The role of the F-Box protein Frp1 in pathogenicity of fusarium oxysporum
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Chapter II

Lessons from fungal F-box proteins

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Introduction: The F-box hypothesis

The F-box domain - so called after a conserved domain found in human Cyclin F (Bai et al., 1994) - was described in 1996 (Bai et al., 1996) after first being denoted as a conserved N-terminal domain found in a subset of proteins (Kumar & Paietta, 1995b). The F-box hypothesis was introduced shortly after (Skowyra et al., 1997, Patton et al., 1998), and holds that F-box-containing proteins (henceforth F-box proteins) act as scavengers in the cell, collecting ‘junk’ proteins to deliver to a ‘waste processor’, called the SCF complex, to which they dock through their F-box domain. In the SCF complex, the junk proteins are marked with ubiquitin for ‘incineration’ in the proteasome. F-box proteins do not act indiscriminately but recruit specific, often modified proteins to the SCF-complex and in this way regulate the level of certain proteins in a cell. F-box proteins are found in all eukaryotes and display a large variety of functions. In fungi they are, for example, involved in controlling the cell division cycle, glucose sensing, mitochondrial connectivity and control of the circadian clock.

F-box proteins are commonly identified by the presence of a stretch of primary sequence that matches the consensus for an F-box (Figure 1). However, it can be questioned whether just the occurrence of an F-box domain in a protein sequence is sufficient to assume compliance with the F-box hypothesis. The F-box hypothesis is based on the assumption that an F-box mediates assembly into an SCF-complex through binding to the Skp1 subunit (Figure 2). The SCF complex consists of Skp1 (suppressor of kinetochore protein mutant (Connelly & Hieter, 1996)), Cul1 (Cullin (Mathias et al., 1996)), Rbx1 (ring-box protein (Kamura et al., 1999)) and an F-box protein and catalyses, like other E3-ligases, in co-operation with the E1 and E2 enzymes, the transfer of the small protein ubiquitin to the target protein (Patton et al., 1998, Krek, 1998).

![Figure 1: The F-box consensus sequence.](image)

The motif is about 45 amino acids long and based on the HMM-logo for the F-box motif (Schuster-Bockler et al., 2004). Highly conserved amino acids are underlined and the two most conserved amino residues, the leucine and proline at position six and seven, are indicated in red. At each position, amino acids are ordered from top to bottom in decreasing occurrence in F-box domains.

![Figure 2: The SCF complex and ubiquitination of target proteins.](image)

The SCF-complex functions within the ubiquination reaction through combined action with the E1 and E2 enzymes. F-box proteins bind to Skp1 via their F-box domain and to targets via their C-terminal domain, thereby presenting the target for ubiquitination.

Among fungi, the regulation of the SCF complexes and hence the regulation of the F-box proteins in these complexes appear to differ. SCF complexes are likely activated and
regulated through a recycling mechanism (Cope & Deshaies, 2003), which involves three main contributors: the neddylation protein DCN1, responsible for the transfer of Nedd8 to Cul1 (Lyapina et al., 2001, Pan et al., 2004, Saha & Deshaies, 2008, Kurz et al., 2005, Osaka et al., 2000, Yang et al., 2007), the de-neddylation CSN (COP9 signalosome) (Schwechheimer, 2004, Maytal-Kivity et al., 2003, Maytal-Kivity et al., 2002, Mundt et al., 1999, Wee et al., 2002) (reviewed in (Wu et al., 2006, Wei & Deng, 2003, Pan et al., 2004)) and the CAND1 protein (Zheng et al., 2002, Liu et al., 2002), which binds to de-neddylated Cul1 and competes out the Skp1-F-box complex from the core of the SCF. A new round of neddylation removes CAND1 and thereby creates binding space for a new Skp1-F-box complex. In budding yeast (Saccharomyces cerevisiae), deletion mutants for Nedd8 as well as CSN5, the CSN subunit responsible for the de-neddylation reaction, are both viable (Cope et al., 2002, Lammer et al., 1998, Liakopoulos et al., 1998). This means that although the components of the SCF recycling mechanism are present, this process is not required for survival. A second difference in budding yeast in comparison to other fungi is that is does not have the CAND1 protein, adding to the notion that in budding yeast recycling acts differently. In fission yeast (Schizosaccharomyces pombe), CAND1 is present, and Nedd8 is required for survival (Osaka et al., 2000), but not the CSN5 subunit (Mundt et al., 2002, Zhou et al., 2001). Apparently in fission yeast, the neddylation reaction is required for proper SCF function, but de-neddylation is not, suggesting that in fission yeast an alternative de-neddylation may be present. In Neurospora crassa, a deletion mutant for subunit 2 of the CSN, Δcsn-2, is viable but lacks a normal circadian rhythm and conidiation (He et al., 2005) while in Aspergillus nidulans four CSN mutants, including one for subunit 5, (Δcsne) all lack fruit body development (Busch et al., 2003, Busch et al., 2007). Together, these data suggest that, in filamentous fungi, proper recycling of the SCF is only strictly required for certain developmental processes, in accordance with the requirements of the CSN in development in more complex multicellular organisms (reviewed in (Schwechheimer, 2004)).

Some F-box proteins appear to function without binding to Skp1, suggesting that not all F-box proteins take part in an SCF-complex. This also means that not all proteins interacting with an F-box protein will be ubiquinated and proteasomally degraded. In another deviation from the F-box hypothesis, some F-box protein/Skp1 complexes do not seem to be involved in ubiquitination. Furthermore, even when an F-box domain mediates assembly into an SCF complex, the result may be self-ubiquitination rather than fulfilling a scavenger function.

Since 1996 several review articles have been published covering the emerging theme of ubiquitin-mediated protein degradation and the widespread occurrence of F-box proteins (Kipreos & Pagano, 2000, Ho et al., 2006, Ho et al., 2008, Hermand, 2006, Vierstra, 2003, Willems et al., 2004, Craig & Tyers, 1999, Lechner et al., 2006, Willems et al., 1999). Here, we discuss fungal F-box proteins including their targets (if identified) and, when possible, classify these F-box proteins according to degree of compliance with the F-box hypothesis. Most literature on fungal F-box proteins covers those found in budding yeast and, to a lesser extent, fission yeast, but important findings have also been reported for filamentous ascomycetes. In Table 1 fungal F-box proteins described in literature are listed according to their main cellular function. The distantly related budding and fission yeasts share ten (likely) orthologous F-box proteins. Budding yeast contains additional eleven F-box proteins and fission yeast seven (Katayama et al., 2002). Cdc4, Grr1 and Met30 from budding yeast and their counterparts in other fungi are the most studied fungal F-box proteins and are conserved throughout the fungal kingdom. In total, 31 F-box proteins will be discussed, exclusively from ascomycetes: the ‘model’ fungi Saccharomyces cerevisiae, Schizosaccharomyces pombe, Kluyveromyces lactis, Aspergillus nidulans, Hypocrea jecorina and Neurospora crassa, and the pathogenic fungi Candida albicans, Fusarium graminearum, Fusarium oxysporum and Magnaporthe grisea.
F-box proteins complying with the F-box hypothesis

To date, the best-described fungal F-box proteins comply with the F-box hypothesis: the targets of these F-box proteins are commonly first phosphorylated before being recognized and ubiquitinated by the SCF-complex and finally degraded by the proteasome. In a study (Kus et al., 2004) in which interactors of Skp1 and the SCF-complex were identified in budding yeast, thirteen F-box proteins were found to bind Skp1 and these thirteen could all be co-purified with an SCF complex. These are Cdc4, Ctf13, Dia2, Grr1, Hrt3, Mdm30, Rcy1, Ufo1, and five uncharacterized F-box proteins. In another study investigating the binding partners of Skp1 and Cul1 (Seol et al., 2001), Met30 and Saf1 were also found to bind Skp1 and two more uncharacterized F-box proteins were found to bind Skp1 and/or Cul1. In the study mentioned above (Kus et al., 2004), auto-ubiquitination of F-box proteins was also investigated using two different E2-enzymes, Cdc34 and Ubc4.

Twelve out of the thirteen F-box proteins showed self-ubiquitination – only Grr1 was found not to be ubiquitinated and Dia2 and Mdm30 showed very little ubiquitination. Also, it was demonstrated that these F-box proteins were differentially ubiquitinated by the two different E2-enzymes and that different numbers of ubiquitin molecules were attached to the F-box proteins. In other reports, ubiquitination and degradation of Cdc4, Met30 and also Grr1 was demonstrated (Galan & Peter, 1999, Zhou & Howley, 1998). It is still unknown whether F-box proteins are ubiquitinated and degraded together with their targets in each degradation round. Another possibility is that they are recycled after recruiting their targets and only ubiquitinated and degraded when unbound to a target protein.

In a study with fission yeast, eleven F-box proteins investigated (Pop1/2, Pof1, Pof3, Pof5, Pof7, Pof8, Pof9, Pof10, Pof12, Pof13 and Fbh1/Pof15 (Lehmann A., 2004)) could all bind to Skp1. The interactions were further studied with a temperature sensitive mutant of Skp1 with point mutations in the Skp1-F-box interaction core. Only the binding to Pof1, Pof3 and Pof10 was weaker with this mutant. The effect of this weakened binding on the function of the individual F-box protein is not known and targets of most of these fission F-box proteins remain to be identified.

C-terminal protein-protein interaction domains

Most of the fungal F-box proteins that comply with the F-box hypothesis have a recognizable C-terminal protein-protein interaction domain (Table 1). Four F-box proteins carry a WD40 domain: Cdc4 (and its orthologs), Fwd1, Ufo1 and Met30 (and its orthologs). Grr1 and its orthologs carry an LRR (leucine-rich repeat) domain (Kajava, 1998), a repeat of about 25 amino acids forming a non-globular, crescent-shaped structure. Saf1 carries a RCC1 repeat (Renault et al., 1998), another domain forming a beta-propeller structure involved in protein-protein interactions. Of the F-box proteins that comply with the F-box hypothesis, only Mdm30 does not contain any known protein-protein interaction motif and it is unknown how it interacts with its targets Fzo1, Mdm34 and Gal4c. The presence of a recognizable protein-protein interaction domain might be an indication that an F-box protein complies with the F-box hypothesis. One of the uncharacterized budding yeast F-box proteins (Ylr352w) that binds Skp1 and Cul1 also contains an LRR domain, suggesting that this F-box protein might also comply with the F-box hypothesis. For Cdc4, Dia2, Grr1, Met30, Mdm30, Saf1 and Ufo1 of budding yeast and Fwd1 from N. crassa one or more targets are known, and most of these targets are degraded via the SCF-complex. This suggests that at least these seven F-box proteins completely fulfill the F-box hypothesis For Cdc4, Dia2, Grr1 and Met30, homologs in other fungal species have been found and characterized, in some cases together with their targets. The degree of conservation of the functions of these F-box proteins between the different species can now be assessed by comparing the different phenotypes of deletion mutants and the conservation of targets.
Table 1: Fungal F-box protein described in literature and discussed in this review (see text for references).

<table>
<thead>
<tr>
<th>Cellular function</th>
<th>F-box protein</th>
<th>Fungal Species</th>
<th>Additional motif¹</th>
<th>Target(s)</th>
<th>Skp1 binding</th>
<th>SCF assembly</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell division cycle, morphological switch, nutrient and calcium sensing</td>
<td>Cdc4</td>
<td>S. cerevisiae</td>
<td>WD40</td>
<td>Sic1, Swi5, Far1, Cdc6, Cbl6?, Tec1, Gcn4,Hac1 Rcn1, Ctf13</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Pop1</td>
<td>S. pombe</td>
<td>WD40</td>
<td>Rum1, Cdc18, Cig2 Sol1, Far1</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Pop2</td>
<td>C. albicans</td>
<td>WD40</td>
<td>Yes</td>
<td>yes</td>
<td></td>
</tr>
<tr>
<td>DNA replication</td>
<td>Dia2</td>
<td>S. cerevisiae</td>
<td>TPR +LRR</td>
<td>Tec1</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Pof3</td>
<td>S. pombe</td>
<td>TPR +LRR</td>
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<tr>
<td></td>
<td>Grr1</td>
<td>S. cerevisiae</td>
<td>LRR</td>
<td>Cln1/2, Gic2, Ime2, Hof1, Std1, Mth1, Gis4, Pfk27, Tye7, Gala/b, MsK1</td>
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<td>yes</td>
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<td></td>
<td>Grr1</td>
<td>K. Lactis</td>
<td>LRR</td>
<td>Sms1</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>Grr1</td>
<td>C. albicans</td>
<td>LRR</td>
<td>Ccn1, Cln1, Hof1</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>GrrA</td>
<td>A. nidulans</td>
<td>LRR</td>
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<td>?</td>
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<td></td>
<td>Fbp1</td>
<td>F. graminearum</td>
<td>LRR</td>
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<td></td>
<td>Pth1</td>
<td>M. grisea</td>
<td>LRR</td>
<td>?</td>
<td>?</td>
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<tr>
<td>Methylmercury resistance</td>
<td>Hrt3</td>
<td>S. cerevisiae</td>
<td>-</td>
<td>?</td>
<td>yes</td>
<td>yes</td>
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<tr>
<td>Mitochondrial function</td>
<td>Mdm30</td>
<td>S. cerevisiae</td>
<td>-</td>
<td>Fzo1, Gal4c, Mdm34</td>
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<td>yes</td>
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<tr>
<td>Quiescence</td>
<td>Saf1</td>
<td>S. cerevisiae</td>
<td>RCC1</td>
<td>Aah1, Ura7?</td>
<td>yes</td>
<td>yes</td>
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<tr>
<td>Genome stability</td>
<td>Ufo1</td>
<td>S. cerevisiae</td>
<td>WD40 + UIM</td>
<td>Ho, Rad30</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Circadian clock</td>
<td>Fwd1</td>
<td>N. crassa</td>
<td>WD40</td>
<td>Frq</td>
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<td>yes</td>
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<tr>
<td>Sulphur metabolism</td>
<td>Met30</td>
<td>S. cerevisiae</td>
<td>WD40</td>
<td>Met4</td>
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<td>yes</td>
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<tr>
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<td>Pof1</td>
<td>S. pombe</td>
<td>WD40</td>
<td>Zip1</td>
<td>?</td>
<td>yes</td>
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<tr>
<td></td>
<td>Scon2</td>
<td>N. crassa</td>
<td>WD40</td>
<td>Cys3</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td></td>
<td>SconB</td>
<td>A. nidulans</td>
<td>WD40</td>
<td>MetR</td>
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<td>?</td>
</tr>
<tr>
<td></td>
<td>Lim1</td>
<td>H. jecorina</td>
<td>WD40</td>
<td>?</td>
<td>?</td>
<td></td>
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<tr>
<td>DNA repair</td>
<td>Fbh1</td>
<td>S. pombe</td>
<td>helicase</td>
<td>?</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td>Root infection</td>
<td>Frp1</td>
<td>F. oxysporum</td>
<td>-</td>
<td>?</td>
<td>yes</td>
<td>?</td>
</tr>
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<td>Peroxide resistance</td>
<td>Pof14</td>
<td>S. pombe</td>
<td>-</td>
<td>Erg9 (inhibited, not degraded)</td>
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<td>?</td>
</tr>
<tr>
<td>Kinetochore assembly</td>
<td>Ctf13</td>
<td>S. cerevisiae</td>
<td>-</td>
<td>?</td>
<td>yes</td>
<td>no</td>
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<tr>
<td>Membrane trafficking</td>
<td>Rcy1</td>
<td>S. cerevisiae</td>
<td>SEC10 + CAAX</td>
<td>Snc1, Kex2 (both recycled, not degraded)</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td></td>
<td>Pof6</td>
<td>S. pombe</td>
<td>SEC10 + CAAX</td>
<td>Sip1 (not recycled or degraded)</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>DNA repair</td>
<td>Ela1</td>
<td>S. cerevisiae</td>
<td>ElonginA + coiled coil</td>
<td>Rpb1</td>
<td>no</td>
<td>no</td>
</tr>
<tr>
<td>Mitochondrial function</td>
<td>Mfb1</td>
<td>S. cerevisiae</td>
<td>-</td>
<td>?</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Exit from mitosis</td>
<td>Amn1</td>
<td>S. cerevisiae</td>
<td>LRR</td>
<td>-</td>
<td>Not in insect cells</td>
<td></td>
</tr>
</tbody>
</table>

¹Abbreviations of additional motifs: WD40 = tryptophan-aspartic acid terminating domain, TPR = tetratricopeptide repeat, LRR = leucine rich repeat, RCC1 = Regulator of chromosome condensation 1, UIM = ubiquitin interaction motif, SEC10 = a domain of approximately 650 residues long found protein of the eukaryotic exocyst complex, which specifically affects the synthesis and delivery of secretory and basolateral plasma membrane proteins (Lipschutz et al., 2003), CAAX motif = a C-terminal motif involved in post-translational modifications (where C is cysteine, A is usually aliphatic, and X may be many different residues) (Ashby, 1998). ²? = unknown.
Cdc4: an F-box protein controlling the cell division cycle, morphogenesis, nutrient sensing and calcium signalling

In Saccharomyces cerevisiae, Cdc4 (cell division cycle 4) regulates multiple processes in the cell by recruiting various proteins for degradation (Figure 3) and especially in the cell cycle process, Cdc4 plays an important role by recruiting different cell cycle inhibitors, a transcription factor, a cyclin and a replication factor for degradation. The CDC4 gene was first identified from a yeast mutant unable to initiate DNA replication during transition from the G1 to the S phase (Yochem & Byers, 1987).

Cdc4 and cell division cycle

Regulation of transition from one cell cycle phase to the next involves proteins that inhibit or promote progression. Proteins from both categories have to be degraded at some point, either to ensure progression or to prevent premature initiation of a new phase. Cdc4 is required for the degradation of Sic1 and Far1, proteins that inhibit cell cycle progression, and for the degradation of Cdc6, a protein that promotes progression (Koepp et al., 1999). Sic1 (Substrate/Subunit inhibitor of cyclin-dependent protein kinase 1) is phosphorylated by its inhibition target, Cdc28, and another kinase, Pho85 (Wysocki et al., 2006, Nishizawa et al., 1998). Phosphorylated Sic1 is recognized by Cdc4 and marked for degradation by poly-ubiquitination (Verma et al., 1997, Feldman et al., 1997). Recently, the transcription factor Swi5, which activates transcription of SIC1 (Kishi et al., 2008) was also found to be degraded through interaction with Cdc4. Degradation of the transcription factor Swi5 via Cdc4 during the early G1 phase allows efficient removal of Sic1 in the late G1 phase (Kishi et al., 2008).

This means that Cdc4 is responsible for Sic1 removal by degrading both the protein itself and the activator of its transcription. Through Sic1 degradation, Cdc4 also regulates expression of OCH1, a gene encoding alpha-1,6-mannosyltransferase, suggesting that Cdc4 is involved in regulation of cell wall composition during the cell cycle (Cui et al., 2002). Far1 (Factor arrest 1) is also phosphorylated by the Cdc28 kinase complex and then recognized by Cdc4 (Henchoz et al., 1997). Degradation of Far1 is nucleus-specific, suggesting that Cdc4 may act specifically in the nucleus (Blondel et al., 2000). Cdc6 (cell division cycle 6), is a DNA replication initiation factor that is degraded via Cdc4 in the late G1/early S phase as well as in the G2/M phase. Phosphorylation of Cdc6 to ensure recognition by Cdc4 at both time points requires the Cdc28 kinase. Cdc6 degradation at these two time points differs in the degradation rate and in the cyclins that take part in the Cdc28 kinase complex (Drury et al., 2000). The first difference is probably due to the fact that degradation depends on two different interaction domains in Cdc4 (Perkins et al., 2001). It has also been suggested that Cdc4 has a role in the degradation of Clb6 (Jackson et al., 2006), a cyclin that triggers, together with Clb5, progression from G1 into S-phase. Clb6 is rapidly degraded at the end of the S-phase and stabilized in cdc4 mutants. Moreover, its sequence harbors Cdc4 degron motifs. Direct interaction however, is not yet been demonstrated. Cdc4 also targets another F-box protein involved in kinetochore assembly and function, Ctf13 (see section on Ctf13 below). Schizosaccharomyces pombe contains two homologs of CDC4, called POP1 and POP2 (polyploidy 1 and 2 (Kominami & Toda, 1997)). POP2 was also discovered in another study, where it was called SUD1 (stops unwanted diploidization 1) (Jallepalli et al., 1998). Pop1 and Pop2 are structurally related, but function independently from each other. The phenotypes of both deletion mutants are comparable in that both display polyploidization, but the two proteins cannot fully take over each other’s function since overexpression of POP1 or POP2 could not suppress the defects caused by loss of the other gene (Kominami et al., 1998). The polyploidization phenotype is caused by the accumulation of the CDK-inhibitor Rum1 (Kominami & Toda, 1997, Jallepalli et al., 1998) (homolog of budding yeast Sic1) and S-phase regulator Cdc18 (Wolf et al., 1999, Jallepalli et al., 1998) (homolog of budding yeast Cdc6). These two proteins are normally degraded during defined stages of the cell cycle, but in the pop1, pop2 and pop1/pop2 double mutant, the levels of these proteins are high when
compared to wild type. The accumulation and poly-ubiquitination of Rum1 and Cdc18 in a proteasomal deficient mutant support the notion that Pop1 and Pop2 recruit these two proteins for degradation. Also, direct interaction between Pop1 and Cdc18 was found using co-immunoprecipitation. Pop1 and Pop2 can form homodimers and heterodimers, resulting in three alternative SCF complexes, SCF$^{\text{Pop1/Pop1}}$, SCF$^{\text{Pop1/Pop2}}$ and SCF$^{\text{Pop2/Pop2}}$, but the different molecular functions of these three complexes remain unclear (Kominami et al., 1998). The S-phase cyclin Cig2 (homolog of budding yeast cyclin Cln2), is also stabilized in pop1 and pop2 deletion mutants, suggesting a role of both proteins in Cig2 degradation (Yamano et al., 2004). Co-immunoprecipitation revealed that Pop1 and Cig2 interact in the cell independently from Pop2 and that this interaction requires phosphorylation of Cig2 and at least the central 93 amino acid residues (181-273). In fungi, Pop1 and Pop2 are currently the only examples of two homologous F-box proteins functioning in the same degradation pathway. The advantage of having two F-box proteins for the same function might be that degradation of certain proteins can be fine-tuned and regulated at an extra level. The degradation of Cig2 is remarkably different between budding and fission yeast since Cln2 is degraded through Grr1 in budding yeast (see section on Grr1) but its fission yeast homolog Cig2 by Pop1 (the role of Pop2 in degradation of Cig2 is still unclear).

Cdc4 and pseudohyphal growth
Cdc4 is also involved in degradation of transcription factors that regulate pseudohyphal growth. The transcription factor Tec1 (Transposon enhancement control 1) is phosphorylated by MAP kinase Fus3 and then recognized by Cdc4 promoting its degradation (Chou et al., 2004). Tec1 is responsible for the onset of filamentous growth in S. cerevisiae. This morphological switch is made when nutrient availability is low, but the switch needs to revert after pheromone sensing to allow mating. Whether Cdc4 is solely responsible for degradation of Tec1 is disputable, because it has been shown that another F-box protein, Dia2, is also able to induce degradation of Tec1 after pheromone sensing (Bao et al., 2004). In the human pathogen Candida albicans, Cdc4 plays a role in the switch from hyphal to yeast-like growth as demonstrated by deletion of CDC4, which results in constitutive hyphal growth (Atir-Lande et al., 2005). This dimorphic switch is important for pathogenicity as the hyphal form contributes to the ability to penetrate the body and cause candidemia. In contrast to budding yeast, the CDC4 deletion mutant of C. albicans is viable and does not display an arrest in the G1 phase. Possibly, in C. albicans Cdc4 may have fewer targets than its budding yeast counterpart, or accumulation of the same targets in a C. albicans Δcdc4 mutant does not (fully) inhibit growth. Similarity between the Cdc4 proteins of the two yeasts is 18% in the first 300 amino acids and 48% from residue 360-743, which includes the F-box domain and the WD40 motif. The two proteins indeed appear to have different functions since CDC4 from C. albicans cannot complement the cdc4 strain of budding yeast (Shieh et al., 2005). The constitutive hyphal growth phenotype of the C. albicans cdc4 strain is not due to the accumulation of CaFar1, the homolog of the Cdc4 target Far1 in budding yeast. Sol1, the closest homolog of Sic1 in C. albicans is degraded via Cdc4, but high levels of Sol1 are not responsible for the constitutive hyphal growth of the cdc4 mutant. Another possible target of Cdc4 involved in filamentous growth is Tec1, but no elevated levels were observed in C. albicans cdc4 mutants (Atir-Lande et al., 2005). This means that the target of Cdc4 in C. albicans whose removal is required for the dimorphic switch has not yet been identified, and could be different from a Cdc4 target in budding yeast.

Cdc4 and growth responses after nutrient sensing
The transcription factors Hac1 (Homologous to Atf/Creb1) and Gcn4 (General control nonderepressible 4) are both involved in the activation of unfolded protein responsive (UPR) genes whose products assist in the folding of proteins in the ER lumen upon ER stress. Hac1, a basic leucine zipper transcription factor, is degraded in the nucleus via Cdc4 when
ER stress is removed (Pal et al., 2007). Gcn4 is also required for the activation of transcription of amino acid and purin biosynthesis genes during starvation. After the switch from poor to rich medium, Gcn4 is degraded via Cdc4, likely after being phosphorylated by Pho85 (Meimoun et al., 2000). The degree of conservation of this Cdc4 function is unclear because the respective targets have not been studied in this respect in other fungi.

**Cdc4 and calcium sensing**

Cdc4 acts in calcium homeostasis by targeting Rcn1 for destruction upon calcium availability (Kishi et al., 2007). Rcn1 (Regulator of calcineurin 1) inhibits calcineurin, a phosphatase that mediates cellular responses after stress and Ca\(^{2+}\) uptake (Cyert, 2003). Calcineurin mediates its own inhibition by a negative feedback loop: it stimulates the expression of RCN1 and stabilizes Rcn1 by dephosphorylation. Phosphorylation of Rcn1 makes it recognizable for Cdc4 and marks it for degradation, allowing Calcineurin to break out of its negative feedback loop and increase its activity.

![Figure 3: Overview of Cdc4 targets in S. cerevisiae. Negatively regulating targets are depicted in red, positively regulating targets in green, Ctf13 in white and transcription factors in blue. See text for description of the different targets.](image)

From the above it is clear that between yeasts, Cdc4 is conserved in some functions like the degradation of the CDK inhibitors Sic1 and Far1. Whether the function of Cdc4 in regulation of pseudohyphal growth is conserved between budding yeast and *C. albicans* is uncertain, since the target protein in this pathway in *C. albicans* has not yet been found. The involvement of Cdc4 in nutrient sensing and calcium signalling in other fungi is unlikely, since deletion of *CDC4* in these fungi has not been reported to lead to defects in these processes. A main difference between budding yeast and the other fungi is that in budding yeast the search for targets has been more intensive, for example by using yeast two-hybrid screens (Kishi et al., 2008). Application of such screens to other fungi would be helpful to more fully evaluate the conservation of targets between the different fungi.

Although no genetic studies have been reported on the Cdc4 homologue in *Neurospora crassa*, it was found to be targeted by a plant defensive peptide. Using a yeast two-hybrid screen, defensin 1 from *Pisum sativum* was shown to interact specifically with Cdc4 (Lobo et
Defensins are plant peptides exhibiting an antifungal activity as part of the plant innate immune system. Interaction with Cdc4 can explain why defensin 1 inhibits fungal growth, namely by interference with the fungal cell cycle. Using microscopy, it was observed that defensin 1, tagged with a fluorophore, was localized in the nucleus of *N. crassa* and *Fusarium solani* suggesting that defensin 1 can enter the fungal cell and interfere in the nucleus with the cell division cycle.

**Dia2: an F-box protein involved in DNA replication**

The F-box protein Dia2 (Digs into agar 2) plays a role in DNA replication in *Saccharomyces cerevisiae* and is thereby also involved in cell growth and division. As mentioned earlier, Tec1, a transcription factor regulating filamentation genes, is degraded via Dia2 (Bao et al., 2004), probably in joint action with Cdc4 (Chou et al., 2004). These two F-box proteins are also both capable of degrading ectopically expressed human Cyclin E (Koepp et al., 2001), eventhough they bear different protein-protein interaction domains (an LRR and WD40, respectively). Deletion of *DIA2* in budding yeast causes a defect in invasive and pseudohyphal growth, slower growth at low temperatures, early entry into the S-phase and accumulation of DNA damage (Koepp et al., 2006). These defects were also observed in *DIA2ΔF-box* mutants, suggesting that binding of Dia2 to Skp1 is necessary for these functions. Dia2 binds both early and late firing origins and is thereby involved in resetting the origin. It recruits the SCF complex to the replication origins, suggesting that a possible target becomes ubiquitinated there. Yra1, previously described as a protein involved in mRNA export (Strasser & Hurt, 2000), is an interaction partner of Dia2 and is required for Dia2 function at replication origins (Swaminathan et al., 2007). Possibly, the SCF\(^{\text{Dia2}}\) complex binds origins with the assistance of Yra1. In another study (Blake et al., 2006), deletion of *DIA2* resulted in accumulation of DNA damage after the collapse of replication forks. This suggests that a possible target of Dia2 may be found among proteins that interfere with replication fork stability in certain genomic regions. Also, genetic interactions of Dia2 were found with DNA replication, repair and checkpoint pathways (Blake et al., 2006). A role of Dia2 in DNA repair was suggested as well by the requirement of Dia2 for resistance to certain DNA damaging compounds. These observations indicate that there are likely more targets or functions of Dia2 than only targeting Tec1 for degradation.

Pof3 from *Schizosaccharomyces pombe* is the ortholog of Dia2 from budding yeast. Deletion of *POF3* results in multiple phenotypes: G2-phase delay (probably due to activation of the DNA damage checkpoints), hypersensitivity to UV radiation, telomere dysfunction and also chromosome instability and segregation defects (Katayama et al., 2002). Targets of Pof3 are not yet known, but may be found among proteins playing a role in chromatin structure and/or function. Fission yeast does not have a Tec1 ortholog, so targets different from Tec1 must be responsible for the phenotype of the deletion mutant. A protein that was found to interact with Pof3 is Mcl1, ortholog of the budding yeast S-phase regulator Ctf4 (Mamnun et al., 2006). Mcl1 is a protein essential for chromosome maintenance and contains WD40 repeats and SepB boxes (Williams & McIntosh, 2002, Kohler et al., 1997). A \(^{\text{Δmcl}}\) strain shows similar phenotypes as the \(^{\text{Δpof3}}\) mutant. Normally, Mcl1 is not rapidly degraded in wild type cells and no ubiquitination of Mcl3 could be demonstrated, suggesting that Mcl1 is not a target of Pof3. This is also in accordance with the fact that the two deletion mutants share the same phenotype, something that is not expected when Mcl1 would be a target of Pof3. Dia2 is not only conserved in fission yeast, but also in filamentous fungi, suggesting a well conserved function (BLAST searches, our observations). It would be worthwhile to investigate whether and how Dia2 regulates DNA replication in filamentous fungi.

**Grr1: an F-box protein involved in glucose and amino acid sensing, cell division cycle, meiosis and retrograde signalling.**
Grr1 (Glucose repression resistant 1) in *Saccharomyces cerevisiae* plays a role in a large number of cellular processes: retrograde signaling, pheromone sensitivity and cell cycle regulation, nutritionally controlled transcription, glucose sensing, and cytokinesis (Li & Johnston, 1997) (Figure 4). Grr1 was initially found in budding yeast through a mutation causing resistance to glucose repression, with a deletion mutant showing additionally growth defects (Bailey & Woodward, 1984, Flick & Johnston, 1991).

**Grr1 and the cell cycle**

That Grr1 is involved in cell cycle control was shown by the accumulation of the cyclins Cln1 and Cln2 in a *GRR1* deletion mutant (Barral et al., 1995, Skowyra et al., 1999, Kishi & Yamao, 1998, Schweitzer et al., 2005, Schneider et al., 1998, Lanker et al., 1996). Degradation of the cyclins Cln1 and Cln2 via Grr1 is required after completion the G1 phase or when cells have to arrest in G1, for instance after pheromone sensing. The binding of Grr1 to Cln2 has been established in multiple ways, but binding to Cln1 has never been detected, suggesting that Grr1 might target Cln1 indirectly. Another target of Grr1 is Gic2, a protein that accumulates throughout the G1 phase and reaches its peak just before bud emergence. At that time, Cdc42, a Rho-related GTP-binding protein required for polarized growth of the cytoskeleton during bud emergence, is activated and binds Gic2. When the bud has emerged, polarized growth ceases and Gic2 is degraded to avoid morphological defects. Only Gic2 bound to Cdc42 can be phosphorylated and eventually recognized by Grr1 (Jaquenoud et al., 1998). During cytokinesis, the process of cell separation, Grr1 is responsible for the degradation of Hof1. Hof1 first forms a ring around the bud neck of the mother cell and then another ring in the daughter cell. Just after septum formation and separation, Hof1 normally disappears (Vallen et al., 2000). Grr1 is recruited to the mother bud neck and binds to Hof1 after the activation of the mitotic exit network (Blondel et al., 2005). This suggests that Grr1 is not only active in the nucleus and cytoplasm, but can be recruited to specific cellular structures. The Grr1 protein of *Candida albicans* is 46% identical to Grr1 and mediates degradation of Ccn1 and Cln3. That Grr1 mediates degradation of Ccn1 and Cln3 was demonstrated by the fact that both cyclins are stabilized and additionally, Cln3 was found as a hyperphosphorylated protein in the *Δgrr1* strain. Elevated levels of Hof1 were also detected in the *Δgrr1* strain. These data suggest that Grr1 function in degradation of cyclins and Hof1, as well as glucose uptake, is conserved between the two yeast species. The fact that the genes from *C. albicans* can functionally replace budding yeast *GRR1* suggests that interactions of Grr1 with its targets are conserved between the two yeasts.

**Grr1 and meiosis**

Generally, the cell cycle is closely connected to the availability of nutrients. In low glucose medium, diploid cells tend to undergo meiosis and sporulation rather than grow and divide. A role of Grr1 in preventing untimely meiosis and sporulation was demonstrated in a study of the degradation of Ime2, a protein kinase required for multiple steps throughout the sporulation process (Purnapatre et al., 2005). In a *Δgrr1* mutant accumulation of (non-ubiquitinated) Ime2 was found and meiosis still occurred, even under high glucose conditions. In *Aspergillus nidulans*, *GRR1*, the ortholog of budding yeast *GRR1*, was found in a subtraction hybridization screen aimed at identification of genes that are specifically expressed during fruiting body development (Krappmann et al., 2006). *GRR1* is able to partially complement the *Δgrr1* phenotype in yeast, or almost fully when the gene is overexpressed. Complemented phenotypes of the yeast deletion mutant include the morphological abnormalities and changes in gene expression upon a carbon source shift...
This demonstrated that GrrA from A. nidulans is probably able to bind endogenous targets in budding yeast, suggesting that these interactions are still conserved within GrrA. However, the phenotype resulting from deletion of GRR1 in A. nidulans is quite different from that of the yeast mutants. A. nidulans ΔgrrA mutants showed impaired ascus sporogenesis, asexual conidiation and sexual development, while displaying a normal vegetative growth. A similar phenotype was observed in CSN subunit mutants in A. nidulans (Busch et al., 2003, Busch et al., 2007). This suggests that the CSN may be involved in the functioning of GrrA in A. nidulans. From further cytological examination it was concluded that meiosis, giving rise to crozier-like structures that contain diploid nuclei, does not take place in the grrA mutant. In striking contrast, meiosis does occur in the budding yeast grr1 mutant, even under meiosis suppressing conditions. In light of this, it would be interesting to investigate whether and how the Ime2 orthologue in A. nidulans is involved in GrrA-controlled sexual development. In the plant pathogenic fungus Fusarium graminearum (Gibberella zeae), the ortholog of budding yeast Grr1, denoted as Fbp1, was found in a REMI screen for non-pathogenic mutants (Han et al., 2007). The virulence of fbp1 mutants on barley heads was severely reduced compared to wild type and growth on potato dextrose agar and carrot agar produced less mycelium. Furthermore, Fbp1 plays a role in sexual reproduction. FBP1 deletion caused loss of perithecia formation as females in self-crosses, and a reduced number of smaller perithecia as a male in the outcross. The asci contained incomplete octads of abnormal spores and did not segregate in a one-to-one manner. Deletion constructs lacking the F-box, the LRR or both domains were non-functional, both in the interaction with endogenous Skp1 and yeast Skp1, and in the ability to complement the sexual reproduction deficiency. Clearly, also in F. graminearum protein turnover is required for sexual reproduction, but whether the ortholog of budding yeast Ime2 is involved is not known. Grr1 from budding yeast was unable to complement the knock out phenotype. Conversely, FBP1 from F. graminearum was able to partially complement the yeast grr1 mutant. This suggests that during evolution, Grr1 has retained the ability to bind at least some heterologous targets, despite their diversification. Grr1 is likely conserved as a pathogenicity factor in plant pathogenic fungi. In a screen searching for pathogenicity genes of Magnaporthe grisea using insertional mutagenesis, one mutant, called PTH1 (pathogenicity 1), had a disruption in GRR1 and a subsequent deletion of this gene resulted in reduced disease symptoms towards barley (Sweigard et al., 1998). How Grr1 functions in causing disease in this fungus is not known. It is likely that in the Δpth1 mutants, the sexual reproduction system is impaired as is demonstrated for A. nidulans and F. graminearum.

Grr1 in growth on glucose and non-glucose carbon sources
In addition to regulating meiosis, Grr1 from S. cerevisiae conducts other glucose availability-related functions. When high levels of glucose are sensed, Grr1 not only initiates the degradation of Ime2, but also activates hexose permeases (HXT) that allow the rapid import of glucose. Activation of HXT expression is achieved by the degradation of Std1 and Mth1, which promote the repression of HXT genes by binding to the repressor Rtg1. Upon glucose sensing, Std1 and Mth1 are phosphorylated, recognized by Grr1 and degraded. Free, unbound Rtg1 can then be phosphorylated, promoting an intramolecular interaction in Rtg1 that prevents DNA binding (Polish et al., 2005), thereby releasing repression of the HXT genes (Kim et al., 2006). Although, not yet fully understood, the process of Snf1 protein kinase inactivation is also required for degradation of Std1 and Mth1 (Pasula et al., 2007). In Kluyveromyces lactis, Grr1 was characterized as an F-box protein required for glucose signaling just as described in budding yeast (Hnatova et al., 2008). Complementation of ΔScgrr1 with KIGRR1 showed full restoration of the growth and morphological defects of the deletion strain, demonstrating that GRR1 from K. lactis is a functional homolog of budding yeast GRR1. It was also shown that in K. lactis Grr1 controls the levels of Sms1, the single ortholog of the budding yeast Grr1 targets Mth1 and Std1. Sms1 level decreased...
dramatically after glucose addition, suggesting rapid degradation of Sms1 to allow expression of the hexose transporter genes. Other targets of Grr1 in K. lactis are not found yet, but as the complementation of ΔScgrr1 with KlGRR1 shows, Grr1 from K. lactis is probably able to bind targets in budding yeast. These targets also include Mth1 and Std1, suggesting that these interactions are still conserved, even though in K. lactis only Sms1 is present.

Additionally to activating genes required for glucose uptake, Grr1 from S. cerevisiae is also required for the assimilation of alternative carbon sources (Flick & Johnston, 1991). Grr1 mediates this process by recruiting Gis4, a target that is ubiquitinated but not degraded (La Rue et al., 2005). The ubiquitinated form of Gis4 binds and activates phosphorylated forms of Snf1, which results in derepression of several genes required for the assimilation of alternative carbon sources. Gis4 is a rare example of a target that is not degraded after ubiquitination, but instead activated. This shows that although Grr1 function generally complies with the F-box hypothesis, ubiquitination of Gis4 is an exception to this rule. Whether and how Gis4 is phosphorylated before recognition by Grr1 and how it is rescued from degradation after addition of ubiquitin is not known.

Grr1 also regulates other metabolic processes in the cell, through its involvement in the degradation of Tye7 (Corinna Löhning, 1994) and Pfk27 (Benanti et al., 2007). Tye7 is a transcription factor that activates several glycolytic genes (Nishi et al., 1995) and Pfk27 synthesizes the second messenger fructose-2,6-biphosphate (Okar & Lange, 1999). After glucose depletion the removal of these proteins via Grr1 probably facilitates the switch from glycolysis to gluconeogenesis. This shows that Grr1 is not only active during glucose availability, but also during glucose depletion.

Furthermore, together with Mdm30, another F-box protein, Grr1 regulates the activation of the Gal4 transcription activation complex. This complex regulates the transcription of genes involved in galactose assimilation. Degradation of the Gal4 isoforms Gal4a and Gal4b via Grr1 is required when glucose becomes available and galactose assimilation is shut down (Muratani et al., 2005). This was demonstrated by deletion of GRR1, which results in stabilization of Gal4a/b and increased activation of Gal4 targets.

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Figure 4: Overview of the Grr1 targets in S. cerevisiae.
Negatively regulating targets are depicted in red, positively regulating targets in green and transcription factors in blue. See text for description of the different targets.
Grr1 and amino acid sensing
Grr1 also plays a role in amino acid sensing by promoting the expression of several amino acid permease genes upon amino acid availability (Bernard & Andre, 2001, Iraqui et al., 1999). Activation of amino acid permease genes is mediated by the transcription factors Stp1 and Stp2. These two proteins are cleaved after activation of the Ptr3/Ssy5 amino acid sensing pathway and transported to the nucleus (Liu et al., 2008). Stp1 cleavage depends on Grr1, suggesting that Grr1 targets a protein that normally inhibits cleavage. A candidate might be Ssy5, which is involved in the amino acid permease expression pathway and that is normally degraded upon amino acid availability. On the other hand, higher protein levels of Stp2 were also found in Δgrr1 cells (Benanti et al., 2007).

Grr1 and retrograde signalling
Mitochondrial retrograde signaling (RTG) is a pathway connecting mitochondria to the nucleus allowing cells to react to changes in the functional state of mitochondria. The RTG pathway targets two transcription factors, Rtg1 and Rtg3. These two proteins form heterodimers and activate RTG responsive genes (Liu & Butow, 2006). Grr1 functions in this pathway by degradation of Msk1, a negative regulator that inhibits localization of Rtg1 and Rtg3 to the nucleus (Liu et al., 2005). Grr1 targets Mks1 only when it is unbound to either Rtg2 or Bmh1, a 14-3-3 protein. When the RTG pathway is off, Bmh1 protects Mks1 and allows it to inhibit Rtg1 and Rtg3. When the pathway is on, Mks1 instead binds to Rtg2 and is thereby inactivated. Degradation of free Mks1 via Grr1 ensures that the switch is quick and under tight control.

Grr1 seems to be conserved among yeasts considering the cell cycle and glucose uptake. Regarding meiosis however, the role of Grr1 between budding yeast and filamentous fungi seems opposite of each other. Another difference between Grr1 in yeasts and filamentous fungi is its involvement in glucose uptake since for example Mig1 and Snf1 repression is in filamentous fungi different from budding yeast (Carlson, 1999, Ruijter & Visser, 1997). From other functions like amino acid sensing and retrograde signalling, hardly anything is known about Grr1 involvement in other fungi. Partly because of lack of knowledge, but probably also partly because these processes are differently regulated. Once again, in other fungi, less studies are carried to find Grr1 targets as performed in budding yeast (Benanti et al., 2007).

Hrt3: an F-box protein enhancing methylmercury resistance
Hrt3 (High level expression reduces Ty3 transposition) and Ylr224w in Saccharomyces cerevisiae both promote resistance to methylmercury, a highly toxic compound (Hwang et al., 2006). Overproduction of these two F-box proteins elevated resistance to the toxic compound in contrast to fifteen other F-box proteins studied. This resistance required the F-box domain of these two proteins and also the proteasome, suggesting that degradation of a target protein is involved. Targets of Hrt3 or Ylr224w that could explain the roles of these F-box protein in methylmercury resistance have not yet been identified. Interactions of Hrt3 other than with ubiquitin conjugation proteins were with alcohol dehydrogenase (Adh2) and Idh1, a subunit of mitochondrial NAD(+) -dependent isocitrate dehydrogenase, which catalyzes the oxidation of isocitrate to alpha-ketoglutarate in the TCA cycle (Ho et al., 2002, Krogan et al., 2006). The biological relevance of the interaction with these two catabolism-related proteins is not clear, but they might be involved in sensitivity to methylmercury. Other interactions were found with ribosomal proteins Rpl12A and Guf1, and a phosphatase functioning in the G1/S phase transition.

Hrt3 is conserved in the entire fungal kingdom (BLAST searches, our observations), suggesting that it serves a fundamental function in fungi. Still, its characterization is limited; only the overexpressing phenotype was investigated in S. cerevisiae. Investigation of a (conditional) deletion mutant in S. cerevisiae or other fungi will be crucial to further explore the functions of Hrt3.
Mdm30: a mitochondria-associated F-box protein

Mdm30 (Mitochondrial distribution and morphology) and Mfb1 (Mitochondria-associated F-Box protein) in *Saccharomyces cerevisiae* both control membrane fusion dynamics of mitochondria. The membranes of mitochondria continuously undergo fusion and fission to maintain a dynamic morphology. A target of Mdm30 is Fzo1 (mitofusin), a membrane-bound GTPase involved in membrane fusion. Fzo1 is ubiquitinated and targeted to the proteasome in an Mdm30-controlled manner (Cohen et al., 2008). Another target of Mdm30 is Mdm34, a mitochondrial outer membrane protein (Ota et al., 2008). Interaction between Mdm30 and Mdm34 is essential for growth on non-fermentable carbon sources and for normal mitochondrial morphology. If and how ubiquitination of Mdm34 contributes to these functions is not yet understood.

Additionally, Mdm30 (alternatively called Dsg1 - Does something to Gal4), is required for destruction of Gal4c, the inhibitory isoform of Gal4, and thereby plays a role in carbon assimilation (Muratani et al., 2005) together with Grr1, which is required for the degradation of Gal4a/b, the Gal4 active isoforms. A Δ*dsg1* strain shows elevated levels of Gal4c, which results in the inability to use galactose as a carbon source.

Mdm30 binds to Skp1 via its F-box domain, and these two protein together with other components of the SCF complex participate in Fzo1 degradation (Cohen et al., 2008). This means that Mdm30, in contrast to earlier views (Ho et al., 2008, Hermand, 2006), can be part of an SCF-complex and conforms to the F-box hypothesis. Mdm30 is not conserved in other fungi, but insight into how mitochondrial morphology is regulated by F-box proteins in *S. cerevisiae* is valuable, as in other fungi alternative F-box proteins or at least ubiquitination and protein turnover could also be involved in this intriguing process.

Saf1: an F-box protein involved in entry into quiescence

Saf1 (SCF associated factor 1) is an F-box protein required for the degradation of adenine deaminase 1 (Aah1) in *Saccharomyces cerevisiae*. A microarray study showed clear *AAH1* downregulation during the shift from proliferation to quiescence (Escusa et al., 2007, Escusa et al., 2006). Quiescence, also known as the stationary phase, is a state that yeast cells enter when nutrients are limiting. A *SAF1* deletion mutant showed no down-regulation of *AAH1* expression and stabilized protein levels of Aah1 were detected upon entry into quiescence. Degradation of Aah1 relies on Saf1, Skp1 and the proteasome, and is dependant on interaction between Saf1 and Skp1 via the F-box domain of Saf1. Saf1 interacts in a yeast two-hybrid experiment with both Aah1 and Skp1. Loss or mutation of the F-box domain of Saf1 abolished the interaction with Skp1, but not with Aah1, although the latter interaction was slightly weakened. Mutation of the lysine at position 329 of Aah1 did not affect the interaction with Saf1, but increased the stability of Aah1, suggesting that this lysine might be the ubiquitination site. Other targets of Saf1 are not known yet, but a candidate might be Ura7, a protein that is present at reduced levels in *SAF1* overexpressing strains and is stabilized in *saf1* strains (36). Curiously, although the known target(s) of Saf1 are conserved among fungi, Saf1 itself is not. In fungi other then budding yeast, degradation of these Saf1-targeted proteins might not be required upon quiescence or they are turned over in a different manner.

Ufo1: an F-box protein involved DNA damage response

In *Saccharomyces cerevisiae* Ufo1 (UV-F-box-HO 1) targets the endonuclease Ho for proteasomal degradation and functions in genome stability and in response to DNA damage (Kaplun et al., 2006). After DNA damage, the MEC1/RAD9/CHK1 pathway phosphorylates Ho stimulating its recognition and degradation. Ufo1 itself is also degraded via self-ubiquitination. This ubiquitination reaction is mediated by the UIMs (ubiquitin interaction motifs) in the C-terminus of Ufo1 that bind during assembly in the SCF complex to Ddi1, a
protein containing ubiquitin-like (UBL) and ubiquitin associated (UBA) domains (Ivantsiv et al., 2006). Removal of the UIM domain in Ufo1 stabilizes the protein and inhibits the degradation of other proteins normally degraded by SCF complexes. It therefore seems that Ufo1Δuim may prevent assembly of other F-box proteins into an SCF complex. Ufo1 also appears to regulate the degradation of Rad30, since that protein is stabilized in proteasomal mutants and in cells lacking Skp1 or Ufo1. Direct interaction between Ufo1 and Rad30 has, however, not yet been demonstrated. Rad30 is a polymerase eta necessary for DNA replication near damaged DNA (Skoneczna et al., 2007) and is removed again after replication because of its higher error frequency. Recently, a study of the interactome of GFP-labeled Ufo1 identified new proteins taking part in Ufo1 function (Keren Baranes-Bacher, 2008). The proteins interacting specifically with GFP-Ufo1 and bearing PEST degrons - potential phosphorylation sites and often found in proteins targeted for degradation (Rechsteiner & Rogers, 1996) - are Rbp2, Spt5, Fas2 and Gip2. Rpb2 is a RNA polymerase II subunit (Cramer et al., 2000) and Spt5 a protein that mediates both activation and inhibition of transcription elongation (Lindstrom et al., 2003). Fas2 is a fatty acid synthetase component (Mohamed et al., 1988) and Gip2 a putative regulatory subunit of the protein phosphatase Glc7p, involved in glycogen metabolism (Tu et al., 1996). Whether these proteins are targets of Ufo1 is not known. Ufo1 is not conserved in other fungi, suggesting that this type of regulation of the DNA damage response is restricted to (close relatives of) budding yeast.

Fwd1: an F-box protein controlling the circadian clock.

In Neurospora crassa, Fwd1 (F-box protein containing a WD40 repeat) was found to be involved in controlling the circadian clock via degradation of Frequency (Frq) (He et al., 2003, He & Liu, 2005). Circadian clocks regulate a wide variety of physiological and molecular processes during oscillation between day to night. Besides being regulated by Frq, the circadian clock in Neurospora is further regulated by light and controlled by the transcription factors Wc-1 and Wc-2. (Dunlap & Loros, 2006). Frq inhibits its own transcription by inhibiting Wc-1 and Wc-2 (Aronson et al., 1994b, Aronson et al., 1994a). When Frq is hyperphosphorylated by CK1 and CKII (He et al., 2006) it is recognized by Fwd1 and degraded. This releases Wc-1 and Wc-2 activity, leading to the production of new Frq. The function of Fwd1 in the SCF complex is regulated by the COP9 signalosome (CSN). Disruption of a subunit of the CSN impaired the degradation of Frq, probably because reduced amounts of Fwd1 were present in the csn mutant: the half life of Fwd1 is reduced from 6-9 hr to 45 minutes, and also other components of the SCF-complex proved to be unstable. In a Δcsn-2 mutant SCF is constitutively neddylated, which enhances the degradation rate of Fwd1. This degradation is probably independent of binding of Frq to Fwd1, resulting in reduced amounts of Fwd1 and impaired degradation of Frq1 (He et al., 2005). The CSN-2 deletion mutant also exhibits slower growth and reduced production of aerial hyphae, suggesting that other F-box proteins might also be affected. N. crassa is the main model organism for the investigation of circadian rhythms in fungi and only in this fungus Fwd1 has been intensively studied. Nevertheless, this protein as well as circadian rhythms are present in other filamentous fungi (Bell-Pedersen et al., 1996, Lakin-Thomas & Brody, 2004) as are homologs of N. crassa clock components like Frq, WC-1 and WC-2 (Lombardi & Brody, 2005). However, in A. nidulans no homolog of FRQ is present (Greene et al., 2003) even though an FWD1 homolog is (our observations). This suggests that at least in some fungi, Fwd1 has other targets. Interestingly in plants, involvement in rhythmic processes has also been demonstrated for several F-box proteins like ZEITLUPE, FKF1 and AFR (Harmon & Kay, 2003, Nelson et al., 2000, Somers et al., 2000), which play a role in photocontrol of the circadian period, the circadian clock and phytochrome A-mediated light signaling respectively.
Met30: an F-box protein involved in sulphur metabolism

As described above, the F-box hypothesis states that the targets of F-box protein are degraded after ubiquitination, but ubiquitination of the Grr1 target Gis4 does not lead to degradation. Met30 is another example of an F-box protein whose target is not necessarily degraded. Met30 is an F-box protein from *Saccharomyces cerevisiae* that can recruit its target to the SCF for degradation, but it can also activate its target by ubiquitination when the target is assembled into a transcription activation complex.

A major Met30 target is Met4, a transcriptional activator of the sulfate assimilation pathway controlling *MET* and *SAM* genes for uptake and biosynthesis of sulphur containing compounds (Thomas et al., 1995). Met4 is also required for cadmium tolerance by activating the expression of genes involved in the glutathione biosynthesis (Barbey et al., 2005, Yen et al., 2005, Brennan & Schiestl, 1996).

The transcription of Met30 is regulated through a feedback loop as Met4 controls the activation of Met30 (Rouillon et al., 2000). The way Met4 is regulated by Met30 has been under discussion for several years (Menant et al., 2006a, Brunson et al., 2004, Kaiser et al., 2000, Brunson et al., 2005, Menant et al., 2006b). A picture has emerged in which Met30 regulates Met4 in multiple ways (Chandrasekaran & Skowyra, 2008). Firstly, Met30 activates Met4 when low levels of methionine are available resulting in the expression of *MET* and *SAM* genes. When through *MET* and *SAM* activation higher intracellular levels of methionine are obtained the intracellular concentration of cysteine also increases through the S-adenosyl-methionine and cysteine biosynthesis pathways. High levels of cysteine again lead to the inactivation of Met4 by Met30. The alternative activation and inactivation of Met4 by Met30 is explained in a two-step model: in its inactive state, dimerization of Met4 causes low interaction with cofactors leading to intermediate expression of *MET* and *SAM* genes. Met30 relieves this dimerization through degradation of one of the dimerized Met4 proteins leaving the other Met4 subunit free to assemble into an activation complex, thus triggering expression of the *MET* and *SAM* genes (step one). When higher levels of sulphur containing amino acids are present, Met30 binds to Met4 in the assembled promoter complex leading to Met4 ubiquitination (step two), this ubiquitinated promoter complex represses transcription of the *MET* and *SAM* genes. Eventually, Met4 is degraded and the complex disassembles, making space for new complexes to form on the promoter when levels of sulphur containing amino acids are low again.

In *Schizosaccharomyces pombe*, the Met30 homolog Pof1 is an essential protein that targets the Met4 homolog Zip1 (a basic leucine zipper) (Harrison et al., 2005). Like in *S. cerevisiae*, Zip1 mediates cadmium tolerance by activation of cadmium response genes. Regulation of Met4 and Zip1 by Met30 and Pof1, respectively, shows a similar pattern. However, one difference between the two systems is that Zip1 is only required for the biosynthesis of sulphur containing amino acids under low levels of sulphur and is not required during normal growth conditions, as Met4 is (Baudouin-Cornu & Labarre, 2006). In *Neurospora crassa*, the Met30 homolog Scon2 (Sulphur controller) is also required for sulfur uptake and assimilation (Kumar & Paietta, 1995a, Kumar & Paietta, 1998). Cys3, the Met4 ortholog of *N. crassa*, is degraded via Scon2 and regulates the entire set of sulfur uptake and assimilation genes. Interaction between Scon2 and Scon3 (*N. crassa* Skp1) was observed using yeast two-hybrid and co-immunoprecipitation and was dependant on the F-box motif in Scon2 (Sizemore & Paietta, 2002). Cys3 activates not only sulfur utilization genes, but also the transcription of *CYS3* itself and of the *SCON2* gene. Therefore, when Scon2 targets Cys3 for proteolysis, its own activation is also reduced ensuring the possibility to rapidly activate Cys3 again. Mutational analysis of Scon2 showed that the F-box domain plays an important role in regulation of Cys3. Eleven out of fourteen mutations in the F-box domain gave rise to a constitutively repressed phenotype, corresponding to the Δcys3 phenotype. This is at first sight surprising since mutation of the F-box is expected to impair Skp1 binding (although loss of binding to Skp1 was not verified), and thereby decrease the ability to degrade Cys3. This
would in turn be expected to lead to a constitutive activation phenotype of CYS3. It is possible that Scon2 is not only required for degradation of Cys3, but also for its activation, as demonstrated for Met30 and Met4 in budding yeast. The role of the Met30 homolog SconB (Natorff et al., 1998) in Aspergillus nidulans has proven to be similar to Scon2 in N. crassa, including binding to Skp1, called SconC in A. nidulans (Piotrowska et al., 2000). Still, differences have also been found: SCONB is not transcriptionally activated by the Cys3 counterpart of A. nidulans, MetR (Natorff R., 2003) and although MetR and Cys3 both recognize the same DNA sequences, full-length CYS3 cannot fully complement the ΔmetR phenotype.

Additionally to its role in sulphur metabolism, Met30 is also essential for cell cycle progression. It regulates multiple aspects of the cell cycle including the expression of cyclins required for G1-phase progression and the accumulation of proteins involved in replication and progression through the M phase (Su et al., 2005, Patton et al., 2000). A target of Met30 was believed to be Swe1 (34), a Wee1-family kinase that inhibits Cdc28 by phosphorylation since higher activity of Swe1 and non-ubiquitinated forms of Swe1 were found in Δmet30 cells. An in vivo interaction between Met30 and Swe1 was also demonstrated (34). A later study concluded, however, that Met30 is not responsible for degradation of Swe1, but that degradation is a result of the interaction between Swe1 and Hsl7 (McMillan et al., 2002, Kaiser et al., 1998). This interaction with Hsl7 mediates the translocation of Swe1 out of the nucleus to the mother-bud neck where its degradation takes place in an unknown manner. The involvement of Met30 in Swe1 degradation is therefore disputable but cannot entirely be ruled out. Regardless of the exact mechanisms, it is now clear that the activation and degradation of proteins involved in the cell cycle is under the control of multiple F-box proteins (Cdc4, Gr1 and Met30). These F-box proteins either directly target cell cycle proteins or regulate their levels indirectly.

Finally, recently a Met30 homolog, Lim1, was found in Hypocrea jecorina through a yeast one-hybrid screen and it was demonstrated that Lim1 can bind promoter sequences of the cellobiohydrolase gene CBH2 (Gremel et al., 2008). Clarification of the role in transcription of Lim1 and involvement of possible targets awaits further investigation.

F-box proteins without identified targets

In the previous section, F-box proteins are described from which targets are known to be ubiquitinated through binding to Skp1 and assembly into an SCF complex. For several other fungal F-box proteins, binding to Skp1 does not appear to be required for function or target inactivation. The functions of these proteins, then, seem to fall outside the F-box hypothesis as probably no targets are recruited to an SCF complex. The F-box domain in some of these proteins could interact with proteins other than Skp1. Alternatively, interaction with Skp1 is only required for self-ubiquitination of the F-box protein to control protein levels.

Fbh1: a DNA repair F-box protein

Fbh1 (F-box DNA helicase) is an F-box protein from Schizosaccharomyces pombe involved in the regulation of recombination levels and DNA repair (Sakaguchi et al., 2008, Osman et al., 2005, Morishita et al., 2005). Like its human homolog, Fbh1 contains a helicase domain to unwind DNA. Human Fbh1 functions downstream of the recombinase enzyme Rhp51 (the ortholog of S. cerevisiae Rad51). Although Fbh1 binds Skp1, it appears that the F-box is not necessary for Fbh1 to promote DNA repair, since two mutations in the F-box domain did not alter growth nor genotoxin resistance (Lehmann A., 2004). A mutation in the helicase domain however, did affect the DNA repair function. The human homolog assembles into an SCF complex, but its targets, if any, are also still unknown. Possibly, Skp1 binding only mediates self-ubiquitination of Fbh1.Remarkably, Fbh1 is the only fungal protein that contains an F-box combined with a helicase domain, and it is only found in fission yeast.
**Frp1: an F-box protein required for root invasion**

In *Fusarium oxysporum* f.sp. *lycopersici*, a vascular wilt pathogen of tomato, Frp1, (F-box protein required for pathogenicity 1), was found using an insertional mutagenesis screen for pathogenicity genes (Duyvesteijn et al., 2005). Frp1 is required for assimilation of various (non-sugar) carbon sources as well as induction of genes for cell wall degrading enzymes, which would explain the deficient plant root colonization and penetration by the Δfrp1 mutant (Chapter V). Frp1 binds to Skp1 in yeast two-hybrid and pull-down assays, but mutations in the F-box domain of Frp1 that impair binding to Skp1 do not affect phenotype, suggesting that the main function of Frp1 does not depend on ubiquitination of targets (Chapter IV). Because *FRP1* orthologs are present in other plant pathogenic fungi, it will be interesting to study its role in pathogenicity in these fungi. The deletion of the *FRP1* ortholog in *F. graminearum*, has been reported but an initial characterization revealed no obvious differences with wild type (Han et al., 2007).

**Pof14: an F-box protein that inhibits ergosterol synthesis**

Pof14 is an F-box protein in *Schizosaccharomyces pombe* required for survival upon hydrogen peroxide stress (Tafforeau et al., 2006). In response to such stress Pof14 binds and inhibits Erg9, a squalene synthase involved in ergosterol synthesis. Ergosterol enhances the permeability of the membrane and thereby the uptake of hydrogen peroxide. Pof14 and Erg9 bind to each other in a membrane bound complex, as was demonstrated by tagging both proteins with fluorescent tags. Binding of Pof14 to Erg9 inhibits the activity of Erg9 and overexpression of *POF14* leads to decreased levels of squalene synthase activity and ergosterol. Transcription of *POF14* is induced after treatment with hydrogen peroxide, and deletion of *POF14* decreases viability after hydrogen peroxide treatment (Tafforeau et al., 2006). Decreased viability was not observed upon deletion of the F-box domain, suggesting that binding of Pof14 to Skp1 is not required for peroxide resistance. However, binding of Pof14 to Skp1 may promote degradation of Pof14 itself. In wild type cells, Pof14 has a half-life time of 20-40 min, but in temperature sensitive mutants of Skp1, Pof14 is stable for at least 60 minutes (Tafforeau et al., 2006). Whether this stabilization is due to defective assembly of Skp1 and Pof14 into an SCF-complex for self-ubiquitination is not known.

**Non-SCF F-box proteins**

Ctf13 and Rcy1 are two F-box proteins that were found to bind Skp1 but nevertheless function independently of an SCF-complex and probably also don’t have targets to be ubiquinated and are therefore unlikely to be involved in protein inactivation. The binding of these proteins to Skp1 may be evolutionary conserved but may have acquired an alternative function.

**Ctf13: a kinetochore assembly F-box protein**

In *Saccharomyces cerevisiae* Ctf13 (Chromosome transmission fidelity 13) is part of the CBF3 complex, which in turn is part of the centromere-bound scaffold where the microtubule binding components of kinetochores assemble. The CBF3 complex consists of four components: Skp1, Ctf13, p64 (encoded by *CEP3* and containing a zinc finger centromere binding domain) and p110, (a protein complex encoded by three genes CBF2/NDC10/CTF14) (Kopski & Huffaker, 1997, Yoon & Carbon, 1995). The binding of Ctf13 to Skp1 requires phosphorylation of Ctf13 (Kaplan et al., 1997, Russell et al., 1999) and the interaction to Sgt1 and Hsp90 (Bansal et al., 2004, Stemmann et al., 2002) for the assembly and function of the kinetochore complex. When mutations were introduced that prevent binding of Ctf13 to Skp1, severely impaired cell growth was observed. Interestingly, Ctf13 is targeted by another F-box protein, Cdc4, for degradation, which is in accordance with Ctf13 not being part of an SCF-complex itself. Binding of Ctf13 to p64 rescues Ctf13
from degradation. Probably only free Ctf13 is degraded via Cdc4, which might be required to tightly regulate kinetochore assembly.

**Rcy1: an F-box protein involved in vesicle trafficking**

Rcy1 (Recycling 1) was found in a genetic screen for *Saccharomyces cerevisiae* mutants defective in membrane trafficking through the endocytic pathway. Deletion of *RCY1* results in an arrest of the endocytic pathway and leads to accumulation of enlarged compartments close to areas of cell expansion (Wiederkehr *et al.* 2000). For Rcy1 to function it needs to bind Skp1, but other components of the SCF are not required for recycling nor for degradation of Rcy1 itself. Rcy1 contains two SEC10 domains and a CAAX box, implicated in mediating interaction with membranes and also needed for recycling. Rcy1 is required for the recycling of the v-SNARE Snc1p, a membrane protein that fuses exocytic vesicles with the plasma membrane (Galan *et al.*, 2001). During vegetative growth Snc1p is localized at the plasma membrane and continually recycles through the Golgi (Masayo Morishita, 2007, Lewis *et al.*, 2000). Rcy1 binds via its C-terminal domain to two GTPases, proteins that regulate vesicle transport during exo- and endocytosis and are required for Golgi function in yeast (Benli *et al.*, 1996). Rcy1 interacts specifically with the active forms of the two GTPases and together they co-localize to the Golgi and endosomes.

A second recycled protein by Rcy1 is Kex2, a calcium dependent serine protease involved in preprotein processing. Kex2p is a membrane-bound protein cycling between trans-Golgi vesicles and late endosomal compartments (Fuller *et al.*, 1989, Wilcox & Fuller, 1991). These studies suggest that the involvement of Rcy1 in vesicle transport is not related to protein degradation. Indeed, ubiquitination of the interacting proteins seems unlikely since assembly into an SCF-complex is not required for Rcy1 function. The F-box of Rcy1 is required for binding to Skp1, but the biochemical function of this small complex during vesicle trafficking remains unclear. Perhaps surprisingly, Rcy1 has been found in an SCF-complex (Kus *et al.*, 2004), but it remains unknown whether this is a functional complex. The *Schizosaccharomyces pombe* homolog of Rcy1, Pof6, also forms a complex with Skp1 and does not function in an SCF-complex (Hermand *et al.*, 2003). Pof6 is required for septum processing and sporulation - deletion of *POF6* results in the formation of a thick septum and absence of viable spores, which differens from the deletion phenotype of Rcy1, which is not lethal. Recently, a specific Pof6 interactor identified as Sip1, was found using TAP purification an MudPIT analysis, (Jourdain *et al.*, 2009). It was shown that Pof6 and Sip1 form a non-SCF complex with Skp1, and both proteins need the interaction to Skp1 for stability. Sip1 is a widely conserved protein in eukaryotes and consists of HEATS-repeats required for interaction to other proteins. Like Pof6, Sip1 is essential and plays a role in endocytosis and cytokinesis. The budding yeast ortholog of Sip1, Laa1, is notidentified yet as an interactor of Rcy1, but might also be part of the Rcy1-Skp1 mini complex as it also mediates protein transport between the trans-Golgi network and endo-somes (Fernandez & Payne, 2006).

This suggests that the role of Rcy1 is conserved between budding yeast and fission yeast. In fact, Rcy1 seems to play a fundamental role in trafficking, since it is conserved throughout the fungal kingdom (BLAST searches, our observations).

**Non-Skp1 binding F-box proteins**

Some proteins with an F-box domain do not bind Skp1 but another E3-ligase subunit instead. For other F-box proteins binding to Skp1 could not be demonstrated or has not been investigated. The F-box domain in some of the latter proteins may also mediate assembly into different complexes.
Ela1: an Elongin complex F-box protein

Ela1 (Elongin A 1) and Elc1 (a Skp1 homolog) were identified in *Saccharomyces cerevisiae* as the homologs of mammalian Elongin complex components (Koth et al., 2000). Also in yeast, Ela1 and Elc1 are present in the same complex. Probably, Ela1 does not act in a SCF complex, since it binds Elc1 instead of Skp1 and Elc1 does not bind Cul1. Ela1 and Elc1 likely bind to Cul3 instead. Cul3 is a Cul1 that is normally part of an E3-ligase complex called BC3B, consisting of Cul3, a BTB-domain containing protein and Rbx1. Apparently, Ela1 exists in a complex (Ela1/Elc1/Cul3/Rbx) that is a combination of the human E3 ligase Von Hippel-Lindau (VHL) and the BC3B complexes. This new combinatory complex was not reported earlier and it shows the possibility that subunits from different complexes can interchange to form new complexes, potentially broadening the arsenal of ubiquitin ligase superfamilies.

Ela1 and Cul3 were found to be required for cell survival after treatment with UV or the mutagen 4-nitroquinoline 1-oxide. Both proteins are also required for degradation and poly-ubiquitination of subunit Rpb1 of RNA polymerase II (Pol II). Pol II is normally removed from damaged DNA to make room for the nuclear excision repair (NER) machinery to assemble at that site and repair damaged DNA strands (Ribar et al., 2007).

Mfb1: A mitochondria associated F-box protein

Mfb1 (Mitochondria-associated F-box protein) in *Saccharomyces cerevisiae* controls membrane fusion dynamics of mitochondria, like Mdm30 described above. Deletion of *MFB1* results in abnormal mitochondrial morphologies, including short tubules, aggregates and fragments in different combinations (Kondo-Okamoto et al., 2006). Binding to Tom71 localizes Mfb1 to mitochondria and binding to Tom70 ensures stable association with these organelles (Kondo-Okamoto et al., 2008). The paralogous TPR repeat proteins Tom70 and Tom71 are both associated with mitochondrial protein import (Brix et al., 2000, Schlossmann et al., 1996). Loss of *MDM30* also results in short tubules, aggregates and fragments, but in a different distribution than in /mfb1 mutants (Fritz et al., 2003). A double knock-out of *MFB1* and *MDM30* results in a decreased number of short tubules, but more aggregates and fragments. On rich dextrose and glycerol plates, an /mfb1 mutant grows like wild type, an /mdm30 mutant grows slower, and a double mutant displays a severe growth problem, probably due to mtDNA instability (Durr et al., 2006). Possible targets of Mfb1 are proteins involved in mitochondrial morphogenesis, but for none of the candidate proteins higher amounts were seen in /mfb1 mutants. This observation and the lack of demonstration that Mfb1 binds Skp1 suggest that Mfb1 may not function as part of an SCF-complex.

Amn1: a mitosis exit state F-box protein.

Amn1 (Antagonist of mitotic exit network 1) from *Saccharomyces cerevisiae* is listed as one of the 21 budding yeast F-box proteins in an earlier review (Willems et al., 2004). The protein shares homology with another F-box/LRR protein, Pof2 of fission yeast, with little conservation of the F-box domain, in part because of an interspersed region of 56 amino acids in the motif. Although a genetic interaction has been found, physical interaction between Amn1 and Skp1 could not be demonstrated (in insect cells), perhaps because of the interspersed region in the F-box domain. Amn1 itself might be targeted for SCF-mediated proteolysis, since stabilized forms of Amn1 were found in /cul1 and /skp1 mutant strains. *AMN1* expression peaks at the M/G1 phase and Amn1 is normally degraded when cells enter the S phase, showing a similar accumulation pattern as the Cdc4 target Sic1. Amn1 is required to turn off the mitotic exit pathway after is has completed and it inhibits the function of Tem1, a small GTPase that activates the mitotic exit network (MEN), which causes spindle breakdown, degradation of mitotic cyclins, cytokinesis and cell separation (Bardin et al., 2000). It was shown that Amn1 binds to Tem1 and inhibits its function by obstructing the binding of Tem1 to Cdc15. This ensures that the cell can exit from mitosis and enter the G1
phase (Wang et al., 2003). Tem1 levels are elevated in a \( \Delta amn1 \) mutant (153) suggesting that Amn1 may regulate Tem1 levels. Since Amn1 apparently does not bind Skp1, this regulation may not involve ubiquitination.

Other fungal F-box proteins

Broad, genomics based interaction and localization studies have provided some information on F-box proteins that have not been investigated individually (Table 2). Most of these proteins bind Skp1 and can assemble into an SCF-complex (Seol et al., 2001, Kus et al., 2004), and some also interact with ribosomes (Ynl311c (Fleischer et al., 2006)) or other proteins like Sg1 (Ynl311c and Ydr306c (Dubacq et al., 2002). Sg1 binds to Skp1 and other SCF components (Kitagawa et al., 1999) and acts as a "client adaptor" linking the chaperone Hsp90 to SCF- and CBF3 complexes containing Skp1 (Catlett & Kaplan, 2006). For one F-box protein (Ymr258c) it was determined that it localizes to the cytoplasm and nucleus using a green fluorescent protein (GFP)-fusion (Huh et al., 2003) and for another (Ylr224w) it was demonstrated that it is readily mono-ubiquitinated in vitro by SCF-Ubc4 complexes (Kus et al., 2004).

Table 2: Additional F-box protein in \( S. \) cerevisiae and \( S. \) pombe without targets and ascribed cellular function or Skp1/SCF binding data.

<table>
<thead>
<tr>
<th>F-box protein</th>
<th>Yeast</th>
<th>Additional motif</th>
<th>Skp1 binding</th>
<th>SCF binding</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cos111</td>
<td>( S. ) cerevisiae</td>
<td>-</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Das1*</td>
<td>( S. ) cerevisiae</td>
<td>-</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Ydr306c</td>
<td>( S. ) cerevisiae</td>
<td>RNI-like</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Pof5</td>
<td>( S. ) pombe</td>
<td>RNI-like</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td>Ynl311c</td>
<td>( S. ) cerevisiae</td>
<td>-</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Ylr224c</td>
<td>( S. ) cerevisiae</td>
<td>-</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Ymr258c</td>
<td>( S. ) cerevisiae</td>
<td>-</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td>Ydr131c</td>
<td>( S. ) cerevisiae</td>
<td>-</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Ylr352w</td>
<td>( S. ) cerevisiae</td>
<td>LRR</td>
<td>yes</td>
<td>yes</td>
</tr>
<tr>
<td>Pof10</td>
<td>( S. ) pombe</td>
<td>WD40</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td>Pof11</td>
<td>( S. ) pombe</td>
<td>-</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>Pof12</td>
<td>( S. ) pombe</td>
<td>-</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td>Pof13</td>
<td>( S. ) pombe</td>
<td>-</td>
<td>yes</td>
<td>?</td>
</tr>
<tr>
<td>Pof16</td>
<td>( S. ) pombe</td>
<td>-</td>
<td>?</td>
<td>?</td>
</tr>
</tbody>
</table>

* Das1 (Dst1delta 6-azauracil sensitivity) from \( S. \) cerevisiae is a putative SCF ubiquitin ligase F-box protein of which a null mutant suppresses \( \Delta dst1 \) sensitivity to 6-azauracil (Chavez, 2007). \(^{1}\)RNI-like = Ribonuclease inhibitor-like, LRR = leucine rich repeat, WD40 = tryptophan-aspartic acid terminating domain. \(^{2}\)? = unknown.

Pof10 from Schizosaccharomyces pombe is an F-box/WD40 protein that binds Skp1 via its F-box domain (Ikebe et al., 2002). Deletion of \( POF10 \) does not result in an obvious phenotype, which is remarkable since \( POF10 \) is conserved between fission yeast and filamentous fungi (BLAST search, our observations). On the other hand, overexpression of
POF10 results in lethality, probably due to sequestration of Skp1, thereby preventing the formation of other SCF-complexes. Viability was restored by concomitant overexpression of SKP1, presumably by making more Skp1 available for formation of other SCF complexes. Binding to Skp1 may not lead to self-ubiquitination because Pof10 is highly stable in contrast to other F-box proteins. Although Pof10 bears a protein-protein interaction domain (a WD40 motif), targets of Pof10 have not been identified.

Concluding remarks

Fungal F-box proteins take part in highly diverse cellular processes, but most share the same molecular function: removal or inactivation of specific proteins. Loss of a fungal F-box protein often results in a pleiotropic phenotype, especially when the F-box has multiple targets. For Cdc4, which has ten known targets, a null mutation is lethal. Conversely, when a gene deletion shows no or little effect, the F-box protein may target only one or a few proteins. For example, the original deletion mutant of COS111 did not show any phenotype, but it was later demonstrated that COS111 may have a function in tolerance to an antifungal agent. Targets of F-box proteins can vary from transcription factors, enzymes, DNA-repair proteins, structural proteins and cyclins to inhibitors and/or activators of various other processes. These targets can operate at an intermediate level of a signaling pathway, for example Rcn1 and Sic1 that are degraded via Cdc4, and Msk1 and Ime2 that are degraded via Grr1. Other targets function at the end of a pathway, examples of which are the transcription factors Tec1 and Gcn1, degraded via Cdc4 and Frq, degraded via Fwd1.

Targets of F-box proteins are mostly recognized when phosphorylated. Such phosphorylation can be performed by many different protein kinases like CDK’s, MAPK’s, Pho kinases and CK’s, depending on the pathway or process in which the target protein functions. Different forms of phosphorylation can be required for recognition. For instance, a requirement for hyper-phosphorylation of a target causes a threshold before a target is being degraded and ensures that multiple phosphorylation steps control degradation, like for Cdc4-mediated degradation of Sic1 and Fwd1-mediated degradation of Frq. An exceptional case of an unphosphorylated target is Msk1, which is degraded by Grr1 when it is unbound to either Rtg2 or Bmh1. Apparently, the site on Msk1 recognized by Grr1 is masked by these interacting proteins.

In addition to these well-studied F-box proteins, several other F-box proteins interact with proteins without targeting them for disposal, examples being Ctf13, Rcy1 and Pof14. For still other F-box proteins no targets or interacting proteins have been found, and these are often referred to as “orphan F-box proteins”. It might be that the targets are yet to be found, or no targets exist for these orphan F-box proteins. Especially for those of which mutation of the F-box domain does not (greatly) affect function (like Frp1 and Fbh1) the F-box domains may serve as degradation motifs solely required for self-ubiquitination. Instead of targeting other proteins, they may, for instance, function as DNA binding proteins or perform an enzymatic reaction. Another variation on the F-box hypothesis is seen for Ela1, an F-box protein that does recruit targets for degradation, but assembles in a complex different from the SCF.

The levels of free and SCF-bound F-box proteins in the fungal cell are regulated by recycling of Skp1-F-box complexes within the SCF core via Nedd8 and CAND1 and by self-ubiquitination. It seems that in unicellular fungi like budding and fission yeast, recycling is less important and F-box proteins are mostly regulated by auto-ubiquitination as shown for several budding yeast F-box proteins. The difference in regulation could be related to the relatively small number of F-box protein in these two yeasts (21 and 16, respectively), in contrast to filamentous fungi which harbor up to hundred or more (BLAST searches, our observations).

F-box proteins are probably also regulated by recycling and auto-ubiquitination to remove “free” F-box proteins (i.e. unbound to a target) from SCF-complexes, so that these
complexes become available for other F-box proteins. Such a scenario was supported by overexpression of POF10, probably leading to constitutive occupation of Skp1, which results in lethality (Ikebe et al., 2002) and for the UFO1Δuim mutant lacking the UIMs. Normally, the UIMs are required for self-ubiquitination of this F-box protein – a mutant lacking the UIMs cannot be ubiquinated anymore and therefore remains in the SCF-complex (Ivantsiv et al., 2006). In a variation on self-ubiquitination, Ctf13 is targeted by another F-box protein, Cdc4, when unassembled into a CBF.

Besides being regulated on the protein level by ubiquitination and recycling, F-box proteins can also be regulated at the transcriptional level. An example is Met30, which creates a negative feedback loop by degrading the transcription factor Met4, inactivating its own transcription. Another potential mechanism of regulation is localization. Some F-box proteins function specifically at certain sites in the cell or at certain regions on chromosomal DNA. To be transported to these sites, interacting partners can play an important role, as demonstrated for Mfb1 and Dia2/Pof3.

In Fungi, regulation of the activity of F-box proteins themselves is usually not an integral part of a signal transduction pathway, in contrast to some cases in plants where it was demonstrated that F-box proteins could be activated by direct binding to a small molecule. These F-box proteins act as receptors, with direct hormone binding triggering their activation (reviewed in (Haichuan Yu et al., 2007)). Such a mechanism remains a possibility also in fungi, for instance for Met30 which is activated when high levels of methionine, S-adenosyl-methionine or cysteine are present. Binding studies of these sulphur containing amino acids or derivatives to Met30 could confirm this possibility.

Perhaps less sophisticated, many fungal F-box proteins appear to function simply as garbage collectors, removing waste proteins that have been marked for degradation. However, several variations on this theme have emerged. For instance, Met30 regulates the transcription factor Met4 in complex ways and does not simply follow the standard F-box hypothesis. Further in-depth investigations of F-box proteins and their potential targets or other functions may reveal more such variations.

Most F-box proteins discussed in this review are from S. cerevisiae, providing a fairly comprehensive overview of the variety of functions that F-box proteins perform in an eukaryotic cell. The additional results obtained with orthologs and other F-box proteins from fission yeast and filamentous fungi gives an impression of the degree of functional conservation of F-box proteins between fungal species. For example, functional conservation of Grr1 is, not unexpectedly, less when species are more distantly related – in contrast to the GRR1 ortholog of C. albicans, the orthologs from two filamentous fungi could not fully complement the Δgrr1 mutant in yeast. Since Skp1 is highly conserved between species, this is probably due to differences in target recognition. Evolution of an F-box protein is constricted by the requirement to recognize diverse targets. When an F-box protein encounters orthologs of its natural target in another fungus, or a ‘novel’ target (i.e. not present in its ‘natural environment’), recognition might be less efficient, despite overall sequence conservation in the target recognition domain of the F-box protein. Conservation of F-box protein function between different fungal species can also be assessed by the conservation of targets and the pathways leading to the phosphorylation of these targets. Fission yeast and C. albicans harbour orthologs of targets of budding yeast Cdc4 and Grr1, but these have not yet been found in other fungal species (Table 1). Of Met4, a target of Met30, homologs are present in fission yeast and in the filamentous fungi A. nidulans and N. crassa. Apparently, the Met30-Met4 interaction system has remained relatively stable during fungal evolution.

Searches in fungal genome sequences allow an estimation of the number of genes encoding F-box proteins in different fungal species. Aspergillus nidulans, for example, contains about 50 genes encoding F-box proteins and in different Fusarium species 60 to 95 genes encoding F-box proteins are present (Chapter III). When compared to the smaller numbers in
yeast (21 in budding yeast and 16 in fission yeast) it becomes clear that the potential variation of processes regulated by F-box proteins in filamentous fungi is much more extensive than in yeasts. Examples of this are regulation of the circadian clock in *N. crassa* and plant infection in *F. oxysporum*.

In fungi it is relatively easy to investigate F-box proteins due to availability of knock out strains and accessibility to molecular manipulations. Sophisticated screens for target identification and deletion studies of all genes encoding F-box proteins present in a fungal genome, combined with detailed investigation of protein-protein interactions and post-translational modifications will promote a deeper and broader insight in the diverse functions of F-box proteins in eukaryotic cells.

Among the general lessons already learned from investigation of the fungal F-box arsenal are that these proteins function in a very broad array of cellular functions and can target many different proteins for degradation. Furthermore, clearly not all F-box proteins comply to the F-box hypothesis and the regulation of at least some of these proteins is more complex than expected. Concerning the conserved F-box proteins found in budding yeast and filamentous fungi, we learned that they can have both conserved and diversified targets, and accumulation of conserved targets in deletion mutants of F-box proteins can sometimes result in different phenotypes. Fungi remain a rich source for the discovery and understanding of a great variety of intricate cellular processes that F-box proteins are involved in.