MOB1, an Essential Yeast Gene Required for Completion of Mitosis and Maintenance of Ploidy

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Mob1p is an essential *Saccharomyces cerevisiae* protein, identified from a two-hybrid screen, that binds Mps1p, a protein kinase essential for spindle pole body duplication and mitotic checkpoint regulation. Mob1p contains no known structural motifs; however *MOB1* is a member of a conserved gene family and shares sequence similarity with a nonessential yeast gene, *MOB2*. Mob1p is a phosphoprotein in vivo and a substrate for the Mps1p kinase in vitro. Conditional alleles of *MOB1* cause a late nuclear division arrest at restrictive temperature. *MOB1* exhibits genetic interaction with three other yeast genes required for the completion of mitosis, *LTE1*, *CDC5*, and *CDC15* (the latter two encode essential protein kinases). Most haploid mutant *mob1* strains also display a complete increase in ploidy at permissive temperature. The mechanism for the increase in ploidy may occur through *MPS1* function. One *mob1* strain, which maintains stable haploidy at both permissive and restrictive temperature, diploidizes at permissive temperature when combined with the *mps1-1* mutation. Strains containing *mob2Δ* also display a complete increase in ploidy when combined with the *mps1-1* mutation. Perhaps in addition to, or as part of, its essential function in late mitosis, *MOB1* is required for a cell cycle reset function necessary for the initiation of the spindle pole body duplication.

INTRODUCTION

Proper cell division requires precise coordination and execution of several events in the cell cycle, including centrosome duplication, DNA replication, mitotic spindle assembly, chromosome segregation, and cytokinesis. A failure in the execution or proper timing of any of these events could lead to chromosome segregation defects resulting in aneuploidy or polyploidy. Such genomic instability is the hallmark of transformed cells but has also been observed in various mutant strains of yeast (Tlsty *et al.*, 1995; Romanowski and Madine, 1996; Stillman, 1996). Analysis of mutant yeast strains that give rise to aneuploid or polyploid cells has led to an understanding of some of the mechanisms that cause genomic instability.

Endomitosis, DNA replication in the absence of karyokinesis and cytokinesis, is one mechanism that

causes an increase in ploidy to yield polyploid cells. In some cells, such as developing endosperm of plant seeds (Grafi and Larkins, 1995) or mammalian megakaryocytes (Jackson, 1992), endomitosis is normal and appears to require the inhibition of M phase-promoting kinases and the activation of S phase-promoting kinases (Grafi and Larkins, 1995; Datta *et al.*, 1996). In rapidly dividing cells such as yeast, overreplication can be induced by a number of conditions that decrease G2 cyclin/p34^{cdc2/Cdc28} activity or keep the levels of certain components of the DNA replication complex high (Chevalier and Blow, 1996; Romanowski and Madine, 1996). This suggests that the mechanism preventing rereplication of DNA is dependent on G2 cyclin/p34^{cdc2/Cdc28} activity. It is thought that in normal cells, the decrease in G2 cyclin/p34^{cdc2/Cdc28} activity at the end of mitosis stimulates the reassembly of a prereplicative complexes on DNA origins, thereby rendering the DNA competent for replication (Romanowski and Madine, 1996). In *Saccharomyces cerevisiae*, the assembly of prereplicative complexes and origin recognition complexes requires the activity of

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CDC14 and *CDC5*, both of which also have roles in the completion of mitosis (Hardy, 1996; Hardy and Pautz, 1996; Kroll *et al.*, 1996).

A second class of defects that can give rise to an increase in ploidy are those that affect processes required for chromosome segregation such as centrosome duplication, kinetochore attachment and mitotic motor function, among others. Mutations causing defects in each of these processes can be found in yeast. Failure in proper spindle pole body (SPB, the yeast centrosome equivalent) duplication can lead to the generation of monopolar or multipolar spindles, both of which can give rise to aneuploid or polyploid cells (Schild *et al.*, 1981; Thomas and Botstein, 1986; Rose and Fink, 1987; Baum *et al.*, 1988; Levine *et al.*, 1991; Winey *et al.*, 1991). Improper kinetochore attachment, if left uncorrected, leads to aneuploidy (Doheny *et al.*, 1993; Goh and Kilmartin, 1993). Defects in mitotic motors responsible for chromosome segregation or nuclear migration may also result in aneuploidy, polyploidy, or, in some cases, multinucleate cells (Hoyt *et al.*, 1993; Saunders *et al.*, 1995; Yeh *et al.*, 1995). This is also true for defects in proteins that affect microtubule function or integrity (Hoyt and Geiser, 1997). One yeast gene required for the maintenance of ploidy is *IPL1*, which encodes an essential protein kinase that is required, in some way, for mitotic spindle function (Chan and Botstein, 1993; Francisco *et al.*, 1994). Strains defective in this kinase become aneuploid.

Checkpoints are an important line of defense against genomic instability. They are signaling pathways that elicit temporary delays in cell cycle progression in response to a defect in DNA integrity, DNA synthesis, spindle function, or other cellular functions (for review see Elledge, 1996; Paulovich *et al.*, 1997). The transient arrest allows time for the completion of some delayed process or for activation of repair mechanisms to attempt to correct the defects. If the defect is irreparable or there is a failure in the checkpoint or repair pathways, cells can suffer a loss in genomic stability. This is manifested by the segregation of defective chromosomes (i.e., unrepaired DNA) or the generation of aneuploid and polyploid cells (i.e., misattached kinetochore or defective spindle). Checkpoints that respond to DNA damage, under-replication, and defects in the mitotic spindle have been identified in budding yeast (Elledge, 1996; Rudner and Murray, 1996; Stewart and Enoch, 1996). Several of the genes involved in these checkpoint pathways have homologs in other eukaryotes, including mammals (Rudner and Murray, 1996; Stewart and Enoch, 1996; Paulovich *et al.*, 1997).

MPS1 is an essential yeast gene that has two important functions with regard to genomic stability. It is required for SPB duplication and for mitotic checkpoint regulation (Winey *et al.*, 1991; Weiss and Winey,

1996). Consequently, *mps1* mutant strains, at the restrictive temperature, not only fail to duplicate their SPBs, but also fail to arrest the cell cycle in response to the defective spindle, leading to aneuploidy and a rapid loss in viability. However, a transient loss in *MPS1* activity can lead to cells with twice the DNA content, attributable to a failure in one round of SPB duplication (Winey *et al.*, 1991). The resultant monopolar mitotic spindle is incapable of chromosome segregation, thereby leaving all the chromatin in one cell.

MPS1 encodes an essential dual specificity protein kinase (Lauzé *et al.*, 1995). Mps1p kinase activity is required for both SPB duplication and for the mitotic checkpoint. Proper activation of the Mps1p kinase requires Cdc37p, which is thought to act as a molecular chaperone (Schutz *et al.*, 1997). To date, no substrate for Mps1p relevant to SPB duplication has been identified. However, Mps1p is required for the increased phosphorylation of the checkpoint gene product, Mad1p, which correlates with the activation of the mitotic checkpoint (Hardwick and Murray, 1995; Hardwick *et al.*, 1996). Mad1p is also phosphorylated by purified Mps1p *in vitro*, but it is not known whether Mad1p is a direct substrate for Mps1p *in vivo* (Hardwick *et al.*, 1996).

To identify potential substrates or effectors that interact with Mps1p kinase, we have employed the two-hybrid system. Here we describe *MOB1* (Mps One Binder), which encodes an essential 314-amino acid protein containing no known structural motifs. *MOB1* is a member of a large, conserved gene family with members present in a wide variety of eukaryotes. Its gene product, Mob1p, is a phosphoprotein *in vivo* and is an *in vitro* substrate of Mps1p. Unlike *MPS1*, *MOB1* has no apparent role in the spindle assembly checkpoint; however, it is required for the completion of mitosis and maintenance of ploidy, the latter perhaps through *MPS1* function. Strains harboring conditional *mob1* mutations arrest in late nuclear division at the restrictive temperature. Several alleles of *mob1* also cause a complete increase in ploidy at permissive temperature, which may suggest a role in SPB duplication (see DISCUSSION). In these cells, haploid strains appear to become diploid, a phenotype observed in cells defective in SPB duplication (Schild *et al.*, 1981; Thomas and Botstein, 1986; Rose and Fink, 1987; Winey *et al.*, 1991).

MATERIALS AND METHODS

Strains, Cell Culture, and Genetic Techniques

The yeast strains used in this study are listed in Table 1. Yeast media, growth conditions, and genetic and molecular techniques were as described (Guthrie and Fink, 1991; Lunblad, 1997). Selected yeast cultures were arrested in α -factor, hydroxyurea, or nocodazole (Sigma Chemical, St. Louis, MO) as previously described (Weiss and

Table 1. Yeast strains

Name	Genotype	Source
D8BX5CA	<i>a/α, ura3-52/ura3-52, trp1Δ1/trp1Δ1, his3Δ200/his3Δ200, leu2-3,112/leu2-3,112</i>	Winey
WX257-5c	<i>a ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	Winey
WX257-8b	<i>α ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	Winey
FLY10	<i>a/α mob1Δ::HIS3/MOB1 ura3-52/ura3-52 trp11/trp1Δ1 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112</i>	This study
FLY12A	<i>a mob1Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112 [pGST-MOB1]</i>	This study
FLY15A	<i>a mob2Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY15B	<i>α mob2Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY26	<i>a/a mob1-34/mob1-34 ura3-52/ura3-52 trp1Δ1/trp1Δ1 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112</i>	This study
FLY29	<i>a/a mob1-46/mob1-46 ura3-52/ura3-52 trp1Δ1/trp1Δ1 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112</i>	This study
FLY30	<i>a mob1-77 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY31	<i>a/a mob1-83/mob1-83 ura3-52/ura3-52 trp1Δ1/trp1Δ1 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112</i>	This study
FLY32	<i>a mob1-95 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY33	<i>a mob1-67 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY40	<i>a/a mob1-55/mob1-55 ura3-52/ura3-52 trp1Δ1/trp1Δ1 his3Δ200/his3Δ200 leu2-3,112/leu2-3,112</i>	This study
FLY59	<i>a mob1Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112 [pRS316-MOB1]</i>	This study
FLT60	<i>a mob1-46::LEU2::mob1Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112 [pRS316-MOB1]</i>	This study
FLY61	<i>a mob1-55::LEU2::mob1Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112 [pRS316-MOB1]</i>	This study
FLY62	<i>a mob1-83::LEU2::mob1Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112 [pRS316-MOB1]</i>	This study
FLY64	<i>α mob1-77 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY66	<i>α mob1-95 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY67	<i>a mob1-77 mps1-1 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY69	<i>α mob1-95 mps1-1 ura3-52 trp1Δ1 his3Δ200</i>	This study
FLY70	<i>a mob1-95 mps1-1 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY71	<i>a mob2Δ::HIS3 mps1-1 ura3-52 trp1Δ1 his3Δ200</i>	This study
FLY72	<i>a mob2Δ::HIS3 mps1-1 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY111	<i>a lte1Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY112	<i>α lte1Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112</i>	This study
FLY113	<i>a mob1-95 lte1Δ::HIS3 ura3-52 trp1Δ1 his3Δ200 leu2-3,112 [pRS316-MOB1]</i>	This study
FLY114	<i>a mob1-77 cdc15-2 ura3-52 trp1Δ1 leu2-3,112 [pRS426-MOB1]</i>	This study
FLY115	<i>a mob1-95 cdc15-2 ura3-52 trp1Δ1 leu2-3,112 [pRS426-MOB1]</i>	This study
H5C1A2	<i>a cdc5-1 ura1 his7</i>	Raymond
H5C1B1	<i>α cdc5-1 ura1 his7</i>	Raymond
WR142	<i>α cdc14-1 his7 leu2 gal1 can1</i>	Raymond
4078-15-3	<i>a cdc15-2 ura3 his7 leu2 tyr1 gal1 can1</i>	Raymond
4078-15-2	<i>α cdc15-2 trp1 his7 leu2 gal1 can1</i>	Raymond
WX241-7a	<i>α mps1-1 ura3-52 trp1Δ1 his3Δ200</i>	Winey
AS132-10a	<i>α mps1-737 ura3-52 his3Δ200 leu2-3,112 lys</i>	Schutz
AS131-5b	<i>α mps1-3796 ura3-52 trp1Δ1 his</i>	Schutz
AS181-12b	<i>α mps1-6 ura3-52 trp1 leu2-3,112</i>	Schutz
EGY40	<i>α ura3-52 his3 leu2</i>	Brent
BJ2168	<i>a ura3-52 trp1 leu2 pep4-3 prb1-1122 prc1-407</i>	Jones

Winey, 1996). Counterselection for yeast harboring *URA3*-containing plasmids was achieved by growth in media containing 1 mg/ml 5-fluoroorotic acid (5-FOA) (United States Biological, Swampscott, MA) as described (Sikorski and Boeke, 1991). The DH5 α strain of *Escherichia coli* (Bethesda Research Laboratories, Bethesda, MD) was transformed with plasmid DNA by electroporation using a Bio-Rad Gene Pulser electroporator (Bio-Rad, Hercules, CA) as described by the manufacturer.

Two-Hybrid Screen

Plasmids and yeast strains for the two-hybrid system were provided by Roger Brent and used as described (Golemis *et al.*, 1997). This version of the two-hybrid system employs the use of LexA-Mps1p fusion protein as “bait” encoded by pLexA- Δ 63 MPS1 (Lauzé *et al.*, 1995) to isolate and identify MOBs (Mps One Binders). The LexA protein is the DNA-binding domain that specifically binds the LexA operon upstream of the LacZ reporter gene, encoded by pSH18-34 (Golemis *et al.*, 1997). pLexA- Δ 63 MPS1 does not activate transcription of the reporter by itself. The “prey” genomic DNA library was

constructed and provided by P. Watt (Watt *et al.*, 1995). It consists of *S. cerevisiae* genomic DNA inserted into a plasmid downstream of a transcriptional activation domain. The yeast strain, EGY40, harboring pSH18-34 and pLexA- Δ 63 MPS1, was transformed with the yeast prey library. Activation of the reporter gene was monitored either by plating transformants onto media containing X-gal or by colony-filter-lift assays (Golemis *et al.*, 1997). The prey plasmids were rescued and introduced into *E. coli* as described (Hoffman and Winston, 1987). Rescued prey plasmids were screened for specificity of interaction by introduction into yeast harboring the reporter plasmid and pRHJM (encoding LexA-Bicoid [Golemis *et al.*, 1997]), or pLexA- Δ 63 MPS1. Only prey plasmids that were specifically dependent on pLexA- Δ 63 MPS1 for activation of the reporter gene were kept for further analysis.

MOB1 Sequence Analysis

MOB (Mps One Binder) DNA was sequenced from pAD-MOB using oligonucleotide AD-1 (Table 2), and the Sequenase II DNA sequencing kit (United States Biochemical, Cleveland, OH) performed ac-

Table 2. PCR primers

Name	DNA sequence
1-A	ACA AAC AAT CCA GTA AAC GGA TAT AAT GAA AGC GAC CAT GGA AGG TCG TTC AGA ATG ACA CG
1-B	AAG AAT ACA ACC TAC AAG CAG ACT TAT ATA AAT ATA CAA TAC TAC CTC TTG GCC TCC TCT AG
1-C	CGG AAT TCC AAG ATC AAG TTC CC
1-D	CGG GAT CCA GCA TGT CTC CCG TCC TCA CTA CGC
1-E	CCC GGA TCC AAA CCC TTC TTC TAC GCC
1-G	GCG TTT ATC GTT TGC AT
1-I	ACT TCA ATT TCC ATG TC
1-K	CAT TTT GTA ATT GTT CAG CG
VI-B	GAA AAA TAA AAA CCA GCC CCT TAA TGT TGC TCA GCC GCC AGC ACT CTT GGC CTC CTC TAG
VI-C	ACC AAG CAT TTA TCA GC
VI-D	GCG TTT ATC GTT TGC AT
VI-E	TAA TTT TGC CCT GTT TTT CAA AGC TTT CAA TTA ACG GTA GCA GTC GTT CAG AAT GAC ACG
VI-F	CAA AGG ATC CTT CTA TCA AGT CAC CCC
VI-G	CCG GGA AGG TAC CGG TG
VI-H	GTT TGA GAA TGC CTC TTA GG
LTE1-A	GCT TTA CAT AGC CTC TAG CCA TTC AAA GAT TCG CTA CCC TGA ACC CTC TTG GCC TCC TCT AG
LTE1-B	GGA AAG TGG CAC AAT ACC TCA TGT GGA TAG TTC ATT AAT CTC TTC TCG TTC AGA ATG ACA CG
LTE1-C	CTC CTT ACT CAT CTT TGC
HIS3A	GAT TAG CGA CCA GCC GG
AD-1	AAT TCG GCA CGA GGC G
T3	ATT AAC CCT CAC TAA AGG GA

cording to the manufacturer's instructions. Five different *MOB* genes were obtained from this screen. Only *MOB1* was novel. *MOB1* DNA was excised from the prey plasmid pAD-MOB1 with *EcoRI*, purified and labeled with α -³²P-dCTP (New England Nuclear, Boston, MA) as described by Brown (1997). Labeled *MOB1* DNA was hybridized on *S. cerevisiae* genomic DNA-mapping filters (Riles *et al.*, 1993). The hybridization pattern revealed that *MOB1* resides on chromosome IX. The entire *MOB1* open reading frame (ORF) and flanking sequences was provided by Bart Barrell as part of the Sanger Centre yeast genome project. *MOB1* is the same as *YIL106w* listed in the *Saccharomyces* Genome Database (SGD) and has the GenBank accession number Z47147x72. It shares sequence similarity with *YFL035c* on chromosome VI (GenBank accession number D50617x34), which we have named *MOB2*. *MOB2* was not isolated in this two-hybrid screen. Various databases, including dbEST and GenBank, were scanned for genes that share sequence similarity to *MOB1*. Sequence comparisons and manipulations were performed using GCG sequence analysis program (Genetics Computer Group, Madison, WI).

Polymerase Chain Reaction (PCR) Conditions

Oligonucleotides were obtained from United States Biochemical (Cleveland, OH), Life Technologies/BRL (Grand Island, NY), or Operon (Alameda, CA) and are listed in Table 2. Deoxynucleoside triphosphates were from Life Technologies/BRL. Genomic DNA was prepared from yeast strains as described by Hoffman and Winston (1987). High-fidelity PCR was performed using Vent DNA polymerase (New England Biolabs, Beverly, MA) to amplify yeast genomic DNA. Mutagenic PCR was performed using *Taq* DNA polymerase (Promega, Madison, WI, or Life Technologies/BRL) as described (Fromant *et al.*, 1995).

To determine whether *MOB1* has a functional intron, *MOB1* cDNA was amplified from a cDNA library (provided by S. Elledge)

using 1-I and 1-G oligonucleotide primers and sequenced using the 1-K oligonucleotide primer and the Sequenase PCR sequencing kit (United States Biochemical, Cleveland, OH). *MOB2* cDNA was amplified and sequenced from a cDNA library (Liu *et al.*, 1992) in a similar manner using T3 and VI-G oligonucleotide primers and sequenced using the VI-H primer.

MOB1, *MOB2*, and *LTE1* Deletions

MOB1, *MOB2*, or *LTE1* (Wickner *et al.*, 1987; Keng *et al.*, 1994) were deleted from the wild-type diploid strain D8BX5CA (Table 1) using a one-step gene replacement technique (Baudin *et al.*, 1993). *HIS3* flanked by sequences of the targeted gene was amplified by PCR from plasmid DNA, such as pEG202 (Golemis *et al.*, 1997), using oligonucleotides 1-A and 1-B for *MOB1*, VI-B and VI-E for *MOB2*, and LTE1-A and LTE1-B for *LTE1* (Table 2). This method yields *HIS3* DNA flanked by the 5' and 3' sequences of *MOB1*, *MOB2*, or *LTE1*. Coding regions upstream of the *MOB1* and *MOB2* introns were not deleted using these PCR products, leaving coding regions intact for the first 78 and 28 codons of *MOB1* and *MOB2*, respectively. The PCR products were individually transformed into D8BX5CA to yield integrative recombinants that were selected for growth in media lacking histidine. Correct integration was confirmed by PCR amplification of genomic DNA with an internal *HIS3* primer, *HIS3A*, and a primer external to the *MOB1*, *MOB2*, or *LTE1* ORF: oligonucleotide 1-C, VI-C, and LTE1-C, respectively (Table 2). PCR product was only generated by these oligonucleotides when *HIS3* was integrated at the targeted locus.

MOB1, *MOB2*, and *MPS1* Plasmids

Restriction nucleases and T4 DNA ligase were obtained from New England Biolabs. Most of the coding region for *MOB1* (amino acids 79–314) was amplified by PCR using oligonucleotides 1-C and 1-D.

The PCR product was purified and cut with *Bam*HI and *Eco*RI and ligated to the yeast expression vector pEG(kt) (provided by R. Deschenes [Mitchell *et al.*, 1993]) to yield pGST-MOB1. The same PCR product was ligated to the *E. coli* expression vector, pRSETA (Invitrogen, San Diego, CA) to create pH6-MOB1. *MOB1* and *MOB2* were subcloned into low-copy and high-copy yeast shuttle vectors as follows. pRS314-MOB1, pRS316-MOB1, pRS424-MOB1, and pRS426-MOB1 were constructed by insertion of the entire ORF and flanking sequences of *MOB1*, amplified by oligonucleotides 1-C and 1-E and cut with *Bam*HI and *Eco*RI, into the same sites of pRS314, pRS316, pRS424, and pRS426 (Sikorski and Heiter, 1989; Christianson *et al.*, 1992). The entire ORF and flanking sequences of *MOB2* were amplified with oligonucleotides VI-E and VI-F, cut with *Bam*HI and *Sal*I, and inserted into the same series of vectors to yield pRS314-MOB2, pRS424-MOB2, pRS316-MOB2, and pRS426-MOB2. *MPS1* was subcloned into the high-copy yeast shuttle vector pRS424 as follows. *MPS1* was excised from pEcoΔMPS1 (Lauzé *et al.*, 1995) with *Eco*RI and *Sal*I and ligated to the same sites of pRS424 to yield pRS424-MPS1.

Generation of Conditional Alleles of *MOB1*

To create a library of mutagenized *MOB1* DNA, *MOB1* was amplified from genomic DNA using oligonucleotides 1-C and 1-E under conditions of poor fidelity (above). The PCR product was purified, cut with *Bam*HI and *Eco*RI, and ligated to the same sites of pRS314. After electroporation into the *E. coli* strain, DH5α, transformants were pooled and grown for 3 h in Luria-Bertani broth (Lech and Brent, 1997) supplemented with 100 μg/ml ampicillin. The pRS314-mob1 DNA library was introduced into FLY12A (Table 1). Yeast transformants were selected for growth in media lacking tryptophan. The *URA3*-containing plasmid also harboring *MOB1* was counterselected by replica plating the yeast colonies onto media containing 1 mg/ml 5-FOA (United States Biochemical, Swampscott, MA) to give rise to *mob1Δ* cells kept alive by the pRS314-mob1 plasmids. These cells were then screened for conditional growth at 15° and 37°C. pRS314-mob1 plasmids causing conditional lethality were rescued as above and retransformed into FLY12A to confirm the mutant phenotype. Mutant *mob1-* containing plasmids were sequenced using the Sequenase PCR sequencing kit (United States Biochemical, Cleveland, OH) as described by manufacturer.

Targeted integration of mutant alleles of *MOB1* was carried out as described (Rothstein, 1991). Conditional alleles of *MOB1* were subcloned from pRS314-mob1 plasmids and ligated to the *URA3*-marked pRS306 integrating vector (Sikorski and Heiter, 1989; Christianson *et al.*, 1992). The pRS306-mob1 plasmids were linearized with *Pml*I and transformed into FLY12A. Transformants were grown on 5-FOA plates and were selected for colonies that were uracil auxotrophs and exhibited the *mob1* temperature-sensitive phenotype.

SDS-PAGE and Immunoblots

SDS-PAGE was performed as described (Anderson *et al.*, 1973). Immunoblots were carried out in a Hoefer Transphor transfer apparatus (Hoefer Scientific Instruments, San Francisco, CA) based on the method of Towbin (Towbin and Gordon, 1984). Immunoblots were blocked 30 min or greater within blocking buffer (3% bovine serum albumin, 2% nonfat dry milk, 0.1% Tween-20, 50 mM Tris, 150 mM NaCl, pH 7.4) at room temperature. They were probed with primary antibody diluted in blocking buffer for 1 h followed by four 15-min washes in TBST (0.1% Tween-20, 50 mM Tris, 150 mM NaCl, pH 7.4). Secondary antibody was also diluted in blocking buffer and reacted with the immunoblots for 1 h. After four more washes in TBST, the immunoblots, depending on the secondary antibody, were visualized by Enhanced Chemiluminescence System (ECL, Amersham, Arlington Heights, IL) according to manufacturer's instruction or reacted with nitro blue tetrazolium/bromochloroindolyl phosphate (Promega) as described (Harlow and Lane, 1988).

Immunoblots of glutathione-S-transferase (GST) and GST-Mob1p were probed with goat anti-GST antibody (Pharmacia, Piscataway, NJ) followed by alkaline phosphatase-conjugated rabbit anti-goat IgG (Pierce Chemical, Rockford, IL). Myc-Mps1p was probed with mouse monoclonal anti-myc antibody, 9E10 (provided by M. Klymkowsky) followed by peroxidase-conjugated anti-mouse IgG (Amersham). Autoradiograms and ECL-treated immunoblots were visualized by exposure to Hyperfilm-MP film (Amersham).

Expression and Purification of Recombinant Proteins

Six histidine-tagged Mob1p (H6-Mob1p) encoded by pH6-MOB1 was expressed in the BL21(DE3) strain of *E. coli* (Studier *et al.*, 1990). Cells were grown to an OD₆₀₀ of 1 and induced for 4 h at 37°C with 1 mM IPTG (Sigma Chemical). Cells were harvested by centrifugation and lysed by sonication in urea-containing buffer as described (Luca *et al.*, 1991). The cell lysate was centrifuged at 15,000 × *g* in a SS-34 rotor (Dupont, Wilmington, DE) to rid it of cell debris and unlysed cells. Recombinant H6-Mob1p was precipitated from the clarified cell extract by incubation with 1 ml of a 1:1 slurry of Ni-agarose (Qiagen, Chatsworth, CA). H6-Mob1p-bound resin was washed several times in lysis buffer followed by several washes in TBS (50 mM Tris, 150 mM NaCl, pH 7.4). Bound protein was eluted in TBS containing 1 mM EDTA and dialysed in TBS. Estimates of protein concentration were obtained by comparison to known amounts of bovine serum albumin on an SDS-PAGE.

GST, GST-Mob1p, and myc-Mps1p were inducibly expressed under the control of the *GAL10* promoter in yeast and purified as previously described for GST-MPS1 with a few modifications (Lauzé *et al.*, 1995). A 1-l culture of BJ2168 harboring pEG(kt), pGST-MOB1, or pELW325, encoding myc-Mps1p (Hardwick *et al.*, 1996), was grown in selective media containing 2% raffinose to OD₆₀₀ of 0.5. The cells were induced with 4% galactose (Sigma Chemical) for 6–8 h at 30°C, harvested by centrifugation, pooled, and washed one time in sorbitol break buffer (0.3 M sorbitol, 0.1 M NaCl, 5 mM MgCl₂, 10 mM Tris, pH 7.4) at 4°C (Mitchell *et al.*, 1993). They were then resuspended in 10 ml cold sorbitol buffer and lysed in a French press under 900 pounds per square inch. Cell debris was pelleted by centrifugation at 15,000 × *g* at 4°C to yield clarified extract. GST, GST-Mob1p, or GST-Mps1p was precipitated from the extract by incubation with glutathione-Sepharose (Pharmacia) (1 ml packed resin per 10 ml cell extract) for 1 h at 4°C. Bound protein was washed six times in phosphate-buffered saline (PBS) (0.15 mM NaCl, 50 mM sodium phosphate, pH 7.4) + 0.1% Tween 20 followed by six washes in PBS or kinase buffer (50 mM HEPES, pH 7.5, 10 mM MgCl₂, 2 mM MnCl₂, 0.5 mM dithiothreitol) (Lauzé *et al.*, 1995).

Myc-Mps1p was immunoprecipitated from yeast extracts with the 9E10 mouse monoclonal antibody using the following protocol: 0.5 ml of 1:10 diluted extract was first "precleared" by incubation for 1 h at 4°C with 0.1 ml gamma-bind G Sepharose (Pharmacia). The Sepharose beads were pelleted and the supernatant was incubated with 5 μg/ml 9E10 mouse monoclonal antibody for 1 h at 4°C. The antibody-antigen complex was precipitated with 50 μl of gamma-bind G Sepharose for 1 h at 4°C. Protein-bound resin was washed as above and analyzed by *in vitro* kinase assays or by immunoblots probed with 9E10 antibody.

Mob1p/Mps1p Coprecipitation and *In Vitro* Kinase Assays

Yeast extract (0.15 ml) containing overexpressed myc-MPS1 was diluted 10-fold and precleared with 0.15 ml GST-bound glutathione-Sepharose. The precleared extract was then split into three equal aliquots and incubated for 1 h at 4°C with either 5 μg 9E10 antibody and gamma-bind G Sepharose as outlined above, 50 μl GST immobilized onto glutathione-Sepharose, or 50 μl GST-MOB1 immobilized onto glutathione-Sepharose. All were washed six times in PBS

Figure 1. MOB1 sequence analysis. (A) MOB1 DNA sequence and ORF. Underlined sequences represent the donor, branch, and acceptor sites for mRNA splicing. Note that the branch site is noncanonical. The position of the initiator methionine of an unspliced MOB1 is boxed. The arrow indicates the fusion junction in the two-hybrid prey plasmid. (B) Aligned sequences of a subset of class 1 Mob1p-related ORFs from various genome databases. Comparison of Mob1p (ScMob1) and Mob2p (ScMob2) to predicted amino acid sequences from *A. thaliana* (At#1 represents a contig of GenBank accession numbers H77164 and T04732; At#2, GenBank accession no. Z46539); Rice (GenBank accession no. C19722); human (Hs#1 represents a contig of GenBank accession numbers F11288, F11866, R19220, R13096, and R59435); mouse (Mm#1, GenBank accession no. AA118366); and *C. elegans* (Ce#1, GenBank accession no. Z69788; Ce#2, GenBank accession no. Z77660). Sequences shaded in black are identical. Sequences shaded in gray are conserved. X's represent ambiguous sequence. Note that many of the sequences derive from expressed sequence tags (ESTs) and therefore may be incomplete. (C) Aligned sequences of a subset of Class 2 Mob1p-related ORFs from various genome databases. Mob1p (ScMob1) sequence is compared with predicted amino acid sequences of the class 2 Mob1p-related sequences isolated from mouse (Mm#2 represents a contig of GenBank accession numbers U01138, AA125007, and AA208960); human (Hs#2 represents a contig of GenBank accession numbers W76032, D59087, N55945, and T79518); *C. elegans* (Ce #3,

+ 0.1% Tween-20 and three times in PBS, followed by a final wash in kinase buffer; 3.5 μ l of each were then loaded onto SDS-PAGE for Coomassie brilliant blue staining and immunoblot analysis. A portion of resin was used for *in vitro* kinase assay performed as described (Lauzé *et al.*, 1995). Resin (12.5 μ l) was added to 12.5 μ l of kinase reaction buffer $\pm$ 2 μ g myelin basic protein (MBP) (Sigma Chemical) and incubated for 30 min at 30°C. The kinase reactions were terminated by boiling in an equal volume of protein sample buffer. Ten microliters of kinase reaction were loaded onto 15% SDS-PAGE (Anderson *et al.*, 1973) and processed for autoradiography.

GST-Mps1p *in vitro* kinase assays were performed as described (Lauzé *et al.*, 1995). GST-Mps1p (2.5 μ l) bound to glutathione Sepharose was incubated in a total of 25 μ l kinase reaction buffer $\pm$ 2.5 μ g MBP or 5 μ g H6-Mob1p. Reactions were terminated and analyzed as above.

In Vivo Labeling

³²P labeling of yeast was done as described (Lauzé *et al.*, 1995). Cultures (50 ml) of BJ2168 cells harboring pEG(kt) or pGST-MOB1 were grown to OD₆₀₀ of 0.5. The cells were then transferred to phosphate-depleted media (Lauzé *et al.*, 1995) and grown for 1.5 h. The cells were labeled for 3 h at 30°C with 5 mCi of [³²P]orthophosphate (Amersham) while induced for expression of GST or GST-Mob1p by the addition of 4% galactose. ³²P-labeled cells were harvested by centrifugation and lysed by vortexing in 0.4 ml sorbitol break buffer and 0.2 ml of acid-washed glass beads. Extracts were centrifuged and GST-Mob1p precipitated on glutathione-Sepharose as above. Mob1p was cleaved from GST by incubating 10 μ l of immobilized GST-Mob1p with 4 U thrombin (Boehringer Mannheim, Indianapolis, IN) for 1 h at 37°C in TBS, pH 8.8. Free Mob1p was separated from the immobilized GST-Mob1p by centrifugation. ³²P-labeled proteins were analyzed on an SDS-PAGE processed for autoradiography.

Cytological Techniques

Yeast cells were prepared for flow cytometry as described by Weiss and Winey (1996) using the DNA stain propidium iodide (Sigma Chemical). Stained cells were analyzed on a FACScan flow cytometer using CELLFIT and LYSYS software packages (Becton Dickinson, San Jose, CA) to obtain and analyze data. Budding indices were performed as described (Weiss and Winey, 1996). Viability of yeast cells was done by counting colony forming units from serial dilutions of a briefly sonicated cell culture. Immunofluorescence was performed as described by Winey *et al.* (1991) using rat monoclonal antitubulin, YOL 1/34, as primary antibody and fluorescein isothiocyanate-conjugated goat anti-rat IgG as secondary antibody (both from Accurate Biochemical, Westbury, NY). DNA was stained with 1 μ g/ml 4,6-diamidino-2-phenylindole. Cells affixed to polylysine-coated (1 mg/ml) microscope slides and visualized with a CCD camera (Empix Imaging, Mississauga, Ontario, Canada) on a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY). Images were captured and manipulated using Metamorph software (Universal Imaging, West Chester, PA).

RESULTS

Screen for Mps One Binding Proteins

Although *S. cerevisiae* Mps1p protein kinase is known to be essential for SPB duplication and for mitotic

checkpoint regulation, its molecular interactions remain largely unknown. In an effort to identify Mps1p-interacting proteins, potential substrates, or effectors, we performed a two-hybrid screen (see MATERIALS AND METHODS). We screened a yeast genomic DNA library (provided by R. Brent and P. Watt) producing hybrid-activating proteins for interactions with a LexA-Mps1p fusion protein. We had previously shown that LexA-Mps1p is functional by its ability to complement *mps1* mutations and by its activity in *in vitro* kinase assays of immunoprecipitated protein (Lauzé *et al.*, 1995). We identified five genes encoding LexA-Mps1p-interacting proteins that failed to interact with an unrelated fusion protein, LexA-Bicoid (our unpublished results).

One of the genes encoding a protein that interacts with LexA-Mps1p is *MOB1*. We mapped *MOB1* to chromosome IX using an *S. cerevisiae* physical mapping filter (provided by L. Riles and M. Olson). We then sequenced a portion of the *MOB1* DNA and obtained the complete ORF and flanking sequences from Bart Barrel of the Sanger Centre Sequencing Group, Cambridge, United Kingdom (SGD ORF = YIL106w). The *MOB1* prey plasmid from the two-hybrid screen encodes an N-terminal truncation (starting at valine 145, VKLP. . .) (Figure 1A). *MOB1* was independently identified by K. Chun and M. Goebel in a screen designed to identify essential yeast genes (Chun and Goebel, 1996). Initial analysis of *MOB1* genomic DNA revealed a putative 85-nucleotide intron with canonical donor and acceptor sites but a noncanonical branch site, TATTAAC instead of the canonical TACTAAC sequence (Ruby and Abelson, 1991) (Figure 1A). If the intron were functional, spliced *MOB1* mRNA would encode a 314-amino acid protein (35,859 Da) (Figure 1A). An unspliced *MOB1* mRNA would encode a 236-amino acid protein (27,405 Da), assuming that translation starts at codon 79. We sequenced *MOB1* cDNA amplified by PCR and found that the intron is spliced. However, using this strategy, we cannot distinguish whether *MOB1* mRNA is differentially spliced.

Sequence analysis of Mob1p reveals no known structural motifs. *MOB1* shares sequence similarity with another predicted *S. cerevisiae* ORF located on chromosome VI (SGD ORF = YFL035c), which we have named *MOB2*. Like *MOB1*, *MOB2* also contains a putative intron; however its donor, acceptor, and branch sequences are canonical. We have confirmed that *MOB2*'s intron is functional by sequencing *MOB2* cDNA. Mob1p shares 43% similarity and 33% identity with Mob2p at the amino acid level, as determined by BESTFIT analysis using GCG sequence analysis program. Mob1p also shares sequence similarity with a variety of predicted ORFs of unknown functions, from a variety of organisms, as diverse as *Arabidopsis* and humans (Figure 1, B and C). The predicted Mob1p-like

Figure 1 (cont). GenBank accession no. L10990); *C. briggsae* (Cb, GenBank accession no. R03084); and *S. masoni* (Sm, GenBank accession no. AA1468).

Figure 2. GST-Mob1p precipitates Mps1p protein kinase. (A) Expression and purification of GST-Mob1p from yeast (see MATERIALS AND METHODS). Lane 1, total yeast protein containing expressed GST-Mob1p stained with Coomassie brilliant blue on SDS-PAGE. Lane 2, GST-Mob1p purified from yeast extract on glutathione-Sepharose. Positions of molecular weight markers are shown. (B) Protein from yeast extract containing myc-epitope-tagged Mps1p was precipitated with anti-myc antibody (MYC IP), GST-Mob1p-bound Sepharose (GST-Mob1p), or GST-bound Sepharose (GST). CBB, Coomassie brilliant blue-stained gel of precipitants. Kinase assay, autoradiogram showing ^{32}P -labeled proteins in corresponding precipitants after performance of an Mps1p protein kinase assay. Immunoblot, precipitated protein were immunoblotted and probed with 9E10. The prominent ~ 60 kDa band below myc-Mps1p on the immunoblot of MYC-IP is IgG heavy chain of the primary antibody recognized by the secondary antibody. Note that active Mps1p protein kinase is present in the anti-myc and GST-Mob1p precipitants but not in the GST precipitants. Also note that in addition to myc-Mps1p autophosphorylation, GST-Mob1p is phosphorylated in vitro. Molecular weight markers (M) and the positions of GST, GST-Mob1p, and myc-Mps1p are shown.

proteins fall into two similar, but distinct, classes by sequence comparisons (Figure 1, B and C). Proteins of the class I of Mob1p-like sequences share sequence similarity with Mob1p ranging from 37–68% similarity and 27–56% identity at the amino acid level. Mob1p shares about 45% similarity and 29% identity to proteins of the class II Mob1p-like proteins. Strikingly, proteins of this class share about 75% identity to each other.

MOB1 Is an Essential Gene, whereas MOB2 Is not Essential

To determine whether *MOB1* is an essential gene, we replaced most of one of the *MOB1* genes in a wild-type diploid cell with the *HIS3* gene (FLY10, Table 1; see MATERIALS AND METHODS). Upon sporulation and dissection of the tetrads, we found that only the two spores containing the wild-type *MOB1* gene were viable ($n = 10$ tetrads). No *HIS3*-containing spores were viable. Spores containing *mob1* Δ ::*HIS3* could be recovered only if they contained a plasmid, such as pRS316-MOB1, encoding wild-type *MOB1*. Furthermore, no viable *mob1* Δ ::*HIS3*-containing cells can be recovered after counter selection of the *URA3* gene

containing pRS316-MOB1 plasmid using 5-FOA (see MATERIALS AND METHODS). These results indicate that *MOB1* is essential, which is in agreement with previous data (Chun and Goebel, 1996). We used the same strategy to delete the *MOB2* gene and determined that *MOB2* is not essential. Spores containing deletions of *MOB2* were viable and displayed no temperature sensitivity for growth ($n = 20$ tetrads).

Mob1p Is a Phosphoprotein and Precipitates Active Mps1p Protein Kinase

We expressed the majority of Mob1p (amino acids 79–314) in yeast as a GST-Mob1p fusion protein under control of the inducible *GAL10* promoter in yeast. GST-Mob1p can be easily purified by precipitation with glutathione-bound Sepharose (Figure 2A). We determined the fusion protein to be functional in vivo because the encoding plasmid complements the *mob1* Δ strain both in glucose (repressing)- and in galactose (inducing)-containing media. This indicates that the 78 N-terminal amino acids of Mob1p encoded by spliced *MOB1* mRNA are nonessential. We were unable to detect any growth or cell cycle defect as a result of the overexpression of this GST-Mob1p.

Figure 3. Mob1p is a phosphoprotein. (A) Autoradiogram of precipitated ^{32}P -labeled GST-Mob1p from extracts of cells grown in the presence of [^{32}P]orthophosphate (see MATERIALS AND METHODS). Lane 1, ^{32}P -labeled GST-Mob1p precipitated by glutathione-Sepharose; lane 2, ^{32}P -labeled Mob1p separated from GST by thrombin cleavage; lane 3, remainder of ^{32}P -labeled protein on glutathione-Sepharose post-thrombin cleavage. (B) H6-Mob1p is phosphorylated by GST-Mps1p in vitro (see MATERIALS AND METHODS). Mps1p kinase assays of GST-Mps1p $\pm$ H6-Mob1p and $\pm$ MBP were separated by SDS-PAGE and processed for autoradiography. Positions of molecular weight markers are shown.

To confirm the two-hybrid interaction between Mps1p and Mob1p, we used GST-Mob1p immobilized on glutathione-Sepharose as an affinity reagent to precipitate active Mps1p protein kinase from cell extracts. Extracts made from yeast overexpressing Mps1p that bore the myc epitope were first precleared with GST bound to glutathione-Sepharose and then incubated with GST or GST-Mob1p-bound glutathione-Sepharose. After extensive washing, we assayed the Sepharose beads for the presence of bound Mps1p by immunoblot and by in vitro kinase assays. GST-bound Sepharose did not precipitate any detectable Mps1p; however, GST-Mob1p-bound Sepharose precipitated active Mps1p protein kinase (Figure 2B). Immunoblot analysis revealed that myc-Mps1p was present on the GST-Mob1p-bound Sepharose. The corresponding in vitro kinase assay indicated that the precipitated myc-Mps1p protein kinase was active as noted by Mps1p autophosphorylation (Figure 2B). Note that only a portion of myc-Mps1p binds the GST-Mob1p-Sepharose (compare levels of myc-Mps1p in myc-Ip to GST-Mob1p on the immunoblot probed with anti-myc antibody, Figure 2B). Perhaps Mob1p binds additional proteins that preclude the binding of Mps1p or maybe only a portion of Mob1p is posttranslationally modified in such a way to interact with Mps1p. Conversely, it is possible that only a subset of Mps1p is modified in such a way to render it capable of binding Mob1p. In fact, myc-Mps1p containing cell extracts was made from cells predominantly in late G2 early M phase, as overproduction of active Mps1p kinase causes a G2/M arrest (Hardwick *et al.*, 1996). Perhaps the Mob1p/Mps1p interaction is not so prominent at this

stage of the cell cycle. Note also that GST-Mob1p was phosphorylated during the in vitro kinase assay, (50–60 kDa region, center lane of kinase assay, Figure 2B); however, we cannot rule out the possibility of a coprecipitating kinase in addition to Mps1p.

GST-Mob1p, purified on glutathione-Sepharose, appears as multiple electrophoretic forms on an SDS-PAGE, a feature suggestive of phosphorylation (Figure 2A). In contrast, GST alone migrates as a single electrophoretic band (our unpublished results). Some of the electrophoretic forms of GST-Mob1p can be eliminated by treatment with protein phosphatase 2A or calf intestinal alkaline phosphatase, suggesting that in vivo phosphorylation alters Mob1p's mobility on SDS-PAGE (our unpublished results). To directly test whether Mob1p is phosphorylated in vivo, we labeled yeast expressing GST-Mob1p with [^{32}P]orthophosphate. We then precipitated GST-Mob1p on glutathione-Sepharose (Figure 3A, lane 1), cleaved Mob1p from GST (which remains immobilized on glutathione-Sepharose) with thrombin (Figure 3A, lane 2), and analyzed the soluble Mob1p by SDS-PAGE and processed for autoradiography. The resultant autoradiograph revealed that thrombin-cleaved Mob1p was ^{32}P -labeled revealing that Mob1p is phosphorylated in vivo.

To address whether Mob1p is phosphorylated by Mps1p protein kinase in vitro, we expressed 6H-Mob1p, (six histidine N-terminally tagged to amino acids 79–314 of Mob1p), in *E. coli* and purified it to use as substrate in an in vitro Mps1p kinase assay (performed as described by Lauzé *et al.*, 1995). We chose 6H-Mob1p for these experiments instead of yeast-

<i>mob1-34</i> <i>mob1-46</i>	1	MSFLQNFHIS	PGQTIRSTRG	FKWNTANAAN	NAGSVSPTKA	TPHNNTINGN
			V			
<i>mob1-55</i> <i>mob1-77</i>	51	NNNANTINNR	ADFTNNPVNG	YNESDHGRMS	PVLTPPKRHA	PPPEQLQNVN
			I		A	
	101	DFNYTPSHQK	PFLQPQAGTT	VPTHQDIKQI	VENTLGSEGV	LNQAVKLPRG
<i>mob1-77</i> <i>mob1-95</i> <i>mob1-34</i> <i>mob1-46</i> <i>mob1-55</i> <i>mob1-83</i>	151	<u>EDENEWLA</u> VH	<u>CVD</u> FYNQINM	LYGSITEFCS	PQTCPRMIAT	NEYEV L WAFQ
		K	PI			
			W		L	
			W			
				R		H
						P
<i>mob1-83</i>	201	KGQPPVSVA	PKYVECLMRW	CQDQFDES	L FFSKVETGTFP	EGFIQRVIQP
			L			
<i>mob1-34</i> <i>mob1-46</i> <i>mob1-67</i>	251	ILRLRLFRVTA	HIYCHHFNEI	LELNLOTVLN	TSFRHFCLFA	QEFELLRPAD
				S		
			Q			
	301	FGPLELLVME	LRDR			

Figure 4. Positions of *mob1* lesions on Mob1p. Amino acid substitutions in *mob1* alleles are noted under wild-type Mob1p sequence. Amino acids represented in bold print are conserved among the class 1 Mob1p-like proteins (see Figure 1). Underlined sequences represent the amino acids changed in *mob1* mutants.

made GST-Mob1p because Mob1p synthesized in *E. coli* migrates as a single electrophoretic band on an SDS-PAGE and is not coexpressed with any yeast kinases (our unpublished results). Upon incubation with GST-Mps1p and [γ^{32} P]ATP, 6H-Mob1p is phosphorylated in vitro (Figure 3B). 6H-Mob1p had no effect on the ability of GST-Mps1p to autophosphorylate or to phosphorylate an equivalent amount of MBP, an exogenous substrate of Mps1p. We feel it is unlikely that H6-Mob1p is phosphorylated by a kinase that coprecipitates with GST-Mps1p. Previous work suggests that an inactive form of GST-Mps1p, when expressed and purified using the same procedure used for active GST-Mps1p, harbors no detectable coprecipitating kinase activity (Lauzé *et al.*, 1995). These results suggest that Mob1p is phosphorylated by Mps1p in vitro; however, further analysis is required to determine whether Mob1p is a substrate of Mps1p in vivo.

MOB1 Is Required for Completion of Mitosis and Maintenance of Ploidy

To explore the essential function(s) of *MOB1* in vivo, we generated conditional alleles of *MOB1*. We used random PCR-mediated mutagenesis to create a library of mutagenized *mob1* genes in a yeast shuttle vector (see MATERIALS AND METHODS). We introduced the *mob1* library into a *mob1* Δ strain (FLY12A, Table 1) using a plasmid shuffle strategy and identified several temperature-sensitive alleles of *MOB1*, all of which have been sequenced. Most of the alleles are point mutations that cause amino acid changes in the conserved domains of Mob1p (Figure 4). Upon shift to the restrictive temperature, 37°C, all of the *mob1* mutants arrest as large budded cells with 2 N DNA content and

separated chromatin as shown for *mob1-77* (Figure 5). Analysis of microtubule structures via antitubulin immunofluorescence reveals that the mutants arrest with long bipolar mitotic spindles (Figure 5C). This cell cycle arrest is reversible with high viability (>95%) if returned to the permissive temperature within 3–4 h. These data suggest that *MOB1* is required for the completion of mitosis.

When the *mob1* mutations are present on low-copy centromeric plasmids in a *mob1* Δ yeast strain, all of the cells are maintained as stable haploids at permissive temperature. However, when present as a single copy integrated into the genome, five *mob1* alleles (*mob1-34*, *mob1-46*, *mob1-55*, *mob1-67*, *mob1-83*) cause an increase in ploidy at the permissive temperature (Figure 6A). The cells appear to have twice the amount of DNA, suggesting that they have become diploids. In agreement, when the diploidized *mob1* strains are mated to haploid cells of the opposite mating type, the mating products appear triploid by flow cytometric analysis and produce very few viable spores (our unpublished results). The observed increase-in-ploidy phenotype at permissive temperature is unusual; however, it has been seen in several mutants defective in SPB duplication (Schild *et al.*, 1981; Thomas and Botstein, 1986; Rose and Fink, 1987; Winey *et al.*, 1991) and in mutants defective in other cellular processes (Chan and Botstein, 1993). All *mob1* integrants, whether haploid or diploid, continue to arrest in late mitosis at the restrictive temperature but display a lower minimal restrictive temperature than when present on low-copy plasmids (34°C instead of 37°C).

MOB1 and MOB2 Share Genetic Interactions with MPS1

The late nuclear division arrest of *mob1* mutants suggests that the execution point for *MOB1* occurs in late mitosis, a time for which there is no evidence of *MPS1* function. In an effort to identify the role for the Mob1p/Mps1p interaction, we looked for genetic interactions between *MOB1* and *MPS1*. We crossed the two integrated alleles of *mob1* that behave as stable haploids at permissive temperature (*mob1-77* and *mob1-95*), to *mpe1* mutants in order to examine the phenotypes of the double mutants. We detected no synthetic lethality between either *mob1-77* or *mob1-95* and several *mpe1* alleles (summarized in Table 3). All of the *mob1 mpe1* double mutants grew without any detectable growth defect. However, *mob1-95 mpe1-1* double mutants diploidized at permissive temperature (Figure 6B). The degree of diploidization within a population of cells harboring both mutations is variable. This interaction appears to be allele specific for both *mpe1-1* and *mob1-95* (Table 3). The increase-in-ploidy phenotype observed in the double mutants may suggest that the mechanism for the increase in

ploidy displayed in some *mob1* single mutants occurs through *MPS1* function. Similarly, populations of *mob2Δ mps1-1* double mutants display an increase in ploidy, suggesting a role for *MOB2* in the maintenance of ploidy (Figure 6C; Table 3).

The observation that both *mob1-95 mps1-1* and *mob2Δ mps1-1* double mutants display an increase in ploidy may suggest that *MOB1* and *MOB2* perform similar or overlapping roles. In an attempt to establish additional genetic interactions shared between *MOB1* and *MOB2*, we looked for synthetic phenotypes displayed by *mob1 mob2Δ* double mutants and we found none. *mob1-77 mob2Δ* and *mob1-95 mob2Δ* double mutants are maintained as stable haploids and grow at wild-type rates at permissive temperature. They arrest in late mitosis, as do the *mob1* single mutants, at restrictive temperature (our unpublished results). Furthermore, high-copy plasmids harboring wild-type *MOB2* are unable to suppress either the increase-in-ploidy phenotype or the late nuclear division arrest of *mob1* mutants at restrictive temperature (our unpublished results).

The Cell Cycle Arrest of mob1 Cells Does Not Require MPS1

Although *MPS1* has no known function in late mitosis, it was possible that *MOB1* requires *MPS1* for its role in late mitosis. To address this possibility, we tested whether cells harboring *mob1* mutations require *MPS1* to arrest in late nuclear division at restrictive temperature. We first synchronized *mob1-77 mps1-1* and *mob1-95 mps1-1* double mutants in G1 with the mating pheromone, α factor, and released them into media containing hydroxyurea. This allows cells to execute *MPS1*'s SPB duplication function and arrest in early S phase. The cells were then released into fresh media without hydroxyurea at the restrictive temperature to inactivate *MPS1*'s checkpoint function (Weiss and Winey, 1996). If *mob1* mutant cells require *MPS1* to arrest in late mitosis, then *mob1 mps1* double mutants should fail to arrest at the restrictive temperature. This is not the case. Both *mob1-77 mps1-1* and *mob1-95 mps1-1* double mutants arrest as large budded cells with G2 DNA content, similar to the *mob1* single mutants (our unpublished results). Furthermore, high-copy plasmids harboring wild-type *MPS1* were unable to suppress *mob1* mutations (our unpublished results). Together, these data suggest that the late nuclear division arrest, caused by *mob1* mutations, is independent of *MPS1* function, as has been shown for other late nuclear division *cdc* mutants (Weiss and Winey, 1996; E. Weiss, personal communication).

Figure 5. Cells harboring *mob1* mutants arrest in late nuclear division. (A) Flow cytometric analysis of DNA content of a wild-type yeast strain grown at 25°C or 37°C for 3 h. The biphasic peaks represent cells with G1 (1N) and G2/M (2N) DNA content. (B) Flow cytometric analysis of DNA content of yeast harboring the *mob1-77* allele grown at 25°C or 37°C for 3 h. At 37°C cells harboring *mob1-77* mutation arrest as large budded cells with G2/M DNA content. Percent large-budded (LB) cells are noted. (C) Arrested *mob1-77* cells (3 h at 37°C) were stained with anti-tubulin antibody (left panels) and 4,6-diamidino-2-phenylindole (right panels). The cells arrest with long mitotic spindles and separated chromatin masses. Three representative cells are shown.

MOB1 and MOB2 Are Not Required for the Spindle Assembly Checkpoint

MPS1 is required for SPB duplication and for mitotic checkpoint control (Winey *et al.*, 1991; Hardwick *et al.*, 1996; Weiss and Winey, 1996). To test whether *MOB1* and *MOB2* are required for *MPS1* function in the spindle assembly checkpoint, we assayed *mob1* mutants and *mob2Δ* for their ability to arrest in nocoda-

Table 3. Synthetic growth phenotypes of double mutants

	<i>mps1-1</i>	<i>mps1-737</i>	<i>mps1-3796</i>	<i>mps1-6</i>	<i>cdc5-1</i>	<i>cdc14-1</i>	<i>cdc15-2</i>	<i>lte1Δ</i>
<i>mob1-77</i>	+H	+H	+H	+H	SL	+	SL	SL
<i>mob1-95</i>	+D	+H	+H	+H	SL	+	SL	SL
<i>mob2Δ</i>	+D	+H	+H	+H	+	+	+	nd

+, no detectable synthetic growth defect; SL, synthetic lethality; H, haploid; D, diploid; nd, not determined.

zole, a microtubule-disrupting agent that triggers the checkpoint pathway causing a cell cycle arrest. All of the *mob1* and *mob2Δ* strains arrest in nocodazole at 25°C, indicating that they are not defective in the spindle assembly checkpoint at permissive temperature (our unpublished results). These results do not completely rule out the involvement of *MOB1* in mitotic checkpoint regulation as it is possible that *mob1* is defective at mitotic checkpoint control at the restrictive temperature. However, it cannot be distinguished cytologically whether nocodazole-treated *mob1* cells arrest at restrictive temperature in response to nocodazole treatment or as a consequence of the defect in *MOB1*'s essential function, executed later in mitosis. To assay mitotic checkpoint activation in *mob1* mutants at restrictive temperature, we monitored Mad1p hyperphosphorylation as a molecular marker for checkpoint activation. When the mitotic checkpoint is triggered, Mad1p becomes hyperphosphorylated, an event dependent on active Mps1p protein kinase and is easily detectable by a shift in mobility by SDS-PAGE (Hardwick and Murray, 1995; Hardwick *et al.*, 1996). We first synchronized *mob1-77* and *mob1-95* cells at permissive temperature in early G1 with a mating pheromone, α factor. The cells were then released into media containing nocodazole at the restrictive temperature. Samples of *mob1* mutants were taken after nocodazole treatment at restrictive temperature and were analyzed for Mad1p hyperphosphorylation by immunoblot as described by Hardwick and Murray (1995). By this analysis, we observed that Mad1p is hyperphosphorylated in nocodazole-treated *mob1-77* and *mob1-95* cells at restrictive temperature (Figure 7). The arrested cells also contained elevated levels of Clb2p (our unpublished results). Mad1p from cells treated similarly but in the absence of nocodazole was not hyperphosphorylated (our unpublished results). These results suggest that *MOB1* is not required for the spindle assembly checkpoint. However, it remains possible that we do not possess the appropriate *mob1* mutant alleles to demonstrate a checkpoint role. It is also possible, although unlikely, given its association with Mps1p, that Mob1p is required for a step in the spindle assembly checkpoint that is downstream of Mad1p hyperphosphorylation.

MOB1 Has Genetic Interactions with CDC5, CDC15, and LTE1

The late nuclear division arrest phenotype of *mob1* mutants is similar to that described for cells harboring mutations in a number of genes, including *CDC5*, *CDC14*, *CDC15*, and *LTE1* (Pringle and Hartwell, 1981; Wickner *et al.*, 1987). *CDC5* and *CDC15* encode essential protein kinases, *CDC14* encodes an essential protein tyrosine phosphatase, and *LTE1* encodes a GDP/GTP exchange protein (Schweitzer and Philippsen, 1991; Wan *et al.*, 1992; Kitada *et al.*, 1993; Keng *et al.*, 1994). Deletion of *LTE1* yields cells that are cold sensitive for growth. To investigate whether *MOB1* function is linked to the function of these four genes, we looked for genetic interactions between *MOB1* and those four genes. We assayed for synthetic interactions between double mutants and observed no synthetic lethality or growth defects in cells harboring *mob1* (using *mob1-77* and *mob1-95*) and *cdc14-1* mutations (Table 3). However, we were unable to obtain double mutants between either allele of *mob1* (*mob1-77*, *mob1-95*) and *cdc5-1*, *cdc15-2*, or *lte1Δ* strains, suggesting that there are synthetic lethal interactions between *mob1* and the three genes. To confirm these results, we crossed *mob1* strains harboring pRS316-MOB1 or pRS426-MOB1 with *cdc15-2* or *lte1Δ* strains. The resultant double mutants were plasmid dependent for viability. We conclude that both the *mob1-77* and *mob1-95* alleles are synthetically lethal with *cdc5-1*, *cdc15-2* and *lte1Δ* strains, suggesting that *MOB1* functions in the same pathway as *CDC5*, *CDC15*, and *LTE1*. Cells harboring *mob2Δ* did not display any synthetic lethality or growth defects when combined with *cdc5-1*, *cdc14-1*, or *cdc15-2* (summarized in Table 3).

DISCUSSION

MOB1 is an essential yeast gene required for the completion of mitosis and for the maintenance of ploidy. It is a member of a conserved, and previously unrecognized, gene family, likely to be present in all eukaryotes. In addition to *MOB1*, budding yeast contain *MOB2*, a nonessential member of the *MOB1* gene family. We have been unable to detect any direct genetic interactions (high-copy suppression and synthetic le-

Figure 6. *MOB1* and *MOB2* are required for the maintenance of ploidy. (A) Flow cytometric analysis of DNA content of yeast harboring various alleles of *MOB1* grown at 25°C. Cells harboring *mob1-77* or *mob1-95* mutations contain haploid biphasic peaks representing G1 (1N) and G2/M (2N) DNA content. Cells harboring *mob1-34*, *mob1-46*, *mob1-55*, or *mob1-83* mutations contain diploid biphasic peaks (2N and 4N) indicating that they have diploidized. Populations of yeast harboring *mob1-67* mutation contain both haploid and diploid cells. (B) Flow cytometric analysis of DNA content of tetraploid tetrad resulting from crossing *mob1-95* and *mps1-1* strains. Populations of wild-type cells and cells harboring *mob1-95* or *mps1-1* mutations are haploids containing both G1 (1N) and G2/M (2N) DNA content. Cells harboring both *mob1-95* and *mps1-1* have diploid DNA content (2N and 4N). (C) Flow cytometric analysis of DNA content of tetraploid tetrad resulting from crossing

thality) between *MOB1* and *MOB2*. Although conserved, the predicted amino acid sequences of the *MOB1*-like gene products offer no insight to their molecular function. We have demonstrated by two independent methods, two-hybrid and coprecipitation, that Mob1p binds Mps1p, an essential protein kinase required for SPB duplication and mitotic checkpoint regulation. In addition, Mob1p is a phosphoprotein in vivo and a substrate of Mps1p protein kinase in vitro, raising the possibility that Mob1p is a substrate of Mps1p in vivo.

Cells harboring defective *MOB1* genes arrest in late mitosis at restrictive temperature. This role of *MOB1* in the completion of mitosis is probably mediated through its association with genes other than Mps1p. There are a number of genes, encoding regulatory proteins, that, when mutated, cause a cell cycle arrest similar to *mob1* mutants. There is an extensive array of genetic interactions linking them together, suggesting that they belong to a common pathway (Pringle and Hartwell, 1981; Wickner *et al.*, 1987; Johnston *et al.*, 1990; Parkes and Johnston, 1992; Kitada *et al.*, 1993; Molero *et al.*, 1993; Spevak *et al.*, 1993; Keng *et al.*, 1994; Shirayama *et al.*, 1994a, 1994b, 1996; Toyn and Johnston, 1994). We have begun to establish that *MOB1* shares genetic interactions with several members of this class of genes. Mutations in *MOB1* exhibit synthetic lethality with mutants in genes encoding two protein kinases (*CDC15*, *CDC5*) and a GDP/GTP exchange factor (*LTE1*). In addition, high-copy plasmids encoding *MOB1* suppress cells lacking *DBF2*, a gene encoding another protein kinase required for the completion of mitosis. Furthermore, Mob1p physically interacts with Dbf2p protein kinase (C. Denis, personal communication). These results suggest *MOB1* participates in a common regulatory event required to complete mitosis. Perhaps Mob1p is a substrate or effector for one or more of the kinases required for completion of mitosis (i.e., Cdc5p, Cdc15p, Dbf2p).

The specific roles for *MOB1* and the other genes required for the completion of mitosis are unknown. However, the late nuclear division arrest has been characterized, to some extent, as the failure to complete a number of events. These events include the inactivation of the M phase-promoting protein kinase complex, Clb2p/Cdc28p, the disassembly of the mitotic spindle, the formation of prereplicative complexes on DNA origins, the lowering of cAMP levels, and the initiation of events responsible for cytokinesis (Smith *et al.*, 1990; Russell *et al.*, 1993; Spevak *et al.*,

Figure 6 (cont). *mob2Δ* and *mps1-1* strains. Populations of wild-type cells and cells harboring *mob2Δ* or *mps1-1* mutations are haploids containing both G1 (1N) and G2/M (2N) DNA content. Cells harboring both *mob2Δ* and *mps1-1* have diploid DNA content (2N and 4N).

Figure 7. Mad1p is hyperphosphorylated in *mob1* mutants. Cells were synchronized in α -factor at 25°C and released in media containing 15 μ g nocodazole (NZL) and 30 μ g benomyl at 36°C. Samples were taken before (–) and after (+) treatment with nocodazole and processed for 10% SDS PAGE and immunoblotted. The immunoblot was probed with affinity-purified antiMad1p antibody. The lane loaded with extract from a strain deleted for *MAD1* (*mad1Δ*) reveals an immunoreactive background protein. Lanes loaded with extract from wild type (WT), *mob1-77*, and *mob1-95* cells are indicated. Mad1p hyperphosphorylation is detectable only in nocodazole-treated cells by the presence of slower migrating forms.

1993; Romanowski and Madine, 1996; Juang *et al.*, 1997). These events may be regulated by the same or separate pathways that are triggered by a master switch at the end of anaphase.

Perhaps *MOB1* and the other genes required for completion of mitosis mediate the transition from M phase to G1 by regulating the anaphase promoting complex (APC)/cyclosome (King *et al.*, 1996). The APC/cyclosome targets several substrates for ubiquitin-mediated proteolysis, carried out by the 26S proteasome, which is crucial for the completion of mitosis. It is responsible for targeting degradation of the B-type cyclin, Clb2p, which is the regulatory subunit of the M phase-inducing kinase, Cdc28p. This event is a prerequisite for the completion of mitosis, as expression of an indestructible form of Clb2p in yeast causes a late nuclear division arrest (Surana *et al.*, 1993). Strains harboring mutations in *DBF2*, *CDC5*, *CDC15*, *CDC14*, *TEM1* or harboring triple deletions in the *RAS1Δ*, *RAS2Δ*, *RSR1Δ* genes all arrest in late nuclear division with high Clb2p/Cdc28p kinase activity, suggesting they may be required for cyclin degradation and/or Cdc28p inactivation (Surana *et al.*, 1993; Shirayama *et al.*, 1994; Toyn and Johnston, 1994; Morishita *et al.*, 1995). The APC/cyclosome is also necessary for targeting the spindle-associated protein, Ase1p, for degradation at the end of mitosis (Juang *et al.*, 1997). Ase1p degradation is a prerequisite for the disassociation of the mitotic spindle at the end of mitosis, and it is probable that Ase1p degradation is inhibited in the late mitotic mutants. Recently, a component of the 26S proteasome has been implicated in an early step in SPB duplication, suggesting that regulated proteolysis is also required for SPB duplication

(McDonald and Byers, 1997). Perhaps all of the above mentioned regulated proteolytic events are coordinated to trigger the exit from mitosis and prepare cells to enter the next cell cycle (see below).

The role in the completion of mitosis for *MOB1* may be independent of *MPS1*. There is no evidence for any genetic interactions between *MPS1* and any of the genes, other than *MOB1*, required for the completion of mitosis (E. Weiss, personal communication). Moreover, cells harboring mutations in both *MPS1* and *MOB1* arrest with phenotypes similar to *mob1* single mutants when shifted to the restrictive temperature. This suggests that the mitotic checkpoint function of *MPS1* is not required for cells to arrest in late mitosis. Likewise, we have no evidence that *MOB1* is required for the spindle assembly checkpoint, as determined by monitoring Mad1p hyperphosphorylation as molecular marker for checkpoint activation.

In addition to its role in late nuclear division, *MOB1* is required for the maintenance of ploidy, which may reflect its interaction with *MPS1*. Most *mob1* mutants display a complete increase in ploidy at permissive temperature, i.e., haploid cells become diploid. The increase in ploidy phenotype is unique to *MOB1* among the collection of genes required for the completion of mitosis and may suggest an additional, perhaps separable, role for *MOB1*. The increase in ploidy phenotype in the *mob1* mutants is sensitive to gene dosage. When present as a single integrated copy, many of the conditional alleles of *MOB1* cause an increase in ploidy at a temperature permissive for growth. However, when the mutant *mob1* alleles are present on low-copy number plasmids, which presumably express more Mob1p than the integrated gene, cells remain haploid. To date, we have been unable to study the increase in ploidy in isolation using the available alleles in synchronized cells or using other methods designed to inactivate Mob1p.

Although this type of increase-in-ploidy phenotype, complete diploidization, is unusual, there are several possible mechanisms that may bring it about. One such mechanism is a failure to undergo budding and cytokinesis. Such a defect would yield binucleate cells, which we have not observed for *mob1* mutants. Another possible mechanism for a complete increase in ploidy is a failure to properly segregate the duplicated DNA at mitosis. This could occur as a result of a defect in nuclear migration, chromosome segregation, or mitotic spindle formation. Perhaps proper mitotic spindle formation is affected in select *mob1* mutants (see below). Cells that overreplicate their DNA, meaning that DNA replication occurs twice in one cell cycle, may also lead to a complete increase in ploidy. Such a defect might occur in *mob1* mutants if *MOB1* were required to limit DNA replication to once per cell cycle.

There is a precedent for genes that are required for completion of mitosis that also have a role in initiation of DNA synthesis. Several genes required for the completion of late mitosis are also required for the formation of prereplicative complexes. Prereplicative complexes are essential for "licensing" DNA for replication in the following S phase and form in late mitosis (Chevalier and Blow, 1996; Romanowski and Madine, 1996; Stillman, 1996). The formation of prereplicative complexes requires the activities of late-division gene *CDC14* and probably the late-division gene *CDC5*, which encode a protein phosphatase and a protein kinase, respectively (Hardy, 1996; Hardy and Pautz, 1996; Kroll *et al.*, 1996). Furthermore, several genetic interactions have been established between *CDC5* and *CDC14* and components of the origin recognition complex (ORC) (Hardy, 1996; Hardy and Pautz, 1996; Kroll *et al.*, 1996). Recently it has been shown that Cdc5p physically interacts with Dbf4p, the regulatory subunit of the S phase-activating protein kinase, Cdc7p, supporting previous evidence of a genetic interaction (Kitada *et al.*, 1993; Hardy and Pautz, 1996). Whether this signifies an S phase execution point for Cdc5p or a late mitotic role for Dbf4p remains to be seen. Nonetheless, at least some of the late nuclear division genes are required to reset the DNA synthesis cycle in late mitosis. Although it is possible that, in addition to a role in exiting mitosis, *MOB1* is required for maintaining proper ploidy through a role in DNA replication, it is the only gene reported of this class that, when mutated, causes a complete increase in ploidy.

Perhaps the mechanism of the increase in ploidy of *mob1* mutants derives from the association between *MOB1* and *MPS1*. Strains harboring both *mob1-95* and *mps1-1* become diploid while strains harboring either mutation alone remain haploid. The complete increase in ploidy could be the result of synergistic damage to parallel pathways, one being the control of the initiation DNA synthesis and the other being the regulation of SPB duplication; or it could be caused by additive damage to a common pathway. This common pathway could be the regulation of SPB duplication. Although *MPS1* has no role in DNA synthesis, it has been well established that Mps1p is required for SPB duplication (Winey *et al.*, 1991). A complete increase in ploidy can occur in mutants that affect SPB duplication (Schild *et al.*, 1981; Thomas and Botstein, 1986; Rose and Fink, 1987; Winey *et al.*, 1991). In these mutants, cells progress through the cell cycle in the absence of SPB duplication. The cells complete S phase and undergo mitosis with a monopolar spindle, which is incapable of properly segregating the replicated DNA. After completion of mitosis, all of the replicated genome localizes to one cell, while the sister cell becomes aploid. This mechanism for an increase

in ploidy also occurs in cells that have transiently lost Mps1p activity (Winey *et al.*, 1991). Given the association of *MOB1* and *MPS1*, reported here, a plausible mechanism for the increase in ploidy for *mob1* single mutants and *mob1-95 mps1-1* double mutants is the failure of SPB duplication. Further, Mob1p may be required to physically associate with Mps1p for proper SPB duplication. Support for this comes from the observation that, in a two-hybrid assay, Mps1p binds poorly to a mutant Mob1p (from *mob1-55*) that causes strains to become diploid. However, Mps1p binds strongly to two other mutant Mob1p proteins that do not cause an increase in ploidy (C. Denis, personal communication).

Genetic interactions between *MOB1* and *MPS1*, other than the diploidization of *mob1-95 mps1-1* mutants, have not been detected. Nonetheless, the increase-in-ploidy phenotype is also observed in *mob2Δ mps1-1* double mutants, but not in the single-mutant strains. This suggests a role for the *MOB1* family in the maintenance of ploidy and may suggest an overlapping role for *MOB2*. However, more work will be required to understand the elusive relationship between *MOB1* and *MOB2*.

All of the genes required for the completion of mitosis, discussed above, may be collectively viewed as performing cell cycle resetting functions. These cell cycle resetting functions may be necessary not only to allow the cell to exit mitosis, but may also be required for progression into and through the next cell cycle. Support for this concept comes from data indicating that prereplicative complexes, required to render chromosomes competent for replication, are assembled in late mitosis (Chevalier and Blow, 1996; Romanowski and Madine, 1996; Stillman, 1996). In this vein, there may be an event that occurs at the M/G1 transition responsible for rendering SPBs competent for duplication. That event may involve targeted proteolysis, since a defect in the cap subunit of the 26S proteasome causes a failure in SPB duplication (McDonald and Byers, 1997). Moreover, the hypothetical SPB "licensing" may require *MOB1* function through its association with *MPS1*.

Whether the mechanism of the complete increase in ploidy of *mob1* mutants occurs through a failure in SPB duplication, over-replication of DNA, or some other mechanism, *MOB1* remains unique in having an additional requirement for the completion of mitosis. *MOB1* appears to reveal a link between the completion of the cell cycle and events in the subsequent cell cycle. Further work on *MOB1* will be necessary to understand how its highly conserved gene product and associated proteins coordinate the timing and execution of these different cell cycle events.

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