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THE ROLE OF MOB PROTEINS IN PROTOZOAN CELL CYCLE REGULATION

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The role of Mob proteins in protozoan cell cycle regulation

ABSTRACT

Proper cell division and control of cell proliferation are critical aspects in cell biology, with implications during embryonic development and in the maintenance of organisms' homeostasis. Mob1 is a core protein of the Mitotic Exit Network and of the Hippo pathway, fundamental signaling cascades for the correct metaphase to anaphase transition and for the proper balance between cell proliferation and death. In this work we took advantage of two protozoan organisms to investigate the role of Mob1, the most ancient protein of the Hippo pathway. In the ciliate *Tetrahymena thermophila* we demonstrated that Mob1 has a polarized subcellular distribution, concentrating in the basal bodies of the cell posterior pole. During cell division, the protein also localizes in the region where the division plane is formed and its absence in this specific place leads to the mispositioning of division axis and cytokinesis impairment. These results revealed that Mob1 directly links proper cell polarity to correct cell division. Our studies of Mob1 in the apicomplexan parasite *Toxoplasma gondii,* also a permanent polarized unicellular organism, contributed to a better understanding of how parasites may regulate cell proliferation inside the host cell, a critical aspect for the course of infection. In *T. gondii*, Mob1 also localizes preferentially in the posterior pole of the cell, where the basal complex, which is essential for cytokinesis, is localized. Interestingly, in agreement with a role for Mob1 in proliferation control in *T. gondii*, we observed that *mob1* mRNA levels are dramatically diminished when parasites are actively replicating inside the cell and that Mob1 overexpression leads to a delay in the parasite replication rate. Altogether, the work presented clearly positions Mob1 as an ancestral molecule playing a critical role in the cross-road of cell polarity establishment, correct cell division and proliferation control.

Keywords: Mob1 proteins, cell polarity, cell division/proliferation, *Tetrahymena thermophila*; *Toxoplasma gondii*

O papel das proteínas Mob1 na regulação da divisão celular em protozoários

RESUMO

A divisão celular e o controlo da proliferação são aspectos fundamentais em biologia celular com implicações no desenvolvimento embrionário e na manutenção da homeostasia nos organismos. A proteína Mob1 é uma componente de duas vias de sinalização celular, a *Mitotic Exit Network* e a via de sinalização Hippo, cascatas de fosforilação essenciais para a correcta transição entre a metáfase a e a anáfase e para o balanço entre a proliferação/morte celular. Neste trabalho, utilizámos dois protozoários modelo para investigar a função da proteína Mob1, a mais ancestral das proteínas nas vias de sinalização referidas. No ciliado *Tetrahymena thermophila*, demonstrámos que a proteína Mob1 apresenta uma localização polarizada, estando principalmente concentrada nos corpos basais do polo posterior das células. Aquando da divisão celular, a Mob1 também é observada na região da célula onde se forma o eixo de divisão. Esta localização é essencial visto a ausência de Mob1 no local conduzir ao deslocamento do eixo e impedir a citocinese. O nosso estudo no parasita apicomplexa *Toxoplasma gondii*, um organismo também permanentemente polarizado, contribuiu para compreender melhor, o possível mecanismo de regulação da proliferação dos parasitas dentro da célula hospedeira, um aspecto essencial no desenvolvimento da infecção. Em *T. gondii*, a proteína Mob1 também se concentra no polo posterior da célula onde se localiza o complexo basal, uma estrutura envolvida na citocinese. Claramente suportando a nossa hipótese que a Mob1 desempenha um papel no controlo da proliferação, observámos que os níveis de RNA mensageiro do gene *mob1* são drasticamente diminuídos quando os parasitas estão no período de replicação activa dentro das células hospedeiras. Adicionalmente, a acumulação da proteína no citoplasma dos parasitas provoca um atraso significativo na sua taxa de replicação. Em conjunto, o trabalho apresentado posiciona a proteína Mob1 como uma molécula ancestral envolvida na conexão entre o estabelecimento da polaridade, a correcta divisão e o controlo da proliferação celular.

Palavras-chave: Proteínas Mob1; Polaridade Celular; Controlo da Divisão/Proliferação; *T. thermophila; T. gondii*

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ABBREVIATIONS

APC/C – anaphase promoting complex/cyclosome

aPKC – atypical protein kinase

ATCC - American Type Culture Collection

BAG1 – bradyzoite antigen 1

BB – basal body

BIRC5 - Baculoviral IAP (inhibitor of apoptosis protein) Repeat-Containing 5

BLAST - Basic Local Alignment Search Tool

BSA – bovine serum albumin

Bub1/2 - budding uninhibited by benzimidazole-1 1/2

cAMP - Cyclic adenosine monophosphate

Cdc14 – cell division cycle 14

Cdc42 - Cell division cycle protein 42

CDKs – Cyclin dependente kinases

cDNA – coding DNA

CENP-E/A – cetromeric protein E/A

CEP55 – centrosomal protein 55

cGMP – cyclic guanosine monophosphate

CPC - chromosomal passenger complex

CPC – chromosome passenger complex

CVPS – contractile vacuole pores

DAPI - 4', 6-Diamidino-2-Phenylindole

dd- destabilization domain

DIAP1- - Drosophila Inhibitor of Apoptosis Protein 1

DLG – discs-large

DMEM - Dulbecco's modified Eagle's médium

DNA - Deoxyribonucleic acid

DP – division plane

eIF2 - eukaryotic initiation factor-2

ESCRT - endosomal sorting complex required for transport

G0; G1; G2 – gap 0; 1; 2

GAP – GTP activating protein

Gapdh - glyceraldehyde-3-phosphate dehydrogenase

GFP – green fluorescent protein

GST - Glutathione S Transferase

GTP - Guanosine triphosphate

HFF - Human foreskin fibroblast

IF – immunofluorescence

IMC – inner complex membrane

IPTG - isopropyl-β-D-thiogalactopyranoside

KD – knock down

kDa - 10^3 Dalton

LATS1/2 – large tumor suppressor ½

LGL – lethal giant larvae

M –Mitosis

MAC – macronucleus

Mad1/2 – Mitosis arrest deficiency 1/2

MATS – Mob as tumor suppressor

MEN – mitotic exit network

MIC – (Chapter III) microneme proteins

MIC – micronucleus

ml – milliliter

Mob1 – Mps one binder 1

Mps1 – multipolar spindle 1

mRNA - messenger ribonucleic acid

MTT1 - metallothionein gene 1

NCBI - National Center for Biotechnology Information

NDR – nuclear dbf2-related

Neo – neomycin

NIMA – never in mitosis protein

NOA – new oral apparatus

NuMa1 - Nuclear mitotic apparatus protein 1

OA – oral apparatus

ORF – open reading frame

PAR proteins - partitioning-defective proteins

PBS – phosphate-buffer saline

PCR – polymerase chain reaction

PFA – paraformaldehyde

Plk1 – Polo-like kinase 1

RNA - Ribonucleic acid

RON – rhoptry neck protein

ROPs – rhoptries proteins

RT-PCR – reverse transcriptase PCR

S – Synthesis

SAC – spindle assembly checkpoint

Shd – shiled

SIN – Septation initiation network

siRNA – short interference Ribonucleic acid

SPB – spindle pole body

SPOC – Spindle positioning checkpoint

SPP – super proteose peptone

Tg ADF – T. gondii actin depolymerizing factor

TgMorn1 – *T. gondii* Membrane Occupation and Recognition Nexus 1

UTR – untranslated region

WB – western blot

wt – wyild type

YAP1 – Yes associated protein 1

µg – microgram

µl – microliter

µm – micrometer

µM – micromolar

CHAPTER I: GENERAL INTRODUCTION

1. Cell Cycle: Brief Description

From the more simple unicellular organisms, to higher eukaryotes as mammals, the cell is the basic element whose main function is to maintain its specific genetic information and to allow its propagation to future generations. In the case of multicellular organisms, the adult arises from a more or less extended developmental period in which a unique original cell, the zygote, originates the several specialized tissues and organs, through multiple cell divisions. After the end of this developmental process, cell division process allows, in the adult, for cell renovation and, being so, the maintenance of organism homeostasis.

Regarding the unicellular organisms, they regulate their cell cycle according to the specific environment in which they live. For example, free living unicellular can control their cell proliferation relatively to the nutrient abundance, and physical stresses such as temperature. A very particular case is the one of parasitic unicellular. Here, cell cycle regulation is a crosstalk between the mechanisms of parasite survival and the host's defense mechanisms.

Globally, the cell cycle is divided into two distinct periods: the interphase, a longer phase that concerns most of the cell's life and mitosis, a faster phase were cells undergo division (Fig. 1). Briefly, the interphase can be divided into three distinct and consecutive stages: G1, S and G2. In G1, the cell presents a very active metabolism and growth and there is synthesis of molecules and organelles, needed for the different cell functions. At this time, cells can receive external signals that dictate their future: entering G0, a non-proliferative phase or continuing cycling and enter S phase (Alberts et al., 2007; Cooper & Hausman, 2007).

Being so, if the environment is favorable, cells go to S phase where DNA replication takes place. At this point, each chromosome is duplicated which allows that the future daughter cells receive the same DNA content initially present in the mother cell. After DNA replication the cell is almost ready for division. Right before mitosis, cells undergo a G2 period where the molecules necessary for division are synthetized. Therefore, G2, as G1, is a growth period where cell activities enable cell to divide.

Mitosis occurs in a short period of time in the life of the cell. It has, as main function, nuclear division and the equal distribution to the daughter cells of DNA molecules replicated in the S phase. Formally, mitosis is divided in four distinct stages: prophase, metaphase, anaphase and telophase. At the end of these sequentially occurring phases, cytokinesis, the individualization of the two daughter cells, takes place.

At prophase, the duplicated chromosomes start to condense, being this condensed state of DNA a critical point for its correct segregation. In fact, longer interphase chromosomes would be very difficult to equally segregate, and cytokinesis would not occur properly if DNA portions were still in the cleavage furrow region.

In a typical mitosis, during prophase, besides this chromatin reorganization, the duplication cycle of the centrosome (the main microtubule organizing center in animal cells) also occurs. Thus, each of the newly duplicated centrosomes migrates towards opposite poles of the cell while the mitotic spindle, which results from the reorganization of interphase microtubules, begins to be assembled. At the end of prophase nuclear envelope breakdown can be observed. The sister chromatids are connected at the specific region of the centromere, a heterochromatin rich region where the proteins of the kinetochore are recruited and assembled. In fact, it is on this specific transient structure that spindle microtubules are going to be connected.

Metaphase follows prophase and this phase is characterized by the equatorial positioning of the mitotic chromosomes in the middle region of the spindle, forming the metaphasic plate. Depending if it is a symmetric or an asymmetric mitosis, the spindle is positioned in different regions of the cytoplasm. Cells arrest in metaphase until all the kinetochores are stably connected to the spindle microtubules. Upon the fulfillment of this connection, cells can go through the anaphase. At this point, the sister chromatids of each chromosome are segregated to opposite poles of the cell.

The mitotic spindle is elongated and the centrosomes are now localized at the distal regions of the cell. At the end of mitosis, after the segregation of sister chromatids, telophase takes place. The DNA, equally distributed to daughter cells, starts to decondense and the new nuclear envelopes are assembled around it. Simultaneously, the cytoplasm of the mother cell is divided into two, in a process known as cytokinesis, which will be later described in more detail (Alberts et al., 2007; Cooper & Hausman, 2007).

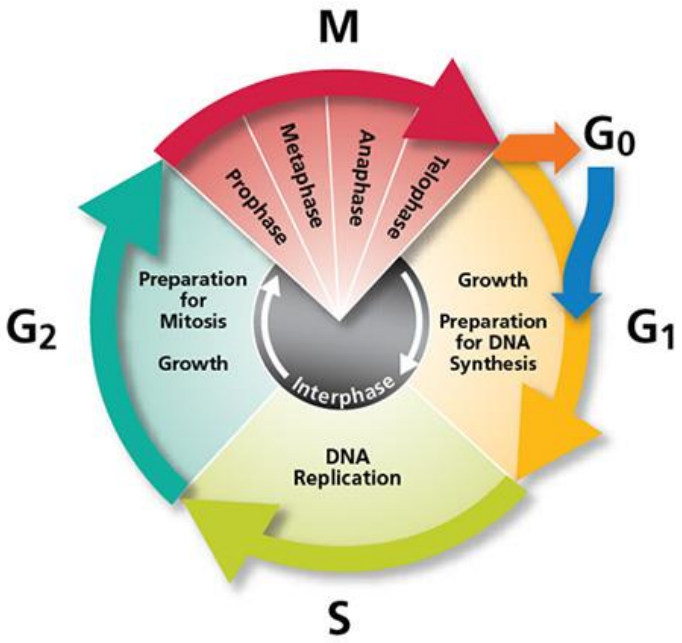

Figure 2 - Eukaryotic cell cycle. The cycle begins in G1, the first period after last cell division. At this time, there is a very active growth and metabolism. During S phase, replication of DNA molecules, which will be equally distributed to daughter cells, takes place. In G2 cells produce all the components that will be needed for mitosis. At this point, protein synthesis is very active. Finally, mitosis occurs and nuclear and cytoplasmic division takes place. (From (BD Biosciences)).

Although the steps briefly described above are observed in a typical cell cycle, namely in animal cells, there are several organisms which present striking differences in this process. For example, the centrosome is not present in all organisms and being so there are alternative strategies to build the mitotic spindle. This is the case for *Saccharomyces cerevisiae* cells, which possess a structure homologue to centrosome, the spindle pole body. Also the ciliate *Tetrahymena thermophila* builds the mitotic spindle possessing neither centrosomes nor a spindle pole body. Other remarkable difference is the case of organisms that present closed mitosis. In this particular situation, the nuclear envelope breakdown does not take place. Examples of organisms with closed mitosis are the two models used in the present study: the ciliate *T. thermophila* and the apicomplexa *T. gondii*.

This analysis of the different details in the cell cycle/mitotic process is surely simplistic and merely illustrative. Nevertheless, for the purpose of this work it matters to be stressed that, regardless of the different strategies, in all cases, the final objective is to properly segregate the DNA content and to distribute the other cell components, namely cytoplasmic organelles.

Taking into account the importance of correctly transmitting the genetic information to the daughter cells, cell division is a tightly controlled process, existing

several mechanisms that work throughout the cell cycle and that guarantee, in normal conditions, its success.

1.1 The importance of regulation in cell division

During the cell cycle, either in interphase as in mitosis, there are several checkpoints that verify the precision of some of the phases described before. These checkpoints, a total of three in all cell cycles are: the G1 checkpoint, the DNA replication checkpoint (S to G2) and the Spindle Assembly checkpoint (Mitosis). The G1 checkpoint ensures that in G1 to S transition the cellular environment is suitable to initiate DNA replication and verifies if the DNA has lesions that can compromise the genetic information transmitted to next generation. In this situation, if the cell machinery does not ensure that DNA molecules are intact and without errors before the restriction point (after which cells are irreversibly committed to enter S phase), DNA will be replicated with the eventual errors. This fact implies that undetected point mutations, insertions or deletions will be transmitted to daughter cells. DNA errors accumulation, due to G1 checkpoint deregulation, is intimately connected to cancer development (Alberts et al., 2007; Cooper & Hausman, 2007).

The DNA replication checkpoint occurs at the end of S phase in transition to G2. The checkpoint machinery evaluates if the DNA was correctly and completely replicated. The consequences of G2 checkpoint failure are very similar to the previous one. Even though DNA molecules can be intact before replication, the replication machinery may itself compromise genetic information by the insertion of errors in the newly synthetized DNA molecules (Alberts et al., 2007; Cooper & Hausman, 2007).

Finally, the spindle assembly checkpoint (SAC) ensures that all the chromosomes are correctly bound to spindle microtubules to allow an even distribution to the daughter cells. Indeed, it is crucial to ensure that homologous chromatids are correctly segregated to opposite sides of the cell. For that, cells present a mechanism that in metaphase, verifies if all the chromosomes are connected, by the kinetochore, to the spindle microtubules, arresting cells in this phase until this condition is achieved. In this particular case, the deregulation of SAC may originate daughter cells with abnormal DNA content, i.e. aneuploid cells, and consequently, genomic instability (Alberts et al., 2007; Cooper & Hausman, 2007).

The malfunctioning of any of these control mechanisms has potentially the formation of daughter cells with severe genetic defects.

1.2 Regulatory molecular mechanisms of cell cycle – a brief overview

After presenting a framework of the problem, it now matters to address the molecular mechanisms at the basis of cell cycle dynamics. Actually, these different control mechanisms acting during the cell cycle can be interpreted as a set of biochemical switches that are negatively regulated. The molecular complexity of this process is massive, but, in a basic view, all the steps involve a protein kinase (from the serine/threonine kinases family) broadly known as CDKs (cyclin dependent kinases), whose activity oscillates during the cell cycle. These proteins are responsible for the phosphorylation and activation of the molecular players of processes such as DNA replication, mitosis and cytokinesis and are in turn regulated by another family of proteins, known as cyclins. Each cyclin binds to the respective CDK protein which activates its catalytic function. In summary, different cyclin-CDK pairs act in different cell cycle stages (Alberts et al., 2007; Cooper & Hausman, 2007). This is a negatively regulated action since, for cells to proceed in the cycle, the specific cyclin at each step has to be targeted for degradation in an ubiquitination dependent mechanism. As a consequence, the interacting CDKs are inactivated and cell cycle progresses (Alberts et al., 2007; Cooper & Hausman, 2007).

CDKs may be classified into two groups: interphase CDKs and mitotic CDKs. In G1 phase, CDK4 and CDK6 bind to cyclin-D type cyclins as a response to growth factor stimuli, typical of this point in cell cycle (for review see (Enders, 2012)). Later in the end of G1, transition to S phase, the complex CDK2-Cyclin E is formed. This complex is present at the centrosome and is involved both in DNA replication and the centrosome duplication cycle, which evidences the coordination of these two processes (Hinchcliffe, Li, Thompson, Maller, & Sluder, 1999; Matsumoto, Hayashi, & Nishida, 1999; Matsumoto & Maller, 2004; H. Zhao, Chen, Gurian-West, & Roberts, 2012). In G2 to M transition, a CDK2 is activated by the connection to cyclin A. In mitosis, the degradation of this CDK2-cyclin A complex allows the establishment of the main complex for cell division completion, the CDK1-cyclin B complex. Just before anaphase, cyclin B is targeted for degradation, which inactivates CDK1 and cells undergo DNA segregation (for review (Malumbres & Barbacid, 2009)).

Given the complexity of the mechanism responsible for metaphase to anaphase transition, this specific point of the cell cycle will be addressed in more detail.

Interestingly, although cell cycle transitions are the ones described above, studies in mice have shown that alone the CDK1-cyclin B complex is capable of promoting all cell cycle transitions. Thus, CDK1 emerge as the unique kinase essential for cell cycle to proceed (Santamaria et al., 2007).

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1.3 The Metaphase to anaphase transition

One of the most critical steps for mitotic precision is the transition from metaphase to anaphase. In fact, the unbalanced distribution of chromosomes to the new cells has severe consequences as aneuploidy and genomic instability. The SAC is the mechanism in the cells that is responsible for the control of this specific step. As described, SAC machinery ensures that all the chromosomes are stably connected to spindle microtubules by the kinetochore, a transient protein scaffold assembled during mitosis. A single unattached kinetochore is capable of emitting enough signaling to maintain SAC active, which implies that the signal must be amplified (Rieder, Cole, Khodjakov, & Sluder, 1995) (Jia, Kim, & Yu, 2013). The two sister chromatids are maintained together by a protein named cohesin which persists, at least in the centromeric region, until just before anaphase onset. This physical connection of sister chromatids is critical to chromosome biorientation and consequently to chromosome segregation (for review see (Peters & Nishiyama, 2012)). Cell division proceeds until metaphase, time at which SAC activation is catalyzed by the presence of unattached kinetochores. SAC is formed by several proteins, Mad1, Mad2, Bub1, Bub2 and Mps1, firstly identified by genetic screening done in *Saccharomyces cerevisiae* (Li & Murray, 1991; Roberts, Farr, & Hoyt, 1994; Weiss & Winey, 1996), which are highly conserved throughout all the eukaryotic lineage (for review see (Jia, Kim, & Yu, 2013).

SAC activation's main consequence is the inhibition of another protein complex, the APC/C (Anaphase promoting complex/ cyclosome). This complex is composed by more than 15 proteins which function is the ubiquitination of several substrates, as for example securin and cyclin B (for review see (Kim & Yu, 2011; Lara-Gonzalez & Taylor, 2012)). After all the chromosomes are properly attached, SAC signaling is extinguished and cells can undergo anaphase. Although object of intense study, the mechanism by which SAC is inactivated is not yet fully understood. Nevertheless, it has been shown that microtubules attachment to the kinetochore and chromosome

biorientation towards opposite sides of the cell generate tension at the kinetochore which in turn inactivates SAC (Maresca & Salmon, 2009; Uchida et al., 2009). Once SAC is silenced, the APC/C is no longer inhibited and becomes active. This complex promotes anaphase by targeting for degradation core proteins that maintain cells in metaphase. Cyclin B degradation implies the inactivation of CDK1 which allows metaphase to anaphase transition.

1.4 Cytokinesis: the final step of cell division

The last step of cell division is the physical separation of the two daughter cells by a process known as cytokinesis. The abscission of cell membrane and so, the individualization of the newly formed cells, is a process intimately associated with the corrected segregation of genetic material and is coordinated in time and space with anaphase progression.

In animal cells, after chromosome segregation, the microtubules of the anaphase spindle, in the central spindle region, serve as a platform to accumulate key regulatory and structural proteins in the place where division plane is to be formed. The major protein complex involved in the process is the chromosomal passenger complex (CPC) which is formed by the kinase Aurora B, the inner centromere protein INCENP and other two molecules, Survivin (BIRC5) and Borealin (CDCA8) (for review see (van der Horst & Lens, 2014)). Also Polo-like kinase 1 accumulates at the spindle midzone which leads to the activation of specific proteins in this region. Aurora B activates the central spindilin complex and the activity of these regulatory proteins culminates with the activation of the *in locus* of the small GTPase Rho (A) which in turn associates to the cell membrane in the equatorial region of the cortex, where the cleavage furrow will be formed (for review see (Green, Paluch, & Oegema, 2012; Lacroix & Maddox, 2012)).

Basically, the presence of Rho A in an active form in cell´s equator allows the establishment of a multiproteic contractile ring, which constriction based on a actomyosin-based force will lead to alterations in cell shape and simultaneously the maturation, by post-translational modifications, of central spindle microtubules. Even though contractile ring function is based on actomyosin forces, septins are also present in the structure via recruitment made by anillins that works as a linker protein to bind the components of the ring (for review see (Green et al., 2012)). Recently, using *Drosophila melanogaster*, it has been shown that septins are responsible by the ring

curvature of F-actin filaments, demonstrating its critical role in the constriction process (Mavrakis et al., 2014).

At the end of cytokinesis, it can be observed the formation of other ring-like structure, the midbody, which is formed in the region where the central spindle microtubules plus ends overlap (for review see (Schiel, Childs, & Prekeris, 2013)). This protein structure is composed by several molecules that concentrate in the intercellular bridge, which can maintain the two daughter cells connected by several hours, until abscission occurs. The specific function of most of the proteins that accumulate in midbody is currently unknown, nevertheless midbody is seen as a platform for the abscission machinery (for review see (Fededa & Gerlich, 2012)). The final step of cytokinesis, the abscission of the intercellular bridge resulting in the total individualization of the two cells, is far from only being a simple cut. In fact, this process is controlled by several protein complexes that involve both lipid and cytoskeleton dynamics remodeling and occurs asymmetrically in the intercellular bridge. Microtubules severing and actin depolymerization are observed in one or both sides of the midbody, which originates a secondary ingression in the region, placing the abscission local. This fact implies that either one of the daughter cells inherits the midbody and the molecules concentrated there or that it is discarded (for review see (Agromayor & Martin-Serrano, 2013; Schiel et al., 2013)). The biological relevance of this is still unknown.

The abscission process is initiated by the localization of the centrosomal protein CEP55 at the midbody. This localization is tightly time controlled since during anaphase PLK1 phosphorylates CEP55 which inhibits out of proper time recruitment to anaphase spindle. Later, when Plk1 is degraded, CEP55 is dephosphorylated and can relocalize in the midbody to promote abscission (Bastos & Barr, 2010). Finally, the endosomal sorting complex required for transport (ESCRT) machinery is recruited to Cep55 already present in the midbody, and mediates abscission in a mechanism that is still elusive.

2. The Mob1 Proteins

As previously mentioned, the control of cell division is a major issue during the life of the cell and several molecules and signaling pathways are implicated in this process. This regulation has consequences in all living organisms. From asexual reproduction in unicellular protozoan to the most complex metazoan where cells have to live in

organized tissues, cell division is tightly regulated. To this regulation, both cell external and internal factors play a critical role. In fact, new cells are only formed when nutrients and space conditions are favorable. In any case, cells that undergo division, intrinsically regulate genetic material segregation in proper quality and quantity.

Mob proteins are a small family of kinase activators whose role in cell division control has been emerging in the past years. Most of the eukaryotes analyzed to date have more than one Mob protein and the presence and function of these proteins is highly conserved. The first described member of the family, *S. cerevisiae* Mob1, is implicated in mitotic exit control being part of the signaling cascade known as Mitotic Exit Network (MEN) that controls anaphase to interphase transition. In multicellular organisms, Mob1 is also part of the Hippo pathway, a molecular cascade that controls cell proliferation versus cell death. Both pathways are going to be described in detail later in the present work, but for now it is worth to explore what is known about Mob1 proteins in terms of their distribution throughout eukaryotic lineage, their cellular localization in different organisms and finally their molecular functions in activation of specific NDR (nuclear dbf2-related) kinase family members.

2.1 The Mob1 in the eukaryotic lineage – conserved functions

Mob1 proteins are conserved and present in all eukaryotes. The first identified protein of the family was described as a binder of Mps1 protein in *S. cerevisiae* (Mob1p). This protein was implicated in spindle pole body duplication and mitotic checkpoint signaling (Luca & Winey, 1998). In this first study it was shown that yeast Mob1p is essential, required to maintain ploidy levels and for successful mitosis. Moreover, the authors also identified another Mob protein, Mob2p that contrary to Mob1p is not essential for yeast survival. This second protein of the family is involved in asymmetric budding by inducing a daughter-specific gene expression as a consequence of Cbk1/Mob2-dependent activation and localization of the Ace2 transcription factor to the daughter nucleus (Colman-Lerner, Chin, & Brent, 2001; Weiss et al., 2002). Later, the role of Mob1 in mitosis completion was dissected when Mob1 was implicated in MEN signaling control. Shortly, after the study in budding yeast, data from a study using the fission yeast *Schizosaccharomyces pombe* supported a role for Mob1 in septum formation, as spores in *mob1* mutants failed to divide which led to highly elongated cells (Salimova, Sohrmann, Fournier, & Simanis, 2000).

Further studies then showed a conservation of Mob1 proteins' role in cell division also in metazoans. The first study of Mob1 in *D. melanogaster* was performed by Lai, ZC and co-workers (2005). These authors demonstrated that Mob1 works as a tumor suppressor protein since mutations in the protein led to cell over proliferation and tumor development in many fly organs. Due to this phenotype, in the fly, Mob1 was named Mats, which stands for Mob as tumor suppressor. The wide variety of tumors formed indicated that this role in tissue proliferation control is not tissue specific but a general effect.

A parallel issue in cell proliferation control is apoptosis. In fact, the balance between cell proliferation and cell death is crucial to maintain tissue and organism homeostasis. Interestingly, in the same study involving *D. melanogaster*, it was also demonstrated that Mats facilitates cell death by negatively regulating DIAP1, a caspase inhibitor essential for cell survival. Several posterior studies showed that this role of Mats in cell proliferation control is due to its core role in the Hippo signaling pathway that will be address in detail later. This function in proliferation control is also conserved in mammals. In fact, the fly Mats and its ortholog in humans, Mob1A, share 87% identity of the aminoacid sequence and Mob1A could rescue pupal lethality in Mats mutants (Lai et al., 2005).

As stated before, Mob proteins are a small family of kinase activators, more exactly, Mob proteins are involved in the activation of NDR kinases, proteins highly conserved from yeast to humans. Human cells express four related NDR kinases – NDR1, NDR2, LATS1 (Large tumor suppressor 1) and LATS2, all involved in cell division control (for a review see (A. Hergovich, 2013)). In fact, the first study to address the function of human Mob1 (Mob1A) was about the activation of NDR kinases and the authors demonstrated that human Mob1 stimulates NDR kinases activity both *in vitro* and *in vivo* (Bichsel, Tamaskovic, Stegert & Hemmings, 2004). The activation of LATS1 kinase by Mob1 was reported later and the data suggested that the interaction between these two proteins was required for proper cytokinesis as LATS1 or Mob1A depletion by siRNAs increased telophase duration (Bothos, Tuttle, Ottey, Luca, & Halazonetis, 2005). The functional relevance of these observations was addressed later in a study also using human cell lines where it was observed that Mob1A/B depletion disrupts the normal localization of CPC at spindle midzone during early anaphase which seems to be related to the larger telophase duration (Wilmeth, Shrestha, Montaño, Rashe & Shuster, 2010). More recently, it was shown that the cytokinesis failure observed in Mob1 depleted cells is related to an over stabilization of microtubules at the end of

telophase, in the midbody region. In fact, Mob1A depleted cells show higher levels of acetylated microtubules (a post translational modification present in more stable microtubules) and also microtubules with higher resistance to cold and nocadozole treatment. In addition to this, Mob1 depleted cells also showed increased motility. It was observed that the resulting daughter cells were kept together by ultrafine bridges. At a first glance, one could think that the increased mobility could be attributed to a response to the intercellular cytoplasm bridge formed due to cytokinesis failure in these cells. In this case, cells would exert a mechanical pulling force to promote abscission. However, the authors observed that the increased motility was also present after abscission (Florindo et al., 2012). These findings support the notion that Mob1A and/or the kinases regulated by it have biological roles beyond cell division, such as in microtubule dynamics and cell polarization, important for cell movement.

Even though Mob1 has been studied in mammalian systems, the role of this protein is still poorly understood. The role of Mob1 in MEN and Hippo pathways, which components are conserved from yeast to humans has been a matter of great interest. In metazoans Mob1 was described as a tumor suppressor protein, mainly because of its involvement in the Hippo signaling cascade. Supporting this fact, there are some more clinical studies showing that *mob1* expression is reduced in non-small-cell lung and colorectal cancers (Kosaka et al., 2007; Sasaki et al., 2007). Finally, a study performed on Mob1A/B double mutant mice demonstrated that Mob1 is needed for embryogenesis and also that mice without Mob1A/B protein are prone to tumor formation, demonstrating that its activity depends on development stage (Nishio et al., 2012).

Apart from the described studies in model organisms and human cells, the role of Mob1 proteins was also addressed in protozoans. Actually, taking into account Mob1 involvement in cell division control and the fact that this protein is deeply conserved among eukaryotes, it is an excellent molecule to explore also in protozoans. Protozoans are ancient organisms, mostly unicellular, which present a plethora of different cell shapes and different life styles. Some of these organisms are excellent models for cell biology studies since they present complex cell structures without the complexity of tissue environment. In the particular case of Mob1 proteins studies, our knowledge about its mechanism of action is still sparse. The use of more simple organisms, which nevertheless present the same basic cell characteristics of metazoan cells, will allow us to address better the fundamental role of Mob1 proteins. Besides that, the study of Mob1 in parasitic protozoa, such *T. gondii* will allow us to address the cell proliferation

control in a unicellular organism that forms multicellular associations inside the host cell. This particular aspect of *T. gondii* life style will allow the study of Mob1's function in a very different cell organization context when compared to free living unicellular or multicellular organisms.

The first study of Mob1 in a protozoan was done in the parasite *Trypanosoma brucei*, the African trypanosome that presents two different Mob1 proteins: Mob1-A and Mob1-B. In this study, the authors showed that both in the bloodstream and procyclic forms of the parasite life cycle, Mob1 depletion by siRNA causes cytokinesis defects. In the bloodstream form, the infective form for mammalians, cytokinesis was delayed in the early stage of furrow ingression. In contrary, parasites in the procyclic form also presented defective cytokinesis due to an incorrect positioning of the cleavage furrow (Hammarton et al., 2005). More recently, in *T. brucei*, it was also demonstrated that NDR-like kinases (PK50 and PK53) are critical for cytokinesis. Interestingly, in this parasite NDR kinases are active even in Mob1 depletion scenario and the authors could not co-precipitate Mob-GST and PK50 and PK53. Nevertheless, it is possible that Mob1 interacts with PK50 and PK53 but does so in a transient manner incompatible with co-precipitation (Ma et al., 2010).

Except for these studies in *T. brucei*, the investigation of Mob1 proteins in protozoa is inexistent. In this context, in the present work, it will be presented and discussed the results obtained from the study of Mob1 proteins in the two different protozoa: the free living ciliate *Tetrahymena thermophila* (Chapter II) and the apicomplexa parasite *T. gondii* (Chapter III). This is a significant contribute to our knowledge concerning Mob1 in complex, but unicellular organisms, that present different life styles.

2.2 Mob1 proteins – subcellular localization

The critical role that Mob1 proteins have in the cell division, may already suggest that its subcellular localization is probably associated with structures involved in mitosis progress.

As mentioned before, Mob1 was first described in the budding yeast, *S. cerevisiae*. In this organism, Mob1 shows a dynamic localization throughout the cell cycle. Using cells expressing Mob1-GFP, it was observed that at mid-anaphase, Mob1 localizes to the spindle pole bodies, the yeast functional equivalents of centrosomes. At the end of cell budding (the specific process of division in this organism) Mob1 is then re localized to the bud neck, the region where abscission will take place, where it stays

until cytokinesis and abscission occurs (Luca et al., 2001). Another study showed that Mob1 also colocalizes with Cdc14 at the kinetochores (Stoepel, Ottey, Kurischko, Hieter & Luca, 2005). Similarly, in the fission yeast *S. pombe*, Mob1 is present at spindle pole bodies during mitosis and accumulates in the medial ring where the septum is formed and posteriorly, where daughter cell separation follows (Salimova et al., 2000)

In *D. melanogaster*, Mats is localized at the plasma membrane in developing tissues. This specific localization in tissues has a functional significance for the role of Mats in Hippo signaling, since it inhibits tissue growth even in the absence of Hippo. This shows that the specific membrane localization and activation of Mats can bypass the need of Hippo activation (Ho, Wei, Shimizu, & Lai, 2010).

In humans, the subcellular localization of Mob1 protein is also known. The first study that investigated Mob1A cellular localization pattern, in human cell cultures, analyzed transfected HeLa cells expressing a Mob1A-GFP fusion protein. Beyond its distribution in the cytoplasm, Mob1A-GFP localizes at the centrosome in early mitosis and at the spindle midzone and midbody later in mitosis. Mob1A presence at the centrosome is dependent on Plk1, since Plk1 depleted cells do not exhibit Mob1-GFP at the spindle poles. Moreover, Mob1-GFP is also present at the kinetochores (Wilmeth et al., 2010). More recently, using an anti-Mob1A specific antibody, Mob1A localization at the centrosome and at the midbody was confirmed (Florindo et al., 2012).

Interestingly, in the parasite *T. brucei*, Mob1 presents a cytoplasmic distribution in the entire parasite, being excluded from the nucleus during division (Hammarton, Lillico, Welburn & Mottram, 2005). This data show that the conserved function of Mob1 is not completely dependent on a conserved cellular localization.

3. The Mitotic Exit Network

One of the signaling cascades in which Mob1 protein is involved is the Mitotic Exit Network (MEN), a signaling pathway controlling mitosis to G1 transition and so coordinating chromosome segregation with cytoplasmic constriction and cytokinesis. MEN was firstly described on budding yeast. However, the molecules involved in the signaling cascade in this organism have orthologs in higher eukaryotes which are part of the Hippo pathway (see below).

The final outcome of MEN is the inactivation of Cdk1, induced by mitotic cyclins degradation, which in turn allows mitosis ending (Fig.2). The core components of MEN

pathway are the Tem1, a GTPase protein, the kinases Cdc15, Dbf2 and Dbf20 and their activator protein, Mob1. Although Dbf2 and Dbf20 present redundant functions, the most prominent kinase in MEN is Dbf2. The majority of MEN proteins are localized at the spindle pole bodies in yeast (for review see (Alexander Hergovich & Hemmings, 2012; Meitinger, Palani, & Pereira, 2012)) which act as a platform where these molecules can concentrate, possibly allowing their activation.

Several genetic and biochemical studies in the past years allowed to establish the order of the signaling cascade events. In a timely coordinated manner, MEN signaling starts with the activation of the GTPase Tem1 at the spindle poles where the protein Nup1 acts as scaffold that assists Tem1 activation. GTP bound Tem1 can then activate the Ste20-like kinase Cdc15 which in turn promotes the up-regulation of the activity of the complex formed by the NDR/LATS kinase Dbf2 and its activator Mob1 (Bardin, Boselli, & Amon, 2003; Mah, Jang, & Deshaies, 2001).

When active, the Dbf2-Mob1 complex can promote Cdc14 phosphorylation, a reaction that triggers the release of Cdc14 from the nucleolus to the nucleus and the cytoplasm. In the cytoplasm, Cdc14 exerts its phosphatase activity and so neutralizes Cdk1, inducing cells to end mitosis (for review see (Queralt & Uhlmann, 2008)) (Fig. 2).

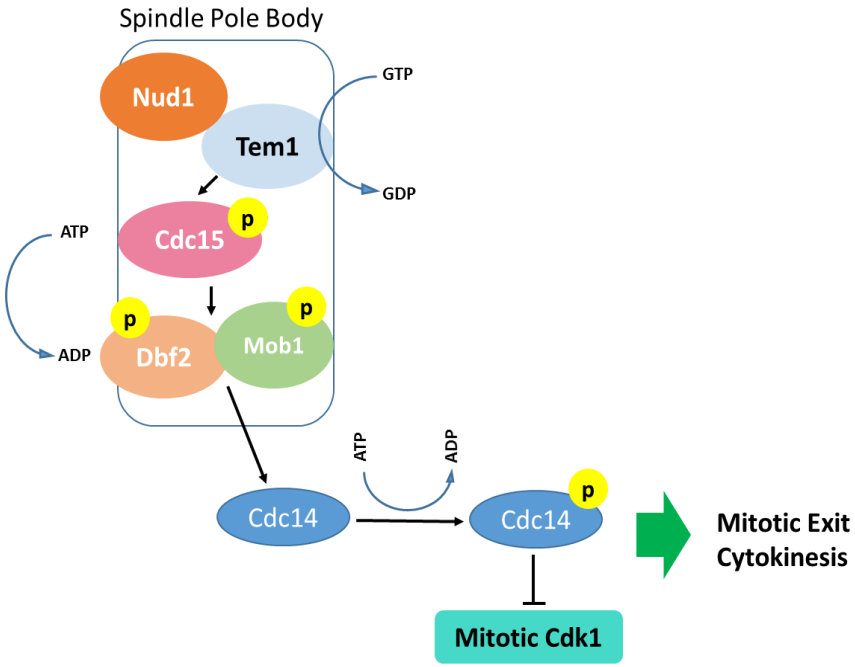


Figure 3 - The Mitotic Exit Network**.** Schematic representation of the signaling cascade of MEN as described in the budding yeast. The GTPase Tem1, the kinase Cdc15 and the complex Dbf2-Mob1 are localized at the spindle pole body. A cascade of phosphorylation events culminates with the activation of the phosphatase Cdc14 that is released into the cytoplasm where it counteracts Cdk1 activity promoting mitotic exit and cytokinesis.

4. The Septation Initiation Network

In the fission yeast *S. pombe*, mitotic process is spatially different from budding yeast. Nevertheless, the molecules involved in the regulation of cell division are deeply conserved. Studies using this model organism have demonstrated that most of the proteins involved in MEN in budding yeast have counterparts in fission yeast. Here, the signaling cascade that coordinates cell division in which Mob1 protein is involved is the Septation Initiation Network (SIN). SIN has a role in actin-myosin ring contraction and in cytokinesis, being involved in the correct formation of the septum in time and space (for review see (Hergovich & Hemmings, 2012)). The mechanics of the SIN cascade are very similar to MEN. As in the case of MEN, the core components of the SIN network are also a GTPase Spg1, the effector kinases Cdc7 and Sid1 that belong to the Ste20 kinase family, the NDR family kinase and its activator Mob1. The final outcome of the SIN is also the phosphorylation, by Sid2-Mob1 complex, of the Cdc14-like phosphatase Cip1p (for review see (Johnson, McCollum, & Gould, 2012)) (Fig.3).

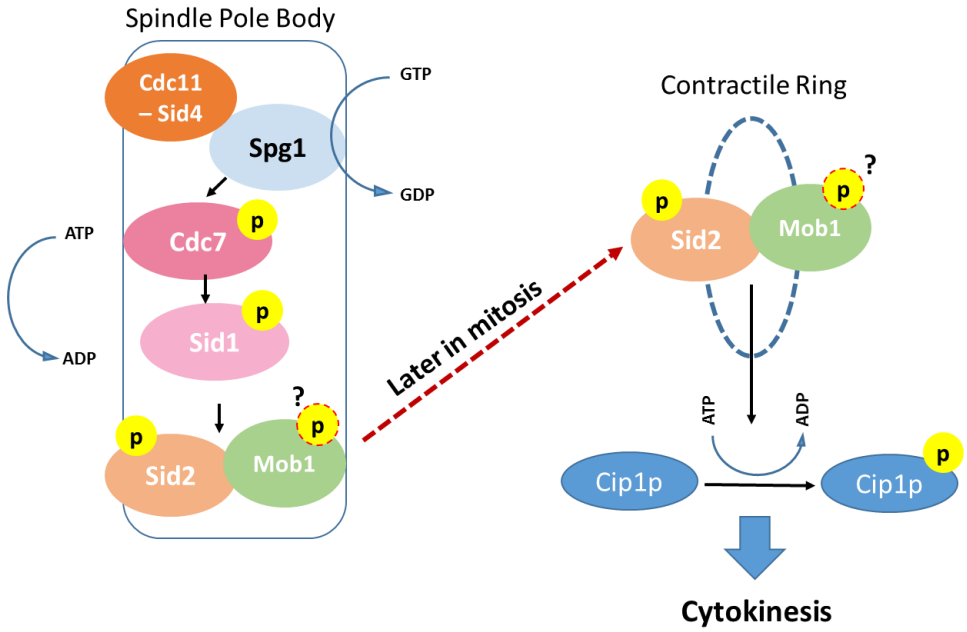


Figure 4 - The Septation Initiation Network – schematic representation of the *S. pombe* SIN. The core components of the SIN show a spindle pole body localization throughout mitosis. The activation of the GTPase Spg1 consequently activates the Cdc7 kinase which in turn phosphorylates the Sid1 kinase. At the end of this cascade, Sid2 is activated by phosphorylation and this activation depends on its binding partner Mob1. This complex relocalizes to the region where the septum and the actin-miosin contractile ring is formed, being involved in cytokinesis completion. One of the effects of the active Sid2-Mob1 complex is the maintenance of the Cdc14-like phosphatase in the cytoplasm, which is critical for cytokinesis progression.

Similarly to what occurs in MEN, the scaffold bipartite protein complex Cdc11-Sid4 is requested to concentrate SIN molecules at the spindle pole bodies. There, the GTPase Spg1 is activated which in turn leads to the activation of the Sid1 kinase that consequently activates by phosphorylation Sid2 protein in the Sid2-Mob1 complex. The activity of the complex Sid2-Mob1 reaches its maximum level just before septation, and it localizes at the septum at the end of mitosis. Still, the details of this relocalization of Sid2-Mob1 are unknown. Active Sid2-Mob1 is critical to maintain the Cdc14-like phosphatase Cip1p in the cytoplasm which allows cytokinesis completion (Chen et al., 2008). Further investigation regarding Sid2 substrates at the contractile ring will certainty contribute to a better understanding of cytokinesis regulation by this molecule.

5. The Hippo pathway

In multicellular organisms, orthologs of the core proteins of both MEN and SIN are also present and most of them are implicated in the major signaling cascade controlling the balance between cell proliferation and cell death, the Hippo pathway. This complex signaling pathway was firstly described in *D. melanogaster* and has since been described also in mammalians (for review see (Mo, Park, & Guan, 2014)). Taking into account the fundamental role of the Hippo pathway it is not surprising that the correct function of this cascade is critical both for embryonic development and adult tissues homeostasis.

In *D. melanogaster*, the core components of the Hippo pathway were identified in genetic screenings searching for tumor suppressor genes, and in fact, inactivation of any of these core proteins always results in cell over proliferation. The fundamental players of this pathway in the fly are the Ste20-like kinase Hippo (Hpo), its scaffolding protein Salvador (Sav), the NDR family kinase Warts (Wts) and finally, its activator Mats (Mob1 nomenclature in the fly) (Harvey, Pfleger & Hariharan, 2003; Justice, Zilian, Woods, Noll & Bryant, 1995; Lai et al., 2005; Pantalacci, Tapon & Leopold, 2003; Udan, Kango-Singh, Nolo, Tao & Halder, 2003). The final target of the pathway is the transcription factor activator Yorkie (Yki) whose nuclear localization depends on its phosphorylation status. Yki acts as an oncongene, since it promotes the transcription of pro-mitotic and anti-apoptotic genes (Huang, Wu, Barrera, Matthews & Pan, 2005; Oh & Irvine, 2008) (Fig. 4).

During development, the Hippo pathway kinase module is inactive, Yki is not phosphorylated by Wts and can migrate to the nucleus to exert its transcriptional

activation function. In this case, cells proliferate until tissues and organs reach their normal size. When proliferation needs to be stopped, the core kinases of the Hippo pathway are sequentially activated, as follows: Hippo is activated upon receiving upstream information that proliferation should be repressed. Active Hippo forms a complex with the scaffold protein Sav and the complex can in turn phosphorylate the Wts kinase that, after forming a complex with its activator Mats, can phosphorylate Yki (Zeng & Hong, 2008). Phosphorylated Yki is sequestered in the cytoplasm mainly via the binding to 14-3-3 proteins (Ren, Zhang & Jiang, 2010).

All the core proteins described in the fly have homologues in mammals, that work in the same fashion as described in *D. melanogaster* (for a review see (Mo et al., 2014)). In mammals, the core components of the Hippo pathways are the ste-20 kinase MST1/2 (Hippo in the fly), the scaffolding protein Sav (also known as hWW45), the NDR family kinase Lats1/2 (stands for large tumor suppressor) and finally, its activator Mob1. The final effector of the Hippo cascade in mammalians is the protein YAP or TAZ (Yki in the fly) that are also transcription activator factors whose activity in the nucleus induces the expression of anti-apoptotic and proliferation associated genes such YAP family members *BIRC5/survivin* and *BIRC2/cIAP1* and the *BCL2* family gene *MCL1* (Dong et al., 2007; Zhang, Smolen & Haber, 2008). To facilitate the understanding of the Hippo pathway cascade both in *D. melanogaster* and in mammals, comparative schemes of the two pathways containing their core molecules are shown in figure 4.

Taking into account the role that Yki/YAP activity has on proliferation induction, it is not surprising that the deregulation of its activity is associated with cancer development. Effectively, while the activity of the Yap protein as a transcription activator is critical for growth and correct development in different organs (Gee, Milgram, Kramer, Conlon & Moody, 2011; Septer et al., 2012; Zhang et al., 2010) and also for stem cell pluripotency (Lian et al., 2010), a great amount of studies have shown that the uncontrolled activity of YAP is associated with tumor formation and cancer progression in several different tissues such as the liver, intestine, lung, skin and brain (Camargo et al., 2007; Orr et al., 2011; Wang et al., 2010; Zhang, Wu, & Xing, 2011). As the cellular effector of Hippo signaling is an oncoprotein it is easy to understand why the deregulation of any of the Hippo pathway core kinases leads to uncontrolled cell proliferation. In fact, while YAP is classified as oncogene, all the other Hippo core proteins that regulate its activity are well established as tumor suppressors.

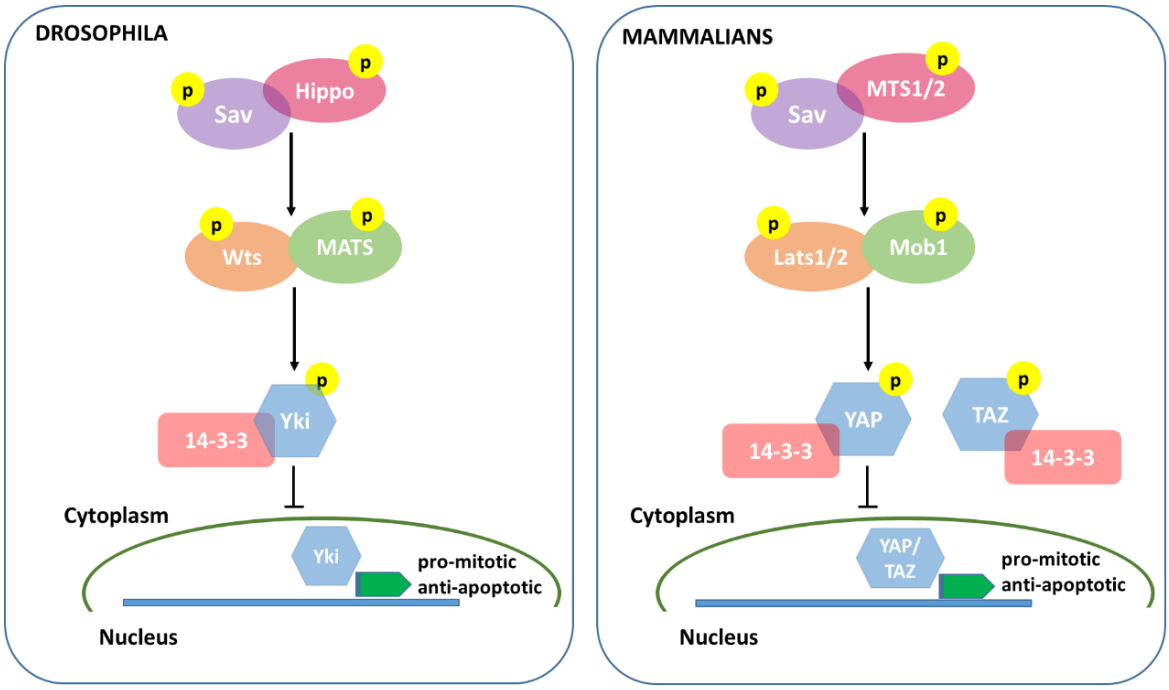


Figure 5 - The Hippo pathway in *D. melanogaster* and Mammals. A schematic representation of the core molecules involved in Hippo signaling both in *D. melanogaster* and in mammals. The pathway impinges on the transcription factor activator Yki/YAP that in its phosphorylated form is maintained in the cytoplasm not inducing pro-mitotic genes transcription. So, when activated, the Hippo cascade inhibits cell proliferation and increase of tissues/organs size.

Even though the main events involving the Hippo pathway central kinase module are the ones described above, the importance of the pathway in tissues homeostasis led to an increasing interest in investigating the upstream regulators of the pathway. Which external signals or signaling pathways impinge on the Hippo cascade to promote its activation?

In recent years, several molecules and pathways have been implicated in the Hippo signaling response, most of them related to cell and tissue organization.

Indeed, the Hippo pathway can accommodate information coming from cell pathways responsible for maintenance of the polar structure of epithelia such as components of the apical-basal polarity protein complexes and also components of the planar cell polarity machinery.

Furthermore, the Hippo pathway can also respond to mechanical alterations or tensions, transducing signals coming from cytoskeleton molecules, and the extracellular matrix (for review see (Yu & Guan, 2013)).

The details of this regulation will be discussed later in more detail. Understanding the regulation of tissue homeostasis and how individual cell function/structure contributes to it is a major issue both in basic cell biology as well as in the understanding of some human diseases. Indeed, the disruption of the tightly controlled structure of tissues such as epithelia can lead to cellular transformation that, when uncontrolled, is associated, for example, with cancer development.

OBJECTIVES

Considering the available data on cell cycle and cell death control signaling pathways, namely the Mitotic Exit Network in yeast and the Hippo pathway in metazoan, and also the fact that Mob proteins are functionally conserved among eukaryotes, we hypothesize that Mob proteins are critical players in the control of cell cycle, proliferation and cell death in protozoa.

The presence of Mob1, a protein that is well established as a tumor suppressor, in unicellular organisms raised several striking questions. What is the functional meaning of a tumor suppressor in a unicellular organism? What is the functional meaning of a protein involved in cell proliferation/apoptosis balance, in organisms with apparently no intrinsic need to control cell numbers? Indeed, these questions can be addressed using the model unicellular organisms: *Tetrahymena thermophila* and *Toxoplasma gondii*.

From a basic point of view, both organisms present a permanently polarized cell with a complex structure that resembles intracellular organization in metazoans. In addition to this, the lack of cell-cell contacts is also an advantage to address the function of Mob1 proteins. In unicellular protozoa Mob1 has most probably a fundamental role that cannot be easily dissected in multicellular environment. Indeed, Mob1 was already investigated in the budding yeast, also a unicellular. However, this organism only polarizes during cell division, which is asymmetric. In this context, the question of how cell polarity features impacts on cell division regulation, a major problem in very organized tissues such as metazoan epithelia, could only be addressed in permanently polarized cells. The deregulation of cell polarity/structure in epithelia is a major risk factor, for example, in cancer development, however studying epithelial tissues is technically very demanding. Thus, the use of polarized single cell organisms may facilitate the investigation of higher cellular organization mechanisms, at least those that do not depend on cell-to-cell interactions.

Apart from these basic questions, the study of Mob1 in the cyst-forming parasite *T. gondii* constitutes a step forward in the understanding how these parasites may regulate cell proliferation inside the host cell. Interestingly, contrary to the parasite *T. brucei*, which lives "free" inside the host, *T. gondii* parasites divide inside a closed structure, the parasitophorous vacuole that is established inside the host cell after invasion (details will be addressed in Chapter III). This active stage of the infection can evolve to a chronic form of the disease either by the pressure of the host immune system or by mechanisms of the parasite not fully understood. In these cases, the vacuole gives

origin to a cyst that can be maintained for long periods of time. Despite being single celled organisms, inside the vacuole *T. gondii* parasites live in close association.

Remarkably, the cyst size varies significantly between different Apicomplexa species, such as *Neospora caninum* and *Besnoitia besnoiti* which suggests that parasite number is probably regulated not only by the pressure exerted by the immune system of the host, but also by the parasites' own means. Since Mob1 is implicated in cell number regulation in metazoans, it is a very interesting molecule to study cell proliferation control in this context.

To test our hypothesis that Mob1 is a critical player in protozoa cell division and proliferation, we searched for the presence of predicted Mob1 proteins in the available genomes of *T. thermophila* and *T. gondii*. In fact, based on the BLAST (Basic Local Alignment Search Tool) preformed using the human and budding yeast Mob1 protein sequences in the TGD (*Tetrahymena* Genome Database) and in the ToxoDB (*Toxoplasma gondii* genome database), the genome of the two protozoa possess one gene encoding a Mob1 protein, the one that was shown to be implicated in MEN and Hippo pathways.

In this context, the present work comprises three major goals:

1. Search for the presence of Mob proteins in protozoa, namely in parasitic ones and address their phylogenetic relationships, using a bioinformatics approach.

2. Investigate the function of Mob1 in *T. thermophila* using the following approaches:

2.1 Investigate the subcellular localization of Mob1 in *T. thermophila.*

2.2 Functionally characterize the role of Mob1 in *T. thermophila* by overexpression and loss of function approaches.

3. Investigate the function of Mob1 in *T. gondii* by:

3.1 Study *T. gondii mob1* expression at different times of parasite proliferation in mammal cells.

3.2 Investigate the subcellular localization of Mob1 in *T. gondii*

3.3 Address the effect of Mob1 overexpression in *T. gondii* (invasion and replication essays).

CHAPTER II: MOB1 IN THE BIOLOGICAL MODEL PROTOZOA *Tetrahymena thermophila*

PART 1: INTRODUCTION

1. Introduction: Cell polarity

Cell polarity, both permanent and transient polarization, is a fundamental characteristic of all different cell types and plays a critical role in cell function. There is a plethora of different cells in which this importance is very present. For example, in epithelial cells, the apical-basal polarity of each individual cell is critical for the maintenance of tissue structure, directional vesicle traffic and morphogenesis (for review see (Rodriguez-Boulan & Macara, 2014)). Also in mesenchymal cells the polarization of the cells allows cell movement and directional migration, where cells present a front-to-back organization. Other clear example of the importance of cell polarity can be observed in the neurons. In fact, the very specialized function of this nervous system cell type is only possible because of its highly polarized structure (for review see (Thompson, 2013)).

Actually, there are several different cell functions, such as cell migration, cell fate during development and morphogenesis, cell adhesion, tissue formation and cell molecular communication that rely on the correct establishment of cell polarity. Therefore, understanding the molecular basis of the establishment of cell polarity is a critical issue both for basic cell biology, development biology and disease development.

1.1 Intrinsic cell polarity

There are numerous molecules and structures that are involved in the spatial organization of the intracellular environment of every type of cells. In fact, even the non-polarized cells present an internal organization that is important for several aspects of the cell's life and that is determined by the position of different cell structures. The cytoskeleton network, both of microtubules and actin, plays a fundamental role in cellular organization. Also, centrosome positioning relatively to the nucleus is an indicator of the cell's orientation and it was shown to be critical in several functions such as development, cell migration, synapsis establishment and others. Interestingly, this internal cell polarity can respond to external clues. Specialized structures of the cytoskeleton as it is the case of primary cilia and the axis formed by it and the Golgi

apparatus-nucleus may act as a receptors and transducers of external signaling (for review see (Soares, Marinho, Real & Antunes, 2014)).

Despite of this structural organization, transversal to almost all types of animal cells (here used as example), in the present work we will be focused in the molecules/mechanisms involved in the establishment of intrinsic cell polarity on epithelial cells, whose internal apical-basal organization has been deeply investigated.

The polarized phenotype of epithelial cells results from an asymmetric distribution of different molecules. Both proteins and lipids concentrate in distinct manners on the two poles forming different domains in the cell. These domains are involved in the formation and maturation of tight junctions that are responsible for blocking lateral movement of particles in the epithelial sheet and creating specific cell membrane domains with different compositions, which impair, for example, protein diffusion (for review see (Rodriguez-Boulan & Macara, 2014)). The first studies concerning the identification of these critical molecules come from a screening aimed at identifying genes involved in the correct partitioning of *Caenorhabditis elegans* embryo components. In these studies several genes were identified and called partitioning defective (PAR) (Kemphues, Priess, Morton, & Cheng, 1988). Across the years, the importance of the PAR proteins in cell polarization has been well documented being these proteins strongly conserved in terms of their presence and function in metazoan (for review see (Rodriguez-Boulan & Macara, 2014)).

Several PAR proteins, such as PAR1, PAR4 (LKB1 or STK11 in mammals), PAR5 (homologue of 14-3-3), and two scaffold and adapter proteins that contain PDZ domains, PAR3 and PAR6, have been implicated in epithelial polarity establishment. Beyond the proteins from the PAR family, also a protein known as atypical protein kinase c (aPKC) and the CDC42 GTPase play a role interacting with PAR proteins (for review see (Rodriguez-Boulan & Macara, 2014)). The core complex of PAR proteins associated with polarization is formed by the PAR3 and PAR6 molecules that interact with aPKC and CDC42, a cytoskeleton regulator. In *C elegans*, PAR3 co-localizes with aPKC in the anterior region of the one cell embryo, and their specific localization is mutually dependent. Furthermore, in this study the authors observed that aPKC depletion leads to embryonic death due to polarity defects and that the specific localization of PAR3-aPKC also depends on other PAR proteins (PAR2, PAR5 and PAR6) (Tabuse et al., 1998).

Shortly after, PAR6 was also described to interact with PAR3 and aPKC which activity is critical to maintain PAR6 specific localization (Hung & Kemphues, 1999).

After these findings, it was demonstrated that CDC42 was also part of this complex activity. In fact, the demonstration that CDC42 is critical for the localization and activity of PAR proteins established a link between cytoskeleton dynamics and polarity factors' distribution throughout the cytoplasm (Kay & Hunter, 2001).In *D. melanogaster*, the homologous of PAR complex, Bazooca (PAR3 homologous in the fly), PAR6, aPKC and CDC42 are critical for generating and also maintaining the apical domain identity in epithelia (Bilder, 2003; Hutterer, Betschinger, Petronczki & Knoblich, 2004; Tanentzapf & Tepass, 2003).

The exact same complex was also described in mammalian cells, where PAR6 acts as an adaptor protein to connect PAR3 to the aPKC and to the CDC42, interactions that are important to maintain tight junctions in epithelial cells (Joberty, Petersen, Gao & Macara, 2000).Beyond the PAR complex, there are other molecules involved in intracellular organization and epithelial tissue structure. The Crumbs (CRB) and the Scribble (SCRIB) complexes, both well conserved in metazoan, are major examples of these molecules. Both PAR and CRB complexes are responsible for the establishment of apical identity in the epithelial cells. On the other hand, the SCRIB complex is required to establish basolateral domains (for review see (Royer & Lu, 2011)) (Fig.5).

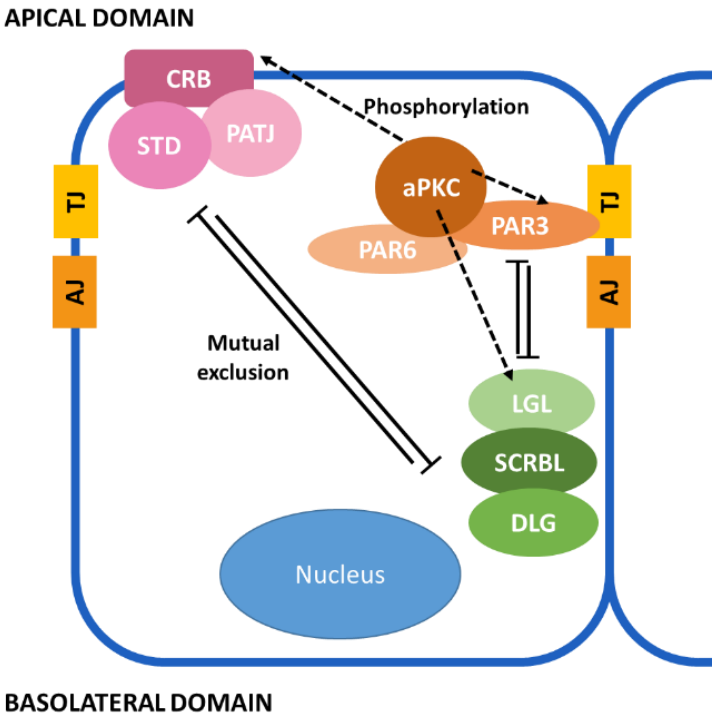


Figure 6 - The main protein complexes involved in epithelial polarity establishment. The CRB and the aPKC/Par3/Par6 complexes are localized in the apical side of the cell, while the SCRBL complex is responsible for the basal/lateral domain identity.

The CRB complex is composed by three proteins, the CRB, a transmembrane protein, the Stardust (STD) and the Patj protein (previously known as Discs lost). CRB and STD were identified in *D. melanogaster* where it was established that mutations in both proteins lead to the formation of very irregular cell clusters and severe cell death in epithelia (Knust, Tepass & Wodarz, 1993; Tepass & Knust, 1993). These two proteins are crucial for the establishment of adherens junctions and the compartmentalization of the apical region (Grawe, Wodarz, Lee, Knust & Skaer, 1996). Interestingly, PAR and CRB complexes mutually regulate each other in their spatial distribution within the cytoplasm. Indeed, PAR3 binding to the stable aPKC/PAR6 complex is very dynamic and competes with the binding of PAR6/aPKC with the CRB in the apical membrane region. aPKC can phosphorylate PAR3 and this phosphorylation promotes its exclusion of the apical domain, by diminishing its affinity to bind PAR6, in the exact same domain where CRB and STD can also interact (Morais-de-Sá, Mirouse & St Johnston, 2010).This spatial exclusion of PAR3 from the more apical domain is then responsible for the positioning of apical-lateral border and for the placement of adherens junction in epithelia.Finally, the SCRIB module was first identified in a genetic screening in the fly (Bilder, Li, & Perrimon, 2000). In this study, the authors demonstrate that SCRIB interacts with two other proteins, LGL (stands for lethal giant larvae) and DLG (discs-large) and that the three proteins of the complex show a similar phenotype of polarity defects (Bilder et al., 2000).

Among the three mentioned core complexes involved in cell polarity, the SCRIB module is the less described. Nevertheless, the same type of exclusive position regulation was also observed in what concerns the SCRIB complex. LGL also competes with PAR3 for the PAR6/aPKC complex and doing so, restricts it to the apical domain (Hutterer et al., 2004; Yamanaka et al., 2006). Moreover, the aPKC can also phosphorylate LGL in the apical region which is important for the establishment of apical-basal domains (Plant et al., 2003).

In conclusion, these data support that cellular polarity is mainly a consequence of asymmetric distribution of different protein complexes that are mutually regulated and also by the spatial distribution of different cellular structures, such as the nucleus the Golgi apparatus and the cytoskeleton related structures like centrosome, and cilia.

1.2 Influence of cell polarity in cell division

Cell polarity can influence several features of cell life, such as vesicle traffic, movement, interphase structure and also cell division, namely, by influencing mitotic spindle positioning and consequently, the cleavage plan. Mitotic spindle positioning is a critical aspect for cell division both in symmetric and asymmetric cell divisions, and also both in polarized and non-polarized cells.

There are several molecules already described to be involved in spindle positioning. The Pins/Mud/Gαi complex (*D. melanogaster* nomenclature; in vertebrates Pins homolog is LgN and Mud homolog is NuMa) is a well conserved set of proteins with a fundamental role in the process as they work to recruit dynein to the cell cortex. The dynein, a motor protein, can then capture astral microtubules from the spindle poles and trigger the proper spindle alignment. Mud/NuMa protein binds to microtubules through the interaction with a dynein/dynactin dimer. Pins/LgN is a scaffold protein that connects both to Mud/NuMa and Gαi and is localized at the cell cortex (for review see (Bergstralh, Haack, & St Johnston, 2013)). The concentration of these proteins at the cell cortex determines the pulling forces that position the aster microtubules and ultimately the spindle. In the single celled *C. elegans* embryo, a well characterized system, during metaphase and at early anaphase, complexes of GPR-1/2 (Pins/LgN) and Lin-5 (Mud/NuMa) are more concentrated at the posterior pole of the embryo which leads to a greater pulling force towards that pole and to the asymmetric localization of the spindle within the cell, resulting in daughter cells of different sizes (asymmetric division) (Grill, Gonczy, Stelzer & Hyman, 2001).

But also in the human cells that present a symmetric division, the establishment of these cortical gradients governs the positioning of the mitotic spindle, but in a different fashion. In Hela cells, the gradient of LgN/NuMa /Gαi is similar in both sides of the cell cortex. Nevertheless, an oscillation of the spindle between the two poles is observed until it presents a correct position in the middle of the cell. This movement is controlled by the Dynein/Dynactin system that shows an unbalanced concentration between the two poles of the cell and that is negatively regulated by Plk1 localized at the spindle poles. In summary, if the spindle pole is closer to one of the cell sides, the Dynein/Dynactin is delocalized to the opposite side, which in turn generates oscillatory movements until the spindle is centered. Specifically, the complex formed by Dynein/Dynactin and LGN/NuMa at the cell cortex is disrupted in the presence of Plk1 (Kiyomitsu, 2012).

Not surprisingly, the molecules involved in establishing cell polarity, namely proteins from the PAR complex, present a crosstalk with spindle position machinery. In fact, several studies have already implicated Par proteins in this critical aspect of cell division. Almost 20 years ago, Etemad-Moghadam and co-workers (1995) reported a correlation between Par-3 localization pattern and spindle positioning in *C. elegans* embryo. Later, also in *D. melanogaster*, the involvement of Bazooka (Par-3) in spindle orientation was demonstrated, as mutant embryos presented severe defects in the coordination of cell polarity, visible in the mispositioning of the mitotic spindle and of the daughter cells after cell division (Kuchinke, Grawe, & Knust, 1998).

1.3 The influence of cell polarity on Mitotic Exit Network and Hippo pathways

1.3.1 Cell polarity and the Mitotic Exit Network

As described in the last sections of the introduction, the asymmetric distribution of molecular complexes inside the cell is determinant for the establishment of cytoplasm organization with implications in cell division and cell functions.

The yeast *S. cerevisiae* presents an asymmetric cell division, in which the resulting daughter cell (bud) is much smaller than the mother. In order to segregate correctly the DNA content, the mitotic spindle must be properly aligned with the axis defined by the localization of the budding daughter. In the case of spindle misalignment, cells have a safety mechanism to correct it, known as Spindle Positioning Checkpoint (SPOC). The SPOC inhibits cells from exiting mitosis, by inactivating MEN, until the mitotic spindle is properly aligned along the mother to daughter cell polarity axis (for review see (Caydasi, Ibrahim, & Pereira, 2010)).

The main molecules involved in this checkpoint are the Bub2-Bfa1 complex, the polo-like kinase Cdc5 and the kinase Kin4. Bub2-Bfa is a GAP (GTP activating protein) bipartite complex that promotes the Tem1 GTP hydrolysis, maintaining this protein inactive and so inhibiting MEN. In turn, Cdc5 is needed to inactivate the Bub2-Bfa1 complex allowing mitotic exit.

Importantly, and as stated before, the asymmetric distribution of this molecules is a critical issue for correct cell division. In fact, in normal conditions, the SPB from the mother and the daughter cells present a different molecular localization since Kin4 and Tem1 are localized in the mother cell SPB and Bub2-Bfa1 and a larger amount of Tem1 are present in the daughter SPB. Besides this specific protein localization at the SPB, a

cytoplasmic protein gradient of Kin4 and Lte1 are also critical for the correct establishment of spindle axis in budding yeast. Briefly, Kin4 is present in the mother cell compartment while Lte1 is restricted to the daughter cell bud. In fact, Kin4 is also present, though at very low concentrations in the bud, but its presence at the daughter SPB is inhibited by the presence of Lte1. This promotes the stabilization of the spindle, the inactivation of Bub2-Bfa1 complex and finally the activation of MEN (for a review see (Caydasi et al., 2012)).

1.3.2 Cell polarity and the Hippo Pathway

As previously described, the Hippo pathway is a major signaling cascade critical for the balance between cell proliferation and cell death, assuming a crucial role during development and in organisms' homeostasis. Although the major players involved in the pathway have been already described, it matters now to present the upstream regulators involved in Hippo pathway control, namely the ones related to cell and tissue polarity.

It is now well-known that the molecules involved in the establishment of epithelial apical-basal polarity regulate the Hippo cascade. The apical determinant CRB was identified as a Hippo signaling regulator since the manipulation of its protein levels in *D. melanogaster* led to over proliferation phenotypes resulting from Hippo cascade deregulation (Chen et al., 2010; Robinson, Huang, Hong & Moberg, 2010).

This regulation is not directly exerted in the core components of the pathway but instead is done through the regulation of another apically localized protein, Expanded. This protein is a molecular link between cell polarity and cell proliferation control, since it acts directly upstream the Hippo core components and its localization is dependent on the polarity factor CRB. Importantly, in a CRB overexpression scenario, a mislocalization of Expanded and the inactivation of the Hippo pathway were observed (Chen et al., 2010; Grzeschik, Parsons, Allott, Harvey, & Richardson, 2010; Robinson et al., 2010). In fact, Expanded is a tumor suppressor protein that is part of a protein complex formed by Merlin and Kibra, which together regulate the Hippo pathway (Yu et al., 2010).

In addition to CRB, Lgl and aPKC polarity proteins were also implicated in Hippo signaling regulation, however by a distinct mechanism. In 2010, Grzeschik and co-workers have demonstrated that in Lgl mutant flies, several targets of the Hippo pathway, such as DIAP (*Drosophila* inhibitor of apoptosis) and Cyc E were down-

regulated. This effect was dependent on the relocalization of Yki (Hippo pathway effector) that in the Lgl depletion scenario was hyper activated, since Yki phosphorylation levels decreased by 35%. Interestingly, the depletion of Lgl to levels where there is only a mild loss of cell polarity does not lead to the increase of Yki levels, suggesting that is indeed the polarity defects which impinge on Hippo signaling regulation (Grzeschik et al., 2010).

In the same study, the authors also explored the influence of aPKC in Hippo regulation. Consistent with the Lgl and aPKC antagonistic role in the establishment of apical-basal polarity, aPKC mutants present a strong suppression of the Hippo cascade targets DIAP and CycE.

Thus, the regulation of Hippo cascade targets depends on a balance between Lgl and aPKC activity. Recently, it was also shown that the role of Lgl and aPKC in Hippo signaling regulation is independent of the apical proteins Kibra, Merlin, Expanded and others, being this independent mechanism still to be elucidated (Parsons, Grzeschik & Richardson, 2014).

Importantly, it is not only cell polarity that influences and regulates the Hippo pathway. Hippo components also play a role in cell polarity establishment. In fact, it was shown in *D. melanogaster* that mutations in several members of Hippo signaling, such as Merlin/Expanded, Hippo and Warts lead to an increase of the levels of several polarity determinants, namely aPKC and CRB. Accordingly, Yki overexpression presents the same phenotype. This effect is specific of Hippo signaling and not only a consequence of cell uncontrolled growth since, for example, overexpression of the Cyc D-Cdk4 does not present the same results (Genevet et al., 2009; Hamaratoglu, 2009).

All these data clearly show the intimate relation between cell polarity establishment and cell division/proliferation, two major features of the cell's life that are mutually regulated.

2. Cell polarity in unicellular protozoans – the case of the ciliate *Tetrahymena thermophila*

Until this point, the establishment of cell polarity was addressed basically regarding the cell structure of epithelial cells and also in the particular case of yeast asymmetric cell division, where the polarization of an interphase unpolarized cell is critical for the correctness of the process. At this point, and considering the two unicellular models

used in the present study, the free living ciliate *T. thermophila* and the apicomplexan parasite *T. gondii*, it is relevant to discuss the establishment and maintenance of cell structure and polarity in protozoa. Both in *T. thermophila* and in *T. gondii*, the cells are permanently polarized with and apical and a basal region well defined by structural markers. Even though these organisms are polar, cell division, contrary to what happens in yeast, is symmetrical, and so, they are good models to explore symmetrical cell division in polarized cells, similar to the metazoan epithelia structure. At this point, it will only be addressed the cell structure and morphogenesis of the ciliate *T. thermophila*, being the case of *T. gondii* presented in the respective introduction section.

3. Cell Polarity in Ciliates

Cell polarity in ciliates is mostly determined by the existence of different landmarks in the two poles of the cell and also by the uniform orientation of the cytoskeleton structure below the cell membrane. In the case of *T. thermophila*, each cell presents on average 17-21 longitudinal microtubule rows that are positioned next to thousands of basal bodies, distributed in a deeply organized manner, and that constitute the structures responsible for cilia assemble. These microtubules, that present a vectorial polarity, define the anterior posterior axis of the cell, while the positioning of the oral apparatus (OA) localized sub-apically, determines the ventral side. The OA is a very important landmark in *T. thermophila* cell structure, since it defines the position of other cortical features (Wloga & Frankel, 2012) (Fig.6).

The structural vectorial polarity of ciliary rows in ciliates is transmitted to daughter cells vegetatively. Since ciliates growth happens along their anterior-posterior axis and cell division occurs transversely, the propagation of the preexisting structure can occur across an indefinite number of generations (Jerka-Dziadosz et al., 1995).

Elegant experiments in the 70's showed that after a 180º degrees rotation of the ciliary rows in *T. thermophila* (done by *quasi*-surgical techniques), the nascent structures in these cells presented also an inverted conformation. These observations, previously collected in the ciliate *Paramecium*, are a clear proof of the cytotaxis phenomena, *i.e.*, that preexisting structures control the development and orientation of new ones, in a non-DNA based inheritance manner (Ng & Frankel, 1977).

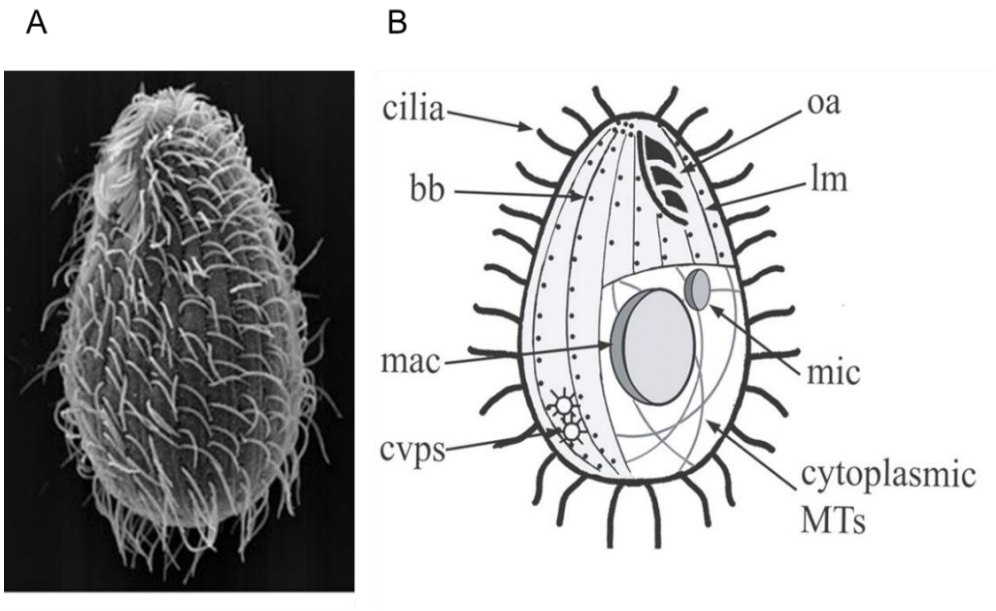


Figure 7 - The ciliate *Tetrahymena thermophila*. (A) *T. thermophila* cell observed in a scanning electron microscope. In the picture, it is visible the longitudinal ciliary rows of the cell. The oral apparatus is placed in the anterior region of the cell and is formed by a set of modified and fused cilia with feeding purposes. (B) Schematic representation of the *T. thermophila* cell with the main structures represented. oa – oral apparatus; bb – basal body; mac – macronucleus; mic – micronucleus; lm – longitudinal microtubules and cvps - contractile vacuole pores. (Adapted from(Sharma et al., 2007)).

In ciliates, the nascent basal bodies appear just above the preexisting ones in an intercalated manner (Fig.7).

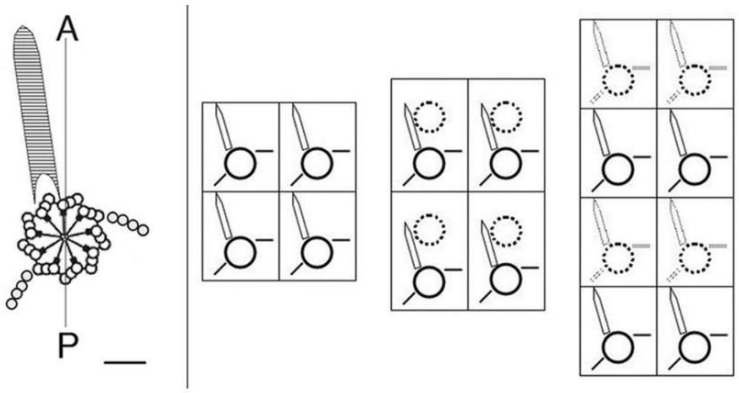


Figure 8 – Schematic representation of basal body structure and formation pattern. (Left) The basic unit of *T. thermophila* cell cortex where it is shown the basal body structure, and the cilia axoneme. In the scheme, it is visible the anterior-posterior orientation (A-P). (Right) Orientation of *T. thermophila* basal bodies during duplication. Two adjacent units along two parallel rows are represented. The new basal bodies (dotted lines) are formed in an intercalated manner in relation to old ones (full lines) allowing the elongation of the cell and the maintenance of the pattern (Adapted from (Beisson, 2008)).

This configuration allows the development of the cells to conserve the initial pattern throughout the cell body, in a manner oriented with the anterior-posterior axis of the cell.

Besides the morphogenesis pattern of these cells in relation to the cortical longitudinal rows, it also matters the formation of specific landmarks such as the oral apparatus and the contractile pores. In the first events of *T. thermophila* division, the region where the new oral apparatus (NOA) will be formed is composed by an anarchic field of basal bodies just below the future fission line, the region where the intercalated insertion of new basal bodies occurs (Kaczanowska et al., 2003; Kaczanowski, 1978). The specific position of the NOA determines the ventral side of the cell and after the concentration of this basal bodies' field, stomatogenesis in *T. thermophila* proceeds in a very well defined manner (Fig.8).

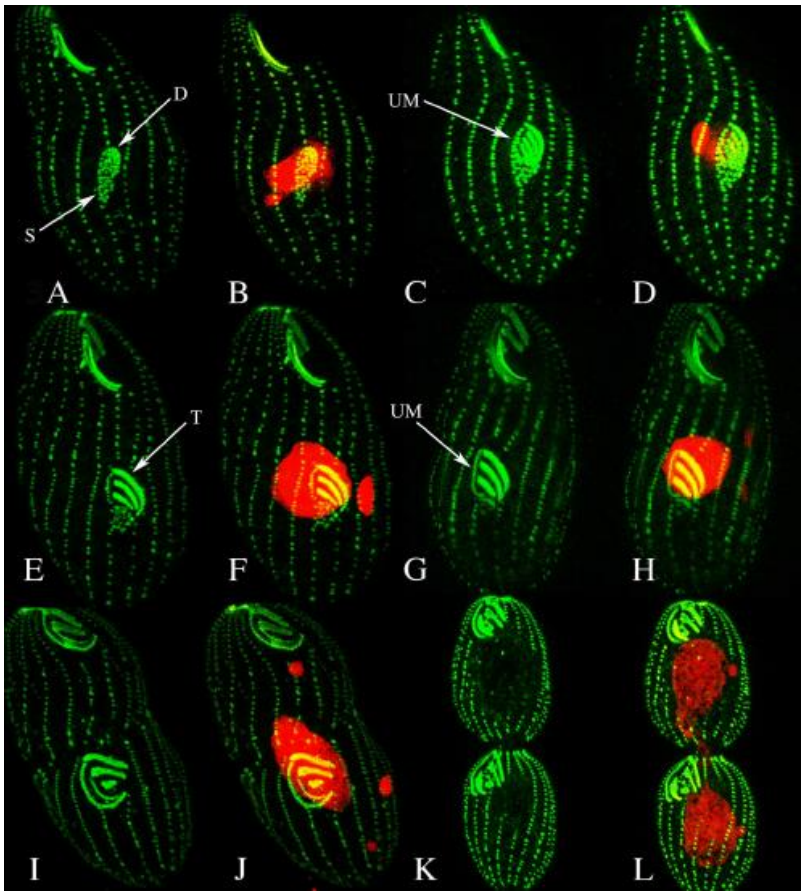


Figure 9 - Sequential steps of oral apparatus formation in *Tetrahymena thermophila* during cell division (A-L). During this process, single basal bodies (green) aggregate in the mid region of the cell, just below the region where the fission line will be defined. The single basal bodies undergo a fusion event, forming basal body doublets (D) and triples (T) and the undulating membrane (UM). *T. thermophila* stomatogenesis is deeply coordinated with micro and macronucleus division (chromatin in red) and with cytokinesis (Image from (Kirk et al., 2008)).

4. *Tetrahymena thermophila* as a model organism

The ciliate *T. thermophila* has been used for many years as a model organism in several different subjects in cell biology. Several specific characteristics of this free living organism make it suitable for studies of cell cycle, cytoskeleton and cilia biology, epigenetics, morphogenesis among many other potential fields of basic and applied research, such as environmental studies and biotechnology. Although *T. thermophila* is a unicellular, its cell structure is highly complex. Being so, cellular studies can conjugate the handiness of *T. thermophila* culture conditions (affordable culture medium, fast generation time, non-pathogenic organism, etc.) with the similarities that *T. thermophila* cell complexity (polarity; cytoskeleton; cilia; DNA/RNA biology, etc.) share wither higher eukaryotes. Other aspect that makes this ciliate such an attractive model is the big dimensions of the cell (~ 60x30 μm), which are very convenient for imaging.

Much before the sequencing of the genome, that become available in 2006 (Eisen, 2006) studies using *T. thermophila* had led to major breakthroughs in fundamental research. For example, the discovery of the telomere structure, of the telomeric enzyme and of its function, was achieved in this organism (Blackburn & Gall, 1978; Greider & Blackburn, 1989). Moreover, the studies that led to the discovery of ribozymes in eukaryotes were also done in *T. thermophila* (Kruger et al., 1982). Importantly, both of these studies were highly recognized as fundamental discoveries and the authors were awarded with the Nobel Prize of Physiology and Medicine and of Chemistry, respectively. In the cytoskeleton field, it is worth to highlight the discovery of the motor protein Dynein and also the directionality of its movement in microtubules (Gibbons, 1963; Gibbons & Rowe, 1965; Sale & Satir, 1977).

Being such an important model, several tools for *T. thermophila* manipulation are available and well established. The complete sequencing of the *T. thermophila* genome was a major advance in the usefulness of this organism in cell biology. In fact, as a result of the effort of a big scientific community that uses this ciliate as a model organism, its genome is now fully sequenced and very well annotated, which greatly facilitates genomics and molecular biology studies.

These cells are easy to manipulate genetically, and genome transformation can be achieved by biolistic techniques, electroporation and microinjection (for a review see (Turkewitz, Orias, & Kapler, 2002)). The biolistic transformation (Cassidy-Hanley et al., 1997) is the most used method to obtain different types of modified *T. thermophila*

strains, such as overexpression (gene expression under the control of well-established and specific promoters), and somatic and germ line knock-outs (replacement of the gene of interest by a resistance cassette by homologous recombination).

Being so, it is clear that this complex unicellular organism represents a valuable biological model for several fundamental and applied studies in different fields.

As stated by Turkewitz "*T. thermophila* is a highly developed unicellular, belonging to a clade of ancient lineage whose cellular complexity rivals that of highly differentiated tissues. It is precisely this complexity, the presence of features that are characteristic of metazoans but that are absent or less easily studied in other simple eukaryotic systems that makes *Tetrahymena* so well suited for a wide range of fundamental problems." (Turkewitz et al., 2002).

PART 2: Mob1: defining cell polarity for proper cell division

1. Abstract

Mob1 is a component of both the mitotic exit network and Hippo pathway, being required for cytokinesis, control of cell proliferation and apoptosis. Cell division accuracy is crucial in maintaining cell ploidy and genomic stability and relies on the correct establishment of the cell division axis, which is under the control of the cell's environment and its intrinsic polarity. The ciliate *T. thermophila* possesses a permanent anterior–posterior axis, left–right asymmetry and divides symmetrically. These unique features of *T. thermophila* prompted us to investigate the role of its Mob1 protein. Unexpectedly, we found that Mob1 accumulated in basal bodies at the posterior pole of the cell, and is the first molecular polarity marker so far described in *T. thermophila*. In addition, Mob1 depletion caused the abnormal establishment of the cell division plane, providing clear evidence that Mob1 is important for its definition. Furthermore, cytokinesis was arrested and ciliogenesis delayed in *T. thermophila* cells depleted of Mob1. This is the first evidence for an involvement of Mob1 in cilia biology. In conclusion, we show that Mob1 is an important cell polarity marker that is crucial for correct division plane placement, for cytokinesis completion and for normal cilia growth rates.

2. Introduction

The accuracy of cell division is crucial in maintaining cell ploidy and genomic stability. In fact, events like DNA replication, chromosome segregation, mitosis completion and cytokinesis must be tightly controlled, and their deregulation is closely associated with cancer development (Kops, Foltz, & Cleveland, 2004; Weaver & Cleveland, 2007). The correct positioning of the division plane of the cell, which is dependent on the polarity axis, is crucial in ensuring proper cell division. This polarity axis is established both by internal and external clues, and the mitotic spindle must be oriented along it, allowing chromosomes to be equally segregated into the two daughter cells (Caydasi et al., 2010; Chan & Amon, 2010).

In *S. cerevisiae*, which presents an asymmetric cell division, whenever the mitotic spindle is mispositioned, the spindle position checkpoint (SPOC) pathway prevents the activation of the mitotic exit network (MEN) until the correct orientation of the spindle is achieved (Caydasi et al., 2010). Therefore, the success of mitosis completion and cytokinesis is dependent on correct spindle orientation, itself a consequence of correct cell polarization.

MEN is a signaling cascade that controls mitosis to interphase transition. The final outcome of MEN signaling is the activation of the Cdc14 phosphatase, which then promotes the degradation of mitotic cyclins. MEN-regulated Cdc14 activation is dependent on the kinase activity of the complex Mob1–Dbf2 (Bardin & Amon, 2001). In yeast, Mob1 mutant cells arrest in late anaphase and show impaired cytokinesis (Francis C. Luca et al., 2001). Most components of the MEN pathway, such as Cdc15, Mob1 and Dbf2, have metazoan homologs. Importantly, the mammalian nuclear Dbf2-related kinases (NDR) LATS1 and LATS2, and MST1/2 (Cdc15 orthologous), or the *D. melanogaster* Hippo and Warts form, together with Mob1 and Sav1 (the core kinase module of the Hippo pathway), were recently established as being part of a major conserved mechanism governing cell contact inhibition, organ size control and cancer development both in mammals and in fly (for a review (Zeng & Hong, 2008)). Furthermore, most MEN proteins are associated with the centrosome, the major microtubule organizing center in animal cells (Glover, Hagan, & Tavares, 1998); (Mailand et al., 2002); (Wilmeth et al., 2010). Human MOB1 was also described as a tumor suppressor and is required for centrosome duplication (Wilmeth et al., 2010); (Hergovich et al., 2009). A phylogenetic analysis showed that Mob proteins are usually

encoded by more than one gene, ranging from two in yeast (mob1 and mob2), to three in *D. melanogaster* (Mob1, Mob2 and Mob3) and at least six human Mob homologous genes (MOB1A and MOB1B that encode two closely related proteins, MOB2, MOB3A, MOB3B and MOB3C) (Chow, Hao & Yang, 2010; Hergovich, 2011).

Although a complete picture of the role of the distinct Mob proteins *in vivo* is still missing, some of these proteins seem to have specialized functions. For example, in budding and fission yeasts, Mob2 interacts with the Cbk1 and Orb6 kinases, respectively, which is required for cell cycle progression and regulates polarized growth and morphogenesis (Hou, Wiley, Verde, & McCollum, 2003; Weiss et al., 2002; Colman-Lerner et al., 2001). Interestingly, Mob1 and Mob2 proteins are not functionally interchangeable (Hou, Guertin, & McCollum, 2004); (Hou et al., 2003). In *D. melanogaster*, although the role of Mob2 is far from being understood, this protein it is known to interact with Trc kinase, playing a role in wing hair morphogenesis (He et al., 2005) and photoreceptor cell development (Liu, Lin, & Fan, 2009). In humans, MOB2 seems to be involved in the regulation of MOB1 by competing for the NDR1/2 kinases (Kohler, Schmitz, Cornils, Hemmings & Hergovich, 2010). The existing data on Mob3 proteins is sparse and their role elusive (Hergovich, 2011).

By contrast, the ciliate *T. thermophila* possesses only one gene (mob1) encoding a Mob1-like protein. *T. thermophila* is a highly differentiated organism that, in contrast with budding yeast, divides symmetrically and possesses a permanent anterior–posterior axis and left–right asymmetry. Moreover, this unicellular organism contains a vegetative macronucleus (MAC) that divides amitotically and a germ line micronucleus (MIC) presenting an acentrosomal spindle (Frankel, 1999). These unique features of *T. thermophila* prompted us to investigate the functions of *T. thermophila* Mob1.

Here, we report that in *T. thermophila* Mob1 is a molecular polarity marker of the posterior pole basal bodies, the first so far described in this organism. Furthermore, *T. thermophila* Mob1 is involved in cilia biogenesis and is a crucial factor for the correct establishment of the cell division plane and cytokinesis.

3. Material and Methods

3.1. Plasmid construction

To construct the *T. thermophila* Mob1-KD plasmid, we amplified the 5′ and 3′ untranslated regions (UTRs) of the *T. thermophila* mob1 gene (5′-mob1UTR and mob1UTR-3′), using specific primers with attached restriction sites. The two homology arms were cloned in the pNeo4 vector (a kind gift from Jacek Gaertig, University of Georgia, Athens, GA) that has an inducible promoter responsive to Cd2+ (MTT1) and a neomycin resistance marker (Neo4). The resulting plasmid was as follows: 5′-mob1UTR–MTT1–Neo4–mob1UTR-3′. For the *T. thermophila* Mob1–GFP construct, we amplified the *T. thermophila* mob1 gene open reading frame (ORF) (TTHERM_00716080; XP_001031965) with specific primers (Table 1) containing the restriction sites to clone the mob1 coding sequence in the pMTT1–GFP vector (kind gift from Jacek Gaertig), in frame with GFP in the C-terminal region. This plasmid contains the 5′ and 3′ UTRs of one of the two β-tubulin genes of *T. thermophila* (BTU1) and also the inducible promoter MTT1. The Mob1–GFP construct used was as follows: 5′-BTU1UTR–MTT1–mob1–GFP–BTU1UTR-3′.

Table 1 - Nucleotide sequences of the primers used in the study of Mob1 in *Tetrahymena. thermophila*.

Tt-Mob1-GFP	
TtMob1 coding sequence Forward	5’ GAT GGT AAG CTT ATG AGT TAG AAG ACA TAT AAG 3’
TtMob1 coding sequence Reverse	5’ TAT TAT ACG CGT TGT TAA GTT TGA GGA ACT TC 3’
TtMob1-KD	
TtMob1 5’ UTR Forward	5’ TAT TAT GGT ACC TGA TGT ACC AAG GCT TAT TT 3’
TtMob15’ UTR Reverse	5’ GT GGT ATC GAT TTT AAC TTT TTT CTT TCA ATA 3’
TtMob13’ UTR Forward	5’ TAT TAT GGA TCC TTA CAC AGC TTC TAA TAA AG 3’
TtMob13’ UTR Reverse	5’ TAT TAT CCG CGG TCT TTT GTA TAT TAA AAC TT 3’
RT-PCR	
cDNA_TtMob1 Forward	5’ CAT GCT AAA TAG GTG TGC CTT TCC CT 3’
cDNA_TtMob1 Reverse	5’ TAT TAT ACG CGT TGT TAA GTT TGA GGA ACT TC 3’
Control L32 Forward	5’ ATG GCT ATT AAA CCC GTT GCC 3’
Control L32 Forward	5’ TGT TCA TTA AGA GAA TTT CGA GAT CA 3’

3.2. *Tetrahymena thermophila* strains

All cell strains used were grown in super proteose peptone (SPP) medium (1% proteose peptone, 0.1% yeast extract, 2.2% glucose, and 0.003% Fe-EDTA) at 30°C, with gentle shaking. The constructs described were used to biolistically transform the MAC of exponentially growing *T. thermophila* cells (Cassidy-Hanley et al., 1997). For the *T. thermophila* Mob1-KD strain, Cu428 cells were transformed with the *T. thermophila* Mob1-KD plasmid, which was inserted in the endogenous locus of mob1. Transformed cells were selected with rising doses of paromomycin, until the sub-lethal concentration of 2.8 mg/ml, to force an increase in the number of disrupted copies by phenotypic assortment. Cu522 cells were transformed with the Mob1–GFP construct, which was inserted in the ectopic locus of BTU1. Transformants were selected with 20 μM paclitaxel. To obtain the *T. thermophila* Mob1–GFP ShuttON/OFF strain, the Mob1–GFP-expressing strain was transformed with the Mob1-KD construct and transformants selected with paromomycin until the sub-lethal dose of 9 mg/ml. The expression and repression of Mob1–GFP in *T. thermophila* Mob1–GFP ShuttON/OFF cells was controlled by the addition of 2.5 μg/ml of CdCl2 or depletion of Cd2+. Cd2+ was eliminated from SPP by depleting the medium with 5% Chelex-100 beads (Bio-Rad) followed by complementation with trace metals (Seixas, Cruto, Tavares, Gaertig & Soares, 2010).

3.3. Nocodazole treatment

T. thermophila exponentially growing cells expressing Mob1–GFP were treated with 30 μM of nocodazole for 30 minutes. After the nocodazole treatment, 2.5 μg/ml of CdCl2 was added to the medium. After 30 minutes, cells were washed in 10 mM Tris-HCl (pH 7.5) and processed for immunofluorescence. Nocodazole-untreated Mob1–GFP-expressing cells with 2.5 μg/ml of CdCl2 in the medium were used as control.

3.4. Immunofluorescence assays

To stain *T. thermophila* Mob1-KD cells, 40–60 cells were isolated into 20 μl of 10 mM Tris-HCl (pH 7.5) on a coverslip previously coated with poly-L-lysine (Sigma). To stain *T. thermophila* Mob1–GFP-expressing and Mob1–GFP ShuttON/OFF cells,

exponentially growing cultures were washed and diluted in 10 mM Tris-HCl (pH 7.5). Then, 20 µl of cells at 20,000 cells/ml were placed on a poly-L-lysine-coated coverslip. The following primary antibodies were used: mouse 20H5 anti-centrin (1:100; kind gift from Jeffrey Salisbury, Mayo Clinic, Rochester, MN), mouse 12G10 anti-α-tubulin (1:10; University of Iowa, Developmental Studies Hybridoma Bank), anti-glutamylated-tubulin (GT335) (kind gift from Carsten Janke, Institut Curie, Orsay, France) and rabbit anti-GFP (1:200; A11122, Invitrogen). Secondary antibodies used were Alexa-Fluor-488-conjugated goat anti-mouse, Alexa-Fluor-594-conjugated goat anti-mouse and Alexa-Fluor-488-conjugated goat anti-rabbit (1:500; Molecular Probes). Coverslips were processed for immunofluorescence labeling as described by Thazhath and co-workers (Thazhath, Liu, & Gaertig, 2002). Cells were viewed using a Leica SP5 Spectral Confocal with resonant scanner (using a 63× oil immersion lens). Images were assembled using NIH ImageJ 1.42q and Adobe Photoshop CS4 extended version 11.0.

3.5. Western blot and electrophoresis analysis

To analyze the expression of the tagged Mob1–GFP protein in *T. thermophila* cells expressing Mob1–GFP and in *T. thermophila* Mob1–GFP ShuttON/OFF cells (with/without Cd2+), total protein extracts from 120,000 cells of both strains and from wild-type cells were prepared. Cells were pelleted at 1600 g for 3 minutes, suspended in 1 ml of 10 mM Tris-HCl (pH 7.5) and concentrated in a final volume of 50 µl. Cell pellets were lysed with 50 µl of lysis buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% β-mercaptoethanol and Bromophenol Blue). Protease inhibitors were added at a final concentration of 0.5 mg/ml leupeptin, 10 mg/ml chymostatin and 15 mg/ml antipain. The mixture was boiled for 5 minutes at 95°C and 50 µl of each sample was used for SDS-PAGE. Electrophoresis and western blot analysis of SDS-PAGE (12% w/v) gels were carried out as described by Soares and colleagues (Soares et al., 1997). Primary antibody used was rabbit anti-GFP (1:2000; A11122, Invitrogen). The secondary antibody used was goat anti-rabbit IgG (H+L) (1:1000; Zymed). The LMW-SDS Marker Kit (GE Healthcare) was used to mark molecular mass.

3.6. Deciliation assay

Exponentially growing *T. thermophila* Mob1-KD and wild-type cells were washed in 10 mM Tris-HCl (pH 7.5). Cells were concentrated in one twentieth of the initial volume and deciliated in drops. Briefly, cells from both strains were transferred from 25 µl of Tris-HCl to 50 µl of 50 mM sodium acetate, 10 mM EDTA-Na2 pH 6; then after 30 seconds 25 µl of water was added. After a further 30 seconds, 2.5 µl of 0.4 M CaCl2 was added to the drop and after 15 seconds the cells were transferred to SPP drops and allowed to recover cilia. Cells were observed over time using bright field microscopy to evaluate movement. Immunofluorescence was performed as described above at different time points of cilia recovery.

3.7. Gene expression analysis by RT-PCR

Total RNA samples from *T. thermophila* wild-type, Mob1-KD, reciliating and Mob1–GFP ShuttON/OFF cells (with/without Cd2+) were prepared using the RNeasy Mini Kit (Qiagen, Germantown, MD) followed by cDNA synthesis using Superscript II RT (Invitrogen). Primers were designed to amplify *T. thermophila* Mob1 and L32, which was used as an internal control. PCR was performed for 30 cycles.

4. Results

4.1. *Tetrahymena thermophila* Mob1 downregulation leads to incorrect cell division planes and impaired cytokinesis

To investigate the function of *T. thermophila* Mob1, we constructed a *T. thermophila* Mob1 knockdown strain (Mob1-KD). Using this strategy, we disrupted the endogenous mob1 locus in the polyploid macronucleus MAC (45n) by the introduction in the coding region of a Neo4 cassette that confers resistance to paramomycin (Gaertig, Gu, Hai & Gorovsky, 1994). Transformants were selected with increasing paromomycin doses until the sub-lethal concentration of 2.8 mg/ml, thus ensuring, by phenotypic assortment (Sonneborn, 1974), the maximum number of *mob1* disrupted alleles (Fig. 9). The correct insertion in the MAC genome was confirmed by PCR analysis, which showed the presence of the *mob1* wild-type allele with the expected size of 3.5 kb and the corresponding knockout allele of 4.6 kb containing the inserted Neo4 cassette (Fig. 10A). The presence of the *mob1* knockout allele was further confirmed by sequence analysis using the primers described in Table 1 (see Materials and Methods). In the resulting *T. thermophila* strain, we achieved an average decrease of 70% of the mob1 mRNA levels (Fig. 10B).

A morphological analysis by light microscopy of the *T. thermophila* Mob1-KD population revealed the presence of cells with dramatically abnormal shapes. A range of defects were observed in these cells (see later) including ‘heart shape’, ‘boomerang shape’ and big cell masses or ‘monsters’. None of these phenotypes was observed in wild-type cells. These classes of defects might be explained by the phenotypic assortment that originates cell populations with individual cells presenting different ratios of wild-type to disrupted alleles.

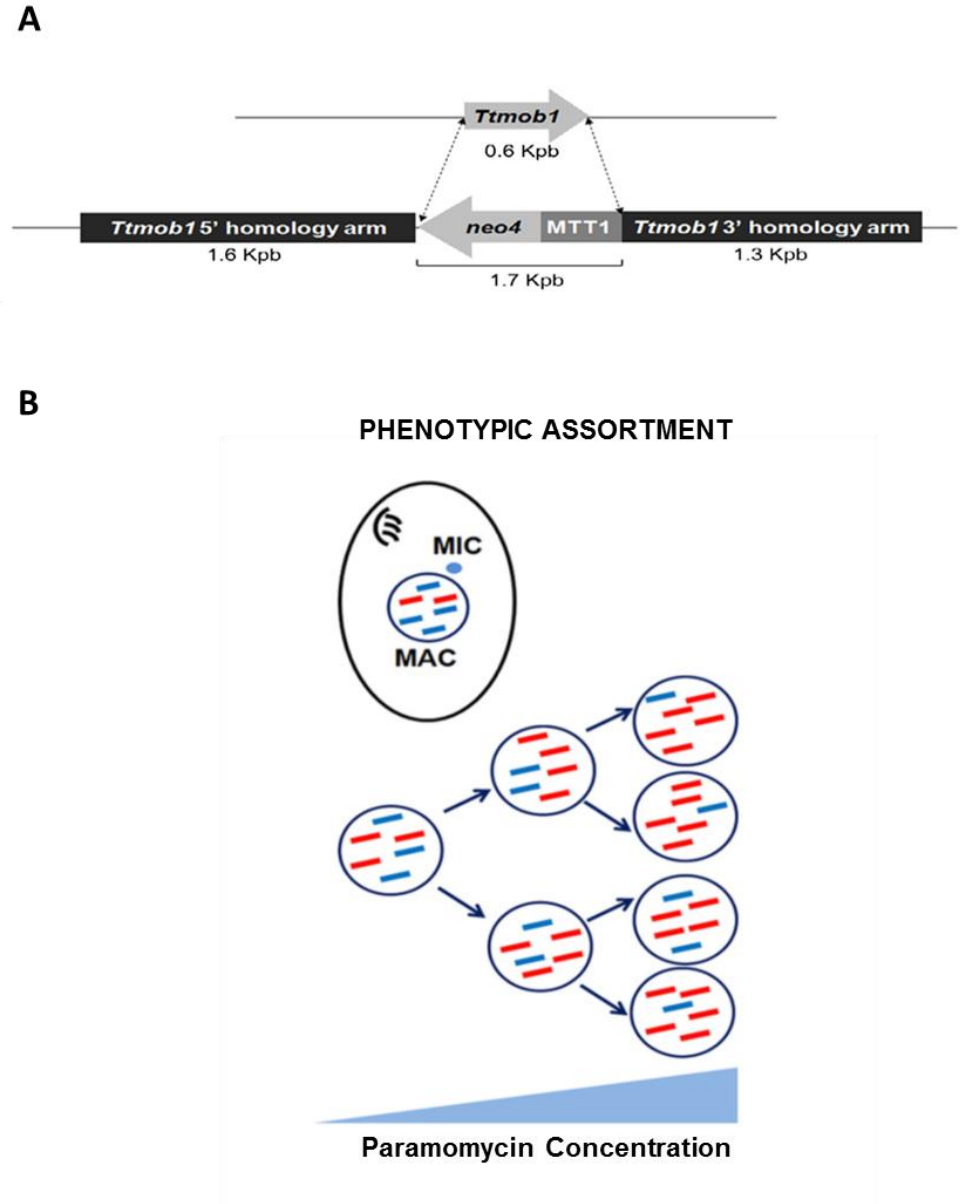


Figure 10 – Strategy used for obtaining the Mob1 knockdown strain of *Tetrahymena thermophila*.

A - Schematic representation of the construct used to disrupt the Ttmob1 locus, originating the TtMob1-KD strain. B - Representation of phenotypic assortment in the *T. thermophila* macronuclear genome. The larger circle represents the MAC and the small blue circle indicates the MIC. For simplicity the MAC genetic content is represented by six copies of one autonomous replicating piece with WT Ttmob1 allele (blue lines) and the knockout Ttmob1 allele (red lines). In cells reproducing asexually, the MAC (45n) divides amitotically, which leads to a random distribution of allelic copies into daughter cells. Genetically transformed cells in the MAC can be pressed to increase the number of disrupted allelic copies by increasing the concentration of the selection drug, paromomycin, in the growth media.

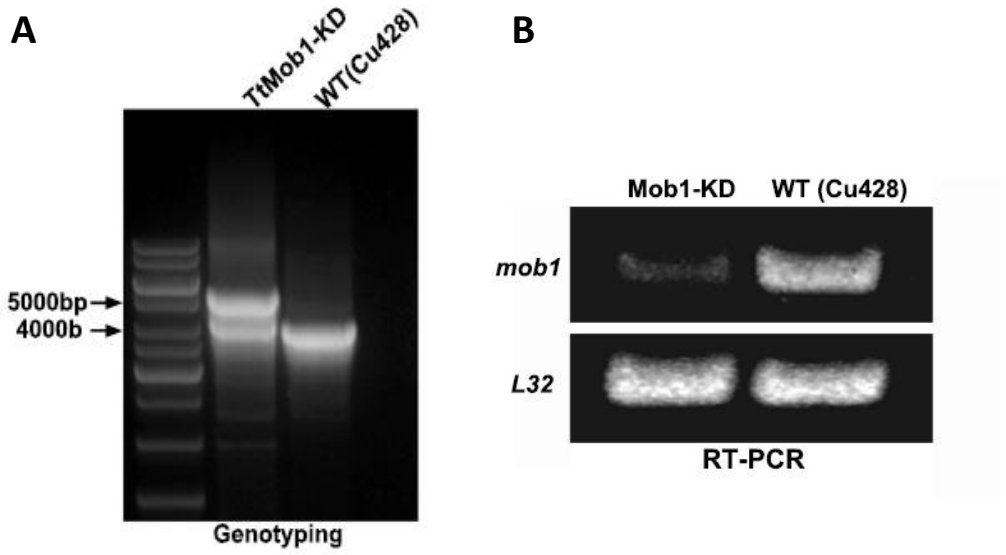


Figure 11 – Validation of the *Tetrahymena thermophila* knockdown strain. A- PCR analysis of genomic DNA (gDNA) obtained from WT (Cu428 strain) and TtMob1-KD to confirm their genotype. gDNA was amplified with specific primers to the homology arms of 5' and 3' flanking regions of Ttmob1 gene. For WT strain it was observed only one band corresponding to WT allele (4 kb), whereas in TtMob1KD cells an additional band corresponding to the disrupted-alleles Ttmob1 is visible (5 kb). B- RT-PCR analysis of Ttmob1 expression in TtMob1-KD and in WT (Cu428 strain) cells showing the Ttmob1 decreased levels in TtMob1-KD cells. The ribosomal protein L32 coding gene was used as control

Immunofluorescence confocal microscopy of *T. thermophila* Mob1-KD cells, using antibodies against α-tubulin and centrin, indicated that the different morphologies are due to the failure of cell division. Wild-type *T. thermophila* cells divide symmetrically, the division plane being perpendicular to the anterior–posterior axis in the midzone of the cell, just above the newly formed oral apparatus (Fig. 11A, B). We observed that in *T. thermophila* Mob1-KD cells the division plane is frequently displaced anteriorly along the anterior–posterior axis. Moreover, the angle that the division plane established with the anterior–posterior axis was severely affected, ranging from 90° to 0° (Fig. 12).

In a dividing wild type cell, the new oral apparatus is the first visible structure assembled prior to cell division and its position is determined by the distance to the old oral apparatus, which is directly proportional to the cell length (Lynn & Tucker, 1976).

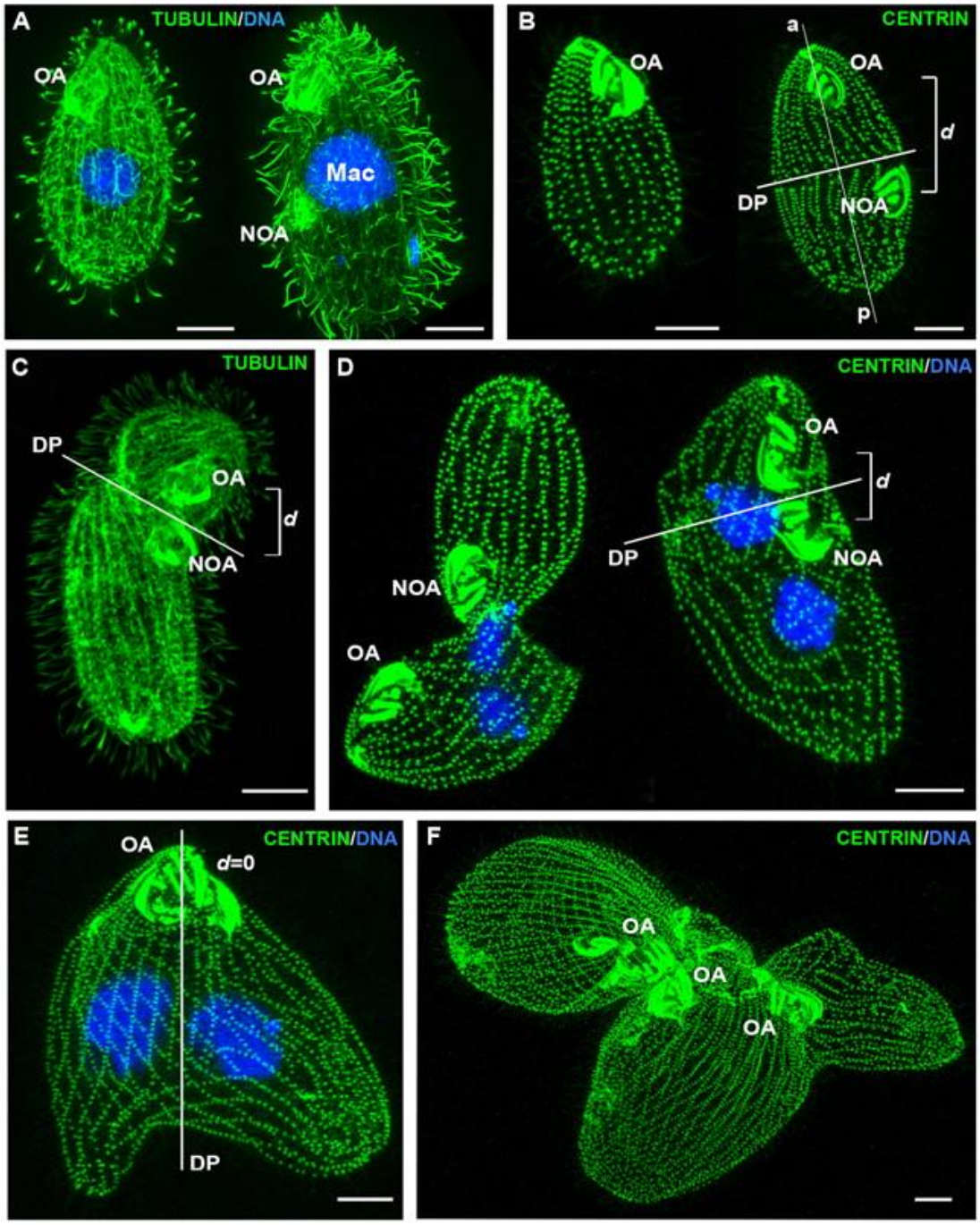


Figure 12 - Mob1 depletion in *Tetrahymena thermophila* causes incorrect positioning of the cell division plane and impaired cytokinesis. (A–F) Immunofluorescence of α-tubulin and centrin in *T. thermophila* wild-type (Cu428 strain) (A, B) and Mob1-KD cells (C–F), co-stained with TOTO-3-iodide to label DNA (A, D, E). In wild-type cells, the division plane (DP) is established perpendicularly to the anterior (a)–posterior (p) axis, in the midzone of the cell. The oral apparatus (OA) and the new oral apparatus (NOA) show a conserved distance (d) that depends on cell length (B). Mob1-KD cells present a DP angle deviation relatively to the anterior–posterior axis and fail cytokinesis (C–F).

We observed that in the majority of *T. thermophila* Mob1-KD cells this distance was reduced (Fig. 11C, D) or abolished (Fig. 11E), suggesting that the new oral apparatus was either assembled at a wrong place or had migrated towards the old oral apparatus during the elongation process (Frankel, 2008). Being the oral apparatus an anterior polarity landmark in *T. thermophila*, the occurrence of these Mob1-KD phenotypes suggested that these cells possess altered polarity, which compromised the correct definition of the division plane. Importantly, Mob1-depleted cells seemed to have started the formation of the cleavage furrow but then aborted division, failing cytokinesis (Fig. 11C–F). In these cases, the normal gap observed between the cilia longitudinal rows was not formed and the rows were continuous across the cleavage furrow (Fig. 11D, right, 11E). Strikingly, these errors did not arrest the cell cycle because the cells continued trying to divide, originating gigantic monsters (Fig. 11F). Also, basal body duplication was not impaired, evidenced by a decreased distance between them over the longitudinal cilia rows (Fig. 11F). Moreover, the MAC and the MIC divisions seemed to occur normally, although the two MAC could fail to segregate correctly (Fig. 11D, left).

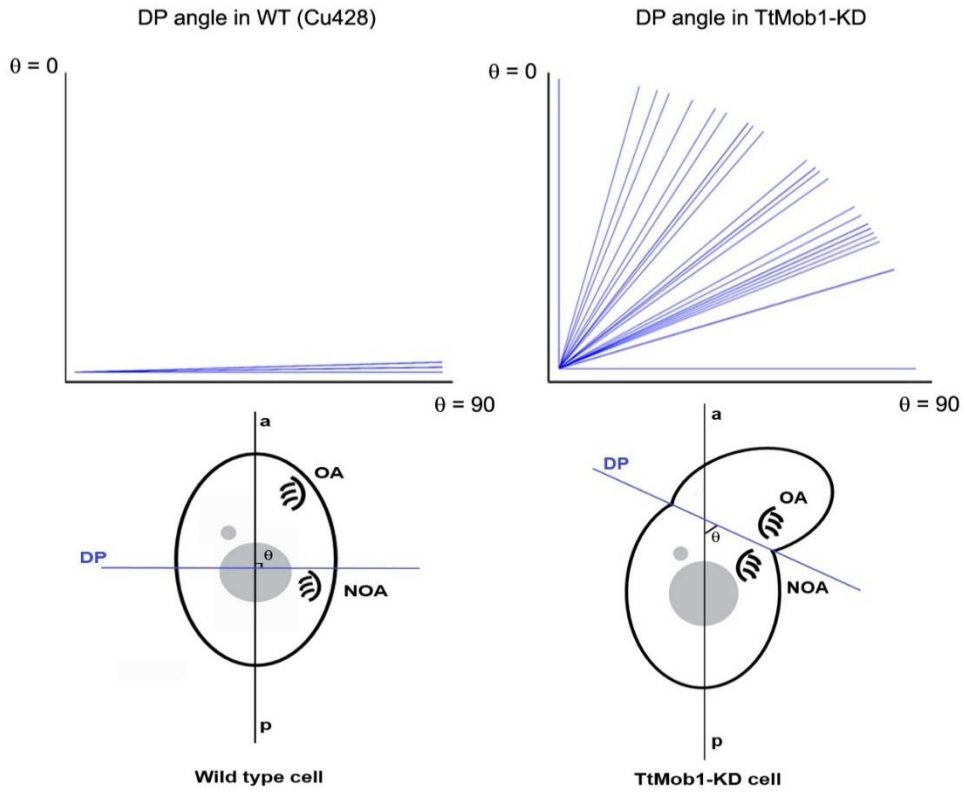


Figure 13 - Graphic representation of the angle (θ) established between the DP and the anterior–posterior axis in wild-type (n514) and Mob1-KD (n533) cells. Measurement of angle (θ) was carried out using ImageJ software.

4.2. *Tetrahymena thermophila* Mob1 accumulates at basal bodies with a polarized gradient distribution towards the posterior pole

In order to further define the function of *T. thermophila* Mob1, we analyzed its cellular distribution. For that purpose we constructed a *T. thermophila* strain expressing Mob1–GFP through the introduction, by DNA homologous recombination, of a Mob1–GFP construct into a β-tubulin locus, under the control of the cadmium (Cd2+)-inducible promoter metallothionein 1 (MTT1) (Fig. 13A). The levels of Mob1–GFP in response to Cd2+ addition for different times were evaluated by western blot using an anti-GFP antibody (Fig. 13B). Mob1–GFP was detected at 15 minutes and reached the highest levels after 30 minutes of Cd2+ induction. As a control, a *T. thermophila* strain expressing just GFP was also created.

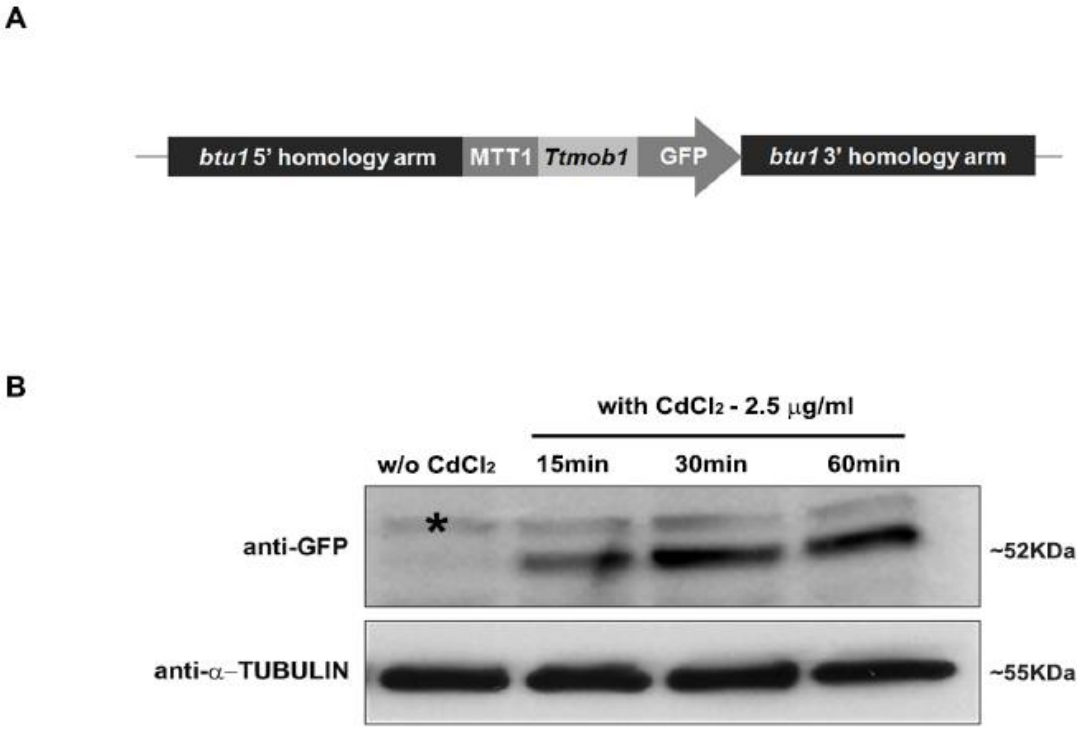


Figure 14 - A) Schematic representation of the DNA construct used to create the TtMob1 GFP strain. DNA was inserted by homologous recombination in the endogenous locus of *the BTU1* gene in the macronucleus of Cu522 strain cells **(B)** Western blot of total proteins obtained from TtMob1-GFP growing in medium without/with Cd2+ (2.5μg/ml) at 15, 30 and 60 min of induction. Protein extracts were probed with anti-GFP antibody and with anti-α-tubulin antibody, used as loading control. Unspecific band (*).

An immunofluorescence confocal microscopy analysis of control cells expressing GFP alone showed it to be distributed in the cytoplasm (Fig. 14A). Unexpectedly, Mob1–GFP, was clearly accumulated in posterior pole basal bodies (Fig. 14B–E), as confirmed by colocalization with centrin (Fig. 14C, E). Remarkably, Mob1–GFP follows a gradient along the anterior–posterior axis of the cell, with a high signal at the posterior pole that progressively and substantially decreases towards the anterior pole (Fig. 14B–E).

Therefore, in *T. thermophila*, distinct basal bodies clearly present different molecular compositions and abilities to recruit and/or concentrate pools of proteins involved in establishing cell polarity, which suggests that, in addition to cilia assembly, they might present specialized functions inside a single cell. Mob1–GFP was also detected in some posterior transversal microtubules, contractile vacuole pores and in the oral apparatus (Fig. 14B–E).

Next, we investigated whether the localization of basal bodies and oral apparatus in *T. thermophila* expressing Mob1–GFP was dependent on microtubules by treating the cells with the microtubule-depolymerizing agent nocodazole. In nocodazole-treated *T. thermophila* cells, Mob1–GFP had the same localization pattern as in non-treated cells, indicating that this specific localization of *T. thermophila* Mob1 is independent of intracytoplasmic and cortical nocodazole-sensitive microtubules (Fig. 15).

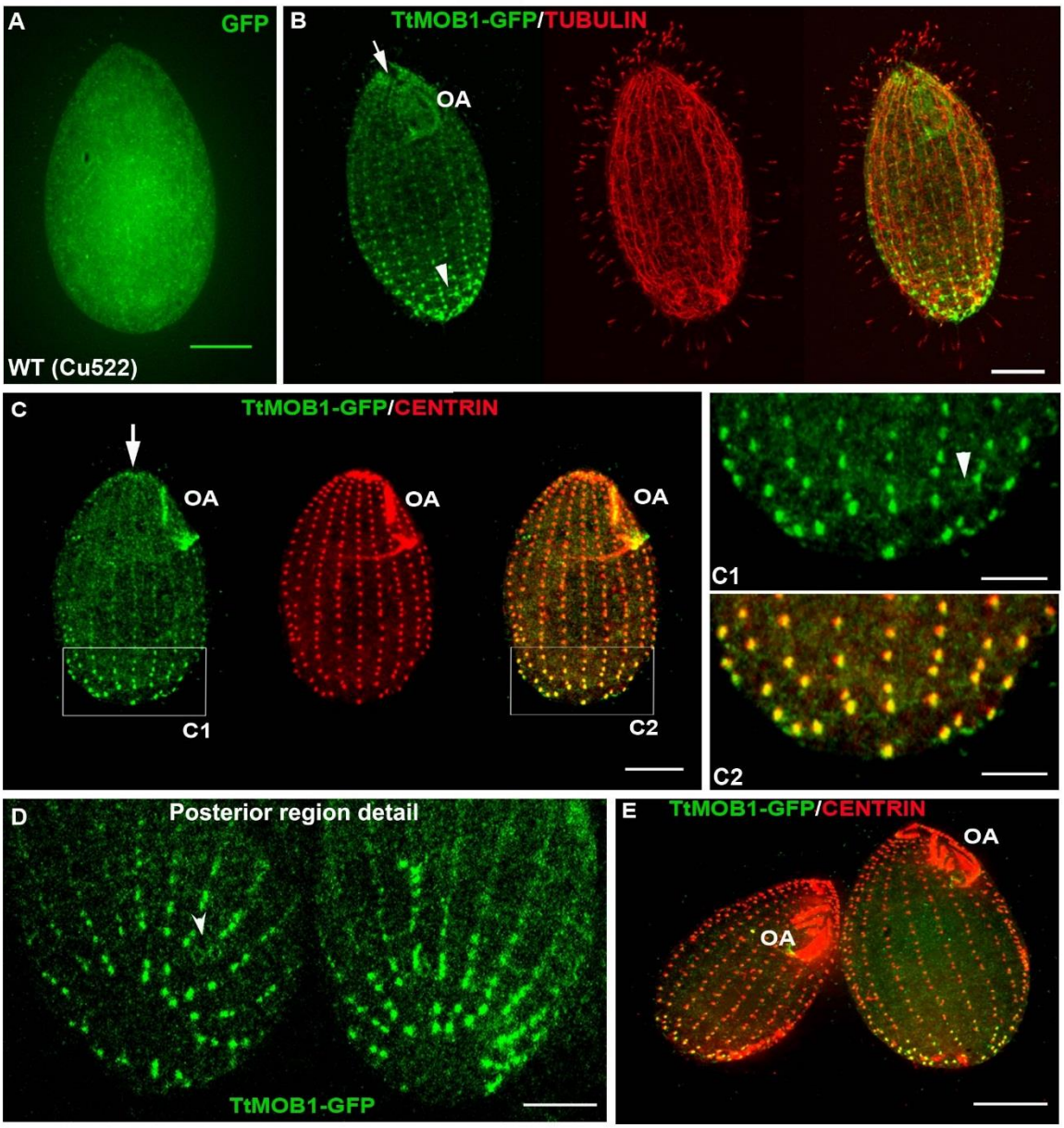


Figure 15 - *Tetrahymena thermophila* Mob1 localizes preferentially at the posterior pole basal bodies. (A) *T. thermophila* cells expressing GFP were stained only with anti-GFP antibody. (B–E) Immunofluorescence microscopy of *T. thermophila* cells expressing Mob1–GFP using anti-GFP (all images), anti-α-tubulin (B) and anti-centrin (C–E) antibodies. Mob1–GFP accumulates preferentially at the posterior pole basal bodies, co-localizing with centrin (C,E). Panoramic view of the posterior region of Mob1–GFP-expressing cells is shown in D; arrowhead indicates where contractile vacuole pores are visible. Mob1–GFP could also be detected at the oral apparatus (OA), at the posterior pole transversal microtubules (indicated by arrowheads in B and C1) and at the asymmetric anterior crown (arrows in B and C). Scale bars: 10 μm (A,B,C,E); 2 μm (C1,C2,D).

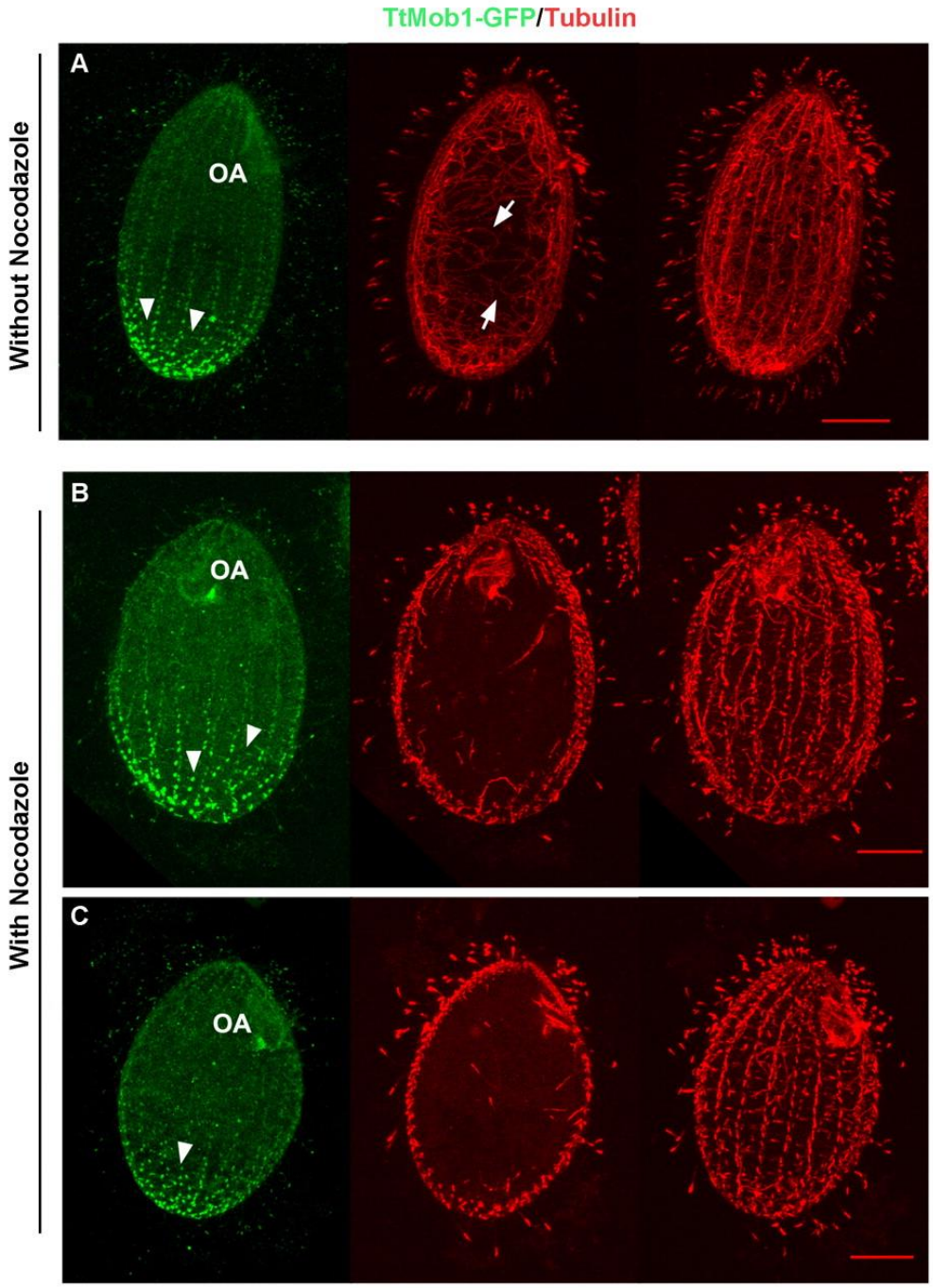
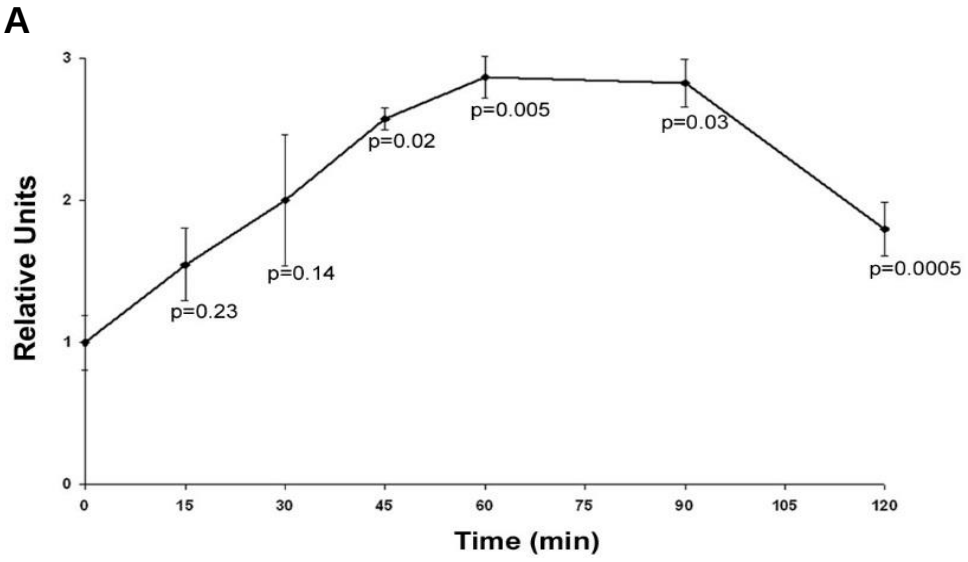


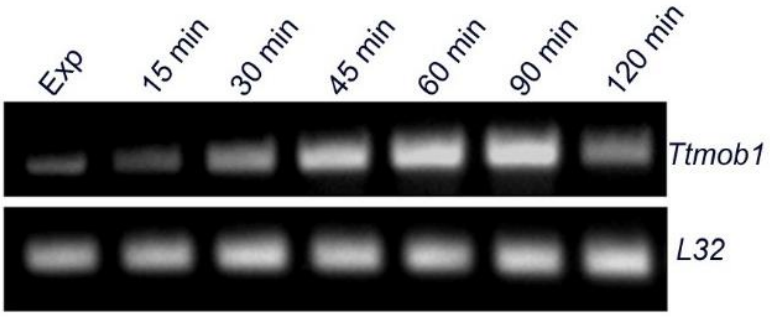
Figure 16 - *Tetrahymena thermophila* Mob1 basal body localization does not depend on intracytoplasmic microtubules. (A) *T. thermophila* cells expressing Mob1–GFP were grown in culture medium supplemented with Cd2+ (2.5 μg/ml) and show the typical polar localization of Mob1–GFP in the oral apparatus (OA) and at the basal bodies of the posterior pole. (B, C) Mob1–GFP-expressing cells were treated with nocodozole (30 μM) to depolymerize internal cytoplasmic microtubules (arrows). In these cells, internal microtubules are not visible, but Mob1–GFP maintains the same pattern of localization in the OA and in posterior basal bodies (arrowheads). Scale bars: 10 μm.

4.3. *Tetrahymena thermophila* Mob1 depletion leads to a delay in cilia recovery

Given the localization of *T. thermophila* Mob1 at basal bodies, we wondered whether it had an involvement in cilia biology. *T. thermophila* has a complex cortical microtubule cytoskeleton where basal bodies and cilia are found in repeated units (see Fig. 11A, B), and is capable of reciliation if cilia are removed (Soares et al., 1994). Therefore, we investigated the behavior of *T. thermophila* Mob1-KD cells deciliated by osmotic or mechanical stress. Interestingly, we found that *T. thermophila mob1* expression was upregulated during cilia recovery after ablation (Fig. 16A), responding to cilia biogenesis, with the highest levels detected after 45–90 minutes of reciliation. In agreement, we observed that *T. thermophila* Mob1-KD cells have delayed cilia recovery compared with wild-type cells (Fig. 16B,C). The swimming behavior of *T. thermophila* Mob1-KD cells was also investigated using bright field microscopy and, whereas wild-type reciliating cells are able to swim in a similar fashion to non-deciliated cells after 87.5±9.6 minutes, the Mob1-KD cells take longer to reach normal swimming behavior (126.7±20.8 minutes) (Fig. 16B).

Noteworthy, the analysis of these cells by immunofluorescence microscopy, using an antibody against glutamylated tubulin, showed no difference in the recovery rates for cilia of different cell regions (Fig. 16C), suggesting that high Mob1 levels at the posterior basal bodies are not important for cilia biogenesis.





B

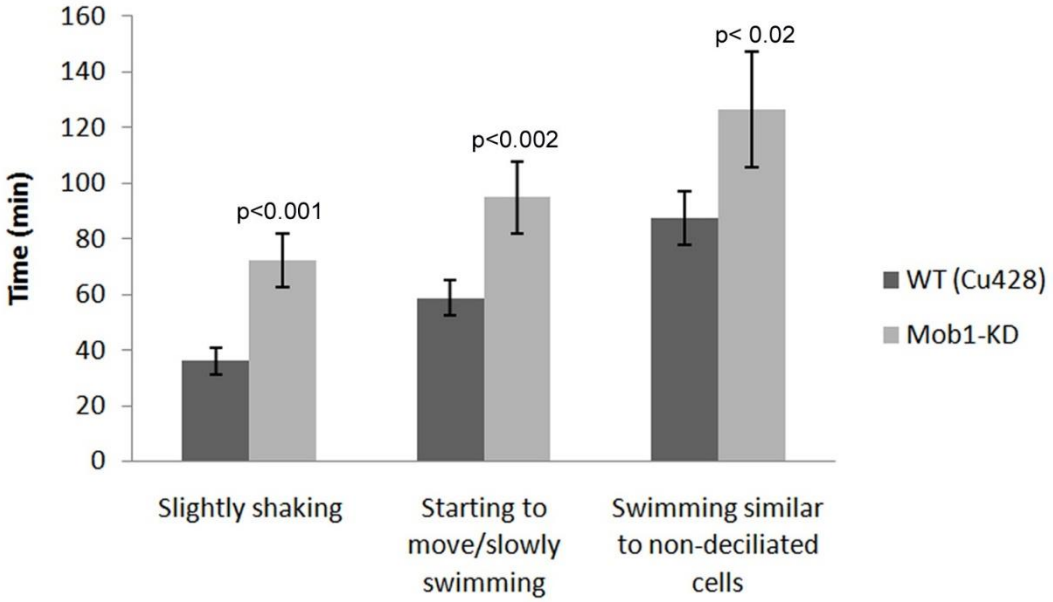


Figure 17 - *Tetrahymena thermophila* Mob1 depletion leads to a delay in cilia recovery. (A) RT-PCR analysis shows the expression pattern of mob1 gene in *T. thermophila* wild-type (Cu428 strain) exponentially growing cells (Exp) and cells recovering their cilia for different times (15–120 minutes). The gene encoding ribosomal protein L32 was used as internal control. Time-course analysis of changes in mob1 mRNA levels during reciliation. The data represent average values from four independent experiments and are expressed relative to the amount of mRNA in Exp cells. Error bars indicate s.d. Statistical significance was calculated using the Student's t-test. (B) Comparison of the time required by *T. thermophila* wild-type (Cu428 strain) and Mob1-KD cells to acquire different swimming behaviors during cilia recovery. The deciliated populations were continuously followed over time until swimming behavior was similar to that of non-deciliated cells. Values show the specific time points at which the majority of the population of cells presented the swimming behaviors indicated. The data are from four independent experiments.

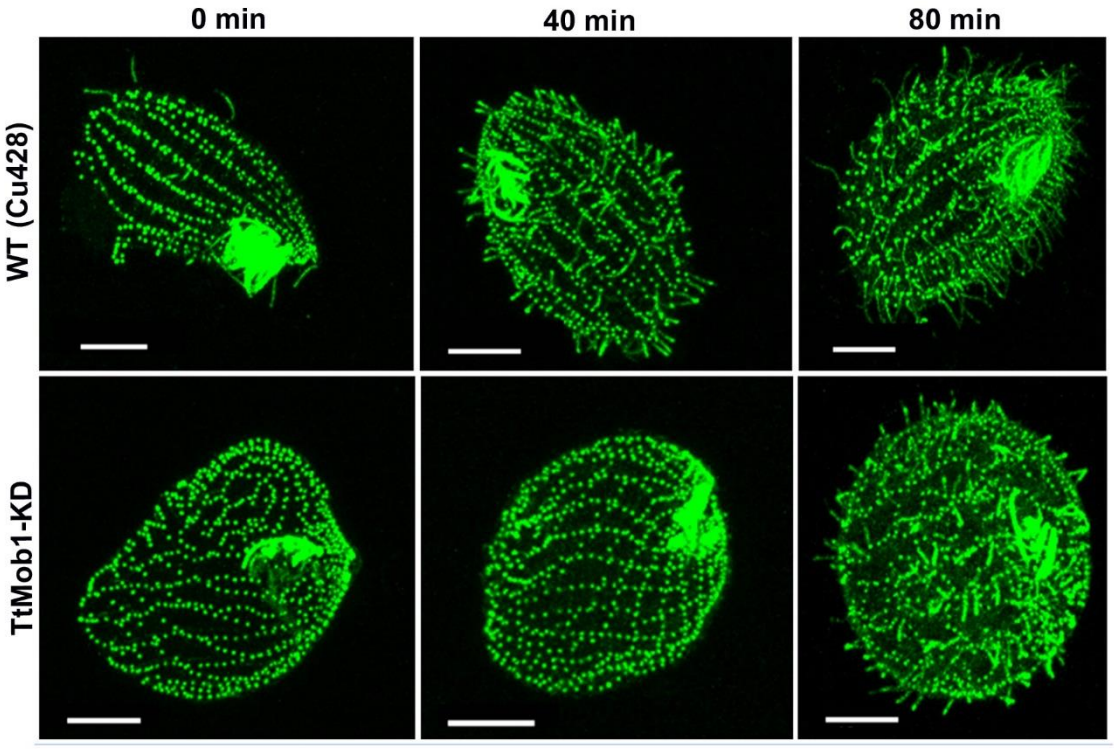


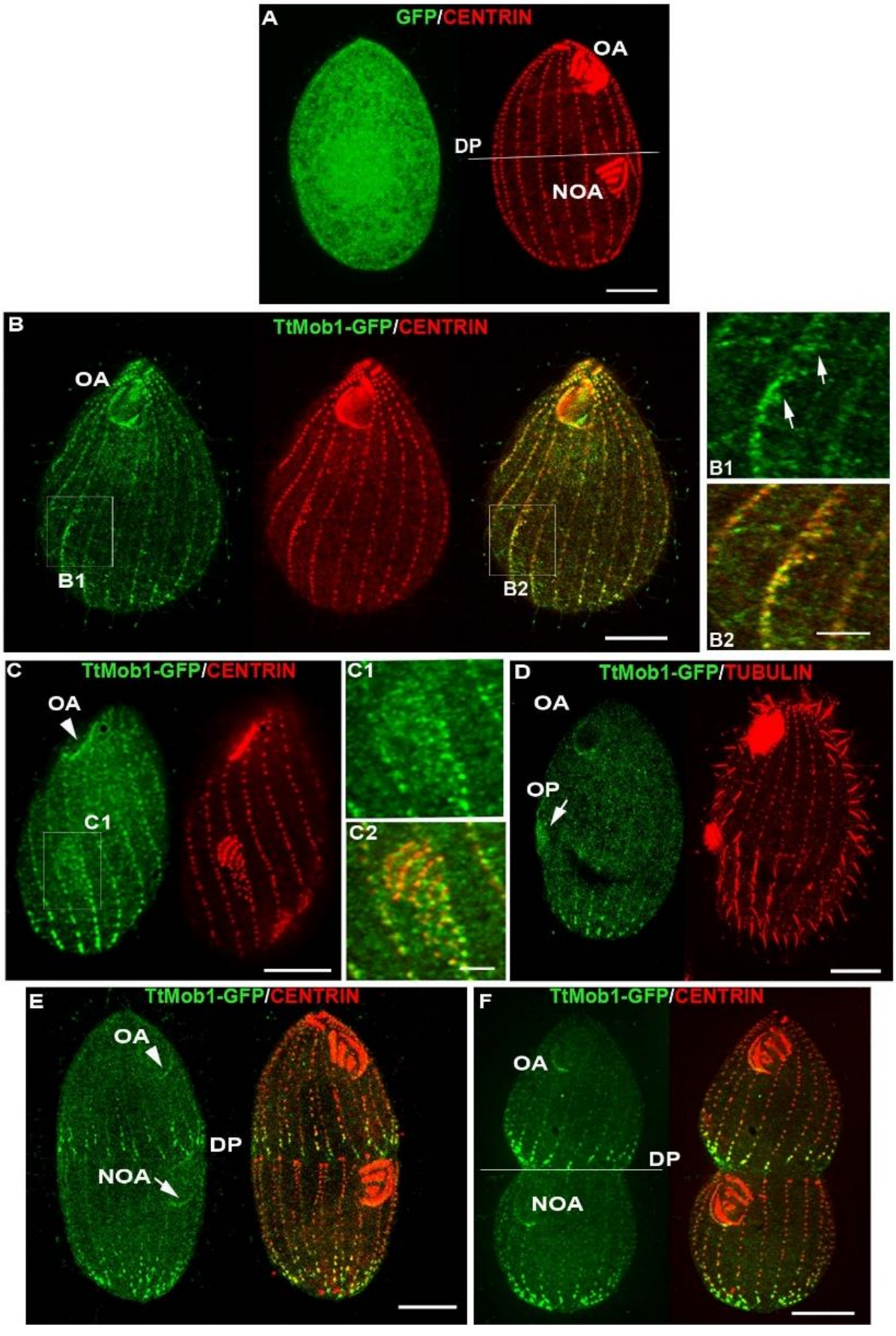
Figure 16 (cont) - (C) Immunofluorescence microscopy of wild-type (Cu428 strain) and Mob1-KD cells with anti-glutamylated-tubulin to evaluate cilia recovery. At 40 minutes, many Mob1-KD cells have no visible cilia. Scale bars: 10 μm.

Our results do not rule out the hypothesis that the delayed cilia biogenesis is related to an abnormal spatial organization and/or altered polarity of the cell. Nonetheless, this is the first time that Mob1 has been implicated in cilia biology. Additional investigation would allow a better understanding of this new function.

4.4. *Tetrahymena thermophila* Mob1 recruitment to the cell midzone defines the division plane

Normally in eukaryotic cells the cell division plane, where the cleavage furrow forms and ultimately cytokinesis occurs, bisects the preformed mitotic spindle (Oliferenko et al., 2009). In *T. thermophila*, macronuclear chromosomes lack centromeres and the MAC divides amitotically without a typical spindle being assembled. However, intranuclear microtubules that organize perpendicularly to the cleavage region have been implicated in MAC division (Smith, Yakisich, Kapler, Cole & Romero, 2004); (Fujiu & Numata, 2000); (Wunderlich & Speth, 1970); (Tamura, Tsuruhara & Watanabe, 1969).

These microtubules seem to be nucleated at a large number of sites inside of the nucleus (Fujiu & Numata, 2000). By contrast, the MIC divides mitotically and the spindle also assembles perpendicularly to the furrow but does not present organized structures at its poles (Frankel, 1999). Also, *T. thermophila* cell division involves several morphological events at the cell cortex, beginning with the formation of an oral primordium, which starts with an anarchic field of basal bodies that will develop into the new oral apparatus (Fig. 17A–C) (Frankel, 1967). In dividing cells, we detected *T. thermophila* Mob1–GFP in the oral apparatus immediately at the beginning of its assembly (Fig. 17B1), where it remained (Fig. 17B–F). As the cell cycle progressed, Mob1–GFP started to accumulate in the midzone basal bodies, just above the region where the cleavage furrow would form (between the old oral apparatus and the new one) (Fig. 17B–F). Therefore, Mob1–GFP accumulates where the new posterior pole of one of the siblings is to be formed (Fig. 17G). These observations, together with the knockdown results, strongly suggest that *T. thermophila* Mob1 is involved in the establishment and maintenance of the anterior–posterior polarity of the cell.


A
GFP/CENTRIN
OA
DP
NOA
B
TtMob1-GFP/CENTRIN
OA
B1
B2
B1
B2
C
TtMob1-GFP/CENTRIN
OA
C1
C1
C2
D
TtMob1-GFP/TUBULIN
OA
OP
E
TtMob1-GFP/CENTRIN
OA
DP
NOA
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OA
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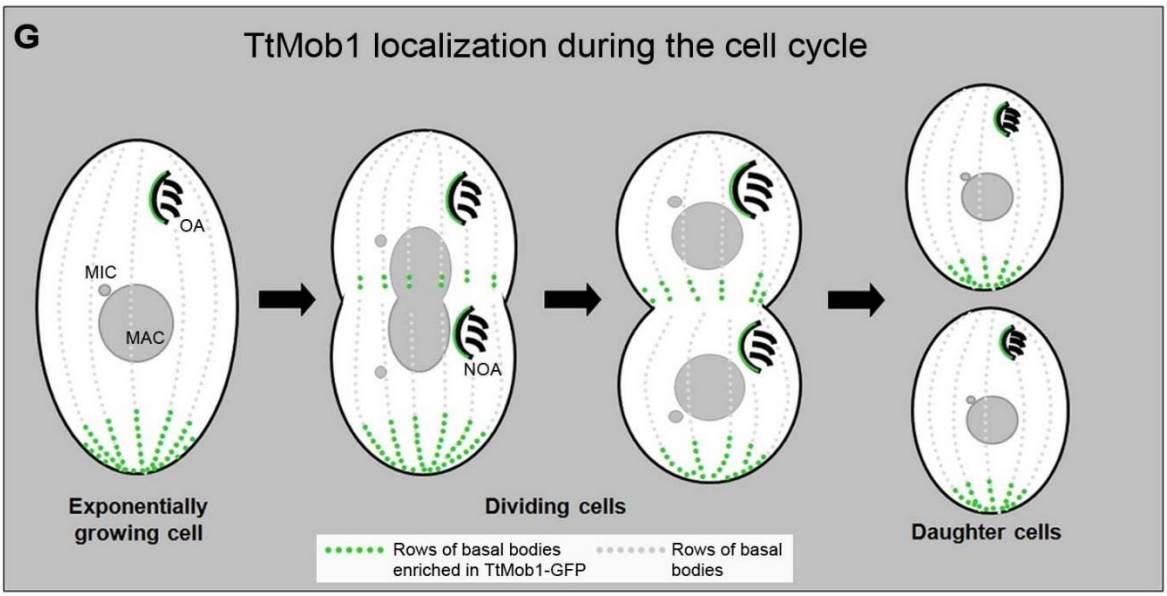


Figure 18 - *Tetrahymena thermophila* Mob1 localizes at the division plane basal bodies and new oral apparatus in dividing cells. (A–F) Immunofluorescence microscopy of *T. thermophila* cells labeled with anti-GFP antibody (A–F), anti-centrin (A–C,E,F) and anti-glutamylated-tubulin (D). (A) In a dividing cell expressing GFP, no specific GFP localization is observed. (B–F) Mob1–GFP-expressing cells through different stages of cell division. In dividing cells, Mob1–GFP is visible in different stages of development of the oral primordium (OP; arrow in D) (B–D) and in the equatorial region where the division plane (DP) will be established (E,F). Magnifications of B and C (B1,B2 and C1,C2) show details of the first steps of the new oral apparatus (NOA; arrow in E) formation. Note that in B1, Mob1 is already recruited to the first basal bodies of NOA (arrows). Arrowheads point to the oral apparatus (OA). (G) Schematic representation of Mob1–GFP localization during the *T. thermophila* cell cycle. Scale bars: 10 μm; 2 μm (in magnifications).

Next, we created a *T. thermophila* Mob1–GFP ShuttON/OFF strain for two different purposes: firstly, to confirm that the *T. thermophila* Mob1-KD phenotypes were specific and due to the downregulation of Mob1; and secondly, to investigate whether Mob1–GFP recruitment to the cleavage furrow is crucial for defining the cell division plane. For this, the *T. thermophila* Mob1–GFP-expressing strain previously described was transformed with the *T. thermophila* Mob1-KD DNA construct (Fig.18).

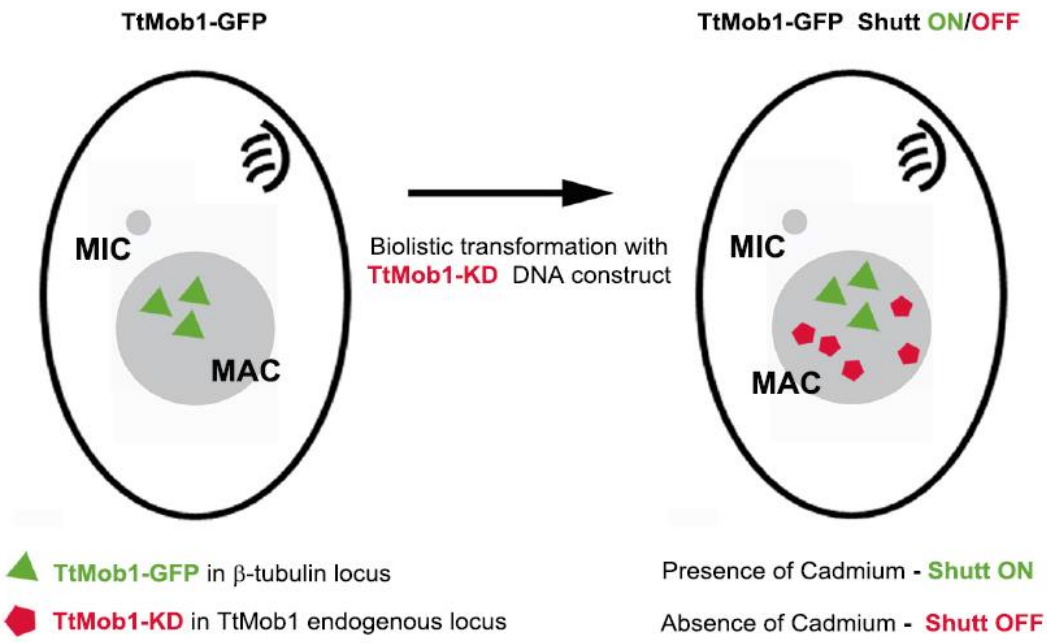
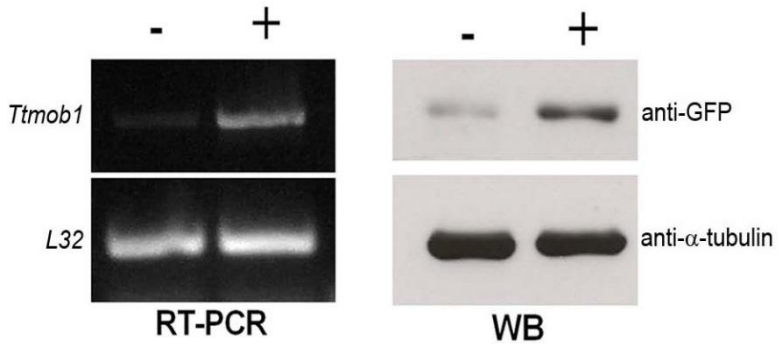


Figure 19 - TtMob1-GFP cells were biolistically transformed with the DNA construct to disrupt the endogenous Ttmob1 locus. Significantly, during transformants' selection with paromomycin, Cd2+ was added to the growth medium (see material and methods) to drive the expression of the NEO4 cassette. Consequently, TtMob1-GFP was also induced, which might compensate for the increasing disruption of Ttmob1 alleles. Thus, as expected, the sub lethal dose of paromomycin achieved for this strain was considerably higher (9000µg/ml) than the obtained for TtMob1-KD cells (2800 µg/ml), indicating that the number of Ttmob1 disrupted copies was higher. In support of this view, the TtMob1 depletion phenotypes frequencies were higher in TtMob1 ShuttON/OFF in comparison to TtMob1-KD cells (see Fig. 19 - Table 1).

With this strategy, we were able to induce or repress Mob1–GFP expression in an endogenous Mob1 depletion background by addition or removal of $Cd2^{+}$ (Fig. 19A). We observed that in the presence of $Cd2^{+}$ (ShuttON), the cells were indistinguishable from wild-type cells (Fig. 19B,C). On the other hand, cells growing in Cd2+-depleted medium (ShuttOFF) showed the same phenotypes as *T. thermophila* Mob1-KD cells (Fig. 19B,D–G), clearly demonstrating that the phenotypes result from low levels or absence of Mob1. In abnormally dividing cells, low levels of Mob1–GFP could be seen in old posterior poles. In addition, the abnormal establishment of the division plane and failure of cytokinesis clearly correlated with the absence or trace levels of Mob1–GFP at the oral apparatus and cell midzone where the cleavage furrow should be formed (Fig. 19D–G).

Whenever ShuttOFF cells showed some Mob1–GFP at the equatorial region, cell division seemed to occur symmetrically (Fig. 19D). These results strongly reinforce the idea that the polarized distribution of *T. thermophila* Mob1 plays a critical role in the definition of the division plane and, consequently, in cytokinesis.

A



B

Table 1

	WT (Cu428)	TtMob1-KD	TtMob1-ShuttON/OFF strain ON With Cadmium	TtMob1-ShuttON/OFF strain OFF Without Cadmium
Normal looking	% 98,6	65,8	% 99,5	45
Heart shape(early)	1,4	22	0,5	25,6
Heart shape(late)	0	4,5	0	7,4
Boomerang shape	0	5,6	0	18,4
Monsters	0	2,1	0	3,6
Number of Cells	**722**	**586**	**824**	**718**

Phenotypic classes

Figure 20 - *Tetrahymena thermophila* Mob1-KD phenotype rescue in a Mob1–GFP ShuttON/OFF strain. (A) Left: RT-PCR analysis of mob1-GFP expression in *T. thermophila* Mob1–GFP ShuttON/OFF cells grown in the absence (−) or presence (+) of Cd2+. Right: Western blot (WB) of total proteins obtained from Mob1–GFP ShuttON/OFF cells under the same conditions. Both mRNA and protein levels of Mob1 are decreased in the absence of Cd2+, which supports the efficiency of the ShuttON/OFF strategy. (B) Frequency of Mob1 depletion phenotypes in *T. thermophila* wild-type (Cu428 strain), Mob1-KD, Mob1–GFP ShuttON and Mob1–GFP ShuttOFF cells. (C–G) Immunofluorescence microscopy of *T. thermophila* Mob1–GFP ShuttON/OFF cells with anti-α-tubulin and anti-GFP antibodies.

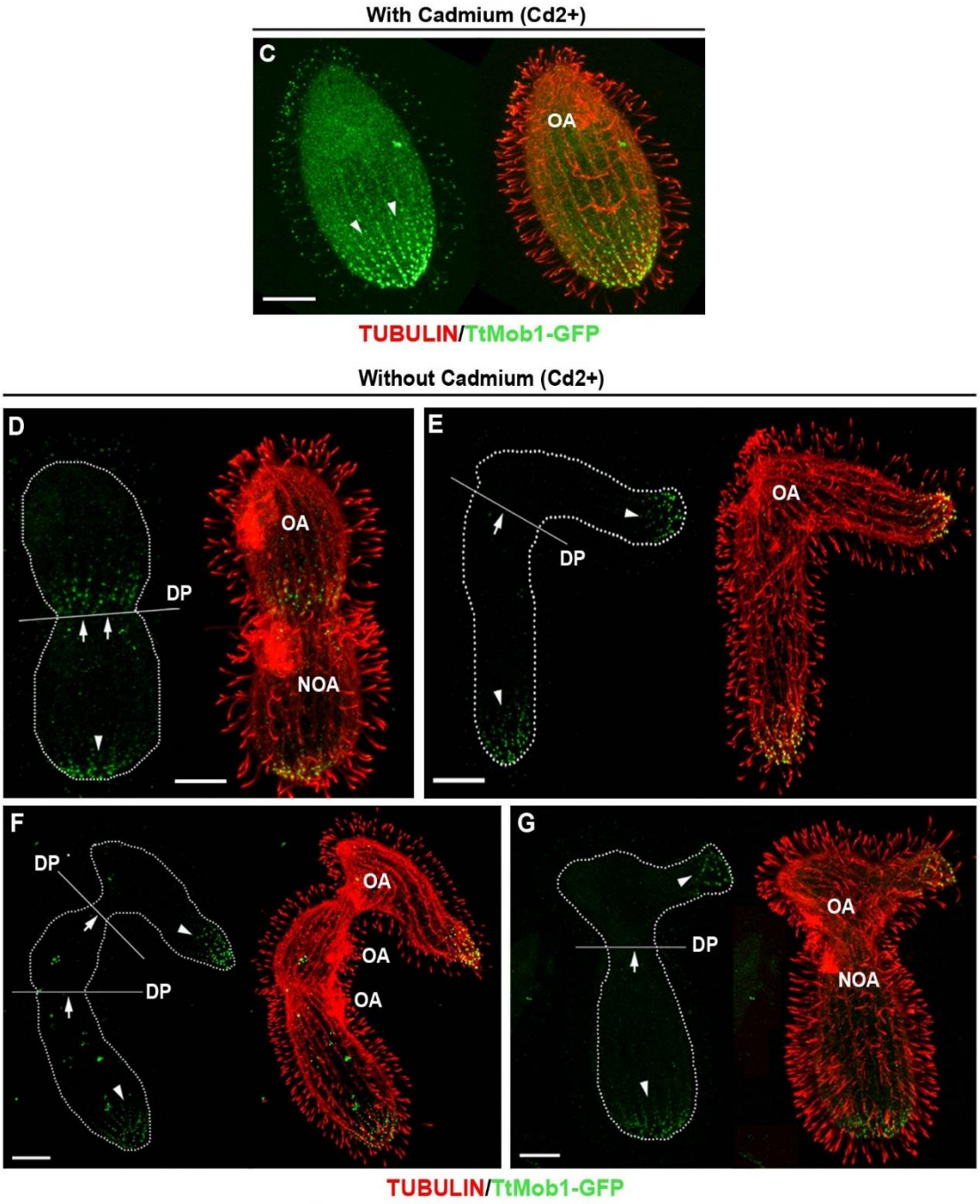


Figure 19 (contin) - (C) In the presence of Cd2+, Mob1–GFP is expressed, suppresses the Mob1-KD phenotypes, resulting in cells that are phenotypically indistinguishable from Mob1–GFP-expressing cells. (D–G) *T. thermophila* cells grown for 18 hours in Cd2+-depleted medium show Mob1-KD phenotypes, including normal-looking cells (D). Mob1 levels are almost inexistent at the division plane (DP) (arrows), indicating a requirement of the protein for correct establishment of the cleavage furrow. At the posterior pole, Mob1–GFP levels are also decreased (arrowheads). Scale bars: 10 μm.

5. Discussion

Because *T. thermophila* is a permanently polarized unicellular protozoa that divides symmetrically, it is an attractive model for investigating how intrinsic cell polarity is related to symmetric cell division. Here we present the first studies concerning the function of the unique *T. thermophila* Mob1-encoding gene (*mob1*) that presents 61% identity with the human counterpart, MOB1, which is a member of the MEN cascade.

Strikingly, *T. thermophila* Mob1 accumulates in the posterior pole basal bodies, creating a gradient through the anterior–posterior axis. Moreover, during cell division *T. thermophila* Mob1 is also recruited to the basal bodies at the cell equatorial zone where the cleavage furrow will be formed. To our knowledge, this is the first molecular polarity marker of the posterior pole localized at the posterior pole basal bodies so far described in *T. thermophila*. This specific polarized localization of *T. thermophila* Mob1 points to the importance of basal bodies possessing distinct compositions, creating specialized landmarks inside a single-celled organism. In fact, the presence of cortical gradients in *T. thermophila* was previously postulated based on the spatial patterns of basal body proliferation that precede cleavage furrow constriction (Kaczanowski, 1978; Kaczanowska et al., 1999). In addition, studies of structural pattern mutants (Joseph Frankel, 2008) and on fenestrin, which localizes below the fission line and accumulates at the anterior region in dividing cells (Nelsen, Williams, Yi, Knaak, & Frankel, 1994); (Cole et al., 2008), indicate the existence of an asymmetry in the cleavage furrow region.

The observed phenotypes in the *T. thermophila* Mob1-KD cells and Mob1–GFP ShuttON/OFF strain clearly show that the Mob1 gradient throughout the anterior–posterior axis is essential for maintaining cell polarity, which is crucial for the establishment of the cell division plane (see Fig. 14). As described, *T. thermophila* cells lacking Mob1 at the constriction region present dramatically altered orientations of the division planes. In these cells, cytokinesis aborts, although the cells continue trying to divide and generate giant monsters with incorrect polarity axes. In yeast, Mob1 mutants also arrest in late anaphase but continue proliferation after cytokinesis failure and undergo several mitotic cycles, resulting in cell chains (Luca et al., 2001). Impaired cytokinesis was likewise described in both *Trypanosoma brucei* and human MOB1-depleted cells, which suggests a conserved role for Mob1 throughout the eukaryotic lineage (Hammarton et al., 2005; Florindo et al., 2012).

Although the basal body localization of *T. thermophila* Mob1 and its recruitment to the constriction zone in dividing cells parallels the yeast spindle pole body localization and recruitment to the neck bud (Francis C. Luca et al., 2001; Visintin & Amon, 2001), mutations in yeast Mob1 do not affect the spindle orientation, showing that Mob1 is only required for cytokinesis and mitotic exit (Luca et al., 2001). Interestingly, in yeast, MEN is only activated when a set of proteins, like Tem1 and Bub2/Bfa1 (SPOC pathway), are asymmetrically distributed in the two spindle pole bodies such that they are more abundant at the one that will migrate to the bud (Caydasi et al., 2010). This suggests that MEN is under the control of polarity factors. Our results show that the *T. thermophila* Mob1 is itself a polarity factor required for the establishment of the polarity axis and consequently for the correct orientation of the division plane and successful cytokinesis.

The differences between *T. thermophila* and *S. cerevisiae* could be related to the fact that yeast possesses two distinct Mob genes (*mob1* and *mob2*). *T. thermophila* Mob1 presents 49% and 42% amino acid sequence identity with yeast Mob1p and Mob2p proteins, respectively. Interestingly, in yeast, Mob2p localizes at the growing bud tip and is required for polarized cell growth and activation of daughter-specific genes necessary for cell separation after cytokinesis (Colman-Lerner et al., 2001); (Weiss et al., 2002). Furthermore, in fission yeast, Mob2p is required for coordinated polarized growth with the onset of mitosis (Hou et al., 2003), and for the maintenance of polarisome components at hyphal tips in *Candida albicans* (Gutiérrez-Escribano et al., 2011). In neuronal mouse cells, Mob2 localizes at the base of the protrusive neurite, at the branching point and at the tip of the neuritis, promoting neuritis differentiation (Lin, Hsieh, & Fan, 2001). These data clearly show that Mob2 is a key factor for cell polarity establishment. At a first glance it seems that the *T. thermophila* Mob1 fulfills all the Mob protein functions. If this is true, it is tempting to speculate that the duplications of Mob-encoding genes throughout the eukaryote lineage lead to the distribution of specific functions of the ancestral gene throughout the distinct members of the gene family. However, this analysis might be simplistic. In humans, MOB1 localizes at the centrosome, at the spindle midzone and midbody (Wilmeth et al., 2010; Florindo et al., 2012). It is also worth mentioning that at the end of mitosis Mob1 is detected only in the centriole that moves closer to the midbody (Florindo et al., 2012), which is probably the mother centriole. The asymmetry of mother and daughter centrioles in a centrosome, and consequently between duplicated centrosomes, is also a crucial feature of asymmetric cell divisions (Yamashita, Mahowald, Perlin, & Fuller,

2007). Interestingly, it was recently demonstrated that proteins related to cell and tissue polarity associate asymmetrically with the mother centriole (Jakobsen et al., 2011). Therefore, it is tempting to suggest that the role of Mob1 in *T. thermophila* could also be replicated in metazoan cells.

Collectively, our data clearly supports the view that *T. thermophila* Mob1 is a key factor for the establishment of intrinsic cell polarity and this is probably its most ancient role. Moreover, our results also show that Mob1 is involved in cilia biogenesis because its depletion delays cilia recovery, which is well correlated with the fact that *T. thermophila mob1* is upregulated in response to cilia regeneration. Finally, we wonder whether tumor formation in Mob1 mutants (Lai et al., 2005) is related to possible alterations in cell polarity. In fact, several studies implicate cell polarity pathways in tumor formation and progression, as in the case of the human polarity protein LKB1 (PAR-4 ortholog) whose mutations cause the Peutz–Jeghers Syndrome, which is characterized by benign hematomas and a high frequency of carcinomas (Baas et al., 2004). Our work, therefore, brings new clues about how cell polarity is linked with accurate cell division and cytokinesis and how it can be related to cancer.

CHAPTER III: MOB1 IN THE APICOMPLEXAN PARASITE *Toxoplasma gondii*

PART 1: INTRODUCTION

1. The *Toxoplasma gondii* – an apicomplexan parasite

T. gondii is an obligate intracellular eukaryotic parasite of the phylum Apicomplexa. The phylum Apicomplexa belongs to the Alveolata group together with the Dinoflagelates and the Ciliates (Burki, 2014) (Fig. 20).

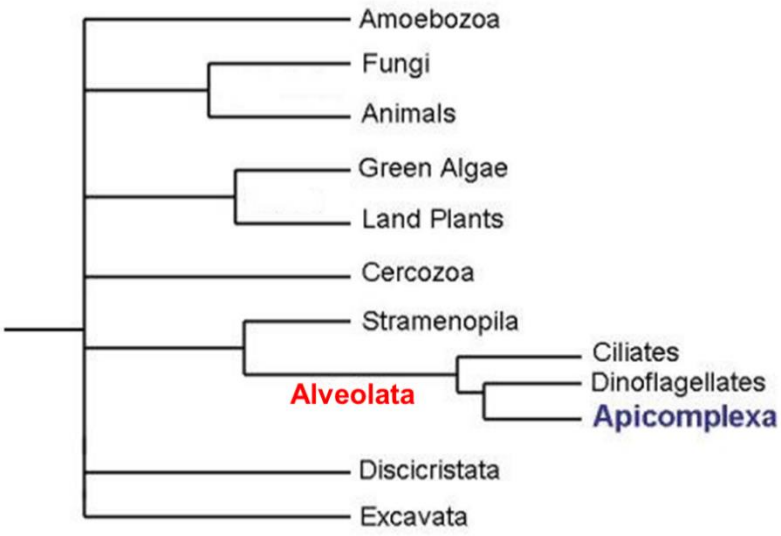


Figure 21 - The Alveolata monophyletic group is composed by several different species of single-celled eukaryotes that present very diverse and distinct life styles. The Ciliates, Dinoflagellates and Apicomplexa are the phyla that constitute this group. Adapted from http://bioweb.uwlax.edu/bio203/s2008/parks_chri/Classification.htm

All the members of the phylum Apicomplexa share an evolutionary unique structure in the anterior pole of the cell, the apical complex, which is involved in several processes of the parasites' life, such as host cell invasion and proliferation. Despite the presence of the apical complex and the parasitic life style, the biodiversity within this group is huge (Morrison, 2009). Apicomplexa parasites, that evolved from a free-living photosynthetic ancestor and are closely related to free living single-cell predators are divided in four clearly distinct groups: the coccidians, the hematozoa, the cryptosporidia and the gregarines (Šlapeta & Morin-Adeline, 2011). Besides *T. gondii*, the phylum Apicomplexa also encompasses many others coccidian parasites such as those of the genera *Neospora*, *Besnoitia*, *Sarcocystis* and *Eimeria*; hematozoan parasites in which are included the genera *Plasmodium*, *Theileria and Babesia*, and the cryptosporidian

genera *Cryptosporidium*, among others. All of these genera are responsible for important animal and human diseases.

Plasmodium parasites are the causative agent of malaria, a disease responsible for severe human life losses, being estimated that during 2013 more that 198 million people were infected, mainly in African countries (World Health Organization, 2014). Regarding *Neospora*, parasites of this genus are a major cause of abortion in cattle, being also a significant cause of abortion in sheep and goats (Dubey & Schares, 2011). *Besnoitia besnoiti* is a cattle parasite that also may lead to abortion in females infected during pregnancy, and male infertility in chronic infections. In the acute phase of the disease, the infected animals present clinical signs such as respiratory disorders, loss of body weight, loss of milk production and generalized decline of fitness. Although not lethal to adult animals in the majority of the cases, besnoitiosis is responsible for significant economic losses in cattle production (Cortes, Leitão, Gottstein & Hemphill, 2014). Parasites of the genus *Babesia* provoke severe disease in cattle. Bovine babesiosis is transmitted by ticks and also in this case, the economic losses associated with the disease are linked to loss of milk and meat production, abortion and mortality and, as expected, cattle trading limitations (Schnittger, Rodriguez, Florin-Christensen & Morrison, 2012). To illustrate another example of this complex group of parasites, it is worth mentioning *Cryptosporidium*, enteric parasites that are transmitted by the consumption of contaminated water. *Cryprotsporidium* species can affect both animals and humans and cause severe diarrhea and vomits among other clinical signs. It is estimated that cryptosporidiosis is the second major cause of diarrhea and death in children under 5 years old (Ryan & Hijjawi, 2015). Apart from the diversity that Apicomplexa present, being indicated here only some examples of the group, the present work was focused on the parasite *T. gondii*, which is going to be presented further in more detail.

T. gondii is considered an opportunistic parasite that has the ability to infect all warm blooded animals. However, these parasites only can reproduce sexually inside felines, most importantly in the domestic cat, which are the definitive hosts (Montoya & Liesenfeld, 2004). *T. gondii* is the etiological agent of toxoplasmosis and is transmitted to humans mainly by the consumption or handling of infected meet (namely pork and lamb) and also by the consumption of water contaminated with oocysts released from infected cats (Montoya & Liesenfeld, 2004). *T. gondii* infections are mostly asymptomatic in immunocompetent individuals, however, in patients with compromised

immunity, such as AIDS patients, the parasite can lead to severe problems that may culminate in death. Beyond this, toxoplasmosis is also an important congenital disease. The effects of congenital transmission are devastating leading to mental retardation, visual and hearing problems in the newborns or even abortion during gestation (Weiss & Dubey, 2009). These symptoms are also observed in animals, namely in domestic animals and in sheep and pigs, where *T. gondii* infection is a significant cause of abortion, especially in sheep (Hill & Dubey, 2013). Although toxoplasmosis is an important zoonosis, our understanding of the disease is still sparse, namely in respect to understanding the mechanisms of parasite biology and infection. In fact, the cellular organization, cell cycle and cell proliferation control of this parasite are deeply complex and full comprehension is far from being achieved. Also, effective therapies to control this disease are still missing. Taking this into account, the study of the mentioned aspects of parasite biology are required and will contribute to our understanding of its mode of action and will eventually lead to effective ways of prevention and treatment.

In the next sections, some of the present knowledge of *T. gondii* biology will be presented namely regarding its life cycle, cell structure and infection biology.

1.1. The life cycle of *Toxoplasma gondii*

T. gondii, presents different cell stages that are linked to specific life cycle stages: the tachyzoites, the bradyzoites (inside tissue cysts), gametes and the consequent sporozoites (inside the oocysts). This parasite presents both asexual (tachyzoites and bradyzoites) and sexual replication (gametes that origin sporozoites), that change according to the host organism. *T. gondii* only undergoes sexual reproduction in feline hosts, but it can infect virtually all warm-blooded animals (Fig. 21) (for review see (Hill, Chirukandoth, & Dubey, 2005)).

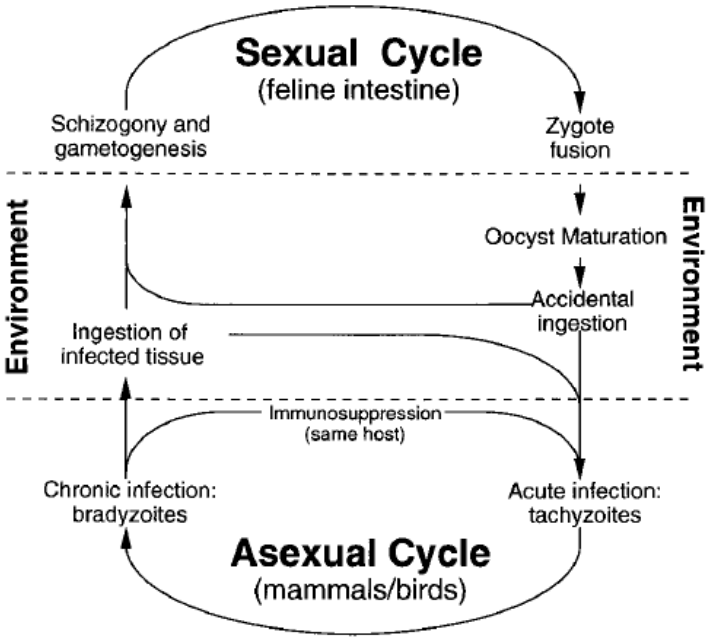


Figure 22 - *Toxoplasma gondii* life cycle. The sexual reprodution of *T. gondii* only occurs in the feline intestine that shed the oocysts to the enviroment where they mature and can be mantained for large periods of time. When ingested, the sporozoits are released from the oocysts and, when inside the new host and during the accute stage of infection are called tachyzoites. This tachyzoite active form, either by the pressure of the host immune system or by some intrinsic parasite features, is then converted to the bradyzoite stage, that establish cysts in the host´s different tissues. Upon a descrease in host immune defenses, the infection can be reactivated and bradyzoites can convert to the tachyzoite form again (adapted from (Black & Boothroyd, 2000)).

Briefly, after the ingestion of infected tissues by the felines, the cyst wall suffers proteolisis by the felines' digestive enzymes which leads to the release of the bradyzoites. These bradyzoites then penetrate the cell membranes of feline epithelial tissues and go through numerous generations of sexual and asexual cycles. The replication rate of *T. gondii* in the felines' intestine is very high and gives origin to the merozoites that produce male and female gametes. The male gamete is bi-flagelated which allows it to swim toward the female one and then fertilization occurs. After fertilization, the oocyst wall starts to be formed around the zygote. Later, mature oocysts provoque the lysis of intestine epithelial cells and are released to the intestinal lumen, and ultimately to the enviroment through the feces. Finally, they sporulate in the environment and become infective (Montoya & Liesenfeld, 2004).

The released oocysts can then be ingested by other animals (birds/mammals), in contaminated water or food. The oocyst wall is degraded by the proteolytic enzymes of the digestive tract and the parasites are released inside the new host. The parasites at this specific stage are called tachyzoites and constitute the most active form of infection. After the acute period of infection, the pressure of the host immune system and likely some intrinsic mechanims of the parasite, lead to the establishement of a chronic stage of the disease. In this stage, the actively asexualy dividing tachyzoites are converted into bradyzoites, that are much less active and that form cysts in the host. This cysts can be mantained for long periods of time, but an active phase of the disease may arise whenever the host immune system is depressed (for review see (Black & Boothroyd, 2000; Hill et al., 2005)).

Although from a structural point of view bradyzoites and tachyzoites are very close forms of the parasite, they present a different transcriptome and proteome, a reflection of different metabolic rates and behavioural differences (for review see (White, Radke & Radke, 2014)). The present work is focused in the asexual stages of *T. gondii* life cycle and being so, the specificities of the tachyzoites and bradyzoites forms will be adressed in more detail.

1.2. The single cell organism *Toxoplasma gondii* – the complexity of a permanent polarized cell strucuture

As is the case of the ciliate *T. thermophila*, *T.gondii* is also an unicellular organism with a permanently polarized cell in which an anterior-posterior axis is easily recognized. This parasite presents a cell shape that is narrower in the anterior pole where the apical complex is a landmark with a more rounded posterior region where we can find the posterior complex.

1.2.1. The apical complex

In the *T. gondii* anterior pole, some particular structures that confer the apical identity of the cell, can be observed. The most apical region of the cell possesses the apical complex that is formed by the polar rings, the conoid, the rhoptries and the micronemes (Fig. 22). These two last structures form a highly specialized secretory system, crucial for several aspects of the parasite's life, such as host cell invasion and the establishment of the parasitophorous vacuole once inside the host cell (for review see (Souza, 2010)).

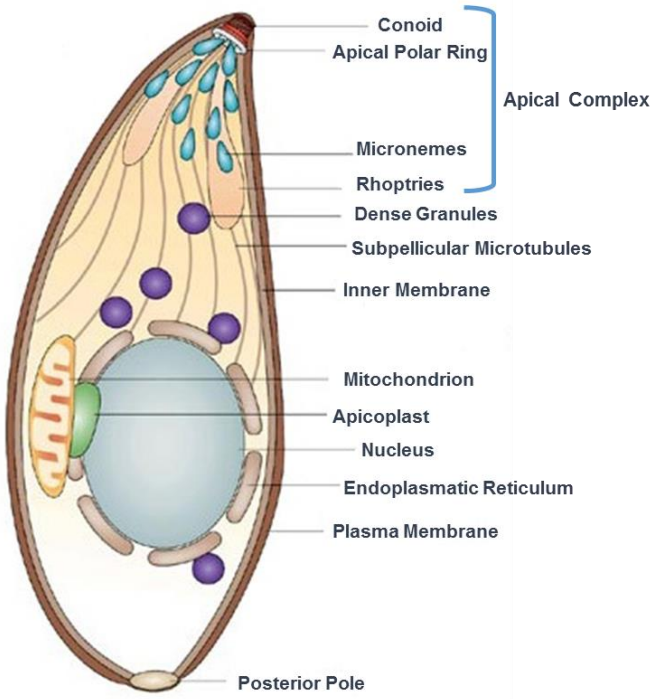


Figure 23 - *Toxoplasma gondii* cell ultra-structure (adapted from (Baum, Papenfuss, Baum, Speed, & Cowman, 2006)).

Besides these Apicomplexa specific secretory structures, also the parasite cytoskeleton, in particular the microtubules, presents a particular organization that is crucial for the cell shape and cell polarity. Thus the subpellicular microtubules (Fig. 22) emerge and radiate from the apical polar ring and extend until the post-nucleus region, being distributed over about two thirds of the cell. This peculiar array of microtubules closely trails the cell shape and confers rigidity to the cell body, typical of the anterior region of the parasite (for review see (Morrissette & Sibley, 2002b)). Apart from this structure, also the conoid is assembled with α/β tubulin dimers. Interestingly, despite the conoid being a microtubule based-structure, it presents an atypical configuration of the microtubule protofilaments that are organized in 9 instead of the typical 13 protofilaments that usually form the wall of the microtubule (Hu, Roos, & Murray, 2002). The conoid is a mobile structure that can be extruded or retracted, which is dependent on actin dynamics and calcium signaling (Mondragon & Frixione, 1996). The exact function of the conoid is still unknown but evidences suggest that this organelle is involved in secretory pathways and in the first steps of host cell invasion (Katris et al., 2014).

1.2.2. The *Toxoplasma gondii* posterior pole

In comparison to the *T. gondii* anterior pole, much less is known regarding the posterior pole composition. To date, most of the proteins described to be present in this region are proteins associated with the cytoskeleton microtubules, such as Tg centrin2, Tg dynein light chain and the TgMorn1 (Membrane Occupation and Recognition Nexus 1) (Hu, 2006); (Heaslip, Dzierszinski, Stein, & Hu, 2010). In *T. gondii*, TgMorn1 forms a ring that occupies the posterior end of the cell, filling the gap left by the interruption of the inner membrane in that region (Hu, 2006). Together, these proteins constitute to the basal complex of the parasite, and TgMorn1 is essential to the recruitment of the other components (Hu, 2008). The basal complex is physically separated from other cytoskeleton structures since in these parasites the cortical microtubules are elongated only to approximately 2/3 of the cell.

This complex is crucial for cytokinesis in *T. gondii*. The structure acts as a contractile ring that enables daughter cell individualization. More recently, two other proteins of the basal complex were identified, the protein TgMsc1, which is a new protein with no known domains and the protein Tg14-3-3 (Lorestani et al., 2012). Interestingly, 14-3-3 proteins are well conserved in eukaryotes and are associated with different signaling pathways in the cell, binding to a plethora of molecules such as kinases and phosphatases (for review see (Gardino & Yaffe, 2011)).

1.2.3. The apicoplast

The apicoplast is a plastid organelle present in most of the parasites from the Apicomplexan group. This organelle had origin in the same evolutionary branch that gave origin to plants and like its plant homologues possesses a DNA chromosome, which confers the parasites three different genomes (nuclear, mitochondrial and apicoplastid). Thus, although some of the plastid proteins are encoded from nuclear genes (for review see (McFadden, 2011)), the apicoplast is a semi-independent organelle whose DNA encodes most of the genes required for its function.

In an evolutionary point of view, it is now established that the apicoplast was acquired by the parasites in an endossymbiotic event that occurred before apicomplexans and dinoflagellates diverged. This hypothesis is supported by the fact that dinoflagellate plastids and apicoplasts share the same ancestral plastid with the

alveolata *Chromera velia*, that harbor a red algal endosymbiont (Moore et al., 2008; Janouškovec, Horák, Oborník, Lukeš, & Keeling, 2010). Despite of the known origin of the apicoplast in Apicomplexa parasites, namely in *T. gondii*, the exact function of this organelle is still a matter of debate. In this discussion, at least one thing is clear: the apicoplast is an essential organelle for parasite survival (Fichera & Roos, 1997).

2. *Toxoplasma gondii* inside the host cell

As an obligatory intracellular parasite, the *T. gondii* only undergoes cell division inside the host cells. After the host cell invasion, a complex process involving several parasite organelles as well as the cytoskeleton, *T. gondii* starts to establish a parasitophorous vacuole inside of which the parasites will proliferate. In this section, some information regarding host cell invasion, the establishment of the parasitophorous vacuole and the parasite replication will be introduced.

2.1. Host cell invasion

In order to invade the host cell, *T. gondii* relies in a very complex secretory system, composed by the micronemes, the rhoptries and the dense granules. *T. gondii* shows a cellular movement, known as gliding, which allows the approach of the parasite to the host cell. The production of microneme proteins (MIC) is essential for this movement and also for the parasite attachment to the host cell. After the first contact is established, several surface proteins are essential for the interaction, as it is the case of a membrane binding protein (PLP-1) that is produced by the parasites. Already in physical contact with the host cell, parasites reorient their position, to allow the apical pole to contact the host cell membrane, which will facilitate and allow host cell penetration. At this point the complex parasitic secretory system comes into action by producing several molecules that are needed for invasion. In fact, after the micronemes proteins, critical for the first steps of host cell approaching, the rhoptries produce a huge amount of molecules that are localized in the parasite-host cell interface. These molecules are also released into the host cell cytoplasm and will take part in the establishment of parasitophorous vacuole. The first rhoptry proteins produced come from the rhoptry neck and are broadly known as RONs. These molecules are initially secreted to the host membrane where they are key players in the formation of the

moving junction, established between both cells. In concrete, the moving junction is composed by the rhoptries originated proteins RON2, RON4 and RON5 and also by the micronemal protein AMA1 (Besteiro, Dubremetz & Lebrun, 2011); (Shen & Sibley, 2012). Posteriorly, the rhoptry bulb starts to segregate other type of proteins, known as ROPs that will be localized in the parasite vacuole surface once inside the cell (Boothroyd & Dubremetz, 2008); (Bradley & Sibley, 2007).

2.1.1. Cytoskeleton role in invasion

The cytoskeleton dynamics of *T. gondii* play a major role in the host cell invasion. In fact, the gliding movement of the parasite, which allows host cell approach and invasion, is maintained by an actomyosin motor complex (actin ACT1 and myosin A MyoA) and two glideosome associated proteins (GAP45 and GAP50), that couple the glideosome machinery to the inner membrane complex (Frénal, Marq, Jacot, Polonais, & Soldati-Favre, 2014). In order to move and invade the host cell, the parasite's actin cytoskeleton has to be highly dynamic, presenting a high turnover of the filaments. In fact, the knockout of the *T. gondii* actin depolymerizing factor (TgADF), which leads to more stable actin filaments, presents reduced movement and severe defects in invasion and egress (Mehta & Sibley, 2011). Interestingly, even though this actomyosin based system is critical for the movement, a recent study suggested that the parasites probably have a non-canonical system that can substitute the Act1-MyoA based movement. The conditional knockout of the MyoA protein does not impair invasion, which support the view that *T. gondii* is capable of using an Actin/MyoA/MIC2-independent mechanism to invade the host cell (Andenmatten, 2013). Supporting this idea, is the fact that *T. gondii* parasites possess two independent glideosome machineries, based on the MyoA and MyoC proteins that can compensate for each other to ensure parasite survival (Frénal et al., 2014).

Regardless of the fundamental role of the actin/myosin cytoskeleton in the process of invasion, the tubulin cytoskeleton also plays a critical role. Recently, it was shown that the spiral movement of gliding parasites relies on the subpelicullar microtubules, in an interdependent relation with the actomyosin motor, in a not understood way (Leung et al., 2014). The parasite *T. gondii* has two distinct sub-populations of microtubules: the subpelicullar set (that are nucleated and organized by the apical polar ring) and the spindle microtubules (nucleated by the centrosome). These two microtubule sub-

populations respond differently to microtubule polymerizing/depolymerizing drugs and present independent activities. Indeed, after the treatment of the parasites with oryzalin they can still assemble short microtubules and undergo nuclear division, but fail to re-invade host cells because of the dysfunctional subpelicullar microtubules (Morrissette & Sibley, 2002b).

Despite the complexity of the invasion process, that involves several organelles and the dynamic cytoskeleton, this occurs very rapidly, within a range of few minutes.

2.2. *Toxoplasma gondii* establishment and replication inside the host cell

2.2.1. The parasitophorous vacuole

Already in the first steps of host cell invasion, the parasite establishes its own semi-independent environment, inside the parasitophorous vacuole. This environment will allow the parasite to undergo active proliferation. For those organisms that establish a vacuole inside the invaded host cells, its more fundamental role is the protection against acidification and hydrolysis by the lysosomes of host cells (Mordue, Håkansson, Niesman, & Sibley, 1999). Some of the biological material that constitutes the vacuole membrane originates from the host cell membrane. Being so, how can this structure be resistant to the cell's lytic machinery? In fact, during invasion and during the establishment of the parasite vacuole, *T. gondii* secretory system (rhoptries, micronemes and the dense granules) secrete molecules that are incorporated in the PV and alter its internal composition (for review see (Souza, 2010)). Besides the protective function, the parasitophorous vacuole membrane also allows the importation of nutrients from the cytoplasm of the host cell into the vacuole lumen, to be used in the parasite's metabolism. In fact, the parasitophorous vacuole membrane is now seen as an extracellular organelle of the *T. gondii* and of other apicomplexan parasites. It constitutes the interface between the parasites and the host, and it is critical for the maintenance of the vacuole shape, the parasite metabolism and the manipulation of several host organelles that are recruited to the proximity of the vacuole in benefit of the parasite (Sinai, 2008).

2.2.2. *Toxoplasma gondii* Replication

After *T. gondii* invades the host cell, the parasites, safe inside the parasitophorous vacuole, may start the process of replication. The Apicomplexan parasites present a multiplicity of cell division strategies that differ from metazoan cells. For the purpose of this work's introduction we will only focus in the particular case of *T. gondii*.

T. gondii divides by endodyogeny, a process in which each DNA replication cycle is followed by nuclear division and budding (Fig. 23). In *T. gondii*, the cell cycle is divided mainly into three steps, G1, S and M, with a very brief or almost inexistent stop in G2 (Radke, 2001). Usually, in each parasitophorous vacuole, where *T. gondii* is in active proliferation, the number of parasites per vacuole is a multiple of 2, since division is synchronous and each mother cell gives origin to two daughters which form inside the mother. The number of parasites indicates the number of cell divisions that they undergone since entering the host (Hu, 2002).

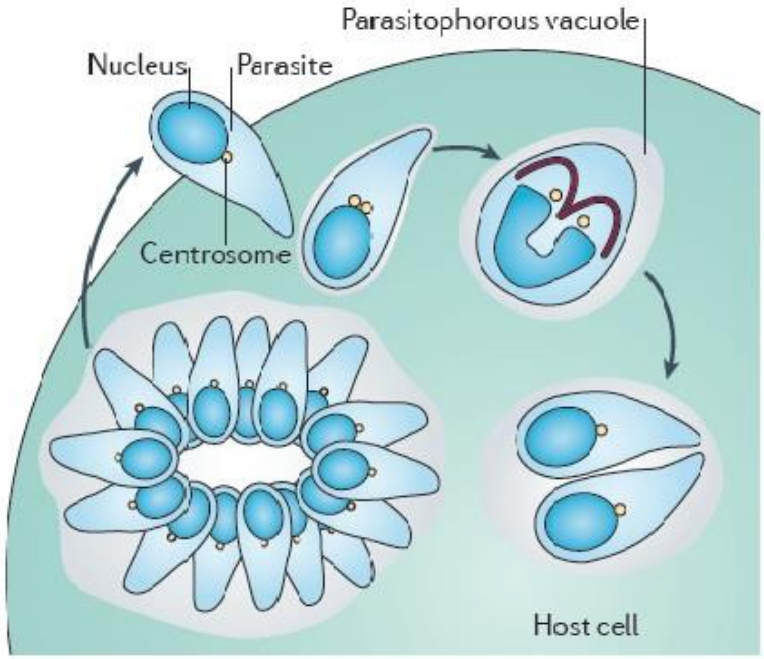


Figure 24 - The replication cycle of *Toxoplasma gondii* occurs by endodyogeny. In this particular mode of apicomplexan replication, DNA replication is always followed by nuclear division and budding. The newly formed daughter cells are assembled inside the mother cell that disappears in the end of the budding (adapted from (Francia & Striepen, 2014)).

Other particular aspect of *T. gondii* cell division, which differs from the typical cell division of the host, is that the parasite presents a closed mitosis. In this case, the nuclear envelope is not disassembled during division. Actually, in interphase, the nucleus of these parasites is highly organized, with the centromeres of the chromosomes clustering in the nuclear periphery in the place where the centrosome is localized. In this region, there is an opening of the nuclear envelope, where on one side is localized the centrosome, and on the other side the endoplasmic reticulum. It is exactly in this region that a tunnel structure will be formed where, during mitosis, the spindle microtubules enter to allow DNA segregation by connecting to the centromeres. This tunnel structure also defines the budding plane. It was recently hypothesized that the clustering of the centromeres next to the region where the centrosome is localized may facilitate the connection between kinetochores and spindle microtubules during division in a nucleus with a huge amount of uncondensed chromatin (Francia & Striepen, 2014).

Similarly to mammalian cells, the centrosome cycle in *T. gondii* is also tightly coordinated with cell division. The centrosome duplication marks the transition between G1 and S phase but contrary to what happens in the case of mammalian cells, the knowledge about centrosome cycle in apicomplexa is sparse. Nevertheless, in *T. gondii*, it was shown that the Nek1 kinase, homologous of Nek2 (NIMA, never in mitosis, related proteins), is essential for centrosome replication. Interestingly, the failure in centrosome splitting in *T. gondii*, gives rise to only one daughter cell, impairing the budding (Chen & Gubbels, 2013). Also the apicoplast, divides in association with the centrosome and its division is impaired upon the disruption of the microtubule network (Striepen, 2000). This clearly demonstrates the importance of the centrosome in *T. gondii* replication.

2.2.2.1. Cytokinesis in *Toxoplasma gondii*

Since apicomplexan parasites divide by budding, the course of cytokinesis differs substantially from the typical cytokinesis in other metazoan cells. In apicomplexans, the beginning of the assembly of the daughter cells which is the beginning of cytokinesis, starts very early in cell division after centrosome duplication, i.e. in the beginning of S phase. This means that the coordination of cell cycle with cytokinesis is differently controlled in the parasites. The shape of the daughter cells in formation is determined by their inner membrane and also by the subpellicular microtubules that are nucleated by the apical polar ring. Each of the daughter cell possesses a complete set of the apical

and other organelles (Morrissette & Sibley, 2002a). Inside the mother parasite, the new apical polar rings and the new subpelicullar microtubules present the same orientation as the old parasite, implying that the budding occurs in a plane that is coincident with the anterior/posterior axis of the mother. Therefore, as is the case of the ciliate *T. thermophila*, cell structure and polarity is inherited (Fig. 24).

As already mentioned, the basal end of the parasite also plays a role in cytokinesis, mainly in the final events of the daughter's individualization process. One of the critical proteins for basal complex assembly is TgMorn1. Parasites lacking TgMorn1 present impaired daughter separation which results in double headed parasites, supporting that successful completion of the budding is not required for cell cycle progression. In fact, these mutants could undergo several rounds of replication, resulting in parasites with multiple apical structures. In this case, the contractile nature of the basal complex was abolished (Lorestani, 2010).

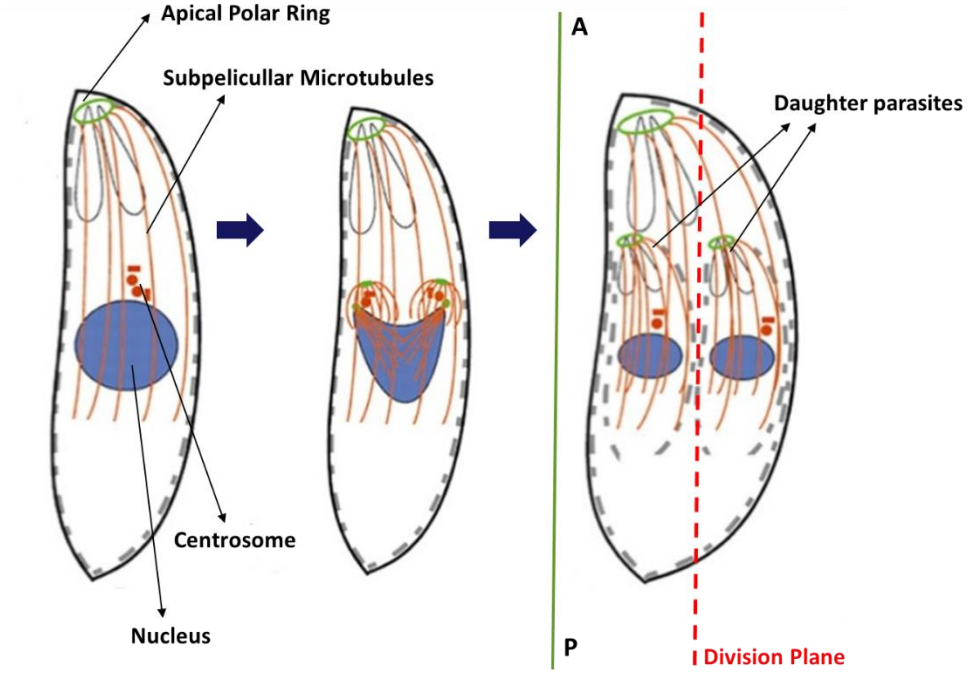


Figure 25 - *Toxoplasma gondii* division and budding. The two daughter parasites (each possessing a set of apical organelles) are enclosed by individual inner membrane complexes and associated subpellicular microtubules that are nucleated by the apical polar ring. The anterior/posterior (A-P) axis of this permanently polarized cell is coincident with the cell division plane (adapted from (Morrissette & Sibley, 2002a)).

The microtubule lattice of the budding daughters also interacts with the inner complex membrane (IMC) and the outermost part of the parasite, the cytoplasmic membrane that is acquired from the mother cell. This network also anchors other organelles such as the secretory system and the gliding machinery, which will allow the full independence of the daughter parasites (for review see (Francia & Striepen, 2014)).

The parasites can undergo several rounds of replication inside the host cell until the moment of egress. When the host cell is not able to sustain the infection anymore, the parasites provoke the lysis of the host, are released to the extracellular environment and invade the neighboring cells. *In vivo*, this active proliferation of the tachyzoites occurs in the acute phase of the infection. At a certain point, environmental properties of the host cell and/or intrinsic changes on the parasites, lead to the stage conversion of the parasites from tachyzoites to bradyzoites. Nevertheless, the molecular mechanisms underlying this transition are not still fully understood.

3. Proliferation control in *Toxoplasma gondii* – the ability of stage conversion

Like other apicomplexan parasites, *T. gondii* relies on different developmental stages in order to maintain a sustainable infection inside the host. In this context, the change from the rapid replicative form of tachyzoites to the cyst-forming bradyzoites constitutes a critical ongoing area of research. As already mentioned briefly above, the parasites can interchange between these two stages depending on host environmental conditions, and some of the parasite steps that are involved in the regulation of this process were already described. Nevertheless much more is yet to be discovered, particularly at the molecular level.

A broad study of *T. gondii's* transcriptome done in 2005 (Radke et al., 2005) showed that gene expression in *T. gondii*, as it is the case for the parasite *Plasmodium falciparum*, is tightly regulated in a stage dependent manner, being some of the genes only expressed in certain stages of parasite development. As expected, the stage conversion is deeply associated with the cell cycle, and one of the first observations during transition is a slowing down of the cell cycle, with an increased population of cells in the G2 phase, which is almost absent in the tachyzoite stage. These differentiating parasites may continue the replication process until the conversion is

complete, time when they enter a quiescent mode in G0 (Radke, Guerini, Jerome & White, 2003).

In the first investigation of the stage conversion in *T. gondii* at the molecular level, BAG1 protein was identified as a bradyzoite specific marker. Interestingly, this protein belongs to the family of small heat shock proteins, which already suggested that bradyzoite transformation could be a consequence of stress response (Bohne, Gross, Ferguson, & Heesemann, 1995). Accordingly, one of the best established ways to convert tachyzoites into bradyzoites *in vitro*, consists on changing the pH of the medium (Soete, Camus, & Dubrametz, 1994). Despite being a specific bradyzoite marker, BAG1 is not necessary for the conversion, appearing only after 2-3 days post-conversion (Bohne et al., 1998). Since the transition in *T. gondii* may be induced by exogenous stress, it was expectable that signaling pathways involved in stress induced differentiation in other organisms, such as the cyclic nucleotide-dependent ones (cGMP, cAMP and respective dependent protein kinases G and A, PKG and PKA), could also play a role here. In fact, the inhibition of cAMP and cGMP dependent kinases leads to parasites differentiation (Eaton, Weiss, & Kim, 2006). Also the eukaryotic initiation factor-2 (eIF2), another molecule involved in stress response in eukaryotes, was implicated in tachyzoite to bradyzoite conversion in Apicomplexa, namely by reducing translation overall (Sullivan, Narasimhan, Bhatti, & Wek, 2004).

More recently, it was also shown that apicomplexan parasites possess transcription factors that are homologous of the plant AP2 and participate in developmental and stress response. This ApiAP2 transcription factor is overexpressed at the bradyzoites stage and ApiAP2 knockout parasites do not undergo alkaline induced differentiation (Walker et al., 2013). Most of the studies done to date regarding *T. gondii* stage differentiation were focused on molecules that were involved in stress response in other eukaryotes. Although it is undeniable that stress is a major feature in this process, the hypothesis that other proteins controlling cell proliferation/apoptosis in eukaryotes may also be involved should not be discarded. As stated before, Mob1 proteins are critical players in cell proliferation control in several eukaryotes and were already investigated in metazoans. In protozoa, our work in *T. thermophila* (Tavares et al., 2012) and, more recently, work developed by others in *Stentor coeruleus* (Slabodnick et al., 2014) have shown that Mob1 accounts for a mechanistic link between cytokinesis and morphogenesis. In both ciliates, Mob1 cellular localization and its depletion showed that Mob1 is essential for the maintenance and regeneration of cell polarity, correct placement of the division plane and for cytokinesis. Thus, these two

studies have highlighted that in a unicellular organism Mob1 is a global patterning protein that is required for proper development and regeneration (Chalker & Frankel, 2014).

These studies, together with that of *T. brucei*, were the first demonstrating the critical role of Mob1 in protozoa. These data and that coming from other eukaryotes clearly suggest that Mob1 may also be a critical factor in the control of stage transition and replication of *T. gondii* and other cyst-forming parasites. In fact Mob1 may also be the missing link between morphogenesis and the control of proliferation integrating information coming from environment. This in *T. gondii* may be translated "as a critical factor to regulate sustained infection in the host". Therefore this prompted us to investigate the role of Mob1 in *T. gondii* which results are described in the next section of this chapter.

PART 2: MOB1 IS A CRITICAL PLAYER IN TOXOPLASMA GONDII REPLICATION

1. Abstract

T. gondii, an obligate intracellular protozoa parasite, is a serious animal health problem and a critical issue for food safety, being a major cause of congenital neurological defects. T. *gondii* presents two inter-converting developmental stages: rapidly growing tachyzoites and slowly replicating bradyzoites that form cysts. This inter-conversion ability and parasite proliferation rate are critical for the course of the disease. Although host immunity plays a major role in stage conversion, recent findings showing that protozoa parasites possess cell death markers led to the discussion that parasites must also control their number in order to maintain a sustainable infection. Therefore, molecules involved in cell cycle, proliferation and death are excellent candidates to be regulators of parasite number. Mob1 is key component of the Hippo pathway, a signaling cascade controlling cell proliferation/cell death in mammals, and the mitotic exit network, which controls the anaphase to interphase transition during cell division in yeast. In protozoan, there are only two studies on Mob1. One in *T. brucei*, demonstrating that Mob1 depleted parasites show inaccurate cytokinesis, and our studies in the ciliate *T. thermophila*. *T. thermophila* Mob1 depletion leads to the incorrect positioning of the division plan, polarity defects and impaired cytokinesis. Here we show that *T. gondii* also possess only one mob1 gene, that is phylogenetically very close to Mob1 proteins of other Apicomplexa parasites. In *T. gondii*, Mob1 presents a polarized subcellular localization, being concentrated mainly in the posterior pole of the cell, where it is localized the basal complex. Importantly, *mob1* gene expression levels decreases when parasites initiate replication inside host cells and Mob1 overexpression decreases the parasite proliferation rate. Altogether our results clearly show that Mob1 is a critical player in the regulation of cell division and proliferation in the parasite *T. gondi,* processes with direct implications in infection control.

2. Introduction

T. gondii, an obligate intracellular protozoan parasite of the phylum Apicomplexa, is a serious animal health problem and a critical problem in terms of food safety. In humans, it is the most common cause of congenital neurological defects and a devastating opportunistic infection in immuno-compromised patients (Montoya & Liesenfeld, 2004). This parasite presents a complex cell cycle with major implications in invasion and prevalence within the hosts. *T. gondii* infection most likely occurs by contact with oocysts containing sporozoites present in cat´s feces, or by the ingestion of contaminated meat or water, containing bradyzoites, the parasite's latent form. Inside the host organism, sporozoites and bradyzoites rapidly differentiate into the actively growing form of the parasite, the tachyzoites, which undergo fast replication until the response of the host immune system eventually kills the majority of the parasites. This response leads to alterations in the environmental conditions inside the host, which probably trigger the conversion of the surviving parasites into bradyzoites (Skariah, McIntyre & Mordue, 2010); (Sullivan & Jeffers, 2012). This conversion allows *T. gondii* to evade from the host immune system and doing so, to maintain a latent infection.

Although the molecular mechanisms of tachyzoite to bradyzoite conversion are far from being understood, the presence of cyclin-dependent kinases, the APC degradation machinery and of other cell division regulators in *T. gondii* support that at least some of the control mechanism in this parasite are conserved among other eukaryotes (White et al., 2014). Being so, proteins involved in cell division control are excellent candidates to regulate stage conversion in *T. gondii*.

One of the major pathways controlling cell proliferation in higher eukaryotes is the Hippo pathway. The core proteins of this phosphorylation cascade, which is conserved between *D. melanogaster* and mammals, are Mob1, Sav1 and the nuclear Dbf2-related (NDR) kinases, LATS1/2 and MST1/2. During cell proliferation the Hippo pathway is activated and its core kinases negatively regulate, by phosphorylation, the transcription factor YAP, an activator of pro-mitotic genes (Zhao, Tumaneng, & Guan, 2011; Zeng & Hong, 2008). Moreover, also in cell cultures, the Hippo signaling is activated in response to cell-cell contacts which demonstrates that the role of this

pathway in the control of cell proliferation is preserved even in a non-tissue environment (Kim, Koh, Chen, & Gumbiner, 2011; Zhao et al., 2007).

Besides its role in the Hippo pathway, Mob1 is also involved in the mitotic exit network (MEN). The Mob1/Dbf2 complex activates the Cdc14, which promotes mitotic cyclins' degradation, and consequently mitotic exit and cytokinesis (MEN and SIN). Mob1 is therefore an excellent candidate to regulate cell proliferation also in *T. gondii.*

Despite the increasing knowledge on the referred pathways, the exact role of Mob1 is still unknown. Also in protozoan organisms, the role of Mob1 protein is still elusive. In fact, the study of Mob1 in these organisms represents an opportunity to dissect the involvement of the protein in distinct but interconnected processes such as cytokinesis, the establishment of cell polarity and morphogenesis. Interestingly, a recent study investigating the evolution of Hippo pathway proteins in premetazoan organisms showed that Mob1 is the most ancient protein of the signaling pathway, being present in unicellular protozoa which supports a fundamental role for this protein (Sebé-Pedrós, Zheng, Ruiz-Trillo, & Pan, 2012). In *T. brucei*, Mob1 is needed for the regulation of cell cycle and for cytokinesis completion (Hammarton et al., 2005). Besides the role of Mob1 in cytokinesis, our work in the ciliate *T. thermophila* also demonstrated that Mob1 is essential for the establishment of cell polarity, namely for the correct positioning of cell division axis (Tavares et al., 2012). A recent work in *S. coeruleus* shows that Mob1silencing by RNAi in this organism leads to morphogenic defects and abnormal body axes (Slabodnick et al., 2014). All together these findings reinforce the interest on studying the different roles of Mob1 in these more simple and polarized organisms.

Taken together the existing knowledge about Mob1's functions, we propose that it presents the same fundamental roles in *T. gondii* and it is also an excellent candidate to be involved in parasite number control in this and other cyst-forming parasites.

Here we describe for the first time the presence of Mob1 and address its function in this parasite. Indeed, by preforming a bioinformatics analysis, we found that *T. gondii* possesses one *mob1* gene. We analyzed the expression of *mob1* by quantitative real time PCR and our data show that *mob1* expression is strongly decreased upon host cell invasion. *T. gondii* only divides inside the host cell and so, this reduction of *mob1* levels within the first hours inside the host, suggested that this gene regulation is associated with cell division.

To evaluate the subcellular localization of Mob1 in *T. gondii*, we raised a polyclonal antibody against the protein. Our immunofluorescence assays demonstrated

that similarly to what we described in *T. thermophila*, in *T. gondii*, Mob1 also presents a polarized distribution in the cell, being present in both poles but enriched in the posterior pole, where the basal complex is localized.

Furthermore, we constructed a *T. gondii* strain overexpressing a Mob1-GFP fusion protein. Using this strain we investigated the ability of the parasites to replicate in a Mob1 overexpression scenario. Importantly, parasites with higher levels of Mob1 showed a significant delay in their replication rate, again supporting the involvement of Mob1 in *T. gondii* proliferation.

3. Material and Methods

3.1.Sequence alignment and phylogenetic analysis

The Mob1 protein sequences of the different organisms used in the phylogenetic analysis were obtained by doing a BLAST, using *Homo sapiens* Mob1 (NCBI Reference Sequence: NP_060691.2) and *S. cerevisiae* (NCBI Reference Sequence: NP_012160.2). Protein-protein BLAST searches (with a threshold of 0.0001 and remaining options as default) were performed against the UniProtKB database (www.uniprot.org) and accession numbers are present in Table 2.

Table 2 – List of the accession numbers of the Mob proteins of the organisms used in the phylogenetic analysis

MOB1 PROTEINS USED IN THE PHYLOGENETIC ANALYSIS			
ORGANISM	**ACCESSION NUMBER**	**ORGANISM**	**ACCESSION NUMBER**
Toxoplasma gondii	XP_002371898.1	***Leishmania braziliensis***	XP_009309166.1
Hammondia hammondi	XP_008885988.1	***Trypanosoma brucei***	AAL10513.1
Neospora caninum	XP_003881413.1	***Trypanosoma brucei***	AAL10512.1
Eimeria maxima	CDJ56791.1	***Trypanosoma cruzi***	XP_819607.1
Eimeria tenella	CDJ41792.1	***Tetrahymena thermophila***	XP_001031965.1
Cryptosporidium muris	XP_002140442.1	***Stentor coeruleus***	AIA82416.1

Cryptosporidium parvum	XP_001388077.1	***Chlamydomonas reinhardtii***	XP_001699958.1
Giardia lamblia	XP_001710048.1	***Medicago truncatula***	KEH37576.1
Saccharomyces cerevisiae	NP_012160.2	***Arabidopsis thaliana***	AED95267.1
Candida albicans	XP_719093.1	***Drosophila melanogaster***	AHN57464.1
Neurospora crassa	XP_956516.2	***Danio rerio***	NP_956208.1
Entamoeba nuttalli	EKE41106.1	***Mus musculus***	NP_663546.1
Naegleria gruberi	XP_002675417.1	***Homo sapiens***	NP_060691.2
MOB2 PROTEINS USED AS AN OUT GROUP			
Danio rerio: NP_001002364.1			
Mus musculus: NP_082584.1			
Homo sapiens: CAE45271.1			

Protein sequence alignments were performed using M-COFFEE (Moretti et al., 2007) and curated with TrimAl (Capella-Gutiérrez, Silla-Martínez, & Gabaldón, 2009), both with default settings. Analysis of the alignment and comparison of sequences was performed using Jalview (Waterhouse, Procter, Martin, Clamp, & Barton, 2009) and Base-By-Base (Hillary, Lin, & Upton, 2011). ProtTest 3.0 (Darriba et al., 2011) was used to select the best model for phylogenetic tree construction. Maximum likelihood (ML) trees with 1000 bootstrap replicates were constructed using a combination of PhyML (Guindon et al., 2010), with settings indicated by previous determination of the best phylogenetic model, and the tools seqboot, consense and retree from the PHYLIP package version 3.695. Trees were edited using MEGA 6 (Tamura, Stecher, Peterson, Filipski, & Kumar, 2013).

3.2. DNA Constructs

The *T. gondii mob1* gene open reading frame (ORF) (TGME49_104730) was amplified by PCR with specific primers (Table 3) containing the restriction sites to clone it in frame with GFP in the N-terminal region using the pddFKBP-myc-GFP-

TgStx6-CAT vector (kind gift from Markus Meissner, Glasgow Biomedical Research Centre, University of Glasgow, UK). This plasmid contains a gene that encodes a protein destabilization domain (dd) that was fused with the N-terminal region of GFP-Mob1-. The *T. gondii* GFP-Mob1Tg construct used was as follows: 5'-ddFKBP–GFP-*mob1*–CAT-3′. The dd domain allows the control of protein overexpression by using a shield molecule that binds to the domain, preventing protein target for degradation. In the absence of shield the over-expressed GFP-Mob1 is labelled and degraded by the proteasome. In the presence of shield, the produced GFP-Mob1 protein is protected against degradation and accumulates inside the parasites (Herm-Götz et al., 2007).

3.3. Cell and parasite cultivation

Human foreskin fibroblast cells (HFF-1) (from ATCC) were grown in DMEM media (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and non-essential amino acids (Invitrogen). Cells were used as confluent monolayers and cultured in a 5% CO2 humidified atmosphere at 37ºC. For all the experiments, *T. gondii* parasites from the RH strain were maintained in HFF-1 cells.

3.4. Quantitative Real-time PCR

Total RNA was extracted from HFF cells cultured in T25 flasks at the different time points after infection with $2.5x10^5$ *T. gondii* parasites. RNA extracted from free parasites was used as control. To obtain the RNA extracts we used the E.Z.N.A.® Total RNA Kit I, according to the manufacturer's instructions. To synthetize cDNA, 1 μg of RNA from each condition was used. The RNA was treated with DNase I (Invitrogen) and reverse transcribed using SuperScript III (Invitrogen) and random primers (Invitrogen). Real-time PCR was performed for 40 cycles on an ABI Prism 7700 Sequence using SYBR Green detection system (Applied Biosystems).

Primer sequences used are presented in Table 3. *gapdh* and *α-tubulin* were used as endogenous controls in relative quantification using the standard curve method. Primers were designed using the Roche Design Centre. All samples were run in duplicate and the data show results from three independent experiments.

3.5.*Toxoplasma gondii* Mob1 Antibody Production

T. gondii mob1 cDNA was cloned in the vector pGEX4T.2 suitable for bacteria expression. The vector pGEX 4T.2-Mob1Tg was transformed into *E. coli* DH5α strain

and purified using the High Pure Plasmid Isolation Kit (Roche). For the recombinant protein expression and purification, the resulting construct was transformed into *E. coli* BL21+ strain. Protein expression was induced with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside). Mob1Tg in fusion with glutathione S-transferase (GST) (pGEX 4T.2-Mob1Tg) was purified with gluthatione sepharose 4BT beads (Amersham), according to manufacturer's instructions. The protein extract was analysed in a 12% SDS-PAGE gel and protein bands were stained with Coomassie Brilliant Blue R-250. To validate the Mob1Tg-GST protein production, protein bands were manually excised from the gels, digested using trypsin and analyzed by mass spectrometry. Mob1Tg-GST protein was used to immunize Balb-C mice.

3.6. Immunofluorescence Assays

For immunofluorescence assays (IF), HFF-1 cells were grown on coverslips until confluence and inoculated with freshly egressed parasites. Cells were fixed either with 4% w/v paraformaldehyde (PFA) in phosphate-buffered saline (PBS) for 20min at room temperature or 100% ice-cold methanol for 10min at −20◦C. In the case of PFA fixation, cells were then permeabilized with 0.2% Triton X-100. Blocking was done during 30 min with 3% BSA (bovine serum albumin) in PBS 0.5% Tween-20. The primary and secondary antibodies were diluted in the blocking solution and incubated with the preparations for 1h at room temperature. Primary antibodies used were: mouse anti-Mob1Tg serum (1:100); mouse anti-polyglutamylated tubulin (Adipogen) (1:200); and rabbit anti-human Centrin (kindly provided by Dr. Iain Cheeseman, Whitehead Institute for Biomedical Research, Cambridge, USA) (1:200). Secondary antibodies used were: Alexa Fluor 488 and Alexa Fluor 594, at 1:500 dilution (Invitrogen). DNA was stained with DAPI, present in the mounting media (DABCO). Images were acquired on an Applied Precision DeltavisionCORE system, mounted on an Olympus inverted microscope, equipped with a Cascade II 2014 EM-CCD camera, using the a 100x Oil immersion objective.

3.7. Invasion Assays

To evaluate the ability of ddGFP-Mob1Tg parasites to invade the monolayers of HFF cells, ddGFP-Mob1Tg *T. gondii*, freshly released from cells, were previously incubated during 6h with or without shield (1µM). Parasites at a $1x10^7$ parasites/ml

density were then allowed to contact with HFF cells grown in coverslips (24 wells plates) for 1h. After this invasion time, the wells were abundantly washed 4x with PBS to remove extracellular parasites and, after 24h, cells were fixed with ice-cold methanol. IF was performed using an anti-polyglutamylated tubulin antibody, and the number of established parasitophorous vacuoles in host cells treated and non-treated with shield was counted.

3.8. Replication Assay

To evaluate the parasites' replication ability, freshly released ddGFP-Mob1Tg parasites were previously incubated with or without shield (1 μM) for 6 h. $2x10^4$ parasites/well were then allowed to invade fresh HFF monolayers grown in 24 wells plates. The parasites were allowed to replicate inside the cells during 24h and subsequently cells were fixed with ice-cold methanol and IF was performed using a mouse anti-polyglutamylated tubulin antibody. The number of parasites treated or non-treated with shield inside the parasitophorous vacuoles was counted.

Table 3 – Nucleotide Sequences of the primers used in the study of Mob1 in *Toxoplasma gondii*

dd-GFP-Mob1Tg	
Tg Mob1 coding sequence Forward	5' ATGAACTACTGGACGTCTTGG 3'
Tg Mob1 coding sequence Reverse	5' CGGGATCCTCAAAAACTGGCCTGAGAG 3'
pet3a-Mob1Tg	
Forward	5' CGGGATCCATGAACTACTGGACGTCTTGG 3'
Reverse	5' CCCAAGCTTAAAACTGGCCTGAGAGCTC 3'
Real Time PCR	
mob1Tg Forward	5' CTGCCACATCTACAGACAAC 3'
mob1Tg Reverse	5' CATTGTCTCCGTACCACTC 3'
a-tubulin Forward	5' CGCCTGCTGGGAGCTCTT 3'
a-*tubulin* Reverse	5' GAAGGTGTTGAAGGCGTCG 3'
gapdh1 Forward	5' CGTGGAGGTTTTGGCGATC 3'
gapdh1 Reverse	5' GACTTCGCCGGGGTAGTG 3'

4. Results

4.1.Phylogenetic analysis of Mob1 protein in *Toxoplasma gondii*

Our first approach to search for the presence of Mob proteins in the parasite *T. gondii* was to perform a bioinformatics analysis with the available genomic DNA database for this parasite (http://toxodb.org/toxo/). For that, we used the amino acid sequences of human Mob1 (NP_060691.2) and *S. cerevisiae* Mob1p (NP_012160.2), the best characterized Mob1 proteins. Interestingly, this search revealed the presence of only one Mob1 protein in *T. gondii* (TGME49_304730). In fact, most of the eukaryotes (from budding yeast to humans) present more than one Mob1 (Hergovich, 2011), being this also the case of *T. brucei* parasite (possesses two Mob1 proteins), the only parasitic organism in which Mob1 was investigated up to date (Hammarton et al., 2005). As far as we know, the only studied organism with only one Mob1 protein was the ciliate *T. thermophila.*

After this, we performed a phylogenetic analysis to investigate the evolutionary relationship between Mob1 proteins from different organisms. The phylogeny of Mob1 proteins was already deeply investigated in a previous study (Vitulo et al., 2007). However, in this revision, Mob1 proteins of parasitic eukaryotes were not taken into account. Therefore, we searched for the presence of Mob1 in some of the parasites for which genomic sequences are available and annotated and compared them with those of some model organisms. The obtained sequences (accession numbers in Table 2) were aligned and used to perform a maximum likelihood phylogenetic analysis. In this phylogenetic tree Mob2 proteins were used as an out group (Fig. 25).

Our analysis shows that Mob1 proteins from animals are evolutionarily very distant from those of unicellular organisms. We could observe that the first node separates animals from two other groups that have diverged later during evolution: i) the group containing plants and unicellular organisms such as microalgae (*Chlamydomonas reinhardtii*) and ciliates (*T. thermophila*, *S. coeruleus*); ii) the group containing the remaining organisms.

Focusing on the unicellular organisms included in the phylogenetic analysis, we could observe that free living and parasitic organisms are located in different clades of the tree.

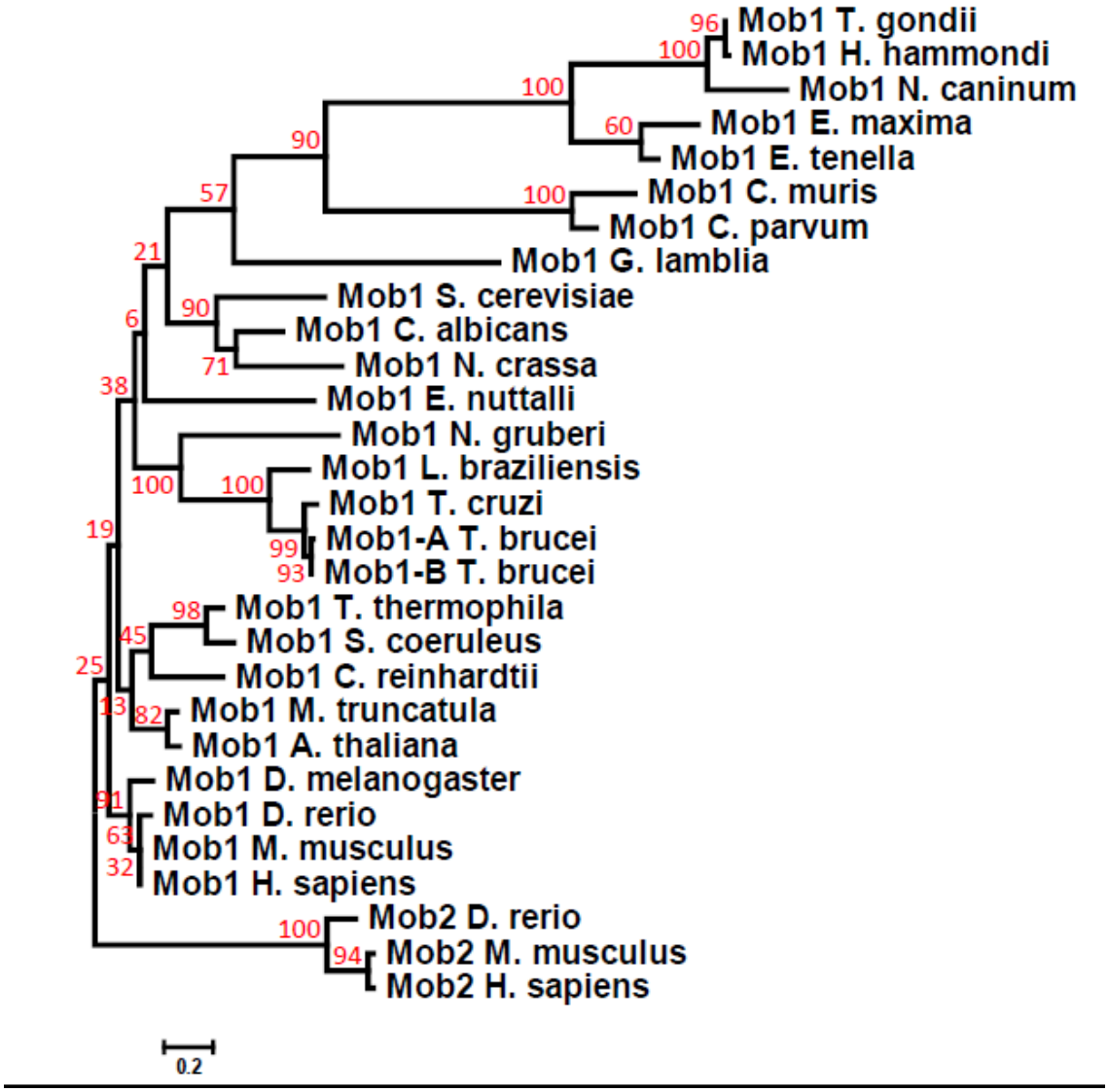


Figure 26 - Phylogenetic analysis of Mob1 proteins from different parasitic organisms. The predicted aminoacid sequence of *T. gondii* Mob1 and those of different parasitic organisms were compared with Mob1 proteins from model organisms throughout the eukaryotic tree of life. These sequences were aligned and used to perform a maximum likelihood phylogenetic analysis. Mob2 proteins were used as an out group.

Regarding parasites, a distinction between the *Trypanosoma* and *Leishmania* species, that belong to the Excavata domain, and parasites belonging to the phylum Apicomplexa (*T. gondii, H. hammondi, N. caninum, E. tenella, E. maxima, C. parvum* and *C. muris*) was noticed. Curiously, in our study, the Excavata *Giardia lambia* appears closer to the Apicomplexa than to the Trypanosomatida. The Apicomplexa form a monophyletic group in which cyst forming coccidia (*T. gondii, H. hammondi, N. caninum*) appear very close to each other and separated from the monoxenous genus *Eimeria* while *C. muris* and *C. parvum* forms a more distant group.

4.2. Upon host cell entrance, *mob1* expression in *Toxoplasma gondii* is greatly diminished

As a first approach to investigate the role of *mob1* gene in *T. gondii* we studied the expression profile of the gene during different phases of parasite host cell invasion by quantitative real time PCR using specific primers for the gene.

T. gondii is an intracellular obligate parasite and thus only replicates inside host cells, after establishing the parasitophorous vacuole. This vacuole starts to be formed right after the first steps of invasion (Francia & Striepen, 2014). For this analysis, total RNA was extracted from freshly released parasites (control) and from parasites inside host cells at 1, 4, 8 and 24h after cell invasion (Fig.26).

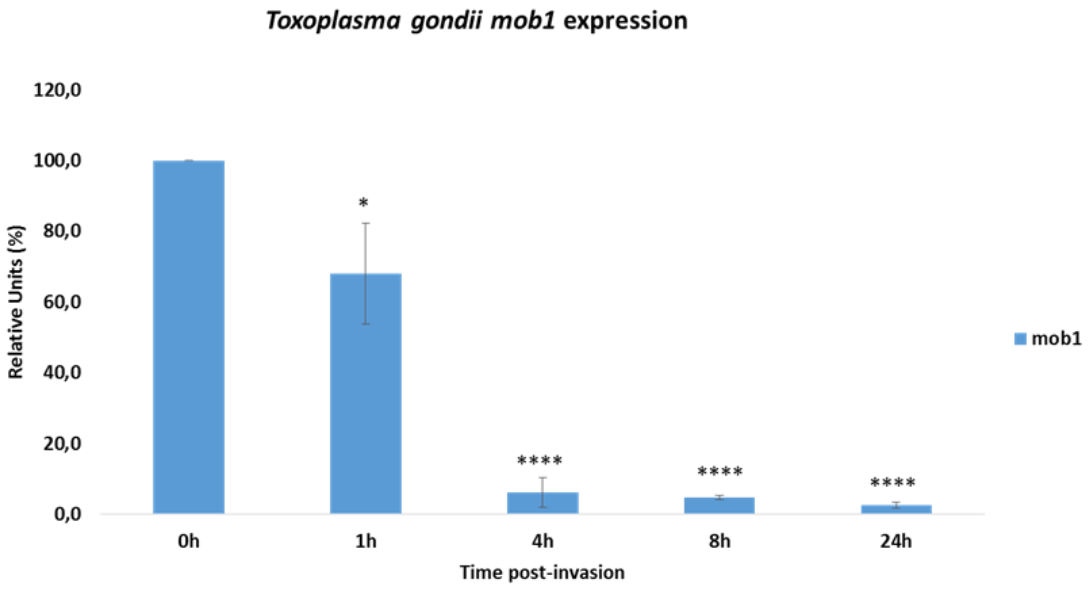


Figure 27 - Quantitative Real-Time PCR analysis of *mob1* expression in *Toxoplasma gondii* after host cell invasion. Total RNA was extracted from freshly released parasites (control) and from parasites inside host cells at 1, 4, 8 and 24h after cell invasion. RNAs were extracted from three independent experiments and data obtained for each time point was normalized to *mob1* levels in the free and freshly released parasites (0h). *tubulin* and *gadph* genes were used as internal controls (* $p< 0.05$; **** $p< 0.001$).

mob1 RNA levels were evaluated from three independent experiments and data obtained to each time point was normalized to *mob1* levels in the free and freshly released parasites (0h). In these experiments, we used the *tubulin* and *gadph* transcripts levels as internal control and to access the real *T. gondii mob1* expression, we normalized the obtained *mob1* RNA levels were evaluated in three independent

experiments and data obtained for each time point was normalized to *mob1* levels in the free and freshly released parasites (0h). In these experiments, we used the *tubulin* and *gadph* transcripts levels as internal control and to access the *T. gondii mob1* expression, we normalized the obtained *mob1* values to the mean value obtained for the two internal controls. Interestingly, we could observe that *mob1* mRNA levels are already reduced in the parasite at 1h post-invasion, 68% (±14.3) comparing to the control (corresponding to 100%, relative units) (Fig. 26). At 4h post invasion, time at which the parasites start to replicate, *mob1* mRNA levels, either by a downregulation of transcription or by the degradation of transcripts, are almost vestigial comparing to free parasites (6.2%±4.1). These levels continue to decrease at least until 24 h after host entrance. In fact, at 8h these levels were 4.7%±0.7, whereas at 24h were only 2.5%±0.8 of those found in control parasites (Fig. 26). The observed decrease of *mob1* transcripts during parasite parasitophorous establishment suggests that this gene is strictly regulated during this process.

Although this study only addresses the mRNA levels and not the real amount of the protein that is still present, it clearly shows that the replication of *T. gondii* parasites inside of the host cell requires mob1 gene downregulation. This downregulation is so drastic that one can assume that protein levels are also affected. This fact implicates Mob1 and associated signaling pathways in the replication control of the parasite, supporting our working hypothesis.

4.3. Mob1 presents a polarized localization in *Toxoplasma gondii* and is associated with key structures involved in cell division

Previous data concerning Mob1 in higher eukaryotes demonstrate that this protein localizes in fundamental structures for cell division. In fact, in human cultured cells, Mob1 localizes to centrosomes in late G2 and is associated with spindle poles and kinetochores during mitosis. At the end of mitosis, during cytokinesis, Mob1 is present at the midbody (Wilmeth et al., 2010), a transient structure formed in this late step of cell division, in the region where cleavage occurs. Also in budding yeast, Mob1p localizes in the spindle pole bodies (a structure functionally equivalent to the centrosome) and at the bud neck, just before and during cytokinesis (Luca et al., 2001). In the protozoan *T. brucei*, Mob1 is distributed in a punctate fashion in the cytoplasm being excluded from the nucleus throughout the cell cycle (Hammarton et al., 2005). Our study in *T. thermophila*, showed that the unique Mob1 protein of this ciliate as a

cytoplasmic pool but is enriched in the basal bodies (structurally similar to centrioles in animal cells and their probable ancestors) of the posterior pole and in the region where the division plan will be established (Tavares et al., 2012). Since protein localization impinges on function, we decided to investigate the subcellular localization of Mob1 in the parasite *T. gondii*. For this purpose, we raised a polyclonal antibody against the protein. The anti-Mob1Tg was tested in a western blot where total protein extracts obtained from fresh released dd-GFP-Mob1Tg parasites were incubated with (+) or without (-) shield (shd) for a period of 6 hours. The produced serum recognized a protein with a molecular weight just below the protein marker of 35 kDa which corresponds to the expected molecular mass of the endogenous Mob1Tg protein with a predicted molecular size of 34 kDa. Besides Mob1Tg, the antibody also recognized a ~75 kDa protein, corresponding to the fusion protein GFP-Mob1Tg (Fig.27).

Consistently with antibody specificity, this band only appeared in the parasites that were incubated with shield which allowed the protein not to be targeted for degradation, accumulating into the cytoplasm of the recombinant parasites (Herm-Götz et al., 2007).

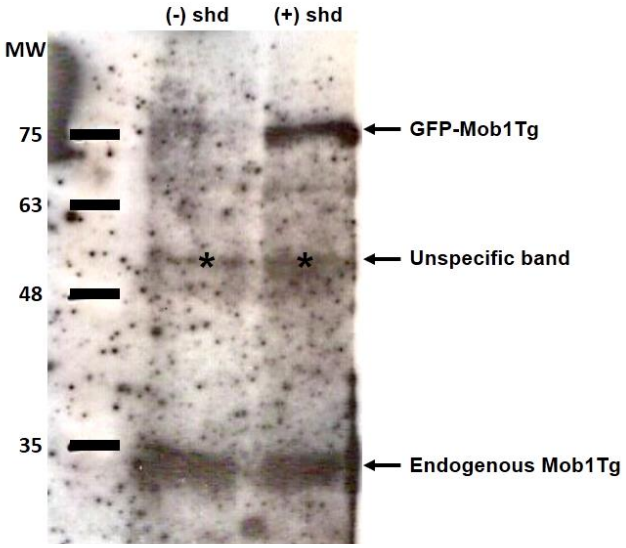


Figure 28 - *Toxoplasma gondii* anti-Mob1Tg polyclonal serum. Total protein extracts of *Toxoplasma gondii* dd-GFP-Mob1Tg parasites, with (+) or without (-) 1 µM of shield (shd) where used to test the anti-Mob1 serum specificity. In both lanes, the anti-Mob1Tg recognizes the endogenous Mob1Tg protein (34kDa) and the fusion protein GFP-Mob1Tg (75kDa).

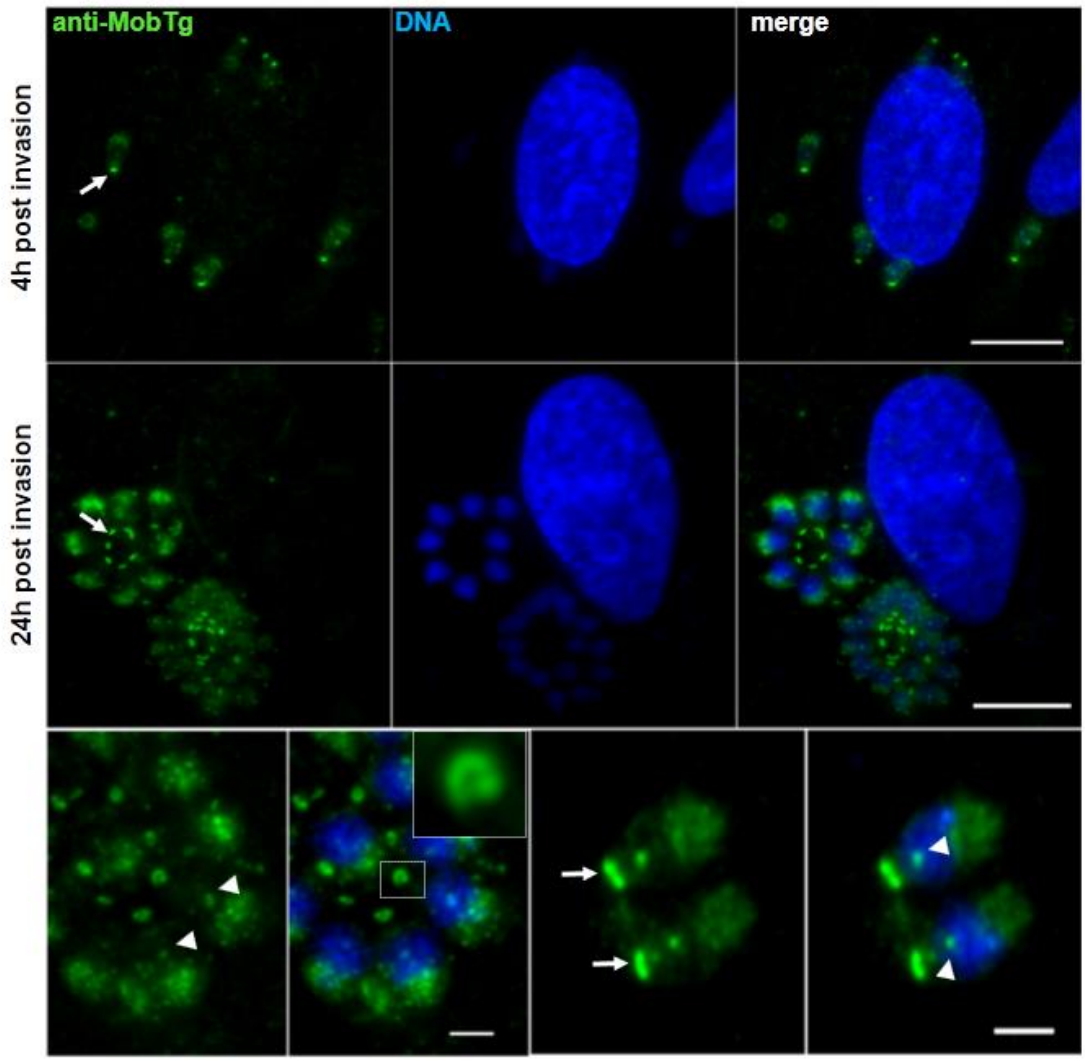


Figure 29 - Analysis of Mob1 subcellular localization and distribution in *Toxoplasma gondii* inside host cell. Mob1 is excluded from the nucleus and presents a polarized distribution, being more concentrated at the basal complex (arrows). Arrowheads point to a specific punctate localization of Mob1 in *T. gondii* which resembles centrosomal localization (Scale bar: 10 µm; last row 2 µm).

An IF analysis showed that Mob1 in *T. gondii* presents a cytoplasmic pool being also, as in *T. brucei*, excluded from the nucleus (Fig. 28, 30). Interestingly, we could observe a very clear localization of the protein in the parasite basal pole, where the basal complex, a structure involved in cytokinesis in *T. gondii* is localized (Fig 28– arrow). A closer look into this structure showed that Mob1 forms a ring-like structure that can be clearly observed in the last row magnification. Furthermore this localization of the protein in the basal complex is maintained at several times after parasite host cell invasion (4h and 24h time points illustrated).

The immunolabeling of the parasites with the Mob1Tg antibody also showed that Mob1 accumulates in a confined region localized in the middle of the parasite cell originating a bright dot, which resembled the centrosome (Fig. 28-arrowhead). In order to investigate if Mob1 in *T. gondii* also presented a centrosomal localization, we performed an IF using an anti-centrin antibody (kind gift from Ian Cheeseman, Whitehead Institute for Biomedical Research, MIT, US) that specifically stains the centrioles. Surprisingly, even though in other eukaryotes Mob1 is localized at the centrosome, in *T. gondii* this is not the case as the IF analysis showed that the Mob1Tg signal did not co-localize with the anti-centrin staining (Fig. 29, 30).

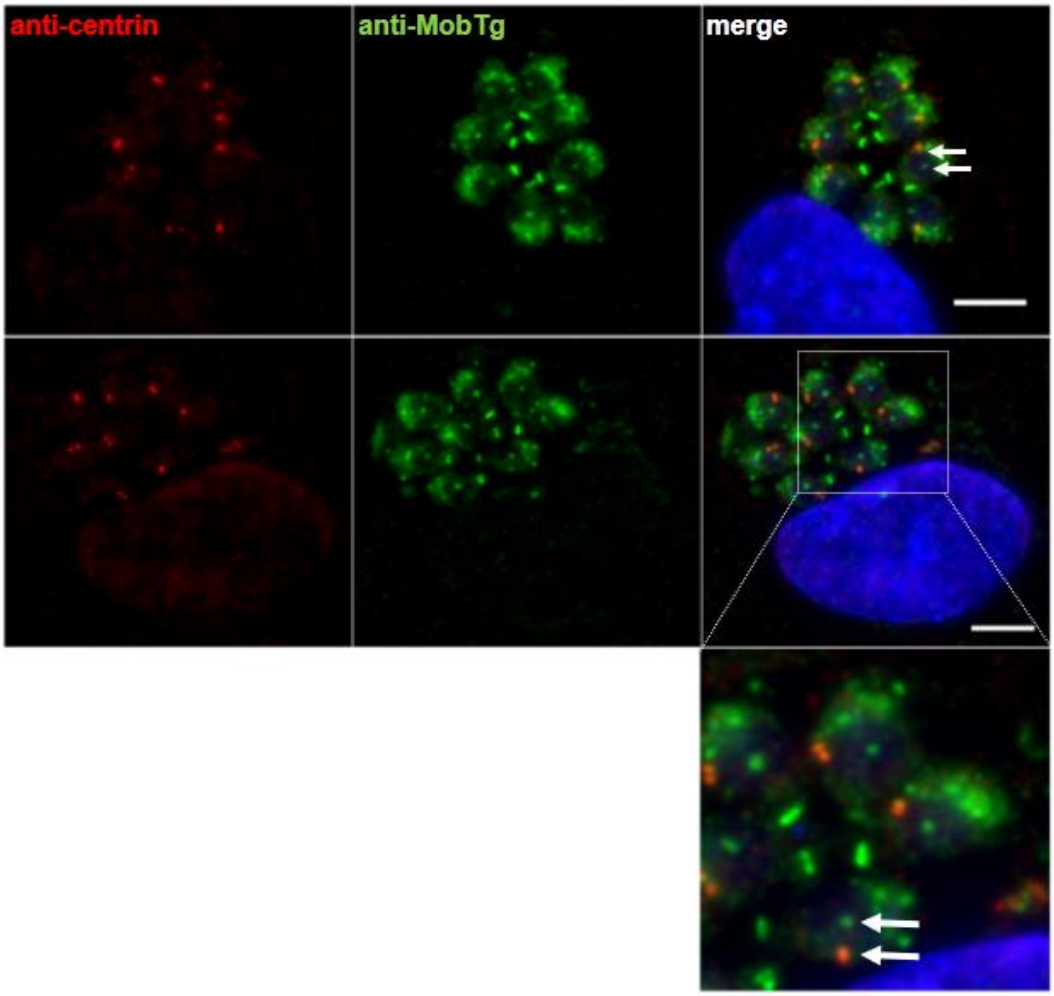


Figure 30 - Mob1 is not a centrosomal protein in *Toxoplasma gondii*. Mob1 signal does not colocalize with centrin in *T. gondii*, and two distinct dots are visible (arrows). Scale bar 10 µm.

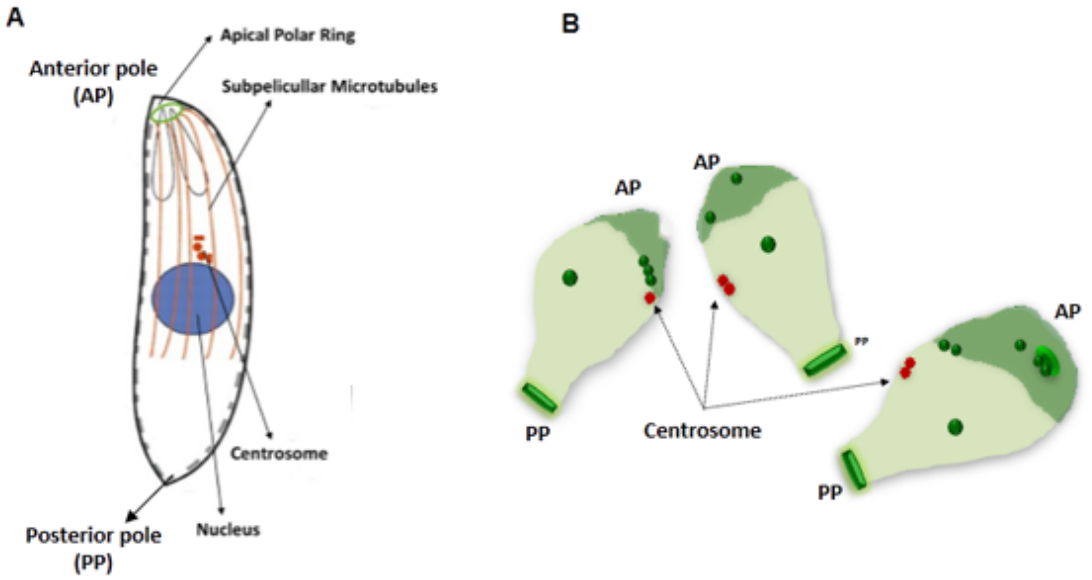


Figure 31 – Schematic representation of Mob1 localization in *Toxoplasma gondii* (A) is showed as a reference for internal organization. (B) Schematic representation of *T. gondii* cells stained with antibodies against TgMob1 (green) and centrin (red). The schemes were drawn based in the IF images in Fig 29. Note the strong TgMob1 labeling on the posterior pole in a ring like structure. Also, a strong spot is always observed in a median region of the cell corresponding to an unknown structure/compartment. A disperse staining is observed in the anterior pole of the cell where several strong dots emerge.

This striking result led us to further investigate the structure, if any, where Mob1 is localized in *T. gondii*. Besides the Mob1 localization in the centrosome, basal bodies and spindle pole bodies, this protein is also present at the kinetochore both in yeast and in human cells (Stoepel et al., 2005); (Wilmeth et al., 2010). The kinetochore is a transient structure that is assembled at the chromosome centromeric region of mitotic chromosomes, functioning as a platform for spindle microtubules binding. In *T. gondii*, which undergoes a closed mitosis, the centromeres form a cluster in a single apical region of the nucleus (Brooks, 2011; Farrell & Gubbels, 2014). Being so, the punctuated pattern of centromeric/kinetochore proteins observed in other organisms is not visible in this parasite. Interestingly, the IF labeling for the centromeric protein Cen-H3 in *T. gondii* (Brooks, 2011) strongly resembles the Mob1 localization in this parasite, which is in agreement with Mob1 involvement in the cell division process. Unfortunately, our attempts to do an immunofluorescence using specific antibodies against human kinetochore proteins (anti-CENP-E and anti-CENP-A) failed since these antibodies did not cross react with the homologue protein in *T. gondii* kinetochore structure.

4.4. Mob1 increased levels lead to a delay in *Toxoplasma gondii* replication

The previously described roles for Mob1 in other organisms, its involvement in cell division/proliferation processes, its specific localization in cell structures critical for mitosis and also the pattern of expression of the T. *gondii mob1* gene led us to hypothesize whether this protein could be involved in replication of this parasite. To test this hypothesis, we constructed a transgenic *T. gondii* line in which the expression of a fusion protein GFP-Mob1Tg is controlled by a destabilization domain (dd) that targets the tagged protein to degradation in the absence of shield (Herm-Götz et al., 2007) (see M&M).

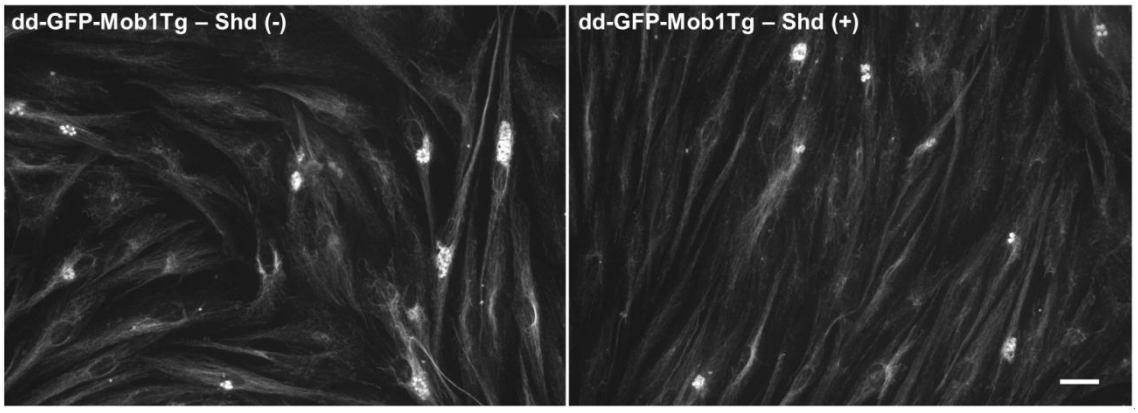


Figure 32 - Representative images of those used to quantify the ability of *Toxoplasma gondii* parasites to replicate in a Mob1 overexpression condition**.** IF using anti-polyglutamylated tubulin in dd-GFP-Mob1Tg parasites that were incubated for 4 hours, without [shd (-)] or with [shd (+)]. After the treatment parasites were allowed to invade and replicate inside the host cells. In the case of shd (+) parasites, the consequent accumulation of Mob1Tg in the cytoplasm led to a delay in replication rate, visible by the accumulation of parasitophorous vacuoles with 1, 2 or 4 parasites. Scale bar: 25µm.

This strategy is preferable to a constitutive expression of the fusion protein since it permits to precisely control the stability of the protein, avoiding the possible toxicity of a constant overproduction.

With this engineered *T. gondii* GFP-Mob1Tg line, we performed replication assays that allow the evaluation of the ability of the parasites to undergo proliferation inside host cells in a Mob1 overexpression background (with shield) in comparison to cells that have only the endogenous Mob1 levels (without shield). To follow the replication process in these parasites, freshly released parasites were allowed to invade host cells and were grown for 24h in the presence or absence of shield (control). After this 24h period, cells were fixed and processed for immunofluorescence and the number

of parasites per parasitophorous vacuole was counted in the two conditions (Figs. 31, 32).

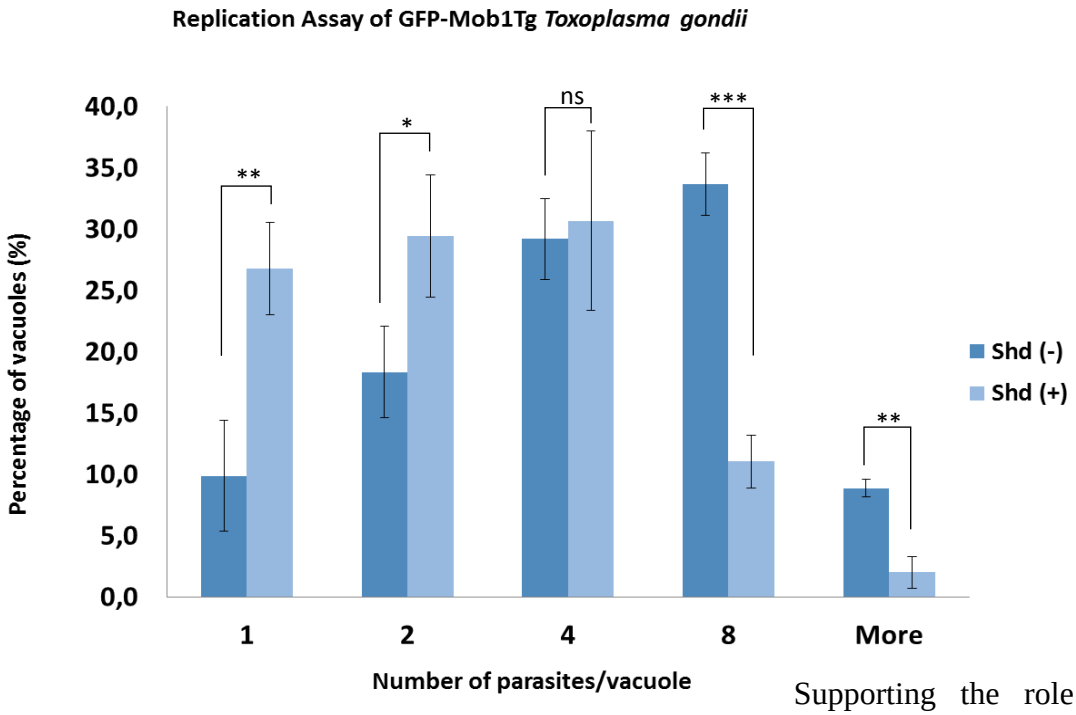


Figure 33 - Overexpression of Mob1 leads to a replication delay in the parasite *Toxoplasma gondii*. Parasites treated with or without, shd(+) or shd(-), respectively, were allowed to invade the host cells. After 24h, the number of parasites in each vacuole was counted. The results are the obtained mean value from three independent experiments and, in each experiment, at least 120 vacuoles per condition were counted (*p≤0.1; ** p≤0.01; *** p≤0.001)

Supporting the role for Mob1 in *T. gondii* control of proliferation, we observed that in the presence of shield, GFP-Mob1Tg parasites present a significant delay in replication capacity. In the presence of shield, we observed a significant enrichment of vacuoles with 1 and 2 parasites (26.8%±3.8 and 29.4%±5, respectively) in comparison to cells without shield treatment (9.9%±4.5 and 18.4%±3.8) (Fig. 32). In control cells, *i.e.* invaded with parasites not treated with shield, the majority of the vacuoles presented 4 (29.2%±3.3), 8 (33.7±2.5) or more (8.9%±0.7) parasites, which corresponds to the normal rate of division inside the host cells.

This transgenic GFP-Mob1Tg line was also used to explore if the invasiveness ability of the parasites was altered under Mob1 overexpression. For this, we allowed freshly released parasites to be in contact with host cells during one hour, after which cells were extensively washed to remove free parasites. The number of vacuoles formed was counted after 24h, independently of the number of parasites per vacuole. Contrary

to what was observed in replication, GFP-Mob1Tg parasites with shield did not present any difference in invasion ability in comparison to non over expressing parasites (without shield) (Fig. 33), supporting the hypothesis that the phenotype observed in the replication assays is a consequence of Mob1Tg overexpression and not caused by parasite genetic manipulation.

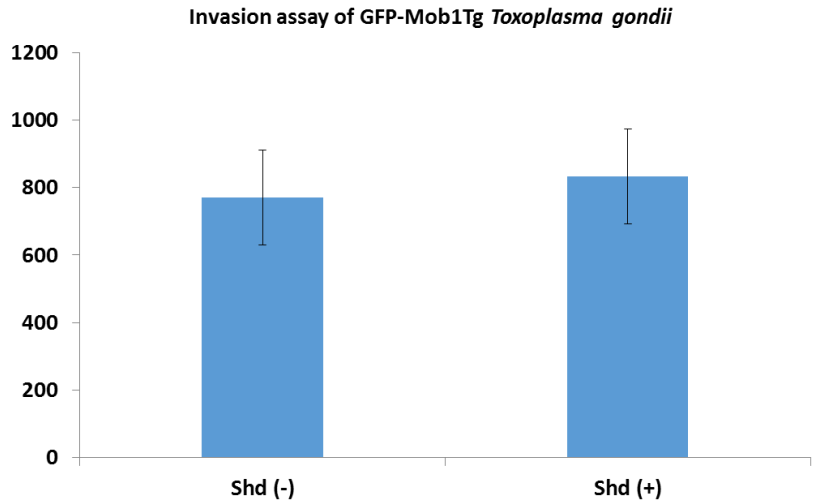


Figure 34 - Overexpression of Mob1 does not affect invasion ability of the parasite *Toxoplasma gondii*. Parasites treated with or without, shd (+) or shd(-), respectively, were allowed to invade the host cells. After 24h, the number of parasites in each vacuole was counted. The results are the obtained mean value from three independent experiments and, in each experiment, at least 120 vacuoles per condition were counted ($p>0.5$, the difference is not statistically significant.

Moreover, we also performed the replication assays with two other different clones of dd-GFP-Mob1Tg parasites. Also here we could see the same phenotype of replication delay in parasites treated with shield comparing to the non-treated ones (data not shown). Each clone is the result of an independent and random insertion of the dd-GFP-Mob1Tg in the genome, so the observation of the same phenotype in three different clones rules out the possibility that the phenotype is a result of the disruption of critical sequences during genetic transformation. Finally, the hypothesis that the shield itself could be the cause of the replication delay can also be excluded since independent studies in our laboratory studying different proteins with the same system do not show any replication phenotype.

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5. Discussion

Mob1's function in cell division seems to be deeply conserved throughout the eukaryotic tree of life (Tavares et al., 2012; Hammarton et al., 2005; Luca et al., 2001; Lai et al., 2005; Wilmeth et al., 2010; Florindo et al., 2012). Interestingly, a recent study showed that among the proteins that constitute the Hippo signaling pathway, Mob1 is the one that appears earlier in eukaryotes, supporting a core role for this protein (Sebé-Pedrós et al., 2012). Up to the present date, there are no published studies about this protein in any member of the phylum Apicomplexa. This taxon groups a large number of protistan parasites for which *T. gondii*, has been regarded as model. In *T. gondii* we found only one Mob1 protein that in a phylogenetic analysis, clustered with Mob1 of other apicomplexan parasites such *as Neospora caninum, Eimeria tenella, Eimeria. maxima, Hammondia hamondii*, *Cryptosporidium parvum* and *Cryptosporidium muris* (Fig. 25) as expected. *T. gondii* Mob1 is evolutionarily distant from the ones of other protistan parasites, such as the Trypanosomatida *Trypanosoma brucei*, *Trypanosoma. cruzi* and *Leishmania braziliensis*. In fact, even though all of these organisms present a parasitic life style, the *modus operandi* inside the host organism is very different. Apicomplexan parasites form oocysts, containing sporozoites and, in some cases, tissue cysts containing bradyzoites inside the host, where parasites are in close relation to each other. The apicomplexan parasites present in the phylogenetic analysis only divide inside the parasitophorous vacuole. In contrast, the parasite *Trypanosoma brucei* and *Leishmania* species do not form these structures and, in their mammal hosts, divide either in body fluids such as blood and lymph, or in the phagolysosomes of cells such as macrophages, respectively (Li, 2012). To which extent this divergent evolution of Mob1 protein in different parasite species can be associated with their different life styles is unknown. Nevertheless, Mob1 is involved in the control of cell division and, being so, it is tempting to speculate that this evolutionarily distant Mob1 proteins can be related to the specific needs of each organism's different cell division regulation according to external conditions.

One of the most significant aspects of the Mob1 study in *T. gondii* came from its specific cellular localization. Mob1 subcellular localization was investigated by taking advantage of the anti-Mob1Tg antibody raised for the present study (Fig. 27). Interestingly, we observed that Mob1 presents a cytoplasmic pool, but presents a polar distribution inside the cell, being clearly concentrated in the posterior pole of the

parasite. This polar distribution of Mob1 in *T. gondii* parallels with our previous observations in the ciliate *T. thermophila* where Mob1 is also enriched in the basal bodies of the posterior pole of the cell (Tavares et al., 2012). Both organisms are permanently polarized cells in which cell polarization is inherited across generations. Curiously, in *T. thermophila* the subcellular distribution of Mob1 likely has a direct role in correct cell division, since its localization in the middle region of the cell is essential for the correct definition of the plane. In the absence of Mob1 in this region, *T. thermophila* cells present a misplaced division axis, with severe consequences in cell shape. Our study in this ciliate allowed us to establish a link between polarity and proper cell division, suggesting that in unicellular organisms with permanently polarized cells, the protein Mob1 is essential for the inherited cell structure and ultimately, morphogenesis. This new role of Mob1 as a linker between cell polarity and correct cell division was also supported by a later study using another unicellular polarized organism, the ciliate *S. coeruleus.* Here the authors also show that Mob1 is essential for the maintenance and regeneration of cell polarity and proper cell proportions, being Mob1 RNAi depleted cells defective in cytokinesis, as is the case of Mob1 depleted *T. thermophila* (Tavares et al., 2012; Slabodnick et al., 2014). Also in *T. brucei* Mob1 seems to be involved in the link between the placement of the division furrow and cell polarity (Hammarton et al., 2005). The functional significance of this asymmetric distribution of the protein is therefore most likely conserved. The specific localization of Mob1 at *T. gondii's* posterior pole, where the basal complex is localized, clearly supports the role of this protein in cell division. Actually, the basal complex is a cytoskeleton structure assembled in the posterior pole of the parasites, likely to be present in all Apicomplexa and is involved in the cell division process (Hu, 2002). Besides the first identified components of the basal complex in *T. gondii*, the proteins TgMorn1, TgCentrin 2 and TgDLC, a dynein light chain, several intermediate filaments-like proteins (IMC proteins), conserved in the Alveolates, are also present in this structure (Hu, 2006); (Anderson-White, 2012). Interestingly, TgMorn1 and TgCentrin2 are also components of the apical complex and of the spindle pole, both structures involved in cell division (Hu, 2008). Recent studies concerning TgMorn1 have demonstrated that this protein in critical for the assembly and maintenance of the parasite basal end structure and also for cytokinesis, since TgMorn1 defective parasites do not segregate daughter cells correctly that fail to separate at the end of cell division (Heaslip et al., 2010); (Lorestani, 2010). Curiously, as it is the case in *T. thermophila*, the impairment of cytokinesis in *T. gondii* does not arrest the organism in interphase,

since the non-segregated daughter cells can undergo cell division again (Lorestani, 2010; Tavares et al., 2012).

Taking into account the previously described role of Mob1 in cytokinesis in several organisms and the particular localization of the protein in *T. gondii*, we suggest that this function is conserved and further investigation needs to be done to evaluate it. Furthermore, the localization of *T. gondii* Mob1 in structures critical for cell division is most likely not exclusive of the basal complex. In fact, Mob1 also presents a punctuated localization in a more apical region of the cell, just above the nucleus (Fig. 28). A careful search in the literature showed that in *T. gondii* this is the region where kinetochores are assembled and connected to the spindle pole microtubules. *T. gondii* parasites present a closed mitosis, i.e. there is no nuclear envelope breakdown during chromosome segregation. Instead, the spindle microtubules enter into a specialized region of nucleus membrane and form the centrocone. Indeed, in *T. gondii*, the centromeres, and consequently the kinetochores are sequestered to this confined region where they are clustered during cell division (Brooks, 2011; Farrell & Gubbels, 2014). Importantly, during division, TgMorn1 also localizes in the apical region of the early forming daughter cells (Gubbels, Vaishnava, Boot, Dubremetz, & Striepen, 2006). In *T. gondii*, the budding of the daughter cells occurs in a plane that is coincident to the anterior posterior parasite axis, and a ring formed by TgMorn1 and IMC protein moves along the longitudinal axis of the mother cell constricting in the posterior end to allow cytokinesis (Gubbels et al., 2006). In this study we could not dissect the specific localization of this punctuated protein pool in the parasites. However, since the presence of Mob1 in the kinetochores was already described in yeast and mammals, it is tempting to speculate that this may also be the case in *T. gondii* (Stoepel et al., 2005; Wilmeth et al., 2010).

In addition to the discussed Mob1 role in cell polarity and how it impinges on correct cell division and cytokinesis, Mob1, as a fundamental protein of the Hippo pathway, is also involved in cell proliferation control (for review see (Hergovich, 2011)). Our first approach to investigate the involvement of Mob1 in *T. gondii* replication was to study the mob1 gene expression in a time course after host cell invasion by real-time PCR analysis (Fig. 26). We observed that *mob1* expression was dramatically reduced after the parasites entered the host cell, specifically in the period when the parasites are actively replicating. Even though this is a response at the gene mRNA expression levels, this observation is consistent with a conserved function of Mob1, since in metazoans, this is a tumor suppressor protein, which when

mutated/depleted leads to cell overgrowth and the occurrence of tumors (Zeng & Hong, 2008; Hergovich, 2011). Moreover, during embryonic development, when tissues and organs are actively growing, Mob1 and the rest of the Hippo pathway core proteins are repressed, being only activated when organs reach their proper size (Zhao et al., 2011). This specific role of the Hippo pathway in cell proliferation control is an essential issue for multicellular organisms, with a supracellular organization in tissues and organs. *T. gondii*, despite being a unicellular organism as is the case of *T. thermophila*, only divides inside the parasitophorous vacuole that is established in the host cell's cytoplasm, where parasites are in close association. Furthermore, *T. gondii* tachyzoites can differentiate to bradyzoites that can be maintained in the cysts for long periods of time. In this case, the parasite replication rate is strictly controlled and the number of parasites inside the cyst is overall constant. Thus, the parasite replication (or eventual cell death) as to be adjusted to spatial constrains, positioning *T. gondii* mode of proliferation between those of free living unicellular and tissues organizing cells.

If Mob1 in *T. gondii* is also important to repressively control cell proliferation, we would expect that in a Mob1 overexpression background the parasites have reduced ability to replicate inside the cells. To test our hypothesis, we constructed a GFP-Mob1 transgenic *T. gondii* line, in which the overexpressed fusion protein is maintained stable in the presence of shield (Herm-Götz et al., 2007) (see material and methods for further details). With these genetically modified parasites, we performed replication assays to evaluate their ability to undergo replication within the host. In clear support to our hypothesis, we observed that parasites where GFP-Mob1Tg was accumulated (with shield), presented lower rates of replication since 24h after they were allowed to invade host cells, the vacuoles had mainly 1 to 4 parasites. In the case of the control parasites (without shield) most of the vacuoles showed more than 8 parasites after the same period (Fig. 30, 31). To rule out the hypothesis that this phenotype could be associated with invasion capacity, rather than only a replication defect, we did invasion assays with this line. Indeed, the phenotype observed is specific to the replication process, as in invasion assays we did not find any difference between control and GFP-Mob1 parasites (Fig. 32).

All together, these data deeply support a conserved role for Mob1 in different but interdependent aspects of cell structure and division. The study of this protein in unicellular but complex organisms allowed us to unravel the role of Mob1 in cell polarity establishment and morphogenesis, namely in the maintenance of cell structure across generations. This fact is particularly evident in our studies using *T. thermophila*,

but the conserved polarized localization in *T. gondii* is a strong evidence for a conserved function also in this parasite. Although Mob1 has been previously implicated in correct cell division, namely in cytokinesis, its clear role in linking cell polarity with proper cell division, through the correct establishment of cell division plane only now is starting to be explored.

In this *T. gondii* Mob1 study, we did not explore directly this issue, since the *T. thermophila* and *S. coeruleus* references correspond to loss of function studies. Nevertheless, Apicomplexa parasites present a very polarized cell structure with a well-defined internal anterior-posterior organization. This polarity is important in various cell functions such as invasion of the host cell. Since we did not observe any difference in invasion rates in the parasites with unbalanced Mob1 levels, and in *T. thermophila*, other permanently polarized organism, this polarity defects were only evident during cell division, it is tempting to speculate that this function of Mob1 in intracellular organization is much more critical during mitosis. Importantly, MEN is also controlled by polarity factors in yeast (Monje-Casas & Amon, 2009) and in multicellular organisms, the Hippo pathway also receives signals from cell junction molecules and other proteins involved in tissue organization that work upstream of the Hippo cascade.

Concluding, all these findings place Mob1 as a key player in cell polarity establishment, that is critical for cell division completion and cell structure maintenance. This link is particularly important in supra cellular structures organizations as is the case of *T. gondii* tissue cysts containing bradyzoites and oocysts containing sporozoites. Our studies of the parasite *T. gondii* Mob1 clearly support a role for this protein in cell proliferation control. As is the case of tissue growth and correct size maintenance in multicellular organisms, the number of parasites inside the confined structure of the vacuole has also to be tightly controlled. Indeed, *T. gondii* parasites have the ability to form cysts with a characteristic size inside the host organism in which proliferation is abolished. So, apart from the fundamental role in cell polarity and cytokinesis, our results strongly suggest that, in *T. gondii*, Mob1 is probably involved in the control of cell proliferation rates.

Although still deserving further investigation, the study of Mob1 in parasites, namely in the cyst forming ones, may open a new avenues ultimately leading to a potential new target for therapeutics and the development of an attenuated vaccine.

CHAPTER IV: CONCLUDING REMARKS AND FUTURE PERSPECTIVES

1. Concluding Remarks

The process of cell division and proliferation control is a fundamental issue in cell biology with great relevance during development, cell and tissue structure maintenance and overall organisms' homeostasis. Apart from the normal processes in which the regulation of cell division is crucial, this is also an important feature in disease development. One of the most discussed cases is cancer development. In fact, the deregulation of cell proliferation and cell death levels can give rise to tumorogenesis. It is now well established that one of the major causes of tumor development is the loss of epithelial organization. Epithelia are a very organized tissue structure in which cells are permanently polarized, with well-established apical and basal poles. In these tissues, cells present very well defined cell-to-cell interactions, positioned by the intracellular distribution of polarity factors and critical for the maintenance of tissue integrity. The disruption of this intracellular organization has consequences, for example, in the positioning of the cell division plane that, when affected, disrupts tissue organization and leads to the disease (see Chapter I).

As stated in the introduction of the present work, Mob1 is a core protein of the Hippo pathway, a fundamental signaling cascade involved in the balance of cell proliferation and apoptosis, being activated when cell proliferation needs to cease. Most of the proteins of this phosphorylation cascade, as is the case of Mob1, are therefore well established as tumor suppressors (see Chapter I). Remarkably, it was recently described that the Hippo pathway responds to the polarity factors of epithelial tissues, clearly linking both processes (see Chapter II).

Taking these data into account, we decided to take advantage of two protozoan unicellular organisms to investigate the connection between cell polarity and cell division and its implications in cell proliferation control.

First we investigated the role of Mob1 in the ciliate *T. thermophila*, a well-established unicellular model that presents a permanently polarized cell. Our studies in *T. thermophila* clearly demonstrated that the protein Mob1 is critical for the establishment and maintenance of cell polarity that is built at each cell division using the mother cell structure as a model. In *T. thermophila*, Mob1 has a polarized distribution, being mainly concentrated in the basal bodies of the posterior pole of the cell. During cell division, Mob1 also localizes in the region where the division plane is established, more precisely in the new posterior pole of the daughter cell. When this localization is inhibited, cells divide with a severe disorientation of the plane and fail to

undergo cytokinesis (see Chapter II). To our knowledge, this was the first time that Mob1 was established as being critical for cell polarity with profound implications in morphogenesis, as the depletion of Mob1 impaired the correct development of new daughter cells. Later, these results were supported by others that studied the role of Mob1 in the ciliate *S. coeruleus* (see Chapter II and III). With no doubts, the question of how the cell polarity impinges in the process of cell division and proliferation could be more easily addressed in these unicellular organisms. Polarized unicellular organisms have a complex and polarized internal organization that recalls epithelial cell structure. With our work we could conclude that Mob1 is a critical player in this issue.

As already described, the implications of this basic question go much further than basic cell biology processes. The control of cell proliferation/cell death is also a fundamental issue in infection biology as the number of parasites inside the host is directly linked to the development and severity of the infection. Knowing that Mob1 is a critical player in cell division/proliferation control in metazoans and also in the protozoan organisms studied to date, it was very likely that Mob1 function in Apicomplexa parasites was also conserved. *T. gondii* is an Apicomplexa parasite that besides being the causing agent of toxoplasmosis is also well established as a model for other Apicomplexa, all of them parasites that provoke human and animal diseases (Chapter III).

Also in *T. gondii,* Mob1 presents a polarized intracellular distribution, being localized in cell structures involved in cell division. As it is the case of *T. thermophila*, *T. gondii* is a permanently polarized cell in which the cell structure in inherited during division. Interestingly, our results concerning Mob1 in *T. gondii* support that the described role of the protein in metazoan cell proliferation control is conserved. Our observations that mRNA levels of *mob1* gene are drastically reduced during parasite replication inside the host cell and that increased levels of Mob1 cause a delay in parasite proliferation rate are in agreement with the tumor suppressor role of Mob1 (see Chapter III). These observations are particularly interesting since *T. gondii* is a cyst-forming coccidian parasite. *T. gondii* have the ability to convert the actively replicating tachyzoites inside the parasitophorous vacuole into an encysted bradyzoite form, where parasites are in close association inside the cyst that overall has a constant size during very long periods in the host. Given that, bradyzoites cysts can be considered as a tissue like structure in between the free living unicellular and the supracellular organization of multicellular organisms. In these cysts parasite number (ultimately cell number) has to be tightly regulated as it is the case of tissues. One may think that this number control

results exclusively from the immune system pressure. However, this regulation is also in the “best interest” of the parasite itself, since it allows a longer survival of the host and consecutively of the parasite (Chapter III).

Here, we showed that Mob1, as hypothesized, has a conserved role also in protozoans. Interestingly, this is the most ancient protein of the Hippo pathway which is in agreement with a fundamental role for this protein that seems also to be transversal throughout the phylogenetic tree. Our results strongly suggest that Mob1 function as a hub that connects intrinsic cell polarity to correct cell division, which has obvious implications in morphogenesis. Since Mob1 is a strongly conserved protein across the eukaryotic tree of life, it is tempting to speculate that this fundamental role in unicellular organisms may be at the base of tissues and multicellular organization, in which Mob1 is a critical player during development and tissue’s homeostasis. Therefore, our work contributed to a better understanding of Mob1 function in two different but intimately interconnected aspects. In one hand, we could access a more fundamental role of this protein as a tumor suppressor by linking cell polarity to proper cell division. On the other, we identified a potential therapeutic target of cell proliferation control, critical in many characteristics of disease such as cancer development and uncontrolled parasite replication during infection.

2. Future Perspectives

Apart from the contribution that the present work already gave to our understanding of Mob1 proteins in protozoan, the research presented and discussed here open new doors of investigation with a great potential.

Regarding our work in *T. thermophila*, there are two major aspects that would deserve further investigation. It is worth mention that the involvement of Mob1 in cilia biogenesis is a totally new data that at a first glance do not seem to match with the already described functions for this protein. Indeed, this unexpected role of Mob1 brings up the idea that the link between cell polarity and morphogenesis is probably not confined to cell division time. Also, other very curious result of our studies in *T. thermophila* is the fact that inside the cell there are distinct populations of basal bodies, at least with different molecular compositions, that are not a result of different age. In fact, Mob1 is enriched in the posterior pole basal bodies and in this ciliate, new basal bodies arise in an intercalated fashion along the ciliary rows.

This polarized distribution of a protein, not in the cytoplasm, but in a specific structure has certainly a functional relevance that should be investigated. In both cases, the next step in the way to clarify these aspects would be to:

- Search and characterize the Mob1 partners, namely the ones involved in cilia biology and morphogenesis;
- Investigate if any of Mob1 partners also present a localization concentrated in the posterior pole basal bodies to access the biological significance of this observation, namely in processes not directly involved in cell division.

These new investigation would allow to dissect unexplored but fundamental roles for Mob1 most likely conserved throughout the eukaryotic tree of life.

In the particular case of the study of Mob1 protein in *T. gondii*, the obtained results clearly demonstrate that further investigation of Mob1 function in this apicomplexan parasite, namely in the regulation of cell proliferation control, would allow to dissect the specific role of the protein in bradyzoite cysts differentiation, a matter of open debate. The ability to convert highly proliferative tachyzoites into the latent bradyzoites that can live encysted in the host for long periods of time in a “tissue-like” structure confers an

adaptive advantage to this parasite in terms of long term survival. In this context, to decipher the cellular and molecular mechanisms of this conversion would allow developing more effective strategies to fight infection.

In a direct follow up of the present work it would be crucial to:

- Study the Mob1 loss of function phenotype in *T. gondii*. The construction and characterization of knockout (KO) strains would allow to investigate if also in this scenario the role of the protein is conserved
- Perform stage conversion assays *in vitro* using genetically modified *T. gondii* parasites (both overexpression and KO) to dissect the involvement of the protein in the process.
- Investigate the Mob1 interacting proteins, namely the potential Hippo pathway homologue proteins, to explore the mechanistic way of action of Mob1 in *T. gondii*.

Together, these experiments would allow to obtain a widespread view of Mob1 mode of action in *T. gondii*.

As a more long term goal, it would be very important to transpose all this questions into *in vivo* experiments, namely in the mouse model. Only in the animal context the question of Mob1 involvement in cyst formation, by controlling proliferation rates, could be fully dissected. In this context, it would be of great significance to investigate Mob1 protein levels and expression dynamics in bradyzoites cysts in comparison to tachyzoites. Furthermore, apart from the study of endogenous levels in non-transformed parasites, the use of genetically modified strains to induce infection in the animal would allow to acutely comprehend the involvement of Mob1 in the infection biology of *T. gondii*.

Altogether, the data to be obtained with these further experiments would allow opening new opportunities for controlling *T. gondii*, and ultimately Apicomplexa parasites, proliferation with direct impact on infection control.

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