Methods and Materials

Cell culture

A total of 11 human fibroblast cultures, established from skin samples of Caucasian males with ages ranging from neonatal to octogenarian (two biological samples per age range), were acquired from cell biobanks as summarized: Neonatal (C-0004-5C lot 74812, Invitrogen; DFM021711A, Zen Bio), 8y (GM08398, Coriell Cell Repository), 10y (GM03348, Coriell Cell Repository), 22y (DFME102710, Zen Bio; GM23976, Coriell Cell Repository), 52y (GM28967, Coriell Cell Repository), 54y (DFM062509, Zen Bio), 84y (AG11488, Coriell Cell Repository), 87y (AG10884, Coriell Cell Repository), 8y HGPS (Hutchinson-Gilford progeria syndrome) (AG03513, Coriell Cell Repository). Several time points over the human lifespan were included to reinforce the validity of any correlation found. All donors were reported as “healthy”, except the 8-year-old donor diagnosed with the Hutchison–Gilford progeria. HDFs were seeded at 1×10^4 cells per cm^2 of growth area in minimal essential medium Eagle–Earle (MEM) supplemented with 15% fetal bovine serum (FBS), 2.5 mM L-glutamine, and 1× antibiotic–antimycotic (all from Gibco, Thermo Fisher Scientific, CA, USA). Only early passage dividing fibroblasts (up to passage 3–5) were used in all experiments. Murine adult fibroblasts (MAFs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with nutrient mixture F12, 10% FBS, L-glutamine, and antibiotic–antimycotic (all from Gibco). All cells were grown at 37 °C and humidified atmosphere with 5% CO₂.

Isolation of mouse fibroblasts

Sv/129 mice were housed and handled accordingly to European Union and MAFs were collected from ears of $n > 3$ sv/129 females of the same litter at their age of 8 weeks, 6 months, 1 year, 1.5 years, and 2 years. Ears were washed with phosphate-buffered saline (PBS), cut into small pieces, and incubated with 1 mg/ml collagenase D and 1 mg/ml collagenase/dispase (both from Roche Applied Science, Germany) in DMEM:F12 without FBS, for 45 min at 37 °C and 5% CO₂. Cells were then grown on a six-well dish containing DMEM:F12, supplemented with 10% FBS and antibiotic–antimycotic.

Drug treatments

Fibroblasts were incubated for 24 h in medium containing 2 µg/ml cytochalasin D (CytoD) (C8273, Sigma-Aldrich, MO, USA) to block cytokinesis. To inhibit the SAC, cells were treated with 5 µM MPS1 inhibitor (AZ3146, TOCRIS, USA). To inhibit Kinesin-5, S-Trityl-L-cysteine (STLC) (Tocris) was used at 5 µM during 16 h, to enrich the MI for mitotic cell shake-off.

Fluorescence in situ hybridization (FISH)

FISH analysis was used to measure aneusomy index (Interphase FISH) and chromosome mis-segregation rate (CytoD FISH). “Interphase FISH” measures the prevalence of somatic aneuploidy by the ratio of aneusomic cells for chromosomes 7, 12, and 18 to the total cell count for a sample (> 2500 nuclei) (aneusomy index). “CytoD FISH” measures the rate of chromosome mis-segregation (number of events in which two sister chromatids co-segregate to the same daughter cell) by combining a cytokinesis-block assay (cytochalasin D 24 h treatment) with FISH staining. “CytoD FISH” measures the percentage of BN telophases exhibiting chromosome 7, 12, and 18 mis-segregation over the total BN cell count for a sample (> 200 BN cells). For both Interphase and CytoD FISH, fibroblasts were grown on Superfrost™ Plus microscope slides (Menzel, Thermo Scientific, CA, USA) placed in a quadriperm dish (Sarsted, Nümbrecht, Germany). Cells were given a hypotonic shock during 30 min (0.03 M sodium citrate, Sigma-Aldrich, MO, USA), followed by fixation in ice-cold Carnoy fixative added drop-wise and incubated for 5 min. This step was repeated two more times. FISH was performed with the Vysis centromeric probes CEP7 Spectrum Aqua, CEP12 Spectrum Green, and CEP18 Spectrum Orange (Abbott Laboratories, Chicago, IL, USA) according to manufacturer’s instructions. Slides were mounted with mounting medium containing DAPI (Vectashield, Vector Laboratories, CA, USA) and scored blindly.

SA-β-gal assay

In fixed-cell analysis, cells were incubated for 90 min in medium with 100 nM Bafilomycin A1 (B1793, Sigma-Aldrich, MO, USA) to induce lysosomal alkalinization. Fluorogenic substrate

(33 μ M) for β -galactosidase, fluorescein di- β -d-galactopyranoside (F2756, Sigma-Aldrich, MO, USA), was then added to the medium, and incubation carried out for 90 min. Cells were fixed in 4% paraformaldehyde for 15 min, rinsed with PBS, and permeabilized with 0.1% Triton-X100 in PBS for 15 min. Finally, cells were counterstained with 1 μ g/ml DAPI (Sigma-Aldrich, MO, USA). For FACS, cells were incubated in Bafilomycin A1 as described above and then exposed to 10 μ M of fluorogenic substrate for β -galactosidase, DDAOG (9H-(1,3-dichloro-9,9-dimethylacridin-2-one-7-yl) β -d-Galactopyranoside), for 90 min (Setareh Biotech LLC, USA).

Fluorescence-activated cell sorting (FACS)

FACS sorting was used to isolate subpopulations of senescent (SA- β -gal positive) live fibroblasts. FACS sorting was performed in FACS Aria™ I Cell Sorter (BD Biosciences, CA, USA), using the laser line of 633 nm. All cells within a single experiment were detected using the same voltage settings and sorted using an 85 μ m nozzle. Cells were initially gated by forward scatter area (FSC-A) vs. side scatter area, which excludes dead cells and subcellular debris, with subsequent exclusion of cell doublets and clumps through FSC-A vs. FSC-width plot. The signal was detected using the APC-A channel. The relative β -galactosidase activity was inferred from the median fluorescence intensity of the population. The sorting gates were designed accordingly to the respective auto-fluorescent control. Cells were sorted directly into MEM with 15% FBS and seeded in Superfrost™ Plus microscope slides for subsequent FISH analysis. Analysis of FACS data was done using FlowJo v10 software (TreeStar, Inc., Ashland, OR).

Immunostaining

Fibroblasts were grown on sterilized glass coverslips coated with 50 μ g/ml fibronectin (F1141, Sigma-Aldrich, MO, USA). Cells were fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min. Following fixation, cells were rinsed in PBS and permeabilized in PBS + 0.3% Triton-X100 for 7 min. Cells were next blocked in 10% FBS in PBS-T (PBS + 0.05% Tween-20) for 1 h and then incubated overnight at 4 °C with primary antibodies diluted in PBS-T + 5% FBS as follows: rabbit anti-53BP1 (4937, Cell Signalling Technology, MA, USA), 1:100; mouse anti-p21 (SC-6246, Santa Cruz Biotechnology, CA, USA), 1:1000; rabbit anti-FOXM1 (13147, ProteinTech Group, Inc., IL, USA), 1:1500; mouse anti-Cdc20 (SC-5296, Santa Cruz Biotechnology), 1:100; mouse anti-Cyclin B (SC-245, Santa Cruz Biotechnology), 1:1000 secondary antibodies AlexaFluor-488 and -568 (Life Technologies, CA, USA) were diluted 1:1500 in PBS-T + 5% FBS. DNA was counterstained with 1 μ g/ml DAPI (Sigma-Aldrich, MO, USA). Coverslips were mounted in slides with mounting solution (90% glycerol, 0.5% *N*-propyl-gallate, 20 mM Tris, pH 8).

Phase-contrast live-cell imaging

Fibroblasts were grown in glass-bottom 35 mm μ -dishes (Ibidi GmbH, Germany), coated with 50 μ g/ml fibronectin (F1141, Sigma-Aldrich, MO, USA). Images were acquired on a Zeiss Axiovert 200 M inverted microscope (Carl Zeiss, Oberkochen, Germany) equipped with a CoolSnap camera (Photometrics, Tucson, USA), XY motorized stage and NanoPiezo Z stage, under controlled temperature, atmosphere, and humidity. Neighbor fields (20–25) were imaged every 2.5 min for 2–3 days, using a $\times 20/0.3$ numerical aperture (NA) A-Plan objective. Stitching of neighboring fields was done using the plugin “Stitch Grid” (Stephan Preibisch) from ImageJ/Fiji software.

Live-cell and fixed-cell correlative microscopy

Eighty-seven-year-old fibroblasts expressing H2B–GFP were grown in μ -Slide 2 Well ibiTreat (Ibidi GmbH). Images were acquired on a Leica DMI6000b inverted microscope (Leica Microsystems, Germany), equipped with an ORCA-Flash4.0 camera (Hamamatsu, Japan), using the laser line 488 nm for the excitation of green fluorescent protein (GFP), and under controlled temperature, atmosphere and humidity. Neighbour fields (60–70) were imaged every 5 min for 3–4 days, using a $\times 20$ LD/0.4 NA objective (Leica Microsystems). Following the long-term live-cell imaging, cells were processed for SA- β -gal assay and 53BP1/p21 double immunostaining as described elsewhere in this section, and the same neighbour fields acquired using a $\times 40$ LD/0.6 NA objective. LAS X software (Leica Microsystems) was used for image acquisition and analysis.

Fluorescence microscopy

Analysis of the SA- β -gal fluorescence assay was carried out on a Zeiss AxioImager Z1 (Carl Zeiss) equipped with an AxioCam MR and using an EC-Plan-Neofluor $\times 40/1.3$ NA objective. Cells displaying > 5 fluorescent granules were considered positive for SA- β -gal activity. Image acquisition of FOXM1, Cyclin B, and CDC20 immunostaining was performed on a Zeiss AxioImager using a Plan-Apochromat $\times 63/1.4$ NA objective. Prometaphase cells were acquired with 0.24 μ m Z-stacks. User-defined fluorescence intensity thresholds were set and used consistently for samples within each experiment. AutoQuant X2 (Media Cybernetics, Rockville, USA) was used for image deconvolution.

Automated microscopy

Image fields of 53BP1/p21 double immunostaining and FISH staining were acquired on IN Cell Analyzer 2000 (GE Healthcare, UK), equipped with a Photometrics CoolSNAP K4 camera and using a Nikon $\times 20/0.45$ NA Plan Fluor objective. User-defined fluorescence intensity thresholds were set and used consistently for samples within each experiment.

Image analysis

Live-cell phenotypes (mitotic duration) and fixed-cell experiments (FISH, SA biomarkers, and micronuclei) were blindly quantified using ImageJ/Fiji software.

Western blotting

Mitotic cell populations were collected by shaking-off cell culture flasks enriched for MI by a 16 h treatment with STLC. MI > 95% was determined by visual scoring of cells with condensed chromosomes after Carnoy fixation, followed by with 1 µg/ml DAPI in PBS. Lysis buffer (150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 0.5% IGEPAL) with protease inhibitors was added to mitotic cell pellets, and lysates quantified for protein content by the Lowry Method (DC™ Protein Assay, Bio-Rad, CA, USA). Twenty micrograms of total extract were then loaded in SDS-polyacrylamide gel electrophoresis gels and transferred onto nitrocellulose membranes for western blot analysis. Membranes were blocked during 1 h with TBS containing 5% low-fat milk. Primary antibodies were diluted in TBS containing 2% low-fat milk as follows: rabbit anti-FOXM1 (13147, ProteinTech Group, Inc.), 1:1000; mouse anti-Cyclin B (SC-245, Santa Cruz Biotechnology), 1:1000; mouse anti-NDC80 (clone 9G3, Abcam, Cambridge, UK), 1:1750; mouse anti-PLK1 (SC-17783, Santa Cruz Biotechnology), 1:100; mouse anti- α -Tubulin (T5168, Sigma-Aldrich, CA, USA), 1:50,000; and mouse anti-GAPDH (60004, ProteinTech Group, Inc.), 1:30,000. Goat anti-rabbit (SC-2004, Santa Cruz Biotechnology) and goat anti-mouse (SC-2005, Santa Cruz Biotechnology) horseradish peroxidase-conjugated secondary antibodies were diluted at 1:3000 in TBS containing 2% low-fat milk. Signal was detected using Clarity Western ECL Substrate reagent (Bio-Rad Laboratories, CA, USA) according to manufacturer's instructions. A GS-800 calibrated densitometer with Quantity One 1-D Analysis Software 4.6 (Bio-Rad Laboratories) was used for quantitative analysis of protein levels.

Lentiviral plasmids

H2B–GFP (Orth et al., 2011) was amplified as a BglII–H2B–GFP–T2A–BamHI–NotI fragment. This PCR fragment was digested with BglII + NotI and ligated into pRetrox-Tight-Puro (Clontech, CA, USA) digested with BamHI + NotI, thus destroying the 5' BamHI/BglII site, while reintroducing a BamHI site 3' of H2B–GFP–T2A. In parallel, α -Tubulin (Orth et al., 2011) was amplified as a BglII– α -Tubulin–BamHI–NotI fragment and ligated into pRetrox-Tight-Puro digested with BamHI + NotI, again destroying the 5' BamHI/BglII site, while reintroducing a BamHI site 3' of α -Tubulin. mCherry was amplified from pExchange-1-Cherry (Agilent Technologies, CA, USA) as a BglII–mCherry–NotI fragment and was ligated into pRetrox– α -Tubulin digested with BamHI–NotI, yielding pRetrox– α -Tubulin–mCherry. Finally, α -Tubulin–mCherry was PCR-amplified as a BglII– α -Tubulin–mCherry–NotI fragment and ligated into pRetrox–H2B–GFP–T2A digested with BamHI + NotI, yielding pRetrox–H2B–GFP–T2A– α -

Tubulin–mCherry. To obtain pLVX–Tight–Puro–H2B–GFP–T2A– α -tubulin–mCherry, a BglII–H2B–GFP–T2A– α -Tubulin–mCherry–NotI fragment was amplified from pRetroX–H2B–T2A–GFP– α -Tubulin–mCherry and ligated into pLVX–Tight–Puro (Clontech) digested with BamHI + NotI. To generate pLVX–Tight–Puro–FOXN1–dNdK, a BglII–FOXN1–dNdK–NotI fragment was amplified from pcDNA3–Flag– Δ N– Δ KEN–FOXN1 (Laoukili, Alvarez, et al., 2008) and ligated into pLVX–Tight–Puro digested with BamHI + NotI. Primers used are described in Table 2.1.

Table 2.1. Primers used for cloning in pRetrox or pLVX vectors.

PCR fragment	PCR fragment name	Forward primer	Reverse primer
H2B–GFP– α -tubulin–mCherry	BglII–H2B–GFP– α -tubulin–mCherry–BamHI–NotI	5'-TATATAAGATCTATGCCAGAGCCAGCGAAGTCT-3'	5'-TATATAGCGGCCGCGGATCCCTACTGTAGCTCAGGAATAAAC-3'
FoxM1– Δ NAKEN	BglII–FoxM1– Δ NAKEN–BamHI–NotI	5'-TATATAAGATCTATGTGTACCTGGAGCAGCGACAGG-3'	5'-TATATAGCGGCCGCGGATCCTTAGTATTCTCTCTCTTCC-3'
H2B–GFP–T2A	BglII–H2B–GFP–T2A–BamHI–NotI	5'-TATATAAGATCTATGCCAGAGCCAGCGAAGTCT-3'	5'-TATATAGCGGCCGCGGATCCGGGGCCGGGGTTCTCTCCACGTCGCGCAAGTACAGCTCGTCCATGCC-3'
mCherry	BglII–mCherry–BamHI–NotI	5'-TATATAAGATCTATGGTGAGCAAGGGCGAGGAG-3'	5'-TATATAGCGGCCGCGGATCCCTCTCCGGTCTCTCTCTGTACAGCTCGTCCATGCC-3'
α -tubulin	BglII– α -tubulin–BamHI–NotI	5'-TATATAAGATCTATGCGTGAGTGCATCTCCATC-3'	5'-TATATAGCGGCCGCGGATCCTTAGTATTCTCTCTCTTCC-3'
α -tubulin–mCherry	BglII– α -tubulin–mCherry–BamHI–NotI	5'-TATATAAGATCTATGGTGAGCAAGGGCGAGGAG-3'	5'-TATATAGCGGCCGCGGATCCTTAGTATTCTCTCTCTTCC-3'

Lentiviral production and infection

Lentiviruses were produced according to the protocol described in Lenti-X Tet-ON Advanced Inducible Expression System (Clontech). Lentiviruses carrying empty pLVX–Tight–Puro, pLVX–Tight–Puro–FOXN1–dNdK, as well as lentiviruses carrying pLVX–Tet-On Advanced (which expresses rTTA), were generated in HEK293T helper cells transfected with packaging plasmids (pMd2.G and psPAX2) using Lipofectamine 2000 (Life Technologies, Thermo Scientific, CA, USA). Human fibroblasts were co-infected for 12–16 h with responsive and transactivator lentiviruses at 2:1 ratio, in the presence of 8 μ g/ml polybrene (AL-118, Sigma-Aldrich, MO, USA). In the following day, 750 ng/ml doxycycline (DOX) (D9891, Sigma-Aldrich, MO, USA) was added to the medium to induce co-transduction. Phenotypes were analyzed and quantified 48–72 h later, and transfection efficiency monitored by scoring the number of fluorescent cells or by western blotting.

FOXN1 RNA interference

Cells were transfected 1 h after plating, with 45 nM FOXN1 small interfering RNA (SASI_Hs01_00243977 from Sigma-Aldrich, MO, USA) using Lipofectamine RNAiMAX (Thermo Scientific) according to manufacturer's instructions. Phenotypes were analyzed and quantified 72 h post transfection, and depletion efficiency monitored by western blotting.

Real-time PCR (RT-PCR, qPCR)

Total RNA was obtained from mitotic fibroblasts using the RNeasy® Mini kit (Qiagen, Hilden, Germany). RNA purity and integrity were confirmed in the Experion™ system (Bio-Rad Laboratories). cDNA was synthesized from total RNA (1 µg) using iScript Advanced Select cDNA Synthesis kit (Bio-Rad Laboratories). The $2^{-\Delta\Delta C_t}$ method was used to quantify the transcript levels of *FOXM1*, *CDKN1A*, *MMP1*, *CXCL8*, *TSPAN13*, and *FAM214B* against the transcript levels of the housekeeping gene (*TBP*). Primers were designed to span at least one exon–intron junction (Table 2.2). Amplification was performed in a C1000 Touch Thermal Cycler (CFX384 Real-Time System, Bio-Rad Laboratories) and analysed using CFX Manager Software (Bio-Rad Laboratories).

Table 2.2. Primers used for qPCR.

Gene Name	Gene Symbol	Forward primer	Reverse primer	Length (bp)	Assay Design	Specie
Forkhead box M1	FOXM1	5'-ATACGTGGATTGAGGACCACT-3'	5'-TCCAATGTCAAGTAGCGGTG-3'	175	Intron-spanning	Human
TATA box binding protein	TBP	5'-GAGCCAAGAGTGAAGAACAGTC-3'	5'-GCTCCCCACCATATTCTGAATCT-3'	116	Intron-spanning	Human
Cyclin dependent kinase inhibitor 1A	CDKN1A/p21	5'-TGGACCTGGAGACTCTCAGG-3'	5'-CGGATTAGGGCTTCCTCTGG-3'	104	Intron-spanning	Human
C-X-C motif chemokine ligand 8	CXCL8/IL8	5'-GCCTTCCTGATTCTGCAGCT-3'	5'-GCACTGACATCTAAGTCTTTAGCA-3'	75	Intron-spanning	Human
Family with sequence similarity 214 member B	Fam214b	5'-CACCATCCAAGTGACCTTATTTAACC-3'	5'-AGAAGTCAAAGGTCACAAGGAACAT-3'	71	Intron-spanning	Human
Tetraspanin 13	TSPAN13	5'-CGCCATGTGCTCCAATCAG-3'	5'-GTAGGTCAGCCAAACACCCA-3'	110	Intron-spanning	Human
Matrix metalloproteinase 1	MMP1	5'-AGCGTGTGACAGTAAGCTAACC-3'	5'-AACTCCGGGTAGAAGGGATTG-3'	109	Intron-spanning	Human
Forkhead box M1	FOXM1	5'-ATCGCTACTTGACATTGGACCA-3'	5'-GATTGGGTCGTTTCTGCTGTG-3'	96	Intron-spanning	Mouse
TATA box binding protein (Tbp)-associated factor, RNA polymerase I	TBP	5'-CTTCCTGCCACAATGTCACAG-3'	5'-CCTTCTCATGCTTGCTTCTCTG-3'	118	Intron-spanning	Mouse

RNA-sequencing (RNA-seq) and bioinformatics

RNA was isolated from mitotic human fibroblasts and validated as described above for Real-time PCR. RNA-sequencing libraries were prepared using TruSeq Stranded Total RNA with Ribo-Zero Human/Mouse/Rat (RS-122-2201; Illumina, CA, USA) according to manufacturer's protocol. Pooled libraries were sequenced on an Illumina HiSeq 2500 (single-end 50 bp). Raw unaligned sequencing reads (fastq-format) were deposited in the European Nucleotide Archive under the accession number PRJEB27047. Reads were aligned to the human genome (hg19) using a splicing-aware aligner (StarAligner). Aligned reads were fragments per million (FPM) normalized, excluding low abundance genes (mean FPM > 1). Differential gene expression analysis was performed using a likelihood ratio test constructed under the R-package edgeR (v3.14.0 available from Bioconductor at <http://www.bioconductor.org/packages/release/bioc/html/edgeR.html>). Significant differential gene expression of aged fibroblasts was defined as p -value < 0.05 and 2logFC cut-off

value < -0.5 or > 0.5 ; and in case of genetic manipulations as p -value < 0.05 , 2logFC cut-off value < -0.5 or > 0.5 , and false discovery rate (FDR) < 0.05 . For generation of RNA-seq heatmaps, transcript counts were normalized to the sample library size. These values were subsequently scaled by gene using normalized scores or z-scores (i.e. a value of 0 corresponds to the mean gene expression of that gene across all libraries, and ± 1 , ± 2 , etc. represent 1, 2, etc. SDs from the gene mean). Hierarchical clustering was performed using the R library heatmap.2. RNA-seq data represent two independent experimental replicates of each biological sample. GO term enrichment analysis of differentially expressed genes was performed using Database for Annotation, Visualization and Integrated Discovery Functional Annotation Tool v6.7 (D. W. Huang et al., 2009) (www.david-d.ncicrf.gov). To calculate the significance of gene set enrichment, empirical p -values were generated using DAVID tool.

Statistical analysis

Sample sizes and statistical tests for each experiment are indicated in the figure legends. p -values were obtained using GraphPad Prism version 6 (GraphPad, San Diego, CA, USA). Data were tested for parametric vs. non-parametric distribution using D'Agostino–Pearson omnibus normality test. Spearman's rank correlation, Mann–Whitney, two-tailed χ^2 , or one-way analysis of variance for multiple comparisons tests were then applied accordingly. NS, $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$. Values are shown as mean $\pm$ SD or mean $\pm$ SEM.

2.3 Results

Aneuploidy increases during natural ageing

In our study, we used HDFs collected from healthy Caucasian males with ages ranging from neonatal to octogenarian, to test whether aneuploidy increases during natural ageing and if this is a consequence of a general dysfunction of the mitotic machinery in aged cells. As inter-individual differences exist in the rate at which a person ages (biological age), we included two donors of each age in a total of five chronological ages to increase the robustness of any correlation found with ageing. Chronic accumulation of macromolecular damage during natural ageing induces a cellular stress response known as senescence (Childs et al., 2015), and accumulation of senescent cells has been widely reported for chronological ageing and age-related disorders (Baker et al., 2011; G P Dimri et al., 1995). Accordingly, we found higher levels of senescence-associated (SA) biomarkers (Rodier & Campisi, 2011), measured under strict quantitative parameters by microscopy analysis, in the proliferating fibroblast cultures

from older individuals and the Hutchinson-Gilford progeria syndrome patient (**Fig. 2.1a-e**), thus validating their suitability as models of advanced and premature ageing, respectively. To determine whether aneuploidy increases with ageing, we performed fluorescence *in situ* hybridization for three chromosome pairs in asynchronous cell populations from different age donors and scored all samples blindly. We found significantly higher (two-tailed χ^2 test) aneusomy indices (ratio of aneusomic cells for chromosomes 7, 12, and 18 to the total cell count for a sample) in the middle-aged, old-aged, and progeria samples (**Fig. 2.2a**). Moreover, as aneuploid cells are most likely outcompeted in culture by diploid cells (B. R. Williams et al., 2008), thereby diluting the aneuploidy index, we additionally measured the rate of chromosome mis-segregation (number of events in which two sister chromatids co-segregate to the same daughter cell) by combining a cytokinesis-block assay with FISH staining. In this approach, we scored all binucleated (BN) cells generated during a 24 h treatment with cytochalasin D (cytokinesis inhibitor; see Methods). We found the percentage of BN cells with chromosome mis-segregation over the total BN cell count for a sample to be significantly higher (two-tailed χ^2 test) in the middle-aged, old-aged, and progeria cultures (**Fig. 2.2b**). Taken together, these FISH experiments show that increased mis-segregation rates are associated with mild aneuploidy levels in elder cultures, supporting the idea of an age-associated loss of mitotic fidelity.

Elder cells divide slower with increased rate of mitotic defects

To gain insight into how old cells divide, we followed individual mitotic cells by long-term phase-contrast time-lapse imaging. Interestingly, we found the interval between nuclear envelope breakdown (NEB) and anaphase onset to increase steadily with advancing age (**Fig. 2.2c,d**). To exclude any effects due to genetic heterogeneity between the Caucasian donors and/or biobank discrepancies in culture set up, we used mouse adult fibroblasts recurrently sampled from female mice over a period of 2 years. In this model, culture conditions were highly homogenous, thereby providing a solid “*ex vivo*” model of chronological ageing. Again, both mitotic duration (**Fig. 2.3a-c**) and SA biomarkers (**Fig. 2.3d,e**) progressively increased with ageing.

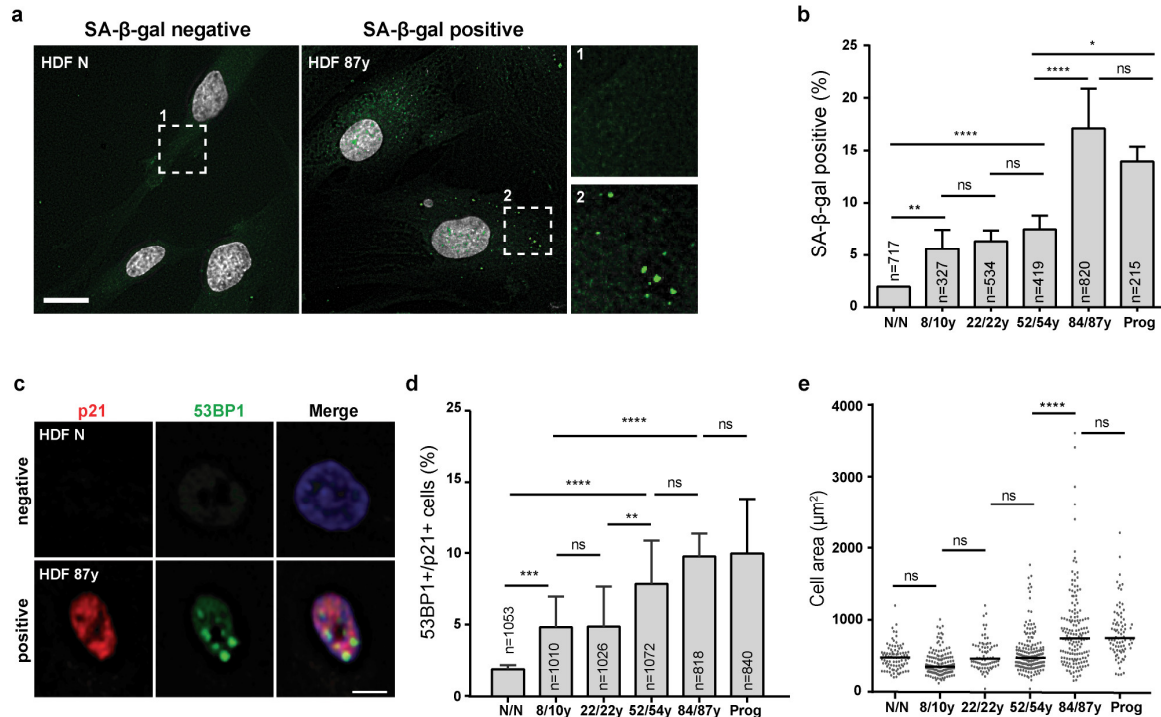


Figure 2.1. Senescence markers in human dermal fibroblasts from different age donors. **a** Representative images of senescence-associated β -galactosidase (SA- β -gal) negative (HDF N) and positive (HDF 87y) cells. Insets are 2x magnifications. Scale bar, 20 μ m. **b** Percentage of cells staining positive for SA- β -gal assay. **c** Representative images of cells negative (HDF N) and positive (HDF 87y) for double immunostaining of Cdkn1a/p21 (cell cycle inhibition) and 53BP1 (>1 foci; DNA damage) senescence markers. Scale bar, 10 μ m. **d** Percentage of Cdkn1a/p21 positive cells with 53BP1 foci. **e** Cellular area as readout for senescence-associated enlarged cell morphology. Scatter plots show mean of n>50 cells. In all graphs, bars represent mean $\pm$ s.d. values from three independent experiments using two biological samples of similar age (except for progeria). Sample size (n) is indicated in each graph. ns: $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ by two-tailed χ^2 (b, d) and Mann-Whitney (e) statistical tests.

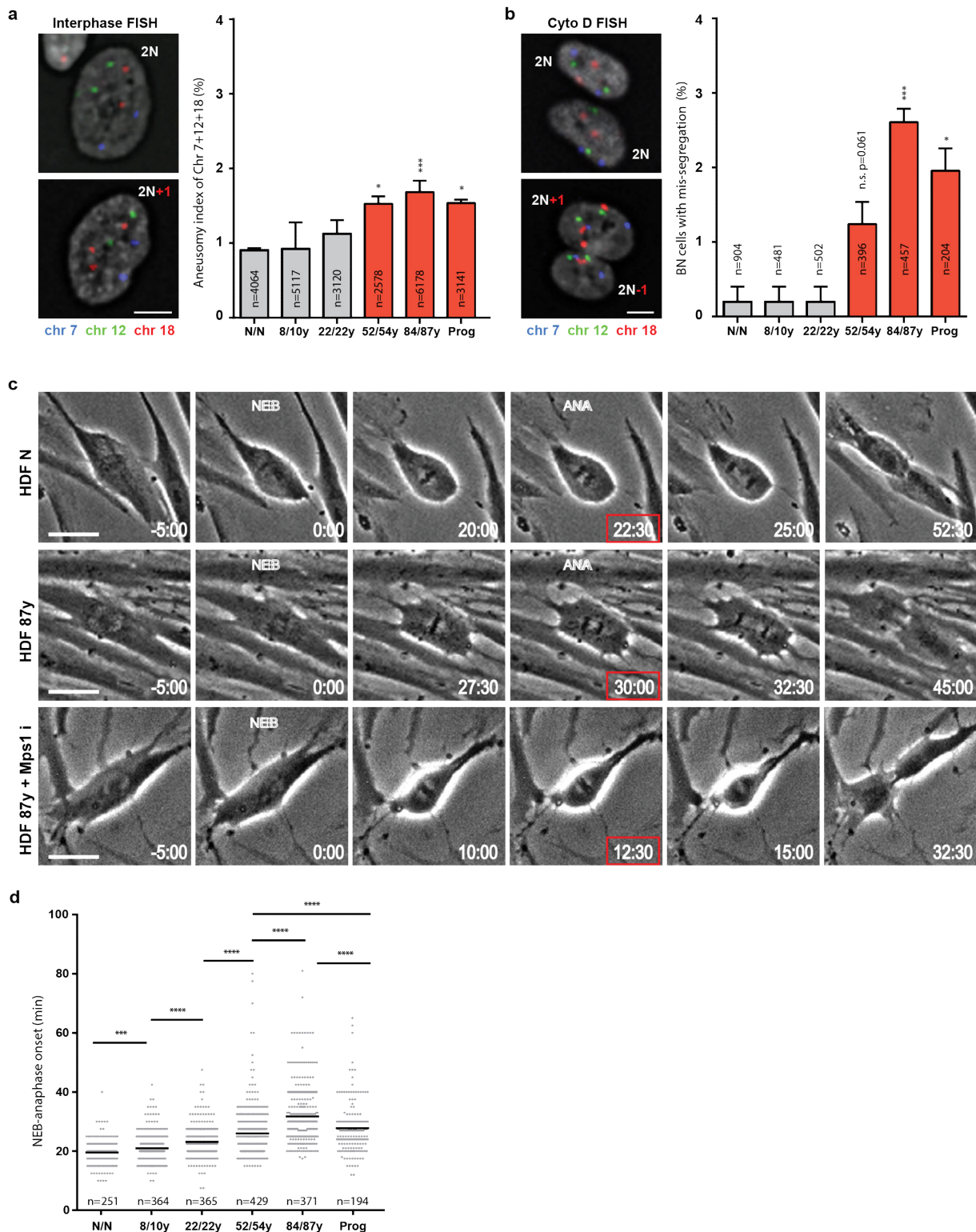


Figure 2.2 Aneuploidy and mitotic duration increase during normative ageing. **a** Aneusomy index of three chromosome pairs (7, 12, and 18) in interphase cells from different age donors. **b** Percentage of cytochalasin D-induced binucleated cells with chromosome 7, 12, and 18 mis-segregation. Representative images of euploid/2N and aneuploid/2N ± 1 cells are shown in **a**, **b**, with chromosome-specific centromeric probes as indicated. Scale bars, 5 μ m. **c** Frame series of time-lapse phase-contrast movies of mitotic neonatal and elderly cells $\pm$ Mps1 inhibitor. Time, min:s. Scale bar, 20 μ m. **d** Mitotic duration of individual fibroblasts from different donors, measured from nuclear envelope breakdown (NEB) to anaphase onset (ANA). Values are mean $\pm$ SD from at least three independent experiments, using two biological samples of similar age (except for progeria). Sample size (*n*) is indicated in each graph. NS, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ in comparison with neonatal (N/N) by two-tailed χ^2 (a, b) and Mann-Whitney (d) statistical tests.

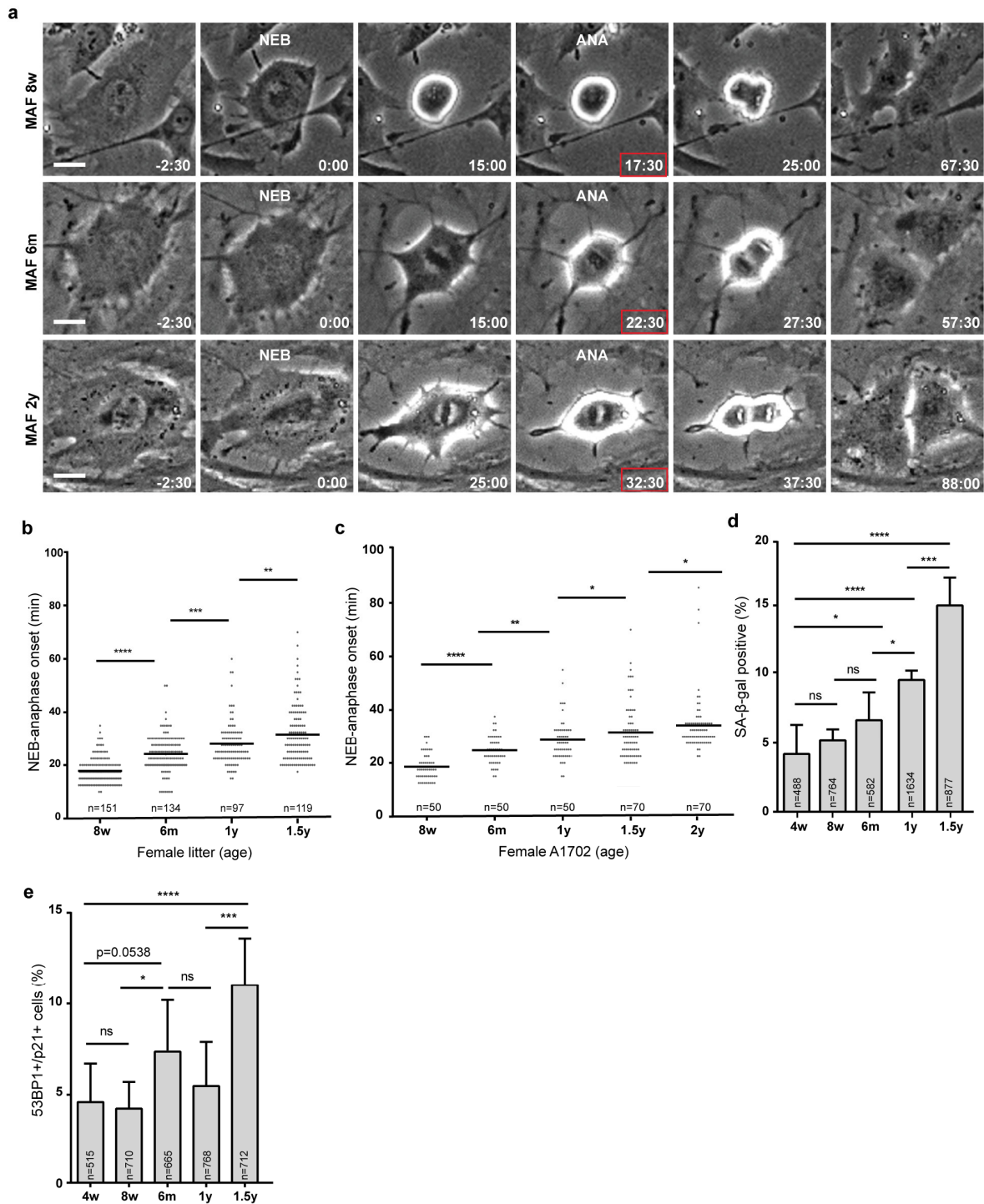


Figure 2.3. Mitotic delay, cellular senescence and FOXM1 repression during mouse normative ageing. **a** Movie frames of mitosis in MAFs collected at different age time-points as indicated. NEB, nuclear envelope breakdown. ANA, anaphase onset. Time min:sec. Scale bar, 20 μ m. **b** Mitotic duration (NEB to anaphase onset) of individual MAFs collected at different age time-points from ≥ 3 sv/129 sister females. **c** Mitotic duration of individual MAFs collected at different age time-points from one randomly chosen single female (A1702). **d** Percentage of cells staining positive for senescence-associated β -galactosidase assay. **e** Percentage of Cdkn1a/p21 positive cells with 53BP1 foci. In all graphs, bars represent mean $\pm$ s.d. values from three independent experiments. Sample size (n) is indicated in each graph. ns: $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ by two-tailed χ^2 (d, e) and Mann-Whitney (b, c) statistical tests.

Mitotic gene transcriptional shutdown and SASP in elderly mitotic cells

To identify the molecular mechanisms behind ageing-associated mitotic defects, we performed RNA-sequencing gene expression profiling of cells captured in mitosis, accordingly to the experimental layout shown in **Fig. 2.4a**. Fibroblast cultures from neonatal and octogenarian donors were treated with STLC to enrich for MI, and mitotic cells collected by mechanical detachment. As cell synchronization (mitotic enrichment) was limited in elderly vs. neonatal cultures, we used a higher number of cell culture flasks with octogenarian fibroblasts treated with STLC to collect equivalent numbers of mitotic elderly and neonatal cells for RNA-seq. The procedure yielded purified cell populations with mitotic indices >95% in both neonatal and 87 y donor cultures (**Fig. 2.4b,c**), and importantly, it uncoupled any changes found in mitotic gene expression from the samples' differences in MI and overall proliferation capacity. RNA-seq revealed that the abundance of 3309 gene transcripts was altered in octogenarian mitotic fibroblasts compared to neonatal mitotic fibroblasts (**Fig. 2.4d**). Principal component analysis showed that two independent experiments from the same donor were consistent for the main component (**Fig. 2.4e**). In agreement with the elderly cell defects in mitosis, the top 10 most altered Gene Ontology (GO) terms included six cell cycle-related and mitosis-related gene ontologies (**Fig. 2.4f**). Seventy-one "mitosis" GO genes were altered in octogenarian mitotic fibroblasts (p -value < 0.05; likelihood ratio test), representing a 1.97-fold enrichment of alterations in this gene set (p -value = $9.73\text{E}-9$, Fisher exact test) (**Fig. 2.4g**). Moreover, a comprehensive list of 51 SA genes, including *CDKN1A*, *CDKN2A*, *GLB1*, *LMNB1*, and SASP genes (i.e., chemokines, cytokines, metalloproteinases, and others) (Coppé et al., 2008; Hernandez-Segura et al., 2017), was interrogated in the RNA-seq data set. We found altered expression of 26 genes in the elderly vs. neonatal mitotic cells, with 19 genes behaving as previously reported (Coppé et al., 2008; Hernandez-Segura et al., 2017) (**Fig. 2.4h**). As the transcriptome of senescent cells is highly heterogeneous and depends on the cell type and senescence stimulus, we additionally extended our analysis into 55 genes recently identified as comprising a "senescence core signature" (Hernandez-Segura et al., 2017). Indeed, we found 19 genes of this signature to be differentially regulated in the RNA-seq data set of mitotic octogenarian dermal fibroblasts, with 16 genes behaving as reported (Hernandez-Segura et al., 2017) (**Fig. 2.4i**). Alterations in the expression levels of *CDKN1A*, *MMP1*, *CXCL8*, *TSPAN13*, and *FAM214B*, were further confirmed by real-time PCR analysis (**Fig. 2.4j**). Thus, an unforeseen SASP phenotype evolves in elderly dividing cells, alongside a global transcriptional shutdown of mitotic genes, likely accounting for ageing-associated mild aneuploidy levels.

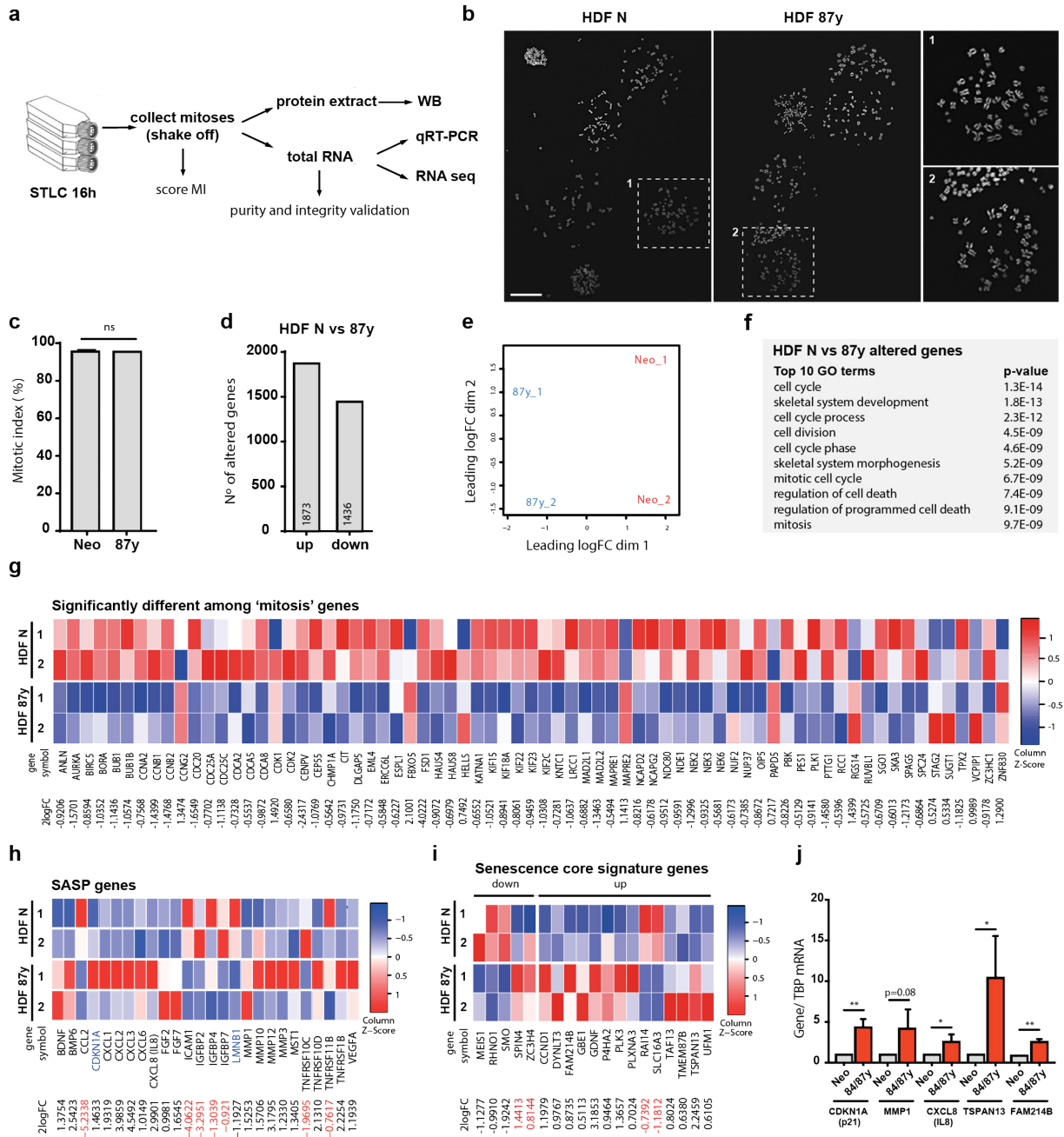


Figure 2.4. Mitotic gene downregulation and senescence gene signature in elderly mitotic fibroblasts. **a** Experimental layout. Fibroblast cultures from different age donors were treated with kinesin-5 inhibitor (STLC) to enrich for mitotic index (MI). Mitotic (detached) cells were collected and inspected for MI. Protein extracts and total RNA were prepared for gene expression analyses. **b** Representative images of neonatal (HDF N) and elderly (HDF 87y) mitotic cell shake-offs. Insets are $\times 2$ magnifications. Scale bar, 50 μ m. **c** MI quantification of neonatal and octogenarian mitotic cell shake-offs. **d** Number of altered genes in HDF 87y RNA-seq comparing to HDF N. **e** RNA-seq multidimensional scaling (MDS) plot. Axes in MDS plot represent leading log-fold changes (logFCs) calculated by the root-mean-square of the largest absolute logFCs between each pair of libraries. **f** Top ten altered GO terms organized by p -values using the DAVID Functional Annotation tool. **g** Heatmap of genes within the "mitosis" GO term differentially expressed between HDF N and HDF 87y. **h** Heatmap of SASP and senescence-associated (*CDKN1A*, *LMNB1*) genes differentially expressed between HDF N and HDF 87y; 2logFCs in red indicate genes behaving differently from expected (Coppé et al., 2008; Hernandez-Segura et al., 2017). **i** Heatmap of senescence core signature genes differentially expressed between HDF N and HDF 87y; 2logFCs in red indicate genes behaving differently from expected (Hernandez-Segura et al., 2017). In all heatmaps, 2logFC cut-off value ≤ -0.5 and ≥ 0.5 , p -value < 0.05 ; genes represented in columns and technical replicates represented in rows; Z-score column color intensities representing higher (red) to lower (blue) expression. **j** Transcript levels of senescence genes in total RNA from mitotic elderly samples normalized to total RNA.

TBP transcript levels and compared with neonatal. NS, $p > 0.05$, $*p \leq 0.05$, and $**p \leq 0.01$ by two-tailed Mann–Whitney statistical test (c, j).

FOXM1 repression dictates mitotic decline during natural ageing

RNA-seq analysis also disclosed *FOXM1*, the transcription factor that primarily drives the expression of G2/M cell cycle genes (Fischer, Grossmann, et al., 2016; Sadasivam et al., 2012) (**Fig. 2.5a**), to be downregulated in elderly mitotic cells (2logFC: -0.85 ; FDR $< 5\%$; likelihood ratio test). Indeed, 36 out of the 71 mitotic genes altered in octogenarian mitotic cells (**Fig. 2.5b**) have been reported as targets of the MMB-FOXM1 transcription complex containing the CHR motif in their promoters (Fischer, Grossmann, et al., 2016; Müller et al., 2016; Sadasivam et al., 2012). In addition, loss of *FOXM1* has previously been shown to cause pleiotropic cell cycle defects leading to embryonic lethality (Laoukili et al., 2005). We therefore asked whether loss of mitotic proficiency during normative ageing was due to *FOXM1* downregulation. Indeed, increasing age correlated with decreased FOXM1 transcript and protein levels in our human mitotic cell samples (**Fig. 2.5c-e**). Importantly, unlike previous studies linking FOXM1 repression and ageing (Smirnov et al., 2016; X. Wang et al., 2001), by analysing mitotic cell samples, we uncoupled FOXM1 downregulation from decreased cell proliferative capacity, demonstrating that elderly cells divide with intrinsically low levels of FOXM1. To get evidence that FOXM1 repression actually accounts for an elderly cell mitosis with low mitotic protein levels, we measured the protein levels of FOXM1 and some of its known mitotic targets (Cyclin B1, PLK1, NDC80, and CDC20) (Fischer, Quaas, et al., 2016) by western blotting analysis of mitotic cell extracts (**Fig. 2.5f,g**), as well as by immunofluorescence analysis of single mitotic cells (**Fig. 2.5h-j**). These quantitative analyses demonstrated that in elderly mitotic cells, important protein players acting from mitotic entry (e.g., Cyclin B1) to anaphase onset (e.g., CDC20) are in fact expressed at low levels. We thus conclude that an ageing-associated FOXM1 repression likely accounts for an extensive transcriptional downregulation of mitotic genes and the phenotypes observed in early senescent dividing cells. To test the correlation between FOXM1 repression and age-associated phenotypes further, we RNAi-depleted FOXM1 in fibroblasts from the 10-year-old donor (**Fig. 2.6a**). Again, to uncouple gene expression analysis from the reduction in MI following FOXM1 RNAi, we performed RNA-seq profiling of cells captured in mitosis accordingly to the experimental layout in **Fig. 2.4a**. RNA-seq revealed that FOXM1 repression enriched for mitosis genes (143 genes, GO analysis; FDR $< 5\%$, likelihood ratio test) (**Fig. 2.7a**), out of the altered 5841 gene transcripts (**Fig. 2.9a,b**). This constituted a 2.41-fold enrichment of alterations in this gene set (p -value = $7.43E-32$, Fisher exact test). The top ten most altered GO terms included six related to mitosis (**Fig. 2.9d**), and 224 out of 249 G2/M cell cycle genes previously reported as targets of the DREAM and MMB-FOXM1 transcriptional complexes (Fischer, Grossmann, et al.,

2016), were downregulated following FOXM1 depletion (**Fig. 2.9d**). Moreover, we found 1937 out of the 3309 genes altered in old-aged fibroblasts, to be also altered in FOXM1-depleted cells (**Fig. 2.7b**). Thus, 58.5% of the age-dependent transcriptional changes are FOXM1-dependent and enriched for cell cycle/mitosis GO terms (**Fig. 2.7b**). In agreement, FOXM1-depleted young fibroblasts displayed lower mitotic index (**Fig. 2.6b,c**), decreased protein levels of FOXM1 targets (**Fig. 2.6d,e**), a mitotic delay (**Fig. 2.7c**), and increased aneuploidy (**Fig. 2.7d,e**), similar to old-aged fibroblasts. Interestingly, the percentage of cells with SA biomarkers also increased following FOXM1 repression (**Fig. 2.7f,g**), further supported by changes in the expression levels of SASP genes and genes of the senescence core signature (**Fig. 2.7h,i**). Altogether, these results show that young cells with induced low levels of FOXM1, equivalent to those in octogenarian cells (**Fig. 2.8f,g**), recapitulate the ageing-associated mitotic defects, aneuploidy, and senescence phenotypes.

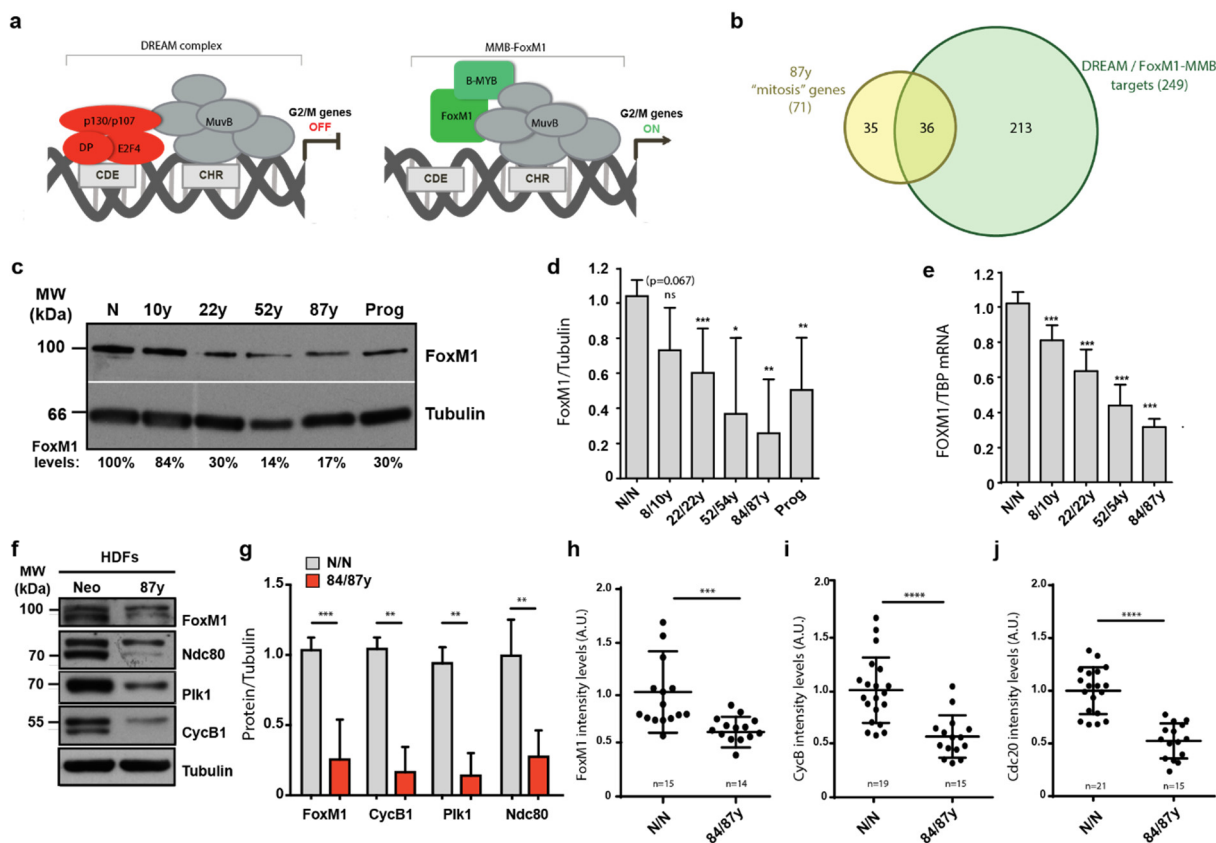
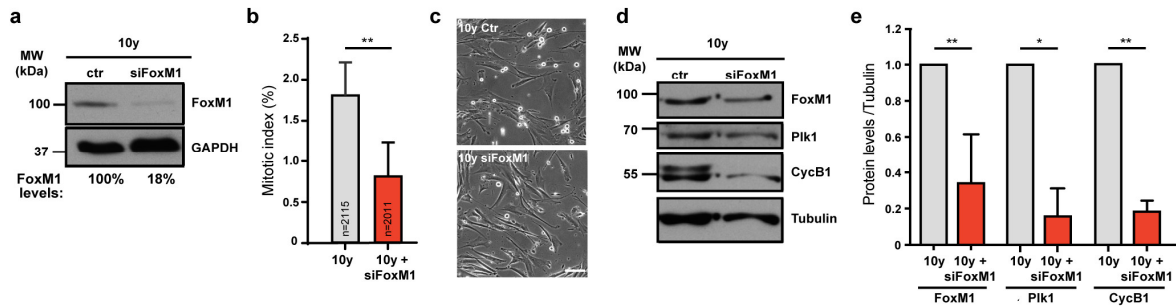


Figure 2.5. Ageing cells express low levels of FOXM1 and mitotic transcripts during mitosis. **a** Regulation of G2/M cell cycle genes by competitive binding of DREAM and FOXM1-MMB transcriptional complexes to CDE/CHR promoter elements, as described in ref. (Müller et al., 2016). **b** Venn diagram displaying the overlap between mitosis genes altered in elderly fibroblasts (87 y) and targets of DREAM/FOXM1-MMB complex (Müller et al., 2016). **c**, **d** FOXM1 protein levels in mitotic extracts of different age samples. FOXM1 levels were normalized to α -tubulin levels and to the neonatal sample (N/N). **e** FOXM1 transcript levels in mitotic total RNA from different age samples normalized to TBP transcript levels and compared to neonatal. **f**, **g** Protein levels of FOXM1 transcriptional targets in mitotic extracts of neonatal and elderly fibroblasts. Values are

normalized to α -tubulin levels and to the neonatal sample (N/N). **h–j** Quantitative analysis of FOXM1 (**h**), Cyclin B (**i**), and Cdc20 (**j**) immunofluorescence intensity levels in n = single mitotic cells (N/N and 84/87 y). Relative intensity levels are indicated as arbitrary units (A.U.). Values are mean $\pm$ SD of two (**h–j**) or three (**d, e, g**) independent experiments. NS, $p > 0.05$, $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$, and $****p \leq 0.0001$ in comparison with N/N by two-tailed Mann–Whitney test.



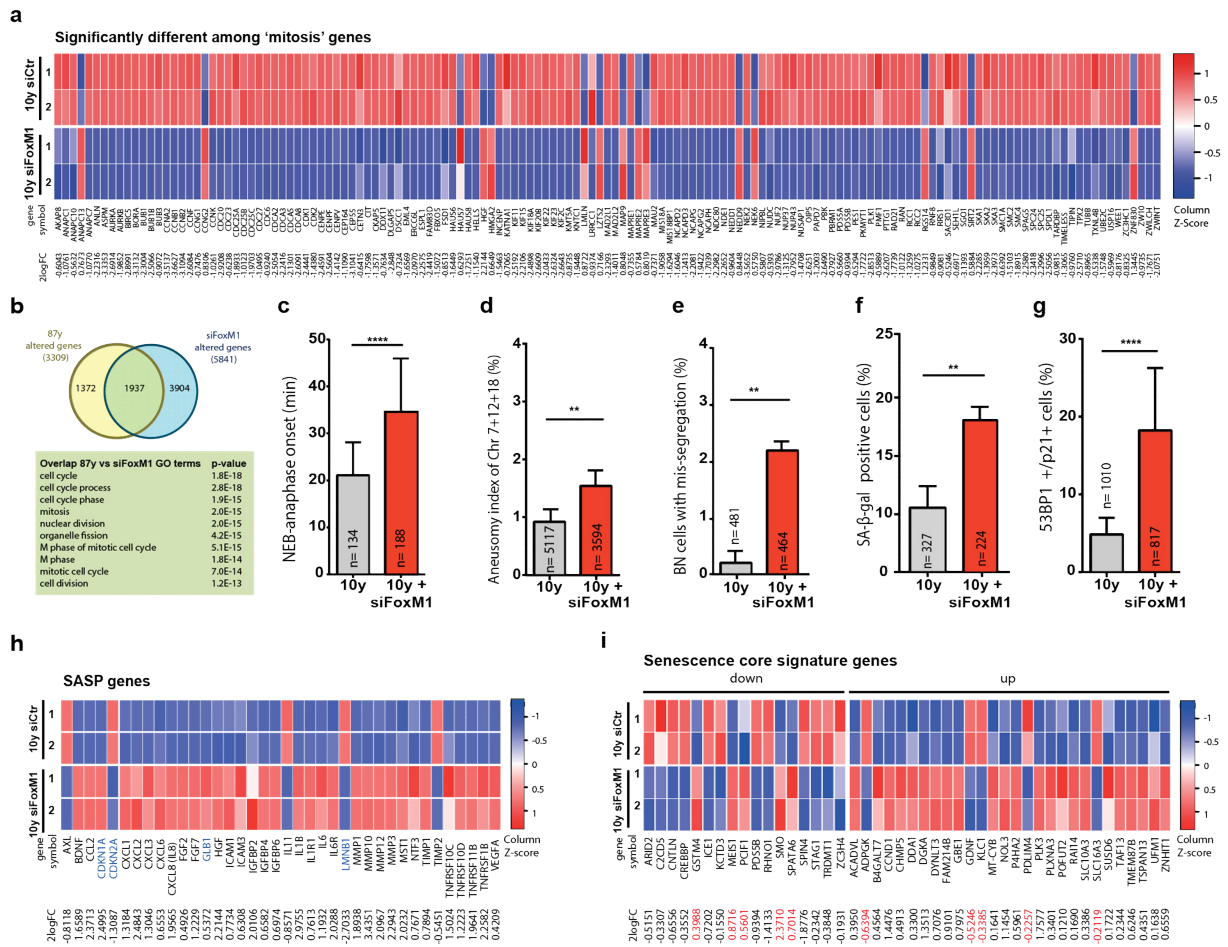


Figure 2.7. FOXM1 repression dictates cellular phenotypes associated with ageing. **a** Heatmap of genes within the “mitosis” GO term differentially expressed between control and FOXM1 siRNA-depleted 10-year-old fibroblasts (2logFC cut-off value ≤ -0.5 and > 0.5 , p -value < 0.05 , FDR $< 5\%$). **b** Venn diagram illustrating the overlap between genes altered in 87y and FOXM1 siRNA-depleted fibroblasts and the top ten altered GO terms. **c** Mitotic duration (NEB-anaphase onset) of control and FOXM1 siRNA-depleted fibroblasts. **d** Aneuploidy index in interphase FISH. **e** Chromosome mis-segregation rate in CytoD-FISH. **f**, **g** Percentage of cells staining positive for the senescence markers β -galactosidase (**f**) and 53BP1/p21 (**g**). **h** Heatmap of SASP and senescence (*CDKN1A*, *CDKN2A*, *GLB1*, *LMNB1*) genes differentially expressed in HDF 10y and 10y siFOXM1 (Coppé et al., 2008; Hernandez-Segura et al., 2017). **i** Heatmap of senescence core signature genes differentially expressed in HDF 10y and 10y siFOXM1; 2logFCs in red indicate genes behaving differently from expected (Hernandez-Segura et al., 2017). In all heatmaps: p -value < 0.05 ; genes represented in columns and technical replicates represented in rows; Z-score column color intensities representing higher (red) to lower (blue) expression. Values are mean $\pm$ SD of three independent experiments. Sample size (n) is indicated in each graph. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ by two-tailed Mann–Whitney (c) and χ^2 (d–g) statistical tests.

FOXM1 induction rescues ageing phenotypes in elderly cells

As FOXM1 depletion reduced mitotic fidelity and induced senescence in young cells, we hypothesized that FOXM1 overexpression in elderly cells should counteract ageing phenotypes. Expression of a constitutively active truncated form of FOXM1 (FOXM1-dNdK) in old-aged fibroblasts (Laoukili, Alvarez-Fernandez, et al., 2008; Laoukili, Alvarez, et al., 2008) (**Fig. 2.8a**) increased MI (**Fig. 2.8b,c**) and resulted in altered expression of 2966 gene

transcripts as determined by RNA-seq profiling of cells captured in mitosis accordingly to experimental layout in Fig. 2.4a (**Fig. 2.9a,c**). Altered gene transcripts were enriched for “mitosis” GO genes (79 genes; **Fig. 2.10a**). To gain insight into the mitosis genes found altered in elderly cells that are dependent on FOXM1 transcriptional activity, we performed comparative analysis with mitosis genes altered in 87 y FOXM1-dNdK and 10 y siFOXM1 samples (**Fig. 2.10b**). Fifty genes out of the 71 mitosis genes altered in 87 y cells were also altered in FOXM1-dNdK and siFOXM1 experimental conditions, suggesting that FOXM1 primarily accounts for age-related mitotic phenotypes. Concordantly, mitotic protein levels (**Fig. 2.8d,e**) and age-associated mitotic delay (**Fig. 2.10c**) were rescued in elderly fibroblasts expressing FOXM1-dNdK, resulting in decreased aneuploidy levels (**Fig. 2.10d,e**). Furthermore, the percentage of cells with SA biomarkers was decreased (**Fig. 2.10f,g**), consistently to amelioration of SASP and senescence core transcriptional signatures (**Fig. 2.10h,i**). Interestingly, the ageing phenotypes were rescued by restoring FOXM1 levels in elderly cells to the levels as present in young cells, indicating that only mild overexpression of FOXM1-dKdK is sufficient to overcome the ageing phenotypes (**Fig. 2.8j,k**). To gain insight into the SA genes found altered in elderly cells that rely on FOXM1 expression levels, we compared the senescence response between 87 y FOXM1-dNdK and 10 y siFOXM1 samples (**Fig. 2.9e,f**). We found that both SASP and senescence core signature genes are largely regulated by FOXM1 levels, with 19 SASP genes and 14 senescence core signature genes consistently altered in all experimental conditions. Further validating our experimental conditions of FOXM1 expression modulation, we found extensive overlap between genes altered in 10 y siFOXM1 and 87 y FOXM1-dNdK (1915 genes; **Fig. 2.9d**), with 158 out of the 249 DREAM/MMB-FOXM1 gene targets included in this overlap. Overall, these data demonstrate that modulation of mitotic efficiency through FOXM1 induction in elderly cells prevents aneuploidy and delays cellular senescence.

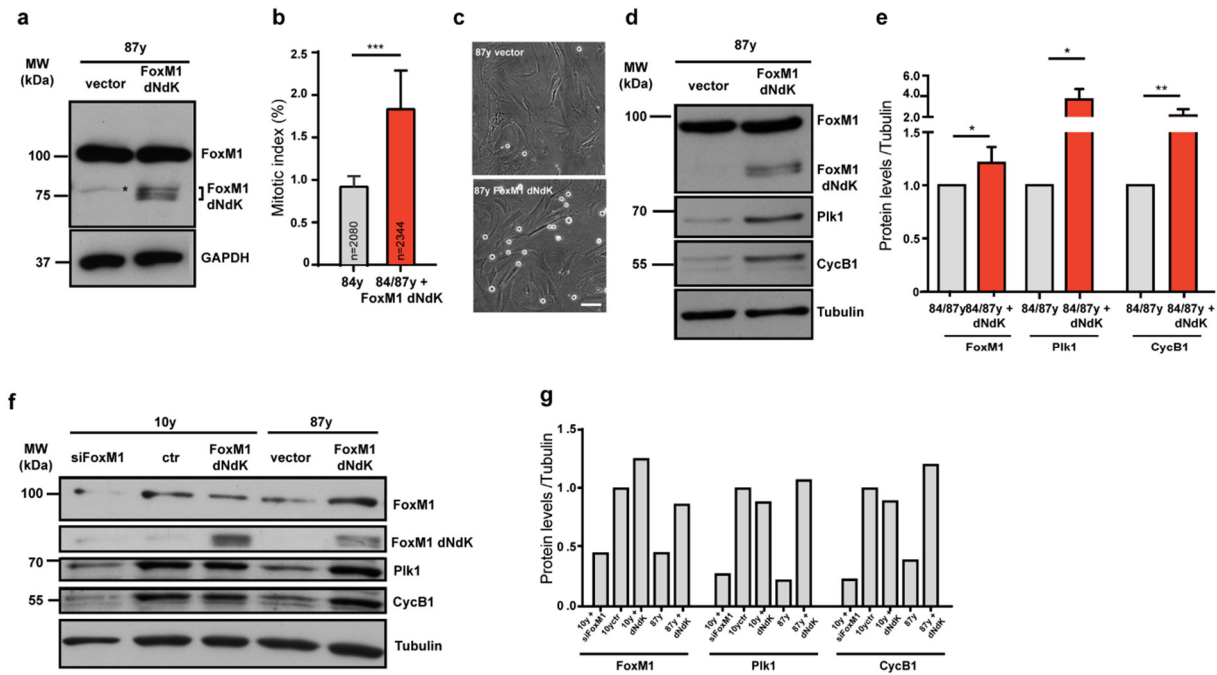


Figure 2.8. Expression of constitutively active FOXM1 (FOXM1dNdK) in elderly cells. **a** Western blot analysis of FOXM1dNdK expression. Asterisk indicates an unspecific band. GAPDH was used as loading control. **b** FOXM1dNdK expression increases mitotic index. **c** Representative phase-contrast images of 87y fibroblasts transduced with empty vector or FOXM1dNdK lentiviruses and treated with STLC for mitotic enrichment. **d-e** Western blot analysis of FOXM1, Plk1 and Cyclin B1 protein levels in mitotic cell extracts of 87y fibroblasts expressing FOXM1dNdK. Tubulin was used as loading control. **f** Western blot comparative analysis of FOXM1 and its mitotic targets, Plk1 and Cyclin B1, in 10y vs. 87y fibroblasts under different experimental conditions as indicated. **g** Quantification of FOXM1, Plk1 and Cyclin B1 protein levels in the western blot shown in f), normalized to tubulin levels and compared to control (ctr) 10y sample. Scale bar, 100 μ m. Values are mean $\pm$ s.d. from two (b) or three (e) independent experiments. Sample size (n) is indicated in each graph. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ and **** $p \leq 0.0001$ by Mann-Whitney (b, e) statistical test.

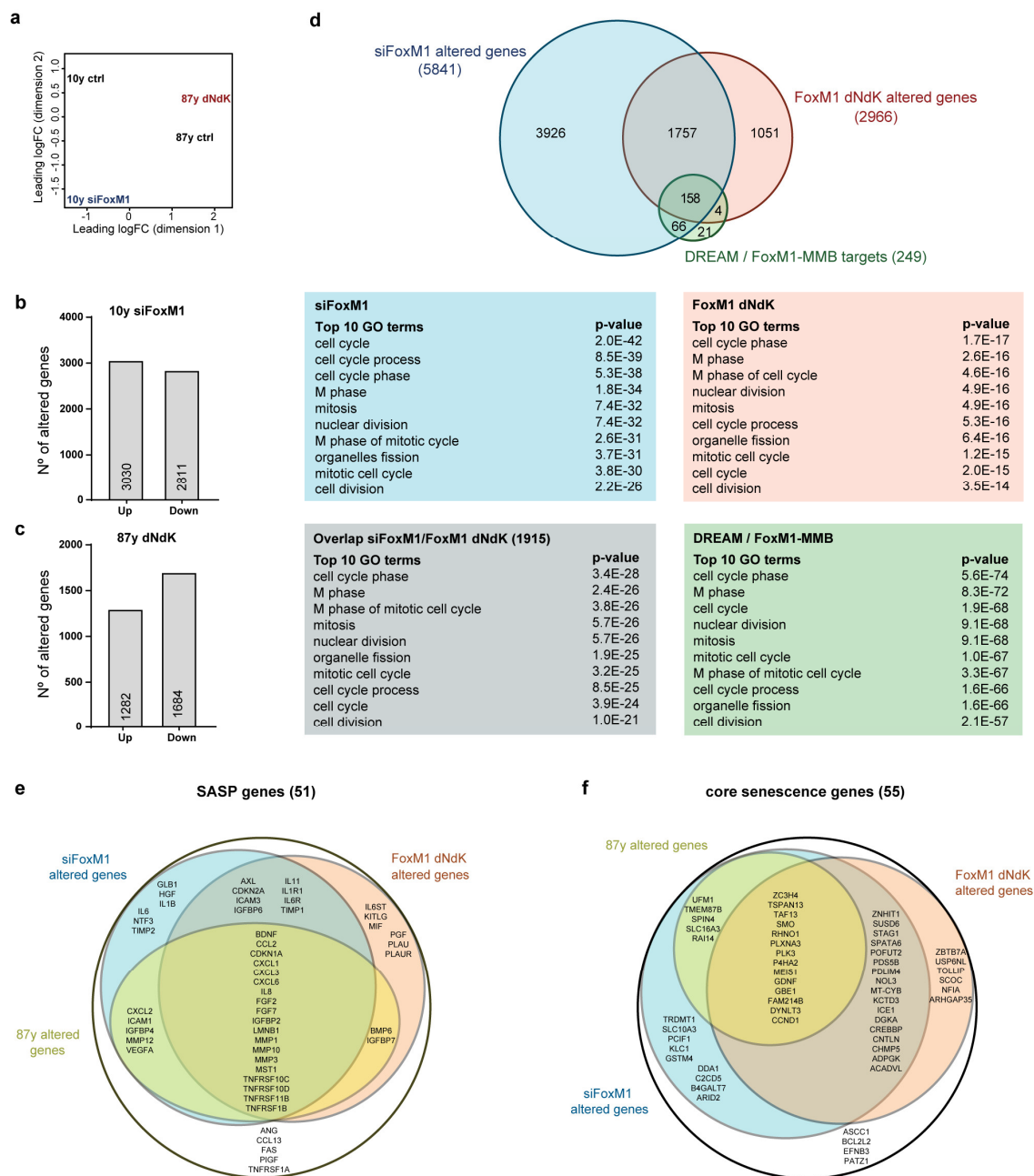


Figure 2.9. Meta-analysis of differentially expressed genes upon modulation of FOXM1 levels. **a** Multidimensional scaling (MDS) plot of RNA-sequencing samples. Axes in the MDS plot represent leading log-fold changes (logFCs) calculated by the root-mean-square of the largest absolute logFCs between each pair of libraries for each sample. **b** Number of altered genes upon FOXM1 RNAi in 10y fibroblasts (Supplementary Data 7). **c** Number of altered genes upon lentiviral transduction of 87y cells with FOXM1dNdK (Supplementary Data 12). **d** Venn diagram displaying the overlaps between siFOXM1 altered genes, FOXM1dNdK altered genes and targets of the DREAM/FOXM1-MMB complexes. The top 10 GO terms for each overlap are organized by *p*-values, using the DAVID Functional Annotation tool. **e** Venn diagram displaying the overlaps between 51 SASP genes interrogated from the RNA-seq datasets of HDF 87y, 10y siFOXM1 and 87y FOXM1dNdK (Supplementary Data 4, 10 and 15). **f** Venn diagram displaying the overlaps between 55 genes of the “senescence core signature” (Hernandez-Segura et al., 2017) interrogated from the RNA-seq datasets of HDF 87y, 10y siFOXM1 and 87y FOXM1dNdK.

Aneuploidy triggers full senescence following old cell division

To directly demonstrate that aneuploidy acts as ultimate trigger to elderly cell permanent cycle arrest, and thus the establishment of a full senescent state, we performed the experimental layout shown in **Fig. 2.11a**. Octogenarian fibroblasts expressing H2B–GFP were followed under long-term time-lapse microscopy. Daughter cell fate of mitotic cells with chromosome segregation defects (anaphase lagging chromosome and/or micronucleus generation), as well as of mitotic cells without apparent chromosome mis-segregation, was tracked individually during 2–3 days for cell death, cell cycling or senescence (SA- β -gal assay at the end of the movie and immunostaining for 53BP1/p21) (Methods). Using this live-cell fixed-cell correlative microscopy analysis, we found that daughter cells from apparently normal mitoses most often kept on cycling ($71.7 \pm 0.4\%$; **Fig. 2.11b,c**) and cell death was never observed. Among the $28.3 \pm 0.4\%$ daughter cells from apparently normal mitoses that stopped cycling, $< 25\%$ exhibited SA biomarkers (β -gal + or 53BP1 + /p21 +) (**Fig. 2.11b,c**). In contrast, daughter cells from mitoses with mis-segregation events consistently stopped proliferating (100%; **Fig. 2.11b,c**). Moreover, when stained for SA- β -gal and 53BP1/p21 SA biomarkers, aneuploid daughter cells were significantly positive ($46.4 \pm 3.6\%$, $p < 0.05$ and $85.7 \pm 7.1\%$, $p < 0.0001$, respectively; two-tailed χ^2 test) in comparison with non-cycling daughter cells produced by apparently normal mitoses (**Fig. 2.11b,c**).

Altogether, our data support a model in which proliferating naturally aged cells with SA gene expression signature (early senescence) lose mitotic fidelity, and generate aneuploid progeny that significantly accounts for the accumulation of full senescent phenotypes (permanent cell cycle arrest) (**Fig. 2.12**). Reinstating FOXM1 transcriptional activity in elderly cells ameliorates cell autonomous and non-autonomous feedback effects between mitotic fidelity and senescence, suggesting that this mechanism evolved as a positive feedback loop between cellular ageing and aneuploidy.

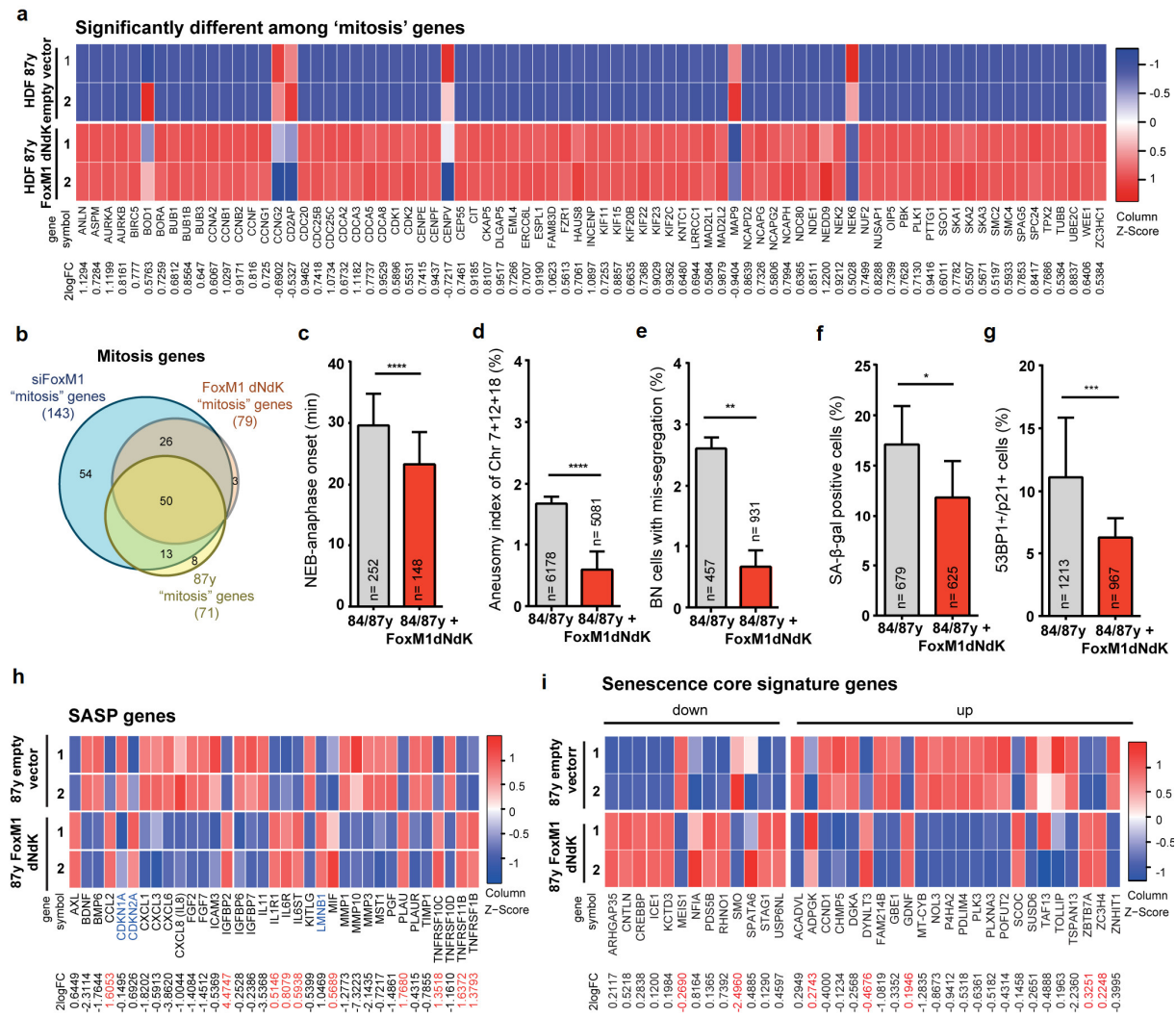


Figure 2.10. Constitutively active FOXM1 ameliorates mitotic fitness and ageing markers in elderly cells. **a** Heatmap of genes within the "mitosis" GO term differentially expressed in mitotic elderly cells transduced with lentiviral empty vector or vector expressing FOXM1-dNdK (2logFC cut-off value < -0.5 and > 0.5 , p -value < 0.05 , FDR $< 5\%$). **b** Venn diagram illustrating the overlap between mitosis genes altered in 87y, 10y FOXM1 siRNA-depleted, and 87y FOXM1-dNdK-expressing fibroblasts. **c** Mitotic duration (NEB to anaphase onset) in elderly fibroblasts infected with control and FOXM1-dNdK lentiviruses. **d** Aneusomy index in interphase FISH. **e** Chromosome mis-segregation rate in CytoD-FISH. **f**, **g** Percentage of cells staining positive for the senescence markers β -galactosidase **f** and 53BP1/p21 **g**. **h** Heatmap of SASP and senescence (*CDKN1A*, *CDKN2A*, *LMNB1*) genes differentially expressed in HDF 87y and 87y FOXM1-dNdK (2logFCs in red indicate genes behaving differently from expected (Coppé et al., 2008; Hernandez-Segura et al., 2017)). **i** Heatmap of senescence core signature genes' expression in 87y and 87y FOXM1-dNdK; 2logFCs in red indicate genes behaving differently from expected (Hernandez-Segura et al., 2017). In all heatmaps, p -value < 0.05 ; genes represented in columns and technical replicates represented in rows; Z-score column color intensities representing higher (red) to lower (blue) expression. Values are mean $\pm$ SD of three independent experiments. Sample size (n) is indicated in each graph. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$ by two-tailed Mann-Whitney (c) and χ^2 (d-g) statistical tests.

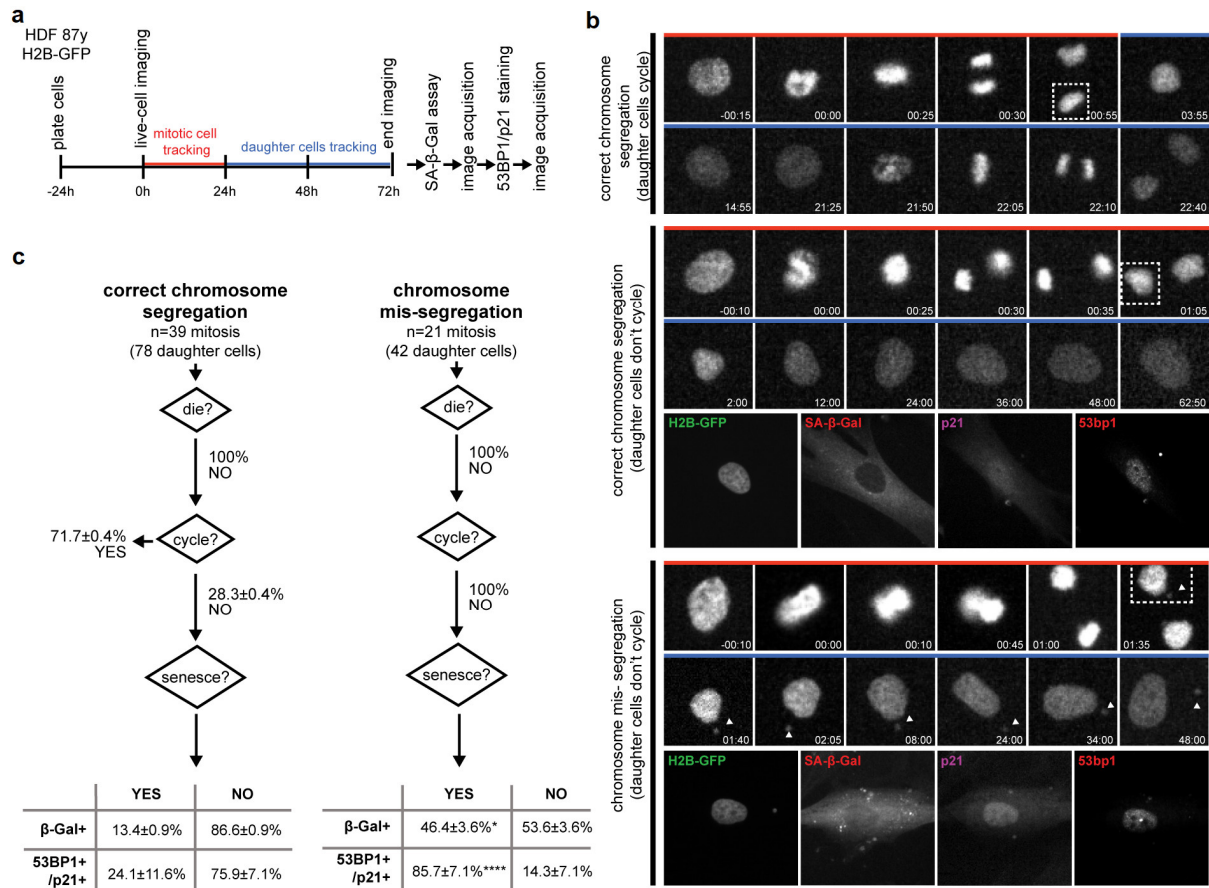


Figure 2.11. FOXM1 governs aneuploidization-driven cellular senescence in elderly cells. **a** Experimental layout of live-cell/fixed-cell correlative microscopy analysis shown in **b**. Mitotic elderly fibroblasts expressing H2B-GFP and respective daughter cells were imaged for 72h. SA-β-Gal assay and immunostaining for 53BP1/p21 were performed at the end of imaging. **b** Movie frames of representative phenotypes observed for elderly cells expressing H2B-GFP. Top panel, correct chromosome segregation, with cycling daughter cells. Middle panel, correct chromosome segregation with non-cycling daughter cells staining negative for SA-β-Gal and 53BP1/p21. Bottom panel, chromosome mis-segregation leading to micronuclei formation (arrowheads), with non-cycling daughter cells staining positive for senescence markers (SA-β-Gal and 53BP1/p21). Dashed line indicates the tracked daughter cell. Time, h:min. Scale bar, 30μm (movie frames) and 15μm (immunostaining). **c** Single-cell analysis of daughter cell fate (cell death, cell cycle arrest, and cell senescence) from mitoses with apparent correct chromosome segregation or with mis-segregation (leading to micronuclei formation). Non-cycling daughter cells were stained for senescence markers (β-gal and 53BP1/p21). Values are mean±SD of two independent experiments. Sample size (*n*) is indicated for **c**. NS, $p>0.05$, * $p\leq0.05$, ** $p\leq0.01$, *** $p\leq0.001$, and **** $p\leq0.0001$ by two-tailed χ^2 statistical tests.

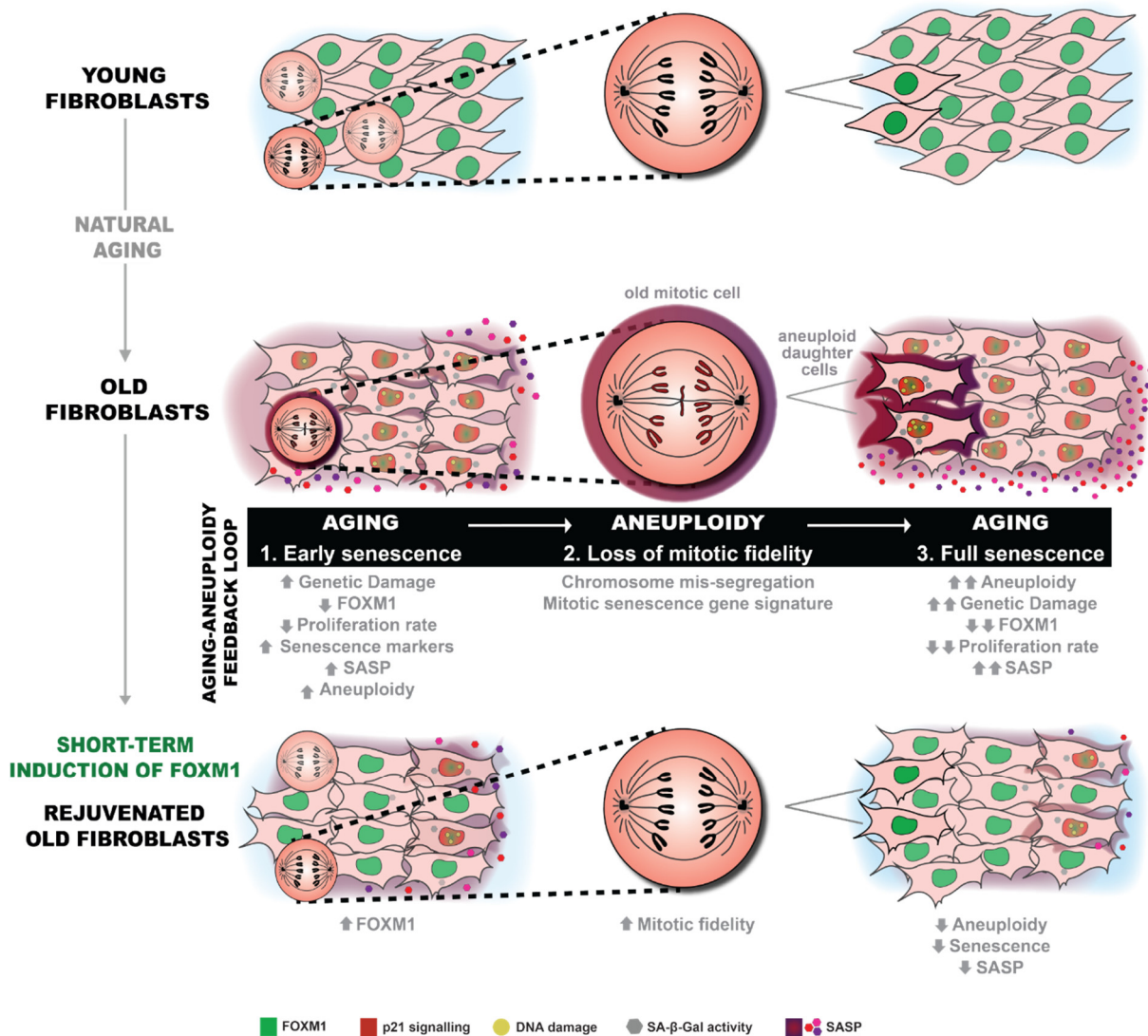


Figure 2.12. Positive feedback loop between ageing and aneuploidy. Model summarizing the molecular basis of age-associated mitotic decline and accumulation of senescent cells induced by aneuploidy. FOXM1 repression in aged dividing cells with senescence gene signature (1. early senescence) leads to general dysfunction of the mitotic machinery and increased chromosome mis-segregation rate (2. loss of mitotic fidelity). The aneuploid progeny stops proliferating exhibiting aggravated senescence phenotypes (3. full senescence) with pro-inflammatory impact in neighboring pre-senescent cells. FOXM1 induction in old cells improves mitotic fitness and delays the accumulation of senescent cells.

2.4 Discussion

Ageing is the largest risk factor for most diseases (Partridge, 2014). In recent years, seminal studies have shown that the accumulation of senescent cells in tissues over time shortens healthy lifespan and that selective clearance of senescent cells can greatly postpone ageing (Baker et al., 2011; Childs et al., 2017). Our results bring insight into how senescent cells arise and provide a molecular basis to delay senescence and thus, a potential strategy against ageing and age-associated diseases.

Previous studies have suggested G2 and 4N G1 (mitosis skip) as the phase at which senescent cells exit the cell cycle (Johmura et al., 2014; Krenning et al., 2014). However, these studies made use of the acute senescence stimuli, gamma irradiation, and oncogenic Ras overexpression, respectively. In contrast to acute senescence, chronic senescence results from long-term, gradual macromolecular damage caused by stresses during lifespan (e.g., loss of proteostasis, DNA damage, epigenetic changes). As shown for oncogene-induced senescence and replicative senescence (Dikovskaya et al., 2015; Johmura et al., 2014), one round of cell division is likely required for a transition from early to fully senescent state. Consistent with this idea of senescence phenotype evolution, our data disclosed the interesting finding that increased pro-inflammatory response is a characteristic of aged cells that are still proliferating, and that chromosome mis-segregation events in these cells are sufficient to trigger transition into permanent cell cycle arrest (full senescence). This is in line with recent findings supporting that micronuclei generated during defective mitoses are a key source of immunostimulatory cytosolic DNA that triggers a cGAS-STING-mediated pro-inflammatory response (Dou et al., 2017; Selene Glück et al., 2017; H. Yang et al., 2017). Whether the evolution of SASP during natural ageing is dependent of cytosolic DNA signaling is an interesting question to address in the future.

We demonstrated that re-establishment of FOXM1 expression in elderly and HGPS fibroblasts can rescue mitotic decline and delay senescence. Fibroblasts are the main constituents of connective tissue and are important factors in extracellular matrix production and tissue homeostasis. Therefore, delayed accumulation of senescent fibroblasts might counteract a SASP-induced inflammatory microenvironment in these tissues and help to protect stem cell and parenchymal cell functions. Re-expression of FOXM1 could thus protect against ageing of adult stem cell and post-mitotic tissues. Indeed, increased expression of one FOXM1 transcriptional target, BUB1B (Fischer, Grossmann, et al., 2016), was previously shown to prevent aneuploidization and delay ageing (Baker et al., 2013). However, other aneuploidy mouse models have not been reported to exhibit premature ageing. Possible explanations are the premature killing of mice before they start developing ageing phenotypes later in life and the cursory analysis for overt age-related degeneration missing tissue-specific phenotypes (Ricke & van Deursen, 2013). Alternatively, aneuploidy-associated genes that are strongly linked with premature ageing might require induction of mild levels of aneuploidy or counteracting functions in additional cellular stresses that engage senescence response pathways. This appears to be the case of FOXM1. FOXM1 repression translates into mild aneuploidy levels and may also further act by counteracting age-associated cellular damage caused by genotoxic and oxidative stresses (Khongkow et al., 2014; Smirnov et al., 2016). Interestingly, increased FOXM1 expression was shown to improve liver-regenerating capacity

in older mice (X. Wang et al., 2001) and lung regeneration following injury (Kalinichenko et al., 2003). However, further evidence shows that FOXM1 is elevated in human cancers, thus questioning its potential for anti-ageing therapy. Thus far, FOXM1 has only been shown to be tumorigenic if tumour suppressor genes are lost or oncogenic mutations (such as K-RAsG12D) are present (Cai et al., 2013; I. C. Wang et al., 2014; I. Ching Wang et al., 2010). As we found that brief reactivation of FOXM1 activity back to the levels of young cells in elderly cells rescues the observed ageing phenotypes, we propose that a cyclic FOXM1 induction scheme, starting in adulthood, could work safely *in vivo*. FOXM1 induction acts not only through modulation of mitotic fidelity and senescence pro-inflammatory phenotype, but also by improving the proliferative capacity of fitter (undamaged) cells in the elderly cell population that dilute, rather than totally clearing, the full-blown senescent cells, thereby coping with the detrimental but also beneficial (wound healing, tumour suppression) effects of senescence. Thus, our findings disclose a molecular mechanism with potential clinical benefit to healthy lifespan extension.

C H A P T E R

I I I

The FOXM1 inhibitor RCM-1 decreases carcinogenesis

The following chapter was published in:

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3.1 Introduction

Robert Costa Memorial drug-1 (RCM-1) is a small-molecule compound that has been identified by high throughput screening as a potent inhibitor of FOXM1 transcription factor (Sun et al., 2017). The RCM-1 compound inhibits the nuclear localization of FOXM1 protein, increasing its ubiquitination and proteasomal degradation (Sun et al., 2017). While in mouse asthma models RCM-1 effectively inhibits FOXM1 without altering expression of other transcription factors or causing toxicity, the efficacy of the RCM-1 compound in cancer models remains unknown. FOXM1, a member of the winged helix or Forkhead Box (FOX) family of transcription factors, has been implicated in activation of cellular proliferation in multiple cell types, making it a promising target for anticancer therapy (D. Balli et al., 2012; David Balli et al., 2011; Kalin et al., 2011; Kalinichenko & Kalin, 2015; Milewski et al., 2017). Unlike other FOX proteins, FOXM1 is expressed in highly proliferative cells but is inhibited during cellular differentiation. FOXM1 is increased in various progenitors and in regenerating adult tissues (X. Huang et al., 2016; Ustiyani et al., 2016; N. Yang et al., 2017). FOXM1 is overexpressed in a variety of human tumours and is used as biomarker for multiple types of cancer (Kalin et al., 2011; Nandi et al., 2018). FOXM1 overexpression increases lung tumour growth in transgenic mice mutant for oncogenic Kras (G12D), while lung epithelial-specific deletion of FOXM1 inhibits pulmonary tumorigenesis (Kim et al., 2006; I.-C. Wang et al., 2009; I. C. Wang et al., 2008, 2014). FOXM1 has been reported to regulate progression of carcinogenesis, and its increased expression is correlated with poor prognoses in patients with prostate cancer (Aytes et al., 2014; Cai et al., 2013; Kalin et al., 2006), lung cancers (Milewski et al., 2017), glioblastoma (N. Zhang et al., 2011), rhabdomyosarcoma (Kuda et al., 2016), melanoma (Kruiswijk et al., 2016), colorectal cancer, breast cancer (N. Yang et al., 2017), liver cancer (Gusarova et al., 2007), pancreatic cancer and gastric cancers (J. Cui et al., 2016; C. Huang et al., 2012). FOXM1 has been also implicated in chemo- and radio-resistance in various cancer types (J. Cui et al., 2016; Y. Lee et al., 2015; Nestal de Moraes et al., 2015; Tassi et al., 2017).

In addition to its prognostic significance, the almost exclusive expression of FOXM1 in the cancer tissue makes it an excellent cancer drug target (Kalinichenko & Kalin, 2015). Inhibition of FOXM1 transcriptional activity led to significant inhibition of carcinogenesis and rendered the tumours responsive to standard chemotherapeutic drugs and radio-therapy (Cai et al., 2013; Koo et al., 2012; Kwok et al., 2008; I. C. Wang et al., 2008). Pharmacological treatment with antibiotics and proteasome inhibitors, such as bortezomib, sialomycin, thiostrepton, MG115 and MG132, decreased FOXM1 expression in cancer cell lines and *in vivo* (Gartel, 2010; Kwok et al., 2008). However, while proteasomal inhibitors efficiently inhibit FOXM1, they affect multiple signalling pathways and cannot be viewed as specific inhibitors of FOXM1.

Development of specific pharmacological inhibitors of FOXM1 represents a considerable clinical value. However, pharmacological targeting of transcription factors has been difficult due to the lack of enzymatic activity (Hagenbuchner & Ausserlechner, 2016). This issue accounts for the lack of advanced target-specific inhibitors of previously characterized transcription factors. The current study was designed to examine the efficacy of RCM-1 in the inhibition of carcinogenesis in different tumour models.

3.2 Methods and Materials

Cell lines and reagents

The cell lines, mouse melanoma B16-F10 and mouse prostate cancer MyC-CaP, were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured with RPMI-1640 (Gibco, Thermo Fisher Scientific, CA, USA) supplemented with 10% FBS and 1x antibiotic-antimycotic (both from Gibco, Thermo Fisher Scientific, CA, USA). Rd76–9 rhabdomyosarcoma cell line (kind gift from Tim Cripe) was cultured with Dulbecco's modified Eagle's medium GlutaMAX (Gibco, Thermo Fisher Scientific, CA, USA) supplemented with 10% FBS and 1x antibiotic-antimycotic. All cells were grown in ventilated flasks at 37 °C and humidified atmosphere with 5% CO₂. The compound RCM-1 (2-[[2-oxo-2-(thiophen-2-yl)ethyl]sulfanyl]-4,6-di(thiophen-2-yl)pyridine-3-carbonitrile) was synthesized by Vitas-M Laboratory (95% purity). The structure of RCM-1 was previously published (Sun et al., 2017).

EdU incorporation assay and mitotic index analysis

Cells were grown on sterilized glass coverslips coated with 50 µg/ml fibronectin (F1141, Sigma-Aldrich, MO, USA) and treated with Dimethyl Sulfoxide (DMSO) or 20 µM of RCM-1. For EdU incorporation analysis (n>500 cells/ cell line/ condition), cells were incubated 1.5 hours with EdU and then processed according to the manufacturer's instructions of Click-iT® EdU Alexa Fluor® 647 Imaging Kit (C10340, Thermo Fisher Scientific, CA, USA). Mitotic index was scored by anti-phospho-Histone H3 (Ser10) (pS10-H3) (Millipore, Merck, Germany) staining (n>1000 cells/ cell line/ condition). In brief, cells were fixed in freshly prepared 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.3% Triton-X100 for 7 min, blocked for 1 hour with 10% FBS, and incubated overnight at 4°C with the primary antibody at 1:1000. Secondary antibody AlexaFluor-647 (Life Technologies CA, USA) was diluted at 1:1500. DNA was counterstained with Hoechst 33342 (Thermo Fisher Scientific, CA, USA). Coverslips were mounted on slides with mounting solution (90% glycerol, 0.5% N-propyl-gallate, 20 nM Tris, pH 8). All slides were acquired on a Leica DMI 6000b inverted microscope (Leica

Microsystems, Germany), equipped with an ORCA-Flash4.0 camera (HAMAMATSU, Japan), using a 20x/0.4NA objective (Leica Microsystems, Germany).

Phase-contrast live cell imaging

Rd76-9, B16-F10 and MyC-CaP cells were grown in Nunc Delta Surface 24-well plates. After treatment with DMSO or RCM-1, microscopic images were acquired on a Leica DMI 6000b motorized inverted epifluorescence microscope under controlled temperature, atmosphere and humidity (Leica Microsystems, Germany), equipped with an ORCA-Flash4.0 camera (HAMAMATSU, Japan). A total of 4 fields per well were photographed every 5 min for 2-3 days, using a 20x/0.4NA objective. Mitotic duration was defined as the interval between nuclear envelope breakdown and anaphase onset. Cell cycle duration was defined as the interval between consecutive mitoses, i.e., from the anaphase onset of a mother cell to the anaphase onset of one of its daughters (n=100 cells/ cell line/ condition).

Mouse models

Mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animal studies were approved by Animal Care and Use Committee (IACUC) of Cincinnati Children's Research Foundation. For rhabdomyosarcoma model, 1×10^6 Rd76-9 cells were injected intramuscularly in the flanks of C56Bl/6J mice. For melanoma model, 1×10^6 B16-F10 cells were injected subcutaneously into C56Bl/6J mice. The tumour bearing mice were randomly divided into two groups (n=5-8) and treated with equal volumes of vehicle (DMSO) or RCM1. RCM1 was dissolved in DMSO and delivered intraperitoneally (IP) in a small volume of 40 μ l at a dose of 20 mg/kg of body weight. 20 mg is equal to 47.1 μ M of the compound. The tumour volume was measured using a digital caliper.

Statistical analysis

Student's t test, Fisher's exact test and one-way analysis of variance (ANOVA) were used to determine statistical significance. *p* values <0.05 were considered significant. Values were shown as mean $\pm$ SD or mean $\pm$ SEM. For the cell cycle duration, EdU and pS10-H3 staining experiments, *p*-values were obtained using GraphPad Prism version 6 (GraphPad, San Diego, CA, USA).

3.3 Results

RCM-1 inhibits cancer cell proliferation rate *in vitro*

To assess the effect of RCM-1 on the proliferative capacity of cancer cells *in vitro*, we performed EdU incorporation assay for DNA synthesis in mouse rhabdomyosarcoma Rd76-9, melanoma B16-F10 and prostate adenocarcinoma MyC-CaP treated with RCM-1 vs. DMSO. The results revealed decreased levels of positive cells in Rd76-9, B16-F10 and MyC-Cap cell lines (**Fig. 3.1a,b**), suggesting that RCM-1 inhibits the growth of several tumour cell lines. Since the inhibition of cell growth in RCM-1-treated cell cultures can be due to delayed progression of the cell cycle or increased apoptosis, we performed long-term phase-contrast live-cell imaging to follow individual cell behavior (**Fig. 3.2a**). 3-day records of Rd76-9, B16-F10 and MyC-Cap cell cultures treated with RCM-1 vs. DMSO control treatment, revealed negligible levels of cell death suggesting cell cycle delay as the major cause of growth defects. Indeed, when measuring cell cycle duration, the interval between anaphase onset of one mother cell and the anaphase onset of a daughter cell (n=100), we found a delay in Rd76-9, B16-F10 and MyC-Cap cell cultures treated with RCM-1 vs. untreated controls (**Fig. 3.2b**). We also measured mitotic duration as the interval between nuclear envelope breakdown and anaphase onset. RCM-1 significantly delayed the completion of mitosis in Rd76-9, B16-F10 and MyC-CaP cell lines (**Fig. 3.2c**), which is consistent with difficulties in the correct attachment of chromosomes to the spindle, a known phenotype cause by FOXM1 inhibition (Macedo et al., 2018). Also, as expected for decreased proliferative capacity, the mitotic indexes of all cell cultures, were significantly lower (**Fig. 3.3a,b**). Thus, treatment with RCM-1 globally inhibited tumour cell growth *in vitro* by delaying cell cycle progression.

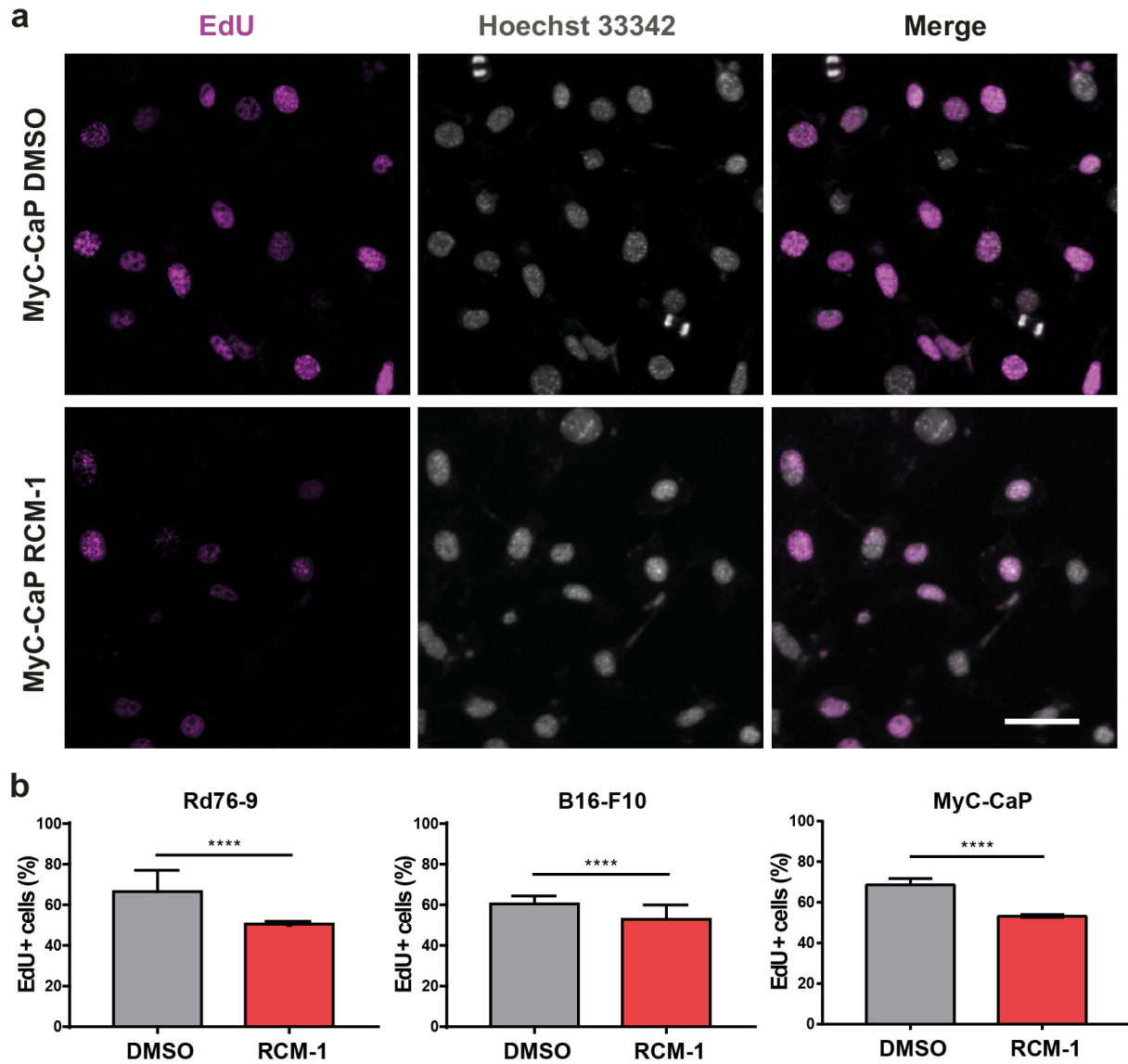


Figure 3.1. RCM-1 decreases the proliferative capacity of cancer cells *in vitro*. **a** Representative fields of DMSO- and RCM-1-treated MyC-CaP cells following EdU incorporation assay (EdU staining, magenta; DNA staining, grey). Scale bar, 100µm. **b** Percentage of DMSO- and RCM-1-treated Rd76-9, B16-F10 and MyC-CaP tumour cells staining positive for EdU ($n > 500$ cells per condition). **** $p \leq 0.0001$ by Fisher's exact statistical test. Values are shown as mean $\pm$ SD from 3 independent experiments.

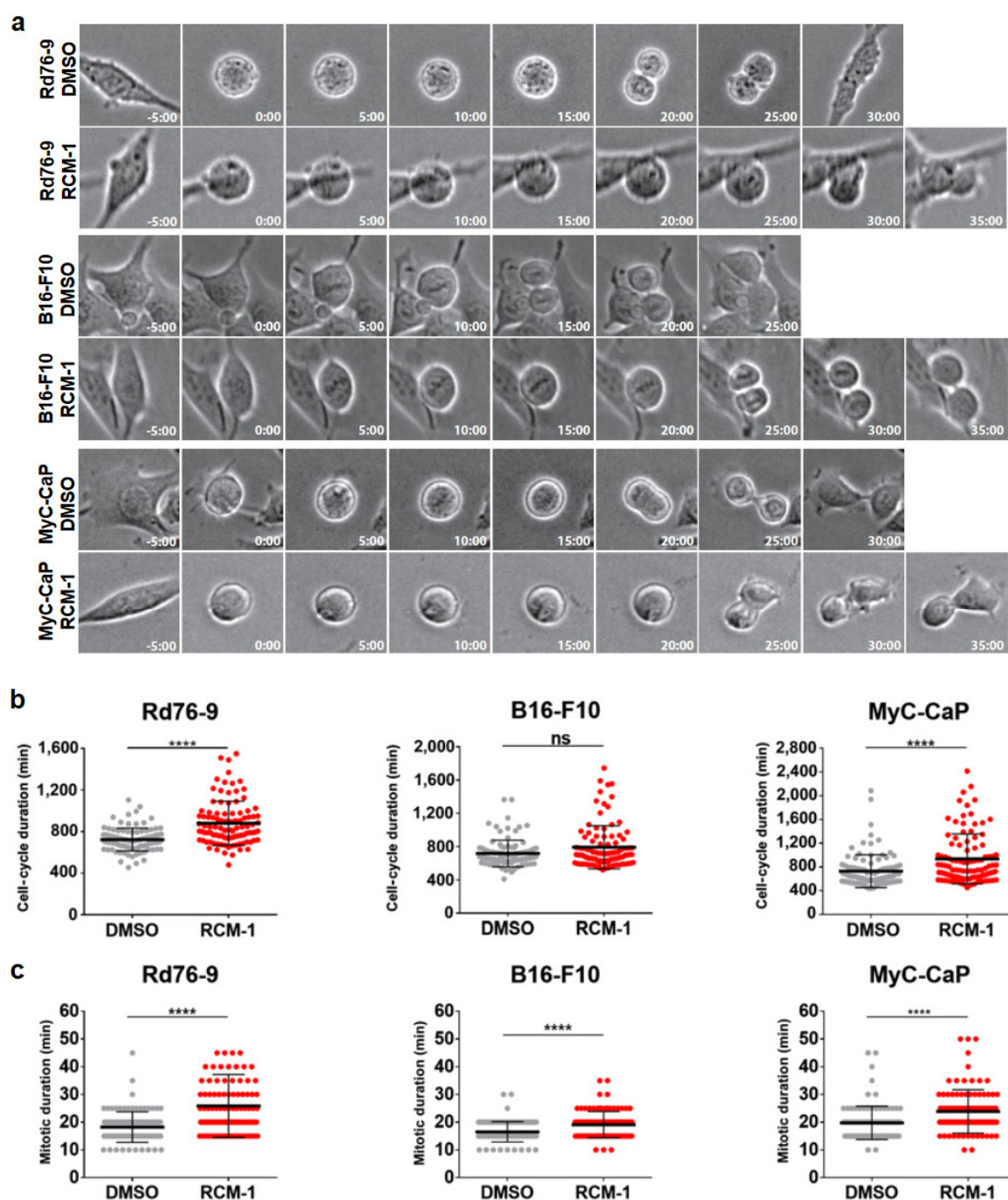


Figure 3.2 RCM-1 increases cell cycle and mitotic duration *in vitro*. **a** Representative stills of DMSO- and RCM-1-treated Rd76-9, B16-F10 and MyC-CaP cells, imaged live every 5 min under phase-contrast microscopy for 2-3 days. **b** Cell cycle duration was measured as the interval between two consecutive mitoses, more specifically from the anaphase onset of a mother cell to the anaphase onset of one of its daughters. **c** Mitotic duration was measured from the nuclear envelope breakdown to anaphase onset. Graphs represent average cell cycle duration of DMSO- and RCM-1-treated cells ($n=100$). ns $p>0.05$, and **** $p\leq 0.0001$ by Mann-Whitney statistical test.

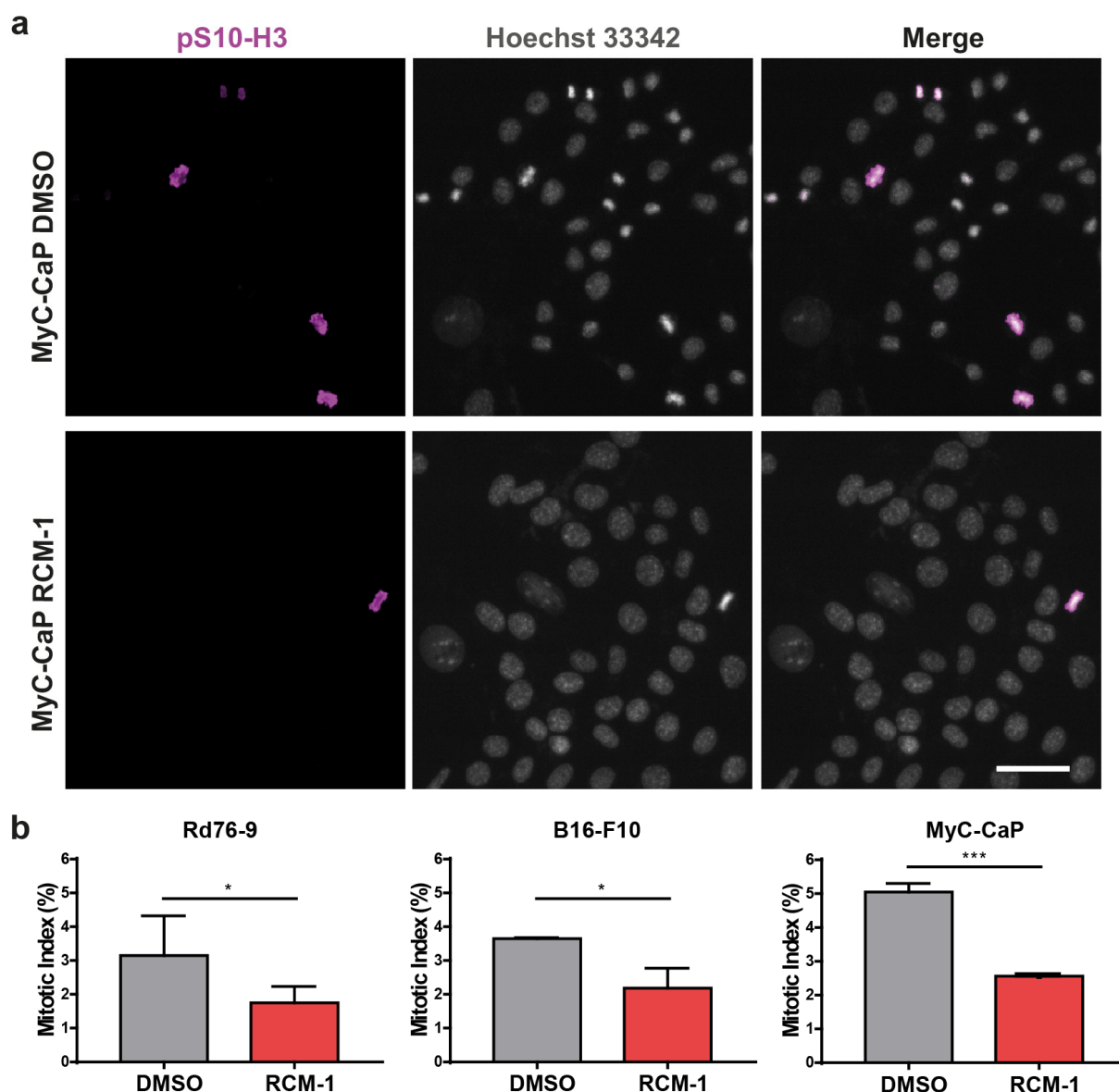


Figure 3.3 RCM-1 decreases the mitotic index of cancer cells *in vitro*. **a** Representative fields of DMSO- and RCM-1-treated MyC-CaP cells immunostained for the phospho-serine 10-Histone 3 (pS10-H3) mitotic marker (pS10-H3 staining, magenta; DNA staining, grey). Scale bar, 100µm. **b** Percentage of DMSO- and RCM-1-treated Rd76-9, B16-F10 and MyC-CaP tumour cells staining positive for pS10-H3 ($n > 1000$ cells per condition). * $p \leq 0.05$, *** $p \leq 0.001$ by Fisher's exact statistical test. Values are shown as mean $\pm$ SD from 3 independent experiments.

RCM-1 inhibits tumour growth in animal models

To examine the efficacy of RCM-1 in the mouse tumour models *in vivo*, rhabdomyosarcoma Rd76–9 cells were injected into the muscle of syngeneic mice, and seven days later, RCM-1 was given to tumour-bearing mice (**Fig. 3.4a**). RCM-1 reduced the Rd76–9 tumour size compared to the vehicle-treated control group (**Fig. 3.4b**). The volumes of RCM-1-treated rhabdomyosarcoma tumours were significantly decreased (**Fig. 3.4c**). To demonstrate that the effect of RCM-1 is not limited to the rhabdomyosarcoma Rd76–9 tumour model, we also used mouse melanoma B16-F10 model. RCM-1 also significantly inhibited tumour growth in B16-

F10 melanoma consistent with loss of FOXM1 (**Figure 3.5a,b**). Consistent with our *in vitro* findings, RCM-1 effectively inhibited FOXM1 in tumour-bearing mice without visible toxicity even with prolonged treatment. Altogether, RCM-1 reduced FOXM1 decreased tumour cell proliferation and growth in rhabdomyosarcoma and melanoma tumours *in vivo*.

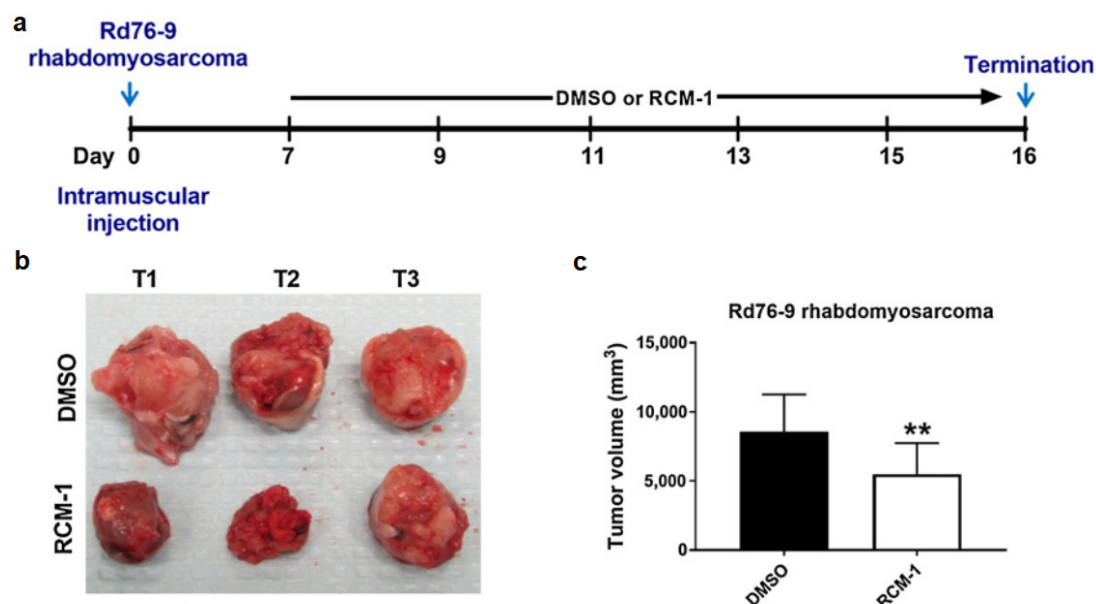


Figure 3.4. RCM-1 treatment reduces growth of orthotopic Rd76-9 rhabdomyosarcoma in mice. **a** Schematic diagram of experimental protocol. **b, c** RCM-1 treatment decreased Rd76-9 tumour growth in mice. DMSO-treated animals were used as control. Data presented as average tumour volume (mean±SD). ** $p < 0.01$, with Student's *t* test.

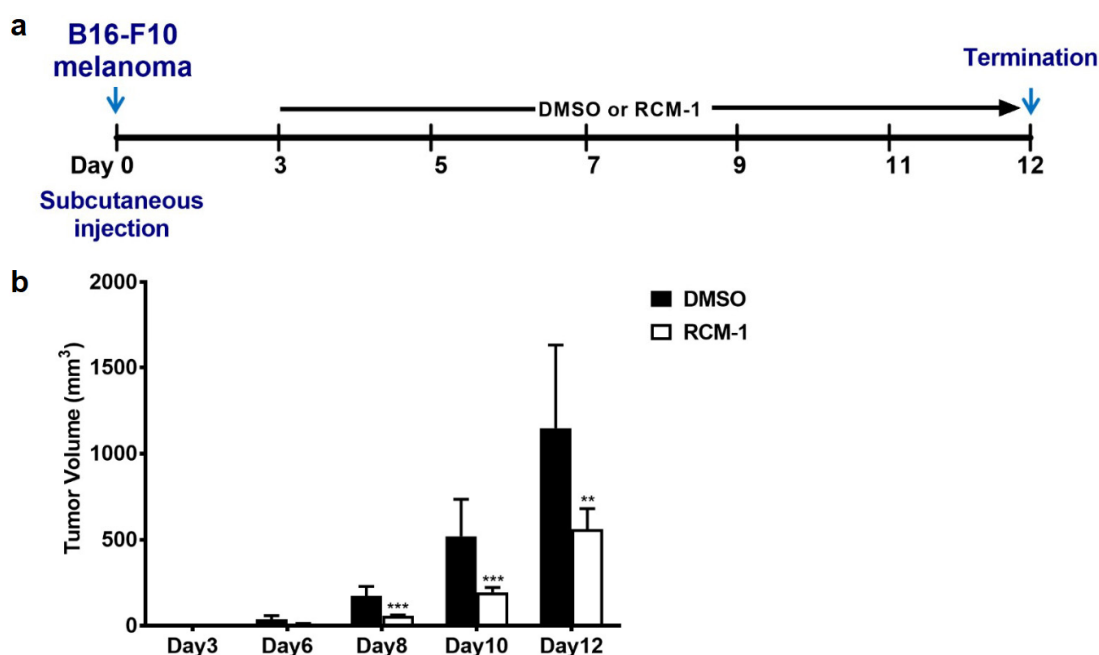


Figure 3.5. RCM-1 treatment reduces growth of B16-F10 melanoma tumours in mice. **a** Schematic representation of experimental protocol. **b** RCM-1 decreased B16-F10 orthotopic tumour growth. Graph shows average tumour volume per group (mean±SD). ** $p < 0.01$, *** $p < 0.001$ with Student's *t* test.

3.4 Discussion

Previous studies demonstrated that FOXM1 is critical for embryonic development and organ regeneration following tissue injury (Kalin et al., 2011). While FOXM1 is decreased after tissue repair, aberrantly high levels of FOXM1 are common for many different types of cancers. Overexpression of FOXM1 increases the initiation, growth, and metastasis in different types of tumours in orthotopic and transgenic mouse models as well as human xenografts (Kalin et al., 2006; Kim et al., 2006; Koo et al., 2012; Milewski et al., 2017; I.-C. Wang et al., 2009; I. C. Wang et al., 2008). Diverse and extensive involvement of FOXM1 in the process of carcinogenesis of different tissue origins suggests that FOXM1 inhibition can be utilized for anti-cancer therapy. A recent study identified the small-molecule FOXM1 inhibitor RCM-1 in a high-throughput screen (Sun et al., 2017). RCM-1 inhibited nuclear localization and increased ubiquitination of FOXM1 in cultured epithelial cells and in the mouse model of goblet cell metaplasia (Sun et al., 2017). In the present study, RCM-1 inhibited tumour growth and decreased tumour cell proliferation in the mouse Rd76–9 rhabdomyosarcoma and B16-F10 melanoma models. FOXM1 is known to function as a typical proliferation-specific transcription factor. It stimulates cellular proliferation by promoting entry into S-phase and M-phase of the cell cycle and controls proper execution of mitotic cell division (I.-C. Wang et al., 2005). RCM-1-mediated inhibition of FOXM1 expression was associated with the inhibition of cell cycle progression. Targeted inhibition of proteins involved in the regulation of the cell cycle has been linked to delays in the duration of mitosis in cancer cells. Similar to previous studies with siRNA inhibition of FOXM1 (Kalinichenko et al., 2004; I.-C. Wang et al., 2005), RCM-1 increased the duration of mitotic division and overall length of the cell cycle and inhibited DNA replication in tumour cells. However, we cannot rule out the possibility that RCM-1 affects other molecular targets in addition to FOXM1.

In summary, the small-molecule compound RCM-1 delays tumour cell proliferation, reducing tumour growth both *in vitro* and *in vivo*. Anticancer effects of RCM-1 are not limited to one tumour type, suggesting a potential for broad use of this small-molecule FOXM1 inhibitor in anti-cancer therapy.

C H A P T E R

I V

FOXM1 repression increases mitotic death upon
antimitotic chemotherapy through BMF upregulation

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4.1 Introduction

Drugs interfering with spindle microtubule (MT) dynamics, MT poisons, have been effectively used as chemotherapeutic agents targeting cancer cell proliferation (Jordan & Wilson, 2004). Notwithstanding substantial research elucidating their mechanism of action, how clinical response is achieved remains elusive. MT poisons, also known as antimitotic drugs, inhibit the metaphase-to-anaphase transition by activating the spindle assembly checkpoint (SAC) due to production of defective chromosome attachments to the spindle (Weaver & Cleveland, 2005). Yet, cancer cells were found to display profound intra- and inter-line variation on their response to antimitotic drugs. This variability is determined by two competing networks: one that protects Cyclin B1 from degradation and another that leads to caspase activation (Gascoigne & Taylor, 2008). If Cyclin B1 levels drop below the threshold needed to maintain Cdk1 activity during prolonged exposure to antimitotics, the cell exits mitosis as a polyploid entity (slippage). If apoptotic signals accumulate faster than Cyclin B1 degradation, the cell dies in mitosis (death in mitosis, DiM). Consequently, understanding the crosstalk between these two pathways is critical to foresee and modulate chemotherapy efficacy.

The FOXM1 transcription factor primarily regulates the proliferation-associated gene cluster critical to G2/M transition, mitotic spindle assembly and chromosome segregation (Fischer & Müller, 2017; Laoukili et al., 2005). Aberrant FOXM1 overexpression is found in most human cancers and is a major adverse prognostic marker (Gentles et al., 2015; Pilarsky et al., 2004). Moreover, FOXM1 upregulation has been implicated in the development of chemotherapeutic resistance, viz. to antimitotic paclitaxel (Arceci et al., 2019; Carr et al., 2010; Chiu et al., 2015; Hou et al., 2017; Khongkow et al., 2016; Na Yang et al., 2019; Zhao et al., 2014), even though the molecular mechanisms remain largely unknown. Resistance to paclitaxel has been associated with evasion to anoikis (J. Chen et al., 2018; Deschesnes et al., 2007; Tamura et al., 2013; M. K. S. Tang et al., 2010), an apoptotic process that adherent cells normally undergo upon loss of contact with the extracellular matrix or neighboring cells (Schmelzle et al., 2007). Cancer cells evade this process to survive after detachment from the primary sites and be able to metastasize (Nagaprashantha et al., 2011). Anoikis execution has been linked to the BH3-only pro-apoptotic proteins of the mitochondrial (intrinsic) apoptotic pathway. In particular, BMF (BCL-2-modifying factor) and BIM, which counteract the anti-apoptotic activity of BCL-2, BCL-XL and MCL-1 (Girnius & Davis, 2017; Hausmann et al., 2011; Hornsveld et al., 2016; Schmelzle et al., 2007), or directly activate the pore-former proteins at the mitochondria (K. Ye et al., 2020). In addition, BMF interacts with the dynein light chain DYNLL1 and DYNLL2 isoforms (P. K. Singh et al., 2019), suggesting for a putative role in cytoskeleton stress sensing (Puthalakath et al., 2001).

Here we investigated whether FOXM1 expression levels determine cell fate decision upon antimitotic drug treatment. Using time-lapse live-cell imaging to monitor individual mitotic cells, we found that FOXM1 repression leads to increased DiM due to upregulation of the pro-apoptotic BMF gene. FOXM1 binding to the BMF promoter and an intronic *cis*-regulatory element acts to repress BMF expression while enhancing the expression of the BUB1B neighbor gene. Through this essential mechanism, mitotic cells are able to circumvent anoikis induction during prolonged arrest unless FOXM1 is repressed. However, this mechanism is partially disrupted in anoikis-resistant tumour cells. Resistance to anoikis-inducing drugs, including paclitaxel, correlates with lower levels of BMF in a FOXM1-independent manner. This provides a useful biomarker for stratification of tumour response to antimitotic chemotherapy.

4.2 Methods and Materials

Cell culture

Human dermal fibroblasts from healthy Caucasian males were obtained from the Coriell Cell Repository (NJ, USA) (HDF 10y GM03348, HDF 87y AG10884) and Zen Bio (NC, USA) (HDF N DFM021711A) and kept at low passage number. HDFs were cultured in Minimal Essential Medium Eagle–Earle supplemented with 15% foetal bovine serum (FBS), 2.5 mM L-glutamine, and 1x antibiotic-antimycotic (Gibco, Thermo Fisher Scientific, CA, USA). MCF-7 cells (ATCC HTB-22) and MDA-MB-231 (HTB-26) were obtained from ATCC (VA, USA) and cultured with Dulbecco's modified Eagle's medium GlutaMAX (Gibco, Thermo Fisher Scientific, CA, USA) supplemented with 10% FBS and 1x antibiotic-antimycotic. All cells were grown in ventilated flasks at 37 °C and humidified atmosphere with 5% CO₂.

Drug treatments

S-Trityl-L-cysteine (STLC, Tocris, UK) was used at 5μM, and paclitaxel (TX, Sigma-Aldrich, MO, USA) at 500nM. For mitotic shake-off, cells were treated with the antimitotic drugs for 16h. For live-cell imaging, cells were treated for 24h with FDI-6 (Axon Medchem, VA, USA) and RCM-1 (kindly provided by Vladimir Kalinichenko, OH, USA). 1mM thymidine block for 20h followed by a 7h washout (WO) into fresh medium was used for G2 cell synchronization.

RNA interference

Human dermal fibroblasts were transfected with 45nM siRNA Universal Negative Control #1 (Sigma-Aldrich, MO, USA), 45nM FOXM1 small interfering RNA (SASI_Hs01_00243977, Sigma-Aldrich, MO, USA), or 45nM FOXO3 small interfering RNA (SASI_HS01_00119127,

Sigma-Aldrich, MO, USA) using Lipofectamine RNAiMAX (Thermo Fisher Scientific, CA, USA) according to manufacturer's instructions. MCF-7 and MDA-MB-231 were transfected with 20nM siRNA. Cells were analysed 72h post-transfection, and depletion efficiency was validated by qPCR.

Plasmid transfection

Human dermal fibroblasts were transfected with 500ng of pcDNA3/CyclinB1-GFP (#39871, Addgene) using Lipofectamine 3000 (Life Technologies, Thermo Scientific, CA, USA) according to manufacturer's instructions. Cells were analyzed 48h post-transfection.

Lentiviral production and infection

pLVX-FOXM1-dNdK was generated as previously described in (Macedo et al., 2018). pBabe HA human BMF plasmid (#17239, Addgene) was digested with BamHI and EcoRI and the coding sequence inserted into the pLVX-Tight-Puro Vector (PT3996-5, Clontech, USA) to generate the pLVX-BMF-Flag construct. Human *BUB1B* was cloned from the cDNA of HDFN into the previously mentioned pLVX-Tight-Puro Vector. Lentiviruses were produced in HEK293T cells transfected with pLVX-empty, pLVX-BMF-Flag, pLVX-FOXM1-dNdK (Macedo et al., 2018) or pLVX-Tet-On Advanced (PT3990-5, Clontech, USA) and the packaging plasmids (pMd2.G and psPAX2, #12259 and #12260 respectively, Addgene) using Lipofectamine 2000 (Life Technologies, Thermo Scientific, CA, USA) according to manufacturers' instructions. HDFs were transduced with inducible and transactivator viruses at 2:1 ratio in media containing 8µg/ml polybrene (Sigma-Aldrich, MO, USA). 500ng/ml doxycycline (Sigma-Aldrich, MO, USA) was added to fresh media and phenotypes analyzed 24h later for BMF overexpression (OX) and 72h later for FOXM1 OX. Transduction efficiency was measured by Flag immunostaining or by FOXM1 qPCR.

CRISPR/Cas9-mediated deletions and validation

Single guide RNAs (sgRNAs) upstream and downstream of the target region were designed using the Benchling online platform (www.benchling.com) (see Table 4.1), and inserted in pSpCas9(BB)-2A-GFP (PX458) (#48138, Addgene) and pU6-(BbsI) CBh-Cas9-T2A-mCherry (#64324, Addgene) plasmids digested with BbsI. Cells were plated in 6-well plate and transfected with 500ng of each plasmid using Lipofectamine 3000 (Life Technologies, Thermo Scientific, CA, USA) according to manufacturers' instruction. After 72h, cells were sorted in a FACSaria II Cell Sorter (BD Biosciences, CA, USA) using an 85µm nozzle and the laser lines 488nm and 561 nm. Cells were gated by forward scatter area (FSC-A) vs. side scatter area and FSC-A vs. FSC-width plot to exclude dead cells and doublets/clumps, respectively. The positive gates were established based on autofluorescence control. GFP/mCherry positive

cells were either collected into a 24-well plate to establish polyclonal cell cultures or collected for qPCR analysis. DNA deletions were validated immediately after FACS (1000 cells) or upon establishment of polyclonal cell cultures. DNA was extracted by boiling the samples in Tris-HCl pH 8.0 for 15 min, incubating with 1µg/µl proteinase K at 56°C, and inactivating at 95°C during 5 min. Genotyping was performed with i-MAX II DNA Polymerase (High Fidelity) (iNtRON Biotechnology, South Korea) according to manufacturers' instruction. Primers used are listed in Table 4.2.

Table 4.1. sgRNAs used for CRISPR/Cas9.

Target	Location	Primer orientation	Primer sequence
BMF KO	upstream target	Fw	caccGCTCCATCTCTCCTGTGAGG
		Rv	aaacCCTCACAGGAGAGATGGAGC
	downstream target	Fw	caccgACTCCTCCCATAGAGACTGG
		Rv	aaacCCAGTCTCTATGGGAGGAGTc
CRE#1 KO	upstream target	Fw	caccGCACTGCAGCTTATGGAGGG
		Rv	aaacCCCTCCATAAGCTGCAGTGC
	downstream target	Fw	caccgTCTGGGTTGAGCAAAGTAAG
		Rv	aaacCTTACTTTGCTCAACCCAGAc
CRE#2 KO	upstream target	Fw	caccgAGACTGTAGCCTCTCCCGAG
		Rv	aaacCTCGGGAGAGGCTACAGTCTc
	downstream target	Fw	caccgTAAATAGCTTGACCATGACT
		Rv	aaacAGTCATGGTCAAGCTATTTAc
CRE#3 KO	upstream target	Fw	caccgCAAGACAAGCCTGACCGACA
		Rv	aaacTGTCGGTCAGGCTTGCTTTGc
	downstream target	Fw	caccgTCTCAATATGCTCTGCAACA
		Rv	aaacTGTTGCAGAGCATATTGAGAc

Table 4.2. Primers used for genotyping.

Target	Primer orientation	Primer sequence
BMF	Fw	ACCGGCCTAGGTCAGAAAAC
	Rv	TAAAGGCCAGGCCACTCAAG
CRE#1	Fw	TCCAAGGCTAAGACAAGACCC
	Rv	GCCAGCCTTCCGTCCTTAT
CRE#2	Fw	TTTGGTTATGGCGGTTCCCA
	Rv	TTCCCCTGGCACTGGTAATG
CRE#3	Fw	CACAAAAGGATCCTAGACTT
	Rv	GTGGCAATGGAGTCTAAAAG

Time-lapse live-cell imaging

Cells were plated in 4-well or 8-well μ -slides (Ibidi, Germany). Time-lapse images were either acquired in a Leica DMI6000b (Leica Microsystems, Germany) or in a Zeiss Axiovert 200M (Carl Zeiss, Oberkochen, Germany) inverted microscope. All experiments were done under controlled temperature, atmosphere, and humidity. Neighbor fields were imaged every 5-10 min for 48-72h. Images were then analyzed in ImageJ/Fiji software.

Western blotting

Western blotting was performed as previously described in (Macedo et al., 2018).

Apoptosis assay

Apoptosis was assessed by flow cytometry using the BD Accuri C6 (BD Biosciences, CA, USA) and the (FITC)-conjugated Annexin V/Apoptosis detection kit (BioLegend, Inc. San Diego, CA). Data were analyzed using the FlowJo v10 software and the gates defined accordingly to the autofluorescence control.

Immunostaining

Cells were fixed in 4% paraformaldehyde in PBS for 20 min, permeabilized with 0.3% Triton-X100 in PBS for 7 min, blocked in 10% FBS and incubated overnight at 4°C with mouse anti-Flag M2 (Sigma-Aldrich, MO, USA) diluted in 5%FBS/PBS. Cells were then incubated for 1h at room temperature with the secondary antibody AlexaFluor-568 (Life Technologies, CA, USA), and DNA was counterstained with 1 μ g/ml DAPI (Sigma-Aldrich, MO, USA). Images were acquired in a Zeiss AxioImager Z1 (Carl Zeiss, Oberkochen, Germany) and analyzed using ImageJ/Fiji software.

Quantitative real-time PCR

Total RNA was extracted using Quick-RNA Microprep Kit (Zymo Research, CA, USA). cDNA synthesis was performed using NZY First-Strand cDNA Synthesis Kit (NZYtech, Portugal). qPCR analysis was performed in a CFX384 Touch Real-Time PCR Detection System using iTaq Universal SYBR Green Supermix accordingly to manufacturers' instruction, and data analysed using the CFX Maestro Software (all from Bio-Rad Laboratories, CA, USA). The $2^{-\Delta Ct}$ or $2^{-\Delta\Delta Ct}$ methods were used to quantify the transcript levels against three housekeeping genes (GAPDH, HPRT1, TBP). Primers used are listed in Table 4.3.

Table 4.3. Primers used for RT-qPCR.

Target	Primer orientation	Primer sequence
BMF	Fw	ACCGGCCTAGGTCAGAAAAC
	Rv	TAAAGGCCAGGCCACTCAAG
BUB1B	Fw	AAATGACCCTCTGGATGTTTGG
	Rv	GCATAAACGCCCTAATTTAAGCC
FOXM1	Fw	ATACGTGGATTGAGGACCACT
	Rv	TCCAATGTCAAGTAGCGGTTG
GAPDH	Fw	GAAGGTGAAGGTCGGAGTCAAC
	Rv	GGTCATTGATGGCAACAATATCCAC
HPRT1	Fw	TGACCAGTCAACAGGGGACA
	Rv	CTGCATTGTTTTGCCAGTGTCAA
TBP	Fw	GAGCCAAGAGTGAAGAACAGTC
	Rv	GCTCCCCACCATATTCTGAATCT

Bisulphite sequencing

The EpiTect Bisulphite Kit (Qiagen, DE) was used accordingly to the manufacturers' instructions. Primers used are listed in Table 4.4.

Table 4.4. Primers used for bisulphite sequencing.

Target	Primer orientation	Primer sequence
Region 1	Fw	TTTTTATGGAAATATTGTAAGGGGT
	Rv	TCCAAAACCTATATTA AAAACAAAATC
Region 2	Fw	GGGGTTTTATTAGTTGTTTG
	Rv	CAATTCTTCATAATCACCCAA
Region 3	Fw	TTTTTTAATAGTTGGGTTTTTTTT
	Rv	ACACACCCCAAACCTAAATAACTC

Cell cycle profiling

Cells were harvested, centrifuged at 1500 rpm for 5min and rinsed in ice-cold PBS. After centrifugation, cells were resuspended in 500µL of ice-cold PBS. An equal volume of 75% cold ethanol was added drop-by-drop, while gently vortexing. Samples were chilled on ice for 30min. Cells were centrifuged at 2000 rpm for 5 minutes and 200µg/µL of RNase A in PBS was added for 3 hours at 37°C. Propidium iodide was used at 50µL/mL for DNA counterstaining. Cells were filtered and the cell cycle profile was assessed by flow cytometry

using the BD Accuri C6 (BD Biosciences, CA, USA). Data were analyzed using the FlowJo v10 software.

Luciferase activity assays

The BMF promoter and CRE#3 regions were amplified from HDF N genomic DNA by PCR. The BMF promoter amplicon was cloned into pNL1.1 plasmid [Nluc] (Promega #N1441) to generate Nluc/BMF. The CRE#3 amplicon was inserted into pCR8/GW/TOPO vector and then cloned into pGL4.23-GW plasmid (Addgene #60323) via Gateway cloning. siNeg- and siFOXM1-depleted MCF-7 cells were used for the luciferase reporter assays. For the promoter reporter assay, cells were transfected with 3 µg of pGL4.54 [luc2/TK] (Promega #E5061) and 300 ng of either pNL1.1 (negative control) or Nluc/BMF using Lipofectamine 3000 (Life Technologies, Thermo Scientific, CA, USA) accordingly to manufacturers' instruction. For the enhancer reporter assay, cells were transfected with 30 ng of Nluc and 3 µg of either pGL4.23-GW (negative control), luc2/TKp (positive control) or luc2/CRE#3 plasmid. pGL4.54 [luc2/TK] and pNL1.1 [Nluc] plasmids were used as transfection controls in the promoter and enhancer assays, respectively. Data were acquired using the Nano-Glo® Dual-Luciferase® Reporter Assay System kit (Promega, WI, USA) in a Synergy™ 2 Multi-Mode Microplate Reader (BioTek, VT, USA). The luciferase activity was normalized to the activity of the transfection control and is presented as a relative response ratio, in which the responses to the empty vector and positive control vector were normalized to 0 and 1, respectively.

4C-Sequencing

Ten million HDF N cells were used for 4C-seq analysis as previously described (Van De Werken et al., 2012) with minor adaptations. Restriction digestions were done with the enzymes DpnII and Csp6I. The final template was purified using an Amicon Ultra-15 Centrifugal Filter Device (Milipore, MA, USA). Two libraries were independently prepared with the Expand Long Template polymerase (Roche, CH) using primers with adaptors (Table 4.5). The resulting 4C libraries were purified with the QIAquick PCR Purification kit (QIAGEN, DE) followed by the Agencourt AMPure XP reagent (Beckman Coulter, CA, USA). Libraries were sequenced on an Ion S5XL System (Ion Torrent, Thermo Fisher Scientific, MA, USA). Processing and alignment of the sequencing data to the human genome (hg38) was performed as previously described (Eufrásio et al., 2020).

Table 4.5. Primers used for RT-qPCR.

Target	Name	Primer sequence
BMF CRE#3	BMF_Read1 (replicate 1)	CCATCTCATCCCTGCGTGTCTCCGACTCAGTTCTAAGAGACGATTCAAAGCAGAAAGTCAGGGATC
	BMF_Read2 (replicate 2)	CCATCTCATCCCTGCGTGTCTCCGACTCAGCTGGCAATCCTCGATTCAAAGCAGAAAGTCAGGGATC
	BMF_Non-read	CCTCTCTATGGGCAGTCGGTGATAACGCAACTGGAAGACACTGG

Association of drug sensitivities with gene expression levels

The mRNA and protein expression levels were obtained from Cancer Cell Line Encyclopedia (CCLE) mRNA expression (11/2020 DepMap release; CCLE_ExpressionI.Full.csv) and CCLE proteomics (01/2020 release; protein_quant_current_normalized.csv) datasets, respectively. The drug sensitivity levels were obtained from the CTRP Cancer Target Discovery and Development (CTD²) dataset (12/2015 DepMap release; CTRPv2.0_2015_ctd2_ExpandedDataset.zip). Spearman's correlation coefficient values and linear regression p-values were calculated using the default association analysis incorporated in the DepMap portal (<https://depmap.org/portal/interactive/>). The mRNA expression levels of clinical samples were obtained from the Gene Expression Omnibus portal (accession number GSE22358) (Stefan Glück et al., 2012). The relative expression levels of BMF (probe ID: 37059) and BUB1B (probe ID: 20572) were compared between the tumours with complete or near-complete response and those with no response to a combination of docetaxel and capecitabine, as was previously done for other genes (Topham et al., 2015). The statistical analysis between the groups was performed using a two-tailed Mann-Whitney test, in GraphPad Prism 9.0.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 8 (GraphPad, CA, USA). Sample sizes and statistical tests are indicated in the figure legends for each experiment. Parametric and non-parametric tests were applied adequately. For pairwise comparisons, either two-tailed t-test, two-tailed Mann-Whitney or two-tailed χ^2 -square was used, otherwise two-tailed one-way ANOVA was applied followed by a post-hoc for multiple comparisons test. ns, $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, and **** $p \leq 0.0001$. Values are shown as mean $\pm$ s.d.

4.3 Results

FOXM1 expression modulates cell fate profile in response to antimitotics

We used time-lapse imaging of human dermal fibroblasts (HDFs) (young vs. aged) with distinct FOXM1 levels (high vs. low) (Macedo et al., 2018) to investigate individual cell response (slippage/exit vs. DiM) to antimitotic drugs (**Fig. 4.1a**). To ensure maximal mitotic blockage and apoptosis response, we treated cells with saturating concentrations of different antimitotic drugs, namely S-trityl-L-cysteine (STLC) that inhibits kinesin-5/Eg5, and the classic MT poison

paclitaxel (TX). 87-year-old HDFs (low FOXM1) were lentiviral-transduced to express a constitutively active form of FOXM1 (FOXM1 OX) (**Fig. 4.1b; Fig. 4.2a**), whereas 10-year-old HDFs (high FOXM1) were transfected with FOXM1 siRNA (siFOXM1) (**Fig. 4.1c; Fig. 4.2b**). We found that FOXM1 induction in elderly cells shifted their cell fate profile towards mitotic exit in both STLC (**Fig. 4.1d,e**) and TX treatments (**Fig. 4.2c,d**). Conversely, siRNA-mediated FOXM1 repression in young fibroblasts shifted their cell fate profile towards DiM in comparison to controls (**Fig. 4.1g,h; Fig. 4.2f,g**), which is in agreement with increased DiM also found in aged vs. young HDFs. The siRNA-driven shift towards DiM was not due to any differences in antimitotic drug action (**Fig. 4.2i**), and was specifically due to FOXM1 repression, as evaluated by FOXM1 OX rescue experiment (**Fig. 4.2j,k**).

We additionally measured the time cells took to execute exit or DiM in response to STLC and TX. We found FOXM1 induction to accelerate slippage and FOXM1 repression to accelerate DiM (**Fig. 4.1f,i; Fig. 4.2e,h**), thus suggesting that FOXM1 modulates both competing networks. Moreover, we assessed the degradation rate of GFP-tagged Cyclin B1 in young and elderly cells (**Fig. 4.3a,b**). Despite the lower Cyclin B1 levels in elderly cells as previously reported (Macedo et al., 2018), we observed that Cyclin B1 is degraded slower in elderly cells (**Fig. 4.3b**) thereby explaining why these cells are not more slippage-prone than younger cells. This is possibly due to downregulation of the CDC20 co-factor of APC/C activity (Macedo et al., 2018). In addition, we tested non-RNAi modalities for FOXM1 repression, namely the small-molecule inhibitors RCM-1 (Sun et al., 2017), which inhibits FOXM1 nuclear localization, and FDI-6 (Gormally et al., 2014), which inhibits FOXM1 binding to DNA. Both inhibitors shifted cell fate profiling of young cells towards increased DiM in response to paclitaxel (**Fig. 4.3c-h**), supporting that FOXM1 repression promotes DiM via its canonical role as transcription factor.

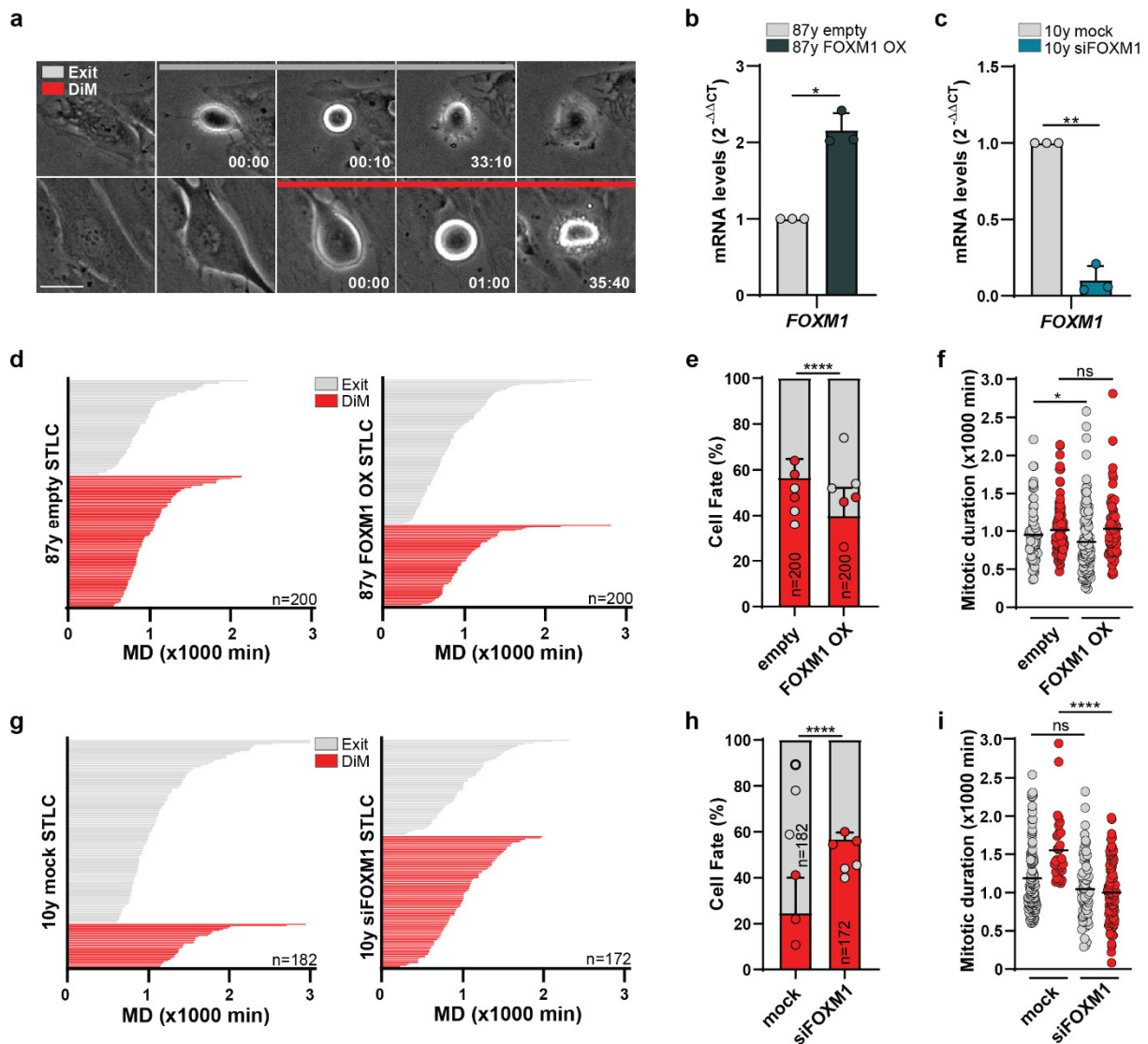


Figure 4.1. FOXM1 levels modulate cell fate profile in response to antimetotics. **a** Representative time-lapse sequences of slippage/mitotic exit (in grey) and death in mitosis (DiM, in red) cell fates of HDFs treated with antimetotics. Time in hr:min, from nuclear envelope breakdown (NEB) to each cellular outcome. Scale bar, 20 μ m. **b** FOXM1 transcript levels in 87-year-old HDFs transduced with control (empty vector) and FOXM1-overexpressing lentiviruses. **c** FOXM1 transcript levels in mock- and FOXM1 siRNA-depleted 10-year-old HDFs. **d** Individual cell fate profiling (exit vs. DiM) of control (empty) (n=200) or FOXM1-overexpressing 87y HDFs (n=200) treated with 5 μ M STLC. MD, mitotic duration. **e** Cell fate percentage of the experiments described in d. **f** Mitotic duration of the individual cell fates shown in d. **g** Individual cell fate profiling (exit vs. DiM) of mock- (n=182) and siFOXM1-depleted (n=172) 10y HDFs treated with 5 μ M STLC. MD, mitotic duration. **h** Cell fate percentage of the experiments described in g. **i** Mitotic duration of the individual cell fates shown in g. Data information: In b, c data are mean $\pm$ s.d. from n=3 independent experiments; *p<0.05, **p<0.01 (two-tailed paired t-test). In e, h data are mean $\pm$ s.d. from n=3 independent experiments; ****p<0.0001 (two-tailed χ^2). In f, i values are mean. *p<0.05, ****p<0.0001 (two-tailed Mann-Whitney test).

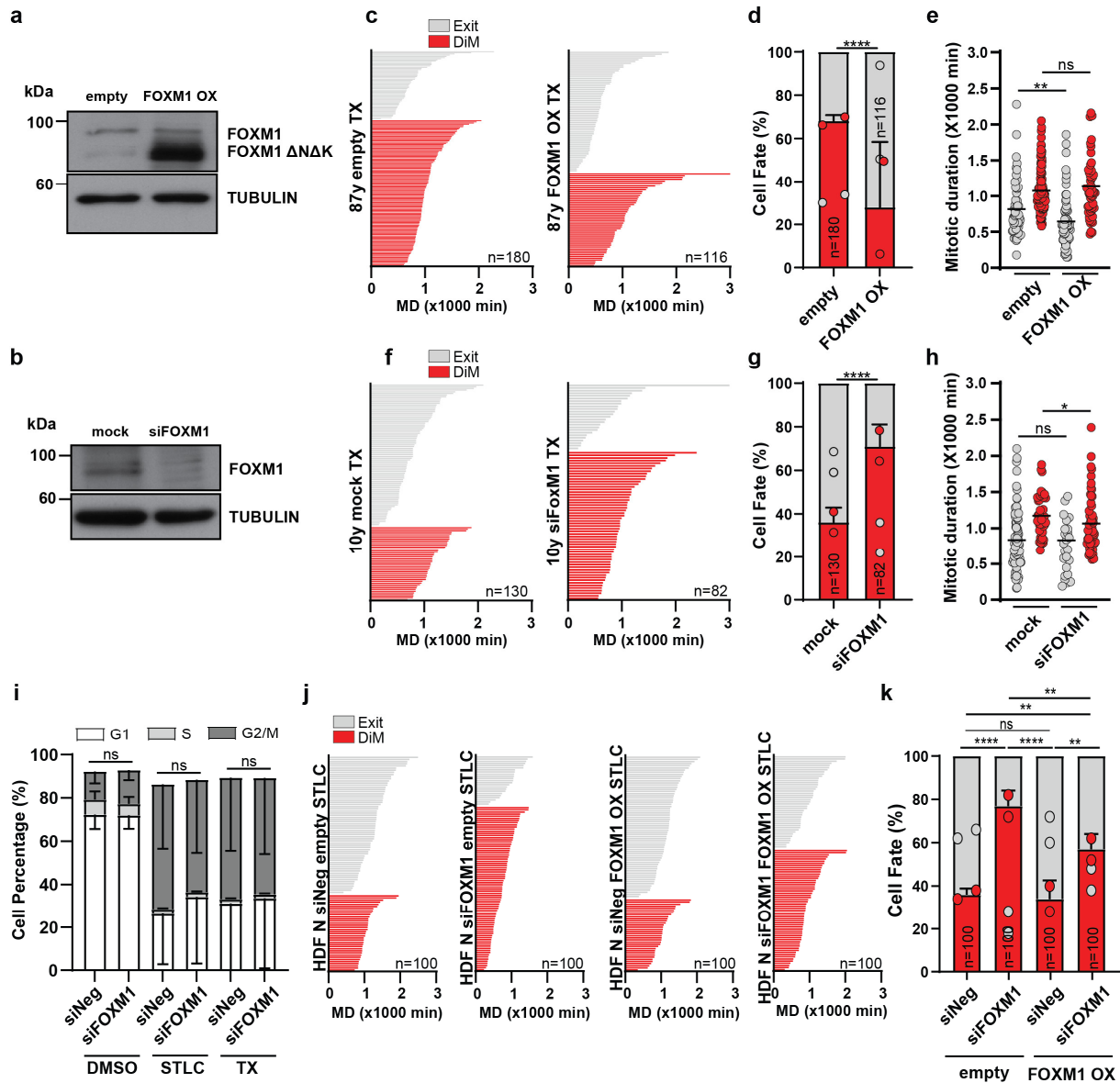


Figure 4.2. Modulation of FOXM1 expression levels in HDFs impact cell fate profiling in response to antimitotics. **a** Western blot analysis of FOXM1 protein levels in 87y HDFs transduced with empty and pLVX-FOXM1-dNdK plasmids. **b** Western blot analysis of FOXM1 protein levels in mock and siFOXM1-depleted 10y HDFs. **c** Individual cell fate profiling (exit vs. DiM) of control (empty vector) (n=180) and FOXM1-overexpressing (n=116) 87y HDFs treated with 500nM paclitaxel (TX). MD, mitotic duration. **d** Cell fate percentage of the experiments described in c. **e** Mitotic duration of the individual cell fates shown in c. **f** Individual cell fate profiling of mock- (n=130) and siFOXM1-depleted (n=82) 10y HDFs treated with 500nM TX. MD, mitotic duration. **g** Cell fate percentage of the independent experiments described in f. **h** Mitotic duration of the individual cell fates shown in f. **i** Cell cycle profile of siNeg- and siFOXM1-depleted young HDFs, treated with DMSO, STLC or TX. **j** Individual cell fate profiling of control (empty) or FOXM1 overexpressing (FOXM1 OX) neonatal HDFs, depleted with Neg or FOXM1 siRNAs, and treated with 5 μ M STLC. MD, mitotic duration. n=cell number. **k** Cell fate percentage of the experiments described in j. Data information: In a, b data are representative images of n=3 independent experiments. In d, g, k data are mean $\pm$ s.d. of n=2 independent experiments; ns p > 0.05, **p < 0.01, ****p < 0.0001 (two-tailed χ^2). In e, h mean values are indicated; ns p > 0.05, *p < 0.05, **p < 0.01 (two-tailed Mann-Whitney test). In i data are mean $\pm$ s.d. from n=3 independent experiments; ns p > 0.05 (two-tailed two-way ANOVA and Tukey multiple comparison test).

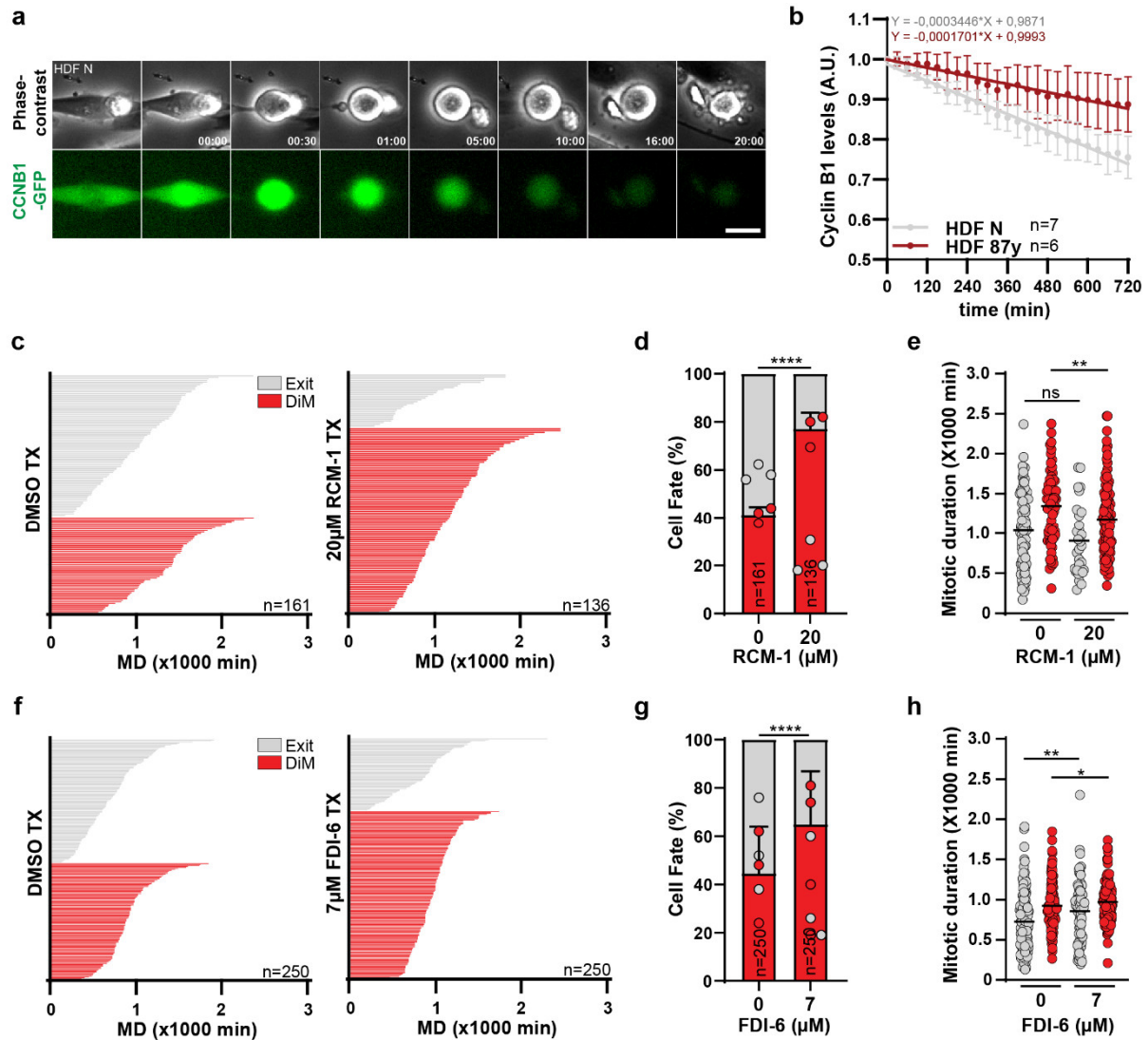


Figure 4.3. Pharmacological modulations of FOXM1 activity impact cell fate profiling in response to paclitaxel. **a** Representative phase-contrast and fluorescent microscopy time-lapse sequences of a neonatal fibroblast expressing cyclin B1-GFP and treated with 5 μ M STLC. Time in hr:min, starting at NEB. Scale bar, 20 μ m. **b** Cyclin B1-GFP levels decay in young (HDF N, n=7) and elderly (HDF 87y, n=6) fibroblasts during prolonged mitotic arrest. For each cell, timepoint fluorescence intensity levels were normalized to the levels at NEB (time 0). **c** Individual cell fate profiling of neonatal HDFs treated with 500nM TX in the absence (DMSO, n=161) or presence of FOXM1 inhibitor (RCM-1, n=136). MD, mitotic duration. **d** Cell fate percentage of the experiments described in c. **e** Mitotic duration of the individual cell fates shown in c. **f** Individual cell fate profiling of neonatal HDFs treated with 500nM TX in the absence (DMSO, n=250) or presence of FOXM1 inhibitor (FDI-6, n=250). MD, mitotic duration. n=cell number. **g** Cell fate percentage of the experiments described in f. **h** Mitotic duration of the individual cell fates shown in f. Data information: In b values are arbitrary units (A.U.) mean $\pm$ s.d.. In d, g data are mean $\pm$ s.d. of n=3 independent experiments; **** $p \leq 0.0001$ (two-tailed χ^2). In e, h mean values are indicated; ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$ (two-tailed Mann-Whitney test).

BMF is an effector of DiM driven by FOXM1 inhibition in response to antimitotics

Since FOXM1 inhibition accelerates DiM, this suggests that FOXM1 influences the death pathway of the competing networks model. Thus, we interrogated the 'regulation of cell death' GO term in our previous RNA-seq datasets generated from mitotic HDFs blocked with STLC (Macedo et al., 2018) (**Fig. 4.4a**). We found 64 differently altered genes across the neonatal

vs. 87y HDFs, mock vs. siFOXM1 10y HDFs, and empty vs. FOXM1 OX 87y HDFs RNA-seq datasets. 48 out of these 64 genes exhibited similar transcriptional changes in elderly and siFOXM1-depleted cells, which were reverted upon FOXM1 overexpression (**Fig. 4.4b**). The most downregulated gene in FOXM1 RNAi was BIRC5, that encodes the well-established pro-survival factor known as Survivin. The most upregulated gene was BMF, which similarly to BBC3 (PUMA) and PMAIP1 (NOXA), encodes for a BH3-only pro-apoptotic protein (R. Singh et al., 2019). BMF captured our attention because, like BIM, it is reported as an effector of anoikis (Schmelzle et al., 2007), and anoikis has been associated with resistance to paclitaxel (J. Chen et al., 2018; Deschesnes et al., 2007; Tamura et al., 2013; M.K.S. Tang et al., 2010). Thus, we wondered whether FOXM1 repression in cells under prolonged mitotic arrest shifts their fate towards DiM through BMF upregulation and anoikis induction. qPCR analysis confirmed BMF upregulation in elderly (87y) and siFOXM1-depleted young (10y) cells arrested in mitosis with STLC in comparison to controls (**Fig. 4.4c-d**). BMF protein levels could not be monitored due to lack of available specific antibodies as reported by others (Schmelzle et al., 2007; Shao & Aplin, 2010). To address the functional role of BMF in mitotic cell fate response to antimitotics, neonatal HDFs (HDF N) were lentiviral-transduced to express FLAG-tagged BMF at an efficiency of ~40% (**Fig. 4.5a,b**). We found that BMF overexpression significantly increased and accelerated DiM in response to STLC (**Fig. 4.4e-g**) and TX (**Fig. 4.5c-e**). Conversely, CRISPR/Cas9-mediated knockout of BMF (**Fig. 4.5f,g**) significantly rescued the shift towards DiM observed in siFOXM1- vs. siNeg-depleted cell cultures treated with STLC and TX (**Fig. 4.4h-i**; **Fig. 4.5h-i**). However, BMF knockout did not alter the time cells took to exit or die in mitosis, suggesting that the Cyclin B1 degradation pathway remained unchanged (**Fig. 4.4j**; **Fig. 4.5j**). In addition, we verified that in the absence of antimitotics, neither FOXM1 repression nor BMF overexpression were able to induce DiM (**Fig. 4.6a-c**), thus indicating that a mitotic delay is needed to mount the apoptotic signalling, and that FOXM1 and BMF modulate cell death under priming conditions of prolonged mitotic arrest. Overall, these data show that BMF accounts for increased DiM in response to antimitotic drugs under low FOXM1 transcriptional activity.

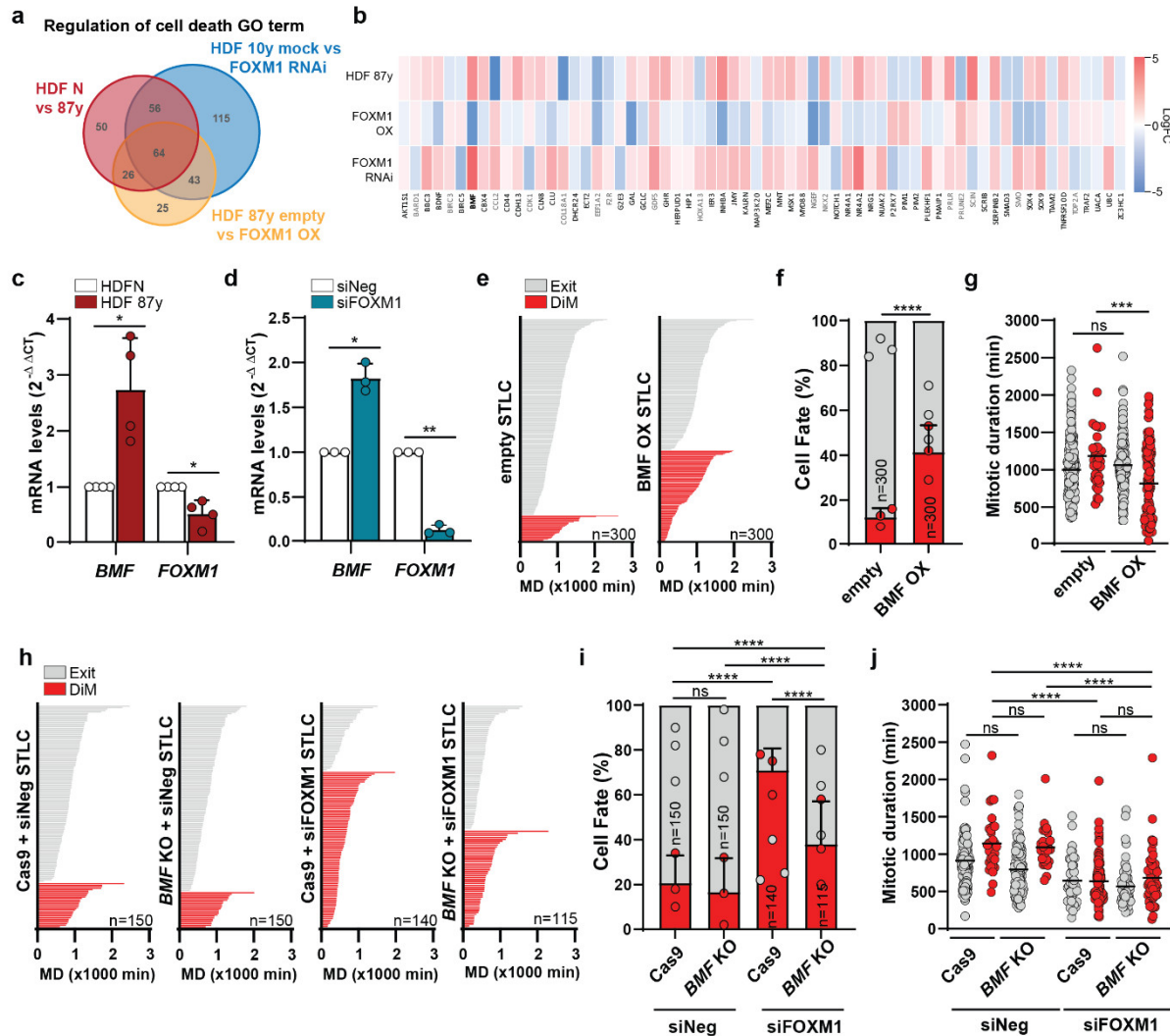


Figure 4.4. BMF upregulation under low FOXM1 levels accounts for the shift towards DiM. **a** Venn diagram of the cell death GO term genes (DAVID Functional Annotation, (D. W. Huang et al., 2009) found significantly altered in the RNA-seq datasets of N vs. 87y, 10y mock vs. FOXM1 RNAi, and 87y empty vs. FOXM1-overexpressing HDFs (Macedo et al., 2018). **b** Heatmap of the 64 overlapping cell death genes shown in A. FC, fold change. **c** *BMF* and *FOXM1* transcript levels in mitotic cells from N vs. 87y HDFs. **d** *BMF* and *FOXM1* transcript levels in mitotic cells from siNeg vs. siFOXM1-depleted neonatal HDFs. **e** Individual cell fate profiling of control (empty vector) (n=300) or BMF-overexpressing neonatal HDFs (n=300) treated with 5μM STLC. MD, mitotic duration. **f** Cell fate percentage of the experiments described in e. **g** Mitotic duration of the individual cell fates shown in e. **h** Individual cell fate profiling of CRISPR/Cas9-edited control (Cas9) or BMF (*BMF* KO) HDFs, depleted with Neg or FOXM1 siRNAs, and treated with 5μM STLC. MD, mitotic duration. n=cell number. **i** Cell fate percentage of the experiments described in h. **j** Mitotic duration of the individual cell fates shown in h. In c, d data are mean ± s.d. from n=4 and n=3 independent experiments; * $p \leq 0.05$, ** $p \leq 0.01$ (two-tailed paired t-test). In f, i data are mean ± s.d. of n=3 independent experiments; **** $p \leq 0.0001$ (two-tailed χ²). In g values are mean ± s.d.; *** $p \leq 0.001$ (two-tailed Mann-Whitney test). In j values are mean ± s.d.; **** $p \leq 0.0001$ (two-tailed Kruskal-Wallis and Dunn's multiple comparison test).

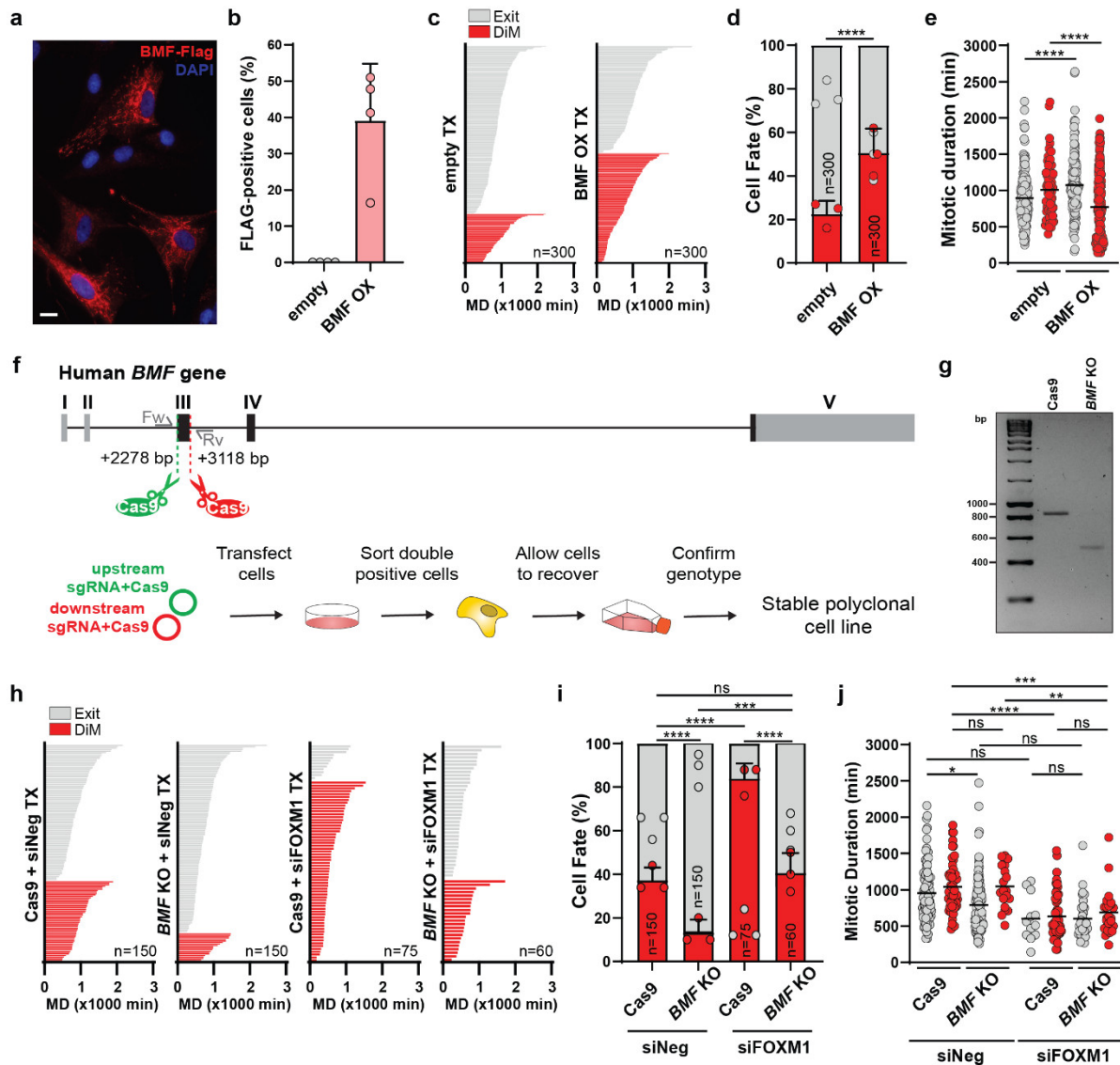


Figure 4.5. BMF expression accounts for increased DiM in response to paclitaxel. **a** Representative image of neonatal HDFs expressing Flag-tagged BMF (in red). Scale bar, 20 μ m. **b** Percentage of cells expressing Flag-tagged BMF upon plasmid transfection. **c** Individual cell fate profiling of control (empty vector) ($n=300$) and BMF-expressing neonatal HDFs ($n=300$) treated with 500nM TX. MD, mitotic duration. **d** Cell fate percentage of the experiments described in c. **e** Mitotic duration of the individual cell fates shown in c. **f** CRISPR/Cas9 gene edition layout for the deletion of exon III in human *BMF*. Upstream and downstream sgRNA plasmids expressing GFP and mCherry, respectively, were used to allow cell sorting of double positive cells (in yellow). Sorted cells were used for establishment of a polyclonal cell culture. **g** Validation by PCR of the efficiency of *BMF* CRISPR/Cas9 genomic deletion in the polyclonal cell population (*BMF* KO). **h** Individual cell fate profiling of siNeg- or siFOXM1-depleted *BMF* KO HDFs treated with 500nM TX. MD, mitotic duration. **i** Cell fate percentage of the experiments described in h. **j** Mitotic duration of the individual cell fates shown in h. In b values are mean $\pm$ s.d. of $n=4$ independent experiments. In d, i values are mean $\pm$ s.d. of $n=3$ independent experiments; *** $p \leq 0.001$, **** $p \leq 0.0001$ (two-tailed χ^2). In e values are mean $\pm$ s.d.; **** $p \leq 0.0001$ (two-tailed Mann-Whitney test). In j values are mean $\pm$ s.d.; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (two-tailed Kruskal-Wallis and Dunn's multiple comparison test).

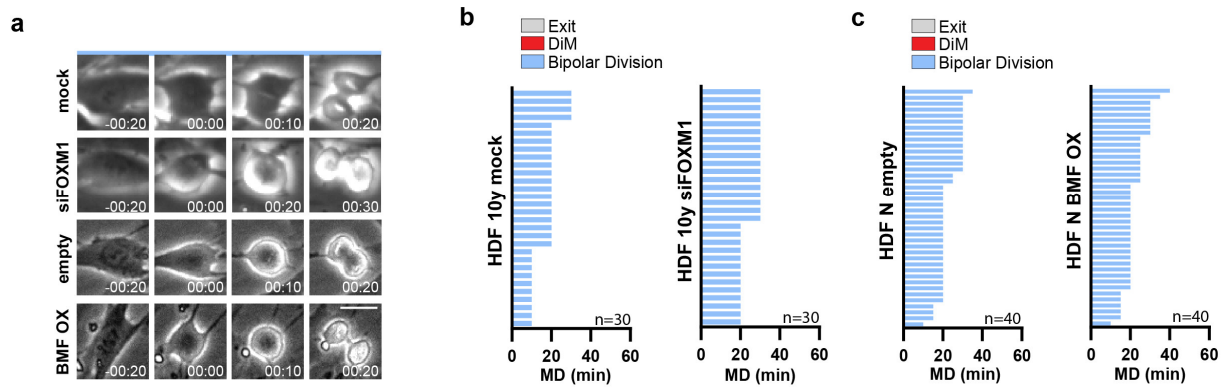


Figure 4.6. FOXM1 depletion or BMF overexpression do not instigate mitotic death during timely mitosis in the absence of antimitotic drugs. **a** Representative time-lapse sequences of HDF bipolar divisions (blue) under the different experimental conditions. Time in hr:min, from nuclear envelope breakdown to anaphase onset. Scale bar, 20 μ m. **b** Individual cell fate profiling of mock (n=30) or siFOXM1-depleted (n=30) 10y HDFs. MD, mitotic duration. Exit/slippage and DiM were never observed. **c** Individual cell fate profiling of HDFs transduced with empty pLVX (n=40) or pLVX-BMF (n=40) plasmids. MD, mitotic duration. Exit/slippage and DiM were never observed.

FOXM1 is required to inhibit BMF expression during mitosis

Since FOXM1 is active at the G2/M cell cycle transition and we found FOXM1 repression to induce BMF upregulation, we hypothesized that BMF gene expression is cell cycle-regulated. To test this, we performed mitotic shake-off of neonatal cultures treated with STLC and TX. qPCR analysis of mitotic cell fractions vs. asynchronous cell population revealed pronounced downregulation of BMF in mitotic cells expressing high FOXM1 levels (**Fig. 4.7a**). Similar results were found using an alternative cellular model, the MCF-7 breast cancer epithelial cell line (**Fig. 4.7b**), in which FOXM1 repression also induces BMF upregulation (**Fig. 4.7c**). In addition, qPCR analysis of an MCF-7 G2-enriched cell population retrieved 7h upon thymidine block washout (before apparent mitotic roundup) (**Fig. 4.8a**) confirmed that BMF is already downregulated in G2 and thus, not merely due to the global transcriptional shutdown during mitosis (**Fig. 4.8b**).

Next we asked whether BMF repression during G2/M is critical to prevent anoikis. To test this idea, we plated asynchronous and mitosis-enriched (6h STLC treatment) MCF-7 cell populations in poly-2-hydroxyethyl methacrylate (poly-HEMA)-coated dishes to measure the extent of anoikis induced by the poly-HEMA inhibition of cell adherence (**Fig. 4.7d**). qPCR analysis of BMF expression (**Fig. 4.7e**) and Annexin-V cytometry (**Fig. 4.7f**) were used as readouts for anoikis after 6h cell culture in poly-HEMA-coated dishes. We found BMF upregulation and increased apoptosis in the MCF-7 asynchronous cell population (mostly G1, see Fig. 4.8a) but not in the mitotic subpopulation. Apoptosis in the MCF-7 asynchronous population was specifically due to BMF, as evaluated by a rescue experiment using BMF KO MCF-7 cells (**Fig. 4.7g**). Conversely, BMF overexpression in MCF-7 cells turned cells more

sensitive to poly-HEMA (**Fig. 4.7h**). Thus, we conclude that BMF expression is cell cycle-regulated, being repressed during G2/M when FOXM1 is transcriptionally active. Importantly, this regulation precludes prolonged mitotic cell rounding from being perceived as cell detachment and from inducing anoikis.

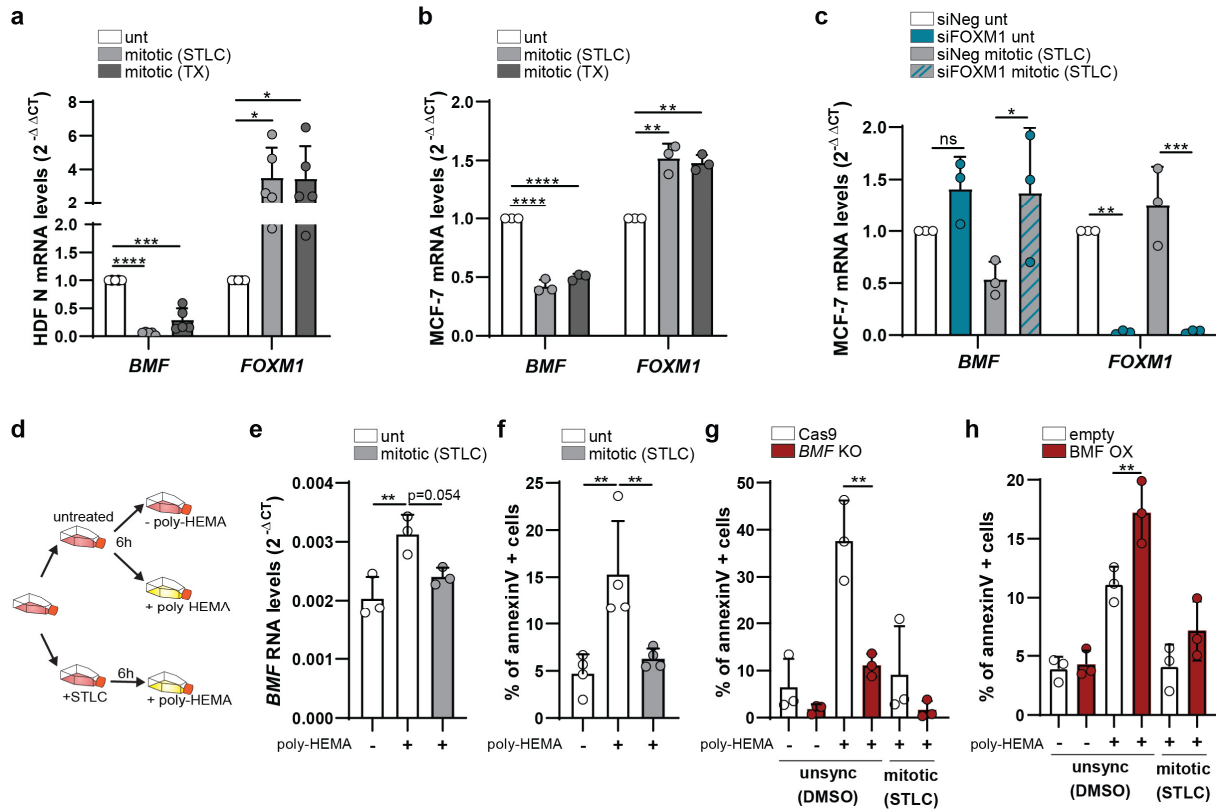


Figure 4.7. FOXM1 represses *BMF* gene expression during mitosis to prevent anoikis. **a** BMF and FOXM1 transcript levels in asynchronous (untreated, unt) and mitotic neonatal HDFs (shake-off upon STLC or TX treatment). **b** BMF and FOXM1 transcript levels in asynchronous (unt) and mitotic MCF-7 cells (shake-off upon STLC or TX treatment). **c** BMF and FOXM1 transcript levels in asynchronous (unt) and mitotic (shake-off upon STLC) MCF-7 cells depleted with Neg or FOXM1 siRNAs. **d** Experimental layout for the assessment of anoikis sensitivity in asynchronous (untreated) vs. mitotic-enriched (STLC treatment) MCF-7 cells cultured in poly-HEMA-coated flasks. **e** BMF transcript levels in the cell culture conditions depicted in d. **f** Annexin V/apoptosis cytometry analysis in the cell culture conditions depicted in d. **g** Annexin V cytometry analysis in the cell culture conditions depicted in d, using CRISPR/Cas9-edited control (Cas9) or BMF (BMF KO) MCF-7 cells. **h** Annexin V cytometry analysis in the cell culture conditions depicted in d, using MCF-7 cells lentiviral-transduced with empty or pLVX-BMF (BMF OX) plasmids. Data information: In a, b data are mean $\pm$ s.d. from n=5 and n=3 independent experiments; * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001 (two-tailed One-way ANOVA and Dunnett multiple comparison test). In c, e-h data are mean $\pm$ s.d. from n=3 or n=4 independent experiments; * p<0.05, ** p<0.01, *** p<0.001 (two-tailed One-way ANOVA and Tuckey multiple comparison test).

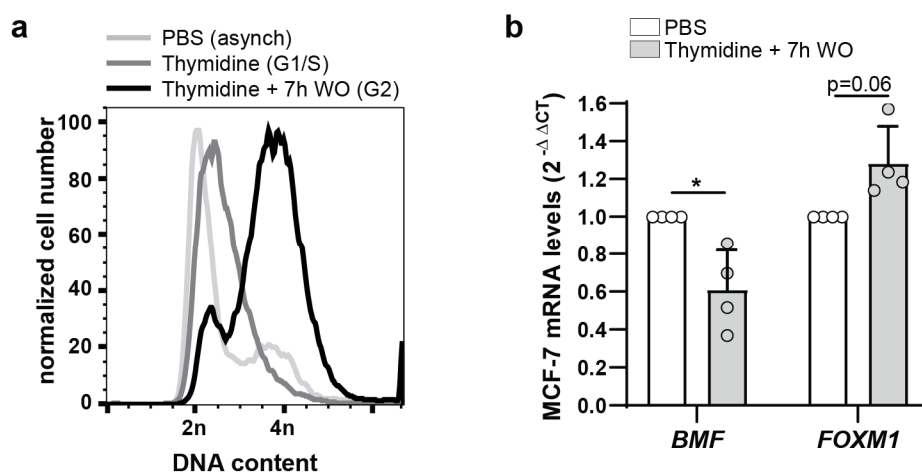


Figure 4.8. Cell cycle regulation of BMF expression. **a** Cell cycle cytometry profiling of MCF-7 cells asynchronous (PBS), under 20h thymidine block (G1/S), and after thymidine washout for 7h (G2). **b** BMF and FOXM1 transcript levels in asynchronous (PBS) and G2-enriched (thymidine + WO) MCF-7 cells. In **b** values are mean $\pm$ s.d. from $n=4$ independent experiments. * $p\leq 0.05$ (two-tailed paired t-test).

FOXM1 acts as a transcriptional repressor of the BMF promoter

To gain insight into the mechanism by which FOXM1 represses BMF transcription during mitosis, we searched for potential FOXM1-binding sites in the BMF genomic region. Interrogation of a publicly available FOXM1 ChIP-seq dataset for MCF-7 cells (Sanders et al., 2013) revealed the BMF promoter, as well as several intronic regions with an H3K27ac mark for active *cis*-regulatory elements (CREs) (Dunham et al., 2012), as FOXM1-binding sites (**Fig. 4.9a**). To assess the role of FOXM1 binding at the BMF promoter, we performed a promoter-luciferase reporter assay in MCF-7 siNeg- and siFOXM1-depleted cells. We found BMF promoter activity in comparison to negative control in siNeg-depleted cells, further enhanced upon siRNA-mediated depletion of FOXM1 (**Fig. 4.9b**). This FOXM1 transcriptional repression at the BMF promoter is not through promoter methylation as previously reported for GATA3, in which FOXM1 was shown to recruit the DNA methylase DNMT3B (Carr et al., 2012) (**Fig. 4.10a**). Also, we excluded transcriptional activation by FOXO3 as a competing mechanism. Although BMF has been described as FOXO3 transcriptional target (Hornsveld et al., 2016), and FOXO3 and FOXM1 to function antagonistically in cancer cells (Yao et al., 2018), we found FOXO3 depletion to induce BMF upregulation and FOXM1 downregulation in HDFs (**Fig. 4.10b**).

As *cis*-regulatory elements can modulate gene expression, we deleted the putative CREs#1-3 (**Fig. 4.9a**) using CRISPR/Cas9-mediated genomic editing. CRE#1 and CRE#2 deletions did not change BMF gene expression in polyclonal MCF-7 cell populations (**Fig. 4.9c,d**; **Fig. 4.10c,d**). Regarding CRE#3 deletion, we repeatedly failed to establish polyclonal cultures from CRE#3-deleted cells (**Fig. 4.10e**). This was not due to sgRNA inefficacy, as successful

genomic deletion was detected immediately after FACS (**Fig. 4.10f**). Importantly, qPCR analysis in these FACS-sorted CRE#3-deleted cells revealed BMF upregulation (**Fig. 4.9e**), thus establishing this FOXM1-binding CRE as a transcriptional BMF silencer. In addition, we confirmed that FOXM1 RNAi, CRE#3 genomic deletion and their combination, all exhibit equivalent effect in BMF upregulation, which supports that FOXM1 represses BMF through its binding to CRE#3 (**Fig. 4.9f**). To confirm that cell death driven by BMF upregulation, and not BUB1B downregulation, was precluding the establishment of a polyclonal cell population deleted for CRE#3, we performed the CRISPR/Cas9-mediated CRE#3 deletion in BMF KO (**Fig. 4.9g**) and in BUB1B OX (**Fig. 4.10g-i**) MCF-7 cells. Under the BMF KO genetic background, but not under BUB1B OX, we were able to establish a polyclonal cell population deleted for CRE#3 (**Fig. 4.9g**), which demonstrates that CRE#3 is required for cell viability. Overall, our data disclosed the FOXM1-binding CRE#3 as a transcriptional BMF silencer.

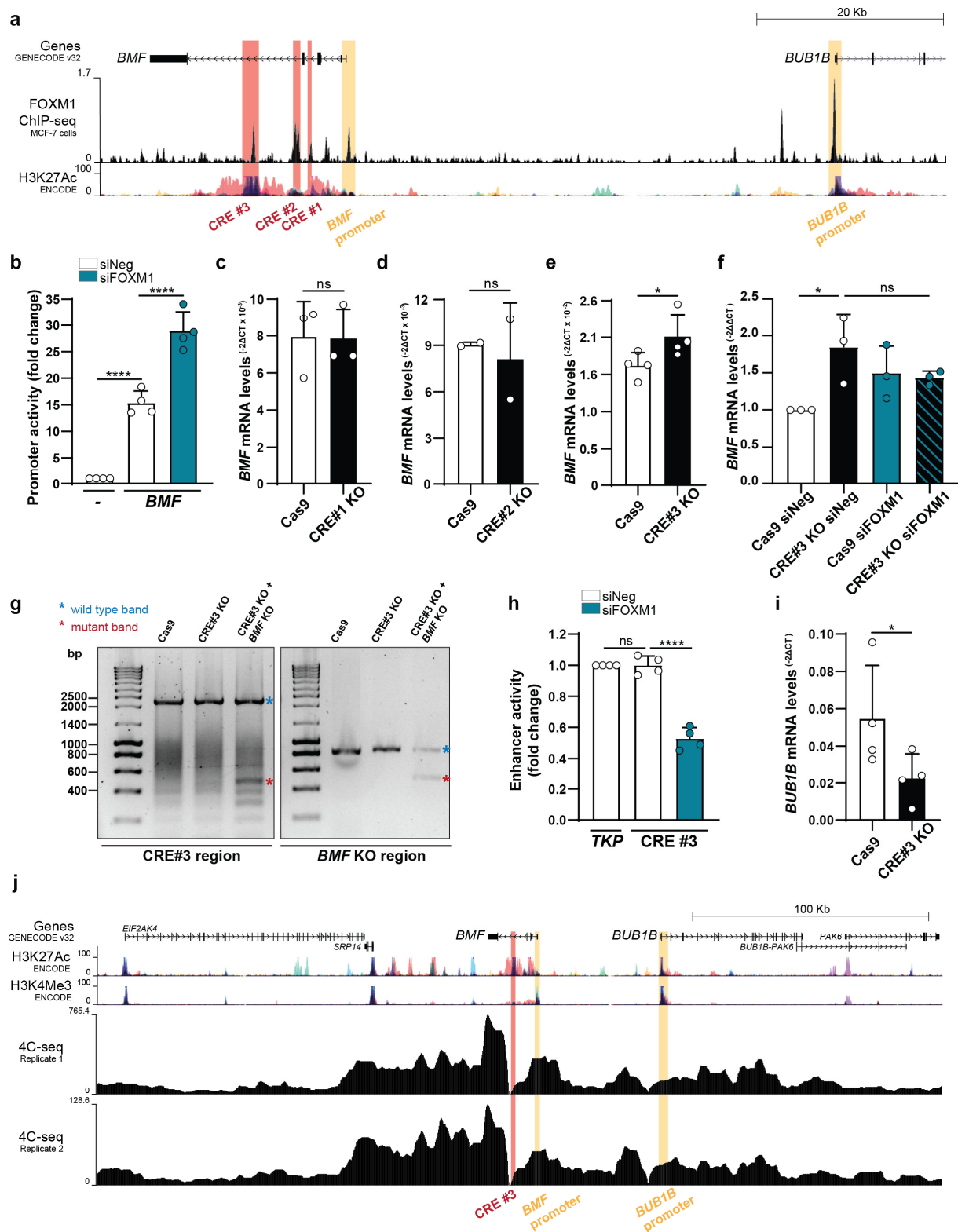


Figure 4.9. FOXM1-binding CRE within *BMF* intronic region represses *BMF* while enhancing *BUB1B* expression. **a** *BMF* and *BUB1B* genomic landscape from UCSC genome browser with tracks of FOXM1 (Sanders et al., 2013) and H3K27Ac (Dunham et al., 2012) ChIP-seq data. **b** *BMF* promoter reporter assay in siNeg- and siFOXM1-depleted MCF-7 cells. **c** *BMF* transcript levels in CRE#1 CRISPR/Cas9-deleted MCF-7 polyclonal cell population. **d** *BMF* transcript levels in CRE#2 CRISPR/Cas9-deleted MCF-7 polyclonal cell population. **e** *BMF* transcript levels in CRE#3 CRISPR/Cas9-deleted MCF-7 cells immediately after sorting. **f** *BMF* transcript levels in CRE#3 CRISPR/Cas9-deleted and siFOXM1-depleted MCF-7 cells immediately after sorting. **g** Genomic PCR validation for the ability to generate CRE#3 CRISPR/Cas9-deleted polyclonal cell population only in a *BMF* KO background. **h** Enhancer reporter assay for CRE#3 in siNeg- and siFOXM1-depleted MCF-7

cells. i) BUB1B transcript levels in CRE#3 CRISPR/Cas9-deleted MCF-7 cells immediately after sorting. j) 4C-seq replicates of neonatal HDFs with a viewpoint in CRE#3 (red). BMF promoter and BUB1B promoter interactions with CRE#3 are highlighted (orange). Data information: In b,f,h data are mean $\pm$ s.d. from n=3 or n=4 independent experiments; **** $p \leq 0.0001$ (two-tailed One-way ANOVA and Tukey multiple comparison test). In c, d data are mean $\pm$ s.d. from n=3 and n=2 independent experiments; ns, $p \leq 0.05$ (two-tailed Mann-Whitney test). In e, i data are mean $\pm$ s.d. from n=4 independent experiments; * $p \leq 0.05$ (two-tailed paired t-test).

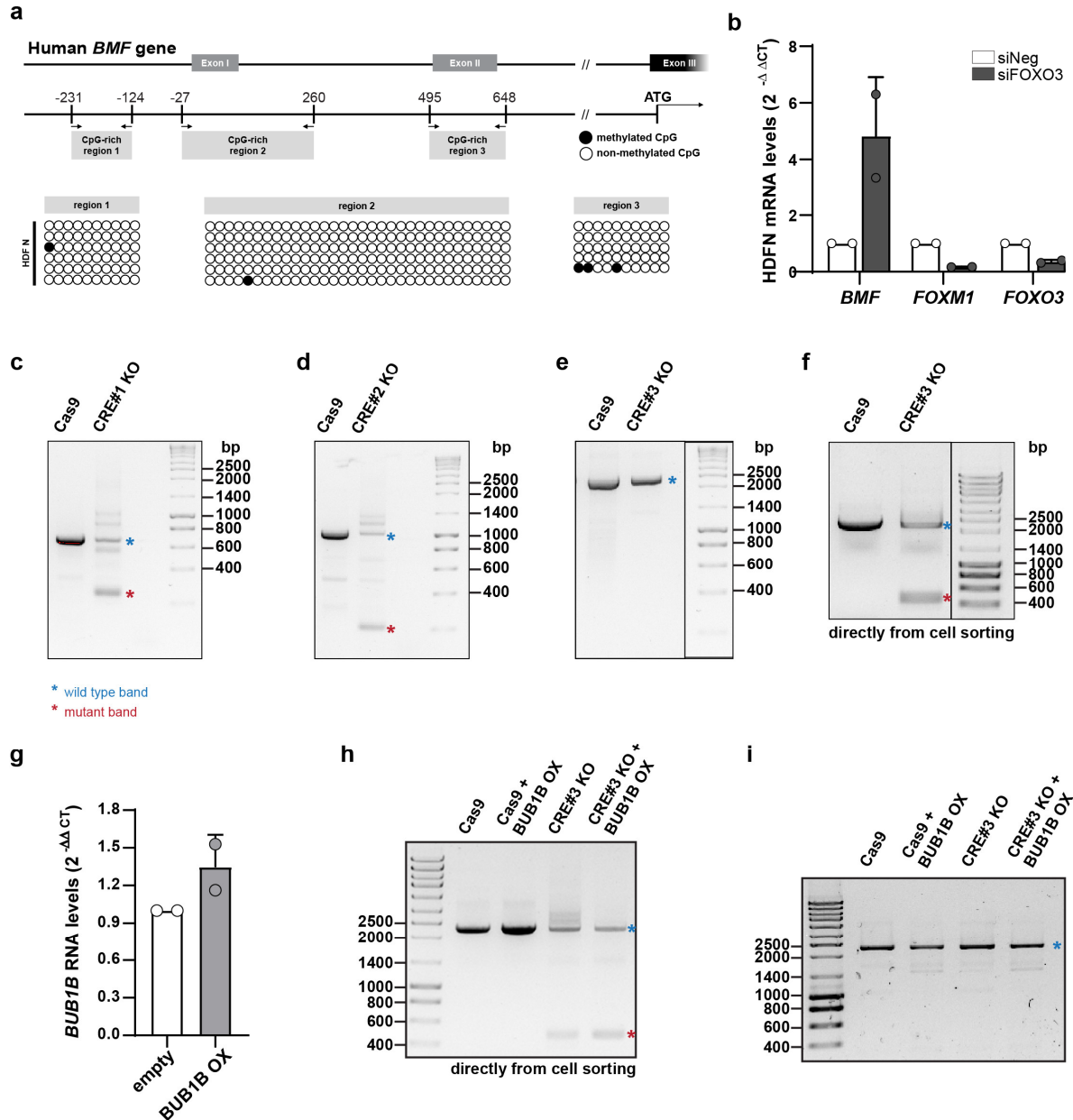


Figure 4.10. Analysis of the DNA methylation and FOXO3 putative roles in *BMF* repression and validation of the CRISPR/Cas9-driven genomic deletions in MCF-7 cells. **a** Bisulphite sequencing of CpG-rich islands near the *BMF* promoter in mitotic neonatal HDFs. **b** *BMF*, *FOXM1* and *FOXO3* transcript levels in siNeg- and siFOXO3-depleted neonatal HDFs. **c-f** Genomic DNA was extracted from polyclonal MCF-7 cell cultures established after cell sorting (c-e) or from cells immediately after sorting (f), and used for PCR detection of the wild type (blue asterisk) and mutant (red asterisk) alleles of the (c) CRE#1, (d) CRE#2 and (e-f) CRE#3 CRISPR/Cas9-mediated genomic deletions. **g** *BUB1B* transcript levels in *BUB1B* overexpressing MCF-7 cells. **h-i** Genomic DNA was extracted from MCF-7 and MCF-7 *BUB1B* overexpressing cells immediately after sorting (h) or from polyclonal cell cultures recovered days after cell sorting (i) and used for PCR detection of the wild type (blue asterisk) and mutant (red asterisk) alleles of the CRE#3 CRISPR/Cas9-mediated genomic deletions. Data information: In b,g values are mean $\pm$ s.d. from n=2 independent experiments.

CRE within BMF intronic region works bi-functionally as BUB1B enhancer and BMF silencer to inhibit anoikis during mitosis

To gain further insight into the functional role of CRE#3, and considering the presence of the H3K27ac mark from available ChIP-seq dataset (Dunham et al., 2012), we performed an enhancer-luciferase reporter assay. We found equivalent enhancer activity in cells transfected with the CRE#3-containing reporter plasmid in comparison to the positive control (Tyrosine kinase promoter) (**Fig. 4.9h**). Moreover, FOXM1 repression reduced the CRE#3 enhancer activity. Interestingly, we noticed that in the vicinity of the BMF genomic region is the BUB1B spindle assembly checkpoint gene, an established FOXM1 transcriptional mitotic target. qPCR analysis of CRISPR/Cas9 CRE#3-deleted cells immediately after FACS revealed decreased BUB1B transcript levels (**Fig. 4.9i**), supporting that CRE#3 is a BUB1B enhancer. It is known that co-regulated genes are normally confined in close proximity and that CREs and promoters are brought together by chromatin looping (Montavon et al., 2011). To ascertain for chromatin interactions between CRE#3 and the BMF and BUBR1 promoter regions, we performed 4C-seq analysis with the viewpoint on CRE#3. We found that BMF promoter, BUB1B promoter and CRE#3 physically interact (**Fig. 4.9j**). Thus, our data disclose an unforeseen FOXM1-binding CRE that acts to induce BUB1B transcription while repressing BMF expression. We propose that through this mechanism, mitotic cells are able to circumvent cell death signaling induced by cell rounding detachment.

High BMF levels correlate with response to anoikis-inducing chemotherapeutic drugs

To extend our findings into antimitotic chemotherapy response, we investigated the FOXM1-dependent co-regulation of BMF and BUB1B expression in the context of tumour cell treatment. As a preliminary approach, we used the MCF-7 and MDA-MB-231 breast cancer cell lines reported as anoikis-sensitive and anoikis-resistant, respectively (Hornsveld et al., 2016). In agreement, we confirmed higher expression of BMF in MCF-7 vs. MDA-MB-231 cells, even though FOXM1 and BUB1B transcript levels were similar in both cell lines (**Fig. 4.11a**). We then evaluated if BMF expression is cell cycle-regulated in a FOXM1-dependent manner in MDA-MB-231 cells, as we did for the MCF-7 cells (see Fig. 4.7c). Interestingly, qPCR analysis of mitotic vs. asynchronous MDA-MB-231 cell populations revealed no significant changes in BMF expression, even upon FOXM1 RNAi (**Fig. 4.11b**). Accordingly, time-lapse imaging of siNeg- and siFOXM1-depleted MCF-7 and MDA-MB-231 cells treated with paclitaxel confirmed a higher resistance of MDA-MB-231 cells in comparison to MCF-7 cells as described (Risinger et al., 2015), and revealed that FOXM1 repression only modestly

increases DiM in the MDA-MB-231 anoikis-resistant cells in comparison to the MCF-7 cells (**Fig. 4.11c-f**). Similar results were obtained when using small-molecule inhibition of FOXM1 with FDI-6 (**Fig. 4.12a-d**). Conversely, we also confirmed FOXM1 overexpression in MDA-MB-231 cells not to change BMF expression (**Fig. 4.12e,f**). Thus, these data suggest that anoikis-resistant cells are able to repress BMF in a FOXM1-independent manner. Still, CRISPR/Cas9-mediated CRE#3 deletion impairs MDA-MB-231 cell viability due to BMF upregulation (**Fig. 4.12g-i**), suggesting that CRE#3 is required for BMF repression, although not through FOXM1 binding as in MCF-7 cells.

To examine BMF expression in a wider tumour context, we interrogated the Cancer Cell Line Encyclopedia (CCLE) (Barretina et al., 2012) for the association between mRNA and protein expression levels with drug responses in the Cancer Target Discovery and Development (CTD2) database (Seashore-Ludlow et al., 2015), specifically for chemotherapeutics most extensively reported as anoikis-inducing: paclitaxel, docetaxel and doxorubicin. We found significant correlation of higher BMF mRNA and protein levels with response to these drugs (**Fig. 4.11g,h; Table 4.6**). Interestingly, higher BUB1B mRNA and protein levels also correlated with increased drug sensitivity as observed for BMF, suggesting the disruption of the FOXM1-dependent co-regulatory mechanism found in untransformed and MCF-7 cells (**Fig. 4.11i-j; Table 4.6**). Accordingly, FOXM1 mRNA and protein levels did not show correlation with any drug (**Table 4.6**). Thus, this analysis supports that tumour cells with lower BMF expression are more resistant to anoikis-inducing drugs, and independently of FOXM1 expression. To further examine the clinical relevance of these findings, we interrogated microarray datasets from XeNA, a clinical trial examining response rates in women with operable, early stage breast cancer receiving neoadjuvant capecitabine plus the antimitotic agent docetaxel (Stefan Glück et al., 2012). Tumours from patients showing no response had diminished BMF expression. Consistent with the premise that BMF expression in these non-responsive patients is FOXM1-independent, we found no significant elevated expression in the case of BUB1B (**Fig. 4.11i,l**).

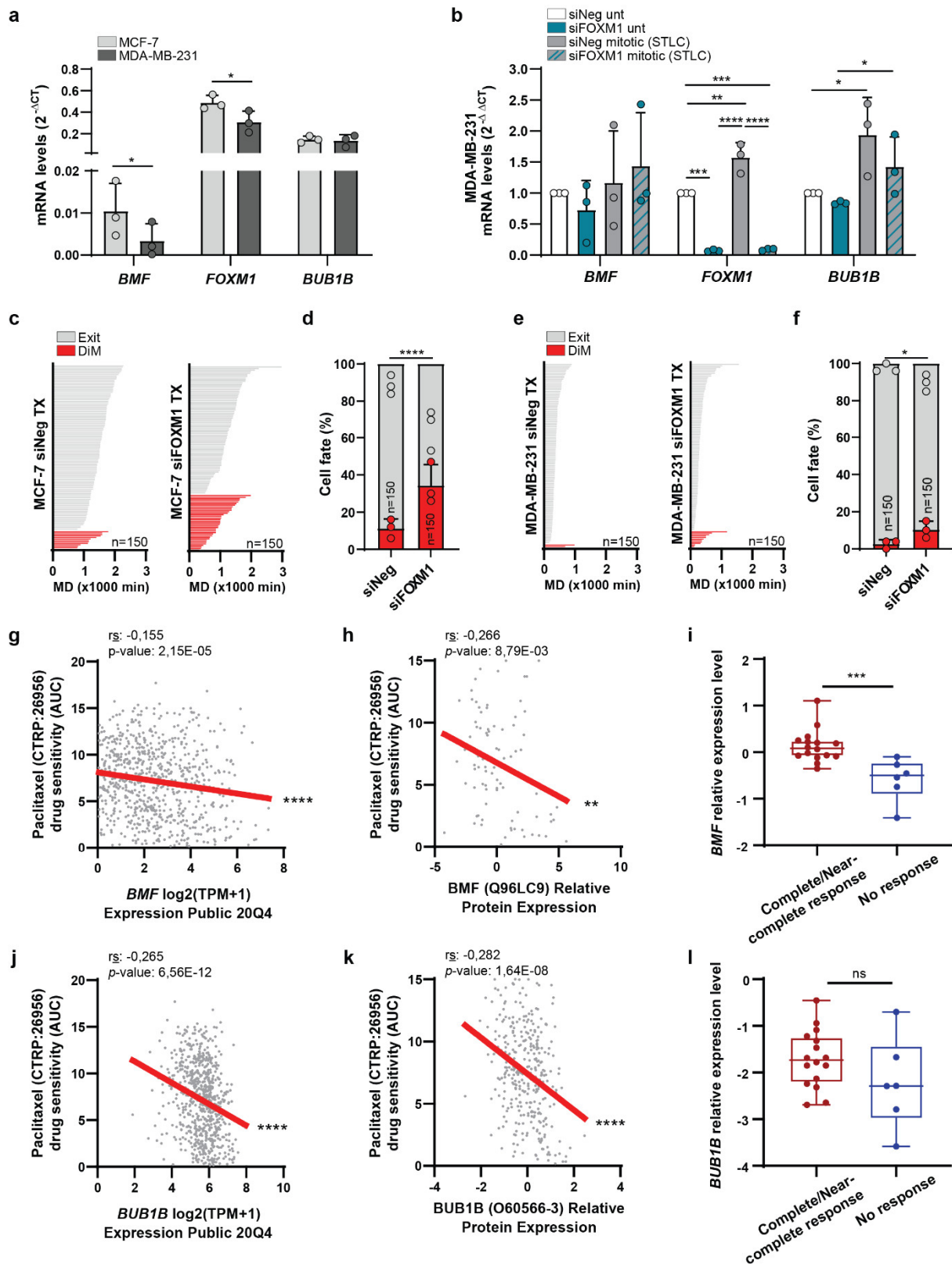


Figure 4.11. *BMF* expression correlates with response to antimitotic drug chemotherapy. **a** *BMF*, *FOXM1* and *BUB1B* transcript levels in MCF-7 and MDA-MB-231 cells. **b** *BMF*, *FOXM1* and *BUB1B* transcript levels in asynchronous (untreated) and mitotic (shake-off upon STLC) MDA-MB-231 cells depleted with Neg or FOXM1 siRNAs. **c** Individual cell fate profiling of siNeg- (n=150) or siFOX1-depleted (n=150) MCF-7 cells treated with 500nM TX. MD, mitotic duration. **d** Cell fate percentage of the experiments described in **c**. **e** Individual cell fate profiling of siNeg- (n=150) or siFOX1-depleted (n=150) MDA-MB-231 cells treated with 500nM TX. MD, mitotic duration. **f** Cell fate percentage of the experiments described in **e**. **g** Correlation of *BMF* mRNA levels and paclitaxel drug sensitivity in the CTRP drug response dataset. **h** Correlation of *BMF* protein levels and

paclitaxel drug sensitivity in the CTRP drug response dataset. **i** Relative *BMF* mRNA expression levels in tumours with complete/near-complete response vs. no response to a combination of docetaxel and capecitabine (Stefan Glück et al., 2012). **j** Correlation of *BUB1B* mRNA levels and paclitaxel drug sensitivity in the CTRP drug response dataset. **k** Correlation of *BUB1B* protein levels and paclitaxel drug sensitivity in the CTRP drug response dataset. **l** Relative *BUB1B* mRNA expression levels in tumours with complete/near-complete response vs. no response to a combination of docetaxel and capecitabine (Stefan Glück et al., 2012). In a data are mean $\pm$ s.d. from $n=3$ independent experiments; * $p \leq 0.05$ (two-tailed paired t-test). In b data are mean $\pm$ s.d. from $n=3$ independent experiments; * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$ (two-tailed One-way ANOVA and Tuckey multiple comparison test). In d, f data are mean $\pm$ s.d. of $n=3$ independent experiments; * $p \leq 0.05$, **** $p \leq 0.0001$ (two-tailed χ^2). In g, h, j, k ** $p \leq 0.01$, **** $p \leq 0.0001$ (<https://depmap.org/portal/interactive/>). In l, **** $p \leq 0.001$, ns $p > 0.05$ (two-tailed Mann-Whitney test).

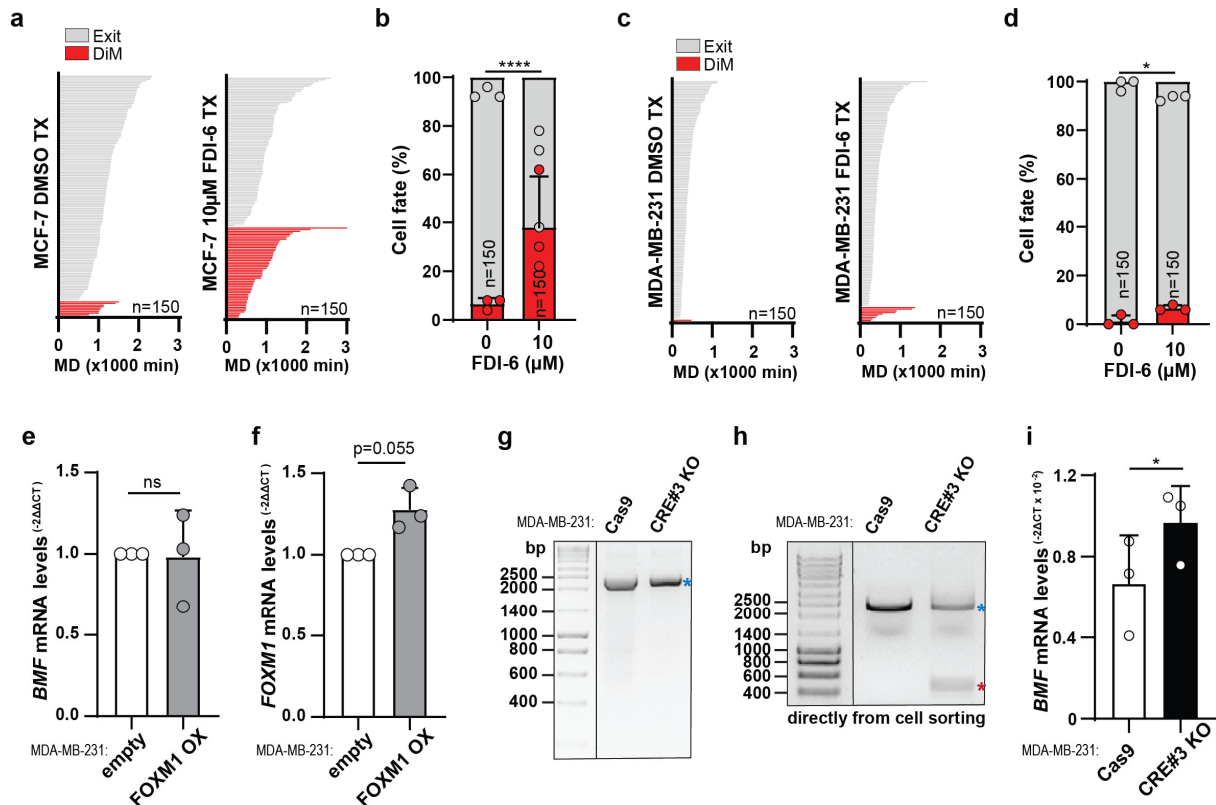


Figure 4.12. Cell fate decision in response taxol plus FOXM1 inhibition and cell unviability upon CRISPR/Cas9-mediated CRE#3 genomic deletion. **a** Individual cell fate profiling of DMSO (n=150) or FDI-6 (n=150) MCF-7 cells treated with 500nM TX. MD, mitotic duration. **b** Cell fate percentage of the experiments described in a. **c** Individual cell fate profiling of DMSO (n=150) or FDI-6 (n=150) MDA-MB-231 cells treated with 500nM TX. MD, mitotic duration. **d** Cell fate percentage of the experiments described in c. **e** BMF transcript levels in FOXM1 overexpressing MDA-MB-231 cells. **f** FOXM1 transcript levels in FOXM1 overexpressing MDA-MB-231 cells. **g-h** Genomic DNA was extracted from polyclonal MDA-MB-231 cell cultures established after cell sorting (g) or from cells immediately after sorting (h), and used for PCR detection of the wild type (blue asterisk) and mutant (red asterisk) alleles of the CRE#3 CRISPR/Cas9-mediated genomic deletions. **i** BMF transcript levels in CRE#3 CRISPR/Cas9-deleted MDA-MB-231 cells immediately after sorting. Data information: In b, d data are mean $\pm$ s.d. of $n=3$ independent experiments; * $p \leq 0.05$, **** $p \leq 0.0001$ (two-tailed χ^2). In e, f, i data are mean $\pm$ s.d. from $n=3$ independent experiments; ns $p > 0.05$, * $p \leq 0.05$ (two-tailed paired t-test).

Table 4.6. Correlation of mRNA or protein levels with dose responses of drugs (CTRP CTD2).

Target	Drug	Analysis	Number of points	Pearson	Spearman	Slope	Intercept	p-value (linregress)
BMF	Paclitaxel	mRNA	746	-0,155	-0,155	-3,80E-01	8,12E+00	2,15E-05
		protein	88	-0,278	-0,266	-5,43E-01	6,78E+00	8,79E-03
	Doxorubicin	mRNA	43	-0,508	-0,451	-9,30E-01	6,42E+00	5,99E-04
		Protein	93	-0,36	-0,3	-4,28E-01	7,55E+00	3,97E-04
	Docetaxel	mRNA	348	-0,195	-0,198	-4,77E-01	7,75E+00	2,44E-04
		Protein	42	-0,508	-0,451	-9,30E-01	6,42E+00	5,99E-04
BUB1B	Paclitaxel	mRNA	746	-0,248	-0,265	-1,15E+00	1,36E+01	6,56E-12
		protein	334	-0,303	-0,282	-1,45E+00	7,41E+00	1,64E-08
	Doxorubicin	mRNA	775	-0,23	-0,231	-6,67E-01	1,15E-01	8,68E-11
		Protein	344	-0,298	-0,26	-8,89E-01	8,04E+00	1,81E-08
	Docetaxel	mRNA	348	-0,303	-0,336	-1,30E+00	1,38E+01	7,63E-09
		Protein	155	-0,355	-0,315	-1,66E+00	6,67E+00	5,85E-06
FOXM1	Paclitaxel	mRNA	746	0,041	0,058	1,83E-01	6,10E+00	2,61E-01
		protein	118	-0,022	-0,004	-7,74E-02	7,30E+00	8,17E-01
	Doxorubicin	mRNA	775	0,057	0,065	1,58E-01	6,87E+00	1,12E-01
		Protein	123	-0,069	-0,038	-1,48E-01	7,94E+00	4,59E-01
	Docetaxel	mRNA	348	0,014	0,028	5,85E-02	6,20E+00	7,98E-01
		Protein	56	-0,181	-0,134	-5,44E-01	6,28E+00	1,82E-01
B2M	Paclitaxel	mRNA	746	-0,008	-0,027	-2,07E-02	7,39E+00	8,35E-01
		protein	334	0,054	0,032	1,46E-01	7,49E+00	3,28E-01
	Doxorubicin	mRNA	775	-0,022	-0,023	-3,75E-02	8,19E+00	5,41E-01
		Protein	344	0,042	0,067	6,92E-02	8,08E+00	4,35E-01
	Docetaxel	mRNA	348	-0,052	-0,055	-1,39E-01	8,01E+00	3,31E-01
		Protein	155	0,01	-0,025	2,89E-02	6,72E+00	8,97E-01

4.5 Discussion

Our study brings insight into the poorly understood mechanisms that define the kinetics of death signalling accumulation and threshold during mitotic arrest induced by antimitotic chemotherapy. We show that FOXM1 transcriptional activity driving G2/M cell cycle gene expression, also modulates the apoptotic pathway of the competing networks model. Both genetic and pharmacological repressions of FOXM1 increased DiM upon antimitotic drug treatment. Previous studies have reported a correlation between FOXM1 repression and paclitaxel response, with targets including genes involved in MT dynamics or drug efflux (Arceci et al., 2019; Carr et al., 2010; Hou et al., 2017; Khongkow et al., 2016; Zhao et al., 2014). Here, we identified BMF as an apoptotic gene repressed by G2/M FOXM1 transcriptional activity. FOXM1 binds to the BMF promoter and an intronic *cis*-regulatory element (CRE#3) to inhibit its expression. Interestingly, we found that transcription of the BMF neighbor gene, BUB1B, which is a FOXM1 transcriptional target, is coupled with BMF repression. We showed that the BMF promoter, BUB1B promoter and CRE#3 physically

interact, with the FOXM1-binding CRE operating bifunctionally to induce BUB1B transcription, while silencing BMF. This mechanism is at the basis of the cell-cycle regulated BMF expression and turns mitotic cells refractory to anoikis, which allows the distinction between cell rounding and cell detachment.

In addition, this mechanism explains how cells with low FOXM1 levels become more prone to DiM upon antimitotic drug treatment. BMF genomic deletion was able to revert increased DiM under low FOXM1 transcriptional activity, disclosing anoikis as an unforeseen apoptotic program in response to antimitotics. This supports the idea of using FOXM1 inhibition to improve antimitotic drug response. However, when extrapolating our findings into wide tumour context, we found that FOXM1-dependent BMF repression appears disrupted in anoikis-resistant cells. Interrogation of the CCLE for the association between FOXM1 and BMF expression levels with anoikis-inducing drug responses in the CTD2 database revealed significant correlation only for BMF. In agreement, we found FOXM1-independent BMF repression in the anoikis-resistant MDA-MB-231 cell line, whereas FOXM1 inhibition still upregulated BMF and increased DiM in the anoikis-sensitive MCF-7 cell line. Although the detailed mechanism of FOXM1-independent BMF repression remains unclear, we found that CRE#3 is still essential. Importantly, our findings disclose BMF levels as predictive of tumour response to antimitotic chemotherapy, with only higher levels supporting combination therapy of FOXM1 inhibitors with antimitotics (**Fig. 4.13**). Curiously, this resembles an antithetical regulation of endothelial ACE and ACE2 by a BRG1-FOXM1 complex previously reported in the context of pathologic cardiac hypertrophy (J. Yang et al., 2016).

Other mechanisms have been reported to influence the timing of mitotic cell death, such as degradation of MCL-1 (Sloss et al., 2016b), CDK1-dependent caspase phosphorylation (Allan & Clarke, 2007), c-MYC overexpression (Topham et al., 2015) and cGAS activation (Zierhut et al., 2019). Whether these mechanisms cross-talk with the FOXM1 transcriptional competence and BMF apoptotic potential remains unknown. FOXM1 has been involved in regulatory loops with CDK1 (Krishnan et al., 2017; Major et al., 2004) and c-MYC (Pan et al., 2018; Wierstra & Alves, 2006a), with CDK1 and c-MYC upregulating FOXM1 activity, as well as the other way round. One interesting aspect about FOXM1 is that it regulates targets of the two competing mechanisms determining fate decision upon mitotic arrest, with this study disclosing its role in the apoptotic pathway. Further investigation on how FOXM1 transcriptional activity becomes uncoupled from BMF-driven apoptosis will contribute to our understanding of chemotherapy response.

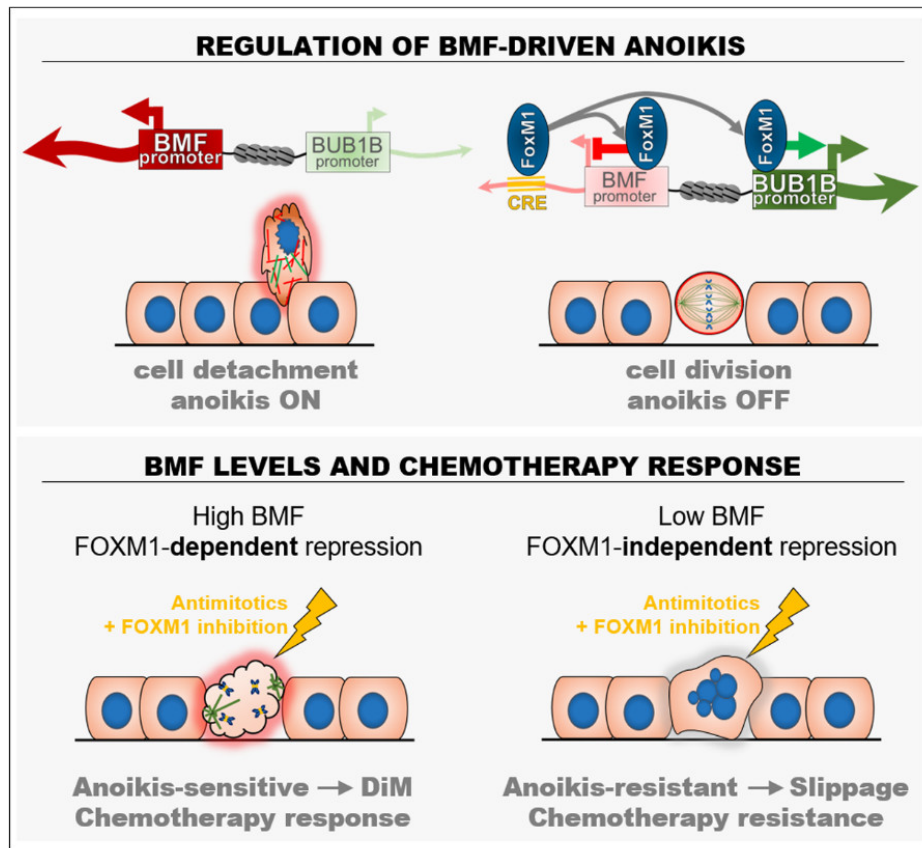


Figure 4.13. Working model integrating BMF transcriptional regulation by FOXM1 in mitosis and antimitotic chemotherapy. Upper panel: FOXM1 inhibits BMF expression and anoikis induction during prolonged mitosis. A FOXM1-binding cis-regulatory element (CRE) in BMF is an enhancer of the BUB1B neighbor mitotic gene. Lower panel: FOXM1 inhibition increases DiM in response to antimitotic drugs in anoikis-sensitive cells (high BMF). FOXM1-independent BMF repression (low BMF) correlates with resistance to antimitotic chemotherapy.

C H A P T E R

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Concluding Remarks

The apparent paradox of how ageing, characterized by reduced cell proliferation, can promote late-life cancer, characterized by uncontrolled cell proliferation, has suggested that ageing and cancer are opposite processes. However, the work here presented supports these events as rather being two sides of the same coin, sharing biological processes that govern mitosis, apoptosis and senescence. Consequently, the study of genes functionally linked to these pathways appear as a valuable strategy to unveil new and more efficient ageing and cancer therapies.

We provided unprecedented functional evidence that, in a natural ageing cellular model, comprising dermal fibroblasts retrieved from healthy donors with ages ranging from neonatal to octogenarian, there is an age-associated loss of mitotic fidelity, due to a global transcriptional shutdown of mitotic genes. Elderly cells exhibit several mitotic defects, which increase chromosome mis-segregation and contribute to the mild aneuploidy levels found in elder cultures. Additionally, we directly demonstrated that chromosome mis-segregation events generate aneuploid senescent cells. It would be interesting to evaluate the paracrine signalling of these aneuploid senescent cells towards their neighbouring cells. We expect that a positive feedback loop occurs, further aggravating the mitotic decline and senescence accumulation of the cell population. Interestingly, we found an unforeseen SASP in elderly mitotic cells. SASP had been strictly associated with cell cycle arrested cells (Juan Carlos Acosta et al., 2013). Thus, our findings support SASP as an evolving phenotype.

We unveiled FOXM1 downregulation as the causal mechanism behind the global transcriptional shutdown of mitotic genes and the SASP in elderly cells. FOXM1-depleted young fibroblasts displayed decreased expression of FOXM1 targets, mitotic defects and increased aneuploidy, similar to old-aged fibroblasts. Moreover, besides impacting mitosis, FOXM1 depletion also increased senescence-associated biomarkers. Concordantly, by RNA-seq profiling, we found 1937 out of the 3309 genes altered in old-aged fibroblasts, to be also altered in FOXM1-depleted cells. Thus, more than half of the age-dependent transcriptional changes are FOXM1-dependent in this research model. Importantly, the re-establishment of FOXM1 transcriptional activity in elderly cells ameliorated cell autonomous and non-autonomous feedback effects between mitotic fidelity and senescence. Hence, our findings disclose a genetic modulation with potential clinical benefit to healthy lifespan extension. *In vivo* studies using a transgenic mouse model for FOXM1 induction are presently running to evaluate if there are benefits of FOXM1 transcriptional rewire at the organismal ageing level. We expect that FOXM1 induction will counteract the low levels of aneuploidy/CIN that arise with age and that contribute to senescence and ageing features. Indeed, a multitude of studies has shown CIN to be a source of senescence (Barroso-Vilares & Logarinho, 2019) and a small-molecule inhibition of chromosomal instability delays this cellular process (Barroso-Vilares et

al., 2020), disclosing modulation of CIN and aneuploidy as an promising therapeutic strategy to slow down ageing. Moreover, besides FOXM1 role in modulating genes involved in CIN, FOXM1 has also been implicated in many other cellular pathways (Halasi & Gartel, 2013) that were shown to be hallmarks of ageing (López-Otín et al., 2013). Of special relevance, FOXM1 controls a DNA damage repair gene expression network (Zona et al., 2014). Therefore, FOXM1 impact on delaying senescence and improving longevity will most likely be due to the modulation of many cellular ageing hallmarks.

Having such a broad impact in cellular processes, it is not surprising that FOXM1 has been implicated directly or indirectly in all cancer hallmarks (Halasi & Gartel, 2013). FOXM1 is typically overexpressed in most solid tumours (Nandi et al., 2018), and its regulatory network is a major predictor of adverse outcome across 39 human malignancies (Gentles et al., 2015). We have also accessed the role of FOXM1 in the apoptotic machinery, in particular, whether FOXM1 could modulate the competing networks model dictating cell fate upon antimitotic chemotherapy. Using single-cell analysis to circumvent the confusing interpretations of population-based assays, we found that both genetic and pharmacological repression of FOXM1 increases death in mitosis upon exposure to different antimitotic drugs. The evidence of FOXM1 as molecular determinant of mitotic cell fate decision is supported by other studies showing a positive correlation between paclitaxel sensitivity and FOXM1 silencing (Arceci et al., 2019; Carr et al., 2010; Hou et al., 2017; Khongkow et al., 2016; Q. Wang et al., 2017; Zhao et al., 2014). However, FOXM1 downstream targets driving paclitaxel induced cell death remained elusive. Here, we identified an apoptotic target, BMF, which overexpression induces DiM and which repression inhibits DiM in response to antimitotics. Our findings disclosed FOXM1 as a transcriptional repressor of BMF gene expression. FOXM1 repression increased BMF transcript levels and the promoter activity in a luciferase reporter assay. Moreover, we found BMF levels to be cell cycle-regulated, i.e., downregulated in late G2/M, which is in line with the temporal frame when FOXM1 is transcriptionally active. Detailed mechanistic insight awaits further investigation to identify the FOXM1-binding repressor protein complexes assembled at the BMF promotor. NOXA, another BH3-only pro-apoptotic protein, has been also described to exhibit low or undetectable levels during mitosis (Haschka et al., 2015), and we found it upregulated upon FOXM1 RNAi in our RNA-seq analysis. Taken together, these observations uncover a FOXM1 functional role in apoptosis, besides its regulatory roles in mitosis and senescence. As cancer cells are highly resistant to intrinsic apoptosis (Hanahan & Weinberg, 2011), it would be relevant to systematically test FOXM1 modulation in luciferase promoter assays for all the other Bcl-2 family members, to fully access FOXM1 apoptotic gene network. Interestingly, we found several FOXM1 ChIP-seq peaks (Sanders et al., 2013) within BMF intronic regions that also had annotated H3K27Ac mark (often found in CREs) (Dunham

et al., 2012). When deleted, one of those regions was found to induce BMF expression, while repressing its neighbouring gene, BUB1B. Furthermore, our study finds evidence that this CRE and the promoters of both BMF and BUB1B are physically interacting. It remains unknown the identity of other factors binding at this CRE. Future work will be required in which factors that exhibit putative binding motifs in this region will be individually depleted to ascertain if BMF and BUB1B levels are still modulated under FOXM1 repression. We found that this CRE is essential to inhibit anoikis death during mitosis, through an opposing effect of FOXM1 in the expression of BMF (anoikis gene) and BUB1B (mitotic gene). During a prolonged mitotic arrest, and under FOXM1 levels, BMF repression is less efficient leading to increased DiM. This supports the idea of using FOXM1 inhibition to improve antimitotic drug response. However, when we interrogated a large dataset of cancer cell lines for gene/protein expression and drug sensitivity, we found that sensitivity to anoikis-inducing drugs revealed significant correlation with BMF but not FOXM1 expression levels. In agreement, we found BMF repression to be FOXM1-independent in the anoikis-resistant MDA-MB-231 cell line, whereas FOXM1 inhibition still upregulated BMF and increased DiM in the anoikis-sensitive MCF-7 cell line. Additional studies will be needed to understand the mechanisms behind the disruption of the FOXM1-BMF axis in anoikis-resistant cells. An unbiased whole transcriptome approach of a large panel of anoikis-resistant cancer cell lines could be useful to address this question. The generation of isogenic cell lines sensitive and resistant to anoikis and/or antimitotics could also be suitable for an easier identification of BMF repression players dictating the disruption of the FOXM1-BMF axis. Nevertheless, our data support BMF levels as a robust predictive biomarker for antimitotic chemotherapy resistance, and grade FOXM1 inhibition as effective combination therapy with antimitotics for BMF expressing tumours. Pre-clinical studies using mouse xenografts should be performed to validate FOXM1 inhibition plus antimitotics as a more efficient strategy than MTA monotherapy in BMF expressing tumours. In this study, we accessed the effect of RCM-1, a novel small-molecule inhibitor of FOXM1 (Sun et al., 2017), in carcinogenesis *in vivo*. Xenograft models of mouse rhabdomyosarcoma and melanoma revealed that the volumes of RCM-1-treated tumors were significantly decreased. Further studies are required to access specifically if RCM-1 affects other molecular targets in addition to FOXM1, and whether combination therapy with antimitotics reveals beneficial.

Our data also revealed cellular aging as contributing to different sensitivity to MTAs, through FOXM1 repression. Indeed, oncologists apprehensively prescribe paclitaxel or docetaxel to older patients (Biganzoli et al., 2009). Elderly patients more likely develop secondary side effects, such as myelotoxicity, neurotoxicity and fatigue (Lichtman, 2003; Socinski, 2014; Walko & McLeod, 2014). Physiological reasons described comprise age-related decreased clearance of taxanes, and in order to prevent major toxicities, lower doses are recommended

for elderly patients (Biganzoli et al., 2009). In this work, we identified age-associated FOXM1 repression as a cell intrinsic reason for increased sensitivity to MTAs. Further data providing evidence for optimal dosage in this population is required (Biganzoli et al., 2009; Lichtman, 2003; Wildiers & Paridaens, 2004), as well as the functional correlation between side effects and FOXM1 expression levels in distressed tissues.

In the next decades the elderly population will increase dramatically, leading to a significant burden on the European health care systems. Consequently, research in ageing biology becomes a priority. Ageing and cancer exhibit a complex cross-talk that is still poorly understood. Our findings brought insight into mechanistic cues of FOXM1, a major player that regulates age-associated changes in cells and sensitivity to chemotherapy. All aims were achieved and the data open valuable therapeutic impact of FOXM1 modulation to delay ageing and treat cancer.

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V I

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