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Identification of Mob1 Protein as a Conserved Centrosome Component

by

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ABSTRACT

The *MOB1* gene was first identified in *S. cerevisiae* and its product, Mob1 protein, was found to be essential for viability. I have identified a cDNA for *MOB1* homolog in *Xenopus laevis*. Both polyclonal and monoclonal antibodies were raised against a bacterially-expressed *Xenopus* Mob1 protein. The specificity of these antibodies was characterized based on the ability to: (1) immunoprecipitate the *in vitro* translated *Xenopus* Mob1 protein; (2) immunoblot the bacterially-expressed Mob1 protein; and (3) detect the GST-tagged human Mob1 protein. Immunoblotting results of *Xenopus* and human cell extracts showed that the molecular size of Mob1 protein is approximately 17 kDa and 26 kDa, respectively. Indirect immunofluorescence experiments demonstrated that Mob1 protein was located on centrosomes during interphase, at spindle poles during mitosis, and within the intercellular bridge during cytokinesis in both *Xenopus* and human cells. In addition, centrosomal Mob1 protein is resistant to extraction with high salt and insensitive to nocodazole treatments.

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Dedicated to

my parents

and my wife, Amanda

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LIST of ABBREVIATION and SYMBOLS

bp	base pair
BSA	bovine serum albumin
CSK	cytoskeleton buffer
DAPI	4', 6-diamidine-2-phenylindole dihydrochloride
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxyribonucleic acid
cDNA	complementary DNA
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetra-acetic acid
EGTA	ethylene glycol tetra-acetic acid
EST	expressed sequence tag
FBS	fetal bovine serum
GST	glutathione S-transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hr	hour
IIF	indirect immunofluorescence
IP	immunoprecipitation
IPTG	isopropylindolyl-β-D-thiogalactopyranoside
kb	kilo-base
kDa	kilo-Daltons
LB	Luria Bertani
mAb	monoclonal antibodies
MBP	maltose binding protein
MOB1	Mps One Binder 1 gene (wild type)
mob1	Mps One Binder 1 gene (mutant)
Mob1	Mps One Binder 1 protein
min	minute

mRNA	messenger RNA
MTOC	microtubule organizing centre
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PMSF	phenylmethyl-sulfonylfluoride
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
sec	second

CHAPTER ONE

Introduction

In most eukaryotes, microtubules are organized by a large proteinaceous structure, termed a microtubule organizing centre (MTOC). The best-studied MTOCs are the centrosomes in animal cells (Mack *et al.*, 2000), the spindle pole bodies in yeast (Adams and Kilmartin, 2000), and the basal bodies of flagellated or ciliated algae (Marshall and Rosenbaum, 2000). Even though the MTOCs from various organisms are quite different in structure, their molecular analysis over the past decade have revealed that the basic functions, such as cell-cycle dependent duplication and microtubule nucleation, are similar on the molecular level.

I. Centrosomes of Animal Cells:

The centrosome is the major microtubule organizing centre in interphase animal cells and duplicated centrosomes serve as the spindle poles for nucleating mitotic microtubules, which are important for sister chromatid separation during mitosis. Therefore, proper cell division requires precise centrosome duplication, mitotic spindle assembly, and chromosome separation in yeast and mammalian cells. Defect in any of these events could lead to loss or gain of the chromosomes, resulting in cell death, birth defects, or cancer (Lingle *et al.*, 1998; Pihan *et. al.*, 1998; Mack *et. al.*, 1998; Lingle and Salisbury, 1999; Tanaka *et. al.*, 1999; Saunders *et. al.*, 2000; reviewed by Hartwell and Kastan, 1994; Brinkkley and Goepfert, 1998).

In most cells, the centrosome, consisting of centriolar domain and pericentriolar

domain, is located at the perinuclear region during interphase. Analysis by electron microscopy has shown that typical animal cell centrosomes are composed of a pair of centrioles surrounded by an amorphous, electron-dense pericentriolar material (PCM) (Wheatley, 1982; Palazzo *et al*, 1992; Kellogg *et al.*, 1994; Balczon, 1996).

I-A. Centriolar Domain:

I-A-1. Centriole Structure:

Generally, centriole duplex composed of two centrioles that are positioned orthogonally to one another (Kuriyama and Borisy, 1981; Kochanski and Borisy, 1990). Each centriole appears as a short, 0.25 μM diameter barrel-shaped structure composed of nine interconnected triplet blades made of a complete 13 protofilament microtubules (the innermost A-tubule) and two incomplete 10 or 11 protofilament microtubules (the B- and C-tubule, respectively) arranged in a cylindrical fashion (Tilney *et al.*, 1973; Wheatley, 1982; Vorobjev and Nadezhdina, 1987; Palazzo *et al*, 1992; Andersen, 1999; Mack *et al.*, 2000). The interior of the centriolar wall forms a lumen, whereas the outside of the wall defines the centriolar surface. According to the regional variation of the centriolar cylinder and the placement of structures found within the lumen, there is a proximal-distal orientation to the centriole. Proximal refers to the end that is closest to the other centriole. A pinwheel, a strictly radial-symmetric structure, connected by the spokes to the triplet is located at the proximal end of the lumen of mature centriole. In contrast, an amorphous ring-like structure is found at the distal end of the lumen (Rattner and Phillips, 1973; Vorobjev and Nadezhdina, 1987). The outer surface of the mature centriole has a series of appendages attached to its distal end, while the appendages are

attached to the proximal end of an immature or daughter centriole during duplication (Vorobjev and Nadezhdina, 1987; Mack *et al.*,2000).

I-A-2. Centriole Proteins:

In the past decade, a number of proteins have been identified and shown to be associated with centriole. These proteins including: alpha- and beta- tubulins, structural protein tektins and 4.1, C-Nap1, Nek2 kinase, cenexin, and centrin (reviewed by Andersen, 1999; Mack *et al.*, 2000).

Alpha- and **beta-tubulins**, proteins of approximately 55 kDa each, share approximately 40% amino acid identity. Recent electron crystallography studies of both α- and β-tubulin monomers revealed that the monomer structures are very similar and can be divided into three functional domains: (1) the amino domain containing the nucleotide-binding region; (2) the intermediate domain containing the Taxol (paclitaxel)-binding site; and (3) the carboxyl-terminal domain may constitute the binding site for motor proteins (Nogales *et al.*,1998). The α- and β-tubulins are the building block of microtubule walls of the centrioles. In most cell types, microtubules are hollow cylinders composed of 13 protofilaments *in vivo*. Each protofilament is made up of tubulin heterodimer consisting of one α-tubulin subunit and one β-tubulin subunit. The protofilaments are arranged so that only like tubulins have lateral contact with each other with the exception of a seam at which α- and β-tubulin monomers interact laterally. α-tubulin is bound to GTP, which is trapped in the middle of heterodimer and is never hydrolysed. In contrast, β-tubulin is a GTPase and unlike other members of the GTPase family, the β-tubulin GTPase does not require a GTPase activation protein. Hence, the

GTP that binds to β-tubulin can be hydrolysed to GDP during microtubule assembly (Amos, 2000). Both α- and β-tubulin are also located at the ends of the centriolar lumen and the pericentriolar domain (Mack *et al.*, 2000).

Tektins were identified by studies on flagellar and ciliary doublet microtubules (Linck and Langevin, 1982; Linck and Stephens,1987; Steffen and Linck, 1988). Three types of tektin protein with relative molecular masses between 46 kDa and 57 kDa have been identified. These proteins form filamentous polymers in the wall of ciliary and flagellar microtubules. The tektins are different than tubulin, but similar to intermediate filament proteins in terms of: amino acid composition, fibrous substructure, high α-helical content, and immunological determinants (Chang and Piperno, 1987; Steffen and Linck, 1988; Chen *et al.* 1993). Therefore, it has been speculated that tektins may also be present in centrioles. Indeed, indirect immunofluorescence results provid evidence showing that tektin is localized to the basal bodies of sea urchin sperm and to the centrioles of Chinese hamster ovary (CHO) and HeLa cells (Steffen *et al.*, 1994). In addition, immunoblotting results demonstrated the presence of tektins in isolated spindles and centrosomes from CHO cells (Steffen *et al.*, 1994). The functions of tektins, however, remain obscured at present.

Protein 4.1, a 80 kDa protein, was first identified in the submembrane cytoskeleton of mature human erythrocyte (Hoover and Bryant, 2000). This protein has also been located in the mammalian centrosome by indirect immunofluorescence and high-resolution whole-mount electron microscopy (Krauss, *et al.*,1997a). Another recent study indicates that protein 4.1 may regulate the assembly of the mitotic spindle by

associating with the protein NuMA (Mattagajasingh, *et al.*,1999). NuMA is mainly nucleus during interphase and localizes to the spindle poles during mitosis. In addition, NuMA forms a protein complex with dynein, which is essential for mitotic spindle assembly (Merdes *et al.*,1996). Overexpression of an isoform of protein 4.1 resulted in NuMA mis-localization and cell death.

Nek2 kinase, the NIMA related kinase 2, and **C-Nap1**, the centrosomal Nek2-associated protein 1, have been shown to co-localize to the proximal ends of the two centrioles using negative staining immunoelectron microscopy of isolated centrosomes (Fry *et al.*, 1998a). C-Nap1 was identified as a substrate of Nek2 kinase in a yeast two-hybrid screen (Fry *et al.*, 1998a) and by human autoimmune sera reactive against centrosomal antigens (Mack *et al.*, 1998). Although the association of Nek2 with the centrosome occurs throughout the cell cycle and is independent of microtubules (Fry *et al.*, 1998b), C-Nap1 antibody staining is stronger on interphase centrosomes than on mitotic spindle poles. Since the overexpression of Nek2 kinase resulted in premature centrosome splitting and disintegration of the entire centrosome structure, Nigg's group proposed that C-Nap1 forms a bridge structure that links the two centrosomes throughout interphase (Fry *et al.*, 2000). Phosphorylation of C-Nap1 by Nek2 causes the removal of the bridge during the G2/M transition (Mayor *et al.*, 1999). In contrast, Rattner's group suggested that C-Nap1/Cep250 may be involved in interphase and post-mitotic nucleation of microtubules, and spindle microtubule formation because no relationship between the pattern of association of C-Nap1 with centrioles and centrosome separation could be seen (Mack *et al.*, 2000). This point of view was recently supported by the

identification of a *Xenopus* homolog of NIMA-related kinase, X-Nek2B (Fry *et al.*, 2000). Immunodepletion of X-Nek2B from *Xenopus* egg extracts significantly delay the assembly of microtubule asters and the recruitment of gamma-tubulin to the basal body of *Xenopus* sperm, suggesting that X-Nek2B is required for the efficient assembly of functional centrosomes from sperm centrioles.

Cenexin is a 96 kDa centriole-associated protein (Lange and Gull, 1995). Even though cenexin has not been cloned, its strict association with mature centrioles during G1 of the cell cycle and throughout the duplication period suggest that this protein may be directly involved in regulating centriole duplication.

Centrin, is a 20 kDa, calcium-binding phosphoprotein that was first identified in the basal bodies of green algae (Salisbury, 1995). Like gamma tubulin (described below), only a fraction of total cellular pool of centrin is associated with centrosomes where it is found in the distal lumen of centrioles (Paoletti, 1996). The precise function of centrin is obscure at the present, but centrin seems to have an important role in microtubule severing during flagella excision in *Chlamydomonas* (Sanders and Salisbury, 1994). In addition, centrin homolog in the budding yeast, CDC31 protein, is a component of the spindle pole body that is required for pole duplication and separation (Baum *et al.*, 1988; Spang *et al.*, 1993). Recently, Middendorp *et al.* (2000) microinjected recombinant human centrin 3 protein (HsCen3p) or RNA encoding HsCen3p into one blastomere of two-cell stage *Xenopus* embryos and found delay in cleavage and inhibition of centrosome duplication in early *Xenopus* embryos. This observation implies that centrin 3 may be crucial for centrosome duplication, although the function of other centrin

members need to be explored in the future.

I-A-3. Centriole Function:

The most obvious function of centrioles is in the assembly of cilia and flagella. Basal bodies at the base of cilia and flagella are equivalent to centrioles and nucleate assembly of ciliary and flagellar microtubules. In human tissue, for example, the precursors of the ciliated epithelial cells that line the trachea and esophagus contain only centrioles. These centrioles migrate from perinuclear position to the luminal plasma membrane as they differentiate. These centrioles then form centriole-like structures, each of which become a basal body of a cilium (Balczon, 1996).

Since centrioles are located at the core of most animal centrosomes, it was hypothesized that centrioles may serve as a discrete scaffold around which all of the microtubule-nucleating material in the cell is recruited into a single focus to form the centrosome (review by Marshall and Rosenbaum, 1999). In some organisms lacking centrioles, higher plants for example, the microtubule-nucleating material is dispersed throughout the cells with no clear single focal point, which supports the view of this hypothesis. In order to provide direct evidence of the hypothesis, Bobinnec *et al.* (1998) loaded HeLa cells with monoclonal antibody against polyglutamylated tubulin, which is found predominantly in centrioles. As a result, the centrioles were disassembled, the microtubule-nucleating materials were dispersed throughout the cytoplasm, and no functional centrosomes were present. Gradually centrioles were reformed, and the nucleating materials re-associated around the centrioles forming discrete centrosomes, as the antibody degraded overtime. This observation supports the view that centrioles are

essential for recruiting microtubule-nucleating materials into discrete foci.

de Saint Phalle and Sullivan (1998) also examined the idea that centrioles are required for the assembly of a functional centrosome in the fly *Sciara coprophila*. Unfertilized embryos of *Sciara* can develop parthenogenetically in the absence of a centriole. These authors showed that unfertilized oocytes undergo multiple rounds of nuclear division and form bipolar spindles assembled by growing out from chromosomes. These spindles, however, lacked astral microtubules, and resulted in defective nuclear spacing and migration during the syncytial divisions. The absence of astral microtubules suggests that the centriole-less oocytes do not have functional centrosomes, which in turn implies that centrioles may be required to recruit microtubule-nucleating materials to form a functional centrosome.

At present, the centriole pair remains without a defined role in the centrosome of animal cells. The only well-established function of the centriole is to act as a template for the growth of the primary or motile ciliary axoneme (Rieder and Borisy, 1982). Although a considerable volume of data suggests that centriole duplication could be used as a morphological indication for the process of centrosome reproduction, the actual role of centrioles in centrosome function remains obscure (Brinkley *et al*, 1981; Sluder and Rieder, 1985; Kochanski and Borisy, 1990; reviewed by Balczon,1996).

I-B. Pericentriolar Domain:

During mitosis the duplicated centrosomes form the poles of the spindle. It is thought that the pericentriolar material (PCM) contains the microtubule nucleating sites, although the centrioles serve as the morphological identification tag for the centrosome

complex (Balczon, 1996). This conclusion is based on the observations of both *in vivo* and *in vitro* centrosome organization. For instance, plant cells and mouse oocytes do not contain identifiable centrioles, even though they are capable of initiating microtubule nucleation and contain molecules that react with antisera raised against animal PCM components (Lambert, 1993; Schatten, 1994).

I-B-1. Pericentriolar Structure:

The pericentriolar material forms around the centriole with a preference for the mature centriole (reviewed by Mack *et al.*, 2000). Analysis of PCM by electron microscopy revealed that the size of PCM varies in different organisms, ranging from 0.5 μm in diameter in mammalian centrosomes (Mitchison and Kirschner, 1984) to 2-3 μm in diameter in marine oocyte and embryos (Sluder and Rieder, 1985; Vogel *et al*, 1997). The size of the pericentriolar domain also varies throughout the cell cycle, reaching its largest dimensions during mitosis (Mack *et al.*, 2000). Electron microscopy studies of sites where PCM nucleates microtubules revealed that PCM consists of two major components: ring-like complex (Zheng *et al.* , 1995; Moritz *et al.*, 1995a; Moritz *et al.*, 1995b) and lattice-like filaments (Dictenberg *et al.*,1998). However, very little is known about either of the structures at present.

I-B-2. Pericentriolar Proteins:

There are a number of proteins that localize to the pericentriolar domain (reviewed by Andersen, 1999; Mack *et al.*, 2000). Among these PCM proteins, γ-tubulin and its associated proteins have attracted the most attention.

γ-tubulin was first identified in *Aspergillus nidulans* in an attempt to find proteins

that rescue β-tubulin mutations (Oakley and Oakley, 1989). Comparison of γ-tubulin with 29 α- and β-tubulin amino acid sequences from various organisms showed approximately 30-40% identity between these proteins, suggesting that the proteins belong to the same tubulin superfamily. The presence of γ-tubulin appears to be ubiquitous in eukaryotic and fungal cells (Zheng *et al.*, 1991; Stearns *et al.*, 1991; Horio *et al.*,1991; Joshi *et al.*, 1992).

Immunolocalization of γ-tubulin showed a striking enrichment at centrosomes during all phases of the cell cycle (Oakley *et al.*,1990; Zheng *et al.*, 1991; Stearns *et al.*, 1991; Horio *et al.*,1991; Joshi *et al.*, 1992) and that it was found in both the centriole domain (Fuller *et al*, 1995) and PCM domain (Stearns *et al.*, 1991), but not on the microtubule lattice itself. On the basis of these observations, it was proposed that γ-tubulin is located at the site of initiation of microtubule-nucleating assembly. Experiments that disrupt the γ-tubulin gene (Oakley *et al.*,1990; Horio *et al.*,1991), or block (Joshi *et al.*, 1992; Stearns *et al.*, 1991) and deplete (Félix *et al.*,1994) γ-tubulin protein using specific anti-γ-tubulin antibodies, all indicated that γ-tubulin is essential for microtubule assembly.

Moritz *et al.* (1995a) used three-dimensional electron tomography to study the PCM microtubule-nucleating sites of isolated *Drosophila* embryonic centrosomes and observed ring-like structures with diameters similar to the microtubules (ie. 25-28 nM) at these sites. By immunoelectron microscopic tomography, the same group also observed that γ-tubulin is localized to ring-structures in the PCM of purified centrosomes without microtubules and that γ-tubulin complexes are located at the minus ends of microtubules

when they are assembled from MTOCs (Moritz *et al.*,1995b). Zheng *et al.* (1995), also showed that γ-tubulin exists in 25S complexes in unfertilized *Xenopus* egg extracts. They were able to purify a soluble, functional protein complex, termed the γ-tubulin ring complex (γ-TuRC) from the extracts, because these components are enriched in *Xenopus* eggs in preparation for repeated rounds of mitosis during early development. The γ-TuRCs have a ring-like structure and consist of about 10-13 γ-tubulin molecules as well as at least seven other proteins. These γ-TuRCs were able to nucleate microtubules and to cap the minus ends of microtubules *in vitro* (Zheng *et al.*, 1995).

Dictenberg et al. (1998), in contrast, observed a reticular lattice structure within the PCM when studying the relationship between γ-tubulin and pericentrin, a 220 kDa conserved centrosome protein involved in microtubule nucleation (Doxsey *et al.*, 1994). This group demonstrated by sucrose gradient sedimentation, gel filtration, and coimmunoprecipitation that pericentrin and γ-tubulin form a soluble protein complex in *Xenopus* egg extracts. Even though pericentrin is not part of the isolated γ-TuRC, identification of pericentrin-γ-tubulin complex implies that these proteins are found together in proximity of the centrosome. The authors then examined the distribution of proteins at the centrosome using conventional indirect immunofluorescence and confocal microscopy. Pericentrin was found to define a lattice-like filament scaffold at the centrosome, and co-localized with γ-tubulin in close proximity within the lattice. Identification of the lattice structure in CHO cells and *Xenopus* spindles that were assembled *in vitro*, suggested that the scaffold was a conserved features of centrosomes in vertebrate organisms. The organization of this lattice seemed to be independent of

centrioles. However, its size and complexity changed dramatically during the cell cycle, increasing from G1 phase to mitosis, but quickly disassembling as cells exit mitosis. Furthermore, these lattice elements appeared to contact the minus ends of nucleated microtubules, indicating that a lattice composed of pericentrin and γ-tubulin may serve as a scaffold for microtubule nucleating at the centrosome. This lattice-like scaffold was also observed by electron microscopy in *Spisula* centrosome extracted with 1.0 M KI, and was called the centromatrix (Schnackenberg *et al.*,1998; reviewed by Schnackenberg and Palazzo, 1999). The centromatrix is unable to nucleate microtubules, although it contains the γ-TuRC and has been shown to provide the structural basis to bind to proteins associated with microtubule nucleating.

Attempts were taken to identify proteins that form the γ-TuRC in order to determine how γ-tubulin becomes localized to the centrosome in a microtubule-independent manner (Murphy *et al.*, 1998; Tassin *et al.*, 1998; Martin *et al.*, 1998; Moritz *et al.*, 1998). Murphy *et al.* (1998) cloned human cell lines that stably expressed epitope-tagged human γ-tubulin. The tagged γ-tubulin was then immunoprecipitated from cells and found to associate with seven other proteins. Two of them, hGCP2 and hGCP3, were cloned and shown to be homologs to the yeast Spc97p and Spc98p, respectively (Murphy *et al.*, 1998; Tassin *et al.*, 1998). Together with γ-tubulin, hGCP2 and hGCP3 form a trimeric complex that is the minimum building block of γ-TuRC. A fourth component of γ-TuRC, hGCP4, was also identified (Fava *et al.*, 1999). The hGCP4 is a 76 kDa protein and is necessary for *in vitro* aster nucleation by *Xenopus* sperm centioles. Martin *et al.* (1998) also identified a protein called Xgrip109, homologous to Spc98p in yeast, from

Xenopus egg extract. They showed that the γ-TuRC was not able to reassemble if Xgrip109 was immunodepleted from salt-disrupted γ-TuRC, suggesting that Xgrip109 is required for γ-TuRC assembly. This finding is in accordance with the data of Murphy *et al.* (1998). Moritz *et al.* (1998) demonstrated that the microtubule nucleating capability of 2M KI salt-treated centrosome scaffolds (ie. centrosomes lack of γ-tubulin and other centrosomal proteins) could be restored by the addition of γ-TuRC and other unknown proteins in *Drosophila* extracts. This study shows that microtubule nucleation requires γ-tubulin and its associated proteins, as well as other unknown factors.

Isolated basal bodies from *Chlamydomonas* have been shown to contain approximately 200 different proteins (Dutcher, 1995; Marshall and Rosenbaum, 2000). Recently, a genetic screen in *Chlamydomonas* identified a new class of tubulin, δ-tubulin, along with α- and β-tubulins required to assemble the triplet microtubules (Dutcher and Trabuco, 1998). The δ-tubulin is encoded by *UNI3* gene and *uni3* defective mutants contain centrioles with doublet microtubules instead of tripltet. The δ-tubulin and a new class of tubulin, ϵ-tubulin, were subsequently identified in human cells (Chang and Stearns, 2000). Chang and Stearns also showed that the localization of δ- and ϵ-tubulins to centrosome is independent of microtubules. However, δ-tubulin is associated with centrioles, whereas ϵ-tubulin is found in the PCM domain. The localization of ϵ-tubulin is cell-cycle dependent, first associating with only the older centrosome in a newly duplicated pair and then latter associating with both centrosomes. This localization pattern is significantly different from γ-tubulin indicating that ϵ-tubulin is not a component of γ-TuRC, but may play a role in some, as yet unkown, centrosome

maturation event. However, the relationship between δ-tubulin and γ-TuRC, and the defined function of both δ- and ϵ- tubulins need to be further clarified.

There are also a number of other proteins that have been identified at the PCM domain, such as p53 (Blair Zadjel and Blair,1988), the BRCA1 (Hsu and White, 1998) and its associated protein pRb (Thomas *et al.*, 1996), cdc2 (Bailly *et al.*, 1989), and cyclin A and B (Bailly *et al.*, 1992). However, the function of these proteins at centrosomes is unclear at present.

I-C. Centrosome Duplication:

I-C-1. Centriole Cycle:

In order to understand better centrosome assembly and function, it is crucial to know the mechanism of centriole assembly. Duplication and maturation of centrioles occur in a cell cycle dependent manner (Vorobjev and Nadezhdina, 1987). Centrioles are the only organelle, other than chromosomes, that replicate precisely once per cell division. A pair of centrioles is delivered to each daughter cell after cell division. The orthogonal orientation of the centriole pair is lost during early G1 phase and no changes take place until late G1 phase, when small protrusions form orthogonally to the proximal end of the parent centriole just before or about the time the cells enter S phase. The daughter centrioles grow perpendicularly on the parent centrioles through S and G2 phases in a semiconservative manner (ie. the mother centriole is completely stable, whereas the daughter centriole is generated *de novo*; Kochanski and Borisy, 1990). The daughter centriole is matured by the time the cell enters mitosis. The mother centriole then acquires cenexin during the G2-M transition. Subsequently, the two centrosomes

separate at M phase and each centrosome harbors a centriole pair consisting of one parent and one daughter centriole. The separated centrosomes then migrate to opposite ends of the cell, where they serve as the spindle poles that are responsible for nucleating mitotic spindle microtubules (reviewed by Brinkley, 1985; Vorobjev and Nadezhdina, 1987; Balczon, 1996).

I-C-2. Proteins that Control Centrosome Duplication:

I-C-2-a. Cdk2 kinase and Centrosome Duplication:

Early studies on the requirement for centriole assembly have shown that centriole duplication is independent of DNA replication in both somatic and embryonic cells (Rattner and Phillips, 1973; Balczon *et al.*,1995; Debec *et al.*, 1996). Nevertheless, centrosome duplication, like chromosome replication, normally occurs only once during each cell division cycle, suggesting the existence of a common regulatory machinery that controls the cell cycle regulating DNA replication and centrosome duplication. A series of four recent papers have revealed the requirement of cyclin-dependent kinase, Cdk2, activity and Cdk2-cyclin complexes to drive centrosome duplication (Lacey, *et al.*, 1999; Hinchcliffe *et al.*, 1999; Matsumoto, *et al.*, 1999; reviewed by Winey, 1999; Meradi *et al.*, 1999).

It was shown that hydroxyurea inhibition of DNA synthesis in CHO cells resulted in accumulation of centrosomes in the absence of cell cycle progression (Balczon *et al.*, 1995). Matsumoto *et al.* (1999) then compared the effect of hydroxyurea and mimosine, a cell cycle inhibitor that arrests cells at late G1 phase prior to the onset of DNA synthesis, on centrosome duplication in CHO cells. They observed centrosome reduplication in

hydroxyurea-treated cells, but not in mimosine-treated cells, suggesting the correlation of centrosome duplication with events occurring at the G1-S transition. Because Cdk2-cyclin E complex activity is found at the G1-S transition and Cdk2-cyclin A complex activity is present during S phase, they examined the Cdk2 activity in CHO cells treated with mimosine or hydroxyurea, and found that Cdk2 activity was significantly higher in the presence of hydroxyurea than in the presence of mimosine. Furthermore, centrosome duplication was inhibited by addition of butyrolactone I and roscovitine, small molecule-inhibitors of the protein kinase activity of Cdk2, Cdc2, and Cdk5. In order to show the specific role of Cdk2 in centrosome duplication, they expressed the Cdk2 inhibitor, p21, in hydroxyurea-treated cells and found that centrosome duplication was blocked in p21-expressing cells. They also showed that overexpression of Cdk2 activity reversed the inhibition of centrosome duplication by mimosine treatment. Taken together, these results indicate that Cdk2 activity is required for centrosome duplication.

Based on the observation that centrosome replication occurs in *Xenopus* blastulae treated with cycloheximide, a protein synthesis inhibitor that blocks the synthesis of cyclin A and B (Gard *et al.*, 1990), Lacey *et al.* (1999) looked for conditions that would block centrosome reproduction. They found that centrosome duplication is blocked in *Xenopus* embryos injected with N-terminal of p21 or p27, protein inhibitors of Cdk2-cyclin A or Cdk2-cyclin E complexes, respectively. In addition, they co-injected Cdk2-cyclin E complexes with p21 into *Xenopus* embryos and found that centrosome duplication was restored. These results suggest that Cdk2-cyclin E is required for centrosome replication. In another experiment, Lacey *et al.* (1999) demonstrated the

requirement of Cdk2 activity for centriole separation by observing the morphology of centriole pairs purified from *Xenopus* XTC cells incubated in *Xenopus* extract. Depletion of Cdk activity in *Xenopus* egg extract by either p21 or $p13^{suc1}$ beads could prevent centriole separation, indicating that Cdk2 activity might play a role in the separation event. Specific depletion of either Cdk2-cyclin A or Cdk2-cyclin E from the *Xenopus* egg extract did not block centriole separation, while depletion of both Cdk2-cyclin complexes inhibited it. These results indicate that the requirement for cyclin A and cyclin E for centrioie separation is redundant.

Hinchcliffe *et al.* (1999) has independently observed multiple rounds of centrosome duplication when demembranated sperm nuclei were incubated in *Xenopus* egg extracts arrested at S phase with aphidicolin. In their system, the centrioles of sperm are converted to centrosomes and then undergo duplication. By using this *in vitro* system, they found that the addition of Δ34Xic1, a specific inhibitor of Cdk2-cyclin E complex, prevented centrosome duplication, whereas addition of recombinant *Xenopus* Cdk2-cylin E to Δ34Xic1-treated S phase extract could resume centrosome duplication. They also showed that cyclin E is localized to centrosomes in *Xenopus* embryonic blastomeres. These data indicate that Cdk2-cyclin E might interact directly with centrosomal components and its activity is important for centrosome duplication. These authors also proposed that Cdk2-cyclin E may be the "licensing factor" that ensures the centrosome is duplicated only once each cell division cycle since inactivation of Cdk2-cyclin E by Δ34Xic1 did not inhibit the first round of duplication *in vitro*, but did block the subsequent rounds of duplication. However, the universality of this "licensing event"

needs to be explored in both embryonic and somatic cell cycles *in vivo* because the centrosomes arise from basal bodies of sperms may be subjected to different rules for duplication. The different rule for centrosome duplication is evident in the case that centrosome duplication occurs in the absence of nucleus and protein synthesis in embryonic system (Sluder *et al.*, 1990; Gard *et al.*, 1990; Hinchcliffe, 1999), whereas centrosome duplication requires an intact nucleus and protein synthesis in somatic cells (Phillips and Rattner, 1976; Kuriyama and Borisy, 1981).

On the other hand, Meraldi *et al.* (1999) has demonstrated the requirement of E2F transcription factors, Cdk2-cyclin A activity, and the phosphorylation of retinoblastoma (Rb) protein for centrosome duplication in mammalian somatic cells (Meradi *et al.*, 1999). Because treatment of CHO cells with hydroxyurea does not inhibit multiple rounds of centrosome duplication and progression into S phase is regulated by the phosphorylation state of the Rb protein, a substrate of Cdk4/6-cyclin D kinase complex, Meraldi *et al.* (1999) looked at the effect of blocking Cdk4/6-cyclin D kinase activity on centrosome duplication. They found that centrosome duplication was greatly inhibited in CHO cells expressing $p16^{INK4}$, a Cdk4/6 specific inhibitor. In order to confirm the result, they expressed RbΔCdk, a Rb mutant which lacks sites for Cdk phosphorylation and blocks cell-cycle progression downstream of Cdk4/6, and found that the expression also prevented centrosome duplication as $p16^{INK4}$ overexpressed cells. Since the phosphorylation state of Rb positively regulates the activity of E2F transcription factors, these researchers looked for the possible requirement of E2F activity in centrosome duplication. Indeed, significant inhibition of centrosome duplication was observed when

a dominant-negative mutant defective in E2F function was overexpressed in CHO cells. This inhibition was in turn rescued by E2F-2 and E2F-3, suggesting that among the proteins controlled by Rb, E2F is sufficient to drive centrosome duplication. Taken together, these data show that centrosome duplication in somatic cells is regulated through an Rb-dependent pathway.

Meraldi *et al.* (1999) also observed the effect of overexpressing p27 and dominant negative mutants of Cdk and found that centrosome duplication was inhibited in cells with overexpressed p27 and with those expressing Cdk2 mutations, but was not inhibited in the dominant negative mutants of Cdk1 and Cdk3. These results indicate that Cdk2 activity is important for centrosome duplication. Then, they went on to see if Cdk2 regulates centrosome duplication in association with cyclin A or cyclin E, or both. They found that even though overexpression of either cyclin could overcome the $p16^{INK4}$ block, only cyclin A was able to override the RbΔCdk inhibition in a Cdk2 dependent manner, suggesting that Cdk2-cyclin A complex, rather than Cdk2-cyclin E complex, is responsible for regulating centrosome duplication in somatic cells. This intriguing discrepancy between the finding of Meraldi *et al.* (1999) and others (Lacey *et. al.*,1999; Hinchcliffe *et. al.*,1999) may be explained by the differences of experimental systems used. These systems are different in that (1) the mammalian somatic cells have prolonged G1 and G2 phases which are missing in rapid dividing *Xenopus* embryonic cells; and (2) cyclin A is the major S-phase partner of Cdk2 in mammalian somatic cells, whereas cycin E is the major Cdk2 partner until mid-blastula transition in *Xenopus* embryos. Therefore, it is possible that Cdk2-cyclin A activity plays a major role in mammalian

somatic cell, while Cdk2-cyclin E activity is the major player in embryonic centrosome duplication.

I-C-2-b. SCF Ubiquitin Ligase Complex and Centrosome Duplication:

The SCF (Skp1-Cullin-F-box) ubiquitin ligase, which determines the specificity for protein ubiquitinylation, is implicated in regulating G1-S progression (review by Deshaies, 1999). Using immunofluorescence microscopy, the mammalian Skp1 (Freed *et al.*, 1999; Gstaiger *et al.*, 1999) and Cul1 (Freed *et al.*, 1999) proteins were shown to be localized to interphase centrosomes and to mitotic spindle poles. Immunodepletion of *Xenopus* egg extracts with antibodies against Skp1 or Cul1 blocked centriole separation (Freed *et al.*, 1999). In addition, inhibitors of the 26S proteasome blocked centriole separation *in vitro* and prevented centrosome duplication when they were microinjected into *Xenopus* blastomeres. These data together suggest that Skp1 and Cul1 have a major function in centrosome duplication cycle. It is possible that the SCF complex regulates centrosome replication by monitoring the abundance of G1 cyclin, since Dealy and colleagues used mice deficient in Cul1 to show that Cul1 is required for early mouse development and that Cul1 mutants fail to regulate the abundance of the cyclin E (Dealy *et al.*, 1999).

The F-box protein serves as the substrate recognition subunit in the SCF complex by binding to Skp1 through its F-box motif. Two recent papers have shown the importance of the *Drosophila* F-box protein, Slimb (Wojcik *et al.*, 2000), and the mouse F-box protein, Skp2 (Nakayama *et al.*,2000), in regulating centrosome replication. During a search for *Drosophia* mutants defective in centrosome replication, Wojcik *et al.*

(2000) identified a hypomorphic mutant called "*Slimb*^{crd}" that resulted in hypernumerous centrosomes and mitotic defects in larval neuroblasts. The target of Slimb, however, is unclear at the moment. Nakayama *et al.* (2000), on the other hand, generated mice lacking Skp2 and showed the derived mouse embryo fibroblasts (MEFs) exhibited hyperaccumulation of cyclin E and p27, polyploidy and multiple centrosomes. In addition, these researchers demonstrated that Skp2 mediated ubiquitin-dependent degradation of cyclin E, and that the binding of Cdk2 to cyclin E prevented cyclin E from interacting with Skp2 and from undergoing ubiquitylation. Therefore, they suggested that Skp2 regulates centrosome duplication by controlling the abundance of cell cycle regulatory proteins, such as cyclin E, at the G1-S transition. Yet the observation that multiple centrosomes result from the accumulation of cyclin E in MEFs derived from Skp2 gene knockout mice seems to contradict with the findings of Meraldi *et al.* (1999), who proposed that cyclin A-Cdk2 complex, rather than cyclinE-Cdk2 complex, is the principle kinase responsible for regulating centrosome duplication. Other proteins have also been shown to be involved in the regulation of centrosome duplication. These proteins include: centrin 3 (Middendorp *et al.*,2000); γ-tubulin (Ruiz *et al.*, 1999), p53 (Fukasawa, 1996; Mussman *et al.*, 2000); polo-like protein kinases (reviewed by Mayor *et al.*, 1999), protein kinase STK15/BTAK (Zhou *et al.*,1998), aurora-related protein kinases (Roghi *et al.*, 1998; Tanaka *et. al.*, 1999), mitotic cyclins and Cdc20^{fizzy} (Vidwans *et al.*, 1999). However, the precise mechanism of how these proteins regulate centrosome duplication remain obscure at the moment.

II. Yeast Spindle Pole Bodies:

In the fission yeast, *Saccharomyces pombe,* and budding yeast, *Saccharomyces cerevisiae,* the spindle pole body (SPB) serves as a MTOC *in vivo* and as a structure capable of nucleating microtubule assembly *in vitro* (reviewed by Winey and Byers, 1993; Sobel, 1997; Adams and Kilmartin, 2000). The spindle pole body is different from centrosome in structure (see below). The suitability of yeast for structural, biochemical, and genetic analysis has led to the organism becoming a popular model for the study of MTOC biology. Therefore, there is perhaps more information available about SPB structure and function in yeast than for centrosome structure/function in any other organism.

II-A. SPBs of *Saccharomyces pombe*:

II-A-1. SPB Structure of *S. pombe*:

Electron micrographs have shown that during G1 phase, the SPB of the fission yeast is a small, multi-layered structure in the cytoplasm adjacent to the nuclear envelope but distinct from it. There are no centioles associated with it. In addition, the interphase SPB does not possess any nuclear microtubule-nucleating activity. However, some cytoplasmic microtubules are located in the immediate vicsinity to the SPB, suggesting that interphase SPB plays a role in the organization of cytoplasmic microtubules, but not nuclear microtubules.

II-A-2. SPB Duplication in *S. pombe:*

The process of SPB duplication in *S. pombe* occurs in the cytoplasm in a semiconservative manner, meaning that the old SPB is retained while a new SPB is

formed beside it (Adams and Kilmartin, 2000). A bridge-like structure is seen beside the SPB in G1 phase in the absence of the electron-dense structure named the "satellite". The SPB is then duplicated in the cytoplasm and the duplicated SPBs are connected by the bridge during G2 phase of the cell cycle. Initially the duplicated SPBs are smaller than the corresponding structure from the G1 phase, suggesting that components are taken from the old SPB to synthesis the new SPB, or that the older SPB is dynamically losing subunits. Soon after duplication, both SPBs enlarge in the plane parallel to the nuclear envelope. In the early mitosis, the nuclear envelope then form fenestrae into which the two SPBs are inserted. The SPBs are then separated, the bridge is severed and microtubules are nucleated from the nuclear surface of both SPBs into the nucleoplasm. The nuclear microtubule arrays interdigitate to form a bipolar spindle for chromosome segregation. The fenestrae that into which the two SPBs are located begins to close during anaphase, while the SPBs are extruded back into the cytoplasm. The constriction of fenestrae and the extrusion of SPBs are complete by the end of telophase. The nuclear microtubule arrays are then disappear and the cells return to interphase. Each daughter cell inherits a SPB and a half-bridge (Ding *et al.*, 1997; West *et al.*, 1998; Adams and Kilmartin, 2000).

II-B. SPBs of *Saccharomyces cerevisiae*:

II-B-1. SPB Structure of *S. cerevisiae*:

Electron micrographs have shown that the *S. cerevisiae* SPB also lacks centrioles and that it has a disk-like structure embedded in the nuclear envelope throughout the cell cycle (Winey and Byers, 1993). The trilaminar morphology of the SPB is made up of a

central plaque flanked on either side by the inner (nuclear) and outer (cytoplasmic) plaques, which are the sites for nuclear and cytoplasmic microtubule nucleation, respectively (Snyder, 1994). The inner and outer nuclear envelope membranes are connected to the central plaque by filamentous spacers (Sobel, 1997). A membrane structure known as the "half-bridge" lies in the plane of the nuclear envelope beside the central plaque. The size of SPBs can change with the ploidy of the cell and the phase of the cell cycle (Adams and Kilmartin, 2000). In hapolid cells, the SPB is about 150 nm high and 110 nm in diameter. The diameter of the SPB decreases to about 80 nm in G1 phase cells. In diploid cells, the height of the SPB remains the same, while the diameter increases to about 160 nm.

II-B-2. SPB Duplication in *S. cerevisiae*:

In *S. cerevisiae*, the structure of the SPB varies greatly during the cell cycle and the process of SPB duplication is not fully understood at present. The SPB duplication begins at G1 phase, when the satellite structure appears on the cytoplasmic face of the nuclear envelope distal to the half-bridge and the existing SPB. During S phase, the satellite enlarges to form a structure called the duplication plaque, which is similar to the central plaque of the existing SPB in both size and appearance. The half-bridge also enlarges and its distal end fuses to the duplication plaque. SPB duplication is completed during S phase, and the duplicated SPBs remain attached side-by-side by a full bridge. Subsequently, the bridge is servered, the SPBs are separated, and short spindles are formed between the two separated SPBs during late G2 phase. The spindles then elongate during chromosome segregation in mitosis. The spindles disassemble at the M-G1

transition, and each daughter cell inherits a SPB and a half-bridge after nuclear division and cytokinesis (Knop *et al.*,1999; Adams and Kilmartin, 2000).

II-C. Proteins that Control SPB Duplication:

Studies in the yeast *S. cerevisiae* have identified *MPS*1 (monopolar spindle 1) as an essential gene required for replication of SPBs and as a component of the mitotic checkpoint (Winey *et. al.*, 1991; Weiss and Winey, 1996). Analysis of temperature sensitive mutant strains has shown that *mps1* mutants contain only monopolar mitotic spindles due to a failure in SPB duplication (Winey *et. al.*, 1991). These monopolar spindle mutants are incapable of segregating their chromosomes, thereby leaving all the chromatin in one cell and results in a cell with twice the normal DNA content. *mps1* mutants also fail to arrest the cell cycle in response to the defective spindle, suggesting that the product of *MPS1* plays a crucial role in mitotic spindle checkpoint (Weiss and Winey, 1996). In fact, Mps1 protein has been found to be an essential protein kinase (Lauzé *et. al.*, 1995), whose activity is required for both SPB duplication and mitotic checkpoint functions.

Recently, a yeast two-hybrid screen was used to identify potential substrates or effectors that interact with Mps1 (Luca and Winey, 1998), and a protein called Mob1 (Mps One Binder 1), which encodes an essential 314-amino acid (ie. 36 KDa) protein, was found to bind Mps1. Sequence analyses of the cDNA encoding Mob1 reveal that sequences similar to *S. cerevisiae* Mob1 can be found in a variety of predicted proteins of unknown functions in cells from organisms as diverse as *Arabidopsis* and human. Proteins with Mob1-like sequences share sequence identities with *S. cerevisiae* Mob1

ranging from 26-59% at the amino acid level. Cells harbouring different *mob1* alleles arrest as large budded cells with 2N DNA content (*S. cerevisiae* is usually haploid), separated chromatin, and long bipolar mitotic spindles at restrictive temperature. The arrest in late mitosis and the increase-in-ploidy phenotype suggests that *MOB1* is required for completion of mitosis and for maintaining genetic stability. In order to determine the requirement of Mps1 for late mitotic arrest in *mob1* mutant cells, Luca and Winey proposed that if *mob1* mutants require Mps1 in late mitosis, then *mob1 mps1* double mutant should fail to arrest in late mitosis at permissive temperatures (Luca and Winey, 1998). The double mutants, however, arrest in late mitosis with a phenotype similar to the *mob1* singe mutant cells. In addition, overexpression of wild-type Mps1 was unable to alleviate the phenotype of *mob1* mutation. These results suggest that Mps1 is not required for late mitotic arrest caused by *mob1* mutations. Conversely, they suggested that if Mob1 is required for spindle assembly checkpoint pathway then *mob1* mutant strains should progress through the cell cycle if they are treated with nocodazole, a microtubule-disrupting agent that triggers the spindle assembly checkpoint. They found that these nocodazole-treated *mob1* mutants were arrested at permissive temperature, suggesting that they are not defective in the spindle assembly checkpoint pathway. Mad1, a molecular marker for activation of the spindle assembly checkpoint (Hardwick and Murray, 1995; Hardwick *et. al.*, 1996), was hyperphosphorylated (ie. activated) in nocodazole-treated cells, but not in control cells or *mob1* mutant cells arrested at restrictive temperature. These data together suggest that Mob1 is not required for the spindle assembly checkpoint. However, it is also possible that the requirement of Mob1

in the spindle assembly checkpoint is downstream of Mad1 hyperphosphorylation.

Subsequently, Denis and colleagues demonstrated, by using a yeast two-hybrid assay, that Mob1 was able to interact with Dbf2, a protein kinase and a component of the CCR4 transcriptional complex, which regulates cell cycle progression during late mitosis and gene expression (Komarnitsky *et. al.*, 1998). Co-immunoprecipitation experiments were performed to further confirm the physical interaction between Mob1 and Dbf2. In addition, it was also shown that *dbf2* mutant strains have a phenotype that is similar to *mob1* mutant cells and *MOB1* mRNA expression was cell cycle controlled in a manner similar as *DBF2* mRNA, peaking at G2/M boundary and decreasing at the end of mitosis. These results indicate that Mob1 and Dbf2 function at the same stage of the cell cycle. Rescue studies suggest that overexpression of Dbf2 was not able to complement *MOB1* deletion mutant strain, but Mob1 overexpression was capable of rescuing strains with either *DBF2* single deletion or *DBF2 DBF20* double deletion. These results suggest that elevated levels of Mob1 are able to overcome the requirement of Dbf2 and that Dbf2 may regulate Mob1 function in late mitosis. Nevertheless, *in vitro* phosphorylation experiments were unable to show that Mob1 is a substrate of Dbf2 (Komarnitsky *et. al.*, 1998). Using the two-hybrid assay, three *mob1* point mutations (Luca and Winey, 1998) blocked binding to Dbf2 but not to Mps1, suggesting that the late mitotic arrest caused by these mutant alleles may result from defects in Dbf2 binding and not Mps1 binding (Komarnitsky *et. al.*, 1998).

Salimova *et al.* (2000) isolated the *MOB1* homolog from *S. pombe* during a search to find septation mutants. These investigators looked for proteins that could rescue a

cold-sensitive mutant stain, SP2198, defective in septum formation, and found that the *S. pombe* homolog of the budding yeast gene *MOB1* was able to suppress the multiseptate phenotype of SP2198. When function of Mob1 in *S. pombe* cell was examined, they found that *MOB1* deletion mutants were lethal, and that microscopic examination of the germinating spores showed that they were highly elongated with multiple nuclei and without septa. In contrast, a mutant strain harboring a partial *MOB1* deletion showed higher viability than the full deletion mutant, even though the germination rate was low. Mutants containing thermosensitive alleles of the *MOB1* gene were also binucleate and lacked a septum at the non-permissive temperature. These results suggested that *MOB1* is an essential gene and its gene product is not required for nuclear cycle, but is essential for cell division. The nuclei of these mutants, however, showed a postmitotic configuration, which is different than the increase-in-ploidy phenotype observed in *S. cerevisiae*, as reported by Luca and Winey (1998). Salimova *et al.* (2000) then examined the effect of overexpressing Mob1 protein. They found that cells with increased expression of the *MOB1* gene displayed a variety of phenotypes, such as failure to septate, formation of one or more septa without undergoing cytokinesis, condensed chromosomes, and formation of thick, aberrant septa. Taken together, these data suggested that in *S. pombe* Mob1 may play a role in signalling the onset of septum formation.

Indirect immunofluorescence studies of fixed *S. pombe* cells and observation of Mob1-GFP fusion protein revealed that Mob1 co-localized with Sid2, a potential *S. pombe* homolog of Dbf2 that localizes to SPBs during mitosis (Salimova *et al.*, 2000) and regulates the onset of cytokinesis (Balasubramanian *et al.*,1998; Guertin *et al.*, 2000).

They were unable to detect Mob1 in interphase SPBs, however, and a generalized cytoplasmic staining was seen throughout the interphase of cell cycle. Later in mitosis, Mob1 also co-localized with the medial ring and flanked the septum as the medial ring contracted. The subcellular localization of Mob1 at SPBs during mitosis was not affected in *sid2-250* mutants at the non-permissive temperature, but Mob1 ring staining was lost in *sid2-250* even at the permissive temperature, suggesting that formation of a stable Mob1 ring requires full Sid2 function. These researchers also looked for a requirement for Mob1 function in Sid2 localization by documenting the subcellular localization of Sid2-GFP in the *mob1-M17* mutant. They observed that both the rings and SPB signals of Sid2-GFP were greatly reduced in *mob1-M17* mutant at the non-permissive temperature, implying that Mob1 function is essential for the stable association of Sid2 with the SPB and for formation of the medial ring. Finally, Salimova and colleagues went on to identify the relationship between Mob1 and Sid2, using protein extracts prepared from cells containing either epitope tagged Mob1 or epitope tagged Sid2 (Salimova *et al.*, 2000). Immunoprecipitates using anti-HA antibodies from *MOB1-HA SID2-GFP* strain contained Sid2-GFP, while immunprecipitation using anti-MYC antibodies from *MOB1-GFP SID2-MYC* cells contained Mob1-GFP. These results indicate that Mob1 interacts with Sid2 in a complex. Using a yeast two-hybrid screen, however, these investigators were not able to detect any interaction between Mob1 and Mph1, a *S. pombe* homolog of Mps1 (He *et al.*, 1998).

The identification of the *S. pombe MOB1* gene and formation of *S. pombe* Mob1-Sid2 complex were also reported by Hou *et al.* (2000). They found that deletion of the

MOB1 gene was lethal and growth of *mob1-1* mutant strain at restrictive temperature resulted in cells that had multiple nuclei, lacked septa and failed to undergo cytokinesis. Unlike the *S. cerevisiae mob1-77* mutant (Luca and Winey, 1998), *mob1-1 S. pombe* mutants had normal mitotic spindles indicating that they may be defective in cytokinesis rather than in mitotic exit. The interaction between Mob1 and Sid2 was confirmed by the yeast two-hybrid system and by co-immunoprecipitation experiments using lysates prepared from cells expressing both GFP-Mob1 and Sid2-MYC. GFP-Mob1 localized to SPBs during both interphase and mitosis, with a stronger signal at mitosis. The presence of Mob1 at interphase SPBs was a novel observation, which was not made by Salimova *et al.* (2000). The localization of Mob1 to SPB was independent of microtubules and Sid2, but was severely affected in septation signalling mutants *sid4, cdc7,* and *cdc11.* In contrast, Mob1 is required for Sid2 localization to SPB as observed by Salimova *et al.*, (2000). Mob1 was found at the medial ring during septum formation, before cell separation. The localization of Mob1 to the cell-division site was dependent on the medial ring component, Cdc15, and on the septation signal proteins, Cdc7, Cdc11, Cdc14, Sid1, Sid2, Sid4 and Spg1. Taken together, these results suggest that *S. pombe* Mob1 may be involved in transducing a signal from the SPB to the septation site thereby regulating the onset of cytokinesis.

The above discussion suggests that Mob1 forms a complex with the related protein kinases, Sid2 and Dbf2, in *S. pombe* and *S. cerevisiae*, respectively. However, the function of Mob1 in *S. pombe* appears to be somewhat different from its homolog in *S. cerevisiae*. *S. cerevisiae mob1* mutants arrest in late nuclear division with an increase-in-

ploidy phenotype, while *S. pombe mob1* mutants are defective in septum formation with post-mitotic nuclear morphology. This observation suggests that Mob1 is required for mitotic exit in budding yeast, but plays a role in cytokinesis of fission yeast.

III. Objective of the Study:

Analysis of the expressed sequence tag (EST) database has identified putative homologs of *S. cerevisiae MOB1* in a varieties of organisms, as diverse as *Arabidopsis* and humans (Luca and Winey, 1998; Salimova *et al.*,2000; Hou *et al.*, 2000). At present, the function for these *MOB1* homologs in vertebrate cells remains unclear. The aims of this thesis are to test the following hypotheses: (1) There is a Mob1 homolog in *Xenopus laevis*; (2) The *Xenopus* Mob1 is localized to the spindle poles of mitotic cells; and (3) The *Xenopus* Mob1 is an integral component of centrosome. The approach chosen was to isolate a fragment of *Xenopus* DNA containing sequence conserved in Mob1 in the majority of species using polymerase chain reaction (PCR). The PCR amplified fragment was used in hybridization screening of a *Xenopus* oocyte cDNA library to isolate the full-length *Xenopus MOB1* homolog. Fusion protein between a maltose-binding protein (MBP) bacterial expression system (New England BioLabs) and the homolog was prepared. Bacterially-expressed proteins were isolated and injected into mice to raise specific antibodies against the Mob1 protein. The antibodies were used for immunofluorescence analyses of Mob1 in both *Xenopus* and human tissue culture cells.

CHAPTER TWO

Methods and Materials

I. Isolation of *Xenopus MOB1* cDNA:

I-A. Amplification of *Xenopus MOB1* Fragment:

Mr. X.C. Wu and I collaborated to identify *Xenopus laevis* cDNA sequence related to *S. cerevisiae* and a putative human *MOB1*. Two degenerate primers (sense: 5' GAC/T C/TTI AAC/T GAA/G TGG C/TTI GC 3'; antisense: 5' GTI GGA/G AAI ACA/G TCC/T TCA/G TC 3') were synthesized based on the conserved regions of peptide sequence between *S. cerevisiae* Mob1 (GenBank accession no. Z47147x72) and human Mob1 (a contig of GenBank accession numbers F11288, F11866, R19220, R13096, and R59435; Luca and Windy, 1998) was designed. A polymerase chain reaction (PCR) was performed with the degenerate primers using *Xenopus* oocyte cDNA library (a gift from Mrs. E. Eriksen and Dr. J.L. Maller) as the template (0.2 μg per reaction). A PCR procedure was modified so that the annealing reaction was decreased by 1 °C for each of the first 15 cycles (ie. from 60 °C to 45 °C, 1 min per cycle) and the following 20 cycles were kept at an annealing temperature of 54 °C. The denaturation reaction was at 94 °C (1 min) and the extension reaction at 72 °C (1 min) when the reaction products were separated. The PCR reaction produced a single 0.22 Kb, DNA fragment on an 1 % DNA agarose gel (1 g DNA gel grade agarose (GIBCO-BRL) in 100 ml TAE buffer (40 mM Tris-acetate pH 8.0, 1 mM EDTA)). The amplified product was purified by gel electroelution (Sambrook *et. al.*, 1989) and labelled with [α-^{32}P]dCTP

(ICN Pharmaceuticals) using the random primed DNA labeling kit (Boehringer Mannheim), according to the manufacturer's instruction.

I-B. Screen Library for Full-Length *Xenopus MOB1* cDNA:

To search the full-length *X. laevis MOB1* cDNA, a *X. laevis* oocyte cDNA library in *pSport 1* vector (GIBCO-BRL) was transformed into *Escherichia coli* strain DH5α competent cells (GIBCO-BRL) and streaked on Luria Bertani (LB) agar plates (1 % tryptone, 0.5 % yeast extract, 0.5 % NaCl, pH 7.2, 1.5 % bacterial agarose) containing 75 μg/ml of ampicillin (Boehringer Mannheim). Approximately 200,000 colonies were transferred to nitrocellulose membranes. The cells were lysed by submerging the membranes into 10 % SDS for 3 min. The DNA was denatured in a solution containing 0.2 N NaOH and 1.5 M NaCl for 5 min. The membranes were neutralized in solution containing 1.5 M NaCl and 0.5 M Tris, pH 7.4 for 5 min followed by washing in 2 X SSC (20 X SSC: 3 M NaCl, 0.3 M sodium citrate-$2H_2O$, pH 7.0) for 5 min. The nitrocellulose filters were allowed to air dry for 1 hr and the nucleic acids were immobilized on the filters by baking in a vacuum oven at 80 °C for 1 hr. The membranes were re-wetted in 2X SSC and then moved to wash buffer (5 X SSC, 0.5 % SDS, 1 mM EDTA) at 50 °C for 1 hr with gentle shaking. The bacterial debris was gently removed from the membrane surfaces with kimwipes. The filters were incubated in pre-hybridization solution (6 X SSC and 0.05 X BLOTTO) at 68 °C for 2.5 hr followed by hybridizing with the [α-^{32}P]dCTP labeled probe (1 X 10^6 cpm per ml of hybridization solution) at 67 °C for overnight. The membranes were subsequently washed in 2 X SSC with 0.1 % SDS at room temperature for 5 min and then in 1 X SSC with 0.1 % SDS at

67 °C for 30 min. Finally, the membranes were allowed to air dry and exposed to X-ray films (Kodak Canada Inc.). After 5 days of exposure, one positive colony was visible on the film. The clone was selected and the plasmid was isolated from the colony and subjected to PCR to confirm the result using the two degenerated primers and to check the cDNA size using both universal and reverse primers for *pSport 1* vector.

I-C. DNA Sequencing of *Xenopus MOB1* cDNA:

Manual sequencing of the putative *X. laevis MOB1* cDNA was carried out using the double-stranded dideoxy chain termination method (Sanger *et. al.*, 1977) using a T7 sequencing kit (Amersham Pharmacia Biotech) according to the manufacturer's instructions.

I-D. DNA and Amino Acid Sequence Analysis:

The DNA sequence and the amino acid sequence of the open reading frame (ORF) were analyzed by ClustalX (Thompson *et al.*, 1997; Aiyar, 2000), GeneDoc (Nicholas and Nicholas, 1997), PepTool (Wishart *et al.*, 1997; Wishart *et al.*, 2000), and GenScape, a web-based software developed by CuraGen (Uetz *et al.*, 2000).

II. Expression and Purification of *Xenopus* Mob1 Recombinant Protein:

II-A. Expression and Purification of Maltose Binding Protein (MBP) Fusion Protein:

A full-length cDNA encoding the *X. laevis MOB1* homologue was cloned into the MBP fusion vector *pMAL-C2* (New England Biolabs). MBP and MBP-fusion protein (MBP-XlMob1) were expressed in *E. coli* strain DH5α and were affinity-purified from the bacterial lysate using amylose column chromatography (New England Biolabs). The

cells were grown in 50 ml rich medium (2 % trypotone, 1 % yeast extract, 50 mM potassium phosphate, 0.2 % glycerol and 0.5 % NaCl, pH 7.4) containing 75 μg/ml ampicillin at 37 °C for overnight in a shaking incubator. At an OD_{600} of 0.6, 150 ml of fresh medium containing 75 μg/ml was added, followed by addition of Isopropyl-1-thio-β-D-galactopyranoside (IPTG, Boehringer Mannheim) to a final concentration of 0.4 mM. For induction, cells were cultured at 25 °C for overnight. The cells were then harvested at 4 °C by centrifugation at 10,000 g for 10 min in a Sorvall GSA rotor (Dupont). The cells were disrupted in 40 ml ice cold lysis buffer (30 mM NaCl, 10 mM EDTA, 10 mM EGTA, 0.25 % Tween 20, 10 mM sodium phosphate, pH 7.2) containing 1 mM PMSF by sonication at 100 % output (Kontes) five 1 min cycles with a 1 min interval of cooling on ice to prevent overheating. The cell debris was removed by centrifugation at 10,000 g for 20 min using a Sorvall HB4 rotor (Dupont) at 4 °C. The supernatant, which contained the MBP fusion protein, was diluted with the column buffer (0.5 mM NaCl, 1 mM EGTA, 1 mM sodium azide, 10 mM sodium phosphate, pH 7.2) at 1:5 dilution and then loaded onto a 3 ml amylose agarose (New England Biolabs) packed in a 1.5 x 12 cm polypropylene column (Bio-Rad Laboratories) that had been equilibrated with the column buffer. The column was washed with 30 ml column buffer and the fusion protein was eluted with 10 ml of 10 mM maltose in the column buffer. The samples were collected in 1 ml fractions. To determine the protein purity and the peak of protein samples, 2 μl sample from each tube was mixed with 5 X SDS-PAGE sample buffer and loaded onto an 10 % SDS-PAGE gel (Laemmli, 1970). The gel was stained with Coomassie brilliant blue (Bio-Rad Laboratories). Pooled elutes were dialyzed against

phosphate-buffered saline (PBS, 137 mM NaCl, 10 mM Na_2HPO_4-$12H_2O$, 2.5 mM KCl 1.4 mM KH_2PO_4) to remove the maltose used for elution. The protein concentration was determined by Bradford method (Bradford, 1976) using bovine serum albumin (BSA, Boehringer Mannheim) as the standard. Aliquots of purified recombinant protein were frozen and stored at -80 °C for further use.

II-B. Cleavage of *Xenopus* Mob1 From Its MBP Tag:

Xenopus Mob1 was separated from its MBP tag by incubating 20 μg of the MBP-XlMob1 protein in the presence or absence of 0.2 ng of Factor Xa (New England Biolabs) for 3 hr at room temperature. At the end of incubation, 5 μl of the Factor Xa reaction was taken out and added into 5 μl 5 X SDS-PAGE sample buffer. The samples were boiled for 2 min and loaded on an 12 % SDS-PAGE gel.

III. Expression and Purification of Human Mob1 Recombinant Protein:

JM109 *E. coli* strain harboring glutathione S-transferase (GST)-human Mob1 recombinant construct (*pGEX-5X1-HsMOB1*, a gift from Dr. J.B. Rattner) was inoculated into 50 ml rich medium containing 75 μg/ml ampicillin. The culture was incubated overnight at 37 °C in a shaker incubator. Next morning, 150 ml of the fresh rich medium containing 75 μg/ml ampicillin was added. The fusion protein was expressed upon 0.4 mM IPTG induction as described above in II-B-1 followed by 16 hour incubation at 25 °C.

GST-HsMob1 fusion protein was purified following the protocol described by Frangioni and Neel (1993). The bacterial cells were harvested at 4 °C by centrifugation at 10,000 g for 10 min in a Sorvall GSA rotor. The pellet was resuspended in 30 ml ice cold

MTPBS lysis buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4, 1 % Triton X-100) containing 1 mM PMSF. The cells were then sonicated for 5 X 1 min with 100% output control. The cell suspension was centrifuged for 20 min at 4 °C at 10,000 g in a Sorvall HB4 rotor. The supernatant was diluted at 1:5 dilution with MTPBS column buffer (150 mM NaCl, 16 mM Na_2HPO_4, 4 mM NaH_2PO_4) and then loaded into a glutathione agarose column (Sigma Chemical). The column was washed with 30 ml of cold MTPBS column buffer. The GST-fusion protein was eluted using 10 ml of 5 mM glutathione in 50 mM Tris-HCl, pH 8 buffer. The samples were collected in 1 ml fraction and the proteins were resolved on an 10 % SDS-PAGE gel to determine the purity. The protein concentration was determined by Bradford method using BSA as the standard. Aliquots of purified recombinant protein were kept frozen at -80 °C for further use.

The *pGEX-2T* vector encoding the control GST protein was transformed into *E. coli* strain DH5α. The protein was expressed and purified as the GST-HMob1 described above.

IV. Production of Polyclonal Antibodies:

The production of polyclonal antibodies was carried out by immunizing Swiss Webster mice with the affinity-purified *Xenopus* Mob1-fusion protein followed by the protocol described by Harlow and Lane (1988). For the immunization, the mice received 3 boosts of 50 μg protein mixed with Freund's complete adjuvent (Sigma Chemical). The interval between immunizations was 2 weeks. To obtain the antibodies, the blood was incubated at 4 °C for overnight and the serum was separated by centrifugation at 3,000 rpm at 4 °C for 10 min. The antibodies was stored at 4 °C with 0.2 % sodium azide.

V. Production of Monoclonal Antibodies:

The production of monoclonal antibodies was carried out in collaboration work with Ms. Warren. Female Swiss Webster mice were immunized with the affinity-purified *Xenopus* Mob1-fusion protein. For the immunization, 50 μg protein mixed with Freund's complete adjuvent (Sigma Chemical) was given 3 times. The interval between immunizations was 2 weeks. After the positive test bleed, the mouse received an additional boost of 50 μg MBP-XlMob1 fusion protein. The mouse was sacrificed and the spleen was removed. The splenocytes were fused with FOX NY myeloma cells (a gift from Dr. B. Burke) in 24 well dishes containing the macrophage feeder cells. The hybridoma cells were grown in HAT media (GIBCO-BRL) at 37 ° C with 5 % CO_2 (IR Autoflow CO_2 water-jacketed incubator; Nuaire) and screened by 3 cycles of indirect immunofluorescence using A6 (*Xenopus* adult kidney) tissue culture cells (see procedures described below) followed by growing the hybridoma cells in soft agar (Harlow and Lane, 1988). The positive hybridoma cell line (3-24-3) was expanded in DMEM/NCTC media (9.0% DMEM, 10% NCTC, 15% fetal bovin serum (FBS), 10 mM HEPES, 0.1% ml β-mercaptoethanol, 4 mM L-glutamine, 75 μg/ml Penicillin and Streptomycin). The cells were grown to high cell densities, in order to optimize the antibody production. The culture supernatant was collected by centrifuging the culture media with Beckman TJ-6 centrifuge (Beckman) at 3,000 rpm and stored at 4 °C. The isotype of 3-24-3 monoclonal antibody was identified by IsoStrip mouse monoclonal antibody isotyping kit (Roche Molecular Biochemicals) according to the manufacturer's instructions.

VI. Characterization of Anti-Mob1 Antibodies by *In Vitro* Translation Assay Followed by Immunoprecipitation:

VI-A. Isolation of *pSport 1-Xenopus MOB1* Plasmid:

The *pSport 1-Xenopus MOB1* plasmid was isolated by the mini-scale preparation method described in Ausubel *et. al.* (1995). *E. coli* strain DH5α harboring *pSport 1-Xenopus MOB1* plasmid was grown in 1.5 ml LB mediun containing 75 μg/ml of ampicillin and incubated overnight at 37 °C in a shaker incubator. The 1.5 ml of bacterial culture cells were harvested by centrifuging with an Eppendorf 5413C (Eppendorf) at 16,000 g for 20 sec. The supernatant was discarded and the cell pellet was resuspended by vortex in 200 μl SET buffer (20 % sucrose, 50 mM Tris-HCl pH 7.6, 50 mM EDTA) and 10 μl DNase-free RNase I (10 mg/ml DNase-free bovine pancreas ribonuclease I (Amersham Pharmacia Biotech) in 0.1 M Na acetate, pH 4.8, 0.3 mM EDTA, and boiled at 100 °C for 10 min). The cells were broken by addition of 400 μl NaOH/SDS lysis buffer (1 % SDS, 0.2 N NaOH) at 4 °C for 5 min on ice. The plasmid DNA was denatured by addition of 300 μl 3 M potassium acetate buffer, pH 5.2 at 4 °C for 10 min. The lysate was centrifuged at 16,000 g for 10 min and 750 μl of the supernatant was removed into a clean tube. To precipitate the plasmid, an equal volume of isopropanol was added to the 750 μl supernatant, and the solution was mixed and centrifuged at 16,000 g for 10 min. The supernatant was gently decanted and 1 ml of ice-cold 70 % ethanol was added to the pellet to wash the plasmids. The reaction was centrifuged again at 16,000 g for 5 min. The supernatant was gently decanted and the plasmids were speed dried for 20 min using a Speed Vac Concentrator (Savant). The isolated plasmids were

resuspended in 100 µl TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA) and 10 µl DNase-free RNase I. After incubating the resuspended plasmids at 37 °C for 30 min, 5 volume of 1:1 (v/v) phenol/chloroform was then added and mixed by vortex. The solution was centrifuged at 16,000 g for 5 min followed by removal of 100 µl supernatant into a clean tube. The supernatant was vigorously mixed with equal volume of isopropanol and centrifuged at 16,000 g for 10 min. The plasmid pellet was washed in 1 ml ice-cold 70% ethanol and centrifuged at 16,000 g for 5 min. Then, the plasmid pellet was speed dried for 20 min, dissolved in 20 µl TE buffer, and kept at -20 °C for further use. The concentration of DNA was determine by measuring the optical density wavelength at 260 nm (Ausubel *et. al.*, 1995).

VI-B. *In Vitro* Transcription and Translation of *Xenopus MOB1*:

Xenopus MOB1 was *in vitro* translated by TNT coupled reticulocyte lysate system (Promega). In the reaction, 12.5 µl TNT reticulocyte lysate, 0.5 µl RNA guard, 1.0 µl TNT reaction buffer, 0.5 µl of 1 mM amino acid mixture (minus methioine), 1 µg *pSport 1-Xenopus MOB1* plasmid, 7 µl nuclease-free water, 20 µCi of [^{35}S] methionine, and 0.5 µl TNT T7 RNA polymerase were added. The reaction was then incubated at 30 ° C for 90 min. At the end of the incubation, sample was kept frozen at -80 ° C until use. A sample of 2 µl was solubilized in 5 X SDS-PAGE sample buffer and analyzed by electrophoresis on an 12 % polyacrylamide SDS-PAGE gel to determined if *Xenopus* MOB1 had been synthesized. The gel was stained with Coomassie Blue staining solution (50 % v/v methanol, 10 % acetic acid, 2.5 g Coomassie Brillian Blue R-250, 40 % v/v water) for 5 min and destained with destaing buffer (87.5 % water, 5 % methanol, 7.5 %

acetic acid) for 10 min followed by enhancing the radioactive signal in 200 ml of 1 M salicylic acid for 40 min (Chamberlain, 1979). The gel was then dried and exposed to X-ray film (Fuji Photo Film) for overnight at -80 ° C with intensifying screens. The film was developed on the following day.

VI-C. Immunoprecipitation of *In Vitro* Translated *Xenopus* Mob1 Protein:

Immunoprecipitation was carried out in two stages. In the first stage, 30 µl of mouse polyclonal serum or 200 µl of hybridoma culture supernatant was diluted in 500 µl PBS and coupled to 20 µl of 1:1 slurry of protein G-Sepharose (Sigma Chemical) by end-to-end rotation at 4 °C for 2 hr. The Sepharose was washed 6 times with 1 ml PBS each. In the second step, 5 µl of *in vitro* translated XlMob1 was diluted in 500 µl PBS and incubated with antibody-conjugated Sepharose by end-to-end rotation at 4 °C for 2 hr. The immunoprecipitates were washed 6 times with 1 ml PBS each, dissolved in 20 µl of 5 X SDS-PAGE sample buffer, and the analyzed on an 12 % SDS-PAGE.

VII. Cell Line and Cell Culture:

Xenopus A6, adult kidney, cells (a gift from Dr. B. Burke) were grown in 60 % concentration of Dulbeccos modified Eagle medium (DMEM/60, 500 ml contains 3.0 g DMEM, 1.04 g HEPES, pH 7.5, 50 ml FBS, 4 mM L-glutamine, 75 µg/ml Penicillin and Streptomycin) (GIBCO-BRL) at room temperature.

A431, human epidermoid carcinoma, was obtained from the American Type Culture Collection and cultured in RPMI-1640 (GIBCO-BRL) supplemented with 10 % FBS, 4 mM L-glutamine (GIBCO-BRL), and 1.5 g/L $NaHCO_3$ at 37 ° C with 5 % CO_2 (IR Autoflow CO_2 water-jacketed incubator; Nuaire).

VIII. Immunoblotting Analysis:

VIII-A. Cell Extacts:

Both A6 and A431 were grown in 100 mm tissue culture dishes (VWR Canlab) until 80 - 90 % confluency. The A6 cells and A431 cells were washed with 3 changes of 10 ml 0.7 X PBS or 1 X PBS, respectively. The cells were lysed by adding 250 µl 5 X SDS-PAGE sample buffer into each culture dish. The cell lysate was then sonicated for 3 x 30 sec with 40 % output control to disrupt DNAs. The crude lysates were then centrifuged at 16,000 g for 10 min and the supernatant was removed for immunoblotting analysis.

VIII-B. Polyclonal Antibodies Blocking:

The mouse polyclonal antibodies were absorbed by MBP or MBP-XlMob1 protein before useage for immunoblotting. To block the antibodies, 500 µg of MBP or MBP-XlMob1 was diluted in 500 µl amylose column buffer and conjugated to 200 µl of 1:1 amylose slurry at 4 ° C for 2 hr by end-to-end rotation. The amylose beads were washed with 5 charges of 1 ml PBS. The antibodies were diluted at 1:200 in PBS containing 5 % non-fat milk to make a final volume of 10 ml. The diluted antibodies were then incubated with the protein-conjugated amylose beads by end-to-end rotation at 4 ° C for 4 hr. The supernatant was removed and stored at 4 ° C for further use.

VIII-C. Immunoblotting Analysis:

To carry out immunoblotting analysis, purified proteins and cell extracts were resolved on 12 % SDS-PAGE. The electrophoresis was performed on a mini-gel apparatus (Idea-Scientific) with a current of 15 mA/gel in a SDS-PAGE running buffer (1

L of 8 X running buffer contains 24 g Tris, 115.2 g glycine, and 8 g SDS). Proteins were then transferred onto a 0.45 μm PVDF-Plus transfer membrane (MSI) using a semi-dry Novablot Electrophoretic Transfer Kit (Amersham Pharmacia Biotech) in a transfer buffer (39 mM Tris-HCl, 48 mM glycine, 20 % methanol, 0.0375 % SDS) with a current of 0.8 mA/cm^2 of gel area for 50 min.

After proteins were transferred, membranes were incubated in blocking solution (5 % skim milk in TBS) for 1 hr at room temperature. The membranes were then incubated with 10 ml of pre-absorbed polyclonal antibodies at 1:200 dilution or 10 ml of full-strength hybridoma culture supernatant at 4 ° C for overnight. The membranes were washed with 3 changes of TBS (10 min/charge) followed by incubation with the secondary antibody. For secondary antibody, 1:3000 dilution of goat anti-mouse conjugated with horse radish peroxidase (Bio-Rad Laboratories) was incubated with the membranes at room temeprature for 1.5 hr. After washing the membranes with 3 changes of TBS (10 min/charge), enhanced chemiluminescent substrate system (ECL, Amersham Pharmacia Biotech Inc.) was used to detect the reaction of the antibody with the proteins. The image was exposed to X-ray film (Fuji Photo Film).

IX. Indirect Immunofluorescence Studies:

IX-A. Monoclonal Antibody Blocking:

The monoclonal antibodies, designated 3-24-3, were pre-absorbed by MBP or MBP-XMOB1 protein as a control for indirect immunofluorescence studies. To block the antibodies, 500 μg of MBP or MBP-XMob1 was diluted in 500 μl amylose column buffer and conjugated 200 μl of 1:1 amylose slurry at 4 ° C for 2 hr by end-to-end

rotation. The protein-conjugated amylose beads were washed with 5 changes of 1 ml PBS and then incubated with 1 ml of hybridoma culture supernatant by end-to-end rotation at 4 ° C for 4 hr. The supernatant was removed and stored at 4 ° C for further use in indirect immunofluorescence studies.

IX-B: Indirect Immunofluorescence Studies of *Xenopus* A6 Cells:

Xenopus A6 cells were cultured on 10 mm round coverslips (Bellco Glass, Inc.) treated with both poly-L-lysine (Sigma Chemical) and collagen (GIBCO-BRL) in 4-well plates (Nunc) until 90-95 % confluency. Coverslips were briefly washed once in 1 ml 0.7 X PBS and cells were extracted in 0.7 X PBS containing 0.1 % Triton X-100 (Sigma Chemical) for 5 min at room temperature. Cells were then washed once in 1 ml 0.7 X PBS followed by fixing in 0.7 X PBS containing 3.7 % paraformaldehyde (BDH Inc.) for 30 min at room temperature. After washing the coverslips with 4 changes of 1-2 ml PBS, non-specific binding sites were blocked with 3 % (w/v) BSA in 0.7 X PBS for 30 min. The coverslips were incubated overnight at 4 °C with full strength 3-24-3 hybridoma culture supernatant. Next day, the coverslips were washed in 3 changes of 1-2 ml 0.7 X PBS (5 min each) and blocked in 3% BSA blocking solution for 30 min. The cells were subsequently incubated with anti-centrosome human auto-immune antibody (M-4491, a gift from Dr. J.B. Rattner) at 1:1000 dilution in blocking solution for 1 hr at room temperature. Cells were then washed in 3 changes of 1-2 ml 0.7 X PBS (5 min each) and counter stained with Cy2-conjugated goat anti-human IgG secondary antibodies (Jackson ImmunoResearch Laboratories Inc.) at 1:1000 dilution in blocking solution for 45 min followed by washing in 3 changes of 1-2 ml 0.7 X PBS (5 min each). The cells were

incubated in blocking solution for 30 min, and counter stained with Texas-Ted conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.) or Alexa Fluor 594 rabbit anti-mouse IgG conjugate (Molecular Probes Inc.) at 1:1000 dilution in blocking solution for 45 min . Subsequently, the coverslips were washed in 3 changes of 1-2 ml 0.7 X PBS (5 min each). Nuclear DNA was visualize by 4', 6-diamidino-2-phenylindole (DAPI) stain and coverslips were mounted on slides using mounting media containing 10 mM NaHCO3, 1 mg/ml phenylenediamine, and 70% glycerol. The coverslips were sealed by clear nail polish. Cells were observed by fluorescence microscopy.

IX-C: Indirect Immunofluorescence Studies of Human A431 Cells:

To study the localization of Mob1 in mammalian cells, the A431 cells were grown on 10 mm round coverslips coated with poly-L-lysine in 4-well plates. Processing of A431 cells for indirect immunofluorescence studies was done similarly as the procedures described above for *Xenopus* A6 cells. Coverslips having A431 cells grown at 50%-60% confluency were washed once with cytoskeleton (CSK) buffer (10 mM Pipes pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$) (Fey *et. al.*, 1986; Todorov *et. al.*, 1995) and then permeabilized with CSK buffer containing 0.15% Triton X-100 at room temperature for 5 min. After a brief rinse with CSK buffer, cells were fixed on coverslips with CSK buffer containing 3.7% paraformaldehyde. Coverslips were then washed with 3 changes of 1-2 ml PBS and blocked with blocking buffer (3% bovine albumin fraction V (Sigma Chemical) dissolved in PBS). Cells were incubated with 3-24-3 culture supernatant at 4 °C for overnight and then washed with 3 changes of PBS (5

min each). The next morning, cell were co-stained with either M-4491 at 1:5000 or rabbit anti-γ-tubulin antibodies (Sigma Chemical) at 1:2500 for 1 hr at room temperature. Coverslips were rinsed 3 times with PBS and then blocked for 30 min at room temperature. Cells were then incubated in secondary antibodies: Cy2 conjugated goat anti-human IgG at 1:1000 dilution for M-4491 staining or FITC goat anti-rabbit at 1:250 dilution for anti-γ-tubulin antibodies staining. Followed by blocking cells in the blocking buffer for 30 min, cells were further incubated with Alexa Fluor 594 rabbit anti-mouse IgG conjugate or Alexa Fluor 594 goat anti-mouse IgM conjugate (Molecular Probes Inc.) at 1:1000 or 1:2500, respectively. Finally, coverslips were washed with 3 changes of PBS (5 min each), incubated with DAPI for 5 min at room temperature, washed with PBS (5 min each) twice, and then mounted on slides and sealed with clear nail polish. All the dilutions of antibodies were done by dilution the antibodies in blocking buffer.

IX-D: Nocodazole Treatement of A431 Cells:

A431 cells were blocked at mitosis by growing in RPMI-1640 medium containing 20 μM (5,2-thienylcarboxyl-1H-benzimidazole-2-YL)-carbamate (nocodazole) (Sigma Chemical) for 4 hr (Jordan and Wilson, 1999). Immediately after removal of the mitotic block, the cells were processed for indirect immunofluorescence studies as described above. Mouse anti-α-tubulin antibodies (a gift from Dr. J.B. Rattner) was used at 1:1000 dilution as a marker for the action of nocodazole.

IX-E: Salt Treatement of A431 Cells:

A431 cells were grown to 50-60% confluency and permeabilized in CSK buffer containing 0.15% Triton X-100 and various NaCl concentrations (ie. 0.1 M, 0.25 M, 0.3

M, or 0.5 M). The cells were fixed and processed for indirect immunofluorescence as described above.

X. Image Processing:

The indirect immunofluorescence results were viewed using a Leitz Aristoplan microscope equipped with a Leica DX camera. The results were photographed in appropriate channels with a X100 objective using Kodak Elite Chrome 400 film (Kodak Canada Inc.). The color slides and immunoblot results were scanned by HP Photo Smart Photo Scanner (Hewlett-Packard Co.) using Adobe Photoshop 5.0 software (Adobe Systems Incorporated). All of the images in the figures in Chapter Three were obtained by scanning slides and blots using the same conditions and organizing the scanned images in WordPerfect 8.0 software (Corel Co.). Final figures were printed with a Epson Stylus Photo 700 printer (Epson America Inc.).

CHAPTER THREE

Results

I. Isolation of cDNA Encoding *Xenopus* Mob1 Homolog:

I-A. Library Screening Using Degenerate Oligonucleotides:

A *Xenopus* homolog of the *S. cerevisiae MOB1* was isolated by screening a *Xenopus* oocyte cDNA library. First, two degenerate oligonucleotides were designed (Figure 1a) based on the amino acid sequences conserved between *S. cerevisiae* and a human contig from the EST sequence database (Luca and Winey, 1998). A PCR reaction was performed with these primers using *Xenopus* oocyte cDNA library as the template. The reaction produced a single DNA fragment of approximately 0.22 kb, of the DNA sequence between the primer set used (Figure 1b). The fragment was then radioactively labelled by random labelling method and used as the probe to screen 2 X 10^5 colonies plated from the same oocyte library by DNA hybridization screening technique. As a result, a single positive colony was obtained. The plasmid was then isolated and the cDNA was confirmed by PCR reaction. The clone was sequenced (Figure 2) using the double-stranded dideoxy chain termination method according to Sanger *et al.* (1977). Of the 2 X 10^5 clones screened, only one cDNA of 1.35 kb was identified, indicating that the cDNA of this protein was not abundant in the oocyte library.

I-B. Analysis of *Xenopus laevis MOB1* cDNA Sequence:

The putative full-length *Xenopus MOB1* cDNA is 1,354 base pairs (bp) long and contains an open reading frame (ORF), which encodes a protein with a predicted

Figure 1. Polymerase chain reaction of a *Xenopus laevis MOB1* cDNA using two degenerate oligonucleotids.

(A) The degenerate oligonucleotides designed based on conserved regions of the *S. cerevisae* Mob1 (GenBank accession No. P40484) and the human Mob1-related contig (Luca and Winey, 1998). (B) PCR amplification of a *Xenopus* oocyte cDNA library using the degenerate primers resulted in identification of a DNA fragment with a molecular size of approximately 220 bp.

A.

Sense: 5' GAC/T C/TTI AAC/T GAA/G TGG C/TTI GC 3'

Antisense: 5' GTI GGA/G AAI ACA/G TCC/T TCA/G TC 3'

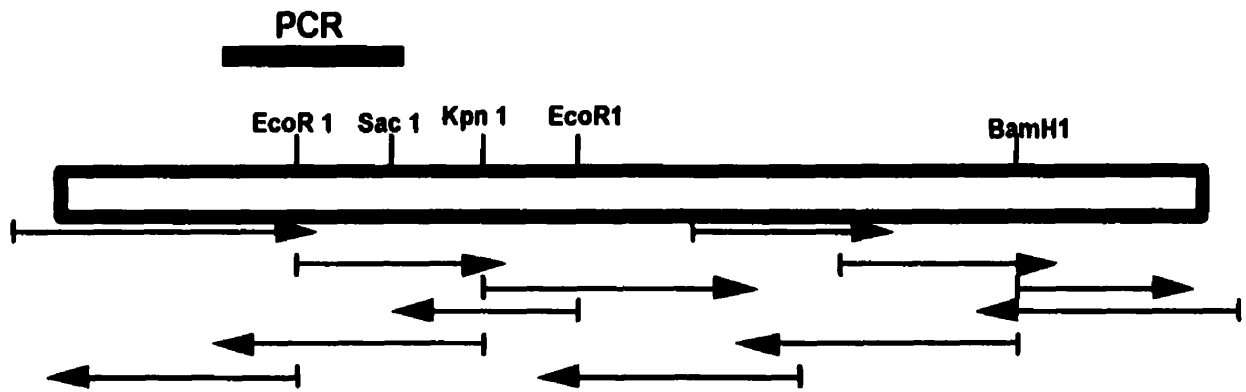
B.

Figure 2. Schematic of *Xenopus MOB1* cDNA sequencing strategy.

The *Xenopus laevis MOB1 (XlMOB1)* cDNA isolated from the oocyte cDNA library was sequenced from both ends. The arrows represent the primers used for the manual sequencing. The bar (▬) indicates the relative position of the 220 bp PCR amplified fragment.

DNA SEQUENCING STRATEGY FOR *XIMOB1* cDNA

DNA sequece : 1354bp



molecular mass of 17.11 KDa, starting with an ATG at position 28 and ending with a stop codon at position 474 (Figure 3). The theoretical pI is 8.78, as predicted by the program of Wilkins *et al.* (1998). DNA sequence analysis revealed that the 3' untranslated region (3' UTR) is 877 bp long and AT rich with a polyadenylation signal AATAAA at position 1315 and a potential consensus sequence (TTTTAT) for cytoplasmic polyadenylation of mRNA during *Xenopus* oocyte maturation at position 953 (McGrew *et. al.*, 1989; Roghi *et. al.*, 1998).

I-C. Analysis of *Xenopus laevis* Mob1 Protein Sequence:

Computer searches of protein sequence databases using the BLAST program (Altschul *et al*, 1990) revealed that cDNA that was isolated was very similar to Mob1 protein conserved from *Arabidopsis* to human. The identification of Mob1 homologs in a variety of organisms suggests that these proteins may form a conserved protein family. Alignment results of *Xenopus* Mob1 amino acid sequence with some of the Mob1 homologs showed that the *Xenopus* Mob1 ORF has 75% and 40% identities with the human (J.B. Rattner unpublished result) and *S. cerevisiae* (GenBank accession no. P40484) Mob1, respectively. The *Xenopus* Mob1 also shows 41% identities with a putative Mob1 homolog in *S. pombe* (GenBank accession no. CAA22288). Complete alignment of Mob1 proteins revealed that the central and C terminal regions of Mob1 proteins are the most highly conserved (Figure 4).

Further analysis identified a cullin-homology region (Yu *et al.*, 1998) in Mob1 proteins. In *Xenopus* Mob1, for example, the cullin homology region is located at the C-terminus from amino acid residue 125 to amino acid residue 149. The cullin homolog

Figure 3. Amino acid sequence of *Xenopus* Mob1.

Nucleotide sequence of *Xenopus MOB1* cDNA and deduced amino acid sequence of the Mob1 open reading frame. Sequences underlined in the 3' UTR are a polyadenylation signal (-AATAAA-) and a potential consensus sequence for cytoplasmic polyadenylation during oocyte maturation (-TTTTAT-)

```
      10        20        30        40        50        60        70        80        90       100
CTGTTTGCAAAGCTGCTTTCCTTTATTATGAGCATCAGGCGCAGTGGCTCCTACACCGTGCAGAAGAAATCGAAAGGAAAGCCGAATGGGAAGAAACCAG
                          M  S  I  R  R  S  G  S  Y  T  V  Q  K  K  S  K  G  K  P  N  G  K  K  P

      110       120       130       140       150       160       170       180       190       200
CGTCGGAGGAAAAGAGTTGTACCTGGAGCCAGAATACCACCCGAGTCCGAGTCACTGACGTGGAATTCAAGCAGCTGGTGACCTTGCCCCAGGAAATCGA
A  S  E  E  K  S  C  T  W  S  Q  N  T  T  R  V  R  V  T  D  V  E  F  K  Q  L  V  T  L  P  Q  E  I  D

      210       220       230       240       250       260       270       280       290       300
CCTGAATGAGTGGCTGGCTAGTAACATTACGACTTTCTTCAACCACATTAACCTTCAGTACAGCACCATCTCCGAATTCTGCACGGGGGAAACCTGCCAG
  L  N  E  W  L  A  S  N  I  T  T  F  F  N  H  I  N  L  Q  Y  S  T  I  S  E  F  C  T  G  E  T  C  Q

      310       320       330       340       350       360       370       380       390       400
ACTATGGCCGCCTGCAATACACAATACTACTGGTATGATGAGCGGGGTAAGAAGGTAAAATGCACAGCCCCACAGTACATTGATTTCGTCATGAGCTCTA
 T  M  A  A  C  N  T  Q  Y  Y  W  Y  D  E  R  G  K  K  V  K  C  T  A  P  Q  Y  I  D  F  V  M  S  S

      410       420       430       440       450       460       470       480       490       500
TACAGAAGCTGGTGACGGATGAGGATGTCTTCCCCACCAAATACGGGAATTCCCTAGCTCCTTCGAGTCGTTGGTGAAGAAGATCTGCCGGTACCTGTTT
I  Q  K  L  V  T  D  E  D  V  F  P  T  K  Y  G  N  S  L  A  P  S  S  R  W  *

CACGTTGTGGCTCACATCTACTGGGCGCATTTCAAGGAAATCACGGTGCTGGAGCTGCACGGACACTTAAACACTCTCTTAATCCACTTCCTCTTATTCT

TGCGGGAATTCAGCCTGCTGGACCCCAAGGAGACCTCCGTCCTGGACGACCTGTCGGAAATCCTATTCTCAGAGGAAAACAGGGAGGCGGTGGGGGCTAC

AGGGGAGGCACAGAACCACGTGAAGGAGAGATGAGCCCCCCCCCCCCTTAATCTCCTAATTCCTGTCCTACCACTTTTCCTTTCCAGCTTACCCCACACC

CGACCGAGAGCTGACCCAGCACAGGGGAGAAGGGGGTTCAGGGACAGCTTTTGGGGTGTAGGAAGGGAGGACTGCCTGTTATACCCCCCGCCTGAGGGGG

AGGAAACCTCGCTCTTCTCTTTAGCAGAAACTTGCACCGAGCATCACCTTAATTTTATTTTGTGTCTTGCGTTTGTCTCTTTTTTTCTTTCCTCTCCTTT

TCCTTCCTTTATTCATCCCCTTCTTCTCATCATGGGCGGCAGGTTCATTCTCTTATTCCGAGGAAACATTTGCATCTTCTCTTTTATTTTGCGGTGGGTT

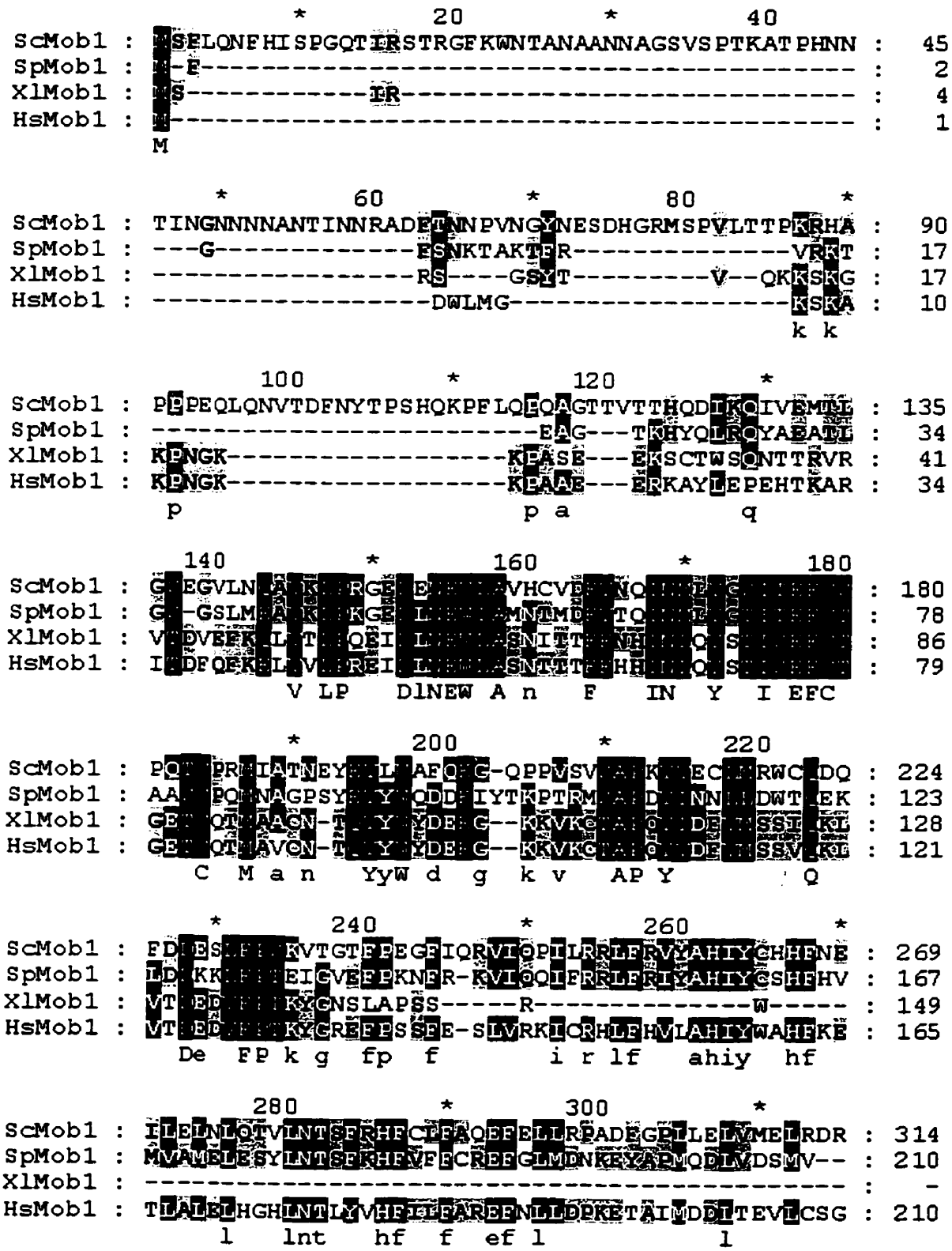
TTCCAAGAAATGTTTGAGGCTCCGGAGTCGGATGATTGGGCTGGAGAGATCGAGGCTCCGGAATTGGATCCGTCCTCCCGCCCCATCCTGGGCAACAACT

TCTCTCCCCGCTTCACGGGATCGGGGGAAAGCCCCGGATGCTCGCCCCTCATTGGGGTGCAAAAATACTGTCGGCTTCTTTTAGTTTATCTGTTAAAATA

ATGATAATATTAATAATAAAAAACAGATTCTATTAAAAAAAAAAAAAAAAAAAA
```

Figure 4. Protein sequences alignment of Mob1-related proteins.

The open reading frames of Mob1-related proteins identified from various organisms were aligned. *Xenopus laevis* Mob1 (XlMob1) sequence was compared with the Mob1 protein isolated from *Saccharomyces cerevisiae* (ScMob1, GenBank accession No. P40484), *Saccharomyces pombe* (SpMob1, Genbank accession No. CAA22288), and human (HsMob1, J.B. Rattner unpublished data) at the amino acid level. The alignment was generated with the Clustal method by the Clustal X program (Thompson *et al.*, 1997; Aiyar, 2000) and edited by GenDoc software (Nicholas and Nicholas, 1997). Residues identical to the consensus sequence were shaded in 4 gray levels as: (1)100% identity were shaded in black; (2) 80% identity were shaded in dark gray; (3) 60% identity were shaded in light gray; and (4) lower than 60% identity were coloured in white. The consensus line was located below the sequence lines and was not shaded. In the consensus line, the 100% conserved residues were printed as upper case , whereas the residues with less than 100% identity were printed as lower case.


ScMob1 : MSELQNFHISPGQTIRSTRGFKWNTANAANNAGSVSPTKATPHNN : 45
SpMob1 : M-F---------- : 2
XlMob1 : MS----------IR---------- : 4
HsMob1 : M---------- : 1
ScMob1 : TINGNNNNANTINNRADETNNPVNGYNESDHGRMSPVLTTPKRHA : 90
SpMob1 : ---G----------FSNKTAKTFR----------VRKT : 17
XlMob1 : ----------RS-----GSYT---------V--QKKSKG : 17
HsMob1 : ----------DWLMG----------KSKA : 10
ScMob1 : PEPEQLQNVTDFNYTPSHQKPFLQLQAGTTVTTHQDIKQIVEMTL : 135
SpMob1 : ----------EAG---TKHYQLRQYAEATL : 34
XlMob1 : KPNGK----------KPASE---EKSCTWSQNTTRVR : 41
HsMob1 : KPNGK----------KPAAE---ERKAYLEPEHTKAR : 34
ScMob1 : : 180
SpMob1 : : 78
XlMob1 : : 86
HsMob1 : : 79
ScMob1 : : 224
SpMob1 : : 123
XlMob1 : : 128
HsMob1 : : 121
ScMob1 : : 269
SpMob1 : : 167
XlMob1 : : 149
HsMob1 : : 165
ScMob1 : : 314
SpMob1 : : 210
XlMob1 : : -
HsMob1 : : 210

```
          320         *        340
ScMob1 : -------------------------- :   -
SpMob1 : -------------------------- :   -
XlMob1 : -------------------------- :   -
HsMob1 : AGGVHSGGSGDGAGSGGPGAQNHVKER : 237
```

domain of XlMob1 shares about 35% identity, and 59% to 77% similarity with the same domain identified from other organisms listed in Figure 5a. Phylogenetic analysis revealed that the cullin homolog region of XlMob1 is most closely related to the *S. cerevisiae* Cdc53 (Figure 5b). A number of protein kinase recognition sequences were also identified, including three sites for protein kinase C (Woodget *et al.*, 1986) in the N- and C-termini of the molecule, one site for cGMP-dependent protein kinase, two sites for cAMP-dependent protein kinase (Glass and Smith, 1983), one site for multifunctional calmodulin-dependent kinase (Pearson *et al.*, 1985), two sites for myosin 1 heavy chain kinase (Brzeska *et al.*, 1990), one site for casein kinase I (Kemp and Pearson, 1990), and three sites for casein kinase II (Kuenzel *et al.*, 1987).

II. Preparation and Characterization of Mouse Anti-XlMob1 Polyclonal Antibodies and Mouse Anti-XlMob1 Monoclonal Antibodies:

The main focus of this project then shifted to the characterization of the Mob1 protein. Attempts were made to characterize the size of the protein in cell extracts, its subcellular localization within cells, and its presence in organisms other than *Xenopus*.

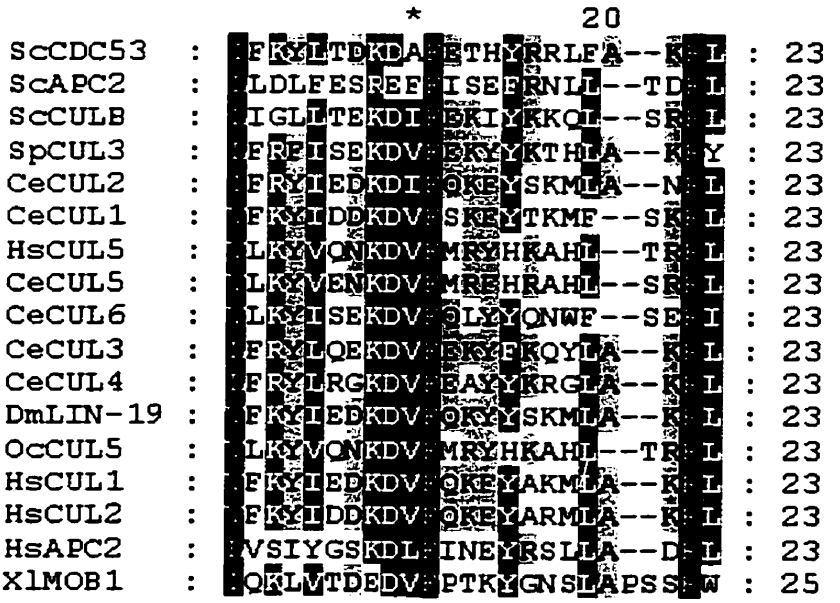
II-A. Expression and Purification of MBP-XlMob1 Recombinant Protein:

For the purpose of expression, purification and characterization of Mob1, a maltose-binding protein (MBP) construct was synthesized (Wu, unpublished result). The full-length cDNA of *Xenopus MOB1* was inserted in-frame into pMAL-c2 vector at the C-terminus of the MBP gene (Figure 6a). The MBP encoded by the pMAL-c2 vector and the MBP-XlMob1 recombinant construct were expressed in *E. coli* strain DH5α. Both MBP and the fusion protein were expressed in the soluble fraction of the cell lysates. The

Figure 5. Sequence comparison of the cullin homolog region.

(A) Alignment of the Cullin homolog domain from Cullin-related proteins identified in *S. cerevisiae* (Sc), *S. pombe* (Sp), *Caenorhabditis elegans* (Ce), *Drosophila melanogaster* (Dm), *X. laevis* (Xl), rabbit (Os), and human (Hs). The alignment was produced with the Clustal method by the program Clustal X (Thompson *et al.*, 1997; Aiyar, 2000) and edited by GeneDoc software (Nicholas and Nicholas, 1997). Residues identical to the consensus were shaded in 4 gray levels as: (1)100% identity were shaded in black; (2) 80% identity were shaded in dark gray; (3) 60% identity were shaded in light gray; and (4) lower than 60% identity were coloured in white. (B) Phylogenetic tree of cullin homolog domains. The tree was generated with neighbor-joining distance method using DAMBE software (Xia, 2000).

A.


* 20
ScCDC53 : FKYLTDKDA ETHYRRLFA--K L : 23
ScAPC2 : LDLFESREF ISEPRNLL--TD L : 23
ScCULB : IGLLTEKDI EKIYKKQL--SR L : 23
SpCUL3 : FRFISEKDV EKYYKTHLA--K Y : 23
CeCUL2 : FRYIEDKDI QKEYSKMLA--N L : 23
CeCUL1 : FKYIDDKDV SKEYTKMF--SK L : 23
HsCUL5 : LKYVQNKDV MRYHKAHL--TR L : 23
CeCUL5 : LKYVENKDV MREHRAHL--SR L : 23
CeCUL6 : LKYISEKDV QLYYQNWF--SE I : 23
CeCUL3 : FRYLQEKDV EKYFKQYLA--K L : 23
CeCUL4 : FRYLRGKDV EAYYKRGLA--K L : 23
DmLIN-19 : FKYIEDKDV QKYYSKMLA--K L : 23
OcCUL5 : LKYVQNKDV MRYHKAHL--TR L : 23
HsCUL1 : FKYIEDKDV QKEYAKMLA--K L : 23
HsCUL2 : FKYIDDKDV QKEYARMLA--K L : 23
HsAPC2 : VSIYGSKDL INEYRSLLA--D L : 23
XlMOB1 : QKLVTDEDV PTKYGNSLAPSS W : 25

B.

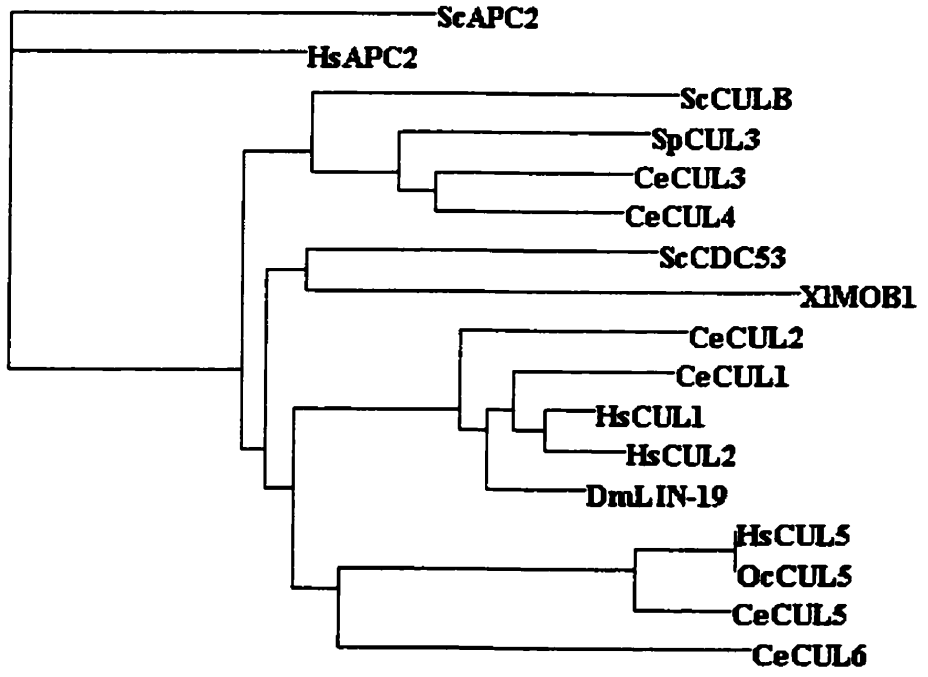

ScAPC2
HsAPC2
ScCULB
SpCUL3
CeCUL3
CeCUL4
ScCDC53
XlMOB1
CeCUL2
CeCUL1
HsCUL1
HsCUL2
DmLIN-19
HsCUL5
OcCUL5
CeCUL5
CeCUL6

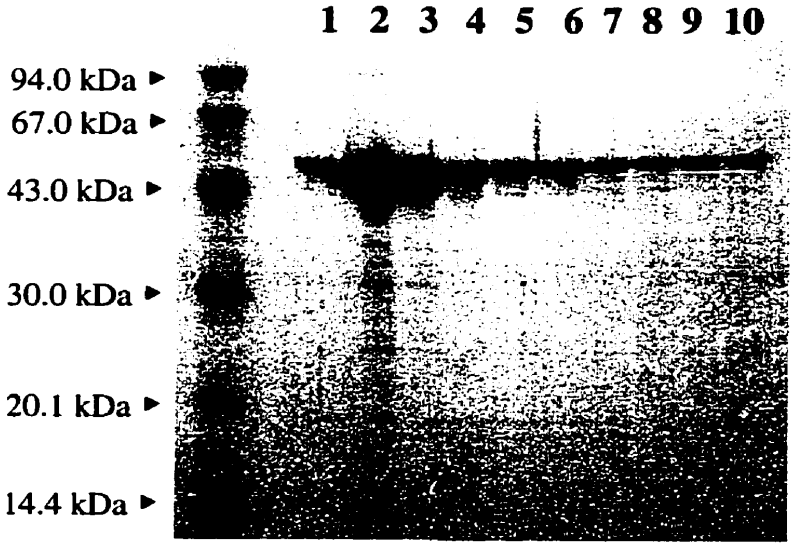
Figure 6. Schematic representation of maltose-binding protein (MBP)-*Xenopus* Mob1 recombinant construct and affinity purification of the bacterially-expressed MBP-XlMob1 fusion protein.

(A) Full-length *Xenopus MOB1* was inserted into the C-terminus of *MBP* tag system. (B) The bacterially-expressed MBP protein was eluted from an amylose column, and the fractions were analyzed on an 12% SDS-PAGE gel stained with Coomassie blue. The purified MBP protein came out primarily in fractions 2 and 3, and exhibited a molecular mass of approximately 45 kDa. (C) Similarly, the bacterially-expressed MBP-XlMob1 fusion protein was eluted from amylose column, and the fractions were analyzed on an 12% SDS-PAGE gel stained with Coomassie blue. The purified recombinant protein came out primarily from fractions 1 to 4, and exhibited a molecular mass of approximately 62 kDa.

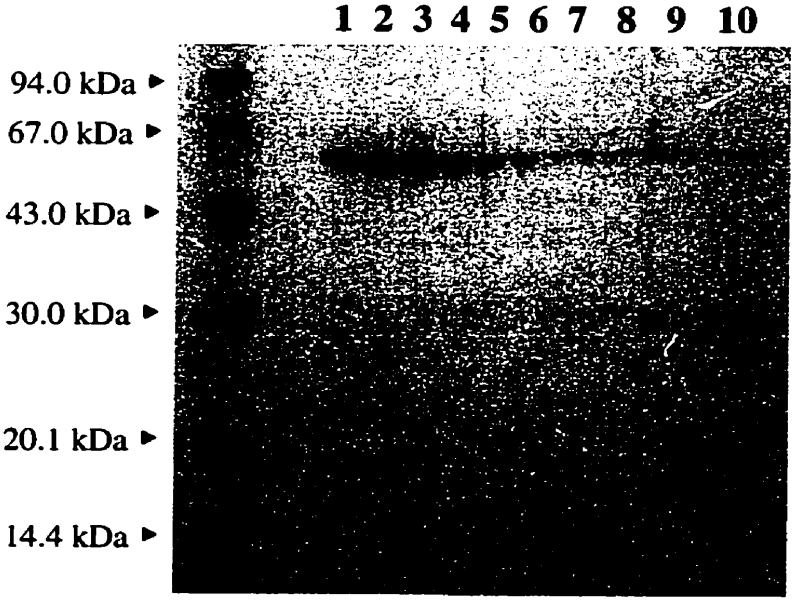
A.


NH2
MBP
XlMob1
COOH

B.


1 2 3 4 5 6 7 8 9 10
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa

C.


1 2 3 4 5 6 7 8 9 10
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa

proteins were then purified with an amylose column. On average, a yield of approximately 12 mg of the purified MBP (Figure 6b) and 4 mg of the MBP-XlMob1 fusion protein (Figure 6c) was obtained from 500 ml of bacterial culture.

II-B. Production of Mouse Anti-XlMob1 Polyclonal Antibodies and Mouse Anti-XlMob1 Monoclonal Antibodies:

In order to characterize the biological properties of a protein, such as its molecular mass, subcellular localization, and possible functions, an antibody that can specifically recognize the protein is required. Therefore, both polyclonal and monoclonal antibodies were raised in mouse. The polyclonal antibodies against Mob1 were produced by immunizing Swiss Webster mice with the full-length affinity-purified MBP-XlMob1 fusion protein. In addition, mouse anti-Mob1 monoclonal antibody was produced (Warren and Wang, unpublished result). To generate the monoclonal antibody, Balb/c mice were immunized with the MBP-XlMob1 recombinant protein and test bleeds were screened for their antibody titre using indirect immunofluorescence (IIF) and by immunoblotting against the bacterially-expressed antigen. IIF was used for antibody screening because specific immunostaining patterns of *Xenopus* Mob1 were obtained with polyclonal antibodies. A positive mouse was sacrificed and the spleen cells were fused with FOX NY myeloma cells. The fusion mixture was cultured together with feeder cells to secrete growth factors that will aid in the growth of the hybridoma cells and to eliminate the parental myeloma cells in the 24-well dishes at 37 °C for 24 hours. The medium was then removed and replaced with HAT selection media and incubated at 37 °C for 24 hours. This procedure was repeated twice to get cells into full selection media.

Once the cells were expanded to a reasonable level, the culture supernatant was removed and tested for the presence of the antibody using IIF. After a positive tissue culture supernatant had been identified, the next step was to isolate a stable, single clone of hybridoma cells that secretes the anti-Mob1 antibody. Thus, the positive hybridoma cells were diluted and plated onto soft-agar plates, and incubated at 37 °C. Upon appearance of clones, each plug of agarose containing the colony was removed with a sterile Pasteur pipet and transferred to a 24-well plate containing 1 ml of HAT selection media. Supernatant from each clone was removed sterilely and screened for antibody production with IIF technique using *Xenopus* A6 tissue culture cells. The positive clones were screened twice more as described above. After going through three cycles of screening assays, we have obtained a clone designated 3-24-3 that consistently showed positive staining in A6 cells throughout the three cycles of IIF assays. Finally, this clone was expanded and cultured in normal expansion medium for hybridoma cells.

II-C. Characterization of Mouse Anti-XlMob1 Polyclonal Antibodies and Mouse Anti-XlMob1 Monoclonal Antibodies:

After generation of both mouse polyclonal and monoclonal anti-Mob1 antibodies, several experiments were performed to demonstrate the specificity of the resultant antisera and hybridoma supernatant.

II-C-1. Characterization of Anti-Mob1 Antibodies Using *In Vitro* Translated *Xenopus* Mob1 Protein Followed by Immunoprecipitation:

In order to assess the ability of anti-Mob1 antibodies in recognition of the Mob1 protein but not the MBP tag, the *Xenopus* Mob1 was translated *in vitro* from *pSport1*-

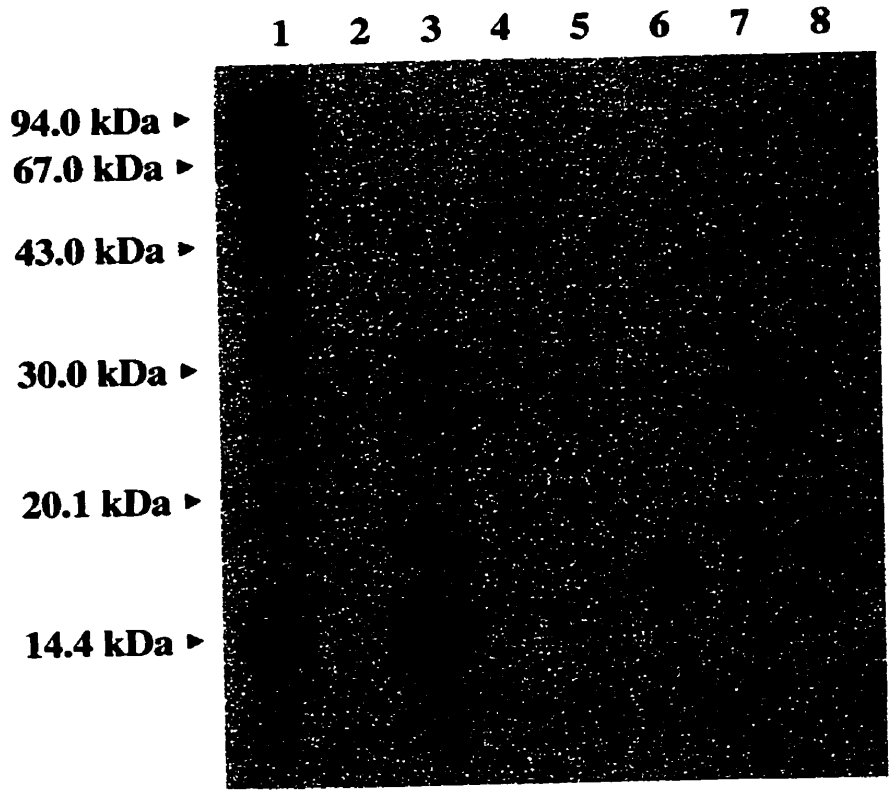
XlMOB1 plasmid using Promega TNT coupled reticulocyte lysate system. *In vitro* translation of *Xenopus MOB1* mRNA produced a major protein with a molecular size of approximately 17 kDa (Figure 7, lane 3), which is in good agreement with the theoretical molecular mass calculated from *Xenopus MOB1* cDNA coding region. There were also few minor proteins of various sizes *in vitro* translated from the *MOB1* mRNA, the presence of these proteins is thought to represent usage of internal AUG codons or partial degradation . This observation is consistent with previous reports for other proteins synthesized in reticulocyte lysates (Aplan *et al.*, 1990; Murrell *et al.*, 1995). The *in vitro* translated proteins were immunoprecipitated by immune mouse polyclonal antibodies (Figure 7, lane 8) but not by non-immune sera (Figure 7, lane 7). Furthermore, the mouse monoclonal antibody, 3-24-3, was able to immunoprecipitate the *in vitro* translated Mob1 (Figure 7, lane 6), whereas the protein A (Figure 7, lane 4) and the hybridoma culture medium alone (Figure 7, lane 5) were not able to precipitate the protein. The 3-24-3 monoclonal antibody (mAb), however, was not able to immunoprecipitate the *in vitro* translated *Xenopus* Cdc27 protein, a subunit of anaphase promoting complex isolated from our laboratory (Wu and Lohka, unpublished result), as shown in Figure 7 lane 1 and 2. Taken together, these data suggest that both the mouse polyclonal antisera and monoclonal antibodies are capable of recognizing the *Xenopus* Mob1 protein specifically.

II-C-2. Characterization of Anti-Mob1 Antibodies Using Factor Xa Treated MBP-XlMob1 Immunoblot Analysis:

Even though both mouse polyclonal antisera and monoclonal anti-Mob1 antibodies were able to immunoprecipitate the *in vitro* translated *Xenopus* Mob1 protein,

Figure 7. Characterization of anti-Mob1 antibodies by immunoprecipitation of ^{35}S labelled *in vitro* translated proteins.

In vitro translated *Xenopus MOB1* products were run on an 12% SDS-PAGE gel before (lane 3) and after (lane 4-10) immunoprecipitation with protein A (lane 4), monoclonal culture medium (lane 5), anti-Mob1 monoclonal (3-24-3) antibody (lane 6), mouse non-immune serum (lane 7), anti-Mob1p mouse polyclonal antibody (lane 8). Lane 1, reticulocyte lysate primed with *Xenopus CDC27* template. Lane 2, *In vitro* translated *Xenopus CDC27* product immunoprecipitated with anti-Mob1p monoclonal (3-24-3) antibody. The protein of approximately 17 kDa in lane 3, 6, and 8 is the *X. laevis* Mob1 protein.


1 2 3 4 5 6 7 8
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa

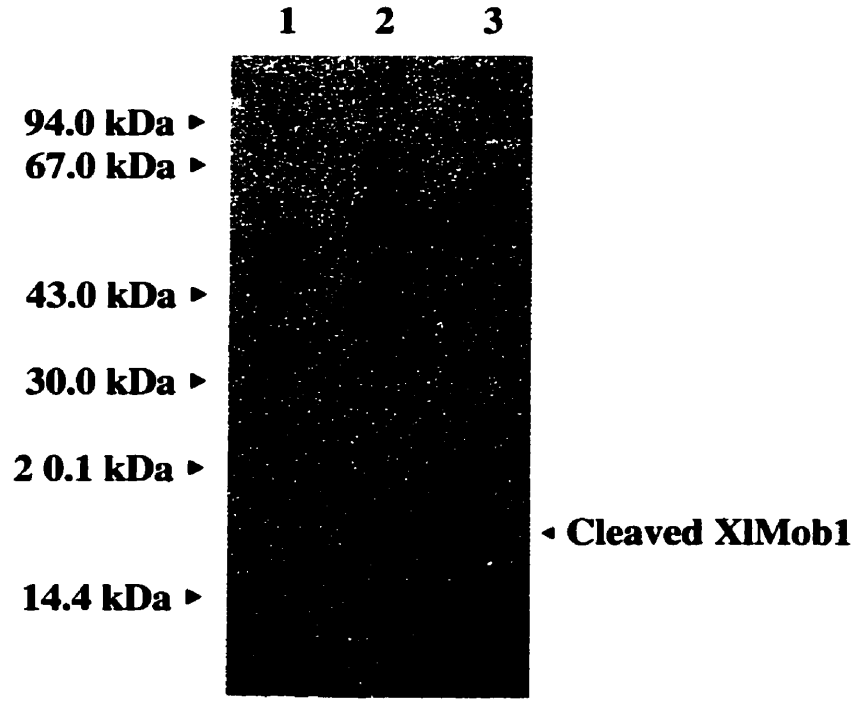
the binding ability of an antibody to its antigen may depend upon the conformation of the antigen (Birkenmeier and Stigbrand, 1993; Zhang *et. al.*, 1994). Therefore, the two antibodies were tested for their ability to recognize the denatured antigen.

The pMAL-c2 vector contains the sequence coding for the recognition site of a specific protease, Factor Xa, 5' to the polylinker insertion sites. This allows the MBP to be cleaved from XlMob1 protein after purification. Factor Xa cleavage occurs after its four amino acid recognition sequence, which is located next to the *XlMOB1* insertion site. Hence, no vector-derived amino acids are attached to XlMob1 protein after Factor Xa digestion and, therefore, it is not possible for the antisera raised against XlMob1 to cross-react with the vector-derived amino acid sequences. The affinity purified MBP protein and MBP-XlMob1 fusion protein were digested with Factor Xa or water (negative control) according to the manufacturer's suggestion. Five microliters of each sample was loaded on an 12% SDS-PAGE gel. As indicated in Figure 8a, a 17 kDa protein was cleaved from the fusion protein (lane 2), but not from the MBP digestion or the recombinant mock digestion (land 1 and lane 3, respectively). The coincidence of the molecular size between the 17 kDa cleaved protein and the *in vitro* translated *Xenopus* Mob1 protein indicates that the 17 kDa cleaved protein is the *Xenopus* Mob1 protein. The digested fusion proteins were then transferred to PVDF membrane and immunoblotted with 3-24-3 mAb, crude mouse anti-Mob1 polyclonal antibodies, or mouse anti-MBP monoclonal antibodies obtained during the production of the 3-24-3 mAb. The bacterially-expressed *Xenopus* Mob1 protein was detected by the mouse polyclonal antibodies (Figure 8b, lane 4), whereas the protein was not detected by either 3-24-3

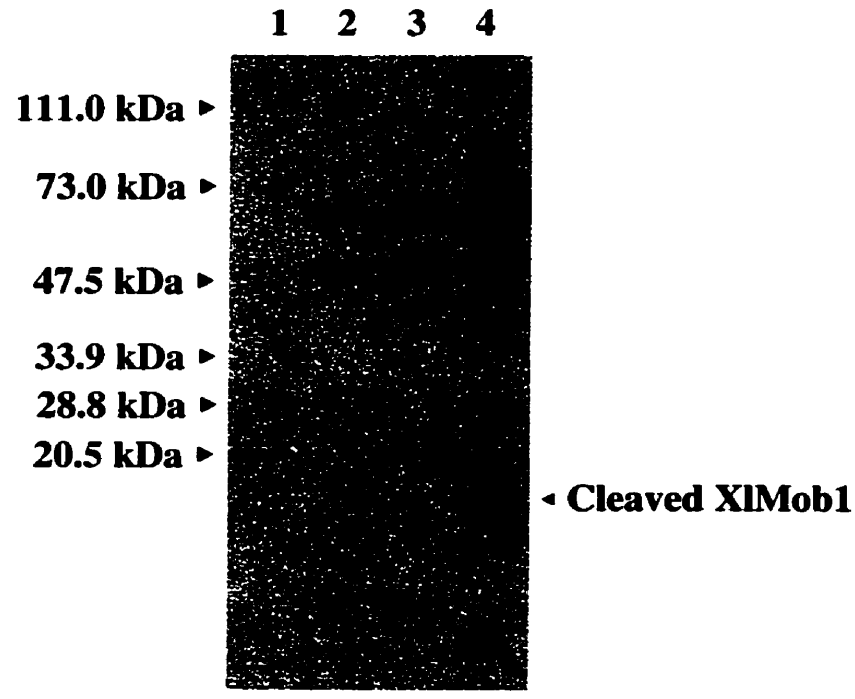
Figure 8. Immunoblotting of Factor Xa digested recombinant Mob1 protein.

(A) The MBP protein (lane 1) or the MBP-XlMob1 (lane 2) was incubated with Factor Xa, MBP-XlMob1 was incubated with water (lane 3) as a mock digestion control. Five microlitters of each sample was loaded on an 12% SDS-PAGE gel. (B) The MBP-XlMob1 fusion protein cleaved by Factor Xa was immunoblotted with anti-Mob1 (3-24-3) mouse monoclonal antibodies (lane 1), anti-MBP monoclonal antibodies (lane 2 and 3), and anti-Mob1 mouse polyclonal antibodies (lane 4).

A.


1 2 3
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
2 0.1 kDa
14.4 kDa
Cleaved XlMob1

B.


1 2 3 4
111.0 kDa
73.0 kDa
47.5 kDa
33.9 kDa
28.8 kDa
20.5 kDa
Cleaved XlMob1

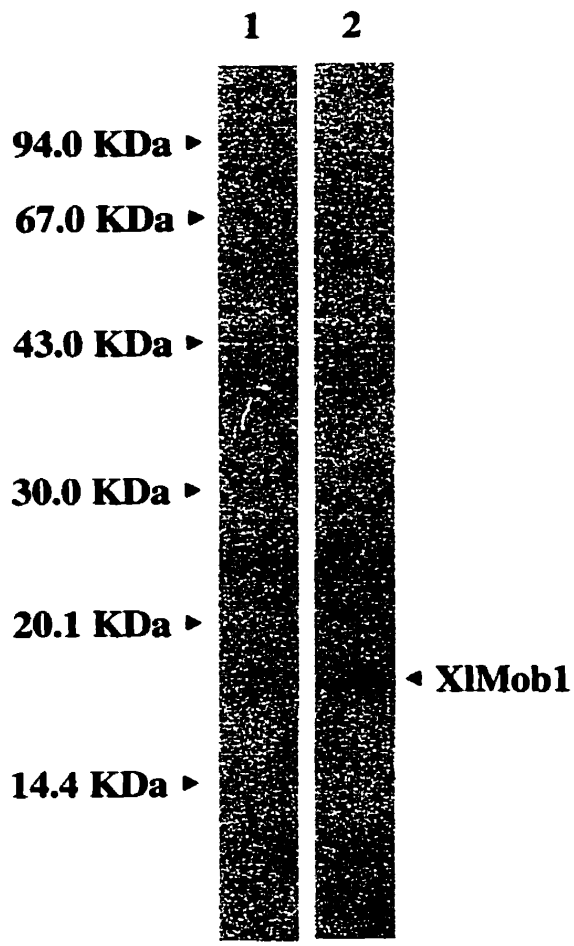
mAb or anti-MBP mAb (Figure 8b, lane1 to land 3). Together with the data on *in vitro* translated proteins, these results indicate that 3-24-3 mAb was able to recognize the native Mob1 protein but not the denatured protein. The mouse polyclonal antibodies, on the other hand, can be used for detection of the protein in both native and denatured conformations.

II-C-3. *Xenopus* Mob1 Is a 17 kDa Protein:

Since the mouse polyclonal antisera were able to recognize the antigen in denatured form, the antisera were used to identify the endogenous Mob1 protein in a *Xenopus* A6 tissue culture cell lysate. *Xenopus* A6 cells (a gift from Dr. B. Burke) were grown on two petri dishes until approximately 80% confluency. The cells on each petri dish were solublized with 250 μl 5X Laemmli sample buffer. The crude lysates from two petri dishes were pooled together and subjected to sonication to shear the DNAs. The supernatant was collected after microcentrifugation of the crude lysate and 20 μl of the extract were loaded onto each lane of an 12% SDS-PAGE gel. The proteins were transferred to PVDF membrane and immunoblotted with mouse anti-Mob1 polyclonal antibodies pre-blocked with either MBP or MBP-XlMob1 fusion protein. Immunoblotting results indicated that anti-Mob1 antibodies blocked with MBP recognized a protein of 17 kDa in A6 cell lysate, but the antisera blocked by the fusion protein did not (Figure 9). Pre-incubation of the antisera with the affinity-purified MBP-XlMob1 fusion protein, but not with the MBP protein, resulted in the loss of recognition of the 17 kDa band, indicating that the antibody reacted specifically with a protein of molecular weight of 17 kDa. The coincidence of the molecular mass with the *in vitro*

Figure 9. Immunoblot of *Xenopus* A6 Cell Extract.

Immunoblot of cell lysate isolated from *Xenopus* A6 cells using mouse polyclonal anti-Mob1 antibodies pre-blocked with either MBP protein (lane 2) or MBP-XlMob1 recombinant protein (lane 1). Twenty microlitters of the cell extract were loaded for each lane of the gel.


1
2
94.0 KDa
67.0 KDa
43.0 KDa
30.0 KDa
20.1 KDa
14.4 KDa
XlMob1

translated *Xenopus* Mob1 and the Factor Xa cleaved fusion Mob1 protein indicates that the protein recognized by the polyclonal antibodies represented the endogenous Mob1 protein in *Xenopus* A6 cells.

II-C-4. Characterization of Mouse Polyclonal Anti-Mob1 Sera Using Bacterially-Expressed GST-HsMob1 Recombinant Protein:

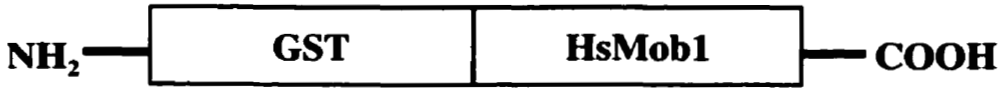
The results of the BLAST search suggested that Mob1 is conserved in a wide variety of organisms including human. The antigen used to raise the antibodies in our laboratory, however, was originally from a bacterially-expressed *Xenopus* protein. Therefore, it would be interesting to examine if the antisera were specific to *Xenopus* Mob1 protein or also could be used to detect Mob1-related protein in other vertebrates.

In order to address this question, a *GST*-tagged human *MOB1* construct was obtained from Dr. J.B. Rattner's lab (Figure 10a, J.B. Rattner's unpublished result). The recombinant protein or its tagging protein, GST, alone were independently expressed in *E. coli* and affinity purified with a glutathione column (Figure 10b and 10c, respectively). The proteins were transferred to PVDF membrane and then incubated with the mouse polyclonal anti-Mob1 antisera. The antisera were able to recognize the fusion protein (Figure 11a lane 2), but not the GST tag (Figure 11a lane 1), suggesting that the mouse polyclonal anti-Mob1 antisera reacted specifically with the bacterially-expressed human Mob1 protein and that the antisera can be used to detect the Mob1-related proteins in organisms other than *Xenopus*.

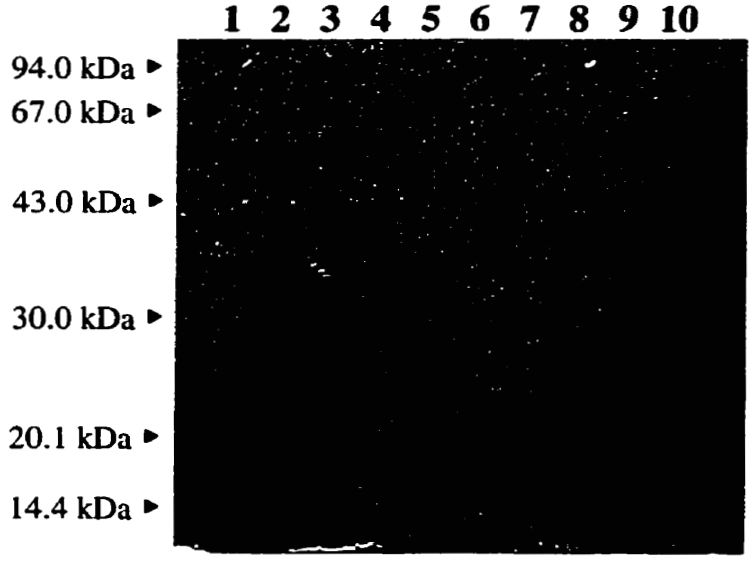
Figure 10. Schematic representation of glutathione S-transferase (GST)-human Mob1 recombinant construct and affinity purification of the bacterially-expressed GST-HsMob1 fusion protein.

(A) Full-length human *MOB1* was inserted into the C-terminus of *GST* tag system. (B) the bacterially-expressed GST-HsMob1 fusion protein was eluted from a glutathione column, and the fractions were analyzed on an 12% SDS-PAGE gel stained with Coomassie blue. The purified GST protein came out primarily in fractions 2 and 3, and exhibited a molecular mass of approximately 26 kDa. (C) Similarly, the bacterially-expressed GST protein was eluted from glutathione column, and the fractions were analyzed on an 12% SDS-PAGE gel stained with Coomassie blue. The purified recombinant protein came out primarily from fractions 2 to 5, and exhibited a molecular mass of approximately 52 kDa.

A.


NH2
GST
HsMob1
COOH

B.


1 2 3 4 5 6 7 8 9 10
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa

C.

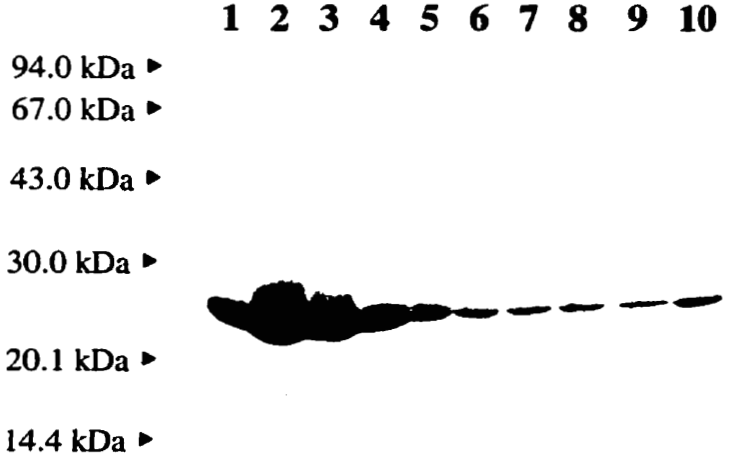
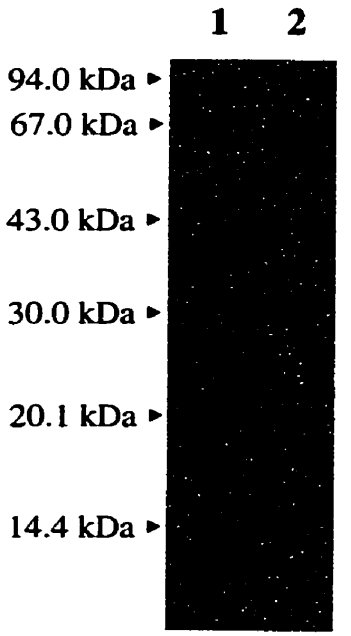

1 2 3 4 5 6 7 8 9 10
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa

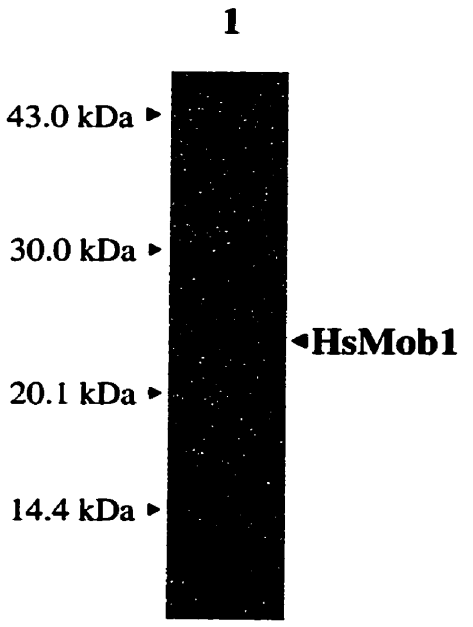
Figure 11. Immunoblot of GST-tagged human Mob1 fusion protein and human Mob1 in A431 Cell Extract.

(A) Immunoblotting of GST (lane 1) or GST-human Mob1 recombinant protein (lane 2) by mouse anti-Mob1 polyclonal antibodies pre-incubated with MBP protein. Approximately 0.2 μg of each GST eluted on fraction 9 (Figure 10c) or the the recombinant protein eluted on fraction 3 (Figure 10b) was loaded onto the gel. (B) Immunoblot of cell lysate isolated from human A431 cells using mouse polyclonal anti-Mob1 antibodies pre-blocked with MBP protein. Fifteen microliters of the cell extract were loaded in each lane.

A.


1
2
94.0 kDa
67.0 kDa
43.0 kDa
30.0 kDa
20.1 kDa
14.4 kDa

B.


1
43.0 kDa
30.0 kDa
HsMob1
20.1 kDa
14.4 kDa

II-C-5. Anti-Mob1 Polyclonal Antibodies Detected a 26 kDa Protein in Human A431 Cell Lysate:

The ability of the mouse polyclonal anti-Mob1 antisera to recognize the human Mob1 protein was further characterized with an extract from human A431 cells. The A431 cell lysate was obtained as described previously for A6 cells, and 15 μl of the A431 cell lysate was loaded onto an 12% SDS-PAGE gel followed by transfer to PDVF membrane. The membrane was incubated with the mouse polyclonal anti-Mob1 antibodies pre-blocked with MBP protein. The immunoblot showed a band at approximately 26 kDa (Figure 11b), which corresponds to the predicted molecular weight of the full-length human Mob1 protein encoded by the *MOB1* cDNA isolated by Dr. J.B. Rattner's lab (unpublished result). The ability of the anti-*Xenopus* Mob1 polyclonal antibody to detect the human Mob1 protein in a A431 cell extract suggests that *Xenopus* Mob1 shares a significant homology with the human Mob1 homolog, as predicted by the BLAST search and sequence comparisons.

III. Indirect Immunolocalization Studies of Mob1 Protein:

In order to analyze the location of Mob1 protein in the cell, the distribution of Mob1 in both *Xenopus* A6 and human A431 culture cells was examined by conventional indirect immunofluorescence microscopy.

III-A. *Xenopus* Mob1 Protein Localizes to Centrosomes in *Xenopus* A6 Cells Throughout the Cell Cycle:

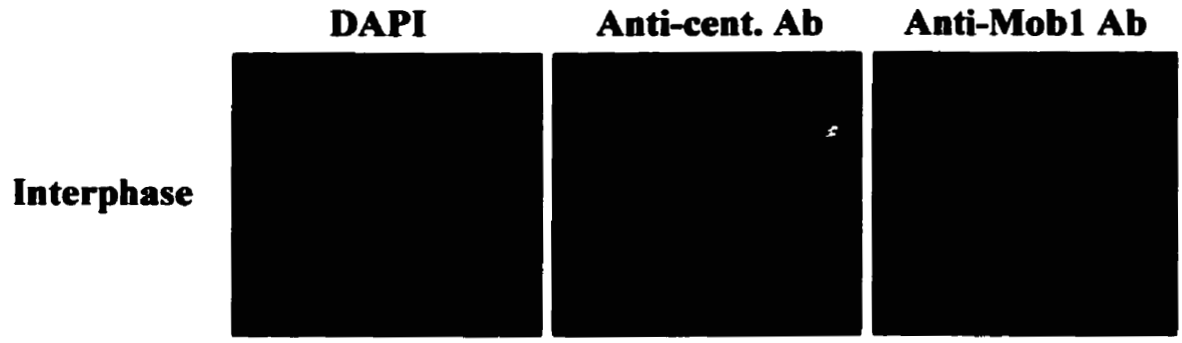
To determine the location of Mob1 in *Xenopus* cells, the A6 cells were grown on coverslips treated with both poly-L-lysine and collagen until approximately 90%

confluency. The cells were first permeabilized with 0.1% Triton X-100 and then fixed with 3.7% paraformaldehyde. The reason to extract cells prior to fixing them is that permeabilization removes lipids and soluble proteins, which provides better view of some cellular structures, such as the cytoskeleton, spindle apparatus, and centrosomal components (Wagner *et al.*, 1986; Hollenbeck, 1989; Hoffman and Mullins,1990; Chen *et al.*, 1996). This procedure was based on the methods used to extract soluble cellular proteins during *in situ* preparations of nuclear matrix or other nuclear structures and is referred to as *in situ* extraction (Fey *et al.*, 1986; Gerdes *et al.*, 1994; Todorov *et al*, 1995). Because the extraction before fixation washes off a large number of the cells from the coverslips, especially mitotic cells, the coverslips were pre-treated with both poly-L-lysine and collagen, and the cells were grown to a high density. Since the monoclonal antibody produced by the 3-24-3 clone can be used to detect the *Xenopus* Mob1 protein in native form, its hybridoma cultural medium was incubated with the A6 cells for overnight at 4 °C. Detection of the Mob1 immuno-complexes was achieved with a secondary antibody of goat anti-mouse IgG labelled with either Alexa 594 dye or Texas Red dye. Also, the cells were co-stained with M-4491, a human auto-immune serum that recognizes a panel of centrosome proteins (a gift from Dr. J.B. Rattner; Mack *et al.*, 1998). A secondary antibody of goat anti-human IgG conjugated with Cy2 fluorochrome was used to detect the M-4491. Chromosome morphologies were revealed by DAPI staining to identify cells in interphase and at different stages of mitosis.

In interphase cells of an asynchronous A6 culture (Figure 12), 3-24-3

Figure 12. Immunolocalization of Mob1 to interphase centrosomes in *Xenopus* A6 cells.

Xenopus A6 cells were extracted *in situ* with 0.1% Triton X-100 in PBS, fixed with 3.7% paraformaldehyde, and processed for IIF. Mob1 was detected by 3-24-3 mAb (Anti-Mob1 Ab) and a secondary antibody, goat anti-mouse IgG conjugated to the Alex 594 fluorochrome. The centrosomes were detected with human anti-centrosome autoimmune antisera (Anti-cent. Ab) and stained with goat anti-human IgG conjugated to Cy2. Nuclear DNA was stained with DAPI.


DAPI
Anti-cent. Ab
Anti-Mob1 Ab
Interphase

predominantly stained the centrosome, with minor staining in the nucleus. During mitosis, XlMob1 was also detected at the mitotic spindle poles (Figure 13). The staining was blocked if the antibodies were first incubated with recombinant XlMob1 protein (Figure 14b), but not with the MBP protein alone (Figure 14a), indicating that the staining was specific to XlMob1. In comparison, Mob1 staining was more pronounced at mitotic spindle poles than at interphase centrosomes. Moreover, Mob1 staining was greatest at metaphase and lowest at late anaphase (Figure 13), suggesting that the localization of Mob1 to centrosomes may be controlled in a cell cycle dependent manner.

III-B. Mob1 Protein Localizes to Centrosomes in Human A431 Cells Throughout the Cell Cycle:

In order to determine if Mob1 is also present at interphase centrosomes and mitotic spindle poles in other cells, A431, the human epidermoid carcinoma cell line, was primarily chosen for indirect immunofluorescence. These cells have abundant desmosomes that increase the adhesion of cells to each other and the attachment to the extracellular matrix (Bornslaeger *et al.*, 1996), even during mitosis. Thus, mitotic A431 cells would attach better to the substrate even after *in situ* extraction. A431 cells were grown to approximately 50% confluency and extracted with 0.15% Triton X-100 dissolved in cytoskeleton (CSK) buffer. The use of CSK buffer for *in situ* extraction was previously reported by Fey *et al.* (1986) and Todorov *et al.* (1995), and this buffer seemed to help keep cells attached to the substrate. The cells were fixed in CSK buffer containing 3.7% paraformaldehyde, blocked and incubated overnight with 3-24-3 mAb. Mob1 antibodies were detected by a secondary antibody of goat anti-mouse IgG labelled

Figure 13. Immunolocalization of Mob1 to mitotic spindle poles in *Xenopus* A6 cells.

The *Xenopus* A6 mitotic cells were *in situ* extracted with 0.1% Triton X-100 in PBS, fixed with 3.7% paraformaldehyde, and processed for IIF. Mob1 was detected by 3-24-3 mAb (Anti-Mob1 Ab) and a secondary antibody, goat anti-mouse IgG conjugated with Texas Red fluorochrome. The centrosomes were detected with human anti-centrosome autoimmune antibody (Anti-cent. Ab) and stained with goat anti-human IgG conjugated with Cy2 fluorochrome. The mitotic stages were monitored by staining with DAPI.

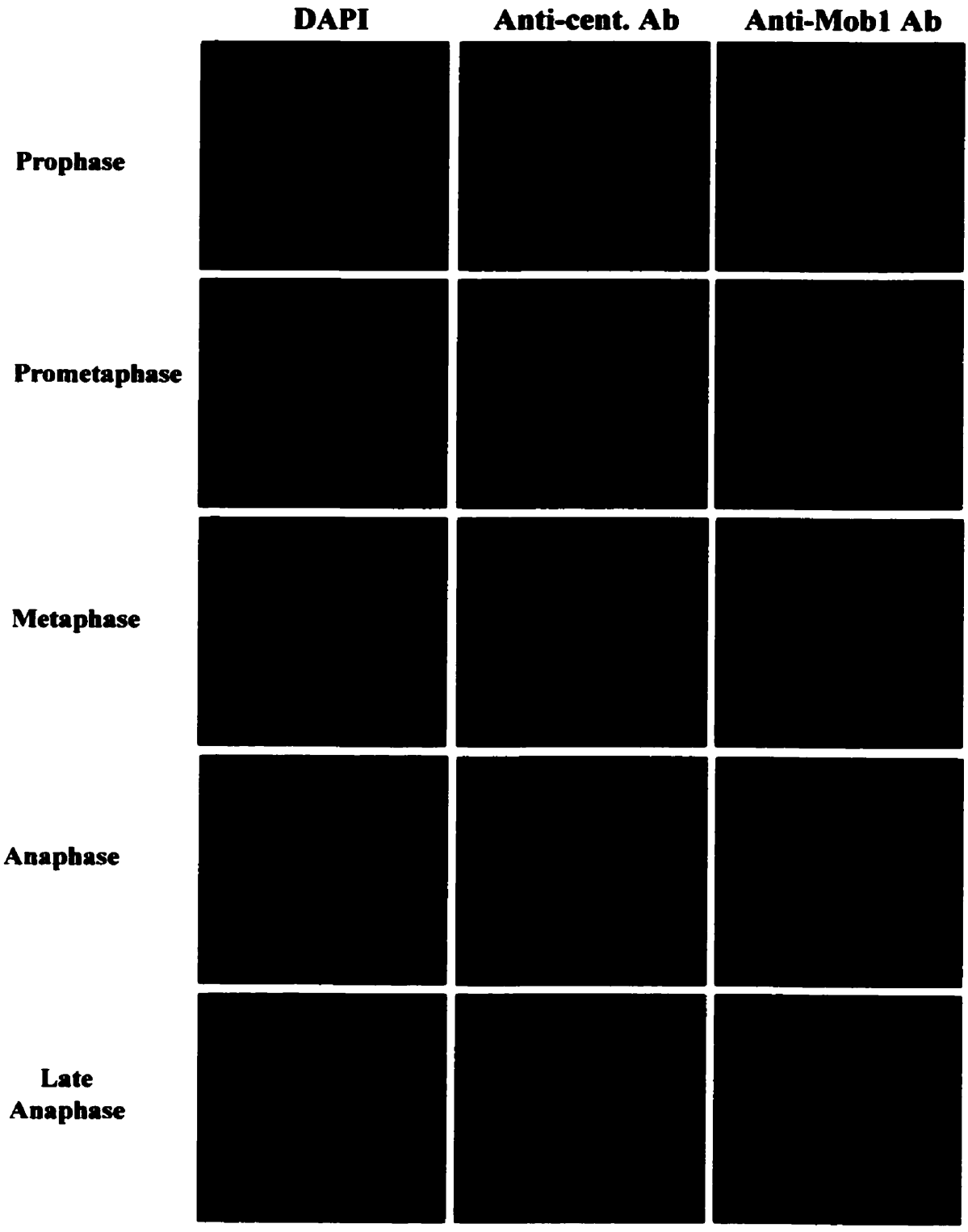
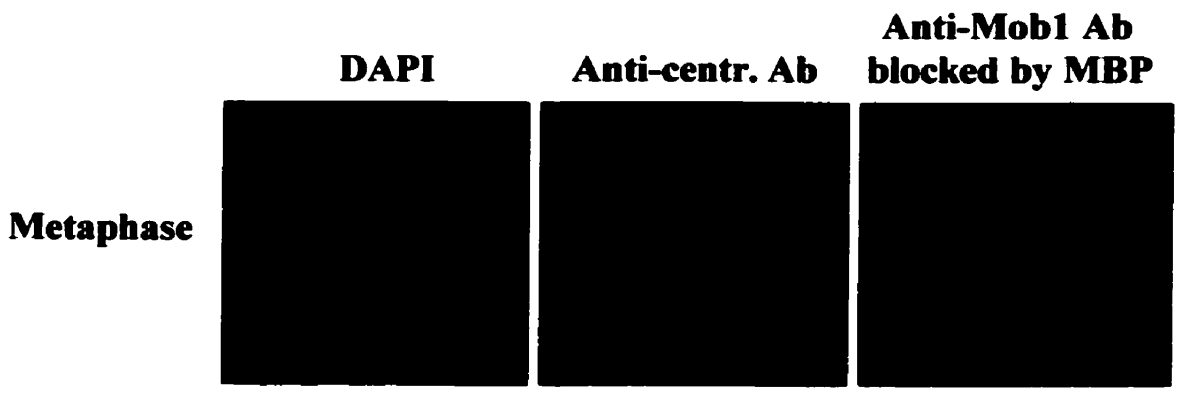

DAPI
Anti-cent. Ab
Anti-Mob1 Ab
Prophase
Prometaphase
Metaphase
Anaphase
Late Anaphase

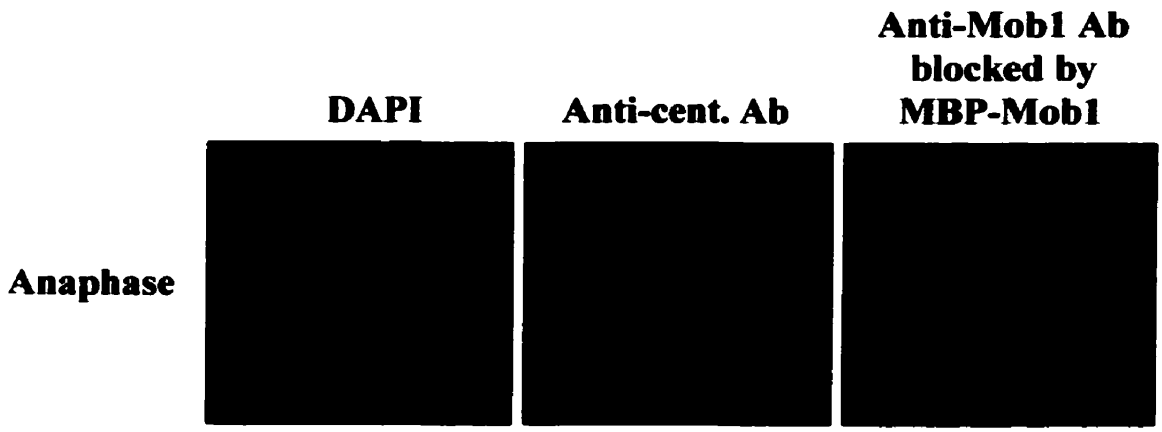
Figure 14. Control experiments for immunolocalization of *Xenopus* Mob1 protein during mitosis.

Subcellular localization of Mob1 in *Xenopus* A6 cells was examined by indirect immunofluorescence microscopy with 3-24-3 mAb blocked by MBP alone (A) or MBP-Mob1 recombinant protein (B). The Mob1 signal was detected with a secondary antibody, goat anti-mouse IgG conjugated with Alexa 594 fluorochrome. The centrosomes were detected with human anti-centrosome autoimmune antiserum and stained with goat anti-human IgG conjugated with Cy2 fluorochrome. Nuclear DNA was stained with DAPI.

A.


DAPI
Anti-centr. Ab
Anti-Mob1 Ab blocked by MBP
Metaphase

B.


DAPI
Anti-cent. Ab
Anti-Mob1 Ab blocked by MBP-Mob1
Anaphase

with Alexa 594 dye The centrosomes were stained with M-4491 auto-immune sera and the appropriate Cy2 conjugated secondary antibodies. Finally, DNA was stained with DAPI to identify interphase and mitotic cells.

Similar to the results obtained from immunolocalization studies of *Xenopus* A6 cells, staining of human A431 cells with 3-24-3 mAb showed that the Mob1 protein was localized to the centrosomes of interphase cells (Figure 15) and to the spindle poles of mitotic cells (Figure 16). Pre-incubation of antibodies with MBP did not affect staining (Figure 17a), but antibodies pre-blocked by the recombinant Mob1 protein gave no staining (Figure 17b), demonstrating that the antibody staining pattern was specific. In addition, control staining where either 3-24-3 mAb (Figure 18a) or M-4491 (Figure 18b) primary antibodies were left out showed no staining of Mob1 or centrosome, respectively, suggesting that the two secondary antibodies used were specific for their appropriate primary antibodies and that Mob1 staining was not due to fluorescence leaking through the two filters in the microscope.

The 3-24-3 mAb gave the characteristic centrosome staining pattern of one focus (Figure 15 upper panel) and two foci (Figure 15 lower panel) in interphase cells, suggesting that Mob1 is present at interphase centrosome before and after centrosome duplication. During mitosis, Mob1 was found at the mitotic spindle poles from prophase to telophase (Figure 16). By comparing the signal intensity, Mob1 staining increased as cells entered mitosis, with the strongest staining during metaphase and early anaphase, and then decreased by telophase (Figure 15 and 16). Together with the observations from A6 cells, the results imply that localization of Mob1 to centrosomes may be controlled

Figure 15. Immunolocalization of Mob1 to interphase centrosomes in human A431 cells.

Human A431 interphase cells were extracted *in situ* with 0.15% Triton X-100 in PBS, fixed with 3.7% paraformaldehyde, and processed for IIF. Mob1 was detected by 3-24-3 mAb (Anti-Mob1 Ab) and a secondary antibody, goat anti-mouse IgG conjugated with Alexa 594 fluorochrome. The centrosomes were detected with human anti-centrosome autoimmune antibody (Anti-cent. Ab) and stained with goat anti-human IgG conjugated with Cy2 fluorochrome. Nuclear DNA was stained with DAPI.

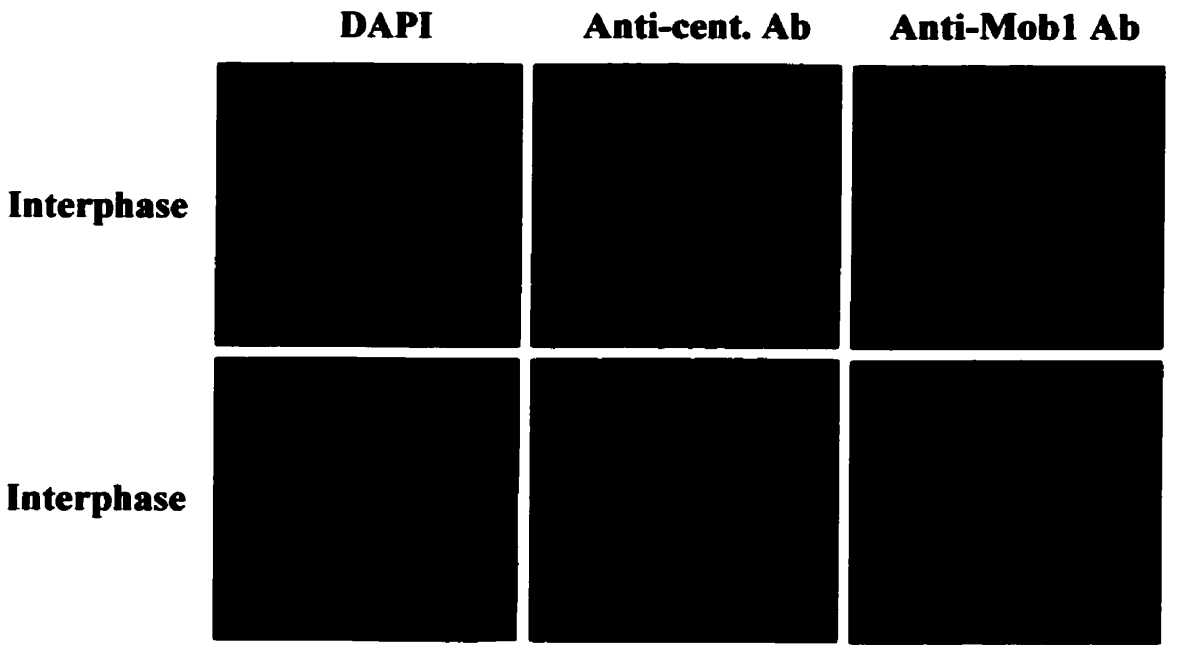

DAPI
Anti-cent. Ab
Anti-Mob1 Ab
Interphase
Interphase

Figure 16. Immunolocalization of Mob1 to mitotic spindle poles in human A431 cells.

Human A431 mitotic cells were extracted *in situ* with 0.15% Triton X-100 in PBS, fixed with 3.7% paraformaldehyde, and processed for IIF. Mob1 was detected by 3-24-3 mAb (Anti-Mob1 Ab) and a secondary antibody, goat anti-mouse IgG conjugated with Alexa 594 fluorochrome. The centrosomes were detected with human anti-centrosome autoimmune antibody (Anti-cent. Ab) and stained with goat anti-human IgG conjugated with Cy2 fluorochrome. The mitotic stages were monitored by staining with DAPI.

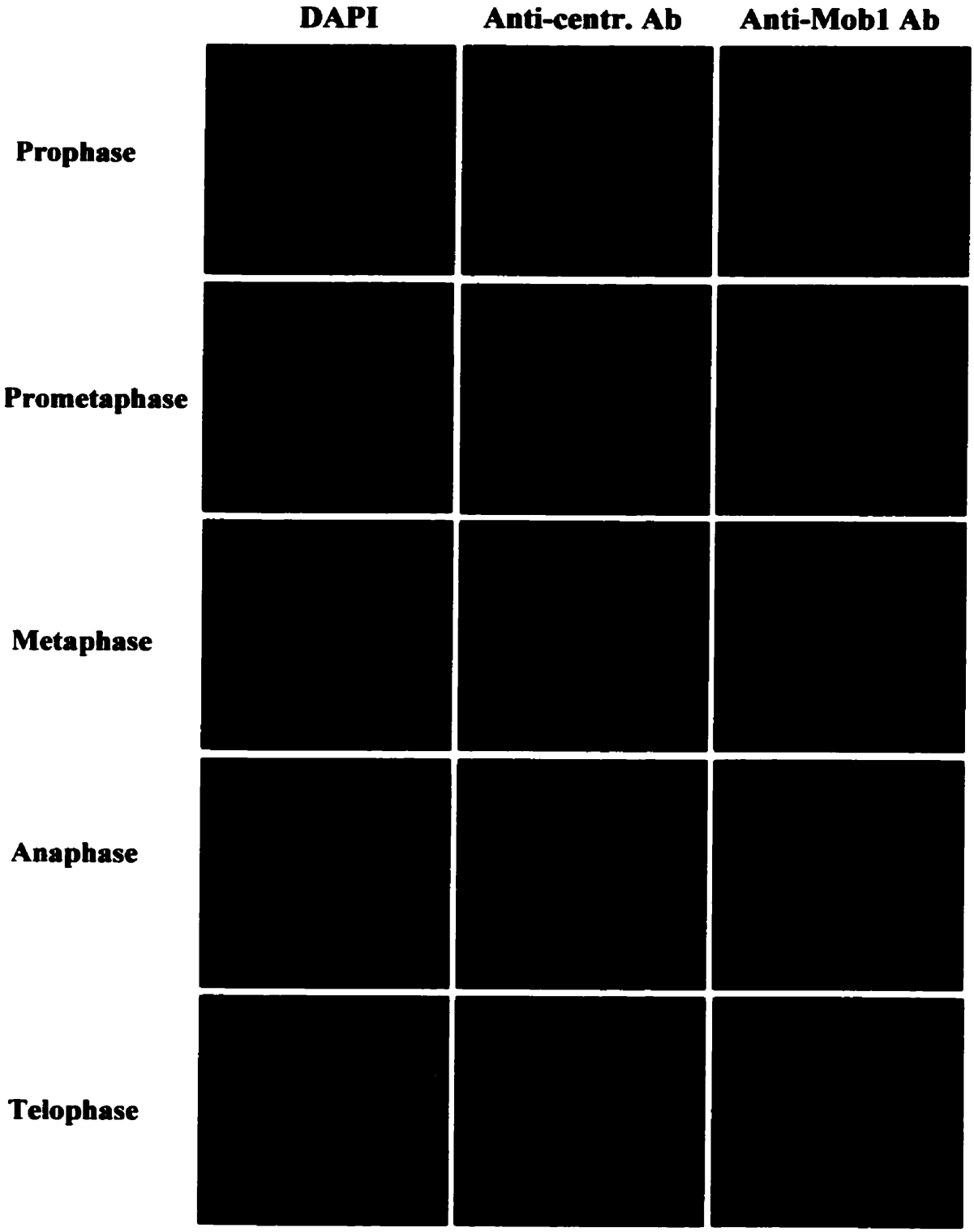
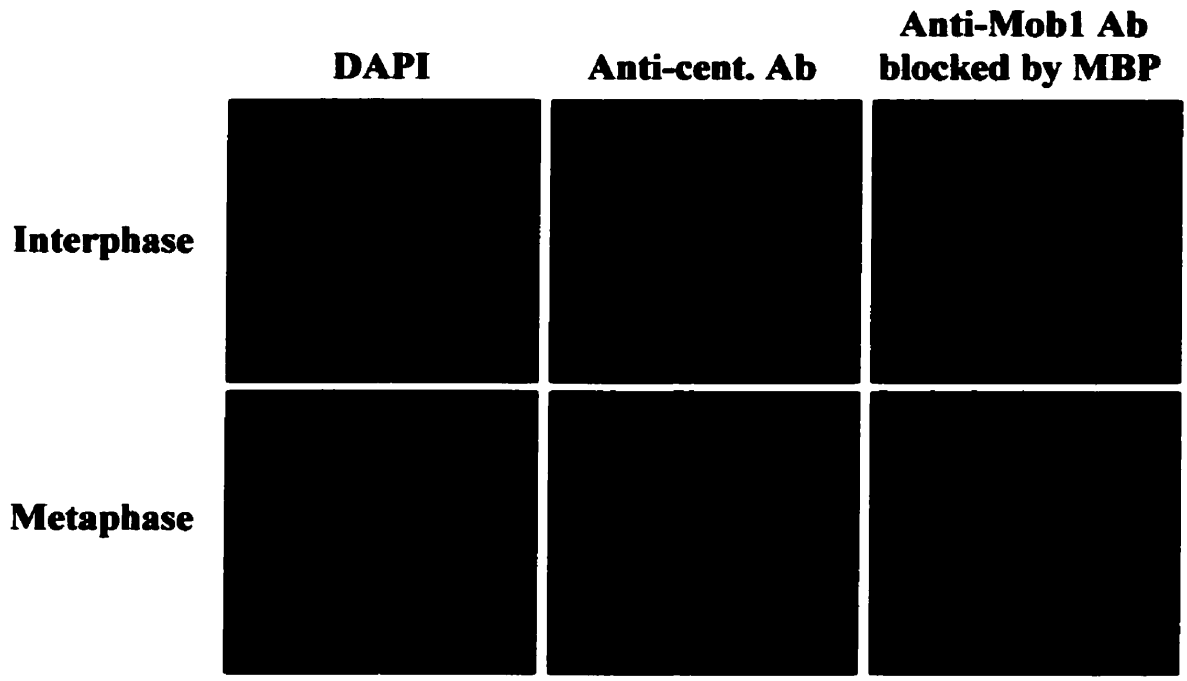

DAPI
Anti-centr. Ab
Anti-Mob1 Ab
Prophase
Prometaphase
Metaphase
Anaphase
Telophase

Figure 17. Control experiments for immunolocalization of Mob1 in A431 cells.

Subcellular localization of MOB1p in human A431 cells was examined by indirect immunofluorescence microscopy with anti-Mob1 3-24-3 mAb (anti-Mob1 Ab) blocked by MBP alone (A) or MBP-Mob1 recombinant protein (B). The Mob1 signal was detected with a secondary antibody, goat anti-mouse IgG conjugated with Alexa 594 fluorochrome. The centrosomes were detected with human anti-centrosome autoimmune antiserum and stained with goat anti-human IgG conjugated with Cy2 fluorochrome. Nuclear DNA was stained with DAPI.

A.


DAPI
Anti-cent. Ab
Anti-Mob1 Ab blocked by MBP
Interphase
Metaphase

B.

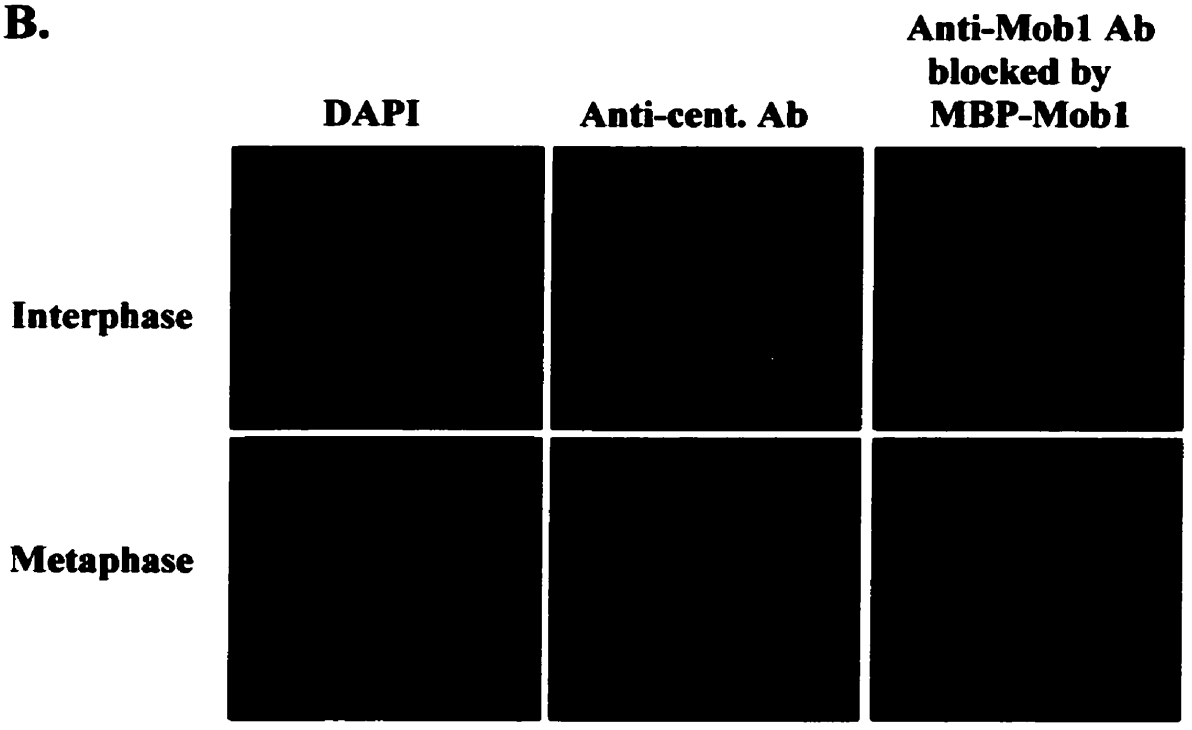
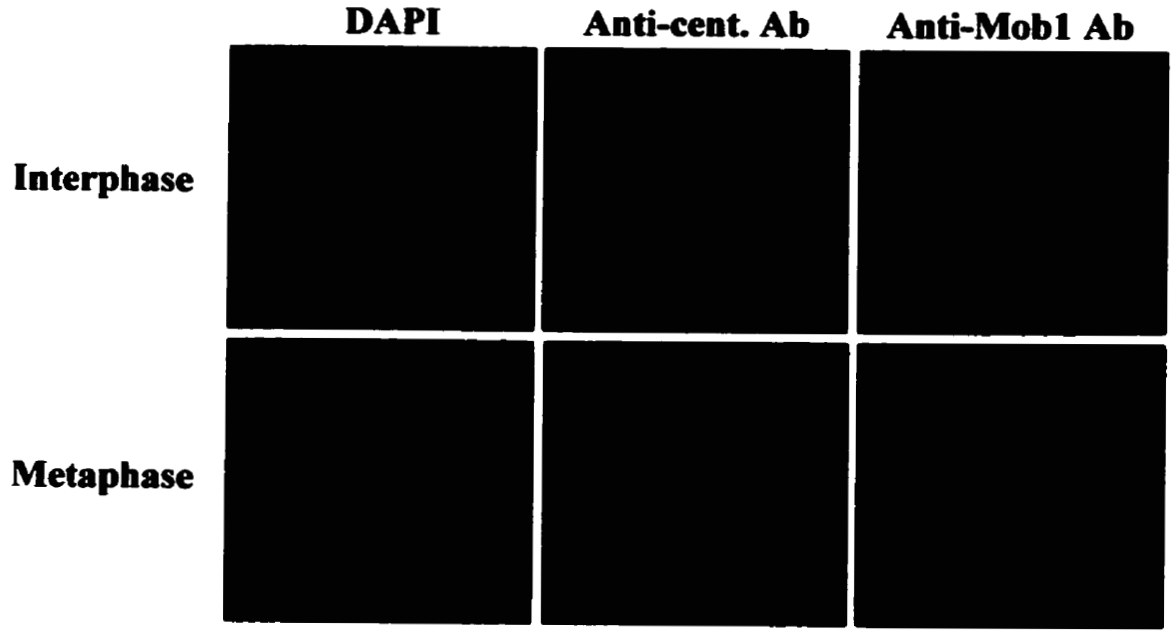

DAPI
Anti-cent. Ab
Anti-Mob1 Ab blocked by MBP-Mob1
Interphase
Metaphase

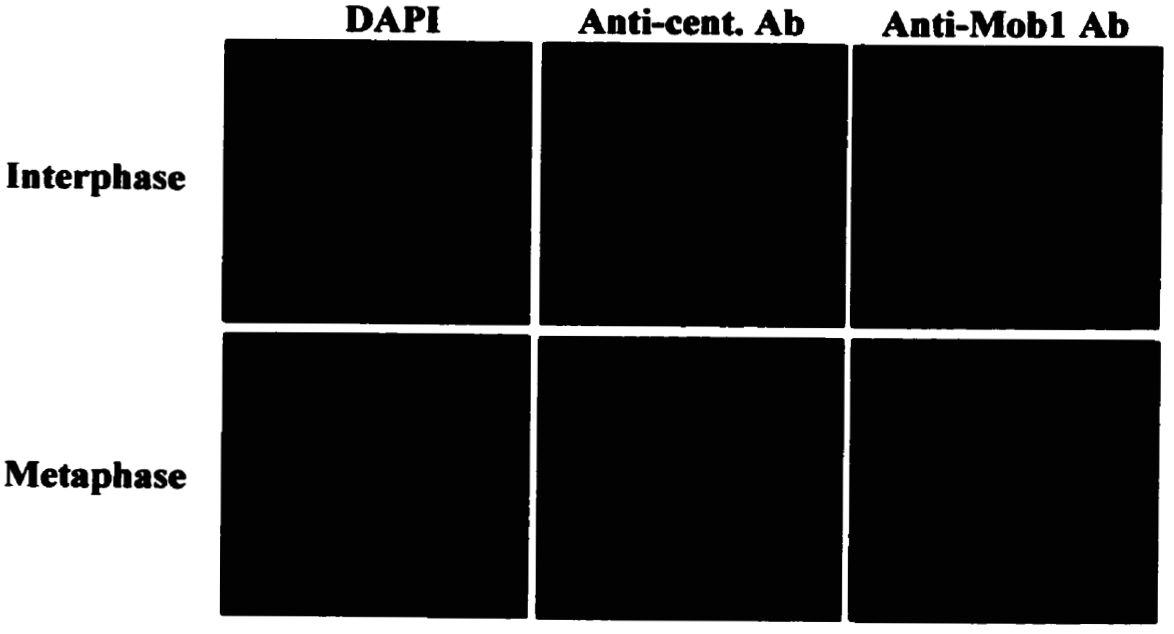
Figure 18. Control experiment for immunolocalization of Mob1 in A431 cells by leaving out the primary antibody.

A431 cells were extracted *in situ* with CSK buffer containing 0.15% Trition X-100 and fixed with 3.7% paraformaldehyde in CSK buffer. (A) The cells were incubated with M-4491 human autoimmune antibody (anti-cent. Ab), followed by detection with goat anti-human IgG secondary antibody conjugated with Cy2 and goat anti-mouse IgG secondary antibody conjugated with Alexa 594. (B) The cells were incubated with 3-24-3 mAb (Anti-Mob1 Ab), followed by staining with goat anti-mouse IgG secondary antibody conjugated with Alexa 594 and goat anti-human IgG secondary antibody conjugated with Cy2. Nuclear DNA was stained with DAPI.

A.


DAPI
Anti-cent. Ab
Anti-Mob1 Ab
Interphase
Metaphase

B.


DAPI
Anti-cent. Ab
Anti-Mob1 Ab
Interphase
Metaphase

during the cell cycle. In addition to staining the interphase centrosome and mitotic spindle poles, anti-Mob1p antibodies stained the cytoplasmic bridges that connect newly divided A431 cells (Figure 16). The localization of Mob1 at intercellular bridge was also observed from *Xenopus* A6 cells (Figure 19), indicating that the staining pattern is not specific to A431 cells. The ability of the 3-24-3 monoclonal antibody to stain both *Xenopus* A6 and human A431 cells strongly supports the immunoblot results obtained with mouse polyclonal antibodies.

III-C. Localization of Mob1 to Centrosomes in HeLa Cells Immunolabelled With Mouse Polyclonal Antibodies:

To test whether antibodies other than 3-24-3 can be used to visualize Mob1 at centrosomes, mouse polyclonal anti-Mob1 antisera were used for IIF. HeLa cells were grown on microscopic slides and processed for IIF as previously described for A431 cells. The cells were immunostained with the polyclonal antibodies, which were detected with goat anti-mouse IgG conjugated with Texas Red dye. Again, like the observations from A6 and A431 cells, Mob1 was shown to localize to mitotic spindle poles (Figure 20). The centrosomal localization of Mob1 was specific because pre-incubation of the antibodies with the MBP protein had no influence on the centrosomal staining (Figure 21a), whereas pre-incubation of the antisera with the recombinant antigen gave no staining (Figure 21b).

IV. Mob1 Is an Centrosome Component:

An increasing number of proteins have been shown to associate with the centrosome and MTOC since 1990. Many of these papers report that proteins are

Figure 19. Localization of Mob1 to the intercellular bridge in *Xenopus* A6 cells.

Asynchronous *Xenopus* A6 cells were extracted *in situ* with with 0.1% Triton X-100 in PBS, fixed with 3.7% paraformaldehyde, and processed for IIF. Mob1 was detected by 3-24-3 mAb (Anti-Mob1 Ab) and a secondary antibody, goat anti-mouse IgG conjugated to the Alex 594 fluorochrome. The centrosomes were detected with human anti-centrosome autoimmune antisera (Anti-cent. Ab) and stained with goat anti-human IgG conjugated to Cy2. The mitotic stages were monitored by DAPI staining

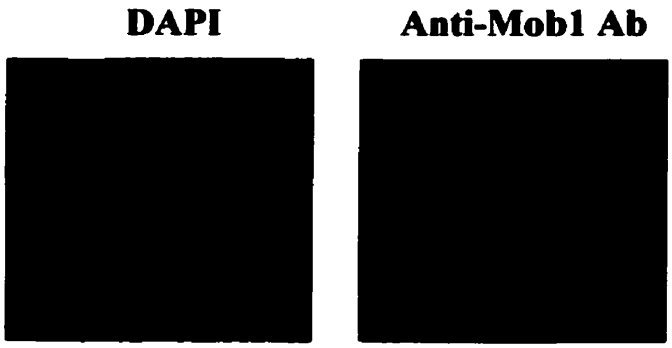

DAPI
Anti-Mob1 Ab

Figure 20. Immunolocalization of Mob1 to mitotic spindle poles using polyclonal anti-Mob1 antisera.

Subcellular localization of Mob1 in human HeLa mitotic cells were *in situ* extracted with 0.15% Triton X-100 in PBS, fixed with 3.7% paraformaldehyde, and processed for IIF. Mob1 was detected by mouse anti-Mob1 polyclonal antibody (anti-Mob1 Ab) and a secondary antibody, goat anti-mouse IgG conjugated with Texas Red fluorochrome. The centrosomes were detected with human anti-centrosome autoimmune antibody (Anti-cent. Ab) and stained with goat anti-human IgG conjugated with Cy2 fluorochrome. The mitotic stages were monitored by staining with DAPI.

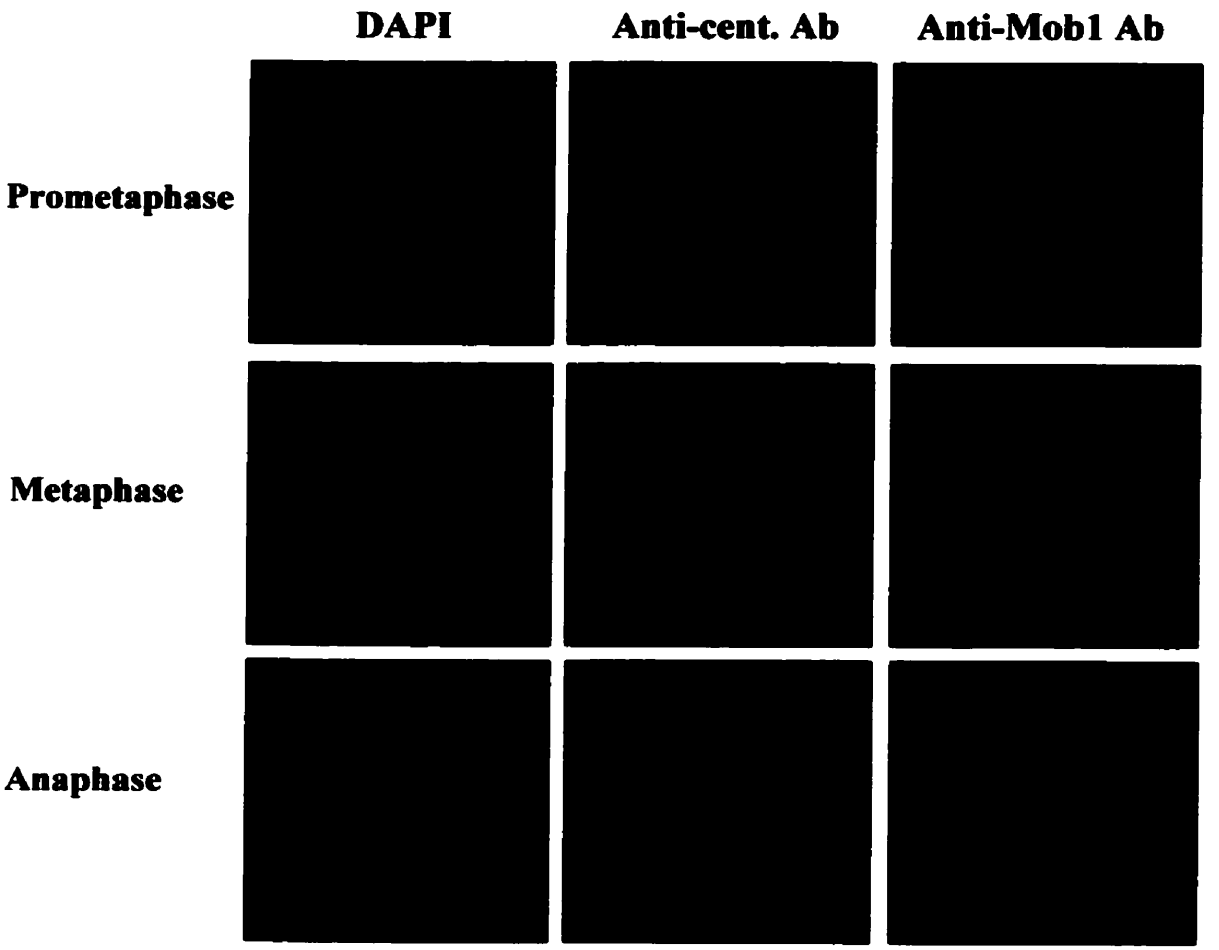
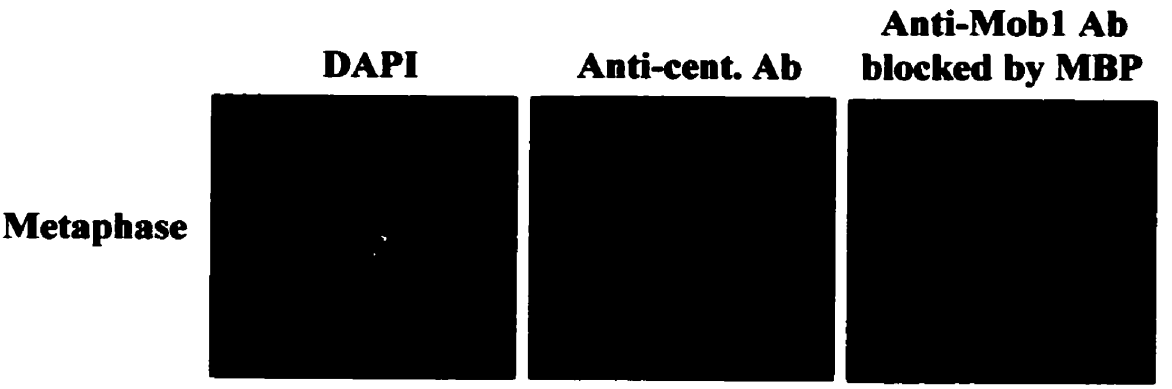

DAPI
Anti-cent. Ab
Anti-Mob1 Ab
Prometaphase
Metaphase
Anaphase

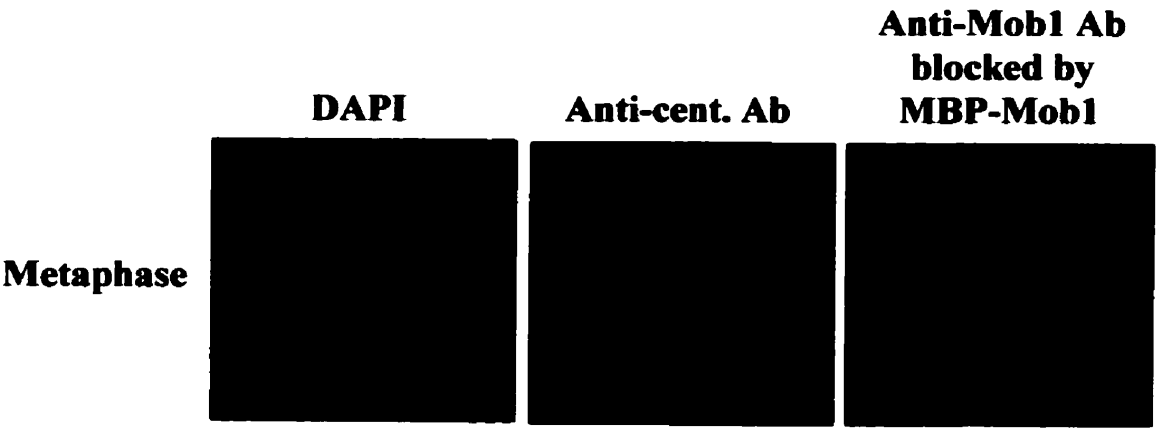
Figure 21. Control experiments for immunolocalization of Mob1 in HeLa cells.

Subcellular localization of MOB1p in human HeLa cells was examined by indirect immunofluorescence microscopy with anti-Mob1 polyclonal antibody (anti-Mob1 Ab) blocked by MBP alone (A) or MBP-Mob1 recombinant protein (B). The Mob1 signal was detected with a secondary antibody, goat anti-mouse IgG conjugated with Texas Red fluorochrome. The centrosomes were detected with human anti-centrosome autoimmune antiserum and stained with goat anti-human IgG conjugated with Cy2 fluorochrome. Nuclear DNA was stained with DAPI.

A.


DAPI
Anti-cent. Ab
Anti-Mob1 Ab blocked by MBP
Metaphase

B.


DAPI
Anti-cent. Ab
Anti-Mob1 Ab blocked by MBP-Mob1
Metaphase

centrosomal simply because they can localize to centrosome by IIF. However, assigning a protein as a centrosome component by IIF alone is not a rigorous criteria because the centrosome is a single focal point, and any minor amount of protein associated with this point would give the impression that the protein is associated with the centrosome by IIF observation. In addition, mitotic spindle poles serve as focal points for mitotic spindle nucleation, and many proteins that are not really centrosomal are probably fluxed to the centrosome by microtubule motors (Andersen, 1999). The minus end-directed microtubule motor proteins, for instance, are also localized to the centrosome (Kimble and Kuriyama, 1992). Therefore, it is important to distinguish between proteins that are integral components of the centrosome and the ones that are loosely associated with it. A series of experiments were designed to test whether Mob1 is a *bona fide* component of the centrosome. The experiments were designed to answer the following questions: (1) Is the Mob1 protein localization to centrosome spindle poles dependent upon microtubules? and (2) Is Mob1 tightly associated with the centrosomes?

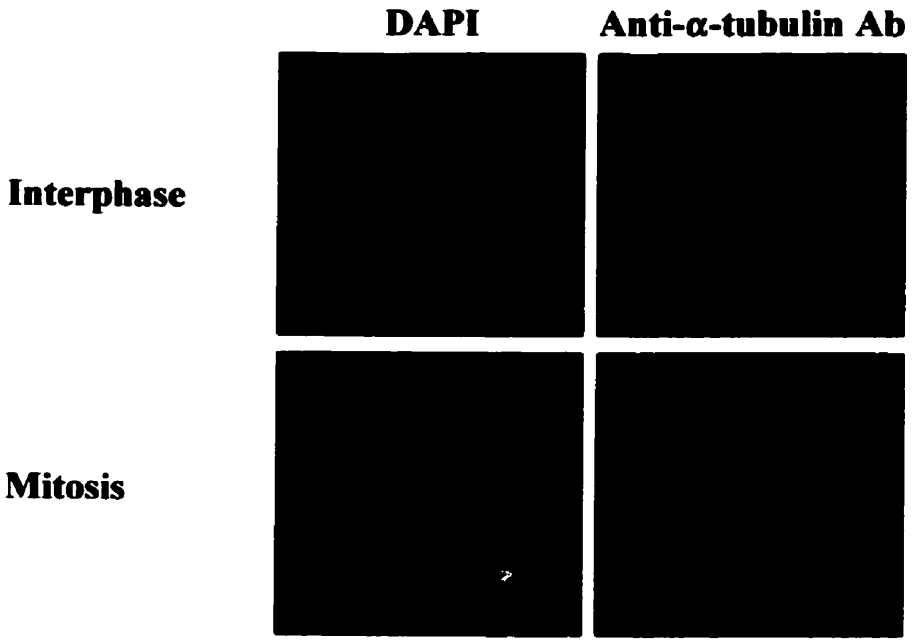
IV-A. Localization of Mob1 to Centrosomes Does Not Require Microtubules:

To determine whether the centrosomal association of Mob1 was dependent upon the continued presence of microtubules, immunostaining was performed on cells in which the microtubule network was disrupted. In cells treated with nocodazole for 4 hours, both interphase and mitotic microtubules were completely depolymerized (Figure 22). Yet, in those cells treated with nocodazole, Mob1 protein was found at interphase and mitotic centrosomes (Figure 23). These results suggested that Mob1 associates with centrosomes independently of microtubules, and by this criterion, Mob1 should be

Figure 22. Control experiment for nocodazole treatment in A431 cells.

Asynchronous A431 cells were grown in RPMI 1640 medium in the absence (A) or the presence (B) of 20 μg of nocodazole for 4 hours. Upon removal of the medium, the cells were extracted *in situ* with CSK buffer containing 0.15% Trition X-100 and fixed with 3.7% paraformaldehyde in CSK buffer. The microtubules were stained with mouse anti-α-tubulin antibodies (a gift from Dr. J.B. Rattner) and detected with a secondary antibody, goat anti-mouse IgG conjugated with Alexa 594 dye. Nuclear DNA was stained with DAPI.

A.


DAPI
Anti-α-tubulin Ab
Interphase
Mitosis

B.

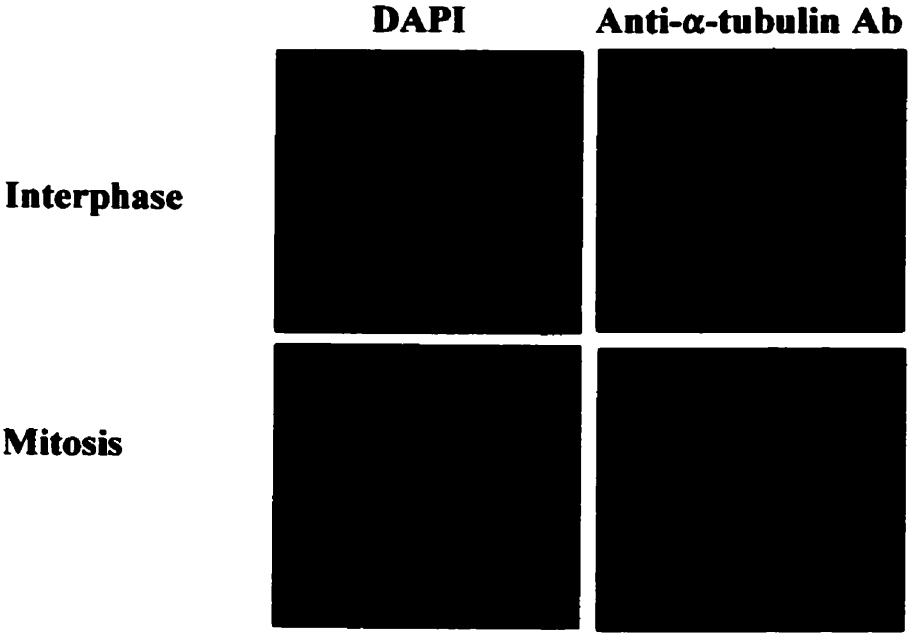
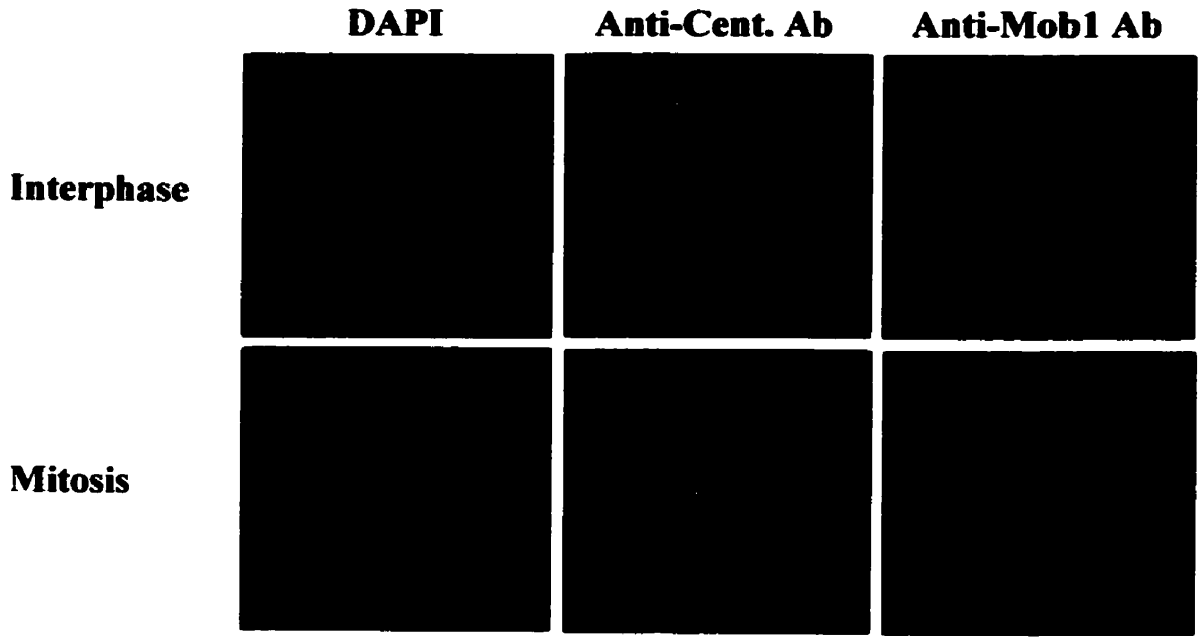

DAPI
Anti-α-tubulin Ab
Interphase
Mitosis

Figure 23. Immunolocalization of Mob1 to interphase centrosome and mitotic spindle poles in nocodazole-treated A431 cells.

Asynchronous A431 cells were grown in RPMI 1640 medium containing 20 μM nocodazole for 4 hours. Upon removal of the medium, the cells were extracted *in situ* with CSK buffer containing 0.15% Trition X-100 and fixed with 3.7% paraformaldehyde in CSK buffer. Mob1 was detected with 3-24-3 mAb (Anti-Mob1 Ab), followed by staining with a secondary antibody, goat anti-mouse IgG conjugated with Alexa 594 dye. The centrosomes were detected with M-4491 human autoimmune antibody (anti-cent. Ab) and stained with goat anti-human IgG secondary antibody conjugated with Cy2 dye. Nuclear DNA was stained with DAPI.


DAPI
Anti-Cent. Ab
Anti-Mob1 Ab
Interphase
Mitosis

consider as a integral component of the centrosome (Oegema *et al.*, 1995; Fry *et al.*, 1998; Roghi *et al.*, 1998; Freed *et al.*, 1999).

IV-B. Association of Mob1 With Centrosomes Is Resistant to Salt Extraction Prior To Fixation:

In order to determine whether Mob1 is a salt-stable centrosome component, A431 cells grown on microscopic slides were treated with various *in situ* extraction procedures before they were immunostained for Mob1 protein. These experimental results were summarized on Figure 24 and 25, showing representative cells in interphase (Figure 24) and mitosis (Figure 25). In each column the same cell was visualized by triple fluorescence labelling of DNA (column 1), centrosomes (column 2), and Mob1 (column 3). Mob1 protein remained bound to the salt-stable centrosome core in both interphase and mitotic A431 cells after 0.5 M NaCl extraction, providing evidence that Mob1 is tightly associated with the centrosome.

V. Classification of 3-24-3 mAb Isotype:

During the last year of my M. Sc. program, a collaboration was carried out with Dr. J.B. Rattner's laboratory to determine the function of Mob1 protein by microinjection of 3-24-3 mAb into HeLa cells. In order to obtain high quality of the mAb for injection, the 3-24-3 mAb was affinity purified from the cultural supernatant with protein A or G immobilized to agarose. These affinity columns were used to purify the antibody because (1) protein A agarose-bound 3-24-3 mAb was successful in immunoprecipitating the *in vitro* translated *Xenopus* Mob1 protein, as previously described, which implied that the

Figure 24. Immunolocalization of Mob1 to interphase centrosome in salt-extracted A431 cells.

Interphase A431 cells were extracted *in situ* with CSK buffer containing 0.15% Trition X-100 and various concentrations of NaCl as indicated on the left margin of the figure. The cells were fixed with 3.7% paraformaldehyde in CSK buffer. Mob1 was detected with 3-24-3 mAb (Anti-Mob1 Ab), and a secondary antibody, goat anti-mouse IgG conjugated with Alexa 594 dye. The centrosomes were detected with M-4491 human autoimmune antibody (anti-cent. Ab), and stained with goat anti-human IgG secondary antibody conjugated with Cy2 dye. Nuclear DNA was stained with DAPI.

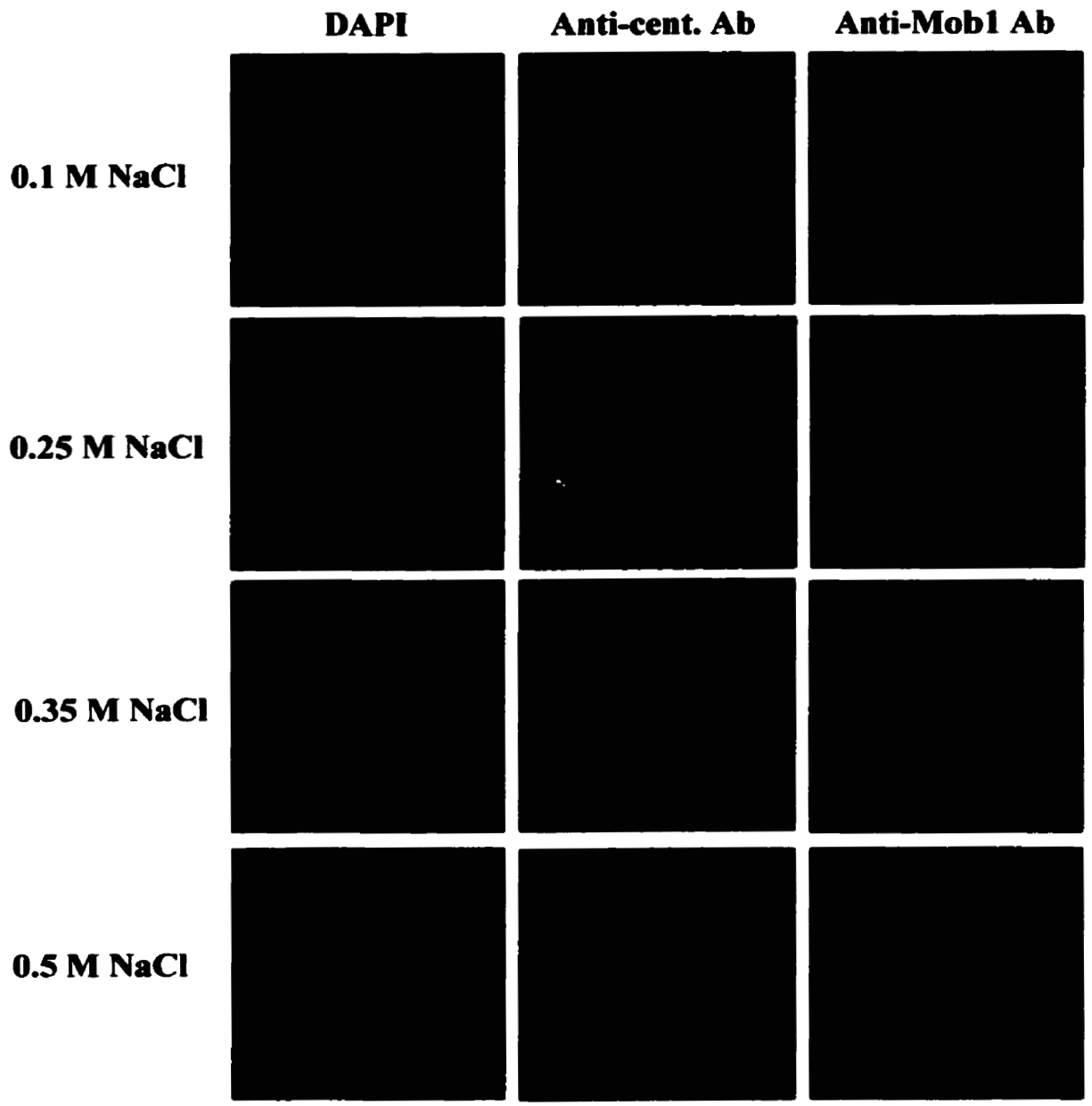
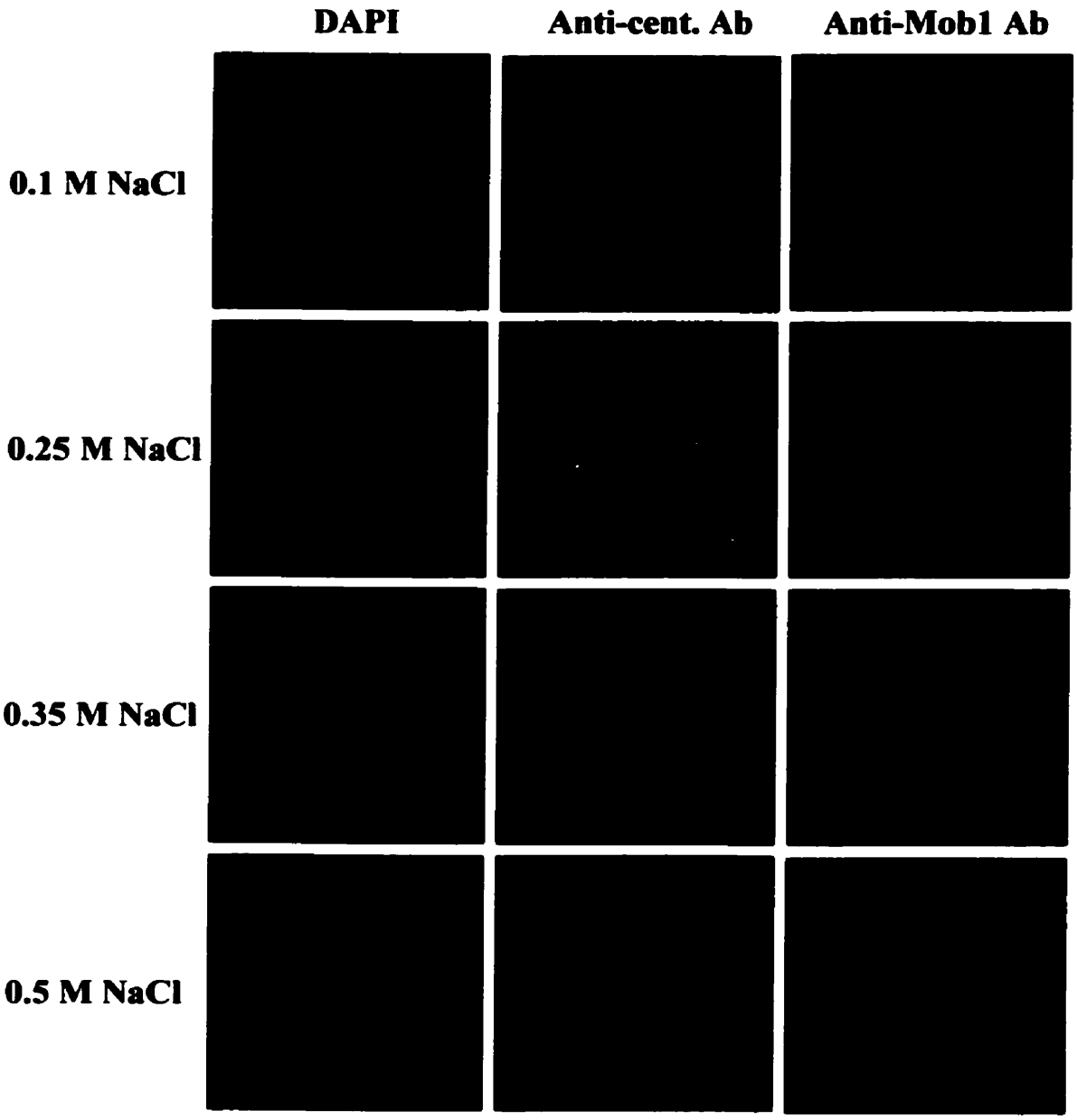

DAPI
Anti-cent. Ab
Anti-Mob1 Ab
0.1 M NaCl
0.25 M NaCl
0.35 M NaCl
0.5 M NaCl

Figure 25. Immunolocalization of Mob1 to mitotic spindle poles in salt-extracted A431 cells.

Mitotic A431 cells were extracted *in situ* with CSK buffer containing 0.15% Trition X-100 and various concentrations of NaCl as indicated on the left margin of the figure. The cells were fixed with 3.7% paraformaldehyde in CSK buffer. Mob1 was detected with 3-24-3 mAb (Anti-Mob1 Ab) and stained with a secondary antibody, goat anti-mouse IgG conjugated with Alexa 594 dye. The centrosomes were detected with M-4491 human autoimmune antibody (anti-cent. Ab) and stained with goat anti-human IgG secondary antibody conjugated with Cy2 dye. The mitotic stages were monitored by staining with DAPI.


DAPI
Anti-cent. Ab
Anti-Mob1 Ab
0.1 M NaCl
0.25 M NaCl
0.35 M NaCl
0.5 M NaCl

mAb is one of the IgG subclass, (2) the anti-mouse IgG secondary antibodies had shown to be specific for 3-24-3 mAb in A431 IIF results; and (3) protein G column has been shown to have higher affinity to mouse IgG subclasses than protein A agarose (Harlow and Lane, 1988). However, I encountered a great deal of difficulty during the monoclonal antibody purification using either affinity chromatographic method. The main difficulty was the loss of antibody function after low pH elution of the antiserum from the column. Thus, the isotype of 3-24-3 mAb was examined to determine whether knowing the isotype and subclass of the mAb would help in determining the best process for antibody purification.

V-A. 3-24-3 mAb Is an IgM Class:

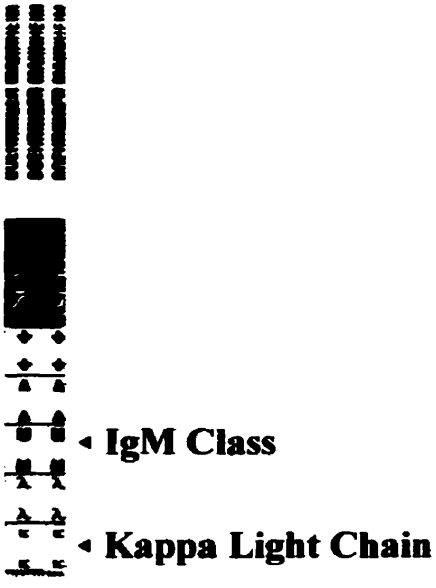
The culture medium supernatant of 3-24-3 mAb was diluted at 1:10 dilution in PBS and was used to develop the IsoStrip mouse monoclonal antibody isotyping kit (Roche Molecular Biochemicals) according to the manufacturer's instructions. The test result demonstrated that 3-24-3 mAb is an IgM class with a kappa light chain (Figure 26), and not an IgG as first believed.

V-B. Indirect Immunofluorescence Using Anti-IgM Secondary Antibodies Also Revealed that Mob1 Protein Localizes to Centrosomes in Human A431 Cells Throughout the Cell Cycle:

Since the first series of experiment described in section III used goat anti-mouse IgG secondary antibody conjugated with Alexa 594 dye to detect the presence of Mob1 immunoconjugate, a goat anti-mouse IgM secondary antibody conjugated with Alexa 594 fluorochrome was used to confirm the Mob1 IIF results. Again, A431 cells were used for

Figure 26. The immunoglobulin of 3-24-3 monoclonal antibody is an IgM class.

The isotype of 3-24-3 mAb was classified by the IsoStrip mouse monoclonal antibody isotyping kit (Roche Molecular Biochemicals). The test result demonstrated that 3-24-3 mAb is an immunoglobulin M class with a kappa light chain


IgM Class
Kappa Light Chain

this purpose and the cells were processed for IIF as previously described. with the exception that goat anti-mouse IgM secondary antibody was used instead of the goat anti-mouse IgG, and anti-γ-tubulin antibody was used instead of the human auto-immune serum as the centrosome marker. Similar to the observations obtained using anti-IgG antibody, anti-IgM antiserum also detected Mob1 at interphase centrosomes (Figure 27) and at mitotic spindle poles (Figure 28). In addition, Mob1 was also found to localize to the intercellular bridge connecting two newly divided cells in telophase (Figure 28). Using anti-IgM antibody, Mob1 staining also increased as cells entered mitosis, with the strongest staining during metaphase and early anaphase, and then decreased by telophase. No new staining pattern was observed with anti-IgM antibody compared to the anti-IgG antiserum used previously, suggesting that the IIF results obtained with anti-IgG antibody were valid.

However, the use of 3-24-3 mAb with an anti-mouse IgM secondary antibody to immunoblot the bacterially-expressed antigen still showed a negative result (data not shown), suggesting that the antibody was able to recognize the native Mob1 protein but not the denatured protein.

VI. Summary of the Results:

1. The screening a *Xenopus laevis* oocyte cDNA library with a set of degenerate oligonucleotides designed from the conserved regions of yeast *MOB1* and its human homologs resulted in the isolation of a full-length *Xenopus MOB1* homolog.

2. The predicted sequence of XlMob1 protein is highly conserved when compared

Figure 27. Indirect immunofluorescence of interphase A431 cells using anti-IgM secondary antibodies revealed that Mob1 is localized to centrosome.

Human A431 interphase cells were extracted *in situ* with 0.15% Triton X-100 in PBS, fixed with 3.7% paraformaldehyde, and processed for IIF. Mob1 was detected by 3-24-3 mAb (Anti-Mob1 Ab) and a secondary antibody, goat anti-mouse IgM conjugated with Alex 594 fluorochrome. The centrosomes were detected with rabbit polyclonal anti-γ-tubulin antibodies (Anti-γ-tubulin Ab) and stained with goat anti-rabbit IgG conjugated with FITC fluorochrome. Nuclear DNA was stained with DAPI.

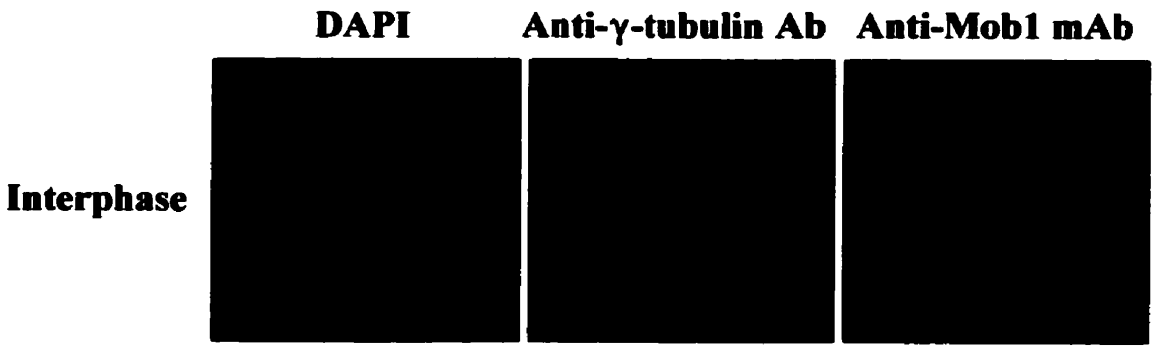
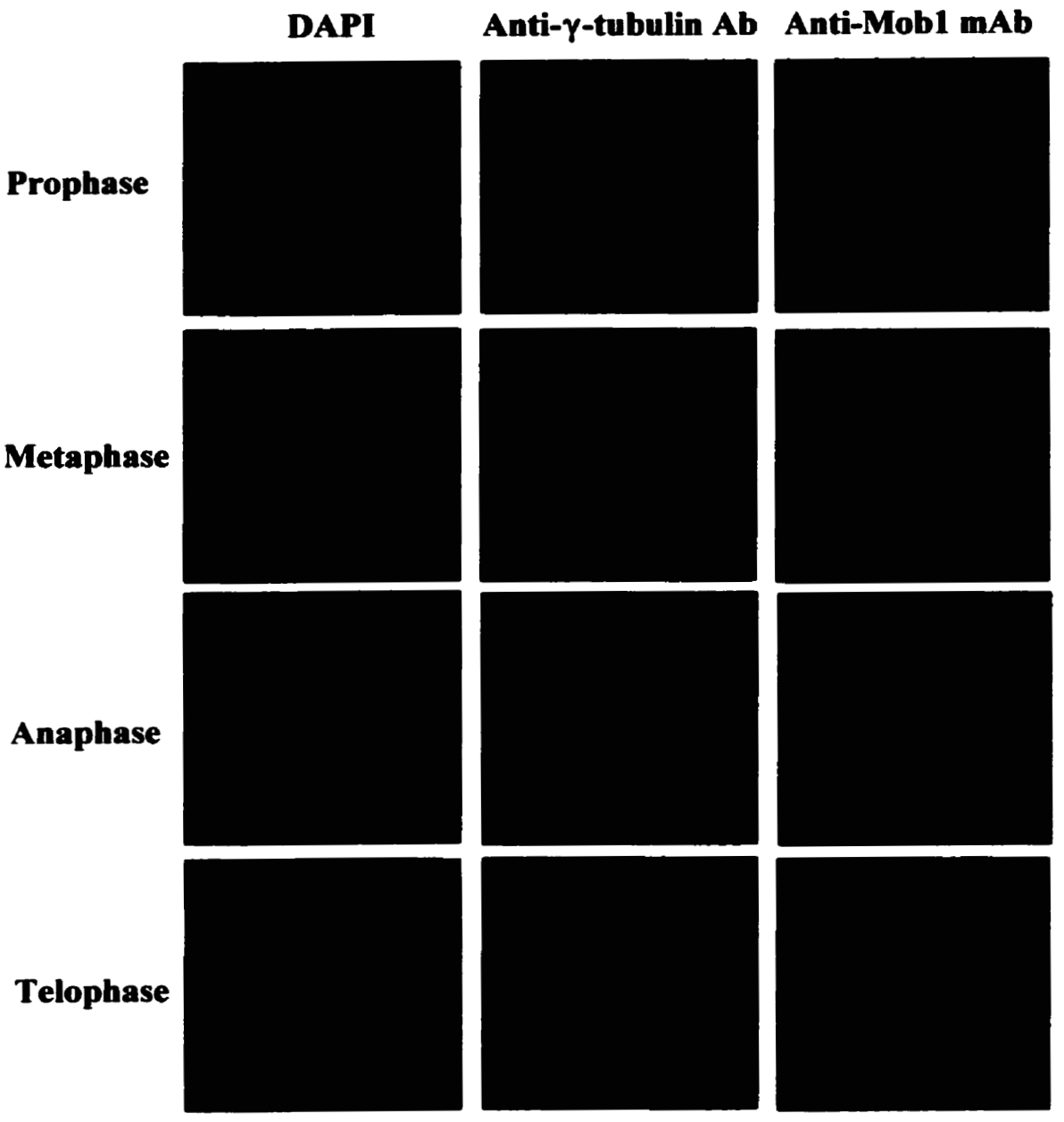

DAPI
Anti-γ-tubulin Ab
Anti-Mob1 mAb
Interphase

Figure 28. Indirect immunofluorescence of mitotic A431 cells using anti-IgM secondary antibodies revealed that Mob1 is located at spindle poles throughout the mitotic stages and at the intercellular bridge in telophase.

Human A431 mitotic cells were extracted *in situ* with 0.15% Triton X-100 in PBS, fixed with 3.7% paraformaldehyde, and processed for IIF. Mob1 was detected by 3-24-3 mAb (Anti-Mob1 Ab) and a secondary antibody, goat anti-mouse IgM conjugated with Alexa 594 fluorochrome. The centrosomes were detected with rabbit polyclonal anti-γ-tubulin antibodies (Anti-γ-tubulin Ab) and stained with goat anti-rabbit IgG conjugated with FITC fluorochrome. The mitotic stages were monitored by staining with DAPI.


DAPI
Anti-γ-tubulin Ab
Anti-Mob1 mAb
Prophase
Metaphase
Anaphase
Telophase

to *S. cerevisiae* Mob1 protein and human Mob1 protein, and possesses a cullin-homolog domain.

3. Both polyclonal and monoclonal antibodies against Mob1 protein were raised in mice and their specificities were characterized.

4. The Mob1 homologs had a molecular weight of approximately 17 kDa and 26 kDa in *Xenopus* A6 cell extracts and human A431 cell extracts, respectively.

5. Mob1 protein was observed at interphase centrosomes and mitotic spindle poles in both *Xenopus* A6 cells and human A431 cells. The centrosome staining was greatest at metaphase. In addition, Mob1 protein was observed at intercellular bridge in telophase cells.

6. Mob1 protein was observed to be a centrosome component resistant to NaCl and nocodazole treatments.

CHAPTER FOUR

Discussion

Studies of the centrosome began more than a hundred years ago, but it is likely that some of its components still await identification, and the mechanisms regulating centrosome function during the cell cycle remain largely unknown. Recently, rapid progress has been made on the identification of proteins present in SPBs purified from the yeast *Saccharomyces cerevisiae*, by combining genomic sequence information with analytical mass spectrometry (Andersen, 1999). Even though there are substantial morphological differences between fungal SPBs and higher eukaryotic centrosomes, at least four similarities can be observed when comparing the yeast SPB and the vertebrate centrosome. First, the process of microtubule nucleation is conserved between the SPB and the centrosome. Second, the machinery that regulates SPB duplication also has homologous components in the centrosome duplication machinery. Third, both SPB and centrosome are dynamic in structure and composition. Fourth, many yeast SPB components have homologs that have been conserved during evolution. These similarities suggest that the yeast SPB and mammalian centrosome are functionally equivalent structures with many similarities in their compositions. However, the functions of many of the SPB components that have been identified at present remain obscured and the universality of these proteins still need to be elucidated. Hence, the approach for identifying novel centrosome components that are homologous to yeast SPB components may provide better understanding of the universal machinery of the centrosome.

I have used the *S. cerevisiae* Mob1 protein sequence to identify related proteins in a variety of organisms by searching the EST databases. The conserved domain was used as a template in a hybridizational screen of a *Xenopus laevis* oocyte cDNA library. Through this approach, I have identified XlMob1, a novel low molecular weight component of the centrosome. Immunocytological studies demonstrated that Mob1 co-localizes with proteins recognized by a human centrosomal autoimmune sera and with gamma tubulin at the centrosomes in *Xenopus* A6, and human A431 and HeLa cells, suggesting that Mob1 proteins are conserved during evolution. Since the localization of Mob1 to the centrosome is not dependent on the presence of microtubules and is resistant to high salt extraction, these results support the hypothesis that Mob1 protein is a novel integral centrosomal component in vertebrates.

I. Cloning and Sequence Analysis of *Xenopus MOB1* cDNA:

In *S. cerevisiae*, *MOB1* is an essential gene required for the completion of mitosis and for the maintenance of ploidy (Luca and Winey, 1998). Analysis of EST databases suggested that *MOB1* is a member of a conserved gene family, likely to be present in all eukaryotes. However, the core function of budding yeast Mob1 and its homolog in vertebrates were not identified. Thus, Mr. Wu and I initiated the project to clone the S. cerevisiae *MOB1* homolog in *Xenopus* by screening a *X. laevis* oocyte cDNA library. After obtaining a positive clone from the library, the cDNA was sequenced from both the 5' and 3' ends using specific primers to ensure the accuracy of the sequencing results (Figure 2). The *Xenopus MOB1* cDNA that we isolated consists of 1,349 nucleotides and the single open reading frame encodes a protein of 149 amino acids (Figure 3).

The 3' UTR of *Xenopus MOB1* cDNA contains a polyadenylation signal and a potential consensus sequence for cytoplasmic polyadenylation during oocyte maturation, suggesting that Mob1 may be synthesized during oocyte maturation (Roghi *et al,* 1998).

I-A. Amino Acid Sequence Alignement of Mob1-Related Proteins:

Protein sequence alignments showed that the central region and the C-terminus of the *Xenopus* Mob1 share a high degree of identity with the Mob1 in *S. cerevisae*, *S. pombe* and a human (Figure 4). However, many gaps were introduced into the *Xenopus* Mob1 sequence while aligning the Mob1-related proteins together. Thus, in order to appreciate the degree of homology shared between *Xenopus* Mob1 and other Mob1-like proteins, I compared the *Xenopus* Mob1 sequence with each of the Mob-like protein separately and attached the alignment results in an appendix section. As shown in Appendix I, *Xenopus* Mob1 shares approximately 40% identity with the *S. cerevisiae* Mob1 (GenBank acession No. P40484). The most conserved region in these two proteins is located between amino acid residue 49 and amino acid residue 138 of the frog Mob1. In addition, *Xenopus* Mob1 shares approximately 50% identity with a *MOB1*-related gene product in *Drosophila melanogaster* (GenBank acession No. AAF50052). The homology domain is located from amino acid residue 41 to amino acid residue 146 of *Xenopus* Mob1 (Appendix II). Strikingly, the frog Mob1 shares about 75% identity with the predicted human *MOB1* product identified in Dr. J.B. Rattner's lab. These two sequences are most homologous between amino acid residue 42 and amino acid residue 140 of *Xenopus* Mob1 protein (Appendix III). The high degree of conservation among Mob1-like proteins identified from a variety of organisms suggests that these proteins form a

protein family, like cyclins and Cdks, and that their function may also be conserved across species.

The *S. cerevisiae* and human Mob1 have predicted molecular size of 36 kDa (Luca and Winey, 1998) and 26 kDa (Dr. J.B. Rattner's unpublished result), respectively, whereas the theoretical molecular size of *X. laevis* Mob1 is only 17 kDa. Amino acid sequence comparisons showed that the *Xenopus* Mob1 lacks the C-terminal region of yeast and human Mob1 (Figure 4). A possible reason for this difference is that the *S. cerevisiae* and human Mob1 C-terminus may perform functions not seen in XlMob1 protein such as interacting with proteins that are species specific. In budding yeast, for example, Mob1 interacts with Mps1 (Luca and Winey, 1998) and Dbf2 (Komarnitsky *et al.*, 1998), but these two proteins have not been identified in vertebrates. Although reason for the discrepancy of these proteins is unknown, the high degree of conservation in Mob1-related sequences suggests that the core function of these proteins may be conserved across species.

I-B. Identification of a Cullin Homolog Region in Mob1-Related Proteins:

The predicted amino acid sequences of the *MOB1*-like gene products offer very little insight to their molecular function. Database search for the functional domains of *Xenopus* Mob1 have identified a region located at the last 25 amino acid residues of the C-terminus that is homologous to cullin homolog (CH) domain, which is the potential functional domain for regulating the ubiquitination of G1 phase cyclins and cyclin-dependent kinase inhibitors (Figure 5). The CH domain was also found in yeast and human Mob1 proteins (Appendix IV). Cullin is a subunit of SCF ubiquitin-ligase

complex that regulates centrosome duplication, possibly through mediating the cyclin E degradation in centriole separation (Freed *et al.*, 1999; Wang *et al.*, 1999). The biochemical purification of centrosomes demonstrated the presence of Cul1, a mammalian cullin family member, suggesting that Cul1 is also a core component of centrosomes (Freed *et al.*, 1999). Like Mob1, indirect immunolocalization studies showed that Cul1 was localized to centrosomes during interphase and to spindle poles during mitosis. In addition, Cul1 has also been shown to localize to the intercellular bridge in telophase. Together, the presence of a CH domain in Mob1 protein and the coincidence of immunolocalization pattern suggest that Mob1 protein, like Cul1, may play a role in regulating centrosome duplication.

Even though the *Xenopus* Mob1 protein consists of CH domain, it is very unlikely that the 3-24-3 mAb would recognize cullin proteins and that the immunolocalization staining pattern with the mAb is due to recognizing cullin protein. This is because the polyclonal antibodies showed similar immunolocalization staining pattern of Mob1 protein as the 3-24-3 mAb (Figure 16 and 20), and because the MBP blocked polyclonal antibodies detected a single protein of 17 kDa in *Xenopus* A6 cell lysate on immunoblots (Figure 9).

I-C. *Xenopus* Mob1 is a Candidate Phosphoprotein:

Phosphorylation seems to be an important mechanism for regulating the microtubule nucleating capability of the centrosome at mitosis (reviewed by Whitehead and Salisbury, 1999; Mayor *et al.*, 1999). Among the large number of centrosome components, several are phosphoproteins, protein kinases and protein phosphatases

(Vandré *et al.*, 1984; Whitehead and Salisbury, 1999). Protein kinase activities have been shown to play a central role in mediating the transition between interphase and mitosis (Karsenti *et al.*, 1984). For instance, phosphorylation of *Xenopus* interphase extracts by mitotic Cdc2 kinase resulted in higher dynamics of microtubule nucleation (Ohta *et al.*, 1993).

In budding yeast, Mob1 was shown to be a phosphoprotein *in vivo* and could be phosphorylated by Mps1 *in vitro* (Luca and Winey, 1998). The phosphorylation level of Mob1 is higher during mitosis than in interphase (Luca, personal communication). Moreover, analysis of the amino acid composition of the *Xenopus* Mob1 protein showed that serine and threonine are the major amino acids (together making up approximately 21% of *Xenopus* Mob1 amino acid residues; Appendix V). A computer search for consensus phosphorylation motifs in Mob1 identified serveral protein kinase recognition sequences, including potential sites for phosphorylation by multifunctional calmodulin-dependent kinase; protein kinase C, myosin 1 heavy chain kinase, casein kinase I and II, and cGMP- and cAMP-dependent protein kinase.

Another possible protein kinase that could phosphorylate Mob1 is the Polo-like protein kinase (Plk). Plks are regulators of cell cycle progression and named after the *POLO* gene of *Drosophila melanogaster*. Members of this family are now being studied in many species including yeast, frog, and human (reviewed by Glover *et al.*, 1998; Nigg, 1998). All of the Plks examined so far share the common property of localization to spindle poles during early mitosis and to the intercellular bridge during cytokinesis (Shirayama *et al.*, 1998; Logarinho and Sunkel, 1998; Qian *et al.*, 1999). The similarities

in protein localization patterns between Mob1 and Plks suppots the notion that Mob1 is a possible substrate of Plks during mitosis and that the localization of Mob1 may be regulated by phosphorylation. Indeed, the *S. cerevisiae MOB1* exhibited genetic interaction with *CDC5*, the budding yeast *PLK* homolog (Luca and Winey, 1998), and mutation of *CDC5* reduced the phosphorylation level of Mob1 (Luca, unpublished result). Nevertheless, according to my knowledge, the consensus phosphorylation sequence for Plks is not known. Therefore, I cannot predict whether there is a Plk phosphorylation site on *Xenopus* Mob1 protein by searching the Mob1 sequence.

II. Production and Characterization of Anti-Mob1 Antisera:

II-A. Generation and Characterization of Mouse Anti-Mob1 Polyclonal Antibodies:

In order to further characterize the *Xenopus* Mob1 protein, specific antisera were raised in mice. The polyclonal antibodies were shown to be specific for Mob1 protein by: (1) immunoprecipitation of *in vitro* translated *Xenopus* Mob1 (Figure 7); (2) the ability to immunblot the Mob1 protein cleaved from the MBP tag (Figure 8); and (3) the ability to detect the GST-tagged human Mob1 protein, but not the GST alone, by immunoblot (Figure 11a). In addition, these antibodies recognized a 17 kDa (Figure 9) and a 26 kDa (Figure 11b) proteins in asynchronous *Xenopus* A6 and human A431 cell extracts, respectively, which are in good agreement with the theoretical molecular masses of Mob1 in these cells. The weak immunoblot signals suggest that Mob1 is a low abundance protein in cells. The presence of 26 kDa band was also detected in purified centrosomes from HeLa cells (Ou and Rattner, unpublished result). The polyclonal antibodies detected the presence of Mob1 protein in mitotic spindle poles of A6 and HeLa cells by IIF

technique. Taken together, these data suggest that the polyclonal anti-Mob1 antisera raised are specific for Mob1 protein. The coincidence in molecular size for both endogenous and *in vitro* translated *Xenopus* Mob1 proteins suggests that the isolated *Xenopus MOB1* cDNA encodes a full-length version of the protein.

II-B. Generation and Characterization of Mouse Anti-Mob1 Monoclonal Antibodies:

Subsequently, Ms. Warren and I generated a mouse monoclonal antibody against the Mob1 protein. We used indirect immunofluorescence to screen for positive clones during the process of making the antibody. As a result, we have obtained a positive clone designated as 3-24-3. The antibody was able to immunoprecipitate the *in vitro* translated *Xenopus* Mob1 (Figure 7), but cannot be used for immunoblotting (Figure 8). As pointed out by Benjamin *et al.* (1984) and Laver *et al.* (1990), the great majority of the antigenic determinants recognized by antibodies are conformational, thereby, it is likely that the antigenic determinant recognized by 3-24-3 mAb is a conformational epitope since we used the native antigen to raise the antibodies and the antigen structure may be preserved during the screening processes by IIF technique (Harlow and Lane, 1988; Pines, 1997). However, using of the 3-24-3 mAb in IIF detected Mob1 protein in mitotic spindle poles of frog and human tissue culture cells, similar to the findings when polyclonal antisera were used. Taken together, the immunoprecipitation and IIF results suggest that the 3-24-3 mAb is able to recognize Mob1 protein specifically. Furthermore, the ability of 3-24-3 mAb to immunolocalize the Mob1 protein in *Xenopus* and human cells suggests that the epitope recognized by the antibody is located in the conserved region.

The mAb could be precipitated by protein A agarose in immunoprecipitation experiments and detected by goat anti-IgG (heavy + light chains) secondary antibody in IIF experiments, suggesting that the antibody is a subclass of immunoglobulin G antibody. The isotype analysis, however, indicated that the 3-24-3 mAb is an immunoglobulin M antibody with kappa light chain (Figure 26). Two possible reasons could account for the cross-reaction between the anti-IgG secondary antibody and the IgM 3-24-3 antibody. First, both mouse immunoglobulin G and M molecules are made up of different heavy chain, but possess either kappa or lambda light chains (Harlow and Lane, 1988). Since the secondary antibodies used were polyclonal antibodies and were able to recognize both heavy and light chains, it is very likely that the antibodies recognized the IgG light chains and bound to the kappa light chain of the 3-24-3 mAb. Second, the immunoglobulin G molecule is a single valency structure, whereas the immunoglobulin M is a pentameric or hexameric molecule. Hence, the large number of binding sites in IgM will greatly increase the total strength of binding, which in turn increases the chance of cross-reaction with the anti-IgG antibody. However, no difference in staining pattern was observed by comparing the IIF results obtained from the anti-IgG antibody (Figure 15 and 16) with the anti-IgM antibody (Figure 27 and 28), suggesting that both antibodies were able to recognize the 3-24-3 mAb specifically and that the observations made with 3-24-3 were valid.

III. Immunolocalization Studies of Mob1 in Vertebrate Cells:

In budding yeast, conditional Mob1 mutants arrested in late nuclear division with long bipolar mitotic spindles, and large-budded cells with separated chromatin masses

and an increase-in-ploidy phenotype (Luca and Winey, 1998). In addition, the budding yeast Mob1 protein has been shown to transiently localize to SPBs in late mitosis and to a ring at the bud neck at the end of mitosis, where cytokinesis takes place, suggesting that Mob1 protein may regulate cytokinesis (Luca, unpublished result). Indeed, strains harboring conditional *mob1* alleles displayed dramatic cytokinesis defects (Luca, unpublished result). On the other hand, fission yeast Mob1 homolog was shown to localize at interphase SPB (Hou *et al,* 2000) and mitotic SPBs, and play a essential role in septum formation (Hou *et al,* 2000; Salimova *et al,* 2000). In order to obtain insights into the function of the vertebrate Mob1 homologs, indirect immunofluorescence was used to examine the subcellular localization of Mob1 protein in *Xenopus* A6, and human HeLa and A431 culture cells.

III-A. Subcellular Localization Studies of *Xenopus* A6 Cells:

Since both budding and fission yeast Mob1 proteins were shown to localize to SPBs during mitosis, it is very possible that the vertebrate Mob1 homologs are also found at the mitotic spindle poles. *Xenopus* A6 cells were extracted *in situ* to provide better view of centrosomal structure and processed for indirect immunofluorescence studies. As a result, *Xenopus* Mob1 protein was found to co-localize with the human anti-centrosome autoimmune sera at interphase centrosome (Figure 12) and at mitotic spindle poles (Figure 13) by staining with the 3-24-3 mAb. The specificity of the monoclonal antibody was demonstrated by blocking the antibody with either MBP or the recombinant Mob1 protein before applying for IIF (Figure 14). The fusion Mob1 protein was able to block the staining, whereas the staining was positive after MBP blocking, suggesting that the 3-

24-3 mAb was able to detect the *Xenopus* Mob1 protein at centrosomes specifically. The distribution of *Xenopus* Mob1 at centrosomes in both interphase and mitosis suggests that the protein may play a role in centrosome function. Since cells were extracted with non-ionic detergent before fixing for IIF, the mitotic cells were flattened onto the coverslips. Therefore, the brightness of immunostaining may reflect the highest protein concentration. During mitosis, Mob1 staining was found to increase from prophase to metaphase and reduced at late anaphase. Together, with the observation that Mob1 staining was more pronounced at mitotic spindle poles than at interphase centrosome, these data suggest that (1) a greater recruitment of Mob1 protein to spindle poles during mitosis; (2) Mob1 and another centrosomal proteins are recruited equally when the centrosomes enlarge in mitosis; (3) the synthesis of *MOB1* mRNA is cell cycle regulated; or (4) an increase in *MOB1* mRNA translation coupled with a recruitment to the spindle poles during mitosis. These possibilities, however, cannot be distinguished because I may only be examining a subset of total Mob1 protein pool by extracting cells with non-ionic detergent before fixation. These cells were extracted *in situ* to provide better view of the Mob1 subcellular localization, since staining was observed everywhere in the cells that were fixed and then permeabilized.

III-B. Subcellular Localization Studies of Human A431 Cells:

The subcellular localization of Mob1 protein was also examined in human A431 cells. In these cells, the Mob1 protein was shown to co-localized with the human anti-centrosome autoimmune sera and γ-tubulin at the interphase centrosome (Figure 15 and 27) and at the mitotic spindle poles (Figure 16 and 28). During interphase, the presence

of Mob1 was detected as a single focal point and two foci, indicating that the protein is present before and after centrosome duplication (Figure 15). The degree of centrosome staining at single or duplicated centrosomes seems to be the same, suggesting that the protein level of Mob1 at interphase centrosomes remains constant.

Mob1 staining at centrosomes increased as cells entered mitosis, with the greatest staining at metaphase and lowest level at telophase. Moreover, the mammalian Mob1 protein was seen at the intercellular bridge in telophase (Figure 16 and 28). The intercellular bridge staining was also observed in A6 cells as illustrated in Figure 19, suggesting that the staining pattern is conserved. The reduction of Mob1 staining at late mitotic spindle poles and its appearance at intercellular bridge in telophase suggest that: (1) the protein moved from the poles to the intercellular bridge region; or (2) the protein located at the intercellular bridge represents a new Mob1 protein pool recruited to this site. These two possibilities cannot be distinguished because I may only observe a fraction of total Mob1 protein pool after the cells were *in situ* extracted. In order to distinguish these two possibilities, real-time images of a GFP tagged Mob1 fusion protein taken by time lapse microscopy may provide better insights into the movement of Mob1 protein in cells. A number of mitotic exit regulators, such as Plks (Qian *et al.*, 1999) and a subunit of anaphase-promoting complex (Kurasawa and Todokoro, 1999), have also been shown to localize at mitotic spindle poles during early mitosis and at intercellular bridge during cytokinesis. In budding yeast, for example, phosphorylation of Mob1 by Cdc5 seems to play a role in localization of Mob1 to bud neck and thereby regulating the mitotic exit event (Luca, unpublished results). Whether the vertebrate Mob1 protein

undergoes same regulation as the *S. cerevisiae* Mob1 at late mitosis still awaits future studies. It is also noteworthy that often I observed the metaphase spindle poles stained like V shape, presumably staining the outer microtubules of the spindle which extend from the pole, when A431 cells were stained with anti-Mob1 antibodies (Figure 16).

The temporal and spatial distribution of Mob1 protein to centrosomes during the cell cycle are similar to the observations of centrosome proteins such as tektins (Hinchcliffe and Linck 1998), protein 4.1 (Krauss *et al.*, 1997a; Krauss *et al.*, 1997b), Nek2 (Fry *et al.*, 1998b; Fry *et al.*, 2000), C-Nap1 (Fry *et al.*, 1998a), centrin (Salisbury,1995), and γ-tubulin (Schnackenberg and Palazzo, 1999.). Like Mob1 protein, in addition, C-Nap1 (Mack *et al.*, 2000), protein 4.1 (Krauss *et al.*, 1997b) and γ-tubulin (Julian *et al.*, 1993) have been demonstrated to localize to intercellular bridge during cytokinesis. However, Mob1 protein localizes to centrosomes throughout the entire cell cycle and is strongly detected at metaphase spindle poles, whereas the localization of C-Nap1 to centrosomes occurs in a cell cycle dependent manner and is weakly detected at the mitotic spindle poles (Fry *et al.*, 1998a; Mack *et al.*, 2000). Moreover, centrin stains one spindle pole more intensely than the other (Salisbury,1995), whereas the Mob1 staining intensity between the two mitotic spindle poles remains the same, suggesting that the protein is equally distributed between the two mitotic spindle poles.

In order to demonstrate that the localization of Mob1 to centrosomes can be seen with more than one antibody, I have also studied the distribution of Mob1 in HeLa cells using mouse polyclonal antibodies. As shown in Figure 20 and 21, the polyclonal anti-Mob1 antibodies specifically detected the presence of Mob1 in mitotic spindle poles.

Together with the immunolocalization results obtained from *Xenopus* A6 and human A431 cells, these data suggest that the observation obtained with the 3-24-3 mAb are valid, and Mob1 is localized to interphase centrosome and mitotic spindle poles of vertebrate cells.

IV. Mob1 Protein is a Novel Centrosome Component:

The A431 cells were treated with nocodazole (Figure 22 and 23) or permeabilized cells were treated with high salt buffers (Figure 24 and 25) before fixation to examine the localization of Mob1 after microtubule disruption or after the removal of loosely-bound centrosome components, respectively. Localization of Mob1 to the interphase centrosome and to mitotic spindle poles is resistant to NaCl extraction, suggesting that the protein is tightly associated with the centrosome component. Also, Mob1 was found at the centrosome of interphase cells and spindle poles of mitotic cells after treating the cells with nocodazole, indicating that the localization of Mob1 protein to centrosomes is independent of microtubules. Moreover, immunoblotting of the biochemically purified HeLa centrosomes with anti-Mob1 polyclonal antisera had identified a protein at 26 kDa region (Dr. J.B. Rattner's unpublished result). These data together suggest that Mob1 is a novel centrosome component identified in vertebrate cells.

V. Future Perspectives on Mob1:

The *in situ* extraction technique used in immunolocalization studies allows better visualization of centrosomes in rounded, mitotic cells by removing most soluble proteins

and membranes. However, this method only permits the observation of a fraction of the total protein pool. Thus, it is not clear whether all of the Mob1 proteins are located at centrosomes or some of them remained in cytoplasm. Examination of cells that are fixed and permeabilized for conventional IIF under confocal microscopy may provide better understanding of the overall distribution of Mob1 proteins in cells.

In budding yeast, Mob1 protein was demonstrated to interact with Dbf2 protein by yeast-two hybrid screen and co-immunoprecipitation (Komarnitsky *et. al.*, 1998). In fission yeast, on the other hand, Mob1 protein was shown to form a complex with Sid2 protein by immunoprecipitation (Salimova *et. al.*, 2000; Hou *et. al.*, 2000). Together, these data suggest that Mob1 may play a role in late mitosis. Currently, the identity of proteins associated with vertebrate Mob1 is unclear. To determine if there are proteins associated with vertebrate Mob1, we could immunoprecipitate the endogenous Mob1 protein from ^{35}S-labelled *Xenopus* or human tissue culture cells with the 3-24-3 mAb, and analyse the resultant precipitate by SDS-PAGE and autoradiography. Further, we could identify the Mob1-interacting proteins by either yeast-two hybrid assay using vertebrate Mob1 as a bait, or by purifying the vertebrate Mob1 complex with column chromatography and analyse the composition of the complex by mass spectrometry or protein sequence. The results from these experiments will provide answer to which proteins are associated with Mob1 protein and may give better insights into the function of vertebrate Mob1 protein.

Even though it may be a little too early to discuss the function of Mob1 protein, some speculations can be made based on our data, as well as the data reported by other

research groups. The centrosome cycle requires the duplication of centrioles and accumulation of PCM, separation of duplicated centrosomes at prophase to form the poles of the mitotic spindle, and segregation of centrosomes to daughter cells at mitotic exit. The mammalian cullin protein, Cul1, has been shown to localize to centrosomes and intercellular bridge like Mob1, and plays an essential role in the centrosome duplication cycle (Freed *et al*, 1999). Both Mob1 and Cul1 proteins consist of a consensus CH domain in addition of having similar immunolocalization profiles. As a result, one may speculate that Mob1 protein plays a role in regulating the centrosome duplication machinery. Indeed, preliminary results showed that when affinity purified monoclonal anti-Mob1 antibodies were microinjected into mitotic HeLa cells, the cells proceeded to the next cell cycle, but centrosome duplication was delayed (Ou and Wang, unpublished result), suggesting that Mob1 protein regulates centrosome duplication cycle.

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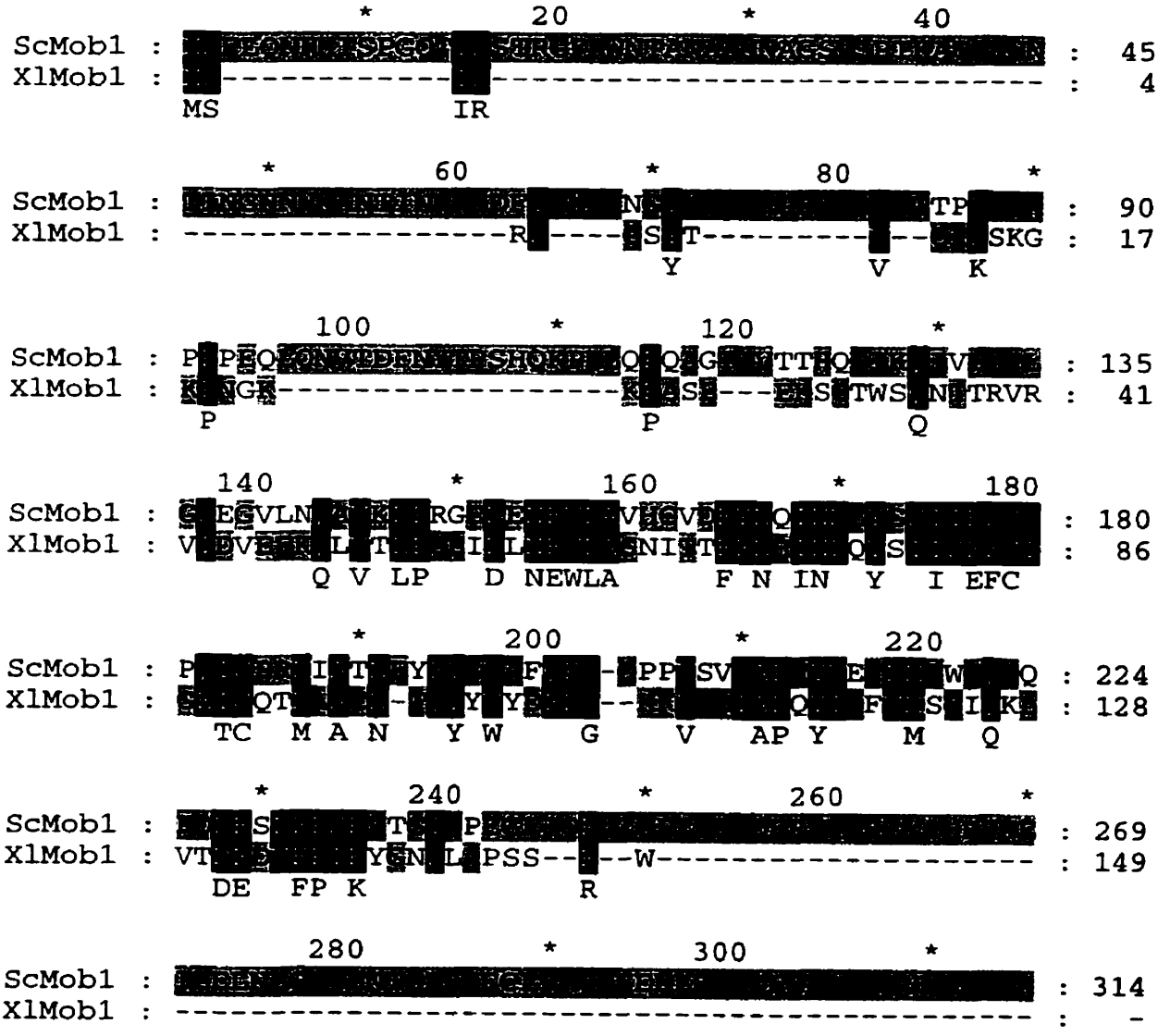
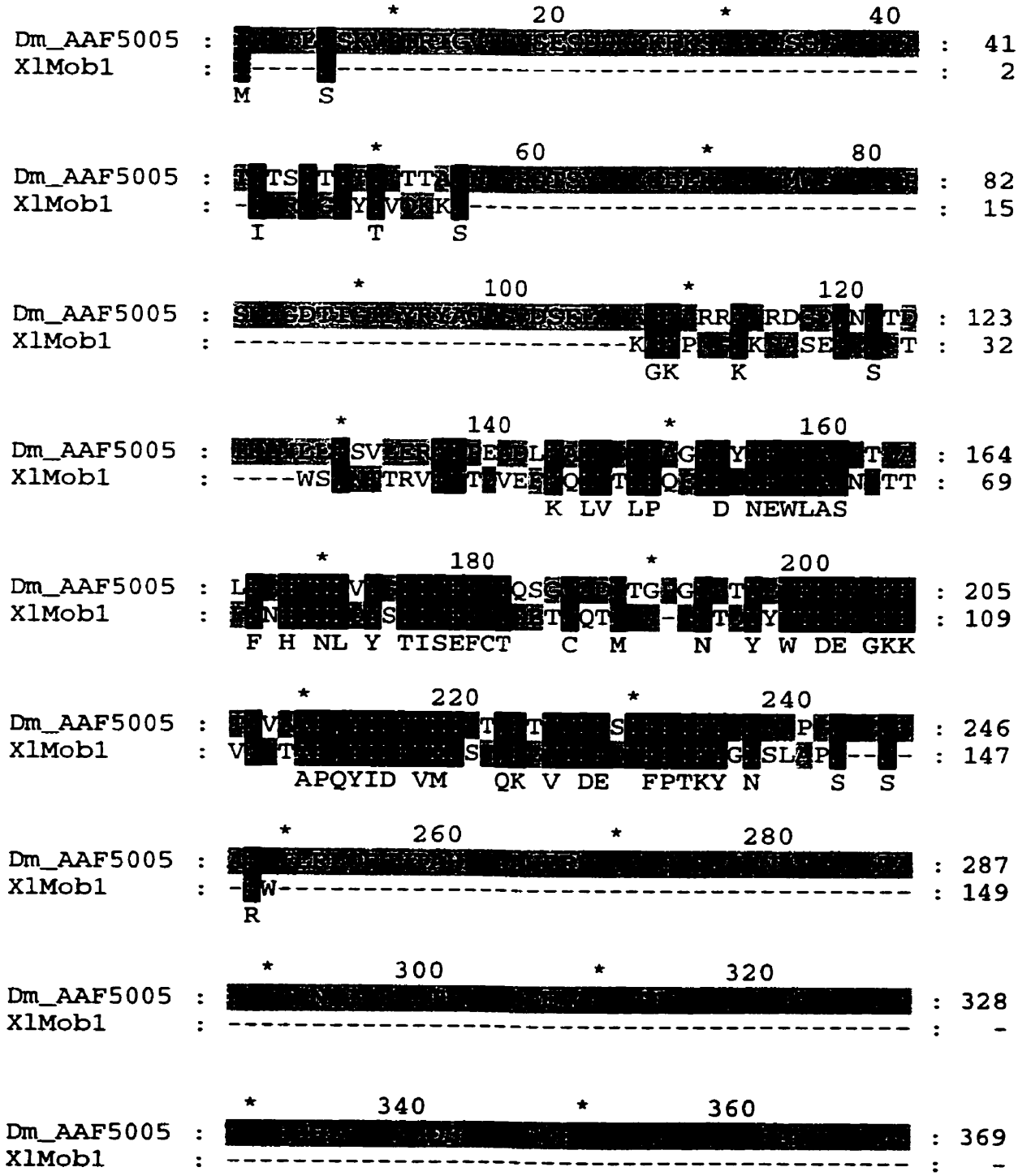
Appendix I

S. cerevisiae Mob1 and *X. laevis* Mob1 Sequence Alignment



Appendix II

D. melanogaster Mob1 and *X. laevis* Mob1 Sequence Alignment



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                  *        380          *        400          *
Dm_AAF5005  :  NNGSTSASASGVSLIDGDAVAPPICTQPEAGAGGKPAGSSGL  : 410
XlMob1      :  ------------------------------------------  :   -

                        420          *        440          *
Dm_AAF5005  :  LGGILGDLTSGEFGDTTRYGTSAVPQAAAAAAGAGVGGTAIG  : 451
XlMob1      :  ------------------------------------------  :   -

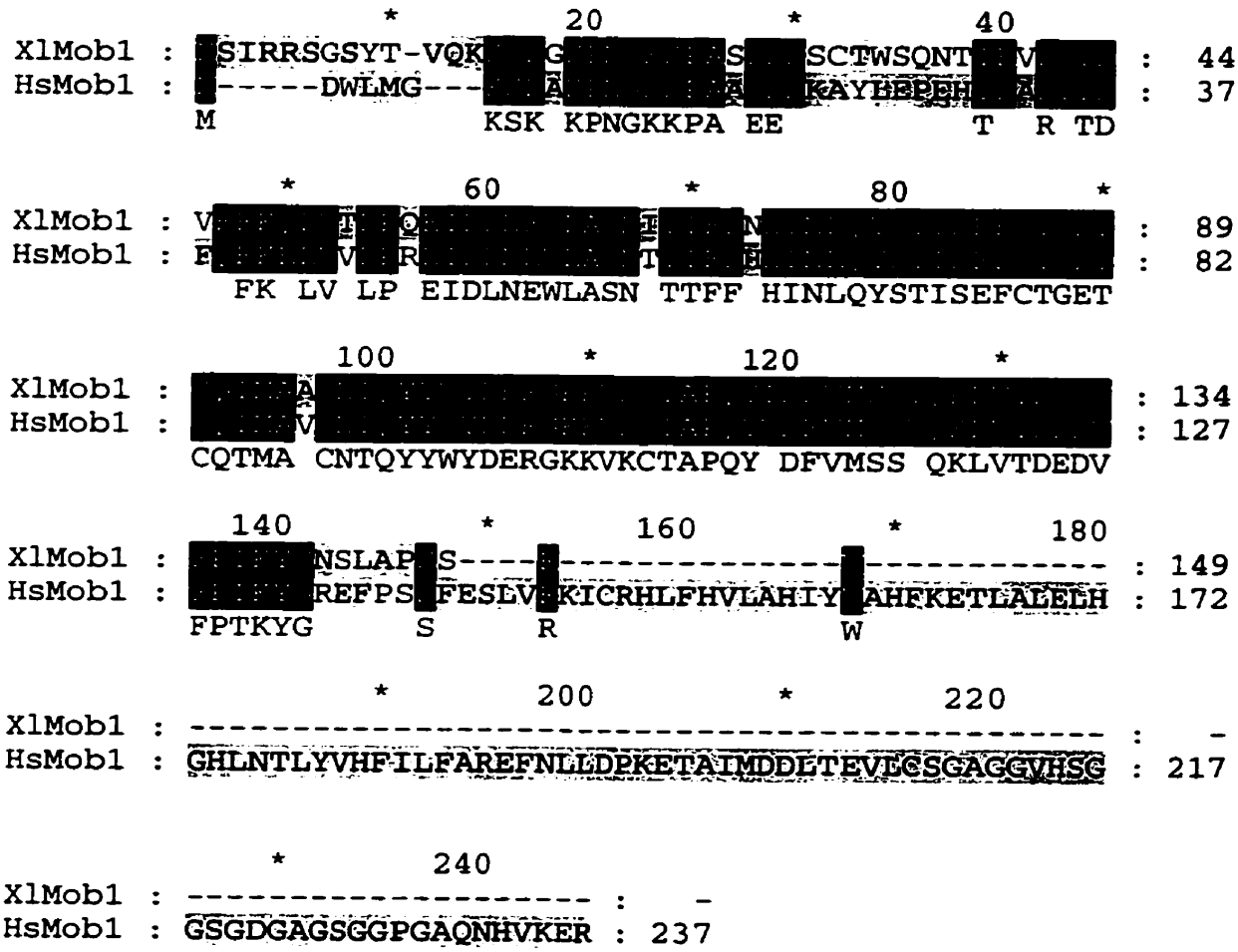
                      460          *        480          *
Dm_AAF5005  :  ATDAAALNNGAGALHENFSNNNNNNHNLNHHHHHHHHHHGHH  : 492
XlMob1      :  ------------------------------------------  :   -

                    500          *        520          *
Dm_AAF5005  :  GHHHAAQQQQQHSGLIQGNAAGGGNATGVATGGATAAASS  : 533
XlMob1      :  ------------------------------------------  :   -


Dm_AAF5005  :  TTTA  :  537
XlMob1      :  ----  :    -
```

Appendix III

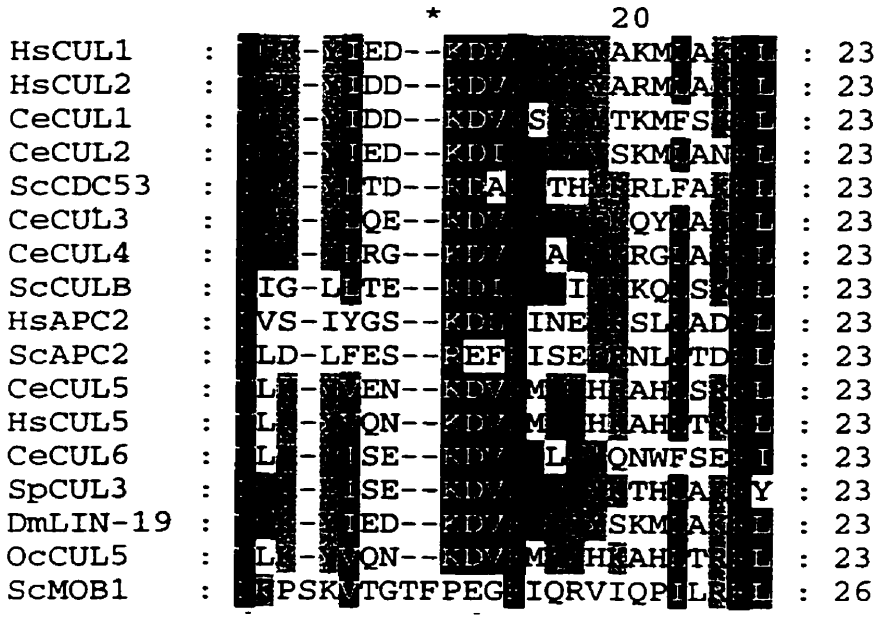
X. laevis Mob1 and *H. sapiens* Mob1 Sequence Alignment



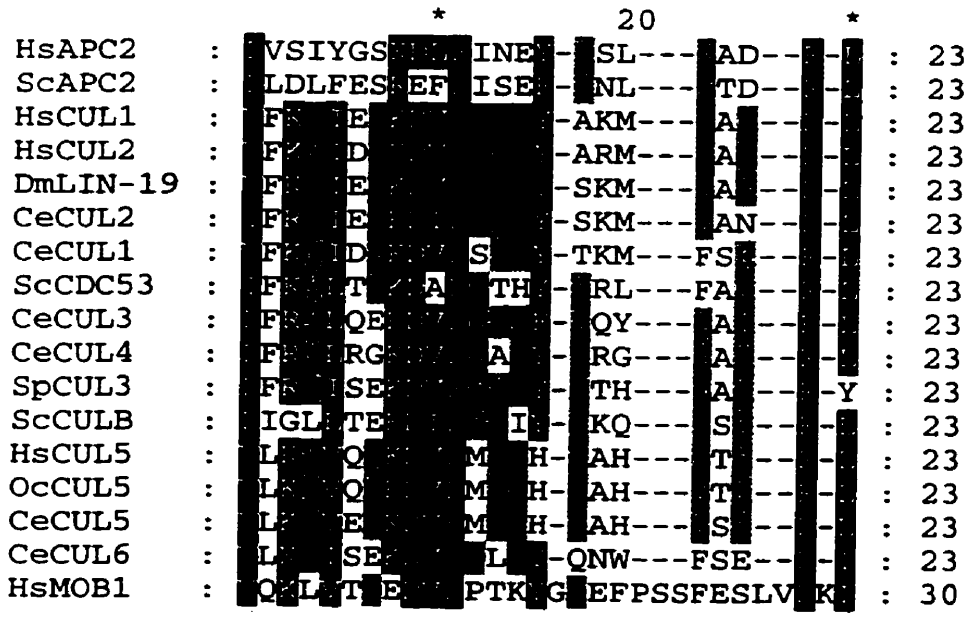
Appendix IV

Cullin Homolog Region Sequence Alignment

A. *S. cerevisae* Mob1 Protein:



A. Human Mob1 Protein:



Appendix V

Amino Acid Composition of the *Xenopus* Mob1 Protein