Functional characterisation of Spc29p,

a component of the *Saccharomyces cerevisiae*

spindle pole body.

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Elliott, S., Knop, M., Schlenstedt, G. and Schiebel, E. (1999) Spc29p is a component of the Spc110p subcomplex and is essential for spindle pole body duplication. *Proc Natl Acad Sci U S A*, **96**, 6205-6210.

Schramm, C., Elliott, S., Shevchenko, A. and Schiebel, E. (2000) The Bbp1p-Mps2p complex connects the SPB to the nuclear envelope and is essential for SPB duplication. *Embo J*, **19**, 421-433.

Abbreviations

A. nidulans	*Aspergillus nidulans*
APC	anaphase promoting complex
APS	ammomium persulphate
ATP	adenosine 5' triphosphate
bp	base pairs
BSA	bovine serum albumin
°C	degrees centigrade
CNBr	cyanogen bromide
Da	daltons
DAPI	4,6-diamidino-2-phenylindole
ddH$_2$O	double distilled water
dNTPs	deoxy nucleotide triphosphates
DMF	N, N-dimethyl formamide
DMP	dimethyl pimelimidate
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DTT	1, 4 dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis (2-aminoethyl) N, N, N', N',-tetraacetic acid
FACS	fluorescence-activated cell sorter
FITC	fluorescein isothiocyanate
5-FOA	5-fluoroorotic acid
g	gram(s)
GFP	green fluorescent protein
3GP	3-glycerophosphate
GST	glutathione S transferase
HA	haemagglutinin
HRP	horse radish peroxidase
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo bases
kDa	kilo daltons
kV	kilo volts
M	molar
mA	milli amps
MALDI	matrix-assisted laser desorption ionisation
MATa	mating type **a**

MATα	mating type α
μF	micro Faraday
μg	micro grams
mg	milli grams
min	minute(s)
μl	micro litres
ml	milli litre(s)
mM	milli molar
MOPS	morpholino-propane sulphonic acid
mRNA	messenger RNA
MTOC	microtubule organising centre
ng	nano gram
nm	nano meters
Ω	ohms
OD	optical density
%	percent
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulphonyl fluoride
ProA	protein A
rpm	revolutions per minute
RNA	ribonucleic acid
RNase	ribonuclease
RT	room temperature
S	sediment coefficient
SCF	Skp1p cullin F-box protein complex
S. cerevisiae	*Saccharomyces cerevisiae*
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
S. pombe	*Schizosaccharomyces pombe*
sec	second(s)
SPB	spindle pole body
TCA	trichloroacetic acid
TEMED	N, N, N', N'- tetramethylethylenediamine
ts	temperature sensitive
U	universal units
Ura	uracil
V	volts
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

Amino acids

A	Ala	alanine
C	Cys	cysteine
D	Asp	aspartic acid
E	Glu	glutamic acid
F	Phe	phenylalanine
G	Gly	glycine
H	His	histidine
I	Ile	isoleucine
K	Lys	lycine
L	Leu	leucine
M	Met	methionine
N	Asn	asparagine
P	Pro	proline
Q	Gln	glutamine
R	Arg	arginine
S	Ser	serine
T	Thr	threonine
V	Val	valine
W	Trp	tryptophan
Y	Tyr	tyrosine

Nomenclature

The nomenclature used to describe the genes and proteins of *Saccharomyces cerevisiae* is different to that used for other organisms, therefore a brief explanation is appropriate. Genes and their encoded protein are given the same name consisting of three letters and a number. The authors that describe identification of a new gene or protein designate these letters and numbers. Often genes are identified through screens for mutants, therefore the name will come from the screen (*CDC31*, <u>c</u>ell <u>d</u>ivision <u>c</u>ycle mutant number <u>31</u>; *MPS2*, <u>m</u>onopolar <u>s</u>pindle mutant number <u>2</u>). Alternatively, the name may be a description of the protein (*SPC29*, <u>s</u>pindle <u>p</u>ole <u>c</u>omponent with a molecular weight of <u>29</u> kDa). Gene names are given in upper case italics (*SPC29*) unless the gene has been mutated, then the gene name is given in lower case italics (*spc29*), if the mutation results in a temperature sensitive phenotype the gene name is followed with *(ts)*, for example *spc29(ts)*. Mutants are often given additional numbers to distinguish between different mutations in the same gene, for example *spc29-2*. If a gene is completely deleted from the genome or contains a deletion within the coding sequence, this is described with *Δ*, for example *Δspc29::HIS3* describes a strain in which the *SPC29* gene has been deleted from the genome and replaced (*::*) with the *HIS3* gene. When referring to the protein the name is not given in italics, the first letter is given in upper case, the following two letters are given in lower case and the number is followed with a p (for protein), for example Spc29p.

Yeast strains are usually given names that describe the identity of the individual that constructed the strain, followed by a number (SEY14, <u>S</u>arah <u>E</u>lliott <u>y</u>east strain number <u>14</u>). The same applies for plasmids, however most plasmid names contain the pre-fix 'p' to designate it as a plasmid name (pSE1, <u>p</u>lasmid constructed by <u>S</u>arah <u>E</u>lliott, plasmid number <u>1</u>).

Abstract

In the budding yeast *Saccharomyces cerevisiae*, microtubule organising functions are carried out by the spindle pole body (SPB). The SPB is a disk-like, multilayered structure that is embedded in the nuclear membrane throughout the cell cycle. The major substructures of the SPB are the outer, central and inner plaques and the half-bridge. The central plaque is embedded in the nuclear membrane. The outer and inner plaques are positioned on the cytoplasmic and nuclear side of the central plaque where they organise the cytoplasmic and nuclear microtubules respectively. The half-bridge extends from the central plaque along the nuclear envelope and is involved in SPB duplication and organises cytoplasmic microtubules during G1 of the cell cycle and during mating.

Described within is the identification and characterisation of a novel SPB component, Spc29p. Spc29p was found as a component of a salt stable SPB subcomplex composed of central and inner plaque SPB components. This complex contained Spc29p, the central plaque component Spc42p, the γ-tubulin-complex-binding protein Spc110p and calmodulin. Spc29p acts as a linker between Spc42p and the globular C-terminus of Spc110p at the central plaque. Evidence is provided that the calmodulin-binding site of Spc110p influences the binding of Spc29p to Spc110p. In addition, co-overexpression of Spc29p, Spc42p and Spc110p resulted in a three fold increase in the diameter of the SPB, indicating that expression levels of SPB components may be involved in the size control of the SPB.

Construction and analysis of *spc29* temperature sensitive mutants uncovered another important function of Spc29p. Using immunofluorescence, FACS analysis and electron microscopy the *spc29(ts)* mutants were found to have an SPB duplication defect. A role for Spc29p in SPB duplication was supported by the genetic and biochemical interactions between Spc29p and other SPB components involved in SPB duplication, namely the half-bridge components Cdc31p and Kar1p. Evidence is also provided for an interaction between Spc29p at the periphery of the central plaque, with the Bbp1p/Mps2p complex, which is involved in embedding the SPB in the nuclear envelope.

Spc29p was also found to be phosphorylated in a cell cycle dependent manner.

Introduction

Yeast as a model system

The budding yeast *Saccharomyces cerevisiae* is a unicellular, eukaryotic organism that is used as a model system to study cellular structures and processes for example, the cytoskeleton, the endoplasmic reticulum and the Golgi apparatus, cell cycle control, mitosis, transcription, protein synthesis and vesicle trafficking to name but a few. The reason the budding yeast has become so popular as a model system is due to the ease in which it can be manipulated genetically and biochemically. The sixteen chromosomes of the haploid budding yeast have been fully sequenced and the genome sequence is easy to access. Yeast can be genetically manipulated, for example chromosomal genes can be specifically deleted, mutated or epitope-tagged with ease enabling the study of gene and protein function. There is also a host of vectors designed for yeast that can be used to construct plasmids for the study of genetic interactions in the two hybrid system, suppression analysis and in determining synthetic lethality between mutants. In addition, experimental techniques for studying the budding yeast are well established and described in the literature. The study of budding yeast can be easily justified by the enormous number of homologues found in higher eukaryotes including humans, in fact the similarities can be so high that some human genes can substitute for their yeast homologues without any loss of function.

The budding yeast life cycle

Budding yeast can proliferate in either a haploid or diploid state. A diploid cell can divide either by mitosis or, in times of nutrient starvation, can go through meiosis and sporulation to form four haploid spores (Kupiec *et al.*, 1997). Haploid cells can divide by mitosis or can undergo conjugation (mating) to form diploid cells (Marsh and Rose, 1997) (Figure 1). Haploid budding yeast cells exist in two mating types, *MATa* or *MATα*, *MATa* cells mate with *MATα* cells, cells with the same mating type cannot mate with each other. However, due to the *HO* endonuclease, haploid yeast cells can
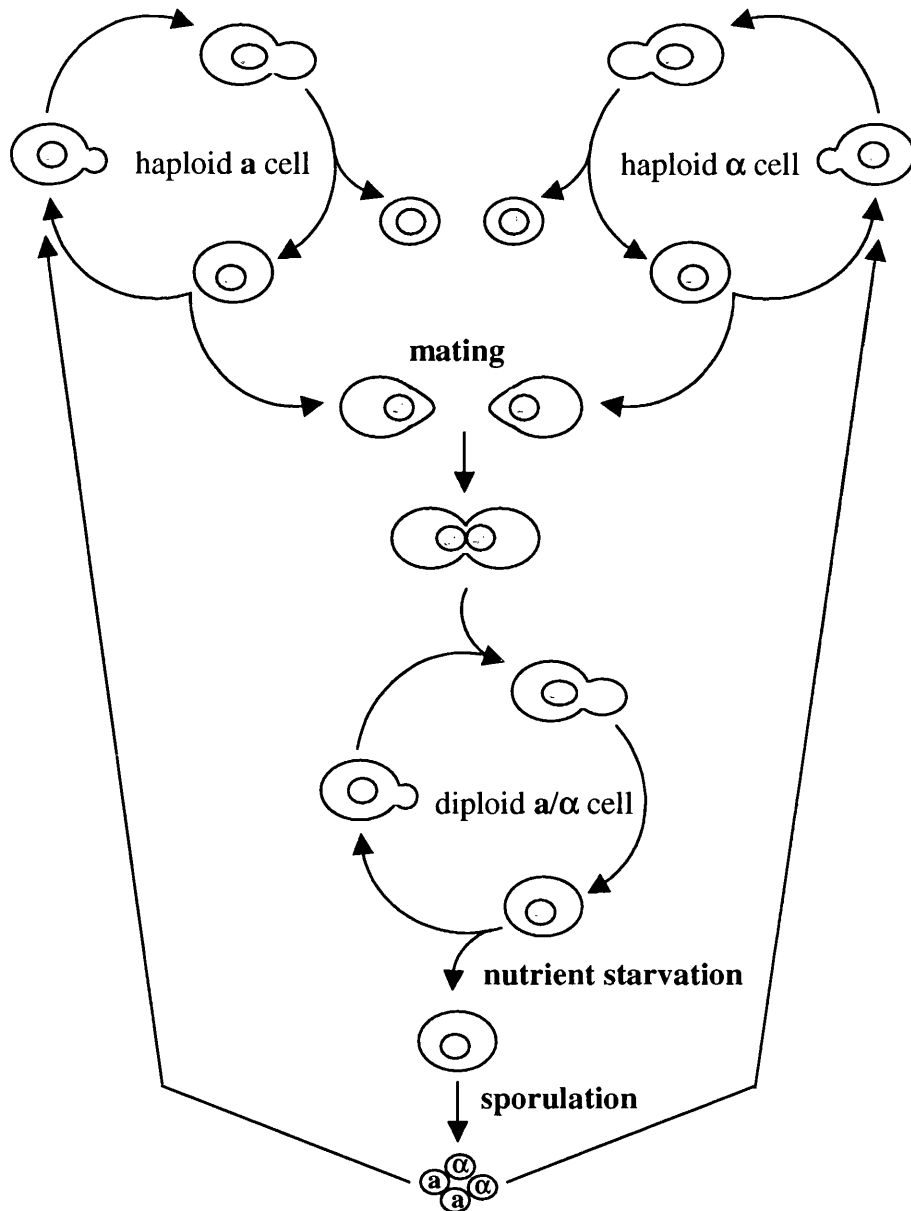
Figure 1 The budding yeast life cycle

Diploid cells sporulate when there is a lack of nutrients in the growth medium, producing haploid spores. These spores can proliferate as haploid cells or can mate to form diploid cells.

switch from one mating type to the other. Therefore, "wild type" laboratory strains are *HO⁻* to prevent the mating type switch. Haploid cells are easier to manipulate genetically as they have only one copy of each gene and different mating types are kept separate to avoid formation of diploids. *MATa* cells are used more frequently than *MATα* cells as cultures of *MATa* cells can be synchronised in G1 using the synthetic mating pheromone, α-factor. Also, "wild type" laboratory strains are in fact mutant strains that have certain growth requirements (for example YPH499, Table 2), these are usually addition of specific amino acids or the nucleotide base uracil to the growth medium. The major advantage of this is that marker genes can be used to follow genetic manipulations.

The cell cycle

The cell cycle of all eukaryotic cells consists of two stages, interphase and mitosis (Alberts *et al.*, 1994). Interphase is the time when a cell is not dividing, it is composed of the two "gap" phases, G1 and G2, that occur before and after S phase, in which DNA replication occurs. M phase occurs after G2 and makes up the rest of the cell cycle. M phase is when the cell segregates its contents, including the duplicated chromosomes into two daughter cells. The cell cycle is therefore composed of four phases, G1, S, G2 and M phase. This is of course, a simplified view of the cell cycle, but suffices for general purposes. *S. cerevisiae* cells divide by budding, which means that a bud forms in late G1 and grows while the mother cell remains the same size. As the bud grows, cytoplasmic components are transported into the bud, then during mitosis the nucleus elongates so that it is positioned along the mother bud axis with the centre of the nucleus in the bud neck. Therefore, after chromosome separation and cytokinesis, the nucleus is split between mother and daughter cells. This means that by looking at the morphology of a cell it can be, to some extent, determined which stage of the cell cycle that cell is in (Figure 2).

When a cell is in the early G1 growth phase there are a number of paths it can follow, determined mainly by the external environment (Lew *et al.*, 1997). If there is a lack of nutrients in the growth medium, haploid G1 cells will enter a quiescent, stress resistance state in which protein synthesis is greatly reduced. Diploid cells in early G1 will undergo meiosis and sporulation in the absence of nutrients. When nutrients are abundant, haploid cells in early G1 are capable of mating. Mating pheromones secreted
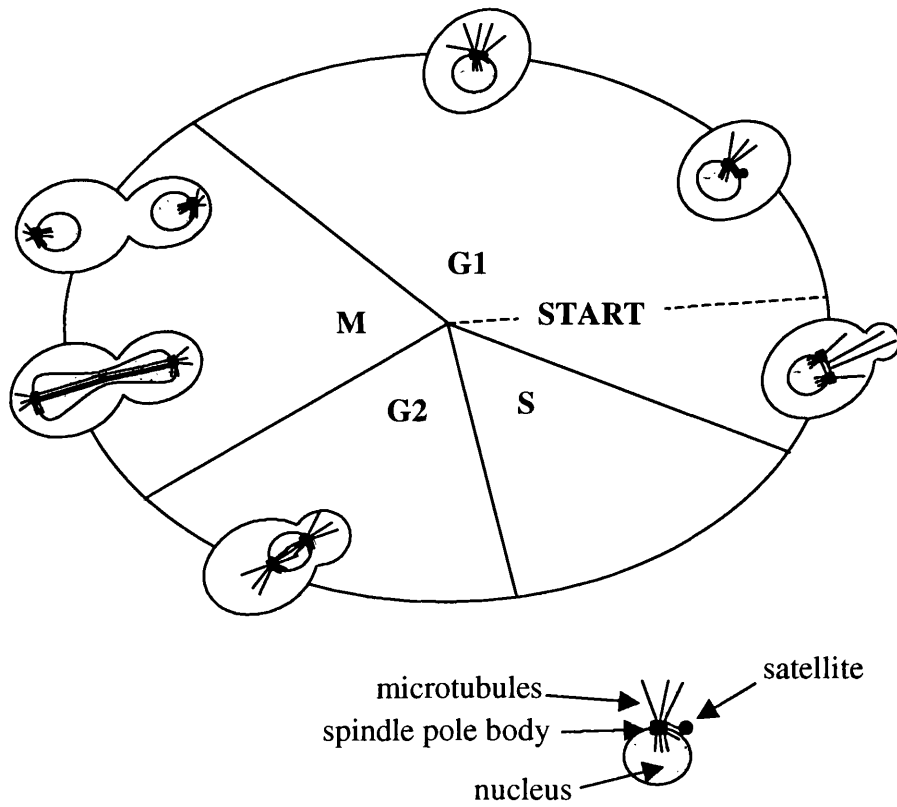
Figure 2 The budding yeast cell cycle

The morphology of a budding yeast cell changes as the cell progresses

through the cell cycle.

from cells of opposite mating types will arrest mating partners in G1, preventing entry into S phase until after mating has occurred. However, in late G1 there is a point that once passed commits the cell to completing the cell cycle, at this point cells are no longer capable of mating until G1 of the next cell cycle. This point in late G1 is termed START.

Cell cycle regulation

The cell cycle can be described as a series of events that occur in a defined order to achieve the accurate division of a cells contents into two daughter cells (Alberts *et al.*, 1994; Lew *et al.*, 1997). Each distinct event cannot occur until the previous event has been completed, for example mitosis will not commence until DNA replication is complete. The progression of the cell cycle in eukaryotic cells is controlled by cyclins. Cyclins exert their control by initiating the transcription or degradation of proteins involved in the different stages of the cell cycle. Cyclins were first identified in rapidly dividing early embryonic sea urchin cells and were named according to the cyclic increase and decrease in their protein levels during cycles of cell division (Evans *et al.*, 1983). Since then cyclins have been identified as components of all eukaryotic cells. The function of cyclins is to activate and provide substrate specificity to cyclin dependent kinases (CDKs). The cyclin/CDK complexes act by phosphorylating target proteins, for example transcription factors, which are activated by the phosphorylation event. Phosphorylation by the cyclin/CDK complexes also targets some proteins for degradation. Degradation is an irreversible way of "switching off" modified proteins and therefore helps to give directionality to the cell cycle.

A protein targeted for degradation by a cyclin/CDK complex is first ubiquitinated by one of the ubiquitin ligase enzymes (or 'E3' enzymes), either the APC (anaphase promoting complex) or the SCF (Skp1p-cullin-F-box protein complex) (Morgan, 1999; Peters, 1998; Zachariae and Nasmyth, 1999). This ubiquitination then allows the protein to be recognised and hence degraded by the proteosome. The activity of the APC and SCF is increased by the binding of activator proteins. In the case of the SCF the activators are members of the F-box protein family, for example Cdc4p and Grr1p (Skowyra *et al.*, 1997). The activity of the APC is dependent on the association of one of two additional subunits, these activating proteins are Cdc20p and Cdh1p/Hct1p (Visintin *et al.*, 1997). Association of the different activator proteins with the SCF and

the APC changes during the cell cycle, hence changing their substrate specificity and adding another level of control to the cell cycle (Morgan, 1999; Peters, 1998; Zachariae and Nasmyth, 1999).

In budding yeast the major CDK involved in cell cycle control is Cdc28p. Cdc28p is present in the cell as an inactive monomer throughout the cell cycle. Activation of Cdc28p is dependent on its association with one of a number of different cyclin subunits. The cyclins in budding yeast are divided into two groups, the G1 cyclins (Cln1p, Cln2p and Cln3p) and the B-type cyclins (Clb1p-6p) (Lew *et al.*, 1997; Nasmyth, 1993; Nasmyth, 1996a; Nasmyth, 1996b). The G1 cyclins are essential for START. Cln1p and Cln2p in association with Cdc28p, increase the levels of the B-type cyclins by turning off their degradation and by turning on degradation of the cyclin B inhibitor, Sic1p. Ubiquitination of phospho-Sic1p by the SCF^{Cdc4p} complex, followed by degradation by the proteosome is essential for the transition from G1 to S phase (Dirick *et al.*, 1995; Peters, 1998; Schneider *et al.*, 1996; Schwob *et al.*, 1994; Tyers, 1996; Verma *et al.*, 1997a; Verma *et al.*, 1997b). Cln1p and Cln2p also turn off the ability of cells to react to mating pheromone (Oehlen and Cross, 1994) by phosphorylating Far1p (the protein required for G1 arrest in response to mating pheromone) thereby targeting it for ubiquitination by the SCF^{Cdc4p} complex followed by proteolysis by the proteosome (Henchoz *et al.*, 1997). Another function of Cln1/2p is to trigger the polarisation of the cytoskeleton required for bud formation (Lew and Reed, 1993). Cln3p activates the transcription of proteins required for continuation of the cell cycle, including *CLB5* and *CLB6* (Dirick *et al.*, 1995). The G1 cyclins eventually switch themselves off by autophosphorylation followed by ubiquitination by the SCF^{Grr1p} complex and degradation by the proteosome (Koepp *et al.*, 1999; Skowyra *et al.*, 1997; Skowyra *et al.*, 1999). The B-type cyclins in association with Cdc28p carry out a number of different roles in S phase, G2 and mitosis. Due to some functional redundancy between the Clbs, distinct roles for the individual Clbs has been difficult to determine. Some of the roles established so far are; Clb5p and Clb6p trigger DNA replication (Schwob *et al.*, 1994; Schwob and Nasmyth, 1993), Clb3p and Clb4p initiate formation of the mitotic spindle (Fitch *et al.*, 1992; Richardson *et al.*, 1992) and Clb1p and Clb2p trigger bud growth in G2, anaphase and nuclear division (Fitch *et al.*, 1992; Lew and Reed, 1993; Richardson *et al.*, 1992; Surana *et al.*, 1991). More recently it has been suggested that Clb5p is involved in positioning of pre-anaphase spindles (Segal *et al.*, 1998). In late mitosis, after chromosome segregation, the B-type cyclins are destroyed, allowing

the cell to exit mitosis (Ghiara *et al.*, 1991; King *et al.*, 1996a; King *et al.*, 1996b; Lew and Reed, 1993).

Cyclins and Cdc28p are important regulators of the cell cycle, however opinions on their exact functions and importance is changing all the time as more discoveries are made about the complex control of the cell cycle. For example, during mitosis the metaphase to anaphase transition seemed to coincide with a drop in B-type cyclin levels, which then leads to inactivation of Cdc28p which triggers mitotic exit (Holloway *et al.*, 1993; Surana *et al.*, 1993). This drop in B-type cyclin levels is due to ubiquitination by the APC^{Cdc20p} followed by degradation by the proteosome (King *et al.*, 1995; Sudakin *et al.*, 1995; Yeong *et al.*, 2000; Zachariae *et al.*, 1996). The APC is required for the onset of anaphase (Irniger *et al.*, 1995) and exit from mitosis (Morgan, 1999; Shirayama *et al.*, 1999). However, it is not the destruction of B-type cyclins that is required for onset of anaphase as expression of high levels of non-degradable cyclin fails to block anaphase (Surana *et al.*, 1993), but arrests cells in late mitosis. The protein that must be destroyed, in an APC^{Cdc20p}-dependent manner, before anaphase can occur is Pds1p. The presence of indestructible Pds1p prevents anaphase onset, therefore Pds1p is termed an anaphase inhibitor (Cohen-Fix and Koshland, 1997a; Cohen-Fix and Koshland, 1997b; Cohen-Fix *et al.*, 1996; Yamamoto *et al.*, 1996a; Yamamoto *et al.*, 1996b). More specifically, the role of Pds1p is as a separin inhibitor. The separin in budding yeast is Esp1p, which forms a stable complex with Pds1p. When Pds1p is destroyed, Esp1p is then free to cleave Scc1p, a component of the cohesin complex that holds sister chromatids together (Ciosk *et al.*, 1998; Uhlmann *et al.*, 1999; Uhlmann *et al.*, 2000). Therefore, ubiquitination of Pds1p by the APC^{Cdc20p} followed by its degradation allows separation of sister chromatids to occur. Pds1p has also been shown to inhibit cyclin degradation and therefore must be destroyed before cyclin degradation, followed by mitotic exit, can occur (Cohen-Fix, 2000; Cohen-Fix and Koshland, 1999; Morgan, 1999; Shirayama *et al.*, 1999; Tinker-Kulberg and Morgan, 1999).

Mitotic exit occurs when the mitotic cyclins are destroyed (Glotzer *et al.*, 1991; Holloway *et al.*, 1993; Irniger *et al.*, 1995; Surana *et al.*, 1993). More specifically the destruction of Clb2p by the APC^{Cdh1p} seems to be the critical event (Visintin *et al.*, 1997). This destruction occurs as a result of activation of the mitotic exit network (MEN). The MEN includes the phosphatase Cdc14p, the GTPase Tem1p, Lte1p (the Tem1p GDP/GTP exchange factor) and the kinases Cdc5p (polo-like kinase), Cdc15p, Dbf2p and Dbf20p (Charles *et al.*, 1998; Glover *et al.*, 1998; Jaspersen *et al.*, 1998; Shirayama *et al.*, 1998; Toyn and Johnston, 1994; Visintin *et al.*, 1998). The mechanism

of action of the MEN is to trigger mitotic cyclin destruction. This is achieved by tipping the balance between phosphorylation and dephosphorylation of the APC activator Cdh1p (also known as Hct1p). Exit from mitosis is triggered first by the destruction of Pds1p (Shirayama et al., 1999) and then by the activation of the GTPase, Tem1p. This occurs when the nucleus migrates into the bud bringing Tem1p at the spindle pole into contact with Lte1p in the bud (Bardin et al., 2000; Pereira et al., 2000). Activated Tem1p, then facilitates the release of the phosphatase, Cdc14p, from the nucleolus (Shirayama et al., 1994; Shou et al., 1999). Pds1p inhibits release of Cdc14p from the nucleolus (Shirayama et al., 1999), therefore must be destroyed before Cdc14p can be released. Cdc14p released from the nucleolus dephosphorylates Cdh1p allowing it to bind to the APC and direct the ubiquitination and consequent destruction of Clb2p (Jaspersen et al., 1999; Visintin et al., 1998). However, Cdh1p is phosphorylated by Clb/Cdc28p therefore, if a cell is to exit mitosis, a mechanism must exist to tip the balance in favour of dephosphorylation of Cdh1p by Cdc14p. This is achieved by the biphasic destruction of Clb/Cdc28p and the activation, by Cdc14p, of the Clb inhibitor Sic1p (Morgan, 1999). APC^{Cdc20p}, as well as mediating the destruction of Pds1p, is also responsible for the destruction of the cyclin Clb5p at the metaphase to anaphase transition which provides the drop in Cdc28p activity required for the success of Cdc14p in promoting mitotic exit (Shirayama et al., 1999).

Cell cycle checkpoints

Another level of cell cycle regulation is checkpoint control (Burke, 2000; Elledge, 1996; Gardner and Burke, 2000; Hoyt, 2000; Lew, 2000; Lew et al., 1997; Longhese et al., 1998; Murakami and Nurse, 2000; Nurse, 1997; Weinert, 1998). Cell cycle events are monitored to ensure that each stage is accurately completed before the next commences and that any defects are corrected. The most important checkpoint is START, it is the point of no return in the cell cycle and a cell must reach a minimum size before it can pass START (Johnston, 1977; Nurse, 1975). Other checkpoints after START can only delay or arrest the cell cycle. For example, DNA replication must be complete before mitosis begins and the chromosomes are separated (DNA replication checkpoint) and the chromosomes must be segregated before cytokinesis occurs (spindle checkpoint). There is also a checkpoint to detect DNA damage and delay the cell cycle until the damage has been repaired (DNA damage checkpoint). These

checkpoints are important to ensure the integrity of chromosomes and the accurate segregation of sister chromatids to mother and daughter cells.

The DNA replication checkpoint is activated by the presence of replication forks (Piatti *et al.*, 1995; Toyn *et al.*, 1995). Components of the protein replication fork complex activate the checkpoint via the kinases Mec1p and Rad53p (Allen *et al.*, 1994; Weinert, 1998; Weinert *et al.*, 1994) resulting in a cell cycle arrest until DNA replication is complete. The DNA damage checkpoint is activated by double strand DNA breaks (Foiani *et al.*, 1998; Mills *et al.*, 1999; Sandell and Zakian, 1993; Siede *et al.*, 1994; Weinert *et al.*, 1994) and can arrest the cell cycle at a number of different stages, again via Mec1p and Rad53p (Allen *et al.*, 1994; de la Torre-Ruiz and Lowndes, 2000; Weinert, 1998). Components of the DNA damage checkpoint also activate the transcription of DNA damage repair genes (Aboussekhra *et al.*, 1996; Allen *et al.*, 1994; Foiani *et al.*, 2000; Kiser and Weinert, 1996; Navas *et al.*, 1996). The mechanism of action of these checkpoints is yet to be confirmed, however, it makes sense for a target of these checkpoints to be stabilisation of Pds1p. Stabilisation of Pds1p would results in arrest of the cell cycle in metaphase as sister chromatids would remain attached and cyclin levels would remain high.

Accurate segregation of chromosomes in mitosis requires the bipolar attachment of replicated chomosomes, via their kinetochores, to the mitotic spindle. If mitosis occurs in the absence of these connections the result would be aneuploidy and death. The nucleus must also migrate into the bud to ensure that the chromosomes are equally segregated between mother and daughter cells. The spindle checkpoint monitors attachment of kinetochores to spindle microtubules and movement of the nucleus into the bud via two separate pathways (Amon, 1999; Burke, 2000; Hoyt, 2000; Lew, 2000). The pathway monitoring attachment of spindle microtubules to the kinetochores involves proteins encoded by *MAD1, MAD2, MAD3, BUB1, BUB3* and *MPS1* (Brady and Hardwick, 2000; Chen *et al.*, 1999; Hardwick *et al.*, 1999; Hardwick and Murray, 1995; Hardwick *et al.*, 1996). The *MAD* and *BUB* genes are non-essential, but the *MPS1* gene is essential and appears to have dual functions in spindle pole assembly and spindle checkpoint signalling (Weiss and Winey, 1996). When an unattached kinetochore is detected by a complex of Mad and Bub proteins localised at the kinetochore, the APC activator Cdc20p is inhibited by Mad2p. Hence, Cdc20p cannot direct the destruction of Pds1p and cells arrest at the metaphase to anaphase transition. Therefore, sister chromatids remain attached until all kinetochores are connected to the spindle. The second spindle checkpoint pathway involves the other Bub protein, Bub2p,

and Bfa1p (Fesquet *et al.*, 1999; Fraschini *et al.*, 1999; Pereira *et al.*, 2000). Bub2p and Bfa1p together form the GAP that activates the GTPase activity of Tem1p when the checkpoint is activated. Tem1p is localised at the spindle pole body that is destined to travel into the bud via its interaction with Bub2p/Bfa1p. Therefore, Tem1p is held in its inactive GDP-bound form until the nucleus is properly positioned and Tem1p is brought into contact with its exchange factor (GEF) Lte1p, which is localised in the bud (Bardin *et al.*, 2000; Pereira *et al.*, 2000; Shirayama *et al.*, 1994). In other words mitotic exit is prevented until the nucleus is properly oriented between mother and bud.

The budding yeast cytoskeleton

The cytoskeleton is an internal array of protein filaments that is a part of all eukaryotic cells (Alberts *et al.*, 1994). The cytoskeleton organises the cytoplasm, generates force within the cell, determines the shape of the cell and maintains the structural integrity of the cell. There are three types of cytoskeletal filaments, microfilaments, intermediate filaments and microtubules. Microfilaments (5-7 nm in diameter) are long flexible polymers of actin, intermediate filaments (~10 nm in diameter) are based on polymers of any of a number of related fibrous coiled-coil proteins and microtubules are based on more rigid polymers of tubulin (hollow tubes 25 nm in diameter). Cells of the budding yeast do not appear to contain intermediate filaments *per se*, however the microfilaments and microtubules of *Saccharomyces cerevisiae* closely resemble those of higher eukaryotes. The functions of these cytoskeletal components have been extensively studied in budding yeast (Adams, 1984; Adams and Pringle, 1984; Amberg, 1998; Ayscough and Drubin, 1996; Barnes *et al.*, 1990; Botstein, 1986; Botstein *et al.*, 1997; Cid *et al.*, 1995; Kilmartin and Huffaker *et al.*, 1987; Stearns, 1990; Stearns *et al.*, 1990; Welch *et al.*, 1994).

Microfilaments in budding yeast are formed by the polymerisation of actin, the product of the single actin gene, *ACT1* (Gallwitz and Seidel, 1980; Gallwitz and Sures, 1980). Actin forms patches as well as filaments, the distribution of both alters through the cell cycle. Actin functions in maintenance of cell polarity, changes in cell shape (for example, during mating) and resistance to osmotic forces. Microtubules are formed by the polymerisation of α- and β-tubulin, in budding yeast these proteins are encoded by the *TUB1, TUB3* (α-tubulin, (Schatz *et al.*, 1986a; Schatz *et al.*, 1986b)) and *TUB2* (β-tubulin, (Neff *et al.*, 1983)) genes.

Microtubules and microtubule organising centres

Microtubules are dynamic structures that fulfil numerous different roles throughout the cell cycle (Alberts *et al.*, 1994). During interphase microtubules are distributed throughout the cytoplasm where they are involved in vesicle trafficking and organelle positioning. At the onset of mitosis, the chromosomes condense and the microtubule network is reorganised to form the mitotic spindle, which separates the duplicated chromosomes into mother and daughter cells (Figure 3. Note, a mammalian cell is used to demonstrate the organisation of the microtubule network throughout the cell cycle as microtubules and DNA are easier to distinguish by immunofluorescence in mammalian cells than in yeast). Microtubules consist of α- and β-tubulin heterodimers (Weisenberg *et al.*, 1968). These tubulin heterodimers come together *in vitro* to form protofilaments, which in turn form the cylinders known as microtubules (Figure 4). The orientation of the α and β subunits give microtubules biochemically distinct ends termed the plus and minus ends. Therefore, microtubules can be considered as "polar" structures. *In vitro* microtubules will self-assemble when the concentration of the tubulin subunits reaches the critical concentration (Weisenberg, 1972). Once a microtubule has formed, tubulin can associate and dissociate with the microtubule ends. Assembly of tubulin into microtubules is more rapid at the plus end than the minus end (Allen and Borisy, 1974; Bergen and Borisy, 1980). Rapid disassembly also occurs at the plus end, these disassembly events are called catastrophes. The resulting alternation between rapid growth or rescue, followed by catastrophes is termed dynamic instability (Desai and Mitchison, 1997; Mandelkow *et al.*, 1991; Mitchison and Kirschner, 1984). The dynamic nature of microtubules is due to GTP hydrolysis (Desai and Mitchison, 1997; Erickson and O'Brien, 1992; Mitchison, 1993; Weisenberg *et al.*, 1976). GTP-bound tubulin assembles at the plus end of growing microtubules where the GTP bound to β-tubulin is rapidly hydrolysed to form GDP. GDP-bound tubulin is less stable and therefore more likely to dissociate from the microtubule ends. Exchange of GDP for GTP only occurs after depolymerisation. Growth occurs when addition of GTP-tubulin is faster than GTP hydrolysis, forming a cap of GTP-tubulin at the growing end, therefore stabilising the microtubule (Desai and Mitchison, 1997; Inoue and Salmon, 1995). *In vivo* the concentration of free tubulin is lower than the critical concentration for spontaneous microtubule assembly, therefore initiation of microtubule formation occurs at specific nucleation sites within the cell. These sites are termed microtubule organising centres (MTOCs) (Pickett-Heaps, 1969). MTOCs dictate the number, orientation and polarity of the microtubules that make up the microtubule network of a
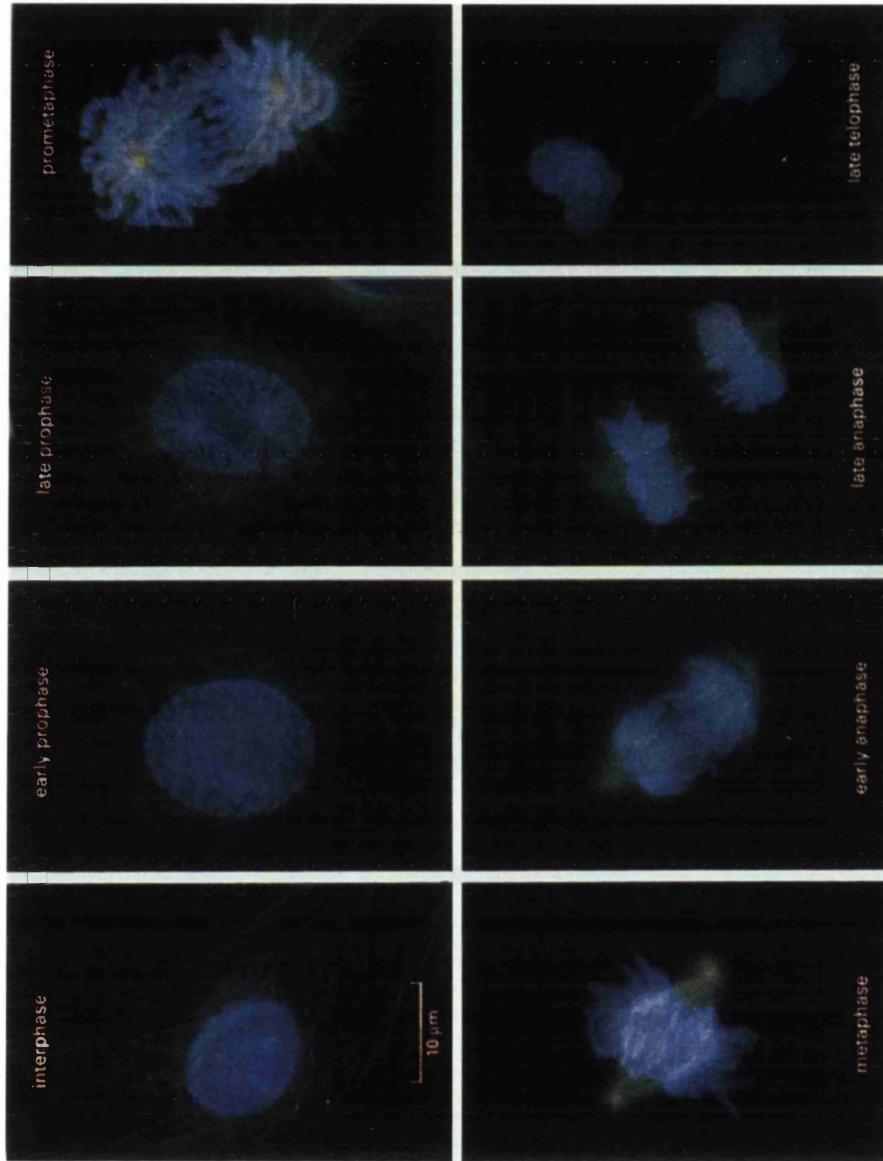
Figure 3 The microtubule network (Alberts *et al.*, 1994)

Visualisation of microtubules (green) and DNA (blue) in a mammalian cell at different stages of the cell cycle
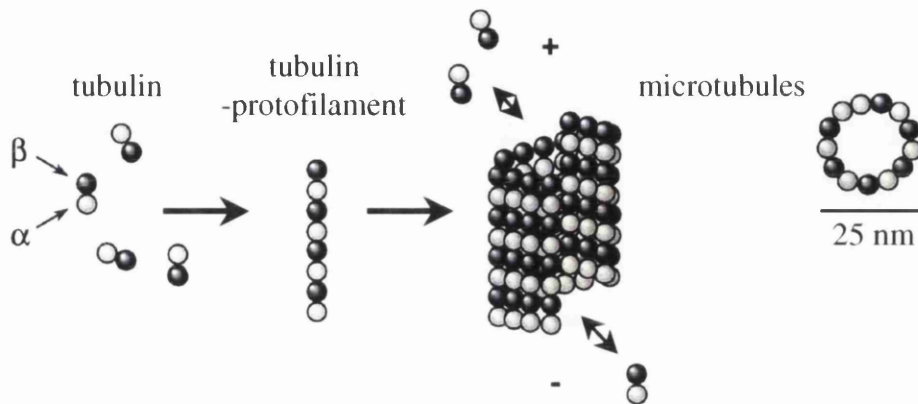
Figure 4 Assembly of Microtubules

In vitro, when the concentration of free tubulin heterodimers reaches a critical concentration, tubulin protofilaments begin to form. These protofilaments then form tubes, the walls of which consist of 13 protofilaments giving a diameter of 25 nm. Due to the arrangement of the α- and β-tubulin subunits, microtubules have distinct ends, termed the plus and minus ends. Once these microtubules have formed, there is continuous exchange of tubulin heterodimers from both ends of the microtubule. This exchange is more rapid at the plus ends of the microtubules.

cell. Dynamic instability is important *in vivo* as it enables the dynamic processes like mitosis to occur. However, many microtubule based processes require microtubules to be stabilised, this stabilisation is controlled by microtubule motors and microtubule binding proteins (MAPs) (Desai and Mitchison, 1997).

In mammalian cells the major MTOC is the centrosome (Figure 5). The centrosome consists of a pair of centrioles surrounded by a dense matrix called the pericentriolar material from which microtubules emanate. A centriole consists of a cylindrical array of nine short microtubules triplets arranged symmetrically to form a short tube. The pericentriolar material contains microtubule nucleating factors, for example γ-tubulin. γ-Tubulin is the third member of the tubulin family (Oakley and Oakley, 1989). It has approximately 30% identity to the α- and β-tubulins. γ-tubulin is a universal component of MTOCs (Oakley *et al.*, 1990; Stearns *et al.*, 1991; Stearns and Kirschner, 1994) which is present at MTOCs in a complex with other proteins (Pereira and Schiebel, 1997). This complex, the γ-tubulin-complex, varies in size between different organisms and is essential for microtubule nucleation *in vivo* (Schiebel, 2000; Tassin and Bornens, 1999).

An additional function of microtubules is in motility, in this case microtubule organising functions are carried out by the basal body. Basal bodies direct the formation of flagella and cilia in many different cell types from single cellular organisms to human cells. For example, the flagella of sperm cells and the cilia lining the respiratory and digestive tracts as well as sensory cilia found in the ears and eyes are all formed around basal bodies (Alberts *et al.*, 1994).

In the budding yeast *Saccharomyces cerevisiae* microtubule organising functions are carried out by the spindle pole body (SPB) (Knop *et al.*, 1999).

The Spindle Pole Body

SPB Morphology

The spindle pole body is a multilayered structure that is embedded in the nuclear envelope throughout the cell cycle (Byers and Goetsch, 1975b). This is possible as yeast undergo closed mitosis, which means that the nuclear envelope stays intact during mitosis. The morphology of the SPB has been well documented by electron microscopy

Centrosomes
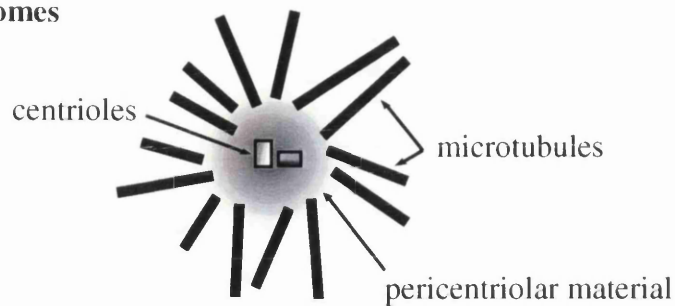
Basal bodies
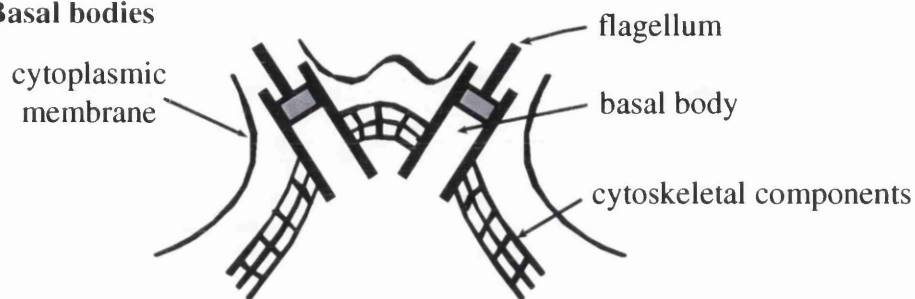
SPB
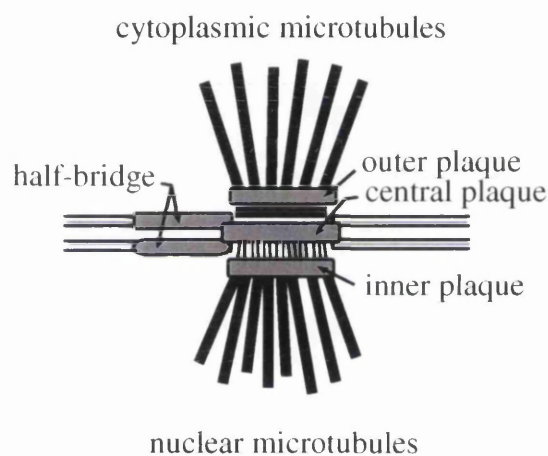
Figure 5 Microtubule organising centres (MTOCs)

The centrosome is the major MTOC of mammalian cells, it is a cytoplasmic organelle that is generally found in the centre of the cell, near the nucleus. The centrosome consists of a pair of centrioles surrounded by a pericentriolar matrix from which microtubules emanate. Basal bodies are found at the base of cilia and flagella and are responsible for generation of movement. The spindle pole body (SPB) of *S. cerevisiae* is the functional equivalent to the mammalian centrosome. The SPB is a multi layered structure that is embedded in the nuclear membrane, from where it nucleates spatially distinct nuclear and cytoplasmic microtubules.

studies (Adams and Kilmartin, 1999; Bullitt *et al.*, 1997; Byers, 1981a; Byers, 1981b; Byers and Goetsch, 1975b; Byers *et al.*, 1978; Moens and Rapport, 1971; O'Toole *et al.*, 1999; Peterson *et al.*, 1972; Peterson and Ris, 1976; Robinow and Marak, 1966; Rout and Kilmartin, 1990). The SPB consists of a number of substructures. The major substructures are the central plaque, the outer plaque, the inner plaque and the half-bridge (Figure 5). The central plaque is embedded in the nuclear envelope, the outer plaque is located on the cytoplasmic side of the SPB and organises cytoplasmic microtubules while the inner plaque is on the nuclear side where it organises nuclear microtubules. The half-bridge extends from one side of the SPB over the cytoplasmic and nuclear surface of the nuclear membrane. The half-bridge is more dynamic than the other SPB substructures and is involved in SPB duplication. Microtubules are associated with the inner plaque at all stages of the cell cycle, however the association of the cytoplasmic microtubules with the SPB substructures changes. During G1 and mating the half-bridge appears to be the major site of cytoplasmic organisation. Throughout the rest of the cell cycle cytoplasmic microtubules are organised by the outer plaque (Knop *et al.*, 1999; Knop and Schiebel, 1998; Pereira *et al.*, 1999).

The minor substructures, as seen by electron microscopy, are the intermediate layers IL1 and IL2, which are present between the outer and central plaques. The inner plaque can also be split into two minor layers, IP1 and IP2. IP1 appears as a beaded layer and IP2 is formed by the capped minus ends of the nuclear microtubules. This gives a final count of six layers making up the SPB (Bullitt *et al.*, 1997).

SPB duplication

Although the SPB is structurally different to the centrosome it is functionally equivalent as both nucleate and organise microtubules and both duplicate once per cell cycle (Figure 6). SPB duplication occurs in G1 of the cell cycle. In early G1 the half-bridge elongates and the precursor of the new SPB, the satellite, forms at the end of the half-bridge furthest from the mother SPB. After START, with the appearance of Cdc28p/Clns, SPB duplication occurs rapidly, therefore intermediates in SPB duplication have only recently been observed (Adams and Kilmartin, 1999) (Figure 7). The satellite extends to form the duplication plaque, which gains the outer plaque and is then inserted into the nuclear envelope. On addition of the inner plaque SPB duplication is complete, however the side by side SPBs are still attached to each other by the
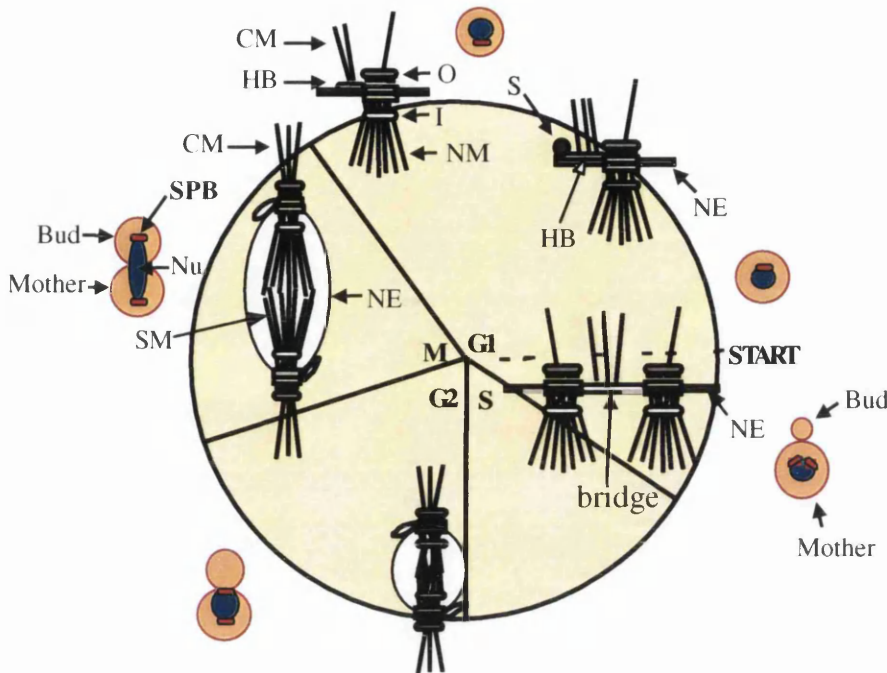
Figure 6 The SPB and the cell cycle

G1 cells are unbudded and contain a single SPB with its associated half-bridge. Before START of the cell cycle SPB duplication begins with the formation of the satellite. SPB duplication is completed before the end of G1 when the bud begins to appear. During S phase the duplicated SPBs separate and migrate to opposite sides of the nucleus. During mitosis the SPBs organise the mitotic spindle and one SPB moves into the bud. Therefore when cell division occurs each daughter cell will contain a single SPB with its associated half-bridge.

CM, cytoplasmic microtubules; HB, half-bridge; I, inner plaque; NE, nuclear envelope; NM, nuclear microtubules; Nu, nucleus; O, outer plaque; S, satellite; SM, spindle microtubules; SPB, spindle pole body.
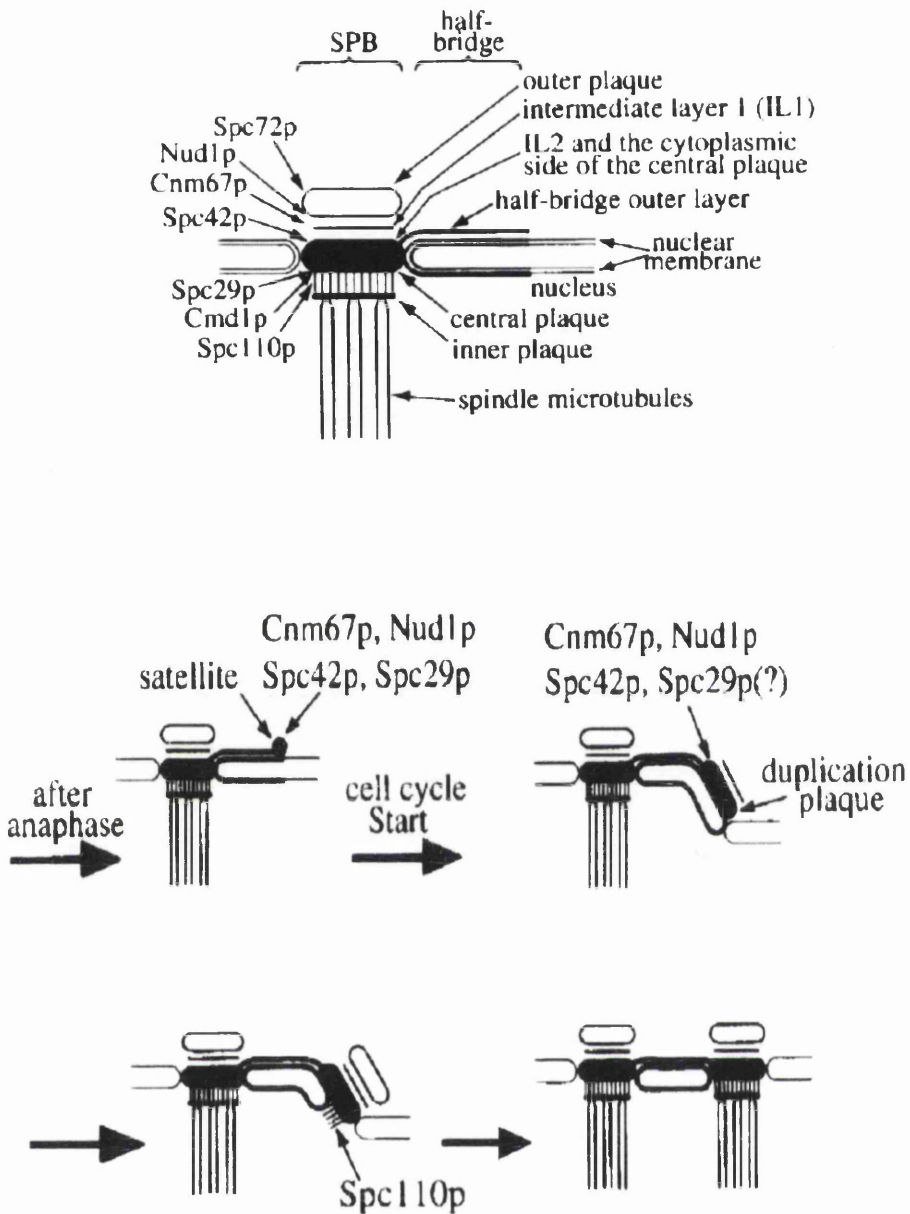
Figure 7 SPB duplication

Intermediate structures seen during SPB duplication. After START the satellite extends to form the duplication plaque, which contains components of the central and outer plaques. As the duplication plaque is inserted into the nuclear membrane, nuclear SPB proteins form the inner plaque. Reproduced from **The Journal of Cell Biology, 1996, 145, p821.** by copyright permission of The Rockefeller University Press.

bridge. As DNA duplication occurs, the bridge is cleaved by an unknown process allowing the two SPBs, each associated with a half-bridge, to separate and move to opposite sides of the cell. This is thought to be a microtubule based movement involving nuclear and cytoplasmic microtubules and microtubule motors. Once the SPBs are correctly positioned, one in the mother cell, one in the bud and joined by the mitotic spindle, the chromosomes are separated. In haploid cells each of the sixteen yeast chromosomes are attached to the poles via a single microtubule from each SPB. There are also a few microtubules that interconnect the two SPBs, therefore the mitotic spindle of haploid cells consists of approximately forty microtubules (Byers and Goetsch, 1975b; Peterson and Ris, 1976; Winey *et al.*, 1995). Once the duplicated chromosomes are separated cytokinesis occurs. After cytokinesis each cell will contain a single SPB and a full set of chromosomes.

SPB duplication is conservative as the new SPB forms adjacent to the old SPB and there does not appear to be a "template" for SPB duplication. However, it has recently been suggested that pre-formed SPBs can incorporate new components due to turn over of core SPB components throughout the cell cycle (Davis and Yoder, abstract, Colorado meeting, 2000), indicating that the SPB may be more dynamic that earlier imagined.

SPB composition

The γ-tubulin-complex

γ-tubulin was first identified as a suppresser of a β-tubulin mutation in *Aspergillus nidulans* (Oakley and Oakley, 1989). Since then γ-tubulins have been identified in many different organisms including human, *Schizosaccharomyces pombe, Drosophila melanogaster, Xenopus laevis, Saccharomyces cerevisiae* and plant cells (Horio *et al.*, 1991; Liu *et al.*, 1994; Lopez *et al.*, 1995; Stearns *et al.*, 1991; Sobel and Snyder, 1995; Zheng *et al.*, 1991). The role of γ-tubulin in microtubule nucleation has been demonstrated by disrupting γ-tubulin function in a number of organisms. For example, when antibodies to γ-tubulin were injected into mammalian cells, microtubule nucleation by the centrosome was prevented throughout the cell cycle (Joshi *et al.*, 1992). In the *S. Cerevisiae tub4(ts)* mutant strain (*TUB4* encodes the yeast γ-tubulin), when incubated at the non-permissive temperature, SPB duplication occurred normally, but the newly formed SPB was unable to nucleate microtubules (Marschall *et al.*, 1996).

In addition, γ-tubulin-containing ring complexes isolated from *Xenopus* egg extracts nucleated microtubules *in vitro* (Zheng *et al.*, 1995). It is now established that γ-tubulin is localised at the MTOCs in many different organisms and its role in microtubule nucleation has been clearly demonstrated. The γ-tubulin open ring complex (γTuRC) has been observed in a number of organisms (Moritz *et al.*, 1995; Murphy *et al.*, 1998; Vogel *et al.*, 1997) since first visualised by electron microscopy (Zheng *et al.*, 1995). In *Xenopus*, γ-tubulin is present in a 25S ring complex containing at least 6 other proteins including α- and β-tubulins. Cryo-electron microscopy studies revealed that the *Drosophila* γTuRC consists of approximately 13 radially arranged structural repeats and has a diameter of ~25 nm. Upon treatment with salt the γTuRC is disrupted giving rise to the γ-tubulin small complex (γTuSC) (Oegema *et al.*, 1999).

The γ-tubulins of *A. nidulans* and *S. pombe* show great similarity to the γ-tubulins of higher organisms, in fact the human γ-tubulin can substitute for the fission yeast γ-tubulin (Horio and Oakley, 1994). For a long time γ-tubulin could not be found in budding yeast casting doubt on the importance of γ-tubulin and its function in microtubule nucleation *in vivo*. Finally, the *S. cerevisiae* γ-tubulin was identified by the yeast genome sequencing project. The reason that the budding yeast γ-tubulin had not been identified by conventional methods was because it only exhibits approximately 40% identity to the γ-tubulins from other organisms (Sobel and Snyder, 1995), whereas γ-tubulins from different species usually have around 65% homology. Despite this difference, the budding yeast γ-tubulin exhibits the same characteristics as γ-tubulins from other organisms. Since the discovery of Tub4p, the budding yeast spindle pole body has become one of the most studied and understood MTOCs.

By studying the SPB in budding yeast it is hoped that a greater insight will be gained into the function, composition and duplication processes of centrosomes and microtubule organising centres in general. Already human homologues of SPB components have been identified, as mentioned above, Tub4p is the yeast γ-tubulin. Tub4p forms a complex with Spc97p and Spc98p which also have homologues in humans (hGCP2 and hGCP3, (Murphy *et al.*, 1998; Tassin *et al.*, 1998)), *Xenopus* (Xgrip109, (Martin *et al.*, 1998)) and *Drosophila* (Dgrip84 and Dgrip91, (Oegema *et al.*, 1999)). In each of these organisms the Spc97/98p homologues were found to co-localise with γ-tubulin at the centrosomes and co-sediment with γ-tubulin on sucrose gradients.

SPC98 was first identified as a multicopy suppresser of a *tub4(ts)* mutant strain (Geissler *et al.*, 1996) and was found to encode the 90 kDa spindle pole body protein described by Rout and Kilmartin (1990). Subsequent screens identified *SPC97* as a multicopy suppresser of an *spc98(ts)* mutant strain (Knop *et al.*, 1997). As expected the *Saccharomyces cerevisiae* γ-tubulin-complex is present at the inner and outer plaques of the SPB where it is involved in microtubule nucleation (Rout and Kilmartin, 1990; Spang *et al.*, 1996a). Further investigation found that the budding yeast γ-tubulin was present in a 6S (~200 kDa) complex consisting of one molecule of Spc97p, one molecule of Spc98p and two or more molecules of Tub4p (Knop and Schiebel, 1997). No γ-tubulin ring structure has been observed in budding yeast. However, it seems that the Tub4p-complex may be the equivalent of the *Drosophila* γTuSC, which contains one molecule of Dgrip84, one molecule of Dgrip91 (homologues of Spc97p and Spc98p respectively) and two molecules of γ-tubulin. The γTuSC is capable of nucleating microtubules *in vitro*, but is much less active than the γTuRC in promoting nucleation. At this time it is estimated that the γTuRC consists of approximately six γTuSCs units (Oegema *et al.*, 1999). In budding yeast the Tub4p-complex is suspected to form the cap structures at the microtubule minus ends situated at the inner plaque of the SPB as observed by electron microscopy (Byers *et al.*, 1978). It is possible that these caps are based on a ring structure, formed by approximately six Tub4p-complexes, which then acts as a seed for nucleation of a microtubule. Recently the γTuRC of *Drosophila* has been shown to form the cap-like structure at the minus ends of microtubules and to nucleate microtubules at subcritical α-/β-tubulin concentration (Keating and Borisy, 2000; Moritz *et al.*, 2000).

As the Tub4p-complex is present at the inner and outer plaques of the SPB there are likely to be control mechanisms in place to mediate the localisation of the Tub4p-complex. Preliminary investigation into the regulation and assembly of the Tub4p-complex suggests the involvement of the nuclear localisation signal (NLS) of Spc98p and phosphorylation (Pereira *et al.*, 1998). The Tub4p-complex appears to assemble in the cytoplasm before binding to the outer plaque or import into the nucleus where it binds to the inner plaque. Import of the Tub4p-complex is mediated by the essential NLS of Spc98p. Spc98p is also phosphorylated in a cell cycle dependent manner. Phosphorylation of Spc98p possibly only occurs at the inner plaque leaving Spc98p at the outer plaque in the dephosphorylated form. The precise function of Spc98p phosphorylation is yet to be determined, however it has been suggested that the kinase Mps1p is involved.

A screen for suppressers of the *tub4-1* mutant identified *GIM1-5*. The Gim proteins form a non-essential complex (GimC) in wild type cells, however *Δgim* mutants have microtubule defects and elevated sensitivity to temperature and microtubule and actin depolymerising drugs. Further analysis of the Gim proteins revealed them to be members of a phylogenetically conserved family of proteins involved in the efficient folding of α-tubulin, γ-tubulin and actin (Geissler *et al.*, 1998). More specifically Siegers *et al* (1999) found that GimC is a co-chaperone that interacts with the chaperonin TRiC to ensure the proper and efficient folding of actin and tubulin *in vivo*.

γ-Tubulin-complex-binding proteins

The γ-tubulin-complex is tethered to the SPB via γ-tubulin-complex-binding proteins at the inner and outer plaques and the half-bridge (Knop and Schiebel, 1997; Knop and Schiebel, 1998; Pereira *et al.*, 1999). At the outer plaque and the half-bridge the γ-tubulin-complex-binding protein is Spc72p (Knop and Schiebel, 1998). Spc72p is a coiled-coil protein with an N-terminal globular domain that localises to the outer plaque of the SPB (Soues and Adams, 1998; Wigge *et al.*, 1998). Spc72p was found to interact with Spc97p and Spc98p of the Tub4p-complex using the two hybrid system (Knop and Schiebel, 1998). *spc72(ts)* cells failed to organise cytoplasmic microtubules at the non-permissive temperature, therefore, these cells were unable to move the nucleus into the bud. However this did not cause a cell cycle block, so cytokinesis occurred resulting in a population of cells containing two or more nuclei (Chen *et al.*, 1998). *spc72(ts)* mutants also exhibited defects in spindle elongation and karyogamy suggesting roles for Spc72p and cytoplasmic microtubules in these processes. Spc72p was then shown to bind the half-bridge component Kar1p and this interaction mediates microtubule nucleation from the half-bridge in G1 and during mating (Pereira *et al.*, 1999) explaining the karyogamy defect observed in *spc72(ts)* and *kar1(ts)* mutants (Conde and Fink, 1976; Pereira *et al.*, 1999).

At the inner plaque the γ-tubulin-complex-binding protein is Spc110p. Spc110p was first identified by Rout and Kilmartin (1990) when they used enriched SPBs to raise monoclonal antibodies. Of the antibodies raised 14 identified three different SPB components of 110 kDa, 90 kDa and 80 kDa. Antibodies against the 110 kDa protein were used to localise the protein by immunoelectron microscopy. It was found that the 110 kDa protein was present on the nuclear side of the SPB at all stages of the cell

cycle. Kilmartin *et al* (1993) later used the antibodies against the 110 kDa SPB protein to screen an expression library and cloned *SPC110*. *SPC110* was found to be identical to the *NUF1* (nuclear filament related) gene. *NUF1* was found in a similar way using antibodies prepared against fractions of yeast nuclei and was predicted to encode a coiled-coil protein (Mirzayan *et al.*, 1992). Further analysis by Kilmartin et al (1993) showed that the *SPC110* transcript is cell cycle regulated and when the putative coiled-coil domain was expressed in *E. coli* it formed rods of the expected length. The anti-Spc110p antibodies labelled the space between the central and inner plaques where filaments could be seen by electron microscopy. The coiled-coil domain was then shown to act as a spacer between the central and inner plaques, as deletions in the coiled-coil domain resulted in a proportional decrease in the distance between the central and inner plaques (Kilmartin *et al.*, 1993). Since the identification of Spc110p and its initial characterisation as a coiled-coil spacer protein, mutational analysis of *spc110* and *cmd1* (yeast calmodulin) mutants has given a greater insight into the function of Spc110p.

Calmodulin is a calcium-binding protein involved in numerous cellular processes. Calmodulin is highly conserved for example the human calmodulin can substitute for the budding yeast calmodulin (Davis and Thorner, 1989). The essential functions of calmodulin in budding yeast are calcium independent as *cmd1* mutants that cannot bind calcium *in vitro* are functional *in vivo* (Geiser *et al.*, 1991). One of the functions of calmodulin is in mitosis. This was shown by *cmd1* mutants that exhibited defects in chromosome segregation (*cmd1-1*, (Davis, 1992)), spindle formation and SPB duplication (*cmd1-101*, (Sun *et al.*, 1992)). In some *cmd1-101* cells nuclear microtubules could be seen detached from the inner plaque. Systematic mutagenesis of calmodulin revealed four diverse essential functions in yeast (Ohya and Botstein, 1994a; Ohya and Botstein, 1994b). One of these groups of mutants (*cmd1C*) showed a severe defect in mitosis, arresting as large budded cells with monopolar spindles. Spc110p was then found to be one of the calmodulin target proteins. First *SPC110* was identified as a suppresser of *cmd1-1* (Geiser *et al.*, 1993) and in a separate screen the C-terminus of Spc110p interacted with calmodulin in the two hybrid system. Direct binding of calmodulin to C-Spc110p was shown by overlay assays. To further support this data a fraction of calmodulin localised to the SPB as shown by immunofluorescence (Geiser *et al.*, 1993; Stirling *et al.*, 1994). More specifically calmodulin localised to the central plaque of the SPB as shown by immunoelectron microscopy (Spang *et al.*, 1996b), this localisation was dependent on the C-terminus of

Spc110p. Therefore, if calmodulin localises to the central plaque and binds to the C-terminus of Spc110p, this gives the orientation of Spc110p within the SPB. This is supported by the labelling of the inner plaque by antibodies raised against the N-terminus of Spc110p (Spang *et al.*, 1996b). The calmodulin binding site of Spc110p was determined using the two hybrid system (Geiser *et al.*, 1993) and by introducing point mutations into the C-terminus of Spc110p and studying the ability of these Spc110p mutant proteins to bind calmodulin *in vitro* (Stirling *et al.*, 1994). Point mutations in the calmodulin binding site of Spc110p abolished calmodulin binding *in vitro* and prevented Spc110p from functioning *in vivo*. These Spc110p mutant proteins were present in lower levels in the cell, but elevation by gene dosage resulted in partial recovery of Spc110p function. Overexpression of calmodulin also suppressed the defects in these *spc110* mutants, supporting the notion that Spc110p stability is a consequence of calmodulin binding and pointing to a role for calmodulin in Spc110p function (Stirling *et al.*, 1994). Interestingly, C-terminal truncation of Spc110p resulting in deletion of the calmodulin-binding site suppresses the *cmd1-1* mutation.

Temperature sensitive alleles of *SPC110* exhibit very similar phenotypes to the *cmd1-1* and *cmd1-101* mutants (Kilmartin and Goh, 1996; Stirling *et al.*, 1996; Sundberg *et al.*, 1996). *spc110(ts)* mutants arrest as large budded cells with a G2 DNA content, there is failure of chromosome segregation and spindle breakage. Closer analysis by electron microscopy showed detachment of nuclear microtubules from the SPB (Kilmartin and Goh, 1996). Overexpression of *SPC110* caused the self-assembly of Spc110p-calmodulin into ordered spheroidal polymers in the nucleus (Kilmartin and Goh, 1996). Similar aggregates were seen in *spc110-220* cells at the non-permissive temperature (Sundberg *et al.*, 1996). These aggregates were capable of microtubule nucleation and so were named intranuclear microtubule organisers (IMOs). The IMO is a transient structure, appearing in the nucleus at the time of SPB duplication and disappearing after SPB separation. Spc110p and the 90 kDa protein (Spc98p) were localised to these IMOs, giving the first indication of a direct interaction between Spc110p and the Tub4p-complex. Spc110p can be divided into three functional regions (Sundberg and Davis, 1997). Mutations in region I (N-terminal) were suppressed by overexpression of *SPC98*. Region II is the carboxy-region of the coiled-coil domain, mutations here lead to loss of SPB integrity during mitosis suggesting it is involved in attachment of Spc110p to the central plaque. Region III is the calmodulin binding site.

These results suggest roles for calmodulin and Spc110p within the SPB. Calmodulin binding to Spc110p appears to relieve some inhibition, probably caused by the very C-

terminus of Spc110p, allowing Spc110p to incorporate into the new SPB. To summarise the functions of Spc110p, the C-terminus contains a calmodulin-binding site and is positioned at the central plaque, the coiled-coil region acts as a spacer between the central and inner plaques and the N-terminus is required to anchor nuclear microtubules to the SPB (Figure 8). Evidence to support this final point came from biochemical and genetic studies. The N-terminal domain of Spc110p interacted with the Tub4p-complex components Spc98p and Spc97p, but not Tub4p in the two hybrid system. An interaction between the N-terminus of Spc110p and the Tub4p-complex was also shown using the purified components (Knop and Schiebel, 1997).

Spc110p is also a phosphoprotein that is phosphorylated in a cell cycle dependent manner (Friedman *et al.*, 1996; Stirling and Stark, 1996). Spc110p is phosphorylated during spindle formation and dephosphorylated as cells enter anaphase. Transport of Spc110p into the nucleus occurs via a nuclear localisation signal (NLS). Deletion of the NLS inhibits SPB duplication (Adams and Kilmartin, 1999).

It may be that γ-tubulin-complex-binding proteins are conserved components of MTOCs as a centrosomal protein, p116, related to Spc110p has been identified in vertebrates. p116 also co-purifies with γ-tubulin (Tassin *et al.*, 1997). Also, Flory *et al* recently identified a human coiled-coil, calmodulin-binding centrosomal protein, named kendrin, which is encoded by the same gene as pericentrin. Pericentrin is known to interact with the γ-tubulin complex (Dictenberg *et al.*, 1998), it is therefore very likely that kendrin/pericentrin is a human orthologue of Spc110p.

The central and outer plaques

As mentioned above the calmodulin-binding C-termini of Spc110p are positioned at the central plaque. For general purposes the central plaque as a term includes the IL2 layer. This is due to the appearance of SPBs in whole cells when observed by electron microscopy where the minor layers of the SPB are hard to distinguish from the major layers. The IL2 layer consists of a crystal of Spc42p (Bullitt *et al.*, 1997). Spc42p was first identified in a similar way to Spc110p, by using enriched SPBs to raise monoclonal antibodies (Rout and Kilmartin, 1991). Further analysis showed that Spc42p and Spc110p have a number of common features, both contain coiled-coil regions and both appear to be cell cycle regulated at the mRNA level with expression occurring at the
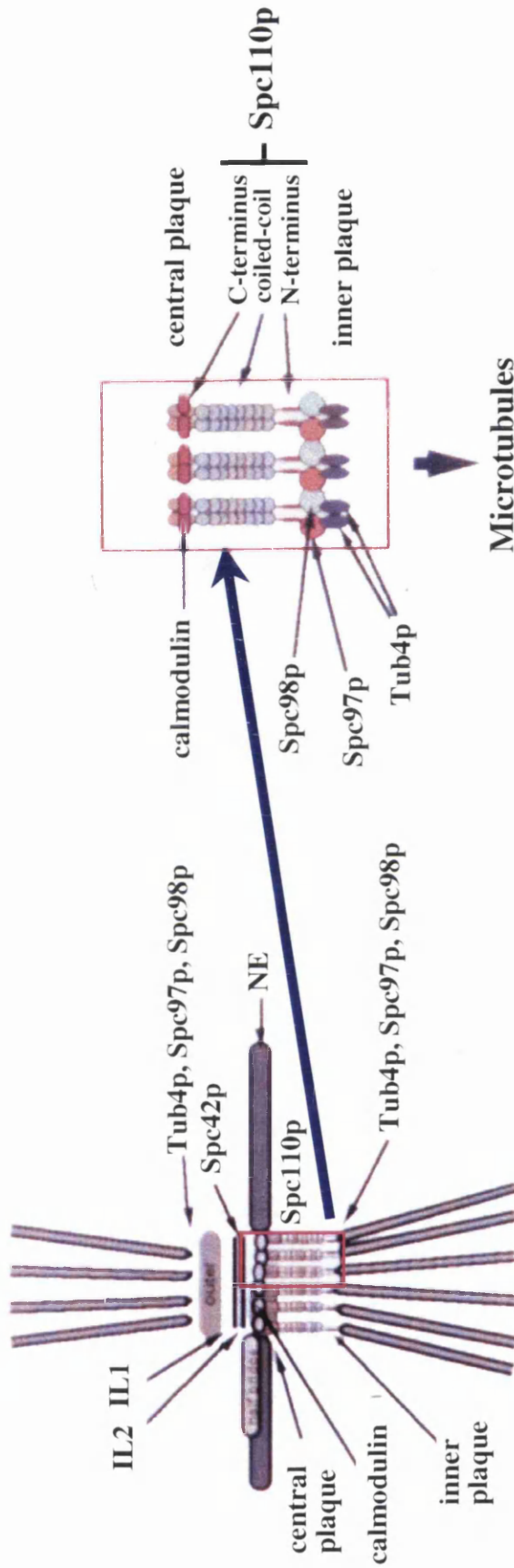
Figure 8 Spc110p binds the Tub4p-complex at the inner plaque of the SPB

Spc110p is a coiled-coil protein that forms dimers. The C-terminal globular domain of Spc110p is positioned at the central plaque, the coiled-coil domain separates the central and inner plaques. The N-terminal domain is also globular and interacts with Spc97p and Spc98p of the Tub4p-complex at the inner plaque. Therefore the N-termini of Spc110p act as docking sites for the Tub4p-complex and hence nuclear microtubules.

time of SPB duplication. Spc42p is also a phosphoprotein (Donaldson and Kilmartin, 1996), but the role of phoshorylation in the function of Spc42p is unclear. Spc42p is important for SPB integrity and plays a role in SPB duplication as seen by analysis of *spc42(ts)* mutants (Donaldson and Kilmartin, 1996). One of the phenotypes observed was detachment of SPBs from the nuclear envelope, suggesting a role for Spc42p in embedding the SPB in the nuclear envelope. Other phenotypes were failure of SPB duplication and accumulation of an electron dense mass on the cytoplasmic side of the half-bridge. This mass may result from the disassembly of the satellite due to loss of Spc42p function explaining the defect in SPB duplication. Spc42p self-assembles into a hexagonal crystal as seen by cryo-electron microscopy of isolated diploid SPBs and SPBs from cells overexpressing *SPC42* (Bullitt *et al.*, 1997). Overexpression of *SPC42* caused this central crystal to extend out laterally from the central plaque to form a dome-like structure around the SPB (Donaldson and Kilmartin, 1996). In these cells the dimensions of the inner and outer plaques remain as wild type. The coiled-coil region of Spc42p is probably important for formation of the Spc42p crystal at the central plaque as point mutations that disrupt the α-helical structure cause a temperature sensitive phenotype (Donaldson and Kilmartin, 1996).

The known components on the cytoplasmic side of the SPB in order from Spc42p are Cnm67p, Spc94p/Nud1p, Spc72p, Stu2p, the Tub4p-complex and the cytoplasmic microtubules. At the cytoplasmic side of the SPB Spc42p interacts with the coiled-coil protein Cnm67p (Elliott *et al.*, 1999). Deletion of *CNM67* is not lethal, but results in SPBs lacking an outer plaque, therefore in *Δcnm67* cells cytoplasmic microtubules are organised entirely by the half-bridge (Brachat *et al.*, 1998). Cytoplasmic microtubules nucleated by the half-bridge cannot fully compensate for the lack of the outer plaque, therefore, *Δcmn67* cells have misorientated spindles during mitosis leading to bi- and multi-nucleated cells. Cnm67p is not strictly required for mitosis, however, Cnm67p is essential for sporulation. Spore wall formation is initiated by an enlargement of the outer plaque which acts as a nucleation site for spore wall assembly (Byers, 1981a; Kupiec *et al.*, 1997; Moens and Rapport, 1971). In *Δcmn67* cells there is no outer plaque, therefore sporulation cannot occur (Brachat *et al.*, 1998). Cnm67p localises close to Spc94p/Nud1p at the outer plaque (Wigge *et al.*, 1998), so it was not surprising to find that Cnm67p and Spc42p co-purified with protein A-tagged Spc94p. Two-hybrid studies gave the order of the three proteins within the SPB as given above (Elliott *et al.*, 1999). Spc94p has also been shown to interact with Spc72p using the two hybrid system and by immunoprecipitation (Gruneberg *et al.*, 2000).

Stu2p is a microtubule binding protein that localises to the SPB and to a lesser extent along spindle microtubules (Wang and Huffaker, 1997). *STU2* was identified as a suppresser of a β-tubulin cold sensitive mutant. Stu2p was later used to identify Spc72p in a two hybrid screen (Chen *et al.*, 1998). The theory for Stu2p function is that it forms a lateral attachment to microtubules which anchors and stabilises microtubules while allowing subunit exchange at the minus ends.

The half-bridge

The only known components of the half-bridge are Cdc31p and Kar1p. Cdc31p is a member of the calcium-binding protein family known as centrins which also includes components of the human centrosome (CEN1, 2 and 3 (Errabolu *et al.*, 1994; Middendorp *et al.*, 1997)). Cdc31p forms a complex at the half-bridge with Kar1p, which is a membrane protein. Both *CDC31* and *KAR1* were first identified in genetic screens. *CDC31* was identified in a screen for cell cycle mutants (Hartwell *et al.*, 1970), whereas *KAR1* was identified in a screen for mutants defective in nuclear fusion (karyogamy) during mating (Conde and Fink, 1976). Cdc31p and Kar1p are known to be involved in SPB duplication as demonstrated by analysing temperature sensitive mutants. The phenotype of *cdc31(ts)* and *kar1(ts)* cells are very similar (Byers, 1981a; Byers, 1981b; Rose and Fink, 1987; Winey *et al.*, 1991a). Both arrest with a single, abnormally large SPB that has failed to duplicate. This phenotype probably results from accumulation of SPB components around the pre-existing SPB due to the absence of a satellite. In support of this, Cdc31p action was found to be at the time of satellite formation (Winey *et al.*, 1991a). *CDC31* was first cloned by complementation of the *cdc31* mutants from the original screen for cell cycle mutants (Baum *et al.*, 1986). Sequence analysis showed that Cdc31p shows significant homology with calmodulin and other calcium-binding proteins. Cdc31p was identified as a member of a growing family called the centrins and as with other centrins, Cdc31p undergoes a calcium-dependent conformational change (Geier *et al.*, 1996; Wiech *et al.*, 1996). Cdc31p was localised to the SPB half-bridge by immunoelectron microscopy (Spang *et al.*, 1993).

Study of *kar1* mutants has shown that Kar1p is involved in mitosis and karyogamy. This was confirmed when mutational analysis of *KAR1* defined three functional regions (Vallen *et al.*, 1992). The first region is required for karyogamy, region II is essential for mitosis and region III is essential for both functions and encodes a membrane

spanning domain. These three regions are distinct, the rest of the protein is non-essential suggesting that Kar1p contains distinct protein binding domains flexibly connected to a membrane anchor. Kar1p was then localised to the half-bridge (Spang *et al.*, 1995). The localisation of Kar1p and Cdc31p to the same substructure of the SPB along with the similar phenotypes of *kar1(ts)* and *cdc31(ts)* mutants and genetic interactions between the two genes (Vallen *et al.*, 1994) led to investigation of a possible direct interaction between Kar1p and Cdc31p. [^{35}S]-labelled Cdc31p was shown to interact with Kar1p *in vitro* using an overlay assay. In addition, the Cdc31p-binding site of Kar1p is part of the region identified as essential for mitosis (Biggins and Rose, 1994; Spang *et al.*, 1995). The region essential for karyogamy coincides with the region of Kar1p that binds to Spc72p. Both *spc72(ts)* and *kar1(ts)* mutants have defects in karyogamy. This is now understood to be due to a defect in microtubule nucleation by the half-bridge. If microtubules are not nucleated by the half-bridge during mating then the two nuclei from the mating cells cannot be brought together, therefore nuclear fusion does not occur (Pereira *et al.*, 1999). The localisation of Cdc31p was claimed to be dependent on Kar1p as Cdc31p was no longer detectable at the SPB after depletion of Kar1p (Biggins and Rose, 1994; Spang *et al.*, 1995). However, a dominant mutation in *CDC31* (*CDC31-16*) allows cells to tolerate a complete deletion of *KAR1* (Vallen *et al.*, 1994). This suggests that there are other proteins involved in the Cdc31p/Kar1p complex. One candidate protein is Dsk2p (Biggins *et al.*, 1996; Vallen *et al.*, 1994). Dsk2p is a non-essential nuclear enriched protein that has an N-terminal ubiquitin-like domain. *DSK2-1* was found in a genetic screen for mutations that suppress the *kar1Δ17* mutation. In *kar1Δ17* cells Cdc31p is mislocalised, however in *kar1Δ17 DSK2-1* cells Cdc31p is relocalised to the SPB. Another member of the family of proteins containing a ubiquitin-like domain is Rad23p, which is involved in DNA repair (Watkins *et al.*, 1993) and interacts with the 26S proteosome (Schauber *et al.*, 1998). Cells containing *DSK2* or *RAD23* deletions are viable, however the double deletion strain (*Δdsk2 Δrad23*) is temperature sensitive due to a block in SPB duplication which can be suppressed by overexpression of *CDC31* or by *CDC31-16* (Biggins *et al.*, 1996). The phenotype of this double deletion mutant is similar to the phenotype of *cdc31(ts)* and *kar1(ts)* mutants, however Cdc31p is not mislocalised in these cells. In addition, overexpression of *DSK2-1* (the allele that suppressed the *kar1Δ17* mutation) also caused a block in SPB duplication, providing further evidence of a role for Dsk2p in this process. Overexpression of *DSK2* caused accumulation of cells with short bipolar spindles, suggesting that Dsk2p may play a number of different roles in the cell cycle.

There are homologues of *DSK2* in other organisms, for example *dhp1⁺* in *S. pombe* (He *et al.*, 1998) and XDRP1 (*Xenopus* Dsk2-related protein (Funakoshi *et al.*, 1999)). In *S. pombe*, overexpression of dph1⁺also causes mitotic arrest with short bipolar spindles. In *Xenopus*, microinjected XDRP1 blocked cell division in cleaving *Xenopus* embryos and inhibited cyclin A degradation. XDRP1 interacted with cyclin A in the two hybrid system and in *Xenopus* egg extracts. Cyclin A seems to play a role in microtubule nucleation and mitotic spindle formation (Maldonado-Codina and Glover, 1992; Verde *et al.*, 1992) and the role of XDRP1 seems to be to protect cyclin A from degradation until these essential functions have been carried out.

Regulation of SPB duplication

Although it is well established that Cdc31p and Kar1p are involved in SPB duplication, the actual mechanisms involved have proved more difficult to elucidate. There are a host of other mutants that exhibit SPB duplication defects, for example a screen for mutants with monopolar spindles identified *MSP1* and *MPS2* which when mutated result in SPB duplication defects (Schutz and Winey, 1998; Winey *et al.*, 1991b). Mps1p is a protein kinase that may be involved in phosphorylation of SPB components (Pereira *et al.*, 1998) and formation of the satellite (Winey *et. al.*, 1991b). These functions may be part of a checkpoint that monitors SPB duplication (Fesquet *et al.*, 1999; Schutz *et al.*, 1997; Schutz and Winey, 1998). Mps2p is a membrane protein that is involved in inserting the newly formed SPB into the nuclear membrane (Munoz-Centeno *et al.*, 1999; Schramm *et al.*, 2000; Winey *et al.*, 1991b). It also appears that there is some functional overlap between nuclear pore components and the SPB, for example Ndc1p localises to both nuclear pores and the SPB (Chial *et al.*, 1998). When mutations were introduced into *NDC1*, the cells exhibited SPB duplication defects (Chial *et al.*, 1998; Thomas and Botstein, 1986; Winey *et al.*, 1993). Interestingly, both Ndc1p and Mps2p are membrane proteins and when mutated, cells exhibit almost identical defects in SPB duplication. Both arrest with a newly formed SPB attached to the mother SPB by the bridge, that is unable to insert into the nuclear membrane and therefore lacks an inner plaque. What is not yet understood is why the deletion of *POM152* which encodes a nuclear pore component (Wozniak *et al.*, 1994) rescues the SPB duplication defects of *ndc1(ts)* and *mps2(ts)* mutants (Chial *et al.*, 1998; Schramm, unpublished data). However, Pom152p co-fractionates with SPBs (Wigge *et al.*, 1998), but at the time this was explained as contamination.

Separation of SPBs by cleaving the bridge is a process which is not yet understood, however homologues of the SCF^{Cdc4p} complex in mammalian cells appear to be involved in separation of centrioles *in vitro* and centrosome duplication in *Xenopus* embryos (Freed *et al.*, 1999). Also, mutations in *CDC4, CDC34* and *CDC53* (components of the SCF^{Cdc4p} complex) result in cells that arrest with SPBs that have duplicated, but not separated (Chun *et al.*, 1996; King *et al.*, 1996a; Mathias *et al.*, 1996). There is also evidence for participation of the proteosome in SPB duplication, mutations in one of the cap structure proteins (Pcs1p) results in cells arrested with a single, enlarged SPB (similar to the phenotype of *cdc31(ts)* and *kar1(ts)* mutants). This suggests that the role of the proteosome is in an early step in SPB duplication and that this role is distinct to cyclin degradation (McDonald and Byers, 1997).

As mentioned at the start of this chapter the cell cycle contains checkpoints that monitor cell cycle events and arrest the cell cycle if defects are detected, but how is SPB duplication monitored? In order for a cell to form a bipolar mitotic spindle SPB duplication must occur, therefore the spindle checkpoint will arrest cells that have not properly duplicated or separated their SPBs.

Aims

When this study commenced, the spindle pole body of *Saccharomyces cerevisiae* was not as well understood as it is at the present time. The only components identified were Spc42p, Spc110p, Cmd1p, Cdc31p, Kar1p and the components of the γ-tubulin complex. Little was know about the components of the outer plaque, how the known components interacted with each other within the SPB or the processes involved in SPB duplication. The aim of the project here in was to identify and characterise additional components of the SPB in the hope of gaining an increased knowledge of the structure of the SPB and perhaps to elucidate further the mechanism of SPB duplication.

Materials and Methods

Media and Plates

All chemicals were from Fisher chemicals unless otherwise indicated.

LB	10 g Bacto-tryptone (DIFCO)
	5 g Bacto-yeast extract (DIFCO)
	10 g NaCl
	Made up to 1 litre with distilled water then autoclaved
	For plates 15 g Bacto-agar (DIFCO) was added
Selection	100 mg/l Ampicillin (SIGMA) diluted from 1000 x stock in ddH$_2$O, stored at -20°C
	50 mg/l Kanamycin monosulphate (SIGMA) diluted from 400 x stock in ddH$_2$O, stored at -20°C
Induction	1 mM IPTG (Melford Laboratories, Ltd.) diluted from 100 x stock in ddH$_2$O, stored at -20°C
SOC Medium	2 g Bacto-tryptone (DIFCO)
	0.55 g Bacto-yeast extract (DIFCO)
	1 ml 1 M NaCl
	1 ml 1 M KCl
	97 ml dd H$_2$O
	This solution was autoclaved, then allowed to cool.
	To the cooled solution was added:
	1 ml 2 M Mg^{2+} (1 M MgCl$_2$, 1 M MgSO$_4$)
	1 ml 2 M Glucose
	The final solution was filter sterilised and stored in 10 ml aliquots at room temperature.

PSi Broth

5 g Bacto-yeast extract (DIFCO)

20 g Bacto-tryptone (DIFCO)

5 g Magnesium sulphate

Adjusted to pH 7.6 with potassium hydroxide

Made up to 1 litre with distilled water then autoclaved

YPD

10 g Bacto-yeast extract (DIFCO)

20 g Bacto-peptone (DIFCO)

20 g Glucose

Made up to 1 litre with distilled water then autoclaved

For plates 20 g Bacto-agar was added

YPD-KanMx plates

YPD containing 20 g Bacto-agar

0.2 g/l Kanamycin (Geneticin G-418 Sulphate from GIBCO). The YPD was autoclaved then cooled. Kanamycin was dissolved in 20 ml ddH$_2$O, filter sterilised, then added to the cooled YPD, the plates were poured immediately after mixing.

YPAD

YPD containing 100 mg/l Adenine (SIGMA)

YPAD for immunofluorescence

10 g Bacto-yeast extract (DIFCO)

20 g Bacto-peptone (DIFCO)

500 mg Adenine (SIGMA)

900 ml distilled water

The above was mixed and divided equally into four 250 ml bottles then autoclaved. Before use 25 ml 20% Glucose (sterile filtered) was added.

SC Medium

6.7 g Bacto-yeast nitrogen base without amino acids (DIFCO)

20 g Glucose

2 g Drop out mix

Made up to 1 litre with distilled water then autoclaved

SC Plates   A   6.7 g Bacto-yeast nitrogen base without amino acids

                              (DIFCO)

                              20 g Glucose

                              2 g Drop out mix

                              Made up to 500 ml with distilled water

                   B   20 g Bacto-agar (DIFCO)

                              Made up to 500 ml with distilled water

          A and B were autoclaved separately and mixed before pouring plates.

SC medium and plates containing galactose/raffinose (SIGMA) instead of glucose were made as above, minus glucose. After autoclaving and cooling, filter sterilised 20% raffinose and/or 20% galactose was added to a final concentration of 2%.

Drop out mix: A mix of amino acids and other growth requirements used to make SC-medium and plates. All the contents of the drop out mix were from SIGMA.

Adenine	5 g	Isoleucine	20 g
Alanine	20 g	Lysine	20 g
Arginine	20 g	Methionine	20 g
Asparagine	20 g	para-Aminobenzoic acid	2 g
Aspartic acid	20 g	Phenylalanine	20 g
Cysteine	20 g	Proline	20 g
Glutamine	20 g	Serine	20 g
Glutamic acid	20 g	Threonine	20 g
Glycine	20 g	Tyrosine	20 g
Inositol	20 g	Valine	20 g

The following was added to 36.7 g of the above mix as required, to allow selection of cells containing specific marker genes.

Histidine	2 g
Leucine	4 g
Uracil	2 g
Tryptophan	2 g

5-FOA plates	A	6.7 g Bacto-yeast nitrogen base without amino acids (DIFCO)

                                       20 g Glucose

                                       2 g Drop out mix minus Uracil

                                       50 mg Uracil

                                       1 g 5-FOA (Melford Laboratories Ltd.)

                                       Made up to 500 ml with distilled water

                                       Filter sterilised

          B   20 g Bacto-agar (DIFCO)

                                       Made up to 500 ml with distilled water

                                       Autoclaved

        A and B were mixed before pouring plates

SPO plates   2.5 g Bacto-yeast extract (DIFCO)

                                       15 g Potassium acetate

                                       0.5 g Glucose

                                       40 mg Adenine

                                       40 mg Uracil

                                       40 mg Tyrosine

                                       20 mg of amino acids: Arg, His, Leu, Lys, Met and Trp.

                                       100 mg Phenylalanine

                                       350 mg Threonine

                                       20 g Bacto-agar (DIFCO)

                                       Made up to 1 litre with distilled water and autoclaved

X-Gal plates (3 plates)   20 ml 5 x SC-His-Leu

                                       10 ml 20% Galactose (filter sterilised)

                                       10 ml 20% Raffinose (filter sterilised)

                                       10 ml BU salts (46.5 g $Na_2HPO_42H_2O$, 33.9 g

                                               $NaH_2PO_41H_2O$ in 1 litre of ddH_2O)

                                       50 ml 4% Bacto-agar in ddH_2O (agar was autoclaved

                                           and stored solid, agar was melted before use using

                                           a microwave)

                                     200 µl X-Gal (Melford Laboratories, Ltd.) (40 mg/ml in

                                           DMF (SIGMA), stored at -20°C)

        X-Gal plates were made fresh for each experiment.

X-Gal top agar (per plate)   9.3 ml 0.5M KPi, pH 7

600 µl DMF (SIGMA)

100 µl 10% SDS (ICN)

50 mg ultrapure LMP agarose (GIBCO)

Solution was heated briefly (30 sec - 1 min) using a microwave, then left to cool.

To the warm solution was added 5 µl β-mercaptoethanol and 100 µl of 40 mg/ml X-Gal (Melford Laboratories, Ltd.) in DMF (SIGMA).

Solutions

TBS   25 mM Tris (GIBCO)

137 mM NaCl

2.7 mM KCl

pH 7.55 (adjusted with HCl)

PBS   137 mM NaCl

2.7 mM KCl

10 mM Na_2HPO_4

1.76 mM KH_2PO_4

pH 7.2 (adjusted with HCl)

50 x TAE stock   2 M Tris

50 mM EDTA (MERCK)

pH 7.7 (adjusted with acetic acid)

Laemmli running buffer   25 mM Tris

192 mM Glycine

0.1% SDS (ICN)

Blotting buffer   25 mM Tris

192 mM Glycine

0.025% SDS (ICN)

20% Methanol

HU buffer

8 M Urea

5% SDS (ICN)

200 mM Tris-HCl, pH 8.0

0.1 mM EDTA (MERCK)

0.5% Bromophenol blue (MERCK)

100 mM DTT (BIOMOL)

Coomassie blue stain

0.25% Coomassie Brilliant blue R250 (Serva)

7% Acetic acid

50% Methanol

Destain solution

10% Acetic acid

50% Methanol

Ponceau S stain

0.2% Ponceau S (SIGMA)

3% TCA

10x PCR buffer

(Perkin Elmer)

100 mM Tris-HCl, pH 8.3

500 mM KCl

10x Ligation buffer

(EPICENTRE Technologies)

50 mM Tris-HCl, pH7.8

10 mM $MgCl_2$

1 mM DTT

1 mM ATP

TfbI

30 mM Potassium acetate

100 mM Rubidium chloride

10 mM Calcium chloride

50 mM Manganese chloride

15% Glycerol (ALDRICH)

Adjusted to pH 5.8 with dilute acetic acid

TfbII   10 mM MOPS

10 mM Rubidium chloride

75 mM Calcium chloride

15% Glycerol (ALDRICH)

Adjusted to pH 6.5 with sodium hydroxide

QIAGEN Plasmid Purification:

Buffer P1   50 mM Tris-HCl, pH 8.0

10 mM EDTA

100 μg/ml RNase A

Buffer P2   200 mM NaOH

1% SDS

Buffer P3   3 M potassium acetate, pH 5.5

Buffer QBT   750 mM NaCl

50 mM MOPS, pH 7.0

15% isopropanol

0.15% Triton X-100

Buffer QC   1 M NaCl

50 mM MOPS, pH 7.0

15% Isopropanol

Buffer QF   1.25 M NaCl

50 mM Tris-HCl, pH 8.5

15% Isopropanol

TE   10 mM Tris-HCl, pH 8.0

                            1 mM EDTA (MERCK)

DNA sample buffer   0.8% Bromophenol blue (MERCK)

                            0.8% Xylene cyanole (SIGMA)

                            15% Ficoll 400 (Pharmacia Biotech)

LiSorb   100 mM Lithium acetate (SIGMA)

                            10 mM Tris-HCl, pH 8.0

                            1 mM EDTA (MERCK)

                            1 M Sorbitol (SIGMA)

                            Filter sterilised

LiPEG   100 mM Lithium acetate (SIGMA)

                            1 M Tris-HCl, pH8.0

                            1 mM EDTA (MERCK)

                            40% PEG 3350 (SIGMA)

                            Filter sterilised

S buffer   10 mM K_2HPO_4, pH 7.5

                            10 mM EDTA (MERCK)

                            50 mM β-Mercaptoethanol (SIGMA)

                            50 μg/ml Zymolyase 50T (Seikagaku Corporation,Tokyo)

                            Prepared fresh

Lysis solution   25 mM Tris-HCl, pH 7.5

                            25 mM EDTA (MERCK)

                            2.5% SDS (ICN)

NaPi buffer (0.1 M)   6.8 ml NaH_2PO_4 (1 M)

                            93.2 ml Na_2HPO_4 (1 M)

                            Made up to 1 litre with distilled water

                            pH 8

KPi buffer   32.95 ml K_2HPO_4 (500 mM)

                                  67.05 ml KH_2PO_4 (500 mM)

                                  pH 6.5

SP buffer   40 mM KPi

                                  0.5 mM $MgCl_2$

                                  1.2 M Sorbitol (SIGMA)

Wickerman's solution   YPAD

                                  1.1 M Sorbitol (SIGMA)

                                  Filter sterilised

PBS-B   PBS

                                  1% BSA (SIGMA)

DAPI-mounting solution   75% Citifuor (Agar Scientific)

                                  25% PBS

                                  0.25 µg/ml DAPI (SIGMA)

IP buffer   50 mM Tris-HCl, pH 7.4

                                  10 mM EDTA (MERCK)

                                  1 mM EGTA (SIGMA)

                                  5% Glycerol (ALDRICH)

IP buffer (low salt)   IP buffer

                                  100 mM NaCl

IP buffer (high salt)   IP buffer

                                  1 M NaCl

TST buffer   50 mM Tris-HCl pH 7.4

                                  150 mM NaCl

                                  0.05% Tween 20 (SIGMA)

Protease Inhibitors	Complete, Mini, EDTA-free, Protease Inhibitor Cocktail Tablets (Boehringer Mannheim). One tablet was added per 10 ml of buffer. Once the tablets were added, buffers were stored on ice and used within 6 hours.
PMSF stock	200 mM PMSF (Fluka) in DMSO, stored at -20°C
Benzamidine stock	500 mM Benzamidine (SIGMA) in ddH$_2$O, stored at -20°C
Phosphate-citrate buffer	170 mM KH$_2$PO$_4$ 30 mM sodium citrate (weighed out) to yield pH 5.8
Phosphate-Mg buffer	40 mM KPi, pH6.5 0.5 mM MgCl$_2$
Pretreatment solution	0.2 M Tris-HCl, pH 9.0 20 mM EDTA (MERCK) 0.1 M β-Mercaptoethanol (added immediately before use)
Lead citrate	1.33 g Lead nitrate (TAAB) 1.76 g Sodium citrate (TAAB)

30 ml CO$_2$-free ddH$_2$O was added and the solution was shaken vigorously for several minutes, then 5 times over 30 min. The milky solution was then cleared with the addition of 5 to 8 ml of 1 N sodium hydroxide, which also altered the pH to 12.0. The solution was then made up to 50 ml with CO$_2$-free ddH$_2$O.

CB buffer	100 mM NaHCO$_3$ 0.5 M NaCl Adjusted to pH 8.3 with HCl
AP buffer	50 mM Tris-HCl, pH 8.0 150 mM NaCl 1 mM MgCl$_2$ 0.1 mM ZnCl$_2$ 1 mM PMSF added from stock immediately before use

Primers

Primers used for cloning *SPC29*

Primers designed for cloning *SPC29* contained sequences approximately 400 bp upstream of the START codon and a *Bam*H1 restriction site (SPC29-A) or approximately 200 bp downstream of the STOP codon and a *Xho*1 restriction site (SPC29-B). Chromosomal DNA was used as the template. The PCR product was then inserted into pRS315 and pRS425.

Name	Sequence
SPC29-A	5'CGggatccGCAAAAACAATGATCCAGTGC3' (forward)
SPC29-B	5'TCCGctcgagAACGAAAGGAGGAGACGGATA3' (reverse)

Primers used to construct *spc29(ts)* alleles

These primers were designed to contain sequence homology with the pRS315 vector. Therefore, these primers could be used to amplify any gene in pRS315. Also, the PCR product would have end regions that could facilitate homologous recombination with the cut pRS315 when both were transformed into yeast cells. pRS315-*SPC29* was used as a template.

Name	Sequence
M1	5'GGATGTGCTGCAAGGCG3'
M2	5'TTGTGTGGAATTGTGAGCG3'

Primers used to sequence *SPC29*

Name	Sequence
SPC29-1	5'TAGGAAAGTGAAGAGCCCG3'
SPC29-2	5'CATCAAGAATAACAAGACTGG3'
SPC29-3	5'TTTATCTCCTCTGTCTGAG3'
SPC29-4	5'AAATGGTGGAGGAGGTAGT3'

Primers used to construct *SPC29* and *SPC110*[2433-2832] two hybrid plasmids

Two hybrid plasmids were designed to contain short sequences of the gene from the START codon and containing a *Bam*H1 site (SPC29-2H1) or ending at the STOP codon and containing a *Xho*1 site (SPC29-2H2, SPC110-2H8). The forward primer used to

create *SPC110²⁴³³⁻²⁸³²* was designed to contain the appropriate sequence, a START codon and a *Bam*H1 site (SPC110-5B). Additional bases are required to ensure the correct reading frame is maintained. The PCR products were inserted into the two hybrid plasmids. These PCR products could also be inserted into expression vectors.

Name	Sequence
SPC29-2H1	5'CGggatccGT<u>ATG</u>GATTATAGTAACTTTGGAAA3' (forward)
SPC29-2H2	5'TCCGctcgag<u>TCA</u>ATTGTGAAAAGACGAAGAC3' (reverse)
SPC110-5B	5'GGTCggatccAGT<u>ATC</u>ACTCAGAAGTAAGAC3'(forward)
SPC110-2H8	5'CTGGCctcgag<u>CTA</u>CCAAGATATACGCCCG3' (reverse)

Primers used for *SPC29* disruption cassettes and tags

The S-primers were designed to amplify epitope tags and disruption cassettes from plasmids. The primers have 'tails' which do not bind to the template DNA, but are amplified as part of the PCR product. These 'tails' are homologous to regions of the gene of interest allowing homologous recombination either at the 3' end to give gene fusions (S2 and S3) or at the start and end of the gene to give a gene disruptions (S1 and S2). The S1 'tail' contains the 45 bp before START and the START codon. S2 is the reverse primer used with both S1 and S3 primers. It contains the STOP codon and the 45 bp after the STOP codon. S3 contains the 45 bp before the STOP codon. The PCR products are transformed into the yeast strain of interest to form gene fusions or knockouts in the genome.

Name	Sequence
S1-SPC29	5'TGTAAAAGTTTAACCAAAGATCCAAAGTGGCTTGTAGGAAT A<u>ATG</u>cgtacgctgcaggtcgac3' (forward)
S2-SPC29	5'TTAGTTACGATTATGCTGGTATTATTTAGTTAAGTACTTAAT <u>TCA</u>atcgatgaattcgagctcg3' (reverse)
S3-SPC29	5'AGTACGGAGGATATACTAAAAATATTGTCTTCGTCTTTTCAC AAT cgtacgctgcaggtcgac3' (forward)
S4-SPC29	5'GAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGA ctcgag<u>TCA</u>atcgatgaattcgagctcg3' (reverse)
	(S4 replaces S2 for *Gal1-SPC29* construct only)

Vectors and plasmids

Table 1 Plasmid backbones

Name	Description	Source or Reference
pRS305	*LEU2*-based yeast integration vector	Sikorski and Hieter (1989)
pRS306	*URA3*-based yeast integration vector	Sikorski and Hieter (1989)
pRS315	*CEN6, LEU2*-based yeast-*E. coli* shuttle vector	Sikorski and Hieter (1989)
pRS316	*CEN6, URA3*-based yeast-*E. coli* shuttle vector	Sikorski and Hieter (1989)
pRS414	*CEN6, TRP1*-based yeast-*E. coli* shuttle vector	Christianson *et al* (1992)
pRS423	2 μm, *HIS3*-based yeast-*E. coli* shuttle vector	Christianson *et al* (1992)
pRS425	2 μm, *LEU2*-based yeast-*E. coli* shuttle vector	Christianson *et al* (1992)
pRS426	2 μm, *URA3*-based yeast-*E. coli* shuttle vector	Christianson *et al* (1992)
p413Gal1	*CEN6, HIS3*-based yeast-*E. coli* shuttle vector containing the *Gal1*promoter	Mumberg *et al* (1994)
pACT2	Two hybrid vector: 2 μm, *LEU2*-based vector carrying the *GAL4* activation domain	Durfee *et al* (1993)
pEG202	Two hybrid vector: 2 μm, *HIS3*-based vector carrying the lexA DNA-binding domain	Gyuris *et al* (1993)
pET28c	*E.coli* expression vector containing *HIS6* under the control of the T7 promoter	Novagen
pGEX-5X-1	*E.coli* expression vector containing GST under the control of the lacZ promoter	Pharmacia
pMM5	Two hybrid vector: pRS423 containing *Gal1-LexA-Myc*	M. Knop
pMM6	Two hybrid vector: pRS425 containing *Gal1-GAL4-HA*	M. Knop
YDpW	*TRP1*-based integration plasmid	Berben *et al* (1991)

Table 2   Plasmids used as templates for PCR to construct tags for chromosomal integration.

Name	Description	Source or Reference
pYM1	pFA6a-3HA-kanMX6	Knop *et al* (1999)
pYM4	pFA6a-3MYC-kanMX6	Knop *et al* (1999)
pYM7	pFA6a-ProA-kanMX6	Knop *et al* (1999)
pYM12	pFA6a-GFPS65T-kanMX6	Wach *et al* (1997)

Table 3   Plasmids used as templates for PCR to construct disruption cassettes

Name	Source or Reference
pFA6akanMX4	Wach *et al* (1994)
pFA6aHis3MX6	Wach *et al* (1994)

Table 4 Plasmids

Name	Description	Source or Reference
pSE1	pRS315-*SPC29*	this study
pSE3	pRS425-*SPC29*	this study
pSE4	pEG202-*SPC29*	this study
pSE7	pACT2-*SPC29*	this study
pSE11	pRS316-*SPC29*	this study
pSE16	pRS425-*SPC110*	this study
pSE17	pRS425-*SPC42*	this study
pSE19	pRS305-Gal1-*SPC42*	this study
pSE20	pRS306-Gal1-*SPC29*	this study
pSE29	pEG202-*SPC110*$^{2433\text{-}2832}$	this study
pSE32	pEG202-*spc110-120*$^{2433\text{-}2832}$	this study
pSE33	pEG202-*spc110-124*$^{2433\text{-}2832}$	this study
pSE39	pEG202-*spc110-116*$^{2433\text{-}2832}$	this study
pSE40	pEG202-*spc110-118*$^{2433\text{-}2832}$	this study
pSE41	pET28c-*SPC29*	this study

Table 4 Plasmids continued

Name	Description	Source or Reference
pSE63	pMM5 containing *SPC29*	this study
pSE64	pMM6 containing *SPC29*	this study
pSE65	pMM5 containing *BBP1*	this study
pSE66	pMM6 containing *BBP1*	this study
pSE67	pMM5 containing $MPS2^{1-307}$	this study
pSE68	pMM6 containing $MPS2^{1-307}$	this study
pSE72	pMM6 containing *KAR1-ΔTM*	this study
pAL2	pET28c containing $MPS2^{1-307}$	A. Camasses
pBG2A-9	pRS425 containing *DSK2*	B. Geier
pCM108	pRS315 containing *SPC110*	E. Schiebel
pCMD1	pRS425 containing *CMD1*	E. Schiebel
pCS11	pGEX-5X-1 containing *BBP1*	C. Schramm
pCS14	pRS425 containing *BBP1*	C. Schramm
pDS110	YDpW containing *SPC110*	Stirling *et al* (1994)
pDS116	YDpW containing *spc110-116*	Stirling *et al* (1994)
pDS118	YDpW containing *spc110-118*	Stirling *et al* (1994)
pDS120	YDpW containing *spc110-120*	Stirling *et al* (1994)
pDS124	YDpW containing *spc110-124*	Stirling *et al* (1994)
pMK82	p413Gal1 containing *SPC110*	M. Knop
pMK150	pACT2-$SPC110^{2433-2832}$	M. Knop
pMK151	pACT2-$SPC110^{1-612}$	M. Knop
pMK174	pACT2-$SPC42^{1-415}$	M. Knop
pMK175	pEG202-$SPC42^{1-415}$	M. Knop
pMK176	pACT2-$SPC42^{291-1092}$	M. Knop
pMK177	pEG202-$SPC42^{291-1092}$	M. Knop
pMK296	pRS425-*CDC31-16*	M. Knop
pSG11	pEG202- $SPC110^{1-612}$	S. Geissler
pSG13	pEG202-$SPC110^{2433-2832}$	S. Geissler
pSM119	pRS315-*KAR1*	E. Schiebel
pSM192	pRS414-*ATG-ProA-SPC110*	E. Schiebel
pSM234	pRS425-*CDC31*	E. Schiebel

E. coli strains

Plasmids listed above were transformed and amplified in one or more of the following strains:

DH5α   *deoR endA1 gyrA96 hsdR17*($r_k^-m_k^-$) *recA1 relA1 supE44 thi-1 Δ(lacZYA-argFV169)* $\phi80\delta lacZ\Delta M15$ F⁻ λ⁻; Clontech, Palo Alto, USA

SURE   e14⁻(McrA⁻) Δ(*mcrCB-hsdSMR-mrr*)171 *endA1 supE44 thi*-1 *gyrA96 relA1 lac recB recJ sbcC umuC*::Tn5 (Kanʳ) *uvrC* [F' *proAB lacI^qZΔ(M15* Tn*10* (Tetʳ)]; Stratagene, La Jolla, USA

BL21   *E. coli* B F⁻ λ *dcm ompT hsdS*($r_\beta^-m_\beta^-$) *gal* (DE3); Stratagene, La Jolla, USA

Yeast strains

Table 5 Background strains

Name	Genotype	Source or Reference
CRY1	*MATa ade2 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1*	M. Stark
ESM356-1	*MATa ura3-52 his3Δ200 leu2Δ1 trp1Δ63*	E. Schiebel
ESM357-9	*Matα ura3-52 leu2Δ1 his3Δ200 trp1Δ63*	E. Schiebel
SGY37VII	*MATa ura3-52::URA3-lexA-op-LacZ trp1 his3 leu2*	Geissler *et al* (1996)
YPH499	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)
YPH501	*MATa/α ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1*	Sikorski and Hieter (1989)

Table 6 Constructed strains

Name	Genotype	Source or Reference
SEY7	*MATa ura3-52 leu2Δ1 his3Δ200 trp1Δ63 SPC29-GFP-kanMX6*	this study
SEY13	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 SPC29-3HA-kanMX6*	this study
SEY14	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 SPC29-3Myc-kanMX6*	this study
SEY23	*MATa his3Δ200 trp1Δ63 leu2Δ1 ura3-52::pSE20*	this study
SEY27	*MATa his3Δ200 trp1Δ63 leu2Δ1::pSE19 ura3-52::pSE20 pMK82*	this study
SEY33	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc98-1 Δspc29::HIS3* pSE11	this study
SEY35	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc97-14 Δspc29::HIS3* pSE11	this study
SEY37	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc110-2 Δspc29::HIS3* pSE11	this study
SEY39	*MATa ade2 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 cmd1-1 Δspc29::HIS3* pSE11	this study
SEY40	*MATa ade2 can1-100 his3-11, 15 leu2-3, 112 trp1-1 ura3-1 Δspc29::HIS3* pSE11	this study
SEY41	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc29::HIS3* pSE11	this study
SEY47	*MATa his3Δ200 leu2Δ1 trp1Δ63 ura3-52::pSE20 Gal1-SPC29-GFP*	this study
SEY56	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc42-9 Δspc29::kanMX4* pSE11	this study
SEY63	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 mps2-1 leu2Δ1::pRS305*	this study
SEY64	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 mps2-1 leu2Δ1::pSE19*	this study
SEY80	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc29-2 SPC72-GFP-kanMX6*	this study

Table 6 Constructed strains continued

Name	Genotype	Source or Reference
SEY82	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc29-2 SPC42-GFP-kanMX6*	this study
SEY83	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc29-2 SPC110-GFP-kanMX6*	this study
spc29-2	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc29-2*	this study
spc29-3	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc29-3*	this study
spc29-9	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc29-9*	this study
spc42-9	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc42-9*	this study
spc42-10	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 spc42-10*	this study
spc110-118	*MATa ade2-1 his3 trp1-1::pDS118 leu2 ura3 spc110::LEU2*	M. Stark
spc110-124	*MATa ade2-1 his3 trp1-1::pDS124 leu2 ura3 spc110::LEU2*	M. Stark
ESM172	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc110::HIS3 pSM192*	Knop *et al* (1997)
ESM473	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δspc29::HIS3 pSE11*	this study
ESM504	*Matα ura3-52 leu2Δ1 his3Δ200 trp1Δ63 SPC72-GFP*	E. Schiebel
YMK230	*MATa ura3-52 lys2-801 ade2-101 trp1Δ63 his3Δ200 leu2Δ1 Δkar1::HIS3 pMK296*	M. Knop

Antibodies

Table 7 Primary antibodies

Antibody	Species	Dilution	Source
anti-HA (12A5)	mouse (monoclonal)	1:10	E. Schiebel
anti-Myc (9E10)	mouse (monoclonal)	1:20	E. Schiebel
anti-tubulin (Wa3)	mouse (monoclonal)	1:20	E. Schiebel
anti-90 kDa SPB protein	mouse (monoclonal)	1:5	J. Kilmartin
anti-Bbp1p	rabbit (polyclonal)	1:200	C. Schramm
anti-Cdc31p	goat (polyclonal)	1:400	E. Schiebel
anti-Cnm67p	rabbit (polyclonal)	1:1000	E. Schiebel
anti-Kar1p	goat (polyclonal)	1:200	E. Schiebel
anti-Kar1p	rabbit (polyclonal)	1:400	E. Schiebel
anti-Mps2p	rabbit (polyclonal)	1:200	C. Schramm
anti-Spc29p	rabbit (polyclonal)	1:100	this study
anti-Spc42p	rabbit (polyclonal)	1:10 000	E. Schiebel
anti-Spc72p	rabbit (polyclonal)	1:250	E. Schiebel
anti-Spc98p	rabbit (polyclonal)	1:1000	E. Schiebel
anti-Spc110p	rabbit (polyclonal)	1:1000	E. Schiebel
anti-Tub2p	rabbit (polyclonal)	1:3000	E. Schiebel

Table 8 Secondary antibodies

Antibody	Species	Coupled to	Dilution	Source
anti-goat	rabbit	HRP	1:5000	Dianova
anti-mouse	sheep	HRP	1:5000	Dianova
anti-rabbit	goat	HRP	1:5000	Dianova
anti-goat	donkey	Cy3	1:1000	Dianova
anti-rabbit	goat	Cy3	1:1000	Dianova
anti-mouse	goat	FITC	1:250	Dianova
anti-mouse	rabbit	FITC	1:250	Dianova

Methods

For all centrifugation steps; Eppendorf mini centrifuges were used for small volumes. Larger volumes were centrifuged in Sorvall rotors in Sorvall RC-5B Refrigerated Superspeed Centrifuges or in falcon tubes in a Heraeus Varifuge 3.2RS.
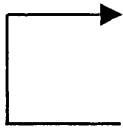
DNA Manipulations

PCR

Template (0.15 µg)	1 µl
Primer 1 (10 µM)	1 µl
Primer 2 (10 µM)	1 µl
10 x PCR buffer	10 µl
dNTPs (each dNTP is present at 2 mM)	10 µl
Magnesium chloride (25 mM)	4 - 24 µl
ddH$_2$O	made up to 100 µl

2 U *Taq* polymerase and 0.4 U Vent polymerase was added per PCR tube (multiply safecups from Sarstedt), just before starting the reaction. PCR machines were from Biometra.

Programme: 3 min 94°C

      1 min ~54°C (depended on calculated annealing temperature of primers)

x 25   elongation 72°C (time depended on length of expected product)

      1 min 94°C

      1 min 55°C

      3 min 72°C

PCR products were analysed by agarose gel electrophoresis, then extracted from the gel using the Gel Extraction Kit from Qiagen. The PCR products were then transformed directly into yeast competent cells or digested with restriction enzymes then ligated into plasmid backbones and transformed into *E. coli* competent cells.

Restriction digests

All digests were carried out using BioLabs or Gibco enzymes and their recommended reaction buffers and BSA, if recommended. 3 U of enzyme was used per μg of DNA. Control digests were carried out using 3 μl mini DNA, the restriction reaction was carried out in a final volume of 20 μl and incubated at 37°C for approximately 1 hour. Plasmid DNA (3 μg) and PCR fragment (10 μl of gel extracted fragment) digests were carried out in a final volume of 100 μl and incubated at 37°C overnight. Incubation temperatures were changed when appropriate. Digests were analysed by agarose gel electrophoresis.

DNA agarose gel electrophoresis

0.8% agarose (Gibco) in TAE (solublised by heating in a microwave) was used for most DNA analysis. DNA was mixed with 0.2 volumes DNA sample buffer and loaded into the wells of the gel. Gels were run at 90 V for 30 to 60 minutes in TAE. Gel apparatus was from BioRad. DNA bands were visualised by UV (254 nm) after staining in an ethidium bromide bath (1 ng/ml). The 1 kb ladder from Gibco was used as a DNA marker.

Isolation of DNA fragments from agarose gels

Gel Extraction Kit from Qiagen was used for all gel extraction. DNA bands were excised from the agarose gel using a clean, sharp scalpel. Three volumes of buffer QX1 was added to each gel slice in 1.5 ml Eppendorf tubes, 10 μl of resuspended QIAEX II was added and the samples were incubated at 50°C for 10 min with shaking. Samples were then centrifuged (14 000 rpm, 30 sec, RT), the pellets were washed once with QX1 and twice with PE, each time the pellet was resuspended by vortexing. After washing the pellets were air-dried for 10 to 15 min. To elute the DNA, 20 μl of ddH$_2$O was added to the pellets and incubated at room temperature for 5 min. The samples were centrifuged for 30 sec and the supernatant, containing the DNA, was pipetted into clean Eppendorf tubes.

Phenol/chloroform extraction

Backbone vectors were extracted from restriction enzyme reactions by phenol/chloroform extraction. To the restriction enzyme reactions was added 0.5 volumes phenol and 0.5 volumes chloroform. The samples were vortexed 3 times for 20 sec with 2 min intervals. Samples were then centrifuged (14 000 rpm, 5 min, RT). After centrifugation, the top layer was transferred into a clean Eppendorf tube and 0.5 volumes of chloroform was added to remove traces of phenol. Samples were centrifuged again and this time the bottom layer was transferred into a clean Eppendorf tube, to this was added 0.1 volumes 3M sodium acetate, 0.005 volumes glycogen (Gibco) and 2 volumes 100% ethanol, samples were mixed and incubated at -80°C for 30 min. Samples were then centrifuged (14 000 rpm, 30 min, 4°C), the pellets were air-dried and resuspended in 20 μl ddH$_2$O or TE.

Ligations

Vector	0.5 μl
Insert	5 - 7.5 μl
ATP (25 mM)	0.4 μl
10 x ligation buffer	1.0 μl
T4 ligase (Epicentre Technologies)	0.5 μl
dH$_2$O	made up to 10 μl

Ligation reactions were incubated at 14°C overnight or at room temperature for 1 to 3 hours then transformed into *E. coli* competent cells.

Preparation of *E.coli* electro-competent cells

For the preparation of electro-competent cells, cultures of DH5α *E.coli* cells were grown in LB medium at 37°C with shaking, to an OD$_{600}$ of 0.5 to 1.0. Cells were harvested by centrifugation (5000 rpm, 15 min, 4°C) in sterile centrifuge tubes. The cell pellet was washed, in decreasing amounts, twice with cold, sterile water and once with cold 10% glycerol. The pellet was then resuspended in an approximately equal volume of 10% glycerol. Cells were divided into aliquots in pre-cooled Eppendorf tubes on dry ice and stored at -80°C.

Transformation of *E.coli* electro-competent cells

One 50 μl aliquot of DH5α competent cells per transformation was thawed on ice and 3 μl of the ligation reaction or yeast mini DNA was added. The cells were then transferred to a pre-chilled electroporation cuvette and electroporated using a BioRad Gene Pulser (400 Ω, 1 kV, 25 μF). Immediately after electroporation 1 ml sterile SOC medium was added. Cells were resuspended and recovered at 37°C for 1 hour in Eppendorfs. After recovery, 200 μl was plated out directly onto selective LB plates. The remaining cells were pelleted (5000 rpm, 5 min, RT), most of the SOC medium was removed, leaving approximately 200 μl in which the cells were resuspended then plated out on selective LB plates. Transformation plates were incubated at 37°C overnight, then stored at 4°C for no longer than 2 weeks.

Preparation of *E.coli* rubidium chloride competent cells

Cultures of Sure, DH5α or BL21 *E.coli* cells were inoculated from an overnight pre-culture and grown in PSi Broth at 37°C with shaking, to an OD_{550} of 0.48. Cultures were then chilled on ice for 15 min, then pelleted at 5000 rpm, 4°C for 5 min. All the following steps used ice cold buffers, tubes and pipettes. The pellet was resuspended in 0.4 volumes (here volume = original culture volume) of cold TfbI. Cells were pelleted as before and the pellet was resuspended in 0.04 volumes of cold TfbII. Cells were divided into aliquots in pre-cooled Eppendorf tubes, snap-frozen in liquid nitrogen and stored at -80°C.

Transformation of *E.coli* rhubidium chloride competent cells

One 100 μl aliquot per transformation, of Sure, DH5α or BL21 *E.coli* competent cells was thawed on ice. The thawed cells were mixed with the 10 μl ligation reaction or 0.5 μg plasmid DNA and left on ice for 30 min. The cells were then heat shocked at 42°C for 1 min (in a waterbath) and placed back on ice for 2 min, 1 ml of SOC medium was then added to the cells and they were recovered at 37°C for 1 hour. After recovery, 200 μl was plated out directly onto selective LB plates. The remaining cells were pelleted (5000 rpm, 5 min, RT), most of the SOC medium was removed, leaving approximately 200 μl in which the cells were resuspended then plated out on selective LB plates.

Transformation plates were incubated at 37°C overnight, then stored at 4°C for no longer than 2 weeks.

Mini and Midi preps

Mini preps

LB containing the appropriate antibiotic was used to make 1.5 ml aliquots, which were inoculated with single *E. coli* colonies from transformation plates and incubated at 37°C overnight with shaking. The cells were harvested and resuspended in 300 μl P1. P2 was added, 300 μl per sample, and mixed by inverting 5 to 10 times. Then 300 μl P3 was added and mixed in the same way. Samples were centrifuged at 14 000 rpm for 5 min and the supernatants containing the DNA were transferred into fresh tubes. To each sample 500 μl isopropanol was added and the DNA was precipitated by centrifugation (14 000 rpm, 10 min). The DNA pellet was washed with cold 70% ethanol, air-dried and resuspended in 20 μl ddH$_2$O. DNA was analysed by restriction enzyme digests and agarose gel electrophoresis. Strains containing the plasmid of interest were used to make glycerol stocks (1 ml *E. coli* culture mixed with 500 ml 50% glycerol). Glycerol stocks were stored at -80°C

Midi preps

The *E.coli* strain containing the plasmid of interest was used to inoculate 50 ml LB containing the appropriate antibiotic. Cultures were grown overnight at 37°C with shaking. The bacteria were harvested by centrifugation (5000 rpm, 15 min, 4°C) and the pellets were resuspended in 4 ml of P1. The cells were lysed by addition of 4 ml of P2, the samples were then mixed by inverting and incubated at room temperature for 5 min. 4 ml of P3 was added, the samples were mixed by inverting and chilled on ice for 15 min. During this time, one QIAGEN-tip 100 per sample was equilibrated by applying 4 ml of QBT and allowing the columns to empty by gravity flow. The lysates were cleared by filtration using QIAfilter Midi Cartridges and the liquid was applied to the columns. Once the columns had emptied they were washed twice with 10 ml QC. Plasmid DNA was eluted from the columns with 5 ml QF, the eluate was collected in glass centrifuge tubes. The DNA was precipitated by adding 3.5 ml isopropanol, mixing

and centrifuging at 11 000 rpm for 30 min at 4°C (glass tubes were placed in rubber adapters then centrifuged in a Sorvall SS-34 rotor). The DNA pellets were washed with 2 ml 70% ethanol, air-dried, resuspended in 200 µl ddH$_2$O and stored at -20°C.

Preparation of yeast competent cells

A single colony of the yeast strain of interest was used to inoculate a 10 ml YPD pre-culture which was grown overnight at 30°C (or 23°C for temperature sensitive strains) with shaking. The pre-culture was then used to inoculate 50 ml YPAD to an OD$_{600}$ of 0.15. This culture was grown to an OD$_{600}$ of 0.5 to 0.6, then harvested by centrifugation in a 50 ml falcon tube (all centrifugation steps: 3200 rpm, 5 min, RT, using a Heraeus centrifuge). The cells were washed with 20 ml sterile water and then 20 ml LiSorb. After all the residual LiSorb was removed the cell pellet was resuspended in 300 µl LiSorb and 30 µl carrier DNA was added (Salmon Sperm DNA from Gibco was heated at 95°C for 5 min then cooled on ice before adding to cells as carrier DNA). Cells were divided into 50 µl aliquots and stored at -80°C.

Transformation of yeast competent cells

Yeast competent cells were thawed at room temperature (one 50 µl aliquot per transformation). DNA (0.5 to 1 µg) was added to the cells followed by 300 µl LiPEG and brief vortexing to mix. The cells were incubated at room temperature with shaking. After 20 min, 35 ml DMSO was added to each transformation and mixed immediately by vortexing. Cells were heat shocked at 42°C, in a waterbath, for 20 min (5 min for temperature sensitive strains). Cells were pelleted by centrifugation (1500 rpm, 2 min, RT), resuspended in 200 µl selective medium and plated out on selective plates. Transformation plates were incubated at 30°C (23°C for temperature sensitive strains), then stored at 4°C for no longer than 1 month.

For transformation of PCR fragments, one 50 µl aliquot of competent cells was used per transformation. For plasmid DNA, as little as 10 µl of competent cells could be used per transformation and all volumes were altered accordingly.

For transformations that relied on kanamycin selection, a recovery time of at least 2 hours (in 2 ml YPD, 30°C with shaking) was needed before plating out cells.

Glycerol stocks were made of the strains of interest. To do this blocks of cells were grown on selective plates, then scraped off and resuspended in 15% glycerol and frozen on dry ice. Glycerol stocks were stored at -80°C.

Preparation of yeast mini DNA

To isolate plasmid DNA from yeast cells 1 ml of the overnight cultures of the yeast strains of interest was pelleted by centrifugation. The cell pellets were resuspended in 500 µl freshly prepared S buffer and incubated at 37°C for 30 min. 100 µl lysis solution was added to each, the samples were mixed by vortexing and incubated at 65°C for 30 min. 166 µl 3 M potassium acetate was added and the samples were chilled on ice for 10 min. The samples were centrifuged (14 000 rpm, 10 min, 4°C) and the DNA-containing supernatants were transferred into clean tubes. The DNA was precipitated by adding 800 µl cold 100% ethanol, chilling on ice for 10 min and centrifuging (as above). The DNA pellets were washed with 70% ethanol, air dried and resuspended in 40 µl sterile water. Yeast mini DNA (1 to 5 µl) was then transformed into *E.coli* by electroporation.

Preparation of yeast chromosomal DNA

Wild type (YPH499) yeast cells were grown to early stationary phase (OD_{600} of 1 to 2) in 30 ml YPAD at 30°C with shaking. Cells were harvested by centrifugation (5000 rpm, 5 min, 4°C, in a Sorvall SS-34 rotor, using polypropylene tubes with screw caps) and resuspended in 3 ml of 0.9 M sorbitol, 0.1 M EDTA, 50 mM DTT, pH 7.5. Cells were spheroplasted by adding 0.5 mg of zymolyase 50T (Seikagaku Corporation, Tokyo) and incubating at 37°C for 30 min with occasional shaking. Spheroplasting was followed using the light microscope. The spheroplasts were pelleted by centrifugation (5000 rpm, 5 min, 4°C), resuspended in 3 ml 50 mM Tris-HCl, 50 mM EDTA, pH 8.0 then mixed with 300 µl of 10% SDS and incubated at 65°C for 30 min. Then 1 ml of 5 M potassium acetate was added and the solution was chilled on ice for 1 hour. The sample was cleared by centrifugation (15 000 rpm, 30 min, 4°C) and the supernatant

was transferred to a clean glass tube and 4 ml cold 100% ethanol was added to precipitate the DNA and RNA. The nucleic acids were pelleted by centrifugation (10 000 rpm, 10 min, 4°C). Salt was removed from the pellet by a 5 min incubation with 4 ml of 50% ethanol and vortexing to break up the pellet. The sample was centrifuged again (10 000 rpm, 10 min, 4°C), the supernatant was removed and the pellet was dried under reduced pressure. The DNA and RNA was then dissolved by incubating in 3 ml of 10 mM Tris, 1 mM EDTA, pH 7.5, for 1 hour. RNA was removed from the sample by addition of 150 μl of 1 mg/ml RNase (Boehringer Mannheim) and incubating for 30 min at 37°C. The DNA was precipitated by adding 3 ml isopropanol and centrifugation (10 000 rpm, 10 min, 4°C). The DNA pellet was washed with 50% isopropanol and dried under reduced pressure. The DNA was then dissolved in 500 μl TE and stored at 4°C or -80°C.

Construction of strains expressing tagged proteins

Plasmids containing the 3HA, 3Myc, protein A or GFP coding regions and the kanamycin marker were used as templates for PCR. The tags with the marker were amplified by PCR using primers designed to contain sequences homologous to the 3' region of the gene of interest. The PCR products were excised from an agarose gel, then transformed into the strain of interest. The kanamycin marker was used to select for cells that had successfully integrated the tag into the genome via homologous recombination. The strains were checked by immunofluorescence and by preparing cell extracts which were analysed by SDS-PAGE followed by Western blotting. Glycerol stocks were made of the strains of interest.

Construction of the *SPC29* shuffle strain

Shuffle strains are yeast strains in which the chromosomal copy of the gene of interest has been replaced with a marker gene and a wild type copy of the gene of interest is present in the cell on a *URA3*-plasmid. First a diploid strain with one copy of *SPC29* replaced with a marker gene (*Δspc29::HIS3*) was constructed. This strain was then plated out on SPO plates, which induce sporulation due to lack of nutrients. Formation of spores was followed using the light microscope. When a cell sporulates it divides into four spores, these four spores are known as a tetrad. Tetrads (2 mm^3) were scraped

off the SPO plate and resuspended in 200 μl of sterile water. 3 μl of zymolyase (27 mg/ml) was added and the cells were incubated at room temperature for 10 min. The tetrads were separated into 4 single spores on YPD plates with the dissecting microscope. Only two of the four spores grew on YPD plates, but these two spores could not grow on SC-His plates, therefore they did not contain the *SPC29* disruption cassette. This shows that *SPC29* is essential (see Results). Therefore to construct the shuffle strain, the diploid strain containing the *SPC29* disruption cassette was transformed with *SPC29* on a *URA3*-plasmid (pRS316-*SPC29*). This strain was subjected to spore analysis as above. Colonies that evolved from a single spore and carried the deletion marker (*HIS3*) as well as the *URA3* marker were selected as these cells would be *spc29::HIS3* pRS316-*SPC29* (the *SPC29* shuffle strain).

Construction of temperature sensitive *SPC29* mutants

SPC29 was amplified using PCR under mutagenic conditions (in the presence of decreased dATP concentration and/or manganese chloride and Goldstar polymerase). The PCR products were cloned into pRS315 by homologous recombination. This was achieved by designing the primers (M1 and M2) so that the sequence at the ends of the PCR product overlapped with the vector pRS315. The PCR product was then co-transformed with pRS315 (restricted with *Sac*1/*Xho*1 then purified) into the *Δspc29::HIS3* pRS316-*SPC29* shuffle strain. Transformants were selected for on SC-Leu plates at 23°C. The *SPC29* shuffle strain contains a *HIS3* disruption cassette in the native *SPC29* locus, growth of the shuffle strain is dependent on the *URA3*-plasmid (pRS316) containing a wild type copy of *SPC29* (see Results). Therefore, the transformation plates were replica plated onto 5-FOA plates at 23°C to select for cells that could grow without the wild type *SPC29* (present on pRS316). The 5-FOA plates were replica plated onto SC-Leu, SC-Ura and SC-His plates at 23°C and YPD plates at 23°C and 37°C. Plasmid DNA (pSR315-*spc29*) was isolated from cells that were *LEU⁺ URA⁻, HIS⁺* (cells that still contained the *HIS* disruption cassette) and temperature sensitive for growth (grow at 23°C, but not 37°C). Plasmid DNA was amplified by transforming 3 μl of yeast mini DNA into DH5α *E.coli* cells by electroporation. Mini DNA was checked by restriction enzyme digests. Plasmids containing the potential temperature sensitive alleles of *SPC29* were then transformed again into the *Δspc29::HIS3* pRS316-*SPC29* shuffle strain and subjected to the same selection process as described above. The *spc29(ts)* alleles were then integrated into the yeast genome at

the native *SPC29* locus. This was achieved by restricting the mini DNA of plasmids containing *spc29(ts)* alleles (pSR315-*spc29*) with the enzymes *Kpn*1 and *Xba*1 to liberate the *spc29(ts)* alleles which were then co-transformed with pRS414 (contains a *TRP1* marker) into the *Δspc29::HIS3* pRS316-*SPC29* shuffle strain. Cells that had taken up DNA were selected for on SC-Trp plates. Cells were then replica plated onto 5-FOA (to select for cells that had lost the *URA3* plasmid containing wild type *SPC29*). The 5-FOA plates were then replica plated onto YPD plates at 23°C and 37°C and SC-His plates at 23°C to detect cells that had lost the *HIS3* cassette due to integration of *spc29(ts)* in the native *SPC29* locus. *HIS⁻* temperature sensitive colonies were streaked out (cells taken from the YPD plate at 23°C) on YPD at 23°C and 37°C to double check the temperature sensitive phenotype. Finally, the YPD (23°C) plates were replica plated on to YPD plates and SC-Trp plates to identify colonies that had lost the plasmid pRS414. Glycerol stock cultures were made of cells that were *URA⁻*, *HIS⁻*, *TRP⁻* and temperature sensitive. Temperature sensitive alleles *spc29-2*, *spc29-3* and *spc29-9* were chosen for further analysis. The temperature sensitive phenotype of *spc29-2*, *spc29-3* and *spc29-9* cells was rescued by pRS315-*SPC29*. The *spc29(ts)* alleles were sequenced, *spc29-2* and *spc29-3* were found to contain point mutations resulting in R161S and L158Q respectively and *spc29-9* carries multiple mutations.

Synthetic lethality

To investigate the function of *SPC29*, *spc29(ts)* alleles were tested for synthetic lethality with temperature sensitive alleles of other SPB components. Temperature sensitive strains containing *spc42-9*, *spc110-2*, *cmd1-1*, *spc97-14* or *spc98-1* alleles were made into *SPC29* shuffle strains. This was done by transforming the strains first with pRS316-*SPC29*, then with the *SPC29* disruption cassette (*HIS3Mx6* or *KanMx6* marker). The strains were checked for the appropriate markers and for growth on 5-FOA (a correct *SPC29* shuffle strain does not grow on 5-FOA). All work was done at 23°C as all strains were temperature sensitive. These shuffle strains were then transformed with *spc29-2* or *spc29-3* on a *LEU2*-plasmid (pRS315) or control plasmids, then grown on 5-FOA to test for cells that could still grow in the presence of the two temperature sensitive alleles (see Results).

The two hybrid system

SPC29, SPC42, SPC110 and *BBP1* were amplified by PCR and inserted into the two hybrid vectors pEG202 and pACT2 or pMM5 and pMM6. pEG202 and pMM5 contain a sequence encoding the LexA DNA-binding protein and a *HIS3* marker, pACT2 and pMM6 contain a sequence encoding the activation domain of *GAL4* and the *LEU2* marker. The plasmids were designed in such a way that cloning via the *Bam*H1 and *Xho*1 restriction sites produces gene fusions between LexA or *GAL4* and the gene of interest. The two plasmids were then co-transformed into the yeast strain SGY37VII which contains the reporter construct *lexA-operator-lacZ*. Transformants were selected for on SC-His-Leu plates. Interactions were detected by streaking transformants on to X-Gal plates or SC-His-Leu plates then overlaying with X-Gal top agar.

Overexpression experiments

Yeast strains were constructed that contained combinations of *SPC29, SPC42* and *SPC110* under the control of the *GAL1* promoter either on a plasmid (*GAL1-SPC110-HIS3*) or integrated into chromosomal markers (*GAL1-SPC29-URA3, GAL1-SPC42-LEU2*) using integration vectors. Cells were grown in selective media containing 2% raffinose instead of glucose. Overexpression via the *GAL1* promoter was achieved by transferring the cells into selective media containing 2% raffinose and 2% galactose instead of glucose and incubating at 30°C for up to 3 hours with shaking.

Sequencing

Sequencing of DNA was provided as a service by The Beatson Institute for Cancer Research.

Protein Techniques

Preparation of yeast cell lysates

To prepare whole cell lysates 1 to 3 OD_{600} were harvested from overnight cultures of the yeast strains of interest. The cell pellets were resuspended in 1 ml cold, sterile water, 150 µl of 1.85 M sodium hydroxide containing 7.5% β-mercaptoethanol (prepared immediately before use) was added per sample and mixed by vortexing. The samples were chilled on ice for 15 min, vortexed, and then 150 µl 55% TCA was added, the samples were again mixed by vortexing and chilled on ice for a further 10 min. The precipitated protein was pelleted by centrifugation (14 000 rpm, 10 min, 4°C). The supernatants were discarded, the samples were centrifuged briefly and the residual supernatant was removed. The protein pellets were resuspended in 300 µl HU buffer and analysed by SDS-PAGE followed by Western blotting.

SDS-PAGE

Protein samples were separated by SDS-PAGE and then analysed by Coomassie blue staining or Western blotting.

Mini gels

	Separating gels			Stacking gels
	10%	12%	15%	4%
30% Acrylamide (Severn Biotech Ltd.)	3.35 ml	4.0 ml	5 ml	650 µl
ddH$_2$O	4.05 ml	3.4 ml	2.35 ml	3.1 ml
1.5 M Tris, pH 8.8 (separating buffer)	2.5 ml	2.5 ml	2.5 ml	-
0.5 M Tris, pH 6.8 (stacking buffer)	-	-	-	1.25 ml
10% SDS	100 µl	100 µl	100 µl	50 µl
10% APS	50 µl	50 µl	50 µl	25 µl
TEMED (SIGMA)	5 µl	5 µl	5 µl	5 µl

Volumes given are sufficient for two mini gels made using SDS-PAGE apparatus from BioRad. Protein samples were loaded into the wells of the mini gel and the protein was separated by running the gel at 25 mA, 200 V for approximately 1 hour in Laemmli running buffer. Gels were then either subjected to Western blotting or stained with Coomassie blue stain (30 min, RT with gentle rocking) followed by destaining until the

background staining was reduced to an acceptable level (approximately three times 30 min incubations in destain at RT with gentle rocking). The gels were then dried using a gel dryer.

Protein molecular weight markers were from BioRad. These markers consist of myosin (200,000 Da), β-galactosidase (116,250 Da), phosphorylase B (97,400 Da), serum albumin (66,200 Da), ovalbumin (45,000 Da), carbonic anhydrase (31,000 Da), trypsin inhibitor (21,500 Da), lysozyme (14,400 Da) and aprotinin (6,500 Da).

Gradient gels

	Separating gel		Stacking gel
	8%	18%	4%
30% Acrylamide (Severn Biotech Ltd.)	8.1 ml	18 ml	1.95 ml
ddH$_2$O	14.1 ml	4.05 ml	9.3 ml
1.5 M Tris, pH 8.8 (separating buffer)	7.5 ml	7.5 ml	-
0.5 M Tris, pH 6.8 (stacking buffer)	-	-	3.75 ml
87 % Glycerol (ALDRICH)	-	1.7 ml	-
10% SDS	300 μl	300 μl	150 μl
10% APS	150 μl	150 μl	75 μl
TEMED (SIGMA)	22.5 μl	15 μl	15 μl

The above volumes are sufficient for two gradient gels made using gradient gel equipment from Biometra. Gels were run at 25 mA, 200 V per gel for up to 6 hours in Laemmli running buffer. Gradient gels were stained using the Fairbanks staining method as it is more sensitive than the Coomassie blue stain.

Fairbanks solutions: A) 25% isopropanol, 10%acetic acid, 0.05% Serva Blue G250

B) 10% isopropanol, 10% acetic acid, 0.005% Serva Blue G250

C) 10% acetic acid, 0.002% Serva Blue G250

D) 10% acetic acid

E) 7% acetic acid, 50% methanol

Gels were incubated as follows: 15 min in E, 2 hours in A, 1 hour in B, 1 hour in C, then destained in D.

Western blotting

For Western blot analysis polyacrylamide gels were blotted onto Protran nitrocellulose transfer membranes (Schleicher and Schuell) using blotting apparatus made at the Max-Planck Institute for Biochemistry, Martinsreid, Germany. After SDS-PAGE, the gels, membranes and 6 Whatman papers per gel were soaked in blotting buffer for 15 min prior to blotting. A blotting sandwich was then assembled using three pieces of Whatman paper followed by the membrane and the gel then another three pieces of Whatman paper. Gels were blotted for 1 to 1.5 hours at 100 mA, 5 V per gel. After blotting, membranes were stained with Ponceau S and the molecular weight markers were marked with a ball point pen.

After Western blotting, membranes were blocked with TBS/PBS, 10% powdered milk (Marvel). Membranes were then incubated with the appropriate primary antibody in TBS/PBS, 5% powdered milk. After extensive washing (5 times 5 min TBS or PBS) the membranes were incubated with the appropriate HRP-coupled secondary antibody (Dianova) in TBS/PBS, 5% powdered milk. Membranes were washed as before then antibody binding was visualised by enhanced chemiluminescence (ECL, Amersham Life Science) followed by exposure to FUJI medical X-ray film (SUPER RX). All incubations were carried out at room temperature for 1 to 2 hours with rocking or overnight at 4°C.

Immunofluorescence

For immunofluorescence, a pre-culture of the yeast strain of interest was used to inoculate 15 ml of YPAD (sterile filtered). Cells were grown to an OD_{600} of 0.3 to 0.6 then prepared for immunofluorescence in one of the following ways:

Preparation for Methanol/Acetone fixation only

Once the yeast culture had reached an OD_{600} of 0.3 to 0.6, 10 ml was harvested in a 15 ml falcon tube by centrifugation (3200 rpm, 3 min, RT using a Heraeus centrifuge). The cell pellet was resuspended in SP buffer and transferred to a 2 ml tube (Sorenson BioScience). The cells were spheroplasted by adding 80 µl glusulase (NEN), 22 µl zymolyase (Seikagaku Corporation, Tokyo) (25 mg/ml in ddH$_2$O) and 10 µl β-

mercaptoethanol (SIGMA) per sample and incubating at 30°C for up to 90 min (spheroplasting was followed using the light microscope). Glusulase and zymolyase were always centrifuged before use (14 000 rpm, 5 min, 4°C). Spheroplasts were pelleted by centrifugation (2500 rpm, 3 min, RT) then resuspended in Wickerman's solution and incubated at room temperature on a rotating wheel for 30 to 60 min. Spheroplasts were washed three times with SP buffer and finally resuspended in about 500 µl (depending on size of pellet) SP buffer.

Formaldehyde fixation

Alternatively, cells could be fixed with formaldehyde. Once the yeast culture had reached an OD_{600} of 0.3 to 0.6, 10 ml was transferred to a 15 ml falcon tube. Cells were fixed by addition of 1 ml KPi and 1 ml 39% formaldehyde (SIGMA). Fixation was carried out for 10 to 60 min at room temperature on a roller or on the bench with occasional gentle mixing. Fixed cells were pelleted by centrifugation (2200 rpm, 3 min, RT, using a Heraeus centrifuge). The cells were resuspended in 1.5 ml 40 mM KPi and transferred to a 2 ml tube. Cells were washed twice with SP buffer (2200 rpm, 3 min, RT) and finally resuspended in 1 ml SP buffer. Cells were spheroplasted by adding 10 µl zymolyase (25 mg/ml) and 3 µl β-mercaptoethanol per sample and incubating for 15 to 30 min at room temperature. Spheroplasting was checked using the light microscope. Spheroplasts were washed three times with SP buffer and then resuspended in about 500 µl (depending on size of pellet) SP buffer.

Fluorescence labelling

During incubations or fixation, the 12 well (7 mm) slides (Polyscience) were prepared, 10 µl of 1% polylysine (SIGMA) was placed on each well and left at room temperature for 10 min, wells were then washed three times with ddH_2O. 15 µl of the spheroblast suspension was placed on each well and left at room temperature for 10 min. Excess cells were then carefully removed by aspiration. Slides were gently immersed first in methanol (-20°C) for five minutes, then in acetone (-20°C), for 30 seconds and air-dried at room temperature. From this point the slides were kept in a moist chamber. The cells were blocked with PBS-B (20 µl per well) for 30 min at room temperature. During this time the appropriate primary antibodies were diluted in PBS-B. The cells were

incubated with the primary antibodies (20 μl per well), after removal of the blocking buffer by aspiration, for 1 hour at room temperature or overnight at 4°C. Wells were washed 3 to 5 times with PBS-B (5 min incubations, 40 μl per well). The primary antibodies were detected by incubation for 1 to 2 hours with the appropriate fluorescently labelled secondary antibodies (Dianova) (diluted in PBS-B, 20 μl per well). Cells were washed twice with PBS-B and 3 to 5 times with PBS (5 min incubations, 40 μl per well). Slides were then air-dried and 4 μl DAPI-mounting solution was placed in the centre of each well. A clean coverslip (24 mm by 60 mm) was fitted on each slide and sealed with nail varnish.

Cells were viewed using the appropriate fluorescent filters and images were saved using the NIH Image programme.

Preparation of anti-Spc29p antibodies

Preparation of antigen

GST-Spc29p was expressed in BL21 *E.coli*. The BL21 cells containing the pGEX-5X expression plasmid of interest was used to inoculate an over night pre-culture of LB medium containing ampicillin (0.1 mg/ml). The pre-culture was diluted 1:50 using 400 ml LB medium containing ampicillin. The bacteria were grown for 4 hours at 30°C with shaking. Expression of fusion proteins was induced by the addition of IPTG to the cultures (final concentration of IPTG was 1 mM) and further incubation at 30°C for 3 hours with shaking. The cells were harvested (6000 rpm, 5 min, 4°C), the cell pellet was resuspended in 20 ml PBS containing the protease inhibitors PMSF (1 mM) and benzamidine (5 mM) and chilled on ice.

The cells were lysed by ultra sonication (2 times 4 min, 50% output, sonicator from Bandelin electronic, Berlin). During lysis cells were kept on ice. Lysis was followed using the light microscope and sonication was repeated if necessary. Protease inhibitors were added as before. When lysis was complete, the lysate containing the GST-Spc29p fusion protein was adjusted to 1% Triton X-100 and incubated on ice for 30 min with occasional mixing. The lysate was centrifuged (12 000 rpm, 5 min, 4°C, Sorvall SS-34 rotor) and the supernatant was incubated with 1 ml pre-washed (2 times PBS, 2000 rpm, 3 min) glutathione sepharose for 2 hours at 4°C on a roller. The sepharose was then washed twice with PBS, 1% Triton X-100 and loaded onto a column (Mini columns

from Pierce) The following steps were carried out at room temperature. The column was washed twice with PBS and the GST-Spc29p fusion protein was eluted with 10 times 1 ml 50 mM Tris-HCl (pH 8.0), 10 mM glutathione. Each 1 ml was incubated in the column for 10 min before elution. Each fraction was tested for protein with Bradford Reagent (100 μl Bradford Reagent (SIGMA) was mixed with 10 μl of the fraction, blue colour indicates presence of protein). Fractions were also analysed by SDS-PAGE followed by Coomassie blue staining. Fractions containing GST-Spc29p were pooled and stored at -20°C.

Rabbit immunisation protocol

For the first immunisation, 1 ml of the pooled fractions was mixed with 1 ml Freud's complete adjuvant (SIGMA) and, after a pre-bleed, the mixture was injected subcutaneously into the rabbit. The rabbit was boosted 3 times at 4 week intervals with 1 ml of the pooled fractions mixed with 1 ml Freud's incomplete adjuvant. A test bleed was taken and tested between boosts 2 and 3, the final bleed was taken 10 days after the third boost.

The test bleed and final bleed were incubated at 37°C for 1 hour, then overnight at 4°C. The serum was separated by centrifugation (3000 rpm, 5 min, RT) and carefully pipetted into a clean falcon tube. Serum was stored at -20°C. The serum was tested for antibodies by using it to probe a Western blot containing cell extracts of wild type cells, cells expressing tagged *SPC29* and cells overexpressing *SPC29*.

Affinity purification of anti-Spc29p antibodies

Anti-Spc29p antibodies were affinity purified from the serum of the final bleed. This was achieved by running the serum through a GST pre-column to remove anti-GST antibodies. The eluate from the pre-column runs directly into the main column containing GST-Spc29p, to which the anti-Spc29p antibodies bind. The anti-Spc29p antibodies were then eluted from the main column, dialysed against PBS and stored at -80°C.

Preparation of GST and GST-Spc29p protein

For the pre-column, a 200 ml culture of BL21 *E.coli* cells containing the plasmid pGEX-5X were induced (as above) to express GST. The cells were harvested (6000 rpm, 5 min, 4°C) and the pellet was resuspended in 20 ml CB buffer. The cells were lysed by sonication (as above). For the main column GST-Spc29p was freshly purified as above. The GST-containing lysate and the pooled fractions of GST-Spc29p were transferred into dialysis tubing (SIGMA) which had been boiled in distilled water for 10 min, and dialysed against CB buffer (3 times 1.5 litres at 4°C, one incubation can be carried out over night).

Preparation of CNBr-sepharose

4 g of CNBr-sepharose freeze-dried powder (Pharmacia) was swelled with 500 ml 1 mM HCl then transferred to a glass filter funnel and the liquid was removed by applying the vacuum. The resin was washed in a further 500 ml 1 mM HCl then removed from the filter (resin was removed by resuspending in 5 ml of 1 mM HCl then transferring into a falcon tube, this was repeated twice to give a 15 ml sepharose suspension). The resin was divided into four (for 2 pre-columns and 2 main columns) 15 ml falcons and centrifuged (3200 rpm, 2 min, 4°C), HCl was removed and the resin was washed with 10 volumes CB buffer.

Coupling

The dialysed samples were added to the CNBr-sepharose immediately after the CB buffer was removed from the sepharose (sample volume:sepharose volume was 2:1). The coupling reactions were incubated at room temperature for 2 hours on a roller. Coupled samples were centrifuged (3200 rpm, 2 min, RT) and the supernatant was removed by aspiration. The samples were blocked by incubating with 0.2 M glycine, pH 8.0, overnight at 4°C.

Preparation of affinity columns

Columns from Pierce were assembled (filled with PBS and fitted with a filter at the bottom then left to empty by gravity flow). The blocked sepharose was added to the columns (2 pre-columns and 2 main columns). The columns were washed with:

1) volumes 100 mM sodium acetate, pH 4.0, 0.5 M NaCl
2) 2 volumes PBS
3) 2 volumes PBS, 1% SDS, when the second volume had run into the column, the column was closed and incubated at 65°C for 40 min.
4) 1 volume PBS, 1% SDS
5) Main column: 2 volumes PBS, 1% Triton X-100. Pre-column: 4 volumes PBS, 1% Triton X-100.
6) Main column only: 2 volumes PBS, 1% Triton X-100, 1% BSA

Purification of antibodies

The pre-column was placed over the main column (using a clamp stand). The centrifuged serum (5 ml, 14 000 rpm, 15 min, 4°C) was added to the pre-column and allowed to run through the pre-column and into the main column. Once all the serum had run into the pre-column, 2 volumes PBS were added and allowed to run through into the main column. The pre-column was then removed and the main column was washed with 3 volumes PBS, 1% Triton X-100, followed by 4 volumes PBS. Before all the PBS had run through, the column was closed and stored at 4°C for 30 min. The anti-Spc29p antibodies were eluted with 8 times 1 ml cold glycine buffer (0.2 M glycine, 1 mM EGTA, pH 2.5). The eluate was collected in 1.5 ml Eppendorf tubes containing 300 µl 1 M Tris-HCl, pH 8.0, to neutralise the glycine buffer. The eluate was mixed immediately by vortexing and stored on ice. Then 5 times 1 ml guanidium-HCl, pH 7.0, was added to the column and the eluates were collected in Eppendorf tubes and stored on ice. The Bradford spot test was used to test all the fractions for protein. The protein containing fractions were pooled (glycine fractions were kept separate from guanidiun fractions) and adjusted to 0.2% BSA. The pooled fractions were then dialysed twice against PBS at 4°C. The affinity purified antibodies were divided into aliquots and stored at -80°C.

Coupling of antibodies to Protein A beads

For immunopurification experiments, the appropriate antibodies were first coupled to Protein A Sepharose Fast Flow (Amersham Pharmacia Biotech Inc.) or Protein A Dynabeads (DYNAL).

Coupling of antibodies to Protein A Sepharose

This was achieved by incubating 2 ml of the washed Protein A Sepharose beads (beads were washed twice with PBS) with affinity purified antibodies diluted in 5 ml PBS overnight at 4°C on a roller. The beads were then washed twice with 20 ml of 0.2 M sodium borate (pH 9.0) by centrifugation (2000 rpm, 5 min, RT, using a Heraeus centrifuge). The beads were resuspended in 20 ml of 0.2 M sodium borate and 20 mM of the crosslinker DMP (104 mg solid DMP) was added. The beads were incubated for 30 min, at room temperature, on a roller. The reaction was stopped by washing the beads with 0.2 M ethanolamine (pH 8.0) and then incubating for 2 hours, at room temperature on a roller in 0.2 M ethanolamine. Finally the antibody-coupled beads were washed twice with PBS and stored in PBS, in the presence of sodium azide, at 4°C. Before use, beads were washed twice with PBS and once with IP buffer (low salt).

Coupling of antibodies to Protein A Dynabeads

The magnetic Protein A Dynabeads were resuspended in the vial and 1 ml was transferred into an Eppendorf tube. The beads were washed twice with 1 ml NaPi buffer. Dynabeads in small volumes (<1.5 ml) were pelleted using the Dynal MPC (magnetic particle concentrator). Beads were then resuspended in 1 ml of affinity purified antibodies diluted in PBS. The beads were incubated with the antibodies for 1 hour at room temperature on a roller. The beads were then washed twice with NaPi buffer and twice with Tris buffer (0.2 M Tris-HCl, pH 9.0). The beads were resuspended in 10 ml Tris buffer and transferred to a falcon tube. 20 mM of the crosslinker DMP (52 mg solid DMP) was added and the beads were incubated at room temperature, for 45 min on a roller. The beads were pelleted by centrifugation (2000 rpm, 2 min, RT). The beads were then resuspended in 0.2 M ethanolamine, pH 9.0 and incubated for 2 hours at room temperature. Beads were again pelleted by centrifugation, resuspended in 1 ml PBS and transferred to an Eppendorf tube. Beads were then washed

twice with PBS and stored in PBS in the presence of sodium azide, at 4°C. Before use, beads were washed twice with PBS and once with IP buffer (low salt).

Immunopurification

A pre-culture of the yeast strain of interest was used to inoculate 200 ml of YPAD. The cells were grown to an OD_{600} of 0.5 to 1.0 and then harvested. The cell pellet was washed once with sterile water in a 50 ml falcon tube. From this point on all steps were carried out on ice and all buffers contained protease inhibitors. The cell pellet (~1 gram) was resuspended in 1 to 2 ml of IP buffer (low salt). Cells were lysed by adding glass beads (SIGMA) and vortexing. Glass beads were added until ~3 mm of buffer was visible above the level of the beads, samples were vortexed 10 to 20 times for 30 seconds with the cells chilling on ice for 30 to 60 seconds between vortexing. Lysis was followed using the light microscope. After lysis, the solution was altered to 0.2% Triton X-100 and the lysed cells were left on ice for 20 min. The lysed cells were then transferred into 1.5 ml Eppendorf tubes and centrifuged at 12 000 rpm for 20 min at 4°C. The supernatant containing the soluble cellular fraction was removed, the pellets were resuspended in 500 μl of IP buffer (high salt), 1% Triton X-100 and incubated for 40 min, at 4°C on a rotating wheel. After this time 1 ml of IP buffer was added and the samples were centrifuged at 10 000 rpm for 15 min at 4°C. The supernatant was removed and incubated with ~50 μl antibody-coupled sepharose beads or ~15 μl antibody-coupled Dynabeads, for 2 to 4 hours at 4°C on a rotating wheel. The beads were then washed 5 times with IP buffer (low salt). The bound protein was eluted from the beads by adding 100 μl HU buffer, incubating at 65°C for 5 to 10 min, then centrifuging (14 000 rpm, 5 min, RT). Samples were analysed by SDS-PAGE, followed by Coomassie blue staining or Western blotting.

For purification of ProA-Spc110p, sepharose coupled to rabbit IgGs (Dianova) was used and the purification was carried out on a larger scale. This was done by growing large cultures of yeast cells in YPAD using Microferm fermentors (New Brunswick Scientific Co. Inc.) The cells were harvested using the continuous centrifuge then washed once with ddH$_2$O (3200 rpm, 5 min, 4°C). The cell pellet was then resuspended in approximately 10 ml ddH$_2$O to make a thick paste that was then dripped in to liquid nitrogen, the frozen cells were stored at -80°C. For the ProA-Spc110p purification 60 g of *ProA-SPC110* cells and 60 g of wild type cells was used. All buffers contained

protease inhibitors. The frozen cells were mixed with 70 ml of TBS and glass beads. The cells were lysed using a bead beater (Biospec Products) (10 times 1 min, on ice, lysis was followed by light microscopy). The lysed cells were adjusted to a volume of 150 ml with TBS and Triton X-100 was added to a final concentration of 1%. The lysed cells were then left on ice for 20 min. The cell lysates were then transferred to centrifuge tubes and centrifuged at 12 000 rpm for 20 min at 4°C (Sorvall rotor and centrifuge). The supernatants were removed and the pellets were slowly solubilised in IP buffer (high salt), 1% Triton X-100, to a final volume of 130 ml, then stirred on ice for 40 min. To the solubilised pellet was added 260 ml IP buffer, 1% Triton X-100. The samples were then centrifuged (9000 rpm, 15 min, 4°C). During this time the columns (Pierce) were prepared, 1 ml of IgG-sepharose was added to each column. The columns were washed with 2 ml TST buffer, 2 ml 0.5 M acetic acid pH 3.4, then again with 2 ml TST buffer, then 2 ml 0.5 M acetic acid pH 3.4 and finally with 5 ml TST buffer. The following steps were carried out in the cold room. The supernatants from the last centrifugation step were added to the columns (one for wild type and one for *ProA-SPC110*) and allowed to run through. The columns were then washed with:

1) 20 ml IP buffer (low salt), 1% Triton X-100
2) 5 ml IP buffer (0.5 M KCl), 1% Triton X-100
3) 10 ml IP buffer (0.5 M NaCl), 1% Triton X-100
4) 20 ml TST buffer
5) 5 ml 5 mM ammonium acetate pH 6.5
6) 5 ml 5 mM ammonium acetate pH 5.5
7) 5 ml 5 mM ammonium acetate pH 5

The bound protein was eluted from the columns with 4.5 ml 0.5 M acetic acid pH 3.4. The eluate from each column was collected in a falcon tube, then divided into 800 μl aliquots in Eppendorf tubes. The samples were lyophilised using a speed vac (Bachhofer Vacuum Concentrator) then washed with distilled water. The samples were finally resuspended in 100 μl HU buffer and stored at -20°C. Samples were analysed using 8-18% SDS-PAGE gradient gels, followed by staining using the Fairbanks staining method or Western blotting.

Preparation of yeast cells for electron microscopy

Preparation of samples

Cells of the yeast strains of interest were grown to an OD_{600} of 0.2 to 0.6. 15 ml of these cultures were harvested and all residual medium was removed. The cells were gently resuspended in Phosphate-Mg buffer, 2% glutaraldehyde (TAAB) and incubated at room temperature for 30 min with occasional inverting. Cells were washed with Phosphate-citrate buffer and transferred into a 2 ml tube. Cells were washed with pre-treatment buffer, followed by a 5 min incubation at room temperature in pre-treatment buffer. The cells were then washed twice with Phosphate-citrate buffer. Spheroplasting was achieved by resuspending the cells in 900 µl Phosphate-citrate buffer, 100 µl glusulase and incubating overnight at 30°C with shaking, if samples were prepared early in the day, an additional 50 µl glusulase was added in the evening, or the following morning (glusulase was always centrifuged before use, 14 000 rpm, 5 min, 4°C) Spheroplasting was followed using the light microscope. After spheroplasting, the cells were washed twice with 0.1 M sodium acetate, (pH 6.1). Cells were then incubated with osmium tetroxide (SIGMA) and then with uranyl acetate (Agar Scientific), both are toxic, therefore safety precautions were used and both were handled in the fume hood. 4% osmium tetroxide was diluted 50:50 with 0.1 M sodium acetate and the cells were resuspended in 500 µl of the mix, then incubated for 15 min at room temperature, before washing twice with ddH_2O. Cells were then incubated with 1 ml 1% aqueous uranyl acetate for 1 hour at room temperature in the dark then washed twice with ddH_2O. Cells were dehydrated by incubating twice with 95% ethanol, followed by two incubations with 100% ethanol (5-15 min incubations). Spurr resin (Agar Scientific) was used to embed the cells, this was done by resuspending the dehydrated cells in 200 µl Spurr resin and incubating for 1 hour at room temperature, incubation was then repeated with fresh Spurr resin. Cells were pelleted and resuspended again in fresh Spurr resin, then divided into two BEEM capsules (Polysciences, Inc.), before pelleting using a horizontal centrifuge (Beckman Microfuge 11) at 10 000 rpm for 15 min. The BEEM capsules were topped up with Spurr resin and an identification label was inserted into the top of the resin. The capsules were then incubated overnight at 65°C to harden.

Sectioning of samples

Samples were cut into serial sections (70 nm) using a diamond knife (TAAB) and mounted on formvar (TAAB) coated grids. This was provided as a service by the technical support team at The Beatson Institute of Cancer Research.

Contrasting of samples

Samples were contrasted first by submerging the grids in uranyl acetate (2% uranyl acetate in 70% methanol) for 5 min in the dark. Grids were washed 10 times with 70% methanol using a dimpled porcelain dish, each dimple was filled with 70% methanol and the grids were transferred through all the dimples before being left to air-dry on Whatman paper. The grids were then floated, sample side down, on a drop of lead citrate stain in a sodium hydroxide atmosphere for 5 min. This was achieved by using a petri dish with a circle of parafilm in the middle, the parafilm was surrounded with sodium hydroxide pellets to scavenge carbon dioxide thereby preventing precipitation of lead salts. The inside of the lid of the petri dish was covered with a round piece of Whatman paper soaked in 5 M sodium hydroxide. Therefore, when the lead citrate drops were placed on the parafilm and the grids were floated on the drops, the lid was placed on the dish creating a chamber containing a sodium hydroxide atmosphere. The grids were then transferred onto a drop of ddH$_2$O then washed individually by holding the grid in forceps over a beaker and allowing a fine stream of ddH$_2$O to gently flow down the forceps and over the grid. The grids were then air-dried and stored in a grid holder.

Samples were visualised using the electron microscope (Zeiss 902) at Glasgow University.

FACS analysis

Cells were prepared for FACS analysis as follows. For FACS analysis cells were grown to an OD$_{600}$ of 0.2 to 2.0 and 5 ml of the yeast culture was used for each sample. Cells were pelleted in 15 ml falcon tubes by centrifugation (3200 rpm, 5 min, RT using a Heraeus centrifuge) then resuspended in 70% ethanol and left on a rotating wheel overnight at room temperature. The cells were then washed twice with 1 ml Tris buffer

(50 mM Tris-HCl, pH 7.8) and transferred to a 2 ml tube. To the cell pellet was added 800 μl Tris buffer and 200 μl of 5 mg/ml RNaseA (SIGMA) followed by incubation at 37°C overnight. Cells were pelleted and resuspended in 500 μl of freshly prepared 5 mg/ml pepsin (Roche) in 55 mM HCl, then incubated in a 37°C water bath for 30 min. Cells were washed in TNM buffer (200 mM Tris-HCl pH 7.5, 211 mM NaCl, 78 mM MgCl$_2$), resuspended in 500 μl TNM buffer and 55 μl of 500 μg/ml propidium iodide (SIGMA) in water was added. To read the samples on the FACS machine (FACScan from Becton and Dickinson) the samples were first sonicated for 30 sec in a water bath sonicator (Transsonic T420 from Elma), then 50 μl was transferred into a FACS tube (Falcon 2052) containing 2 ml Tris buffer. 20 000 events were read per sample. Files were processed using the Cell quest programme.

In vitro labelling of Spc29p with [^{35}S]

In vitro labelling of Spc29p with [^{35}S] was achieved using the TNT Coupled Reticulocyte Lysate System (Promega). The following components were added in the order given.

1. TNT buffer	2 μl
2. DEPC H$_2$O	16 μl
3. Amino acid mix (minus methionine), 1 mM	1 μl
4. RNasin Ribonuclease inhibitor, 40 U/μl	1 μl
5. TNT Rabbit Reticulocyte Lysate	25 μl
6. TNT T7 RNA Polymerase	1 μl
7. pET28c based DNA template (0.5 μg/μl)	2 μl
8. [^{35}S]methionine (>1 000 Ci/mmol at 10 mCi/ml)	2 μl
Total	50 μl

The translation reaction was incubated in a waterbath at 30°C for 3 hours. 1 μl of each reaction was taken for analysis by SDS-PAGE. Control reactions, containing an empty plasmid, were carried out in parallel.

In vitro binding assay

Expression of GST and Histidine fusion proteins in E.coli

Fusion proteins were expressed in BL21 bacteria. The bacteria of interest were used to inoculate over night pre-cultures of LB medium containing the appropriate antibiotics. The pre-cultures were diluted 1:50 using 200 ml LB medium containing the appropriate antibiotics. The bacteria were grown for 4 hour at 30°C with shaking. Expression of fusion proteins was induced by addition of IPTG to the cultures (final concentration of IPTG was 1 mM) and further incubation at 30°C for 3 hours with shaking. The cells were harvested (6000 rpm, 5 min, 4°C) and the cell pellet was resuspended in 5 ml PBS and stored at -20°C.

Lysis

The bacterial cell pellets were thawed and a further 15 ml PBS was added along with the protease inhibitors PMSF (to a final concentration of 1 mM) and benzamidine (to a final concentration of 5 mM). The cells were lysed by ultra sonication (3 times 1 min, 50% output, sonicator from Bandelin electronic, Berlin). During lysis cells were kept on ice, all further steps were also carried out on ice. Lysis was followed using the light microscope and sonication was repeated when necessary. Protease inhibitors were added as before. When lysis was complete, the lysate containing GST/GST-Bbp1p protein was adjusted to 1% Triton X-100 and incubated on ice for 15 min with occasional mixing. The lysate containing the His-Mps2p fusion protein was adjusted to 0.1% Triton X-100 and incubated on ice for 1 to 2 hours with occasional mixing.

Preparation of glutathione sepharose

500 μl of glutathione sepharose (Amersham Pharmacia Biotech Inc.) per binding assay was washed with 5 volumes PBS followed by three times 5 volumes PBS, 1% Triton X-100 (2000 rpm, 3 min, 4°C).

Binding assay

After 15 min on ice 1.5 ml (per binding assay) of the GST containing lysates were transferred into Eppendorf tubes and centrifuged (14 000 rpm, 5 min, 4°C). 1 ml of the supernatant was mixed with an aliquot of washed glutathione sepharose. Then 2 mM Mg^{2+} (1 mM $MgCl_2$, 1 mM $MgSO_4$) and 2 mM ATP was added and GST/GST-Bbp1p was allowed to bind to the glutathione sepharose by incubating at 4°C for 45 min on a roller. The sepharose was washed once with PBS, 0.1% Triton X-100, followed by three times with PBS (2000 rpm, 3 min, 4°C). The lysate containing the His-Mps2p was prepared for the binding assay (1.5 ml, per binding assay, was transferred into Eppendorf tubes and centrifuged, 14 000 rpm, 5 min, 4°C). The supernatant (1 ml) was then mixed with washed glutathione sepharose (+/- GST/GST-Bbp1p) and incubated for 1 hour at 4°C on a roller (Mg^{2+} and ATP were added as before). The sepharose was washed (once with PBS, 0.1% Triton X-100, followed by three times with PBS) then incubated with *in vitro* translated [^{35}S]-Spc29p for 1 hour at 4°C on a roller (Mg^{2+} and ATP were added as before). The sepharose was then washed twice with PBS, 0.1% Triton X-100 and transferred into spin columns (Bio101). The sepharose was then washed a further two times with PBS, 0.1% Triton X-100 and three times with PBS (10 000 rpm, 3 min, 4°C). Spin columns were transferred to clean Eppendorf tubes and the proteins were eluted from the sepharose by adding 100 μl HU buffer, heating at 65°C for 5 min, then centrifuging (14 000 rpm, 5 min, RT). The eluted proteins were analysed by SDS-PAGE and Western blotting or autoradiography.

Alkaline phosphatase treatment

The yeast cells of interest were lysed using glass beads. Spc29p was then purified from the soluble cellular fraction and the extracted pellet using ~60 μl Protein A sepharose coupled to anti-Spc29p antibodies. The purification was taken to the last wash, but the protein was not eluted, instead the sepharose was washed twice with AP buffer and divided into three tubes. The beads were then resuspended in an equal volume of AP buffer, 0.4% SDS (20 μl beads plus 20 μl buffer) and incubated at 65°C for 3 min. The tubes were cooled, and the following was added;

tube 1) 10 U alkaline phosphatase (Roche)

tube 2) 10 U alkaline phosphatase, 100 mM 3GP (SIGMA)

tube 3) AP buffer only.

The tubes were incubated at 30°C for 30 min. The reaction was stopped by adding an equal volume of HU buffer (40 μl), and incubating at 65°C for 15 min. Samples were centrifuged (14 000 rpm, 5 min, RT) and analysed by SDS-PAGE and Western blotting.

RESULTS

Identification of Spc29p

Purification of the ProA-Spc110p subcomplex

At the inner plaque of the SPB, the γ-tubulin-complex binding protein is Spc110p. Spc110p is an important structural component of the SPB and therefore worth further investigation. In an attempt to find novel Spc110p-interacting proteins a protein A-tagged Spc110p (ProA-Spc110p) was purified from salt extracted SPBs using an IgG-sepharose column to which protein A binds with high affinity (Figure 9). The purified proteins were then run on an SDS-PAGE gradient gel which was then stained using the Fairbanks method (Figure 9A). This allowed identification of seven bands specifically present in the ProA-Spc110p purification. Bands 1-4 were derived from ProA-Spc110p, whereas band 5 was identified as Spc42p by immunoblotting. Bands 6 and 7 were sent to A. Shevchenko at the EMBL in Heidelberg for identification by MALDI peptide mapping. Band 7 was identified as calmodulin, which is known to bind to the C-terminus of Spc110p (Geiser *et al.*, 1993; Spang *et al.*, 1996b; Stirling *et al.*, 1994). Band 6 was a previously uncharacterised protein that was then named Spc29p. There were no components of the γ-tubulin complex detected in the Spc110p-complex, this is probably due to the harsh conditions used in the purification.

To confirm the presence of Spc29p in the Spc110p-complex, a strain was constructed that expressed *SPC29-3HA* and *ProA-SPC110*. The ProA-Spc110p purification was then repeated using the strain containing Spc29p-3HA (Figure 9B). The precipitate from this purification and the control purification (*SPC110 SPC29-3HA*) were probed for the presence of numerous SPB components. Positive results were seen for ProA-Spc110p, Cnm67p (a component of the outer plaque), Spc42p and Spc29p-3HA. Only small amounts of Cnm67p were detected in the Spc110p-ProA purification, this was due to the interaction of Cnm67p with Spc42p as Cnm67p was later shown to be an outer plaque component that interacts with Spc42p (Elliott *et al.*, 1999).

The results of these purifications show the identification of a salt stable SPB sub complex containing Spc110p, calmodulin, Spc42p, low amounts of Cnm67p and the novel SPB component Spc29p.
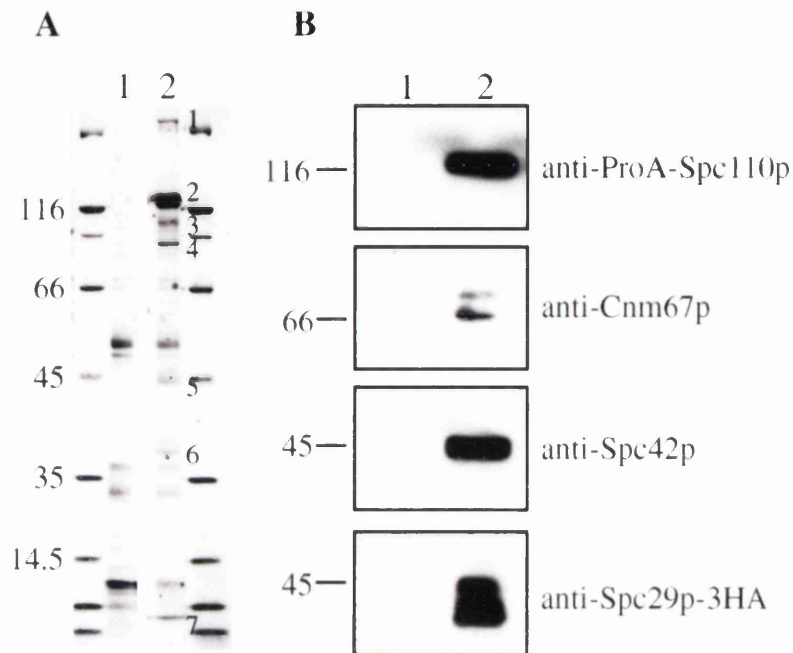
Figure 9 Spc29p is a component of the Spc110p subcomplex

A   An Spc110p-containing SPB subcomplex was purified from cells with a protein A tagged Spc110p, ProA-Spc110p (lane 2), using IgG sepharose. Wild type cells, containing Spc110p without the ProA-tag (lane 1), were treated identically to identify proteins that bound non-specifically to the IgG resin. The protein bands present only in the ProA-Spc110p lane were identified by immunoblotting or matrix-assisted laser desorption ionisation (MALDI) peptide mapping. The major proteins in lane 2 were encoded by *SPC110* (band 1, ProA-Spc110p homodimer; band 2, ProA-Spc110p; bands 3 and 4, ProA-Spc110p degradation products), *SPC42* (band 5), *SPC29* (band 6) and *CMD1* (band 7).

B   Co-immunoprecipitation of Spc29p and Spc110p. High salt extracts from cells expressing *SPC110 SPC29-3HA* (lane 1) or *ProA-SPC110 SPC29-3HA* (lane 2) were incubated with IgG sepharose. The immunoblots were probed with the indicated antibodies.

Spc29p is essential

When beginning work on a novel protein it is important to know if that protein is essential for viability. To determine whether Spc29p is essential, a wild type diploid strain was transformed with a *HIS3Mx6* disruption cassette that was designed specifically to integrate into the native *SPC29* locus. The *HIS⁺* colonies were sporulated and the resulting tetrads were dissected and grown on YPD plates (Figure 10A). Each tetrad contained two viable and two non-viable spores. The viable spores were unable to grow on SC-His plates, therefore they did not contain the *HIS3Mx6* disruption cassette. This is a strong indication that *SPC29* encodes an essential protein. However, this result does not discount the possibility that the defect is only in sporulation, therefore a shuffle strain was made. To make an *SPC29* shuffle strain the diploid strain containing a *HIS3* cassette in one of the *SPC29* loci was transformed with a *URA3*-based plasmid containing a wild type copy of *SPC29*. The resulting strain was sporulated and tested for growth on selection plates, all *HIS⁺* spores were also *URA⁺* again indicating that *SPC29* is essential. One of the *HIS⁺ URA⁺* spores was taken for use as a shuffle strain (*Δspc29::HIS3Mx6* pRS316-*SPC29*). If *SPC29* is essential, viability of the shuffle strain should depend on the presence of the plasmid. To test this the shuffle strain was grown on 5-FOA plates. 5-FOA is lethal to cells containing a *URA3* marker, therefore only cells that can grow without the *URA3*-based plasmid will grow on 5-FOA plates. The *SPC29* shuffle strain was unable to grow on 5-FOA, however when the shuffle strain was transformed with *SPC29* on a *LEU2*-based plasmid, growth on 5-FOA was possible (Figure 10B). Therefore Spc29p is an essential SPB component.

Localisation of Spc29p

Co-purification of Spc29p with Spc110p strongly suggests that Spc29p is an SPB component. To confirm this notion, the localisation of Spc29p was established. This was done by tagging the single chromosomal copy of *SPC29* in wild type haploid cells with *GFP* and the *3Myc* epitope. When observed by fluorescence microscopy Spc29p-GFP exhibited typical SPB behaviour throughout the cell cycle (Figure 11A). In G1 cells there is no bud and a single GFP signal in the form of a dot. As the cell moves through the cell cycle, the bud appears and begins to grow as the GFP signal becomes two dots positioned close to each other. As the bud grows the two GFP signals move apart and one GFP dot moves into the bud, therefore when cytokinesis occurs each cell
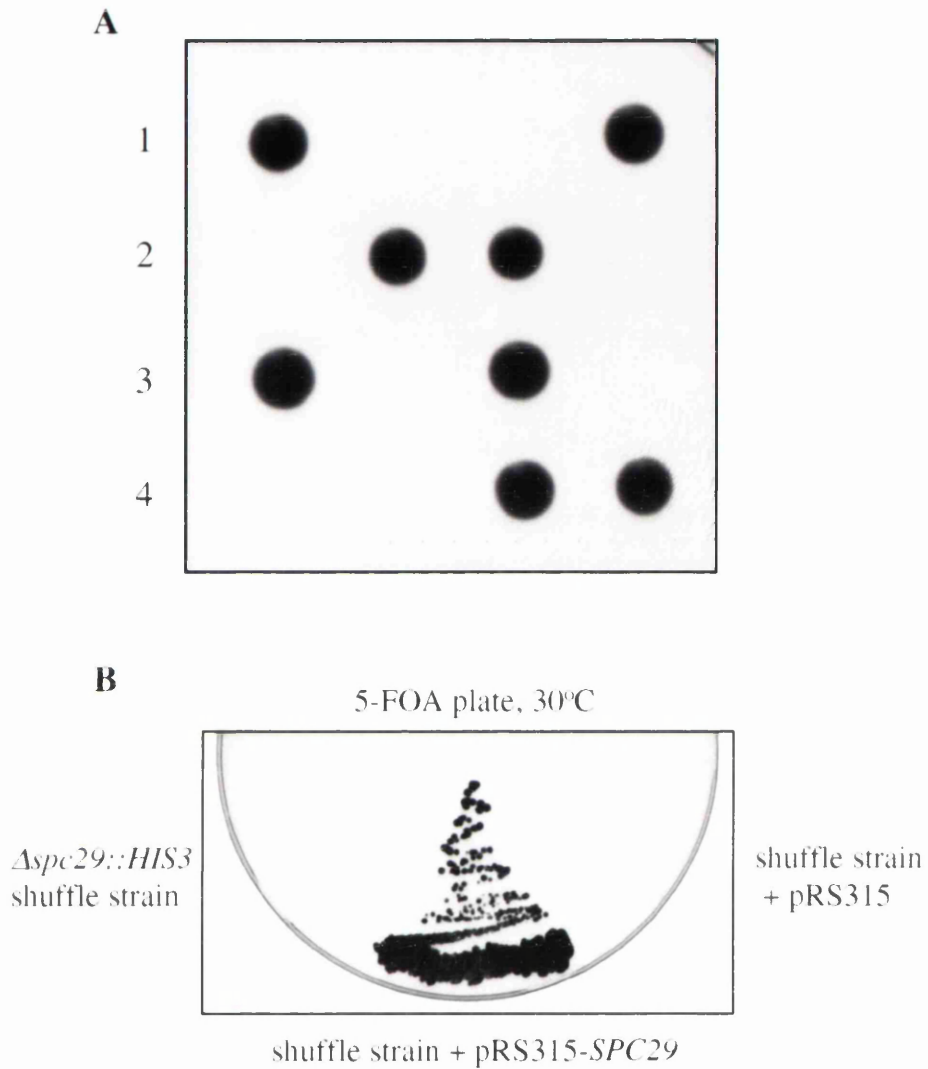
A

B

5-FOA plate, 30°C

Δspc29::HIS3
shuffle strain

shuffle strain
+ pRS315

shuffle strain + pRS315-*SPC29*

Figure 10 *SPC29* is essential

A Spore analysis of four tetrads formed by sporulating a diploid strain containing a *HIS3* cassette specific for disrupting the *SPC29* gene. From each tetrad only two spores grow, indicating that *SPC29* is essential (spores that grow are *HIS*).

B Growth of the Δ*spc29::HIS3* shuffle strain on 5-FOA is dependent on the presence of *SPC29* on pRS315, a *LEU2*-based plasmid (shuffle strain + pRS315-*SPC29*), confirming that *SPC29* is essential.

A   Spc29p-GFP

B   anti-   anti-
         Spc98p   Spc29p-3Myc   DAPI
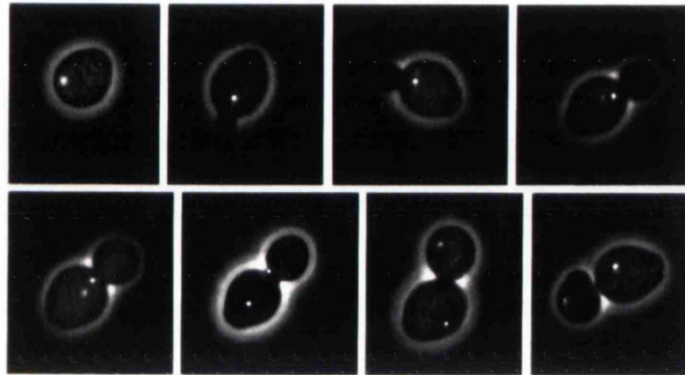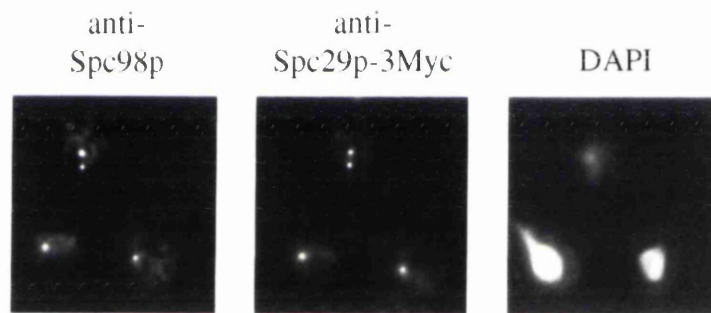
Figure 11 Spc29p localises to the SPB

A   Spc29p-GFP exhibits typical SPB behaviour throughout the cell cycle in live cells.

B   Localisation of Spc29p by indirect immunofluorescence. Spc29p-3Myc was detected in fixed cells with mouse anti-Myc (9E10) antibodies. The Spc29p-3Myc signal co-localised with a known SPB component (Spc98p detected with rabbit anti-Spc29p antibodies). DNA was stained with DAPI.

will contain one GFP signal. In addition, immunofluorescence using the strain expressing *SPC29-3Myc* showed that Spc29p-3Myc co-localised with a known SPB component in formaldehyde fixed cells (Figure 11B). Therefore, Spc29p is indeed a component of the SPB that is localised at the SPB throughout the cell cycle.

Genetic interactions between *SPC29*, *SPC42*, *SPC110* and *CMD1*

Synthetic lethality

An advantage to working with yeast is that genetics can be used as a tool to study functional interactions between gene products. Synthetic lethality is one such genetic approach, it occurs when two temperature sensitive mutants are lethal in combination at the permissive temperature of the individual mutants. If a defined combination is lethal to the cell it suggests an interaction between the gene products. For example, if two proteins interact in an essential pathway, a mutation in one may be tolerated, however if both binding partners are mutated the interaction between them may be compromised causing a lethal phenotype. To gain an insight into the biological significance of Spc29p's presence in the ProA-Spc110p subcomplex, *spc29(ts)* mutants (see Figures 18, 19 and 20 for phenotype of *spc29(ts)* mutants) were tested for synthetic lethality with mutants of other SPB components. This investigation was carried out by making the SPB mutant strains into *SPC29* shuffle strains then introducing a *LEU2*-based plasmid containing either *spc29-2* or *spc29-3*. These strains were then grown on 5-FOA at 23°C to select for cells that could grow in the presence of the two mutant genes and the absence of wild type *SPC29*. Strains that can not grow with the defined combination of mutations at the permissive temperature of the mutants (23°C) are said to be synthetically lethal.

Synthetic lethality was observed when *spc29-2* or *spc29-3* was combined with *spc42-9*, *spc110-2* or *cmd1-1* (Figure 12, compare sectors *ts/spc29-2* with *wt/spc29-2* and *ts/spc29-3* with *wt/spc29-3*). However no synthetic lethality was observed when *spc29-2* or *spc29-3* was combined with mutants of the γ-tubulin-complex components *spc97-14* or *spc98-1*, indicating that the synthetic lethality observed between the *spc29(ts)* mutants and mutants of the other Spc110p subcomplex components is specific. This

Figure 12 *spc29(ts)* **mutants exhibit synthetic lethality with** *cmd1-1, spc110-2* **and** *spc42-9,* **but not with** *spc97-14* **or** *spc98-1*

The chromosomal *SPC29* of *cmd1-1, spc110-2, spc42-9, spc97-14* and *spc98-1* (sectors "*ts*") mutants or wild type cells (sector "*wt*") was deleted in the presence of a plasmid containing *SPC29* (*URA3*-based). Growth of mutant and *SPC29* wild type cells with the control plasmid pRS315 (sector "pRS315"), or *spc29-2* (sector "*spc29-2*"), *spc29-3* (sector "*spc29-3*"), or *SPC29* (sector "*SPC29*") on pRS315 were tested for growth at 23°C (the permissive temperature of the mutants) on plates containing 5-FOA, which selects against the *URA3*-based pRS316-*SPC29* plasmid. Lack of growth on 5-FOA in the presence of pRS315 (sector "*ts*/pRS315") shows that *SPC29* is an essential gene. All cells grow with *SPC29* on pRS315 (sectors "*ts*/*SPC29*" or "*wt*/*SPC29*"). The reduced growth of *cmd1-1, spc110-2* and *spc42-9* cells with pRS315-*spc29-2* (*ts*/*spc29-2*) or pRS315-*spc29-3* (*ts*/*spc29-3*) indicates synthetic lethality. In contrast, *spc97-14, spc98-1* and wild type (*wt*/*spc29-2, wt*/*spc29-3*) cells with *spc29-2* or *spc29-3* on pRS315 grow on 5-FOA. As a control, the *ts* strains were complemented with *CMD1, SPC110, SPC42, SPC97* or *SPC98* (sector "*ts*/*TS*") on pRS315, which allowed growth on SC-Leu plates at 37°C, however these cells did not grow on 5-FOA as *SPC29* is essential for growth.
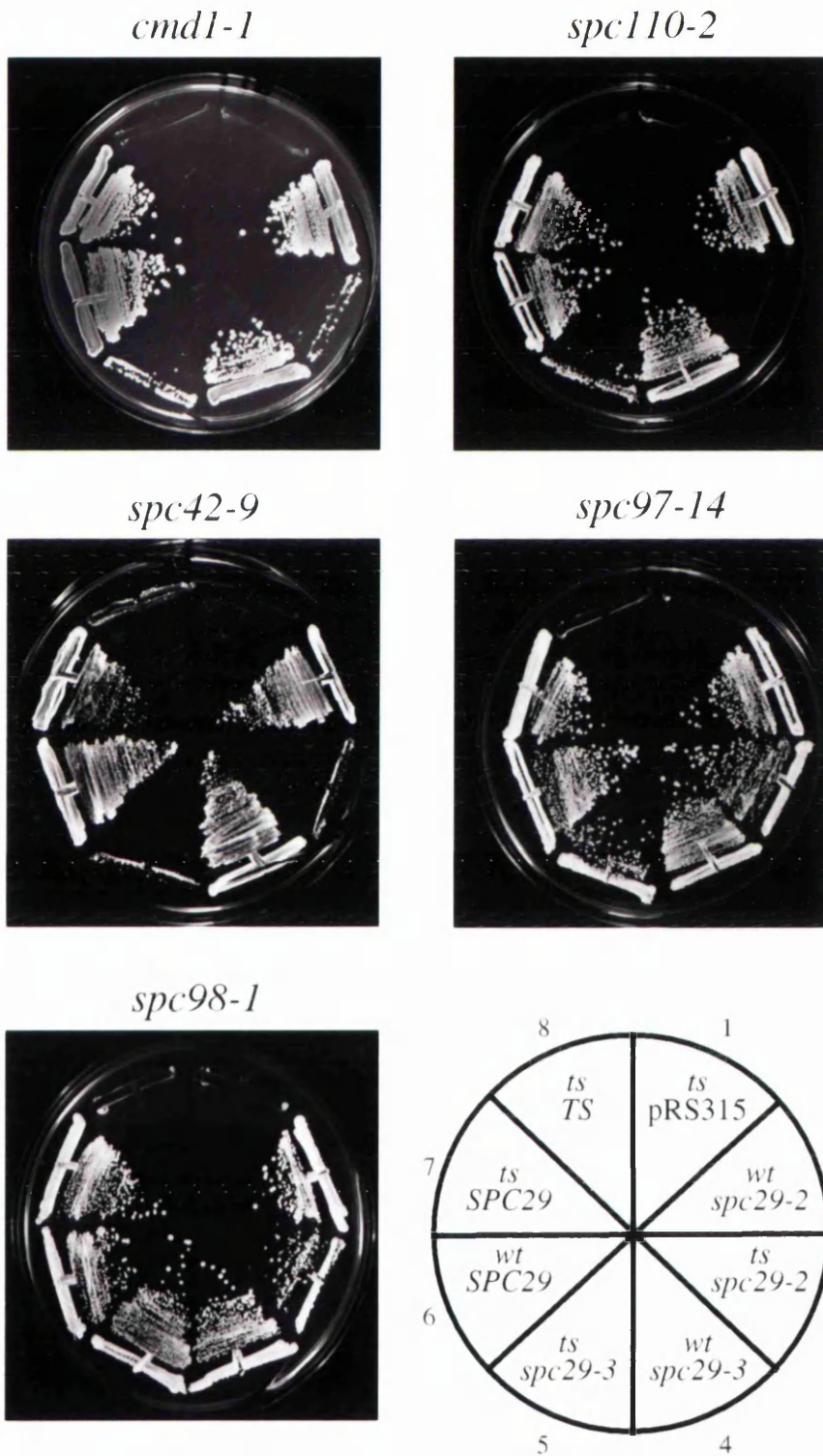
Figure 12 *spc29(ts)* mutants exhibit synthetic lethality with *cmd1-1*, *spc110-2* and *spc42-9*, but not with *spc97-14* or *spc98-1*

confirmed that the gene products of *SPC29*, *SPC42*, *SPC110* and *CMD1* function together. Also, it was interesting to find that calmodulin (*CMD1*) and *SPC29* mutants were synthetically lethal as the role of calmodulin at the SPB is poorly understood.

The two hybrid system

At this point it was clear that Spc29p was an SPB component and was part of a subcomplex with other well characterised SPB components. To further characterise the interaction of Spc29p with Spc42p and Spc110p, the two hybrid system was used. The two hybrid system identifies protein-protein interactions *in vivo*. This system is based on the construction of a transcription factor from the activation domain of the transcriptional activator Gal4p and the bacterial DNA-binding protein LexA. Interaction of the activation domain and the DNA binding protein can be obtained by fusing two proteins to the Gal4 and LexA proteins. If the two fusion proteins interact, the complex can activate the transcription of β-galactosidase in a specially constructed yeast strain bearing a *lexA-operator-lacZ* reporter construct. β-Galactosidase activity can be determined using plates containing X-Gal or by overlaying the yeast plate with top agar containing X-Gal. When an interaction occurs β-galactosidase hydrolyses X-Gal resulting in accumulation of an blue dye within the cells.

Two hybrid plasmids containing *SPC29* were constructed and combined with two hybrid plasmids containing fragments of other SPB component genes. All two hybrid plasmids were also tested with the empty two hybrid vectors as controls. Spc29p interacts specifically with the N-terminus of Spc42p and the C-terminus of Spc110p as shown by the blue colour of the colonies (Figure 13). These interactions are specific as all the controls are white. This result suggests that Spc29p is localised at the central plaque between the Spc42p layer and the C-terminal domain of Spc110p.

Spc110p has a calmodulin-binding site at its C-terminus between residues 900 and 914 (Geiser *et al.*, 1993; Stirling *et al.*, 1994). Mutations within this region result in lethality or in a temperature sensitive phenotype (Geiser *et al.*, 1993; Stirling *et al.*, 1994; Sundberg *et al.*, 1996). However, STOP codons introduced into *SPC110*, N-terminal to the calmodulin binding site, do not affect viability (Geiser *et al.*, 1993; Stirling *et al.*, 1994) and can rescue the temperature sensitive phenotype resulting from a defect in calmodulin binding to Spc110p (Geiser *et al.*, 1993). This indicates that a region of
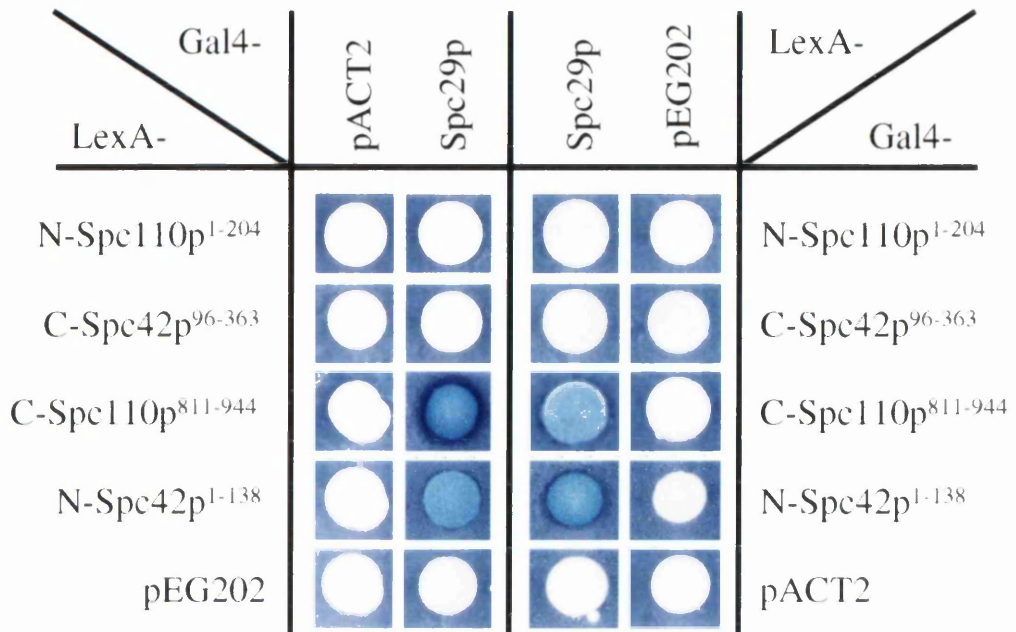
Figure 13   Spc29p interacts with C-Spc110p and N-Spc42p in the two hybrid system

The indicated pACT2 and pEG202 derivatives were transformed into the indicator strain SGY37VII. β-Galactosidase activity was determined by an X-Gal plate assay. Blue colour indicates interaction.

Spc110p, N-terminal of the calmodulin-binding site, is responsible for the binding of Spc110p to the spindle pole body. As Spc29p and calmodulin both interact with the C-terminus of Spc110p, the two hybrid system was used to investigate whether the Spc29p and Cmd1p-binding sites of Spc110p are adjacent or overlapping. To test this Spc29p was combined with the C-terminal truncation of Spc110p that lacked the Cmd1p-binding site (*spc110-120*). An interaction was still seen between the truncated C-terminus of Spc110p and Spc29p (Figure 14). This suggests that Spc29p interacts with Spc110p at a site adjacent to the calmodulin-binding site. There was also an indication that calmodulin affects Spc29p binding to Spc110p as Spc110p mutants with a defect in calmodulin binding (*spc110-116, spc110-118* and *spc110-124*) no longer showed an interaction with Spc29p (Figure 14). However, when the entire calmodulin-binding site of Spc110p was removed (*spc110-120*), binding to Spc29p was restored. This result supports the theory that the C-terminus of Spc110p has an inhibitory role that is relieved by calmodulin binding or truncation mutations deleting the calmodulin-binding site of Spc110p (Stirling *et al.*, 1994).

Multicopy suppression

Suppression occurs when a growth defect, resulting from a mutation in a gene, is rescued either by a second mutation or by increased gene dosage of a second gene. Suppression indicates an interaction between the protein products of the genes involved. For example if a mutant protein has a defect resulting in reduced stability or a reduced affinity for a binding partner, this may be over come by increasing the concentration of the binding partner. Therefore, genetic interactions can be shown by the rescue of temperature sensitive mutants by the introduction of multiple copies of another gene. This is known as multicopy suppression. To further investigate the function of Spc29p numerous SPB components were tested for multicopy suppression of *spc29(ts)* mutants. In addition, multiple copies of *SPC29* were introduced into *spc110(ts)* and *spc42(ts)* mutants and growth was tested at permissive and non-permissive temperatures (Table 9). Genetic interactions were seen by the allele specific multicopy suppression of the temperature dependent growth defect of *spc42-10* but not *spc42-9* by *SPC29*. In contrast the presence of multicopy *SPC42* or *SPC110* increased the growth defect of some *spc29(ts)* alleles. In addition the presence of multiple copies of *SPC29* increased the growth defect of *spc110-118* and *spc110-120* cells, whereas multiple copies of
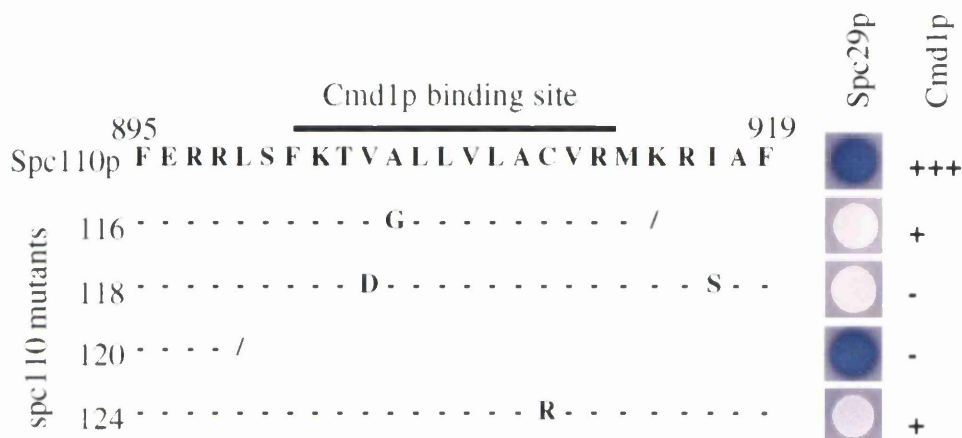
Figure 14 Spc29p interacts with Spc110p at a site adjacent to the Cmd1p binding site.

Plasmid pEG202 containing $SPC110^{811-944}$ of wildtype *SPC110* (top) or of *spc110* mutants (*spc110-116, spc110-118, spc110-120* and *spc110-124*) carrying the indicated mutations in the Cmd1p-binding site were tested in the two hybrid system for interaction with *SPC29* in pACT2 using a plate assay. Cmd1p binding to the Spc110p mutants has been determined before using an *in vitro* binding assay (Stirling *et al*, 1994). / indicates truncated protein resulting from the introduction of a STOP codon. +++ indicates strong binding, + indicates weak binding, - indicates no binding.

Table 9 Multicopy suppression analysis

Mutant	Multicopy gene	Temperature				
		23°C	30°C	33°C	35°C	37°C
spc29-2	*SPC29*	+++	+++	+++	+++	+++
	SPC42	+++	+++	+++	-	-
	SPC110	+++	+++	+++	-	-
	CDC31	+++	+++	+++	+++	+++
	pRS315-*KAR1*	+++	+++	-	-	-
	control	+++	+++	+++	-	-
spc29-3	*SPC29*	+++	+++	+++	+++	+++
	SPC42	+++	+++	-	-	-
	SPC110	+++	+	-	-	-
	CDC31	+++	+++	-	-	-
	pRS315-*KAR1*	+++	-	-	-	-
	control	+++	+++	-	-	-
spc29-9	*SPC29*	+++	+++	+++	+++	+++
	SPC42	+++	+	-	-	-
	SPC110	+++	+	-	-	-
	CDC31	+++	+++	-	-	-
	pRS315-*KAR1*	+++	+++	-	-	-
	control	+++	+++	-	-	-
spc42-9	*SPC42*	+++	+++	+++	+++	+++
	SPC29	+++	+++	+++	+++	-
	SPC110	+++	+++	+++	+++	-
	control	+++	+++	+++	+++	-
spc42-10	*SPC42*	+++	+++	+++	+++	+++
	SPC29	+++	+++	+++	+++	+++
	SPC110	+++	+++	+++	-	-
	control	+++	+++	+++	-	-
spc110-118	*SPC110*	+++	+++	+++	+++	+++
	SPC29	+++	+++	+	-	-
	CMD1	+++	+++	+++	+++	+++
	control	+++	+++	+++	+	-
spc110-124	*SPC110*	+++	+++	+++	+++	+++
	SPC29	+++	+++	+++	-	-
	CMD1	+++	+++	+++	+++	+++
	control	+++	+++	+++	+++	-

The conditional lethal *spc29-2*, *spc29-3*, *spc29-9*, *spc42-9*, *spc42-10*, *spc110-118* and *spc110-124* mutants were transformed with either an empty 2μ plasmid (control) or with a 2μ plasmid carrying one of the indicated genes. Transformants were tested for growth at various temperatures. +++, good growth; +, reduced growth; -, no growth.

CMD1 rescued the defect. These genetic interactions support the theory that Spc29p functions with Spc42p and Spc110p at the SPB.

Overexpression experiments

Overexpression has given valuable data about SPB components in the past (Bullitt *et al.*, 1997; Donaldson and Kilmartin, 1996; Kilmartin and Goh, 1996). Overexpressing Spc42p (Figure 15) shows that Spc42p is a component of the central plaque and that Spc42p forms a polymer which extends out from the central plaque forming a dome like structure around the SPB. However, the dimensions of the outer and inner plaque remain as wild type. This shows that the expression levels of a single SPB component does not dictate the size of the entire SPB. Nevertheless, the size of the SPB must be tightly controlled as the SPBs in haploid yeast cells are relatively uniform in size (80-110 nm) and in diploid cells the SPBs are approximately twice the size of SPBs in haploid cells (~160 nm) (Byers and Goetsch, 1974).

SPC29, *SPC42* and *SPC110* were overexpressed in various combinations. Initially *SPC29-GFP* was expressed under the control of the *GAL1* promoter. This caused accumulation of Spc29p-GFP in the nucleus and at the SPB (Figure 16A). However, looking at these cells using the electron microscope the structure of the SPB is not drastically changed (Figure 16B). The accumulation of overexpressed GFP-Spc29p in the nucleus suggests that Spc29p contains a nuclear localisation signal or is imported into the nucleus via interaction with another protein. If Spc29p is a nuclear protein then Spc29p would assemble at the SPB from the nuclear side of the SPB. During SPB duplication the spatial arrangement of the soluble SPB components may be important for the proper assembly of the SPB. Spc110p is already known to be a nuclear protein (Adams and Kilmartin, 1999; Sundberg *et al.*, 1996). To determine whether Spc42p is incorporated into the SPB from the nucleus or the cytoplasm, *SPC42* was overexpressed in an *MPS2* mutant strain. *mps2-1* cells arrest at the non-permissive temperature with an enlarged satellite that is unable to embed in the nuclear envelope (Winey *et al.*, 1991b). When *mps2-1 GAL1-SPC42* cells were incubated at 37°C in the presence of galactose and were observed by electron microscopy, an Spc42p polymer could be seen extending from the satellite into the cytoplasm (Figure 17, compare A to B). This confirms that Spc42p is a cytoplasmic protein and is a component of the satellite. These results also suggest that Spc29p interacting with Spc42p brings the cytoplasmically organised SPB
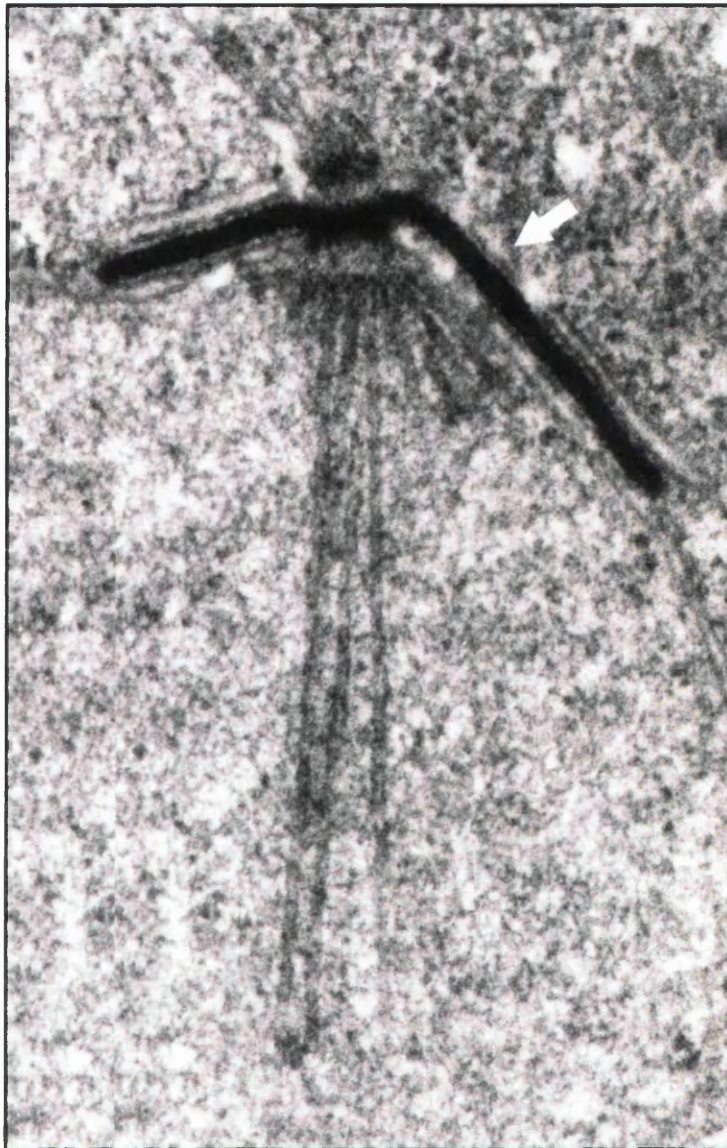
Figure 15 Overexpression of *SPC42*

Electron micrograph showing an SPB from a cell overexpressing *SPC42*. Arrow indicates the Spc42p polymer extending from the central plaque.

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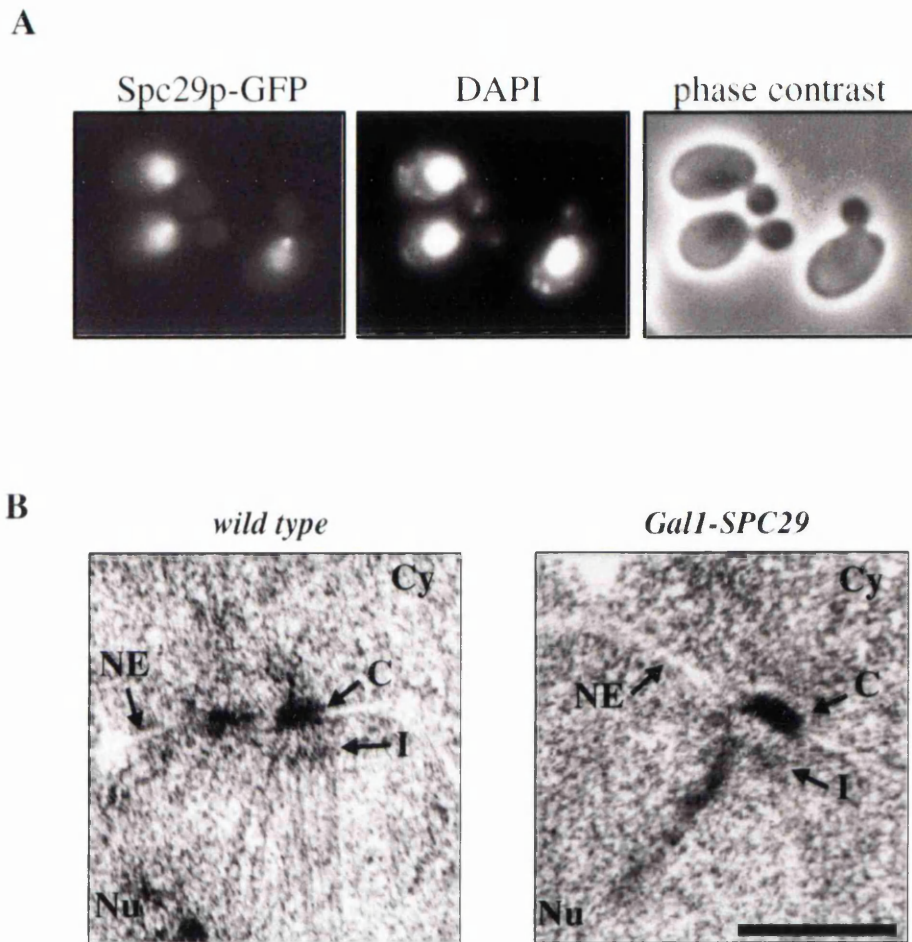
A

Spc29p-GFP   DAPI   phase contrast

B

wild type   *Gal1-SPC29*

Figure 16 Overexpression of *SPC29*

A Localisation of Spc29p-GFP after its overexpression from the *Gal1* promoter for 3 hours at 30°C was determined by fluorescence microscopy. Cells were fixed in 4% para-formaldehyde for 10 min, DNA was stained with DAPI.

B Electron micrographs of SPBs from a wild type cell and from a cell overexpressing *SPC29* from the *Gal1* promoter. C, central plaque; Cy, cytoplasm; I, inner plaque; NE, nuclear envelope; Nu, nucleus. Bar = 0.25 µm, both micrographs are the same magnification.
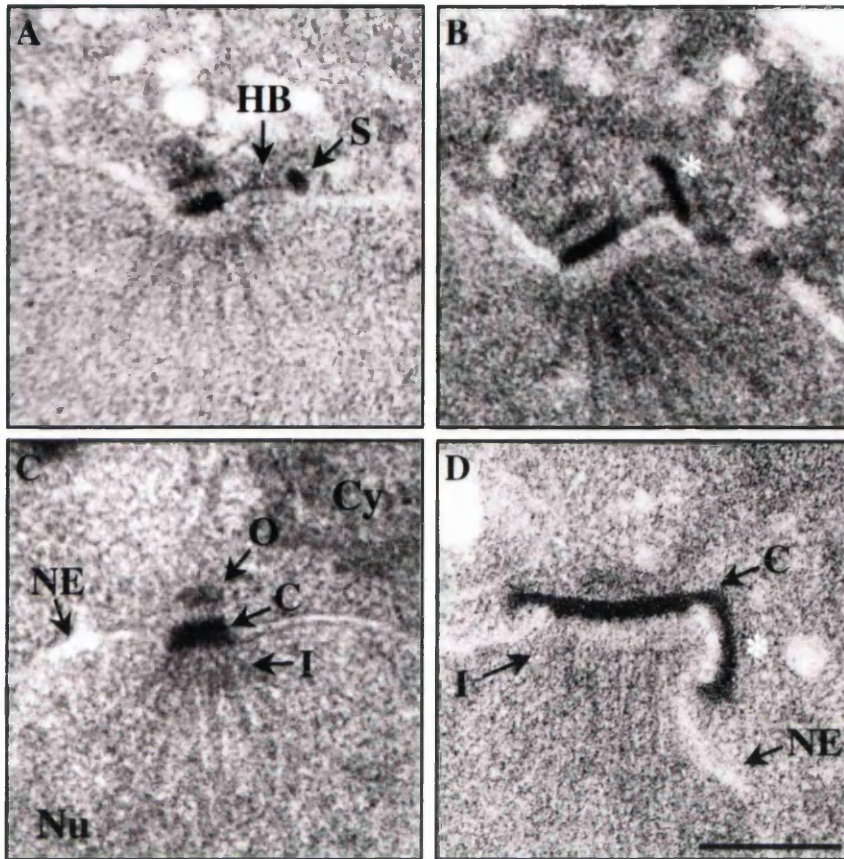
Figure 17 Overexpression of *SPC29*, *SPC42* and *SPC110*

A and B Electron micrographs of SPBs of *mps2-1* cells with (**B**) or without (**A**) overexpression of *SPC42*. Cells of *mps2-1* with or without *Gal1-SPC42* were shifted to 37°C for 30 min in 2% raffinose medium followed by induction of the *Gal1* promoter by addition of 2% galactose. * in **B** indicates the Spc42p polymer.

C and D Electron micrographs of SPBs from cells with (**D**) or without (**C**) co-overexpression of *SPC29*, *SPC42* and *SPC110*. Cmd1p is present in excess in the cell and was therefore not overexpressed. * in **D** indicates the Spc42p polymer.

C, central plaque; Cy, cytoplasm; HB, half-bridge; I, inner plaque; NE, nuclear envelope; Nu, nucleus; O, outer plaque; S, satellite. Bar = 0.25 µm, **A-D** are all the same magnification.

substructures and the nuclear SPB substructures together during SPB duplication. However, recent immunoelectron microscopy studies have localised Spc29p to the satellite as well as the central plaque (Adams and Kilmartin, 1999). Therefore, it may be that an interaction of Spc29p retained at the satellite with Spc29p in the nucleus forms this interface. This is a possibility as Spc29p interacts with itself in the two hybrid system (Figure 25C). Alternatively, overexpression of *SPC29* may cause mislocalisation of Spc29p and the interface could be between Spc29p at the satellite interacting with Spc110p-Cmd1p in the nucleus.

In addition, triple co-overexpression of *SPC29*, *SPC42* and *SPC110* caused a dramatic increase in the size of the SPB (Figure 17, compare C to D). As mentioned above overexpression of *SPC42* causes a polymer of Spc42p to extend from the central plaque, however the dimensions of the inner and outer plaques remain the same as those of wild type cells. Overexpression of *SPC29* or *SPC110* alone has little effect on the SPB proper, *CMD1* was not overexpressed as Cmd1p is present in excess in the cell. However, triple co-overexpression of *SPC29*, *SPC42* and *SPC110* dramatically increases the diameter of the inner plaque as well as the central plaque. This indicates that expression levels of the components of the Spc110p-complex contribute to size regulation of the SPB.

Analysis of *spc29(ts)* mutants

SPC29 is an essential gene, therefore to study the function of Spc29p, conditional lethal mutants of *SPC29* were made using mutagenic PCR. Alleles that conferred temperature sensitivity were selected for and sequenced. Three *spc29(ts)* alleles were chosen for further analysis and integrated into the wild type *SPC29* locus. *spc29-2* and *spc29-3* contain single point mutations, resulting in R161S and L158Q respectively, *spc29-9* contains multiple point mutations. To gain an insight into the function of Spc29p the phenotype of wild type, *spc29-2* and *spc29-3* cells was observed after incubation at the non-permissive temperature in order to identify any physical differences between *spc29(ts)* mutants and wild type cells. Cultures of wild type, *spc29-2* and *spc29-3* cells were synchronised at 23°C using the pheromone α-factor, the synchronised cultures were then washed to release the cell cycle block and shifted to the non-permissive temperature. Sample were taken at hourly intervals after the shift for analysis by FACS,

Figure 18 Analysis of *spc29(ts)* mutants by FACS and immunofluorescence

Wild type cells (*SPC29*) and cells of *spc29-2* and *spc29-3* were incubated for 2.5 hours at 23°C with 0.01 mg/ml α-factor. α-factor was removed by washing with pre-warmed YPD medium (37°C) to release the cell cycle block. Cells were incubated for 3 hours (**B**) or for up to 4 hours (**A**) at 37°C.

A The DNA content of cells of *SPC29*, *spc29-2* and *spc29-3* was determined by FACS analysis before release (0 hours) and 1, 2, 3 and 4 hours after release of the cell cycle block.

B After 3 hours at the non-permissive temperature, *SPC29* and *spc29-2* cells were fixed with formaldehyde for 15 min then prepared for immunoflourecence. Microtubules were detected with the anti-tubulin antibody Wa3, the SPB was detected using anti-Spc72p antibodies and DNA was stained with DAPI. In wild type cells 100% of large budded cells had two Spc72p signals. Microtubule staining shows a bipolar spindle in wild type cells, connecting the two SPB signals. However, in *spc29-2* cells only ~30% of large budded cells contained two Spc72p signals (arrow indicates example of second Spc72p signal). In cells with one SPB signal, the SPB was localised near the bud neck, microtubule staining showed monopolar spindles and DAPI staining showed that the chromosomes had not been equally segregated between the mother and bud.
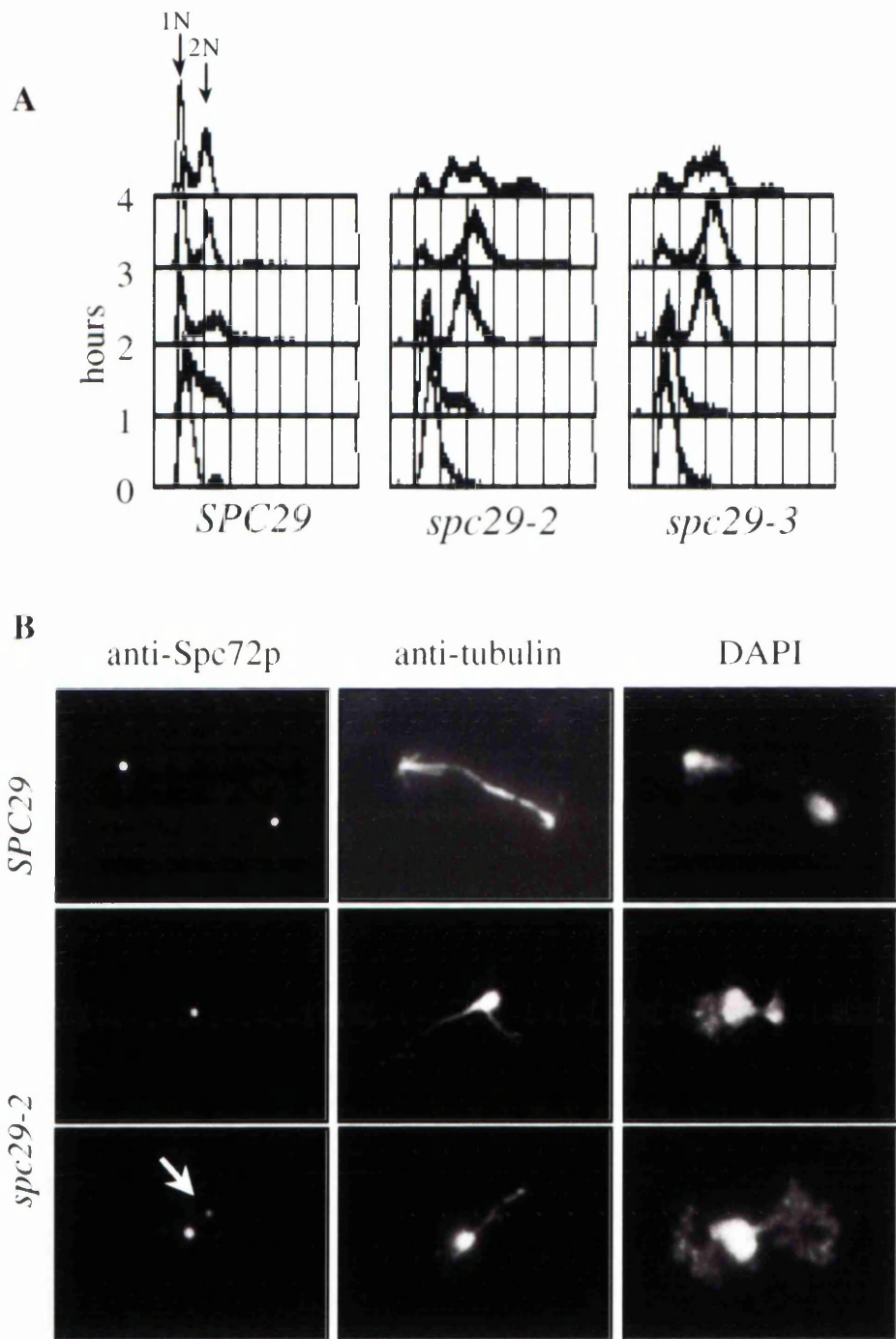
Figure 18 Analysis of *spc29(ts)* mutants by FACS and immunofluorescence

immunofluorescence and electron microscopy. Wild type cells, when analysed by FACS (Figure 18A), could be seen to be synchronised in G1 at the time of α-factor release (0 hours) as seen by the large 1N peak signifying that the cells had not moved into S phase. The wild type cells then moved into S phase, as seen by the increased number of cells with 2N DNA (1 hour), followed by mitosis (2 hours) shown by the 1N (new G1 cells) and 2N (cells in mitosis) peaks. At 3 hours the synchrony of the cultures is lost as newly born daughter cells in G1 are smaller than the mother cells in G1, therefore must grow for longer before they can enter into another cell cycle. Cells of *spc29-2* and *spc29-3* strains are viable at 23°C, but when an α-factor synchronised culture of either mutant was incubated at the non-permissive temperature (37°C) the cells arrested in G2 of the first cell cycle with a large bud and a 2N DNA content (Figure 18A). This arrest was only transitory as DNA contents larger than 2N were seen at later time points.

Wild type cells in late mitosis have a 2N DNA content and appear as large budded cells, mother and daughter cells have a single SPB each which are connected by the mitotic spindle and sister chromatids have been separated to the spindle poles. This can be seen by observing cells by immunofluorescence (Figure 18B, *SPC29*). When the *spc29(ts)* cells were observed by immunofluorescence after 3 hours at the non-permissive temperature, they showed chromosomal segregation defects (Figure 18B, *spc29-2*). This can be seen by looking at the DAPI staining of the DNA (note the DAPI staining in the mutants is not separated equally into the mother and daughter cells, this indicates a defect in chromosomal segregation). Also, by using an antibody against the SPB component Spc72p (an outer plaque component), it was observed that the mutants have a defect in SPB duplication or separation as most mutant cells have only one SPB signal, whereas wild type large budded cells have two SPB signals separated by the mitotic spindle. In 30% of *spc29(ts)* cells there was a second, fainter SPB signal close to the first (Figure 18B, arrow).

To ensure that the localisation of Spc72p in the *spc29(ts)* mutants is a true representation of SPB localisation, the *spc29(ts)* strains were modified by fusing GFP to SPB components of the outer, central and inner plaques. These strains were then synchronised with α-factor and incubated at 37°C for up to 3 hours then observed by fluorescence microscopy. A similar result to the Spc72p immunofluorescence, regarding SPB number and intensity of signal, was seen when the SPBs of *spc29(ts)* cells were marked using GFP fusions of the outer plaque component Cnm67p or the

Figure 19 Analysis of *spc29(ts)* mutants using GPF fusions of SPB components

α-factor synchronised *spc29-2* cells containing Cnm67p-GFP, Spc42p-GFP or Spc110p-GFP were shifted to the non-permissive temperature (37°C) for up to 3 hours. Samples were taken after 15 mins, 30 mins, 1, 2 and 3 hours. Cells were fixed with 4% paraformaldehybe for 10 min and DNA was stained with DAPI. The GFP signal was then observed by fluorescence microscopy.

A A wild type budded cell showing typical SPB staining by Spc72p-GFP.

B Examples of *spc29-2* cells at the non-permissive temperature expressing the indicated GFP fusion protein. In ~ 70% of *spc29-2 CNM67-GFP* and *spc29-2 SPC42-GFP* large budded cells only one SPB signal could be seen. In cells where a second SPB was observed, the two signals were close together and one was weaker than the other (arrow). When Spc110p-GFP was used as an SPB marker only one SPB signal was observed in ~95% of large budded *spc29-2* cells.
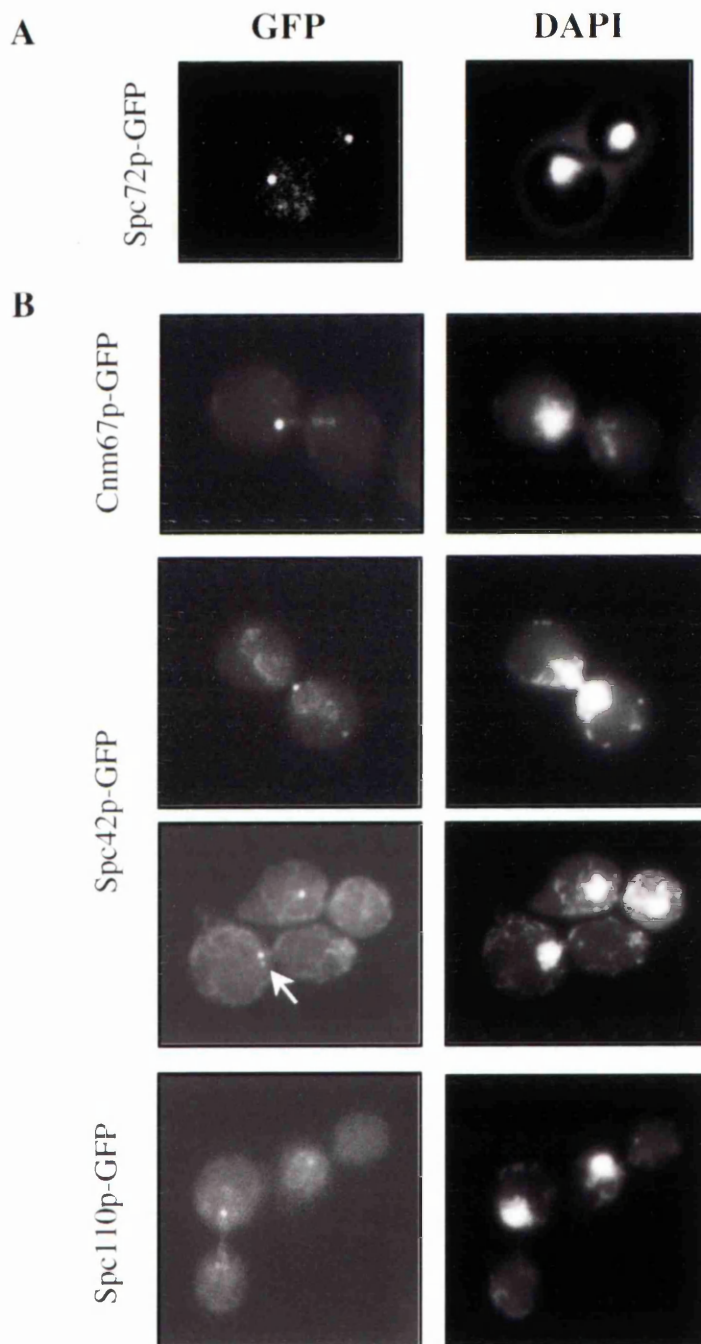
Figure 19 Analysis of *spc29(ts)* mutants using GFP fusions of SPB components

central plaque component Spc42p (Figure 19B). However, when the inner plaque component Spc110p was expressed as a GFP fusion protein in the *spc29(ts)* strains, 95% of cells contained only one SPB signal (Figure19B). These results confirm that there is an SPB duplication or separation defect in *spc29(ts)* cells incubated at the non-permissive temperature.

To understand the SPB defect in *spc29(ts)* cells, wild type and *spc29(ts)* cells were prepared for electron microscopy after 3 hours at the non-permissive temperature (Figure 20). Large budded wild type cells contain two SPBs, with a diameter of approximately 80 nm, one in the mother and one in the bud. Each SPB has an outer and inner plaque with associated microtubules and a central plaque with associated half-bridge (Figure 20A). When serial sections of *spc29(ts)* cells were observed using electron microscopy (Figure 20B-F) only one SPB was found per cell, despite the appearance of two SPB signals in 30% of cells as seen by immunofluorescence. This difference may be due either to detachment of the outer plaque or disintegration of the newly formed SPB (a phenotype described for some *SPC110* mutants). In addition, the morphology of the single SPB in *spc29(ts)* cells was not as wild type. For example the SPB shown in Figure 20B appears to have two inner plaques, whereas the SPB in Figure 20C is greatly enlarged. In Figure 20D the SPB has an associated half-bridge with, what appears to be a malformed satellite, whereas in Figure 20E SPB duplication appears to have taken place, but the two SPBs remain attached. It should also be noted that all of the SPBs observed in *spc29(ts)* cells were associated with microtubules, therefore *spc29(ts)* mutants do not have a microtubule nucleation defect. Taken together, these results suggest that Spc29p is involved in SPB duplication.

In conclusion, analysis of *spc29(ts)* cells by FACS, immunofluorescence and electron microscopy showed that these cells are able to replicate their DNA, but arrest in G2 with unsegregated chromosomes, probably due to a defect in SPB duplication which results in triggering of the spindle assembly checkpoint pathways.

Figure 20 Analysis of *spc29(ts)* mutants by electron microscopy

Wild type cells and cells of *spc29-2* and *spc29-3* were incubated for 2.5 hours at 23°C with 0.01 mg/ml α-factor. α-factor was removed by washing with prewarmed YPD medium (37°C) to release the cell cycle block. Cells were incubated for 3 hours at 37°C then prepared for electron microscopy followed by serial sectioning.

A Wild type SPB. **B and C** SPBs from *spc29-2* cells. **D-F** SPBs from *spc29-3* cells.

C, central plaque; Cy, cytoplasm; HB, half-bridge; I, inner plaque; NE, nuclear envelope; Nu, nucleus; O, outer plaque. Bar = 0.25 µm, **A-F** are all the same magnification.
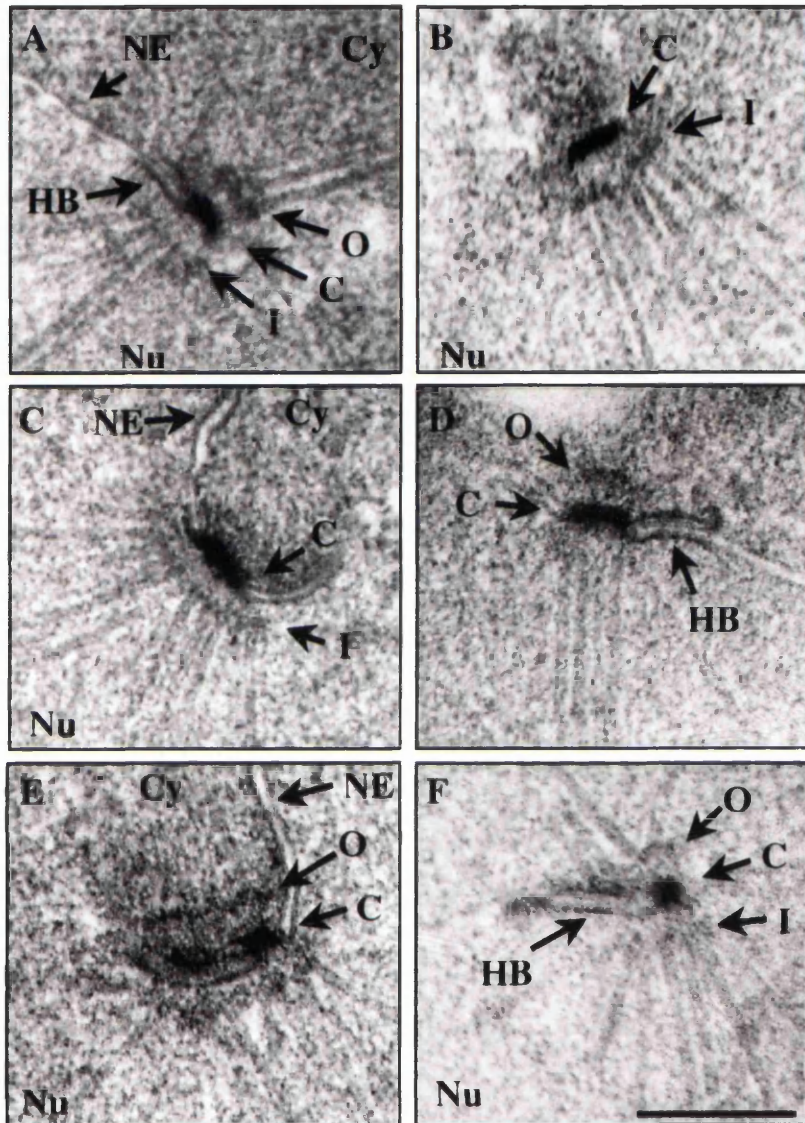
Figure 20 Analysis of *spc29(ts)* mutants by electron microscopy

Spc29p is present in a subcomplex containing Cdc31p and Kar1p

Genetic interactions

Spc29p appears to have a role in SPB duplication, therefore other SPB components known to be involved in SPB duplication were tested for genetic interactions with *spc29(ts)* alleles. The half bridge components Cdc31p and Kar1p are essential for SPB duplication (Byers, 1981a; Rose and Fink, 1987; Winey *et al.*, 1991b). When *CDC31* was introduced into *spc29(ts)* strains on a multicopy plasmid, it rescued the temperature dependent growth defect of *spc29-2*, but not *spc29-3* or *spc29-9* cells. However, a single additional copy of *KAR1* increased the temperature sensitivity of *spc29-2* and *spc29-3*, but not *spc29-9* cells (Table 9). These allele specific genetic interactions indicate a functional relationship between Cdc31p, Kar1p and Spc29p.

There have been numerous genetic interactions reported between *CDC31*, *KAR1*, and *DSK2* (Biggins *et al.*, 1996), therefore *DSK2* was tested for genetic interactions with *spc29(ts)* mutants. *DSK2* was found to be a multicopy suppresser of *spc29-2* and *spc29-3* mutants (Figure 21). This again indicates a connection between Cdc31p, Kar1p and Spc29p.

Localisation of Cdc31-16p

Cdc31p and Kar1p are essential and are the only two known components of the half-bridge. Kar1p has a membrane anchor and it has been shown that localisation of Cdc31p at the half-bridge was dependent on its interaction with Kar1p (Biggins and Rose, 1994; Spang *et al.*, 1995). *CDC31-16* contains a gain of function mutation that allows a strain carrying this allele to survive when *KAR1* is deleted. *CDC31-16 Δkar1* cells were observed by immunofluorescence to localise Cdc31-16p. In wild-type cells Cdc31p localises to the SPB and the nuclear envelope, Kar1p localises to the SPB (Figure 22A). However, in *CDC31-16 Δkar1*, where there is no Kar1p, Cdc31-16p also localises to the SPB (Figure 22B). This indicates that Cdc31-16p interacts with at least one other SPB component.

As Cdc31p and Kar1p are the only known components of the half-bridge, the morphology of the half-bridge of *CDC31-16 Δkar1* cells was examined for defects or
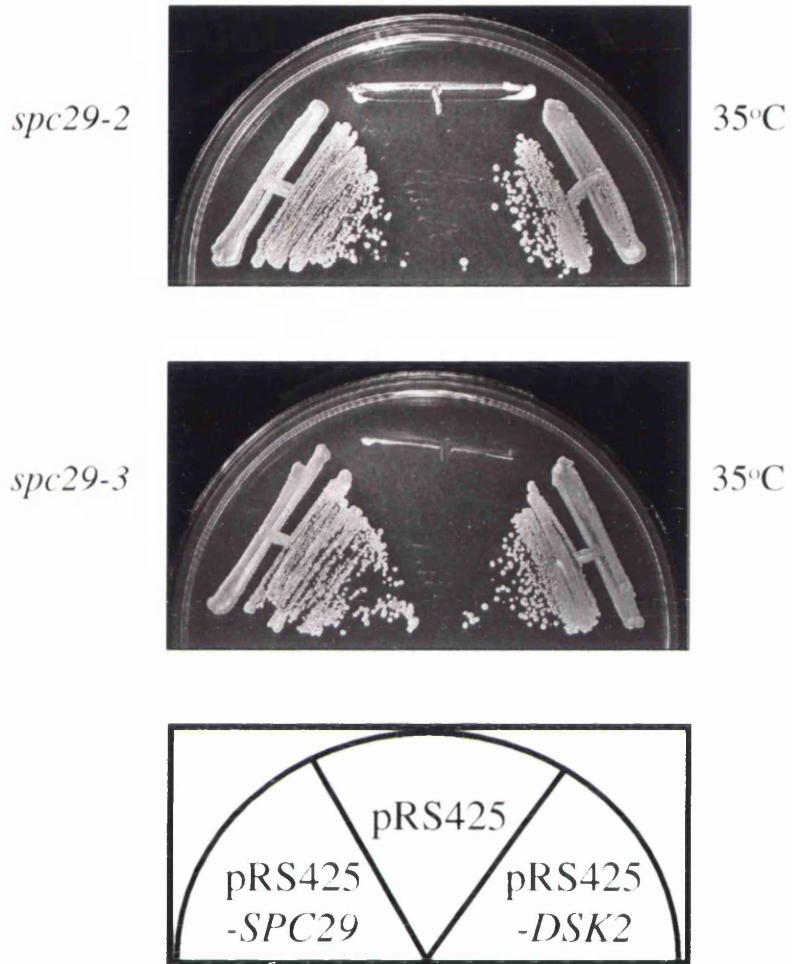
Figure 21 *DSK2* is a multicopy suppressor of *spc29(ts)* mutants

DSK2 present on the multicopy plasmid pRS425 allows growth of *spc29-2* and *spc29-3* at the non-permissive temperature (35°C).

A
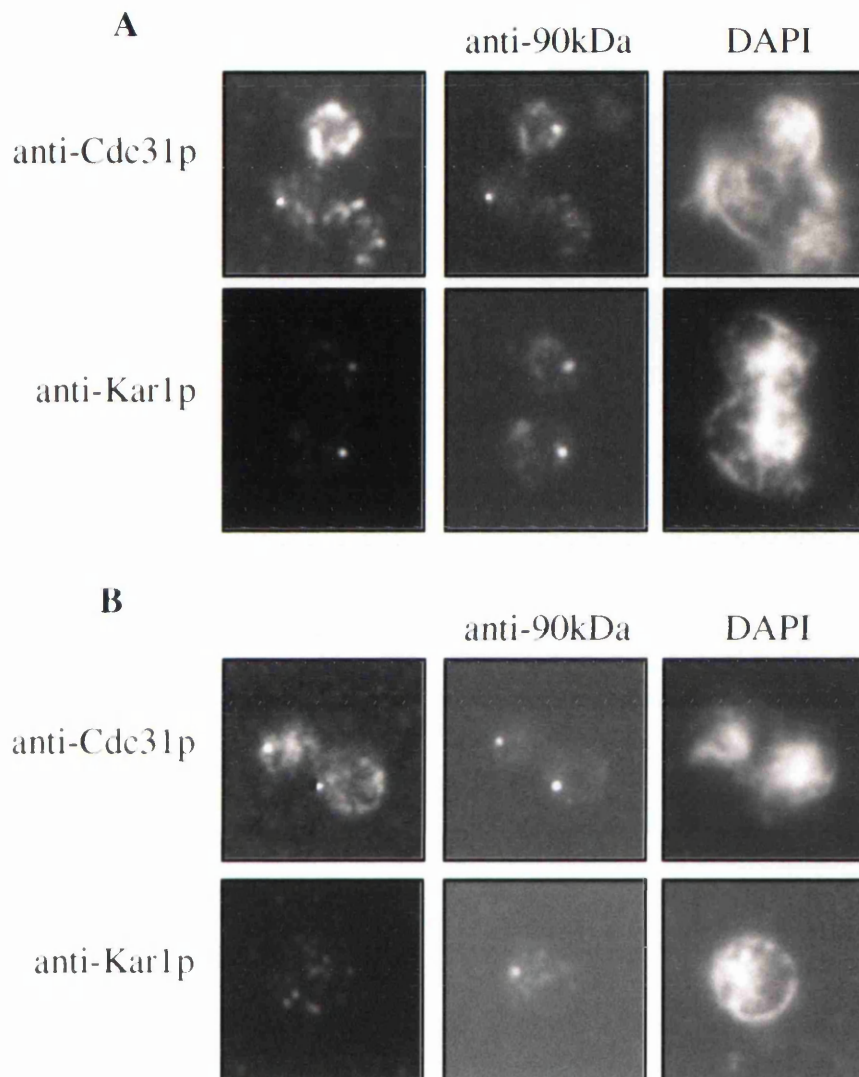

B

Figure 22 Localisation of Cdc31p

Wild type and *CDC31-16 Δkar1* cells were fixed with methanol/acetone then prepared for immunofluorescence. DNA was stained with DAPI.

A   Kar1p and Cdc31p co-localise with Spc98p (anti-90kDa (Rout and Kilmartin, 1990)) in wild type cells.

B In *CDC31-16 Δkar1* cells Cdc31-16p still localises to the SPB, even in the absence of Kar1p.

abnormalities using electron microscopy (Figure 23). Wild type and *CDC31-16 Δkar1* cells were arrested with α-factor, to increase the number of cells containing single SPBs with associated half-bridges, then prepared for electron microscopy. *CDC31-16 Δkar1* cells still contained a half-bridge, however the morphology was a little unusual. The half-bridge of *CDC31-16 Δkar1* cells appeared thicker when compared to wild type, also the half-bridge only extended over the cytoplasmic side of the nuclear membrane whereas in wild type cells the half-bridge extends over both the nuclear and cytoplasmic sides of the nuclear membrane. The important observation though is that *CDC31-16 Δkar1* cells do contain a half-bridge, therefore the half-bridge in these cells must either contain additional proteins or consist exclusively of Cdc31-16p. So how is Cdc31-16p connected to the central plaque and the satellite? Does Cdc31p bind directly to satellite and central plaque components or is the interaction via another protein?

Spc29p immunoprecipitation

Spc29p is a component of the central plaque, where it interacts with Spc42p and Spc110p. Spc29p is also a component of the satellite (Adams and Kilmartin, 1999) where it may play its role in SPB duplication and interact with components of the half-bridge. To further investigate the role of Spc29p, Spc29p was purified from wild type cells using anti-Spc29p antibodies coupled to Protein A Dynal magnetic beads in the presence of 1M NaCl and 1% Triton. Cdc31p and Kar1p specifically co-purified with Spc29p, as shown by immunoblotting (Figure 24, compare lane 1 to lane 3). However, when Spc29p was purified, under the same conditions, from *CDC31-16 Δkar1* cells, Kar1p did not co-purify, as expected, but neither did Cdc31-16p (Figure 24, compare lane 4 to lane 6). These results suggest that Spc29p, Cdc31p and Kar1p interact *in vivo*. The interaction of Spc29p with Cdc31p may be via Kar1p as in the absence of Kar1p Cdc31-16p does not co-purify with Spc29p. Alternatively, the interaction of Spc29p with Cdc31-16p may be weaker than the interaction with Cdc31p and therefore was disrupted by the harsh conditions used in the purification or simply, the stability of the complex is reduced in the absence of Kar1p.

Cdc31p was purified in the same experiment using anti-Cdc31p antibodies, again coupled to Protein A Dynal magnetic beads, in the presence of 1M NaCl and 1% Triton. Kar1p specifically co-purified with Cdc31p, but Spc29p was not detected in the Cdc31p-purification (Figure 24, compare lanes 2 and 3). As expected, when Cdc31-16p
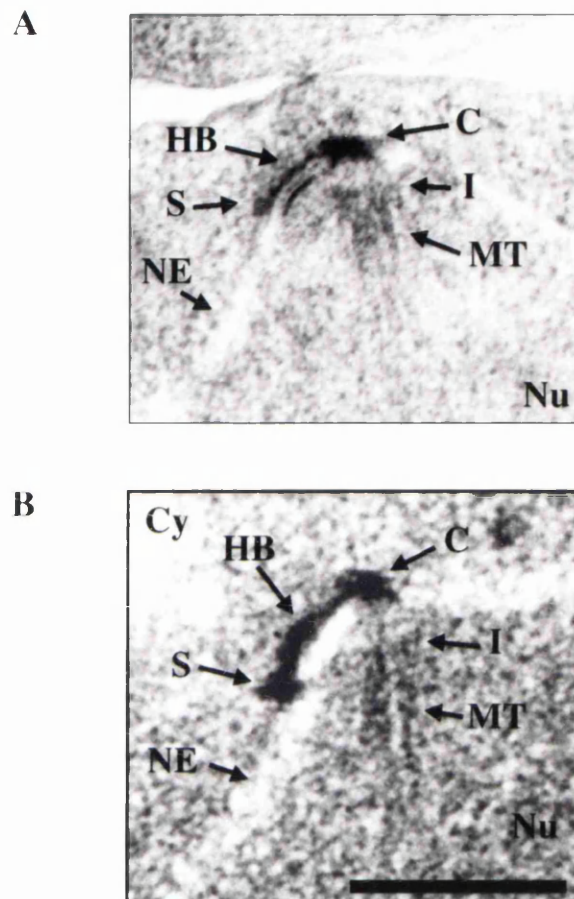
Figure 23 Morphology of the SPB of *CDC31-16 Δkar1* cells

A Wild type SPB

B SPB of a *CDC31-16 Δkar1* cell.

C, central plaque; Cy, cytoplasm; HB, half-bridge; I, inner plaque; MT, microtubules; NE, nuclear envelope; Nu, nucleus; O, outer plaque; S, satellite.

Bar = 0.25 μm, **A** and **B** are the same magnification.

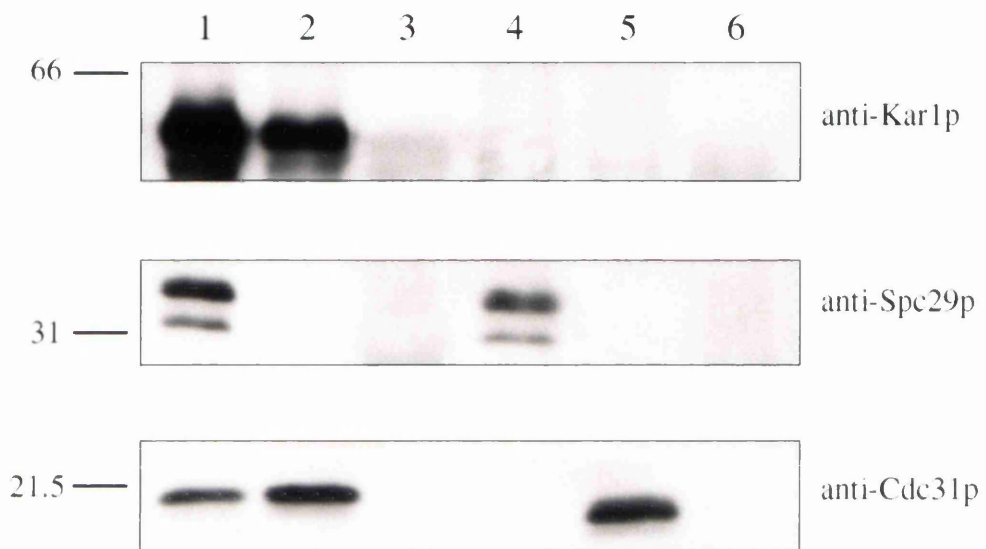**Figure 24   Co-immunoprecipitation of Cdc31p and Kar1p with Spc29p in wild type cells**

High salt extracts of wild type (lanes 1-3) or *CDC31-16 Δkar1* (lanes 4-6) cells were incubated with control beads bound to non-specific IgGs (lanes 3 and 6) or anti-Spc29p (lanes 1 and 4) or anti-Cdc31p (lanes 2 and 5) antibody-coupled magnetic beads. Immunoblots were probed with the indicated antibodies.

was purified from *CDC31-16 Δkar1* cells, there was no Kar1p in the precipitate, but there was no Spc29p either (Figure 24, compare lanes 5 and 6).

Despite this, the finding that Cdc31p and Kar1p co-purify with Spc29p indicates an interaction *in vivo* between half-bridge components and central plaque components. This interaction may be important during SPB duplication.

Additional interactors of Spc29p

Bbp1p was identified as a novel SPB component when its presence was identified in a highly enriched spindle pole body preparation (Wigge *et al.*, 1998). At this time the function of Bbp1p was not known, however *BBP1* was found as a multicopy suppresser of *spc29(ts)* mutants (Figure 25A). To further investigate the relationship between Bbp1p and Spc29p, Bbp1p-ProA was immunoprecipitated using an IgG sepharose column. Spc29p was not detected in the precipitate, however a protein of 45 kDa was specifically present in precipitates from *BBP1-ProA* cells, but not in *BBP1* cells (Schramm *et al.*, 2000). This protein was identified as Mps2p by MALDI peptide mapping. Mps2p is a nuclear membrane protein that is involved in embedding the SPB in the nuclear envelope (Winey *et al.*, 1991b). Immunoelectron microscopy studies have shown that both Bbp1p and Mps2p are located at the periphery of the SPB, close to the central plaque (Schramm *et al.*, 2000; Winey *et al.*, 1991b). These results, along with numerous genetic interactions, indicate that Bbp1p and Mps2p form a complex at the periphery of the central plaque where they have a function in embedding the central plaque in the nuclear membrane. In order to carry out this role it would be expected that the Bbp1p/Mps2p complex would interact with the central plaque. Further attempts to co-precipitate Spc29p and Bbp1p gave inconclusive results, therefore alternative methods were employed to investigate the possible physical interaction between Bbp1p and Spc29p. Interactions were tested using an *in vitro* binding assay and the two hybrid system. *SPC29* was translated and transcribed *in vitro* in the presence of [^{35}S]-labelled methionine to give [^{35}S]-Spc29p. GST, GST-Bbp1p and His-Mps2p were expressed in *E.coli*. GST and GST-Bbp1p were bound to glutathione sepharose beads. These GST and GST-Bbp1p beads were then incubated with [^{35}S]-Spc29p alone, to test for direct binding of [^{35}S]-Spc29p to GST-Bbp1p (Figure 25B compare lanes 1 and 3). GST and GST-Bbp1p beads were also incubated with His-Mps2p followed by incubation with [^{35}S]-Spc29p to test whether [^{35}S]-Spc29p binds to the Bbp1p/Mps2p complex (Figure

Figure 25 Spc29p interacts with Bbp1p

A *BBP1* is a multicopy suppresser of the temperature dependent growth defect of *spc29(ts)* cells. Cells of *spc29-2* and *spc29-3* with the indicated plasmids were incubated at 35°C for 3 days.

B Spc29p interacts with Bbp1p *in vitro*. GST (lane 1 and 2) or GST-Bbp1p (lane 3 and 4) was bound to glutathione sepharose beads. These beads were incubated with (lanes 2 and 4) or without (lanes 1 and 3) His-Mps2p. The beads were washed then incubated with [^{35}S]-labelled Spc29p (lanes 1-4). The bound proteins were eluted from the beads with HU buffer and analysed by immunoblotting with anti-Bbp1p and anti-Mps2p antibodies or by autoradiography ([^{35}S]-Spc29p).

C Two-hybrid interactions between Spc29p and Bbp1p. β-Galactosidase activity was determined using an X-Gal overlay assay, blue colour indicates interaction. The control cells carry, for example, the pMM5 derived *LexA-BBP1* plasmid combined with pMM6 without an insert.
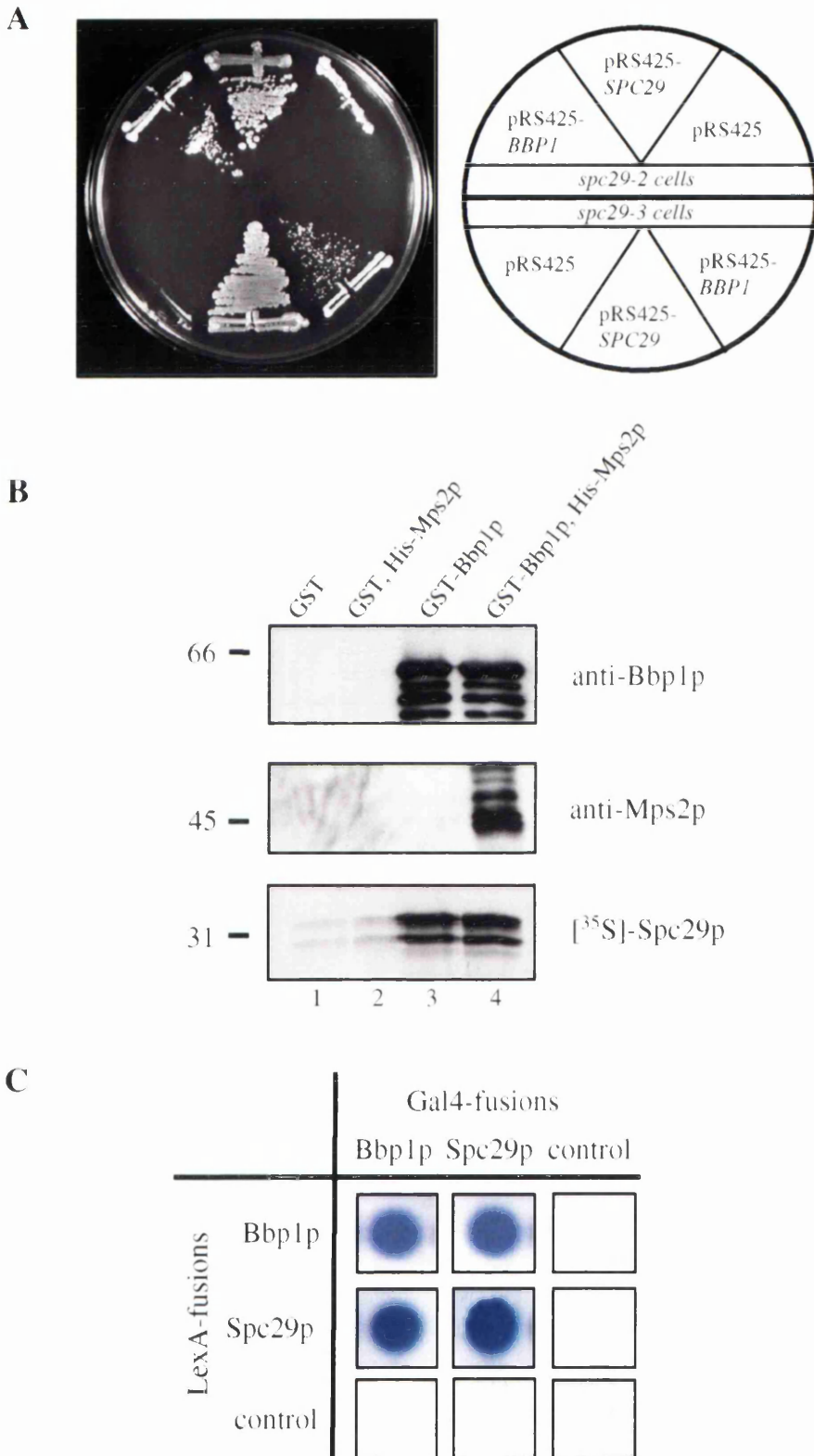
A

B

C

Figure 25 Spc29p interacts with Bbp1p

25B compare lanes 2 and 4). [^{35}S]-Spc29p was found to interact with GST-Bbp1p in the presence and absence of His-Mps2p, but neither [^{35}S]-Spc29p nor His-Mps2p interacted with the GST beads. Therefore Spc29p interacts with Bbp1p and the Bbp1p/Mps2p complex *in vitro*. Further support for an interaction between Spc29p and Bbp1p came from the two hybrid system. Interactions were seen when Bbp1p was combined with Spc29p (Figure 25C), Mps2p or Kar1p in the two hybrid system (Schramm et al, 2000) Taken together, these results are the first to give an insight into the proteins involved directly in embedding the SPB in the nuclear membrane and connecting the half-bridge to the central plaque.

Spc29p is a phosphoprotein

During the study of Spc29p it was noted that Spc29p appeared as a doublet when identified by immunoblotting. As a number of SPB components are phosphoproteins, it was predicted than the appearance of Spc29p as a doublet was due to phosphorylation. This was tested by purifying Spc29p from the soluble cellular fraction of lysed wild type cells and the salt extracted pellet containing the insoluble cellular material (including SPBs). Spc29p purified from these two cellular fractions was treated with alkaline phosphatase, which dephosphorylates proteins, then visualised by immunoblotting. The Spc29p purified from the soluble cellular fraction appeared as a single band, independent of alkaline phosphatase treatment, indicating that soluble Spc29p is not phosphorylated *in vivo*. Spc29p purified from the salt extracted pellet appeared as a doublet in the absence of alkaline phosphatase, however after treatment with alkaline phosphatase the upper band of the Spc29p doublet was greatly reduced. This was due to dephosphorylation by alkaline phosphatase as when the phosphatase inhibitor 3GP was added, at the same time as alkaline phosphatase, the appearance of the Spc29p doublet was the same as the buffer only control (Figure 26A). Therefore, Spc29p is a phosphoprotein.

In addition, Spc29p phosphorylation is cell cycle dependent. This was shown by taking samples from an α-factor synchronised culture at 15 minute intervals after release. Immunoblots of the cell extracts showed that Spc29p is phosphorylated at the same point of the cell cycle as Spc110p (Figure 26B-D, note the upper band of the Spc29p and Spc110p doublets is stronger in intensity between 45 min and 75 min after release from α-factor arrest). Spc110p phosphorylation has previously been shown to occur at

Figure 26 Spc29p is a phosphoprotein

A Wild type cells were lysed with glass beads, then separated into a soluble fraction and an insoluble pellet (containing SPBs) by centrifugation. The pellet was extracted with high salt, 1% Triton X-100. Spc29p was purified from the soluble fraction and the extracted pellet using anti-Spc29p antibodies coupled to protein A sepharose. Immuno-precipitated Spc29p was incubated with alkaline phosphatase (lanes 1, 2, 4 and 5), with (lanes 2 and 5) or without (lanes 1 and 4) the phosphatase inhibitor 3GP. Lanes 3 and 6 contain buffer only as an additional control. Spc29p was detected by immunoblotting with anti-Spc29p antibodies.

B and C Phosphorylation of Spc29p changes during the cell cycle. Wild type cells were synchronised with α-factor, samples were taken at 15 minute intervals after release and analysed by immunoblotting with anti-Spc29p antibodies. **B** and **C** represent two independent experiments. The control lane (c) contains overexpressed Spc29p (**B**) or Spc29p-3HA (**C**).

D The samples used in **B** were loaded identically on a second gel and probed with anti-Tub2p antibodies as a loading control. Spc110p was detected with anti-Spc110p antibodies for comparison with Spc29p.
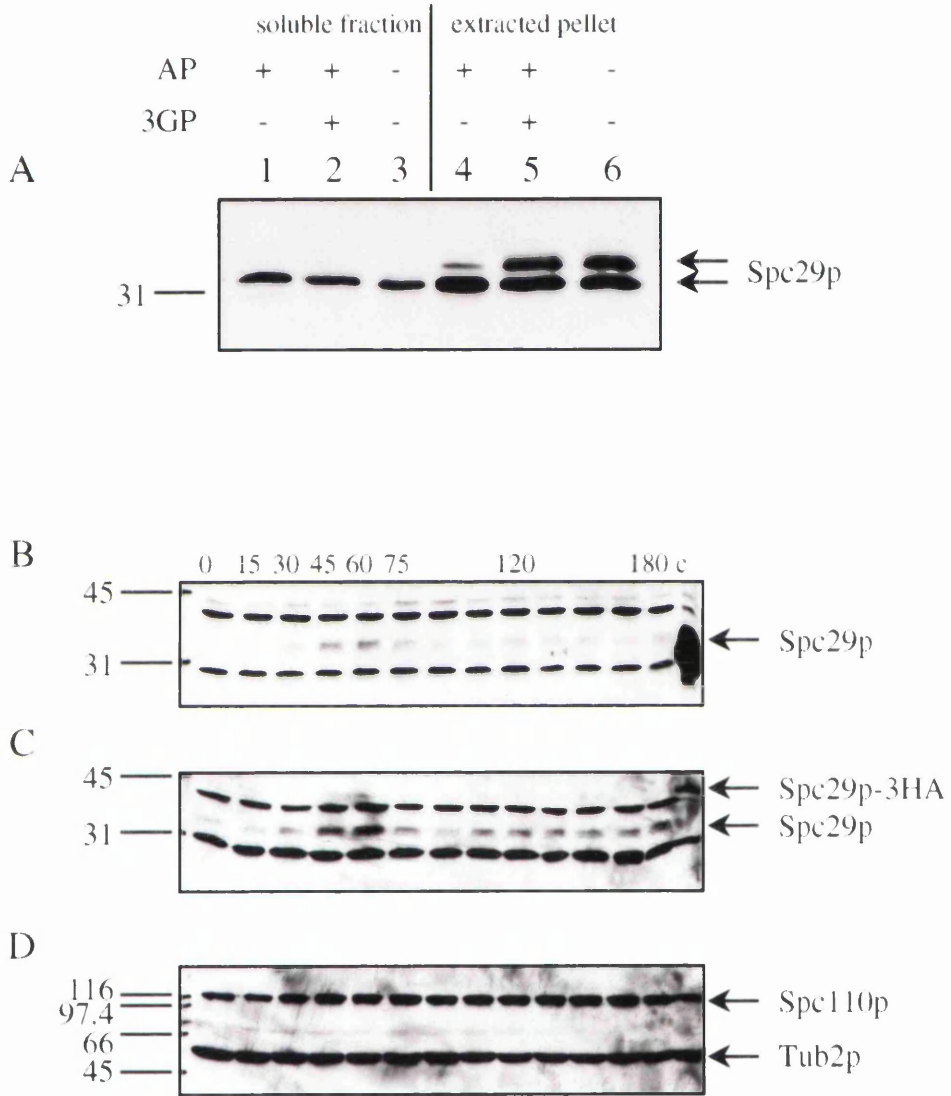
Figure 26 Spc29p is a phosphoprotein

the time of spindle assembly, the level of phosphorylated Spc110p then diminishes as the cells enter anaphase (Friedman *et al.*, 1996). As well as being phosphorylated, the protein levels of Spc29p appear to increase and decrease during the cell cycle reaching a peak at 45-60 minutes after release from α-factor arrest (Figure 26B and C).

Discussion

Spc29p is an essential component of the spindle pole body

Spc29p was first identified as a spindle pole body component by its presence in a salt stable SPB subcomplex containing ProA-Spc110p, calmodulin, Spc42p and small amounts of Cnm67p. Spc29p had previously been named Nip29p (Nup1p interacting protein with a molecular weight of 29 kDa) as it was found to interact with the nuclear pore component Nup1p in a two hybrid screen carried out by G. Schlenstedt. However this interaction was described as "of no biological significance", therefore Nip29p was renamed Spc29p (spindle pole component). *SPC29* is an essential gene as its deletion is lethal. This was confirmed by Wigge et al (1998) when they identified Spc29p as an essential component of purified SPBs. The fact that Spc110p, Cmd1p, Spc42p and Spc29p were present in the same complex indicates that at least some of these proteins directly interact with each other. The C-terminus of Spc110p contains a calmodulin binding site, explaining the presence of Cmd1p in the complex. The C-terminus of Spc110p is also known to be positioned at the central plaque, close to the IL2 Spc42p layer (Bullitt *et al.*, 1997; Spang *et al.*, 1996b; Sundberg *et al.*, 1996) suggesting that Spc110p and Spc42p interact either directly or via an additional protein. Spc110p can be truncated at its C-terminus, by introducing a STOP codon just before the calmodulin binding site, without consequence (Geiser *et al.*, 1993). Therefore, it is unlikely that the calmodulin binding site is involved in anchoring Spc110p to the Spc42p layer. By using the two hybrid system Spc29p was shown to interact with the C-terminus of Spc110p$^{811-898}$ adjacent to the calmodulin binding site and the N-terminus of Spc42p. The interaction of Spc29p with Spc110p and Spc42p has been supported by the results of genetic studies. *spc29-2* and *spc29-3* exhibit synthetic lethality with *spc110-2*, *spc42-9* and *cmd1-1*. Also, multiple copies of *SPC110* or *SPC42* increase the growth defects of some *spc29(ts)* alleles. These data suggest that Spc29p interconnects Spc110p with Spc42p at the central plaque (Figure 27). In support of this Spc29p has been localised to the central plaque of the SPB by immunoelectron microscopy (Adams and Kilmartin, 1999). No other proteins have been identified as integral components of the central plaque.

The observations that mutations in Spc110p that reduce calmodulin binding are lethal or conditional lethal to yeast cells and that the deletion of the entire calmodulin binding
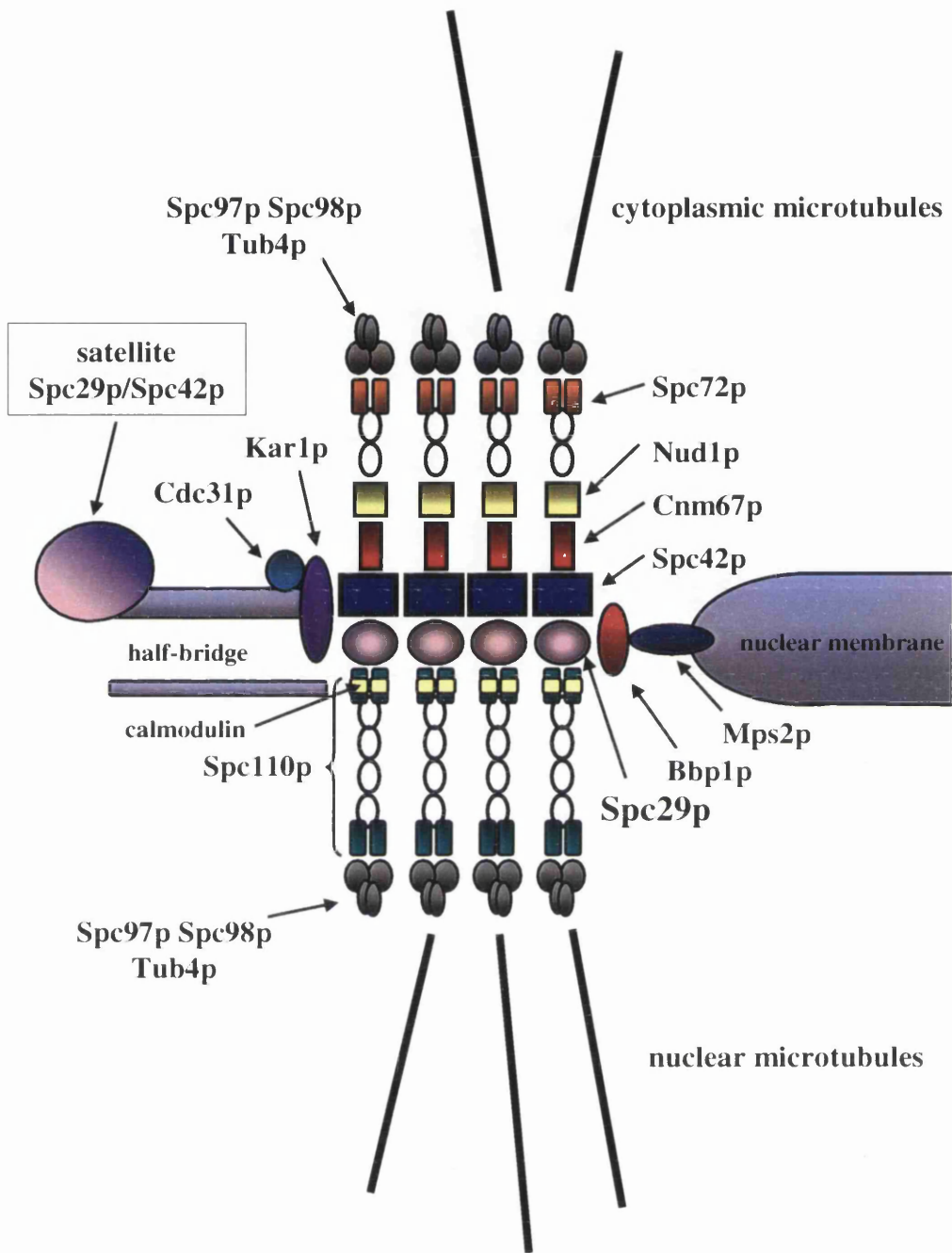
Figure 27 Schematic diagram of the spindle pole body

site is not lethal, but suppresses the *cmd1-1* mutation led to the suggestion that the calmodulin binding site of Spc110p has an inhibitory function that is relieved by its deletion or by calmodulin binding (Geiser *et al.*, 1993; Stirling *et al.*, 1994). The two hybrid interactions between Spc110p mutants and Spc29p presented here are in agreement with this model and raise the possibility that Spc29p binding to Spc110p is regulated by calmodulin. It is likely that calmodulin binds first to Spc110p reliving the inhibition caused by the very C-terminus of Spc110p, therefore allowing Spc29p to bind to Spc110p (Figure 28). This ordered assembly of the Spc110p/calmodulin/Spc29p complex may be important before or during SPB duplication. It is also interesting to find that the region of Spc110p that contains the Spc29p-binding site overlaps with Region II described by Sundberg and Davis (1997) as being important for binding of Spc110p to the central plaque.

Size regulation of the SPB is an area of regulation that is not really understood. It has been observed that diploid cells have SPBs that are twice the size of SPBs in haploid cells (Byers and Goetsch, 1975a) and that the size of the SPB correlates with the number of nuclear microtubules organised. Also, expression of the components of the Spc110p-containing SPB subcomplex fluctuates during the cell cycle, mRNA abundance of *SPC110, SPC42* and *SPC29* increases in G1 at the time of SPB duplication (Cho *et al.*, 1998; Zhu and Davis, 1998). However, do the expression levels of SPB components control the size of the SPB? Overexpression of *SPC42* has been shown to drastically increase the size of the Spc42p polymer at the central plaque (IL2 layer) (Donaldson and Kilmartin, 1996), but there is no effect on the inner or outer plaques. Overexpression of *SPC110* causes aggregation of Spc110p in the nucleus (Kilmartin and Goh, 1996; Sundberg *et al.*, 1996) and overexpression of *SPC29* causes accumulation of Spc29p in the nucleus. However, when all three are co-overexpressed in the same cell there is a dramatic increase in the size of both the central and inner plaques. This indicates that the expression levels of SPB components may contribute to the size regulation of the SPB. If this is so, there must also be additional controls in place to direct SPB components to the satellite and prevent SPB components from assembling at the mother SPB during duplication. These controls have not been identified, however in mutants that prevent formation of a satellite (for example *cdc31(ts)* and *kar1(ts)*), the cells arrest with a single enlarged SPB (Byers, 1981a; Rose and Fink, 1987). Even more confusing is the recent suggestion that there is turnover of SPB components throughout the cell cycle and that the protein level of GFP-Spc29p peaks in mitosis (Davis and Yoder, Colorado meeting, 2000). In a way, this certainly
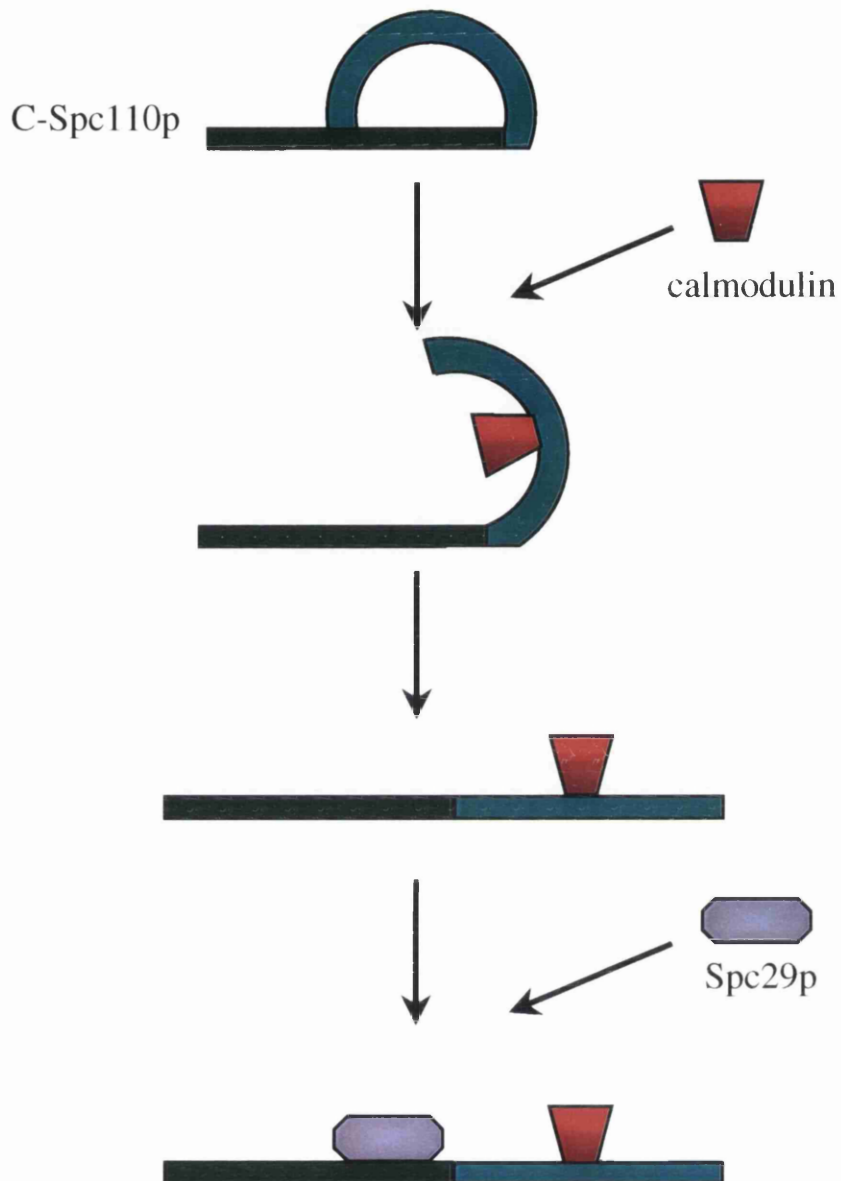
Figure 28 A model for binding of Cmd1p and Spc29p to Spc110p

Binding of Cmd1p to the C-terminus of Spc110p relieves a self-inhibition loop within Spc110p, allowing Spc29p to bind.

makes sense, as during numerous divisions it is hard to imagine that an SPB can survive for generations without turnover of its core components, however why there would be an increase in the protein levels of SPB components in mitosis is yet to be investigated.

Spc29p is involved in SPB duplication

To investigate the role of Spc29p, temperature sensitive mutants were constructed. The mutations introduced into *SPC29* allowed the cells to grow as wild type at the permissive temperature, but caused lethality at the non-permissive temperature. The cause of this lethality can be elucidated by analysing the mutant cells after short incubations at the non-permissive temperature. Synchronised *spc29(ts)* mutant cells were analysed by FACS and, after 3 hours at the non-permissive temperature, by immunofluorescence and electron microscopy. This line of investigation showed that Spc29p is involved in SPB duplication as most *spc29(ts)* mutants arrested in G2 as large budded cells with replicated, but not segregated chromosomes and monopolar spindles emanating from a single SPB signal. This single SPB was usually positioned in an invagination of the nuclear membrane close to the bud neck and showed abnormal morphology as seen by electron microscopy. A similar result was reported by Adams and Kilmartin (1999) who analysed *spc29-20* cells after 2 or 4 hours at the non-permissive temperature. After two hours at the non-permissive temperature, a second smaller SPB could be observed close to, but not connected to the larger SPB. However, after 4 hours at the non-permissive temperature only one SPB could be detected by electron microscopy indicating that the smaller SPB was unstable and had most likely disintegrated. This SPB duplication defect indicates that Spc29p may not be solely a structural protein and due to the variety of different SPB morphologies, may be involved in more than one step in SPB duplication. These observations led to the investigation of a possible interaction between Spc29p and proteins known to be involved in SPB duplication.

Initial studies investigated a possible link between Spc29p and components of the half-bridge. The presence of multiple copies of *CDC31* was found to suppress the defects of *spc29-2* cells, but a single extra copy of *KAR1* increased the temperature sensitivity of *spc29-3* cells. These genetic interactions point to a possible functional relationship between Spc29p and Cdc31p/Kar1p. This was further investigated by immuno-precipitation experiments. When Spc29p was purified, Kar1p and Cdc31p both co-

purified with Spc29p in wild type cells. Spc29p was also purified from *CDC31-16 Δkar1* cells, but Cdc31-16p did not co-purify with Spc29p. This result indicates that either Spc29p interacts with Cdc31p via Kar1p or that the stability of the complex is compromised in the absence of Kar1p. However, the *CDC31-16* mutation is termed a "gain of function" mutation as it allows the deletion of the essential *KAR1* gene. This led to the hypothesis that Cdc31-16p is able to interact with another SPB component independently of Kar1p, this interaction may be weaker in wild type cells or may not occur at all. This hypothesis is supported by the finding that Cdc31-16p localises to the SPB in the absence of Kar1p, whereas in wild type cells localisation of Cdc31p to the SPB has been reported to be dependent on Kar1p (Biggins and Rose, 1994; Spang *et al.*, 1995). An interesting observation was that *CDC31-16 Δkar1* cells contained SPBs with half-bridges that appeared thicker and longer than in wild type cells, but only extended over the cytoplasmic side of the nuclear membrane. Centrins in other organisms are present at the centrosome and basal bodies (Salisbury, 1995). The function of centrins at the centrosome is yet to be fully understood, however centrin present in the flagella apparatus of unicellular green algae forms contractile fibres that connect basal bodies to one another and to the nucleus (Salisbury *et al.*, 1984). These fibres are involved in basal body localisation, orientation and segregation (McFadden *et al.*, 1987; Taillon *et al.*, 1992; Wright *et al.*, 1989; Wright *et al.*, 1985). Centrins form fibres *in vitro* (Wiech *et al.*, 1996) however, fibre formation is prevented by addition of Kar1p peptides to the reaction. In the absence of Kar1p, a membrane protein, there is no known connection between Cdc31p and the nuclear membrane, so how is the half-bridge formed in *CDC31-16 Δkar1* cells? There is little evidence for formation of Cdc31p polymers in budding yeast, however in the absence of Kar1p, which inhibits centrin filament formation *in vitro*, it is possible that the half-bridge seen in *CDC31-16 Δkar1* cells is a filament of Cdc31-16p anchored at the central plaque and the satellite. If this theory is true then Cdc31-16p must interact with at least one other SPB component, most likely is the formation of a complex that includes Spc29p due to its localisation at the central plaque and the satellite. In further support of an interaction between Spc29p and Cdc31p, Spang et al (1995), reported that a protein of approximately 30 kDa binds to Cdc31p as demonstrated by crosslinking studies.

One protein that has been proposed to be involved in the gain of function interaction with Cdc31-16p is Dsk2p. Dsk2p was proposed due to numerous genetic interactions between *CDC31*, *KAR1* and *DSK2* alleles (Biggins *et al.*, 1996). *DSK2* was also found as a multicopy suppresser of *spc29-2* and *spc29-3* mutants, indicating that Dsk2p

functions with Spc29p. Purification of Dsk2p-3Myc and Dsk2p-ProA gave inconclusive results, due to insufficient protein levels (Dsk2p-3Myc) or the presence of IgGs in the precipitate (in the Dsk2p-ProA purification). Although attempts to identify a Dsk2p-containing complex by immunopurification yielded no convincing results, further investigation may shed light on the function of Dsk2p and the involvement of Dsk2p with Spc29p and the components of the half-bridge. Work by Khalfan et al (2000) identified genetic interactions of *karl∆17*, *cdc31-2* and *∆dsk2∆rad23* mutants with components of the *PKC1* cell integrity, signal transduction pathway, though the significance of these interactions is unclear.

Spc29p interacts with Bbp1p

BBP1 is an essential SPB component (Wigge *et al.*, 1998) that was identified as a multicopy suppresser of the temperature dependent growth defect of *spc29-2* and *spc29-3* cells. This interaction raised questions regarding the function of Bbp1p. Bbp1p was then found to interact with Spc29p in the two hybrid system. Purification of Bbp1p failed to identify a complex containing Spc29p, however a complex consisting of Bbp1p and Mps2p was identified (Schramm *et al.*, 2000). Mps2p and Bbp1p both localise to the periphery of the SPB (Munoz-Centeno *et al.*, 1999; Schramm *et al.*, 2000) where they are involved in inserting the newly formed SPB into the nuclear membrane during SPB duplication. This can be seen in the *bbp1-1* and *mps2-1* mutants which arrest at the non-permissive temperature with a duplication plaque that is unable to insert into the nuclear membrane (Schramm *et al.*, 2000; Winey *et al.*, 1991b). It is the central plaque that is embedded in the nuclear membrane, therefore it is probable that Mps2p in the membrane interacts with a central plaque component via Bbp1p. Therefore Spc29p was tested for direct binding to Bbp1p. *In vitro* translated Spc29p interacted with recombinant GST-Bbp1p and the Bbp1p-Mps2p complex. It is possible then to suggest that the Mps2p-Bbp1p-Spc29p complex forms the hook-like structures seen connecting the SPB to the nuclear membrane by high voltage electron tomography (O'Toole *et al.*, 1999).

BBP1 interacts with *KAR1* genetically, as with *spc29(ts)* mutants, a single additional copy of *KAR1* increased the temperature dependent phenotype of *bbp1-1* cells (Schramm *et al.*, 2000). Bbp1p also interacted with Kar1p in the two hybrid system, this is not necessarily a direct interaction as the two hybrid system identifies interactions *in*

vivo, therefore any interaction seen may be via other proteins present in the yeast cells. However, it is possible that the Bbp1p/Mps2p complex interacts with the half-bridge where it meets the central plaque (Figure 29). Two hybrid interactions were also seen when Spc29p was combined with Cdc31p or Kar1p, however these results were not consistent. Despite this there are now indications, for the first time, of a link between core SPB components and half-bridge components.

Conclusions

Spc29p appears to interact with a number of different proteins (Figure 30) This has been shown using numerous genetic and biochemical methods. During these studies it appeared that some interactions were more obvious and more easily explained than others. For example it is easy to state that Spc29p is a structural component of the central plaque where it interacts with Spc42p and Spc110p. However this simple model does not explain the interactions with Cdc31p, Kar1p and Bbp1p, how do these interactions fit into the model? It may be that multiple proteins interact with Spc29p at the periphery of the Spc29p layer within the central plaque. These interactions would be important during SPB duplication and/or insertion of the newly formed SPB into the nuclear membrane. In this model the strongest interactions would be Spc29p with Spc110p and Spc42p at the central plaque, Bbp1p with Mps2p in the membrane at the periphery of the SPB and Cdc31p with Kar1p at the half-bridge. The weaker interactions would involve Spc29p, around the rim of the central plaque or at the satellite, interacting with Bbp1p/Mps2p and Cdc31p/Kar1p. These weaker interactions may explain why Spc29p was not present in precipitates from Bbp1p and Cdc31p purifications. Also, the Spc29p immunoprecipitate was probed for the presence of Bbp1p, Mps2p, Spc42p and Spc110p, unfortunately the only antibodies available for these proteins were rabbit antibodies, the secondary antibody therefore detected contamination by rabbit IgGs washed from the beads. These signals were strong and present on the gel in the areas where Bbp1p, Mps2p and Spc42p would be found, identification of Spc110p was inconclusive. However, Cdc31p and Kar1p could be detected specifically in the Spc29p purification using goat antibodies.

The observation that Spc29p is phosphorylated allows the formation of a hypothesis in which phosphorylation of Spc29p may regulate interactions of Spc29p with different proteins at different cell cycle stages.

A

cytoplasmic microtubules

Spc42p

Spc29p

Bbp1p

Mps2p

nuclear membrane

calmodulin

Spc110p

nuclear microtubules

B

Kar1p-Cdc31p complex
at the half-bridge

Spc29p at the
central plaque

Spc29p at the
satellite

Bbp1p-Mps2p
complex
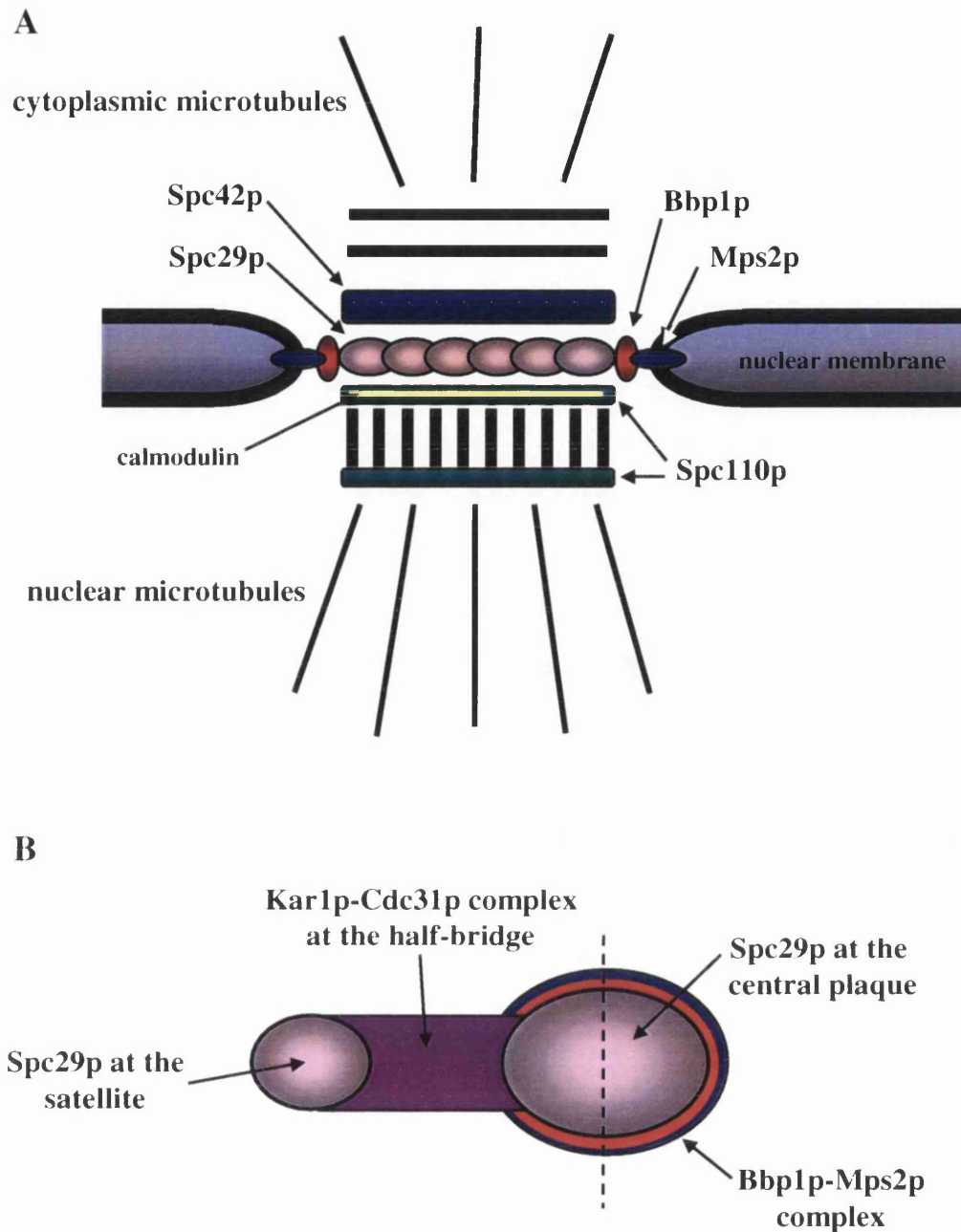
Figure 29   Model describing the interactions between Spc29p, the Bbp1p/Mps2p complex and the Cdc31p/Kar1p complex

A Cross section through an SPB showing Bbp1p-Mps2p binding Spc29p at the central plaque.

B   Horizontal cross section through Spc29p layer at the central plaque showing that the Bbp1p-Mps2p complex forms a ring around the periphery of the central plaque. Dotted line shows position of cross section in **A**

Figure 30 Summary of Spc29p interactions

This research into the structure of the budding yeast spindle pole body and the control of its duplication have significance in other fields and to 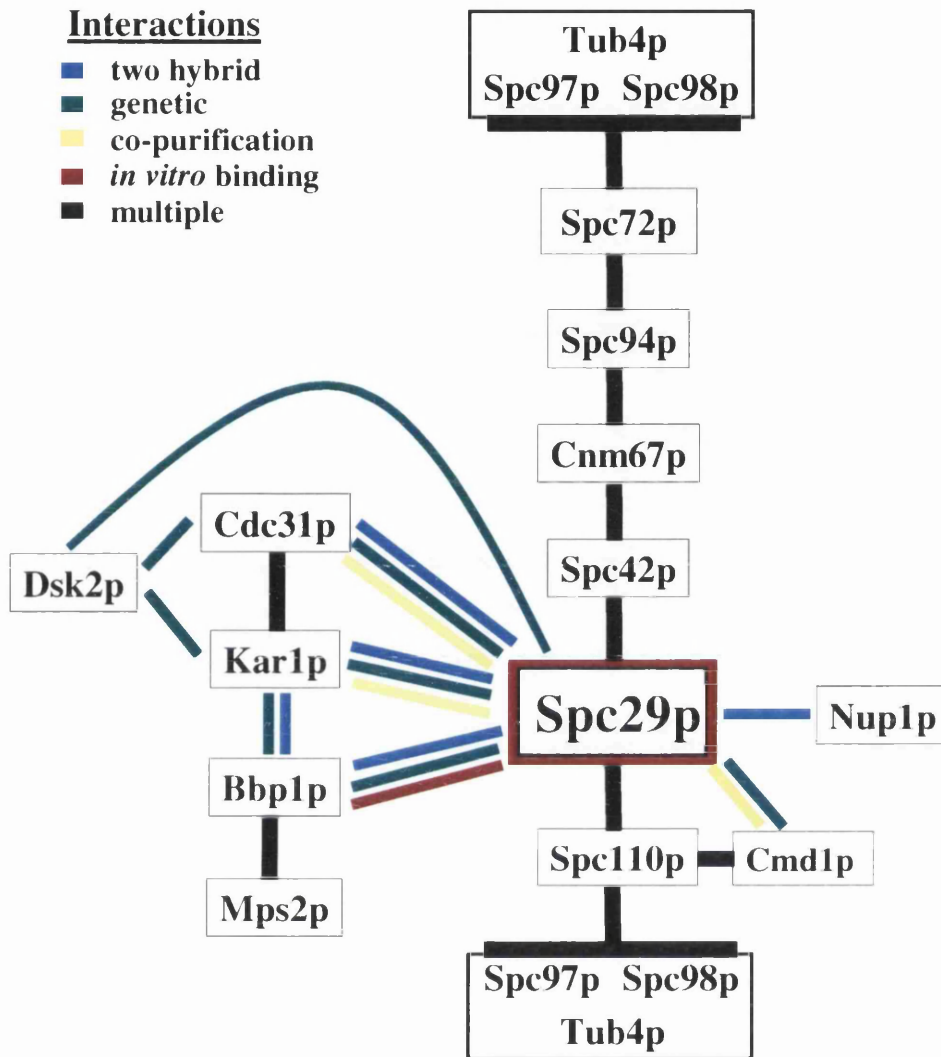other organisms. Since the discovery of Tub4p (γ-tubulin) the budding yeast spindle pole body has become one of the most studies microtubule organising centres. Despite its structural difference to the centrosome, the SPB and centrosome share homology in their component proteins. Understanding the centrosome and how its duplication is controlled is an integral part of cancer research. Fidelity of centrosome duplication is important to ensure that the replicated genome is correctly separated during mitosis and that mother and daughter cells each receive a full set of chromosomes. It has been shown that centrosomes are abnormal in number, form and function in a wide variety of human malignant tumours (Lingle *et al.*, 1998; Pihan *et al.*, 1998). It is easy to see how a defect in centrosome duplication could lead to aneuploidy and genetic instability, resulting in overexpression of oncogenes or loss of tumour suppresser genes and therefore contribute to the progression of cancer (Doxsey, 1998). The identification of pathways and proteins that regulate centrosome function and duplication would provide an opportunity to determine the origins of cancer and develop novel treatments. Recently a protein related to Spc110p has been implicated in the development of cancer (Doxsey *et al*, ASCB meeting, San Francisco, 2000). As yet no Spc29p homologues have been identified, however Spc29p interacts with two proteins (Spc110p and Cdc13p) that are known to have homologues in higher eukaryotes, therefore it is more than likely that as more discoveries are made and more genomes are sequenced, that homologues will be identified.

The Future

Were this project to continue, it would focus on elucidating the functions of the Spc29p interactions with Cdc31p/Kar1p, Dsk2p and Bbp1p/Mps2p, with the hope of gaining a greater understanding of spindle pole body duplication and of microtubule organising centres in general.

Further to this aim, a search for homologues of Spc29p would be carried out. This could be done by screening for suppression of *spc29(ts)* mutants using human, *Xenopus* or *S. pombe* cDNA libraries.

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