Master of Science Dissertation

Unravelling novel pathways of spindle orientation defects in *Drosophila*

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Abstract

Proper spindle orientation in mitosis is critical for faithful distribution of the genetic material to daughter cells and for determination of the progeny fate. Spindle mis-orientation is intimately associated with cancer progression as it is proposed to generate aneuploidy, potentiate tissue disorganization, metastization and cancer stem cell pool expansion.

Here, we show *in vitro* and *in vivo*, that inducing overexpression of MPS1, a checkpoint kinase frequently overexpressed in colorectal cancer, causes massive spindle orientation defects in *Drosophila*. Live cell imaging of S2 cells in mitosis revealed that MPS1 overexpression leads to a pronounced rotation of the spindle. Importantly, overexpressing a kinase-dead version of MPS1 or active MPS1 tethered to kinetochores failed to impose a similar phenotype, indicating that spindle rotation is caused by excessive activity of cytosolic MPS1. Importantly, rotation of the mitotic spindle was also observed in *Drosophila* follicular epithelium cells overexpressing Mps1. This resulted in loss of normal tissue architecture, forming a multilayered epithelium as opposed to the monolayer observed under control conditions.

In metazoans, spindle orientation and positioning is controlled by pulling forces exerted on astral microtubules by dynein/dynactin motor complex present at the cell cortex and by the evolutionarily conserved Gαi/LGN/NuMA complex that polarizes cortical force generators. RNAi-mediated depletion of dynein, dynactin or Mud/NuMA prevented spindle rotation in MPS1-overexpressing cells, suggesting that excessive MPS1 deregulates spindle orientation through the canonical orientation pathway. Spindle and astral microtubules stability was not affected by MPS1 overexpression as well as Mud localization pattern and levels. Moreover, expressing a phosphomimetic version of Dynein Light Intermediate Chain (DLIC), whose phosphorylation on Ser^{432} was shown to be under control of MPS1, did not cause an evident rotation of the mitotic spindle.

The present work provides data describing an abnormal spindle rotation in both S2 and follicular epithelium cells and its consequences for tissue organization. We uncovered preliminary insights underlying the observed rotation, yet molecular mechanisms are still elusive. We believe that unravelling those molecular cues will generate critical knowledge to understand the causes of spindle mis-orientation in cancer and provide a well-characterized set of molecular signatures that might be used to assess tumor aggressiveness and invasion potential.

Keywords: MPS1 Kinase, Spindle rotation, tissue disruption, Mud/Dynein/Dynactin complex.

Resumo

A orientação correta do fuso acromático na mitose é essencial para a fiel distribuição do material genético pelas células filhas e para o seu destino. A orientação errada do fuso está intimamente associada com a progressão do cancro, uma vez que pode gerar aneuploidia, potencializar a desorganização do tecido, a metastização e a expansão do número de células estaminais cancerígenas.

Neste estudo, mostramos *in vitro* e *in vivo* que a sobreexpressão de MPS1, uma cinase envolvida na regulação da mitose e frequentemente sobreexpressa em cancro colorretal, provoca defeitos de orientação do fuso em *Drosophila*. Microscopia ao vivo de células S2 mitóticas revelou que a sobreexpressão de MPS1 leva a uma rotação pronunciada do fuso. Além disso, a sobreexpressão de uma versão inativa da cinase de ou de uma versão ativa e presa aos cinetocoros não conseguiu reproduzir um fenótipo semelhante, indicando que a rotação do fuso é causada por atividade excessiva do MPS1 citosólico. A rotação do fuso mitótico também foi observada nas células do epitélio folicular de *Drosophila* que sobreexpressam MPS1. Isso resultou na perda de arquitetura de tecido normal, formando um epitélio em multicamadas por oposição à monocamada observada sob condições controlo.

Nos animais, a orientação e o posicionamento do fuso são controlados por forças de tração exercidas sobre microtúbulos astrais pelo complexo motor de dineína / dinactina presente no córtex celular e pelo complexo Gαi / LGN / NuMA. Este complexo é conservado evolutivamente, e polariza as proteínas geradoras de força mecânica, no córtex celular. A depleção, por RNAi, de dineína, dynactin ou Mud / NuMA reduziu o fenótipo de rotação do fuso nas células que sobreexpressam MPS1, sugerindo que elevados níveis de MPS1 desregulam a orientação do fuso através da via de orientação canónica. A estabilidade dos microtúbulos astral e do fuso central não foi afetada pela sobreexpressão da MPS1, bem como o padrão e níveis de localização da proteína Mud. Além disso, expressando uma versão fosfomimética da cadeia intermédia-leve da dineína (DLIC), cuja fosforilação na Ser^{432} tinha sido descrita como estando sob controlo de MPS1, não causou uma rotação evidente do fuso mitótico.

O presente trabalho fornece dados que descrevem uma rotação anormal do fuso mitótico tanto nas células S2 como no epitélio folicular e as suas consequências para a organização do tecido. Descobrimos informações preliminares subjacentes à rotação observada, mas os mecanismos moleculares ainda são desconhecidos. Acreditamos que desvendar essas pistas moleculares gerará conhecimento crítico para entender as causas da má orientação do fuso no cancro e fornecer um conjunto de assinaturas moleculares bem caracterizadas que podem ser usadas para avaliar a agressividade e o potencial de invasão do tumor.

Palavras-chave: Cinase MPS1, rotação do fuso mitótico, disrupção do tecido, complexo Mud/Dineína/Dinactina.

Table of Contents

Table of Figures

List of Abbreviations

APC	Adenomatous Polyposis Coli
APC/C	Anaphase Promoting Complex / Cyclosome
aPKC	atypical Protein Kinase C
AurA	Aurora A
Bub1	Budding uninhibited by benzimidazole
BubR1	Bub1-Related protein kinase
C. elegans	*Caenorhabditis elegans*
Cdc16	Cell division cycle protein 16
Cdk1	Cyclin-dependent kinase
CID	Centromere identifier
CycB	Cyclin B
DAPI	4,6-diamidino-2-phenylindole
DHC	Dynein Heavy Chain
DLIC	Dynein Light Intermediate Chain
$DLIC^{S432D}$	DLIC mutated on Serine 432 to Aspartate
$DLIC^{WT}$	Wild-type version of DLIC
Dlg	Discs large
Dm	*Drosophila melanogaster*
DNA	Deoxyribonucleic acid
dsRNA	double stranded RNA
ECM	Extracellular matrix
E. coli	Escherichia coli
EGFP	Enhanced Green Fluorescent Protein
EMT	Epithelial to Mesenchymal Transition
FBS	Fetal Bovine Serum
Gαi	Heterotrimeric G protein

GAL4 / G4	Galactose-responsive transcription factor
GAP	GTPase Activating Protein
GDI	Guanine Dissociation Inhibitory
GDP	Guanosine diphosphate
GEF	Guanine exchange factor
GFP	Green Fluorescent Protein
GPR1/2	G-Protein Regulator 1/2
GTPase	Guanosine triphosphate hydrolase
GUK	Guanylate Kinase domain
HeLa cells	Cells isolated from Henrietta Lacks' cervical cancer
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
Hsp70	70kDa Heat shock proteins family
IF	Imunofluorescence
Insc	Inscuteable
KD	Kinase-Dead
LB	Luria Bertani medium
LGN	leucine–glycine–asparagine
LIN5	abnormal cell lineage 5
Mad	Mitotic arrest deficient
MAGUK	Membrane Associated Guanylate Kinase
MCC	Mitotic Checkpoint Complex
mCherry / mCh	Monomer red fluorescent protein
MDCK cells	Madin-Darby canine kidney cells
Mis12	Mis-segregation 12
MOPS	3-(N-Morpholino)propanesulfonic acid
MPS1	Monopolar spindle 1
$MPS1^{KD}$	Kinase-dead version of MPS1

$Mps1^{WT}$	Wild-type version of MPS1
mRNA	messenger RNA
Mud	Mushroom body defect
NEB	Nuclear Envelope Breakdown
NuMA	Nuclear and Mitotic Apparatus
OD_{595}	Optic Density at 595nm
Par3	Partitioning defective protein 3
PBS	Phosphate-buffered saline
PBT	PBS 0.05% Tween20
PBSTF	PBS 0.05% Tween20 10% FBS
PDZ	PSD-95/Dlg/ZO-1 domain
PFA	4% Paraformaldehyde in PBS
Pins	Partner of Inscuteable
PIPES	Piperazine-1,4-bis(2-ethanesulfonic acid)
$Pins^{TPR}$	Pins TPR domain
RFP	Red Fluorescent Protein
RGS14/Loco	Regulator of G protein signaling 14
Rho	Ras homolog
Ric8a	Resistance to inhibitors cholinesterase 8
RNA	Ribonucleic acid
RNAi	RNA interference
ROI	Region of interest
S2 cells	*Drosophila* Schneider cells
SAC	Spindle Assembly Checkpoint
S. cerevisiae	*Saccharomyces cerevisiae*
SH3	Src homology 3 domain
SOP	Sensory Organ Precursor

Tfb	Transformation buffer
TJ	Traffic jam
TPR	Tetratricopeptide domain
TRITC	Tetramethylrhodamine isothiocyanate
U2OS	Human Bone Osteosarcoma Epithelial Cells
UAS	Upstream Activation Sequence
VHL	von Hippel-Lindau

CHAPTER 1

INTRODUCTION

1.1. Mitosis Overview

In 1839, Schleiden and Schwann postulated 3 tenets: (i) all living organisms are composed of one or more cells, (ii) the cell is the basic unit of structure and organization in organisms, and (iii) cells arise from pre-existing cells, in what has become known as the Cell Theory. Rudolf Virchow has also contributed to the widespread acceptance of this theory publishing, in 1855, the epigram *omnis cellula e cellula*, meaning “all cells arise from other cells” (Nurse, 2000). The process by which cells are generated can be either symmetrical, for proliferation of a certain cell type and tissue growth, or asymmetrical, for differentiation to specialized cells. Both cases require the equal separation of the genetic material to the two daughter cells. The process of genome partitioning was named mitosis (from the Greek *mitos*: threads) by Walther Flemming in 1879, after observing what seemed to be threads in dividing cells (Mitchison et al., 2001).

Mitosis can be decomposed in five morphologically distinct stages: prophase, prometaphase, metaphase, anaphase and telophase (Figure 1). Mitotic commitment is signaled by the activation of Cyclin-dependent kinase 1 (CDK1)/Cyclin B leading to nuclear envelope breakdown (NEB) (Gavet et al., 2010; Virshup et al., 2010). During prometaphase, the chromosomes are brought to the cell equator and are aligned to form the metaphase plate. When the chromosomes are properly aligned the anaphase promoting complex/cyclosome (APC/C) is able to trigger the degradation of cyclin B and securin. The sister chromatids are then separated (anaphase onset) and equally distributed to the daughter cells. It is then important to keep the APC/C unable to degrade cyclin B and securing until all chromosomes are properly aligned and bioriented (kinetochores on the sister chromatids must be attached to opposite spindle poles). That regulation is ensured by the spindle assembly checkpoint (SAC). During prometaphase, SAC proteins concentrate at kinetochores and use them as catalytic platforms for the assembly of the mitotic checkpoint complex (MCC) that will bind to the APC/C (Musacchio et al., 2007). Monopolar spindle 1 (MPS1)/TTK is a serine-threonine kinase present in eukaryotes fundamental for SAC signaling. MPS1 is required for SAC function by acting at multiple points along the signaling pathway. MPS1 checkpoint function is attributed to its upstream role in the recruitment of Mad1, Mad2, BubR1, Bub1 and Bub3 to unattached kinetochores (Lan et al., 2010). This protein has been found overexpressed in different tumors and deemed a promising target for cancer therapy (Yuan, 2006; Malumbres et al., 2007; Landi et al., 2008). It is then critical to understand how is MPS1 regulated and the consequences of its mis-regulation.

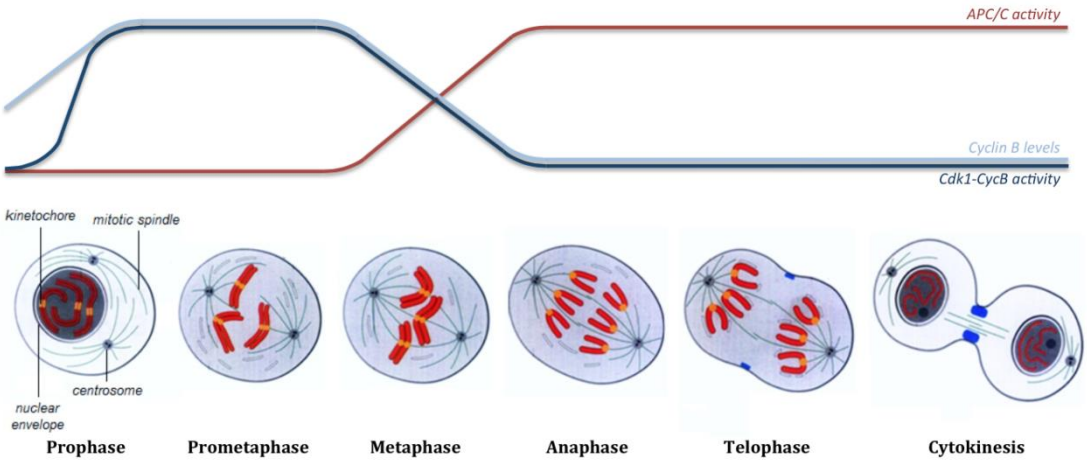

Figure 1 - Mitotic stages. DNA condensation becomes visible and centrosomes begin to separate in prophase. Rising Cyclin B levels activate Cdk1, which drives mitotic progression. Prometaphase is initiated with NEB, which allows bipolar spindle formation. In metaphase, all chromosomes align at the metaphase plate and kinetochore-microtubule are correctly attached. At this point APC/C activity rises, leading to Cdk1-CycB inactivation and beginning of anaphase. After segregation of sister chromatids to opposite poles, nuclear envelope reforms during telophase. The resulting two daughter cells emerge at cytokinesis after constriction of a contractile ring, which separates their cytoplasmic content (adapted from Morgan, 2007).

1.2. Spindle Orientation

The segregation of chromosomes during mitosis is mediated by kinetochores attached to the mitotic spindle (Tanaka, 2010). The mitotic spindle is composed by an intricate microtubule network radiating from the spindle poles: (i) kinetochore microtubules attach the chromosomal kinetochores to the spindle poles (Tanaka, 2010), (ii) interpolar and central microtubules extend to the opposite spindle pole serving as the structural backbone that connects the two spindle poles (Sharp et al., 1999) and (iii) astral microtubules anchor spindle poles to the cell cortex (Hayden et al., 1990).

Proper spindle orientation in mitotic cells is critical to ensure correct segregation of the genetic material and for determination of the progeny fate. Spindle orientation was shown to be required for asymmetric segregation of polarized cell fate determinants in *Drosophila* neuroblasts (Kraut et al., 1996). Moreover, the spindle must be correctly oriented to ensure that daughter cells are placed in the right position within the tissue to maintain its structure or help in tissue morphogenesis. A paradigmatic example is the epithelium, where the spindle must be positioned in the center of the cell and the orientation of the division must be planar and follow an axis parallel to the epithelium plane, guaranteeing symmetric cell division

(identical daughter cells) and the maintenance of those cells in the plane of the tissue, respectively (Peyre et al., 2011; Zheng et al., 2010).

The correlation between the orientation of the plane of division and cell fate was first established in asymmetrical divisions of *Drosophila* and *C. elegans* models, where cell polarity regulators were shown to control spindle orientation. In most of the animal cells with oriented cell divisions the spindle position is achieved by the transmission of localized pulling forces in the cell cortex to astral microtubules. For those reasons, the cell cortex, the specific mechanisms that recruit and localize force generators, and the astral microtubule network are regarded as the three essential levels of regulation for spindle orientation. This complex apparatus has the function of ensuring correct positioning and orientation of the microtubule spindle during metaphase in many cell types and organisms. Here, I describe how that apparatus and its many pathways work, as well as what may happen as consequence of their failure.

1.2.1. Gαi/LGN/NuMA/dynein complex

Several studies have described an evolutionarily conserved complex as having fundamental functions in spindle positioning and orientation in many tissues of both vertebrate and invertebrate species. This complex is composed by the heterotrimeric Gα protein Gαi, the adaptor molecule LGN (leucine–glycine–asparagine), and NuMA (Nuclear and Mitotic Apparatus), respectively Gαi, Pins (Partner of Inscuteable), and Mud (Mushroom body defect) in *Drosophila*, and GOA1/GPA16, GPR1/2 (G-Protein Regulator 1/2), and LIN5 (abnormal cell lineage 5) in *C. elegans* and herein referred to as LGN complex (Figure2) (di Pietro et al., 2011). In mitosis, this complex is localized in a specific cortical region of the cell and is responsible for the recruitment of dynein, a molecular motor directed to microtubules minus-end (Figure 2 A) (Nguyen-Ngoc et al., 2007). Once cortically anchored, dynein directed movement along astral microtubules generates pulling forces on the spindle poles to achieve the correct positioning and orientation of the spindle. For this reason, LGN complex localization determines the subcortical domain for force concentration and the axis of the spindle orientation. Consistently, *Drosophila* neuroblasts (Figure 2 B) and mouse skin progenitors present an apical-basal axis for spindle orientation as consequence of Pins/Mud and LGN/NuMA apical localization (Williams et al., 2011; Yu et al., 2000). In the *C. elegans* zygote, enrichment of GPR1/2 at the posterior cortex is needed to achieve a antero-posterior axis for spindle orientation (Figure 2 B) (Srinivasan et al., 2003). In addition, LGN complex

lateral localization determines planar spindle orientation in both mouse neuroepithelium (Konno et al., 2008) and *Drosophila* epithelial morphogenesis (Bergstralh et al., 2013).

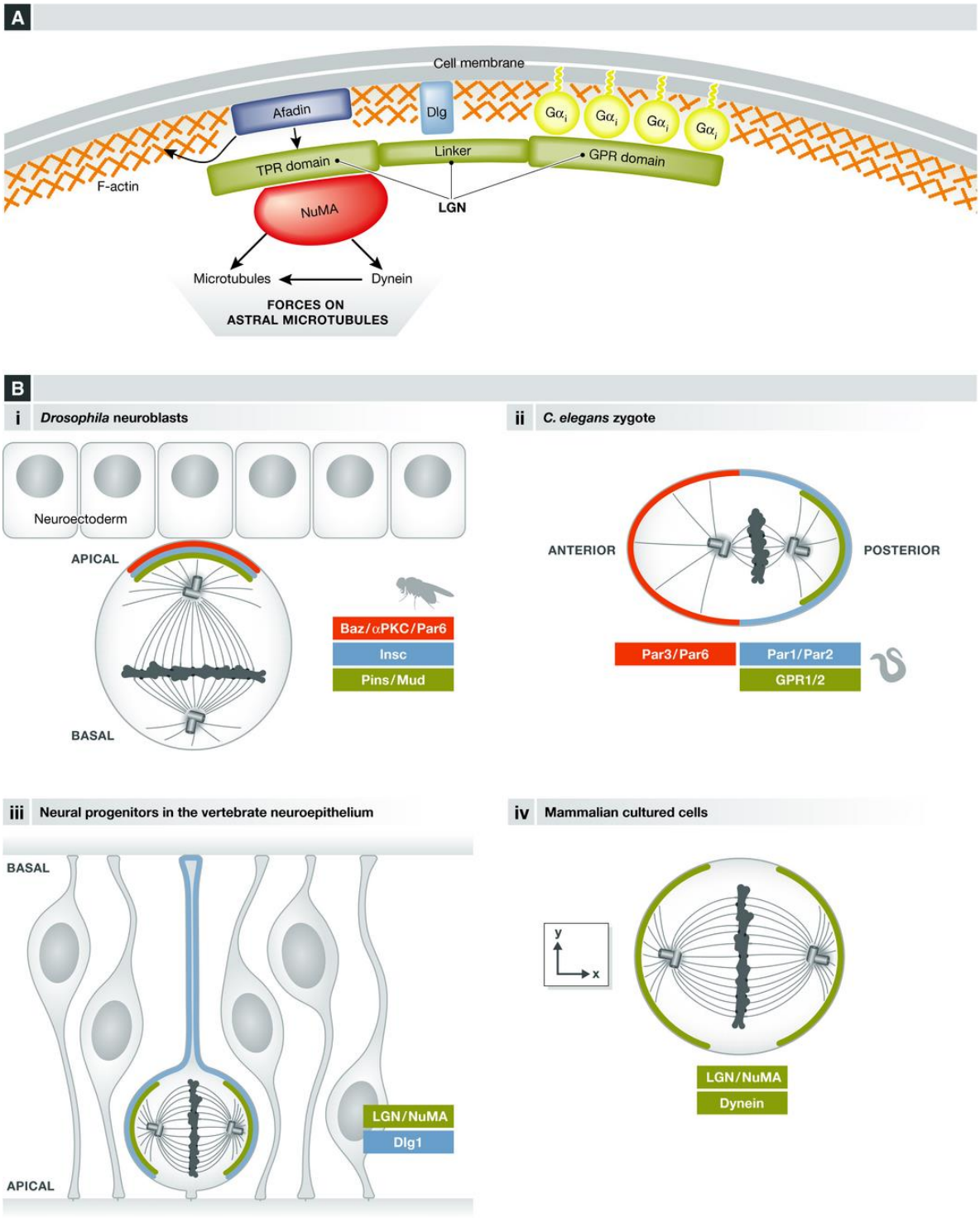

Figure 2 – The LGN complex. (A) The scheme shows the LGN domains and its interactions with Gαi membrane-anchored subunits, and with NuMA, as well as the interaction with cortical proteins (Dlg, Afadin) that regulate LGN cortical localization. (B) LGN complex localization in different systems, showing the polarity

proteins regulating this specific localization when applicable. (i) Drosophila embryonic neuroblasts, (ii) C. elegans zygote, (iii) neural progenitors in the vertebrate neuroepithelium, and (iv) mammalian cell lines (adapted from di Pietro et al., 2016).

Pins/LGN is a protein that encompasses three main domains (Figure 2 A). Its N-terminal is enriched in tetratricopeptide repeats (TPR domain) and has a regulatory function in mediating interactions with several binding partners, including NuMA (Du et al., 2001; Izumi et al., 2006). The central “linker” domain has not been described as having any recognizable organization or binding motif, but is nevertheless required for LGN/Pins function. Finally, the C-terminal GPR (G-protein regulator) domain has 4 GoLoco domains (3 in *Drosophila* Pins and 1 in *C. elegans* GPR1/2) that mediate the interaction with Gαi subunits (Johnston et al., 2009a). This interaction is exclusive for GDP-bound Gαi subunits, having a guanine dissociation inhibitory (GDI) activity. For that reason, the interaction between Gαi subunits and LGN, as well as the complex stability, is controlled by the phosphorylation state of bound guanosine. The modulation is achieved through the balance between the guanine exchange factor (GEF) Ric8a and the GTPase activating protein (GAP) RGS14/Loco (in vertebrates and *Drosophila*, respectively) activities (Tall et al., 2005). Pins binds Mud and interacts with the Gαi at the plasma membrane via its N-terminal and C-terminal domains, respectively, acting as a molecular scaffold of the spindle orientation apparatus (Izumi, 2006; Johnston, 2009a).

NuMA was initially shown to bind LGN in early 2000s, later its *Drosophila* homolog Mud was decribed as a key protein of the spindle orientation in *Drosophila* neuroblasts (Du, 2001; Izumi, 2006). Mud, similarly to Pins/LGN, localizes both in the cortex and spindle (where it recruits Pins) of mitotic cells. Mud binds dynein, and exerts its function in the spindle orientation through the dynein/dynactin complex.

Gαi subunits bind to the cell membrane through their myristoyl groups and anchor the LGN complex to it. Since Gαi subunits completely cover the inner surface of the cell, polarized cortical localization of the LGN complex requires specific polarity proteins (Yu et al., 2003).

Dyneins and kinesins are ATP-fueled motor proteins, that use microtubule as trails to ensure the active and accurate transportation of cargoes in opposite directions. Most kinesins move towards microtubule plus-ends, while all known dyneins move towards minus-ends. Cytoplasmic dynein (dynein) is a multisubunit complex composed of heavy chains (DHC), intermediate chains (DIC), light intermediate chains (DLIC) and light chains (DLC) (di Pietro et al., 2017). Besides cargo transportation, dynein pulling forces are critical for spindle

orientation and positioning. Dynein binds to the LGN complex via Mud/NuMA and accumulates in specific cortical regions. Astral microtubules are captured by dynein which move towards their minus-end. However, while anchored to the cortex, dynein is unable to move along the microtubules. Instead, it pulls the microtubules towards the cortex (Roberts et al., 2013). In HeLa cells, the Plk1 kinase, localized at the spindle poles, regulates the proximity to the cortex, as it negatively controls the cortical localization of dynein/dynactin. When a spindle pole is close to the cortex it dissociates the dynein from cortical NuMA, excluding dynein from this cortical site. This process stops the spindle movement towards that direction and ensures central positioning (Kiyomitsu et al., 2012). Dynein activity relies on many proteins that are not part of the dynein complex but are crucial for adapting the motor to the specific cellular function. Dynactin is one of the most well characterized dynein adaptors and it has been described to be important for nearly every cellular dynein function (Walker et al., 2009). Dynactin inhibition or depletion studies report phenotypes similar to complete loss of dynein function (Walker, 2009).

1.2.2. Molecular regulation of LGN complex recruitment and stability at the cortical region

Pins interacts with inscuteable (Insc), an apical protein described as having a role in the apical-basal orientation of the spindle in *Drosophila* neuroblasts (Yu, 2000). In turn, apical localization of Insc, and therefore of Pins, is dependent of presence of Bazooka (*Drosophila* homolog of Par3) and of atypical protein kinase C (aPKC) (Wodarz et al., 2000; Yu, 2000). Interestingly, although aPKC is needed for the recruitment of Pins to the apical region in *Drosophila* neuroblasts, it is responsible for the lateral enrichment of LGN in MDCK cells cystogenesis (Figure 3 B), as it inhibits its apical localization in these cells. This inhibition is achieved through phosphorylation of LGN by apical aPKC, which leads to an enhanced affinity of LGN for a 14-3-3 protein. This interaction competes with the interaction between LGN and Gαi localized in the apical region thus, increasing the lateral localization of LGN and resulting in a planar spindle orientation (Hao et al., 2010a). Nonetheless, it is important to understand that the role for aPKC in spindle orientation is not universal. The disruption of aPKC in the chick neuroepithelium, *Drosophila* follicular epithelium and *Drosophila* imaginal wing disc does not affect spindle orientation (Bergstralh, 2013; Bergstralh et al., 2016; Peyre, 2011).

Detailed structural analysis of the bindings between Pins and Inscuteable and Pins and Mud revealed that theses bindings share the same sites and are therefore exclusive. These results suggest that although Inscuteable may be responsible for Pins recruitment to the apical cortical region, it has to release it and hand it to Mud (Culurgioni et al., 2011; Yuzawa et al., 2011; Zhu et al., 2011). Even though Pins is released from its binding to Insc it does not lose its localization as it remains bound to the Gαi. This model is well established in Drosophila neuroblasts, however Insc is absent in epithelial cells, for that reason the correct localization of Pins must be ensured by other means in those systems.

1.2.2.1. DLG

Discs large (Dlg) (Figure 2A) is a well-known *Drosophila* tumor suppressor (Bergstralh, 2013). Dlg is a protein from the membrane associated guanylate kinase (MAGUK) family, defined by a specific architecture including GUK, PDZ and SH3 domains. These proteins bind to the plasma membrane through their PDZ domains via intermolecular interactions. The GUK domain has no catalytic activity known but is known to bind some phosphorylated partners (Lars Funke, Srikanth Dakoji, 2005).

Dlg is responsible for Pins/LGN anterior and apical localization, in *Drosophila* larval sensory organ precursor (SOP) cells (pI cells), where this interaction was first described, and neuroblasts, respectively (Bellache et al., 2001; Siegrist et al., 2005). In the latter case, Dlg is a member of a non-crucial microtubule-based mechanism that works alongside with the main inscuteable recruitment pathway above mentioned (Siegrist, 2005). Dlg/Dlg1 depletion studies were described as affecting Pins/LGN specific cortical localization and leads to defects in planar orientation in Drosophila epithelia and in chick neuroepithelium (Bergstralh, 2013; Saadaoui et al., 2014). Dlg depletion in *Drosophila* follicular epithelia resulted in defects in the planar orientation of the spindle, as Pins becomes localized all over the cell cortex, instead of its regular lateral cortical localization typically observed in these cells. These results suggest that Dlg may have a role in restricting Pins localization to the lateral cortex (Bergstralh, 2013). Dlg1 depletion in human HeLa cells reduces LGN and NuMA cortical localization and leads to defects in micropattern-guided spindle orientation (Figure 3 C,D) (Saadaoui, 2014). In neither of the cases, Dlg/Dlg1 depletion resulted in noticeable tissue polarity defects, indicating that the role of Dlg/Dlg1 in spindle orientation is independent of its role in cell polarity (Bergstralh, 2013; Saadaoui, 2014).

The interaction between Dlg/Dlg1 and Pins/LGN (Figure 2 A) is made between GUK domain of Dlg and Pins linker domain and depends on the phosphorylation of the conserved serine residue S436/S401 in Pins (Johnston, et al., 2009; Hao et al., 2010b). In *Drosophila*, Pins S436 was described as an *in vitro* direct target of Aurora A and that Aurora A depletion leads to spindle misorientation in S2 induced polarity cells (Johnston, 2009a). Furthermore, Pins phosphomimetic form was shown to rescue spindle orientation in Aurora A depletion in polarity induced S2 cells and *pins* mutant *Drosophila* neuroblasts (Johnston, 2009a).

1.2.2.2. E- Cadherin

In many tissues, cell-cell adhesion between neighboring cells is sustained by a class of calcium-dependent transmembrane proteins called cadherins. Its cytosolic domain is bound to the actin cytoskeleton through catenin proteins (α-, β- and p120-catenin) and makes a signaling bridge between transcellular stimuli at the cadherin extracellular domain and the intracellular responses (Beavon, 2000) . Interestingly, the loss of E-cadherin results not only in disruption of cell-cell adhesion, but also in spindle misorientation (Le Borgne et al., 2002). This observation led to the hypothesis that E-cadherin could be important for regulation of the LGN complex. Immunofluorescence analysis revealed that LGN in MDCK epithelial cell monolayers accumulates at cell–cell contacts and is absent from membranes that have no cell-cell contact. The accumulation of LGN at cell–cell contacts was even more prominent after cells had entered mitosis (Gloerich et al., 2017).

To further address this, U2OS cells missing endogenous E-cadherin and expressing the E-cadherin cytosolic domain mistargeted to the mitochondria surface were used. This experiment led to the recruitment of the TPR repeat of LGN (LGN-TPR) to the mitochondria surface, as well (Gloerich, 2017). A mutant E-cadherin with a truncated extracellular domain and, consequently, nonfunctional in cell-cell adhesion was also expressed in MDCK cells and was seen distributed through the whole cell membrane, independently of any cell-cell contact. Consistently with the previous results, LGN localized equally around the plasma membrane and the spindle planar orientation was lost (Gloerich, 2017). Additionally, E-cadherin mutant that maintained its cell-cell adhesion function, but lacking the LGN binding motif was also used. Even though the cell-cell adhesions were similar to the wild-type, the spindle planar orientation was not induced. This phenotype was very similar to the one presented by cells with RNAi-mediated LGN depletion (Gloerich, 2017). Finally, it also demonstrated that NuMA co-localized with LGN, forming the LGN complex and that cadherin

was also capable of stabilizing cortical astral microtubules associations. Altogether, these results indicate that LGN localization requires the interaction with the E-cadherin cytosolic tail and that the E-cadherin-mediated spindle orientation is dependent of the formation of this complex. Thus, E-cadherin has a role in instructing LGN complex assembly at cell–cell contact regions, mediating cell division orientation to intercellular adhesion.

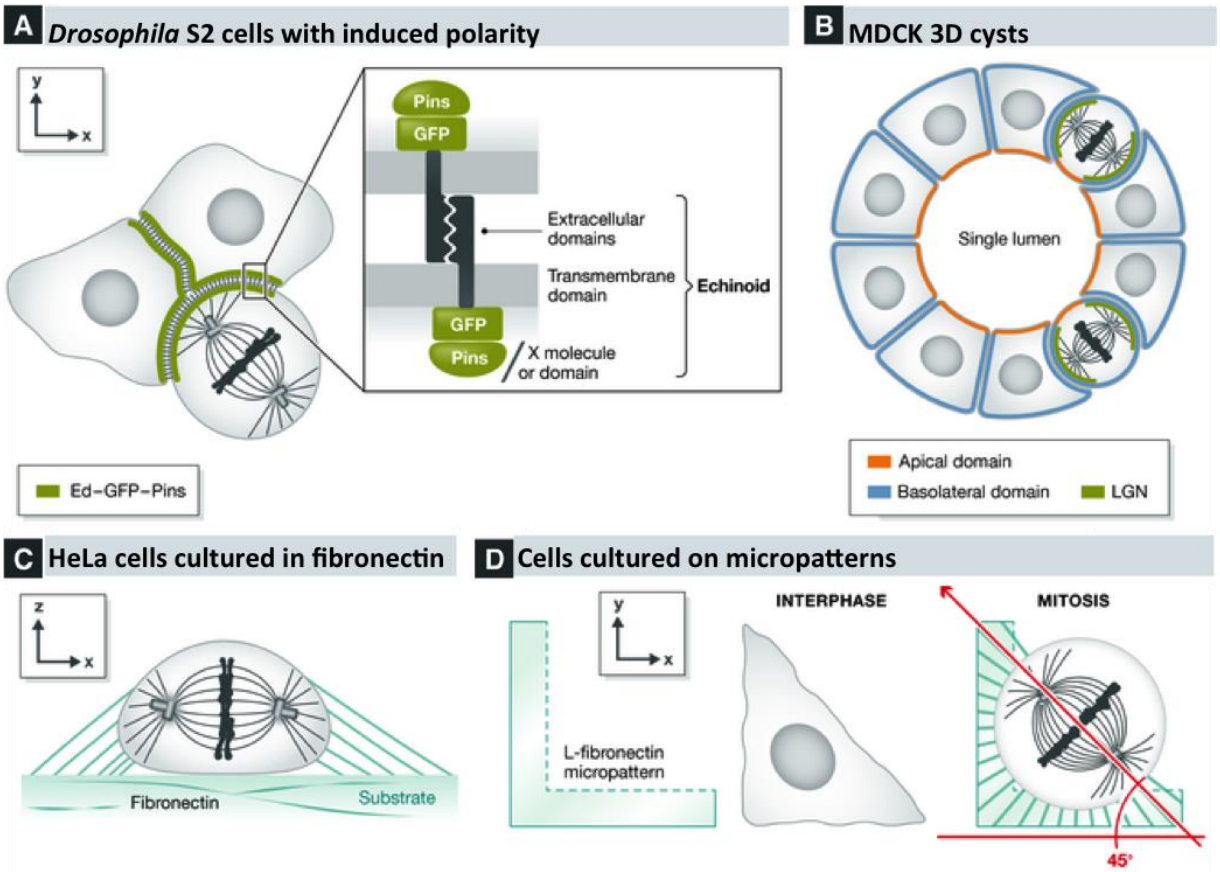

Figure 3 – Models used for Sipndle Orientation studies. (adapted from di Pietro et al., 2016). (A) - MDCK cysts, a well-known 3D model of epithelial morphogenesis. Dog MDCK cells seeded in Matrigel develop cysts with a central lumen and defined polarity domains. Spindle orients parallely to the plane of the epithelium, in a LGN-dependent fashion, which localizes to the lateral cell cortical region (Zheng, 2010). Errors in the spindle orientation regulation leads to multiple lumina cysts. (B) - Induced polarity assay in Drosophila S2 cells. Fusion protein of the transmembrane and extracellular domains of the echinoid (Ed) homophilic cell–cell adhesion protein and Pins is used. This method localizes Pins to the cell cortex near the contact regions between clustered cells, creating a polarized distribution in each cell. This method results in spindle orientation in the direction of the Ed-Pins-enriched cortical region. This cell model allows the assessment of the function molecules downstream of Pins (Johnston et al., 2009; Wee et al., 2011) (C) - HeLa cells culture on a fibronectin substrate. The mitotic spindle of this cell line has been described as orienting parallel to the fibronectin substrate (Toyoshima et al., 2007) (D) - Cells cultured on micropatterns. Individual cells are cultured on defined geometry micro surfaces, which induce a specific shape, adhesion pattern and spindle orientation (Théry et al., 2007).

1.2.2.3. Canoe

The *Drosophila* adherens junctions scaffolding protein Canoe (*Drosophila* homolog of mammals' Afadin) has also an important role in LGN complex formation and spindle orientation. This role was described in *Drosophila* neuroblasts, where Canoe localizes to the apical cortical region of the cells and regulates spindle orientation in an apical–basal axis (Speicher et al., 2008). Further studies in S2 cell induced polarity assays (Figure 3 A) (Wee et al., 2011) have dissected the molecular underpinnings of the pathway where Canoe interacts with Pins and acts specifically in the spindle orientation pathway mediated by Pins TPR domain ($Pins^{TPR}$) and Mud (Johnston et al., 2009). Afadin depletion decreases LGN cortical recruitment, and prevents NuMA and dynein recruitment, resulting in spindle misorientation, both in adherent cells and in 3D cell cultures (Carminati et al., 2016). Since NuMA localizes in the nucleus throughout interphase and is only released after nuclear envelope breakdown (NEB), is hypothesized that Afadin has a role in the initial recruitment of LGN to the cell cortex and interacts with Gαi subunits in early mitosis, before LGN interacts with NuMA at the cell cortex (di Pietro, 2016).

Recently, the phosphorylation of Mud/NuMA has been described as needed for its localization at the cell cortex. In *Drosophila*, Warts, a kinase of the Hippo pathway, phosphorylates Mud in its coiled-coil domain. This induces conformational change that, exposes the Pins binding domain thus, allowing Mud interaction with cortical Pins (Dewey et al., 2015). In human cells, the centrosome-associated AurA kinase also phosphorylates NuMA. This phosphorylation occurs in a different domain and is equally necessary for NuMA recruitment to the cell cortex (Gallini et al., 2016). Interestingly, both Warts and AurA kinases localize to spindle poles in mitotic cells, suggesting that this phosphorylation works as a molecular switch to activate cortical Mud function, releasing it from the spindle pole and promoting the interaction with Pins in the cell cortex (Dewey, 2015; Gallini, 2016).

1.3. Consequences of spindle misorientation

Spindle misorientation is thought to be related to cancer in some extent. However, is still unclear if spindle misorientation by itself causes cancer or if is it more likely to help in the evolution of aggressive cancer phenotypes in conjunction with the well-known changes in tumor suppressors or oncogenes (Pease et al., 2011).

Assessing a potential role for spindle misorientation in tumorigenesis is challenging, as the well-described tools to disturb spindle orientation would also affect other spindle functions. For instance, mutation of adenomatous polyposis coli (APC), a microtubule plus-end-tracking-protein involved in microtubule dynamics, or using microtubule-destabilizing drugs are two possible ways to disrupt astral microtubules. However, these strategies are also likely to disrupt both astral and kinetochore microtubules, and, consequently, cause defects in spindle orientation and chromosome segregation (Green et al., 2005). In addition, some tumor suppressors like E-cadherin and von Hippel-Lindau (VHL) have also been shown to have important roles in spindle orientation through the stabilization of astral microtubules and the regulation of epithelial polarity (Green et al., 2005; den Elzen et al., 2009; Thoma et al., 2009).

Some authors believe that there is enough evidence indicating that spindle misorientation is not tumorigenic by itself. An example of those evidences is a study where mice with polycystic kidney disease show spindle misorientation and consequent morphological changes in their kidneys, but still those animals are not regarded as tumor prone (Fischer et al., 2006; Patel et al., 2008). These findings led to hypothesis that spindle misorientation disturbs tissue morphology during development but does not contribute directly to the neoplastic transformation. However, it is believed that, due to the important role in tissue organization and morphogenesis, spindle orientation may have a considerable synergy with the several other changes during tumor development and progression (Pease, 2011). In this section, we will discuss the possible effect of spindle misorientation on several levels of tumor evolution, namely, (i) increased aneuploidy, (ii) tissue disorganization and (iii) expansion of the cancer stem cell pool.

1.3.1. Aneuploidy and spindle misorientation

Aneuploidy is characterized by the presence of an abnormal number of chromosomes and is a hallmark of cancer (Chandhok et al., 2009; Weaver et al., 2006). Although establishing aneuploidy as directly responsible for cancer phenotypes is technically challenging, some evidences indicate that it can rush genetic changes, which may, ultimately, lead to cancer (Chandhok, 2009).

Spindle misorientation and aneuploidy are related in two different ways. Firstly, the interaction between microtubules subset and other cellular structures is critical in both spindle orientation and appropriate chromosome segregation. Several proteins responsible

for microtubules plus-end dynamics are also implicated in chromosome segregation fidelity (Green et al., 2005; Vallee et al., 2001). For those reasons, disruption of these factors would be likely to cause aneuploidy and spindle misorientation simultaneously. Secondly, spindle misorientation has the potential to exacerbate, or even facilitate, the development of aneuploidy due to its implication on cytokinesis. This idea was verified in a study using RNAi-mediated APC depleted cells, in which not only spindle misorientation was induced but also cytokinesis failure was detected, leading to a tetraploidic phenotype (Figure 4 B) (Caldwell et al., 2007). The same phenotype was demonstrated in dividing crypt cells in the small intestines APC mutant mice (Caldwell, 2007). These two mitotic erroneous phenomena are likely to be further increased by the quick cell proliferation observed in most tumors, as the number of mitotic events increases and, consequently, the time to correct mitotic errors decreases.

1.3.2. Disruption of tissue organization and morphology and promotion of metastasis

Planar spindle orientation results in the maintenance of daughter cells proximity or contact with the extracellular matrix (ECM). Contrarily, when there is spindle misorientation, one daughter cell can be actually separated from the ECM. This phenomenon may lead to different outcomes (Figure 4 C). Firstly, the mispositioned daughter cell may apoptosis as a result of extrusion and anchorage loss. However some defects found in cancer cells, such as the loss of correct apoptotic mechanisms may prevent its death (Slattum et al., 2009). Alternatively, the cell can establish cell-cell interactions with the cell below it, leading to vertical tissue expansion and tissue hyperplasia, which is regarded as a premalignant change (Jones et al., 1994; Kanitakis et al., 2015). Finally, if the tissue geometry formed favors the establishment of a lumen, it might result in the formation of a gland. This event has not been demonstrated *in vivo*, however studies using three-dimensional cultures show that spindle misorientation is related to epithelial organization remodeling, often resulting in multiple lumen phenotypes (Qin et al., 2010; Rodriguez-Fraticelli et al., 2010). For these reasons, tissue organization and morphology can be critically modified as a result of spindle misorientation and those changes can lead to increased propagation of cells with abnormal genomes propagation.

It has been also hypothesized that spindle misorientation has the potential to assist in the process of metastization. Cultured cells with Rho GTPase overexpression were shown to

have the spindle misoriented and to produce daughter cells lacking ECM contact and floating away in the culture media without losing its viability and re-adhering to the dish at another site of the culture plate (Vasiliev et al., 2004). Despite the simplicity of this model, that prevents it from simulating many lymphovascular metastasis features that occur with solid tumors, it recapitulates some characteristics of metastasis, such as loss of cell–cell cohesion, and the potential to cause tumor dissemination in fluid compartments, such as the ascites and effusions present in the tumor-promoted neovascularized blood vessels. Alternatively, basal extrusion could enable daughter cells to initiate metastasis (Slattum, 2009). Metastization could be particularly enhanced by mutations that promote both spindle misorientation and epithelial to mesenchymal transition (EMT).

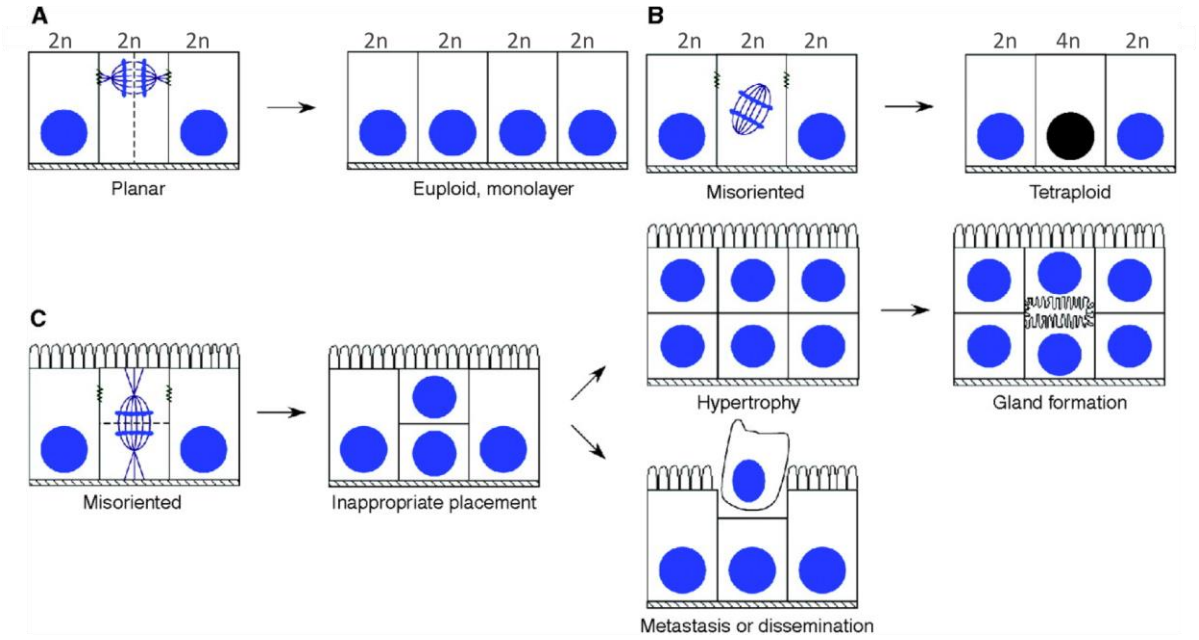

Figure 4 – Spindle orientation effects on the organization of a precancerous tissue or tumor. (A) Proper planar spindle orientation in an epithelium would produce a simple monolayer of euploid daughter cells. (B) Aneuploidy. Spindle misorientation due to the loss of astral microtubules could cause failure of cytokinesis, which would prevent cell division and lead to tetraploidization. The resulting single tetraploid daughter cell (black nucleus with twice the appropriate number of chromosomes, designated 4n) is more likely to acquire further genetic changes that promote tumorigenesis. (C) Tissue disorganization and metastasis. Loss of planar spindle orientation in an epithelium could result in a vertical cytokinesis leading to daughter cells placed inappropriately one on top of the other. Many potential outcomes could include tissue hypertrophy, formation of a new gland (generation of a new lumen) and the detachment of the apically placed daughter cell, which could contribute to dissemination in fluid compartments, such as ascites and effusions. The scalloped section on the apical surface of the cells represents the brush border. (Adapted from Pease & Tirnauer, 2011)

The correlation between spindle misorientation and tissue disorganization may also be bidirectional. Not only the spindle misorientation may have consequences on tissue disorganization, but also polarity defects may correlate with additional spindle misorientation. Some studies suggest that tumor formation, on its own, is not enough to promote spindle misorientation (Fleming et al., 2009; Pease & Tirnauer, 2011). Nonetheless, the conjugation between specific mutations or microenvironmental changes in tumors have the potential to promote spindle misorientation along with other defects and, consequently, induce a positive feedback loop between spindle misorientation and an altered microenvironment in premalignant tissues and tumors.

1.3.3. Cancer stem cell compartment expansion

Cancer prognosis is related to its aggressiveness and drug resistance capacity, two parameters that seem to be correlated with the number of cancer stem cells in the tumor (Alison et al., 2010). Intestinal stem cells of heterozygotic APC mutant mice were found to lose their apico-basal axis for spindle orientation (Quyn et al., 2010), suggesting that spindle misorientation may be responsible for disrupting of the balance between asymmetric and symmetric divisions in cancer stem cells and promote the alteration in the number of cancer stem cells.

Studies in flies were conducted to assess the role of spindle misorientation in increasing the number of cancer stem cells. It was shown that disruption of spindle orientation regulators in larval neuroblasts (Knoblich, 2008) leads to tumor development and aneuploidy. This further accentuates the overlapping functions of several spindle regulators, such as Polo kinase (a key regulator of the spindle checkpoint) and Aurora A kinase, and the possibility that spindle misorientation in stem (or progenitor) cells could be responsible for tumor formation and expansion (Caussinus & Gonzalez, 2005). Such results have huge importance as they suggest that spindle misorientation may contribute to the increase of the number of cancer stem cells in human tumors, thus, increasing its aggressiveness and drug resistance. In the future, three major aspects must be addressed: (i) mechanisms promoting spindle misorientation in cancer stem cells, (ii) the impact of misorientation in the balance between symmetric and asymmetric cell divisions, and (iii) its function in tumor formation.

1.4. *Drosophila* oogenesis: a powerful model for epithelial cell and spindle orientation

Drosophila oogenesis, which started as a useful system to study the embryo patterning, is also regarded as a powerful tool to study many other features in cell and developmental biology. Oogenesis is responsible for the development of a mature egg from a single stem cell and requires many cellular processes, from cell cycle control and differentiation to polarization and tissue morphogenesis (Bastock et al., 2008).

A female fly has two ovaries containing approximately 18 egg ovarioles. Ovarioles are composed of linearly distributed egg chambers in increasing developmental stages (Figure 5). New egg chambers are formed from the progeny of both somatic and germline stem cells present in the germarium, at the anterior end of the ovariole (Figure 5). The germline progenitor cells undergo asymmetrical division, resulting in new stem cell and a cystoblast. The cystoblast forms 16 cell cysts after experiencing four rounds of incomplete cell divisions. One of them differentiates to oocyte and the other 15 are kept as nurse cells. These cells are responsible for the support of the oocyte supplying it with nutrients, proteins and RNAs. Nurse cells and oocyte, are the germline cells of the egg chamber. These cells are enveloped by a monolayered epithelium that comprises the somatic cells of the egg chamber, named follicular epithelium (Figure 5).

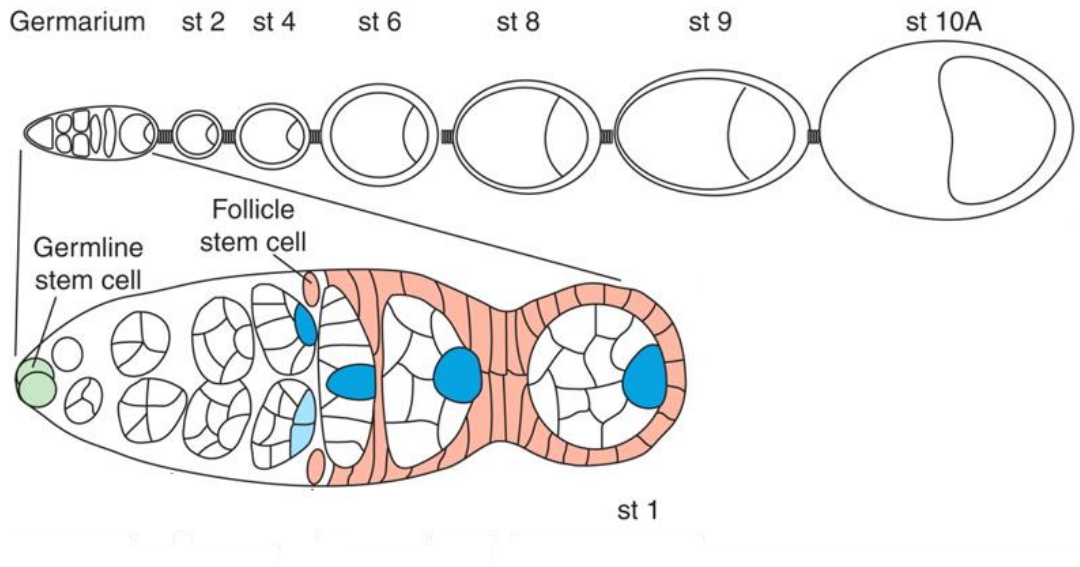

Figure 5 – The ovariole. *Top*: schematic drawing of ovariole with the germarium at the anterior tip and egg chambers of increasing developmental stage. *Bottom*: Magnified view of germarium (Adapted from Roth et al., 2009)

Egg chambers bud off the germarium and mature throughout the ovariole until they reach posterior end as mature eggs ready for fertilization. The whole process takes approximately a week and is divided in 14 distinct morphological stages, from the budding of the egg chamber from the germarium (Stage 1) to the mature egg (Stage 14). The development between stage 1 and stage 14 takes roughly 3,5 days.

The follicular epithelium is a highly organized and polarized tissue along its apical-basal axis. The apical side contacts intimately with the germline through most of oogenesis and the basal side contacts with the extracellular matrix. Follicular cells divide laterally and symmetrically in the early stages of oogenesis. Combining the ability to track the mitotic spindle by live imaging of integral tissues and the *Drosophila* genetic tractability this system becomes a valuable model for the study of spindle orientation in a tissue context (Bastock, 2008; He, 2011).

1.5. Objectives

The accurate distribution of the chromosomes during mitosis is essential for the formation of genetically identical daughter cells. This relies on the attachment of sister kinetochores to microtubules of opposite spindle poles, thus allowing sister chromatids to be pulled to opposite sides during cell division. To prevent errors in genome partitioning eukaryotic cells have evolved a surveillance mechanism known as Spindle Assembly Checkpoint (SAC). MPS1 is a serine/threonine kinase that has emerged as its master regulator. It accumulates at unattached kinetochores, where it phosphorylates several substrates creating docking sites for the hierarchical recruitment of additional SAC components required for the assembly of anaphase inhibitory complexes (Shepperd et al., 2012; Vleugel et al., 2015; Yamagishi et al., 2012). Trans-autophosphorylation of MPS1 T-loop has been shown to be critical for full kinase activity in vitro and robust SAC signaling (Kang et al., 2007; Mattison et al., 2007). MPS1 is frequently overexpressed in cancer cells. However, the relevance of this for tumorigenesis remains unclear. Preliminary data obtained in the host laboratory revealed that overexpression of MPS1 in *Drosophila* S2 cells leads to massive spindle rotation.

The present thesis aims to unravel the molecular defects underlying spindle rotation upon MPS1 overexpression and its relevance for tissue organization. For that, we will combine live-cell confocal imaging with cell biology and *Drosophila* genetics. Spindle orientation during mitosis will be examined in *Drosophila* cultured S2 cells and in follicular

epithelium of adult flies. Overexpression of EGFP-MPS1 will be induced and immunofluorescence and live cell imaging will be used to monitor the spindle behavior during mitosis in comparison to cells expressing endogenous levels of MPS1 or overexpressing a catalytically inactive version of MPS1. The ability to rescue MPS1-induced spindle rotation by RNAi depletion will allow to identify the pathways underlying the described phenotype. Following up these results, we aim to identify MPS1 targets in those pathways upon overexpression and understand the molecular mechanisms responsible for the spindle positioning defects. Results from this project are expected to generate critical knowledge to understand the causes of spindle misorientation in cancer and provide a well-characterized set of molecular signatures that might be used to assess tumor aggressiveness and invasion potential.

CHAPTER 2

MATERIALS AND METHODS

2.1. Rubidium Chloride competent cells generation and transformation

To produce competent E. coli TOP10 cells (Invitrogen), 5mL of a starting culture (grown overnight, at 37ºC) was used to inoculate 500mL of LB medium, which were grown at 37ºC until they reached an OD_{595} of 0.5. At this time, cells were cooled down on ice, for 15 minutes. Afterwards, the culture was centrifuged for 10 minutes at 4500rpm and 4ºC to collect the cell pellet, which was resuspended in 30mL of Tfb I (100mM RbCl, 50mM $MnCl_2$ ・$4H_2O$, 30mM Potassium Acetate, 10mM $CaCl_2$ ・2 H_2O and 15% Glycerol, prepared in deionized H2O) and incubated for another 15 minutes on ice. Then, cells were centrifuged for 5 minutes at 4000rpm and 4ºC and the cell pellet was resuspended in 6mL Tfb II (0.2M MOPS, 10mM RbCl, $CaCl_2$ ・2 H_2O and 15% Glycerol). Aliquots were frozen in dry ice and stored at -80ºC.

For each transformation, one 50µL aliquot of TOP10 competent cells was defrosted on ice for a short period of time. About 5µL of a recombination reaction were then added to the cells and the mixture was incubated on ice for 20 minutes

Transformation was induced through heat-shock at 42ºC for 45-60 seconds. After 2 minutes on ice, 200µL of LB medium were added and the culture was incubated at 37ºC for 1h. To select transformed bacteria, cells were plated on LB agar supplemented with specific antibiotics (ampicillin or kanamycin were used at the final concentrations of 100µg ・mL-1 and 50µg ・mL-1, respectively).

Plasmid extractions were done with Fast-n-Easy Plasmid Mini-Prep Kit (Jena Bioscience GmbH) according to the manufacturer's instructions.

2.2. RNA interference (RNAi) synthesis

To deplete Mud, DHC and $p150^{glued}$ from Drosophila S2 cells, double stranded (ds)RNA was synthesized, targeting Mud, DHC and $p150^{glued}$ mRNAs. RNAi synthesis was performed with T7 Megascript kit (Ambion), according to the manufacturer's instructions. The resulting single stranded RNA was denaturated at 96ºC for 5 minutes, in order to eliminate secondary structures, and gradually cooled down (2ºC every minute) to allow the formation of dsRNA duplex. The integrity, concentration and purity degree of the synthetized dsRNA duplexes was evaluated by 1% agarose gel electrophoresis. The

primers used to produce the DNA strands used as targeting region in the RNAi synthesis were:

Mud coding region:

Forward: 5'-TAATACGACTCACTATAGGGAGATAGAGGAACTGCAGGCAAAGCTG-3'

Reverse: 5'-TAATACGACTCACTATAGGGAGATGGTCTGTGCCTTCAATGACAGAC-3'

P150^{glued} coding region:

Forward: 5'-TAATACGACTCACTATAGGGAGAACTGCCTCCAGCAGCAGTAT-3'

Reverse: 5'-TAATACGACTCACTATAGGGAGAAGCACCCATGATTTTCGTTC-3'

2.3. *Drosophila* S2 cell transfection

Stable transfection of recombinant plasmids into S2 cells was performed using Effectene Transfection Reagent (QIAGEN). For each transfection, 10^6 *Drosophila* S2 cells were seeded in a twelve-well plate in 800µL Schneider's medium (Gibco, BRL) supplemented with 10% FBS. One hour later, the medium was replaced with 500µL of fresh Schneider's medium with 10% FBS and a plasmid mix (0.5µg of pHGW-DLIC and 0.5 µg of pAC-Tub-mCherry or 0.5µg of pHGW-DLIC S432D and 0.5 µg of pAC-Tub-mCherry in 75µL of EC Buffer, 8µL of Enhancer, 10µL of Effectene and 1mL of Schneider's medium with 10% FBS prepared according to the manufacturer's instructions). For stable transfections, after a three-day period the selection was started with blasticidin, at a final concentration of 25 µg/mL.

2.4. S2 cell culture, RNAi-mediated depletion and drug treatment

Drosophila S2 cells were cultured at 25°C in Schneider's medium (Gibco, BRL) supplemented with 10% fetal bovine serum (FBS). For each depletion, 10^6 S2 cells/mL of Schneider's medium (Gibco, BRL) were plated in twelve-well plates (0.5 mL) and 15 µg (for DHC) or 20 µg (for p150^{glued} or Mud) of the respective dsRNAi were added. After one hour at 25°C, cells were supplemented with 1mL of Schneider's medium (Gibco, BRL) 10% FBS. The incubation period was 120 hours (for DHC and p150^{glued}) or 96h (for Mud). At the selected time points, cells were collected and processed for time-lapse microscopy. When

required, cells were subjected to several drug treatments before being collected and processed. To prevent mitotic exit, in a checkpoint independent manner, cells were incubated with 20 μM of the proteasome inhibitor MG132 (Calbiochem).

Expression of EGFP-Mps1, EGFP-Mps1-KD transgenes was induced by treatment of the S2 transfected cells with 100μM $CuSO_4$ at least 8 hours before analysis. To induce expression of EGFP-DLIC, EGFP-DLIC^{S432D} and Mis12-MPS1, under regulation of Hsp70 promoter, cells were incubated for 30 minutes at 37°C 6 to 8 hours prior to cell analysis.

2.5. Immunofluorescence analysis in *Drosophila* S2 cells

To analyze αTubulin levels, 10^5 *Drosophila* S2 cells were seeded in glass coverslips, previously treated with concanavalin A. One hour after the seeding, cells were treated with 20μM MG132 for 2 hours. Taxol was added to the medium at a final concentration of 10 nM. 5 minutes later, the medium was changed for the permeabilization buffer (100 mM PIPES, 1 mM $MgCl_2$, 0,1 mM $CaCl_2$ and 0,1% Triton X-100). Two minutes later, the fixation was started with 4% paraformaldehyde in the same buffer for 10 minutes. Finally, cells were washed three times with TBS with Triton X-100 0,1%.

To reveal spindle morphology and assess Mud levels, *Drosophila* S2 cells (10^5 cells in 120 μL) were collected and centrifuged onto slides for 5 min, at 1000 rpm (Cytospin 2, Shandon). Cells were fixed with 3.7% Formaldehyde in PHEM (60 mM PIPES, 25 mM HEPES pH 7.0, 10 mM EGTA and 4 mM MgSO4) for 12 minutes and extracted with PBS Triton X-100 0.05% thrice for 5 minutes.

Fixed cells were blocked for one hour in PBS with 0.05% Tween20 and 10% fetal bovine serum (PBSTF) at room temperature and then incubated overnight at 4°C with primary antibodies (prepared in blocking solution). After three five-minute washes in PBS with 0.05% Tween 20 (PBT), cells were incubated with fluorescent-labeled secondary antibodies diluted in PBSTF for one hour at room temperature. Finally, cells were washed thrice with PBT for 5 minutes and slides were mounted with Vectashield mounting medium for fluorescence with 1 mg/mL of DAPI (Vector Laboratories, UK).

Images were collected in a Leica TCS SP5 II laser scanning confocal microscope (Leica Microsystems, Germany). Data stacks were analyzed using ImageJ 1.49k software (http://rsb.info.nih.gov/ij/).

For immunofluorescence quantification of proteins, the mean pixel intensity obtained from maximum projected raw images acquired with fixed exposure acquisition settings.

Kinetochore location was defined manually based on CID staining within a specific predefined region of interest (ROI). After subtraction of background intensities, estimated from regions outside the cell, the intensity relative to CID signal was determined for each kinetochore.

2.6. Transgene expression using the GAL4 – UAS system

The GAL4 – UAS system has been one of the most helpful and established tools used by *Drosophila* geneticists. This system ensures a targeted and regulated induction of transgene expression. It takes advantage of an adapted transcription factor – recognition element couple from the yeast *S. Cerevisiae* to the fruit fly (Brand et al., 1993). This dual system comprises by the GAL4 protein that acts as a transcription factor able to recognize an Upstream Activation Sequence (UAS), normally essential for the transcriptional activation of the GAL4 activated genes. Expression of GAL4 can be made under the regulation of a tissue/temporal specific endogenous promoter (driver line, Figure 6). When the driver line is crossed with a second line containing a UAS dependent transgene (responder line, Figure 6) the proper progeny will contain both the GAL4 and UAS element. The GAL4 protein drives the expression of the target UAS dependent transgene in a pattern specific manner and it does not activate any endogenous *Drosophila* genes. The main advantages of this couple system are: (i) The absence of toxic transgene expression in the absence of the GAL4 driver, since the UAS dependent transgene element is silent until the responder line is crossed with an appropriate GAL4 driver line; (ii) The extensive diversity of GAL4 driver lines, under the regulation of different endogenous promoters, allows researchers to express UAS transgenes in an array of tissues with different temporal patterns (Elliott & Brand 2008).

To induce the expression of the UAS transgenes in the desired tissue we used GAL4 under the regulation of the traffic jam promoter (traffic jam::GAL4, tj::G4). The traffic jam GAL4 driver line has been previously described as being optimal for RNAi expression in the follicular epithelial cells (Olivieri et al. 2010). The traffic jam GAL4 driver exhibits ever increasing expression throughout oogenesis and regular levels of expression in stages 8-9. The GAL4 system is temperature dependent, thus by simply altering the temperature at which the flies are kept, it is possible to model the expression levels of the interest transgene. GAL4 activity is minimal at 16ºC and maximal at 29ºC without compromising fly viability due to growth at high temperature. Flies with transgenes driven with traffic jam

GAL4 were grown and kept at 18ºC and switched to 29ºC one or three days before dissection, depending on the desired analysis.

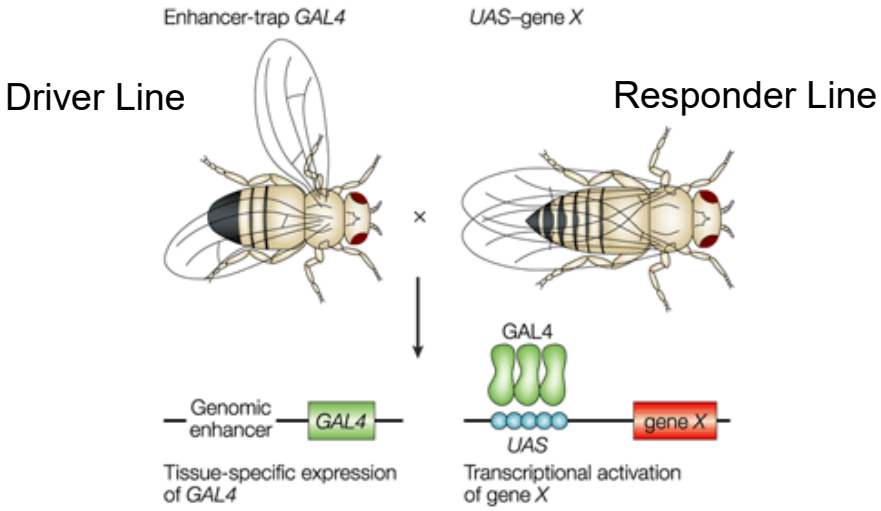

Figure 6 – Schematic representation of the GAL4-UAS system for transgene expression. After proper crossing and fly selection, a fly geneticist can obtain flies containing both the GAL4 and the UAS-transgene. The GAL4 transcription factor drives the transcription of the target UAS-transgene. An endogenous tissue specific promoter drives the expression of the GAL4 that acts on existing UAS sequences, resulting on the expression of the orange transgene in all stages of oogenesis. Adapted from (St Johnston, 2002).

2.7. Ovary F-actin staining

Ovaries of well-fed *Drosophila* females were dissected in (PBT) and fixed in 4% paraformaldehyde in PBS (PFA) for 20 minutes. Ovaries were washed 2 times x 10 minutes in PBT and then incubated in PBT supplemented with 1/250 TRITC-Phalloidin (Molecular Probes), which selectively marks filamentous actin (F-actin), for 30 minutes. Ovaries were then washed 2 times x 5 minutes in PBT and then incubated overnight in Vectashield with 1mg/mL DAPI (Vector Laboratories). Finally, slides were mounted.

Images were collected using a 63x oil objective in a Leica TCS SP5 II laser scanning confocal microscope (Leica Microsystems, Germany). 1,7x zoom was used for all ovaries to facilitate developmental stages comparison. Data stacks were analyzed using ImageJ 1.49k software (http://rsb.info.nih.gov/ij/).

2.8. Time-lapse microscopy

Live analysis of mitotic *Drosophila* S2 cells was done in cell lines co-expressing Tub-mCh with EGFP-Mps1, CID-mCh with EGFP-Tub and Tub-mCh with EGFP-DLIC or $DLIC^{S432D}$. After the desired treatment, $7{,}5x10^5$ cells were plated in MatTek glass bottom dishes (MatTek Corporation) previously treated with Concanavalin A at 0,25 mg/mL (Sigma). Six-dimensional datasets were collected at 25ºC with a spinning disc confocal system (Revolution; Andor) equipped with an electron multiplying charge-coupled device camera (iXonEM+; Andor) and a CSU-22 unit (Yokogawa) based on an inverted microscope (IX81; Olympus). Two laser lines (488 and 561 nm) were used for near-simultaneous excitation of EGFP and mCherry, respectively. For S2 cells, a UPLSAPO 100x/ NA 1.40 Oil objective was used and the system was driven by iQ software (Andor). Time-lapse imaging of z stacks with 0.8 µm steps covering the entire volume of the cell were collected every 180 seconds.

Live imaging analysis of the follicular epithelium was performed with a PLAPON 60x/NA 1.42 objective using the same software. Time-lapse imaging of z stacks was collected as 11 optical sections along 10 µm every 30 seconds.

Image sequence analysis and video assembly was done with ImageJ and Andor iQsoftware.

2.9. Antibodies

The primary antibodies used were mouse anti-αtubulin B512 (Sigma) at 1:4000 for immunofluorescence (IF), chicken anti-GFP ab13970 (abcam ®) at 1:2000 for IF and rabbit anti-Mud (aa375-549) (gift from Floris Bosveld, Bosveld et al., 2016) 1:2000. The secondary antibody anti-chicken 488. The others secondary antibodies conjugated with fluorescent dyes are from the Alexa series (Invitrogen) and were used according to the manufacturer's instructions. Fluorescent secondary antibodies were used at 1:2000 except for Alexa 647 conjugated ones, which were used at 1:1000.

CHAPTER 3

RESULTS AND DISCUSSIONN

3.1. MPS1 overexpression induces abnormal spindle rotation in mitotic S2 cells

Proper spindle orientation in mitotic cells is critical (i) to avoid genomic instability, (ii) for determination of the progeny fate, and (iii) to ensure that daughter cells are placed in the right position within the tissue. While studying the role of MPS1 kinase in the spindle assembly checkpoint an interesting and unexpected phenotype was observed. Live-cell imaging of *Drosophila* S2 cells revealed an evident rotation of the mitotic spindle in cells overexpressing MPS1 (Figure 7 A,B). To quantify the extent of spindle rotation, the spindle poles were tracked and the corresponding (x, y) coordinates determined for each time frame. A straight line between the spindle poles was drawn and its slope was calculated. The line between the spindle poles in the first frame was considered as reference (0 degrees) (Figure 7 C). For each frame, the angle between the line traced and the line traced for the first frame (Figure 7 C) was measured and plotted through time (Figure 7 B). To exclude from the quantification the initial spindle poles movement upon mitotic entry and the presence of multiple centrosomes in some cells, the first frame was always considered to be the one in which a properly formed bipolar spindle could be observed. MPS1 overexpression causes a mitotic arrest due to constitutive SAC signaling. To assess whether the observed spindle rotation was a consequence of the prolonged mitotic delay we monitored the spindle behavior in S2 cells expressing endogenous levels of MPS1 but treated with the proteasome inhibitor MG132 to prevent mitotic exit. As expected, MG132 arrested S2 cells in metaphase for long periods of time. However, these cells failed to exhibit a discernable spindle rotation. Tracking the movement of spindle poles in these cells revealed a minor oscillation that is typically observed during mitosis (Figure 7 A,B). This result allows us to conclude that the spindle rotation observed upon MPS1 overexpression is not caused by the mitotic arrest.

To test whether the spindle rotation phenotype relies on MPS1 catalytic activity we overexpressed a kinase-dead version of MPS1 ($MPS1^{KD}$) (Moura et al., 2017) in S2 cells treated with MG132. Unlike the wild-type version of MPS1, overexpression of EGFP-$MPS1^{KD}$ failed to cause an evident rotation of the mitotic spindle (Figure 7 A,B). Next, we sought to assess the relevance of MPS1 subcellular localization for spindle rotation. When expressed at endogenous levels in mitosis, MPS1 preferentially accumulates at unattached kinetochores to promote SAC activation (Conde et al., 2013; Moura et al., 2017). When overexpressed, EGFP-MPS1 also

decorates the mitotic spindle and is uniformly distributed in the cytoplasm (Figure 7 A). To test if spindle rotation is caused by an excess of non-kinetochore MPS1, we overexpressed active MPS1 tethered to kinetochores. MPS1 fused to the structural kinetochore component Mis12 is enriched at kinetochores in S2 cells with only a faint signal of EGFP-Mis12-MPS1^{WT} detected in the cytosol (Figure 7 A). Interestingly, no striking spindle rotation was detected upon overexpression of EGFP-Mis12-MPS1^{WT} (Figure 7 A,B).

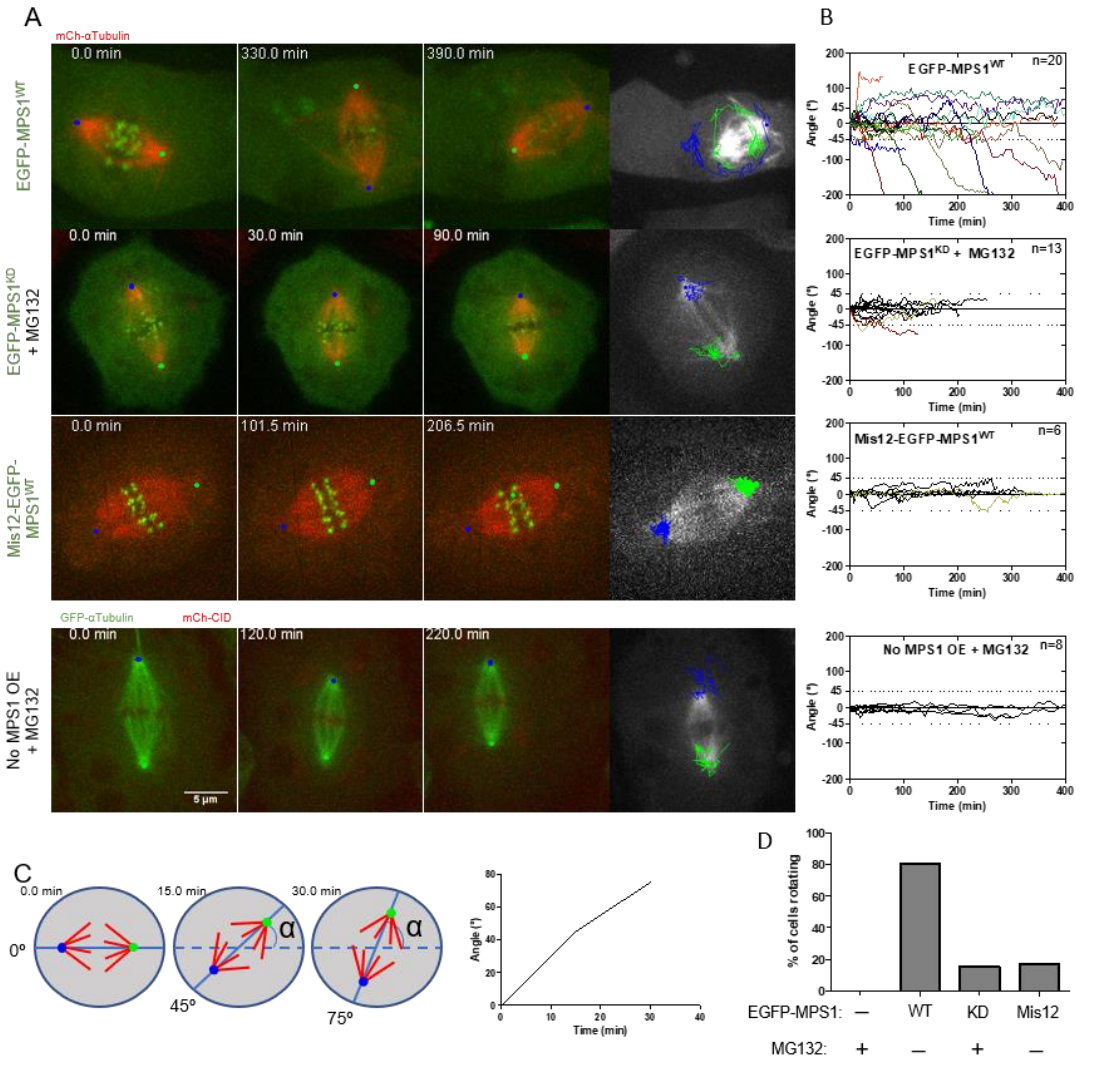

Figure 7 - Increased levels of non-kinetochore MPS1 induce abnormal spindle rotation in mitotic S2 cells. (A) Representative spindle positioning in mitotic S2 cells expressing EGFP-Mps1, Wild Type (WT), Kinase Dead (KD) and kinetochore-tethered versions, and mCherry-αTubulin (upper panel) or GFP-αTubulin and mCherry-CID (lower panels) monitored by time-lapse microscopy. Selected stills of live-cell imaging are depicted and respective time shown in min. Spindle poles positions are represented by the green and blue spots. The green and blue lines represent all the positions tracked along the movie, respectively (right) (B) Time-course quantification of the angle between the pole-to-

pole axis in each time-frame and the initial position for the indicated conditions. Black lines represent cells that never exceeded the 45 degrees threshold relative to their initial position. (C) Schematic representation of the measurement and plotting of the angle between the pole-to-pole axis in each time-frame and the initial position (α). (D) Percentage of cells exceeding 45 degree threshold for each condition in B. Scale bar, 5μm. OE stands for overexpression.

To compare the penetrance of the spindle rotation phenotype, the spindle was considered to rotate when its pole-to-pole axis moved by at least 45 degrees from its initial tracking position. Thus, under this criterion, the spindle rotates in approximately 80% of mitotic S2 cells overexpressing EGFP-MPS1^{WT}. This represents a 4-fold increase in the percentage of cells with rotating spindles relative to the EGFP-MPS1^{KD} and EGFP-Mis12-MPS1^{WT} overexpressing conditions (Figure 7 D). Collectively, these results indicate that excessive activity of non-kinetochore MPS1 induces abnormal spindle rotation in mitotic S2 cells.

Endogenously regulated MPS1 has limited impact outside the kinetochore in mitotic cells. However, its overexpression is likely causing an ectopic increase in MPS1 activity that leads to spindle rotation defects. It would be interesting to target endogenous MPS1 to the cell cortex and/or spindle poles, structures with well-established functions in controlling spindle orientation, and assess whether this induces abnormal spindle rotation.

3.2. MPS1 overexpression induces spindle rotation along the apico-basal axis in *Drosophila* follicular epithelium cells

Correct spindle orientation is essential in a tissue context, being the epithelium a paradigmatic example of it. To keep the daughter cells within the tissue, cells must divide parallel to the plane of the epithelium. *Drosophila* follicular epithelium is a valuable system to study the orientation of the spindle due to the ability to track the spindle by live-imaging of the integral tissue and its simple genetic tractability (Bastock, 2008; He, 2011). This tissue consists of an epithelial monolayer, with cells that proliferate extensively from stage 2 to 6. (Bastock, 2008). Thus, we sought to overexpress MPS1 in the follicular epithelium cells to examine its impact on spindle orientation *in vivo*. To produce adult flies overexpressing the MPS1 kinase, we needed to use an inducible system for transgene expression. For that purpose, we

used the temperature dependent GAL4 – UAS system. To induce the expression of the GAL4 only in the desired tissue we used the *traffic jam* promoter (*traffic jam*::GAL4), allowing a targeted and time regulated induction of the UAS-transgene expression (Olivieri et al., 2010). Depending on the chosen plane view, this tissue allows the observation of spindle movements along the lateral axis (basal view) or along the apical-basal axis (transverse view) (Figure 8 A).

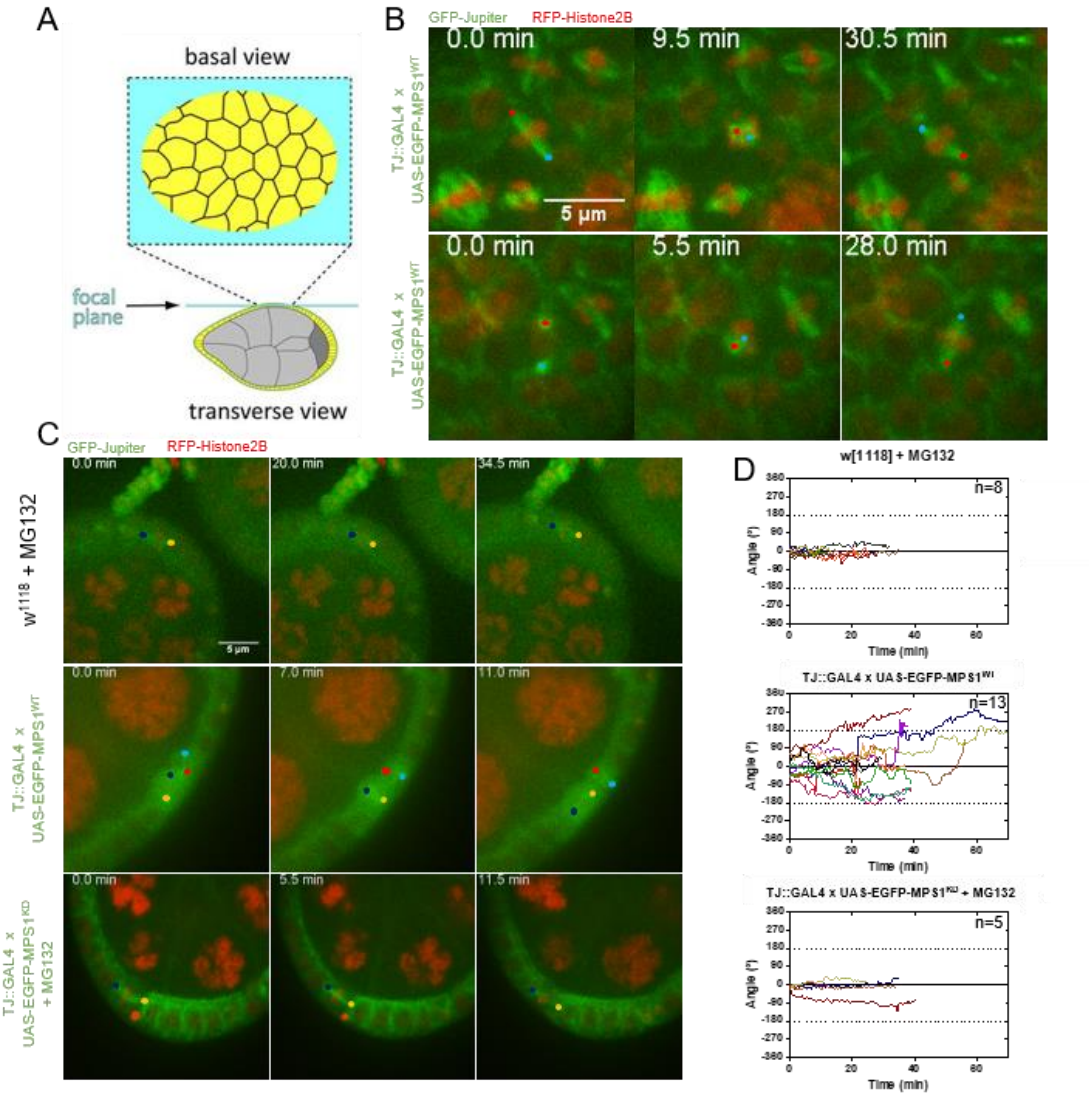

Figure 8 - MPS1 overexpression induces spindle rotation along the apico-basal axis in *Drosophila* follicular epithelium cells. (A) Schematic representation of the basal and transverse view of a *Drosophila* egg chamber. (B) Representative spindle positioning in follicular epithelium (basal view). Mitotic cells expressing EGFP-MPS1, GFP-Jupiter and mRFP-Histone2B were monitored by time-lapse microscopy. Red and light blue spots indicate spindle poles positions. The two cells highlighted appear to have spindle rotation along their apico-basal axis. (C) Representative spindle positioning in follicular epithelium (transverse view). Mitotic cells expressing GFP-Jupiter and mRFP-Histone2B in the three

indicated conditions were monitored by time-lapse microscopy. Red, yellow, dark and light blue spots indicate spindle poles positions. Two mitotic cells are displayed in EGFP-MPS1 condition; light blue and red spots represent the poles from the cell on the right, dark blue and yellow spots represent the poles from the cell on the left . (D)Time-course quantification of the angle between pole-to-pole axis in each time-point and the epithelium axis for the indicated conditions. Scale bars, 5µm.

To keep track of the spindle in the mitotic follicular cells we used flies expressing RFP-Histone 2B (DNA-associated protein) and GFP-Jupiter (spindle-associated protein) for live tissue imaging. Initially, follicular cells expressing EGFP-MPS1^{WT} were imaged at the basal view (Figure 8 B). We were able to observe some rotation from this point of view. Most of those rotations are made maintaining the spindle laterally oriented (parallel to the epithelium), as it should. Theoretically, cells could divide in any of those orientations and the daughter cells would still be rightfully placed in the tissue. However, two cells caught our attention, as their spindles seemed to flip (Figure 8 B), spindle poles in bright blue and red). Their spindles are laterally oriented and, suddenly, rotate along their apical-basal axis. This result is intriguing, because normal follicular cells should not align their spindles along the apical-basal axis.

To properly track the spindle poles and be sure of this apical-basal rotation we decided to image this tissue in its transverse view. Flies expressing GAL4 under the TJ promoter, GFP-Jupiter and RFP-Histone2B were used as background setting and three conditions were imaged: (i) no UAS transgene (w^{1118}), (ii) UAS-EGFP-MPS1^{WT} and (iii) UAS-EGFP-MPS1^{KD} (Figure 8 C). Ovaries were dissected 24 hours after TJ::GAL4 induction in Scheiner 2 medium supplemented with FBS and Insulin. Egg chambers were carefully separated and imaged. Similar to S2 cells, MG132 was added to the medium used during imaging in order to delay control and EGFP-MPS1^{KD} expressing cells in mitosis. Spindle poles were tracked and the angle between their position in each frame and the epithelial plane was plotted through time (Figure 8 D). Cells overexpressing EGFP-MPS1^{WT} exhibited massive spindle rotation along the apical-basal axis, reaching, in some cases, above 200 degrees of angle variation (Figure 8 D). This phenotype was not observed in the control nor upon EGFP-MPS1^{KD} overexpression (Figure 8 D). These two conditions, maintained most of their spindles beneath the 45 degrees range. The rotation analyzed here, when above the 45 degrees threshold may have consequences for tissue integrity. If cells divide when their spindles are oriented above that threshold they will likely originate one daughter cell outside the plane of the tissue. Molecular mechanisms

responsible for the reintegration of misplaced cells into epithelium monolayers have been proposed (Bergstralh et al., 2015). However, it is important to keep in mind that those mechanisms may fail under given conditions and may not be able to reposition all cells originated outside the epithelium. Thus, it is critical to understand if MPS1 overexpression causes spindle misorientation and if this has any consequence on tissue organization.

3.3. MPS1 overexpression leads to epithelium multilayering

The results described so far indicate that excessive MPS1 activity disrupts the spindle orientation signaling leading to abnormal spindle rotation both *in vitro* and *in vivo*. This represents an interesting finding that might assume pathophysiological relevance as MPS1 overexpression is frequently observed in cancer cells (Landi, 2008; Yuan, 2006). Thus, we envisage that MPS1 overexpression may lead to erroneous orientation of division and consequently generate daughter cancer cells outside its original tissue context (Pease, 2011). To test if MPS1 overexpression leads to the disruption of the tissue organization, we decided to induce MPS1 expression in follicular cells over a period of 72 hours. Flies were dissected and the ovaries fixated and stained with DAPI and phalloidin-red for DNA and F-actin, respectively. Egg chambers in later stages of development were imaged by confocal microscopy (Figure 9 A,B). F-actin staining allows us to delineate the cell cortex and analyze tissue organization. The egg chambers expressing UAS-EGFP-MPS1^{WT} (in green) displayed follicular epithelium disruption. Overall, the epithelia exhibited heterogeneous diameter throughout the egg chamber, presenting some regions with absence of epithelial cells and other regions with multilayered epithelia (Figure 9 A). Some follicular cells were even imaged inside the region intended for the nurse cells and oocyte (Figure 9 B, arrows). Note that the TJ::GAL4-UAS system drives the expression of EGFP-MPS1^{WT} exclusively in the follicular epithelium, thus all the cells expressing EGFP are follicular epithelial cells. On the other hand, epithelia overexpressing the catalytically inactive version of MPS1 (EGFP-MPS1^{KD}) showed an integral monolayer just like the wildtype egg chambers. Once again, to be sure that the mitotic delay induced by MPS1 is not the reason for the phenotype observed we had to find a way to produce a mitotic delay. This time the MG132 treatment was not an option because we needed to induce the delay for 3 days and only then

dissect the flies. With that in mind, we opted for the RNAi-mediated depletion of CDC16, an APC/C subunit. The mitotic delay was confirmed by live cell imaging (data not shown). Flies ovaries were dissected 72 hours after induction and egg chambers expressing CDC16 RNAi were imaged using confocal imaging to check the integrity of their epithelium (Figure 9 A). These egg chambers also displayed tissue disruption with loss of epithelial cell from the tissue (Figure 9 A). However, it is important to notice that no evident multilayering could be observed (Figure 9 A), suggesting that the morphological alterations observed upon EGFP-MPS1^{WT} are not a consequence of a prolonged mitotic delay.

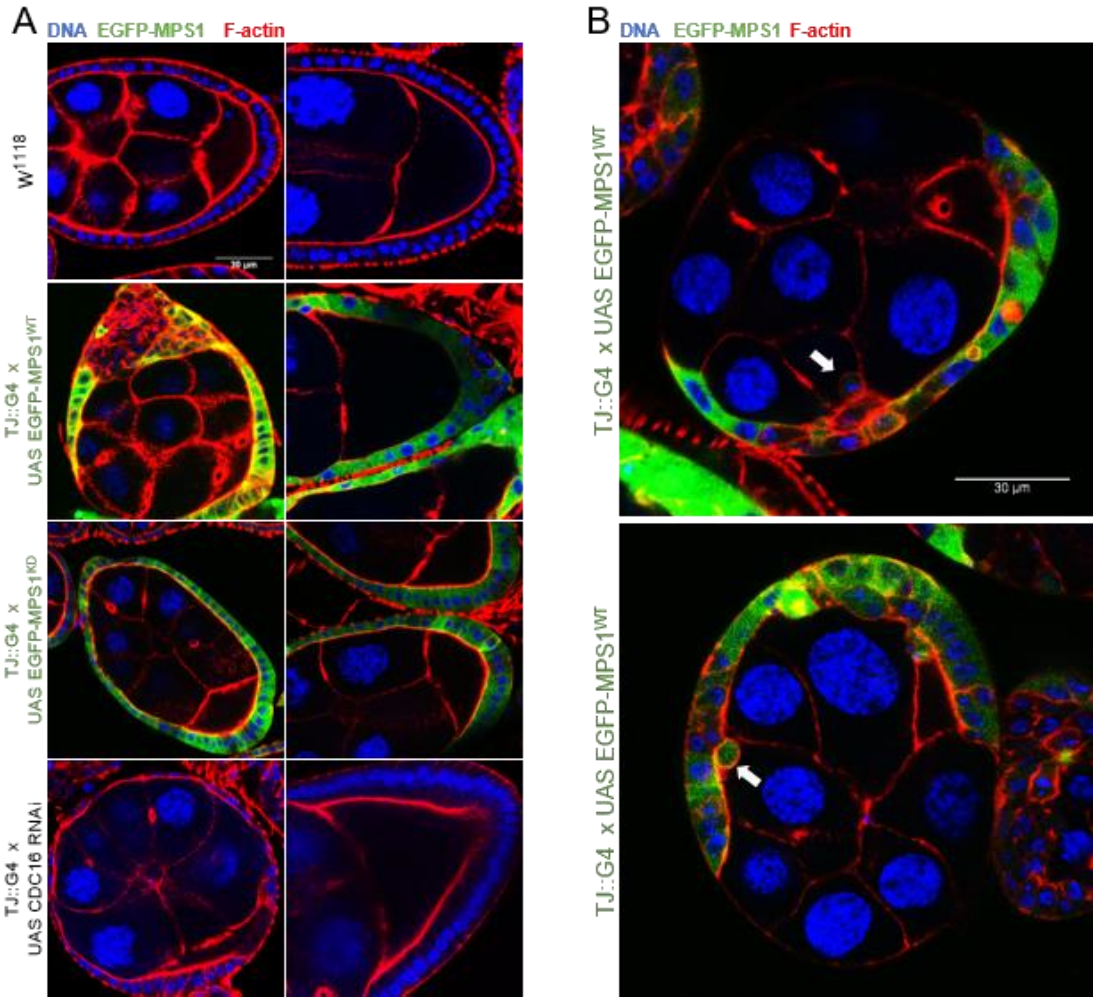

Figure 9 – MPS1 overexpression leads to epithelium multilayering. (A,B) Representative transverse images of egg chambers from flies in the indicated conditions. The egg chambers displayed belong to the Stage 7-10 range. DNA and F-actin (cell cortex) were stained with DAPI and Phalloidin-red, respectively. (B) Arrows highlight cells outside the epithelial monolayer. Scale bars, 30µm.

Collectively, these results strongly suggest that the spindle orientation defects caused by MPS1 overexpression lead to epithelium multilayering and neighbor tissues invasion.

Note that the egg chambers displayed here (Figure 9) have no dividing follicular cells, due to their developmental stage. The disruptions observed here must be related to errors that occurred in earlier stages and that have accumulated through the development. With these results in mind is fair to assume that tissues with high MPS1 levels may experience errors in division orientation. The accumulation of those errors may lead to tissue disruption and colonization of neighbor tissues by those cells. If we look at this possibility in a tumor context, increased MPS1 levels may actually help triggering invasion and metastization processes. If this correlation comes to proof, MPS1 levels in tumor cells might come to be used as a molecular signature to assess tumor aggressiveness and invasion potential.

3.4. Depletion of Mud, Dynein or Dynactin ameliorates the spindle rotation defects caused by MPS1 overexpression

Correct spindle orientation and positioning relies on the balance between pushing and pulling forces applied on the spindle poles through the astral microtubules. That balance is achieved through the concentration of motor proteins in specific regions of the cell cortex (di Pietro, 2016). One of the motor complexes described to be important in spindle orientation and positioning is the Dynein/Dynactin complex (di Pietro, 2016).

To understand if the spindle rotation observed upon MPS1 overexpression is produced by dynein/dynactin we depleted either dynein heavy chain (DHC) or the dynactin subunit $p150^{glued}$ from S2 overexpressing EGFP-$MPS1^{WT}$ (Figure 10 A). Mitotic progression was monitored by live-cell imaging and spindle poles position tracked as previously described. The angle variation of the pole-to-pole axis relative to the orientation of the spindle in the initial frame was plotted through time. Mitotic spindles exhibiting an angle variation above 45 degrees were scored as rotating. RNAi-mediated depletion of DHC or $p150^{glued}$ partially rescued the MPS1 overexpression spindle phenotype as revealed by the significant reduction in the

percentage of cells with rotating spindles (Figure 10 A-C). Moreover, for the cells in which spindle rotation was considered to occur, the average rate of angle variation was significantly reduced in the absence of Dynein (Figure 10 D).

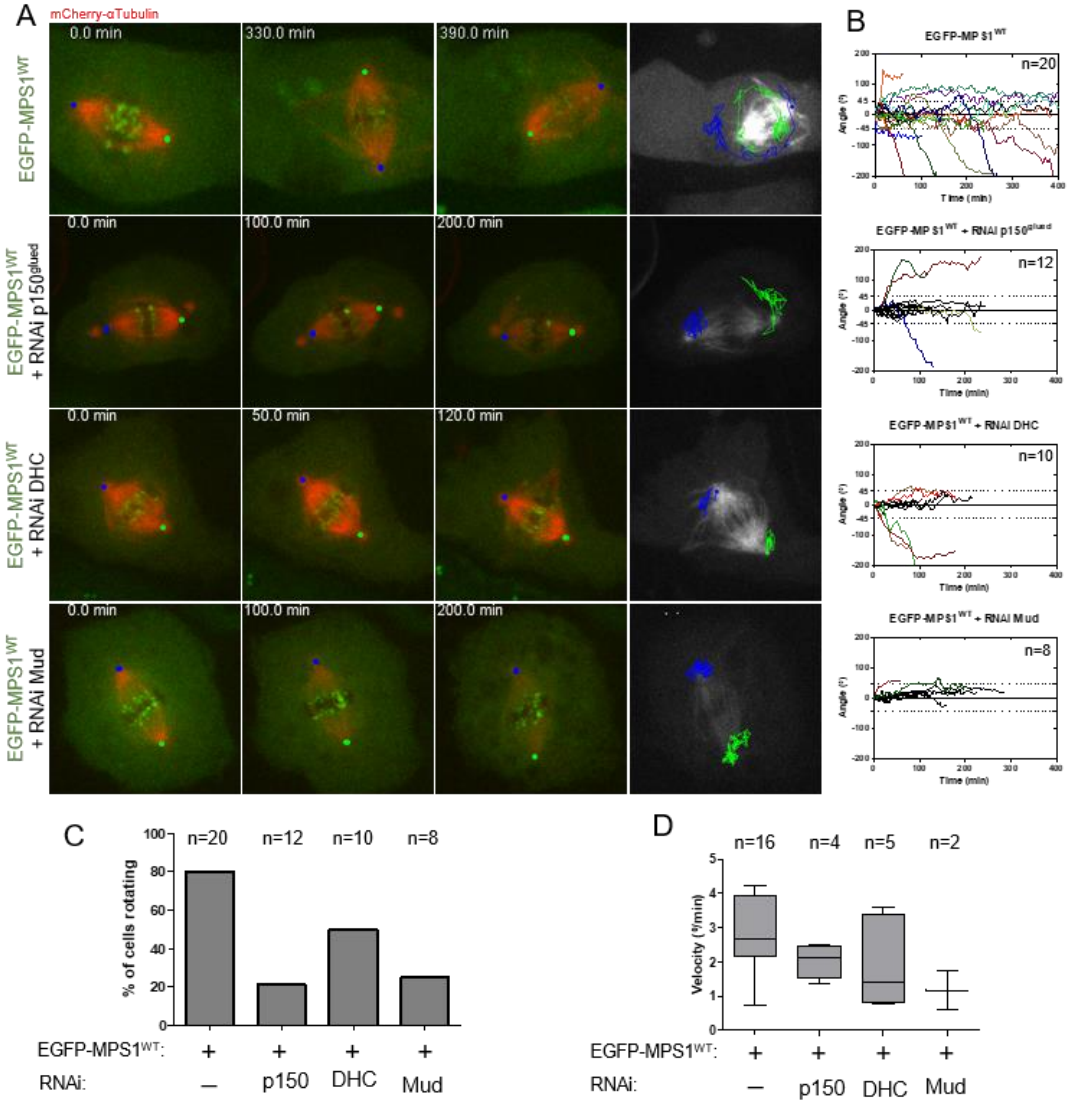

Figure 10 - Depletion of Mud, Dynein or Dynactin ameliorates the spindle rotation defects caused by MPS1 overexpression. (A) Representative spindle positioning in S2 cells expressing EGFP-MPS1 and mCherry-αTubulin with depletion of the indicated proteins monitored by time-lapse microscopy. Selected stills of live-cell imaging are depicted and respective time shown in min. Spindle poles positions are represented by the green and blue spots. The green and blue lines represent all the positions tracked along the movie, respectively (right). (B) Time-course quantification of the angle between the pole-to-pole axis in each time-frame and the initial position for the indicated conditions. Black lines represent cells that never exceeded the 45 degrees threshold relative to their initial position. (D) Percentage of cells exceeding 45 degree threshold for each condition in B. (G) Representation of the rotation rate for the cells scored in C. Scale bar, 5μm. p150 stands for p150glued.

The Gαi, LGN and NuMA (Gαi, Pins and Mud in *Drosophila,* respectively) complex is crucial for proper localization of Dynein at the cell cortex. In this evolutionary conserved complex, NuMA/Mud is the protein responsible for microtubule binding and dynein recruitment to the cell cortex (di Pietro, 2016). Hence, we decided to assess how depletion of Mud affects spindle rotation in S2 cells overexpressing MPS1. Live-cell imaging of mitotic cells and spindle pole tracking showed that depletion of Mud efficiently prevents spindle rotation. In the few rotation events that were occasionally detected, the rate of angle variation was significantly reduced, paralleling the decrease observed upon Dynein depletion (Figure 10 D).

Taken together, these data indicate that the spindle orientation defects resulting from excessive activity of non-kinetochore MPS1 require the canonical Mud/dynein/dynactin orientation pathway.

3.5. Microtubules density and Mud localization are not affected by MPS1 overexpression

Our data indicate that spindle rotation is caused by MPS1 activity away from the kinetochore. Thus, we hypothesized that MPS1 might be disrupting orientation pathways at the cortex or at the spindle poles. The number of astral microtubules and their stability near the cell cortex are important for proper spindle positioning (di Pietro, 2017). Therefore, we investigated microtubule stability upon MPS1 overexpression in mitotic S2 cells. Antibody staining for α-Tubulin did not show any significant difference in the density of central spindle microtubules or astral microtubules in comparison to cells expressing endogenous levels of MPS1 (Figure 11 A-D). Likewise, no discernable differences in spindle size and morphology were observed (Figure 11 A).

Proper anchoring of microtubules is also critical to ensure correct spindle positioning. The astral microtubules anchors are the force generator complexes such as the Dynein/Dynactin complex and other spindle-associated proteins like Mud. For that reason, we assessed Mud levels by immunofluorescence in variable MPS1 expression levels in dividing S2 cells (Figure 11 A,E,F). Mud levels normalized for the α-Tubulin levels displayed no difference across the different levels of MPS1 both

at the spindle poles and central spindle (Figure 11 A,E,F). In our hands, the Mud antibody failed to detect the protein at the cell cortex in all the conditions tested.

Due to the similar phenotypes in astral and spindle microtubules density for both conditions, is unlikely that the rotation phenotype is consequence of alterations in microtubule stability. Moreover, MPS1 overexpression does not seem to affect Mud levels or localization pattern.

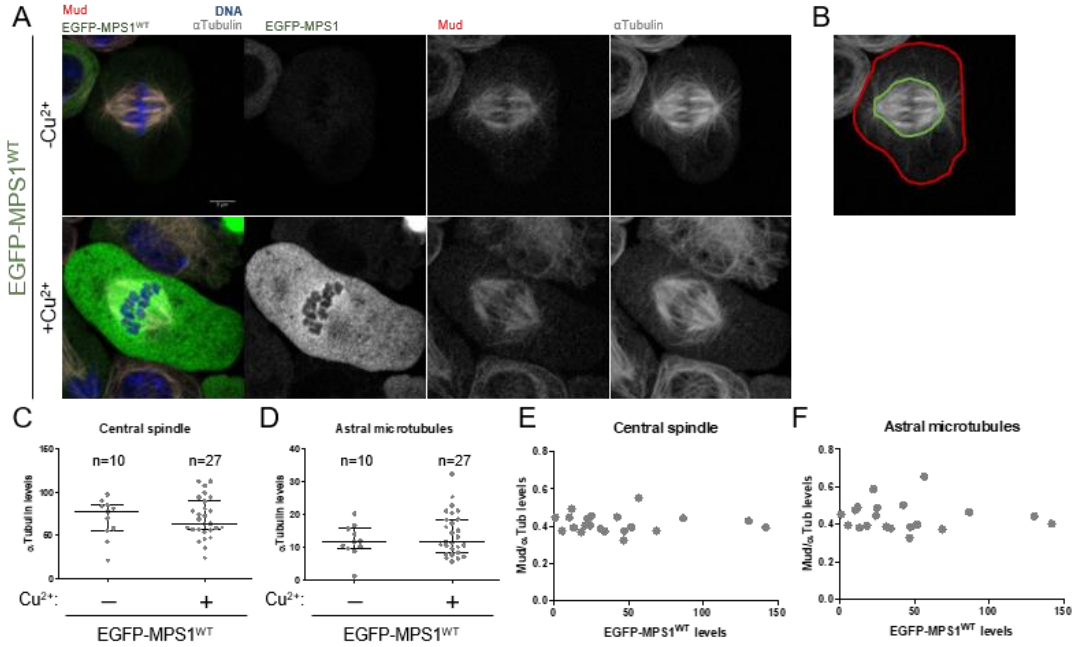

Figure 11 - Microtubules density and Mud localization are not affected by MPS1 overexpression. (A) Representative immunofluorescences of αTubulin localization in no overexpressing and EGFP-MPS1 overexpressing Drosophila S2 cells. (B) Schematic representation of the quantification performed in C–F. The red line represents was drawn along the membrane and the green on around the central spindle. Central spindle levels are considered the levels inside the green line and astral microtubules levels are considered between both lines. (C) αTubulin levels in central spindle microtubules. (D) αTubulin levels in astral microtubules. (E-) Mud levels in the central spindle (E) and on astral microtubules (F) of EGFP-MPS1 expressing cells. Scale bars, 5μm

3.6. Spindle rotation is not caused by MPS1-dependent phosphorylation of DmDLIC on S432

Recent work using chemical genetics and phosphoproteomics led to the identification of several direct and indirect downstream targets of MPS1 in human cells (Maciejowski et al., 2017). From the identified putative MPS1 substrates, the cytosolic dynein light intermediate chain (DLIC) was the only one whose function disruption had been shown to affect spindle orientation (Mahale et al., 2016). DLIC is a subunit of the dynein complex thought to be involved in linking dynein to cargos and to adapter proteins that regulate dynein function (Tan et al., 2011). The MPS1-dependent phosphorylation site detected in human DLIC and its adjacent motif is conserved in the *Drosophila* orthologue. Therefore, we mutated the serine^{432} of DmDLIC to aspartate to mimic its constitutive phosphorylation and expressed the transgene tagged with EGFP in S2 cells (Figure 12 A). We treated cells with MG132 to arrest cells in mitosis and monitored the spindle behavior over time by live-cell imaging (Figure 12 A,B). The wild type and phosphomimetic versions of DmDLIC were expressed at similar levels and exhibit the same localization pattern during mitosis. Both EGFP-DLIC^{WT} and EGFP-DLIC^{S432D} accumulate at kinetochores during prometaphase and as chromosomes congress to the metaphase plate kinetochore levels dramatically decrease. This correlates with the expected behavior of the dynein complex and its role in stripping SAC proteins to allow SAC silencing (Musacchio, 2007). It is therefore likely that these proteins are properly folded and functional. Moreover, both EGFP-DLIC^{WT} and EGFP-DLIC^{S432D} associate with the spindle and could be detected in the cytoplasm throughout mitosis (Figure 12 A). The spindle behavior in S2 cells expressing EGFP-DLIC^{S432D} was similar to the observed upon expression of EGFP-DLIC^{WT} or in S2 cells with endogenously regulated MPS1 (Figure 12 B,C). This suggest that the molecular mechanism by which the excess of non-kinetochore MPS1 promotes abnormal spindle rotation does not involve the phosphorylation of DmDLIC on Ser^{432}, or at least is not sufficient on its own. However, it is important to mention that in our experimental setting, S2 cells expressing EGFP-DLIC^{S432D} still retained the endogenous form of the protein, whose function may still sustain a normal spindle behavior.

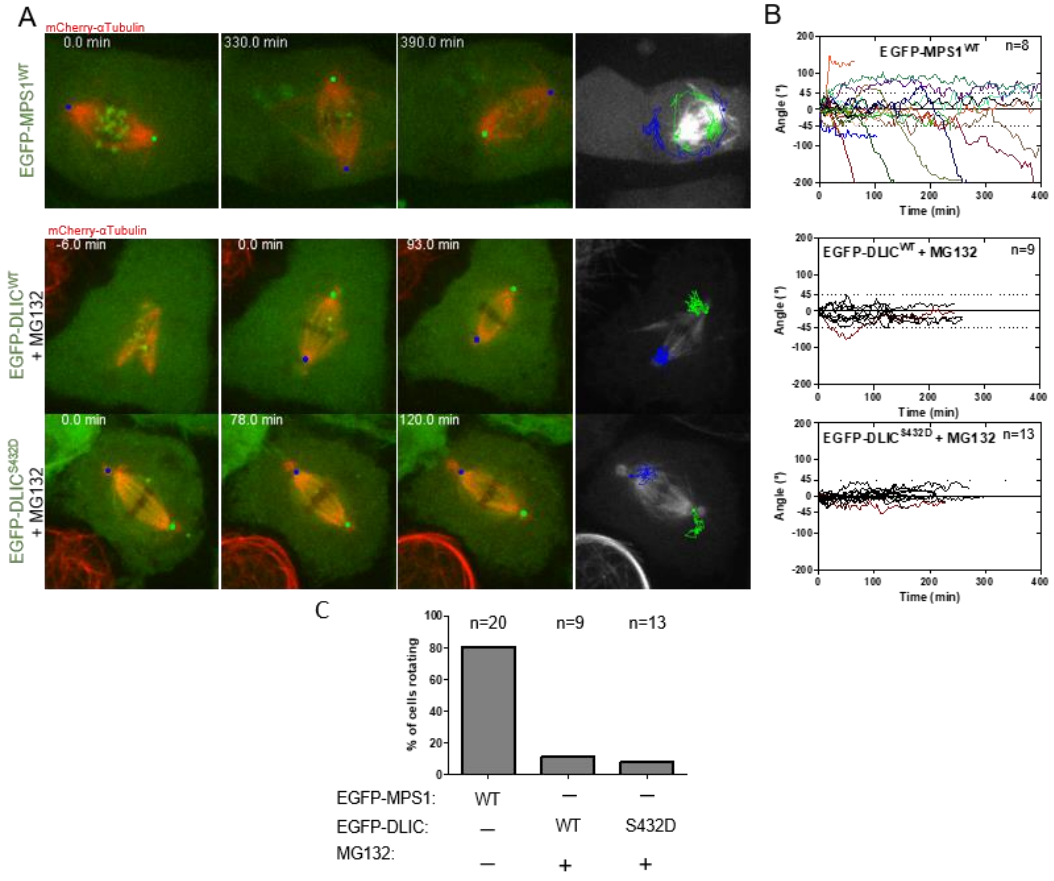

Figure 12 - Spindle rotation is not caused by MPS1-dependent phosphorylation of DmDLIC on S432. (A) Representative spindle positioning in S2 cells expressing EGFP-MPS1WT and mCherry-αTubulin (upper panel) or EGFP-DLIC, Wild Type and S432D versions, and mCherry-αTubulin (lower panels) monitored by time-lapse microscopy. Selected stills of live-cell imaging are depicted and respective time shown in min. Spindle poles positions are represented by the green and blue spots. The green and blue lines represent all the positions tracked along the movie, respectively (right) (B) Time-course quantification of the angle between the pole-to-pole axis in each time-frame and the initial position for the indicated conditions. Black lines represent cells that never exceeded the 45 degrees threshold relative to their initial position. (C) Percentage of cells exceeding 45 degree threshold for each condition in B. Scale bar, 5μm.

3.7. Concluding Remarks

In this work we report an anticipated link between the mitotic checkpoint and the spindle orientation pathway. We found that overexpression of the master SAC regulator, MPS1 kinase, causes massive rotation of the mitotic spindle in *Drosophila* S2 cells. Importantly, we were able to demonstrate that the abnormal spindle behavior is not an indirect consequence of the mitotic arrest imposed by constitutive SAC function resulting from MPS1 overexpression. Our data demonstrate that it is

the excessive catalytic activity of non-kinetochore pools of MPS1 that induce abnormal spindle rotation.

Proper control of spindle orientation is particularly important within the context of a tissue to ensure that its normal architecture and morphology are maintained as well as to prevent aneuploidy and possible malignant transformation or progression. The abnormal spindle rotation caused by MPS1 overexpression in S2 cells was also recapitulated *in vivo* in the follicular epithelium of adult flies. Follicular cells overexpressing MPS1 exhibited spindle rotation along their apical-basal axis. In some cells, we could observe more than 180 degrees of rotation, which we confirmed not to be caused by a prolonged mitotic delay. Importantly, we were able to analyze the consequences of MPS1 overexpression for tissue integrity and organization at later stages of oogenesis. We observed regions of the epithelium lacking cells, probably due to long periods in mitosis and apoptosis. However, we also found regions where the epithelium was no longer an organized monolayer. Instead, we could clearly see that the epithelium was now presented as a multilayered tissue. This finding strongly suggests that the spindle orientation defects caused by MPS1 overexpression lead to epithelium multilayering and probably favors tissue evasion. Interestingly, MPS1 overexpression is frequently detected in several tumors and often correlates with advanced stages of malignancy.

Aiming to dissect the molecular underpinnings of MPS1-induced spindle rotation we depleted several well-established regulators of spindle orientation. RNAi-mediated depletion of Dynein, Dynactin or Mud/NuMA decreased the frequency and the velocity of spindle rotation in MPS1-overexpressing cells, suggesting that excessive MPS1 deregulates spindle orientation through the conserved Mud/Dynein/Dynactin pathway. Spindle and astral microtubules density was not affected by MPS1 overexpression as well as Mud localization pattern and levels. Moreover, expressing a phosphomimetic version of Dynein Light Intermediate Chain (DLIC), in a MPS1 putative phosphorylation site (Ser^{432}), did not cause an evident rotation of the mitotic spindle. Thus, further studies are required to understand the molecular mechanism by which excessive activity of non-kinetochore MPS1 deregulates spindle orientation. Identifying MPS1 targets responsible for this phenotype and understanding the molecular insights of their interaction is critical to understand the results obtained. If these observations are replicated in human tissues or organoid models, MPS1 levels may be used, in the future, to assess tumor aggressiveness and invasion potential.

CHAPTER 4

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