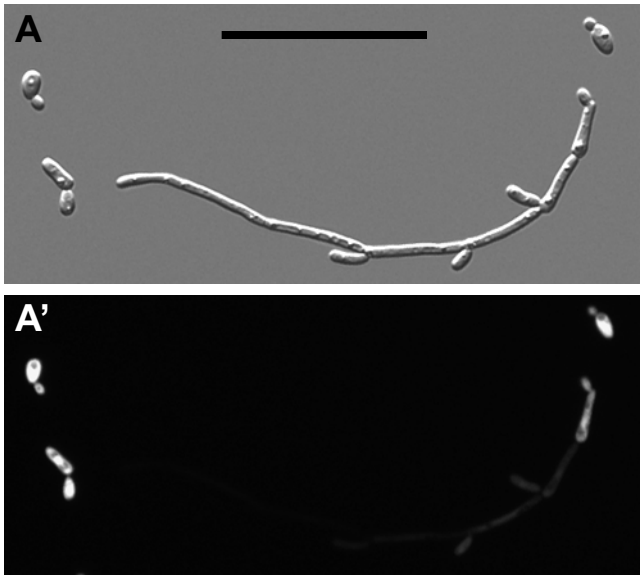
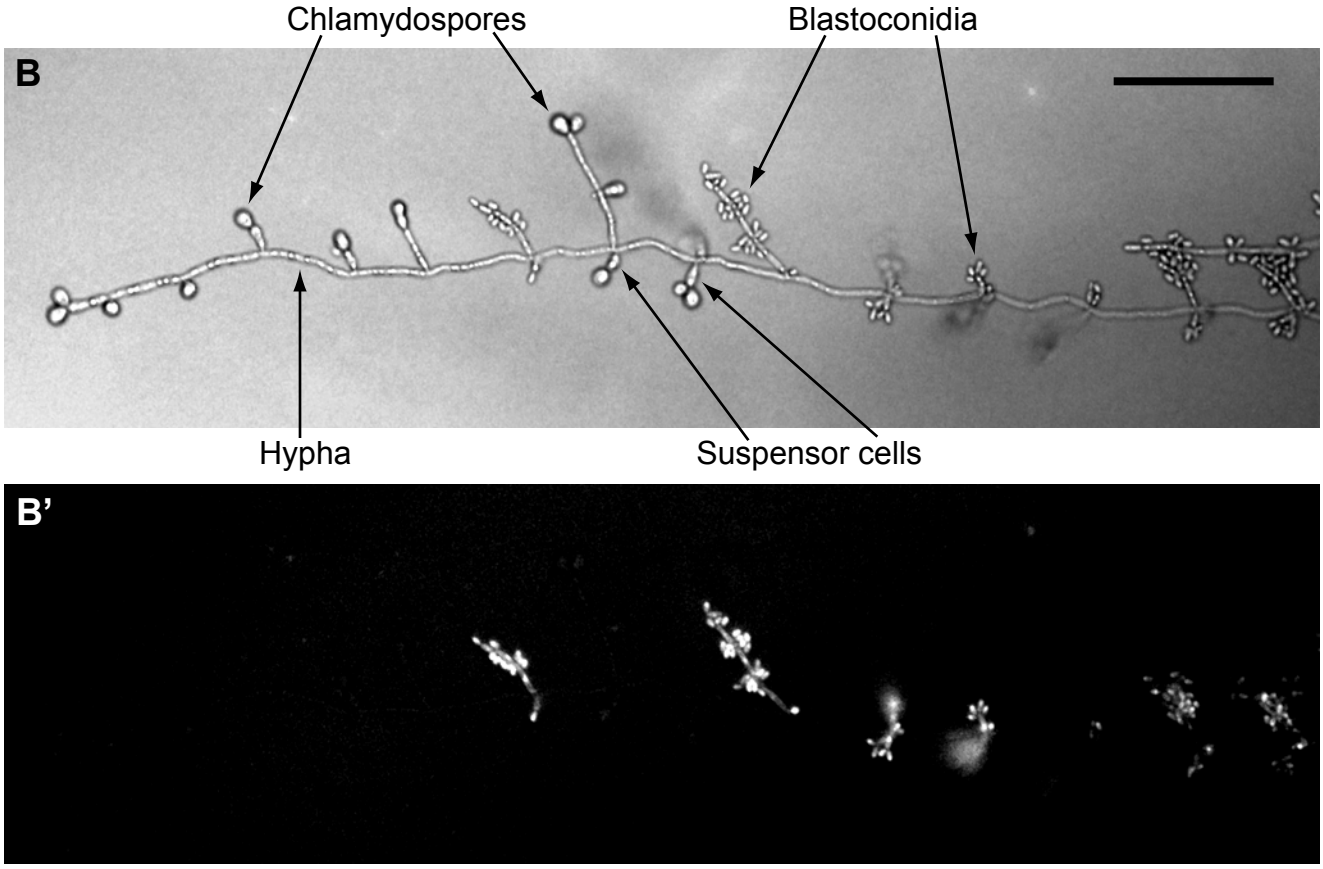
Figure S1

(BL Granger 2012 Eukaryotic Cell)



Expression patterns of *YWP1* as reported by cytosolic GFP

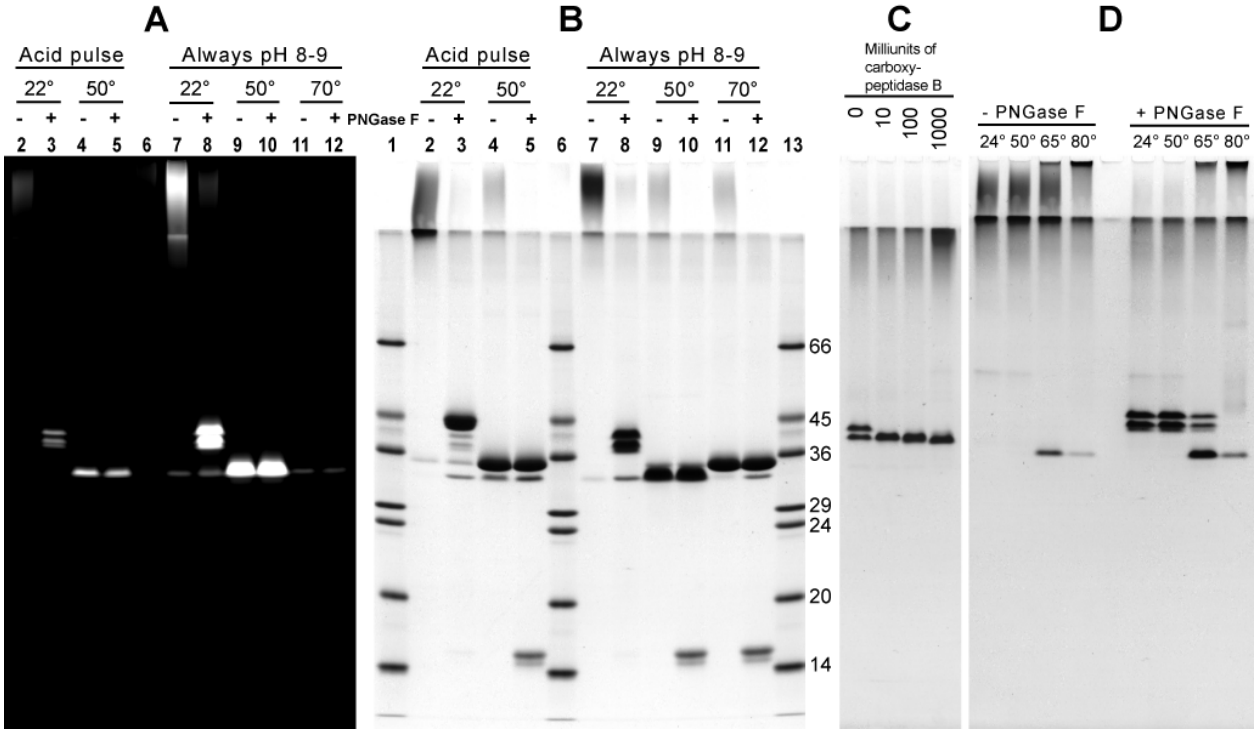
A: Strain BJ3a1a was grown for one day at 37°C in the filamentation medium of Lee prior to visualization by light (A) and fluorescence (A') microscopy. The GFP is prominent in yeast forms (blastoconidia), but not in filamentous forms. The cytosolic GFP reporter in these cells may have passively entered daughter germ tubes by diffusion, but it eventually diluted to undetectable levels in filaments that do not express *YWP1*. Once incorporated into the cell wall, Ywp1 itself likely shows little or no movement into daughter cells. Bar = 50 μm.



B: Strain BJ3a1a was grown for 12 days at room temperature under a coverslip on cornmeal agar containing 0.2% Tween 80 prior to visualization by light (B) and fluorescence (B') microscopy. The GFP is prominent in yeast forms (blastoconidia), but not in filamentous forms or chlamydospores. Blastoconidia that arose from the same hyphae as the suspensor cells and chlamydospores show strong GFP fluorescence, demonstrating that there was sufficient oxygen present in this environment for GFP chromophore maturation. Bar = 50 μm.

Figure S2

(BL Granger 2012 Eukaryotic Cell)



Analysis of secreted Ywp165-GFP

Ywp165-GFP samples (culture supernatants concentrated by ultrafiltration) were first digested with PNGase F (or left undigested), then given SDS and either kept at 22°C, heated to 50°C for 5 min (which dissociated the propeptide from the GFP moiety), or heated to 70°C for 5 min (which denatured both polypeptides); before adding the SDS, one set of PNGase F digests was acidified with HCl to pH 1 for 5 min and then realkalinized with Tris base, as indicated ("acid pulse"). SDS-PAGE was followed by laser scanning for GFP fluorescence (panel A) and then staining for protein with Coomassie Blue R-250 (panel B). The N-glycosylated propeptide runs as a smear in the stacking gel, whereas the deglycosylated propeptide runs slightly above the 14 kDa marker in these samples that were reduced with DTT at the time of SDS addition. In panel C, PNGase F-digested Ywp165-GFP samples were digested with 0-1000 mU carboxypeptidase B for 70 min, mixed with SDS (without disulfide reduction), resolved by PAGE, and stained with Coomassie Blue. In panel D, Ywp165-GFP samples were digested with PNGase F or left undigested; they were then incubated for 5 min at the indicated temperatures prior to resolution by PAGE in the absence of any SDS.

Detailed Explanation

When mixed with SDS at room temperature, the Ywp165-GFP chimera penetrated the polyacrylamide gel poorly during electrophoresis, remaining largely in the stacking gel (panel A, lane 7). When treated with PNGase F, however, which removed the N-glycan from the Ywp1 propeptide, the GFP fluorescence and the Coomassie-stainable protein migrated to the center of the resolving gel (panels A and B, lane 8). This demonstrated that the large N-glycan retarded movement of the chimera through the gel, and that the propeptide remained associated with the GFP under these conditions. Heating of the SDS-containing sample to 50°C before electrophoresis allowed the GFP to migrate even farther into the resolving gel, as it separated the GFP from the N-glycosylated Ywp1 propeptide (panels A and B, lane 9). Pre-digestion with PNGase F freed the propeptide from its N-glycan, allowing the deglycosylated propeptide to migrate to the bottom part of the gel (panel B, lane 10); otherwise, it remained in the stacking gel (panel B, lane 9). Heating in SDS to 70°C denatured the GFP, resulting in the loss of its fluorescence (panel A, lanes 11 and 12) and a slightly altered mobility (panel B, lanes 11 and 12). As shown in lanes 2-5 of panels A and B (compared to lanes 7-10), briefly subjecting the chimera to a pH of 1 prior to SDS exposure at pH 8 denatured most of the GFP, resulting in fluorescence loss and a mobility shift; the Ywp1 propeptide, either with or without its N-glycan, remained

associated with the GFP-containing moiety during this treatment. Subsequent treatment with SDS at 50°C, but not at room temperature, dissociated the propeptide.

Additional experimentation explained the unexpected multiplicity of some of the bands. The deglycosylated (PNGase F digested) Ywp165-GFP chimera routinely appeared as a doublet in this gel system (*e.g.*, panel C, left lane); a third band under this doublet appeared and increased in quantity as samples aged during storage at 0-4°C (*e.g.*, panels A and B, lane 8). The two bands of the doublet were excised from a gel and subjected separately to mass spectrometric analysis, which dissociated the non-covalently associated propeptides from the GFP-containing moieties, but did not cleave the peptides (data not shown); remarkably, the masses were measured as 11,058 for the upper band and 10,902 for the lower band, differing by the mass of one arginine (156 Da), and exactly corresponding to the predicted masses of the propeptides with the two cysteines disulfide bonded to each other and the asparagine converted to aspartate by PNGase F. Thus, the two bands of the gel doublet represent propeptides with either one or no arginine at the C-terminus (…STAAIR and …STAAI), and lengths of 100 aa and 99 aa. For both samples, the dissociated GFP moiety gave a peak at 30,060, close to the predicted 30,211. No peak was seen that might correspond to the peptide that lies between the tribasic site and dibasic sites of Ywp1 ([R][R]LMGETPIV[K][R]), suggesting that it does not remain associated after the two cleavage events.

These findings were confirmed with carboxypeptidase B, which preferentially cleaves C-terminal lysines and arginines. Carboxypeptidase B converted the upper band of the doublet into the lower band (panel C), consistent with removal of the C-terminal arginine of the upper band. The lower band of the doublet was relatively stable under these conditions, but much greater concentrations of enzyme resulted in trimming of additional amino acids, giving a band corresponding to the third band seen in aged samples (which was presumably created by endogenous proteases). Such end-nibbling also explains variations in the mobility of the deglycosylated propeptide near the bottom of the gel (*e.g.*, panel B, lanes 5, 10 and 12). The mobility of the GFP moiety was not changed by any of the carboxypeptidase B treatments, as evidenced by heating of digests to 50°C prior to SDS-PAGE in order to dissociate the propeptide and view the GFP moiety by itself (data not shown). All independent transformants secreting Ywp165-GFP that were examined (five), as well as numerous subclones, had roughly equimolar amounts of the two bands of the doublet; the significance of this is unknown.

That a single charged amino acid can result in a mobility shift of this magnitude is supported by two additional observations. First, the band patterns seen on gels were independent of the presence of SDS, except when thermal denaturation of the proteins caused aggregation in the absence of SDS. In panel D, glycosylated and deglycosylated Ywp165-GFP were heated prior to PAGE in the absence of any SDS. The GFP moiety did not dissociate from the propeptide at 50°C in either case, but there was substantial dissociation at 65°C; at 80°C, most of the polypeptide appeared to have aggregated and barely entered the gel. SDS facilitated the dissociation of the propeptide from the GFP moiety at lower temperatures (it was virtually complete at 50°C), but it did not affect the mobility of the bands on the gel. The samples in panel D were never exposed to SDS, while those in panels A-C were; the gel and cathode buffer in panels A and B contained SDS, while those in panel C did not. These results indicate that native (non-denatured) Ywp165-GFP does not bind SDS, and that its migration during PAGE (in both the presence and absence of SDS) is due only to its intrinsic net negative charge. An extra positive charge (arginine) thus significantly retards that mobility.

Second, using Endo H instead of PNGase F for deglycosylation of Ywp165-GFP preserved the underlying asparagine (uncharged) instead of converting it to aspartate (negatively charged); this caused the doublet to migrate more slowly, shifting upwards (toward the cathode) by a distance equivalent to the spacing of the doublet, further demonstrating that a single charge can result in a significant mobility shift in this gel system (data not shown).

Interestingly, the mobility of the doublet was not changed by treating the samples with disulfide-reducing agents. In contrast, the deglycosylated and SDS-dissociated propeptide showed a sizeable mobility shift upon reduction (Fig. S3 and ref. (1)); this is presumably because the latter was denatured, while the former retained a more native conformation.

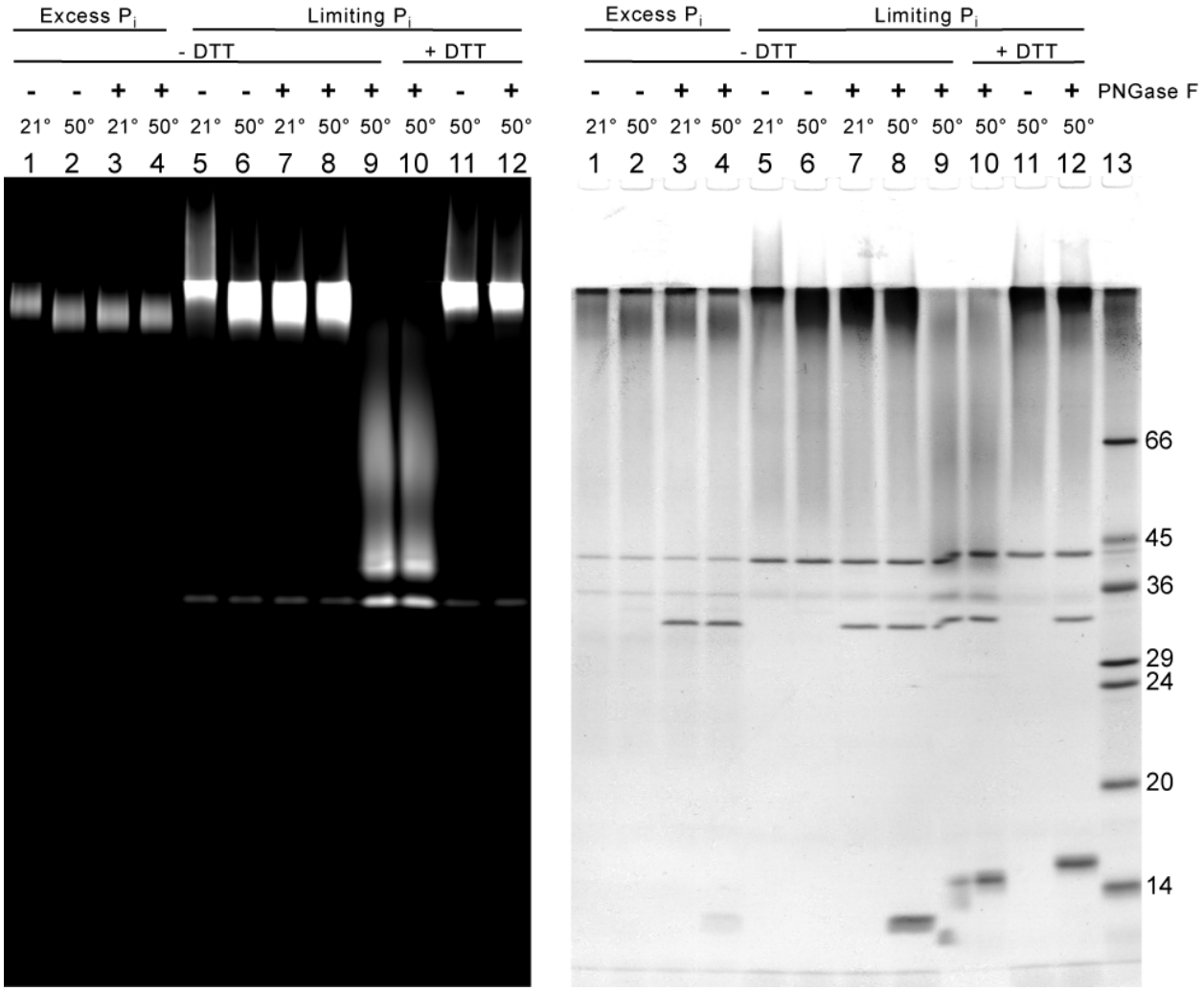
Additional method details: While still between the glass plates, the gel in panel A was imaged with a GE Typhoon Trio flatbed scanner with its PMT set at 350, laser excitation at 488 nm, and emission detection at 510 nm. Carboxypeptidase B from porcine pancreas (Sigma C9584; EC 3.4.17.2), treated with the protease inhibitor AEBSF, was used in 20 mM NaCl, 24-28 mM Tris, 10-14 mM HCl, 0-2 mM EDTA, 0.02% Tween 80 (pH 8.3) at room temperature (23°C); digestion showed little inhibition by 6 M urea, but was rapidly blocked by SDS at room temperature.

Reference

1. **Granger BL, Flenniken ML, Davis DA, Mitchell AP, Cutler JE**. 2005. Yeast wall protein 1 of *Candida albicans*. Microbiology **151**:1631-1644.

Figure S3

(BL Granger 2012 Eukaryotic Cell)



Analysis of secreted Ywp520-GFP

Phosphate was limiting or in excess during growth of the cultures, as indicated. Ywp520-GFP samples (culture supernatants concentrated by ultrafiltration) were first digested with PNGase F or left undigested, then given SDS and either kept at 21°C or heated to 50°C for 5 min; three samples were given DTT before heating, as indicated. SDS-PAGE was followed by laser scanning for GFP fluorescence (left) and then staining for protein with Coomassie Blue R-250 (right). One of the initial samples (run in lanes 9 and 10) had sat at 4°C for several months and experienced significant degradation from endogenous protease activity.

Detailed Explanation

The secreted Ywp520-GFP chimera had properties similar to the Ywp165-GFP chimera, but the heavily O-glycosylated stalk region prevented migration as a discrete, sharp band during PAGE. Nevertheless, similar experimentation demonstrated that the cleaved propeptide, whether with or without its N-glycan, remained associated with the downstream Ywp1 segment attached to GFP. In SDS, dissociation also occurred at 50°C and above; the deglycosylated propeptide then ran as a sharp band near the bottom of the gel, with its mobility dependent on the status of its disulfide bond and whether endogenous proteases had nibbled its end(s) (lanes 8 and 12). The longer segment of Ywp1 attached to GFP either afforded specific sites or more opportunities for cleavage by endogenous proteases, as Ywp520-GFP was more prone to degradation than was Ywp165-GFP upon storage. As even a single cleavage within the stalk region would separate the large N-glycan of the propeptide from the GFP-containing moiety, these degraded forms of the GFP-containing moiety all had much greater mobility during electrophoresis, as shown by the fluorescence in lanes 9 and 10; the lowest fluorescent band corresponded to residual GFP that had little or no Ywp1 left, and thus no glycans, and therefore appeared sharp (compact). This degradation was strongly inhibited by alkalinizing the supernatants to pH 8, or by heating them briefly to 70°C (which has been shown to inactivate secreted aspartyl proteases Sap1, 2 and 3; ref. (2)). However, there was little inhibition of this degradation by addition of the acid protease inhibitor Pepstatin A to cultures or culture supernatants, nor by other protease inhibitors (EDTA, Leupeptin, Aprotinin, AEBSF or TLCK), nor by adding additional ammonium ion to the culture medium to suppress secreted protease production (data not shown). Similar insensitivity has been observed for cleavage of the extracellular domain of *C. albicans* Msb2 (3). Note that the DTT in lane 10 diffused into lane 9 during electrophoresis and caused mobility shifts in an unidentified band at 42 kDa, in PNGase F (running here at 33 kDa), and in the deglycosylated Ywp1 propeptide at the bottom of the gel, as noted previously (1). The propeptide also experienced slight degradation in the stored samples, as it is slightly smaller than in the fresher samples (lanes 8 and 12).

As noted previously, phosphate limitation during growth enhances expression of *YWP1* and generates Ywp1 with decreased mobility during SDS-PAGE (1); this was also reflected here for Ywp520-GFP (lanes 5-8 *vs.* lanes 1-4), and suggests that the mobility shift is due to differences in the O-glycans (since the N-glycan was separated from the GFP moiety in lanes 2-4 and 6-8 either by PNGase F or by heating in SDS to 50°C).

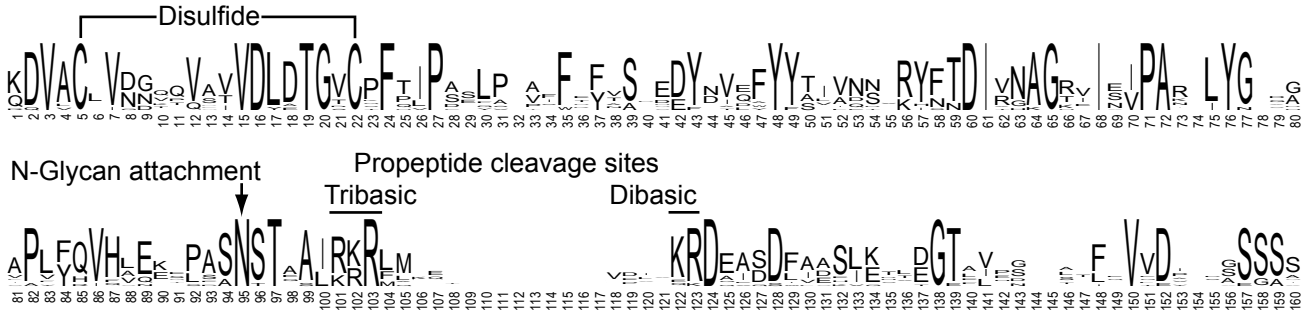
For both Ywp165-GFP and Ywp520-GFP, the strong association of the Ywp1 propeptide with the downstream segment of Ywp1 was additionally demonstrated by other assays (see File S1). Freeze/thaw cycles did not dissociate the propeptide, but there may have been a slight thermal destabilization (on the order of a few °C) of the propeptide association as a consequence of either N-glycan removal or disulfide reduction (data not shown).

References

1. **Granger BL, Flenniken ML, Davis DA, Mitchell AP, Cutler JE**. 2005. Yeast wall protein 1 of *Candida albicans*. Microbiology **151**:1631-1644.
2. **Smolenski G, Sullivan PA, Cutfield SM, Cutfield JF**. 1997. Analysis of secreted aspartic proteinases from *Candida albicans*: purification and characterization of individual Sap1, Sap2 and Sap3 isoenzymes. Microbiology **143**:349-356.
3. **Szafranski-Schneider E, Swidergall M, Cottier F, Tielker D, Román E, Pla J, Ernst JF**. 2012. Msb2 shedding protects *Candida albicans* against antimicrobial peptides. PLoS Pathog. **8**:e1002501.

Figure S4

(BL Granger 2012 Eukaryotic Cell)



Sequence logo of 12 Ywp1 orthologs in 9 species

The N-terminal domains (without signal peptides) of predicted Ywp1 orthologs of *Candida albicans, C. dubliniensis, C. guilliermondii, C. lusitaniae, C. parapsilosis, C. tropicalis, Debaryomyces hansenii, Lodderomyces elongisporus and Pichia stipitis* are shown schematically as a Web Logo. The numbering does not match any of the individual sequences, since a few gaps were introduced into each to facilitate alignment. Features that are absolutely conserved include the N-glycan attachment site, the tribasic site for propeptide cleavage, and the two cysteines that have been shown to form a reversible disulfide in *C. albicans* Ywp1; the dibasic site for propeptide cleavage is found in 10 of the 12 sequences. The segment between propeptide cleavage sites (comprising 6-20 amino acids in the individual sequences) is least conserved; 23 amino acids are absolutely conserved in the remainder of this N-terminal domain.

This sequence logo (1) was created using WebLogo (2).

1. **Schneider TD, Stephens RM.** 1990. Sequence logos: a new way to display consensus sequences. Nucleic Acids Res. **18**:6097-6100.
2. **Crooks GE, Hon G, Chandonia J-M, Brenner SE.** 2004. WebLogo: a sequence logo generator. Genome Res. **14**:1188-1190.

File S1

(BL Granger 2012 Eukaryotic Cell)

Additional clues about *YWP1* expression and Ywp1 function, largely mined from published omics data

Genomic, transcriptomic and proteomic studies continue to clarify the circumstances of *YWP1* expression and modify expectations for its encoded protein, but substantial changes in *YWP1* expression that have correlates beyond cell morphology remain uncertain at this time, as detailed below. More moderate changes may well have unrecognized correlates, however, and the existing data conceivably hold overlooked clues about the function of Ywp1. Comparisons of the structure and properties of Ywp1 to those of other wall proteins of *Candida* have been instructive, but have not yet revealed likely activities or functions.

Analyses of *Candida albicans* transcripts by deep sequencing (6, 64) and array hybridization (53) have revealed that the *YWP1* mRNA has a 5' UTR of typical length at 92 (64) or 98 (6) bases, and a 3' UTR that is unusually long at 399 (6), 527 (64) or about 600 (53) bases. (The respective 5' and 3' UTR medians are 99 and 136 bases for polyadenylated transcripts (64).) The regulatory factors that bind these *YWP1* UTRs are beginning to be identified. For example, the morphogenetic regulator Efg1 is necessary for *YWP1* expression in yeast as well as for its repression in hyphae (58), and its physical interaction with the *YWP1* UTR is now being defined (34). Six transcription factors that have roles in biofilm formation (Bcr1, Brg1, Efg1, Ndt80, Rob1 and Tec1) have all been found to bind upstream from the *YWP1* coding region during one defined *in vitro* biofilm growth condition that was examined (43). In addition, many other transcription factors and signaling proteins have been found to directly or indirectly regulate the expression of *YWP1*. For example, *YWP1* expression is activated by Nrg1 (24, 32), a hyphal suppressor that facilitates dispersal of yeast cells from biofilms (66). Pes1 is involved in the hypha-to-yeast transition, and overexpression increases production of lateral yeast from hyphae and the release of yeast from biofilms (56, 65), but the effect of Pes1 on *YWP1* expression has not been reported. Tsa1 (67), Yak1 (25), Zap1 (22, 44), Brg1, Rob1 (43), Ada2, Gin4 and Taf14 (20) appear to suppress *YWP1* expression upon filamentation and/or biofilm formation, while Snf5 and Zcf34 (and five others to a much lesser extent) appear to induce it (20). Ssk1 appears to have a suppressive effect on *YWP1* in yeast forms (11).

It has been reported that the *YWP1* mRNA binds to the She3-dependent RNA transport system in filamentous but not budding yeast forms of *C. albicans*, where it is thought to be transported to the tips of hyphae (18). As there is currently no evidence that *YWP1* mRNA might be translated at (and Ywp1p incorporated into) hyphal tips, it is conceivable that this is a mechanism for removal of residual *YWP1* mRNA from the lengths of the hyphae. This would be consistent with the ability of Ywp1 to interfere with normal hyphal adhesion, but also raises the possibility that Ywp1 might have a previously undetected and unexplored role at the hyphal tip. At this position, as in rapidly proliferating yeast forms, Ywp1 might be involved in the rapid expansion of existing cell walls; hyphal expression of *YWP1* would appear to be very low because hyphal tips would comprise a very small fraction of mycelial cultures, but this scenario would also require that the Ywp1 not be incorporated into the hyphal wall to the same extent as in yeast walls. Thus, these observations and ideas require further confirmation and testing.

Estimates of the relative levels of *YWP1* mRNA have been obtained for numerous growth conditions through microarray hybridization, Northern blotting, quantitative RT-PCR, and deep sequencing (RNA-seq), and conditions that promote filamentation have consistently been found to strongly downregulate *YWP1* expression (6, 30, 32, 39, 40, 53, 58). In rich yeast medium, adding serum and raising the growth temperature from 30°C to 37°C have similar, additive effects on this downregulation (32, 40). Relative to the exponential growth of yeast forms, *YWP1* is downregulated in yeast stationary phase (49), as shown

previously with a fluorescent reporter (26). Excessive dilution of stationary phase yeast into yeast-favoring media can trigger filamentation through dilution of quorum signals such as farnesol (19), and thus rapidly but temporarily downregulate *YWP1* expression (25, 32, 39). For planktonic yeast, hypoxia roughly doubles *YWP1* expression (2, 54, 63). Caspofungin, which inhibits the synthesis of β-1,3-glucan in the cell wall, has been reported to upregulate *YWP1* in biofilms (68), but to downregulate it in planktonic yeast (5, 36), likely through transcription factor Cas5 (5). Perhaps the latter finding correlates with the observation that when the yeast cell wall is removed by digestion with protease and β-glucanase, even though *CAS5* is not concurrently upregulated, *YWP1* is downregulated as the resulting protoplast regenerates its wall (9). Amphotericin and azole drugs appear to have little short-term effect on relative *YWP1* expression (36, 63, 68); it is conceivable that strong upregulation reported for *YWP1* in a fluconazole-treated culture at 72 hours (14) was an artifact of the untreated comparison culture growing more rapidly and being more fully into stationary phase (with more strongly downregulated *YWP1*). The phenotypic switch from white to opaque cells was accompanied by a nearly 2-fold downregulation of *YWP1* in one study (64), and fell below the 2-fold significance cutoff in another (33). *YWP1* is moderately downregulated during mating of opaque cells, consistent with the upregulation of hypha-associated genes during this process (72). Pheromone treatment "induces mating-incompetent white cells to become adhesive and cohesive, and form thicker biofilms that facilitate mating" (51); as expected, this did not induce *YWP1* expression, but whether it downregulated *YWP1* expression was not reported (51). As for exposure of receptive cells to mating pheromone, *YWP1* expression changes fell below the 3-fold significance cutoff in another study (3).

YWP1 transcript profiling under more complex or conflicting growth conditions is more difficult to interpret, especially when cell morphologies are not quantified at the time of RNA harvest. Also, comparison cultures often have multiple, uncontrolled variables that cloud interpretation. For example, acidic pH typically promotes yeast morphology, while alkaline pH favors filamentation (7); in combination with mammalian culture media (M199) and mammalian core temperature (37°C), both of which favor filamentation, *YWP1* expression was found to be similar at pH 4 and pH 8 (4, 6); the cell morphologies in these respective media at the time of RNA harvest were not reported, however. Biofilm formation is typically accompanied by extensive filamentation of adherent yeast cells, resulting in *YWP1* downregulation (39, 43, 53); upregulation has also been reported (23), however, perhaps because the biofilms had matured to an increasingly dispersive state in a continuous-flow bioreactor and/or because the planktonic culture used for comparison had reached stationary phase and thus downregulated *YWP1*. Another study looked at three strains at three times under three conditions and found less than a 2-fold difference in *YWP1* transcript levels between biofilms and planktonic cells, but up to a 3.4-fold difference depending on the biofilm substrate (71). *YWP1* expression in a central venous catheter biofilm composed of both yeast and hyphae was similar to that in a planktonic culture in a defined mammalian medium, but the morphologies of the latter cells were not specified (41). As a commensal in the mouse cecum, *C. albicans* grows primarily in the yeast form, and *YWP1* expression is relatively high, intermediate between that of exponential phase and stationary phase yeast growing in YPD at 37°C *in vitro* (49), but 4-fold less than exponential phase yeast in YPD at 30°C (53); whether sub-maximal rates of *YWP1* expression promote commensal yeast adhesion in the intestine or elsewhere remains to be determined. The cecal environment also induces genes such as *ECE1* that are usually associated with filamentation, exemplifying distinct requirements for filamentation, colonization and invasion (45, 49). There appears to be little or no difference in total *YWP1* expression between *C. albicans* after 3 days in the kidneys of systemically infected rabbits and after 6 hours in a defined mammalian culture medium at 37°C *in vitro* (69); although these two conditions are too disparate for a meaningful comparison, they nevertheless fail to raise the possibility that Ywp1 has a major role in growth *in vivo* or pathogenesis. Thus, in assessing the accumulated transcript data, it appears that cell morphology currently remains the predominant correlate of *YWP1* expression. Modest changes in expression may well have unrecognized correlates, however. Independent and unequivocal correlates to adhesion have not yet been demonstrated or refuted by transcript profiling alone, but none of these findings appear to be inconsistent with an anti-adhesive effect for Ywp1 in yeast cells.

The abundance of Ywp1 compared to other yeast wall proteins is not known, but various lines of evidence based on transcript levels suggest that it is high. A strong promoter is thought to be required for visibility of GFP reporters in cells by fluorescence microscopy (15), and the *YWP1* promoter easily fulfills this criterion (26). Based on RNA-seq, the transcript levels of *YWP1* can indeed be high, 12-13 times the average transcript of yeast cells growing in YPD at 30°C, and within a factor of 2 of the transcripts for Cht2, Bgl2, MP65, Sun41, Sim1/Sun42, Tos1, Phr2 and Rhd3/Pga29 under these same conditions (6). (For reference, mass spectrometry of cell wall proteins has suggested that Rhd3/Pga29 is one of the most abundant yeast wall proteins (16)). However, the transcripts for Pga59 and Pga62, two small GPI-proteins that are important for cell wall integrity (38, 47), are an order of magnitude greater than the Ywp1 transcript, perhaps consistent with the observation that the loss of Ywp1 has no obvious effect on wall resistance properties (26, 47) and the implication that Ywp1 does not have a major structural role in the wall.

Transcript profiling provides a snapshot of relative mRNA abundance, but this may correlate poorly with the abundance of the encoded proteins (28), especially wall proteins that may have low turnover rates (12, 13, 50). Proteomics approaches utilizing mass spectroscopy to identify tryptic peptides have provided valuable information about Ywp1, including the first direct evidence for its predicted GPI-wall-anchorage and the O-glycosylation of its Thr/Ser-rich core (17). Detection of Ywp1 peptides has been facilitated by chemical deglycosylation prior to trypsinization and MS, and has allowed identification of four peptides from the 378 aa core of Ywp1 as well as two from its 100 aa propeptide. As detection of Ywp1 peptides is unreliable in any given run (and Ywp1 has unexpectedly remained undetected in some samples), multiple runs must be performed to build semi-quantitative datasets; these have supported the assumption that Ywp1 is present in the yeast cell wall but not the hyphal cell wall (29, 37, 62), and have confirmed the liberation of Ywp1 into yeast culture media (37, 60). Fluconazole appears to modestly decrease the amount of Ywp1 in the cell wall (59). The current study presents evidence that treatment with 70°C SDS dissociates the Ywp1 propeptide from its 378 aa core, yet in some MS runs the propeptide is detected in cell wall samples that have been extensively pre-extracted with boiling SDS, usually in the presence of 2-mercaptoethanol and EDTA (8, 29, 59); whether this reveals incomplete propeptide cleavage, an alternative mechanism of covalent wall anchorage (such as through the N-glycan of the Ywp1 propeptide), or merely incompleteness in the extraction process, awaits further investigation.

The spatial distribution and orientation of Ywp1 within the cell wall is presumably relevant to its function. The 378 aa core of Ywp1 is theoretically long enough to position its N-terminus and attached propeptide at the outer surface of the wall (35). Indeed, a 6xHA tag placed 9 aa into the N-terminus of the core is accessible to externally-applied antibodies (52). In contrast, a set of antibodies specific for the N-terminal portions of the Ywp1 polypeptide showed no significant binding to intact cells (26). The difference may be explained by differences in epitope accessibility or the non-quantitative nature of such assays, and do not rule out the possibility that some or all Ywp1 N-terminal regions are exposed at the outer surface of the wall. The sole N-glycan of Ywp1 would then be predicted to contribute to the wall's outer mannan layer.

Alcian Blue can be used to quantify mannosylphosphate in the cell wall (21, 31); strains that have had one *YWP1* allele converted to an anchor-minus version consistently show about 10% less Alcian Blue binding (unpublished observations), suggesting that, despite its single N-glycan, a significant fraction of the yeast cell's mannosylphosphate may be carried by Ywp1. (Whether this is related to the observation that *YWP1* is upregulated upon phosphate limitation (26) is an intriguing possibility that remains to be investigated.) Thus, Ywp1 appears to be a relatively abundant yeast cell wall protein. Notably, the ease with which the Ywp1 propeptide can be selectively stripped from intact cells with 70°C SDS suggests utility in the study of N-glycosylation by *Candida*. Purification of the propeptide would yield a single N-glycan whose structure and properties could be studied as they vary under different growth conditions, phases, and residence times in the cell wall, obviating interference from N-glycans attached to other proteins or to other

sites within the same protein. A similar goal for glycan analysis is being explored with engineered proteins that possess affinity tags and single N-glycans and are secreted from *C. albicans* into the culture medium (55).

To our knowledge, the cleaved propeptide of Ywp1 is the only *C. albicans* propeptide that has been visualized experimentally. Secreted proteins with predicted Kex2 (KR-containing) cleavage sites are not uncommon in *C. albicans* (42), however, raising the possibility that many externalized propeptides remain to be documented. Relaxation of the cleavage specificity to RR-containing sites and mere Kex2 dependency, as documented for Ywp1, further expands this list. All of the predicted Ywp1 orthologs in different members of the *Candida* clade have KR or RR within their tribasic site, and 9 of the 12 have KR as their dibasic sequence. Among the 115 predicted GPI-proteins of *C. albicans* (48), 37 have KR sequences in the N-terminal 65% of their length (measured after removal of the signal peptide), which, if cleaved, would result in propeptides of 6-304 aa. This calculation is based on the distance between the predicted signal peptide cleavage site (46) and the first KR site; 15 of these propeptides would be 6-20 aa long, 10 would be 33-73 aa, and 8 (including Ywp1) would be 88-180 aa. Cysteines and consensus sites for N-glycan attachment are not found in any of the putative propeptides that have fewer than 40 aa, but, as for Ywp1, are found in 14 (with 1-7 cysteines) or 15 (with 1-8 N-glycan sites) of the remaining 21 predicted propeptides. Including RR-containing cleavage sites would shorten 3 of these propeptides and add 5 more, ranging in length from 25 to 303 aa. Cleavage at additional downstream KR-containing sites would add 17 peptides from 9 proteins, ranging in length from 14 to 177 aa. Thus, just among the GPI-proteins of *C. albicans*, there is considerable potential for propeptide production, but in reality this must depend upon the actual specificities of Kex2 and Kex2-dependent proteases, and the accessibility of their cleavage sites. In fact, MS analysis has detected some of these potential propeptide sequences (for Phr1, Phr2, Crh11, Hwp2, Pga4 and Plb5) in cell walls that have been stripped with hot SDS and reducing agent (8, 16, 17, 29, 61, 62), suggesting that their KR sites have remained partially or completely uncleaved. A cleaved, KR-terminated 33 aa propeptide of Sap9 has not been detected experimentally in *C. albicans*, but an additional (possibly autocatalytic) cleavage at one of Sap9's many downstream monobasic (K) sites creates a small fragment that remains associated with the core polypeptide when synthesized as an anchor-minus version ectopically in *Pichia pastoris*, and is visible by protein staining after SDS-PAGE (1, 10). It is expected that further empirical identification of such classical propeptides and unpredictable cleavages will begin to shed light on the functional relevance of these cell wall protein fragments, heretofore undetected and uninvestigated.

Some of the unusual features and expression patterns of Ywp1 are shared by other GPI-proteins of the *C. albicans* cell wall. For example, like Ywp1, Rhd3 (Pga29) is strongly expressed in yeast, but downregulated upon filamentation (16), and expressed less at pH 7 than at pH 4 (62); it has no obvious effect on adhesion, however (16). As observed for Ywp1, loss of Als5, Als6 or Als7 results in increased adhesiveness (73); these proteins may be upregulated upon filamentation (27), but overall their expression is quite low, and morphological correlates of their expression are not yet well established (6, 27). Also, ectopic expression of these proteins in *S. cerevisiae* results in increased adhesiveness (57), a test that has not yet been performed for Ywp1. Als4 is abundant in the yeast cell wall, but in contrast to Ywp1, its expression increases rather than decreases as the cell progress from exponential growth to stationary phase (12, 74). Als4 is far more abundant in yeast grown at 30°C than at 37°C (12, 74); interestingly, when Ywp1 is absent, yeast cells are more adherent when grown at 30°C than at 37°C, with an especially sharp drop-off in adhesion in going from 36°C to 37°C. Whether Als4 has a role in yeast adhesion that is somehow counteracted by Ywp1 remains to be determined. Exploration of such possibilities is further complicated by findings that patterns of expression observed *in vivo* may unexpectedly not match those *in vitro* (12, 13, 70). It thus appears that Ywp1 has a unique role in the cell wall that is not similar to any other protein that has been characterized to date.

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File S2

(BL Granger 2012 Eukaryotic Cell)

Optimization of the production and analysis of secreted Ywp1-GFP chimeras

Cell culture. The *GFP* coding sequence was inserted at various positions into the *Candida albicans YWP1* coding sequence, so expression of the chimeras was driven solely by the *YWP1* promoter. Yields of Ywp1-GFP in culture supernatants were maximized by taking into consideration the following factors: (i) expression of *YWP1* (and thus, the chimeras) is greatest in yeast forms and least in filamentous forms of *C. albicans* (2, 3, 5, 6, 9); (ii) acidic media favor growth of the cells as yeast, while neutral and alkaline media favor filaments; (iii) GFP is unstable at acidic pH, denaturing and losing its fluorescence below pH ~5; and (iv) unbuffered yeast cultures may drop the pH of the medium below 2 at stationary phase. Therefore, batch production cultures were routinely buffered to maintain the pH around 6, which preserved the high expression of *YWP1* in yeast forms as well as the GFP fluorescence. This was accomplished with a combination of MES and Bis-Tris in the culture medium that totaled 150-180 mM (usually 100 mM MES and 80 mM Bis-Tris). As the optimum pH for GFP fluorescence proved to be around 8, which resulted in ~3 x greater fluorescence than pH 6, culture supernatants were routinely alkalinized to pH 8 after harvest. The fluorescence of the secreted Ywp1-GFP chimeras was readily detectable in culture supernatants (even after 100-fold dilution) by fluorimetry using excitation at 468 nm and emission at 511 nm. The Ywp1-GFP chimeras were concentrated from these supernatants by centrifugal ultrafiltration in devices with nominal cutoffs of 10-50 kDa, and the chimeras represented the predominant protein species in these samples. The chimeras were also precipitable with 1-2 volumes of ethanol, producing visibly green pellets after centrifugation; this treatment did not destroy the GFP fluorescence or dissociate the Ywp1 propeptide from the chimeras.

Yields of secreted fluorescent Ywp1-GFP chimeras were additionally increased in two ways: reducing the initial concentration of phosphate in the medium from 5 mM to 0.3 mM, which increased expression of *YWP1* (2), and including Tween 80 in the medium at a concentration of 0.02%. The Tween 80 may have served simply as a carrier that inhibited loss of secreted proteins by adsorption to various accessible surfaces, as yields were also increased by inclusion of similar concentrations of Tween 20, PEG 8000, Pluronic F-68, polyvinylpyrrolidone, octyl-glucoside, saponin, CHAPS or Nonidet P 40 in the culture medium. Yields were not increased by inclusion of RNA, DNA, dextran, Ficoll 400 or linear polyacrylamide.

Finally, yields were doubled for strains that spontaneously converted from heterozygous (*YWP1 / ywp1::YWP1-GFP-HIS1*) to homozygous (*ywp1::YWP1-GFP-HIS1 / ywp1::YWP1-GFP-HIS1*). This gene conversion also made the yeast forms more adhesive, since wall-anchored Ywp1 was no longer produced. Even though the chimeric “Ywp165-GFP” and “Ywp520-GFP” proteins were not retained by the cells that produced them, such convertants were found as sectors with increased fluorescence in rapidly-growing colonies on nutrient agar (but not in older colonies in which most of the GFP had transited the secretory pathway and diffused away from the cells). These convertants were thus more difficult to screen for and isolate than those that retained their GFP within their cytosol (2), but several convertants were isolated in this manner. These genetic changes were confirmed by numerous PCR analyses.

Electrophoretic analysis: For the Ywp1-GFP fusion proteins, the discontinuous (1, 7) system of Laemmli (4) was modified to simultaneously resolve 10-100 kDa polypeptides at moderate acrylamide concentrations, avoid expensive Tricine (8), and maintain the stability and fluorescence of GFP by avoiding acidic phases. Specifically, slab gels consisted of the following:

Stacking gel: 5% acrylamide, 0.13% bis-acrylamide, 200 mM Tris, 100 mM HCl (pH 8.3).

Resolving gel: 15% acrylamide, 0.15% bis-acrylamide, 400 mM Tris, 100 mM HCl (pH 8.8).
Cathode (upper) buffer: 100 mM Tris, 100 mM glycine (pH 9.1).
Anode (lower) buffer: 200 mM Tris, 50 mM HCl (pH 8.8).

Polymerization of degassed solutions was initiated with ammonium persulfate and TEMED. Samples were loaded in 200 mM Tris, 100 mM HCl (pH 8.3), with 2% Ficoll 400 or glycerol as density-increasing agents, occasionally 40 μM Phenol Red or Bromophenol Blue as tracking dyes, and occasionally 1 mM EDTA as a chelator; none of these additives modified protein mobility or fluorescence. For SDS-PAGE, SDS was included at 1% in the samples, and at 0.1% in the cathode buffer and stacking gel. Sample heating and disulfide reduction were as indicated in the main text and figure legends. Resolving gels were 140 mm x 135 mm x 0.75 mm, and electrophoresis proceeded at 16 mA for 4 h.

Liquid chromatographic analysis: In some cases, the strong association of the Ywp1 propeptide with the downstream segment of Ywp1 in the Ywp1-GFP chimeras was demonstrated by size-exclusion chromatography utilizing conditions that maintained the fluorescence of GFP but were sometimes incompatible with electrophoresis. LKB Ultrogel AcA34, a beaded acrylamide-agarose matrix that separates globular proteins in the range of 400 kDa (void volume) to 20 kDa (included volume), was found to give good separation of the secreted Ywp1-GFP chimeras that possessed the large N-glycan from those that lacked it (data not shown). Thus, under mild conditions, PNGase F treatment shifted the GFP fluorescence of theYwp165-GFP chimera from close to the void volume to closer to the included volume, as assayed by fluorimetry of the eluted fractions. When the elution buffer was changed to 4 M NaSCN, 8 M urea, or 1% SDS, or was alkalinized to pH 12, the fluorescence was found to elute close to the void volume, indicating that the GFP was not denatured by these conditions, and that the propeptide with its large N-glycan remained associated with the GFP-containing moiety. If the N-glycan were first removed by PNGase F, however, or if samples in SDS were first heated to 50°C, the fluorescence eluted closer to the included volume. Ywp520-GFP fluorescence showed a smaller shift upon loss of the N-glycan, but degraded forms showed much of the fluorescence eluting near the included volume.

Anti-GFP: Western blotting with monoclonal or polyclonal antibodies against GFP proved to be an extremely sensitive method for studying the Ywp1-GFP chimeras (data not shown). It had the advantage of detecting non-fluorescent forms and fragments of GFP after PAGE, and it confirmed the conclusions derived by other means. However, it was often extremely misleading because of its non-quantitative nature. Minor forms and fragments sometimes produced extremely strong signals, either because they happened to have exceptional epitope exposure or because they transferred out of the gel and bound much better to the nitrocellulose and PVDF filters. Major forms sometimes gave weak signals because they transferred poorly, bound poorly, or were insufficiently denatured. The technique was thus of limited use for the current studies.

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Table S1

(BL Granger 2012 Eukaryotic Cell)

Strains of *Candida albicans* used in this study

Designation in manuscript	Laboratory name	Parental strain	Relevant portion of genotype	Reference
SC5314	SC5314	(clinical isolate)	(Wild type)	(3)
BWP17	BWP17	SC5314	*arg4/arg4 his1/his1 ura3/ura3*	(6)
Ca#12	Ca#12	BWP17	*ywp1Δ::ARG4/ ywp1Δ::URA3-dpl200*	(4)
BJ3a1a	BJ3a1a	BWP17	*ywp1Δ::GFP-HIS1/ywp1Δ::GFP-HIS1*	(4)
Als3Δ	1843	CAI4	*als3laΔ/als3saΔ-URA3*	(8)
Als3Δ parent	CAI4	SC5314	*ura3Δ/ura3Δ*	(8)
Als3 restored	2322	1843	*als3laΔ/als3saΔ-ALS3LA-URA3*	(7)
Als3Δ (independent)	CAYF178U	BWP17	*als3::ARG4/ als3::HIS1 URA3-IRO1*	(5)
Hyr1Δ	FJS2	BWP17	*hyr1::Tn7-UAU1/hyr1::Tn7-URA3*	(5)
Cht2Δ	FJS5	BWP17	*cht2::Tn7-UAU1/cht2::Tn7-URA3*	(5)
Ece1Δ	FJS6	BWP17	*ece1::Tn7-UAU1/ece1::Tn7-URA3*	(5)
Rbt5Δ	FJS8	BWP17	*rbt5::Tn7-UAU1/rbt5::Tn7-URA3*	(5)
Ecm331Δ	FJS10	BWP17	*ecm331::Tn7-UAU1/ecm331::Tn7-URA3*	(5)
Als1Δ	als1null	CAI4	*als1/als1*	(2)
Als1 restored	ALS1REV	als1null	*als1/als1 ALS1*	(2)
Figures 1 & 2:				
WT	DAY185	BWP17	*ARG4*, *HIS1* and *URA3* restored	(1)
ΔY	13s1	Ca#12	*YWP1-HIS1* inserted at *ywp1* locus	(4)
ΔΔY	4L1	Ca#12	*his1::hisG::HIS1-4* (*ywp1/ywp1*)	(4)
HY	HY13a	Ca#12	*HWP1/hwp1::YWP1-HIS1*	(0)
HYΔY	HY13aΔU1ΔYA	HY13a	*HWP1/hwp1::ywp1-HIS1::URA3*	(0)
HYU	HY13aΔU1ΔYB	HY13a	*HWP1/hwp1::YWP1-HIS1 URA3*	(0)
Figures 3 & 4:				
WT / WT	BQ1a	BWP17	*upy1(orf19.3621)::URA3*	(0)
WT / AM	YAM-BQ-4a	BQ1a	*YWP1/YWP1(anchor-minus)*	(0)
WT / Δ	YAM-H3-4	H3	*YWP1/ywp1Δ::ARG4 HIS1*	(0)
WT / Δ	YAM-TU-3	CaTU15x	*YWP1/ywp1Δ::URA3 HIS1*	(0)
AM / Δ	YAM-H3-22a	H3	*YWP1(anchor-minus)/ywp1Δ::ARG4*	(0)
AM / Δ	YAM-TU-30a	CaTU15x	*YWP1(anchor-minus)/ywp1Δ::URA3*	(0)
Figure 5: Same as Figs 3 & 4, plus:				
WT / Δ	H3a	BWP17	*YWP1/ywp1Δ::ARG4*	(4)
WT / Δ	CaTU15x	BWP17	*YWP1/ywp1Δ::URA3*	(0)
Δ / Δ	3L1	Ca#12	*his1::hisG::HIS1-3* (*ywp1/ywp1*)	(4)
Figures S2 & S3:				
BF2d or BF2ih	Ywp165-GFP	BWP17	*YWP1(codons1-165)-GFP* fusion	(0)
BG2d or BG2d1	Ywp520-GFP	BWP17	*YWP1(codons1-520)-GFP* fusion	(0)

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Table S2

(BL Granger 2012 Eukaryotic Cell)

DNA primers that were used to create PCR amplicons

that were transfected into *Candida albicans* auxotrophs
to effect stable genetic modification through homologous recombination

All primer sequences are written in the 5'→ 3' orientation.
Abbreviations used: **aa** (amino acids), **bp** (base pairs), **cds** (coding sequence [translated sequence]), **UTR** (untranslated region)

Ectopic (hyphal) Ywp1

For placing expression of *YWP1* under the control of the *HWP1* promoter
(by replacing the initial coding sequence of one allele of *HWP1* with the full coding sequence of *YWP1*)
and then testing phenotypic reversibility by disrupting the ectopic *YWP1* with *URA3*

HWP-5Y (underlined 65 bases match *HWP1* 5' UTR; italicized 19 bases match *YWP1* cds; bold = start codon)
TCTCAACAATATCAAACACAACAGGAATCTCCTATAGTCACTCGCTTTTAGTTTCGTCAAT***ATGA***AAGGTATCTACTATTT

HWP-3H (underlined 61 bases match *HWP1* cds; italicized 20 bases match *HIS1* 3'UTR)
TCCATAGGTGGAATGGAAGCTTCTGGAGTTGAATCAGTACCTGGAGTGTTTTCAGTCAATG*TAGATCTATAAGTATATAT*

HWP-3H2 (underlined 61 bases match *HWP1* cds; italicized 19 bases match *HIS1* 3'UTR)
TGTGGGTAATCATCACATGGTTCTTGATAGTAATCATAAGATCTCTTTTGAATAAGAGCTT*GTAGATCTATAAGTATATA*

HWP-5H (underlined 66 bases match *HWP1* cds; italicized 19 bases match *HIS1* 5'UTR)
TCCATAGGTGGAATGGAAGCTTCTGGAGTTGAATCAGTACCTGGAGTGTTTTCAGTCAATG*GACAG*CAAGACAATTTATT

YWP-5U (underlined 60 bases match *YWP1* cds; italicized 21 bases match *URA3* 5' UTR)
TGGACACTGGTGTTTGTCCATTCACCATTCCAGCTTCATTAGCTGCTTTCTTCACATTTG*AGTAATTACAAAGTACTAAT*

YWP-3U (underlined 60 bases match *YWP1* cds; italicized 20 bases match *URA3* 3' UTR)
TGAACCTGGAACGTAATCAACAACTTCAAGAGTAGAACCTTCAAGAGCAGTACCTTCAGT*CCTGTCATGATTTCTAGAAG*

	Primer pair used for PCR amplification	Template(s) used	Amplicon size (bp)	Replaced *HWP1* cds nucleotides	Replaced *YWP1* cds nucleotides	Strains generated
(1)	HWP-5Y + HWP-3H	"5" & "9"	3789*	+1 to +1238		HY5a; HY9a,c,d
(2)	HWP-5Y + HWP-3H2	"5" & "9"	3790*	+1 to +97		HY5b,c,d,e,g; HY9e,f,g
(3)	HWP-5Y + HWP-5H	"13" & "16"	3790*	+1 to +1238		HY13a ("**HY**"); HY16b
(4)	YWP-5U + YWP-3U	*URA3*	1508		+173 to +435**	*** (see below)

* For allele *YWP1*-1; 4 bp fewer than this for allele *YWP1*-2. (The two alleles of *YWP1* have identical coding sequences, but are distinguishable by an upstream *Hin*dIII site in allele 1.)

** Parental strain Ca#12 had *YWP1* cds nucleotides +64 to +815 deleted from both alleles, so this amplicon could recombine homologously only with the full *YWP1* that was inserted ectopically into the *HWP1* gene.

*** Correct insertion (*YWP1* disrupted): HY5cΔU1ΔYA; HY9e'ΔU1ΔYK,P; HY13aΔU1ΔYA ("**HYΔY**"); HY13aΔU2bΔYB; HY16bΔU2ΔYC,E. Incorrect insertion (*YWP1* not disrupted): HY5cΔU1ΔYB; HY9e'ΔU1ΔYA; HY13aΔU1ΔYB ("**HYU**"); HY13aΔU2bΔYA; HY16bΔU2ΔYB.

Templates used for PCR amplification:

"5", "9", "13" & "16": *YWP1* was amplified from *C. albicans* strain BWP17 genomic DNA with primers TEP1-F7 (GTTCAAATAAAGAATAGTCAAGCTC) and TEP1-R8 (GTTTCCAATTTTGTGATGTTTTCAAGTGC) and cloned into pGEM-T Easy (Promega) to give pGTE-TEP1F7R8-24 and -25. (*YWP1* allele 1 [*YWP1*-1], which has an upstream *Hin*dIII site, is represented by -25, and *YWP1* allele 2 [*YWP1*-2], which lacks the upstream *Hin*dIII site, is represented by -24.) *YWP1* was excised from these plasmids with *Not*I and ligated into the *Not*I site of pGT-HIS1-3ΔN1 and -4ΔN1 (each lacking the *Nsi* I site of pGEM-T, with *HIS1* [amplified from *C. albicans* strain 3153A genomic DNA] in opposite orientations), giving pGT-HIS1-TEP1-5, -9, -13 and -16, with the following relative orientations:

"5" series: >*YWP1*-2> >*HIS1*-3>
"9" series: >*YWP1*-1> >*HIS1*-3>
"13" series: >*YWP1*-2> <*HIS1*-4<
"16" series: >*YWP1*-1> <*HIS1*-4<

The *YWP1-HIS1* cassettes were excised with *Apa*I + *Sac*I prior to PCR amplification.

URA3: A gel-purified, 1389 bp *URA3* amplicon from *C. albicans* strain 3153A genomic DNA, as described in ref. (1).

Strains transfected:

Amplicons (1), (2) & (3) were used to transfect *C. albicans* strain Ca#12, giving HY_ transformants (listed above).
Amplicons (2) & (3) were used to transfect *C. albicans* strain BWP17, giving BHY_ transformants (BHY13D,F; BHY16A,B,G,H,J).
Amplicon (4) was used to transfect five HY_ strains that had lost their *URA3* (selected with 5-FOA): HY5cΔU1, HY9e'ΔU1, HY13aΔU1, HY13aΔU2b & HY16bΔU2.

Anchor-minus Ywp1

For creation of a stop codon in *YWP1* that is immediately upstream from the codon for the GPI-anchorage omega site.
Specifically, to convert glutamate codon 510 to a TAA stop codon. (Glycine 511 is the omega site.)

YWP1STOP-5H (underlined 58 bases match *YWP1* cds; italicized 19 bases match *HIS1* 5' UTR; bold = stop codon)
TTCTCAAACCACTGTTGCTAAAGCTTCAGGTTCTGGTAAAGCTGCCATTAGTACTTTT**TAA***GACAGCAAGACAATTTATT*

TEP1-3H2 (underlined 59 bases match *YWP1* 3' UTR; italicized 21 bases match *HIS1* 3' UTR)
GACTCTTCAACTTCTGTTCATGATAGTTGGTATAATGATTGTAAGGACAGAATTGAAGC*GTAGATCTATAAGTATATATG*

Templates used for creation of the 1299 bp *YWP1-HIS1-YWP1* amplicons:

Gel-purified *HIS1* amplicons that were amplified either from *C. albicans* strain 3153A genomic DNA [ref. (1)] for the BQ series, or from plasmid pGEM-HIS1 [ref. (3)] for the H3 and TU series, using primers HIS1-F1 (GACAGCAAGACAATTTATTTATTTAATAG) and HIS1-R1 (GTAGATCTATAAGTATATATGTATAA).

Transfected strain	**Parental strain**	**Relevant genotype of transfected strain**
BQ1a	BWP17	*upy1::URA3* (*UPY1* is the upstream pseudogene paralog of *YWP1*)
H3a	BWP17	*ywp1::ARG4*
CaTU15x	BWP17	*ywp1::URA3* (not *URA3dpl200*)

***HIS1* and the *YWP1* stop codon inserted into↓**	BQ1a derivatives	H3a derivatives	CaTU15x derivatives
An unknown location:	BQ-1,2,3,5,14,20,25	H3-1,5,13,15,17,19,29	TU-1,4,17,33
An unmodified *YWP1* allele:	BQ-4,6,24,27	H3-22,24,26,28,30,33	TU-30,32,38
The previously-disrupted *YWP1* allele:		H3-2,3,4,6,11,23,27	TU-2,3,5,6,12,22

Code in manuscript	**Strain in Figures 3 & 4**	**Strains in Figure 5**
WT / WT	BQ-1a	BQ-1a
WT / AM	YAM-BQ-4a	YAM-BQ-4a
WT / Δ	YAM-H3-4	YAM-H3-4, H3a, YAM-TU-3, CaTU15x
AM / Δ	YAM-H3-22a	YAM-H3-22a, YAM-TU-30a
Δ / Δ		3L1

Ywp1-GFP fusion proteins

For inserting *GFP-HIS1* into *YWP1* in four different places to encode four different Ywp1-GFP chimeras.

TEP1-5GFP (underlined 60 bases match *YWP1* cds; italicized 23 bases match *GFP* cds)
GCCGTTCCATTTTTCCAAGTTCATTTAGAAAAACAATTAGAAGCTAATTCTACTGCTGCT*TCTAAAGGTGAAGAATTATTCAC*

T1G1-5DR' (underlined 60 bases match *YWP1* cds; italicized 23 bases match *GFP* cds)
ACTGAAGGTACTGCTCTTGAAGGTTCTACTCTTGAAGTTGTTGATTACGTTCCAGGTTC*AATGTCTAAAGGTGAAGAATTAT*

TEP1-5GFP2 (underlined 58 bases match *YWP1* cds; italicized 23 bases match *GFP* cds)
CTGGTAAAGCTGCCATTAGTACTTTTGAAGGTGCCGCCGCCGCTTCAGCTGGTGCC*TCTAAAGGTGAAGAATTATTCAC*

TEP1-5GFP3 (underlined 56 bases match *YWP1* cds; italicized 24 bases match *GFP* cds)
CTTCAGCTGGTGCCTCAGTCTTGGCTTTAGCTTTGATTCCATTAGCTTATTTCATT*ATGTCTAAAGGTGAAGAATTATTC*

TEP1-3H (underlined 63 bases match *YWP1*; italicized 22 bases match *HIS1* 3'UTR; bold = complement of *YWP1* stop codon)
GTTAAAC**TTA**AATGAAATAAGCTAATGGAATCAAAGCTAAAGCCAAGACTGAGGCACCAGC*TGTAGATCTATAAGTATATATG*

TEP1-3H2 (underlined 59 bases match *YWP1* 3'UTR; italicized 21 bases match *HIS1* 3'UTR)
GACTCTTCAACTTCTGTTCATGATAGTTGGTATAATGATTGTAAGGACAGAATTGAAGC*GTAGATCTATAAGTATATATG*

Template used for creation of all four *YWP1-GFP-HIS1-YWP1* amplicons:
Gel-purified *Apa*I + *Hin*dIII insert of pGT-GFP-HIS1-8 [GenBank accession AY656807; ref. (1)]

	Primer pair used for PCR amplification	Amplicon size (bp)	Chimeric protein	Ywp1 aa	GFP aa	Ywp1-GFP splice sequence	Strains with correct insertions
(E)	TEP1-5GFP + TEP1-3H	2015	**Ywp119-GFP**	1-119	2-238	QLEANSTAA-SKGEEL	E3, E4
(F)	T1G1-5DR' + TEP1-3H	2018	**Ywp165-GFP**	1-165	1-238	VVDYVPGS-MSKGEEL	BF2,3,5,7; CF3,4,6,7,9,10
(G)	TEP1-5GFP2 + TEP1-3H2	2008	**Ywp520-GFP**	1-520	2-238	GAAAASAGAS-KGEEL	BG2,3; CG1,2,4,5,7,8
(H)	TEP1-5GFP3 + TEP1-3H2	2011	**Ywp533-GFP**	1-533	1-238	LALIPLAYFI-MSKGEEL	BH9; CH3,11

Transfected strain	Parental strain	Relevant genotype of transfected strain	Transfected with
BWP17	SC5314	*ura3/ura3 arg4/arg4 his1/his1*	E,F,G,H amplicons (giving B_ series)
CaAU2	BWP17	*PHO100 / pho100::URA3*	F,G,H amplicons (giving C_ series)

Strains in which the unmodified *YWP1* allele was spontaneously replaced with the *YWP1-GFP-HIS1* cassette through gene conversion or mitotic recombination were isolated from transformants BF2 and BG2. Multiple subclonings generated pure cultures with one or both *YWP1* alleles so modified, as confirmed by PCR analyses, adhesion assays and GFP secretion for the following strains:

One allele encodes Ywp1-GFP:	BF2d11,13; BF2ih; BF2j2g	BG2c1b,d; BG2x; BG3a
Both alleles encode Ywp1-GFP:	Bf2d6,9; Bf2j2b,h,k	BG2c1a,c,f; BG2d1; BG2f1

NOTES:

Ywp165-GFP and **Ywp520-GFP** were secreted into the culture medium, but **Ywp119-GFP** and **Ywp533-GFP** were not:

Ywp119-GFP included the signal peptide and propeptide of Ywp1, but not the tribasic or dibasic cleavage sites (giving a splice sequence of …QLEANSTAA-Gfp, where the underlined N is the sole N-glycan attachment site of Ywp1). Fluorescence microscopy of these transformants showed weak GFP fluorescence in punctate intracellular compartments, but no secreted GFP was detected by Western blotting with anti-GFP (data not shown). This chimera was presumably degraded internally, and was not investigated further.

Ywp533-GFP included all 533 amino acids of Ywp1, thus placing GFP downstream from the hydrophobic GPI anchor signal of Ywp1; this was predicted to trap the GFP domain in the cytosol, and indeed no secreted GFP was detected by Western blotting (data not shown). Fluorescence microscopy showed weak intracellular fluorescence that was more uniformly distributed than that of the punctate **Ywp119-GFP** chimera (suggesting that some of the GFP may have been cleaved from Ywp1), but occasional cells showed fluorescence that was faintly enriched at the plasma membrane in a uniform distribution in mother cells and buds (data not shown), similar to the pattern seen with an epitope-tagged version of Ywp1 (2). Again, low fluorescence levels suggested that the **Ywp533-GFP** chimera was relatively unstable, and it was not investigated further.

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