Filamentous growth of the budding yeast *Saccharomyces cerevisiae* induced by overexpression of the *WHI2* gene

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The *WHI2* gene of the budding yeast *Saccharomyces cerevisiae* is required for the arrest of cell proliferation upon nutrient exhaustion: *whi2* mutants carry on dividing and in the absence of growth become abnormally small. It is reported here that overexpression of *Whi2* from the *GAL1* promoter results in filamentous growth – cells fail to complete cytokinesis, the budding pattern changes from axial to polar, cells become elongated and cell size increases threefold. In many ways, these filaments resemble the pseudohyphae which result from nitrogen-limited growth and the filaments seen during the invasive growth of haploids. However, *Whi2*-induced filament formation is reduced, but not blocked, by mutations in *STE7*, *STE12* or *STE20* which do block pseudohypha formation. Furthermore, pseudohypha formation can still occur in a diploid in which both copies of the *WHI2* gene have been deleted. Thus *Whi2*-induced filament formation and pseudohypha formation must come about through the action of different pathways. Despite this, a mutation in the *STE11* gene, which is required for pseudohypha formation, does block *Whi2*-induced filament formation. Concanavalin A pulse-chase experiments show that new cell wall material is incorporated only into the tips of the apical cells. An extragenic suppressor of the *whi2* allele also results in filamentous growth. These results suggest that *Whi2* negatively regulates a function required for the budding mode of cell proliferation including cytokinesis. This function is defined wholly or in part by the *fsw1* allele.

Keywords: cell cycle, filamentous growth, pseudohyphae, *Saccharomyces cerevisiae*, *WHI2*

INTRODUCTION

When the budding yeast *Saccharomyces cerevisiae* is starved of nutrients, cells arrest in G1 as unbudded, phase-bright cells and undergo a series of changes that allow them to survive the adverse environmental conditions – they contain elevated levels of carbohydrates such as glycogen and trehalose, they are resistant to environmental stresses such as a 50 °C heat shock and UV irradiation, and cell wall changes result in resistance to cell-wall-degrading enzymes such as β -glucuronidase

or zymolyase (Hartwell, 1974; Pringle & Hartwell, 1981; Saul *et al.*, 1985; Werner-Washburne *et al.*, 1993). There is no strict coupling between stress resistance and G1 arrest, since slow-growing cells in all phases of the cell cycle acquire stress resistance (Elliott & Futcher, 1993). Yeast cells may respond in other ways to nutrient limitation. Diploid cells sporulate when growing on an aerobic carbon source such as acetate in the absence of nitrogen. When diploid cells are grown on agar plates containing nitrogen-limiting medium they form pseudohyphae, in which budding switches from a bipolar to a unipolar pattern, cytokinesis is not completed and cell growth becomes highly polarized (Gimeno *et al.*, 1992; Gimeno & Fink, 1992; Wright *et al.*, 1993). As a result filaments of cells protrude from colonies and invade the agar. This response is considered to be a means by which colonies may forage for new sources of nutrients.

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Abbreviations: DAPI, 4',6-diamidino-2-phenylindolyl; FITC, fluorescein isothiocyanate; TPCK, *N*-tosyl-L-phenylalanine chloromethylketone.

Haploid cells may also show a similar response known as invasive growth, in which filaments of cells protrude in the agar underneath colonies (Wright *et al.*, 1993; Roberts & Fink, 1994). Like pseudohyphal growth, invasive growth also involves a switching of the budding pattern, in this case from an axial to a bipolar form. The production of filaments in both pseudohypha formation and invasive growth depends on the functioning of the *STE7*, *STE11*, *STE12* and *STE20* genes which are also elements of the pheromone response pathway (Liu *et al.*, 1993; Roberts & Fink, 1994).

The *WHI2* gene is required for cell cycle arrest and the acquisition of stress resistance upon nutrient deprivation (Kelly *et al.*, 1988; Mountain & Sudbery, 1990a, b; Rahman *et al.*, 1988; Saul *et al.*, 1985; Saul & Sudbery, 1985; Sudbery *et al.*, 1980). *whi2* mutants continue to divide upon carbon exhaustion in a YEPD batch culture and the resulting division without growth results in abnormally small cells which finally arrest at random stages of the cell cycle. They also retain the properties of exponentially growing cells and fail to acquire stress resistance. Transcripts of the G1 cyclins, *CLN1* and *CLN2*, persist upon starvation in a *whi2* cell and this is responsible for the inappropriate continuation of cell division (Radcliffe *et al.*, 1997). In this paper we report an involvement of *WHI2* in determining whether cells proliferate in a unicellular budding or filamentous mode. We found that overexpressing *Whi2* results in filamentous growth. Furthermore, *fsu1*, which is a second site suppressor of *whi2*, also causes a similar phenotype. We propose that a function or functions required for the unicellular, budding mode of cell division, including *FSW1*, are negatively regulated by *WHI2*.

METHODS

Strains and culture conditions. Strains used in this paper are described in Table 1. They were routinely cultured in YEPD broth (1% Difco Yeast Extract, 2% Difco peptone, plus 2% glucose) at 26 °C with shaking. Plates were solidified by the addition of 2% Difco agar. YEPGal and YEPRAF contained 2% galactose and 2% raffinose respectively in place of the glucose. CASGal, CASRAF and CASGLU consist of 2% Difco Casamino acids, 0.67% Difco Yeast Nitrogen Base and respectively 2% galactose, raffinose or glucose. CAS-based media select for the maintenance of a *URA3*-containing plasmid in a *ura3* host as Casamino acids do not contain uracil. Cells transformed with pWHIM5 were routinely induced to form filaments by inoculation from a fresh CASGLU plate into 2.5 ml CASGal in a 50 ml Erlenmeyer flask; filaments were then visible after overnight culture at 26 °C. For Western blot experiments cells were pre-grown in CASRAF or CASGal as described in the text and figure legends. For measurements of the percentage of cells showing filament formation cells were grown on CAS-based medium to select for the maintenance of the *URA3*-based pWHIM5.

Epitope-tagging of *Whi2* and the construction of pWHIM3, pWHIM4, pWHIM5 and pWHIM7. The overall strategy was to use YCpmyc111, a yeast centromeric plasmid engineered to facilitate the C-terminal addition of the *c-myc* epitope, EQKLISEEDLN (Reisdorf *et al.*, 1993). It contains a multiple cloning site with a 3' *SmaI* site followed by the nucleotide sequence encoding the epitope. The following primers were

used to amplify the *WHI2* gene by PCR: 5'-GTAGTCGACAC-GACCCGAAGGAGGGCAAT (upstream), and 5'-ACTCCCGGGTCTGCACCCCAATAACGCTC (downstream). The 3' end of the upstream primer hybridized 505 bp upstream of the ATG. The downstream primer had a 5' extension which contained a *SmaI* site and was designed so that when the PCR product was cut with *SmaI* and ligated to *SmaI*-cut YCpmyc111, the *WHI2* ORF would be fused in-frame at its 3' end to the *c-myc* epitope. After the PCR product had been cloned into YCpmyc111 the reading frame at the junction was verified by sequencing. Although the whole *WHI2* ORF was amplified by PCR, the subsequent cloning was performed so that the *WHI2* ORF in the final constructs contained only 328 bp derived from PCR-amplified material, that is from a *XhoI* site to the end of the ORF. The nucleotide sequence of this DNA was checked by sequencing for mutations introduced by the PCR reaction. Plasmids pWHIM3 and pWHIM4 contained 1307 bp upstream of the ATG as far as a *Sall* site. Unpublished experiments show that this is sufficient for normal *WHI2* expression. The vector for pWHIM3 is the centromeric YCpmyc111. The vector for pWHIM4 is the 2 µm-based pYES2 (Invitrogen). In pWHIM5 the *WHI2* ORF is joined to the *GAL1* promoter in pYES2 using a *BamHI* site 258 bp upstream of the ATG and 10 bp downstream of the normal transcription start site. Both Northern and Western hybridization experiments showed that *WHI2* expression in pWHIM5 is under the control of the *GAL1* promoter. Plasmid pWHIM5 complemented a *WHI2*-deleted strain, showing that the epitope does not affect gene function. A triple *c-myc* cassette cloned into pUC119 (this was constructed by S. Kron and kindly provided by R. Jansen, IMP, Vienna), was employed to create pWHIM7. The cassette was amplified by PCR using primers 5'-TATGCCCCGGGTGTTCAAGTCTTC and 5'-ATTGCCCCGGAACAAAAGTTG. The primers were designed so that the product could be digested with *XmaI* and ligated to *XmaI*-digested pWHIM3 to create four tandem copies of the epitope, all of which were in-frame with the *WHI2* ORF. The structure of the resulting construct was confirmed by sequencing.

Construction of L5366W containing a homozygous deletion of the *WHI2* gene. Haploid segregants of L5366 with complementary mating types were isolated; these were all *ura3* since the diploid parent was homozygous for this mutation. They were transformed with a linear DNA fragment consisting of the *WHI2* region in which 1420 bp was replaced with the *URA3* gene, that is from a *BamHI* site 258 bp upstream of the start of the *WHI2* ORF to a *XhoI* site 328 bp before the end of the ORF. *Ura*⁺ transformants with complementary mating types were crossed to form diploids. As expected these had a strong *whi2* phenotype. The deletion was confirmed by Southern hybridization. The results presented here have been repeated with four independently isolated diploid strains homozygous for the *WHI2* deletion.

Detection of epitope-tagged *Whi2* by Western blotting. Cells were grown to an OD₆₀₀ of 0.3 in CASRAF, harvested by centrifugation, resuspended in an equal volume of YEPGal or YEPD and incubated for a further 3 h. Approximately 10⁹ cells were harvested by centrifugation, washed in ice-cold water and resuspended in lysis buffer consisting of 50 mM Tris/HCl pH 7.5, 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 0.1% NP40, 1 mM freshly prepared PMSF, 0.6 mM dimethylamino-purine, 1 µg leupeptin ml⁻¹, 10 µg TPCK ml⁻¹, 10 µg soybean trypsin inhibitor ml⁻¹ (all protease inhibitors were purchased from Sigma). To this was added an equal volume of acid-washed glass beads (40 mesh BDH) and the mixture vortexed five times for 30 s with 30 s intervals on ice. Cell lysis was

Table 1. *S. cerevisiae* strains used

Strain	Genotype	Source
W303a	<i>a ade2-1, trp1-1, can1-100, leu2-3, his3-11, ura3, GAL1 SSD1-1D</i>	M. Tyers, Samuel Lunenfeld Research Institute, Toronto
L5366	<i>a/α ura3/ura3</i>	H.-U. Mosch & G. R. Fink
L5626	<i>a/α ura3/ura3 ste7/ste7</i>	H.-U. Mosch & G. R. Fink
L5625	<i>a/α ura3/ura3 ste11/ste11</i>	H.-U. Mosch & G. R. Fink
L5627	<i>a/α ura3/ura3 ste12/ste12</i>	H.-U. Mosch & G. R. Fink
L5624	<i>a/α ura3/ura3 ste20/ste20</i>	H.-U. Mosch & G. R. Fink
L5366h	<i>a ura3</i>	This work
L5626h*	<i>ura3 ste7</i>	This work
L5625h*	<i>ura3 ste11</i>	This work
L5627h*	<i>ura3 ste12</i>	This work
L5624h*	<i>ura3 ste20</i>	This work
L5366W	<i>a/α Δwhi2::URA3/Δ whi2::URA3</i>	This work

* The mating type of these strains could not be determined because of the respective *ste* mutations.

confirmed by microscopic examination. The beads and cell debris were pelleted for 2 min in a microfuge and the supernatant cleared by a further 10 min centrifugation. Protein content of the sample was determined using a commercial protein assay reagent (Bio-Rad). Forty-five micrograms of the sample was separated on a 7.5% SDS-polyacrylamide gel using a Bio-Rad Miniprotein II gel tank. The proteins in the gels were transferred to GeneScreen nylon membranes (DuPont) using a Bio-Rad TransBlot apparatus. Ponceau S (Sigma) staining was used to confirm equal loading of the sample in each lane. Blocking reactions, incubation with primary and secondary antibodies and washes were performed as described in the GeneScreen manual. The primary antibody was the *c-myc* monoclonal antibody, 9E10 (Santa Cruz Biotechnology) diluted 1:100; the secondary antibody was anti-mouse IgG (whole molecule) peroxidase conjugate (Sigma) diluted 1:2000. Immunoreactive material was visualized using enzyme chemiluminescence (ECL; Amersham International).

Cell protein was fractionated into soluble and insoluble fractions as follows. Induced cells were harvested and disrupted with glass beads as described above. The crude mixture of glass beads and cell debris was cleared by passing the suspension through a Pierce & Warriner disposable polystyrene column containing a sintered glass plug, by centrifuging for 10 s at 1000 r.p.m. The filtrate was then centrifuged for 10 min at 15000 r.p.m. in a microfuge. The supernatant was reserved as the soluble fraction. The pellet was washed twice with 0.5 ml lysis buffer, resuspended in SDS loading buffer containing 10 mM β -mercaptoethanol, boiled for 5 min, centrifuged for 5 min and the supernatant retained as the insoluble fraction. Samples of the soluble and insoluble fractions were adjusted so that, when loaded onto an SDS gel as described above, each sample would originate from an equal amount of cells. After Western blotting, staining with Ponceau S revealed that the soluble fraction contained much more material than the insoluble fraction, distributed into a large number of bands; in contrast the insoluble fraction contained a small number of bands.

Cytological methods and microscopy. Immunofluorescence was carried out according to Pringle *et al.* (1991) using the *c-*

myc monoclonal antibody 9E10 (Santa Cruz Biotechnology), diluted 1:100, as the primary antibody and fluorescein isothiocyanate (FITC) conjugated to rabbit anti-mouse whole molecule IgG (Sigma), diluted 1:64, as the secondary antibody. Cells were examined with a Leitz DMRBE microscope connected to a Leica TCS4D confocal scanning module. Although images presented here show fluorescence in one focal plane, images were examined in other planes to confirm that there was no other out of focus fluorescence. Images were recorded as Tiffs.

FITC conjugated to Concanavalin A (Sigma) was used to visualize cell walls as described by Adams & Pringle (Adams & Pringle, 1983). One microgram DAPI per millilitre (Sigma) was incorporated into the mounting medium to visualize nuclei. To visualize bud scars cells were fixed for 2 h at room temperature in 3.7% formaldehyde, washed twice in distilled water and resuspended in 1 mg Calcofluor-white ml⁻¹ (Sigma). Cells were incubated for 10 min in the dark, then washed three times in distilled water before viewing. The fluorescent images from Concanavalin A-FITC, DAPI and Calcofluor-white labelled cells were examined using an Olympus BH2 microscope with a BH2 RFCA reflected light fluorescent light attachment. Images were recorded as Tiffs using a video camera and microcomputer. Colonies growing on plates were examined and photographed using a Nikon Labphot microscope.

Cell volume. The radii of the major (*a*) and minor (*b*) axes of at least 40 mother cells were measured. Assuming cells approximate to a prolate spheroid, their volume (*V*) can be calculated according to the formula $V = 4/3 \times \pi \times b^2 \times a$ (Sudbery *et al.*, 1980).

RESULTS

Epitope-tagged *Whi2*

To identify *Whi2* and locate it within the cell it was tagged at its C-terminus with a single copy of the *c-myc* epitope recognized by the 9E10 monoclonal antibody (see Methods). The tagged protein complemented the size defect of a *whi2* deletion allele (*whi2Δ*) in strain

Fig. 1. Overexpression of epitope-tagged Whi2. (a) W303a cells were transformed with pWHIM3 (lanes 1, 2), pWHIM4 (lanes 3, 4), pWHIM5 (lanes 5, 6) or pYES2 (lanes 7, 8). Cells were pre-grown in CASRaF medium then incubated for 3 h in either YEPD (lanes 1, 3, 5, 7) or YEPGal (lanes 2, 4, 6, 8) medium as described in Methods. Proteins were extracted and Western blots prepared using the 9E10 monoclonal antibody. The specific band described in the text is indicated by an arrow. (b) W303a cells were grown overnight on CASRaF and resuspended ($OD_{600} = 0.3$) in YEPGal medium for 3 h. Lanes: 1, pWHIM5; 2, pYES2; 3, pWHIM7; 4, pWHIM4; 5, pWHIM3; 6, untransformed. In both parts the molecular mass in kDa of marker proteins is shown on the left.

W303a (data not shown). The tagged gene also complemented a variety of novel phenotypes which we have recently observed in a W303a *whi2* Δ strain – hypersensitivity to caffeine, a glycerol growth defect, sensitivity to 1 M NaCl and a 37 °C growth defect (unpublished). In fact, in all respects, W303a *whi2* Δ cells transformed with plasmids containing the tagged gene had an identical phenotype to the same cells transformed with an equivalent plasmid containing the unmodified gene. Moreover, the effects of overexpressing the tagged gene described below were also observed when the unmodified gene was overexpressed. Thus the epitope tag does appear to interfere with, or modify the function of Whi2.

The tagged gene was incorporated into the following constructs. In pWHIM3 it was expressed from its own promoter on a low-copy centromeric plasmid. In pWHIM4 it was expressed from its own promoter on a multicopy 2 μ m vector. Finally, in pWHIM5 it was under the control of the *GAL1* promoter on a multicopy 2 μ m vector. Wild-type cells (strain W303a) were transformed with each of the plasmids and Western blots were carried out using protein extracts of cells growing on either galactose or glucose. The results are shown in Fig. 1(a). A number of bands were present in both the negative controls and the experimental samples, these presumably arose by non-specific binding of the monoclonal antibody to proteins present in the total extract. In addition to these non-specific bands, a band of the predicted molecular mass of epitope-tagged Whi2 was evident in pWHIM4-transformed cells growing on galactose or glucose and pWHIM5-transformed cells growing on galactose (indicated by an arrow in Fig. 1a).

The band was absent in pWHIM5-transformed cells growing on glucose and cells transformed with the pYES2 vector. Furthermore, the molecular mass of this band increases by an appropriate amount when four copies of the epitope instead of one are present (see Fig. 1b and below). We concluded that this band represents epitope-tagged Whi2. The amount of Whi2 in pWHIM5-transformed cells growing on galactose was considerably higher than that from pWHIM4-transformed cells growing on either galactose or glucose.

The level of Whi2 produced by the low-copy pWHIM3 is presumably similar to the normal level of Whi2 in untransformed cells. Since no band was observed in pWHIM3-transformed cells, this level is apparently too low to measure by these means. Because of this we constructed pWHIM7, which consists of a low-copy centromeric vector carrying the *WHI2* gene tagged with four copies of the *c-myc* epitope. The tagged protein was now clearly visible in Western blots (Fig. 1b); indeed the band of immunoreactive material was more intense than that from pWHIM4, the multicopy vector containing a single copy of the tag. The increased molecular mass of the band is consistent with the extra copies of the *c-myc* epitope.

Overexpression of *WHI2* causes filament formation

During the course of experiments to detect epitope-tagged Whi2 we observed that its overexpression from the strong *GAL1* promoter resulted in the formation of filaments, both in liquid culture and on the surface of an agar plate (Fig. 2). The results are described here for strain L5366h but similar results have been obtained for strain W303a (Fig. 6b). L5366h is a haploid strain derived by sporulation from the diploid L5366 kindly provided by H.-U. Mosch & G. R. Fink, Massachusetts Institute of Technology, MA, USA, as an example of a strain capable of forming pseudohyphae. Normally, L5366h cells grow in liquid culture as spherical clumps of cells due to the failure of cells to separate after cytokinesis; such clumping is common in haploid strains of *S. cerevisiae*. Fig. 2(a) shows pWHIM5-transformed L5366h cells, growing in liquid culture on galactose to induce the expression of Whi2. It can be seen that the cells grew in filaments. Approximately 40% of cells responded in this way (Table 2). Cells transformed with the pYES2 vector had a normal morphology (inset, Fig. 2a). Cell dimensions were measured from this and other similar photomicrographs. The axial ratio (longer axis/shorter axis) of non-filamenting cells was 1.05 ± 0.039 , while the axial ratio of filamenting cells was 2.27 ± 0.12 . A similar elongation of cell shape occurs during pseudohypha formation in nitrogen-limited diploids and the invasive growth of haploids, although the shape change is less severe in the latter case. Overexpression of Whi2 also results in a three-fold increase in cell volume from 15.5 to $48.3 \mu\text{m}^3$ (Fig. 2a) showing that overexpression of Whi2 has reset the size control mechanism (Johnston *et al.*, 1977). A *whi2* null mutation resets this ratio in the opposite direction (Sudbery *et al.*, 1980); thus Whi2 clearly plays a crucial role in controlling cell size. Most

Fig. 2. Effect on cell morphology of *Whi2* overexpression. (a) L5366h cells transformed with pWHIM5 (main picture) or pYES2 vector (inset) were grown in liquid CASGal medium, stained with both Concanavalin A-FITC and DAPI and examined with a fluorescence microscope with the filter optimized for DAPI fluorescence. The cell walls are outlined by the residual fluorescence from FITC-Concanavalin A. Note both the main picture and the inset were photographed at the same magnification (bar, 10 µm). L5366h-pWHIM5 cells (b) or L5366h-pYES2 cells (c) were streaked onto the surface of a minimal medium plus galactose agar plate, incubated overnight at 30 °C and examined by transmitted light microscopy. (b, c) Bar, 20 µm.

Table 2. Percentage of clumps or microcolonies containing filamenting cells

Cells were cultured overnight on CASGal medium which selects for the presence of the *URA3*-based pWHIM5 plasmid, harvested, resuspended in fresh CASGal medium to $OD_{600} = 0.2$, incubated for a further 4 h and scored for the percentage of clumps containing filaments; 200 clumps of cells were counted for each value. Generally, either all or none of the cells in a clump showed filament formation; so the presence of a single distinct filament in a clump was used as the criterion to score it as forming a filament. On plates, cells were streaked from a fresh YEPD plate culture onto CasGal, to produce well-separated colonies. After 16 h incubation the percentage of microcolonies containing filaments was scored in the part of the plate where they were well separated. As in liquid culture, generally either all or none of the cells in a microcolony showed filament formation.

Strain	Relevant genotype	Filaments on plates (%)	Filaments in liquid culture (%)
L5366h-pWHIM5	WT	100 ± 7	40 ± 3
L5626h-pWHIM5	<i>ste7</i>	39 ± 3	25 ± 2
L5625h-pWHIM5	<i>ste11</i>	0	0
L5627h-pWHIM5	<i>ste12</i>	36 ± 3	24 ± 2
L5624h-pWHIM5	<i>ste20</i>	63 ± 5	20 ± 2

of the cells contain nuclei except for the terminal cell in the filament, which is often anucleate; this is presumably the equivalent of a bud still physiologically connected to the adjacent compartment. In addition to nuclei, mitochondria arranged in necklaces are visible around the perimeter of the cells. On plates, filaments form overnight in all of the colonies (Fig. 2b and Table 2). Chains of long thin cells can be seen to radiate from a central clump. After 3 d the colonies frequently overgrow these extended filaments but, in a sample taken from such

colonies, cells can still be seen to be growing in a filamentous fashion. As reported above, overexpression of *Whi2* in strain W303a also resulted in filamentous growth both in liquid culture and on plates (see Fig. 6b). This strain does not show the clumping evident in L5366h, and cells normally complete cytokinesis. Thus the failure of cells to separate after cytokinesis, evident in the filaments, represents a real change in the pattern of cell proliferation and is not a result of the tendency of strain L5366h to clump.

Fig. 3. Bud scar patterns of normal and filamenting L5366h cells. L5366h-pYES2 (a) and L5366h-pWHIM5 (b) cells were grown overnight in YEPGal liquid medium. They were stained with Calcofluor and examined in a fluorescence microscope.

Many of the changes described above are characteristic of cells forming pseudohyphae. However, unlike pseudohyphae, the filamentous growth was not repressed by excess nitrogen, as filaments formed on rich YEP-based, synthetic minimal and nitrogen-limited SLAHD (Gimeno *et al.*, 1992) media (data not shown). Cells transformed with pWHIM5 remained viable and formed colonies at a normal rate on galactose. To assess the viability of filamenting cells, individual filaments were transferred by microdissection to a glucose plate. They formed colonies, consisting of normal cells, with a frequency of 100%. The response is specific to *Whi2*. Cells transformed with the vector alone failed to form filaments [Fig. 2a (inset), c], so it was not an indirect consequence of inducing multiple copies of the *GAL1* promoter or of growth on galactose. Nor was the response caused by the *c-myc* epitope tag since overexpression of untagged *Whi2* also caused identical filaments to form (data not shown). Although induced pWHIM5 cells make *Whi2* at greatly elevated levels, it does not constitute a significant fraction of total cell protein since no extra band is visible in Coomassie-stained gels; thus it is not a non-specific response to protein overexpression. Cells transformed with pWHIM4, which produce more *Whi2* than normal cells, but less than pWHIM5-transformed cells on galactose, did not filament on glucose or galactose.

Filament formation results from a change in budding pattern

Normally, the pattern of budding in a haploid cell is axial; that is, the site of bud formation in a mother cell is adjacent to the site of bud formation in the previous generation or, in a virgin mother cell, at the birth pole of

the cell (Freifelder, 1960; Gimeno & Fink, 1992). If cells fail to separate after cytokinesis, axial budding will result in clumps of cells. Filamentous growth requires both a failure of cells to separate after cytokinesis and a polar pattern of budding, where the daughter bud forms at the free end opposite the birth pole, resulting in linear chains of cells. Calcofluor-staining of non-filamenting cells shows that the bud scars are clustered together in a pattern typical of axial budding (Fig. 3a). Filamenting cells are arranged linearly, with a single bud scar at each junction between the cells, which is clearly not an axial pattern (Fig. 3b). On plates, branches formed two or more cells away from the apical cell and invariably extended in the same direction as the main filament, indicating that the pattern of budding is unipolar; that is, the second and subsequent buds are also restricted to the non-birth pole.

Filament formation is blocked by a mutation in the *STE11* gene but not the *STE7*, *STE12* or *STE20* genes

Pseudohypha formation in diploid cells in response to nitrogen-limited growth and the invasive growth of haploids have previously been shown to depend on the functioning of the *STE7*, *STE11*, *STE12* and *STE20* genes (Liu *et al.*, 1993; Roberts & Fink, 1994). These genes were first described as elements of the pheromone-response pathway (for a review see Kurjan, 1993). Haploid strains containing disruptions in these genes (derived from diploids provided by H.-U. Mosch & G. R. Fink) were tested to see if they still produced filaments in response to *Whi2* overproduction. A mutation in the *STE11* gene completely abolished the filamenting response both in liquid culture and on the surface of an agar plate (Table 2). However, in *ste7*, *ste12* or *ste20* mutants, filaments formed which were similar in appearance to those that occur in wild-type cells, although the frequency of filamenting cells was reduced (Table 2). It should be noted that Table 2 shows that different *ste* mutants appear to have a different residual capacity for filament formation on plates but apparently equal capacity in liquid culture. Since the function of *STE7*, *STE12* and *STE20* is required for pseudohypha formation we conclude that *Whi2* operates through a different pathway. This conclusion was confirmed by the observation that a diploid strain, in which both copies of the *WHI2* have been deleted (see Methods), still forms pseudohyphae in response to nitrogen-limited growth (Fig. 4). Thus pseudohypha formation does not require *WHI2* function.

The filaments show strongly polarized growth

The elongated appearance of the cells suggested that they were growing in a polarized fashion. To determine if this was so, the Concanavalin A procedure described by Adams & Pringle (1983) was employed. Cells were grown in YEPGal to allow filaments to form. They were then resuspended in FITC-Concanavalin A to allow existing cell walls to be labelled, the cells were then harvested by centrifugation, washed and resuspended in

Fig. 4. Pseudohypha formation does not require the function of the *WHI2* gene. A diploid homozygous for a *WHI2* deletion was cultured for three days on the surface of a SLAHD plate (Gimeno et al., 1992). Bar, 80 μ m.

YEPGal in the absence of FITC-Concanavalin A. After 3 h they were harvested and the extent of labelled and unlabelled cell wall material determined by fluorescence microscopy. Unlabelled material, representing new growth, occurred exclusively at the tips of the filaments (Fig. 5). This result shows growth of the filaments is strongly polarized, consistent with the elongated appearance of the cells.

When overexpressed, *Whi2* is concentrated at the tips of the filaments

Experiments were carried out to locate epitope-tagged *Whi2*. No signal was detected in cells transformed with the low-copy plasmids pWHIM3 or pWHIM7, despite the latter containing four tandem copies of the tag which allowed it to be visualized in Western blots. Clearly, in normal cells *Whi2* is present in very small amounts. *Whi2* was detected when pWHIM5-transformed cells growing on raffinose were induced by the addition of galactose. Five hours after induction it was observed only in the apical cells of the resulting filaments (Fig. 6). When cells were grown for prolonged periods in galactose, some filaments became uniformly labelled, but we never observed filaments in which the internal cells, but not the apical cells, were labelled. In such cultures, not all of the cells show the filamenting response (see above); some, but not all, of the remaining cells showed a positive signal. In cells that were labelled, the protein appeared to be uniformly located throughout the cell. These observations should be interpreted with caution, since it is possible that a specific location was being masked by overproduction of *Whi2*. No signal was seen in untransformed cells or pWHIM5-transformed cells growing on glucose (data not shown).

Since *Whi2* could be detected from the low-copy pWHIM7 in Western blots, but its location not resolved by immunofluorescence, we carried out a simple fractionation of pWHIM7-transformed cells (see Methods). The immunoreactive material was strongly concentrated in the insoluble fraction (Fig. 7).

An extragenic suppressor of *whi2* also forms filaments.

We shall describe elsewhere that *whi2 ssd1-d* strains have a number of characteristics that suggest cytoskeletal defects (unpublished) including hypersensitivity to caffeine. Growth is inhibited when patched onto plates containing 8 mM caffeine; however papillae of clones carrying a suppressor are evident. Many of these clones show a phenotype which is very similar to that seen on overexpression of *WHI2*. One such clone has been characterized and shown to be due to a single recessive nuclear mutation which we designate *fsw1* (filamenting suppressor of *whi2*). The appearance of *fsw1* cells is shown in Fig. 8. We argue below that the *fsw1* allele identifies a downstream, negatively regulated target of *WHI2*.

DISCUSSION

Overexpression of *Whi2* results in a concerted reorganization of the cell cycle. The budding pattern changes from axial to polar, cytokinesis fails to proceed to completion, cells show hyperpolarized growth, new cell wall synthesis is confined to apical cells, cells become elongated in shape – the axial ratio doubles, and volume increases threefold. The change is reversible: isolated filaments when placed on glucose can act as founders of colonies of yeast-like cells. Taken together these results suggest that a switch to an alternative form of cell proliferation has occurred. These changes could be a non-specific pathology brought about by the effect of overexpressing a gene normally present in low abundance. There are two reasons arguing that this is not the case. Firstly, the level of overexpression is still too low for detection on a Coomassie-Blue-stained gel. Thus the results of overexpression must be a consequence of the specific properties of *Whi2* and not due to a general burden on the cell arising out of unbalanced protein synthesis. Perhaps more importantly, a suppressor of the caffeine sensitivity of a *whi2 ssd1-d* strain has a filamenting phenotype which is very similar to that seen upon *WHI2* overexpression. We suggest that the simplest explanation for these observations is that, directly or indirectly, *WHI2* negatively regulates a function required for the correct completion of cytokinesis encoded by the *FSW1* gene.

This reorganization of the cell cycle described above is also a central feature of pseudohyphal development. However, there are three important differences. Firstly, diploids homozygous for a deletion of the *WHI2* gene still form pseudohyphae in response to nitrogen limited growth suggesting that *Whi2*-induced filament formation acts through a different pathway. Secondly, *Whi2*-induced filament formation does not result in cells invading the agar. Thirdly, *Whi2*-induced filament formation is not blocked in *ste7*, *ste12* or *ste20* mutants, although it is reduced. The filamenting response is blocked by an *ste11* mutation. *STE11* encodes a protein kinase homologous to mammalian MEK kinases and to the *BCK1* component of the *PKC1/MPK1* pathway. A

Fig. 5. New cell wall material is incorporated only into the tips of filaments. A Concanavalin A pulse-chase experiment was carried out as described in the text. The figure shows a selection of filaments after the end of the chase period. The dark region at the end of each filament shows new cell material incorporated during the chase period. Bar, 10 μ m.

Fig. 6. Epitope-tagged Whi2 is concentrated in the apical cells of the filaments. W303a-pWHIM5 cells were grown to an OD_{600} of 0.6 on YEPRaf; they were harvested by centrifugation and resuspended in YEPGal and incubated for 5 h. Immunofluorescence was carried out as described in Methods: (a) confocal fluorescence image, (b) confocal phase-contrast. The images show a single clump of filamenting cells, of which only the tips are in focus. The two brightly fluorescing patches shown in (a) correspond to the tips of the cell chains shown in (b). The samples were examined in a series of focal planes; fluorescence was only seen in the apical cells. Control L5366h-pYES2 cells showed no detectable fluorescence (not shown).

Fig. 7. Whi2 is recovered in the insoluble cell fraction. Insoluble (lane 1) and soluble (lane 2) protein fractions were prepared from W303a cells transformed with pWHIM7 induced as described in the legend to Fig. 1. Samples originating from equal amounts of cells were loaded onto an SDS gel and Western blotted and probed with 9E10 monoclonal antibody.

straightforward interpretation of this observation would place *WHI2* upstream of *STE11*. However, the response is not blocked by *ste7* or *ste12* mutations which lie downstream of *STE11* in both the mating pheromone-transduction pathway and pseudohypha formation (Liu *et al.*, 1993). Either the response to Whi2 overproduction does involve the mating pheromone-response pathway but the order of function of its components is different from that seen previously, or *STE11* has an additional function in another pathway.

There has been considerable interest recently in the mechanisms involved in bud site selection. *BUD1* (also

Fig. 8. Appearance of *fsw1* cells. Cells were grown overnight in liquid YEPD and examined with a phase-contrast microscope.

known as *RSR1*), *BUD2* and *BUD5* are required for both bipolar and axial budding patterns, since mutations lead to random bud-site selection (Bender & Pringle, 1989; Chant & Herskowitz, 1991; Chant *et al.*, 1991). *BUD3*, *BUD4* and *BUD10* are required specifically for the axial budding pattern, as mutations do not affect the bipolar budding pattern in α/α diploids but cause α and α cells to bud in a bipolar fashion (Bender & Pringle, 1989; Chant & Herskowitz, 1991; Chant *et al.*, 1991; Halme *et al.*, 1996). A search for mutants specifically affected in the bipolar pattern yielded *SPA2* (Zahner *et al.*, 1996), a non-essential gene previously identified because mutant alleles result in an altered budding pattern and display weak defects in cytokinesis (Snyder, 1989). The search also yielded novel alleles of *BUD2* and *BUD5* and identified mutant alleles of four novel genes. Among these were *bud6* and *bud7* which budded randomly, *bud8* which bud exclusively at the proximal pole and *bud9* which bud only at the distal pole. A recent model (Chant & Pringle, 1995) for bud formation proposes that axial specific signals (*Bud3*, *Bud4*, etc.) and bipolar-specific signals both act upstream of general site-selection functions (*Rsr1/Bud1*, *Bud2/Bud5*). These act upon polarity establishment functions (*Cdc24*, *Cdc42*, *Bem1*) which in turn act upon the cytoskeleton. Overexpression of *Whi2* modifies this pathway in at least three different ways: it changes the budding pattern from axial to unipolar, it results in hyperpolarized growth and it results in a failure to complete cytokinesis, which suggests that some essential function in budding has not been completed.

The change in budding pattern could result from down regulation or inactivation of *BUD3* and/or *BUD4* which

would abrogate the axial budding pattern. It is unclear what causes the polarized growth. *spa2* alleles are co-lethal with *slk1*, a component of the *PKC1/MPK1* signalling pathway. We shall describe elsewhere extensive genetic interactions between *WHI2* and this pathway and furthermore how *whi2* cells have a disorganized actin cytoskeleton (unpublished). These observations suggest that both *Whi2* and known components of the bud selection and formation pathways are part of a common system that interacts with the cytoskeleton. The cytokinesis defect resembles the phenotype of *cdc3*, *cdc10*, *cdc11* and *cdc12* alleles. These genes encode septins, which associate in a complex of 10 nm filaments that forms a ring defining the size and position of the bud neck at an early stage in bud formation, coincident with the assembly of the actin cap (reviewed by Sanders & Field, 1994). *cla* mutants were isolated in a screen for mutations synthetically lethal in combination with *cln1* and *cln2* (Cvrckova *et al.*, 1995). They also have a cytokinesis defect, and in fact *cla10* is allelic to *cdc12*. *CLA4* encodes a kinase that is similar to *Ste20* and the mammalian-brain-specific Pak kinase (Manser *et al.*, 1994). *cla4* is synthetically lethal in combination with *ste20*, but interestingly, not with *ste11*, *ste7*, *kss1* or *fus1*, whose wild-type alleles encode members of a mitogen-activated protein kinase cascade which normally acts downstream of *Ste20*. Thus it is thought that *Ste20* may participate in a second signalling pathway (c.f. the role of *Ste11* in *WHI2* overexpression). Since the *cla* mutants were uncovered in a synthetic lethal screen involving mutant alleles of *CLN1* and *CLN2*, it has been suggested that both the *Cln1/2*-activated p34 kinase and the *Cla4/Ste20* kinases are required to complete an event in bud formation. Thus the failure to complete cytokinesis is seen as a late consequence of an earlier defect.

We report elsewhere that, directly or indirectly, *WHI2* regulates G1 cyclins since *whi2* cells have elevated levels of *CLN1* and *CLN2* transcripts which persist longer upon starvation than *WHI2* cells (Radcliffe *et al.*, 1997). There may thus be a connection between the observations reported here and the *cla* mutants – in both cases *CLN* activity is likely to be compromised and both result in a failure to complete cytokinesis. Characterization of the *FSW1* gene, which is currently underway, will test these speculations.

ACKNOWLEDGEMENTS

P.A.R. and K.M.B. were in receipt of BBSRC Research Training Awards. We gratefully acknowledge strains provided by Dr H.-U. Mosch and Professor G. R. Fink. We thank Dr Martin Cambray-Deakin for assistance with the confocal microscopy.

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Received 14 October 1996; revised 8 January 1997; accepted 16 January 1997.