The role of the fission yeast Wis1 pathway in stress response and cell cycle control.

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To Yerma, Yerpa and Yersis

"all a genetic heritage has to do is transmit what is transmitted to it for transmitting, not giving a damn about how it's received."

Priscilla: I. Mitosis in Time and the Hunter, Italo Calvino, 1967, Torino.

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"It takes a good bit of gear and a lot of knowledge."

Just think about it.

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Abbreviations used in this thesis

°C	degrees Centigrade
μ	micro-
Amp	ampicillin
ars	autonomously-replicating sequence
ATP	adenosine tri-phosphate
CAK	cdk-activating kinase
cdi	cdk inhibitor
cdk	cyclin-dependent kinase
CS	cold sensitive
DAPI	4',6-diamidino-2-phenylindole dihydrochloride
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide-triphosphate
EDTA	ethylenediaminetetraacetic acid
EMS	ethylmethylsulphonate
g	gramme
HOG	High-Osmolarity Glycerol
hsp	heat-shock protein
JNK	c-Jun N-terminal Kinase
k	kilo-
kb	kilo base
l	litre
m	metre, milli-
M	Molar
MAPK	Mitogen-Activated Protein-Kinase
MAPKK	MAPK Kinase
MAPKKK	MAPKK Kinase
ME	Malt extract medium
MM	Edinburgh Minimal Medium
MMK	Edinburgh Minimal Medium supplemented with 1.2M KCl
MMPB	Edinburgh Minimal Medium supplemented with phloxin B
MNNG	1-Methyl-3-nitro-1-nitrosoguanidine
mol	moles
mw	molecular weight
NPD	Non-parental ditype
OD	optical density

ORF	Open Reading Frame
ori	origin of replication
p	pico-
PB	phloxine B
PCR	Polymerase Chain Reaction
PD	parental ditype
SAPK	Stress-Activated Protein-Kinase
SD	budding yeast minimal medium
SDW	sterile deionised water (autoclaved)
T	threonine
T_m	melting temperature
TAE	tris acetate EDTA
TBE	tris borate EDTA
TE	tris EDTA
Thr	threonine
Tris	tris (hydroxymethyl) aminomethane
ts/TS	temperature sensitive
TT	tetratype
Tyr	tyrosine
U.V.	ultra-violet
Y	tyrosine
YE	Yeast Extract (supplemented with adenine and uracil)
YEK	Yeast Extract Medium supplemented with 1.2M KCl.
YEPB	Yeast Extract containing Phloxin B
YES	Yeast Extract supplemented with 0.9M sorbitol

Abstract

The *wis1* gene of fission yeast encodes a Mitogen-Activated Protein Kinase Kinase (MAPKK). These enzymes are part of architecturally- and functionally- conserved signal transduction modules, which have been found in all eukaryotes studied. Several such pathways exist in a single cell type and each responds to a different signal, which activates a MAPKKK. This kinase phosphorylates and activates a MAPKK, such as Wis1, which in turn phosphorylates and activates a MAPK. Activated MAPKs phosphorylate transcription factors and other proteins. The MAPKKKs upstream of Wis1 are Win1 and Wis4; the MAPK downstream is Sty1.

wis1 is not an essential gene, but if deleted (*wis1*Δ) it causes defects in cell-cycle and mating and gives rise to sensitivity to stresses such as high temperature and high osmolarity. The *wis1* MAPK pathway regulates genes such as *ste11*, *fbp1*, *ctt1*, *gpd1*, *pyp2* and *tps1* and acts antagonistically to the cAMP pathway. This thesis consists of work on the *wis1* pathway: the analysis of some of the upstream components and the isolation and characterisation of genes that lie downstream of *wis1*.

The *mcs4*, *win1* and *wis4* genes had already been shown to lie upstream of *wis1*. Strains were constructed with different combinations of mutations in these genes. *fbp1* transcription was assayed in these strains. An additive effect was seen in *win1 wis4* double mutants, suggesting that *win1* and *wis4* act in parallel.

To identify functionally-related genes downstream of *wis1*, the stress sensitivity of *wis1*Δ cells was exploited. A screen for extragenic suppressing mutations was carried out. Several hundred heat resistant mutants were isolated. Some also suppressed the salt sensitivity and/or cell length defect of *wis1*Δ. Twelve such *sow* (for suppressor of *wis1*Δ) mutants, each containing a single suppressing mutation, were analysed further. They fell into two linkage groups: *sow1* (nine strains) and *sow2* (three strains).

When the *sow* mutations were crossed into a *wis1*$^+$ background, both *sow1* and *sow2* were able to grow at temperatures above the usual range for *S. pombe*. In addition, *sow1* strains divide at a shorter length than wild type, indicating a mitotic advance, and *sow2* cells have a slightly aberrant morphology.

To determine whether the *sow* mutations corresponded to any known genes, crosses were carried out between the *sow* mutants and mutants in the following genes: *wis1* pathway genes (*sty1*, *atf1*, *ppa1*, *ppa2* and *ppe1*), cAMP pathway genes (*cyr1*, *pka1*), cell cycle regulation genes (*cdc2*, *cdc25*, *wee1*, *cdc13*), a heat shock protein (hsp90) gene (*swo1*) and a gene required for maintenance of the mitotic cell cycle (*pat1*). No linkage to *sow1* or *sow2* was observed, so a mechanism for the genetic interaction between the *sow* mutants and *wis1* remains unknown.

Furthermore, in most cases, no striking genetic interaction was seen in the double mutants. However, a genetic interaction was seen with TS alleles of *cdc13*, *wee1* and *cdc27*: the introduction of a *sow1* or *sow2* mutation partially rescues the mutant phenotype, which suggests that the *sow* genes may have a role in regulating internal osmolarity. This is interesting as the osmotic stabiliser, sorbitol, suppresses the temperature sensitivity of a *wis1*Δ strain, conceivably in the same way as a *sow* mutation.

Chapter 1: Introduction

All living organisms are made up of cells. Whether the organism is a yeast, consisting of a single cell or a human being with many millions of millions of cells, new cells needed for growth and the replacement of old cells arise from existing cells dividing to produce two new cells.

Cells in the laboratory are well fed, sheltered from unpleasant changes in environmental conditions; and encouraged to live it up a little. The wild is drastically less forgiving: hazards are encountered frequently and suddenly and cells have had to evolve stress response mechanisms for tolerating myriad dangers so that they can survive even under harsh conditions.

These two processes, stress response and cell cycle control, are crucially important for ensuring continued survival. What is more, the processes interact. One gene that has a crucial involvement in this cross-talk is *wis1* and its investigation is the subject of this thesis.

1.1: The Cell Division Cycle.

1.1.1: The importance of regulating the Cell Cycle.

Cell division is a complex process. At the end of each cycle a single cell must divide to give rise to two cells that can themselves divide and so on. To achieve this, the parental cell must contain enough components to make two viable daughter cells. The cytoplasm and its contents (mitochondria, ribosomes and so on) can be split roughly between the daughter cells, but each daughter must inherit exactly one copy of the genome in a single intact nucleus.

It is the role of a process known as the nuclear division cycle to ensure this exact duplication of the genetic material and its equal segregation to two daughter nuclei. This process would not be an ubiquitous part of cellular biology if cells had not evolved a careful regulation mechanism.

The cell must make sure that it has sufficient resources to complete division before it embarks on the process. To ensure this, the nuclear

division cycle is tightly linked to cell growth (ie accumulation of cytoplasm). If this were not the case, cells would gradually increase or decrease in size with each successive division which would inevitably lead to death.

In metazoans, the situation is complicated further by the need for cells to coordinate growth and division with that of their neighbours (a process which is cerntral to development). In these organisms, loss of tight cell cycle control, leading to inappropriate cell division gives rise to tumours as well as developmental abnormalities, and is thus a major cause of disease in humans. In single-celled organisms, aberrations in cell cycle control also lead to inappropriate division, and a state that is conceptually similar to cancer. However, as they are easier to study, unicellular eukaryotes such as the yeasts offer an excellent experimental system for studying the control of the cell cycle.

1.1.2: The eukaryotic cell cycle.

In all eukaryotes, the cell division cycle runs in parallel to the nuclear division cycle. The cell division cycle involves the continuous accumulation of cytoplasm and cell division itself, but the nuclear division cycle (or cell cycle) is regarded as the more important of the two. In eukaryotes, the cell cycle is divided into separate phases, with checkpoints that regulate the transitions between them. Once cells embark on a new cell cycle, they are committed to completing it.

The beginning of the cell cycle is taken to be a point called Start in yeasts and the restriction point in higher animal cells (see Figure 1.1). This takes place in a phase called G1 (for Gap phase). Straight after passing Start, cells enter S phase (DNA Synthesis) during which they duplicate their DNA.

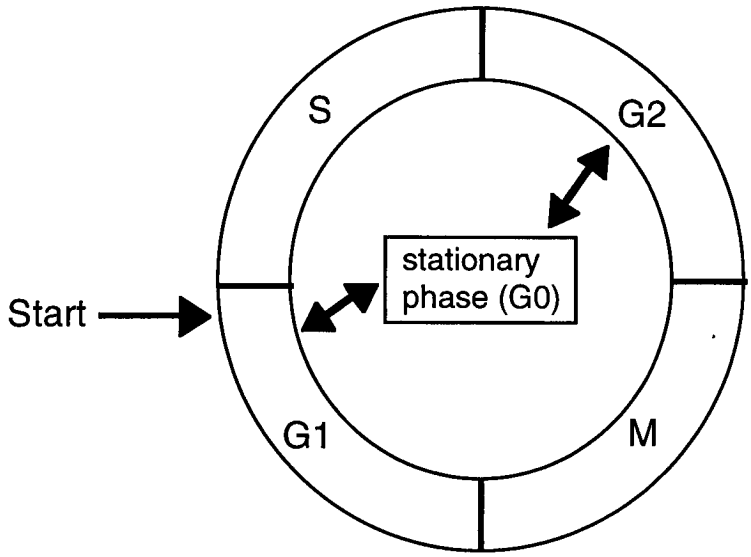

Figure 1.1: The eukaryotic cell cycle.
If the size and nutritional conditions are satisfied, cells may pass Start (or the restriction point in higher cells) and enter S phase (DNA Synthesis); cells lacking sufficient nutrients temporarily exit the cell cycle and pass into G0 (stationary phase). After S phase, cells remain in G2 until they satisfy the conditions of the G2-M checkpoint, when they enter M phase (mitosis). Certain types of cells can enter stationary phase from G2. After M phase, the daughter cells return to G1, and the cycle begins again.

Following S phase, there is a second gap phase, called G2, and cells enter M phase (mitosis). During mitosis, the chromosomes condense, the nuclear membrane breaks down (except in yeasts and some other organisms) and the sister chromatids separate and two new nuclei form around them. What follows is cell division itself (cytokinesis), when the cytoplasm divides to form two daughter cells.

In order to enter S or M phase, the cell must be large enough. This size checkpoint is the method cells use to gauge whether they have enough resources to complete the process. Thus at each of these two points (the G1-S (Start) and G2-M transitions) cell cycle is linked to cell growth. This also ensures that cells maintain a constant size at division.

In eukaryotes, cells which are not actively dividing enter a quiescent state (G0), from which they may later re-enter the cell cycle. In yeasts, this phase is called stationary phase and it is a distinct, highly stress-resistant part of the cell cycle (discussed further in Section 7.1) In the absence of an appropriate mating partner, yeast cells enter stationary phase if they do not have sufficient nutrients to pass Start. However, if a

partner is present, then the sexual development pathway is entered at this point. The end product of this is spores, another stress-resistant developmental stage.

1.1.3: Studying the cell cycle in yeasts

Yeasts are ideal for genetic studies as they are eukaryotes with small genomes, have haploid stages in their life cycles and quick replication times. The two yeasts used extensively are the budding yeast, *Saccharomyces cerevisiae*, and fission yeast, *Schizosaccharomyces pombe* (see Figure 1.2).

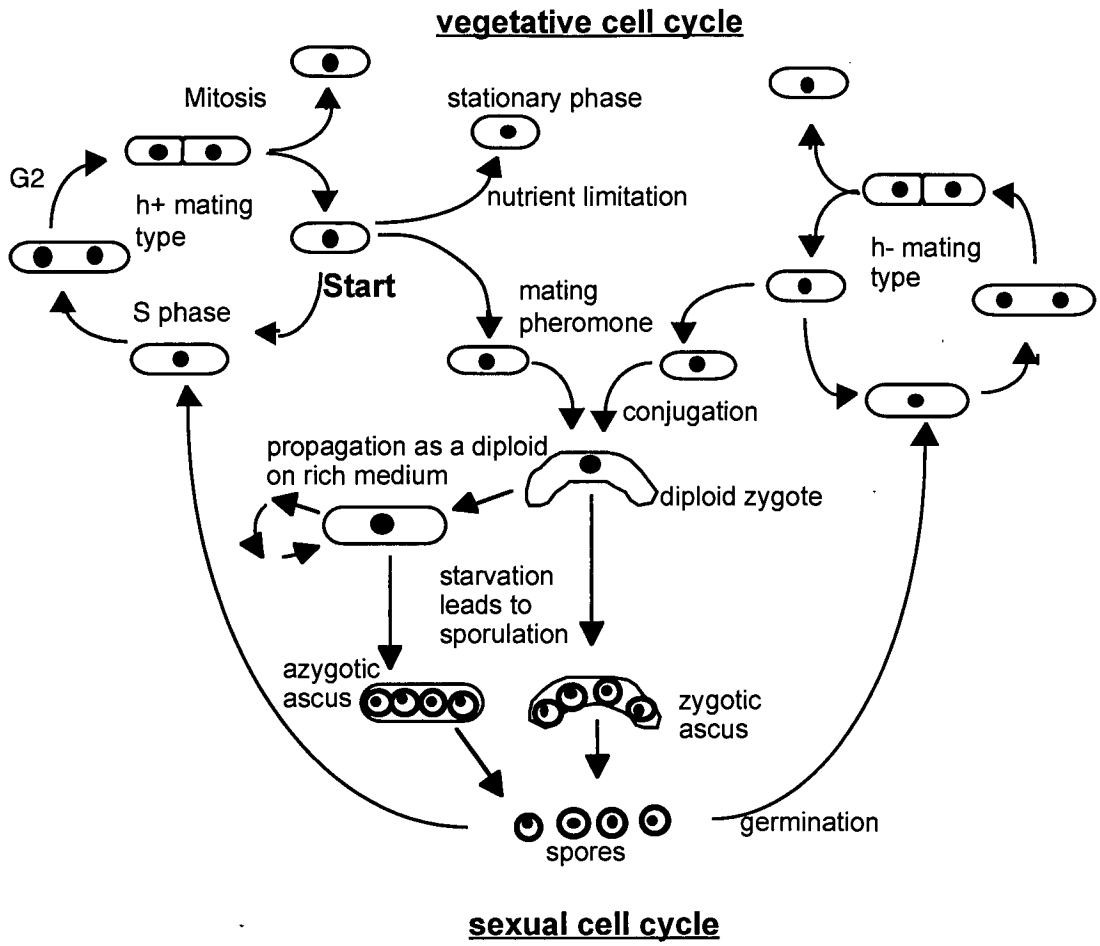

Figure 1.2: The life cycle of the fission yeast, *Schizosaccharomyces pombe*.
Schizosaccharomyces pombe cells normally proliferate mitotically as haploids in one of two mating types, h^+ and h^- (true wild type cells (h^{90}) switch mating type). Under nutrient limitation cells enter stationary phase unless a mating partner of the opposite mating type is present. In this case reciprocal emission and receipt of pheromone induces cell cycle arrest just before Start and conjugation, which gives rise to a diploid zygote. If nutrients are made available at this point, the diploid can proliferate mitotically, but under continued starvation, meiosis takes place immediately, giving rise to an ascus containing four haploid spores. These hatch and re-enter the haploid mitotic cell cycle once nutrients become available.

The two yeasts are distantly related, and probably as close to each other as they are to higher eukaryotes (reviewed in Sipiczki, 1995). The cell cycle in both organisms is regulated by external environment (nutrition and stress) and cell-cell interactions (mating), so the yeasts are choice organisms for studying how these signals affect the cell cycle. Furthermore, given the universal nature of cell cycle regulation, observations from studies in yeasts may act as a guide to more complex systems, such as humans.

A key contribution to understanding cell cycle in fission yeast came from a screen for temperature sensitive cell division cycle (*cdc*) mutants (Nurse et al., 1976).

Fission yeast cells grow solely by tip extension. Therefore, because *cdc* mutants fail to divide but continue to grow, *cdc* strains that are maintained at the restrictive temperature become very long without septa (the *cdc* phenotype). In addition to this, any acceleration or retardation in the timing of cell division (ie entry into M phase) is visible as a shortening or a lengthening, respectively, of the size at which the cells divide (Mitchison, 1957; Mitchison, 1990; Nurse and Thuriaux, 1980). These properties make *S. pombe* particularly useful for studying cell cycle control at the G2-M transition, and is the reason why the organism was used to study this important aspect of biology in the first place.

1.1.4: Regulation of the cell cycle.

The proteins that are responsible for cell cycle regulation are highly conserved in all eukaryotes: entry into M phase is regulated by a complex consisting of two proteins, Cdc2 and cyclin (Lohka et al., 1988). The abundance of cyclin varies during the cell cycle (Evans et al., 1983) and as cyclin binding activates Cdc2, the activity of Cdc2 also oscillates during the cell cycle (Gerhart et al., 1984). Cdc2 is also referred to as a cyclin-dependent kinase (cdk), because it must bind cyclin to be active.

Not only is there a Cdc2 homologue in all eukaryotes, but its sequence is highly conserved, with roughly 65% identity. The various cyclin subunits and many other components of the machinery that regulates the cell cycle are also conserved. This is not surprising, given the importance of the process.

1.1.5: Regulation of Cdc2 in fission yeast.

In the fission yeast, Cdc2 regulates both the G1-S and G2-M transitions. There is also a single M-phase cyclin, Cdc13 (Moreno et al., 1989).

Like many proteins, Cdc2 is regulated by phosphorylation. There are two key residues. Phosphorylation on tyrosine 15 (Y15) is inhibitory and phosphorylation on threonine 167 (T167) is necessary for activation (see

Figure 1.3). In addition, T14 phosphorylation has been reported in *S. pombe*, but plays no clear biological role (Carr et al., 1989; MacNeill and Nurse, 1993)

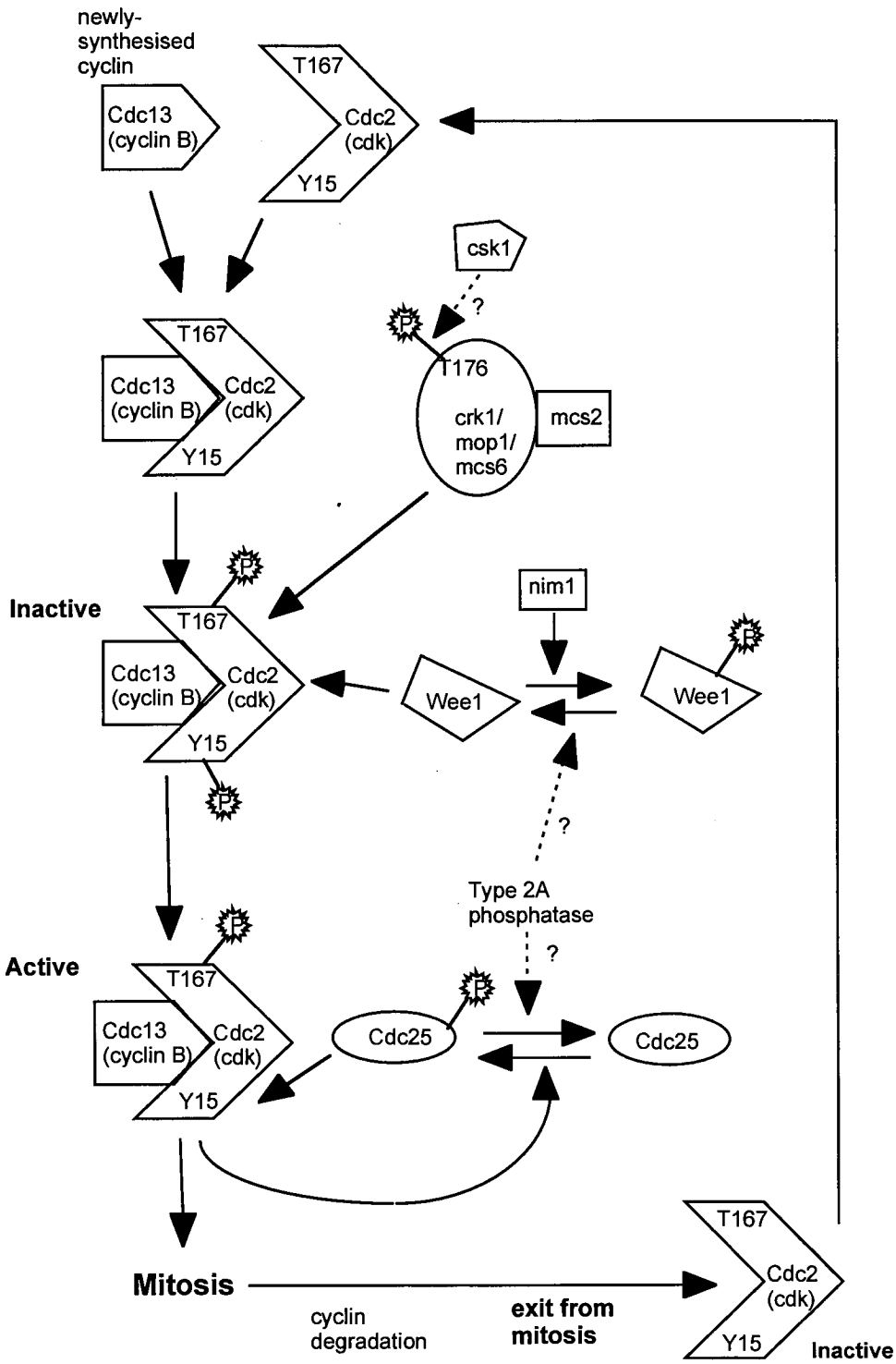

Figure 1.3: regulation of Cdc2/Cdc13 at G2-M.
A Cdc13 cyclin and a Cdc2 cdk subunit form a complex which is phosphorylated on T167 by CAK and on Y15 by Wee1 and Mik1. Dephosphorylation of Y15 by Cdc25 and Pyp3 activates this cdk/cyclin and brings about entry into mitosis. Type 2A phosphatases may help regulate this step by activating Wee1 and inactivating Cdc25 by dephosphorylating these proteins.

Regulation of the phosphorylation on tyrosine-15 (Y15) is best understood. This residue is phosphorylated by Wee1 and Mik1 kinases from the end of S phase. Completely unphosphorylated Y15 (for example in a *wee1 mik1* double mutant) leads to immediate entry into mitosis (mitotic catastrophe) (Lundgren et al., 1991). The activities of Wee1 and Mik1 kinases are balanced by at least two phosphatases: Cdc25 (Russell and Nurse, 1986) and Pyp3 (Millar et al., 1992), and perhaps Spt1 (Mondesert et al., 1994). Of these proteins, at least Cdc25 and Wee1 are known to be regulated by phosphorylation. This phosphorylation could be reversed by the Type 2A phosphatases, Ppa1 and Ppa2, which are mitotic inhibitors (Kinoshita et al., 1993).

The kinase that phosphorylates Wee1 is Nim1 kinase (Coleman et al., 1993). Interestingly, *nim1* is allelic to *cdr1* which had previously been isolated in cells deficient in altering cell cycle regulation in response to a change in nutritional conditions (Young and Fantes, 1987), so it appears conceivable that nutritional signals are fed into the cell cycle mechanism through the Wee1 pathway (Fantes et al., 1991).

The role and regulation of phosphorylation on T167 is becoming clearer. An enzyme called Cdc2-Activating Kinase (CAK) phosphorylates this residue. This CAK is encoded by *crk1/mop1/mcs6* (Buck et al., 1995; Damagnez et al., 1995) and the cyclin subunit by *mcs2* (Molz and Beach, 1993). Both are members of the basal TFIID transcription complex, as well as cell cycle regulators, which may explain why they are essential, and why the loss of function phenotype is not *cdc*.

In a manner that parallels that of Cdc2/Cdc13 activation, CAK (Mcs6/Mcs2) is itself subject to activational phosphorylation on a conserved threonine (T176) (Fisher and Morgan, 1994), which, in fission yeast, may be mediated by the Csk1 protein kinase (Molz and Beach, 1993).

1.1.6: Regulation of Cdc2 in other systems

In higher animal cells, the mechanism of cdk/cyclin regulation is analogous to that of the fission yeast, but in contrast to the single cdk of the unicellular eukaryote (Cdc2), there are at least seven cdks in human cells (Cdc2 corresponds to cdk1). In addition, there are families of

different cyclins that can assemble with the cdks in higher systems, whereas only four have been found in fission yeast so far.

Experiments in *Xenopus* addressing the regulation of cdk1 have shown that it is not phosphorylated unless complexed with cyclin (Meijer et al., 1991; Solomon et al., 1990). Yet, once it has formed a complex with a cyclin, it becomes an efficient substrate for phosphorylation. Furthermore, Type 2A phosphatases appear to play a role in cell cycle control in this organism (Clarke et al., 1993; Felix et al., 1990; Lee et al., 1991), as does CAK (cdk7) which binds a cyclin H homologue (Fesquet et al., 1993; Solomon, 1994) and phosphorylates T161 on cdk1 (the equivalent of T167 on *S. pombe* Cdc2). CAK/cyclin H is, like cdk1/cyclin, itself subject to activational phosphorylation on a conserved threonine (T176) (Fisher and Morgan, 1994),

At the end of mitosis, deactivation of *Xenopus* cdk/cyclin involves dephosphorylation on T161 in cdk1 as well as cyclin degradation (Lorca et al., 1992). The degradation of cyclin is mediated by Anaphase-promoting complex (APC) and involves the ubiquitin-mediated proteolysis pathway. The destruction of the cyclin in the cdk-cyclin complex is necessary for exit from the end of mitosis (telophase) and the return to interphase, presumably as it allows access to T161 by phosphatases, thus completing inactivation of the cdk.

1.1.7: Other regulators of the G2-M transition.

In addition to the core Cdc2/Cdc13 cdk/cyclin B and its direct regulators, many other proteins playing roles in the process have been identified. These proteins have several different functions: checkpoint function (DNA replication checkpoint: Cdc1, Cdc27; DNA damage/repair checkpoint: Rad1, Rad3, Rad9, Rad17, Rad26 and Hus1); phosphatases (Tyrosine phosphatases Pyp1 and Pyp2; Serine/Threonine phosphatases Type 2A: Ppa1, Ppa2, Ppe1, Type 1: Dis2, Sds21 and perhaps Type 2C: Ptc1, Ptc2, Ptc3) and a pathway consisting of stress response genes with a cell cycle role (the Wis1/Sty1 pathway). The Wis1 pathway is the subject of Section 1.3.3.

Protein serine/threonine phosphatases of Type 1, Type 2A and Type 2C have been found to exert a role in the G2-M transition in fission yeast. As

discussed earlier, Type 2A phosphatases (PP-2A) have also been implicated in cell cycle control in *Xenopus* (see for example Clarke et al., 1993). In fission yeast, the PP-2A phosphatases are encoded by *ppa1*, *ppa2* and *ppe1* (Kinoshita et al., 1990; Shimanuki et al., 1993). Double mutants including *ppa2*Δ are lethal, but other combinations are viable showing that these genes encode redundant functions. A *ppa2*Δ strain divides at reduced cell length, indicating mitotic advance. This advance may be mediated by Cdc25 (as is the case in *Xenopus*). *ppe1*Δ cells divide at a reduced cell length showing that Ppe1 plays a role in mitosis (Shimanuki et al., 1993).

Type 1 phosphatases (PP-1) are encoded by the *sds21* and *dis2* genes. *bws1* (*dis2*) was isolated as a multi-copy suppressor of the *wee1* bypass (suppression) of *cdc25* cell cycle block, showing that Bws1/Dis2 plays a role in mitotic control (Booher and Beach, 1989). The type 2C phosphatase gene *ptc3* is the only PP-2C that leads to reduced cell length when mutated. Furthermore *ptc3* suppresses a *swo1-26* mutation (Shiozaki and Russell, 1995b).

The tyrosine phosphatases also play a role in mitotic timing (Millar et al., 1992), by counteracting the Wis1 pathway. This will be discussed in Section 1.3.3. Pyp3 has been shown to dephosphorylate Cdc2 on Y15 (Millar et al., 1992).

1.2: Signal transduction pathways.

One way that individual cells ensure that they divide only when they are ready is by including checkpoints in the cell cycle. However, even single-celled organisms must communicate with their neighbours. In a metazoan, precisely and predictably coordinating the cell cycles of the myriad cells that make up the organism (ie the process of development) is crucial. Any serious deviation will almost always be detrimental, unless of course it produces a new species.

In order to interact with their surroundings and their neighbours, cells have evolved signal transduction pathways. The role of these pathways is to carry information from outside the cell to the nucleus, where that information affects cellular decisions. Most signalling pathways consist of transmembrane receptor proteins whose extracellular portion reacts to

external signals and causes the activation of a signal transduction module via intermediary adaptor proteins.

For example, the pathway which transfers mitogenic signals to the nucleus is well understood, and consists of a Mitogen-Activated Protein Kinase (MAPK) cascade. This signal transduction module transmits signals into the nucleus, where proteins and transcription factors required for mitosis are regulated.

1.2.1: The classical Mitogen-Activated Protein Kinase (MAPK) signal transduction cascade.

The MAPK cascade is a highly-conserved signal transduction unit, with a core made up of three proteins. Furthest downstream lies a MAPK, a proline-directed protein kinase whose targets include kinases and transcription factors (see Figure 1.4). The MAPK is activated by a MAPK kinase (MAPKK), which in turn is activated by MAPKK kinase (MAPKKK).

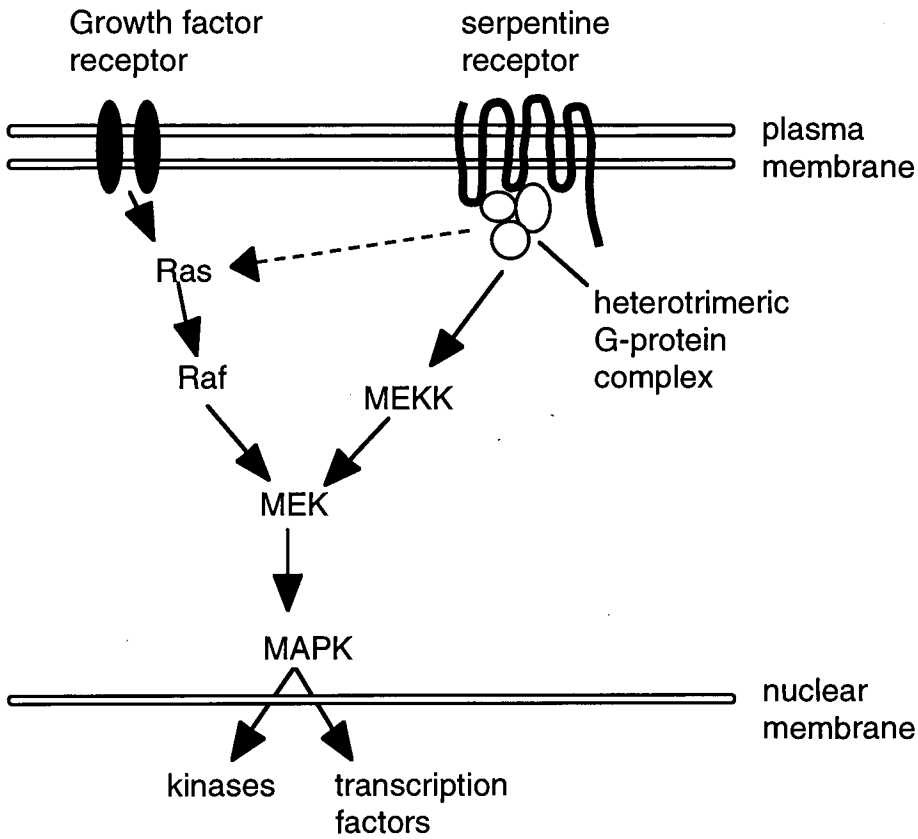

Figure 1.4: A schematic diagram of the metazoan MAPK pathway. MAPK is activated by the MAPKK MEK (Mitogen-Activated and Extracellular Signal Regulated Protein Kinase Kinase), which is activated by Raf and the MAPKKK, MEKK. Raf is activated through a receptor tyrosine kinase binding growth factor and thus activating Ras, as, well as via G-protein-linked receptors; MEKK1 is activated via a G-protein-linked receptor with seven membrane-spanning regions.

MAPKs are unusual in that they require phosphorylation on two conserved residues (a tyrosine and a threonine) for activation (Anderson et al., 1990). Both these activating phosphorylations are provided by MAPKK. In addition, evidence from *Xenopus* suggests that the N-terminal Nuclear Export Signal (NES) on MAPKK causes it to be excluded from the nucleus (Fukuda et al., 1996). Prior to activation, the MAPK is bound to the MAPKK and is hence restricted to the cytoplasm (Fukuda et al., 1997). On activation it is released and enters the nucleus (Fukuda et al., 1997; Lenormand et al., 1993).

MAPKKs are activated by phosphorylation on serine and threonine by MAPKKK. MAPKKKs are activated by various types of activated GTP-bound G-proteins. These are released from receptor complexes on binding

the appropriate ligand. Receptor tyrosine kinases (RTKs) are activated by the binding of such ligands as growth factor. RTKs are associated with small monomeric G-proteins like Ras, which are activated as a result of ligand binding. Trimeric G-proteins are associated with receptors whose amino acid chain crosses the plasma membrane seven times (Seven Trans-membrane Motif Receptors (STMR) or serpentine receptors). One of the G-proteins in the heterotrimer is activated and released when the receptor is activated.

A potential extra level of control could involve the negative feedback loop which appears to exist between MAPK and MAPKK: MAPKs can phosphorylate MAPKKs in both yeasts (Errede et al., 1993) and higher eukaryotes (Gonzalez et al., 1991). In the budding yeast pheromone pathway, this down-regulates them (Zhou et al., 1993) although the biological significance of this loop has not been determined.

1.2.2: Cross-talk and amplification by MAPK pathway.

The trio of MAPKKK / MAPKK / MAPK is a conserved element in signal transduction. The presence of three elements allows regulation by and signal integration with other pathways at several different levels in the pathway. Although there is evidence of cross-talk in higher cells (for areview, see Cooper, 1994) and perhaps in fission yeast (Yanagida, M.; pers. comm.), the only evidence of this in budding yeast, to date, is the observation that the pheromone pathway MAPKKK, Ste11, can phosphorylate the Hog1 pathway MAPKK, Pbs2 (Posas and Saito, 1997).

Furthermore, the presence of three kinases could in theory allow a small input signal to be amplified, thus producing a large effect. In the *S. cerevisiae* MAPK pathway, a scaffold protein Ste5 tethers a single molecule of each of the three kinases together, which prevents amplification of this sort (Faux and Scott, 1996), but which does not preclude the possibility that activated MAPKK can activate more than one molecule of MAPK.

1.2.3: Conservation of MAPKs

MAPK pathways have been found in all eukaryotes investigated and are highly conserved, both at the level of overall architecture and functional

homology between equivalent proteins in different organisms. This is demonstrated by the fact that the yeast MAPKKKs Ste11 (Rhodes et al., 1990) (*Saccharomyces*) and Byr2 (Wang et al., 1991) (*Schizosaccharomyces*) are functional homologues (Neiman et al., 1993; Styrkarsdottir et al., 1992). More impressively, the fission yeast mutation *spk1* (a MAPK) can be complemented by MAPKs from budding yeast, *Xenopus* and mammals (Neiman et al., 1993).

The conserved threonine and tyrosine residues that are phosphorylated in MAPK to activate it are separated by a single amino acid residue, making a TXY motif. The middle residue in this motif correlates with the class the protein belongs to: TEY in the mitogenic MAPKs (ERK1, ERK2, ERK5, Fus3, Kss1 and Spk1); TGY for the osmotic tolerance MAPKs (p38, RK, Hog1, Sty1); TPY for the stress-activated protein kinases (SAPKs) and c-Jun N-terminal Kinases (JNKs) and TNY for the budding yeast spore formation MAPK, Smk1. ERK3 is unusual in that it has an SEG motif. Furthermore, the TXY motifs found in plant MAPKs do not follow this correlation (reviewed in Hirt, 1997).

1.2.4: The Diversity of MAPK proteins and pathways.

The "classical" MAPK pathway is the mitogenic pathway, which is involved in proliferation and differentiation in higher eukaryotes and pheromone response in the yeasts. MAPK pathways that are involved in transducing other signals have been discovered. This is perhaps best demonstrated in budding yeast, for which the complete sequence of the genome exists. In this organism there appear to be a total of five pathways (reviewed in Hunter and Plowman, 1997). They are involved in pheromone response, high osmolarity tolerance, cell wall integrity, pseudohyphal development and spore formation.

1.2.5: The mating pheromone response MAPK pathways in yeasts.

In yeasts, the mating pheromone response pathway (the equivalent to the "classical" mitogenic mammalian pathway) lies downstream of a serpentine receptor. The activation of the MAPK cascade by pheromone binding the receptor is analogous to the mammalian system discussed above. This pathway is extremely well understood in budding yeast and fairly well in fission yeast (see Figure 1.5).

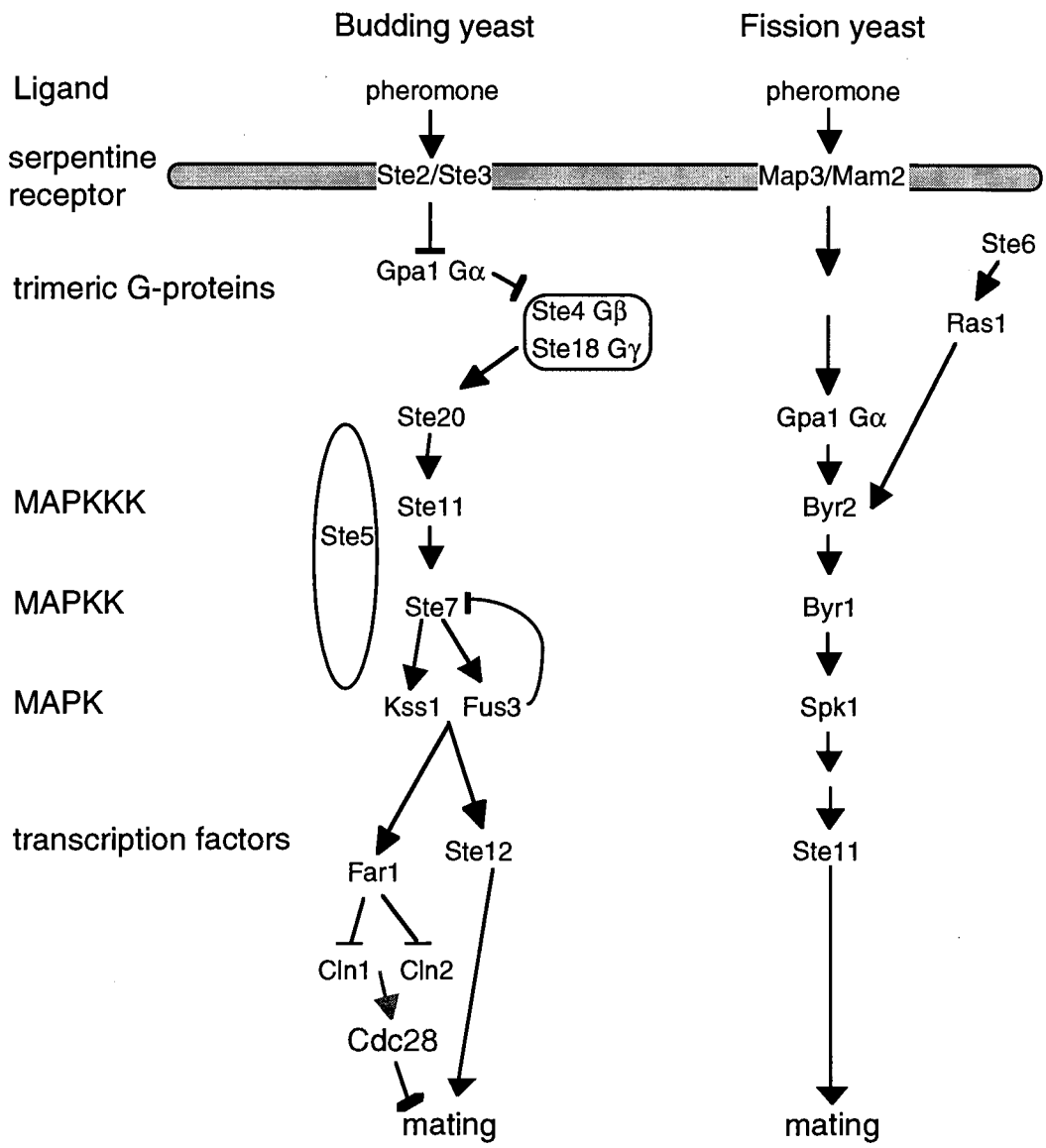

Figure 1.5: The mating pathways of yeasts.
Binding of pheromone to a serpentine transmembrane receptor activates a G_{α} subunit in fission yeast or $G_{\beta\gamma}$ subunits in budding yeast. These in turn activate a MAPK cascade which activates transcription factors and other proteins, leading to the initiation of mating.

In budding yeast the MAPK trio consists of the MAPKK Ste11, the MAPKK Ste7 and two MAPKs, Fus3 and Kss1 (for review, see Kurjan, 1993). The MAPK cascade activates the transcription factor Ste12 (Elion et al., 1993). Ste12 is responsible for the transcription of FAR1 and various genes required for mating. Far1 causes cell cycle arrest by inhibiting the activity of cdks Cdc28-Cln1 and Cdc28-Cln2 (Peter and Herskowitz, 1994).

The fission yeast Byr2/Byr1/Spk1 cascade is analogous to the budding yeast Ste11/Ste7/Fus3-Kss1 pathway (reviewed in Errede and Levin, 1993), as shown in Figure 1.5.

1.2.6: The mammalian MAPK pathway.

The yeast mating MAPK pathways are activated by a single receptor and are essentially linear. In contrast, MAPK pathways of metazoans are activated by more than one mechanism in each cell, in a cell-type dependent manner. For example, vertebrate MEK1 (MAPKK) can be activated via Raf, the MAPKKK, MEKK1, (Langecarter et al., 1993) and mos (Shibuya and Ruderman, 1993) (see Figure 1.6). Raf is principally activated by receptor tyrosine kinases (RTKs), whereas MEKK1 is activated by serpentine receptors coupled to heterotrimeric G-proteins. mos may be regulated by cdk1 (the equivalent of fission yeast Cdc2) (Van Renterghem et al., 1993), and thus be under cell cycle control. So, three different pathways converge at this point and their signals are integrated into the MAPK cascade.

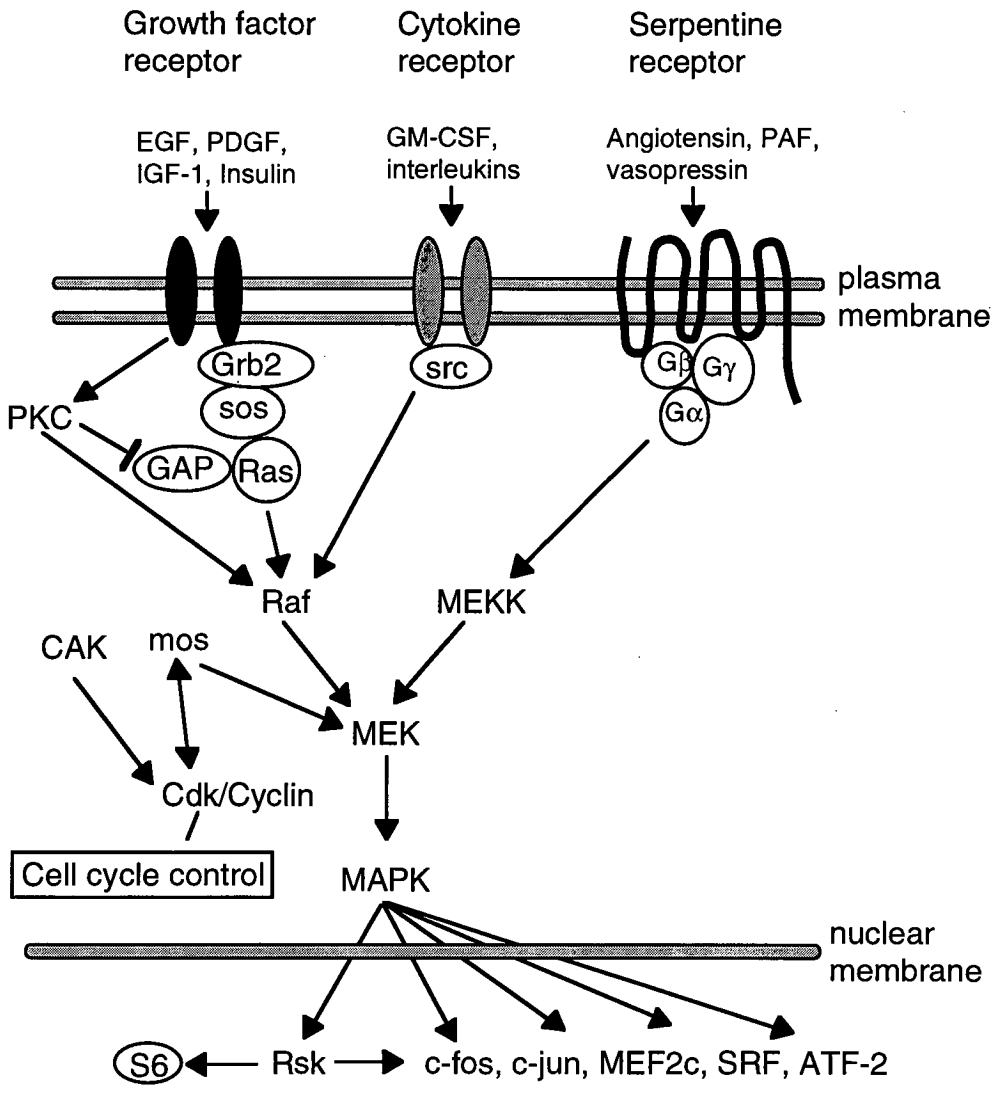

Figure 1.6: The "classical" metazoan MAPK pathway.
The Raf/MEKK/MEK/MAPK cascade is activated by growth factor, cytokine/lymphokine and serpentine trimeric G-protein-linked receptors. In addition, mos can activate the pathway, integrating cell cycle signals. Activated MAPK activates nuclear kinase Rsk and several transcription factors, leading to proliferation and differentiation. Abbreviations in this figure: EGF Epidermal Growth Factor; PDGF Platelet-derived Growth Factor; IGF Insulin-Like Growth Factor-2; PKC Protein Kinase-C; Grb2 Growth Factor Receptor-bound protein-2; sos son of sevenless; GAP GTPase activating protein; CAK cdk-activating kinase; Rsk Ribosomal S6 subunit Kinase; SRF Serum Response Factor; ATF-2 Activating Transcription Factor-2; MEK (MAPK (Mitogen-Activated Protein Kinase) and ERK (Extracellular Signal Regulated Protein Kinase) Kinase); MEKK (MEK kinase).

A vast range of different RTKs is capable of activating Raf, including cytokine-, lymphokine- and growth factor-type receptors. Ligand binding to an RTK causes dimerisation of the receptor, which leads to activating tyrosine phosphorylations. This promotes the binding of adaptor proteins

containing SH2-domains (which bind phospho-tyrosine) and SH3 domains. The SH3 domains facilitate the binding of other proteins which regulate Ras, a small monomeric G-protein which is active when bound to GTP. This is encouraged by the activated regulators. Active Ras-GTP binds to Raf and activates it, a step that is inhibited by cyclic AMP dependent protein kinase (cA-PK or protein kinase A) (Hafner et al., 1994).

Activated MAPK activates transcription factors such as MEF2C (Han et al., 1997), serum response factor (SRF), which is made up of Elk-1 and ATF-2. SRF initiates transcription from genes such as Elk-1 itself (Hill and Treisman, 1995) and fos that are downstream of a serum response element (SRE). fos together with Jun make up the AP-1 protein which regulates many genes involved in proliferation and differentiation.

MAPK also activates ribosomal S6 kinase II (Rsk), which phosphorylates a component of the 40S ribosomal subunit (Sturgill et al., 1988). This leads to increased protein synthesis during mitogenesis and meiotic maturation.

1.3: Stress-Activated MAPK pathways.

In addition to the "classical" mitogen-activated MAPKs discussed so far, there is a different type of MAPK pathway that is activated by environmental stresses, the stress-activated MAPK pathway. This type of MAPK pathway has been found in yeasts as well as higher eukaryotes. The simplest example is the budding yeast Hog1 pathway.

1.3.1: The budding yeast Hog1 Pathway.

The *Saccharomyces cerevisiae* Hog1 MAPK pathway responds only to osmostress (Brewster et al., 1993). Its architecture is analogous to that of the "classical" MAPK pathways. It consists of two MAPKKKs Ssk2 and Ssk22, MAPKK Pbs2 and MAPK Hog1 (Brewster et al., 1993; Brewster and Gustin, 1994; Maeda et al., 1995) (see Figure 1.7). However, the upstream regulation is unusual. In the only known example of its kind, a transmembrane protein, Sho1 allows activation of the MAPKK Pbs2 by MAPKKK Ste11 (Maeda et al., 1995; Posas and Saito, 1997).

Furthermore, the MAPKKKs are activated by a bacterial two-component signal transducer.

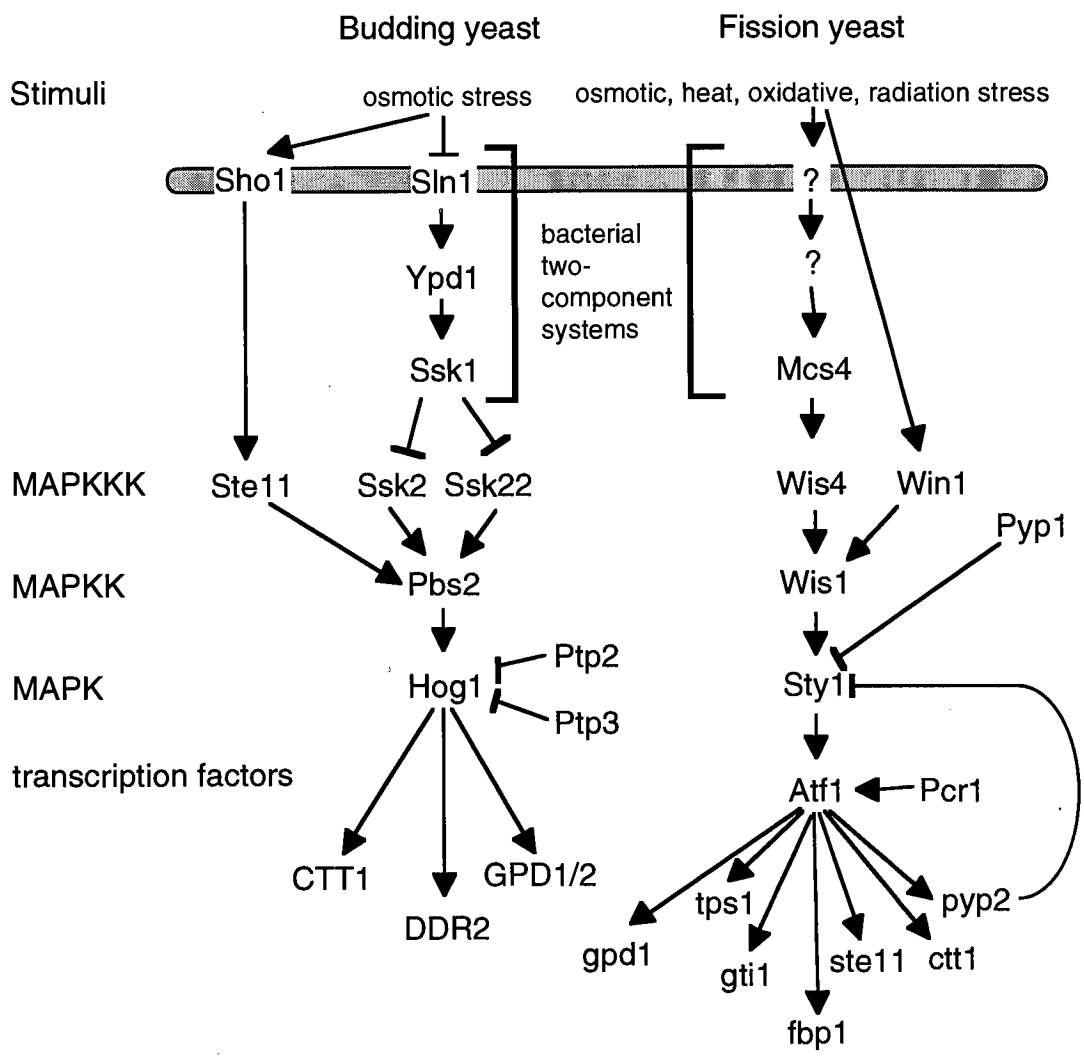

Figure 1.7: The Hog1 and Wis1 pathways.
The Saccharomyces cerevisiae Hog1 pathway responds to salt stress. Activation is via bacterial two-component systems (Sln1/Ypd1/Ssk1) or Sho1 mediating activation by Ste11 (Ste11 normally acts in the mating response pathway). In *Schizosaccharomyces pombe*, different stresses activate the Wis1 pathway in different ways. Salt stress acts through Win1; heat shock through Pyp1. The pathway is responsible for transcription of many genes under the transcriptional regulation of Atf1.

1.3.2: A bacterial two-component system activates the Hog1 pathway.

These systems are ubiquitous in bacteria and control chemotaxis, sporulation, osmoregulation, transformation efficiency, virulence, heavy

metal tolerance and response to changes in nutrient availability (reviewed in Burg et al., 1996; Egger et al., 1997; Silver and Phung, 1996). They have now been found in eukaryotes such as *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, *Arabidopsis thaliana* (Mizoguchi et al., 1994), *Neurospora crassa* (Alex et al., 1996), *Plasmodium falciparum* (Doerig et al., 1996) and *Dictyostelium discoidum* (Gaskins et al., 1996), but have not so far been reported in higher animal systems.

Normally, the two components involved are a Sensor Kinase protein (made up of input and transmitter domains) and a Response Regulator protein (consisting of receiver and output domains) (reviewed in Appleby et al., 1996) and see Figure 1.8. The input domain is extracellular and responds to a signal which activates (or inhibits) the cytosolic kinase transmitter domain, which leads to transfer of a phosphate group to the receiver domain of the response regulator, thus activating (or repressing) the output domain. This usually involves regulation of transcription.

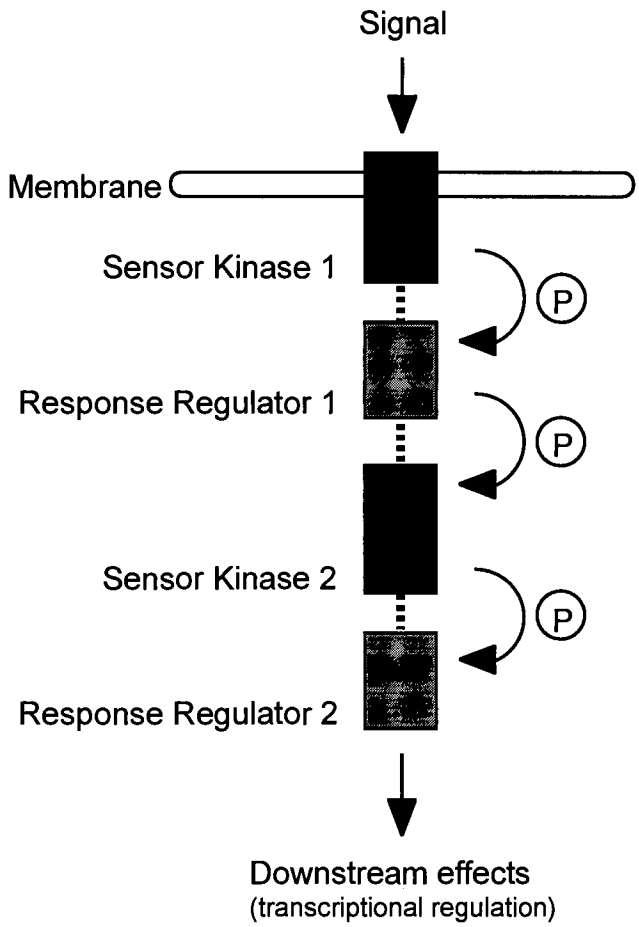

Figure 1.8: Schematic diagram of bacterial two-component system.
The two components are a Sensor Kinase (SK) and a Response Regulator (RR), These two components are repeated. Extracellular signals are sensed by the membrane-spanning SK1. This leads to a sequence of phosphate transfers to RR1, then SK2 and then RR2, which elicits the appropriate response, usually transcriptional activation. The four domains in the phospho-relay (SK1, RR1, SK2 and RR2) may be part of a single polypeptide or may be on two, three or four separate polypeptides.

The budding yeast Hog1 pathway contains two sequential two-component systems. Sln1 represents a complete two-component system itself: this protein contains both Sensor Kinase and a Response Regulator in a single polypeptide (Ota and Varshavsky, 1993). Sln1 transfers a phosphate to Ypd1, which is the sensor kinase (Posas et al., 1996) of the next phospho-relay. Ypd1 transfers its phosphate to the aspartate of Ssk1 (Posas et al., 1996).

Phosphorylated Ssk1 inhibits the activation of the MAPKKKs Ssk2 and Ssk22. Sln1 is inactivated by high osmolarity (Ota and Varshavsky,

1993), which causes the dephosphorylation of Ssk1 and therefore renders the MAPKKKs active, most likely by interfering with their inhibitory N-terminus (Maeda et al., 1995). Activation of the pathway is also reversed by the tyrosine phosphatases, Ptp2 and Ptp3 (Jacoby et al., 1997).

The Hog1 pathway activates transcription of a variety of genes such as *HSP12* (Varela et al., 1995), *CTT1* (Schuller et al., 1994) and *GPD1* (Hirayama et al., 1995) Gpd1 is required for glycerol synthesis and yeasts accumulate glycerol to resist high osmolarity (see Section 1.4).

1.3.3: The Wis1 pathway.

The closest homologue to Pbs2 is the fission yeast protein Wis1 (Warbrick and Fantes, 1991). The *wis1* gene was isolated during a screen for genes which act at the G2-M transition. It encodes a MAPKK which acts as a dose-dependent initiator of mitosis (Warbrick and Fantes, 1991).

The *wis1* gene is not essential, but loss of function leads to many phenotypes. In addition to a cell cycle defect, *wis1*Δ cells exhibit sensitivity to environmental stress (heat, osmotic and oxidative stress), sensitivity to starvation (stationary phase), defects in conjugation and sporulation as well as defects in transcription (Degols et al., 1996; Kato et al., 1996; Millar et al., 1995; Shiozaki and Russell, 1995a; Stettler et al., 1996).

The Wis1 MAPK pathway consists of two MAPKKKs, Wis4 (also known as Wik1 and Wak1 (Shieh et al., 1997; Shiozaki et al., 1997a)) and Win1, which lie upstream of Wis1 (Samejima, I., pers. comm. and Samejima et al., 1997), which activates the MAPK Sty1 (Degols et al., 1996; Kato et al., 1996; Millar et al., 1995; Shiozaki and Russell, 1995a) (see Figure 1.7). The protein tyrosine phosphatases Pyp1 and Pyp2 oppose the activation of Sty1 by Wis1 (Millar et al., 1995).

There is a bacterial two-component sensor upstream of the Wis1 pathway. The *mcs4* gene encodes a response regulator (Cottarel, 1997; Shieh et al., 1997; Shiozaki et al., 1997a), equivalent to Ssk1. The two genes *mak1* and *mak2* encode homologues of Sln1 sensor kinase (Millar, 1997) that lie upstream of Mcs4. No fission yeast homologue of Ypd1 has yet been found, but the available data strongly predicts that one will exist.

The MAPK Sty1 activates two CREB/ATF transcription factors, Atf1(Gad7) (Shiozaki and Russell, 1996; Wilkinson et al., 1996), and Pcr1 (Watanabe and Yamamoto, 1996). Both transcription factors are homologues of human ATF-2.

Downstream of fission yeast Atf1 lie several genes that are required for different cellular functions, including stress response. The following genes are transcriptionally regulated by the Wis1 pathway: *ctt1* (Schuller et al., 1994; Wilkinson et al., 1996), *tps1* (Degols et al., 1996), *gti1* (Caspari, 1997), *gpd1* and *pyp2* (Degols et al., 1996; Wilkinson et al., 1996), *ste11* (Kanoh et al., 1996; Takeda et al., 1995) and *fbp1*(Stettler et al., 1996).

There is strong evidence for a bifurcation in the Wis1 pathway downstream of Sty1. *atf1*Δ cells are partially heat sensitive (personal observation), but show no cell cycle defect (Kanoh et al., 1996). *sty1*Δ and *wis1*Δ cells on the other hand are profoundly heat sensitive and show a cell cycle defect (Millar et al., 1995; Shiozaki and Russell, 1995a). This strongly suggests that the cell length defect seen in *wis1*Δ and *sty1*Δ strains occurs via an Atf1-independent mechanism and furthermore, part of the temperature sensitivity phenotype must arise via a pathway that does not involve Atf1.

1.3.4: The SAPK/JNK and p38(HOG1)/RK pathways are activated by environmental stress.

In contrast to the single stress-activated MAPK pathway in yeasts, there are two overlapping pathways in higher eukaryotes (see Figure 1.9). One pathway consists of Stress-Activated Protein Kinases (SAPKs) (which are also known as c-Jun N-terminal Kinases (JNKs)) and the other contains $p38^{HOG1}$ (or MAPKAP kinase-2 Reactivating Kinase (RK)).

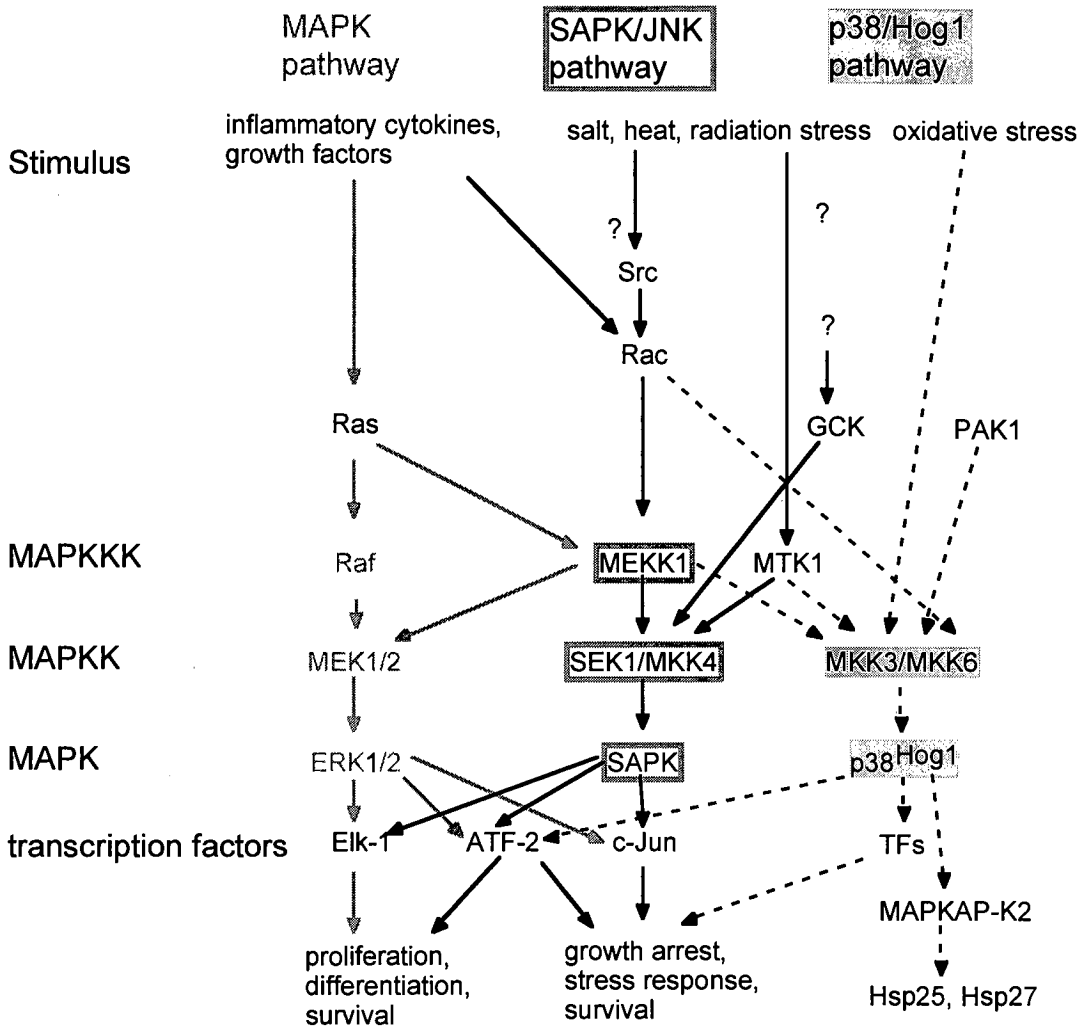

Figure 1.9: Mammalian stress-activated MAPK pathways.
The two mammalian stress-activated pathways (SAPK/JNK and p38^{HOG1}) are activated by a variety of stress stimuli and interact with the proliferative MAPK at several levels to generate a cellular response that depends on the balance of activation of the different pathways. The proliferative MAPK cascade and signals are grey; the SAPK/JNK cascade is boxed in grey, with black arrows for signals and the p38^{HOG1} cascade is shaded with signals as dotted lines. Abbreviations: PAK (p21-Activated Kinase); GCK (Germinal Centre Kinase); TF (Transcription Factor); MAPKAP-K2 (MAPK Activated Protein Kinase-2).

The SAPK and p38^{HOG1} pathways share many stimuli, but have some cell-type specific differences. Both the SAPK and the p38^{HOG1} pathways are activated by heat shock, osmotic shock and UV (see Figure 1.9). In addition to these stimuli, the SAPK and p38^{HOG1} pathways have pathway-specific stimuli, for example, the vertebrate p38^{HOG1} pathway responds to ionising radiation and oxidative stress.

The ability of many upstream elements to activate both pathways may explain the overlap observed in activating stimuli. For example, the MAPKKK MTK1, (a homologue of the yeast stress-activated MAPKKKs (see Sections 1.3.2 and 1.3.3)), activates both the SAPK and the $p38^{HOG1}$ pathways in response to stress (Takekawa et al., 1997).

One way in which the signals from upstream G-proteins are fed into the SAPK or $p38^{HOG1}$ MAPK cascades is analogous to the mechanism seen in the yeast mating pheromone response pathways. The human small monomeric G-protein, Cdc42 activates $p65^{PAK1}$ (p21-activated kinase). PAK1 is a human homologue of budding yeast Ste20 and fission yeast Shk1 (Marcus et al., 1995)). Ste20 carries signals from the upstream G-proteins to the MAPKKK Ste11 in the budding yeast pheromone response pathway.

Following activation of the MAPKKK member of the SAPK or $p38^{HOG1}$ pathway, the MAPKK and the MAPK are sequentially activated. Once activated, SAPK phosphorylates transcription factors, such as ATF-2, which is a member of the CREB sub family of bZIP proteins (Gupta et al., 1995) and a homologue of the fission yeast Atf1 (Kanoh et al., 1996; Takeda et al., 1995).

Phosphorylation of ATF-2 leads to dimerisation with c-Jun (also activated by SAPK (Adler et al., 1992)) and hence transcriptional activation (Pombo et al., 1994).

1.4: Stress Survival and the role of the Stress-Activated MAPK cascades.

Cells must react to stress in order to survive. Linked to this, stress factors play a role in many detrimental processes in cell biology, many with important clinical ramifications, such as cell ageing, the ætiology of cancer and hypoxia (reviewed in Ames et al., 1993; Welch, 1993; Young and Elliott, 1989), and the survival of drought, salt and heat in plants, which are important considerations for agriculture (Boyer, 1982).

Most studies on environmental stresses have been carried out in budding yeast, and from this it is apparent that this organism and perhaps others have evolved two lines of defence against stress: a constitutive system

and an inducible system. The constitutive system provides minimal protection, but will cope with most stresses in the immediate term. It is mediated by protein kinases and protectants such as trehalose and glycerol, present at basal levels. In addition, the constitutive system induces the inducible system, which allows adaptation to stress in the longer term. Induction involves synthesis of heat shock proteins, oxygen scavengers and other stress-resisting molecules, as well as changes in enzyme activities, membranes, the availability of intracellular water and an increase in the levels of stress protectants such as trehalose and glycerol (reviewed in Ruis and Schuller, 1995).

In bateria, compounds called compatible solutes in bacteria, such as glycerol and trehalose, play a crucial role in stress resistance. Cells accumulate them in response to both osmotic (Aiba et al., 1995; Sunder et al., 1996) and heat stress. These compounds have been shown to stabilise proteins *in vitro* and *in vivo*.

Heat and salt stress weakens hydrophobic bonds in proteins. This is counteracted by glycerol which reduces water availability, thus strengthening the hydrophobic bonds and stabilising proteins. Trehalose is implicated in resistance to heat stress in many systems (de-Araujo, 1996; Strom and Kaasen, 1993). Trehalose is able to stabilise hydrogen-bonds, thus protecting proteins from denaturation *in vitro*.

When a form of stress induces the stress response, this leads to resistance to that particular stress as well as to other forms of stress. For example, exposure of budding yeast to mild heat shock (Craig and Gross, 1991; Lindquist and Craig, 1988; Mager and Varela, 1993) induces tolerance of heat, oxidative and salt shock. Conversely, osmo-stress leads to tolerance of at least heat and salt shock (Trollmo et al., 1988). Nutrient limitation leads to entry into stationary phase and associated heat tolerance (Plesset et al., 1987) and resistance to other stresses (reviewed in Werner-Washburne et al., 1993).

The above physiological observations are supported by molecular data. For example, the heat shock gene, *CTT1*, which is induced to deal with both oxidative and osmotic stress, must also be induced for cells to survive stationary phase (see Ruis and Schuller, 1995). These

observations provide overwhelming evidence for a common stress-survival mechanism.

It is conceivable that Heat Shock Factor (HSF) may play a role in this, as it binds to Heat Shock Elements (HSEs) upstream of the promoters of genes in response to heat shock, thus regulating them. However, the fact that HSF is essential in yeasts (Gallo et al., 1993; Sorger and Pelham, 1988; Wiederrecht et al., 1988), suggests it plays a role more in the constitutive minimal stress tolerance system and indeed HSF is only required for the transcription of a few heat shock genes. Furthermore, HSE does not mediate the heat-induced transcription of *CTT1* (Wieser et al., 1991). This suggests the existence of an alternative control element.

In budding yeast, it is the Stress Response Element (STRE) that regulates transcription of genes essential for surviving stress, such as *CTT1* (Marchler et al., 1993; Wieser et al., 1991), which encodes catalase, trehalose phosphate phosphatase (*TPS2*) (Gounalaki and Thireos, 1994) and genes involved in glycogen metabolism. Transcription from an STRE is also activated by ethanol and the responses to ethanol and heat stress are similar (reviewed in Piper, 1993).

Thus, a partial explanation for the cross-protection seen following stress conditions is that many of the genes required for stress protection are transcriptionally co-regulated via the STRE element, which is itself under control of the osmotic-stress-activated Hog1 MAPK pathway (Schuller et al., 1994).

1.5: The role of the cyclic-AMP Protein Kinase (cA-PK) in stress tolerance.

Evidence that the cAMP pathway is linked to stress comes from the observation that *S. cerevisiae* strains deficient in cAMP production show reduced viability in stationary phase (Werner-Washburne et al., 1993). Furthermore, the induction of thermotolerance is sensitive to cAMP levels, and cAMP plays a role in the induction and activation of heat shock proteins (Piper, 1993).

In addition to transcriptional regulation of the STRE by the Hog1 pathway in budding yeast, an important role is played by the cA-PK

pathway. In response to nutritional conditions, cyclic AMP (cAMP) is produced by the enzyme adenylate cyclase from ATP and activates the cyclic-AMP dependent protein kinase (cA-PK). This down-regulates transcription from STREs (Belazzi et al., 1991).

It seems, at least in budding yeast, that the cAMP pathway plays a role, more as a volume control, up- or down-regulating transcription generally in response to nutrient availablility (reviewed in Piper, 1993), while the Hog1 pathway actually transduces the signals generated by environmental stress (Brewster et al., 1993; Schuller et al., 1994). The same appears to be true for fission yeast (Stettler et al., 1996).

1.6: Aims of this thesis.

The processes of cell division and stress tolerance are essential to cell survival. Moreover, these two processes interact with eachother, yet the mechanism is unknown. The isolation of the fission yeast Wis1 MAPKK, a protein which appears to play a role in both processes, was an important first step in characterising the interaction between cell cycle and stress response.

This thesis describes studies on Wis1 and the signal transduction pathway it lies in. These were undertaken in an attempt to reveal further information on the function of Wis1 and its role in the connection between cell cycle and stress response.

The first experimental section (see Chapter 3) describes the physiology and genetics of *wis1*Δ mutants. The second part of this thesis (see Chapters 4 to 6) discusses the isolation and characterisation of mutants that suppress *wis1*Δ phenotypes (the *sow* mutants). It was hoped that these mutants would lie in genes downstream of *wis1*, thus enabling the downstream architecture of the Wis1 pathway and importantly, the mechanism that connects the Wis1 pathway to cell cycle control, to be elucidated.

Chapter 2: Materials and Methods

2.1: *Schizosaccharomyces pombe* methods.

2.1.1: Yeast Strains used in this work.

Strains were derived from 972 h^- and 975 h^+ (Leupold , 1950) (see Table 2.1).

Table 2.1: Strains used in this thesis.

strain number	full genotype
ED812	*h*$^-$
ED878	*h*$^+$
5.7	*sow2-7 wis1::his1*$^+$ *his1-102 ura4::fbp1-lacZ ade6-M216 h*$^-$
5.11	*sow2-11 wis1::his1*$^+$ *his1 ura4::fbp1-lacZ ade6-M216 h*$^-$
5.22	*sow1-22 wis1::his1*$^+$ *his1 ura4::fbp1-lacZ ade6-M216 h*$^-$
ED632	*win1-1 h*$^+$
ED683	*cdc2-1w leu1-32 h*$^+$
ED716	*cdc13-117 h*$^-$
ED752	*mat2-102 ade6-M210*
ED815	*ppa1::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$
ED817	*ppa2::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$
ED900	*ura4::fbp1-lacZ h*$^-$
ED907	*cdc2-33 h*$^-$
ED932	*his1-102 h*$^-$
ED933	*his1-102 leu1-32 h*$^+$
ED942	*wis1::LEU2 wee1-50 leu1-32 h*$^+$
ED952	*git2-1*int::*LEU2 leu1-32 h*$^+$
ED957	*git2-1*int::*LEU2 leu1-32 ura4::fbp1-lacZ h*$^+$
ED961	*wis1::his1*$^+$ *his1-102 ura4::fbp1-lacZ h*$^-$
ED975	*wis1::his1*$^+$ *his1-102 h*$^+$
ED976	*wis1::his1*$^+$ *his1-102 h-*
ED1006	*wee1-50 h*$^-$
ED1010	*wis1::his1*$^+$ *his1-102 leu1-32 h*$^+$

ED1011	*wis1::his1*$^+$ *his1-102 leu1-32 h*$^-$
ED1039	*cdc2-3w h*$^-$
ED1052	*pat1-114 wis1::LEU2 leu1-32 h*$^+$
ED1053	*pat1-114 wis1::LEU2 leu1-32 h*$^-$
ED1060	*ppe1::ura4*$^+$ *leu1 ura4* [pPH101 (*ppe1*$^+$)]h$^+$
ED1063	*git6-261 h*$^+$
ED1073	*git6-261 leu1-32 h*$^-$
ED1076	*wis1::his1*$^+$ *his1-102 ade6-M210 leu1-32 mat2-102*
ED1085	*=ED961*
ED1090	*leu1-32 ura4-D18 h*$^-$
ED1118	*cdc27-P11 h*$^-$
ED1119	*cdc27-P11 h*$^+$
ED1126	*wee1-50 his1-102 h*$^+$
ED1135	*wis1::his1*$^+$ *his1102 ura4::fbp1-lacZ* ade6-M216 *h*$^-$
ED1147	*=ED1076*
ED1151	*sty1-1 ura4-D18 leu1-32 h*$^-$
ED1177	*sty1-1 ura4::fbp1-lacZ leu1-32 h*$^-$
ED1185	*cgs1::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 h*$^+$
ED1186	*mcs4::ura4*$^+$*ura4::fbp1-lacZ ade6-M216 h*$^-$
ED1188	*win1-1 ura4::fbp1-lacZ leu1-32 h*$^+$
ED1207	*win1-1 mcs4::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 h*$^-$
ED1209	*wis4::ura4*$^+$ *ura4::fbp1-lacZ h*$^-$
ED1211	*win1-1 wis4::ura4*$^+$ *ura4-D18 leu1-32 h*$^-$
ED1225	*mcs4::ura4*$^+$ *wis4::ura4*$^+$ *ura4::fbp1-lacZ h*$^-$
ED1234	*win1-1 mcs4::ura4*$^+$ *wis4::ura4*$^+$ *ura4::fbp1-lacZ leu1-32 h*$^-$
ED1256	*wis1::his1*$^+$ *his1? sty1*$^+$*::ura4*$^+$ *h*$^+$
ED1274	*pat1-114 leu1-32 h*$^-$
ED1275	*pat1-114 his1-102 leu1-32 ura4-294 h*$^+$
ED1278	*atf1::ura4*$^+$ *leu1-32 ura4-D18 h*$^-$
ED1281	*atf1::ura4*$^+$ *ura4-D18 leu1-32 h*$^-$ [pREP41-*atf1*$^+$]
ED1286	*pat1-114 wis1::his1*$^+$ *his1-102 leu1-32 h*$^+$
ED1287	*pat1-114 wis1::his1*$^+$ *his1-102 leu1-32 ura4-294 h*$^+$
ED1339	*cdc25-22 h*$^+$
ED1340	*swo1-26 leu1-32 ura4-D18 h*$^-$
ED1364	*ppa1::ura4*$^+$ *ura4-D18 wis1::LEU2 leu1-32 h*$^-$
ED1365	*ppa1::ura4*$^+$ *ura4-D18 wis1::LEU2 leu1-32 h*$^-$
ED1366	*ppa2::ura4*$^+$ *ura4-D18 wis1::LEU2 leu1-32 h*$^-$

ED1433	*tps1::ura4*$^+$ *ura4-D18 ade6-M216 leu1-32* h^+
SP3	*sow1-T20 wis1::his1*$^+$ *his1-102* h^-
SP5	*sow1-T20 wis1::his1*$^+$ *his1-102 leu1-32* h^-
SP19	*sow2-11 wis1::his1*$^+$ *his1-102* h^-
SP31	*sow2-11 wis1::his1*$^+$ *his1-102 ura4::fbp1-lacZ* h^-
SP32	*sow2-11 wis1::his1*$^+$ *his1-102 ura4::fbp1-lacZ* h^+
SP38	*sow2-7 wis1::his1*$^+$ *his1-102* h^+
SP39	*sow2-7 wis1::his1*$^+$ *his1-102 ura4::fbp1-lacZ* h^-
SP55	*sow1-T20 wis1::his1*$^+$ *his1-102 leu1-32 ura4::fbp1-lacZ* h^+
SP60	*sow1-22 wis1::his1*$^+$ *his1-102 ura4::fbp1-lacZ* h^-
SP64	*sow1-22 wis1::his1*$^+$ *his1-102* h^-
SP75	*sow1-22 wis1::his1*$^+$ *his1-102 pat1-114 leu1-32 ura4::fbp1-lacZ* h^-
SP80	*sow2-7 wis1::his1*$^+$ *his1-102 pat1-114 leu1-32 ura4::fbp1-lacZ* h^-
SP93	*sow2-11 wis1::his1*$^+$ *his1-102 pat1-114 leu1-32 ura4::fbp1-lacZ h?*
SP99	*sow2-11 wis1::his1*$^+$ *his1-102 pat1-114 leu1-32 ura4::fbp1-lacZ h?*
SP100	*sow1-22 wis1::his1*$^+$ *his1-102 pat1-114 leu1-32 ura4::fbp1-lacZ h?*
SP133	*sow1-22 his1-102* h^-
SP148	*sow2-7 his1-102* h^-
SP165	*sow2-11 his1-102* h^-
SP232	*sow2-7* h^-
SP237	*sow1-22* h^-
SP239	*sow2-11* h^+
T20	*sow1-T20 wis1::his1*$^+$ *his1-102 ura4::fbp1-lacZ* h^-
T20.16	*sow1-T20* h^-
T20.23	*sow1-T20 ura4::fbp1-lacZ* h^+

2.1.2: Media and supplements used for yeast growth and matings.

Nutritional Supplements

Uracil, adenine, lysine, histidine and leucine were used to supplement *ura*, *ade*, *lys*, *his* and *leu* strains on minimal medium respectively. 50x (3.75 g/l) stock solutions were made for adenine and uracil; 100x (7.5 g/l) stock solutions were made for the remaining supplements. Supplements were added to cooled media to a final concentration of 75μg/ml. Stock solutions were autoclaved and stored at room temperature.

Osmotic and selective supplements

To make medium containing sorbitol or KCl, the compound was added to hot medium, microwaved to ensure dissolution. All other chemicals were added to cooled media just before pouring.

A stock solution of paraquat was made at 0.1g/ml in SDW and stored at -20°C.

Normal handling of *S. pombe*, and media used were as described in (Alfa et al., 1993), with exceptions described below.

YE

Yeast extract medium is supplemented with adenine (75mg/l) and uracil (75mg/l).

SPA Mating media

SPA contains: 10g/l glucose; 1g/l KH2PO4; 10mg/l biotin; 1mg/l calcium pantothenate; 10mg/l nicotinic acid; 10mg/l meso-inositol and 30g/l agarose.

Phloxine B

This dye stains dead cells in a colony. Thus it can be used to differentiate between diploid and haploid colonies (as diploids are slightly sicker than haploids) or to gauge the health of cells under deleterious conditions. A stock solution (x500) of 10mg/ml in ethanol was made and used at a final concentration of 20μg/ml in solid or liquid medium.

2.1.3: Propagation of yeast strains

Strains were defrosted from -70°C onto YE (or appropriately supplemented MM for strains harbouring plasmids) and streaked straight to single colonies. Strains were stored at 5°C and replicated to fresh YE every week (for stationary phase sensitive strains) or every three-four weeks for hardier strains. This was repeated, three or four times, then fresh material was isolated from -70°C glycerol stocks.

2.1.4: Procedure for mating yeast strains

A very small amount (less than a toothpick end) of very freshly growing material of each of the h^+ and h^- strains to be crossed was placed on mating medium and mixed with a toothpick with approximately 7µl SDW; allowed to dry briefly then incubated at 20°C or 25°C for strains which mate poorly, or 28°C for others.

Tetrad dissection was performed after 1.5 to 3 days.

2.1.4.1: Diploid selection

Two strains were selected with at least one unique marker each, such that the diploid zygote formed would be prototrophic for these markers, but the individual haploid strains would be auxotrophic. The strains were mated on SPA as described above and after one, two and three days, a small amount of material was taken from the mating patch and streaked to single colonies at 28°C on MMPB supplemented appropriately to ensure only diploid strains can grow.

Diploid colonies appear much darker pink on PB than haploid colonies. Microscopic examination was used to confirm suspected ploidy, as diploid cells are longer and somewhat fatter than haploids. Diploid strains were propagated for a few days at 25°C, 28°C or 32°C and then abandoned.

2.1.4.2: Random Spore analysis

The enzyme used is from the gut of the snail *Helix pomatia* (supplied as Suc d'*Helix pomatia*, Industrie Biologique, France) A small amount of material from the cross was suspended in 1ml of x50 dilution of a stock solution of snail gut enzyme (The stock solution is a 10x dilution of the

concentration it is supplied at, and is stored at 5°C). The suspension was incubated at 37°C overnight, pelleted (30s. at 13,000 rpm), the supernatant removed and the pellet resuspended in 1ml fresh SDW, then diluted x100. 10µl and 100µl aliquots were plated onto appropriate medium, spread, and the plates were incubated until colonies appeared.

2.1.4.3: Tetrad analysis

A Singer MSM tetrad dissector was used. Very thin YE plates were poured and dried thoroughly prior to dissection. The manufacturer's instructions were followed for the manipulation of asci. Spores were hatched overnight at 20°C before dissection and then incubated until colonies appeared.

2.1.4.4: Iodine Staining of spores

An even 2-3mm layer of solid iodine (Sigma I-3380) was spread in the inverted lid of a petri dish; the plate with the crosses to be tested was inverted over this for 5 mins, then allowed to destain if necessary. Plates were viewed against a black background. Successful matings (ie those containing spores) stain dark brown or black.

2.1.5: *Schizosaccharomyces pombe* physiology

2.1.5.1: Microscopic examination of *S. pombe* on plates

Cells on plates were examined with a microscope with a x20 objective and x10 eyepiece, under bright field illumination.

2.1.5.2: Sampling cell number

Cell number was counted on a Coulter counter. 100µl of culture were added to 9.9ml Isoton II (Coulter Electronics 8448011). The sample was sonicated continuously for 10s on power setting 1 with a large probe and then counted immediately.

For rough estimations of cell number, the optical density at 595 nm (OD_{595}) was measured on a Hitachi U-2000 spectrophotometer. An OD_{595} of 0.25 was taken to correspond to $5x10^6$ cells/ml.

2.1.5.3: Plate assay of viability

At each time point cell number in the culture was counted and a sample was serially-diluted with distilled water and plated onto duplicate YE plates and incubated until the appearance of colonies. The proportion of viable cells in the original culture was hence calculated.

2.1.5.4: Exponentially-growing liquid cultures

An over-night 10ml starter culture of the appropriate medium was inoculated with material (preferably from a single colony) on an agar plate. This culture was then diluted into a main culture so that when grown overnight, it would yield a culture of roughly 2-5x10^6 cells/ml. Mid-log phase was taken as 5x10^6 cells per ml, and entry into stationary phase above roughly 1.5x10^7 cells/ml.

2.1.5.5: Measuring Cell length

A 1ml sample of an exponentially-growing MM culture was centrifuged for 10s and approximately 950µl of supernatant discarded. The loose pellet was resuspended in the remaining medium and then pipetted onto a slide and allowed to dry briefly before covering.

Cell length at division was measured using phase microscopy with a x100 objective and an eyepiece graticule. The length of cells that had formed a septum but had not yet begun cytokinesis were measured, as described in (Alfa et al., 1993). As far as possible, all measurements were made in the same focal plane to ensure repeatability.

2.1.5.6: ß-galactosidase assay

The methods are derived from (Miller, 1972).

The presence or absence of the *lacZ* gene can be assayed on plates, but quantitative measurements of ß-galactosidase activity must be done in liquid culture.

Plate ß-galactosidase assay

Immediately prior to the assay, ß-mercaptoethanol was added to Z-buffer to a final concentration of 2.6% by volume.

Cells growing on a plate were transferred onto filter paper. To do this, two circles of filter paper (Whatman 1001 150) cut to size were placed on top of a velvet on a replicating block. The petri dish was inverted over the paper and even pressure applied until the whole of the paper had made contact with the surface of the agar. The plate was then removed and the filter paper with the cells adhered to it carefully peeled off the agar with tweezers.

The filter and cells were frozen at -70°C for 15 minutes then allowed to thaw at 37°C for 15 minutes. 10ml Z-Buffer and 0.25 ml X-gal (40mg/ml) in DMSO were mixed and the filter paper was briefly immersed in this solution. The filter was incubated at 28°C until the appearance of a blue colour in patches that contain the *lacZ* gene (10 to 30 minutes).

Liquid culture ß-galactosidase assay

Immediately prior to the assay, ß-mercaptoethanol was added to Z-buffer to a final concentration of 2.6% by volume.

100µl of cells (concentrated by centrifugation if necessary) were added to 900µl Z-buffer in an Eppendorf. 30µl chloroform and 20µl 0.1% sodium dodecyl sulphate (SDS) were added and the tube was vortexed vigorously for ten seconds and then pre-warmed in a water bath at 28°C for ten minutes.

200µl ONPG solution were added, the tube was vortexed and incubated at 28°C for 10 to 60 minutes. The reaction was stopped by adding 250µl Na_2CO_3 and vortexing briefly. The tube was then centrifuged at 13,000 rpm for 10 minutes, and 1ml of supernatant pipetted into a 2ml disposable cuvette (Fisons CXA-100-030R). Optical density at 420 nm was measured on a Hitachi U-2000 spectrophotometer against a blank which was identical except that it contained no cells.

X-gal solution

5-bromo-4-chloro-3-indoyl-ß-D-galactopyranoside (X-gal) (Biosynth AG) was dissolved in dimethyl sulphoxide (DMSO) at 40mg/ml and stored at -20°C.

Z-buffer

Disodium hydrogen orthophosphate dihydrate (5.12g), sodium dihydrogen orthophosphate dihydrate (3.1g), potassium chloride (376mg), magnesium sulphate heptahydrate (125mg) were dissolved in deionised water and made up to 500ml and autoclaved.

ONPG

A solution of 4mg/ml o-nitrophenyl ß-D-galactopyranoside (ONPG) (Sigma N 1127) was made up in water and stored at -20°C.

2.1.5.7: DAPI staining of DNA

Cells were fixed and their nuclei were stained as described by MacNeill (MacNeill and Fantes, 1993)

2.1.5.8: Mutagenesis of fission yeast

The protocol was adapted from the method described for budding yeast (Lawrence, 1991). A Stratalinker was used to provide UV radiation with wavelength 254µm, and cells were placed in an open glass petri dish. Stirring was effected by swirling the petri dish halfway through the irradiation until Mutagenesis V, when the culture was stirred very vigorously magnetically. After irradiation cells were kept in the dark for at least twenty-four hours to prevent photoreactivation.

2.1.6: Manipulation of *Schizosaccharomyces pombe* DNA

2.1.6.1: The plasmid used for transformation of *S. pombe*

pON160 was obtained from Olaf Nielsen. It contains fission yeast *ars1* (autonomously replicating sequence) and *ura4*$^+$ sequences.

2.1.6.2: Electroporation of *S. pombe*

This was performed as described by Prentice (Prentice, 1992).

2.1.6.3: Lithium acetate transformation of *S. pombe*

The strain to be transformed was grown in YE to 10^7 cells/ml. The cells were collected by centrifugation at 3,000 rpm for 10' at room temperature, washed twice with 10 ml TE (pH 8.0), resuspended in 10ml TE (pH 8.0)

containing 0.1 M lithium acetate, and left at room temperature for one hour.

The cells were collected by centrifugation and resuspended at approximately $3.5x10^8$ cells per ml in TE (pH 8.0) containing 0.1 M lithium acetate. 150µl aliquots of this suspension (roughly $5x10^7$ cells) were mixed with 350µl 50% PEG (mw 3350) and 0.1 to 1µg plasmid DNA and left for one hour at room temperature.

The cells were then pelleted, resuspended in SDW and plated on selective media.

2.1.6.4: Plasmid stability assay

A 10ml YE culture was inoculated with a very small amount of the plasmid-bearing strain using a sterile wire loop and then incubated overnight. The cell density of the culture was estimated and a sample of the culture was diluted and plated on YE to obtain plates with roughly 100 colonies.

Once colonies had formed, the plates were replicated to MM with and without the supplement synthesised by the product of the marker gene on the plasmid. Thus colonies that had lost the plasmid would not grow without the appropriate supplement and those that had retained it would grow without the supplement.

The numbers of colonies with and without the plasmid were counted. If all colonies had retained the plasmid it was assumed to have integrated, and if some of the colonies had lost the plasmid it was assumed to be unintegrated.

2.2: Bacterial procedures

Escherichia coli bacteria were grown and manipulated as described by Sambrook (Sambrook et al., 1989).

2.2.1: *E. coli* Plasmids

Dr. Gancedo kindly sent the plasmid pMR821 at 2µg/µl which contained the *tps1*$^+$ open-reading frame (see Figure 3.7).

2.2.2: Bacterial Strains

Electrocompetent XL1 Blue *E. coli* were kindly made by Michael Carr (according to Sambrook et al., 1989).

Heat shock competent JM109 *E. coli* cells were obtained from Promega (High Efficiency Competent Cells #L2001) or kindly made by Ifat Ahmed (according to Sambrook et al., 1989).

2.3: Molecular Biology

1.5ml Eppendorf tubes were used wherever convenient.

SDW refers to sterile (autoclaved) deionised water.

2.3.1: Solutions and chemicals used

Chemicals were analytical grade unless otherwise specified and obtained from Fisons, Sigma or BDH.

3M sodium acetate (pH 4.6/5.2)

1xTE, 50xTAE (Tris-acetate) and 50xTBE (Tris-borate) were made according to Sambrook et al. (1989).

dNTPs a solution of 10mM of each dNTP (stored at -20°C)

2.3.1.1: DNA solutions

Isolated DNA was dissolved in TE or SDW and stored at -20°C.

2.3.2: Agarose Gels

0.8% agarose gels were made with TBE containing 0.5μg/ml ethidium bromide (from a 10mg/ml aqueous stock solution (Sigma E-1510)). TAE buffer was used occasionally.

Loading Buffer was added to samples prior to loading.

2.3.3: Amplification of DNA

Small-scale: Promega Wizard Plus minipreps (cat. no. A1330) were used according to manufacturer's instructions.

Medium-scale: Promega Wizard Plus midipreps (cat. no. A7640) were used according to manufacturer's instructions.

2.3.4: Gel purification of restriction digests.

Gel purification was performed using a Qiagen Qiaquick gel extraction kit (cat. no. 28704) according to the manufacturer's instructions.

2.3.5: Polymerase Chain Reaction

Taq polymerase (Promega #M1861) was used as per the protocol given in Promega manual (see Beckler et al., 1996).

2.3.5.1: PCR conditions.

The protocol given in the Promega guide (Beckler et al., 1996) was followed exactly. A heated-lid thermal cycler was used with the following programme:

95°C 1'
35 cycles of (95°C 1'; 45°C 1'; 72°C 3')
72°C 10'

2.3.5.2: Primers for PCR amplification of *S. pombe tps1*$^+$.

The sequence of the 5' and 3' primers used were "AGC TGT CGA CAT GTC GGA TGC TCA TGA T" and "GAT CGC ATC CTC ACC GAC AAA GCT TTG G" respectively, with calculated T_ms of 52°C and 54°C. They were diluted to 100pmol/µl and stored at -20°C.

2.3.6: Restriction Digestion of DNA

Digestion mixes were made according to instructions from the supplier of the enzyme, in a total volume of 20µl. The state of the reaction was

judged by agarose gel electrophoresis in the presence of ethidium bromide and visualised by UV transillumination.

2.3.7: Ligation of DNA fragments.

Ligations of PCR fragments into the pGEM-T Easy vector (Promega #A1360) were set up according to the manufacturer's instructions and allowed sixteen to twenty hours at 18°C to 20°C. The ligation mix was de-salted with a Millipore 0.025µm dialysis membrane (cat. no. VSWP 025 00) before being transformed into E. coli cells.

Ligations of fragments that had been cut from vectors were performed in a volume of 10µl at 18°C with T4 DNA ligase (Promega #M1801) (as described in Beckler et al., 1996).

2.3.8: Blue/White colour screening for ligations

Carried out on LB+Amp plates containing 0.1mM IPTG (ß-D-isopropyl-thiogalactopyranoside) and 40µg/ml X-Gal (5-bromo-4-chloro-3-indolyl-ß-galactopyranoside) (as described in Beckler et al., 1996).

Chapter 3:

Characterisation of Wis1 and its pathway.

3.1: Introduction

In an attempt to add to the paltry amount known about Wis1 when this project started, two different approaches were decided upon and this chapter deals with both: first, a detailed investigation into the stress sensitivity of a *wis1*Δ strain was undertaken in an attempt to understand the mechanism involved (see Section 3.2). Second, as Wis1 is a MAPKK (Warbrick and Fantes, 1991), it would almost certainly lie in a signal transduction pathway. Attempts were therefore made to discover whether any known genes with promising phenotypes or genetic interactions could be placed in such a pathway (see Section 3.3).

3.2: *wis1*Δ stress sensitivity: a study.

3.2.1: Ultra-violet sensitivity.

Before this project was started, it was known that high salt, high temperature (Degols et al., 1996; Millar et al., 1995; Shiozaki and Russell, 1995b; Stettler and Fantes, 1994) and stationary phase (Kato et al., 1996; Warbrick and Fantes, 1991) were lethal to *wis1*Δ cells. I wondered whether they might also be sensitive to ultra-violet radiation.

To examine this, liquid YE cultures of a wild type strain (ED812) and a *wis1*Δ strain (ED1135) were set up and grown to mid-log phase at 32°C. Cells were harvested and 10^2 to 10^6 were plated per petri dish. The plates were exposed to U. V. radiation at 254nm varying from zero to 2000 J/m^2 and then incubated in the dark at 32°C. The number of colonies that formed was counted and the results plotted on a graph (see Figure 3.1).

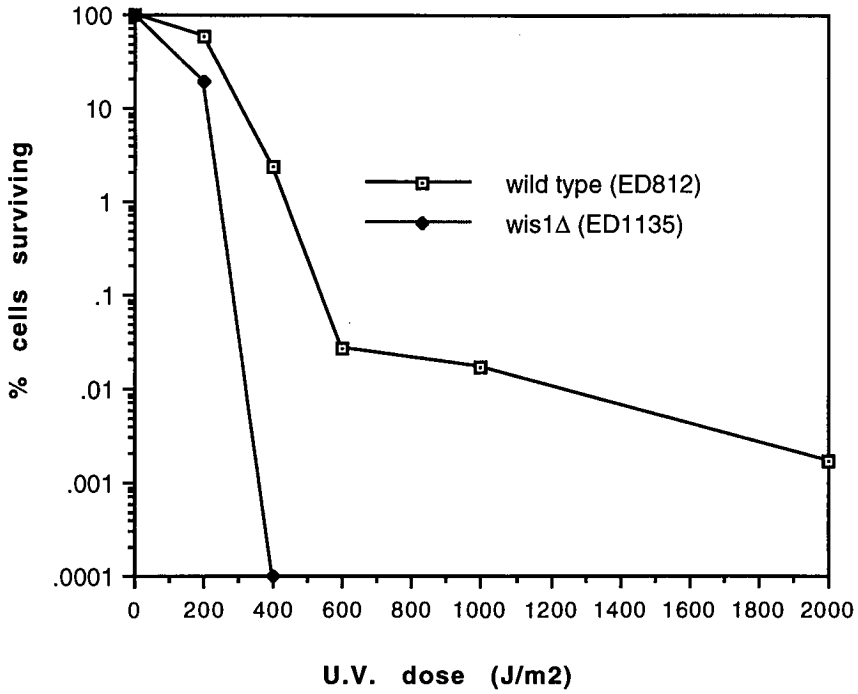

Figure 3.1: A *wis1Δ* strain is hyper-sensitive to ultra-violet radiation.
See text and Chapter 2 for experimental details.

As can be seen from this graph, the *wis1Δ* strain is much more sensitive to U.V. radiation than the wild type.

3.2.2: Investigation of *wis1Δ* heat sensitivity.

3.2.2.1: Shift to 36°C.

As discussed above, *wis1Δ* strains are unable to proliferate to give rise to colonies at 36°C. Microscopic examination after two days at this temperature, reveals that the cells have become very long and branched (Stettler et al., 1996). This phenotype is reminiscent of cell division cycle (*cdc*) mutants under the same conditions.

This suggests that one possible reason for the loss of viability seen at 36°C is that under these conditions, *wis1Δ* cells behave like *cdc* mutants at the restrictive temperature, ie cell growth continues yet division is blocked.

To investigate whether this was the case, cell number was counted in a wild type (ED812) and a *wis1*Δ (ED976) culture following a shift to 36°C from 32°C in liquid MM medium. The results are shown in Figure 3.2.

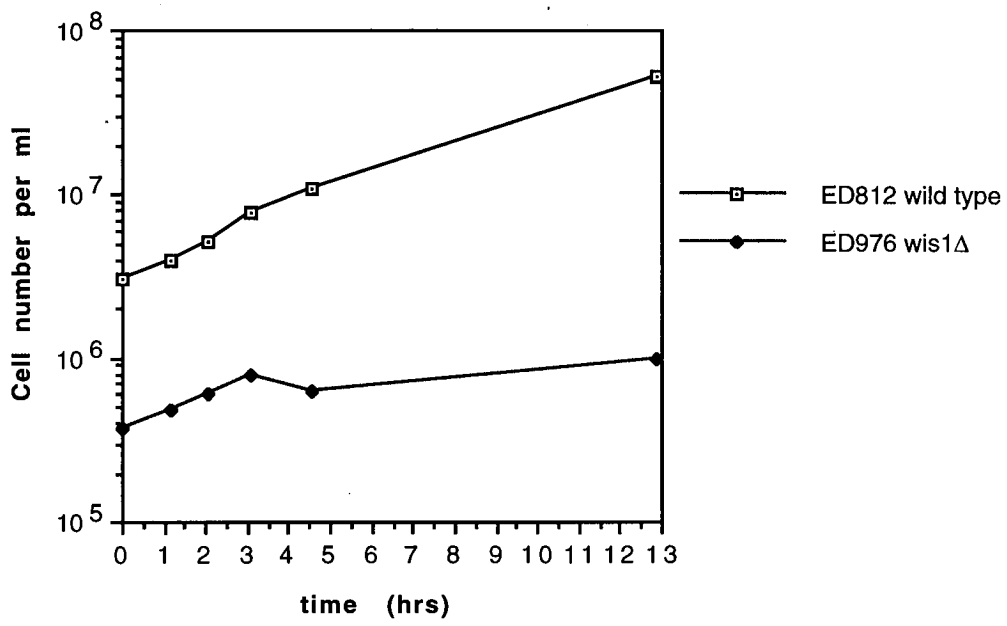

Figure 3.2: Cell number increase following shift
Cell number was counted with a Coulter Counter following a shift from 32°C to 36°C at time = 0.

Following a shift up, wild type cells continue to divide normally, until by thirteen hours, they have entered stationary phase. In contrast, *wis1*Δ cell number plateaus some three hours after the shift, having increased by a factor of about two. Thus, each cell in the *wis1*Δ culture divides about once, suggesting that cells initially in G2 complete the next M phase and then arrest in the cycle following that.

This experiment shows that *wis1*Δ cells stop dividing approximately one cell cycle after the shift. But to determine whether cell growth continues over the same time period, cell length was measured and is shown in Figure 3.3.

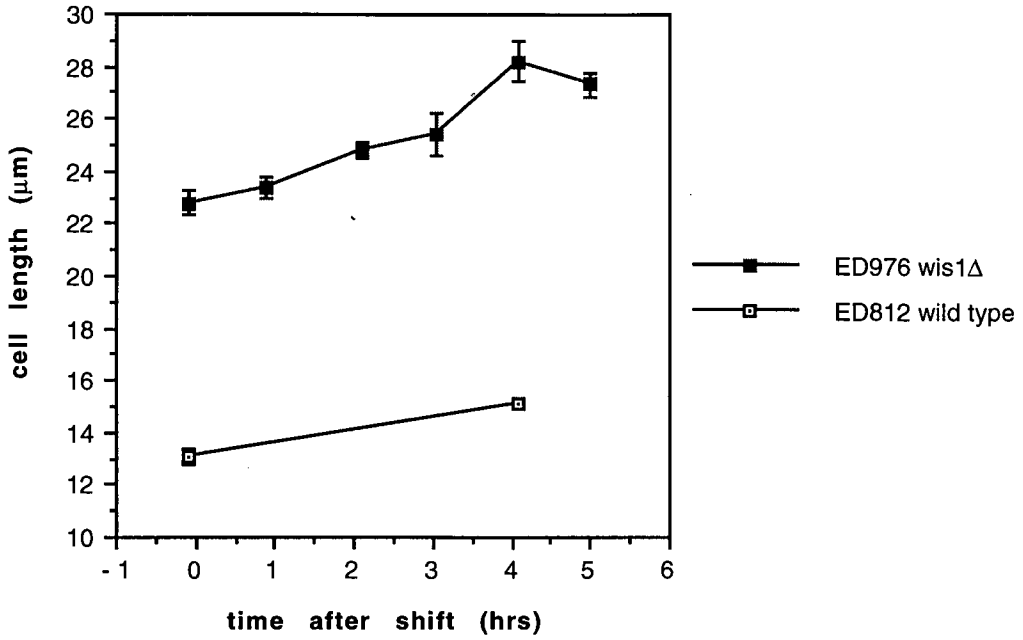

Figure 3.3: Cell length of *wis1*Δ cells after a shift to 36°C.
The lengths of cells that had just completed septum formation were measured until two hours after the shift, thereafter, all cells that appeared to be still growing were measured. The error bars are the Standard Error of the mean.

As the graph shows, *wis1*Δ cells continued to grow. Furthermore, in an independent experiment, which showed identical behaviour over the first five hours, the length of septated *wis1*Δ cells at 12.85 hours had further increased to (55±8) µm (see below).

The data presented in these two experiments are consistent with the failure of *wis1*Δ cells to proliferate at high temperature being due to a *cdc* arrest. For the first five or so hours a shift to restrictive temperature is reversible for many of the *cdc* mutants (Nurse et al., 1976). To see at what time after the shift *wis1*Δ cells lost the ability to proliferate, cells were plated for viability. The data obtained are presented in Figure 3.4.

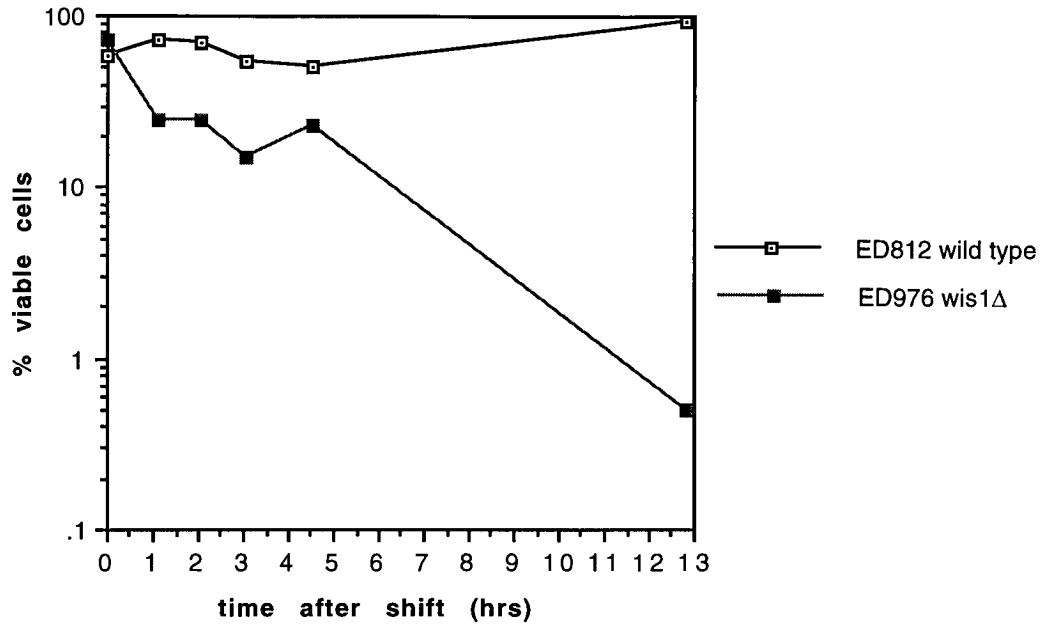

Figure 3.4: Cell viability for *wis1*Δ cells shifted to 36°C.
See text and Chapter 2 for details.

A three-fold decrease in viability was seen within an hour of shifting *wis1*Δ cells to the higher temperature. Viability remained steady for three hours and then decreased dramatically. Wild type cells remained fully viable throughout the experiment.

The two-stage decrease in viability seen in Figure 3.4 suggests that at least two different effects are the cause: the first initial drop is likely due to the heat shock caused when *wis1*Δ cells are suddenly exposed to 36°C. This appears to kill roughly two-thirds of the cells immediately. The remainder survive for a few hours and then begin to die. This later behaviour is similar to that of a typical *cdc* mutant (see Nurse et al., 1976).

These two stages of cell death mean that there will be two populations of cells: those that are killed instantly, and therefore do not have a chance to elongate; and those that survive the heat shock and continue to grow, but fail to divide.

In case the use of distilled water as a dilutant was responsible for the loss of viability seen, this experiment was repeated using YE to dilute the

culture for viability plating assays (data not shown). Although this second treatment led to slightly higher survival of the *wis1*Δ strain, there was no real difference between the two treatments.

3.2.2.2: *wis1 wee1* mutants at 36°C.

A possible conclusion from these experiments is that *wis1*Δ cells die at 36°C from an irreversible cell cycle block. Strains containing the *wee1* mutation divide at a reduced length relative to *wee1*$^+$ strain and furthermore, *wis1*Δ *wee1-50* double mutants are "wee" in length (Warbrick and Fantes, 1991), showing that a *wee1* mutation is epistatic to *wis1*, at least as far as cell division is concerned. An interesting question is whether in a *wis1*Δ background, the presence of the *wee1* mutation is capable of suppressing stress sensitivity, as well as the cell length defect if the *wis1*Δ mutant.

The stress resistance of *wis1Δ wee1* double mutant strains was therefore examined.

The *wee1 wis1* strain (ED942), together with wild type (ED812), *wis1*Δ (ED976) and *wee1-50* (ED1006) control strains were grown on YE plates at 32°C and then streaked to single colonies at 36°C and on 1.2M KCl YE. Under both sets of conditions, the wild type and *wee1-50* strains had formed colonies of cells; whereas neither the *wis1*Δ, nor the *wee1 wis1Δ* strains had formed any colonies. On microscopic inspection, the double mutant cells showed some variation in cell length, but the vast majority were short (or "semi-wee").

This experiment seemed to show that although the presence of the *wee1* mutation was preventing most of the *wis1*Δ cells from elongating, it was not preventing them from dying.

To investigate the timing of the cell death seen on plates, a *wee1 wis1*Δ culture was grown in liquid MM, with wild type, *wee1* and *wis*Δ controls. Cell number following a shift to 36°C was followed and is graphed below in Figure 3.5.

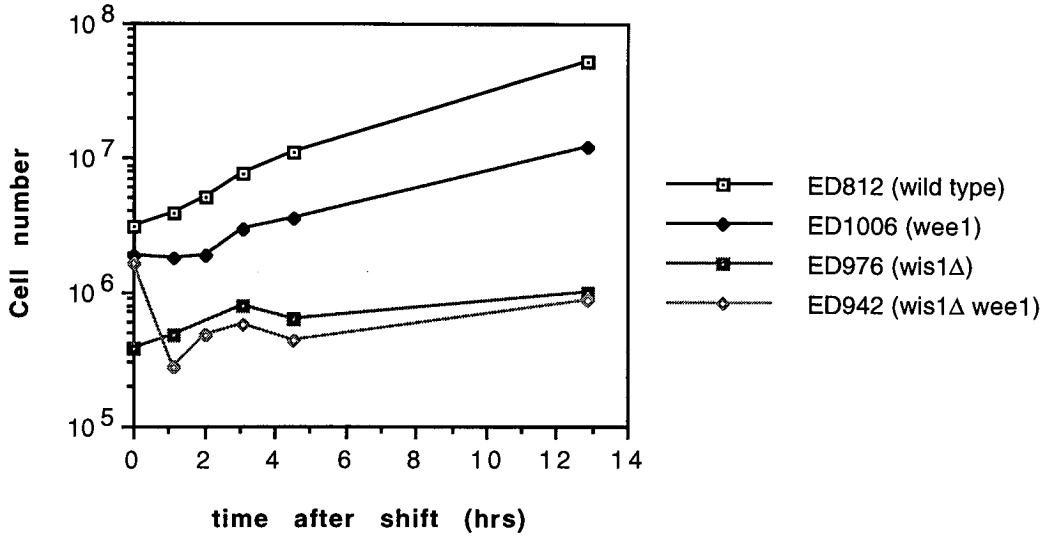

Figure 3.5: Cell number increase for *wee1* and *wis1* strains following a shift to 36°C.
Strains were grown in MM at 32°C and shifted to 36°C at time=0. Cell number was counted with a Coulter Counter.

Cell division in the *wee1 wis1*Δ culture ceased roughly three hours after the shift up, which is similar to a *wis1*Δ strain (see Figure 3.5 above). The *wee1-50* strain continued to divide, just like the wild type control.

Thus the lack of Wee1$^+$ does not affect the timing or the extent of the heat sensitivity of a *wis1*Δ strain. But, at 32°C, *wee1-50* strains have almost no Wee1 activity, and are therefore in a *wee1*$^-$ steady state. A more revealing experiment to address the issue of whether forcing cell division rescues the temperature sensitivity of a *wis1*Δ strain would involve shifting *wee1-50 wis1*Δ cells up from 25°C (which is the permissive temperature for *wee1-50*) to 36°C and following viability, cell length and number.

3.2.3: DAPI staining at 37°C

From these results, it was expected that other phenotypes would be apparent when *wis1*Δ cells were incubated at high temperature. To examine the DNA under these conditions, *wis1*Δ and wild type cells were stained with DAPI (which stains DNA blue under fluorescence) and examined microscopically at various times after a shift to 37°C. For the

first two hours after the shift, *wis1*Δ nuclei remained indistinguishable from those of wild type cells. However, between this time and five hours, they became gradually more diffuse (data not shown). The disruption of the nucleus seen at later time points appears to go hand in hand with the second stage of loss of viability (see Section 3.2.2.1 and Figure 3.4).

3.2.4: Investigation of osmoremediability of *wis1*Δ

*wis1*Δ mutants display heat sensitivity. To investigate this, Sophie Stettler tried to rescue this phenotype by the addition of sorbitol, which is a known osmoprotectant. *wis1*Δ cells were streaked onto YE supplemented with 1M sorbitol and incubated at 36°C. Surprisingly, the *wis1*Δ cells formed colonies, showing that the heat sensitivity of *wis1*Δ was indeed osmoremediable.

Dr. Chales Hoffman suggested that the observation that a considerable proportion of temperature sensitive mutations are osmoremediable might throw some light on the mechanism that lies behind the osmoremediability of *wis1*Δ. I decided to investigate the range of concentrations of sorbitol that would allow *wis1*Δ strains to grow at 36°C.

Freshly growing strains ED976 (*wis1*Δ) and wild type ED812 (h^-) were streaked to single colonies at 36°C on YE supplemented with various concentrations of sorbitol from 0 to 1.2M. It was already known that 1.5M sorbitol was lethal, even at normal temperatures (Millar et al., 1995; Shiozaki and Russell, 1995a). After two days the plates were examined for colony formation and, for the *wis1*Δ strain, cell morphology, as described in Table 3.1 and Figure 3.6.

Table 3.1: *wis1*Δ can grow at 36°C on sorbitol-supplemented YE medium.

test from bindab4...

	wild type	*wis1*Δ	
sorbitol concentration (M)	growth	growth	cell morphology
0	+	-	very long, branched, swollen cells
0.5	+	(+)	very long, swollen, sick cells
0.8	+	+	elongated, but healthy and dividing normally
0.9	+	+	elongated, but healthy and dividing normally
1.0	+	+	elongated, but healthy and dividing normally
1.2	+	(+)	very long, swollen, sick cells

- indicates no colony formation; (+) indicates the formation of some micro-colonies; + means all cells formed normal colonies.

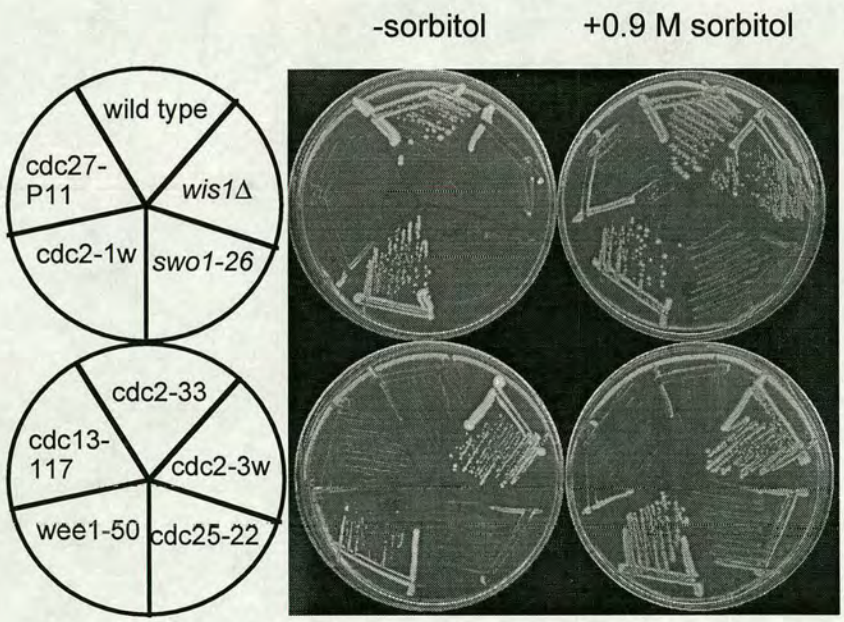

Figure 3.6: The effect on growth at 35°C of adding sorbitol to the medium.

Strains used: ED812 wild type; ED976 *wis1*Δ; ED1340 swo1-26; ED683 cdc2-1w; ED1119 cdc27-P11; ED907 cdc2-33; ED1039 cdc2-3w; ED1338 cdc25-22; ED1006 wee1-50; ED716 cdc13-117. They were grown on YE + 0.9M sorbitol at 35°C.

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The addition of intermediate concentrations of sorbitol (0.8M to 1.0M) to the medium allows *wis1*Δ strains to grow at 35°C, and furthermore, reduced the length they divide at. At concentrations outside this range, the typical *wis1*Δ high temperature phenotype was seen. Clearly, in a *wis1*Δ mutant, Wis1 protein itself cannot be rescued by an osmoprotectant, but one or more of its targets could be. Osmolarity and high temperature both affect protein folding. One possible explanation for the effect seen is that the addition of sorbitol is capable of reversing the deleterious effects on protein structure of high temperature. Furthermore, both the lethality and the cell cycle defect are being rescued.

Sorbitol is also capable of at least partially rescuing growth at 35°C of strains containing the following mutations: *wee1-50*, *cdc27-P11* and *cdc25-22*.

3.2.5: The role of trehalose-6-P synthase in heat shock survival

Over-expression of glycerol-3-phosphate dehydrogenase, encoded by the *gpd1* gene was shown to be sufficient to rescue the salt sensitivity of a *wis1*Δ strain (Aiba et al., 1995). But, unlike sorbitol, this was incapable of rescuing the cell length defect and temperature sensitivity associated with lack of *wis1*$^+$.

Given that over-expression of *gpd1*$^+$ is sufficient to suppress the salt sensitivity of a *wis1*Δ strain, I wondered whether *wis1*Δ temperature sensitivity could be suppressed in a similar manner. An obvious experiment to investigate this would be to over-express *tps1*$^+$, which encodes trehalose-6-phosphate synthase, as this enzyme is implicated in heat shock survival (Piper, 1993).

The plasmid pMR821 (see Figure 3.7), which contains the *tps1*$^+$ open-reading frame was amplified.

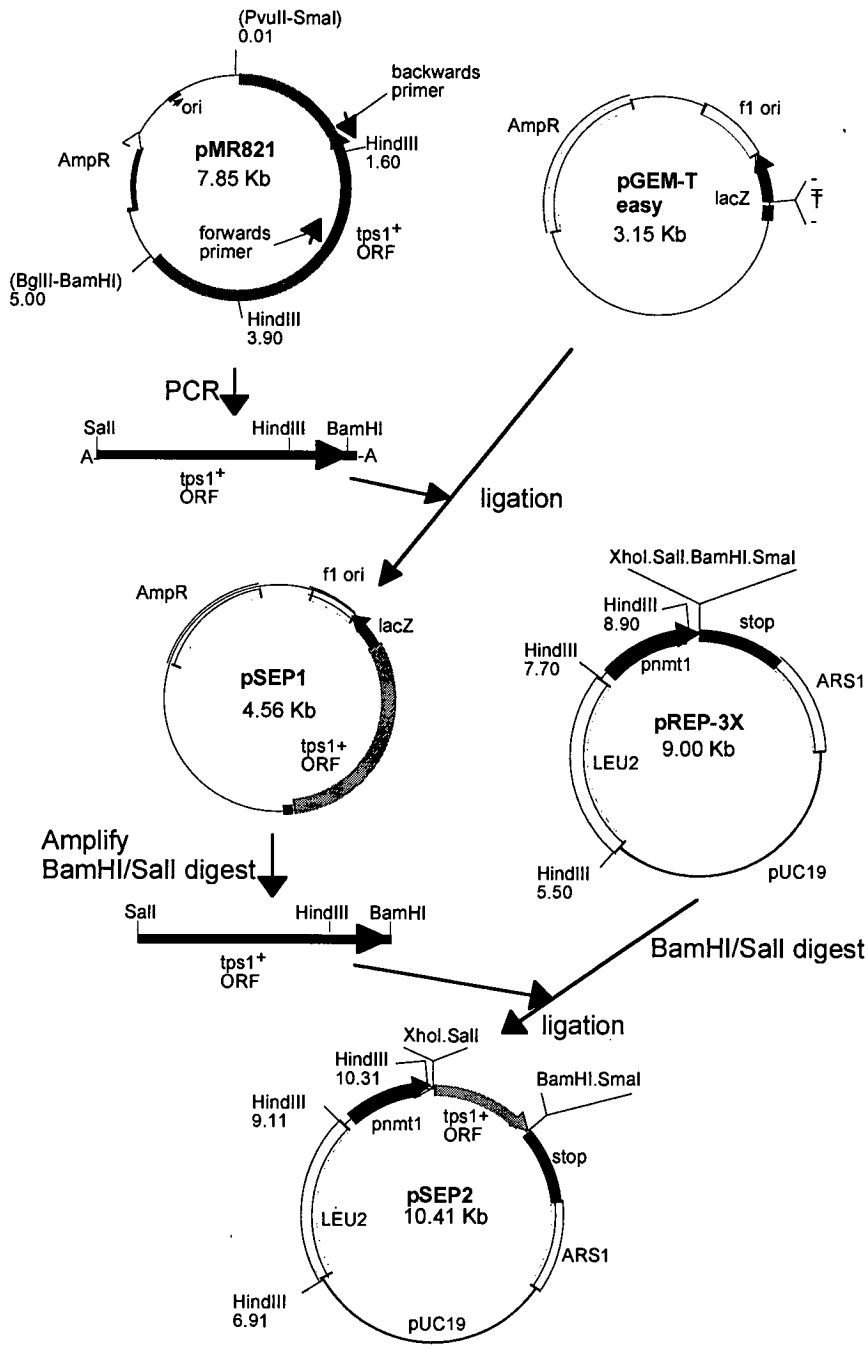

Figure 3.7: Scheme of plasmid design.

Primers were designed to produce a *SalI-BamHI* PCR product containing the *tps1*$^{+}$open reading frame.fragment. This was ligated into a Promega pGEM-T easy vector, to make pSEP1, which was amplified and digested with *SalI* and *BamHI*. The *tps1*$^{+}$-containing fragment was ligated into *BamHI/SalI* digested pREP-3X to make pSEP2.

A PCR approach was adopted to amplify the *tps1*$^+$ open-reading frame (ORF). Primers were designed to enable the amplified ORF to be ligated between the *SalI* and *BamHI* sites in the pREP-3X plasmid (see Figure 3.7).

A sample of the PCR was run on a gel and produced a single band at about 1400bp, which is consistent with the expected size of the ORF of 1439 bp (see Figure 3.8).

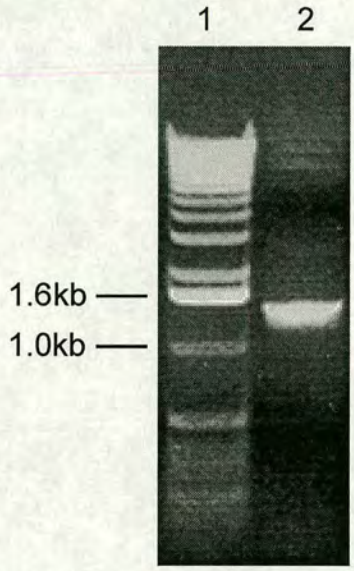

Figure 3.8: PCR to amplify the *tps1*$^+$ ORF.
lane 1: 1 kb ladder; lane 2 PCR mix. The band in lane 2 at approximately 1.4kb corresponds to the size expected for the *tps1*$^+$ open reading frame.

The PCR product was gel purified and transferred into pGEM-T EASY (supplied by Promega) in three different reactions, with the following molar ratios of insert to vector DNA: 4:1, 1:1 and 1:4. XL1 Blue *E. coli* were transformed with the ligation mix and plated onto LB Amp plates overnight at 37°C. Of the five colonies that grew in the 4:1 ratio transformation, a single one was white. This colony was picked into LB Amp medium and the plasmid DNA contained was isolated using a Qiagen MIDI prep kit. This plasmid was named pSEP1.

Samples of this DNA were run on a gel after restriction digestion with *BamHI* and/or *SalI*, to give the gel shown in Figure 3.9.

Figure 3.9: *BamHI* and *SalI* single and double digests on pSEP1.
lane1: 1kb ladder; 2: *BamHI* single digest; 3: *SalI* single digest; 4: double digest.

The 1.45kb fragment representing the *tps1*$^+$ ORF was gel purified from a *BamHI/SalI* double digestion, and transferred into gel purified pREP-3X which had also been double digested with *BamHI* and *SalI*.

This ligation was used to transform a JM109 strain and DNA was recovered from twenty-three of the resulting colonies by mini-prep. After *HindIII* digestion the DNA was run on a gel. An extra band at 1.6kb from inclusion of the *tps1*$^+$ ORF insert appeared in samples 8, 17 and 27.

To analyse these plasmids, DNA from sample number 8 was digested with *BamHI/SalI* and *HindIII* and run on a gel with various controls (see Figure 3.10)

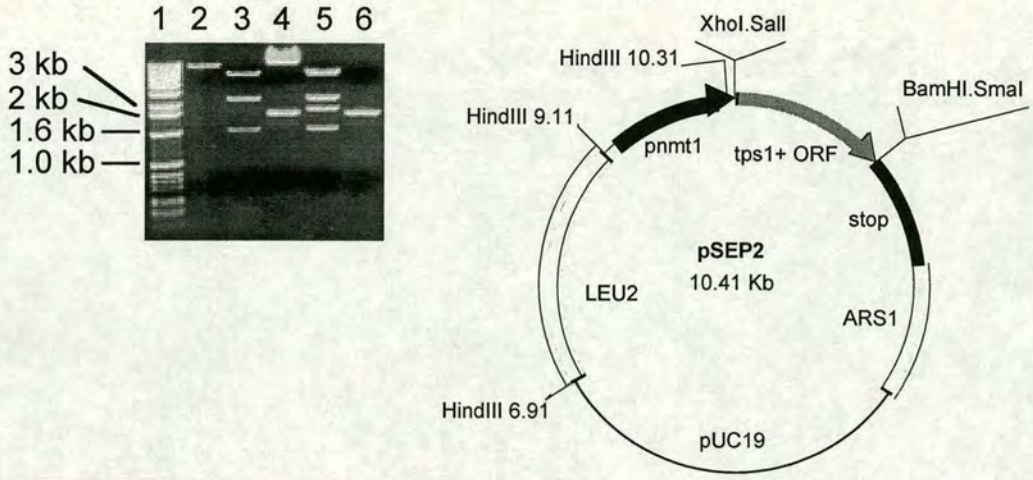

Figure 3.10
lane1: 1kb ladder; 2: pREP-3X *BamHI* digest; 3: pREP-3X *HindIII* digest; 4: pSEP2 *BamHI/SalI* digest; 5: pSEP2 *HindIII* digest; 6: tps1 ORF.

This band pattern was consistent with the *tps1*$^+$ ORF having integrated into the pREP-3X vector. In this vector, transcription is under the control of the *nmt1* promoter and is repressed in the presence of thiamine. All three plasmids (8, 17 and 27) were used to transform *wis1*$^+$ and *wis1*Δ *S. pombe* strains. Six resulting strains from each of the transformations were streaked (± thiamine) to single colonies on MM, on MM + 1.2 M KCl and on MM at 36°C.

None of the *wis1*Δ phenotypes was rescued in the transformants: they showed a cell length defect, and were salt and temperature sensitive. This suggests that over-expression of *tps1*$^+$ is not sufficient to rescue *wis1*Δ temperature sensitivity, although sequencing of the *tps1*$^+$ ORF in plasmid pSEP2 would be required to confirm that the correct sequence has been PCR amplified and cloned.

3.3: Investigating the architecture of the Wis1 pathway.

3.3.1: Are *win1* and *sty1* allelic?

The *sty1* gene encodes the MAPK downstream of the MAPKK Wis1 (Millar et al., 1995). One of the genes that was thought to be in the pathway is *win1*, which had not been cloned yet. The only allele of it was the mutation *win1-1*, whose molecular nature was unknown. The cell

length defect phenotype of *win1-1*, and its genetic interaction with *wis1* were consistent with it being a partial loss of function allele of *sty1*. To investigate this, a *sty1-1* strain (JM 1144) was crossed to a *win1-1* strain (ED632).

Five four-spored asci were obtained: the segregants were streaked onto YE KCl plates. After two days, colony growth and cell morphology were examined. By examining control strains, which had been streaked under these conditions, it was clear that wild type cells were distinguishable from the two mutants, the former being short and able to form colonies, whereas *sty1-1* cells were unable to form colonies and died highly elongated. In contrast, *win1-1* cells formed colonies, but the cells were long and slightly swollen.

In one of the tetrads there were two wild type segregants; in three, there was one wild type segregant and in the fifth, there was none, indicating that the tetrads fall into NPD, TT and PD classes, respectively. This shows that *sty1-1* and *win1-1* are different genes which are not closely linked.

win1 has since been cloned by Itaru Samejima and he has shown that it encodes a MAPKKK which activates Wis1 (pers. comm.).

3.3.2: Stress sensitivity, *fbp1*, and the Wis1 pathway.

3.3.2.1: Growth at high temperature and on high salt.

wis1 and *wis4* were cloned as genetic interactors with *win1* and were also shown to interact genetically with *mcs4* (Warbrick and Fantes, 1992). In addition, the three mutants *mcs4*, *win1* and *wis4* are elongated at division. Taken together, these data suggest that these three genes might lie somewhere in the Wis1 pathway.

Genetical experiments were undertaken in collaboration with Itaru Samejima on epistasis relationships between these putative Wis1 pathway genes to try to understand how they might fit into the pathway. Single, double and triple mutants containing the genes *mcs4*, *wis4* and *win1* were constructed. The resulting strains were tested for sensitivity to high temperature and high salt, as shown in Table 3.2.

Table 3.2: *wis1* pathway mutants under stress.

Strain	genotype	growth on KCl at 32°C	growth at 36°C
ED900	wild type	+	+
ED1186	*mcs4*	+	(+)
ED1209	*wis4*	+	+
ED1188	*win1*	+	+
ED1207	*mcs4 win1*	-	-
ED1211	*wis4 win1*	-	(+)
ED1225	*wis4 mcs4*	+	(+)
ED1234	*wis4 win1 mcs4*	-	-
ED1151	*sty1*	-	-
ED1085	*wis1*	-	-

Freshly-growing material was streaked to single colonies under the conditions shown.
+ indicates that many single colonies of healthy cells formed; (+) that some single colonies formed and cells in them were slightly sick; - indicates that no colonies formed.

The presence of a single *win1*, *wis4* or *mcs4* mutant does not make cells stress sensitive. The same is true of the *wis4 mcs4* double mutant. However, the three multiple mutants that contained the *win1-1* mutation (*win1 mcs4*, *win1 wis4* and *win1 mcs4 wis4*) were stress sensitive and were phenotypically identical to the *wis1* and *sty1* controls.

The observations that *win1 wis4* and *win1 mcs4* double mutants are stress sensitive are consistent with *win1* lying parallel to *mcs4* and *wis4* in the *wis1* pathway. The stress-resistance shown by the *wis4 mcs4* double mutant suggests that these two genes lie in series. Consistent with this, over-expression of an activated allele of *wis4*$^+$ can rescue the cell cycle defect of an *mcs4* mutation (Warbrick and Fantes, 1992), which places *wis4* downstream of *mcs4* (see Figure 3.11).

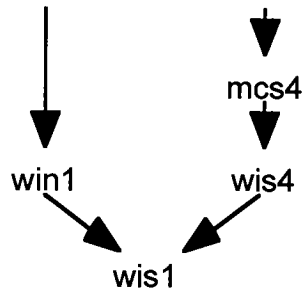

Figure 3.11: Schematic model of Wis1 pathway.
Wis4 and Mcs4 lie upstream of Wis1 in a separate branch of the pathway from Win1.

The *pka1* and *cyr1* genes lie in the cA-PK pathway which acts antagonistically to the *wis1* pathway. Thus, it is surprising that both *pka1* and *cyr1* mutants should be salt sensitive.

In addition to this study of stress sensitivity, Wis1 activity was monitored by assaying *fbp1* induction in the strains shown in Table 3.2.

3.3.2.2: *fbp1* induction following a shift to low glucose.

The *fbp1* gene encodes fructose-1,6-bisphosphatase, which is required for gluconeogenesis. *wis1*$^+$ is required for its transcription (Stettler et al., 1996), which is also regulated by the cA-PK pathway (Hoffman and Winston, 1991). The level of *fbp1* transcription in mutants of interest can therefore be assayed (Hoffman and Winston, 1990) to determine whether the mutation defines a gene in the *wis1* pathway Furthermore, genetic epistasis relationships can be studied in multiple mutants (Stettler et al., 1996).

Single or multiple *wis1* pathway mutants were grown in liquid MM in the presence of glucose and then shifted to low glucose medium, and transcription from the *fbp1* promoter was monitored by assaying activity of ß-galactosidase activity from the *fbp1-lacZ* construct included in these strains. The results are shown in Figure 3.12.

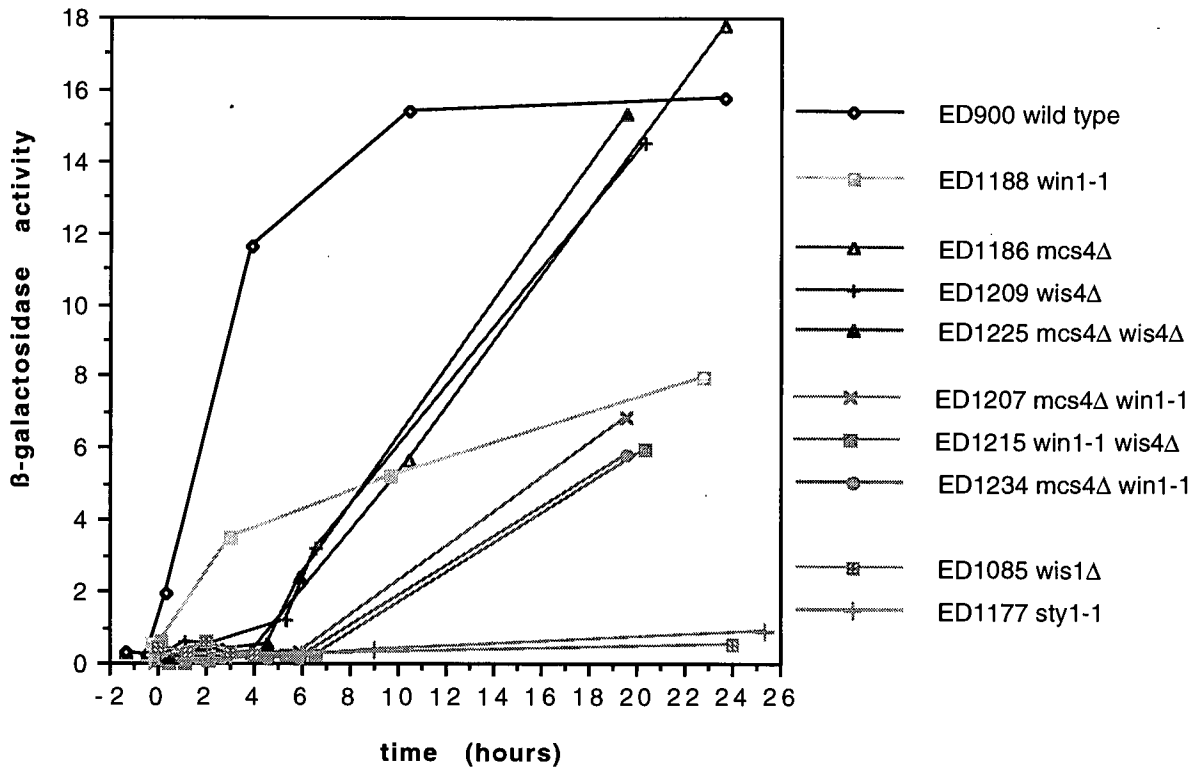

Figure 3.12: Induction of *fbp1* expression following shift to low glucose.
Strains were grown in MM and shifted to MM low glucose (0.1%) at time = 0.

The wild type strain (ED900) shows a rapid and strong induction of *fbp1* expression following the shift. Although all the mutants tested showed a reduced induction, patterns emerged and will be discussed in turn.

In considering these observations, it is useful to remember that Wis4 acts downstream of Mcs4 and that Win1 acts in parallel to these two proteins.

The induction seen in the *win1* strain was approximately a quarter that of the wild type, but took place with the same timing. In contrast, the *mcs4* and *wis4* strains showed no response for four hours and then showed a large response roughly equal in size to the wild type. Unsurprisingly, the *mcs4 wis4* double mutant showed identical behaviour to this.

Interestingly, when the *win1* mutation was introduced into the *mcs4*, *wis4* and *mcs4 wis4* strains, a roughly four-fold reduction in the level of

induction was seen, but there was no change in its timing. This is similar to the effect seen when the *win1* mutation is introduced into a wild type strain.

Both the *sty1* and the *wis1* strains showed no induction at all, whereas the *win1 mcs4 wis4* triple mutant had shown some induction. This suggests that Win1-1 protein has some residual activity, or that there may be another protein acting in parallel to Win1 and Mcs4/Wis4.

The pattern that emerges from consideration of the *win1*, *wis4* and *mcs4* mutants is consistent with the model presented above, namely that Win1 acts in parallel to the other two proteins. Furthermore, *mcs4*$^+$ and *wis4*$^+$ seem to be responsible for a swift, if small-scale response to a shift in nutritional conditions, whilst *win1*$^+$ appears to transduce the signal more slowly, even though the signal is larger.

To determine whether these three genes lie upstream or downstream of *wis1*, wild type, alleles of *wis1* that encode activated and catalytically inactive versions of Wis1 were over-expressed in these strains.

3.3.3: Interactions between the Wis1 and cA-PK pathways.

Over-expression of *wis1*$^+$ is lethal in a wild type strain. This lethality will be abrogated in a mutant strain that no longer allows the signal resulting from the over-activation of the Wis1 pathway. Thus if over-production of Wis1 in a given mutant background is no longer lethal, then the mutation lies in a gene via which Wis1 signals are normally transmitted.

Using this approach, in conjunction with the observation that over-expression of an activated allele of *wis1* was still lethal, the three genes, *mcs4*, *win1* and *wis4* were placed upstream of *wis1* by Itaru Samejima. Additional strains were transformed as part of the work in this thesis: I chose two cA-PK pathway mutants, namely *pka1* and *cgs1*, to investigate how this pathway interacted with the *wis1* pathway. Table 3.3 presents the results obtained.

Table 3.3: over-expression of *wis1*$^+$ in cA-PK mutants.

host strain	vector alone		*wis1*$^+$ over-production	
	cols.	cells	cols.	cells
wild type	+	normal	-	lysis, swollen, fat, short
pka1	+	short, round	-	no division, some lysis, short, round
cgs1	+	long	-	hetero-geneous, some lysis, fat

The formation of colonies and appearance of cells are given for *pka1* and *cgs1* mutants transformed with a control vector and over-expressing *wis1*$^+$.

Loss of either *pka1* or *cgs1* does not relieve the lethality of over-expression of *wis1*$^+$. In fact in a *pka1* background it seems to be even more deleterious to the cells than in wild type: they die before the first division. This synthetic interaction suggests that the two pathways lie in parallel, rather than in series. Furthermore, the presence of a *cgs1* mutation reduces the amount of lysis seen and allows cells to grow further before dying, suggesting this gene acts antagonistically to *wis1*. This is understandable, as Pka1 and a Cgs1 act in opposite directions in the cA-PK pathway.

These observations are consistent with others (eg Stettler et al., 1996), which suggest that the *wis1* and *pka1* pathways act in opposite directions. For example, the cell length of *cgs1Δ wis1Δ* double mutants is longer than that of the two single mutants. Consistent with this, I observed that while *cgs1*Δ and *wis1*Δ strains are partially sterile, the double mutant is completely sterile. These data strongly imply that the two pathways must at least act in parallel at some point.

3.4: Conclusions for Chapter 3.

The purposes of the experiments in this chapter were twofold: first to try to understand the nature of the stress sensitivity of a *wis1*Δ and second to throw light onto the architecture of the *wis1* pathway.

In addition to heat, salt and starvation sensitivity, *wis1*Δ cells are more susceptible to U.V. radiation than wild type, as found during this work and by two other groups (Degols and Russell, 1997; Kato et al., 1996).

But, *wis1*Δ stress sensitivity is not limited to the environmental challenges discussed so far: the mutant also tolerates oxidative stress (Degols et al., 1996), gamma rays and bleomycin (Kato et al., 1996) much less well than wild type.

Although *wis1*Δ cells are known to die from a variety of stresses, little is known about how this comes about. It seems that, at least for heat stress, the cells die in two stages. The first, being instant, seems to be caused by the failure of the cells to tolerate the shock of sudden transfer to a high temperature. Indeed, *wis1*Δ cells are highly sensitive to heat shock at 47°C (Stettler et al., 1996).

The second wave of death, interestingly, involves both a cell cycle arrest after approximately one division, and cellular elongation, owing to continued growth. Furthermore, this arrest is reversible for several hours. This behaviour is highly similar to that of a typical *cdc* mutant (Nurse et al., 1976). This suggests that a component of the cell cycle machinery becomes inactive in a *wis1*Δ strain at 36°C. This could be a protein that becomes temperature sensitive in the absence of Wis1 function.

Interestingly, the introduction of a *wee1* mutation into a *wis1*Δ strain rescues the cell cycle defect resulting from lack of functional Wis1, but does not have any effect on the stress sensitivity of the strain, at least as judged by behaviour at high temperature. This suggests that Wee1 only plays a role in the cell cycle signalling of the *wis1* pathway. A further experiment examining the effect of a shift from Wee1-50 permissive temperature (25°C) to the restrictive temperature for both *wee1* and *wis1* strains (36°C) would be useful, as this would be able to answer the question of whether accelerating cell division can rescue the stress sensitivity.

The experiments with *wee1* and *wis1* mutants strongly suggest that the extreme stress sensitivity caused by lack of Wis1 is not merely due to a cell cycle defect. Although there was no obvious defect in the DNA immediately following a shift to high temperature, the morphology of the nucleus became increasingly aberrant. Again, this hints at a general cellular breakdown, caused by a failure to adapt to prolonged stress conditions.

A clue to the nature of this breakdown may come from the observation that an osmoprotectant such as sorbitol is capable of reversing the heat sensitivity of a *wis1*Δ strain. Perhaps stressed *wis1*Δ cells die because of a failure to regulate internal osmolarity. This will have detrimental effects on cellular proteins and could thus lead to death. This ties in with the possibility discussed earlier that proteins in the cytoplasm become temperature sensitive without Wis1$^+$, and that at least one of these proteins is involved in cell cycle regulation.

Restoring the cell's ability to synthesise glycerol by over-expressing *gpd1*$^+$ is sufficient to rescue osmosensitivity, but not other phenotypes, which suggests that the model of osmoregulation mooted above is too simplistic, especially as trehalose is known to play an important role in heat tolerance in yeasts (Fernandez et al., 1995; Solapenna and Meyer-Fernandes, 1994), yet over-expression of trehalose synthesis gene *tps1*$^+$ was not able to rescue *wis1*Δ temperature sensitivity.

The genetical analysis on the putative genes in the *wis1* pathway described in this chapter and elsewhere (Samejima et al., 1997) revealed that *mcs4*, *win1* and *wis4* all lie upstream of *wis1*. Data from the stress sensitivity shown by *win1 mcs4* and *win1 wis4* mutants demonstrates that *win1* lies parallel to *mcs4* and *wis4*. The results from the experiment on the induction of *fbp1* following a shift to low glucose show that Win1$^+$ acts in a nutrient signalling pathway that responds more slowly to the shift to low glucose than does the pathway that comprises Mcs4$^+$ and Wis4$^+$.

When these experiments were carried out nothing was known about what lay downstream of *wis1*. To learn more about the function of Wis1, it was important to discover which genes *wis1* interacted with. To do this a genetic search for interacting mutations was embarked upon and this is the subject of the next chapter.

Chapter 4:

Isolation of extragenic suppressors of *wis1Δ* and phenotypic characterisation.

4.0: Introduction: Why look for mutants?

As discussed in Chapter 3, *wis1Δ* strains are sensitive to a variety of environmental stresses, such as high salt (1.2M KCl, 0.9M NaCl, 1.5 M sorbitol) and high temperature (36°C) (Millar et al., 1995; Shiozaki and Russell, 1995b; Stettler and Fantes, 1994). At the time this project was started, the molecular mechanisms accounting for these and other *wis1Δ* phenotypes were unknown; the only clue available was that Wis1 had sequence homology to the Mitogen-Activated Protein Kinase Kinase (MAPKK) family of proteins (Warbrick and Fantes, 1991), suggesting a signalling role for the fission yeast protein. More intriguingly, its closest homologue was Pbs2, a budding yeast MAPKK which is involved in osmotolerance (Brewster et al., 1993). This suggested that Wis1 might be involved in transmitting environmental stress signals to the nucleus, thus allowing the cell to adapt to inhospitable surroundings.

A further interesting phenotype of *wis1Δ* strains is the doubling of cell length at division on minimal medium (Warbrick and Fantes, 1991). This demonstrates that Wis1 regulates cell division to some extent, but is clearly not essential for completing the cell cycle under normal conditions. On the other hand, the greatly elongated cells seen when *wis1Δ* strains are stressed certainly resemble *cdc*$^-$ cells, so it is conceivable that Wis1 is essential for cell division under stressful conditions.

This project is concerned with investigating the fission yeast cell cycle. It is therefore interesting that the *wis1* gene was isolated during a screen in such a manner that suggests it plays a role in cell cycle control. This further suggests that mutations that suppress the *wis1Δ* cell cycle phenotype would be interesting, as they might define genes which integrate signals from a stress-activated MAPK pathway into the

machinery which controls cell division, one area of cellular signalling which was at the time (and still is) wholly perplexing.

Given that the questions this project sought to answer were fairly open-ended, a genetical approach seemed the most promising one to take.

4.1: Exploiting *wis1*Δ salt and heat sensitivity to select for suppressors.

One potential approach to exploring what genes lie downstream of *wis1* would be to exploit the stress sensitivities of *wis1*Δ strains in designing a screen for extragenic chromosomal mutations that are capable of suppressing the fragility of the original *wis1*Δ strain. Furthermore, an open-ended genetical approach to uncovering downstream elements might reveal more about the *wis1* pathway than a more focused method.

In addition to showing sensitivity to various stresses, *wis1*Δ strains show two phenotypes that were interesting as far as this study is concerned: the cell-division length defect and the failure to induce *fbp1* expression in response to glucose starvation. Selecting for mutations which suppress or enhance the cell length defect would not be an efficient way to generate mutants, so this was not chosen as a primary screen, but rather as a secondary phenotype to screen for.

Similarly, as Dr. Charles Hoffman had already performed extensive screens focusing on the regulation of *fbp1* (Hoffman and Winston, 1990), it seemed pointless to risk repeating his work. Therefore, it was decided to use *fbp1* expression as a tool, rather than as a primary selection. To assist with this, Charles Hoffman kindly sent us a strain containing a strain containing the *ura4::fbp1-lacZ* reporter construct consisting of the *lacZ* gene under control of the *fbp1* promoter, allowing regulation of the *fbp1* promoter to be monitored by assaying ß-galactosidase activity.

Thus, the *wis1*Δ phenotypes that will be exploited in this work are temperature sensitivity (36°C) and salt sensitivity (1.2M KCl) as primary selections and doubling of cell length at division and inability to induce *fbp1* transcription following glucose starvation as secondary phenotypes. (see Figure 4.1)

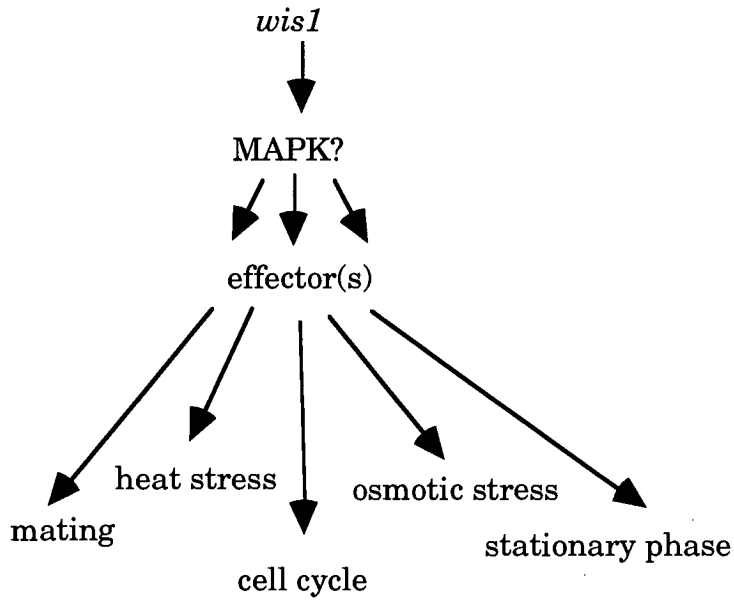

Figure 4.1: schematic diagram of *wis1* pathway and downstream cellular processes.
Wis1 was known to encode a Mitogen-Activated Protein Kinase Kinase (MAPKK). This strongly suggested a role in signalling and the existence of a MAPK directly downstream. Deletion of *wis1* leads to pleiotropic phenotypic effects and these suggest that the protein plays a role in many processes: cell cycle, mating and heat, salt and nutritional stress survival.

4.1.1: Rationale of mutagenesis and mutations expected

To avoid generating backmutations, the screen selected for mutagenesis contained a deletion of the *wis1* gene. Thus the sorts of mutations that were expected were loss of function mutations in genes counteracting Wis1 function, or gain of function mutations (activated alleles) in genes acting downstream (or in parallel). From sequence homology, Wis1 appeared to lie in a MAP kinase cascade. This strongly suggested that there would be a MAP kinase directly downstream of Wis1. A likely target gene could be this MAPK.

By considering the number and type of *wis1*Δ phenotypes suppressed in each mutagenised strain, it was hoped that information could be gleaned about where in the pathway the affected gene lay. One such target gene would be the MAPK which was predicted to lie directly below Wis1 in the MAPK cascade. Since the project was started, a MAP kinase has indeed

been found to lie directly downstream of Wis1 and it is called Sty1 (Millar et al., 1995), Spc1 (Shiozaki and Russell, 1995a), or Phh1 (Kato et al., 1996). An activating mutation in this gene would be predicted to suppress all *wis1*Δ phenotypes, and this should be possible to produce, as it was expected that an activated allele of a MAP kinase could be generated by changing a single base (Nishida, E., pers. comm.)

Mutations that only reverse the phenotype used in the initial selection of mutants might lie far downstream in a pathway that is responsible solely for tolerating that sort of stress and would tell us little about Wis1 function, whereas suppressing mutations which are capable of suppressing several or maybe all phenotypes should map to genes which lie at the heart of the Wis1 pathway and give important information on the cellular rôle of the pathway.

4.1.2: Optimising conditions for mutagenising *wis1*Δ cells.

Some pilot experiments were undertaken to establish mutagenesis conditions that could be scaled up. The two important points to take into account were cell plating density and conditions of selection.

High densities of *wis1*Δ cells "protect" each other and will therefore grow (to some extent) under conditions that would normally be toxic (Sophie Stettler, pers. comm.). Therefore, an appropriate plating density had to be found, before mutagenesis could be undertaken.

It was already known that 1.2M KCl and growth at 36°C was toxic to *wis1*Δ strains (Stettler, S., pers. comm.), but as mentioned above, it was not known what density of cells could be plated before the protection became a problem, nor what the frequency at which a *wis1*Δ strain acquires spontaneous suppressing mutations was.

Liquid YE was inoculated with a *wis1*Δ strain, ED961 (*wis1::his1*$^+$ *his1 ura4::fbp1-lacZ h*$^-$), and a wild type strain, ED812 (*h*$^-$) at 32°C. Cells were harvested and plated to 10^6, 10^7, $3x10^7$ and 10^8 cells per plate. Plates with these densities of cells were incubated at 34°C and 35.5°C; and at 32°C with 1.1M KCl and 1.2M KCl added to the medium. Under all these conditions, wild type cells were able to grow and formed a confluent lawn.

The "protection" mentioned above was visible for *wis1*Δ cells when plated at 10^7 per plate or more: a confluent lawn of cells was seen. This was also the case for selection at 34°C and 1.1 M KCl. From these plating experiments, it was concluded that good selection could be obtained at 35.5°C or on 1.2M KCl. Under these conditions, almost all cells died at or before the micro-colony stage. Occasionally, one or two larger colonies were seen (under both salt and temperature selection) and these were assumed to contain spontaneous suppressing mutations.

These colonies seemed to grow well under conditions that were toxic to *wis1*Δ cells, so twenty-four such colonies were picked and kept. Their ability to grow was assumed to be due to a genetic change, rather than the protection mentioned above (see next section). From this, the spontaneous mutation frequency can be estimated to be of the order of 10^{-6}.

4.1.2.1: Spontaneous suppressors.

Twenty-four supposedly *wis1*Δ colonies had grown at 35.5°C, a temperature at which normal *wis1*Δ cells are unable to proliferate. In order to investigate whether these colonies had grown because they harboured a spontaneous suppressing mutation, they were picked and restreaked to 35.5°C. Ten of them were able to form colonies well, and two were able to form a few colonies (weak suppression) (see Table 4.1; isolates 0-12 to 0-27). These isolates were numbered 0-12 to 0-27, ("zero-twelve" to "zero-twenty-seven") the zero denoting the absence of any artificial mutagenesis, to contrast these strains with those generated later by U.V.-induced mutagenesis (see Section 4.1.3 onwards).

Table 4.1: Summary of phenotypes of spontaneous suppressors.

	Selection	[a]Growth on KCl	[a]Growth at 35.5°C	*fbp1* induction[b]	*fbp1* induction[c]	cell length[d]
Control strains						
wild type	n/a	++	++	3.6	++	++
*wis1*Δ	n/a	-	-	0.15	-	-
Spontaneous suppressor strains						
0-1	KCl	+	++	0.22	-	-
0-2	KCl	+	++	0.21	-	-
0-3	KCl	+	+	0.26	-	-
0-4	KCl	+	-	0.26	-	-
0-5	KCl	++	+	0.13	-	-
0-6	KCl	+	+	0.65	(+)	-
0-7	KCl	(+)	++	0.26	-	-
0-8	KCl	(+)	-	0.25	-	-
0-9	KCl	(+)	+	0.24	-	-
0-12	35.5°C	-	++	0.26	-	-
0-15	35.5°C	-	++	0.39	-	-
0-16	35.5°C	-	++	0.39	-	-
0-17	35.5°C	-	++	0.47	-	-
0-19	35.5°C	-	++	0.68	(+)	-
0-20	35.5°C	-	++	0.90	(+)	-
0-22	35.5°C	-	+	1.81	+	-
0-23	35.5°C	-	++	0.03	-	-
0-24	35.5°C	-	++	0.26	-	-
0-25	35.5°C	-	++	0.03	-	-
0-26	35.5°C	-	++	0.78	(+)	-
0-27	35.5°C	-	+	0.31	-	-

[a]single colony forming ability (see below for key)
[b]in stationary phase (OD420 per 10^8cells)
[c]previous column summarised
[d]suppression of *wis1*Δ cell length defect on minimal plates
n/a = not applicable; ++ = identical to wild type; + = significant growth/suppression, but less than wild type; (+) = some growth/suppression; - = no growth/suppression.

As twelve interesting heat resistant mutants had been fortuitously recovered as a by-product of the investigation into plating densities, it was decided to see if any spontaneous salt-resistant mutants could be obtained using a similar approach. *wis1*Δ cells were grown on YE overnight at 32°C and then spread over four 1.2M KCl plates and incubated at 32°C. After eight days, thirteen colonies had grown and they were picked, regrown overnight and then streaked to single colonies on 1.2M KCl to check that the suppression was stably inherited. Nine of the

thirteen grew (to various degrees) when restreaked. (Table 4.1; isolates 0-1 to 0-9)

The best six salt resistant strains (0-1 to 0-6) together with the twelve temperature resistant strains were frozen as -70°C glycerol stocks.

As described in Section 4.1.1, I was interested in generating and investigating mutations that could suppress as many *wis1*Δ phenotypes as possible. Thus, the twenty-one spontaneous mutants that had so far been isolated were tested for suppression of various *wis1*Δ phenotypes in addition to the one used to select them. In all, the following phenotypes were investigated: temperature sensitivity, salt sensitivity, cell length at division and induction of *fbp1* in stationary phase (see Figure 4.2). The results from all four suppression assays are summarised in Table 4.1.

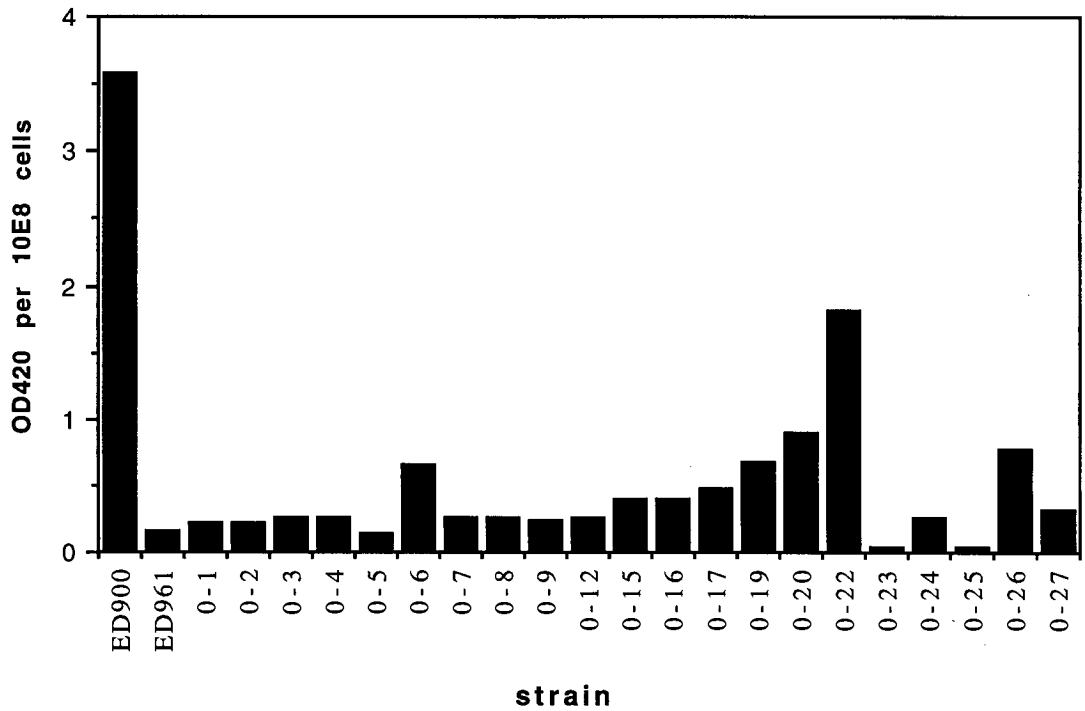

Figure 4.2: ß-galactosidase assays in stationary phase.
Cells were grown overnight in 1ml cultures to >1 x 10^7 per ml and assayed for ß-galactosidase activity. ED900 is wild type; ED961 *wis1Δ*; 0-1 etc are spontaneous suppressor strains.

Several of the mutants obtained (such as strains 0-4 and 0.12; Table 4.1) only suppressed the phenotype that was used in their selection. This

suggests that the mutation lies fairly far downstream of *wis1*, most likely in a part of the pathway that is solely responsible for tolerating salt stress (0-4) or heat stress (0-12). This kind of mutation is unfortunately not particularly interesting from the point of view of this project.

Some of the spontaneous suppressors isolated, however, suppress several phenotypes (e.g. 0-6). This suggests they contain mutations that lie in genes further upstream, before the pathway bifurcates. In fact, when measured microscopically, the cell length of strain 0-6 is also suppressed (see Section 4.3). Mutations of this kind are particularly important for this project, as they may define genes responsible for integrating different types of stress signals and the cell division machinery.

Of these mutants, the most interesting seemed to be 0-6, as the strain was capable of at least partially suppressing three of the four *wis1*Δ phenotypes examined. It is important to note that the cell length defect did not seem to be suppressed in the plate assay used. For this reason, more accurate cell length measurements were carried out (see Section 4.3). No other strains were taken forwards at this stage as an investigation into the types of mutants that could be obtained from U.V. mutagenesis was underway.

4.1.2.2: U.V. mutagenesis conditions

Although a small-scale investigation into spontaneous suppressors of *wis1*Δ had revealed some interesting strains, it seemed that a programme of induced mutagenesis would be productive for several reasons, the most important of which being that the frequency of mutation is greatly increased.

U.V. was chosen initially from the range of mutagens available as it produces a greater range of mutations than EMS or MNNG. The latter give rise to little apart from transitions at G.Cs (Kohalmi and Kunz, 1988), whereas U.V. produces transitions and transversions and single nucleotides deletions. This gives U.V. the potential to generate a wide spectrum of mutations. Furthermore, it is safer to use, and the equipment required was readily available.

It can be seen from Table 4.1 that a variety of different types of mutation were obtained spontaneously, but so far, a crucial class was missing: the mutation that is capable of suppressing all *wis1*Δ phenotypes. If it is possible to create such a mutation, it should be possible to isolate it, as well as others, by U.V.-induced mutagenesis.

4.1.2.3: Calibrating kill curve for U.V. mutagenesis.

From studies on the budding yeast, *Saccharomyces cerevisiae*, a kill rate of 50-90% is required to give rise to a large number of mutants in the surviving population; higher kill rates result in an increased frequency of strains with multiple mutations (p. 274 in Lawrence, 1991). These values were used as a guide to the kill rates to aim for in the fission yeast, *Schizosaccharomyces pombe*.

Cells from a wild type strain (ED812) and a *wis1*Δ strain (ED961) were grown at 28°C overnight in 20 ml YE liquid to mid-log phase. The cultures were diluted. 10^3, 10^4 and 10^5 cells of the *wis1*Δ strain were spread on separate YE plates. A subset of these conditions was used for the wild type control strain. The plates were then exposed to a range of doses of U.V. irradiation in a Stratalinker (wavelength 254nm), wrapped in foil to keep the plates in the dark, thus preventing photo-reactivation (p. 278 in Lawrence, 1991) and incubated until colonies had formed. The number of colonies that grew up was counted. Data obtained are shown in Figure 4.3.

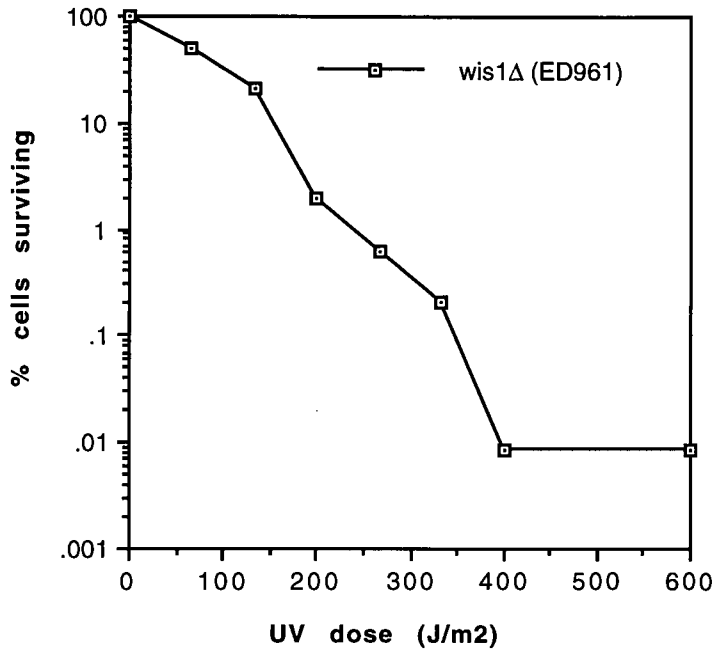

Figure 4.3: fraction of *wis1*Δ cells surviving U.V. irradiation.
Known numbers of exponentially-growing cells were plated onto YE and exposed to the doses of U.V. irradiation shown and then incubated until colonies formed. The proportion of cells that survived was calculated from the number of colonies that grew on each plate.

A further point to take into account is the fact that cells in suspension scatter U.V. light as a function of the density of the culture (Lawrence, 1991) so that cells deeper in the culture are exposed to less radiation than expected. From *S. cerevisiae*, as a rough, guide, conversion factors are: x1.5 for cultures of ~10^7 cells ml^{-1} and x10 for cultures of ~10^8 cells ml^{-1} (Lawrence, 1991).

4.1.3: Mutagenesis I

A kill rate of 75% was chosen to aim for as it lies approximately in the middle of the range of ideal kill rates, which goes from 50% to 90%. From Figure 4.2, this corresponds to about 130 J m^{-2} or about four seconds' irradiation. In a pilot experiment, *wis1*Δ cells (strain ED961) were grown in YE liquid medium to mid-log phase ($5x10^6$ cells/ml) and mutagenised using 254nm U.V. at a dose of 130 Jm^{-2}. The mutagenised cells were allowed to recover in the dark for 24 hours and then plated on YE supplemented with 1.2M KCl. Surprisingly, the kill rate was close to 0%, as opposed to the expected estimate of 75%. Twelve colonies grew on the

1.2M KCl plates and were picked and restreaked to single colonies on KCl. None of them grew after restreaking, so the strains were abandoned.

Two further mutagenesis experiments were carried out using similar conditions, but the kill rate was still close to 0% and no interesting mutants were recovered in either screen.

From these initial experiments, it seemed that the low kill rate achieved went hand in hand with poor recovery of mutants, and those that had been recovered probably contained spontaneous mutations, rather than induced ones.

4.1.4: Mutagenesis II

*wis1*Δ cells (strain ED961) at 10^7 ml^{-1} were exposed to 400, 600 and 800 Jm^{-2} irradiation (and given a swirl halfway through the exposure to mix the cells), allowed to recover in the dark at 32°C for seven hours and then plated at 10^6 cells per plate (seven plates at 35.5°C; three on 1.2M KCl). Kill rate was 43% for the cells that had been irradiated with 800 Jm^{-2}. This was surprisingly much less than predicted, but seemingly acceptable, as shown below.

From the cells that were subjected to KCl selection, 215 primary colonies were picked. When retested, 78 grew well (osmotic resistant, Osm^R), nine grew poorly and 51 did not grow at all. In the case of 35.5°C selection, 227 high temperature resistant ($Temp^R$) isolates were picked and restreaked to 35.5°C. At this temperature, 24 of these strains grew well and one strain grew poorly. These data are summarised in Table 4.2.

Table 4.2: summary of mutant colonies from Mutagenesis II (Mut II)

Selection	primary colonies	grow well when restreaked	grow partially when restreaked	no growth when restreaked
1.2M KCl	215	78	9	51
35.5°C	201	24	1	176

As can be seen from the Table above, rougly three times more Osm^R isolates were recovered than $Temp^R$ isolates. Both the Osm^R and $Temp^R$

suppressing strains were tested for suppression of other *wis1Δ* phenotypes (see Table 4.3). Several strains were isolated that were capable of suppressing other phenotypes in addition to the one that had been used in the primary selection (e.g., K44, K46, T16, T20). This had not been the case when the spontaneous mutants had been isolated, except for 0-6. Strain T20 was particularly interesting because it suppressed all the *wis1Δ* phenotypes it had been tested for so far.

Table 4.3: Summary table of Mutagenesis II revertants that suppress at least two phenotypes.

Isolate	Selection	KCl[a]	35.5°C[b]	*fbp1* [c]	length[d]
wild type control		+	+	+	+
wis1Δ control		-	-	-	-
K23	KCl	+	-	+	-
K37	KCl	+	+	-	-
K44	KCl	+	+	-	+
K46	KCl	+	+	+	+
K47	KCl	+	+	-	-
K49	KCl	+	+	-	-
K61	KCl	+	+	(+)	-
K62	KCl	+	+	(+)	-
K74	KCl	+	-	(+)	-
T3	35.5°C	+	+	-	-
T5	35.5°C	+	+	-	-
T6	35.5°C	+	+	-	-
T9	35.5°C	+	+	-	-
T11	35.5°C	+	+	-	-
T16	35.5°C	+	+	+	-
T17	35.5°C	+	+	-	-
T18	35.5°C	+	+	-	-
T19	35.5°C	+	+	-	-
T20	35.5°C	(+)	+	+	+

[a]ability to form colonies on YE + 1.2M KCl; + = form colonies well; (+) = form some colonies; - = form no colonies.

[b]ability to form colonies on YE at 35.5°C; same classification as for 1.2M KCl.

[c]ability to induce *fbp1* in stationary phase; + = good induction of *fbp1*; (+) = some induction; - = no induction.

[d]suppression of cell length defect on minimal medium; + = ~20% shorter than *wis1Δ*; (+) = ~10% shorter; - = no shorter.

Another improvement over the spontaneous mutants (with the exception of 0-6) was that some of the Mut II mutants were capable of suppressing the *wis1*Δ cell length defect. These are summarised in Table 4.4.

Table 4.4: Summary of short Mut II mutants

Selection	~10% shorter[a] than *wis1*Δ	~20% shorter[a] than *wis1*Δ
1.2M KCl	1, 13, 62, 67, 78	44, 46
35°C	-	20

[a]to estimate this, cells were grown overnight at 28°C on MM and length was examined microscopically, compared to *wis1*Δ and *wis1*$^+$ control strains. See Chapter 2 for details.

Furthermore, the extent of suppression of *wis1*Δ stress sensitivity afforded by induced suppressors was greater than for spontaneous suppressors. This is demonstrated in the following experiment.

The proportion of cells able to form colonies under stress conditions was estimated by examining cells in streaks microscopically. Whilst no *wis1*Δ cells form colonies under stressed conditions, the spontaneous mutants (eg 0-6) had been able to form colonies; but a background of dead cells surrounding the colonies that had formed was observed, which suggested that most cells were unable to grow. This dead background was reduced amongst the strains from Mutagenesis II: a greater proportion of the cells were able to form colonies. This suggests that the suppression of *wis1*Δ phenotypes was stronger in these strains.

The most promising mutants from Mutagenesis IIwere frozen down: K23, K46, K61, K62, K74; T16 and T20. It now seemed that the experimental approach adopted was capable of producing mutants of the desired types.

In contrast to the first few mutagenesis experiments, Mut II had both generated an interesting collection of mutants and a significant kill rate. This clearly demonstrated the importance of avoiding low mutation rates. It also seemed that that the relationship between U.V. dose and kill rate was a non-linear. So, to ensure that low mutation rates were avoided in the future, details of the experimental method being used were reviewed before further mutagenesis was undertaken.

4.1.5: Mutagenesis V

From the literature, vigorous mixing of the cells during irradiation is important as this achieves even exposure to the U.V. and minimises the scattering effect of cells in the top of the culture (p. 278 in Lawrence, 1991). It is clear from the above results that swirling the culture halfway through the time allotted is not sufficient to achieve even radiation of all cells. Thus, new mutagenesis experiments were planned in which the cells would be stirred vigorously during irradiation: the first was Mutagenesis V which would only use selection at 36°C selection.

For Mutagenesis V, strain ED1135 was grown to mid-log phase and U.V.-irradiated with 670 Jm^{-2} at 10^7 cells per ml with vigorous magnetic stirring of the culture. The cells were allowed to recover for eight hours at 32°C then plated to 36°C. Encouragingly, the kill rate was 86%.

The twenty-seven plates at 36°C yielded 123 suppressor strains, all of which grew when restreaked to 36°C. This was in stark contrast to previous mutagenesis experiments. Furthermore, the colony-forming ability of the mutants obtained was markedly improved: in streaks at 36°C, nearly all the cells grew to form colonies, whereas in previous studies, less than half of the cells were able to form colonies.

The Mutagensis V strains were next streaked to KCl to investigate salt resistance and to MM to examine suppression of cell length (see Table 4.5).

Table 4.5: Summary of mutants obtained in Mutagenesis V that suppress at least two phenotypes.

Isolate	KCl[a]	36°C[b]	length[c]
5.7	++	++	(+)
5.10	++	++	(+)
5.11	+	++	-
5.13	-	++	+
5.15	++	++	+
5.16	++	++	-
5.17	++	++	-
5.22	-	++	+
5.31	+	++	-
5.32	++	++	-
5.33	-	++	(+)
5.35	-	++	(+)
5.36	+	++	-
5.38	++	++	-
5.39	-	++	(+)
5.41	++	++	-
5.47	++	++	-
5.49	++	++	+
5.51	+	++	-
5.54	++	++	-
5.56	++	++	-
5.57	-	++	(+)
5.60	+	++	-
5.61	++	++	-
5.63	++	++	-
5.64	++	++	-
5.67	-	++	(+)
5.69	++	++	-
5.71	++	++	-
5.74	+	++	(+)
5.76	++	++	-
5.79	++	++	-
5.81	++	++	-
5.97	-	++	+
5.102	-	++	+
5.110	-	++	(+)
5.112	-	++	(+)
5.115	-	++	(+)
5.123	+	++	-

[a]ability to form colonies on YE + 1.2M KCl; ++ = nearly all cells form colonies; + = most cells form colonies; (+) = some cells form colonies; - = no colonies.

[b]ability to form colonies on YE at 36°C; same classification as for 1.2M KCl

[c]suppression of cell length defect on minimal medium; + = ~20% shorter than *wis1Δ*; (+) = ~10% shorter; - = same legth.

A further mutagenesis, using only salt selection, gave a promising kill rate of 88%, but, even so, produced no interesting mutants.

4.2: *fbp1* induction assays in suppressor strains.

The *fbp1*$^+$ gene encodes fructose-1,6-bisphosphatase, which is involved in gluconeogenesis. The gene is subject to glucose repression, mediated via the cA-PK pathway (Hoffman and Winston, 1991). Furthermore, the Wis1 pathway is required for its transcription (Stettler et al., 1996). An *fbp1-lacZ* reporter construct (Hoffman and Winston, 1990) had already been used to assay activity of the Wis1 pathway (see Chapter 3). Using this method, Sophie Stettler had investigated *fbp1* induction following a shift to low glucose. It was clear this treatment gave a different response to that seen in stationary phase (Stettler et al., 1996). For this reason two of the strains that had given positive results in the *fbp1* assay in stationary phase (T20 and K46) were retested in an assay which involved shifting exponentially growing cells from high to low glucose and following induction of *fbp1* over four hours.

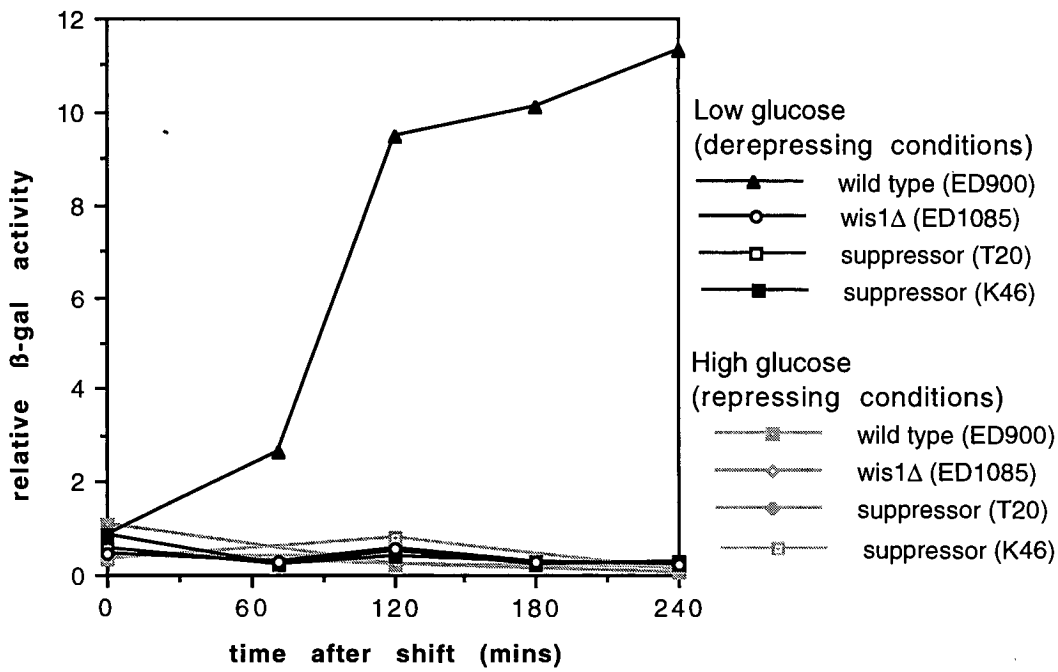

Figure 4.3: *fbp1* induction following a shift to low glucose
Exponentially-growing cultures were harvested by centrifugation from high (2%) glucose and resuspended in fresh pre-warmed low glucose (0.1%) medium. Strains T20 and K46 contain *wis1Δ* and a suppressing mutation. See Chapter 2 for description of ß-galactosidase assay.

Both the suppressor strains (T20 and K46) were able to induce strong expression of *fbp1* in stationary phase. However, when assayed under more defined conditions, by shifting cultures from high to low glucose, there was no evidence of any ability to express *fbp1* (see Figure 4.3). Sophie Stettler noticed that unsuppressed *wis1*Δ strains showed some induction of *fbp1* in stationary phase, conditions under which the suppressed strains induce well (Stettler et al., 1996). This suggests that there may be (an) alternative mechanism(s) for inducing *fbp1* in stationary phase.

4.3: Cell lengths

In Section 4.1, cells were examined on MM plates to determine whether the cell length defect of *wis1Δ* was suppressed. Minimal medium was chosen, as this exacerbates the *wis1*Δ cell length defect and hence significant differences in cell length are easy to see. The local nutritional conditions cells are exposed to on plates vary widely. Cells at the centre of colonies are starved of nutrients and grow slower. This leads to a heterogeneous cell population and thus differences in cell length at division. It is, therefore, more rigorous to measure cell lengths microscopically in a homogeneous exponentially-growing culture. This is particularly important when working with *wis1*Δ strains which are highly sensitive to nutritional conditions.

The best spontaneous and Mut II strains were grown to mid-log phase in supplemented minimal medium at 28°C and cell lengths were measured microscopically (see Table 4.6). Strain 0-6 did not proliferate healthily the first time, so the culture had to be reinoculated.

Table 4.6: Cell lengths of Mut II strains in minimal medium

Strain	genotype	length ± standard error (μm)	no. cells measured
ED900	*wis1*$^+$ *ura4::fbp1-lacZ*	15.6 ± 0.4	5
ED1085	*wis1::his1*$^+$ *his1-102 ura4::fbp1-lacZ h*$^-$	23.0 ± 0.4	20
0-6	as ED1085 + suppressing mutation	18.0 ± 0.3	15
T20	as ED1085 + suppressing mutation	17.0 ± 0.3	15
K46	as ED1085 + suppressing mutation	20.5 ± 0.4	12

Strains were measured in exponential growth phase in minimal medium growing at 28°C. Only cells that had just formed a septum but had not yet started to pinch in were chosen for length measurement.

T20 and 0-6 showed the greatest decrease in cell length compared to an unsuppressed *wis1*Δ strain. K46 also showed some suppression of the cell length defect.

As a result of genetic analysis of the best mutants produced in the mutagenesis experiments (see Chapter 5), the strains shown in the table below were selected for in-depth analysis and backcrossed to unmutagenised *wis1*Δ strains. This procedure is important, as it removes extraneous mutations and auxotrophic markers. Cells from these additional strains were grown in minimal medium at 32.5°C to mid-log phase and their lengths were measured microscopically (see Table 4.7).

Table 4.7: Cell lengths of Mut V strains (and T20) in minimal medium

Strain	genotype	length ± standard error (μm)	no. cells measured
ED812	wild type	15.1 ± 0.3	10
ED812	wild type	14.5 ± 0.2	16
ED976	*wis1::his1*$^+$ *his1-102*	24.5±0.7	14
derivative of T20 (SP3)	*wis1::his1*$^+$ *his1-102* *sow1-T20*	17.7±0.3	20
derivative of 5.22 (SP64)	*wis1::his1*$^+$ *his1-102* *sow1-5.22*	18.3±0.3	20
derivative of 5.7 (SP38)	*wis1::his1*$^+$ *his1-102* *sow2-5.7*	19.3±0.3	20
derivative of 5.11 (SP19)	*wis1::his1*$^+$ *his1-102* *sow2-5.11*	19.0±0.3	22

Measurements were made on cells growing exponentially in liquid minimal medium at 32.5°C

All four strains (SP3, SP64, SP38 and SP19) showed a reduction in cell length relative to an unsuppressed *wis1*Δ strain.

4.4: Selecting Mutants for further investigation

Once Mutagenesis II had been performed, strains 0-6, K46 and T20 seemed promising enough to warrant genetic analysis. These mutants comprised the first set of mutants to be analysed genetically. Suppressor strains from a later set (generated in Mutagenesis V) were, in turn, analysed genetically. These experiments are discussed in Chapter 5.

4.5: Conclusions for Chapter 4.

This project set out to obtain and characterise mutations which are able to suppress *wis1*Δ phenotypes. From Section 4.4 above, it is clear that such mutants can be generated. Furthermore, it is possible to suppress more than one *wis1*Δ phenotype in a single mutant strain (as can be seen from those mutants listed in Table 4.8) although fewer strains of this type were recovered than strains that can only suppress the phenotype they were selected for.

Table 4.8: Summary tables of suppressors strains made

Strain	Muta-genesis	Selection	[a]Growth on KCl	[a]Growth at 36°C	Suppression of cell length defect[b]	Suppression of length defect[c]
0-6	sponta neous	KCl	+	+	-	+
K46	Mut II	KCl	+	+	+	+
T16	Mut II	35.5°C	+	+	-	N.D.
T20	Mut II	35.5°C	(+)	+	+	+
5.7	Mut V	36°C	+(+)	+	(+)	+
5.10	Mut V	36°C	+(+)	+	(+)	N.D.
5.11	Mut V	36°C	+	+	-	+
5.13	Mut V	36°C	-	+	+	N.D.
5.15	Mut V	36°C	+(+)	+	+	N.D.
5.22	Mut V	36°C	-	+	+	+
5.31	Mut V	36°C	+	+	-	N.D.
5.36	Mut V	36°C	+	+	-	N.D.
5.49	Mut V	36°C	+(+)	+	+	N.D.
5.51	Mut V	36°C	+	+	-	N.D.
5.60	Mut V	36°C	+(+)	+	-	N.D.
5.74	Mut V	36°C	+	+	(+)	N.D.
5.97	Mut V	36°C	-	+	+	N.D.
5.102	Mut V	36°C	-	+	+	N.D.
5.123	Mut V	36°C	+	+	-	N.D.

[a] as judged by formation of single colonies
[b] on plates
[c] in liquid culture
N.D. not determined
+(+) indicates almost all cells form colonies; + = most cells form colonies; (+) = some cells form colonies and - = no colonies formed.

For there to be so many phenotypes in a *wis1*Δ strain, many cellular processes must lie downstream of Wis1. This implies that downstream of Wis1, the pathway becomes increasingly branched. Thus, mutations that suppress several phenotypes presumably encode proteins that act sooner after Wis1 in the pathway (and hence on more cellular processes) than those that suppress a single phenotype. This makes the former class more interesting to work on and is why members of this class was selected for further analysis.

The hypothetical suppressor of all *wis1*Δ phenotypes, such as an activated allele of *sty1*, was not recovered. Nevertheless, two sets of seemingly promising mutants were isolated (see Table 4.8) and they, together with

the mutations they harbour, will be analysed further. This is the subject of the next chapter.

One final conclusion from these studies is that selection on 1.2M KCl in general yielded mutants of poorer quality than selection at 36°C. This is perhaps because plating mutant strains straight onto medium containing 1.2M KCl is too harsh. Perhaps a better selection would be to arrange for the salt concentration (ie strength of selection) to increase gradually, as temperature does for plates in an incubator.

Chapter 5:

Genetic Characterisation of Suppressor Strains.

5.1: Introduction.

The previous chapter described the isolation and initial characterisation of a large number of suppressor strains. The first set of mutants to be isolated comprises the spontaneous mutant, 0-6 (see Section 4.1.2.1) and strains T20, K46 and T16, from mutagenesis II (see Section 4.1.4). This set, having been isolated first was already being analysed genetically by the time the second set was generated. The second set consists of mutants from mutagenesis V: 5.7, 5.10, 5.11, 5.13, 5.15, 5.22, 5.31, 5.36, 5.49, 5.51, 5.60, 5.74, 5.97, 5.102, 5.123 (see Section 4.1.5). Table 4.8 summarises the *wis1*Δ phenotypes suppressed by these strains.

Given that the nature of the suppressing mutations these strains contained was entirely unknown, I chose a genetic approach to analyse them in the first instance. First, strains were backcrossed to unmutagenised *wis1Δ* strains to determine the number of mutations that had been induced and to encourage the elimination of extraneous mutations. Next, the strains were crossed to eachother to determine how many different suppressing mutations had been isolated; then diploids were constructed to investigate whether the suppressors were dominant or recessive.

The suppressor strains were then crossed to *wis1*$^+$ strains, to investigate the phenotype of the induced mutations in the absence of *wis1Δ*. The primary aim of these experiments was to analyse the mutations, but the important secondary objective was to design a cloning strategy.

5.2: Genetic Analysis of 0-6, T20, K46 and T16

5.2.1: Backcrosses to *wis1*Δ strains

5.2.1.1: Strain 0-6 contains two suppressors.

In order to determine how many mutations were responsible for the suppression observed in strain 0-6, it was backcrossed to ED1010 (*wis1*Δ). Having dissected just seven tetrads, and streaked the progeny to YEK and YE at 36°C, it was clear that at least two genes were segregating: the suppression of temperature and salt sensitivities segregated independently.

Furthermore, when 0-6 was later crossed to a *wis1*$^+$ strain, a third phenotype segregated: sick, round, short cells were seen. This could be due to an interaction between the suppressors already identified, or the presence of a third suppressor. As this strain contains at least two mutations, it was not analysed further.

5.2.1.2: The suppression in strain K46 is too weak to be analysed.

Similarly, strain K46 was backcrossed to ED1010, and eight tetrads dissected. The *wis1*Δ phenotypes suppressed by this strain are salt sensitivity, heat sensitivity and cell length defect. As far as they could be classified, the suppressing mutations for the three phenotypes investigated appeared to be segregating separately. But, it was very hard to differentiate unambiguously between suppressed and unsuppressed segregants. Further evidence of the weakness of the suppression was seen when the strain was outcrossed into a *wis1*$^+$ background (see Section 5.2.4).

Furthermore, as it is unlikely that the strain could have acquired three independent mutations, all of which happen to suppress *wis1*Δ phenotypes, I concluded from this experiment that it would not be profitable to investigate this strain any further.

5.2.1.3: Strains T16 and T20 contain a single mutation.

Five asci of the cross T16 x ED1010 were dissected and although the KCl resistance was too weak to score unambiguously, temperature resistance

segregated 2:2, showing that a single gene is responsible for this phenotype.

Strain T20 was backcrossed to ED1010 in two separate experiments, in which a total of thirteen asci were dissected. Suppression of cell length segregated faithfully with suppression of temperature sensitivity showing that these two phenotypes are probably due to the same mutation. Suppression of salt sensitivity, was hard to score, but as far as I could tell, it seemed likely that it was due to the same mutation that was responsible for suppression of temperature sensitivity and cell length.

5.2.2: Analysis of linkage between the mutation in strains T16 and T20.

Each of these two strains contained a single suppressing mutation that gave rise to slightly different phenotypes in a *wis1*Δ background. An interesting question was whether these two mutations lay in the same gene. To investigate this, appropriate derivatives from the two strains were crossed to eachother.

A *wis1*Δ h^+ derivative of strain T20 (from the backcrosses in Section 5.2.1) was crossed to strain T16 (h^-) by random spores. 32 colonies from amongst the segregants were picked and streaked to 36°C. 24 of these colonies grew at 36°C; eight did not. This 3:1 ratio is as expected for the segregation of two unlinked genes (see Figure 5.1 for scheme of intercross).

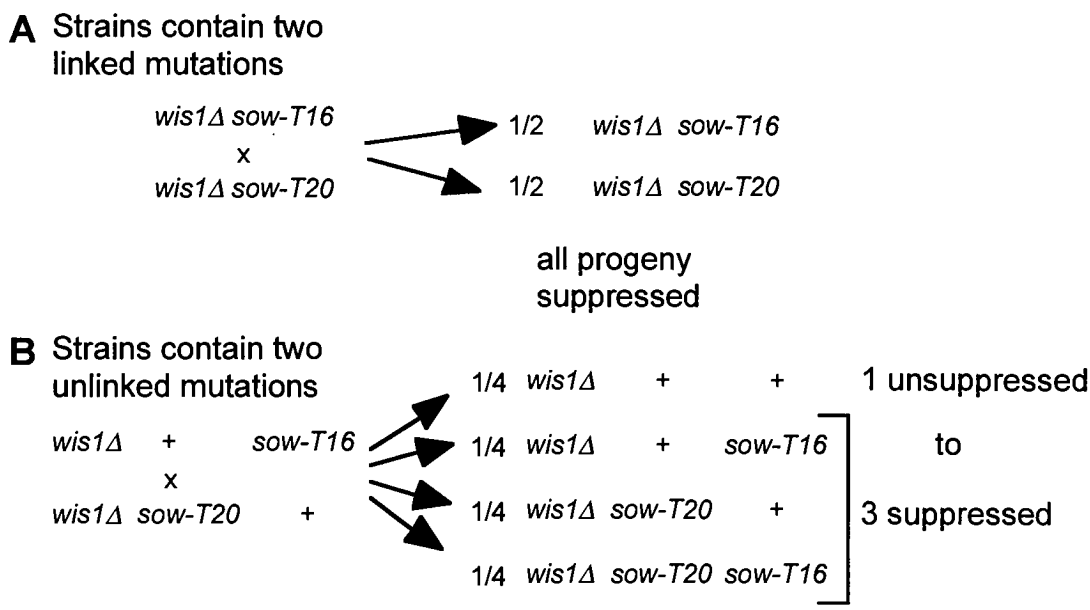

Figure 5.1: Scheme of intercross between T16 and T20.
sow = suppressor of *wis1Δ*

From this experiment and the previous one (see Section 5.2.1.3), the strains T16 and T20 each contain a single mutation in unlinked loci. To make it simpler to refer to these mutations, the genes they lie in were named *sow* (for suppressor of *wis1Δ*). The mutation in T20 will, for the time being, be referred to as *sow-T20* and the mutation in T16 as *sow-T16*.

5.2.3: Studying the *sow* mutations in heterozygous diploids.

The dominance or recessivity of a *sow* mutation can be assessed experimentally by making a homozygous *wis1Δ* diploid strain which contains one wild-type and one mutant allele of the gene of interest. Stable diploids can be made in *S. pombe* by mating an h^- strain with a strain carrying a *mat2-102* mutation, which prevents the resulting diploid from progressing past conjugation and into meiosis.

To make diploids that were heterozygous for a *sow* mutation, strains T16 (*wis1::his1*$^+$ *his1 ura4::fbp1-lacZ sow-T16* h^-) and T20 (*wis1::his1*$^+$ *his1 ura4::fbp1-lacZ sow-T20* h^-) were crossed to strain ED1076 (*wis1::his1*$^+$

his1 ade6 leu1 mat2-102) and after one, two and four days, diploids were selected on MM supplemented with Phloxin B at 32°C.

Once diploid colonies were large enough, they were streaked (with controls given in Table 5.1) to single colonies on YE plus various concentrations of KCl and to YE at 34°C and at 36°C (see Table 5.1). Neither *sow-T16-* nor *sow-T20-* containing heterozygous diploids were able to suppress lethality at salt concentrations above 0.6M, yet single colonies formed well at 0.5M and 0.6M KCl, concentrations that are toxic for an unsuppressed *wis1Δ/wis1Δ* diploid strain.

Table 5.1 heterozygous *sow* diploids.

conditions	*wis1Δ* / *wis1Δ* *sow-T16* / +	*wis1Δ* / *wis1Δ* *sow-T20* / +	*wis1Δ* / *wis1Δ*	w.t. / w.t.
0.3M KCl	N.D.	N.D.	+	++
0.4M KCl	N.D.	N.D.	(+)	++
0.5M KCl	+	+	-	++
0.6M KCl	+	+	-	++
0.7M KCl	-	-	-	++
0.8M KCl	N.D.	-	-	++
1.0M KCl	-	-	-	++
1.2M KCl	-	-	-	++
34°C	N.D.	++	-	++
36°C	N.D.	+	-	++

Various diploid strains were streaked onto media under the conditions shown. ++ means wild type behaviour (all cells formed colonies); + strong suppression (most cells form colonies); (+) some suppression; - unsuppressed *wis1Δ* phenotype (no colonies); N.D. = not determined.

The *sow-T20* heterozygous diploid was able to suppress, partially or better, the temperature sensitivity of a *wis1Δ/wis1Δ* diploid at both 34°C and 36°C. (Table 5.1). Furthermore, both *sow-T16* and *sow-T20* heterozygous diploids are shorter than *wis1Δ/wis1Δ* diploids.

These data show that the presence of a single copy of either of these *sow* mutations is enough to suppress *wis1Δ* phenotypes. This suggests that the mutant alleles are dominant over the wild type versions.

Given this, *sow-T16* and *sow-T20* could be fully or semi-dominant over the wild type alleles. To distinguish between these possibilties,

homozygous *wis1Δ* diploid strains were made that contained zero, one or two copies of each of these relevant *sow* mutations.

As Table 5.2 shows, the diploids heterozygous for *sow-T16* have, under appropriate conditions, a phenotype that lies between the two homozygotes. For example, at 36°C in a *wis1Δ/wis1Δ sow-T16/sow-T16* diploid, two copies of the *sow-T16* mutation suppress better than one (*wis1Δ/wis1Δ sow-T16/+*) (see Table 5.2). The same can be seen for 0.8M KCl.

Table 5.2: Diploids containing *sow-T16*

conditions	genotype of diploid			
	wis1Δ sow-T16 / *wis1Δ sow-T16*	*wis1Δ sow-T16* / *wis1Δ +*	*wis1Δ +* / *wis1Δ +*	w.t. / w.t.
0.7M KCl	+	+	-	++
0.8M KCl	+	(+)	-	++
36°C	+(+)	+	-	++

Diploid strains were streaked under the conditions shown. ++ means wild type behaviour (all cells formed colonies); +(+) nearly wild type (almost all cells forming colonies); + strong suppression (most cells form colonies); (+) some suppression/colonies; - unsuppressed *wis1Δ* phenotype (no colonies).

Similarly, *wis1Δ/wis1Δ sow-T20/sow-T20* diploids grow better on 0.8M KCl and at 36°C than *wis1Δ/wis1Δ sow-T20/+* diploids (see Table 5.3).

Table 5.3: Diploids containing *sow-T20*.

conditions	genotype of diploid			
	wis1Δ sow-T20 / *wis1Δ sow-T20*	*wis1Δ sow-T20* / *wis1Δ +*	*wis1Δ +* / *wis1Δ +*	w.t. / w.t.
0.7M KCl	+	+	-	++
0.8M KCl	+	(+)	-	++
36°C	++	+	-	++

See Table 5.2 for experimental details and key.

For both *sow-T16* and *sow-T20* two copies of the mutation suppress *wis1Δ* better than a single copy. This suggests that either the mutations are semi-dominant or that the effects seen are due to gene dosage, in which case the mutations could be recessive.

The dominance/recessivity of these two *sow* mutations has implications for the sort of gene they might lie in. Wis1 is an activator in the pathway it lies in, so a *sow* mutation could suppress *wis1*Δ if it was an activated allele of a gene encoding a protein which acts in the same direction as Wis1, ie downstream of (or in a parallel pathway to) Wis1. This sort of mutation should be at least partially dominant. An alternative type of mutation could be a loss of function mutation in a gene lying in a counteracting pathway. One would expect this type of mutation to recessive. See Section 5.4 for more discussion of these points.

5.2.4: Outcrossing the *sow* mutations from the *wis1*Δ background.

The *sow* mutations were isolated in *wis1*Δ strains and so far, they had only been studied in this background. The mutations exhibited interesting phenotypes in the absence of *wis1*, but would a *sow* mutation alone have a phenotype and, if so, what would it be like? What is more, if either *sow-T16* or *sow-T20* had a phenotype in a *wis1*$^+$ background, it might hint at the wild type function of the genes.

To generate *sow wis1*$^+$ derivatives, T20 (*wis1*::*his1*$^+$ *his1 ura4*::*fbp1-lacZ sow h*$^-$) was outcrossed to ED933 (*wis1*$^+$ *leu1 his1 h*$^+$), as illustrated in Figure 5.2. Strain ED933 was chosen because it is *his*$^-$ so that *wis1*::*his1*$^+$ segregants would be *his*$^+$ and *wis1*$^+$ strains would be *his*$^-$. The segregation of the *ura* and *leu* markers was as expected, verifying that meiosis had taken place normally in the cross.

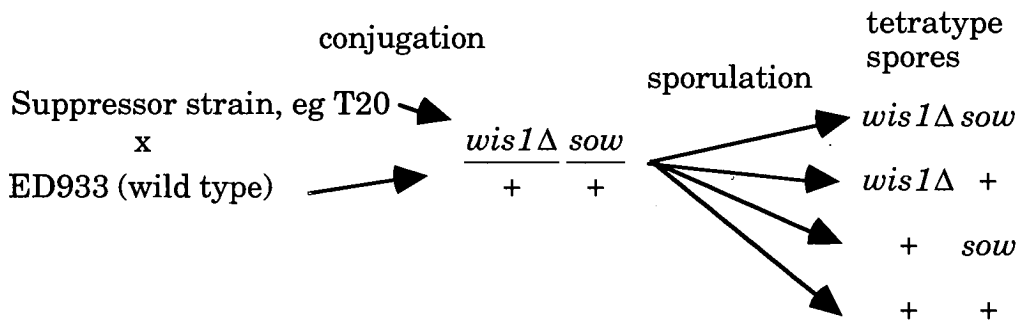

Figure 5.2: scheme for outcrossing a *sow* mutation from *wis1*Δ background to *wis1*$^+$.
A suppressor strain such as T20 is genotypically *wis1*Δ *sow*. When crossed to a wild type (*wis1*$^+$ *sow*$^+$), four different genotypes of spore are produced: *wis1*Δ *sow*, *wis1*Δ, *sow* and

wild type. Phenotypically, the double mutant segregant will behave like the suppressor parent; the *wis1*Δ single mutant like an unsuppressed *wis1*Δ strain; the wild type like the wild type parent. Only the phenotype of the *sow* segregant cannot be predicted.

The segregants resulting from two of these crosses were analysed for their phenotypes on minimal medium (to gauge cell length) and on high salt and at high temperature, with wild type, suppressed and unsuppressed *wis1*Δ strains acting as controls. The results for the four-spored asci obtained are given in Table 5.4; six three-spored asci were also analysed and data from these is consistent with the results given below.

Table 5.4: Tetrad analysis of T20 x ED933.

			spore	cell length	36°C	KCl
			ED933 control	wt	++ W	++ W
			wis1Δ control	v. long	-	-
cross	tetrad	type	T20 control	s. long	++ L	++ L
13/1	98	TT	A	wt	++ W	++ W
			B	s. long	++ L	++ L
			C	v. long	-	-
			D	short?	++ S?	++ W
3/2	110	PD	A	s. long	++ L	++ L
			B	wt	++ W	++ W
			C	s. long	++ L	++ L?
			D	wt	++ W	++ W
	126	TT	A	wt	++ W	++ W
			B	v. long	-	-?
			C	short	++ S	++ S
			D	s. long	++ L	-?
	130	PD	A	wt	++ W	++ W
			B	s. long	++ L	++ L
			C	s. long	++ L	++ L
			D	wt	++ W	++ W

Four-spored asci from two independent crosses are shown. Strains were crossed on SPA at 25°C. cell length = cell length at division; 36°C = single colony formation at 36°C; KCl = single colony formation on 1.2M KCl; TT = tetratype; PD = parental ditype; NPD = non-parental ditype.
Cell lengths: wt = wild type cell length; v. long = twice as long as wild type; short = shorter than wild type; s. long = slightly longer than wild type;
Colony formation at 36°C and on KCl: ++ W = many single colonies, cells wild type length; ++ S = many single colonies formed, cells short; ++ L = many single colonies formed, cells slightly long; - = no single colonies formed, cells very long and branched.

The segregants in tetrads 98 (13/1) and 126 (3/2) were interesting as each of the four spores clearly had a different phenotype. This allowed them to be classed as tetratypes (see Figure 5.2). This was most obvious for tetrad 126 (see Table 5.4), in which segregant 126A produced cells that were indistinguishable from the wild type control under all conditions tested; 126B was indistinguishable from a *wis1*Δ control strain and 126D was identical to the T20 parent strain. However, a novel phenotype was seen in the cells that grew from segregant 126C. This colony contained cells which that grew like wild type, but which divided at a slightly shorter length (see Section 5.3.4.2). The cells were also somewhat more rounded than normal cells and had thicker cell walls and were therefore reminiscent of cells in stationary phase.

All data so far suggested that tetrad 126 was tetratype. This implies that one of the *wis1*$^+$ segregants (126A and 126C) should be *sow*$^+$ and the other should be *sow*. The unusual short cells seen in 126C made it likely that the *sow* mutation lay in this strain, rather than 126A. To confirm this, strain 126C was backcrossed to a normal *wis1*Δ strain to see whether a suppressed *wis1*Δ strain would segregate (see Figure 5.3). 126A, which was not expected to contain a *sow* mutation, was also crossed to a *wis1*Δ strain to act as a control.

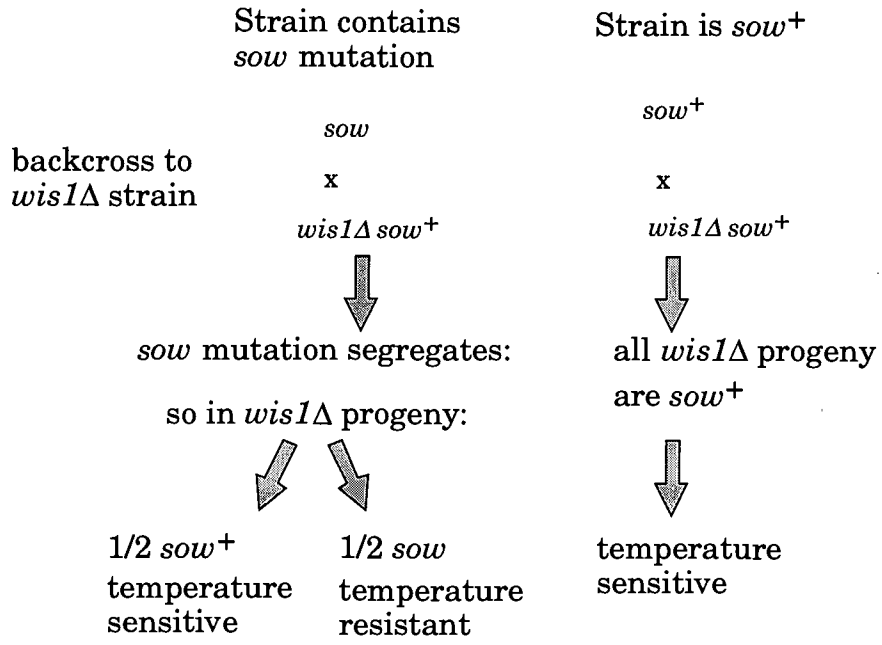

Figure 5.3: scheme of backcross of putative *sow* strains to a *wis1*Δ strain.
A strain containing a *sow* mutation will segregate *sow wis1*Δ progeny when crossed to a *wis1*Δ strain. These segregants will be phenotypically like a suppressed *wis1*Δ strain and will therefore be expected to grow at 36°C.

Strains 126A and 126C were backcrossed to strain ED1058 (*wis1*::*his1*$^+$ *his1*). After two days at 32°C, when colonies were just begining to appear, the plate was replicated to MM-his, to select for *wis1*::*his1*$^+$ (ie *wis1*Δ) segregants. Both this MM-his plate and the master YE plate were incubated further. Colonies that continued to grow on MM-his were examined microscopically to determine whether cell length was suppressed.

For the backcross to strain 126A, all colonies that grew on MM-his (ie *wis1*::*his1*$^+$) were made up of cells as long as *wis1*Δ cells. In the 126C cross, half of the *his1*$^+$ colonies contained cells as long as *wis1*Δ cells; the remainder were shorter than *wis1*Δ cells. This is consistent with there being no suppressor gene in strain 126A and there being a single suppressor gene segregating in the 126C backcross.

To confirm this, approximately 80 separate colonies were picked from the YE master plates and streaked to 1.2M KCl and patched to MM ± his. None of the *wis1*Δ segregants from the 126A cross were able to suppress *wis1*Δ osmosensitivity, whereas approximately half the *wis1*Δ progeny segregating from the 126C backcross were able to grow on high salt and half were not (see Table 5.5). This is consistent with there being a single suppressing *sow* mutation in strain 126C and none in strain 126A.

Table 5.5: segregation of salt sensitivity in backcrosses of 126A (putative *sow*$^+$) and 126C (putative *sow*).

	wis1::*his1*$^+$ (*wis1*Δ *his1*$^+$) segregants		*wis1*$^+$ *his1* segregants		
Strain crossed	KCl^R	KCl^S	KCl^R	KCl^S	Total
126A	0	44	42	0	86
126C	21	16	50	0	87

The fact that tetratype asci containing four viable spores could be dissected from the T20 outcross allows three important conclusions to be drawn: the *sow-T20* mutation alone is not essential under any of the conditions tested and, second, it leads to a reduction in cell length at division in a *wis1*$^+$ background, which suggests the gene may regulate the timing of mitosis. Lastly, the *sow-T20* locus is not linked to *wis1*.

Work on strain T20 had shown some interesting initial data on the *sow-T20* mutation it contained. Strain T16 was not investigated further, as the new set of mutants from mutagenesis V were more productive to work on and they are the subject of the next section.

5.3: Analysis of *sow* Mutants from Mutagenesis V.

The mutants had been made in Mutagenesis V suppressed better than most earlier mutants, which made them much easier to work on. I chose fifteen mutants to work on, selecting those that suppressed as many phenotypes as possible as well as mutants showing particularly strong suppression.

T20, being the best (and only) early mutant still worth investigating, was added to this later group of fifteen strains, making sixteen in all. In previous analysis, strains had been backcrossed to a *wis1Δ* strain first. This time, I decided to investigate linkage first. By crossing each strain to a derivative of T20, I would discover whether the *sow-T20* locus had also been mutated in the strains from Mutagenesis V or not. At the same time it should show which strains contained more than one mutation.

5.3.1: Intercrosses between *sow* mutants to define linkage groups

Each Mut V strain was crossed to T20.126B (*wis1Δ sow-T20* h^+). Intially, twenty spores from each cross were picked and streaked to 36°C. In some crosses, phenotypes segregated in an ambiguous way (as discussed below), so more progeny from these crosses were streaked and analysed for growth at 36°C. The results are given in Table 5.6.

Table 5.6: Mut V mutants crossed to T20

strain	no. of spores picked	no growth at 36°C	growth at 36°C	linked to *sow-T20*?	notes
5.7	20	4	16	no	
5.10	88	0	96	yes	
5.11	20	4	16	no	
5.13	50	0	50	yes	
5.15	20	0	20	yes	
5.22	59	0	59	yes	3 segregants suppressed only weakly
5.31	20	0	20	?	partially sterile (see text)
5.36	33	1	32	yes?	6 segregants suppressed only weakly
5.49	90	13	77	no	>1 unlinked genes (see text)
5.51	70	0	70	yes	
5.60	60	21	39	?	partially sterile (see text)
5.74	30	0	30	yes	
5.97	60	0	60	yes	
5.102	60	1	59	yes?	probably linked
5.123	20	5	15	no	

Initially, twenty spores were picked and streaked to 36°C. No growth at this temperature indicates *wis1Δ* behaviour; growth indicates suppressed *wis1Δ*.

If a particular MutV strain contains a suppressor which is unlinked to *sow-T20*, a quarter of the progeny is expected to be *sow*$^+$ (see Figure 5.1). As both parents are *wis1*Δ, these *sow*$^+$ segregants will not grow at 36°C, and these will be the only segregants in the cross unable to grow, giving rise to a 1:3 segregation of unsuppressed to suppressed. Strains such as 5.7, 5.11 and 5.123 showed a 1:3 segregation, indicating that the suppressor gene in these strains is unlinked to *sow-T20*.

The other strains did not show this 1:3 ratio, and so more progeny were analysed for growth at 36°C. For strains 5.10, 5.13, 5.15, 5.22, 5.51, 5.74 and 5.97 no unsuppressed progeny segregated indicating that the mutations in these strains are probably allelic to *sow-T20*.

The suppressing mutations in strains 5.36 and 5.102 are probably also allelic to *sow-T20*, but as one unsuppressed segregant was observed in each cross, this is not entirely certain.

The remaining strains either crossed poorly or seemed to contain more than one suppressing mutation. For 5.31 and 5.60, nearly all the spores plated contained parental markers suggesting that they were not the product of normal meiosis. Strain 5.49 showed approximately 1:7 segregation. This suggested that this strain had two unlinked suppressors and that they were segregating as well as the single suppressor from strain T20. These three strains was abandoned.

Of the strains that could be analysed straightforwardly, nine contained mutations that were linked to *sow-T20*, thus forming a linkage group, which was named *sow1*. This group consists of: T20, 5.10, 5.13, 5.15, 5.22, 5.36, 5.74, 5.97 and 5.102 (see Table 5.8). The *sow-T20* mutation in strain T20 was accordingly renamed *sow1-T20*.

The remaining strains (5.7, 5.11 and 5.123) were crossed to eachother to see if they formed a second linkage group. An *h*$^+$ derivative of 5.7 was crossed to 5.11 and 5.123. Sixty progeny from each cross were assayed for growth at 36°C, as shown in Table 5.7.

Table 5.7: linkage analysis between putative *sow2* alleles

cross	no growth at 36°C	growth at 36°C	linked?
5.7 h^+ x 5.11 h^-	1	59	yes?
5.7 h^+ x 5.123 h^-	0	60	yes

No unsuppressed progeny segregated in the cross between 5.7 and 5.123, showing that the *sow* mutations these strains contain are at least closely linked and therfore probably allelic. This is most likely the case for strains 5.7 and 5.11 too, even though a single unsuppressed segregant was observed. As both 5.11 and 5.123 are linked to 5.7, these three strains form a second linkage group, *sow2*. These results are summarised in Table 5.8.

Table 5.8: strains in the *sow* linkage groups.

locus	strains	*sow* alleles
sow1	T20, 5.10, 5.13, 5.15, 5.22, 5.36, 5.74, 5.97, 5.102	*sow1-T20*, *sow1-10*, *sow1-13*, *sow1-15*, etc.
sow2	5.7, 5.11, 5.123	*sow2-7*, *sow2-11*, *sow2-123*

5.3.2: Backcrossing suppressed strains to a *wis1*Δ strain

Three strongly-suppressing strains from each of the *sow1* and *sow2* linkage groups were picked as representative mutants. From the crosses to a T20 h^+ strain, which were described in Section 5.3.1, it appeared that the strains contained a single suppressor. To verify this, they were backcrossed to a *wis1*Δ strain, the results of which are given in Table 5.9.

Table 5.9: new mutants backcrossed to ED1010.

		no. of asci dissected		
strain	linkage group	4-spored	3-spored	no. of asci segregating 2:2
T20	*sow1*	4	3	7[a]
5.10	*sow1*	2	1	3[a]
5.22	*sow1*	1	1	2[a]
5.7	*sow2*	5	0	5[a]
		5	3	8[b]
5.11	*sow2*	14	0	14[a]
		4	5	9[b]
5.123	*sow2*	3	4	7[b]

All strains were crossed to ED1010 (*wis1*Δ) on SPA for three days at 20°C. Strains 5.10 and 5.22 crossed poorly. Growth at 36°C was tested by streaking to YE at 36°C for three days. Cell length was examined on MM at 28°C.

[a]segregation as per single mutation (2:2) and cosegregation of temperature resistance and reduced cell length.

[b]2:2 segregation of temperature supression.

The data presented in Table 5.9 confirmed that all of the above strains (T20, 5.7, 5.10, 5.11, 5.22 and 5.123) contained a single suppressing mutation. Some of the segregants from these crosses had combinations of markers in them that would make them useful for future work, so -70°C glycerol stocks were made from them. Details are given in Table 5.10.

Table 5.10: backcrossed strains that were frozen for future use.

Strain	from cross	genotype
SP31	5.11 x ED1010	*sow2-11 wis1::his1*$^+$ *his1 ura4::fbp1-lacZ* h^-
SP39	5.7 x ED1010	*sow2-7 wis1::his1*$^+$ *his1 ura4::fbp1-lacZ* h^-
SP60	5.22 x ED1010	*sow1-22 wis1::his1*$^+$ *his1 ura4::fbp1-lacZ* h^-

5.3.3: *sow1* and *sow2* show synergistic suppression.

During the intercrosses described in Section 5.3.1, it was observed that some of the progeny grew better than normal suppressed *wis1*Δ strains at 36°C and were practically identical to wild type under these conditions. These progeny came from crosses in which unsuppressed *wis1*Δ progeny were seen to segregate. The crosses must therefore have been between unlinked suppressors ie *wis1 sow1* x *wis1 sow2*. Segregation of *wis1 sow1*

sow2 triple mutants would also be expected in these crosses. It seemed that it was the triple mutant that was growing better than the *wis1Δ sow* double mutants at 36°C.

To investigate, *wis1 sow1-T20* was crossed to *wis1 sow2-11* and tetrads were analysed. Seventeen tetrads were isolated and the segregants were streaked to single colonies at 36°C with wild type, *wis1Δ*, *wis1Δ sow1-T20* and *wis1Δ sow2-11* control strains.

Three phenotypic classes were seen amongst the progeny: the first class was unable to grow at 36°C and at this temperature, the cells became highly elongated and branched. This class was indistinguishable from the *wis1Δ* control strain, so it was assigned the *wis1Δ* genotype.

The second class could grow at 36°C, and produced slightly elongated cells at this temperature. The behaviour of this class and of the two *wis1 sow* double mutant controls, was indistinguishable. It was therefore deduced that this class represented *wis1Δ sow1* and *wis1Δ sow2* double mutants.

The remaining class grew very well at 36°C, producing larger colonies after one day than the *wis1 sow* double mutants. These colonies consisted of cells that were almost indistinguishable from wild type. This class was deduced to represent the *wis1Δ sow1 sow2* triple mutant.

Tetrads from the *wis1 sow1* x *wis1 sow2* cross contained all three phenotypic classes consisted of one unsuppressed segregant (ie *wis1Δ*); two suppressed segregants (*wis1 sow1* and *wis1 sow2*) and one nearly wild type segregant (*wis1 sow1 sow2*). These tetrads were presumably tetratype. The segregants from one such tetrad (10F-I) were streaked to single colonies at 36°C with control strains and growth after one and three days is shown in Figure 5.4.

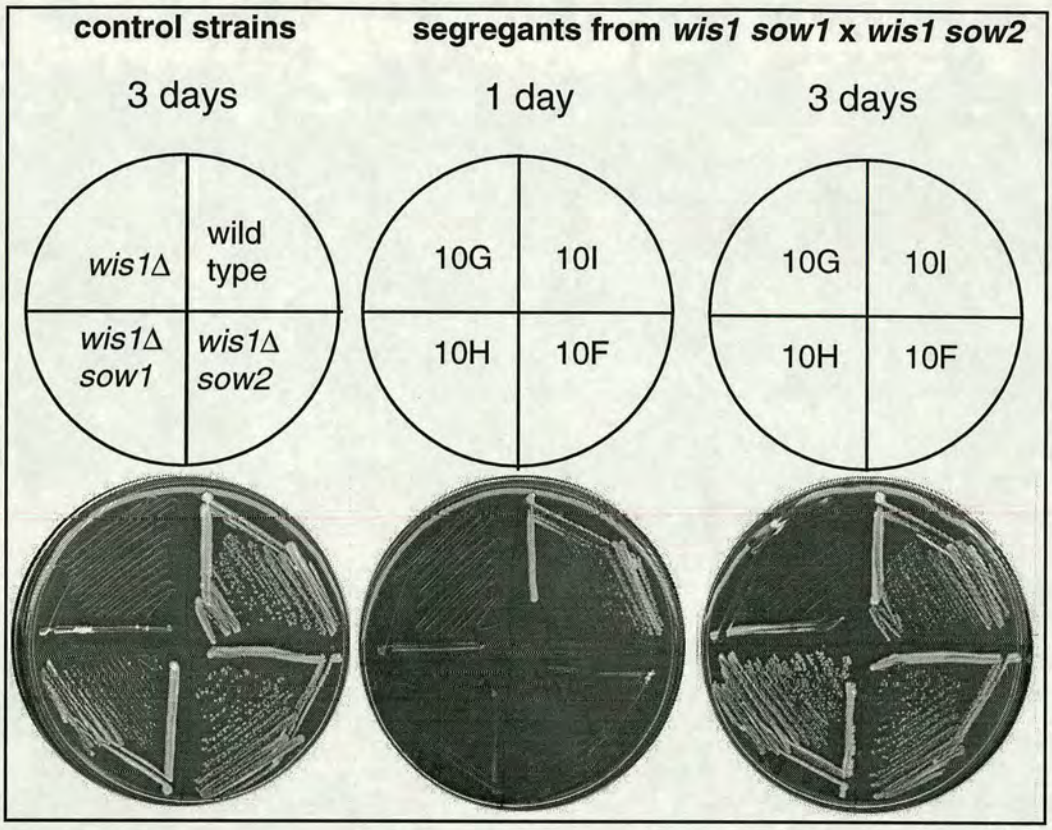

Figure 5.4: suppression of *wis1*Δ temperature sensitivity by *sow1* and *sow2*.
Wild type, *wis1*Δ, *wis1 sow1* and *wis1 sow2* control strains were streaked with the segregants from tetrad 10F-I from the cross *wis1 sow1* x *wis1 sow2* to YE at 36°C. Growth after one or three days is shown.

Segregant 10G does not grow at 36°C, even after three days' incubation and was indistinguishable from the *wis1*Δ control strain, so, presumably, it corresponds to *wis1*Δ; 10F and 10H form colonies after three days and resemble the *wis1 sow1* and *wis1 sow2* control strains. They were therefore assigned the *wis1 sow* genotype, although it was not possible to tell whether these strains contained the *sow1* or *sow2* suppressor.

The remaining segregant (10I) formed colonies at 36°C after one day (see Figure 5.4). At this time, the *wis1 sow* strains had not yet formed colonies, but the wild type control strain had (data not shown). The genotype of this strain was deduced to be *wis1 sow1 sow2* because this segregant grew better than *wis1 sow* strains.

The segregants from this tetrad were grown on MM to determine whether cell length suppression was affected in the triple mutant strains. The *wis1 sow1 sow2* triple mutant consisted of cells that were the same length as wild type, whereas the double mutants were slightly elongated compared to wild type.

These observations show that *sow1* and *sow2* suppress *wis1*Δ temperature sensitivity and cell length defect additively.

5.3.4: Making *wis1*$^+$ *sow1* and *sow2* derivatives.

When *sow1-T20* had been crossed out of a *wis1*Δ background in Section 5.2.4, it generated a *sow1-T20 wis1*$^+$ strain, that divided at a reduced cell length. To investigate whether this was the case for any of the other *sow* alleles, the strains in Table 5.10 were crossed to ED933 (*leu1 his1 h*$^+$). The numbers of asci dissected for each of these crosses is given in Table 5.11 below.

Table 5.11: outcrosses to ED933.

cross	4-spored asci	3-spored asci	total asci
SP31 x ED933	4	8	12
SP39 x ED933	4	4	8
SP60 x ED933	6	4	10

The *wis1*Δ and *wis1*Δ *sow* segregants resulting from these crosses could be unambiguously classified, as they could be compared to previously isolated control strains of known genotype (see Table 5.12). The fact that *sow1-T20* had a very similar, but subtly different phenotype to wild type predicted that distinguishing strains of these genotypes would be rather hard. However, as shown in Table 5.13 - Table 5.15, segregants arose in these outcrosses that were distinguishable from wild type, albeit with difficulty.

Table 5.12: Phenotypes of control strains used in *sow* outcrosses to ED933.

		phenotypes			
control strain	relevant genotype	36°C → 36°C	streaks at 36°C	colour on phloxin B	cell length on MM
SP31, SP39, SP60	*wis1Δ sow*	+	++	P	~1.5 x w.t.
ED1135	*wis1Δ*	-	-	P	~2 x w.t.
ED933	wild type	+	++	W	w.t.

36°C → 36°C: patches were grown on YE at 36°C for three days, then replicated to YE at 36°C.

Consider a sample tetratype tetrad (7A-D) from the cross between SP60 (*wis1Δ sow1-22*) and ED933 (wild type) first. The following classes of segregants were found in this tetrad (see Table 5.13): the colony that grew from segregant 7B contained cells that grew well at 36°C, but were pink on Phloxin B and were roughly 1.5x longer than wild type cells. This class therefore comprised suppressed *wis1Δ* cells. 7C contained cells that would not grow at 36°C, were pink on Phloxin B and were twice as long as wild type cells on minimal medium, and are thus indistinguishable from *wis1Δ* cells. 7D contained cells that were grew just like wild type under the conditions examined.

Table 5.13: Tetratype tetrad 7A-D from SP60 x ED933.

segregant	deduced genotype	36°C → 36°C	streaks at 36°C	colour on phloxin B	cell length on MM
7A	*sow1-22?*	+	++	W	< w.t.[a]
7B	*sow1-22 wis1Δ*	+	++	P	~1.5 x w.t.
7C	*wis1Δ*	-	-	P	~2 x w.t.
7D	*wild type*	+	++	W	w.t.

36°C → 36°C: patches were grown on YE at 36°C for three days, then replicated to YE at 36°C.

[a]this segregant consisted of slightly rounded cells which divided at a reduced cell length compared to wild type.

Thus, the three segregants dealt with so far each matched one of the three control strains exactly. This left segregant 7A, which gave rise to cells which resembled wild type, but were slightly rounded and divided at

a shorter length. By a process of elimination, the genotype of this segregant must be *wis1*$^+$ *sow1-22*.

Tetrad 7A-D is used here as an example. Patterns of segregation in the other nine tetrads that were dissected in this cross were consistent with this tetrad. From this it was concluded that the *sow1-22* allele, like the *sow1-T20* allele gives rise to a reduction in cell length at division as well as making cells rounded.

A representative tetratype tetrad from the SP31 (*wis1Δ sow2-11*) x ED933 (wild type) outcross is shown in Table 5.14.

Table 5.14: Tetratype tetrad 10A-D from SP31 x ED933.

segregant	deduced genotype	36°C → 36°C	streaks at 36°C	colour on phloxin B	cell length on MM
10A	*w.t.*	+	++	W	w.t.
10B	*wis1Δ sow2-11*	+	++	P	~1.5 x w.t.
10C	*sow2-11*?	+	++	W	~ w.t.[a]
10D	*wis1Δ*	-	-	P	~2 x w.t.

36°C → 36°C: patches were grown on YE at 36°C for three days, then replicated to YE at 36°C.
[a]these cells were rounded and slightly bulgy compared to wild type.

Again three of the four segregants (10A, 10B and 10D) corresponded to the three control strains (wild type, SP31 (*wis1Δ sow2-11*) and ED1135 (*wis1Δ*) respectively), leaving one segregant, 10C, with a novel phenotype of rounded and bulgy cells. This segregant must therefore have the genotype *wis1*$^+$ *sow2-11*. The other tetrads that were analysed in this cross showed a consistent pattern of segregation of phenotypes.

The other *sow2* strain that was outcrossed was SP39 (*wis1Δ sow2-7*). Like the SP31 (*wis1Δ sow2-11*) cross above, this cross gave rise to rounded and bulgy progeny (10D in Table 5.15) which are predicted to be *wis1*$^+$ *sow2-7*.

Table 5.15: Tetratype tetrad 10A-D from SP39 x ED933.

segregant	deduced genotype	36°C → 36°C	streaks at 36°C	colour on phloxin B	cell length on MM
10A	*w.t.*	+	++	W	w.t.
10B	*wis1Δ* *sow2-7*	+	++	P	~1.5 x w.t.
10C	*wis1Δ*	-	-	P	~2 x w.t.
10D	*sow2-7*?	+	++	W	~ w.t.[a]

36°C → 36°C: patches were grown on YE at 36°C for three days, then replicated to YE at 36°C.
[a]these cells were rounded and slightly bulgy compared to wild type.

Although both *sow1* and *sow2* lead to aberrant cell morphology, it is interesting that *sow2* does not seem to accelerate cell division and therefore porbably plays no rôle in the timing of mitosis.

Furthermore, these crosses show that, like *sow1-T20*, the other *sow* alleles are not lethal and both *sow1* and *sow2* are unlinked to *wis1*. These outcrosses led to the creation of strains that were used in future work and their genotypes are given in Table 5.16.

Table 5.16: *sow* strains generated during outcrosses

Strain	genotype
SP133	*sow1-22 his1* h^-
SP148	*sow2-7 his1* h^-
SP165	*sow2-11 his1* h^-

5.3.4.1: Generation of *sow* strains for future work.

Judging by phenotype, some of the strains discussed in the previous section, namely SP148, SP165 and SP133 (see Table 5.16), seemed highly likely to contain *sow* mutations. To confirm this, these strains were studied as described below (refer to Figure 5.5).

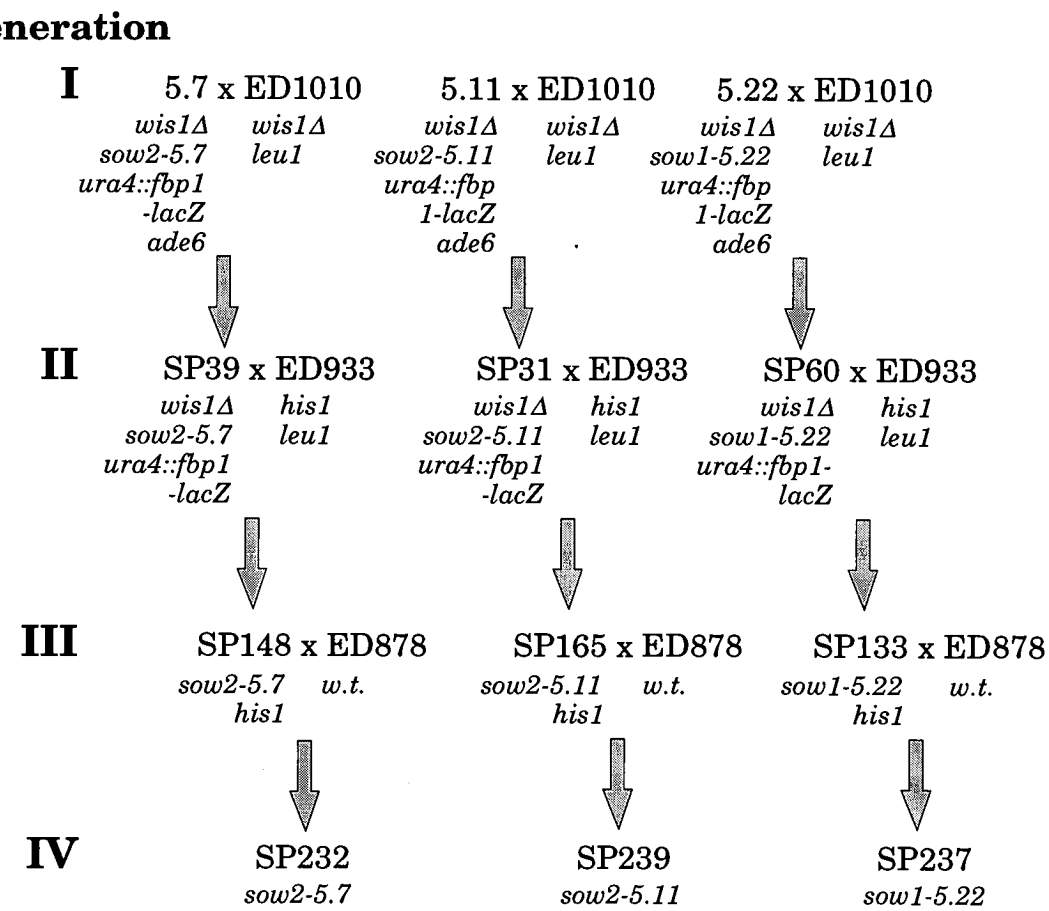

Figure 5.5: derivation of strains used.
wis1$^+$ *sow* strains were obtained from the *wis1Δ sow* mutants, 5.7, 5.11 and 5.123, that were obtained in Mutagenesis V.

Strains SP148 (*sow2-5.7 his1 h*$^-$), SP165 (*sow2-5.11 his1 h*$^-$) and SP133 (*sow1-5.22 his1 h*$^-$) (generation III in Figure 5.4) were all crossed to wild type strain ED878 (h^+) to generate strains SP232, SP239 and SP237 respectively (generation IV in Figure 5.4). To confirm that these putative *sow* strains contained a *sow* mutation they were each backcrossed to a *wis1Δ* strain.

SP232, SP239 and SP237 were crossed to ED975 (*wis1Δ*). Resulting asci were digested and roughly 100 spores from each cross were plated on YE + phloxin B at 32°C. After six hours, the plates were shifted to 36°C. The classes of colonies that grew up in each of the crosses are given in Table 5.17.

Table 5.17: results of backcross plating experiment.

ED975 crossed to:-	genotype	large white colonies	large pink colonies	tiny dark pink colonies
ED812	wild type	+	-	+[b]
T20.16	*sow1-T20*	+	+[a]	+[b]
SP232	putative *sow2-5.7*	+	+[a]	+[b]
SP239	putative *sow2-5.11*	+	+[a]	+[b]
SP237	putative *sow1-5.22*	+	+[a]	+[b]

[a]Cells in these colonies grew well, were approximately 1.5 x wild type length.
[b]Cells in these tiny colonies were extremely long and branched, and had ceased division.

Cells in the large white colonies were all rougly wild type in length and grew well. I therefore concluded that they were *wis1*$^+$. The cells in the tiny dark red colonies very closely resembled *wis1*Δ cells at 36°C. The large pink colonies were made up of cells that were growing well, but slightly elongated and red in colour, indicating that they were *wis1*Δ *sow* cells. The backcross involving the known *sow* strain T20.16 produced progeny of this class, so the presence of this class of progeny in the backcross of SP232 to *wis1*Δ demonstrates that there must be a *sow* mutation in strain SP232.

Similarly, SP239 and SP237 must also be *sow*. Furthermore, as expected, there were roughly twice as many white colonies as there were large pink ones. It was hard to count small dark pink colonies as many of them were too small to see with the naked eye.

It can hence be concluded that strains SP232, SP239 and SP237 all contain *sow* mutations. As they came from a cross between wild type ED878 and strains SP148, SP165 and SP133 respectively, this implies that the latter strains must also contain *sow* mutations. Hence the strains SP148, SP165 and SP133 have been assigned the genotypes shown in Figure 5.4.

5.3.4.2: Measuring cell length at division of *sow* strains.

In Section 5.2.4, it was shown that *sow1-T20* makes cells divide at a reduced length. It appeared that at least *sow1-5.22* did the same (see

Section 5.3.3). To verify this apparent shortening of cell length at division, cell lengths were measured microscopically, as described in Table 5.18.

Table 5.18: lengths at division of *sow* mutants.

Strain	genotype	length ± standard error (μm)	no. cells measured
ED812	h^-	15.1 ± 0.3 14.5 ± 0.2	10 16
T20.16	sow*1-T20* h^-	13.8±0.2	21
SP237	*sow1-22* h^-	13.3±0.2	25
SP232	*sow2-7* h^-	15.5±0.1	24
SP239	*sow2-11* h^+	14.5±0.2	20

Strains were grown in MM at 32°C to mid-log phase and septated cell lengths were measured microscopically.

This experiment shows that *sow1* mutants divide at a shorter length than wild type cells. If this is taken with previous cell length measurements in a *wis1*Δ background (see Section 4.3), it appears that a *sow1* mutation causes a reduction in cell length regardless of Wis1 activity, and the mutation may therefore accelerate mitosis independently of Wis1.

In contrast to this, the presence of a *sow2* mutation only has an effect on cell length in a *wis1*Δ backgound. This implies that *sow2* may require Wis1$^+$ for its function.

5.4: Conclusions for Chapter 5.

Having completed initial genetical investigation, it it was clear that the mutations in the suppressor strains defined two *sow* loci and that a single *sow* mutation was sufficient to suppress several *wis1*Δ phenotypes.

Having said this, the experiments on diploids containing different doses of *sow1-T20* showed only that this mutation was semi-dominant. Furthermore, nine different alleles of this gene were isolated, all with fairly similar phenotypes. As a loss of function mutation is much easier to generate than an activated allele, the most likely solution to the dominance question is that *sow1-T20* is in fact a recessive mutation and that the phenotypes seen in the various diploids that were constructed

are due to dosage effects. This implies that in wild type cells, precise levels of *sow1*$^+$ are probably important for at least one cellular process.

A similar argument can be constructed for the three *sow2* alleles. Although less convincing than for *sow1*, it is perhaps likely that these are also loss-of-function mutations.

Given that *sow1* and, perhaps, *sow2* are most likely loss-of-function mutations, the additive suppression of *wis1*Δ shown by *sow1-T20* and *sow2-11*, suggests that the two *sow* genes lie in different pathways, which can suppress *wis1*Δ independently.

So far, the only difference seen between *sow1* and *sow2* is that *sow1* can reduce cell length independently of *wis1*, whereas, for the alleles obtained so far, *sow2* affects mitotic timing only in a *wis1Δ* background, yet leads to aberrant cell shape on its own. This has implications for the relationship between the *sow* genes and Wis1 pathway, which will be discussed later (see Chapter 7).

To investigate these points, the *sow* mutations were characterised physiologically and genetically and these experiments form the topics considered in the next chapter.

Chapter 6: *sow1* and *sow2*.

6.1: Environmental Stress and the *sow* mutants.

The previous chapter discussed the phenotypes of *sow1* and *sow2*. In a *wis1*Δ background, a *sow1* or a *sow2* mutation reduces cell length and increases stress resistance. In a *wis1*$^+$ background, *sow1* affects cell length and *sow2* alters cellular morphology, a phenotype which conceivably could be connected to cell cycle control. The observation that *sow* mutants affect cell length independently of *wis1* suggest that the stress resistance shown by *sow* mutants (so far only seen in a *wis1*Δ strain) may also be independent of *wis1*. This would predict that a *sow wis1*$^+$ strain could show different stress resistance from a wild type strain. It was hoped that information gleaned from this sort of exploration would hint at *sow*$^+$ gene function.

6.1.1: The *sow* mutants on stress-inducing media.

To determine whether the *sow* mutants showed altered osmotolerance, *sow1-T20* (strain T20.16) and wild type (ED812) strains were streaked to single colonies on YE supplemented with 1.5M, 1.75M, 2.0M and 2.25M KCl both at 32°C and at 36°C. At 32°C, both strains were able to form colonies on up to 1.75M KCl, whereas at 36°C, the strains grew poorly on 1.75M KCl. No difference in the behaviour of the two strains was seen.

In budding yeast, four *uth* mutants were obtained during a screen for stress-resistant mutants (Austriaco, 1997). One of them shows sensitivity to the weedkiller, paraquat. Using the behaviour of budding yeast as a guide, the *sow* mutants were tested for paraquat sensitivity. Four *sow* strains: *sow1-T20*, *sow1-22*, *sow2-7* and *sow2-11* and a wild type control strain were streaked to single colonies on YE plates containing 0.33, 0.033 mg/ml paraquat. The plates were incubated for three days at 32°C.

A paraquat concentration of 0.033 mg/ml had no effect on growth; growth at 0.33 mg/ml is shown in Figure 6.1.

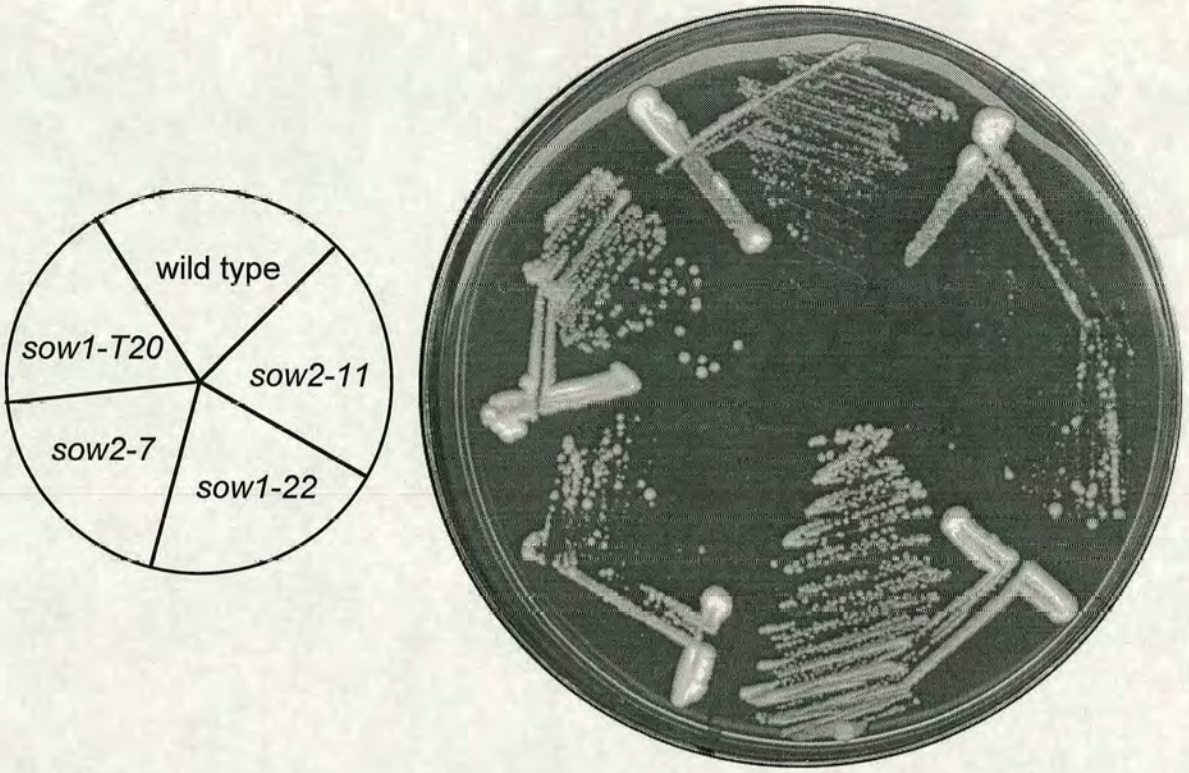

Figure 6.1: Growth of *sow* mutants on medium containing paraquat.
The figure shows growth after three days at 32°C. The medium is YE supplemented with 0.33 mg/ml paraquat.

The *sow1* strains formed colonies well, the wild type poorly and the *sow2* very poorly. From the work on *Saccharomyces* described above, one would expect stress resistant mutants to be more sensitive to paraquat than wild type. This is what was seen in the case of *sow2* mutants. However, *sow1* mutants grew better than the wild type, which is in contrast to what might be predicted.

6.1.2: *sow* mutants grow at 39°C

One possibility was that the *sow* mutants might show an increased heat tolerance relative to wild type, especially as the *wis1*Δ *sow* mutants grew better at high temperature than on high salt, demonstrating better suppression of *wis1*Δ temperature sensitivity than salt sensitivity. The first experiment performed investigated the viability of wild type, *sow1* and *sow2* mutants grown at temperatures above the normal range for *Schizosaccharomyces pombe*. The maximum temperature fission yeast

will grow at is 36°C; the wild type control strain was therefore not expected to remain viable at this temperature.

A wild type strain and both *sow1* and *sow2* mutants were streaked to single colonies at 38.5°C and 39°C. After a day, the plates were examined for formation of colonies, as described in Table 6.1.

Table 6.1: growth of *sow* mutants at high temperature

Strain	genotype of strain	YE 38.5°C	YE 39°C
ED812	*sow*+	-	-
T20.16	*sow1-T20*	+	+
SP133	*sow1-22 his1*	+	+
SP148	*sow2-7 his1*	not done	+
SP165	*sow2-11 his1*	+	+

The table shows the ability of wild type and sow strains to form colonies on YE at 38.5°C and at 39°C after two days. - indicates no colony formation at all; + indicates the formation of small colonies.

Surprisingly, both *sow1* and *sow2* strains were able to form small colonies at 38.5°C and at 39°C, whereas wild type formed none at all (see Figure 6.2).

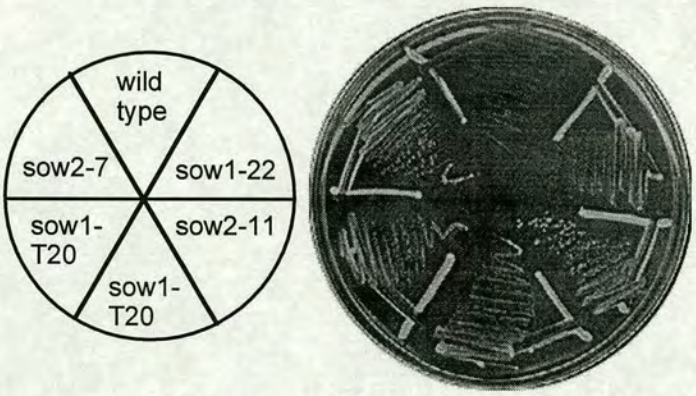

Figure 6.2: *sow1* and *sow2* at 38.5°C.
The figure shows the formation of colonies of wild type, *sow1* and *sow2* strains after two days on YE at 38.5°C.

However, after two days, division of all three strains had ceased. Wild type had divided only a few times and many of the cells had lysed and those that were still intact were very swollen. On the other hand, the *sow*

mutants showed essentially no lysis, although the cells were swollen, and many of them had one, or occasionally more, prominent septa across their centre. These observations show that *sow1* and *sow2* mutants are able to grow at temperatures above the normal range for *S. pombe*.

6.1.2.1: Phloxine B staining

The dye phloxine B is taken up by cells whose membrane has lost its integrity, and is a good indicator of how healthy a colony is: sick and dead cells lose membrane integrity and hence stain red; whereas healthy cells exclude the dye and remain white.

Freshly-growing material from wild type, *sow1* and *sow2* strains was patched to YEPB at 39°C. After two days, the patches were examined for colour, as shown in Table 6.2.

Table 6.2: Colour of *sow* strains on phloxine B at 39°C.

Genotype of strain	colour of patch
wild type	dark pink
sow1-T20	white
sow1-22	pale pink/whitish
sow2-7	pale pink
sow2-11	pink

The table shows the colour of patches of sow^+ and sow^- strains after two days at 39°C on YE containing phloxine B.

To investigate this further, 10ml cultures of MM including phloxine B were inoculated with wild type, *sow1* and *sow2* at 39°C at approximately 2×10^5 cells per ml. The purpose of this experiment was to investigate cellular morphology rather than cell growth. Even so, when samples were taken for microscopic examination after incubation for one day, it was noticed that the cell number of the *sow* strains had increased during the incubation to $2\text{-}6 \times 10^6$ cells per ml whereas the wild type culture had only increased roughly three-fold.

Wild type, *sow1* and *sow2* cells are shown after one day at 39°C in Figure 6.3.

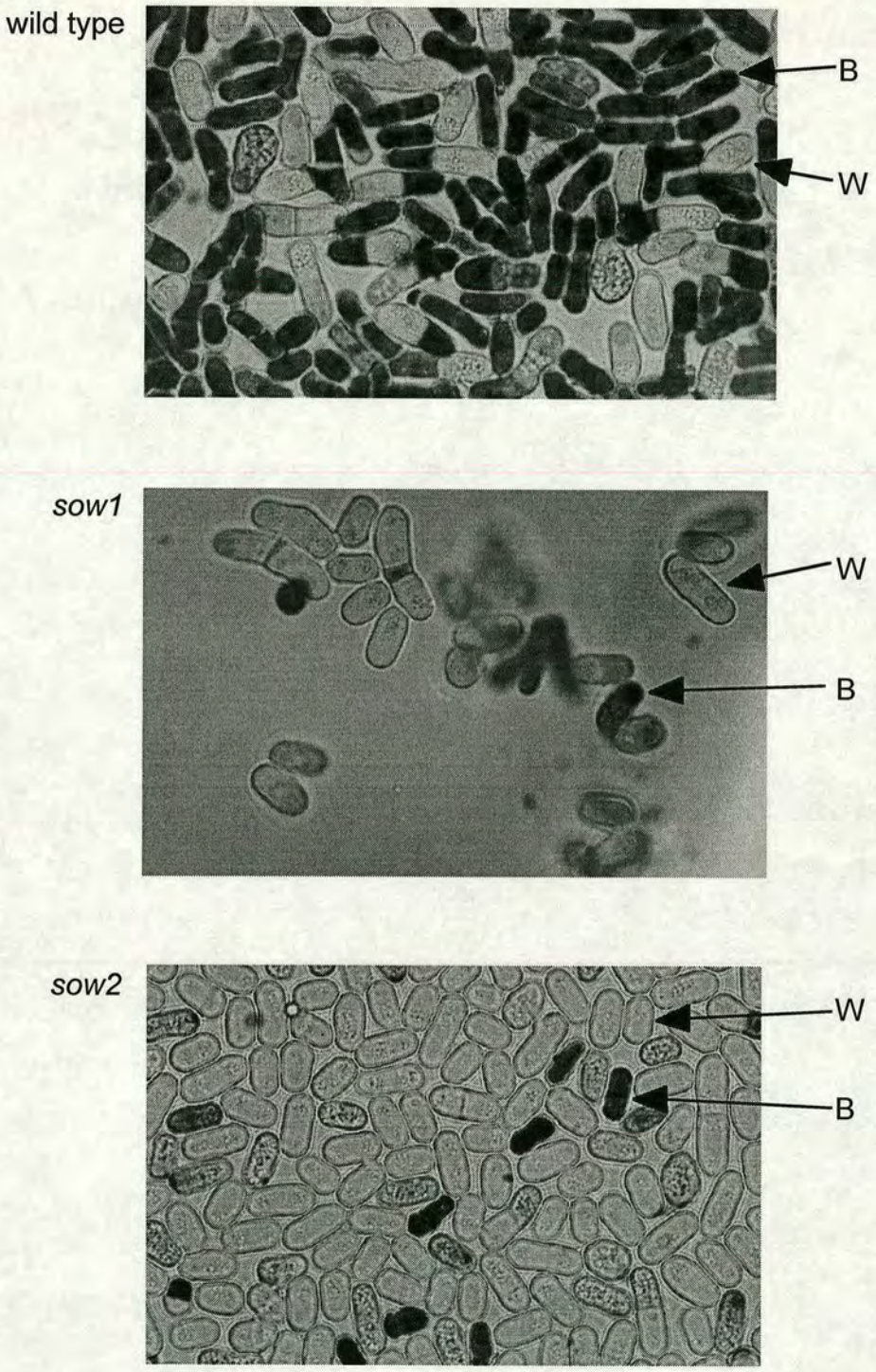

Figure 6.3: Photomicrographs of phloxine B stained cells at 39°C. Photomicrographs of these cells were taken with a H/DIC objective using a green filter, which makes red phloxine B stained cells appear black (B); unstained cells were green and appear white (W).

As Figure 6.3 shows, many of the cells in the wild type culture were dark and therefore must have taken up the phloxine B, implying loss of membrane integrity; both *sow1* and *sow2* mutants mainly remained light, excluding the dye, demonstrating that the cells were still healthy. This constitutes further evidence that *sow* mutants are more heat resistant than wild type cells.

6.1.2.2: Investigation of viability.

The previous experiment demonstrated that *sow* mutants are healthier and formed colonies better than wild type at 39°C. It would therefore be interesting to compare their viability to wild type at this temperature. In order to do this, freshly-growing wild type and *sow* strains were spread onto YE at 39°C. The cells were spread as thinly as possible over a large area of the plate to ensure that they had a sufficient supply of nutrients, thus preventing starvation.

After one, two and three days at 39°C, small samples of cells were taken using the end of a sterile toothpick from the edge of the patches. These samples were streaked to single colonies on YE at 32°C.

Once single colonies had formed, the plates were examined microscopically to determine what proportion of cells were able to form colonies after various times at 39°C, thus allowing the viability at 39°C to be estimated. Data are presented in Table 6.3.

Table 6.3: Proportion of cells forming colonies at 32°C after incubation at 39°C.

	time at 39°C		
Genotype of strain	one day	two days	three days
wild type	≤ 10%	~ 0%	~ 0%
his1	~ 0%	~ 0%	~ 0%
sow1-T20	~ 100%	~ 100%	≥ 90%
sow1-T20 his1	~ 100%	~ 100%	≥ 90%
sow1-22 his1	~ 100%	~ 100%	≥ 90%
sow2-7 his1	~ 100%	~ 100%	≥ 90%
sow2-11 his1	~ 100%	~ 100%	≥ 90%

Cells were streaked to single colonies at 32°C after the number of days indicated at 39°C. The approximate proportion of cells that had formed colonies, as judged by microscopic examination of the plates, is given in the table.

Neither the wild type nor the *his1* strain was able to form colonies after more than a day at 39°C. In contrast, the *his1*$^+$ *sow* as well as the *his1 sow* strains were able to form colonies very efficiently, even after three days at 39°C. This shows that the *sow* mutation (rather than the histidine auxotrophy) in these strains is responsible for their survival.

6.1.3: *sow1-T20* is resistant to heat shock.

As *sow* mutants were able to tolerate steady-state high temperatures, one possible prediction from this would be that their heat shock tolerance would be improved relative to wild type. Cultures of three *sow* strains (*sow1-T20, sow1-22* and *sow2-7*) were grown with wild type and *wis1*Δ controls in MM at 31°C. Cells were collected by centrifugation and resuspended in pre-warmed medium at 47°C. Samples were taken over approximately fifty minutes to assay viability. Cell number was counted at the beginning and the end of the experiment. The number of viable cells in the culture was assessed by plating cells at 32°C, counting colonies that grew up and hence calculating the viability. The results are plotted on the graph in Figure 6.4.

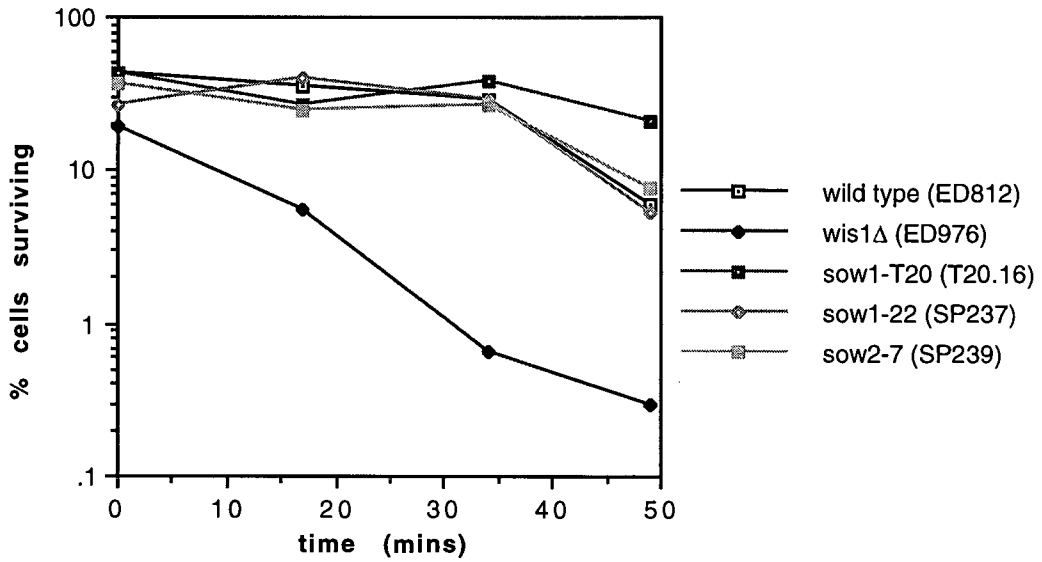

Figure 6.4: Heat shock survival of *sow* strains.
Exponentially-growing cultures were shifted to 47°C at time = 0.

The behaviour of the *sow1-22* and *sow2-7* mutants under heat shock was indistinguishable from that of the wild type control strain: these three strains start to lose viability after 34 minutes at 47°C. In contrast, the *sow1-T20* strain appears to show only a slight loss of viability even after 49 minutes' heat shock. This suggests that *sow1-T20* increases heat shock tolerance, although later time points would be required to confirm this.

6.1.4: *sow1 sow2* double mutants.

In Section 5.3.3 it was shown that a *wis1Δ sow1 sow2* triple mutant shows better suppression of the *wis1Δ* TS phenotype than the *wis1Δ sow1* and *wis1Δ sow2* double mutants. This implies a synergistic interaction between *sow1* and *sow2* in a *wis1Δ* background. Now, having found that the *sow* mutations make cells resistant to high temperature (a phenotype that could be followed easily), I wondered whether a *sow1 sow2* strain would also exhibit synergism in a *wis1*$^+$ background.

A cross was set up to generate a *sow1 sow2* double mutant: T20.16 (*sow1-T20)*x SP233 (*sow2-7)*. Fourteen tetrads were obtained, of which nine were four-spored. The colour exhibited on YEPB at 38°C was used to distinguish the segregants, as fortunately, subtle differences between the various strains were perceivable, as described in Table 6.4.

Table 6.4: Phloxine B staining of strains from *sow1* x *sow2* at 38°C.

Strain	Colour of patch
wild type	dark pink
sow1-T20	light pink
sow2-7	light pink; dark pink edge to patch
sow1-T20 sow2-7 (putative)	light pink; dark pink edge to patch

The *sow1-T20* x *sow2-7* cross produced eight tetrads that contained one segregant which was dark pink (like wild type), one which was light pink (*sow1-T20*) and two segregants that were light pink with a dark edge (*sow2-7*). These tetrads are tetratype, and furthermore, one of the two *sow2-7*-like progeny must be the *sow1 sow2* double mutant. These two segregants were indistinguishable, which suggests that, as far as ultra-

high temperature growth is concerned, the *sow2-7* mutation is epistatic to the *sow1-T20* mutation.

This concludes the investigation into the *sow* mutants and the effects they exert on their own. The next section describes experiments that looked at the effects additional mutations have on *sow* mutants.

6.2: Genetic Investigation of the *sow* mutants.

A series of genetic experiments was undertaken to try to investigate the nature of the *sow* genes genetically. The first aim of these experiments was to cross both *sow1* and *sow2* to known genes and use tetrad analysis to determine whether either of the *sow* genes was allelic to any known gene. Either the presence of non-parental tetrads or the segregation of wild type progeny in a cross is sufficient to demonstrate that two genes in a cross are unlinked and therefore cannot be allelic. Interestingly, in every case tested no linkage was observed, so both *sow1* and *sow2* appear to correspond to novel genes.

The second aim of this investigation was to investigate possible genetic interactions between known genes and the *sow* genes. Because none of the genes tested for allelism was linked to either of the *sow* genes, there will be double mutants for both a *sow* gene and the gene being tested amongst the progeny. By studying the phenotypes of these double mutant strains, it should be possible to see whether the *sow* genes interacted genetically with the genes they had been crossed to. It was hoped that a study of this sort would reveal more about the *sow* genes.

Moreover, if any of the double mutants had a phenotype that could be selected against, it might be possible to exploit this in designing a strategy for cloning *sow1* and/or *sow2*.

The genes that were chosen to cross to *sow1* and *sow2* came from those that are known to interact with *wis1* (see Figure 6.5). The reason for this was that *sow1* and *sow2* were isolated owing to their genetic interaction with *wis1*. It is therefore likely that some of the *wis1*-interacting genes that were already known would also interact with *sow1* or *sow2*.

The hsp90 gene *swo1* (see Figure 6.5) was chosen because of its role in stress survival (reviewed in Buchner, 1996). The *sty1* and *atf1* genes were chosen because they lie downstream in the Wis1 pathway. Wis1 is a mitotic initiator, so *cdc2*, *cdc25*, *wee1* and *cdc13* were chosen as they are central to mitotic control. The type 2A phosphatases, *ppa1*, *ppa2* and *ppe1* also have an effect on cell cycle, and furthermore, as the Wis1 pathway is a phosphorylation cascade, phosphatases are needed for down-regulation. *wis1* is also known to interact with the cAMP pathway, so *pka1* and *cyr1* from this pathway were chosen. In addition, both the cAMP pathway and *wis1* interact with *pat1*.

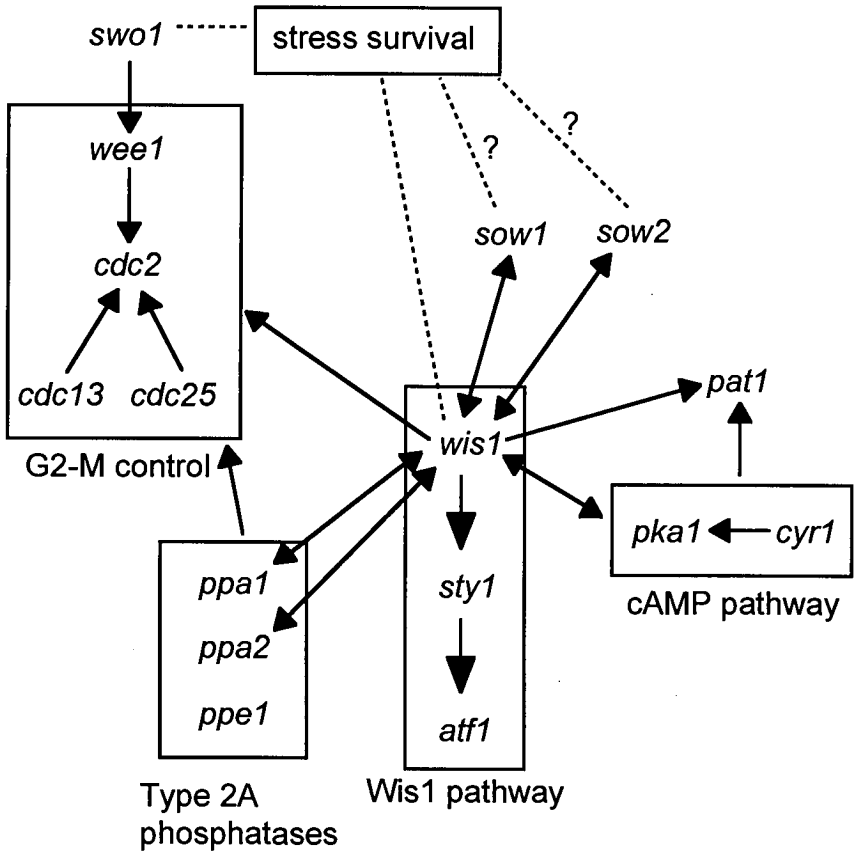

Figure 6.5: The network of interactions with *wis1*.
A double headed arrow indicates a genetic interaction between two genes; a single headed arrow denotes the action of one gene product on another and a dotted line indicates a link between genes and processes.

In addition to the genes described above, a particular allele of *cdc27* (*cdc27-P11*) was chosen to be crossed to the *sow* genes. In Chapter 3, the observation that *wis1*Δ is osmoremediable was described. It is possible

that the sow mutations suppress *wis1Δ* phenotypes by altering cellular osmolarity. The *cdc27-P11* mutation is also osmoremediable and it was chosen for this reason, rather than for the indirect role it plays in cell cycle progression.

6.2.1: *swo1*

As discussed in Section 6.1.2, *sow* mutants are unusually resistant to high temperature. One of the ways heat stress causes damage to cells is by increasing protein denaturation. This is resisted in part by chaperones such as the heat shock protein 90 (hsp90), which helps to refold proteins (reviewed in Johnson and Craig, 1997). It is conceivable that the *sow* mutations suppress *wis1Δ* phenotypes by interfering with this process. The *S. pombe* hsp90 gene, *swo1* (Aligue et al., 1994) would therefore be an interesting gene to test for linkage to and for a genetic interaction with *sow1* and *sow2*.

Strains containing the *swo1-26* allele are temperature sensitive and at 35°C arrest without dividing, with swelling and extensive lysis. This cellular breakdown worsens as the temperature increases (Aligue et al., 1994).

To investigate possible allelism and genetic interactions, *swo1-26* was crossed to *sow1-22*, *sow2-7* and *sow2-11*. Each cross produced roughly ten tetrads with enough viable progeny to interpret. First, the resulting tetrads were analysed for linkage between the *swo1* and *sow* genes. Segregants were present in both the *sow1* and *sow2* crosses that grew at 35°C (which shows that they are *swo1*$^+$), but which died swiftly and became dark red on phloxine B at 39°C, (ie *sow*$^+$). These segregants must therefore be wild type. The presence of wild type recombinants demonstrates that *swo1* is not allelic to either *sow1* or *sow2*.

Next, the segregants in the crosses were analysed for genetic interactions between *swo1* and the *sow* genes. In all four-spored tetrads in the *swo1* x *sow1* crosses, there were two segregants that would grow at 35°C (ie *swo1*$^+$) and two that would not (ie *swo1*$^-$). Judging by the presence of a single wild type segregant, some were TT. Those that had no wild type segregants were classified as NPD.

In NPD tetrads there are two double *swo1 sow1* mutants. At 35°C and at 32°C, no difference was seen between the behaviour of the *swo1 sow1* mutants from these tetrads and control *swo1* strains. Thus at these temperatures, *swo1* is epistatic to *sow1* and there is no other genetic interaction between *swo1* and *sow1*. This rules out the conjecture that *sow1* might suppress *swo1*.

No interaction was seen in the *swo1* x *sow2* crosses either. There does not therefore seem to be any interaction between the *swo1* and *sow* genes.

6.2.2: Genes downstream of Wis1.

Next *sow1* and *sow2* were crossed to two genes encoding proteins that lie downstream of Wis1 in the pathway: *sty1* and *atf1*.

6.2.2.1: *sty1*

The *sty1* gene encodes the MAPK which is phosphorylated by Wis1 (Kato et al., 1996; Millar et al., 1995; Shiozaki and Russell, 1995a). Sty1 then phosphorylates the transcription factor, Atf1 (Wilkinson et al., 1996).

As discussed in Chapter 4, an activated allele of *sty1* might be able to suppress *wis1*Δ phenotypes. As the *sow* mutations only suppress some of these, namely temperature sensitivity, salt sensitivity and cell cycle delay, it would seem that for either of the *sow* genes to be allelic to *sty1*, the *sow* mutants isolated would have to be partial gain of function mutations, such that they were activated for some, but not all Sty1 functions.

To investigate this, first *sow1* was crossed to *sty1*. All resulting segregants were replicated to MM and streaked to single colonies on YE at 36°C, with a *wis1*Δ *sow1-T20* strain, a *sty1* strain, a *sow1-T20* strain and a wild type as controls.

Four different phenotypic classes were observed. One class gave highly elongated progeny on MM. As the *sty1* mutation gives rise to progeny with this phenotype (Shiozaki and Russell, 1995a), they were deduced to be *sty1* mutants. Segregants that were indistinguishable from wild type were also observed, as well as some that seemed slightly shorter than wild type, presumably corresponding to *sow1-T20* mutants. An

interesting fourth phenotype was also seen: slightly elongated cells, very similar to the suppressor strains streaked as controls. These were inferred to be *sty1 sow1-T20* double mutants.

Like *wis1Δ sow1-T20*, the *sty1 sow1-T20* double mutants formed colonies at 36°C, whereas *sty1* strains are unable to. Thus, *sow1-T20* suppresses both the cell length and the high temperature defects of *sty1*, and is clearly not the same gene.

To investigate whether *sow2* was linked to *sty1*, a *wis1Δ ura4*$^-$ strain with a copy of the *ura4*$^+$ gene integrated at the *sty1*$^+$ locus was crossed to the strains *wis1Δ ura4*$^-$ *sow2-7* and *wis1Δ ura4*$^-$ *sow2-11*. As the only functional *ura4*$^+$ gene in the cross is integrated at the *sty1* locus, *ura*$^+$ segregants from this cross can be assumed to be *sty1*$^+$. All the *ura*$^+$ segregants from the tetrads were therefore streaked to 36°C and replicated to MM.

On MM, half the segregants were highly elongated (like unsuppressed *wis1Δ*) and half were only slightly elongated (like suppressed *wis1Δ*); at 36°C half died (like unsuppressed *wis1Δ*) and half grew (like suppressed *wis1Δ*). This shows that the *sow2* gene segregates independently of the *sty1* locus, which implies that the two must be different genes. As the *ura4*$^+$ marked *sty1* strain contained the *sty1*$^+$ gene, it was not possible to investigate interactions between *sty1* and *sow2*.

6.2.2.2: *atf1*

The *atf1* gene (also known as *gad7*) encodes an ATF/CREB transcription factor (Kanoh et al., 1996; Takeda et al., 1995) which is regulated by phosphorylation by Sty1 (Wilkinson et al., 1996). In this manner, Atf1 regulates the transcription of several genes in response to activation of the Wis1 pathway. In contrast to *wis1Δ*, *atf1Δ* cells show no cell cycle defect, yet both *wis1Δ* and *atf1Δ* strains are more sensitive than wild type cells to environmental stresses such as stationary phase (Takeda et al., 1995) and high salt (Kanoh et al., 1996; Wilkinson et al., 1996) and low temperature (Kanoh et al., 1996; Takeda et al., 1995).

Whether *atf1Δ* is sensitive to high temperature or not had not been reported. So, I streaked freshly-growing wild type, *wis1Δ* and *atf1Δ*

strains to single colonies on YE at 36°C. The wild type formed colonies efficiently; the *wis1*Δ not at all and the *atf1*Δ strain very poorly. On microscopic inspection, these *atf1*Δ colonies contained heterogeneous cells that were round and swollen. There was also some lysis. Although *atf1Δ* strains grow better than *wis1*Δ at high temperature, the fact that *atf1*Δ is partially heat sensitive shows that a significant component of *wis1*Δ temperature sensitivity acts through Atf1.

To investigate whether *atf1* was linked to *sow1*, an *atf1::ura4*$^+$ strain (ED1278) was crossed to *wis1::his1*$^+$ *his1 ura4 sow1-T20* (strain T20). In this cross, the only *ura*$^+$segregants must be *atf1*Δ. Three four-spored asci (and eight with three or two spores) were obtained. All segregants were streaked to YE at 36°C, and examined microscopically after four days.

As a typical example of the segregation of phenotypes seen in this cross, the data from one four-spored ascus, 2A-D, are presented in Table 6.5 below:

Table 6.5: phenotypes of segregants of cross of *atf1* to *wis1*Δ *sow1-T20*.

segregant	*atf1*	*wis1*	36°C	cell length	*sow1*
2A	+	+	+	w.t.	?
2B	+	+	+	w.t.	?
2C	Δ	Δ	-	highly elongated	+
2D	Δ	Δ	+	slightly elongated	*sow1-T20*

The genotypes of the segregants in this cross were deduced from their phenotypes. ura+ segregants are *atf1*Δ and *ura*$^-$ are *atf1*$^+$. Pink segregants on phloxine B are *wis1*Δ; white are *wis1*$^+$. The unsuppressed *wis1*Δ segregant dies at 36°C and is long on MM, whereas, the *wis1*Δ strain that grows at 36°C and is shorter on MM must contain a suppressing *sow1* mutation. See text for details.

The three genes segregating in this cross (*atf1*, *wis1* and *sow1*) were followed by the following characteristic phenotypes. Growth on medium lacking uracil was used to determine the *atf1* allele: *atf1* strains are *ura4*$^+$ and will grow on -ura medium, whereas *atf1*$^+$ strains will be *ura*$^-$. The colour of the segregants on medium containing phloxine B was used to determine the *wis1* allele they contained. *wis1*Δ cells are pink *wis1*$^+$ cells are white. From these two tests, segregants 2C and 2D could be shown to be *atf1*Δ *wis1*Δ double mutants (see Table 6.5).

In *wis1*Δ strains, the presence of the *sow1* mutation was determined by its suppression of the *wis1*Δ mutation. *wis1*Δ strains cannot grow at 36°C and the cells are highly elongated on MM. However, segregant 2D (which is *wis1*Δ) could grow at 36°C and its cells were only slightly elongated on MM showing that this segregant also contained the *sow1* suppressor (see Table 6.5). On the other hand, segregant 2C (which is also *wis1*Δ) was unable to grow at 36°C and produced highly elongated cells on MM. This strain therefore does not contain a suppressor of *wis1*Δ.

The presence of both *atf1* and *sow1* in the same strain (segregant 2D) shows that *sow1* and *atf1* do not correspond to the same gene. Furthermore it is interesting that *sow1-T20* does not require Atf1 activity to suppress *wis1*Δ temperature sensitivity. This suggests that Sow1 acts in a different pathway from Atf1, and conceivably Wis1. Given that Atf1 does not play a role in cell cycle control it is not surprising that the *sow1-T20* does not require Atf1 to suppress the *wis1*Δ cell length defect.

6.2.3: Phosphatase genes.

As the Wis1 pathway activates its downstream targets via a cascade of phosphorylation, it is clear that phosphatases must act on the pathway to provide down-regulation. Moreover, activated MAPKs such as Sty1 are known to activate other kinases, it is likely that counteracting phosphatases also act downstream of Sty1.

It is conceivable that the loss of one of these phosphatases could suppress *wis1*Δ, and thus *sow1* or *sow2* could correspond to a mutation in a downstream phosphatase.

Given that there is no phosphorylation of Sty1 in a *wis1*Δ strain, the *sow* mutations cannot correspond to a Sty1-specific phosphatase, such as Pyp1 or Pyp2, so these genes were not crossed to *sow1* or *sow2*. The Type 2A phosphatase genes, *ppa1*, *ppa2* and *ppe1*, were chosen to be crossed to *sow1* and *sow2*. To facilitate identification of the *sow* segregants, a *wis1*Δ background was chosen for these crosses.

6.2.3.1: *ppa1* and *ppa2*

First, *ppa1 wis1* and *ppa2 wis1* strains were constructed, from the crosses *ppa1::ura4*$^+$ x *wis1::LEU2* and *ppa2::ura4*$^+$ x *wis1::LEU2*. The choice of

leu and *ura* markers ensured that both the *wis1* and the *ppa* genes could be followed unambiguously, as *wis1*Δ strains will be *leu*$^+$ and *ppa* strains will be *ura*$^+$. (The *S. cerevisiae LEU2* gene rescues the *S. pombe leu1* mutation.)

The *ppa1 wis1* and *ppa2 wis1* double mutants obtained from these crosses were shorter than *wis1*Δ cells, showing that the *ppa* mutants suppressed the *wis1*Δ cell length defect, as shown previously (Warbrick and Fantes, 1991). This is similar to *sow1 wis1* and *sow2 wis1* strains. I was therefore interested in whether the *ppa* mutations could suppress other *wis1*Δ phenotypes, particularly stress sensitivity, as this would make these phosphatase genes very likely to be allelic to the *sow* genes.

To examine this, the *ppa1/2 wis1*Δ mutants were streaked to single colonies on YEK and YE at 36°C. Under both conditions, the *ppa1/2 wis1*Δ mutants failed to form colonies, as shown in Table 6.6.

Table 6.6: *ppa1/2 wis1*Δ mutants under stress conditions.

Genotype of Strain	YEK 32°C	YE 36°C
wild type	+	+
*wis1*Δ	-	-
*ppa1*Δ	+	+
*ppa1*Δ *wis1*Δ	-	-
*ppa2*Δ	+	+
*ppa2*Δ *wis1*Δ	-	-

The strains were scored for growth on YEK at 32°C and YE at 36°C. + indicates the formation of colonies; - indicates no colonies were formed.

The *ppa1* and *ppa2* mutants grow on YEK and YE at 36°C, whereas the *ppa1/2 wis1* mutants do not, showing that the presence of either *ppa* mutation does not allow *wis1* strains to tolerate stressful conditions. Therefore *ppa1* and *ppa2* can only suppress the *wis1*Δ cell cycle defect.

Next, both *ppa1 wis1* and *ppa2 wis1* strains were crossed to a *sow1 wis1* strain. The two genes segregating in these crosses are *sow1* and a *ppa* gene. The *sow1* gene allows *wis1*Δ strains to form colonies at high temperature. As the crosses were performed in a *wis1*Δ background, and the *ppa* genes are not able to suppress *wis1*Δ temperature sensitivity, the only strains that will be able to form colonies must contain a *sow1*

mutation. The *ura*$^+$ marker was used to follow the *ppa1*Δ and *ppa2*Δ genes as *ppa*$^+$ strains will be *ura*$^-$.

Using these two methods to score for *sow1* and *ppa* genes, it was seen that both crosses produced wild type segregants as well as segregants containing both *sow1* and a *ppa* gene. The presence of non-parental segregants shows that *sow1* is not linked to *ppa1* or *ppa2*.

Furthermore, the *ppa sow1 wis1* triple mutants grew at 36°C and were shorter than *wis1*Δ strains. As both *ppa* and *sow* mutations suppress the *wis1*Δ cell length defect to the same degree, it was only possible to conclude that the presence of both *sow1* and a *ppa* mutation does not suppress additively. Furthermore, the observation that the triple mutants were temperature resistant may suggest that for this phenotype *sow1* is epistatic to the *ppa* mutations. It is possible that this is also the case for cell length suppression, but given that *ppa wis1* and *sow1 wis1* divide at similar lengths, this phenotype cannot be used to test this hypothesis.

Next, *sow2 wis1* was crossed to *ppa1 wis1* and *ppa2 wis1*. Non-parental asci segregated, showing that *sow2* is not allelic to *ppa1* or *ppa2*. Also, like *sow1*, *sow2* was epistatic to *ppa1* and *ppa2* in a *wis1*Δ background, at least as far as temperature resistance is concerned.

6.2.3.2: *ppe1*

ppe1 encodes a somewhat diverged Type 2A phosphatase. Its sequence has less similarity to Type 2A phosphatases than Ppa1 and Ppa2. As loss of *ppe1*$^+$ causes sterility, a strain carrying a *LEU2* plasmid encoding the *ppe1*$^+$ gene was used in crosses.

First, a *ppe1*Δ *wis1*Δ double mutant was constructed.

A *wis1*::*his1*$^+$ *his1 ura leu* strain (ED1008) was crossed to a *ppe1*::*ura4*$^+$ *ura leu* [p(*ppe1*$^+$ *LEU2*)] strain (ED1060) by tetrads. Fourteen tetrads were obtained. Half of the segregants in this cross were *leu ura* and the other half were *leu*$^+$ *ura*$^+$. Furthermore, it was impossible to obtain *leu*$^-$ derivatives from the *leu*$^+$ strains, showing that the *ppe1*$^+$ plasmid was now stably inherited. The simplest explanation for these observations was that the *LEU2 ppe1*$^+$ plasmid had integrated homologously at the *ppe1*::*ura4*$^+$ locus in the *ppe1*Δ strain.

As the *ppe1*Δ *wis1*Δ strains had integrated the *ppe1*$^+$ plasmid, it was not possible to examine the phenotype of the *ppe1*Δ *wis1*Δ double deletion. Despite this, a cross between *ppe1* and *sow1* or *sow2* would still reveal linkage if it existed. Therefore a *ppe1*Δ *wis1*Δ strain was crossed to both *sow1 wis1*Δ (SP55) and *sow2 wis1*Δ (SP32).

In these crosses, *ppe1*Δ was followed by its accompanying *ura*$^+$ marker. The crosses were performed in a *wis1*Δ background, so *sow1* and *sow2* could be followed by their ability to allow *wis1*Δ to form colonies at 36°C. Amongst the progeny, four different phenotypic classes were seen, corresponding to the following genotypes: *wis1*Δ, *sow wis1*, *ppe1 wis1* and *sow ppe1 wis1* The presence of non-parental segregants in the crosses involving both *sow1* and *sow2* demonstrates that the genes in each cross are unlinked and therefore that neither *sow1* nor *sow2* corresponds to *ppe1*.

6.2.4: Genes in the cyclic-AMP protein kinase (cA-PK) pathway.

Two genes from the cA-PK pathway were tested for genetic interactions with the *sow* genes: *pka1* (also known as *git6*) and *cyr1* (also known as *git2*).

6.2.4.1: *cyr1*.

A *cyr1* mutant was crossed to *sow1-T20*. The crosses were dissected and the segregants were replicated to YEK and YEPB at 39°C. These conditions allowed the segregation of the two genes in this cross to be followed as *cyr1* strains are unable to grow on YEK and *sow* segregants are white and swollen with little lysis at 39°C on YEPB.

The segregants from this cross fell into four phenotypic classes. The corresponding genotypes could be unambiguously assigned. These classes were: salt resistant and red on YEPB at 39°C (wild type); salt sensitive and red on YEPB at 39°C (*cyr1*); salt resistant and white on YEPB at 39°C (*sow1*) and salt sensitive and white on YEPB at 39°C (*cyr1 sow1*). The presence of non-parental progeny demonstrates that the two genes are unlinked. Furthermore, the double mutant had characteristics of both the single mutants, namely it was salt sensitive and heat resistant. This demonstrates that *sow1* and *cyr1* do not interact.

When *cyr1* was crossed to *sow2-7*, the segregation of phenotypes was identical to that seen in the cross to *sow1*. Thus *sow2* is also not linked to *cyr1* and does not interact with it.

6.2.4.2: *pka1.*

*pka1*Δ spores germinate poorly, and when they do, they take approximately three times longer to grow into a colony than *pka1*+ siblings. Despite this, enough progeny were obtained from both *sow1* x *pka1* and *sow2* x *pka1* crosses to analyse.

pka1 cells are round and *sow* cells are white on YEPB at 39°C. Amongst the progeny from the two crosses were cells that were of wild type length and red on YEPB. These were deduced to be wild type. The presence of these segregants is sufficient to ascertain that both *sow1* and *sow2* are unlinked to *pka1*.

The predicted double mutants in tetratype asci from both crosses resembled *pka1* mutants in that the cells were very short and rounded. This suggests that *pka1* is epistatic to both *sow1* and *sow2* .

6.2.5: Cell cycle genes.

Given that the *sow* mutants are capable of advancing mitosis, they might correspond to cell cycle mutations that make the cells divide at a shorter length. This can be brought about by loss-of-function mutations in mitotic inhibitors, such as the *wee* mutations, *wee1* and *cdc2-1w*, or by gain-of-function mutations in genes which are required to enter mitosis, such as *cdc2*, *cdc13* and *cdc25*.

6.2.5.1: *wee1-50*

wee1 reverses the cell cycle defect of *wis1*Δ, making the cells phenotypically "wee" (Warbrick and Fantes, 1991), but does not suppress the salt and temperature sensitivity (Stettler, S., pers. comm.). *sow1* and *sow2* were therefore not expected to be allelic to *wee1*, but as all three genes advance mitosis, there might be an interesting interaction between *wee1* and the *sow* genes.

sow1-T20 was crossed to *wee1-50* and tetrads were dissected. The two genes segregating in this cross (*sow1* and *wee1-50*) were followed by the

following phenotypes: *sow1* is white on YEPB at 39°C, whereas *sow1*$^+$ is pink. *wee1-50* strains show a temperature sensitive reduction in the cell length at division, dividing at almost wild type length at 25°C, and being "wee" at 32°C (Nurse and Thuriaux, 1980). Furthermore, *wee1-50* strains are nearly white on YEPB at 25°C, and become progressively darker pink on YEPB as the temperature is increased.

In the five four-spored tetrads that were obtained, the progeny fell into four phenotypic classes. Using the phenotypes described above, three classes could be assigned to the following genotypes: wild type, *wee1*, *sow1*. The fourth phenotypic class was novel and was assumed to represent *sow1 wee1* double mutants. It is described below.

On YEPB at 28°C, 32°C, 35°C and 38.5°C, a difference was seen between the appearance of *wee1* and *wee1 sow1* segregants, as described in Table 6.7.

Table 6.7: *wee1-50* vs. *wee1-50 sow1*.

genotype	*wee1-50*		*wee1-50 sow1-T20*	
temp.	colour†	cell length	colour†	cell length
28°C	light P	semi-wee	W	slightly short
32°C	P	wee	light P	semi-wee
35°C	dark P	wee (heterogeneous)	light P	semi-wee/wee
38.5°C	dark P	wee (heterogeneous)	dark P	wee (heterogeneous)

†colour on YE supplemented with phloxine B (YEPB): P = pink; W = white.

A *wee1-50* strain at 32°C is phenotypically "wee" (see Table 6.7), *wee1-50* column, 32°C). At the same temperature, a *wee1 sow1* strain is semi-wee (see *wee1 sow1* at 32°C), which suggests it is closer to wild type activity.

Now, a *wee1-50* strain is semi-wee at the cooler temperature of 28°C. This implies that *sow1-T20* has the same effect on *wee1-50* as lowering the temperature. This effect was seen throughout the range of temperatures used.

To investigate this behaviour further, the following 10ml liquid cultures were set up: *wee1-50* at 25°C, *wee1 sow1* at 32°C and *wee1-50* at 32°C. The strains were inoculated thinly and grown overnight to approximately

$5x10^6$ cells per ml. Samples of the cells in these cultures were examined microscopically (see Figure 6.6).

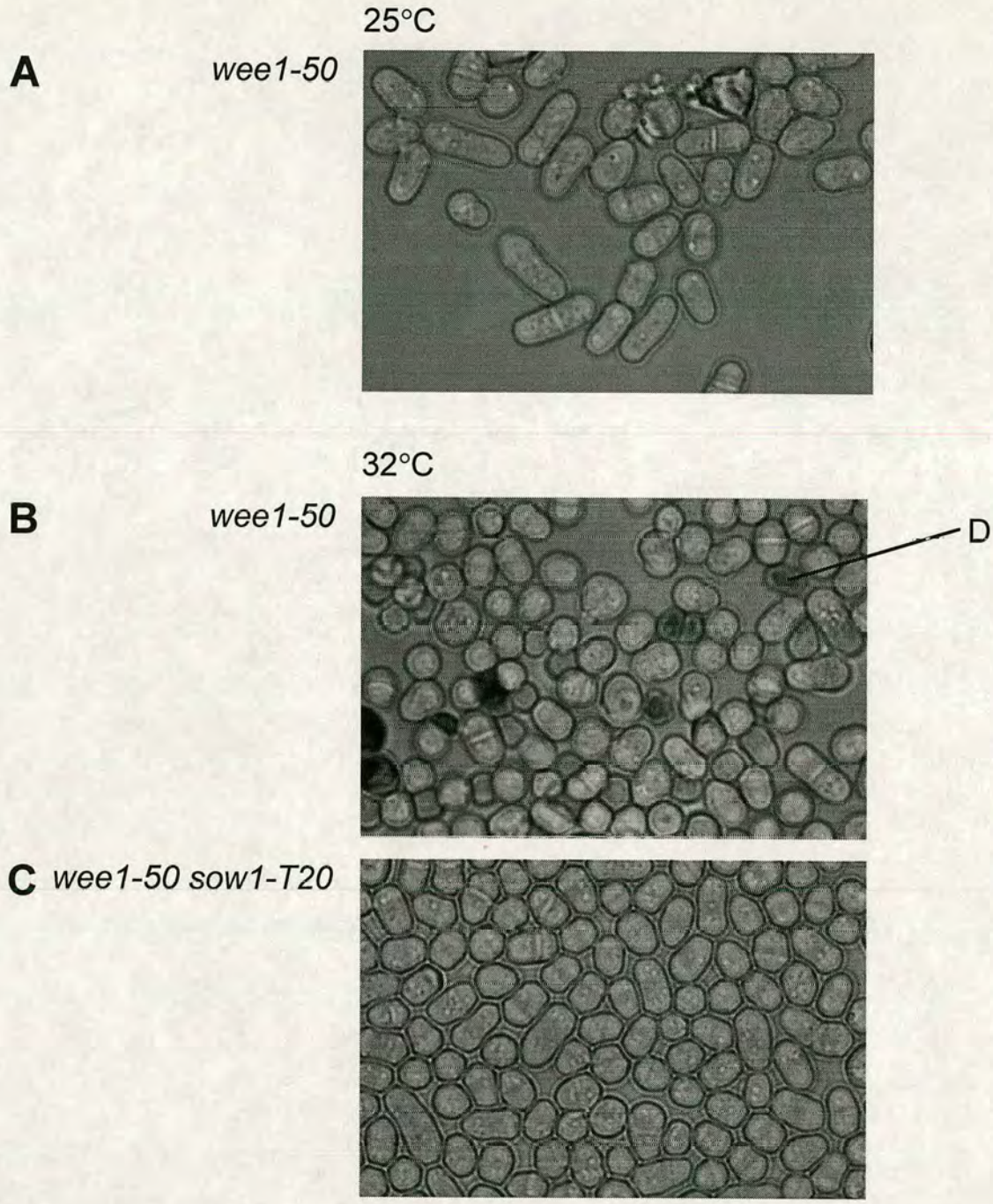

Figure 6.6: suppression of *wee1* by *sow1* .
Cells were grown in YEPB and examined microscopically using bright field with a green filter. Cells that were stained with phloxine B therefore appear dark or black. *wee1-50* cells at 25°C divide at roughly wild type length and exclude phloxine B so no dark cells are seen (A); at 32°C *wee1-50* cells divide at a much reduced cell length and appear round ("wee"), some cells also stain with phloxine B and appear dark (D) under these conditions (B). In contrast, a *wee1 sow1* double mutant at 32°C divides at a longer cell length than the *wee1* single mutant and no stained cells are seen (C).

At 25°C (the permissive temperature for *wee1-50*) the length *wee1-50* cells divide at is only slightly shorter than wild type (A, Figure 6.6) and no darkly-stained cells are seen, which indicates that the cells have an intact membrane and therefore exclude phloxine B. At 32°C, many round and short cells were seen (the "wee" phenotype). Moreover a proportion of the cells appeared dark, from phloxine B, showing that the strain is fairly sick at this temperature (B, Figure 6.6).

In contrast, few cells of the *sow1-T20 wee1-50* double mutant were round, and most were longer than *wee1-50* cells at this temperature. (C, Figure 6.6) Furthermore, no darkly-staining cells were seen. This shows that the introduction of a *sow1-T20* mutation into this strain partially suppresses a *wee1-50* mutation.

Next, *sow1-22, sow2-7* and *sow2-11* were each crossed to *wee1*. In all three crosses, very similar results to the *sow1-T20* cross were obtained. Thus, the presence of either *sow1* or *sow2* makes *wee1-50* strains behave as if they are at a lower temperature.

6.2.5.2: *cdc2-1w* and *cdc2-33.*

cdc2-1w was crossed to *sow1-22* and *sow2-11*. In these crosses, the *cdc2-1w* gene was followed owing to its making cells pink on YEPB at 28°C, and "wee" at 32°C. The *sow* mutations could be identified because they make cells white on YEPB at 39°C.

Owing to poor spore viability in both crosses, it was hard to classify some of the tetrads. However, in both crosses, close to a quarter of all the segregants were white on YEPB at 28°C (= $cdc2^+$) and pink on the same medium at 39°C (= sow^+), and are therefore wild type. This clearly shows that neither *sow* gene is *cdc2* nor linked to it.

One four-spored ascus from *sow1-22* x *cdc2-1w* produced progeny with four different phenotypes. While one of the segregants was "wee" at 32°C, and therefore a *cdc2-1w* mutant, another, was semi-wee. Deduction from the other segregants predicts that this should be *sow1 cdc2-1w*. Thus, the *sow1* mutation seems to be having a somewhat restorative or suppressive effect on the *cdc2-1w* mutation. In contrast, the *sow2 cdc2-1w*: double

mutants looked like *cdc2-1w* mutants, showing that *cdc2-1w* is epistatic to *sow2*.

sow1 and *sow2* were next crossed to *cdc2-33*, in order to investigate possible genetic interactions, as it had already been demonstrated that the *sow* genes were not linked to the *cdc2* locus. The *cdc2 sow* double mutants were indistinguishable from *cdc2*, showing that neither *sow1* nor *sow2* can affect the phenotype of a *cdc2-33* mutant.

6.2.5.3: *cdc13*

cdc13 was crossed to *sow1-22*, *sow1-T20* and *sow2-11*. As in previous crosses, the *sow* mutants were identified by the presence of white patches on YEPB at 39°C; *cdc13* could be followed because it makes cells pink on YEPB at 32°C.

For all three crosses, close to a quarter of the segregants were white at 32°C and pink at 39°C, showing that they were wild type, and hence that neither *sow* gene is, or is linked to, *cdc13*.

A subtle interaction was seen between the *sow* genes and *cdc13*. Cells in the *cdc13* control strain were uniformly long (*cdc*$^-$) at 32°C, whereas putative *cdc13 sow* segregants (as deduced from the other progeny in a tetrad) were long, but rather heterogeneous, and thus on average, they were shorter than the *cdc13* strain.

6.2.5.4: *cdc25*.

cdc25-22 was crossed to *sow1-T20*. From three tetratype tetrads obtained, it was clear that these two genes are not allelic, and at 35°C, the double mutant was indistinguishable from *cdc25-22*.

6.2.6: *cdc27-P11*

The *cdc27-P11* mutation was chosen as it represented a temperature sensitive allele that was known to be osmoremediable (Hughes et al., 1992), rather than for its role in controlling cell cycle.

cdc27-P11 was first crossed to *sow1-T20*. The resulting tetrads were dissected, and the segregants were replica plated to YE at 36°C. Two segregants in each tetrad grew and two died as highly elongated cells.

This showed that there was clear 2:2 segregation of *cdc*$^+$ and *cdc*$^-$. Thus the presence of the *cdc27-P11* allele could be clearly followed. The *sow1* mutation was followed by its white colour on YEPB at 39°C.

Using these two tests, three phenotypic classes could be discerned amongst the progeny: those that were white on YEPB (ie *sow1*); those that were red on YEPB, but grew at 36°C (ie wild type) and those that were *cdc* at 36°C (ie *cdc27*). From the presence of wild type segregants, it was clear that *cdc27* and *sow1* were not linked and therefore correspond to different genes.

The phenotypically *cdc* class contained twice as many progeny as the other two classes and therefore contains both *cdc27* and *cdc27 sow1* progeny. No difference between the two had been seen on YE at 36°C. To see whether there might be a difference at lower temperatures, the cdc progeny (ie *cdc27* and *cdc27 sow1*) were streaked to single colonies at 33.5°C, 34°C and 35°C and on YE containing sorbitol (YES) at 36°C (see Table 6.8).

Table 6.8: comparison of *cdc27-P11* and *cdc27-P11 sow1* segregants.

Strains[c]	33.5°C	34°C, 35°C, 36°C	36°C + sorbitol
cdc27-P11 control strain	*cdc* (small cols.)	*cdc*	weakly suppressed *cdc*
cdc27 segregant	*cdc* (small cols.)	*cdc*	weakly suppressed *cdc*
cdc27 sow1 segregant	suppressed *cdc*; med. cols.	*cdc*	weakly suppressed *cdc*

[a] *sow1-T20* and *sow1-22* crosses were indistinguishable
[c] see text

Two clearly distinguishable types of behaviour emerged at 33.5°C. One class of *cdc* segregants formed small colonies of very long (*cdc*) cells and were indistinguishable from a *cdc27-P11* control. This class was deduced to be *cdc27* (see Figure 6.7). The other class formed larger colonies of shorter cells. In this class the *cdc* phenotype was partially suppressed and their genotype was deduced to be *cdc27 sow1*.

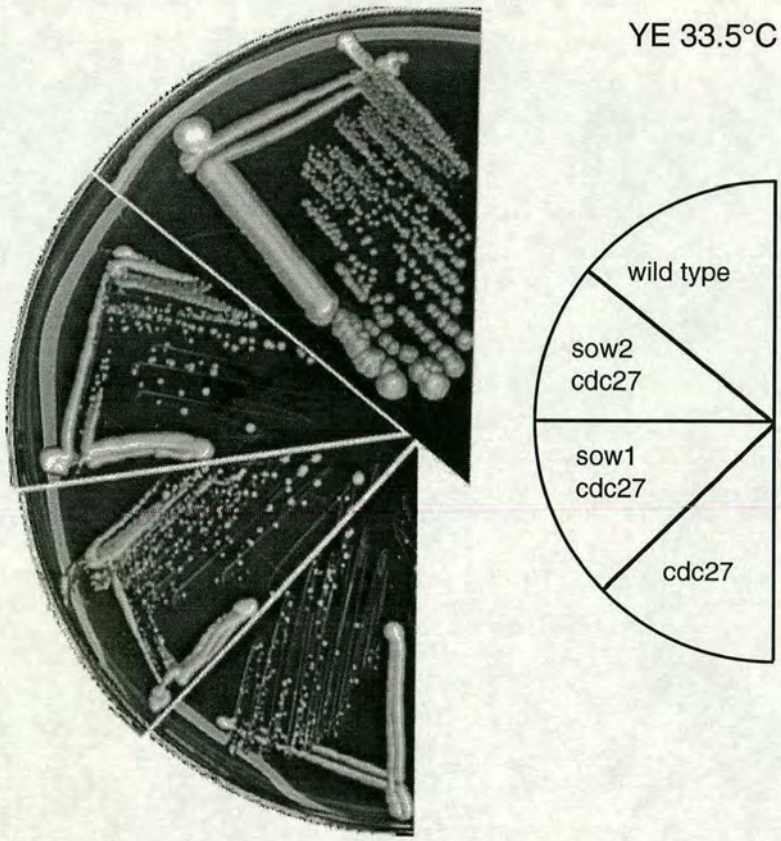

Figure 6.7: *cdc27* and *cdc27 sow* strains.
cdc27 sow1, *cdc27 sow2*, *cdc27* and wild type strains were streaked to single colonies at 33.5°C on YE medium and photographed when colonies had formed.

Next *cdc27* was crossed to *sow1-22*. The behaviour of the *sow1-22* allele was indistinguishable from that of the *sow1-T20* allele. Thus, the suppression of the cdc phenotype seen in the *cdc27 sow1* double mutant is not due to allele specific effects.

The two *sow2* alleles, *sow2-7* and *sow2-11*, were now crossed to *cdc27*, and analysed in the same manner as the *sow1* crosses had been. Like the two *sow1* alleles, these two *sow2* alleles behaved identically to each other.

The segregation of wild type progeny was seen in the *sow2* crosses to *cdc27*, showing that *sow2* is not linked to *cdc27*. Comparison of *cdc27-P11* and *cdc27-P11 sow2* strains at 33.5°C, 34°C, 35°C and 36°C and at 36°C on YE + sorbitol showed interactions between *cdc27-P11* and *sow2* (see Table 6.9).

Table 6.9: comparison of *cdc27-P11* and *cdc27-P11 sow2* segregants

Strains[c]	33.5°C	34°C	35°C, 36°C	36°C + sorbitol
cdc27-P11 control strain	*cdc* (small cols.)	*cdc*	*cdc*	weakly suppressed *cdc*
cdc27 segregant	*cdc* (small cols.)	*cdc*	*cdc*	weakly suppressed *cdc*
cdc27 sow2 segregant	suppressed *cdc*; med. cols.	weak suppression of *cdc*; hetero-geneous cols.	*cdc*	moderately suppressed *cdc* (small cols.)

[b]both *sow2* crosses were identical.
[c] see text

At 33.5°C, the *cdc27-P11 sow2* double mutant could form colonies of elongated cells, whereas *cdc27-P11* can only form very small colonies of *cdc* cells (see Figure 6.7). This shows that the presence of a *sow2* mutation partially suppresses *cdc27-P11*.

In addition to this effect, a further effect was seen at 34°C. Normally, adding sorbitol to the medium at this temperature can only effect partial suppression of the *cdc* phenoptype (see the *cdc27-P11* control strain in Table 6.9). This suppression was significantly enhanced in *cdc27-P11 sow2* segregants.

The data presented in this section demonstrate that *sow2*, and *sow1* to a lesser extent, can both suppress a *cdc27* mutation and interestingly, the effect of the *sow* mutation is, phenotypically, very similar to the osmoremediability seen when sorbitol is added to the medium.

6.2.7: *pat1*

Investigating genetic interactions between *pat1* (also known as *ran1*), *wis1* and the *sow* genes was suggested by Itaru Samejima. *pat1-114* is a temperature sensitive mutation which leads to lethal haploid sporulation at the restrictive temperature of 34°C (Iino and Yamamoto, 1985a; Iino and Yamamoto, 1985b; Nurse, 1985). This aberrant entry into meiosis is suppressed by *wis1*Δ, which allows the double mutant to form colonies at 32°C and 34°C, temperatures which are lethal to a *pat1-114* strain (Stettler et al., 1996).

The *sow* mutants were isolated as suppressors of the temperature sensitivity of *wis1*Δ. They also suppress the salt sensitivity and the cell length defect resulting from a *wis1*Δ. In addition to these phenotypes, *wis1*Δ suppresses the lethality of *pat1* at 34°C (Stettler et al., 1996). It would be interesting to see whether the *sow* mutants were also capable of suppressing this *wis1*Δ phenotype. If they are, this would predict that the *pat1 sow wis1*Δ triple mutant would be lethal at the restrictive temperature of *pat1*, whereas the *pat1 wis1* double mutant would still be viable. If this was the case, then it provides selective conditions, which would allow the cloning of the *sow* genes.

6.2.7.1: Making a *pat1 wis1* strain.

First a *pat1 wis1*Δ strain was constructed. The phenotype of the *pat1 wis1* double mutant was varied: four of the five strains obtained consisted of highly elongated cells with lysis and branching. They grew poorly at 32°C on YE. In contrast, the fifth strain grew well, although the cells were highly elongated. Analysis of the five strains revealed that the progeny that were growing poorly were *ura*$^-$, and that the strain that grew well was *ura*$^+$. This is interesting, particularly as the YE medium used is supplemented with uracil.

The *ura*$^+$ *pat1 wis1* strain was able to form colonies on YE at 32°C, a temperature that is lethal to *pat1-114*, and thus behaved like the prototrophic *pat1 wis* strain that had previously been constructed (Stettler et al., 1996).

6.2.7.2: Crossing *pat1 wis1* to *sow1 wis1* and *sow2 wis1*

A *wis1 sow1-T20* strain was crossed to a *pat1 wis1* strain and tetrads were dissected. The resulting segregants were grown at 32°C and five four-spored tetrads were examined. These tetrads only produced three viable colonies, the fourth having died as highly elongated, swollen cells.

There are four possible genotypes resulting from this cross: *pat1 wis1*, *wis1*, *wis1 sow1* and *pat1 wis1 sow1*. Three of these can grow at 32°C, namely *pat1 wis1*, *wis1* and *wis1 sow1*, so it seemed very likely that the inviable segregant corresponded to a *pat1 wis1 sow1* triple mutant. This genotype was therefore lethal at 32°C.

Next, crosses to *sow1-22*, *sow2-7* and *sow2-11* were set up. The resulting tetrads were dissected and progeny classified phenotypically, with the phenotype of the putative triple mutant judged from TT or NPD tetrads, as shown in Table 6.10.

Table 6.10: progeny from *pat1 wis1* x *wis1 sow* crosses.

sow allele in cross					*wis1 pat1 sow* triple mutant phenotype on YE at 32°C	
	strains	PD	TT	NPD	cols.	cells
sow1-T20	T20.126B x ED1052	0[a]	5	0[a]	-	highly elongated and swollen
sow1-22	SP60 x ED1286	1	9	1	-	highly elongated; extensive lysis
sow2-7	SP39 x ED1286	0	5	2	-	highly elongated; extensive lysis
sow2-11	SP31 x ED1286	1	9	1	-	highly elongated, grainy; extensive lysis

[a] only five of the tetrads from this cross were analysed.

As this Table shows, all four *pat1 wis1 sow* triple mutants were unable to form colonies on YE at 32°C. Two independent isolates of each of the triple mutants containing *sow1-22*, *sow2-7* and *sow2-11* were confirmed to be triple mutants by backcrossing.

Given that the interaction between the *pat1*, *wis1* and *sow* genes is lethal, it might be expected that *pat1* would interact with *sow1* and *sow2* in a *wis1*$^+$ background. *pat1* was therefore crossed to *sow1-T20* to investigate whether these two genes interacted. The progeny were examined at 25°C, 28°C, 32°C, 36°C and 39°C. At all temperatures, the progeny that had been deduced to be *pat1 sow1-T20* double mutants were indistinguishable from *pat1* single mutants. Thus, surprisingly, the only interaction observed is that *pat1* is epistatic to *sow1-T20*.

6.2.7.3: Trying to exploit the lethality of *pat1 wis1 sow* triple mutants.

pat1 wis1 double mutants will grow on YE at 32°C. whereas *pat1 wis1 sow1* and *pat1 wis1 sow2* mutants will not. Thus if triple mutants are transformed with a wild type library, those transformants carrying wild type copies of the *sow* genes should be able to grow. However, the

available library carries the *ura4*$^+$ marker and a dosage effect had been seen with the *ura4* gene.

In order to investigate whether these conditions would be viable for cloning the *sow* genes, *pat1 wis1*, *pat1 wis1 sow1* and *pat1 wis1 sow2* strains were transformed at 25°C with a multi-copy plasmid, containing only the *ura4*$^+$ marker (pON160).

If selection based on the lethality of the triple mutant is to work, it is important that the *pat1 wis1* [+pON160] transformants grow well and the *pat1 wis1 sow1* [+pON160] and *pat1 wis1 sow2* [+pON160] strains do not grow. Several isolates of each of three strains were streaked to single colonies on MM - ura at 28°C (see Table 6.11). *ura*$^-$ derivatives could be obtained from all strains tested, demonstrating that the plasmids had not integrated.

Table 6.11: The effects of *ura* dosage on the colony formation of *pat1 wis1* strains.

genotype	effective *ura* genotype	MM - ura 28°C
wild type control	+	++
pat1 wis1 + pON160	MC	+(+)
pat1 wis1 sow1-22 + pON160	MC	+
pat1 wis1 sow2-11 + pON160	MC	+(+)

MC = multi-copy; ++ = all cells form colonies; +(+) = nearly all cells form colonies; + = some cells form colonies

The transformed *pat1 wis1* strain grew nearly as well as the wild type control. However, the *pat1 wis1 sow2-11* transformant grew as well as the *pat1 wis1* transformant, and the *pat1 wis1 sow1-22* transformant grew fairly well. This is unfortunate, as it is crucial for the desired selection that the triple mutant strains cannot form colonies when transformed with a plasmid that does not rescue a *sow* mutation. The same strains were streaked to single colonies at 30°C and at 32°C in an attempt to alleviate this problem, but at these temperatures, the *pat1 wis1* transformant was unable to form colonies.

Sadly these observations mean that this screening approach is useless, as there would be little differentiation between positive transformants and the background for *sow1-22* and *sow2-7* and none at all for *sow2-11*.

6.2.7.4: *pat1-114* is osmoremediable

Although the genetic interaction between *wis1*, *pat1* and *sow1* and *sow2* did not permit the cloning of the *sow* genes, it would be interesting to learn something about its mechanism.

All the mutations that had shown an interaction with the *sow* genes so far had also been osmoremediable to some extent. Although *pat1* did not show any suppression by *sow1-T20*, it would be worth knowing whether *pat1-114* was osmoremediable. To test this, freshly-growing *pat1* and wild type cells were streaked to single colonies on YE and YES at 32°C. The plates are shown after three days' incubation in Figure 6.8.

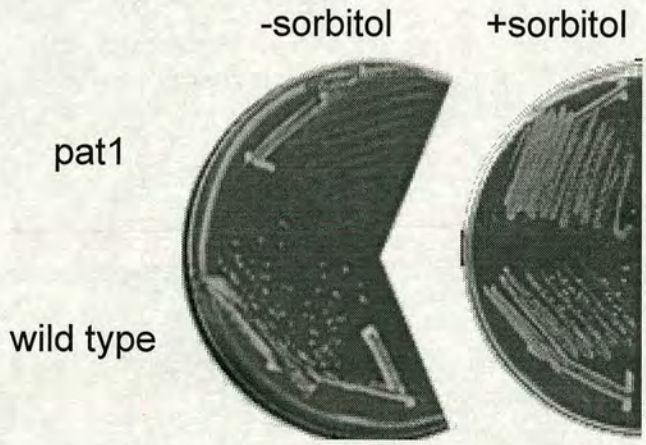

Figure 6.8: The effect of growing a *pat1* strain on sorbitol.
pat1-114 and wild type cells growing at 32°C on YE with and without 0.9M sorbitol.

On YE at 32°C, *pat1* cells are unable to form colonies at all and most cells undergo aberrant sporulation. However, on YES at 32°C, *pat1* cells are able to form colonies of normal cells with no sign of the sporulation. Thus, sorbitol is able to rescue the TS phenotype of the *pat1* mutation. Perhaps this is achieved via the increase in osmolarity the solute creates. This was perhaps an unexpected result and it will be discussed in the next section.

6.3: Discussion

The experiments in this final Chapter of results had two aims: to investigate the functions of the *sow1* and *sow2* genes, with a view to cloning the genes themselves, if possible. To what extent were they able to answer these questions?

The first set of experiments focused on the stress tolerance of *sow1* and *sow2*. They tolerate heat better than wild type, which suggests the *sow* genes play a role in the heat shock response pathway. This is to some extent supported by the apparent improvement in heat shock survival seen in a *sow1-T20* strain. Further evidence would be needed to clarify this.

In the genetic investigation, it was found that *sow1* and *sow2* are not allelic to any of the following genes: *swo1*, *sty1*, *ppa1*, *ppa2*, *ppe1*, *cyr1*, *pka1*, *wee1*, *cdc2*, *cdc13*, *cdc27*, *pat1*; and that *sow1* is not allelic to *atf1* or *cdc25*.

The genetic interactions fell into the following three classes: suppression of the temperature sensitive phenotypes of *sty1*, *wee1*, *cdc2-1w*, *cdc13*, *cdc27*; suppression of the cell length defect of *sty1* and no suppression (*swo1*, *atf1*, *ppa1*, *ppa2*, *ppe1*, *cyr1*, *pka1*, *cdc2-33* and *pat1*).

For *wee1*, and the *cdc* genes, the effect the *sow* mutations have on these mutations is very similar to lowering the temperature, or, interestingly, adding sorbitol (an osmo-stabiliser) to the medium. The stability of proteins decreases at high temperature as well as in response to changes in osmolarity. It is therefore conceivable that the *sow* mutations suppress by increasing protein stability, presumably, in much the same way as sorbitol. This ties in intriguingly with the fact that they suppress *wis1*Δ, a genetic background that is itself osmoremediable, even though there is no Wis1 protein present in the strain.

The Wis1 pathway bifurcates just below Sty1. Non cell cycle phenotypes are to a very great extent dependent on Atf1 activity; cell cycle effects and some temperature sensitivity act through a separate branch (or perhaps branches) of the pathway. Suppression of both the cell cycle and heat sensitive *sty1* phenotypes by the *sow* mutants suggests that they lie

outwith the known Wis1 pathway. This is supported by the fact that Atf1 is not needed for Sow1 to reduce the length *wis1* cells divide at.

As far as the relationship between *sow1* and *sow2* is concerned, something can be gleaned from the following: in *wis1*Δ strains, the effects of *sow1* and *sow2* are additive, which implies that they lie in parallel pathways, acting on a common target. Yet, at 39°C, the double mutant appears to act like *sow2*. If we assume the mutations are loss of function (as discussed in Section 5.4), then this suggests either that Sow2 lies downstream of Sow1 or that Sow1 requires Sow2 to act. As far as heat tolerance is concerned, their role is very likely inhibitory, given that a loss of function mutation makes cells more tolerant of stress.

Experiments with *pat1* gave rise to interesting results: although *sow1-T20* does not interact with *pat1* in a *wis1*$^+$ strain, it is conceivable that other *sow* alleles might, especially given that *pat1* is osmoremediable. The genetic interaction seen between *pat1*, *wis1* and *sow1* and *sow2* looked like it might provide a promising strategy for cloning the *sow* genes; however on closer investigation the selection afforded was not sufficient.

This situation could perhaps be remedied by the use of the milder *pat1-6* allele, or a change to SD (a synthetic *S. cerevisiae* minimal medium), which showed no ura-dependent growth differences. Alternatively, a leucine-based library could be used.

Thus, cloning proved elusive, but with additional time, the *sow* genes could be mapped (using the phenotype of extreme heat resistance) and hence cloned. This would perhaps provide better answers to some of the questions tackled during this project.

Chapter 7: Discussion

The overall aim of this project was to uncover the mechanism of stress sensitivity and cell cycle defect of *wis1*Δ strains, and if possible to learn why these two seemingly unconnected phenotypes were downstream of the same gene. Two different experimental approaches were used. The experiments described in Chapter 3 used the tools available at the time to examine the physiology of *wis1*Δ strains and the genetics of the Wis1 pathway. The remaining chapters describe the isolation and characterisation of new mutants which suppress *wis1*Δ phenotypes.

7.1: How Wis1 mediates stress resistance.

*wis1*Δ cells are unable to grow at 36°C, and furthermore, become highly elongated, a phenotype which is reminiscent of *cdc* mutants. Therefore, to investigate whether *wis1*Δ cells die at high temperature from cell cycle arrest, their behaviour was closely examined following a shift to 36°C (see Section 3.2.2.1).

During this treatment, cell number increased by a factor of roughly two and cell length continued to increase. A striking observation was that *wis1*Δ cells lose viability in two stages following shift to high temperature.

Normally, cells have two lines of defence against stress. The first is a minimal constitutive system which will tolerate the immediate inception of stress, and which induces the second system: a stress response, which involves the production of new proteins and which can tolerate stress in the longer term (reviewed in Ruis and Schuller, 1995).

When *wis1*Δ cells are shifted to high temperature, those that are not killed instantly continue to grow, and are viable for at least five hours, but divide only once. This leads to highly elongated cells and suggests that while cell growth (ie accumulation of cytoplasm) is not immediately affected under these conditions, cell division is.

S. pombe cells spend roughly 70% of their cell cycle in G2 (Nasmyth et al., 1979), so nearly all cells in a population will be in G2. Thus, if *wis1*Δ cells at high temperature can divide only once, it appears that high

temperature prevents progression through the following cell cycle, in a manner similar to a *cdc* mutant. It is likely that this reflects the temperature sensitivity of at least one protein that is required for cell cycle progression. Normally the stability of this protein is maintained by functioning Wis1, but this fails in a *wis1*Δ strain under stress conditions. The identity of this protein is unknown.

To investigate further the conjecture that *wis1*Δ cells die from a cell cycle defect (ie because they are unable to divide) the *wee1-50*^{ts} mutation was used (see Section 3.2.2.2). This mutation forces early division at the restrictive temperature so the cells are short. They are also stress-resistant. *wee1 wis1* double mutant cells are also short, but stress-sensitive, and die without the elongation seen in a *wis1*Δ strain when stressed.

This observation may suggest that *wis1Δ* cells do not die only because of a cell cycle defect, because when the cell cycle is artificially accelerated by the *wee1* mutation, they still are stress sensitive. A more likely explanation is that as Wee1 plays such a key role in Y-15 phosphorylation that its cell cycle effect is epistatic over many other mechanisms of regulating cell cycle and this merely reflects its acting directly on Cdc2 and playing a key regulatory role in Cdc2 activation.

*wis1*Δ cells are longer than wild type and furthermore become longer and longer under increasing stress (Millar et al., 1995; Stettler et al., 1996). As the Wis1 pathway is non-functional in a *wis1*Δ strain, the increase in cell length seen as stress is increased cannot be mediated by the Wis1 pathway itself. This implies that this increase in cell length is due to the effects of a protein with a role in cell cycle, which is increasingly inactivated by stress in the absence of Wis1.

One possible way the Wis1 pathway could affect the stability of proteins would be by regulating the amounts of compatible solutes in the cytoplasm. Compatible solutes are highly soluble, polar molecules that are compatible with the cell's metabolism. They add structure to cytoplasmic water and (likely as a result of this property) are effective stabilisers of protein structure (Galinski, 1993).

Sorbitol can function as a compatible solute when added to medium at certain concentrations and is therefore routinely used as an osmoprotectant in techniques such as electroporation. Often, mutations that arise from misfolded proteins (typically, but not exclusively, temperature sensitive mutations) can be rescued by the addition of a compatible solute, such as sorbitol, to the medium.

In Section 3.2.4, the sorbitol-mediated osmoremediability of certain mutations was investigated and it was observed that *wis1*Δ and other mutations (*wee1-50*, *cdc27-P11*, *cdc25-22* and *pat1-114*) were osmoremediable. However, as there is no Wis1 protein in a *wis1*Δ strain, sorbitol is clearly not rescuing a mutant Wis1 protein, and must therefore be acting on tagets of the Wis1 pathway (as discussed above). This suggests that one of the roles of the Wis1 pathway is to moderate cytoplasmic osmolarity. During times of stress, this function would appear to be indispensable as *wis1*Δ cells die when stressed.

It is known that yeasts mainly use glycerol as a compatible solute to resist changes in osmolarity (Aiba et al., 1995; Albertyn et al., 1994; Blomberg, 1997). In addition to this, the non-reducing sugar, trehalose affects protein stability. Trehalose synthesis requires the Tps1 protein in fission yeast (Blazquez et al., 1994)). This compound is involved in heat stress resistance in many systems (de-Araujo, 1996; Ribeiro et al., 1997; Strom and Kaasen, 1993). and for maintaining protein stability in response to heat in both budding (Solapenna and Meyerfernandes, 1994) and fission (Fernandez et al., 1995) yeasts. Trehalose is capable of stabilising hydrogen bonds in proteins in vitro. Conceivably, this is the mechanism by which it stabilises proteins in response to heat stress in the yeasts.

Interestingly enzymes for synthesising both glycerol and trehalose are transcriptionally regulated by the Wis1 pathway. In addition, the catalase encoding gene, *ctt1* is also under the transcriptional regulation of the Wis1 pathway (Degols and Russell, 1997). *ctt1*$^+$ is required for resistance to oxidative stress (Wieser et al., 1991).

Activation of the Wis1 pathway follows heat, osmotic, oxidative, and radiation stresses (Degols and Russell, 1997; Degols et al., 1996; Kato et

al., 1996; Samejima et al., 1997; Shiozaki and Russell, 1995a; Shiozaki and Russell, 1996) and, via transcription of *gpd1*, *tps1* and *ctt1*, leads to an increase in the intracellular levels of glycerol (an osmo-protectant) and trehalose (a thermo-protectant) and catalase (which protects against oxidative damage) respectively. This presumably results in cells being protected from osmotic, heat and oxidative stresses in response to a single (or multiple) stress stimulus.

This model of a Wis1 pathway-mediated mechanism for multiple stress tolerance is supported by evidence from, for example the observation that over-expression of *gpd1*$^+$ in fission yeast is sufficient to rescue the osmotic sensitivity of the strain (Aiba et al., 1995) and that *tps1*$^+$ is required for heat shock resistance in fission yeast (Ribeiro et al., 1997).

Cells have a two-tier system for tolerating stresses (see Section 1.4). A minimal constitutive system protects cells from stresses in the immediate term, and an inducible system, which allows adaptation to prolonged stress. The inducible system is induced by the constitutive system, and leads to cross-protection, for example, heat stress can lead to osmotic stress resistance.

The model of the Wis1 pathway proposed above, ties in very closely with the function of the inducible stress response system observed in cells. Furthermore, it is formally possible that in the absence of stress, the basal activity of the Wis1 pathway, provides the constutive basal stress tolerance system that cells rely on to tolerate the immediate effects of stress.

In support of this, when *wis1*Δ cells were transferred to 36°C, a proportion died instantly (see Section 3.2.2.1). This suggests that the minimal constitutive pathway is defective, and thus many cells died immediately they are exposed to stressful conditions. Clearly further experiments would be needed to corroborate this tenet.

Stress conditions are usually associated with cell cycle delays (heat shock causes a delay at Start in *Saccharomyces cerevisiae* (Johnston and Singer, 1980) and a G2 delay in S. pombe (Polanshek, 1977)). The rationale is that this presumably allows organisms long enough to repair damage before dividing. In direct contravention of this conjecture is the

observation that activation of the Wis1 stress response pathway leads to mitotic advance (Warbrick and Fantes, 1991).

Why cells should have evolved a mitotic advance as part of a stress response is an interesting question. It hints that there might be cell-cycle dependent responses to stress, ie responses that can perhaps only be ellicited during certain stages of the cell cycle.

Stationary phase is entered just before Start and is separate from the rest of the cell cycle (as shown by the budding yeast *gcs1* mutants (Drebot et al., 1987)). Furthermore, stationary phase is a highly stress resistant state (see Piper, 1993). Another stage of the yeast life cycle that is highly stress-resistant is the spore, which is the end product of entering the sexual differentiation pathway.

Moreover, the processes of stress response, mating and stationary phase entry are profoundly affected by the Wis1 MAPK pathway. Wis1 is required for stress resistance, stationary phase survival and efficient mating (Kato et al., 1996; Shiozaki and Russell, 1995a; Shiozaki and Russell, 1996; Stettler et al., 1996). This leads to the conjecture that all three processes can be considered to be different approaches to stress survival that will be more or less appropriate choices for the organism to take, depending on mate and nutrient availability.

Normally, when fission yeast cells are starved for nitrogen, mitosis is accelerated relative to growth and they therefore undergo two rapid divisions, until the cells are too small to pass the G1-S size control. This ensures cell cycle arrest at the appropriate point in the cell cycle (just before Start) for mating with a partner.

This acceleration is not seen in *wis1*Δ cells (personal observation). These cells fail to divide under nitrogen starvation, and elongate instead. As the Wis1 pathway is activated by nitrogen starvation (Kato et al., 1996; Shiozaki and Russell, 1996; Stettler et al., 1996), this suggests that the acceleration normally seen is mediated by activation of the Wis1 pathway. The acceleration seen following activation of the Wis1 pathway, may therefore exist in order to speed cells through mitosis until they are small enough to arrest at G1, thus reducing the delay between the onset of starvation and G1 arrest. In addition to the transcriptional defects

already known (Kanoh et al., 1996; Shiozaki and Russell, 1995b; Wilkinson et al., 1996), the failure of *wis1*Δ cells to accelerate mitosis in response to starvation may explain why they conjugate so poorly.

In mammalian systems, stress-mediated activation of the $p38^{HOG1}$ and SAPK pathways influences the developmental decisions taken by cells, in conjunction with signals from the "classical" MAPK pathways (Xia et al., 1995). It appears that a similar relationship might exist between the fission yeast Wis1 and mating MAPK pathways.

7.2: The Wis1 pathway.

In order to investigate the architecture of the Wis1 pathway, multiple mutants for *mcs4*, *wis4* and *win1* were made (see Section 3.2.2). The stress sensitivity of multiple mutants containing *win1* suggested that Win1 acted in parallel to Mcs4 and Wis4. Over-expression of *wis1*$^+$ is lethal and this was suppressed in *win1*, *mcs4* and *wis4* mutants (Samejima et al., 1997) confirming that these mutants lie in the Wis1 pathway. Over-expression of the Wis1EE allele (which is partially activated but unregulatable) is also lethal. And lethality was not suppressed in *win1 mcs4* and *wis4* strains. This shows that the *win1 wis4* and *mcs4* genes lie upstream in the pathway and are responsible for activating Wis1.

All three genes have now been cloned. *win1*$^+$ encodes a MAPKKK (Samejima, I., pers. comm.) and so does *wis4*$^+$ (Samejima et al., 1997; Shieh et al., 1997; Shiozaki et al., 1997a). Mcs4 is homologous to a bacterial two-component response regulator and activates Wis4 (Cottarel, 1997; Shieh et al., 1997; Shiozaki et al., 1997a). Furthermore, two putative histidine kinases (Mak1 and Mak2) have recently been identified in fission yeast based on sequence similarity to budding yeast Sln1 (Makino, K. and Millar, J.A., unpublished observations in Shieh et al., 1997)

A Sensor Kinase of a two-component system encoded by *YPD1* (Posas et al., 1996) lies between Sln1 and Ssk1 in the budding yeast Hog1 pathway. Given that the Wis1 MAPK pathway in fission yeast has so far mirrored the budding yeast Hog1 pathway exactly, the existence of a fission yeast homologue of Ypd1 is strongly predicted.

Furthermore, an osmosensor, Sho1 directly activates Pbs2 (the *S. cerevisiae* homologue of Wis1), by interacting with the N-terminus of Pbs2 (Maeda et al., 1995). The N-terminus of Wis1 contains a consensus SH3 binding motif (Mackie, S., pers. comm.), which could hint at the existence of a fission yeast Sho1 homologue, but it seems unlikely that even if it exists this Sho1 homologue plays an important role in signalling osmotic stress. In fission yeast loss of both MAPKKKs (*win1-1 wis4*Δ) leads to a osmotically-sensitive phenotype as serious as that seen when *wis1* alone is mutated, suggesting that Wis4 and Win1 are sufficient between them to transmit osmostress signals to Wis1 (Samejima et al., 1997). In contrast, a *ssk2*Δ *ssk22*Δ double mutant in budding yeast is osmotically resistant unles *sho1* is also mutated (Maeda et al., 1995).

Both the Hog1 and Wis1 pathways are activated by osmostress. However, the only role that the Hog1 pathway plays in budding yeast is in regulating osmostress. In contrast, the Wis1 pathway responds to and is needed for the survival of many different types of stress. This situation is analagous to that found in the SAPK and $p38^{HOG1}$ pathways in metazoans.

Although it is clear that osmostress is transduced by the Wis1 pathway, some controversy exists over exactly how osmostress is transduced. *wis4*Δ cells show a wild type activation of Sty1 following osmotic shock and furthermore do not elongate under these conditions (Samejima et al., 1997). Thus it seems that $win1^{+}$ is sufficient for osmostress signalling and the role Wis4 plays is primarily in the basal activity of the pathway (loss of both *win1* and *wis4* is necessary to make cells osmosensitive (Samejima et al., 1997)). However, Russell and Shiozaki failed to detect activation of Sty1 following osmotic shock in an *wis4* mutant and therefore concluded that this stress (and others) are mediated by Wis4 (Shiozaki et al., 1997a).

In addition, heat shock and oxidative stress lead to activation of Sty1 in a *wis4 win1* double mutant in a Pyp1-dependent manner (Samejima et al., 1997), which strongly suggests that these two stresses are not transmitted through the MAPKKKs (Wis4 and Win1) or the MAPKK (Wis1), but rather enter the pathway at the MAPK level via regulation of the tyrosine phosphatase Pyp1.

The Wis1 pathway also interacts with the cAMP pathway. This pathway counteracts the Wis1 pathway: for example, upregulation of the cAMP pathway in the *cgs* mutants, or down-regulation of the Wis1 pathway (*wis1*Δ) both lead to a cell cycle delay (Devoti et al., 1991; Millar et al., 1995; Shiozaki and Russell, 1995a). The converse is also true. The situation is the same in mammalian system, where both the SAPK and p38HOG1 MAPK pathways and the cyclic AMP pathway regulate transcription of target genes via CREB/ATF elements that lie upstream of genes (Clerk and Sugden, 1997; Gupta et al., 1995). In budding yeast there is also antagonistic regulation of STREs (the equivalent of CREB/ATFs) by the Hog1 and cAMP pathways (Belazzi et al., 1991; Schuller et al., 1994; Varela et al., 1995).

The Wis1 pathway bifurcates just below Sty1 (see Figure 7.1). Cell cycle regulation and a component of heat stress tolerance lie in a branch (or branches) that does not involve Atf1. On the other hand, other stress responses and mating lie donwstream of Atf1. The part of the pathway mediated by Atf1 is well understood, and parallels the architecture of stress-activated pathways in metazoans. However, the mechanism by which the Wis1 pathway controls the G2-M transition is still completely unknown.

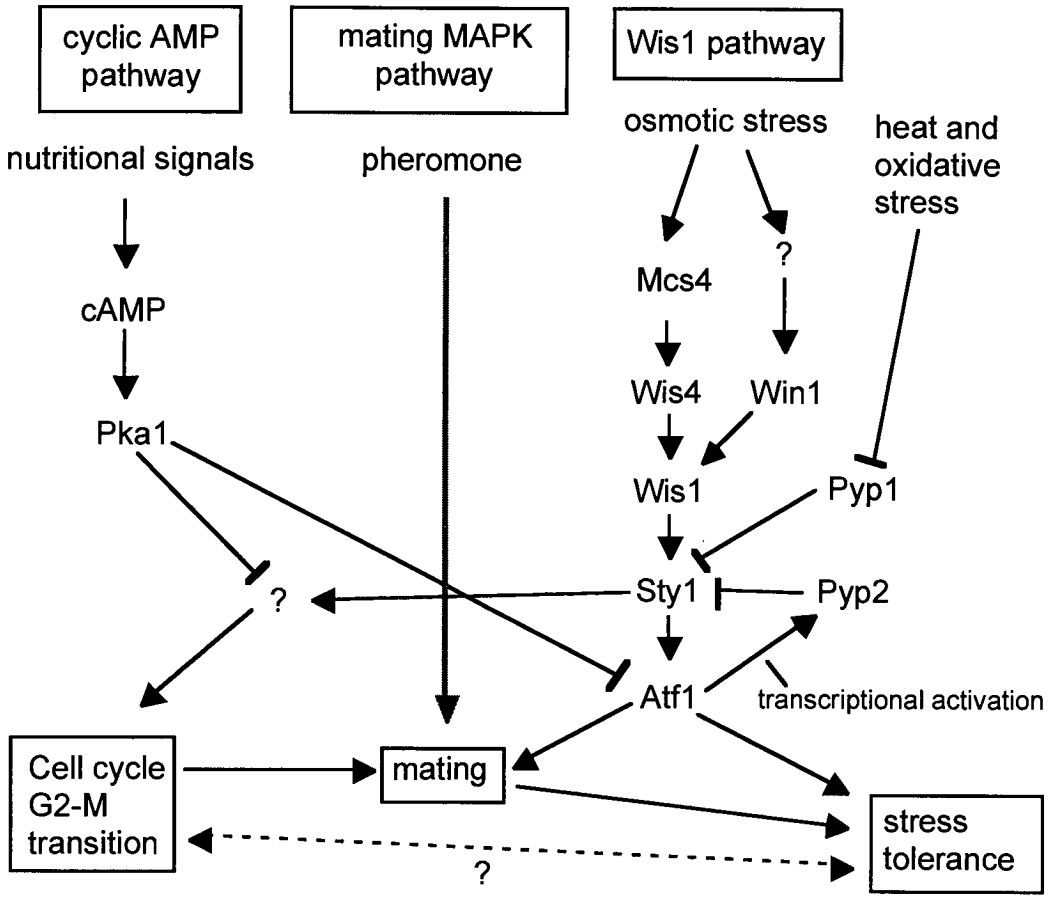

Figure 7.1: architecture of the Wis1 pathway and interaction with other pathways.
Activation of the Wis1 pathway by stressful environments leads to mitotic advance, and induction of stress tolerance. The cyclic AMP pathway responds to nutritional signals and reduces stress tolerance and the propensity to mate under favourable nutritional conditions. Stress conditions can affect cell cycle control.

Although Wis1 does not act on the cell cycle via Atf1, clearly it must act on the cell cycle at some point. One possible target of Wis1 activity is CAK (Mcs2/Mcs6), although phosphorylation on T167 is not capable of inducing mitosis in the presence of Y15 phosphorylation, so this is an unlikely scenario.

Wis1 could exert its effect on cell cycle via a cdk inhibitor (cdi). Rum1 functions as a cdi (Correa-Bordes and Nurse, 1995), but *rum1 wis1* double mutants are the same length as *wis1*Δ cells, showing that Rum1 does not act downstream of Wis1 (Fantes, P.A., pers. comm.). However, a different cdi could mediate the Wis1 cell length defect.

Altering the expression of *wis1* has the same effect on cell cycle no matter whether the major regulators of Y-15 (Pyp3, Cdc25, Wee1 and Mik1) are functional or not (Cripps, K., pers. comm., Fantes laboratory, unpublished observations, Warbrick and Fantes, 1991). This suggests that Wis1 does not appear to act on Cdc2/Cdc13 tyrosine-15 phosphorylation via a single regulatory protein. This leaves open the possibility that Wis1 acts on more than one of these regulators, or that Wis1 acts via a novel mechanism.

7.3: Suppressors of *wis1*Δ: function of Sow1 and Sow2 .

In order to elucidate the Wis1 pathway, extragenic suppressors of *wis1*Δ were isolated using heat sensitivity as selection (see Chapter 4). It was also possible to obtain mutants by using *wis1*Δ osmotic sensitivity as selection, although this rarely gave rise to strong enough suppression to work with. This may be because it is simply harder to mutate the genome of fission yeast to generate efficient suppressors of osmotic sensitivity than heat sensitivity, or it may reflect the difference in exposure to the selection: when cells are plated on salt containing medium, they are immediately exposed to the high osmolarity, treatment which likely constitutes an osmotic shock, whereas in an incubator, plates are gradually heated to the required temperature.

A minority of the strains contained mutations that also suppressed one or more other the other *wis1*Δ phenotypes such as osmotic sensitivity and cell length defect. Mutants that strongly suppressed more than one *wis1*Δ phenotype were chosen for further analysis (see Section 4.4).

Genetic analysis revealed that twelve of the strains analysed contained a single mutation (see Section 5.2.1.3 and Section 5.3.2). These fell into two linkage groups, which were named *sow* for suppressor of *wis1*Δ. Nine strains made up the *sow1* linkage group (T20, 5.10, 5.13, 5.15, 5.22, 5.36, 5.74, 5.97 and 5.102) and *sow2*, three strains (5-7, 5-11 and 5-123) (see Section 5.3.1).

As all Wis1 signals appear to act through Sty1 (the MAPK directly downstream by Wis1 (Kato et al., 1996; Millar et al., 1995; Shiozaki and Russell, 1995a)), an activated allele of *sty1* should suppress all *wis1*Δ phenotypes. Yet, neither *sow1* nor *sow2* mapped to *sty1* (see Section

6.2.2.1). This might be explained by the following observation: a MAPK from Xenopus has been shown to require two sequential conformational changes for activation (Canagarajah et al., 1997). This would be hard to mimic mutationally, which would explain why no activated alleles of *sty1* were recovered.

7.4: Sow1 and Sow2 probably lie outside the Wis1 pathway

Several different experiments provided evidence that could be used tentatively to explain how Sow1 and Sow2 might act on Wis1. The first such experiment (in Section 5.2.3) involved the construction of heterozygous diploids for *sow1-T20* to examine whether this mutation was dominant. This allele appeared to be semi-dominant (ie the phenotype of the heterozygous diploid was intermediate between the phenotypes of the two homozygous diploids).

Several different alleles of each of *sow1* and *sow2* were obtained (see Section 5.3.1). This suggests that the mutations are loss of function to varying degrees, especially for *sow1* which has nine alleles that are phenotypically very similar. Given this, the apparent semi-dominance seen with *sow1-T20* in heterozygous diploids is most likely due to a gene dosage effect, suggesting that regulation of precise levels of *sow1*$^+$ transcription is important under stress conditions. This is reminiscent of proteins like budding yeast Hsp82 (Hsp90 in other organisms) for which levels are crucial for survival at high temperature (Borkovich et al., 1989).

The *sow1* mutation makes *wis1*Δ strains grow at higher temperature and divide at shorter cell length (see Section 4.3). It has the same effects on *wis1*$^+$ cells (see Section 5.3.4.2 and Section 6.1.2). This implies that Sow1 does not require Wis1 to act, and suggests therefore that it acts in a separate pathway to Wis1.

During intercross experiments linkage groups of *sow1* and *sow2* mutants were defined. *wis1 sow1 sow2* triple mutants were also constructed in these crosses and it was noted that they grew better at high temperature and divided at a shorter length than *wis1 sow1* and *wis1 sow2* mutants (see Section 5.3.3). This additive suppression shown by *sow1* and *sow2*

was interesting because it corroborates the suggestion that Sow1 and Sow2 act in parallel.

In Chapter 6, *sow1* and *sow2* were crossed to a set of genes that are known to interact with *wis1* in order on the one hand to determine whether any were linked to a *sow* gene and on the other hand whether they interacted with *sow1* or *sow2*.

sow1 and *sow2* were found not to be allelic to the following genes: *swo1*, *sty1*, *ppa1*, *ppa2*, *ppe1*, *cyr1*, *pka1*, *wee1*, *cdc2*, *cdc13*, *cdc27*, *pat1*; and that *sow1* is not allelic to *atf1* or *cdc25*. These genes were as far as possible a complete set of the genes that are known to interact genetically with *wis1*.

sow1 was observed to suppress the cell length defect and temperature sensitivity of *sty1*Δ (see Section 6.2.2.1), and this did not require Atf1 (see Section 6.2.2.2). This implies that Sow1 does not lie downstream of Atf1 in the Wis1 pathway

The other genetical interactions seen with the *sow* genes were a shift towards wild type behaviour of *wee1-50*, *cdc2-1w* (*sow1* only), *cdc13-117* and *cdc27-P11*. *wee1-50* and *cdc27-P11* were also shown to be osmoremediable (as well as *cdc25-22* and *pat1-114*). As sorbitol mimics the effects of a *sow* mutation in a *wis1*Δ background, perhaps it also does in a *wis1*$^+$ background, and therefore, the *sow* mutation is partially rescuing these mutant alleles by an equivalent mechanism.

However, *cdc13* was partially rescued by *sow1* and *sow2* (see Section 6.2.5.3), and *cdc2-1w* was suppressed by *sow1* only (see Section 6.2.5.2). Neither *cdc13* nor *cdc2-1w* is osmoremediable (see Section 3.2.4). Furthremore, *pat1-114* is osmoremediable but shows no genetic interaction with the *sow* genes (see Section 6.2.7).

This suggests that the similarity seen between the effects of sorbitol and *sow* mutations may not reflect a common mechanism, but may reflect two separate processes that can achieve the same result. This may suggest that the sows play a role in regulating chaperone-like proteins such as Hsp70 and Hsp90, perhaps transcriptionally.

It is unlikely that the *sow* genes act through *swo1* as they were unable to rescue the *swo1* temperature sensitive phenotype at any temperature (see Section 6.2.1). Hsp90 (encoded by *swo1*) forms many large complexes, which include many accessory proteins including p50 (*CDC37*), the immunophilins Cyp-40, fkbp51 and fkbp52 (Bose et al., 1996; Kimura et al., 1997). Interestingly, a *Schizosaccharomyces pombe* Cyp-40 homologue, Wis2 was identified in the same genetic screen as *wis1*$^+$ (Warbrick and Fantes, 1992; Weisman et al., 1996).

Interesting further experiments to explore this would involve investigating whether there were genetic interactions between the *sow* genes and *wis2*, and furthermore, it is possible that the transcriptional activation of certain genes involved in the heat shock response is altered in sow mutants. Northern analysis *sow* mutants would address this issue.

While genetical data of the sort presented in this project can be useful in uncovering novel genes and investigating their interactions with known genes, it is impossible to define the cellular roles of Sow1 and Sow2 any more accurately without molecular work. Clearly the cloning of the sow genes would be a first step in this direction. However, in the absence of another plausible strategy, genetic mapping followed by positional cloning seems the best option.

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"Did all that really just happen or have I wandered into a Dada exhibition?"

Notes from a Small Island, Bill Bryson, 1995, London.

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The wis1 signal transduction pathway is required for expression of cAMP-repressed genes in fission yeast

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SUMMARY

The wis1 protein kinase of *Schizosaccharomyces pombe* is a member of the MAP kinase kinase family. Loss of wis1 function has previously been reported to lead to a delay in the G_2-mitosis transition, loss of viability in stationary phase, and hypersensitivity to osmotic shock. It acts at least in part by activating the MAP kinase homologue sty1; loss-of-function *sty1* mutants share many phenotypes with *wis1* deletion mutants.

We show here that, in addition, loss of wis1 function leads to defective conjugation, and to suppression of the hyperconjugation phenotype of the *pat1-114* mutation. Consistent with this, the induction of the *mei2* gene, which is normally induced by nitrogen starvation, is defective in *wis1* mutants. In wild-type cells, nitrogen starvation leads to *mei2* induction through a fall in intracellular cyclic AMP (cAMP) level and activity of the cAMP-dependent protein kinase. We show here that wis1 function is required for *mei2* induction following nitrogen starvation. Expression of the *fbp1* gene is negatively regulated by cAMP in response to glucose limitation: induction of *fbp1* also requires wis1 and sty1 function. Loss of *wis1* is epistatic over increased *fbp1* expression brought about by loss of adenylate cyclase (git2/cyr1) or cAMP-dependent protein kinase (pka1) function. These observations can be explained by a model in which the pka1 pathway negatively regulates the wis1 pathway, or the two pathways might act independently on downstream targets. The latter explanation is supported, at least as regards regulation of cell division, by the observation that loss of function of the regulatory subunit of the cAMP-dependent protein kinase (cgs1) brings about a modest increase in cell length at division in both *wis1*$^+$ and *wis1*Δ genetic backgrounds.

Key words: Fission yeast, Cell cycle, MAP kinase, cAMP, Stress response, Transcriptional control

INTRODUCTION

Cells respond to their environments in a variety of ways: by starting or stopping proliferation, by embarking on a new developmental course, or by varying the level of expression of particular genes. The transduction of signals from outside the cell to effect changes within it has been investigated in a range of cell types. In the past few years it has become clear that one type of signalling pathway, containing a MAP kinase (mitogen activated protein kinase) homologue and its activators, is of central importance. Within a single cell type there may be more than one such kinase cascade operating (reviewed by Blumer and Johnson, 1994; Johnson and Vaillancourt, 1994; Marshall, 1995). In yeasts, largely independent pathways respond to different environmental stimuli, for example the presence of mating pheromone and extremes of growth conditions such as the osmotic strength of the medium. Only appropriate responses are elicited by the different stimuli (Herskowitz, 1995). In contrast, in higher cells different cascades are often activated in response to a single stimulus (Cano and Mahadevan, 1995).

We previously reported that the *wis1*$^+$ gene of *S. pombe* encoded a protein kinase which regulated entry into mitosis and cell viability in stationary phase. At that time, as now, the most similar protein in the databases was the *S. cerevisiae* Pbs2 protein kinase; more recently both wis1 and Pbs2 have been shown to be highly homologous to the MAP kinase kinase (MAPKK) subfamily (Kosako et al., 1993). Pbs2 has since been shown to be part of a MAP kinase signalling pathway activated in response to high osmotic stress: Pbs2 phosphorylates the MAP kinase homologue Hog1, thereby activating it (Brewster et al., 1993; Schüller et al., 1994). Three gene products involved in the activation of Pbs2 have recently been isolated, including two MAPKK kinases (MAPKKKs) (Maeda et al., 1995). In *S. pombe*, wis1 has been implicated in osmotic and temperature stress responses (Millar et al., 1995; Shiozaki and Russell, 1995a; S. Stettler and P. A. Fantes, unpublished results) and a MAP kinase homologue called sty1, spc1 or phh1 has been identified as a major substrate (Millar et al., 1995; Shiozaki and Russell, 1995a,b; Kato et al., 1996). The protein tyrosine phosphatases pyp1 and pyp2 have been shown to dephosphorylate and inactivate sty1 (Millar et al., 1995; Shiozaki and Russell, 1995b).

In this manuscript we show that in addition to its role as a

component of a stress-responsive pathway, wis1 is required for transcriptional induction of two genes (*fbp1*$^+$ and *mei2*$^+$) by starvation conditions, a process that is known to be mediated by the cAMP-protein kinase (PKA) signalling pathway. Probably as a consequence of this requirement, cells lacking wis1 function are deficient in sexual differentiation. In the accompanying paper (Dal Santo et al., 1996), the protein tyrosine phosphatases pyp1 and pyp2 are shown to play a role in regulating *fbp1*$^+$ expression and sexual differentiation. These observations indicate that the wis1-sty1 pathway and the cAMP-PKA pathway interact and play different roles in regulating gene expression and sexual development in *S. pombe*.

MATERIALS AND METHODS

Media and general techniques

All the media used in this study are derived from the *S. pombe* media described by Moreno et al. (1991). The minimal medium used, which specifically contains 2% glucose and 5 g/l NH_4Cl, corresponds to the EMM medium with the following modifications: no KCl, 144 μg/l molybdic acid instead of 40 μg/l and 10 μg/l KI instead of 100 μg/l. Minimal media with either no nitrogen (EMM-N) or limited nitrogen source (EMM lowN) are minimal medium as described above, with either no or 5 mg/l NH_4Cl instead of 5 g/l. Similarly, minimal medium with limited carbon source (EMM lowG) is minimal medium with 0.1% glucose instead of 2%. Complex medium, which contains 3% glucose, is YE medium supplemented with 75 mg/l adenine and uracil.

Glycerol containing medium is as supplemented YE but contains 3% glycerol instead of 3% glucose and is supplemented with 2% casaminoacids (Difco).

The solid sporulation and conjugation medium was either MEA (Moreno et al., 1991) or SPA (Gutz et al., 1974). General molecular techniques were performed as described by Sambrook et al. (1989). Standard genetical procedures for *S. pombe* were according to Gutz et al. (1974) and Kohli et al. (1977).

Yeast strains

The genotypes of the strains used in this study, otherwise described in the text, are given in Table 1. Genetic nomenclature for *S. pombe* follows the rules proposed by Kohli (1987). All strains were derived from the haploid wild-type *S. pombe* strains 972 (h^-; =ED812), 975 (h^+) and 968 (h^{90}) (Leupold, 1970). Strain SP259, carrying the *pat1-114* allele (Iino and Yamamoto, 1985a), was a gift of Maureen McLeod; other *pat1-114* strains were derived from SP259 by crossing. The double mutant *wis1Δ pat1-114* was identified at 32°C on minimal medium as elongated cells which sporulated upon entry into stationary phase. FWP139 and FWP191, containing *pka1-261* (=*git6-261*) and *git2* deletion alleles, respectively, were gifts from C. S. Hoffman (Byrne and Hoffman, 1993; Dal Santo et al., 1996). Other strains containing this mutant allele were obtained by crossing (see below). The *ura4::fbp1-lacZ* allele (in FWP77) is a disruption of the *ura4* gene by the *fbp1-lacZ* translational fusion (Hoffman and Winston, 1990). Strain FYC71 carries the *E. coli lacZ* gene under the control of the *mei2* promoter integrated at the *mei2* locus (Wu and McLeod, 1995). Strains moderately overexpressing *wis1*$^+$ were derived from the OP2 strain (Warbrick and Fantes, 1991) in which an extra copy of the *wis1*$^+$ gene, under the control of the thiamine-repressible *nmt1* promoter (Maundrell, 1990, 1993), is integrated at the *wis1* locus.

Construction of the *wis1::his1*$^+$ allele

The *wis1::his1*$^+$ allele was constructed in order to facilitate genetic interaction studies. A 2.2 kb *Bam*HI-*Sac*I DNA fragment bearing the *his1*$^+$ gene of *S. pombe* (Hagan and Yanagida, 1990; Iain Hagan, unpublished results) was subcloned into pTZ19R (Pharmacia Biotech Ltd) to give plasmid pTZH. The *wis1*$^+$ gene on plasmid pwis1-XP (Warbrick and Fantes, 1991) was subsequently disrupted by deletion of an internal 1.2 kb *Bgl*II-*Eco*RI fragment and its substitution with a 2.2 kb *Bam*HI-*Eco*RI *his1*$^+$ fragment isolated from plasmid pTZH. This removed most of the conserved kinase domain of the protein, leaving the first 93 and last 120 amino acids intact. The chromosomal *wis1*$^+$ gene was then inactivated by single step gene transplacement (Rothstein, 1983). A diploid strain homozygous for the *his1-102* mutation (*h*$^+$/*h*$^-$ *his1-102/his1-102 ade6-M210/ade6-M216 leu1-32/leu1*$^+$ *ura4-D18/ura4*$^+$) was transformed by electroporation (Prentice, 1992) with a 4.5 kb *Pst*I-*Xba*I fragment bearing the *wis1::his1*$^+$ allele. Integration of the mutant allele at the *wis1* locus was confirmed by tetrad analysis and Southern blot analysis. As expected, the resulting *wis1::his1*$^+$ allele conferred on *S. pombe* cells the same mutant phenotype as the previously described *wis1::LEU2* allele (Warbrick and Fantes, 1991).

Table 1. *S. pombe* strains

Strain	Genotype	Source*
ED800	h^- (=972; standard wild type)	
ED895	h^+ *mei2::mei2-lacZ*	
ED898	h^+ *wis1::LEU2*$^+$ *mei2::mei2-lacZ leu1-32*	
ED900	h^- *ura4::fbp1-lacZ*	
ED903	h^- *wis1::LEU2 leu1-32*	
ED905	h^- *wis1::LEU2 leu1-32 ura4::fbp1-lacZ*	
ED906	h^+ *wis1::LEU2 leu1-32 ura4::fbp1-lacZ*	
ED957	h^- *git2-1 int::LEU2 leu1-32 ura4::fbp1-lacZ*	
ED961	h^- *wis1::his1*$^+$ *his1-102 ura4::fbp1-lacZ*	
ED965	h^- *wis1::his1*$^+$ *git2-1 int::LEU2 ura4::fbp1-lacZ his1-102 leu1-32*	
ED976	h^- *wis1::his1*$^+$ *his1-102*	
ED983	h^+ *git2-1 int::LEU2 leu1-32 mei2::mei2-lacZ*	
ED985	h^+ *wis1::his1*$^+$ *git2-1 int::LEU2 mei2::mei2-lacZ his1-102 leu1-32*	
ED988	h^- *wis1 int::nmt-wis1*$^+$ *-ura4*$^+$ *git2-1 int::LEU2 ura4::fbp1-lacZ leu1-32*	
ED1010	h^+ *wis1::his1*$^+$ *his1-102 leu1-32*	
ED1049	h^- *leu1-32*	
ED1053	h^- *wis1::LEU2 pat1-114 leu1-32*	
ED1065	h^+ *pka1-261 ura4::fbp1-lacZ*	
ED1077	h^+ *wis1::LEU2 pka1-261 ura4::fbp1-lacZ leu1-32*	
ED1091	h^- *pat1-114*	
ED1093	h^- *wis1 int::nmt-wis1*$^+$*-ura4*$^+$ *ura4::fbp1-lacZ*	
ED1177	h^+ *sty1-1 ura4::fbp1-lacZ leu1-32*	J. Millar
ED1185	h^+ *cgs1::ura4 ura4::fbp1-lacZ leu1-32*	
ED1248	h^+ *cgs1::ura4*$^+$ *wis1::LEU2 leu1-32 ura4-D18*	
FWP77	h^- *ura4::fbp1-lacZ leu1-32*	C. S. Hoffman
FWP139	h^- *pka1-261 ura4::fbp1-lacZ leu1-32 ade6-M216 his7-366*	C. S. Hoffman
FWP191	h^- *git2-1 int::LEU2 leu1-32 ura4::fbp1-lacZ ade6-M210*	C. S. Hoffman
Fy149	h^+ *his1-102 ade6-M210 leu1-32 ura4D-18*	R. Allshire
FYC71	h^{90} *mei2::mei2-lacZ ade6-M216 leu1-32*	M. McLeod
OP2	h^- *wis1 int::nmt-wis1*$^+$ *-ura4*$^+$ *ura4-294 leu1-32*	Warbrick and Fantes (1991)
SP259	h^{90} *pat1-114 ade6-M216*	M. McLeod

*Except where indicated, all strains were constructed in the course of this work.

Construction of the *wis1Δ pka1-261* double mutant

Double mutant *wis1Δ pka1-261* strains were constructed as follows. An *h*$^+$ *pka1-261 leu1-32* strain derived from FWP139 was crossed with ED905 (*h*$^-$ *wis1::LEU2 ura4::fbp1-lacZ leu1-32*) and the progeny analysed by tetrad analysis. Because double mutant progeny were indistinguishable phenotypically from single mutant *wis1Δ* progeny, putative *wis1Δ pka1-261* spores isolated from non-parental

itype tetrads were tested for their capacity to give *pka1* spores in heir progeny when crossed with a *wis1*Δ strain.

ell number, viability and length

ell number/ml of liquid culture was determined from 0.1 ml samples Isoton (Coulter Electronics). Following sonication, cells were ounted electronically with a Coulter Counter (Coulter Electronics).

Cell viability was determined by the capacity of cells to form olonies on supplemented YE at 32°C.

Cell length was determined on 25 live septated cells from cultures exponential growth (1-3×10^6 cells/ml), using a 100× phase contrast bjective.

-galactosidase assays

-galactosidase assays were performed essentially as described by Miller (1972), on samples of 0.1 ml or 1 ml of culture (~10^6-10^7 cells, epending on the strain and the nutritional conditions) after cell permeabilization with chloroform and sodium dodecyl sulphate. Specific -galactosidase activity was calculated as activity (expressed as $_{420nm}$)/hour per 10^7 cells.

ESULTS

vis1+ is required for nutritional induction of onjugation

Ve first reported the *wis1*+ gene as affecting progress through he G_2-mitosis transition in a nutritionally sensitive manner, nd as being required for maintenance of cell viability in tationary phase (Warbrick and Fantes, 1991). These phenoypes suggested a role for *wis1*+ in monitoring the environmental status of the cell. In the course of constructing strains arrying a *wis1* deletion (*wis1*Δ) allele we observed difficulty crossing such strains, and microscopic examination uggested a defect in conjugation. Since conjugation and neiosis in *S. pombe* are subject to nutritional control (Egel, 989) we carried out a quantitative analysis of conjugation in *is1*Δ strains.

S. pombe cells are able to undergo sexual differentiation as n alternative to progressing through the cell division cycle. This occurs in response to nutrient deprivation: media limiting or nitrogen are conventionally used for experimental urposes. Under these conditions, haploid cells of opposite nating type can conjugate to form a diploid zygote, which apidly undergoes meiosis and spore formation to give a nature ascus (Egel, 1989). We tested the ability of homothalic (h^{90}) haploid *wis1*Δ cells to conjugate following starvation or either nitrogen or carbon sources. The results (Fig. 1) show hat *wis1*Δ cells are able to conjugate after nitrogen limitation, lthough at a reduced level (20%) compared with *wis1*+ cells 70%). However, *wis1*Δ cells were completely unable to onjugate when carbon (glucose) availability was limited, hile *wis1*+ cells showed a similar response to either glucose r nitrogen limitation.

Suppression of *pat1-114* defect by loss of *wis1*+ unction

The *pat1*+ gene (also called *ran1*+) is required for negative egulation of sexual differentiation to maintain cells in the nitotic cycle. Partial or complete loss of *pat1*+ function in a emperature-sensitive *pat1* mutant at elevated temperatures nduces sexual development (Nurse, 1985; Iino and Yamamoto, 1985a,b). At 30°C, even when nutrients are not limiting, conjugation is stimulated in h^{90} *pat1-114* cells and vegetative growth is inhibited (Nielsen and Egel, 1990; Beach et al., 1985). Because *wis1*Δ cells are defective in conjugation, and because of phenotypic similarities between *wis1*Δ mutants and mutants defective in *cgs1* and *cgs2* (which were isolated as suppressors of *pat1-114*; DeVoti et al., 1991), we investigated whether loss of *wis1*+ function would suppress *pat1-114* mutant phenotypes.

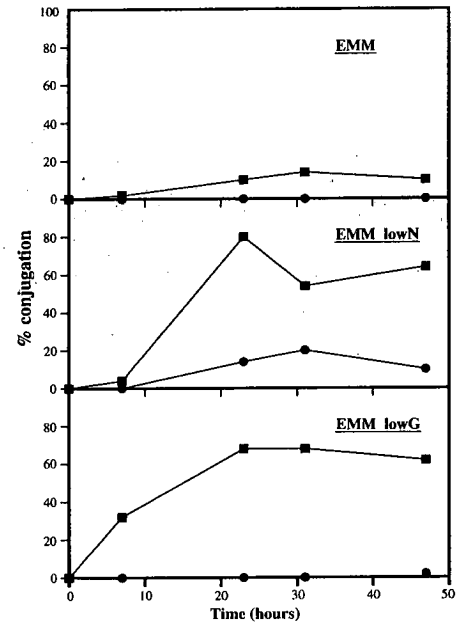

Fig. 1. Defective conjugation of *wis1*Δ cells. Homothallic (h^{90}) *wis1*+ (■) and *wis1::LEU2* (●) strains were grown exponentially at 25°C in minimal medium to a cell density of ~4×10^6 cells/ml. Cells were then washed and resuspended at the same cell density in either the same medium (EMM) or in minimal medium containing a limiting concentration of nitrogen source (EMM lowN) or glucose (EMM lowG) (see Materials and Methods). Each culture was incubated at 25°C and at the times indicated, samples were examined microscopically and scored for zygote formation. Each zygote was scored as two cells.

We first investigated the effect of *wis1*Δ on the growth defect of *pat1-114* cells at 34°C (Fig. 2A). The *pat1-114* strain did not grow under these conditions, while in contrast the *pat1-114 wis1*Δ double mutant was able to grow, indicating suppression of the *pat1* growth defect by loss of *wis1*+ function. We then examined whether loss of *wis1*+ function would also suppress the hyperconjugation defect of *pat1-114* at 30°C. Cultures of *pat1-114* h^{90} and *wis1*Δ *pat1-114* h^{90} cells were grown in minimal medium at 25°C and shifted to 30°C. Within 6 hours a third of the *wis1*+ cells had conjugated, and this increased to a final value of about 55% by 10 hours. In contrast, the *wis1*Δ *pat1-114* cells showed a greatly reduced frequency of conjugation, with a maximum level of around 5% (Fig. 2B), indicating that *wis1*Δ suppresses this aspect of *pat1-114* mutant phenotype also.

wis1 is required for expression of cAMP-regulated genes

The initiation of sexual differentiation in *S. pombe* following

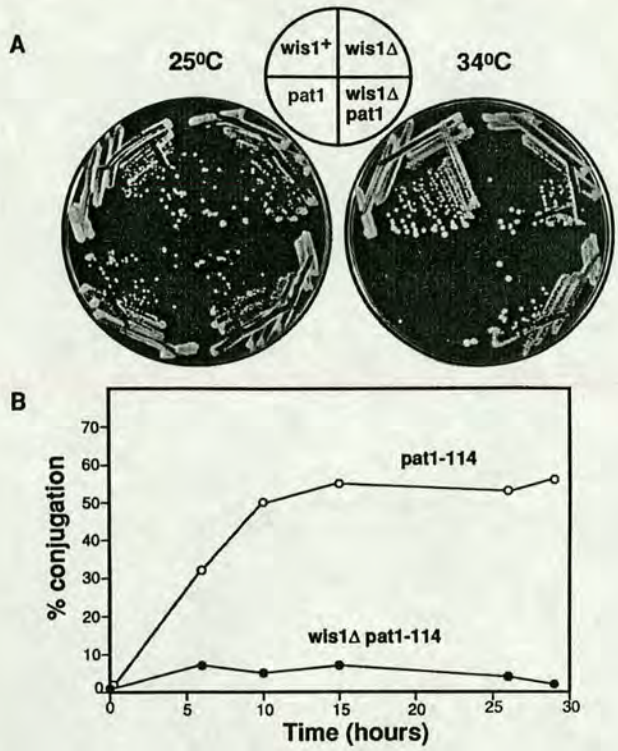

Fig. 2. Suppression of *pat1-114* growth defect and hyperconjugation phenotype by loss of *wis1* function. (A) Growth of ED812 (*wis1*$^+$), ED903 (*wis1*Δ), ED1091 (*pat1-114*) and ED1053 (*wis1*Δ *pat1-114*) strains at 25°C and 34°C on minimal medium. (B) Homothallic *pat1* (h^{90} *pat1-114*) and *wis1*Δ *pat1* (h^{90} *pat1-114 wis1::LEU2*) strains were grown in minimal medium at the permissive temperature (25°C) to ~5×10^5 cells/ml. Then half of each culture was shifted to 30°C. For each time point, the percentage of cells that had conjugated was determined by scoring 200 (cells + zygotes) under the microscope. No conjugation was observed in the control cultures remaining at 25°C.

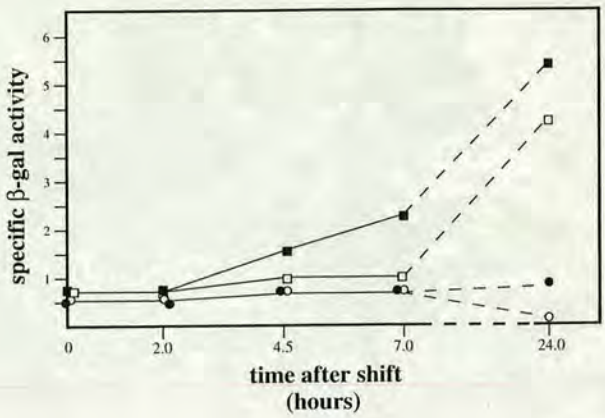

Fig. 3. Defective induction of the *mei2-lacZ* gene in response to nitrogen starvation in *wis1*Δ cells. ED895 (*wis1*$^+$) (□,■) and ED898 (*wis1*Δ) (○,●) cells growing exponentially in minimal medium at 30°C were harvested, washed and resuspended at a cell density of ~2×10^6 cells/ml in either the same medium (+ NH_4Cl (□,○); repressing conditions) or in the same medium but without the nitrogen source (–NH_4Cl (■,●); derepressing conditions). Cells were incubated at 30°C and at the time points indicated cell number and β-galactosidase activities were measured. Cell viability was also determined 7 and 24 hours after transfer. Specific β-galactosidase activities are expressed as A_{420nm}/hour per 10^7 cells. It should be noted that under nitrogen starvation, cell division continues for many hours even though biomass accumulation stops almost immediately (Fantes, 1984). Therefore the increase in specific β-galactosidase activity as presented here underestimates the accumulation of β-galactosidase in the culture as a whole. The specific β-galactosidase activities at the time of shift were: *wis1*$^+$, 0.75; *wis1*Δ, 0.55. The specific activities shown are based on total cells: precise interpretation of the 24 hour time points is complicated by the loss of viability of *wis1*Δ cells at this time.

nitrogen starvation has been shown to be associated with a reduction in cAMP level (Maeda et al., 1990; Mochizuki and Yamamoto, 1992). This leads to a decrease in cAMP-dependent protein kinase (PKA) activity and ultimately to the induction of mating-specific genes (Sugimoto et al., 1991). The conjugation defect of *wis1*Δ strains led us to investigate the relationship between *wis1*$^+$ and the cAMP signalling pathway of *S. pombe*. For this purpose we first investigated the regulation in *wis1*Δ cells of the *mei2*$^+$ gene, which encodes an essential activator of meiosis and is required for premeiotic DNA replication (Shimoda et al., 1985; Watanabe and Yamamoto, 1994). Expression of *mei2*$^+$ is normally induced in response to nitrogen starvation, the usual experimental stimulus for initiating sexual development. Expression of *mei2*$^+$ is negatively regulated at the transcriptional level by the cAMP-PKA pathway. In exponential growth, the high cAMP level represses expression of *mei2*$^+$, but following nitrogen starvation the cAMP level falls and allows *mei2*$^+$ induction (DeVoti et al., 1991; Watanabe et al., 1988). We investigated the regulation of *mei2*$^+$ expression in *wis1*Δ cells, using an integrated *mei2-lacZ* reporter construct in which the *E. coli lacZ* gene is under the control of the *mei2* promoter (Wu and McLeod, 1995). *mei2*$^+$ expression was monitored by assaying β-galactosidase activity in *wis1*$^+$ and *wis1*Δ cells after transfer from minimal medium containing ammonium to nitrogen-free medium. Fig. 3 shows that the specific β-galactosidase activity of the wild-type cells increased, after a lag, by threefold during the first 7 hours of nitrogen starvation, and had increased eightfold at 24 hours. In contrast, in the nitrogen-starved *wis1*Δ culture, no induction of β-galactosidase was observed during the first 7 hours, with little or no induction by 24 hours (see legend to Fig. 3). The *wis1*$^+$ cells grown in the presence of ammonium showed a low level of β-galactosidase activity during exponential growth, which increased by sixfold at 24 hours, by which time growth and cell division had ceased. In contrast, the *wis1*Δ cells showed no increase in activity throughout. These observations show that induction of *mei2*$^+$ in response to nitrogen starvation requires *wis1*$^+$ function. Nitrogen starvation acts at least in part by reducing the intracellular level of cAMP, suggesting a connection between the cAMP signalling pathway and the wis1 pathway.

Another gene whose expression is under negative regulation by cAMP and PKA is the *fbp1*$^+$ gene, which encodes fructose-1,6-bis-phosphatase, an enzyme of the gluconeogenic pathway essential for utilisation of glycerol as sole carbon source (Hoffman and Winston, 1990). A high level of glucose in the medium leads to high intracellular cAMP levels, which repress *fbp1*$^+$ expression, whereas low glucose concentration or entry into stationary phase lead to *fbp1*$^+$ induction (Hoffman and

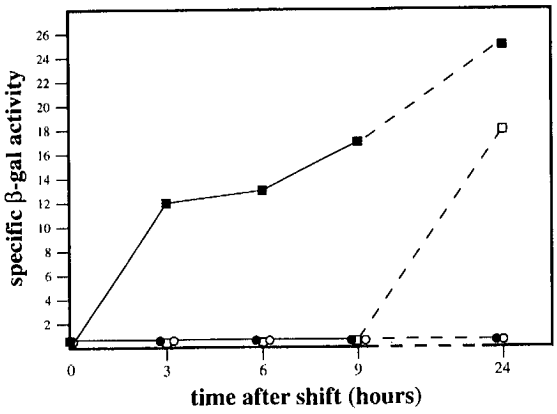

Fig. 4. Defective induction of the *fbp1-lacZ* gene in *wis1*Δ cells in response to glucose limitation. ED900 (*wis1*$^+$) (□,■)and ED905 (*wis1*Δ) (○,●) cells growing exponentially at 30°C in supplemented minimal medium (containing 2% glucose) were washed and resuspended at ~2×10^6 cells/ml in either the same medium (□,○; repressing conditions) or an otherwise identical medium containing only 0.1% glucose (■,●; derepressing conditions). Cells were incubated at 30°C and at the time points indicated, samples of each culture were removed and cell densities and β-galactosidase activities were determined (see Materials and Methods). Specific β-galactosidase activities are expressed as A_{420nm}/hour per 10^7 cells. The specific β-galactosidase activities at the time of shift were: *wis1*$^+$, 0.15; *wis1*Δ, 0.08. The specific activities shown are based on total cells: precise interpretation of the 24 hour time points is complicated by the loss of viability of *wis1*Δ cells at this time.

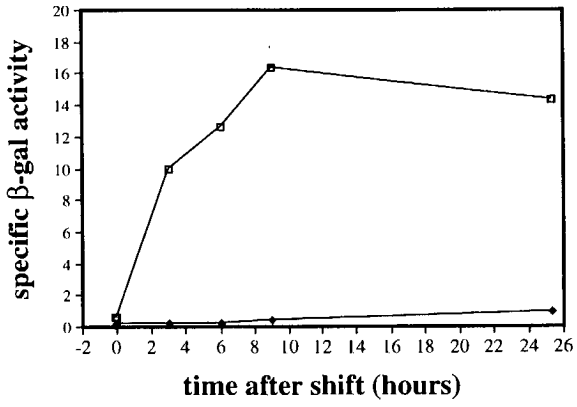

Fig. 5. Defective induction of *fbp1-lacZ* gene in *sty1-1* cells in response to glucose limitation. ED900 (*sty1*$^+$) (□) and ED1177 (*sty1-1*) (◆) cells were grown and starved for glucose essentially as described for Fig. 4: cell densities at the time of transfer were 4×10^6 for ED900 and 8×10^5 for ED1177; β-galactosidase activities were assayed as before. Specific β-galactosidase activities are expressed as A_{420nm}/hour per 10^7 cells. The specific β-galactosidase activities at the time of shift were: *sty1*$^+$, 0.58; *sty1-1*, 0.24.

Winston, 1989). In order to investigate *fbp1*$^+$ regulation in *wis1*Δ cells, we used a chromosomally integrated *fbp1-lacZ* reporter gene (Hoffman and Winston, 1990). *wis1*$^+$ and *wis1*Δ cells growing exponentially in supplemented minimal medium containing 2% glucose (repressing conditions) were transferred to the same medium but containing only 0.1% glucose (derepressing conditions). Fig. 4 shows that in wild-type cells *fbp1*$^+$ expression is strongly and rapidly derepressed in response to glucose limitation, with the specific β-galactosidase activity increasing 80-fold during the first 3 hours, and then a further twofold by 24 hours. In contrast *wis1*Δ cells showed essentially no increase in β-galactosidase activity throughout the first 9 hours, with only a fourfold increase by 24 hours.

As expected, the *wis1*$^+$ cells growing under repressing conditions (2% glucose) showed a low level of β-galactosidase activity during exponential growth. However by 24 hours, as the cells entered stationary phase, the activity had increased 100-fold, presumably because of glucose deprivation (glucose is the primary limiting nutrient in this medium). In contrast, no *fbp1-lacZ* derepression was observed in *wis1*Δ cells on entry into stationary phase (Fig. 4).

The *sty1*$^+$ gene has been shown to encode a MAP kinase homologue, which is a substrate of the wis1 protein kinase (Millar et al., 1995; Shiozaki and Russell, 1995b). *sty1* mutants show a very similar set of phenotypes to *wis1*Δ mutants, and we therefore tested the induction of *fbp1*$^+$ in a *sty1-1* mutant strain. As shown in Fig. 5, the effect of *sty1-1* is very similar to that of *wis1*Δ in that essentially no β-galactosidase induction takes place after glucose limitation.

These results show that the *wis1*$^+$/*sty1*$^+$ pathway is required for *fbp1* derepression in response to glucose limitation. Wild-type cells express *fbp1*$^+$ during growth on glycerol (Hoffman and Winston, 1990). Consistent with their inability to derepress *fbp1*$^+$ expression under normally derepressing conditions, *wis1*Δ and *sty1-1* mutants are unable to utilise glycerol as sole carbon source (data not shown).

wis1 is epistatic over mutants defective in the cAMP-PKA pathway

The experiments described above show that *wis1*$^+$ is required for the transcriptional induction of *fbp1* and *mei2* in response to nutrient deprivation, which is mediated by a low cAMP signal via a reduction in PKA activity (Byrne and Hoffman, 1993; Hoffman and Winston, 1991; Watanabe et al., 1988). This suggested a connection between the wis1 pathway and the cAMP-PKA signalling pathway. We therefore tested the relationships between *wis1* and genes encoding known elements of the cAMP pathway by genetic epistasis analysis. Mutants defective in the genes coding for adenylate cyclase (*git2/cyr1*), which converts ATP into cAMP, or the catalytic subunit of PKA (*pka1/git6*) have little or no PKA activity in vivo, and show phenotypes opposite in sense to those shown by *wis1*Δ mutants. Specifically, loss-of function mutants in these genes divide at reduced cell length (Dal Santo et al., 1996), and are derepressed for sexual development and for *fbp1* and *mei2* expression (Maeda et al., 1990, 1994; Kawamukai et al., 1991; also see below). Conversely, *wis1*Δ cells are elongated, and defective in conjugation and in *fbp1* and *mei2* derepression, similar to the effects of overexpressing *cyr1*$^+$/*git2*$^+$ or *pka1*$^+$ (Kawamukai et al., 1991; Maeda et al., 1994). We therefore constructed a series of double mutant strains containing *wis1*Δ and a mutation in either the *git2* or *pka1* gene, and performed epistasis tests by assaying the expression of β-galactosidase from the *fbp1* and *mei2* reporter genes.

We first investigated the regulation of *mei2* expression in a *wis1*Δ *git2*Δ double mutant. Table 2 shows that *wis1*Δ cells showed little or no increase in *mei2* expression during the transition from exponential to stationary phase of growth, in

Table 2. *mei2-lacZ* gene expression in a *wis1*Δ *git2*Δ double mutant

Strain	Genotype		β-galactosidase activity	
	wis1	*git2*	Exponential	*Stationary
ED895	+	+	0.7	3.8
ED898	Δ	+	0.7	0.9
ED983	+	Δ	5.0	15.9
ED985	Δ	Δ	0.8	1.0

Cells were grown at 30°C in supplemented YE medium (repressing conditions) and samples were removed to estimate the specific β-galactosidase activities (expressed as A_{420nm}/hour per 10^7 total cells). The values given for each strain during exponential growth phase are average values from several time points.
*Calculated on the basis of total cells/ml: see legend to Fig. 3.

Table 3. *fbp1-lacZ* gene expression in a *wis1*Δ *git2*Δ double mutant

Strain	Genotype		β-galactosidase activity	
	wis1	*git2*	Exponential	*Stationary
ED900	+	+	0.2	19
ED961	Δ	+	0.05	0.5
ED957	+	Δ	30	65
ED965	Δ	Δ	0.08	0.7

For experimental details, see legend to Table 2.
*Calculated on the basis of total cells/ml: see legend to Fig. 4.

contrast to the *wis1*$^+$ control where a greater than fivefold induction was observed, similar to the results in Fig. 3. The *git2*Δ strain showed a high level of *mei2* expression during exponential growth, consistent with the reported alleviation of nutritional repression (DeVoti et al., 1991); *mei2* expression increased further in stationary phase. The double *wis1*Δ *git2*Δ mutant behaved as the *wis1*Δ single mutant, showing the normal level of *mei2* expression during exponential growth, and failing to derepress in stationary phase, indicating that *wis1*Δ is epistatic over *git2*Δ for regulation of *mei2* expression.

The regulation of *fbp1* in a *wis1*Δ *git2*Δ double mutant was investigated: Table 3 shows that, again, the double mutant behaved as the single *wis1*Δ mutant parent in showing a slightly reduced level of *fbp1* expression during exponential growth. The *git2*Δ mutant showed greatly increased *fbp1* expression in exponential phase, as previously reported (Hoffman and Winston, 1990, 1991). A parallel set of experiments was carried out using the *pka1-261* mutation, which is defective in pka1 activity (Jin et al., 1995; Dal Santo et al., 1996). The effect of the *pka1-261* mutation was very similar to that of *git2*Δ in showing elevated *fbp1* expression during

Table 4. *fbp1-lacZ* gene expression in a *wis1*Δ *pka1* double mutant

Strain	Genotype		β-galactosidase activity	
	wis1	*pka1*	Exponential	*Stationary
ED900	+	+	0.12	9.0
ED906	Δ	+	0.08	0.30
ED1065	+	−	77	95
ED1077	Δ	−	0.15	1.8

For experimental details, see legend for Table 2.
*Calculated on the basis of total cells/ml: see legend to Fig. 4.

Table 5. *fbp1-lacZ* gene expression in *wis1*$^+$ overexpressing strains

Strain	Genotype		β-galactosidase activity	
	wis1	*git2*	Exponential	*Stationary
ED900	+	+	0.3	23
ED1093	OP	+	1.5	45
ED957	+	Δ	25	43
ED988	OP	Δ	40	55

Cells were grown at 32°C in minimal medium rather than supplemented YE medium in order to allow full derepression of *wis1*$^+$ expression from the thiamine repressible *nmt1* promoter (OP). Specific β-galactosidase activities are expressed in A_{420nm}/hour per 10^7 cells. The values given for each strain during exponential growth phase are average values from several time points.
*Calculated on the basis of total cells/ml: see legend to Fig. 4.

exponential growth (Table 4). Wild-type cells showed a large (~100-fold) increase in *fbp1* expression during the transition into stationary phase, due at least in part to depletion of glucose in the medium. The *git2* and *pka1* single mutants showed a modest increase from an already very high level. *fbp1* expression in the the *wis1*Δ single and double mutants increased by between four- and tenfold in stationary phase. Although *fbp1* was expressed at very low levels in *wis1*Δ and *sty1* mutants during exponential growth, we consistently observed an increase of up to ninefold in stationary phase cells (Tables 3 and 4; Fig. 5). This suggests the existence of a regulatory system for *fbp1* that acts independently of the wis1-sty1 pathway.

In summary, for all the phenotypes that we examined, i.e. cell length, starvation-induced sexual differentiation and expression of *mei2* and *fbp1*, the double *wis1*Δ *git2*Δ and *wis1*Δ *pka1* mutants resembled *wis1*Δ cells and differed from *git2*Δ and *pka1* cells. In addition, the double mutants were unable to utilise glycerol as sole carbon source, like *wis1*Δ. Thus loss of *wis1*$^+$ function is epistatic over several effects of a defective cAMP signalling pathway.

A modest increase in *wis1*$^+$ expression causes a reduction in cell length, indicating that wis1 has a rate-limiting role in regulating entry into mitosis (Warbrick and Fantes, 1991). We therefore investigated whether wis1 abundance might also be limiting for *fbp1* expression by comparing β-galactosidase activities in *git2*$^+$ and *git2*Δ cells overexpressing *wis1*$^+$. Table 5 shows that under normally repressing conditions, increasing *wis1*$^+$ expression led to fivefold-increased *fbp1-lacZ* expression in the *git2*$^+$ control strain, indicating that the level of wis1 protein is indeed limiting under normal conditions.

The opposite effects of loss of function of the cAMP-PKA and wis1 pathways, and the epistasis of *wis1* over *git2* and *pka1* mutations, suggested that either the PKA pathway negatively regulated the wis1 pathway, or that the two pathways exerted opposite effects on a common downstream target or targets (see Discussion). To try to resolve this question, we tested the effect of simultaneously activating the PKA pathway and eliminating wis1 function, in two ways. First, we constructed double mutant strains lacking both wis1 function and the regulatory subunit of PKA, cgs1 (loss of cgs1 function leads to increased activity of pka1; DeVoti et al., 1991). We determined the length of dividing cells of this strain, and of the parent single mutants (Table 6). *cgs1*Δ cells showed a small but repeatable increase in cell length compared with their *cgs1*$^+$

Table 6. Cell length at division of *wis1Δ* and *cgs1Δ* strains

Strain	Genotype *wis1*	Genotype *cgs1*	Experiment 1 −cAMP/ caffeine	Experiment 1 +cAMP/ caffeine	Experiment 2 −cAMP/ caffeine
ED1049	+	+	13.4±0.5	15.2±0.5	13.5±0.8
ED1010	Δ	+	21.1±1.0	24.6±1.8	
ED905	Δ	+			23.7±1.4
ED1185	+	Δ	15.6±0.5	ND	15.5±0.8
ED1248	Δ	Δ	23.6±1.5	ND	26.2±2.2

Cells were grown at 29°C in minimal medium to 1.5-3×10^6 cells/ml. The lengths of 25 septated cells were determined; mean ± standard deviation is shown for each condition. Where indicated, cAMP (10 mM) and caffeine (5 mM) were included in the growth medium.

counterparts, in both *wis1*$^+$ and *wis1*Δ genetic backgrounds. Second, we investigated the effect of adding cAMP and caffeine to the growth medium of *wis1*$^+$ and *wis1*Δ strains, a procedure which has been shown to increase pka1 activity (Watanabe et al., 1988). Again, a modest increase in cell division length was observed when pka1 was activated.

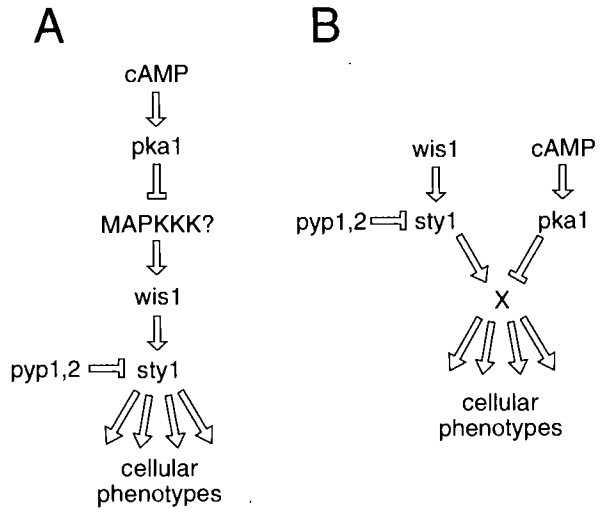

Fig. 6. Models for the mode of action of the wis1 and pka1 pathways in *S. pombe*. See text for details.

DISCUSSION

We reported previously that the MAPKK homologue wis1 regulates the timing of the G_2-M transition and is required for maintenance of cell viability in stationary phase (Warbrick and Fantes, 1991). In this paper we show that wis1 has other cellular roles: loss of *wis1*$^+$ impairs conjugation and suppresses *pat1-114* hyperconjugation phenotype. In addition, *wis1*$^+$ is required for transcriptional induction of reporter genes driven by the *mei2*$^+$ or *fbp1*$^+$ promoters. *wis1*Δ cells are unable to induce either gene, and are unable to grow on glycerol as carbon source. Recently a MAPK substrate of wis1, known variously as sty1, phh1 or spc1, has been isolated (Millar et al., 1995; Shiozaki and Russell, 1995b). We show here that, consistent with this, loss of *sty1*$^+$ function abolishes *fbp1*$^+$ inducibility by glucose deprivation, and the ability to grow on glycerol. Other phenotypic similarities are described elsewhere (Millar et al., 1995; Shiozaki and Russell, 1995b; Kato et al., 1996).

The expression of both *mei2*$^+$ and *fbp1*$^+$ is repressed by the cAMP-PKA pathway (DeVoti et al., 1991; Watanabe et al., 1988; Hoffman and Winston, 1991), and most of the phenotypic effects of wis1 described above can be explained on the basis of an interaction between the cAMP-PKA signalling pathway and the wis1 pathway. This is most directly shown by the opposite effects of mutants in the wis1 and PKA pathways on regulation of *mei2* and *fbp1*. *wis1* mutants fail to activate transcription of these genes, whereas in mutants defective in adenylate cyclase (*git2*) or cAMP-dependent protein kinase (*pka1*) they are constitutively expressed. However, the apparent relationship between the wis1 and cAMP-PKA pathways extends beyond this. cAMP is a repressor of sexual differentiation in *S. pombe*: loss of *pka1*$^+$ or *git2*$^+$/*cyr1*$^+$ function allows initiation of sexual differentiation under nutritional conditions that prevent this in wild-type cells (Kawamukai et al., 1991; Maeda et al., 1990). Conversely, overexpression of *pka1*$^+$ or *git2*$^+$/*cyr1*$^+$ leads to sterility (Kawamukai et al., 1991; Maeda et al., 1994). Also, cAMP prevents hyperconjugation induced by a temperature-sensitive *pat1* mutation (Beach et al., 1985). We show here that loss of *wis1*$^+$ function impairs conjugation, and blocks *pat1*-induced hyperconjugation. Furthermore, mutants lacking either adenylate cyclase or PKA show reduced cell size (Jin et al., 1995; Dal Santo et al., 1996), similar to the effect of moderate overexpression of *wis1*$^+$ (Warbrick and Fantes, 1991); we show here that hyperactivation of PKA during exponential growth leads to a modest increase in cell length. We conclude that the wis1-sty1 and PKA pathways have a close functional relationship, acting in opposite senses on several distinct target systems.

In principle, the wis1 pathway could act either upstream or downstream of PKA, or the two pathways could act on a common downstream substrate in opposing senses. It seems unlikely that wis1 acts upstream of pka1 (or adenylate cyclase, encoded by *git2*$^+$) in a linear pathway, because double mutants lacking both wis1 and pka1 show phenotypes very similar to the *wis1*Δ parent, with respect to inducibility of *mei2* and *fbp1*, cell size and conjugation phenotypes. Two basic possibilities remain: wis1 acts downstream of pka1 and is (directly or indirectly) negatively regulated by it (Fig. 6A), or that the wis1 and PKA pathways converge on a common substrate or substrates (Fig. 6B). It is of course possible that the PKA pathway interacts with the wis1 pathway at more than one point.

Model A provides an economical explanation of the opposing effects of the wis1-sty1 and PKA pathways, and in particular the complete epistasis of *wis1*Δ mutations over *pka1* and *git2* mutations for cell length, conjugation and *fbp1* inducibility phenotypes. Also, *cgs1* mutants, in which pka1 is more active, resemble *wis1* and *sty1* mutants in showing cell elongation and reduced ability to induce *fbp1* (DeVoti et al., 1991; Hoffman and Winston, 1991). An analogous situation to model A is found in mammalian cells, where PKA negatively regulates Raf-1, an activator of MEK (equivalent to MAPKK) (Hafner et al., 1994; Faure and Hafner, 1995). However, three sets of observations are inconsistent with the simplest form of this model. Firstly, both *wis1* and *cgs1* mutants show increased cell length at division, and double mutants show an additive

effect, although the effect of *cgs1* is modest (Table 6). This indicates that the PKA pathway acts on a component(s) that is (are) downstream of sty1; it does not formally exclude the possibility that PKA might also act on wis1 or further up the pathway (see below). Secondly, *cgs1* mutants, unlike *wis1* or *sty1* mutants, are neither heat- nor osmotically sensitive (S.P. and P.A.F., unpublished observations). This could be explained if there were a mechanism distinct from the PKA pathway that regulated wis1 activity in response to heat and osmotic stresses. Thirdly, we noted during the construction and testing of the *wis1 cgs1* double mutant strain that the double mutant was completely sterile, in contrast to the parent single mutants, which, although deficient in mating, were still able to cross. This suggests that the effects on mating efficiency of the PKA and wis1-sty2 pathways are independent.

An alternative possible relationship between the PKA and the wis1-sty1 signalling pathways is that the two act in parallel (model B), and converge on a common component (or components) which in turn regulate(s) various cellular activities. Dual regulation by the PKA and wis1-sty1 pathways of several different aspects of cellular behaviour might be mediated by convergence of the pathways on several different targets, each controlling a single cellular activity, although this seems intrinsically unlikely. Alternatively these might be controlled indirectly through a single regulatory molecule, which is under dual PKA and wis1-sty1 control (Fig. 6). Recently, two transcription factors related to the mammalian CREB/ATF family have been identified in *S. pombe*: atf1 (Takeda et al., 1995) and pcr1 (Watanabe and Yamamoto, 1996). Loss of function of either of these factors leads to phenotypes very similar to those of *wis1* and *sty1* mutants: partial sterility, inability to derepress genes under cAMP-PKA regulation, and loss of viability in stationary phase of growth. This suggests that either atf1 or pcr1 or both may be targets of the wis1-sty1 pathway, perhaps requiring phosphorylation by sty1 for activity. For some phenotypes under the control of both the cAMP-PKA pathway and of atf1 and pcr1, the effects of mutations in the two pathways appear to be additive, indicating that inhibition of atf1/pcr1 activity by the PKA pathway acts independently of the activation of these transcription factors. Thus, if either atf1 or pcr1 is indeed activated by wis1-sty1, then at least one point of interaction between the PKA and wis1-sty1 pathways would be below the level of sty1. Regulation of this type is shown by the *CTT1* gene in *S. cerevisiae*, which is subject to independent regulation by cAMP-PKA and the Pbs2-Hog1 pathway (Schüller et al., 1994; Marchler et al., 1993).

One important role of the wis1 pathway is as a stress response system. Cells lacking wis1 or sty1 are very sensitive to conditions of high osmolarity or elevated temperature (Millar et al., 1995; Shiozaki and Russell, 1995a,b; S.S. and P.A.F., unpublished observations), and the activity of the pathway has been shown to respond to changes in external osmolarity (Millar et al., 1995; Shiozaki and Russell, 1995b; Kato et al., 1996). The relationship between the stress response role of wis1 and its connection with the cAMP signalling pathway is unclear. While a functional wis1 pathway is necessary for proper control of expression of cAMP-regulated genes, sexual differentiation and correct mitotic timing, it is not known whether signals for these events are actually transmitted through the pathway. It is possible that signalling is mediated through the cAMP-PKA pathway, which exerts its effect by interacting with the wis1-sty1 pathway (model B, Fig. 6). However, the observations that moderately increasing the level of *wis1*$^+$ expression affects both entry into mitosis (Warbrick and Fantes, 1991) and the level of expression of *fbp1* (Table 5) suggests that wis1 does indeed have a regulatory role. Strong overexpression of *wis1*$^+$ causes cell swelling and lysis (our unpublished observations; Millar et al., 1995), and loss of sty1 function blocks this lethality (Shiozaki and Russell, 1995b), indicating that the effect is mediated through sty1. However overexpression of *sty1*$^+$ is not lethal, suggesting that the phosphorylation and activation of sty1 by wis1, rather than sty1 abundance, limits the activity of the pathway.

Dal Santo et al. (1996) show that increased activity of either of two protein tyrosine phosphatases, pyp1 and pyp2, represses *fbp1*$^+$ expression and has other effects similar to loss of wis1 or sty1 function. Together with the recent demonstration (Millar et al., 1995; Shiozaki and Russell, 1995b) that these phosphatases act on sty1 and inactivate it, these observations provide strong support for an important role of the wis1 pathway in nutritional modulation of cellular activity. Interestingly, pyp2 contains two consensus PKA phosphorylation sites, suggesting that part of the interaction between the wis1 and PKA pathways might be mediated at the sty1 level by cAMP-regulated dephosphorylation. Other regulatory mechanisms must operate, however, since deletion of *pyp2* has no effect on *fbp1* regulation (Dal Santo et al., 1996).

In this paper we address one aspect of wis1-sty1 function, the relationship with cAMP-regulated gene expression. It is not clear at present whether or how this group of effects is related to other cellular roles, such as response to osmotic or temperature stress, maintenance of cell viability in stationary phase and the traverse of major cell cycle transitions, although cross-connections between these aspects of cellular behaviour have been reported in budding yeast (Werner-Washburne et al., 1993; Tokiwa et al., 1994).

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