SAPIENZA
Università di Roma
Facoltà di Scienze Matematiche Fisiche e Naturali

DOTTORATO DI RICERCA
IN GENETICA E BIOLOGIA MOLECOLARE

XXV Ciclo
(A.A. 2011/2012)

Novel mitotic roles of Aurora-A and implications for tumorigenesis and anti-cancer strategies

Italia Anna Asteriti

Docente guida
Dr. Giulia Guarguaglini

Tutore
Dr.Silvia Soddu

Coordinatore
Prof. Irene Bozzoni

Cover image: merged immunofluorescence image of a metaphase U2OS osteosarcoma human cell stained for Aurora-A (red) and TPX2 (green). Chromosomes are in blue.

Alla mia famiglia

ACKNOWLEDGMENTS

My PhD project was carried out at the Institute of Molecular Biology and Pathology of the National Research Council of Italy, c/o Sapienza University of Rome, under the supervision of Dr. Giulia Guarguaglini.

I want to thank especially Giulia, who has guided me with her sweetness and professionalism and has made me grown professionally. Giulia has been following me since the First Level degree; I'll always carry with me the precious advices she gave me in hard times and in the future I'll try to improve on the basis of her teachings.

I am very grateful to Dr. Patrizia Lavia, who has been a reference point for me for her experience and enthusiasm for science and has always been ready to help and support me. The exchanges of ideas during our meetings have often been the starting point for reflections and gave impetus to my work.

Thanks to my tutor, Dr. Silvia Soddu, for her availability at any time, and for her suggestions that helped me to improve my work.

Thanks to past and present members of Dr. Lavia's lab, especially Maria D.L., Maria G., Willeke and Erica for their collaborative efforts and for a stimulating and pleasant environment. Thanks to Emanuele for the animated discussions during the lab meetings.

Thanks to the Nikon team for the assistance, especially to Dr. Cirigliano for continuously collaborating and helping with microscopy instrumentations and softwares.

Thanks also to Eurobioimaging and to the members of the Microscopy Facility at EMBL of Heidelberg, in particular to Prof. Pepperkok, Dr. Neumann and Dr. Tischer, who welcomed me into the Facility and gave me the opportunity to approach to new techniques and increase my knowledge and skills. Thanks to SIBBM and EMBO for supporting the visit.

Regione Calabria and Fondazione Monte dei Paschi di Siena supported me during the PhD.

INDEX

- LIST OF ABBREVIATIONS AND ACRONYMS.................p. 8

- SUMMARY...p. 10

- INTRODUCTION:
1. Mitotic spindle assembly in mammalian cells……………..p. 13
1.1. Microtubule-associated forces and motor proteins...…..p. 15
1.2. Non-motor proteins associated to the mitotic spindle......p. 19
2. Aneuploidy and cancer………………………………......p. 20
3. The Aurora kinase family of mitotic regulators……….......p. 23
3.1. The Aurora-A kinase……………………...…...…......p. 25
3.2. TPX2: the major Aurora-A regulator…..……...……... p. 28
3.3. Aurora-A and cancer………………..…...……………..p. 31
4. Aurora-A as a novel target in anti-cancer terapy
4.1. Traditional anti-mitotic agents in anti-cancer therapy....p. 33
4.2. Aurora-A inhibitors and novel strategies in tumor treatments..p. 34

- AIM…………..…………………...………………………....p. 37

- RESULTS:
1. Aurora-A controls spindle pole integrity by regulating microtubule-associated forces…………………..…………p. 39
1.1. Aurora-A inactivation yields hyperstabilisation of mitotic spindle microtubules…………………..…………………..….p. 39
1.2. Microtubules hyperstabilisation in Aurora-Ai mitoses: the contribution of ch-TOG. ………………………...……..…..p. 41
1.3. Eg5 is required for spindle pole fragmentation induced by Aurora-A inactivation…………………………………….....p. 44
1.4. Interfering with kinetochore-microtubule attachments prevents spindle pole fragmentation induced by Aurora-A inactivation…………………………………...………………p. 47

2. Effects of a small molecule inhibitor of Aurora-A activity at the single cell level in human cells…………………………..……p. 50
2.1. MLN8237 inhibition of Aurora kinases in pre-synchronised cultures……………………………………………………..…p. 50
2.2. Delayed mitotic entry and prolonged mitosis in cells treated with MLN8237………………………………………....…..p. 54
2.3. Multiple spindle defects and mitotic outcome after MLN8237 treatment……………………….……………..………….........p. 59
3. Aurora-A/TPX2 as an oncogenic holoenzyme…………….…p. 62
3.1. TPX2 is overexpressed in many tumor types……......... p. 64
3.2. Aurora-A and TPX2 coexpression in tumors…...……... p. 67

- DISCUSSION……………………...…………………..........p. 70

- MATERIALS AND METHODS…………………………....p. 80

- REFERENCES……...……………………………………....p. 85

- LIST OF ALL PUBLICATIONS...p. 109

LIST OF ABBREVIATIONS AND ACRONYMS

APC/C: Anaphase Promoting Complex/Cyclosome
Arpc1b: actin related protein 2/3 complex, subunit 1B
BRCA1: Breast Cancer Associated gene 1
BTAK: Breast Tumor Activated Kinase
BUB1: Budding Uninhibited by Benzimidazoles 1
BUB3: Budding Uninhibited by Benzimidazoles 3
BUBR1: BUB1B: budding uninhibited by benzimidazoles 1 homolog beta
CDC25: Cell Division Cycle 25
CDK1: Cyclin Dependent Kinase 1
CENP-E: centromere associated protein E
Chfr: Checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase
ch-TOG: colonic and hepatic Tumor Over-expressed Gene
CIN: Chromosomal Instability
CPC: Chromosomal Passenger Complex
Eg5: kinesin family member 11
Gadd45a: Growth Arrest and DNA-Damage-inducible, Alpha
Hec1: Highly Expressed in Cancer protein 1
HEF1: Human Enhancer of Filamentation 1
IF: immunofluorescence
KT: kinetochore
LATS2: Large Tumor Suppressor 2
MAD1: Mitotic Arrest Deficient 1
MAD2: Mitotic Arrest Deficient 2
MAPs: Microtubule Associated Proteins
MCAK: Mitotic Centromere Associated Kinesin
MON: monastrol
MT: microtubule
NDEL1: Nuclear Distribution protein nudE-Like 1
NLS: Nuclear localisation Signal
NOC: Nocodazole
Nuf2: NDC80 kinetochore complex component

NuMA: Nuclear Mitotic Apparatus protein
PAK1: p21 Protein (Cdc42/Rac)-Activated Kinase 1
PCM: pericentriolar material
PLK1: Polo Like Kinase 1
PM: prometaphase
PM/M: prometaphase/metaphase
PP1: Protein Phosphatase 1
PP2A: Protein Phosphatase 2 A
RNAi: RNA interference
SAC: Spindle Assembly Checkpoint
siRNA: small interfering RNA
Spc24: NDC80 kinetochore complex component
Spc25: NDC80 kinetochore complex component
TACC3: Transforming Acidic Coiled-Coil containing protein 3
TPX2: Targeting Protein for Xklp2
WB: Western Immunoblotting

SUMMARY

Faithful segregation of the replicated genome in mitosis is orchestrated by the mitotic spindle: mis-assembly or mis-function of the spindle can yield aneuploidy and cell transformation. Mitotic regulators are often abnormally expressed in cancer cells and are considered attractive targets in anti-cancer therapy. Specific inhibitors are being developed, some of which are under clinical evaluation. Aurora-A, a mitotic centrosomal kinase regulating several aspects of spindle assembly, is overexpressed in many tumor types.

In our laboratory we had previously investigated the role of Aurora-A in spindle pole formation, finding that Aurora-A inactivation by RNA interference (RNAi) induces the appearance of multipolar spindles, characterised by fragmentation of centrosomes. We also found that Aurora-A regulates the localisation of ch-TOG (colonic and hepatic tumor over-expressed gene) and MCAK (mitotic centromere associated kinesin) [respectively, a microtubule (MT) stabilizer and a microtubule-depolymerising kinesin] at spindle poles: in Aurora-A depleted cells we observed an abnormal accumulation of ch-TOG, and the decrease of MCAK, at spindle poles (De Luca et al., 2008).

To follow up my interest in the mechanisms of centrosome control by Aurora-A I decided to take a PhD project with the aim to investigate the underlying molecular mechanisms, and their potential relevance in tumorigenesis and cancer therapy.

I have shown a correlation between mitotic spindle pole fragmentation induced by Aurora-A inactivation and hyperstabilisation of spindle MTs. Co-silencing of ch-TOG prevents spindle pole fragmentation caused by inactivation of Aurora-A alone and concomitantly reduces the hyperstabilisation of MTs. Furthermore, inhibition of the Eg5 kinesin using monastrol (MON) (which decreases pole-directed spindle forces), or RNAi against the kinetochore (KT) protein Nuf2 (which destabilises MT-KT attachments), also prevent pole fragmentation

in Aurora-A-inactivated mitoses. In this part of my project, therefore, I have shown that MT-generated forces are unbalanced in Aurora-A-inactivated cells and exert an abnormal pressure towards spindle poles, ultimately causing centrosome fragmentation (Asteriti et al., 2011). These results identify a novel function of Aurora-A but also raise concern about the proposed use of Aurora-A inhibitors in anti-cancer therapies.

I have therefore undertaken a comparison between Aurora-A inactivation by RNAi and by the specific Aurora-A inhibitor MLN8237 in human U2OS osteosarcoma cells. I used both high-resolution and quantitative microscopy with fixed samples and medium- and high-throughput time-lapse microscopy to study the effects of MLN8237 treatment at the single cell level. Results confirm and extend previous observations on Aurora-A inactivation: MLN8237 treatment impairs mitotic entry, induces prometaphase (PM) delay and formation of spindles disorganised or with additional poles, in a dose-dependent manner. In addition, I identified novel phenotypes, suggestive of poorly explored Aurora-A roles: cells without MTs that display only little asters or spots of tubulin, and cells that divide in a mis-oriented manner; at high concentrations of MLN8237, I observed the absence of chromosome segregation and the re-adhesion into one single cell. I never observed death from mitosis and analysis of interphase cells originating from MLN8237-treated mitoses reveals DNA abnormalities with dose-dependent severity, and hence aneuploidy induction. It has recently emerged that the extent of aneuploidy can determine subtle genetic instability (tumorigenic) or massive cell death: further studies will therefore be needed to predict the cellular contexts where Aurora-A inhibition in cancer therapy may prove successful.

In parallel work, I have studied Aurora-A in complex with its major regulator TPX2 (Targeting Protein for Xklp2). I have contributed to characterise a novel mechanism by which TPX2 regulates Aurora-A protein stability by protecting Aurora-A from protesome-dependent degradation (Giubettini et al., 2011). Based

on these observations I have carried out literature and data-mining searches to assess the oncogenic potential of TPX2 or of the Aurora-A/TPX2 complex. I have found that TPX2 is overexpressed in many tumors and, moreover, it is frequently co-overexpressed with Aurora-A. I therefore propose that the association between Aurora-A and TPX2 generates a functional complex with oncogenic properties and that some roles that are conventionally attributed to Aurora-A in cell transformation and tumorigenesis are in fact a consequence of the oncogenic activation of this complex (Asteriti et al., 2010).

INTRODUCTION

1. Mitotic spindle assembly in mammalian cells

Mitosis is the phase of the cell cycle in which replicated molecules of DNA, compacted into chromosomes, are distributed in the two daughter cells. This distribution must be equal and precise: the consequences of one single error can generate aneuploid cells, with unbalanced chromosome sets, that can in turn become the first step of cancerous transformation.

A bipolar spindle is assembled during mitosis in order to ensure balanced chromosome segregation (Figure 1A). The mitotic spindle is a dynamic, complex macromolecular machine made of MTs, polymers of alpha and beta tubulin. In animal cells spindle MTs are mainly nucleated from centrosomes (Doxsey, 2001), also called MT organising centers, but evidence have emerged that mitotic spindles assemble through a cooperative mechanism, in which chromatin-mediated pathways of MT nucleation and capture also play major roles (O'Connell and Khodjakov, 2007).

Centrosomes are the only non-membranous organelles in vertebrate cells and are composed of two centrioles and surrounding pericentriolar material (PCM), which is the site of MT nucleation (Doxsey, 2001; Mardin and Schiebel, 2012). At mitosis, duplicated centrosomes organise the spindle poles; MTs between spindle poles are organised into an antiparallel array, and MTs directed towards the cortex form two radial asters (Figure 1B). MTs undergo periods of polymerisation and depolymerisation and interconvert randomly between these states, a property known as "dynamic instability". MTs are intrinsically "polarised" and are organised so that most of the less dynamic and anchored MT "minus" ends are near the poles of the spindle, whereas the more dynamic and quickly growing "plus" ends extend towards the spindle equator or the cell cortex (Figure 1) (Walczak et al., 2010).

A.

B.

modified from Gadde and Heald, 2004

Figure 1. Spindle assembly and dynamics. A) Mitotic stages. At prophase, chromosomes condensate, centrosomes separate and the nuclear envelope breaks down. Chromosomes, captured by MTs in PM, align at metaphase, move apart in anaphase and segregate in the re-forming daughter nuclei at telophase. **B) The mitotic spindle.** MT minus ends are at spindle poles, while plus ends extend towards the cell cortex (astral MTs) or KTs; KT fibers contact chromosomes, while interpolar MTs form an overlapping antiparallel array, ensuring spindle stability.

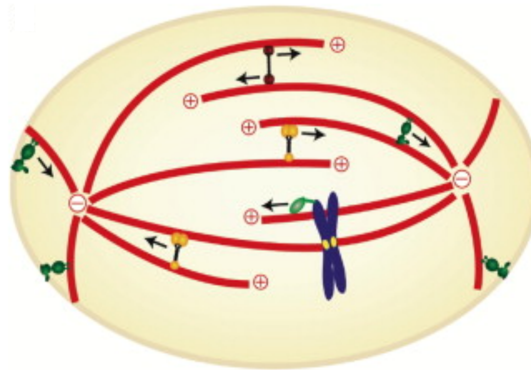
The establishment of a proper spindle geometry and bipolar organisation requires a regulated balance between opposite directional forces exerted along growing MTs (Kapoor et al., 2000; Sharp et al., 2000; Tanembaum et al., 2008). Given that improperly assembled or multipolar spindles can drive chromosome mis-segregation, there is a growing research focus on the mechanisms through which this balance is generated.

1.1. Microtubule-associated forces and motor proteins

Mechanical forces are involved in the separation of the two duplicated centrosomes and in the organisation of a bipolar spindle. There are two types of active spindle-associated forces created by polymerisation dynamics and motor proteins and two types of passive spindle forces generated by elasticity and friction of the MTs (Dumont and Mitchison, 2009).

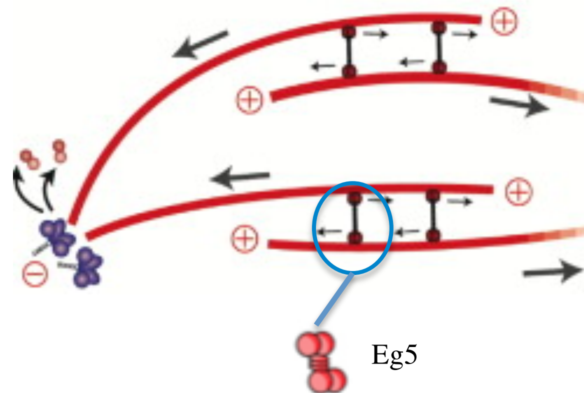
The most important forces involved in spindle assembly are the active forces. One of the generators of active forces is represented by the dynamic properties of the MTs. The concept that spindle fibers could push by polymerising and pull by depolimerysing was proposed in yeast in 1967 (Inouè and Sato, 1967). These forces are not dominant in larger mammalian cells, where the key generators of forces on MTs during spindle formation are motor proteins. Motors convert energy released by ATP hydrolysis into movement along MTs. They move towards the fast growing plus ends (plus end-directed kinesins) or towards the relatively stable minus ends (minus-end directed dyneins) (Figure 2), directing the transport of associated cargoes. Motors can also act as dynamic cross-linkers, moving MTs relative to each other: this generates a “sliding-filament” mechanism that importantly contributes to the organisation of MTs during spindle assembly. It is driven predominantly by members of the plus-end tetrameric kinesin-5 family of MT motors: among these, Eg5 has a prominent role (Gatlin and Bloom, 2010) (Figure 3). The tetrameric structure of Eg5 makes it a particularly attractive candidate for binding antiparallel MTs and sliding them apart; its activity in sliding anti-

parallel MTs is linked to both the establishment of bipolarity and the regulation of steady-state spindle length. The selective inhibition or pertubation of Eg5 significantly affects spindle assembly: it prevents centrosome separation, resulting in the formation of monopolar spindles (Blangy et al., 1995; Kapoor et al., 2000).



modified from Gadde and Heald, 2004

Figure 2. Motor proteins organise the mitotic spindle. Plus- (red) and minus (yellow) -end directed motors increase or decrease the overlap of antiparallel MTs, thus contributing to spindle pole separation. Dynein (dark green) at the cortex can pull on astral MTs or focus MT minus-ends into poles. Chromokinesins (light green) mediate chromosome attachment and movement along MTs.

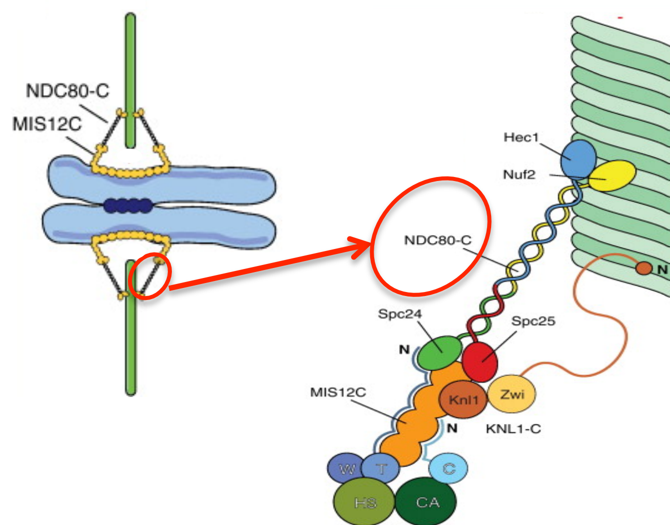


modified from Gadde and Heald, 2004

Figure 3. The Eg5 motor protein. Eg5, in red, has a tetrameric structure that allows it to bind antiparallel MTs and slide them apart. By this sliding mechanism, Eg5 contributes to establish the spindle bipolarity and regulates steady-state spindle length.

Some spindle forces are also associated to chromosomes: chromokinesins, which bind both MTs and chromosome arms, and KT motors (see Figure 2), such as dynein and CENP-E (centromere associated protein E), importantly contribute to move chromosomes along the spindle and therefore to chromosome congression. By regulating MT plus-end dynamics they determine a push of MT minus-ends toward the poles and in this way they also contribute to the spindle organisation (rev. in Mazmudar and Misteli, 2005; Mao et al., 2010).
Recently, the role in spindle formation of KT pushing forces, already observed early in 1996 by Waters and collegues (Waters et al., 1996), is being clarified. KTs are macromolecular complexes

assembled at the centromeric regions of chromosomes, and are responsible for the interaction with MTs; the NDC80 complex (formed by four proteins: Ndc80, Nuf2, Spc24 and Spc25), harbouring both MT- and KT-binding sites, plays a key role in establishing KT/MT binding (Figure 4; Santaguida and Musacchio, 2009).



modified from DeLuca and Musacchio, 2012

Figure 4. The KT/MT attachment. Left: during mitosis, KTs (yellow) assemble on centromeric chromatin and create a contact with MTs (green). KT components required for MT binding are represented on the right. The NDC80 complex is a tetramer. The Spc24 and Spc25 subunits interact with the MIS12-C, that is a component of the KT, whereas the Hec1 (Highly Expressed in Cancer protein 1)/Ndc80 and Nuf2 subunits face the MT.

Recent studies demonstrated that KTs promote centrosome separation after nuclear envelope breakdown by exerting a pushing force through the KT fibers (K-fibers), bundles of MTs connected to the KTs. This force is generated by the incorporation of tubulin subunits at the plus ends of kinetochore MTs and becomes essential when other factors (for example Aurora-A) that are involved in centrosome separation are altered (Toso et al., 2009; Mchedlishvili et al., 2012).

1.2. Non-motor proteins associated to the mitotic spindle

The organisation of MTs into the highly ordered bipolar mitotic spindle depends on the activities of motor proteins (see previous paragraph) but also on the action of non-motor MT-associated proteins, that are mostly involved in structural organisation (Manning and Compton, 2008).
Among non-motor proteins, microtubule associated proteins (MAPs) have a central role in the formation and maintenance of the mitotic spindle, through different mechanisms including the nucleation and organisation of MTs; they also act by mechanically crosslinking MTs, thus providing structural support for the mitotic spindle (Manning and Compton, 2008). NuMA (nuclear mitotic apparatus protein) and TPX2 are two non-motor proteins that bind MTs and are able to crosslink them at the level of centrosomes, thus ensuring focusing and integrity of the spindle poles. Perturbation of NuMA or TPX2 function results in poorly focused spindle poles leading to multipolar spindles (Compton and Luo, 1995; Garret et al., 2002; Haren et al., 2009). The MT crosslinking activity of NuMA is dominant to that of TPX2, but TPX2 becomes essential for spindle pole organisation under conditions where NuMA function is perturbed (Manning and Compton, 2007).
Many non-motor proteins also control the density of MTs by regulating MT nucleation and/or stability; an example of this class of factors is ch-TOG that promotes the assembly of MTs by ushering tubulin subunits into the growing MT end and

antagonising the depolymerising activity of the kinesin-13 protein MCAK (Cassimeris and Morabito, 2004).

2. Aneuploidy and cancer

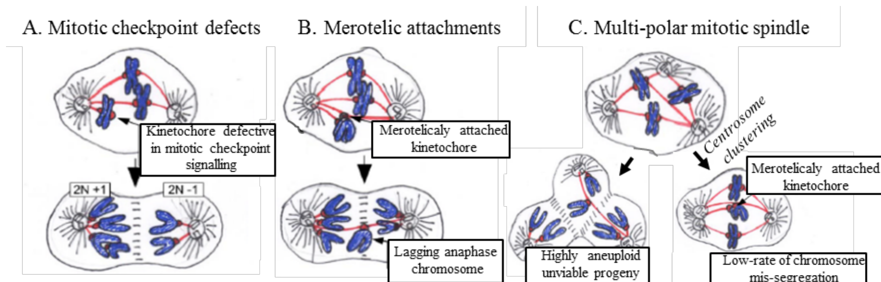
Millions of cells divide every minute in humans and the fidelity of chromosome segregation is critical for maintaining the diploid status along cell generations. The mitotic checkpoint, also known as the spindle assembly checkpoint (SAC), has evolved as a surveillance mechanism. It is a complex signalling network that consists of several proteins, including MAD1 (mitotic arrest deficient 1), MAD2 (mitotic arrest deficient 2), BUB1 (budding uninhibited by benzimidazoles 1), BUBR1 (budding uninhibited by benzimidazoles 1) homolog beta, BUB3 (budding uninhibited by benzimidazoles 3) and CENP-E. The SAC delays the irreversible transition from metaphase to anaphase until the KTs of each replicated sister chromatid have correctly attached to spindle MTs. Impairment of checkpoint signalling determines premature mitotic exit before complete KT attachment and thus significantly increases the probability of chromosome mis-segregation (Figure 5A).

Mitotic errors can generate aneuploidy also with an intact mitotic checkpoint signalling (Figure 5B and C; Weaver and Cleveland, 2006; Holland and Cleveland, 2012):

-mis-segregation events occur when the KT of a single replicated chromosome becomes attached to MTs from both spindle poles, a situation known as merothelic attachment. Since the chromosome is attached and under tension, no mitotic checkpoint signal is generated. This attachment can produce a lagging chromosome that remains in the spindle midzone, becoming excluded from both daughter cells during cytokinesis, or segregating into one daughter cell, where it may form a micronucleus;

- segregation errors resulting from multipolar spindles also cannot be prevented by the mitotic checkpoint, because the chromosomes make productive attachments;

- when three or more daughter cells are created by multiple cytokinetic furrows, aneuploid progeny is produced.

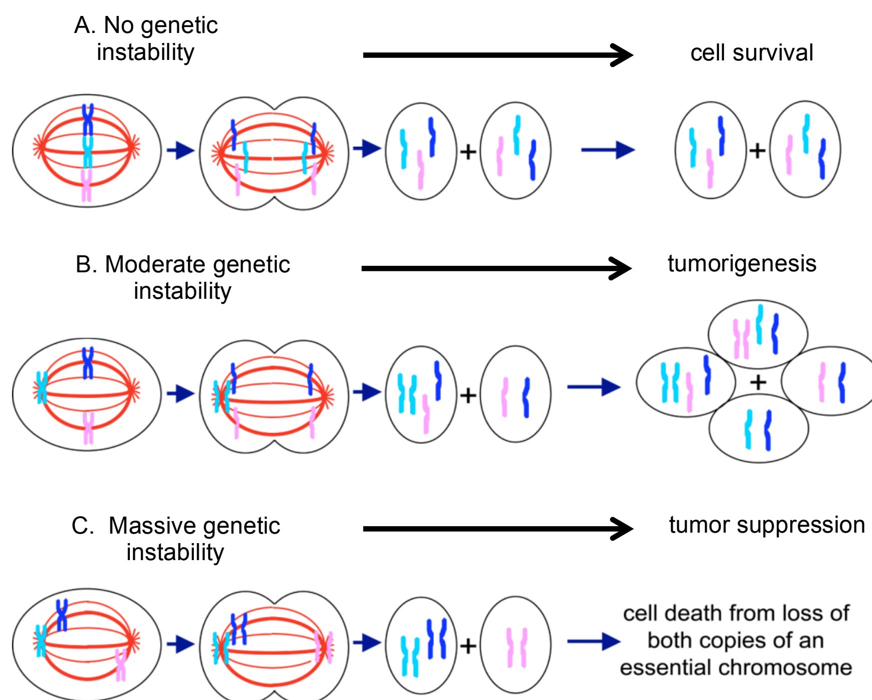


modified from Holland and Cleveland, 2009

Figure 5. Different pathways inducing aneuploidy. A) When the mitotic checkpoint is defective cells enter anaphase in the presence of unattached chromosomes, yielding unbalanced chromosome segregation. B) One KT may attach to MTs from both poles (merotelic): resulting lagging chromatids may mis-segregate or remain excluded from both daughter cells. C) Multipolar spindles can give rise to highly aneuploid and often unviable daughter cells. Centrosome clustering yields a bipolar division even in the presence of multiple centrosomes, but produces merotelic attachments, as in B.

Over a century ago the german zoologist Boveri described the effect of aneuploidy on organism development, proposing that aneuploidy has a detrimental effect on cell and organism physiology (Boveri 1902 and 1914). Today we know that aneuploidy is a common feature of human cancer, present in ~90% of solid human tumors and >50% of haematopoietic cancers (Mitelman et al., 2012). In addition to simple aneuploidy caused by rare mis-segregation of one or a few chromosomes, many tumor cells acquire chromosomal instability (CIN), a condition characterised by a high percentage of chromosome gain and loss during divisions (Holland and Cleveland, 2012). However, whether

aneuploidy is a cause or a consequence of malignant transformation remains hotly debated. The involvement of aneuploidy in driving or inhibiting tumorigenesis is related to its extent: moderate aneuploidy may be compatible with cell survival, with loss or gain of one or a few chromosomes promoting cell growth and tumorigenesis; on the contrary, high levels of aneuploidy determine cell death and tumor suppression (Figure 6; discussed in Weaver and Cleveland, 2007).



(modified from Weaver and Cleaveland, 2007)

Figure 6. Aneuploidy can drive or inhibit tumorigenesis. A) Normal cells maintain a diploid genome with intact growth regulatory pathways, consistent with continued cell survival. B) Moderate aneuploidy can promote cell growth and tumorigenesis. C) High levels of genetic instability, or massive mis-segregation (10-15 chromosomes per division), result in cell death and tumor suppression.

3. The Aurora kinase family of mitotic regulators

Phosphorylation-based signalling networks play a key role in orchestrating spindle assembly and ensuring the correct sequence of mitotic events. Among mitotic kinases, Aurora-A plays a prominent role.

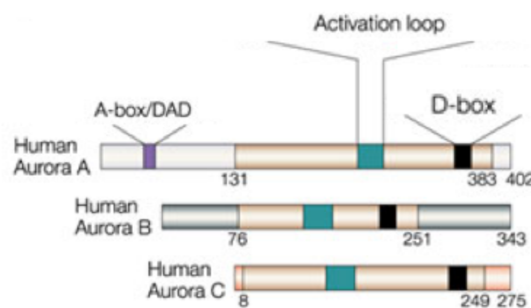
Aurora-A belongs to the family of Aurora kinases: these serine/threonine kinases are key regulators of cell division, involved in many aspects of mitosis, including centrosome maturation, mitotic spindle formation, chromosome alignment, mitotic checkpoint activation: overall the are very important factors for accurate and equal segregation of chromosomes in the two daughter cells (Carvajal et al., 2006).

The founding member of the family was identified in a screen for mitotic mutants that failed chromosome segregation in *Saccharomyces cerevisiae* (Chan et al., 1993), and independently in *Xenopus laevis* (Paris and Philippe, 1990) and *Drosophila melanogaster* (Glover et al., 1995*)*: *Xenopus* Eg2 gene encodes for a kinase that was defined as a regulator of the G2/meiosisI transition in *Xenopus* oocytes and of mitotic spindle function in *Xenopus* eggs extracts (Andresson and Ruderman, 1998; Roghi et al., 1998). Severe mutations at the Aurora locus in *Drosophila* determined pupal lethality and a mitotic arrest characterised by the presence of monopolar spindles (Glover et al., 1995). Together, these early studies indicated a requirement for the kinase in mitotic progression.

In mammalian cells three Aurora kinases have been described: Aurora-A, Aurora-B and Aurora-C (Figure 7). The genes have been mapped on chromosomes 20q13.2, 17p13.1 and 10q13 respectively; proteins have an aminoacid sequence length ranging from 309 to 403. The catalytic domain is in the C-terminal region; Aurora-A and B have more than 70% of identity in their C-terminal catalytic domain (Kollareddy et al., 2008; Nikonova et al., 2012).

Human Aurora-A and Aurora-B were first isolated in PCR screens to identify protein kinases that are overexpressed in breast and

colon carcinoma, respectively (Sen et al., 1997; Bischoff et al., 1998; Bischoff and Plowman, 1999). It was evident that transcripts of both kinases were abundant (i) in tissues with a high mitotic index such as thymus primary epithelial cells and fetal liver, and (ii) in human tumor cell lines compared with matched normal controls. These profiles suggested that both kinases play a role in cell proliferation.



modified from Carmena and Earnshaw, 2003

Figure 7. Aurora kinases. Human Aurora-A, -B and -C kinases are schematically represented. The A-box/D-box-activating domain (DAD) at the N-terminus, the D-box at the C-terminus and the position of the activation loop (green) within the catalytic domain (light brown) are shown.

Although the three members of the Aurora family have a high level of similarity, they display distinct localization patterns and functions.

- Aurora-C: Aurora-C was isolated in 1999 (Kimura et al., 1999) but it has been less characterised compared to the other family members, mainly due to its specificity of expression and function: Aurora-C expression is specifically limited to testis although overexpression in cancer cell lines is observed (Kimura et al., 1999). Indeed a role of the kinase during spermatogenesis has been shown (Dieterich et al., 2007; Kimmins et al., 2007). Studies in HeLa cells showed that Aurora-C protein levels are up-regulated in

mitosis and that the kinase localises at centrosomes from anaphase to cytokinesis suggesting roles in centrosome function at late mitotic stages (Kimura et al., 1999).
-Aurora-B is the "equatorial Aurora". It localises at chromosomes during prophase and, in a dynamic manner, at the inner centromere region between sister chromatids during PM and metaphase; the kinase localises at central spindle MTs during anaphase, when its mobility is drastically reduced (Terada et al., 1998). This dynamic localisation gained to Aurora-B, with its regulatory and targeting components INCENP (inner centromere protein), survivin and borealin, the name of "Chromosomal passenger complex" (CPC). The CPC has pleiotropic mitotic roles (for a recent and extensive review see Carmena et al., 2012), briefly summarised below. It is essential for accurate chromosome segregation during cell division: via Aurora-B activity it has a key role in regulating KT/MT attachments, and in particular in the "correction" function, by destabilising unproperly connected KT/MT. Major Aurora-B targets relevant to this function are KT components responsible for MT binding and regulators of MT dynamics, such as the kinesin 13 family member MCAK. Aurora-B is also involved in control of the SAC, by recruiting the SAC components and thus contributing to delay cell cycle progression until all KTs attain bipolar MT attachments. Finally, the CPC is involved in cytokinesis, by regulating central spindle formation, furrow ingression and abscission.
In the next paragraph I will describe in more detail the regulation and roles of the Aurora-A member, which has been the object of this thesis.

3.1. The Aurora-A kinase.

Among the three mammalian Aurora kinases, Aurora-A has attracted significant attention, based on the evidence that it is overexpressed in many tumor types (Nikonova et al., 2012).
Aurora-A associates with duplicated centrosomes during late S and G2 phases: immunofluorescence (IF) staining is detected at the

PCM of centrosomes and increases as the cell cycle progresses. As the nuclear envelope breaks down and the bipolar spindle starts to form, the Aurora-A signal increases at centrosomes and it remains associated with the mitotic poles, but also to the regions of MTs that are proximal to the centrosomes, until telophase. Then, low levels of the kinase can be detected at MTs of the central spindle (Figure 8) (Kimura et al., 1997). The bulk of the kinase is instead degraded at ana-telophase through an Anaphase Promoting Complex/Cyclosome (APC/C) and Cdh1 ubiquitin-dependent mechanism (Crane et al., 2004; Floyd et al., 2008).

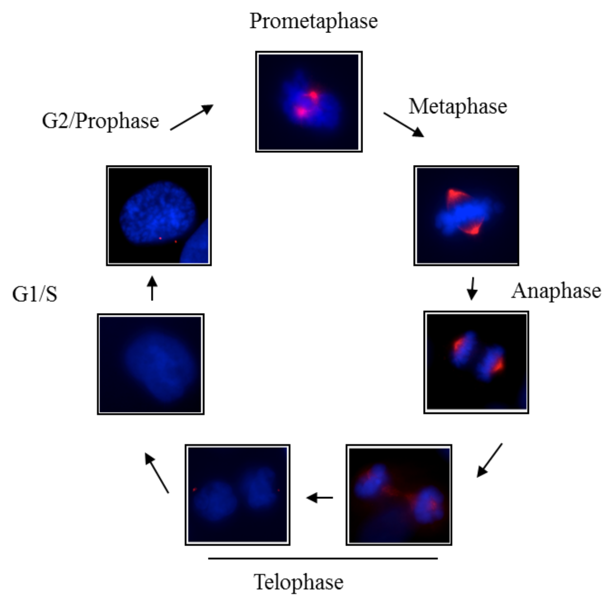


Figure 8. Aurora-A in the cell cycle. Aurora-A (in red) strongly accumulates at centrosomes and is activated between the G2 and M phases. Aurora-A localises at the poles and at the spindle polar MTs. In late mitosis a fraction of Aurora-A is at the spindle midzone, while most of the protein is degraded before cytokinesis with only low levels detectable in G1 cells. DNA is in blue.

Aurora-A is a key regulator of mitosis and plays roles in different steps of mitotic progression.

- **Centrosome maturation and separation**: the high frequency of monopolar spindles initially observed in *Drosophila* Aurora-A mutants indicated a role of Aurora-A in centrosome separation, that was later on confirmed in *C. elegance* and mammalian cells (Hannak et al., 2001; Giet et al., 2002; Marumoto et al., 2005). After they duplicate and separate, centrosomes recruit a number of proteins in a process known as maturation, which renders them capable of driving the abundant nucleation of MTs typical of mitosis. In late G2, Aurora-A contributes to centrosome maturation, by recruiting the PCM components γ-tubulin, centrosomine, LATS2 (Large Tumor Suppressor 2), TACC3 (transforming acidic coiled-coil containing protein 3), NDEL1 (nuclear distribution protein nude-like 1). Aurora-A phosphorylation of the LATS2 kinase promotes its recruitment to centrosomes, which is in turn required for the recruitment of γ-tubulin (Toji et al., 2004). NDEL1, an evolutionarily conserved coiled-coil-containing protein, and TACC3, member of the transforming acidic coiled-coil family, form a complex with Aurora-A at centrosomes, that has a role in mitotic spindle formation, interacting with the ch-TOG/XMAP215 family (see paragraph 1.3) to stabilise MTs at centrosomes (Mori et al., 2007).
- **Mitotic entry**: mitotic entry is promoted by the activation of CDK1 (Cyclin Dependent Kinase 1)/cyclin B1, which occurs initially at centrosomes. Aurora-A contributes to CDK1 activation by (i) phosphorylating the CDK1-activating phosphatases CDC25 (Cell Division Cycle 25) B and C; (ii) activating PLK1 (polo like kinase 1), that induces the degradation of the CDK1-inhibitory kinase WEE1 (van Vugt et al., 2004). Aurora-A also controls the G2/M transition via interaction with BRCA1 (breast cancer associated gene1) mediating its localisation to the centrosome. The BRCA1 ubiquitin ligase interacts during mitosis with γ-tubulin and other centrosomal components to regulate G2/M progression and spindle assembly (Ouchi et al., 2004).

- **Construction and control of a bipolar spindle**: mutation or depletion of Aurora-A in different model systems causes formation of spindles with abnormally organised poles, including multipolar spindles, and mitoses with weak, sparse or short astral MTs (Hannak et al., 2001; Marumoto et al., 2003; De Luca et al., 2006). Aurora-A role in spindle pole formation may depend in part on its action in complex with the TACC proteins, as described above, in MT stabilisation at centrosomes, thus opposing the activity of the MT-destabilising kinesin MCAK; Aurora-A can also directly phosphorylate and regulate MCAK (De Luca et al., 2008; Zhang et al., 2008). Still, much has yet to be learned on how Aurora-A controls spindle pole organisation.

3.2. TPX2: the major Aurora-A regulator

Several factors modulate the activity, localisation and stability of Aurora-A in human cells (rev. in Carmena et al., 2009). The centrosomal pool of Aurora-A is activated by specific factors, i.e. Ajuba, HEF1 (Human Enhancer of Filamentation 1), PAK1 [p21 Protein (Cdc42/Rac)-Activated Kinase 1] and Arpc1b (actin related protein 2/3 complex, subunit 1B); phosphatase PP2A (protein phosphatase 2A) shows mutual interdependence with Aurora-A for their co-localisation at the centrosome (which is also regulated by Plk1) and modulates Aurora-A stability through control of phosphorylation of the key residue Ser51. Other interactors regulate the stability [Cdh1, Chfr (checkpoint with forkhead and ring finger domains, E3 ubiquitin protein ligase), AURKAIP1] or activity [PP1 (protein phosphatase 1), PP1 Inhibitor-2, Gadd45a (growth arrest and DNA-damage-inducible, alpha)] of Aurora-A, but it is not clear whether they regulate specifically localised fractions or not. The spindle-associated fraction of Aurora-A is largely regulated by the MT-binding protein TPX2.

TPX2 was originally identified in *Xenopus* as a spindle pole organizer involved in the recruitment of the kinesin Xklp2 (Xenopus kinesin-like protein2) to MTs (Wittman et al., 1998) and subsequently in mammalian cells (Gruss et al., 2001); *C. Elegans*

and *Drosophila* TPX2-like proteins have been also described (Ozlu et al, 2005; Goshima, 2011). TPX2 is a100 kDa protein: the Xklp2 MT-targeting domain, as well as an Eg5 binding region, are in the C-terminus (Brunet et al., 2004; Ma et al., 2010); MT binding domains are distributed along the protein sequence (Brunet et al., 2004); the central region contains a NLS (nuclear localisation signal; Schatz et al., 2003); a KEN Box, important for TPX2 degradation (Stewart and Fang, 2005), and the region required for Aurora-A binding, are located in the N-terminus (Figure 9A).
TPX2 levels are cell cycle-regulated, with a peak in mitosis when it associates with spindle MTs, whereas during S and G2, it is diffusely distributed in the nucleus (Figure 9B; Gruss et al., 2002).
TPX2 is required for stability of the spindle poles (Wittman et al., 2000; Gruss et al., 2002; see paragraph 1.3), but also for activation, localisation and stability of Aurora-A (Figure 9C). In interphase, TPX2 is inhibited by the interaction with importin-alpha and -beta (cargo receptors for nuclear transport). After nuclear envelope breakdown, importins are displaced by the small GTPase Ran (a regulator of nuclear transport) and TPX2 is free to bind Aurora-A, which gets activated (Gruss and Vernos, 2004). The mechanism through which TPX2 activates Aurora-A has been characterised: TPX2 binding induces a conformational change in the kinase, modifying the position of a key residue (Thr288) in the Aurora-A three-dimensional structure and interfering with the inhibitory activity of PP1 (Protein phosphatase 1) upon Aurora-A (Figure 9C). Thus, TPX2 binding stabilises Thr288-phosphorylated Aurora-A, which represents the active form of the kinase (Bayliss et al., 2003; Eyers et al., 2003; Tsai et al., 2003). TPX2 is also required to target Aurora-A to the spindle (Kufer et al., 2002; De Luca et al., 2006). We recently reported that TPX2 controls Aurora-A stability in human cells: Aurora-A protein levels decrease in PMs lacking TPX2, in a proteasome- and Cdh1-dependent manner, and the interaction between Aurora-A and TPX2 is required for protecting Aurora-A from degradation (Giubettini et al., 2011).

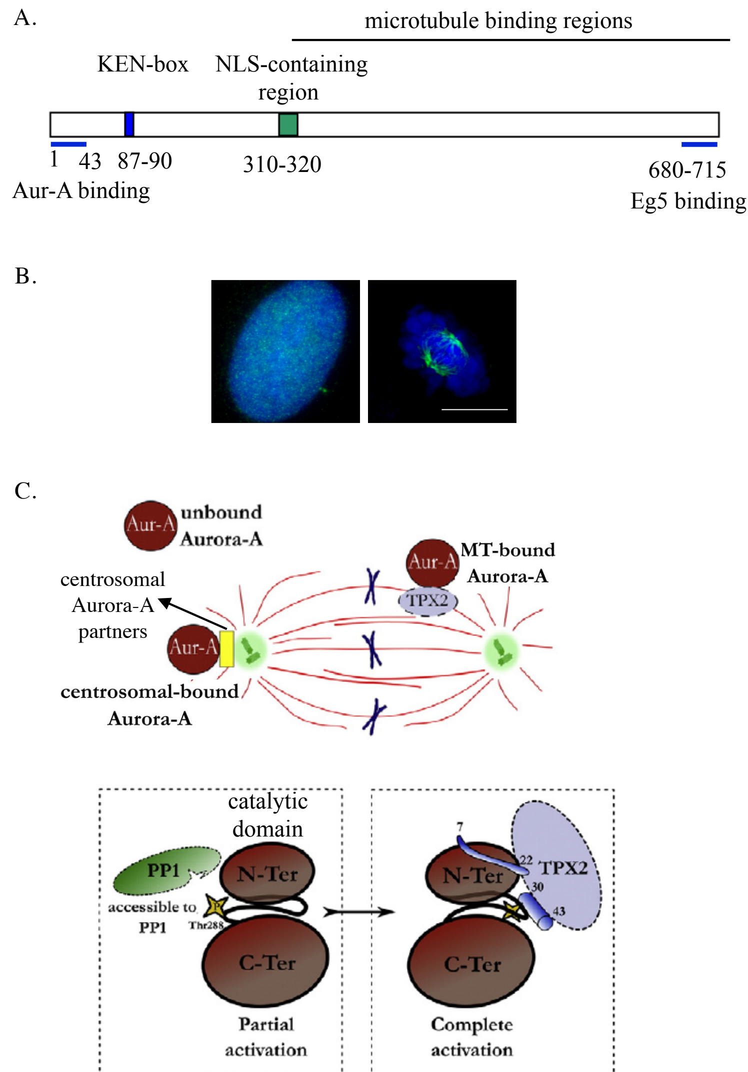


Figure 9. The Aurora-A activator TPX2. A) Representation of TPX2 functional domains. B) TPX2 (green) in interphase and mitotic cells. IF images show merge with DNA (in blue). Bar: 10 μm. C) Schematisation of TPX2 regulation on Aurora-A. High: TPX2-mediated Aurora-A localisation to spindle MTs. Low: TPX2 interferes with the inhibitory activity of protein phosphatase PP1 upon Aurora-A.

3.3. Aurora-A and cancer

The *Aurora-A* gene locus is on chromosome 20q13.2, within a region that is frequently amplified in many tumor types.

Indeed, the Aurora-A gene was first named as BTAK (Breast Tumor Activated Kinase), because its mRNA was found to be overexpressed in breast tumors (Sen et al., 1997); next, other independent studies identified the up-regulation of Aurora-A as a common feature of multiple solid cancers, including colorectal, breast, ovarian, gastric, prostate, neuroblastoma and cervical, in both primary tumor tissues and cell lines (Bischoff et al., 1998; Zhou et al., 1998; Tanaka et al., 1999; Pihan et al., 2001; Sakakura et al., 2001; Gritsko et al., 2003; rev. in Vader and Lens, 2008).

Aurora-A protein levels can be altered as a consequence of gene amplification, changes of gene expression or protein stabilisation. Aurora-A transcription is regulated by different pathways (STA5a, estrogen/GATA3, HIF1) (Lee et al., 2008; Jiang et al., 2010; Xu et al., 2010) that are frequently elevated in cancer. In addition many Aurora-A interactors, that normally protect Aurora-A from protein degradation (IQ-GAP1, NEDD9/HEF1) are up-regulated in cancer (Pugacheva et al., 2005; Tikhmyanova et al., 2010). On the contrary, many factors involved in the physiological Aurora-A degradation during the cell cycle, such as Chfr, are downregulated in many cancers (Sanbhnani et al., 2012).

Aurora-A overexpression may contribute to cell transformation through different routes. For instance, unlike in normal cells, in some tumor cells Aurora-A is detected diffusely in the cytoplasm (Gritsko et al., 2003), giving rise to aberrant phosphorylation of cytoplasmic proteins. In addition, Aurora-A phosphorylates the p53 tumor suppressor protein, thus inactivating it (Liu et al., 2004); a large number of studies have reported complex direct and indirect relationships between Aurora-A, p53 and carcinogenesis (Chiang, 2012). Aurora-A also regulates factors involved in cancer-related pathways, e.g. BRCA1 (Ouchi et al., 2004; Sankaran et al., 2007), N-Myc (Otto et al., 2009), NF-kB and IkBα (Briassouli et al., 2007).

Given that Aurora-A is a key regulator of mitosis, cell division defects may also account for the tumorigenic potential of its overexpression. Following elevated Aurora-A expression in cell cultures, mitosis is characterised by the presence of multiple centrosomes, overriding of the spindle checkpoint and failure of cytokinesis, developing polyploidy and progressive chromosomal instability (Zhou et al., 1998; Meraldi et al., 2002; Anand et al., 2003).
Despite these evidences, a direct correlation between a sufficient and direct role of Aurora-A overexpression and cell transformation remains controversial. Transformation assays with Aurora-A in different cell lines as well as mouse models designed to assess the direct contribution of Aurora-A to tumorigenesis yielded controversial results and only one set of in vivo models to date clearly supports the notion that Aurora-A overexpression can drive tumor formation in mice (Bischoff et al., 1998; Zhou et al., 1998; Anand et al., 2003; Zhang et al., 2004; Fukuda et al., 2005; Wang et al., 2006; Warner et al., 2008; Li et al., 2009) Thus, evidences supporting the definition of Aurora-A as an oncogene are contradictory; the specific molecular background and/or gene profile of the experimental system under examination could modulate the ultimate effect of Aurora-A overexpression.
Studies of the best characterised allelic variants of Aurora-A (Phe31Ile and Val57Ile) have confirmed the idea that concomitant alterations in other factors modulate the oncogenic capacity of Aurora-A: although there is an overall consensus that these alleles confer increased risk of breast and colon cancer (Gautschi et al., 2008) and promote genetic instability and malignant transformation (Hienonen et al., 2006; Torchia et al., 2009), their effects are influenced by the tissue type, patient age and tumor stage (Ju et al., 2006; Chen et al., 2007; Gu et al., 2007). Interestingly, neither of these mutations affects the Aurora-A catalytic domain directly, but both can potentially affect the interaction of Aurora-A with its regulators, partners or substrates. Thus, studies on Aurora-A overexpression alone indicate a clear

potential, and yet a broad variability, as an oncogenic agent, leaving much room for partners to modulate the full oncogenic potential of Aurora-A.

4. Aurora-A as a novel target in anti-cancer terapy

4.1. Traditional anti-mitotic agents in anti-cancer therapy

Many drugs that are used in anti-cancer therapy are "anti-proliferative" drugs. They act by perturbing the proliferative cycle of tumor cells: tipically, DNA-damaging agents and inhibitors of cyclin-dependent kinases arrest cell cycle progression at different stages. Anti-mitotic drugs selectively perturb progression through mitosis (Janssen and Medema, 2011). Currently, the anti-mitotic drugs that have been approved for clinical use target MTs. The two conventional MT-targeting drugs are taxanes and Vinca alkaloids, both of which have proven effective in the treatment of different types of cancer: Taxol is frequently used in the treatment of breast and ovarian cancers, while Vinca alkaloids are used in combination with other drugs for the treatment of hematological cancers. The Vinca alkaloids at low concentrations inhibit MT dynamics, and at high concentrations induce MT depolymerisation. Taxol stabilises MTs, by altering depolymerisation dynamics and thus preventing normal spindle assembly (Gascoigne and Taylor, 2009; Janssen and Medema, 2011). As a consequence of the absence of MT dynamics, tension is not produced across sister chromatids (Kelling et al., 2003) and correct chromosome bi-orientation is not achieved. This situation determines the chronic activation of the spindle assembly checkpoint that leads to mitotic arrest (Musacchio and Salmon, 2007).

Although these anti-mitotic drugs have been used with clinical success, they also display negative effects: i) some patients respond well to the treatment, but others rapidly acquire resistance to the treatment; ii) toxicity is a major limitation: anti-MT drugs affect the division of normal cells too, as well as MT-related functions in non-dividing cells, like peripheral neurons, with consequent neurotoxicity and permanent damage (Rowinsky et al.,

1993; Gascoigne and Taylor, 2009). To minimise neurological effects, new agents are being developed that interfere with mitotic progression without altering MT dynamics in non-dividing cells. This new class of potential therapeutic agents include compounds that inhibit the activity of mitotic kinases.

4.2. Aurora-A inhibitors and novel strategies in tumor treatments.

Many mitotic inhibitors that are under clinical evaluation for anti-cancer therapy are directed against the three members of the Aurora family. These kinases are proposed as potential targets given their clear association with tumorigenesis (Lapenna et al., 2009). The proof of concept study to address the possibility to use Aurora-A as a therapeutic target was performed by Hata and collaborators in 2005 (Hata et al., 2005). Using RNAi to knockdown Aurora-A expression in cultured pancreatic cancer cells, in which the kinase is overexpressed, they observed that in vitro cell growth as well as tumorigenicity in mice were strongly suppressed. This pioneering study was the starting point for a large number of studies characterising Aurora-A as a target in cancer therapy. Supporting this idea, early *in vitro* experiments with the first Aurora kinase chemical inhibitors demonstrated that they affect viability only on the dividing cells while the non dividing cells remained viable (Ditchfield et al., 2003). The anti-tumor activities of these inhibitors have also been evaluated in xenografts and a few preclinical studies have progressed to the early stages of clinical trials (Katayama and Sen, 2010). All the Aurora kinase inhibitors currently in development for clinical use are small molecules designed to bind to the ATP-binding pocket that is highly conserved among kinases, and for this reason many of these compounds are active, to some extent, against multiple structurally related kinases (ABL, SRC, JAK2, VEGFR2, FLT3 and FGFR1); this observation influenced their clinical development towards certain tumor types with relevance to their off-target activity (Nikonova et al., 2012). Even more, it has been very difficult to

find compounds which are able to discern between Aurora-A and Aurora-B. Given the different roles of these kinases in cell division, discussion is ongoing on the therapeutic value of targeting either of them specifically, or both. Initially, Aurora-A was considered as a therapeutically more valuable target, given its clear involvement in tumorigenesis and the protective role of Aurora-B against aneuploidy generation; still, no clinical data have shown specific inhibitors to be more or less therapeutically valuable than multi- or pan-Aurora inhibitors so far (rev. in Green et al., 2011).
Aurora-A kinase inhibitors activity has been studied in a broad range of hematological and solid tumors with good results in disease stabilisation in a minority of patients (Table 1).

Inhibitor	**Tumor types**	**Current status**
ENMD-2076	myeloma, breast cancer, leukemia, colorectal cancer, ovarian cancer	phase I/II
MLN-8237	lymphoma, leukemias, myeloma, breast cancer, prostate cancer	phase I/II
MK-5108	breast cancer, cervix cancer, colorectal cancer, ovarian cancer, pancreas neoplas	phase I
XL-228	lung, leukemia	phase I
KW-2449	leukemia	phase I

Table 1. Aurora-A inhibitors. The most specific Aurora-A inhibitors under evaluation in cinical trials are listed. The central column shows the tumor types in which the effects of the inhibitor are being evaluated. In the right column the current clinical evaluation status is represented.

MLN8054 was the first specific Aurora-A inhibitor studied, which is approximately 40 fold more active towards Aurora-A compared to Aurora-B. It has completed three phase I studies with promising

early indication of anti-tumor activity. However somnolence was observed associated with the treatment, that was unexpected and remains poorly understood. This Aurora-A inhibitor, that is structurally similar to benzodiazepines, may act on GABA alpha1 receptor and consequent benzodiazepine-like central nervous system effects may be involved (Macarulla et al., 2010; Chakravarty et al., 2011; Dees et al., 2011). At present, trials involving MLN8054 have been arrested.

Numerous clinical trials are ongoing using a second generation compound, MLN8237, the most specific inhibitor of Aurora-A to have arrived to phase II. It shares structural homology with MLN8054, but has 4-fold greater inhibitory potency for Aurora-A kinase and a decreased tendency to cause somnolence. MLN8237 is being tested both as a single agent and in combination with other anti-cancer compounds (reviewed in Green et al., 2011). Abundant preclinical data had provided support for combining Aurora-A inhibitors with a wide variety of existing agents targeting the Aurora-A partners and effectors; indeed, phase I trials with MLN8237 have shown that its combination with Taxol is well tolerated and that anti-tumor activity is observed in ovarian and breast cancer (Kelly et al., 2012). Modest clinical effects have been observed in the solid tumors studies with MLN8237 alone; the reasons are unclear, particularly with respect to results obtained in cell lines and xenografts. One possibility for this discrepancy might be the existence of redundant signalling pathways in tumor cells allowing for bypass signalling (i.e., drug-selected activation of Aurora-A partners or effectors) to drive cellular proliferation despite the blockade of Aurora-A. Active research is ongoing to understand the cellular basis of Aurora-A inhibitors mode of action and thus improve on their use in the clinics.

AIM

The aim of my PhD project was to investigate the mitotic roles of the Aurora-A kinase in human cells and the effects of its deregulation on cell division.

In previous works (De Luca et al., 2006 and 2008) we had observed that depletion of Aurora-A by RNAi in human U2OS osteosarcoma cells determines the formation of spindles with multiple poles. We had also observed that under these conditions regulators of MT dynamics are mis-localised: ch-TOG, a MT-stabilising factor, increases at spindle poles; its major antagonist, MCAK, a kinesin with MT-destabilising activity, decreases at poles. Spindle pole fragmentation is abolished when ch-TOG and Aurora-A are co-inactivated, indicating a correlation between the increase of ch-TOG at spindle poles and the formation of extrapoles. Building on these observations, in the first part of my PhD project, I investigated the molecular mechanisms through which Aurora-A exerts this newly identified function in control of spindle pole integrity.

The observation that Aurora-A inhibition induces supernumerary spindle poles also raises concern about Aurora-A inhibition-based therapeutic protocols that are under evaluation in anti-cancer therapy: indeed, unforeseen dangerous effects may occur, due to aneuploidy induction. An in-depth understanding of the effects of Aurora-A inhibition may help to interpret the results from ongoing clinical trials and to improve on the use of Aurora-A inhibition as an anti-cancer strategy. During my PhD I therefore evaluated the cellular effects of the Aurora-A inhibitor MLN8237, which is being tested in clinical trials and has been mainly characterised using whole cell population approaches. Cellular responses to anti-mitotic drugs are heterogeneous, and single cell analysis can reveal stochastic events and cell-to-cell variability that cannot be appreciated otherwise, providing a better understanding that can importantly contribute to predict whether these compounds will have clinical efficacy. I therefore focused on single-cell

microscopy-based approaches, with the final aim of identifying relevant parameters determining either effective cell death, or, on the contrary, the generation of aneuploid cells, and rationalise the therapeutic use of Aurora-A inhibitors.

Data from the literature suggest that the specific molecular background modulates the ultimate effect of Aurora-A overexpression in tumor formation: expression levels of activators or substrates may therefore be relevant. Stemming from the observation that the MT-associated protein TPX2, besides its known roles in regulating the activity and the localisation of Aurora-A, also modulates its protein stability (Giubettini et al., 2011), I hypothesised that its deregulation may contribute to elicit the full oncogenic potential of Aurora-A in transformed cells. In a data mining effort, I searched for evidence of TPX2 overexpression, and Aurora-A/TPX2 co-overexpression, and suggest that the complex, as a unit, may contribute to tumor formation.

RESULTS

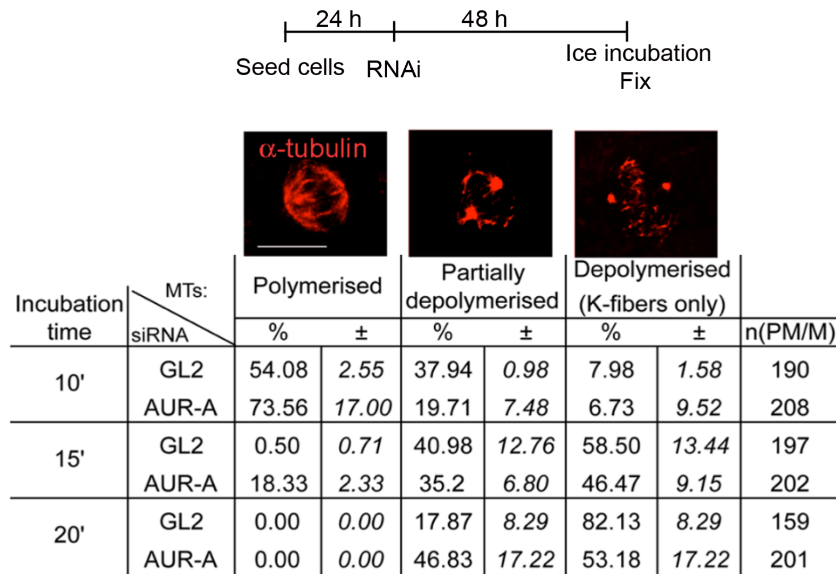
1. Aurora-A controls spindle pole integrity by regulating microtubule-associated forces

As recalled in the Aim, spindle poles are fragmented in mitotic cells where Aurora-A is inactivated by RNAi; concomitant accumulation of the MT-stabilising factor ch-TOG is observed (De Luca et al., 2008). The first aim of my PhD project was to elucidate the mechanisms by which Aurora-A regulates the organisation of spindle poles.

1.1. Aurora-A inactivation yields hyperstabilisation of mitotic spindle microtubules.

I first asked whether the altered levels of ch-TOG at spindle poles in Aurora-A-interfered (Aurora-Ai) mitoses modify the dynamic properties of spindle MTs. MTs are quickly depolymerised when incubated on ice. Assaying the sensitivity of MTs to depolymerisation at low temperatures, in the presence or absence of a specific protein, can be used to assess the "protective" effect conferred by the factor, or absence thereof. Thus, I used this assay to observe the effect of Aurora-A inactivation on MT dynamic properties. I performed a time-course analysis, incubating the cultures for 10, 15 and 20 minutes on ice, and observed the status of polymerisation of MTs in prometa- and metaphase (PM/M) cells (Figure 10). I classified the cells as: a) with a normal spindle ("polymerised"), b) with partially depolymerised MTs and c) with fully depolymerised MTs. In GL2-interfered (GL2i) cultures, interfered for 48 hours with neutral small interfering RNA (siRNA), cells with polymerised MTs quickly disappeared (less than 1% after 15 minutes of ice incubation). Cells with partially depolymerised spindles, displaying disorganised MTs with clear centrosomal origin, are present at 10 to 15 minutes of ice incubation; after 20 minutes on ice there is a decrease of this class of cells and the predominant status (over 80% of all PM/M) is represented by cells with fully depolymerised MTs in which

centrosomes are totally devoid of MTs and only the more stably KT- attached MTs (K-fibers) are evident (Figure 10).



Incubation time	MTs: / siRNA	Polymerised %	±	Partially depolymerised %	±	Depolymerised (K-fibers only) %	±	n(PM/M)
10'	GL2	54.08	*2.55*	37.94	*0.98*	7.98	*1.58*	190
	AUR-A	73.56	*17.00*	19.71	*7.48*	6.73	*9.52*	208
15'	GL2	0.50	*0.71*	40.98	*12.76*	58.50	*13.44*	197
	AUR-A	18.33	*2.33*	35.2	*6.80*	46.47	*9.15*	202
20'	GL2	0.00	*0.00*	17.87	*8.29*	82.13	*8.29*	159
	AUR-A	0.00	*0.00*	46.83	*17.22*	53.18	*17.22*	201

Figure 10. Aurora-A inactivation induces hyperstabilisation of spindle MTs. A schematisation of the protocol is shown. PM and M cells from control (GL2) and Aurora-Ai cultures incubated on ice for the indicated times were classified according to the status of MTs, exemplified in the IF panels. The rightmost column indicates the number (n) of scored PM/M cells in 2 independent experiments; mean values (%) and s.d. (italics) are indicated.

In Aurora-Ai PM/M the kinetics of depolymerisation is significantly slower compared to control cells. After 20 minutes of ice incubation the percentage of cells with fully depolymerised MTs is only about 50%, while the remaining 50% still display partially depolymerised MTs, a condition observed in <20% of mitoses in control cultures at this time point (Figure 10).

Thus, the ice-induced depolymerisation assay indicates a resistance of MTs to depolymerise, indicative of a MT hyperstabilisation, in cells depleted of Aurora-A.

1.2. Microtubules hyperstabilisation in Aurora-Ai mitoses: the contribution of ch-TOG.

In order to investigate whether ch-TOG, which accumulates at poles in Aurora-Ai mitoses (De Luca et al, 2008), is also involved in the observed hyperstabilisation of MTs, I performed a set of experiments in which I co-depleted ch-TOG and Aurora-A by RNAi. I then observed the status of polymerisation of MTs in the double-interfered cultures compared with control and with the single-interfered cultures (Aurora-Ai or ch-TOGi). I analysed PM/M cells after 15 minutes of ice incubation (Figure 11).

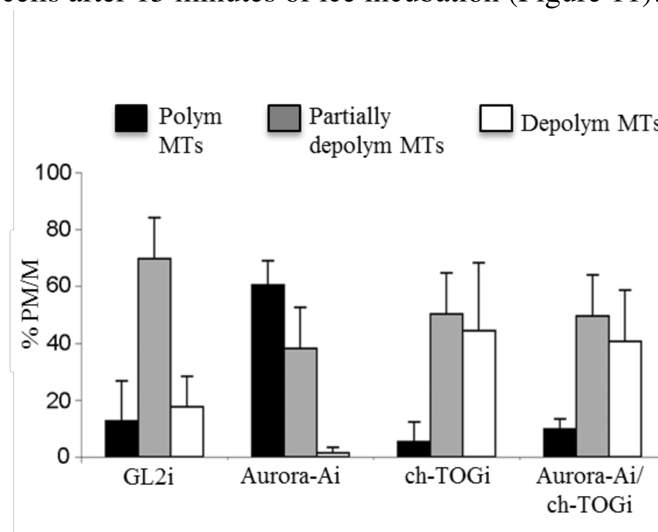


Figure 11. Aurora-A and ch-TOG modulate the stability of spindle MTs in opposite manners. Cells interfered (i) with the indicated siRNAs were incubated on ice for 15 minutes. Histograms represent the distribution of PM/M in the three MT categories identified in Figure 10 (at least 350 counted cells per condition, 2 experiments). Error bars denote s.d.

In ch-TOGi cultures, ice-induced MT-depolymerisation is faster than in control cultures; this is consistent with the loss of the MT-stabilising activity of ch-TOG. In cell cultures in which both Aurora-A and ch-TOG are inactivated, the MT hyperstabilisation caused by Aurora-A inactivation alone (see Figure 10) is reduced and sensitivity to ice-induced depolymerisation is restored.
This result suggests a role of ch-TOG in the altered stability of MTs in Aurora-A-depleted cells. We had previously shown that ch-TOG activity is required for spindle pole fragmentation in Aurora-Ai mitoses, and that ch-TOG co-depletion reduces the percentage of mitoses with spindle extrapoles in Aurora-Ai cultures (De Luca et al., 2008).
To test whether there is a correlation between the altered stability of MTs and the formation of mitotic spindle extrapoles, I examined how Aurora-Ai PM/M cells with extrapoles are distributed in the three classes of MT polymerisation (polymerised, partially depolymerised, depolymerised) after 15 minutes of ice incubation (Figure 12). Pole fragmentation was particularly represented (16%) and statistically significant compared to GL2i, among Aurora-Ai PM/M with ice-resistant MTs. By contrast, mitoses with partially or fully depolymerised MTs in Aurora-Ai cultures do not display a statistically significant occurence of pole fragmentation compared with controls. Mitoses where ch-TOG and Aurora-A were co-inactivated generally assemble a normal bipolar spindle; the rare cells that displayed spindles with extrapoles showed no specific association with the status of depolymerisation of MTs and were uniformly distributed among the different categories similarly to control cultures.
This shows that the absence of Aurora-A yields mitotic spindle pole fragmentation in those mitoses in which MTs are hyperstabilised.

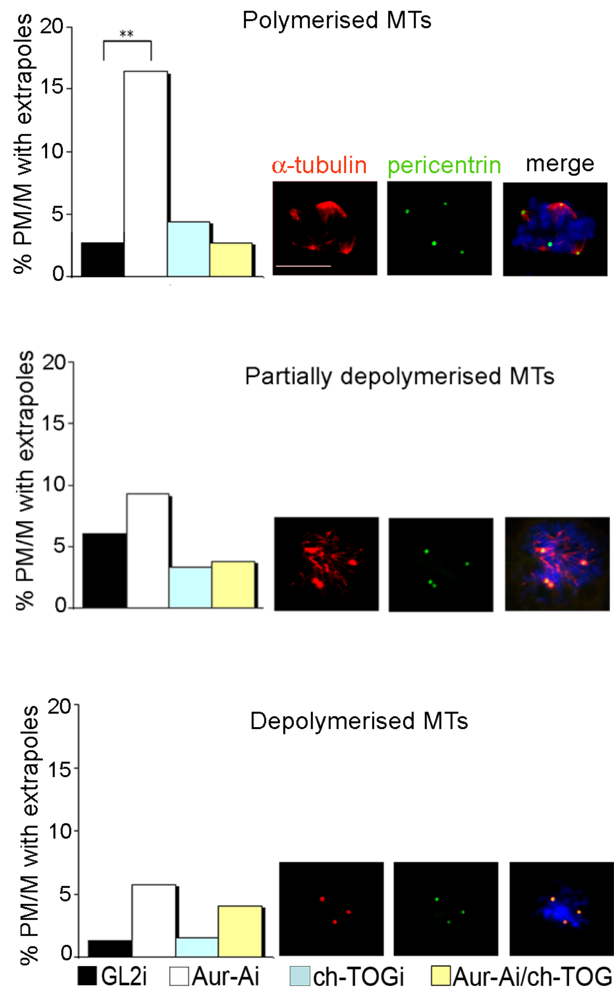


Figure 12. Correlation between PM/M cells with extrapoles and MT stability. Histograms represent the distribution of mitoses with fragmented spindle poles among the MT stability categories shown in Figure 10 (examples are shown in the IF panels). Around 700 PM/M were counted for each interference (2 experiments). Error bars represent s.d. **: $p < 0.001$, $\chi 2$ test. Scale bar: 10 μm.

1.3. Eg5 is required for spindle pole fragmentation induced by Aurora-A inactivation.

In previous work we observed that spindle pole fragmentation in Aurora-A-depleted mitoses occurs in a MT-dependent manner (De Luca et al., 2008); results presented in the previous paragraph demonstrate that spindle extrapoles manifest preferentially in PM/M cells harbouring hyperstable MTs. This evidence suggests that when Aurora-A is inactivated, spindle poles may be subjected to an excessive MT-originated pressure (see Introduction for a discussion of MT-associated forces), due to the altered stability of MTs, which is responsible for fragmentation of centrosomes. If this hypothesis is correct, inactivation of centrosome-directed forces, that contribute to generate pressure on centrosomes, should inhibit spindle pole disruption in Aurora-A-depleted cells (Figure 13).

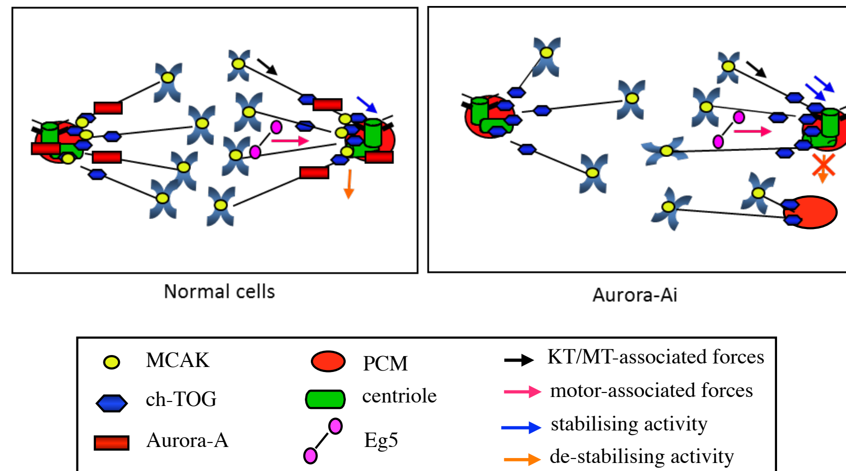


Figure 13. Hypothesised mechanism for spindle pole fragmentation in Aurora-Ai mitoses. MT-associated forces are normally balanced with respect to each other and with the action of MT stabilysing factors. In Aurora-Ai cells an imbalance in the forces directed towards centrosomes may determine an excessive pressure on poles and their fragmentation. Factors and forces investigated in the experiments described in this thesis are schematised.

The Eg5 kinesin is a motor protein that moves along antiparallel MTs in the plus-end-direction, thus generating a force directed towards centrosomes (Sharp et al., 2000; see Introduction). Eg5 activity is specifically inhibited by MON (Mayer et al., 1999; Kapoor et al., 2000); in order to asses whether Eg5-generated forces affect spindle pole fragmentation in a condition of Aurora-A depletion, I treated Aurora-Ai cultures with MON. Simultaneous staining of MTs (using an alpha-tubulin-directed antibody) and centrosomes (with a pericentrin-directed antibody) (Figure 14) showed that MON treatment of Aurora-Ai cells significantly reduces the fragmentation of centrosomes and the formation of spindle extrapoles compared with Aurora-Ai cells with active Eg5, with a parallel increase of mitoses with monopolar spindles, the typical phenotype observed following MON treatment (Kapoor et al., 2000).

To further confirm the role of Eg5-mediated forces in the formation of fragmented poles in Aurora-Ai mitoses, I took advantage of the reversibility of MON to restore Eg5 activity. I washed-out MON with the addition of MON-free medium and Eg5 activity was rapidly resumed, as monitored by the recovered ability of centrosomes to move apart; in parallel, spindle pole fragmentation rapidly returned to comparable levels to those seen in Aurora-Ai cultures (Figure 14). This result shows that, in Aurora-A-depleted cells, restoring Eg5 activity is sufficient to generate spindle pole fragmentation induced by inactivation of the kinase. This is consistent with the idea that MT-associated forces directed towards centrosomes are imbalanced in Aurora-Ai mitoses and underlights the key role of MT-associated forces in the spindle pole fragmentation event caused by Aurora-A inactivation.

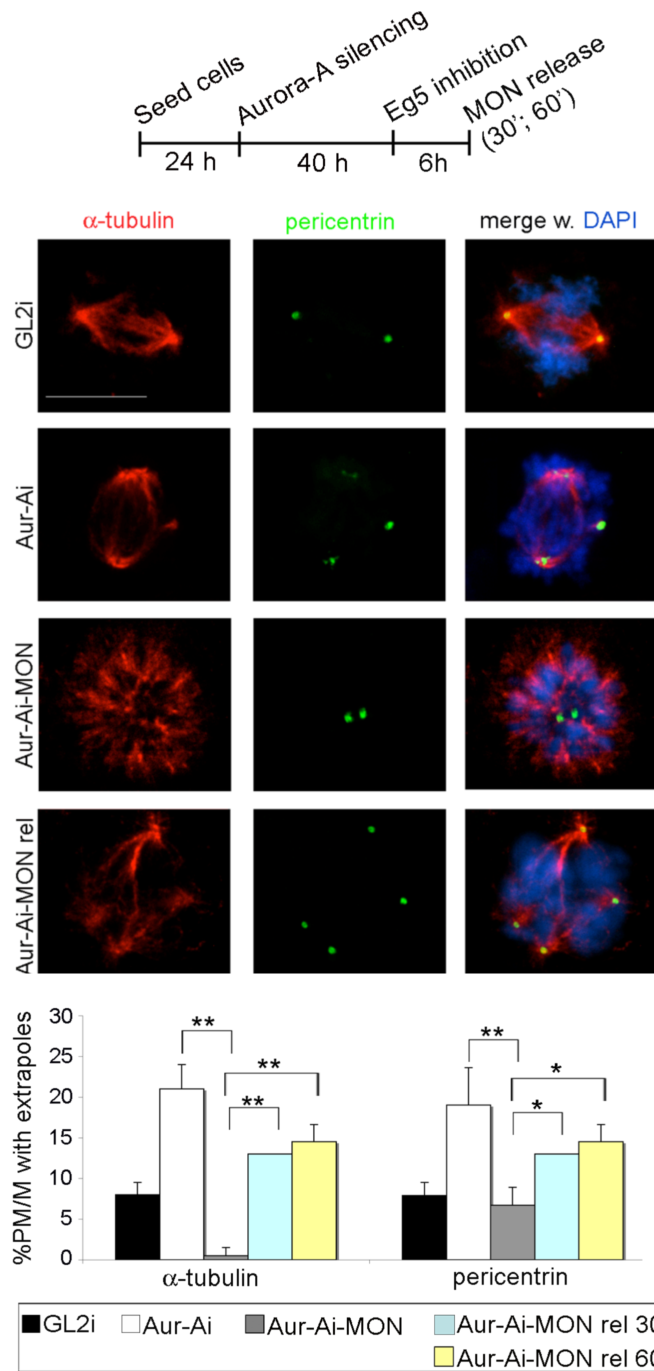
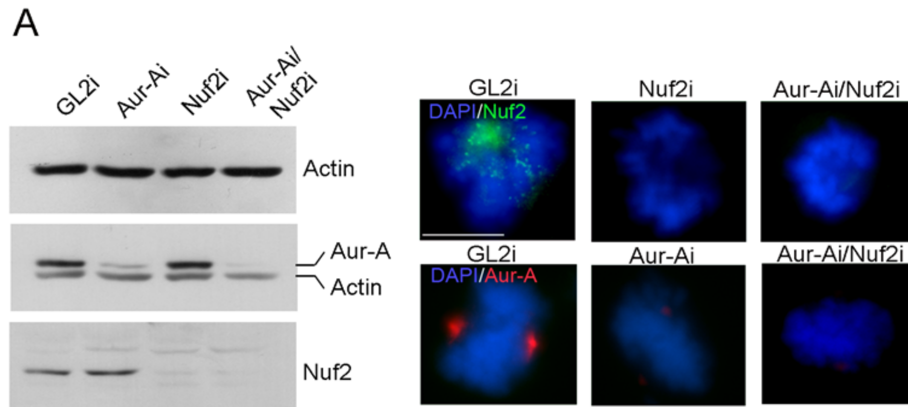

Seed cells
Aurora-A silencing
Eg5 inhibition
MON release
(30'; 60')
24 h
40 h
6h
α-tubulin
pericentrin
merge w. DAPI
GL2i
Aur-Ai
Aur-Ai-MON
Aur-Ai-MON rel
%PM/M with extrapoles
30
25
20
15
10
5
0
**
**
**
**
*
*
α-tubulin
pericentrin
GL2i
Aur-Ai
Aur-Ai-MON
Aur-Ai-MON rel 30'
Aur-Ai-MON rel 60'

Figure 14. Spindle pole fragmentation in Aurora-Ai mitoses depends on Eg5 activity. A schematisation of the protocol is shown (time intervals not represented to scale). IF panels show spindles displaying normal or fragmented poles in control and Aurora-Ai cells, respectively (first and second row); monopolar spindles in Aurora-Ai cells treated with MON (third row); spindles displaying pole fragmentation in Aurora-Ai cells after MON release (MON-rel; lower row). Histograms represent the percentage of PM/M displaying fragmented poles, as assessed by alpha-tubulin (left) and pericentrin (right) staining (200 to 400 counted cells per condition in 2-4 experiments; s.d are shown). *: $p < 0.01$, **: $p < 0.001$, $\chi 2$ test. Scale bar: 10 μm.

1.4. Interfering with kinetochore-microtubule attachments prevents spindle pole fragmentation induced by Aurora-A inactivation

In the experiments in which I inhibited Eg5 it was still possible that spindle formation was blocked at a stage that preceded the fragmentation of poles induced by Aurora-A inactivation. To distinguish between a specific temporal implication of Eg5 and a global alteration of spindle forces orchestration in Aurora-A-depleted cells, I used an independent approach to reduce MT-associated forces directed towards poles. During normal spindle assembly, K-fibers-generated forces contribute to centrosome separation during PM and need counteracting by motor-associated MT-focusing activities at poles (see paragraph 1.3 in Introduction): thus, destabilisation of KT-MTs attachments would reduce KT-generated forces directed towards centrosomes and hence the pressure applied on spindle poles. To test whether this condition restores spindle bipolarity in Aurora-Ai cells, I co-depleted by RNAi Aurora-A and the KT-protein Nuf2, a key factor involved in the formation of stable KT-MTs attachments (see paragraph 1.3 in Introduction; results are shown in Figure 15). The occurrence of PM/M with spindle extrapoles was significantly reduced in Nuf2i/Aurora-Ai cultures compared to those defective for Aurora-A alone. This evidence confirms that KT-MT attachments are

involved in the generation of mitotic spindle pole fragmentation in a condition of Aurora-A depletion.



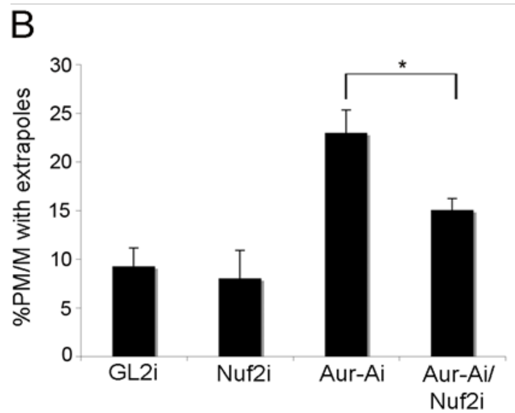


Figure 15. Spindle pole fragmentation in Aurora-Ai mitoses depends on active Nuf2. A. The efficiency of Aurora-A and Nuf2 depletion after RNAi was assessed by WB (left panels) and IF (right panels) analyses. B. Histograms represent the percentage of PM/M displaying fragmented spindle poles (alpha-tubulin staining) after transfection with the indicated siRNAs (at least 200 cells per condition, 2 experiments). Error bars denote s.d. *: $p < 0.01$, **: $p < 0.001$, $\chi 2$ test. Scale bar: 10 μm

It is known that Nuf2 interference yields the appearance of elongated mitotic spindles (DeLuca et al., 2002; Manning and Compton, 2007). These elongated figures were unaffected by the simultaneous inactivation of Aurora-A (Figure 16). Thus, the co-inactivation of Aurora-A and Nuf2 does not restore the overall spindle shape but specifically restores spindle bipolarity, by preventing spindle pole fragmentation.

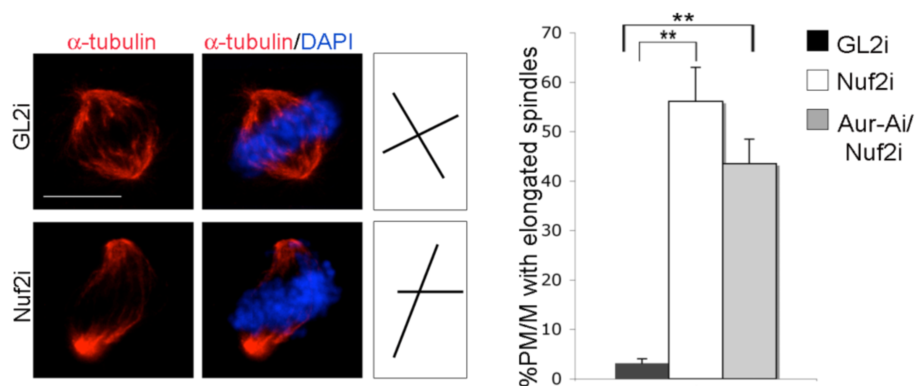


Figure 16. Elongated spindles induced by Nuf2 RNAi are not rescued in Aur-A/Nuf2 co-inactivated cells. IF images show spindles (alpha-tubulin) in control and Nuf2i mitoses. Spindle axes are schematised on the right: the pole-to-pole axis is longer in Nuf2i cells compared to controls. Histograms show the percentage of mitoses displaying such abnormal spindles under the distinct examined conditions.About 200 counted PM/M per condition in 2 experiments. Error bars denote s.d. *: $p < 0.01$, **: $p < 0.001$, $\chi 2$ test. Scale bar: 10μm.

In conclusion, results shown in this first part highlight a novel function of Aurora-A in orchestrating the balance between MT-associated forces that ensure the proper organisation of mitotic spindle poles.

2. Effects of a small molecule inhibitor of Aurora-A activity at the single cell level in human cells

Our RNAi data in human U2OS cells indicated that inhibition of Aurora-A induces the fragmentation of mitotic spindle poles as a consequence of the key role of the kinase in the control of the balance of MT-associated forces within the spindle. Mis-assembled spindles can determine mis-segregation of chromosomes in the two daughter cells and hence aneuploidy that, if moderate, can drive tumorigenesis (see Figure 6). This may be a critical issue in the proposed use of Aurora-A chemical inhibitors in cancer therapy (Green et al., 2011) and deserves better insight. Since the overall response of cultures to anti-mitotic compounds is in fact a “profile of cell fates”, single-cell analysis, e.g. live cell imaging, may add relevant information on cell-to-cell variability and stochastic events that would go unnoticed in whole cell population studies (Gascoigne and Taylor, 2009). I therefore decided to undertake single cell analyses in human cells using MLN8237, one of the most specific inhibitors of Aurora-A that are under evaluation in clinical trials.

2.1. MLN8237 inhibition of Aurora kinases in pre-synchronised cultures

Unlike Aurora-A inhibition by RNAi, chemical inhibition of Aurora-A affects the activity of the kinase, while leaving the total levels of the protein unaltered (Figure 17). I set up a protocol in which U2OS cells pre-synchronised at the G1/S transition by thymidine treatment were treated with MLN8237 after 6 hours from thymidine release, when cells were in late S-phase or early G2. Cells were then harvested after 4 hours in order to analyse mitotic cells (Figure 17).

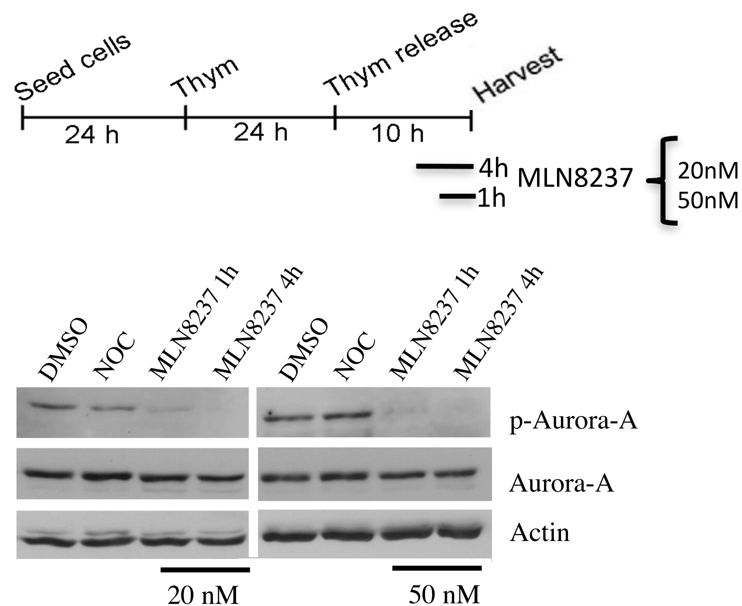


Figure 17. MLN8237 treatment drastically reduces Aurora-A activity. A. The experimental protocol is shown (time intervals not represented to scale). The efficiency of Aurora-A activity inhibition after MLN8237 treatment was assessed by WB analysis using phospho-Thr288-Aurora-A (p-Aurora-A); Aurora-A total levels were checked for control; actine was the loading control; extracts are obtained from mitotic cells harvested by shake off. One series of extracts was treated with nocodazole (NOC) for 4 hours before harvesting in order to enrich in PM figures.

To test the effectiveness of MLN8237 treatment, I first performed a Western immunoblotting (WB) analysis using two different concentrations (20 and 50 nM) of MLN8237, which had proven effective in previous publications (Zeng et al., 2010; Manfredi et

al., 2011). 4 hours after treatment with both doses of MLN8237 no phospho-Thr288 (active)-Aurora-A was detectable in mitotic cell extracts (Figure 17). Aurora-A protein levels were instead unchanged.
To obtain more detailed information, I measured Aurora-A activity at the single cell level using the anti-Aur-A-phospho-Thr288 IF staining in dose-response assays (from 5 nM to 250 nM). Treatments with 5 nM and 10 nM MLN8237 yields a significant inhibition of Aurora-A auto-phosphorylation, compared to controls (DMSO alone), but a residual fraction (25-30%) of active Aurora-A is still evident; at 20 nM there is a virtually complete Aurora-A inactivation (Aurora-A auto-phosphorylation is about 10% compared to controls) and with higher concentrations the average residual Aur-A-phospho-Thr288 signal at spindle poles was below 5% compared to controls (Figure 18). In order to confirm the specificity of action of the MLN8237 inhibitor in single cell analyses I measured Aurora-B activity by staining cultures with the anti-phospho-Thr232 (active)-Aur-B antibody (Figure 18). It is known that high concentrations, i.e. 250 nM, of MLN8237 inhibit Aurora-B activity too (Manfredi et al., 2011). Surprisingly, I noticed that partial but highly significant Aurora-B inhibition was already occurring when using 50 nM MLN8237 (residual phospho-Thr232-Aur-B signal was below 45% compared to controls), indicating that in U2OS cells there is a narrow window (20 to 50 nM) in which the inhibitor is really specific for Aurora-A.

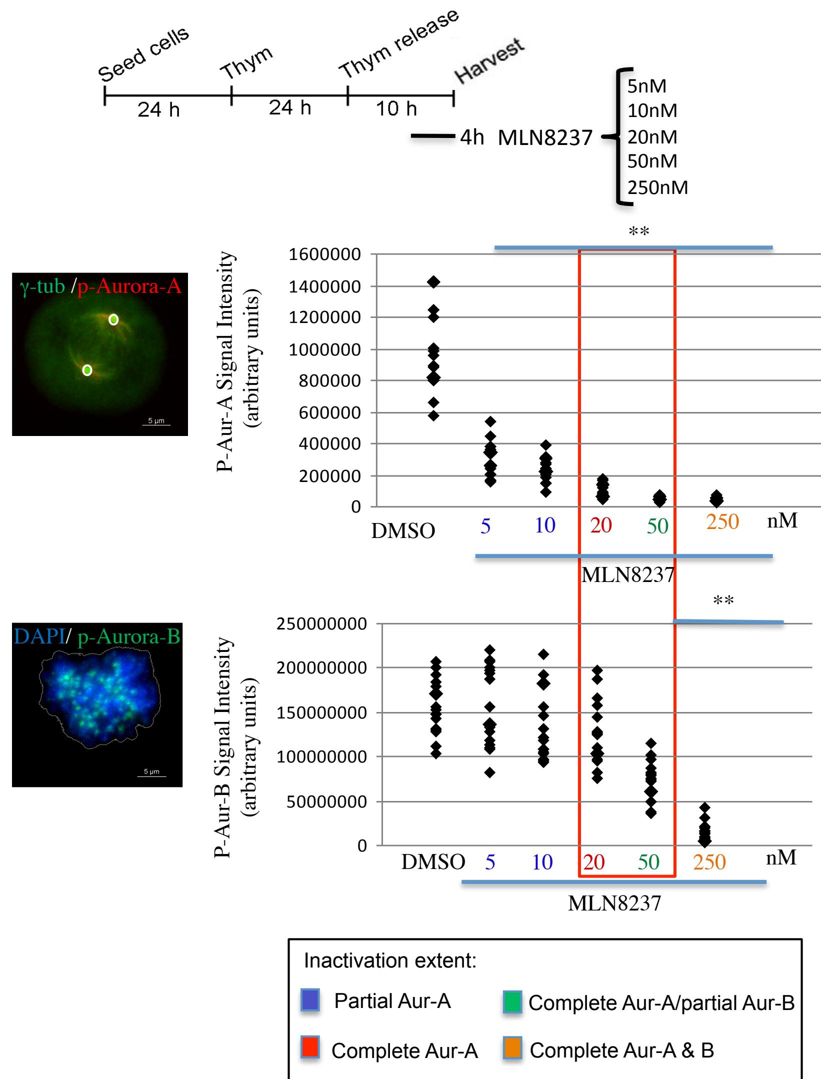


Figure 18. Effects of MLN8237 treatment on Aurora-A and Aurora-B activity. A schematisation of the experimental protocol is shown (time intervals not represented to scale). The efficiency of Aurora-A and Aurora-B activity inhibition after MLN8237 treatment was assessed by IF analysis. Selections (at poles and chromosome respectively) used for

measuring signal intensity are shown in the IF panels (scale bar: 5 μm). Each diamond in the graphs represents the signal intensity of phospho-Thr288-Aurora-A (p-Aurora-A) or phospho-Thr232-Aur-B (p-Aurora-B) in a single cell. Experiments were repeated 2/3 times and 20 cells per experiment were measured; one representative experiment is shown. **p<0.0001, Student's t-test was applied.

2.2. Delayed mitotic entry and prolonged mitosis in cells treated with MLN8237.

To study the biological effects of MLN8237 at the single cell level, I started a collaboration with the Advanced Light Microscopy Facility (ALMF) of EMBL of Heidelberg, thanks to the selection within the Proof of concept studies of the Euro-BioImaging project (http://www.eurobioimaging.eu). I performed a short visit to the ALMF, during which I could use high-throughput microscopy instrumentations, to get a first "global view" of the effects of MLN8237 in pre-synchronised U2OS cultures in dose response experiments; sample numerosity associated with the possibility to observe the long term outcome of the cultures were important features of the high throughput microscopy experiments. Cultures treated as in Figure 18 were video-recorded from the moment of treatment until 48-60 hours later; concomitant recording of all analysed MLN8237 doses (about 20 fields per condition) was possible, enabling me to collect a large amount of data within each experiment. A dose-dependent increase of mitotic length was evident up to the highest dose (250 nM) when mitotic duration starts decreasing (Figure 19). Two additional observations were obtained (Figure 19): (i) multiple and dose-dependent phenotypes are induced, ranging from multipolar divisions to cells that re-adhere without dividing; (ii) poor cell death induction was observed after MLN8237 treatment, both from mitoses and from the subsequent interphases. Finally, MLN8237-treated cultures appeared to enter more slowly in mitosis (not shown).

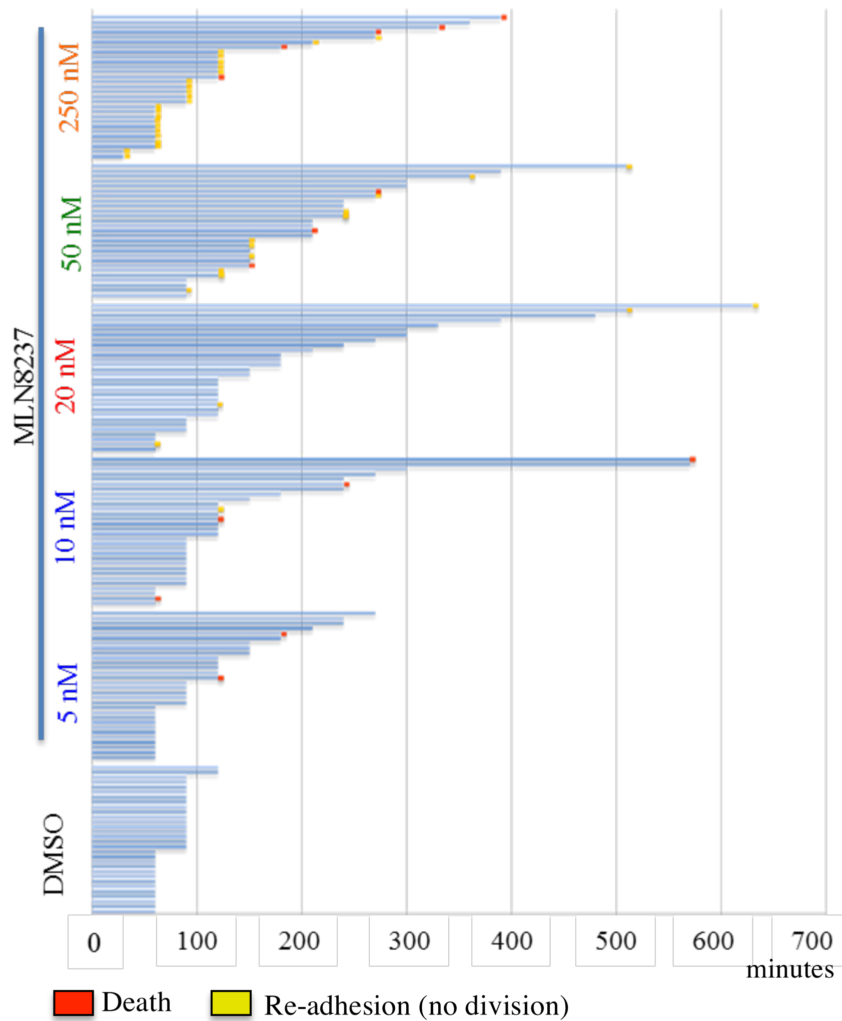


Figure 19. Effects of MLN8237 treatment on mitotic timing in high-throughput time-lapse live cell imaging. The timing of mitosis from round-up to re-adhesion is shown. Each horizontal bar represents 1 cell; 30 cells per condition are shown. Occurrence of extreme phenotypes i.e. cell death and re-adhesion without division are indicated by red or yellow rectangles at the end of the row. The experimental protocol and the color code (extent of Aurora kinases inhibition) are as in Figure 18.

To clarify effects caused by MLN8237 treatment I then analysed mitotic progression in dose-response assays using a medium-throughput approach in my laboratory in Rome, in order to obtain a major optical and temporal resolution despite of a reduction in sample numerosity; parallel high resolution observations of fixed samples were routinely performed.
First, I observed a reduction of cells that enter mitosis compared to control cultures (Figure 20). The analysis of samples fixed after 4 hours of treatment with MLN8237 in pre-synchronised cultures progressing towards mitosis (Figure 20A; see protocol in Figure 18) shows that this reduction occurs in a dose-dependent manner.
In order to clarify whether cells were permanently arrested before entry into mitosis or rather delayed in progression through the G2/M transition, I recorded cultures from the moment of the treatment with the inhibitor until 16 hours later (Figure 20B). Time-lapse analysis confirmed a dose-dependent reduction of the percentage of cells that enter in mitosis within 4 hours from MLN8237 treatment. Still, when observing cultures during the subsequent 12 hours, entering of MLN8237-treated cells into mitosis was apparent, although strongly delayed compared to control cultures (Figure 20B).
Once in mitosis, MLN8237-treated cells are also delayed in PM execution (Figure 21A), recalling the prolonged duration of mitosis observed in the high-throughput experiments (see Figure 19); interestingly, though, this was consistently dose-dependent, including at 250 nM, a condition that in high-throughput experiments restored a total mitotic duration comparable more similar to controls. The PM delay is reflected in an accumulation of PM figures in fixed samples (Figure 21B), as already observed in previous works (Marumoto et al., 2003; De Luca et al., 2006).

Together, these results show a dose-dependent delay in passing through the G2 to M transition and in progression through the mitotic division, in Aurora-A-inhibited MLN8237-treated cells.

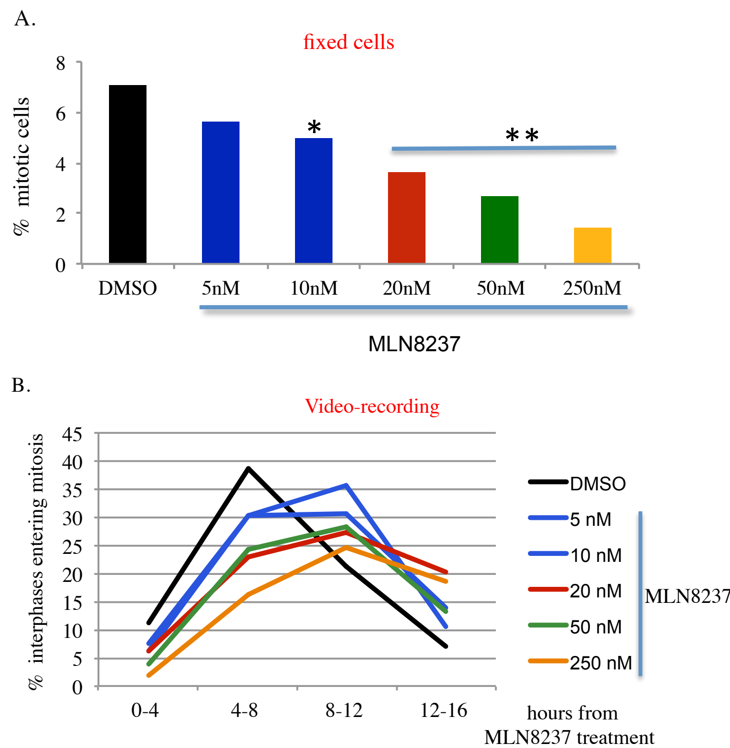


Figure 20. Aurora-A inhibition by MLN8237 delays mitotic entry. A) Histograms represent the percentage of mitoses scored in different conditions in fixed samples. At least 350 counted cells per condition, 2 experiments. *: $p < 0.01$, **: $p < 0.001$, $\chi 2$ test. **B.** The graph represents the percentage of interphases present at the beginning of video recording (at least 250 cells per condition from 3 experiments) that enter mitosis during the following 16 hours, under the different conditions. The experimental protocol and the color code (extent of Aurora kinases inhibition) are as in Figure 18.

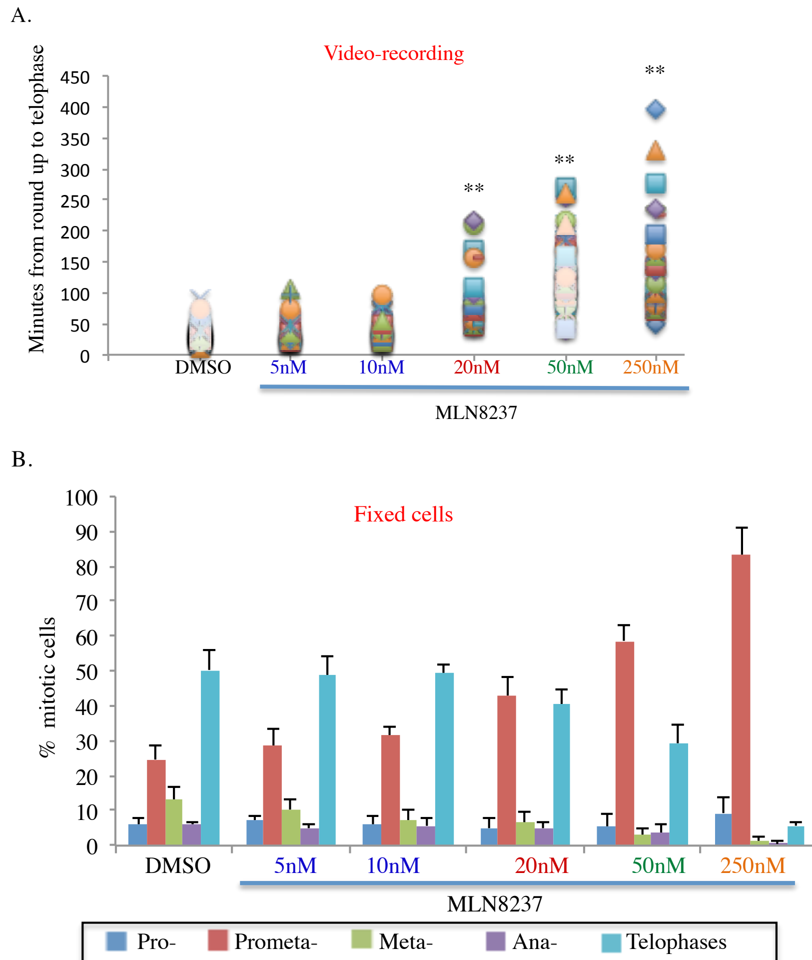


Figure 21. Prometaphase is prolonged following Aurora-A inhibition. A. The time required to complete PM is indicated. Each symbol in the graph represents 1 cell; at least 40 cells per condition from 3 experiments are represented. **: p < 0.001, Mann–Whitney test. **B.** Histograms represent the percentage of mitotic cells in different mitotic phases under the analysed conditions (at least 250 scored mitoses per condition, 2 experiments). Error bars denote s.d. The experimental protocol and the color code (extent of Aurora kinases inhibition) are as in Figure 18.

2.3. Multiple spindle defects and mitotic outcome after MLN8237 treatment.

In order to clarify the reason for the delayed PM progression in MLN8237-treated mitoses, I analysed the effects of the treatment on mitotic spindle assembly and organisation. Cells that entered mitosis with different extent of Aurora-A or Aurora-A + Aurora-B inhibition displayed a variety of spindle defects (Figure 22).

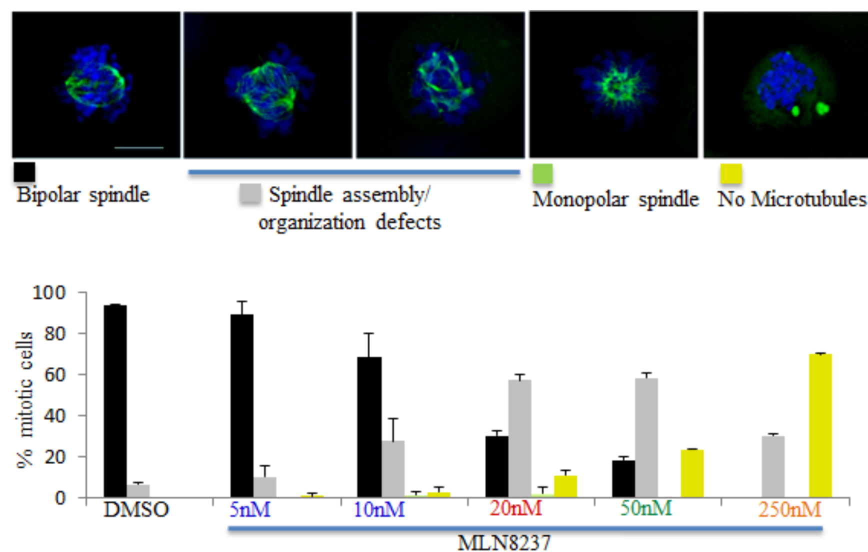


Figure 22. Mitotic spindle defects in Aurora-A-depleted cells. The IF panels (alpha tubulin in green, DNA in blue) show the different observed mitotic spindle defects. Histograms represent the percentage of mitotic cells displaying those abnormalities at different MLN8237 doses. Error bars denote s.d. 200 counted cells per condition, 2 experiments. The experimental protocol is as in Figure 18. Scale bar: 10 µm.

Partial Aurora-A inhibition induced the formation of mitotic spindles with supernumerary poles and/or largely disorganised: interestingly, these phenotypes recalled those described following Aurora-A RNAi (De Luca et al., 2008; see also Figure 12 and 14), which indeed leaves a residual Aurora-A cellular activity (Marumoto et al., 2003; De Luca et al., 2006). When Aurora-A

activity is virtually completely inhibited, at 20 nM MLN8237 and higher, less than 30% of PM/M cells displayed normal spindles. Interestingly, a MT nucleation defect became apparent at these doses, which increased in a dose-dependent manner, representing the almost totality (about 70%) of the spindle defects at 250 nM, when Aurora-A and Aurora-B are concomitantly inhibited.

To investigate how the spindle defects observed in fixed samples treated with MLN8237 influence cell division, I analysed mitotic progression and outcome by time-lapse analysis (Figure 23).

In addition to the PM delay (Figure 21A), several phenotypes were observed, depending on MLN8237 dose:

1) mis-orientation of division (Figure 23A, second row): ~10-15% of mitoses divided with a normal timing, but perpendicularly respect to the growth surface. This determined a loss of synchrony at mitotic exit: one daughter cell re-adheres while the other is still rounded on top of it. This phenotype was observed already when only partial Aurora-A inhibition is achieved (5-10 nM MLN8237) and not when complete Aurora-A inhibition is accompanied by Aurora-B inactivation (50-250 nM; see Figure 18).

2) Multipolar division (Figure 23A, third row): about 15% of mitoses proceed to a multipolar telophase (generally tripolar) after a prolonged PM, yielding division into three or more daughter cells with mis-segregated chromosomes. This phenotype was observed at all tested conditions, with prevalence in the 20 and 50 nM MLN8237-treated cultures.

3) No division (Figure 23A, fourth and fifth row): an extreme phenotype was observed, with complete failure of division and re-adhesion in large interphase cells from a state of PM. Sometimes cells re-adhere after a long PM, during which attempts to divide occur, with evident membrane blebs (top panels); other cells re-adhere more rapidly without any attempt to divide (lower panels). This phenotype was observed with high MLN8237 doses (50-250 nM), representing the vast majority of mitoses (>80%) at 250 nM.

Importantly, I never observed cell death from mitosis after MLN8237 treatment in time-lapse analysis.

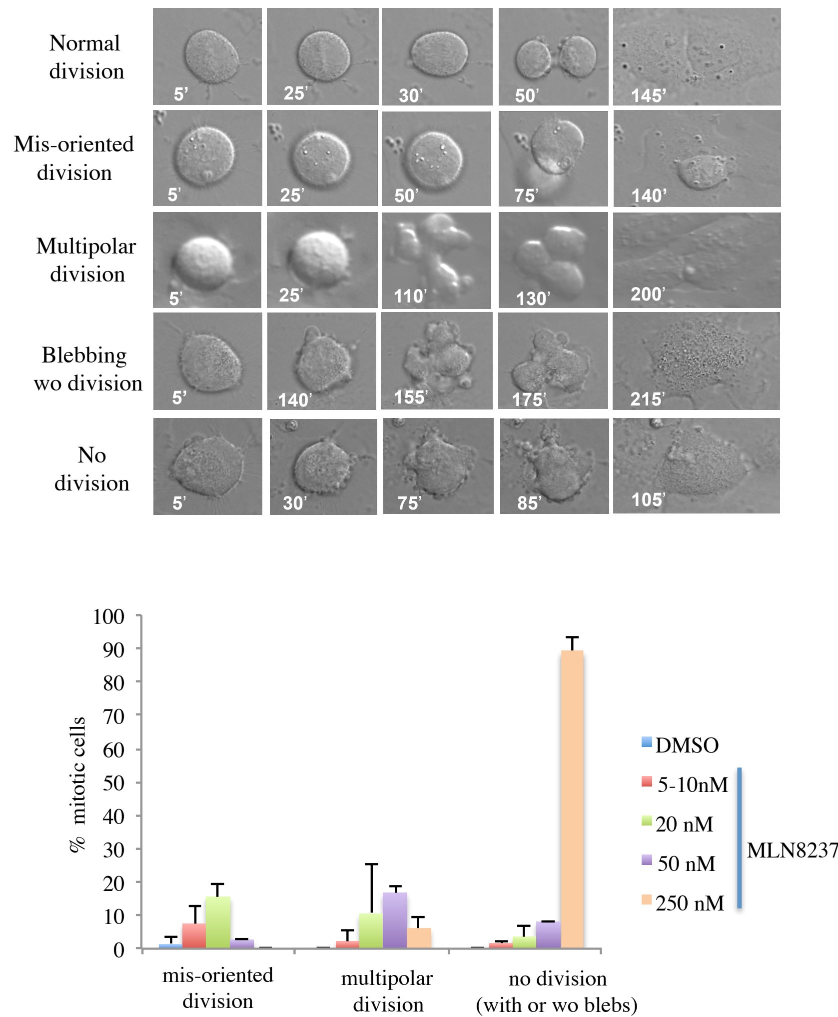


Figure 23. Defects during mitotic progression in Aurora-A depleted cells. The plate shows the different observed mitotic defects (DIC images); timing from mitotic onset is indicated. Histograms represent the percentage of mitotic cells displaying those abnormalities at different MLN8237 doses. Error bars denote s.d. 100 counted cells per condition, 2 experiments. The experimental protocol is as in Figure 18.

In conclusion the experiments with MLN8237 in synchronously cycling cultures showed multiple and dose-dependent defects in mitotic entry, spindle assembly and mitotic progression; these are associated with aberrant chromosome segregation, while they do not significantly trigger cell death from mitosis. Importantly, although MLN8237 is regarded as highly specific for the Aurora-A kinase, my analyses call for an involvement of Aurora-B in some of the induced phenotypes.

3. Aurora-A/TPX2 as an oncogenic holoenzyme

As recalled in the Introduction, Aurora-A overexpression has been detected in many cancer types and the kinase has been proposed as a potential oncogene, although available data suggest that the specific molecular background and/or the gene profile can modulate the effect of Aurora-A overexpression in tumor progression. Aurora-A activity, localisation and stability are modulated by several factors; among these, the MT-binding protein TPX2 plays a major role in mammalian cells (see paragraphs 1.2 and 3.2 in Introduction).

Recent work in our laboratory, to which I contributed during my PhD project, identified a novel level of control of TPX2 on Aurora-A: TPX2 controls not only the localisation and the activation of the kinase but also its stability (Giubettini et al., 2011). This evidence prompted me to investigate whether TPX2 levels can influence the oncogenic potential of Aurora-A: high expression of TPX2, which would lead to abnormal activation and stability of Aurora-A, may contribute to modulate the transforming potential of Aurora-A. TPX2 overexpression may represent an independent route, besides Aurora-A gene amplification or overexpression, to increased Aurora-A levels in tumors. Besides, co-overexpression of the two genes may exacerbate the effect of Aurora-A overexpression, yielding excess amount of active Aurora-A/TPX2 complex, which can be more deleterious than overexpression of the kinase alone (Figure 24).

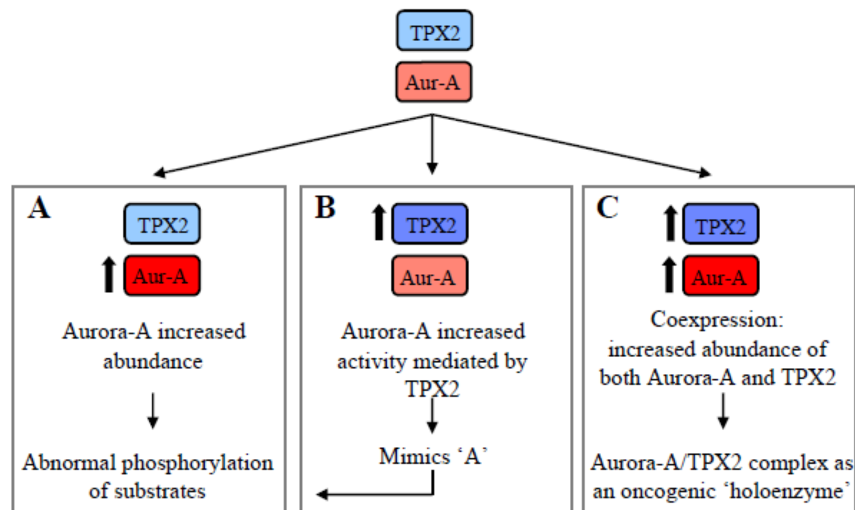


Figure 24. Scenarios for Aurora-A and TPX2 deregulation leading to tumorigenesis. Alterations in the Aurora-A/TPX2 complex with respect to a normal condition (top panel) can take place in three hypothetical manners: increased Aurora-A (A) or TPX2 (B) abundance, both expected to cause abnormal phosphorylation of Aurora-A substrates, or overexpression of both (C); the latter scenario yields excessive abundance of the whole complex, which can then act as an oncogenic holoenzyme.

This hypothesis is strengthened by the evidence that both genes (Aurora-A and TPX2) are located on the long arm of chromosome 20, in a region that is frequently amplied in many tumor types, such as breast, ovarian, cervical, colon, bladder (Knuutila et al, 1998; Hodgson et al., 2003; Scotto et al., 2008; Beroukhim et al., 2010): thus co-amplification of both genes can occur in cancer.

To substantiate this idea I searched for evidence of TPX2 overexpression, and TPX2/Aurora-A co-overexpression, in human cancer cells.

3.1. TPX2 is overexpressed in many tumor types.

First, I analysed the literature for specific reports of TPX2 overexpression in tumors. TPX2 is included in a list of more than 50 mitotic genes with altered expression in cancer cells (Perez de Castro et al., 2007). Recently, by computational means, a highly significant correlation between TPX2 overexpression, CIN and cancer has been highlighted (Carter et al., 2006). Furthermore, several instances of TPX2 overexpression in tumor tissues and cell lines emerge from specific studies and in some cases this overexpression correlated positively with tumor grade and stage, with lympho-metastasis and negatively with the survival rate (Table 2).

Based on the results obtained from the literature search I extended the analysis of TPX2 overexpression in cancer using the Oncomine database, a very useful tool to evaluate the expression of specific genes in many cancer types relative to their non-transformed tissue counterparts. Oncomine collects data from several datasets (484 datasets when this analysis was performed) in which mRNA expression or DNA copy number were measured in primary tumors, cell lines or xenografts. Cancer microarray data are carefully reviewed before inclusion in the Oncomine database; computational analyses (e.g. differential expression analysis) can be performed and results can be filtered using specific criteria (e.g. tumor type, dataset type, clinical outcome and others). My search revealed significant TPX2 overexpression in tumors in 27% of all comparative analyses of tumor versus normal tissues (Table 3).

TPX2 ranked among the first 10% or even 5% of all measured genes in virtually all differential expression analyses in which it was significantly overexpressed. Cancer types in which TPX2 overexpression was observed include lung carcinoma, cervical carcinoma and sarcoma (>50% of the analyses), and head and neck, gastro-intestinal, kidney, liver, breast, ovarian and bladder cancer (between 25-50% of the analyses). There is a good overlap between cancer types extracted from the Oncomine analysis and those reported in specific literature studies (see Table 2).

Together these data suggest an oncogenic potential of TPX2, or an ability to predispose or cooperate with other genes in cell transformation and tumorigenesis when overexpressed.

Cancer type	Cell line (C) or tissue (T) (tumor/control)	Overexpression	Reference
Brain	T (52/5) C (2/1)	protein[f,g] and transcript[a] protein[f] and transcript[a]	Li et al., 2010
Oral SCCs	T (43/7)	transcript[a]	Shigeishi et al., 2009
	T (59/10)	protein[h]	Fenner et al., 2005
Salivary gland	T (20/6)	transcript[b]	Shigeishi et al., 2009
Lung	C (31/0) T (31/3)	transcript[a]	Tonon et al., 2005
	C (1/1) T (6/6)	protein[f,g,h] and transcript[a] protein[h]	Zhang et al., 2008
	C (3/4) T (21/21) T (595/42)	protein[f] and transcript[b] transcript[a] protein[h]	Ma et al., 2006
	C (9/3)	transcript[c,d]	Manda et al., 1999
	C (1/1)	transcript[b]	Kadara et al., 2009
Colon	T (1/1)	transcript[e]	Hufton et al., 1999
Liver	T (10/10)	transcript[b]	Wang et al., 2002
	T(20/20) T(64/64) T(19/19)	transcript[b] transcript[a] protein[g]	Satow et al., 2010
Pancreas	C (9/1) T (40/31)	protein[f] and transcript[a] protein[h]	Warner et al., 2009
Ovarian	T (9/10) * T (4/4) *	transcript[b] transcript[a]	Sharer et al., 2008
Cervix	C (9/0) T (20/20)	transcript[b] transcript[b]	Scotto et al., 2008
Mesothelial	T (36/22) *	protein[h]	Taheri et al., 2008

Table 2. TPX2 overexpression in human cancer. Studies in which TPX2 expression was assessed in tumor compared to normal samples are listed. (*) indicate studies comparing malignant vs benign, instead of tumor vs normal tissues. Overexpression is reported according to the method employed for analysis: "transcript"results were obtained from a) RT-PCR, b) microarrays (mRNA, cDNA), c) Northern blot, d) mRNA differential display or e) Reverse Northern blotting. "Protein" results were obtained by f) Western blot, g) immunocytochemistry, h) two-dimensional electrophoresis.

Cancer type	**Total analyses**	**Analyses in which TPX2 is significantly overexpressed**				
	(N)	(N)	(%)	Gene rank threshold (%)		
				first 10%	*first 5%*	*first 1%*
Brain and CNS cancer	22	5	23	*18*	*9*	*0*
Head and neck cancer	13	4	31	*31*	*23*	*0*
Lung cancer	13	9	69	*69*	*69*	*46*
Gastrointestinal cancer	22	11	50	*50*	*45*	*14*
Liver cancer	6	2	33	*33*	*33*	*0*
Kidney cancer	10	3	30	*20*	*10*	*10*
Pancreatic cancer	6	1	17	*17*	*17*	*0*
Breast cancer	7	3	43	*43*	*43*	*0*
Ovarian cancer	11	3	27	*27*	*27*	*18*
Cervical cancer	1	1	100	*100*	*100*	*0*
Bladder cancer	7	2	29	*14*	*14*	*14*
Prostate cancer	14	0	0	*0*	*0*	*0*
Lymphoma	11	0	0	*0*	*0*	*0*
Leukemia	12	1	8	*8*	*0*	*0*
Myeloma	2	0	0	*0*	*0*	*0*
Sarcoma	8	6	75	*75*	*75*	*63*
Melanoma	6	0	0	*0*	*0*	*0*
Other cancer	22	2	9	*9*	*9*	*5*
Total	**193**	**53**	**27**	***26***	***23***	***10***

Table 3: TPX2 overexpression in tumor versus normal tissues using Oncomine. Oncomine™ (Compendia Bioscience) database was searched for TPX2 differential expression in tumor versus normal samples. The number and percentages of total examined analyses and cancer types in which TPX2 is highly significantly overexpressed ($p<0.0001$, Student's t-test) are indicated. The rightmost columns show the percentages obtained after applying a second threshold (in addition to the p-value threshold) based on the gene rank, taking into account TPX2 ranking among the first 10%, 5% or 1% analysed genes.

3.2. Aurora-A and TPX2 coexpression in tumors

The simultaneous overexpression of both TPX2 and Aurora-A can determine a particularly relevant situation in which the high abundance of Aurora-A protein would find non-limiting amounts of its activator TPX2: under these conditions, the Aurora-A/TPX2 complex would yield abnormal phosphorylation of downstream targets. Concomitant overexpression of Aurora-A and TPX2 could therefore confer a proliferative advantage over normal cells and promote tumor growth (see Figure 24).

I found direct evidence in the literature for Aurora-A and TPX2 coexpression in tumors. Aurora-A is included in the cancer-associated chromosomal instability CIN70 signature, in which TPX2 ranks first, identified in the computational study previously mentioned (Carter et al., 2006). In addition, overexpression of both Aurora-A and TPX2 has been shown in a study on lung cancer, comparing cancer vs normal lung cells, and was also detected in samples from carcinoma vs adenoma ovarian cancer patients in a combination of genome-wide expression data, in silico interaction network analysis and real time PCR mRNA measurements (Scharer et al, 2008; Kadara et al., 2009).

To assess the general validity of these observations, I again screened the Oncomine database (Table 4). First, I noticed that in around 70% of the analyses in which TPX2 was significantly overexpressed, Aurora-A was overexpressed as well. Then I used the Oncomine coexpression analysis tool, which assigns correlation values (cv) to all genes in a dataset with respect to a gene of interest (cv = 1 indicates genes that are co-overexpressed).

I used Aurora-A and TPX2 as references genes (see Materials and Methods for details): Aurora-A cvs with respect to TPX2 were >0.5 in 19 out of 22 datasets; cvs were remarkably high (≥ 0.8) in 12 out of 22 datasets, indicating that Aurora-A and TPX2 are expressed with a similar pattern. In the reciprocal analysis, setting Aurora-A as the “reference gene” and examining TPX2 coexpression, cvs were >0.5 in 16/20 and ≥0.8 in 11/20 datasets.

Cancer type	Coexpression with TPX2: AurkA correlation value	Coexpression with TPX2: AurkA gene position	Coexpression with AurkA: TPX2 correlation value	Coexpression with AurkA: TPX2 gene position	N control/ N tumor	Data- set legend
Head and neck	0.84	5	0.84	6	12/26	Ye et al., 2008
	0.86	2	0.86	2	13/41	Ginos et al., 2004
Lung	0.74	17	0.74	27	6/67	Garber et al., 2001
	0.88	1	0.88	1	19/20	Stearman et al., 2005
	0.78	105	0.78	105	31/35	Su et al., 2007
	0.84	44	0.84	23	49/58	Landi et al., 2008
	0.58	32	0.58	27	17/186	Bhattacharjee et al., 2001
	n.i.	n.i.	<0.44	>400	5/5	Wachi et al., 2005
Gastro-intestinal	<0.46	>400	<0.38	>400	41/82	Ki et al., 2007
	0.86	2	0.86	3	5/100	Kaiser et al., 2007
	<0.15	>400	<0.44	>400	22/56	Gaspar et al., 2008
	0.81	6	0.81	5	12/48	Graudens et al., 2006
	0.95	238	0.95	221	94/94	Kurashina et al., 2008
	<0.36	>400	<0.14	>400	29/103	Chen et al., 2003
Liver	0.80	128	0.80	21	10/65	Wurmbach et al., 2007
Kidney	0.66	>400	n.i.	n.i.	10/10	Gumz et al., 2007
	0.85	15	n.i.	n.i.	11/59	Beroukhim et al., 2009
Breast	0.86	32	0.86	22	7/40	Richardson et al., 2006
	0.64	101	0.64	94	3/61	Zhao et al., 2004
Ovarian	0.70	43	n.i.	n.i.	5/45	Lu et al., 2004
	0.87	23	n.i.	n.i.	10/38	TCGA
Bladder	0.83	11	0.83	10	48/109	Sanchez et al., 2006
Prostate	n.i.	n.i.	0.81	4	23/89	Yu et al., 2004
Sarcoma	0.73	75	0.73	82	15/39	Detwiller et al., 2005

Table 4: Co-overexpression of TPX2 and Aurora-A in cancer. Coexpression of Aurora-A (AurkA) and TPX2 was evaluated using Oncomine by (i) correlation values (see https://www.oncomine.org/content/org/help/OncomineHelpFile.htm for how these values are calculated; 1 indicates a perfect positive correlation), and (ii) position of Aurora-A in the list of TPX2 coexpressed genes and viceversa (position 1 is the most coexpressed gene). In each analysis, only datasets in which the reference gene was significantly and frequently overexpressed (as explained in detail in the Materials and Methods) were considered; n.i. (not included) indicates datasets excluded in one or the other analysis due to these criteria. TCGA (The Cancer Genome Atlas): Ovarian Serous Cystadenocarcinoma Gene Expression Data. Note that controls (non tumor) in each analysis are often less numerous than tumor samples.

Since cvs may be influenced by the overall background noise within a dataset, we also used a second parameter to evaluate coexpression, i.e. Aurora-A position among TPX2-coexpressed genes and viceversa. We found that in 13 out of 22 datasets Aurora-A was among the first 50 TPX2-coexpressed genes (of at least 6000 measured genes), while TPX2 ranked among the first 50 Aurora-A coexpressed genes in 12/20 analyses, again indicating a positive correlation between Aurora-A and TPX2 overexpression in tumors (see Table 4).
To control for the specificity of the co-overexpression I assessed coexpression of TPX2 with two additional mitotic genes, Aurora-B (which is highly similar to Aurora-A in sequence, but acts in a different pathway) and the unrelated Plk1 kinase. Neither gene displayed good coexpression with TPX2: cvs were ≥ 0.8 in 3 out of 22 datasets for Plk1 and in 2 out 22 for Aurora-B; furthermore, Aurora-B and Plk1 were within the first 50 TPX2 coexpressed genes in only 3 and 6 datasets, respectively (not shown).
These data reinforce the idea that the Aurora-A/TPX2 complex may act as a novel functional unit, or “holoenzyme”, with specific consequences in cell transformation and tumorigenesis.

DISCUSSION

My PhD thesis focused on the Aurora-A kinase, with the aim to clarify its mechanisms of action in mitosis and the effects of its deregulation on cell division, also with respect to its possible involvement in cancer.

Results obtained in my PhD project indicate that Aurora-A is needed for establishing a finely-tuned balance among MT-associated forces operating in bipolar spindle formation. I observed that in Aurora-A defective cells an imbalance among MT forces directed towards centrosomes determines the fragmentation of mitotic spindle poles:

- Aurora-A-defective mitoses display hyperstable MTs associated with spindle pole fragmentation; the co-inactivation of ch-TOG, a MT-stabiliser, rescues spindle pole fragmentation as shown in previous work (De Luca et al., 2008), but also counteracts the abnormal MT hyperstabilisation caused by Aurora-A inactivation.
- Inhibition of the kinesin Eg5 reduces the occurrence of the spindle pole fragmentation phenotype induced by Aurora-A depletion.
- Reduction of spindle extrapoles is also evident in Aurora-A-inactivated cells after silencing of the KT protein Nuf2, a condition in which MT-KT attachments are destabilised.

The evidence that loss of activity of ch-TOG, Eg5 and Nuf2 prevents Aurora-Ai-dependent pole abnormalities, despite of their otherwise divergent biological functions, strengthens the idea that the rescuing activity is associated with their shared property to modulate pole-directed forces. I schematised these data in a model (Figure 25) in which I show how Aurora-A interplays with forces regulating spindle pole formation. It is conceivable that in Aurora-A-defective cells the pressure directed towards poles is excessive, or is not properly counteracted at the centrosome, and spindle poles fragment.

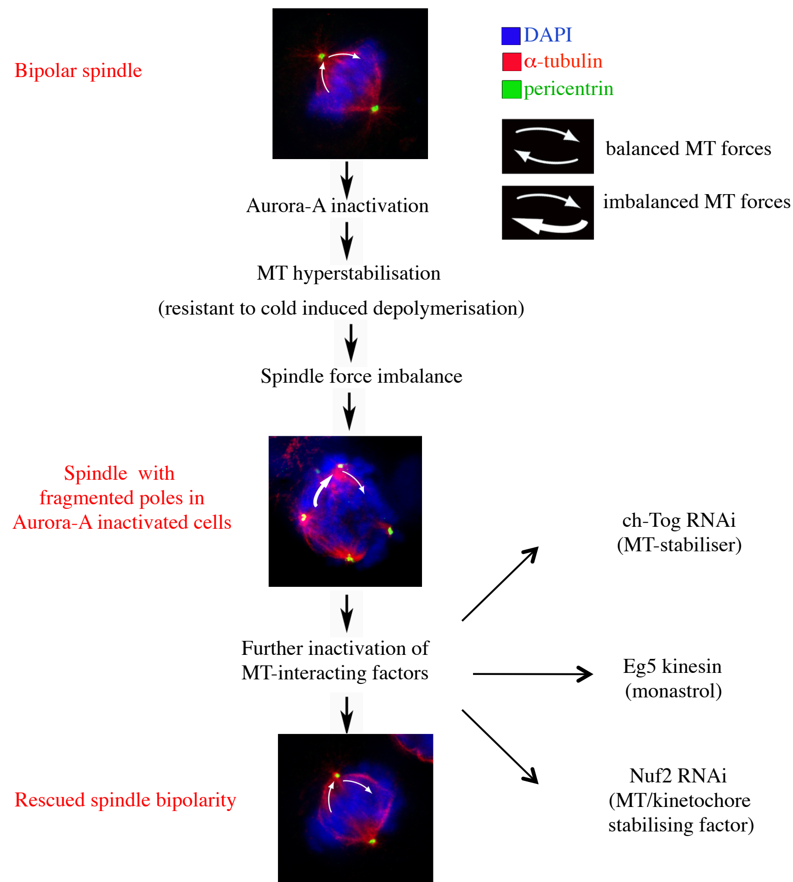


Figure 25. Aurora-A modulates the balance of forces required for spindle pole integrity: a model. Upper panel: in a normal mitosis balanced MT forces determine the formation of a symmetrical bipolar spindle. Arrows represent opposite-directed MT forces. Central panel: in Aurora-A-defective mitoses, spindles display fragmented poles. Lower panel: pole disruption is prevented by either inactivating a MT stabiliser (ch-TOG), or weakening KT-generated (Nuf2 silencing), or inhibiting (monastrol) Eg5-associated centrosome-directed MT forces in the absence of Aurora-A activity. The model suggests therefore that spindle poles fragment consequently to the imbalance in MT-generated forces in Aurora-A-defective mitoses.

ch-TOG and Aurora-A are part of a complex in human cells (Fielding et al., 2008) and in *Xenopus* extracts (Koffa et al., 2006). In *Xenopus* the complex also contains Eg5 that is a substrate of Aurora-A (Koffa et al., 2006; Giet et al., 1999). Therefore it is possible that Aurora-A regulates Eg5, and possibly ch-TOG, by modulating their interactions, and, in the case of Eg5, its phosphorylation status. The rescue obtained with Aurora-A/Nuf2 co-depletion does not necessarily imply a direct link between Aurora-A and the KT protein Nuf2, but is more likely to reflect a global re-balancing of MT forces in Aurora-A-defective cells following the destabilisation of MT-KT attachments, adding support to the notion that K-fibers directly contribute to spindle pole organisation (Manning and Compton, 2007; Toso et al., 2009).
The present data suggest that investigating the status of spindle forces in Aurora-A-overexpressing cells may be relevant for understanding the mechanisms of the Aurora-A transforming ability; at the same time they raise a concern that Aurora-A inhibitors, currently under evaluation in anti-cancer therapy, may facilitate the formation of multipolar spindles and hence chromosome segregation defects. The extent of chromosome mis-segregation may determine opposite outcomes: on the one hand, mild aneuploidy facilitates tumorigenesis; on the other hand, massive aneuploidy generated by strong mis-segregation defects yields cell death and it is therefore regarded as a potential strategy to kill cancer cells (see Figures 5 and 6 in Introduction).
In order to investigate the cellular effects of Aurora-A inhibition by chemical compounds used in clinical trials, I used the MLN8237 inhibitor in human cells. High-resolution and quantitative microscopy with fixed samples, as well as medium- and high-throughput time-lapse observations enabled me to obtain information at single cell level. The combination of these approaches has enabled me to gain insight both in potential novel mitotic functions of Aurora-A and in the cellular response to Aurora-A inhibition, and together have underlined the importance

of accompanying cellular analyses to clinically directed investigations.

MLN8237 is considered the most specific inhibitor of Aurora-A that has arrived to phase II evaluation in clinical trials. The dose-response assays that I have performed indicate partial specificity of action of MLN8237, when used in cultured cells, on Aurora-A vs Aurora-B: 20 nM MLN8237 is the only tested condition with virtually complete Aurora-A inhibition and unaltered Aurora-B activity. Partial Aurora-B inhibition observed at 50 nM MLN8237 is less evident with the widely used anti-phospho-Ser10-Histone H3 marker of Aurora-B activity (not shown), maybe reflecting kinase redundancy or a differential sensitivity of auto-phosphorylation vs targets phosphorylation; this may represent a reason for not having detected this partial specificity of action in previous studies (Sloane et al., 2010; Manfredi et al., 2011).
Spindle abnormalities and mis-aligned chromosomes have been described following MLN8237 treatment (Manfredi et al., 2011), although the majority of the studies carried out with this inhibitor are clinical. I characterised in depth mitotic entry and progression in the presence of different MLN8237 concentrations. Dose-response assays enabled me to assess processes that are more robust in respect to Aurora kinases inhibition, compared to others which on the contrary are quickly affected.
I first confirmed that Aurora-A inhibition impairs entry into mitosis, that was previously observed by either antibody microinjection (Marumoto et al., 2002) or RNAi in synchronised cultures (Hirota et al., 2003). This was under-appreciated in experiments performed in asynchronous cultures (Sloane et al., 2010; Manfredi et al., 2011), but may be relevant for the use of Aurora-A inhibition in cancer therapy: indeed, G2 arrest is transient, thus not impairing cell division; in addition, non-physiological divisions may follow this “pausing” period, during which alterations may accumulate.

Phenotypes previously described using RNAi or chemical inhibitors were reproduced, i.e PM delay, spindles displaying additional poles or disorganised spindles (Marumoto et al., 2003; De Luca et al., 2006 and 2008). In addition, novel phenotypes were apparent:
1) cells totally devoid of MTs, that only display spots of tubulin or little asters, that I regarded as a MT nucleation defect; this may well relate to the proposed function of Aurora-A in centrosome maturation. It remains to be clarified whether the occurrence of this phenotype at the highest doses of MLN8237 is dependent on a more complete inhibition of Aurora-A activity under these conditions, or on the contribution of Aurora-B which is also inhibited at those doses; indeed, no evidence so far supports the involvement of Aurora-B in MT nucleation.
2) As soon as Aurora-A is partially inhibited, many cells perform a mis-oriented division, perpendicular to the growth surface; this can reflect a role of Aurora-A in the orientation of mitotic spindle and/or an involvement in regulating cell adhesion to the substrate. Interestingly, Aurora-A was shown to contribute to spindle orientation in Drosophila S2 cells and neuroblasts (Johnston et al., 2009; Yamada et al., 2010).
3) Finally, by time-lapse imaging I observed the absence of chromosome segregation and the re-adhesion into one single cell at high concentrations of MLN8237 inhibitor. Similar defects have been described with the Aurora-A inhibitor MLN8054 (Chakravarty et al., 2010; Sloane et al., 2010). It is known that Aurora-B inhibition is dominant on Aurora-A inhibition, given its effects on mitotic checkpoint overcoming and failure of cytokinesis (Yang et al., 2005); in addition, a similar defect in cell division was described in chicken cells as typical of Aurora-A+B inactivation (Hégarat et al., 2011). Together with my finding on the partial aspecificty of MLN8237 (see above), these observations suggest that this extreme phenotype is dependent on the concomitant inhibiton of the two kinases.

In cell cultures experiments, cells are continually exposed to a constant concentration of anti-mitotic agents, while the concentration of the drug in vivo will vary. The majority of anti-mitotic agents are administered intravenously, and therapies are based on cyclic treatment of only a few hours, on a weekly basis: thus, drug concentration increases, peaks and falls and exposure of the tumor to an effective dose is likely to be transient (Gascoigne and Taylor, 2008). Thus, the large spectrum of mitotic phenotypes, mirroring the dose-dependent inhibition levels of Aurora kinases, observed in my experiments in cultured cells, may all contribute to the response in patients, and hence have relevant consequences for the outcome of therapies.

The dose-dependency of mitotic outcomes may also be highly relevant for therapy. I never observed death from mitosis, which is the desirable outcome of an anti-mitotic treatment: chemical compounds that act specifically in mitosis determine alterations in mitotic progression and, if cells do not die, chromosome mis-segregation and generation of aneuploid daughter cells is likely to occur. If the aneuploidy is not massive, but compatible with the cell survival, cells will go through different cell cycles with high probability to accumulate defects and mutations that can drive tumorigenesis. Indeed, a preliminary analysis of interphase cells originating from MLN8237-treated mitoses reveals that these cells accumulate DNA abnormalities in a dose-dependent manner (Figure 26): at 20 nM cells have normal nuclei and display only a low percentage (10% of cells) of chromosome loss or gain, comparable to control cultures. Treatment with 50 nM MLN8237 yields binucleated or polyploid cells (40%) and multinucleated cells (23%). Together, these results indicate that increasing MLN8237 concentration does not induce mitotic death, but rather the accumulation of DNA abnormalities in the subsequent interphases. The collaboration with the ALMF of the EMBL of Heidelberg will give me the opportunity to use high-throughput microscopy and to perform analysis of the data with automated methods (using softwares such as CellCognition): this will be for

me an essential support to measure the influence of multiple parameters on the effects of Aurora-A inhibition on cell division, in particular with respect to cell death induction, and identify cell types where intervention by Aurora-A inhibition may be effective.

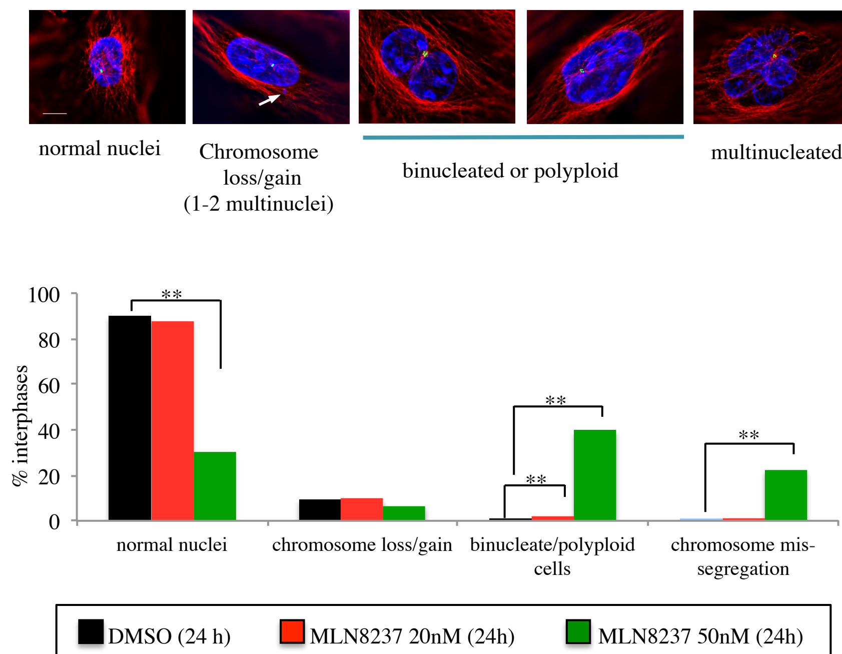


Figure 26. Outcome of mitoses treated with the MLN8237 inhibitor. The IF panels show the different scored categories. DNA is in blue, α-tubulin is in red, pericentrin, used to identify the presence of multiple centrosomes (indicative of failed division) is in green. Histograms represent the percentage of interphases, under two different MLN8237 dose conditions, in the classes shown in the IF panels. At least 100 counted cells per condition, 1 preliminary experiment, **: $p < 0.001$, $\chi 2$ test. Scale Bar: 10 μm

In parallel to studies to understand the relevance of Aurora-A inhibition in anti-cancer therapies, I investigated novel aspects

linked to the role of its overexpression in tumorigenesis: specifically, I addressed the possibility that the oncogenic potential of Aurora-A can be modulated by altered levels of its interactors/regulators. I focussed on TPX2, that is the major regulator of Aurora-A, and hypothesised that its overexpression may be relevant to elicit Aurora-A transforming potential.

Data discussed in chapter 3 of the Results suggest that elevated TPX2 levels provide an additional route to increased Aurora-A activity in cells. By a search in the literature and using the Oncomine database it was evident that TPX2 is overexpressed in many tumor types; more, the co-overexpression of Aurora-A and TPX2 has emerged as a feature of many tumors. TPX2 overexpression alone in cultured cells mainly induces the appearance of monopolar spindles associated with mitotic arrest, and induction of apoptosis has also been reported (Gruss et al., 2002; Stewart and Fang, 2005). These phenotypes can hardly support completion of an abnormal cell division that would then give rise to viable genetically imbalanced cells. These observations suggest that concomitant mitotic defects, such as a concomitant weakening of the mitotic checkpoint for example, can contribute to cell transformation. The simultaneous overexpression of both TPX2 and Aurora-A can indeed determine a particularly relevant situation; in that case the high abundance of Aurora-A protein, which is critical for proper exit from mitosis (Meraldi et al., 2002), would be accompanied by high levels of its activator TPX2. Consequently, spindle formation and function and balanced chromosome segregation would be deregulated, and aneuploid daughter cells can be originated. The concomitant overexpression of Aurora-A and TPX2 could therefore confer a proliferative advantage over normal cells and favor tumor growth. A recent study supports this hypothesis. Aurora-A and TPX2 are both on the q arm of the chromosome 20, which is frequently amplified in cancer; Sillars-Hardebol and collaborators analysed several colorectal carcinoma and adenoma, and identified *TPX2* and *AURKA* as two genes on the 20q amplicon important to promote

colorectal adenoma to carcinoma progression. They also show that down-modulation of TPX2 and Aurora-A inhibits invasion (Sillars-Hardebol, 2011).

The observation that Aurora-A and TPX2 are frequently co-overexpressed, together with our recent finding that TPX2, besides its ability to up regulate the kinase activity, also regulates Aurora-A protein stability (Giubettini et al., 2011), suggest that protein stabilisation, even in the absence of gene amplification, represents an additional route to Aurora-A increased levels in cancer. Growing evidence correlate abnormal Aurora-A protein stability to tumors and are consistent with this view:

(i) increased Aurora-A protein levels, without amplification or overexpression of the gene, have been reported in head and neck cancers (Kitajima et al., 2007), and are associated with abnormal phosphorylation on Ser51, a modification that protects Aurora-A from degradation (Crane et al., 2004);

(ii) the Aurora-A allelic variants associated with increased risk of cancer involve amino acids 31 and 57, both of which lie in the proximity of the A-box degradation motif, and may therefore render the A-box less accessible or less effective, again suggesting a possible contribution of Aurora-A stabilisation to tumorigenesis.

Based on my observations that current Aurora-A inhibitors display critical specificity issues, associated with aneuploidy induction, and that the Aurora-A/TPX2 complex may act as an oncogenic unit, I would like to propose that the Aurora-A/TPX2 complex may also represent a potential target in cancer therapy. Recent TPX2 inactivation experiments have yielded anti-proliferative effects in cancer cells (Morgan-Lappe et al., 2007; Zhang et al., 2008; Warner et al., 2009; Li et al., 2010; Satow et al., 2010), suggesting a potential value of TPX2 as an anti-cancer target; the effectiveness of TPX2 inactivation or downregulation may reflect, at least in part, the ensuing down-regulation of the Aurora-A/TPX2 holoenzyme. Direct approaches to develop specific inhibitors of the Aurora-A/TPX2 complex formation, which would not be active

against the related kinase Aurora-B, may constitute a novel strategy in the field of Aurora-A targeting in cancer therapy.

MATERIALS AND METHODS

Cell cultures, synchronisation protocols and treatments

U2OS cells (ATCC: HTB-96) were cultured at 37 ^{o}C in a 5% CO2 atmosphere in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 2% L-glutamine, 2% penicillin/streptomycin. For synchronisation, cells were subjected to a 24 hours treatment with 2 mM thymidine. Cultures were then released from the G1/S arrest by washing away the thymidine (3 washes with DMEM for 5 minutes at 37 ^{o}C) and adding fresh medium containing 30 μM deoxycytidine to restore the synthesis of deoxyribonucleotide triphosphates; about 10 hours post-release, mitoses-enriched cultures were fixed and processed for IF, WB or video recorded. MLN8237 (5-250 nM, as indicated; Selleck Chemicals), NOC (0,1 μg/ml) and MON (100 μM) were added to thymidine-released cultures at the indicated times before harvesting. When indicated, asynchronously growing cultures were treated with MON for 6 hours; after 3 washes with DMEM as above, cultures were released in complete medium for 30 and 60 minutes. For MT depolymerisation experiments, cells were incubated on ice for 10, 15 and 20 minutes, then fixed and processed for IF.

RNAi

cDNA sequences targeted by small interfering (si)RNA oligonucleotides (QIAGEN or Applied Biosystems/Ambion) are: 725- ATGCCCTGTCTTACTGTCA-743 (Aurora-A), 126-GAGCCCAGAGTGGTCCAAA-144 (ch-TOG), 397-GCATGCCGTGAAACGTATA-415 (Nuf2). A GL2 siRNA duplex targeting the luciferase gene was used for control. Final concentrations of siRNA oligonucleotides were: 80 nM (Aurora-A and GL2), 40 nM (ch-TOG) and 60 nM (Nuf2). In co-transfections of Aurora-A and either ch-TOG or Nuf2 siRNAs the final amount of siRNA oligonucleotides in control cultures was balanced (to 120 or 140 nM, respectively) by adding GL2 oligo. Transfection

reagent was Oligofectamine (Invitrogen). Cultures were analysed 48 hours after transfection. Experiments were repeated 2 to 4 times; statistical analysis of data was performed using the χ^2 test and calculating standard deviations (s.d.), as indicated.

IF

Cells grown on coverslips were fixed as follows: (a) -20°C methanol, 6 minutes; (b) 3,7% PFA plus 0.2% TritonX-100 in 20 mM Pipes pH 6.9, 1 mM MgCl2, 10 mM EGTA, 10 minutes at room temperature; or c) fixed in 3.7% PFA (10') and then permeabilised in 0.1% Triton-X100–PBS1X (5'). Blocking and all antibody incubations were performed at room temperature in PBS containing 0.05% Tween 20 and 3% BSA. Cells were counterstained with 4,6-diamidino-2-phenylindole (DAPI, 0.05 µg/ml) and mounted using Vectashield Mounting Medium (Vector MLaboratories) that prevents rapid photobleaching of several fluorophores. Primary antibodies were: anti-alpha-tubulin (1:2000, B-5-1-2, Sigma), anti-Aurora-A (0.5 µg/ml, BD Transduction Laboratories), anti-phospho-Aurora-A (Thr288) (1:250; C39D8; Cell Signaling Technology), anti-pericentrin (2 µg/ml, ab4448; Abcam), anti-ch-TOG (1:25, ab18320, Abcam), anti-Nuf2 (1:300, ab17058; Abcam), anti phospho-Aurora-B (Thr232) (1:50; Poly6361 BioLegend); anti phospho-Histone H3 (Ser10) (0,25 µg/ml; 3H10; Millipore).

Fixed samples were analysed using a Nikon Eclipse 90i microscope equipped with a Qicam Fast 1394 CCD camera (Qimaging). An oil immersion 100X (N.A. 1.3) objective was used. Image acquisition was performed using NIS-Elements AR 3.2 (Nikon); three-dimensional deconvolution of the 0.4-µm or 0.6-µm Z-serial optical sections and reconstruction was performed using the "Legacy" or "AutoQuant" deconvolution modules of Nis-Elements AR 3.2/4.0. Creation of image projections from z-stacks was also performed using the Maximum Intensity Projection (for quantitative analyses), and Extended Depth of Focus functions

of NIS-Elements AR 3.2/4.0. Images were processed also with Adobe Photoshop CS 6.0.
Signals were measured using NIS Elements AR 3.2/4.0 (nd2 file format), using the “manual measurements” function: external background correction was applied and the sum intensity of signals on indicated selected area was measured. Values were statistically analysed using the InStat3 software, using either (i) the unpaired Student’s *t-test* (for Gaussian distributions), applying the Welch correction when required, or (ii) the Mann–Whitney test, when the populations did not follow a Gaussian distribution.

Time-lapse live cell imaging
Cells were seeded in wells/dishes with glass bottom or with bottom made of 0,18 mm microscopy plastic with highest optical quality (both from Ibidi) and cultured as described above. Cultures were video recorded from the moment of treatment (6 hours after thymidine release) for the indicated times. The microscope was a Nikon Ti Eclipse automated inverted microscope, equipped with a water jacket CO_2 stage incubator **(**Okolab), designed to mantain cultures under controlled environmental conditions right on the microscope stage. Before imaging, medium was replaced with DMEM without Phenol Red. Acquisition was performed without lids to optimise image quality and anti-evaporation oil (Ibidi) was added on top of the medium in order to avoid evaporation. Image acquisition was performed using a DS-Qi1MC (Nikon) camera and the software NIS-Elements AR 3.2 (Nikon) at 5 minutes intervals for 16 or 24 hours. Plan Fuor 40x (N.A. 0,60) and Plan Apo 60x (oil, N.A. 1,3) objectives were used and acquisitions were performed using differential interference contrast (DIC).
At the Advanced Light Microscopy Facility of Heidelberg, cells were seeded in 96-well dishes with glass surface. Images were acquired every 30 minutes for 48-65 hours with a 10x objective using an Olympus Cell^R Scan^R automated microscope with EMBL Environment box to control environmental conditions (http://www.embl.de/almf/almf_services/booking/micro_olympus_

scan/index.html). Hoechst (1:1000, Sigma) was added for 1 hour prior to video recording (and then removed), in order to stain DNA. Transmitted light and fluorescence images were acquired. Videos were mounted and analysed using Fiji (an image processing package of ImageJ) and NIS-Elements AR 4.0 (Nikon). Preliminary analyses were also performed using the software CecogAnalyzer (http://cellcognition.org).

WB

Cells were lysed in RIPA buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP40, 1 mM EGTA, 0.25% sodium deoxycholate) supplemented with protease and phosphatase inhibitors. Proteins were resolved by electrophoresis on 10% Laemmli gel and transferred on a nitrocellulose membrane (Protran BA83, Whatman) using a semi-dry system (BIO-RAD). 30 µg of extract per lane were loaded. Blocking and antibody incubations were performed at room temperature in TBS containing 0.1% Tween-20 and 5% low fat milk. Antibodies were: anti-Aurora-A (0.5 µg/ml), anti-Nuf2 (1:1 000, kind gift of V. Draviam), anti-phospho-Aurora-A (Thr288) (1:500; C39D8; Cell Signaling Technology), anti-actin (0.5 µg/ml, I-19; SantaCruz Biotechnology). Signals were visualised by enhanced chemiluminescence detection (ECL plus, GE Healthcare, and Protein Detection System, GeneSpin).

Oncomine™ (Compendia Bioscience) analyses

TPX2 overexpression studies were perfomed using the Oncomine™ database (www.oncomine.org), searching for TPX2 differential expression in tumor versus normal samples. A first threshold was set for highly significant overexpression ($p<0.0001$, Student's t-test); a second threshold was based on the gene rank, taking into account TPX2 ranking among the first 10%, 5% or 1% overexpressed genes in each analysis.
Co-expression analyses of Aurora-A and TPX2 were also performed. In each analysis, only datasets in which the reference gene was highly significantly ($p<0.0001$) and frequently

overexpressed were considered. Datasets include 6000–20000 genes. Datasets from tissues in which overexpression occurred in less than 25% (TPX2) or 20% (Aurora-A) of all examined cases, or containing data from non homogeneous tissues, were not considered.

REFERENCES

Anand S, Penrhyn-Lowe S, Venkitaraman AR. (2003) AURORA-A amplification overrides the mitotic spindle assembly checkpoint, inducing resistance to Taxol. *Cancer Cell*. 3:51-62.

Andresson T, Ruderman JV (1998) The kinase Eg2 is a component of the Xenopus oocyte progesterone-activated signaling pathway. *EMBO J* 17:5627-37.

Bayliss R, Sardon T, Vernos I, Conti E. (2003) Structural basis of Aurora-A activation by TPX2 at the mitotic spindle. *Mol. Cell* 12: 851-62.

Beroukhim R, Brunet JP, Di Napoli A, Mertz KD, Seeley A, Pires MM et al. (2009) Patterns of gene expression and copy-number alterations in von-hippel lindau disease-associated and sporadic clear cell carcinoma of the kidney. *Cancer Res*. 69: 4674-81.

Beroukhim R, Mermel CH, Porter D, Wei G, Raychaudhuri S, Donovan J et al. (2010) The landscape of somatic copy-number alteration across human cancers. *Nature*. 463: 899-905.

Bhattacharjee A, Richards WG, Staunton J, Li C, Monti S, Vasa P et al. (2001) Classification of human lung carcinomas by mRNA expression profiling reveals distinct adenocarcinoma subclasses. *Proc Natl Acad Sci U S A*. 98:13790-5.

Bischoff JR, Anderson L, Zhu Y, Mossie K, Ng L, Souza B et al. (1998) A homologue of Drosophila aurora kinase is oncogenic and amplified in human colorectal cancers. *EMBO J*. 17:3052-65.

Bischoff JR, Plowman GD. (1999) The Aurora/Ipl1p kinase family: regulators of chromosome segregation and cytokinesis. *Trends Cell Biol*. 9:454-9.

Blangy A, Lane HA, d'Hérin P, Harper M, Kress M, Nigg EA. (1995) Phosphorylation by p34cdc2 regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. *Cell*. 83:1159-69.

Boveri T. (1902) Ueber mehrpolige Mitosen als Mittel zur Analyse des Zellkerns. Verh Phys-med Ges.Würzburg NF; 35:67-90.

Boveri T. (1914) Zur Frage der Entstehung maligner Tumoren. (The origin of malignant tumors). Gustav Fischer, Jena.

Briassouli P, Chan F, Savage K, Reis-Filho JS, Linardopoulos S. (2007) Aurora-A regulation of nuclear factor-kappaB signaling by phosphorylation of IkappaBalpha. *Cancer Res*. 67:1689-95.

Brunet S, Sardon T, Zimmerman T, Wittmann T, Pepperkok R, Karsenti E, Vernos I. (2004) Characterization of the TPX2 domains involved in microtubule nucleation and spindle assembly in Xenopus egg extracts. *Mol Biol Cell*.15: 5318-28.

Carmena M, Earnshaw WC. (2003) The cellular geography of aurora kinases. Nat Rev *Mol Cell Biol*. 4:842-54.

Carmena M, Ruchaud S, Earnshaw WC. (2009) Making the Auroras glow: regulation of Aurora A and B kinase function by interacting proteins. Curr Opin Cell Biol. 21:796-805.

Carmena M, Wheelock M, Funabiki H, Earnshaw WC. (2012) The chromosomal passenger complex (CPC): from easy rider to the godfather of mitosis. *Nat Rev Mol Cell Biol*. 13:789-803.

Carter SL, Eklund AC, Kohane IS, Harris LN, Szallasi Z. (2006) A signature of chromosomal instability inferred from gene expression profiles predicts clinical outcome in multiple human cancers. *Nat. Genet*. 38: 1043-48.

Carvajal RD, Tse A, Schwartz GK. (2006) Aurora kinases: new targets for cancer therapy. Clin Cancer Res. 12:6869-75.

Cassimeris L, Morabito J. (2004) TOGp, the human homolog of XMAP215/Dis1, is required for centrosome integrity, spindle pole organization, and bipolar spindle assembly. *Mol Biol Cell*. 15:1580-90.

Chakravarty A, Shinde V, Tabernero J, Cervantes A, Cohen RB, Dees EC et al. (2011) Phase I assessment of new mechanism-based pharmacodynamic biomarkers for MLN8054, a small-molecule inhibitor of Aurora A kinase. *Cancer Res*. 71:675-85.

Chan CS, Botstein D. (1993) Isolation and characterization of chromosome-gain and increase-in-ploidy mutants in yeast. *Genetics* 135: 677-691.

Chen J, Sen S, Amos CI, Wei C, Jones JS, Lynch P, Frazier ML. (2007) Association between Aurora-A kinase polymorphisms and age of onset of hereditary nonpolyposis colorectal cancer in a Caucasian population. *Mol. Carcinog*. 46: 249-256.

Chen X, Leung SY, Yuen ST, Chu KM, Ji J, Li R, Chan AS, Law S, Troyanskaya OG, Wong J, So S, Botstein D, Brown PO. (2003) Variation in gene expression patterns in human gastric cancers. *Mol. Biol. Cell*. 14: 3208-15.

Chiang CM. (2012) p53-Aurora A mitotic feedback loop regulates cell cycle progression and genomic stability. *Cell Cycle*. 11:3719-20.

Compton DA, Luo C. (1995) Mutation of the predicted p34cdc2 phosphorylation sites in NuMA impair the assembly of the mitotic spindle and block mitosis. *J Cell Sci*.108:621-33.

Crane R, Kloepfer A, Ruderman JV. (2004) Requirements for the destruction of human Aurora-A. *J Cell Sci*.117:5975-83.

De Luca M, Brunetto L, Asteriti IA, Giubettini M, Lavia P, Guarguaglini G. (2008) Aurora-A and ch-TOG act in a common pathway in control of spindle pole integrity. *Oncogene*.27:6539-49.

De Luca M, Lavia P, Guarguaglini G. (2006) A functional interplay between Aurora-A, Plk1 and TPX2 at spindle poles: Plk1 controls centrosomal localization of Aurora-A and TPX2 spindle association. *Cell Cycle*. 5:296-303.

Dees EC, Infante JR, Cohen RB, O'Neil BH, Jones S, von Mehren M et al. (2011) Phase 1 study of MLN8054, a selective inhibitor of Aurora A kinase in patients with advanced solid tumors. *Cancer Chemother Pharmacol*. 67:945-54.

DeLuca JG, Moree B, Hickey JM, Kilmartin JV, Salmon ED. (2002) hNuf2 inhibition blocks stable kinetochore-microtubule attachment and induces mitotic cell death in HeLa cells. *J Cell Biol*. 159:549-55.

DeLuca JG, Musacchio A. (2012) Structural organization of the kinetochore-microtubule interface. Curr Opin Cell Biol. 24:48-56.

Detwiller KY, Fernando NT, Segal NH, Ryeom SW, D'Amore PA, Yoon SS. (2005) Analysis of hypoxia-related gene expression in sarcomas and effect of hypoxia on RNA interference of vascular endothelial cell growth factor A. *Cancer Res*. 65: 5881-9.

Dieterich K, Soto Rifo R, Faure AK, Hennebicq S, Ben Amar B, Zahi M et al. (2007) Homozygous mutation of AURKC yields large-headed polyploid spermatozoa and causes male infertility. *Nat Genet*. 39:661-5.

Ditchfield C, Johnson VL, Tighe A, Ellston R, Haworth C, Johnson T et al. (2003) Aurora B couples chromosome alignment with anaphase by targeting BubR1, Mad2, and Cenp-E to kinetochores. *J Cell Biol*. 161:267-80.

Doxsey S. (2001) Re-evaluating centrosome function. *Nat Rev Mol Cell Biol*. 2:688-98.

Dumont S, Mitchison TJ. (2009) Force and length in the mitotic spindle. *Curr Biol*. 19:R749-61.

Eyers PA, Erikson E, Chen LG, Maller JL. (2003) A novel mechanism for activation of the protein kinase Aurora A. *Curr. Biol*. 13: 691-97.

Fenner M, Wehrhan F, Jehle M, Amann K, Radespiel-Tröger M, Grabenbauer G et al. (2005) Restricted-expressed proliferation-associated protein (Repp 86) expression in squamous cell carcinoma of the oral cavity. *Strahlenther. Onkol*. 181: 755-61.

Fielding AB, Dobreva I, McDonald PC, Foster LJ, Dedhar S. (2008) Integrin-linked kinase localizes to the centrosome and regulates mitotic spindle organization. *J Cell Biol*. 180: 681-89.

Floyd S, Pines J, Lindon C. (2008) APC/C Cdh1 targets aurora kinase to control reorganization of the mitotic spindle at anaphase. *Curr Biol*.18:1649-58.

Fukuda T, Mishina Y, Walker MP, DiAugustine RP. (2005) Conditional transgenic system for mouse aurora a kinase: degradation by the ubiquitin proteasome pathway controls the level of the transgenic protein. *Mol. Cell. Biol*. 25: 5270-81.

Gadde S, Heald R. (2004) Mechanisms and molecules of the mitotic spindle. Curr Biol.14:R797-805.

Garber ME, Troyanskaya OG, Schluens K, Petersen S, Thaesler Z, Pacyna-Gengelbach M et al.(2001)Diversity of gene expression in adenocarcinoma of the lung. *Proc. Natl. Acad. Sci. U. S. A.* 98: 13784-89.

Garrett S, Auer K, Compton DA, Kapoor TM. (2002) hTPX2 is required for normal spindle morphology and centrosome integrity during vertebrate cell division. *Curr Biol.* 12:2055-9.

Gascoigne KE, Taylor SS. (2009) How do anti-mitotic drugs kill cancer cells? *J Cell Sci*.122: 2579-85.

Gaspar C, Cardoso J, Franken P, Molenaar L, Morreau H, Möslein G et al. (2008) Cross-species comparison of human and mouse intestinal polyps reveals conserved mechanisms in adenomatous polyposis coli (APC)-driven tumorigenesis. *Am. J. Pathol.* 172: 1363-80.

Gatlin JC, Bloom K. (2010) Microtubule motors in eukaryotic spindle assembly and maintenance. *Semin Cell Dev Biol.* 21:248-54.

Gautschi O, Heighway J, Mack PC, Purnell PR, Lara PN Jr., Gandara DR. (2008) Aurora kinases as anticancer drug targets. *Clin. Cancer Res.* 14: 1639-48.

Giet R, McLean D, Descamps S, Lee MJ, Raff JW, Prigent C, Glover DM (2002) Drosophila Aurora A kinase is required to localize D-TACC to centrosomes and to regulate astral microtubules. *J Cell Biol.* 156:437-51.

Giet R, Uzbekov R, Cubizolles F, Le Guellec K, Prigent C. (1999) The Xenopus laevis aurora-related protein kinase pEg2 associates with and phosphorylates the kinesin-related protein XlEg5. *J Biol Chem.* 274:15005-13.

Ginos MA, Page GP, Michalowicz BS, Patel KJ, Volker SE, Pambuccian SE et al. (2004) Identification of a gene expression signature associated with recurrent disease in squamous cell carcinoma of the head and neck. *Cancer Res*. 64: 55-63.

Giubettini M, Asteriti IA, Scrofani J, De Luca M, Lindon C, Lavia P, Guarguaglini G. (2011) Control of Aurora-A stability via interaction with TPX2. *J Cell Science* 124: 113-22.

Glover DM, Leibowitz MH, McLean DA, Parry H (1995) Mutations in aurora prevent centrosome separation leading to the formation of monopolar spindles. *Cell*. 81:95-105.

Goshima G. (2011) Identification of a TPX2-like microtubule-associated protein in Drosophila. *PLoS One*.6: e28120.

Graudens E, Boulanger V, Mollard C, Mariage-Samson R, Barlet X, Grémy G et al. (2006) Deciphering cellular states of innate tumor drug responses. *Genome Biol*. 7: R19-R39.

Green MR, Woolery JE, Mahadevan D. (2011) Update on Aurora Kinase Targeted Therapeutics in Oncology Expert Opin Drug Discov. Mar; 6(3):291-307.

Gritsko TM, Coppola D, Paciga JE, Yang L, Sun M, Shelley SA et al. (2003) Activation and overexpression of centrosome kinase BTAK/Aurora-A in human ovarian cancer. *Clin. Cancer Res*. 9: 1420-26.

Gruss OJ, Carazo-Salas RE, Schatz CA, Guarguaglini G, Kast J, Wilm M, Le Bot N, Vernos I, Karsenti E, Mattaj IW. (2001) Ran induces spindle assembly by reversing the inhibitory effect of importin alpha on TPX2 activity. *Cell*. 104:83-93.

Gruss OJ, Wittmann M, Yokoyama H, Pepperkok R, Kufer T, Silljé H et al. (2002). Chromosome-induced microtubule assembly mediated by TPX2 is required for spindle formation in HeLa cells. *Nat. Cell Biol.* 4: 871-79.

Gruss, O J, Vernos I. (2004). The mechanism of spindle assembly: functions of Ran and its target TPX2. *J. Cell Biol.* 166: 949-55.
Gu J, Gong Y, Huang M, Lu C, Spitz MR, Wu X. (2007) Polymorphisms of STK15 (Aurora-A) gene and lung cancer risk in Caucasians, Carcinogenesis 28. 350-55.

Gumz ML, Zou H, Kreinest PA, Childs AC, Belmonte LS, LeGrand SN et al. (2007) Secreted frizzled-related protein 1 loss contributes to tumor phenotype of clear cell renal cell carcinoma. *Clin Cancer Res.* 13:4740-9.

Hannak E, Kirkham M, Hyman AA, Oegema, K. (2001) Aurora-A kinase is required for centrosome maturation in Caenorhabditis elegans. *J. Cell Biol.* 155, 1109-16.

Haren L, Gnadt N, Wright M, Merdes A. (2009) NuMA is required for proper spindle assembly and chromosome alignment in prometaphase. *BMC Res Notes.* 2:64.

Hata T, Furukawa T, Sunamura M, Egawa S, Motoi F, Ohmura N et al. (2005) RNA interference targeting aurora kinase a suppresses tumor growth and enhances the taxane chemosensitivity in human pancreatic cancer cells. *Cancer Res.* 65:2899-905.

Hégarat N, Smith E, Nayak G, Takeda S, Eyers PA, Hochegger H. (2011) Aurora A and Aurora B jointly coordinate chromosome segregation and anaphase microtubule dynamics. *J Cell Biol.* 195:1103-13.

Hienonen T, Salovaara R, Mecklin JP, Järvinen H, Karhu A, Aaltonen LA. (2006) Preferential amplification of AURKA 91A (Ile31) in familial colorectal cancers. *Int. J. Cancer*. 118: 505-08.

Hirota T, Kunitoku N, Sasayama T, Marumoto T, Zhang D, Nitta M et al. (2003) Aurora-A and an interacting activator, the LIM protein Ajuba, are required for mitotic commitment in human cells. *Cell*. 114: 585-598.

Hodgson JG, Chin K, Collins C, Gray JW. (2003) Genome amplification of chromosome 20 in breast cancer. *Breast Cancer Res. Treat*. 78 337-45.

Holland AJ, Cleveland DW. (2009) Boveri revisited: chromosomal instability, aneuploidy and tumorigenesis. *Nat Rev Mol Cell Biol*. 10: 478-87.

Holland AJ, Cleveland DW. (2012) Losing balance: the origin and impact of aneuploidy in cancer. *EMBO Rep*.13:501-14.

Hufton SE, Moerkerk PT, Brandwijk R, de Bruïne AP, Arends JW, Hoogenboom HR. (1999) A profile of differentially expressed genes in primary colorectal cancer using suppression subtractive hybridization. *FEBS Lett*. 463: 77-82.

Inoué S, Sato H. (1967) Cell motility by labile association of molecules. The nature of mitotic spindle fibers and their role in chromosome movement. *J Gen Physiol*. 50 (Suppl):259-92.

Janssen A, Medema RH. (2011) Mitosis as an anti-cancer target. *Oncogene*. 30:2799-809.

Jiang S, Katayama H, Wang J, Li SA, Hong Y, Radvanyi L et al. (2010) Estrogen-induced aurora kinase-A (AURKA) gene

expression is activated by GATA-3 in estrogen receptorpositive breast cancer cells. *Horm Cancer*. 1:11-20.

Johnston CA, Hirono K, Prehoda KE, Doe CQ. (2009) Identification of an Aurora-A/PinsLINKER/Dlg spindle orientation pathway using induced cell polarity in S2 cells. Cell. 138:1150-63.

Ju H, Cho H, Kim YS, Kim WH, Ihm C, Noh SM et al. (2006) Functional polymorphism 57 ValNIle of Aurora kinase A associated with increased risk of gastric cancer progression. *Cancer Lett*. 242: 273-79.

Kadara H, Lacroix L, Behrens C, Solis L, Gu X, Lee JJ, et al. (2009) Identification of gene signatures and molecular markers for human lung cancer prognosis using an in vitro lung carcinogenesis system. *Cancer Prev. Res*. 2: 702-11.

Kaiser S, Park YK, Franklin JL, Halberg RB, Yu M, Jessen WJ et al.(2007) Transcriptional recapitulation andsubversion of embryonic colon development by mouse colon tumor models and human colon cancer. *Genome Biol*. 8 R131.

Kapoor TM, Mayer TU, Coughlin ML, Mitchison TJ. (2000) Probing spindle assembly mechanisms with monastrol, a small molecule inhibitor of the mitotic kinesin, Eg5. *J Cell Biol*. 150:975-88.

Katayama H, Sen S. (2010) Aurora kinase inhibitors as anticancer molecules. *Biochim Biophys Acta*. 1799:829-39.

Kelling J, Sullivan K, Wilson L, Jordan MA. (2003) Suppression of centromere dynamics by Taxol in living osteosarcoma cells. *Cancer Res*. 63: 2794-801.

Kelly KR, Nawrocki ST, Espitia CM, Zhang M, Yang JJ, Padmanabhan S et al. (2012) Targeting Aurora A kinase activity with the investigational agent alisertib increases the efficacy of cytarabine through a FOXO-dependent mechanism. *Int J Cancer*. 131:2693-703.

Ki DH, Jeung HC, Park CH, Kang SH, Lee GY, Lee WS et al (2007) Whole genome analysis for liver metastasis gene signatures in colorectal cancer. *Int J Cancer*. 121: 2005-12.

Kimmins S, Crosio C, Kotaja N, Hirayama J, Monaco L, Höög C, et al. (2007) Differential functions of the Aurora-B and Aurora-C kinases in mammalian spermatogenesis. *Mol Endocrinol*. 21:726-39.

Kimura M, Kotani S, Hattori T, Sumi N, Yoshioka T, Todokoro K, Okano Y. (1997) Cell cycle-dependent expression and spindle pole localization of a novel human protein kinase, Aik, related to Aurora of Drosophila and yeast Ipl1. *J Biol Chem*. 272:13766-71.

Kimura M, Matsuda Y, Yoshioka T, Okano Y. (1999) Cell cycle-dependent expression and centrosome localization of a third human aurora/Ipl1-related protein kinase, AIK3. *J Biol Chem*. 274:7334-40.

Kitajima S, Kudo Y, Ogawa I, Tatsuka M, Kawai H, Pagano M, Tarata T. (2007) Constitutive phosphorylation of aurora-a on ser51 induces its stabilization and consequent overexpression in cancer, PLoS ONE 2: e944.

Knuutila S, Björkqvist AM, Autio K, Tarkkanen M, Wolf M, Monni O, Szymanska J et al. (1998) DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies, *Am. J. Pathol*. 152: 1107-23.

Koffa MD, Casanova CM, Santarella R, Köcher T, Wilm M, Mattaj IW. (2006) HURP is part of a Ran-dependent complex involved in spindle formation. *Curr Biol*. 16: 743-54.

Kollareddy M, Dzubak P, Zheleva D, Hajduch M. (2008) Aurora kinases: structure, functions and their association with cancer. *Biomed Pap Med Fac Univ Palacky Olomouc Czech Repub*. 152: 27-33.

Kufer TA, Silljé H HW, Korner R, Gruss O J, Meraldi P, Nigg EA. (2002) Human TPX2 is required for targeting Aurora-A kinase to the spindle. *J. Cell Biol*. 158: 617-23.

Kurashina K, Yamashita Y, Ueno T, Koinuma K, Ohashi J, Horie H, et al. (2008) Chromosome copy number analysis in screening for prognosisrelated genomic regions in colorectal carcinoma. *Cancer Sci*. 99: 1835-40.

Landi MT, Dracheva T, Rotunno M, Figueroa JD, Liu H, Dasgupta A, Mann FE (2008) Gene expression signature of cigarette smoking and its role in lung adenocarcinoma development and survival. PLoS ONE 3: e1651.

Lapenna S, Giordano A. (2009) Cell cycle kinases as therapeutic targets for cancer. *Nat Rev Drug Discov*. 8:547-66.

Lee HH, Zhu Y, Govindasamy KM, Gopalan G. (2008) Downregulation of Aurora-A overrides estrogen-mediated growth and chemoresistance in breast cancer cells. *Endocr. Relat. Cancer*. 15:765-75.

Li B, Qi XQ, Chen X, Huang X, Liu GY, Chen HR et al. (2010) Expression of targeting protein for Xenopus kinesin-like protein 2 is associated with progression of human malignant astrocytoma. *Brain Res*.1352: 200-7.

Li CC, Chu HY, Yang CW, Chou CK, Tsai TF. (2009) Aurora-A overexpression in mouse liver causes p53-dependent premitotic arrest during liver regeneration. *Mol. Cancer Res.* 7: 678-688.

Liu Q, Kaneko S, Yang L, Feldman RI, Nicosia SV, Chen J, Cheng JQ.(2004) Aurora-A abrogation of p53 DNA binding and transactivation activity by phosphorylation of serine 215. *J. Biol. Chem.* 279:52175-182.

Lu KH, Patterson AP, Wang L, Marquez RT, Atkinson EN, Baggerly KA et al. (2004) Selection of potential markers for epithelial ovarian cancer with gene expression arrays and recursive descent partition analysis. *Clin Cancer Res.* 10:3291-300.

Ma N, Tulu US, Ferenz NP, Fagerstrom C, Wilde A, Wadsworth P. (2010) Poleward transport of TPX2 in the mammalian mitotic spindle requires dynein, Eg5, and microtubule flux. *Mol Biol Cell.* 21:979-88.

Ma Y, Lin D, Sun W, Xiao T, Yuan J, Han N et al. (2006) Expression of targeting protein for xklp2 associated with both malignant transformation of respiratory epithelium and progression of squamous cell lung cancer. *Clin. Cancer Res.* 12: 1121-27.

Macarulla T, Cervantes A, Elez E, Rodríguez-Braun E, Baselga J, Roselló S et al. (2010) Phase I study of the selective Aurora A kinase inhibitor MLN8054 in patients with advanced solid tumors: safety, pharmacokinetics, and pharmacodynamics. *Mol Cancer Ther.* 9:2844-52.

Manda R, Kohno T, Matsuno Y, Takenoshita S, Kuwano H, Yokota J. (1999) Identification of genes (SPON2 and C20orf2) differentially expressed between cancerous and noncancerous lung cells by mRNA differential display. *Genomics* 61: 5-14.

Manfredi MG, Ecsedy JA, Chakravarty A, Silverman L, Zhang M, Hoar KM et al. (2011) Characterization of Alisertib (MLN8237), an investigational small-molecule inhibitor of aurora A kinase using novel in vivo pharmacodynamic assays. *Clin Cancer Res*. 17:7614-24.

Manning AL, Compton DA. (2007) Mechanisms of spindle-pole organization are influenced by kinetochore activity in mammalian cells. *Curr Biol*. 17:260-5.

Manning AL, Compton DA. (2008) Structural and regulatory roles of nonmotor spindle proteins. *Curr Opin Cell Biol*. 20:101-6.

Mao Y, Varma D, Vallee R. (2010) Emerging functions of force-producing kinetochore motors. *Cell Cycle*. 9:715-9.

Mardin BR, Schiebel E. (2012) Breaking the ties that bind: new advances in centrosome biology. *J Cell Biol*.197:11-8.

Marumoto T, Hirota T, Morisaki T, Kunitoku N, Zhang D, Ichikawa Y et al. (2002) Roles of aurora-A kinase in mitotic entry and G2 checkpoint in mammalian cells. *Genes Cells*. 7:1173-82.

Marumoto T, Honda S, Hara T, Nitta M, Hirota T, Kohmura E, Saya H. (2003) Aurora-A kinase maintains the fidelity of early and late mitotic events in HeLa cells. *J Biol Chem*. 278:51786-95.

Marumoto T, Zhang D, Saya H (2005) Aurora-A—a guardian of poles. *Nat Rev Cancer* 5:42-50.

Mayer TU, Kapoor TM, Haggarty SJ, King RW, Schreiber SL, Mitchison TJ. (1999) Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. *Science*. 286:971-4.

Mazumdar M, Misteli T. (2005) Chromokinesins: multitalented players in mitosis. *Trends Cell Biol*. 15:349-55.

McHedlishvili N, Wieser S, Holtackers R, Mouysset J, Belwal M, Amaro AC, Meraldi P. (2012) Kinetochores accelerate centrosome separation to ensure faithful chromosome segregation. *J Cell Sci*.125:906-18.

Meraldi P, Honda R, Nigg EA. (2002) Aurora-A overexpression reveals tetraploidization as a major route to centrosome amplification in p53–/– cells. *EMBO J*. 21: 483-92.

Mitelman F, Johansson B, Mertens FE. (2012) Mitelman Database of Chromosome Aberrations and Gene Fusions in Cancer. http://cgap.nci.nih.gov/Chromosomes/Mitelman

Morgan-Lappe SE, Tucker LA, Huang X, Zhang Q, Sarthy AV, Zakula D et al. (2007) Identification of Ras-related nuclear protein, targeting protein for xenopus kinesin-like protein 2, and stearoyl-CoA desaturase 1 as promising cancer targets from an RNAi-based screen. *Cancer Res*. 67: 4390-4398.

Mori D, Yano Y, Toyo-oka K, Yoshida N, Yamada M, Muramatsu M et al. (2007) NDEL1 phosphorylation by Aurora-A kinase is essential for centrosomal maturation, separation, and TACC3 recruitment. *Mol Cell Biol* 27:352-67.

Musacchio A, Salmon ED. (2007) The spindle-assembly checkpoint in space and time. *Nat Rev Mol Cell Biol*. 8:379-93.

Nikonova AS, Astsaturov I, Serebriiskii IG, Dunbrack RL Jr, Golemis EA. (2012) Aurora A kinase (AURKA) in normal and pathological cell division. *Cell Mol Life Sci*. [Epub ahead of print]

O'Connell CB, Khodjakov AL. (2007) Cooperative mechanisms of mitotic spindle formation. *J Cell Sci*. 120:1717-22.

Otto T, Horn S, Brockmann M, Eilers U, Schuttrumpf L, Popov N, et al.. (2009) Stabilization of N-Myc is a critical function of Aurora A in human neuroblastoma. *Cancer Cell* 15: 67-78.

Ouchi M, Fujiuchi N, Sasai K, Katayama H, Minamishima YA, Ongusaha PP et al.(2004) BRCA1 phosphorylation by Aurora-A in the regulation of G2 to M transition. *J Biol Chem* 279:19643–48.

Ozlü N, Srayko M, Kinoshita K, Habermann B, O'toole ET, Müller-Reichert T et al. (2005) An essential function of the C. elegans ortholog of TPX2 is to localize activated aurora A kinase to mitotic spindles. *Dev Cell*. 9:237-48.

Paris J, Philippe M (1990) Poly(A) metabolism and polysomal recruitment of maternal mRNAs during early Xenopus development. *Dev Biol* 140:221-24.

Pérez de Castro I, de Cárcer G, Malumbres M. (2007) A census of mitotic cancer genes: new insights into tumor cell biology and cancer therapy. *Carcinogenesis*. 28: 899-912.

Pihan GA, Purohit A, Wallace J, Malhotra R, Liotta L, Doxsey SJ. (2001)Centrosome defects can account for cellular and genetic changes that characterize prostate cancer progression. *Cancer Res*. 61:2212-9.

Pugacheva EN, Golemis EA. (2005) The focal adhesion scaffolding protein HEF1 regulates activation of the Aurora-A and Nek2 kinases at the centrosome. *Nat. Cell Biol*.7:937-46.

Richardson AL, Wang ZC, De Nicolo A, Lu X, Brown M, Miron A, Liao X, Iglehart JD, Livingston DM, Ganesan S. (2006) X

chromosomal abnormalities in basal-like human breast cancer. *Cancer Cell*. 9:121-32.

Roghi C, Giet R, Uzbekov R, Morin N, Chartrain I, Le Guellec R et al. (1998) The Xenopus protein kinase pEg2 associates with the centrosome in a cell cycle-dependent manner, binds to the spindle microtubules and is involved in bipolar mitotic spindle assembly. *J Cell Sci* 111:557-72.

Rowinsky EK, Wright M, Monsarrat B, Lesser GJ, Donehower RC. (1993) Taxol: pharmacology, metabolism and clinical implications. *Cancer Surv*.17:283-304.

Sakakura C, Hagiwara A, Yasuoka R, Fujita Y, Nakanishi M, Masuda K et al. (2001) Tumour-amplified kinase BTAK is amplified and overexpressed in gastric cancers with possible involvement in aneuploid formation. *Br. J. Cancer* 84: 824-31.

Sanbhnani S, Yeong FM. (2012) CHFR: a key checkpoint component implicated in a wide range of cancers. *Cell Mol. Life Sci*. 69:1669-87.

Sanchez-Carbayo M, Socci ND, Lozano J, Saint F, Cordon-Cardo C. (2006) Defining molecular profiles of poor outcome in patients with invasive bladder cancer using oligonucleotide microarrays. *J Clin Oncol*. 24:778-89.

Sankaran S, Crone DE, Palazzo RE, Parvin JD. (2007) Aurora-A kinase regulates breast cancer associated gene 1 inhibition of centrosome-dependent microtubule nucleation. *Cancer Res*. 67:11186-94.

Santaguida S, Musacchio A. (2009) The life and miracles of kinetochores. *EMBO J*. 28:2511-31.

Satow R, Shitashige M, Kanai Y, Takeshita F, Ojima H, Jigami T, et al. (2010) Combined functional genome survey of therapeutic targets for hepatocellular carcinoma. *Clin. Cancer Res.* 16: 2518-28.

Scharer CD, Laycock N, Osunkoya AO, Logani S, McDonald JF, Benigno BB, Moreno CS. (2008) Aurora kinase inhibitors synergize with paclitaxel to induce apoptosis in ovarian cancer cells, *J. Transl. Med.* 6:79.

Schatz CA, Santarella R, Hoenger A, Karsenti E, Mattaj IW, Gruss OJ, Carazo-Salas RE. (2003) Importin alpha-regulated nucleation of microtubules by TPX2. *EMBO J.* 22: 2060-70.

Schumacher JM, Ashcroft N, Donovan PJ, Golden A. (1998) A highly conserved centrosomal kinase, AIR-1, is required for accurate cell cycle progression and segregation of developmental factors in Caenorhabditis elegans embryos. *Development*.125:4391-402.

Scotto L, Narayan G, Nandula SV, Arias-Pulido H, Subramaniyam S, Schneider A et al. (2008) Identification of copy number gain and overexpressed genes on chromosome arm 20q by an integrative genomic approach in cervical cancer: potential role in progression. *Genes Chromosom. Cancer.* 47: 755-65.

Sen S, Zhou H, White RA. (1997) A putative serine/threonine kinase encoding gene BTAK on chromosome 20q13 is amplified and overexpressed in human breast cancer cell lines. *Oncogene*. 14:2195-200.

Sharp DJ, Rogers GC, Scholey JM. (2000) Microtubule motors in mitosis. *Nature*. 407:41-7.

Shigeishi H, Fujimoto S, Hiraoka M, Ono S, Taki M, Ohta K et al. (2009) Overexpression of the receptor for hyaluronan-mediated motility,correlates with expression of microtubule-associated protein in human oral squamous cell carcinomas. *Int. J. Oncol*. 34: 1565-71.

Shigeishi H, Ohta K, Hiraoka M, Fujimoto S, Minami M, Higashikawa K, Kamata N. (2009) Expression of TPX2 in salivary gland carcinomas. *Oncol. Rep*. 21: 341-44.

Sillars-Hardebol AH, Carvalho B, Tijssen M, Beliën JA, de Wit M, Delis-van Diemen PM, Pontén F, van de Wiel MA, Fijneman RJ, Meijer GA. (2012) TPX2 and AURKA promote 20q amplicon-driven colorectal adenoma to carcinoma progression. Gut. 61:1568-75.

Sloane DA, Trikic MZ, Chu ML, Lamers MB, Mason CS, Mueller I, et al. (2010) Drug-resistant aurora A mutants for cellular target validation of the small molecule kinase inhibitors MLN8054 and MLN8237. *ACS Chem Biol*. 5:563-76.

Stearman RS, Dwyer-Nield L, Zerbe L, Blaine SA, Chan Z, Bunn PA Jr. et al. (2005) Analysis of orthologous gene expression between human pulmonary adenocarcinoma and a carcinogeninduced murine model. *Am. J. Pathol*. 167: 1763-75.

Stewart S, Fang G. (2005) Anaphase-promoting complex/cyclosome controls the stability of TPX2 during mitotic exit. *Mol Cell Biol*. 25:10516-27.

Su LJ, Chang CW, Wu YC, Chen KC, Lin CJ, Liang SC et al. (2007) Selection of DDX5 as a novel internal control for Q-RT-

PCR from microarray data using a block bootstrap re-sampling scheme. *BMC Genomics* 8: 140.

Taheri ZM, Mehrafza M, Mohammadi F, Khoddami M, Bahadori M, Masjedi MR. (2008) The diagnostic value of Ki-67 and repp 86 in distinguishing between benign and malignant mesothelial proliferations. *Arch. Pathol. Lab. Med.* 132: 694-7.

Tanaka T, Kimura M, Matsunaga K, Fukada D, Mori H, Okano Y. (1999) Centrosomal kinase AIK1 is overexpressed in invasive ductal carcinoma of the breast. *Cancer Res.* 59: 2041-44.

Tanenbaum ME, Macůrek L, Galjart N, Medema RH. (2008) Dynein, Lis1 and CLIP-170 counteract Eg5-dependent centrosome separation during bipolar spindle assembly. *EMBO J.* 27:3235-45.

Terada Y, Tatsuka M, Suzuki F, Yasuda Y, Fujita S, Otsu M. (1998) AIM-1: a mammalian midbody-associated protein required for cytokinesis. *EMBO J.* 17:667-76.

Tikhmyanova N, Little JL, Golemis EA. (2010) CAS proteins in normal and pathological cell growth control. *Cell Mol. Life Sci.* 67:1025-48.

Toji S, Yabuta N, Hosomi T, Nishihara S, Kobayashi T, Suzuki S et al. (2004) The centrosomal protein Lats2 is a phosphorylation target of Aurora-A kinase. Genes Cells 9:383-97.

Tonon G, Wong KK, Maulik G, Brennan C, Feng B, Zhang Y et al. (2005) High-resolution genomic profiles of human lung cancer. *Proc. Natl Acad. Sci. USA* 102: 9625–9630.

Torchia EC, Chen Y, Sheng H, Katayama H, Fitzpatrick J, Brinkley WR et al. (2009) A genetic variant of Aurora kinase A

promotes genomic instability leading to highly malignant skin tumors. *Cancer Res*. 69: 7207-15.

Toso A, Winter JR, Garrod AJ, Amaro AC, Meraldi P, McAinsh AD. (2009) Kinetochore-generated pushing forces separate centrosomes during bipolar spindle assembly. *J Cell Biol*.184:365-72.

Tsai MY, Wiese C, Cao K, Martin O, Donovan P, Rudermann J, et al. (2003) A Ran signalling pathway mediated by the mitotic kinase Aurora-A in spindle assembly. *Nat. Cell Biol*. 5: 242-48.

Vader G, Lens SM. (2008) The Aurora kinase family in cell division and cancer. *Biochim. Biophys. Acta*.1786:60-72.

van Vugt MA, Bras A, Medema RH (2004) Polo-like kinase-1controls recovery from a G2 DNA damage-induced arrest in mammalian cells. Mol Cell 15:799-811.

Wachi S, Yoneda K, Wu R. (2005) Interactome-transcriptome analysis reveals the high centrality of genes differentially expressed in lung cancer tissues. *Bioinformatics* 21: 4205-08.

Walczak CE, Cai S, Khodjakov A. (2010) Mechanisms of chromosome behaviour during mitosis. *Nat Rev Mol Cell Biol*. 11:91-102.

Wang X, Zhou YX, Qiao W, Tominaga Y, Ouchi M, Ouchi T, Deng CX. (2006) Overexpression of aurora kinase A in mouse mammary epithelium induces genetic instability preceding mammary tumor formation. *Oncogene*. 25: 7148-58.

Wang Y, Han KJ, Pang XW, Vaughan HA, Qu W, Dong XY et al. (2002) Large scale identification of human hepatocellular

carcinoma-associated antigens by autoantibodies. *J. Immunol*. 169: 1102-09.

Warner SL, Muñoz RM, Bearss DJ, Grippo P, Han H, Von Hoff DD. (2008) Pdx-1-driven overexpression of aurora a kinase induces mild ductal dysplasia of pancreatic ducts near islets in transgenic mice. *Pancreas*. 37: e39-e44.

Warner SL, Stephens BJ, Nwokenkwo S, Hostetter G, Sugeng A, Hidalgo M et al. (2009) Validation of TPX2 as a potential therapeutic target in pancreatic cancer cells. *Clin. Cancer Res*. 15: 6519-28.

Waters JC, Mitchison TJ, Rieder CL, Salmon ED. (1996) The kinetochore microtubule minus-end disassembly associated with poleward flux produces a force that can do work. *Mol Biol Cell*. 7:1547-58.

Weaver BA, Cleveland DW. (2006) Does aneuploidy cause cancer? Curr Opin Cell Biol. 18:658-67.

Weaver BA, Cleveland DW. (2007) Aneuploidy: instigator and inhibitor of tumorigenesis. Cancer Res. 67:10103-5.

Wittmann T, Boleti H, Antony C, Karsenti E, Vernos I. (1998) Localization of the kinesin-like protein Xklp2 to spindle poles requires a leucine zipper, a microtubule-associated protein, and dynein, *J. Cell Biol*. 143 673-85.

Wittmann T, Wilm M, Karsenti E, Vernos I. (2000) TPX2, A novel xenopus MAP involved in spindle pole organization, *J. Cell Biol*. 149 1405-18.

Wurmbach E, Chen YB, Khitrov G, Zhang W, Roayaie S, Schwartz M et al (2007) Genome-wide molecular profiles of HCV-

induced dysplasia and hepatocellular carcinoma. *Hepatology*. 45:938-47.

Xu J, Li H, Wang B, Xu Y, Yang J, Zhang X, Harten SK et al. (2010) VHL inactivation induces HEF1 and Aurora kinase A. *J. Am. Soc. Nephrol*. 21: 2041-46.

Yamada M, Hirotsune S, Wynshaw-Boris A. (2010) The essential role of LIS1, NDEL1 and Aurora-A in polarity formation and microtubule organization during neurogensis. *Cell Adh Migr*. 4:180-4.

Yang H, Burke T, Dempsey J, Diaz B, Collins E, Toth J, Beckmann R, Ye X. (2005) Mitotic requirement for aurora A kinase is bypassed in the absence of aurora B kinase. *FEBS Lett*. 579:3385-91.

Ye H, Yu T, Temam S, Ziober BL, Wang J, Schwartz JL, Mao L, et al. (2008) Transcriptomic dissection of tongue squamous cell carcinoma. *BMC Genomics* 9: 69.

Yu YP, Landsittel D, Jing L, Nelson J, Ren B, Liu L et al. (2004) Gene expression alterations in prostate cancer predicting tumor aggression and preceding development of malignancy. *J Clin Oncol*. 22: 2790-9.

Zeng K, Bastos RN, Barr FA, Gruneberg U. (2010) Protein phosphatase 6 regulates mitotic spindle formation by controlling the T-loop phosphorylation state of Aurora A bound to its activator TPX2. *J Cell Biol*. 191:1315-32.

Zhang D, Hirota T, Marumoto T, Shimizu M, Kunitoku N, Sasayama T et al. (2004) Cre-loxP-controlled periodic Aurora-A overexpression induces mitotic abnormalities and hyperplasia in mammary glands of mouse models. *Oncogene*. 18: 8720-8730.

Zhang L, Huang H, Deng L, Chu M, Xu L, Fu J et al. (2008) TPX2 in malignantly transformed human bronchial epithelial cells by anti-benzo[a]pyrene-7, 8-diol-9, 10-epoxide. *Toxicology*. 252: 49-55.

Zhang X, Ems-McClung SC, Walczak CE. (2008) Aurora A phosphorylates MCAK to control ran-dependent spindle bipolarity. Mol Biol Cell. 19:2752-65.

Zhao H, Langerød A, Ji Y, Nowels KW, Nesland JM, Tibshirani R, Bukholm IK et al. (2004) Different gene expression patterns in invasive lobular and ductal carcinomas of the breast. *Mol Biol Cell*. 15:2523-36.

Zhou H, Kuang J, Zhong L, Kuo WL, Gray JW, Sahin A et al. (1998) Tumour amplified kinase STK15/BTAK induces centrosome amplification, aneuploidy and transformation. *Nat. Genet*. 20: 189-93.

LIST OF ALL PUBLICATIONS

1. **I.A. Asteriti**, M. Giubettini, P. Lavia and G. Guarguaglini. (2011) Aurora-A inactivation causes mitotic spindle poloe fragmentation by unbalancing microtubule-generated forces *Mol Cancer* 10:131.

2. M. Giubettini, **I.A. Asteriti**, J Scrofani, M. De Luca, C. Lindon, P. Lavia and G. Guarguaglini. (2011) Control of Aurora-A stability via interaction with TPX2. *J Cell Science* 124: 113-122.

3. **I.A. Asteriti**, W. M.Rensen, C. Lindon, P. Lavia and G. Guarguaglini (2010). The Aurora-A/TPX2 complex: a novel oncogenic 'holoenzyme'? *BBA Rev Cancer* 1806: 230-239.

4. M. De Luca, L. Brunetto**, I.A. Asteriti**, M. Giubettini, P. Lavia and G. Guarguaglini (2008) Aurora-A and ch-TOG act in a common pathway in control of spindle pole integrity. *Oncogene* 27: 6539-6549.