Supplemental Information

Structurally unique interaction of RBD-like and PH domains is crucial for yeast pheromone signaling

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**Figure S2**
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**TABLE S1.** Structural statistics for the RBL$^{Ste11}$ domain.

| Restraints for structure calculation                        |       |
|-------------------------------------------------------------|-------|
| Total                                                       | 1696  |
| Total NOE                                                   | 1492  |
| Intra-residual                                              | 358   |
| Sequential $|i - j| = 1$                                                  | 431   |
| Medium range $1 < |i - j| < 5$                                                | 256   |
| Long range $|i - j| ≥ 5$                                                  | 447   |
| Hydrogen bonds                                             | 12    |
| Dihedral angles                                            | 172   |

**RMSD of the 20 lowest energy structures from the mean coordinates (Å)**

- Average backbone RMSD to mean: 1.02 +/- 0.35
- Average heavy atom RMSD to mean: 1.49 +/- 0.29

**Ramachandran plot statistics**

- Most favoured (%): 77.2
- Additionally allowed: 20.6
- Generously allowed: 1.1
- Disallowed: 1.0
Supplemental figure legends

Figure S1. Multiple sequence alignments of predicted PH domains of fungal Ste5 and Far1 proteins. (a) Multiple-query/multiple-template sequence alignment of PH domains from *S. cerevisiae* and *C. albicans* Ste5 and Far1 proteins with similar PH domain template structures. Color shading represents secondary structure elements, β-strands (cyan) and α-helices (yellow), predicted for queries and observed experimentally for templates (PDB codes in red). (b) Extensive multiple sequence alignment of PH domains from fungal Ste5 and Far1 proteins. Marked residues correspond to those mutated in this study, with colors corresponding to those in Figure 5A.

Figure S2. Multiple sequence alignments of predicted RBD-like domains of fungal Ste11 proteins. (a) Multiple sequence alignment of the RBD-like domain from *S. cerevisiae* with similar structures adopting ubiquitin fold (including various RBD domains and ubiquitin). Color shading represents secondary structure elements, β-strands (cyan) and α-helices (yellow), observed experimentally for the RBL<sub>Ste11</sub> domain from *S. cerevisiae* (this work; labeled on top), and for other structures (PDB codes in red). (b) Extensive multiple sequence alignment of RBD-like domains from fungal Ste11 proteins. Residues marked by triangles are functionally important as determined by random mutagenesis in this study, with symbol colors according to residue colors in Figure 5B (dotted connecting lines indicate double mutants). Residues marked by cyan squares interact with the PH<sub>Ste5</sub> domain according to the NMR experiments in this study (also shown in Figure 5C).

Figure S3. Stereoview showing the backbone superposition of 20 lowest-energy NMR solution conformations of the RBL<sub>Ste11</sub> domain structure. Flexible regions are labeled.

Figure S4. *In vitro* interaction of the RBL<sub>Ste11</sub> domain with the PH<sub>Ste5</sub> domain. (a) Surface plasmon resonance (SPR) analysis of bacterially expressed and purified RBL<sub>Ste11</sub> and PH<sub>Ste5</sub> domain. (b) Expanded region of NMR HSQC spectra of the RBL<sub>Ste11</sub>:PH<sub>Ste5</sub> complex. 15N-labeled RBL<sub>Ste11</sub> domain alone (red spectrum) and in complex with the unlabeled PH<sub>Ste5</sub> domain (black spectrum).

Figure S5. Structural convergence of the MD simulation of the PH<sub>Ste5</sub> domain. (a) Backbone root-mean-square deviations (RMSD) from the initial structure. (b) Backbone root-mean-square fluctuations (RMSF) averaged per each residue over the last 5 ns of MD trajectory (16<sup>th</sup> ns to 20<sup>th</sup> ns). The residues 517-527 show backbone RMSF values larger than 3 Å. (c) Overlay of 5 average 1-ns structures from the last 5 ns of MD trajectory (16<sup>th</sup> ns to 20<sup>th</sup> ns). The residues with backbone RMSF values larger than 3 Å are indicated.

Figure S6. Quality validation tests for the PH<sub>Ste5</sub> structural model. (a) Ramachandran plot from PROCHECK analysis (Laskowski et al., 1993), showing that the backbone phi and psi torsion angles for 98% of modeled residues occupy the most favoured and additionally allowed regions of the conformational space. (b) Global quality from ProSA analysis (Wiederstein and Sippl, 2007). The global Z-score is -2.6 for 164 residues (black dot), which lies within the space occupied by native protein structures determined by X-ray and NMR methods. (c) Local quality from Verify3D analysis of Profiles-3D scores (Luthy et al., 1992) in Discovery Studio v2.5 (Accelrys, Inc., San Diego, CA). Averaging over a 10-residue sliding window was applied. The backbone ribbon rendering of the model in the panel on right has the color and width proportional with the Verify3D score. Negative values (full red, widest ribbon) may represent regions of lower quality, which in the present model structure are the flexible, solvent exposed
segments with weak intramolecular packing: the flexible linker between the C-terminal αC and αC1 helices, and the tip of the longer β3-β4 hairpin loop.

**Figure S7.** Assessing potential PI-binding sites on the modeled PHSte5 domain. (a) Global views. PI binding sites are identified by PI molecules (yellow CPK models) extracted from PH domains superimposed onto the modeled PHSte5 domain. No structural refinement was attempted in order to optimize the interactions between PHSte5 domain and each PI. The positions of the canonical (PIA) and non-canonical (PIB) binding sites are obtained from the PH domains of DAPP1 (PDB code 1FAO) and β-spectrin (PDB code 1BTN), respectively. The PI shown is PI(4,5)P2 at both the PIA site (obtained after removal of the 3-phosphate from the PI(3,4,5)P3 bound in the template structure 1FAO) and PIB site (as complexed in the template structure 1BTN). The side-chains of four basic residues of Ste5 mutagenized previously in order to study PI-binding capacity of the PHSte5 domain (Garrenton et al., 2006) are represented as CPK models and labelled. (b) Close-up views of PHSte5 domain interactions at the canonical and non-canonical PI-binding sites. PI models are shown as ball-and-stick models, and select side-chains of charged residues in the vicinity of these sites are shown as sticks and labeled.

**Supplemental Experimental Procedures**

**Reagents**

Restriction endonucleases and DNA-modifying enzymes were obtained from New England Biolabs (Beverly, MA) and GE Healthcare (Quebec, Canada). High fidelity Expand thermostable DNA polymerase, and tablet protease inhibitors were purchased from Roche Molecular Biochemicals (Quebec, Canada). Acid-washed glass beads (450-600 μm), synthetic α-mating factor, protease inhibitors, and bovine serum albumin were purchased from Sigma (Ontario, Canada). Geneticin was purchased from Life Technologies (Ontario, Canada), and nourseothricin (clonNAT) from Werner BioAgents (Jena-Cospeda, Germany). Plasmids pGEX-4T-3 and pGEX-2TK, glutathione-Sepharose beads, glutathione, NiNTA resin, and protein A/G Sepharose beads were obtained from GE Healthcare. Vector pET15b was from EMD4Biosciences. The antibody against GST was described previously (Wu et al., 1999), and horseradish peroxidase conjugated secondary antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Nitrocellulose and FVDF membranes were purchased from Millipore. The enhanced chemiluminescence (ECL) assay system was purchased from Roche Molecular Biochemicals.

**Yeast strains and manipulations**

Yeast media, culture conditions and manipulations of yeast strains were as described (Rose et al., 1990). Yeast transformations with circular or linearized plasmid DNA were carried out after treatment of yeast cells with lithium acetate (Gietz et al., 1992; Rose et al., 1990). The yeast GST-ORF library was purchased from Invitrogen (Carlsbad, CA), and the yeast deletion strain collection was purchased from ATCC. The yeast strains used in this study are listed below:

| Strain     | Relevant genotype       | Source         |
|------------|-------------------------|----------------|
| W303-1A    | MATα ade2 ura3 his3 leu2 trp1 can1 | R. Rothstein   |
| W303-1B    | MAT ade2 ura3 his3 leu2 trp1 can1  | R. Rothstein   |
| BY4741     | MATα ura3 his3 leu2 met15      | ATCC           |
Plasmid construction and mutagenesis

To map protein-interaction boundary of PH\textsuperscript{Ste5} domain with Ste11, we used the yeast two-hybrid system (Y2H) developed recently in our lab for the detection of protein-protein interactions in cytoplasm (Cote et al., 2011); detailed information of this Y2H system will be published elsewhere. Briefly, this yeast two-hybrid system is based on the interaction of Ste11 (MAPKKK) and Ste50 that is required for the HOG pathway activation and osmoadaptation, which is critical for the survival of yeast cells under hyperosmotic stress. The interaction of Ste11 and Ste50 through their respective SAM domains that is required to activate the HOG pathway can be replaced by interaction of other protein interacting modules (Wu et al., 2006). We used this property to analyze the interaction of the PH\textsuperscript{Ste5} domain with Ste11. The PH\textsuperscript{Ste5} domain fragments were cloned into the SmaI site of plasmid pYL45 containing the fragment of \textit{STE50} encoding Ste50 protein without its SAM domain (aa 115-346) at the SalI site of pGREG503 (Jansen et al., 2005) through in vivo recombination (IVR) in yeast strain YCW1476. All the primers used for the PCR amplification reactions contain gene specific sequences and common sequences used for IVR in a layout as follows: 5’-ATTCTAGAGCGGCCGCACTAGTGGATCCCCGG-gene specific sequence (starting with ATG)-3’ for the forward orientation, and 5’-TCGATAAGCTTGATATCGAATTCCTGCAGCCGG-gene specific sequence (delete the stop codon)-3’ for the reverse orientation. To query the bait-prey interaction, IVR positive clones were selected and their ability to activate the HOG pathway measured as the ability to grow on hyperosmolarity media was scored.

For random mutagenesis of the PH\textsuperscript{Ste5} domain and to effectively select against nonsense mutations, we first generated the IFM-Ste50\Delta SAM construct in which the NAT1 (nourseothricin resistance marker) was cloned into pYL45 into the AatII site in frame with Ste50\Delta SAM, and the resulting plasmid was digested with Smal to add a stuffer URA3 marker as Smal fragment, which
replaces the N-terminal part (1-16aa) of Nat1 to create pVY0017. The error prone PCR of the PHSte5 domain (corresponding to aa 373 to 537) was performed with the primers OCW357 and OVY13 (the low case letters corresponding to the sequence of PHSte5 domain). Error prone PCR was carried out with Taq DNA polymerase with 5 mM MgCl2, 0.2 mM dNTP (each) mix, BSA (0.8 ug/μl) for 30 cycles. The PCR products were cloned into SmaI digested pVY17 through in vivo recombination in yeast strain YCW1476. Recombinants were first selected on SD-his plates, then on YP/Gal-nourseothricin plates for nourseothricin resistance (IMF function), and finally on SD/Gal –his + sorbitol hyperosmolarity plates. PHSte5 domain mutants that were unable to grow on high osmolarity media but were nourseothricin resistant indicative of defective binding to the RBLSte11 domain were picked. The PHSte5 domain mutants were transferred through IVR with pCW789 to make a CEN plasmid-born STE5 under its own promoter for further analysis. GFP-tagged Ste5 alleles were constructed by cloning the Ste5 mutants into pGREG576 according to the procedure as described (Jansen et al., 2005).

To create a Ste11 mutant with an internal in-frame deletion of the region encompassing amino acid residues 117-240 corresponding to the predicted RBL domain, primer OCW331 was used with pCW199 (Wu et al., 1999) as template using a Quickchange kit (Strategene). The mutagenesis also introduced unique restriction enzyme sites for XhoI and HpaI, and all the modifications were confirmed by DNA sequencing. This gave plasmid pCW850. A Smal-site ended URA3-stuffer was then put into the HpaI site of pCW850 to create pCW719 to be used for the mutagenesis analysis of the RBLSte11 domain. Random mutagenesis of RBLSte11 was performed by IVR of XhoI-digested pCW719 with error-prone PCR product of Ste11 fragment encompassing the RBL region. The loss of the URA3-stuffer marker was used as the selection of IVR product at the Ste11 BRL region.

To make Ste5 constructs carrying the SAM domain (corresponding to aa 29 - 131) of Ste50, we amplified the SAM domain using PCR with the primers OCW541 and OCW542R, and inserted it into STE5 at the XhoI site through in vivo recombination with XhoI digested Ste5 plasmids. The manipulation resulted in the Ste50-SAM domain fused to Ste5 C-terminally at the XhoI site.

Site-directed mutagenesis of STE5 and STE11 were performed either with either mutagenic sewing PCR (Ho et al., 1989) followed by in vivo recombination (IVR) in yeast, or with a Quick-change mutagenesis kit according to manufacturer’s protocol. All desired mutations were confirmed by sequencing. Oligonucleotides and plasmids used in this study are listed below:

### Oligonucleotides used in this work.

| Name    | Sequence                                                                 |
|---------|--------------------------------------------------------------------------|
| OCW331  | CGATGAATTCCAGAATTGTGATTCTCCTTctcgagttaaacTCCTCGTCAAATTTGGTTTGGCA          |
| OCW357  | ATTCTAGAGCGGCGCGCGACTAGGTGGATCCCCCCCAGGATGacacggtgcggtgta               |
| OCW358R | TCGATAAGCTTGTATATCGAATTCTCTGCGAGCCCGGAGGATTGgaatatataatctcgatt          |
| Plasmids used in this work. |
|-----------------------------|
| **Name** |                                                                 |
| pCW199 | pRS313-STE11::HIS3, own promoter, CEN/ARS, Wu et al, 1999. |
| pCW51 | pRS316-STE5::URA3, own promoter, CEN/ARS. |
| pCW185 | YCP50-STE11-1, CEN/ARS, (Stevenson et al., 1992). |
| pVY2 | pFO1a-Ste5(373-537), N-terminally His-tagged |
| pVY3 | pFO1a-Ste5(373-523), N-terminally His-tagged |
| pVY4 | pFO1a-Ste11(116-236), N-terminally His-tagged |
| pVY6 | pGEX-Ste5(373-537), N-terminal GST-fusion |
| pVY7 | pGEX-Ste5(373-523), N-terminal GST-fusion |
| pVY8 | pGEX-Ste11(116-236), N-terminal GST-fusion |
| pVY-1-18 | pGAL1-GFP-Ste5<sup>1504I</sup>::URA3, CEN/ARS |
| pVY31-11-11 | pGAL1-GFP-Ste5<sup>Q501K</sup>::URA3, CEN/ARS |
| pVY31-11-17 | pGAL1-GFP-Ste5<sup>F514L</sup>::URA3, CEN/ARS |
| pVY31-8-5 | pGAL1-GFP-Ste5<sup>wt</sup>::URA3, CEN/ARS |
| pVY13 | Derivative of pGAL1- PH<sup>Ste5</sup>-Ste50ΔSAM::HIS3 where NalI was |

Plasmids used in this work.
inserted into AatII site in frame with upstream PH<sup>Ste5</sup> domain and downstream Ste50ΔSAM, CEN/ARS and HIS3 as plasmid selection marker.

| Plasmid | Description |
|---------|-------------|
| pVY17   | Derivative of pVY0013 where PH<sup>Ste5</sup> domain and the part of Nat1 were replaced by URA3-stuffer marker for IVR selection at SmaI sites. |
| pCW719: | p<sup>STE11</sup>RBL(117-240)-<sup>URA3</sup>::HIS3, own promoter, CEN/ARS, URA3-stuffer marker for IVR selection, HIS3 for plasmid selection, XhoI digestion for IVR. |
| pCW789  | p<sup>STE5</sup>ΔPHD(373-538)-<sup>HIS3</sup>:<sup>URA3</sup>, own promoter, CEN/ARS, HIS3-stuffer marker for IVR selection, <sup>URA3</sup> for plasmid selection, Smal digestion for IVR. |
| pCW790  | p<sup>STE5</sup>ΔPHD(373-538)-<sup>URA3</sup>::HIS3, own promoter, CEN/ARS, URA3-stuffer marker for IVR selection, HIS3 for plasmid selection, Smal digestion for IVR. |
| pCW850: | p<sup>STE11</sup>RBL(117-240)::HIS3, CEN/ARS, HIS3 for plasmid selection, Hpa1/XhoI sites introduced at the deletion. |

**Yeast HOG, pheromone response and other assays**

Halo assays to test cell growth inhibition in response to α-mating factor, assays for the ability of cells to grow on hyperosmotic media to test the function of the HOG pathway, and yeast extract preparation and Western blot analyses were performed as described previously (Wu et al., 1999). Quantitative β-galactosidase reporter assays for the pheromone response and HOG pathways were performed as described (Tatebayashi et al., 2006; Wu et al., 1999). GFP fluorescence photomicroscopy was performed as previously described (Wu et al., 1999).

**Protein expression and purification**

Various Ste5 and Ste11 fragments were cloned into pGEX or pFO1a (a derivative of pET15b) vectors, and expressed in the BL21 or Rosetta pLys strains of *E. coli*. Protein was expressed in *E. coli* grown in 2YT medium supplemented with 200 mg/l ampicillin. Cultures were grown at 25°C to an OD<sub>600</sub> of 0.5-0.6. The temperature was then decreased to 15°C and protein expression was induced with 1 mM IPTG. After incubation at 15°C overnight, cells were harvested by centrifugation. Cells were re-suspended in PBS supplemented with 300 mM NaCl, 10 mM imidazole and 1 mM DTT, and lysed by sonication. The lysate was cleared of cell debris by centrifugation at 37,000 x g for 1 hour at 4°C. All His-tagged fusion proteins were purified by affinity chromatography using NiNTA resin (GE Healthcare) using standard protocols. The GST fusion proteins were purified by affinity chromatography on glutathione Sepharose according to a modification of the manufacturer’s recommendation. Cleared lysates were incubated with resin on ice for 30 minutes. Resin was then collected in a column and washed with 50 ml of 20 mM HEPES, pH 7.5, 300 mM NaCl, 5 mM DTT. For resin binding assay, the beads were blocked for 30 min with the same buffer supplemented with 1% BSA and 0.1% Triton X-100 before incubation with binding partners to be tested. For further purification of proteins for biophysical studies, the bound fusion protein was cleaved with thrombin for 4 hours at room temperature and the protein was eluted in the HEPES buffer. Size exclusion
chromatography was done on a Superdex 75 10/30 column (GE Healthcare) equilibrated with 20 mM HEPES, pH 7.5, 300 mM NaCl, 5 mM DTT. The buffer was sparged with argon for 30 minutes before use. The flow rate was 0.5 ml/min and the sample volume was 200 μl. Protein concentrations were estimated by the Bradford method and the BCA (Pierce) method following the protocols supplied by the manufacturers.

Yeast GST fusions of the small GTPases of Ras/Rho family were cloned from the GST-ORF library collection. The expression and purification of the GST fusion onto glutathione Sepharose beads, and subsequent resin binding assay with bacterially expressed His-tagged RBL\(^{\text{Ste11}}\) were carried out in conditions as previously described (Annan et al., 2008). The presence His-tagged protein and GST fusions were analyzed by Western-blotting with anti-His and anti-GST antibodies respectively.

Surface plasmon resonance analysis of protein-protein interaction

Surface plasmon resonance analysis of the RBL\(^{\text{Ste11}}\).PH\(^{\text{Ste5}}\) domain interaction was performed using a Biacore 3000 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ). The PH\(^{\text{Ste5}}\) domain was covalently immobilized to a CM-5 sensorchip using standard amine coupling methods. Briefly, a 35 μL injection of a mixture of 0.05 M N-hydroxysuccinimide (NHS) and 0.2 M N-ethyl-N'-(3-diethylaminopropyl) carbodiimide hydrochloride (EDC) was followed by the manual injection of His-tagged PH\(^{\text{Ste5}}\) domain diluted 100-fold in 10 mM sodium acetate pH 5 until approximately 700 RU immobilization level was reached. The remaining activated surface groups were inactivated by a 35 μL injection of 1 M ethanolamine. Similarly, a blank control surface was created by NHS / EDC treatment followed immediately with ethanolamine.

Using the KINJECT command, various concentrations of RBL\(^{\text{Ste11}}\) domain in running buffer containing 10 mM HEPES, 0.02% Tween 20, 50 μM EDTA, pH 7.4 were injected over the PH\(^{\text{Ste5}}\) surface for 2 minutes at a flow rate of 25 μL/min and a dissociation of 300 s. The PH\(^{\text{Ste5}}\) surface was regenerated for subsequent RBL\(^{\text{Ste11}}\) domain injections using a 15 s pulse of 20 mM HCl. Sensorgrams were aligned and double referenced to the control surface using buffer injections, and analyzed by both global fitting to a 1:1 interaction and steady state analysis with BiaEvaluation (v3.2) software.

Structural bioinformatics

Homologous protein sequences were retrieved by either BLASTP or TBLASTN search (Altschul and Lipman, 1990) in the Fungal genome database (http://seq.yeastgenome.org/) and by protein domain architecture search in the SMART database (http://smart.embl.de/; (Letunic et al., 2006)). Comparative sequence analysis of the assembled datasets (29 Ste11-like, 27 Far1-like and 18 Ste5-like sequences) was carried out with MUSCLE (Edgar, 2004a, b) and MAFFT v6 (Katoh et al., 2005) for deriving multiple sequence alignments with the L-INS-i iterative refinement algorithm and to generate phylogenetic trees, and visualized using Jalview v2.4.0B2 (Waterhouse et al., 2009).

Structural fold detection for regions of representative Ste5, Far1 and Ste11 fungal proteins was carried out at the Structure Prediction Meta Server (http://bioinfo.pl/meta/), which assembles state-of-the-art fold recognition methods, and provides a consensus sequence-to-structure scoring using the 3D-Jury meta-predictor (Ginalska and Rychlewski, 2003). Multiple-queries/multiple-templates sequence alignments were assembled using a combination of: (i) multiple sequence alignment of the queries using MAFFT v6 (Katoh et al., 2005), (ii) structural-
based sequence alignment of the templates using 3D-Coffee (Armougom et al., 2006), (iii) 3D-Jury consensus fold recognition based alignment between queries and templates, (iv) manual minor local improvements in the overall alignment of secondary structure elements predicted using SAM-T02 with DSSP and STRIDE alphabets (Karplus et al., 2005), and (v) 3D-structural alignment by alternate domain fit in Swiss-PdbViewer v4.0.1 (Guex and Peitsch, 1997).

**Model building and refinement**

Homology modeling of the PH domain of *S. cerevisiae* Ste5 was done in MODELLER v9.1 (Fiser and Sali, 2003a, b; Marti-Renom et al., 2000). Using a multiple-queries/multiple-templates sequence alignment (Figure S1) several hundreds of initial models were built and ranked based on global and residue-based DOPE scores (Shen and Sali, 2006). Based on this analysis, the structure of PH domain of the guanine nucleotide exchange factor collybistin (PDB code 2DFK) (Xiang et al., 2006) was selected as the most suitable template structure, providing a good overall sequence and secondary structure alignment together with structural homology in the functionally important N- and C-terminal extensions beyond the canonical PH fold. A stepwise loop refinement protocol was devised, consisting of sequential refinement of structurally adjacent loop pairs, using the DOPE score to select best the model at each stage.

The structure resulted from the loop refinement protocol was used as input for a classical MD simulation carried out under the AMBER force field with the FF03 parameters (Duan et al., 2003; Lee and Duan, 2004) using the AMBER 9 software (Case et al., 2005). The structure was solvated in a truncated octahedron TIP3P water box (Jorgensen et al., 1983), and electroneutrality was achieved by adding Na\(^+\) counterions. Applying harmonic restraints with force constants of 10 kcal/(mol Å\(^2\)) to all solute atoms, the system was energy-minimized first, followed by heating from 100 to 300 K over 25 ps in the canonical ensemble (constant number of particles, volume, and temperature, NVT) and by equilibrating to adjust the solvent density under 1 atm pressure over 25 ps in the isothermal-isobaric ensemble (constant number of particles, pressure, and temperature, NPT) simulation. The harmonic restraints were then gradually reduced to 0 with four rounds of 25-ps simulations. A 20-ns production NPT run was obtained with snapshots collected every 1 ps, using a 2-fs time step and 9-Å nonbonded cutoff. The Particle Mesh Ewald method (Darden and Pedersen, 1993) was used to treat long range electrostatic interactions, and bond lengths involving bonds to hydrogen atoms were constrained by SHAKE (Ryckaert et al., 1977). Standard analyses of MD trajectories were carried out with PTRAJ in AMBER 9. Model validation was carried out with PROCHECK (Laskowski et al., 1993), ProSA (Wiederstein and Sippl, 2007), and Verify3D (Luthy et al., 1992).

**Protein production for NMR spectroscopy**

Constructs expressing the RBL\(^{Ste11}\) domain (aa 116-236) were prepared using the expression vector pFO1, a derivative of pET15b (Novagen), to obtain an N-terminal His\(_8\)-tagged thrombin-cleavable construct. After verification by DNA sequencing, the constructs were transformed into *E. coli* Rosetta pLysS (Novagen) for protein expression. The RBL\(^{Ste11}\) domain was produced in minimal medium (M9) enriched with \(^{15}\)N-ammonium chloride or \(^{15}\)N-ammonium chloride /\(^{13}\)C-glucose and expression was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside at 20 ºC for 18 hrs. The recombinant protein was purified by standard immobilized metal affinity chromatography using Ni\(^{2+}\)-NTA resin (Qiagen). The protein was eluted with buffer containing 350 mM imidazole, and the His-tag was cleaved using thrombin.
For studies of RBL/PH domain interactions, His-tagged PH$^{\text{Ste5}}$ domain (aa 373-537) was used, produced and purified as described above.

**NMR spectroscopy**

Samples for NMR measurements were prepared in 50 mM sodium phosphate buffer pH 6.8, 200 mM NaCl, 5 mM DTT, 0.02% (w/v) NaN$_3$ at protein concentrations of 1.2 – 1.5 mM. All NMR data were collected at 300 K on a Bruker Avance500 spectrometer equipped with a triple-resonance cryoprobe and with z-gradient pulse field gradient accessories.

Sequence-specific backbone and aliphatic side chain chemical shift assignments for the RBL$^{\text{Ste11}}$ domain were obtained using combined HNCA, CBCA(CO)NH, HNCACB, H(CCCO)NH, HBHA(CO)NH and (H)CC(CO)NH experiments. The $^1$H chemical shift assignments for aromatic side chains were based primarily on 2D DQF COSY, TOCSY and NOESY experiments. The $^1$H chemical shifts were referenced directly to internal DSS at 0 ppm and the $^1$C and the $^{15}$N chemical shifts were referenced indirectly to DSS. NMR spectra were processed using XWINNMR (Bruker Biospin) and analysed with programs CARA, XEASY (Bartels et al., 1995), Sparky (Goddard and Kneller), SPARTA and MARS (Jung and Zweckstetter, 2004). The interaction between the $^{15}$N-labeled RBL$^{\text{Ste11}}$ and the PH$^{\text{Ste5}}$ domains was studied by recording the HSQC spectra of $^{15}$N-labeled RBL domain, titrated with unlabeled PH domain. To assist in assigning shifted NMR signals in $^1$H-$^{15}$N HSQC spectra, amino acid specific $^{15}$N-enriched samples were prepared using defined media and $^{15}$N-labeled valine and leucine.

**NMR structure calculation**

The 3D $^1$H-$^{15}$N NOESY-HSQC, 3D $^1$H-$^{13}$C NOESY-HSQC (in $^2$H$_2$O) and 2D homonuclear NOESY experiments were used to collect NOE-restraints for structure calculation. A mixing time of 100 ms was used in all experiments. Dihedral restraints were derived from $^{13}$C$^\alpha$, $^{13}$C$^\beta$, $^{13}$C$'$, $^1$H$^\alpha$ and $^{15}$N chemical shifts using the program TALOS (Cornilescu et al., 1999). Structure calculations were performed using CYANA 2.1 (Güntert, 2004). NOE upper limit distances were used together with dihedral angles in the standard CYANA protocol of seven interactive cycles of calculations. Automatic NOE assignments from CYANA were manually verified/corrected. The 20 final structures with lowest energy or violations were retained for refinement. Hydrogen bond constraints derived from $^1$H, $^{15}$N HSQC exchange experiment were added at the last stage of calculations. A summary of the results is given in Table S4, each structure was refined by conjugate-gradient energy minimization using the AMBER force field (Hornak et al., 2006) and a distance-dependent dielectric constant in a stepwise protocol in which the flexible termini were relaxed first, followed by minimization of the side-chains, and finally full relaxation of the structure.
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