Spitzenkörper assembly mechanisms reveal conserved features of fungal and metazoan polarity scaffolds

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The Spitzenkörper (SPK) constitutes a collection of secretory vesicles and polarity-related proteins intimately associated with polarized growth of fungal hyphae. Many SPK-localized proteins are known, but their assembly and dynamics remain poorly understood. Here, we identify protein-protein interaction cascades leading to assembly of two SPK scaffolds and recruitment of diverse effectors in *Neurospora crassa*. Both scaffolds are transported to the SPK by the myosin V motor (MYO-5), with the coiled-coil protein SPZ-1 acting as cargo adaptor. Neither scaffold appears to be required for accumulation of SPK secretory vesicles. One scaffold consists of Leashin-2 (LAH-2), which is required for SPK localization of the signalling kinase COT-1 and the glycolysis enzyme GPI-1. The other scaffold comprises a complex of Janus-1 (JNS-1) and the polarisome protein SPA-2. Via its Spa homology domain (SHD), SPA-2 recruits a calponin domain-containing F-actin effector (CCP-1). The SHD NMR structure reveals a conserved surface groove required for effector binding. Similarities between SPA-2/JNS-1 and the metazoan GIT/PIX complex identify foundational features of the cell polarity apparatus that predate the fungal-metazoan divergence.

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Eukaryotic diversity is manifest in a stunning variety of cellular form and function. From unicellular yeasts to multicellular plants and animals, the ability to polarize signaling, cytoskeleton and endomembrane trafficking underlies the fundamental processes of morphogenesis and differentiation. In a given cell type, polarization appears to involve the combined action of ancient functional modules, such as Rho GTPases and cytoskeletal elements, operating under the control of lineage-specific regulatory components. Intensively studied in the yeasts, polarization is a sequential process consisting of the selection of a defined cortical site, recruitment of polarity establishment proteins, F-actin polymerization, cytoskeleton-dependent recruitment of secretory and endocytic machineries, and reinforcement of the polarity axis through scaffold assembly and transport-mediated positive feedback (reviewed in refs. 5,16).

In certain cell types, such as neurons and fungal hyphae, persistent vesicle organizing centres assemble at sites of polarization. In the fungi, the Spitzenkörper (SPK) is a phase-dark structure seen by light microscopy at the growing hyphal tip in multicellular Ascomycetes (Pezizomycotina) and Basidiomycetes (Agaricomycotina) (reviewed in refs. 5,12). Electron microscopy reveals an actin filament-containing core, which is likely the phase-dark region, surrounded by ~70–100 nm secretory vesicles. In the Pezizomycotina, the core also contains ~40 nm micro-vesicles. Interestingly, macro- and micro-vesicles appear to transport distinct secreted cargoes, suggesting that they comprise different types of post-Golgi secretory vesicles.

Vesicles are delivered to the SPK through long-range microtubule-mediated transport, followed by short-range transport via type V myosin motors and actin filaments. The conserved exocyst complex promotes vesicle fusion with the plasma membrane and also appears to be required for SPK stability. Various polarity-related signaling proteins accumulate at the SPK. These include the nuclear dbf2-related (NDR) kinase COT-1 and associated proteins, and the polarisome protein SPA-2. In budding yeast, the polarisome comprises Spa2, Pea2, and the F-actin polymerization factors Bni1 and Bud6, all of which colocalize at sites of cell growth and are required to maintain proper cell shape. Recent work has identified an additional polarisome component, Aip5, which synergizes with Bni1 to promote F-actin polymerization, and Spa2 binds to Bni1 and Aip5 through a C-terminal domain, while its conserved Spa homology domain (SHD) interacts with MAP kinase components and Rab GTPase activating (GAP) proteins, suggesting that it plays a central scaffolding role. Pea2 is required for SPA-2 tip-localization. However, the precise function of Pea2 is unknown. The majority of characterized SPK proteins are conserved in budding yeast, which does not produce a persistent vesicle supply centre. In Neurospora crassa (N. crassa) and Aspergillus nidulans, BUD-6/BudA do not colocalize with SPA-2/SpaA in the SPK. Moreover, Pea2 has not been identified outside close relatives of budding yeast. Thus, the role of the polarisome, and determinants of the SPK’s unique features remain unclear.

Using the N. crassa model system, we previously identified Spitzenkörper-1 (SPZ-1) as a novel coiled-coil SPK protein present in the SPK-containing multicellular Ascomycota, but absent in budding and fission yeasts. Here, we show that SPZ-1 acts as a cargo adaptor allowing the MYO-5 motor to transport two distinct scaffold complexes to the SPK. One consists of Leashin-2 (LAH-2), which is required for SPK-residency of the signalling kinase COT-1, and the glycolysis enzyme GPI-1. The other is made up of a megadalton hetero-oligomer composed of SPA-2 and Janus-1 (JNS-1). SPA-2 employs its conserved SHD to recruit a novel calponin domain-containing F-Actin effector, CCP-1. The SHD NMR structure reveals a conserved surface groove required for effector binding. Similar interactions and sequence features of SPA-2/JNS-1 and the mammalian G protein-coupled receptor kinase interacting ArfGAP (GIT) p21-activated kinase-interacting exchange factor (PIX) scaffold suggests an ancestral relationship that predates the fungal/metazoan split. By contrast, SPZ-1 and LAH-2 appear to have evolved at key junctures leading to multicellularity in the Ascomycota.

**Results**

**Identification of SPZ-1 interacting proteins.** To identify N. crassa SPZ-1 interacting proteins, we employed immunoprecipitation (IP) and mass spectrometry. SPZ-1 co-precipitating proteins include MYO-5, SPA-2, and an uncharacterized protein, NCU03458. Based on its role in SPA-2 transport (see below), we name the latter JANUS-1 (JNS-1) after the Roman god of passages and transition. An epitope-tagged version of each pulls down the others (Fig. 1a and Supplementary Table 1), suggesting that they form a stable complex. SPZ-1 and LAH-2 are co-precipitate (Fig. 1a and Supplementary Table 1). All of these proteins possess conserved predicted coiled-coil domains (Fig. 1b), suggesting a basis for their interaction. In keeping with their co-precipitation, mGFP fusions produced from chromosomal loci all localize to the SPK (Fig. 1c). Deletion strains reveal diminished growth rates for Δspz-1, Δspa-2, Δjns-1, and Δlah-2, indicating that each plays an important cellular role (Fig. 1d). To assess their ability to nucleate complex assembly, we ectopically targeted SPZ-1, SPA-2 and JNS-1 to the peroxisome surface (see Methods). Each protein is sufficient to recruit GFP-tagged versions of the others to the peroxisome membrane (Fig. 1e). Moreover, each promotes the aberrant accumulation of peroxisomes at the SPK, indicating that the complexes formed are competent for MYO-5 engagement and transport (Fig. 1f).

**Ordered dependencies leading to SPK-localization.** We next employed sexual crossing to combine the tagged proteins and deletion strains in all possible combinations. These data reveal hierarchical relationships leading to SPK-residency (Fig. 2a). MYO-5 localizes to the SPK independently of all the other proteins. SPZ-1 only depends on MYO-5, while all others depend on SPZ-1. These findings suggest that SPZ-1 acts as a cargo adaptor allowing MYO-5 to transport SPA-2, JNS-1 and LAH-2. Relationships between SPA-2, JNS-1 and LAH-2 are more complex. SPA-2 depends on JNS-1, but JNS-1 retains weak SPK-residency in the absence of SPA-2. LAH-2 also retains weak SPK-localization in the absence of SPA-2 and JNS-1. None of the other proteins depend on LAH-2, suggesting that it is a terminal component of the localization pathway. Together, these data place MYO-5 upstream of SPZ-1, SPA-2 upstream of SPA-2, JNS-1 and LAH-2, and JNS-1 upstream of SPA-2.

Native polyacrylamide gel electrophoresis (native PAGE) was next used to investigate the formation of complexes by SPZ-1 interacting proteins (Fig. 2b and Supplementary Fig. 1). MYO-5 and SPZ-1 both migrate at approximately 800 kDa and their banding patterns are unaffected in the absence of the other proteins (Supplementary Fig. 1). By contrast, the banding patterns of JNS-1 and SPA-2 are interdependent (Fig. 2b). Both migrate as three bands of approximately 600, 900 and 1100 kDa. When either JNS-1 or SPA-2 is absent, the larger species of the other collapse to the 600 kDa band. This molecular weight is significantly higher than the ~100 kDa predicted molecular weights of SPA-2 and JNS-1, suggesting that they may both form homo-oligomers. To examine this possibility, we used heterokaryons to combine mGFP- and HA-tagged versions in the deletion background of the other. For both proteins, when precipitation is carried out with anti-GFP antibodies, HA-tagged versions are co-precipitated (Fig. 2c).
Together, these data show that SPA-2 and JNS-1 form homo-oligomers, which further associate to form two stable hetero-oligomeric species (Fig. 2b). The intimate relationship between JNS-1 and SPA-2 is further demonstrated by their dependency on one-another for pull-down by SPZ-1 (Fig. 2d). For JNS-1, this does not appear to be consistent with its weak SPK-localization in the SPA-2 mutant, which is presumably dependent on SPZ-1. Steady-state levels of JNS-1 appear to be diminished in the SPA-2 mutant (Fig. 2b), suggesting that it requires SPA-2 for stability. Thus, in the absence of SPA-2, JNS-1’s SPK-localization may be the result of its aberrant interactions. Alternatively, weak binding to SPA-1 may not be captured by IP.

Two functionally distinct domains in SPZ-1, SPA-2 and JNS-1. Data presented thus far show that SPZ-1 acts as a cargo adaptor allowing MYO-5 to transport SPA-2/JNS-1 and LAH-2 to the SPK. To investigate the basis for these interactions, we deleted discrete regions of SPZ-1 selected based on a combination of coiled-coil prediction and evolutionary conservation (Fig. 3a). Deletions were constructed at endogenous loci by replacing selected coding sequences with an in-frame mCherry-selectable marker fusion 37. The resulting variants were analysed for loss-of-function, localization, interaction, and steady-state protein levels. This analysis identifies two key regions whose absence leads to distinct SPZ-1 loss-of-function phenotypes (Fig. 3b, c and d).
Supplementary Fig. 2a). Deletion of the highly conserved region 3 coiled-coil domain abolishes the ability of SPZ-1 to precipitate SPA-2/JNS-1 and LAH-2. However, MYO-5 interaction (Fig. 3c) and SPK-localization (Fig. 2e) are retained, albeit at somewhat diminished levels, possibly due to diminished steady-state accumulation of this variant as compared to wild-type SPZ-1 (Supplementary Fig. 2a). By contrast, the coiled-coil region 6 deletion variant retains cargo binding, but abolishes MYO-5 binding (Fig. 3c) and SPK-localization (Fig. 2b), indicating that it is responsible for motor engagement. These data show that SPZ-1 binds to cargos and MYO-5 through distinct coiled-coil domains. JNS-1 and SPA-2 can also be dissected into two discrete functional regions (Fig. 3d, g and Supplementary Fig. 2b,c). N- and C-terminal coiled-coil domains of JNS-1 are essential for SPK-residency (Fig. 3e). However, only the N-terminal domain is required for hetero-oligomer formation with SPA-2 (Fig. 3f). SPA-2 possesses an essential coiled-coil domain and neighbouring sequences (regions 3 and 4), required for hetero-oligomer formation with JNS-1 (Fig. 3h, j) and SPK-residency (Fig. 3i). By contrast, deletion of the N-terminal SHD containing region 1 results in loss-of-function, but does not impair SPK-residency or hetero-oligomer formation with JNS-1, suggesting its exclusive association with effector recruitment. IP of variants corroborates these conclusions: the SPA-2 region 3/4 deletions impair precipitation with JNS-1, SPZ-1 and MYO-5, while the region 1 SHD deletion retains this ability (Fig. 3j).

NMR structure of the SPA homology domain. The SHD was originally identified as a direct repeat conserved between the mammalian polarity scaffold protein GIT and yeast Spa239. Because of its conserved and apparently central role in effector recruitment, we purified the N. crassa SHD and determined its NMR solution structure (Fig. 4 and Supplementary Fig. 3, PDB ID: 6LAG). The overall fold consists of six alpha-helical segments (Fig. 4b). The conserved direct repeats encode α-2 and α-3 (repeat 1), and α-4 and α-5 (repeat 2) (Fig. 4b, c). Conserved residues in these segments form a surface groove with a partially hydrophobic base and positively charged rims. Antiparallel arrangement of α-3 and α-5 form the groove base, while antiparallel α-2 and α-4 form the rims (Fig. 4b–d). In the mammalian SHD, the L288A mutation abolishes binding to Piccolo and FAK, but not to GIT39. Sequence alignment shows that L288 is conserved in the N. crassa SHD (L133) where it contributes hydrophobicity to the groove’s base (Fig. 4c, d). The L133A mutation in N. crassa SPA-2 leads to a full loss-of-function (Supplementary Fig. 4a), suggesting that fungal and metazoan SHD domains recruit effectors through a similar structural moiety.

Identification of a new SHD effector. Initial SPA-2 IP experiments did not identify N. crassa SHD effectors. We reasoned that this might be due to interference of detergents with binding to the SHD groove. Indeed, when detergent is excluded from IP washes, an uncharacterized protein (NCU00277) co-precipitates with SPA-2 (Fig. 5a, b). NCU00277 contains an N-terminal calponin homology domain and central coiled-coil domain (Fig. 5c). We therefore named it calponin-coiled-coil protein-1 (CCP-1). The L133A mutation significantly diminishes the ability of SPA-2 to bind CCP-1, indicating that they interact through the SHD (Fig. 5a, b). The calponin homology domain and coiled-coil region are both essential for CCP-1 function and localization to the SPK (Fig. 5c). Calponin homology domains occur in diverse actin regulatory proteins40. Thus, we next examined the impact of ccp-1 deletion on F-actin distribution. Loss of CCP-1 and its upstream regulators, lead to significantly diminished levels of SPK F-actin (Fig. 5d, e). Interestingly, CCP-1 loss-of-function also impairs SPK-incorporation of LAH-2, but does not affect SPK-residency of SPZ-1, SPA-1 or JNS-1 (Fig. 5f). Together, these data...
suggest that CCP-1 participates in transport-mediated positive feedback to stabilize SPK F-actin.

Identification of LAH-2 effectors. Data presented thus far identify an ordered cascade of protein-protein interactions leading to assembly and SPK-residency of two distinct SPK scaffolds. The SHD domain of the JNS-1/SPA-2 complex recruits the actin effector CCP-1 to the SPK (Fig. 5). By contrast, the role of LAH-2 remains unclear. We therefore screened proteins known to reside at the SPK for LAH-2 dependency (Fig. 6a). This identified the polarity-associated NDR kinase, COT-1 and its regulatory binding partner MOB-2A24 as LAH-2 clients. In unrelated work we found that the glycolysis enzyme glucose-6-phosphate isomerase (GPI-1) is localized to the N. crassa SPK. It also depends on LAH-2. All three proteins depend on SPZ-1, and like LAH-2 show diminished SPK-residency in the absence of SPA-2 and JNS-1 (Fig. 6a).

Scaffold mutants are not impaired in vesicle accumulation. Scaffold clients identified here are associated with signaling (COT-1/MOB-2A), actin regulation (CCP-1), and metabolism (GPI-1). However, none appears to be directly associated with vesicle trafficking. We concluded this study by examining the effect of scaffold loss-of-function on secretory markers. Remarkably, SPK-localization of markers associated with post-Golgi micro- (CHS-1) and macro-vesicles (GS-1)15 as well as markers of early- (YPT-1)
and late-Golgi/post-Golgi vesicles (YPT-31 and SYN-1), do not appear to be significantly altered in the mutants (Fig. 6b). Moreover, the inner and outer layers of the SPK also appear to form normally (Fig. 6c). Thus, while MYO-5 is known to be required for delivery of post-Golgi secretory vesicle delivery to the N. crassa SPK, the scaffolds identified here do not appear to be directly related to this process.

**Discussion**

Cell polarity requires the coordinated regulation of signaling, cytoskeletal dynamics, and membrane trafficking. Protein scaffolds act as points of convergence to organize these diverse activities. However, an overall understanding of these complex systems is lacking. Here, we characterize the assembly of Spitzenkörper scaffold complexes and effectors associated with F-actin reinforcement (CCP-1), signalling (COT-1) and metabolism (GPI-1). SPZ-1 plays a key role as cargo adaptor allowing MYO-5 to promote SPK-residency of the ancient polarisome-related SPA-2/ JNS-1 complex and the Pezizomycotina-specific LAH-2 scaffolds. Neither appears to be associated with MYO-5 dependent transport of vesicles to the SPK (Fig. 7). This has important implications, suggesting that parallel transport pathways can allow for SPK-specific regulatory interactions between scaffold clients and vesicles (see below).

Scaffold effectors support distinct activities associated with cell polarity. CCP-1 loss-of-function leads to significantly diminished SPK F-actin, suggesting that it acts as part of a transport-mediated positive feedback loop (Fig. 7a). Such a role for CCP-1 is consistent with findings in metazoan systems where non-muscle calponin proteins stabilize F-actin networks. COT-1 is a member of the ancient NDR kinase family and plays an essential role in cell polarity. By contrast, LAH-2 is non-essential, suggesting that its scaffold function plays a regulatory role to promote COT-1 activity. GPI-1 catalyses the second step in glycolysis. Its association with the SPK suggests coordination between metabolism and tip-growth. Resolving the functional consequence of this intriguing association will require more work.

Several findings support a model in which overall SPK assembly occurs through the concerted action of independent functional modules. The SPK-residency of LAH-2 depends on
SPA-2/JNS-1 (Fig. 2a). However, this relationship is not reciprocal. Loss of the SPA-2 effector CCP-1 also leads to diminished SPK-localization of LAH-2, but does not affect SPA-2/JNS-1. Together, these results support a model in which LAH-2’s dependence on SPA-2/JNS-1 is an indirect consequence of CCP-1’s absence at the SPK and a resulting diminishment in SPK F-actin. This may also be true of BNI-1 which displays similar dependencies as LAH-2 (Supplementary Fig. 4b). The apparently normal stratification of SPK secretory markers in scaffold deletion mutants (Fig. 6b, c) further attests to modular SPK assembly and the independent accumulation of scaffolds and vesicles, as well as differential sensitivity of SPK constituents to levels of SPK F-actin.

Scaffold deletion mutants retain residual levels of SPK F-actin (Fig. 5d) and BNI-1 (Supplementary Fig. 4b). The ability of Formin proteins like BNI-1 to nucleate F-actin polymerization is well-established, and in N. crassa BNI-1 is known to be an effector of RHO-1 and its nucleotide exchange factor LRG, which localize to the SPK and cell cortex, respectively. Thus, normal levels of SPK F-actin appear to be a product of the converging activities of BNI-1 controlled polymerization, originating from the cell cortex, and CCP-1 mediated stabilization, which depends on MYO-5 transport from sub-apical regions of the hypha.

A number of observations support an ancestral relationship between SPA-2/JNS-1 and the mammalian GIT/PIX polarity scaffolds. The SHD was first identified as a sequence repeat shared by metazoan GIT and yeast Spa-2 proteins. Previous work showed that Spa-2 and GIT recruit a variety of effectors through this domain. Here we show that as with GIT and PIX, C. neoformans SPA-2-1 (r1-r3) were deleted using an mCherry-selectable marker fusion as described in Materials and Methods. Variant functionality is scored according to the scale shown in the legend. Lower panels show localization of the indicated deletion variants. Dotted white lines show the hyphal outline. Scale bar = 10 µm. Source data are provided as a Source Data file.
JNS-1 possesses two essential coiled-coil domains. The N-terminal region 1 is required for complex formation with SPA-2, while both region 1 and the C-terminal region 3 are required for SPK-localization (Fig. 3d–f). These data suggest that JNS-1 has a primary function in promoting SPA-2 accumulation at the SPK. Despite an absence of primary sequence similarity, several commonalities suggest that budding yeast Pea2 performs an analogous function to JNS-1. Both possess predicted coiled-coil domains, and as with N. crassa SPA-2 and JNS-1, yeast Spa2 depends on Pea2 for localization to the bud tip33, Spa2 and Pea2 co-sediment in a large complex (12S by velocity sedimentation)28, and as with JNS-1, steady-state levels of Pea2 are diminished in the absence of Spa233. Furthermore, in yeast, Spa2 co-precipitates with Myo2 and depends on it for tip-localization48. Whether the Myo2 interaction is direct or requires Pea2 or another inter- mediary remains unclear. Nevertheless, combined with findings presented here, these observations suggest that SPA-2 proteins generally require coiled-coil binding partners and Myosin V motors for polarized accumulation. Future work can address whether a similar relationship exists with the GIT/PIX complex.

Surface features and dimensions of the SHD groove suggest a potential to bind an amphipathic alpha-helical segment through hydrophobic contacts with the base and charged interactions with the rim (Fig. 4). GIT forms a parallel dimer through its coiled-coil domain47. This arrangement positions two SHDs to potentially bind clients cooperatively. Interestingly, the GIT SHD linked to the dimerization domain interacts with piccolo more strongly than monomeric SHD39, suggesting that this may indeed be the case. Alternatively, the dimeric arrangement of SHDs could be exploited to promote interaction between distinct SHD-bound clients. In the future, powerful haploid genetics of N. crassa can be used to investigate how the oligomeric presentation of SHD influences its activity.

In metazoans, GIT and PIX regulate diverse polarity-related processes that include focal adhesion dynamics and cell migration49–51, organ development52–54, and synapse formation55 and dynamics56–58. From the perspective of domain organization, GIT/PIX are significantly more complex than their fungal counterparts. In addition to the coiled-coil, SHD and FAT domain, GIT proteins contain Arf GAP and Ankyrin repeat domains, while PIX contains calponin homology, SH3, Rho GEF, and PH domains59. These domain gains are likely to re- fuse its activity. 

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Interestingly, mammalian PIX contains a calponin domain, while in *N. crassa* and presumably other filamentous fungi, the calponin domain in CCP-1 is recruited through the SHD (Fig. 5).

With respect to regulation of membrane trafficking, in the GIT/PIX complex, the GIT ARF-GAP domain\(^6\) influences a variety of membrane trafficking events at the plasma membrane\(^6\). In yeast, the Spa2 SHD binds two related Rab GTPase activating proteins (GAPs), Msb3 and Msb4\(^7,8\), which display GAP activity towards Sec4\(^9\). In budding yeast, Myo2 transports post-Golgi secretory vesicles through sequential association with activated...
Rab GTases, Ypt31/32 and Sec465,66. Neurospora encodes a single Msh homolog, GYP-3, which was not captured in our pull-down experiments. However, recent work has shown that it indeed depends on SPA-2 for SPK-residency67 (Supplementary Fig. 4d). Furthermore, GYP-3 SPK-residency is also abolished in the SPA-2 L133A mutant (Supplementary Fig. 4e), indicating that recruitment occurs through the SHD. N. crassa MYO-5 has been shown to deliver post-Golgi secretory vesicles to the SPK23. However, scaffolds identified here do not appear to play a role in vesicle transport (Fig. 6b). These observations lead to a model in which MYO-5/SEC-4 tethered post-Golgi vesicles are likely to encounter high concentrations of SPA-2 clients such as GYP-3 only after delivery to the SPK. Such an arrangement could ensure that the link between vesicle and motor is only terminated in close proximity to the site of exocytosis.

In budding yeast, polarisome loss-of-function leads to abnormally shaped and enlarged cells, suggesting a primary function in morphogenesis2. By contrast, in N. crassa polarisome loss-of-function leads to diminished growth rate (Fig. 1d). An analysis of hyphal shape reveals minor defects in morphology as compared to loss-of-function in the endocytic component coronin (Supplementary Fig. 7), which affects both growth rate and morphogenesis68. Filamentous fungi such as N. crassa can display remarkably high rates of tip-growth, which can approach 1 μm per second69 and unlike yeast, morphogenesis and nuclear division are not coupled. Thus, the polarisome is likely to have diverged at the level of effectors to accommodate differing exigencies in hyphal budding and budding yeast. This idea is further supported by work in Ashbya gossypii and N. crassa, where SPA-2 localization transitions from a yeast-like tip crescent to a SPK sub-apical dot as the rate of hyphal tip growth increases27,70.

To better understand the evolutionary history of SPK components, we searched for related sequences in representative fungal and metazoan proteomes (Fig. 7b). SPA-2 SHD and FAT domains identify relatives throughout the fungi and metazoans. JNS-1, Pca2 and PIX distribution suggests that SPA-2/GIT proteins generally require a coiled-coil binding partner. Together, these findings point to an ancestral hetero-oligomeric polarisome scaffold that predates the divergence of fungi and metazoans. The analysis further implicates the serial advent of new protein domains that predates the divergence of fungi and metazoans. The analysis further implicates the serial advent of new protein domains that predates the divergence of fungi and metazoans.
Solutions nuclear magnetic resonance (NMR) analysis. The SHD from *N. crassa* SPA-2 (from glycine-8 to serine-217) was expressed as a HIS-tagged protein in E. coli BI21 in the presence of 12.8 g/L NaCl, 0.2 M HEPES-7H2O, 3 g/L KH2PO4, 0.5 g/L Na2CO3, 2 mM MgSO4, 0.1 M NaCl, 0.2% C12E6-glucose (Cambridge Isotope Laboratories, Inc. CLM-1396-5) and 0.1% W12E6-NaHCO3 (Sigma-Aldrich, 299321). The protein was purified under native conditions using Ni-NTA resin (QIAGEN, 30320), followed by gel filtration using Superdex75 column (GE Healthcare). Fractions containing the SHD protein were concentrated to 2 mM in 10 mM phosphate buffer at pH 6.5 with 1 mM EDTA, 1 mM DTT, 0.05% NaN3 and 5% D2O. All NMR experiments were performed on a Bruker Avance 800 spectrometer equipped with a cryo-probe at 25 °C. 2D HSQC, 3D HNCA77, HNCO, MQ, MQ-HSQC, TOCSY79 and 4D NOE60 were recorded using TOPSPIN software (www.bruker.de) without non-uniform sampling scheme. NMR spectra were processed using NMRPipe v10.8a and analysed using NMRFAME–Sparky v3.1082. Backbone and side-chain resonance assignments were achieved using the 4D NOEY-based strategy53. Unambiguous NOEs were obtained from three sub-spectra: 1 3C, 2 15N, 3 1H. Distance constraints were obtained from the NOEs assigned, while dihedral angle restraints of φ and ψ were calculated with TALOS+44 using the assigned chemical shifts of Cα, Cβ, N, Hα and HN. The structure was determined using distance and dihedral angle constraints derived from NOEs and chemical shifts (Supplementary Table 2). Except 15 proline residues, five N–H correlations (M1, R25, N34, K35 and G103) were not observed in the 2D HSQC spectrum and thus could not be assigned. The initial structure calculation was employed with Xplor-NIH using the conventional simulated annealing protocol from an extended conformation of SHD. Then the best folded models with the lowest total energy were selected for EEFX force-field minimization using Xplor-NIH. Both protocols employ the internal variable module and share the same basic scheme: (i) torsion angle dynamics at high-temperature (3,500 K) for 15,000 timesteps; (ii) torsion angle dynamics with simulated annealing, where the temperature is reduced from the initial high temperature value to 25 K in steps of 12.5 K, for a time of 0.4 ps per temperature step (respectively); (iii) 500 steps of torsion angle minimization; and (iv) 500 steps of Powell Cartesian minimization.

In the high temperature stage, experimental dihedral angle restraints and distance restraints were applied with respective force constants of kDNN = 10 kcal mol−1 rad−2 and kNN = 2 kcal mol−1 rad−2. In the simulated annealing stage, kDNN was set to 200 kcal mol−1 rad−2 and kNN was increased geometrically from 2 to 30 kcal mol−1 rad−2. The torsionDB statistical torsion angle potential was included with a force constant set to kφ = 0.02 kcal mol−1 rad−2 in the high temperature stage and ramped geometrically from 0.02 to 2 kcal mol−1 rad−2 during simulated annealing. A total of 100 EEFX force-field refined structures were calculated and 20 conformers with the lowest total energy were deposited with the PDB ID 6LAG and the assigned chemical shifts are deposited with the BMRB ID 36299.

Bioinformatics. Sequence conservation shown in Figs. 1b, 4a, 5a, d, and 7c was determined from the multiple sequence alignments which were constructed using MAFFT v6.2405. For each position in the alignment, the percentage of amino acids in each of the following groups was calculated: aromatic (phenylalanine, tyrosine, tryptophan), polar (serine, threonine, glutamine, asparagine), negatively charged (aspartic acid, glutamic acid), positively charged (lysine, arginine, histidine), hydrophobic (alanine, valine, leucine, isoleucine), others (glycine, cysteine, methionine, proline). The highest percentage will be used as the conservation score for that position. Potential metazoan homologs of fungal SPA-2 were identified by searching the human reference proteome (UP000000540) using an hhm profile constructed from the alignment of fungal SPA-2 sequences (Supplementary Fig. 7). Phylogenetic distribution, and sequence divergence of proteins indicated in Fig. 7b were determined from branch lengths of maximum likelihood trees inferred with RAxML v8.1.158 using input alignment constructed with MAFFT v6.240 and trimmed with TrimAl v1.2 as previously described33. Individual protein domains shown in Fig. 7b correspond to the following regions of the *N. crassa* sequences, SPA-2 SHD: amino acids 118–180, SPA-2 FAT: amino acids 753–873 (Uniprot ID: VISHM7), CCP-1 Pezizomycotina-specific coiled-coil containing region: amino acids 274–709. All proteomes used in these analyses are listed in Supplementary Table 3.

Statistics and Reproducibility. When representative images are shown (Fig. 1c, e, f; 2a, 3b, e, i; 5c, d; 6a–c and supplementary fig. 4b–e), five independent hyphae were imaged and one representative image is shown. The full dataset is available in the Source data file. Error bars in Figs. 1d, 5e and Supplementary Fig. 4a represent mean ± SD. Each measurement was made independently at least three times.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. A reporting summary for this Article is available as a Supplementary Information file. The source data underlying Figs. 1c–f, 2a–c, 3b, e, f, h, i; 5b–d, f; 6a–c and Supplementary Figs. 1a, b; 2a–c and 4a–e are provided in the Source data file. The *N. crassa* SPA-2 Spa Homology Domain (SHD) NMR structure is deposited with the Protein Data Bank under accession number PDB 6LAG [https://doi.org/10.2210/pdb6LAG/pdb] and the assigned chemical shifts are deposited with the Biological Magnetic Resonance Bank under accession number BMRB ID 36299 [https://doi.org/10.13018/BMRB36299]. Other data supporting the findings of this manuscript are available from the corresponding author upon request. Source data are provided with this paper.

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Author contributions
G.J. conceived the project. P.Z., J.Y.W., M.L., and T.A.N. performed experiments. T.A.N. performed bioinformatics analysis. D.W.Y. and J.S.F. performed the NMR structure analysis. G.J. wrote the manuscript with input from all authors.

Competing interests
The authors declare no competing interests.

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