Review

Knr4: a disordered hub protein at the heart of fungal cell wall signalling

Hélène Martin-Yken,1* Jean Marie François1 and Didier Zerbib1,2
1 LISBP, Université Fédérale de Toulouse, CNRS, INRA, INSA, 135 Avenue de Rangueil, F-31077 Toulouse, France. 2 Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, CNRS, UPS, F-31077 Toulouse, France.

Summary

The most highly connected proteins in protein–protein interactions networks are called hubs; they generally connect signalling pathways. In Saccharomyces cerevisiae, Knr4 constitutes a connecting node between the two main signal transmission pathways involved in cell wall maintenance upon stress: the cell wall integrity and the calcium–calcineurin pathway. Knr4 is required to enable the cells to resist many cell wall-affecting stresses, and KNR4 gene deletion is synthetic lethal with the simultaneous deletion of numerous other genes involved in morphogenesis and cell wall biogenesis. Knr4 has been shown to engage in multiple physical interactions, an ability conferred by the intrinsic structural adaptability of major disordered regions present in the N-terminal and C-terminal parts of the protein. Taking all together, Knr4 is an intrinsically disordered hub protein. Available data from other fungi indicate the conservation of Knr4 homologs cellular function and localization at sites of polarized growth among fungal species, including pathogenic species. Because of their particular role in morphogenesis control and of their fungal specificity, these proteins could constitute interesting new pharmaceutical drug targets for antifungal combination therapy.

Introduction

Highly connected proteins located at nodes in interactions networks are referred to as hub proteins. In addition to their central position in protein–protein interaction (PPI) networks, hub proteins are frequently found at the crossroads of signalling pathways. Hub proteins are often intrinsically disordered proteins (IDPs; i.e. proteins that do not possess any defined secondary structure in solution) or contain intrinsically disordered regions (IDRs), which allow them to fulfil their multiple interactions (Dunker et al., 2005; Uversky, 2013). IDPs and IDRs are involved in numerous essential biological processes (Dunker et al., 2002, 2006) through PPI involving transient or permanent interaction with multiple protein partners. The conformational flexibility of these IDPs/IDRs gives them the ability to be involved in multiple binding (a single disordered domain being able to bind several structurally diverse partners) and multiple functions. The strategic positions of hub IDPs make them very attractive targets for inhibitors development. In addition, their intrinsic versatility renders their structural study both arduous and fascinating (Oldfield and Dunker, 2014). In this review, we summarize two decades of work showing that Saccharomyces cerevisiae Knr4 is a characteristic IDP hub that connects signalling pathways affecting cell wall synthesis, morphogenesis and cell cycle progression (Martin-Yken et al., 2003; Dagkessamanskaia et al., 2010a,b). Most of the data available in the literature and reported here have been obtained on the model budding yeast protein counterpart. However, several pieces of evidence from other yeasts and fungi clearly demonstrate that the function and localization of this protein are conserved among the fungal kingdom. Discussion of whether or not this family of homologous proteins can be considered as potential antifungal targets involves considering their specificity to this kingdom, their peculiar structure and their role as hubs in cell signalling related to morphogenesis and cell wall synthesis.

Isolation of KNR4 gene and functional analysis

Genetic screens

Saccharomyces cerevisiae KNR4/SMI1 double gene name comes from its isolation in 1993 as SMI1 (for Suppressor of Mar Inhibition), in a colorimetric screen for suppressors of transcription inhibition associated to DNA Matrix Associated Regions, a screen that also led to the simultaneous isolation of Calmodulin (Fishel et al., 1993). Almost at the...
same time, the gene was identified as KNR4 (Killer Nine Resistant 4), in a screen based on resistance to the killer toxin K9 of Hansenula mrakii, a phenotype likely linked to the reduced β(1,3)-glucan synthase activity in a knr4 mutant (Hong et al., 1994). The same authors showed that indeed the colorimetric test used by Fishel et al. was possibly influenced by the increased permeability of the knr4 mutant cell wall. Hence, the name KNR4 should be used to refer to this gene. The KNR4 gene was then re-isolated as a suppressor of the calcofluor white (CFW) hypersensitive phenotype of cell wall mutants (Martin et al., 1999) as overexpression of this gene was found to cause strong transcriptional repression of the three chitin synthase genes, significantly reducing chitin content in the wall and thus rendering these mutants less sensitive to CFW. In most S. cerevisiae genetic backgrounds including BY4741, KNR4 gene is not essential for growth under standard laboratory conditions (30°C, rich medium). However, its deletion leads to growth defects under numerous stresses such as elevated temperature, presence of sodium dodecyl sulfate (SDS), caffeine, various antibiotics and cell wall-affecting drugs. It also leads to a significantly increased release of polysaccharides and mannanproteins into the culture medium, which is of special interest for oenological fermentation processes (Gonzalez-Ramos et al., 2008). Indeed, mannanproteins released by yeast cells are key determinants of the stability of wine against haze and positively impact several other quality properties of wine including their aroma, colour, astringency, mouthfeel, malolactic fermentation and the formation of foam by sparkling wines (Gonzalez-Ramos and Gonzalez, 2006). Homozygous deletions of the KNR4 gene have been conducted in industrial (wine) yeast strains and shown to ensure improved oenological parameters (Gonzalez-Ramos et al., 2008, 2009; Penacho et al., 2012). In addition, the surface properties of KNR4 deletion strains affect their sorption capacities, which are also relevant to oenology because yeast cell walls are well known and widely used to reduce the concentration of undesirable molecules in wine, such as fatty acids and volatile phenols (Pradelles et al., 2008).

**Cell wall integrity and calcineurin pathways**

In S. cerevisiae, two main pathways control cell wall synthesis and integrity in response to stress: the CWI pathway and the calcineurin pathway (Fig. 1A). Remarkably, these two pathways are mutually essential: if one is interrupted, the other becomes crucial for cell survival. The Knr4 protein physically interacts with a key component in each of these two pathways: the Slt2 MAP kinase for CWI (Martin-Yken et al., 2003) and the phosphatase calcineurin of the calcium responsive pathway (Dagkessamanskaia et al., 2010a,b) (Fig. 1A). Similarly, the presence of Knr4 is essential in mutants affected in either one or the other of these two pathways. This points to the functional role of this protein as either a coordinator between these two signalling pathways or possibly a common scaffold for them. Knr4 interaction with Slt2 has been shown to affect the transcriptional outputs of CWI pathway (refer to Transcription paragraph). The functional consequences of Knr4 interaction with calcineurin have not yet been fully disclosed; however, KNR4 deletion renders cells hypersensitive to Ca²⁺ without affecting the intracellular level of calcium in response to calcium stress (Zhao et al., 2013), a phenotype that can be suppressed by the presence of Mg²⁺ but not by the calcineurin inhibitor cyclosporine A, and hence is not due to hyperactivity of calcineurin.

**Progression through checkpoints**

In the genetic background, W303 (SSD1 deficient) knr4 mutants arrest growth in S-phase with 80% small-budded cells and DNA content between 1 and 2 N (Fishel et al., 1993). Knr4 participates with Bck2 in the Cln3-Cdc28-dependent gene transcription at G1/S transition (Martin-Yken et al., 2002; Kuravi et al., 2011), and in the absence of Knr4, the SBF transcription factor is constitutively hyper-activated throughout the cell cycle instead of peaking at the G1/S transition. This is likely caused by inappropriate phosphorylation of Serine 238 of Swi6 by the Slt2 MAP kinase, which normally occurs in response to heat shock (Kim et al., 2010b). In addition, the absence of Knr4 leads to dysfunction of at least two cell cycle checkpoints: the morphogenesis checkpoint – in which both calcineurin and Slt2 are involved, which couples cell division with bud growth (Harrison et al., 2001; Mizunuma et al., 2001; Miyakawa and Mizunuma, 2007) – and the mechanism controlling daughter cell size at cytokinesis (Dagkessamanskaia et al., 2010a,b). Finally, KNR4 gene deletion results in short telomeres by affecting the level of telomerase mRNA (Askree et al., 2004; Mozdy et al., 2008).

**Transcription**

A role for Knr4 in gene expression was first revealed by its ability to simultaneously repress all three chitin synthase genes of S. cerevisiae upon overexpression (Martin et al., 1999). Global transcriptomic profiles of knr4Δ mutant and overexpression mutant further revealed a wide range of genes whose expression is directly or indirectly affected by its absence or its gene dosage (Martin-Yken et al., 2002; Lagorce et al., 2003; Penacho et al., 2012), most of which have functions in cell cycle, cell wall synthesis and morphogenesis. In addition to the transcriptional control of G1/S transition mentioned earlier, Knr4 also participates in the transcriptional response to heat and cell wall stress. It is specifically required to allow Slt2 MAP kinase of the CWI pathway to correctly activate its targets (Fig. 1A). The physical interaction of Knr4 with Slt2 is in fact required for the correct signal transmission from the activated kinase to the transcription factors Rlm1 and SBF. Hence, Knr4 seems to act
Fig. 1. Knr4 is an intrinsically disordered Hub protein. (Central panel) The protein Knr4 is represented by a filled box. The disordered domains are in green and the central structured domain is in blue. The numbers of the amino acids residues limiting the domains are indicated. Red arrows indicate the positions of the phosphorylated Serine (S) or Threonine (T) residues. The functions of each domain are listed below. PPI is for protein–protein interactions, CWI is for cell wall integrity signalling pathway.

A. Knr4 is at crossroads of the calcineurin and CWI pathways. The proteins of the CWI pathway are depicted in green, those of the calcineurin pathway in blue. Proteins involved in the transcriptional regulation are represented in orange. When an essential phosphorylation is present, it is depicted by a circled P. The red arrows indicate the presence of a physical and/or genetics interaction with Knr4 figured in purple. The functions affected by these pathways are shown below the diagram.

B. Schematic representation of the main physical and genetic interactions with Knr4. The proteins of the calcineurin pathway are represented in blue, of the CWI pathway in green, the ones involved in stress response and transcription regulation in orange, and those of the polarisome in grey. Knr4 is depicted in purple.

C. Prediction of disordered regions of Knr4. The sequence of the protein Knr4 as represented in the central panel has been analysed using the VSL predicteur 2B (various short long 2B,(Obradovic et al., 2005)). The result of the prediction is represented by the plot in red. The disordered regions are represented in green and their limits are indicated. Three major disordered regions (above the average value of 0.5) are presented: the disordered domains 1 and 2 (respectively DD1 and DD2) and the disordered loop 1 (DL1).
as a modulator of Slt2 kinase activity, directing the kinase towards one or the other of its substrates during cell cycle progression and stress response, particularly heat shock (Martin-Yken et al., 2003; Dagkessamanskaia, 2010b). This functional interaction has been confirmed and enlarged to include the Cbk1 kinase, the terminal protein kinase in the RAM network (Kuravi et al., 2011). Thus, it is a group of the three proteins Knr4, Cbk1 and Bck2 that are actually critical for Slt2/Mpk1-dependent Rlm1 activation upon heat shock and cell wall stress conditions.

**Phosphorylation sites**

Knr4 is a phosphoprotein *in vivo*, as established by numerous global studies (Ficarro et al., 2002; Chi et al., 2007; Li et al., 2007; Smolka et al., 2007; Albuquerque et al., 2008). Several residues, which were identified as phosphorylated *in vivo* in *S. cerevisiae*, are conserved in its fungal homologs, and some have also been identified as phosphorylated in the model fission yeast *Schizosaccharomyces pombe* (Wilson-Grady et al., 2008) and the human pathogen *Candida albicans* (Beltrao et al., 2009). The distribution of these phosphorylated residues along the protein sequence is likely significant: most of them are located in the C-terminal domain of the protein within a stretch of 30 amino acids, predicted to constitute a PEST sequence (rich in Proline, Glutamic Acid, Serine and Threonine), and which is typical of rapidly degraded proteins (Rogers et al., 1986). Thus, the *in vivo* phosphorylation of these residues could be involved in the rapid degradation rate observed for Knr4 (Durand et al., 2008). This is fully consistent with its function in signalling and cell cycle progression, which generally require short lifespan proteins. Alternatively, they could mediate the removal of the C-terminal domain to allow the rest of the protein to interact with its partners at the right time and the right place, according to the proposed role of this domain (Dagkessamanskaia et al., 2010a,b). Moreover, two of these phosphosidues (S381 and S400) actually lie in consensus sites for phosphorylation by Pck1.

On the other hand, one threonine and two *in vivo* phosphorylated serine residues are located in the functional core of the protein (T198, S200 and S203) in the disordered loop 1 (DL1, Fig. 1). Specific replacement of Serine S200 and S203 by non-phosphorylatable alanine residues renders the mutant protein unable to suppress the *S. cerevisiae* null mutant sensitivity to drugs affecting cell wall, and to interact with a subset of its partners, notably the MAP kinase Slt2 and the calcineurin catalytic sub-unit Cna1 (Dagkessamanskaia et al., 2010a,b). In the global kinase phosphatase interactions network, Knr4 was placed as a direct neighbour to both Pck1 and Bck2. According to Breitkreutz and colleagues (Breitkreutz et al., 2010), Knr4 is a substrate of Pck1 in an endocytosis-related module and interacts with Bck2, a non-kinase/phosphatase hub in the network, responsible for integrating several different cell cycle and environmental inputs to determine the timing of cell cycle commitment.

**Multiple partnerships**

A strikingly high number of interactions (641 so far) have been described for Knr4, concerning 298 unique genes/features (*Saccharomyces Genome Database*, http://www.yeastgenome.org/). They include a huge number of genetic interactions (275), most of which are synthetic lethal interactions that highlight the crucial role played by this protein in the yeast cell (Goehring et al., 2003; Lesage et al., 2005; Costanzo et al., 2010). However, the number of pure physical interactions reported for Knr4 are also remarkably high: 38 different protein partners so far (Fig. 1B). These data come from several global interactions screens, conducted by different labs using distinct methods (Uetz et al., 2000; Ito et al., 2001; Goehring et al., 2003; Martin-Yken et al., 2003; Tong et al., 2004; Basmaji et al., 2006). These protein partners are involved in different cellular processes, but all related to morphogenesis and stress response. Many of them belong to signalling pathways, notably to CWI (Mid2, Pck1, Slt2, Bck2, Rlm1, Pil1) and the calcineurin pathway (Cna1) (Fig. 1B), including several kinases and phosphatases. Others Knr4 proteins partners have functions in bud emergence, development of cell polarity and secretion (Bud6, Act1, Cin8, Rvs167, Jnm1, Dnf1, Sec6, Sec26, Sec27, Gga2), transcription (Bas1, Cin5, Rlm1) and ubiquitination (Ubi4, Ubc1).

Another protein showing a strong physical interaction with Knr4 is Tys1, the only *S. cerevisiae* tyrosyl-tRNA synthase (Dagkessamanskaia et al., 2001, 2010a). It has been proposed that the two proteins are required for the formation of the mature spore wall, notably the specific dityrosine layer. Sporulation efficiency, tetrads formation and spore wall synthesis in diploid cells are all affected by the copy numbers of *KNR4* and *TYS1* (Dagkessamanskaia et al., 2001).

**Cellular localization**

A functional C-terminal GFP fusion of Knr4 displays a dynamic localization throughout the *S. cerevisiae* cell cycle. The protein is visible as foci at the presumptive bud site during G1 phase, then at the tip of small buds in G2, and later on relocates at the mother–daughter neck during M phase and cytokinesis (Martin et al., 1999). Upon pheromone exposure, the protein localizes as a bright focus at the shmoo tip (Dagkessamanskaia, 2010b). This localization is mostly similar to the one reported for the polarisome complex, both during mitosis and shmoo formation, which is coherent with the physical interaction observed between Knr4 and Bud6 as well as with the participation of Knr4 in the functioning of the morphogenesis checkpoint (Dagkessamanskaia, 2010b). Indeed, a recent global screen for genes required for the correct cellular localization of Knr4 confirmed the
direct or indirect involvement of BEM1, BEM2, BNI1, BUD6, PCL1, PCL2, SPA2, YCK1, RRD1 and TPD3 in this localization process (Liu et al., 2015a). Remarkably, this specific cellular localization pattern appears conserved among the fungal kingdom for Knr4 homologs, as indicated by fluorescent microscopy images from S. pombe, Neurospora crassa and Aspergillus nidulans (Matsuyama et al., 2006; Verdin et al., 2009; Schultzhaus et al., 2015).

Very interestingly, the cellular localization of Knr4 during the cell cycle might be required for its function in signalling and morphogenesis control. Detailed morphological data from the Saccharomyces cerevisiae Morphological Database (http://scmd.gi.k.u-tokyo.ac.jp/, recorded by the CalMorph Software (Ohtani et al., 2004; Ohya et al., 2005)) show that the knr4Δ mutant displays defects including abnormal bud neck width and mother-bud orientation regarding the mother-cell axis, possibly connected with a partial disorganization of the polarisome and/or other elements of the polarized growth machinery. In addition, Knr4 likely participates in the formation of shmoos by MATa cells upon exposure to α factor, because its absence strongly delays this phenomenon (Martin et al., 1999). Two different mechanisms might explain this effect: either the presence of Knr4 at the incipient shmoos site is necessary for the further onset of polarized growth in response to pheromone, or it could be connected to the function of Knr4 in cell cycle progression, which has to be arrested in G1 for shmoos formation to proceed.

Knr4 is an intrinsically disordered protein

The conformational flexibility of IDPs gives them the ability to be involved in multiple binding, where a single disordered region binds to several structurally diverse partners. Hence, hub proteins might fulfil their multiple signalling functions thanks to their intrinsically disordered nature (Dunker et al., 2005; Uversky, 2013). Multiple partners interactions are based on two mechanisms: the ‘one-to-many binding’ consist in the binding of a disordered region to different partners and the ‘many-to-one binding’ in different disordered regions binding to one structured partner (Dunker et al., 2005). As proposed in the review from Oldfield and Dunker (2014), a very good example of the former mechanism is given by the interactions between the tumour suppressor p53 (an IDP) and its multiple partners. Disordered regions of p53 are folded upon binding into alternative specific structures to fit with their multiple partners. In addition, post-translational modifications like phosphorylation are often involved in the structuration of unfolded regions. This has been nicely illustrated by the study of the completely disordered protein 4E-BP1 that binds the eukaryotic initiation factor 4F (eIF4E), folds upon binding (Gosselin et al., 2011) and becomes a regulator switch upon phosphorylation (Bah et al., 2015). We hypothesize here that a similar mechanism might take place between Knr4 and its partners, because it is also a partial IDP phosphorylated in a disordered loop.

Structural characteristics of Knr4

Sequence alignment of Knr4 with protein databases (Swissprot, MIPS, RCSB PDB, Pfam and ProDom) only reveals a ‘calcineurin-like phosphoesterase domain’ common to the Knr4 protein family. In silico analysis, combined with biophysical and biochemical methods, have shown that N-terminal (DD1, Fig. 1C) and C-terminal domains (DD2, Fig. 1C) of Knr4 are disordered, while the central core of the protein (aa 80–340) is structured with a small internal disordered loop (DL1, Fig. 1C). The three-dimensional structure of Knr4 is unknown to date, as well as those of its fungal homologs. However, the central core of Knr4 has been recently crystallized and secondary structure elements revealed, indicating that this domain is indeed able to adopt a stable conformation (Julien et al., 2015). This central core holds the essential of the protein biological function as attested by its ability to complement most of the S. cerevisiae null mutant phenotypes, while the two terminal parts are involved in ensuring and controlling the interactions of Knr4 with its protein partners (Durand et al., 2008) (Fig. 1). The physical interaction of the N-terminal domain (aa 1–80) with protein partners is necessary for the correct localization of the protein (Dagkessamanskaia et al., 2010b). Remarkably, N-terminal fusion with a GFP tag leads to a non-functional Knr4 protein, likely because it prevents the proper protein interactions. On the opposite, the large, massively disordered and phosphorylated C-terminal domain (aa 340–505) inhibits these interactions, possibly preventing non-specific interactions (Dagkessamanskaia et al., 2010a). It is thus possible that this domain is cleaved in vivo, to allow some specific functional interactions to happen. There are two indications of this: first, the C-terminal domain contains several PEST sequences, with multiple phosphorylated residues. Second, structural disorder is known to also serve as a weak signal for protein degradation (Tompa et al., 2008). Altogether, these data suggest that the central core might be sufficient to ensure Knr4 function and that PPI would be dispensable. However, global analysis of the complementation of the synthetic lethal interactions with KNR4 deletion by gene constructs expressing different parts of the protein contradicts this interpretation. While the central core is sufficient to rescue all the other knr4 synthetic lethal interactions, the N-terminal domain (aa 1–80) is indeed specifically required for the rescue of double mutants deleted for genes encoding members of the CWI pathway (Durand et al., 2008). Hence, the N-terminus of Knr4 becomes essential when CWI pathway is impaired. We can hypothesize that Knr4 interacts through this domain with another or other protein(s) whose functions allow cells to bypass an impaired CWI pathway.
Two known interactors of Knr4 may fulﬁl this role: Cna1, the catalytic subunit of calcineurin, because calcineurin becomes essential in the absence of a functional CWI pathway (Garrett-Engele et al., 1995), or Bck2, originally isolated as Bypass of C Kinase 2 (Lee et al., 1993).

Knr4 is a hub protein conserved among fungi

Hub proteins in eukaryotic PPI networks are signiﬁcantly enriched in functional categories related to signalling pathways and transcription control. Accordingly, the range of cellular functional activities affected by Knr4 protein includes signal transmission, interconnection or common scaffolding of different pathways, transcription, and progression through checkpoints. The presence of disordered domains in the Knr4 protein is also in agreement with the idea of this protein being a hub, because disorder is signiﬁcantly increased among eukaryotic PPI networks hubs (Dunker et al., 2005; Patil et al., 2010). Finally, another common characteristic of hub proteins is their rapid turnover and regulation (Batada et al., 2006), as was shown for Knr4.

Alignment of Knr4 sequence with all available ORF sequence translations in the databases shows homologs in the whole fungal kingdom, but none among higher eukaryotes. This contributes to the idea that the Knr4 family is a target of choice for developing new antifungal agents. Although no sequence homology is found within prokaryotes, predictions of tertiary structure have revealed some similarities with bacterial counterparts (Zhang et al., 2011). Phylogenetic analysis conducted with PhylomeDB v4 (Huerta-Cepas et al., 2014) indicate that the S. cerevisiae KNR4 gene is closely related to Ashbya gossypii NP_983957.1 and to C. albicans ortholog SMI1B, Fig. 2. Indeed, these two genes complement, to different extents, the cell wall-related phenotypes of a S. cerevisiae knr4 null mutant, while more distant genes such as those from N. crassa, and Aspergillus fumigatus fail to do so (Martin-Yken, FCWB 2012). However, functional and genomic data on homolog genes from N. crassa GS1 and S. pombe indicate that their products could perform very similar cellular functions in these organisms (see succeeding sections). The absence of complementation of the S. cerevisiae knr4 null mutant observed with these genes is likely caused by sequence differences in the unstructured domains of the protein, notably the N-terminal domain, which is responsible of establishing the interactions with partner proteins (Dagkesamanskaya et al., 2010a,b). Remarkably, the localization of Knr4 at the sites of polarized cell growth in S. cerevisiae appears conserved among the fungal kingdom, as attested by all data available at the moment (see succeeding sections), notably those on the product of S. pombe gene homolog (SPBC30D10.17c) and GS1 in N. crassa.

Candida albicans

The human pathogen C. albicans possesses 2 orthologs of KNR4: CaSMI1 and CaSMI1B. Global analysis of gene expression indicate that SMI1 expression is induced in hyphal cells by Cyr1, a Class III adenyl cyclase involved in the Ras-cAMP-PKA signalling pathway that controls C. albicans hyphal growth and virulence (Harcus et al., 2004), and repressed by the Hap43 iron-dependant transcription repressor and by caspofungin (Liu et al., 2005; Bonhomme et al., 2011; Singh et al., 2011). On the other hand, SMI1B transcription appears negatively regulated by Rim101p, a transcription factor required for alkaline-induced hyphal growth and involved in C. albicans virulence (Lotz et al., 2004). Separate deletions of the two coding genes have been conducted. They result in different phenotypes: smi1ΔΔ (homozygous deletion mutant) shows a clear hypersensitivity to CFW or sodium dodecyl sulfate treatment, whereas smi1BΔΔ is not affected (Lavie-Richard M., personal communication). Moreover, the smi1ΔΔ mutant is affected in cell wall β-glucan synthesis, biofilm formation and biofilm extracellular matrix production, as well as in the associated drug resistance (fluconazole) (Nett et al., 2011). Because these two C. albicans genes are able to complement the cell wall-related phenotypes of S. cerevisiae knr4 null mutant, the function of the proteins seems conserved between the two species. This is also supported by the finding that in S. cerevisiae, KNR4 affects filamentous growth, mucin secretion and agar invasion (Birkaya et al., 2009). Hence, the functional interaction observed in S. cerevisiae between Knr4 and calcineurin highlights the importance of studying the homologous proteins in C. albicans. Indeed, calcineurin is known to play an essential role in the molecular mechanisms that control morphogenetic switches (yeast–hyphae transition) at the basis for virulence in pathogenic fungi, such as C. albicans and Candida neoformans (Fox and Heitman, 2002; Kraus and Heitman, 2003).

Schizosaccharomyces pombe

The fission yeast genome contains only one homolog of KNR4, SPBC30D10.17c. This gene is essential according to S. pombe genome database (Kim et al., 2010a), www.pombase.org, and our unpublished data). Cellular localization of the corresponding protein O14362 (UniProt access number) has been investigated as part of a global ‘orfeome’ localization project and shown to occur at the cell division site (Matsuyama et al., 2006). Moreover, this gene belongs to a set of genes crucial for entry and maintenance of fission yeast cells in quiescence (Sajiki et al., 2009). Remarkably, this phenotype is reminiscent of one observed in S. cerevisiae: in an elegant screen for genes affecting senescence in budding yeast, Chang and colleagues have shown that deletion

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of KNR4 causes accelerated senescence and failure to recover from it, as well as a short telomeres phenotype in an est1.1 telomerase deficient background (Chang et al., 2011).

Kluveyromyces lactis

Interestingly, the Kluveyromyces lactis genome possesses two KNR4 gene homologs, one of which could have been acquired recently by horizontal gene transfer from bacteria, possibly from Aeromonas salmonicida or Bacillus species (Rolland et al., 2009). No functional characterization of either of these two homologs has been conducted to our knowledge.

Neurospora crassa

The homolog of the KNR4 gene in the model filamentous ascomycete fungus N. crassa has also been shown to encode a protein involved in cell wall morphogenesis, more specifically in β(1–3) glucans synthesis, and was thus named GS-1 for Glucan Synthase 1 (Enderlin and Selitrennikoff, 1994). Point mutants of this gene exhibit a typical hyperbranched phenotype, especially pronounced at high temperature (Seiler and Plamann, 2003; Reshefat-Eini et al., 2008) and a significantly enhanced sensitivity to caspofungin. In fact, the presence of caspofungin induces extensive hyperbranching of a gs-1 mutant even at 25°C. Remarkably, truncated versions of the gene are sufficient

Fig. 2. The Knr4 (Smi1) tree in Phylome 4. The KNR4 gene of S. cerevisiae (SMI1) was used as a seed sequence in Yeast phylome (P12b) using the PhylomeDB v4 server (http://phylomedb.org/, (Huerta-Cepas et al., 2014)).

A. The tree is represented by using the default settings of the server. The speciation nodes are in violet; the duplications nodes are in red.

B. Sequence alignments used to construct the tree. Each homology block is symbolized by a specific colour. The Smi1_Knr4 PFAM domain is symbolized by a purple diamond.
to complement the different mutants defects in cell wall synthesis (Enderlin and Selitrennikoff, 1994) and in elongation/branching of hyphae (Reshef-Eini et al., 2008). Thus, the C-terminal region of the protein seems unnecessary for the functioning of the GS-1 protein in morphogenesis, as for Knr4 in S. cerevisiae (Durand et al., 2008; Dagkessamanskaya et al., 2010a,b). GS-1 localizes as a bright spherical structure at the tip of growing hyphae, at the outer layer of the Spitzenkörper (Verdin et al., 2009). The Spitzenkörper – or apical body – is a fungal structure specific for true hyphae, located at the hyphal tip and responsible for their dramatic growth polarization (Girbardt, 1957; Harris et al., 2005). It is thought to function as a vesicle supply centre that regulates the delivery of cell wall-building vesicles to the apical cell surface (Bartnicki-Garcia et al., 1995; Virag and Harris, 2006). This extremely dynamic structure is absent in S. cerevisiae, but present in C. albicans hyphae and shares at least one common element with the polarisome, the Bni1 formin (Crampin et al., 2005; Jones and Sudbery, 2010). Fluorescent microscopy images of GS-1-GFP fusions at the tip of N. crassa hyphae (Sánchez-León et al., 2011) are strikingly reminiscent of the cellular localization of Knr4-GFP fusions at the tip of elongated shmoo in S. cerevisiae. In addition, very recent data (Schultzhaus et al., 2015) revealed a perfect conservation of this cellular localization in another ascomycete filamentous fungus, A. nidulans (also called Emericella nidulans as in Fig. 2). In the work of Schultzhaus and colleagues, the GsaA protein tagged with GFP can be observed as a sphere at the tip of growing hyphae (see Fig. 4 therein, and proposed model Fig. 4H).

**Distant structural homology to bacterial proteins**

Although classical sequence similarity search software only reveals fungal homologs for Knr4, novel sensitive sequence profile analysis methods recently led to the characterization of a structural superfamily named SUKH (for Syd, US22, Knr4 homology) that unites a diverse group of proteins including fungal homologs of Knr4, PGs2, FBXO3, SKIP16, Syd, herpesviral US22, IRS1 and TRS1, and several bacterial proteins (Zhang et al., 2011). Those in silico analyses of structural predictions indicate that SUKH proteins possess a versatile scaffold that can be used to bind a wide range of protein partners. Zhang and colleagues proposed that Knr4 homologs originally derived from bacterial toxin–immunity systems that had been recruited by double-stranded DNA viruses to perform multiple roles in intracellular survival and morphogenesis of these viruses. Then they would have transferred to lower eukaryotes, which re-used the versatile binding ability of this particular protein structure.

**Concluding remarks and outlook**

The most recent data available on KNR4 gene in S. cerevisiae and its homologs in other fungi finally make it possible to understand the multiplicity of its associated mutants phenotypes and synthetic lethal interactions. Indeed, it is now clearly established that the members of this protein family act as intrinsically disordered hub adaptors, allowing specific functional interactions to take place along stress signalling pathways, morphogenesis and cell wall synthesis events, which have to be tightly correlated with cell cycle progression. In addition, this ubiquitous role is coherent with the recent finding of more distant structural homologs in the bacterial kingdom. These structural similarities, so far only inferred in silico, do raise a puzzling question: by which mechanism did the SUKH gene family come to the fungal kingdom? Either by horizontal transfer or through viruses? In any case, fungi seem to have taken hints from smaller and more efficient organisms to help maintaining their shape in stressful conditions.

Nowadays, instead of looking for an idealistic unique and essential protein target for antibacterial, antitumor or antifungal agents, medical research has started targeting key proteins located at the junction of several essential biological processes. In that regard, hub proteins represent the Achilles’ heel of pathogenic organisms. Interconnecting several essential cellular processes such as resistance to cell wall stress, regulation of cell wall biosynthesis genes, cell integrity and calcium signalling pathways, cell cycle progression and morphogenesis, Knr4 is typically one of these relevant hub proteins. Because its functions are conserved in the fungal kingdom and specific to this kingdom, and given the importance of these functions in fungal processes involved in pathogenicity, targeting Knr4 connections with its partners may represent an efficient way to inhibit fungal growth.

Although the gene is not essential for growth in S. cerevisiae, it is in S. pombe, and hence could also be in other fungi. Moreover, even if KNR4 is not essential per se, its absence is lethal in numerous conditions, and notably when calcineurin and CWI pathways are weakened. Combination therapy using two or more antifungals to more efficiently treat patients with invasive fungal infections is already widely used (Johnson et al., 2004). We could thus imagine that inhibiting either the calcineurin or CWI pathways together with Knr4 would be an interesting fungicide strategy. Although some elements of these two pathways are not easily targetable due to their conservation in higher eukaryotes and accessibility issues, some others are already considered as very interesting (Liu et al., 2015b). Anyhow, the multiplicity of synthetic lethal interactions observed with the KNR4 gene deletion opens a search for a wide range of other targetable pathways. Hence, deciphering the 3D structure of Knr4 in complexes involving different partners might bring invaluable pieces of new information on the precise functions of this fascinating protein family in the near future, and the definition of its interacting domains will identify regions that can be targets for future antifungal agents.

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