Master of Science Dissertation

CHROMOSOMAL INSTABILITY AND KEY MITOTIC REGULATORS IN LOW PASSAGE COLORECTAL TUMOR CELL LINES

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Abstract

Cell division is fundamental for growth, maintenance and repair of cells and tissues. Dividing cells must distribute one copy of the duplicated genome into each new daughter nucleus. The fidelity of chromosome segregation relies on the correct attachment of sister kinetochores (KTs) to microtubules (MTs) of opposite spindle poles (amphitelic attachments) and on the Spindle Assembly Checkpoint (SAC), a biochemical pathway that prevents anaphase until the former state is achieved. Accurate KT-MT attachments result from a tightly controlled balance of microtubule stabilizing and destabilizing forces at KTs to allow the release of incorrectly attached MTs and selectively stabilize amphitelic attachments.

Cancer cells are prone to chromosome segregation errors in mitosis. This was shown to cause chromosomal instability (CIN), the continuous gain and loss of chromosomes during cell division, which is intimately linked to aneuploidy and a proposed driver of tumorigenesis. Aneuploidy is observed in 65%–70% of sporadic colorectal cancers (CRCs), but whether CIN represents its underlying cause is unclear due to the challenges associated with the analysis of mitotic fidelity *in situ*. Hence, the occurrence of CIN *in vivo*, its molecular underpinnings and its impact for CRC remain elusive. *In vitro* studies indicate that CRC cell lines have hyperstable KT-MT interactions that prevent the correction of erroneous attachments and thereof, thought to represent a major cause of CIN. This was however observed in immortalized cell lines, which typically accumulate genetic aberrations with increasing passage numbers. This causes variability, limits conclusion accuracy and precludes physiological/clinic correlation. In the present work we resorted to novel low passage primary cultures of CRC cell lines derived from primary tumors to examine the occurrence of CIN and characterize the genetic integrity and expression pattern of key mitotic regulators known to be involved in KT-MT attachments and SAC signaling. Human fibroblasts, a microsatellite instable (MSI) cell line and microsatellite stable (MSS) cell lines with varying levels of aneuploidy were used. Next-generation sequencing (NGS) revealed absence of mutations in the coding sequence of *Aurora A*, *Aurora B*, *Plk1* and *Mps1* genes. Expression of these genes at the transcript and protein levels was monitored by RT-qPCR and Western Blotting, respectively. The mitotic kinases were found to be prevalently overexpressed in MSS cell lines with higher levels of aneuploidy.

This work sets the ground for future studies aiming to examine the cause of chromosome segregation errors in CRCs and their contribution for CIN. Importantly, as these novel CRC cell lines closely resemble the biology of the original tumor, a detailed knowledge of their mitotic behavior will be critical to design anti-cancer therapeutic strategies that will have an impact *in vivo*.

Resumo

A divisão celular é fundamental para o crescimento, manutenção e reparação de células e tecidos. Este processo deve distribuir uma cópia do genoma já duplicado para cada um dos núcleos das novas células filhas. A fidelidade da segregação cromossómica depende não só de uma ligação correta entre os cinetocoros e os microtúbulos de polos opostos do fuso (ligações anfitélicas), mas também do *Spindle Assembly Checkpoit* (SAC), uma via bioquímica que previne a anafase até o estado anterior ser atingido. Uma ligação correta entre os cinetocoros e os microtúbulos resulta de um balanço equilibrado nos cinetocoros de forças estabilizantes e destabilizantes de microtúbulos, de forma a permitir que estes últimos se libertem quando incorretamente ligados e que as ligações anfitélicas sejam seletivamente estabilizadas.

As células cancerígenas são propensas a erros de segregação cromossómica durante o processo de divisão mitótica. Tais erros podem causar instabilidade cromossómica, isto é, ganhos ou perdas contínuos de cromossomas durante a divisão celular. Este fenómeno está intimamente ligado a aneuploidia e poderá conduzir a tumorogénese. A aneuploidia é observada em 65%-70% dos cancros colorretais esporádicos, mas não é claro se a instabilidade cromossómica representa a sua causa subjacente, devido aos desafios associados à análise da fidelidade mitótica *in situ*. Assim, a ocorrência de instabilidade cromossómica *in vivo*, os seus fundamentos moleculares e o seu impacto para o cancro colorretal são ainda pouco descritos. Estudos *in vitro* indicam que as linhas celulares de cancro colorretal têm interações hiperestáveis entre cinetocoros e microtúbulos, que impedem a correção de ligações erradas e, assim, pensa-se que representam uma das principais causas de instabilidade cromossómica. No entanto, isto foi observado em linhas celulares imortalizadas que normalmente acumulam aberrações genéticas com o crescente número de passagens. Este facto causa variabilidade, limita a precisão da conclusão e impede a correlação fisiológica/clínica. No presente trabalho, recorremos a culturas primárias de baixa passagem de linhas celulares de cancro colorretal derivadas de tumores primários para examinar a ocorrência de instabilidade cromossómica e caracterizar a integridade genética e o padrão de expressão dos principais reguladores mitóticos conhecidos por estar envolvidos em ligações cinetocoros-microtúbulos e sinalização SAC. Foram utilizados fibroblastos humanos, uma linha celular *microsatellite instable* (MSI) e linhas celulares *microsatellite stable* (MSS) com diferentes níveis de aneuploidia. *Next generation sequencing* (NGS) revelou a ausência de mutações na sequência codificante dos genes *Aurora A*, *Aurora B*, *Plk1* e *Mps1*. A expressão desses genes a nível de transcrição e a nível proteico foi monitorizada por *RT-qPCR* e *Western Blot*, respetivamente. As cinases mitóticas revelaram-se predominantemente sobre-expressas nas linhas celulares MSS com graus mais elevados de aneuploidia.

Este trabalho estabelece a base para estudos futuros com o objetivo de examinar a causa dos erros de segregação cromossómica em cancro colorretal e a sua contribuição para a instabilidade cromossómica. De realçar que como as linhas celulares de cancro colorretal utilizadas se assemelham bastante à biologia do tumor original, um conhecimento detalhado do seu comportamento mitótico será crítico para projetar estratégias terapêuticas anticancerígenas que terão um impacto *in vivo*.

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List of Abbreviations

AurkA	Aurora A
AurkB	Aurora B
APC	Adenomatous polyposis coli
APC/C	Anaphase Promoting Complex/Cyclosome
Bub	Budding uninhibited by benzimidazoles
BubR1	Bub1-related protein kinase
C-Mad2	closed Mad2
Cdc	Cell division cycle
Cdk	Cyclin-dependent kinase
CNA	Copy number alteration
CPSF6	Cleavage And Polyadenylation Specific Factor 6
cDNA	complementary DNA
Cenp	Centromere protein
CIN	Chromosomal instability
CRC	Colorectal Cancer
CPC	Chromosomal passenger complex
DNA	Deoxyribonuclein Acid
EDTA	ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
Hec1	Highly expressed in cancer 1
IGV	Integrative genomics viewer
IF	Immunofluorescence
INCEP	Inner Centromere Protein
KMN	Knl-1/Mis12 complex/Ndc80 complex
Knl-1	KT null-1
KT	Kinetochore
Mad	Mitotic arrest deficient
MCC	Mitotic checkpoint complex
MSI	Microsatellite instable
MSI-H	MSI high
MSI-L	MSI low
MSS	Microsatellite stable

MT	Microtubule
Mps1	Monopolar spindle 1
Ndc80	Nuclear division cycle 80
NGS	Next Generation Sequencing
O-Mad2	open Mad2
p	phosphorylated
PBS	Phosphate-buffered saline
PBST20	PBS 0,05% Tween20
PCR	Polymerase Chain Rection
Plk	Polo-like kinase
PP2A	Protein phosphatase 2A
RT-qPCR	Quantitative reverse transcription PCR
RNA	Ribonucleic acid
RPE	Retinal Pigment Epithilium
S-CIN	structural CIN
SAC	Spindle Assembly Checkpoint
SDS	Sodium dodecyl sulfate
SNP	Single Nucleotide Polymorphism
TPX2	Targeting protein for Xklp2
w-CIN	whole CIN
WB	Western Blot
%GC	Guanine-cytosine content

CHAPTER 1

INTRODUCTION

1.1. Cell division

Cell division is critical for growth, maintenance and repair of cells and tissues (Clift et al., 2013; Ohkura, 2016). The eukaryotic cell cycle is made up of two main phases, interphase and mitosis. Interphase prepares the cell for the actual genome division that takes place in mitosis. It consists of 3 sub-phases termed G1, S and G2. Firstly on G1, the cellular contents are duplicated and cells synthetize enzymes required for the next step (Lian et al., 2016; Salazar-Roa et al., 2016). Secondly in S phase, the chromosomes and centrosomes are duplicated. Finally in G2 phase, the duplicated centrosomes separate and cells inspect for replication errors (Lian, 2016; Salazar-Roa, 2016). In mitosis (M phase) the duplicated genome is segregated into two new daughter cells. The cell cycle is tightly regulated by checkpoint mechanisms that delay progression into the next stage until errors are corrected. Thus, checkpoints ensure that genomic stability is maintained in dividing cells (Lian, 2016).

1.2. Mitosis

The purpose of mitosis is to distribute one copy of each chromosome that has been duplicated in S phase into each one of the two daughter cells (Foley et al., 2013). It is a complex and highly regulated process encompassing five steps: prophase, prometaphase, metaphase, anaphase, telophase, **(Figure 1)** followed by cytokinesis (Lian, 2016). The major components of the mitotic apparatus are the kinetochores (KTs), spindle microtubules (MTs) and the spindle assembly checkpoint (SAC) (McEwen et al., 2007).

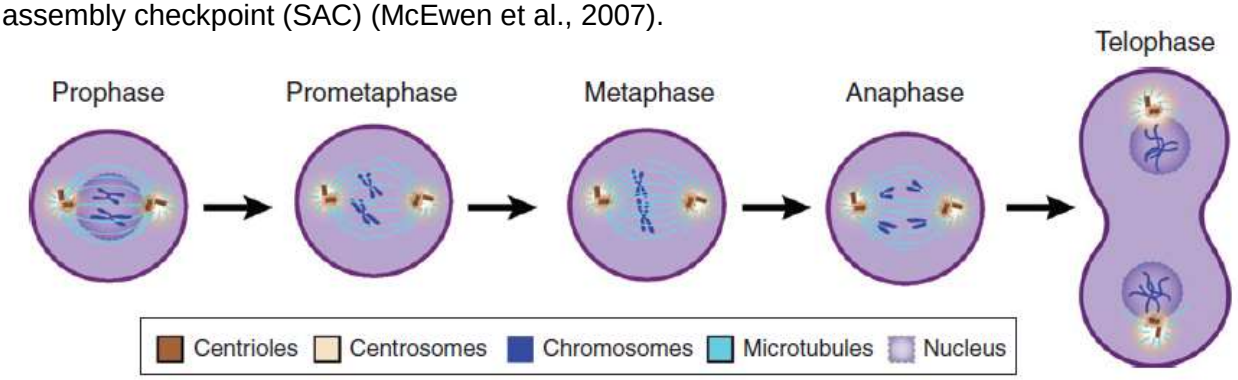

Figure 1. Mitotic stages. Mitosis begins with **prophase**, where chromosomes condensation starts and nuclear envelope breaks down. The microtubule cytoskeleton begins to re-form as a bipolar spindle. Then, in **prometaphase**, KTs start to interact with the spindle MTs so chromosomes are directed to the equatorial plan of the cell. **Metaphase** describes the stage in which all chromosomes are aligned at the spindle equator and connected to MTs from opposite spindle poles. This biorientation is critical to ensure equal partitioning of the genome to the dividing daughter cells. Sister chromatids are separated in **anaphase**, moving to opposite spindle poles. Finally, during **telophase**, chromatids decondense and the nuclear envelope re-forms. As illustrated in Schatten, 2013.

1.2.1. Mitotic apparatus components: kinetochore, microtubules and SAC

The structure of the KTs began to be elucidated through electron microscopy (McEwen, 2007). The observation of vertebrate cells chromosomes showed that KTs have a trilaminar morphology composed by the inner KT, the outer KT and the fibrous corona **(Figure 2).** The inner plate contacts with the centromeric chromatin and the outer plate contacts with the spindle MTs. The fibrous corona is a dense array of fibers that extend away from the outer KT (Cheeseman et al., 2008; Gascoigne et al., 2011).

Today it is known that KTs are macro-molecular complexes composed of more than 90 proteins (McEwen et al., 2010). KTs mediate the attachment of chromosomes to spindle MTs, allowing their biorientation on the metaphase plate and the correct segregation of sister chromatids at anaphase (Gascoigne, 2011; McEwen, 2010).

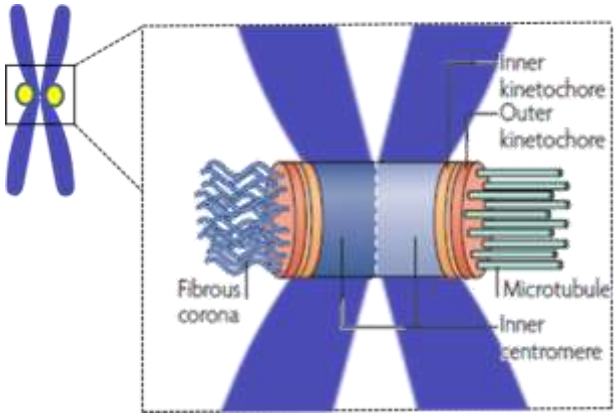

Figure 2. Scheme of the vertebrate kinetochore structure. In the mitotic chromosome illustrated, one of the sister chromatids (**right**) is attached to MTs, unlike the other (**left**), which is unattached. The inner and outer kinetochore, the inner centromere and the fibrous corona, detectable on the unattached kinetochore, are also represented. Adapted from Cheeseman, 2008.

MTs are 25-nm diameter polymers of α-β-tubulin dimers (Cheeseman, 2008). During mitosis, KT establish a direct physical connection with the bipolar spindle-shaped structure built from MTs (Cheeseman, 2008). This process requires a large number of structural and regulatory proteins. One of the most relevant players is the four-subunit Ndc80 complex, located on the KT outer plate (Cheeseman, 2014; McEwen, 2010). This hetero-tetramer is composed by Ndc80 (Hec1), Nuf2, Spc24 and Spc25 in a 1:1:1:1 stoichiometry. It forms an extended rod-shaped structure that binds directly to microtubule polymers through a Calponin (calcium binding protein) homology domain and a positively charged amino-terminal tail (Cheeseman, 2014).

Except for budding yeast, eukaryotic KTs bind to multiple spindle MTs. In humans 15-20 MTs, known as kinetochore fibers (K-fibers), are able to bind to each KT (Cheeseman, 2008;

McEwen, 2010). The interaction between KTs and MTs is complex, as MTs are highly dynamic polymers undergoing constant changes in their length due to polymerization and depolymerization events (Cheeseman, 2014; McEwen, 2010). To establish stable KT-MT attachments, KT must have mechanisms that control the growth and shrinkage cycles of MTs accordingly to the direction of chromosome motion (Cheeseman, 2014; McEwen, 2010).

The major cell cycle control mechanism that acts during mitosis is the mitotic checkpoint, also known as the spindle assembly checkpoint (SAC) (Rao et al., 2005; Weaver et al., 2006; Weaver, Silk, et al., 2007). It is a conserved molecular pathway that ensures the fidelity of chromosome segregation by delaying anaphase onset until all sister-KTs have made attachments to MTs from opposite spindle poles (Bakhoum, Thompson, et al., 2009; Malumbres, 2011; Weaver, 2006). This mechanism prevents chromosome mis-segregation and consequently aneuploidy in the newly formed cells (Malumbres, 2011; Weaver, 2006; Weaver & Cleveland, 2007). Core components of SAC are the Ser/Thr kinases Mps1 (monopolar spindle protein 1, also known as Mps1 in humans) and Bub1 (budding uninhibited by benzimidazoles 1 homolog), as well as non-kinase components BubR1 (budding uninhibited by benzimidazoles 1 homolog β, also known as Mad3), Bub3 (budding uninhibited by benzimidazoles 3 homolog), Mad1 (mitotic arrest deficient-like 1) and Mad2 (mitotic arrest deficient-like 2) (Combes et al., 2017; Foley, 2013; Heinrich et al., 2013; Ricke et al., 2008). Protein kinases Bub1 and Mps1 are critical for SAC activation; they are responsible for kinetochore recruitment of BubR1, Mad1 and Mad2 proteins (Diogo et al., 2016). Mad1 and Mad2 form a complex in which Mad2 adopts a closed conformation (C-Mad2) (Heinrich, 2013). The localization of all these proteins at the kinetochore is transient and Mad1, Mad2 and BubR1 are progressively dislodged as microtubule attach (Cheeseman, 2008).

The target of the SAC is the anaphase-promoting complex also known as the cyclosome (APC/C). It is a E3 ubiquitin ligase that targets Securin and Cyclin B for degradation by the 26S proteasome. Degradation of these proteins allows cells to undergo anaphase and exit mitosis (Diogo et al., 2016).

The SAC is activated at unattached KTs **(Figure 3, A)** (Diogo et al., 2016; Hernando et al., 2004). Mad1-Mad2 heterodimers localized at unattached kinetochore recruit and convert cytosolic “open” inactive Mad2 into “closed” active Mad2. This interacts with cell division cycle 20 (Cdc20), Bub3 and BubR1, to form the mitotic checkpoint complex (MCC), which acts as a pseudo-substrate of APC/C, hence blocking its activity (Diogo et al., 2016; Heinrich, 2013; Tanaka et al., 2009). This prevents the APC/C from targeting Cyclin B and Securin for

degradation by the 26S proteasome. The result is the inhibition of sister chromatids separation and of mitotic exit (Diogo et al., 2016; Hernando, 2004).

When all chromosomes become attached to spindle MTs and achieve biorientation the SAC is silenced **(Figure 3, B)**. MCC assembly ceases, which allows APC/C activation by Cdc20 and consequently ubiquitination of Securin and Cyclin B. APC/C-mediated ubiquitylation of Securin (Diogo et al., 2016) results in the activation of separase, the protease that cleaves the cohesion complexes linking sister chromatid pairs, and triggers their separation (Foley, 2013; Lu et al., 2014). Degradation of cyclin B renders cyclin-dependent kinase 1 (CDK1) inactive, inducing late mitotic events and promoting mitosis exit (Diogo et al., 2016; Tanaka, 2009).

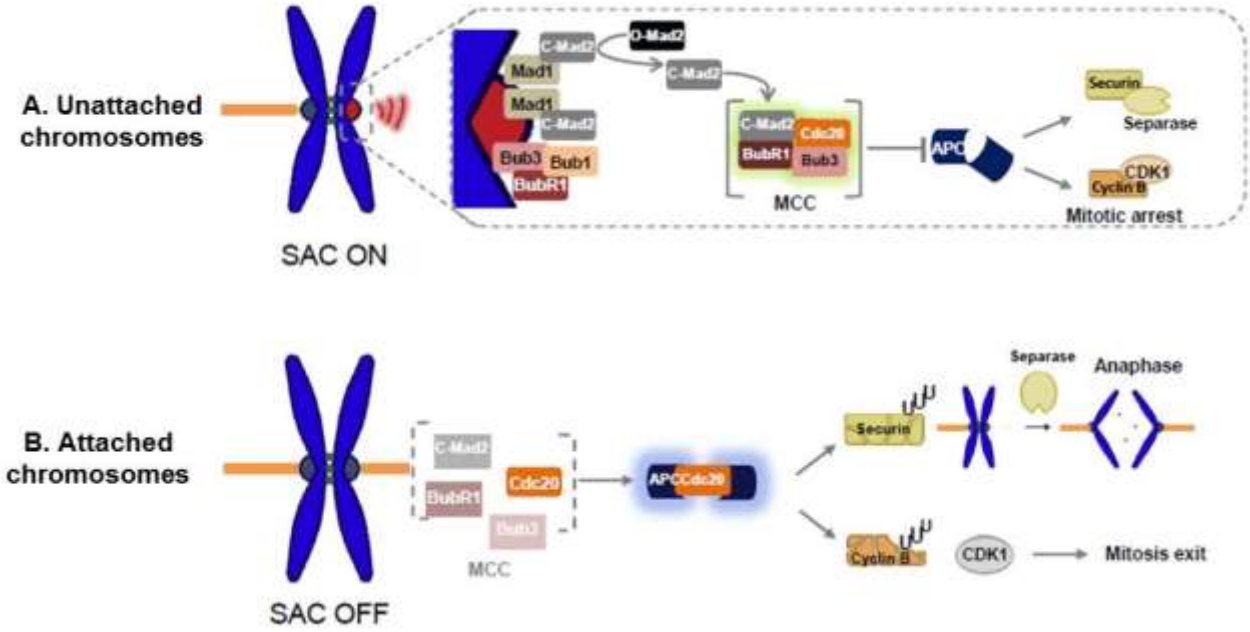

Figure 3. Molecular View of the Spindle Assembly Checkpoint (SAC) Pathway. (A) Unattached KTs engage the SAC by promoting MCC assembly. Binding of the MCC to the APC/C precludes it from targeting Cyclin B and Securin for degradation, hence halting anaphase onset and mitotic exit. **(B)** When KTs attach to MTs, MCC formation ceases allowing APC/C activation. Active APC/C ubiquitinates Securin and Cyclin B for degradation which allows separase to cleave the cohesion complex linking sister chromatid pairs and renders CDK1 inactive. Adapted from Diogo et al., 2016**.**

1.2.2. The mitosis process

Mitosis begins in prophase when the replicated chromosomes become condensed within the nucleus (Cheeseman, 2008; McEwen, 2007). Nuclear envelope breakdown sets the beginning of prometaphase, when KTs start to interact with the spindle MTs (Cheeseman, 2008). As KTs attach to MTs, chromosomes are directed to the equatorial plan of the cell. Metaphase describes the stage in which all chromosomes are aligned at the spindle equator and connected

to MTs from opposite spindle poles (biorientation). This is critical as it ensures equal partitioning of the genome (McEwen, 2007; Ye et al., 2015). After all chromosomes have attached to MTs, cohesin is cleaved and the sister chromatids are separated in anaphase (Foley, 2013). Then, during telophase, chromatids decondense and the nuclear envelope re-forms. Finally in cytokinesis an actin myosin ring bisects the cell between the separated chromatid masses, generating two daughter cells which have an exact copy of the duplicated genome (Cheeseman, 2008).

1.2.3. Mitotic kinases in the spindle assembly checkpoint

The reversible phosphorylation of proteins carried out by kinases and phosphatases represents a regulatory strategy common to most intracellular signaling pathways (Combes, 2017). Several mitotic kinases required to control KT-MT attachments and SAC signaling are phosphorylated during mitosis, including Aurora A, Aurora B, Plk1 and Mps1 (Nigg, 2001). Their main functions are synthetized in **Table I**. A more detailed description will be discussed in the following sections.

Table I. Key mitotic players and respective main functions during cell division

PROTEIN	MAIN FUNCTIONS	REFERENCES
Mps1	Mitotic progression Centrosome duplication Chromosome congression SAC	(Conde et al., 2013) (Tandle et al., 2016) (Combes, 2017) (Sherr et al., 2017)
Plk1	Centrosome maturation Chromosome segregation Chromosome congression Cytokinesis SAC	(Archambault et al., 2012) (Penna et al., 2017) (Otto et al., 2017) (Sherr, 2017) (Combes, 2017)
AURKA	Mitotic entry Chromosome alignment Centrosome maturation Centrosome separation SAC	(Goldenson et al., 2014) (Otto, 2017) (Sherr, 2017) (Yu et al., 2017)
AURKB	Sister chromatid cohesion Bipolar spindle assembly KT-MT attachment correction Cytokinesis SAC	(Goldenson, 2014) (Pitts et al., 2016) (Sherr, 2017) (Penna, 2017)

1.2.3.1. Aurora B

The Chromosomal Passenger Complex (CPC) is a major mitotic regulator, which ensures faithful chromosome segregation through the regulation of different mitotic events (reviewed in Ruchaud et al., 2007; van der Waal et al., 2012). The complex is composed by Aurora B, a serine/threonine kinase, and three non-enzymatic subunits, Inner Centromere protein (INCENP), Borealin and Survivin, which function as a structural unit to control Aurora B location, activity and stability (Jeyaprakash et al., 2007).

In early mitosis, the CPC moves from the chromosome arms to the centromeres. This location is mediated by Survivin, which recognizes the phosphorylation of histone H3 by Haspin kinase (Yu, 2017). Then, and until metaphase the complex localizes in the inner centromeres (Goldenson et al., 2015; Musacchio, 2015; Yu, 2017) where it is mainly involved in the correction of aberrant KT-MT attachments (Diogo et al., 2016; Moutinho-santos et al., 2012; Musacchio, 2015). Centromeric Aurora B directly phosphorylates KT proteins that mediate the attachment to MTs, leading to the destabilization of incorrect KT-MTs attachments and further establishment of new and correct KT-MT interactions (Cimini et al., 2006; Krenn et al., 2015; Lin et al., 2012). This correction mechanism involves a transient state of unattached KTs, which can activate the SAC. Thus, Aurora B was initially proposed to have an indirect role in SAC function (Pinsky et al., 2006). However, Aurora B-dependent Mps1 KT recruitment indicates a more direct involvement of this kinase in SAC signaling (Santaguida et al., 2010; Saurin et al., 2011).

1.2.3.2. Mps1

The serine-threonine kinase Mps1 was first identified in budding yeast where it was shown to be important for spindle pole body (SPB) duplication (Lauze et al., 1995; Winey et al., 1991). Later, it was discovered that Mps1 has critical roles in SAC function (Liu et al., 2012). Upon mitotic entry, Mps1 is recruited to unattached KTs in a Hec1/Ndc80 and Aurora B dependent manner, which correlates with a peak in its kinase activity (Higgs et al., 2005; Martin-Lluesma, 2002; Santaguida, 2010; Saurin, 2011; Stucke et al., 2004). The dimerization and subsequent *trans* autophosphorylation of its activating T-loop renders Mps1 fully active (Hewitt et al., 2010; Kang et al., 2007). At the onset of mitosis, Mps1 promotes Mad1/C-Mad2 recruitment to unattached KTs (Hewitt, 2010; Tighe et al., 2008). Afterwards, Mps1 kinase activity continuously promotes O-Mad2 recruitment to the Mad1/C-Mad2 complex at KT, in order to promote the conformational change of Mad2 and subsequent Cdc20 binding to form the MCC (Hewitt, 2010; S. Kim et al., 2011). Additionally, studies in human cells and yeast have shown that Mps1

phosphorylates Met-Glu-Leu-Thr (MELT) motifs in Spc105/Blinkin/Kn11, which creates docking sites for recruitment of Bub3-Bub1 and possibly Bub3/BubR1 to the KTs (London et al., 2012; Shepperd et al., 2012; Yamagishi et al., 2012).

Mps1 also has important roles in KT-MT attachments. It regulates Aurora B activity in at least two distinct ways. Mps1 can directly phosphorylate Borealin, which was shown to enhance Aurora B activity in human cells (Bourhis et al., 2009; Jelluma et al., 2008; Sliedrecht et al., 2010). Mps1 can also contribute to CPC accumulation in the centromeres (van der Waal et al., 2012). Both processes ensure an efficient correction of aberrant KT-MT attachments by Aurora B during the mitotic process (van der Waal et al., 2012).

1.2.3.3. Polo kinase

The polo-kinase (Plk) family was discovered in *Drosophila* approximately three decades ago (Llamazares et al., 1991; Sunkel et al., 1988). In humans, there are five polo kinases (Plk1-5) with different and specialized roles, from cellular proliferation to tissue-specific functions. Plk1 is the most studied member of the Plk family and it has a central role in cell cycle regulation and chromosome segregation.

Plk1 contribution to SAC signaling is not fully understood (Archambault, 2012; Schmucker et al., 2014). Studies using a RPE-1 cell line demonstrated that Plk1 directly phosphorylates Mps1 and stimulates its autophosphorylation in vitro. Moreover, it can also phosphorylate MELT motifs, promoting the recruitment of SAC components, just previously described for Mps1 (Schubert et al., 2015). Studies showed that Aurora A, together with its cofactor Bora, can directly phosphorylate Plk1 before mitotic entry (Archambault, 2012; Goldenson, 2015; Yu, 2017). Once activated, Plk1 directly phosphorylates Cdc25B, a phosphatase needed for Cyclin B/Cdk1 centrosomal activation (Vader et al., 2008).

The expression of Plk1 is tightly regulated during cell division, increasing during G2. The phosphorylation of the conserved Thr210 in the activation loop of the kinase domain reaches its maximum during mitosis (Bruinsma et al., 2012; Schmucker, 2014). In prometaphase and metaphase, Plk1 is preferentially in the KTs, where it was associated with the attachment of chromosomes to the spindle microtubules (Combes, 2017; Schmucker, 2014). It was demonstrated that Plk1 phosphorylation of BubR1 on Ser676 is important for the stability of KT-MT interactions and chromosome alignment, as it promotes PP2A-B56α (protein phosphatase A) recruitment. This phosphatase counteracts Aurora B activity, stabilizing initial KT-MT attachments (Suijkerbuijk et al., 2012).

1.2.3.4. Aurora A

Aurora A kinase is required for efficient mitotic entry and chromosome alignment. However, it is not so well study as Aurora B (Vader, 2008). This kinase can be activated by two distinct ways (Vader, 2008). The autophosphorylation of the kinase in its T288 segment usually occurs in the centrosomes and promotes Aurora A phosphorylation of downstream targets (Vader, 2008; Zorba et al., 2014). Nevertheless, Aurora A can also be activated by other proteins. Among them, TPX2 (Targeting protein for Xklp2) is considered its best characterized cofactor. TPX2-mediated Aurora A activation is tough to occur in the spindle microtubules (Zorba, 2014).

The levels of Aurora A expression reach a maximum at the centrosomes in G2 phase (Goldenson, 2015; Zorba, 2014). This peak in early mitosis is justified by the involvement of the kinase in promoting timely mitotic entry (Goldenson, 2015). As previously described, Aurora A promotes Plk1 activation in G2 (Nikonova et al., 2013). Moreover, it was shown to directly phosphorylate Cdc25, leading to Cyclin B/Cdk1 activation at the centrosomes (Nikonova, 2013; Vader, 2008).

A recent study demonstrated that Aurora A has also a role in chromosome segregation. During early mitosis, Aurora A phosphorylates and activates Haspin. In turn, Haspin phosphorylates histone H3. As previously said, Survivin recognizes the phosphorylated histone H3, leading to CPC accumulation in the centromeres, where it will correct possible aberrant KT-MT attachments (Yu, 2017).

1.3. Chromosomal instability and aneuploidy

Aneuploidy describes the incorrect genomic content that occurs when there is a loss or gain of one or more chromosome, or a chromosome portion (Bakhoum, 2009; Lacroix et al., 2012; Zasadil et al., 2013). It frequently results from chromosome mis-segregation events during mitosis, (Bakhoum, 2009; Britigan et al., 2014; Mankouri et al., 2013). To prevent aneuploidy, cells evolved mechanisms to ensure that chromosome segregation is tightly regulated during mitosis **(Figure 4, A)**. Erroneous KT-MT attachments are a commonplace but are usually corrected prior to anaphase. Thus, the rates of chromosome mis-segregation in normal diploid cells follow below 2% (Nicholson et al., 2013; Ye, 2015).

Aneuploid cells, including near-polyploid cells, can maintain a stable karyotype or exhibit chromosomal instability (CIN) (Zasadil, 2013). CIN can be present in two forms: structural chromosome instability (S-CIN, also referred as segmental chromosome instability) and whole chromosome instability (W-CIN) (Nicholson, 2013; Ricke, 2008; Schvartzman et al., 2010). S-CIN is represented by chromosome rearrangements such as reciprocal or nonreciprocal

chromosomal translocations, deletions of chromosome arms and amplifications of large chromosome regions; it is caused by inappropriate DNA damage response/repair (Kamata et al., 2011; Schvartzman, 2010). W-CIN is the major form of genomic instability (Kamata, 2011; Terradas et al., 2009) in which there is a persistent mis-segregation with gain or loss of entire chromosomes, leading to an unstable karyotype (Britigan, 2014; Ganem et al., 2009; Orr et al., 2013; Weaver, 2006). The general cause of this type of CIN are defects during mitosis (Mankouri, 2013; Orr, 2013), namely lagging chromosomes **(Figure 4, B)** (Cimini et al., 2001) resultant of persistent errors in KT-MT attachments (Bakhoum, Genovese, et al., 2009; Kleyman et al., 2014; Malterer et al., 2008).

Cancer cells, which are generally aneuploid, (Britigan, 2014; Mantripragada et al., 2008; Weaver, 2006) often contain both W-CIN and S-CIN (Ricke, 2008; Schvartzman, 2010; Terradas, 2009). This stage likely contributes to the maintenance of transformed cell populations, drug resistance, and disease progression (Mooney et al., 2016). The CIN phenotype was also recently been associated with tumor metastasis (Gao et al., 2016).

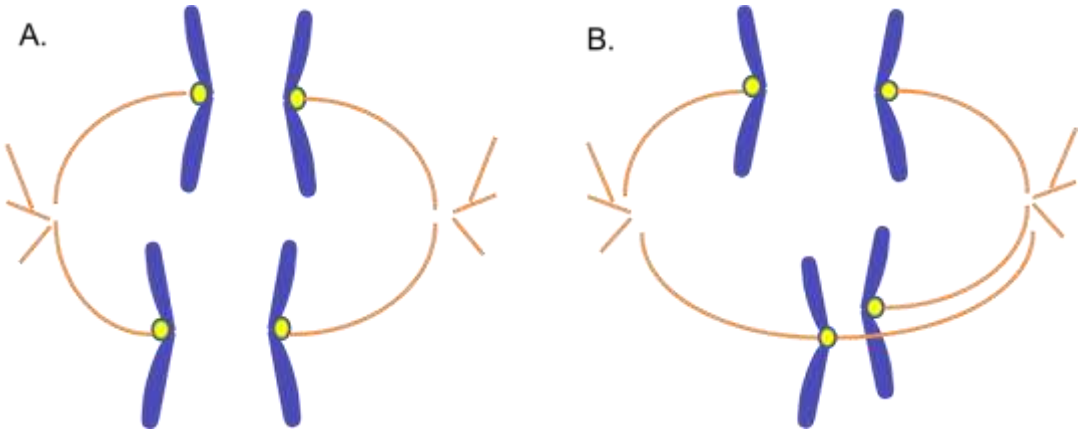

Figure 4. Comparison a normal anaphase with an anaphase lagging chromosome. Chromosomes are represented in blue, KTs in yellow and MTs in orange. **(A)** In normal anaphase, chromosomes are equally segregated to opposite poles of the mitotic spindle. **(B)** However, some dividing cells display anaphase lagging chromosomes, single chromosomes that are left behind during anaphase, typically near the spindle equator, while all the others move to the spindle poles. This occurs due to defects in KT-MT attachments and leads to W-CIN.

1.4. Causes of CIN

1.4.1. Defects in SAC signaling

The SAC is a highly robust surveillance mechanism. A single unattached kinetochore is sufficient to delay anaphase onset for several hours. However, there are several ways to slip

past the SAC (Subramanian et al., 2013). First, an improper regulation of the abundance of mitotic key players can lead to cell errors in chromosome segregation (Heinrich, 2013; Subramanian, 2013). Second, merotelic attachments are not detected by the SAC and can lead to lagging chromosomes (Subramanian, 2013). Third, if chromosomes detach from the spindle immediately before anaphase, there is insufficient time to block the cell cycle and prevent mis-segregation (Subramanian, 2013).

Mutations in genes encoding SAC related proteins have been observed at very low frequency in CIN tumors (Bakhoum, 2009; Hernando, 2004; Orr, 2013; Ricke, 2008). This might be explained by the inability of cells to cope with complete loss of SAC function as this results in massive segregation defects (Orr, 2013). Instead, misexpression of SAC genes is more frequently observed in cancer **(Table II)** (Faisal et al., 2017).

Table II. Key mitotic players overexpression was detected in different types of cancer

Protein	Expression level	Type of cancer	References
Mps1	Overexpression	Bladder Thyroid Breast Glioblastoma Pancreatic **Colorectal** Liver	(Thykjaer et al., 2001) (Salvatore et al., 2007) (Daniel et al., 2010; Richardson et al., 2013; B. Yuan, 2006) (Tannous et al., 2013) (Kaistha et al., 2014; Slee et al., 2014) (Ling et al., 2014; Zhang et al., 2016) (Miao et al., 2016)
Plk1	Overexpression	Lung Head and neck Endometrium **Colorectal** Prostate Ovary Breast Thyroid Glioma	(Wolf et al., 1997) (Knecht et al., 1999) (Takai et al., 2001) (Takahashi et al., 2002) (Weichert et al., 2004) (Dietel et al., 2004) (Weichert et al., 2005) (Salvatore, 2007) (Cheng et al., 2012)
Aurora A	Overexpression	Pancreatic **Colon** Thyroid Head and neck Breast Bladder	(Li et al., 2003) (Belt et al., 2012) (Reiter et al., 2006) (Ulisse et al., 2006) (Treekitkarnmongkol et al., 2016; Xia et al., 2003) (Mobley et al., 2017)
Aurora B	Overexpression	Thyroid Prostate **Colorectal** Lung Breast	(Ulisse, 2006) (Chieffi et al., 2006) (Subramaniyan et al., 2016; Tuncel et al., 2012) (Takeshita et al., 2013) (Zhang et al., 2015)

Mps1 is overexpressed in several types of cancer, including breast (Daniel et al., 2010; Richardson et al., 2013; B. Yuan, 2006), colorectal (Ling, 2014; Zhang, 2016), pancreatic (Kaistha, 2014; Slee, 2014), thyroid (Salvatore, 2007), glioblastoma (Tannous, 2013), bladder (Thykjaer et al., 2001) and liver (Miao et al., 2016) cancers. This overexpression is usually related with a poor prognosis (Faisal, 2017). In CRC cell lines, Mps1 overexpression led to genomic instability and contributed to tumorigenesis due to SAC weakness. Moreover, it allowed the survival of the tumor cells with higher degrees of aneuploidy (Ling, 2014). This was also verified in glioblastoma, the most common and lethal type of primary brain tumor, where a high Mps1 expression was linked to an increase in tumor grade and a decrease on patients' survival (Tannous, 2013).

Like Mps1, Plk1 is also overexpressed in different types of human cancers (Schubert, 2015; Takai et al., 2005). Among them, in glioma (Cheng, 2012), head and neck (Knecht, 1999), colorectal (Takahashi, 2002), ovary (Dietel, 2004), thyroid (Salvatore et al., 2007), breast (Weichert, 2005), lung (Wolf, 1997), endometrium (Takai, 2001) and prostate (Weichert, 2004) cancers Plk1 overexpression was associated with cancer progression, aggressiveness, higher recurrence risk and, thus, a poor prognosis (Z. Liu et al., 2017; Rodel et al., 2010; Takai, 2005). A study analyzed Plk1 expression in a cohort of 158 benign and malignant colon tumors; in 66,7% of them, Plk1 was not only overexpressed, but also linked to advanced tumor stages of the disease (Weichert et al., 2005). Overexpression of Plk1 was also observed in a cohort of 70 sporadic human CRC; in 77,1% of the cases, Plk1 was overexpressed in the primary CRC tissues, when in comparison with normal mucosa (Macmillan et al., 2001). A similar study was made using 78 primary CRCs and 15 normal colorectal specimens. Overexpression of Plk1 was observed in 73,1% of the CRC cases (57 out of 78) (Takahashi, 2002). Thus, it can be used as target for cancer therapy (Z. Liu, 2017; Rodel, 2010; Takai, 2005; Weichert, 2005).

As the previous mitotic regulators, Aurora A and Aurora B overexpression has been detected in several types of solid tumors and associated cancer progression and poor prognosis (Takeshita, 2013). Prostate (Chieffi, 2006) , colon (Belt, 2012), head and neck (Reiter, 2006), lung (Takeshita, 2013), breast (Xia, 2003), pancreas (Li, 2003) and thyroid (Ulisse, 2006) cancers are some examples. Additionally, an aberrant expression of both kinases was also found in some types of leukemia, as reviewed by (Goldenson, 2014).

Overexpression of Aurora A is related with chromosomal instability, oncogenic transformation and chemoresistance (Jin et al., 2015; Treekitkarnmongkol, 2016). Aurora A activates Plk1 and downregulates tumor suppressor genes, like *TP53*, by phosphorylation (Ikezoe, 2008; Treekitkarnmongkol, 2016). The kinase can also phosphorylate BRCA1, a breast cancer

susceptibility gene, blocking its function as a G2/M checkpoint keeper (Ikezoe, 2008). A study using a transgenic mouse model showed that Aurora A overexpression in the mammary gland led to several genetic alterations, culminating in the development of mammary adenocarcinomas (Treekitkarnmongkol, 2016). Moreover, high levels of Aurora A kinase were detected in patients with advanced colon cancer and associated with an increase of disease recurrence (Belt, 2012). Aurora A overexpression was also related with colorectal adenoma to carcinoma progression (Carvalho et al., 2016). A recent study also reports that Aurora A overexpression is related with bladder cancer aggressiveness and poor prognosis (Mobley, 2017).

Aurora B was found overexpressed in non-small-cell lung cancer and associated with vascular invasion, poor differentiation, larger tumor size and lymph node metastasis (Takeshita, 2013). The elevated expression of Aurora B together with elevated levels of cytoplasmic Survivin and INCENP has been associated to lymph node metastasis in CRC (Subramaniyan, 2016; Tuncel, 2012). Additionally, using tumor tissues from 312 invasive breast cancer patients, overexpression of Aurora B was correlated with poor survival and chemoresistance (Zhang, 2015).

1.4.2. Defects in KT-MT Attachments

The most common cause of whole chromosome mis-segregation is thought to be the persistence of errors in chromosome-MTs attachments (Kleyman, 2014). In early mitosis, highly dynamic MTs search and capture KTs (Gregan et al., 2011; Lin, 2012). Each sister KT must bind MTs emanating from opposite spindle poles – amphitelic attachment **(Figure 5, A)** - in order to ensure proper chromosome segregation in anaphase (Gregan, 2011; Kleyman, 2014; Lin, 2012). However, due to the dynamic and stochastic nature of the attachment process, errors can take place.

During early stages of mitosis, it is common for chromosomes to establish erroneous attachments. Usually, MTs bind first to one of the sister KTs in what is called a monotelic attachment **(Figure 5, B).** This represents an intermediate state that results from the difficulty of both sister KTs in establishing a simultaneous attachment to the mitotic spindle (Gregan, 2011). In this configuration, the unattached KT engages the SAC to prevent premature anaphase onset. Other types of erroneous KT-MT interactions are the syntelic and the merotelic attachments **(Figure 5, C and D, respectively)**. The first one occurs when both sister KTs interact with MTs from the same spindle pole (Ganem, 2009; Gregan, 2011). As in monotelic attachments, syntelic attachments also lead to SAC activation due to the low centromeric

tension (Gregan, 2011). Merotelic attachments take place when a single kinetochore attach to MTs emanating from both spindle poles (Bakhoum, 2009; Kleyman, 2014; Lin, 2012). This KT-MT configuration is not detected by the SAC (Bakhoum, 2009; Ganem, 2009; Lin, 2012), as KTs attain full (however improper) occupancy of MTs (Gregan, 2011; Orr, 2013).

Aurora B activity is critical to ensure proper KT-MT attachments (Britigan, 2014; Kops, 2014; Lampson et al., 2017). When KTs are not under tension from opposing microtubule-based pulling forces, as in syntelic attachments, centromeric Aurora B directly phosphorylates kinetochore proteins that mediate the attachment to MTs. This results in the destabilization of incorrect KT-MTs attachments, hence providing opportunity to establish new and correct KT-MT interactions (Cimini, 2006; Krenn, 2015; Lin, 2012).

Moreover, it was shown that partial inhibition of Aurora B results in an increase in the number of merotelic attachments, compromising appropriate chromosome segregation (Cimini, 2006).

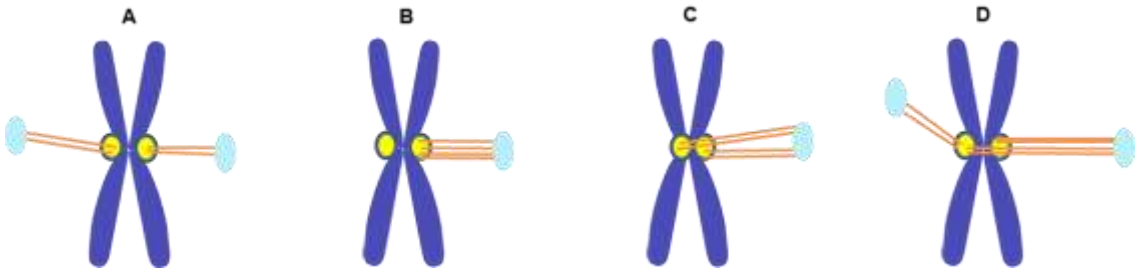

Figure 5. Types of KT-MT attachments during mitosis. Chromosomes are represented in dark blue, KTs in yellow, MTs in orange and spindle poles in light blue. **(A) Amphitelic**. Each sister kinetochore must bind MTs emanating from opposite spindle poles in order to ensure proper chromosome segregation in anaphase. **(B) Monotelic**. Nevertheless, at early stages of mitosis, it is common that only one of the two sister KTs is attached to spindle MTs **(C) Syntelic**. Occurs when both sister KTs interact with MTs from the same spindle pole. **(D) Merotelic**. It takes place when a single kinetochore attach to MTs emanating from both spindle poles. Unlike the other types of attachments, merotelic attachments are not detected by SAC, compromising chromosome segregation.

If merotelic attachments are not corrected before anaphase, lagging chromosomes will occur (Ganem, 2009; Gregan, 2011). This is the primary cause of mis-segregation events and aneuploidy chromosomal-instable cancer cell lines (Ganem, 2009; Gregan, 2011; Kleyman, 2014; Lin, 2012).

Interestingly, KT-MT attachments in cancer cells with CIN were found to be inherently more stable than those in normal diploid retinal pigment epithilium (RPE-1) cells (Bakhoum, 2009; Orr, 2013). Mad2 overexpression might contribute for this hyperstabilization, which occurs independently of SAC (Kabeche et al., 2012). Thus, cancer cells have a diminished capacity to

correct merotelic attachments, which explains the widespread occurrence of CIN in tumors. Importantly, increasing the detachment rate of KT-MT improves error correction and is sufficient to restore faithful chromosome segregation in CIN cancer cells (Orr, 2013).

1.5. CIN and Cancer

Genetic instability is a hallmark of cancer (Maser et al., 2002; Rao, 2005). The mechanisms responsible for the maintenance of genome integrity and growth arrest are suppressed in cancer cells (Maser, 2002). In 1914, Theodor Boveri proposed the *aneuploidy hypothesis* (Boveri, 1902; Weaver, 2007a). According to his observations, chromosome mis-segregation leading to aneuploidy was the cause of tumorigenesis (Boveri, 1902; Weaver, 2007b). However, in the 1970s and 1980s, with the discovery that oncogenes and tumor suppressors can be the point of transformation initiation, Boveri hypothesis lost part of the interest (Weaver, 2007a). Several theories have emerged, and a great deal of controversy has arisen in part due to the inability of generating and testing aneuploidy without causing other damage (Weaver, 2007a; Weaver, 2007b). In order to try to clarify the relation between aneuploidy and tumorigenesis, experiments using animals with reduced expression of mitotic checkpoint genes took place. However, this was difficult, as these genes are not only important for cell division, but also for other essential cellular processes (Weaver, 2007a; Weaver, 2007b).

Aneuploidy is a hallmark of cancer and a proposed driving force of tumor progression (Britigan, 2014; Davoli et al., 2013; Nicholson, 2013). Inducing aneuploidy leads to higher level of spontaneous lymphomas and lung tumors in aged animals (Weaver, 2007b) Most solid tumors have an abnormal chromosome number (Orr, 2013; Tu et al., 2003; Zhang et al., 2008) with aneuploid karyotypes ranging from 40 to 90 or more chromosomes (Bakhoum, 2009); some of them are capable of accurately segregate their improper chromosome number, remaining genetically stable (Bakhoum, 2009). Nevertheless, others are genetically unstable and mis-segregation events occur, leading to CIN (Bakhoum, 2009). Additionally, they display heterogeneity, not only in comparison with other tumors but also within the tumor itself (Burrell et al., 2013; Orr, 2013; Thompson et al., 2008). Heterogeneity is mainly caused by genomic instability (Burrell, 2013). The increasing rate of mutation generates a genetically extended and diverse pool of tumor cells (Dewhurst et al., 2014). Aneuploidy can stimulate the overexpression of loci responsible for cell growth leading to a selection of the most aggressively cells (Dewhurst, 2014; Mantripragada, 2008).

As aneuploidy, W-CIN is a distinguishing feature of most cancer cells (Cresta, 2012; Dewhurst, 2014; Ganem, 2009; Kamata, 2011; Orr, 2013). Experiments using mutant mice with

W-CIN demonstrate that these animals have a tendency to develop cancer (Ricke, 2008). Chromosome mis-segregation occurs 10 to 100 times more in CIN cells, comparing to diploid cancer cells chromosomally stable (Ganem, 2009; Thompson, 2008). As a cause of CIN, chromosome mis-segregation can be important in cancer adaptation and evolution (Covo et al., 2014; Ganem, 2009). It may be correlated with high cell proliferation, metastasis, drug resistance, poor prognosis (Covo, 2014; Dewhurst, 2014; Ganem, 2009), tumor aggressiveness and recurrence (Kleyman, 2014), bringing therapeutic challenges (Covo, 2014; Ganem, 2009; Thompson, 2008). As tumor heterogeneity, genomic instability is also the responsible of cancer cells expansion because it allows cells to reach a higher proliferation capacity (Mantripragada, 2008). Thus, understanding how genomic instability modulates tumor evolution is crucial for the development of cancer therapeutics (Burrell, 2013).

1.5.1. Possible strategy to approach erroneous cell death: increase CIN through key mitotic players inhibition

Although aneuploidy can drive tumorigenesis, recent studies describe a threshold level from which a higher aneuploidy can work as inhibitor for tumor development (Lee et al., 2016; Weaver, 2007b). Thus, increasing CIN as a mean to block tumor cells proliferation and tumor growth can be therapeutically attractive (Sansregret et al., 2017). As Mps1, Plk1, Aurora A and Aurora B kinases are critical mitotic regulators and frequently overexpressed in human tumors, it is tempting to envisage them as promising targets to increase chromosome segregation errors (Penna, 2017; Sansregret, 2017). Several of the inhibitors that have been developed bind to kinases in their ATP binding site, preventing its normal catalytic activity (Weiß et al., 2012).

A small-molecule inhibitor for Mps1, SP600125, used in breast cancer cells decreased their viability and increased cell death, particularly in TP53 defective cells (Gyorffy et al., 2014). More recently, another inhibitor named NTRC 0066-0 showed also promising results in several human cancer cell lines. Moreover, the combination of this inhibitor with docetaxel, a chemotherapy medication, increased survival and extended tumor remission without toxicity in a murine breast cancer model (Maia et al., 2015). Another study with orthotopic mouse models tested the Mps1 inhibitor MPS1-IN-3 together with vincristine, another chemotherapeutic drug, in glioblastoma cells. The result was an increase in segregation defects, aneuploidy and tumor cells death (Tannous et al., 2013). Recently, it was described a Mps1 inhibitor, CC8271850, applied in several cancer cell lines (Faisal, 2017). The cells submitted to this drug lost KT location of important mitotic proteins (BubR1, Mad1, Mad2) and start to mis-segregate their

chromosomes. Cell death was observed after one or two mitosis; among the different cancer cell lines tested, the effect was higher in the colon cancer cell ones (Faisal, 2017).

Inhibitors of Plk1 have also been developed. The combination of the small-molecule inhibitor Volasertib (BI 6727) with low doses of the chemotherapy agent Cytarabine in acute myeloid leukemia patients lead to higher response rates and improved survival (Michael et al., 2017; Penna, 2017). A small-molecule inhibitor for Plk1, GSK461364, was administered to patients with solid tumors. It was verified an overall decrease in the number of tumor cells (Olmos et al., 2011). Moreover, the inhibition of Plk1 can increase the sensitivity of cancer cells to chemo/radiotherapy (Z. Liu, 2017; Takai, 2005).

As for Aurora B, Barasertib (AZD1152) stands as a promising potent and selective inhibitor (Tsuboi et al., 2011). When tested in immunodeficient mice with human colon, lung and hematologic tumor xenografts, Barasertib efficiently inhibited tumor growth (Ikezoe, 2008; Wilkinson et al., 2007). When administered to patients with advanced myeloid leukemia, Barasertib exhibited preliminary efficacy (Falchook et al., 2015; Tsuboi, 2011). More recently, a combination of Barasertib with low doses Cytarabine was tested also in myeloid leukemia patients and showed higher responses when compared to the administration each one of the agents alone (Penna, 2017).

The main Aurora A inhibitors currently in clinical trials are MLN8237 (Alisertib), MK-5108 and ENMD-2076 (Damodaran et al., 2017). Alisertib was already tested in Phase I trials with solid tumors and lymphomas, and some good responses were observed (Falchook, Bastida, & Kurzrock, 2015). MK-5108 is the most promising Aurora A inhibitor, as it proved to be the least toxic, even for higher doses than the therapeutic ones (Amin et al., 2016). ENMD-2076 has also low toxicity levels (Damodaran, 2017). It was tested in a cohort that included patients with ovarian cancer, CRC and refractory solid tumors. Promising anti-tumor activity was observed, particularly for ovarian cancer (Diamond, 2010).

Despite the encouraging outcomes of some of the mitotic drugs described, they are still below the response levels needed in clinical trials for single agents (Faisal, 2017; Z. Liu, 2017; Penna, 2017). On one hand, it is not possible to increase the administrated doses of this drugs, as the majority of them are toxic (Faisal, 2017). On the other hand, the cancer cells can develop resistance against a certain drug (Sansregret, 2017; Yuan et al., 2016). However, the combination of those drugs with conventional treatments like chemotherapy or radiotherapy can be a promising strategy to overcome both of these problems (Falchook, 2015; Penna, 2017; Yuan, 2016). Additionally, a better comprehension of the functions of this kinases represents a key-point to develop specific and effective inhibitors (Damodaran, 2017).

1.6. Colorectal Cancer

The incidence of cancer and consequent mortality have been decreasing as a result of a more preliminary detection and treatment development (Miller et al., 2016). Moreover, the availability of information led to an increase of the lifestyle care by the population; for example, all the campaigns against tobacco smoking (Siegel et al., 2017). However, cancer is still the second leading cause of death in the United States (Siegel et al., 2016). Among the variety of cancers, CRC is now the third most commonly diagnosed and the fourth leading cause of death in both women and men (Siegel, 2017; Vasuri et al., 2017). The majority of CRC metastases are located in the liver, followed by the lungs (Goos, 2013). About 95% of CRC are sporadic, meaning that the individuals have a predisposition that can turn into cancer as a consequence of the exposure to certain environmental factors (Watson et al., 2011). As the incidence is higher in the developed countries, diet factors can be a main cause. There is a correlation between the consumption of red meat, fat and alcohol and the incidence of CRC (Siegel, 2017; Watson, 2011).

1.6.1. From normal tissue to cancer

CRC is originated from the epithelial cells lining the colon or rectum of the large intestine and its main cause are mutations in Wnt Signaling Pathway (Choudhry et al., 2015). The evolution of CRCs begins with alteration of this normal colonic epithelium to an adenomatous polyp intermediate. Then, it can develop to an advanced adenoma with high dysplasia and finally become an invasive cancer (Markowitz et al., 2009; Pino et al., 2010). There are five general distinguishable stages **(Figure 6)**. The stage 0 corresponds to cancer in situ, when cancer cells are in the mucosa or in the colon or rectum inner lining (Choudhry, 2015). If the tumor has grown through the mucosa and through the colon or rectum walls, it is classified as stages I and II, respectively (Markowitz, 2009). If untreated, it can extent to regional lymph nodes and become a stage III CRC. At this point, surgery with adjuvant chemotherapy is recommended. Lastly, if metastasis is generated in other organs, it constitutes a stage IV CRC, usually not treatable (Choudhry, 2015; Markowitz, 2009).

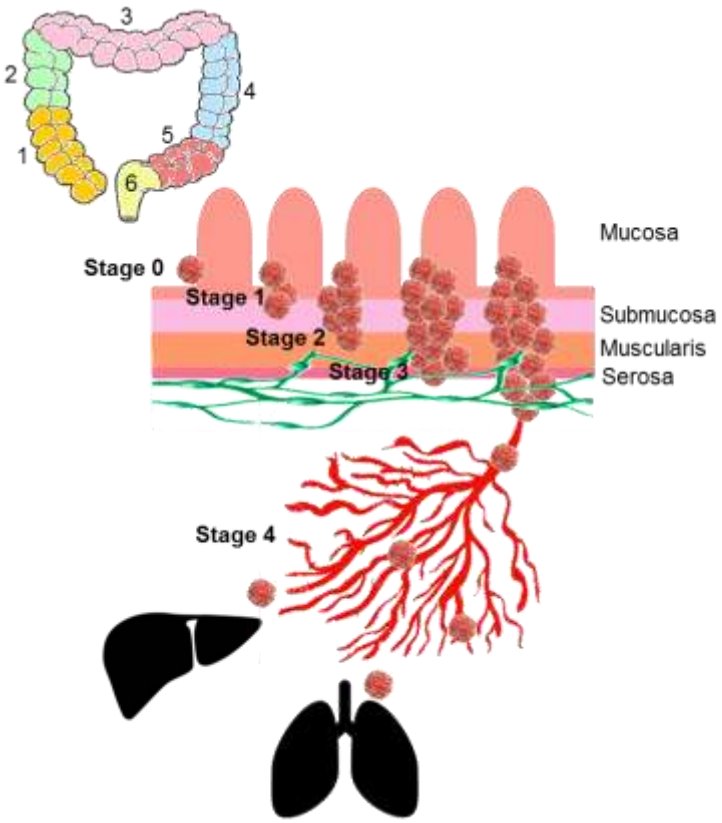

Figure 6. Illustration of the large intestine anatomy and CRC evolution. 1. Cecum, **2**. Ascending colon, **3.** Transverse colon, **4.** Descending colon, **5**. Sigmoid colon, **6**. Rectum. The stage 0 is characterized by cancer cells in the inner lining of the colon or rectum. The disease can grow through the mucosa and reach the muscle layer; the CRC is now in the **stage I** and **II**, respectively. Further proliferation of the cancer cells can spread the disease to regional lymph nodes, leading to a **stage III** disease. Lastly, reaching the blood vessels, metastasis can be generated in other organs, which constitutes a **stage IV** CRC.

1.6.2. CRC classification: MSS vs MSI-H

Genomic instability, namely aneuploidy, can contribute for tumor progression through two main pathways, microsatellite stability (MSS) or microsatellite instability (MSI) (Barresi et al., 2017; Pino, 2010; Rao, 2005). These pathways are not mutually exclusive, and some tumors can be positive for both forms (Barresi, 2017; Pino, 2010), being the prognosis better for MSI CRC patients (Watson, 2011).

Microsatellites are small mono-, di-, tri- or tetra-nucleotide repetitive sequences present in the genome. These locations are more prone to accumulate mutations (Kim et al., 2014; Parine et al., 2016). During DNA synthesis the DNA polymerase has difficulties to bind to the microsatellites leading to an incorrect reannealing. Consequently, base-base mismatches and insertion-deletion loops arise. When in an exonic region, those alterations will cause frameshift mutations (Parine et al., 2016). To prevent this, there is a system called DNA mismatch repair system. In human, it includes three main phases: recognition, excision and resynthesis **(Figure 7)** (Groothuizen et al., 2016; Parine et al., 2016). Firstly, a protein complex formed by MutS - either MutSα, the MSH2-MSH6 heteroduplex **(Figure 7 i.A)** or MutSβ, the MSH2-MSH3 heteroduplex **(Figure 7 i.B)** - together with proliferating cell nuclear antigen (PCNA) moves through DNA until it recognizes a base-base mismatch or a base insertion-deletion, respectively

(Goellner et al., 2014; Groothuizen, 2016). At this point, MutS conformation change and another protein complex, MutLα (MLH1-PMS2) is recruited (it can also be MutLγ, MLH1-MLH2, when MutLα levels are low) (Groothuizen, 2016; Kolodner, 2017). The nucleotides excision is made by exonuclease 1 (Exo1) **(Figure 7 ii)**. By this time, replication protein A (RPA) stabilizes the single-stranded DNA and after the excision it inhibits Exo1 to prevent further degradation. PCNA together with replication factor C (RFC) helps in the resynthesizes step carried out by DNA polymerase δ (Kolodner, 2017). Finally, DNA ligase ligates remaining leaks (Goellner, 2014; Parine et al., 2016) **(Figure 7 iii)**. When there are mutations in DNA mismatch repair system genes, the system function is compromised and microsatellite instability arises. This instability form is present in about 15% of CRC (Orsetti et al., 2014; Parine et al., 2016); within these, 20% are hereditary and named Lynch syndrome, as a consequence of germline mutations mainly in the MLH1, MLH2, MSH6 or PMS2 genes. The 80% left are sporadic, mainly due to MLH1 promotor hypermethylation (Kim et al., 2014; Watson, 2011).

Approximately 85% of CRCs are microsatellite stable, revealing instead different levels of CIN (Barresi, 2017). Moreover, studies suggest that it may occur at early stages of the tumorigenesis (Rao, 2005). However, CIN evaluation is not a focus in clinical practice (Barresi, 2017). The explanation lies on the absence of an effective approach to quantify CIN and standardize a criterion to define that a tumor is chromosomally instable, as only a few genes responsible for this phenotype were identified (Barber et al., 2008; Barresi, 2017). Adenomatous polyposis coli (*Apc*) gene encodes a 310-kDa cytoplasmatic protein, APC protein, responsible for prevention of β-catenin accumulation and thus controlling the Wnt output signaling (Choudhry, 2015; Dow et al., 2015; Rao, 2005). Mutations in *Apc* gene, in the tumor suppressor gene *TP53* and in *Kras* are the most frequent ones in colon cancer, being present in about 80-90%, 54% and 45% of the cases, respectively (Dow, 2015; Watson, 2011). Using a CRC mouse model it was demonstrated that *Apc* suppression leads to adenomas formation in the colon and the small intestine; moreover, it can turn into an invasive carcinoma when *Kras* and *TP53* mutations are also present (Dow, 2015). As previously mentioned (section 4.1.), mutations in genes encoding SAC related proteins are rare in CIN tumor cells (Bakhoum, 2009; Hernando, 2004; Orr, 2013; Ricke, 2008), as the absence of SAC function leads to cell death (Orr, 2013). However, the overexpression of several SAC components is observed in several cancer types, including CRC. Mps1 (Ling, 2014), Plk1 (Takahashi, 2002), Aurora A (Belt, 2012) and Aurora B (Subramaniyan, 2016; Tuncel, 2012) are found to be overexpressed in several CRC tissues and cell lines.

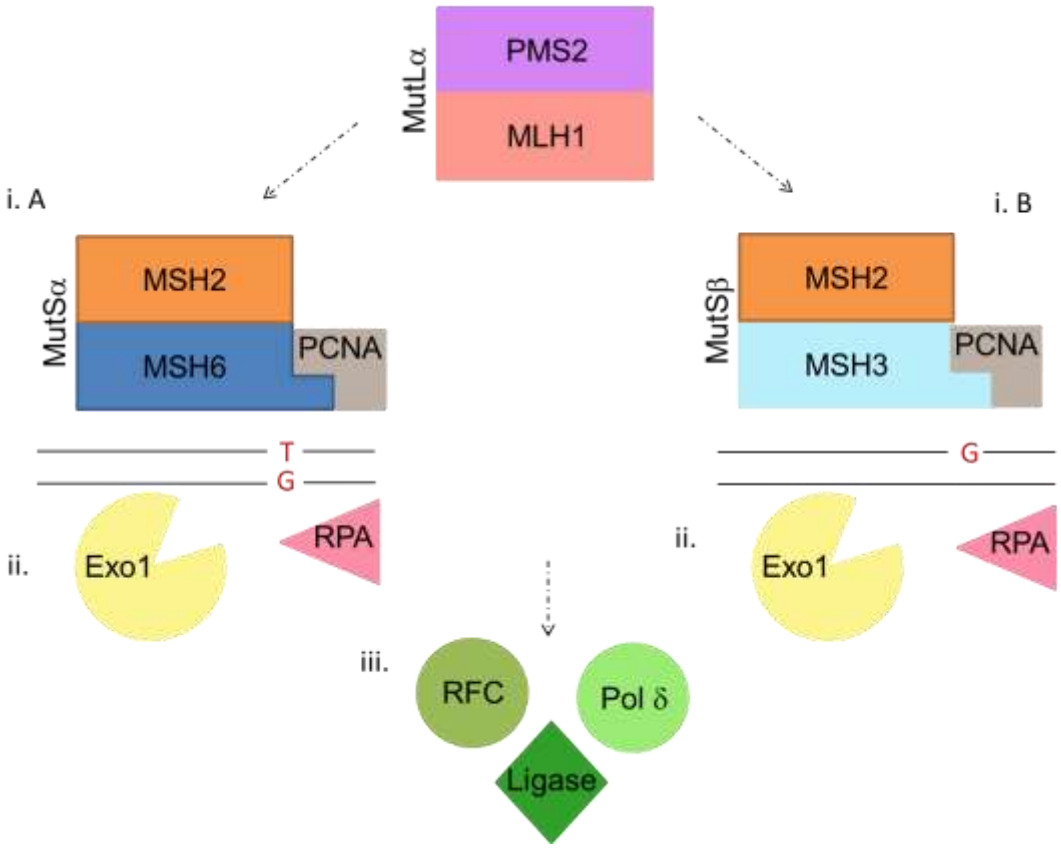

Figure 7. **Illustration of the DNA mismatch repair system in humans.** The process can be divided in three main phases. **i. Recognition**; base-base mismatches or base insertions-deletions are recognized by MutSα **(i.A)** or MutSβ **(i. B)**, respectively, together with PCNA and MutLα. **ii. Excision;** Exo1 is responsible for taking the wrong nucleotides off the chain. RPA stabilizes this process. **iii. Resynthesizes**; DNA polymerase δ, helped by RFC, fills the chain with the right missing nucleotides and DNA ligase ligates remaining leaks.

Despite being the major phenotype present in CRC cells the causes and consequences of CIN are not clearly understood (He et al., 2015). It is still unknown if CIN drives tumorigenesis, or if it happens in the other way around (Pino, 2010). There are some models suggesting CIN as the tumor initiator. However, this is not stablished for CRC (Pino, 2010). Further investigation may let us understand if the alteration in the expression of key mitotic regulators is responsible for the CIN acquired by cancer cell lines. If so, key mitotic player's inhibition can be a promising approach in cancer therapeutics.

1.7. Objectives

Analysis of whole chromosome karyotypic variability indicates that 44% of solid tumors and 14% of hematopoietic cancers exhibit CIN (Zasaldi et al., 2013). The continuous gain or loss of chromosomes generates phenotypic variation within the tumor, which is thought to allow for adaptation to environmental stress, hence contributing for the maintenance of transformed cell populations, drug resistance, and disease progression (Mooney, 2016). Particularly, the majority of CRCs exhibit aneuploidy. However, the occurrence of CIN *in vivo*, its molecular underpinnings and its impact for CRC remains elusive (He, 2015). Although it has been demonstrated that CRC cell lines often exhibit erroneous KT-MT attachments that contribute for chromosome segregation errors, these findings were obtained in immortalized cell lines with high passage numbers (Baron et al., 2016). These cells have been in culture for decades and most likely diverged from initial cultures at both the genetic and epigenetic levels. Moreover, in most cases, the clinicopathological parameters and patient information from which these cells were established are incomplete. This causes variability, limits conclusion accuracy and precludes physiological/clinic correlation.

Therefore, in the present work we resorted to novel low passage primary cultures of CRC cell lines derived from primary tumors to examine the occurrence of CIN and characterize the genetic integrity and expression pattern of key mitotic regulators known to be involved KT-MT attachments and SAC signaling. Cell lines exhibiting different levels of numerical aneuploidy were used and the mutational profiles of *Mps1*, *Plk1 Aurora B* and *Aurora A* kinases determined by NGS. The transcript and protein levels of each kinase were monitored by RT-qPCR and western blotting, respectively.

Contrasting with cell lines of high passage, the low-passage CRC cell lines used in this study well reflect the biology of the original tumor, such as growth behavior, morphology, and mutational profile. Thus, results obtained in the present thesis will set the ground to understand how deregulation of critical mitotic regulators might impact CIN in CRC.

CHAPTER 2

MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. General reagents and materials

A list of all reagents and materials, as well as supplier and catalogue number can be found in **Table III.**

Table III. Reagents and materials used

Reagent	Supplier	Catalogue number
Acetic acid	Merck	1.00063.1011
Acrylamide mix	Protogel	EC-890
Agarose	Eurogentec	EP-0010-05
Ammonium persulfate	Sigma	431532
Ampure XP beads	Beckman	15580500
AMV-RT buffer	Roche	11465368001
Barcoded Primer	NS*	NS*
100 Base Pair Ladder	NEB New England Biolabs	390892
cOmplete	Roche	11697498001
Deoxycholic acid sodium salt, 99%	ACROS	AC21859250
Cell Culture dishes, 60x15mm	CELLSTAR	628160
DMEM/F12 (1:1) 1x	Gibco	1830681
dNTPs 10mM	GE Healthcare	US77119-500UL
Ethanol	VWR	20823-293
Eppendorfs	NS*	61724987
Falcons (15mL, 50mL)	CELL STAR	227261,188271
FastStart HiFi Reaction buffer + 18mM MgCl2	Roche	03698203001
FastStart HiFi Enzyme Blend	Roche	03553361001
FBS	Greiner bio one	750093
HANKs'balanced salt solution 10x	SIGMA	RNBD7502
Hard-shell PCR 96 well plate	BIORAD	HSP9635
iQ SYBR mix	BIORAD	170-8887
4x Laemelli sample buffer	BIORAD	161-0747

Magnet	ALPAQUA	A32782
β-Mercaptoethanol	Baker	02-002-079
Methanol	Merck	1.06009.2511
Molecular Weight marker	nzytech	MB17601
Molico (powder milk)	Nestlé	-
MicroAmp Optical 8-cap strip	Applied Biosystems	4316567
Microseal 'B' seal	BIORAD	MSB1001
OdT	NS*	NS*
P1-M13 primer	Eurofins MWG operon	Custom oligonucleotide order
Pasteur pipette	Wu Mainz	NS*
PBS	B/BRAUN	163448081
Penicillin/Streptomycin	Gibco	15140-122
PhosSTOP	Roche	04906837001
Ponceau S	Sigma	P-3504
Primer A	Eurofins MWG operon	Custom oligonucleotide order
Primer P1	Eurofins MWG operon	Custom oligonucleotide order
2-Propanol	SIGMA	STBG3188V
RNase inhibitor	Promega	N251A
RT enzyme	Roche	10109118001
Scraper	Greiner bio one	541070
SDS	Promega	H5115
Sodium Chloride	EMSURE	1.06404.1000
Sodium Hydroxide (powder)	Merck	B0428998 943
SYBR Gold	ThermoFisher	S11494
T-flasks (50mL and 200mL)	CELL STAR	690160
TBE	NS*	NS*
TEMED	BIORAD	161-0801
10x TGS	BIORAD	161-0772

Tris	Merck	648311
Triton X-100	Sigma	93443
Tris	Merck	648310
2,5% Trypsin	Gibco	15090-046
Tween 20	Merck	817072
X-ray film	Fuji Medical X-Ray Film	47410 19318

***NS:** not specified

2.1.2. Kits

A list of all the kits used, as well as supplier and catalogue number can be found in **Table IV.**

Table IV. Kits used

Kit	Supplier	Catalogue number
High sensivity DNA Reagents	*Agilent Technologies*	5067-4626
Genomic DNA from Tissue, NucleoSpin® Tissue	Macherey-Nagel	740952.250
iBlot 2 NC Regular Stacks	*Invitrogen*	IB23001
PCR clean-up Gel extraction kit, NucleoSpin® Gel and PCR clean-up	Macherey-Nagel	740609.50
Qubit® 2.0 Fluorometer	*Invitrogen*	Q32854
RNA isolation kit*, NucleoSpin® RNA*	*Macherey-Nagel*	740955.250
Agarose gel kit	BIORAD	-

2.1.3. Solutions

Frequently used solutions were prepared as follows:

- Blocking solution: 5% powder milk in PBST20
- DMEM/F12 (1:1) 1x: add 10-20% FBS and 1:200 P/S
- Hans 1x: Hanks 10x diluted in demi water
- PBS 0,05% Tween: Tween 20 dissolved in 1xPBS
- Ponceau staining: 0.25% Ponceau S in 40% methanol and 15% acetic acid
- Trypsin (total volume of 50mL): 2,5mL 2,5% trypsin, 0,5mL TRIS EDTA, Hans medium to dilution
- RIPA buffer: 150mM sodium chloride, Triton X-100, 0,5% sodium deoxycholate, 0,1% SDS, 50Mm Tris, pH 8,0

2.1.4. Antibodies

Primary and secondary antibodies used in the Western Blot are listed in **Table V** and **Table VI,** respectively.

Table V. Primary antibodies used in the Western Blot

Antibody	Host	Type	Molecular weight (kDa)	Dilution	Supplier	Catalogue number
Aurora A	Rabbit	Monoclonal	48	1:1000	CST	14475
Aurora B	Mouse	NS*	41	1:1000	Biosciences	611082
Plk1	Rabbit	Monoclonal	62	1:1000	CST	4513
Mps1	Mouse	Monoclonal	97	1:500	*merckmillipore*	05-682
α-Tubulin clone DM1A	Mouse	Monoclonal	55	1:10000	Sigma	T9026
Cyclin B	Mouse	Monoclonal	55	1:1000	CST	4135

***NS: not specified**

Table VI. Secondary antibodies used in the Western Blot

Antibody	Dilution	Supplier	Catalogue number
Goat anti-mouse IgG-HRP	1:2500	Santa Cruz Biotechnology	sc-2031
Goat anti-rabbit IgG-HRP	1:5000	Santa Cruz Biotechnology	sc-2004

2.2. METHODS

2.1. Cell lines

A total of seven cell lines, six colorectal cancer cell lines (JVE187, JVE207, KP363T, JVE528, JVE774, JVE059) and one commercial human dermal fibroblast cell line (VH10) were used in the present work. The CRC cell lines were obtained from colorectal cancer tissues of patients of the Leiden University Medical Centrum. The cell lines were cultured in DMEM/F12 (1:1) medium supplemented with FBS and Penicillin/Streptomycin, and incubated at 37ºC.

2.2. Cell lines lysis

At passage time, each cell line was not only passed to a new T-flask, but also to two culture petri dishes (*CELLSTAR*). When those become ≈70% full (after ≈3 days), the medium was taken from them using a vacuum pipette. The cells were then washed once with PBS. The PBS was discarded. To each one of the petri dishes, one lysis buffer was added:

- DNA lysis buffer: 180µL Lysis 1 (in Genomic DNA from Tissue, NucleoSpin® Tissue, Macherey-Nagel)
- RNA lysis buffer: 350µL RA1 (in *RNA isolation* kit*, NucleoSpin® RNA,* Macherey-Nagel) plus 3,5µL β-mercaptoethanol

The petri dishes were scraped. The content was aspirated to the respective *eppendorf*, previously labeled. The RNA and DNA *eppendorfs* were stored in the freezer at -20ºC.

2.3. DNA

2.3.1. DNA isolation

The DNA isolation was performed using the *Genomic DNA from Tissue, NucleoSpin® Tissue kit* from *Macherey-Nagel*. The *Standard protocol for human or animal tissue and cultured cells* was followed.

2.3.2. DNA quantification

Concentration measurements were performed using the nanodrop device and Qubit® 2.0 Fluorometer from *Invitrogen*, by *life technologies* (according to manufacturer instructions). For the samples that missed a minimal concentration, a CentriVap concentrator (*LABCONCO*) was used.

2.3.3. M13 Ion Torrent NGS

NGS is a powerful technique that allows the sequencing of thousands to billions of samples at the same time. Sample preparation for NGS requires a library, prepared by PCR technique. During the library preparation, unique index sequences – M13 barcodes in this case - are added to each sample. After the 2nd round PCR, libraries are pooled together, which is called multiplexing. The whole procedure can now be made with one sample that will be sequenced in one single time. Once NGS is finished, de-multiplexing takes place; this means that the pooled libraries are computationally identified and the samples with the same barcode are set together. Finally, the results are aligned with the reference sequence and possible mutation sites can emerge. Those can be analyzed with IGV software and classified as positive or false positive by a process called visual inspection (Goodwin et al., 2016).

Here, Ion Torrent was the type of NGS used as sequencing method. It uses the release of hydrogen ions (H^+) during the sequencing reaction. Each cluster of DNA, resultant from the library amplification, is located directly above a semiconductor transistor, which is capable of detecting changes in the pH of the solution. During nucleotide incorporation a single hydrogen ion is release into the solution, which makes a 0,02 unit change in the pH that is detected. If it's not the correct nucleotide, no voltage will be found. In case there are two nucleotides added, the voltage will be doubled. In the end a collection of DNA sequences that were generated at each cluster is obtained (Goodwin, 2016).

2.3.3.1. Panel test: 1st round PCR

The panel was obtained from *Integrated DNA Technologies* in a total of 108 amplicons divided between two pools.

In order to find the optimal primer concentration, three different concentrations (25nM, 50nM, 100nM) were tested with one cell line isolated DNA. A sample and panel that worked before were also used as positive control. A first round PCR was performed using 1x FS HiFi Reaction Buffer + 18mM MgCl2, 200µM dNTPs, 0,05U/µL FastStart Hifi Enzyme Blend, 2ng/µL DNA, 25nM OR 50nM OR 100nM of each primer, and prefacing with water to a final volume of 25µL. The PCR plate was covered with a Microseal 'B' seal, centrifuged briefly at 1000g and run (≈1,5h) according to the following programme.

- 95ºC, 10min
- 95ºC, 15s
- 60ºC, 30s
- 72ºC, 1min

30x

- 72ºC, 5min
- 15ºC, hold

2.3.3.2. Panel test: Electrophoresis in agarose gel (SYBR gold)

The PCR products were run in an electrophoresis in 2% agarose gel was performed. The gel was made mixing agarose and TBE in a flask (amounts calculated according to the volume of the plastic mold). After boiling the mixture in the microwave, the flask was cooled down using cold water in the outer part of it. The gel was introduced in the mold with the comb and possible bubbles were pushed away. When the gel became ready, it was introduced inside the BIORAD construct. The last one was filled with TBE, until the gel was covered. Once the loading buffer was added to the samples and the comb of the gel was removed, the samples were loaded. Finally, the construct was closed. A 120V run was performed.

The staining was made using SYBR® Gold. A box with 1000x dilution SYBR Gold in TBE was prepared. The SYBR was added drop by drop in different spots of the box, and mixed with the TBE by gently shacking.

Once the run was complete, the gel was placed inside the box. After 20 minutes, the gel was analyzed in UV light using the proper protection.

2.3.3.3. Library preparation

Having the optimal primer concentration (100nM) and using the DNA samples isolated from each one of the cell lines of study a 1st round PCR was performed just as described in section **2.3.3.1**. An hour before the 1st PCR ending, Ampure XP beads were placed at RT for half an hour.

Once the first round PCR was finished, 20µL of each primer pool reaction were combined per sample (40µL in total) in a 96 well plate. Purification of the PCR product was made using the Ampure XP beads. The purification is important because the primer dimers and all the products with less than ≈150bp are discarded, while the PCR product remains attached to the beads. Firstly, 48µL (1,2x sample volume) of Ampure XP beads were added per sample and well mixed. While 5 minutes of incubation at RT were taking place, a solution of 80% ethanol was prepared. After the incubation period, the plate was placed on a magnet for 3 minutes and the supernatant was discarded. An amount of 150µL of ethanol was added per sample, followed by 30 seconds incubation. The supernatant was then discarded. This process was repeated two times. The samples were dried at RT for 5 minutes. 25µL of water were added and

resuspended on each sample. After 2 minutes of incubation, the plate was placed again in the magnet. 10µL of the supernatant were transferred twice to a new well.

A second round PCR was then prepared for the Forward and Reverse setup, using 1x FS HiFi Reaction Buffer + 18mM MgCl2, 0,3µM P1-M13 primer, 200µM dNTPs, 0,05U/µL FastStart Hifi Enzyme Blend, 0,3µM of M13 Barcoded Primers, and prefacing with water to a final volume of 25µL.

The following PCR program was run:

- 95ºC, 10min
- 95ºC, 15s
- 60ºC, 30s
- 72ºC, 1min

  } 5x
- 72ºC, 5min
- 15ºC, hold

When the run was finished, a new purification of the PCR product was performed exactly as previously described, using the Ampure XP beads. In the end, 24µL of the supernatant were transferred to a new well.

2.3.3.5. Sample pooling and qPCR

The next step was the sample pooling and qPCR. A 1:1000 dilution of the previously PCR product was aliquoted.

The qPCR reaction was prepared using 1x iQ SYBR mix, 250nM primer A, 250nM primer P1 and prefacing with water to a final volume of 9µL. 1µL of the 1:1000 library dilution was added to the mixture.

The following program was run:

- 95ºC, 5min
- 95ºC, 10s
- 60ºC, 10s
- 72ºC, 10s
  Plate read

  } 30x
- 72ºC, 10min
- Melt curve 70ºC to 95ºC, ↑ 0,2ºC, 10s + plate read

2.3.3.6. Electrophoresis in agarose gel

In order to verify the 2nd round PCR product amplification, an electrophoresis in 2% agarose gel was performed, like described in **2.3.3.2.**

2.3.3.7. DNA extraction from the agarose gel

Having the expected product, DNA extraction was performed using the PCR clean-up Gel extraction kit, NucleoSpin® Gel and PCR clean-up according to the manufacturer's instructions. The *DNA extraction from agarose gels* protocol was followed. Alteration to the protocol: for each 100mg of agarose gel, 300µL (instead of 200µL) of Buffer NTI were used.

2.3.3.8. Lab on a chip run

In order to determine the DNA concentration, a Lab on a chip was performed according to the *Agilent High sensivity DNA kit, Agilent Technologies.*

2.3.3.9. Ion Torrent chip run

The sample was run in an Ion Torrent chip.

2.4. RNA

2.4.1. RNA isolation

The RNA isolation was performed using the *RNA isolation* kit*, NucleoSpin® RNA* from *Macherey-Nagel* according to the manufacturer's instructions. The *RNA purification from cultured cells and tissue* protocol was followed.

2.4.2. RNA quantification

Concentration measurements were performed using the nanodrop device.

2.4.3. cDNA synthesis

A master mix containing the following reagents was made (20µL per reaction): 2µL dNTPs, 1µL OdT, 4µL 5x AMV-RT buffer, 1µL RNase inhibitor, 0,2µL RT enzyme in a total volume of 11,8µL of 1µg RNA in water. The *eppendorf* strip was heated 1h at 42ºC. 80µL of water were added to each sample, and the stripe was stored in the freezer.

2.4.4. SYBR Green RT-qPCR

For each gene (*AurkA, AurkB, Plk1, Mps1*) a forward and reverse primer was designed using the Primer BLAST software (**Table VII**). In order to test the primers, standard dilutions (1:5, 1:25, 1:125, 1:625, 1:3125; water) of the DNA mix of the 5 samples were made. A reaction mix containing cDNA (25x diluted), IQ sybr. Master mix (1x) and Primermix (3pmol) was prepared in a final volume of 10µL. The eppendorfs were vortexed and spun down before use. The Hard-Shell PCR 96 well-plate was prepared on an ice block, sealed and spun down before the run. The following PCR program was introduced in the Bio-RAD CFX96 machine:

- 95ºC, 5min
- 95ºC, 10s
- 60ºC, 30s
  + Plate read
- GOTO 2, 38 more times
- 95ºC, 1min
- Melt curve 65ºC to 95ºC, ↑ 0,5ºC, 10s + plate read
- 15ºC, hold

For the final qPCR, a 1:25 dilution of each one of the cDNA samples was prepared. The previous protocol was then followed. Additionally to the 4 primermix (AurkA, AurkB, Plk1, Mps1) that were used in the primer test, housekeeping gene CPSF6 primers were used as control. The results were analyzed using CFX Manager, Microsoft Excel and GraphPad Prism v7.0.

Table VII. Set of forward (F) and reverse (R) primers used in the qPCR

Gene	Sequence (5'→ 3')
Aurora A	F: CAGTCCCACCTTCGGCATC
	R: GTTCCAAGTGGTGCATATTCCAG
Aurora B	F: CGACATCTTAACGCGGCACT
	R: GACGCAGGATGTTGGGATGG
Plk1	F: GTATTCCCAAGCACATCAACCC
	R: GGCAGTGGGATCTGTCTGAA
Mps1	F: GATTCTCAGGTTGGCACAGTT
	R: CATCCTAAGGACCAAACATCACT

2.5. Protein isolation

At passage time, each cell line was not only passed to a new T-flask, but also to a culture petri dish (*CELLSTAR*). When the cells were ≈70% confluent, the medium was taken from them using a vacuum pipette. The cells were then washed with PBS once. The PBS was discarded using again the vacuum system. 200µL of RIPA lysis buffer was added to the petri dish. Using a scraper, the petri dish was scraped. The content was aspirated to an *eppendorf*, previously labeled and placed 5 minutes at 95ºC in a heating block, in order to improve the lysis process. After that, it was placed at -80ºC.

A different method to obtain the protein lysates was also performed. During cell passage, $1x10^8$ cells of each cell line were counted and placed inside an individual eppendorf. The eppendorf was centrifuged 5min, 500g. The supernatant was discarded and the pellet was stored at -20ºC.

2.5.1. Western Blot

Protein extracts were resolved in 8% SDS-PAGE. The resolved proteins were transferred to a nitrocellulose membrane, using the iBlot Dry Blotting System according to the manufacturer's instructions. Transferred proteins were confirmed by ponceau staining. Membranes were blocked for 1 hour at room temperature with 5% powder milk prepared in PBST20. After an overnight incubation at 4ºC with the primary antibodies diluted in blocking solution, membranes were washed three times for 10 minutes with PBST20 and incubated for one hour at room temperature with the HRP-conjugated secondary antibodies diluted in blocking solution. Blots were developed with ECL Chemiluminescent Detection System (Amersham) according to manufacturer's protocol and detected on X-ray film. Quantification was made using Image J software.

2.5.2. Stripping membranes

To remove the primary and secondary antibodies from the membranes and detect other proteins, an agitating incubation of 2-3min with NaOH 2M was the starting point. Then, the membranes were washed 2 times, 3 minutes each with distilled water. After another washing step of 2 times, 3 minutes each, with PBST20 the membranes were ready for the blocking step. The next steps were performed like previously described in section **2.5.1.**

CHAPTER 3

RESULTS

3.1. Low Passage CRC cell lines classification

Six low passage CRC cell lines and one fibroblast cell line were used in the present work. The morphology of all them can be observed in **Figure 8**, while in **Table VIII** a more detailed data about the patient and the tumor from which each cell line was established is specified.

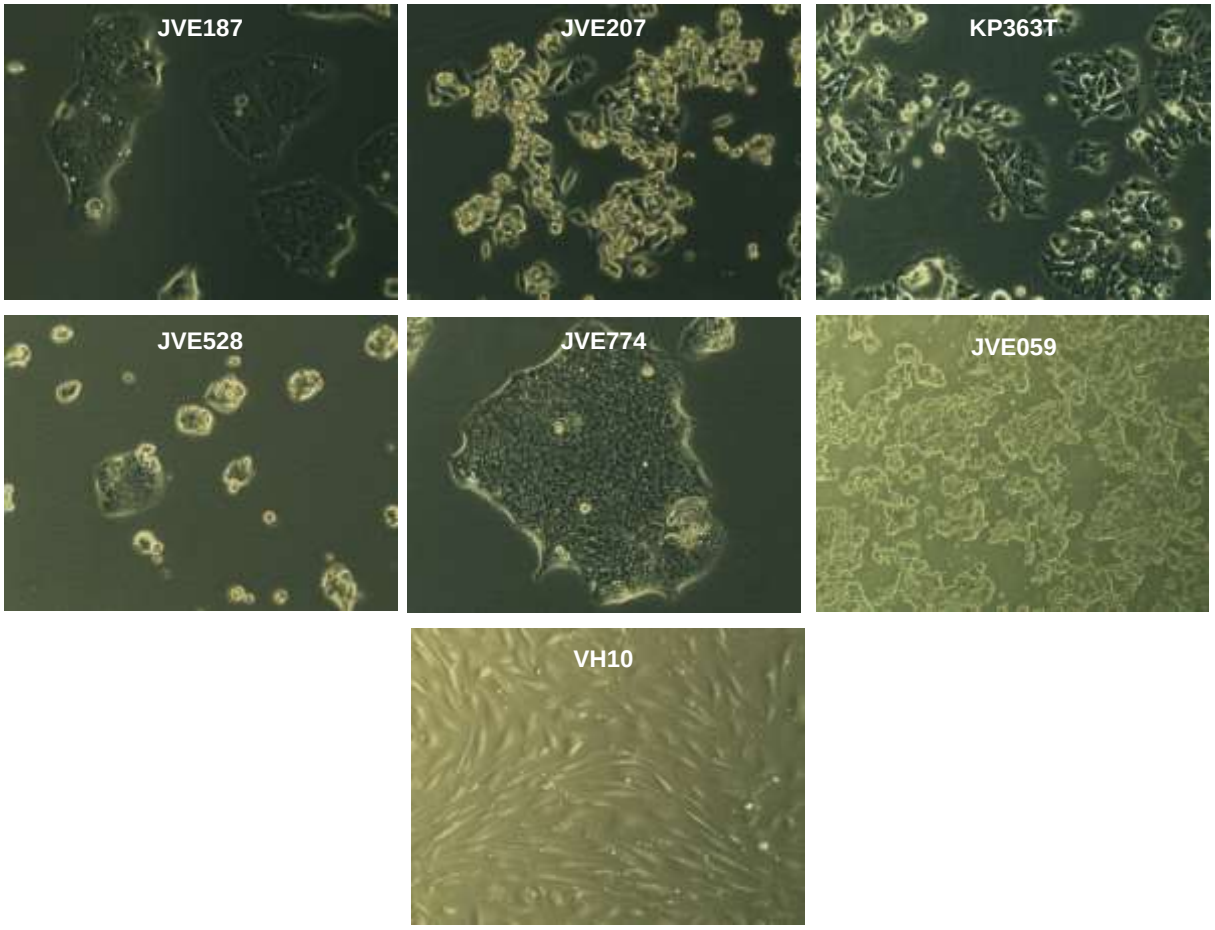

Figure 8. Optical microscope images of the cell lines used in the present work. JVE187, JVE207, KP363T, JVE528 and JVE774 are MSS CRC cell lines. JVE059 is a MSI CRC cell line. VH10 is an immortalized non-malignant fibroblast cell line. Total magnification: 400x

Colorectal cancer cells can display microsatellite stability (MSS) or microsatellite instability (MSI) (Barresi, 2017; Pino, 2010; Rao, 2005). MSS tumors may present different degrees of CIN and are associated with a worse prognosis in CRC (Barresi, 2017; Watson, 2011). High-throughput SNP genotyping arrays can be used to measure instability degrees, through copy number alterations (CNA) analysis (Corver et al., 2008; Wu et al., 2014). CNAs are gains and/or losses of large segments of the genome, from a few kilo-bases to whole

chromosomes. Somatic CNAs that occur during a lifetime of an individual are a major contributor to cancer development (Wu, 2014). Thus, the study of specific chromosomal aberrations in different tumor samples can lead to the identification and validation of important cancer genes (Venkatraman et al., 2007). For example, recurrent aberrations in CRC include gains of chromosomes 7, 8q, 13, and 20q and losses of 4q, 8p, and 18q (De Angelis et al., 1999; E. H. Lips et al., 2007; Esther H. Lips et al., 2005).

MSI results from mutations in the DNA mismatch repair genes (Parine et al., 2016). A panel of five markers is recommended as reference to classify a tumor as microsatellite stable or instable. The mononucleotides BAT25 and BAT26 and the dinucleotides D5S346, D2S123 and D17S250 constitute the reference panel (Boland et al., 1998). If two or more of those five markers are detected, the tumor is microsatellite instable high (MSI-H). Those tumors have unique histopathological features and are associated with a better prognosis; if only one of the five markers is detected, the tumor is microsatellite instable low (MSI-L); if none of the five markers is detected, the tumor can be microsatellite instable low or microsatellite stable. In order to distinguish these two genetic states, a greater number of markers should be used (Boland, 1998).

Table VIII. Cell lines used in this work. Patient and tumor data from which the cell lines used in the present work were established. Adapted from Boot et al., 2016.

CELL LINE	PATIENT		TUMOR		MSI
	Age	Gender	Morphology	Location	
JVE059	58	M	Adenocarcinoma	Transverse Colon	MSI-H
JVE187	60	F		Liver metastasis	MSS
JVE207	67	M		Descending colon	
JVE528	57	F		Ascending colon	
JVE774	61	M		Rectum	
KP363T	80	M		Left-sided colon	
VH10	(anonymous; commercial cell line)		Immortalized non-malignant fibroblast cell line		NA*

***NA:** not applicable*

From the six low passage CRC cell lines used in the present work, five of them are MSS (JVE187, JVE207, JVE528, JVE774, KP363T), while JVE059 is a MSI cell line. **Figure A 1** illustrates the CNA plots of each cell line, resultant from a SNP array. JVE207 and KP363T seem to have the higher degrees of aneuploidy, translated by gains and losses of genome segments in all chromosomes. JVE528, JVE774 and JVE187 have more than one chromosome without any/almost any gain or loss of genome fragments. Thus, the aneuploidy degree of this three cell lines should be lower than KP363T and JVE207. Finally, in JVE059 at least half of the chromosomes show total integrity. Moreover, in the remaining chromosomes, the uneven blue lines are not so evident as in JVE528, JVE774 and JVE187. Thus, JVE059 should have the lowest degree of aneuploidy.

3.2. Aurora A, Aurora B, Plk1 and Mps1 are not mutated in CRC cell lines

Mutations in genes encoding SAC related proteins have been observed at very low frequency, even in aneuploid tumor cells with CIN (Bakhoum, 2009; Hernando, 2004; Orr, 2013; Ricke, 2008). In order to see if there was any mutation in the genes that codify for the key mitotic players of interest, Ion Torrent Next Generation Sequencing (NGS) was performed as detailed in Materials and Methods section. A schematic representation of the procedure may be found in **Figure 9**.

The NGS primer panel was obtained from *Integrated DNA Technologies* in a total of 108 amplicons divided between two pools. The primers had the coding sequences of *Aurora A*, *Aurora B*, *Plk1* and *Mps1* as target. The primer panel was tested by PCR followed by electrophoresis in agarose gel. Three different concentrations (25nM, 50nM, 100nM) were tested with one cell line isolated DNA. Two positive controls, a sample and a panel that worked in previous experiments, were also used. The electrophoresis results are illustrated in **Figure 10**.

The concentration of 100nM was the primers concentration that worked the best. Using this concentration, a ≈200bp product can be detected not only in the sample used as positive control, but also in the cell line tested (JVE187). In the panel used as positive control there were no results because it was used a different PCR program from the one that worked before using that same panel. Having a proper primer concentration, a first and second round PCR were performed, each one followed by a beads purification step. After sample pooling, a qPCR was performed to confirm the PCR amplification, before running it on gel. Once verified that the PCRs worked, the sample pooling of the 2nd round PCR product was run in a 2% agarose gel electrophoresis (**Figure 11**). A clear band between 200 and 300bp can be observed.

The product was extracted from the agarose gel, digested and run in a lab-on-chip. With the previous result the sample concentration was adapted to the Ion Torrent chip run. The Ion Torrent NGS results show all the possible mutation sites, in a total of 72 positions. The sites were individually analyzed by visual inspection in order to classify them as mutation or false positive. An example of these positions is illustrated in **Figure 12.** For the complete file, please consult the Appendix data (**Figure A 2**). Moreover, a graph that translates the amount of reads per amplicon is represented in **Figure 13.**

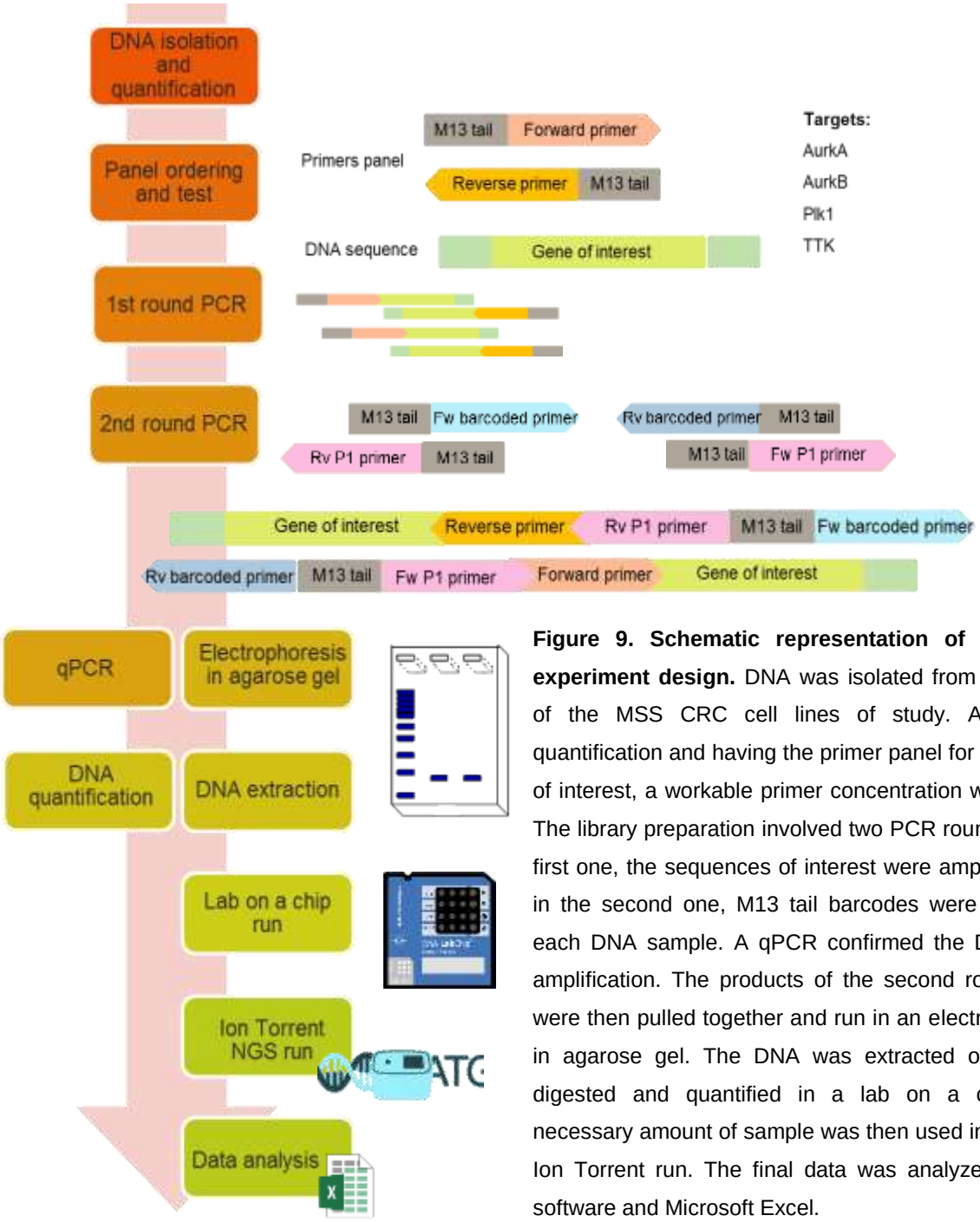

Figure 9. Schematic representation of the NGS experiment design. DNA was isolated from each one of the MSS CRC cell lines of study. After DNA quantification and having the primer panel for the genes of interest, a workable primer concentration was found. The library preparation involved two PCR rounds; in the first one, the sequences of interest were amplified; and in the second one, M13 tail barcodes were added to each DNA sample. A qPCR confirmed the DNA PCR amplification. The products of the second round PCR were then pulled together and run in an electrophoresis in agarose gel. The DNA was extracted of the gel, digested and quantified in a lab on a chip. The necessary amount of sample was then used in the NGS Ion Torrent run. The final data was analyzed by IGV software and Microsoft Excel.

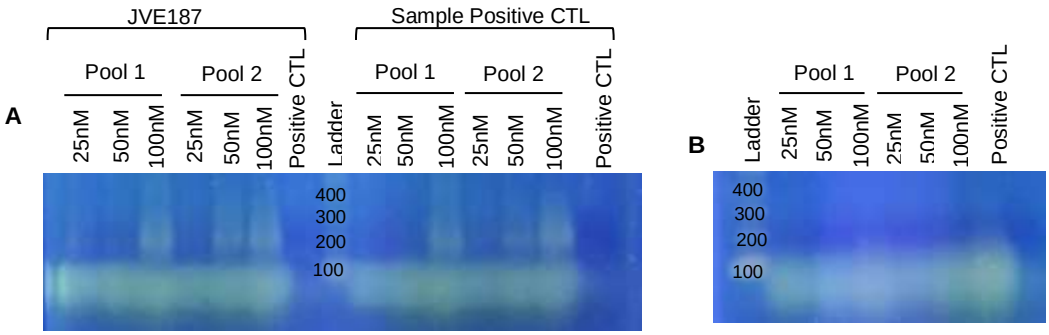

Figure 10. SYBR Gold electrophoresis on agarose gel. A. Three different concentrations (25nM, 50nM, 100nM) of the primer panel were tested with the DNA isolated from the cell line JVE187. An old DNA sample (Sample positive CTL) and a primer panel (Positive CTL) were also used as positive controls. The PCR results were run on 2% agarose gel. **B.** The blank, without DNA, is also illustrated.

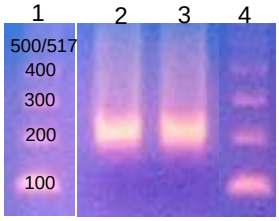

Figure 11. SYBR Gold electrophoresis results from the second round PCR samples after pooling. The second round PCR product containing the DNA sequences of interest amplified from all samples was run in an agarose gel. A clear product between 200-300bp can be observed. 1, 4 – Ladder; 2, 3 – Sample (duplicated)

	Chr	Start	End	Ref	Alt	Func.refGene	Gene.refGene
1	chr6	80751897	80751897	A	-	exonic	TTK
2	chr6	80751905	80751905	A	G	exonic	TTK
3	chr6	80751909	80751909	G	A	exonic	TTK
4	chr6	80751910	80751910	A	G	exonic	TTK
5	chr6	80751942	80751942	G	A	UTR3	TTK

Figure 12. Excel fragment showing five possible mutation sites, in a total of 72. Ion Torrent NGS results showed all the possible mutation sites, in a total of 72 positions The sites were individually analyzed by visual inspection in the IGV software in order to classify them as mutation or false positive. The complete file can be found in the Appendix section. Chr: chromosome, Ref: reference, Alt: alteration, Func.refGene: Function in the reference gene; Gene.refGene: Gene of reference

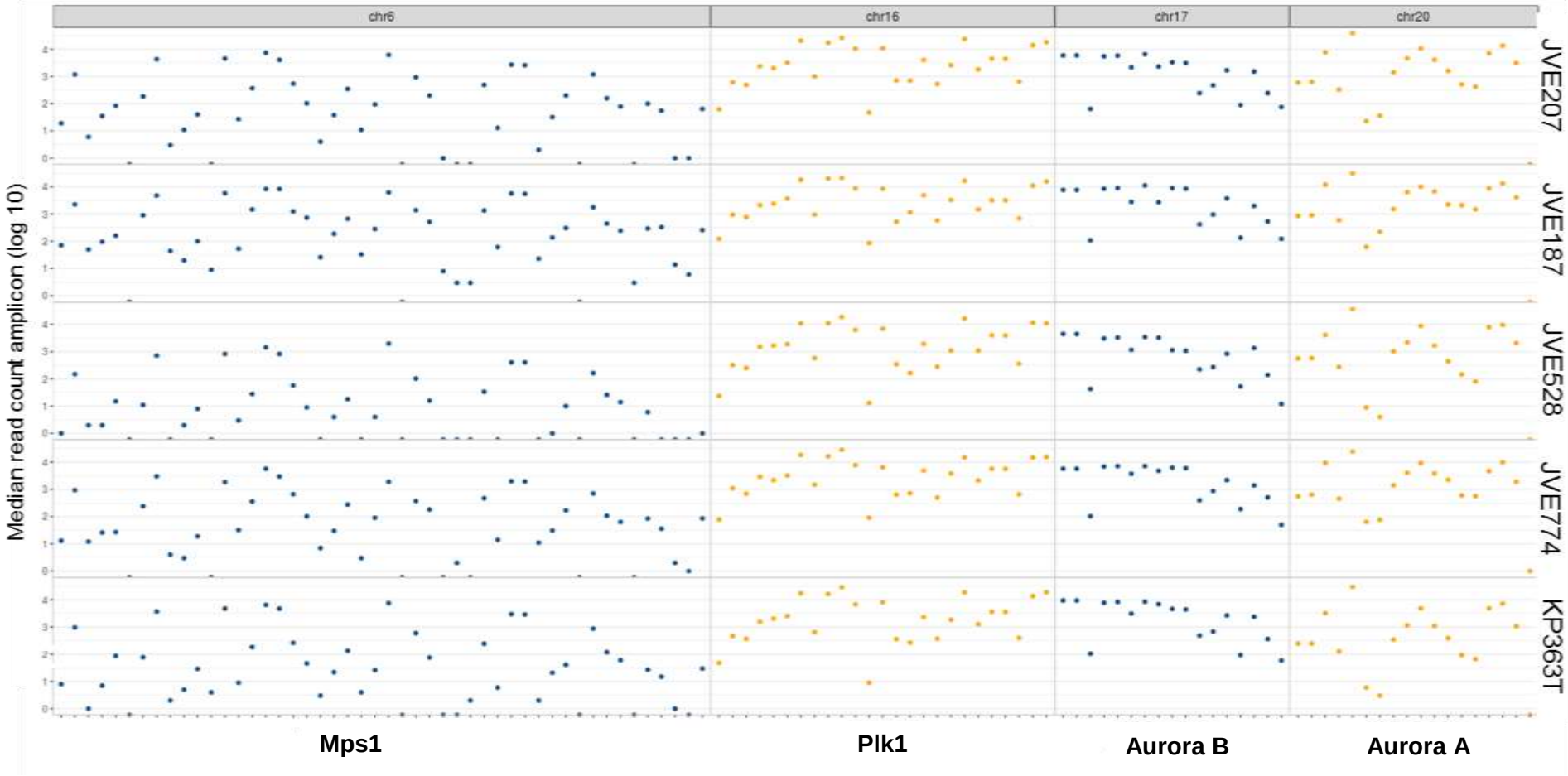

Figure 13. Ion Torrent NGS coverage graph. The MSS CRC cell lines (JVE207, JVE187, JVE528, JVE774, KP363T) were tested for mutations in key mitotic player genes. *Mps1, Plk1, Aurora B* and *Aurora A* genes are located in chromosome 6, 16, 17 and 20 of the human genome, respectively. A panel of 108 amplicons, each one represented here by a dot, was used. The data shown is in logarithmic scale. For a number of reads below 10 it is not possible to take a viable conclusion about the amplicon of analysis.

3.3. Key mitotic players expression at transcript level is higher in MSS CRC cell lines

The NGS experiment revealed the absence of mutations in *Aurora A, Aurora B, Plk1* and *Mps1* genes in all cell lines under study. Next, we were interested in comparing the expression level of these genes in the different cell lines. To do so, a SYBR Green RT-qPCR based method was performed. For each gene (*AurkA, AurkB, Plk1, Mps1*) a forward and reverse primer were designed using the Primer BLAST software (see **Table VII** in Materials and Methods section). The house keeping gene CPSF6 was used to normalize the results. The relative transcript levels for each gene in the different CRC cell lines are summarized in **Figure 14.**

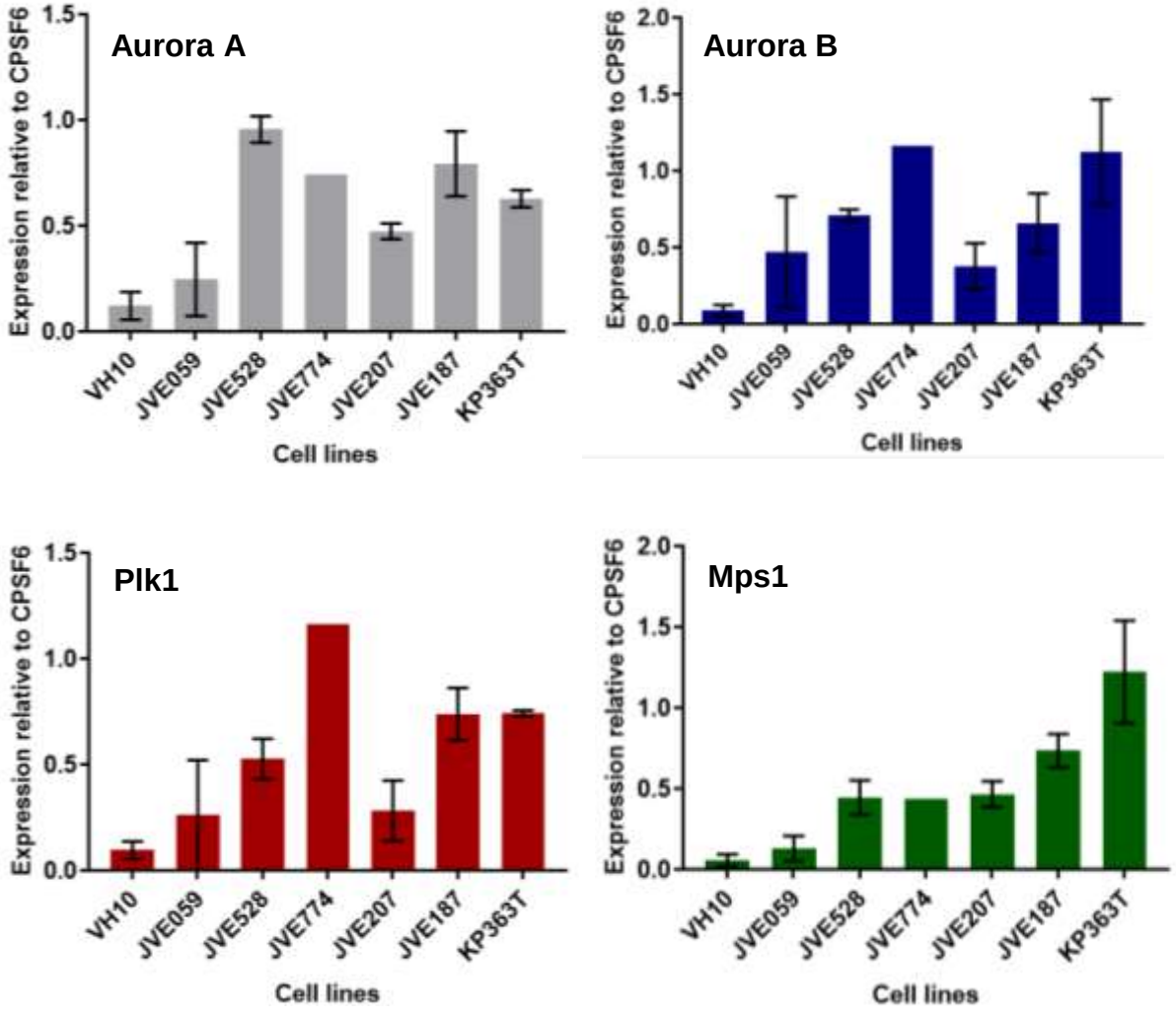

Figure 14. Transcript levels of *Aurora A, Aurora B, Plk1* and *Mps1* in different CRC cell lines. Graphs show the relative transcript levels of *Aurora A, Aurora B, Plk1* and *Mps1* in the cell lines of study assessed by RT-qPCR. For JVE774 only one replicate is represented. As this was a slow growth CRC

Figure 14 continued on next page

Figure 14 continued

cell line, it was not possible to repeat the essay due to time restrictions. For all the other cell lines the values are expressed as the mean ± standard deviation.

Aurora A expression is higher in the CRC cell lines, in comparison to the fibroblast cell line used as control (VH10). The transcript level of this kinase in JVE059 and JVE207 cell lines is similar, although slightly lower in the MSI cell line. In the other four MSS cell lines, Aurora A expression in higher. JVE528 is the one where the difference in this kinase expression is more significant.

Interestingly, JVE059 and JVE207 are also the cell lines with an Aurora B and Plk1 expression closer to VH10. Regarding Aurora B, JVE774 and KP363T are the MSS cell lines with an expression more significantly different than VH10. Concerning Plk1, JVE774 is the MSS cell line with the higher expression.

Mps1 expression is higher in JVE528, JVE774 and JVE207 in comparison to VH10 and JVE059. JVE187 has a slightly higher Mps1 expression than the three MSS CRC cell lines previously referred. The highest Mps1 expression is observed for KP363T.

The transcript levels for each gene were higher in the CRC cell lines than in the fibroblast cell line used as control, VH10. Moreover, those levels were in general higher in the MSS cell lines than in the MSI cell line, JVE059. JVE207 constitutes an exception to this statement in terms of Aurora B expression, as in this case JVE059 has a slightly higher Aurora B transcript level than JVE207.

3.4. Higher expression of the key mitotic players in MSS CRC cell lines is confirmed by protein analysis

To monitor the expression at the protein level we performed western blot analysis for each kinase in the different cell lines.

Aurora A, Aurora B, Plk1 and Mps1 protein expression was analyzed in four MSS CRC cell lines (JVE528, JVE207, JVE187, and KP363T), in one MSI CRC cell line (JVE059) and compared with a human fibroblast cell line (VH10) (**Figure 15**). The results were normalized for α-Tubulin as well as for Cyclin B (**Figure 16**).

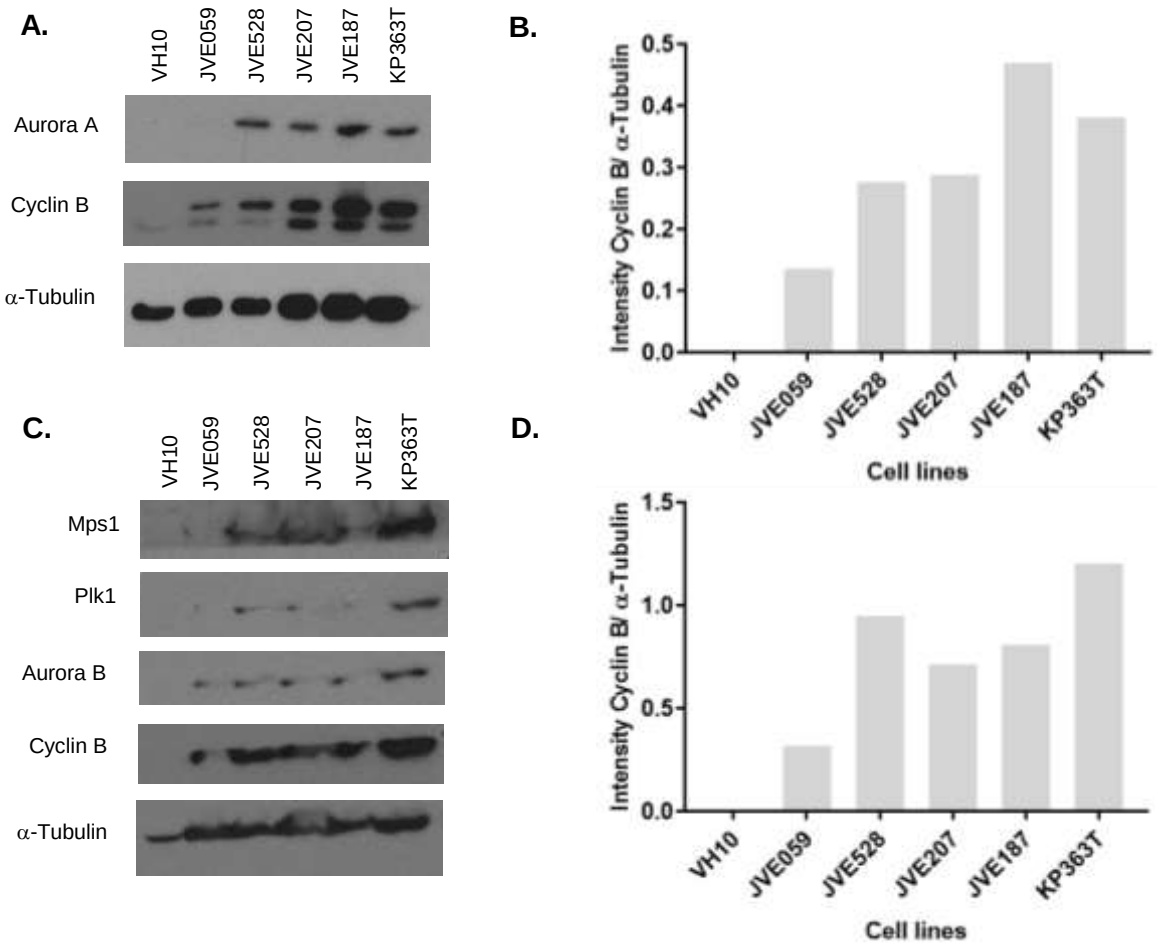

Figure 15. Western blot analysis. Protein cell lysates of a fibroblast cell line, VH10, and five CRC cell lines were run in gel. (**A**) Aurora A levels were assessed by western blotting. Cyclin B is a cell cycle marker. α-Tubulin was set as loading control. (**B**) The ratio between the intensities of Cyclin B and α-Tubulin was calculated. (**C**) Mps1, Plk1 and Aurora B levels were assessed by western blotting. Cyclin B is a cell cycle marker. α-Tubulin was set as loading control. (**D**) The ratio between the intensities of Cyclin B and α-Tubulin was calculated.

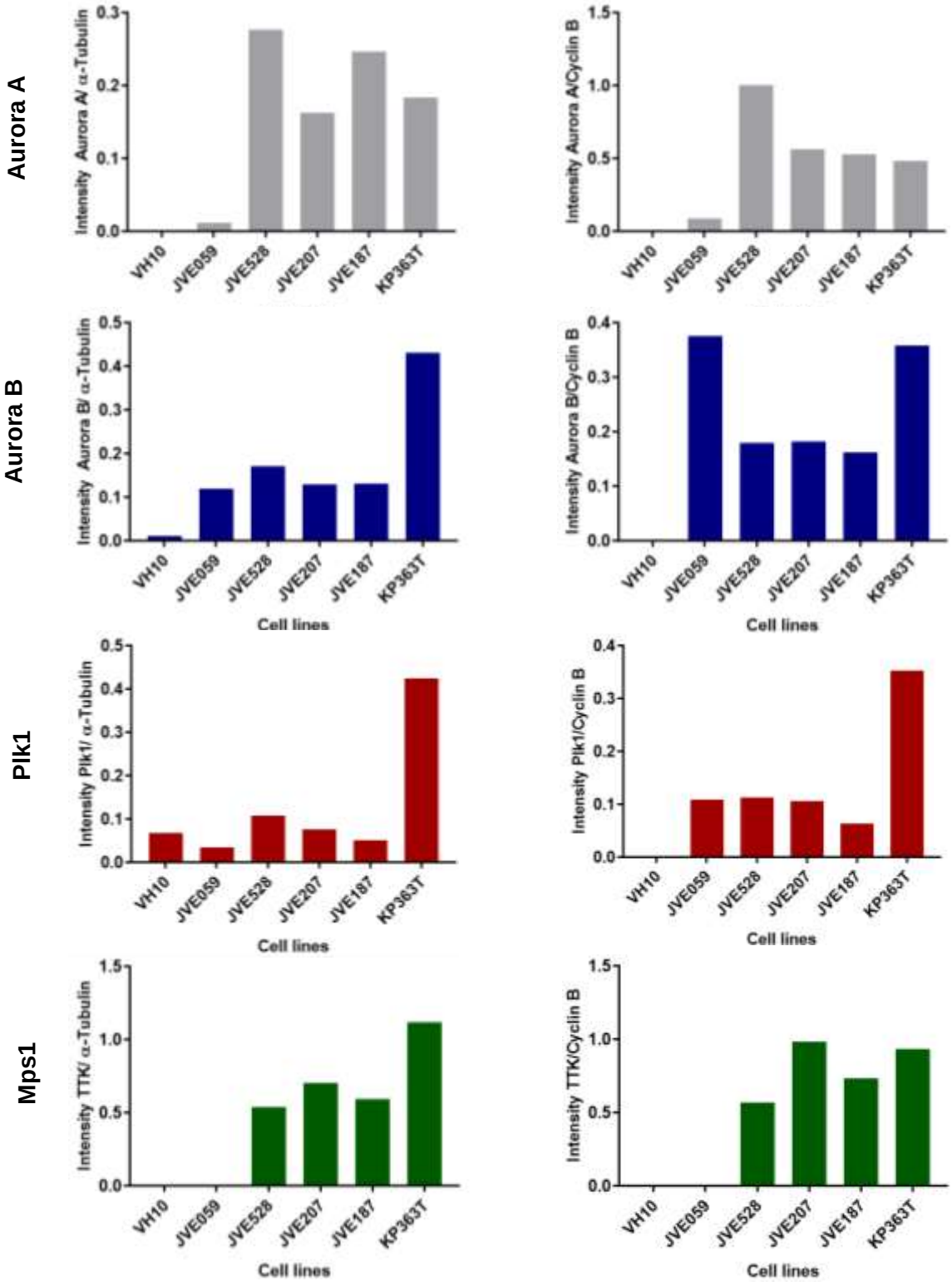

Figure 16. Relative expression of Aurora A, Aurora B, Plk1 and Mps1 in each cell line of study. Graphs show the intensity ratio between each one of the kinases detected in the western blot analysis and α-Tubulin (**left**) or Cyclin B (**right**) for each cell line of study.

After quantification of the proteins detected in both membranes, normalization calculations were made. On one hand, dividing the Aurora A, Aurora B, Plk1 or Mps1 expression by α-Tubulin, the total relative expression of each protein is obtained. On the other hand, the normalization with Cyclin B gives the relative expression of each protein taking into consideration the number of mitotic cells in each protein lysate. Analysing **Figure 16**, expression of Aurora A is higher in the MSS cell lines (JVE528, JVE207, JVE187, KP363T) in comparison with the MSI cell line (JVE059) and the control (VH10). This is not dependent on the number of mitotic cells, as when the normalization with cyclin B is made, those cell lines maintain a higher Aurora A expression. JVE528 shows the highest expression of Aurora A, taking into account the number of mitotic cells. Aurora B expression seemed also higher in the MSS CRC cell lines. However, the normalization with cyclin B revealed the highest Aurora B expression by JVE059, the MSI CRC cell line. KP363T also shows a very high expression, close to the one of JVE059. The normalization with cyclin B in the Plk1 quantifications revealed a higher expression in the CRC cell lines, comparing with the fibroblast cell line. This expression is particularly higher for KP363T. Finally, for Mps1, MSS CRC cell lines showed an overall higher expression, which was confirmed by normalization with cyclin B.

CHAPTER 4

GENERAL DISCUSSION

In this work, we studied four key mitotic regulators, Aurora A, Aurora B, Plk1 and Mps1, in low passage CRC cell lines derived from primary tumors. The majority of the data published in the literature indicate that CRC cell lines have hyperstable KT-MT interactions that prevent the correction of erroneous attachments and thereof, thought to represent a major cause of CIN. However, this was observed in CRC cell lines that were established several decades ago. As the number of passages increases, there is a higher probability of errors occurrence during cell division. This causes variability, limits conclusion accuracy and precludes physiological/clinic correlation. Using low passage primary cultures of CRC cell lines derived from primary tumors, either MSI or MSS, and a human fibroblast cell line we characterized the genetic integrity and expression pattern of four key mitotic players known to be involved KT-MT attachments and SAC signaling. At genetic level, next-generation sequencing (NGS) revealed absence of mutations in the coding sequence of *Aurora A*, *Aurora B*, *Plk1* and *MPS1* genes. At transcript and protein level, expression of these genes was monitored by RT-qPCR and Western Blotting, respectively. The mitotic kinases were found to be prevalently overexpressed in CRC MSS cell lines with higher levels of aneuploidy.

4.1. NGS revealed lack of mutation in *Aurora A, Aurora B, Plk1 and Mps1* exons in several CRC cell lines

NGS was performed using the DNA isolated from the CRC cell lines characterized by different degrees of aneuploidy.

A total of 72 possible mutation sites initially detected (see Appendix, **Figure A 2**) but subsequently found to be false positives after detailed visual inspection. This is not uncommon with the Ion Torrent sequencing, especially when the genes under analysis contain a lot of homopolymers, as in case of the mitotic regulators in this study. Homopolymers may lead to errors in the sequencing process. In fact, when one nucleotide aligns in Ion Torrent NGS, the change in pH, translated by a change in voltage, is very low. In the particular case of a homopolymer (X)n, there are n alignments of X following each other. It is difficult for the system to sum up all those small changes in voltage. Thus, errors like mismatches may occur, leading to false mutations in the analysis. Another common error occurs when a single nucleotide polymorphism (SNP) takes place. It is a variation in a single nucleotide at a specific position in the genome that is present in more than 1% of the population. Those are the most common type of genetic variation among people (Zeng et al., 2013).

The coverage graph (**Figure 13**) shows that there are amplicons with a number of reads below 1 (corresponds to 10, as the graph is in the logarithmic scale). Those amplicons were not

read enough times, so it is not possible to withdraw any conclusion about them. This situation happens particularly for Mps1. As the %GC on the *Mps1* gene is low, the annealing temperature used for primer hybridization was likely higher than the optimal, which possibly underlies the higher number of inconclusive amplicons.

To sum up, the NGS shows that there are no mutations in the DNA that codify our proteins of interest so far. This was predictable, once the proteins that emerge from those genes are very important in cell division. Without them, cells fail division and, inevitably, end up dying. Despite the low frequency of mutations in the genes that codify key mitotic players, Sanger sequencing of the amplicons with a number of reads below 10 should be performed.

4.2. Aurora A, Aurora B, Plk1 and Mps1 are prevalently overexpressed in MSS cell lines with higher levels of aneuploidy

The expression of Aurora A, Aurora B, Plk1 and Mps1 in different CRC cell lines was monitored by RT-qPCR and Western blotting. Transcript levels of all the key mitotic regulators are higher in CRC cell lines when compared with genomic stable human fibroblasts (**Figure 14**). For all other kinases, transcript levels are higher in the MSS CRC cell lines, than in the MSI CRC cell line. This is in line with previous studies describing the overexpression of several mitotic regulators in different tumor types (Faisal, 2017). Analysis of 18 expression data sets from 6 different tumor types revealed 70 genes that are overexpressed in CIN tumors. The so called CIN70 includes several genes related with the cell cycle control, namely *Aurora A*, *Aurora B* and *Mps1* (Carter et al., 2006). In another study, qPCR analysis showed a higher expression of *Plk1* in 41 of 56 colorectal cancer tissues, which was additionally correlated with an increase in the tumor size and lymphatic metastasis (Han et al., 2012).

The western blot analysis confirmed the expression pattern obtained by RT-qPCR (**Figure 16**). The consistency of these results both at the transcript and protein levels highlight the higher expression of Aurora A, Aurora B, Plk1 and Mps1 in the CRC cell lines, particularly in the MSS ones with higher levels of genomic instability (**Figure 17**). The cell line KP363T stands out as the one with higher expression of Aurora B, Plk1 and Mps1. Interestingly, this cell line is the one with higher proliferation rate in culture (Boot, 2016). Our findings are in line with previous studies reporting the overexpression of these proteins in CRCs (Han, 2012; Katayama et al., 1999; Ling, 2014).

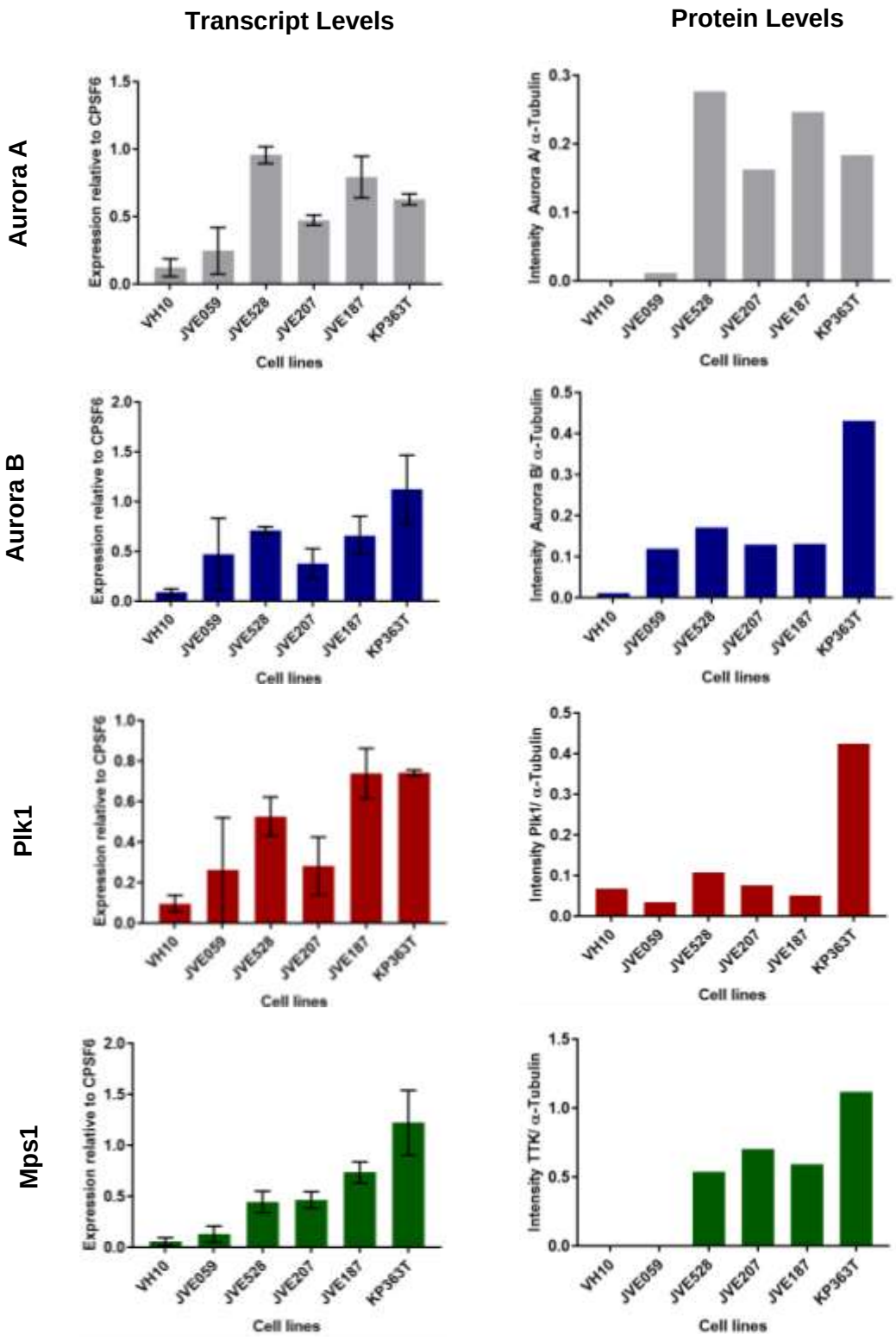

Figure 17. Comparison of Aurora A, Aurora B, Plk1 and Mps1 expression pattern obtained by RT-qPCR (left) and Western Blot (right). JVE774 cell line was excluded from the RT-qPCR graphs so it is easier to compare both analyses (note that JVE774 is a CRC slow growth cell line that was not analyzed by Western Blot due to time restrictions). The RT-qPCR values (**left**) are expressed as the mean ± standard deviation.

4.3. Concluding remarks

In this work, we analysed four key mitotic players in terms of gene integrity and at expression level. Aurora A, Aurora B, Plk1 and Mps1, known to be involved in SAC activity and in KT-MT attachments, were studied in low passage CRC cell lines. Despite the absence of mutations so far, those genes were shown to be overexpressed, not only at transcript level, but also at protein level. Moreover, the upregulation observed seems to be directly related with the degree of instability found in the cells. Together, these findings are in line with what is currently described in the literature for CRC commercially cell lines. However, because we used novel CRC cell lines that closely resemble the biology of the original tumor, the results obtained regarding the expression profile of Aurora A, Aurora B, Plk1 and Mps1 assume higher relevance. More work is needed to dissect the importance of the results obtained. These findings set the ground for future studies aiming to examine the cause of chromosome segregation errors in CRCs and their contribution for CIN. A detailed knowledge of the mitotic behavior of these novel low passage CRC cell lines will be critical to design anti-cancer therapeutic strategies that will have an impact *in vivo*.

CHAPTER 5

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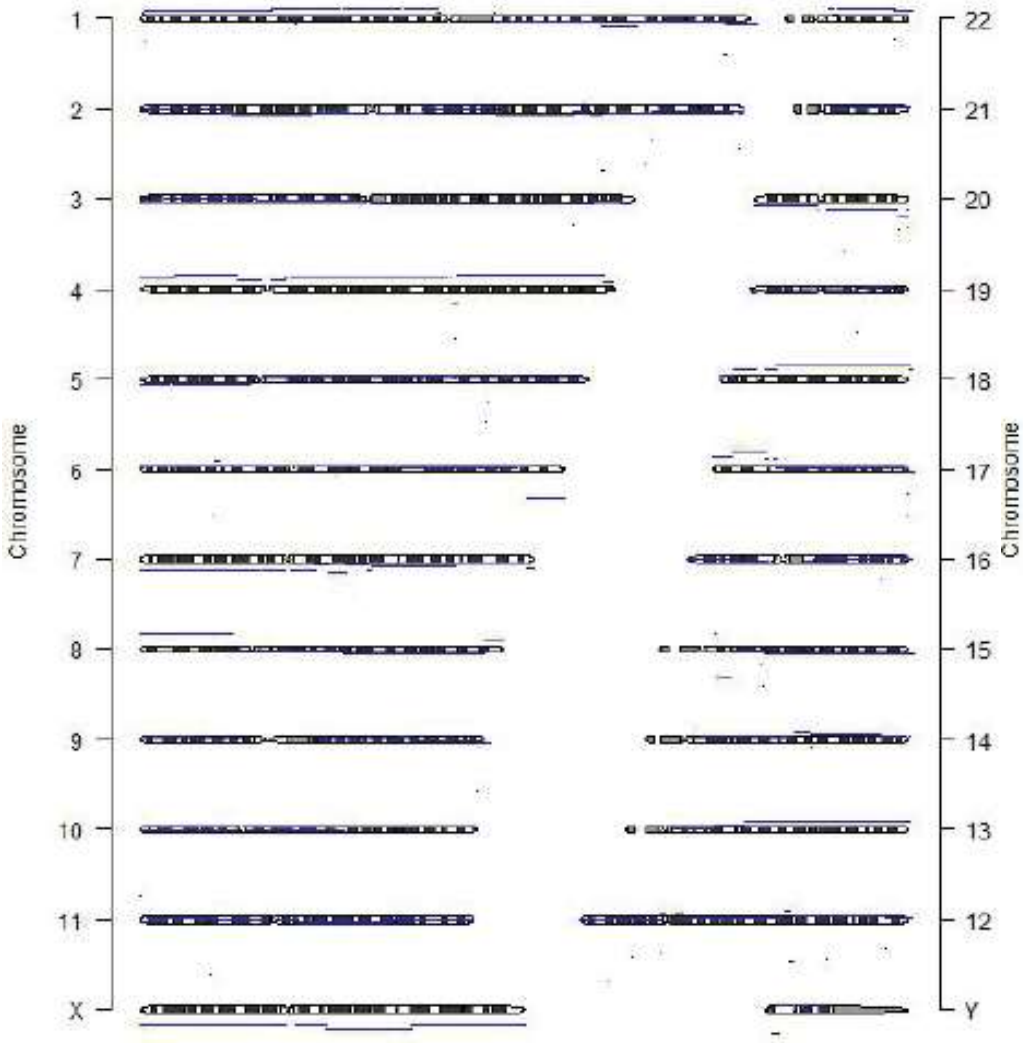
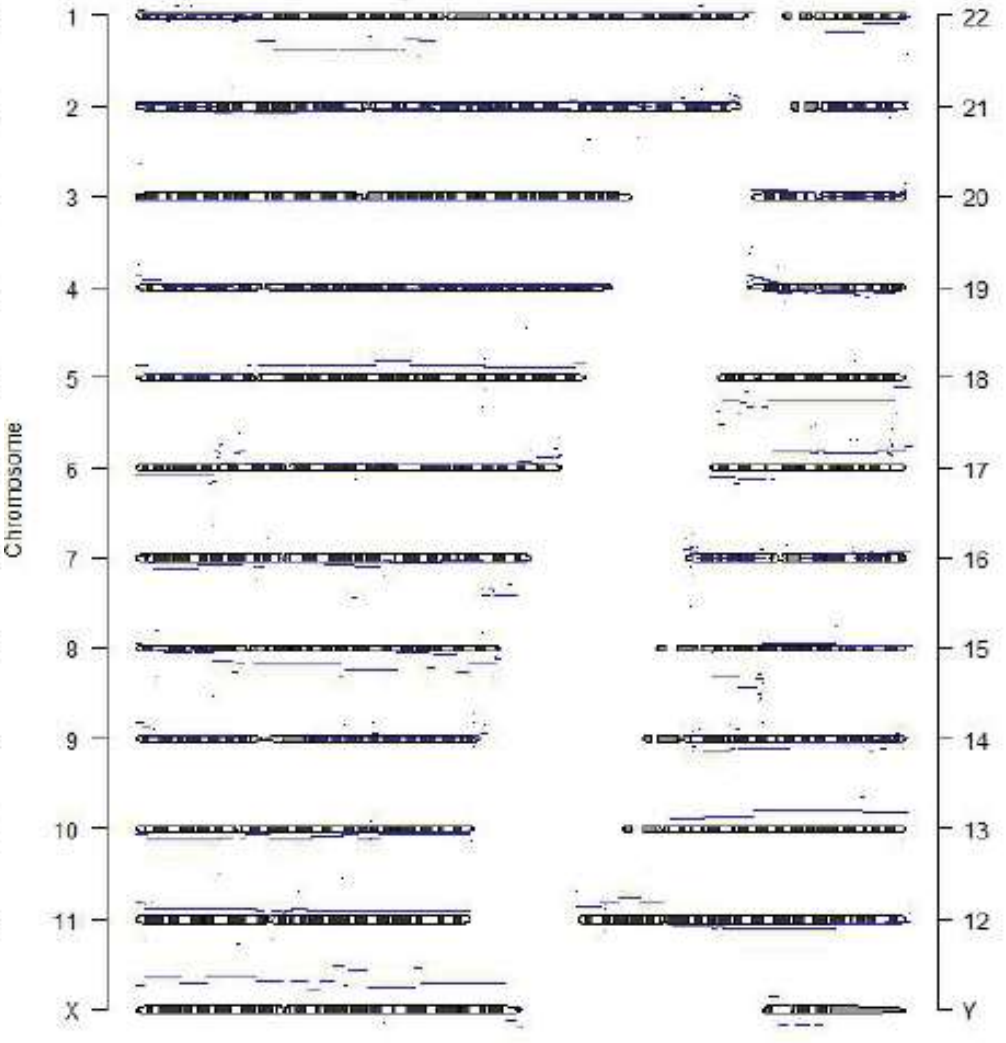
APPENDIX

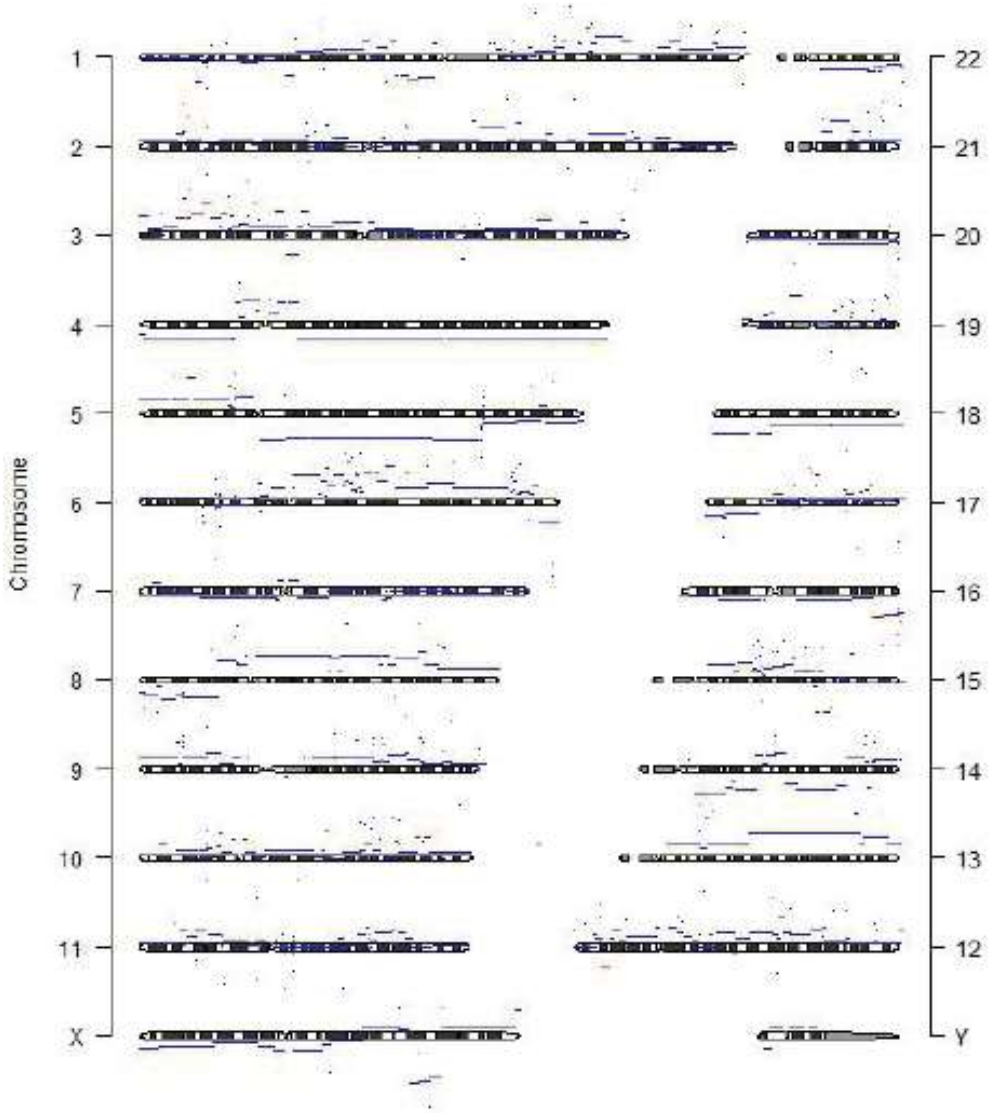
JVE059



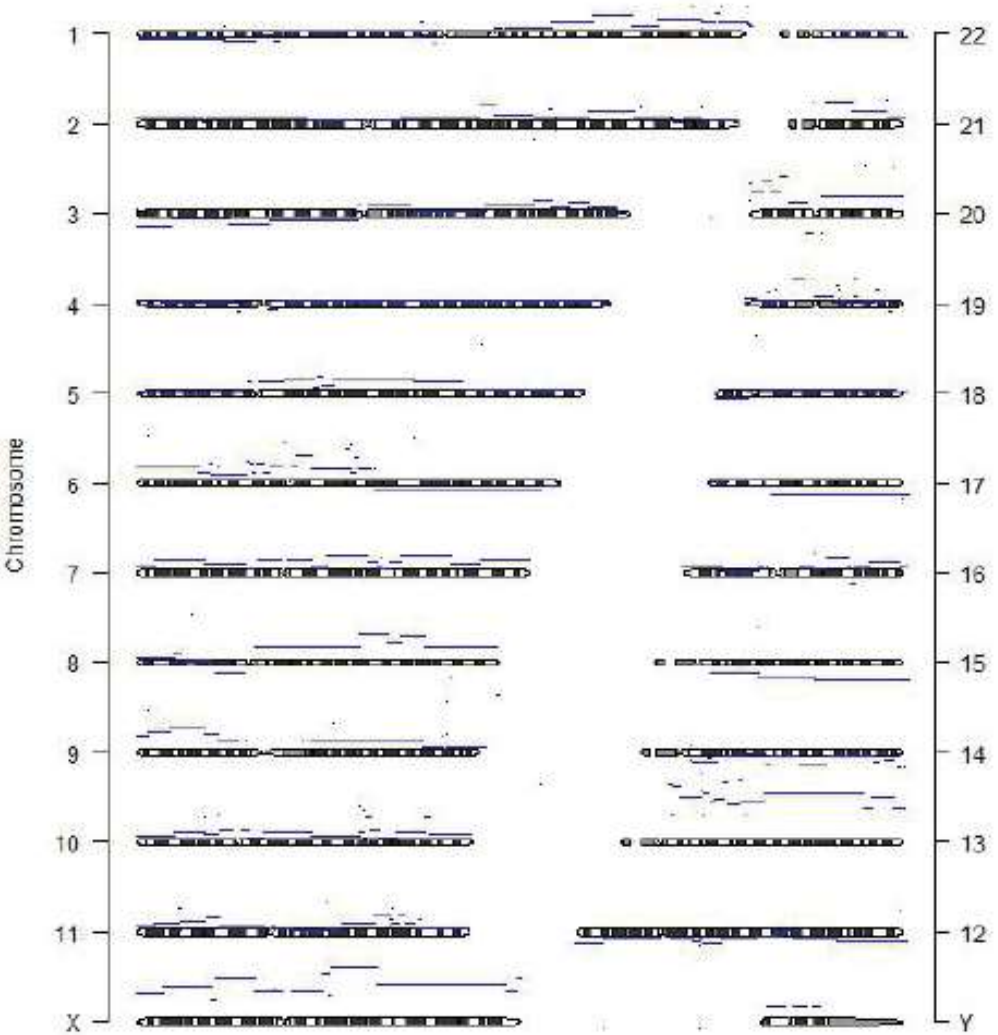
JVE187



JVE207



JVE528



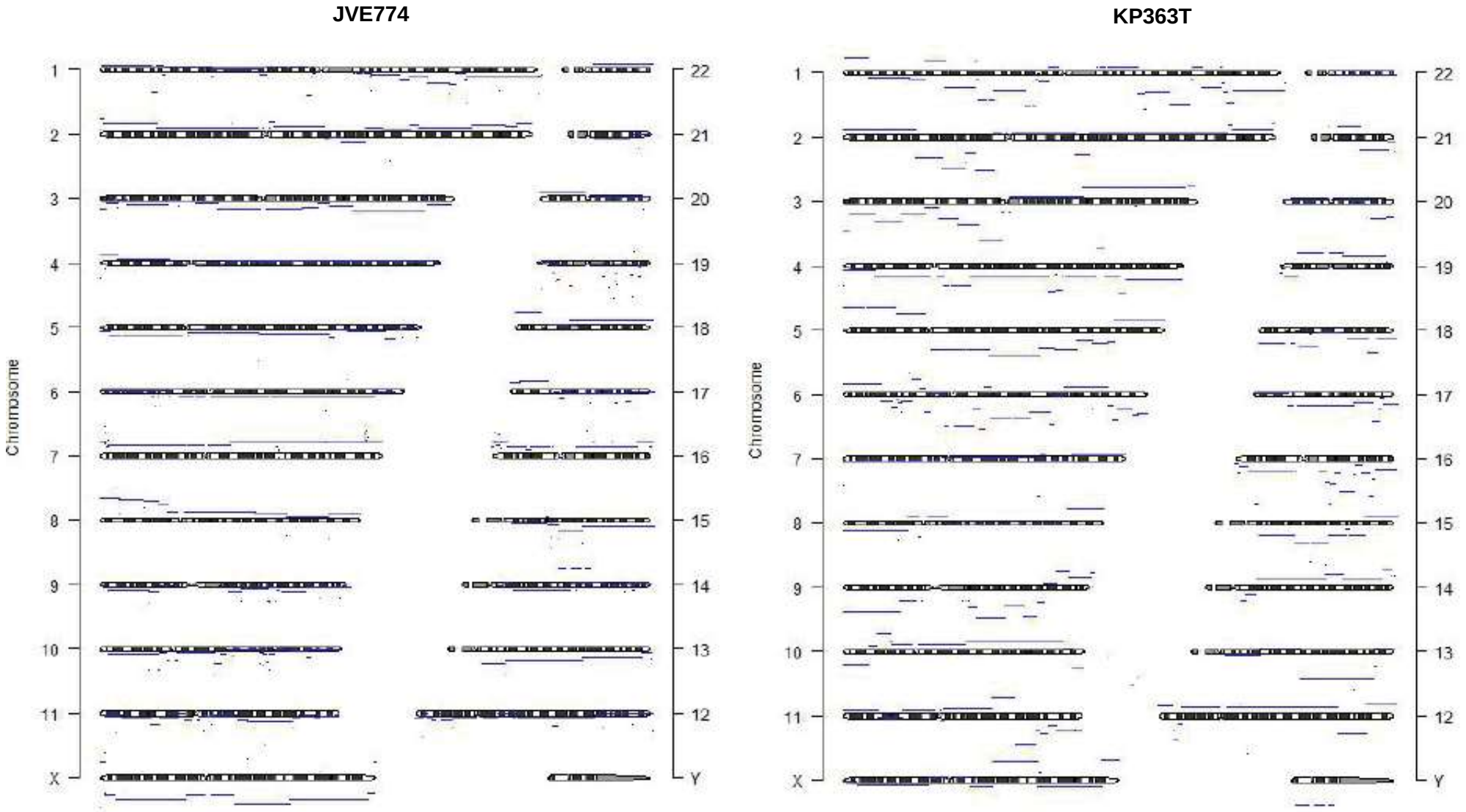


Figure A 1. CNA plots of the low passage CRC cell lines used as model of study in the present work. For each CRC cell line (JVE059, JVE187, JVE207, JVE528, JVE774, KP363T) the 23 chromosomes that constitute the karyotype are represented. The blue lines translate the gains and losses (CNAs) in a specific chromosome; if the line is above the chromosome, it represents a gain; if the line is below the chromosome, it translates a loss. More gains and losses translate an increase in genetic instability.

Chr	Start	End	Ref	Alt	Func.refGene	Gene.refGene
chr16	23692405	23692405	A	G	intronic	Plk1
chr16	23695198	23695198	-	C	exonic	Plk1
chr16	23695212	23695212	-	C	exonic	Plk1
chr16	23698772	23698772	T	-	intronic	Plk1
chr16	23698823	23698823	A	-	exonic	Plk1
chr16	23698857	23698857	G	-	exonic	Plk1
chr16	23698944	23698944	-	G	exonic	Plk1
chr16	23698952	23698952	-	G	intronic	Plk1
chr16	23698954	23698954	-	G	intronic	Plk1
chr16	23700009	23700009	-	C	exonic	Plk1
chr16	23700020	23700020	-	G	exonic	Plk1
chr16	23700637	23700637	G	-	exonic	Plk1
chr16	23700740	23700740	-	G	intronic	Plk1
chr16	23701329	23701329	-	C	exonic	Plk1
chr17	8108272	8108272	G	-	exonic	AURKB
chr17	8108331	8108331	A	G	exonic	AURKB
chr17	8108339	8108339	G	A	exonic	AURKB
chr17	8108473	8108473	-	C	intronic	AURKB
chr17	8108480	8108480	-	C	intronic	AURKB
chr17	8108576	8108576	A	-	exonic	AURKB
chr17	8108592	8108592	-	C	exonic	AURKB
chr17	8109964	8109964	G	-	intronic	AURKB
chr17	8110043	8110043	-	C	intronic	AURKB
chr17	8110130	8110130	G	-	exonic	AURKB
chr17	8110135	8110135	G	C	exonic	AURKB
chr17	8110140	8110140	-	G	exonic	AURKB
chr17	8110208	8110208	-	G	exonic;splicing	AURKB;AURKB
chr17	8110510	8110510	T	-	exonic	AURKB
chr17	8110856	8110856	-	C	intronic	AURKB
chr17	8111099	8111099	-	G	exonic	AURKB
chr17	8113555	8113555	-	G	UTR5	AURKB
chr17	8113568	8113568	-	G	splicing	AURKB
chr20	54945231	54945231	C	T	exonic	AURKA
chr20	54945248	54945248	C	G	exonic	AURKA
chr20	54945254	54945254	G	A	exonic	AURKA
chr20	54945681	54945681	G	C	exonic	AURKA
chr20	54945683	54945683	-	G	exonic	AURKA
chr20	54948466	54948466	G	-	exonic	AURKA
chr20	54956635	54956635	A	-	intronic	AURKA
chr20	54961315	54961315	G	-	exonic	AURKA
chr20	54961422	54961422	C	T	exonic	AURKA
chr20	54961463	54961463	T	C	exonic	AURKA
chr6	80715553	80715553	T	C	UTR5	Mps1
chr6	80715554	80715554	-	T	UTR5	Mps1
chr6	80718066	80718066	-	T	intronic	Mps1
chr6	80718168	80718168	-	T	exonic	Mps1
chr6	80718231	80718231	-	A	intronic	Mps1
chr6	80720488	80720488	C	A	intronic	Mps1
chr6	80720490	80720490	T	G	intronic	Mps1
chr6	80720491	80720491	T	A	intronic	Mps1
chr6	80720630	80720630	A	-	exonic	Mps1
chr6	80721045	80721045	A	G	intronic	Mps1
chr6	80721180	80721180	T	-	exonic	Mps1
chr6	80721299	80721299	-	T	intronic	Mps1
chr6	80721693	80721693	A	-	exonic	Mps1
chr6	80724156	80724156	T	C	intronic	Mps1
chr6	80724312	80724312	A	G	intronic	Mps1
chr6	80736148	80736148	A	G	exonic	Mps1
chr6	80744866	80744866	-	T	intronic	Mps1

chr6	80744870	80744870	T	-	intronic	Mps1
chr6	80745095	80745095	A	-	exonic	Mps1
chr6	80746182	80746182	-	TA	intronic	Mps1
chr6	80749972	80749972	A	C	exonic	Mps1
chr6	80750015	80750015	A	T	intronic	Mps1
chr6	80750290	80750290	C	T	intronic	Mps1
chr6	80750315	80750315	T	-	intronic	Mps1
chr6	80751897	80751897	A	-	exonic	Mps1
chr6	80751905	80751905	A	G	exonic	Mps1
chr6	80751909	80751909	G	A	exonic	Mps1
chr6	80751910	80751910	A	G	exonic	Mps1
chr6	80751942	80751942	G	A	UTR3	Mps1

Figure A 2. All possible mutation sites after NGS data analysis. These 72 positions were analyzed one by one in the IGV software. Chr: chromosome, Ref: reference, Alt: alteration, Func.refGene: Function in the reference gene; Gene.refGene: Gene of reference