UNIVERSITÉ DE GENÈVE

Archive ouverte UNIGE
https://archive-ouverte.unige.ch

Thèse 2020

Open Access

This version of the publication is provided by the author(s) and made available in accordance with the copyright holder(s).

Investigating the mitotic role of Retinoblastoma protein and its impact on chromosomal instability

Stingi, Aureliano

How to cite

STINGI, Aureliano. Investigating the mitotic role of Retinoblastoma protein and its impact on chromosomal instability. Doctoral Thesis, 2020. doi: 10.13097/archive-ouverte/unige:153795

This publication URL: https://archive-ouverte.unige.ch/unige:153795
Publication DOI: 10.13097/archive-ouverte/unige:153795

© This document is protected by copyright. Please refer to copyright holder(s) for terms of use.

UNIVERSITÉ DE GENÈVE FACULTÉ DE MÉDICINE

Section de médecine fondamentale
Département de physiologie cellulaire et métabolisme

Professeur Patrick Meraldi

Investigating the mitotic role of Retinoblastoma protein and its impact on chromosomal instability

THÈSE

présentée aux Facultés de médecine et des sciences de l'Université de Genève
pour obtenir le grade de Docteur ès sciences en sciences de la vie,
mention Sciences biomédicales

par

Aureliano Stingi

de

Italie

Thèse N°

GENÈVE

2020

Résumé

Le corps humain est composé de 30 trillions de cellules qui produiront au court de sa vie environ 10 quadrillons de divisions cellulaires. La division cellulaire est une étape cruciale pour toutes les créatures vivantes car elle assure leur reproduction, croissance, différentiation ainsi que la réparation de leur tissus. Grâce aux premières observations de Boveri, nous savons que les erreurs de division cellulaire sont une des causes majeures du cancer. En plus d'une division cellulaire infidèle, les mutations génétiques alimentent le cancer humain. Une question se pose donc : combien de mutations sont-elles nécessaires pour qu'une tumeur maligne se déclare ? Dans le cancer humain, le nombre de mutation estimé se situe entre trois et douze. La probabilité que six mutations différentes se produisent dans une même cellule sont extrêmement rare, mais certaines mutations spécifiques augmentent considérablement cette probabilité. La liste de gènes perdus ou mutés dans le cancer humain est limitée et plusieurs de ces gènes sont impliqués dans différents cancers. Curieusement, peu de gènes mitotiques sont perdus ou mutés dans le cancer humain. Cette observation suggère que des gènes de l'interphase mutés dans le cancer peuvent être également impliqués dans la mitose.

Par exemple, le gène responsable du rétinoblastome (RB1) est un gène d'interphase qui serait également engagé dans le processus de division cellulaire.

Le gène RB1 code pour un suppresseur de tumeur qui est la protéine appelé pRB.

pRB réprime les gènes impliqués dans la réplication de l'ADN et empêche la progression du cycle cellulaire depuis la phase G1. Un large pourcentage de cancers humains comporte une mutation dans la cascade de signalisation de pRB, dérégulant fréquemment la famille de facteurs de transcription E2F qui initie la transcription de gènes engagés dans la phase S. Malgré le rôle bien caractérisé de RB en interphase, il a été supposé que sa perte mènerait à un gain ou à une perte de

chromosomes durant la mitose, causant par la suite une instabilité chromosomique. Cependant, les mécanismes conduisant à cette dernière sont encore mal compris. Le but de mon travail de thèse (PhD) est donc d'évaluer le rôle de pRB pendant la mitose, mais également de comprendre comment son absence provoque une instabilité chromosomique.

Dans la première partie de mon PhD, j'ai obtenu une réduction de l'expression de pRB de 60% grâce à l'emploi de siARN dans les cellules RPE1. La déplétion de pRB a augmenté la durée du temps en mitose ainsi que l'incidence du retard de ségrégation des chromosomes après traitement au Monastrol, soulignant la contribution de pRB dans l'instabilité chromosomique.

Un retard de ségrégation de chromosomes peut survenir lors de dérégulation dans l'attachement de microtubule aux kinétochores, lors de défauts dans la structure du kinétochore, lors d'une détérioration des points de contrôles menant au fuseau mitotique, ou lors d'une condensation incomplète de l'ADN.

La compétence du point de contrôle pour l'assemblage du fuseau mitotique a été testée et se trouve inaltérée dans les cellules déplétées pour pRB. Lors de l'analyse d'imagerie sur cellules vivantes j'ai constaté que l'attachement microtubules-kinétochores ainsi que la condensation de l'ADN n'étaient pas affectés par la déplétion de pRB. Par contre, une déplétion aiguë de pRB diminue les niveaux de CENP-A aux centromères, l'histone centromérique requis pour l'assemblage des kinétochores. Mes expériences suggèrent donc que la réduction des niveaux de CENP-A n'affecte pas directement l'attachement des microtubules aux kinétochores, mais corrèle avec une réduction de la capacité de correction d'attachements erronés. AuroraB possède un rôle clé dans le mécanisme de correction d'erreur d'attachement et se trouve hyperactivé dans les cellules déplétées pour pRB. Il est intéressant de constater que l'hyperactivation d'AuroraB mène à un retard dans la ségrégation des chromosomes et peut être pharmacologiquement inhibé. Ceci suggère un lien entre la déplétion

partielle de pRB, la présence de retard dans la ségrégation des chromosome et l'hyperactivation d'AuroraB.

Dans la deuxième partie de mon PhD, j'ai établi une lignée cellulaire avec un knockout complet de pRB afin d'évaluer l'impact de l'absence de cette protéine par rapport à sa déplétion partielle. J'ai constaté que l'absence complète de la protéine pRB par knockout présentait des caractéristiques drastiquement différentes par rapport à sa déplétion partielle. Cette découverte met en évidence le rôle crucial de la déplétion partielle de pRB dans le cas de l'instabilité chromosomique. En conclusion, ma thèse a contribué à comprendre comment l'absence de pRB influence l'instabilité chromosomique.

Summary

The human body is composed of 30 trillion cells and during our lifetime we experience circa 10 quadrillion cell divisions. Cell division is a crucial step for all living creatures and ensures reproduction, growth, differentiation and tissue repair. Thanks to Boveri's original observations we know that erroneous cell division is one of the first causes of cancer. Besides unfaithful cell division, gene mutations promote human cancer. How many mutations are necessary for a malignancy to occur? For human cancer, the estimated number of mutations is between three and twelve. The probability that 6 different mutations happen in the same cell is extremely low, but some specific mutations increase that probability. The list of genes lost or mutated in human cancer is limited and many genes are involved in several cancers. Interestingly, few mitotic genes are lost or mutated in human cancer. This observation suggests that cancer mutated interphase genes could also be involved in mitosis. The retinoblastoma gene (RB1) is an example of an interphase gene also involved in the cell division process.

RB1 gene encodes for a tumor suppressor called Retinoblastoma protein 'pRB'.

pRB represses genes involved in DNA replication and prevents cell cycle progression in G_1 phase. A large percentage of human cancers bear a mutation in the pRB pathway, these mutations usually deregulate the E2F family of transcription factors. E2F initiates the transcription of genes involved in S phase. Despite the well-characterized role of pRB in interphase, it has been proposed that its loss leads to gain or loss of chromosomes in mitosis, causing chromosomal instability, but the mechanisms are not well understood. My PhD aimed to assess the role of pRB in mitosis and understanding how its absence causes chromosomal instability.

In the first part of my PhD, I achieved a 60% reduction in pRB expression by RNAi in RPE1 cells. pRB depletion extended the duration of mitotic timing and increased the incidence of lagging chromosomes after a Monastrol-release, highlighting its contribution to chromosomal instability.

Lagging chromosomes can arise from deregulated kinetochore-microtubule attachments, defective kinetochore structure, impaired spindle assembly checkpoint or incomplete DNA condensation. Spindle assembly checkpoint proficiency was tested and was found unaffected in pRB depleted cells. Using live-cell imaging-based assays I found that microtubules-kinetochore attachments and DNA condensation were not affected by pRB depletion. In contrast, acute pRB depletion lowered the levels of CENP-A at centromeres, the centromeric histone required for the assembly of kinetochores. My experiments further suggest that this reduction in CENP-A levels, does not affect kinetochore-microtubule attachments directly, but correlates with a reduced ability to correct erroneous kinetochore-microtubule attachments. AuroraB is a key component of the error correction machinery and is hyperactivated in pRB depleted cells. Interestingly, AuroraB hyperactivation leads to lagging chromosomes and can be pharmacologically inhibited. The last finding suggested a connection between pRB partial depletion, the presence of lagging chromosomes and AuroraB hyperactivity.

In the second part of my thesis, I established a pRB full knockout cell line to assess the impact of pRB knockout compared to partial depletion. I found that pRB full knockout shows completely different features compared to pRB partial depletion. This finding highlights the crucial role played by pRB partial depletion in term of chromosomal instability.

Overall my thesis contributed to understanding how pRB absence influences chromosomal instability.

Contents

Figures

Introduction

Life origin

Life can be defined in several ways and a consensus on it is still missing, nonetheless, one feature is shared by all living organisms: the ability of single individuals to propagate their genetic material through generations. We can imagine that the transition from LUCA (Last Unknown Common Ancestor) to us happened in billions of years through evolution.

Evolution, by definition, is the change in characteristics of a species over several generations and relies on the process of natural selection. How species propagate themselves is explained by genome duplication and segregation. Every living organism's goal is to duplicate the genome and propagate it to the next generation, and this process happens with different extent of complexity. The event of genome duplication is cyclical and is part of the cell cycle (The Cell, 2nd edition, 2000). The cell cycle is a series of events that prepares the cell to the duplication and segregation of the genome. Cell cycle control was discovered and understood thanks to a small unicellular yeast by the Nobel laureates Lee Hartwell first in Budding yeast and later confirmed by Paul Nurse in Fission Yeast (Nurse, 2000). Despite the phylogenetic distance between humans and yeast, we share many cell cycle control mechanisms.

Cell cycle complexity normally reflects the complexity of the organism, for the purpose of this thesis I will focus my attention on the animal eukaryotic cell cycle.

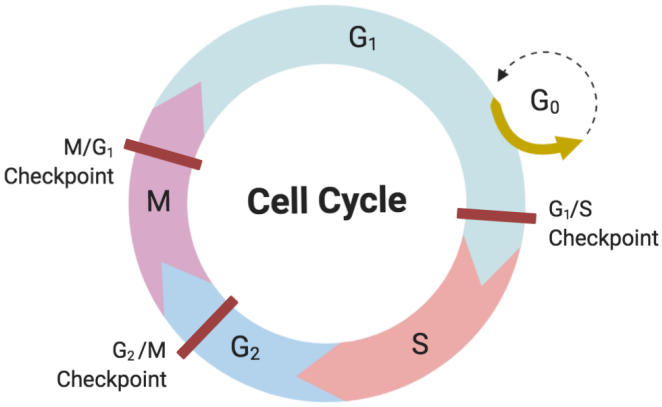


Figure 1 Schematic representation of cell cycle.

Checkpoints are depicted in red. In G_1 the cell grows and is metabolically active, in S phase DNA is replicated. In G_2 the cell grows and repairs eventual DNA damage, and finally in mitosis, the cell segregates the genetic material in two daughter cells.

Thesis outlook

In my thesis, I will focus my attention on the cell cycle and specifically on mitosis, a crucial step in which the genetic material is segregated into two daughter cells. Since Boveri's original observations we know the connection between mitosis and cancer, therefore I will discuss cancer and mitosis (Lee, 2014). I will describe each step of mitosis and how it is regulated. I will focus my attention on the error correction mechanisms and all the possible conditions leading to errors. Finally, I will introduce the retinoblastoma protein. The retinoblastoma gene (RB1) was the first tumour suppressor identified more than 25 years ago, thanks to extensive research we know now that it is the causal agent of Retinoblastoma and also a critical cell cycle regulator (Knudson, 1971). Moreover, recent publications indicate that pRB can also undermine mitotic fidelity.

I will describe in details what is known about pRB and how it could influence mitosis.

The eukaryotic cell cycle

The cell cycle is a highly controlled process that ensures DNA duplication and segregation (Hartwell, Weinert, Hartwell, & Weinert, 1989). DNA duplication and DNA segregation are two error prone events therefore both are preceded by two gap phases, G_1 and G_2. During the gap phases, the cell is growing and preparing for the two major events: DNA duplication and DNA segregation. In the majority of adult tissues, cells are fully differentiated and are blocked in a phase called G_0. According to the cell fate, G_0 can be subdivided in senescence if the cells stop indefinitely, or quiescence if the stop is temporary (Rando, 2013). Some specific cell types can be even stuck in meiosis, for example, oocytes are blocked indefinitely in prophase II of meiosis. Gap phases and S phase together fall under the name of interphase, that can be defined as the time between one mitosis and the next. Human cells in culture employ approximatively 20-24 hours to complete a cell cycle, G_1 phase is the longest with 11 hours, S phase 8 hours, G_2 4 hours and finally mitosis in circa 1 hour (The Cell, 2nd edition, 2000). Mitosis is the shortest event of the cell cycle and it has been observed that longer mitosis increases the chance to accumulate errors (Stevens, Gassmann, Oegema, & Desai, 2011) (Potapova & Gorbsky, 2017). This can be easily explained by the fact that during mitosis the DNA condensates becoming fragile and particularly prone to breakages (M. S. Levine & Holland, 2015). Moreover, mitosis length reflects the presence of errors or anomalies. In 2008 Yang et al. elegantly demonstrated that additional centrosomes and/or chromosome can extend mitosis to different extents (Zhenye Yang, 2008).

Historically, at least two divergent models described cell cycle regulation and progression: the clock and dominos model. Those two models were discovered and studied in two different model organisms: the Xenopus egg, that was used to describe to clock model and on the other hand Yeast that was used to characterize the dominoes model. According to the first model the embryo division

follow clock-like progression, fast and without gap phases. According to this model mitosis entrance happens independently from other cell cycle events. Xenopus eggs represent a perfect example of the clock model, in fact Xenopus eggs undergo rapid cleavage without experiencing any cell growth or gap phases. Furthermore, pioneering experiments from Marc Kirschner demonstrated that Xenopus egg are synchronized by an internal clock independent from the nucleus.

Conversely, the dominoes model is characterized by inter-dependence of cell cycle events on phase transitions and notably each event to start it needs the completion of the previous. Several experiments on Yeast mutants showed genes involved in cell cycle transition and in checkpoints. Interestingly, the meaning of checkpoints is to 'buy time' before cell cycle progression to detect and eventually correct defects (Murray & Kirschner, 2008).

How can those two divergent models be reconciled?

Interestingly, several key components of embryonic and somatic cell cycle are the product of homologous genes. This finding suggest that the same molecules can exerts different roles in different situations, furthermore cell cycle regulation is orchestrated according to both models (Murray & Kirschner, 1989).

The eukaryotic cell cycle is regulated by molecular switches that ensure orderly progression through it. The molecular switches normally trigger dichotomic responses, (on/off) that ensure irreversible events. For example, nuclear envelope breakdown is an irreversible event that cannot be partially activated otherwise it would lead to deleterious consequences. Also, cell cycle system controls are adaptable and robust (Harashima, Dissmeyer, & Schnittger, 2013).

The total length of the cell cycle, therefore, is determined by the length of single phases but more specifically by three factors; checkpoints, extracellular signals and cyclins.

As suggested by the name checkpoint are barriers between different phases in which highly sophisticated and redundant mechanisms control the cell status to proceed to the next step.

External signals modulate both initiation and inhibition of cell cycle and the checkpoints work as an accelerator or brake pedal in a car. Cyclins is a family of proteins that regulates cell cycle length thanks to the interaction with a group of kinases called CDKs. (Vermeulen, Bockstaele, & Berneman, 2003) (Murray, 2004)

A stereotypical example of CDKs activation is CDK1, CDK1 is activated by the binding partner CyclinB and subsequently by the phosphorylation of a residue on the T-loop (Thr160). CyclinB levels accumulates throughout the cell cycle, peaks at the end of G_2 and it is finally degraded during mitosis. The initial phosphorylation to the CDK1/CyclinB complex is ensured by CDK7 that is the catalytic subunit of the CAK complex. CDK1/CyclinB complex regulates the transition from G_2 to M phase of the cell cycle, giving a crucial role in cell cycle regulation. Given the important role in cell cycle transition CDK1 is tightly regulated. As previously described the first layer of regulation is represented by its binding partner CyclinB, CyclinB is produced in cyclical manner during the cell cycle and is rapidly degraded during mitosis by the anaphases promoting complex (APC/C). Second, CAK phosphorylates the CDK/CyclinB complex on the threonine residue 160 on the activation domain. This phosphorylation prevents the inhibitory phosphorylation exerted by the CKIs. In case of DNA damage ATM/ATR through Chek1/2 activates Wee1 kinase that in turn inactivate CDK1 blocking the cell cycle progression. In case of repair the kinase PLk1 will activate the phosphatase CDC25 that in turn will remove the inhibitory phosphorylation from CDK1 (Chow, Poon & Ma, 2011).

Cyclins and Cyclin-Dependent Kinases

CDK as suggested by the name are kinases involved in cell cycle regulation, their activity increases throughout it. CDKs are strictly regulated during the cell cycle mostly by cyclins and by other

mechanisms. In yeast, a single CDK binds all the cyclins classes, whereas in human cells we encounter 4 CDKs involved in cell cycle regulation (Satyanarayana & Kaldis, 2009).

- CDK4/6 activated by cyclins D during G1
- CDK2 activated by cyclin E for the G1/S transition
- CDK2 activated by cyclin A ensuring the transition from S phase to G2
- CDK1 activate by cyclin A during G2 phase
- CDK1 activated by cyclin B stimulates M phase

Cyclins and CDKs form a specific complex during the cell cycle, also, cyclins can target CDKs toward their targets.

To prevent nonspecific binding during cell cycle Cyclins are degraded immediately after they have exerted their role. The degradation is mediated by the ubiquitin pathway and is crucial for cell cycle progression. For example, the degradation of CyclinB is required for the kinase inactivation and mitosis exit (Murray, 2004).

Beyond the cyclins regulations, CDKs are further regulated by at least 3 mechanisms: (Morgan, 1995)

1. CDK-activating kinases (CAK) phosphorylation
2. Regulatory inhibitory phosphorylation
3. Binding of CDK inhibitory subunits (CKIs)

As previously described the binding between cyclins and CDKs represent the major regulation nonetheless the binding only partially activates the CDKs. CDK-activating kinases (CAK) are required to provide activating phosphorylation. On the other hand, cyclin-CDK inhibitors (CKI) acts in two ways: first preventing CDKs phosphorylation, second inhibiting CDK binding to its substrates. CKI

directly inhibits CDKs kinase activity and are activated by external stimuli or DNA damage. CKIs can be divided into two main families: INK4 that inhibits CDK4/6 and CIP/KIP that mostly inhibits CDK2. For example, p21 transcription is p53 mediated, that in turn is active in presence of DNA damage. p21 directly inhibit CDK2 and CDK4 preventing pRB phosphorylation and cell cycle progression moreover p21 can inhibit Cdk1 blocking mitosis entrance (Wenzel & Singh, 2018) (Satyanarayana & Kaldis, 2009).

Cell cycle checkpoints

The cell cycle is a sequence of cyclical events that take place in a dividing and growing cell, requiring optimal conditions.

The cell cycle must stop in case of errors, depletion of nutrients, DNA damage, etc., and the possibility of 'choosing' is provided by the checkpoints (Hartwell et al., 1989).

Checkpoints are roadblocks along the cell cycle in which cells gain time to set everything to proceed further. Checkpoints are often lost or deregulated in cancer, highlighting their crucial role (Wenzel & Singh, 2018). Eukaryotic checkpoints can be classified in intrinsic and extrinsic. Intrinsic checkpoints are always active and regulate cell cycle progression whereas extrinsic are activated by DNA damage therefore not always active. Intrinsic checkpoints share the same structure in which there is one sensor that triggers a signalling cascade, that in turn activates an effector that finally will activate the target.

The G_1/S checkpoint can be used to describe a stereotypical intrinsic checkpoint. External growth signals are sensed by the transmembrane receptors (sensors) and transduced into a signalling cascade that starts the transcription of several genes and amongst them CyclinD. CyclinD coupled with CDK4/6 phosphorylates the effector, pRB that in turn releases the target E2F. E2F release causes its activation that in turn allows cycle progression from G_1 to S (Harashima et al., 2013).

Besides G1/S transition, checkpoint control is exerted during G2/M transition and in mitosis.

On the other hand, extrinsic checkpoints are activated only when DNA damage is detected and it requires an ATM/ATR activation (Mogila, 2011). DNA damage is a detrimental event that poses a serious risk for genome stability and cell homeostasis (Friedberg, 2003). ATM and ATR are two proteins involved in DNA damage response; both can stop the cell cycle or activate p53. ATM and ATR activates CHK1/2, that in turn phosphorylates p53 preventing its ubiquitin-dependent degradation Mdm2 mediated (Ishak et al. 2017).

p53 also called 'the guardian of the genome' is a well-known onco-suppressor responsible for genome stability and capable of cell cycle regulation through its checkpoint activity (A. J. Levine, 2020).

Extracellular signals

Extracellular signals are stimuli that come from outside the cell and their role is to communicate and coordinate multicellular processes (Mogila, 2011). Growth factors, cytokines, hormones, ligands are extracellular signals that can travel between organs and tissue. Extracellular signals play a crucial role at the beginning of the cell cycle in a pre-phase called 'start'. The primary G1/S checkpoint controls the commitment of a human cell to enter into the cell cycle. This checkpoint represents a bifurcation where the cell 'decides' its fate. Extracellular signals play a crucial role in this phase in fact, the amount and the type of extracellular signals determine cell cycle entrance and duration.

Growth factors are captured by the transmembrane extracellular receptor that activates a signalling cascade that ultimately induces cyclin transcriptions. The transcribed cyclins drive the activity of CDKs that subsequently start the phosphorylation of several targets indispensable for cell cycle progression (Murray, 2004).

G1/S checkpoint

The G_1/S checkpoint is also called restriction point because it represents a 'point of no return' in term of cell cycle commitment. During G1 the cell gathers extracellular grow factors and proliferation signals, also called mitogen signals. Interestingly, cells are dependent on those mitogen signals during G1, whereas after the entrance in the cell cycle and the commitment they become independent (Rescan et al., 1999). Mitogenic signals like growth factors (PDGF, VEGF, EGF) through a signalling cascade, normally mediated by the MAPK /ERK pathway, induce the transcription of cyclins D1-3. Cyclins D1-3 activate CDK4/6 that subsequently phosphorylate the retinoblastoma protein pRB. Cyclin D1-3 activity is normally counterbalanced by the activity of the CKI inhibitor p16 (Cobrinik, 2005).

The retinoblastoma protein pRB with the other member of the pocket protein family binds the transcription factor E2F and the binding prevents its activity. Besides the physical binding between pRB and E2F, pRB recruit chromatin remodeling factors that prevent E2F activation. pRB contains at least 16 phosphorylation sites that are phosphorylated in a sequential manner in different phases of the cell cycle by different kinases (Mittnacht, 1998). The partial pRB phosphorylation exerted by CDK4/6 activates E2F that starts the transcription of more than 1000 genes involved in S phase, one of those genes is CyclinE. CyclinE activates the kinase CDK2 forming the active complex CDK2/CyclinE that in turn contributes to phosphorylation of pRB causing its complete inactivation. Normally, this complex is inhibited by p27. Finally, CyclinE is rapidly degraded in a ubiquitin-dependent manner causing the inactivation of the CDK2/CyclinE complex.

E2F controls the transcription of many more genes involved in S phase, DNA replication and cell cycle progression, including CycilnA (Cobrinik, 2005).

CyclinA transcription is regulated via E2F in a negative feedback loop, as CDK2/CyclinA can phosphorylate and directly inhibit E2F.

pRB is part of the pocket protein family, that is composed of 2 partners: p130 and p107. Pocket proteins control cell cycle progression through the interaction with the E2F transcription factor protein family (Graña, Garriga, & Mayol, 1998).

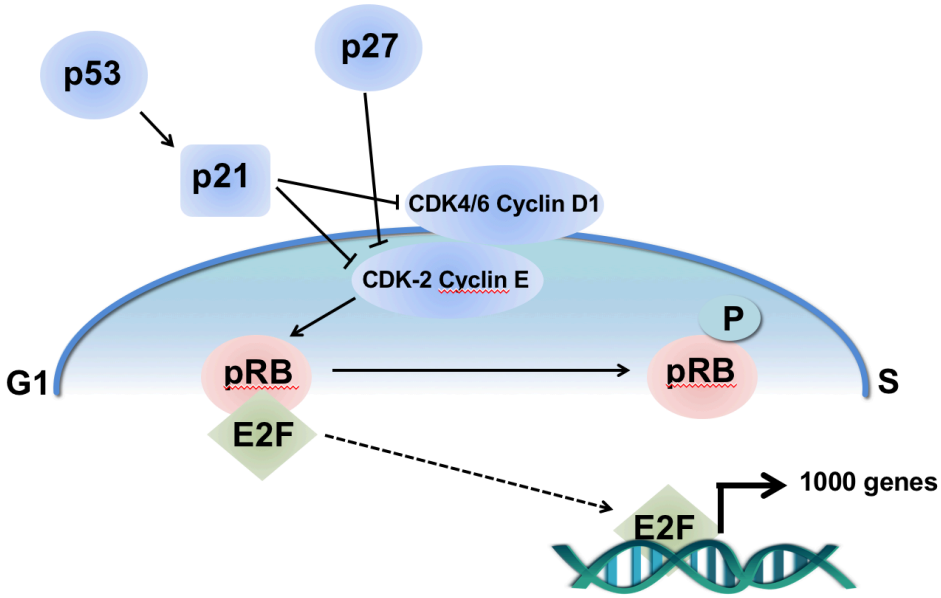


Figure 2 Schematic representation of the simplified pRB pathway.

Arrows and lines represent the interaction between the proteins. The complete pRB phosphorylation determines E2F release and the transition in the S phase.

G2/M checkpoint

This checkpoint occurs after DNA replication, making it fundamental for DNA damage detection. DNA damage is a detrimental consequence of DNA replication, caused by several internal and external events. A common endogenous source of DNA damage is the deamination of cytosine to uracil, producing a mismatch. Conversely, and external source of DNA is the UV light that can generate pyrimidine dimers from cytosine and thymine through a photochemical reaction. The

most dangerous DNA damage is the double-strand break (DSB) in which both DNA strands are severed and it can lead to genome rearrangements (Hakem, 2008).

The role of the G2/M checkpoint is to detect and possibly repair DNA damage before DNA segregation. Cells with defective DNA damage response either undergo apoptosis or proceed to erroneous DNA segregation and therefore genomic instability. Specialized proteins detect DNA damage sites and through a negative loop mechanism block the progression to M phase. This additional time helps the cells to establish all pathways for DNA repair.

DNA damage is sensed by the ATM/ATR pathway that can elicit a fast and slow response through different pathways. In the fast response, ATM/ATR activates the kinases ChK1/2 that in turn through phosphorylation inhibits Cdc25c and consequently the activation of CDK1/CyclinB, preventing the entrance into mitosis. Cdc25c is a phosphatase that counteracts protein kinases such as Wee1 that with their phosphorylation prevent CDK1 activity. The slow pathway stabilizes p53 that consequently activates p21 that finally inhibits pRB phosphorylation. Once DNA damage is repaired, ATM/ATR stop their activity through Chk1/2 and consequently the Cdc25c phosphorylation is removed. Finally, Cdc25 dephosphorylates CDK1 promoting CDK1/CyclinB nuclear translocation and cell cycle progression (Florensa, Bachs, & Agell, 2003).

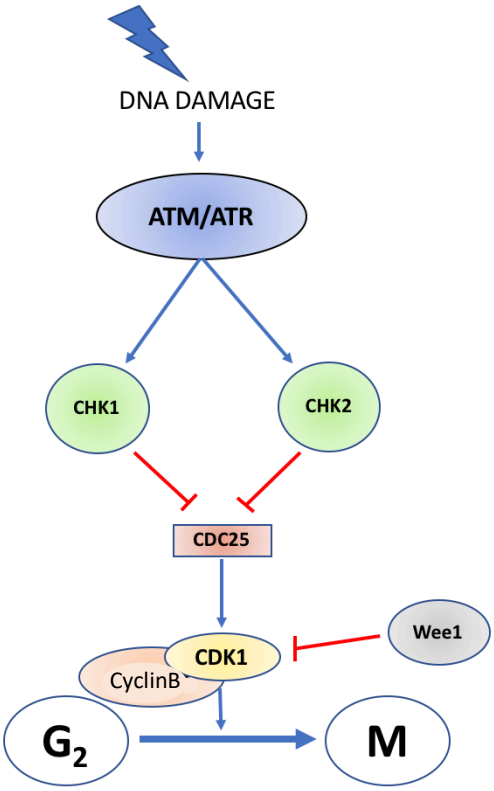


Figure 3 Schematic representation of the G2/M checkpoint

A simplified version in which blue lines means activation, red lines means inhibition. DNA damage causes cell cycle arrest.

Spindle Assembly Checkpoint

This checkpoint operates in the transition from metaphase to anaphase. During metaphase, chromatids are aligned along the metaphase plate, and they maintain their position thanks to the pulling forces exerted by the microtubules. The goal of the spindle assembly checkpoint is to ensure that chromosomes are bipolarly attached to the mitotic spindle before chromosome segregation. Chromosomes need to be bipolarly and properly attached to avoid erroneous segregation. SAC senses unattached kinetochores and blocks anaphase onset. The spindle assembly checkpoint will be extensively discussed in the mitosis section and the error correction part (Lara-gonzalez & Westhorpe, 2012).

Mitosis

Mitosis is a phase of the cell cycle in which the replicated genome is segregated into two identical daughter cells. Mitosis derives from an ancient Greek term ‘mitos’ that means a line, wire, string due to the filamentous shape of the chromosomes during cell division.

During mitosis the condensed DNA become part of a structure called the spindle, were the chromosomes are attached to tubulin wires called microtubules. Microtubules are characterized by a continuous shrinking and lengthening called dynamic instability and this specific feature is crucial in the different sub-phases of mitosis. Microtubules are formed by Tubulin dimers which are bound to the energy carrier guanosine triphosphate (GTP). The process of polymerization (growing) hydrolyze GTP in GDP causing the release (shrinking) (Mitchinson, 1989). This process contributes to the dynamic instability that characterize microtubules. Mitosis can be divided into several sub-phases with different meaning and roles we encounter, in chronological order:

prophase, prometaphase, metaphase, anaphase and telophase (Biology of the cell, 2004)

Prophase

The main goal of prophase is to condensate the chromosomes DNA in visible X shape structures. Chromosomes are made of two sister chromatids and appear in a x shape. The condensation process is carried out by a protein family named ‘Condensin’ (Antonin & Neumann, 2016). The sister chromatids are attached in a central region called ‘centromere’, this region will be crucial for the subsequent assembly of the kinetochore. The sister chromatid linkage happens thanks to another important complex called Cohesins. Cohesin complex is literally a ring that binds together both sister chromatids. Interestingly, Cohesins are present all along the chromosomes and are removed at mitotic onset during prophase except at the centromere (Nakajima et al., 2007) (Gligoris & Löwe, 2016).

During prophase, besides DNA condensation, the cell starts to self-organize in a structure called spindle (Nedelec et al. 1997). The spindle is formed immediately after NEBD and is composed by two poles constituted by the centrosomes and different types of microtubules. Microtubules attached to chromosomes are called k-fibers, microtubules anchoring the centrosome to the cell cortex are called astral and finally microtubules connecting the poles are called inter-polar. Centrosomes are a membrane-less structure that consists of two centrioles surrounded by pericentriolar material, responsible for the organization of the microtubules (they are also called Micro-Tubule Organization Center, MTOC).

Prometaphase

The end of the prophase corresponds to the nuclear envelope breakdown (NEBD), in which the nuclear envelope dissolves and the chromosomes become exposed to the cytosol. There are at least two models that describe how the spindle organize itself. The first called 'search and capture' the chromosomes are captured by the microtubules with a stochastic process (Heald & Khodjakov, 2015). This model focuses the attention on the centrosomes from where the microtubules are nucleated, and thanks to the continuous growth and catastrophe search they will capture the chromosomes. Conversely, the second model focuses on the chromatin and claims that microtubules are nucleated acentrosomally near the chromatin where they spontaneously assemble into anti-parallel bundles adopting a spindle-like structure. Due to this feature, the second model is called microtubule self-organization model (Nedelec et al. 1997).

Interestingly, not only centrosomes can nucleate microtubules but also kinetochores under certain conditions . Kinetochore-nucleated microtubules may speed up kinetochore capture and spindle assembly (Pavin et al. 2014).

The attachment between chromosomes and microtubules happens thanks to a structure previously cited, the kinetochore. In human cells kinetochore is assembled in a specialized chromosomal

region called centromere, that links a pair of sister chromatids. The kinetochore is the interface between the DNA of the centromere and the microtubules. The kinetochore is a multi-protein structure in which we recognize at least three regions: the inner kinetochore, outer kinetochore and the corona (Musacchio & Desai, 2017).

Each of these regions has different functional tasks: inner kinetochore serves as a platform for the entire structure, the outer kinetochore is crucial for microtubule attachments and finally the corona is involved in spindle assembly checkpoint and in microtubule anchoring. The kinetochore structure and function will be extensively analyzed and discussed later in a specific section of the thesis.

Microtubules are structural elements of the spindle playing a crucial role in mitosis. Microtubules are part of the cytoskeleton and are made of Tubulin dimers that polymerize into cylindrical hollow polymer. Microtubules ability to grow and shrink is exploited in the establishment of the bipolar spindle first, and later to segregate genetic material (Mitchison, 1989).

As previously mentioned, the spindle is formed by two poles, the duplicated centrosomes, from which microtubules emanate and between them the chromosomes reside.

During prometaphase chromosomes are bipolarly attached and subjected to opposite forces, in normal condition the resultant of those forces brings them to the spindle centre. In specific conditions, some chromosomes may not be orthogonally attached to the microtubules and therefore the spindle forces are useless. Those chromosomes are brought to the spindle centre by molecular motors like CENPE or Dynein. Dynein is a molecular motor able to 'walk' on microtubules and is required for initial attachment of polar chromosomes. Dynein ability to move toward the minus end of microtubules is exploited to drive polar chromosomes at poles. On the other hand, CENPE is a microtubule plus end directed kinetochore motor that drives chromosomes toward the metaphase plate using preexisting kinetochore fibres (Wood, Sakowicz, Goldstein, & Cleveland, 1997) (Figueiredo & Maiato, 2019).

Metaphase

Once all the chromosomes are bipolarly attached they can start their alignment on the equatorial plane also called metaphase plane. Attached chromosomes in metaphase are characterized by an oscillation along the spindle axis, this oscillation reflects the dynamic instability of spindle microtubules. The oscillation period and periodicity reflect the propensity of kinetochore-microtubules to switch from polymerization to depolymerization and vice-versa (Maddox et al. 2003).

Metaphase is the last step before the irreversible chromosome segregation, therefore a control mechanism is required. The presence of unattached kinetochore is sensed by the Spindle assembly checkpoint that can repress mitotic progression (Lara-gonzalez & Westhorpe, 2012).

SAC is not the only complex ensuring correct cell division, its activity being coupled by the Chromosome Passenger Complex (CPC). CPC destabilizes weak kinetochore-microtubule attachments triggering SAC activation (Carmena, Wheelock, Funabiki, & Earnshaw, 2012). CPC role and structure will be analyzed in a specific chapter of the thesis.

Once all the chromosomes are aligned and properly bipolarly attached cells can proceed to anaphase.

Anaphase

Anaphase happens if the spindle assembly checkpoint is satisfied. Sister chromatids are attached by their central region thanks to a protein complex called Cohesin. To separate sister chromatids the Cohesin link has to be removed by an enzyme called Separase, that directly cleaves the Cohesin ring. The final stage of mitosis is characterized by a drop of CyclinB levels and consequently the inactivation of CDK1. Normally, CDK1 and the SAC inhibit APC to prevent the degradation of Securin. Securin prevents the activity of Separase, a specific enzyme that cleaves the connection between the sister chromatids. To summarize, exit from mitosis reduces CDK1 and SAC activity allowing APC

to target Securine for degradation, therefore Separase is free to remove the chromosomes connection. The pulling of microtubules results in the movement toward the poles. Anaphase happens in two steps: first, anaphase A in which the kinetochore-microtubules shorten and pull apart the chromatids, and second anaphase B where the astral microtubules that are anchored to the cell membrane pull apart the poles and the interpolar microtubules slides past each other. Erroneous kinetochore-microtubule attachment, hyper stable or hypo stable microtubules, defective DNA condensation or architectural kinetochore defects may lead to segregation errors. Erroneous segregation will be addressed extensively later in the thesis (Cimini, 2011) (Silk, Zasadil, Holland, Vitre, & Cleveland, 2013) (Schvartzman, Sotillo, & Benezra, 2010) (Negrini, Gorgoulis, & Halazonetis, 2010).

Telophase

The final phase of mitosis is telophase, in which chromatids are completely segregated, the mitotic spindle dissolves and chromatids dissociate from microtubules. In addition, during telophase chromosomes decondense and nuclear envelope reform. All these events are triggered by CDK1 deactivation that happen due to CyclinB degradation and APC/C activity.

The cytoplasm is divided in two parts by a contractile ring previously formed. During telophase DNA decondensates and organelles as well as the nuclear envelope reform including nuclear pores that re-establish the separation between nuclear space and cytoplasm (Liu et al. 2018).

Cytokinesis

The final goal of mitosis is the creation of two identical daughter cells, the exact process that creates two daughter cells is called cytokinesis.

The process of cytokinesis can be divided in several sub-phases: anaphase spindle reorganization, division plane specification, actin-myosin ring assembly and contraction and abscission (Barr et al. 2007).

A contractile actin ring cuts the cytoplasm exactly in the middle, on a structure called midbody that represents the remnant of the mitotic spindle. The midbody plays a crucial role in cytokinesis and its absence prevents cell division. The contractile ring is powered by a Type II Myosin ATPase, that convert ATP into physical force. The contractile ring shrinks at the cell equator forming the cleavage furrow to the point which physically cut in two the cytoplasm. The final stage of cytokinesis is called abscission.

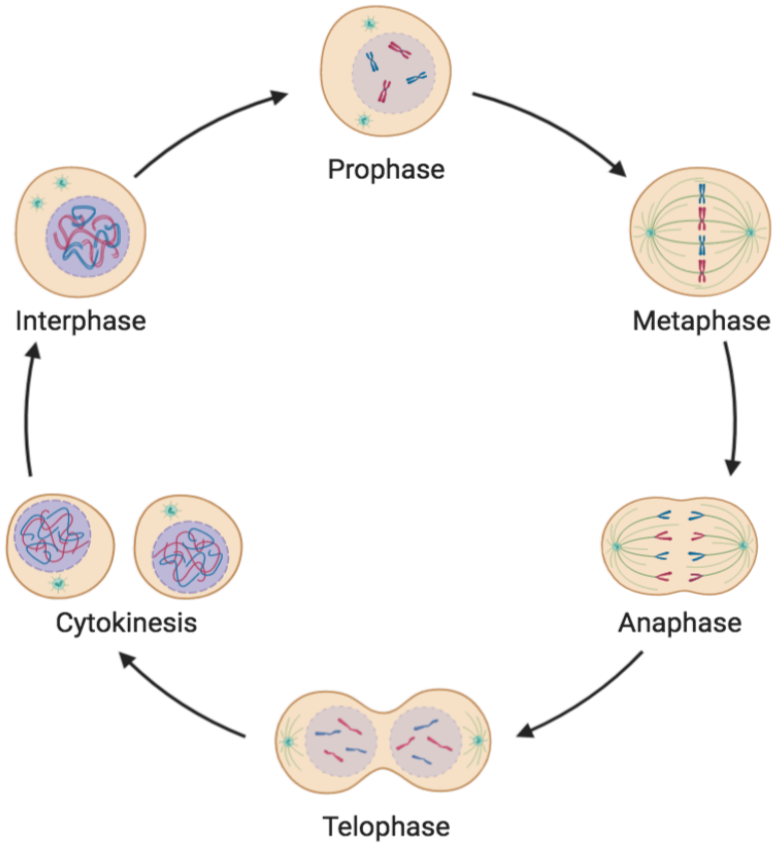


Figure 4 Schematic simplified representation of mitosis

From top left (clockwise) cells in interphase are metabolically active and DNA is duplicated. In prophase, the DNA start condensing and nuclear envelope break down. In metaphase chromosomes are bipolarly attached and aligned on the metaphase plate. During anaphase, sister chromatids are separated by the pulling microtubules. During telophase, the cytoplasm is cut by an actin ring. Finally, cytokinesis marks the end of mitosis in which two small daughter cells are generated.

Chromosome segregation defects and genomic instability

Despite control mechanisms, feedback loops and checkpoints, segregation errors may happen. Segregation errors is a broad term that includes several defects that cells may encounter during mitosis with different origins and with several potential outcomes: cell death, mitotic slippage, senescence and chromosomal instability. Moreover, those type of outcomes in turn depend on the genetic context, error severity and the cell type.

According to the origin segregation errors can be divided in several types:

- Nondisjunction
- Lagging chromosomes
- Multipolar spindle
- Monopolar spindle
- Cytokinetic failure

Nondisjunction happen when the link between sister chromatids is not removed and therefore a single chromosome is segregated in one of the two daughter cells creating an imbalance in chromatids number.

Lagging chromosomes are single chromatids that lag behind to the spindle equator, while the other chromatids move toward the poles. When the lagging chromosome is not included in one of the daughter nuclei may give rise to a high instable structure called micronuclei (Luzhna, Kathiria & Kovalchuk, 2013). Micronuclei may encounter different fates: nucleus reincorporation, degradation, cell extrusion, chromothripsis and finally apoptosis (Hintzsche et al. 2017).

Multipolar spindles happen when mitotic cells form more than two spindle poles. Multipolar divisions give rise to more than 2 daughter cells and this process leads to severe aneuploidy resulting in cell death.

Similarly, a monopolar spindle failed to separate spindle poles and therefore chromosome segregation is impossible. This specific conformation leads to a mitotic arrest and apoptosis (Yang et al. 2003)

Cytokinetic failure is a condition where the duplicated DNA is not segregated into the daughter cells resulting in one binucleated cell or a cell with a tetraploid genome.

One possible outcome of segregation defects is chromosomal instability. Several mechanisms are responsible for chromosomal instability, like defects in the spindle assembly checkpoint, sister chromatid cohesion, the regulation of microtubule attachments to chromosomes at kinetochores, centrosome duplication, telomere maintenance, and pre-mitotic replication stress (Funk, Zasadil, & Weaver, 2016) (Cimini, 2011) (Bakhoum & Swanton, 2014).

Chromosomal instability is a type of genomic instability which in turn is defined by an extremely high rate of mutations inside an organism genome.

Genomic instability is considered a cancer hallmark and it can concern nucleotides (NIN), microsatellite (MIN) or chromosomes (CIN) (Yao, 2014) (Negrini et al., 2010) (Hanahan & Weinberg, 2011).

Segregations defects mostly induce CIN that in turn can be divided into structural CIN (part of a chromosome is lost: inversion, deletion, mutation, translocation) or numerical (entire chromosome is lost). Numerical CIN finally can lead to aneuploidy, that is the presence of an unbalanced number of chromosomes in one genome (Mcgranahan, Burrell, Endesfelder, Novelli, & Swanton, 2012).

Aneuploidy is a consequence of CIN and often lead to cell cycle arrest or apoptosis (Wenzel & Singh, 2018). Interestingly, not all segregation defects cause aneuploidy, in fact, a single premature

separated chromatid may segregate in the right pole with a frequency of 50% (Potapova & Gorbsky, 2017). Although aneuploidy is present in the vast majority of human cancers, we can also encounter it elsewhere. Diseases like down syndrome, Edwards syndrome and Patau syndrome are example of aneuploidy not linked to cancer (Weaver & Cleveland, 2008).

Interestingly, Nicholson et al. observed a unidirectional relationship between CIN and aneuploidy. They observed that diploid cells, subjected to CIN, once they became aneuploid they also became genomically instable. This mechanism drives cancer mutation and evolution and will select the fittest karyotype (Nicholson & Cimini, 2013).

Genomic instability driven by CIN leads to aneuploidy that can turn into cancer. The following three elements are crucial for cancer formation and progression; Genomic instability, CIN and aneuploidy are connected and influences each other. Nonetheless, they are not accountable for cancer initiation and they cannot be considered the cancer causal agent.

We know the causal agent of a few human cancers and a relatively small number of genes are always mutated in cancer. p53 can be used as a paradoxical example, p53 is mutated or lost in the vast majority of human cancers but p53 knock out is insufficient for cancer initiation (Yao, 2014). p53 KO mice develop cancer only in the presence of other mutations, suggesting that p53 cannot be considered a causal agent. The p53 example shows us that our comprehension of cancer initiation is still limited.

Nonetheless, there are at least two hypotheses trying to elucidate cancer origins.

The mutator phenotype hypothesis claim that genomic instability is present in precancerous lesions and drive tumorigenesis through mutation in caretaker genes. The primary function of Caretaker genes is to maintain genome stability and they are mutated in the germline of inherited cancer patients. A single mutation in the second allele of a caretaker gene will cause a loss of heterozygosity (LOH). LOH is a common event in cancer and normally corresponds to the loss of

function of a tumour suppressor gene. When LOH affects a caretaker gene, it results in higher genomic instability and eventually cancer (Genet, 2001). The mutator phenotype can explain familiar breast cancer and other hereditary tumours. The former cancer is characterized by the mutation of a well-known caretaker gene BRCA1/2. BRCA1/2 is involved in DNA double-strand break (DSB) repair, regulating homologous recombination (HR) (Zámborszky et al., 2017). Many other tumour suppressor genes can be inherited already mutated, for example: p53, VHL, APC and ATM.

The second model connecting genomic instability and cancer is called the Oncogene Induced DNA Replication Stress Model. According to this model, mutated oncogenes induce DNA replication stress and consequently genomic instability. Activated oncogenes increase the proliferation rate that cause DNA replication stress. This type of stress, in particular genomic sites called common fragile sites, is detrimental. It has been observed that those sites are mutated in precancerous lesions in many tissues (Yao, 2014) (Negrini et al., 2010).

Although our knowledge about cancer causal agents is still limited and needs further investigation, the correlation between erroneous segregation and tumour formation is well accepted (Bakhoum & Swanton, 2014). This correlation prompted researchers to invest time in understanding exactly how DNA segregation works and how it is regulated. One mitotic structure deserving attention for its strategical role is the kinetochore. The kinetochore is not only the platform for microtubule attachment but is also directly involved in error correction (Cimini et al., 2001).

The interface between microtubules and DNA: the kinetochore

The chromosome is composed of duplicated DNA organized in an identical structure called sister chromatids.

To segregate DNA into two daughter cells during mitosis microtubules pull apart the sister chromatids. Microtubules do not attach directly on the DNA but on a protein structure called the kinetochore. The kinetochore has two main functions: first, it converts the microtubules dynamics into a force, specifically in tension exerted on sister chromatids. Second, kinetochore work as sensor in case of unattached kinetochore and is directly involved in the spindle assembly checkpoint (SAC). In order to exert their main function, human kinetochores normally are attached by 15 to 35 k-fibres (microtubules specialized in kinetochore capture). Furthermore, kinetochores controls and supervise all chromosome movements during mitosis (Dudka et al., 2018) (Heald & Khodjakov, 2015).

Kinetochores transiently auto-assemble on the centromere during mitosis, ensuring correct segregation. Some proteins are recruited to the centromere one by one whereas other kinetochores proteins such as the CCAN complex are already present before mitosis and are simply recruited to the centromere. Furthermore, the kinetochore is a multi-protein complex composed of hundreds of proteins organized in at least three portions:

- inner kinetochore: DNA embedded serves as a 'basement' for the kinetochore structure composed of proteins like CENPA, CENPB, CENPC
- outer kinetochore: microtubule binding site in which we find proteins like Hec1 and Mps1
- corona: a network of permanent and temporary proteins involved in checkpoint activity, microtubule anchoring. Moreover, the corona allows unattached kinetochores to generate a larger interaction surface for microtubules. CENPF and CENPE are members of the kinetochore corona (Musacchio & Salmon, 2007) (Musacchio & Desai, 2017).

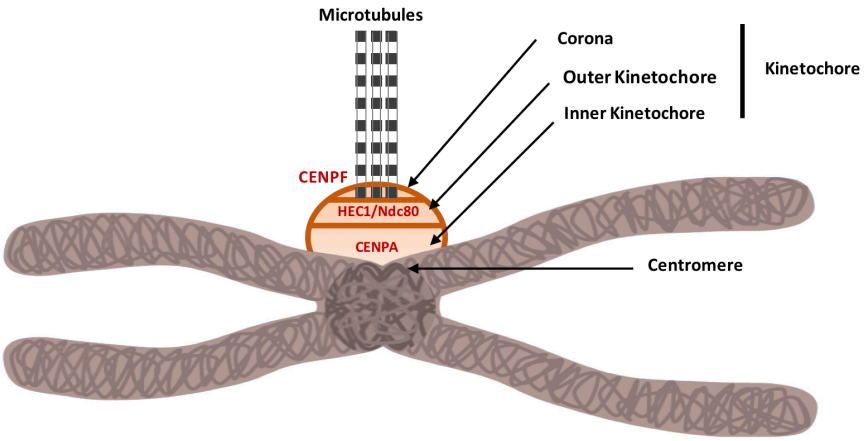


Figure 5 Schematic representation of human kinetochore

Kinetochore and chromosomes are not in scale to facilitate vision.

The kinetochore is temporarily assembled on the centromeric DNA before mitosis, and its positioning is crucial for faithful segregation.

Proper kinetochore localization is ensured by a H3 histone variant that acts as an epigenetic marker: CENPA (Bodor et al., 2014) (Valdivia et al. 2009).

CENPA serve as a platform for kinetochore deposition, consequently it is the first recruited protein and its absence precludes kinetochore formation. Human cells lacking CENPA and kinetochore die in mitosis. (Hoffmann et al., 2016) CENPA is recruited to the centromere between telophase and early G1. In this phase, the chaperone HJURP plays a crucial role as chaperon protein, in fact, HJURP absence causes CENPA degradation (Barnhart-dailey, Trivedi, Stukenberg, & Foltz, 2017). CENPA recruitment happens thanks to the 'old' CENPA pool. After recruitment CENPA is incorporated and stabilized in the centromere. During S phase DNA is duplicated in identical copies and CENPA is split between the newly synthesized centromeric regions. This feature is called semi-quantitative inheritance and is crucial to maintain the centromeric epigenetic marker. During mitosis, duplicated DNA condensates into chromatids to which CENPA is equally distributed. Human cells contain circa 150000 CENPA molecules per centromere, this large amount ensures that kinetochore will form in

the right place. Interestingly, Fachinetti et al. demonstrated that only 5% of CENPA is sufficient to establish a functional kinetochore, confirming that CENPA is recruited in excess at the centromere given its crucial role (Musacchio & Salmon, 2007) (Fachinetti et al., 2013) (Bodor et al., 2014).

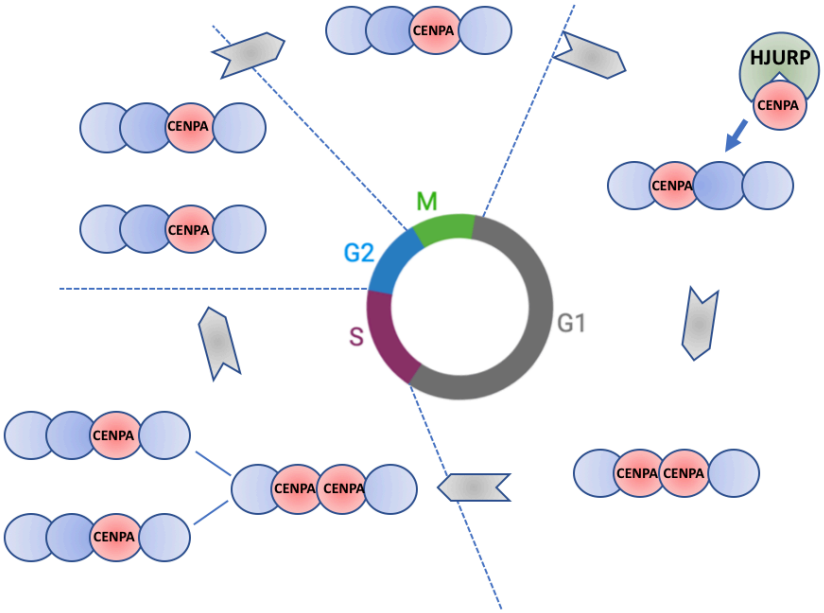


Figure 6 Schematic representation of CENPA cycle

Blue circles represent histones, red circles represent the modified histone CENPA. The sequences of circles represent the chromatin. CENPA loading happens at beginning of G_1, thanks to the chaperon protein HJURP. Subsequently, CENPA pool is split in two during S phase. During G_2 CENPA is maintained, finally in mitosis CENPA serves as an epigenetic marker for centromeric DNA and will be the basement for kinetochore assembly.

Chromosomes passenger complex

The chromosomes passenger complex (CPC) acts as a master regulator of cell division through its ability to control several processes, such as correction of microtubule-kinetochore errors, activation of the spindle assembly checkpoints and construction and regulation of the contractile apparatus regulating cytokinesis (Broad, Deluca, & Deluca, 2020).

The CPC components AuroraB, Borealin, INCENP and Survivin are strictly regulated in terms of time and space during mitosis. We can recognize one localization module and one kinase module, the connection between those two is represented by INCENP. (M. Lampson & Grishchuk, 2017)
Inner Centromere Protein (INCENP) as suggested by the name is localized in the centromere and is fundamental for AuroraB localization and activation. First, the INCENP IN domain recruits AuroraB to the centromere in early mitosis, second the INCENP TSS motif contributes to AuroraB activation. In turn, INCENP centromeric localization is regulated by AuroraB and Cdk1/cyclin B.

Survivin is the second component of the CPC, it contributes to AuroraB activation and localization.

Survivin, like INCENP, is tightly regulated during mitosis by several post-translational modifications. Finally, Borealin is involved in the cytokinesis and in centromere docking.
AuroraB represents the kinase module of the CPC, is part of the Ser/Thr kinase family and belongs to the Aurora kinase family that with Plk1 and CDKs can be considered master regulators of mitosis (Broad et al., 2020).
AuroraB regulation is an elaborate multistep process, in which many actors are involved.

To ensure proper localization AuroraB binds to INCENP, this binding activates the low level of kinase activity. Subsequently, AuroraB phosphorylates both INCENP and itself causing its full activation. AuroraB activation follows a model called 'density-dependent', according to this model AuroraB is not activated improperly, but only when the conditions are met (Carmena et al., 2012). The main role of CPC is to ensure proper chromosome bi-orientation and correction of erroneous kinetochore-microtubule attachments. During early mitosis, chromosomes must be bipolarly attached to ensure faithful segregation. Erroneous attachments like synthelic or merotelic may occur. In the first case, sister chromatids are attached to the same pole, in the latter the same kinetochore is attached to both poles. AuroraB ability to sense and correct erroneous attachments

is known, nonetheless the exact mechanisms are still unclear (Cimini, Moree, Canman, & Salmon, 2003).

It is believed that once bi-polar attachments are established, the pulling physical forces stretch the kinetochores out of the AuroraB activity zone. This removal protects kinetochore-microtubule attachments from AuroraB destabilizing activity and therefore stabilizes it. Conversely, incorrect kinetochore microtubule attachment fails to generate enough tension between sister chromatids to escape from the activity range of AuroraB. AuroraB kinetochore targets are part of the KMN network, a specialized group of proteins responsible for kinetochore microtubule attachment.

The KMN network is formed by Kln1 complex, Mis12 complex and NDC80 complex, and together they represent the outer kinetochore. In the case of erroneous attachment, AuroraB phosphorylates kinetochores reducing microtubule binding affinity. This reduced affinity facilitates the release of erroneous attachments and consequently their correction. Once tension is established phosphorylated KMN network proteins are pulled away from the AuroraB activity range and PP1y can exert its role dephosphorylating kinetochore targets.

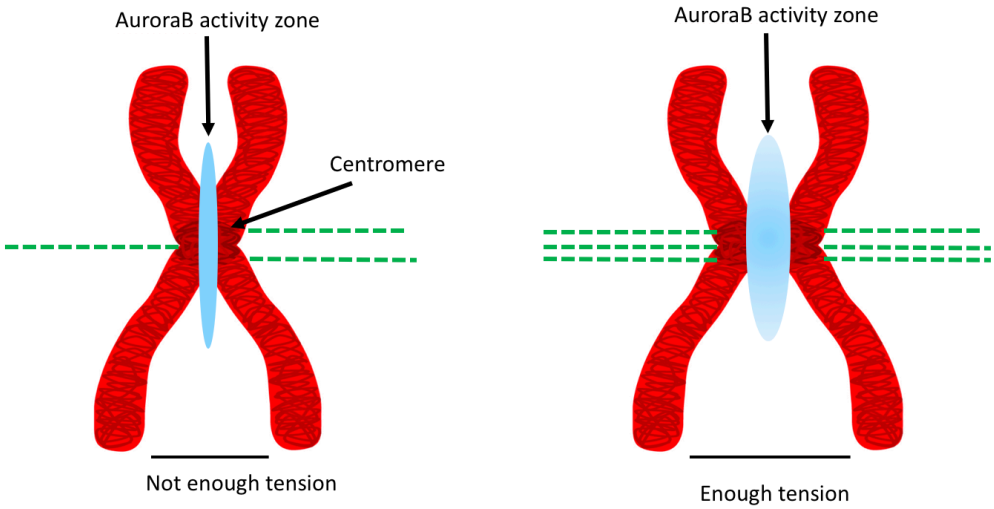


Figure 7 Schematic representation of AuroraB working models

Green lines represent microtubules, the light blue oval represent AuroraB activity zone. On the left is represented a situation of low tension due to incomplete microtubule-kinetochore occupancy. On the right, the full occupancy ensures proper tension and AuroraB targets are out of its activity (Broad, Deluca & Deluca, 2020).

This entire model is based on tension but was not widely accepted by the scientific community. Recently, Dudka et al. proposed a new model in which tension play a minor role (Dudka et al.,2018) According to Dudka et al. in fact, low tension is not enough to induce AuroraB-dependent error correction. The authors reproduced the same tension exerted by syntelic attachments reducing the kinetochore occupancy. Dudka et al. observed that in syntelic attachment triggers AuroraB activity and SAC activation, whereas in the low-tension model (drug-induced) AuroraB is partially active and SAC is off. The finding of Dudka et al. suggests that the tension is not sufficient to trigger a SAC mediated response, indicating the possibility that other mechanisms exist.

AuroraB activity generates unattached kinetochores that are sensed by the spindle assembly checkpoint (SAC) component Mps1. Mps1 compete with microtubules to bind Knl1 and the binding, via the recruitment of the checkpoint proteins including the effector Mads, prevents the activation of APC/C E3 ligase. APC/C E3 ligase in turn targets CyclinB and Securin which in turn inhibits Separase (Lara-Gonzales & Westhorpe, 2011). When chromatids are bipolarly attached CDKs phosphorylates APC/C favouring the binding with Cdc20. APC/C ^{cdc20} targets CyclinB and Securin for degradation. This causes the release of Separase that in turn degrades Cohesin, making sister chromatids ready to migrates to the respective pole. This cascade of events marks the transition from metaphase to anaphase (Musacchio, 2015).

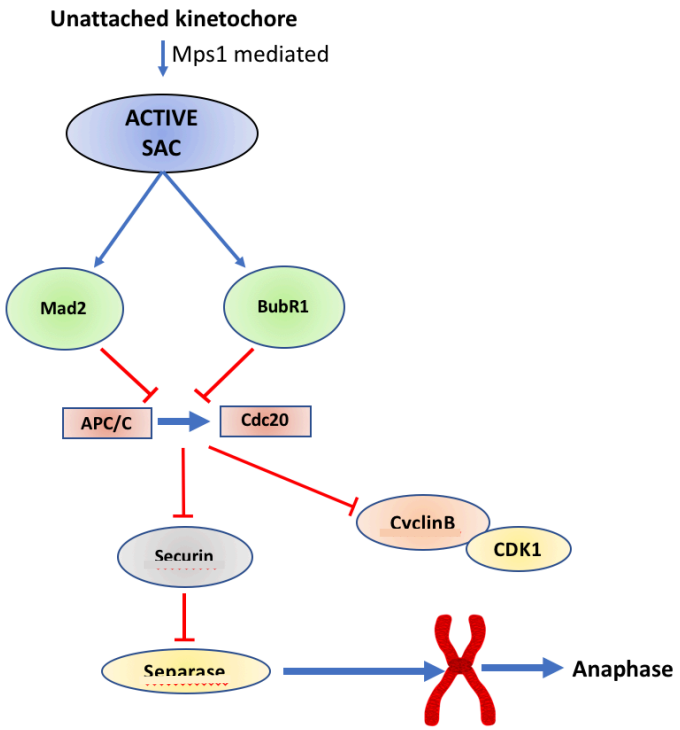


Figure 8 Schematic representation of the SAC pathway

Unattached kinetochores trigger SAC activation, Mad2 and BubR1 inhibit APC/C-Cdc20 complex. When SAC is satisfied APC/C-Cdc20 complex blocks Securin that in turn inhibit Separase. In parallel, APC/C-Cdc20 is involved in CyclinB degradation and therefore cell cycle progression.

Spindle Assembly Checkpoint

As previously mentioned, an additional checkpoint ensuring proper kinetochore microtubule attachments before anaphase, is the spindle assemble checkpoint SAC also called mitotic checkpoint or M-phase checkpoint.

This mechanism was first discovered in budding yeast and the main components were characterized; Mad1, Mad2, Mad3, Bub1, Bub2 and MPS1. The final target of SAC is APC/C (anaphasepromoting complex/cyclosome) an E3 ubiquitin ligase that targets many mitotic proteins including cyclins. Mad2 in concert with other SAC components can inhibit APC/C activity preventing mitosis progression. The main role of APC/C is to target Securin for degradation from Separase that in turn will cleave Cohesin ring causing the detachment of sister chromatids. SAC proteins are recruited on unattached kinetochores and the first recruited protein is MPS1. MPS1 competes with

microtubules for KLN1 binding on kinetochores (Musacchio & Salmon, 2007) (Kabeche & Compton, 2012) (Musacchio, 2015) (Hiruma, Sacristan, Pachis, Perrakis, & Kops, 2015).

Mps1 can phosphorylate Kln1 on the MELT motif creating a binding platform for Bub1 and BubR1. Bub1 and Bubr1 both contain a TPR domain that directly interacts with KNL1 KI motif when this interaction is compromised by a mutation not only Bub1 and Bubr1 are not recruited to the kinetochore, but also SAC functions are impaired (Krenn, Overlack, & Primorac, 2014). This observation supports the hypothesis that KNL1 and Bub1 are crucial for SAC recruitment and activity. Mad2 is a central component of the SAC being direct responsible for APC/C binding and inhibition. After mitotic entry, Mad2 is recruited at the kinetochore and it is removed by microtubules binding.

Mad2 exists in two conformations, open and closed, the closed form binds Mad1 at the kinetochore. Mad1 is a Mad2 kinetochore receptor and is anchored before the arrival of the microtubules. The Mad2 open form, is floating in the cytosol and is capable of APC/C binding and therefore stop mitosis. In the case of unattached kinetochore, the closed form recruits the open form to the kinetochore blocking the mitotic progression.

Partial Mad2 loss leads to premature Securin degradation and therefore premature sister chromatid separation. Pharmacological Mad2 inhibition causes lagging chromosomes, defective segregation and catastrophic cell death (Kastl et al., 2015). Interestingly, Mad2 is more often overexpressed than downregulated in the cancer context; the literature does not report cancers with a Mad2 loss of function (Lara-gonzalez & Westhorpe, 2012).

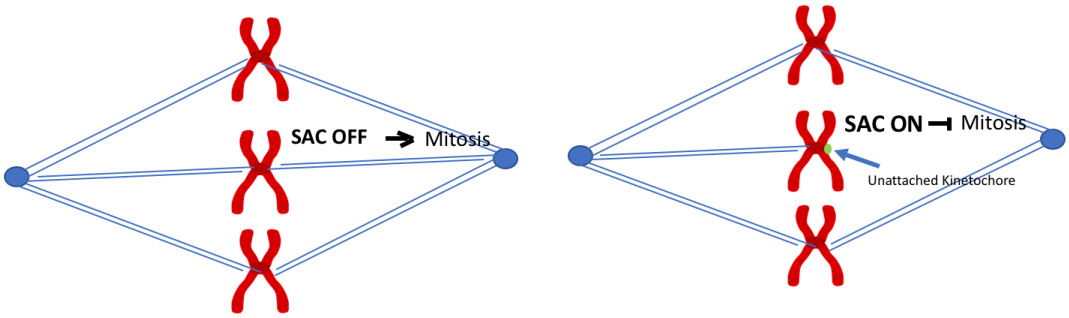


Figure 9 Schematic representation of the spindle assembly functioning

Unattached kinetochore triggers SAC activation that halts mitotic progression. Elements are not in scale

Mad2 and cancer

Several studies show that Mad2 is overexpressed in many human cancers, which is associated with a poor prognosis. To assess Mad2 overexpression role in tumour progression and initiation, Sotillo et al. created a transgenic mouse overexpressing Mad2 (Sotillo et al., 2008).

The authors found that Mad2 overexpression is sufficient for cancer initiation in different contexts and cell lines. Moreover, the authors discovered that prolonged Mad2 overexpression is not necessary for tumour maintenance, highlighting the importance of tumour initiation.

Mice overexpressing Mad2 present broken chromosomes, anaphase bridges and entire chromosome gain and loss. Based on these observations Kabeche and Compton built a model trying to explain the Mad2 overexpression phenotype (Godek, Kabeche, & Compton, 2015). The authors show that Mad2 overexpression hyper stabilizes KT-microtubule attachments impairing the error correction machinery. Finally, Mad2 overexpression in a non-SAC dependent mechanism can alter AuroraB centromeric localization. This model helps to understand some phenotypes observed upon Mad2 overexpression, both experimentally and in human tumours.

The mitotic cancer paradox

Mitosis is an extremely delicate step of the cell cycle, even a small event like an erroneous attachment can lead to deleterious consequences like chromosomal instability (Potapova & Gorbsky, 2017).

Although the link between cancer and mitosis is well established, only a few mitotic genes are directly mutated in cancer tissues. Cancer sequencing data reveals that the genes that are most frequently

mutated are: (Bailey et al., 2018)

- caretaker genes, involved in DNA repair (BRCA1/2)
- oncogenes, like growth factors and protein kinases (KRAS)
- checkpoint genes, cell cycle controller (p53, pRB)

One possible explanation for this paradox is that cancer-related interphase genes are also involved in mitosis.

VHL is an example of such an interphase gene also involved in the cell division process. The main function of VHL is to degrade the hypoxia factor HIF1, and its mutation is associated to kidney cancer, in parallel VHL contributes to microtubule stabilization during mitosis (Thoma et al., 2009).

Another prominent example of an interphase gene also involved in mitosis is the retinoblastoma gene RB1.

Retinoblastoma Protein, historical view

The first report of retinoblastoma was made in 1929 by Benedict who observed the presence of retinal tumour in homologous eyes in identical tweens (William Benedict, 1922). This observation suggested that retinoblastoma can be inherited. Later in 1951 Neel and Falls reported a rare ocular cancer occurring in children (The Rate of Mutation in the Gene Responsible for Retinoblastoma in

Man). The malignancy sometimes was inherited and rarely was sporadic. Those first observations paved the way to the discovery of retinoblastoma, a rare aggressive pediatric malignancy. Finally, in 1986 the retinoblastoma gene was identified, cloned and was established as the causal role for retinoblastoma (Friend, 1986). The retinoblastoma can be familiar (60%) or sporadic (40%), the first is characterized by an inherited germline mutation in one of the RB1 copies, a second somatic mutation is required to develop the tumour, this type of cancer occurs very early in young patients (Mastrangelo, De Francesco, Di Leonardo, Lentini, & Hadjistilianou, 2008). The sporadic form is rarer and both mutations are somatic, this type of cancer occurs later in age. The sporadic and the familiar forms also differ in the localization, indeed the familiar form affects both eyes (bilateral) in 35% of cases, conversely, the sporadic form appears mostly in one eye. The difference in localization between the two forms reinforces the two-hit hypotheses formulated by Knudson in 1971 (Knudson, 1971). According to this hypothesis to inactivate an onco-suppressor you need to inactivate both alleles. As a matter of fact, in the familiar form, the children are born with one defective copy of the gene, therefore the likelihood to get a second, somatic, mutation in both eyes is not negligible (bilateral retinoblastoma 35%). Conversely, sporadic retinoblastoma rarely happens in both eyes since two random somatic mutations would be required in both eyes. After the role of pRB was assessed in the context of retinoblastoma, scientists noticed that pRB was widely expressed and regulated cyclically. The two observations suggest that pRB plays a crucial role in the cell cycle.

pRB structure and functions

The retinoblastoma protein is encoded by a 27-exon gene positioned on chromosome 13 called RB1. Germline mutations normally occur along the entire gene without a remarkably mutational hotspot. Interestingly less than 10% of retinoblastoma patients have a structural chromosomal

abnormality, like deletion, detectable by karyotyping. The rest of the mutations affect single nucleotides, resulting in a truncated unstable protein form. The second RB1 allele mutation, Knudson's second hit, normally is due to the chromosomal aberration that determines the loss of heterozygosity (LOH). It is worth remembering that the second type of mutation happens with a higher rate than the first, suggesting that pRB haploinsufficiency may play a role (Ishak & Dick, 2015). pRB is a DNA binding protein involved in the regulation of the cell cycle. pRB is a 928 amino acid protein that exerts its role with E2F transcription factor family and other partners, it localizes in the nucleus and is phosphorylated in a cell cycle-dependent manner. pRB is composed of 3 main domains, C and N terminal domains being separated by a pocket domain. The pocket domain is normally disrupted in pRB malignancies and can be recognized by the LXCXE motif present in HDACs and onco-viruses (Cobrinik, 2005a).

Some viruses, called oncovirus, cause cancer as a consequence of infection: papovaviruses, adenoviruses, herpesviruses and hepatitis B. These oncoviruses could integrate their genome inside the host disrupting two important tumour suppressors: pRB and p53. The E7 papillomavirus, E1A adenovirus and SV40 Large T antigen bind pRB, causing its degradation through the ubiquitin-proteasome system, consequently deregulating cell cycle regulation (Fischer, Uxa, Stanko, Magin, & Engeland, 2017) (DeFilippis, Goodwin, Wu, & DiMaio, 2002).

The pRB pocket domain is used not only for interaction with other proteins but also acts as a molecular switch. Indeed, the hyperphosphorylated pocket domain is no longer able to bind E2F, causing its release. The same mechanism is also used to inhibit the interaction between pRB and HDAC. pRB can regulate the cell cycle in at least two ways: first, directly through its interaction with E2F and second, through the interaction with HDACs (Dick & Rubin, 2013) (Talluri & Dick, 2012).

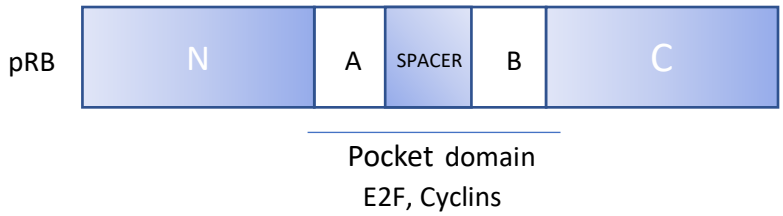


Figure 10 Schematic representation of retinoblastoma protein domains.

The pocket domain allows the E2F finding due to the presence of the LXCXE motif.

HDACs are a class of enzyme responsible for histones post-translational modifications: specifically, HDACs remove acetyl residues from histones making DNA less accessible and more compact.

Thus, pRB through the interaction with HDACs reduces the accessibility to DNA and therefore transcription. On the other hand, when pRB is phosphorylated HDAC is released and, as a consequence, DNA becomes more accessible and transcribable (Vélez-Cruz & Johnson, 2017).

pRB belongs to the pocket protein family, which comprises also p130 and p107. These proteins share extensive homology and interact with E2F family members (Cobrinik, 2005b).

Interestingly, the majority of human cancer does not present relevant or remarkable mutations in p130 and p107 suggesting a minor role in terms of carcinogenesis. Considering the homology, p130 and p107 probably only play an ancillary role in specific situations and have a redundant role in pRB activity (Cobrinik, 2005a).

As previously mentioned, the G_1/S transition is regulated by the pRB pathway. pRB contains 16 phosphorylation sites that are cyclically phosphorylated by CDKs. At least 3 Cyclins/CDKs are involved in pRB phosphorylation: CDK4/6 CyclinD in early G1, CDK2 CyclinE at the end of G1 and CDK2 CyclinA to maintain its phosphorylation in S phase. Moreover, in order to be fully active pRB needs multiple and specific phosphorylation. For example, pRB hyperphosphorylation is achieved by the sequential CDK4/6 and CDK2 phosphorylation.

pRB and genomic instability

pRB is a stereotypical onco-suppressor that acts as a checkpoint control, nevertheless, pRB inactivation not only leads to unregulated cell cycle, but it also affects cell division. Errors in cell division and low mitotic fidelity lead to genomic instability and ploidy changes. Several authors have shown that pRB absence contributes to genomic instability and aneuploidy through mechanisms that are still under investigation (Amity L. Manning & Dyson, 2012a) (Schvartzman, Duijf, Sotillo, Coker, & Benezra, 2011).

The fact that pRB absence can influence cell division suggests a direct role in mitosis. There are multiple hypotheses connecting pRB to mitosis and they are not mutually exclusive. Here I will explore the three major hypotheses.

1. **Mitotic protein expression** pRB is mostly known for its ability to inhibit E2F transcription in G1. E2F as an transcription factor controls the transcription of thousands of genes, some of which are involved in mitosis. The SAC protein Mad2 is an E2F target. It has been observed that Mad2 overexpression leads to chromosomal instability and cancer initiation through a mechanism described by Kabeche et al. (Schvartzman et al., 2011) (Godek et al., 2015). The same observation was made in many human cancers indicating Mad2 as a potential oncogene, on the contrary no tumors show Mad2 downregulation.

Interestingly, pRB depletion is not sufficient to trigger Mad2 overexpression but the concomitant p53 absence is (Sheahan, Bellamy, Treanor, Harrison, & Prost, 2004).

Although pRB absence is not sufficient to trigger Mad2 overexpression, the concomitant pRB and p53 depletion determine Mad2 overexpression. Mad2 is not the only mitotic gene influenced by pRB, in fact also CENPA, AuroraB and Hec1 are E2F regulated. In conclusion, pRB indirectly controls the transcriptions of many mitotic genes nonetheless a direct connection between pRB absence phenotype and a mitotic gene is still missing.

2. Replication control and regulation

Bester and colleagues describe a different type of stress encountered by pRB depleted cells. The authors find that pRB depleted cells experience longer S phase and replication fork stall, both phenotypes can be explained by a suboptimal nucleotides production. In support of this observation, nucleotide supplementation rescues the pRB depletion phenotype. Many E2F target genes are involved in nucleotide production and S phase like MCM, PCNA, TS and RR. Notably, stalling of the replication fork on sensitive sites can lead to double-strand DNA breaks (DSBs) that normally are repaired before S phase exit. In pRB depleted cells those DSBs are not corrected and are propagated in mitosis. Unresolved DNA damage in mitosis can lead to SAC activation, centromere disruption and consequently erroneous segregation (Bester et al., 2011) (Amity L. Manning & Dyson, 2012b).

3. Chromatin remodeling

The last element connecting pRB to mitotic phenotypes is its ability to recruit chromatin modifiers. pRB regulates transcription both directly and indirectly: directly inhibiting E2F activation and indirectly through the chromatin modifier recruitment. Specifically, pRB is able to interact and potentially recruit Cohesin and Condensin complex elements. Condensins are large protein complexes, whose role is to condensate DNA before mitosis and ensure faithful segregation. Centromeric DNA Condensation plays a crucial role in mitosis and it has been observed that uncondensed centromeric DNA leads to erroneous attachments and segregation defects (Samoshkin et al., 2009).

Dyson et al. observed that pRB depleted cells failed to recruit CAP-D2 (Condensin complex subunit) at the centromeric region, resulting in altered chromosome structure in mitosis and lagging chromosomes. (Amity L Manning et al., 2015) (C.H. et al., 2010)

Despite the many hypothesies and observations linking pRB to mitosis, it is hard to identify a single feature that explains how pRB absence increases CIN.

Of note, all the hypotheses discussed before are not mutually exclusive. pRB absence can induce CIN through multiple mechanisms and with a synergistic effect.

Change in mitotic protein expression, altered chromatin structure and impaired DNA replication contributes to a different extent to chromosomal instability observed in pRB depleted cells. Interestingly a 2015 paper from Dick et al. demonstrates that a single RB1 copy is not sufficient to maintain genomic stability. When a single copy of the tumour suppressor gene is not able to maintain its cellular role, one refers to 'haploinsufficiency'. Dick's results suggest a revision of the two-hit hypothesis. (Ishak & Dick, 2015) According to Knudson's two-hit hypothesis, both tumour suppressor alleles need to be inactivated to suppress the protein activity (Mastrangelo et al., 2008). Ishak and colleagues suggest that a partial pRB depletion is sufficient to induce chromosomal instability and cancer initiation (Ishak et al. 2015). The derived genomic instability often inactivates p53 making the process even more tumorigenic. (Sheahan et al., 2004)

A new model in which RB1 single copy contributes to genomic instability may explain several paradoxical features. For example, the second mutation on the RB1 allele happen with a higher frequency compared to the first one (10^{-3} as compared with 10^{-7} for the first mutation) (Knudson, 1971) Consequently, the familiar form of retinoblastoma emerges earlier than the sporadic. Second, Retinoblastoma survivors are more susceptible to develop tumours during their lives. Third, the vast majority of human tumours present a disrupted pRB pathway but only a few tumours are directly linked to pRB: retinoblastoma, osteosarcoma, mesenchymal tumour and some melanomas. All those paradoxes can be explained, at least partially, by pRB haploinsufficiency.

The RB1 gene was cloned more than 30 years ago and its role in cell cycle progression is well characterized. Some recent publications showed that pRB absence can influence mitosis and is

linked to chromosomal instability. This novel pRB role may explain why in many human tumours pRB is lost or mutated. A better understanding of the role of pRB in mitosis will help to elucidate cancer initiation and progression.

pRB pathway and cancer

Rb1 is not the only gene that can be lost or mutated in pRB pathway. The INK4 protein family (p16, p15, p18 and p19) belongs to the pRB pathway and mainly inhibits the activity of the CyclinD dependent kinases, finally preventing pRB phosphorylation and therefore inactivation. Several human tumors present mutation or the loss of INK4 proteins, for example 80% of pancreatic cancers and 60% of glioblastoma multiforme present INK4 loss. Conversely, CyclinD or CDK4 are overexpressed in human cancers: more than 90% of Mantle T cell lymphoma and 50% of breast cancer present a CyclinD1 overexpression. Despite many human cancers present a mutation or the loss of those genes still is not clear if is it a cause or a consequence. Likewise, pRB is highly mutated in human cancers but the high incidence of mutations can be a consequence of the high genomic instability that characterize human cancers. For example, animal models with CyclinD overexpression or INK4 depletion only predispose to cancer development indicating that more players are required and involved (Sherr & McCormik, 2002).

Aim of the project

Retinoblastoma was observed for the first time more than 100 years ago, and since then the amount of information about the disease and the cause dramatically increased (William Benedict, 1922). Retinoblastoma is one of the few cancers in which a single gene is responsible for the disease and this gene is RB1 (Goodrich, 2006)

After the characterization of RB1 and its cloning, scientists started speculating about the gene's physiological role.

Thanks to years of observations and experiments we now know that RB1 produces the pRB protein responsible for the G_1/S transition of the cell cycle making it a stereotypical onco-suppressor. In addition to the well-characterized role in cell cycle transition, the absence of pRB is also linked to the presence of chromosomal instability. Furthermore, pRB is lost or mutated in the vast majority of human cancers, making it extremely appealing in term of cancer biology (Dyson, 2016). At least two non-mutually exclusive hypotheses explain the link between pRB absence and chromosomal instability.

The goal of my project was, first to assess pRB role in mitosis and later understand the impact on chromosomal instability. To do that I tested both the current hypotheses and I elaborated a complementary one. Finally, I tried to understand the impact of pRB partial depletion compared to a full knockout.

Results

Investigating the mitotic role of Retinoblastoma protein and its impact on chromosomal instability

Aureliano Stingi[1,2] and Patrick Meraldi. [1,2]

[1]Department of Cell Physiology and Metabolism, Faculty of Medicine, University of Geneva, 1211 Geneva 4, Switzerland

[2]Translational Research Centre in Onco-hematology, Faculty of Medicine, University of Geneva, 1211 Geneva 4, Switzerland

AUTHORS CONTRIBUTIONS AND PERSPECTIVES

The project was initiated and directed by Patrick Meraldi. Aureliano Stingi performed all experiments. Aureliano Stingi and Patrick Meraldi analyzed and interpreted all the results and wrote the manuscript. The authors are planning to submit the manuscript as soon as possible to an international peer-reviewed journal.

Introduction

The Retinoblastoma protein (pRB) is a tumour suppressor that is lost or mutated in the vast majority of human cancers. pRB represses genes involved in DNA replication and prevents G1/S transition. A part from the well-characterized role in interphase, it has been reported that pRB absence leads to chromosomal instability through at least two different mechanisms.

According to the first mechanism, pRB loss is linked to Mad2 overexpression and SAC deregulation and consequently to genomic instability.

According to the second mechanism, pRB absence interferes with the recruitment of Condensin complex to the centromeric DNA, impairing chromosome segregation.

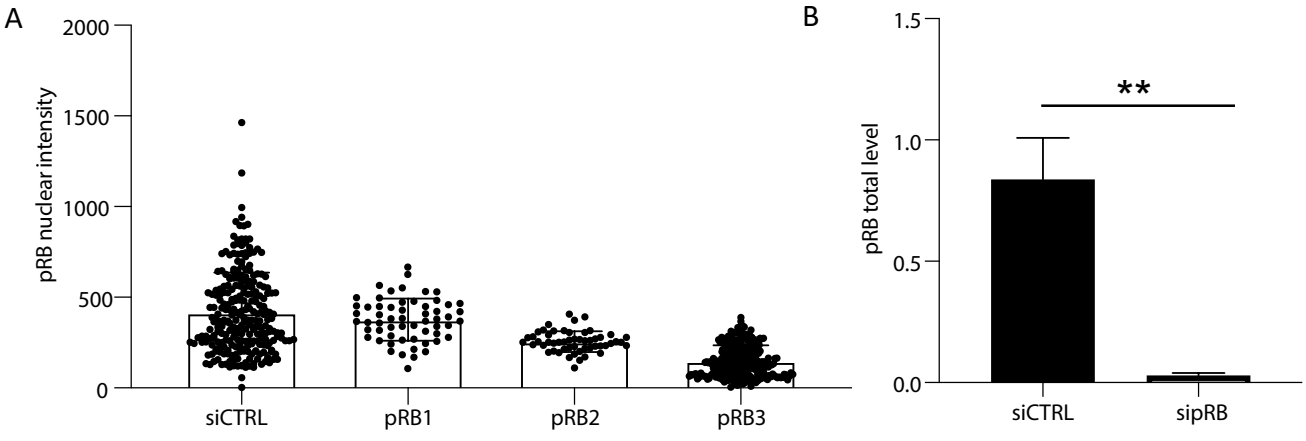
In the experimental part of my thesis, I focused on the non-canonical roles of pRB and I tried to elucidate which mechanism links pRB to chromosomal instability.

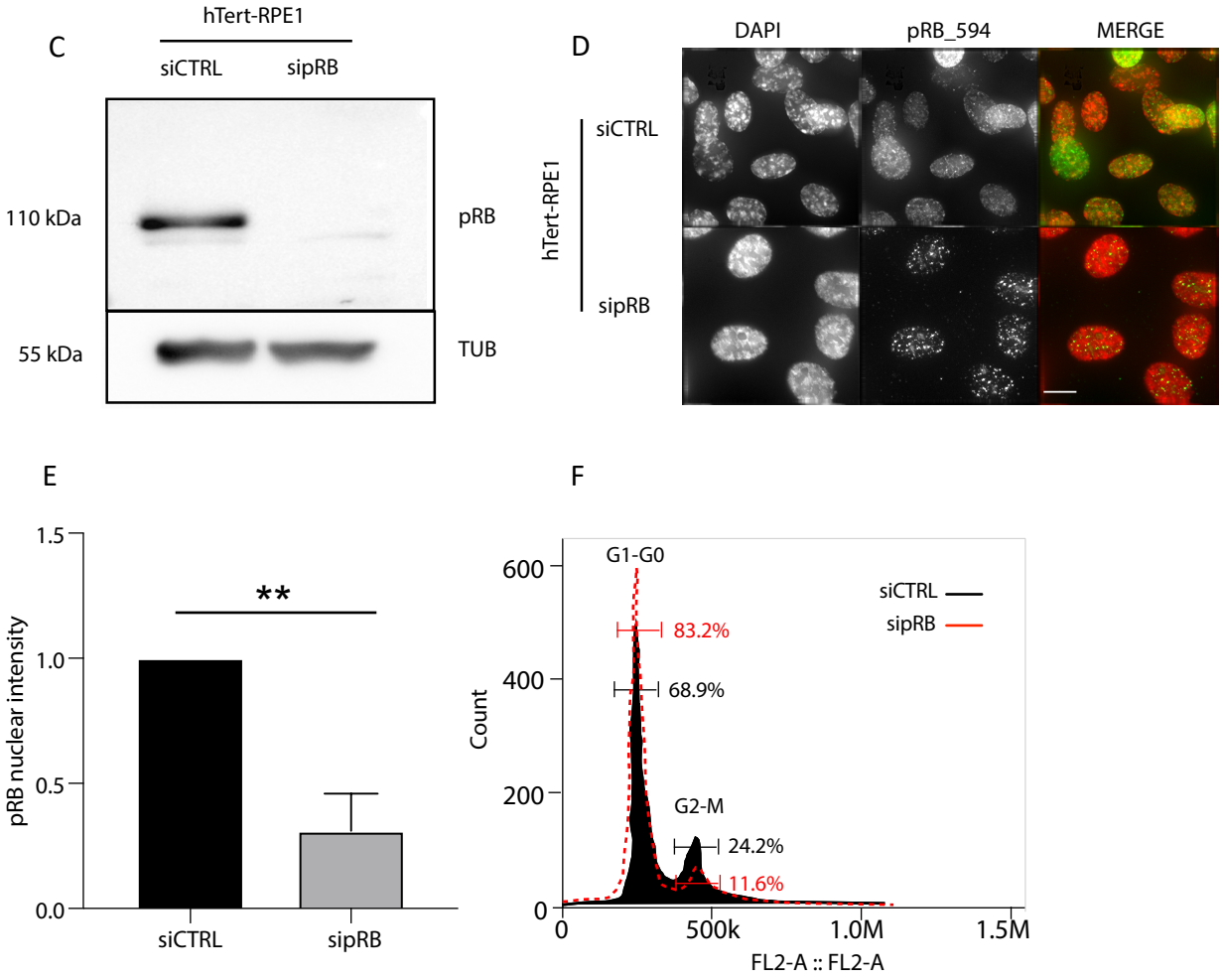
The partial depletion of the retinoblastoma protein pRB prolongs the mitotic duration and increases the probability of lagging chromosomes

To investigate the potential role of pRB during mitosis I depleted the protein, using siRNAs targeting the pRB mRNA in the genetically stable non-cancerous hTERT-RPE1 cell line, that has been immortalized with human telomerase. This cell line has functional cell cycle checkpoints and displays a low incidence of chromosome segregation errors (Wilhelm et al., 2019). Given that pRB is known to have a long half-life (Y. Wang et al., 2015), cells were treated in parallel for 72 hours with 3 siRNAs targeting pRB or a control siRNA. Later cells were fixed with cold methanol and stained with an antibody against pRB. pRB localizes mostly in the nucleus due to its DNA binding ability (Szekely et al., 1991). To quantify pRB levels based on a sufficiently large number of cells, a minimum of 30 random cells were chosen in each depletion and visualized by fluorescence microscopy. For each cell, I quantified the pRB signal in the nucleus and the background intensity outside the nucleus. After background subtraction, I determined the depletion efficiency by

calculating the ratio between the average intensity in siRB-treated cells versus control-transfected cells. Each experiment was performed at least three times independently unless specified.

Based on this quantification protocol I determined that the siRB oligonucleotide pair #1 caused a 12% reduction in the RB nuclear signal, the siRB oligonucleotide pair #2 a 39% reduction and the oligonucleotide pair #3 a 64% reduction when compared to siCTRL-transfected cells (Fig11.1 A). Equivalent results were obtained by western blot analysis on cells treated with the same depletion protocol (Fig11.1 B,C). I concluded that the pRB antibody is specific and that the different pRB siRNAs are all able to partially deplete pRB within the course of 72 hours. Since the siRNA oligonucleotide pair #3 showed the highest depletion efficiency it was used for all the depletions reported in the results section, unless specifically indicated (Fig11.1 D,E). Given the importance of pRB in the control of the G_1/S transition, we compared the cell cycle profile of sipRB- or control transfected cells by FACS analysis (Cobrinik, 2005b). To my surprise, I observed a higher percentage of cells in the G_1/G_0 phase (83.2%) in pRB-depleted cells when compared to control transfection (68.9%). As a consequence, the fraction of mitotic cells was reduced in pRB-depleted cells when compared to control transfection. (Fig11.1 F).





The partial depletion of the retinoblastoma protein pRB prolongs the mitotic duration and increases the probability of lagging chromosomes

Figure11.1 A. Quantification of the pRB signal in the nucleus of cells stained with pRB antibody after background subtraction after indicated treatments. N=1

B. Quantification of the pRB/α-tubulin ratio signal in three independent Western blots as shown in **(C)** and in siCTLR and sipRB cell extracts. Error bars represent standard deviation, statistical significance was determined with an unpaired t test; ns (non-significant), $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ N=3

C. Representative western blot of lysates of hTert-RPE1 cells treated with indicated siRNAs and probed with anti pRB (upper row), anti α-tubulin (lower row)

D. Representative wide field images of hTert-RPE1 stained with anti pRB (green) antibody and counterstained with DAPI (red) Cells were treated with siCTRL (upper row) or sipRB (lower row) for 72 hours. Scale bars=10µm

E. Quantification of the pRB signal in the nucleus of cells stained with pRB antibody after background subtraction in images as shown in **(D)** error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3

F. FACS profile of hTert-RPE1 treated with sipRB or siCTRL. Cells were treated with the indicated siRNAs for 72hours, and stained with DNA-dye propidium Iodide (PI). Events=10000 N=1

To validate these findings, we assessed the number of cells entering mitosis in a given time period in both pRB-depleted cells and siCTRL treated cells. To visualize mitotic progression, hTert-RPE1 cells were stained with the live DNA dye SiR-DNA and monitored by live fluorescence microscopy at 37°C. To determine the mitotic index, several regions of interest were selected, cells were imaged for 24 hours, cells entering mitosis during this time frame were counted by visual inspection, and the number of mitotic cells was divided by the total number of cells in the ROIs. (Fig11.2 A). This quantification revealed a mitotic index of 3% for pRB-depleted cells vs 20% for control-depleted cells, confirming that pRB depletion reduced the number of cells entering mitosis over a given time period.

Theoretically, pRB depletion should lead to uncontrolled cell cycle and proliferation due to the absence of the checkpoint control. Nonetheless, pRB depletion is reported to trigger the activation of the tumour suppressor p53, resulting in a cell cycle arrest via the activation of the Cyclin dependent kinase inhibitor p21 (Zhe Yang, Maciejowski, & Lange, 2017). To confirm that accumulation in G_0/G_1 depended on p53 activation I performed a rescue experiment.

pRB and p53 were depleted individually and together and the mitotic index was assessed by live-cell imaging as previously described. pRB single depletion reduced mitotic index from 20% (siCTRL) to circa 3% (sipRB). Conversely, the double depletion siRB/sip53 partially rescued the mitotic index to a value of 10% (Fig11.2 B). This result suggests that p53 is involved, at least partially, in the cell cycle arrest observed in pRB depletion. pRB depletion dramatically reduces mitotic index, nonetheless 10% of the pRB depleted cells underwent mitosis allowing its analysis.

To understand how pRB depletion impacts genomic instability, it is necessary to clarify how pRB absence impacts mitosis. To visualize chromosomes in mitosis, I took advantage of a stable tagged cell line expressing CENPA YFP. This cell line harbors an endogenous copy of CENPA tagged with YFP. CENPA is a modified H3 centromeric histone crucial for kinetochore formation (Bodor et al., 2014). Thanks to tagged CENPA I followed the cells through mitotic progression and monitored segregation errors.

pRB was depleted in hTERT-RPE1-YFP-CENPA resulting in a 86% reduction in protein abundance compared to control transfection (Fig11.2 C,D). Data were confirmed by western blot analysis performed with the same protocol as previously described (Fig11.2 E,F).

pRB was depleted in hTERT-RPE1-YFP-CENPA and mitotic progression was monitored through live-cell imaging. Only a small fraction of pRB depleted cells underwent mitosis (less than 10%) compared to siCTRL transfected cells, consistent with FACS data (Fig11.1 F). Furthermore, I measured the mitotic timing: the time occurring between nuclear envelope break down and anaphase onset.

Live cell imaging showed that pRB depletion increases mitotic timing, in fact control cells needed 20 minutes to complete mitosis, compared to pRB depleted cells that took 28 min (Fig11.2 G,H). The longer mitotic timing suggested the presence of segregation errors or impairment in the error correction machinery (Meraldi, Draviam, & Sorger, 2004) (Zhenye Yang, 2008). Despite the longer mitotic timing, visual inspection of dividing pRB depleted cells did not reveal any segregation error. This can be explained by the fact that hTERT-RPE1 are particularly genomic stable and in physiological condition they experience an extremely low rate of segregation errors (Wilhelm et al., 2019).

To enrich for the number of segregation errors I used the Monastrol drug (Santaguida et al., 2018). Monastrol exerts its role by inhibiting Eg5, a tetrameric kinesin-5 that pushes apart antiparallel

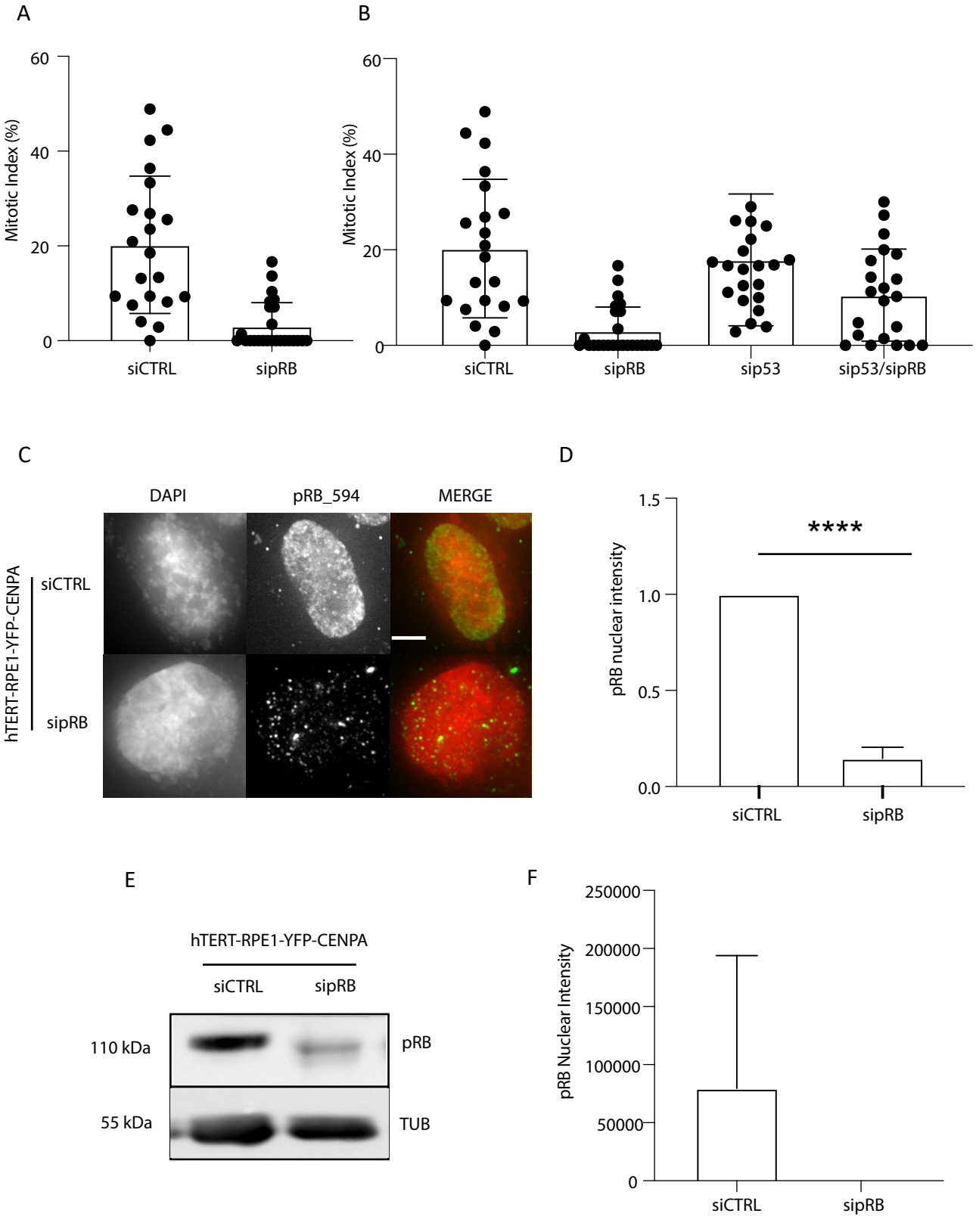
microtubules, driving centrosome separation. Cells treated with Monastrol are organized in a monopolar configuration and the chromosomes are rich in syntelic and merotelic attachments. Syntelic attachment happens when both sister chromatids are attached to the same spindle pole whereas merotelic attachment occurs when a single sister chromatid is bipolarly attached. Remarkably, synthelic attachments normally are detected and corrected, on the contrary merotelic attachments are often undetected (Cimini et al., 2003). Monastrol release allows bipolar spindle formation and consequently chromosomes align on the metaphase plate. (Kaseda, Mcainsh, & Cross, 2000) (Mchedlishvili, Wieser, Mouysset, Belwal, & Amaro, 2012). During the transition from monopolar to bipolar, the cell detects and corrects syntelic attachments activating the spindle assembly checkpoint.

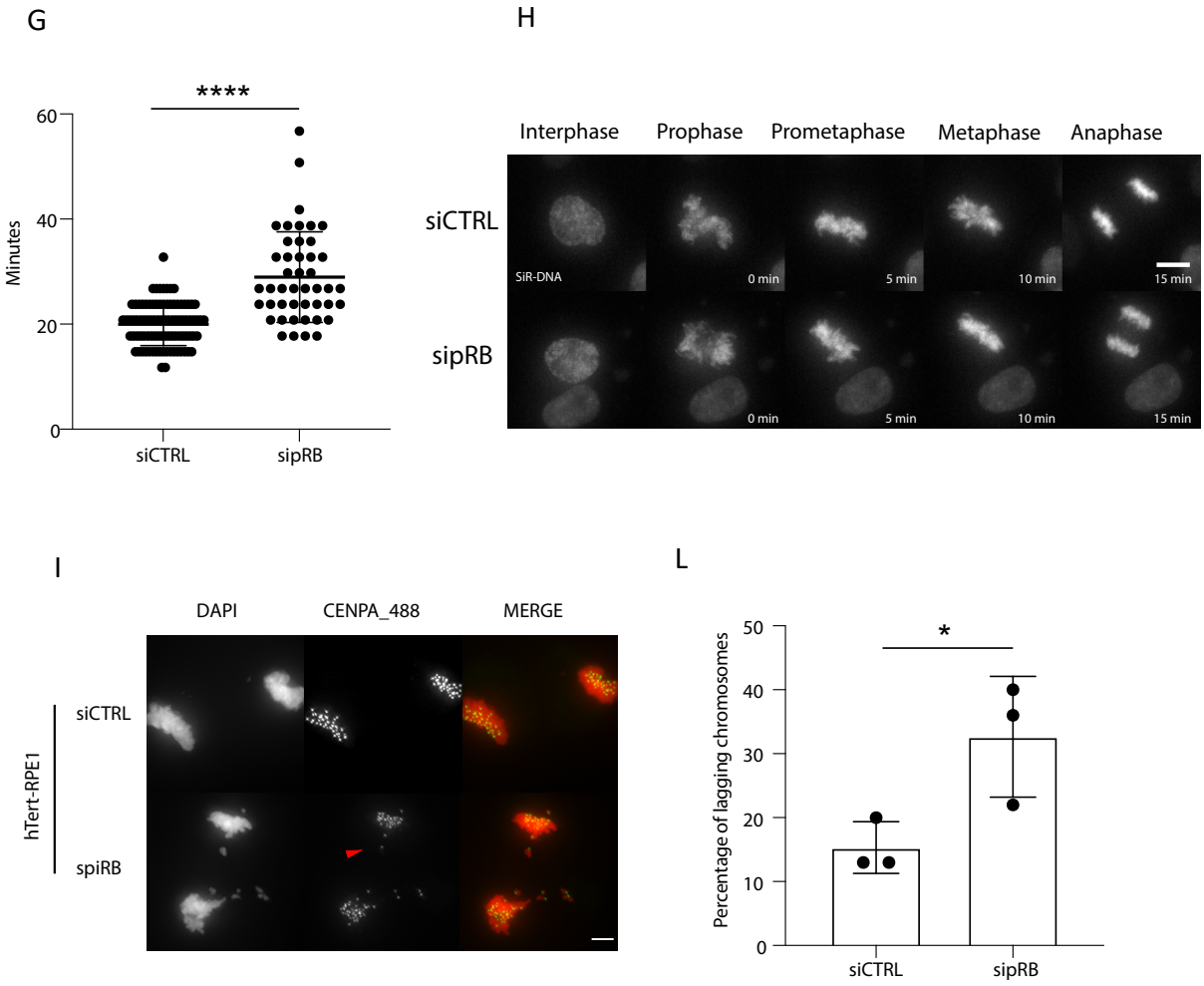
Conversely, due to their geometry merotelic attachments are not detected by spindle assembly checkpoint (Cimini, 2011). Lagging chromosomes are single kinetochore-positive chromosomes that lag between the two masses of segregated chromatids are and their presence is a readout for chromosomal instability (M. A. Lampson, Renduchitala, Khodjakov, & Kapoor, 2004).

I depleted pRB in hTERT-RPE1-YFP-CENPA, after 72 hours, the cells were treated with Monastrol for 4 hours. Finally, I released the cells and imaged them thanks live-cell imaging. Thanks to the CENPA YFP signal single mitosis were analyzed and lagging chromosomes were visually detected. Control depleted cells showed at least one lagging chromosome in 15% of the observed mitosis, whereas pRB depleted cells showed at least one lagging chromosome in 32% of the analyzed mitosis (Fig11.2 I,L). Furthermore, pRB depleted cells employed in average 30 minutes for the transition from monopolar to bipolar, while control cells employed in average 24 minutes. (Fig14 A,B)

The longer mitotic timing and the presence of lagging chromosomes suggested that pRB depletion is associated with a misregulation of kinetochore microtubule attachments, kinetochore structure

or error correction machinery. Finally, I could conclude that pRB depletion after Monastrol release is associated with a high number of lagging chromosomes.





The partial depletion of the retinoblastoma protein pRB prolongs the mitotic duration and increases the probability of lagging chromosomes

Figure11.2 A. Dot plot representing single cell undergoing mitosis divided by the total number of observed cells. Cells were treated with sipRB or siCTLR for 72 hours and subsequently imaged for 24 hours. N=2 **B.** Dot plot representing single cell undergoing mitosis divided by the total number of observed cells. Cells were treated with sipRB, siCTLR, sip53 or sip53/pRB for 72 hours and subsequently imaged for 24 hours. N=2 **C.** Representative wide field images hTERT-RPE1-YFPCENPA stained with anti pRB (green) antibody and counterstained with DAPI (red) Cells were treated with siCTRL (upper row) or sipRB (lower row) for 72 hours. Scale bars=5µm **D.** Quantification of pRB signal in the nucleus of cells stained with anti-pRB antibody after background subtraction in images as shown in **(C)** error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **E.** Western blot of lysates of hTERT-RPE1-YFP-CENPA cells treated with indicated siRNAs and probed with pRB (upper row), α-tubulin (lower row) antibodies. **F.** Quantification of pRB/α-tubulin ratio signal in three independent Western blots as shown in **(E)** and in siCTRL and sipRB cell extracts. Error bars

represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ N=3 **I.** Representative wide field images of hTert-RPE1 after Monastrol release. Cells were either depleted with siCTRL or with sipRB for 72 hours, after cells were treated with 100 uM of Monastrol for 4 hours and finally Monastrol was removed. Cells were stained with anti CENPA (green) and counterstained with DAPI (red) Red arrow indicates the presence of lagging chromosomes. Scale bars=5µm **G.** Dot plot representing single cell mitotic timing, mitotic timing was measured from NEBD to anaphase onset for each cell. Error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ N=4 **H.** Representative wide field images of time-lapse sequence of hTert-RPE1 stained with SiR-DNA and treated with siCTRL or sipRB after 72 hours. T_0= Nuclear envelope breakdown T_1= anaphase onset **L.** Dot plot representing percentage of mitosis containing at least one lagging chromosome per experiment. Cells were treated with 100 uM of Monastrol for 4 hours subsequently Monastrol was washed away and cells fixed in methanol after 40 minutes. Error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ N=3

Retinoblastoma protein partial depletion is not affecting microtubule stability nor Spindle Assembly Checkpoint

The presence of lagging chromosomes is often caused by merotelic kinetochore-microtubule attachments. Merotely can arise as a consequence of different abnormalities: altered microtubule stability, defective kinetochore-microtubule attachment, overexpression of spindle assembly checkpoint proteins or altered DNA condensation (Cimini, 2011). I, therefore, tested these hypotheses to understand how pRB depletion could increase the incidence of lagging chromosomes. The first parameter I tested was microtubule stability.

Altered microtubule stability meaning too stable or unstable microtubules can dramatically influence cell division (Thompson & Compton, 2011). Error correction machinery exerts its role destabilizing the erroneous attachments. Hyper stable microtubules cannot be corrected and may progress to erroneous segregation (Godek et al., 2015). On the other hand, unstable microtubule kinetochore attachments may interfere with the complete kinetochore microtubule occupancy.

Partial binding of kinetochores to microtubules often leads to segregation errors (Bakhoum, Thompson, Manning, & Compton, 2009) (Dudka et al., 2018).

To test microtubule stability, I took advantage of the microtubule dye SiR-tubulin and the potent depolymerizing agent Nocodazole.

SiR-tubulin allows to visualize the spindle structure in live condition and to probe mitotic spindle stability (Magliocca, Petrini, Franchin, & Borghi, 2017). The presence of SiR-Tubulin allowed to visualize and quantify the spindle collapse induced by Nocodazole.

The sensitivity and robustness of the microtubule stability assay was tested by Dudka et al., who detected a minimal perturbation in microtubule stability after depletion of HURP, a stabilizer of kinetochore-microtubules (Dudka, 2019).

I depleted pRB from hTERT-RPE1 cells for 72 hours and later I stained the microtubules with the live-cell imaging dye SiR-tubulin and Nocodazole was applied. Spindle collapse was recorded and the tubulin signal was quantified. siCTRL cells and pRB depleted cells showed the same dynamics in spindle collapse, suggesting that global microtubules stability was unaffected (Fig12 A,B).

Even if global microtubule stability is unaltered, kinetochore microtubule attachment could be impaired. To test this hypothesis, I utilized a kinetochore tracking assay. Dividing cells were recorded during metaphase with high temporal resolution and in 3D. Later, an automatized Matlab code recognized kinetochore pairs and tracked their movements in time and space (Armond, Vladimirou, Mcainsh, & Burroughs, 2016).

Kinetochore pair movements are highly informative in term of kinetochore-microtubule plus end dynamics, microtubule kinetochore attachment and DNA condensation. The code allowed me to extract several parameters like the oscillation period and sister separation.

Sister-kinetochore pairs undergo semi-regular oscillations along the spindle axis reflecting the dynamic instability of the kinetochore-microtubules. By applying an auto-correlation analysis, one

can extract the periodicity of these oscillations, which reflects the propensity of kinetochore/microtubules to switch from polymerization to depolymerization and vice-versa. Proof-of-principle experiments carried out 10 years ago, showed that depletion of microtubule depolymerase at kinetochores lengthens the period of these oscillations, reflecting higher kinetochore-microtubule stability (Armond et al., 2016) (Amaro et al., 2010).

I depleted pRB in hTERT-RPE1-YFP-CENPA and thanks to CENPA YFP signals I imaged the cells during mitosis. Kinetochore pairs oscillations were comparable between siCTRL and pRB depleted cells, indicating that kinetochore microtubule attachment is not affected in the absence of pRB (Fig12 E). Furthermore, this is consistent with the results of the microtubule stability assay (Fig12 A)

The kinetochore tracking assay also provides information about the distribution of inter-kinetochore distance, that can be used as an indirect measure of DNA condensation. Sister kinetochores are pulled apart in a spring-like behaviour, and the inter-kinetochore distance depends on two parameters. First, the pulling forces acting on the kinetochores directly influence the inter-kinetochore distance. The pulling forces depend on microtubule depolymerization of K fibres. Second, the spring constant of the centromeric DNA, defined as K, is a proper feature of the spring. The pulling forces are applied on the centromeric DNA and the spring constant is given by DNA condensation. More condensed DNA reduces interkinetochore distance and vice versa (Bloom, 2010).

I depleted pRB and the Condensin subunit Cap-D3 as a positive control in hTERT-RPE1-YFP-CENPA cells. Condensin depletion resulted in less condensed DNA and therefore higher sister separation (Samoshkin et al., 2009). Cap-D3 is believed to be directly recruited by pRB before mitosis (Longworth, Herr, Ji, & Dyson, 2008) (Amity L. Manning, Longworth, & Dyson, 2010). Cap-D3 depletion resulted in a 1.20 uM inter-kinetochore distance, whereas in control and pRB depleted

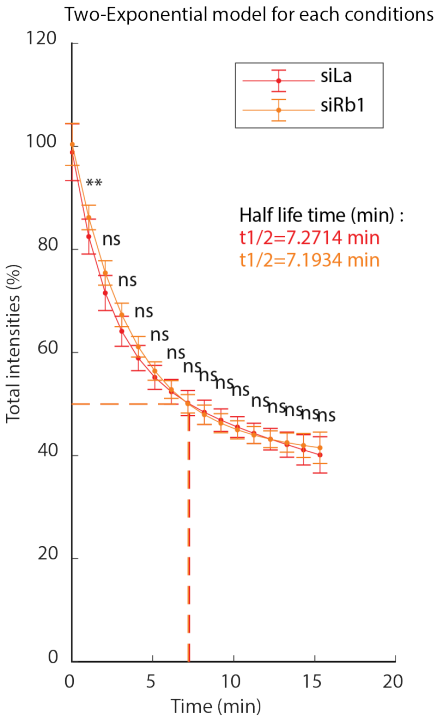
cells the distance was 1 uM (Fig12 C,D). Unaltered interkinetochore distance between siCTRL and sipRB depleted cells suggest that pRB depletion is not affecting DNA condensation.

The third parameter that I tested in pRB depleted cells was Mad2 activity.

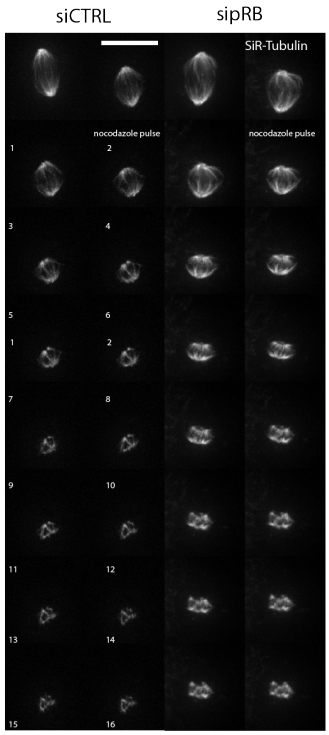
Mad2 belongs to the Spindle Assembly Checkpoint (SAC) that controls the metaphase to anaphase transition. In this transition chromosomes needs to be bipolarly attached and enough tension should be exerted from both poles. In case of unattached kinetochores, MAD2 is recruited and it will prevent mitotic progression, interacting directly with APC/Cdc20 (Musacchio & Salmon, 2007). Mad2 transcription is E2F regulated, therefore pRB absence should cause Mad2 overexpression (Dimova & Dyson, 2005). Moreover, according to previous publications MAD2 overexpression can induce lagging chromosomes (Schvartzman et al., 2011; Sotillo et al., 2007). Sotillo et al. showed that Mad2 overexpression leads to microtubule hyper stabilization through an unknown mechanism and, as previously described, hyper stable microtubules lead to merotelic attachments (Sotillo et al., 2007) (Cimini et al., 2003). I depleted pRB in hTERT-RPE1 cells for 72 hours and later I applied nocodazole to depolymerize microtubules and to form unattached kinetochores. Nocodazole blocked the cells in prometaphase and caused a Mad2 recruitment on the unattached kinetochores (Waters et al., 1998) (Li, Dang, Wood, & Huang, 2017). Finally, the kinetochores were stained with anti Mad2 antibodies. The amount of recruited Mad2 was equivalent between sipRB and control cells (Fig12 F,G). A western blot analysis was performed with the same protocol on pRB depleted cells and control depleted cells. The total Mad2 protein amount was even lower in pRB depleted cells compared to control. (Fig12 H,I)

My data suggest that pRB depletion is not affecting global microtubule stability and DNA condensation. Moreover, I assessed Spindle Assembly Checkpoint activity in pRB depleted cells without finding differences from control cells.

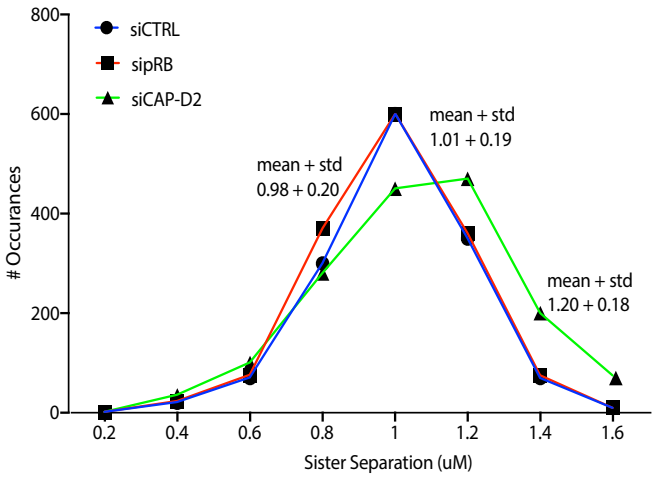
A


Two-Exponential model for each conditions
siLa
siRb1
Half life time (min) :
t1/2=7.2714 min
t1/2=7.1934 min
Total intensities (%)
Time (min)

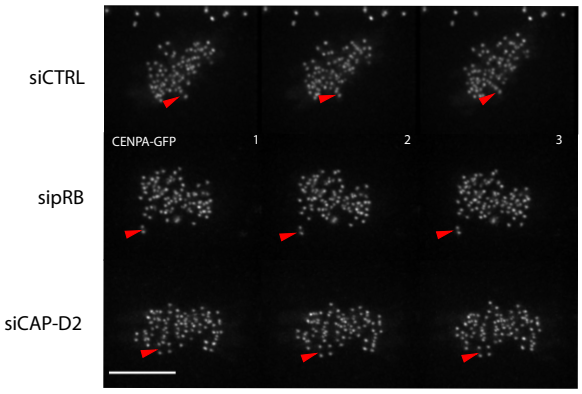
B

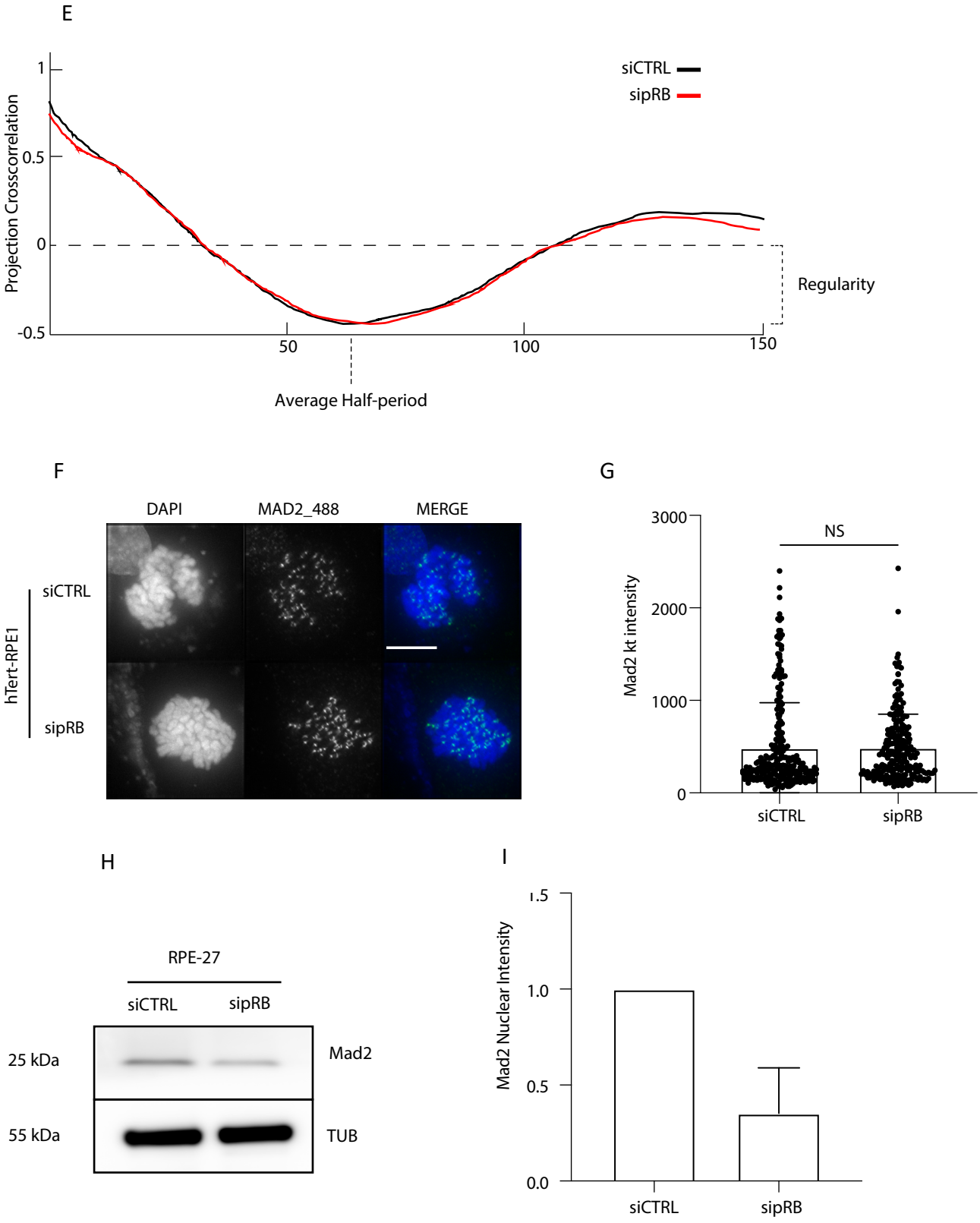

siCTRL
sipRB
SiR-Tubulin
nocodazole pulse
nocodazole pulse

C


siCTRL
sipRB
siCAP-D2
mean + std
0.98 + 0.20
mean + std
1.01 + 0.19
mean + std
1.20 + 0.18
Occurances
Sister Separation (uM)

D


siCTRL
sipRB
siCAP-D2
CENPA-GFP



Retinoblastoma protein depletion is neither affecting microtubules stability nor Spindle Assembly Checkpoint

Figure12 A. Quantification of the spindle intensity over time after a nocodazole (200 nM) pulse, in metaphase hTert-RPE1 cells. Error bars represent standard deviation, statistical significance was determined by Anova repeated test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p <$

0.0001 N=3 **B**. Representative time-lapse images of hTert-RPE1 cells stained with SiR-Tubulin and treated with a 200 nM of nocodazole pulse at t = 0 after indicated siRNAs. Scale bars=10µm **C.** Distribution of inter-KT distances (CENPA-CENPA) in hTERT-RPE1-YFP-CENPA cells treated with siCTRL (blue), sipRB (red) or siCAP-D3 (green), N = 3; n = 600-KT pairs; values are means and error bars represent standard deviation, statistical significance was determined by two-tailed Mann–Whitney U test and ns, p > 0.05, ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001, ∗∗∗∗p < 0.0001 N=3 **D.** Representative time-lapse images of hTERT-RPE1-YFP-CENPA cells treated with different siRNAs after 72 hours. Red arrow indicates sister kinetochores over time. Scale bars=5µm **E.** Autocorrelation curves of sister-KT pairs of hTERT-RPE1-YFP-CENPA cells treated with sipRB (black) or siCTRL (red). n = 700 sister-KT pairs N = 3
F. Representative wide field images of hTert-RPE1 stained with anti Mad2 (green) antibody and counterstained with DAPI (blue) Cells were treated with siCTRL (upper row) or sipRB (lower row) for 72 hours and finally with 200 nM of Nocodazole for 1 hour. Scale bars=5µm **G.** Quantification of Mad2 signal in the nucleus of cells stained with of anti-Mad2 antibody after background subtraction in images as shown in **(F)** error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, p > 0.05, ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001, ∗∗∗∗p < 0.0001. N=3 **H.** Western blot against lysates of hTert-RPE1 cells treated with indicated siRNAs and probed with Mad2 (upper row), α-tubulin (lower row). **I.** Quantification of Mad2/α-tubulin ratio signal in three independent Western blots as shown in **(H)** and in siCTRL and sipRB cell extracts. Error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, p > 0.05, ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001, ∗∗∗∗p < 0.0001 N=3

Retinoblastoma protein partial depletion reduces CENPA protein abundance but is not affecting kinetochore structure

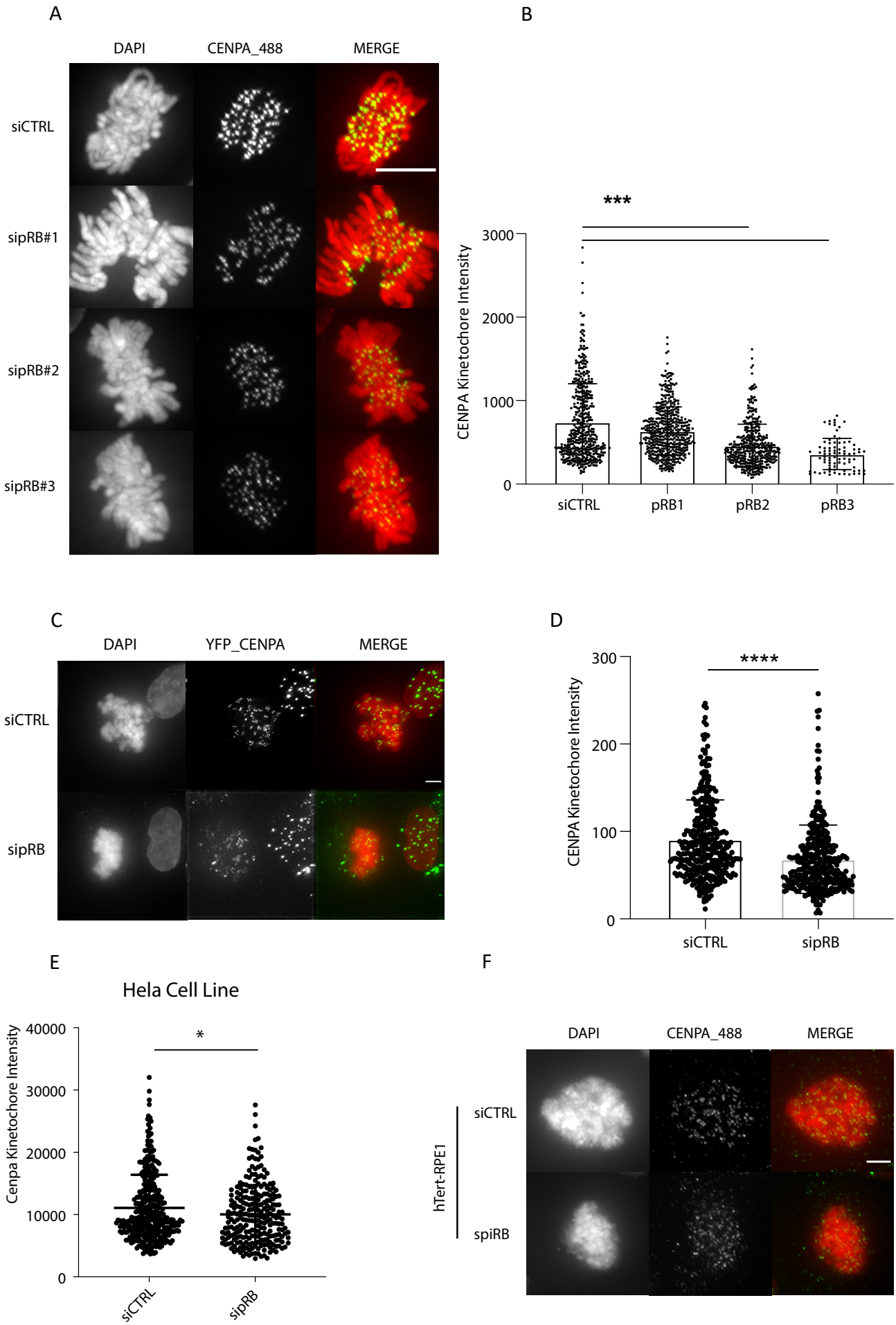
The results collected so far suggested that pRB depletion increases mitotic timing and lagging chromosomes, while not affecting microtubule stability, DNA condensation or Spindle Assembly Checkpoint. Another element responsible for lagging chromosomes is the kinetochore. To understand how pRB absence influences the presence of lagging chromosomes, I focused on the kinetochore structure.

The kinetochore is a protein structure that allows the connection between centromeres and microtubules and is assembled on the modified histone protein CENP-A. CENP-A nucleosome represents the platform on which the entire kinetochore structure is built (Valdivia, Hamdouch,

Ortiz, & Astola, 2009). For live-cell imaging movies I took advantage of hTERT-RPE1-YFP-CENPA, and interestingly when I depleted pRB I noticed a YFP-CENPA signal reduction.

This prompted me to investigate further in details the kinetochore structure and specifically CENPA. I depleted pRB in hTERT-RPE1 cells for 72 hours, later I blocked the cells in mitosis with Nocodazole and finally I stained them with anti CENP-A antibodies. pRB depleted cells showed a 3 fold reduction in CENP-A abundance compared to control-transfected cells (Fig13.1 A,B) To exclude potential off target effects I used three different pRB siRNAs oligos (Fig13.1 A,B) finding the same trend with all oligos. Interestingly, the observed CENPA reduction in mitosis correlates with depletion efficiency exerted by oligos on pRB protein abundance (Fig11.1 A). The same result was observed in hTERTRPE1-YFP-CENPA excluding the possibility of cell line specific off-target effect (Fig13.1 C,D). To further confirm that the observed CENPA reduction is pRB mediated I depleted it in HeLa cells. HeLa cells are pRB positive but not proficient, because they contain the E7 subunit of the HPV that prevents pRB activation and causes its degradation (Dick & Dyson, 2002) (Fischer et al., 2017). According to my hypothesis, pRB depletion in Hela cells should not lead to any significant change in the phenotype.

pRB depletion in HeLa cells only mildly reduced CENPA levels at the kinetochore in mitosis, confirming that CENPA reduction observed in pRB depleted cells is pRB mediated. (Fig13.1 E,F) In conclusion, pRB depletion influences CENPA abundance at the kinetochore.


A
DAPI
CENPA_488
MERGE
siCTRL
sipRB#1
sipRB#2
sipRB#3
B
***
CENPA Kinetochore Intensity
3000
2000
1000
0
siCTRL
pRB1
pRB2
pRB3
C
DAPI
YFP_CENPA
MERGE
siCTRL
sipRB
D
****
CENPA Kinetochore Intensity
300
200
100
0
siCTRL
sipRB
E
Hela Cell Line
*
Cenpa Kinetochore Intensity
40000
30000
20000
10000
0
siCTRL
sipRB
F
DAPI
CENPA_488
MERGE
hTert-RPE1
siCTRL
spiRB

Retinoblastoma protein depletion reduces CENPA protein abundance

Figure13.1 A. Representative wide field images of hTert-RPE1 stained with anti CENPA (green) antibody and counterstained with DAPI (blue) Cells were treated with siCTRL (upper row) or different sipRB oligos (lower row) for 72 hours and finally with 200 nM of Nocodazole for 1 hour. Scale bars=5µm **B.** Dot plot quantification CENPA signal in the nucleus of cells stained with of anti-CENPA antibody after background subtraction in images as shown in **(A)** error bars represent standard deviation, statistical significance was determined by Anova repeated test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **C.** Representative wide field images of hTERT-RPE1-YFP-CENPA stained with anti CENPA (green) antibody and counterstained with DAPI (blue) Cells were treated with siCTRL (upper row) or sipRB (lower row) for 72 hours and finally with 200 nM of Nocodazole for 1 hour. Scale bars=5µm **D.** Dot plot quantification of CENPA signal in the nucleus of cells stained with of CENPA signal in the nucleus of cells stained with of anti-CENPA antibody after background subtraction in images as shown in **(C)** error bars represent standard deviation, statistical significance was determined by Anova repeated test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **E.** Dot plot quantification of CENPA signal in the nucleus of cells stained with of anti-CENPA antibody after background subtraction in Hela-K cells. Error bars represent standard deviation, statistical significance was determined by T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **F.** Quantification of CENPA signal in the nucleus of cells stained with anti-CENPA antibody after background subtraction in Hela-K. Error bars represent standard deviation, statistical significance was determined by unpaired T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3

CENP-A reduction prompted me to investigate other kinetochore proteins, to test whether CENP-A reduction would affect kinetochore structure and overall function. pRB depleted cells were stained with several antibodies against kinetochore proteins: CENP-C from the inner kinetochore, HEC1 from the outer kinetochore and CENP-F from the corona. CENP-C is a direct CENPA interactor and recently its indispensable role for kinetochore formation and function was shown (Hoffmann et al., 2016). Hec1 which is part of the Ndc80 complex, is involved in the kinetochore microtubule attachments, in the spindle checkpoint and is an AuroraB target. Finally, CENPF belongs to the corona that is a fibrous kinetochore domain, involved in spindle checkpoint, microtubule anchoring and regulation of chromosome behaviour (Musacchio & Desai, 2017). Testing three different

candidates from three different portions of the kinetochore helped me to evaluate kinetochore architecture and functionality.

I observed a reduction in protein abundance of the three candidates in sipRB depleted cells compared to control. (Fig13.2 A,B,C,D,E,F). Nonetheless, sipRB depletion caused only a 14% and 11% reduction in protein abundance of HEC1 and CENPF respectively. Even though, this reduction is statistically significant, it is not comparable to the 50% reduction observed in CENPA protein abundance in sipRB depleted cells.

These results suggest that a partial CENPA reduction is sufficient to establish a functional kinetochore and is consistent with previous findings (Hoffmann et al., 2016). Furthermore, the unaffected kinetochore structure is consistent with the previous results on kinetochore microtubule attachment. Altered kinetochore structure would affect metaphase kinetochore oscillation but as previously described pRB depleted cells present normal oscillations (Fig12 E).

My results indicate that pRB depletion reduces CENPA kinetochore levels in mitosis without affecting kinetochore structure or function. The CENPA reduction observed in mitosis prompted me to investigate when and how this reduction happened.

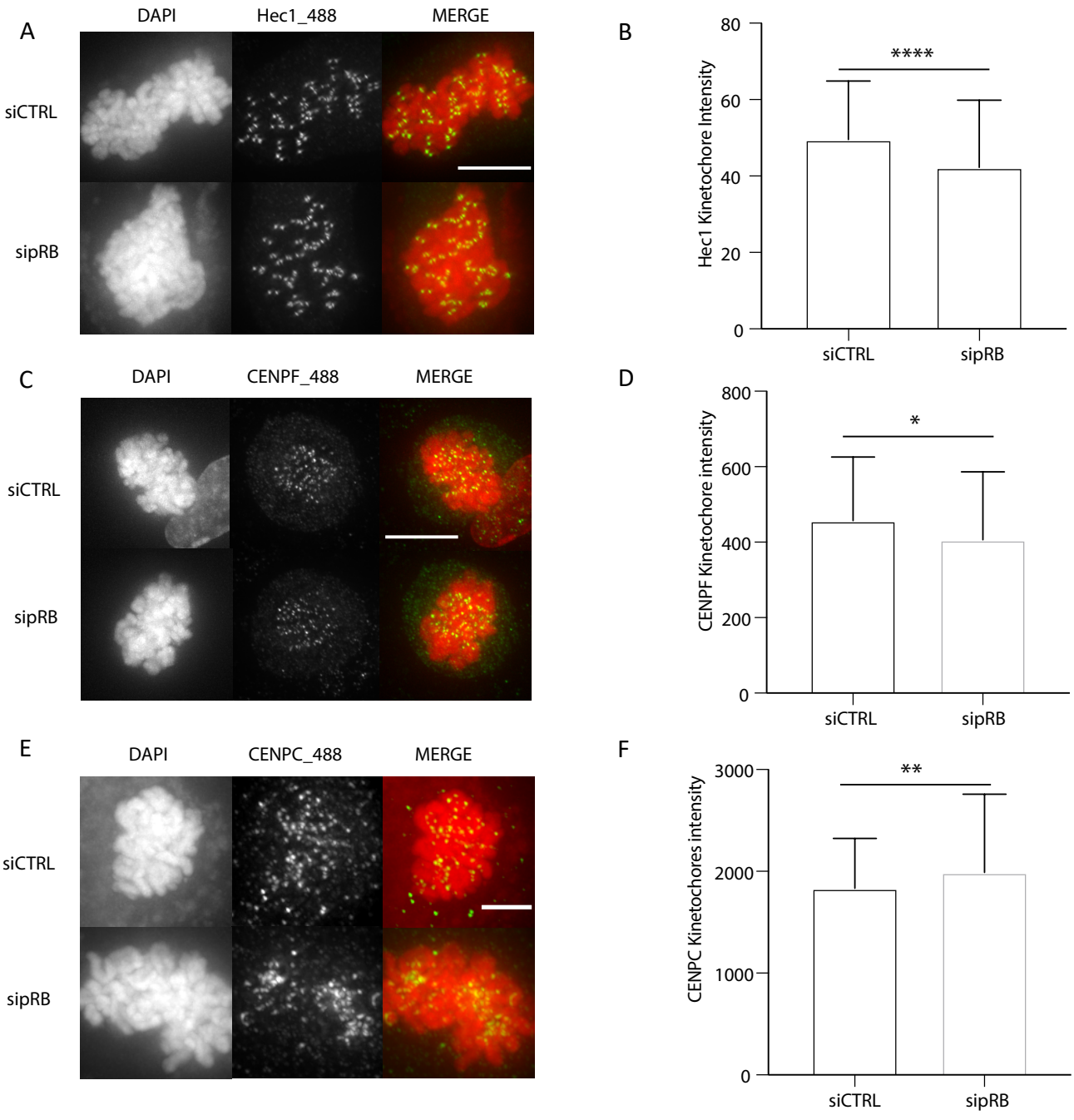
I took advantage of the hTERT-RPE1-YFP-CENPA cells to monitor live CENPA signal. I depleted pRB and thanks to live cell imaging I observed a CENPA reduction after Nuclear Envelope Breakdown compared to control cells. (Fig13.2 G)

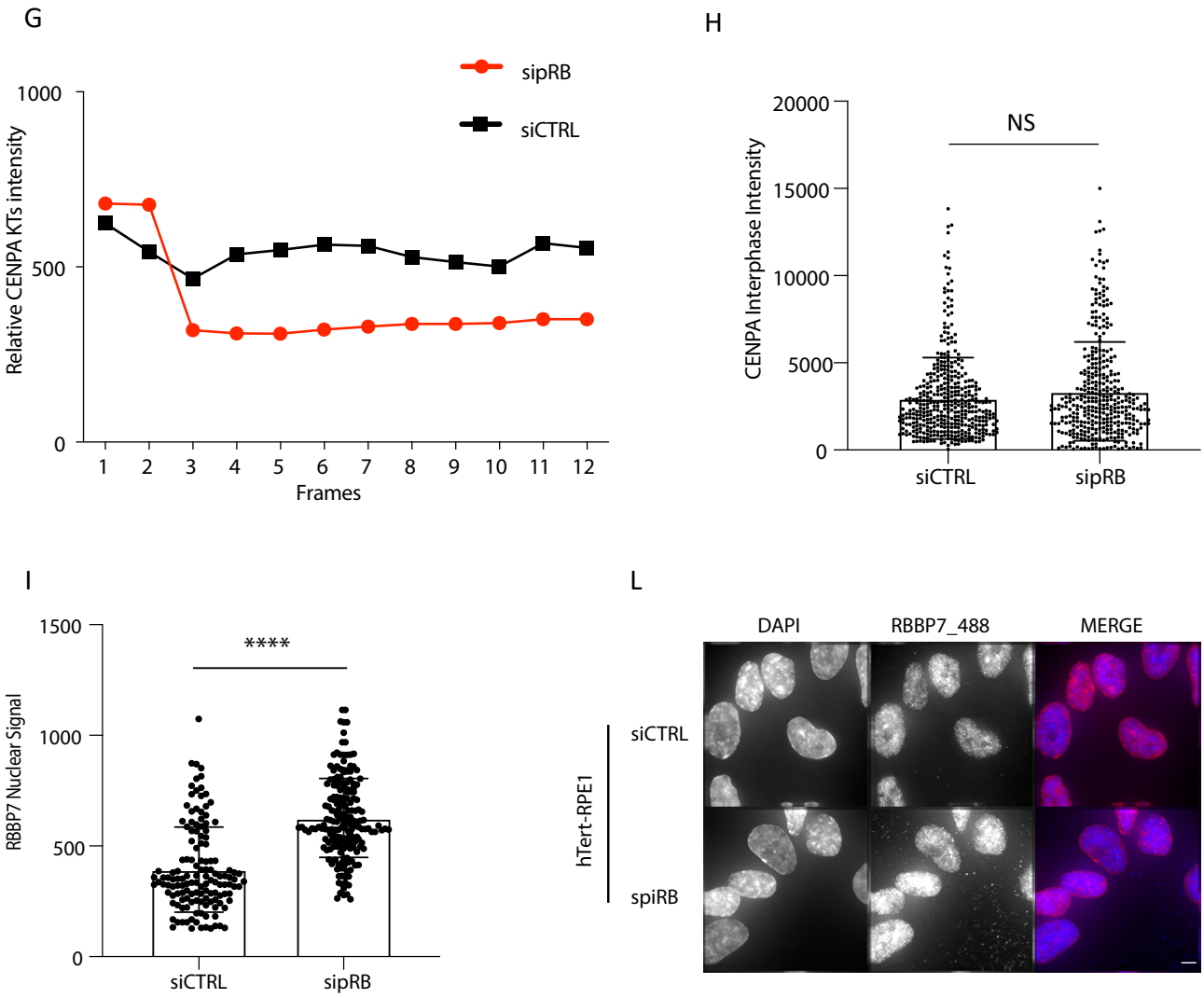
Later, I quantified CENPA levels in siCTLR and sipRB depleted cells in interphase cells finding comparable levels (Fig13.2 H). This observation indicated that the CENPA reduction observed in pRB depleted cells is compensated through the cell cycle by unknown mechanisms.

The hypothesis of a compensatory mechanism acting during interphase prompted me to measure the protein expression level of Rbbp7.

Rbbp7 is a protein involved in CENPA deposition in late mitosis (Mouysset et al., 2015).

Interestingly, pRB depleted cells showed an increase of 77% Rbbp7 protein abundance compared to siCTRL (Fig13.2 I,L). Rbbp7 overexpression may recruit more CENPA to compensate the loss in mitosis. This preliminary finding needs to be addressed in future experiments.





Retinoblastoma protein depletion reduces CENPA protein abundance but is not affecting kinetochore structure

Figure13.2 A. Representative wide field images of hTert-RPE1 stained with anti Hec1 (green) antibody and counterstained with DAPI (blue) Cells were treated with siCTRL (upper row) or sipRB (lower row) for 72 hours and finally with 200 nM of Nocodazole for 1 hour. Scale bars=5µm **B.** Quantification of Hec1 signal in the nucleus of cells stained with anti-Hec1 antibody after background subtraction in images as shown in **(A)** error bars represent standard deviation, statistical significance was determined by T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3

C. Representative wide field images of hTert-RPE1 stained with anti CENPF (green) antibody and counterstained with DAPI (blue) Cells were treated with siCTRL (upper row) or sipRB (lower row) for 72 hours and finally with 200 nM of Nocodazole for 1 hour. Scale bars=5µm **D.** Quantification of CENPF signal in the nucleus of cells stained with of anti-CENPF antibody after background subtraction in images as shown in **(C)** error bars represent standard deviation, statistical significance was determined by T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3

E. Representative wide field images of hTert-RPE1 stained with anti CENPC (green) antibody and counterstained with DAPI (blue) Cells were treated with siCTRL (upper row) or sipRB (lower row) for 72 hours and finally with 200 nM of Nocodazole for 1 hour. Scale bars=5µm **F.** Quantification of CENPC signal in the nucleus of cells stained with of anti-CENPC antibody after background subtraction in images as shown in **(E)** error bars represent standard deviation, statistical significance was determined by T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **G.** CENPA kinetochore signal over time in of hTERT-RPE1-YFP-CENPA. Cells were imaged for 4 hours and CENPA YFP signal was quantified. N=1 **H.** Quantification of CENPA signal in the nucleus of cells stained with of anti-CENPA antibody after background subtraction in hTert-RPE1 treated with sipRB or siCTRL for 72h and collected in interphase. Error bars represent standard deviation, statistical significance was determined by unpaired T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **I.** Quantification of Rbbp7 signal in the nucleus of cells stained with anti-Rbbp7 antibody after background subtraction in hTert-RPE1. Error bars represent standard deviation, statistical significance was determined by unpaired T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **L.** Representative wide field images of hTert-RPE1 stained with anti RBbp7 (green) antibody and counterstained with DAPI (blue) Cells were treated with siCTRL (upper row) or sipRB (lower row) for 72 hours and finally with 200 nM of Nocodazole for 1 hour. Scale bars=5µM

Retinoblastoma protein partial depletion hyperactivates AuroraB kinases

The absence of kinetochore abnormalities and longer mitotic timing measured in Monastrol release experiment, (Fig13.2 A,B,C,D,E,F) (Fig14 A,B) prompted me to investigate the error correction machinery. This machinery is involved in the correction of erroneous attachments in prophase and is coordinated by the protein kinase AuroraB (M. Lampson & Grishchuk, 2017) (Waal, Hengeveld, Horst, & Lens, 2012).

AuroraB is localized at the centromere and acts mostly through its positioning. During metaphase, the chromosomes are aligned along the metaphase plate and are subjected to tension from both poles. Correct kinetochore microtubule attachments generate enough tension and kinetochores are pulled away from the metaphase plate, conversely incorrect attachments fail to generate enough tension. AuroraB senses kinetochores within its activity zone. By the ensuing

phosphorylation, the weak attachments are destabilized. AuroraB has a plethora of substrates, some are involved in DNA condensation like histone H3, other are fundamental for the microtubule-capture activity like DSN1, Hec1 and finally, some are involved in the depolymerization activity like MCAK (Carmena et al., 2012).

Through the direct phosphorylation of kinetochore targets, AuroraB reduces the affinity for microtubules, which disfavors the establishment of kinetochore microtubule attachments.

Considering the crucial role played by AuroraB in error correction, I wanted to test its activity in pRB depleted cells. To this end, I took advantage of Monastrol that prevents centrosomes separation and enriches the number of merotelic attachments, causing AuroraB activation.

I treated hTERT-RPE1 cells with sipRB and siCTRL for 72 hours, later I blocked the cells in Monastrol. Finally, I stained for AuroraB and the AuroraB phosphorylated targets DSN1 and H3. Interestingly, AuroraB kinetochore levels were modestly affected (12% increases) (Fig14 E,F) in pRB depleted cells compared to siCTRL, whereas AuroraB targets were hyperphosphorylated. The pDsn1 signal was 68% higher in pRB depleted cells compared to control. (Fig14 C,D)

This result suggests that pRB depletion increases AuroraB activity during mitosis.

Increased AuroraB activity could explain the presence of lagging chromosomes consistent with previous publications. AuroraB hyperactivation hyper-phosphorylate its substrates preventing proper error correction resulting in segregation errors (Ricke, Jeganathan, & Deursen, 2011) (Aurora, Ricke, & Deursen, 2011). My data suggested that pRB depletion causes lagging chromosomes and AuroraB hyperactivity. I hypothesized that the lagging chromosomes observed in pRB depleted cells were AuroraB mediated. To test this hypothesis, I pharmacologically inhibited AuroraB activity.

If my hypothesis was correct, AuroraB inhibition should reduce the number of lagging chromosomes in pRB depleted cells.

First, I established the right dose of AuroraB inhibitor to reduce the augmented AuroraB activity observed in pRB depleted cells. I treated pRB depleted cells and control-depleted cells with 0.2 uM and 0.7 uM of ZM1 (AuroraB inhibitor) and I stained for phospho-histone 3 (pH-S28) to assess AuroraB activity. I found that 0.2 uM ZM1 is sufficient to rescue the AuroraB hyperactivation induced by pRB depletion. (Fig14 G)

Second, I wanted to assess the number of lagging chromosomes in presence of AuroraB inhibition in pRB depleted cells. If the number of lagging chromosomes observed in pRB depleted cells depends on AuroraB activity, I would expect that AuroraB inhibition would fully or partially decrease it. I performed Monastrol release assay in pRB depleted cells and control depleted cells. Later, cells were treated with 0.2uM of ZM1 for 1 hour and finally, cells were fixed and stained for CENPA to visualize lagging chromosomes in anaphase.

DMSO treated siCTRL cells displayed lagging chromosomes in 11% of the mitosis conversely ZM1 treated cells showed lagging chromosomes in 45% of mitosis. This finding is consistent with the literature and confirms that inhibition of AuroraB activity leads to lagging chromosomes (Trakala, Fernández-miranda, Castro, Heeschen, & Malumbres, 2013). pRB depleted cells treated with DMSO showed lagging chromosomes in 19% of mitosis, confirming my previous data, finally, ZM1 treated pRB depleted cells show lagging chromosomes in 33% of mitosis. (Fig14 H) Considering that both pRB depletion and AuroraB inhibition leads to lagging chromosomes, and assuming that both effects are independent I would expect a higher number of lagging chromosomes. However, my results showed pRB depleted cells treated with ZM1 presents lagging chromosomes only in 33% of mitosis indicating that AuroraB partially influence the presence of lagging chromosomes. I concluded that pRB depletion hyperactivates AuroraB increasing the number of lagging chromosomes and ZM1 partially rescue the phenotype.

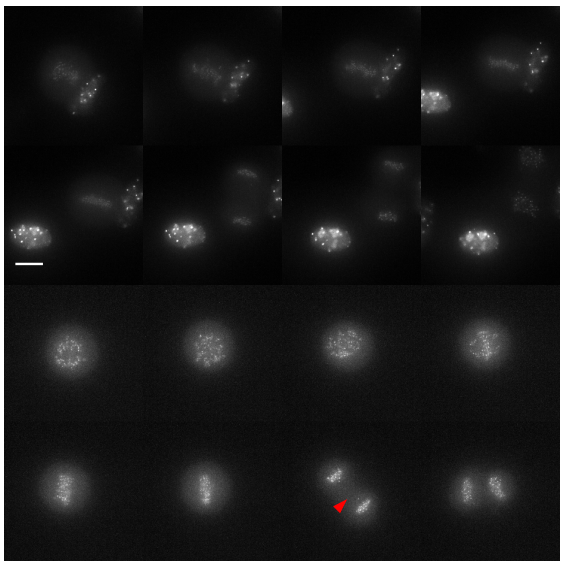
Furthermore, my data are consistent with other publications that showed that AuroraB activity alteration leads to lagging chromosomes (Ricke et al., 2011) (Muñoz-barrera & Monje-casas, 2014) (Huang, Lampson, Efimov, & Yen, 2018).

Finally, I wanted to test the relationship between pRB depletion, CENPA reduction and increased AuroraB activity. pRB depletion increases AuroraB activity and simultaneously decreases CENPA kinetochore abundance. If CENPA reduction is mediated by AuroraB activity, a pharmacological inhibition should rescue it.

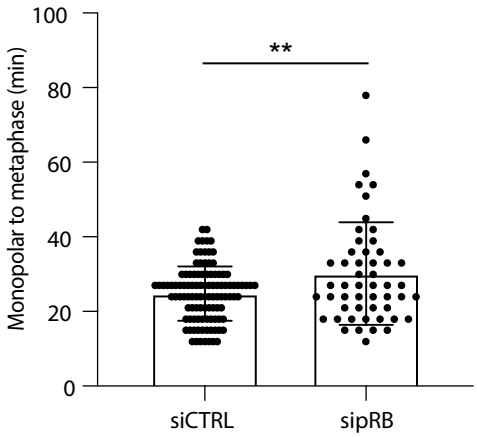
I depleted pRB in hTERT-RPE1-YFP-CENPA cells for 72 hours, subsequently I applied Monastrol for 4 hours and finally released the cells into different doses of ZM1 (0.2 and 0.7 uM) and cells were stained for CENPA. CENPA kinetochore level were consistently lower in pRB depleted cells, circa 60% reduction compared to siCTRL. Surprisingly, CENPA kinetochore levels were partially increased when ZM1 was applied (both doses), siCTRL cells showed 18% increases compared to DMSO whereas sipRB depleted cells showed 24% increases compared to DMSO (Fig14 I).

Why AuroraB inhibition increased CENPA abundance at kinetochore in both siCTRL and sipRB is still unknown. My data suggest that pRB depletion influences centromere biology in at least two distinct ways: first, it increases AuroraB activity and second it reduces CENPA level at kinetochore in mitosis.

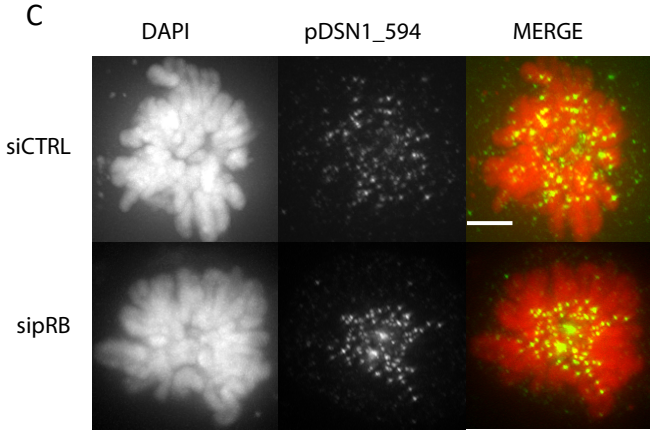
A


siCTRL
sipRB

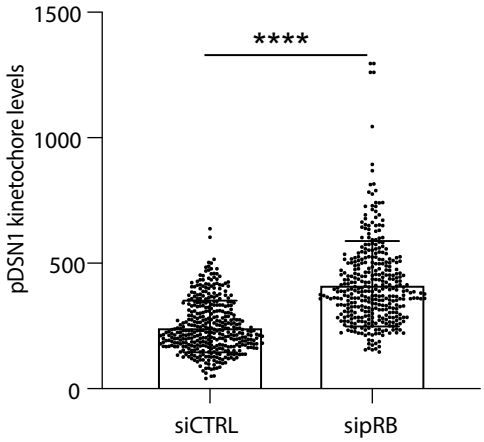
B


Monopolar to metaphase (min)
100
80
60
40
20
0
**
siCTRL
sipRB

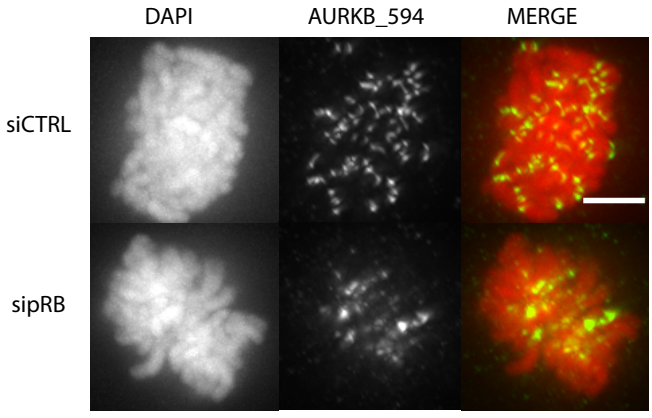
C


DAPI
pDSN1_594
MERGE
siCTRL
sipRB

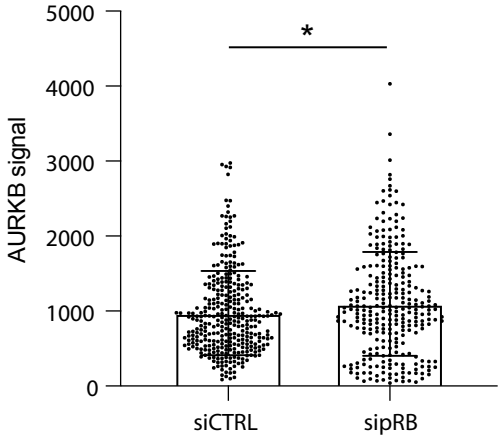
D

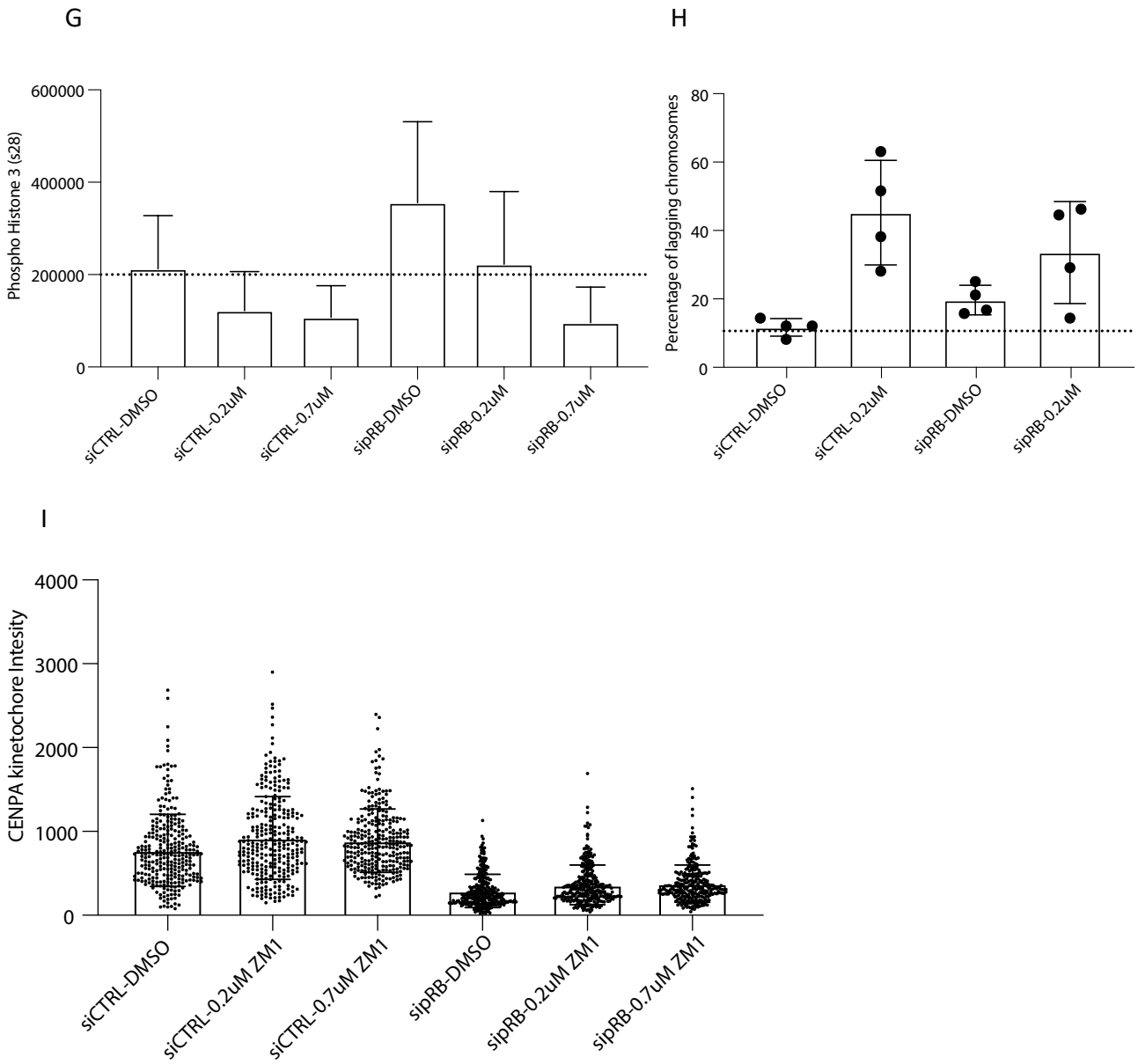

pDSN1 kinetochore levels
1500
1000
500
0
****
siCTRL
sipRB

E


DAPI
AURKB_594
MERGE
siCTRL
sipRB

F


AURKB signal
5000
4000
3000
2000
1000
0
*
siCTRL
sipRB



Retinoblastoma protein partial depletion hyperactivate AuroraB kinases

Figure14 A. Representative time-lapse sequence of hTERT-RPE1-YFP-CENPA cells treated with siCTRL or sipRB after 72 hours subsequently treated with 100 uM of Monastrol for 4 hours and finally released. T_0= monopolar configuration T_1= anaphase onset. Red arrow indicates lagging chromosomes. Scale bars=5µm **B.** Dot plot representing single cell mitotic timing, mitotic timing was measured from monopolar configuration to anaphase onset for each cell and pulled together. Error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ N=4 **C.** Representative wide field images of hTert-RPE1 stained with anti-pDSN1 (green) antibody and counterstained with DAPI (blue) Cells were treated with siCTRL (upper row) or different sipRB oligos (lower row) for 72 hours and finally with 100 uM of Monastrol for 1 hour. Scale bars=5µm **D.** Dot plot quantification of pDSN1 signal in the nucleus of cells stained with of anti-pDSN1 antibody after background subtraction in images as shown in **(C)** error bars represent standard deviation, statistical significance was determined by T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3

E. Representative wide field images of hTert-RPE1 stained with anti-AuroraB (green) antibody and counterstained with DAPI (blue) Cells were treated with siCTRL (upper row) or different sipRB oligos (lower row) for 72 hours and finally with 100 uM of Monastrol for 1 hour. Scale bars=5µm **F.** Dot plot quantification of AuroraB signal in the nucleus of cells stained with of anti-AuroraB antibody after background subtraction in images as shown in **(E)** error bars represent standard deviation, statistical significance was determined by T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **G.** Absolute quantification of pH3(S28) intensity in cells treated first with siCTRL or sipRB for 72 hours later with 100 uM of Monastrol and different concentration of ZM1 (0.2 uM or 0.7uM) for 4 hours. **H.** Dot plot representing percentage of mitosis containing at least one lagging chromosome per experiment. Cells were transfected with siCTRL or sipRB, later treated with 100 uM of Monastrol and 0.2uM of ZM1 or DMSO for 4 hours subsequently Monastrol was washed away and cells fixed in methanol after 40 minutes. Error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ N=4 **I.** Quantification of CENPA signal in the nucleus of cells stained with of anti-CENPA antibody after background subtraction in hTert-RPE1 treated with sipRB or siCTRL for 72h and collected at different time points. Error bars represent standard deviation, statistical significance was determined by unpaired T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3

Retinoblastoma protein knock out cells show different behaviour compared to pRB partial depletion

pRB partial depletion increases AuroraB activity and the number of lagging chromosomes in parallel it reduces CENPA abundance at kinetochore. To understand the long-term consequences of this phenotypes I established a Crispr Cas9 pRB knock out cell line.

I infected an inducible Cas9hTert-RPE1 cell line (kind gift of Ian Cheeseman) (McKinley & Cheeseman, 2017) with 2 sgRNAs against RB1 exon 1 and exon 7. Later, thanks to doxycycline I activated the Cas9 doxycycline-inducible promoter and validated the knockout. Finally, single stable clones were sorted and selected. The selection of clone was based on the viability and on pRB protein level. Single clones were cultured in doxycycline for weeks and periodically pRB protein

abundance was assessed by immunofluorescence and western blot. (Fig15 A,B,C), Finally, a single clone was selected and used for all the experiments later described. (Fig15 A,B,C),

RPE-Cas9i-pRB-KO cells were treated with Monastrol and the number of lagging chromosomes was assessed. Interestingly, the number of lagging chromosomes in the RPE-Cas9i-pRB-KO cell line was lower than the parental cell line 6% vs 10% (Fig15 D). Surprisingly, these results were in contrast with the results previously obtained with partial pRB depletion. This discrepancy may have different explanations: first, the lagging chromosomes observed in pRB depleted cells may be an off-target effect.

Second, pRB depletion and full knockout may have different impacts and phenotypes, the relationship between pRB reduction and lagging may not be linear.

Third, the pRB knock out cell line may have acquired adaptive mutations that reduces the lagging chromosomes observed in pRB depleted cells (El-brolosy & Stainier, 2017).

To exclude off-target effects, I depleted pRB from the knockout cell line. If the lagging chromosomes observed in pRB depleted cells were caused by pRB, then pRB depletion in the knockout cell line should not induce any phenotype. Conversely, if the siRNA against pRB has an off-target effect I should observe lagging chromosomes also in the pRB knockout cell line.

pRB was depleted from RPE-Cas9i-pRB-KO cells and I assessed the number of lagging chromosomes in the Monastrol release assay. pRB depletion in the parental cell line induced lagging chromosomes in 20% of mitosis, conversely pRB depletion in RPE-Cas9i-pRB-KO induces lagging chromosomes only in 10% of mitosis (Fig15 E). This finding confirms that the observed lagging chromosomes in pRB depleted cells were caused by the depletion of pRB.

The second phenotype observed in pRB depleted cells was CENPA reduction at kinetochore.

I tested CENPA kinetochore levels in RPE-Cas9i-pRB-KO and in the parental cell line finding no difference in protein abundance (Fig15 F). This result showed that pRB full knockout does not influence CENPA kinetochore abundance.

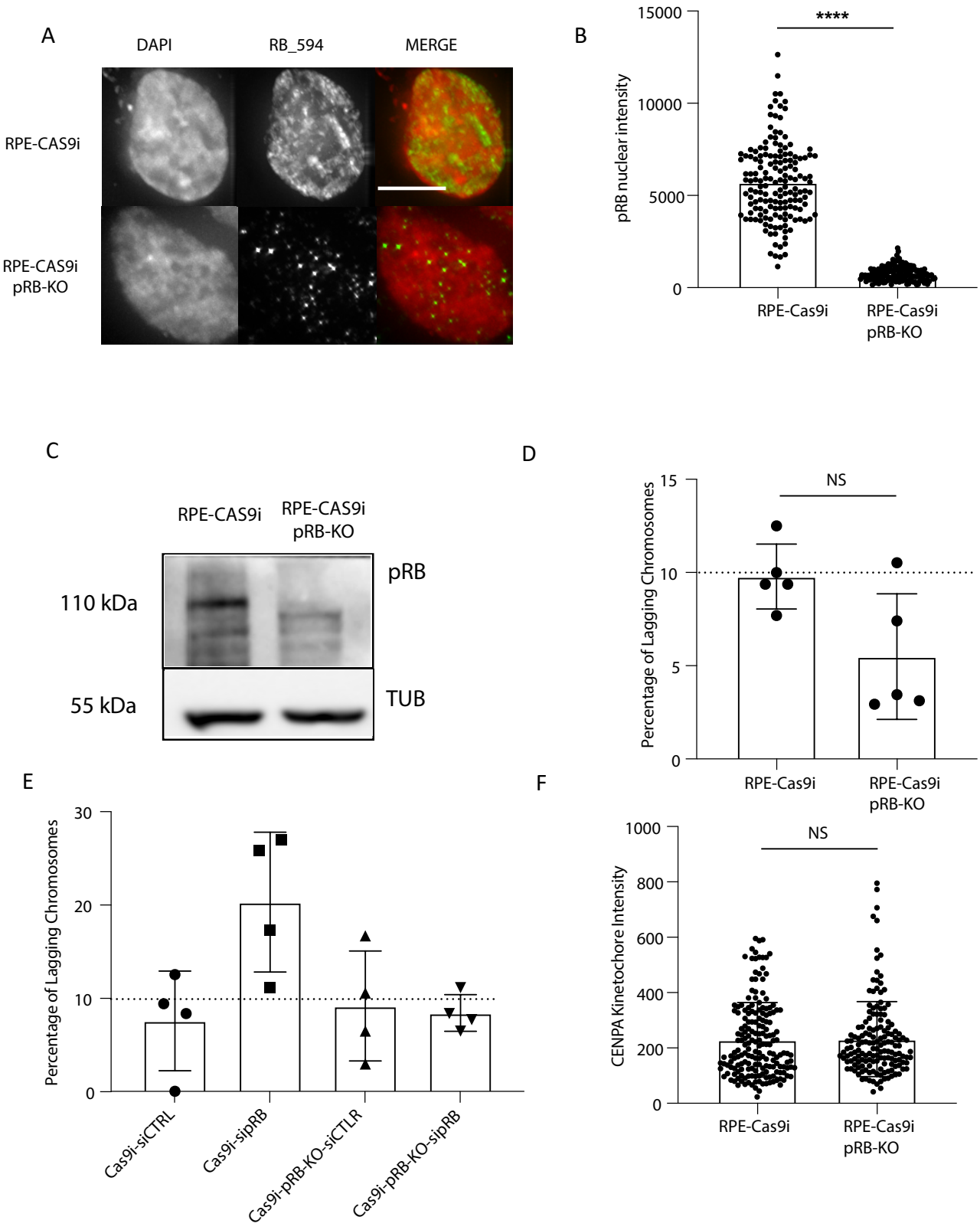
The third phenotype observed in pRB partial depletion was AuroraB hyperactivation. To fully characterize RPE-Cas9i-pRB-KO, AuroraB activity was assessed. AuroraB activity was evaluated through the phosphorylation of histone 3, RPE-Cas9i-pRB-KO showed lower phosphorylation compared to the parental cell line. (Fig15 G). This result suggested that pRB full knockout does not hyper activate AuroraB.

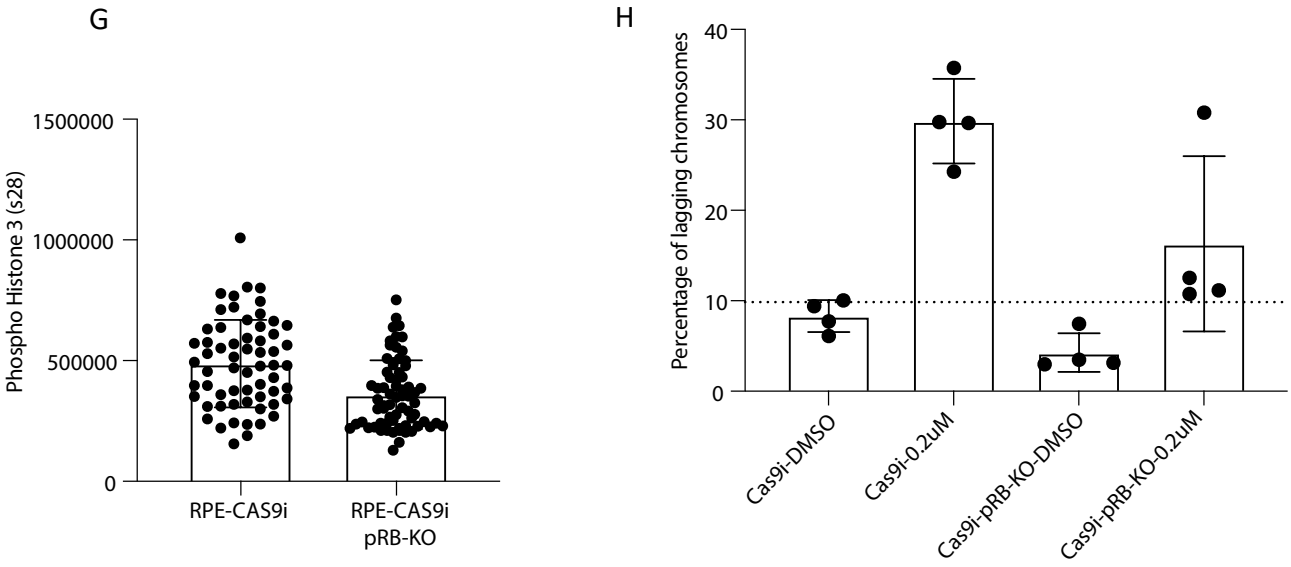
Finally, I evaluated the effect of AuroraB inhibition in RPE-Cas9i-pRB-KO.

I treated RPE-Cas9i-pRB-KO and parental cells either with DMSO or 0.2 uM of ZM1.

DMSO-treated parental cells showed 10% of mitotic cells whereas ZM1 treated showed 30% of lagging chromosomes. RPE-Cas9i-pRB-KO DMSO treated showed 5% of lagging chromosomes, while ZM1 treated 15% (Fig15 H). AuroraB inhibition caused a 3-fold increase in the number of lagging chromosomes in both RPE-Cas9i-pRB-KO and parental cells. Notably, AuroraB inhibition in pRB depleted caused only a 1.7-fold increase in lagging chromosomes. These data indicate that pRB depleted cells are less sensitive to AuroraB inhibition due to its hyperactivation. On the contrary, pRB full knock out does not influence AuroraB activity and therefore its inhibition causes a 3-fold increase in lagging chromosomes.

In conclusion, pRB partial depletion and full knockout showed different phenotypes. pRB knockout cells presented a low number of lagging chromosomes, normal AuroraB activity and normal CENPA kinetochore abundance. Furthermore, my data suggest that pRB partial depletion is more detrimental in term of chromosomal stability compared to pRB full knockout. The discrepancy observed between partial depletion and full knockout could be attributed to unknown adaptive mechanisms rather than off-target effects.


A
DAPI
RB_594
MERGE
RPE-CAS9i
RPE-CAS9i
pRB-KO
B
15000
10000
5000
0
pRB nuclear intensity
****
RPE-Cas9i
RPE-Cas9i
pRB-KO
C
RPE-CAS9i
RPE-CAS9i
pRB-KO
110 kDa
pRB
55 kDa
TUB
D
15
10
5
0
Percentage of Lagging Chromosomes
NS
RPE-Cas9i
RPE-Cas9i
pRB-KO
E
30
20
10
0
Percentage of Lagging Chromosomes
Cas9i-siCTRL
Cas9i-sipRB
Cas9i-pRB-KO-siCTLR
Cas9i-pRB-KO-sipRB
F
1000
800
600
400
200
0
CENPA Kinetochore Intensity
NS
RPE-Cas9i
RPE-Cas9i
pRB-KO



Retinoblastoma protein knock out cells show different behaviour compared to pRB depletion

Figure15 A. Representative wide field images of hTert-RPE1-Cas9i (upper row) and hTert-RPE1-Cas9i-pRB-KO (lower row) stained with anti pRB (green) antibody and counterstained with DAPI (red). Scale bars=5µm **B.** Quantification of pRB signal in the nucleus of cells stained with anti-pRB antibody after background subtraction in images as shown in **(A)** error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **C.** Representative western blot against lysates of hTert-RPE1-Cas9i (left column) and hTert-RPE1-Cas9i-pRB-KO (right column) probed with pRB (upper row), α-tubulin (lower row) antibodies. **D.** Dot plot representing percentage of mitosis containing at least one lagging chromosome per experiment. hTert-RPE1-Cas9i and hTert-RPE1-Cas9i-pRB-KO were treated with 100 uM of Monastrol for 4 hours subsequently Monastrol was washed away and cells fixed in methanol after 40 minutes. Error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ N=5 **E.** Dot plot quantification of pH3(S28) signal in the nucleus of cells stained with anti-pH3(S28) antibody after background subtraction in hTert-RPE1-Cas9i and hTert-RPE1-Cas9i-pRB-KO cells treated with 100uM of Monastrol for 4 hours. Error bars represent standard deviation, statistical significance was determined by T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=2

F. Dot plot quantification of CENPA signal in the nucleus of cells stained with anti-CENPA antibody after background subtraction in hTert-RPE1-Cas9i and hTert-RPE1-Cas9i-pRB-KO cells. Error bars represent standard deviation, statistical significance was determined by T test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$. N=3 **G.** Dot plot representing percentage of mitosis containing at least one lagging chromosome per experiment. hTert-RPE1-Cas9i and hTert-RPE1-Cas9i-pRB-KO were treated with siCTRL or sipRB for 72 hours subsequently treated with 100uM of Monastrol and finally Monastrol was washed away and cells fixed in methanol after 40 minutes. Error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ N=4 **H.** Dot plot representing percentage of mitosis containing at least one lagging chromosome per experiment. hTert-RPE1-Cas9i and hTert-RPE1-Cas9i-pRB-KO were treated with 0.2 uM of ZM1 or DMSO and Monastrol (100 uM) for 4 hours subsequently Monastrol was washed away and cells fixed in methanol after 40 minutes. Error bars represent standard deviation, statistical significance was determined by unpaired t test and ns, $p > 0.05$, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ N=4

Discussion

Introduction

The Retinoblastoma protein is the causal agent of retinoblastoma and is a well-known oncosuppressor that is lost or mutated in the vast majority of human cancers (Takashi, 2015) (Burkhart & Sage, 2008). pRB is a key regulator of cell cycle progression and proliferation, nonetheless several studies indicate that pRB inactivation induces chromosomal instability (Hernando et al., 2004) (C.H. et al., 2010) (Amity L. Manning et al., 2010).

Chromosomal instability contributes to cancer initiation and progression and is linked to poor prognosis (W. Wang et al., 2017) (Negrini et al., 2010) (Silk et al., 2013).

pRB depletion causes defects in cell cycle progression and mitosis. Here, I focused my attention on mitotic defects arising when pRB is partially depleted.

In my thesis, I show that pRB partial depletion affects mitosis and causes lagging chromosomes through an AuroraB mediated mechanism. Furthermore, pRB absence also influences CENPA abundance at the kinetochore without affecting kinetochore functionality or structure. However, both phenotypes cannot be explained by the current hypothesis linking pRB to chromosomal instability.

Finally, I show that pRB partial depletion has unique features compared to the full knockout.

pRB depletion perturbs mitotic progression at several levels

Here, I find that pRB depletion by 60% affects mitosis at different levels. First, I demonstrate that pRB depletion prolongs the time frame between the nuclear envelope breakdown and anaphase onset (mitotic timing) by 10 mins when compared to control depletion. Since this time frame is under the control of the spindle assembly checkpoint, and since its duration is extended in the presence of unattached kinetochores (Meraldi et al., 2004). I postulate that the pRB depletion must

lead to kinetochore-microtubule attachment defects. Second, the Monastrol-release assay indicates that pRB depletion doubles the rate of lagging chromosomes in this sensitized background, implying an increase in chromosomal instability that could result in genomic instability. Interestingly, I observed this phenotype in pRB-depleted cells only in the presence of Monastrol, a drug that in itself induces erroneous kinetochore-microtubule attachments. These divergent results could be due to two different reasons: first, hTertRPE1 cells have a very low chromosome segregation error rate (0.5%) (Kaseda et al., 2000) due to efficient error correction mechanisms, and therefore even a doubling of the chromosome segregation error rate might go undetected (Silk et al., 2013). Second, the presence of pRB might be specifically required in the presence of erroneous kinetochore-microtubule attachments as obtained after a Monastrol release, consistent with my findings that pRB depletion deregulates Aurora B activity (see below). To differentiate between the two possibilities, it might be necessary to quantify the behaviour of a much larger number of unperturbed pRB-depleted cells, or to test whether pRB depletion affects chromosome segregation after other types of stress, such a DNA replication stress, a mild inhibition of the spindle assembly checkpoint, or drugs affecting DNA strand concatenation (I.e. etoposide; (Wilhelm et al., 2019) (Marchetti et al., 2001)). Nevertheless, I conclude that partial pRB depletion can cause chromosome segregation errors in a sensitized background, which allowed us to test the two reported mechanisms that had been postulated for the mitotic errors in RB1-null cells: Mad2overexpression (Sotillo et al., 2008) (Hernando et al., 2004) and deregulation of centromere condensation (C.H. et al., 2010) (Amity L. Manning et al., 2010)

The SAC hypothesis

Sotillo et al. observed that a sustained Mad2 overexpression leads to hyper stabilization of kinetochore microtubule attachments and consequently to lagging chromosomes. The finding was also confirmed in a mouse model, where a transient Mad2 overexpression was sufficient to induce several cancer types (Sotillo et al., 2008). Notably, Mad2 transcription is controlled by the E2F transcription factor and therefore indirectly by pRB (Dimova & Dyson, 2005). Hernando et al. observed that pRB inactivation leads to genomic instability by uncoupling cell cycle progression from mitotic control and by Mad2 overexpression (Hernando et al., 2004).

To test this hypothesis, I checked SAC proficiency after pRB depletion. Surprisingly, pRB depletion does not induce Mad2 overexpression nor is it able to disrupt SAC activity. The discrepancy between my results and the one of Hernando resides mostly in the model system. All the experiments carried out by Hernando are knockouts. Remarkably, a stable knockdown may select clones with a Mad2 overexpression (El-Brolosy & Stainier, 2017). On the other hand, in my partial depletion model, the other pocket protein p130 and p107 may have overcome the pRB role in E2F regulation. This hypothesis can be easily tested, I could deplete pRB and then quantify protein expression and activity of the other pocket proteins. My results suggest that pRB partial depletion can affect chromosomal stability while not being sufficient to disrupt E2F regulation. This finding is not surprising, since it was reported that cells heterozygous for RB1 are still able to proceed into the cell cycle and proficiently regulates G1/S transition (Amity L. Manning & Dyson, 2011).

Furthermore, pRB was reported to influence chromatin structure through an E2F independent mechanism. Manning et al. reported that specific pRB mutations that retain E2F activity can disrupt chromatin structure at the centromere (Ishak & Dick, 2015).

I can conclude that pRB partial depletion is causing chromosomal instability without affecting the spindle assembly checkpoint.

The DNA condensation hypothesis

Manning et al. suggested that pRB depletion induces chromosomal instability, compromising centromere structure. Specifically, the authors observed the pRB is involved in Cap-D3 recruitment. Cap-D3 belongs to the CondensinII complex and is responsible for DNA condensation before mitosis. It has been shown that pRB depletion prevents Cap-D3 recruitment at centromeric DNA, causing condensation defects and consequently unfaithful DNA segregation (Amity L. Manning et al., 2010) (Ishak et al., 2017) (C.H. et al., 2010).

DNA condensation influences chromosome behaviour in mitosis and can be indirectly quantified. During metaphase, chromosomes are subjected to pulling forces, the resultant of those forces determines sister chromatids separation or inter-kinetochore distance. The inter-kinetochore distance can be quantified during metaphase and is determined by two factors: first, the exerted tension and second by DNA condensation. More condensed DNA reduces inter-kinetochore distance, whereas less condensed DNA increases it (Antonin & Neumann, 2016).

The partial pRB depletion does not cause any change in sister separation and consequently in DNA condensation. The unaffected DNA condensation suggests that at least another mechanism links pRB and genomic instability.

A new hypothesis: pRB depletion affects Kinetochore structure

Lagging chromosomes are often a consequence of merotelic attachments, erroneous attachments arising in case of impaired microtubule stability, structural kinetochore defects, and/or error correction deficiency (Cimini et al., 2001) (Cimini, 2011). To confirm that the observed lagging chromosomes are due to merotelic attachments, I could take a cue from the elegant work of Cimini et al. in which they showed merotelic attachments through electron microscopy. EM would allow

to directly visualize DNA and microtubules and due to the geometry, I can recognize merotelic attachments in pRB depleted cells treated with Monastrol.

My microtubule stability assay shows that pRB depleted cells have unaltered microtubule stability. My pRB partial depletion is unable to affect DNA condensation nor microtubule stability or Mad2 expression all these elements prompted me to investigate in more detail the kinetochore structure. The kinetochore is the interface between microtubules and the centromere, it plays a crucial role in mitosis and ensures proper segregation. The kinetochore is assembled on CENPA, a modified histone that serves as an epigenetic marker and a platform for kinetochore assembly (Cleveland, Mao & Sullivan, 2003). CENPA depletion causes, first a high rate of segregation errors and later cell death (Hoffmann et al., 2016).

Amato et al. reported that pRB depletion causes CENPA overexpression and consistently CENPA is overexpressed in many colorectal cancers (Amato, Lentini, Schillaci, Iovino, & Di Leonardo, 2009). The work of Amato suggests that pRB absence can affect CENPA expression and consequently the presence of genomic instability. Counterintuitively, in my experiments the partial pRB depletion reduces CENPA protein abundance at the kinetochore. This finding prompted me to investigate further the kinetochore structure upon pRB depletion.

The kinetochore is organized in 3 different portions: inner kinetochore, outer and corona. (Bodor et al., 2014).

The analysis of three different protein candidates, belonging to different parts of the kinetochore, revealed no significant difference in term of expression in pRB depleted cells. Microtubule stability data and immunofluorescence data are consistent and indicate that the kinetochores in pRB depleted cells are functional. Interestingly, my data show that pRB depletion reduces by 50% CENPA abundance at the kinetochore and this reduction happens right after NEBD. Conversely, CENPA protein abundance in interphase is comparable between sipRB and siCTRL.

How is it possible that only 50% of CENPA protein is sufficient to establish a functional kinetochore? Thanks to Hoffmann et al. we know that only 5% of CENPA copies are sufficient to establish a functional kinetochore. This piece of evidence allows me to speculate about at least two CENPA features. First, CENPA is produced in excess due to its crucial role in kinetochore assembly. Only a small pool of CENPA is necessary to establish the kinetochore. Second, CENPA is an epigenetic marker for centromeric DNA, rather than being a structural element of the kinetochore. Hoffmann et al. demonstrated that after the initial deposition CENPA is dispensable for kinetochore function (Hoffmann et al., 2016).

It is still unknown why CENPA protein abundance drops consistently after NEBD and I can only speculate about the reason. Nuclear Envelope Break Down marks the transition from prophase to prometaphase and corresponds to the nucleus dissolution. Furthermore, NEBD is characterized by DNA condensation, crucial for the subsequent chromosome segregation (Samoshkin et al., 2009). My data suggest that DNA condensation after NEBD reduces CENPA abundance in pRB depleted cells and this can be explained by a reduced CENPA stability at the centromere.

According to my hypothesis, pRB depletion affects chromatin structure and consequently CENPA deposition and/or maintenance. First, I could in the future assess chromatin modifications in pRB depleted cells compared to depletion control. The differences in chromatin modifications may explain the reduced CENPA stability. Second, I could reproduce the observed chromatin modification in non-treated cells and monitor CENPA protein abundance. If my hypothesis is correct the chromatin modifications, induced by pRB depletion, would impair CENPA deposition and/or maintenance. pRB depleted cells show a 50% CENPA reduction in correspondence to NEBD, interestingly no CENPA signal is detectable in the cytosol after NEBD.

When CENPA is not incorporated into the centromere but is bound to protein chaperons like HJURP (Barnhartdailey et al., 2017).

Considering CENPA biology, I can speculate that in pRB depleted cells, the released CENPA pool undergoes degradation (Valdivia et al., 2009). To test this hypothesis, I could mutate CENPA making it degradation resistant. If my hypothesis is correct in pRB depleted cells, I will observe CENPA reduction at the kinetochore, at NEBD, and simultaneously I will observe CENPA release into the cytoplasm.

According to my data, CENPA reduction observed in mitosis in pRB depleted cells is 'compensated' during G1. CENPA levels in interphase are comparable between pRB depleted cells and control, besides, I don't observe a progressive CENPA depletion through cell cycles.

How the cell senses CENPA reduction and compensates for it is still unknown.

We can speculate that the cell can sense CENPA reduction through a positive feedback loop, like for other proteins (REF). To test this hypothesis, I can take advantage of a CENPA SNAP-tag cell line. I could deplete pRB in synchronized cell cycle experiments and activate the fluorescent TAG at the beginning of G1 (Bodor, Rodríguez, Moreno, & Jansen, 2004). Newly incorporated CENPA will be tagged and I can follow it through the cell cycle. I should observe a CENPA drop during mitosis and If my hypothesis is correct pRB depleted cells should load more CENPA molecules to 'compensate' the lost pool. To test the 'compensation' hypothesis I measured Rbbp7 protein abundance in pRB depleted cells.

Rbbp7 in concert with Mis18 complex directly contributes to CENPA deposition in G1, interestingly my preliminary data shows Rbbp7 overexpression in pRB depleted cells. Rbbp7 overexpression may compensate CENPA depletion observed in mitosis. To test this hypothesis, I can overexpress Rbbp7 and quantify CENPA deposition in G1 to understand if a correlation exists.

In conclusion, pRB depletion reduces CENPA protein abundance at the kinetochore, while the kinetochore structure is unaltered and functional. My data suggest that the lagging chromosomes

observed in pRB depleted cells are not linked to kinetochore dysfunction. Therefore, I moved my attention to the error correction machinery.

pRB depletion and the error correction machinery

The presence of lagging chromosomes and delayed mitosis suggests involvement of the error-correction machinery (Meraldi et al., 2004). Chromosome segregation errors give rise to both loss and gain of entire chromatids or part of it contributing to cancer progression and initiation. Several control mechanisms are in place to prevent erroneous segregation, the CPC (chromosome passenger complex) ensures proper mitosis at different levels. CPC controls mitotic checkpoints, destabilizes erroneous attachments and finally ensures proper cytokinesis (Vader, Medema, & Lens, 2006) (Carmena et al., 2012).

CPC consisting of AuroraB, INCENP, Borealin and Survivin localizes inside the centromere and controls error correction through a complex multistep process. AuroraB kinase is the CPC master regulator and corrects erroneous attachments through phosphorylation of several targets;

Histone3, Hec1, DSN1 etc (Waal et al. 2012).

To evaluate AuroraB activity in pRB depleted cells, I quantified pH3(s28) and pHec1 and observed higher phosphorylation compare to siRNA control. The AuroraB gene is under the control of transcription factor E2F and pRB depletion should affect its transcription. Surprisingly, the global level of AuroraB is only mildly affected by pRB depletion, indicating that pRB depletion mostly affects AuroraB activity rather than its expression.

Consistently with my previous results higher AuroraB activity is linked to lagging chromosomes and aneuploidy (Ricke et al., 2011) (Ricke et al., 2011).

To confirm the correlation between pRB depletion, AuroraB overexpression and lagging

chromosomes I pharmacologically inhibited AuroraB. Pharmacological AuroraB inhibition partially reduces lagging chromosomes in pRB depleted cells.

This finding suggests that lagging chromosomes observed in pRB depleted cells are AuroraB mediated, furthermore pharmacological AuroraB inhibition can partially rescue the phenotype reducing the number of lagging chromosomes.

My results show that pRB depletion leads to AuroraB hyperactivation. This phenomenon can be explained in at least two ways: a hyperactivation of AuroraB kinase activity per se or downregulation of the counteracting phosphatase PP1.

To understand which mechanism is involved, I could test PP1 activity in pRB depleted cells: if other PP1 targets were hyperphosphorylated it would suggest that PP1 is involved. Interestingly, PP1 is also responsible for pRB de-phosphorylation and is CDK1 regulated.

PP1 and PP2A control circa 90% of dephosphorylation in eukaryotes, making it an impractical target for therapeutic strategies (Smith et al., 2019). PP1 inhibition would certainly lead to pleiotropic effects.

According to my data pRB depletion hyperactivates AuroraB. AuroraB exerts its error correction role through the phosphorylation of many kinetochore targets (Broad et al., 2020). Interestingly, pRB-induced AuroraB hyperactivation does not influence microtubule stability nor kinetochore microtubule attachments. My data indicate that pRB depletion increases AuroraB activity in the transition from prometaphase to anaphase. Error corrections happen during this transition, emphasizing its crucial role. AuroraB acts through the phosphorylation of many different kinetochore targets, surprisingly my kinetochore tracking assays detected no differences between pRB depleted cells and control depleted cells. Why does AuroraB activation not change microtubule stability?

AuroraB hyperactivity should hyper phosphorylate kinetochore targets like DNS1 or Hec1, resulting in less stable kinetochore microtubule attachments and consequently affecting kinetochore oscillation. Notably, AuroraB hyperactivation was observed only after Monastrol treatment. Also, AuroraB global level was practically comparable between pRB depleted cells and control. This finding suggests that pRB depleted cells hyper activate AuroraB only in presence of merotelic attachments (Monastrol induced). To test this hypothesis, I should perform kinetochore tracking assay in Monastrol treated pRB depleted cells. Monastrol will enrich for merotelic attachments and will cause an AuroraB hyperactivation in pRB depleted cells. Hyperactive AuroraB will affect microtubule stability and therefore kinetochore oscillation, in fact, as observed by Wan et al. chromosomes organized in monopolar spindle maintain the ability to oscillate (Wan, Cimini, Cameron, Salmon, & Doxsey, 2012).

Finally, several lines of evidence indicate AuroraB inhibition as a potential therapeutical target for cancer treatments. Oser et al. reported that tumours lacking pRB are more sensitive to AuroraB inhibition (Oser et al., 2019). My data show that partial pRB depletion induces genomic instability but counterintuitively genomic instability is partially rescued by AuroraB inhibition. AuroraB inhibitor reduces the number of lagging chromosomes in pRB depleted cells compared to control cells. A possible explanation for the rescue is that pRB depletion-evoked genomic instability is AuroraB mediated. My finding seems to be in contrast with the results of Oser at al. This discrepancy can be explained in at least two ways.

First, Oser et al. used two different SCLC cell lines NCI-H82 and NCI-H69, both reported to be p53 mutated. As previously discussed p53 plays a crucial role in the pRB depletion phenotype. P53 absence allows tolerance and proliferation of aneuploid cells. Second, in the current work, I focused

on a partial transient pRB depletion, conversely, Oser and colleagues knocked out pRB through Crispr Cas9. pRB knockout can justify a higher dependency on AuroraB.

To reconcile my results with the one obtained by Oser et al. I could try different experiments. For example, I could treat the pRB KO cell line with AuroraB inhibitors and evaluate genomic instability and viability. My pRB KO cell line will resemble more closely the Oser system. Furthermore, I could deplete p53 in the pRB knockout cell line and apply an AuroraB inhibitor. Later I could evaluate genomic instability and viability. Again, p53 depletion will mimic the genetic background of the used cell lines in the Oser manuscript.

pRB depletion affects AuroraB activity and CENPA abundance, searching the link

pRB depletion leads to AuroraB hyperactivation and CENPA kinetochore reduction. Both AuroraB and CENPA are localized in the centromeric DNA, suggesting that pRB depletion affects centromeric structure. pRB interacts with several chromatin modifiers like HDAC, HAT, SUV39H1, SMCII complex and it has been reported that pRB removal influences chromatin structure (Amity L Manning et al., 2015). To understand how pRB depletion exactly affects chromatin structure, I could perform ChIP analysis over centromeric DNA. ChIP analysis will reveal which modifications are induced by pRB depletion.

Once the modifications are assessed I could try to rescue them. For example, it has been reported that pRB is involved in methylation of the Lysine20 in Histone4 (H4K20 methylation), that in turn is a prerequisite for cohesion establishment at centromeres (Amity L Manning et al., 2015). Manning et al. showed that pRB absence impairs H4K20, compromising cohesion. SUV-20H1, controlling H4K20 methylation, could be overexpressed to rescue pRB depletion. Besides, I could mimic pRB depletion by inhibiting SUV4-20H1 and subsequently check CENPA protein abundance at the kinetochore in mitosis. impaired CENPA recruitment at the kinetochore by SUV4-20H1 inhibition,

would mean that H4K20 methylation is relevant. I could use the same approach to test AuroraB activity and I could try to rescue it.

pRB knockout does not resemble the pRB depletion phenotype

All data presented in this thesis were obtained with a transient partial pRB depletion. Some authors indicate that partial depletion can resemble heterozygous background, notably, Coschi et al. showed that pRB protein abundance in heterozygous pRB +/- is comparable with sipRB (Coschi et al., 2014). The Knudson double hits hypothesis claims that both onco-suppressor alleles need to be inactivated to develop the disease with a process called loss of heterozygosity (LOH)(Mastrangelo et al., 2008) (Knudson, 1971). My data indicate that pRB partial depletion is not sufficient to maintain genome stability and therefore creates chromosomal instability (Ishak & Dick, 2015). Moreover, my findings could explain some pRB paradoxes; why is pRB mutated or lost in the vast majority of human cancers, while only few tumours are directly connected to its absence? Why retinoblastoma patients develop several tumours during their lives? Why the familiar form of retinoblastoma emerges earlier than the sporadic? pRB haploinsufficiency may explain some of these observations and can play a pivotal role in tumour initiation (Ishak & Dick, 2015). Elevated segregation errors lead to chromosomal instability that creates the perfect environment for cancer formation (M. S. Levine & Holland, 2015). On the contrary, a full pRB knockout may force the cell to compensate with other mutations or even to arrest the cycle and undergo apoptosis, a partial depletion goes undetected and pose less evolutive pressure on the cell. pRB haploinsufficiency resembles a phenomenon observed with Mad2 heterozygosity. Mad2 heterozygosity causes premature anaphase and genomic instability in a human cell with a similar impact (Michel et al., 2001).

Also, pRB is often lost or mutated with p53 in a variety of human cancers (Zilfou & Lowe, 2009). Individually pRB and p53 contribute to the maintenance of genome stability, cell cycle control, senescence and cell death. It has been observed that the inhibition of both tumour suppressors has a synergistic effect. P53 depletion alone is not able to induce genomic instability similarly pRB absence only partially induces it (Yao, 2014). Conversely, the double depletion promotes chromosomal instability and cancer formation. The synergistic effect could be explained by the fact that pRB loss influences mitotic fidelity, and p53 absence allows tolerance and proliferation of aneuploid cells (A L Manning, Benes, & Dyson, 2014).

To thoroughly investigate the impact of pRB depletion in cancer initiation I could establish a Crispr Cas9 inducible pRB knockout cell line. The knockout cell line provides useful information about pRB absence over time, highlighting compensatory mutations and adaptive mechanisms. On the other hand, knockout cell lines provide a snapshot of the genetic landscape achieved through many mutations and adaptations.

The pRB knockout clone expresses an extremely low level of pRB conversely CENPA levels are comparable to the parental cell line. Unaffected CENPA levels could suggest an adaptive mechanism occurring after pRB depletion and maintained over time (El-brolosy & Stainier, 2017). Furthermore, pRB knockout cells present a low number of lagging chromosomes, notably less than 10% of mitotic cells contains a lagging chromosome. This specific feature can be attributed to a specific clonal behaviour. AuroraB activity, measured through pH3 phosphorylation, appears unaltered or even lower compared to the parental cell line. A lower number of lagging chromosomes and low AuroraB activity are reciprocally consistent and confirm that pRB knockout cells acquired adaptive mutations to compensate for pRB absence. To exclude any possible off-target effect, I applied pRB siRNA to the pRB knockout cell line. pRB siRNA failed to induce lagging chromosomes in the pRB

knockout cell line confirming that the observed phenotype is pRB specific. Finally, pharmacological AuroraB inhibition was applied in the pRB knockout cell line to induce lagging chromosomes. AuroraB inhibition caused a threefold increase in lagging chromosomes in the pRB knockout cell line, consistent with previous results (Huang et al., 2018) (Ricke et al., 2011).

My last findings suggest that pRB knockout does not induce AuroraB hyperactivation and consequently lagging chromosomes. Finally, AuroraB inhibition induces lagging chromosomes even in pRB absence.

My results indicate a marked difference in term of phenotype between the partial depletion and the knockout. This discrepancy can be explained in several ways. First, the partial depletion reduced pRB protein abundance by 60-70%. Several proteins can exert their role even in a minimal amount. Second, due to the crucial role of pRB in cell cycle regulation genetic compensation may have occurred in the pRB knockout clone. Compensatory mutations are a common and well-known phenomenon, occurring when an essential gene is knocked out (El-brolosy & Stainier, 2017).

Third, the observed phenotypes like increased AuroraB activity and CENPA reduction at the kinetochore may not show a linear response. Phenomena like lagging chromosomes have multiple causes and explanations, not representing a dichotomic response to a perturbation. Therefore, we cannot expect a linear response between the number of observed lagging chromosomes and pRB protein abundance (Cimini, 2011). Finally, partial depletion and knockout act by two completely different timelines.

Partial depletion is established in 72 hours and cells are analyzed after a maximum of 48 hours, this time frame represents roughly 3 cell cycles (The Cell, 2nd edition, 2000). By contrast, knockout cell lines are established over a period of several weeks. Potentially, each cell cycle represents an opportunity to accumulate mutations.

Considering the discrepancy between partial depletion and full knockout, I could perform different experiments aiming two reconcile the two.

I could dose pRB depletion to different extents and analyze the corresponding phenotypes. I could try to establish the minimal pRB protein amount that allows to exert its functions. I could take advantage of sequential partial depletions and monitor pRB protein abundance through western blot. In parallel, I could analyze E2F activity and the presence of segregation errors. This experiment could inform how much pRB is required for the different functions.

Moreover, I could perform RNA-seq in cells pRB depleted versus siCTRL depleted and in parallel in cells pRB knockout. My analysis would highlight gene expression adaptations between the different conditions and will eventually show compensatory mutations.

Finally, taking advantage of the Auxin degron system I could ensure a complete protein degradation in a short period, circa 10 minutes. This system would merge the timing of the depletion with the efficacy of the knockout and would help to discriminate between direct and indirect pRB depletion effects (Nishimura, Fukagawa, Takisawa, Kakimoto, & Kanemaki, 2009).

Materials and Methods

1. Cell culture

Different hTERT-RPE1 cells were employed in this study: hTERT-RPE1 CENPA GFP (Dudka et al 2018), inducible Cas9 hTERT-RPE1 (kind gift of I. Cheeseman), hTERT RPE1 cells CA-/Y CENPA YFP (called hTERT-RPE1-YFP-CENPA)(kind gift of L. Jansen), hTert-RPE1 H2B-mCherry/EB3-eGFP (kind gift of W. Krek), hTERT-RPE1. In addition, HeLa Kyoto (Hela K) were used.

Cells were grown in DMEM (Dulbecco's modified Eagle's medium; GIBCO) supplemented with 10% FBS (Fetal Bovine Serum; GIBCO), 2 mL L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (Invitrogen) inside an incubator kept at 37°C with 5% CO_2. The only exception was the inducible Cas9 hTERT-RPE1cell line that was cultured in a tetracycline free medium.

Cells were split every 2/3 days according to the confluency and were kept in culture for a maximum of 30 days. For live cell imaging experiments, cells were plated inside IBIDI chamber (Vitaris), pre-treated with 25 nM SiR-Hoechst (Spirochrome AG, Switzerland) for at least 4 hours before imaging. SiR-Hoechst served as chromosome marker to monitor chromosome segregation in mitosis. Cells were kept inside imaging medium Leibovitz L-15 (Thermofisher) medium supplemented with 10% FCS.

2. Drug treatments

In order to enrich the number of merotelic attachments, 100µM of Monastrol (Eg5 inhibitor; Sigma Aldrich) was added to the cells for at least four hours before fixation/imaging. For monastrol release assay, cells were released after 3 washes with fresh medium.

In order to inhibit AuroraB activity, ZM1 (Aurora-B inhibitor; Enzo Life Sciences, Switzerland) was used in different concentration (0.2 0.5 and 0.7 µM) at least 4 hours before fixation/imaging. Nocodazole (microtubule depolymerizing agent, Sigma) was used to enrich the number of prophase cells at 10 ng/mL for minimum 2 hours before fixation.

In order to obtain metaphase cells, we used 5 µM of MG132 (protease inhibitor, Sigma) for 1h.

For the microtubule decay assay, cells were treated for 1h with 5 µM MG132 prior to the experiment. 200ng/ml nocodazole was added just before starting the acquisition of the movie, in L15 10% FCS medium on the cells.

3. siRNAs

siRNA treatments were performed for 72 hours. For all the siRNAs a mixture of 40 nmol siRNAs and 1% Lipofectamine RNAimax (Invitrogen) in Opti-MEM (Invitrogen) was added dropwise on cells after 35 min of incubation, in MEM (Thermofisher) supplemented with 10% FCS.

The following oligos were used in this study:

sipRB1 = 5-GAAAGGACATGTGAACTTATT-3

sipRB2 = 5-CGAAATCAGTGTCCATAAATT-3

sipRB3 = 5-CGAAATCAGTGTCCAUAAA-3

siCAP-D2 = 5'-CCATAUGCTCAGUGCTACATT-3'

siCTRL = 5'- AAGGACCTGGAGGTCTGCTGT - 3'

4. Inducible knock out cell line

In order to establish a Crispr-CAS9 inducible cell line we obtained from Ian Cheeseman the inducible Cas9 hTERT-RPE1 line. Subsequently, we infected it with Sanger validated sgRNAs (Sigma) (CTAGATGCAAGATTATTTTTGG, GACGAGAGGCAGGTCCTCCGGG). Sanger Validated sgRNAs were assembled in lentiviral particle containing a puromycin resistance.

Validated sgRNAs were provided inside a lentiviral particle ready for the infection. (https://www.sigmaaldrich.com/pc/ui/crisprgrnahome/crisprgrna)

Inducible Cas9 hTERT-RPE1 cells were plated and infected with different MOIs:

1:0,5 / 1:1 / 1:2 / 1:5 (cell to virus ratio)

in order to find the right ratio between virus and cells. 48 hours after the infection puromycin medium was added in the concentration of 5 ugr/ml in order to apply a positive selection on infected cells. The MOI of 1:5 was the most effective in term of infection. After 2 weeks of culture puromycin was removed. Cas9 activation was achieved with 2ugr of Tetracycline for at least 48 hours and cells were tested. Cells were cultured in Tetracycline for at least one week, subsequently single sorted. Single clones were let grow for at least one week and tested periodically. pRB protein abundance

was assessed via immunofluorescence and western blot.

5. Live cell imaging and KTs tracking

Cells were imaged either using a Nikon Eclipse Ti-E wide-field microscope (Nikon, Switzerland) equipped with a DAPI/eGFP/TRITC/Cy5 filter set (Chroma, USA) and a 40X N.A. 1.3 objective (mitotic timing) and recorded with an Orca Flash 4.0 CMOS camera (Hamamatsu, Japan) and the NIS software; or an Olympus DeltaVision wide-field microscope (GE Healthcare, Switzerland) equipped with a eGFP/RFP filter set (Chroma) and with 40X NA 1.3. (Monastrol release), or 100x NA 1.4 objectives (KT tracking) and recorded with a Coolsnap HQ2 CCD camera (Roper Scientific, USA) and the Softworx software (GE Healthcare).

Mitotic timing quantification was performed as follows, cells were plated in IBIDI chamber, transfected with siRNAs for 72 hours, 25nM SiR-Hoechst was applied 4 hours before imaging in L15 10% FCS medium on the cells. Cells were imaged for 24 hours every 5 minutes with 2 µm z-stacks.

Mitotic timing was assessed by visual inspection, T_0 was assigned to Nuclear Envelope BreakDown and T_1 was anaphase onset.

In Monastrol release experiment hTert- RPE1 GFP-CENPA were imaged every 3 min for 2 hours after Monastrol release using 2 µm z-stacks. Videos were analyzed with ImageJ Fiji, T_0 was the monopolar configuration and T_1 was anaphase onset. Anaphase onset was analyzed in 3D o with Z stack project in order to find lagging chromosomes.

To measure microtubule decay, cells were first treated with the different siRNAs for 72 hours prior to the imaging. Cells were treated for 2 hours with 50 nM sir-Tubulin (SpiroChrome) to stain microtubules, and for 30min with 5µM MG132 to block the cells in metaphase. Before starting the movie, 200 ng/ml nocodazole was added on the cells. The Olympus Deltavision with its Coolsnap H2Q CCD camera was used to take a 24 stack-picture of each cell every minute for 15 min. Microtubule depolymerization rate was then measured by a code developed on Matlab R2016b (Mathworks). Detailed information about code can be found in (Dudka, 2019).

The KT tracking of hTert-RPE1 GFP-CENPA protocol was adapted from our previous work. Single cells were recorded in 0.5mm steps at a sampling rate of 7.5 s over a period of 15 min obtaining kinetochore 3D stacks. The 3D z-stacks were deconvoluted using SoftWorx software and analyzed in MATLAB (The Math Works, Inc, Natic, USA) with an automated KT tracking code (the latest code is available under https://github.com/cmcb-warwick) (Olziersky, n.d.) .

6. Immunofluorescence

Cells were grown on microscopy coverslips pretreated with HCl, treated with different drugs or siRNAs. Cells were fixed with two methods: fixation buffer (20 mM PIPES (pH = 6.8), 10 mM EGTA, 1 mM MgCl2, 0.2 % Triton X-100, 4 % formaldehyde; Sigma Aldrich) for 7 min at room temperature or cold methanol for 7 minutes at -20°C. Later cells were washed with Phosphate-buffered saline

solution (PBS) three times before adding a blocking buffer for 1h at room temperature (RT) (7.5% Bovine Serum Albumin (BSA), 0.25% sodium azide in PBS).

The following primary antibodies were used:

- mouse anti-CENPA (1:1000; Abcam ab13939)
- rabbit anti-MAD2 (1:1000; Bethyl A300-301A)
- rabbit anti-pRB (1:1000 Abcam ab181616)
- mouse anti-Ndc80^{Hec1} (1:1000; Abcam 9G3)
- rabbit anti-pS100- Dsn1 (1:1000; kind gift of I. Cheeseman)
- rabbit anti-AuroraB (1:1000 Abcam ab2254)
- rat anti-Histone 3 (pS28) (1:1000 BD Biosciences 558217)
- guinea pig anti-CENPC (1:1000 Labforce PD030)
- mouse anti-CENPF (1:1000 Abcam ab90)

The coverslips were then washed with PBS and incubated 1 h with secondary antibodies in blocking buffer. Finally, coverslips were mounted on microscopy slides using Vectashield with DAPI mounting medium (ReactoLab).

Microscopy slides were analyzed on Olympus DeltaVision wide-field microscope (GE Healthcare, Switzerland) equipped with a eGFP/RFP filter set (Chroma) and with 60x NA 1.4 or 100X 1.4 NA objectives and recorded with a Coolsnap HQ2 CCD camera (Roper Scientific, USA) and the Softworx software (GE Healthcare). Acquired images were analyzed of ImageJ Fiji. DAPI signal was used to establish a ROI and protein signal and background signal were quantified. Finally, the background signal was subtracted.

7. Western blot

Cells were plated in a 10 cm dish for 72 hours treated with drugs or siRNAs, later harvested and lysed. Lysis buffer containing 20mM Tris pH 7,5, 150 mM NaCl, 20 mM Glycero- -phosphate, 5 mM MgCl2, 10% Glycerol, 0.2% NP-40, 1µM DTT, PhosoStop Phosphatase inhibitor Cocktail, EDTA-free Protease Inhibitor Cocktail. After lysis, protein extract was quantified (Bradford) and protein were separated by SDS-PAGE and transferred onto nitrocellulose. Proteins were detected by immunoblotting and visualized by treating the blots with ECL (Millipore). Several antibodies were used:

- rabbit anti-MAD2 (1:1000; Bethyl A300-301A)
- rabbit anti-pRB (1:1000 Abcam ab181616)

Horseradish peroxidase-conjugated secondary antibodies were incubated for one hour at room temperature: goat anti-rabbit (1:100'000, Thermofischer) and goat anti-mouse (1:10'000, Bio Rad). Blot were imaged with a Syngene PXi imaging system, and signal intensities were quantified using ImageJ Fiji and normalized to -tubulin.

8. Flow cytometry

hTERT-RPE1 cells were grown in 10 cm dishes. Treated with different siRNAs or drugs. On the day of the experiment, cells were harvested, washed with PBS and fixed with 70% ethanol 4 hours -20°C. After fixation, cells were stained 20 minutes with antibodies in stain buffer (PBS, 5% BSA, 0.05% Tween) and counterstained with Propidium Iodide (PI) for additional 20 minutes. Stained cells were acquired using a Accuri C6 flow cytometer (BD bioscience) equipped with 2 lasers and 4 filters. Cell cycle profiles were analyzed using the software FlowJo.

9. Statistical analysis

All experiments are based on at least 3 independent experiments, unless otherwise indicated in figure legends. Numbers of independent experiments (N) and number of analyzed cells/KTs (n) are indicated in the figure legends. All statistical evaluations were run on PRISM 7.02 (GraphPad, USA); the specific statistical tests and the p-values are indicated in the figure legends. Graphs were plotted in PRISM 7.02 and mounted in Adobe Illustrator (Adobe).

References

Amaro, A. C., Samora, C. P., Holtackers, R., Wang, E., Isabel, J., Alonso, M., … Mcainsh, A. D. (2010). *chromosome oscillations*. *12*(4), 319–329. https://doi.org/10.1038/ncb2033.Molecular

Amato, A., Lentini, L., Schillaci, T., Iovino, F., & Di Leonardo, A. (2009). RNAi mediated acute depletion of Retinoblastoma protein (pRb) promotes aneuploidy in human primary cells via micronuclei formation. *BMC Cell Biology*, *10*, 79. https://doi.org/10.1186/1471-2121-10-79

Antonin, W., & Neumann, H. (2016). ScienceDirect Chromosome condensation and decondensation during mitosis. *Current Opinion in Cell Biology*, *40*, 15–22. https://doi.org/10.1016/j.ceb.2016.01.013

Armond, J. W., Vladimirou, E., Mcainsh, A. D., & Burroughs, N. J. (2016). *KiT : a MATLAB package for kinetochore tracking*. *32*(February), 1917–1919. https://doi.org/10.1093/bioinformatics/btw087

Ricke, R. M., & Deursen, J. M. Van. (2011). *Aurora B hyperactivation by Bub1 overexpression promotes chromosome missegregation*. *4101*. https://doi.org/10.4161/cc.10.21.18156

Bailey, M. H., Tokheim, C., Porta-pardo, E., Mills, G. B., Karchin, R., Ding, L., … Bertrand, D. (2018). *Comprehensive Characterization of Cancer Driver Genes and Mutations Article Comprehensive Characterization of Cancer Driver Genes and Mutations*. 371–385. https://doi.org/10.1016/j.cell.2018.02.060

Bakhoum, S. F., & Swanton, C. (2014). *Chromosomal instability , aneuploidy , and cancer*. *4*(June), 9–11. https://doi.org/10.1038/25292

Bakhoum, S. F., Thompson, S. L., Manning, A. L., & Compton, D. A. (2009). *HHS Public Access*. *11*(1), 27–35. https://doi.org/10.1038/ncb1809.Genome

Barnhart-dailey, M. C., Trivedi, P., Stukenberg, P. T., & Foltz, D. R. (2017). *HJURP interaction with the condensin II complex during G1 promotes CENP-A deposition*. https://doi.org/10.1091/mbc.E15-12-0843

Bester, A. C., Roniger, M., Oren, Y. S., Im, M. M., Sarni, D., Chaoat, M., … Kerem, B. (2011). Nucleotide Deficiency Promotes Genomic Instability in Early Stages of Cancer Development. *CELL*, *145*(3), 435–446. https://doi.org/10.1016/j.cell.2011.03.044

Bloom, K. S. (2010). *to mitosis*. *117*(2), 103–110. https://doi.org/10.1007/s00412-007-0138-0.Beyond

Bodor, D. L., Mata, J. F., Sergeev, M., David, A. F., Salimian, K. J., Panchenko, T., … Jansen, L. E. (2014). The quantitative architecture of centromeric chromatin. *ELife*, *3*, 1–26. https://doi.org/10.7554/elife.02137

Bodor, D. L., Rodríguez, M. G., Moreno, N., & Jansen, L. E. T. (2004). *Analysis of protein turnover by quantitative SNAP-based pulse-chase imaging*. 1–42.

Broad, A. J., Deluca, K. F., & Deluca, J. G. (2020). *Aurora B kinase is recruited to multiple discrete kinetochore and centromere regions in human cells*. *219*(3).

Burkhart, D. L., & Sage, J. (2008). Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nature Reviews Cancer*, *8*(9), 671–682. https://doi.org/10.1038/nrc2399

C.H., C., A.L., M., K., R., S.M., F., S., C., N.G., B., … Dick, F. A. (2010). Mitotic chromosome condensation mediated by the retinoblastoma protein is tumor-suppressive. *Genes and Development*, *24*(13), 1351–1363. https://doi.org/10.1101/gad.1917610 LK - http://vu.on.worldcat.org/atoztitles/link?sid=EMBASE&issn=08909369&id=doi:10.1101%2Fgad.1917610&atitle=Mitotic+chromosome+condensation+mediated+by+the+retinoblastoma+protein+is+tumorsuppressive&stitle=Genes+Dev.&title=Genes+and+Development&volume=24&issue=13&spage=1351&epage=1363&aulast=Coschi&aufirst=Courtney+H.&auinit=C.H.&aufull=Coschi+C.H.&coden=GEDEE&isbn=&pages=1351-1363&date=2010&auinit1=C&auinitm=H.

Carmena, M., Wheelock, M., Funabiki, H., & Earnshaw, W. C. (2012). The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. *Nature Publishing Group*,

13(December). https://doi.org/10.1038/nrm3474

Cimini, D. (2011). *Merotelic kinetochore attachment : causes and effects*. *21*(6). https://doi.org/10.1016/j.tcb.2011.01.003

Cimini, D., Howell, B., Maddox, P., Khodjakov, A., Degrassi, F., & Salmon, E. D. (2001). Merotelic kinetochore orientation is a major mechanism of aneuploidy in mitotic mammalian tissue cells. *Journal of Cell Biology*, *152*(3), 517–527.

Cimini, D., Moree, B., Canman, J. C., & Salmon, E. D. (2003). *Merotelic kinetochore orientation occurs frequently during early mitosis in mammalian tissue cells and error correction is achieved by two different mechanisms*. https://doi.org/10.1242/jcs.00716

Cobrinik, D. (2005a). *Pocket proteins and cell cycle control*. 2796–2809. https://doi.org/10.1038/sj.onc.1208619

Cobrinik, D. (2005b). Pocket proteins and cell cycle control. *Oncogene*, *24*(17), 2796–2809. https://doi.org/10.1038/sj.onc.1208619

Coschi, C. H., Ishak, C. A., Gallo, D., Marshall, A., Talluri, S., Wang, J., … Dick, F. A. (2014). *Haploinsuffi ciency of an RB – E2F1 – Condensin II Complex Leads to Aberrant Replication and Aneuploidy*. (July), 840–854. https://doi.org/10.1158/2159-8290.CD-14-0215

DeFilippis, R. A., Goodwin, E. C., Wu, L., & DiMaio, D. (2002). Endogenous Human Papillomavirus E6 and E7 Proteins Differentially Regulate Proliferation, Senescence, and Apoptosis in HeLa Cervical Carcinoma Cells. *Journal of Virology*, *77*(2), 1551–1563. https://doi.org/10.1128/jvi.77.2.1551-1563.2003

Dick, F. A., & Dyson, N. J. (2002). *Three Regions of the pRB Pocket Domain Affect Its Inactivation by Human Papillomavirus E7 Proteins*. *76*(12), 6224–6234. https://doi.org/10.1128/JVI.76.12.6224

Dick, F. A., & Rubin, S. M. (2013). Molecular mechanisms underlying RB protein function. *Nature Reviews Molecular Cell Biology*, *14*(5), 297–306. https://doi.org/10.1038/nrm3567

Dimova, D. K., & Dyson, N. J. (2005). *The E2F transcriptional network : old acquaintances with new faces*. 2810–2826. https://doi.org/10.1038/sj.onc.1208612

Dudka, D. (2019). *Allows Centrosomes to Control Kinetochore-Fiber Spindle-Length-Dependent HURP Localization Allows Centrosomes to Control Kinetochore-Fiber Plus-End Dynamics*. 3563–3578. https://doi.org/10.1016/j.cub.2019.08.061

Dudka, D., Noatynska, A., Smith, C. A., Liaudet, N., Mcainsh, A. D., & Meraldi, P. (2018). Complete microtubule–kinetochore occupancy favours the segregation of merotelic attachments. *Nature Communications*. https://doi.org/10.1038/s41467-018-04427-x

Dyson, N. J. (2016). RB1: A prototype tumor suppressor and an enigma. *Genes and Development*, *30*(13), 1492–1502. https://doi.org/10.1101/gad.282145.116

El-brolosy, M. A., & Stainier, D. Y. R. (2017). *Genetic compensation : A phenomenon in search of mechanisms*. 1–17.

Fachinetti, D., Folco, H. D., Nechemia-arbely, Y., Valente, L. P., Nguyen, K., Wong, A. J., … Cleveland, D. W. (2013). A two-step mechanism for epigenetic specification of centromere identity and function. *Nature Cell Biology*, *15*(9). https://doi.org/10.1038/ncb2805

Figueiredo, A. C., & Maiato, H. (2019). *Europe PMC Funders Group Let there be light on kinetochores*. *13*(9), 1058–1059. https://doi.org/10.1038/nchembio.2464.Let

Fischer, M., Uxa, S., Stanko, C., Magin, T. M., & Engeland, K. (2017). Human papilloma virus E7 oncoprotein abrogates the p53-p21-DREAM pathway. *Scientific Reports*, *7*(1), 1–11. https://doi.org/10.1038/s41598-017-02831-9

Florensa, R., Bachs, O., & Agell, N. (2003). *ATM / ATR-independent inhibition of cyclin B accumulation in response to hydroxyurea in nontransformed cell lines is altered in tumour cell lines*. (February), 8283–8292. https://doi.org/10.1038/sj.onc.1207159

Friedberg, E. C. (2003). *and repair*. *421*(January).

Friend. (1986). *A human DNA segment with properties of the gene that predisposes to*

retinoblastoma and osteosarcoma. 6–9.

Funk, L. C., Zasadil, L. M., & Weaver, B. A. (2016). Review Living in CIN : Mitotic Infidelity and Its Consequences for Tumor Promotion and Suppression. *Developmental Cell*, *39*(6), 638–652. https://doi.org/10.1016/j.devcel.2016.10.023

Genet, M. (2001). *Two hits revisited again*. (1), 81–85.

Gligoris, T., & Löwe, J. (2016). Structural Insights into Ring Formation of Cohesin and Related Smc Complexes. *Trends in Cell Biology*, *26*(9), 680–693. https://doi.org/10.1016/j.tcb.2016.04.002

Godek, K. M., Kabeche, L., & Compton, D. A. (2015). *HHS Public Access*. *16*(1), 57–64. https://doi.org/10.1038/nrm3916.Regulation

Goodrich, D. W. (2006). The retinoblastoma tumor-suppressor gene, the exception that proves the rule. *Oncogene*, *25*(38), 5233–5243. https://doi.org/10.1038/sj.onc.1209616

Graña, X., Garriga, J., & Mayol, X. (1998). Role of the retinoblastoma protein family, pRB, p107 and p130 in the negative control of cell growth. *Oncogene*, *17*(25), 3365–3383. https://doi.org/10.1038/sj.onc.1202575

Hakem, R. (2008). *DNA-damage repair; the good, the bad, and the ugly*. (January), 589–605. https://doi.org/10.1038/emboj.2008.15

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell*, *144*(5), 646–674. https://doi.org/10.1016/j.cell.2011.02.013

Harashima, H., Dissmeyer, N., & Schnittger, A. (2013). Cell cycle control across the eukaryotic kingdom. *Trends in Cell Biology*, *23*(7), 345–356. https://doi.org/10.1016/j.tcb.2013.03.002

Hartwell, L. H., Weinert, T. A., Hartwell, L. H., & Weinert, T. E. D. A. (1989). *Checkpoints : Controls that Ensure the Order of Cell Cycle Events Linked references are available on JSTOR for this article : Checkpoints : Controls That Ensure the Order of Cell Cycle Events*. *246*(4930), 629–634.

Heald, R., & Khodjakov, A. (2015). *Thirty years of search and capture : The complex simplicity of mitotic spindle assembly*. *211*(6), 1103–1111. https://doi.org/10.1083/jcb.201510015

Hernando, E., Nahlé Gloria, Z. 1-J., Diaz-Rodriguez, E., Alaminos, M., Hemann, M., Michel, L., … Cordon-Cardo, C. (2004). Rb inactivation promotes genomic instability by uncoupling cell cycle progression from mitotic control. *Nature*, *430*(7001), 797–802. Retrieved from //www.ncbi.nlm.nih.gov/pubmed/15306814

Hiruma, Y., Sacristan, C., Pachis, S. T., Perrakis, A., & Kops, G. J. P. L. (2015). *Competition between MPS1 and microtubules at kinetochores regulates spindle checkpoint signaling*. *348*(6240).

Hoffmann, S., Dumont, M., Barra, V., Ly, P., Nechemia-Arbely, Y., McMahon, M. A., … Fachinetti, D. (2016). CENP-A Is Dispensable for Mitotic Centromere Function after Initial Centromere/Kinetochore Assembly. *Cell Reports*, *17*(9), 2394–2404. https://doi.org/10.1016/j.celrep.2016.10.084

Huang, H., Lampson, M., Efimov, A., & Yen, T. J. (2018). Chromosome instability in tumor cells due to defects in Aurora B mediated error correction at kinetochores. *Cell Cycle*, *17*(23), 2622–2636. https://doi.org/10.1080/15384101.2018.1553340

Ishak, C. A., & Dick, F. A. (2015). Conditional haploinsufficiency of the retinoblastoma tumor suppressor gene. *Molecular and Cellular Oncology*, *2*(1), 3–5. https://doi.org/10.4161/23723548.2014.968069

Ishak, C. A., Dick, F. A., Wang, W., Zhang, Y., Chen, R., Tian, Z., … Cleveland, D. W. (2017). Public Access NIH Public Access. *Genes and Development*, *8*(1), 1–14. https://doi.org/10.1038/mp.2011.182.doi

Kabeche, L., & Compton, D. A. (2012). Checkpoint-independent stabilization of kinetochore microtubule attachments by Mad2 in human cells. *Current Biology*, *22*(7), 638–644. https://doi.org/10.1016/j.cub.2012.02.030

Kaseda, K., Mcainsh, A. D., & Cross, R. A. (2000). *Dual pathway spindle assembly increases both the speed and the fidelity of mitosis*. https://doi.org/10.1242/bio.2011012

Kastl, J., Braun, J., Prestel, A., Mo, H. M., Huhn, T., & Mayer, T. U. (2015). *Mad2 Inhibitor-1 (M2I-1): A Small Molecule Protein − Protein Interaction Inhibitor Targeting the Mitotic Spindle Assembly Checkpoint*. *1*, 1–6. https://doi.org/10.1021/acschembio.5b00121

Knudson, A. G. (1971). *Mutation and Cancer : Statistical Study of Retinoblastoma*. *68*(4), 820–823.

Krenn, V., Overlack, K., & Primorac, I. (2014). KI Motifs of Human Knl1 Enhance Assembly of Comprehensive Spindle Checkpoint Complexes around MELT Repeats. *Current Biology*, *24*(1), 29–39. https://doi.org/10.1016/j.cub.2013.11.046

Lampson, M. A., Renduchitala, K., Khodjakov, A., & Kapoor, T. M. (2004). *Correcting improper chromosome – spindle attachments during cell division*. *6*(3). https://doi.org/10.1038/ncb1102

Lampson, M., & Grishchuk, E. (2017). Mechanisms to Avoid and Correct Erroneous Kinetochore-Microtubule Attachments. *Biology*, *6*(4), 1. https://doi.org/10.3390/biology6010001

Lara-gonzalez, P., & Westhorpe, F. G. (2012). The Spindle Assembly Checkpoint. *Current Biology*, *22*(22), R966–R980. https://doi.org/10.1016/j.cub.2012.10.006

Lee, H. (2014). *How Chromosome Mis-Segregation Leads to Cancer : Lessons from BubR1 Mouse Models*. *37*(10), 713–718.

Levine, A. J. (2020). p53: 800 million years of evolution and 40 years of discovery. *Nature Reviews Cancer*, *20*(August), 471–480. https://doi.org/10.1038/s41568-020-0262-1

Levine, M. S., & Holland, A. J. (2015). *The impact of mitotic errors on cell proliferation and tumorigenesis*. 620–638. https://doi.org/10.1101/gad.314351.118.620

Li, J., Dang, N., Wood, D. J., & Huang, J. (2017). The kinetochore-dependent and -independent formation of the CDC20-MAD2 complex and its functions in HeLa cells. *Nature Publishing Group*, (December 2016), 1–15. https://doi.org/10.1038/srep41072

Longworth, M. S., Herr, A., Ji, J. Y., & Dyson, N. J. (2008). RBF1 promotes chromatin condensation through a conserved interaction with the Condensin II protein dCAP-D3. *Genes and Development*, *22*(8), 1011–1024. https://doi.org/10.1101/gad.1631508

Magliocca, V., Petrini, S., Franchin, T., & Borghi, R. (2017). *Identifying the dynamics of actin and tubulin polymerization in iPSCs and in iPSC-derived neurons*. *8*(67), 111096–111109.

Manning, A L, Benes, C., & Dyson, N. J. (2014). *Whole chromosome instability resulting from the synergistic effects of pRB and p53 inactivation*. (April 2013), 2487–2494. https://doi.org/10.1038/onc.2013.201

Manning, Amity L., & Dyson, N. J. (2011). PRB, a tumor suppressor with a stabilizing presence. *Trends in Cell Biology*, *21*(8), 433–441. https://doi.org/10.1016/j.tcb.2011.05.003

Manning, Amity L., & Dyson, N. J. (2012a). RB: Mitotic implications of a tumour suppressor. *Nature Reviews Cancer*, *12*(3), 220–226. https://doi.org/10.1038/nrc3216

Manning, Amity L., & Dyson, N. J. (2012b). RB: Mitotic implications of a tumour suppressor. *Nature Reviews Cancer*, *12*(3), 220–226. https://doi.org/10.1038/nrc3216

Manning, Amity L., Longworth, M. S., & Dyson, N. J. (2010). Loss of pRB causes centromere dysfunction and chromosomal instability. *Genes and Development*, *24*(13), 1364–1376. https://doi.org/10.1101/gad.1917310

Manning, Amity L, Yazinski, S. A., Nicolay, B., Bryll, A., Zou, L., & Dyson, N. J. (2015). *enhancement of chromosome cohesion*. *53*(6), 993–1004. https://doi.org/10.1016/j.molcel.2014.01.032.Suppression

Marchetti, F., Bishop, J. B., Lowe, X., Generoso, W. M., Hozier, J., & Wyrobek, A. J. (2001). *Etoposide induces heritable chromosomal aberrations and aneuploidy during male meiosis in the mouse*.

Mastrangelo, D., De Francesco, S., Di Leonardo, A., Lentini, L., & Hadjistilianou, T. (2008). The retinoblastoma paradigm revisited. *Medical Science Monitor : International Medical Journal of Experimental and Clinical Research*, *14*(12), RA231-40. Retrieved from papers://845cdd87-89d0-41ea-a194-dbffe873f166/Paper/p3412

Mcgranahan, N., Burrell, R. A., Endesfelder, D., Novelli, M. R., & Swanton, C. (2012). Cancer chromosomal instability: therapeutic and diagnostic challenges. *EMBO Reports*, *13*(6), 528–538. https://doi.org/10.1038/embor.2012.61

Mchedlishvili, N., Wieser, S., Mouysset, J., Belwal, M., & Amaro, A. C. (2012). *Kinetochores accelerate centrosome separation to ensure faithful chromosome segregation*. 906–918. https://doi.org/10.1242/jcs.091967

McKinley, K. L., & Cheeseman, I. M. (2017). Large-Scale Analysis of CRISPR/Cas9 Cell-Cycle Knockouts Reveals the Diversity of p53-Dependent Responses to Cell-Cycle Defects. *Developmental Cell*, *40*(4), 405-420.e2. https://doi.org/10.1016/j.devcel.2017.01.012

Meraldi, P., Draviam, V. M., & Sorger, P. K. (2004). Timing and checkpoints in the regulation of mitotic progression. *Developmental Cell*, *7*(1), 45–60. https://doi.org/10.1016/j.devcel.2004.06.006

Michel, L. S., Liberal, V., Chatterjee, A., Sorger, P. K., Murty, V. V. V. S., & Benezra, R. (2001). *premature anaphase and chromosome instability in mammalian cells*. 355–359.

Mitchison, T. J. (1989). *Mitosis : basic concepts*.

Mogila, V. (2011). *An Intrinsic Cell Cycle Checkpoint Pathway Mediated by MEK and ERK in Drosophila*. *11*(4), 575–582. https://doi.org/10.1016/j.devcel.2006.08.010.An

Morgan, D. (1995). *Principles of CDK regulation*. *374*(March), 13–16.

Mouysset, J., Gilberto, S., Meier, M. G., Lampert, F., Belwal, M., Meraldi, P., & Peter, M. (2015). CRL4RBBP7 is required for efficient CENP-A deposition at centromeres. *Journal of Cell Science*, *128*(9), 1732–1745. https://doi.org/10.1242/jcs.162305

Muñoz-barrera, M., & Monje-casas, F. (2014). *Increased Aurora B activity causes continuous disruption of kinetochore – microtubule attachments and spindle instability*. https://doi.org/10.1073/pnas.1408017111

Murray, A. W. (2004). *Recycling the Cell Cycle : Cyclins Revisited*. *116*, 221–234.

Murray, A. W., & Kirschner, M. W. (2008). *Dominoes and Clocks : The Union of Two Views of the Cell Cycle Published by : American Association for the Advancement of Science Stable URL : http://www.jstor.org/stable/1704850*. *246*(4930), 614–621.

Musacchio, A. (2015). Review The Molecular Biology of Spindle Assembly Checkpoint Signaling Dynamics. *CURBIO*, *25*(20), R1002–R1018. https://doi.org/10.1016/j.cub.2015.08.051

Musacchio, A., & Desai, A. (2017). *A Molecular View of Kinetochore Assembly and Function*. https://doi.org/10.3390/biology6010005

Musacchio, A., & Salmon, E. D. (2007). *The spindle-assembly checkpoint in space and time*. *8*(May), 379–393. https://doi.org/10.1038/nrm2163

Nakajima, M., Kumada, K., Hatakeyama, K., Noda, T., Peters, J., & Hirota, T. (2007). *The complete removal of cohesin from chromosome arms depends on separase*. 4188–4196. https://doi.org/10.1242/jcs.011528

Negrini, S., Gorgoulis, V. G., & Halazonetis, T. D. (2010). *Genomic instability — an evolving hallmark of cancer*. *11*(March), 220–228. https://doi.org/10.1038/nrm2858

Nicholson, J. M., & Cimini, D. (2013). *Cancer karyotypes : survival of the fittest*. *3*(June), 1–9. https://doi.org/10.3389/fonc.2013.00148

Nishimura, K., Fukagawa, T., Takisawa, H., Kakimoto, T., & Kanemaki, M. (2009). An auxin-based degron system for the rapid depletion of proteins in nonplant cells. *Nature Methods*, *6*(12), 917–922. https://doi.org/10.1038/nmeth.1401

Nurse, P. (2000). *A Long Twentieth Century of the Cell Cycle and Beyond*. *100*, 71–78.

Olziersky, A.-M. P. M. (n.d.). *Mitotic live-cell imaging at different timescales*. *145*, 1–27.

Oser, M. G., Fonseca, R., Chakraborty, A. A., Brough, R., Spektor, A., Jennings, R. B., … Kaelin, W. G. (2019). Cells lacking the RB1 tumor suppressor gene are hyperdependent on aurora B kinase for survival. *Cancer Discovery*, *9*(2), 230–247. https://doi.org/10.1158/2159-8290.CD-18-0389

Potapova, T., & Gorbsky, G. (2017). The Consequences of Chromosome Segregation Errors in Mitosis and Meiosis. *Biology*, *6*(4), 12. https://doi.org/10.3390/biology6010012

Rando, T. C. T. (2013). *Molecular regulation of stem cell quiescence Tom*. *14*(6), 1–26. https://doi.org/10.1038/nrm3591.Molecular

Rescan, C., Cariou, S., Glaise, D., Zanninelli, G., Bilodeau, M., Loyer, P., … Baffet, G. (1999). *The Mitogen-Activated Protein Kinase Kinase / Extracellular Signal- Regulated Kinase Cascade Activation Is a Key Signalling Pathway Involved in the Regulation of G 1 Phase Progression in Proliferating Hepatocytes*. *19*(9), 6003–6011.

Ricke, R. M., Jeganathan, K. B., & Deursen, J. M. Van. (2011). *Bub1 overexpression induces aneuploidy and tumor formation through Aurora B kinase hyperactivation*. *193*(6). https://doi.org/10.1083/jcb.201012035

Samoshkin, A., Arnaoutov, A., Jansen, L. E. T., Ouspenski, I., Dye, L., Mcnally, J., … Strunnikov, A. (2009). *Human Condensin Function Is Essential for Centromeric Chromatin Assembly and Proper Sister Kinetochore Orientation*. *4*(8). https://doi.org/10.1371/journal.pone.0006831

Santaguida, S., Richardson, A., Iyer, D. R., Knouse, K. A., Wong, Y. L., Rhind, N., … Jolla, L. (2018). *HHS Public Access*. *41*(6), 638–651. https://doi.org/10.1016/j.devcel.2017.05.022.Chromosome

Satyanarayana, A., & Kaldis, P. (2009). *Mammalian cell-cycle regulation : several Cdks , numerous cyclins and diverse compensatory mechanisms*. 2925–2939. https://doi.org/10.1038/onc.2009.170

Schvartzman, J. M., Duijf, P. H. G., Sotillo, R., Coker, C., & Benezra, R. (2011). Mad2 is a critical mediator of the chromosome instability observed upon Rb and p53 pathway inhibition. *Cancer Cell*, *19*(6), 701–714. https://doi.org/10.1016/j.ccr.2011.04.017

Schvartzman, J. M., Sotillo, R., & Benezra, R. (2010). Mitotic chromosomal instability and cancer: Mouse modelling of the human disease. *Nature Reviews Cancer*, *10*(2), 102–115. https://doi.org/10.1038/nrc2781

Sheahan, S., Bellamy, C. O. C., Treanor, L., Harrison, D. J., & Prost, S. (2004). Additive effect of p53, p21 and Rb deletion in triple knockout primary hepatocytes. *Oncogene*, *23*(8), 1489–1497. https://doi.org/10.1038/sj.onc.1207280

Silk, A. D., Zasadil, L. M., Holland, A. J., Vitre, B., & Cleveland, D. W. (2013). *Chromosome missegregation rate predicts whether aneuploidy will promote or suppress tumors*. https://doi.org/10.1073/pnas.1317042110

Smith, R. J., Cordeiro, M. H., Davey, N. E., Vallardi, G., Ciliberto, A., Gross, F., … Gross, F. (2019). to Control Distinct Processes at the Kinetochore Article PP1 and PP2A Use Opposite Phosphodependencies to Control Distinct Processes at the Kinetochore. *CellReports*, *28*(8), 2206-
2219.e8. https://doi.org/10.1016/j.celrep.2019.07.067

Sotillo, R., Hernando, E., Díaz-Rodríguez, E., Teruya-Feldstein, J., Cordón-Cardo, C., Lowe, S. W., & Benezra, R. (2007). Mad2 Overexpression Promotes Aneuploidy and Tumorigenesis in Mice. *Cancer Cell*, *11*(1), 9–23. https://doi.org/10.1016/j.ccr.2006.10.019

Sotillo, R., Hernando, E., Díaz-rodríguez, E., Teruya-feldstein, J., Lowe, S. W., & Benezra, R. (2008). *mice*. *11*(1), 9–23.

Stevens, D., Gassmann, R., Oegema, K., & Desai, A. (2011). *Uncoordinated Loss of Chromatid Cohesion Is a Common Outcome of Extended Metaphase Arrest*. *6*(8). https://doi.org/10.1371/journal.pone.0022969

Szekely, L., Uzvolgyi, E., Jiang, W. Q., Durko, M., Wiman, K. G., Klein, G., & Sumegi, J. (1991). Subcellular localization of the retinoblastoma protein. *Cell Growth & Differentiation : The Molecular Biology Journal of the American Association for Cancer Research*, *2*(6), 287–295. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2064997

Takashi, N. H. C. (2015). *iMedPub Journals Pleiotropic Functions of RB Protein in Tumor Suppression Abstract Regulation of G 1 -S Transition by pRB Regulation of Mitosis by pRB*. 1–11.

Talluri, S., & Dick, F. A. (2012). *2012 pRB review.pdf*. *20*, 3189–3198.

Thoma, C. R., Toso, A., Meraldi, P., Krek, W., Thoma, C. R., Toso, A., … Krek, W. (2009). *Doubletrouble in mitosis caused by von Hippel- Lindau tumor-suppressor protein inactivation Doubletrouble in mitosis caused by von Hippel-Lindau tumor-suppressor protein inactivation*. *4101*, 20–22. https://doi.org/10.4161/cc.8.22.9907

Thompson, S. L., & Compton, D. A. (2011). *Chromosome missegregation in human cells arises through speci fi c types of kinetochore – microtubule attachment errors*. *108*(44), 17974–17978. https://doi.org/10.1073/pnas.1109720108

Trakala, M., Fernández-miranda, G., Castro, I. P. De, Heeschen, C., & Malumbres, M. (2013). *Aurora B prevents delayed DNA replication and premature mitotic exit by repressing p21 Cip1*. *12*(7), 1030–1041.

Vader, G., Medema, R. H., & Lens, S. M. A. (2006). *The chromosomal passenger complex : guiding Aurora-B through mitosis*. *173*(6), 833–837. https://doi.org/10.1083/jcb.200604032

Valdivia, M., Hamdouch, K., Ortiz, M., & Astola, A. (2009). CENPA a Genomic Marker for Centromere Activity and Human Diseases. *Current Genomics*, *10*(5), 326–335. https://doi.org/10.2174/138920209788920985

Vélez-Cruz, R., & Johnson, D. G. (2017). The retinoblastoma (RB) tumor suppressor: Pushing back against genome instability on multiple fronts. *International Journal of Molecular Sciences*,

18(8). https://doi.org/10.3390/ijms18081776

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

Waal, M. S. Van Der, Hengeveld, R. C. C., Horst, A. Van Der, & Lens, S. M. A. (2012). Cell division control by the Chromosomal Passenger Complex. *Experimental Cell Research*, *318*(12), 1407–1420. https://doi.org/10.1016/j.yexcr.2012.03.015

Wan, X., Cimini, D., Cameron, L. A., Salmon, E. D., & Doxsey, S. (2012). *The coupling between sister kinetochore directional instability and oscillations in centromere stretch in metaphase PtK1 cells*. *23*, 1035–1046. https://doi.org/10.1091/mbc.E11-09-0767

Wang, W., Zhang, Y., Chen, R., Tian, Z., Zhai, Y., Janz, S., … Yang, Y. (2017). Chromosomal instability and acquired drug resistance in multiple myeloma. *Oncotarget*, *8*(44), 78234–78244. https://doi.org/10.18632/oncotarget.20829

Wang, Y., Zheng, Z., Zhang, J., Wang, Y., Kong, R., Liu, J., … Ke, Y. (2015). *A Novel Retinoblastoma Protein (RB) E3 Ubiquitin Ligase (NRBE3) Promotes RB Degradation and Is Transcriptionally Regulated by E2F1 Transcription Factor *. *290*(47), 28200–28213. https://doi.org/10.1074/jbc.M115.655597

Waters, J. C., Chen, R., Murray, A. W., Salmon, E. D., Schultz, A., Cole, R., & Biol, G. S. J. C. (1998). *Localization of Mad2 to Kinetochores Depends on Microtubule Attachment, Not Tension*. *141*(5), 1181–1191.

Weaver, B. A., & Cleveland, D. W. (2008). The Aneuploidy Paradox in Cell Growth and Tumorigenesis. *Cancer Cell*, *14*(6), 431–433. https://doi.org/10.1016/j.ccr.2008.11.011

Wenzel, E. S., & Singh, A. T. K. (2018). *Cell-cycle Checkpoints and Aneuploidy on the Path to Cancer*. *5*, 1–5. https://doi.org/10.21873/invivo.11197

Wilhelm, T., Olziersky, A., Harry, D., Sousa, F. De, Vassal, H., Eskat, A., & Meraldi, P. (2019).

disengagement. *Nature Communications*, 1–14. https://doi.org/10.1038/s41467-019-11584-0

William Benedict. (1922). *Retinoblastoma in identical twins**.

Wood, K. W., Sakowicz, R., Goldstein, L. S. B., & Cleveland, D. W. (1997). *CENP-E Is a Plus End – Directed Kinetochore Motor Required for Metaphase Chromosome Alignment*. *91*, 357–366.

Yang, Zhe, Maciejowski, J., & Lange, T. De. (2017). *Nuclear Envelope Rupture Is Enhanced by Loss of p53 or Rb*. (6), 1579–1587. https://doi.org/10.1158/1541-7786.MCR-17-0084

Yang, Zhenye. (2008). *Extra centrosomes and/or chromosomes prolong mitosis inhuman cells*. *10*(6), 748–751.

Yao, Y. (2014). *Genomic Instability and Cancer*. https://doi.org/10.4172/2157-2518.1000165.Genomic

Zámborszky, J., Szikriszt, B., Gervai, J. Z., Pipek, O., Póti, Á., Krzystanek, M., … Szallasi, Z. (2017). *Loss of BRCA1 or BRCA2 markedly increases the rate of base substitution mutagenesis and has distinct effects on genomic deletions*. (July 2016), 746–755. https://doi.org/10.1038/onc.2016.243

Zilfou, J. T., & Lowe, S. W. (2009). Tumor suppressive functions of p53. *Cold Spring Harbor Perspectives in Biology*, *1*(5), 1–12.

Ishak, C. A., Dick, F. A., Wang, W., Zhang, Y., Chen, R., Tian, Z., … Cleveland, D. W. (2017). Public Access NIH Public Access. *Genes and Development*, *8*(1), 1–14. https://doi.org/10.1038/mp.2011.182.doi

Ishak, C. A., Dick, F. A., Wang, W., Zhang, Y., Chen, R., Tian, Z., … Cleveland, D. W. (2017). Public Access NIH Public Access. *Genes and Development*, *8*(1), 1–14. https://doi.org/10.1038/mp.2011.182.doi

Ishak, C. A., Dick, F. A., Wang, W., Zhang, Y., Chen, R., Tian, Z., … Cleveland, D. W. (2017). Public Access NIH Public Access. *Genes and Development*, *8*(1), 1–14.

https://doi.org/10.1038/mp.2011.182.doi

"Tieni costantemente a mente in quante cose tu stesso hai testimoniato di essere già cambiato. L'universo è cambiamento, la vita è comprensione"

Marco Aurelio

Acknowledgments

Science is a collective effort and its collegial nature made it reproducible and reliable.

There are many people that I would like to thank and acknowledge but clearly the first one is professor Patrick Meraldi. Almost 5 years ago he convinced me to join his lab and I am very grateful that he did it. Patrick is a great boss, a brilliant scientist, a passionate teacher and a fantastic human being. All these features helped me a lot during my PhD.

I want to thank my jury members Prof Monica Gotta, Prof Florian Steiner and Prof Patrick Heun. Their advices and their help were precious and I did not take it for granted.

Among my jury members I want to especially thank Prof Monica Gotta. I had the chance to share lab meetings and the lab with Monica for 4 years. Her presence was important and useful not only scientifically but also from a 'human' point of view.

A special thank goes to my academic godfather, Prof. Claes Wollheim. Claes our meetings and conversations were extremely enlightening to me. For me you are an example as a scientist should be: curious, open-minded, passionate and committed.

As previously mentioned science is a collective effort hence I need to thank all my colleagues, we did part of journey together and was great!

Thanks to: Amanda (we made it!!), Ennio, Cedric (my mentor), Elena, Amy, Damian, Emeline, Therese, Morgan and Devashish (SVD) for all the support, morally and scientifically. Thanks to the past and present members of Monica Gotta's lab, Ainoa, Simona, Ida, Victoria, Adeline, Sofia, Meghan, Nikola, Luca and Françoise.

Inevitably with some people I created a stronger bound that I'm sure is here to stay: Bjango and Alex you are my older brother and my older (not so older) sister. I thank you mostly for the non-scientific time.

Daniela Harry need a special mention, beyond being a perfect lab manager you are a great human being. You taught me so much and an I'm extremely grateful.

Many other people of the department made my days here full of joy and need to be acknowledged: Nico, Claire, Nico, Raphael, Ebru, Karolina, Miguel, Gonzalo and many others that I'm forgetting.

Thanks to all the PHYM department for the fruitful discussions and feedbacks.

Thanks to my former colleagues in Torino, Simona and Valeria that taught me that science can be also fun.

Thanks to Luca Cirillo, I'm sure our paths will cross again.

Thanks to Visa Suomi, you taught me the passion for science.

Thanks to my former PIs: Vincenzo D'angiolella, Massimo Squatrito and Silvia Deaglio.

Thanks to my Italian friends Tommaso and Marco that despite the distance are always present in my life and they will be in the future.

Thanks to Marco that I met at the beginning of my PhD, our friendship is a journey and so far, one of the best that I ever made.

Thanks to Davide, our similar background and mentality bound us together a lot.

If I'm writing my acknowledgment now is thank to a single person, Martina. Thank you for all, without you nothing would be possible.

Last but not least my family. A big thank to all of you, you believed in me and you are my biggest supporters since 1991. Finally, a special thanks to a person that cannot be here today but she would have loved to be: my grandmother. This thesis is dedicated to you.