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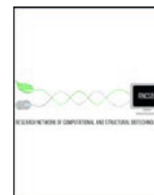

Review

SUMOylation in fungi: A potential target for intervention

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ABSTRACT

SUMOylation is a post-translational, reversible modification process which occurs in eukaryotes. Small Ubiquitin like MOdifier or (SUMO) proteins are a family of small proteins that are covalently attached to and detached from other proteins to modify the target protein function. In pathogenic fungi, SUMO has been identified and preliminary studies indicate its importance either for survival and/or for virulence. In this review we provide an overview of the current state of knowledge of SUMOylation in fungi and the effects on pathogenesis. Subsequently we identify the orthologs of the SUMOylation pathway components across fungi. We also show the level of conservation of the proteins involved and identify the similarities/differences in the orthologs across fungi and the human and plant hosts to identify potential targets of intervention.

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1. Introduction

Fungi are a major eukaryotic kingdom with several hundred thousand species. They are an important part of the biosphere due to their primary role in degrading organic matter. Many fungi live in either symbiotic or commensal relationship with plant and animal hosts. In humans, many yeasts like Candida species are a part of the normal microbiota of the mouth, gastrointestinal and vaginal tracts. In healthy hosts, it is relatively non-pathogenic. However, a few species of fungi are harmful and cause disease in both animals and plants.

Over the past few decades, incidence of serious fungal infections has been increasing. While most fungal infections are superficial, some mucosal infections (Candida) and lung infections (Aspergillus), are also becoming common, particularly in patients with tuberculosis. Invasive infections like candidiasis, which are often hospital acquired, are a major growing threat. They cause death in 30% to 90% of the cases accounting for about 1.5 million deaths per year. Similarly, a large amount of staple crops like rice, wheat, corn and soyabean succumb to fungal attacks and in some cases the loss can be over 70% [1].

Fungal diseases in plants and animals are treated with antifungals that are fairly specific and can protect the individual from disease [2]. However, overuse of these antifungals, especially azoles in crops, has led to the emergence of resistance to these drugs [3]. Although not much appreciated, resistance to antifungal drugs can be life threatening for human as well as for other species [4]. Fungi are under-recognized as pathogens and the research is much less funded than other diseases that cause similar death tolls like malaria [5,6]. Therefore, new antifungals have not been introduced for many years. It is important to discover new targets that can be used for developing antifungals. Given that fungi are eukaryotes, the targets are relatively limited. A successful target should be a) present in the pathogen b) should be critical for pathogenesis c) should be accessible for drugging d) should not be present in the host or should be sufficiently diverged from the pathogen target. This is particularly difficult to find for eukaryotic pathogens as their basic cellular machinery is very similar to animals and plants.

In this context, drugs targeting the post-translational modifications have not been explored much and could be highly effective targets. One such post-translational modification mechanism is SUMOylation, which appears to be unique to eukaryotes. In this review we look at the potential of SUMOylation pathway as a target for intervention in fungal pathogens. After a brief introduction to SUMOylation primarily from *S. cerevisiae*, we first briefly review the literature on what is known about SUMOylation in pathogenic fungi. We follow this up with some detailed bioinformatic analysis to evaluate the SUMOylation pathway in pathogenic fungi. We make several interesting observations with respect to conservation of the SUMO pathway in fungi. We have then compared some of the conserved players in SUMOylation with the human and plant counterparts in an effort to predict potential targets for drug development.

1.1. SUMOylation and SUMO genes

SUMOylation is a post-translational modification that is essential for cell growth, division, and adaptation to stress in most organisms, including fungi. It is a process where a small protein is covalently added to the target protein and this modified protein serves as a stage for interaction with other proteins. SUMOylation of target proteins can lead to multiple consequences (Fig. 1). Addition of a single SUMO moiety to target proteins can alter interactions with other proteins, change protein sub-cellular localization, alter catalytic functions or stabilize protein

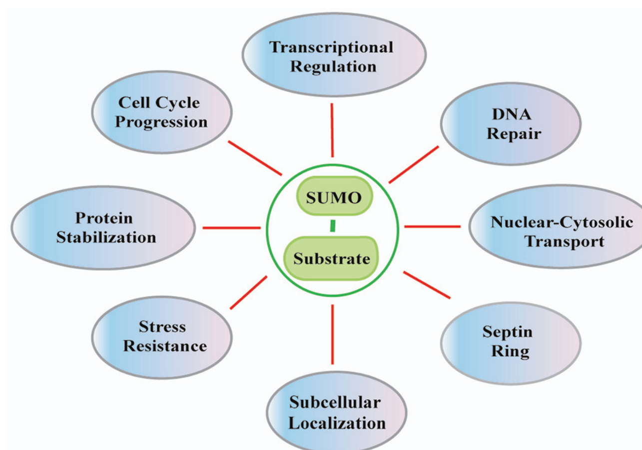

Fig. 1. Schematic representation of various physiological processes that are regulated by SUMOylation.

interactions. SUMOylation plays a key regulatory role in many distinct physiological pathways like DNA repair, transcription, cell-cycle progression, immune response, viral defence and intracellular transport. PolySUMOylation of a target, where SUMO chains are added, is usually a signal for recognition by the ubiquitinating machinery. These polySUMOylated proteins are polyubiquitinated and degraded in the proteasome. Thus while the consequence of monoSUMOylation is target-specific, polySUMOylation regulates the turnover of the target (reviewed in [7])

A cascade of enzymatic actions leads to SUMOylation of targets. While basic machinery appears conserved in all organisms studied so far, the SUMOylation system has expanded in some organisms. For instance, there are potentially 8 isoforms of the SUMO gene in *Arabidopsis thaliana* [8]; humans possess 4 SUMO isoforms, *viz*, SUMO-1, −2, −3 and −4, while *S. cerevisiae* expresses a single SUMO paralogue, called Smt3p.

1.2. Mechanism of SUMO conjugation

SUMOylation is a multistep process; SUMO is first proteolytically processed by the removal of a few C-terminal amino acids to reveal a diglycine motif. SUMO conjugation requires an ATP-dependent E1 activating enzyme (Aos1/Uba2 in yeast), an E2 conjugating enzyme (Ubc9), and one of several SUMO E3 ligases that finally transfer the SUMO moiety to the epsilon amino group of lysine in target proteins (Fig. 2). The yeast SUMO E1 activating enzyme is a heterodimer consisting of Aos1p and Uba2p. A high energy thioester bond is formed between Uba2p and SUMO C-terminus; this reaction involves ATP hydrolysis [9,10]. Through

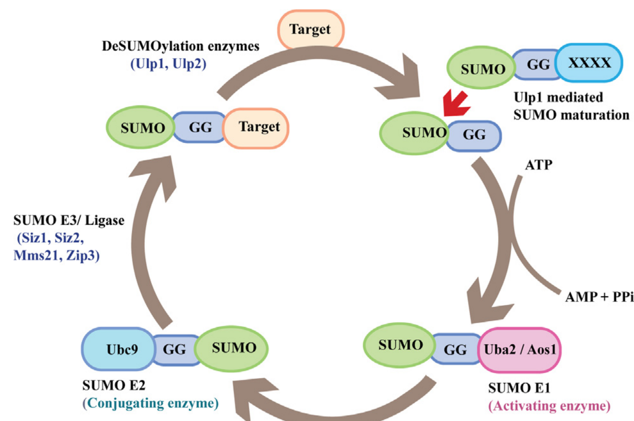

Fig. 2. Schematic depiction of the mechanism of SUMOylation.

thioester linkage, SUMO is then transferred to a cysteine residue of the E2 conjugating enzyme Ubc9. In vitro, the E2 enzyme is sufficient for conjugating SUMO to a lysine residue in the substrate, though in vivo this process is facilitated by E3 ligases [11].

Unlike ubiquitination, which has many E2 conjugation enzymes, Ubc9 is the only SUMO-conjugating enzyme in eukaryotes. Like many SUMOylation components, *UBC9* is essential in *S. cerevisiae* [12,13]. In vivo, SUMO is transferred from Ubc9 to target proteins via E3 ligases. Most organisms have multiple E3 ligases, which confer substrate range and specificity to SUMOylation. The SUMO E3 proteins identified so far include members of PIAS (protein inhibitor of activated STAT) proteins with the SP-RING domain, *viz*, Siz1, Siz2 , Mms21 and Zip3 in *S. cerevisiae.* RANBP2 (Ran binding protein 2) protein that is part of the nuclear pore complex, is a SUMO ligase but does not have the canonical RING domain for catalytic activity [14–17]. Other proteins that have been described as potential SUMO ligases comprise histone deacetylase 4 (HDAC4), KRAB-associated protein 1 (KPA1), Pc2 and Topors [18].

1.3. SUMO deconjugating enzymes

Protein SUMOylation is reversible by the action of deconjugases or deSUMOylating enzymes, namely, Ulp1 and Ulp2, in yeast system. Ulp1 is found at the nuclear pore complex (NPC) and is required for cleaving both the SUMO precursor and also SUMO conjugates from target proteins. Ulp2 localizes in the nucleoplasm [19], and is particularly important for dismantling polySUMO chains [20]. The two proteins also appear to deSUMOylate a distinct set of conjugates [19]. Ulps/SENPs (in higher eukaryotes) share a conserved ˜200-amino-acid catalytic domain that is typically found near their C-terminus.

There is increased complexity in SUMOylation pathway in animals and plants. First, many higher animals and plants have multiple SUMO proteins. Second, the SUMO ligases and SUMO isopeptidases have also expanded. Both the SUMO ligase and SUMO isopeptidase appear to be atleast partially specific for the SUMO isoform in these organisms. Mammals have at least six SENPs: SENP1, SENP2, SENP3, SENP5, SENP6 and SENP7. SENP1-3 and SENP5 are more similar to Ulp1, whereas SENP6 and SENP7 are more Ulp2-like [21]. The SENP proteins can be classified into 3 main families. The first family containing SENP-1 and SENP-2, are efficient at removing all the mammalian SUMO isoforms (SUMO-1–3). The second family contains SENP-3 and SENP-5 which preferentially remove SUMO-2/3 from substrates. Finally, SENP-6 and SENP-7, also preferentially remove SUMO-2/3 from substrates [22]. Recently, two new classes of SUMO proteases have also been identified, a dimer DeSI1(DeSUMOylating isopeptidase 1), located in the cytosol and nucleus, and DeSI2 which is mainly located in the cytoplasm, in contrast to SENPs which are predominantly located in the nucleus [2324]. DeSI1 has isopeptidase activity and can deconjugate both SUMO1 and SUMO-2/3 [23]. In the *Arabidopsis* genome, seven SUMO-specific proteases have been reported. Of them, ULP1a/ELS1 (Ub-like protease 1A/ESD4 like SUMO protease 1), ULP1b, ULP1c/OTS2 (Overly Tolerant to Salt2), ULP1d/OTS1 (Overly Tolerant to Salt1) and ESD4 (Early in Short Days 4) are classified as ULP1-like SUMO protease. ULP2a and ULP2b/ASP1 (*Arabidopsis* SUMO protease1) are identified as ULP2-like SUMO proteases. Furthermore, ULP1d/OTS1 and ULP1c/OTS2 are closer to yeast ScUlp2 as indicated by phylogenetic analysis [25–27]. These studies underscore the increased complexity in the SUMOylation pathway in higher eukaryotes compared to yeast.

1.4. SUMO-dependent ubiquitination mediated protein degradation

SUMO-targeted ubiquitin ligases (STUbLs) are a group of E3 ubiquitin ligases which selectively ubiquitinate polySUMOylated proteins and target them to the 26S proteasome for degradation. In *Saccharomyces cerevisiae*, the STUbL family includes Slx5/Slx8, Uls1 and Rad18 and in *Schizosaccharomyces pombe,* Rfp1, Rfp2 and Slx8 and in humans, RNF4 and RNF111. These have multiple SIMs (SUMO Interacting Motifs) except Slx8 (which has a single SIM) at the N-terminus as well as a RING finger domain at the C-terminal end [28–30]. Uls1 localizes in nucleolus and nucleoplasm and the heterodimer Slx5/Slx8 (together named as Uls2) resides in the nucleus. In mutant strains lacking STUbLs, accumulation of higher molecular weight polySUMOylated proteins is observed [28]. These observations show the connection between SUMOylation and ubiquitination and also underscore the importance of SUMOylation in protein homeostasis.

2. SUMOylation in pathogenic fungi

While studies in *S. cerevisiae* have unravelled both, the mechanisms of SUMOylation and the functional consequences, recently, a number of laboratories have examined SUMOylation in pathogenic fungi. These studies have shown that SUMOylation controls the functional properties of a large number of proteins found in pathogenic fungi and so plays a significant role in the physiology and pathology of these fungi. As other reviews have recently elaborately discussed this [31], below we summarize some of the studies on SUMOylation in pathogenic fungi briefly.

Candidemia, a key part of invasive candidiasis is one of the most common fungal infection in humans. This is especially seen in hospital settings like the Intensive Care Unit, in immunocompromised individuals and the elderly. It is caused by multiple Candida species, with *C. albicans* accounting for about 40–50%, followed by *C. glabrata*, *C. tropicalis* and *C. auris* species. *C. glabrat*a, which is a part of the normal microbiota of the mouth, gastrointestinal and vaginal tracts in humans, is relatively non-pathogenic in healthy hosts. However, it can cause serious candidemia in immunocompromised conditions. The emergence of antifungal resistant *C. glabrata* that is resistant to moderate quantities of antifungal drugs is posing a threat to treat the infections [32]. *C. glabrata* accounts for up to one third of total Candida bloodstream infections across the world [34]. *Candida auris* is an emerging pathogen that appears to be resistant to most antifungal [33]. Protein SUMOylation has been studied in both *C. albicans* and *C. glabrata*. *SMT3*, the gene encoding SUMO is essential for growth in *C. glabrata*. Perturbing SUMOylation affects growth, stress response and DNA repair capacity in *C. glabrata*. Deletion of *ulp2* in *C. glabrata* resulted in increased sensitivity to stress and was found to be required for virulence, adherence and biofilm formation [35]. While *SMT3* is not essential in *C. albicans, smt3* mutants exhibit slow growth that is exacerbated by stress conditions leading to sensitivity to a varied range of perturbations, including temperature, oxidative, and cell wall stresses [36]. The inactivation of *SMT3* in *C. albicans* makes a heterogeneous population of sluggishly growing, enlarged, elongated, pseudohypha-like cells [36]. Loss of SUMO ligases also increased hyphal formation in *C. albicans*, thus making it potentially more virulent [37]. However, these mutants were also sensitive to genotoxic, thermal and cell wall stresses, suggesting that SUMO conjugation may be important for differentiation and for combating stress.

Aspergillus and *Cryptococcus* species are harmful to both animals and plants. *Aspergillus* infections lead to invasive pulmonary aspergillosis (IPA), chronic pulmonary aspergillosis (CPA), simple pulmonary aspergilloma (SPA), and allergic bronchopulmonary aspergillosis (ABPA) [38,39]. *Cryptococcus* has been found to be responsible for cryptococcal meningoencephalitis and pulmonary cryptococcosis. *Cryptococcus neoformans* is an opportunistic human pathogenic fungus, affecting mainly immune compromised

patients [40,41]. Cryptococcus shows resistance to many currently available antifungal drugs and the infection has a high death rate. SUMOylation has not been directly investigated in Cryptococcus [42]. However, in the search of novel drug molecule for *C. neoformans* in a large-scale study, knock out for SUMO activating enzyme (Δ*aos1*) ortholog was found to reduce capsule and biofilm formation by *C. neoformans* in medical devices [43]. Loss of capsule formation reduces the virulence of *C. neoformans*. In this study, multiple other proteasomal components and NEDD (another Ubiquitin like molecule) were also found to be important for capsule formation. This suggests that virulence properties of this pathogen could be compromised in the absence of these pathways.

The most common pathogens among Aspergillus species are *A. fumigatus*, *A. flavus* and *A. carcinogen* that produces aflatoxin. Aflatoxin is a major cause of contamination in food, for example in nuts. Studies in *A. flavus* showed accumulation of SUMO conjugated protein at higher temperature, whereas deletion of SUMO gene adversely affected its pathogenicity and colony forming ability [40]. In *A. nidulans*, the complete SUMOylation machinery has been identified and characterized. In *A. nidulans,* SUMO is encoded by a single gene, SumO and appears to have the E3 ligases SizA , SizB and MmsU [44,45]. Although SumO protein is non-essential for fungal vegetative growth, it is indispensable for cellular differentiation in this fungus. Apart from reduced conidiation, Δ*sumO* cells in *A. nidulans* show increased sensitivity to the DNA-damaging agent methyl methanesulfonate (MMS) and to the DNA synthesis inhibitor hydroxyurea (HU) [46]. Δ*sumO* cells also display self-sterility, suggesting that SUMOylation of key targets is essential for the development of viable meiotic progeny in *A. nidulans*. Using a technique dubbed "SUMOlock", proteins were tagged and a set of 149 SUMOylated proteins (SUMOylome) were identified [45]. This study showed that a large fraction of the SUMOylated proteins were involved in transcription regulation, RNA processing and DNA repair. Interestingly, loss of *UlpB* or all the three SUMO ligases together had very severe effects on growth and conidation. Another interesting observation from these studies was that while *sumO* deletion was fairly well tolerated, deletion of *ulpB* was not, suggesting deSUMOylation of critical substrates is essential for growth.

Magnaporthe oryzae is a filamentous ascomycete plant pathogen and is responsible for causing rice blast disease. In *M. oryzae,* deletion of SUMO (*smt3*), E1 (*aos1, uba2*) and E2 enzyme (*ubc9*) resulted in pleiotropic phenotypes, which included defect in conidiation, septum formation, sensitivity to stress, mycelial growth and pathogenicity [47,48]. At least four septins were demonstrated to be SUMOylated and any changes in consensus SUMOylation sites in each septin led to separation of septins in appressoria and reduced virulence [49]. These mutants were delayed in host penetration and obtrusive development. SUMO pathway mutants (*smt3, aos1, uba2, ubc9*) also exhibited greater sensitivity to DNA damage stress as compared to wild type [48].

Together these studies indicate that while SUMOylation is not essential for survival (except in *C. glabrata*), it is critical for stress response, differentiation and pathogenicity in all fungi studied so far. Therefore, we examined if the SUMO pathway is present in other pathogenic fungi, and asked if there was conservation among fungi between the various components. While earlier studies have performed some analysis [31,48], we have performed a more in depth analysis and included more species to broadly test if SUMOylation pathway could be a target for intervention.

3. Orthologs of SUMO pathway proteins across fungi

In order to identify orthologs in a few selected fungi, the sequence of the proteins involved in the SUMOylation pathway in *S. cerevisiae* from SGD (Saccharomyces Genome Database) was downloaded. Fungal organisms from the five major phyla namely, Ascomycota, Basidiomycota, Chytridiomycota, Mucoromycota and Microsporidia were chosen. These represent fungi from different phyla that are pathogenic to animals and plants. The protein sequences of each of the 13 *S. cerevisiae* proteins were then used to identify orthologs across the 41 fungi shortlisted. For each protein, homologs were obtained by performing BLASTp against NCBI nr database restricted to each of the organisms considered [50]. The top-hits (RefSeq hits) obtained in the BLASTp analysis were then assessed by using reciprocal BLAST analysis (BLASTp restricted to *S. cerevisiae* S288c sequences) to identify true orthologs. Only the hits that returned the *Saccharomyces cerevisiae* query protein as the top hit were considered as true orthologs. The sequences which did not give the *S. cerevisiae* protein in the rBLAST were removed. The domain architecture of the orthologs was analysed using Pfam, CD (Conserved Domain) search and CDvist (Comprehensive domain visualisation tool). The results are presented in Table 1 and Supplementary file S1. In some cases where no orthologs could be found using *S. cerevisiae*, we used sequences from *S. pombe*, or *C. albicans* or other more closely related fungi to identify orthologs and are indicated in the text.

3.1. SUMO

The orthologs of the SUMO protein Smt3, were identified in all the fungi considered in this study. While most fungi have a single gene that encodes Smt3, 5 of them are found to have multiple orthologs with *R. irregularis* possessing 5 orthologs and *B. cinerea, T. terrestris, T. versicolor* and *S. commune* with 2 orthologs each (Table 1). In Agaricomycetes, *T. versicolor* (Polyporales) and *S. commune* (Agaricales) have two orthologs of SUMO protein. However, *A. bisporus* (Agaricales), which is closely related to *S. commune* has only a single ortholog. This suggests the possibility of a duplication event in their common ancestor followed by loss in *A. bisporus.* The *S. punctatus* ortholog and one of the orthologs in *S. commune, T. terrestris and R. irregularis* lacked the typical diglycine motif of the SUMO protein, but had single glycine residue near to the C- terminal end of the sequence. The domain present in the SUMO protein identified in all the organisms considered is approximately 70 aa long with the exception of *Z. rouxii* (78 aa) and *R. irregularis* (61 aa) (Supplementary file S1).

3.2. SUMO activating enzymes

For Aos1 and Uba2, all organisms have one ortholog except *P. nodorum* and *N. crassa,* which have 2 orthologs (Table 1). All the orthologs of Uba2 had a conserved ThiF domain and UAE UbL/UBA_e1 domain, except for *C. dubliniensis*, *Z. rouxii* and Microsporidia. The UAE-ubiquitin like domain is known to be involved in the transfer of the SUMO protein to conjugating enzymes. In *P. nodorum*, while one of the orthologs had only ThiF domain, the other was found to have a Zinc finger domain in addition to ThiF. The conserved domain showed an average length of 338 aa across all organisms. The presence of orthologs of Aos1 and Uba2 indicates a highly conserved SUMO conjugation pathway.

3.3. SUMO conjugating enzymes

A single ortholog of Ubc9 was identified in all the organisms considered except for *R. irregularis*, which has two orthologs. All the organisms have similar domain architectures, with an Ub-conjugating domain. The length of the domain is very small, ranging from 143 to 146 aa for all the organisms considered with the exception of *M. canis* (154 aa) and *T. melanosporum* (92 aa). The overall length of the Ubc9 protein ranges from 150 to 165 with

Table 1
The orthologs of SUMOylation process across fungi. # indicates organisms that are pathogenic to animals, * indicates organisms pathogenic to plants and $ indicates those that are pathogenic to both animals and plants. The columns show number of orthologs identified.

Class	Fungal Organisms	Smt3	Aos1	Uba2	Ubc9	Mms21	Nfi1	Siz1	Zip3	Ulp1	Ulp2	Slx5	Slx8	Uls1
Phylum- Ascomycota														
Eurotiomycetes	* *Aspergillus fischeri*	1	1	1	1	1	0	1	0	1	1	0	0	1
Eurotiomycetes	$ *Aspergillus nidulans*	1	1	1	1	1	1	1	0	1	1	0	1	1
Eurotiomycetes	# *Microsporum canis*	1	1	1	1	1	1	1	0	1	1	0	1	1
Dothideomycetes	* *Leptosphaeria maculans*	1	1	1	1	1	0	1	1	1	1	0	2	1
Dothideomycetes	* *Parastagonospora nodorum*	1	2	1	1	1	0	1	0	1	1	0	1	1
Leotiomycetes	* *Botrytis cinerea*	2	1	1	1	1	0	2	0	1	3	1	1	2
Leotiomycetes	* *Sclerotinia sclerotiorum*	1	1	1	1	1	0	2	0	1	2	1	1	1
Sordariomycetes	* *Fusarium graminearum*	1	1	1	1	1	1	1	0	3	1	0	1	1
Sordariomycetes	* *Colletotrichum graminicola*	1	1	1	1	1	0	1	0	1	1	0	2	1
Sordariomycetes	# *Chaetomium globosum*	1	1	1	1	1	0	1	0	1	1	1	1	1
Sordariomycetes	# *Thielavia terrestris*	2	1	1	1	1	0	2	0	1	1	0	1	1
Sordariomycetes	* *Neurospora crassa*	1	1	2	1	0	0	2	0	1	2	0	1	1
Sordariomycetes	* *Magnaporthe oryzae*	1	1	1	1	1	0	2	0	1	1	0	1	1
Pezizomycetes	*Tuber melanosporum*	1	1	1	1	1	1	1	0	1	1	1	1	1
Saccharomycetes	# *Candida albicans*	1	1	1	1	1	0	1	1	2	1	1	2	1
Saccharomycetes	# *Candida dubliniensis*	1	1	1	1	1	0	1	1	1	1	1	3	1
Saccharomycetes	# *Candida tropicalis*	1	1	1	1	1	1	2	1	2	1	1	2	1
Saccharomycetes	# *Candida parapsilosis*	1	1	1	1	0	0	1	0	1	1	1	1	1
Saccharomycetes	# *Candida orthopsilosis*	1	1	1	1	0	1	1	1	2	1	1	2	1
Saccharomycetes	# *Candida auris*	1	1	1	1	1	0	1	0	1	1	1	1	1
Saccharomycetes	# *Candida glabrata*	1	1	1	1	1	1	1	1	1	1	1	1	1
Saccharomycetes	*Saccharomyces cerevisiae*	1	1	1	1	1	1	1	1	1	1	1	1	1
Saccharomycetes	*Zygosaccharomyces rouxii*	1	1	1	1	1	0	1	1	1	1	1	1	1
Saccharomycetes	*Kluyveromyces lactis*	1	1	1	1	1	0	1	1	1	1	1	1	1
Saccharomycetes	* *Eremothecium gossypii*	1	1	1	1	1	0	1	0	1	1	1	1	1
Saccharomycetes	*Komagataella phaffii*	1	1	1	1	1	0	1	0	1	1	1	2	1
Schizosaccharomycetes	*Schizosaccharomyces pombe*	1	1	1	1	1	0	1	0	1	1	0	1	2
Phylum- Basidiomycota														
Agaricomycetes	*Trametes versicolor*	2	1	1	1	1	0	1	1	2	1	0	1	1
Agaricomycetes	*Schizophyllum commune*	2	1	1	1	1	0	1	0	4	1	0	4	1
Agaricomycetes	*Agaricus bisporus*	1	1	1	1	0	0	1	1	1	1	0	1	1
Tremellomycetes	$ *Cryptococcus neoformans*	1	1	1	1	1	0	1	1	1	1	0	2	1
Ustilaginomycetes	* *Ustilago maydis*	1	1	1	1	1	0	1	1	1	1	0	1	1
Pucciniomycetes	* *Puccinia graminis*	1	1	1	0	1	3	4	1	2	1	0	2	1
Phylum- Mucoromycota														
Glomeromycetes	*Rhizophagus irregularis*	5	1	1	2	1	0	1	1	3	0	0	0	0
Phylum- Chytridiomycota														
Chytridiomycetes	*Spizellomyces punctatus*	1	1	1	1	0	3	4	1	2	2	0	0	2
Chytridiomycetes	# *Batrachochytrium dendrobatidis*	1	1	1	1	1	0	1	0	1	1	0	1	1
Phylum- Microsporidia														
Microsporidia	# *Encephalitozoon intestinalis*	1	1	1	1	1	0	0	0	1	0	0	2	0
Microsporidia	# *Encephalitozoon cuniculi*	1	1	1	1	1	0	0	0	1	0	0	1	0
Microsporidia	# *Encephalitozoon hellem*	1	1	1	1	1	0	0	0	1	0	0	1	0
Ordosporidae	# *Ordospora colligata*	1	1	1	1	1	0	0	0	1	0	0	2	0
Nosematidae	# *Nosema ceranae*	1	1	1	1	1	0	0	1	1	0	0	0	0

the exception of *C. albicans* (219) and *E. cuniculi* (204) and is found to be highly conserved at the sequence level across all fungi (Supplementary file S1).

3.4. SUMO ligases

All organisms have at least one E3 ligase that contain the characteristic SP-RING motif. Most organisms have Mms21 and/or Siz1. Orthologs of Mms21 could not be identified in few organisms, which include *N. crassa, C. parapsilosis, C. orthopsilosis, A. bisporus* and *S. punctatus*. Only orthologs of Mms21 present in Ascomycetes could be detected using the *S. cerevisiae* protein, while others were identified using the *S. pombe* Mms21 (Table 1). All Mms21 orthologs have one Zinc finger domain with Nse subunit. The domain is highly conserved, although the remainder of the sequence is not well- conserved. The sequence length of the domain ranges from 53 to 56 aa with the exception of *A. fischeri* (70) and *B. cinerea* (63) (Supplementary file S1).

In order to find the Mms21 ortholog in *A. nidulans*, we performed a BLAST search using Mms21 of *S. cerevisiae* and found AN10240.4 (genbank sequence - CBF8582.1) as the top hit followed by AN1916.2 (RefSeq sequence - XP_659520.1) as the second hit, although both have exactly the same score (50.4) and E-value (2e-07). The difference between AN10240.4 (502 amino acids) and AN1916.2 (1481 amino acids) is that AN10240.4 is a subsequence of AN1916.2 with AN10240.4 being the last 502 amino acids of AN1916.2. Since AN10240.4 showed Mms21 as the top hit in rBLAST, also contains the zf-Nse domain found in MMS21 orthologs and was reported earlier [45], it is considered as an ortholog of Mms21.

Siz1 and Siz2 (Nfi1) are closely related E3 SUMO conjugating enzymes and Siz2 is a known paralogue of Siz1[51]. In *C. glabrata* and *C. orthopsilopsis*, we could identify two orthologs, one of which returns Nfi1 as a top hit and the other returning Siz1 as the top most hit. However, in most of the other organisms, the homologs obtained return only Siz1 as the top hit. We do not find any organism that has a Nfi1 homologue but not Siz1; thus, it appears that Nfi1/Siz2 is restricted to a few fungal groups, specifically the Saccharomycetes. Of note, a number of Ascomycetes have more than one ortholog of Siz1 class of SUMO ligase. The organisms *P. graminis* and *S. punctatus* have three orthologs each. In *S. cerevisiae*, Siz1 and Nfi1 have similar domain architectures containing SAP

domain, PINIT domain and zf-MIZ domain. The domain architecture is conserved across all orthologs. While, some of the orthologs are found to lack the SAP domain, some of them have just the zf-MIZ domain and lack both PINIT and SAP domains (Supplementary file S1). Additionally, while most organisms do not have Siz2/Nfi1, two organisms, *Puccinia graminis* and *Spizellomyces punctatus* have multiple othologs of both Siz1 and Siz2. Interestingly, no SUMO ligase of the Siz1/Siz2 type was identified in any of the Microsporidia considered in this study and they all have only Mms21 as the SUMO ligase.

The orthologs of the SUMO ligase Zip3 were identified in few fungal organisms considered in this study. Zip3 ortholog in *C. tropicalis* and *S. punctatus* could only be detected using *C. albicans* sequences. In one of the microsporidia (*N. ceranae*), Zip3 ortholog was identified using *A. bisporus* sequences. All the orthologs of Zip3 had a conserved zf-RING domain, except for *A. fischeri, N. crassa* and *C. tropicalis.*

3.5. SUMO proteases

SUMO proteases play critical roles in SUMOylation. First, they are required for the proteolytic processing of SUMO to generate mature SUMO. In most organisms, Ulp1 family of proteins carry out this process. Second, SUMO proteases remove SUMO from the target protein thus ensuring reversibility of this modification. While both Ulp1 and Ulp2 can remove SUMO, they appear to have some distinct targets. Of note, removal of polySUMOylation is preferentially carried out by Ulp2. This is thought to be possible due to the presence of a SIM (SUMO interacting motif) towards the C-terminus in Ulp2 [20]. SUMO protease Ulp1 could be identified in all the organisms considered in this study while Ulp2 orthologs were missing in a few organisms. More than two orthologs of Ulp1 are found in 3 organisms namely, *C. graminicola, S. commune* and *R. irregularis.* Furthermore, Ulp2 ortholog in the Chytridiomycetes was detected using *S. pombe* sequence. No ortholog of Ulp2 could be identified in any Microsporidia considered using *S. cerevisiae, C. albicans, S. pombe* and the Chytridiomycetes (*S. punctatus* and *B. dendrobatidis*) sequences. However, all Microsporidia have the Ulp1 ortholog. There are only 4 organisms, which have multiple orthologs of Ulp2; *B. cinerea* with 3 and *S. sclerotiorum, N. crassa* and *S. punctatus* with 2 each. All the sequences of Ulp1 and Ulp2 orthologs have only one conserved domain, the Peptidase C48 domain. In both Ulp1 and Ulp2, conservation beyond the catalytic domain is limited.

3.6. SUMO-targeted ubiquitin ligases (STUbLs)

STUbLs are ubiquitin ligases that target polySUMOylated proteins for ubiquitination and further degradation to the proteasome. We searched for orthologs of the RING finger containing STUbLs namely, Slx5, Slx8 and Uls1 across 41 organisms. Orthologs of Slx5 and Slx8 were identified in few organisms using *S. cerevisiae* sequences. For some fungal organisms, Slx8 orthologs could be detected using *C. albicans* and *S. pombe*. Few of them were found to have multiple Slx8 orthologs with *S. commune* possessing 4 orthologs, *C. dubliniensis* possessing 3 orthologs and *L. maculans*, *C. graminicola*, *C. albicans*, *C. tropicalis, C. orthopsilosis, K. phaffii, C. neoformans and P. graminis* possesing 2 orthologs each (Table 1). All the sequences of Slx8 orthologs have only one RING finger domain except *M. canis, F. graminearum, C. graminicola, M. oryae*, *C. dubliniensis, C. tropicalis, K. phaffii* and *C. neoformans* which have an additional SPX domain at N-terminus and RING finger motif at the C-terminus. In Microsporidia using *S. cerevisiae* STUbL sequences, we could identify a single ortholog of *Slx8* in *E. intestinalis, E. hellem* and *O. colligata*. Interestingly, using *C. albicans* STUbL Slx8 sequences, we were able to detect an additional Slx8 ortholog in *E. intestinalis* and *O. colligata.* Slx5 was not detected in any Microsporidia neither using *S. cerevisiae* nor *C. albicans*, or other more related organisms.

A large protein, Uls1 (1619 amino acids), whose domain structure comprises Snf2-like translocase in N-terminus and RING finger motif in C-terminus appears to be more wide-spread. A single Uls1 ortholog is present in most of the organisms except *B. cinerea, S. pombe, S. punctatus* which have two orthologs. Interestingly, though Uls1 orthologs are more widespread across other classes of fungi, no ortholog could be identified in any of the Microsporidia shortlisted in our study.

In summary, SUMOylation pathway exists in all fungi. There is variation in the number of and types of E3 ligases in the fungal kingdom. Few organisms have multiple orthologs for ligases which suggest that there could be redundancy and/or functional diversity in terms of substrate range and specificity for these enzymes in these organisms. In this context, unexpectedly, *R. irregularis* has multiple homologs of SUMO, Ubc9 and Ulp1. The Nfi1/Siz2 duplication event appears to be specific to Saccharomycetes. In addition, we find Microsporidia to have a minimal SUMO system and STUbLs have been very difficult to detect in this phyla. This could either be due to a large divergence in sequences or could indicate absence in Microsporidia, which are known to have undergone reductive genome evolution.

4. Can SUMOylation pathway be a drug target?

A few studies have examined the possibility of targeting SUMOylation for intervention. A group of alkyl phenol, ginkgolic acid and its analog, anacardic acid have been found to inhibit SUMOylation. They bind to E1 activating enzyme, consequently interfering with the formation of an E1-SUMO thioester complex [52]. Several small molecule inhibitors that target deSUMOylases and E2 conjugase have been reported [53]. SUMOylation has also been examined as a target in cancer treatment in a couple of studies. Both breast cancer and pancreatic cancer cells appear to respond to the inhibitor of SAE, the E1 activating enzyme for SUMO [54]. Another molecule, N106 (N-(4-methoxybenzo [d] thiazol–2–yl)-5- (4-methoxyphenyl)-1,3,4-oxadiazol-2- amine, was also found to target E1 enzyme and enhance SUMOylation of SERCA2a (sarcoplasmic reticulum calcium ATPase), a calcium regulating pump in cultured cardiomyocyte cells. This resulted in enhanced contractility in vitro and in vivo [55] and therefore is a potential treatment option. However, all these studies were directed towards targeting the human enzymes. While some are likely to inhibit fungal enzymes as well, they cannot serve as good targets for fungal infections as these inhibitors would affect the host SUMOylation pathway as well. Therefore, there is a need to identify specific inhibitors that can target the fungal enzymes without affecting the host enzymes.

SUMOylation pathway is conserved across fungi, plants and animals. With the available limited information on SUMOylation in fungi, it is clear that SUMOylation plays critical roles in fungal physiology and pathogenesis. As fungi and animals belong to the same supergroup of Opisthokonts, it is possible that the proteins conserved in these organisms share a high similarity. Thus designing drugs that target specifically the fungal protein without affecting the human counterparts is a challenge. In order to identify how similar or different each of the SUMO pathway proteins in fungi are in comparison to the human and plant orthologs, we calculated the percentage similarity of the orthologs using EMBOSS stretcher global alignment tool (Table 2A and 2B) [56]. We identified the orthologs of all the 8 proteins in human and in *Arabidopsis thaliana*. We then compared the SUMO pathway proteins of six fungi, viz., *C. glabrata, C. albicans, C. neoformans, M. oryzae, A. nidulans, C. parapsilosis* and *S. cerevisiae* with the human and plant orthologs. Among

Table 2A
The table shows the percentage similarity of the closest (lowest E-value) ortholog of *H. sapiens* with the orthologs identified in selected fungi. Full length protein sequences were considered for comparison.

SUMO pathway genes	*Saccharomyces cerevisiae*	*Candida glabrata*	*Candida albicans*	*Cryptococcus neoformans*	*Magnaporthe oryzae*	*Aspergillus nidulans*	*Candida parapsilosis*
SMT3	57.4	54.7	51.6	48.1	50.0	51.7	39.2
AOS1	52.8	50.4	46.0	48.5	43.7	47.0	46.5
UBA2	50.4	51.0	51.5	51.6	51.7	54.3	50
UBC9	69.9	70.8	51.5	75.1	73.3	67.0	68
MMS21	40.1	40.7	40.3	43.6	36.6	32.8	–
SIZ1	24.6	27.4	17.5	26.1	39.5	31.2	21.9
ULP1	39.3	40.5	47.4	46.0	23.6	30.8	44.0
ULP2	38.9	38.9	38.2	34.1	37.9	36.4	36.8

Table 2B
The table shows the percentage similarity of the closest (lowest E-value) ortholog of *A. thaliana* with the orthologs identified in selected fungi. Full length protein sequences were considered for comparison.

SUMO pathway genes	*Saccharomyces cerevisiae*	*Candida glabrata*	*Candida albicans*	*Cryptococcus neoformans*	*Magnaporthe oryzae*	*Aspergillus nidulans*	*Candida parapsilosis*
SMT3	56.9	56.6	54.9	53.3	53.1	57.4	41.8
AOS1	46.1	48.5	44.3	46.9	41.1	43.9	46.2
UBA2	49.6	51	51.2	50.7	50.4	55.3	48.7
UBC9	70.5	69.8	50.4	67.8	72.8	63.6	68.5
MMS21	40.6	41.7	43.1	40.9	34	32	–
SIZ1	39.8	41.3	30.3	37.7	34.3	38.3	34.6
ULP1	35.1	35.2	39.8	38.8	20.3	26.2	38.5
ULP2	39	38.1	39.1	31.6	37.4	37.3	36.8

Table 3A
Comparison of the percentage similarity of the full-length protein and the catalytic domains of the *SIZ1*, *ULP1* and *ULP2* orthologs of *C. glabrata* and *H. sapiens.*

Candida glabrata	*Homo sapiens*	Full length (% similarity)	Catalytic Domain (% similarity)
CgSIZ1	*PIAS1*	39.7	58.6
CgSIZ1	*PIAS2*	39.1	57.9
CgSIZ1	*PIAS3*	38.6	56.1
CgSIZ1	*PIAS4*	33.3	60.3
CgSIZ1	*ZMIZ1*	35.5	56.1
CgSIZ1	*ZMIZ2*	35.6	56.1
CgULP1	*SENP1*	41.0	49.1
CgULP1	*SENP2*	40.2	47.9
CgULP1	*SENP3*	38.8	45.0
CgULP1	*SENP5*	39.8	47.9
CgULP2	*SENP6*	37.5	31.1
CgULP2	*SENP7*	38.7	42.0

Table 3B
Comparison of the percentage similarity of the full-length protein and the catalytic domains of the *SIZ1*, *ULP1* and *ULP2* orthologs of *M. oryzae* and *A. thaliana.*

Magnaporthe oryzae	*Arabidopsis thaliana*	Full length (% similarity)	Catalytic Domain (% similarity)
MoSIZ1	*SIZ1*	30.9	53.2
MoSIZ1	*SIZ1*	34.5	40.5
MoULP1	*ULP1a*	25.4	46.0
MoULP1	*ULP1b*	20.1	43.1
MoULP1	*ULP1c*	24.4	37.4
MoULP1	*ULP1d*	29.4	40.9
MoULP1	*ESD4*	26.3	45.0
MoULP2	*ULP2a*	38.0	38.4
MoULP2	*ULP2b*	36.1	38.7

humans and plants, which have multiple orthologs, the sequence with the lowest E-value, i.e., the most closely related, is used for the comparisons Supplementary file S2.

As shown in table 2A and 2B, many proteins of this pathway share very high level of similarity with both plant and human orthologs. This is especially true for Smt3, Aos1, Uba2 and Ubc9, with Ubc9 sharing maximum similarity. In case of Smt3, orthologs in fungi are found to share higher similarity with the *A. thaliana* protein compared to that of human. Interestingly, the *C. parapsilosis* Smt3 shares much less similarity with human and plant protein compared to its close relatives *C. albicans* and *C. glabrata*. Aos1 in *M. oryzae* (plant pathogen) shares less similarity with the plant ortholog compared to the human protein where as Uba2 orthologs shares equal similarity with both the human and plant protein. Therefore, Uba2 appears to be more conserved in humans, plant and fungi than its activating enzyme partner Aos1. Irrespective of other SUMO pathway genes, Ubc9 fungal orthologs share very high similarity with the human and plant orthologs.

SUMO ligase (Mms21 and Siz1) and SUMO protease (Ulp1 and Ulp2) orthologs of fungi are relatively less similar to human and plant orthologs. The percentage similarity and identity calculated for the full-length fungal proteins with the human and plant counterparts showed that Siz1, Ulp1 and Ulp2 share the least similarity and identity compared to the other proteins in the SUMOylation pathway. As the catalytic domains of the proteins are crucial for their function and are generally highly conserved, we checked the percentage similarity of the catalytic domains of these proteins in *C. glabrata* and *M. oryzae* with that of the catalytic domain present in all of the human and plant orthologs respectively. For these comparisons, protein accession number, GeneID and catalytic domain residues are available in Supplementary file 3. The results are summarized in Table 3A and 3B. When only the catalytic domains were compared, as expected, the similarities between fungi, plant and animal homologs increased considerably. Despite this, Ulp1 and Ulp2 appear to be distant enough from animal and plant homologs to serve as targets.

The catalytic domain i.e., zf-MIZ present in Siz1 of *C. glabrata* and *M. oryzae* (2 orthologs in Siz1) were compared with that present in the human orthologs PIAS1-4 and ZMIZ1-2 and plant ortholog Siz1. Highest similarity was found with PIAS4 ortholog of human (60.3%) and with Siz1 ortholog of plant (53.2%). Similarly, *M. oryzae* Ulp1 and Ulp2 orthologs share 46% similarity with Ulp1a and 38.7% similarity with Ulp2b ortholog of plant (Table 3B). On comparing the catalytic domains, the SUMO proteases Ulp1 and

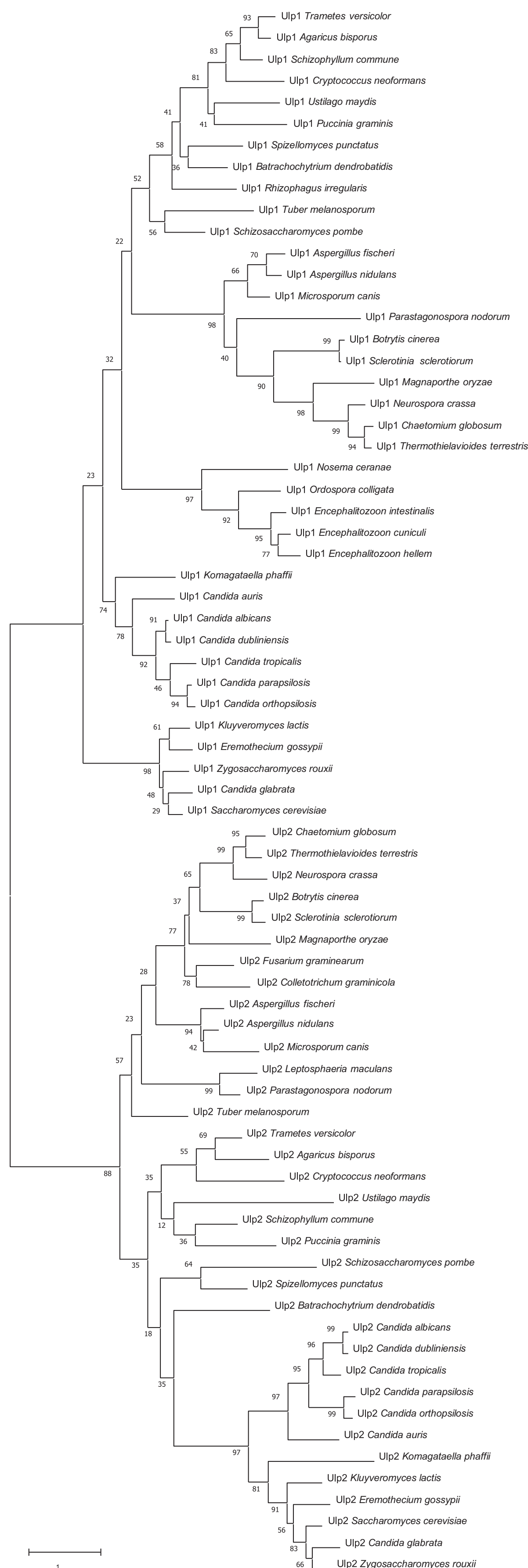

Fig. 3. Maximum likelihood tree of SUMO proteases. The phylogenetic tree was constructed using MEGA X-10.1.8 (Molecular Evolutionary Genetics Analysis) for the catalytic domain of Ulp1 and Ulp2 proteins identified across fungi. Bootstrap values are shown at the nodes. The scale bar represents the average number of substitutions per site.

Ulp2 share lesser similarity with the human and plant counterparts. Furthermore, when the catalytic domain i.e., Peptidase C48 domain present in Ulp1 and Ulp2 of *C. glabrata* and *M. oryzae* were compared with the Peptidase C48 domain of human orthologs (SENP1, 2, 3 and 5 and SENP 6 & 7) and plant orthologs (Ulp1a-d, ESD4 and Ulp2 a-b), it showed *Cg*Ulp1 was 49.1% similar with SENP1 and *Cg*Ulp2 was 42% similar with SENP7 ortholog of human (Table 3A). Thus the Ulp2 orthologs have the least similarity suggesting that they can be potential drug targets.

While deSUMOylating enzymes appear to be least similar to the host deSUMOylases, loss of deSUMOylating enzymes appears to have a significant effect on the viability and virulence of fungal pathogens [35,45]. Loss of deSUMOylating activity could potentially have two consequences: accumulation of monoSUMOylated and/or polySUMOylated proteins that are toxic or alternately, reduction in the level of those proteins due to excess degradation by targeted ubiquitination in the absence of balanced deSUMOylating activity. The reversibility of SUMOylation brought about by Ulps is probably key to protein homeostasis and loss of this activity could lead to phenotypes seen by disrupting Ulp2. This further makes deSUMOylases an attractive target for intervention.

As Ulp2 appears to be particularly important for pathogenesis and is sufficiently different from the human and *Arabidopsis* counterparts, we further investigated the phylogenetic relationship between Ulp1 and Ulp2 in the chosen fungi. We performed a maximum likelihood (ML) analysis using the sequence of the catalytic domains of the two SUMO proteases, Ulp1 and Ulp2, identified across fungi. In the ML tree we find a clear split between the Ulp1 and the Ulp2 proteins suggesting the presence of both the SUMO proteases in the common ancestor of fungi and thus the gene duplication event leading to the Ulp1 and Ulp2 proteins possibly happened before the common ancestor of fungi arose (Fig. 3). As the homologs of both the proteins are found in several organisms across metazoa as well, we speculate the presence of both Ulp1 and Ulp2 proteins in the common ancestor of Opisthokonts itself (the supergroup comprising of fungi and metazoa). In the ML tree, the Ulp2 proteins in all the Saccharomycetes considered clustered together with good bootstrap support. We also find a strong clustering of organisms belonging to the CTG clade in accordance with previous studies. [57,58] In case of Ulp1 protein, monophyly of the Saccharomycetes could not be recovered and we find one set of Saccharomycetes to be distinct from the rest suggesting sequence divergence.

Apart from the SUMO machinery, the target proteins that get SUMOylated could also serve as targets. Components of the DNA repair pathway, transcription, chromatin modifiers, nuclear transport etc are SUMOylated in *S. cerevisiae*. These could be additional targets either independently or in concert with the SUMOylation inhibitors. However, the targets in pathogenic fungi are only now beginning to be identified and once known, could work as specific targets for SUMOylation inhibition.

5. Summary and outlook

SUMO protein and SUMO activating enzymes are found across all the fungi. Orthologs of the SUMO pathway components, Smt3, Aos1, Uba2, Ubc9, Mms21, Siz1, Nfi1, Ulp1 and Ulp2 and the SUMO-dependent ubiquitin ligases have been identified across 41 fungi belonging to the five major phyla. In summary, based on

studies from *S. cerevisiae* and other yeasts and fungi, it is clear that SUMOylation plays an important role in the control of growth, cell division, differentiation and stress adaptation. In all pathogenic fungi, loss of SUMOylation compromises their virulence. By phylogenetic analysis and ortholog studies we find that Ulp2 could be a potential drug target. In parallel, identifying the targets of SUMOylation in all these pathogenic fungal strains would unravel the pathways that are specifically modulated by SUMOylation and these could also eventually be targeted. In addition, once we know the key enzymes in the SUMOylation pathway that are modulated during stress and infection, that particular step could be a potential target. Further work is needed to describe and define the mechanisms by which this post translational modification contributes to disease development.

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CRediT authorship contribution statement

Dipika Hita Gupta Sony Garapati: Data curation, Formal analysis, Writing - original draft, Writing - review & editing, Data curation, Formal analysis, Supervision. **Kakumanu V.S. Akhil:** Data curation, Formal analysis. **Renu Krishnaveni Shukla Mishra:** Visualization, Writing - original draft, Writing - review & editing, Conceptualization, Funding acquisition, Project administration, Supervision, Writing - original draft, 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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2020.10.037.

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