**DEPARTAMENTO DE CIÊNCIAS DA VIDA**

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Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Celular e Molecular, realizada sob a orientação científica da Doutora Ana Rita Ramada Maia (Universidade do Porto) e da Professora Doutora Maria Cármen Alpoim (Universidade de Coimbra)

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# Abstract

Mitosis is the phase of the cell cycle in which the cell divides into two genetically identical daughter cells. The spindle assembly checkpoint (SAC), also known as mitotic checkpoint, safeguards the transition from metaphase to anaphase and is responsible for correct chromosome segregation by delaying the onset of anaphase until the chromosomes are properly attached to the mitotic spindle. The SAC is comprised of the mitotic checkpoint complex (MCC), which is formed by Mad2, BubR1, Bub3 and Cdc20. Apart from the MCC many more proteins are essential for the proper function of the SAC, including the kinases Aurora B and Mps1. Mps1 is an essential threonine/tyrosine kinase that not only controls the recruitment of SAC proteins to unattached kinetochores starting the SAC signal but also monitors the formation of correct kinetochore-microtubule attachments.

Depletion of Mps1 causes shortening of the time cells spend in mitosis due to the premature satisfaction of the SAC. This results in errors in chromosome segregation, which is associated with aneuploidy and chromosomal instability (CIN). Although aneuploidy and CIN have often been associated with tumorigenesis and tumor progression, cells can only cope with a certain level of chromosome imbalances. Overexpression of Mps1 has already been described as a protective mechanism against aneuploidy in several cancer types. These observations led to the development of small molecule inhibitors against cell cycle kinases, such as Mps1, which explore the concept that increased missegregation rates are detrimental for cell survival. Although described to exhibit promising results in cell culture and xenograft models, resistance to some of the developed inhibitors, such as Aurora B and PLK1, has already been reported. On the other hand, resistance to Mps1 inhibitors has not been described so far and is a very relevant point to be addressed. After characterization of four mutations present in the kinase domain of Mps1 and identified in tumor samples, we show that certain mutations can confer tumors resistance to specific Mps1 inhibitors. Besides this, two more mutations also identified in tumor samples are studied to better understand Mps1 contribution to tumorigenesis. However, characterization of these two new Mps1 mutations did not confer new insights on the role of this kinase in the process of tumorigenesis.

**Key words:** Spindle assembly checkpoint, chromosome segregation, aneuploidy, Mps1, mutations, small molecule inhibitors

# Resumo

A mitose é a fase do ciclo celular na qual a célula divide-se em duas células-filhas idênticas. O ponto de controlo mitótico controla a transição de metáfase para anáfase e é responsável pela correta segregação de cromossomas ao atrasar a entrada em anáfase até garantir que todos os cromossomas estão corretamente ligados ao fuso mitótico. O ponto de controlo mitótico é composto por um complexo formado pelas proteínas Mad2, BubR1, Bub3 e Cdc20. Além deste complexo, muitas outras proteínas são essenciais para o seu correto funcionamento, incluindo a Aurora B e a Mps1. A Mps1 é uma quinase treonina/tirosina essencial que não só controla o recrutamento de proteínas do ponto de controlo mitótico para os cinetócoros livres dando início ao sinal ativador deste mas também monitoriza a formação das ligações corretas entre os cinetócoros e os microtúbulos.

Depleção da Mps1 leva ao encurtamento do tempo que a célula passa em mitose devido à satisfação prematura do ponto de controlo mitótico. Isto resulta em erros de segregação dos cromossomas, o que está associado a aneuploidia e instabilidade cromossómica (IC). Apesar da aneuploidia e a IC estarem associados a tumorigénese e progressão tumoral, as células conseguem apenas lidar com certos níveis de desequilíbrios cromossómicos. Sobrexpressão da Mps1 já foi descrita como sendo um mecanismo protetor para a aneuploidia em vários tipos de tumores. Estas observações levaram ao desenvolvimento de inibidores contra várias quinases do ciclo celular, entre as quais a Mps1, que pretendem explorar o conceito de que elevados níveis de erros de segregação são prejudiciais para a sobrevivência das células. Apesar de terem mostrado resultados promissores em cultura de células e modelos xenográficos, resistência contra alguns dos inibidores, como contra a Aurora B e PLK1, já foi reportada. No entanto, resistência a inibidores da Mps1 não foi ainda descrita e parece um ponto bastante relevante que deve ser endereçado. Após caracterização de quatro mutações no domínio quinase da Mps1 identificadas em amostras tumorais, é aqui mostrado que certas mutações conferem aos tumores resistência a inibidores da Mps1. Duas outras mutações, também identificadas em amostras tumorais, são estudadas para melhor compreender a contribuição da Mps1 para a tumorigénese. No entanto, caracterização destas duas mutações não contribuiu para o melhor conhecimento do papel desta quinase neste processo.

**Palavras-chave:** Checkpoint mitótico, segregação de cromossomas, aneuploidia, Mps1, mutações, inibidores

# Abbreviations

| | |
|---|---|
| ACA | Anti-centromere antibodies |
| APC/C | Anaphase-promoting complex or cyclosome |
| APC15 | Anaphase-promoting complex subunit 15 |
| ATM | Ataxia-telangiectasia mutated |
| ATP | Adenosine triphosphate |
| ATR | Ataxia-telangiectasia and Rad3-related protein |
| ATRIP | ATR-interacting protein |
| Blm | Bloom syndrome protein |
| BSA | Bovine serum albumin |
| Bub1/3 | Budding uninhibited by benzimidazoles 1/3 |
| C | Cysteine |
| CCAN | Constitutive centromere-associated network |
| Cdc20 | Cell-division cycle protein 20 |
| Cdc25 | Cell-division cycle protein 25 |
| CDKs | Cyclin-dependent kinases |
| Chk1/2 | Checkpoint kinase 1/2 |
| CIN | Chromosomal instability |
| COSMIC | Catalogue of Somatic Mutations in Cancer |
| CPC | Chromosomal passenger complex |
| Cpd-5 | Compound 5 |
| CRIPSR | Clustered regularly interspaced short palindromic repeats |
| DAPI | 4',6-diamidino-2-phenylindole |
| DMEM | Dulbecco's Modified Eagle Medium |
| DNA | Deoxyribonucleic acid |
| Dox | Doxycycline |
| DSB | Double strand break |
| DTT | Dithiothreitol |
| E | Glutamic Acid |
| ECL | Enhanced chemiluminescence |
| EGTA | Ethylene glycol tetraacetic acid |
| G | Glycine |
| GFP | Green fluorescent protein |
| HeLa | Henrietta Lacks cells |
| I | Isoleucine |
| INCENP | Inner centromere protein |
| IPTG | Isopropyl $\beta$-D-1-thiogalactopyranoside |
| K | Lysine |
| KMN complex | KNL1-Mis12-Ndc80 complex |

| | |
|---|---|
| NL1 | Kinetochore null protein 1 |
| LAP | Localization and affinity purification |
| Mad1/2 | Mitotic arrest deficient 1/2 |
| MAPK | Mitogenic activated protein kinases |
| MCC | Mitotic checkpoint complex |
| MDR pumps | Multi drug resistance pumps |
| $MgCl_2$ | Magnesium chloride |
| Mis12 | Missegregation 12 |
| Mps1 | Monopolar spindle 1 |
| mRNA | Messenger ribonucleic acid |
| MT | Microtubules |
| N | Asparagine |
| NaCl | Sodium chloride |
| Ndc80 | Nuclear division cycle 80 |
| NEB | Nuclear envelope breakdown |
| PBS | Phosphate-buffered saline |
| PBS-T | PBS with addition of Tween-20 |
| pH3 | Phosphorylated histone 3 |
| PLK | Polo-like kinase |
| R | Arginine |
| Rb | Retinoblastoma protein |
| RPA | Replication protein A |
| RPE-1 | Retinal pigment epitelial cells |
| RT | Room temperature |
| RZZ complex | Rod-Zw10-Zwilch complex |
| S | Serine |
| SAC | Spindle assembly checkpoint |
| SDS | Sodium dodecyl sulfate |
| SDS-PAGE | SDS Polyacrylamide gel electrophoresis |
| SMC | Structural maintenance of chromosomes |
| ssDNA | Single stranded DNA |
| STLC | S-trityl-L-cysteine |
| TBS | Tris-buffered saline |
| TBS-T | TBS with addition of Tween-20 |
| Tet | Tetracycline |
| TPR | Tetratricopeptide repeat |
| TTK | Threonine-Tyrosine kinase |
| UbcH10 | Ubiquitin-conjugating enzyme E2 C |
| U2OS | Human bone osteossarcoma epitelial cells |
| WT | Wild-type |
| YFP | Yellow fluorescent protein |

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# 1. Introduction

Cancer is one of the leading causes of death in developed countries with ten million people being diagnosed every year and less than half surviving[1].

The most prevalent sites of cancer are the breast, colon and rectum, prostate and lung and bronchus[2]. Besides being one of the most prevalent cancers with 1.7 million of diagnosed cases worldwide in 2012[3], breast cancer also accounts for the highest cost in years of life per person[2].

For these reasons it is mandatory to improve our knowledge about this disease in order to understand the mechanisms involved in the development and progression so that new therapies can be developed.

Deregulation of the processes that control cell division is one of the main drives of malignancy. If the proteins that ensure the proper set of events are not functioning accurately, cell proliferation can become uncontrolled and give rise to the formation of tumors[4]. In accordance, therapeutic targeting of the cell cycle is one of the most common strategies for cancer treatment with more targeted drugs emerging in the past few years[5].

## 1.1 Cell Cycle

The cell cycle is the process through which cells grow, replicate DNA and organelles and, finally, divide into daughter cells. The cell cycle is divided in different phases – G1, S, G2 and mitosis. Progression from one phase to the other is dependent on the interaction between cyclins and cyclin-dependent kinases (CDKs)[6]. Cyclins form complexes with CDKs modulating their kinase activity and substrate specificity[7]. Once these complexes are assembled and activated, the CDKs can phosphorylate several target proteins and as a consequence, enable or inhibit the progression to the next cycle phase. Different types of cyclins and CDKs are associated with different cell cycles stages[6]. All these stages are controlled by checkpoints in order to ensure accurate cell cycle progression[8]. The cell cycle checkpoints work as 'cell polices' ensuring that the cell cycle is arrested whenever the cell does not have the necessary requirements to proceed to the next stage.

## 1.1.1 G1, S and G2 checkpoints

The first obstacle that the cell has to overcome is called restriction point and occurs during G1[9]. If the cell receives enough mitogenic signals that enable its growth, the cell surpasses this point and keeps cycling. On the other hand, if the conditions gather are not sufficient, the cell will arrest the division and enter G0 phase[9]. The major pathway involved depends on the retinoblastoma protein (Rb) that controls gene expression of necessary components for the next phase of the cell cycle – the S-phase[10]. When the conditions are gathered, Rb is phosphorylated and inhibited by CDK4/6 in complex with cyclin D and is no longer capable of binding and inhibiting E2F transcription factors[11]. E2F transcription factors promote the transcription of essential components for DNA replication.

After the cell ensured that all conditions for the next phase are present, it proceeds to S-phase. In this stage, the DNA is duplicated. The checkpoint that occurs in the end of this phase is essential to ensure that DNA replication is completed and no errors are present[12]. Whenever the cell senses damages in the DNA the ATM/ATR signaling machinery is activated[13]. ATM is found in the cell as a homodimer, in an inactivated state. When double-strand breaks (DSBs) in the DNA are sensed, ATM is phosphorylated promoting its activation which then phosphorylates its targets. On the other hand ATR is found as a heterodimer with ATRIP that is capable of binding RPA, a DNA damage sensor protein. Single-stranded DNA (ssDNA) leads to the recruitment of ATRIP-ATR to the DNA and ATR activation. After activation of both kinases, protein targets are phosphorylated such as CHK1 and CHK2 that further phosphorylate and activate p53 and inhibit CDC25, respectively[12,14]. The consequences are inhibition of cyclin/CDK complexes and cell cycle arrest. The ATM/ATR pathway not only works in S phase, but it can also be activated throughout the cell cycle whenever the cell is exposed to a genotoxic stimulus that induces DNA damage[14] (*Figure 1*).
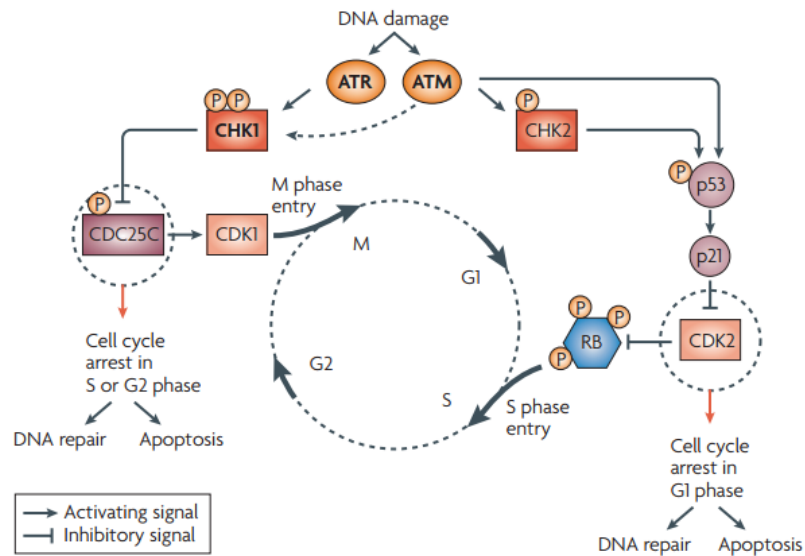
**Figure 1. ATR/ATM signalling pathway.**

If DNA damage is detected the entry of the cell in the next cycle phase is inhibited. Different sensors, transductors and targets are involved according to the cell cycle phase (Adapted from *Lapenna and Giordano 2009 review*[15]).

When the cell reaches mitosis it can finally prepare to segregate its chromosomes into the two daughter cells. This process is controlled by another checkpoint, the spindle assembly checkpoint that ensures that chromosome segregation is delayed until all chromosomes are correctly attached to the mitotic spindle[15]. Errors in chromosome segregation can lead to daughter cells with altered number of chromosomes, and promote cell death or cell transformation.

## 1.1.2 Mitosis

The final stage of the cell cycle is mitosis. Mitosis ensures identical distribution of the genomic material to the daughter cells and is divided into several phases – prophase, prometaphase, metaphase, anaphase and telophase – and is completed by the physical separation of the newly formed daughter cells, called cytokinesis[16].

The mitotic spindle, formed by microtubules (MTs), is the structure that controls the proper partition of the replicated genome. The MTs are highly dynamic structures that can grow or shrink through the addition or loss of tubulin-dimers at their ends. Besides this dynamicity, they also present another essential feature – MTs have structural polarity[17,18]. This means that MTs have a plus and a minus end which have different characteristics. While the plus end is more dynamic and is the end that binds to the kinetochore, the minus end is

attached to the spindle poles and is less dynamic[19]. The structure from which MTs are nucleated is called centrosomes. These are formed by a pair of cylindrical centrioles surrounded by pericentriolar material[16,20]. During chromosome segregation, there are two centrosomes located in opposite poles of the cells. Alterations in the number or function of centrosomes can cause aneuploidy as a result of chromosome missegregation[21]. There are three subclasses of spindle MTs – astral, interpolar and kinetochore-fibers (K-fibers)[22] (*Figure 2*).
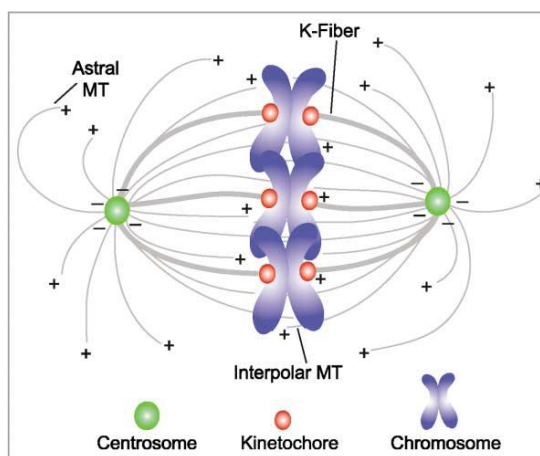


**Figure 2. The Mitotic Spindle.**

Different types of MTs constitute the mitotic spindle – astral MTs, spindle or interpolar MTs and K-Fibers. They all emanate from the centrosomes but have different ending points. Astral MTs connect the centrosomes to the cell cortex playing an important role in spindle positioning. On the other hand, both interpolar MTs and K-fibers extend towards the center of the spindle. While K-fibers bind the kinetochores making them essential in chromosome segregation, interpolar MTs can interact with the interpolar MTs that emanate from the opposite pole and are important in the maintenance of spindle bipolarity (Adapted from *Kline-Smith and Walczak 2004 review*[19]).

The K-fibers connect the chromosomes to the centrosomes. The region where these fibers attach is called kinetochore[19]. The kinetochore is localized in the centromere of mitotic chromosomes[23] and is composed by more than hundred proteins[24]. Kinetochores are formed by two distinct protein networks – the constitutive centromere-associated network (CCAN) and the KMN. The latter includes the kinetochore null protein 1 (KNL1), missegregation 12 (MIS12) and nuclear division cycle 80 (NDC80)[25]. While the CCAN binds centromeric DNA, the KMN is important to bind the MTs[24].

During the cell cycle, chromosomes change their structure. When the cell reaches mitosis, each chromosome is composed of two sister chromatids that are tethered by cohesion[20]. Cohesion is mediated by a structural maintenance of chromosomes (SMC) complex of four proteins – SMC1, SMC3, RAD21 and SA – named cohesin[26,27]. Rad21 belongs to the $\alpha$-kleisin

family and connects the two SMC proteins of the complex forming a ring-like structure[26,28]. At anaphase onset, the kleisin subunit of the ring is cleaved by the enzyme separase which originates the separation of the two sister chromatids[29]. After separation, the sister chromatids can finally start to migrate to opposite poles of the cell.

## 1.1.2.1 Spindle Assembly Checkpoint

Since chromosome segregation is an essential process to maintain genome stability, it is controlled by a checkpoint, called the spindle assembly checkpoint (SAC). This checkpoint controls the activity of the anaphase-promoting complex or cyclosome (APC/C).

Firstly identified in *Saccharomyces cerevisiae*[30], the SAC or mitotic checkpoint is a regulatory mechanism through which the cell ensures that the chromosomes are correctly segregated to the daughter cells[29]. The SAC prevents anaphase entry until all chromosomes are correctly attached to the mitotic spindle[31]. SAC activation is driven by unattached or incorrectly attached kinetochores which leads to the recruitment of mitotic checkpoint components[32] which in turn will inhibit the APC/C[33]. The signal of one single unattached kinetochore can delay the onset of anaphase for hours[34]; However, it was shown that when the signal of a single kinetochore appears in the later stages of the metaphase it is not sufficient to inhibit the APC/C and cells progress to anaphase[35].

*How is the kinetochore able to sense if the spindle microtubules are correctly attached to the chromosome?* The knowledge about this issue is little and whether the signal is generated by attachment or tension is still a point of debate[31]. Limitations such as the fact that attached kinetochores to the spindle MTs are under tension have turned this subject hard to study[31]. Besides all this, tension exerted by the microtubules in the kinetochore is likely to play an important role[36]. Aurora kinases are important in different stages of the cell cycle, including during chromosome segregation[37], with the family member Aurora B working as a tension sensor[29]. Depletion of this kinase increases erroneous attachments between chromosomes and spindle MTs. There are three major types of erroneous attachments – merotelic,syntelic and monotelic attachments. Merotelic attachment describes the attachment of a kinetochore from MTs emanating from both spindle poles, syntelic attachment occurs when sister kinetochores are attached to the same pole and when only one kinetochore is attached to the spindle pole this is called monotelic attachment. Whenever the attachment between the kinetochore and spindle MTs are not bipolar (amphitelic), the tension at the kinetochores

exerted by these attachments is small. This lack of tension enhances the spatial activity of Aurora B at the kinetochore where it phosphorylates several targets to promote the destabilization of these wrong attachments[38].

The SAC is composed by a complex of proteins, Mad2-Cdc20-BubR1-Bub3[39], named the mitotic checkpoint complex (MCC). However, proper SAC function depends on a much more complex group of proteins in which Mps1 kinase is included[40]. It is observed that during mitosis, Mps1 is hyperphosphorylated[40] and that the activation of this kinase is essential for the recruitment of other checkpoint proteins to the unattached kinetochores. Mps1 phosphorylates KNL1 which allows the posterior binding of Bub3-Bub1 complex to KNL1[24]. In fact, KNL1 depletion abolishes the kinetochore localization of BubR1 and Bub3[29]. KNL1 is also essential for the recruitment of the RZZ complex, composed by the proteins Rod, Zw10 and Zwilch, that in turn is responsible for the binding of Mad1-Mad2 complex to the kinetochore[29] (*Figure 3*).
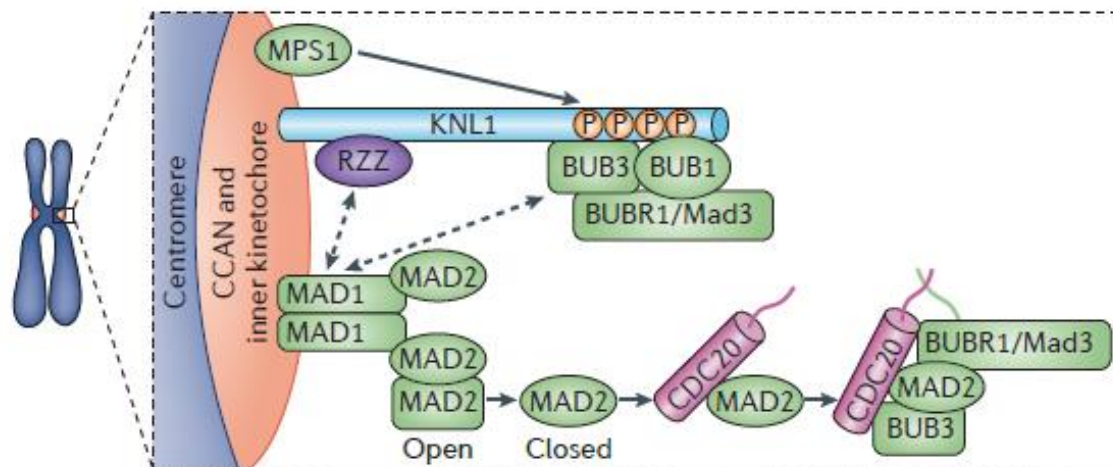


**Figure 3**. **SAC activation by the unattached kinetochore**.

Unattached kinetochore recruits the kinase Mps1 which phosphorylates KNL1 allowing the binding of BUB1 and BUB3 and subsequent recruitment of BUBR1. Phosphorylated KNL1 also allows the binding of RZZ complex that is responsible for the recruitment of the MAD1-C-MAD2 complex (Adapted from *Foley and Kapoor 2013 review*[24]).

In the cell, the protein Mad2 exists in two conformations – open (O) and closed (C)[29] . The Mad2 template model describes the alterations and exchanges that occur in Mad2 that are required for efficient SAC activation and function. The kinetochore bound Mad1-C-Mad2 promote the conformational switch of the cytoplasmic O-Mad to C-Mad2 which can bind to Cdc20[41] (*Figure 4*). The Mad2-Cdc20 complex promotes the binding to BubR1 and Bub3 thereby establishing the MCC. The MCC inhibits APC/C activation by sequestering the co-

activator Cdc20[42]. Once all chromosomes are bi-oriented, mitosis can progress[30,43]. For this, the MCC must be inactivated and Cdc20 released to be able to bind to APC/C.
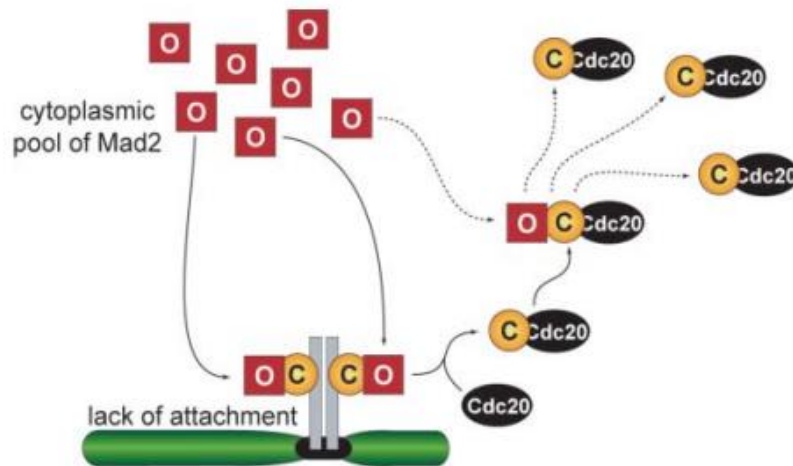


**Figure 4. The Mad2 template model.**

Mad1-C-Mad2 complex bound to the unattached kinetochores recruits O-Mad2 from the cytoplasm. The O-Mad2 is converted into C-Mad2 that is released to the cytoplasm where it bounds Cdc20, clustering it (Adapted from *De Antoni et al 2005*[44]).

Inactivation of the MCC is promoted by the elimination of the SAC ON signal and leads to the disassembly of the MCC. It is thought that three main mechanisms are involved in the inhibition of the MCC – kinetochore stripping, p31[comet] and Cdc20 ubiquitination. Stripping is the name given to the process by which RZZ and Mad1-C-Mad2 complexes are removed from the kinetochores, which is mediated by Dynein, a microtubule motor protein[30,29]. While Dynein is responsible for removing Mad1-Mad2 from the kinetochore, and eliminating the SAC ON signal, the p31[comet] has a role in the inactivation of formed MCC. Many theories exist of how the p31[comet] exerts its function: p31[comet] binds the C-Mad2 interfering with its ability to recruit O-Mad2[45], but it is also able to bind Mad2 localized in the MCC[45,46]. Finally, non-degradative ubiquitination of Cdc20 by UbcH10 promotes the disassembly of Mad2-Cdc20 complexes and, consequently, inactivation of MCC[45] (*Figure 5*). Several experiments have shown that APC15, an APC/C subunit might be responsible for the ubiquitination of Cdc20 and, consequently, MCC disassembly[43].

After SAC silencing, the APC/C targets securin and Cyclin B1 to proteasome degradation[32]. Degradation of securin releases separase that is the enzyme responsible for the degradation of the cohesin complex that keeps the two sister chromatids together, whereas degradation of

Cyclin B1 inactivates CDK1 thereby promoting mitotic exit[47]. After cohesin removal from the chromosomes, the forces exerted by the mitotic spindle promote the separation of the two chromatids.
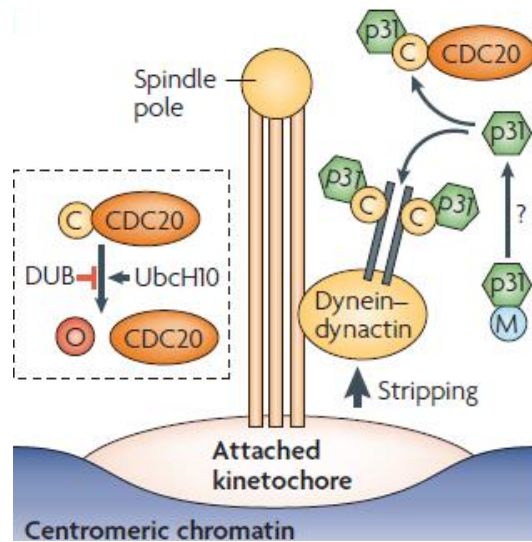


**Figure 5. Model of the mechanisms responsible for the inactivation of the MCC**.
These mechanisms include reactivation of the p31[comet], ubiquitination of Cdc20 and stripping (Adapted from *Musacchio and Salmon 2005 Review*[30]).

Once cells exit mitosis, the Cdc20 in complex with APC/C is replaced by dephosphorylated Cdh1 and the APC/C is inactive. Dephosphorylation of Cdh1 is promoted by Cdk1 inactivation that occurs due to degradation of Cyclin B1[48].

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## 1.2 Aneuploidy, chromosome instability and cancer

One of the main regulators of the cell cycle is the protein kinases. As mentioned before, CDKs regulate the transition from one stage of the cell cycle to the next one. The Aurora kinases are involved in centrosome formation as well as control of MT spindle attachment to the kinetochore[49] with Aurora A and B playing their role, respectively[38]. Other kinases control the SAC such as Bub kinases and Mps1[49].

The SAC is essential to prevent abnormal chromosome segregations and alterations of this checkpoint are linked to genomic instability and aneuploidy – an abnormal content of chromosomes[50] – which may drive tumorigenesis. However, the tumorigenesis process is dependent on the tissue and the affect cell type[51]. Although errors in chromosome segregation occur with low frequency in normal tissue, cells originated from a defective mitosis seem to be efficiently eliminated[52] maybe as a consequence of their limited tolerance to aneuploidy[53,54]; however, the presence of certain conditions may enable the survival and proliferation of these cells[52]. Cancer cells seem to be more fitted to survive aneuploidy by avoiding the process of negative selection that normal cells undergo, which allows cancer cells to divide and generate further aneuploid cells [55]. Consistently, one of the hallmarks of cancer cells is aneuploidy[56].

Cancer cells often lose and gain chromosomes at a very high rate which increases the chances of cells to lose tumor suppressor genes and increases the chances of them to gain oncogenes. This feature is named chromosome instability (CIN)[32] and it is proposed that aneuploidy occurs as a consequence of this process[57]. Moreover, the relation between both mechanisms can promote malignancy. Besides driving aneuploidy, CIN is associated with the high capacity of adaptation to alterations in the environment observed in cancer cells[32]. In turn, CIN and aneuploidy promote tumor development and are involved in chemotherapy resistance[32]. Although an obvious relation seems to exist between CIN and aneuploidy, not all aneuploid cancer cells exhibit CIN[58].

Several reasons can be the causes of CIN and aneuploidy: cells with multipolar spindles, problems with chromosome cohesion and erroneous attachments between the kinetochores and spindle MT[32,52] (*Figure 6*).
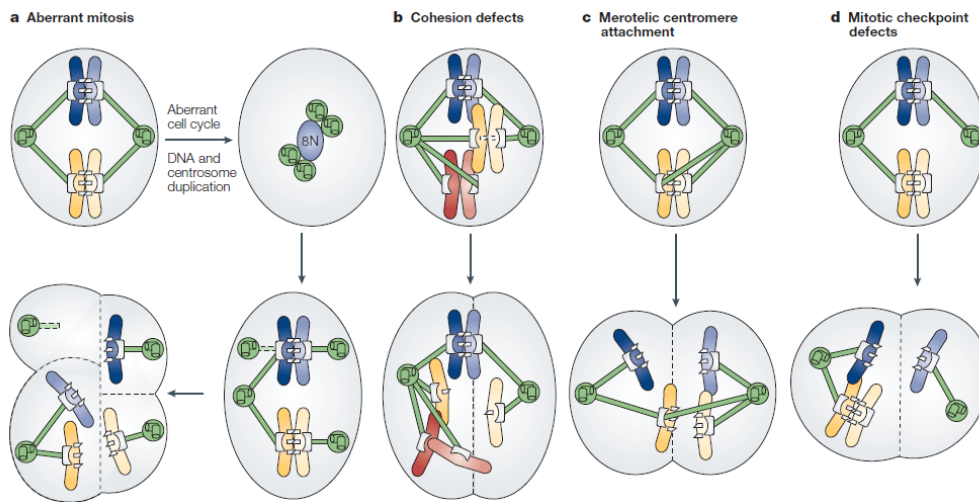
**Figure 6. Different causes for the formation of aneuploid cells.**

a) Cells that underwent defective cytokinesis and possess multipolar spindles usually form cells with aneuploidy. b) Cohesion defects that affect the proper segregation of chromosomes by allowing premature separation of sister chromatids are also an important cause of aneuploidy. c) and d) Abnormal attachments between the MT spindles and chromosomes that are corrected by the spindle checkpoint machinery can inhibit cytokinesis or lead to inappropriate chromosome segregation with the final result of aneuploidy (Adapted from *Kops et al 2005 review*[32]).

In some cancer types such as colon cancer, mutations and altered expression of SAC genes have been identified[58]. The causes of these variations in expression can be of different origin. Alterations in tumor suppressor genes and oncogenes which interfere with the levels of components of the SAC that in turn alter the function of the checkpoint can be one of them[51]. In breast cancer tumors, increased levels of SAC genes are observed[59]. Among these, Mps1/TTK is found[60] which is thought to work as a protective mechanism for these tumor cells. Even though cancer cells present higher tolerance to CIN and aneuploidy than normal cells, they can only survive below a certain threshold level of alterations. Surpass this level turns CIN in an obstacle for tumor progression[61,62] (*Figure 7*).
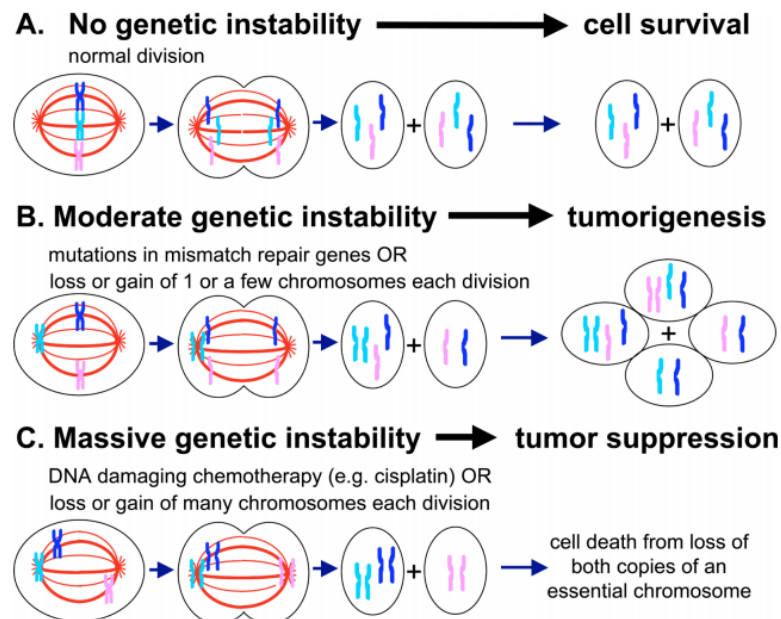
**Figure 7. Role of aneuploidy in tumor development and progression.**

Low levels of aneuploidy can confer growth advantage to cancer cells compared to normal cells maybe through the loss or gain of tumor suppressor genes and oncogenes, respectively. However, cells can only coop with a certain level of aneuploidy and when this threshold is exceed, aneuploidy becomes an obstacle to tumor progression by promoting cell death (Adapted from *Weaver, B. A. A. & Cleveland, D. W.*[62])

Recently, many works exploring the consequences of inhibiting the spindle checkpoint have arisen. The goal of this strategy is to increase aneuploidy to levels that cancer cells can no longer tolerate and, consequently, causing cell death. This idea lead to the development of many mitosis-targeted anti-cancer therapies[63]. Many proteins involved in the SAC can be targeted and inhibited to increase the errors in chromosome segregation in order to increase apoptotic rate in cancer cells[64]. Among these proteins, the selective inhibition of Mps1 with small-molecule compounds has been proven effective in treatment of various xenograft and one breast cancer model [51,57,65,66].

# 1.3 Mitosis as a therapeutic target

Since cancer is associated with deregulation of the normal cell cycle, most therapies for cancer have an anti-proliferative effect[64]. The goal is to interfere with cell cycle progression and to achieve this goal, different processes can be targeted. Targeting mitosis is a widely used strategy and the drugs used for this purpose are commonly called anti-mitotic drugs[5,67].
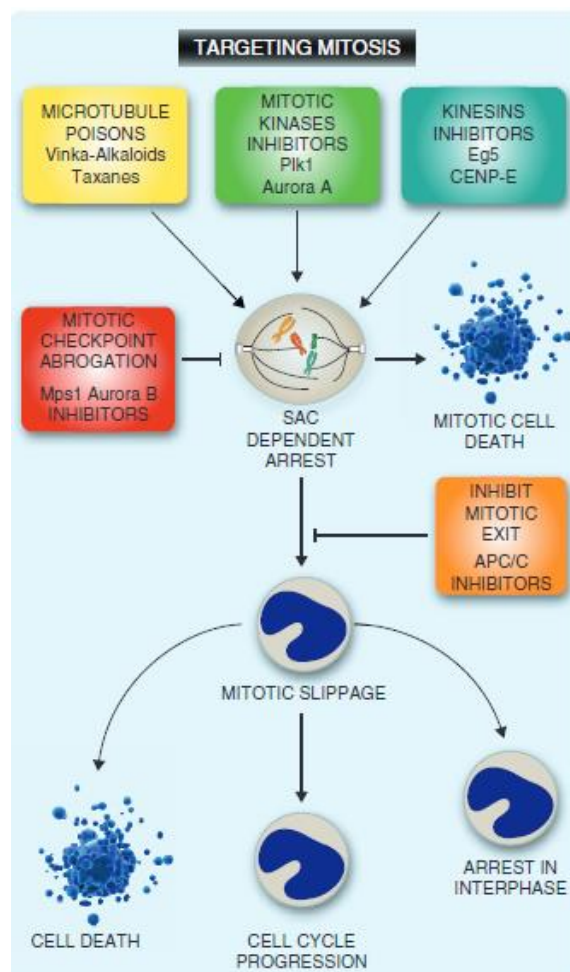


**Figure 8. Different anti-mitotic therapies studied for cancer treatment.**

Several mitotic-targeting drugs can be explored as a therapeutical strategy for cancer. Microtubule poisons are one of the most common therapies in cancer treatment and include several drugs such as taxol. Over the years more targeted drugs were developed and have been studied which include small molecule inhibitors that target specific kinases.

A great number of chemotherapy agents alter the normal dynamics of mitosis by interfering with different aspects essential for the faithful progression of this cell cycle phase[68]. For example, taxol, a very common agent used for breast cancer therapy, affects microtubules dynamics which causes a SAC-dependent arrest. The consequences for cell faith of the

prolonged mitotic arrest can only be two: either the cell undergoes apoptosis or mitotic slippage[63,68]. In the last case, cells exit mitosis without division which leads to the survival of cells with an aberrant DNA content. Although proven quite efficient in cancer therapy, anti-mitotic drugs that interfere with microtubule dynamics have also shown severe toxicity, especially towards the nervous system[68,63,67,69].

A more targeted approached to disturb mitosis that has emerged in recent years is to treat cells with small molecule inhibitors that target kinases with essential roles in mitosis[5,63,67]. Kinases are a very attractive target for therapy not only because alterations in these proteins are usually associated with malignant progression, but also because they are easily targetable with specific drugs. Two kinases with essential roles in mitosis against which small molecule inhibitors have been designed are the Aurora and Polo-like (PLK) kinase families. Although the PLK family is composed of several members, PLK-1 is the one implicated in the control of mitotic progression, and for this reason the one targeted by these inhibitors[5]. While inhibition of this kinase is thought to prevent the formation of a bipolar spindle, inhibition of the Aurora kinase family has different effects. For example, Aurora B inhibition leads to alignment problems and cytokinesis failure which promotes aneuploidy[69]. Loss of Aurora A activity is not only associated with an increase in segregation errors but, since Aurora A is also involved in the formation of the mitotic spindle, also with mitotic spindle problems[5]. Apart from small molecule inhibitors against these kinases, new inhibitors that target Mps1/TTK, have recently drawn the attention as a potential therapeutic strategy for cancer treatment[70,71].

Despite the successful outcome of targeting mitotic kinases with small molecule inhibitors in tissue culture and mouse models, the results obtained by clinical trials in terms of efficiency were however disappointing [63,72,73]. Furthermore, resistance to specific PLK-1[74] and Aurora B inhibitors[75] in cancer cell lines has been described. Resistance to drugs can be primary or acquired when there is no response to the initial treatment or when there is an initial benefit from the treatment but after some time the response to the drug stops, respectively[76,77]. Several mechanisms can drive drug resistance such as overexpression of multi-drug resistant (MDR) pumps[74], amplification of the target gene, or alterations in other molecules of the signaling pathway which can overcome the need for the target kinase[77]. However, when talking about kinase inhibitors there is one mechanism that seems to be more clinically relevant which is the appearance of resistant alleles. This means that cells with specific mutations in the kinase structure, usually in the ATP-binding pocket of the kinase, that

abrogate the activity of the inhibitor, are selected after treatment, can overgrow and give rise to a resistant population[75,77].

## 1.4 Mps1 kinase family

Mps1, also known as TTK, belongs to a subgroup of proteins, named kinases, which regulate a huge variety of processes in the cell. To do this, kinases transfer the γ-phosphate from the ATP molecule that binds to their ATP pocket to other substrates that then become phosphorylated[77]. Several kinases are important for the regulation and assembly of the SAC among which we can find the Mps1[78]. Apart from its well established role in SAC, Mps1 has been implicated in various processes during cell cycle[40,79]. More recently, Mps1 was linked to the DNA damage response since it was shown that Mps1 phosphorylates Chk2 kinase[70]. By phosphorylating the Bloom syndrome protein, Blm, Mps1 is involved in the maintenance of genome stability[49].

Mps1 kinase consists of a N-terminal TPR domain and a C-terminal kinase domain. The TPR domain has been implicated in the formation of homodimers and in regulating Mps1 localization[80]. Dimerization seems to be sufficient for the activation of Mps1[81] by transphosphorylation[29] on the threonine 676 residue (T676) located in the activation loop of Mps1[82,83].

Whereas transphosphorylation is important for kinase activity, it is not the only process by which Mps1 is controlled. Phosphorylation of Mps1 by Chk2[84,85], Aurora B[86] and MAPK[87] contributes for the correct localization of Mps1 at unattached kinetochores. Once localized and activated at unattached kinetochores, Mps1 is essential for proper SAC activation. Mps1-dependent phosphorylation of KNL1 is the event that starts the signaling cascade that culminates with MCC formation. Phosphorylation occurs at specific motifs, named MELT, and allows the recruitment of the important players, BUB1 and BUB3 to the kinetochore[88]. This in turn leads to the recruitment of Mad1-Mad2 complexes to the unattached kinetochore which is an essential event for the formation of the MCC. Inhibition or depletion of Mps1 results in defective cell cycle arrest in the presence of unattached kinetochores leading to apoptosis of the cells formed due to aberrant alterations in the chromosome content[40].

Equally, or even more important than understanding how Mps1 activity is regulated, is to understand what role it has in the cell and how it performs its functions. As mentioned above, Mps1 kinase has an essential role in the recruitment of Mad1-Mad2 complexes to the

unattached kinetochore in a Mps1-mediated KNL1 phosphorylation dependent manner[24]. Recently, Mps1 also seems to have an important function in the correction of erroneous attachments between the kinetochores and MT spindle by indirectly controlling Aurora B[89]. Aurora B is an essential kinase in the correction of misattachments and is the main player of a specialized complex named chromosomal passenger complex (CPC)[90]. Four different proteins – INCENP, Borealin, Survivin and Aurora B, form the CPC. This complex not only ensures proper chromosome segregation by correcting the attachments between the spindle MT and the kinetochores, but is also involved in cytokinesis[91,92]. By phosphorylating one member of the complex, Borealin, Mps1 promotes an increase in Aurora B activity[89,92].

## 1.4.1 Mps1 and cancer

Studies showed that Mps1 is overexpressed in some cancers, such as glioma[65] and breast cancer. In addition, the more aggressive breast cancer, the triple negative breast cancer showed a correlation with increased Mps1 mRNA levels[93]. In breast cancer, Mps1 high expression is thought to act as a protective mechanism to cancer cells by preventing intolerable levels of aneuploidy[60]. Although this seems an attractive hypothesis, lower levels of checkpoint proteins have also been reported in several cancer types[52]. Besides alterations in the levels of this essential kinase, mutations have also been reported, although not so extensively studied. Alterations in the localization and function of this protein can lead to a defective SAC signal and, as consequence, lead to missegregation in normal cells. It would not be very surprising that mutations that impair localization or the kinase activity of Mps1 could contribute to tumorigenesis in normal cells. For this reason, characterization of mutations localized in the N-terminal of Mps1 that were identified in tumor samples seems very important to understand if these can promote malignant transformation and progression. After sequencing of the whole kinome of breast tumor samples from patients, two new mutations were identified in Mps1, one of them located in the TPR domain. **Do these mutations alter Mps1 function and did they drive tumorigenesis in the tumors where they were identified?**

Since Mps1 has been associated with cancer, this kinase has been studied as a possible target in cancer therapy. Development of small molecule inhibitors that compete with ATP for the ATP-binding pocket emerged in the past recent years. Use of Mps1 inhibitors seems to increase the levels of aneuploidy in cancer cells above the sustained threshold causing their

apoptosis[57,64,65,80]. Although these small molecules inhibitors seem a very attractive option for therapeutics it is essential to determine if cancer cells can develop resistance against them. Why is essential to answer to this point? Drug development is not only an extremely expensive process but also very time consuming. Releasing a drug in clinic and after some time finding out that patients become resistant to it shortly after is not only a big loss of investment but also a failure in terms of cancer treatment. Several can be the mechanisms by which a cell becomes resistant to therapy, as described above. For kinase inhibitors the most relevant mechanism seems to be the appearance of mutations in the target gene that confers insensitivity of the kinase to the drug. In fact, Aurora B and Plk-1 mutants that no longer respond to the inhibitors were already described in literature; however, the question '**Can we find mutations in tumor samples from patients that give resistance of these tumors to Mps1 inhibitors?**' remains unanswered.

# 2. Materials and Methods

**Site Directed Mutagenesis**

Site-directed mutagenesis was performed with Phusion Polymerase (NEB) and a complementary primer pair harbouring the desired mutations. The pNIC28-Bsa4-Mps1-519-808aa plasmid served as template for the mutations G534E, E571K, M600I and C604F. The pBABE-Blast-LAP-Mps1 plasmid served as template for the mutations N106S, S403C, G534E, E571K, M600I and C604F. The same construct was used to introduce four silent basepair-mutations in order to obtain a short-hairpin resistant Mps1 construct. The used oligonucleotides are described in Table 1. Mutations were confirmed by DNA sequencing using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems) according to the manufacturer instructions.

**Table 1. Oligonucleotide primers used for site-directed mutagenesis.**

|  | Forward Primer | Reverse Primer |
|---|---|---|
| **N106S** | 5'- CTTCCCCCAGATAAATATGGCCAAAGTGAGAGTTTTGCT – 3' | 5' - AGCAAAACTCTCACTTTGGCCATATTTATCTGGGGGAAG - 3' |
| **S403C** | 5' - CCAGAATCCTGCTGCATGTTCAAATCACTGGCAG - 3' | 5' - CTGCCAGTGATTTGAACATGCAGCAGGATTCTGG - 3' |
| **G534E** | 5'- TTAAAGCAGATAGGAAGTGAAGGTTCAAGCAAGGTATTTC -3' | 3'- GAAATACCTTGCTTGAACCTTCACTTCCTATCTGCTTTAA -5' |
| **E571K** | 5'- CTCTTGATAGTTACCGGAACAAAATAGCTTATTTGAAT -3' | 3'- ATTCAAATAAGCTATTTTGTTCCGGTAACTATCAAGAG -5' |
| **M600I** | 5'- GACCAGTACATCTACATTGTAATGGAGTGTGGAAAT -3' | 3'- ATTTCCACACTCCATTACAATGTAGATGTACTGGTC -5' |
| **C604F** | 5'- CTACATGGTAATGGAGTTTGGAAATATTGATCTT -3' | 3'- AAGATCAATATTTCCAAACTCCATTACCATGTAG -5' |
| **sh Res** | 5'- GTGAAGACGGACGACTCGGTTGTACCTTGTTTTATG -3' | 3'- CATAAAACAAGGTACAACCGAGTCGTCCGTCTTCAC -5' |

**CRISPR/Cas9 mediated genome editing**

In a 6 well-plate 8x10^{5} HeLa cells were plated. The vector pX330 containing the guide against the region of interest (5'-CACGGACCAGTACATCTACA-3') and the endonuclease Cas9 was co-transfected using FuGENE transfection reaction (Promega) together with a 100 bp oligonucleotide to generate the C604F mutation (C604F: 5'-GTTTAATTGCAGTGAAATCACGGACCAGTACATTTAtATGGTAATGGAGTTTGGAAATATTGATCTTAAT

AGTTGGCTTAAAAAGAAAAAATCCATTGATC -3'). Cells were selected with 80 nM of Compound 5 (Cpd-5) (synthesized in the lab) two days after transfection for 2weeks. After single clones isolation cells were grown until confluency and harvested for cDNA synthesis. mRNA from single clones was isolated with RNAeasy Mini Kit (Qiagen) and used as a template for cDNA synthesis using SuperScript III First-Strand Synthesis System (Invitrogen) and random hexamer primers which was sequenced for the desired mutation.

**Cell culture**

Non-transformed Retinal pigment epithelium RPE-1, and human osteosarcoma U2OS cell lines were grown in Dulbecco's modified Eagle's medium F12 (DMEM/F12) and Dulbecco's modified Eagle's medium (DMEM) (Sigma-aldrich, Saint Louis, MO, USA) supplemented with 6% fetal bovine serum (FBS) (Integro), 50 µg/mL penicillin/streptomycin (Invitrogen), and 2 mM-Glutamine (Lonza), respectively. RPE-1 and U2OS cells stably expressing LAP-Mps1 were created by retroviral infection with pBABE-Blast-LAP-Mps1. Infected cells were selected with 5 µg/mL blasticidine (Sigma) for a minimum of 2 weeks. U2OS cell lines stably expressing a tetracycline-inducible short-hairpin targeting Mps1 were incubated with 1 µg/mL doxycycline for a minimum of 48 hours to induce short-hairpin expression.

Cervical adenocarcinoma HeLa cell lines were cultured in DMEM supplemented with 6% FBS, 50 µg/mL penicillin/streptomycin and 2 mM-Glutamine. All HeLa cells lines stably expressed an YFP-tagged histone H2B construct.

**Overexpression and purification of the Mps1 kinase domain**

Mps1 kinase domain constructs (Mps1-519-808aa) were transformed in Rosetta(DE3)pLysS and grown at 37°C in Luria-Bertani (LB) medium containing 50 µg/mL kanamycin until an $OD_{600}$ of ~0.6. After addition of 0.5 mM IPTG, cells were incubated for 5 hours at 20°C, and harvested by centrifugation (Speed, Time, Temp). All following steps were performed at 4°C. Cell pellets were resuspended in lysis buffer (50 mM $NaH_2PO_4$, 300 mM NaCl, 10 mM imidazole at pH 8.0) containing 50µg/mL Lysozyme, incubated 15 minutes on ice and then sonicated with a Branson sonifier. Lysates were centrifuged (14000 g, 15 min, 4°C) and supernatants applied to a column containing Nickel-nitrilotriacetic acid (Ni-NTA)–coupled agarose beads (Sigma). After gravitation dependent removal of the supernatant, the column was washed with a minimum

of six column volumes of washing buffer (50 mM $NaH_2PO_4$, 300 mM NaCl and 20 mM imidazole at pH 8.0). Ni-NTA-bound proteins were eluted with elution buffer (50 mM $NaH_2PO_4$, 300 mM NaCl and 250 mM imidazole at pH 8.0). Finally, the proteins were loaded into a SlideALyzer dialysis cassette (Pierce) and dialysed three times in 500x volume excess in buffer (50 mM Tris, 100 mM NaCl and 20 mM $MgCl_2$ at pH 8.0). For storage of proteins at -80°C, glycerol (final concentration 10%) was added after recovery from the dialysis cassette. To assess purification efficiency, samples of different steps of the purification were loaded in a 15% polyacrylamide gel and stained with Commassie Brillant Blue (CBB). For CCB-staining, the polyacrylamide gel was fixed for a minimum of 1 h in 40% Ethanol/10% acetic acid. After fixation, the gel was rehydrated with dH2O. The staining solution (0.1% CBB G250, 2% ortho-phosphoric acid, 10% ammonium sulphate) was mixed 4:1 with methanol and added to the gel and incubated for a minimum of 5 hours. After staining the gel was washed with dH2O.

**Immunoblotting**

RPE-1, U2OS and HeLa cell lines were harvested and stored at -20°C. Cell pellets were lysed in Lysis Buffer containing 20 mM Tris/HCl (pH7.5), 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 2 mM EDTA, 1 mM NaF, 1 mM Ortho-Vanadate, 25 mM beta-glycerophosphate and protease inhibitor cocktail (Roche). Protein concentration of the samples was measured with Bradford reagent (BioRad) by BSA standard curve (1 µg/mL, 2 µg/mL, 4 µg/mL, 6 µg/mL and 8 µg/mL). Samples were loaded in an 8% polyacrylamide gel and separated by SDS-PAGE. Proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (BioRad). Membranes were blocked with 4% bovine serum albumin (BSA) for 1 h at room temperature (RT) and incubated with the primary antibodies overnight at 4°C or at RT for a minimum of 1 h. After washing a minimum of three times for at least 15 minutes with TBS-T the membrane was incubated with the secondary antibodies (1:2000) at RT for 1 h. Finally, the membranes were washed with TBS-T and afterwards incubated for 2 min with ECL reagent (Amersham). Signal was detected with ChemiDOC XRS+ (Biorad) and images were then processed with Adobe CS6.

*In vitro* **kinase assays**

To determine the kinase activity of the proteins with the different mutations, 1.0 µg of the different versions of Mps1 were incubated with 1.5 µg of KNL1 for 1h at 32°C in a buffer

containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 20 mM MgCl$_2$, 1 mM DTT, and 0.2 mM ATP. Reactions were stopped by addition of SDS-sample buffer. Samples were denatured at 95°C for 5 min. Samples were run in a polyacrylamide gel and immunoblotted for phospho-KNL1 and penta-Histidine.

**Live-cell imaging and Immunofluorescence**

Cells were plated in 4-well or 8-well chamber slides (Ibidi) in DMEM. Before imaging, the medium was replaced by Leibovitz's L-15 medium (GIBCO) or by L-15 with nocodazole (200 ng/mL) (Sigma) supplemented with 6%FCS and cells were imaged in a heated chamber (37°C) with a 20x 0.75 NA lens (Olympus), 40x 0.6 NA lens (Olympus) or 60x 0.85 NA lens (Olympus) on a DeltaVision Elite (Allpied Precision) controlled by SoftWorx Software. Multiple Z-layers were acquired and projected into a single layer by maximum projection. Image analysis was done using ImageJ software.

For immunofluorescence, cells were plated in a 6-well plate with a 1.3 x 10$^5$ cells density per well. The next day cells were treated with 200 ng/mL nocodazole for 3 hours, 2 μM MG132 (Sigma) for 1.5 h and 100 nM of Cpd-5 for 0.5 h. After drug treatment, mitotic cells were collected by mitotic shake-off and centrifuged at 1800 rpm for 5 min against 12 mm coverslips. Cells were treated with 0.2% Triton X-100 in PEM (100 nM Pipes, pH 6.8, 1 mM MgCl$_2$ and 5 nM EGTA) for 1 min before addition of 4% paraformaldehyde and 0.1% Triton X-100 in PEM for 10 minutes, Coverslips were washed with 1xPBS/0.1% Tween (PBS-T) and then blocked with 3% BSA in 1xPBS for 1 hour, incubated with primary antibodies for 1 hour at room temperature or overnight at 4°C. After, they were washed 3 times with PBS-T, incubated with the secondary antibodies and DAPI for 1 h at RT and mounted using ProLong antifade (Invitrogen). Images were acquired on a DeltaVision Elite with 60x 0.85 NA lens (Olympus). Analyzed in ImageJ were maximum projections of deconvolved images.

**Mitotic Index Determination**

Mitotic index was determined by a fixed or a live method. For fixed determination, ~12500 cells were plated at day 0 on 96-well plates (BD Biosciences). At day 1, Thymidine (2.5 nM) (Sigma) was added to cells. After 24h arrest, cells were washed 3 times with PBS and new medium with nocodazole and Cpd-5 or reversine was added for 16 h. After incubations, cells

were fixed in 4% paraformaldehyde and 0.1% Triton for 10 min. Cells were incubated with the primary antibody against phosphorylated histone H3 at Serine 10 (pH3) (antibody concentration - 1:800) (Upstate) for 2 h at RT and washed again 2 times with PBS-T. Finally, cells were incubated for 1 h at RT with the secondary antibody coupled to Alexa 569 fluorophore (1:1000) and DAPI. Image acquisition was done by DeltaVision Elite (Applied Precision) using a 10x 0.4NA objective. 9 images per well were acquired and analyzed with ImageJ. For image analysis an ImageJ macro was used to threshold the pH3 levels to distinguish mitotic and interphasic cells. To determine the amount of mitotic cells, the amount of positive pH3 cells was divided by the total amount of cells, determined by the DAPI positive cells.

## Colony Formation Assays/ Proliferation Assays

In a 96 well plate, 1750 or 2000 cells (HeLa and U2OS, respectively) per well were plated (day 0). At day 1 medium with different concentrations of Reversine and Cpd-5 were added. On day 7 plates were fixed with 96% methanol and stained with 0.1% crystal violet overnight. Plates were washed with dH$_2$O and dried before scanning. Scanned images were analysed with ImageJ software.

For U2OS cell lines, proliferation assays were performed in the presence and absence of doxycycline. In this case, doxycycline was added together with the inhibitors at day 1.

## Flow cytometry

After treatment with nocodazole or STLC for 16 h cells were harvested and pelleted (1500 rpm, 5 min, RT). The supernatant was removed and cells resuspended in the remaining liquid (~100 µL). Cells were fixed with 4 mL of -20°C cold 70% ethanol (dropwise) while vortexing and incubated for a minimum of 30 min at 4°C. After incubation cells were spun down (3000 rpm, 5 min, RT) and the supernatant was removed. Blocking solution (4% BSA in PBS) was added to the cells for a minimum of 1 h. Cells were again spun down and the supernatant was removed. The primary antibody pH3-S10 (1:500) was added to the cells for a minimum of 2 hours at RT or overnight at 4°C. Cells were washed with blocking buffer 2 to 3 times and the secondary antibody added to cells (1:600). After incubation for 2 h at RT cells were washed at least once with blocking buffer. Finally, a solution containing PBS, RNAse (1:40) and DAPI (1:100) was

added to the cells and incubated at 37°C for 15 minutes. Samples were run through a flow cytometer.

**Antibodies**

For immunoblotting, rabbit-GFP (custom made, Geert Kops), rabbit-TTK (C19) (Santa Cruz, sc-540), goat-Actin (I-19) (Santa Cruz, sc-1616), rabbit-CyclinB1 (H-433) (Santa Cruz, sc-752) and rabbit-phospho-KNL1 (MELT 13/17) (custom made, Geert Kops) primary antibodies were used. GFP and CREST (#15-235-0001, Antibodies Inc.) antibodies were used for immunofluorescence imaging. For the mitotic index and flow cytometry assays, an antibody against pH3-Ser10 (#06-570, Merck Millipore) was used. Secondary antibodies for immunoblot detection and secondary antibodies coupled to fluorophores for immunofluorescence were purchased from DAKO.

**Modeling**

Structures of the Mps1 kinase domain in complex with ATP and NMS-P715 were obtained from Protein Data Bank (PDB identifier 3HMN and 2X9E, respectively). Images were obtained using MacPyMol crystallography software. Image processing was done with AdobeCS6 software.

# 3. Results

## 3.1 Mutations in the N-terminus of Mps1

After sequencing the whole kinome (set of kinases present in the genome of an organism) of breast cancer samples from patients, two novel heterozygous mutations in the essential mitotic kinase Mps1 were identified. These mutations, located in the N-terminus of the protein, were hypothesized to be important contributors for tumorigenesis in these tumors. Why did this hypothesis emerge? As mentioned in the introduction, Mps1 function is essential for the proper activation of the SAC, due to its kinetochore localization and the phosphorylation of KNL1 at the MELT repeats[88,94]. KNL1 phosphorylation leads to the recruitment of several important intermediate players which culminates in the formation of the MCC[88]. Without proper kinetochore localization of the MCC, cells undergo an erroneous mitosis leading to CIN and aneuploidy. Moreover, the kinetochore localization of Mps1 is dependent on its N-terminus[95,96].

One of the identified mutations, a substitution of an aspartate for a serine at the position 106 (**N106S**) is located in the TPR domain of Mps1. The location makes it especially interesting, since the TPR domain has been implicated in the control of Mps1 localization at the kinetochores[97]. Sequence alignment of various Mps1 homologs exhibits a high conservation of this residue among several eukaryotic species (**Fig. 9a**) which may indicate an important role of it. The second mutation displays a serine to cysteine replacement at the position 403 (**S403C**). For this residue the sequence alignment shows a rather limited conservation (**Fig. 9a**).

To address the question if these mutations affect Mps1 function and, as a consequence, could have contributed to malignant transformation in the tumors where they were identified, I made use of a LAP (Localization and Affinity Purification) tagged Mps1. This construct comprises a yellow fluorescence protein (YFP) for localization studies and an S-peptide for protein purification purposes (**Fig. 9b**). The LAP tagging of Mps1 was successfully used before to determine the role of specific mutations or truncation in Mps1[96]. After introducing the desired mutations, N106S and S403C in the LAP-Mps1 construct by site directed mutagenesis (**Fig. 9b**), I created RPE-1 cell lines stably expressing the LAP-Mps1 WT, LAP-Mps1 N106S and LAP-Mps1 S403C constructs (**Fig. 9c**). The non-transformed RPE-1 cell lines co-expressing the

LAP-Mps1 construct with endogenous Mps1 provides a perfect tool to investigate the role of the identified heterozygous mutations in the onset of tumorigenesis.
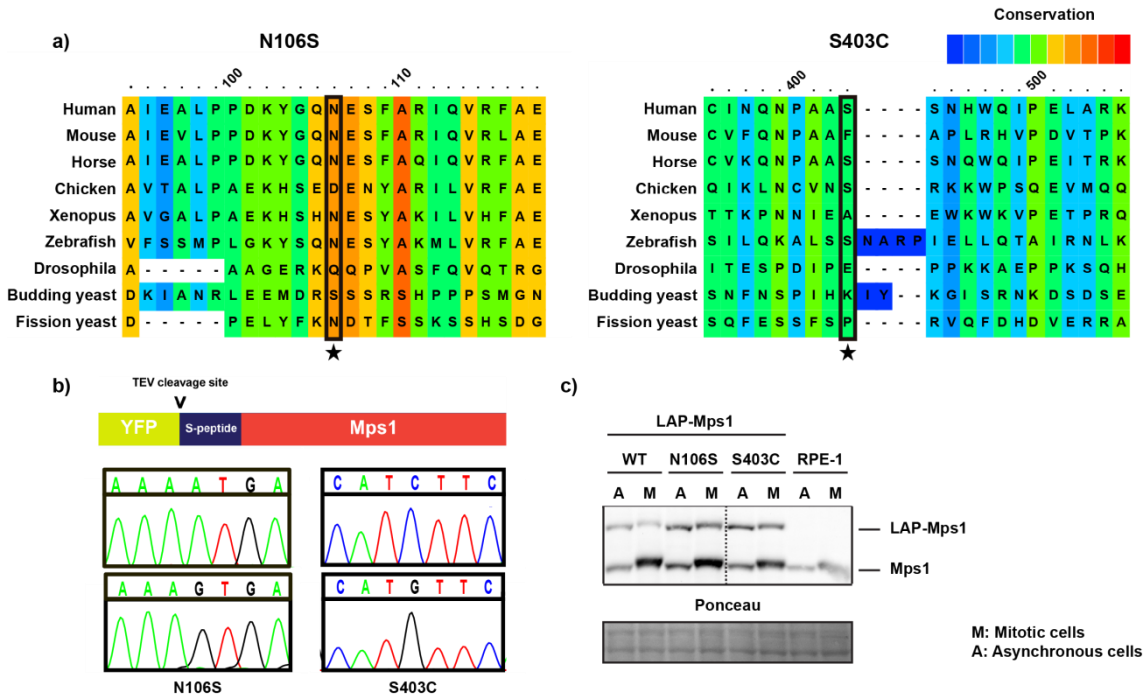


**Figure 9. Ectopic expression of LAP-tagged Mps1 in RPE-1 cells with the N106S and S403C mutations.**

**a)** Multiple sequence alignment of Mps1 homologs across various eukaryotic model organisms. Conservation varies as shown in the color-coded heat map with blue representing the less conserved residues and red the most conserved. **b)** Top: Schematic representation of the LAP-Mps1 construct; Bottom: Sequence traces after site directed mutagenesis of the LAP construct for the two identified mutations. **c)** Immunoblot of whole-cell lysates and mitotic-cell lysates against Mps1.

## 3.1.1 LAP-Mps1 N106S and S403C do not affect Mps1 localization

The first question that needed to be addressed is if the mutants can still localize at the kinetochores. Since the N106S mutation is in the TPR domain we questioned if this mutant has impaired kinetochore localization. To test this hypothesis, live cell imaging and immunofluorescence experiments were performed. Live cell imaging was performed in unperturbed mitosis and cells were followed through mitosis. On the other hand, immunostaining of RPE-1 was done in cells arrested in mitosis by treatment with nocodazole. Both assays showed unperturbed localization of the LAP-Mps1 constructs at the kinetochores (**Figure 10**). In an unperturbed mitosis (**Fig. 10a**) the constructs appear to localize at the kinetochores when the cell goes into mitosis which corresponds to prophase and prometaphase[86] but signal intensity is lost with time, probably due to alignment of the

chromosomes with subsequent silencing of the SAC and removal of Mps1 from the kinetochores.

Since RPE-1 cells do not express any fluorescent kinetochore marker, co-staining of LAP-Mps1 with a centromeric protein was performed (**Fig. 10b**). As described previously, to increase the signal of LAP-Mps1 at the kinetochores[98–100] we used a Mps1 inhibitor in all conditions (100 nM Cpd-5). Whereas no signal is observed for control RPE-1 cells, cells stably expressing the LAP-Mps1 construct showed Mps1 localization (GFP) in proximity of the centromeric protein signal (ACA).

We conclude that in the presence of endogenous Mps1, the mutants have no effect in Mps1 localization during mitosis, which may indicate that these residues are not important for the control of Mps1 localization.
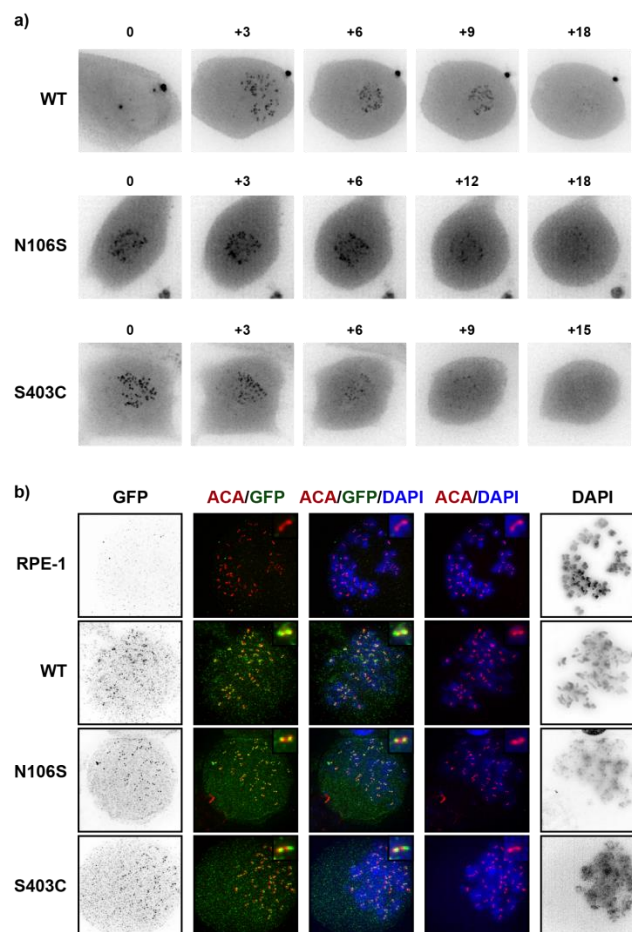


**Figure 10. Localization of LAP-Mps1 WT, N106S, and S403C during mitosis.**

**a)** Time-lapse imaging of single cells of the different mutants. Cells were filmed for YFP-signal with a 60x lens and followed through mitosis, in the presence of endogenous Mps1. **b)** Representative images of immunolocalization of the different LAP-Mps1 construct in cells treated with nocodazole, Cpd-5 and MG132. LAP-Mps1 constructs are

visualized with an antibody against GFP (green). A centromeric protein (red) is stained to assess co-localization of the LAP-Mps1 with the kinetochores. DNA is stained with DAPI. In all cases, LAP-Mps1 localization was assessed in the presence of endogenous Mps1.

Although localization was not impaired for LAP-Mps1 N106S and S403C, the functionality of the constructs remained to be assessed. As mentioned above, several studies have already demonstrated that inhibition of the kinase activity of Mps1 does not prevent its localization at the kinetochores, but leads to an increase of levels at this region. This indicates that, although kinetochore localization is observed for the two mutations, these can have impaired Mps1 activity. As a consequence, the next step in the characterization of these mutants was to determine if they are as functional as the WT protein.

## 3.1.2 LAP-Mps1 mutants have a functional SAC

After showing that the mutants have normal Mps1 localization at the kinetochores, cells were imaged in unperturbed mitosis and the time they spend in mitosis (from nuclear envelope breakdown (NEB) until anaphase onset) was assessed. In the presence of endogenous Mps1, cells expressing LAP-Mps1 WT, N106S and S403C spend on average the same time in mitosis as RPE-1 control cells (20 min) (**Fig. 11a**). Since RPE-1 cells stably expressing the LAP-Mps1 constructs did not express any fluorescent DNA marker, segregation error analysis had to be performed by immunofluorescence. In this case, DNA was stained with DAPI and anaphases were scored for normal segregation or missegregation. Again, in the presence of endogenous Mps1, no significant difference between constructs was found (**Fig. 11b**).
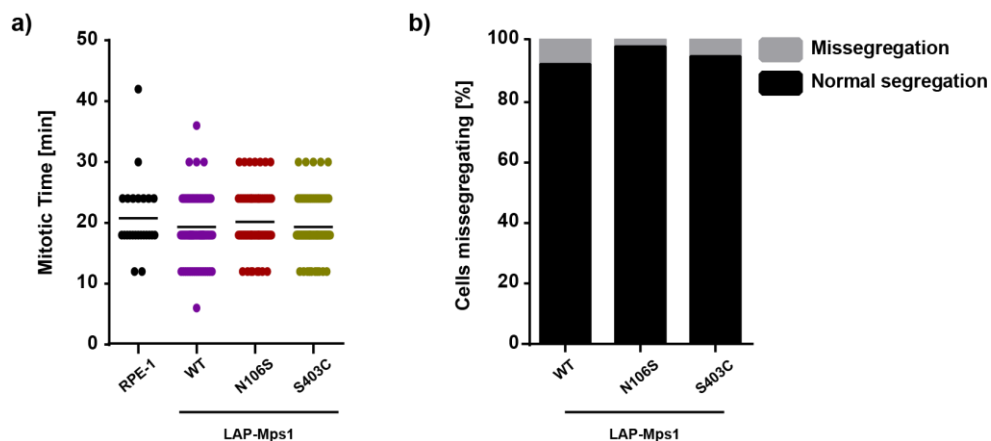


**Figure 11. Activity of the LAP-Mps1 constructs in unperturbed mitosis in the presence of endogenous Mps1.**

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**a)** Time-Lapse analysis of the mitotic timing (NEB-Anaphase) of the different RPE-1 cell lines. 100 cells were analyzed for each condition. **b)** Quantification of the segregation errors by analysis of anaphases in fixed material. DNA was stained with DAPI and anaphases were scored for normal segregation or missegregation according to the presence of lagging chromosomes, anaphase bridges or other segregation errors.

Since RPE-1 cells have a very short mitosis, the fact that all the constructs show similar mitotic time does not tell us that they have the same activity. However, if the mutants are less active than the WT one would expect that cells expressing the LAP-Mps1 mutants have an impaired checkpoint. This means that the cells do not wait until all their chromosomes are properly aligned and attached to microtubules to segregate them to opposite poles, resulting in more segregation errors[100]. The fact that all RPE-1 cell lines show similar missegregation rates can be an indicator that the mutants have similar activity to the WT Mps1 or that any impairment on Mps1 function present in the mutants can be rescued by the presence of a WT copy.

Although LAP-Mps1 N106S and S403C seem to have WT activity in unperturbed mitosis, what happens if the checkpoint is challenged with drugs that affect the normal progression of mitosis? Are the Mps1 mutants able to sustain a checkpoint-dependent arrest to the same extension as the WT Mps1? To address this question, cells were treated either with nocodazole or S-Trityl-L-cysteine (STLC). Nocodazole treatment interferes with microtubules polymerization[101] and leads to mitotic spindle disruption resulting in unattached kinetochores. STLC, an inhibitor of the kinesin-5 Eg5, prevents the separation of the two centrosomes, which creates a monopolar spindle[102]. STLC treated cells have attached kinetochores but that are not under tension due to the absence of forces exerted by the MT of separated centrosomes. In both cases, exposure of cells to these drugs should result in a mitotic arrest dependent of the SAC[30]. This means that if the SAC is activated, cells should not be able to progress into anaphase and arrest in a prometaphase-like state[30,103]. To read out SAC robustness, the percentage of cells that arrest in mitosis as well as the time they can sustain the arrest was quantified (**Figure 12**).

Cells were treated with nocodazole or STLC for 16 h, immunostained for pH3-S10 as a mitotic marker[104] and analyzed by flow cytometry. Cell cycle profiles and the amount of pH3 positive cells was determined (**Fig. 12a, b**). In the cell cycle profile it is possible to see a significant increase in the 4n population in cells treated with the spindle poisons. The 4n population represents not only mitotic, G2, but also tetraploid cells originated from cytokinesis failure or mitotic slippage[68,105] (**Fig. 12b**). To determine the amount of cells in mitosis,

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quantification of the pH3 positive cells was used. Control RPE-1 and all cell lines expressing either LAP-Mps1 or mutated LAP-Mps1 showed an increase in the pH3 positive population when cells are exposed to both drugs (**Fig. 12b**). However, since flow cytometry is an end-point assay, it is not possible to conclude that the different constructs have the same capacity to sustain a mitotic arrest. For this reason time-lapse imaging of RPE-1 cells, RPE-1 LAP-Mps1 WT, N106S and S403C was performed. Cells were treated with nocodazole and the time they sustained the mitotic arrest assessed (**Fig. 12c**). More than 75% of the cells in all conditions arrest in mitosis for longer than 5 hours (**Fig. 12d**). This suggests that none of the residues found mutated in tumor samples have an important role in regulating Mps1 activity in the presence of the endogenous Mps1.
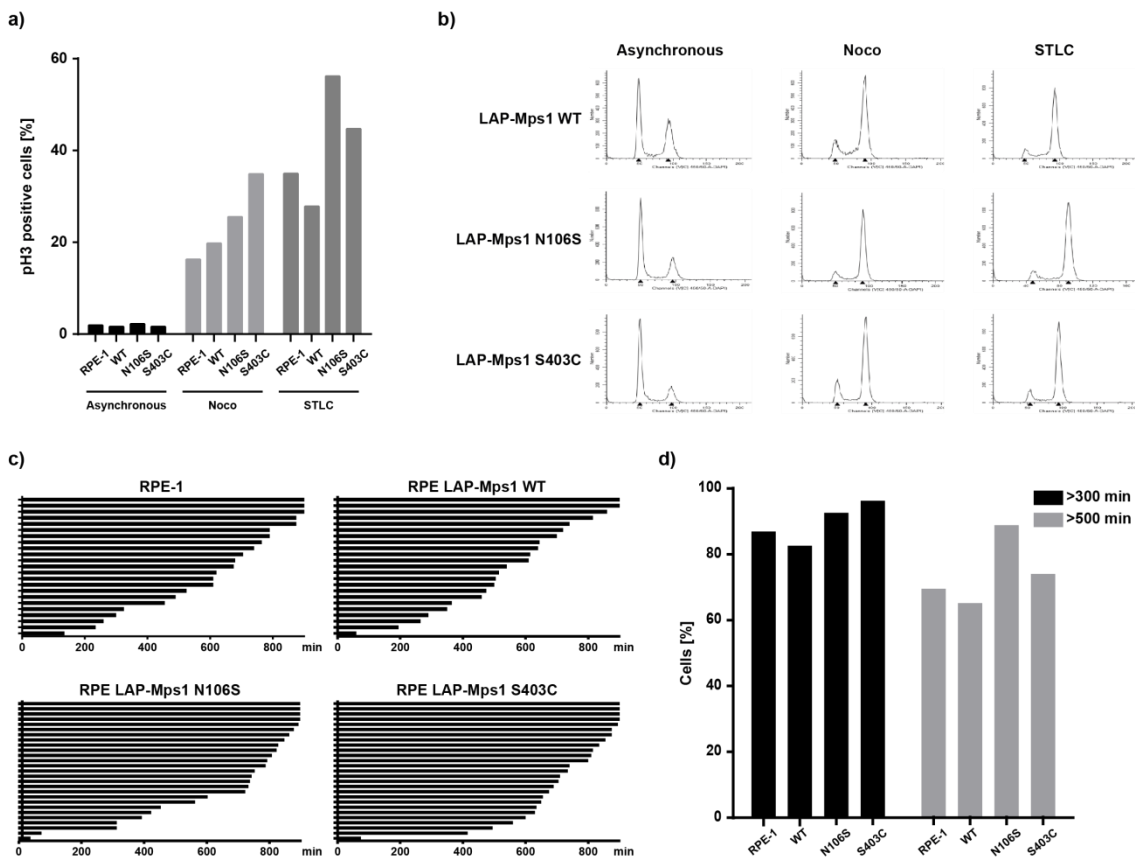


**Figure 12. Cells stable expressing the LAP-Mps1 constructs are able to sustain a prolonged mitotic arrest in the presence of endogenous Mps1.**

**a)** Cell cycle profile of untreated cells and nocodazole or STLC-treated cells. Cells were treated with spindle poisons for 16 hours and after fixed with methanol. Staining with pH3-S10 antibody was performed after fixation. **b)** Quantification of the pH3 positive population in untreated and nocodazole or STLC-treated cells. Two gates were set for analysis of the results. The first gate (Gate 1) includes all single cells and excludes death and duplets and the number of events represented in this gate is the total number of cells analyzed. Another gate (Gate 2) for the positive pH3 cells was established. Number of events present in this gate was considered the number of cells that were in mitosis. Quantification of the percentage of cells in mitosis was done by dividing the total amount of pH3

positive cells by the total amount of cells (Gate 2/Gate 1). **c)** Time-lapse analysis of the amount of time LAP-Mps1 WT, N106S and S403C are able to sustain the mitotic arrest after treatment with nocodazole. Quantification of the number of frames cells are in mitosis was done to determine total amount of time. **d)** Quantification of the % of cells that can sustain a mitotic arrest for longer than 300 min or 500 min.

Although these results do not answer the initial question if these mutations can have a role in tumorigenesis, these data suggest that at least they do not affect the normal progression of mitosis. Not only do N106S and S403C not impair Mps1 localization at the kinetochores in the presence of the endogenous Mps1 but they also do not affect Mps1 checkpoint activity, which is read out by the presence of a fully active SAC.

## 3.1.3 Do the mutations N106S and S403C affect Mps1 activity in the absence of the endogenous Mps1?

As a final question, we asked if these mutations are able to affect Mps1 activity when they are not in the presence of WT of Mps1. Although mutations were found in a heterozygotic background, it is also interesting to determine if these mutations show any phenotype in the absence of endogenous Mps1. As mentioned before, in order to be fully activated, Mps1 must localize to the kinetochores but must also form homodimers[95]. All the assays mentioned before were done in the presence of endogenous Mps1 which may be rescuing any phenotype caused by the two mutations. To determine the impact of the Mps1 mutations in the absence of the endogenous kinase, U2OS cells stably expressing a doxycycline-inducible short-hairpin against Mps1[106] were used. To induce the expression of the short-hairpin and subsequently reduce the expression of Mps1, cells were exposed to doxycycline (**Fig. 13a**). To prevent degradation of the LAP-Mps1 constructs by the short-hairpin targeting Mps1, I created U2OS cell lines stably expressing a LAP-Mps1 constructs that had 4 silent mutations present in the region of the short-hairpin target sequence (**Fig. 13b**). This means that when the short-hairpin is transcribed only endogenous Mps1 is depleted and not the LAP-Mps1 constructs. Before the silent mutations were introduced in the Mps1 sequence, the LAP-Mps1 constructs were also depleted after induction of the short-hairpin (**Fig. 13a**). After infection and selection with blasticidine, the levels of expression of the LAP-Mps1 constructs were too low for cells to survive without endogenous Mps1. To overcome this problem, cells were cultured in medium with 1 µg/mL of doxycycline for two weeks so that only cells with high levels of LAP-Mps1 expression are able to survive (**Fig. 13c**). An increase in the overall expression of the exogenous Mps1 is observed for all the LAP-Mps1 constructs.
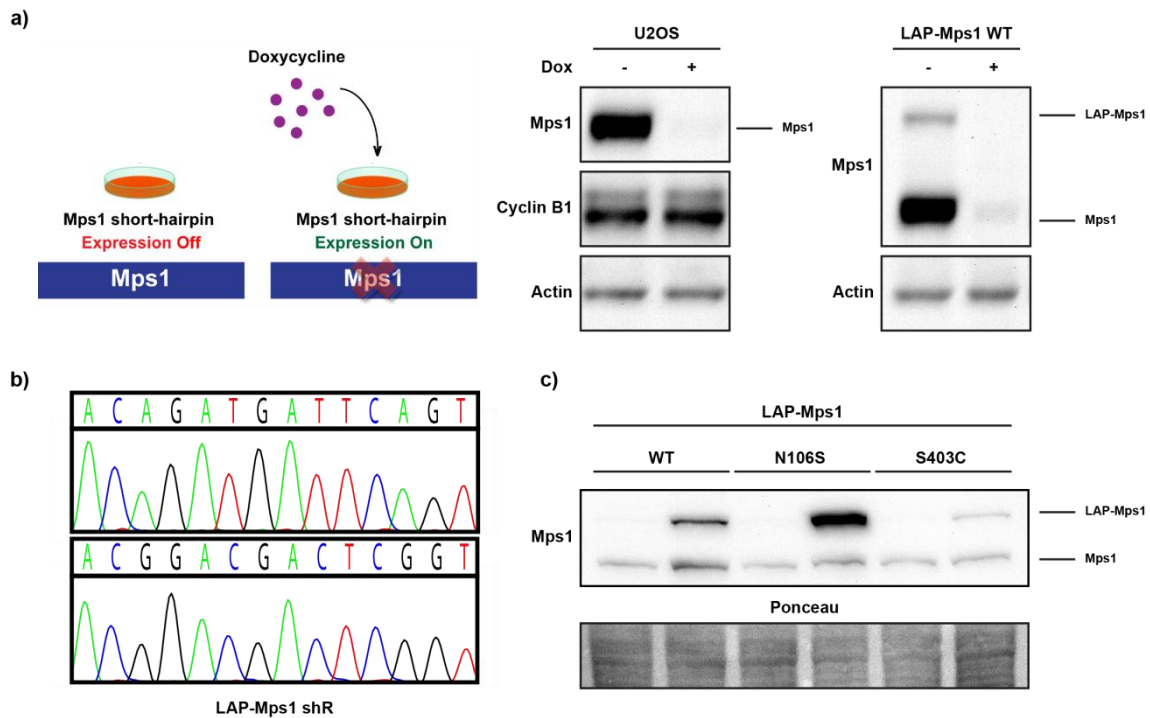
**Figure 13. U2OS cells stably expressing a dox-inducible short-hairpin against Mps1 as a tool to study the LAP-Mps1 N106S and S403C phenotype when endogenous Mps1 is depleted from cells.**

**a)** Schematic representation of the U2OS system. Doxycycline is used to induce the expression of the Mps1 short-hairpin; Immunoblot of whole-cell lysates induced with doxycycline for 48 hours. An antibody against Mps1 was used. U2OS and U2OS LAP-Mps1 cells were used. Depletion of endogenous Mps1 and LAP-Mps1 construct before introduction of the 4 silent mutations. **b)** Site directed mutagenesis to introduce 4 silent mutations in the region recognized by the short-hairpin. **c)** Immunoblot of whole-cell lysates against Mps1. U2OS cells stably expressing the LAP-Mps1 constructs before and after doxycycline selection. Cells were cultured for two weeks in medium containing 1 µg/mL of doxycycline.

# 3.2 Mutations in the kinase domain of Mps1

In recent years, the possibility of using small molecule inhibitors against Mps1 as a cancer therapy has emerged as an attractive therapeutic strategy. However, the main drawback in cancer treatment is that after some time of treatment cells become resistant to the drugs[73]. So, it is essential to study and understand what mechanisms are behind this resistance and what can be done to overcome this problem.

Since the main mechanism of resistance of kinase inhibitors is the appearance of resistant alleles[77], a data base that contains all the somatic mutations identified in cancer samples – COSMIC data base – was consulted[107]. Mutations identified in the kinase domain of Mps1 were selected for further analysis. As it was already described for other kinases[108,109], mutations that confer resistance to small molecule inhibitors are usually located in the kinase domain of the

protein, close or in the ATP-binding pocket region, where the inhibitor binds. After some structural analysis – localization of the mutation in the kinase domain and computational residue change – of Mps1 mutations, four mutations were chosen for further analysis (**Fig. 14**). These mutations are **G534E**, **E571K**, **M600I** and **C604F**. As mentioned before, a study addressing the resistance of cancer cell lines to Mps1 inhibitors has led to the discovery of 4 mutations, namely I531M, I598F, C604Y and S611R, in the Mps1 kinase domain. Therefore **C604F** was a very interesting mutation, since the C604Y mutation is located in the same residue.
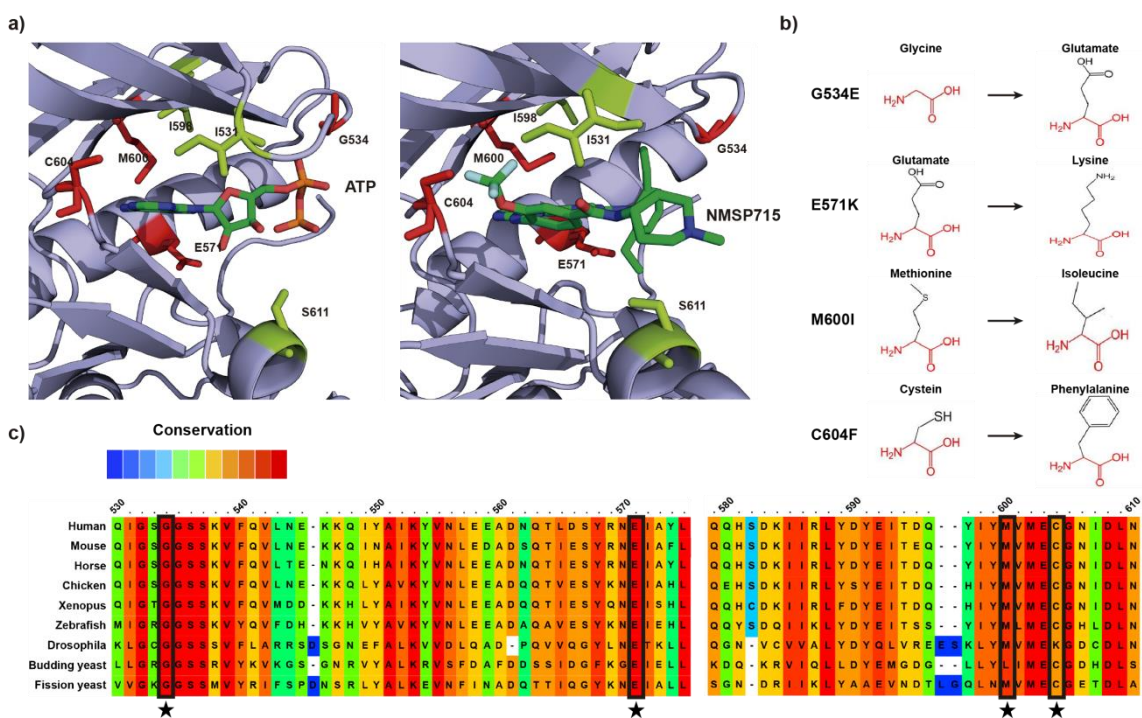


**Figure 14. COSMIC data base mutations selection.**

**a)** Localization of the Mps1 mutations in the X-ray structure of the Mps1 kinase domain in complex with ATP or NMS-P715, a selective inhibitor of Mps1. In yellow are shown the mutations previously identified in cancer cell lines – I531M, I598F, C604Y and S611R. Red residues are the mutated sites selected from COSMIC data base. ATP and NMS-P715 are shown in green. **b)** Structure of the different residues for each mutation. **c)** Multiple sequence alignment of the kinase domain of Mps1. High conservation conservation of the mutated residues, G534, E571, M600 and C604, in different Mps1 homologs.

Again, the short-hairpin resistant LAP-Mps1 construct was used and mutated for the four selected mutations. These mutations were also introduced in the kinase domain of Mps1 cloned in a bacterial expression vector (**Fig. 15a**). U2OS cells stably expressing a dox-inducible short-hairpin were used to create cell lines stably expressing the LAP-Mps1 constructs with the mutations (**Fig. 15b**). Expression levels of LAP-Mps1 were not high enough for cells to survive

without the endogenous Mps1. Selection of these cells with doxycycline for two weeks led to a higher expression of the LAP-Mps1 WT, LAP-Mps1 G534E, M600I and C604F constructs (**Fig. 15c**). However, for two of the mutations, G534E and E571K, the selection was very difficult and even impossible for E571K, with no clones growing out after the two weeks of treatment with doxycycline (**Fig 15d**).

One of the explanations would be that these mutations impair the kinase activity of Mps1 which would lead to severe segregations errors[98] and, as a consequence, cells die after depletion of endogenous Mps1. To test this hypothesis, the kinase domain of Mps1 with the different mutations was overexpressed in bacteria. After purification of the recombinant kinase domains (**Fig. 15e**), *in vitro* kinase assays with the WT and mutants were performed (**Fig. 15f**). As expected, the G534E and E571K mutants have an impaired kinase activity. Surprisingly also the M600I mutant has reduced catalytic activity when compared to the WT and to C604F mutation (**Fig. 15f**).
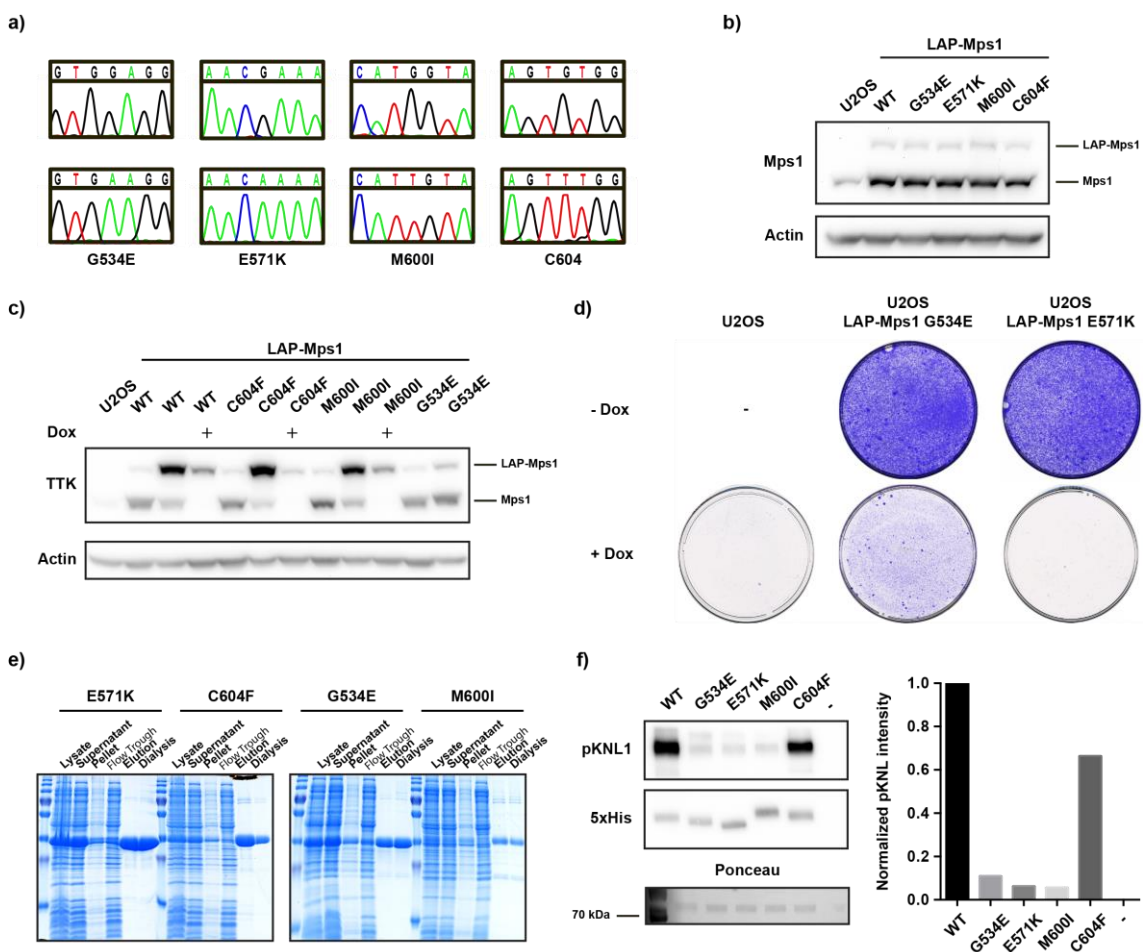


**Figure 15. Characterization of the Mps1-mutants G534E, E571K, M600I and C604F.**

**a)** Mutation of the residues in the sequence of Mps1. Shown are DNA sequence traces from WT (top) or successfully mutated Mps1 constructs. **b)** Immunoblot of whole-cell lysates against Mps1. U2OS cell lines stably expressing the LAP-Mps1 WT and the mutated constructs. **c)** Immunoblot of whole-cell lysates against Mps1 after doxycycline selection. Cells were cultured in medium with doxycycline for two weeks. After two weeks, doxycycline was removed from the medium and after some time, cells were harvested. **d)** U2OS, U2OS LAP-Mps1 G534E and E571K cells after two weeks of doxycycline selection. **e)** Commassie Brillant Blue stained gels for the purification of the kinase domains of Mps1 with the different mutations. Nickel-bead purification of the recombinant mutated kinase domains after overexpression of the proteins in bacteria. Cells were lysed, sonicated and afterwards ran through a column with nickel-beads. After elution of the proteins from the beads, dialysis was performed. **f)** In vitro kinase assays of the WT and mutants kinase domains of Mps1. The mutated kinase domains of Mps1 were incubated with recombinant KNL1 for 1 h at 32°C . Catalytic activity was assessed by quantification of immunoblot against pKNL1.

## 3.2.1 Expression of LAP-Mps1 mutants can confer resistance to Mps1 inhibitors

After assuring that the LAP-Mps1 constructs in the U2OS cell lines do localize to kinetochores and do not affect the progression of cells through mitosis (**Fig. 16**), resistance to a selective Mps1 inhibitor, Cpd-5, was tested in a proliferation assay in the presence and absence of doxycycline (**Fig. 17**). Cells ectopically expressing LAP-Mps1-C604F are more resistant to Cpd-5, surviving to concentrations up to 200 nM of Cpd-5 (**Fig. 17a**). Expression of LAP-Mps1 WT construct does not affect the resistance of U2OS cells to Cpd-5 in the absence of doxycycline and cells die when a concentration of 40 nM is reached. For the other mutation, LAP-Mps1 M600I, we observed a slight increase in the resistance with cells dying at 63 nM of Cpd-5. Quantification of the proliferation assays was performed and a significant shift of the survival curve to the right occurs in the presence of the mutation C604F, meaning that cells can cope with higher concentrations of this inhibitor (**Fig. 17b**). This result shows that mutations identified in tumor samples can confer resistance to Mps1 inhibitors with C604F being the strongest mutation.

As expected, in the presence of doxycycline, U2OS cells without expression of exogenous Mps1 die even in the absence of the inhibitor (**Fig. 17a**). However, even the cells that express the LAP-Mps1 WT show an impaired growth when cultured with doxycycline, showing that this may not be the ideal system to further characterize the C604F mutation.
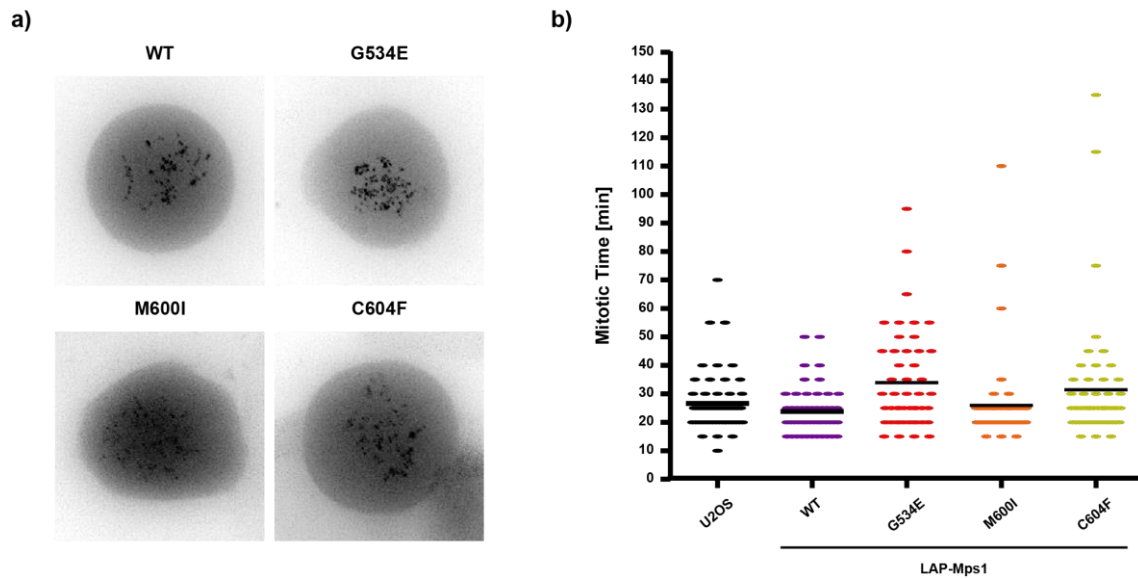
**Figure 16. LAP-Mps1 constructs seem functional when expressed in the presence of endogenous Mps1.**

**a)** Time-lapse imaging of LAP-Mps1 YFP constructs. Nocodazole-treated cells were imaged with a 60x lens. **b)** Time-lapse imaging of U2OS cells stably expressing the different LAP-Mps1 constructs. Time in mitosis is determined by quantification of the number of frames cells took from NEB to anaphase.
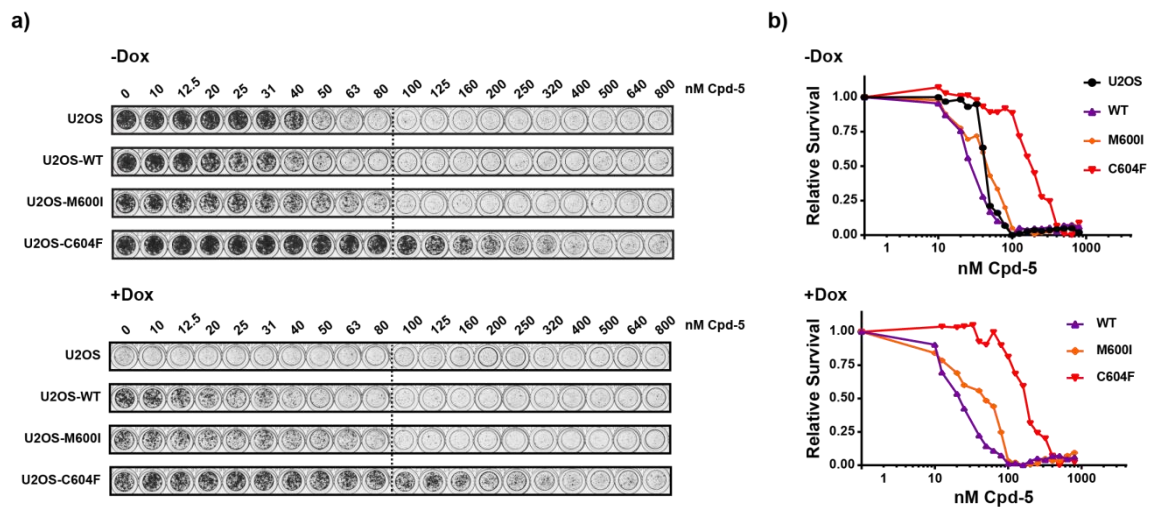


**Figure 17. Ectopic expression of Mps1-C604F confers resistance to the selective Mps1 inhibitor, Cpd-5.**

**a)** Proliferation assays of the U2OS cell lines. Cells were exposed to different concentrations of Cpd-5, in the absence and presence of doxycycline. **b)** Plot of the survival curves of the proliferation assays.

## 3.2.2 Genome editing using the CRISPR-Cas9 system

Since C604F was identified as the strongest mutation in conferring resistance to Mps1 inhibitors, I further characterized this mutation by making use of the CRISPR-Cas9 technology[110]. An oligonucleotide with the mutation C604F was designed and transfected in HeLa cells together with the endonuclease Cas9 and a single-guide RNA, which targets the

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Cas9 molecules to the region of interest. The idea is that the cell uses the oligo as a template to repair the double-strand breaks produced by the Cas-9 molecules (**Fig. 18a**). Cells were selected with 80 nM of Cpd-5 (**Fig. 18b**) so that only cells with the mutation survive. Cells with no mutation and only WT copies of Mps1 will die after exposure to this amount of inhibitor. To assess the efficiency of the system, non-transfected cells and cells transfected only with the oligo were also treated with 80 nM of Cpd-5 (**Fig. 18c**). As expected, no cells were able to survive in these two cases. After 2 weeks of selection with Cpd-5, single cell clones were isolated in the wells transfected with the oligo and the guide (**Fig. 18b**). To assure that the mutation was present in the cells, genomic and cDNA of single cell clones were sequenced for Mps1. Two out of 15 positive C604F clones were chosen for further characterization of the C604F mutation (**Fig. 18d**).
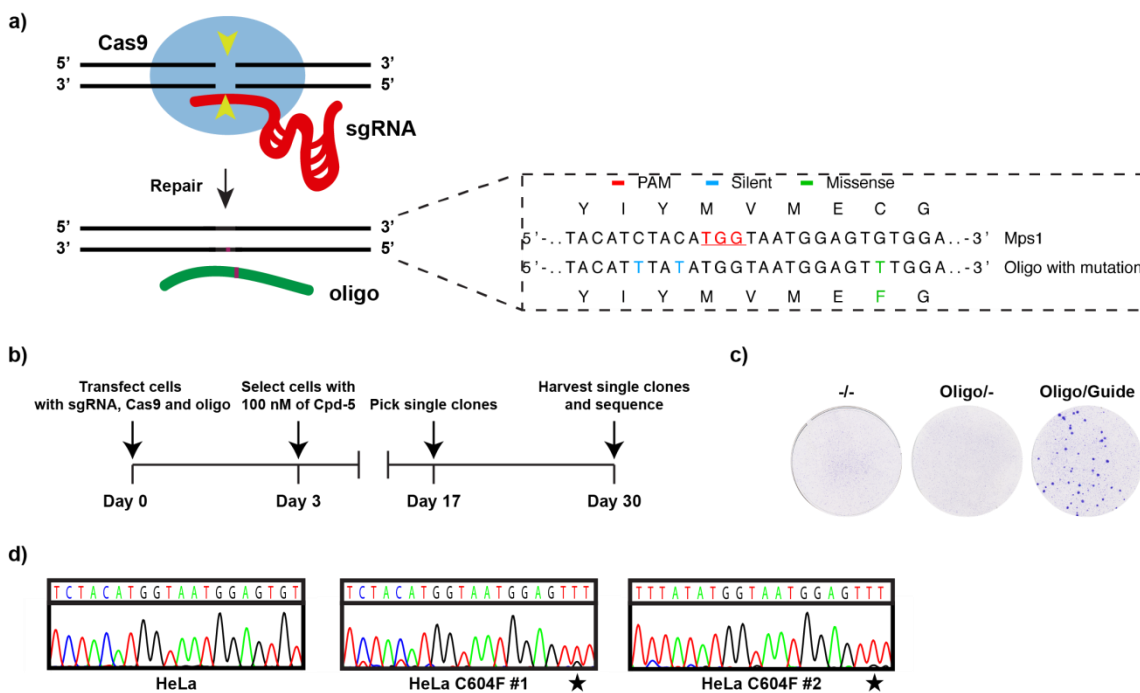


**Figure 18. Genome editing by CRISPR-Cas9.**

**a)** Schematic representation of the CRISPR-Cas9 technology concept. The sgRNA targets the Cas9 molecule to the region of interest, in this case the Mps1 sequence. Cas9 cuts the DNA leading to the formation of a double-strand break which is repaired by homologous recombination using the oligonucleotide as a template. **b)** Schematic representation of the experimental setup used to generate clones with the Mps1-C604F mutation. **c)** Cells after selection with Cpd-5. Cells were selected for two weeks with 80 nM of Cpd-5. Untransfected cells, cells transfected only with the oligo and cells co-transfected with the oligonucleotide, Cas9 and sgRNA went under selection. For all the conditions, the same number of cells was plated. **d)** DNA sequence traces of Mps1 cDNA of single cell clones.

## 3.2.3 The C604F mutation responds differently to different Mps1 inhibitors

After genome editing of HeLa cells, I had a powerful system to characterize the C604F mutation. Previously, I had shown with proliferation assays that U2OS ectopically expressing this mutation are less sensitive to the Mps1 inhibitor Cpd-5 (**Fig. 17**). I repeated this assay for the HeLa-C604F cells with the same concentrations of Cpd-5. HeLa cells mutated for the mutation C604Y previously identified in resistant cancer cell lines was taken along as a control (**Fig. 19**). The tyrosine − Y − and phenylalanine − F − aminoacids are structurally almost identical, which pointed to a similar behavior of the two mutations. However, although HeLa-C604F cells are significantly more resistant to Cpd-5 than HeLa cells, that die at 40 nM of Cpd-5, this mutation does not give such a strong phenotype as the C604Y mutation. HeLa-C604Y can cope with more than 500 nM of Cpd-5 while HeLa-C604F cells only survive concentrations up to 200 nM of Cpd-5 (**Fig. 19a**). To further investigate Cpd-5 resistance of these mutations, live cell imaging was performed. In the presence of different concentrations of Cpd-5, the mitotic timing and segregation errors for the HeLa cells lines were assessed (**Fig. 19b, c**). In the presence of only 100 nM of Cpd-5 it is possible to observe a shortening in the time that cells spend in mitosis, a result of inhibition of the SAC (**Fig. 19b**). Another consequence of the inhibition of the SAC is that HeLa cells exposed to this concentration of Cpd-5 no longer align their chromosomes at the metaphase plate to proceed into anaphase, which results in a dramatic increase of segregation errors (**Fig. 19c**). While control HeLa cells show reduced mitotic timing at 100 nM of Cpd-5, the HeLa-C604F seemed to be unaffected when treated with concentrations up to 300 nM of Cpd-5. The HeLa-C604Y showed no significant reduction in mitotic timing with up to 400 nM Cpd-5, which means that Mps1 is still active and the SAC is not severely affected. Another interesting observation is that, although HeLa-C604F cells already show slight alignment problems and segregation errors at 200 nM, these are mild errors (less than 4 chromosomes are non-aligned) (**Fig. 19e**) and only at 300-400 nM of Cpd-5 severe mistakes start to be significant (**Fig. 19c**). As a final assay to characterize Cpd-5 resistance of the C604F mutation, the mitotic index of cells in the presence of different concentrations of Cpd-5 was determined (**Fig. 19d**). In the presence of 150 nM of Cpd-5, HeLa cells have a reduction of almost 80% in the number of cells that can sustain a mitotic arrest and, although HeLa-C604F also suffers a reduction in the amount of mitotic cells, this is not as

drastic as in control cells (**Fig. 19d**). Again the mutation with the strongest phenotype is the C604Y, which show no reduction in the mitotic index (**Fig. 19d**).
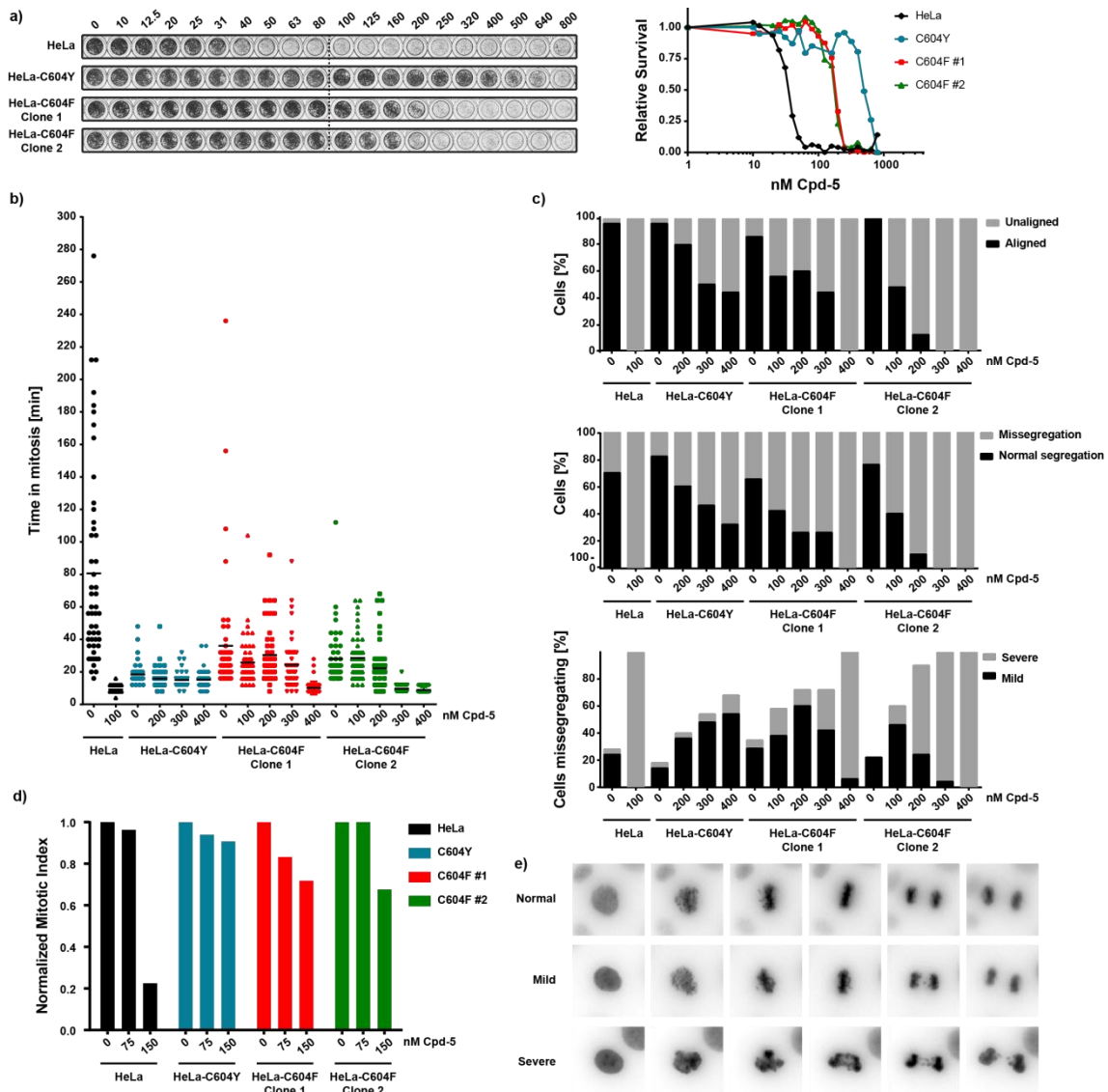


**Figure 19. Characterization of the HeLa-C604F cell line in light of Cpd-5 resistance.**

**a)** Proliferation assays with different concentrations of Cpd-5. Cells were plated with different concentrations of Cpd-5 and grown until confluency in the well with 0 nM of Cpd-5 was reached. Quantification is shown as a relative survival plot. **b)** Time-lapse imaging of HeLa cell lines exposed to different concentrations of Cpd-5. Mitotic timing in the different conditions was assessed by the quantification of the number of frames from NEB to anaphase. **c)** Time-lapse imaging of HeLa cells lines exposed to different concentrations of Cpd-5. Quantification of chromosome alignment and missegregations was done. Proper alignment occurs when all the chromosomes are aligned at the metaphase plate. Missegregations were classified as mild (up to 4 chromosomes) or severe (more than 4 chromosomes) according to the number of missegregated chromosomes. **d)** Mitotic index determination. Cells were synchronized and treated with nocodazole and different concentrations of Cpd-5 for 16h. After, cells were fixed and stained for pH3-S10 and DAPI. **e)** Representative figures of normal segregation, mild and severe missegregation.

In order to investigate whether the C604F mutation not only confers resistance to Cpd-5 but also another Mps1 inhibitor, I characterized the effects of reversine[111] on these cells. Reversine belongs to another class of Mps1 inhibitors and although it binds to the ATP pocket similarly to Cpd-5, is it structurally different. Since reversine is not as potent as Cpd-5, higher concentrations of reversine compared to Cpd-5 are used. Exactly the same assays as above were performed (**Fig. 20**). Contrarily to what was expected, cells with the C604F and C604Y mutations were more sensitive to reversine compared to WT cells. While HeLa cells can survive to concentrations of 200 nM of Cpd-5, HeLa-C604Y and HeLa-C604F died with only 75 nM of Cpd-5 (**Fig. 20a**). Looking at the proliferation assays, C604F and C604Y seem to confer similar sensitivity to reversine; however, after time-lapse analysis of the HeLa cell lines, HeLa-C604Y seems to be affected by lower concentrations of reversine (**Fig. 20b, c**). Quantification of the mitotic timing shows that the SAC of HeLa-C604Y is inhibit with only 75 nM of reversine which is translated by a drastic shortening in the time that cells spend in mitosis. The same reduction for the HeLa-C604F is observed with concentrations of 150 nM. HeLa cells only seem to be affected with 300 nM of drug where a drop in the mitotic timing to less than 20 minutes occurs (**Fig. 20b**). Although changes in the SAC activity can be detected by alterations in the mitotic timing, alignment and segregation analysis detect minor effects in SAC activity (**Fig. 20c**). For example, whereas the drop in mitotic time for the HeLa-C604F only occurs at 150 nM of reversine, it is possible to appreciate that at 75 nM cells already have trouble aligning their chromosomes and, as a consequence, commit more mild segregation errors (**Fig. 20e**). This indicates that at this concentration the SAC is partially inhibited. Full inhibition happens at 150 nM with cells not even being able to align their chromosomes and exhibiting 100% of severe segregation errors (**Fig. 20c, e**). As indicated by the mitotic timing, full inhibition of the SAC occurs in the HeLa cell lines with different concentrations of reversine, with HeLa-C604Y showing the most sensitive phenotype and HeLa. This is also supported by the mitotic index analysis with HeLa-C604F and HeLa-C604Y not being able to sustain a mitotic arrest with 150 nM of reversine, contrarily to what happens with HeLa cells (**Fig. 20d**).
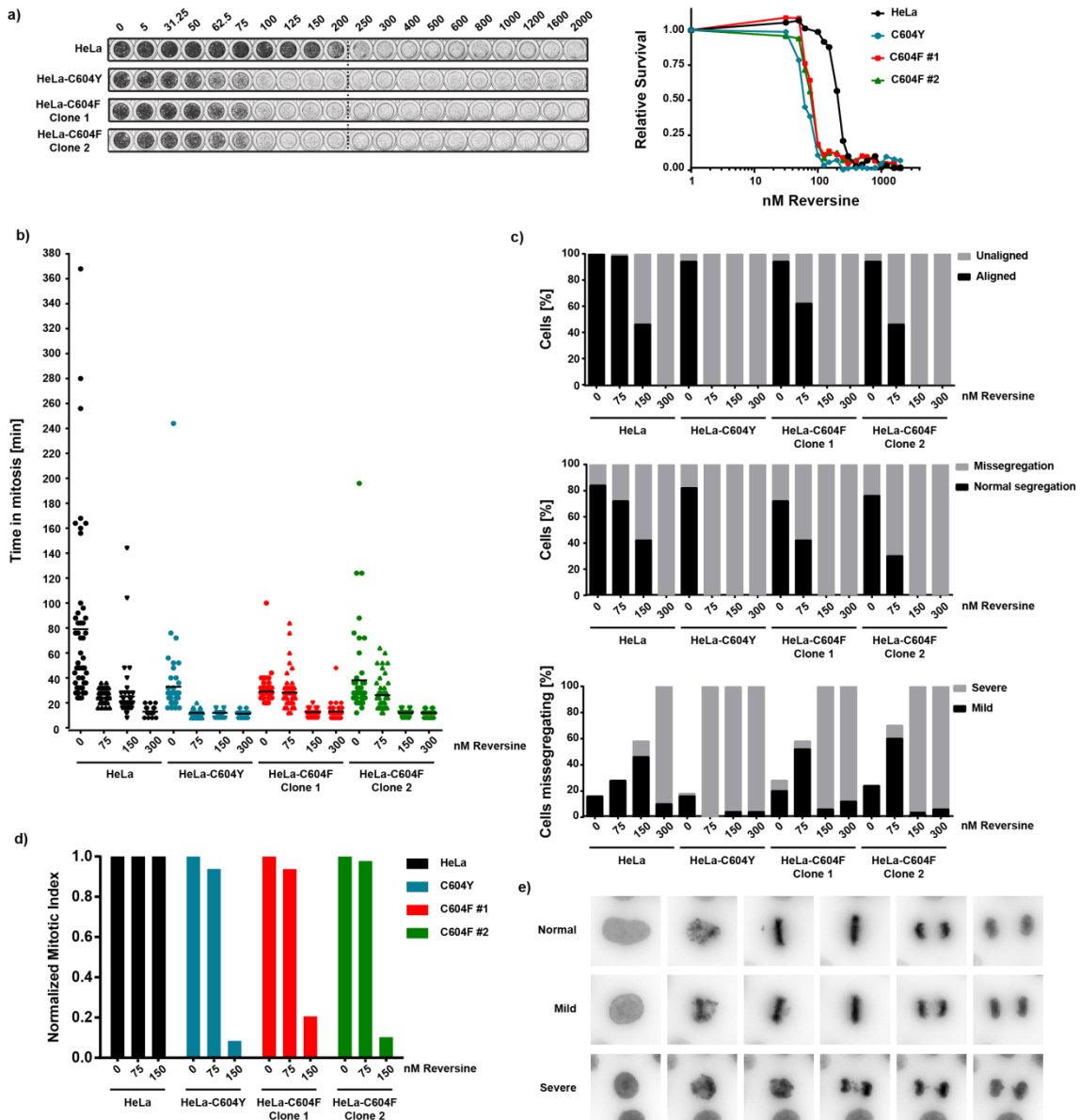
**Figure 20. Characterization of the HeLa-C604F cell line in light of possible reversine resistance.**

**a)** Proliferation assays with different concentrations of reversine. Cells were plated with different concentrations of reversine and grown until confluency in the well with 0 nM of reversine was reached. Quantification is shown as a relative survival plot. **b)** Time-lapse imaging of HeLa cell lines exposed to different concentrations of reversine. Mitotic timing in the different conditions was assessed by the quantification of the number of frames from NEB to anaphase. **c)** Time-lapse imaging of HeLa cells lines exposed to different concentrations of reversine. Quantification of chromosome alignment and missegregations was done. Proper alignment occurs when all the chromosomes are at the metaphase plate. Missegregations were classified as mild (up to 4 chromosomes) or severe (more than 4 chromosomes) according to the number of missegregated chromosomes. **d)** Mitotic index determination. Cells were synchronized and treated with nocodazole and different concentrations of reversine for 16h. After, cells were fixed and stained for pH3-S10 and DAPI. **e)** Representative figures of normal segregation, mild and severe missegregation.

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# Discussion

Identification and characterization of mutations identified in cancer samples has provided many valuable insights on the function of many proteins and has also been of major importance for drug development. Understanding the effect of a mutation in the function of the protein can reveal new or elucidate previously described roles of the protein in the cell. In a more translational view, these studies can have a major impact in the identification of new players in tumor progression which is essential for the development of new targeted drugs. Many players have already been identified and extensively studied. However, not all players are easily targetable and this is what makes Mps1 such an attractive target. Like most kinases, Mps1 is targetable by small molecule inhibitors that compete with ATP for the ATP binding pocket. A very good example that highlights the importance of these studies in drug development is the specific inhibitors that target the B-Raf kinase and are currently used in melanoma treatment. A mutant version of B-Raf (V600E) that makes it constitutively active was identified in tumor cells[112] and inhibitors that exclusively bind the mutated version were developed[112,113] which allows selectivity for the tumor cells, decreasing the side effects of these inhibitors. Another advantage of knowing and studying these mutations is that like in the B-Raf example, only patients that are positive for V600E mutation are treated with the inhibitor. This means that these studies also provide precious insights for more personalized treatments that allow the improvement of cancer treatment. For all these reasons, characterization of Mps1 mutations in tumor samples is a valuable and important study to perform.

Depletion of the N-terminus or TPR domain was already shown to abrogate the SAC resulting in mitotic slippage once cells are exposed to spindle poisons[96]. The first part of my work had as main goal to determine if the two mutations identified in the N-terminus of Mps1 affect Mps1 activity and result in the impairment of the normal function of the SAC. The hypothesis is that if Mps1 function is altered by these two mutations, genetic instability driven by SAC inactivation could contribute to tumorigenesis in the tumor samples where the mutations were identified. Here I show that the expression of two mutant variants of Mps1 in the presence of the endogenous protein does not affect normal Mps1 localization and activity at the kinetochores during mitosis. This might be because these residues are not important for the control of Mps1 during mitosis and so, alterations in these would not cause any

deregulation of the normal mitosis. It can also be postulated that any phenotype associated with mutations is not observed because the presence of a WT copy of Mps1 rescues it, which means that these mutations are not dominant-negative. According to these results, it seems that these mutations do not have a role in promoting malignancy in the tumor where they were found since there was only one mutated allele of Mps1. However, it is relevant to determine whether any phenotype is present when these mutations are found in the cells homozygously. Genetic instability is one of the hallmarks of cancer[56] and, if advantageous for the cell, loss of specific genes can easily occur. In case any of the mutations provides an advantage for tumor growth, loss of the WT allele of Mps1 could happen. It would be interesting to determine if these mutations can promote tumor progression and understand the role of these residues in Mps1 activity.

Although not studied with this aim, three of the mutations selected from the COSMIC data base revealed to be kinase dead *in vitro*. G534E and E571K were present in tumor samples with a low incidence of mutations. As mentioned in the previous paragraph, a high percentage of tumor cells are genetically instable and exhibit a high mutational rate. This might result in aneuploidy tumors with more than 100 mutations, which makes the identification of mutations with a potential role in tumorigenesis very complicated. However, some tumor samples such as the ones where G534E and E571K mutations were identified, present a low number of mutations, which might be an indicator that alterations in these few residues play an important role in tumorigenesis. Based on my results, these two residues seem to be essential for the catalytic activity of Mps1 and the hypothesis that alterations in these could have promoted genetic instability in the tumor cells seems interesting for future investigations.

In the second part of my report, the effect of specific Mps1 mutations in light of inhibitor resistance was explored. Efficacy of small-molecule inhibitors that target Mps1 has been already studied in rodent xenograft models and proven to have high anti-tumor activity, verified by the increase in the overall survival of the mice[65,71]. Small molecule inhibitors against Aurora B, PLK and B-Raf were also described to have promising results for cancer therapy. However, a main drawback with these specific inhibitors is the development of resistance in cancer cells after some time of treatment[74,75,113]. So far, resistance against Mps1 inhibitors has not been reported which makes it essential to further investigate this topic. In my results, at least one mutation identified in tumor samples (C604F) provides resistance to one Mps1

inhibitor named Cpd-5. Although the C604F mutation confers resistance to Cpd-5, sensitivity to the other Mps1 inhibitor reversine is fully restored. C604F expression results even in a hypersensitivity of cells to reversine which means that the effect observed in the kinase activity of the mutated Mps1 in the presence of the inhibitor is likely due to alterations in the structure of its kinase domain as a consequence of the mutated residue. Alterations in residues in or close to the ATP-binding pocket can confer some structural changes which can affect the inhibitor binding to the kinase. Since Cpd-5 and reversine have different structures, the binding of these to the ATP-binding pocket of Mps1 is specific for each inhibitor. While the binding of the first inhibitor can be prevented by the change of the 604 residue from a cysteine to a phenylalanine, this mutation can allow stronger binding of reversine, explaining the observed results. Another way for the mutations to promote resistance to Mps1 inhibitors is to enhance its kinase activity. However, this hypothesis does not seem to be true because of the results in the kinase *in vitro* assays which do not show any increase in kinase activity of the C604F mutation. Another factor that seems to go against this is the different phenotypes showed by the cells in the presence of the two different inhibitors.

# Conclusions and future directions

Mps1 inhibition showed promising anti-tumor activity in preclinical studies. Although overexpression of Mps1 seems to have a protective role against excessive levels of aneuploidy in tumor cells allowing tumor growth and promoting tumor aggressiveness, so far none of the identified mutations in this kinase seems to play any role in tumorigenesis.

On the other hand mutations that confer resistance to Mps1 inhibitors can occur in tumor samples. This means that if Mps1 inhibitors will be used in the clinic, Mps1 mutation status in the tumor should be assessed before administration of the drug. This way, patients that are resistant to the Mps1 inhibitor will not benefit from it, and are not eligible for this treatment. However, another thing to be taking into consideration is that as observed in the results and discussed above, the same mutation can have opposite responses to different inhibitors. This means that although not eligible for the treatment with one specific inhibitor, this patient may be suitable for the treatment with another inhibitor approved in the clinic. For this reason, it is essential to characterize more mutations that can have similar effects in order to improve personalized medicine and increase treatment efficiency. It also seems of major importance to understand how these mutations promote resistance or sensitivity to certain inhibitors in order to design new inhibitors able to target resistant mutations.

In conclusion, screening for mutations and trying to understand their role in the tumor is essential not only for basic knowledge but also for drug development. Knowledge about the mutations allows a more rational therapy for the patients with personalized being the word of order.

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