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THE ROLE OF PHOSPHORYLATION IN THE REGULATION OF THE CHROMOKINESIN XKID

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Para os cúmplices da minha vida: os meus pais.

Para ti, Lili, a pessoa mais maravilhosa que eu conheço,
e responsável de pôr sentido na minha vida.

Juntos, conseguimos qualquer objectivo!

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Recordar es vivir! Yo, recuerdo!

ABSTRACT

Xkid is a *Xenopus* chromokinesin required for metaphase chromosome alignment and for meiosis I to meiosis II transition in oocytes.

The aim of this work was to study the regulation of Xkid by phosphorylation using the *Xenopus* oocyte and egg extract systems. To achieve this, a reliable method to express proteins in egg extract by addition of *in vitro* transcribed mRNAs was established.

Xkid was found to be efficiently phosphorylated in meiosis and mitosis at the cdk1 site. Although phosphoXkid localized efficiently to the mitotic chromosomes, phosphorylation at the cdk1 site had no role in the binding of Xkid to the chromosomes but prevented the protein to localize to the spindle microtubules like the endogenous protein. The dominant negative effect on spindle assembly of a phospho mimicking form of Xkid indicated that phosphorylation plays an important role in the regulation of Xkid function. Several partners for Xkid were identified.

RESUMEN

Xkid es una cromoquinesina del sistema de *Xenopus*, necesaria para el alineamiento de los cromosomas en la placa metafásica y para la transición entre la meiosis I y meiosis II en los oócitos.

El objectivo de este trabajo era estudiar la regulación de Xkid por fosforilación en los oócitos y en el extracto de huevos de *Xenopus*. Para poder cumplirlo se estableció un método para la expresión de proteínas añadiendo al extracto de huevos ARN mensajeros sintetizados *in vitro*.

Los resultados obtenidos sugieren que Xkid es eficientemente fosforilada en el sitio cdk1 durante la meiosis y la mitosis. Aunque la forma de Xkid fosforilada se localiza eficientemente a nivel de los cromosomas mitóticos, esta fosforilación no parece tener ningún papel regulador sobre esta localización. En cambio, parece interferir con la localización de Xkid sobre los microtúbulos mitóticos. El efecto dominante negativo de la forma de Xkid que mimetiza la fosforilación durante la formación del huso mitótico, sugiere además que la fosforilación desempeña un papel importante en la regulación de la función de Xkid. Finalmente, varias proteínas que interaccionan con Xkid han sido identificadas.

PREFACE

The work outlined in this thesis was carried out in the Cell and Developmental Biology Program at the CRG (Center for Genomic Regulation), and finished at the European Molecular Laboratory (EMBL) (Heidelberg, Germany) in the laboratory of Professor Iain Mattaj.

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The thesis provides a detailed description of the establishment of a useful methodology using the *Xenopus* egg extract system.

ABBREVIATIONS

APC	Anaphase promoting complex
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
ºC	Celsius grade
CaMK II	Calmodulin-dependent kinase II
Cdk	Cyclin dependent kinase
cDNA	Complementary DNA
Cenp-E	Centromere protein E
Clasp	Clip-associated protein
CPC	Chromosomal passenger complex
CSF	Cytostatic factor
C-terminal	Carboxy-end of protein
Da	Dalton
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EB	End-binding proteins
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(b-aminoethyl ether) N,N,N´,N´ tetraacetic acid
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
GVBD	Germinal vesivle breakdown
HCG	Human chorionic gonadotropin
HEPES	N-(2-hydroxyethyl)piperizine-N´-(2-ethanesulfonic acid)
His	Histidine
Human	*Homo sapiens*
HRP	Horseradish Peroxidase
IPTG	Isopropyl b-D-thiogalactopyranoside
IF	Immunofluorescence buffer
Kd	Kilo dalton
Kinesin	Conventional kinesin

KLP	Kinesin like-protein
M	Molar
MAP	MT-associated protein
MAPK	Mitogen-activated protein kinase
MCAK	Mitotic Centromere-associated kinesin
MPF	Maturation Promoting Factor
MT	Microtubule
MTOC	Microtubule organizing center
MI	Meioisis I
MII	Meiosis II
mA	MilliAmpere
mg	Milligramme
min	Minute
ml	Milliliter
mM	MilliMolar
µg	Microgramme
µl	Microliter
NIMA	Never in Mitosis A
NuMA	Nuclear mitotic apparatus protein 1
NLS	Nuclear localization signal
N-terminus	Amino-terminal-end of protein
nM	NanoMolar
nm	Nanometer
ORF	Open reading frame
PCM	Pericentriolar material
PCR	Polymerase Chain reaction
PBS	Phosphate Buffered Saline
PGE	Progesterone
PXkid	Phospho-Xkid
PIPES	Piperazine-N,N´-bis(2-ethanesulfonic acid)
PLK1	Polo-like kinase 1
PMSG	Pregnant mare serum gonadotropin

pSer	Phosphoserine
pThr	Phosphothreonine
RCC1	Regulator of chromosome condensation
RNA	Ribonucleic acid
rpm	Rotations per minute
RT	Room temperature
SAFs	Spindle assembly factor
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
sec	Second
Tris	Tris(hydroxymethyl) amniomethane
Xenopus	*Xenopus laevis*

CONTENTS

1. INTRODUCTION

1.1 THE CELL

The cell is the smallest unity that composes the organisms. There are two kinds of cells: the prokaryotic (such as the bacterial cells), and the eukaryotic cells (such as the human cells). The main difference between them is the absence of a membrane surrounding the nucleus, in the case of the prokaryotic cells. Thus, the genetic material is located in the cytoplasm.

The eukaryotic cell contains one nucleus surrounded by a nuclear envelope that encloses the genetic material (the DNA) which is the main component of the chromosomes. In addition, it contains other organelles usually enclosed by a lipid membrane: the endoplasmic reticulum, the Golgi Apparatus, the lysosomes, the peroxysomes, the ribosomes and the endosomes. The intracellular organization of these organelles and their function depend in large part on the cytoskeleton. In most eukaryotic cells, the cytoskeleton is composed of three types of filaments: microtubules, microfilaments (actin) and intermediate filaments.

1.1.1 Microtubules

Microtubules (MTs) are large polymers of α and β tubulin dimers, that bind head-to-tail forming protofilaments (fig 1.1). Each microtubule consists of 13 individual protofilaments that interact longitudinally to form a hollow tube 25 nm in diameter. They have two distinguishable ends: the minus (α-end) which is close to the cell center and the plus-end (β-end), which is close to the cellular membrane. In physiological conditions, polymerization (growth) and depolymerization (shrinkage) occur at both ends of microtubules. The plus-end grows faster and is more dynamic than the minus end.

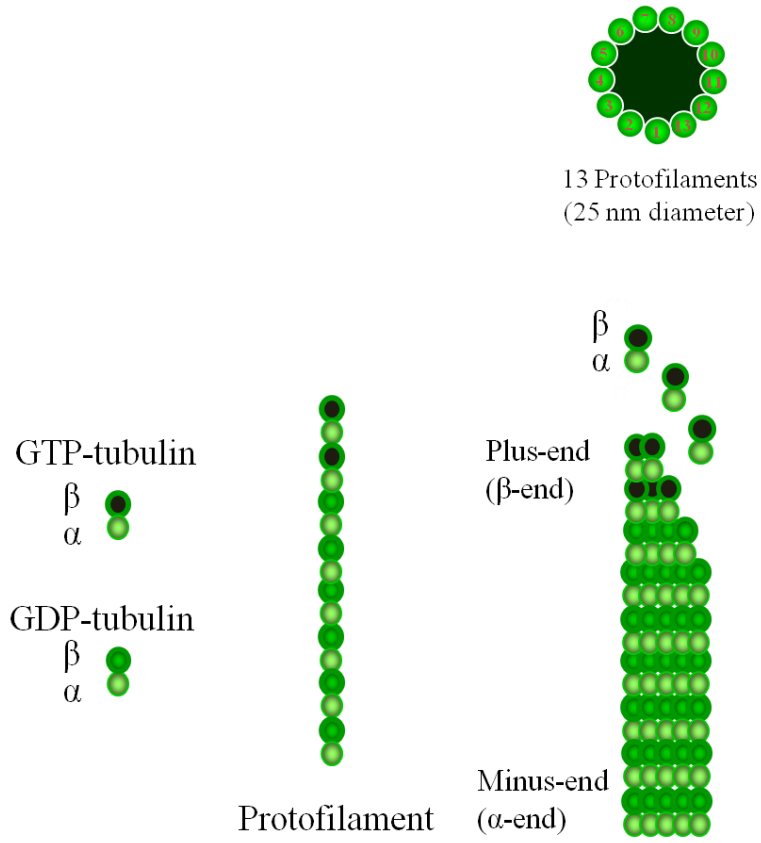


Figure 1.1. Schematic representation of microtubules structure. Microtubules are dynamic polymers that are assembled from tubulin heterodimers, which present intrinsic polarity: the minus-end and the plus-end.

Polymerization occurs by the assembly of tubulin dimers in the presence of GTP (fig 1.2). GTP hydrolysis that occurs only at the β-tubulin subunit, contributes to microtubule properties by releasing energy that is stored in the microtubule lattice, increasing its curvature and generating instability in the growing microtubule (Desai and Mitchison, 1997).

Thus it has been proposed that a cap of GTP-bound tubulin contributes to microtubule polymerization. When GTP hydrolysis takes place at the tip of the microtubule, this promotes a rapid depolymerization and microtubule shrinking. In 1984, Mitchison and Kirschner described this general property of microtubules and named it “dynamic instability” (Mitchison and Kirschner, 1984a) : microtubules are intrinsically dynamic and can undergo stochastically fast transitions from the growing state to the shrinking state (named catastrophe) or from the shrinking state to the growing state (rescue).

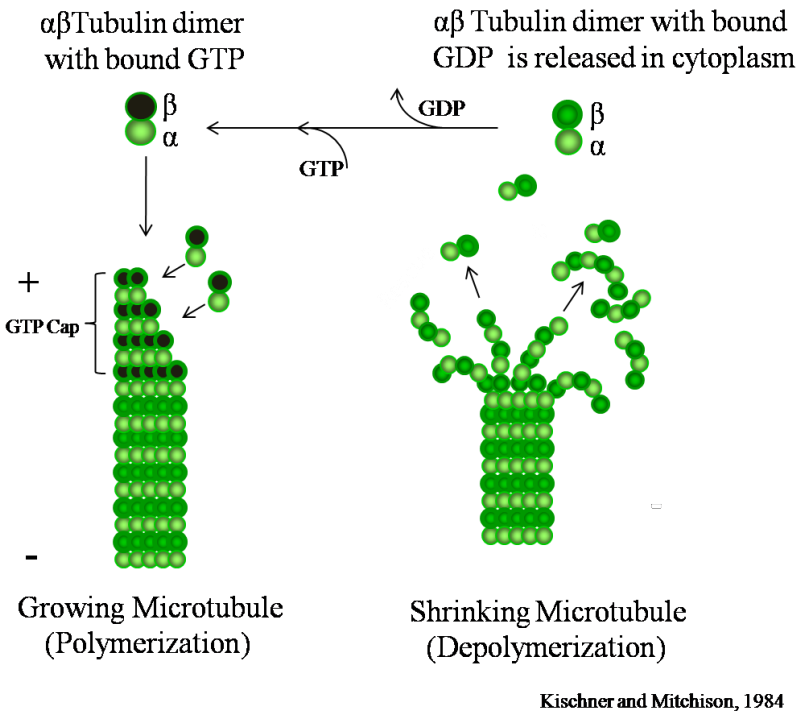


Figure 1.2. Microtubules undergo periods of polymerization and depolymerization and interconvert randomly between these states, a property named as dynamic instability. This property is presented at both ends of microtubules although. However, the plus ends are more dynamic than the minus-ends.

1.1.2 Centrosomes

Microtubules are nucleated and organized by the Microtubule Organizing Centers (MTOCs). One of the components of MTOC is gamma tubulin that provides a minus-end nucleation template for microtubule growth. The inhibition of gamma tubulin results in the inhibition of microtubule nucleation. The gamma-tubulin associates with several other proteins to form a circular structure known as the "gamma-tubulin ring complex." This complex acts as a scaffold for α/β tubulin dimers to begin polymerization; it acts as a cap of the minus end while microtubule growth continues away from the MTOC in the plus direction (fig 1.3 A). Thus, microtubule minus-ends are often stabilized or are anchored at the MTOC whereas the plus-ends explore the cellular space.

Centrosomes (the main type of Microtubule Organizing Center) were first described during the 1800s by Theodor Boveri, based on their central position in the cell. Centrosomes are small cellular non-membranous organelles formed by two centrioles which are surrounded by a mass of proteins forming the pericentriolar material (PCM) (fig 1.3 B). Each centriole is formed by an assembly of nine triplets of microtubules.

Centrosome duplication occurs during the S-phase of the cell cycle. At the end of G2 when the cell is committed to enter mitosis, the duplicated centrosomes separate and reach opposite sites along the nuclear envelope.

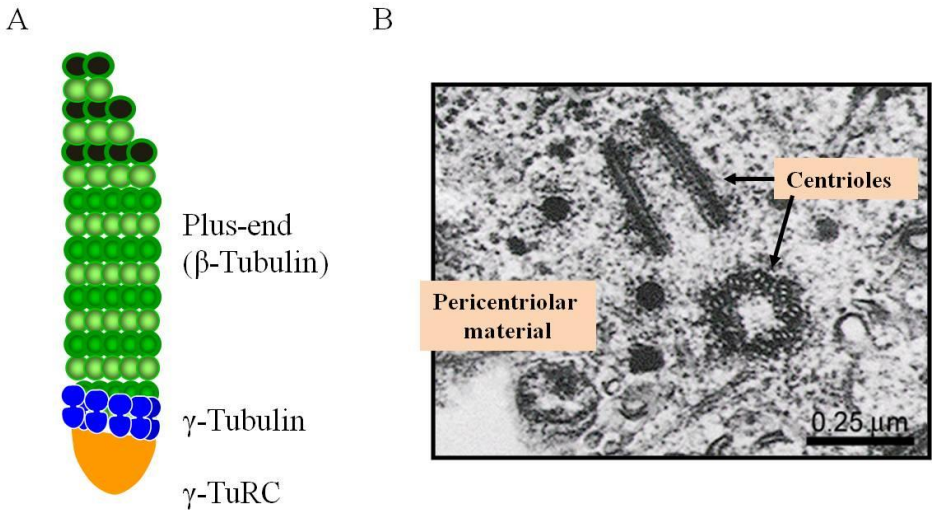


Figure 1.3. The Microtubule Organizing Centers (MTOCs).
(A) The "γ-tubulin ring complex", acts as a scaffold for α/β tubulin to initiate polymerization.
(B) An electron microscopy section showing the two centrioles and the pericentriolar material.

MTOCs have a common conserved function in microtubule nucleation and organization in most animal cells. Therefore they participate in all microtubule-dependent processes, including organelle transport, cell shape, polarity and motility. In interphase, the centrosome generates a polarized radial MT array, with MT minus-ends focused at the centrosome and the rapidly growing plus-ends reaching out into the surrounding cytoplasm. Centrosomes also play a critical role during mitosis when they become tightly associated to each of the spindle poles defining their position and therefore spindle orientation and the cell division axis (Bettencourt-Dias and Glover, 2007; Nigg, 2002).

1.1.3 Microtubule-associated proteins (MAPs and motors).

The different families of microtubule-associated proteins (MAPs) can be subgrouped in two main classes: the structural MAPs and the motor proteins. Structural MAPs usually stabilize and stimulate the assembly of microtubules. The motor proteins move

organelles and proteins along microtubules participating in their organization and dynamics.

1.1.3.1 STRUCTURAL MAPS

The structural MAPs stabilize or destabilize MTs by binding to the sides or ends of microtubules. A number of structural MAPs have been well characterized in the *Xenopus* system. XMAP215 modulates microtubule dynamics by speeding the microtubule plus-end growth and protecting them from the microtubule depolymerizing activity of the motor protein MCAK (Walczak et al., 1996). XMAP230 is needed for normal spindle assembly through MT stabilization activity (by decreasing catastrophes) (Cha et al., 1999) and XMAP310 increases the rescue frequency (Andersen and Karsenti, 1997). The Clasp proteins are plus-end microtubule binding proteins important for kinetochore function during mitosis. In *Xenopus,* XOrbit/Clasp has been proposed to be responsible for a stabilizing activity that is important for spindle assembly and chromosome segregation (Hannak and Heald, 2006).

Recently, using mass spectrometry Popov et al (2010) identified 43 new possible microtubule- bound proteins in the *Xenopus* egg system.

1.1.3.2 MOTORS

Microtubule motors are proteins that use the energy derived by ATP hydrolysis to move along the surface of microtubules. The known motors belong to two structurally different families: the dyneins and kinesins. The dyneins are very large protein complexes composed of two heavy chains, multiple light and intermediate chains (fig 1.4 A). These motors move towards the minus-end of microtubules, and there are two kinds of dyneins: the cytoplasmic dyneins and axonemal dyneins. The cytoplasmic dyneins are involved in the movement of cytosolic vesicles and kinetochores during meiosis and mitosis (Ross et al., 2008).The axonemal dyneins perform their function in cilia and flagella.

In mammalian cells, there are 14 kinesin subfamilies, also known as kinesin-related proteins (KRPs) or kinesin-like proteins (KLPs). All share a conserved motor domain but differ in the amino acid sequence outside the motor domain, suggesting their different roles depend on their non-motor domain. At the molecular level, kinesin 1

(conventional kinesin) is a heterotetramer formed by 2 heayy chains, responsible for the motor activity through its binding to the microtubule, and by two light chains which mediate protein-protein interactions helping to move vesicles and their contents in a directed manner (fig 1.4 A). The kinesin movement along microtubules is powered by the hydrolysis of ATP (fig 1.4 B). Depending on the position of the motor domain different kinesins can move towards the microtubule minus-end (motor domain located at the C-terminus) or towards the plus-end (motor domain at the N-terminal or in the middle of the protein).

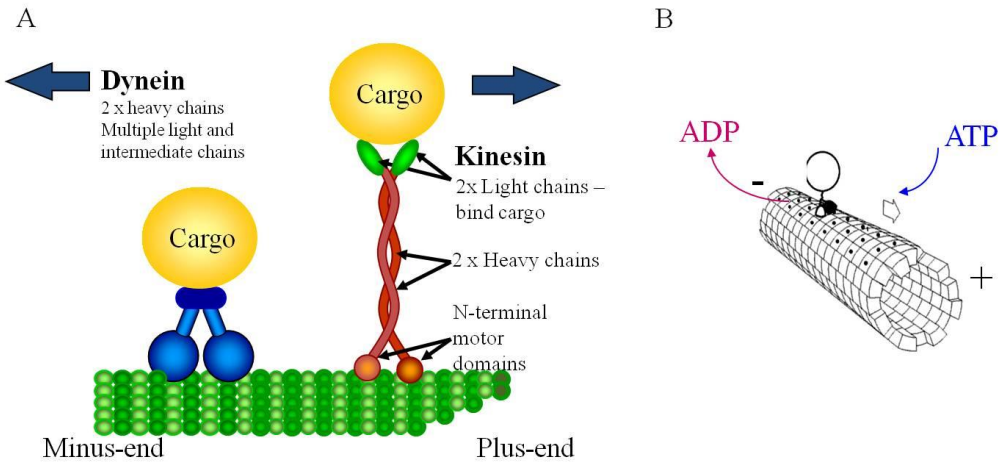


Figure 1.4. Diagram showing the structure and movement of microtubule motors.
(A) Dynein moves towards the minus ends of microtubules. Kinesin-like motors are active as dimers. Some members move toward the plus-end of microtubules, others move toward the minus-end.
(B) Microtubules motors use energy derived from ATP hydrolysis to move along the microtubules.

There are orthologs of the kinesin subfamily in different species. In *Xenopus,* several members of this family have been identified and some of them have been studied functionally (Table I) (Loughlin et al., 2008; Wordeman). The majority have important functions during cell division. They are associated with the spindle or the kinetochores and play a role in the attachment of microtubules to kinetochores, the regulation of microtubule dynamics, the establishment of spindle bipolarity, chromosome movements and cytokinesis. It is noteworthy that, a particular class of kinesin–like proteins are nuclear during interphase and associate with chromosome arms during cell division. These motors called chromokinesins, have been proposed to function in several aspects of spindle assembly and chromosome segregation. They belong to either of two families: Kinesin-4 or kinesin-10 (Mazumdar and Misteli, 2005).

Kinesin-4 motors have been described in human (HKIF4A and HKIF4B), mouse (KIF4), chicken (CHRKIN), *Drosophila* (KLP3A), *C.elegans* (KLP-19), *Xenopus* (XKLP1) and plant. The kinesin-10 family includes *Xenopus* Xkid and orthologs in human (kid), *Drosophila* (Nod) and mouse (skl4).

Table I. Kinesin-like motor proteins described previously in the *Xenopus laevis* system.

Protein Name	Family	Full Name	Direction	MW kDa	Mitotic Localization	Proposed function
XCenp-E	7	Centromere protein E	(+) end	312	Centromere/ Kinetochore region	Chomosome congression (Wood et al., 1997)
XCTK2	14	C-terminal kinesin-like protein 2	(-) end	75	Mitotic spindle	Bipolar spindle assembly (Walczak et al., 1997)
XEg5	5	Kinesin-related motor protein Eg5	(+) end	120	Mitotic spindle	Crosslinks MTs and slides antiparallel Mts outward (Kapitein et al., 2005; Mitchison et al., 2005)
XKCM1	13	kinesin catastrophe modulator-1	Internal motor domain	≈90	Centromeres and centrosomes	Depolymerizes kinetochore MTs (Walczak et al., 1996)
Xkid	10	kinesin-like DNA binding *protein*	(+) end	72	Chromosome arms	Chromosome alignment (Antonio et al., 2000; Funabiki and Murray, 2000)
XKLP1	4	kinesin-like *protein* 1	(+)end	140	Chromosome arms	Bipolar spindle stabilization and chromosome positioning (Vernos et al., 1995)
XKLP2	13	kinesin-like protein 2	(+)end	≈160	Centrosomes and Spindle Poles MTs	Centrosome separation (Boleti et al., 1996)
XKif2A	13	kinesin family 2A	Internal motor domain	95	Centromeres and spindle poles.	MT dynamics regulation (Ganem and Compton, 2004)

Adapted from Loughlin et al, (Cell, 2008) and Wordeman L., (2010,Seminars in cell and developmental biology)

1.1.4 Chromosomes.

Chromosomes are the structures that contain the genetic information of the cell. The eukaryotic chromosomes consist of chromatin, a complex of DNA and proteins that condenses during cell division making the chromosomes to become visible. Thus, chromosome condensation is one of the signals of the start of mitosis (Johnson and Rao, 1971).

Chromosome morphology and behavior changes during the cell cycle. During G1 phase, the cell accumulates resources for DNA replication. At this point, the DNA and its associated proteins are uncoiled in thin strands, the chromatin. At the S phase, DNA

replication takes place in order to create the duplicated DNA strand. They consist of two sister chromatids joined by a centromere. During the M phase, the sister chromatids condense to form the chromosomes. The metaphase chromosomes are formed by two sister chromatids attached via their centromere (fig 1.5). At the beginning of anaphase, the chromatids are separated, becoming free and moving toward opposite poles. At telophase, the chromosomes decondense and form long and thin chromatin threads.

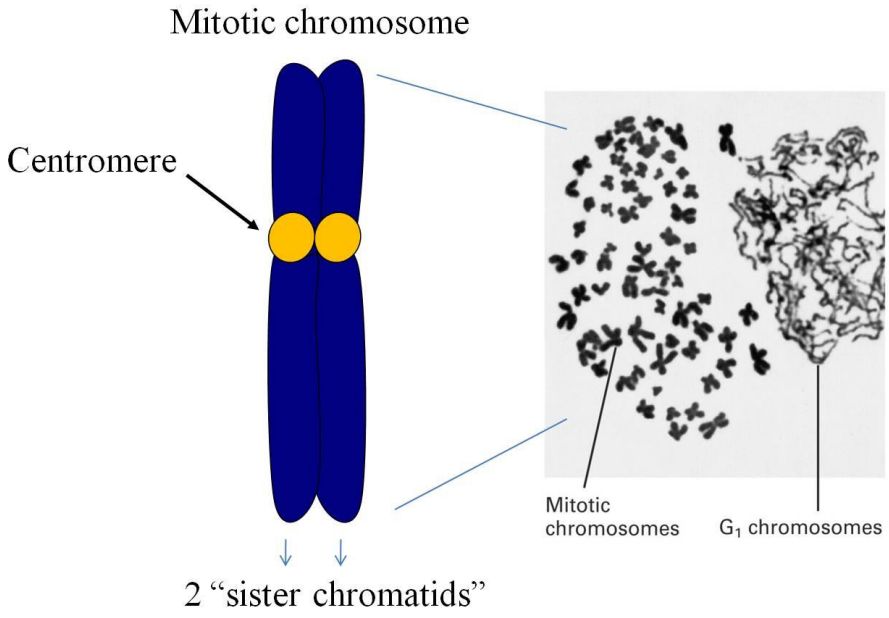


Figure 1.5. The appearance of chromosomes in mitosis and in G1 phase.

1.2 THE CELL CYCLE

"All eukaryotic organisms are composed of cells, and cells are the smallest independent units of life" (Schleiden and Schwann, 1837).

"All cells arise only from pre-existing cells" (Rudolf Virchow,1855).

These two statements in combination with advances in microscopy and cell biology tools have been influential in shaping the biological sciences. Altogether, they provided a basis for understanding the composition of cells and the molecular details of individual processes during the cell cycle.

The German anatomist Walther Flemming was one of the first to describe the cell division process. In 1882 he coined the term "mitosis" to characterize the formation of paired threads (Greek=mitos) during division of the cell nucleus. These threads, which

formed from a substance Flemming called chromatin, came to be known as the "chromosomes". The definition of mitosis has since then been expanded to include "cytokinesis" the process by which the cell cytoplasm is partitioned at the end of nuclear division (Flemming, 1965).

Until 1950, the biologists did not have any information about what happened in between two mitosis. Auto-historadiography and cell culture synchronization techniques, which allowed different cells to be at the same specific stages of the cell cycle in a culture, were crucial to dissect what happens during interphase. In 1953, Howard and Pelc, suggested that interphase is divided in three phases: cell growth (G1-phase), DNA replication (S-phase for DNA synthesis) and cell growth (G2-phase). Their statement survives up to the present day (fig 1.6).

Thus, the cell cycle is the group of events that take place from the moment a cell is formed and then divides again into two daughter cells through mitosis. The cell cycle is divided into two major phases: M-phase and interphase. The M-phase includes the events of mitosis and cytokinesis. During interphase the DNA is diffusely distributed within the nucleus, and individual chromosomes cannot be distinguished. As the cell commits to divide, the chromosomes condense and become segregated by the spindle to form the daughter nuclei.

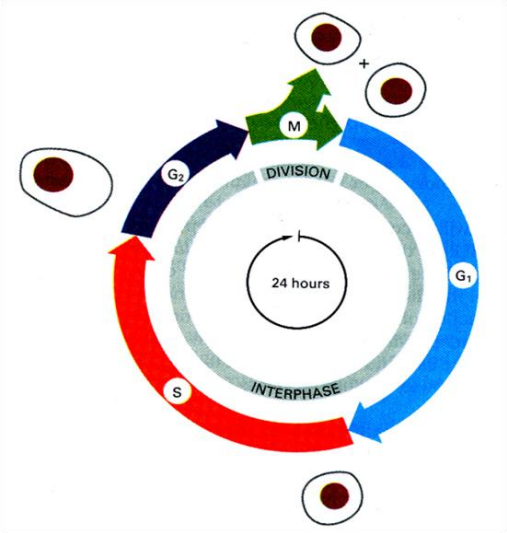


Figure 1.6. The cell cycle is divided in interphase, which includes: the G1 phase, the S phase (synthesis), and the G2 phase. The M-phase is composed of mitosis (the cell chromosomes are divided between the two daughter cell's) and cytokinesis (the cytoplasm is divided between the two daughter cells).

1.2.1 Mitosis

The primary result of mitosis is the division of the parent cell genome into two daughter cells. The equal distribution of the genome during cell division in eukaryotes is ensured by the action of a large macromolecular machine consisting of the mitotic spindle, an ensemble of microtubules, and numerous molecular motors, including kinesins, dyneins and myosins which are responsible for actin-based motility. Spindle assembly and function require localized regulation of microtubule dynamics and the activity of the microtubule-based motor proteins. The number of molecules that contribute to spindle assembly and dynamics continue to expand our understanding of their role and regulation remains still incomplete. To date, different regulatory pathways have been characterized such as, the RanGTP gradient, and a temporal and spatial regulation by kinases and phosphatases that altogether define microtubule nucleation and stabilization. In the last several years, many efforts have been done in order to identify the molecular factors that are regulated by these different mechanisms.

1.2.1.1 PHASES OF MITOSIS

The different stages of mitosis were already very well described by Fleming .The advances in different techniques such as cell culture methods, microscopy and the development of antibodies produced an explosion of information on the composition and modifications of the spindle apparatus (fig 1.7).

Mitosis consists of two important events: Karyokinesis is the division of the nuclear material and cytokinesis, the division of the cytoplasmic material**.**

The first sign that a cell is about to enter mitosis is a period called prophase. The centrosome separation takes place at the very beginning of prophase. The centrioles and other components of the centrosome are duplicated in interphase cells, but they remain together on one side of the nucleus until the beginning of mitosis. Each MTOC or centrosome polymerizes microtubules, forming the astral microtubules. These microtubules and the pericentriolar material constitute the asters. Thus, there is the formation of two asters that progressively migrate to opposite sides of the nucleus to define the poles of the mitotic spindle. Furthermore, the microtubules that are growing between the centrosomes contribute to the mitotic spindle. In prophase, the chromatin starts to condense to form the chromosomes.

The prometaphase is characterized by the abrupt nuclear envelope break down. Each chromosome initially becomes attached to one pole of the spindle, this is mediated by the attachment of MTs to the kinetochores, specialized protein structures localized at the primary constriction of each sister chromatids (centromere).

At metaphase, all chromosomes become bi-oriented and reach the equator of the spindle, midway between the spindle poles. At anaphase, the sister chromatids synchronously separate and move to opposite spindle poles. This stage can be further subdivided into anaphase A and B. During anaphase A chromosomes loose their cohesion, split apart and each chromatid moves towards one spindle pole. During anaphase B, the spindle elongates separating furthermore the two groups of chromatids. In telophase, the two sets of daughter chromosomes that have reached the poles of the spindle, start to decondense and the nuclear envelope assembles around them to form the daughter nuclei.

Cytokinesis consists of the partition of the whole cytoplasm to separate the two daughter cells. The contractile ring gives rise to the midbody, a structure positioned between the two new daughter cells. The whole process is finished when abcission completely separates the cytoplasm of the new cells.

Although the goal of mitosis, the equal segregation of chromosomes, is achieved in anaphase, it is becoming increasingly clear that the proper attachment and alignment of chromosomes, which occurs during prometaphase, is the defining aspect of mitosis that holds the key to understanding the fidelity of genome inheritance.

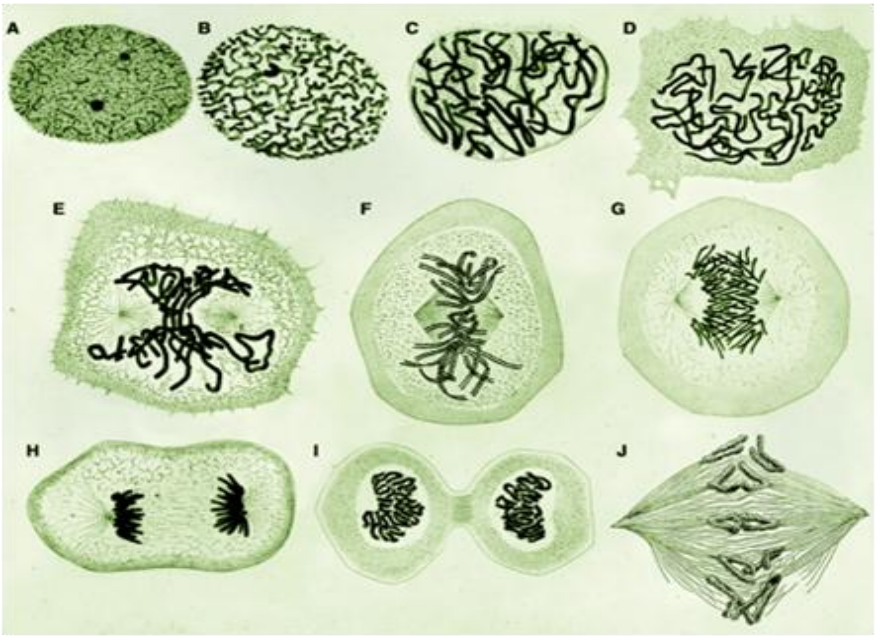


Figure 1.7. Flemming's illustrations for the different stages of mitosis in newt cells. During prophase (A to C) the chromosome start to condense. After nuclear envelope breakdown (D), the chromosomes start to interact with the two separating centrosomes (E) to constitute the spindle-apparatus (E and F). The metaphase (G) is the moment where the chromosomes are lined up on the equator of the cell. In anaphase (H and I) the bounds between the sister chromatids dissolve and they move to the opposite poles. (J) Drawinf from Schrader's showing the kinetichore fibers (K-fiber) during early anaphase in *Lilium.*

1.3 MECHANISM OF SPINDLE ASSEMBLY

1.3.1. The regulation of microtubule dynamics between interphase and mitosis

MTs are continuously being built and depolymerized in living cells. During interphase, the relative stable and long network of filaments radiate from the center of the cells and extend to the periphery of the cells. When mitosis takes place, the first signal in prophase is that the long interphase microtubules disappear and are replaced by short and highly dynamic microtubules that organize a mitotic spindle apparatus. Consequently, a change in cell shape and reorganization of cell cytoplasm is produced (fig 1.8). The MT turnover between interphase to mitosis transition increases dramatically: MT half-life change from 5 minutes to 45 seconds and as a consequence its turnover changes from 5 to 10 folds in the mammalian cells (McIntosh, 1984; Saxton et al., 1984).

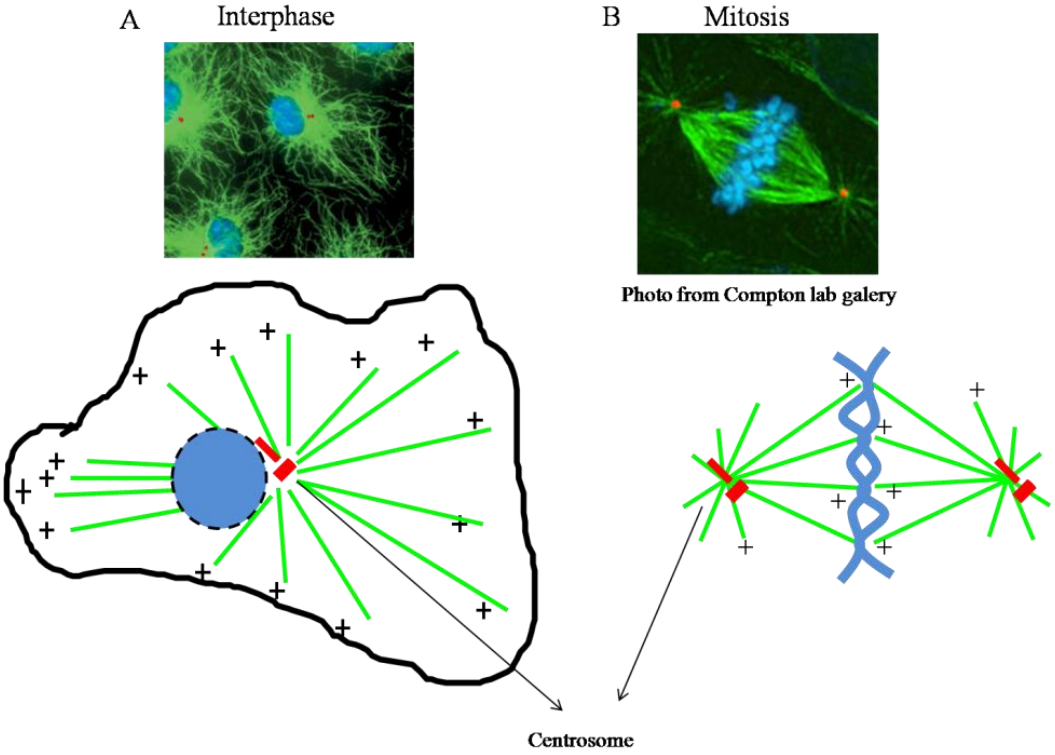


Figure 1.8. Image and cartoon of interphase (A) and mitotic somatic cells (B) showing the structural changes of the MT network during spindle assembly.

Microtubule dynamics, MAPs and motors contribute to the reorganization of the spindle microtubules to form the bipolar spindle (Maddox et al., 2003; Shirasu-Hiza et al., 2003). Some are stabilizing and others are catastrophe factors. During mitosis there is a change in catastrophe and rescue frequencies. The catastrophe frequency is modulated by the activity of "catastrophe" factors (McNally, 2003; McNally, 1999) (fig 1.9).

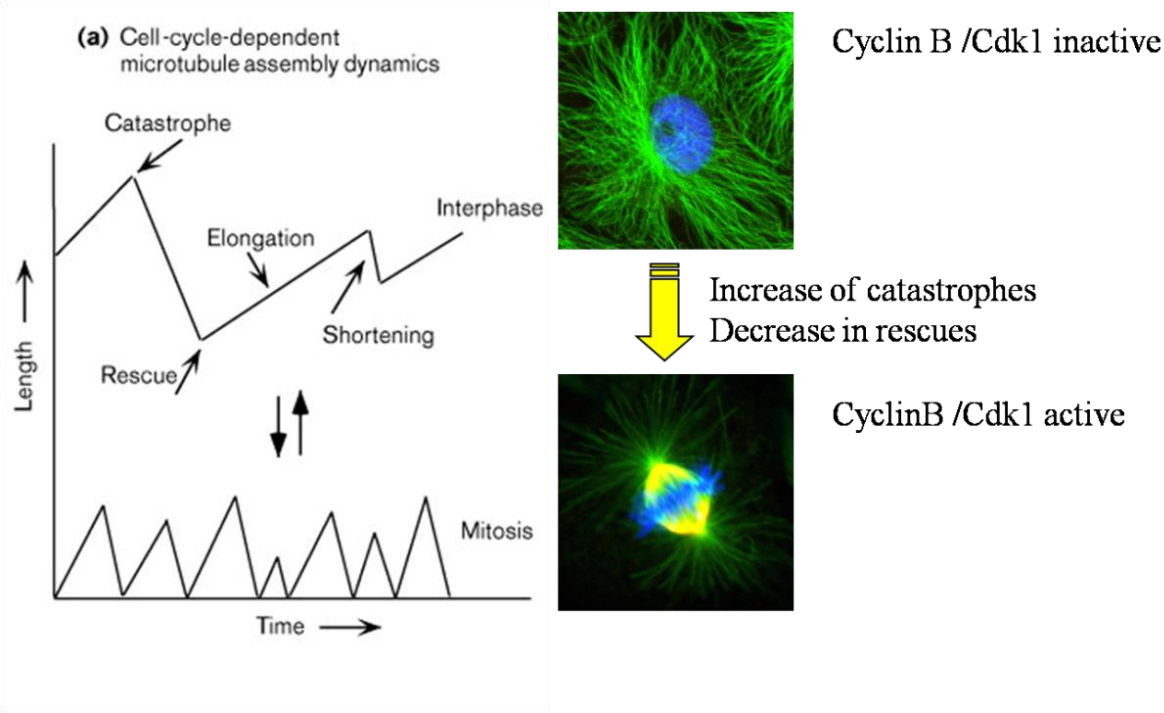


Figure 1.9. Graphic showing the changes in MT turnover during the transition between interphase and mitosis: the relative stable and long MTs are substituted by highly dynamic and short mitotic MTs.

Some have been well characterized in the *Xenopus* egg extract: katanin is responsible for severing MTs (McNally and Thomas, 1998), Op18/Stathmin sequesters tubulin and destabilizes MTs by promoting catastrophes (Belmont and Mitchison, 1996) and XKCM1 promotes MT depolymerisation during mitosis (Walczak et al., 1996). XMAP215, EB1 and CLASP/XOrbit contribute to MT polymerization. XMAP250 localizes along the MTs and stabilize them by cross-linking adjacent tubulin units. The MT-plus end tracking proteins (+TIPs) include end-binding proteins (EBs), Clip associated proteins (CLASPs/ XOrbit) and adenomatous polyposis coli (APC). These proteins accumulate specifically at the plus-end of MTs stabilizing them and showing an anti-catastrophe activity (fig 1.10).

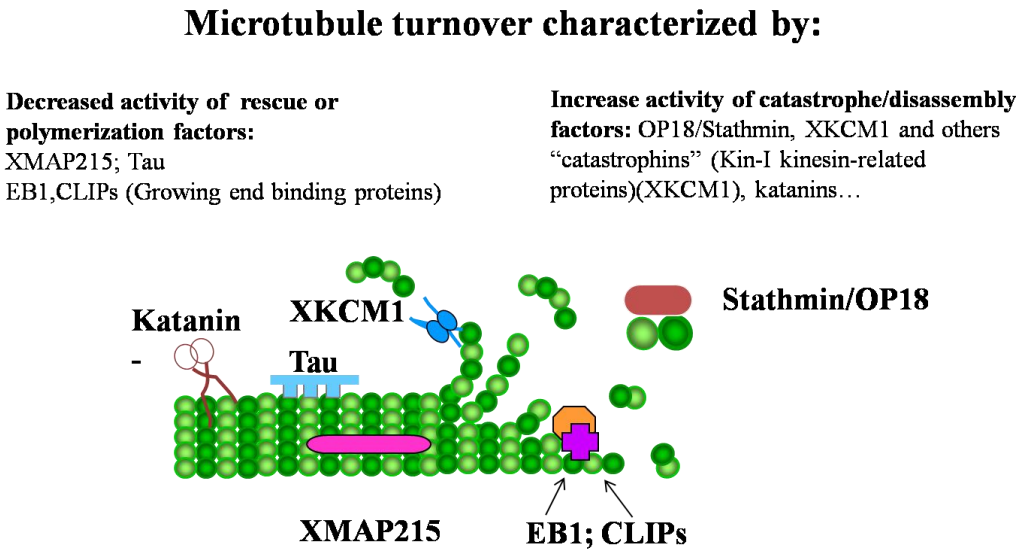


Figure 1.10. Drawing of the different MAPs and Motors that modulate microtubule dynamics: Some promote MT stabilization while others promote MTs desestabilization.

1.3.2 The centrosomal and acentrosomal pathways

How the MTs of spindle apparatus grow and how chromosomes achieve bi-orientation during cell division, are questions that lead researchers to propose different models to explain spindle assembly. Kirschner and Mitchison proposed the "search and capture model", where the centrosomes have a key role for bipolar spindle assembly by nucleating microtubules (Kirschner and Mitchison, 1986; Mitchison et al., 1986; Mitchison and Kirschner, 1984b). Upon mitosis entry the two centrosomes move apart to provide the spindle poles of the spindle. Microtubule associated-proteins (MAPs and Motors) crosslink the antiparallel MT array between the poles and help to focus the spindle poles (Walczak et al., 1998). MTs emanating from a centrosome grow and shrink randomly "searching" the space until they attach to kinetochores. Thus, the sister chromatids become bi-oriented and oscillate near the center of the spindle. At metaphase, the bipolar spindle will be formed by both sister chromatids bi-orientated and by three classes of microtubules (fig 1.11):

-astral microtubules: radial array of microtubules projected from spindle poles bodies
-Interpolar microtubules: emanate from the two centrosomes and are not attached to chromosomes. In addition, they are stabilized by overlapping with each other in the center of the cell.

-Kinetochore-microtubules: attach to the condensed chromosomes of mitotic cells at their centromeres forming the kinetochore fiber.

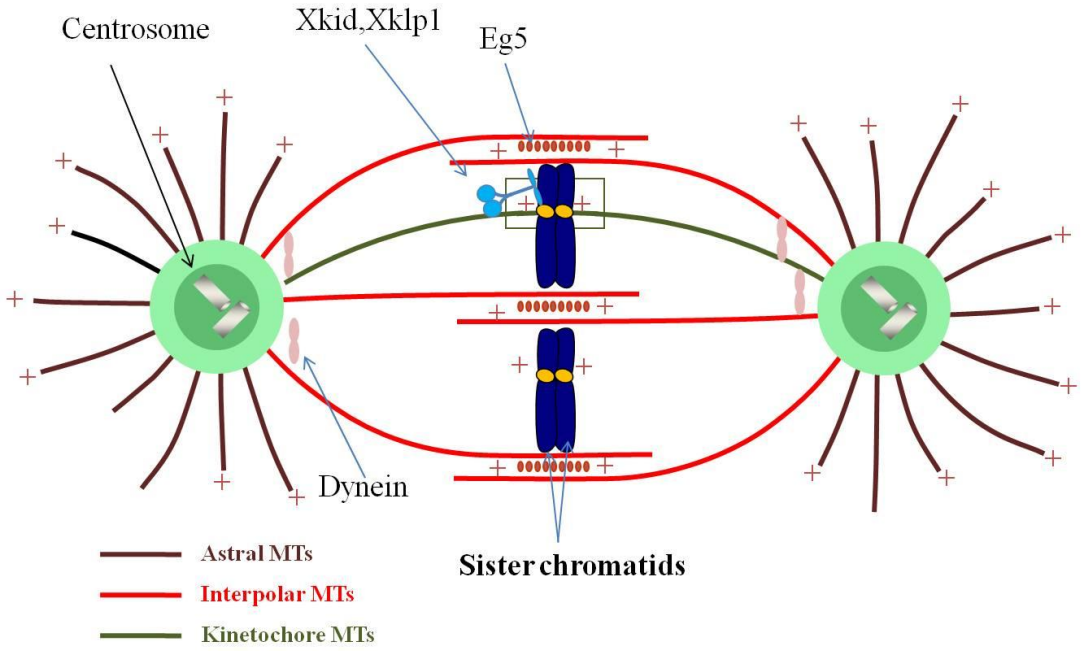


Figure 1.11. A schematic representation of a metaphase spindle is shown. Xkid, Xklp1,Eg5 and dynein are some of the motors that contribute for spindle apparatus formation. The astrasl MTs, interpolar MTs and kinetochores MTs are indicated.

The "search and capture" model was accepted for a long time, although nowadays it is clear that chromatin has an essential role in driving spindle assembly. In 1984, Karsenti et al, demonstrated that the bipolar spindle could be assembled around DNA that was injected in the *Xenopus* egg (Karsenti et al., 1984). This suggested that spindles did not require centrosomes to become bipolar. Furthermore, Heald and collegues (1996) (Heald et al., 1996) demonstrated that chromatin beads added to *Xenopus* egg extracts could trigger the assembly of bipolar spindles in the absence of centrosomes. We know now that this centrosome-independent mechanism is based in large part in the ability of the spindle components to self-organize: microtubules form in the vicinity of the chromatin and then become organized by microtubule-associated proteins (MAPs and Motors) into a bipolar apparatus. In fact, it is the only mechanism present in plant cells and in meiotic oocytes systems (Khodjakov et al., 2000).

Furthermore, we know now chromatin generates a gradient of RanGTP that controls the activity of numerous factors called SAFs (spindle assembly factors) involved in microtubule nucleation, stabilization and organization (Kalab and Heald, 2008).

Ran belongs to the family of GTPases: it is active when bound to GTP, and is inactive when bound to GDP. RanGTP is involved in the nucleo-cytoplasmic transport and in spindle assembly. The SAFs contain a signal sequence for nuclear transport, named NLS (Nuclear Localization Signal). During interphase, the NLS of the cargo protein is recognized by the importin complex. The importin-cargo complex is transported into the nucleus through the nuclear pores. In the nucleus Ran-GTP binds the importin complex, which releases the cargo protein. Then, the Ran-GTP is exported from the nucleus through the nuclear pore. The GTP hydrolysis in the cytoplasm is stimulated by Ran-GAP (Ran GTPase Activating Protein) and consequently, importin is released in the cytoplasm. Ran GDP is transported into the nucleus. Ran-GTP state is restored by RCC1 (Regulator of Chromosome Condensation1) also known as Ran GEF (Ran Guanine nucleotide Exchange Factor) (Carazo-Salas et al., 2001; Carazo-Salas et al., 1999; Carazo-Salas and Karsenti, 2003; Gruss et al., 2001). Finally, the SAF is therefore localized to the nucleus in interphase and perform its role during mitosis (fig 1.12).

Some spindle assembly factors (SAFs) that are regulated by RanGTP are TPX2 (Brunet et al., 2004), NuMA, XCTK2 (Ems-McClung et al., 2004) or human kid (Trieselmann et al., 2003) .

Nowadays, spindle assembly is thought to occur by a combination of the “search and capture” and “self-organization” models.

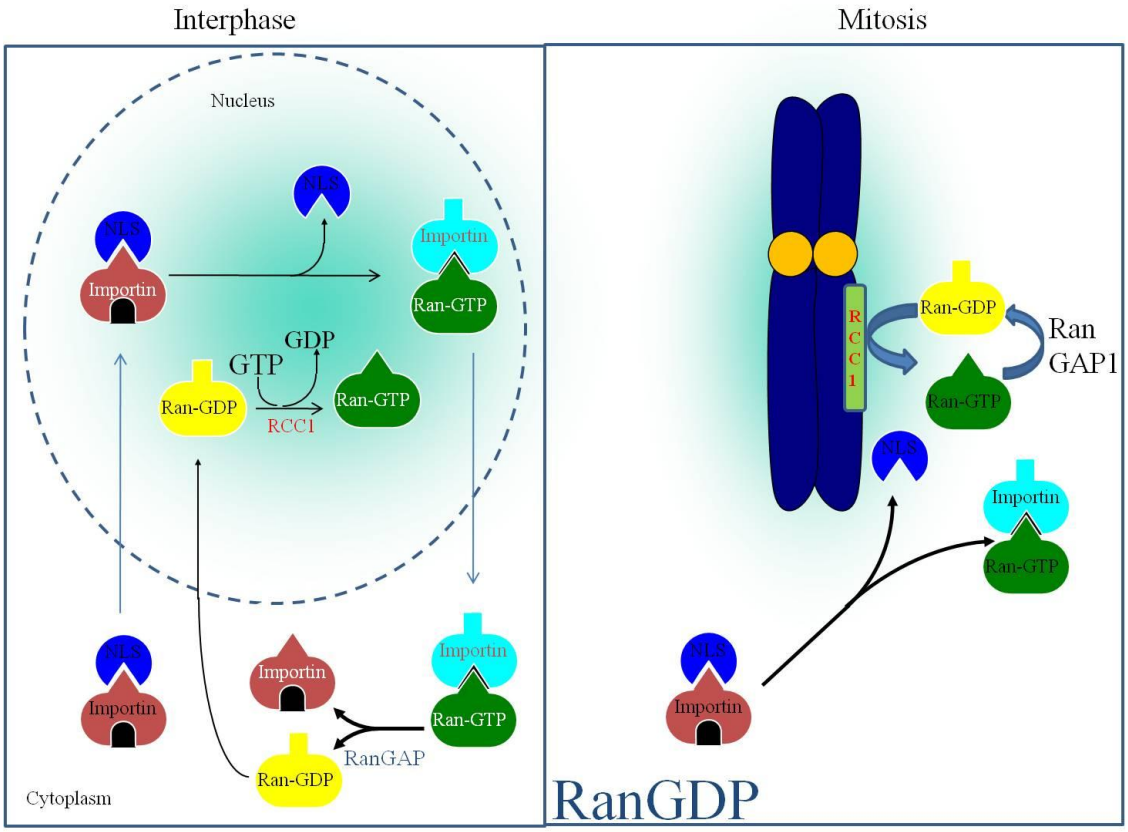


Figure 1.12. Cartoon for Ran functions in interphase and mitosis.
(A) During interphase, Ran is maintain in a GDP form in the cytoplasm by Ran-GTPase-activating enzyme (Ran-GAP) and loaded with GTP by the RCC1 in the nucleus. NLS-containing proteins (cargos) are bound to the importin complex (importin α and importin β) in the cytoplasm. When transported to the nucleus, Ran-GTP dissociates this complex and releases the cargo into the nucleus.
(B) In mitosis, Ran loaded with GTP is concentrated by the activity of RCC1 around the chromosomes, resulting in a steady state Ran-GTP gradient. SAFs are dissociated from their interaction with importins by the presence of Ran-GTP around chromosomes. The release of SAF locally triggers MT nucleation and stabilization around the chromosomes.

1.4. CHROMOSOME-MICROTUBULE INTERACTIONS DURING MITOSIS

1.4.1. Kinetochores and their role in chromosome movements

An important structure on chromosomes for interaction with the mitotic spindle is the kinetochore, a macromolecular complex that is responsible for microtubule binding.

In vertebrate cells, a typical k-fiber comprises 20-25 microtubules. On the contrary *S.cereviseae* kinetochores only bind one microtubule. Despite this difference the protein complexes that assemble to this centromeric heterochromatin is very well conserved along evolution.

Kinetochore fibers (k-fibers) and spindle MTs exhibit an additional dynamic property, known as microtubule flux, in which there is a net addition of tubulin heterodimers at the microtubule plus ends near the kinetochores and a net loss of tubulin subunits at the minus-ends near the centrosomes (Walczak et al.). (fig 1.13 A). At the beginning of prometaphase, the kinetochores capture the plus end of microtubules through the action of dynein. This mono-oriented chromatid pair oscillate close to the spindle pole, and then moves towards the center of the cell (Kapoor et al., 2006)Alternatively, the kinetochore fiber of a mono-oriented chromosome, can bind MTs from the opposite pole. The chromosome becomes bi-oriented, and move toward the spindle center (fig 1.13 B).

It is noteworthy that in *Xenopus* egg extract the non-kinetochore MTs comprise more than 90% of microtubules. Thus, the kinetochore acts as a "hot spot" in the somatic cells contributing for K-fiber oscillation (O'Connell et al., 2009) This is not observed in the *Xenopus* system.

The kinetochore–microtubule linkage is very dynamic, and is responsible for chromosome movement in the direction of the metaphase plate during prometaphase and metaphase.

At the beginning, the chromosome migrates in the direction of the spindle pole. There is an opposite force produced by the growing microtubules. The chromosome arms stay at the spindle poles, meanwhile the kinetochore is moving in the direction of the pole: thus, the centromere is in the pole direction. Once the effect of this force finishes, the activity of motor and structural proteins modify the direction of movement, and the kinetochore starts moving away from the spindle pole, until it reaches the metaphase plate.

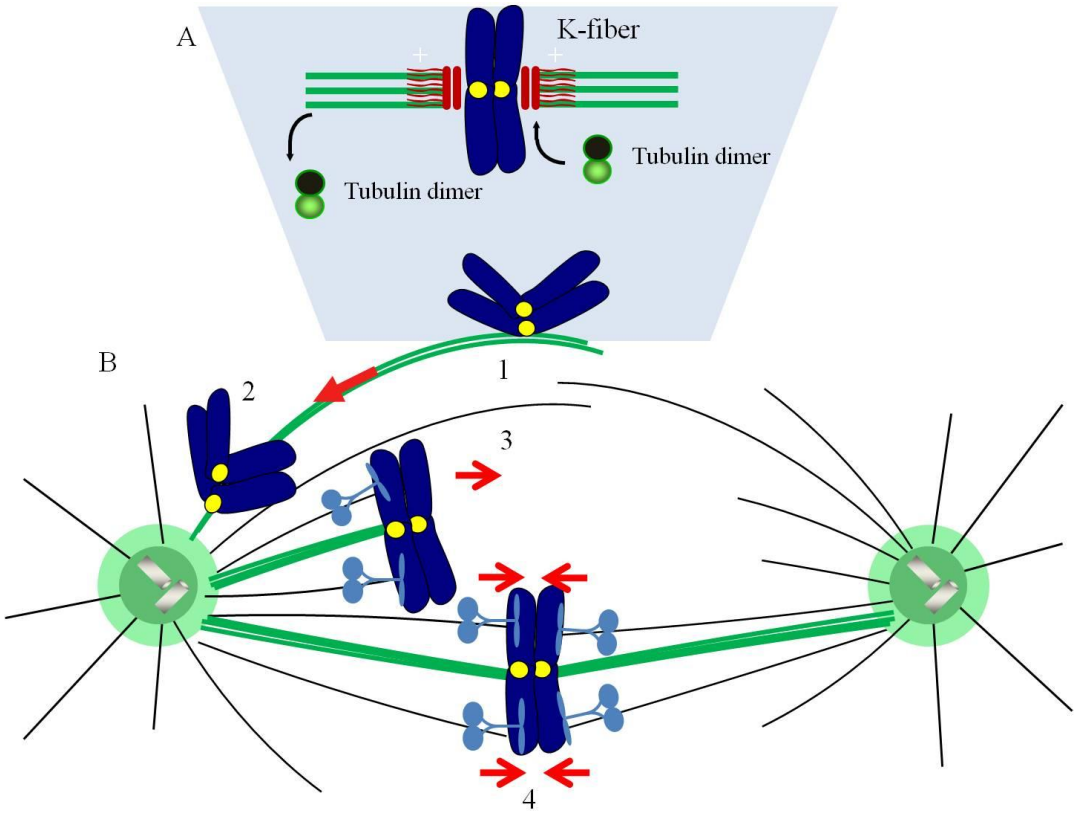


Figure 1.13. (A) The microtubule flusx at kinetochore fiber and spindle MTs**.**
(B) Chromosome movements during mitosis. (1) When the nuclear envelope breaks down, dynamic microtubules contact and eventually get stabilized by the kinetochores. (2) Chromosomes frequently attach first to microtubules from one pole and moves polewards. As the chromomes reach the pole, more microtubules connect to the kinetochores and also to the chromosomes arms. (3) Chromosomes are pushed to the spindle equator. (4) Sister kinetochores attach to microtubules polymerized from the opposite pole. Through a combination of forces, chromosomes congress to metaphase plate.

Different kinetochore proteins are responsible for chromosome movements to the metaphase plate. Both plus end- and minus end-directed motors, such as Cenp-E and cytoplasmic dynein, associate with kinetochores, suggesting that motors could drive the movement of chromosomes either towards or away from the spindle equator (Walczak et al.).

The finding that loss of the plus end-directed kinesin Cenp-E was associated with a failure in chromosome congression provided the first real evidence that motors drive chromosome motility. Cenp-E is implicated in the movement of mono-oriented chromosomes, indicating that chromosome congression can occur in absence of chromosome bi-orientation (fig 1.14).

Cytoplasmic dynein plays a role in capturing microtubules and guiding them rapidly to the spindle pole. Meanwhile the chromosomes are more distant from the spindle pole,

each growing microtubule contact with the outer kinetochore, and binds MCAK, a protein involved in regulating MT attachment and thus translocating kinetochores. MCAK may use its depolymerizing activity to release a small proportion of microtubules from the kinetochore.

This facilitates and synchronizes chromosome movement by modulating the number of attachment sites between the kinetochore and the MTs. Other MAPs including EB1,CLIP170, XMAP215, CLASP, and Ska1/RAMA are as well important for kinetochore function.

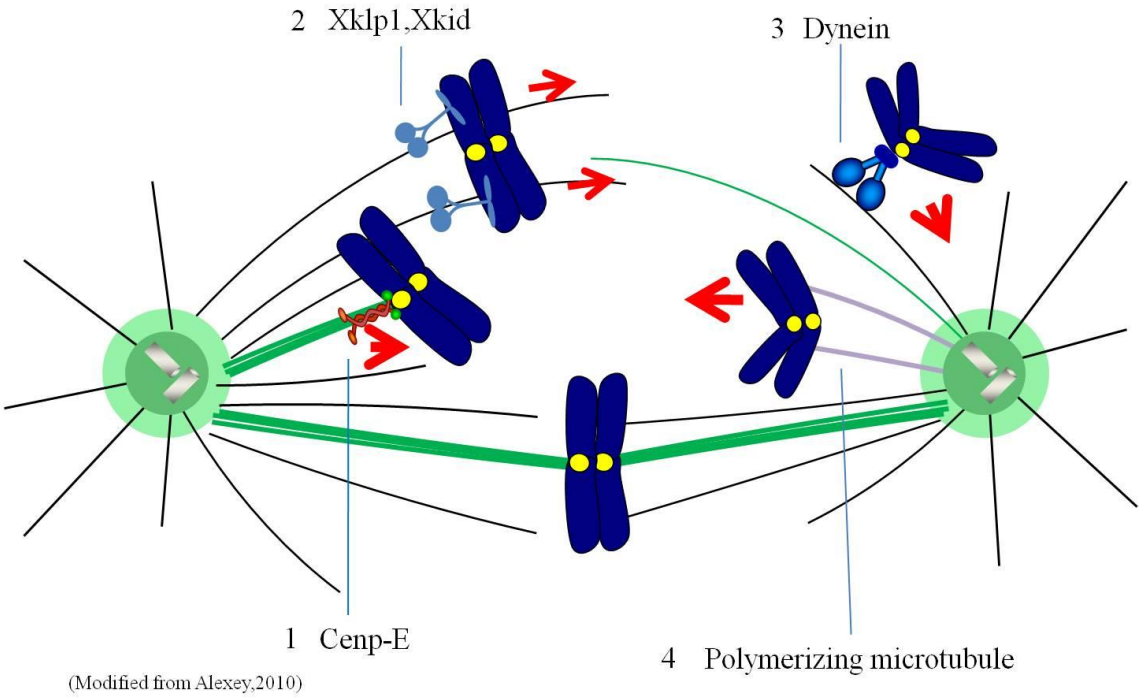


Figure 1.14. Different plus-end and minus-end motor proteins involved in chromosome alignment towards the spindle equator. (1) Cenp-E, is proposed to be involved in chromosome movement towards the spindle equator. (2) Xkid and Xklp1, are motor proteins associated to chromosome arms pushing a chromosome towards the spindle equator. (3) Dynein, have been implicated in the movement of laterally associated kinetochores towards spindle poles during early mitosis. Dynein can also contribute to the poleward chromosome movement. (4) Forces associated with polymerizing microtubules may push a chromosome towards the spindle equator during congression.

1.4.2. Role of the chromosomes arms in chromosome movements

"Indeed, the role in mitosis of the chromosome arms, which carry most of the genetic material, may be compared with that of a corpse at a funeral: they provide the reason for the proceedings but do not take an active part in them." (Mazia, 1961)

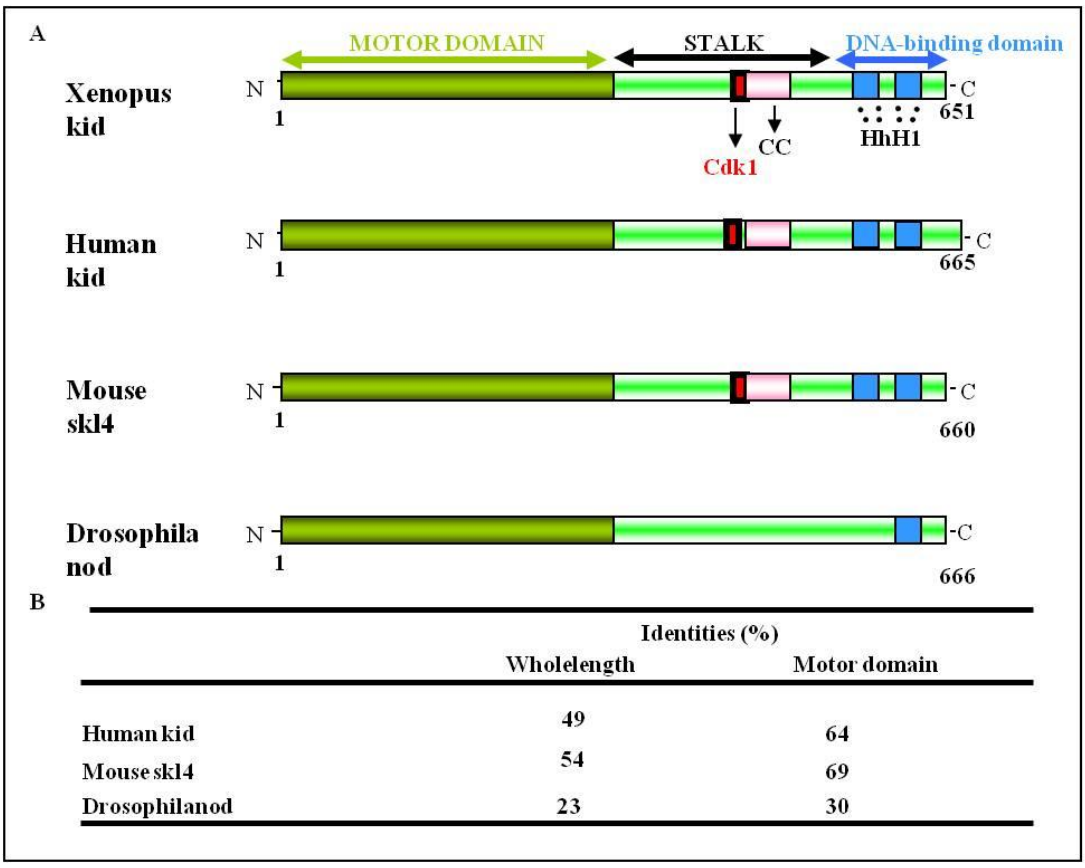
Nowadays, we can say that Mazia's comparison was elegant but it was an overstatement as recent data suggest that forces acting on chromosomes arms strongly influence chromosome behavior. Interestingly, mitotic chromosome movements in vertebrates exhibit distinctive oscillatory movements called "directional instability". The forces underlying these movements depend on interactions of the chromosomes with microtubules (MTs) emanating from the spindle poles, including diffuse interactions between interpolar MTs and the chromosome arms, and interactions of kinetochore MTs attached to the nucleoprotein kinetochores located at a chromosome's primary constriction (Loncarek et al., 2007).

In *Xenopus,* two kinesins are known to localize to chromosome arms: Xkid and Xklp1. They both mediate the interaction between chromosome arms and microtubules but perform different functions. Xklp1 is needed for spindle formation and integrity. Xkid is required for the generation of the polar ejection force and chromosome congression. Recently, Bieling et al (Bieling et al.) demonstrated that both proteins generate a continuously plus-end directed motility on chromatin. Xkid has a major role in chromosome movement. It is still not known how two motor proteins with the same localization and motility direction can perform different roles.

1.4.2.1. THE *XENOPUS* CHROMOKINESIN XKID AND ITS ORTHOLOGHES

Xkid was discovered in a screening to identify proteins synthesized de *novo* during oocyte meiotic maturation. Xkid has an N-terminal motor domain followed by a region containing a stretch of 50 amino acids predicted to be involved in coiled-coil interactions**.** Other interesting features include a consensus site for phosphorylation by cdk1, three putative nuclear localization signals (NLS), and two helix–hairpin–helix DNA binding motifs class I at the C terminus (fig 1.15).

Xkid is more closely related to the chromosome-associated human, kid (49% identity throughout and 64% identity in the motor domain alone). Xkid is 54% identical to the mouse orthologue throughout the whole sequence and 69% in the motor domain. Like Xkid, the human, the mouse and drosophila proteins are short klps of 665, 660 and 666 amino acid respectively with an N-terminal motor domain. The human and mouse proteins have some features of Xkid at approximately the same position on the sequence: a short stretch of 36 and 40 amino acids predicted to be involved in coiled coil interactions, a consensus site for phosphorylation by cdk1 and the two HhH class I domains at their C-terminus (Figure 1.15).



	Identities (%)	
	Wholelength	Motor domain
Human kid	49	64
Mouse skl4	54	69
Drosophilanod	23	30

Figure 1.15. Molecular structure of *Xenopus* Xkid, human kid, the mouse "similar to kinase-like 4" (slk4) and the Drosophila nod protein.
(A) Schematic drawing indicating the motor domain , the cdk1 phosphorylation consensus, the coiled coil sequence (CC) and the DNA-binding domain (two helix-hairpin-helix motifs (HhH1)).
(B) Table 1. Sequence comparison of *Xenopus* Xkid with the human kid, the mouse "similar to kinase-like 4" and the Drosophila nod proteins. Percentage of identical amino acids along the whole length and motor domain.

Xkid is specifically involved in chromosome congression by mediating the polar ejection forces that act on chromosome arms. It does not have a major role in the formation of the bipolar spindle itself although inhibition or depletion of Xkid modifies slightly the morphology of the bipolar spindle maybe as a secondary effect of the altered chromosome positioning (Antonio et al., 2000; Funabiki and Murray, 2000).

In addition, the chromokinesin Xkid plays a fundamental role in the meiotic cell cycle of oocytes, which seems to be independent of its role in chromosome positioning. Cell cycle progression is controlled and driven by the periodic activation of the Cdc2-cyclin B complex, the main regulator of G2/M progression. This kinase controls the activity and/or localization of multiple proteins that are involved in spindle formation and chromosome segregation (Karsenti and Vernos, 2001). Interestingly, Xkid has one consensus site for phosphorylation by this kinase and is therefore one candidate for being regulated by this kinase. In turn it was found that in the absence of Xkid, cyclin B does not accumulate at the transition between MI and MII during *Xenopus* oocyte maturation and therefore the Cdc2-cyclin B is not activated blocking cell cycle progression (Perez et al., 2002). Recently, it has been shown that spindle-localized translational activation of CPE-regulated mRNAs, encoding for proteins with a known function in spindle assembly and chromosome segregation, such as Xkid, is essential for completion of the first meiotic division and for chromosome segregation in *Xenopus* oocytes (Eliscovich et al., 2008).

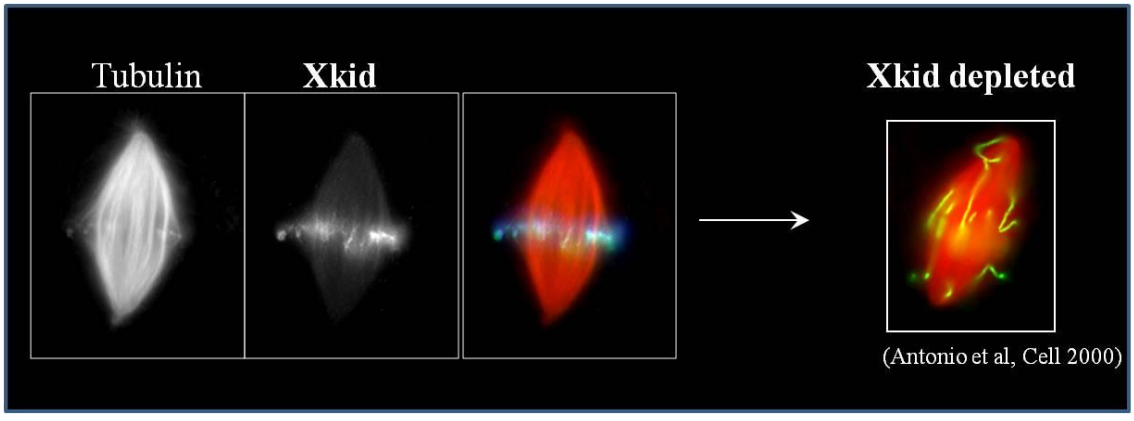


Figure 1.16. Xkid localization to mitotic chromosomes in *Xenopus* egg extract: Cycled spindles were assembled around sperm nuclei (sperm spindles) in *Xenopus* egg extract in the presence of rhodamine-labelled tubulin. Xkid localizes to chromatin.

The human kid (Hkid)is a plus end-directed microtubule motor protein that binds both MTs and chromosomes (Tokai et al., 1996). It is involved in generating the polar ejection force that pushes the chromosomes away from the spindle poles toward the metaphase plate. In mitosis, kid localizes to the chromosomes (fig 1.17). In contrast, with the Xkid, the human orthologue activity causes loss of chromosome oscillation during prometaphase and metaphase and chromosomes aligned efficiently at the spindle equator (Levesque and Compton, 2001). Different mechanisms have been described to be responsible for human kid localization and consequently, for its function. During metaphase, human kid localization to microtubules or chromosomes is subjected to different layers of regulation (fig 1.17). On one hand, its targeting to spindle apparatus is regulated by phosphorylation at the cdk1 site (fig 1.17, 2). The non-phosphorylated form of human kid has stronger affinity for microtubules through a second microtubule-binding site (fig 1.17, 3). (Ohsugi et al., 2003). Furthermore, this protein is required for maintenance of metaphase spindle, probably by contributing to MT stabilization, and consequently MT bundling of the spindle MTs, at least in part, by directly cross-linking parallel MTs through its motor and second MT-binding sites (Tokai-Nishizumi et al., 2005). On the other hand, hkid microtubule binding activity is inhibited by importin-α/β (Trieselmann et al., 2003). Upon RanGTP gradient generation around mitotic chromosomes, the chromosome loading of human kid is enhanced by importin-β (fig 1.17, 4). In addition, human kid and NuMA, a protein that cross-links MT ends at spindle poles, contribute to chromosome alignment and spindle morphology and size (fig 1.17, 5). (Levesque et al., 2003). The efficient interaction between human kid and spindle MTs requires CHICA, which is a spindle protein (fig 1.17, 6). (Santamaria et al., 2008). Cells arrested by nondegradable cyclin B1 maintained the expression of human kid, suggesting that cyclin B1 inactivation during mitosis could be required to allow efficient poleward chromosome movement by turning off hkid (fig 1.17, 7). (Wolf et al., 2006). At anaphase/telophase, is no longer phosphorylated and shows high affinity for microtubules in between adjacent chromosomes, contributing to the shortening of the anaphase chromosome mass along the spindle axis (fig 1.17, 8). Hkid loss in anaphase chromosome very often causes formation of multinucleated cells (Ohsugi et al., 2008). Degradation of human kid is mediated by APC/Cdh1 and takes place late in mitosis or in early G1 and is not essential for mitotic progression (fig 1.17,

9). (Feine et al., 2007). Furthermore, human kid was identified by the two-hybrid system as an interacting partner of SIAH-1, the human member of the highly conserved family of E3 ubiquitin ligases (Germani et al., 2000). SIAH-1 induces its degradation by the ubiquitin proteosome pathway. Recently, it was shown that human kid mRNA levels were decreased in all tumors in which SIAH-1 mRNA was decreased and vice-versa, suggesting the existence of an additional level of control than the previously described protein-protein interaction and protein stability regulation (Bruzzoni-Giovanelli et al.).

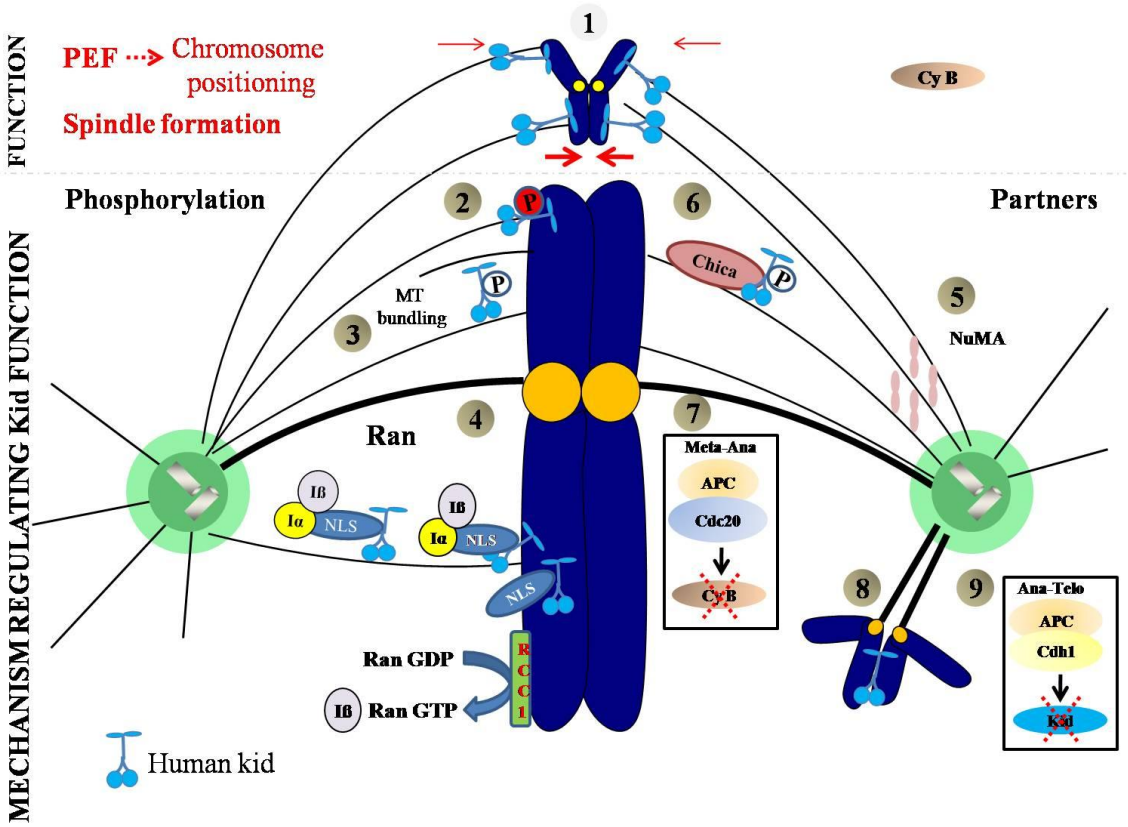


Figure 1.17. Cartoon representing the human kid localization, and proposed model for function and regulation. Kid localizes on or around prometaphase/metaphase chromosome arms to exert force to push the chromosome arms toward the plus-end of spindle Mts (1). Non-phosphorylated human kid might interact with MTs to facilitate microtubule stability (2 and 3). The association of hkid with Importin-β triggers the initial targeting of hkid to mitotic chromosomes, whereas cargo release, mediated by the activity of Ran-GTP, promotes the accumulation of hkid on the chromosomes (4). Kid might recruit other factors to stabilize the spindle MTs (6).

1.5. TEMPORAL REGULATION OF SPINDLE ASSEMBLY: PHOSPHORYLATION AND DEGRADATION CONTROL PASSAGE THROUGH THE CELL CYCLE

Protein phosphorylation is the most common post-translational modification that regulates processes inside cells and plays a key role in regulating the cell cycle engine. Protein kinases add phosphates to proteins by transferring phosphate groups from ATP to hydroxyl groups on amino acids side chains; protein phosphatases remove the phosphate groups.

The protein kinases and phosphatases that govern the cell cycle are divided into three classes, based on the amino acids that they phosphorylate and dephosphorylate. One class recognizes serine or threonine residues, another recognizes tyrosine residues, and a small group recognizes serine, threonine and tyrosine residues.

The cdk's (Cyclin-dependent protein kinases) and cyclins are the main regulators for the events of the eukaryotic cell cycle.

The cdk proteins belong to a family of enzymes that catalyze the reactions needed for the cell to enter into mitosis by triggering a cascade of phosphorylation. Cdk1 is the "master kinase" for mitosis. The activation of cdk1 for the entry to M-phase depends on its association with cyclin B. The cyclinB/cdk1 complex is also known as maturation promoting factor (MPF) (Gautier et al., 1990; Maller et al., 1989).

The mitotic spindle begins to assemble in prometaphase through metaphase, when the major mitotic kinase, cyclin dependent kinase 1 (cdk11) is highly active. Inactivation of cdk1 drives the cell into anaphase and mitotic exit.

The degradation of cyclin B leads to MPF inactivation and it essential for mitotic exit. This degradation is driven by the anaphase promoting complex (APC), a large ubiquitin-ligase complex that targets key cell cycle regulators and other mitotic proteins for degradation through the proteasome (Sullivan and Morgan, 2007). Ubiquitin-mediated proteolysis of cyclin B1 takes place as soon as the last chromosome is aligned on the metaphase plate and the spindle checkpoint is satisfied and promotes the

transition from metaphase to anaphase and mitotic exit (Pines, 1999),(Clute and Pines, 1999).

1.5.1. The role of Cdk1 phosphorylation in governing the localization and/or function of different kinesins

Phosphorylation of a given amino acid in a protein can have a variety of effects: activating or inactivating a protein activity, or increasing or decreasing its affinity for binding to other proteins. Proteins can have multiple sites that act as substrates for different protein kinases.

There are different studies reporting how cdk1 phosphorylation can regulate motor proteins localization and/or activities.

The localization and activity of MCAK (Mitotic centromere-associated kinesin) are controlled by Aurora A/B Kinases and cdk1 phosphorylation. Phosphorylation at T537 by cdk1 attenuates its microtubule-destabilizing activity *in vitro* and *in vivo (Zhang et al., 2007).* In addition, interfering with the regulation of MCAK by cdk1 promotes defects in spindle formation and chromosome positioning (Gorbsky, 2004).

Studies on *Xenopus* kinesin5-subfamily showed that the targeting of Eg5 to spindle microtubules increases upon phosphorylation by cdk1 (Cahu et al., 2008) .

The *Xenopus* Cenp-E protein is involved in chromosome congression and cdk1 phosphorylation at the Cenp-E tail has been proved to reverse the autoinhibition effect produced by the binding between Cenp-E and Cenp-E tail (Espeut et al., 2008). Recently, a role for phosphorylation of Cenp-E by Aurora A has been described (Kim et al., 2010).

1.5.2 Other members of several kinase families regulate cell division

Even though cdk1 is the main regulator of mitosis, other kinases such as Polo-like kinases, the Aurora kinase family and the NIMA family function in various mitotic events including cell cycle progression, spindle assembly and checkpoint activation (Salaun et al., 2008) (fig 1.18).

The aurora kinase family of proteins kinases constitute a family of serine-threonine kinases that are strongly related with cancer. Aurora A and B are fundamental for mitosis. Aurora C function is still unclear, although it is known that can complement Aurora B kinase function in mitosis.

The different Aurora kinases exhibit differential substrate affinity, subcellular localization and associated activities (Table II) (Andrews, 2005).

Table II. Table representing the different substrates, localization and effect of substrates-Aurora binding functions. Many of these substrates are directly involved in spindle assembly. Functions of Aurora family during cell division are related to their distinctive localizations.

	Substrates	**Cell localization**	**Effect of substrates-aurora binding functions**
Aur A	PP1,p53, Cdh-1,TPX-2,Ras GAP, Ajuba,TACC	Mitotic spindle, centrosome	Spindle assembly, cytokinesis, centrosome, maturation and separation
Aur B	Histone H3,INCENP,CENP-A,desmin, Rec8,vimentin,MCAK,survivin	Centrosome, central spindle, chromosome arms	Chromosome alignment and segregation,cytokinesis,microtubule dynamics
Aur C	Aur B, INCENP	Central spindle, chromosome arms?	Role in spermatogenesis, possible role in regulation of chromosmome segregation and cytokinesis

Polo-like kinases 1, are a multitask family of kinases, participating in bipolar spindle assembly, regulation of centrosome maturation, sister chromatid cohesion, activation of APC/C, and initiation of cytokinesis (Archambault and Glover, 2009).

Polo-like kinase 1, has a well characterized polo-box domain which acts as a specific phosphoserine (pSER) or phosphothreonine (pThr) binding domain. This motif is present in many Plk1 substrates, such as Cdc25. Thus, localization of Plk1 varies upon Cdk phosphorylation at those sites.

The *Xenopus* Polo like kinase family is composed by Plx1, Plx2 and Plx3. They all share a conserved amino acid sequence: an N-terminal Serine/threonine kinase domain,

and two conserved C-terminal regions, termed polo box which recognize phosphopeptides with the core consensus motif Ser-pThr,/pSer-Pro-X. Some of the Plx1 binding partners are: Cdc25C,Chk2, Mcm7 and Grasp 25 (van de Weerdt and Medema, 2006).

The NIMA family of serine/threonine kinases is also involved in cell cycle regulation. In human, they are known as Neksand they share considerable homology with the Never in Mitosis gene A (NIMA) kinase from *Aspergillus nidulans*.This family contribute to the timing of mitotic entry through controlling the localization/or activation of the Cdc2/cyclin B kinase to the spindle pole body. In addition, they promote chromatin condensation (O'Regan et al., 2007) .

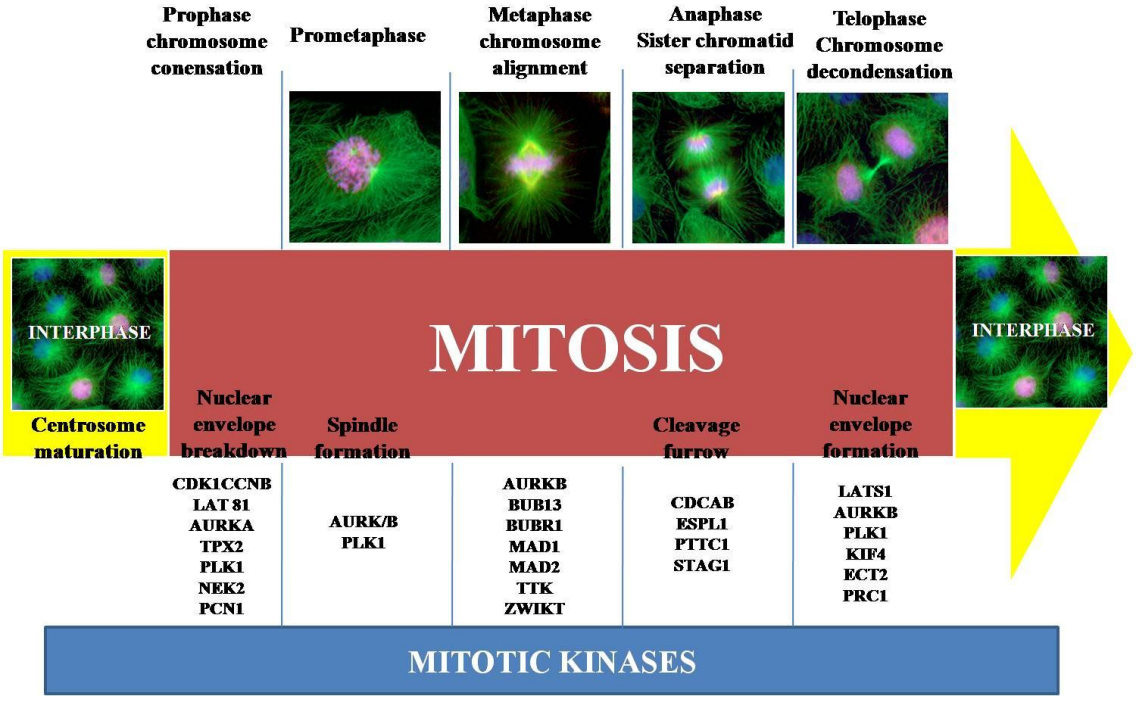


Figure 1.18. Cartoon representing the different mitotic kinases that regulate cell division and the fidelity of chromosome transmission. (Adapted from Erich Nigg, 2001).

1.6 THE *XENOPUS* SYSTEM

1.6.1. The embryonic cell cycles

Biochemical studies of the cell cycle require populations of cells that proceed synchronously through the cell cycle. Cells in the somatic cell cycle are usually not

synchronous, making it necessary to devise techniques to produce populations that are all in the same part of the cell cycle. This disadvantage can be overcome by exploiting the natural synchrony of the early embryonic cell cycles that occur in fertilized eggs.
For biochemical studies of the cell cycle, the eggs and early embryos of amphibians and marine invertebrates are particularly suitable. In these organisms, multiple synchronous cell cycles follow fertilization of a large egg. By isolating large numbers of eggs from females and fertilizing them simultaneously by addition of sperm (or treating them in ways that mimic fertilization), researchers can obtain extracts for analysis of proteins and enzymatic activities that occur at specific points in the cell cycle.

1.6.2. The *Xenopus* system in cell cycle related studies

The oocytes of the South African clawed frog *Xenopus laevis (Xenopus)* provide an excellent model system for studying the biochemical mechanisms regulating the G2/M transition. These oocytes are big (about 1 mm diameter) and can easily be maintained in culture and manipulated by microinjection of mRNA or protein.
On the other hand, the possibility to prepare concentrated egg extract that can recapitulate *in vitro* the events of mitosis has been extremely useful in studies on spindle assembly.

1.6.2.1 MEIOSIS IN *XENOPUS* OOCYTES

Meiosis is the process by which the germ cells reduce the number of chromosomes to haploid prior to fertilization. This genome reduction is accomplished through the omission of DNA replication (S-phase) between two successive cell division phases, meiosis I (MI) and meiosis II (MII). The meiotic resumption of G2-arrested oocytes is induced by hormonal stimuli. Typically, the hormone is progesterone in frog oocytes. Afterwards, with some variability in timing, *Xenopus* oocytes activate MPF, the MAPK/Rsk pathway, and undergo germinal vesicle breakdown (GVBD)/MI approximately 3-5 hours after progesterone stimulation (Figure 1.19 (B)). GVBD/MI is scored morphologically by the appearance of a white spot in the pigmented animal hemisphere of the oocyte (mature oocyte - Fig 1.19 (A)). The MI-MII transition, which occurs two hours after GVBD, is characterized by a 50% drop and subsequent recovery of MPF activity. Then, meiosis II (MII) starts with the disassembly of the nuclear

membrane formed in telophase I and a spindle is formed. The final products of the meiosis II division are haploid cells.

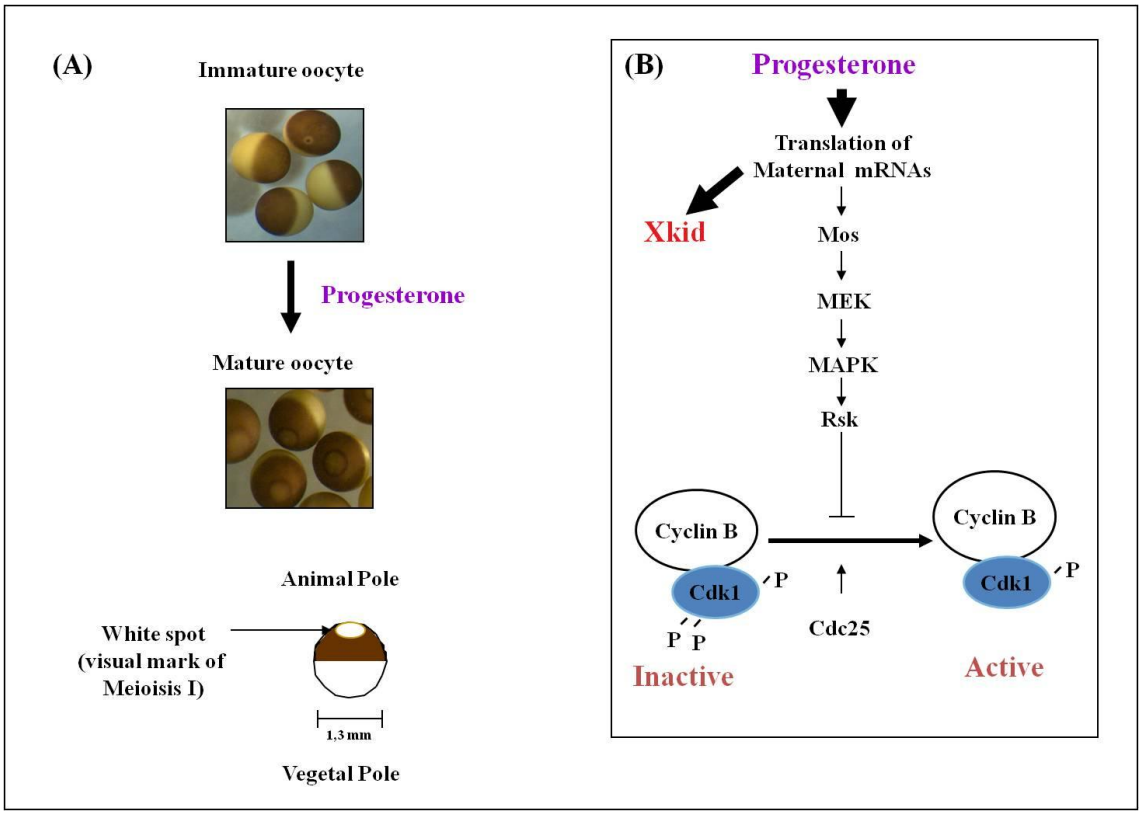


Figure 1.19: (A) Immature and mature *Xenopus laevis* oocytes. The fully-grown *Xenopus* oocytes (Stage VI) are easily identifiable by their specific pigmentation pattern, which defines the radial symmetry of the oocyte. The animal pole is brown, whereas the vegetal hemisphere is weakly pigmented and appears yellow.
(B) Signalling cascade involved in the *Xenopus* oocytes meiotic maturation.

Maturing oocytes are known to be transcriptionally dormant. Over the last few years, it has been established that an essential requirement for meiotic maturation is the translation of maternal mRNAs stored in the oocyte. In fact, we have gained important insights into the nature and function of the proteins synthesised *de novo* that lead to the activation of MPF and meiosis progression in *Xenopus* oocytes. Protein synthesis in these oocytes is enhanced by recruitment of pooled mRNAs and their selective cytoplasmic polyadenylation. One of these mRNAs encodes Xkid.

The meiotic maturation can be studied *in vitro* by adding progesterone to oocytes that have been surgically removed from female frogs.

1.6.2.2 THE *XENOPUS* EGG EXTRACT SYSTEM

1.6.2.2.1 Key experiments in cell cycle research performed in the *Xenopus* egg extract system

Organisms that are suitable for genetic approaches have been fundamental for the identification of many factors involved, but mechanistic studies are often hampered by the complexity of the cellular environment and the checkpoint machinery that controls cell-cycle progression. The only way to do this was to develop extracts that perform the cell cycle in a test tube, where it is more accessible to experimental manipulation. The cell extracts consist of pure cytoplasm prepared from frog eggs. They represent a concentrated cytoplasm that is open to biochemical manipulation and capable of recapitulating many cellular events in a test tube under conditions that lack checkpoints.
A crucial feature of this system is the ability to manipulate its cell-cycle state. When laid, *Xenopus laevis* eggs are arrested in metaphase of meiosis II by an activity called cytostatic factor (CSF). Sperm entry triggers a calcium spike, which induces the destruction of CSF and cyclins, and the subsequent exit from meiosis. Extracts prepared in the presence of the calcium chelator EGTA are able to maintain the metaphase arrest and are called CSF extracts. Importantly, these extracts can be cycled through interphase by adding calcium or its downstream target calcium/calmodulin-dependent kinase II (CaMKII) and can then be driven back into M-phase with fresh CSF extract.
Cytostatic factor extracts do not contain endogenous DNA, as the meiotic chromosomes are pelleted during the crushing spin of the eggs, but addition of any DNA is able to promote spindle formation.
For monitoring ongoing spindle assembly and chromosome segregation reactions, sperm nuclei is added. When *Xenopus* sperm nuclei are added to the cytoplasm, they swell, acquire a nuclear envelope, and replicate their DNA. In fact each spermatid carry one centrosome, that also participate in spindle assembly. Frog eggs are normally fertilized by frog sperm, and the frog egg extracts contain all the components necessary to unpack and remodel sperm chromatin.
In the laboratory, in order to monitor spindle assembly, the egg extract is supplemented with fluorescently labelled rhodamin tubulin which is incorporated into microtubules. In fixed samples, the chromatin is stained with blue fluorescent DNA dye (Hoechst).

Cell cycle extracts showed that cyclin synthesis drives early embryonic cells into mitosis

In 1989, Murray and Kirschner showed that destruction of all the endogenous messenger RNA arrests the extracts in interphase. The addition of exogenous cyclin mRNA was then shown to be sufficient to produce multiple cell cycles. The newly synthesized cyclin protein accumulates during each interphase and is degraded at the end of each mitosis (Murray and Kirschner, 1989).

1.6.2.2.2. Spindle assembly in *Xenopus* egg extracts

Spindles assemble in the mitotic extract either by the addition of demembranated sperm nuclei or DNA coated beads (Figure 1.20). Spindle formation around sperm nuclei follows a pathway similar to the one in mitotic cells allowing the study of mitotic events *in vitro*.

Two pathways can lead to spindle formation around sperm nuclei. Each sperm nucleus has a centrosome that nucleates microtubules when added to mitotic egg extracts. This generates an aster that eventually forms a half spindle in contact with the condensed chromosomes. Two half spindles can eventually fuse and give rise to a "bipolar spindle" (Fig 1.20 (A)). Alternatively, when the extract containing sperm nuclei is driven into interphase by the addition of calcium the chromosomes as well as the centrosomes of each nucleus will replicate once. Mitosis is then induced by addition of M-phase *Xenopus* egg extract and a bipolar spindle will form following a pathway similar to the one followed by animal cells. Anaphase can be induced by a second addition of calcium (Fig .1.20 (B)).

Spindles also assemble around DNA coated beads in the absence of centrosomes and kinetochores (1.20 (C)). DNA coated beads are prepared by coating magnetic beads with plasmid DNA of bacterial origin which is by definition without centromeric sequences.

Hence, the system is appropriate to study the process of meiotic spindle formation since meiosis in most animal oocytes occurs in the absence of centrosomes. DNA coated beads can also be used to analyze questions related to spindle microtubule organization independent of chromosome alignment or centrosome function.

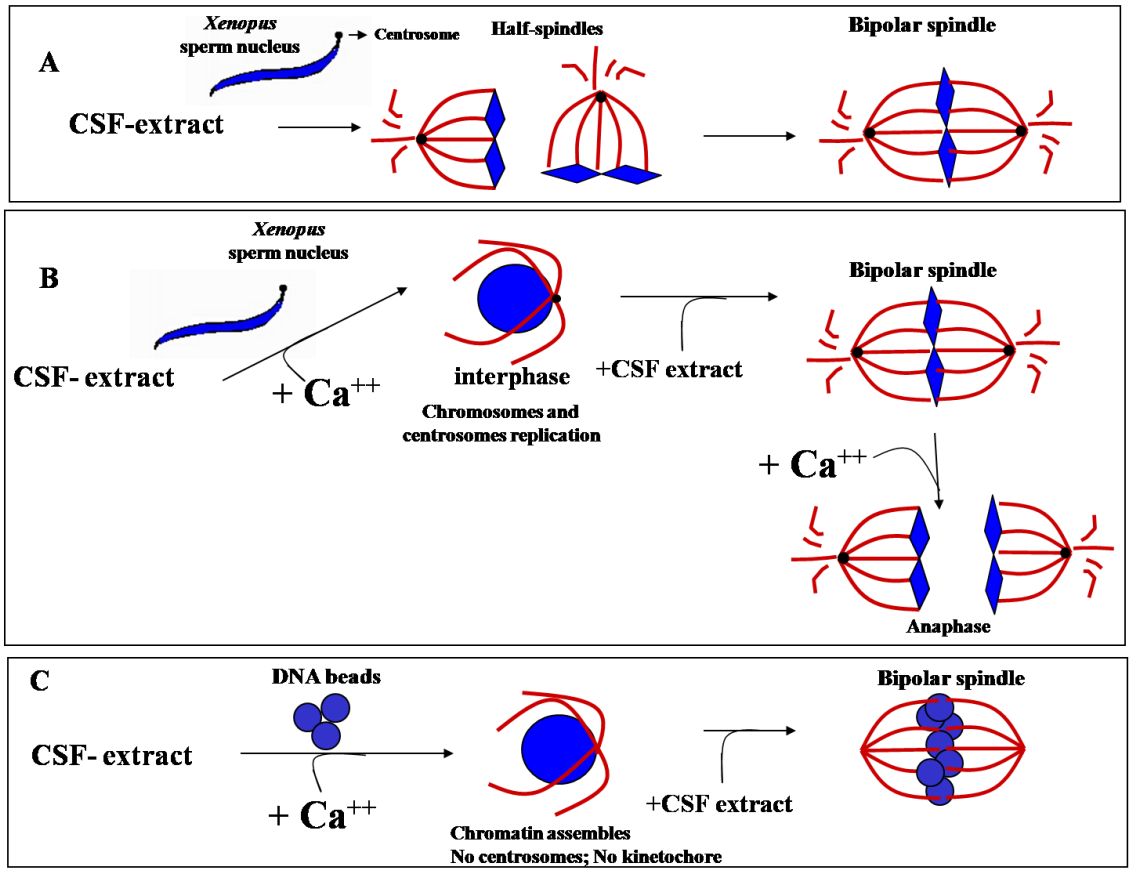


Figure 1.20. Spindle assembly in Xenopus mitotic egg extract.

(A) Half spindle assembly: Half spindles assemble around sperm nuclei at 30 min of incubation in mitotic egg extract and can eventually meet and fuse giving rise to bipolar spindles.

(B) Cycled spindle assembly: Calcium addition releases the CSF-arrest and induces interphase allowing DNA replication and centrosome duplication. After 90 min, fresh mitotic egg extract induces bipolar spindle formation: After 45-60 min of incubation spindles are in metaphase. Addition of calcium will induce anaphase and chromosome segregation.

(C) Bead spindle assembly: Chromatin assembles around DNA. In mitotic egg extract the assembled chromatin induces spindle formation.

1.6.2.2.3. The egg extract as a possible protein expression system overcoming the need of recombinant proteins

The egg extract system has been used to show that Xkid is required for metaphase chromosome alignment to the spindle equator.

Spindle assembly can be easily triggered in this extract in different ways. Several methods have been developed to assess the function of proteins involved in spindle assembly and chromosome segregation: removal of the protein by immunodepletion, addition of specific antibodies and addition of dominant negative proteins to inhibit the function of a certain protein.

The current methods to examine the function of a protein during spindle assembly in this system involves depletion of the protein with specific antibodies and the add-back of the recombinant protein expressed and purified from bacteria or baculovirus. This is very often a limiting factor because some proteins (particularly large ones) are often difficult to express and purify.

In order to overcome the need of recombinant protein, in the bibliography there are at least two articles that use the previously described 'mRNA-dependent' extract system (Murray and Kirschner, 1989). For the Chromosomal Passenger Complex reconstitution, Funabiki et al, used pools of *in vitro* transcribed CPC mRNAs that were added to RNaseA treated, CPC-depleted (ΔIncenp) extracts. The exogenous CPC mRNAs, including Aurora B, Incenp, Dasra A, Dasra B, Survivin, and SIX, a second Survivin-related protein in *Xenopus* were efficiently translated at approximately endogenous levels (Kelly et al., 2007). To examine the role of ε-tubulin in centrosome duplication (Chang et al., 2003) depleted *Xenopus* egg extract and then incubated with exogenously added ε-tubulin mRNA. Immunoblotting demonstrated that add-back of ε-tubulin mRNA to the depleted extract restored ε-tubulin protein levels to normal levels and that ε-tubulin is required for centriole duplication and microtubule organization.

In both studies the addition on exogenous mRNA restored the endogenous protein levels and it was possible to study the localization of that exogenous translated proteins.

This seems to be a good way to study the mechanism behind protein localization and/or function, as sometimes it cannot be performed using recombinant proteins. Thus, in this study, the translation of Xkid variants in order to study the role of phosphorylation in Xkid localization and/or function was set up.

2. MATERIALS AND METHODS

2.1. TOOLS

2.1.1. Plasmid constructions

The complete cDNA sequences are available from GenBank under accession number AJ 249840.1.The plasmids described in this work were obtained with usual DNA recombinant techniques like restriction enzyme digestions, DNA gel extraction, DNA purification, DNA ligation, following the manufacturer's instructions from kits and reagents.

2.1.2. N-terminal Myc tag constructs

2.1.2.1. THE pFTX5 VECTOR

Is composed by the following structural elements:

Size: 3302 base pairs

Resistance: Ampicilin

Tag: Myc at the N-terminus

T7 promoter site at position 1-21

SP6 promoter site at position: 515-538

Poly A tail: 340-490

Polylinker from 122

The pFTX5−Xkid Full Length (pFTX5-FL) was a gift from Ángel Nebreda.

2.1.2.2. N-TERMINAL Myc TAG Xkid MUTANT LACKING THE MOTOR DOMAIN (XkidΔMOTOR DOMAIN)

To generate a Xkid mutant lacking the motor domain, a PCR fragment was made by amplifying the region corresponding to aa351-651 using the primers:

Forward:

5'GC**C ATG G**CG AAC

Reverse:

5' GC**T CGA G**AG AGC

The PCR product was cloned into the *Nco* I–*Xho*I sites of the pFTX5 vector. The resulting construct was fully sequenced to confirm the absence of mutations.

2.1.2.3 N-TERMINAL Myc TAG Xkid PHOSPHORYLATION VARIANTS

pFTX5-Xkid cDNA was mutagenised in the consensus site by changing the predicted phosphorylated threonine at position 450 to an alanine (XkidT450A) to abrogate phosphorylation or to a glutamic acid (XkidT450E) to mimick the phosphorylation state. Mutagenesis was achieved by PCR amplification with specific primers that included in their sequence the desired substitutions (**see table III**) using the QuickChange Site-Directed Mutagenesis Kit from Stratagene following the manufacturer instructions. The cycling parameters for control and single amino acid change reactions were:

Table III. The cycling parameters for control and single amino acid change reactions.

95ºC	30 sec	
95ºC	30 sec	
55ºC	1 min	16 cycles
68ºC	6 min	
4ºC	∞	

The PCR was done using pfu turbo DNA polymerase (Stratagene) for 16 cycles, using the original clone, pFTX5-Xkid Full length, as a template. The constructs were fully sequenced to confirm that the site-directed mutagenesis worked and that no other mutations were introduced by the PCR.

The primers used in the PCR were the following:

Primer	**Sequence**
XkidT450A	Forward 5’GCCCAGCTCTTGAGC**GCC**CCCAAGAGAGAGCGAATG3’ Reverse 5’CAT TCG CTCTCTCTTGGG**GGC**GCTCAAGAGCTGGGC3’
XkidT450E	Forward 5’GCCCAGCTCTTGAGC**GAA**CCCAAGAGAGAGCGAATG3’ Reverse 5’CATTCGCTCTCTCTTGGG**TTC**GCTCAAGAGCTGGGC3’

2.1.3. C-terminal Histidine tag Xkid phosphorylation variants

The Xkid-His Full Length, Xkid T450A-His and Xkid T450E-His were cloned into the pFTX4GH2 vector to express the proteins with a C-terminal tag with a linker of 5x Glycines and 4x Histidines.

2.1.3.1 STRUCTURAL ELEMENTS OF pFTX4GH2:

Size: 3302 base pairs

Resistance: Ampicilin

Tag: No tag

T7 promoter site at position 1-21

SP6 promoter site at position: 515-538

Poly A tail: 340-490

Polylinker from 122

The constructs were generated by cutting out the inserts for Xkid-Full Length, Xkid T450A and Xkid T450E from the pFTX5 vectors with NcoI and Xho I and subcloning them into pFTX4GH2. The constructs were fully sequenced.

2.1.4. Polyclonal anti-Xkid antibody

The sera from the rabbits injected with a tagged fusion protein containing the C-terminal 90 amino acids of Xkid (malE-Xkid tail) were affinity purified on an affinity column of GST-Xkid tail using standard protocols. The eluted antibody was dialysed against PBS buffer. In all purifications, similar results were obtained by Western blotting and immunofluorescence analysis.

2.1.5. Polyclonal anti-phospho Xkid antibody

Polyclonal anti-Phospho Thr 450 Xkid antibodies were generated in rabbits by injection of the phosphopeptide (KKAQLLS (**pT**) PKRERMALLC) corresponding to the cdk1 consensus site of Xkid. The sera were firstly purified by affinity chromatography using the Hi-trap NHS-activated columns (GE healthcare) with the phosphopeptide. Secondly, the antibodies obtained from the first elution were further purified by affinity chromatography using the Hi-trap NHS-activated columns (GE healthcare) with the

non-phospho peptide. The specificity of anti-phosphoXkid antibodies was determined by Western blotting.

2.2. TISSUE CELL CULTURE

Extracts from *Xenopus* XL-177 were prepared as described previously in Le Bot et al.(Le Bot et al., 1998).

XL-177 cells were grown onto coverslips in tissue culture plate, and fixed for 5-10 minutes in cold methanol (-20°C) and then transferred in PBS. Unspecific binding was blocked by incubating 20 minutes at room temperature with IF1 buffer.

Primary antibodies diluted in IF1 buffer at 0,1-10 µg/ml were layered onto the coverslips for 20-30 min. The coverslips were washed 3-4 times with PBS followed by an incubation with the secondary antibodies diluted in IF1 according to the indications of the supplier. DNA was stained by incubation with 5 µg/ml of Hoechst (33342) diluted in IF1 for 5 minutes. The coverslips were mounted on glass slide with 6 µl of Mowiol. Prior to observation, the Mowiol was allowed to set overnight at room temperature or for 30min at 37°C. The images were acquired with a Leica Fluorescence microscope.

IF1 buffer: PBS containing
2 % BSA
0.1 % Triton X-100

Mowiol: 10% Mowiol 4-88 (Hoechst)
25% glycerol
0.1 M Tris pH 8.5

2.3. *XENOPUS* OOCYTES

2.3.1. *Xenopus* oocyte maturation

Stage VI *Xenopus* oocytes were obtained by surgery on anestethysed females by surgery and sorted after incubation with 0,1% collagenase IA (Sigma). Meiotic maturation was induced by incubating the satge VI oocytes in modified Barth medium containing 5 µg ml^{-1} progesterone (Sigma) and scored by the appearance of a white spot at the animal pole of the oocytes. For GVBD synchronization, oocytes that formed a white spot within a 10-min interval were pooled. For the preparation of lysates, oocytes were homogenized in 10 µl per oocyte of H1K buffer. The lysates were centrifuged at 10 000 *g* for 10 min. The supernantants were resuspended in sample buffer and resolved by 8% or 10% SDS-Page. The equivalent of one oocyte was loaded onto each lane.

Barth medium:
10mM KCl
50 mM Hepes (pH, 7,8)
10 mM MgSO4
25 mM NaHCO3
880 mM NaCl

H1K buffer:
80 mM sodium β-glycerophosphate
20 mM EGTA
15 mM MgCl2
0.5 mM Na2VaO2

2.3.2. Antisense experiments

The following morpholino oligonucleotide (from GeneTools) were used for antisense experiments: for Xkid silencing 5'-GCCCAGTAAGAACCATTCCCGCCTC-3'; as control, the sense oligonucleotide 5' GCCgAGTtAGAACCATTCgCGCgTC-3'

(containing four mismatches). Oocytes were injected with 50 nl of each morpholino oligonucleotide (1 mM final concentration).

2.3.3. Rescue experiments in *Xenopus* oocytes

Different *in vitro* transcribed Xkid mRNAs were prepared using the MEGAscript kit (Ambion). Oocytes were microinjected with *in vitro* transcribed mRNAs (diluted DEPC-treated water) and maintained for 1 h before progesterone treatment.

2.4. *XENOPUS* EGG EXTRACTS

2.4.1 *Xenopus* egg extract preparation

CSF-arrested *Xenopus* egg extracts were prepared according to Murray (Murray, 1991)However, some modifications were introduced to improve the reliability of the extracts in forming spindles in the presence of *in vitro* transcribed mRNAs.

Xenopus laevis females were induced to ovulate by a first injection with 100 units pregnant mare serum gonatotropin (PMSG) followed 3 to 14 days later by another injection with 500-1000 units human chorionic gonadotropin (HCG, Sigma Chemical Co.) on the afternoon before the day of extract preparation. The injected frogs were then kept overnight in MMR at 16-18°C. The next morning, the eggs were collected in MMR, washed a few times with MMR and dejellied in 2% Na-cysteine pH 7.8 for 5-10 min until the eggs were tightly packed together.

The dejellied eggs were washed 2-3 times with XB, twice with CSF-XB and finally with CSF-XB containing protease inhibitors. The morphology of the eggs was carefully assessed and all the abnormal eggs were removed. The eggs were carefully transferred a cut Pasteur pipette into Beckman SW50 centrifuge tubes containing 1 ml CSF-XB with protease inhibitors and 10 μg/ml cytochalasin D. Eggs were then packed by centrifugation at 800 rpm (200 g) for 1 minute, followed by 1600 rpm (400 g) for 30 seconds at 16°C in a Table Top Beckman centrifuge. Any buffer at the top of the tube was then removed, The packed eggs were crushed by spinning at 10500 rpm (about 18000 rcf) at 16°C in a HB-6 (Sorval) or a JS 13.1 (Beckman) rotor for 18 minutes. Subsequently, the tubes were transferred on ice and the cytoplasmic layer was collected

by piercing the tube with a syringe and needle from the side. The extract was then transferred into a test tube, and 1:1000 of protease inhibitors solution, 1:500 of cytochalasin D (10 mg/ml in DMSO) and energy mix at 1/40th of the extract volume were added, and carefully mixed. After preparation, the extract maintained on ice, remained competent to assemble spindles for several hours

MMR:

5 mM Na-HEPES pH 7.8

100 mM NaCl

2 mM KCl

1 mM MgCl2

2 mM CaCl2

0.1 mM EDTA

XB:

10 mM K-HEPES pH 7.8

100 mM KCl

0.1 mM CaCl2

1 mM MgCl2

50 mM sucrose

CSF-XB:

XB containing 2 mM MgCl2

5 mM EGTA

2.4.2. Spindle assembly in *Xenopus* extracts

Spindle assembly was carried out following the method of Sawin and Mitchison (Sawin and Mitchison, 1991). To visualize the MT and follow spindle formation, tetramethylrhodamine (rhodamine) labelled bovine brain tubulin was added to the CSF-arrested egg extract at 0.2 mg/ml. The extract was then divided into two aliquots.

The following reagents were added to one of the tubes (tube 1): demembranated sperm nuclei (500 nuclei/µl of final volume of extract reaction) and calcium to a final concentration 0.4 mM. Tube 1 was then incubated at 20ºC for 90 minutes. During this time the extract went into interphase, the DNA replicated and the sperm centrosome duplicated. At this point, the extract contains round shaped nuclei with decondensed DNA and relatively stable long MT.

In the meanwhile the second aliquot of extract (Tube 2) was maintained on ice. For translation of selected proteins, the *in vitro* transcribed mRNAs were added to this extract and incubated for 40min to 1 hour at 20ºC. This tube was then transferred on ice until later use.

After the 90min incubation, the interphase extract in tube 1 was sent into mitosis by addition of the M-phase extract from Tube 2. The mixture was then incubated at 20ºC, and samples were taken at different time points to evaluate the stage of spindle formation (45-60 minutes). For data quantification and localization studies, spindles were pelleted onto coverslips using flat bottom tubes or 15 ml COREX tubes with appropriate adaptors. The flat bottom tubes (Greiner Bio-one Company) with a round glass coverslip (12 mm in diameter) at the bottom were filled with 4 ml of spindle cushion (40% glycerol cushion in BRB80) at room temperature. Alternatively, 15 ml COREX tubes were loaded with plastic adapters, a round ethanol-washed coverslip and 5 ml of spindle cushion. Usually, after 60 min of incubation, the extract was diluted in 1 ml BRB80 containing 30% glycerol, 0.25% glutaraldehyde and 0.1% Triton X-100, carefully layered on top of the cushion using an Eppendorf pipette with a cut 1-ml tip, and centrifuged (Sorvall HB4 rotor at 12,000 rpm for 12 min at 16°C). The cushion was then removed with a vacuum pump and the coverslips were fixed in -20°C methanol for 5-10 min, incubated twice for 10 min in 0.1% $NaBH_4$ in PBS and processed for immunofluorescence. All incubations with antibodies and washes were done sample side up on a piece of parafilm. Finally, the samples were monuted in Mowiol.

For immunolocalization with the anti-Xkid antibody glutaraldehyde fixation or formaldehyde fixation was used. In this case, after 60 min of incubation, the extract was diluted in 1 ml BRB80 containing 30% glycerol, 0.25% glutaraldehyde or 4% formaldehyde and processed as described above.

10x Calcium solution:

4 mM CaCl2

100 mM KCl

1 mM MgCl2

BRB80,:

80 mM Pipes, pH 6.8

1 mM MgCl2

1 mM EGTA

2.4.3. Depletion

Magnetic beads coated with Protein A (Dynal) were washed 3 times with PBS containing 0.1 % Triton X-100. Then the beads were incubated in a final volume of 0.25-0.5ml of PBS-T with antibody (30 µg of antibodies per 60 µl of beads) for 30 minutes at room temperature on a rotating wheel. Unspecific IgGs were used as control. The beads were then retrieved with a magnet (Dynal), washed once with PBS containing 0.1 % Triton X-100 and once with CSF-XB before addition to a freshly prepared CSF-egg extract on ice. The efficiency of depletion was variable depending on the egg extract. Sometimes, a good depletion could be achieved with a short 30 min incubation, mixing the extract 2-3 times with a cut pipette tip. Sometimes, a better depletion was achieved by two successive rounds of depletion. For each experiment, two volumes of beads coated with antibodies were used to deplete one volume of egg extract. To retrieve the beads from the extract, the tube was maintained on the magnet for 10 minutes on ice, with occasional mixing of the extract. The depleted extract was then carefully transferred to a new eppendorf tube and maintained on ice until further use.

2.4.4. Immunoprecipitation

Xkid was immunoprecipitated with Protein A magnetic beads (Dynal) coated with specific ant-Xkid antibodies. Magnetic beads coated with Protein A (Dynal) were washed 3 times with PBS containing 0.1 % Triton X-100. Then the beads were incubated in a final volume of 0.25-0.5ml of PBS-T with anti-Xkid antibodies (30 µg of

antibodies per 60 µl of beads) during 30 minutes at room temperature on a rotating wheel. The beads were washed twice in 1 ml of PBS-T and twice with 1 ml of CFS-XB buffer. Antibody-coated beads were incubated with the *Xenopus* egg extract on ice for 1 h, retrieved on a magnet for 10 minutes and washed twice with 1ml of CSF-XB and one with 1ml of PBS-T, both containing phosphatase inhibitors (100 mM NaF, 80 mM β-Glicerophosphate and 1 mM Na3VO3). Proteins were eluted from the beads by incubation in sample buffer for 10 minutes at room temperature. The samples were then subjected to SDS-PAGE electrophoresis gels and analyzed by Western blotting analysis with corresponding antibodies. For the identifation of Xkid associated proteins by mass spectrometry, 240 µl-900µl of *Xenopus* egg extract were used. To confirm a possible interacting partner by Western blot analysis, the immunoprecipitations were performed with 40µl of *Xenopus* egg. To examine the putative role of RanGTP in regulating a specific interaction the egg extract used was incubated previously incubated with RanQ69L-GTP (15 µM final concentration) for 5min at 20^0C.

2.4.5. Sucrose gradients

160 µ l of CSF egg extract was loaded with a cut pipette tip on the top of 5 ml of 5 to 25% sucrose in CSF-XB, prepared with a gradient maker (Auto Densi-Flow, Labconco). The gradient was centrifuged in a Beckman SW 55 rotor for 16 hours at 4 ºC at 27,000 rpm (g). Fractions of 300 µl were collected using the gradient maker and the percentage of sucrose in each fraction was measured with a refractometer. The pattern of Xkid in the gradient was determined by Western Blot analysis of 10 µl of each fraction.

2.4.6. MT pelleting assay and spindle pelleting

MT pelletting assays were performed from extracts containing either taxol or nocodazole. MTs were polymerized by addition of 20 µM of taxol in 40 µl of CSF egg extract. As a control, 20 µM of Nocodazole were added to another 40 µl of CSF arrested egg extract. After 30 minutes at 20ºC, extracts were diluted in 1ml dilution buffer. Polymerized MTs were sedimented through a 1 ml of Spindle cushion and centrifuged for 12 minutes at 20ºC, 12000 rpm in a TLS-55 rotor. The cushion was washed twice with dilution buffer and the MT pellet was resuspended directly in 40 µl of Sample

buffer. MT pellets were analyzed by Western blot with anti-Xkid and anti-tubulin (DM1A, sigma) antibodies.

For spindle pelleting assays, 60-80 µl of cycled egg extract containing sperm nuclei (with or without 40µM of Nocodazole), were diluted with 1 ml of dilution buffer (without glutaraldehyde). Spindles were sedimented by centrifugation through a Spindle cushion for 12 minutes at 20°C, 12000 rpm in a TLS-55 rotor. The cushion was washed twice with dilution buffer, and the spindle pellet was resuspended in 60-80 µl of Sample buffer. Proteins associated with the spindle were analyzed by Western blotting with anti-Xkid, anti-tubulin (DM1A, sigma) and anti-XCAP-G (as a DNA control) antibodies.

2.5. ADDITIONAL PROTOCOLS

2.5.1. SDS-PAGE, Coomassie staining and Western blots

Protein analysis of equivalent amounts of treated and untreated egg- or cell extracts were resolved on SDS-PAGE according to Laemmli (Laemmli, 1970). The separating gel contained 8-15% acrylamide (BioRad, 30% acrylamide stock containing 0.8% bisacrylamide). The stacking gel contained 4% acrylamide. The gels were usually 0.75-1 mm thick minigels and were run in a Running buffer at 100V. Proteins were stained by incubation of the gels with Coomassie brilliant blue (R250) staining solution for 10-20 minutes and distained. In some cases, the gels were stained overnight with a mixture of Staining and Distaining solutions. Molecular weight markers were from Bio-Rad Laboratories or Fermentas. For Western blot analysis, gels were transferred onto a nitrocellulose membrane (Protan). Western blots were either wet or semidry. The wet transfers were performed using a Wet transfer buffer for 60-120 minutes at a constant voltage of 100 V in a Bio-Rad system. The semidry transfer was performed using the Semidry transfer buffer for 60-120 minutes at 1mA per square cm of nitrocellulose membrane, in a Amersham system. The membrane was stained in ponceau S solution (Serva) to monitor the correct transfer. Membranes were then blocked by incubation in 5% non fat dry milk in PBS for 1 hour at RT or overnight at 4ºC. Antibodies were diluted in PBS, 5% non fat milk and added to the membrane for 1 hour at RT. After each antibody incubation, the membrane was washed 3-4 times with PBS-0.1%Tween

for 30 minutes. The membrane was probed with primary and HRP-conjugate secondary antibodies (Dianova) or using Alexa fluor 680 or 780 labelled antibodies (Molecular Probes and Rockland, respectively). Blots were developed using the ECL chemiluminescence detection system (Amersham) or using the Odyssey Infrared imaging system (Li-Cor). In the case of phospho-Xkid antibody, the blocking from unspecific binding was done by incubating the membrane with 5% BSA in PBS-0.1 Tween for 1 hour at RT or overnight at 4ºC. The phospho-Xkid antibody was diluted in 5% BSA in PBS Tween.

3. RESULTS

3.1. CHARACTERIZATION OF XKID PHOSPHORYLATION AT THE CDK1 CONSENSUS SITE

3.1.1. Sequence analysis of Xkid and its orthologues: conservation of a consensus site for cdk1 phosphorylation

Xkid consensus site for phosphorylation by cdk1 (amino acids 449 and 453:S**T**PKR), is conserved in its homologues in human and mouse (Antonio et al., 2000; Ohsugi et al., 2003).This suggests that the role of phosphorylation by cdk1 may be conserved through evolution. In addition, it has been shown that cdk1-mediated phosphorylation of human Kid controls its distribution to spindle microtubules and chromosomes (Ohsugi et al., 2003).

3.1.2. Temporal pattern of Xkid phosphorylation on Thr450 (cdk1 site)

To study the temporal and spatial pattern of phosphorylation of Xkid on Thr450, a polyclonal antibody was generated against the phosphopeptide (KKAQLLS (**pT**) PKRERMALLC) that includes the predicted cdk1 consensus site.

3.1.2.1. CHARACTERIZATION OF XKID-PHOSPHOPEPTIDE ANTIBODY SPECIFICITY

The specificity of the anti-PXkid antibody was evaluated by Western blot analysis. Endogenous Xkid was immunoprecipitated from a mitotic egg extract using our general anti-Xkid antibody. Half of the immunoprecipitated protein was then incubated with Lambda phosphatase during 30 min at 30ºC.

Western blot analysis showed that the anti-PXkid antibody specifically recognized the Xkid immunoprecipitated from the mitotic egg extract but not the same protein treated with phosphatase. This result indicates that the anti-PXkid recognizes specifically Xkid phosphorylated on Thr450 (fig 3.1).

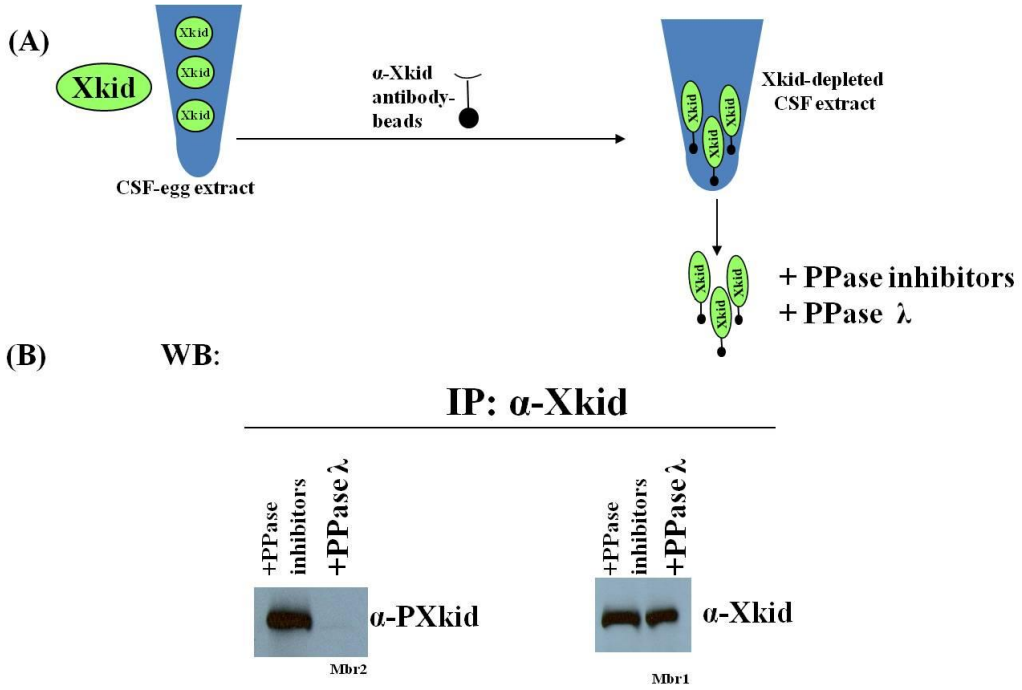


Figure 3.1. Characterization of phospho-Xkid specific antibody.
(A) Schematic representation of the experimental approach that has been performed: endogenous Xkid was immunoprecipitated with a general anti-Xkid antibody from a mitotic egg extract. Immunoprecipitated endogenous Xkid proteins were incubated with phosphatase inhibitors (PPase inhibitors) or with phosphatase lambda (PPase λ).
(B) The western blot analysis showing that the phospho-Xkid antibody (α-PXkid) reactivity was diminished under phosphatase lambda (PPase λ) treatment.

3.1.2.2. TEMPORAL PATTERN OF XKID PHOSPHORYLATION ON THR450 DURING OOCYTE MATURATION

Xkid is not present in stage VI *Xenopus* oocytes. Xkid maternal mRNA becomes translated during oocyte maturation. The protein is first detected at about the time of GVBD or meiosis I entry and continues accumulating until the natural arrest in metaphase II, when it reaches its highest levels.

The pattern of Xkid phosphorylation on Thr450 was monitored throughout oocyte maturation by Western blot analysis of oocytes incubated in progesterone for different times (fig 3.2 A). Phospho-Xkid was first detectable at around the time of GVBD that indicates meiosis I entry. Its levels then decreased during the transition between meiosis I and meiosis II, increasing again to reach the highest level in metaphase of meiosis II (fig 3.2 B).

This pattern follows the pattern of expression of Xkid suggesting that the protein gets phosphorylated efficiently upon expression, reaching a peak of protein level and phosphorylation at MI and MII.

Consistently, phospho-Xkid could be detected in M-phase but not in interphase egg extracts, suggesting that phosphorylation of Xkid at Thr 450 is concomitant with its expression and peaks in M-phase.

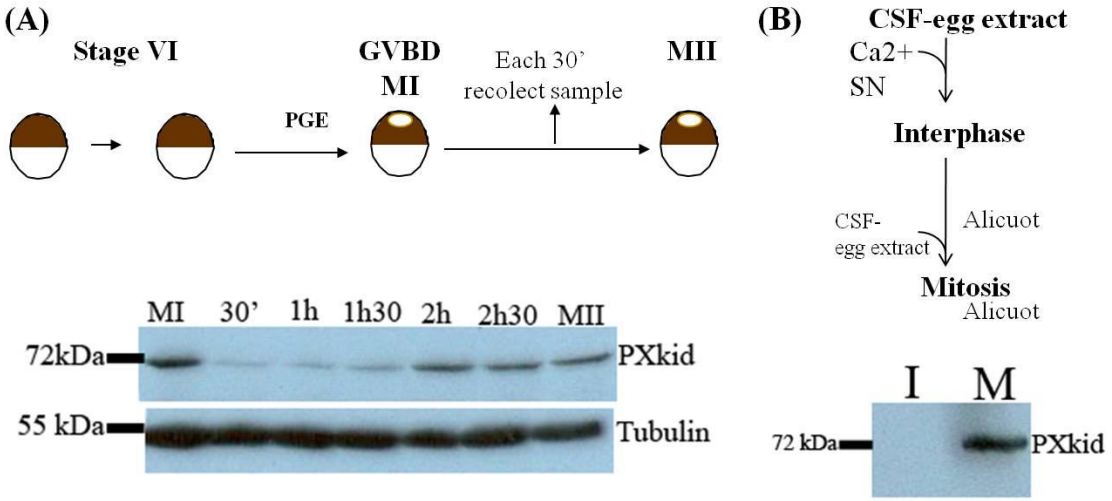


Figure 3.2. The anti-PXkid recognizes specifically the phosphorylated Xkid form in maturing oocytes and in mitotic egg extracts.
(A) Schematic representation of the experimental approach to follow the oocyte maturation: *Xenopus* oocytes stimulated with progesterone (PGE) were collected at different time points and analysed by western blotting using anti-PXkid or anti-tubulin. Immunoblot analysis showing that the anti-PXkid antibody recognized the phosphorylated form of Xkid in matured oocytes.
(B) Cycled egg extract was used to study the phosphorylation of Xkid. Samples were taken at interphase and mitosis. Immunoblot probed with anti-PXkid showing that the antibody recognizes the phosphorylated form of Xkid in mitotic egg extracts.

3.1.3. Subcellular localization of phospho Xkid

3.1.3.1. LOCALIZATION OF PHOSPHO-XKID IN SOMATIC CELLS

The subcellular localization of phospho-Xkid was examined by immunofluorescence in XL177 *Xenopus* tissue culture cells (fig 3.3). Cells were grown on coverslips and fixed with methanol and processed for immunofluorescence using a mouse anti-tubulin monoclonal antibody and the affinity purified anti-PXkid antibody. After incubation with fluorescently labelled anti-mouse and anti-rabbit antibodies and Hoechst, the coverslips were mounted with Mowiol and observed on a fluorescence microscope.

Although Xkid accumulates in the nucleus of interphase cells, no signal for PXkid was detected at this stage. At prometaphase, PXkid colocalized with the condensing chromosomes and this chromosomal localization persisted in metaphase. In addition, a faint signal was observed on the spindle microtubules, suggesting that PXkid is associated to the microtubules, at least to a certain extent. In anaphase, the signal for PXkid disappeared both at the chromosomes and the spindle microtubules.

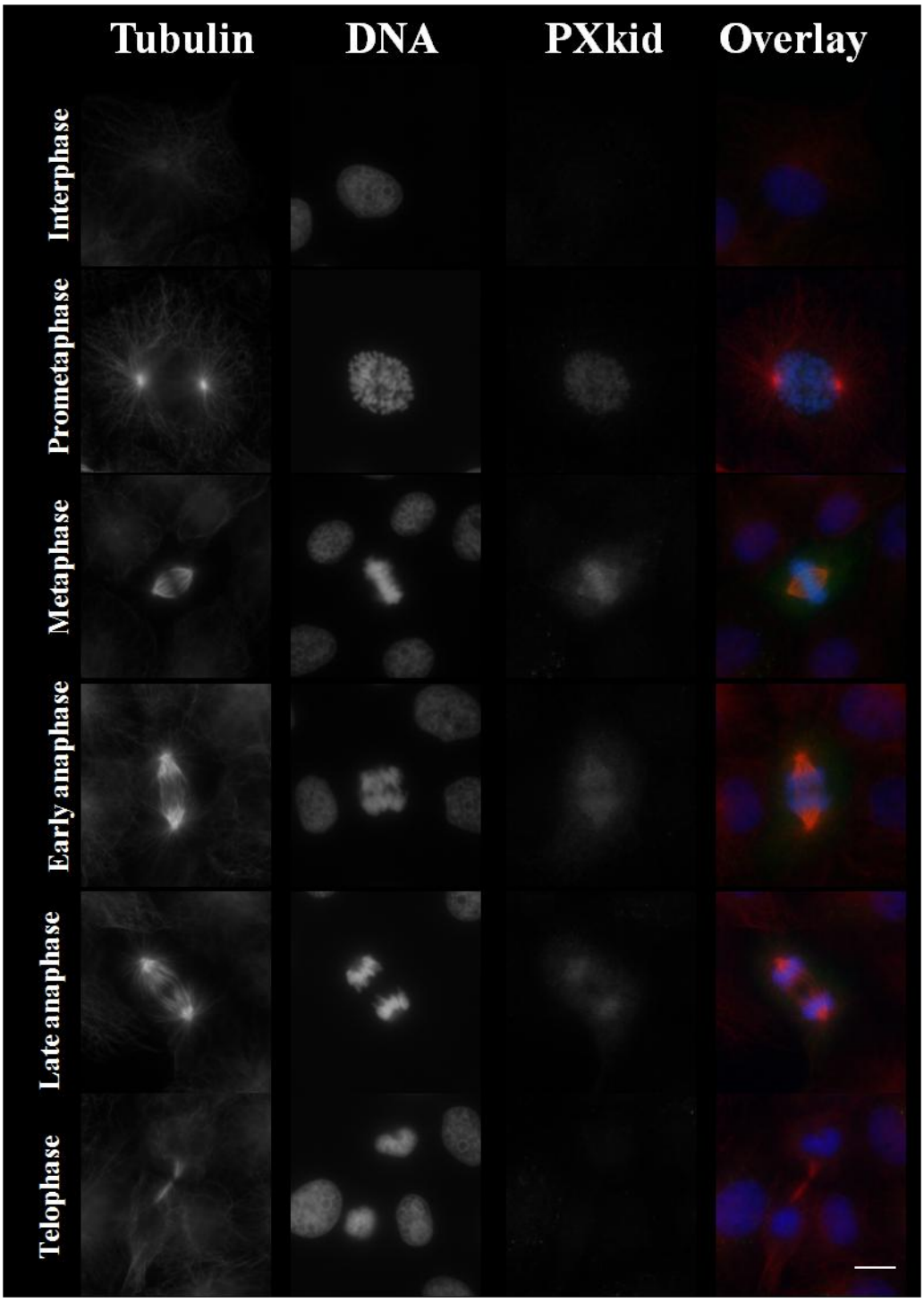


Figure 3.3. Localization of phospho-Xkid (PXkid) throughout the cell-cycle. Methanol-fixed XL177 cells were stained with the anti-PXkid antibody and a monoclonal anti-α-tubulin. Hoeschst was used to visualize the DNA. During mitosis, phospho-Xkid (PXkid) localizes to chromosomes and to spindle microtubules. Xkid (green), tubulin (red) and DNA (blue). (bar,10μm)

3.1.3.2 LOCALIZATION OF PHOSPHO-XKID IN EGG EXTRACT

Spindles were assembled by addition of sperm nuclei to cycled *Xenopus* egg extracts containing rhodamine-labeled tubulin and centrifuged through a cushion onto coverslips (fig 3.4). The samples were fixed in methanol and processed by immunofluorescence using the anti-PXkid antibody and Hoechst (to visualize the chromosomes). Like in tissue culture cells, PXkid localized to the chromatin. Interestingly, the anti-PXkid antibody decorated the chromosomes in a punctuate pattern that did not overlap completely with the pattern observed with the anti-CT-Xkid antibody.

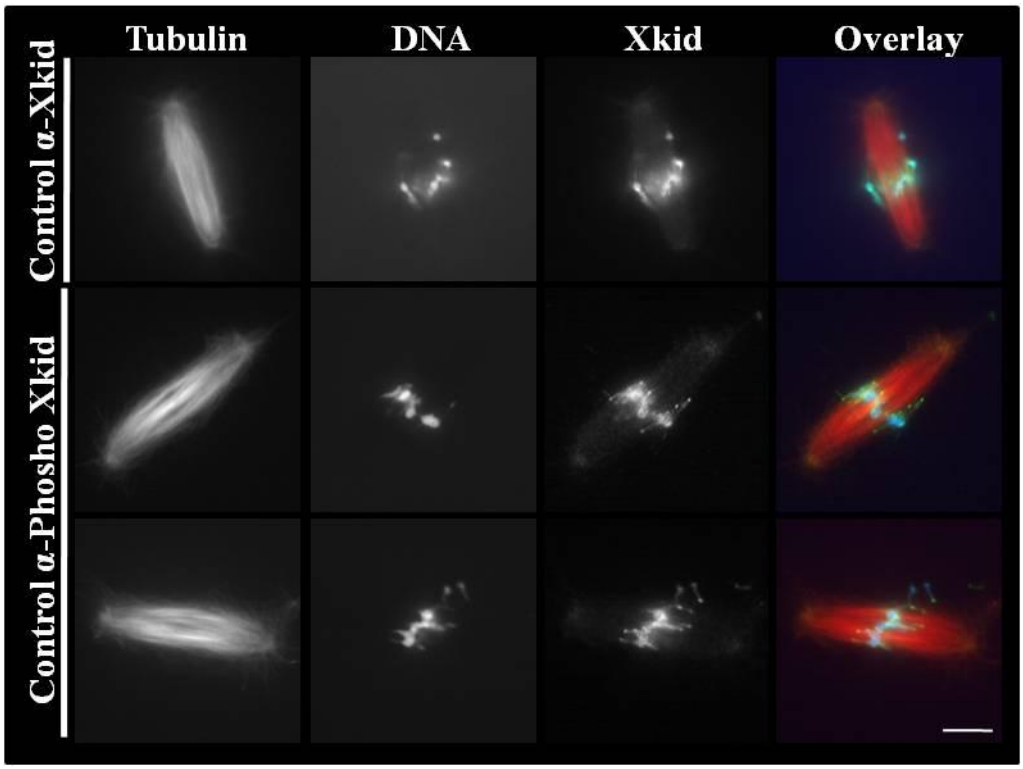


Figure 3.4. Localization of phospho-Xkid in spindles assembled in *Xenopus* egg extracts. Cycled spindles were assembled around sperm nuclei (sperm spindles). The samples were centrifuged onto coverslips, fixed and stained with anti-PXkid antibody and Hoeschst. Phospho-Xkid localizes to chromosomes and a faint signal is detected on the spindle microtubules. (bar, 10µm).

3.2. FUNCTIONAL STUDIES ON THE ROLE OF XKID PHOSPHORYLATION AT THE CDK1 CONSENSUS SITE THR450

The *Xenopus* system offers two complementary systems for studying cell cycle related issues: the maturation of the oocyte and the use of egg extracts. In both cases, the

protein of interest can be eliminated either by injection of anti-sense oligos (to eliminate its expression in oocytes) or by immuno-depletion of the protein (in egg extract). As essential control, the endogenous levels of the corresponding protein (or any selected mutant) has to be restored either using mRNA or purified proteins. As previous attempt to express and purify Xkid proteins mutagenized on Thr450 in the baculovirus system had failed, we decided to work with the corresponding mRNAs transcribed *in vitro* for expression of the mutant proteins upon injection in oocytes or addition to egg extracts.

3.2.1- Production of Xkid mutants in the consensus site for phosphorylation by the Ccd2 Kinase.

Xkid cDNA was mutagenised at the consensus site for phosphorylation by cdk1 to change the predicted phosphorylated residue, Thr450, to an alanine (XkidT450A) to generate a non phosphorylatable protein or to a glutamic acid (XkidT450E) to mimick a constitutively phosphorylated protein (fig 3.5).

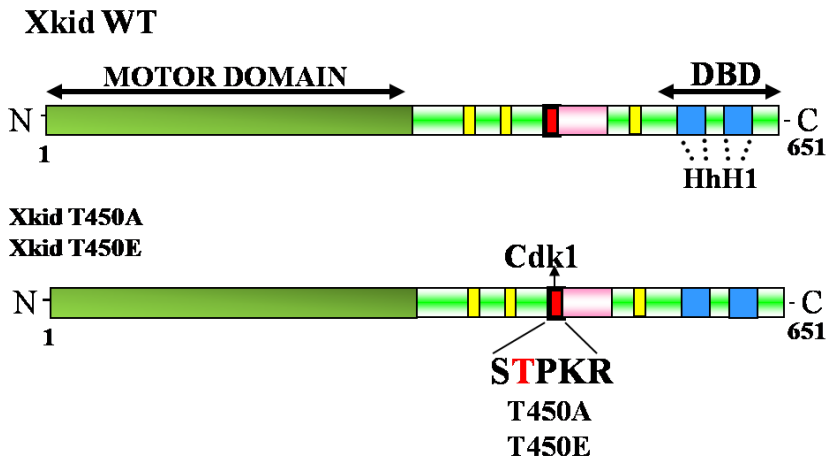


Figure 3.5. Schematic representation of the wild-type Xkid sequence and the different phosphorylation mutants generated.

3.2.2. The role of Xkid phosphorylation at Thr450 during oocyte maturation

The *Xenopus* oocyte has been extensively used as a functional expression system. In fact, transcription (Gurdon et al., 1971; Gurdon et al., 1974) and translation of foreign genetic information can take place in the *Xenopus* oocyte.

Previous studies have shown that Xkid is required for the meiosis I to meiosis II transition in *Xenopus laevis* oocytes (Perez et al., 2002)I therefore injected Stage VI oocytes with Xkid antisense oligonucleotides at 0.5mM has been described. However in my hands this did not block completely the expression of endogenous Xkid in Meiosis II. Therefore, to obtain a more efficient block of Xkid translation I increased the concentration of the injected antisense oligonucleotide (1 mM). In these conditions endogenous Xkid expression was fully abolished as monitored by western blot analysis of Meiosis II oocytes (MII) (fig 3.6 B).

To determine whether phosphorylation of Xkid at Thr450 is important for oocyte maturation, oocytes were injected with the Xkid morpholino antisense oligonucleotides and the mRNA encoding XkidT450A. Oocytes were then incubated with progesterone. Three to four hours after the progesterone addition, all oocytes had performed GVBD and reached meiosis II. Western blot analysis showed that XkidT450A ectopic mRNA was efficiently expressed (Fig 3.6 B). Meiosis II oocytes expressing XkidT450A presented a normal morphology **(**Figure 3.6 C). These results suggest that Xkid phosphorylation at Thr450 is not essential for its role in the meiotic cell cycle progression.

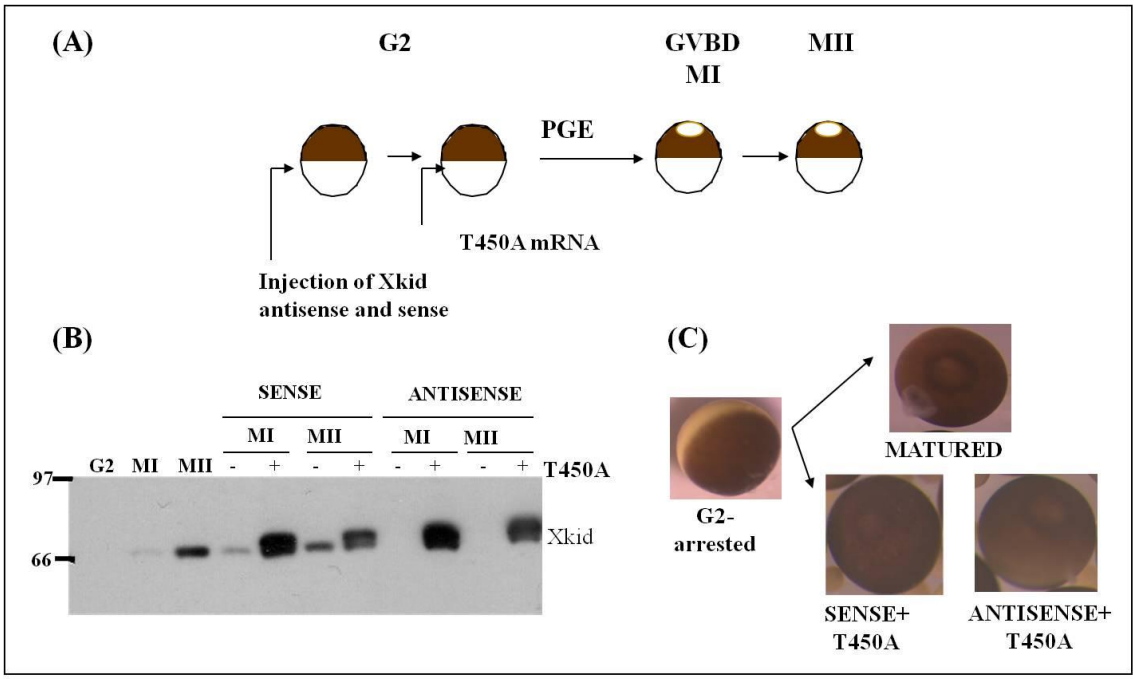


Figure 3.6. Expression of T450A mutant in Xkid-depleted oocytes.
(A) Oocytes were injected with Xkid antisense or control oligonucleotides, after two hours they were injected with *in vitro* transcribed mRNA encoding XkidT450A. Progesterone was added 1 h later and oocytes were collected at GVBD (MI) and 4 h later (MII).
(B) Expression of Xkid T450A in *Xenopus* oocytes was analysed by immnunoblotting. The XkidT450A was efficiently translated.
(C) Morphological appearance of Xkid-depleted and wild-type oocytes during the meiotic cell cycle. Pictures were taken before progesterone stimulation (G2) and 4 h after GVBD (MII).

3.2.3. Establishing a reliable method for performing functional experiments in *Xenopus* egg extracts expressing proteins from *in vitro* transcribed mRNAs

To study the role of Xkid phosphorylation in spindle assembly I used the *Xenopus* egg extract system. As mentioned above functional experiment in this system require the availability of purified protein(s) to perform rescues and/or study specific protein domains or mutants. Previous attempts performed in the lab to purify phospho mutant variants of Xkid in bacculovirus had been unsuccessful and therefore we decided to try another approach. Several years ago, Murray and Kirschner (Murray and Kirschner, 1989), showed that it was possible to rescue the cell cycle progression in an extract depleted of all the endogenous mRNA by adding exogenously an mRNA encoding sea urchin or *Xenopus* cyclin B. This suggested that the addition of *in vitro* transcribed

mRNA to the extract should allow the expression of the protein of interest and therefore we perform the functional experiments that we had planned.

The mRNAs transcribed *in vitro* and used successfully for expression in oocytes were added to *Xenopus* egg extracts that were cycled into interphase and back into M-phase. Expression of the Xkid variants was then evaluated by Western blot analysis. These initial experiments showed that there was a huge variability in the efficiency of translation of the exogenously added mRNAs, as well as in the capacity of these egg extracts to assemble mitotic structures. Therefore, a major effort was directed at establishing a robust protocol and optimize this experimental approach. The improvements were focused on getting better:

(1) mRNA preparation

(2) Cytostatic factor (CSF) extract preparation

and in establishing a standardized flow for the complete depletion-add-back experiment involving:

(3) mRNA expression tests in the egg extract to determine the right concentration of mRNA to be added for each batch of in vitro transcribed mRNA

(4) optimized depletion and add-back in cycled *Xenopus* egg extract.

3.2.3.1. GENERAL CONSIDERATIONS FOR PREPARING GOOD QUALITY MRNA

Transcription yields can differ substantially depending on the individual DNA template. In general, a concentrated and pure enough DNA is easily digested with restriction enzymes. The best transcription yields were obtained using 40μg of DNA template for the linearization reaction resuspended in 15-20 μl of water.

The exogenous transcripts to be added to the egg extract should carry a cap structure and a poly (A) tail. The cap structure improves the mRNA stability and translation efficiency. Usually, translation improves if the mRNA contains a poly (A) tail longer than 31 residues. Optimally, the poly (A) tail is encoded in the DNA construct used to synthesize RNA. All Xkid DNA constructs had included a poly (A) tail (Gebauer and Hentze, 2007).

Two transcription protocols were used in order to obtain large amounts of mRNA: the Hartmann Lab protocol and the mMessage mMachine T7 Ultra Kit. Both protocols were

performed at room temperature, and included a capped transcription reaction and a poly (A) tail procedure. Different methods were initially used to purify and concentrate the mRNA. Finally the most efficient method was to use the RNeasy Micro or Mini Kit.
Initially, the mRNA was then resuspended in CSF-XB, but these mRNAs were not efficiently translated in a consistent manner. CSF-XB contains different salts and salts were described as inhibitors for the translation reaction (Baglioni et al., 1978).
Therefore, the mRNA's were resuspended in RNase-free water, measured by nanodrop, visualized by agarose gel, aliquoted and kept at -80º. The yield of Xkid mRNA's varied between 0.2 µg/µl to 1-2 µg/µl. Ideally, the same Xkid mRNA preparations were used for several independent egg extracts experiments.

3.2.3.2 PREPARATION AND USE OF CYTOSTATIC FACTOR (CSF) EXTRACTS

3.2.3.2.1. Functional assays: the translation of mRNAs was always performed in fresh CSF-extracts.

Translation in frozen extracts was sometimes used to determine the rate of translation of specific mRNA's batches.
In general, Cytostatic factor (CSF)-arrested extracts were prepared following the protocol described by Murray (Murray, 1991).The quality of the egg extract was particularly critical for the translation of *in vitro* transcribed mRNAs . To obtain good extracts, eggs obtained from different frogs were kept separate for close examination. Any batch containing lysed and/or activated eggs, or "puffballs" was discarded. High-quality eggs from different frogs were pooled, dejellied, tightly packed and crushed by low-speed centrifugation. Three layers are then visible: a yellow layer at the top containing lipid droplets, middle layer corresponding to the cytoplasm and a dark layer at the bottom containing yolk granules, heavier membranes and nuclei. The cytoplasmic layer including the grayish material at its bottom that contains mitochondria and vesicles was carefully removed using a needle. Following standard protocols protease inhibitors and cytochalasin D were added to the resulting CSF-extract. The capacity of the extract to translate exogenously added mRNA was also found to be more reliable when adding as well an ATP regenerating system also called energy mix (creatine phosphate, ATP and MgCl2).

The quality of the CSF-extract was always assessed before further use. Extracts were supplemented with rhodamine-labelled tubulin and sperm nuclei, and incubated in a water bath at 20ºC. Small samples were taken at different time points (30, 45 and 60 min) and spotted on a slide. Upon fixation the structures assembled around the sperm nuclei were monitored by fluorescence microscopy. CSF-extracts that formed half-spindles with a clear spindle pole and condensed DNA at around 30 min of incubation were considered competent for further experiments including mRNAs. Egg extracts showing free microtubule nucleation and/or abnormal structures were discarded.

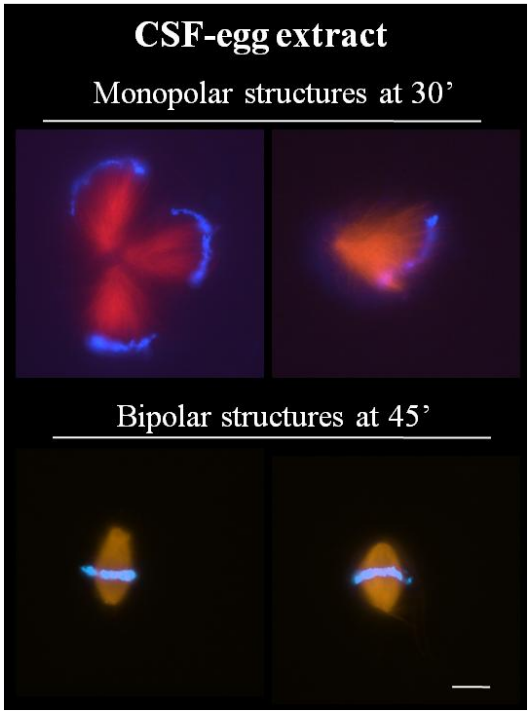


Figure 3.7. Examples of spindle assemblies from independent CSF-egg extracts: The CSF-extract containing rhodamine-labeled tubulin and sperm nuclei were mixed gently, and incubated at 20ºC. At 30 min, 45 min and 60 min, samples were taken to test the progress of CSF spindle assembly. The CSF extracts considered for further experiments were the ones that by 30 min presented the chromatin migrated away from the centrosome, and a clear spindle pole, termed half-spindles. Between 45min and 60 min half spindles begin to fuse to form bipolar structures.

3.2.3.2.2 Optimization of Xkid depletion from CSF-extracts

One main difficulty in immunodepletion experiments using CSF extracts is that these extracts can loose the CSF arrest during or soon after immunodepletion. Therefore, the first step was to optimize the conditions for Xkid immunodepletion in order to minimize the perturbation of the extract. The Xkid immunodepletion was performed using Protein A magnetic beads (Dynal) that have a high affinity for the heavy chain of

immunoglobulins. To coat the beads with the anti-Xkid tail antibody, 20 µl of beads (for depleting 100 µl egg extract) were washed three times with 1 ml of PBS-T and incubated in a final volume of 0.25 ml of PBS-T containing 10 µg of antibodies during 30 minutes at room temperature on a rotating wheel. The beads were then washed twice with 1 ml of PBS-T and twice with 1 ml of CFS-XB buffer. Antibody-coated beads were recovered on a magnet and added to 100µl of CSF-egg extract on ice for 30 minutes. Then, the magnetic beads were retrieved on a magnet for 10 minutes, and the egg extract transferred to another tube with antibody-Xkid coated beads and kept on ice. For control, another aliquot of the same CSF-extract was mock depleted with an equivalent amount of unspecific IgG. Immunoblot analysis of the control and depleted extracts showed that under these conditions, more than 95% of Xkid was removed from the extract (fig 3.8). Therefore this improved previously described protocols (Antonio et al., 2000) by reducing the time of incubation by half and therefore ensuring a minimal manipulation of the egg extract for further experiments.

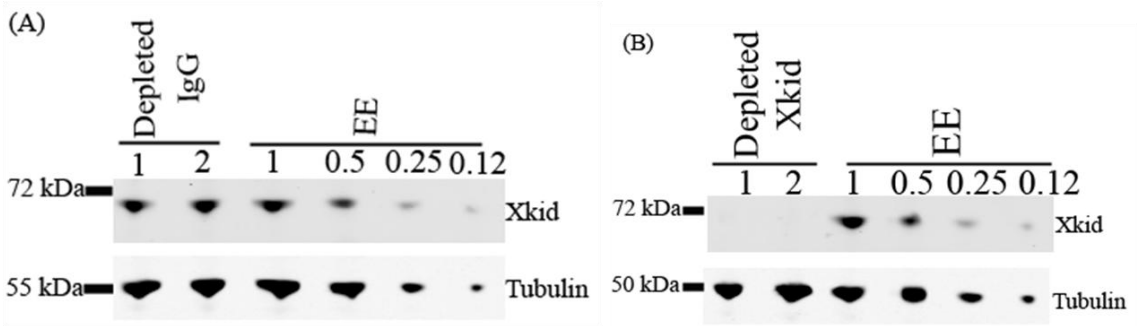


Figure 3.8. Xkid depletion from *Xenopus* egg extract. Immunoblot probed with anti-Xkid antibody showing the efficiency of Xkid depletion. One µl of control (depleted IgG) and Xkid depleted (Depleted Xkid) were run in parallel with 1, 0.5, 0.25 and 0.12 µl of a control egg extract. More that 95% of Xkid is removed after depletion.

3.2.3.2.3. The translation assay in *Xenopus* egg extracts by addition of exogenous mRNA to Xkid depleted egg extracts

As a first step, a translation assay was always performed for every new mRNA preparation to select the optimal concentration to obtain levels of corresponding Xkid protein in the range of the endogenous Xkid concentration (fig 3.9). Usually, the concentration of the added mRNA was between 0, 03-0, 1 µg / µl per reaction. The egg extract incubation was performed at 20ºC in a cooled water bath for 40 minutes to 1 hour.

Although the mRNA volume added to the egg extract varied depending on the experiment, it was never larger than 5% of the final reaction volume. In addition, the egg extract was never diluted by more than 10 % of total volume since greater dilution resulted in poor translation efficiency and/or extract performance.

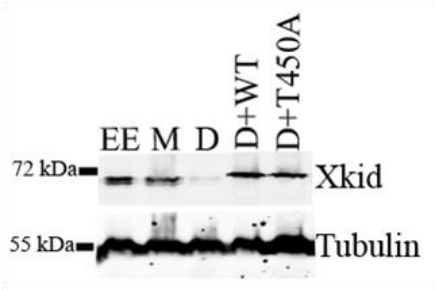


Figure 3.9. Different *in vitro* transcribed mRNA's were used to study the efficiency of translation in *Xenopus* egg extract. Samples correspond to EE: egg extract. M: mock (control) depleted extract. D: Xkid depleted extract. D+WT: Xkid depleted extract containing wild-type form. D+T450A: Xkid depleted extract containing T450A form. From this blot we estimate that all variants of Xkid were efficiently translated.

3.2.3.2.4. Stability of the translated Xkid variants in the egg extract

Since Xkid protein levels are tightly regulated during the cell cycle, we wondered whether the translated proteins, in particular the phospho variants were as stable as the endogenous protein. To investigate this point, Xkid depleted CSF- extracts containing the mRNAs encoding the different variants of Xkid were incubated for 1 hour at 20^0C. Then, the extracts were sent into interphase by addition of calcium and protein synthesis was blocked by addition of cyclohexymide (fig 3.10 A). The stability of the different proteins was then monitored by immunoblot of samples taken at different time points of incubation at 20^0C: 30 min, 1 hour, 1h 30 min and 2 hours (fig 3.10 B).

The levels of endogenous Xkid were reduced after 30min of incubation and then remained constant at longer incubation times. All the translated Xkid variants showed a similar pattern of stability suggesting that these proteins are not susceptible to degradation in the egg extract and that phosphorylation at Thr450 does not play a role in Xkid stability. As a control, the blots were probed with an antibody against Xorbit.

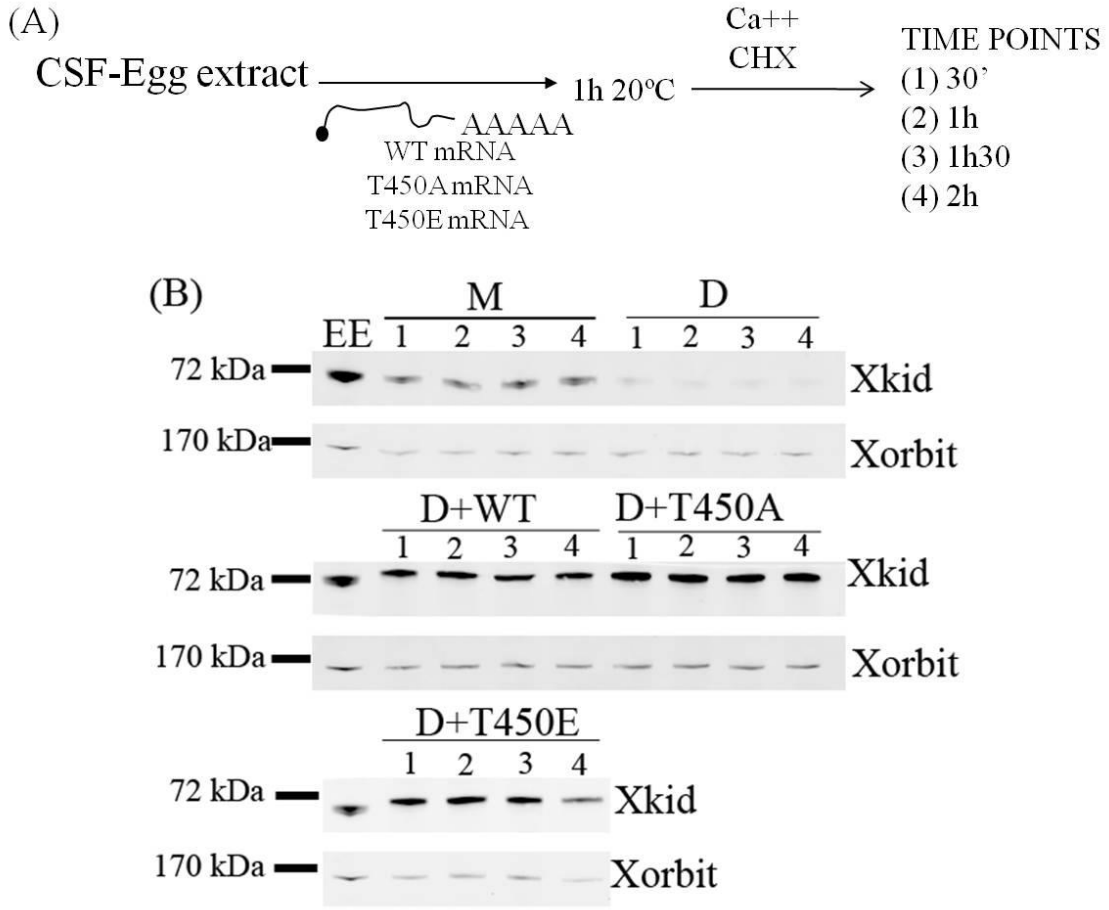


Figure 3.10. In CSF-egg extract, endogenous and exogenous Xkid proteins are stable.
(A) Schema of the experimental approach: CSF- egg extracts were incubated with the different *in vitro* transcribed mRNA for 1h at 20ºC.Then, calcium (Ca++) and cycloheximide (CHX), an inhibitor of biosynthesis were supplemented. Aliquots of samples were taken at 30 min (1), 1h (2), 1h30 min (3) and 2h (4), and runned on an SDS-polyacrylamide gel, for further analysis by immunoblot.
(B) Immunoblot probed with anti-Xkid antibody, and anti-Xorbit as a control. Samples correspond to EE: egg extract. M: Mock (control) depleted extract. D: Xkid depleted extract. D+WT: Xkid depleted extract containing wild-type form. D+T450A: Xkid depleted extract containing T450A form. D+T450E: Xkid depleted extract containing T450E form. From this blot the translated Xkid variants seemed to have a similar pattern of stability.

3.2.3.2.5. Probing the addition of mRNA to egg extract for functional experiments

To validate the approach by performing a depletion add-back experiment in cycled egg extract using the mRNA encoding for the different variants of Xkid, the protocol established was the one represented in fig 3.11.

More reliable results, were obtained with egg extract reaction volumes of either 40 µl (tube 1:15 µl to cycle into interphase and tube 2: 25 µl to sent back to mitosis) or 50 µl (tube 1: 20 µl to cycle into interphase and tube 2: 30 µl to sent back to mitosis).

One aliquot of extract (tube 1) was supplemented with sperm nuclei, sent to interphase by the addition of calcium, and incubated at 20ºC for 90 minutes. Meanwhile, the another aliquot of the same extract (tube 2) was supplemented with mRNA and incubated at 20ºC for 1 hour. The efficiency of translation was later monitored by western blot analysis of aliquots taken at 30-,45-, and 60- minutes of incubation. This extracts was then kept on ice and then added to the interphase extracts from tube 1 to send it into mitosis. The mixture was then incubated further for 60-minutes at 20^0C. Spindles were centrifuged onto a coverslip, fixed in formaldehyde and methanol and the DNA stained with Hoechst.

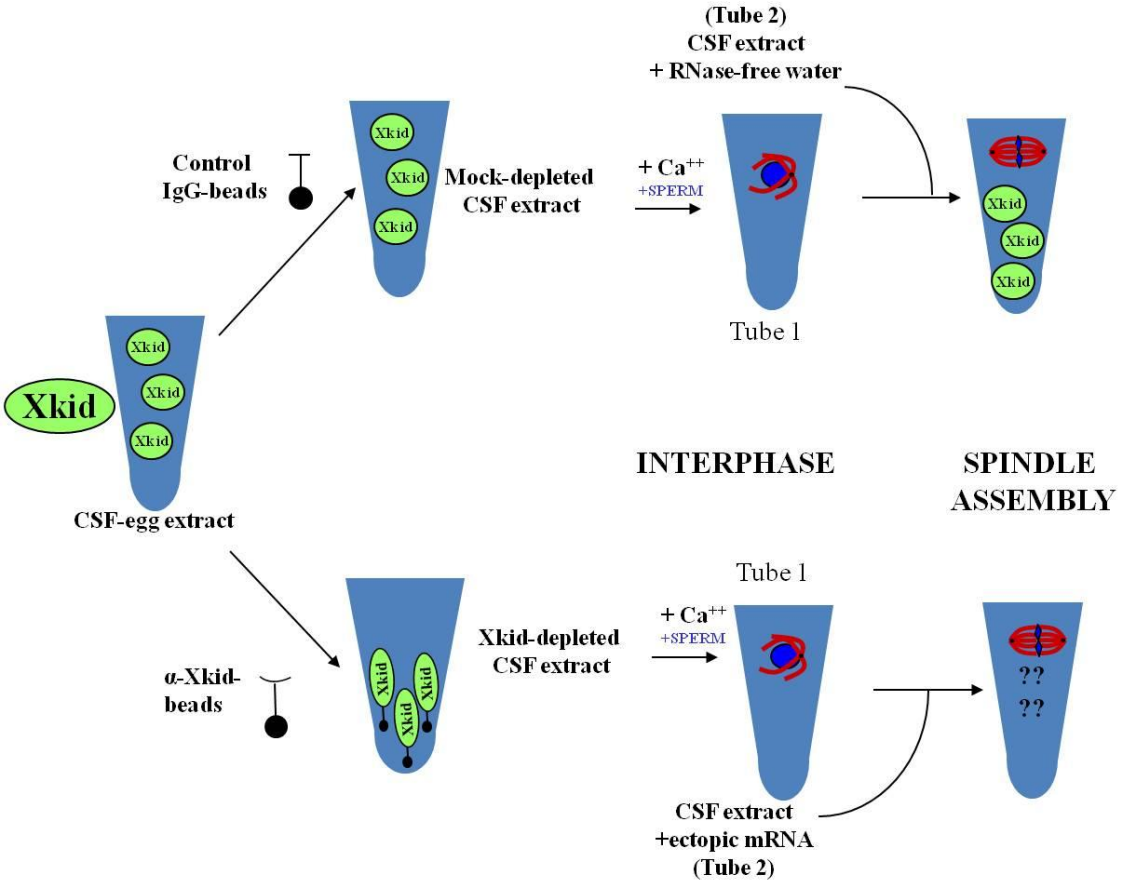


Fig 3.11. A schematic representation of the protocol established to study the addition of exogenous mRNA in Xkid-depleted egg extract.

Western blot analysis showed that endogenous Xkid was depleted from the egg extract and that exogenous amounts of Xkid-WT were expressed in the extract supplemented with the mRNA (fig 3.12 A).

Immunofluorescence analysis (fig 3.12 B), showed that, as expected the anti-Xkid antibody did not generate any signal on spindles assembled in Xkid depleted extracts. By contrast it decorated the chromosomes in depleted extracts expressing Xkid-WT, indicating that this protein is correctly targeted and behaves as the endogenous protein. In this particular case, it seems that the expression of Xkid-WT in Xkid depleted extracts, rescues the chromosome misalignment phenotype. These results indicated that the expression of an exogenously added mRNA does not affect the ability of the egg extract for spindle assembly.

However, in general in these samples there was systematically a lower number of bipolar spindles and an increase in the number and appearance of abnormal structures with some spindles having an increase in width, less microtubule density and not tightly aligned chromosomes. One possibility was that the capacity of extracts containing exogenous mRNAs to form bipolar spindles might be strongly dependent on the egg extract quality.

Our results suggest that, the mRNA add-back methodology can be used as a way to study the function of a protein using the *Xenopus* egg extracts.

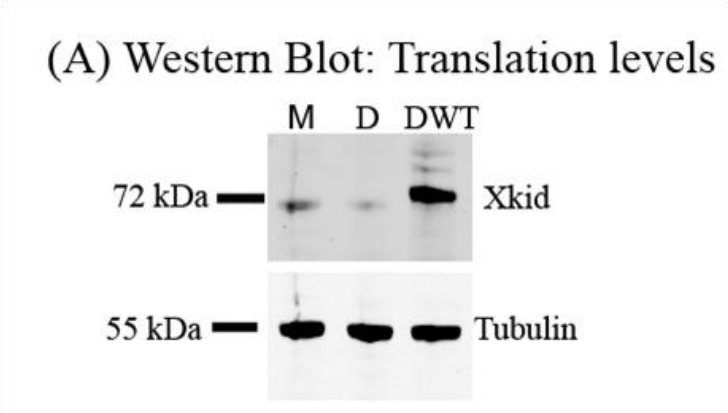


Figure 3.12. Xkid-wild type mRNA addition to the depleted *Xenopus* egg extract.
(A) Immunoblot probed with anti-Xkid antibody and anti-tubulin antibody. Samples correspond to mock (control) (M), Xkid depleted extract (D) and Xkid depleted extract containing Xkid wild-type mRNA (DWT). From this blot, we conclude that Xkid-WT was efficiently translated.

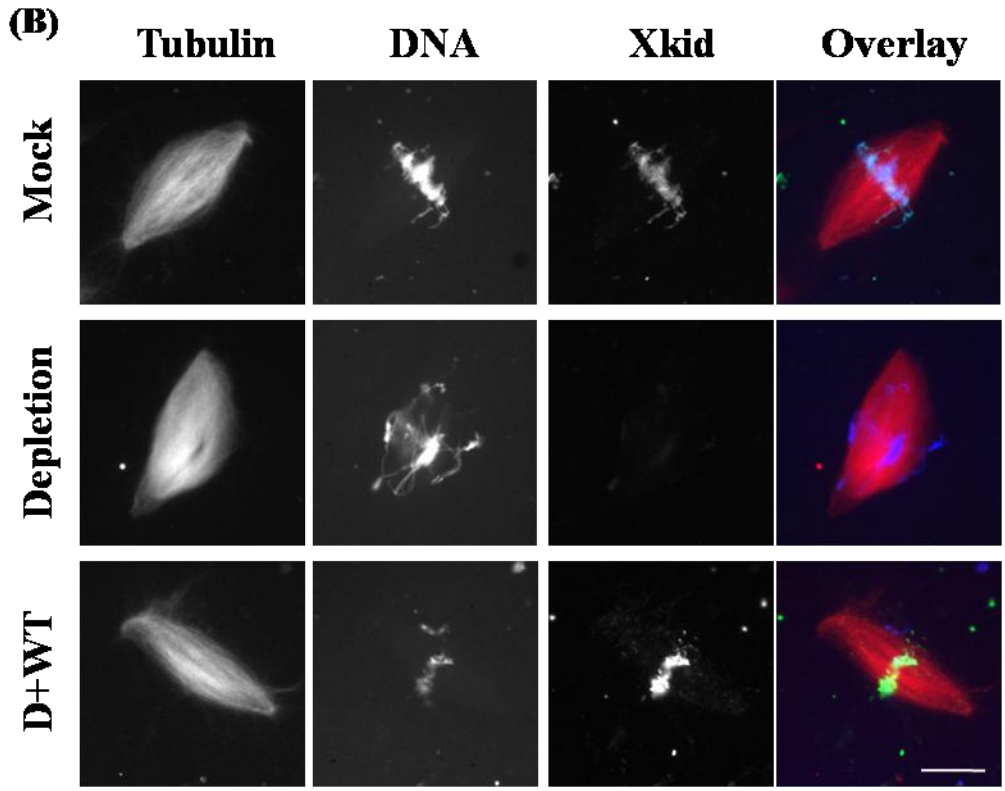


Figure 3.12. Xkid-wild type mRNA addition to the depleted *Xenopus* egg extract.
(B) Spindles assembled in mock (control) depleted, Xkid depleted (Depletion) and Xkid depleted supplemented with Xkid wild type (DWT) are shown. In this particular case, bipolar spindles assemble with aligned chromosomes. Xkid (green), Tubulin (red) and DNA (blue).

3.2.4. The role of Thr450 phosphorylation in Xkid function and localization

To investigate the role of Thr450 phosphorylation in Xkid function and localization, mRNAs encoding Xkid-T450A and Xkid-T450E mRNA's were added to control and Xkid depleted egg extracts. Cycled spindle assembly experiments were performed as described above.

3.2.4.1. THE ROLE OF THR450 PHOSPHORYLATION IN XKID LOCALIZATION

Our previous results showed that Xkid wild type expressed in egg extract from exogenously added mRNA does localize correctly to the chromosomes in M-phase as the endogenous protein. Western blot analysis showed that endogenous Xkid in this particular experiment was not efficiently depleted from the egg extract (fig 3.13 A). This happened with some

frequency in independent experiments, probably due to the Xkid resynthesis capacity. Xkid-T450A and Xkid-T450E were efficiently expressed.

Immunofluorescence analysis (3.13 B), showed that anti-Xkid antibody gave a faint signal on chromosomes assembled in Xkid-depleted egg extract. In this preliminary result, Xkid-depleted egg extracts where the Xkid-WT was expressed, this protein is correctly targeted to chromosomes, behaving as the endogenous protein. The spindles assembled in extracts expressing Xkid-T450A or Xkid-T450E showed that these proteins localized to the chromosomes suggesting that Thr450 phosphorylation has no role in Xkid chromosome localization in mitosis.

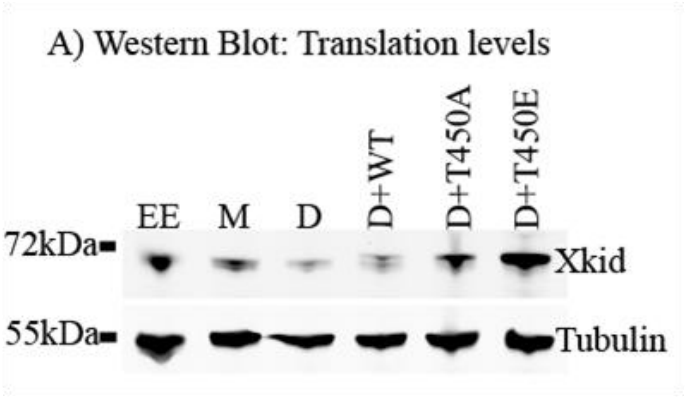


Figure 3.13. Addition of different variants of Xkid to depleted extracts.
(A) Immunoblot probed with anti-Xkid antibody and anti-tubulin antibody. Samples correspond to egg extract (EE),mock (control) (M) , Xkid depleted extract (D) , Xkid depleted extract containing wild-type mRNA of Xkid (DWT), Xkid depleted extract containing T450A mRNA of Xkid (D+T450A) and Xkid depleted extract containing T450AE mRNA of Xkid (D+T450E). The XkidT450A and T450E were efficiently translated.

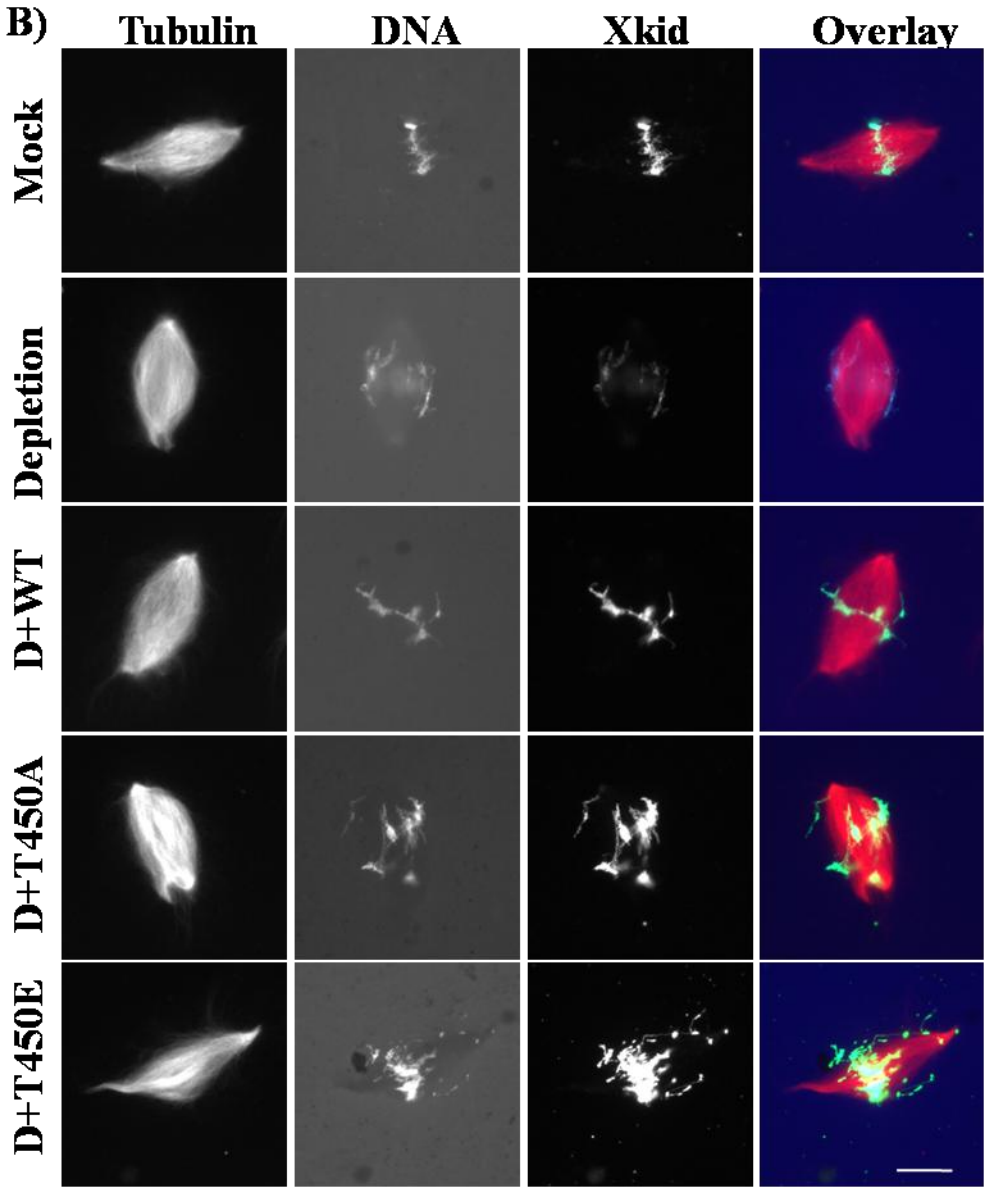


Figure 3.13. Addition of different variants of Xkid to depleted extracts.
(B) Add-back experiment using *in vitro* transcribed mRNA's used to study the role of phosphorylation in Xkid chromosomal localization: Spindles found in mock (control) depleted, Xkid depleted (depletion) extracts and Xkid depleted extracts added-back with wild-type Xkid mRNA (D+WT), T450A mRNA of Xkid (D+T450A) and T450AE mRNA of Xkid (D+T450E) are shown. Tubulin (red) and DNA (blue). (bar,10μm). The nonphosphorylated form of Xkid localized to the chromosomes, suggesting that Thr450 phosphorylation is not needed for Xkid chromosomal localization.

Previous immunofluorescence studies in Xenopus XL177 cells using the anti-Xkid antibody, has shown that in interphase Xkid is a nuclear protein (Antonio et al., 2000). This is consistent with the presence of NLS in Xkid sequence and the enrichment of the protein in the nuclear fraction of cell extracts. We therefore examined whether Thr450

phosphorylation had any influence in this interphase localization using egg extracts expressing Xkid-T450A. The egg extract was supplemented with sperm nuclei, and sent to interphase by the addition of calcium, and incubated at 20ºC for 90 minutes. After 90 min incubation at 20ºC, samples were taken for western blot analysis (3.14 A) and the extract was centrifuged onto coverslips for immunofluorescence with the anti-Xkid antibody (fig 3.14 B). As expected, endogenous Xkid was present inside the nuclei. No labelling was detected in Xkid depleted extract. Xkid-T450A also accumulated inside the nuclei. This result indicated that the T450A mutation does not disrupts Xkid localization in the nuclei during the interphase.

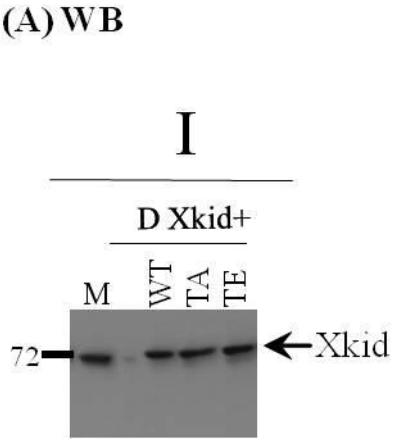


Figure 3.14. T450A mutation does not interfere with Xkid localization to the nuclei during the interphase. (A) Immunoblot probed with anti-Xkid antibody. Interphasic samples (I) correspond to mock (control) (M), Xkid depleted extract (D Xkid) and Xkid depleted extract where the different variants of Xkid (wild type mRNA (WT), T450A mRNA (TA) and T450E mRNA (TE) have been added.

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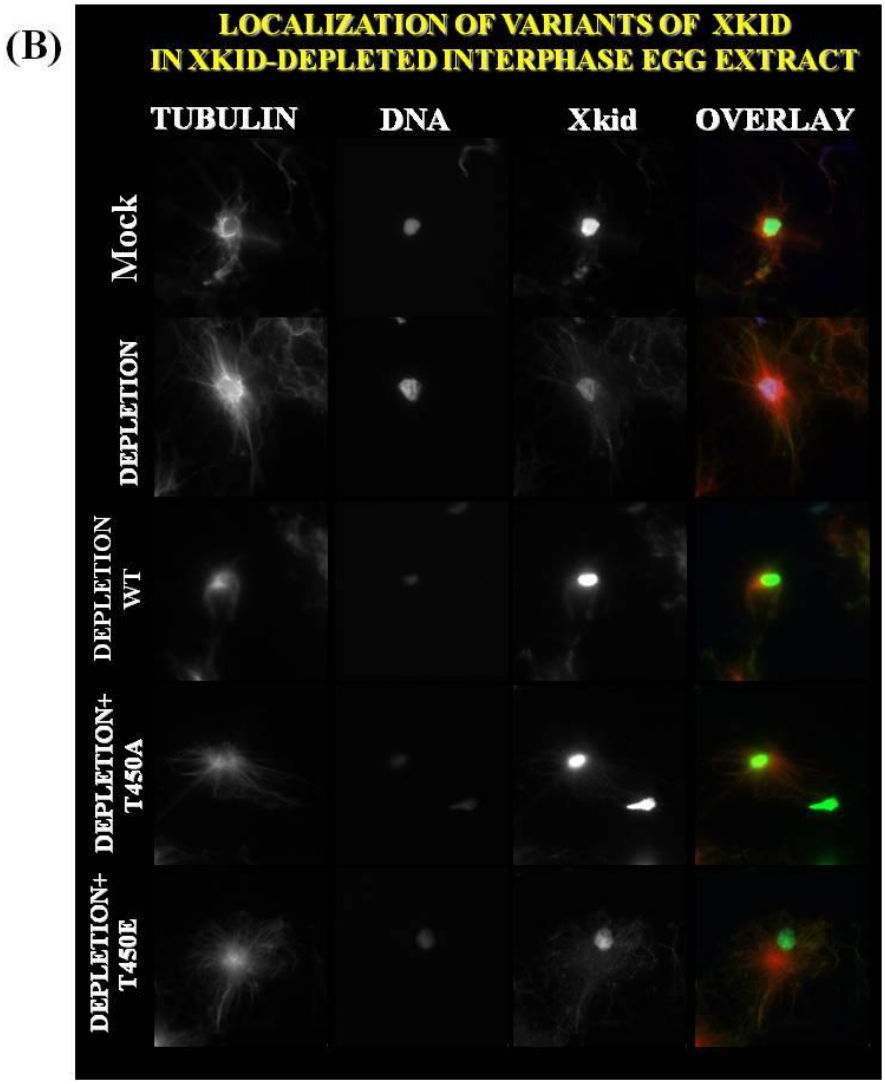

Figure 3.14. T450A mutation does not interfere with Xkid localization to the nuclei during the interphase. (B) The interphase structures found in a mock (control) depleted, Xkid depleted (depletion) extracts , Xkid depleted extracts added-back with wild-type Xkid mRNA (Depletion+WT) ,T450A Xkid mRNA (depletion+T450A) and T450E Xkid mRNA (depletion+T450E) are shown. Tubulin (red), Xkid (green) and DNA (blue).

3.2.4.2. THE ROLE OF THR450 PHOSPHORYLATION IN XKID FUNCITON IN M-PHASE

The majority of the mitotic structures formed in Xkid depleted cycled extract expressing the Xkid- T450A and Xkid-T450E variants were mostly aberrant. Very often they consisted of a mass of microtubules or spindle-like structures with chromosomes that were scattered everywhere. Due to the small number of bipolar structures or spindle-like structures in these samples, it was impossible to conclude if the expressed Xkid proteins were able or not to restore metaphase chromosomes alignment. However, the samples where Xkid-T450E was

expressed the effect was more strong compared with Xkid-T450A, indicating that it could be behaving as a dominant negative (fig 3.15 B). This suggests that phosphorylation might be important for Xkid function. One possibility, is that there is an overexpression effect. Further experiments will be needed to get a clear answer. The translation levels for this experiment is shown (fig 3.15 A).

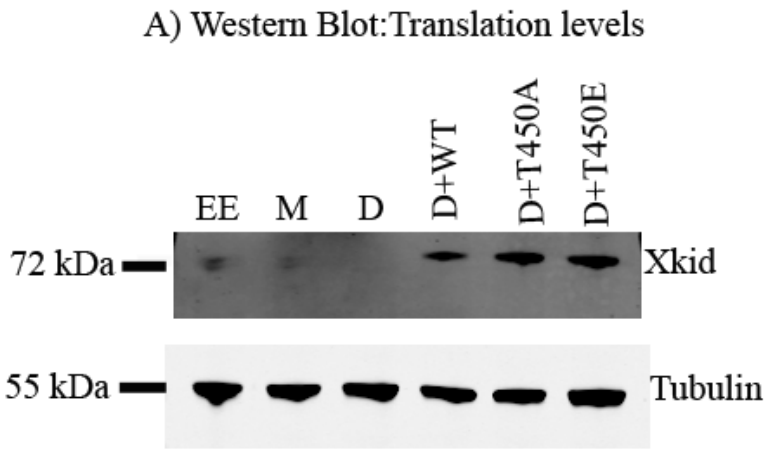

Figure 3.15. Addition of different variants of Xkid to depleted egg extracts.
(A) Immunoblot probed with anti-Xkid antibody and anti-tubulin antibody. Samples correspond to egg extract (EE),mock (M), Xkid depleted extract (D) , Xkid depleted extract added abck with wild-type mRNA of Xkid (D+WT), T450A mRNA of Xkid (D+T450A) and T450AE mRNA of Xkid (D+T450E). The XkidT450A and T450E were efficiently translated. From this blot we estimate that all exogenous forms of Xkid were efficiently translated.

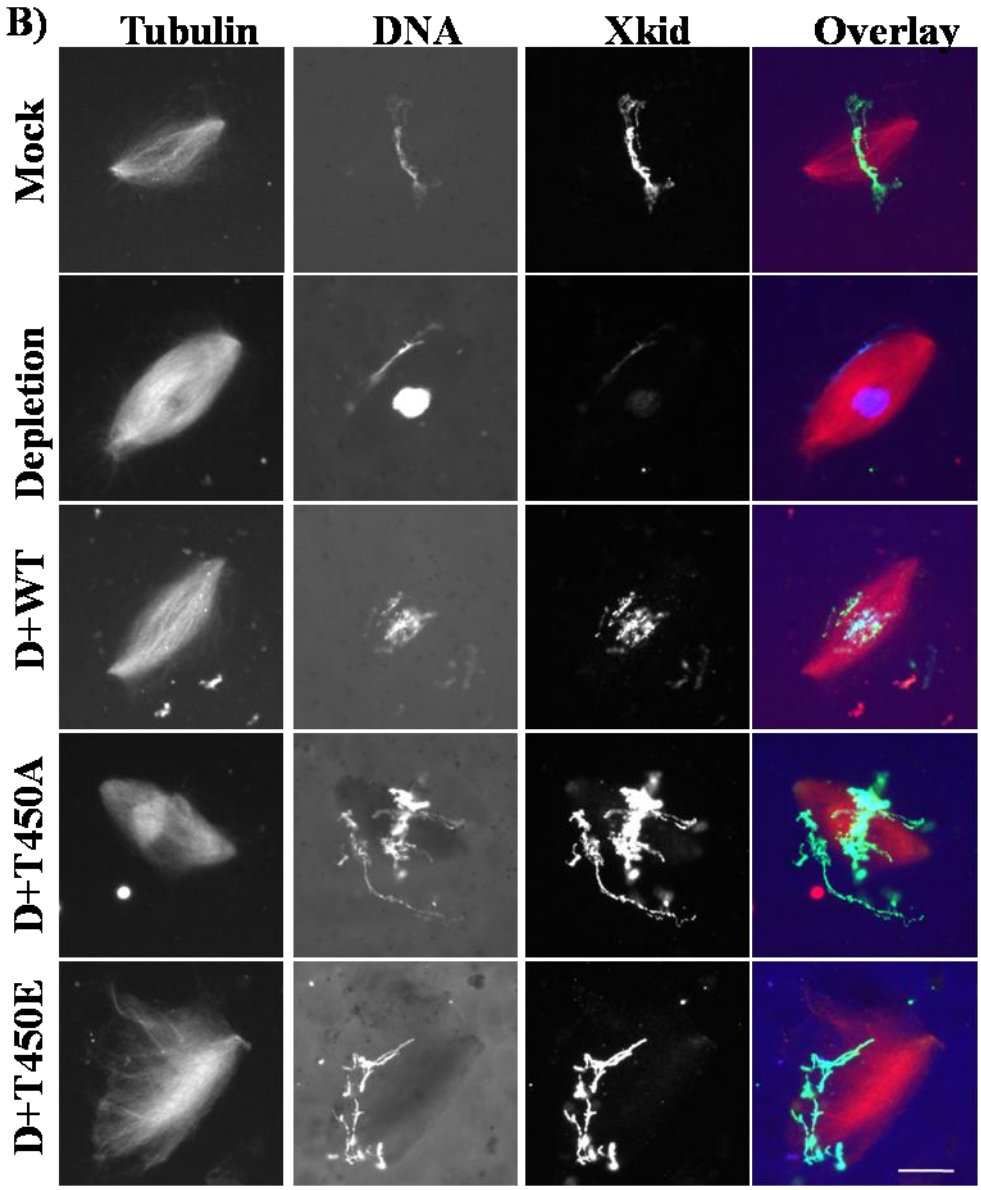

Figure 3.15. Addition of different variants of Xkid to depleted egg extracts.
(B) Add-back experiment using *in vitro* transcribed mRNA's used to study the role of phosphorylation in Xkid chromosomal localization: Spindles found in mock depleted, Xkid depleted (depletion) extracts and Xkid depleted extracts added-back with wild-type Xkid mRNA (D+WT), T450A mRNA of Xkid (D+T450A) and T450AE mRNA of Xkid (D+T450E) are shown. Tubulin (red) and DNA (blue). (bar,10µm). The T450E seems to behave as a dominant negative.

3.2.5 The role of Thr450 phosphorylation in regulating Xkid microtubule and or spindle binding properties

The data obtained so far indicated that phosphorylation at the cdk1 consensus site is not important for Xkid chromosome localization in mitosis. However, the phenotype of the mitotic structures suggested that spindle microtubules were affected. We therefore decided

to test more directly whether Thr450 phosphorylation could regulate the binding of Xkid to microtubule or spindles.

3.2.5.1 ROLE OF THR450 PHOSPHORYLATION IN REGULATING XKID ASSOCIATION WITH SPINDLES

To determine whether Xkid and its phosphorylation variants co-pellet with spindles, Xkid depleted egg extract containing the mRNAs for expression of Xkid-WT or XkidT450A and sperm nuclei were incubated at 20^0C for 60 min. The translation levels were checked by western blot (fig 3.16 A).An half of the reaction was then supplemented with nocodazole to induce microtubule depolymerization. The different extracts (30-60 µl) were then diluted and centrifuged through a sucrose cushion (for 12 minutes at 20ºC, 12000 rpm in a TLS-55 rotor). The pellets were resuspended in 30-60 µl of sample buffer and analyzed by western blotting. As expected, the addition of nocodazole to the extract resulted in the total absence of tubulin in the pellet (fig 3.16 B). These samples therefore should contain only mitotic chromatin devoid of associated microtubules. Consistently, we found that this pellets contained Xcap-G, a subunit of the condensing I complex.

Xkid-WT and XkidT450A co-pelleted with spindles and chromatin alone as efficiently as the endogenous protein. Therefore, Thr450 phosphorylation does not regulate the binding of Xkid to spindles or mitotic chromatin. The higher levels of Xkid associated to full spindles compared to chromatin alone suggests that independently of its phosphorylation on Thr450 some Xkid associates to spindle microtubules and some to the chromosomes.

Interestingly, these results show that the mRNA add-back methodology can be useful for this type of biochemical approaches.

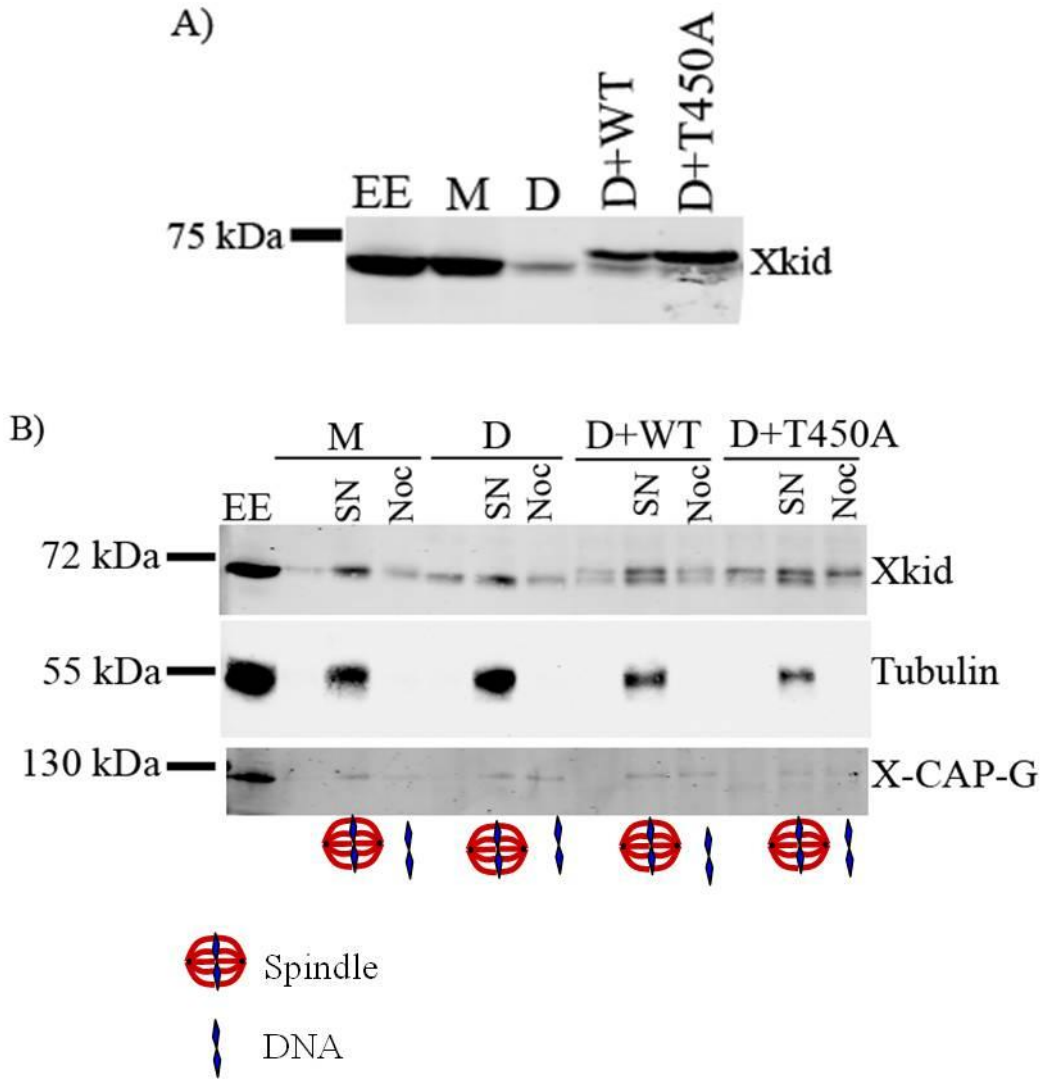

Figure 3.16. Spindle pelleting assay using the mRNA add-back methodology.
(A) Immunoblot showing the efficiently of translation of the exogenous forms of Xkid. Samples correspond to egg extract (EE),mock (control) (M), Xkid depleted extract (D) , Xkid depleted extract added –back with wild-type mRNA of Xkid (D+WT), and T450A mRNA of Xkid (D+T450A).
(B) The Xkid wild type and XkidT450A were recovered with spindles and chromatin alone as efficiently as the Xkid endogenous protein. The blot was probed with specific antibodies as indicated.

3.2.5.2 ROLE OF THR450 PHOSPHORYLATION IN XKID MICROTUBULE BINDING PROPERTIES

To investigate whether Thr450 phosphorylation has any role in regulating Xkid association with microtubules, mock- and Xkid-depleted CSF-egg extracts were supplemented with the different Xkid mRNAs (encoding Xkid-WT or XkidT450A). 30-60 µl of extract from each condition were supplemented with either taxol to stabilize the microtubules or nocodazole to

prevent microtubule polymerization (3.17 A). The extracts were then pelleted through a cushion and analyzed by western blotting with the anti-Xkid and an anti-tubulin antibodies. As shown in (fig 3.17 B), XkidT450A co-pelleted specifically with the microtubules like the endogenous Xkid and the Xkid-WT indicating that Thr450 phosphorylation does not play a major role in regulating the binding of Xkid to the microtubules.

Interestingly, these results show that the mRNA add-back methodology may be extremely useful to study the microtubule binding properties of any given protein without having to express and purify it.

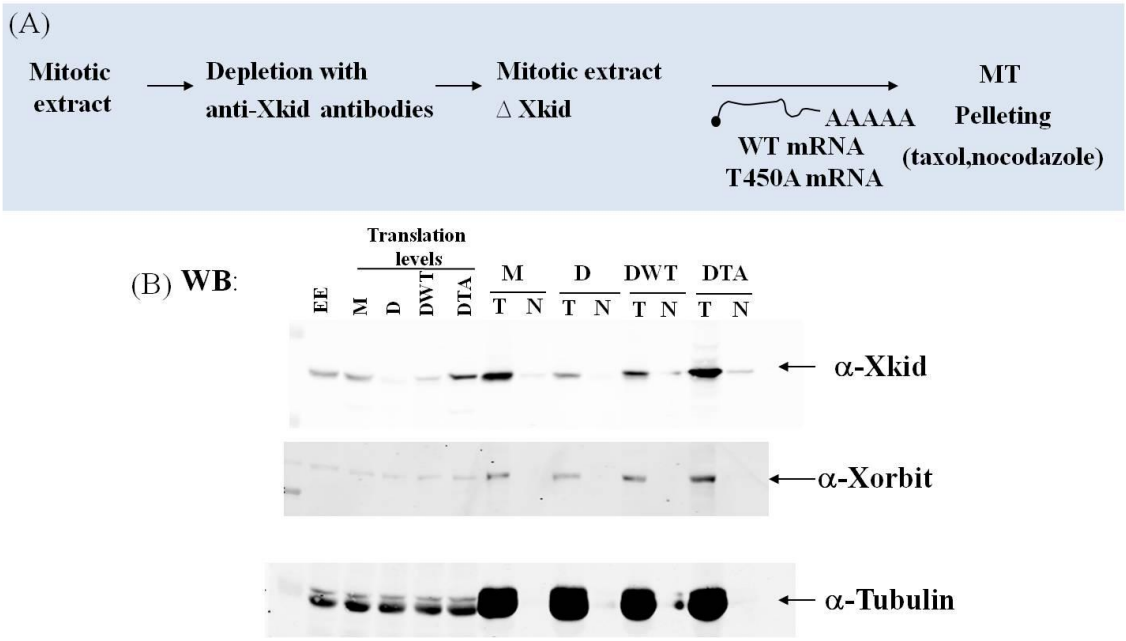

Figure 3.17. Microtubule pelleting assay using the mRNA add-back methodology.
(A) Cartoon of experimental approach used to perform microtubule pelleting assays in presence of *in vitro* transcribed mRNA.
(B) Immunoblot showing the translation of Xkid wild type and Xkid T450A. Samples correspond to egg extract (EE),mock (M), Xkid depleted extract (D) , Xkid depleted extract containing Wild-type mRNA of Xkid (D+WT), Xkid depleted extract containing T450A mRNA of Xkid (D+T450A). The Xkid wild type and XkidT450A were spefically recovered in the MT pellet. The blot was probed with specific antibodies as indicated. Xorbit antibody was used as a control.

3.3. IDENTIFICATION OF XKID PARTNERS DURING M-PHASE AND THE ROLE OF THR450 PHOSPHORYLATION IN THESE INTERACTIONS

One way to improve the understanding on the function of a protein in this case Xkid, is to identify its binding partners. Previous data in the laboratory indicated that Xkid is in a high molecular weight complex in CSF-egg extract. Many KLPs oligomerize through long coiled coil sequences forming a stalk domain that plays an important role in their motor activity and function. Xkid only has a short stretch of 50 amino acids predicted to form coiled coil interactions that might not be sufficient for oligomerization. The sucrose density gradient data obtained previously in the lab showed that the position of Xkid in the gradient cannot be explained only by a dimerization of the monomer that has a predicted MW of 73 kDa. These data therefore suggested that Xkid interacts with other proteins forming a high molecular weight complex in M-phase *Xenopus* egg extract.

Interestingly, Thr450 is located at one end of the sequence predicted to form coil-coiled interactions. To check whether Thr450 phosphorylation may change some interactions of Xkid with itself and/or its partners, sucrose density gradients of Xkid-depleted extracts expressing Xkid-WT or Xkid-T450A were performed. Western blot analysis showed that Xkid was successfully depleted from the extract and all exogenous Xkid variants were efficiently translated. The migration profile of Xkid variants on the sucrose gradient was determined by running the collected fractions on a SDS-PAGE followed by Western blotting and detection of the protein with an Xkid-antibody (fig 3.18). Endogenous Xkid as well as Xkid-WT and XKidT450A were found in the last fractions of the sucrose gradients indicating that they all form high molecular weight complexes in the mitotic *Xenopus* egg extract. This suggests strongly that Thr450 does not impair most of the interactions between Xkid and its partners although this approach does not allow to rule out that some of the interactions are affected.

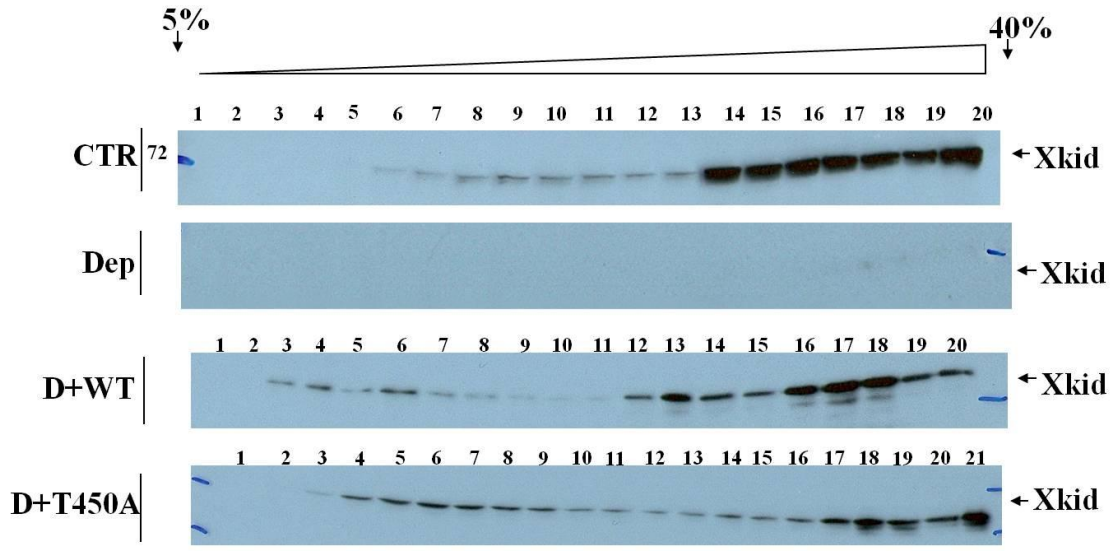

Figure 3.18. Sucrose gradient pattern analysis of *Xenopus* egg extract added back with the different variants of Xkid.The fractions obtained were analysed by SDS-Page followed by western blotting and Xkid was detected with anti-Xkid antibody. The endogenous and exogenous forms of Xkid were found in the last fractions of the sucrose gradient.

3.3.1 Identification of Xkid binding partners in *Xenopus* egg extract

To identify proteins associated with Xkid in the egg extract, several immunoprecipitations were performed using the anti-Xkid antibody.

Anti-Xkid antibodies were coupled to the protein A DynaI beads. The beads were then washed and added to CSF-egg extract and incubated for one hour. The beads were retrieved, washed and then boiled with sample buffer. As control the same protocol was followed using unspecific IgG. Proteins from both Xkid and IgG beads were visualized on SDS gels stained with coomassie blue as shown in fig 3.19. Bands present in the Xkid immunoprecipition and absent in the IgG control were cut and sequenced by mass spectrometry for their identification.

The proteomic analysis revealed that Xkid interacts with:

(1) several microtubule-associated proteins: CLIP-170, XOrbit (CLASP), NuMA, Maskin

(2) several microtubule-dependent motor proteins and their accessory proteins: Xklp1, XCenp-E, Dynein Intermediate chain, Dynein light-intermediate chain, , Dynactin

(3) other cytoskeletal proteins: actin

(4) a chromatin associated protein: SMC6

(5) a tumor suppressor protein: Mdm2

(6) and Importin Beta-1 (in agreement with Xkid being a nuclear protein)

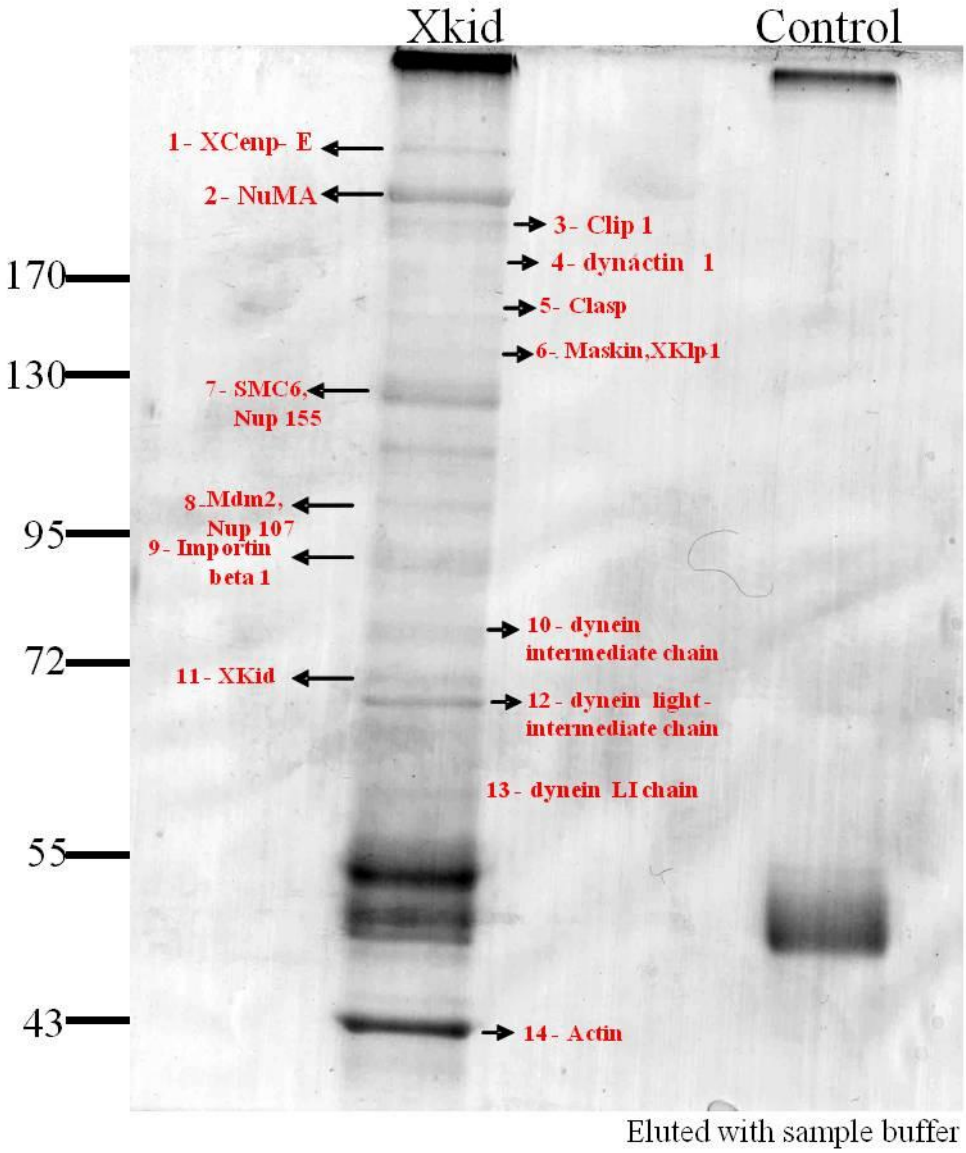

Figure 3.19. Coomassie staining of immunoprecipitation using anti-Xkid antibody and unspecific IgGs as a control (Mock).

To obtain further evidence for these interactions and validate them, I repeated Xkid immunoprecipitations and analyzed them by western blot with available antibodies.

Some candidates could not be further confirmed:
- Maskin and dynactin were not present in Xkid immunoprecipitations.
- The putative interaction of Xkid with structural maintenance chromosomes (SMC6) and Mdm2 (a tumor suppressor protein) could not be tested because no antibodies were available.
- The Dynein light-intermediate chain and NuMA have not been examined yet.

However some of the candidates were found to consistently co-immunoprecipitate with Xkid.
This is the case for XCenp-E, Clip-170 (fig 3.20), Xklp1, and dynein Intermediate chain.

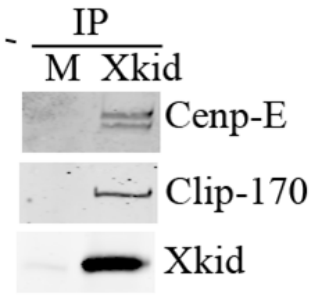

Figure 3.20: Xcenp-E and Clip-170 co-immunoprecipitated with Xkid in CSF- egg extracts. Immunoblot is specific for the antibodies that are showed.

3.3.1.1. XKID-XORBIT INTERACTION

Among the putative interacting proteins, two of them are particularly interesting: XOrbit the *Xenopus* homologue of CLASP (CLIP-associated protein) and CLIP 170. CLASP/XOrbit has been shown to interact with CLIP-170. Interestingly, both proteins are important players during mitosis. The CLASP-CLIP complex associates with microtubule plus-ends, and have a microtubule-stabilizing activity but so far this interaction has only been reported in interphase (Akhmanova et al., 2001). Interestingly, previous work in *Xenopus* egg extracts has shown that Xorbit immunodepletion or inhibition by a dominant-negative fragment resulted in chromosome alignment defects

(Hannak and Heald, 2006).These effects are similar to those of Xkid immunodepletion in *Xenopus*-egg extract, suggesting that both proteins could work in a similar pathway.

Western blot analysis of Xkid immunoprecipitates confirmed XOrbit as putative Xkid partner (Fig 3.21 A). Interestingly, anti-XOrbit antibodies co-immunoprecipitated Xkid from egg extract (Fig 3.21 B) providing further support for an interaction between these proteins. In addition, by immnunoblot, dynein co-immunoprecipitated with Xkid (3.21 A).

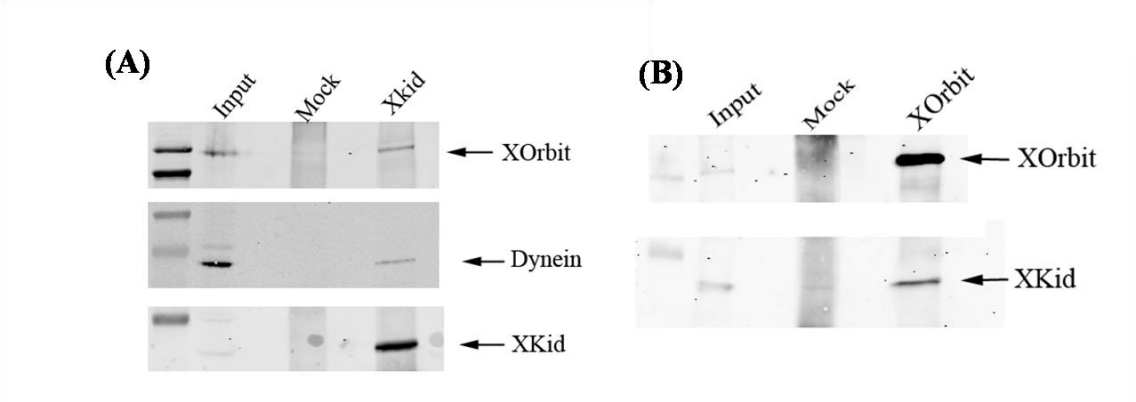

Figure 3.21. Xkid interacts with Xorbit in the *Xenopus* egg-extract.
(A) CSF-egg extract was used for immunoprecipitations with Xkid and rabbit IgGs (mock: negative control), respectively. The immunoprecipitates were separated by SDS-PAGE and probed with Xkid and XOrbit antibodies. Xorbit and dynein co-immunoprecipitate with Xkid.
(B) Xkid co-immunoprecipitates with XOrbit antibodies in the egg extract. CSF-egg extract was used for immunoprecipitations with XOrbit and rabbit IgGs (mock: negative control), respectively. The immunoprecipitates were separated by SDS-PAGE and probed by western blotting with Xkid and XOrbit antibodies.

3.3.2. Establish method to pull down Xkid partners

To determine whether Thr450 phosphorylation may regulate Xkid-Xorbit interaction a protocol for pulldown of the proteins translated in egg extract from exogenously added mRNA was established.

The quantity of mRNA added, was established as previously described with a test translation assay to obtain all the exogenous proteins at close to endogenous levels after 1 hour incubation at 20ºC. Immunoprecipitations were then perfomed by using anti-Xkid antibodies coupled to protein A DynaI beads.

Western blot analysis of the proteins pulled down by the anti-Xkid antibody showed that all the exogenous forms of Xkid proteins expressed in the egg extracts were successfully recovered.

Thus, this protocol will provide a way to check protein-protein interactions and to study whether phosphorylation is involved in their regulation.

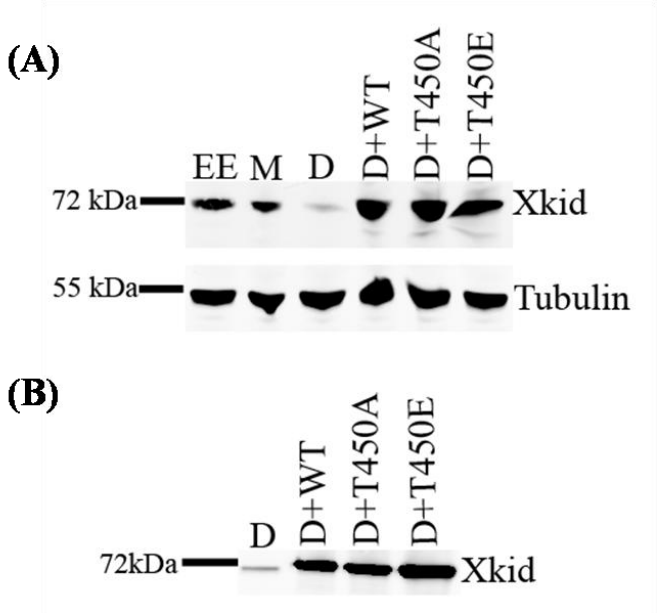

Figure 3.22. Pulldown protocol established using the mRNA add-back methodology.
(A) Immunoblot probed with anti-Xkid and anti-tubulin. All exogenous forms of Xkid were efficiently translated. Samples correspond to egg extract (EE),mock control (M), Xkid depleted extract (D) , Xkid depleted extract containing Wild-type mRNA of Xkid (D+WT), Xkid depleted extract containing T450A mRNA of Xkid (D+T450A) and Xkid depleted extract containing T450E mRNA of Xkid (D+T450E).
(B) The mRNA's previously translated in the CSF-egg extract were efficiently pulldowned by the anti-Xkid antibody.

4. DISCUSSION

The *Xenopus* system and in particular the egg extract system has been very powerful for studying the function of different spindle factors that are very well conserved throughout evolution: TPX2 (involved in MT nucleation and spindle pole formation), Maskin (important for stabilization and anchoring of spindle MTs at poles), Eg5 (important for spindle bipolarity) are just some examples. In addition, the oocytes maturation and egg extract systems have provided a large amount of information on the signaling pathways that control the cell cycle.

4.1. A method to study the function of cell cycle related proteins using *Xenopus* egg extract

The *Xenopus* egg extract system offers an attractive alternative to studies in tissue culture cells or whole organisms because it allows some experimental manipulations that are difficult or impossible in these systems. This is due to some characteristics of the egg system: (1) upon fertilization, the egg divides for several cell cycles in the absence of transcription and all the material required for assembling a very large number of spindles and all the related cell cycle events is stored in the cytoplasm, (2) cytoplasmic extracts prepared from unfertilized eggs are naturally synchronized in metaphase of meiosis II due to the activity of CSF (Cytostatic Factor) and (3) the egg extract is an open system, consequently, amenable to a large number of experimental manipulations.

One of the methods of choice in the egg extract system, is the depletion of the protein of interest and the use of the depleted extract to study the potential role of this protein during spindle assembly. This approach is only reliable if the phenotype observed in depleted extract can be rescued by addition of the corresponding protein to the depleted extract. Thus it is essential to obtain this protein in a purified form. The standard approach is to express and purify the protein in a recombinant form either in bacteria or baculovirus. This is very often time consuming and moreover a limiting factor because some proteins (particularly large ones) are often difficult to express and purify.

Furthermore, sometimes the protein is insoluble or it is impossible to obtain enough amounts of functional protein to perform the rescue experiments in the egg extract.

A large part of my thesis was devoted to establish a reliable method to express proteins in the *Xenopus* egg extract from exogenously added mRNAs in order to overcome these limitations. This approach had been previously described in the literature by Murray and Kirschner and later on by other authors (Chang et al., 2003; Funabiki and Murray, 2000).However initial attempts to use this approach for rescue experiments in egg extract spindle assembly assays proved to be highly unreliable. Therefore, one major aim was to establish the detailed experimental conditions to obtain functional CSF-egg extracts while expressing *in vitro* transcribed mRNA in a consistent way. Hentze and Gebauer (Gebauer and Hentze, 2007) had previously established a series of important considerations to obtain efficient translation of *in vitro* transcribed mRNAs in a *Drosophila* cell-free system. This system was used successfully to study translational control mechanisms during early stages of *Drosophila* development. Interestingly, the *Xenopus* egg extract shares similarities with the Drosophila ovary or embryo extract system as both are powerful systems for translation of mRNAs transcribed *in vitro*. In both cases, the translational efficiency depends on the extract daily variation and the best results are achieved with fresh egg extract preparations (Gebauer and Hentze, 2007).In addition, also in both systems, the following issues are determinant for efficient translation: (1) the DNA template preparation, mRNA preparation, purification and recovery, (2) the preparation of capped and poly-A tailed mRNA, (3) the addition of an ATP regenerating system to the extract.

In addition, for functional experiments it was essential to maintain the capacity of the *Xenopus* egg extract to cycle and assemble bipolar spindles. To this aim, it was important to (1) obtain very good quality egg extract able to assemble half spindles by less than 30min in the presence of sperm nuclei, (2) to prepare a good antibody able to deplete the protein in as little time as possible of incubation (optimally around 30-45 min maximum) and (3) to obtain concentrated mRNA preparations in order not to dilute the extract (maximum of 10% or less of extract volume).

4.2. The role of phosphorylation at the Cdk1 consensus site in Xkid localization to microtubules and chromosomes

In order to analyze the role of phosphorylation in Xkid localization and/or function during spindle assembly, two approaches were used. In one hand, we generated an anti-phospho specific antibody to follow by western blot analysis and immunofluorescence the temporal and localization profiles of the form of Xkid phosphorylated on Thr450. On the other hand, I examined the localization and functionality of the two phospho variants: Xkid T450A and Xkid T450E

4.2.1. TEMPORAL PHOSPHORYLATION PROFILE OF XKID THR450 DURING THE CELL CYCLE

Western blot analysis of extracts from maturing oocytes and from egg extract showed that the phosphorylation of Xkid on Thr450 follows closely the profile of expression of the protein. Since Thr450 is found in a cdk1 consensus site and Xkid expression is cell cycle regulated peaking in meiosis I, meiosis II and in the CSF egg extract, this result is quite consistent as these phases correspond to the highest peak of activity of the cdk1 kinase. This suggests that Xkid is efficiently phosphorylated by cdk1 both in meiosis and mitosis.

4.2.2. LOCALIZATION OF PHOSPHO XKID

Immunofluorescence analysis of XL177 tissue culture cells, showed that the anti-PXkid did not label any structure in interphase cells. This correlates with the cell cycle regulated expression of Xkid and it is also in agreement with the low levels of Xkid found in interphase *Xenopus* egg extract.

By contrast the anti-PXkid antibody produced a very clear decoration of the metaphase chromosomes in spindles assembled in egg extract and XL177 cells. Interestingly, it generated a dotty pattern with thin filament-like threads that was very distinct from the pattern obtained with the general anti-Xkid antibody. The meaning of this very peculiar pattern is not clear and additional experiments will be needed to try to determine if phospho-Xkid associates with specific regions of the chromosomes like for instance, the telomeres.

The clear localization of phospho-Xkid to the chromosomes suggests that phosphorylation may be required for the targeting of Xkid to the chromosomes. However, XkidT450A as well as XkidT450E were both efficiently localized to the chromosomes in egg extract suggesting that phosphorylation is not involved in the targeting of this motor to the chromosomes. Studies performed in human cells had previously proposed that phosphorylation of kid at the corresponding cdk1 site does not regulate the affinity of kid for the chromosomes. However in this system it was difficult to really demonstrate this. Here I could address the question directly and show convincingly that the targeting of Xkid does not depend on Thr450 phosphorylation. Previous work identified the region of Xkid involved in this targeting as a hairpin-helix-hairpin DNA binding motif found at the C-terminus of the protein (Antonio et al., 2000).Thr450 is located in the middle of the sequence of Xkid therefore well away from this motif. Since it is close to a predicted coiled coil region one possibility was that it could regulate the dimerization of the protein. Although I did not address this question directly, the fact that the protein localizes correctly suggests that it is unlikely that Thr450 phosphorylation regulates a major conformational change involving dimerization through this coiled coil.

The localization pattern of phospho XKid both in extract and in XL177 cells suggest that there are two sets of protein: one phosphorylated and one unphosphorylated that could cooperate in the functionality of Xkid. However this is still a matter of investigation.

Antonio et al (Antonio et al., 2000) showed that the general anti-Xkid antibody decorated also the spindle microtubules in metaphase and anaphase in XL177 cells. A similar localization was also described for the human kid in tissue culture cells (Tokai et al., 1996). It is interesting that recently, Bieling et al (Bieling et al.)found that Xkid has a high affinity for microtubules. Their data show Xkid binds more strongly to microtubules (by a factor of approximately 70) as compared to the motor domain of the other chromokinesin Xklp1. The anti-PXkid antibody did not decorate microtubules at any stage of mitosis in egg extract or in XL177 cells. Additionally, Xkid-T450E that should mimick the phosphorylation state was not visualized on the spindle microtubules in egg extract.

Our findings are compatible with previous work showing that the localization of the human kid to spindle microtubules is negatively regulated by cdk1 (Ohsugi et al., 2003). In this work, the authors proposed that phosphorylation of human kid by cdk1 downregulates its affinity for spindle MTs through a second ATP-independent MT binding site. This second MT-binding site is conserved among the species and more specifically in Xkid, suggesting that it may be functionally important (Yajima et al., 2003). The unphosphorylated human kid could interact with MTs to facilitate microtubule stability whereas phospho-kid would preferentially bind to the chromosomes. Although our data using the anti phospho-Xkid antibody and the XkidT450E variant are compatible with this model, it is nonetheless important to point out that XkidT450A was not visualized either on the microtubules in spindles assembled in egg extract. This data however has to be taken with caution because in this system no microtubule localization was detected for Xkid with the general anti-Xkid antibody either, raising the possibility that it does not associate with them or in a very weak manner. However, this suggests that the unphosphorylated form of Xkid does not have a higher affinity for the microtubules as it was suggested for the human orthologue. The difference of Xkid localization in egg extract versus tissue culture cells is unclear but could be due to the quite different size and composition of the spindles assembled in these two systems. *Xenopus* egg extract spindles contain indeed a very large proportion of non-kinetochore microtubules (>90%) and they follow a dominant self-assembly mechanism dominated by the RanGTP pathway.

Our data suggest that phospho-Xkid does not associate more efficiently with the microtubules. Since the conditions to perform MT and spindle pelleting assays in egg extract circumventing the need of recombinant proteins have been established, it will be relatively easy to investigate further the role of the conserved second MT-binding site in Xkid functionality and its putative regulation by cdk1 phosphorylation.

4.2.3. ROLE OF PHOSPHORYLATION IN REGULATING XKID FUNCTION IN EGG EXTRACT

Although the phosphorylation variants of Xkid do not interfere with the localization of the protein to mitotic chromosomes, they seem to be unable to restore Xkid function.

Thus, our results suggest that phosphorylation is not needed for Xkid localization but it is important for Xkid function, maybe more specifically for spindle assembly.

These are still very preliminary conclusions. Wild type Xkid was able to rescue the Xkid chromosome misalignment phenotype. However, we found that increasing the levels of Xkid or its phosphorylation variants seemed to correlate with the formation of less robust bipolar spindles. Previous work had shown that the expression of GFP-Kid-Thr463A in human cells correlated with a thickening of spindle MTs and the breakage of spindles in prometaphase (Ohsugi et al., 2003) . Here we observed an even stronger phenotype in extract containing the XkidT450E variant suggesting that it acts as a dominant negative mutant.

4.3. A general function of chromokinesin Kif22 family members?

The high level of amino acid sequence similarity between the human kid and Xkid suggests that these proteins perform similar functions. However, the results presented in this study, and the work done by different groups on the human and mouse kid, revealed some differences:

- Xkid does not localize to spindle microtubules in egg extract
- Phosphorylation by cdk1 regulates negatively kid association to spindle microtubules but this is not so clear for Xkid
- Xkid has a clear function in metaphase chromosome alignment in egg extract but its human orthologue appears to have a lesser contribution to this process .

These differences in fact may be due to the different experimental systems used. Indeed it is worth noting that:

(1) Centrosomes are present in somatic cells but not in Xenopus and mouse oocytes;

(2) RanGTP is not crucial for assembly of functional Meioisis I spindle in mice and *Xenopus* oocytes. On the contrary, it is very important for Meiosis II and Mitosis (Dumont et al., 2007);

(3) in *Xenopus* eggs the Chromatin-driven spindle assembly (spindle formed in absence of centrosomes and even kinetochores around beads is largely predominant (Heald et al., 1996);

(4) in the *Xenopus* egg extract spindles are formed by more than 90% of non kinetochore MTs.
(5) Chromosome bi-orientation in somatic cells is achieved independently of the polar ejection force (through the k-fiber) (Walczak et al.);
(6) There are k-fiber oscillations in somatic cells (Walczak et al.).

4.3.1. XKID IS PRESENT IN A HIGH MOLECULAR WEIGHT COMPLEX IN *XENOPUS* EGG EXTRACT

Little is known about the protein partner of KIF22 family members. Only a few partners have been described for humnan kid: Importin β and CHICA have been shown to associate with human kid contributing to regulate its binding to spindle MTs or chromosomes. Another partner is SIAH-1 a ubiquitin ligase that mediates human kid degradation by the proteosome.
Sucrose gradient analysis performed in the lab suggested that Xkid is in a high molecular weight complex in mitotic Xenopus egg extract. Preliminary results suggest that cdk1 phosphorylation does interfere with the formation of this complex as the phospho variants of Xkid show similar position in the sucrose gradients as the endogenous protein. Immunoprecipitations experiments in egg extract followed by mass spectrometry analysis have lead to the identification of several proteins as novel potential Xkid partners. Among the identified proteins, several were consistently co-immunoprecipitated with Xkid: Xklp1, XCenp-E , Dynein, XOrbit and Clip170.
XCenp-E, Xklp1 and Dynein are MT motors playing roles in chromosome movement and positioning. Xklp1 is the other chromokinesin known to localize to chromosomes arms in *Xenopus* egg extract. XCenp-E is a kinetochore associated motor that keeps the kinetochores associated with dynamic plus-ends of microtubules and required for the congression of chromosomes on the metaphase plate (Wood et al., 1997). It is somehow puzzling that several chromosome associated motors could be in one complex. The immunoprecipitations were done in CSF-egg extract, which in fact is a meiotic II cytoplasm rich in cytoskeleton and spindle factors needed for the first 12 cell divisions of the embryo (in the absence of transcription and not much of protein synthesis). It is therefore possible that Xkid and the other chromosome associated motors may be stored in a complex to store related proteins needed for cell cycle division. It would be very

interesting to examine whether these interactions also occur in cultured somatic cells to find out if they are really functionally relevant.

Among the possible Xkid interacting partners, XOrbit was particularly interesting. CLASP/XOrbit, was shown to be important for chromosome alignment, therefore it could indeed be functionally related to Xkid. Work done by Heald at al (Hannak and Heald, 2006) showed a biochemical interaction between a COOH-terminal XOrbit fragment and Clip 170 and XCENP-E. Clip 170 is implicated in the control of MT dynamics and dynactin localization. Preliminary data indicated that Xkid-XOrbit-Clip170-Dynein could form a complex to cooperate together during *Xenopus* chromosome congression (Hannak and Heald, 2006). Thus, Xkid could function by binding to chromosomes arms and targeting/regulating other motor and plus-end activities. It would be interesting to map which is the minimal domain of Xkid needed for XOrbit interaction and vice-versa. Furthermore, in order to assess to the functional significance of these interaction, Xkid depletion experiments added-back with XOrbit mRNA, and vice-versa, can be performed.

Interestingly, all these proteins are associated with chromosomes or at the plus-end of microtubules and therefore it is possible that a complex of proteins including Xkid could be involved in mediating the interactions between the chromosome arms and the dynamic plus-ends of microtubules.

Finally, a study on human kid reports a functional relationship between NuMA and human kid that influences the spindle morphology contributing to chromosome alignment in mammalian cells (Levesque et al., 2003). NuMA forms a complex with dynein and dynactin. Although we did not find dynactin as a potential partner of Xkid, it would be interesting to study if upon NuMA-Dynein-Dynactin complex perturbation there are changes in Xkid localization in the *Xenopus* egg extract system.

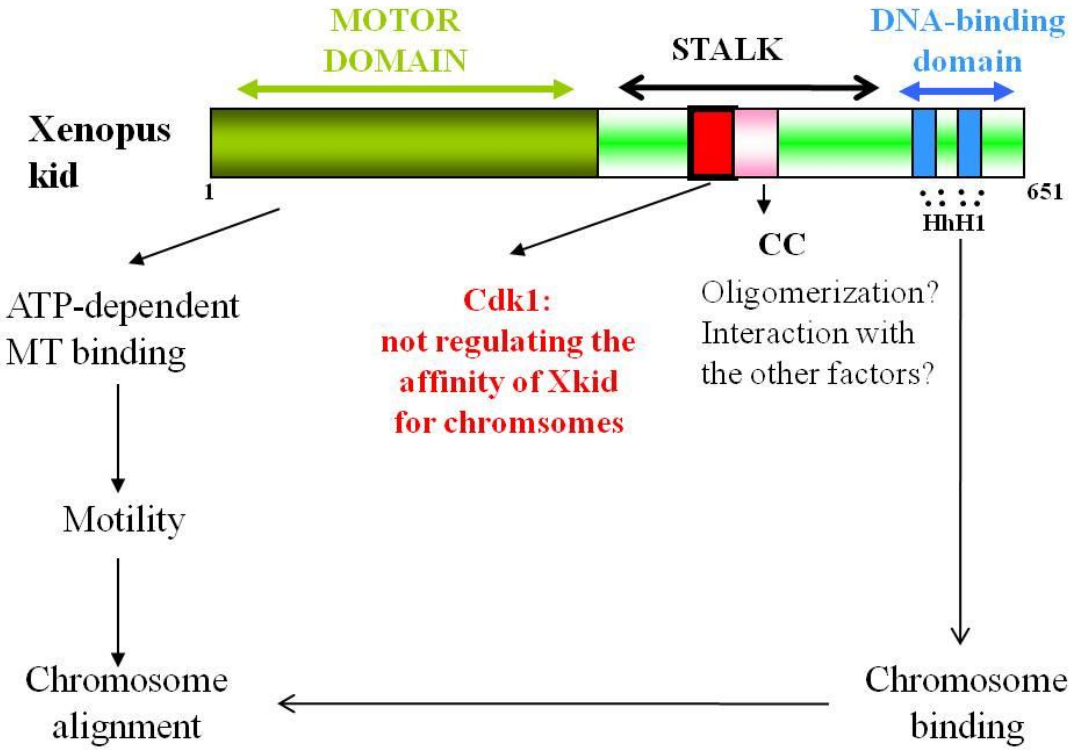

Figure 4.1. Summary of the contribution of the different domains of Xkid in its function during cell division. Together, the motor domain and the DNA-binding domain contribute for Xkid's role during metaphase: at the beginning of mitotic spindle assembly, chromosomes frequently attach first to microtubules emanating from one spindle pole. Xkid binds to chromosomes through its DNA-binding domain and together with the motor domain activity, Xkid exert a force to push the arms of the chromosomes in the direction of the spindle equator. Together with poleward forces acting at the kinetochores, the polar ejection force exerted by Xkid will align the chromosomes at the metaphase plate.

5. CONCLUSIONS AND PERSPECTIVES

The mRNA methodology established in this study makes possible to overcome the need for obtaining purified recombinant proteins to perform full experiments in the *Xenopus* egg extract system. Apart from spindle assembly assays, other biochemical assays can also be performed like microtubule and spindle pelleting, sucrose gradients and GST-pull downs, offering a large number of possibilities for understanding the role of selected proteins during M-phase. One interesting application would be to perform motility assays with motor proteins translated in egg extract and therefore in their natural environment.

Since it is clear that increasing the levels of XKid in the egg extract results in spindle assembly defects, it would be important to perform new experiments with the different phospho variants expressed at endogenous levels. In addition these experiments could be complemented with the biochemical approaches mentioned above to obtain a clear picture of the potential regulatory role of cdk1 phosphorylation in Xkid affinity to microtubules.

One very interesting partner of Xkid is XOrbit/Clasp. It will be interesting to investigate this interaction further, examine whether it is regulated by phosphorylation or the RanGTP pathway and determine its implications in the context of spindle assembly and chromosome movements.

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7. APPENDIX

I. Characterization of the Anti-phospho Xkid antibody: Immunoprecipitation

In order to characterize if the phospho-Xkid antibody could immunoprecipitate selectively the phospho-Xkid protein present in the CSF- egg extract, immunoprecipitation experiments on CSF-egg extract were performed with Protein A magnetic beads (Dynal) coated with anti-PXkid antibodies.

The anti-phospho Xkid antibody co-immunoprecipitated a small amount of phospho-Xkid protein. Thus, this phospho-antibody can be used for future experiments in order to analyze possible interacting Xkid partners under phosphorylation by cdk1 site.

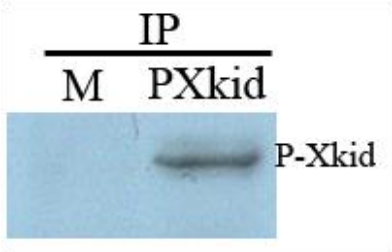
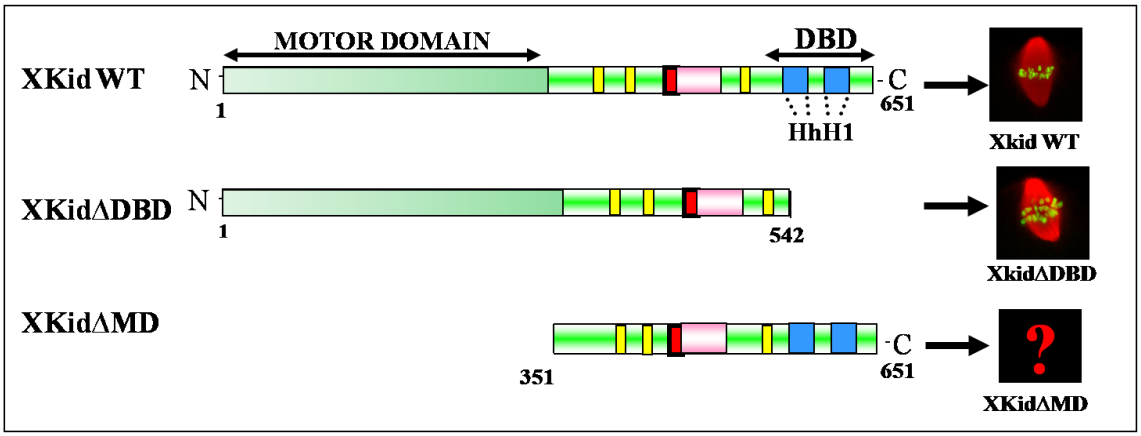


Figure I. Anti-phospho Xkid antibody co-immuniprecipitates a small fraction of phospho-Xkid protein. Immunoblot probed with anti-phospho Xkid, showing the efficiency of phospho Xkid depletion.

II. The *Xenopus* oocyte: System for the study of functional expression of mRNA

Previously, has been shown that expression of an Xkid mutant lacking the DNA-binding domain in Xkid-depleted oocytes rescues meiotic maturation. This result indicated that Xkid has a role in meiotic cell cycle progression that is independent from its role in metaphase chromosome alignment**.**

To define the domain of Xkid required for meiotic cell cycle progression, we generated an mRNA to express a truncated form of Xkid lacking the motor domain (XkidΔMD) (fig II).



FigureII.1 Schematic representation of wild-type Xkid sequence and different Xkid mutants.

Stage VI oocytes were first injected with Xkid antisense oligonucleotides at 1 mM and then with mRNA encoding XkidΔMD before being stimulated with progesterone.

Under these conditions it was possible to prevent endogenous Xkid expression in Meiosis II (MII) in XkidΔMD expressing oocytes (Figure III). The mRNA encoding XkidΔMD was efficiently expressed but "less" stable at meiosis II.

This experimental system can be used to investigate whether the motor domain is required for oocyte maturation examining morphological appearance of the oocytes, by analysis of biochemical markers (such as tyrosine dephosphorylation of cdc2, hyperphosphorylation of the Cdc25 phosphatase, levels of cyclin B1 and cyclin B2, etc.) and histone H1 kinase (H1K) activity.

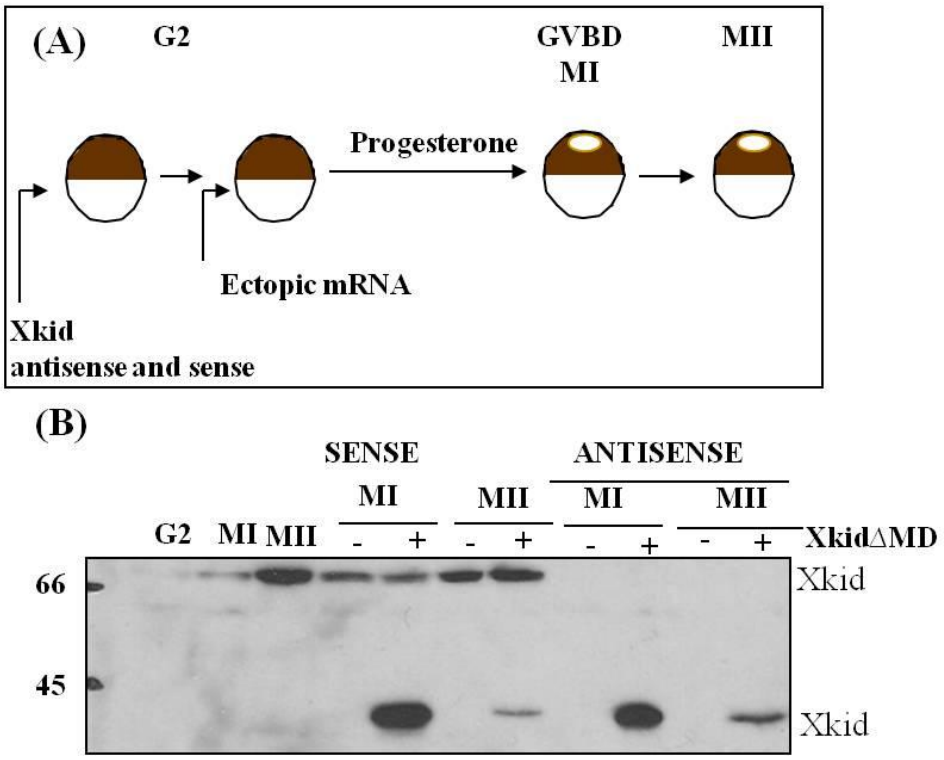


Figure II.2 Expression of a Xkid mutant lacking the Motor domain in Xkid-depleted oocytes.
(A) Schematic of treatments in G2 oocytes. G2 oocytes were injected with Xkid Morpholino antisense and sense oligonucleotide, left for overnight at 18ºC,and then injected with *in vitro* transcribed Xkid mRNA. Progesterone was added 1 h later and samples were taken in syncronized oocytes at GVBD (MI) and 4 h later (MII).
(B) Oocytes were injected with *in vitro* transcribed mRNA encoding a truncated Xkid mutant lacking the motor domain, and collected at GVBD (MI) and 4 h after GVBD (MII). Expression of Xkid was anlaysed by immunobloting.

Furthermore, the XkidΔMD expression does not interfere with the formation of the Xkid high molecular weight complex in egg extract as estimated by sucrose density gradient centrifugation (fig II.3).

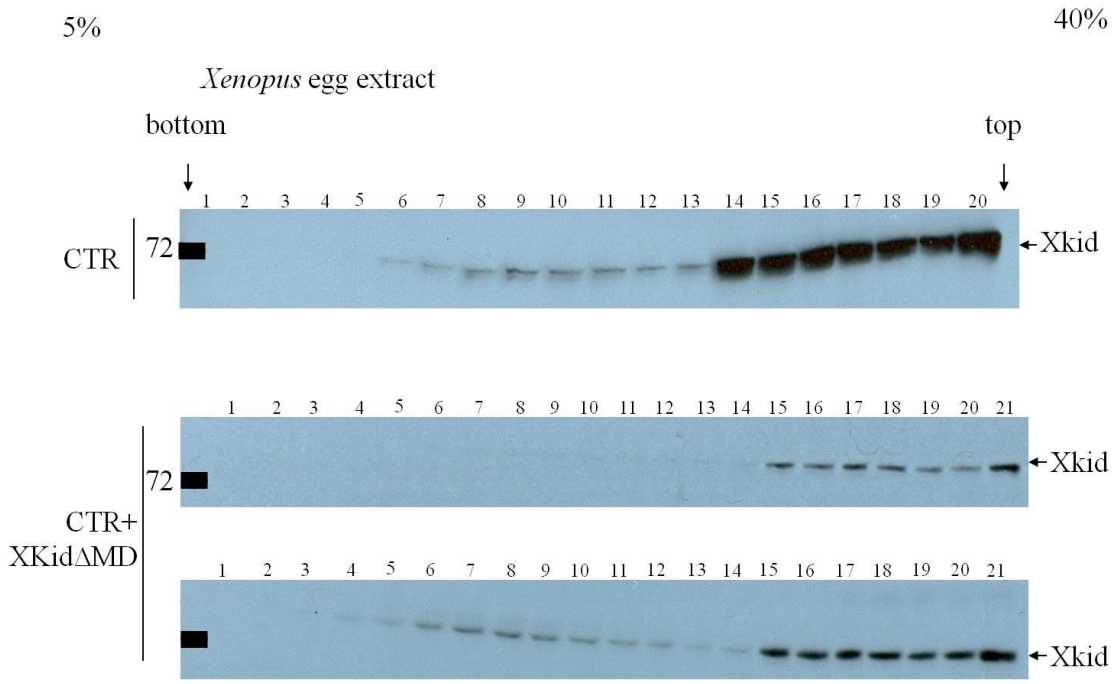


Figure II.3. Sucrose gradient showing that expression of a Xkid mutant lacking Motor domain does not change the formation of Xkid protein complex.