

## The Ubiquitin-Proteasome System and F-box Proteins in Pathogenic Fungi

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The ubiquitin-proteasome system is one of the major protein turnover mechanisms that plays important roles in the regulation of a variety of cellular functions. It is composed of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 ubiquitin ligases that transfer ubiquitin to the substrates that are subjected to degradation in the 26S proteasome. The Skp1, Cullin, F-box protein (SCF) E3 ligases are the largest E3 gene family, in which the F-box protein is the key component to determine substrate specificity. Although the SCF E3 ligase and its F-box proteins have been extensively studied in the model yeast *Saccharomyces cerevisiae*, only limited studies have been reported on the role of F-box proteins in other fungi. Recently, a number of studies revealed that F-box proteins are required for fungal pathogenicity. In this communication, we review the current understanding of F-box proteins in pathogenic fungi.

**KEYWORDS :** *Cryptococcus neoformans*, E3 ligase, F-box, Fungi, Virulence

### The Ubiquitin-Proteasome System

Protein turnover is an important regulatory mechanism of cellular function in eukaryotes. During proteolysis, proteins are hydrolyzed to their constituent amino acids and used for synthesis of new proteins. Proteolysis is highly specific and a diverse group of enzymes designated proteases are involved in this process. In eukaryotic cells, there are two major protein degradation pathways mediating protein degradation: the lysosomal proteolysis and the ubiquitin-proteasome system [1].

Some cytosolic proteins are degraded in lysosomes after being engulfed in autophagotic vacuoles that fuse with lysosomes, which are accelerated by carbon or nitrogen starvation [2]. The proteins susceptible to degradation by this pathway are normally long-lived but dispensable proteins. However, the majority of intracellular proteins are degraded by the ubiquitin-proteasome pathway, in which the intracellular proteins are degraded with a high degree of specificity [1, 3].

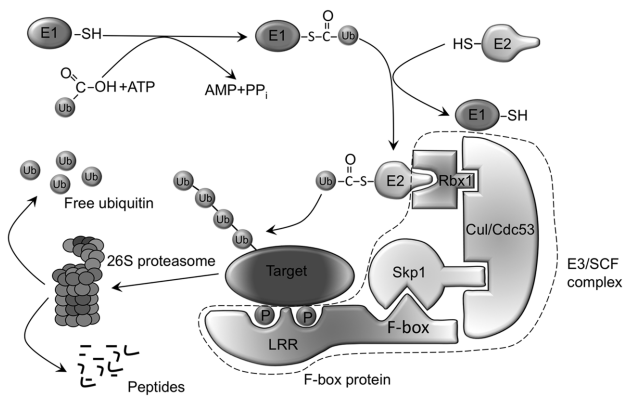
The ubiquitin proteasome pathway uses ubiquitin as a marker that targets proteins for rapid proteolysis. Ubiquitin is a 76 amino acid regulatory protein that is highly conserved in all eukaryotes. Proteins are marked for degradation by the attachment of multiple ubiquitin molecules and are then recognized and degraded to small

peptides by the 26S proteasome, a large multicatalytic protease complex, with the release of free and reusable ubiquitin [3]. Three enzymatic components are required to assemble chains of ubiquitin onto the substrates that are destined for proteolytic destruction. First, ubiquitin is activated by an E1 (ubiquitin-activating enzyme) in an ATP-dependent reaction and forms a thioester linkage between the C-terminal carboxyl group of the ubiquitin and the E1 cysteine sulfhydryl group. Ubiquitin is then transferred to an ubiquitin-conjugating enzyme E2 again via a thioesterification reaction. In the final step, an ubiquitin protein ligase E3 catalyzes the transfer of ubiquitin to specific substrates by creating an isopeptide bond between a lysine of the target protein and the C-terminal glycine of ubiquitin (Fig. 1). Lysines on conjugated ubiquitin molecules can then be targeted for additional ubiquitination via isopeptide bond formation. Poly-ubiquitinated substrates are recognized and subsequently degraded by the 26S proteasome [4, 5].

The importance of the ubiquitin-proteasome system in higher eukaryotes has been recognized following the recent discovery that F-box proteins are receptors of several important plant hormones [6] and mutations in the E3 ubiquitin ligase pathway lead to human diseases [7]. The therapeutic potential of the ubiquitin-proteasome system for human diseases has been actively evaluated, and has

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**Fig. 1.** The Skp1, Cullin, F-box protein (SCF) E3 ligase-mediated ubiquitin-proteasome system of protein degradation. Ubiquitin (Ub) is conjugated to substrate proteins through effort of three enzymes by an ATP-dependent process. First, ubiquitin is activated by an ubiquitin-activating enzyme (E1) in an ATP-dependent way and transferred to its active site through formation of a thiol-ester bond between the ubiquitin and E1. Then the ubiquitin is passed to the second enzyme complex, the ubiquitin-conjugating enzyme (E2), through the same thiol-ester linkage. Finally, the target substrate is recognized by the third enzyme complex, ubiquitin ligase (E3), and labeled with the ubiquitin. This process can be repeated and a multi-ubiquitin chain can be formed, which usually targets the substrate for proteolysis by the 26S proteasome.

resulted in the drug development such as bortezomib, a proteasomal inhibitor that has been used to treat human neoplasias [8].

### E3 Ubiquitin Protein Ligases

The key enzymes in the ubiquitination process are the E3 ubiquitin ligases, because they function as the substrate recognition modules of the system and are capable of transferring the activated ubiquitin to the substrate. Specific E3s are responsible for the selectivity of ubiquitin-protein ligation and degradation by binding specific protein substrates that contain specific recognition signals [4].

Generally, E3s are divided into two broad structural classes: the HECT (homologous to E6-AP C-terminus) domain family, where the HECT domain from a monomeric E3 accepts the activated ubiquitin from E2 and transfers it to the substrate [9], and the RING (really interesting new gene) domain family [10].

The suppressor of kinetochore protein mutant (Skp1), Cullin (Cul1), F-box protein (SCF) E3 ligases are among the best-understood groups of the RING ligases. Three major components of the SCF complex, Skp1, Cul1, and ring-box protein (Rbx1), form an invariable core complex that associates with one of a number of F-box proteins that bind to specific substrates (Fig. 1). In cooperation

with E1 and E2 enzymes, the SCF complex can transfer small ubiquitin proteins to the target protein [11-13].

The SCF E3 ligase was first identified in the budding yeast *Saccharomyces cerevisiae*, where it functions to catalyze the phosphorylation-dependent ubiquitination of G1 cyclins and Cdk inhibitors [14-16]. It also has important roles in regulating transcription factors [17] and regulators involved in G2 and M phases [18, 19], as well as having a role in the labeling of various other cellular proteins for degradation.

### F-box Proteins

F-box proteins are proteins containing the F-box domain, a protein structural motif of approximately 50 amino acids initially identified in human cyclin F [14, 20]. F-box proteins commonly interact with Skp1 of the SCF and E3 ligase complexes to take part in protein ubiquitination and degradation. They are responsible for the substrate specificity of the SCF complexes by interacting with the substrates through their C-terminal protein binding domains, including leucine-rich repeats (LRR), WD40 repeats, as well as other protein-protein interaction domains such as carbohydrate-interacting (CASH), zinc-finger, and proline-rich domains [21-23], and then recruiting the substrates to the SCF core through the interaction of the F-box domain with Skp1 [14]. There are exceptions, in which an F-box protein does not interact with Skp1, such as Ela1 in *S. cerevisiae* [24]. To date, all known SCF substrates must be phosphorylated before being recognized by an F-box protein.

A large number of F-box proteins exist in all eukaryotic model organisms reported so far, and each of them likely binds to multiple substrates for ubiquitination. The fungal F-box proteins are important in regulating cellular functions including cell cycle, circadian clocks, transcription, development, signal transduction, and nutrient sensing [24]. The understanding of SCF E3 ligases has largely come from extensive studies in two model yeasts, *S. cerevisiae* and *Schizosaccharomyces pombe*. *S. cerevisiae* has at least 20 proteins containing an F-box domain and several have been well studied, including glucose repression resistant 1 (Grr1) [24]. Grr1 is part of the SCF<sup>GRR1</sup> E3 ligase that interacts with Skp1 via its F-box domain and downstream targets via its LRR domain. Grr1 was originally identified as a primary element responsive for glucose repression [25], and was also found to control amino acid sensing [26] and cell cycle regulation [27]. *S. pombe* is another yeast in which a number of F-box proteins have been studied [24].

Despite extensive studies in both model yeasts, very limited studies of SCF E3 ligases have been reported in other fungi. Recent studies on the function of F-box proteins in pathogenic fungi have revealed that SCF E3 ligases are required for fungal virulence. Because of the

proven therapeutic potential of the ubiquitin-proteasome pathway for human diseases [8], it would be very important to understand the molecular basis of how this pathway regulates fungal virulence, which may lead to novel approaches in developing new antifungal agents.

### F-box Proteins in Fungal Pathogens

The function of F-box proteins and their potential as part of the ubiquitin-proteasome system (UPS) has been reported in a number of fungal pathogens, including those cause human infections (such as *Cryptococcus neoformans* and *Candida albicans*) or plant diseases (such as *Fusarium* species and *Magnaporthe oryzae*). A number of studies have focused on the fungal homologs of Grr1 in *S. cerevisiae*, and all the studies have demonstrated that these F-box proteins are important for fungal pathogenicity (Table 1).

*C. neoformans* is an encapsulated yeast causing fungal meningitis in immunocompromised patients. There are at least 19 proteins containing the F-box domain in *C. neoformans*. Among them, Fbp1 is identified as the only

F-box protein with additional 12 LRR repeats, a common protein binding domain. Fbp1 shares sequence similarity with Grr1 in *S. cerevisiae* and physically interacts with Skp1 homologues in both *S. cerevisiae* and *C. neoformans* via its F-box domain, suggesting it may function as part of an SCF E3 ubiquitin ligase. Our recent studies also revealed that Fbp1 likely regulates multiple substrates (our unpublished data). Functional studies demonstrated that Fbp1 is essential for fungal virulence in a murine inhalation model. Fungal strains lacking Fbp1 protein produce normal virulence factors (capsule, melanin, and growth at 37°C), but fail to cause any disease symptom. Interestingly, the fungal burden in infected lungs remains at a consistent level from 3~60 days post-infection, which mimics a latent infection (our unpublished data). Fbp1 is also important for fungal sexual reproduction because basidiospore production is blocked in bilateral mating between *fbp1Δ* mutants, even though normal dikaryotic hyphae are observed during mating. Moreover, in a stress response assay, *fbp1Δ* mutants showed hypersensitivity to sodium dodecyl sulfate, but not to calcofluor white or Congo red, indicating that Fbp1 may regulate cell membrane

**Table 1.** F-box proteins reported in pathogenic fungi

Fungi	Predicted total F-box proteins in the genome	Grr1 homologs	CDC4 homologs	MET30 homologs	Fwd1 homologs	Frp1 homologs
<b>Human pathogens</b>						
<i>Candida albicans</i>	21	(Grr1) <sup>a</sup> Pseudo-hyphal production (29)	(Cdc4) Dimorphic switch (30) Hyphal production (31)	NP	NP	NP
<i>Cryptococcus neoformans</i>	19	(Fbp1) Sexual sporulation, cell membrane integrity, virulence (28)	NP	NP	NP	NP
<b>Plant pathogens</b>						
<i>Fusarium graminearum</i>	62	(Fbp1) Sporulation, virulence(34)	NP	NP	NP	(Frp1) Virulence, sexual reproduction, carbon utilization (36)
<i>Fusarium oxysporum</i>	53	NP	NP	NP	NP	(Frp1) Virulence, carbon utilization (35,36)
<i>Botrytis cinerea</i>	40	NP	NP	NP	NP	(Frp1) Sexual reproduction (36)
<i>Magnaporthe oryzae</i>	11	(Pth1) Virulence (37)	NP	NP	NP	NP
<b>Non-pathogen</b>						
<i>Aspergillus nidulans</i>	55	(GrrA) Sexual sporulation (39)	NP	(SconB) Sulfur metabolism (40)		NP

NP, not reported.

<sup>a</sup>Name of the F-box protein, followed by their major role in fungal development and virulence, and references.

integrity [28].

The dimorphic switch in *C. albicans* between yeast form, pseudohyphal form, and true hyphal form in response to different environmental stimuli is important for host invasion and establishment of disease, and is considered as an important virulence trait. Two F-box proteins, Cdc4 and Grr1, have been reported to be required for this morphological transition in *C. albicans*. Deletion of either *CDC4* or *GRR1* from the *C. albicans* genome results in a highly filamentous or pseudohyphal morphology under conditions that normally promote the yeast form of growth [29-31]. The suppression of pseudohyphal production by Grr1 is likely due to its negative regulation on cytokinesis via two G1 cyclins, Ccn1 and Cln3, in a similar way as that of the *S. cerevisiae*. These cyclin proteins are stabilized in a *grr1Δ/Δ* mutant background, which prevents cell separation after cytokinesis, indicating that they are potential substrates of Grr1. Furthermore, the cellular level of Hof1, a protein having a role in cytokinesis, is also significantly increased in *grr1Δ/Δ* cells [32]. Meanwhile, it is less clear how Cdc4 regulates cell morphology. One substrate of Cdc4, Sol1, was identified to play a role in *C. albicans* morphogenesis [30]. Additional efforts to identify Cdc4 associated proteins using *in vitro* affinity purification method revealed several novel proteins, including Gph1 (a putative glycogen phosphorylase) and Thr1 (a putative homoserine kinase). Both Gph1 and Thr1 are regulated by Tup1, a master regulator of hyphal production [33]. These studies suggest that ubiquitin-mediated protein degradation is involved in the regulation of the dimorphic switch of *C. albicans*. However, a direct role of these proteins in fungal virulence has not been established.

Interestingly, filamentous fungi such as *Fusarium* species contain much larger numbers of F-box proteins than yeast: 60~94 in *Fusarium* species compared to around 20 in yeasts (*S. cerevisiae*, *C. albicans*, or *C. neoformans*). This suggests that F-box proteins contribute to the regulation of a more complex developmental and metabolic processes occurring in filamentous fungi. *Fusarium graminearum* is a plant pathogen that infects wheat and barley to cause headblight disease, which not only leads to a significant loss of crop yield, but also produces a variety of harmful mycotoxins. By screening an insertional mutagenesis library, a homolog of Grr1 in *S. cerevisiae*, Fbp1, was identified to be important for fungal pathogenicity. Although Fbp1 is likely a part of the SCF complex since it can bind to *S. cerevisiae* Skp1 protein, Fbp1 can only partially complement several defects of a yeast *grr1Δ* deletion mutant. Functional analyses demonstrated that *FBP1* is required for both sexual development and virulence in *F. graminearum* [34].

The same approach was used to identify another F-box protein required for pathogenicity (Frp1) in *Fusarium oxysporum* f. sp. *Lycopersici*, the causal agent of tomato

wilt disease. Frp1 is filamentous fungi specific, only can be found in fungi belonging to Sordariomycetes, leotiomycetes, and Dothideomycetes. The *frp1Δ* mutant is avirulent due to its failure to colonize roots, confirming that the *FRP1* gene is required for pathogenesis [35]. Interestingly, the homologs of Frp1 in different fungal pathogens that are closely related in evolution have different functions in sexual development, metabolism, and pathogenicity. Frp1 is essential for root infection in *F. graminearum*, but is dispensable for the infection on plant parts above ground in both *F. graminearum* and *Botrytis cinerea*, a common pathogen infecting wine grapes [36]. Frp1 is required for nonsugar carbon catabolism and regulates the growth of *Fusarium* species under a variety of conditions but does not play a role in the growth of *B. cinerea*, indicating a functional diversity on nonsugar carbon catabolism among these fungi.

*Magnaporthe oryzae* causes one of the most devastating diseases on rice. By screening an insertional mutagenesis library, an early study identified one F-box protein, Pth1, which is required for fungal pathogenicity both on rice and barley [37]. Pth1 is a homolog of Grr1 and is required for regulating maturation of the appressorium, a specialized cell structure for penetrating host cells. The *pth1Δ* mutant fails to penetrate the host leaf surface and to establish a successful host-pathogen interaction. Later studies demonstrated that Pth1 is critical for fungal carbohydrate metabolism and turgor pressure generation in appressoria, which could be the cause of producing defective appressoria in *pth1Δ* mutants [38].

There are several human and plant pathogens in *Aspergillus* species, such as *Aspergillus fumigatus* and *A. flavus*. There is no report of F-box proteins in these pathogenic *Aspergillus* species. *A. nidulans* is not a pathogen, but serves as a model system in understanding of other related species and has been extensively studied. Hence, we include the current understanding of F-box proteins in *A. nidulans* in this article.

The F-box protein GrrA identified in *A. nidulans* is a homolog of Grr1 in *S. cerevisiae* [39]. Functional studies have revealed that GrrA is dispensable in hyphal growth, asexual sporulation or development of asci-containing cleistothecia, but that the *grrAΔ* deletion mutant is unable to produce mature ascospores due to a block in meiosis [39]. The regulation of GrrA in meiosis is consistent with the role of other Grr1 homologs in pathogenic fungi, including *C. neoformans*, suggesting a conserved function of these F-box proteins among fungi. It will be interesting to analyze the potential role of GrrA in pathogenic *Aspergillus* species to understand whether they are also involved in regulation of fungal virulence.

The *A. nidulans* SconB is another F-box protein that shares sequence similarity with Met30 in *S. cerevisiae* and Scon2 in *Neurospora crassa*. SconB is required in

regulation of sulphur metabolite repression. Besides the F-box domain, SconB also has seven WD repeats characteristic of the large WD40 family of eukaryotic regulatory proteins. The *SCONB* transcript is derepressed under sulphur limitation conditions and partly repressed by high methionine [40]. No homolog of conB has been reported in pathogenic fungi (Table 1).

### Concluding Remarks and Future Directions

The recognition that the ubiquitin-proteasome system is a principle intracellular route for controlled protein turnover represents a major conceptual advancement in our understanding of cell biology. This protein degradation system has been proposed as the new generation therapeutic targets for human diseases [41]. Extensive studies in this field have been conducted in human [23, 42], as well as several model systems, such as *S. cerevisiae* [24], *Arabidopsis thaliana* [6, 43], and *Caenorhabditis elegans* [44]. The importance of the proteolytic function of the ubiquitin-proteasome system in regulating the virulence of pathogenic fungi has just been realized recently. Current studies of this system in pathogenic fungi are mostly focused on the F-box proteins. The substrates remain to be understood in most cases, which should be the focus of next step to understand the mechanism of the ubiquitin-proteasome system regulation in fungal diseases. An advanced understanding of the SCF E3 ligase-mediated ubiquitin-proteasome system in pathogenic fungi could potentially lead to innovative approaches to understand fungal virulence and novel strategies to explore therapeutic targets.

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