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Identification of SkpA-CulA-F-box E3 ligase complexes in pathogenic Aspergilli

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ABSTRACT

The ubiquitin proteasome system is critical for the regulation of protein turnover, which is implicated in the modulation of a wide array of biological processes in eukaryotes, ranging from cell senescence to virulence in plant and human hosts. Proteins to be marked for ubiquitination and subsequent degradation are bound by F-box proteins, which are interchangeable substrate-recognising receptors. These F-box proteins bind a wide range of substrates and associate with the adaptor protein Skp1 and the scaffold Cul1 to form Skp1-Cul1-F-box (SCF) complexes. SCF complex components are highly conserved in eukaryotes, ranging from yeast to humans. However, information regarding the composition of these complexes and the biological roles of F-box proteins is limited, specifically in filamentous fungal species like the genus *Aspergillus*. In this study, we have identified 51 and 55 *fbx*-encoding genes in the genomes of two pathogenic fungi, *A. fumigatus* and *A. flavus*, respectively. Immunoprecipitations of the HA-tagged SkpA adaptor protein revealed that 26 F-box proteins in *A. fumigatus* and 30 F-box proteins in *A. flavus* are involved in SCF complex formation during vegetative growth. These interactome data also revealed that a diverse array of SCF complex conformations exist in response to various exogenous stressors. Lastly, we have provided evidence that the F-box protein Fbx45 interacts with SkpA in both species in response to Amphotericin B. Orthologs of the *fbx45* gene are highly conserved in *Aspergillus* species, but are not present within the genomes of organisms such as yeast, plants or humans. This suggests that Fbx45 could potentially be a novel F-box protein that is unique to specific filamentous fungi such as *Aspergillus* species.

1. Introduction

In order for cells to maintain homeostasis, complex methods of regulation are required for the control of protein synthesis and degradation. Proteins are readily manipulated via posttranslational modifications (PTMs), which occur at specific amino acid residues. One of the most common and essential PTMs is ubiquitination. This involves the covalent attachment of the small regulatory protein ubiquitin (Ub) onto target proteins at lysine side-chain residues to mark them for proteasomal degradation (Hershko, 1983; Hershko and Ciechanover, 1998; Popovic et al., 2014). In the ubiquitin proteasome system (UPS), Ub is coupled to proteins via an enzymatic cascade in a sequential manner. This involves an activating enzyme (E1), a conjugating enzyme (E2) and a ligating enzyme (E3) (Wilkinson, 1987). The E1 enzyme is required for the initial activation of the C-terminal glycine residue of Ub, which is then transferred to a cysteine residue of the E2 enzyme. The E3 ligase then binds the E2-Ub intermediate and facilitates the coupling of Ub to the lysine residues on target proteins to mark them for degradation (Hershko and Ciechanover, 1998; Wang et al., 2017). The UPS is considered an important target for anticancer therapies as dysfunction or abnormal regulation of the UPS is implicated in various human diseases, such as cancers, diabetes and neurodegenerative conditions (Ciechanover, 2003; Ciechanover and Iwai, 2004; Schwartz and Ciechanover, 2009; Zheng et al., 2016).

In mammalian cells, there are more than 600 types of E3 ligases, which are organised into 4 main categories (Zhao and Sun, 2013). One group is known as the RING (really interesting new gene) finger-containing E3s (Zhao and Sun, 2013; Zheng and Shabek, 2017), which facilitate the transfer of Ub directly from the Ub-loaded E2 to a substrate (Hershko and Ciechanover, 1998; Lipkowitz and Weissman, 2011). The RING-finger E3s are sub-categorised into two groups, one of which is the multicomponent-containing E3s. In this group, the substrate-binding components are assembled together with the RING-finger unit from various E3s such as cullin ring ligases (CRLs) (Sun, 2003; Willems et al., 2004). CRLs are the largest and most diverse class of RING-finger E3 ligases and are involved in marking thousands of proteins (roughly 20% of cellular proteins) for ubiquitination and subsequent degradation via the UPS (Deshaies and Joazeiro, 2009; Sarikas

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et al., 2011; Soucy et al., 2009). CRL complexes consist of 4 proteins, a cullin scaffold protein, an adaptor protein, a substrate recognising receptor and a RING protein which acts as a catalytic subunit (Zhao and Sun, 2013). The cullin scaffold binds an adaptor protein, which itself associates with a variety of interchangeable substrate recognising receptors. Upon binding of the substrate, the cullin scaffold then mediates transfer of ubiquitin from an E2 enzyme via interactions with the RING protein, which directly interacts with the E2 (Rusnac and Zheng, 2020).

Within the CRL group, the mammalian CRL1 complex is the most extensively studied. This complex consists of the s-phase kinase-associated protein 1 (SKP1), which acts as an adaptor protein, the cullin 1 scaffold (CUL1), the RING protein (RBX1) and a wide range of interchangeable F-box proteins that act as substrate recognising receptors, which are essential for mediating substrate selectivity and specificity. Due to the presence of these proteins, the CRL1 complex is often appropriately termed the SCF (Skp1-Cul1-F-box) complex (Cardozo and Pagano, 2004; Feldman et al., 1997; Hua and Vierstra, 2011; Yen and Elledge, 2008; Zheng et al., 2002). F-box proteins consist of a characteristic F-box domain which spans roughly 40–50 amino acids and is required for the interaction of F-box proteins with the Skp1 adaptor (Bai et al., 1996; Kipreos and Pagano, 2000; Skowyra et al., 1997). F-box proteins also possess various substrate-binding domains and are organised into three groups accordingly (Craig and Tyers, 1999). One group of F-box proteins possess a WD40 scaffold domain (Smith et al., 1999), while another group possess a leucine rich repeat (LRR) domain (Enkhbayar et al., 2004; Kobe and Kajava, 2001). Lastly, the final group of F-box proteins are characterised by the presence of various other substrate-binding motifs (such as proline-rich domains or zinc finger domains) but do not possess WD40 or LRR domains (Craig and Tyers, 1999; Yoshida et al., 2002). In humans, 69 F-box proteins have been shown to associate with the CRL1 complex components, signifying that this complex is capable of targeting a wide array of substrates for degradation (Jin et al., 2004).

SCF complex components are highly conserved in eukaryotes. *Saccharomyces cerevisiae, Drosophila melanogaster* and humans possess one or a few SKP1 proteins, as well as 14, 27 and 69 *FBX* genes respectively (Skaar et al., 2009). However, some organisms possess a large array of SCF components and are capable of assembling thousands of different SCF complexes. For example, *Arabidopsis* species such as *A. thaliana* possess 19 *SKP1* genes and roughly 700 *FBX* genes (Farras et al., 2001; Gagne et al., 2002; Hua et al., 2011). F-box proteins play diverse roles in eukaryotic organisms. For example, in yeast, the F-box protein Grr1 is required for the control of the cell cycle and glucose-induced gene expression (Li and Johnston, 1997). In *Neurospora crassa*, the F-box protein MUS-10 functions in the regulation of mitochondrial maintenance and cell senescence (Kato et al., 2010). In *Arabidopsis* species, photoreceptor F-box proteins such as ZTL, FKF1 and LKP2 are critical for the regulation of the circadian clock (Ito et al., 2012; Stefanowicz et al., 2015). The F-box protein Fbp1 in both the human pathogen *Cryptococcus neoformans* and the plant pathogen *Fusarium oxysporum* is essential for regulating virulence (Masso-Silva et al., 2018; Miguel-Rojas and Hera, 2016).

With regards to filamentous fungi like *Aspergillus* species, little is known about the biological roles of F-box proteins and formation of SCF complexes. The model filamentous fungus *A. nidulans* encodes orthologous CRL1/SCF complex components. It has been shown that *A. nidulans* possesses 74 *fbx* genes and the functions of these respective F-box proteins are mostly unknown (de Assis et al., 2018; Galagan et al., 2005; von Zeska Kress et al., 2012). In *A. nidulans,* Fbx23 and Fbx47 have been shown to be required for carbon catabolite repression (de Assis et al., 2018), while Fbx50 (GrrA) is essential for the production of mature ascospores (Krappmann et al., 2006) and Fbx15 is necessary for positive regulation of both asexual and sexual development (von Zeska Kress et al., 2012). Fbx15 is also required for regulating virulence and the response to oxidative stress in the opportunistic pathogen *A. fumigatus* (Johnk et al., 2016).

Despite this knowledge, the understanding of the functions of F-box proteins and the organisation of SCF complexes in pathogenic *Aspergilli* is limited. Particularly, in *A. fumigatus* and the plant pathogen *A. flavus,* little is known about the existence of F-box proteins. Consequently, in this study, we identified orthologous SCF complex components in both *A. flavus* and *A. fumigatus,* using *A. nidulans* as a reference. By tagging the SkpA adaptor protein in both species, we detected protein–protein interactions with a wide range of F-box proteins and the scaffold CulA and provided evidence for the existence of an array of SCF complexes in both species in response to various stress conditions. Overall, these data suggest that SCF complex formation is a conserved mechanism in *Aspergillus* species and is likely required for the maintenance of various biological processes in fungal organisms.

2. Methods

2.1. Strains, growth media and culturing conditions

Fungal strains used in this study are listed in Table S1. The *Aspergillus fumigatus* CEA17 (*pyrG*$^{\triangle}$) strain and the *Aspergillus flavus* TJES19.1 strain served as wild type hosts for all epitope taggings. Various plasmids used for epitope tagging experiments are listed in Table S2. Plasmids were cloned into Stellar (Clontech) and MACH-1 (Invitrogen) competent *Escherichia coli* cells and these cells were cultured in LB medium (1% tryptone, 0.5% yeast extract, 1% NaCl), supplemented with 100 μg/ml ampicillin and SOC medium (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose).

For the asexual growth of fungal strains, Glucose Minimal Medium (GMM) agar plates were used: (6 g/L $NaNO_3$, 0.52 g/L KCl, 1.52 g/L KH_2PO_4, 10 g/L Glucose, 0.24 g/L $MgS0_4$, 0.1% trace element solution). For vegetative growth, liquid complete medium (GMM medium ingredients with the addition of 1 g/L tryptone, 2 g/L peptone and 1 g/L yeast extract) and Sabouraud medium (CMO147 Oxoid, 30 g/L) were used. All appropriate supplements were added to each medium prior to inoculation with fungal spores. These supplements included uracil (1 g/L) and uridine (0.25 g/L). All *A. flavus* strains inoculated on agar plates and liquid media were cultured at 30°C, while all *A. fumigatus* strains were cultured at 37°C.

For stress tests, strains were inoculated in liquid complete medium and left to incubate on a shaker at 180RPM for 24 h. After 24 h of vegetative growth, various stress agents were added to the liquid medium and mycelia were grown for a further 1 h. These stress agents included NaCl (0.5 M), H_2O_2 (5 mM), Congo red (20 μg/ml), Amphotericin B (1 μg/ml) and Miconazole (4 μg/ml).

2.2. Plasmid construction

Details of all plasmids used in this study are given in Table S2 and all oligonucleotide sequences are provided in Table S3. The Lasergene Seqbuilder software was used to design all plasmid maps *in silico*. For all cloning experiments, pUC19 (Fermentas) digested with a *Sma*I restriction enzyme (Thermo Scientific) was used. To create the *sgfp* and *3xha skpA* fusion constructs in both *A. flavus* and *A. fumigatus*, respective WT genomic DNA was isolated and used as a template in various PCR reactions to allow for replication of specific regions of DNA. Designed primers were used to bind the genomic DNA and replicate a 1.5–2 Kb segment of the open reading frame (ORF) of the *skpA* gene in both species. These replicated ORF segments lack the natural stop codon and also contain overhangs for the pUC19 plasmid, as well as overhangs for both the *sgfp* and *3xha* epitope tags. Primers were also used to replicate 1–1.5 Kb segments of the 3′ UTR sequences immediately downstream of the *skpA* gene ORFs. These replicated 3′ UTR segments contain overhangs for both the pUC19 plasmid and the *pyrG* gene. Lastly, segments of DNA containing either *sgfp* or *3xha* epitope tags connected to the *pyrG* selection marker were PCR-replicated from plasmids containing

these cassettes of interest. These three replicated DNA fragments were then cloned into the *Sma*I site of pUC19 by the In-Fusion HD Cloning enzyme (Clontech, 121416), allowing for fusion of each fragment and coupling of the epitope tags to the C-terminal ends of the *skpA* genes. Each ligated plasmid was then transformed into competent bacterial cells to promote replication of the plasmids.

2.2.1. Transformation of bacterial and fungal cells

Transformation of both bacterial and fungal cells was performed as explained in detail (Frawley et al., 2020). *A. flavus* colonies were cultured at 30 °C, while *A. fumigatus* colonies were cultured at 37 °C.

2.3. Hybridization techniques

Labelling of DNA probes and southern blotting was performed as explained in detail (Frawley et al., 2018).

2.4. Extraction of crude and nuclear proteins

Isolation of crude protein extracts was performed as explained in detail (Elramli et al., 2019). Nuclear protein extracts were isolated from mycelia that had been incubated for 24 h in liquid GMM or Sabouraud medium. The mycelia were washed with 1x PBS, snap-frozen using liquid nitrogen and pulverised using a mortar and pestle. 5–6 ml of pulverised mycelial powder was collected per strain in 50 ml falcon tubes. The mycelia were resuspended in 20 ml nuclei isolation buffer (1 M sorbitol, 10 mM pH 7.5 Tris-HCl, 10 mM EDTA), supplemented with 0.15 mM spermine dihydrate, 0.5 mM spermidine, 25 mM PMSF, 1 mM DTT and 1x Roche protease inhibitors immediately before use. Resuspended samples were kept on ice for 5 min. Samples were centrifuged at 1000*g* for 10 min at 4 °C. The supernatant was filtered through two layers of miracloth into SS34 tubes. These filtered samples were then centrifuged at 10,000 RPM for 15 min at 4 °C. The supernatant was removed and pellets were resuspended in 1.5 ml of pre-cooled resuspension buffer (1 M sorbitol, 10 mM pH 7.5 Tris-HCl, 1 mM EDTA), supplemented with 0.15 mM spermine dihydrate, 0.5 mM spermidine, 25 mM PMSF and 1 mM DTT, immediately prior to use. Samples were then transferred to 2 ml microcentrifuge tubes and kept on ice. These samples were then centrifuged at 12,000 RPM for 15 min at 4 °C. The supernatant was removed and the crude nuclei samples were resuspended in 400 µl of ST buffer (1 M sorbitol, 10 mM pH 7.5 Tris-HCl), supplemented with 10 mM PMSF, 0.1 mM DTT and 1× Roche protease inhibitors, immediately prior to use. Samples were centrifuged at 4,800 RPM for 30 s at 4 °C. 50 µl of the supernatant was added to a new microcentrifuge tube and used to determine protein concentration via a Bradford assay. Another 200 µl of the supernatant was added to a separate microcentrifuge tube and 100 µl of 3X SDS loading dye was added, followed by an incubation at 95 °C for 8 min. These samples were then stored at −80 °C until further use.

2.5. Immunoblotting

For all immunoblots, protean membranes (0.45 µm pore size, GE Healthcare) were incubated in blocking solution (5% (w/v) non-fat dry milk solution in 1X TBS with 0.1% Tween 20) for 1 h at room temperature with gentle shaking. For the detection of GFP-tagged proteins, mouse anti-GFP antibody (SC-9996, SantaCruz) was used at 1:1000 dilution in blocking solution for 2 h at room temperature. For HA-tagged proteins, mouse anti-HA antibody (H3663, Sigma) was used at 1:2000 dilution in blocking solution for 2 h at room temperature. Secondary goat anti-mouse (170-6516, Biorad) was used at 1:2000 dilution in blocking solution for 1 h at room temperature. For the detection of Histone 3, rabbit anti-H3 antibody (AB1791, Abcam) was used at 1:4000 dilution in blocking solution for 2 h at room temperature. Goat anti-rabbit (1662408, Biorad) was used as a secondary antibody for H3 detection at 1:2,000 dilution in blocking solution for 1 h at room temperature. After each antibody incubation, membranes were washed three times with 1X TBST (0.1% Tween 20) for 5 min. For visualisation of all membranes, Luminata Crescendo Western HRP Substrate (Millipore) was added and membranes were exposed using the G:BOX Chemi XRQ (Syngene).

2.6. Immunoprecipitation of HA fusion proteins

For the immunoprecipitation of HA fusion proteins, 1 ml protein crude extracts were isolated from vegetative cultures grown for 24 h on a shaker in complete medium, followed by a further 1 h incubation after addition of various stress agents. Per 1 ml protein sample, 10 µl anti-HA magnetic beads (Pierce) were washed twice with 190 µl protein extraction buffer, containing supplements. The anti-HA beads were then resuspended in 50 µl protein extraction buffer and added to 1 ml protein extract. This mixture was left to incubate on a rotator for 3 h at 4 °C. Samples were placed in a magnetic rack and the supernatant was discarded. Beads were washed twice with 1 ml protein extraction buffer (without supplements) and were then washed for a third time with the same buffer containing 1 mM DTT. All liquid was removed and the beads were stored at −80 °C until further use.

2.7. Sample preparation for LC-MS/MS protein identification

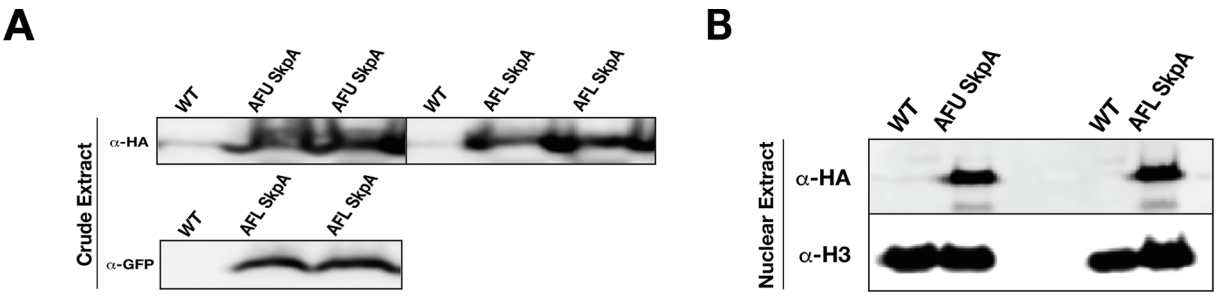
Isolated HA-tagged proteins were resuspended in 50 mM ammonium bicarbonate. 1 µl of 0.5 M DTT was added and samples were incubated at 56 °C for 20 min. 2.7 µl of iodoacetamide (0.55 M) was added and samples were incubated in the dark for 15 min. 1 µl of 1% (w/v) ProteaseMAX (Promega) was added, followed by addition of 1 µl trypsin (1 µg/µl) (Promega). Samples were left to incubate overnight at 37 °C. The next day, 1 µl of Trifluoroacetic acid (TFA) was added to each and samples were vortexed briefly and left to incubate for 5 min at room temperature. Beads were collected on a magnetic rack and the supernatant was transferred to new tubes. The supernatants were centrifuged at 13,000 RCF for 10 min and dried in a SpeedVac for 3 h. Samples were stored at −20 °C until further use.

Peptide samples were resuspended in 20 µl resuspension buffer (0.5% TFA) and sonicated for 3 min, followed by a brief centrifugation. ZipTip C_{18} pipette tips (Millipore) were used to purify peptide samples prior to mass spectrometric analysis. To equilibrate the ZipTips, a wetting solution (0.1%, 80% acetonitrile) was aspirated 5 times, followed by aspiration of an equilibration buffer (0.1% TFA) 5 times. ZipTips were then used to aspirate the peptide samples 15–20 times. Then, the equilibration buffer was aspirated again 5 times, followed by elution of the peptides via aspiration of an elution buffer (0.1% TFA, 60% acetonitrile) 5 times into a new microcentrifuge tube. This solution was dried in a SpeedVac for 2 h and peptide samples were stored at −20 °C.

Immediately prior to loading, peptide samples were resuspended in 15 µl Q-Exactive loading buffer (2% acetonitrile, 0.5% TFA) and 8 µl was added to mass spectrometry vials (VWR). Samples were loaded on a high resolution quantitative LC-MS mass spectrometer (Thermo Fisher Q-Exactive). LC-MS identifications of peptides were performed using the Proteome Discoverer Daemon 1.4 software (Thermo Fisher) and organism-specific taxon-defined protein databases. To act as controls, anti-HA magnetic beads were added to crude protein extracts from wild type strains. These samples were prepared for mass spectrometry analysis as described for the immunoprecipitated HA fusion proteins. Confirmation of protein interactions and unique peptides were determined by isolating only those that appear in the SkpA-HA purifications but do not appear in any of the wild type controls.

2.8. Protein homology searches and generation of interaction networks

Detection of all protein homologs in *A. flavus* and *A. fumigatus* was performed by reciprocal BLAST searches (Altschul et al., 1990). The



C

AN		AFL		AFU	
F-box	ID	F-box	SCF	F-box	SCF
Fbx1	AN6086	AFLA_045780		Afu2g09240	+
Fbx2	AN6816	AFLA_136960	+		
Fbx3	AN6183	AFLA_134460		Afu2g11570	+
Fbx4	AN6552	AFLA_095460		Afu6g04645	
Fbx5	AN7956	AFLA_026350	+	Afu8g00260	
Fbx6	AN9187	AFLA_003770		Afu7g08540	
Fbx7	AN0249	AFLA_029900	+	Afu1g05330	+
Fbx8	AN0353			Afu1g02120	+
Fbx9	AN10061	AFLA_081410	+	Afu1g02050	+
Fbx10	AN0460	AFLA_029080		Afu1g04380	
Fbx11	AN0557	AFLA_108840		Afu6g11400	+
Fbx12	AN2029				
Fbx13	AN2348			Afu4g09270	
Fbx14	AN2353				
Fbx15	AN2505	AFLA_019100		Afu3g14150	
Fbx16	AN2636	AFLA_117270			
Fbx17	AN2861	AFLA_030670		Afu3g11870	
Fbx18	AN4488	AFLA_092310	+	Afu2g03440	+
Fbx19	AN4510	AFLA_092540	+	Afu2g03170	+
Fbx20	AN4535	AFLA_133190		Afu2g02860	
Fbx21	AN5509	AFLA_095650		Afu4g00200	
Fbx22	AN5517	AFLA_033920		Afu6g13030	
Fbx23	AN5593	AFLA_027420	+	Afu4g11440	+
Fbx24	AN6217	AFLA_134910	+	Afu2g12060	+
Fbx25	AN6359	AFLA_138030	+	Afu2g14110	+
Fbx26	AN7086	AFLA_059420		Afu3g01810	
Fbx27	AN9113	AFLA_071170	+	Afu7g02000	+
Fbx28	AN5209	AFLA_087920	+	Afu6g07420	+
Fbx29	AN2933	AFLA_086930	+	Afu3g08170	+
Fbx30	AN9059	AFLA_104080	+	Afu3g04320	
Fbx31	AN0307	AFLA_080800		Afu1g02660	
Fbx32	AN1693	AFLA_111120		Afu4g08560	
Fbx33	AN2106	AFLA_035000	+		
Fbx34	AN2364	AFLA_117140	+	Afu8g06990	
Fbx35	AN2806				
Fbx36	AN3203	AFLA_044660		Afu2g14640	
Fbx37	AN4237	AFLA_127420		Afu1g06160	+

AN		AFL		AFU	
F-box	ID	F-box	SCF	F-box	SCF
Fbx38	AN5075				
Fbx39	AN5161				
Fbx40	AN5568	AFLA_027690	+	Afu4g11700	+
Fbx41	AN5933	AFLA_043900		Afu2g10720	+
Fbx42	AN6777				
Fbx43	AN6999	AFLA_115160	+	Afu4g04510	
Fbx44	AN8008				
Fbx45	AN8098	AFLA_023590		Afu3g00440	
Fbx46	AN8776	AFLA_068320	+		
Fbx47	AN8909	AFLA_094910			
Fbx48	AN10073	AFLA_081270		Afu1g02200	
Fbx49	AN10117	AFLA_017280		Afu1g14050	
Fbx50	AN10516	AFLA_049980	+	Afu1g05970	+
Fbx51	AN8462				
Fbx52	AN2837	AFLA_119030	+		
Fbx53	AN5263				
Fbx54	AN1705				
Fbx55	AN5941				
Fbx56	AN6152				
Fbx57	AN6944			Afu7g01770	
Fbx58	AN8051	AFLA_025290	+	Afu5g02120	+
Fbx59	AN10347	AFLA_030480		Afu3g12050	
Fbx60	AN6887	AFLA_094430	+	Afu5g13280	+
Fbx61	AN7964				
Fbx62	AN8128			Afu3g13590	
Fbx63	AN2540				
Fbx64	AN2808	AFLA_085650	+		
Fbx65	AN3371	AFLA_117680	+	Afu7g01290	+
Fbx66	AN5034				
Fbx67	AN5576	AFLA_027580	+	Afu4g11590	+
Fbx68	AN2183	AFLA_056270	+	Afu6g03800	
Fbx69	AN6634	AFLA_056300		Afu6g03780	+
Fbx70	AN4548	AFLA_133510	+	Afu2g02570	+
Fbx71	AN5714	AFLA_127790	+	Afu1g06762	+
Fbx72	AN4149	AFLA_080510	+		
Fbx73	AN6625	AFLA_056160		Afu6g03900	
Fbx74	AN0703	AFLA_017730	+	Afu1g13690	+

Fig. 1. Comparison of F-box proteins and SCF complexes in *A. nidulans, A. flavus and A. fumigatus.* (A) Detection of GFP and HA-tagged SkpA fusion proteins. Crude protein extracts were isolated from vegetative mycelia cultured in either GMM or Sabouraud liquid medium overnight. 100 μg of protein was loaded on 15% SDS gels. AFU (*Aspergillus fumigatus*), AFL (*Aspergillus flavus*), WT (wild type). (B) Detection of HA-tagged SkpA fusion proteins in nuclear protein extracts. Histone 3 is used as a loading control. 10–15 μg of protein was loaded on 15% SDS gels. (C) F-box proteins 1–74 in *Aspergillus nidulans* (AN) and their respective gene IDs are listed. Homologous F-box proteins in *A. flavus* (AFL) and *A. fumigatus* (AFU) were identified by reciprocal BLAST searches and their respective gene IDs are listed. Yellow boxes containing a '+' symbol represent F-box proteins that were identified in SkpA-HA pulldowns after 24 h of growth in liquid complete medium, indicating that they may exist as part of SCF (SkpA-Cullin-F-box) complexes.

SkpA interaction networks were generated by using the Gephi 0.9.2 software.

3. Results

3.1. The SkpA protein in A. flavus and A. fumigatus recruits various F-box proteins to form SCF E3 ligase complexes

In order to assess whether SkpA in both *A. flavus* (AFLA_048530) and *A. fumigatus* (Afu5g06060) acts as an adaptor protein in SCF complexes, SkpA fusion proteins were generated. The C-terminal ends of both *skpA* genes were fused to *sgfp* and *3xha* epitope tags (Fig. S1). However, all attempts to tag the *A. fumigatus skpA* gene with the *sgfp* epitope tag proved to be unsuccessful, which was validated by both southern blotting and western blotting. To confirm that the remaining fusion proteins were successfully tagged, western blotting was performed (Fig. 1A, Fig. S2), using antibodies specific for both epitope tags. To determine whether these proteins are localised to the nucleus, nuclear protein extracts were isolated from the HA-tagged samples. Western blotting confirmed that the SkpA protein in both *A. flavus* and *A. fumigatus* is localised either in the nucleus or at the nuclear envelope (Fig. 1B, Fig. S2).

To determine whether *A. flavus* and *A. fumigatus* possess F-box proteins that are orthologous to those detected in *A. nidulans* (de Assis et al., 2018; Galagan et al., 2005), reciprocal BLAST searches were performed (Altschul et al., 1990). *A. nidulans* possesses 74 F-box proteins that enable the detection, binding and degradation of a range of substrates (de Assis et al., 2018). BLAST analysis revealed the existence of orthologous *fbx* genes in both *A. flavus* and *A. fumigatus* (Fig. 1C). In *A. fumigatus,* orthologs of 51 *fbx* genes were shown to exist (Table S4), while *A. flavus* was shown to possess 55 orthologous *fbx* genes (Table S5). Of these predicted orthologs, 47 are common to both *A. flavus* and *A. fumigatus.* The 4 *fbx* genes that were shown to exist in *A. fumigatus* but not *A. flavus* were orthologs of *fbx8, fbx13, fbx57* and *fbx62.* The 8

fbx genes that exist in *A. flavus* but not *A. fumigatus* are orthologs of *fbx2, fbx16, fbx33, fbx46, fbx47, fbx52, fbx64* and *fbx72*.

The presence of both *fbx* genes and SkpA adaptor proteins in *A. flavus* and *A. fumigatus* led to the proposal that SCF complexes may be assembled in these organisms to facilitate substrate recognition and protein degradation. To detect the F-box interaction partners of SkpA in both species, SkpA-HA proteins were immunoprecipitated from strains that were cultured vegetatively in liquid complete medium for 24 h. Mass spectrometry analysis was performed on these tagged samples and the F-box proteins found to be interacting with SkpA are tabulated in Fig. 1C. Of the 51 predicted F-box orthologs in *A. fumigatus,* 26 were shown to interact with SkpA (Table S6). For *A. flavus,* 30 of the 55 predicted F-box orthologs were detected in SkpA-HA purifications (Table S13). 19 of these detected F-box proteins are common interaction partners for both *A. flavus* and *A. fumigatus* SkpA. F-box interaction partners that were shown to be unique in *A. flavus* were Fbx2, Fbx5, Fbx30, Fbx33, Fbx34, Fbx43, Fbx46, Fbx52, Fbx64, Fbx68 and Fbx72. F-box interactions that were shown to be unique in *A. fumigatus* were Fbx1, Fbx3, Fbx8, Fbx11, Fbx37, Fbx41 and Fbx69. These results provide evidence that roughly half of the predicted F-box proteins in both *A. flavus* and *A. fumigatus* interact with SkpA during vegetative growth in complete liquid medium. This suggests that a wide range of SCF complexes could be assembled in these two species to regulate protein degradation. However, it can also be noted that additional F-box proteins may interact with SkpA under other growth conditions and developmental programmes.

3.2. The F-box interaction partners of A. fumigatus SkpA vary in the presence of different stress agents

In order to understand the composition of SCF complexes in response to various cellular stresses, the *A. fumigatus* SkpA-HA strain was initially cultured vegetatively for 24 h. Multiple exogenous stress agents were then added and the strain was cultured for an additional 1 h to determine the F-box proteins that are recruited to SCF complexes in response to each respective stressor. The SkpA-HA fusion protein samples were immunoprecipitated and analysed via mass spectrometry. An interaction network of the SkpA-HA fusion protein in the presence of each stress agent is depicted in Fig. 2A, while the F-box proteins detected in each SkpA-HA purification are tabulated in Fig. 2B.

As highlighted in Fig. 1C, A. *fumigatus* SkpA was shown to interact with 26 F-box proteins during vegetative growth in liquid complete medium. In the presence of the osmotic stress agent NaCl, 19 F-box proteins were found to be interacting with SkpA (Table S7). 31 F-box proteins were detected in SkpA-HA immunoprecipitations when the antifungal agent Amphotericin B was added (Table S8). Upon addition of the cell wall stressor Congo red, 26 F-box proteins were interacting with SkpA (Table S9). In the presence of the oxidative stress agent H_2O_2, 28 F-box proteins were detected (Table S10). Lastly, in the presence of the antifungal agent Miconazole, 24 F-box proteins were shown to be interacting with SkpA (Table S11).

By compiling the interactome data for each SkpA-HA purification (Table S12), an interaction network for SkpA in the presence of all stressors tested was generated (Fig. 2A). It was observed that there is significant overlap of interacting proteins with SkpA across all conditions tested. SkpA was found to interact with the cullin scaffold CulA during all conditions (Tables S6–S11), providing further evidence that SCF complexes are formed in *A. fumigatus*. With regards to F-box protein interactions, 19 were shown to interact with SkpA during all culture conditions. Certain F-box proteins were found to be unique to one condition. Two examples of unique F-box proteins were detected in purifications of SkpA cultured in the presence of Amphotericin B, which were Fbx20 and Fbx45 (Table S8). There were many F-box proteins interacting with SkpA that were detected in more than one condition, but not in all. Fbx1 was found to be interacting with SkpA during unstressed vegetative growth and also in the presence of Amphotericin B and H_2O_2. Fbx8 was interacting with SkpA during vegetative growth, as well as in the presence of Amphotericin B, H_2O_2 and Miconazole. Fbx11 and Fbx50 were detected in all SkpA purifications, with the exception of the cultures treated with NaCl. Fbx22 was detected in purifications of SkpA in the presence of Amphotericin B, Congo red and H_2O_2. Fbx24 and Fbx41 were detected in all conditions, aside from NaCl and Miconazole-treated cultures. Fbx37 was only found in SkpA purifications during unstressed vegetative growth and in the presence of Amphotericin B. Fbx43 and Fbx68 were observed to interact with SkpA during all stress conditions, aside from NaCl-treated samples and these proteins were also not detectable during unstressed vegetative growth.

These interactome data suggest that in the presence of various stress conditions, the composition of SCF complexes are variable and are readily altered. While there is significant overlap of the F-box proteins interacting with SkpA between different culture conditions, unique F-box proteins may be recruited to SCF complexes in response to specific stimuli.

3.3. SCF complexes in A. flavus are also variable in the presence of different exogenous stressors

To determine the composition of SCF complexes in *A. flavus* in the presence of various stress agents, the *A. flavus* SkpA-HA strain was cultured according to the steps described for *A. fumigatus* SkpA-HA and the same concentrations of each stressor were added. The F-box interaction partners detected in each SkpA-HA purification were compiled to form an interaction network which is depicted in Fig. 3A. The tabulated lists of F-box proteins detected in each SkpA purification are presented in Fig. 3B. *A. flavus* SkpA was shown to interact with 30 F-box proteins during unstressed vegetative growth (Fig. 1C). Upon incubation in the presence of NaCl, 29 F-box proteins were found to interact with SkpA (Table S14). After addition of Amphotericin B, 35 F-box proteins were detected in SkpA-HA purifications (Table S15). In the presence of Congo red, 31 F-box proteins were detected (Table S16). Upon addition of H_2O_2, 28 F-box proteins were present (Table S17) and lastly, incubation in the presence of Miconazole results in the detection of 31 F-box proteins (Table S18).

These interactome data from each SkpA purification were collated (Table S19) to construct an interaction network for SkpA (Fig. 3A) and to compare the trends of F-box interactions between different culture conditions. Like in *A. fumigatus,* SkpA interacted with many common proteins across all conditions tested. In each culture condition, SkpA interacted with CulA (Tables S13–S18). 28 F-box proteins were found to be interacting with SkpA in each culture condition tested. There were certain F-box proteins that were unique to one condition. Fbx1, Fbx11 and Fbx45 were found to interact with SkpA only when *A. flavus* was cultured in the presence of Amphotericin B (Table S15). Some F-box proteins were detected in more than one culture condition, but not all. For example, Fbx9 was only found to interact with SkpA during unstressed vegetative growth and in the presence of NaCl. Fbx22, Fbx37 and Fbx69 were detected in SkpA purifications from cultures treated with Amphotericin B, Congo red and Miconazole. Fbx46 was present in SkpA purifications during unstressed vegetative growth and in the presence of Amphotericin B.

Overall, these data suggest that SCF complexes are assembled in *A. flavus* and that the composition of these complexes can vary in response to various culture conditions. Like in *A. fumigatus,* a high proportion of F-box proteins are found to interact with SkpA during all conditions tested. However, certain F-box proteins may be unique to one particular stress response.

4. Discussion

Aspergillus species are considered to be both friends and foe of mankind, in most part due to their ability to produce secondary metabolites (SMs) that can positively and negatively influence human and

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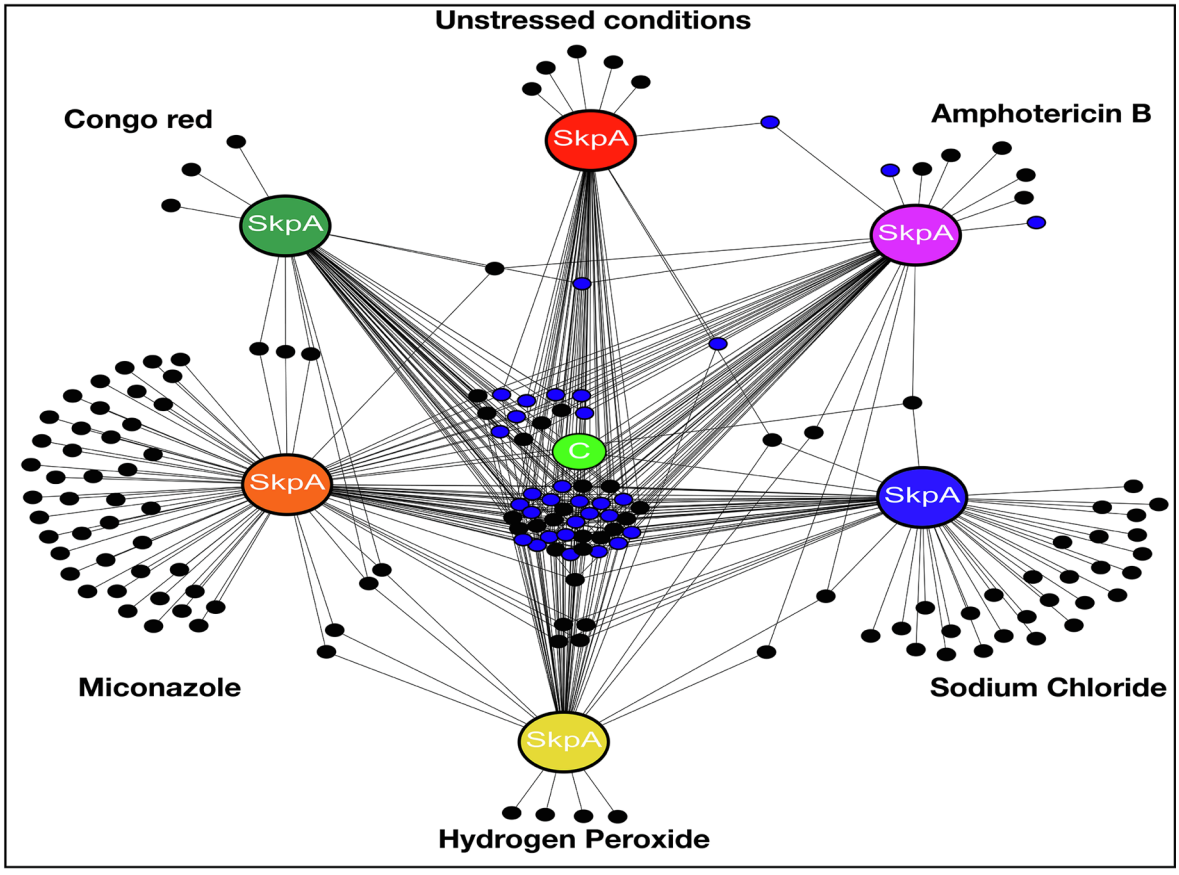

B

SkpA-HA Unstressed	
AN F-box	AFU F-box
Fbx1	Afu2g09240
Fbx3	Afu2g11570
Fbx7	Afu1g05330
Fbx8	Afu1g02120
Fbx9	Afu1g02050
Fbx11	Afu6g11400
Fbx18	Afu2g03440
Fbx19	Afu2g03170
Fbx23	Afu4g11440
Fbx24	Afu2g12060
Fbx25	Afu2g14110
Fbx27	Afu7g02000
Fbx28	Afu6g07420
Fbx29	Afu3g08170
Fbx37	Afu1g06160
Fbx40	Afu4g11700
Fbx41	Afu2g10720
Fbx50	Afu1g05970
Fbx58	Afu5g02120
Fbx60	Afu5g13280
Fbx65	Afu7g01290
Fbx67	Afu4g11590
Fbx69	Afu6g03780
Fbx70	Afu2g02570
Fbx71	Afu1g06762
Fbx74	Afu1g13690

SkpA-HA NaCl	
AN F-box	AFU F-box
Fbx3	Afu2g11570
Fbx7	Afu1g05330
Fbx9	Afu1g02050
Fbx18	Afu2g03440
Fbx19	Afu2g03170
Fbx23	Afu4g11440
Fbx25	Afu2g14110
Fbx27	Afu7g02000
Fbx28	Afu6g07420
Fbx29	Afu3g08170
Fbx40	Afu4g11700
Fbx58	Afu5g02120
Fbx60	Afu5g13280
Fbx65	Afu7g01290
Fbx67	Afu4g11590
Fbx69	Afu6g03780
Fbx70	Afu2g02570
Fbx71	Afu1g06762
Fbx74	Afu1g13690

SkpA-HA Amphotericin B	
AN F-box	AFU F-box
Fbx1	Afu2g09240
Fbx3	Afu2g11570
Fbx7	Afu1g05330
Fbx8	Afu1g02120
Fbx9	Afu1g02050
Fbx11	Afu6g11400
Fbx18	Afu2g03440
Fbx19	Afu2g03170
Fbx20	Afu2g02860
Fbx22	Afu6g13030
Fbx23	Afu4g11440
Fbx24	Afu2g12060
Fbx25	Afu2g14110
Fbx27	Afu7g02000
Fbx28	Afu6g07420
Fbx29	Afu3g08170
Fbx37	Afu1g06160
Fbx40	Afu4g11700
Fbx41	Afu2g10720
Fbx43	Afu4g04510
Fbx45	Afu3g00440
Fbx50	Afu1g05970
Fbx58	Afu5g02120
Fbx60	Afu5g13280
Fbx65	Afu7g01290
Fbx67	Afu4g11590
Fbx68	Afu6g03800
Fbx69	Afu6g03780
Fbx70	Afu2g02570
Fbx71	Afu1g06762
Fbx74	Afu1g13690

SkpA-HA Congo Red	
AN F-box	AFU F-box
Fbx3	Afu2g11570
Fbx7	Afu1g05330
Fbx9	Afu1g02050
Fbx11	Afu6g11400
Fbx18	Afu2g03440
Fbx19	Afu2g03170
Fbx22	Afu6g13030
Fbx23	Afu4g11440
Fbx24	Afu2g12060
Fbx25	Afu2g14110
Fbx27	Afu7g02000
Fbx28	Afu6g07420
Fbx29	Afu3g08170
Fbx40	Afu4g11700
Fbx41	Afu2g10720
Fbx43	Afu4g04510
Fbx50	Afu1g05970
Fbx58	Afu5g02120
Fbx60	Afu5g13280
Fbx65	Afu7g01290
Fbx67	Afu4g11590
Fbx68	Afu6g03800
Fbx69	Afu6g03780
Fbx70	Afu2g02570
Fbx71	Afu1g06762
Fbx74	Afu1g13690

SkpA-HA H_2O_2	
AN F-box	AFU F-box
Fbx1	Afu2g09240
Fbx3	Afu2g11570
Fbx7	Afu1g05330
Fbx8	Afu1g02120
Fbx9	Afu1g02050
Fbx11	Afu6g11400
Fbx18	Afu2g03440
Fbx19	Afu2g03170
Fbx22	Afu6g13030
Fbx23	Afu4g11440
Fbx24	Afu2g12060
Fbx25	Afu2g14110
Fbx27	Afu7g02000
Fbx28	Afu6g07420
Fbx29	Afu3g08170
Fbx40	Afu4g11700
Fbx41	Afu2g10720
Fbx43	Afu4g04510
Fbx50	Afu1g05970
Fbx58	Afu5g02120
Fbx60	Afu5g13280
Fbx65	Afu7g01290
Fbx67	Afu4g11590
Fbx68	Afu6g03800
Fbx69	Afu6g03780
Fbx70	Afu2g02570
Fbx71	Afu1g06762
Fbx74	Afu1g13690

SkpA-HA Miconazole	
AN F-box	AFU F-box
Fbx3	Afu2g11570
Fbx7	Afu1g05330
Fbx8	Afu1g02120
Fbx9	Afu1g02050
Fbx11	Afu6g11400
Fbx18	Afu2g03440
Fbx19	Afu2g03170
Fbx23	Afu4g11440
Fbx25	Afu2g14110
Fbx27	Afu7g02000
Fbx28	Afu6g07420
Fbx29	Afu3g08170
Fbx40	Afu4g11700
Fbx43	Afu4g04510
Fbx50	Afu1g05970
Fbx58	Afu5g02120
Fbx60	Afu5g13280
Fbx65	Afu7g01290
Fbx67	Afu4g11590
Fbx68	Afu6g03800
Fbx69	Afu6g03780
Fbx70	Afu2g02570
Fbx71	Afu1g06762
Fbx74	Afu1g13690

Fig. 2. The F-box interaction partners of *A. fumigatus* SkpA in the presence of various exogenous stressors. (A) Interaction network of SkpA in *A. fumigatus* based on the unique peptides detected in each HA pulldown. The HA-tagged strain was cultured vegetatively for 24 h in complete medium, followed by a 1 h incubation after addition of various stress agents. 'Unstressed conditions' refers to complete medium without any stress agents added. The following stress agents were used: NaCl (0.5 M), Amphotericin B (1 μg/ml), Congo red (20 μg/ml), H_2O_2 (5 mM) and Miconazole (4 μg/ml). The interaction network was generated using the Gephi 0.9.2 software. Each black dot represents a protein detected in at least 1 of 2 independent biological replicates but not in any of the wild type samples. Each blue dot represents an F-box protein. 'C' (CulA). (B) The F-box proteins detected in SkpA-HA pulldowns in each culture condition. The *A. fumigatus* (AFU) F-box proteins and the respective *A. nidulans* (AN) homologs are listed. F-box protein names highlighted in blue represent those that were found to be interacting with SkpA in all culture conditions. F-box proteins in red indicate those that are unique to one culture condition. Proteins in black represent those that are detected in one or more condition but not all.

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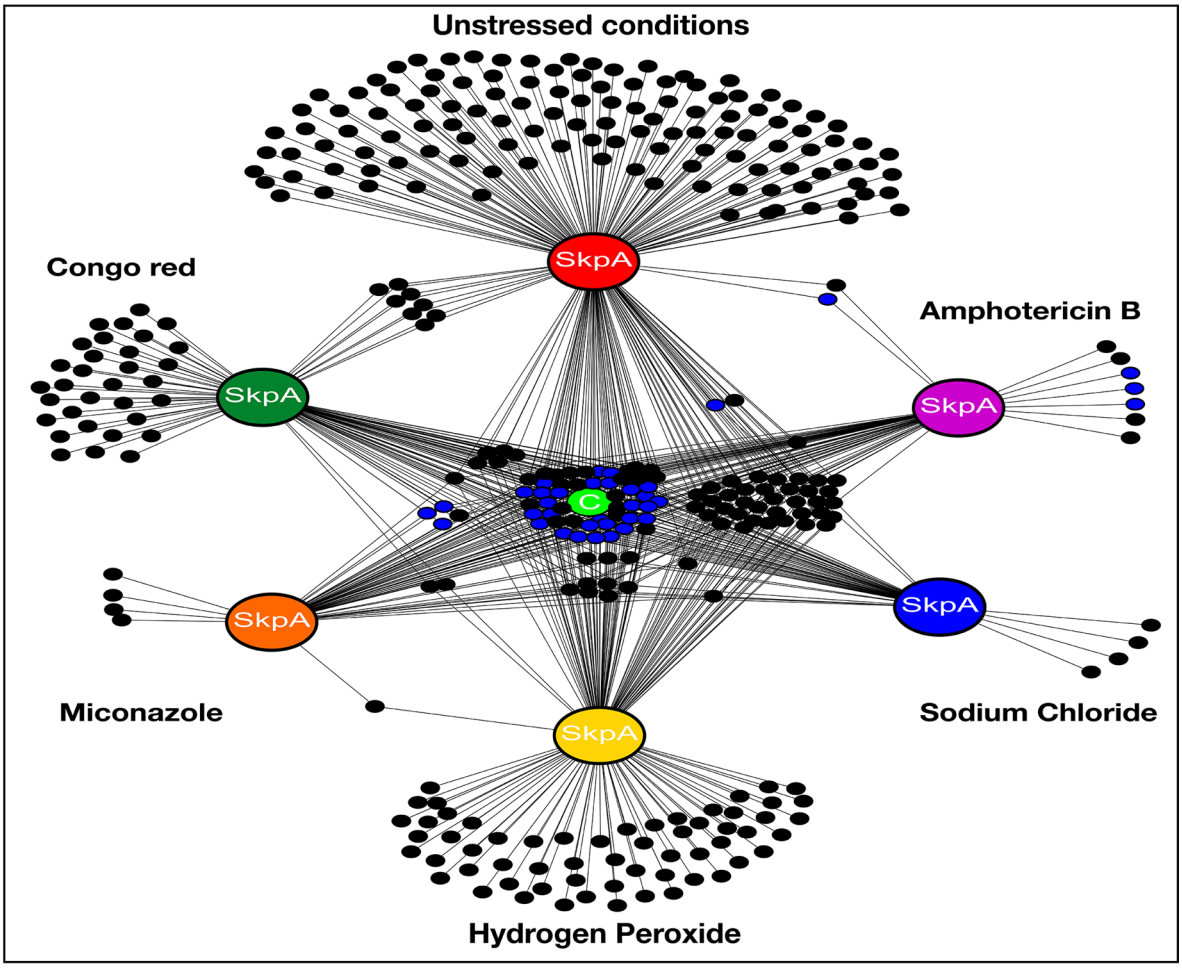

B

SkpA-HA Unstressed	
AN F-box	AFL F-box
Fbx2	AFLA_136960
Fbx5	AFLA_026350
Fbx7	AFLA_029900
Fbx9	AFLA_081410
Fbx18	AFLA_092310
Fbx19	AFLA_092540
Fbx23	AFLA_027420
Fbx24	AFLA_134910
Fbx25	AFLA_138030
Fbx27	AFLA_071170
Fbx28	AFLA_087920
Fbx29	AFLA_086930
Fbx30	AFLA_104080
Fbx33	AFLA_035000
Fbx34	AFLA_117140
Fbx40	AFLA_027690
Fbx43	AFLA_115160
Fbx46	AFLA_068320
Fbx50	AFLA_049980
Fbx52	AFLA_119030
Fbx58	AFLA_025290
Fbx60	AFLA_094430
Fbx64	AFLA_085650
Fbx65	AFLA_117680
Fbx67	AFLA_027580
Fbx68	AFLA_056270
Fbx70	AFLA_133510
Fbx71	AFLA_127790
Fbx72	AFLA_080510
Fbx74	AFLA_017730

SkpA-HA NaCl	
AN F-box	AFL F-box
Fbx2	AFLA_136960
Fbx5	AFLA_026350
Fbx7	AFLA_029900
Fbx9	AFLA_081410
Fbx18	AFLA_092310
Fbx19	AFLA_092540
Fbx23	AFLA_027420
Fbx24	AFLA_134910
Fbx25	AFLA_138030
Fbx27	AFLA_071170
Fbx28	AFLA_087920
Fbx29	AFLA_086930
Fbx30	AFLA_104080
Fbx33	AFLA_035000
Fbx34	AFLA_117140
Fbx40	AFLA_027690
Fbx43	AFLA_115160
Fbx50	AFLA_049980
Fbx52	AFLA_119030
Fbx58	AFLA_025290
Fbx60	AFLA_094430
Fbx64	AFLA_085650
Fbx65	AFLA_117680
Fbx67	AFLA_027580
Fbx68	AFLA_056270
Fbx70	AFLA_133510
Fbx71	AFLA_127790
Fbx72	AFLA_080510
Fbx74	AFLA_017730

SkpA-HA Amphotericin B	
AN F-box	AFL F-box
Fbx1	AFLA_045780
Fbx2	AFLA_136960
Fbx5	AFLA_026350
Fbx7	AFLA_029900
Fbx11	AFLA_108840
Fbx18	AFLA_092310
Fbx19	AFLA_092540
Fbx22	AFLA_033920
Fbx23	AFLA_027420
Fbx24	AFLA_134910
Fbx25	AFLA_138030
Fbx27	AFLA_071170
Fbx28	AFLA_087920
Fbx29	AFLA_086930
Fbx30	AFLA_104080
Fbx33	AFLA_035000
Fbx34	AFLA_117140
Fbx37	AFLA_127420
Fbx40	AFLA_027690
Fbx43	AFLA_115160
Fbx45	AFLA_023590
Fbx46	AFLA_068320
Fbx50	AFLA_049980
Fbx52	AFLA_119030
Fbx58	AFLA_025290
Fbx60	AFLA_094430
Fbx64	AFLA_085650
Fbx65	AFLA_117680
Fbx67	AFLA_027580
Fbx68	AFLA_056270
Fbx69	AFLA_056300
Fbx70	AFLA_133510
Fbx71	AFLA_127790
Fbx72	AFLA_080510
Fbx74	AFLA_017730

SkpA-HA Congo Red	
AN F-box	AFL F-box
Fbx2	AFLA_136960
Fbx5	AFLA_026350
Fbx7	AFLA_029900
Fbx18	AFLA_092310
Fbx19	AFLA_092540
Fbx22	AFLA_033920
Fbx23	AFLA_027420
Fbx24	AFLA_134910
Fbx25	AFLA_138030
Fbx27	AFLA_071170
Fbx28	AFLA_087920
Fbx29	AFLA_086930
Fbx30	AFLA_104080
Fbx33	AFLA_035000
Fbx34	AFLA_117140
Fbx37	AFLA_127420
Fbx40	AFLA_027690
Fbx43	AFLA_115160
Fbx50	AFLA_049980
Fbx52	AFLA_119030
Fbx58	AFLA_025290
Fbx60	AFLA_094430
Fbx64	AFLA_085650
Fbx65	AFLA_117680
Fbx67	AFLA_027580
Fbx68	AFLA_056270
Fbx69	AFLA_056300
Fbx70	AFLA_133510
Fbx71	AFLA_127790
Fbx72	AFLA_080510
Fbx74	AFLA_017730

SkpA-HA H_2O_2	
AN F-box	AFL F-box
Fbx2	AFLA_136960
Fbx5	AFLA_026350
Fbx7	AFLA_029900
Fbx18	AFLA_092310
Fbx19	AFLA_092540
Fbx23	AFLA_027420
Fbx24	AFLA_134910
Fbx25	AFLA_138030
Fbx27	AFLA_071170
Fbx28	AFLA_087920
Fbx29	AFLA_086930
Fbx30	AFLA_104080
Fbx33	AFLA_035000
Fbx34	AFLA_117140
Fbx40	AFLA_027690
Fbx43	AFLA_115160
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Fbx60	AFLA_094430
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Fbx65	AFLA_117680
Fbx67	AFLA_027580
Fbx68	AFLA_056270
Fbx70	AFLA_133510
Fbx71	AFLA_127790
Fbx72	AFLA_080510
Fbx74	AFLA_017730

SkpA-HA Miconazole	
AN F-box	AFL F-box
Fbx2	AFLA_136960
Fbx5	AFLA_026350
Fbx7	AFLA_029900
Fbx18	AFLA_092310
Fbx19	AFLA_092540
Fbx22	AFLA_033920
Fbx23	AFLA_027420
Fbx24	AFLA_134910
Fbx25	AFLA_138030
Fbx27	AFLA_071170
Fbx28	AFLA_087920
Fbx29	AFLA_086930
Fbx30	AFLA_104080
Fbx33	AFLA_035000
Fbx34	AFLA_117140
Fbx37	AFLA_127420
Fbx40	AFLA_027690
Fbx43	AFLA_115160
Fbx50	AFLA_049980
Fbx52	AFLA_119030
Fbx58	AFLA_025290
Fbx60	AFLA_094430
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Fbx65	AFLA_117680
Fbx67	AFLA_027580
Fbx68	AFLA_056270
Fbx69	AFLA_056300
Fbx70	AFLA_133510
Fbx71	AFLA_127790
Fbx72	AFLA_080510
Fbx74	AFLA_017730

Fig. 3. The F-box interaction partners of *A. flavus* SkpA in the presence of various exogenous stressors (A) Interaction network of SkpA in *A. flavus* based on the unique peptides detected in each HA pulldown. Culturing conditions and stress agents used are as described for Fig. 2. Each black dot represents a protein detected in at least 1 of 2 independent biological replicates but not in any of the wild type samples. Each blue dot represents an F-box protein. 'C' (CulA). (B) The F-box proteins detected in SkpA-HA pulldowns in each culture condition. The *A. flavus* (AFL) F-box proteins and the respective *A. nidulans* (AN) homologs are listed. F-box proteins in blue represent those that are detected in all culture conditions. F-box proteins in red indicate those that are unique to one culture condition. Proteins in black represent those that are detected in one or more culture condition but not all.

plant health. *A. fumigatus* is an opportunistic human pathogen and is a major threat to individuals with impaired immune systems. It produces the immunosuppressive SM gliotoxin, which promotes invasive pulmonary aspergillosis (Hof and Kupfahl, 2009; Latge, 1999; Maschmeyer et al., 2007; van de Veerdonk et al., 2017). *A. flavus* is a saprophytic fungus that has the potential to be a major threat to mankind as it is capable of producing dangerous SMs such as the carcinogen aflatoxin B1. This can lead to contamination of various crops and development of hepatocellular carcinomas in humans (Amare and Keller, 2014; Hedayati et al., 2007; Kew, 2013). In order to regulate both development and secondary metabolism, a complex array of signalling pathways are utilised by *Aspergillus* species (Bayram et al., 2012; Bayram et al., 2008; Elramli et al., 2019). However, to ensure these pathways are finely regulated and to maintain cell homeostasis, mechanisms must be employed to promote protein synthesis and degradation. In eukaryotes, the UPS is critical for regulating the life span and turnover of thousands of proteins (Hershko and Ciechanover, 1998). The majority of short-lived proteins are substrates for the CRL1/SCF complex, such as signalling molecules, transcription factors and cell cycle regulators (Sun, 2020).

The SCF complex is required for the regulation of a wide range of biological processes in eukaryotes, ranging from cell senescence to virulence in plant and human hosts (Kato et al., 2010; Masso-Silva et al., 2018; Miguel-Rojas and Hera, 2016). This diversity is made possible due to the presence of a large array of interchangeable F-box proteins which act as substrate-recognising receptors in SCF complexes (Yen and Elledge, 2008; Zheng et al., 2002). Despite these F-box proteins exhibiting high conservation in eukaryotes, little is known regarding the biological roles of F-box proteins and the composition of SCF complexes, specifically in organisms like fungi. In filamentous fungi, such as the genus *Aspergillus,* the influence of these F-box proteins with respect to the regulation of fungal growth, development, secondary metabolism and virulence is poorly understood. However, in the model organism *A. nidulans,* 74 *fbx* genes exist (de Assis et al., 2018; Galagan et al., 2005) and the majority of these respective proteins have unknown functions. Thus, it is possible that *A. nidulans* is capable of assembling a large repertoire of SCF complexes to mark a diverse array of substrates for degradation. Identification of F-box proteins in *A. nidulans* led to the proposal for this study which was to identify orthologous *fbx* genes in both *A. fumigatus* and *A. flavus*, using *A. nidulans* as a reference. Another aim of this work was to detect the presence and composition of SCF complexes in both species in response to various exogenous stresses.

Studies on F-box proteins in *A. fumigatus* are limited but it has been shown that Fbx15 is associated with nuclear SCF complexes and is required for positive regulation of pathogenicity and the oxidative stress response (Johnk et al., 2016). There have been no studies on F-box proteins in *A. flavus* and it is not currently known how many *fbx* encoding genes are present within the *A. flavus* genome. In this study, we have identified *fbx* genes in both *A. fumigatus* and *A. flavus* that are orthologous to those that exist in *A. nidulans* (Fig. 1C), suggesting that these genes are highly conserved in *Aspergillus* species. 51 *fbx* genes were detected in *A. fumigatus* (Table S4), while 55 were detected in *A. flavus* (Table S5). *A. fumigatus* lacks orthologs of 23 *fbx* genes (*fbx2, fbx12, fbx14, fbx16, fbx33, fbx35, fbx38, fbx39, fbx42, fbx44, fbx46, fbx47, fbx51-56, fbx61, fbx63, fbx64, fbx66, fbx72*). *A. flavus* lacks orthologs of 19 *fbx* genes (*fbx8, fbx12, fbx13, fbx14, fbx35, fbx38, fbx39, fbx42, fbx44, fbx51, fbx53-57, fbx61-63* and *fbx66*). A comparison between the two species reveals that 15 of these *fbx* genes are absent from the genomes of both species and 47 *fbx* genes are present in both organisms. *A. fumigatus* possesses 4 *fbx* genes that do not exist in *A. flavus* (*fbx8, fbx13, fbx57* and *fbx62*), while *A. flavus* possesses 8 *fbx* genes that do not exist in *A. fumigatus* (*fbx2, fbx16, fbx33, fbx46, fbx47 fbx52, fbx64* and *fbx72*). By performing SkpA-HA immunoprecipitations, the F-box interaction partners in both species were detected. It was found that during vegetative growth in liquid complete medium, *A. fumigatus* SkpA interacts with 26 F-box proteins (Table S6), while *A. flavus* SkpA interacts with 30 F-box proteins (Table S13). 19 of these F-box proteins, as well as the CulA scaffold are common interaction partners in both species. These data suggest that a diverse array of conserved SCF complexes are formed in *Aspergillus* species and also that unique SCF complex conformations may exist in specific species. It is also possible that separate SCF complexes could be formed under other growth conditions or during specific developmental stages of these fungi.

To determine whether the composition of SCF complexes are altered in response to various stress conditions, the SkpA fusion protein was immunoprecipitated from strains cultured in the presence of a myriad of exogenous stressors (Figs. 2 and 3). In *A. fumigatus,* the number of F-box proteins found to interact with SkpA in the presence of NaCl, Amphotericin B, Congo red, H_2O_2 and miconazole were 19, 31, 26, 28 and 24 respectively (Tables S7–S12). 19 of these proteins, as well as CulA were found to be interacting with SkpA in all conditions tested, including during unstressed vegetative growth. For *A. flavus,* the respective numbers were 29, 35, 31, 28 and 31 (Tables S14–S19) and 28 of these F-box proteins, as well as CulA were found to be interacting with SkpA in all conditions, including during unstressed vegetative growth. This provides evidence that a large repertoire of SCF complexes are active in these species regardless of the presence of an exogenous stress.

By comparing the interactome data from the two species (Figs. 2B and 3B, Tables S6–S19), it is evident that in the presence of NaCl, there are no unique F-box interactions detected in either species. *A. flavus* SkpA recruited considerably more F-box proteins (29) in comparison to *A. fumigatus* SkpA (19) and 17 of these F-box proteins were common to both data sets. In the presence of congo red, H_2O_2 and Miconazole, the number of common F-box proteins found to interact with both *A. flavus* and *A. fumigatus* SkpA were 22, 20 and 20, respectively. In the presence of Amphotericin B, 26 F-box proteins were found to be interacting with SkpA in both species. Interestingly, in *A. fumigatus,* two unique F-box proteins (Fbx20 and Fbx45) were detected, while in *A. flavus,* three unique F-box proteins (Fbx1, Fbx11 and Fbx45) were detected, suggesting that unique SCF complexes may be assembled in response to this antifungal drug and that Fbx45 may be essential for regulating responses to this stress in both species. To date, there have been no studies on the biological roles of Fbx45 in *A. nidulans* and BLAST analysis reveals that orthologs of this protein are mostly conserved within *Aspergillus* species and do not exist in species such as yeast, *N. crassa* or humans.

Overall, this work highlights the identification of *fbx* encoding genes in two pathogenic *Aspergillus* species and provides evidence that a wide variety of SCF complexes are assembled in responses to various stress conditions. The existence of such a large repertoire of SCF complexes in these species could have implications with regards to the regulation of a wide variety of biological processes, such as fungal growth, development, secondary metabolism and virulence. These data also highlight the identification of a novel F-box protein Fbx45, which is highly conserved in the *Aspergilli.* This protein is recruited to SCF complexes via interactions with SkpA in both *A. flavus* and *A. fumigatus* and is potentially involved in the cellular response to antifungal agents like Amphotericin B. Future studies will help elucidate the importance of these F-box proteins with respect to fungal development, SM production and pathogenicity. This could lead to the potential use of these F-box proteins as anti-fungal targets to help reduce the incidence of fungal infections and crop contamination.

CRediT authorship contribution statement

Dean Frawley: Conceptualization, Methodology, Investigation, Validation, Visualization, Formal analysis, Writing - original draft, Writing - review & editing. **Özgür Bayram:** Conceptualization, Methodology, Project administration, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

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