Control of mating in Schizosaccharomyces pombe

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ABSTRACT

Activation of key transcriptional regulators often results in profound changes in the cellular gene expression profile, committing cells to a differentiation pathway. The \textit{Schizosaccharomyces pombe} Ste11 protein is a crucial regulator of the mating and meiotic program. Several genes required for mating-type specific gene expression, conjugation, and meiosisis are transcriptionally activated after nitrogen starvation in a Ste11-dependent manner.

The aim of this thesis was to extend our understanding of how the HMG-box containing Ste11 is regulated at the protein level, and to identify and characterise novel Ste11-target genes involved in the mating pathway. Using a one-hybrid assay, I have shown that Ste11 protein is a strong transcriptional activator, and was able to map its transcriptional activation domain to the C-terminal region. Interestingly, induction of Ste11 target genes upon nitrogen starvation could neither be attributed to regulation of the transcriptional activity of this domain, nor to changes in Ste11 subcellular localisation.

Knowing the DNA sequence necessary for Ste11 binding and for starvation-mediated transcriptional induction, I searched the \textit{S. pombe} genome database for putative Ste11 target genes. One of the genes found was \textit{rgs1}, a member of the Regulator of G-protein Signalling (RGS) family. \textit{rgs1} expression requires both a Ste11-mediated starvation signal and the pheromone-induced activation of the Byr2/Byr1/Spk1 MAPK pathway. Disruption of the \textit{rgs1} gene results in sensitivity to pheromone and in a mating defect. Rgs1 localises to the nucleus and cytoplasm, which is not altered during pheromone treatment. Importantly, Rgs1 function requires its C-terminal RGS domain, as well as a central DEP domain and a novel N-terminal homology domain. In summary, Rgs1 negatively regulates pheromone signalling during mating, acting in a negative feedback loop that is essential for the mating process.

Taken together, these results extend our understanding of the mechanisms regulating the mating pathway in the fission yeast.
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Finally, a very special thanks to my wife Andrea, for being the most important person in my life, and for constantly loving and supporting me without ever asking for anything in return, but for an occasional foot massage.

Dedication
This Thesis is dedicated to all the people that, either through donations or voluntary work, make research at the Imperial Cancer Research Fund a reality.
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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>cAMP</td>
<td>Adenosine 3',5'-cyclic monophosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>2'-Deoxycytidine 5'-triphosphate</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulphoxide</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylene diamine tetra acetic acid</td>
</tr>
<tr>
<td>EMM</td>
<td>Edinburgh minimal medium</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FACS</td>
<td>Fluorescence activated cell sorter</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine 5'-triphosphate</td>
</tr>
<tr>
<td>GTPase</td>
<td>GTP phosphohydrolases</td>
</tr>
<tr>
<td>G protein</td>
<td>Guanine nucleotide-binding protein</td>
</tr>
<tr>
<td>HPR</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pairs</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>ONPG</td>
<td>o-Nitrophenyl-β-D-galactopyranoside</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecy Sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>TRIS</td>
<td>TRIS(hydroximethyl)-aminomethane</td>
</tr>
<tr>
<td>YE5S</td>
<td>Yeast extract with supplements medium</td>
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</table>
CHAPTER 1

INTRODUCTION
1) INTRODUCTION

1.1. Yeast mating pathways

Yeasts can be defined as unicellular fungi without fruiting bodies, and can propagate either by budding (like *Saccharomyces*) or by fission (like *Schizosaccharomyces*). The fission yeast *Schizosaccharomyces pombe* (*S. pombe*) is an important eukaryotic unicellular model organism, and about 50 years of basic research has contributed to the elucidation of several biological mechanisms from general genetics to molecular cell biology (Egel 2000). The importance of *S. pombe* as a model organism has gained considerably from the understanding that many cell biology mechanisms are conserved between unicellular yeast and metazoan organisms.

The two widely studied *Saccharomyces cerevisiae* and *S. pombe* yeasts normally reproduce by closed-mitotic cell division but have also evolved alternative sexual differentiation pathways, where haploid cells of opposite mating types fuse to form a diploid. This mating process involves cell-cell communication via the reciprocal exchange of small diffusible peptides (matting pheromones), which allow cells to locate a prospective partner and to undergo cell changes necessary for mating. The systematic identification and analysis of the genes involved in establishing, sensing and transducing the pheromone signal in both yeasts has established a paradigm for eukaryotic signal transduction.

Herein this Introduction, I would like to review the current understanding of the *S. pombe* sexual differentiation pathway. Comparisons between fission yeast and budding yeast pathways will be presented at specific points, either highlighting conservation or discussing reasons for divergence on physiological and molecular aspects. This comparison will take into account the prediction that the two yeasts ancestors have separated about 330 to 420 million years ago (Sipiczki 2000).
1.2. *S. pombe* life cycle

Under rich nutritional conditions, *S. pombe* propagates exclusively by mitotic cell division as a haploid organism. The fission yeast mitotic cycle has a typical eukaryotic organization having discrete G1, S, G2, and M phases (reviewed by Forsburg and Nurse 1991). Under standard laboratory conditions cells complete a mitotic cell cycle every 2-3 hours. Haploid cells of fission yeast do not display any mating activity before the end of the vegetative phase when nutrients become depleted. The overall organisation of the *S. pombe* life cycle is shown in Figure 1-1. Early studies by Urs Leupold (Leupold 1950) discriminated between self-fertile (homothallic) parental strains and heterothallic derivative strains displaying one of two possible mating-types. Homothallic cells (*h^o*) change their mating-type during mitotic proliferation, while heterothallic cells have fixed Plus (*P* or *h^o*) or Minus (*M* or *h*) mating-type. When haploid cells of opposite mating types, *h^o* and *h*, are starved of nutrients, especially nitrogen, they arrest in G1 and induce genes required for sexual development including pheromones and their receptors (reviewed in Yamamoto et al. 1997; Davey 1998). Pheromone signalling is established, leading to sexual agglutination and conjugation between two mating partners, involving cytoplasmic fusion, and nuclear fusion (karyogamy). The diploid zygote then undergoes meiosis generating four haploid spores. When refed with nutrients, spores germinate and resume mitotic proliferation. Although diploid cells can be grown as diploids if transferred to rich medium, they readily enter meiosis when the nutritional quality of the media is reduced. When heterothallic cells are starved of nitrogen in the absence of sexual partners, they arrest in an uncommitted G1 state. If starvation is prolonged these cells enter a dormant G0 state displaying great resistance to heat shock while undergoing a profound subcellular reorganisation (Su et al. 1996).
1.3. Mating-type genes

The *S. pombe* mating type region consists of three closely-linked cassettes, mat1, mat2-P, and mat3-M. The mating type is determined by the DNA sequence present at the mat1 locus. The transcriptionally silent loci mat2-P and mat3-M serve as donors for copy transposition to the mat1 locus during mating type switching (reviewed in Klar and Bonaduce 1993). Cells that carry the mat1-P cassette are h+ (or P) mating-type, while cells that carry the mat1-M cassette are h- (or M) mating type. The mat1-P segment contains the mat1-Pc and mat1-Pm (Pi) genes, and the mat1-M contains mat1-Mc and mat1-MMm (Mi) (Kelly et al. 1988). The mat1-Pm and mat1-MMm genes are only required for meiosis, but both mat1-Pc and mat1-Mc genes are required for mating and meiosis (Kelly et al. 1988). Furthermore the mat1-Pc and mat1-Mc genes are necessary and sufficient to confer, respectively, h+ or h- mating-type phenotype. Both genes are expressed at basal levels during proliferation in rich medium and induced by nutritional starvation (Kelly et al. 1988). All the mentioned gene functions required for mating and meiosis are summarized in Table 1-1 and 1-2. The mat1-Mc gene encodes a transcription factor containing a High Mobility Group (HMG)-box, while the mat1-Pc encodes a protein with 118 aminoacids, which is likely to be a transcription factor but has no homology to known proteins (Kelly et al. 1988; Willer et al. 1995; Nielsen et al. 1996; Kjaerulff et al. 1997). The mat1-Pm gene encodes a homeobox protein and it has been shown to be a direct transcriptional activator of mei3, a meiotic-inducer gene (Van Heeckeren et al. 1998).

1.4. Nutritional regulation of sexual differentiation

An early insight into the regulation of fission yeast sexual differentiation pathway was the observation that addition of cAMP to the medium inhibited mating and meiosis (Calleja et al. 1980). This observation was shown to be physiologically relevant as cellular cAMP levels decrease by about 50% after nitrogen starvation or upon entry into stationary phase (Maeda et al. 1990; Mochizuki and Yamamoto 1992). These initial observations led to experiments
which defined the cAMP-dependent signal transduction pathway involved in nutrient-stimulated repression of sexual differentiation, which will be described in detail below.

1.4.1. Gpa2 and Git3, a nutrient-sensing heterotrimeric G-protein and its coupled receptor

The mechanisms used by *S. pombe* to monitor the nutritional quality of the extracellular medium have yet to be understood. However, both nitrogen and glucose levels are sensed via the same signal transduction pathway. Two independent approaches contributed to the characterisation of this pathway: the isolation of a number of *git* (glucose-insensitive transcription) genes required for glucose repression of *fbpl* (fructose-1,6-bisphosphatase) transcription (Hoffman and Winston 1990) that turned out to be identical to cAMP-related genes necessary to block mating and meiosis in response to nitrogen (Hoffman and Winston 1991; Isshiki et al. 1992; Nocero et al. 1994).

The picture emerging is that nutrients activate Git3, a seven transmembrane G-protein coupled receptor, by as yet an unclear mechanism. Δ*git3* cells are unable to sense glucose, have a germination delay, and display starvation-independent conjugation and meiosis (Welton and Hoffman 2000). Epistasis analysis reveals that Git3 appears to activate Gpa2 (Git8), a heterotrimeric G-protein α subunit. Firstly, the phenotype resulting from deleting or mutating *gpa2* (git8) is identical to the *git3* deletion (Isshiki et al. 1992; Nocero et al. 1994). Secondly, a loss-of function mutation in *git3* is suppressed by *gpa2*<sup>+</sup> in high copy number (Nocero et al. 1994). Git5 is a β-subunit G-protein also required for the transmission of the nutrient signal (Landry et al. 2000). Multicopy *gpa2* partially suppresses the loss of *git5*, while the converse is not true (Landry et al. 2000). Furthermore *gpa2<sup>R176H</sup>*<sup>+</sup>, an activated allele of *gpa2* whose product is defective in GTPase activity, fully suppresses Δ*git5* (Welton and Hoffman 2000). So Git5 appears not to be required for direct transmission of the stimulus but rather to contribute to Gpa2 activation. Landry
et al. (2000) recently identified a 72 aminoacid protein, Git11, which is a putative Gγ-subunit for the glucose sensing heterotrimeric G-protein. To summarize, in fission yeast a conserved heterotrimeric G-protein coupled receptor pathway is responsible for nutrient-sensing signalling.

1.4.2. Activation of Protein kinase A (Pkal) via the Gpa2/Git5/Git11 heterotrimeric G-protein

Eukaryotic intracellular cAMP levels are regulated both at the level of synthesis by adenylate cyclase and degradation by cAMP-phosphodiesterase. Nutritional signals, through the activity of the Gpa2/Git5/Git11 G-protein, could potentially raise S. pombe cAMP levels by activating the single adenylate cyclase protein (Cyr1/Git2) (Yamawaki-Kataoka et al. 1989; Young et al. 1989; Maeda et al. 1990; Hoffman and Winston 1991; Kawamukai et al. 1991), or by inhibiting the phosphodiesterase Pde1/Cgs2 (DeVoti et al. 1991; Mochizuki and Yamamoto 1992). Disruption of pde1/cgs2 raises cAMP levels fourfold (Isshiki et al. 1992). Surprisingly, combining the constitutively active alleles, gpa2R176H or gpa2Q202L, that can only slightly raise cAMP levels on their own, with pde1 deletion results in a 20 times higher level of cAMP (Isshiki et al. 1992). These results suggest that Ga Gpa2 strongly activates Cyr1/Git2 adenylate cyclase activity. Interestingly, this coupling is conserved in mammalian cells, where adenylate cyclase enzymatic activity is also regulated by heterotrimeric guanine nucleotide-binding proteins (G proteins) (Gilman 1987). In contrast, the budding yeast S. cerevisiae adenylate cyclase activity is regulated by monomeric, guanine nucleotide-binding Ras proteins (Broek et al. 1985; Toda et al. 1985).

The fission yeast pka1 gene encoding the catalytic subunit of the cAMP-dependent protein kinase (protein kinase A) was isolated as a gene blocking mating and meiosis when overexpressed (Maeda et al. 1994). Deletion of pka1 resulted in a similar phenotype to the cyr1 disruption: derepression of sexual development during growth in rich medium. The fission yeast genome encodes two Pka1-related protein kinases, Sck1 and Sck2, that can suppress loss of pka1
gene when over-produced. Interestingly, sck1 or sck2 single deletions have no apparent phenotype, so presumably Pka1 is responsible for the bulk of cAMP-dependent protein kinase activity involved in blocking sexual differentiation (Jin et al. 1995; Soto et al. 1997; Fujita and Yamamoto 1998). Elevated Pka1 activity also inhibits entry into stationary phase, thermotolerance, and the uptake of gluconate as an alternative carbon source (DeVoti et al. 1991; Caspari 1997; Ribeiro et al. 1997).

1.4.3. Pka1-mediated inhibition of sexual differentiation

It has been proposed that Pka1 activity inhibits sexual differentiation by repressing the expression of ste11 (sterile 11) during mitotic proliferation under favourable nutritional conditions (Sugimoto et al. 1991). Ste11 is a crucial regulator of the mating and meiotic pathway; Δste11 cells are completely sterile and overexpression of ste11 drives cells growing in rich medium into mating and meiosis (Sugimoto et al. 1991). In wild-type cells ste11 is only induced upon starvation, while in Δpka1 or Δcyr1 cells it is constitutively expressed (Sugimoto et al. 1991; Watanabe and Yamamoto 1996). Regulation of ste11 transcription is a key regulatory stage of the mating and meiotic pathway, and is controlled by several signalling pathways (Fig. 1-2). In the following section, ste11 gene regulation will be discussed in a detailed way. Gene functions that regulate ste11 expression are listed in Table 1-2.

1.5. ste11, a key regulator of mating and meiosis

ste11 was independently identified by three different laboratories as a gene required for mating and meiosis, and also named steX or aff1 (Sipiczki 1988; Watanabe et al. 1988; Kitamura et al. 1990). Cloning and characterisation of the ste11 gene, revealed that it encodes a DNA-binding factor belonging to the HMG-box family, and that its function is required for transcriptional induction of several mating and meiotic genes (Sugimoto et al. 1991). Several Ste11-dependent genes are expressed in a mating-type specific manner, while
some can be further induced by pheromone signalling (Sugimoto et al. 1991). Due to this complexity, I will now discuss the regulation of \textit{ste11} transcriptional induction, but will only discuss regulation of Ste11 protein after introducing the pheromone signalling pathway.

1.6. Regulation of \textit{ste11} transcriptional induction

\textit{ste11} mRNA levels are present at very low levels during the mitotic cell division cycle but are strongly induced by nitrogen starvation (Sugimoto et al. 1991) or glucose starvation (Okazaki et al. 1998).

1.6.1. Rst2

As mentioned above, Pka1 activity senses a cAMP signal generated in response to nutrients in the medium and represses \textit{ste11} expression during mitotic proliferation. How does Pka1 kinase activity block \textit{ste11} expression? Pka1 is expected to directly modulate the activity of transcriptional activators or repressors through phosphorylation. One putative target is Rst2, a Cys$_2$His$_2$ zinc-finger transcription factor that is absolutely required for \textit{ste11} induction (Kunitomo et al. 2000). Rst2 protein specifically binds a sequence on the \textit{ste11} promoter that resembles a \textit{S. cerevisae} STRE (Stress response element) cis-element (Kunitomo et al. 2000). In budding yeast, the STRE-element is targeted by two Cys$_2$His$_2$ zinc-finger transcription factors, Msn2 and Msn4 (Martinez-Pastor et al. 1996; Schmitt and McEntee 1996). PKA activity inhibits Msn2 and Msn4 function, apparently by promoting cytoplasmic localisation of the two transcription factors (Gorner et al. 1998). In fission yeast, disruption of \textit{rst2} results in sterility and can also suppress hypermating and hypersporulation in the Pka1-null mutant. Kunitomo \textit{et al.} (2000) speculate that Rst2 is a substrate of Pka1 that is negatively regulated by phosphorylation, but biochemical evidence supporting this hypothesis is still missing.
1.6.2. **Atf1/Pcr1 and the stress-activated MAPK pathway**

Another regulator of *ste11* transcription is the heterodimeric complex formed by two basic leucine zipper (bZIP) transcription factors, Atf1 (also called Gad7) and Pcr1. The *atf1* gene was independently identified in three different ways: (1) as a fission yeast open reading frame (ORF) with homology to mammalian ATF/CRE transcription factors (Takeda et al. 1995), (2) as a sterile mutant that failed to arrest in G1 under nitrogen starvation (Kanoh et al. 1996), (3) and in a screening for high-copy suppressors of the mating defect of *sty1* mutants (see below) (Shiozaki and Russell 1996). The *pcr1* gene was isolated as a weak high-copy suppressor of a *spo5* meiosis II defect, although this suppression turned out to be indirect and fortuitous (Watanabe and Yamamoto 1996). Deletion of either *atf1* or *pcr1* results in partial sterility, with about 10% of cells succeeding in mating under conditions where wild-type cells achieve 75-80% mating efficiency (Watanabe and Yamamoto 1996; Kanoh et al. 1996). Both disruptions abolished transcriptional induction of *ste11* and the ability to arrest in G1 under starvation. As bZIP factors have the ability to form heterodimers through their leucine zipper domain, and deletion of *atf1* or *pcr1* resulted in similar phenotypes, Kanoh and colleagues (1996) undertook a biochemical analysis of putative Atf1/Pcr1 heterodimers. The two proteins, that have similar DNA-binding specificity, form a heterodimer complex and are responsible for the cAMP response element (CRE)-binding activity in the cell (Kanoh et al. 1996). *ste11* appears to have a putative CRE site in its 5' regulatory region, but whether or not Atf1/Pcr1 complexes directly regulate *ste11* expression through that site remains to be demonstrated experimentally. Interestingly, Atf1 is phosphorylated in vitro by Pka1 kinase, and deletion of *pka1* affects the Atf1 phosphorylation pattern, as judged by Western blotting. However, Kanoh and colleagues (1996) could not demonstrate the role that Atf1 phosphorylation by Pka1 might play, and suggest that other kinase(s) might be more critical. In contrast to *pcr1* disruption, deletion of *atf1* also results in sensitivity to osmotic and heat stress (Shiozaki and Russell 1996; Wilkinson et al. 1996; Kon et al. 1997). These results imply that besides regulating *ste11*
expression together with Pcr1, Atf1 has a separate role in the transcriptional response to stress. Two Atf1 targets are the catalase gene (ctt1) involved in oxidative stress response, and glycerol-3-phosphate dehydrogenase (gpd1) necessary for osmotic tolerance (Shiozaki and Russell 1996; Wilkinson et al. 1996). Atf1 is directly regulated by the stress-activated Sty1 MAP kinase: following osmotic stress Atf1 is rapidly and transiently phosphorylated by Sty1 MAPK (Shiozaki and Russell 1996; Wilkinson et al. 1996). MAPK (mitogen-activated protein kinase) modules are ubiquitously used by eukaryotic cells to respond to growth modulatory signals (reviewed in Robinson and Cobb 1997). There appear to be multiple mechanisms for signal sensing, but the molecular mechanism for signal transduction through the MAPK module is well conserved. It involves the sequential phosphorylation and activation of three different kinases: the MAPK kinase kinase (MAPKKK), the MAPK kinase (MAPKK), and the MAPK itself. The fission yeast Sty1 MAPK pathway is activated by a variety of stress conditions, including osmotic, oxidative, and heat stress (reviewed in Millar 1999). Wis1 is the MAPKK that phosphorylates and activates Sty1 MAPK, and Wis1 is itself phosphorylated and activated by the MAPKKK Wak1 (also called Wik1 or Wis4). The Wak1/Wis1/Sty1 MAPK pathway plays a crucial role in cell survival upon exposure to the mentioned stress conditions, and is also absolutely required for sexual differentiation. Mutants in this pathway fail to arrest in G1 under nitrogen starvation, and are unable to induce ste11 expression (Kato et al. 1996; Shiozaki and Russell 1996; Wilkinson et al. 1996). Transcriptional regulation by this pathway appears to be extremely complex with different subsets of target genes being induced in a stress specific manner, and depending on distinct transcription factors acting downstream of Sty1 MAPK (Toone and Jones 1998). In summary, ste11 transcriptional induction upon nitrogen starvation is dependent on the Wak1/Wis1/Sty1 pathway that phosphorylates Atf1, allowing the Atf1/Pcr1 heterodimer to activate ste11 transcription.
1.6.3. **Rcd1**

Rcd1 is another regulator of \textit{ste11} induction that appears to act independently of the Pka1 or Sty1 MAP pathway (Okazaki et al. 1998). \textit{Δrcd1} cells are sterile if sexual development is induced by nitrogen starvation, but fertile if it is induced by glucose starvation. This phenotype results from a defect in \textit{ste11} induction by nitrogen starvation. Rcd1 however, is not a component of the general nitrogen sensing pathway since \textit{rcd1}-deleted cells can still arrest normally in G1 in response to nitrogen starvation (Okazaki et al. 1998). Rcd1 is a member of a highly conserved protein family, with close homologues (>70% amino acid identity) in \textit{Caenorhabditis elegans} and \textit{Homo Sapiens}, but the biochemical function of the family members remains unknown. Rcd1 could be a member of a novel signalling pathway responsible for \textit{ste11} induction in response to nitrogen starvation.

1.6.4. **Taf1**

The \textit{taf1} gene was fortuitously identified in a two-hybrid screen for binding partners of the \textit{S. pombe} telomere protein Taz1 (Ueno et al. 2001). Deleting \textit{taf1} results in sterility under nitrogen starvation, resulting from a defect in inducing \textit{ste11} expression. Furthermore, \textit{Δtaf1} cells fail to arrest in G1 under nitrogen-starvation conditions and rapidly lose viability. Surprisingly, \textit{Δtaf1} cells resemble \textit{Δrcd1} cells, in the sense that conjugation efficiency is normal if sexual development is induced by glucose starvation. These results suggest that Taf1 and Rcd1 are involved in a signalling pathway that regulates \textit{ste11} expression in response to nitrogen-starvation, but not glucose-starvation. The protein sequence of Taf1 gives no indication about possible functions.

1.6.5. **Nrd1**

\textit{nrd1} is another gene regulating sexual development onset, independently of the Pka1 kinase, Sty1 MAP kinase, or Rcd1 pathways (Tsukahara et al. 1998).
Nrd1 is an RNA-binding protein with four conserved RRM motifs (RNA recognition motifs), and it has the ability to bind uridine-rich sequences (Tsukahara et al. 1998). Deletion of nrd1 causes cells to initiate sexual development without complete glucose or nitrogen starvation. The function of Nrd1 seems to be to repress ste11-dependent mating and meiotic genes, until cells sense a threshold level of starvation. Nrd1 is expected to be regulated by a nutritional signal at the protein level, as nrd1 is constitutively expressed during nutrient starvation. Tsukuhara and colleagues (1998) suggest that Nrd1 might regulate ste11 at a post-transcriptional step, such as decreasing ste11 mRNA stability or its translation.

1.6.6. Pac2

The fission yeast pac2 gene encodes a protein of 235 aminoacids lacking any structural motif of known function (Kunitomo et al. 1995). Kunitomo and colleagues (1995) show that Pac2 negatively regulates ste11 expression independently of the cAMP/Pka1 kinase signalling pathway. Disruption of pac2 causes upregulation of ste11 expression, leading to entry into sexual development under incomplete starvation.

1.6.7. Ste11 autoregulation

Interestingly, it has been demonstrated that Ste11 directly regulates its own transcription (Kunitomo et al. 2000). Kunitomo and colleagues used DNaseI footprinting analysis to show that the HMG domain of Ste11 could bind and protect an element in the ste11 5' promoter region (TR1: TTCTTTGTTT) conforming to the previously identified Ste11 binding site consensus sequence (Sugimoto et al. 1991). Furthermore, mutation of the TR1 sequence showed that this promoter element is required for ste11 expression. The positive autoregulatory role of Ste11 was confirmed in an elegant experiment analysing the transcription of a ste11 mutant loss-of-function allele (ste11-029). This allele
is transcribed at a very low level, but was induced by ectopic expression of Ste11 from a plasmid. The positive feedback loop function of Ste11 might generate a sharp increase in \textit{ste11} expression, once its protein levels reach a threshold, committing cells to the sexual differentiation pathway.

### 1.7. Mating pheromone signalling

1.7.1. Mating pheromones

Early experiments showed that both P or M cells, when placed in close proximity of cells of the opposite mating type can secrete a diffusible factor that induces morphological changes (elongation of conjugation tubes) on their mating partners (Fukui et al. 1986a; Leupold 1987). Biochemical purification and characterisation revealed that the M-factor (the pheromone released by M cells) is a nonapeptide with a C-terminal cysteine residue that is both carboxymethylated and S-farnesylated (Davey 1992), while the P-factor is a peptide with 23 aminoacid residues that apparently is not modified (Imai and Yamamoto 1994). Three unlinked genes ($\text{mfml}$, $\text{mfml}$, and $\text{mfmS}$) encode a single-copy precursor form of the mature M-factor (Davey 1992; Kjaerulff et al. 1994). As expected, transcription of the $\text{mfm}$ genes is restricted to M cells, and dependent on the M-cell specific Mat1-Mc factor. Furthermore, even though there is a basal level of transcription in rich medium, transcription is upregulated upon nitrogen starvation or exposure to P-factor in a \textit{ste11}-dependent manner (Davey 1992; Kjaerulff et al. 1994). The molecular mechanisms underlying $\text{mfm}$ transcriptional activation by Mat1-Mc, Ste11, and P-factor will be discussed in more detail below. The three genes appear to be functionally redundant as each one, on its own, can support sufficient production of M-factor to sustain high mating efficiency. M cells only became sterile when the three $\text{mfm}$ genes were deleted (Kjaerulff et al. 1994). The $\text{map2}$ gene encodes a 201 aminoacid protein containing four tandem non-identical
copies of the mature P-factor (Imai and Yamamoto 1994). Similarly to \textit{mfm} genes, \textit{map2} transcription is restricted to P-cells and further induced by nitrogen starvation and M-factor signalling (Imai and Yamamoto 1994).

1.7.2. Mating pheromone receptors

P-factor and M-factor receptor genes were identified as mating-type specific genes required for mating. \textit{mam2} (the P-factor receptor), and \textit{map3} (M-factor receptor) encode putative seven-pass transmembrane receptors of the G-protein coupled receptor superfamily (GPCR) (Kitamura and Shimoda 1991; Tanaka et al. 1993). \textit{mam2} and \textit{map3} have significant homology to the \textit{S. cerevisiae} pheromone receptors Ste2 and Ste3, respectively. It has been estimated that \textit{S. cerevisiae} MATa cells contain 8000 alpha factor-binding sites (Ste2) on their surface, and radioligand binding studies demonstrated that purified Ste2 receptor could bind alpha-factor with high affinity (Jenness et al. 1986; David et al. 1997). In fission yeast, however, the direct binding of pheromones to their receptors has not been studied. Confirmation of the expected plasma membrane localisation of the pheromone receptors, Mam2 and Map3, is also missing.

1.7.3. Gpa1, the pheromone receptor coupled G-protein

The Mam2 and Map3 receptors were expected to transmit the pheromone signal to a heterotrimeric guanine nucleotide-binding protein (G-protein). The \textit{gpa1} gene was identified in a cross-hybridisation screen using the rat G\(\alpha\) subunits Gi1\(\alpha\) and Gx\(\alpha\) cDNAs (Obara et al. 1991). Analysis of Gpa1 deduced amino acid sequence revealed that it is closely related to the Gi\(\alpha\) subfamily of G-protein \(\alpha\) subunits. The \textit{gpa1} gene is expressed in both P and M cells, and is required for mating and meiosis. To investigate if Gpa1 is involved in the transmission of the pheromone signal, Obara and colleagues (1991), expressed a constitutively active form of Gpa1, Gpa1\textsuperscript{G224L}, in both P and M cells. Interestingly, upon nitrogen starvation both heterothallic strains elongated
conjugation tubes in the absence of the opposite pheromone. These results suggest that Gpa1 is the α-subunit of the G-protein coupled to the pheromone receptors, and that Gpa1 Gα is responsible for transmission of the signal. This last point is in stark contrast with the situation in S. cerevisiae where expression of constitutively active alleles of Gpa1 confer pheromone resistance, and it is the Gβγ subunits that transmit the pheromone signal (Whiteway et al. 1989; Stone and Reed 1990; Hirsch et al. 1991). To date, no Gβγ partner subunits for Gpa1 have been identified.

1.7.4. Pheromone-activated MAPK cascade

The direct effector target for Gpa1 activation has not been identified, but a MAPK cascade acting downstream of Gpa1 that is required for transmission of the pheromone signal has been characterised. This MAPK module consists of Byr2 (a MAPKKK), Byr1 (MAPKK), and Spk1 (MAPK) (Nadin-Davis and Nasim 1988; Nadin-Davis and Nasim 1990; Wang et al. 1991b; Gotoh et al. 1993). One key regulatory event in the pheromone signal transmission is activation of the Byr2 kinase. Besides a conserved C-terminal catalytic kinase domain, Byr2 has an N-terminal regulatory domain (Tu et al. 1997). The intramolecular interaction between these two domains appears to be autoinhibitory, and the pheromone signal is necessary to disrupt this interaction, thereby resulting in activation of the Byr2 kinase activity (Tu et al. 1997). Detailed analysis of the N-terminal regulatory domain revealed the presence of distinct regions able to bind Ras1, Ste4, and the Byr2 catalytic domain (Masuda et al. 1995; Barr et al. 1996; Tu et al. 1997). What is the molecular mechanism for Byr2 activation? The fission yeast ras1 gene is, apparently, the single homologue of the mammalian ras oncogenes. The small monomeric G-protein, Ras1, is required for transmission of the pheromone signal, and its absence results in a mating defect (Fukui et al. 1986a; Fukui et al. 1986b; Nadin-Davis et al. 1986a). Ras1 is thought to cycle between a GTP-bound active form and a GDP-bound inactive form. S. pombe cells expressing an
activated Ras1^{Val17}, an allele that is unable to hydrolyse GTP, are hypersensitive to pheromone and elongate abnormally long conjugation tubes (Fukui et al. 1986b; Nadin-Davis et al. 1986a). Interestingly, in contrast with the expression of a constitutively active form of Gpa1, Ras1^{Val17} induction of morphological changes is still dependent on the presence of mating pheromone (Fukui et al. 1986b; Nadin-Davis et al. 1986a). These results suggest that Ras1 is a modulator of the pheromone response, and is possibly required for Gpa1-mediated activation of Byr2 MAPKKK. Within the N-terminal regulatory domain of Byr2 there is a distinct region that binds to Ras1, and that is required for Byr2 function (Tu et al. 1997). Furthermore, overexpression of Ras1 causes translocation of Byr2 from the cytoplasm to the plasma membrane periphery, and a constitutively active form of Ras1 was shown to increase the level of Byr2 recruitment (Bauman et al. 1998). It has been suggested that recruitment of Byr2 by plasma-membrane associated Ras1 could be the first event leading to Byr2 activation (Tu et al. 1997). Once localised to the plasma membrane, Byr2 could interact with other signalling components. Pak1 (or Shk1), a fission yeast homologue of the eukaryotic p21-activated serine-threonine kinase (PAK) family is thought to mediate the next activation step. Pak1 is able to disrupt the intramolecular Byr2 interaction, activating Byr2 catalytic activity (Tu et al. 1997). In *S. cerevisiae* the Pak1 homologue Ste20 directly phosphorylates and activates the MAPKKK Ste11 in response to pheromone signalling (Wu et al. 1995). Given the similarity between the mating signalling pathways of the two yeasts, and the conservation of the phosphorylation sites in Byr2 kinase, it is expected that Pak1 also phosphorylates and activates the MAPKKK Byr2 (Tu et al. 1997). Opening Byr2, exposes a Ste4 binding site in the Byr2 regulatory domain (Barr et al. 1996; Tu et al. 1997; Bauman and Albright 1998), that is distinct from the Ras1 binding site. Ste4 is a leucine zipper protein required for mating and meiosis, and is capable of homotypic interaction (Okazaki et al. 1991; Barr et al. 1996). Ste4 could stimulate dimerization of Byr2 molecules, and Tu and colleagues (1997) speculate that Byr2 dimerization could lead to Byr2 autophosphorylation and further activation. Supporting this hypothesis is the
fact that forced dimerization of mammalian RAF-1 kinase leads to activation of its kinase activity (Farrar et al. 1996). The model presented here for the mechanism of Byr2 MAPKKK activation is still highly speculative as several aspects of it remain to be demonstrated directly. Upon activation, Byr2 is thought to directly phosphorylate the Byr1 MAPKK (Styrkarsdottir et al. 1992; Neiman et al. 1993), which then relays the pheromone signal to the Spk1 MAPK, also via phosphorylation-mediated activation (Gotoh et al. 1993). The S. pombe pheromone response pathway is schematically presented in Figure 1-3.

1.8. Pheromone signalling changes

1.8.1. Ste11 regulation and the transcriptional induction by pheromone signalling

The mating pheromones can significantly change the pattern of gene expression in target cells. Analysis of pheromone-dependent transcription is complicated by the fact that all the genes requiring a pheromone signal for transcriptional induction also require a nitrogen starvation signal. Furthermore, some of these target genes are mating-type specific. Presumably, activated Spk1 modulates the transcriptional response to the mating pheromones by directly or indirectly activating one or more transcription factors. One candidate for a pheromone-modulated transcription factor is the Ste11 protein (Sugimoto et al. 1991). Ste11-mediated transcriptional regulation appears to be very complex: Ste11 is required for the initial wave of transcription induced by nitrogen starvation, for subsequent mating-type specific gene induction, and also for pheromone induction of both ubiquitous and mating-type specific genes. Sugimoto and colleagues (1991) showed that the Ste11 protein binds a T-rich DNA sequence, the TR-box, TTCTTTGTTY, present in the promoter regions of genes that are expressed in a Ste11-dependent manner upon nitrogen starvation. These included genes that are expressed in both P and M cells (like
mei2), as well as P-specific genes (mat1-Pc), and M-specific genes (mat1-Mc). Transcriptional activation of these genes requires nitrogen starvation because it absolutely depends on the presence of Ste11 that is itself only induced after nutritional-starvation inhibition of Pka1 activity.

One of the questions raised by these observations is: how does Ste11 function to activate transcription in a mating-type specific manner? A molecular mechanism for the role of Ste11 in activating mating-type specific gene expression has recently been proposed (Kjaerulff et al. 1997). This model developed from the observation that all promoters of genes that are ubiquitously expressed have at least one copy of a long form of the Ste11-binding site (TR-box), TTTCTTTGTT, that is not found in any of six known M-specific genes. Promoters of M-specific genes have a shorter and weaker TR-box, TCTTTGTT, and Ste11 is only able to bind these promoters in a ternary complex with an M-cell specific HMG-box protein, Mat1-Mc, a protein that binds a neighbouring sequence called the M-box, only present in these promoters. Strong TR-boxes are necessary to drive transcription of heterologous reporter genes in both mating types, and mutational conversion of a short TR-box into a long one in the promoter of mfm1, an M-cell specific gene, results in ubiquitous expression (Kjaerulff et al. 1997).

A second issue is that some ste11-dependent genes are transcriptionally activated upon nitrogen starvation and further stimulated by pheromone signalling (like mam2, ste6, and mat1-Pm) (Nielsen et al. 1992; Aono et al. 1994; Hughes et al. 1994; Xu et al. 1994), while others require both signals for transcriptional activation (like sxal and fusl) (Imai and Yamamoto 1992; Petersen et al. 1995). Substantial evidence suggests that transcriptional induction in response to pheromone occurs by stimulation of Ste11 activity. Firstly, pheromone signalling can not stimulate transcription independently of Ste11 function: for fus1, mat1-Pm, and mfm1, mutation of the TR-box that abolishes Ste11 binding results in lack of gene expression even under pheromone stimulation (Aono et al. 1994; Petersen et al. 1995; Kjaerulff et al. 1997). Secondly, deletion analysis of the mat1-Pm promoter shows that a region
with 62 bp, adjacent to the transcription start site, is sufficient to drive transcription of a reporter gene upon nitrogen starvation and to sustain further stimulation after pheromone treatment. Furthermore, this region contains a sequence with 23 bp that includes a Ste11 binding site (TR-box) that is necessary and sufficient for the pattern of mat1-Pm transcription observed in vivo (Aono et al. 1994). A different study went further to narrow the pheromone-responsive sequence to the TR-box itself: eight copies of the TR-box of the mfm1 promoter were sufficient to confer both nitrogen-starvation transcriptional induction, as well as further upregulation by the pheromone signal on a heterologous minimal promoter (Kjaerulff et al. 1997). How does pheromone activation of Spkl MAPK stimulate Ste11 activity? If Spkl stimulates Ste11 activity directly, it could act in a number of ways, for example: raising Ste11 protein levels, increasing its affinity for the TR-box, or stimulating its transactivation activity. If, on the other hand, Spkl acts indirectly, it could activate Ste11 specific co-activators or release Ste11 from inhibitory binding partners. In the budding yeast, the pheromone-responsive transcription factor, Ste12, is inactive in the absence of pheromone signalling, due to complex formation with two inhibitors, Dig1 and Dig2 (Cook et al. 1996; Tedford et al. 1997). Upon pheromone stimulation, the Fus3 and Kss1 MAPKs, phosphorylate the three components of the complex, resulting in release and activation of Ste12. Whether a similar mechanism operates with Ste11 is not known and it still remains to be demonstrated that the pheromone signal transmitted by Spkl directly upregulates Ste11 activity. Analysis of Ste11 regulation by the pheromone response MAPK cascade will be complicated by the fact that positive feedback between both Ste11 and Spkl activation might occur. The spkl gene is a putative Ste11 transcriptional target, due to the presence of two long TR-boxes in its promoter (Kjaerulff et al. 1997), and the Ste11 protein is a putative target for activation by Spkl. Furthermore, even in the absence of pheromone stimulation, Ras1, Gα Gpa1, and Byr1 MAPKK are required for mfm1 transcriptional activation in response to nitrogen starvation (Kjaerulff et al. 1994). This observation implies that in the absence of pheromone
stimulation, the pheromone response pathway might be active at a basal level. Even tough Ste11 appears to be an attractive candidate target for the pheromone-activated MAPK cascade, the possible involvement of other transcriptional regulators cannot be excluded.

1.8.2. Cell cycle arrest

A crucial step in the eukaryotic cell cycle occurs in late G1 where a decision is made as to whether a cell progresses through S-phase or exits the cell cycle and enters a differentiation pathway (Sherr 1994). The fission yeast mating factors induce an arrest of mitotic proliferation at the G1 phase of the cell division cycle (Davey and Nielsen 1994; Imai and Yamamoto 1994). This arrest in G1 is a pre-requisite for initiation of the sexual development pathway, ensuring that both mating partners conjugate in the haploid state to form a diploid zygote. Similarly to the analysis of pheromone-dependent changes in transcription, the study of the G1-arrest induced by pheromones is complicated by the intricate link between nitrogen starvation and pheromone signalling. Physiologically, both P and M factors can only induce G1-arrest in nitrogen-starved cells as components of the pheromone signal transduction pathway are only expressed under those conditions. Furthermore, nitrogen starvation on its own is sufficient to induce a mitotic G1 arrest (Egel and Egel-Mitani 1974; Costello et al. 1986). From a physiological point of view, the role of pheromone stimulation is to accelerate the G1 arrest imposed by nitrogen starvation. An example of this is the fact that when heterothallic strains are nitrogen starved, they divide twice before arresting in G1, whereas homothallic strains (a mixed population of both mating-types where pheromone signalling is active under starvation) arrest after a single division (Egel and Egel-Mitani 1974).

In eukaryotic cells, the G1-S phase transition requires both the activation of cyclin-dependent kinases (CDK) and certain transcription factors necessary for expression of genes involved in S phase. In the fission yeast, the onset of S-phase requires the activation of a G1-specific transcription factor complex
containing Cdc10 and Res1, and the activation of the Cdc2 CDK (Nurse and Bissett 1981; Aves et al. 1985; Lowndes et al. 1992; Tanaka et al. 1992; Caligiuri and Beach 1993). The activation of Cdc2 in G1 requires its association with B-type cyclins: Cig2 is the main partner of Cdc2 in G1, but the mitotic B-cyclin Cdc13 can activate Cdc2 if Cig2 is absent (Fisher and Nurse 1996; Martin-Castellanos et al. 1996; Mondesert et al. 1996). It has been proposed that nitrogen starvation-induced G1 arrest is due to a specific translational block of cig2 and cdc13 mRNAs, encoding S-phase promoting B-cyclins (Grallert et al. 2000). The mechanism for this specific translational block remains to be determined, but might involve inactivation of a general translation factor Sum3 (or Ded1) to which cig2 and cdc13 mRNAs appear to be particularly sensitive (Forbes et al. 1998; Grallert et al. 2000). Mating pheromone induces G1 arrest via a distinct mechanism, by promoting the proteolysis of Cig2 and Cdc13 via the anaphase-promoting complex (APC) (Stern and Nurse 1997; Stern and Nurse 1998). The CDK inhibitor Rum1 is necessary to maintain the G1 arrest induced by pheromone, as it binds Cig2 and Cdc13 and is specifically required for Cdc13 proteolysis (Correa-Bordes and Nurse 1995; Stern and Nurse 1998). Mechanistic details about direct targets of the pheromone-activated Spk1 MAPK involved in inducing the G1 arrest remain unknown.

1.8.3. Morphological response

During the pheromone-induced G1 arrest fission yeast cells agglutinate and continue to grow at one of the cell tips, but this growth no longer occurs necessarily along the long cell axis and can result in a bent conjugation tube (or shmoo) (reviewed in Bahler and Peter 2000). The shmoo elongates towards a pheromone concentration gradient that acts as an extracellular signal to override the intrinsic mitotic cell polarity programme (Fukui et al. 1986a; Leupold 1987; Leupold et al. 1991). The actin cytoskeleton has an important role in the establishment of polarized growth in eukaryotic cells. In the fission yeast, the G1 phase F-actin cortical dots are concentrated at the growing end (Marks et
al. 1986; Petersen et al. 1998b). At an early stage, nitrogen starvation and pheromone signalling switch this distribution to a bipolar one, but later shmoo formation is accompanied by repolarization of the F-actin cytoskeleton to the tip selected for pheromone-induced elongation (Petersen et al. 1998b). 

*S. pombe* cells are able to form shmoos at either end of the cell (Myata and Myata 1981). It has been suggested that the initial F-actin bipolarization occurs during a brief period when the cell is screening for the presence of a pheromone gradient, and so increase the efficiency of mating partners detection (Petersen et al. 1998b). F-actin is required for shmoo formation and cell fusion as treating cells with the depolymerising drug Latrunculin A blocks those processes (Petersen et al. 1998b). Microtubules are also required for shmoo elongation and cell fusion (Petersen et al. 1998a). Interestingly, microtubules are required for localisation of actin to the shmoo tip, whereas the reverse is not true (Petersen et al. 1998a).

How the sensing of a pheromone gradient results in the establishment of positional landmarks at the cell tip nearest to the pheromone source is still unclear. Earlier on in this Introduction, I discussed the role of Ras1 in stimulating a MAPK cascade responsive to the pheromone signal. In addition to that role, Ras1 also regulates cell morphology, as *ras1*-disrupted cells are shorter and rounder compared with wild-type cells (Fukui et al. 1986b; Nadin-Davis et al. 1986a). This role of Ras1 is distinct of its role in activating the MAPK cascade, since mutations in *byr2*, *byrl*, and *spkl* do not affect cell morphology (Nadin-Davis and Nasim 1988; Toda et al. 1991; Wang et al. 1991b). Ras1 binds and activates Ral1 (or Scd1), a putative guanine nucleotide exchange factor for the Rho-like GTPase Cdc42 (Chang et al. 1994; Miller and Johnson 1994). Activation of Ral1 is expected to promote exchange of GTP for GDP on Cdc42 thereby activating Cdc42 function. Mammalian Rho-like GTPases are crucial regulators of the actin cytoskeleton and mediate morphological changes in response to extracellular signals (Hall 1998). One of the targets of Cdc42 appears to be the Pak1 (Shk1) kinase, as the two proteins interact *in vivo* and overexpression of *pak1* rescues the mating defects caused by expression of a dominant negative form of Cdc42 (Marcus et al. 1995; Ottilie et al. 1995).
1.8.4. **Cell fusion**

Upon agglutination and shmoo formation, a cell grows towards a mating partner of opposite mating-type. After the two conjugation tubes contact, localised cell wall degradation occurs followed by plasma membrane fusion. The *S. pombe* fus1 gene is specifically required for cell fusion during mating (Bresch et al. 1968; Petersen et al. 1995). In the absence of Fus1, cells agglutinate and form shmoos but the cell walls separating the mating partners are not degraded (Petersen et al. 1995). Therefore, conjugation is blocked at the prezygotic stage with fus' phenotype (two mating partners attached by their shmoo tips, but unable to dissolve the cell wall between them). As mentioned before, fus1 expression is dependent on Ste11 function and pheromone signalling, and consistent with its role in conjugation, it localises to the shmoo tip following agglutination (Petersen et al. 1995; Petersen et al. 1998c). Fus1 belongs to the formin protein family, and it contains three formin-homology (FH) domains. Members of this eukaryotic protein family are important for actin-dependent polarization events (reviewed by Tanaka 2000). One possible role for formin proteins is to function as scaffolding proteins for cytoskeletal components. As an example, the *S. cerevisiae* Bni1 formin, which is required for the pheromone-induced polarisation, has been shown to interact with Rho1, Cdc42, actin, and two actin-binding proteins (profilin and Bud6) (Kohno et al. 1996; Evangelista et al. 1997). In agreement with this model, the fission yeast Fus1 is required to stabilise the association of polarized F-actin with the shmoo tip (Petersen et al. 1998c).

1.9. **Adaptation to pheromone stimulation**

Stimulation of cells with extracellular ligands for G-protein coupled receptors provokes a physiological response, but continuous stimulation induces an adaptive downregulation of signalling (reviewed by Tsao and Zastrow 2000). Fission yeast cells have the ability to recover from the effects of
pheromone stimulation (including G1 arrest) and resume the mitotic cell cycle (Davey and Nielsen 1994; Imai and Yamamoto 1994). Understanding the molecular mechanisms underlying signal adaptation is extremely important, as the superfamily of GPCR proteins contains >1000 members and the therapeutic effects of many drugs are based on their ability to regulate G-protein signalling. Several mechanisms contribute to signal adaptation including: removal or degradation of the extracellular ligand; GPCR desensitisation, endocytosis, and degradation; and downregulation of heterotrimeric G-proteins (reviewed by Tsao and Zastrow 2000). In the following sections, I will discuss how these mechanisms contribute to pheromone adaptation in S. pombe.

1.9.1. Degradation of extracellular mating pheromones

Degradation of extracellular signals would contribute to adaptation by preventing the cell from further stimulation, and allowing time for other adaptation mechanisms to act. The S. pombe carboxypeptidase Sxa2 plays an important role in the adaptation of M cells to stimulation with P-factor (Imai and Yamamoto 1992; Imai and Yamamoto 1994). Sxa2 is involved in a negative feedback loop that regulates the sensitivity of M-cells to P-factor. Firstly, the sxa2 gene is only expressed in M-cells upon nitrogen starvation and stimulation by P-factor (Imai and Yamamoto 1994). Sxa2 is involved in a negative feedback loop that regulates the sensitivity of M-cells to P-factor. Secondly, biochemical analysis shows that Sxa2 is secreted and inactivates P-factor by removing the C-terminal leucine residue (Ladds et al. 1996; Ladds and Davey 2000). Interestingly, deletion of sxa2 also results in mating deficiency in M cells, demonstrating the important role of timely downregulation of pheromone stimulation during the conjugation event (Imai and Yamamoto 1992).

1.9.2. Downregulation of the pheromone GPCRs

In fission yeast there is, so far, no reported example of adaptation by downregulation of the pheromone GPCRs (Mam2 and Map3). However, that
mechanism has been well characterised in other systems, including budding yeast, so they are likely to be conserved in *S. pombe* as well. In *S. cerevisiae*, a possible sequence of events leading to downregulation of pheromone GPCRs has been proposed (reviewed by Riezman 1998). When pheromones bind their cognate GPCRs, the receptors undergo a conformational change, and are phosphorylated and ubiquitinated on their C-terminal cytoplasmic region. Ubiquitination stimulates internalisation by endocytosis, and the receptors are then delivered to the vacuole where they are degraded. Interfering with the internalisation of the pheromone receptors leads to supersensitivity to pheromone and a delay in recovery upon pheromone removal (Reneke et al. 1988; Hicke and Riezman 1996).

1.9.3. **Negative regulation of G-protein signalling**

In their inactive conformation, heterotrimeric guanine-binding proteins are heterotrimers containing an α, a β, and a γ subunit. In this conformation, the α-subunit (Gα) is bound to GDP. Upon activation the GPCR stimulates the Gα protein to release GDP and bind GTP, acting as a GDP/GTP exchange factor (GEF). When Gα binds GTP the conformation of the primary contact site with the βγ dimer is altered, resulting in dissociation of Gα from the βγ dimer (Wall et al. 1995). This dissociation activates both the Gα-GTP and the Gβγ, as in this conformation they are able to interact and activate downstream signalling effectors. The time length of signal transmission by a heterotrimeric G-protein is determined by the GTPase hydrolysis rate of the Gα subunit. Upon GTP hydrolysis, Gα-GDP binds the Gβγ dimer with high affinity, terminating signal transmission. Gα normally has slow intrinsic GTPase activity, however the deactivation rates for some G-protein signalling pathways, like phototransduction, are much faster than predicted based on those slow hydrolysis rates. This suggested the existence of GTPase-activating proteins (GAPs) able to accelerate the hydrolysis of GTP by Gα. The regulator of G-protein signalling (RGS) protein family appear to perform the role of GAP
proteins for heterotrimeric G-proteins (Fig. 1-4), and will be discussed in the following section.

1.9.4. The Regulator of G-protein Signalling (RGS) protein family

The first RGS proteins were identified genetically in *S. cerevisae* (Sst2) and in *Caenorhabditis elegans* (EGL-10), and shown to function as negative regulators of G-protein signalling (reviewed by Dohlman et al. 1998). A conserved 120 amino acid region (RGS domain) was originally identified in EGL-10, Sst2, as well as in human RGS1 and RGS2 (Koelle and Horvitz 1996). The RGS domain was identified independently in a distinct human RGS protein, GAIP (Gα-interacting protein), and shown to be sufficient to mediate its interaction with Gα3 in a two-hybrid assay (De Vries et al. 1995). Subsequently, three laboratories independently reported that RGS proteins display GAP activity towards Gαi and Gαq sub-family members (Berman et al. 1996a; Hunt et al. 1996; Watson et al. 1996). Since then many members of the family have been identified, and more than 25 human RGS proteins have been reported to date (reviewed by Hepler 1999; Nestler and Landsman 2001). The fission yeast genome appears to contain a single ORF encoding an RGS-domain containing protein (Tesmer et al. 1997).

The mechanism of GTPase activation by RGS proteins:

Biochemical studies on purified GAIP and RGS4 elucidated the mechanism for the GAP activity of RGS proteins (Berman et al. 1996a; Berman et al. 1996b). In *in vitro* assays, either RGS4 or GAIP stimulated the hydrolysis rate more than 40-fold for all Gα subtypes, except Gαi (Berman et al. 1996b). Neither RGS protein affected the steady-state rate of GTP hydrolysis by the targeted Gα subunities (Berman et al. 1996b). Several studies analysed the binding of RGS proteins to Gα proteins in their GDP-bound, GTP-bound, and (GDP-αF4)′-bound (mimicking the transition state complex) conformations (Berman et al. 1996a; Watson et al. 1996; Natochin et al. 1997). RGS proteins
were found to preferentially bind the transition state complex. The structure of rat RGS4 bound to activated G_{ia1} (GDP- AlF_{4}-bound) has been solved at 2.8-Å resolution (Fig. 1-5, A) (Tesmer et al. 1997). Only the RGS domain of RGS4 had an ordered structure in the crystal (besides the central RGS domain, RGS4 has small flanking N- and C-terminal regions). The RGS domain contains nine α-helices that fold into two small subdomains (Fig. 1-5, B) (Tesmer et al. 1997; de Alba et al. 1999). The larger subdomain contains 4 α-helices (α4, α5, α6, and α7), and is a classic right-handed, antiparallel four-helix bundle. Both subdomains are required for RGS4 GAP activity. Tesmer and colleagues (1997) show that unlike Ras-GAPs, RGS4 does not contribute with catalytic residues to the active site of G_{ia1}, and suggest that RGS4 antagonises G_{ia1} signalling by stabilising its transition state for GTP hydrolysis.

S. cerevisae Sst2: a paradigm for RGS proteins function:

SST2, a budding yeast RGS gene, was identified in the 1980s in a screen for mutants hypersensitive to pheromone-induced cell cycle arrest (Chan and Otte 1982b; Chan and Otte 1982a). sst2 loss-of-function mutations increase pheromone sensitivity by 100-fold, and prevent adaptation to continued exposure to mating pheromone (Chan and Otte 1982b; Chan et al. 1983). SST2 is expressed at low basal level and is transcriptionally induced by pheromone signalling, with a kinetics similar to that for pheromone desensitisation (Dietzel and Kurjan 1987). The low basal level of SST2 expression might set the threshold for pheromone sensitivity, with induction of SST2 being responsible for adaptation to pheromone signalling. Genetic analysis revealed that Sst2 probably downregulated pheromone signalling at the level of the pheromone-receptor coupled heterotrimeric G-protein (Dohlman et al. 1995). This suggestion was confirmed by a study showing that Sst2 co-localised and interacted with the pheromone-activated Gα subunit (Gpa1) (Dohlman et al. 1996). The catalytic role of Sst2 was later demonstrated in a biochemical study, using purified Sst2 and Gα Gpa1 proteins, that shows that Sst2 displays strong GAP activity towards activated Gα Gpa1 (Apanovitch et al. 1998).
The Sst2 protein contains 689-amino acid residues, and its RGS domain is localised at the C-terminal between residues 417 to 685 (Dietzel and Kurjan 1987; Dohlman et al. 1996). Interestingly, the RGS domain in Sst2, like in other fungal RGS proteins, is split in subdomains (three in the case of Sst2) separated by spacer sequences of variable length. These spacer sequences lie on the opposite side of the Gα interaction surface, and are predicted not to disturb the RGS-Gα interaction (Fig. 1-5, A). Besides transcriptionally activating SST2, pheromone signalling also regulates Sst2 at the post-translational level. Sst2 is phosphorylated in vivo at Ser-539, within a consensus MAPK phosphorylation site (Pro-X-Ser-Pro) (Garrison et al. 1999). Garrison and colleagues show that this phosphorylation occurs in response to pheromone stimulation and requires the presence of the pheromone-activated MAPKs Fus3 and Kss1. Replacing Ser-539 with Ala, Asp, or Glu, do not significantly alter Sst2 activity as pheromone sensitivity remained unchanged. Consistent with these results, it is predicted that Ser-539 localises at a distal site from the Gα interaction surface, based on the crystal structure determination of rat RGS4 with Giα1 (Tesmer et al. 1997; Garrison et al. 1999). Pheromone stimulation of Ser-539 phosphorylation appears to slow the rate of Sst2 degradation, which has a short half live in unstimulated cells, revealing a feedback regulatory mechanism for pheromone signalling (Garrison et al. 1999).

A recent report has showed that the Sst2 protein (82 kDa) is proteolytic processed in vivo producing two fragments: the C-terminal RGS domain fragment (36 kDa) and a complementary N-terminal fragment (Hoffman et al. 2000). The full-length Sst2 is cleaved after Ser-414 and Ser-416 at the N-terminal boundary of the RGS domain. Interestingly, Sst2 processing does require the presence of components of the pheromone signalling response pathway, however it is not regulated by pheromone signalling. The endoproteolytic processing of Sst2 is not extremely efficient, and both the full-length and the its cleavage products can be detected in vivo. What might be the physiological role of the Sst2 processing? Sst2 had previously been shown to be localised to the microsome, cytosol and plasma membrane compartments (Dohlman et al. 2000).
1996). Hoffman and colleagues (2000) demonstrate that both the N-terminal fragment of Sst2, and the C-terminal RGS domain fragment were absent from the plasma membrane, localising to the microsomal and cytosolic compartments. Co-expression of the two fragments failed to fully restore Sst2 activity. This suggests that the mislocalisation from the plasma membrane resulting from the processing of the two fragments might have an inhibitory effect on Sst2 activity. Pheromone stimulation of Ser-539 phosphorylation appears not to influence the Sst2 processing (Garrison et al. 1999).

Characterisation of Sst2 biochemical activity and regulation has shown that RGS protein function might be regulated at several levels and constitute an important target for regulation of GPCR signalling.

1.10. Nuclear reorganisation and karyogamy

Soon after cytoplasmic fusion between mating partners, the two haploid nuclei migrate towards the fusion site and fuse to form a diploid nucleus, in a process called karyogamy (Calleja et al. 1977). This process is accompanied by a complex reorganisation of the nuclear structure (Chikashige et al. 1994; Chikashige et al. 1997). During the mitotic cell division cycle, chromosome centromeres are clustered near the spindle pole body (SPB; the fungi equivalent of centrosome) whereas telomeres are clearly separated from the SPB. However, when cells enter meiotic prophase a switch between telomeres and centromeres has occurred, and it is the telomeres that associate with the SPB (Chikashige et al. 1994). Taz1, a S. pombe telomere protein, is required for telomeres to associate with the SPB, and disruption of the taz1 gene results in meiotic chromosome segregation and recombination defects, demonstrating the important role of the telomeres/SPB meiotic clustering (Cooper et al. 1998; Nimmo et al. 1998). The telomere-centromere switching at the SPB occurs in two stages: firstly, telomeres associate with SPB, and secondly, centromeres dissociate from the SPB (Chikashige et al. 1997). Interestingly, the first step occurs in haploid cells in response to mating pheromone, while the second step appears to depend on cell-cell contact and cell fusion related events (Chikashige et al. 1997). The
mechanism mediating pheromone-stimulated association of telomeres with the SPB remains unknown.

So far, a single *S. pombe* gene specifically required for karyogamy has been identified, *tht1* (Tange et al. 1998). In a *Δtht1* strain, upon cytoplasmic fusion, the nuclei migrate to the fusion site and the two SPB fuse normally, but karyogamy is blocked as the fusion of nuclear envelopes does not occur. Corroborating the phenotype of *tht1* disruption, *tht1* expression requires nitrogen starvation and pheromone signalling. Tht1 is a type I membrane protein and appears to localise to the nuclear envelope or ER membranes. How Tht1 promotes nuclear membrane fusion is still unknown.

### 1.1. Meiosis

A successful conjugation event generates a single-nucleated diploid zygote. After nuclear fusion, the diploid nucleus initiates premeiotic DNA replication, assumes a horsetail shape and initiates a striking period of strong oscillatory nuclear movements between the cell ends (lasting for 146±14 min) (Bahler et al. 1993; Hiraoka et al. 2000). After coming to rest, the nucleus remains in the center of the cell for 26±5 min before initiating the first meiotic division (Hiraoka et al. 2000). Four haploid nuclei are generated by the two meiotic divisions, and then each of the four nuclei are encapsulated by a double-layered forespore membrane that supports the formation of the spore cell wall during sporulation (reviewed by Yamamoto et al. 1997).

How does mating pheromone signalling regulate meiosis? Unlike *S. cerevisiae*, fission yeast diploid zygotes require the pheromone-responsive signalling pathway to enter the meiotic cell cycle. I will now discuss the control mechanisms that restrict entry in meiosis to mating-type heterozygous diploid zygotes, under nutrient starvation and exposed to pheromone stimulation. The *pat1* (or *ran1*) gene encodes a serine/threonine kinase that acts as a key negative regulator of meiosis (Beach 1985; Beach et al. 1985; Iino and Yamamoto 1985b; Iino and Yamamoto 1985a; McLeod and Beach 1986). Loss of *pat1* gene function allows cells to enter ectopic meiosis and sporulation irrespective of the
nutritional status and cell ploidy (even in the haploid state). The molecular mechanism underlying Pat1 inhibition of entry in meiosis is well characterised: Pat1 inhibits Mei2 (an inducer of meiosis) by phosphorylating its Ser438 and Thr527 residues, and expression of Mei2 with these residues mutated into alanine (Mei2-SATA) causes ectopic meiosis (Watanabe et al. 1997). Furthermore, disrupting the mei2 gene in pat1 loss-of-function mutants abolishes ectopic meiosis (Beach et al. 1985; Iino and Yamamoto 1985a; Iino and Yamamoto 1985b). mei2 is transcriptionally activated by Ste11 under nitrogen starvation conditions in both haploid and diploid cells (Shimoda et al. 1987; Watanabe et al. 1988; Sugimoto et al. 1991). The Mei2 protein carries three RNA-recognition motifs (RRMs), and is required at two meiotic stages: firstly it induces premeiotic DNA synthesis, and later on it is required to promote the first meiotic division (Meiosis I) (Watanabe and Yamamoto 1994). These results suggest that Pat1 phosphorylates and inhibits Mei2 until all the requirements for meiosis entry are met. How is Pat1 activity inhibited in response to diploidy and mating-type heterozygosity? The mei3 gene encodes a positive regulator of meiosis that can induce haploid meiosis, independently of the nutritional status, when ectopically expressed (McLeod et al. 1987; Willer et al. 1995). Mei3 can bind to Pat1 and inhibit its kinase catalytic activity (McLeod and Beach 1988). mei3 transcriptional activation is tightly regulated and only occurs upon nutritional starvation in a heterozygous diploid. Ectopic expression of Mat1-Pm and Mat1-Mm in haploid cells, in the absence of Mat1-Pc and Mat1-Mc, lead to expression of mei3 and consequentially to ectopic meiosis (Willer et al. 1995). A recent analysis of the mei3 promoter showed that a critical element for mei3 expression is a binding site for Mat1-Pm, and that Mat1-Pm functions as a direct activator of mei3 expression in vivo (Van Heeckeren et al. 1998). The dependence of mei3 expression on Mat1-Pm and Mat1-Mm function explains the several requirements for its activation, as induction of mat1-Pm (in P cells) and mat1-Mm (in M cells) require both nitrogen starvation and pheromone signalling (Nielsen and Egel 1990; Nielsen et al. 1992; Willer et al. 1995).
Presence of both Mat1-Pm and Mat1-Mm in the same cell, only occurs upon conjugation of mating partners, so mei3 will only be expressed when the required conditions for meiosis are achieved. Upon conjugation, mei3 is expressed and inhibition of Pat1 by Mei3 results in accumulation of non-phosphorylated Mei2, that is able to promote entry into meiosis.

There is also some evidence that Pat1 negatively regulates sexual differentiation at an early stage. If homothallic cells carrying temperature sensitive pat1 mutants are incubated at a semi-restrictive temperature, presumably resulting in partial inactivation of Pat1 activity, high levels of conjugation occur even in nutrient-rich medium (Beach et al. 1985; Nurse 1985). This effect has been explained by the observation that in those conditions Pat1 inactivation results in ectopic expression of both Mat1-Mc and Mat1-Pc (Nielsen and Egel 1990). Furthermore, if pat1 is overexpressed, it inhibits meiosis in diploid cells as expected, but it also inhibits mating of haploid cells (McLeod and Beach 1988). Pat1 has been shown to phosphorylate Ste11 in vitro at residues Thr-173 and Ser-218 (Li and McLeod 1996). However, the physiological importance of this phosphorylation is unclear as mutation of both residues to aspartic acid (mimicking phosphorylation) or to alanine (non-phosphorylatable) failed to interfere with either mating or sporulation (Li and McLeod 1996). The mechanism by which Pat1 inhibits conjugation has yet to be unequivocally demonstrated.

1.12. Aims and Summary

The aim of the work presented in the following Chapters was to further characterize the function of the Ste11 transcription factor, in order to improve our understanding of how fission yeast cells regulate entry into the sexual differentiation pathway.

An initial approach was to use an established One-hybrid assay to evaluate possible models for transcription activation by the HMG-box containing Ste11 factor. The One-hybrid assay was also used to identify and map an activation domain within Ste11, and to investigate if Ste11 is regulated
by upstream signalling pathways at the level of its transcriptional activity. The analysis of Ste11 protein function was further extended by the study of possible regulatory events at the level of its intracellular localisation pattern or protein expression.

A second experimental approach taken was to identify novel ste11-dependent genes involved in the mating and meiotic pathway, by searching for Ste11-binding sites in the fission yeast genome database. Characterisation of the function of rgs1, a novel Ste11 target gene, revealed that this gene encodes a member of the RGS protein family that negatively regulates pheromone signaling and is essential for mating.
Figure 1-1. Overall scheme of the fission yeast life cycle.
Under rich nutritional conditions, fission yeast cells propagate by mitotic cell division as haploid cells. Nutritional starvation stimulates P and M cells to express their mating-type, resulting in activation of pheromone signalling leading to formation of a diploid zygote by conjugation of two cells of opposite mating-type. Cells that fail to mate enter the stationary phase (G0). The diploid zygote readily enters meiosis and subsequently sporulates producing four haploid ascospores. When re-fed with nutrients ascospores germinate and re-enter growth by mitotic cell cycle.
Germination

Ascopores

Ascus

Mitos

Nutrients

Starvation

Zygote

Mitosis

Go

Meiosis
Figure 1-2. Regulation of *ste11* transcription.

*ste11* expression is regulated by Sty1 stress-activated MAPK pathway and by several nutrient-sensing pathways. *ste11* transcriptional induction upon starvation is dependent on Sty1 activity that phosphorylates Atf1 allowing the heterodimer transcription factor Atf1/Pcr1 to activate *ste11* expression. *ste11* induction by nitrogen starvation, but not by glucose starvation, requires the Rcd1 and Taf1 factors. Repression of *ste11* expression in the presence of nutrients require activation of the GPCR Git3, that results in activation of Pka1 kinase, via activation of the Ga Gpa2, and of the adenylate cyclase Cyr1. Pka1 might inhibit Rst2-mediated activation of *ste11* expression. The Pac2 factor is also necessary for *ste11* repression by nutrients, independently of the Pka1 pathway. Finally, Ste11 functions in a positive feedback loop activating its own transcription.

The Nrd1 RNA-binding protein regulates *ste11* at a post-transcriptional step, possibly decreasing *ste11* mRNA stability or its translation.
**Figure 1-3. The pheromone response pathway in fission yeast.**

P and M cells are stimulated by pheromone factors secreted by cells of opposite mating-type, to undergo changes required for mating. Binding of the mating factors to seven-transmembrane receptors at the plasma membrane results in activation of Gpa1, a Ga protein that transmits the signal to the Byr2/Byr1/Spk1 MAPK pathway. Activation of the MAPKKK Byr2 is tightly regulated and it also requires the input of GTP-bound Ras1, Shk1 (a PAK-family kinase), and of the leucine-zipper Ste4 protein. Both Ras1 and Shk1 are components of a signalling pathway required to mediate the morphological changes involved in the mating process. Pheromone-activated Spk1 MAPK mediates transcriptional induction of several mating and meiotic genes, and is expected to be responsible for the pheromone-induced G1 arrest. The Spk1 pathway might induce transcriptional changes by stimulating the activity of the Ste11 transcription factor. Several of the genes here described, for example ste6, require Ste11 for transcriptional induction, so Ste11 might regulate the pheromone response in a positive feedback way. The red arrows represent transcriptional activation.

The Ste6 protein is a putative GDP-GTP exchange factor for the ras1 gene product. Genetic analysis of the ste6 gene has shown that it is required for mating and for the response to mating pheromones.
Figure 1-4. The role of the Regulators of G-protein Signalling (RGS) proteins (taken from Hepler 1999).

RGS proteins negatively regulate G-protein signalling. (centre) In the absence of extracellular ligand, like neurotransmitter or hormone (H), GPCRs are inactive and G-proteins exist as a αβγ trimer, where Gα is GDP-bound. Ligand (H) binding to the receptor (top) stimulates guanine nucleotide exchange on Gα, resulting in dissociation of Gα-GTP from the βγ dimer. The two released G-protein components (Gα-GTP and Gβγ) activate downstream effectors like ion channels or enzymes that convert substrate molecules (S) into second messenger products (P). Signalling is terminated as a result from the intrinsic GTPase activity of Gα. Most RGS proteins bind and stimulate the intrinsic GTPase activity of Gα-GTP (bottom). GTP hydrolysis by Gα results in reassociation with βγ terminating G-protein signalling.
Effector - R G S

Effector trends in Pharmacological Sciences

+ RGS

- RGS
Figure 1-5. Structural analysis of the RGS domain.

A) The structure of the rat RGS4 (RGS domain)-G<sub>1α</sub> complex.

The RGS domain of RGS4 stabilises the transition state for GTP hydrolysis by G<sub>1α</sub> by interacting with the switch regions of the G<sub>1α</sub> Ras-like domain (Tesmer et al. 1997). However, unlike Ras-GTPase-activating proteins (RAS-GAPs), RGS4 does not contribute to GTP hydrolysis with catalytical residues. Coordinates were obtained from the Protein Data Bank (PDB, www.rcsb.org/pdb/, accession code:1AGR) and prepared for display using the Molecular graphics RasMol software.

B) Solution structure of the RGS domain of human GAIP (Gα Interacting protein).

The solution structure of the RGS-box of GAIP was determined by NMR (de Alba et al. 1999). The RGS box corresponds to nine α-helices that fold into two small subdomains, the larger being a classic right-handed, antiparallel four-helix bundle. This structure is superimposable with the RGS4 RGS-box crystal structure. The three loops that form the Gα interaction surface are labelled with arrows. The diagram was obtained from the Protein Data Bank (PDB, www.rcsb.org/pdb/, accession code:1CMZ).
Table 1-1. Genes involved in mating and meiosis.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Product/function</th>
</tr>
</thead>
<tbody>
<tr>
<td>mat1-Pc</td>
<td>required for mating in P-cells</td>
</tr>
<tr>
<td>mat1-Pm (Pi)</td>
<td>homeobox protein, required for meiosis</td>
</tr>
<tr>
<td>mat1-Mc</td>
<td>HMG-box transcription factor, required in M-cells</td>
</tr>
<tr>
<td>mat1-Mm (Mi)</td>
<td>required for meiosis</td>
</tr>
<tr>
<td>mfm1, 2, 3</td>
<td>M-factor precursors</td>
</tr>
<tr>
<td>map2</td>
<td>P-factor precursor</td>
</tr>
<tr>
<td>mam2</td>
<td>P-factor seven-pass transmembrane receptor</td>
</tr>
<tr>
<td>map3</td>
<td>M-factor seven-pass transmembrane receptor</td>
</tr>
<tr>
<td>gpa1</td>
<td>pheromone-receptor coupled G-protein α-subunit</td>
</tr>
<tr>
<td>byr2</td>
<td>pheromone-activated MAPKK</td>
</tr>
<tr>
<td>byr1</td>
<td>pheromone-activated MAPKK</td>
</tr>
<tr>
<td>spk1</td>
<td>pheromone-activated MAPK</td>
</tr>
<tr>
<td>ras1</td>
<td>small monomeric G-protein; activates pheromone MAPK cascade</td>
</tr>
<tr>
<td>pak1 (shk1)</td>
<td>p21 (cdc42/Rac)-activated kinase; activates pheromone MAPK cascade</td>
</tr>
<tr>
<td>ste4</td>
<td>leucine-zipper protein; regulates Byr2 activation</td>
</tr>
<tr>
<td>ral1 (scd1)</td>
<td>Guanine nucleotide exchange factor for Cdc42</td>
</tr>
<tr>
<td>cdc42</td>
<td>Rho-like small monomeric G-protein; positive role in mating</td>
</tr>
<tr>
<td>sxa2</td>
<td>carboxypeptidase; degrades P-factor</td>
</tr>
<tr>
<td>fus1</td>
<td>formin family; required for cell fusion</td>
</tr>
<tr>
<td>taz1</td>
<td>telomere-binding protein; required for meiosis</td>
</tr>
<tr>
<td>tht1</td>
<td>transmembrane protein; required for karyogamy</td>
</tr>
<tr>
<td>pat1 (ran1)</td>
<td>Ser/Thr protein kinase; negative regulator of mating and meiosis</td>
</tr>
<tr>
<td>mei2</td>
<td>RNA-binding protein; required for premeiotic DNA synthesis and meiosis I</td>
</tr>
<tr>
<td>mei3</td>
<td>inhibitor of Pat1 kinase; induces meiosis</td>
</tr>
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Table 1-2. Genes regulating *ste11* expression in response to nutritional status.

<table>
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<tr>
<th>Gene</th>
<th>Product/function</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>git3</em></td>
<td>heterotrimeric G-protein coupled receptor for glucose</td>
</tr>
<tr>
<td><em>gpa2 (git8)</em></td>
<td>heterotrimeric G-protein α-subunit; activated by glucose</td>
</tr>
<tr>
<td><em>git5</em></td>
<td>heterotrimeric G-protein β-subunit; activated by glucose</td>
</tr>
<tr>
<td><em>git11</em></td>
<td>heterotrimeric G-protein γ-subunit; activated by glucose</td>
</tr>
<tr>
<td><em>pka1 (git6)</em></td>
<td>Catalytic subunit of the cAMP-dependent protein kinase</td>
</tr>
<tr>
<td><em>pde1 (cgsl)</em></td>
<td>3',5'-cAMP phosphodiesterase</td>
</tr>
<tr>
<td><em>cyrl (git2)</em></td>
<td>adenynyl cyclase</td>
</tr>
<tr>
<td><em>sck1</em></td>
<td>putative protein kinase; function related to <em>Pka1</em></td>
</tr>
<tr>
<td><em>sck2</em></td>
<td>putative protein kinase; function related to <em>Pka1</em></td>
</tr>
<tr>
<td><em>ste11 (affl)</em></td>
<td>HMG-box transcription factor</td>
</tr>
<tr>
<td><em>rst2</em></td>
<td>zinc-finger protein (Cys2His2 class), activator of <em>ste11</em> expression</td>
</tr>
<tr>
<td><em>atfl (gad7)</em></td>
<td>leucine zipper transcription factor, positive regulator of <em>ste11</em> expression</td>
</tr>
<tr>
<td><em>pcrl</em></td>
<td>leucine zipper transcription factor, positive regulator of <em>ste11</em> expression</td>
</tr>
<tr>
<td><em>sty1</em></td>
<td>Stress-activated MAPK</td>
</tr>
<tr>
<td><em>wis1</em></td>
<td>Stress-activated MAPKK</td>
</tr>
<tr>
<td><em>wak1 (wik1, wis4)</em></td>
<td>Stress-activated MAPKK</td>
</tr>
<tr>
<td><em>rcdl</em></td>
<td>leucine-rich; required for nitrogen-starvation induction of <em>ste11</em></td>
</tr>
<tr>
<td><em>tafl</em></td>
<td>required for nitrogen-starvation induction of <em>ste11</em></td>
</tr>
<tr>
<td><em>nrd1</em></td>
<td>RNA-binding protein; represses <em>ste11</em>-dependent genes</td>
</tr>
<tr>
<td><em>pac2</em></td>
<td>negative regulator of <em>ste11</em> expression</td>
</tr>
</tbody>
</table>
CHAPTER 2

CHARACTERISATION OF STE11 TRANSCRIPTIONAL ACTIVITY
2) CHARACTERISATION OF STE11 TRANSCRIPTIONAL ACTIVITY

2.1. Introduction

Regulation of transcriptional activation of protein-encoding genes involves interplay between enhancer cis-element binding proteins (activators and repressors) and the general transcription machinery (RNA polymerase II complex) (reviewed by Sauer and Tjian 1997). Therefore, a crucial issue for understanding eukaryotic transcriptional regulation is the elucidation of the mechanism by which the input of activators and repressors is integrated and relayed to the basal transcription machinery, in a specific promoter context. Activators can use distinct mechanisms to relay activating signals to the RNA polymerase II complex (reviewed by Mitchell and Tjian 1989). A class of DNA-binding factors promotes transcriptional activation by interacting directly with the basal transcriptional machinery. One example being the well characterised yeast GAL4 regulator that stimulates transcription by specifically interacting with the general transcription factor TFIIB and increasing the assembly rate of functional pre-initiation complexes (Lin and Green 1991; Lin et al. 1991). GAL4 contains an acidic domain that is sufficient to activate transcription by recruiting TFIIB to promoters, a rate-limiting step in the assembly of pre-initiation complexes. Another type of transcription factor also contains an identifiable activation domain but this domain stimulates transcription by recruiting other activators (or specific cofactors) and/or facilitating their activating function. One example being the recruitment of the TCF factor by the serum response factor (SRF) protein to form an activating ternary complex (together with Elk-1) at the c-fos promoter Serum Response Element (SRE) (Dalton and Treisman 1992; Hill et al. 1993). Even though different mechanisms are employed, a common feature to both classes of transcription factors is the
The fission yeast Ste11 protein belongs to the family of HMG-box containing DNA-binding factors. In particular, Ste11 belongs to the mammalian testis determining factor (SRY) subfamily of HMG proteins that is mainly comprised of transcription factors able to bind DNA in a sequence-specific fashion, unlike other sub-families of HMG-containing factors (Baxevanis and Landsman 1995). Included in this group are the mammalian lymphoid enhancer factors LEF-1/TCF-1, as well as the SRY-related mammalian SOX proteins, and the fungal regulatory factors Mat1-Mc and Mat-a1. Characterisation of the mechanism used by LEF-1/TCF-1 for transcription stimulation raised the possibility that HMG-containing transcription factors could function as "architectural" factors, without containing an identifiable activation domain. Neither LEF-1 nor TCF-1 is able to stimulate transcription from a synthetic enhancer containing multimeric binding sites (Travis et al. 1991). Furthermore, in the context of the T-cell receptor enhancer, LEF-1 transcriptional activity has strict requirements on the presence, arrangement, and occupancy of neighbouring cis-elements (Giese and Grosschedl 1993; Giese et al. 1995). HMG-boxes bind DNA in an unusual way, establishing contacts with the minor groove of DNA and inducing strong bending (between 65° and 130° for the HMG-factors analysed so far). Based on these observations it has been suggested that HMG-box transcription factors act as "architectural" factors binding and bending DNA. The strong DNA bending facilitates the interaction of factors bound to flanking enhancer regions resulting in the assembly of higher-order DNA-multiprotein complexes, and transcription stimulation (reviewed in Grosschedl et al. 1994).

One of the aims of this thesis was to evaluate the "architectural" model for transcription stimulation by HMG-box transcription factors. The approach used was to test if this model is applicable to the fission yeast Ste11 regulator, or whether Ste11 contains a "typical" activation domain essential for its function. A second objective was to investigate if Ste11 is regulated by
upstream signalling pathways at the level of its transcriptional activity. As Ste11 is able to activate its own expression, and its ectopic expression drives cells into mating and meiosis, regulation of Ste11 at the protein level is a crucial step in the control of fission yeast sexual differentiation.

2.2. Results

2.2.1. Ste11 activates transcription during mitotic growth in the LexA One Hybrid assay

To test the ability of Ste11 to function as a classic transactivator, I used a fission yeast one-hybrid assay recently developed in the lab by Peter Stacey (Ph.D. Thesis 1997, University of London). P. Stacey showed that fusions of a S. pombe transcription factor of interest with the E. coli LexA full-length DNA-binding protein can be stably expressed, targeted to reporter constructs and stimulate transcription in fission yeast. In this Thesis, the reporter construct chosen was LopT4LacZ, one of the reporter vectors developed by Peter Stacey (Fig. 2-1, A). LopT4LacZ is a β-galactosidase reporter construct, with a core promoter containing two binding sites for LexA fusion dimers and a mutated (attenuated) TATA box. This particular reporter was chosen because of its low basal activity (or expression) and higher inducibility potential. The full-length Ste11 protein (468 aminoacids) was fused to the LexA protein C-terminal, and expressed under the control of the S. pombe medium strength ADH promoter. LexA fused to the activation domain of the yeast Gal4 regulator (LexA-Gal4AD) and LexA, on its own, were used as positive and negative activating controls, respectively (Fig. 2-1, B). Peter Stacey demonstrated that the LexA-Gal4AD fusion protein is stably expressed and targeted to the nucleus were it can strongly activate transcription of the lacZ ORF from the LopT4LacZ reporter construct, whereas expression of LexA fails to stimulate any detectable expression of the β-galactosidase gene. An h' wild-type strain was transformed
with each of the three activating constructs and the reporter vector, and transcriptional stimulation assessed by a liquid culture β-galactosidase activity assay (Fig. 2-1, B). In a first series of experiments, β-galactosidase activity was quantified in cultures growing mitotically in EMM medium at the mid-log stage. This analysis confirmed the previous observations showing that LexA-Gal4AD strongly activates \textit{lacZ} transcription from the LopT4LacZ reporter construct, while LexA on its own is inactive. I found that LexA-Ste11, the full-length Ste11 fusion, was able to strongly activate \textit{lacZ} transcription during the mitotic cycle at levels similar to the LexA-Gal4AD fusion (145 Miller units vs. 176, respectively).

2.2.2. Fine mapping of transcriptional activation domains within Ste11

To test the possibility that non-specific bending of the DNA helix by the HMG box contributes to the ability of LexA-Ste11 to stimulate \textit{lacZ} transcription, I constructed a LexA-Ste11 fusion lacking the 92 N-terminal aminoacids of Ste11, deleting the HMG-box (LexA-Ste11 ΔN92, see Fig. 2-2). LexA-Ste11 ΔN92 retains full transcriptional activity suggesting that the HMG-box is not required for transcriptional stimulation. Importantly, it has been shown that LexA interacts with its DNA-binding site with high affinity and specificity, without inducing any detectable bend in the DNA helix (Lloubes et al. 1988). These observations demonstrate that for LexA-Ste11 protein, in the conditions of this assay, DNA-bending of the promoter region is not involved in its ability to stimulate transcription.

Since the Ste11 HMG-box DNA-binding domain is, insofar, its only characterised functional domain, I attempted to map its transcriptional activation domain. A series of ten LexA-Ste11 fusions were constructed and tested for activity in the LexA One hybrid assay as described above (Fig. 2-2). As mentioned in the previous section, removal of the Ste11 N-terminal 92 aminoacids does not affect transcriptional activity. Similarly, further N-terminal truncations deleting 260 and 363 aminoacids (ΔN260 and ΔN363) did not
interfere with Ste11 transcriptional activity. By contrast, deletion of the C-terminal 104 amino acids completely abrogated LexA-Ste11 ability to stimulate lacZ transcription. In fact, all the constructs tested that lacked this C-terminal region were inactive in the One Hybrid assay. I conclude that the C-terminal 105 amino acids of Ste11 (i.e. residues 365-468) are necessary and sufficient for its ability to stimulate transcription in this assay. Furthermore, these results suggest that this is the single activation domain present in the Ste11 protein. Suggestively, the C-terminal activation domain on its own (ΔN363) is a significantly stronger activator than either LexA-Ste11 full-length or LexA-Ste11 ΔN92. This observation raises the possibility that the Ste11 central region encompassing residues 93-260 might constitute an intramolecular inhibitory domain targeting the C-terminal transcription activation domain.

2.2.3. The intrinsic transactivation activity of Ste11 is not influenced by stress conditions or nutritional status

I next addressed the question of whether modulation of the activity of this activation domain could contribute to the regulation of Ste11 protein function, and consequently to the control of entry into mating and meiosis. The finding that Ste11 is able to bind its own promoter regulatory region, and can commit cells to the sexual differentiation pathway by stimulating its own transcription in a positive feedback loop, has raised the possibility that upstream signalling pathways previously implicated in the control of stell transcription might actually regulate Ste11 at the post-transcriptional level (Kunitomo et al. 2000).

The Sty1 MAP kinase pathway plays an essential role in the survival of fission yeast cells exposed to multiple environmental insults. Cells lacking Sty1 MAPK or its downstream effector, the Atf1 transcription factor, are also sterile (5-10% efficiency of the wild-type). This mating defect has been attributed to a defect in the induction of ste11 expression during the commitment phase of the sexual development pathway (Kato et al. 1996; Shiozaki and Russell 1996;
Wilkinson et al. 1996). To investigate if Sty1 MAPK activation modulates, or is necessary, for the ability of Ste11 to stimulate transcription (when tethered to an heterologous promoter), I measured β-galactosidase activity resulting from LexA-Ste11 in cells under osmotic stress conditions that result in Sty1 kinase activation (0.5M and 0.9M of NaCl) or in cells lacking sty1 gene function. The transactivation strength of LexA-Ste11 was significantly reduced in wild-type cells subjected to a 4 hours treatment with either 0.5M or 0.9M NaCl, however this effect was not specific to LexA-Ste11, as the activity of the positive control LexA-GAD was also reduced to a similar degree (Fig. 2-3). When I performed the One-hybrid assay in a sty1' genetic background, β-galactosidase activity was detected either for the LexA-GAD or the LexA-Ste11 transactivators. sty1' cells transformed with both reporter and activating constructs grew very poorly and appeared sick (abnormal morphology and septation) under microscopic observation. The reason for the toxic effects of the One-hybrid constructs on sty1' cells, or for the lack of any detectable β-galactosidase activity in these cells is unknown. In summary, osmotic stress and Sty1 kinase activation do not stimulate LexA-Ste11 transcriptional activity when Ste11 is strongly bound to an heterologous promoter, but I could not address the requirement of Sty1 basal activity for LexA-Ste11 activity.

Addition of cAMP to nutrient-low culture medium inhibits fission yeast mating and meiosis (Calleja et al. 1980), which has been suggested to be a consequence of cAMP-Pka1-dependent repression of ste11 expression (Sugimoto et al. 1991). I investigated if nutritional status, and consequently Pka1 kinase activation status, regulated ste11 function at the level of its transcriptional activity. To test this possibility, I assayed for LexA-Ste11 dependent β-galactosidase activity in wild-type cells growing in the mid-log phase that had been treated with 15 mM cAMP for 4 hours. No effect on the transactivation potential of LexA-Ste11 was observed in these conditions, and unlike osmotic stress, cAMP treatment did not interfere with the activity of LexA-GAD in the assay (Fig. 2-3, A). Thus, increasing Pka1 activation status appears not to result in inhibition of Ste11 transactivation potential. Nutritional
starvation decreases the cellular cAMP concentration, and consequently, the level of Pka1 kinase activation. This results in the induction of ste11 expression. When I tested the effect of nitrogen starvation on the full-length LexA-Ste11 construct, no specific alteration of transcriptional activity levels was observed (Fig. 2-3, B). After four hours of growth in EMM(-NH₄Cl), the transactivation activity of LexA-Ste11 was strongly reduced, but a comparable reduction occurred to the activity of the positive control LexA-GAD. In this series of experiments, I also tested three other LexA-Ste11 constructs, including the C-terminal activation domain on its own (LexA-Ste11 ΔN363). The aim was to map approximately a hypothetical region that mediated a starvation-dependent stimulation of Ste11 transcription activity. Interestingly, the transcription activity of LexA-Ste11 ΔN363 is not as significantly reduced after four hours of nitrogen starvation. If the results obtained under starvation conditions are normalised to the activity of LexA-GAD, the conclusion is that the transactivation potential of the Ste11 C-terminal activation domain is induced, while the activity of the full-length Ste11 or of the LexA-Ste11 ΔN260 construct is not significantly affected.

2.2.4. Ste11 C-terminal activation domain is required for its mating function

Having shown that residues 364-468 of Ste11 are essential for its activity in the One-hybrid assay, I decided to investigate if this C-terminal activation domain is essential for Ste11's essential role in mating and meiosis. The functionality of the three LexA-Ste11 fusions were tested in vivo by transformation of an homothallic ste11'' strain (h⁰ ste11::ura4*) and quantification of mating efficiency after two days growth in nitrogen-free media (EMM-NH₄Cl). The three LexA-Ste11 constructs chosen for this experiment were the full-length LexA-Ste11, ΔC104, and ΔC305. The LexA-Ste11 plasmids used in the One-hybrid assay could not be transformed directly in h⁰ ste11::ura4', as these plasmids use the ura4' marker. Therefore, before transformation, the ura4' marker was replaced by the LEU2 marker by DNA
subcloning. Deletion of ste11 makes cells profoundly sterile, and the presence of zygotes, asci, or spores is never detected, even if several thousands of starved cells are observed. In contrast, expression of full-length LexA-Ste11 from the medium strength ADH promoter (comparable to induced nmt41 promoter) results in a weak partial rescue of mating and meiosis, as 6% of the transformed cells succeed in conjugating with a mating partner and entering meiosis (Fig. 2-4, A). However, when either the AC104 or AC305 LexA-Ste11 constructs were tested for their ability to rescue the Astel11 sterility defect, not a single zygote, ascus or spore was observed in three independent transformations, and in three experiments for each transformant (Fig. 2-4, A). To exclude the possibility that truncation of the Ste11 C-terminal region affected protein levels, I assessed the expression of the three LexA-Ste11 protein fusions by immunoblotting analysis (Fig. 2-4, B). I performed this analysis on protein extracts of transformants (h^stel11 background) growing vegetatively (mid-log phase), using both a monoclonal antibody against LexA, and a monoclonal antibody against the N-terminal region of Ste11 (a kind gift from O. Nielsen). The data shows that the lack of functionality of C-terminal truncations cannot be attributed to a reduced level of expression. In fact, LexA-Ste11 AC104 was expressed at a similar level to that of the full-length construct, and LexA-Ste11 AC305 was expressed at a significantly higher level (Fig. 2-4, B).

The reduced functionality of the full-length LexA-Ste11, when compared with other described Ste11 tagged fusion proteins, is surprising since this fusion is expressed and active in the One-hybrid assay. The presence of a second DNA-binding domain might result in a reduced binding of Ste11 to its endogenous binding sites. Nevertheless, the data supports that Ste11's activation domain is not only required for its transactivation ability in the One-hybrid assay, but also for its essential function in promoting mating and meiosis. Removal of the central Ste11 region (residues 164-363) from LexA-Ste11 constructs appears to result in a higher fusion protein expression levels.
2.3. Summary

The main conclusion from the analysis of LexA-Ste11 fusions presented in this Chapter is that Ste11 contains a "classic" transcription activation domain, able to stimulate transcription when bound to a synthetic enhancer. This result goes against the general applicability of an earlier proposed model suggesting that HMG-box transcription factors act as "architectural" activators regulating the assembly of higher-order complexes of transactivators (Travis et al. 1991). Recent reports suggest that Ste11 is not the only HMG-box transcription factor that fails to function as an "architectural" factor, as other murine and human HMG-box factors have also been found to have "classic" activation domains. The transactivating function of SOX9 was mapped to a C terminal, non-acidic domain, which is rich in proline, glutamine and serine (Sudbeck et al. 1996). Haploinsufficiency for SOX9 causes human campomelic dysplasia syndrome, a skeletal malformation condition, associated with XY sex reversal. Interestingly, nonsense and frame shift mutations described in campomelic dysplasia patients result in truncation of the SOX9 activation domain, suggesting that loss of SOX9 transactivation function impairs gonadal and skeletal development (Sudbeck et al. 1996). Murine Sox18 also contains a transactivation domain that has been mapped to a 92 amino acid region immediately C-terminal to the HMG-box (Hosking et al. 2001). The Ste11 activation domain has a high serine content (22% of residues), and is very acidic with an overall net charge of minus 8, and an isoelectric point value equal to 4.5 (Fig. 2-5, A). In comparison to the remaining part of the Ste11 protein, the activation domain is also enriched in arginine residues (9.5% vs. 4.9%) and aspartate residues (6.7% vs. 3.8%). There is a low sequence homology between the SOX9 and Ste11 activation domains (Fig. 2-5, B) but not with other acidic, or serine-rich activation domains (Bours et al. 1993; Tjian and Maniatis 1994). Further elucidation of the molecular mechanisms and interactions used by Ste11 and SOX9 will be required to test the relevance of the limited homology between both activation domains, and the degree to which the function of sequence-specific HMG-box containing activators has been conserved through evolution.
The results presented in this Chapter, also demonstrate that Ste11 is able to activate transcription during the mitotic cell cycle if promoter-bound, and that the function of the activation domain is not stimulated by Sty1 MAPK activation, neither repressed by raising Pka1 activation status. One implication of these observations is that in nutrient-rich conditions, Ste11 protein activity is inhibited at a different level. The issue of whether inhibition of ste11 function during mitotic growth occurs at the level of its transcription or its transcriptional activity remains unclear.

The results also hint that internal residues (residues 164/363) may repress the adjacent C-terminal activation domain both under starving and non-starving conditions. Suggestively, the absence of this internal segment results in a high level of LexA-Ste11 fusion protein expression (Fig. 2-4, B). It will be interesting to study whether this repressive region regulates Ste11 stability or degradation rates. Deletion of the Ste11 C-terminal activation domain results in a complete lack of Ste11 activity both in vivo and in the one-hybrid assay. The possibility that this loss of activity is due to an incorrect targeting of Ste11 to the nucleus can be excluded, as a recent large-scale intracellular localisation study of fission yeast proteins showed that the Ste11 N-terminal HMG-box region is sufficient for nuclear localisation (Ding et al. 2000).

One caveat of the approach used in this Chapter is that the one-hybrid assay used is based on the heterologous targeting of Ste11 to LexA-binding sites, bypassing the analysis of Ste11 binding to TR-boxes in vivo, in the context of its targeted promoters. One important issue that should be addressed in future work is the question of whether endogenous Ste11 binds TR-boxes constitutively during vegetative growth and starvation, or whether Ste11 DNA-binding is regulated by entry into starvation.
Figure 2-1. Ste11 strongly activates transcription in a LexA one-hybrid assay.

A) Scheme of the activating construct (pLexX) used to express LexA-fusion proteins and reporter vector (LopT4LacZ) carrying two binding sites for LexA fusion dimers driving expression of the LacZ ORF.

B) The indicated LexA-Ste11 fusion proteins were constructed (Material & Methods) and assayed for transcriptional activity in the LexA one hybrid assay. β-galactosidase activity was measured in mid-log cultures grown in EMM medium. The activity values shown represent mean values for a minimum of four independent experiments. The N-terminal HMG-box of Ste11 is shown as a red box.
**A)**

**LexA One-hybrid assay vectors**

**Activating construct:** pLex-X

ATG

<table>
<thead>
<tr>
<th>ADH</th>
<th>LexA(1-202)</th>
<th>X</th>
</tr>
</thead>
</table>

promoter

**Reporter construct:** LopT4LacZ

TATA box; ATAAA

<table>
<thead>
<tr>
<th>ura4-*</th>
<th>ATG</th>
</tr>
</thead>
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30bp 11 58bp

<table>
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<tr>
<th>LEU2</th>
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</table>

2x LexA binding site

**B)**

<table>
<thead>
<tr>
<th><strong>β-galactosidase (Miller Units)</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td>LexA</td>
</tr>
<tr>
<td>LexA-Gal4AD</td>
</tr>
<tr>
<td>LexA-Ste11</td>
</tr>
</tbody>
</table>
Figure 2-2. Mapping of a transcriptional activation domain in Ste11.
The indicated LexA-Ste11 fusion proteins were constructed (Material & Methods) and assayed for transcriptional activity in the LexA one hybrid assay using the LopT4LacZ reporter construct. β-galactosidase activity was measured in mid-log cultures grown in EMM medium. The activity values shown represent mean values for a minimum of four independent experiments. The N-terminal HMG-box of Ste11 is shown as a red box.
LexA-Ste11 fusions:

![Graph showing β-galactosidase activity (Miller Units) for different LexA-Ste11 fusions.](image-url)
Figure 2-3. Regulation of Ste11 transcriptional activity.
The indicated LexA fusion proteins were assayed for transcriptional activity in the one hybrid assay under the conditions indicated in the graph legend. The β-galactosidase activity values shown represent mean values for a minimum of four independent experiments.

A) The mid-log cultures of the wild-type host strain transformed with the indicated combinations of activating and reporter vectors were either subjected to osmotic stress treatment (0.5M and 0.9M NaCl) for 4 hours, or to a 4 hours treatment (15mM cAMP and 7.5mM caffeine) that activates the Pka1 kinase pathway. LexA fusions were also tested in a h' sty1' strain background, using samples from mid-log cultures.

B) Transcriptional activity of LexA-Ste11 fusion proteins under nitrogen starvation. Wild-type cells transformed with the indicated combinations of activating and reporter vectors were grown to mid-log phase in EMM, washed, resuspended in EMM(-NH4Cl), and incubated for 4 hours at 30°C. β-galactosidase activity was measured in mid-log and starved culture samples.

In this figure, asterisks represent no detectable signal.
A

LexA

LexA-GAD

LexA-Ste11

β-galactosidase activity (Miller Units)

B

LexA

LexA-Gal4AD

LexA-Ste11

LexA-Ste11 ΔN260

LexA-Ste11 ΔN363

LexA-Ste11 261/364

β-galactosidase activity (Miller Units)
Figure 2-4. Analysis of the functionality and expression of LexA-Ste11 fusions.
A) The functionality of full-length LexA-Ste11, and two C-terminal truncated LexA fusion proteins (ΔC104 and ΔC305) was tested by quantifying their ability to rescue the complete sterility defect of an homothallic Δste11 strain after 2 days growth in EMM(-NH4Cl). Mating and sporulation was quantified as described in Material and Methods. Also shown are phase-contrast micrographs of cells nitrogen-starved for 24 hours in EMM(-NH4Cl), where zygotes, asci and free spores are indicated by black arrows.
B) Immunoblotting detection of the LexA-Ste11 fusions described above. h90 Δste11 cells transformed with plasmids expressing the indicated LexA-Ste11 fusion proteins were grown to mid-log phase and culture samples taken for analysis. Total protein extracts were analysed by immunoblotting with anti-LexA and anti-Ste11 monoclonal antibodies. Black arrows point the migration position of the three LexA-Ste11 fusions. Equal protein loading was confirmed using an anti-α-tubulin monoclonal antibody.
A

**LexA-Ste11:**

- FL
  - LexA: 16-91
  - HMG: 468

- ΔC104
  - LexA: HMG 364

- ΔC305
  - LexA: HMG 163

**EMM - NH4Cl (2 days)**

- Mating:
  - 6.1% (± 2.9%)
  - 0%
  - 0%

B

**h^30 Δste11 + pLexA-Ste11(LEU2) fusions**

- FL
  - α-LexA
  - α-tub

- ΔC104
  - α-LexA
  - α-tub

- ΔC305
  - α-LexA
  - α-tub
Figure 2-5. Analysis of peptide properties and sequence homology of the Ste11 activation domain.

A) Scheme comparing the net charge and isoelectric point values of Ste11 C-terminal activation domain with the ones for the N-terminal region (containing the HMG-box) and for the full-length protein.

B) Identification of sequence homology between Ste11 and Sox9 transcriptional activation domains. Alignment was generated using ClustalX and Boxshade programs. Conserved amino acid similarity is displayed in light blue, and conserved identity in purple. Percentage of similarity and identity between the two domains is indicated. Protein sequences used in the alignment are: *S. pombe* Ste11 (A41518) and *Drosophila* Sox9 (P48436).
Net Charge: +12 -8
pl: 10.8 4.1

pl: 7.8

Identity/Similarity:
28%/18%
CHAPTER 3

REGULATION OF Ste11 FUNCTION
3) REGULATION OF Ste11 FUNCTION

3.1. Introduction

The results presented in the last Chapter demonstrate that Ste11 is able to activate transcription during the mitotic cell cycle if promoter-bound, and that the function of the activation domain is neither stimulated by Sty1 MAPK activation, nor repressed by raising Pkal activation status. One consequence of these observations is that, in nutrient-rich conditions, Ste11 function must be inhibited at a different level. Ste11 protein might be specifically degraded under those conditions, prevented from binding its target cis-element on promoters, or even excluded from the nucleus when expressed at physiological levels. Another possibility is that nutrient-activated Pka1 kinase prevents the reinforcement of ste11 function (by its self-mediated positive feedback loop), at the level of ste11 transcription. For example, Pka1 kinase could directly block Ste11 activity or regulate the activity of a distinct transcription factor playing a crucial role in the regulation of ste11 transcription. Despite the crucial role of Ste11 in the fission yeast sexual differentiation pathway, there has been little detailed analysis of its regulation by upstream signalling pathways. Thus, the aim of the work described in this Chapter was to identify novel aspects of Ste11 protein regulation during the commitment phase to mating and meiosis, including the possibility that it is regulated at the level of intracellular localisation or protein expression, and to identify the signalling pathways that may be responsible for any regulation seen.

3.2. Results

3.2.1. Ste11 intracellular localisation

As described previously, Pat1 kinase (an inhibitor of mating and meiosis) phosphorylates Ste11 in vitro at residues Thr-173 and Ser-218, although the
physiological significance of this phosphorylation is still unclear (Li and McLeod 1996). The same study showed that Ste11, when overexpressed, localised mainly to the nucleus, but that this localisation was blocked when Pat1 was also overexpressed. These results suggested that regulation of Ste11 localisation might be an important mechanism controlling initiation of the mating pathway. To examine the intracellular localisation of Ste11 protein, I expressed Ste11 with an N-terminal green fluorescent protein (GFP) tag from the intermediate strength \textit{nmt41} inducible promoter. GFP-Ste11 was fully functional, as its expression could efficiently rescue the sterility of \textit{\Delta}ste11 cells (Fig. 3-1, A). During vegetative growth, in nutrient-rich conditions, GFP-Ste11 localised mainly to the nucleus (Fig. 3-1, B). GFP-Ste11 localisation in wild-type cells was also followed during nitrogen-starvation induced mating, but no significant change in the localisation pattern was observed (data not shown). These observations do not support the previous suggestion that Ste11 is inactivated by Pat1 kinase during mitosis by being prevented from accumulating in the nucleus (Li and McLeod 1996). The next question I addressed was whether the nuclear accumulation of Ste11 was affected by mutations in different upstream signalling pathways. No discernible alteration in the localisation pattern or in expression levels (judged by the intensity of GFP signal) was observed in strains with deletions affecting the Sty1 MAPK or Pka1 pathways (Table 3-1). Furthermore, nuclear accumulation of Ste11 was not exacerbated by loss-of-function mutation in the \textit{pat1} gene (\textit{pat1-114}).

3.2.2. \textbf{Ste11 is degraded by the 26S proteasome pathway}

Analysis of the expression and intracellular distribution of GFP-Ste11 by fluorescence microscopy suggested that Ste11 might be an unstable protein, as the protein levels of the fusions were significantly lower than GFP alone. Basic cellular processes like regulation of cell cycle, control of signal transduction pathways, and differentiation, can be controlled via specific degradation of key protein regulators (reviewed in Ciechanover et al. 2000). To investigate if Ste11
is a specific target substrate for the 26S proteasome degradation pathway, I analysed GFP-Ste11 intracellular distribution and total protein levels in the temperature-sensitive mutant \textit{mts3-1} strain. \textit{mts3-1} cells are defective in subunit 14 of the 26S proteasome protease, and after growth at the restrictive temperature, mutated Mts3 fails to be incorporated into the 19S regulatory complex rendering the 26S proteasome unable to degrade ubiquitin conjugates (Gordon et al. 1996; Seeger et al. 1996). \textit{mts3-1} cells were transformed with the vector p409GFPSte11, and incubated for 4 hours at the restrictive temperature of 36°C. This resulted in a strong increase in GFP-Ste11 fusion protein levels, as determined by western blotting, when compared with cultures grown at the permissive temperature (Fig. 3-2, A). Fluorescence microscopy analysis of cells in both conditions also revealed an increase in both total and nuclear GFP-Ste11 fluorescent signal (Fig. 3-2, B). This experiment suggested that the proteasome degradation pathway is involved in regulating Ste11 protein levels.

3.2.3. Ste11 regulation during the mitotic cell cycle

Careful observation of the pattern of GFP-Ste11 localisation in vegetatively growing cells (asynchronous culture) suggested that cells in the G1 phase of the mitotic cell cycle (large cells with two divided nuclei that have not initiated septation) have a brighter nuclear GFP-Ste11 signal (Fig. 3-1, A). This intensity increase could potentially result either from an increase in total GFP-Ste11 protein levels, or from a dynamic concentration of GFP-Ste11 in the nucleus. Both mechanisms have the potential to contribute to an increase in the transcriptional activity of Ste11 since its activation domain is fully active during the mitotic cell cycle (see Chapter 2). It is interesting to note that it has been previously shown that pheromone-induced transcription (positively regulated by Ste11) occurs only in the G1 phase in cells where the requirement of nitrogen starvation for mating has been bypassed (Stern and Nurse 1998). Stimulation of Ste11 activity during G1 could be an important mechanism ensuring that transcription of mating genes (and consequently mating itself) only occurs in
G1 arrested cells. To test this hypothesis, I overexpressed GFP-Ste11 in the temperature-sensitive cell cycle mutant strains, \( h^+ \) \( cdc25-22 \) and \( h^+ \) \( cdc10-129 \), and analysed its intracellular distribution at the permissive and restrictive temperatures. G1 arrest was induced in \( cdc10-129 \) cells by shifting cultures from 25°C to 36°C and incubating for 4 hours. Microscopic observation of these cells suggested that arresting the mitotic cell cycle in G1 intensified the GFP-Ste11 nuclear fluorescence signal (Fig. 3-3, A). This enhancement of GFP-Ste11 nuclear accumulation was not observed in G2-arrested \( cdc25-22 \) cells (data not shown). The next question to be addressed was whether the enhanced nuclear signal reflected a redistribution of fusion protein or a cell-cycle specific stabilisation of the GFP-Ste11 fusion protein. To investigate if regulation of Ste11 protein stability is cell-cycle modulated, I performed western blotting analysis of a synchronised cell culture: \( cdc25-22 \) cells transformed with the p409GFP-Ste11 plasmid were grown at the permissive temperature for 12 hours in thiamine-free media, then shifted to the restrictive temperature for 4 hours so that cells accumulated at the G2-M stage of the mitotic cell cycle. The cells were then shifted back to the permissive temperature resulting in synchronised progression through the cell cycle as shown by determination of the septation index (Fig. 3-3, B). Immunoblotting of samples taken at various times, using an anti-GFP monoclonal antibody, revealed that the levels of GFP-Ste11 did not increase significantly during the G1 phase (Fig. 3-3, B). To exclude the possibility that the observed lack of regulation of Ste11 stability in the latter experiment resulted from overexpression of a tagged form of Ste11, I monitored Ste11 protein levels using a monoclonal anti-Ste11 antibody. Immunoblotting comparison of whole cell extracts from non-synchronous and G1-arrested \( cdc10-129 \) cells shows that endogenous Ste11 protein levels are, in fact, not increased during a G1 mitotic block (Fig. 3-3, C, lanes 2 and 3). Similar analysis of cell extracts from \( cdc10-129 \) cells expressing GFP-Ste11 from the nmt41 promoter, also failed to show an increase of Ste11 protein levels in G1 (Fig. 3-3, C, lanes 4 and 5). Interestingly, overexpression of GFP-Ste11 caused an increase in endogenous Ste11 protein levels presumably due to Ste11 activating its own
transcription in a positive feedback manner. This increase in the pool of endogenous Ste11 protein appears to result in an altered electrophoretic migration, as under these conditions, Ste11 runs as multiple bands, the exact number of which could not be readily determined. This result suggested that if Ste11 concentration rises beyond a threshold point it might become a target for protein modifications by upstream regulators. However, the exact reason for migration of Ste11 as multiple bands remains to be determined.

3.2.4. Expression and stability of Ste11 protein after nitrogen starvation

One caveat with the approach of using the temperature-sensitive h^* cdc10-129 mutant strain to obtain a G1 arrest is the qualitative difference between this type of early G1 block and a physiological G1 arrest in an homothallic wild-type strain subjected to nutritional starvation where the pheromone-responsive MAPK cascade is activated. As mentioned previously, Ste11 is required for pheromone-mediated stimulation of mating and meiotic gene transcription, so Ste11 could potentially be stabilised or modified by the Spk1 MAPK pathway. To investigate that hypothesis, I monitored endogenous Ste11 protein levels in an h^90 wild-type strain during the mating process using an anti-Stell monoclonal antibody (Fig. 3-4, A). Surprisingly, Ste11 protein was detected in exponentially growing h^90 wild-type cells but its level fell below detection as rapidly as 3 hours after initiation of nitrogen starvation, and remained undetectable for up to 21 hours post-starvation (Fig. 3-4, A). This result is very surprising since in h^90 wild-type cells ste11 mRNA levels peak after 4 hours of nitrogen starvation and remain high for up to 8 hours further (Benton et al. 1993; Okazaki et al. 1998; Tsukahara et al. 1998). A previous study of Ste11 expression using the same anti-Ste11 antibody showed that Ste11 protein levels rise slightly after one hour of nitrogen starvation and then decline becoming undetectable after a further 3 hours (S. Kjaerulff, Ph.D. Thesis, University of Copenhagen, 1997). To distinguish between transcriptional and protein stability regulatory effects, I overexpressed myc-tagged Ste11 from the intermediate-
strength nmt41 promoter. Myc-Ste11 protein levels were not significantly affected during 4 hours of nitrogen starvation (Fig. 3-4, B). However, if a GFP-tagged Ste11 fusion was expressed at low levels from the weak nmt81 promoter, then during a similar period of starvation a fall in Ste11 levels to below the level of detection occurred (Fig. 3-4, C). One possible interpretation of these results is that if Ste11 levels rise beyond a threshold level it becomes resilient to degradation maybe due to saturation of a specific recognition or modification event. The possibility that Ste11 protein function might only be required during the first few hours of exposure to starvation, while pheromone signalling is being established, is intriguing and requires a more detailed future analysis. It will be important to eliminate the possibility that upon initiation of the mating process by nitrogen starvation, Ste11 associates strongly with chromatin and becomes non-extractable using the adopted protein extraction protocol.

3.2.5. Analysis of ste11 overexpression effects in homothallic Δatf1, Δsty1, and Δbyr1 cells.

As mentioned before, mutations in Sty1 MAPK or in the transcription factor Atf1 result in sterility, and in a failure to arrest in G1 and induce ste11 expression under nitrogen starvation conditions (Kato et al. 1996; Shiozaki and Russell 1996; Wilkinson et al. 1996). The sterile phenotype of these mutants has been explained in terms of a defect in the transcriptional activation of ste11 under nutritional starvation. The failure to arrest in G1 under nitrogen starvation has been interpreted as a ste11-unrelated phenotype, resulting from a defective G2/M transition control, thus allowing G1 cells to retain a cell size larger than the critical size, which would result in cells undergoing an extra round of DNA replication under starvation conditions (Kanoh et al. 1996). However, no direct analysis of a possible contribution of Ste11 towards the ability of cells to arrest in G1 when starved has been reported.

To test the possibility that Ste11 is necessary for the G1 arrest induced by nitrogen starvation, homothallic wild-type and Δste11 cells were grown in EMM
medium to mid-log phase, washed and resuspended in EMM lacking a nitrogen source (EMM -NH4Cl). Cultures were incubated at 30°C, and samples collected at the time points indicated for DNA content analysis by FACS scanning (Fig. 3-5). Wild-type cells sense nitrogen starvation and the majority arrested in G1 with 1C DNA content after 24 hours incubation. Interestingly, h^90 Δste11 cells are consistently slower than WT cells to initiate G1 arrest, and after 24 hours of nitrogen starvation the percentage of the population arrested in G1 is significantly smaller (Fig. 3-5). This result shows that even though Ste11 is not strictly required for cells to arrest in G1 upon nitrogen starvation, it contributes, in homothallic cells, to the extent and speed of that arrest. It is worthwhile mentioning that Ste11 is required for the transcription of genes involved in pheromone signalling, and that it has been shown that heterothallic cells also arrest slower than homothallic cells, due to the lack of pheromone signalling. The slower G1 arrest observed in h^90 Δste11 might be a consequence of the lack of pheromone communication in these cells. In the following experiment, I investigated if overexpressing functional GFP-Ste11 in homothallic Δatfl and Δsty1 strains could rescue the G1 arrest and sterility defects of these cells. As shown in Fig. 3-6A, Δatfl cells arrested poorly in G1 after 24 hours of nitrogen starvation, and overexpression of GFP-Ste11 did not rescue this defect. Similarly, overexpression of GFP-Ste11 also failed to rescue the inability of Δsty1 cells to respond to nitrogen starvation by arresting in G1. In this set of experiments I also examined the effect of overexpressing GFP-Ste11 in homothallic cells deleted for the pheromone-response MAPKK byr1 gene. Surprisingly, h^90Δbyr1 cells (expressing GFP from the p409 vector) did not arrest in G1 after 24 hours of nitrogen starvation. This observation is unexpected since heterothallic cells do arrest in G1 phase upon nitrogen starvation, suggesting that pheromone signalling is not essential for the cell cycle arrest. One possible explanation for this result is that the pheromone-responsive MAPK pathway might be active at a constitutive basal level in the absence of pheromone communication, and that it plays a crucial role in the induction of G1 phase arrest in response to nutritional starvation. After a longer period of nitrogen
starvation (3 days), cultures of the above mentioned strains were examined in order to quantify any mating and meiotic activity (Fig. 3-6, B and C). As reported previously, h^o Aatf1 cells conserve a small residual mating and meiotic ability (7%). Ectopic expression of ste11 significantly improved the mating and meiotic efficiency of h^o Aatf1 cells to 26%. The graph shows that about 55% of the h^o Aatf1 (overexpressing ste11) cells that mated, also concluded meiosis and sporulated (reaching the ascus or free spore stages). Interestingly, in h^o Astyl cells the overexpression of ste11 caused only a slight increase in their very poor mating efficiency (from 3% to 8%), and the vast majority of the cells that did mate failed to sporulate. This was not due to a failure of GFP-Ste11 expression as levels were similar to those obtained in wild-type cells, and furthermore, the fusion protein had the same intracellular distribution pattern (data not shown).

In h^o Abyr1 cells, the overexpression of ste11 did not result in any detectable suppression of their complete sterile defect (not shown), but did have a strong and surprising effect on their morphology upon nitrogen starvation (Fig. 3-6, B). A significant percentage of these cells presented long protrusions on one of the tips, resembling long conjugation tubes. As deletion of byr1 completely abolishes cellular responses to extracellular pheromone, the morphological effect resulting from ste11 overexpression is similar to the pheromone-independent formation of abnormally extended conjugation tubes in heterothallic cells expressing the constitutively active Gpa1^Q6 allele (Obara et al. 1991). One observation that complicates the interpretation of this result is that ectopic expression of ste11 did not induce any morphological alteration (or sterility rescue) on cells deleted for the pheromone-responsive spkl MAPK gene (data not shown). One possible explanation for this result is that byr1 is expressed constitutively, while spkl might be induced by nitrogen starvation in a ste11-dependent manner (see Chapter 4). Ste11 activity might depend on the presence of Spk1, which could be active at a basal level in the absence of Byr1.
3.3. Summary

The results presented in this Chapter show that Ste11 is a protein that accumulates in the nucleus during the mitotic cell cycle and during the mating process. Ste11 is an unstable protein that is targeted by the proteasomal degradation pathway, but this targeting does not appear to correlate with progression through any specific phase of the cell cycle. Nuclear accumulation of Ste11 is intensified during G1, even though total protein levels remain unchanged, so this effect might result from a dynamic relocalisation of a relatively small cytoplasmic pool. The physiological significance and the molecular mechanism of this increase in Ste11 nuclear accumulation in G1 are unknown, but it might help to restrict pheromone-dependent transcription and conjugation to the G1 phase of the cell cycle. G1 arrest is an essential requisite for mating, and mutants like Δrum1, nuc2-663, and Δsty1, that fail to do so are sterile (Moreno and Nurse 1994; Kumada et al. 1995; Shiozaki and Russell 1996). It will be important to confirm these observations with an analysis of the localisation pattern of untagged Ste11 expressed from its own promoter.

Surprisingly, the results presented here also suggest that Ste11 might not be required after the early stages of the mating and meiotic pathway, as Ste11 protein (either endogenous or when expressed from a weak promoter) could not be detected by immunoblotting after three hours of starvation. Ste11-dependent genes reach maximal induction after 4 hours of starvation (maximum), so it will be important to investigate whether Ste11 is continually required to maintain transcription of its target genes or whether it is just required to act as a switch, altering the transactivation state of promoters during early starvation. This analysis could be coupled with the study of in vivo TR-box occupancy by Ste11 in promoters of Ste11 regulated genes, to clarify the mechanisms used by Ste11 to activate transcription.

My results also show that overexpression of ste11 can partially rescue mating and meiosis in Δatf1 cells, and confirm previous observations suggesting that overexpressed ste11 only partially rescues mating in Δsty1 cells (Kato et al. 1996). Thus, while Δatf1 sterility can be explained by the lack of ste11
transcription in these cells, \( \Delta sty1 \) sterility must also be the result of either a specific meiotic defect or of a decrease in general "fitness" that happens to affect meiosis in a non-specific way.
Figure 3-1. Ste11 localises to the nucleus.

A) Functionality of the GFP-Ste11 fusion protein. The functionality of GFP-Ste11 was confirmed in vivo by testing its ability to rescue the complete sterility defect of a homothallic Δste11 strain after 2 days growth in EMM. Phase-contrast micrographs of cells nitrogen-starved for 48 hours in EMM.

B) N-terminal tagged GFP-Ste11 fusion expressed from the nmt41 promoter localises to the nucleus during mitosis. Live cell fluorescence microscopy analysis of \( h^{90} \Delta ste11 \) cells transformed with p409GFP-Ste11 plasmid. Cells were grown to early mid-log stage in the absence of thiamine (for derepression of the nmt41 promoter). DAPI staining was used for DNA visualisation.
A

\( h^{90} \Delta ste11 \)

EMM (2 days)

+p409GFP (Empty vector)

+p409GFP-Ste11


B

\( h^{90}\Delta ste11 \)

+p409GFP-Ste11

<table>
<thead>
<tr>
<th>GFP</th>
<th>DNA</th>
<th>Overlay</th>
</tr>
</thead>
</table>

(EMM, mid-log)
Figure 3-2. Ste11 is a target for the proteasome degradation pathway.

A) GFP-Ste11 is stabilised in the mts3-1 mutant. WT and mts3-1 cells were transformed with either the p409GFP-Ste11 plasmid or p409 empty vector, grown at 25°C (in the absence of thiamine, for nmt41 promoter induction) to early mid-log stage, and either kept growing at 25°C or shifted to 36°C for 4 hours, as indicated. Samples were taken and the levels of GFP-Ste11 and α-tubulin, as loading control, determined by western blotting using anti-GFP (Clontech) and anti-α-tubulin monoclonal antibodies, respectively.

B) Ste11 stabilisation in the mts3-1 mutant results in nuclear accumulation. Live cell fluorescence microscopy analysis of mts3-1 cells transformed with p409GFP-Ste11 plasmid. Cells were grown at 25°C to early mid-log stage in the absence of thiamine (for derepression of the nmt41 promoter), and then either kept growing at 25°C or shifted to 36°C for 4 hours.
**A**

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>mts3-1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>36</td>
</tr>
</tbody>
</table>

- **p409GFP-Ste11**
- **Temp. (°C)**

**anti-GFP**

**GFP-Ste11**

**α-tubulin**

**B**

**h- mts3-1**

+ **p409GFP-Ste11**

- **25 °C**
- **36 °C (4 hours)**
Figure 3-3. Regulation of Ste11 during the mitotic cell cycle.

A) Mitotic G1 arrest induces nuclear accumulation of Ste11. Live cell fluorescence microscopy analysis of $h^+ cdc10-129$ cells transformed with p409GFP-Ste11 plasmid. Cells were grown to early mid-log stage in the absence of thiamine (for derepression of the nmt41 promoter) at 25°C and then either kept growing at 25°C or shifted to 36°C for 4 hours.

B) $h^+ cdc25-22$ cells were transformed with the p409GFP-Ste11 plasmid, grown to early mid-log stage in the absence of thiamine at 25°C and shifted to 36°C for 4 hours to arrest cells in the G2/M stage. Cultures were then shifted back to 25°C to allow cells to resume mitosis in a synchronised manner. The right panel shows the level of synchrony obtained, as indicated by the percentage of septated cells during the time-course of the experiment. Samples from the block-and-release experiment, as well as a sample from a mid-log culture of $h^+ cdc25-22$ cells transformed with p409 empty vector, were taken and the levels of GFP-Ste11 determined by western blotting using an anti-GFP monoclonal antibody (ICRF). This antibody recognises a non-specific protein that was used as an internal loading control.

C) $h^+ cdc10-129$ cells were transformed with either p409GFP-Ste11 or p409 (empty vector) plasmids, grown at 25°C to early mid-log stage in the absence of thiamine, and then either kept growing at 25°C or shifted to 36°C for 4 hours. Samples from those cell cultures, as well as a control sample from a mid-log culture of $h^{90} \Delta ste11$ cells transformed with p409 empty vector, were taken and the levels of GFP-Ste11 and α-tubulin, as loading control, determined by western blotting using anti-Ste11 and anti-α-tubulin monoclonal antibodies, respectively. In the upper panel, arrowheads indicate the migration position of both ectopically expressed GFP-Ste11 and endogenous Ste11 proteins.
A

$h^+\ cdc10-129$  
$+p409GFP-Ste11$

25 °C  
36 °C (4 hours)

B

$h^+\ cdc25-22$  
$+p409GFP-Ste11$

Time (minutes)

GFP-Ste11  
Non-specific

anti-GFP

C

$\Delta ste11$

Endogenous Ste11

anti-Ste11

GFP-Ste11

α-tubulin
Figure 3-4. Expression and stability of Ste11 during nitrogen starvation.

A) Strains \( h^{90} \) wild-type, \( h^{90} \Delta ste11 \) were grown in EMM to mid-log at 30°C, washed and transferred to EMM(-NH\(_4\)) medium (lacking a source of nitrogen). Samples were collected at the time points indicated and the levels of Ste11 protein and \( \alpha \)-tubulin, as loading control, determined by western blotting using anti-Ste11 and anti-\( \alpha \)-tubulin monoclonal antibodies, respectively.

B) \( h^{90} \) wild-type and \( h^{90} \Delta ste11 \) cells were transformed with either pRep41MycSte11 (kind gift from H. Maekawa) or empty vector plasmids, grown in EMM to mid-log at 30°C, washed and transferred to EMM(-NH\(_4\)Cl) medium (lacking a source of nitrogen). Samples from mid-log and starved (4 hours) cell cultures, were taken and the levels of Myc-Ste11 and \( \alpha \)-tubulin, as loading control, determined by western blotting using anti-Myc (9E10) and anti-\( \alpha \)-tubulin monoclonal antibodies, respectively.

C) \( h^{90} \) wild-type and \( h^{90} \Delta ste11 \) cells were transformed with either pRep81Ste11GFP or empty vector plasmids, grown in EMM to mid-log at 30°C, washed and transferred to EMM(-NH\(_4\)Cl) medium (lacking a source of nitrogen). Samples from mid-log and starved (4 hours) cell cultures, were taken and the levels of Ste11-GFP, determined by western blotting using an anti-GFP monoclonal antibody (ICRF). This antibody recognises a non-specific protein that was used as an internal loading control.
Figure 3-5. Deletion of ste11 interferes with G1 arrest induced by nitrogen starvation.

Flow cytometric analysis of DNA content in h90 wild-type and h90 Δste11 cells after nitrogen starvation. Cultures of h90 wild-type and h90 Δste11 strains were grown in EMM to mid-log at 30°C, washed, and transferred to EMM(-NH4Cl) medium (lacking a source of nitrogen) at the same temperature. Samples were collected at the time points indicated in the figure.
$h^{90} \text{ WT} \quad h^{90} \Delta \text{ste11}$

$\text{-NH}_4\text{Cl (hrs)}$

24

6

3

0
Figure 3-6. Analysis of Ste11 overexpression effects in homothallic Δatf1, Δsty1, and Δbyr1 cells.

A) Flow cytometric analysis of DNA content in h^90 wild-type, h^90 Δatf1, h^90 Δsty1, h^90 Δbyr1 cells transformed with either p409GFPSte11 or empty vector plasmids, grown in EMM to mid-log at 30°C, washed and transferred to EMM(-NH4Cl) medium (lacking a source of nitrogen). Samples from cells starved for 24 hours were subjected to FACS analysis.

B) Phase-contrast micrographs of h^90 Δatf1, h^90 Δsty1, and h^90 Δbyr1 cells transformed with either p409GFPSte11 or empty vector plasmids, and nitrogen-starved for 3 days in EMM(-NH4Cl).

C) Quantification of mating and sporulation of h^90 Δatf1 and h^90 Δsty1 cells transformed with either p409GFPSte11 or empty vector plasmids, and nitrogen-starved for 3 days in EMM(-NH4Cl). The graph indicates total percentage of cells that mated, and the partial percentage of cells that successfully concluded meiosis and sporulated.
A.

EMM (-NH4Cl) 24 hrs

B.

EMM (-NH4Cl) 3 days

C.

Mating and sporulation %

- Sporulation partial %
- Total mating and sporulation %
Table 3-1. Regulation of Ste11 localisation.

<table>
<thead>
<tr>
<th>Background</th>
<th>EMM</th>
<th>EMM (-NH₄Cl)</th>
<th>EMM (36°C)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Ste11 localisation</td>
<td>Nuclear accumulation</td>
<td>Nuclear accumulation</td>
</tr>
<tr>
<td></td>
<td>Nucleus</td>
<td>Cyto.</td>
<td></td>
</tr>
<tr>
<td>h⁹⁰ Δste11</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>h⁹⁰ wt</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>h⁻ wt</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>h² Δgit2</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>h⁺ pat1-114</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>h⁹⁰ Δatf1</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>h⁹⁰ Δsty1</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>h⁹⁰ Δspk1</td>
<td>+++</td>
<td>+/-</td>
<td>-</td>
</tr>
<tr>
<td>h⁺ cdc10-129</td>
<td>+++</td>
<td>+/-</td>
<td>nd</td>
</tr>
<tr>
<td>h⁺ cdc25-22</td>
<td>+++</td>
<td>+/-</td>
<td>nd</td>
</tr>
<tr>
<td>h⁻ mts3-1</td>
<td>+++</td>
<td>+/-</td>
<td>nd</td>
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</table>

The indicated strains were transformed with vector p409GFP-Ste11. Cells were grown at 25°C to early mid-log stage in the absence of thiamine (for derepression of the nmt41 promoter), and then either kept growing at 25°C, shifted to 36°C, or nitrogen starved in EMM(-NH₄Cl) as indicated. Live cell fluorescence was performed after 4 hours incubation.

Nuclear accumulation:
nd = not determined, - means no change, + means increase, ++ means strong increase.
CHAPTER 4

IDENTIFICATION OF NOVEL STE11 TARGET GENES
4) IDENTIFICATION OF NOVEL Ste11 TARGET GENES

4.1. Introduction

In order to fully understand the major role that Ste11 plays in the control of *S.pombe* sexual differentiation pathway, it is crucial to identify all its target genes. A genome-wide identification of Ste11 targets became feasible when the *S.pombe* genome sequencing effort approached completion, since the precise sequence requirements for DNA binding and transcriptional regulation of target promoters by Ste11 have been characterised in detail. In *S. cerevisae*, the \(\alpha_2\) and Mcm1 proteins form heterotetramers and repress transcription of cell-type-specific genes. A search of the *S. cerevisae* genome database for potential \(\alpha_2/\text{Mcm1}\) DNA binding sites resulted in the successful identification of a novel \(\alpha\)-specific gene, demonstrating the applicability of this kind of database search (Zhong et al. 1999). Analysis of Ste11 binding sites (TR-boxes) in its target gene promoters revealed two classes of TR-boxes. One class (consensus: TTTCTTTGTT) is present in genes that are expressed in both mating-types, while the second (consensus: TCTTTGTT) is present in M-cell specific target genes. The longer TR-box sequence has been shown to be a Ste11 preferred binding site, to which Ste11 binds strongly on its own, and from which it can stimulate transcription of heterologous reporter genes (Kjaerulff et al. 1997; van Beest et al. 2000). In contrast, Ste11 binding to the shorter TR-box requires the presence of the M-cell specific Mat1-Mc protein. The aim of the work presented in this Chapter was to identify Ste11-dependent genes involved in the mating and meiotic pathway, by searching for long TR-boxes in the fission yeast genome database.
4.2. Results

4.2.1. A computer search for *ste11*-dependent genes in the *S. pombe* genome

As an initial approach to identify novel *ste11*-dependent genes, I used the MatInspector software (release 2.2, Genomatix GmbH, http://genomatix.gsf.de) to quantify the abundance of long TR-boxes (TTTCTTTGTT) in the fission yeast genome database. This preliminary search (not allowing any mismatch) estimated the number of long TR-boxes in the *S. pombe* genome to be 243. The relatively long size (10 bp) of the Ste11 binding site used in this search contributes to the statistical significance of this result, however it is unlikely that all the TR-boxes identified are functional as transcription *cis*-elements. Upstream regulatory regions of characterised *ste11*-dependent genes contain a variable number of TR-boxes that, interestingly, tend to be found clustered together. As an example, four of the five previously reported TR-boxes in the *mei2* promoter region are located in a region of about 400bp, 1.5-1.9kb away from the translation initiation codon (Sugimoto et al. 1991). In order not to identify a large number of false positive *ste11* target genes, I restricted the search to ORFs containing at least two long TR-boxes in the proximal 2.5 kb (from the translation initiation codon) upstream regulatory region. The database search identified TR-boxes in both orientations relative to ORFs, as the function of the TR-box has been shown to be orientation independent (Petersen et al. 1995). Some *ste11*-dependent genes like *ste6* (Hughes et al. 1994) contain only one long TR-box (our data not shown; Kjaerulff et al. 1997) and will escape identification by this search. This implies that this genome database search was obviously expected to identify only a subset of all the *ste11*-dependent genes, but had the advantage of reducing the number of false positive hits. In this analysis, TR-boxes located in ORFs were not considered. In a few cases, the clusters of TR-boxes were located in the intergenic regions of two genes, where they have the potential to control transcription of both genes. If these genes satisfy the criteria outlined above
they were listed as individual hits. Upon the identification of candidate ORFs, the identification of TR-boxes in their upstream regulatory regions was refined by searching these regions using a shorter consensus TR-box sequence (TTCTTTGTT). Overall, the database search identified five previously reported ste11-dependent genes (Table 4-1), and 11 new candidate genes (Table 4-2). Of the 11 putative ste11 target genes, two of them (pat1 and spk1) had been previously characterised and implicated in the control of the sexual differentiation pathway (see Chapter 1). The identification of five TR-boxes in pat1 is very surprising since Pat1 kinase is an inhibitor of mating and meiosis, thought to be inactivated in a stepwise manner to allow cells to proceed from mitosis to conjugation, and subsequently to meiosis.

During the course of this work, the function of four of the 11 candidate ste11-dependent genes was reported in the literature. SPAC30C2.01c was shown to encode the dynein heavy chain (Dhc1) subunit protein, and to function specifically in meiosis (Hiraoka et al. 2000). dhc1 mutant cells are able to complete meiosis but show reduced frequencies of homologous recombination and completely lack the strong oscillatory nuclear movements observed in wild-type cells after nuclear fusion. The meiotic role of dhc1 is compatible with the possibility that its expression is induced during starvation in a ste11-dependent manner but, to our knowledge, no data about dhc1 expression pattern has been reported. The SPAC23E2.03c gene was found to be identical to sk7, a gene essential for both mating and meiosis (Sipiczki 1988; Matsuyama et al. 2000). Ste7 is a serine-rich protein displaying no significant similarity to other proteins, that has been shown to interact with both Pat1 and Mei2, and suggested to be involved in the establishment of mating pheromone signalling. As the presence of two TR-boxes in its upstream regulatory region suggested, ste7 transcription is strongly induced in response to nitrogen starvation in a ste11-dependent manner (Matsuyama et al. 2000). SPAC1F5.09c (shk2/pak2) encodes a second p21(cdc42/rac)-activated protein kinase (PAK) homologue in fission yeast (Sells et al. 1998; Yang et al. 1998). Shk2 was shown to bind to Cdc42 and to participate in the Ras-controlled morphology and mating
response pathways in fission yeast. Unlike shk1/pak1, shk2/pak2 is not essential for vegetative growth or for mating in S. pombe. Shk2/Pak2 function appears to overlap with Shk1/Pak1, as overexpression of shk2 restores viability and normal morphology but does not rescue the sterile defect of fission yeast cells carrying a shk1/pak1 null mutation (Sells et al. 1998; Yang et al. 1998). The analysis of the function of SPAC22F3.12c (rgs1) gene, encoding a Regulator of G-protein Signalling (RGS) family protein, will be the subject of the next Chapter.

The five remaining putative ste11-dependent genes encode uncharacterised proteins with unknown functions. SPAC22F3.13 encodes a protein with significant homology to human hamartin, the protein product of the tumour suppressor gene TSC1. Mutations in TSC1 have been associated with tuberous sclerosis, a human genetic syndrome characterized by the development of tumors in a variety of tissues (Cheadle et al. 2000). Hamartin has been shown to interact with tuberin (encoded by TSC2) (van Slegtenhorst et al. 1998), a protein displaying GTPase activating protein (GAP) activity towards the small GTPase Rap1a (Wienecke et al. 1995). The presence of hamartin is essential for tuberin function suggesting that hamartin might play a role supporting or activating tuberin GAP activity (Potter et al. 2001). Recent genetic studies in Drosophila suggest that both TSC tumor suppressors regulate cell growth and proliferation acting as downstream negative regulators of insulin signalling (Gao and Pan 2001; Potter et al. 2001). Interestingly, the SPBC23G7.08c gene (with 4 TR-boxes in its upstream region) encodes a putative GAP towards small GTPases with strong homology to Rap1GAP over the GAP domain. This observation raises the possibility that SPBC23G7.08c and SPAC22F3.13 proteins might work together to regulate the activity of an unidentified small GTPase with a role in the sexual differentiation pathway. The SPAC6C3.01c gene encodes a hypothetical RNA-binding protein that contains two repeats of the 90 aminoacid long RNA Recognition motif (RRM), which is also present in the Mei2 protein. The remaining two uncharacterised candidate genes encode putative transcriptional regulators: SPCC320.03 encodes a protein with a
fungal-specific zinc-finger motif (Zn(2)-Cys(6) binuclear cluster), and the SPAC31G5.10 gene product contains a Myb-like DNA binding domain.

4.2.2. Functionality of TR-boxes clusters identified in the computer search

To study the ability of TR-boxes to support induction of the uncharacterised putative ste11-dependent genes, I analysed the expression of pat1, shk2/pak2, and rgs1 by northern blotting. Homothallic wild-type and Δste11 cells were grown in EMM medium to mid-log phase, washed and resuspended in EMM lacking a nitrogen source (EMM -NH4Cl). Cultures were incubated at 30°C, and samples collected for northern analysis at the time points indicated (Fig. 4-1, A). As previously described, ste11 is expressed at very low level during mitotic growth and is quickly and strongly induced during nitrogen starvation. The transcript for the shk2/pak2 kinase gene was not detected during mitosis but it appeared 90 minutes after starvation in wild-type cells (Fig. 4-1, A). However, shk2/pak2 was not expressed in Δste11 cells even after 150 minutes of starvation. Similarly, rgs1 was only expressed after 90 minutes under starvation conditions in a ste11-dependent manner, but the expression levels reached during early stages of nitrogen starvation were very low. This analysis of shk2/pak2 and rgs1 expression supports the validity of the criteria used in the database search for novel ste11-dependent genes. As expected from a gene essential for mitotic growth, pat1 has a distinct pattern of expression from shk2/pak2 and rgs1, it is already expressed during mitosis and is gradually upregulated during the first two hours of nitrogen starvation (Fig. 4-1, B).

4.2.3. rgs1 induction requires Ste11 and an intact pheromone-activated MAPK cascade

Because heterotrimeric G-protein signalling plays an important and characterised function in the control of sexual differentiation in fission yeast, I analysed in more detail the expression pattern of rgs1. This novel gene
(rgsl/SPAC22F3.12c), encoding a putative negative regulator of heterotrimeric G-protein signalling, has TR-boxes in its 5' regulatory region clustered at positions -450, -493, and -552 relative to the translation start codon (Table 4-2). The ORF is intronless and is predicted to encode a protein product of 481 amino acids in length. As rgs1 expression was barely detectable during the first two hours of the starvation response, I analysed its expression and ste11-dependance during a longer time-course of nitrogen starvation (Fig. 4-2, A) rgs1 expression was not detectable in wild-type mitotic cells but was strongly activated after three hours of nitrogen starvation. This activation was lost in Δste11 cells, and greatly reduced in Δpcr1 cells (Watanabe and Yamamoto 1996), where ste11 transcription is extremely low (Fig. 4-2, A). Furthermore, overexpression of ste11 is sufficient to drive ectopic expression of rgs1 in vegetatively growing cells (Fig. 4-2, B). These results suggest that Ste11 directly regulates rgs1 expression.

Some of the genes that require Ste11 for induction during nitrogen starvation also require input from the pheromone signalling pathway, either for transcription to occur at all or for full induction (Kitamura and Shimoda 1991; Hughes et al. 1994; Petersen et al. 1995). For example, together with pheromone signalling, Ste11 function and the presence of TR-boxes in 5' regulatory region are necessary for full induction of mat1-Pm expression (Aono et al. 1994). To determine if the same is true for rgs1, I investigated if rgs1 induction by nitrogen starvation still occurred in heterothallic wild-type cells, or in cells carrying deletions in key transducers of pheromone signalling. During 7 hours of nitrogen starvation no rgs1 induction was seen in the h WT strain, or in h Δbyr2 and h Δbyr1 strains, deleted for the pheromone-activated MAPKKK and MAPKK, respectively (Fig. 4-2, C). In the same experiment, a strong induction of rgs1 is observed in homothallic wild-type cells indicating a requirement for the MAPK module that transduces the pheromone signal.
4.3. Summary

In this Chapter, I described a search of the fission yeast genome for potential Ste11 binding sites. Using a restrictive set of parameters, the search identified eleven putative novel ste11-controlled genes. Northern results demonstrated that two of these genes, shk2/pak2 and rgs1, are expressed specifically in response to nitrogen starvation in a ste11-dependent manner, and that Ste11 overexpression results in rgs1 ectopic expression. I also show that rgs1 induction requires an intact pheromone-activated MAPK cascade, as mutations in byr2 and byr1 genes completely abolished rgs1 induction in homothallic cells. These results strongly support the validity of the criteria used in the genome database search, and its applicability in the identification of genes specifically involved in the sexual differentiation pathway. Two independent studies reported that two of the putative Ste11 target genes identified, dhc1 (Yamamoto et al. 1999) and ste7 (Matsuyama et al. 2000), are specifically involved in sexual differentiation events, and furthermore that ste7 is strongly induced in response to nitrogen starvation. As predicted by my results, the ste7 gene is controlled by Ste11; Ste11 is absolutely required for its transcription, and Ste11 overexpression drives ectopic expression of ste7 during vegetative growth. The suggestion by Matsuyama et al. (2000) that ste7 is likely to be involved in the establishment of mating pheromone signalling, taken together with the identification of spkl as a putative Ste11-regulated gene, further reveals the extensive involvement of Ste11 in the transcriptional activation of the pheromone signalling cascade. Thus, as the transcriptional induction of some Ste11-dependent genes, like rgs1 (Fig. 4-2) and fus1 (Petersen et al. 1995), also requires pheromone signalling, it can be inferred that Ste11 activates these genes in both direct and indirect ways. This apparent redundancy adds another layer of complexity to the analysis of the transcriptional control of this class of genes. In particular, one important and crucial question that needs to be addressed is what is the mechanism used by pheromone signalling to stimulate transcription from these genes.
Figure 4-1. Functionality of TR-boxes clusters identified in the computer search.

A) Strains h\textsuperscript{90} wild-type, h\textsuperscript{90} Δste11 were grown in EMM to mid-log at 30°C, washed and transferred to EMM(-NH\textsubscript{4}Cl) medium (lacking a source of nitrogen). Samples were collected at the time points indicated, and RNA extracted for northern analysis. Detection of ste11, shk2/pak2, and rgs1 transcripts was performed as described in Material and Methods. Equal loading was confirmed by ethidium bromide staining (data not shown) and enolase (eno) mRNA detection.

B) pat1 is induced by nitrogen starvation. h\textsuperscript{90} wild-type cells were grown in EMM to mid-log at 30°C, washed and transferred to EMM(-NH\textsubscript{4}Cl) medium (lacking a source of nitrogen). Samples were collected at the time points indicated, and RNA extracted for northern analysis. Detection of ste11 and pat1 transcripts was performed as described in Material and Methods. Equal loading was confirmed by ethidium bromide staining (data not shown) and enolase (eno) mRNA detection.
A

<table>
<thead>
<tr>
<th></th>
<th>( t^0 ) Wild-type</th>
<th>( t^0 ) ( \Delta )ste11</th>
</tr>
</thead>
<tbody>
<tr>
<td>0'</td>
<td></td>
<td>NH_4Cl (min)</td>
</tr>
<tr>
<td>30'</td>
<td>ste11</td>
<td></td>
</tr>
<tr>
<td>60'</td>
<td>shk2/pak2</td>
<td></td>
</tr>
<tr>
<td>90'</td>
<td>rgs1</td>
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<tr>
<td>120'</td>
<td>eno</td>
<td></td>
</tr>
<tr>
<td>150'</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th></th>
<th>( t^0 ) Wild-type</th>
</tr>
</thead>
<tbody>
<tr>
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<td>pat1</td>
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<td>90'</td>
<td></td>
</tr>
<tr>
<td>120'</td>
<td></td>
</tr>
<tr>
<td>150'</td>
<td>eno</td>
</tr>
</tbody>
</table>
Figure 4-2. *rgs1* induction requires Ste11 and an intact pheromone-activated MAPK cascade.

A) *rgs1* expression requires *ste11*. Strains *h*<sup>90</sup> wild-type, *h*<sup>90</sup>Δ*ste11*, *h*<sup>90</sup>Δ*pcr1*, were grown in EMM to mid-log at 30°C, washed and transferred to EMM(-NH<sub>4</sub>Cl) medium (lacking a source of nitrogen). Samples were collected at the time points indicated, and RNA extracted for northern analysis. Equal loading was confirmed by ethidium bromide staining (data not shown) and *cdc2* mRNA detection.

B) Ectopic expression of *rgs1* in *ste11*-overexpressing cells. RNA from strains *h*<sup>90</sup> wild-type, *h*<sup>90</sup>Δ*ste11*, and *h*<sup>90</sup>Δ*ste11* transformed with a pRep41-Ste11 plasmid (a gift from H. Maekawa), was obtained from cells growing vegetatively in thiamine-free EMM medium. Detection of *rgs1*, *ste11*, and *cdc2* transcripts was performed as described in Material and Methods.

C) *rgs1* expression requires pheromone signalling. Strains *h*<sup>90</sup> wild-type, *h*<sup>90</sup> wild-type, *h*<sup>90</sup>Δ*ste11*, *h*<sup>90</sup>Δ*bbyr2*, and *h*<sup>90</sup>Δ*bbyr1* were grown in EMM to mid-log at 30°C, washed and transferred to EMM(-NH<sub>4</sub>Cl) medium (lacking a source of nitrogen). Samples were collected at the time points indicated, and RNA extracted for northern analysis. Equal loading was confirmed by ethidium bromide staining (data not shown) and *cdc2* mRNA detection.
A

<table>
<thead>
<tr>
<th>h*0 WT</th>
<th>h*0 Δste11</th>
<th>h*0 Δpcr1</th>
<th>-NH4Cl (hrs)</th>
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<tr>
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<td>3</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>3</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4</td>
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<td></td>
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</tr>
</tbody>
</table>

rgs1

cdc2

B

Mid-log

rgs1

ste11

cdc2

C
Table 4-1. Identification of TR-boxes in previously identified *ste11*-dependent genes.

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<th>Gene</th>
<th>TR-boxes</th>
<th>Position</th>
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<td>Number</td>
<td>Sequence</td>
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<td>ste11</td>
<td>3</td>
<td>aaTTTCTTTGTTtg</td>
<td>-2338,-2329</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ctTTTCTTTGTTtt</td>
<td>-1827,-1818</td>
</tr>
<tr>
<td></td>
<td></td>
<td>tgTTTCTTTGTTgc</td>
<td>-1379,-1370</td>
</tr>
<tr>
<td>mei2</td>
<td>6</td>
<td>gaTTTCTTTGTTcc</td>
<td>-1894,-1885</td>
</tr>
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<td></td>
<td></td>
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<td>-914,-905</td>
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<td>fus1</td>
<td>3</td>
<td>taTTTCTTTGTTct</td>
<td>-249,-240</td>
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<td></td>
<td></td>
<td>acTTTCTTTGTTcg</td>
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<td>rep1</td>
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<td>ttTTTCTTTGTTta</td>
<td>-582,-573</td>
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<td></td>
<td></td>
<td>ccTTTCTTTGTTtt</td>
<td>-533,-524</td>
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<td>mat1-Mc</td>
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<td>gaaTTTCTTTGTTtg</td>
<td>-1440,-1432</td>
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<td></td>
<td></td>
<td>taTTTCTTTGTTtg</td>
<td>-516,-507</td>
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<td></td>
<td>tcTTTCTTTGTTcc</td>
<td>-498,-489</td>
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<tr>
<td></td>
<td></td>
<td>cgTTTCTTTGTTct</td>
<td>-66,-57</td>
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Position refers to the distance between a TR-box and the open reading frame translation start ATG codon.
Right arrows indicate that the TR-box and ORF are on the same strand.
Left arrows indicate that the TR-box and the ORF are on opposite strands.
Table 4-2. Identification of TR-boxes in putative ste11-dependent genes.

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<th>Gene</th>
<th>Gene product function/homology</th>
<th>TR-boxes</th>
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<th>Orientation</th>
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<td></td>
<td></td>
<td>Number</td>
<td>Sequence</td>
<td></td>
</tr>
<tr>
<td>SPAC22F3.12c (rgs1)</td>
<td>RGS (Regulator of G-protein signalling) domain</td>
<td>3</td>
<td>gtTTTCTTTTGTtca</td>
<td>-561, -552</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>atgTTTCTTTTGTtca</td>
<td>-501, -493</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gatTTTCTTTTGTtca</td>
<td>-459, -450</td>
</tr>
<tr>
<td>SPAC22F3.13</td>
<td>Hamartin homolog, putative coiled coil</td>
<td>3</td>
<td>gatTTTCTTTTGTtca</td>
<td>-1488, -1479</td>
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<td></td>
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<td>atgTTTCTTTTGTtca</td>
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<td>gatTTTCTTTTGTtca</td>
<td>-1386, -1377</td>
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<td>SPCC320.03</td>
<td>Putative transcriptional regulator. Fungal Zn(2)-Cys(6) binuclear cluster</td>
<td>3</td>
<td>caatTTTCTTTTGTtta</td>
<td>-5550, -5542</td>
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<td></td>
<td></td>
<td>gatTTTCTTTTGTtca</td>
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<td></td>
<td></td>
<td>gatTTTCTTTTGTtca</td>
<td>-168, -159</td>
</tr>
<tr>
<td>SPAC1F5.09c (shk2/pak2)</td>
<td>PAK family Ser/Thr kinase</td>
<td>2</td>
<td>caTTTCTTTTGTtta</td>
<td>-113, -104</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>gatTTTCTTTTGTtca</td>
<td>-74, -65</td>
</tr>
<tr>
<td>SPAC30C2.01c (dhc1)</td>
<td>Dynein heavy chain (required for meiotic nuclear movements)</td>
<td>3</td>
<td>gctTTTCTTTTGTtta</td>
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<tr>
<td></td>
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<td>gatTTTCTTTTGTtca</td>
<td>-113, -104</td>
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<td>-2099, -2090</td>
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<td>SPAC23E2.03c (ste7)</td>
<td>Required for mating (serine-rich)</td>
<td>2</td>
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<td>atTTTCTTTTGTtga</td>
<td>-734, -725</td>
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<td>SPAC31G5.10</td>
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<td></td>
<td>agTTTCTTTTGTtta</td>
<td>-486, -477</td>
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<td>-360, -352</td>
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<td>SPBC23G7.08c</td>
<td>Small GTPase-activating protein homolog</td>
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<td>tcTTTCTTTTGTtca</td>
<td>-1099, -1090</td>
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<td>gatTTTCTTTTGTg</td>
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<td>SPBC19C2.05 (pat1)</td>
<td>Negative regulator of mating/meiosis Ser/Thr kinase</td>
<td>5</td>
<td>ccTTTCTTTTGTtca</td>
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<td>Pheromone-activated MAP kinase</td>
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Position refers to the distance between a TR-box and the open reading frame translation start ATG codon.
Right arrows indicate that the TR-box and ORF are on the same strand.
Left arrows indicate that the TR-box and the ORF are on opposite strands.
CHAPTER 5

CHARACTERISATION OF rgs1, A GENE REQUIRED FOR MATING.
5) CHARACTERISATION OF *rgs1*, A GENE REQUIRED FOR MATING.

5.1. Introduction

The Rgs1 protein has significant homology to the metazoan and fungal Regulator of G-protein Signalling (RGS) protein family, that includes approximately 30 mammalian members (reviewed by [Hepler 1999; De Vries et al. 2000]). As mentioned previously (see 1.9.4), RGS proteins promote adaptation to heterotrimeric G-protein-coupled receptor signalling by binding the GTP-bound active form of specific G-protein α-subunits and stimulating their intrinsic GTPase activity. Rgs1 appears to be the single fission yeast protein containing an RGS domain, and this domain is most closely related to the domains of *Saccharomyces cerevisiae* Sst2, and *Aspergillus nidulans* FlbA. Both proteins have been shown to regulate fungal sexual development by negatively regulating Gα-protein signalling. FlbA is required to block proliferation and activate asexual sporulation, and its main role appears to be to antagonise transmission of a proliferative signal by FadA, a heterotrimeric Gα protein ([Lee and Adams 1994; Yu et al. 1996]). In *Saccharomyces cerevisiae*, Sst2 expression is induced by pheromone signalling and acts on Gpa1, a Gα protein, promoting recovery from pheromone induced G1 arrest and re-entry into mitosis ([Dohlman et al. 1996]). The homology of Rgs1 to characterised fungal regulators of sexual differentiation together with its expression pattern strongly implied that *rgs1* could play a similar role in the regulation of fission yeast mating and meiotic pathway. The aim of the work described in this Chapter was to investigate the function of the *rgs1* gene and protein in the sexual differentiation pathway.
5.2. Results

5.2.1. rgs1 is essential for mating and is a negative regulator of pheromone signalling

To investigate the role of rgs1 in the mating and meiotic process, I deleted the full-length rgs1 ORF in a diploid homothallic h^90 wild-type strain, replacing it by the *ura4*^ marker gene. A heterozygous diploid rgs1^+/rgs1::ura4^ was isolated and rgs1 disruption confirmed by PCR. This strain was induced to sporulate and asci dissected for tetrad analysis. A 2:2 co-segregation of *ura4*^ and rgs1 ORF disruption confirmed a correct gene disruption, and that Δrgs1 cells are viable.

In keeping with lack of detectable transcript in mitotic cells (Fig. 4-2, C), Δrgs1 cells display no morphological or growth defect during the vegetative growth phase. Interestingly, the mutant cells showed a severe mating defect: when induced to conjugate, only approximately 4% were found to do so in contrast to 53% for WT cells (Fig. 5-1, A). rgs1 appears not to be essential for meiosis and sporulation, as the diploid zygotes resulting from the rare conjugation events were able to complete meiosis and produce four spores. Furthermore, homozygous Δrgs1 diploid cells showed no defect when induced to enter meiosis and sporulate (data not shown).

Mating between fission yeast cells occurs following a G1 arrest resulting from nutritional starvation and pheromone signalling. To investigate if the mating defect of Δrgs1 cells was due to a defect in the ability of the cells to arrest, wild-type and Δrgs1 cells were grown in EMM medium to mid-log phase, washed and resuspended in EMM lacking a nitrogen source (EMM - NH4Cl). Cultures were incubated for 6 hours and samples collected hourly for DNA content analysis by FACS scanning (Fig. 5-1, B). Δrgs1 cells were able to sense nitrogen starvation and the majority arrested in G1 with 1C DNA content after 6 hours incubation. Interestingly, a small 1C peak was already detectable in mid-log phase, and Δrgs1 cells appeared to accumulate in the G1 phase slightly faster than wild-type cells. h^90 Δste11 cells are completely defective in
entering the sexual differentiation pathway and when starved, arrest with small cell size and round morphology, like heterothallic wild-type cells (Sugimoto et al. 1991). In contrast, $h^{90}$ $\Delta rgs1$ cells arrest with a larger size and elongated morphology resembling abnormal conjugation tubes (Fig. 5-1, C). This is similar to the phenotype described for pheromone-sensitive strains like $\Delta sxa2$, lacking a P-factor protease (Imai and Yamamoto 1994), or cells carrying a constitutively active Gpa1QL allele (Obara et al. 1991). Transcription of $mam2$, encoding the P-factor receptor, is induced in wild-type cells by nitrogen starvation in a $ste11$-dependent manner, and further enhanced by pheromone signalling via the Byr2/Byr1 MAPK pathway (Kitamura and Shimoda 1991; Xu et al. 1994). $mam2$ expression was seen in $\Delta rgs1$ cells following nitrogen starvation, the level of activation being higher than in wild-type cells (Fig. 5-1, D). Similarly, transcription of the $shk2/pak2$ gene, which is dependent on $ste11$ function (Chapter 4, Fig. 4-1), was also induced earlier on $\Delta rgs1$ cells subjected to nitrogen starvation, reaching a higher level of expression upon four hours of starvation when compared with expression on wild-type cells (Fig. 5-1, D). One further characteristic of $h^{90} \Delta rgs1$ cells, was an enhanced level of agglutination when grown to saturation or following nitrogen starvation. The enhanced levels of agglutination are dependent on pheromone signalling as $h^{90} \Delta rgs1$ cells do not display this phenotype (data not shown).

To further investigate the possibility that $rgs1$ has a role in feedback downregulation of pheromone signalling, I tested the response of the heterothallic $h^{90} \Delta rgs1$ strain to synthetic P-factor. Mid-log phase cultures of $h^{90} \Delta rgs1$ and $h^{90}$ wild-type cells were divided, and P-factor (20 $\mu$g/ml) added to one half, upon which cultures were grown to stationary phase. Fission yeast cells enter stationary phase both from C1 and G2 phases of the mitotic cell cycle (Costello et al. 1986). Unlike $S. cerevisiae$, $S. pombe$ cells do not arrest in G1 in response to pheromone when growing in rich medium (Davey and Nielsen 1994; Imai and Yamamoto 1994), as nutritional starvation is required for transcriptional induction of several genes involved in the pheromone response pathway. However, a significant proportion (64%) of $\Delta rgs1$ cells responded to
P-factor by arresting in the G1 phase of the cell cycle (Fig. 5-2, A), while in the absence of P-factor they arrested with a pattern that was similar to that seen in wild-type cells (20% in G1). Additionally, many of the P-factor treated Δrgs1 cells that did arrest in stationary phase were abnormally long or had elongated conjugation tubes (Fig. 5-2, B). In contrast, non-treated Δrgs1 cells arrested with normal morphology.

In the budding yeast, the level of the Rgs1 homologue, Sst2, appears to be rate limiting, as its overexpression accelerates the rate at which cells adapt to pheromone treatment and resume growth (Dohlman et al. 1996). Since the characterisation of rgs1 indicates that this gene negatively regulates pheromone signalling, I decided to investigate whether overexpression of Rgs1 protein interferes with the cellular response to pheromone treatment. Overexpression of GFP-Rgs1 fusion protein from the nmt41 promoter neither reduced the rate of pheromone-induced G1 arrest in Δcyr1Δsxa2 cells (Fig. 5-2, C), nor blocked the extension of abnormally long conjugation tubes by these cells (not shown). This negative result suggests that either Rgs1 levels are not rate limiting or that Rgs1 activity is not effective in attenuating the pheromone response in a hypersensitive strain like Δcyr1Δsxa2.

Together, these results suggest that rgs1 acts downstream of ste11 in the mating pathway, and that it negatively regulates pheromone signalling. Thus in the absence of rgs1, pheromone induced effects such as G1 arrest, mam2 induction, and agglutination are enhanced.

5.2.2. Rgs1 expression and localisation

To examine the intracellular localisation of Rgs1 protein through the sexual differentiation pathway I expressed Rgs1 with an N-terminal green fluorescent protein (GFP) tag from the intermediate strength nmt41 inducible promoter. GFP-Rgs1 appears to be fully functional, as its expression can rescue the mating defect of Δrgs1 cells (Fig. 5-3, A). During the mitotic cell cycle, GFP-Rgs1 mainly localises to the nucleus, but a faint, cytoplasmic signal was also observed (Fig.
As \textit{rgs1} is not expressed in mitosis, this localisation pattern might not reflect the physiological distribution of Rgs1 during starvation and mating. To investigate this possibility, GFP-Rgs1 localisation in wild-type cells was followed during nitrogen-starvation induced mating. No significant change in the localisation pattern was observed (data not shown).

To study the precise timing of Rgs1 expression and its intracellular localisation during a synchronised response to pheromone, I integrated a GFP C-terminal tagged form of \textit{rgs1}, expressed from its native promoter, in an \textit{h\textsuperscript{+}Δcyr1Δsxa2} strain. C-terminal tagging of Rgs1 with GFP does not affect Rgs1 function, as wild-type cells expressing this Rgs1 fusion protein at endogenous levels conserve full mating ability (data not shown). Unlike wild-type cells, \textit{h\textsuperscript{-}Δcyr1Δsxa2} cells are sensitive to P-factor in rich medium, as deletion of \textit{sxa2}, a P-factor protease, enhances P-factor activity (Imai and Yamamoto 1994), and the lack of Cyr1, adenylate cyclase, derepresses expression of genes required for pheromone communication and signal transduction (Maeda et al. 1990; Kawamukai et al. 1991). These cells respond readily to P-factor by arresting in G1 after four hours exposure, and then elongating conjugation tubes. Immunoblotting analysis of Rgs1-GFP protein levels, revealed a very strong induction after two hours of P-factor treatment when a large percentage of the cell population had undergone G1 arrest (Fig. 5-4, A and B). Rgs1-GFP protein levels did not change significantly during longer exposure to pheromone, and it was still present after 24 hours treatment. In contrast, Rgs1-GFP expression was not detected upon nitrogen starvation (data not shown). Rgs1-GFP localised both to the nucleus and to the cytoplasm, although it appears to accumulate at higher levels in the nucleus (Fig. 5-4, C). These results confirmed that Rgs1 expression requires pheromone signalling and that Rgs1 protein localises both to the nucleus and cytoplasm.
5.2.3. Rgs1 functional domains

To investigate how Rgs1 may regulate mating and pheromone sensitivity, I searched the Rgs1 protein sequence for other protein homology domains besides the described C-terminal RGS domain (Tesmer et al. 1997). This analysis revealed the presence of another recognisable feature: a DEP domain (amino acids 232-311) (Fig. 5-5). The DEP domain was initially identified as an 80 residue long homologous domain in *Drosophila* Dishevelled, *C.elegans* EGL-10, and Pleckstrin (Ponting and Bork 1996). It is often found in RGS-containing proteins or in small GTPase regulators. It has been shown that the DEP domain in Dishevelled (Dsh) is required for its plasma membrane recruitment upon activation of the receptor Frizzeled (Axelrod et al. 1998). Mammalian Dishevelled (Dvl) proteins are involved in transducing Wnt signalling, and in this pathway the DEP domain is required for Dvl-mediated activation of c-Jun N-terminal kinase (Li et al. 1999; Moriguchi et al. 1999). However, the precise function or binding targets of the DEP domain are still unknown.

The N-terminal 227 residues of Rgs1 displays significant homology to N-terminal regions in *A. nidulans* FlbA and *S. cerevisae* Sst2, so that the overall domain organisation of the three proteins is very similar, as FlbA and Sst2 also contain a central DEP domain and a C-terminal RGS domain (Fig. 5-5, A). This novel homology domain appears to be only present in these three fungal proteins that regulate differentiation pathways, so I will refer to it as Fungal-Differentiation Regulator (Fungal-DR) domain.

To investigate the functional role of the three sequence domains, I constructed several truncations and deletions of Rgs1 (Fig. 5-6, A) and tested their *in vivo* functionality by measuring complementation of the mating defect of the *h^0^ Arg51* strain. These Rgs1 truncations and deletions were expressed from the *nmt41* promoter, and N-terminal tagged with GFP to assess protein expression levels and intracellular localisation pattern. Removal of the C-terminal RGS domain resulted in total loss of Rgs1 function (Fig. 5-6, B and C) without significantly interfering with Rgs1 localisation or protein expression levels (Fig. 5-6, A and D). Interestingly, the RGS domain on its own (Rgs1 314/481) is not
sufficient to rescue mating, suggesting that N-terminal regions are also required for Rgs1 activity. Moreover, an internal deletion that removes the 80 amino acid DEP domain (Rgs1 Δ232/311), or a truncation of the N-terminal 93 amino acid region that removes part of the Fungal-DR domain, also resulted in total loss of Rgs1 function (Fig. 5-6, C). Both fusion proteins were stably expressed, and localised to the nucleus and cytoplasm just as full-length Rgs1 protein (Fig. 5-6, A and D). Unlike the full-length GFP-Rgs1 fusion, neither the GFP-Rgs1 1/320 protein nor any of the other deletion constructs tested (not shown) could rescue either the agglutination defect or the excessive elongation of conjugation tubes seen in Δrgs1 starved cells (Fig. 5-6, B). This result suggests that there is a tight linkage between the pheromone sensitivity and the agglutination and mating defects of the Δrgs1 cells.

In summary, the results presented in this section show that Rgs1 protein function requires the contribution of its three homology domains (RGS, DEP, and Fungal-DR domains).

5.2.4. **Rgs1 does not interact with Gpa1 in a 2-hybrid assay**

As mentioned previously, several RGS proteins have been shown to function by binding their cognate Gα protein in the transition state for GTP hydrolysis (reviewed by De Vries et al. 2000). In particular, an interaction between Gα3 and the mammalian Rgs1 homologue GAIP has been detected using the two-hybrid system (De Vries et al. 1995). In order to analyse possible interactions between the Rgs1 and Gpa1 proteins, I used the two-hybrid system (Fields and Song 1989; James et al. 1996). As shown in Fig. 5-7, I did not detect an interaction between Rgs1 and Gpa1, or between Rgs1 and the Gpa1 constitutively active allele, Gpa1QL (Obara et al. 1991). In the budding yeast, the Rgs1 homologue Sst2 also fails to interact with the Gα Gpa1 protein in a two-hybrid assay (Chen et al. 1997), even though this interaction has been clearly demonstrated by several cofractionation and binding assays (Dohlman et al. 1996). The lack of a detectable interaction might be due to plasma-membrane
localisation being required for Rgs1 to bind Gpa1, or due to Rgs1 only transiently binding Gpa1 when the G-protein is in the transition state for GTP hydrolysis.

5.3. Summary

Here in this Chapter I show that deletion of the \textit{rgs1} gene results in sensitivity to pheromone and in a mating defect. \textit{\Delta rgs1} cells are able to initiate the sexual differentiation pathway in a normal way by agglutinating, arresting in G1, and inducing pheromone-dependent transcription, but ultimately fail to fuse with a mating partner while elongating abnormal conjugation tubes to mate. My results suggest that the Rgs1 protein acts in a negative feedback loop, as it is expressed in response to pheromone signalling and then acts to downregulate cellular sensitivity towards pheromone. Endogenous Rgs1 tagged with GFP localises to the nucleus and cytoplasm, and this localisation pattern is not altered during pheromone treatment. Importantly, Rgs1 function requires its C-terminal RGS domain, as well as a central DEP domain and a novel homology domain present in its N-terminal region (Fungal-DR domain).
Figure 5-1. Characterization of the *rgs1* deletion phenotype.

A) Mating performance of wild-type and *Δrgs1* cells grown for 4 days in EMM plates at 30°C. Mating was quantified as described in Material and Methods.

B) FACS analysis of DNA content in *h^90* wild-type and *h^90Δrgs1* cells after nitrogen starvation. Cells were grown to mid-log phase in EMM at 30°C, washed and resuspended in EMM(-NH4Cl). Samples were taken hourly until 6 hours of nitrogen starvation.

C) Cell morphology of wild-type and *Δrgs1* cells under mating-inducing conditions. Phase-contrast micrographs of cells grown in EMM plates for 4 days at 30°C.

D) Nitrogen starvation induced expression of *mam2* and *shk2/pak2* is upregulated in *h^90Δrgs1*. *mam2* and *shk2/pak2* mRNA levels in *h^90* wild-type, *h^90Δste11*, *h^90Δrgs1* strains during nitrogen starvation were analysed by northern blotting (upper panel). Equal loading of RNA was evaluated by analysis of *cdc2* mRNA levels (lower panel).
Figure 5-2. *rgs1* deletion causes sensitivity to pheromone.

A). FACS analysis of DNA content in wild-type and Δ*rgs1* cells after 3 days exposure to P-factor. Mid-log phase cultures of *h' Δrgs1* and *h'* wild-type cells were split in two, and P-factor (20 μg/ml) was added to one culture, upon which cultures were grown to stationary phase. Similar results were obtained with a lower concentration of P-factor (1.5 μg/ml).

B) Cell morphology of wild-type and Δ*rgs1* after 24 hours exposure to P-factor. *h' Δrgs1* and *h'* wild-type cells were treated as described in (A), and observed under phase-contrast microscopy.

C) *rgs1* overexpression does not reduce the rate of pheromone-induced G1 arrest in Δ*cyr1Δsxa2* cells. FACS analysis of DNA content in Δ*cyr1Δsxa2* cells expressing N-terminal tagged GFP-Rgs1 fusion protein from the *nmt41* promoter, or empty vector as a control. Cells were treated with P-factor (1.5 μg/ml), upon growth to early mid-log stage in the absence of thiamine (for derepression of the *nmt41* promoter).
**A**

**P-factor**

- WT
- Δrgs1

**B**

**P-factor**

- Δrgs1

**C**

\[ h^- \Delta cry1 \Delta sxa2 \]

- 0 3 7 + P-factor
  (hours)

- + GFP
- + GFP-Rgs1
Figure 5-3. Rgs1 localises to the nucleus and cytoplasm.

A) Functionality of GFP-Rgs1 fusion protein. Phase-contrast micrographs of cells nitrogen-starved for 24 hours in EMM(-NH4Cl).

B) N-terminal tagged GFP-Rgs1 fusion expressed from the nmt41 promoter localises to the nucleus and cytoplasm during mitosis. Live cells fluorescence microscopy analysis of h^90 Δrgs1 nmt41GFP-Rgs1 strain. Cells were grown to early mid-log stage in the absence of thiamine (for derepression of the nmt41 promoter). DAPI staining was used for DNA visualisation.
A

\[ h^{90} \Delta rgs1 \]

<table>
<thead>
<tr>
<th>+ Empty vector</th>
<th>+ p409GFP-Rgs1</th>
</tr>
</thead>
</table>

B

GFP-Rgs1  
DAPI
Figure 5-4. Expression and localisation of Rgs1 during pheromone-induced G1 arrest. Live cell fluorescence microscopy analysis of C-terminal tagged Rgs1-GFP expressed from its endogenous chromosomal locus. h Δcyr1Δsxa2 rgs1-GFP cells were grown to early mid-log phase, treated with P-factor (1.5 μg/ml), and samples were collected every 60 minutes.

A) Expression of Rgs1-GFP protein during pheromone treatment. Protein extracts were analysed by immunoblotting with an anti-GFP antibody (Clontech), as described in Material and Methods.

B) Cellular DNA content was analysed by FACS.

C) Fluorescence microscopy was performed to visualise cellular distribution of Rgs1-GFP (upper panel), and DAPI staining of DNA shown overlapped with contrast-phase (lower panel).
Figure 5-5. Identification of sequence homology domains in Rgs1. Alignments were generated using ClustalX and Boxshade programs. Conserved amino acid similarity is displayed in gray, and conserved identity in black.

A) N-terminal region of Rgs1 contains a novel protein sequence homology domain. Diagram and sequence alignment of the Fungal-DR domain identified in the N-terminal region of three fungal RGS proteins. Percentage of similarity and identity (in brackets) between Fungal-DR domains is indicated. Protein sequences used in the alignment are: *S. pombe* Rgs1 (Q09777); *A. nidulans* FlbA (P38093), and *S. cerevisiae* Sst2 (P11972).

B) Sequence alignment of the DEP domain. BLAST and PFAM databases searches revealed the presence of a DEP domain (aminoacids 232 to 311) as well as an RGS domain in Rgs1 sequence. The RGS domain of Rgs1 (aminoacids 344-479) was previously identified and aligned to other RGS family members by (Tesmer et al. 1997). Protein sequences used in the alignment are: human RGS11 (O94810); *C. elegans* EgI10 (P49809), *S. pombe* Rgs1 (Q09777), *S. cerevisiae* Sst2 (P11972), and *A. nidulans* FlbA (P38093).
**A**

**Fungal-DR Domain**

![Diagram showing the Fungal-DR Domain with sequences and percentages]

1. **A. nid. FlbA**
   - DEP: 46% (28%)
   - RGS: 36.9% (18%)

2. **S. pom. Rgs1**
3. **S. cere. Sst2**

100 a.a.

**B**

**DEP Domain**

![Diagram showing the DEP Domain with sequences and identifiers]

- Human: RG211
- C. ele: Eq110
- S. pom: Eq110
- A. nid: FLBA

![List of sequences for human, C. ele, S. pom, A. nid]
Figure 5-6. Identification of protein domains required for Rgs1 function.

$h^{90}\Delta rgs1$ cells were transformed with empty vector p409 or with p409 vector expressing $rgs1$ truncations or deletions from the inducible $nmt41$ promoter.

A) GFP-Rgs1 mutant proteins localise both to the nucleus and cytoplasm. Fluorescence microscopy analysis was done as described in Material and Methods.

B) The RGS domain is required for Rgs1 function. Unlike the full-length GFP-Rgs1 fusion, the GFP-Rgs1 1/320 protein could not rescue either the agglutination defect (lower panel) or the excessive elongation of conjugation tubes seen in $\Delta rgs1$ starved cells (upper panel). Cells were grown in liquid EMM for 4 days at 30°C and phase-contrast micrographs (upper panel) or photos of cultures transferred to petri dishes (lower panel) were taken.

C) Ability of the GFP-Rgs1 mutants to complement $h^{90}\Delta rgs1$ cells sterility defect. Mating efficiency was measured as described in Material and Methods after growth in EMM plates for 4 days.

D) Expression of GFP-Rgs1 mutant proteins in exponentially growing $h^{90}\Delta rgs1$ cells. Protein extracts were analysed by immunoblotting with an anti-GFP antibody (Clontech), as described in Material and Methods.

The lower panel in B) shows cell clumping in liquid cultures transferred for empty petri dishes and photographed.
A. 

h^{90} \Delta rgs1 +

<table>
<thead>
<tr>
<th>Construct</th>
<th>GFP</th>
<th>DAPI</th>
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<td>1/481</td>
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GFP-Rgs1 constructs

B. 

h^{90} \Delta rgs1

+ GFP  +GFP-Rgs1  +GFP-Rgs1 1/320

C. 

Mating %

D. 

h^{90} \Delta rgs1 +

\( \alpha\)-GFP

\( \alpha\)-tubulin
Figure 5-7. Rgs1 does not interact with Gpa1 in a 2-hybrid assay.

*S. cerevisiae* PJ69-4A strain was transformed with the indicated combinations of Gal4AD and Gal4DBD fusion expression plasmids, or with the plasmid pCL1 (expressing *S. cerevisiae* Gal4) as a positive control, and the β-galactosidase activity of independent transformants measured as described in Material and Methods.
Gal4-DBD fusion:
Gal4-AD fusion:

β-galactosidase activity (Miller units)
CHAPTER 6

DISCUSSION
6) DISCUSSION

In this Chapter, I will attempt to integrate the main results presented in this Thesis, together with results recently reported in the literature. This integration will allow the development of some of the conclusions already hinted at, as well as the definition of future work necessary to extend the characterisation of the function of Ste11 and Rgs1, in particular, and of the sexual differentiation pathway of fission yeast, in general.

6.1. ste11 function

One very important aspect of the regulation of the S. pombe life cycle that is yet not fully understood is the question of how the cell senses nutritional starvation and responds by activating ste11 expression. Transcriptional control of ste11 expression has been considered to be the major point of control of ste11 function and of the commitment to mating and meiosis. Considerable advances have been made in the identification and characterisation of the members of the Gpa2 G-protein coupled receptor pathway that in response to the presence of nutrients plays a key role in repressing ste11 function during the mitotic growth (reviewed in Yamamoto et al. 1997). Activation of this pathway appears to be required to sustain, or maybe stimulate Pka1 kinase activity that results in inhibition of ste11 function. Other signalling pathways, like the Sty1 stress response cascade, and regulators, like Pac2 and Nrd1, have been shown to function independently of the Ga Gpa2 protein in regulating ste11 function. One caveat with most of the studies of the control of ste11 function, is the assumption that ste11 is mainly regulated at the transcriptional level, ignoring the early indication of the presence of a TR-box in ste11 own upstream region (Sugimoto et al. 1991). Thus, if Ste11 self-regulates its own expression, the indicated signalling pathways acting upstream of ste11, could in fact stimulate ste11 at a post-transcriptional stage, and the observed induction of ste11 mRNA be an indirect consequence of that stimulation. Furthermore, until very recently not a single transcription factor or cis-element in the ste11 promoter had been
directly and conclusively implied in the regulation of *ste11* transcription. Importantly, the first molecular analysis of the promoter region of *ste11*, identified a zinc-finger protein, Rst2, as a direct activator of *ste11* transcription, and it also confirmed that Ste11 protein stimulates its own transcription (Kunitomo et al. 2000). Rst2 protein binds a core sequence (CCCTC) at nucleotides –195 to –190 (from transcription start point) on the *ste11* promoter that resembles a *S. cerevisiae* STRE (Stress response element) cis-element. A three-base substitution in the Rst2 core binding element reduced *ste11* expression levels upon nitrogen starvation to one-fourth of that normally seen (Kunitomo et al. 2000). Similarly, DNase I footprint analysis demonstrated that the HMG-box of Ste11 could protect a TR-box element in the *ste11* promoter (TR1), and that mutation of this sequence reduced *ste11* expression to about one-third. Combining this mutation in TR1 with a mutation in a second TR-box (TR2) further upstream in the *ste11* promoter almost completely abolished *ste11* expression, demonstrating the crucial role of Ste11 in the control of its own transcription (Kunitomo et al. 2000). It should be mentioned that this mutational analysis was based on northern analysis of transcripts from a *ste11*-mei2 fusion, where the *ste11* regulatory region (1.4 kb) is fused to a fragment of the mei2 ORF. Using this experimental approach, the Ste11 positive feedback loop is not active. Thus, the role of each TR-box could be even more crucial in vivo as a slight reduction in *ste11* expression due to mutating the TR-box sequence would be amplified (decreasing expression) by the positive feedback loop. The results presented in Chapter 3 (Fig. 3-3, C) support the self-regulatory activity of Ste11 as overexpressing a tagged Ste11 fusion protein from a plasmid resulted in an increase in the expression levels of endogenous Ste11. Furthermore, Kunitomo *et al.* (2000) report that episomal overexpression of Ste11 results in the transcriptional activation of the *ste11* gene, both in the presence and absence of nutrients. Returning to the issue of *ste11* regulation by upstream signalling pathways, is Rst2 protein directly regulated by any of the cascades involved? *S. cerevisiae* studies showed that Pka1 inhibits the activity of the STRE-element regulators, Msn2 and Msn4 (Rst2 homologues), apparently
by promoting nuclear exclusion (Martínez-Pastor et al. 1996; Schmitt and McEntee 1996; Gorner et al. 1998). Based on those studies, Kunitomo et al. (2000) speculate that Rst2 is a substrate of Pka1 and is negatively regulated by phosphorylation. The data clearly demonstrates that rst2 acts downstream of the Pka1 kinase: disruption of rst2 results in sterility and can also suppress hypermating and hypersporulation in the pkal-null mutant. Conversely, overexpression of rst2 rescues the complete sterile defect of Δcgs1 cells, where the gene encoding the Pka1 regulatory subunit is deleted. However, the biochemical evidence supporting a direct regulation of Rst2 by Pka1 is still missing. In fact, Kunitomo et al. (2000) observed that overexpression of rst2 in Δcgs1 rescues mating and sporulation back to wild-type levels in the conditions used (~60%), while overexpressing ste11 in the same strain only results in a low rescue efficiency (10%). Taking into account that the overexpression of ste11 in the Δrst2 strain rescues the sterile defect of these cells, raising the mating and sporulation efficiency from <0.1% to 30%, it is tempting to speculate that Pka1 might, actually, directly inhibit Ste11 activity at the protein level.

Because the core binding sequence of Rst2 and the TR-box, TR1, are separated by just 34 nucleotides, Kunitomo et al. (2000) speculated that Rst2 and Ste11 could act synergistically to activate ste11 transcription. According to the architectural model for transcriptional stimulation by HMG-box factors, it would be tempting to speculate that the role of Ste11 would be to bend this promoter region, allowing Rst2 to contact other unknown regulators or the basal transcription machinery. The Ste11 protein has, indeed, the ability to bend its targeted DNA regions, and it has been estimated using a circular permutation assay that Ste11 bends the mfml TR-box by about 56° (S. Kjaerulff, Ph.D. Thesis, University of Copenhagen, 1997). However, the analysis of transactivation by Ste11 (Chapter 2), does not support this “architectural” model for Ste11 function since I identified and mapped a “classic” activation domain in Ste11, which stimulates transcription constitutively when tethered to a synthetic enhancer. Obviously, it still remains possible that in vivo, DNA bending of the promoter region plays an important role in the context of
regulation of complex promoters. As an example, Ste11 regulates transcription of the M-cell specific mfm1 gene, in a synergistic way with the HMG-box Mat1-Mc factor, which also strongly bends DNA around its cognate DNA-binding element (Kjaerulff et al. 1997).

Close analysis of my results and of other reports, reveals an important lacuna in the knowledge of how S. pombe controls entry into the sexual differentiation pathway. During mitotic growth, Ste11 protein is expressed, localises to the nucleus, and is able to activate transcription if promoter-bound (Chapter 3; K. Kitamura and T. Toda, unpublished observations; S. Kjaerulff, Ph.D. Thesis, University of Copenhagen, 1997; Li and McLeod 1996). Furthermore, Ste11 activates its own transcription and consequently it would be expected that Ste11 levels would increase strongly, committing cells to the mating and meiotic pathway. Since this is not the case, it is likely that during mitotic growth ste11 function might be actively inhibited. This inhibition could occur either at the transcriptional or post-transcriptional level, or even at both levels. An earlier study of Ste11 regulation showed that, at least in vitro, Pat1 kinase phosphorylates Ste11 at residues Thr-173 and Ser-218, although the physiological significance of this phosphorylation could not be demonstrated (Li and McLeod 1996). Li et al. (1996) suggested that Pat1 kinase could inactivate Ste11 during mitosis by preventing Ste11 from accumulating in the nucleus. However, my results (Chapter 2 and 3) do not suggest that Ste11 localisation pattern or intrinsic transactivational potential is regulated by upstream signalling pathways. Interestingly, I could observe that Ste11 was actively targeted by the proteasomal degradation pathway when overexpressed from the intermediate strength promoter nmt41 (Fig. 3-2). Nevertheless, it remains to be demonstrated that Ste11 degradation by the proteasome plays an important physiological role in the control of entry into the sexual differentiation pathway. At the molecular level, it will be important to study the Ste11 polyubiquitination profile during the mitotic cell cycle and upon starvation-mediated induction of mating and meiosis. A second issue that should be addressed is the identification of the complex displaying ubiquitin-
protein ligase activity towards Ste11, and of the sequence determinants and protein modifications, like phosphorylation, that are required for targeted degradation of Ste11. My analysis of the expression of some of the LexA-Ste11 fusion proteins suggests, indirectly, that an internal region of Ste11 (residues 164 to 363) contributes to the regulation of Ste11 levels (Fig. 2-4, B). It is interesting to note that this sequence contains the two phosphorylation sites targeted by the Pat1 kinase. Could Ste11 phosphorylation by Pat1 function by stimulating Ste11 degradation by the 26S proteasome pathway during mitotic cell growth?

Another hypothetical explanation for the interruption of the Ste11 self-regulatory loop, during vegetative growth, is that under those conditions Ste11 might be actively prevented from binding its cognate cis-element, the TR-box. Thus, it would be very interesting to study the pattern of in vivo TR-box occupancy by Ste11 during the fission yeast cell cycle. A possible experimental approach would be to carry out chromatin immunoprecipitation (ChIP) experiments, which basically involve 3 steps: firstly, the formaldehyde cross-linking of intact cells, followed by the immunoprecipitation of protein-DNA complexes with specific antibodies, and finally the PCR-based detection of precipitated specific DNA sequences (Kuo and Allis 1999). This method allows the detailed analyses of protein-DNA interactions in a native chromatin environment, and has been successfully used in fission yeast (Ekwall et al. 1997). An important question concerning the control of Ste11 function is how does the pheromone signalling cascade stimulates transcription of a subset of the ste11-dependent genes, via Ste11 and the TR-box element (Aono et al. 1994; Kjaerulff et al. 1997). The ChIP experiments could also elucidate whether Ste11 binding to promoters is differentially regulated on target genes that require both starvation and pheromone signalling for induction, when compared to genes that are fully induced by nitrogen starvation.

Taking advantage of the fact that the TR-box is a relatively large cis-element and is often found in clusters, the database search described in Chapter 4, allowed the identification of a subset of the ste11-regulated genes.
In particular, it was surprising to identify *pat1* as a putative Ste11 target gene and to observe transcriptional upregulation of *pat1* in response to nitrogen starvation. If at first glance, upregulation of *pat1* transcript levels seems to conflict with its characterised role as an inhibitor of the sexual differentiation pathway, a two-staged analysis of its role in mating and meiosis might remove the apparent conflict. In fact, in response to nitrogen starvation *ste11* strongly induces the expression of the meiosis-promoting *mei2* gene. One of the characterised functions of the Pat1 kinase is to inhibit Mei2 through phosphorylation on residues Ser438 and Thr527, and expression of the Mei2-SATA allele (both residues mutated into Ala) that is not phosphorylated by Pat1 forces cells to bypass the conjugation event and enter haploid meiosis (Watanabe et al. 1997). This implies that in order to inhibit initiation of meiosis before a successful event, Pat1 must be present at a certain level to ensure a transient inhibition of Mei2 activity. When overexpressed, *pat1* has the ability to block mating, and so a gradual upregulation of *pat1* transcription might allow cells to proceed through the conjugation process and at the same time hold initiation of meiosis until a diploid status is reached. Pat1 activity is then inhibited by the activity of the diploid-specific gene product, Mei3, that can bind to Pat1 and inhibit its kinase catalytic activity (McLeod and Beach 1988).

In the near future, it will be very interesting to use microarrays, that allow the examination of the expression profile of every gene in the genome (Cho et al. 1998; Chu et al. 1998), to identify genes that are induced during mating and meiosis, and to compare their expression profile in wild-type and Δ*ste11* cells. In addition, several computational methods based on microarray data could be used to cluster *ste11*-regulated genes according to their expression profile, and to identify novel promoter *cis*-elements characteristic of each cluster (Futcher 2000; Pilpel et al. 2001).
6.2. Characterisation of *rgs1*

6.2.1. *rgs1* identification and expression

In this thesis, I characterised the function and expression pattern of the *S. pombe* *rgs1* gene, which was identified as a candidate during a genome wide screen for genes with clusters of TR-boxes in upstream regulatory regions. I show that *rgs1* expression requires *ste11* function, as well as nitrogen starvation and mating pheromone signalling. Two lines of evidence support the conclusion that *rgs1* induction requires pheromone treatment and an intact pheromone-activated MAPK cascade. Firstly, mutations in *byr2* and *byr1* genes, encoding kinases of the MAPK cascade responsible for transmission of pheromone signalling, completely abolished *rgs1* induction in homothallic cells; secondly, I observed that *h' Δcyr1Δsxa2* haploid cells have very low levels of Rgs1-GFP protein in the absence of pheromone exposure. This result also indicates that nitrogen starvation, by itself, is not sufficient for *rgs1* expression, as the disruption of the adenylate cyclase gene, *cyr1*, results in low levels of cAMP in the cell leading to derepression of genes under nutritional regulation. Therefore, *rgs1* transcription is regulated in a similar manner to *fus1*, another Ste11-dependent gene that is required for fusion.

6.2.2. *rgs1* function

During the course of this work, a description of the *rgs1* deletion phenotype was reported (Watson et al. 1999). Δ*rgs1* strains were found to be hypersensitive to pheromone stimulation, as revealed by the enhanced activation of the *Mat1-Pm* promoter by low levels of M-factor, and were unable to conjugate with a mating partner. My analysis of the *rgs1* deletion phenotype confirms and significantly extends these observations. The ability of cells to respond to nitrogen starvation by arresting in G1 is a pre-requisite for initiation
of the sexual development, ensuring that both mating partners conjugate in the haploid set to form a diploid zygote. I show that \( \Delta rgs1 \) mating defect is not due to a defect in G1 arrest upon nitrogen starvation. Furthermore, I show that unlike wild-type cells, \( \Delta rgs1 \) cells respond to pheromone stimulation in nitrogen-rich medium arresting in G1 and forming conjugation tubes. This characterisation of the pheromone sensitivity of \( \Delta rgs1 \) cells extends the previous observation that \( \Delta rgs1 \) cells increase their median cell volume when treated with pheromone (Watson et al. 1999). A discrepancy between the two analyses is that I observed a strong enhancement of sexual agglutination of the \( h^{90} \Delta rgs1 \) strain, while Watson et al. (1999) report a complete lack of sexual agglutination in their strain. In both strains the \( rgs1 \) ORF was fully deleted, so the reason for the discrepancy remains unclear.

The sterile phenotype of \( \Delta rgs1 \) cells is distinct from the phenotype resulting from \( ste11 \) deletion. In the latter case initiation of a sexual differentiation response is completely blocked, while deletion of \( rgs1 \) appears to derepress early events like the transcriptional induction of pheromone receptor, agglutination, G1 arrest, and pheromone-induced morphological changes but also to produce a block at a late stage of conjugation. These enhanced early events are also seen under conditions that result in abnormally high levels of pheromone signalling. Cells deleted for \( gap1/sar1 \), encoding a GTPase-activating protein for Ras1, or expressing Ras1-Val17, a constitutively active allele of Ras1, form extremely long conjugation tubes when exposed to pheromone and ultimately fail to mate (Fukui et al. 1986b; Nadin-Davis et al. 1986b; Imai et al. 1991; Wang et al. 1991a). Similarly, expression of the constitutively active Gpa1-QL allele, leads to pheromone-independent formation of abnormally extended conjugation tubes and mating failure (Obara et al. 1991). It has been proposed that a subset of RGS proteins could promote desensitisation of G protein-coupled-receptor pathways by acting in a classic negative feedback loop. For example, \( S. cerevisiae \) SST2 transcription is induced
by pheromone treatment, and Sst2 protein downregulates pheromone signalling, by binding and stimulating the intrinsic GTP hydrolytic activity of Gpa1, the pheromone receptor coupled G-protein α-subunit (Dietzel and Kurjan 1987; Dohlman et al. 1995; Apanovitch et al. 1998). Mammalian RGS1 appears to function in a similar way, as it is induced by platelet-activating factor (PAF) via a G-protein coupled receptor (GPCR) pathway, and subsequently acts to downregulate PAF-induced MAPK activity (Druey et al. 1996). \textit{rgs1} is involved in a similar negative feedback loop: \textit{rgs1} mRNA is transcriptionally induced by a pheromone-activated GPCR pathway, and Rgs1 has a role in downregulating signalling activity of the same pathway. This inability to adapt correctly to pheromone stimulation, may result in a deficient orientation of the conjugation tube towards the pheromone gradient. This chemotropism deficiency could be the reason why \textit{Δrgs1} cells fail to find a mating partner. In keeping with the demonstrated role for the RGS proteins, Rgs1 could function as a GTPase-activating protein for the pheromone-activated Gα Gpa1. The fission yeast genome encodes a second Gα, Gpa2, which is activated by high glucose levels and leads to activation of the Pka1 kinase. This results in repression of \textit{ste11} expression and blocks entry into the sexual differentiation pathway. As \textit{rgs1} expression is \textit{ste11}-dependent and induced by nitrogen starvation, Rgs1 would only be present upon starvation-induced deactivation of the Gpa2 pathway. I have also shown that \textit{rgs1} expression requires pheromone signalling which is only possible upon deactivation of Gpa2. These results strongly suggest that Rgs1 does not act as a GTPase-activating protein for Gpa2.

### 6.2.3. Rgs1 localisation and domain requirement

Several genetic, biochemical, and structural studies have suggested that RGS proteins act by directly binding Gα proteins, which localise to the plasma
membrane and, in some cases, to the Golgi apparatus. Interestingly, I show that Rgs1 protein mainly localises to the nucleus, even though a small pool of Rgs1 is also present in the cytoplasm, where potentially it could transiently bind plasma-membrane associated Gpa1. An important question that arises out of these results is whether nuclear Rgs1 performs a second role during mating, or whether it is stored in the nucleus, and therefore unable to bind Gpa1 and stimulate Gpa1-associated GTPase activity. In support of the former possibility, a recent study examining the intracellular localisation of several human RGS proteins showed that while some (RGS4 and RGS16) are predominantly cytoplasmic, shuttling between the nucleus and cytoplasm, others (RGS2 and RGS10) localise exclusively to the nucleus (Chatterjee and Fisher 2000). Interestingly RGS3 and RGS3T, a truncated variant, both inhibit Go(q/11) signalling, despite the fact that RGS3 localises to the cytoplasm and RGS3T mainly to the nucleus. The authors suggest that RGS3T must have other distinct functions in the cell nucleus (Dulin et al. 2000). However, the nature of the putative role of RGS proteins in the nucleus remains unknown.

I have shown that deletion of C-terminal RGS domain of Rgs1 renders Rgs1 non-functional, without significantly affecting its localisation or expression levels. However, the RGS domain alone is not sufficient to rescue Δrgs1 sterility suggesting that other regions of Rgs1 protein are important. The DEP domain of Drosophila Dishevelled protein is required for its plasma membrane recruitment upon Frizzled receptor activation. A subfamily of RGS proteins contains a DEP domain associated with a GGL (G-protein gamma like) domain. The latter domain has been shown to mediate binding to Gβ5 subunits in a similar fashion to conventional Gγ /Gβ (Snow et al. 1998; Snow et al. 1999). It has been suggested that members of this subfamily, like RGS6, RGS7, RGS9, RGS11, and C.elegans EGL-10, could form a plasma-membrane associated complex including receptor, Go-subunit protein, and effectors (Siderovski et al. 1999). The DEP domain would be responsible for membrane localisation of the
RGS protein. Interestingly, the fungal RGS proteins that contain a DEP domain, Rgs1, Sst2, and FlbA, have no recognisable GGL domain. However, these proteins display significant homology in an N-terminal region of about 194 aminoacid in length (Fig. 5-5). This homology domain appears to be only present in these three fungal RGS proteins, so I named it Fungal-Differentiation Regulator domain (Fungal-DR). Interestingly, the Fungal-DR domain has also been shown to be required for the ability of Sst2 to promote adaptation to pheromone signalling: either a short truncation of the N-terminal 45 residues of the Sst2 Fungal-DR domain or a point mutation (G137E) in a residue which is conserved in the three sequences resulted in loss of Sst2 function (Dohlman et al. 1996; Kallal and Fishel 2000). I have not observed plasma membrane recruitment of Rgs1 upon pheromone stimulation, and neither the deletion of the DEP nor of the Fungal-DR domain alter its intracellular distribution, even though both domains are essential for Rgs1 activity. It will be interesting to analyze the function of the DEP domain of Rgs1 as it is conserved in both metazoans and fungal RGS proteins, as well as in other signal transduction regulators, and yet its precise function or the motifs to which it binds remain unknown. Heterotrimeric G-protein signalling is required by several plant and human fungal pathogens to achieve full virulence (Bolker 1998). Analysis of the Fungal-DR domain could potentially reveal novel mechanisms of G-protein regulation that are fungal-specific and may therefore constitute a target for drug therapy.
CHAPTER 7

MATERIAL AND METHODS
7) MATERIAL AND METHODS

7.1. Reagents and Enzymes
Chemical reagents were purchased from BDH Chemicals, UK; Sigma, UK; and FISONS, UK; unless otherwise stated. Restriction endonucleases, DNA polymerases and modifying enzymes were purchased from New England Biolabs, UK; unless otherwise stated.

7.2. Strains and media

7.2.1. *E. coli* Strains

DH5α, supE44, ΔlacU169(f80lacZDM15), hsdR17, recA1, endA1, gyrA96, thi-1, relA1

7.2.2. Bacterial growth media

Bacteria were cultured in Luria broth (LB) prepared by the ICRF central services. LB contained: 1% Bacto-tryptone (Difco), 0.5% Bacto-yeast extract (Difco), 1% NaCl. For solid LB medium, 1.5% Bacto-agar (Difco) was also added. Culture media were supplemented with 50 μg/ml ampicillin where appropriate.

For cell recovery during transformation, BHI medium was used: 3.7% brain heart infusion powder.

7.2.3. *S. cerevisiae* strain

PJ69-4A: MATa trp1-901 leu2-3, 112 ura3-52 his3-200 gal4Δ gal80Δ

GAL2-ADE2 LYS2::GAL1-HIS3 met2::GAL7-lacZ

Generous gift from Philip James, (James et al. 1996).
7.2.4. *S. cerevisiae* media

*S. cerevisiae* cells were grown at 30°C on complete medium YPD (10g/l yeast extract (Difco), 20g/l Bacto-peptone (Difco), 20g/l D-glucose). Transformants were grown on SD synthetic medium (6.7 g/l yeast nitrogen base without amino acids (Difco), and other necessary nutrients as required).

7.2.5. *S. pombe* Strains

Strains used in this study were generally from Nic Jones and Paul Nurse Laboratories (ICRF):

- \( h^{90} \) ade6-M216 leu1-32 ura4-D18
- \( h^{+} \) ura4-D18 leu1-32
- \( h^{90} \) byr2::ura4\(^{+}\) ura4-D18 leu1
- \( h^{90} \) Abyr1 ura4-D18 leu1
- \( h^{90} \) ste11::ura4\(^{+}\) ade6-M216 leu1-32 ura4-D18
- \( h^{+} \) cyr1::LEU2\(^{+}\) sxa2::ura4\(^{+}\) ura4-D18 leu1-32
- \( h^{+} \) git2::his7\(^{+}\) ura4-D18 leu1 his7-366
- \( h^{+} \) pat1-114 ade6-M210 leu1-32 ura4-D18
- \( h^{90} \) atfl::ura4\(^{+}\) ura4-D18 leu1
- \( h^{+} \) styl-1 leu1-32 ura4-D18
- \( h^{90} \) styl::ura4\(^{+}\) ura4-D18 leu1;
- \( h^{90} \) spk1::ura4\(^{+}\) ura4-D18 leu1
- \( h^{+} \) cdc10-129 leu1
- \( h^{+} \) cdc25-22 leu1
- \( h^{+} \) mis3-1 leu1-32
- \( h^{90} \) pcr1::ura4\(^{+}\) leu1-32 ura4-D18

The following strains were newly constructed:

- \( h^{+} \) cyr1::LEU2\(^{+}\) sxa2::ura4\(^{+}\) rgs1GFP(kan) ura4-D18 leu1-32.
- \( h^{90} \) rgs1::ura4\(^{+}\) ade6-M216 leu1-32 ura4-D18;
- \( h^{+} \) rgs1::ura4\(^{+}\) leu1-32 ura4-D18;
7.2.6. *S. pombe* media

YE5S: 0.5% (w/v) oxoid yeast extract, 3% (w/v) glucose, 150mg/l adenine, histidine, leucine, uracil and lysine hydrochloride. For solid medium, 2% Bacto-agar (Difco) was also added.

EMM: 3g/l potassium hydrogen phthalate, 2.3g/l Na$_2$HPO$_4$, 5g/l NH$_4$Cl, 2%(w/v) Glucose, 20ml/l Salts (50xstock), 1ml/l Vitamins (1000xstock), 0.1ml/l Minerals (10,000xstock). For solid medium, 2% Bacto-agar (Difco) was also added.

EMM(-NH$_4$Cl): as EMM but without NH$_4$Cl.

50xStock Salts contains 52.5g/l MgCl$_2$.6H$_2$O, 0.735mg/l CaCl$_2$.2H$_2$O, 50g/l KCl, 2g/l Na$_2$SO$_4$.

1000xStock Vitamins contains 1g/l pantothenic acid, 10g/l nicotinic acid, 10g/l myo-inositol, 10mg/l biotin.

10,000xStock Minerals contains 5g/l boric acid, 4g/l MnSO$_4$, 4g/l ZnSO$_4$.7H$_2$O, 2g/l FeCl$_2$.6H$_2$O, 0.4g/l Molyboric acid, 1g/l KI, 0.4g/l CuSO$_4$.5H$_2$O, 10g/l citric acid.

7.3. Molecular Biology techniques

7.3.1. General techniques

The following techniques were performed essentially as described (Sambrook et al. 1989): preparation of competent bacteria cells, transformation of bacteria with plasmid DNA, ethanol precipitation of nucleic acids, phosphatase treatment of vector DNA to remove 5' terminal phosphate groups, ligation of DNA fragments, minipreps.
7.3.2. DNA sequencing

All sequencing was performed using a PE Applied Biosystems (USA) automated sequencer that was operated by the I.C.R.F. central sequencing service. AmpliTaq DNA polymerase, FS and dichlororhodamine dye terminators were used to generate PCR fragments to be sequenced by fluorescence-based cycle sequencing.

7.3.3. Polymerase Chain Reaction (PCR)

High fidelity Vent (New England Biolabs) or Expand (Boehringer Mannheim) DNA polymerases were used to produce fragments for subcloning by PCR. Typically, reactions were set up according to manufacturers instructions, and the PCR program used was: 5min at 94°C, 30x(1min at 94°C, 1 min at 50°C, 1 min at 72°C) and 10min at 72°C. The annealing temperature was occasionally adjusted for particular combinations of oligonucleotides, and extension times optimized for relatively smaller or larger PCR fragments. When routinely checking yeast genotypes, or when generating DNA fragments to be used as probes for Northern blotting, TAQ polymerase provided by the I.C.R.F. central services was used in a reaction buffer of 10mM Tris-HCl pH8.5, 50mM KCl, 1.5mM MgCl2.

7.3.4. Oligonucleotides used in PCR reactions

All the oligonucleotides were prepared using an Applied Biosystems 380B DNA Synthesiser by the ICRF Oligonucleotide Synthesis Laboratory.

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7.3.5. DNA constructs

LexA-Ste11 fusion expression constructs:

The coding region of full-length, N- and C-terminal Ste11 truncations were amplified by PCR from template pAS1-Ste11 (gift from Mark Toone), using
primers that introduced NotI and SpeI restriction sites at the 5' and 3' ends of the PCR fragment, respectively. The following primers were used:

- **pLexA-Ste11FL:** \([\text{ste11(NotI)} +1] \text{ and } [\text{ste11(Spel)} -468]\)
- **pLexA-Ste11 ΔC104:** \([\text{ste11(NotI)} +1] \text{ and } [\text{ste11(Spel)} -364]\)
- **pLexA-Ste11 ΔC207:** \([\text{ste11(NotI)} +1] \text{ and } [\text{ste11(Spel)} -261]\)
- **pLexA-Ste11 ΔC305:** \([\text{ste11(NotI)} +1] \text{ and } [\text{ste11(Spel)} -163]\)
- **pLexA-Ste11 ΔN92:** \([\text{ste11(NotI)} +93] \text{ and } [\text{ste11(Spel)} -468]\)
- **pLexA-Ste11(93/261):** \([\text{ste11(NotI)} +93] \text{ and } [\text{ste11(Spel)} -261]\)
- **pLexA-Ste11(93/163):** \([\text{ste11(NotI)} +93] \text{ and } [\text{ste11(Spel)} -163]\)
- **pLexA-Ste11 ΔN260:** \([\text{ste11(NotI)} +261] \text{ and } [\text{ste11(Spel)} -468]\)
- **pLexA-Ste11 ΔN363:** \([\text{ste11(NotI)} +364] \text{ and } [\text{ste11(Spel)} -468]\)
- **pLexA-Ste11(261/364):** \([\text{ste11(NotI)} +261] \text{ and } [\text{ste11(Spel)} -364]\)

The PCR fragments were purified, digested with NotI and SpeI, and cloned into pLexA vector (Peter Stacey, Ph.D. Thesis 1997, University of London).

**Construction of GFP fusion expression constructs:**

- **p409GFP-Ste11.** *ste11* ORF was amplified by PCR from template pAS1-Ste11 using a forward primer introducing a terminal *SalI* site \([\text{ste11(SalI)} +1]\) and a reverse primer introducing a *BamHI* site \([\text{ste11(BHIS)} -468]\). Reaction products were digested with *NotI* and *SalI* and ligated into *NotI-SalI* digested p409(*nmt41*)GFP vector (Sawin and Nurse 1996).

- **pRep81 Ste11-GFP.** *ste11* ORF was amplified by PCR from template pAS1-Ste11 using a forward primer introducing a terminal *SalI* site \([\text{ste11(SalI)} +1]\) and a reverse primer introducing a *BamHI* site \([\text{ste11(BHIS)} -468]\). Reaction products were digested with *NotI* and *SalI* and ligated into *NotI-SalI* digested pRep81-GFP (Watanabe et al. 1997).
p409GFP-Rgs1 fusion constructs. rgs1 ORF was amplified by PCR from cosmid c22F3 (from Sanger Centre, Cambridge, U.K.) with primers [rgs1(NotI) +1] and [rgs1 (Sall) –481], and cloned in vector pCR2.1 (Invitrogen, CA) generating pCR-rgs1. Constructs encoding N-terminal GFP-tagged Rgs1 truncations were generated by PCR amplifying rgs1 ORF fragments from template pCR-rgs1 using forward primers with terminal NotI sites and reverse primers with Sall sites. Reaction products were digested with NotI and Sall and ligated into NotI-Sall digested p409(nmt41)GFP vector (Sawin and Nurse 1996). Primers used were as follow:

- p409GFP-Rgs1 1/481: [rgs1(NotI) +1] and [rgs1 (Sall) –481]
- p409GFP-Rgs1 1/320: [rgs1(NotI) +1] and [rgs1(Sall) –320]
- p409GFP-Rgs1 314/481: [rgs1(Not) +314] and [rgs1 (Sall) –481]
- p409GFP-Rgs1 230/481: [rgs1(NotI) +230] and [rgs1 (Sall) –481]
- p409GFP-Rgs1 94/481: [rgs1(NotI) +94] and [rgs1 (Sall) –481]

The p409GFP-Rgs1 Δ232/311 internal deletion was constructed by a PCR method previously described (Russel 1990). N-terminal region was amplified using primers [rgs1(NotI) +1] and [rgs1(A232/311)rev], and for the C-terminal region the primers used were [rgs1(A232/311)for] and [rgs1 (Sall) –481].

7.3.6. RNA preparation and Northern blotting

Total fission yeast RNA was prepared using a glass beads-phenol protocol described previously (Zhu et al. 1997). RNA was prepared from 50ml cultures with an OD of 0.5, washed in RNA extraction buffer (0.1M EDTA, 0.1M NaCl, 0.05M Tris pH8.0), and resuspended in eppendorf tubes using 200 μl cold RNA extraction buffer. For cell lysis, 200 μl phenol/chloroform and 10 μl 10% SDS were added. Glass beads were added to the meniscus level, and tubes vortexed three times (1min each time, with 1min rest inbetween). 600 μl of RNA extraction buffer were added, the aqueous phase was separated and re-extracted twice with phenol/chloroform. RNA was ethanol-precipitated and
stored in RNase-free water at -70°C. For RNA analysis, 8 μl of DMSO, 1.6 μl
0.1M phosphate buffer pH 6.5, and 2.5 μl of 6.6M deionised glyoxal were added
to 5 μl of RNA sample (2μg/μl). The mixture was heated to 50°C for 15min to
denature RNA, briefly cooled on ice and 4 μl of RNA loading buffer added
(50% glycerol, 10mM phosphate buffer pH 6.5, 0.4% bromophenolblue). Samples
were electrophoretic separated on a gel [1.2% agarose, 15 mM sodium
phosphate (pH 6.5)] at 4V per cm, and transferred to a GeneScreen
hybridization membrane (Dupont NEN, Boston, MA, USA). RNA was fixed to
membrane with UV light, using a "Stratalinker" (Stratagene). Probes were
labelled with [α-32P]dCTP by random priming using a DNA labelling kit
(MegaPrimer, Amersham). Hybridisation was carried out overnight at 42°C in 5x
SSPE, 50% formamide, 5x Denhardt's solution, 10% Dextran solution, 1% SDS,
and 100μg/ml heat denatured, sonicated salmon sperm DNA, after 1-3 hours
pre-hybridisation. Blots were washed using two 45min washes at 65°C in
2xSSPE, 2% SDS, followed by two 15min washes at room temperature in
0.1xSSPE.

Probes:
Probes for several genes were made by PCR amplification from template
genomic DNA, using standard PCR techniques. The primers used were as
follows:

- **rgs1** probe (1.0-kb ORF fragment): rgs1-for and rgs1-rev
- **mam2** (1.0-kb full-length ORF): mam2-for and mam2-rev
- **cdc2** (1.2-kb ORF fragment): cdc2-for and cdc2-rev.
- **ste11** (1.4-kb fragment): [ste11(NotI) +1] and [ste11(SpeI) -468]
- **shk2/pak2** (0.9-kb fragment): shk2-for and shk2-rev
- **pat1** (1.4-kb fragment): pat1-for and pat1-rev

The enolase probe was a gift from Mike Samuels.
7.4. Yeast related techniques

7.4.1. Transformation of fission yeast by electroporation

Transformation of fission yeast cells with plasmid DNA was done using electroporation (Toone et al. 1998). 200ml of EMM supplemented with corresponding nutrients was inoculated with 5mls of freshly saturated yeast preculture. The culture was grown for 6 hours, and then harvested by centrifugation at 2000g for 3mins at 4°C. The pellet was washed once in ice-cold water, and twice in ice cold, filter sterilised 1.2M sorbitol. The cells were resuspended in 1.2M sorbitol at approximately $10^9$ cells per ml, and stored in aliquots at -70°C. 2µl of plasmid DNA (approx 0.25-1µg) was placed in a 2mm gap electroporation cuvette and chilled on ice. 200µl of competent yeast were thawed, and added to the cuvette. The cells and DNA were mixed by gentle pipetting. Electroporation was performed immediately, using a Biorad Gene Pulser set at 200Ω, 25uF and 2250V (time constants were typically 4.7-4.9ms). 400µl of ice-cold 1.2M sorbitol was then added directly to the cuvette, and a 200µl aliquot of the transformation was spread on an EMM plate with appropriate selection. The plate was incubated at 30°C for 3-4 days until colonies appeared.

7.4.2. Transformation of fission yeast cells with PCR products

Transformation of S.pombe with relatively short PCR products to tag or delete genes was performed using a lithium acetate/DMSO protocol as previously described (Bahler et al. 1998).

7.4.3. Mating and sporulation quantification assay

Homothallic haploid ($h^+$) cells were induced to mate in EMM or EMM(-NH4Cl) as indicated. Cells were observed by phase-contrast microscopy, and mating
and sporulation frequencies (Mat %) were calculated using the following equation: Mat(%) = (2Z + 2A + 0.5S)/ (H + 2Z + 2A + 0.5S) where Z stands for the number of zygotes, A for the number of asci, S for the number of free spores, and H for the number of cells that failed to mate (Kunitomo et al. 1995).

7.4.4. Flow cytometric analysis

1 x 10^7 cells were centrifuged, washed once with H_2O and fixed in 1 ml of cold 70% ethanol. ~2 x 10^6 cells were then rehydrated in 3 ml of 50 mM Sodium citrate, centrifuged, resuspended in 0.5 ml 50 mM Sodium citrate containing 0.1 mg/ml RNase, and incubated overnight at 37 °C. Propidium iodide was added to achieve a final concentration of 2 μg/ml, and after sonication, analysis was performed with a Becton-Dickinson FACScan.

7.4.5. rgs1 disruption

rgs1 was disrupted using a recently described PCR-based one-step gene disruption protocol (Bahler et al. 1998). Briefly, ura4^ gene was amplified by PCR with 2 primers containing 80-bp tails corresponding to regions immediately upstream and downstream of the rgs1 ORF, rgs1(KO)for and rgs1(KO)rev, respectively. The resulting product was purified and transformed into an h^90/h^90 diploid strain, by lithium acetate protocol. Ura4^ transformants were selected and chromosomal disruption of rgs1 confirmed by PCR as described by Bahler et al. (1998). Tetrads of sporulating heterozygous diploid cells was performed, and I observed that ura4^ segregation was 2:2, suggesting that rgs1 is not an essential gene. All ura4^ segregants were also Δrgs1 and sterile, while ura4^- segregants were rgs1^ and had normal mating frequencies, confirming that sterility is due to rgs1 disruption. rgs1 was independently disrupted in an h^90 strain, and several Δrgs1 sterile strains obtained that showed the phenotype previously observed.
7.4.6. **rgs1 tagging**

Rgs1 was tagged at its COOH terminus with green fluorescent protein (GFP) carrying the S65T mutation by direct chromosomal integration into strain h\(^{+}\)cyr1::LEU2\(^{+}\) sxa2::ura4\(^{-}\) ura4-D18 leu1-32 of a fragment generated by PCR using plasmid pFA6a-GFP(S65T)-kanMX6 as template (Bahler et al. 1998). The two primers, [rgs1(ta)for] and [rgs1(ta)rev], had 80-bp tails corresponding to the regions just upstream and 51-130 bp downstream of the rgs1 ORF stop codon. The resulting strain was checked for correct integration by PCR as described by (Bahler et al. 1998).

7.4.7. **Fluorescence microscopy**

GFP fluorescence was observed in living cells stained with DAPI. Culture samples were gently centrifuged, washed in H\(_2\)O, and centrifuged again. Cell pellets were resuspended in a few μl of H\(_2\)O and spread on poly-L-lysine coated coverslips. 5 μl of DAPI-containing (1.5 μg/ml) mounting medium (Vector Laboratories, Burlingame, CA, USA) was added, and the coverslip mounted on a microscope slide.

7.4.8. **β-galactosidase activity assay in fission yeast cultures**

A 1 ml aliquot of a culture in exponential growth phase, was centrifuged at 2000g for 3 minutes, washed with 1ml of Z buffer [60mM Na\(_2\)HPO\(_4\), 40mM NaH\(_2\)PO\(_4\), 40mM KCl, 1mM MgSO\(_4\) pH 7.5, 0.03M b-mercaptoethanol (added just before use)], and resuspended in 600μl of Z buffer. 50μl chloroform and 50μl 0.1% SDS were added to the tube which was then vortexed for 30secs and then equilibrated at 30°C for 5min. 200μl of ONPG (4mg/ml stock in Z buffer) was added and the sample incubated at 30°C until a yellow colour developed. The reaction was then stopped by adding 400μl of 1M Na\(_2\)CO\(_3\). The sample was centrifuged in a microfuge for 10mins, the OD\(_{420}\) of the clarified supernatant was determined, against a blank of the above mixture, without
cells. A 100μl aliquot of the original cell culture was resuspended in 1ml of water, and the OD\textsubscript{595} determined. β-galactosidase activity expressed in arbitrary "Miller" units was calculated as follows: Units≈1000 x OD\textsubscript{420} / (incubation time in mins x OD\textsubscript{595} x 10)

7.4.9. Two-hybrid analysis

The plasmids and yeast strain used in the two-hybrid analysis are described in James et al. (1996). \textit{rgs1} ORF was generated by PCR amplification from template p409Rgs1 using primers that introduced terminal EcoRI and PstI sites, \texttt{[rgs1(EcoRI)for]} and \texttt{[rgs1(PstI)rev]}, respectively. This product was digested and fused in frame to Gal4-binding domain in pGBD plasmid (sites EcoRI and PstI) generating pGBD-Rgs1. In a similar way, pGAD-Gpa1 and pGAD-Gpa1QL were constructed by PCR amplifying both ORFs from templates pART1-Gpa1 (Obara et al. 1991) and pRep2Gpa1Q244L (kind gift from K. Kitamura), using primers \texttt{[gpa1(EcoRI)for]} and \texttt{[gpa1(PstI)rev]}, and fusing them in frame with Gal4-AD in EcoRI and PstI sites in pGAD plasmid. As a positive control, plasmid pCL1 (Clontech) expressing the full length, wild-type \textit{S. cerevisiae} Gal4 protein was used. \textit{S. cerevisiae} strain PJ69-4A was transformed with the various combinations of plasmids and the β-galactosidase activity measured using liquid assays, as described previously (James et al. 1996).

7.5. Western Blots

About 1x10\textsuperscript{8} cells were harvested by centrifugation at 2000g for 3min at 4°C, washed in 50ml of ice-cold water and resuspended in 200μl of 20% TCA. Cells were lysed using acid-washed glass beads in a Fast-Prep centrifuge (Bio 101, Savant)(3x1 min at fastest speed). 400 μl of 5% TCA were added to the cell lysates, and protein pellets obtained by centrifugation at 10,000g for 3 min at room temperature. Pellets were resuspended in 200 μl of Laemli buffer plus 50 μl of 1M Tris (pH 8.0). Protein extract samples (~100 μg) were electrophoresed
on a 10% SDS-PAGE gel and transferred to Immobilon-P (PVDF) membranes (Millipore, MA). Primary antibodies used were:

- mouse monoclonal anti-α-tubulin (T5168, Sigma)
- mouse monoclonal anti-GFP (8362-1, Clontech).
- mouse monoclonal anti-Myc (9E10, Babco)
- mouse monoclonal anti-LexA (5397-1, Clontech)
- mouse monoclonal anti-Ste11 (a generous gift from O. Nielsen)

Bound primary antibodies were detected using HRP-conjugated goat anti-mouse IgG (Biorad, CA) and enhanced chemiluminescence (ECL, Amersham, U.K.).
CHAPTER 8

REFERENCES
8) REFERENCES


