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Function and localization of the SUN family of proteins in yeast populations

PhD thesis

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Statement

This is to certify that no material appearing in this PhD thesis has previously been submitted and approved for the award of a degree by this or any other university. I furthermore certify that this thesis is the result of independent study, under the supervision of Prof. RNDr. Zdena Palková, CSc. and RNDr. Libuše Váchová, CSc.

In Prague 22.05.2016

Signature _____

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Table of content

1. Abstract

The SUN family of proteins (Uth1p, Sun4p, Sim1p, Nca3p) is a group of fungal proteins similar to cell wall glucanases and highly homologous in their 256 long C-terminal amino acid domain. Our previous studies on yeast colony development revealed that members of the SUN family of proteins may be involved in the aging process and may play important role for survival during the development and grow of multicellular yeast populations. Our lab implemented a microarray analysis of expression changes in Saccharomyces cerevisiae colonies, which showed significant changes in the expression level of the SUN family member - UTH1. In addition, a strain with a disrupted UTH1 gene displayed a poorer grow and rate of survival in yeast colonies in comparison to the wild type. However, in this work, we focused on identifying and better understanding the functions of particular SUN proteins and determitation of their exact localization. Interestingly, some SUN family proteins have dual localization (Uth1p, Sun4p) to the mitochondria and cell wall and may thus be involved in mitochondrial and cell wall function. In this thesis, the "Results and discussion" section is divided into two parts as follows: the first part addresses questions concerning localization, oxygen-depended regulation and the possible involvement of SUN proteins in cell wall remodeling. The second part was preceded by a novel method of visualization of birth scars and of cell wall localized proteins and concerned participation of Sun4p in birth scar composition. In our study we showed that three members of the SUN family of proteins (Uth1p, Sun4p, Sim1p) are released from cells. In additional, we found that expression of UTH1, SUN4 and SIM1 genes regulated differently under different oxygen levels and during particular phases of yeast culture. We suppose that SUN proteins can be involved in remodeling of the cell wall and changes in its resistance to extracellular toxic compounds. We indicate that Uth1p may be an interesting target for study of the mode of boric acid's action. We implemented a novel method of visualization of cell wall proteins using antibodies conjugated with fluorescent dyes. This method allowed us to acquire new knowledge about the birth scar: a relatively little-studied yeast cell wall structure of unknown composition. We determined the localization of Sun4p, Dse2p and Dse4p to the birth scar. We determined the precise localization and co-localization of Dse2p, Dse4p and Sun4p within the yeast cell wall. We showed that the specific localization of Sun4p depends on the presence of Dse2p and that the localization of both Sun4p and Dse2p to birth scars depends on GPI-

anchored Egt2p. Deletion or combined double deletion of any of these predicted glucanases leads to cell separation defects. We hypothesize that these proteins are parts of the septum destruction complex which localizes to the daughter side of the bud neck and the birth scar and are required for mother–daughter cell separation at late mitosis. In addition, we showed that the presence of Sun4p within birth scars and the extracellular matrix depends indirectly on the Ace2p transcription factor

Using a novel immunofluorescence approach to yeast birth scar visualization we found that Aim44p is necessary for correct new bud selection. A strain with a disrupted AIM44 gene showed a so called “budding-within-birth scar” phenotype where new buds always appear within birth scars, the zone restricted for budding. A similar phenotype is seen in a strain deleted for gene SWI5, encoding a transcriptional regulator of AIM44. In summary, our results generate new knowledge about the localization of SUN family proteins and their roles in cell wall biogenesis, cell separation and birth scar composition.

1. Abstrakt

Rodina SUN proteinů (Uth1p, Sun4p, Sim1p, Nca3p) je skupina proteinů vyskytujicích v houbach a podobných glucanazům bunečny steny. SUN proteiny mají vysokou homologie v jejich C-terminalní doméně která je 256 aminokyselin dlouhá. Naše předchozí studium vývoje kvasinkových koloní odhalilo, že členy rodiny SUN proteinů mohou byt zapojený do procesu stárnutí a také mohou hrát důležitou roli pro přežívaní během vývoje a růstu mnohobunečné kvasinkové populace. V naší laboratoři jsme provedli microarray analýzu změn expresí genů v koloních, ktera ukázala výrazné změny v urovni exprese člena SUN rodiny UTH1. Kmen s deletovaným UTH1 genem vykazuje zpomalenější růst a horší přeživaní v kvasinkových koloních při porovnaní s wild type kmenem. Nicméně v své práci jsem se soustředil na identifikaci a ziskaní vice informací o funkcích jednotlivych SUN proteinů a zjištění jejich přesné lokalizace. Je zajimavé, že nekteré SUN proteiny mají duální lokalizaci (Uth1p a Sun4p) v mitochondrích a buněčné stěně a mohou byt spojeny s jejich funkcí. V této disertační práci, kapitola “Results and discussion” byla rozdělena na dvě části: první adresovaná otázkám, které se týkají lokalizace, regulaci zavislé na kysliku a možne zapojení SUN proteinů v remodelaci buněčné stěny. Druhé časti předcházel vývoj nové metody vizualizací jizvy zrodu a proteinů lokalizovaných v buňečné stěně a týkala se účasti Sun4p ve složení jizvy zrodu. Ve své práci jsem ukázal, že tři členy rodiny SUN proteinů (Uth1p, Sun4p a Sim1p) jsou uvolňovány z buňek. Zjistil jsem, že exprese genů UTH1, SUN4 a SIM1 je odlišně regulována při podminkách s různou koncentrací kyslíku v prostředí a během jednotlivých fazí vývoje kvasinkové kultury. Predpokladam, že se SUN proteiny účastni remodelace buněčné stěny a mění rezistenci proti toxickým látkám. Jsem ukázal, že Uth1p může být zajímavým cílem pro studium způsobu učinkovaní kyseliny borité. Zavedli jsme novou metodu vizualizace proteinů buněčné stěny pomocí protilátek s připojenými fluorescenčními barvami. Tato metoda umožní ziskání novych znalostí o jizvé zrodu: poměrně málo studované struktuře s neznamem složením, která je součástí buněčné stěny kvasinek. Určil jsem, že proteiny Sun4p, Dse2p, Dse4p jsou lokalizovány v jizvé zrodu. Zjistil jsem přesnou lokalizaci Sun4p, Dse2p, Dse4p v rámci buněčny stěny. Ukázal jsem, že specifická lokalizace Sun4p zavislá na přítomnosti Dse2p a že lokalizace obojích proteinů Sun4p a Dse2p v jizvé zrodu zavislá na Egt2p které je ukotvěné pomocí GPI. Delece

nebo kombinace dvojité delece těchto predikovaných glukanáz véde k defektu buněčné separace. Předpokládam, že tyto proteiny jsou součástí komplexu degradujícího septum, který je lokalizován v dceřiné části bud neck a v jizvé zrodu a je nezbytný pro buněčnou separaci v pozdní mitóze. Přitomnost Sun4p v jizvě zrodu a v mezibuněčném prostoru je nepřimo zavislá na transcripčním faktoru Ace2.

Použitím nového imunofluorescenčního přístupu pro vizualizaci kvasinkové jizvy zrodu jsem objevil, že Aim44p je nezbytný pro správný výběr nového mista pučení. Kmen s deletovaným genem AIM44 ukazuje tak zvaný “budding-within-birth scar” fenotyp, v kterém nový pupen vždy vzniká v rámci jizvy zrodu neboli oblasti omezené pro pučení. Podobný fenotyp má kmen s deletovaným genem SWI5, kodujicím transkripční regulátor pro AIM44. Ve shrnutí moje výsledky přespívají ke znalostem o lokalizaci rodiny SUN proteinů, jejich roli v biogenezi buněčné stěny, buněčné separaci a složeni jizvy zrodu.

2. General introduction

The cell wall is the outer layer which determines cell shape in the yeast Saccharomyces cerevisiae and in other fungi. The cell wall of yeast is essential for cell integrity, division and growth. Yeast cell wall proteins include the SUN family of 4 genes (SIM1, UTH1, NCA3 and SUN4) coding for homologous proteins similar to cell wall glucanese. The SUN-family of proteins is fungal specific group with homologous that conserved between varied yeasts and fungi. The exact function(s) of these proteins are largely unknown. In previous publications SUN family proteins have been implicated in different, often unrelated cellular processes such as mitochondrial biogenesis or cell wall integrity. Our study is concerned mostly with Sun4p. Cells lacking a SUN4 gene show delayed cell separation without any change in generation time and displayed modifications in the bud scar area. In strains deleted for the SUN family homology genes AfSUN1 in Aspergillus fumigatus (Gastebois et al. 2013), SUN41 in Candida albicans (Norice et al. 2007), and PSU1 in Schizosaccharomyces pombe (Omi et al. 1999) are observed similar to Δsun4 mutants phenotype. In addition to SUN proteins, a group of proteins closely related to glucanases, the so-called “Daughter Expression Specific” proteins Dse2 and Dse4 (Colman-Lerner et al. 2001) and GPI anchored Egt2 (Caro et al. 1997) function exclusively in mother/daughter separation. Genes encoding Dse2p, Dse4p and Egt2p are regulated by the Ace2p transcription factor, which is required for exit from mitosis (Laabs et al. 2003; Nelson et al. 2003; Maerz and Seiler 2010). All of these proteins have predicted localization to the bud neck region. The daughter side of the bud neck forms a birth scar on the cell surface after mother-daughter cell separation. The birth scar is a daughter-specific structure with unknown composition (Barton 1950; Bacon et al. 1966; Belin 1972). The birth scar normally forms a zone restricted from budding and in the wild type no bud scar has ever been observed completely within the birth scar (Tong et al. 2007). This thesis is devoted to the study of SUN family proteins and includes a summarization of previous knowledge concerning SUN proteins in Chapter 4 with results and discussion in Chapter 5. Results described in Kuznetsov et al., (2013, PlosOne) address questions about localization, oxygen-depended regulation and the possible involvement of SUN proteins in cell wall remodeling (Kuznetsov et al. 2013). The results of a second publication (Kuznetsov et al., 2016, Cell Cycle) discussed in Chapter 5 focus on the role of Sun4p in birth scar composition.

3. Aims

1. To ascertain the localization of SUN family proteins and test the hypothesis that SUN proteins are involved in the remodeling of the yeast cell wall under different environment conditional.

Specific aims

- Determination of presence of SUN proteins in extracellular space of developing colony and in liquid yeast culture.
- Assay of cell sensitivity to compounds influencing cell wall composition and integrity, when cells grow on fermentative or respiratory carbon sources.
- Test of effects of oxygen levels on SUN protein production.

2. Determination of cellular localization of Sun4p and its interaction with cell wall proteins or the yeast birth scar.

Specific aims

- Development of a novel method of visualization of cell wall proteins.
- Visualization of localization and co-localization of Sun4p, Dse2p and Dse4p to cell wall and birth scar.
- Provide new data about participation of Ace2p and Swi5p transcription factors in regulation of Sun4p localization, production and release.
- Screening of Sun4p-HA-knockout strains for Sun4p-HA production and cellular localization by western blot and immunofluorescence.

4. Introduction

4.1 SUN family proteins.

The four members of the SUN family of genes (SIM1, UTH1, NCA3, SUN4) encode a group of fungal proteins that are absent in mammals and plants. SUN proteins have high homology in the C-terminal domain. The 258 amino acid long domains share 75-78% identity and are similar to a b-glucosidase. The SUN domain contains four putative iron-binding cysteine residues in Cys-X5-Cys-X3-Cys-X24-Cys motifs which are predicted to bind to Fe-S proteins (Bandara et al. 1998). All SUN family proteins have a putative N-terminal signal peptide (Finn et al. 2006). SUN family proteins, as with most cell wall proteins, have a very high degree of glycosylation. Predicted from amino-acid sequences, their molecular weights are 43 414 kDa (Sun4p), 46 916 kDa (Uth1p) and 48 046 kDa (Sim1p). Real molecular weights demonstrated by SDS PAGE are 96 000 kDa, 60 000 kDa, 120 00 kDa for Sun4p, Uth1p and Sim1p respectively (Velours et al. 2002). There are only a few publications, containing diverse and unclear data about the possible functions of SUN family proteins, which therefore remain largely unknown. SUN proteins belong to a group of predicted gluconases and have been described as having beta-glucosidase enzymatic activity (Skory and Freer 1995). Analysis of proteins sequence homology (http://blast.ncbi.nlm.nih.gov) support hypothetical enzymatic activity in other fungi (including pathogenic Candida species that can cause an infection) such as *Candida wickerhamii*, *Candida albicans, Schizosaccharomyces pombe* and *Neurospora crassa*. Enzyme assays in the cellulolytic fungus *Candida wickerhamii* identified b-glucosidase activity of a cell-bound, extracellular protein BglB (Skory and Freer 1995), which has similarity to the SUN homolog BglA and may indicate a non-glucose-inhibited cellulase system. But SUN proteins cannot be considered as glucosidases because experimental data, supporting enzymatic activity, is lacking (Omi et al. 1999; de Groot et al. 2007). Despite their strong homology within the C-terminal domain, SUN family proteins take part in different cellular processes and functions, such as cell wall and mitochondrial biogenesis (Camougrand et al. 2000; Ritch et al. 2010), the aging process (Kennedy et al. 1995), stress response (Bandara et al. 1998) and cell septation (Mouassite et al. 2000), but exact functions are unknown. Some SUN proteins have unusual dual localization. Uth1p and Sun4p non-covalently bind to the cell wall and have also

been found in mitochondria (Camougrand et al. 2000; Velours et al. 2002; Camougrand et al. 2003; Camougrand et al. 2004; Kissova et al. 2004; Kissova et al. 2006; Kissova et al. 2007; Deffieu et al. 2009)

4.1.1 Localization and Involvement of Uth1p in different cellular process and functions.

Among members of the SUN family proteins (including homolgs in other fungi) Uth1p is the most studied and best described in literature. First the UTH gene (youth) was identified with the aid of the identification, Isolation and characterization of longer-lived mutants as an aging gene connected with replicative life span (Kennedy et al. 1995). The term “Replicative life span” (RLS) can be characterized as the total number of mother cell divisions before division ceases. Deletion of the UTH1 gene leads to an increased number of individual cell divisions and prolongation of RLS (Kennedy et al. 1995; Austriaco 1996; Bandara et al. 1998; Camougrand et al. 2003).

Uth1p has a dual cell wall/mitochondria localization and is an oxidative-stress-response gene. Although Velourz et al 2002 showed localization of Uth1p in outer mitochondrial membrane, later experiments predicted insertion of Uth1p in the inner mitochondrial membrane (Welter et al. 2013). The mutant Δ*uth1* demonstrates higher resistance to peroxide and increased sensitivities to oxidative damage agents such as superoxide and the thiol oxidant, diamide (Bandara et al. 1998). Localization of Uth1p to the mitochondria may indicate a possible role in mitochondrial biogenesis. Camougrand et al showed, that the double deletion Δ*uth1*Δ*nca3* results in a strong decrease in the concentration of mitochondrial cytochromes aa3, c and b. In particular, cytochrome aa3 was reduced by 50% in comparison with the wild type (Camougrand et al. 2000).

KIssova et al. (2004) offer the theory that Uth1p is involved in the autophagic degradation of mitochondria and take part in recognition of mitochondria by the autophagic machinery (Kissova et al. 2004, Camougrand et al. 2004). In experiments yeast cells were grown in conditions of nitrogen starvation. The mitochondria were visualized using mitochondria-targeted green fluorescent protein. As a control for relocalization of mitochondria to vacuoles the autofluorescent, vacuolar membrane probe FM4-64 was used. In starved cells deletion of the UTH1 gene delays degradation of mtGFP (Figure 1).

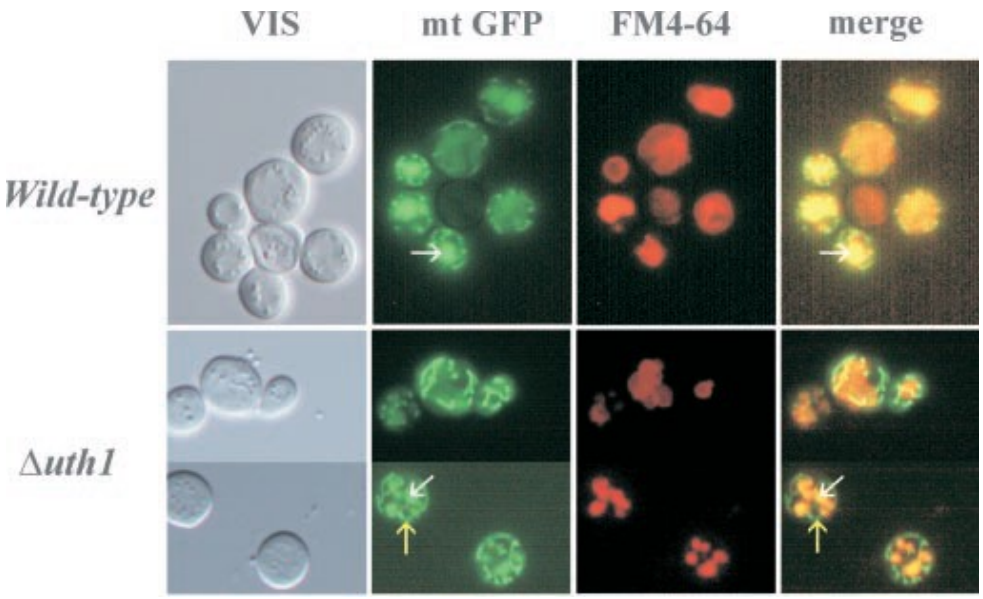


Figure 1. The absence of Uth1p delays the degradation of mtGFP in starved cells. Wild-type, Δuth1 cells were grown in YNB medium supplemented with 2% lactate and starved for 6 days.

The theory that Uth1p is involved in mitophagy is supported by experiments showing the induction of biodegradation processes by rapamycin treatment. The wild-type and Δ*uth1* strains have a normal mitochondrion network, but addition of rapamycin induces disorganization of the mitochondrial network in the wild-type. In the Δ*uth1* strain however the addition of rapamycin did not affect the mitochondrial network within two hours of treatment.

Mitochondria in cells can be degraded by selective mitophagy or nonselective macrophagy. Selective degradation of mitochondria in starved yeast cells was investigated in the Δ*uth1* strain with the aid of electron microscopy (Kissova et al. 2007). Results show an absence of selective mitophagy in yeast strains inactivated in the UTH1 gene (Figure 2).

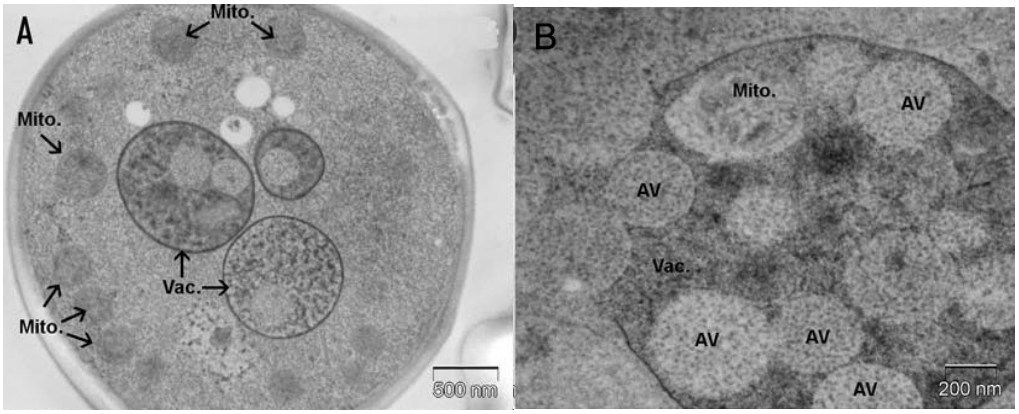


Figure 2. Absence of selective mitophagy in the ∆uth1 mutant.
The *Δuth1* strain was grown in lactate and submitted to nitrogen starvation for two hours (A) or six hours (B). (A) shows the fragmentation of vacuoles, as in the wild-type, but the total absence of vacuole/mitochondria contacts. (B) Mitochondria-containing vesicles in the ∆*uth1* mutant always contained a high proportion of cytosol, the presence of mitochondria in the vacuole remained a rare event.

Additional evidence of the involvement of Uth1p in mitophagy was obtained from experiments in which mammalian BAX-protein was inserted into yeast cells. BAX-protein creates mitochondrial apoptosis-induced channels (Kelly et al.) in mammalian apoptotic cells and is responsible for citochrome c release from mitochondria. When BAX-protein is inserted into the genome and expressed, utilising a BAX-carrier plasmid, giant mitochondrial channels form in yeast cells. Figure 3 shows a scheme for BAX cell death induction.

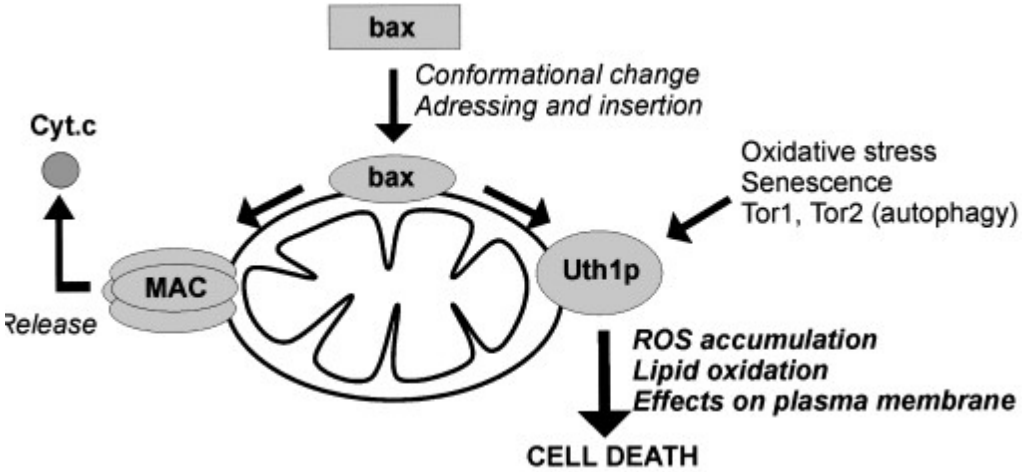


Figure 3. Bax effects on yeast mitochondria.
After insertion in the outer mitochondrial membrane, Bax is involved in MAC formation and cytochrome c release.

Although disruption of UTH1 gene does not modify BAX-mitochondria interaction resistance to BAX-expression was detected in the Δ*uth1* strain (Figure 4).

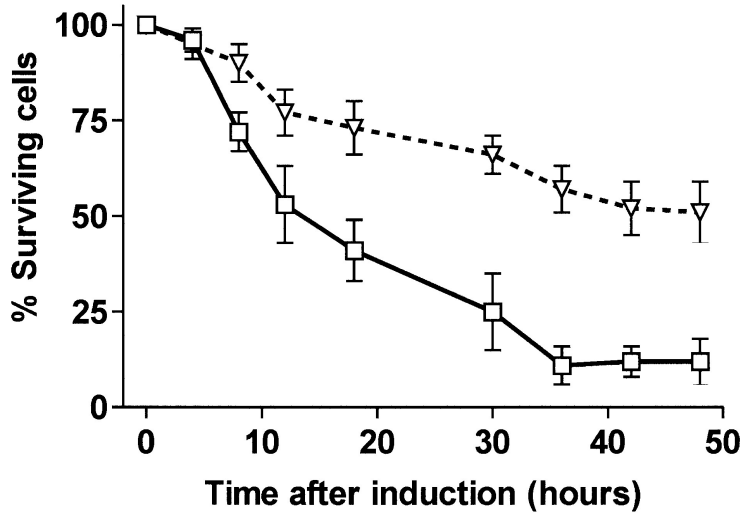


Figure 4. Resistance of Δuth1 strain to Bax expression.
Wild-type (squares) or Δuth1 (inverted triangles) strains carrying the pCM189/ Bax plasmid were grown aerobically in YNB-ura + glucose medium

Sun et al (2013) described the repression effect of anti-aging compounds Nolinospiroside F and Hesperedin on expression UTH1 gene. In additional,

prolongation of life-span in the presence of Nolinospiroside F and Hesperedin, tipically observed in the wild type, is absent in Δ*uth1* strain.

As has been described previously, Uth1p has dual localization (cell wall and mitochondrial membrane)(Velours et al. 2002). The function of Uth1p in the cell wall is relatively unstudied. Ritch et al. (2010) suggested that several phenotypes of ∆*uth1* related to enhanced growth under stress conditions arise from changed cell wall composition. Defects in cell wall structure are often observed by examining the sensitivity to treatment with cell wall disrupting enzymes and agents (Shiozaki and Russell 1995; Kinoshita et al. 1996; Toda et al. 1996; Hirata et al. 1998). Strain lacking the UTH1 gene has more robust cells, altered ratios of β-D-glucan and chitin in the cell wall and greater resistance to different cell wall toxic agents in comparison with the wild type. Spheroplast formation of yeast cells induced by zymolyase treatment significantly decreased in the ∆*uth1* strain. Testing sensitivity to calcofluor white (CFW) and sodium dodecyl sulfate (SDS), two agents which perturb the cell wall, detected decries of sensitivity and enhance cell growth in ∆*uth1* cells. Deleting UTH1 modifies the polysaccharide content of the yeast cell wall, especially glucans and chitin. Significantly higher levels of both alkaline-soluble and alkaline-insoluble β-D-glucan could explain why ∆*uth1* cells are more resistant to zymalyase treatment and cell wall toxic compounds. On the other hand the ∆*uth1* strain has relatively low levels of chitin. Authors have suggested that yeast cells have a compensatory mechanism, whereby decreases in chitin levels lead to a compensatory increase in β-D-glucan levels. In addition Ritch el al. (2010) suspect that resistance to mammalian BAX-mediated toxicity in ∆*uth1* cells is also explained by a strengthened cell wall.

4.1.2 Localization and function of Sun4p

Similarly to Uth1p, another SUN family protein – Sun4p was found to have dual localization to the cell wall and mitochondrial matrix.(Velours et al. 2002). Sun4p has a predicted N-terminal mitochondrial transit signal peptide and was detected in isolated and highly purified mitochondria with the aid of SDS-PAGE/western blot analysis. After carbonate treatment of purified mitochondria, this protein was detected in the supernatant fraction, which indicated a mitochondrial matrix localization.

In the same time the SUN4 gene product was described as a cell wall protein easily released by small-molecule redox reagent known as dithiothreitol (Cappellaro et al. 1998). Transcript profiling data (microarray analyze) link the expression of the SUN4 gene to the Ace2p transcription factor (data from SPELL tool of Yeast Genom Database).

Cells lacking a SUN4 gene show delayed cell separation without any change in generation time. Microscopic analysis of the Δ*sun4* strain reveals modifications in bud scar area. Figure 5 show that Δ*sun4* cells have deformed buds, often possess more than one bud within mother cell and the septum area was very thick (Figure 5) (Mouassite et al. 2000).

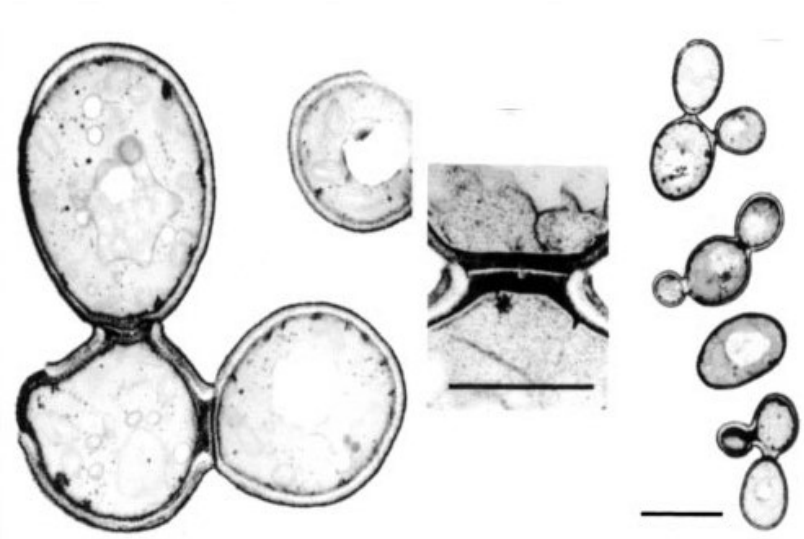


Figure 5. Ultra structural features of the Δ*sun4* strain delaminated by electron microscopy. Cells are often seen with more than one deformed bud, the septum area is very thick. Bar=5 mm or 1 mm.

In cell culture, a greater number of daughter cells remained attached to the mother cell in the Δ*sun4* strain. Furthermore, in the Δ*sun4* strain the daughter cell seemed to be anchored by the ring but this defect did not prevent the mother cell from starting a new cell cycle and forming a new bud. Δ*sun4* cells are slightly enlarged and granulated, often with more than one bud.

Separation defects, observed in Δsun4 were greatly enhanced in a strain with the double inactivation of SUN4 and another SUN family gene UTH1. The double knock-out of UTH1 and SUN4 led to an apparent polyploidy, which corresponded with clumped cells that remain connected after division. The double SUN4 and CTS1

deletion mutant has a similar morphology to the ∆*sun4*/∆uth1 strain (Mouassite et al. 2000).

4.1.3 Homologs of Sun4 in other fungi

A mutant deleted for the SUN family homology gene SUN41 in *Candida albicans* has a similar phenotype to that of the *S. cerevisiae Δsun4*. Disruption of SUN41 leads to a defect in cell separation. The double mutations sun41 and sun42 results in lysis of mother cells after separation (Figure 6) and an increase in sensitivity to toxic for yeast agents that disturb cell wall or membrane (Firon et al. 2007).

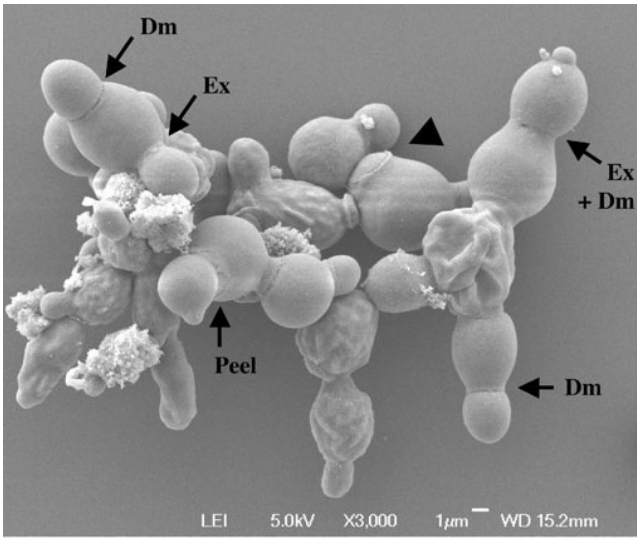


Figure 6. *sun41ΔΔ/sun42Δ* cells show cell wall and septum defects.
Black arrows highlight cell wall alterations in the septum region (Ex: excess of cell wall material; Dm: demarcation between mother and daughter cells; Peel: peeling of the mother cell wall).

Similar phenotypes are seen in *sun41Δ/sun41Δ* and *Δsun4* mutants with defects in cell separation and data from transcriptome analysis link SUN41 (Mulhern et al. 2006) and SUN4 (Colman-Lerner et al. 2001; Nelson et al. 2003) to the Ace2p transcription factor. Ace2p is the main component of the RAM-signaling pathway which controls daughter-specific expression of genes encoding enzymes that degrade the septum.
Experiments with a SUN41-disrupted strain showed that Sun41p is important for biofilm formation and virulence in *Candida albicans* (Norice et al. 2007).

sun41Δ/sun41Δ mutant cells fail to infiltrate host tissue efficiently in mouse models of infection.

The SUN family homolog AfSun1p from *Aspergillus fumigatus* is also connected with cell wall biogenesis and cell separation. AfSun1p is required for correct hyphal morphogenesis and growth. Deletion of the AfSUN1 gene negatively affects hyphal growth resulting in the presence of short hyphal cells, swollen hyphae and ultra-hyphal growth. Substrate binding assays identified hydrolytic activity against β-(1,3)-glucan which is negatively affected by branching of glucan. Authors suggest that SUN proteins could be building blocks for other proteins that participate in cell wall biogenesis and/or the suppression of cell wall degradation enzymes (Gastebois et al. 2013).

A *Schizosaccharomyces pombe* gene was isolated, with homology to SUN family genes and was named PSU1 (S. pombe homologue of the SUN family). Psu1p is essential for cell growth in S. pombe. Microscopic examination of *Δpsu1* cells revealed that PSU1 gene disruption is lethal (Figure. 7).

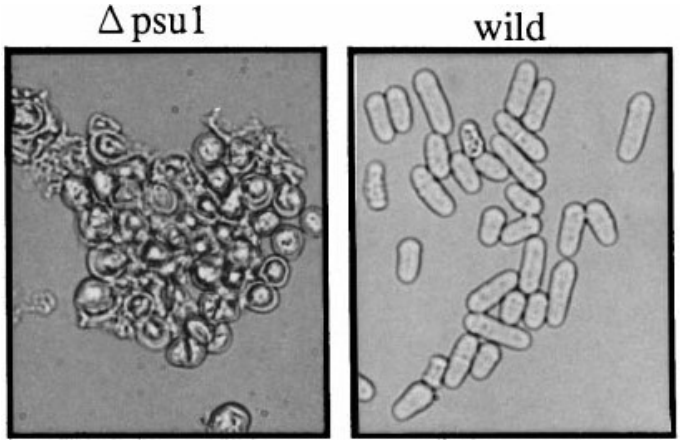


Figure 7. Terminal phenotype of *Δpsu1* cells with swollen spores.

Psu1-deficient cells are resistant to digestion by 1,3-b-glucanase. The composition or organization of the cell wall was altered by the deletion of PSU1 gene (Omi et al. 1999).

4.1.4 Sim1p

SIM1 (start independent mitosis) is a paralog of SUN4 encoding a protein with unknown function. Sim1p participates in regulation of Clb5 kinase activity on a post-transcriptional level. Clb5 kinase is a B-type cyclin which, via Cdc28p activation, is involved in DNA replication during S phase (Schwob and Nasmyth 1993; Mendenhall and Hodge 1998). Cells deleted for the SIM1 gene have a replication defect, and begin a fresh round of replication and re-budding during G2 phase. Sim1p prevents the onset of DNA replication without mitosis and is necessary for the activity of Clb5 kinase.(Dahmann et al. 1995). Overproduced Sim1p from multicopy plasmid, functioned also as a high copy extracellular suppressor of grow defect in *Δpag1* and *Δcbk1* cells. On the other hand *Δpag1* and *Δcbk1* strains with overexpressed Sim1p still have the cell separation defect and cell shape defect. (Du and Novick 2002).

Mouassite et al. (2000) found that inactivation of the SIM1 gene in mutants of another SUN family member – *Δuth1* leads to reduced viability and slower biomass production. In additional, DAPI staining of the *uth1Δ sim1Δ* mutant permitted visualization of a nuclear migration defect. Several cases of defects in nuclear localization and distribution levels were described (Figure 8). The authors proposed that the defect in nuclear migration, in combination with numerous pleiotropic phenotypes (low levels of glycogen, increase of heat shock sensitivity, reduced viability, slow growing, inability to grow on non-fermentable carbon sources and failure of spores to germinate), could be related to a damages in the Ras-adenylate cyclase pathway (Thevelein 1994).

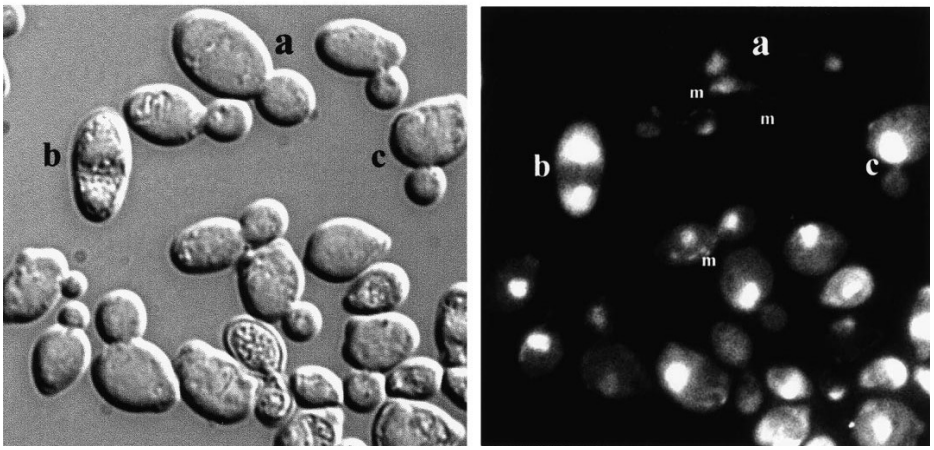


Figure 8. (a) binucleate mother cells and anucleate daughter cells; (b) cytokinesis failed to complete; (c) the nucleus remained undivided at the neck without translocating to a bud.

4.1.5 Nca3p

Nca3p (Nuclear Control of ATPase) is involved in the biogenesis of mtDNA-encoded proteolipids. Nca3p participates in genetic regulation of the mitochondrial synthesis of subunits 6 (Atp6p) and 8 (Atp6p) of the Fo-F1 sector of the ATP synthase (Pelissier et al. 1995).
Inactivation of the NCA3 gene decreased cytochrome aa3 levels by 10-15 % (Camougrand et al. 2000)

4.2 Daughter specific septum destruction enzymes

During cell separation the septum is exclusively disrupted from the daughter cell side. Septum degradation enzymes include chitinase Cts1p and putative hydrolytic enzymes that target wall components other than chitin. Cts1p is a chitin destruction ennzyme with specific localization to the daughter side of the cell septum (Colman-Lerner et al. 2001). Disruption of the CTS1 gene results in a defect in chitin degradation in the septum and a cell phenotype with cells that stay connected after cytokinesis. Other septum destruction enzymes that participate in the process of cell separation are putative hydrolases Dse2p, Dse4p, Egt2p and Scw11 (Dahmann et al. 1995; O'Conallain et al. 1998; O'Conallain et al. 1999; Bidlingmaier et al. 2001;

Colman-Lerner et al. 2001; Doolin et al. 2001; Di Talia et al. 2009). One group of these hydrolases participates in removing cell wall polymers localized to the secondary septum. Another group of hydrolases reorganizes the cell wall in the septum region by transferring glucan chains from one macromolecule to another. SUN family proteins belong to the septum destruction group. As described previously, deletion of SUN genes results in a separation defect. In addition, combined double deletion of genes coding different types of hydrolases enhances the separation defect. (Mouassite et al. 2000).

Closely related to septum destruction enzymes is Gas1p, a GPI-linked protein that contain glucanosyl transferase domains (Popolo et al. 2001; Ragni et al. 2007; Ragni et al. 2007; Popolo et al. 2008; Rolli et al. 2011). Gas1p transfers glucan chains extracted from β1–3 glucan synthase to other cell wall components and probably influences the action of exoglucanases and chitinase. Deletion of Gas1 causes a phenotype that is similar to other strains lacking septum destruction enzymes – again involving cell separation defects.

4.2.1 Dse2p

First DSE2 (Daughter Specific Expression) was identified as a gene regulated by the Ras-cAMP-cPKA signaling pathway and induced in conditions of nitrogen limitation (Tadi et al. 1999). The cyclic AMP messaging system is an important pathway for G1 to S progression of the cell cycle and for exit from mitosis. cAMP represses expression of the DSE2 gene.

DSE2 belong to the daughter-specific group of genes activated by the Ace2 transcription factor during cytogenesis. (Colman-Lerner et al. 2001; Doolin et al. 2001). The possibility that DSE2 is a target of the Ace2 transcription factor was supported by microarray data of cell-cycle regulated genes published by Cho et al. (1998) and Spellman et al. (1998). Disrupting of Ace2 completely abolishes expression of the DSE2 gene. The effect of deleting Ace2 was shown in experiments using Northern blot analyses of *ace2* and *swi5* mutants (Figure 9) (Doolin et al. 2001)

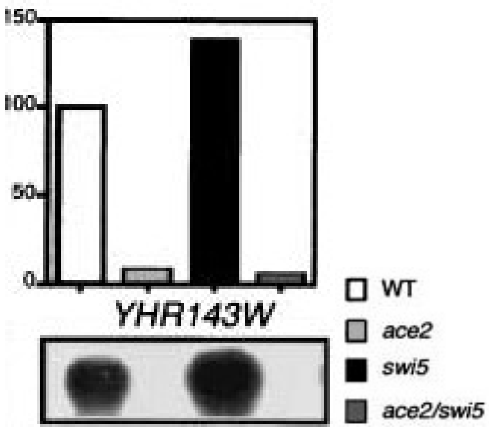


Figure 9. Northern blot analysis of expression of the DSE2/YHR143W gene in *ace2* and *swi5* mutants

Ace2 transcription factor has specific activity during M and G1 phase of the mitotic cell cycle. Cell cycle-dependent expression of DSE2 was demonstrated using cell cultures synchronized by arresting in G1 phase with α-factor. Examination of transcripts shows that DSE2 is expressed during the G1 phase of the cell cycle.
Doolin et al. (2001) described the phenotype of *Δdse2* (Figure.10). Deletion of DSE2 causes defects in cell separation in diploid strains and enchants of pseodohyphae production on low-nitrogen medium.

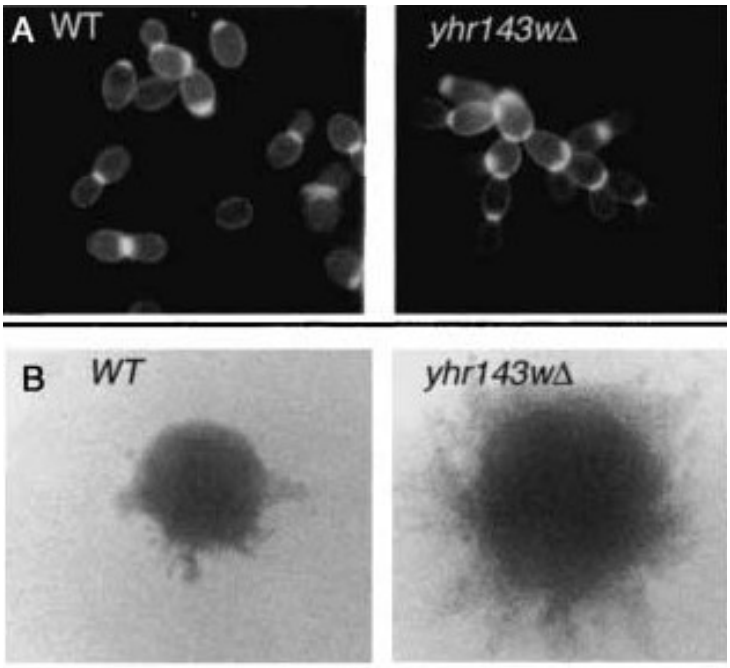


Figure 10. Phenotypic analysis of *dse2/yhr143* mutant. A. Diploid cells stay connected after cytokinesis. Bud scars visualized by calcofluor white. B. Examination of pseudohyphal growth on low-nitrogen medium.

Pseodohyphal growth involves switching from a unipolar budding pattern (with new buds appearing near previous budding sites) to a bipolar budding pattern, resulting in the production of long chains of cells. The DSE2 deficient strain is sensitive to SDS, calcofluor white and caffeine.

Colman-Lerner et al. (2001) suggested that the DSE2 gene product, together with another member of the so called “Daughter Specific Expression” genes, DSE4 participates in degradation of cell wall polymers in the bud neck region during G1 cell cycle phase and in mother-daughter cell separation.

4.2.2 DSE4

DSE4 (Daughter Specific Expression) is similar to the DSE2 daughter cell-specific expression gene encoding a putative glucanase. Similarly to other daughter-specific proteins, expression of DSE4 depends on the Ace2 transcription factor, which is expressed during the G1 cell cycle phase. (Colman-Lerner et al., 2001). Bidlingmaier et al. (2001) noted that DSE4/*YNR067C* is under the control of the serine/threonine protein kinase Cdk1, which is not daughter specific. At the same time Cbk1 controls

the localization and activity of Ace2p and participates in the regulation of polarized growth and septum destruction. DSE4 gene expression is reduced in a *cbk1* mutant (Bidlingmaier et al., 2001).

Dse4p was describedas possessing 1,3-β-glucan-degradative activity against laminarin with an endo-type mechanism of hydrolysis. (Baladrone et al. 2002). Sequence analysis determined several yeast homologs of Dse4p: the predicted *Saccharomyces cerevisiae* gluconase Acf4p, eng1^{+} and eng2^{+} in *Schizosaccharomyces pombe*, Engl1p in *Aspergillus fumigatus* and Dse4p in *Pichia pastoris* (Mattanovich et al. 2009). In addition, Dse4p has significant homology to plant β-glucan binding proteins (Umemoto et al. 1997; Mithofer et al. 2000) and a bacterial protein with unknown function in *Bacillus halodurans* (www.cazy.org/GH81_characterized.html).

Dse4p was detected not only in cell extracts but in the culture liquid medium. While the predicted molecular weight of the DSE4 gene product is 121 kDa, SDS detection shows several bands at 90, 120 and 160 kDa. Dse4p has a high level of N-linked glycosylation.

In cell culture of *dse4* mutants clusters of cells are present that have not completed separation (Figure 11).

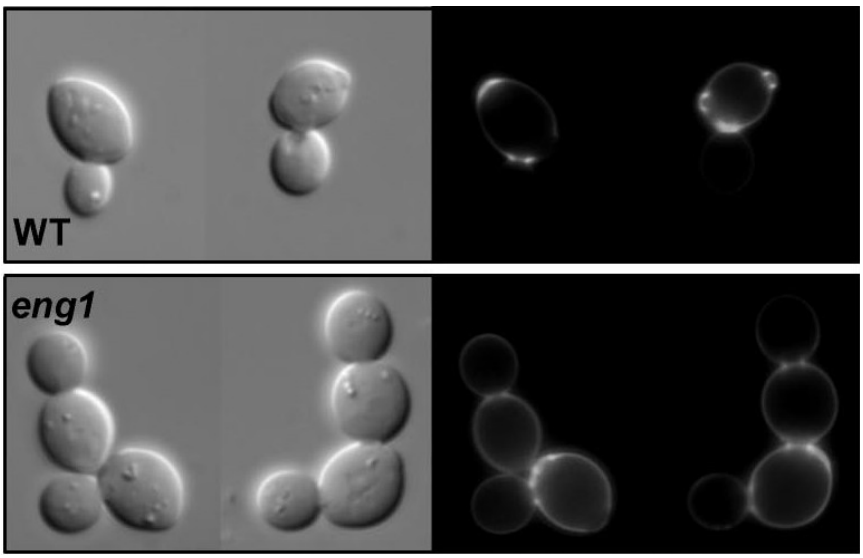


Figure 11. Δ*eng1* cells have a defect in cell separation. Cells were fixed with formaldehyde and stained with Calcofluor White to visualize the chitin.

Dse4p is asymmetrically distributed within the cell wall during cytogenesis with accumulation in the daughter side of the septum. The phenotype with many clusters of connected cells, the predicted endo-1,3-β-glucanase activity and the daughter-specific localization in the bud neck indicates the involvement of Dse4p in the processes of septum degradation and cell separation (Baladrone et al. 2002). Dse4p may contain a GPI anchor that attaches it to the plasma membrane and/or wall matrix (De Groot et al. 2003).

4.2.3 Egt2p

Egt2p is a glycosylphoshatidylinositol (GPI)-anchored cell wall endoglucanase involved in cell separation after cytokinesis. The protein contains a predicted N-terminal signal sequence and a 13 amino acid long C-terminal hydrophobic tail, suggesting that Egt2p may be attached to a membrane. GPI-anchored proteins are divided into two groups of dominant localization: GPI-attached plasma membrane proteins (GPI-PMP) and GPI-dependent cell wall proteins (GPI-CWP). Egt2p was identified as a GPI-CWP protein detached from the plasma membrane to β-glucan of the cell wall (Hamada et al. 1998).

The morphology of *egt2* mutant cells with clusters of more than four cells suggests that EGT2 is involved in cell separation (Figure 12) and is required for degradation of the bud neck between mother and daughter cells.

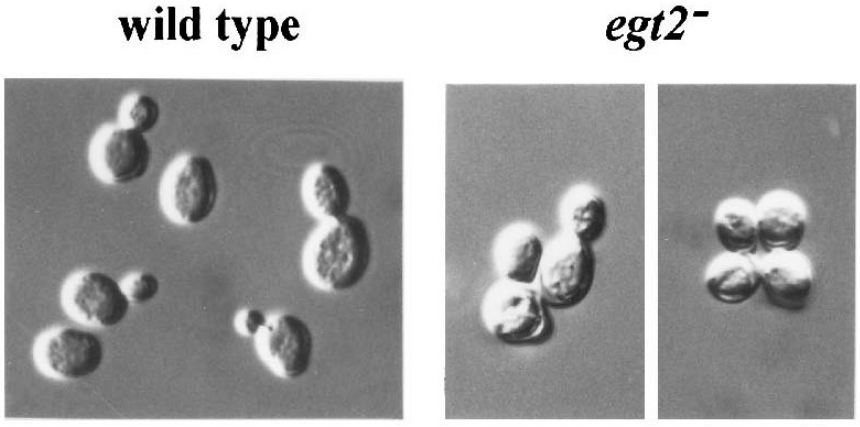


Figure 12. *Δegt2* cells form clusters of up to four members.

Δegt2 cells show a wild type chitin pattern, visualized by Calcoflur White. EGT deficiency does not impact chitin degradation, but it is involved in the degradation of other cell wall components, such as mannoproteins and glucans.

Similar to other proteins involved in cell separation (DSE2, DSE4, CTS1) (Cross and Tinkelenberg 1991; Bulter et al. 2003) EGT2 is an ACE2- and SWI5-dependent gene. Deletion of SWI5 reduces the level of EGT2 expression by more than 50% but in the double mutant *swi5/ace2* EGT2 expression is completely abolished (Figure 13).

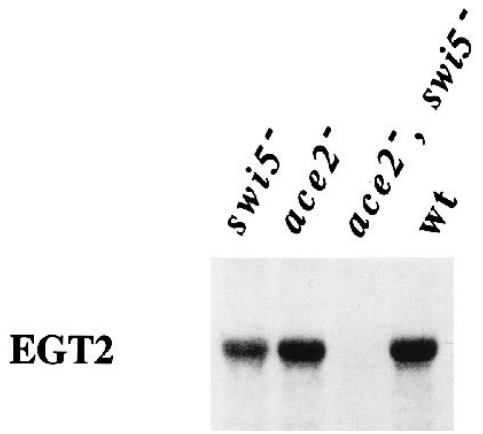


Figure 13. Disruption of Ace2 and Swi5 led to the abolition of EGT2 expression.

Pan and Heitman (2000) described the effect of disruption of the SOK2 transcription factor on EGT2 gene expression. Sok1p is a negative regulator of pseudohyphal differentiation (*Δsok2* mutants are hyperfilamentous) and negatively regulates the expression of the SWI5 transcription factor. Sok2 mutation enhances EGT2, CTS1 and ASH1 gene expression via induction of SWI5 (Pan and Heitman 2000).

The involvement of Egt2p in mother-daughter separation was shown in case of EGT2 expression under the control of a GAL-induced promoter in cells lacking the LRG1 gene (Svarovsky and Palecek 2005). Lrg1p is a bud neck-localized, GTPase-activating protein (GAP) (Lorberg et al. 2001; Roumanie et al. 2001; Watanabe et al. 2001) with a “clustered” phenotype in mutant cells. Plasmid pRS316-GAL1 (Liu et al. 1992), consisting of EGT2 under the control of the GAL promoter, was transformed into *Δlrg1* cells. Expression of EGT2 significantly reduces the number of clustered *Δlrg1* cells, indicating the involvement of endoglucnase Egt2p in septum degradation.

4.3 Fungal cell wall scars.

4.3.1 Bud scars

Collaboration among chitinase and other wall-degrading enzymes within the primary and secondary septum facilitates the release of the daughter cell and the formation of a chitin-rich bud scar on the mother side of the bud neck and a chitin-free birth scar on the daughter side (Shaw et al. 1991) (Figure 14). Bud scars have been described in literature as mother-specific crater-like cell wall rings on the cell surface (Barton 1950; Bacon et al. 1966; Cabib et al. 1997). The number of bud scars always depends on the number of cell divisions (Barton 1950; Egilmez et al. 1990; Sinclair et al. 1998). Bud scars can be visualized using the fluorescent dye Calcofluor White which specifically binds chitin (Pringle 1991; Amberg et al. 2006). Chitin is an important structural component of the fungal cell wall. Fluorescent staining shows that chitin is predominantly located at the site of bud emergence.(Bacon et al. 1966; Cabib and Bowers 1971; Cabib et al. 1974; Cabib et al. 1982; Roberts et al. 1983; Osumi 1998). Chitin is produced in division site prior to and during budding (Cabib et al. 1974; Sloat and Pringle 1978; Cabib et al. 1982; Yamaoka et al. 1989; Cabib et al. 1997).

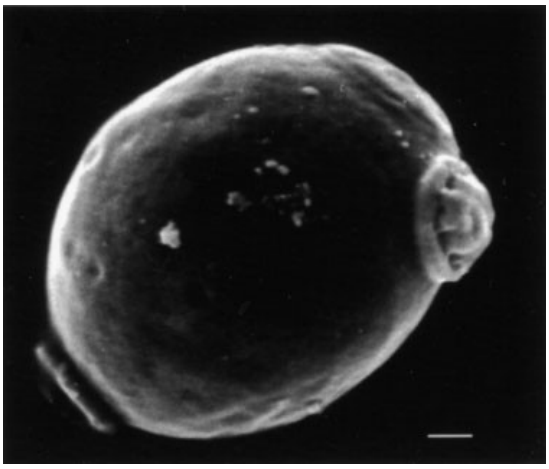


Figure 14. Scanning electron microscopy image demonstrating bud (Laabs et al.) and birth (BiS) scar morphologies. Bar, 1 mm.

Bud scars did not overlap within bipolar or axial budding (Barton 1950; Streiblova 1970) but were located very close to one another. Bud scars expand with age. The diameter of bud scar tissue is approximately 1-2 μm (Barton 1950; Woldringh et al. 1995). The diameter increased from 1,9±1 μm to 2,2±1 μm during next eighth generations. Bud scar microstructure examination showed the presence of breaks within chitin rings (Powell et al. 2003).

4.3.2 Birth scar

The birth scar is a daughter-specific structure exhibited on the cell surface after mother-daughter cell separation (Barton 1950; Bacon et al. 1966; Belin 1972). In contrast to bud scars all fungal cells have only one birth scar. The exact composition of birth scars is unknown. Beran et al. (1969) and Shaw et al. (1991) reported that birth scars consist of little or no chitin and the use of chitin specific Calcofluor staining does not aid visualization of the birth scar (Beran and Rehacek 1969; Shaw et al. 1991). For visualization and examination of both bud and birth scar morphology (Figure 15) FITC-labelled wheat-germ agglutinin (WGA) staining was used (Powell et al. 2003). WGA is highly specific to chitin and reacts weakly with N-acetylneuraminic acid, N-acetylgalactosamine and Man-b-(1R4)-GlcNAcb-(1R4)-GlcNAc-b-N-Asn (Bulawa 1993). Wheat germ agglutinin (WGA) could not be used for specific birth scar visualization because it stains both types of scars. Birth scars have been identified as being single circular rings and in rare cases double rings (approx. 2–3% of the population). The composition of the ring and cell wall inside the ring is little-studied and largely unknown. The number of bud scars on the cell surface is dependent on the number of times a cell divides (Sinclair et al. 1998), while cells always have only one birth scar. Comparing cell populations containing virgin, second, fourth and later generation cells showed that the mean number of bud scars increases with age (Figure 15). The birth scars occupies approximately 5% of the mother cell surface but the proportion of cell wall covered by the birth scar is constant (Powell et al. 2003). These measurements indicates that a birth scar is an integrated component of the cell wall and not a separate structure (Figure 14).

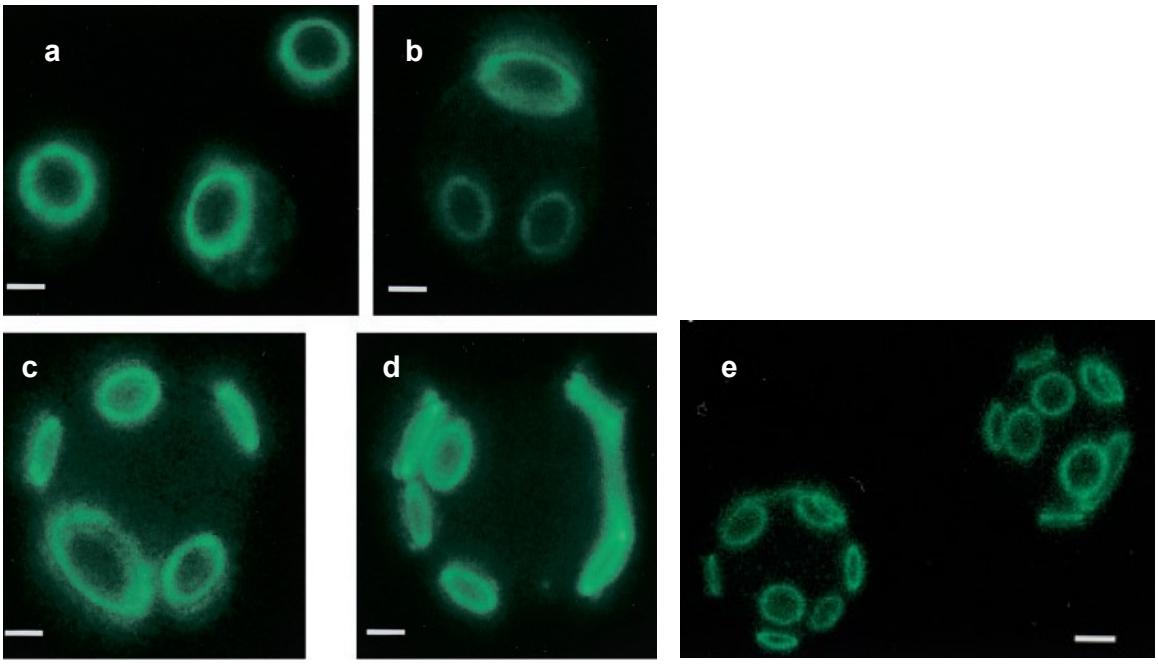


Figure 15. Age-synchronized populations of virgin (a), and second- (b), fourth- (c), sixth- (d) generation cells illustrating birth (a–e) and bud scar (b–e) morphology. Birth scars are distinct due to their large size. Bars, 3 mm (a), 1,5 mm (b, c), 2,3 mm (d)

A new bud never appears within the birth scar area of the wild type (Tong et al. 2007). Aberrant budding in the zone restricted for new bud selection was observed only between strains deleted for certain genes encoding cell wall proteins. The budding-within-the-birth-scar phenotype which is induced by overexpressed daughter-specific Dse1p or by deletion of the ISW2 gene was described (Frydlova et al. 2009). Disruption of the DSE1 gene leads to abnormal budding within the birth scar region. The authors suggested that Dse1p is involved in bud site selection in haploid cells.

4.3.3 Aim44p

Aim44/Gps1p is a bud neck localised protein (Huh et al. 2003) involved in the process of new bud selection during cytokinesis. Expression of the AIM44 gene depends on the Swi5p transcription factor. Swi5p takes part in the regulation of exit from mitosis into the G1 phase of the cell cycle. Doolin et al. (2001) analysed and summarised expression data of cell cycle-regulated genes, previously published by

Cho et al. (1998) and expression data of “SIC1 cluster”. Genes of SIC1 cluster” have a peak of expression in the M/G1 phase border (Cho et al. 1998; Spellman et al. 1998). Between ‘’CIS1 cluster” genes there are potential targets for Ace2p and Swi5p transcription factors. The result of this analysis was a list of genes, including AIM44 (YPL158C), with a high probability of regulation by Ace2p, Swi5p or both. Expression levels of selected genes were measured by northern analysis in *Δace2, Δswi5* mutants and in the double mutant *ace2/swi5*. The level of AIM44 gene expression was significantly reduced in the strains with disrupted SWI5 (Figure16).

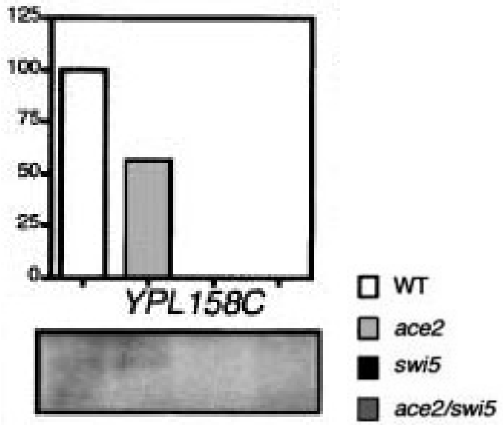


Figure 16. Northern blot analysis of expression of the AIM44 gene in *ace2* and *swi5* mutants

Meitenger et al. (2013) showed that the function of Aim44p/Gps1 (GTPase-mediated polarity switch) is closely connected with the bud-neck-co-localising G-proteins, Rho1 and Cdc42, which have important roles in the maintenance of cell polarity and cell separation (Johnson 1999; Tolliday et al. 2002; Yoshida et al. 2006). Aim44 interacts with Rho1 and Cdc42 and forms a ring-like structure in the septum region. The *aim44* mutant has a phenotype characterised by large amounts of cells which stay connected after mitosis and by budding within old bud site regions (Figure 17).

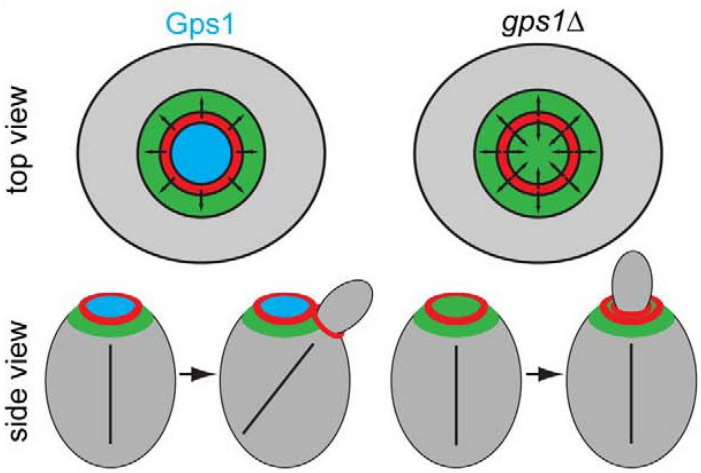


Figure 17. GFP-Cdc42 and Myo1-3Cherry at the bud neck in *Δgps1/aim44* and wild-type cells.

The authors proposed that the *aim44Δ* phenotype resulted from thinning of secondary septal material at the bud/birth scar. The authors further suggested that the impaired secondary septum was explained by decreasing levels of Rho1p at the bud neck which is efficient for septum-synthesizing Fks1p activity (Meitinger et al. 2013). Aim44, independently on GEFs (guanine nucleotide exchange factors) Tus1 and Rom2, regulates localisation of Rho1 to the bud neck. Both GEFs localised in the bud neck and participate in AMR contraction, primary septum formation and control Rho1p localisation (Yoshida et al. 2006; Yoshida et al. 2009).

About 30 % of Aim44-deficient cells have a phenotype involving daughter cells lysing shortly after mitosis, which could be a consequence of mis-regulation of Cdc42. The authors suggest that Aim44 inhibits Cdc42 function and prevents activation of Cdc42 at the bud neck after cytokinesis. This theory corresponds with the ‘‘budding inside the old bud neck’’ phenotype previously described for *rga1* mutants with activated Cdc42 in old cell division sites (Tong et al. 2007)

Aim44 creates a zone restricted for budding around the cell division site via Cdc42p. Aim44 specifically inhibits activation of Cdc42-dependent Cla4 kinase at the old bud neck and prevents budding in the old bud site region.

4.3 Regulation of cell separation

4.3.1 Mitotic exit pathways: APC/C, FEAR, MEN and RAM signaling network

”Mitotic exit” is the part of cell division that begins in anaphase and ends after cell separation. The mitotic exit control system consists of four regulatory pathways (Figure 18.).

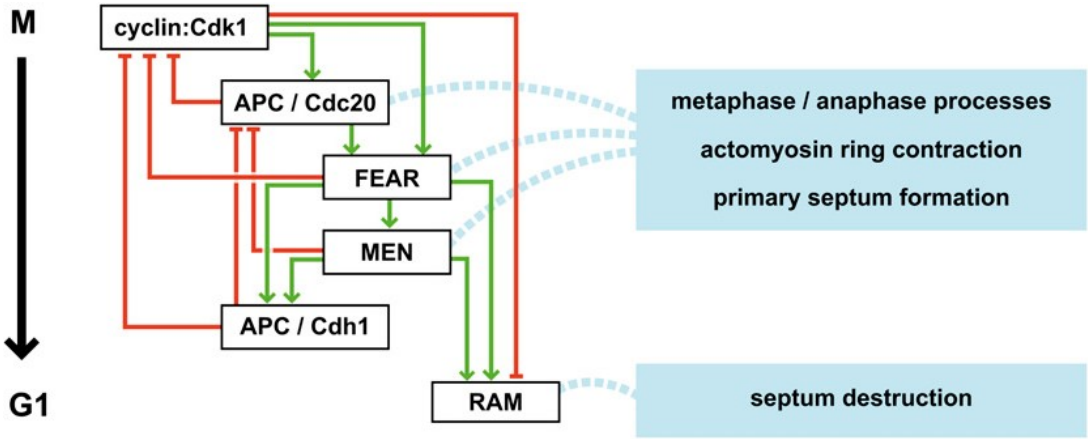


Figure 18. Organization of mitotic exit control. Activation systems (green pointed arrows) and inhibition systems (red block arrows) act during cell-transition from M into G1 phase.

The first is the anaphase promoting complex/cyclosome (APC/C) (Pines 2011; Song and Rape 2011) that in complex with Cdc20 degrades the mitotic cyclins and material that prevent the separation of replicated chromosome (Sethi et al. 1991; Vodermaier et al. 2003; Cooper and Strich 2011). The Cdc fourteen early anaphase release (FEAR) pathway and the mitotic exit network (Mendenhall and Hodge) (Mendenhall and Hodge 1998; Dumitrescu and Saunders 2002; de Bettignies and Johnston 2003; Stegmeier and Amon 2004; Sullivan and Morgan 2007; Queralt and Uhlmann 2008) which control Cdc14. Protein phosphatase Cdc14p is involved in mitotic exit via dephosphorylation of key mitotic targets - mitotic Cyclin-depended kinase substrates. Cdc14 directly regulates processes important for mitotic exit, chromosome segregation, meiotic spindle disassembly, stress response and cytokinesis. The RAM regulation (Regulation of Ace2 and Morphogenesis) (Caro et al. 1998) regulates specific localization and activation of one of the key transcription factors for septum destruction and cytokinesis, Ace2. This transcription regulator

controls expression of genes, including Daughter Specific Expression genes, encoding enzymes which participate in septum destruction. (Nelson et al. 2003; Maerz and Seiler 2010).

4.3.2 RAM network

The RAM network pathway turns on the mechanism of septum degradation via the Ace2p transcription factor that activates transcription of mother/daughter separation genes. The structure of the RAM pathway is shown in Figure 19.

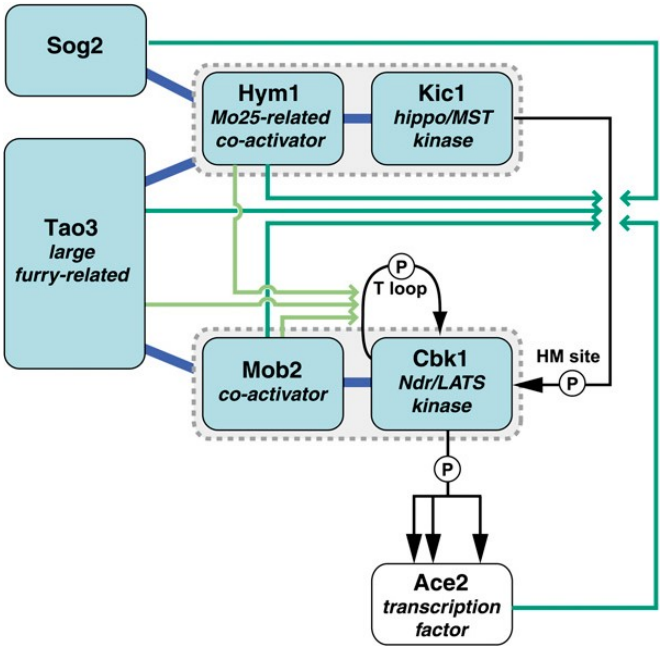


Figure 19. Signalling structure of the RAM network.
Protein–protein interactions (shown as thick blue lines) and the importance of in vivo phosphorylation levels at individual sites (shown as green lines) suggest how modules in the RAM network relate to Cbk1 phosphoregulation.

The Ndr (nuclear dbf2-relate) family protein kinase Cbk1 make a complex with its co-activator Mob2 (Weiss et al. 2002). The Mob2–Cbk1 module functions as a key component in the coordination of Ace2 activity (Weiss et al. 2002; Nelson et al. 2003). Ace2 is a direct target of Cbk1 (Mazanka et al. 2008; Mazanka and Weiss 2010).

The RAM network controls the translation of specific proteins by regulating the mRNA binding protein Ssd1p. Cbk1 protein kinase activates post-transcriptional regulator Ssd1 through phosphorylation. Ssd1p is a relatively poorly studied regulator with unknown machinery of translation control. There is published evidence which shows that Ssd1p associates with mRNAs that encode some of the septum destruction enzymes, such as the chitinase Cts1, the predictet glucanase Dse2 and the SUN-family glucanase Sun4p.(Hogan et al. 2008; Jansen et al. 2009).

4.3.3 Transcription factors Ace2p and Swi5p are required for mother-daughter cell separation.

Ace2p with Swi5p are main components in the regulation of septum destruction and localize to the cytosol and nucleus. Ace2p regulates transcription of genes encoding septum destruction enzymes and participates in the regulation of mother/daughter cell identity. Swi5p is a paralog of Ace2p but functionally distinct, controls the expression of a different set of genes that similar to mitotic exit genes and functions in G1 processes.

Ace2p and Swi5p are zinc finger proteins which have DNA-binding domains and regulate expression of several target genes which are involved not only in cell separation, but also in mating type switching and cell wall function. The ACE2 polypeptide has strong similarity to the SWI5 protein near the carboxyl termini (Butler and Thiele 1991) and within zinc finger domains (Berg 1990). Ace2p has a specific nuclear import (NLS) and a export sequences which binds exportin Crm1/Xpo1 (Jensen et al. 2000; Mazanka et al. 2008). GFP fused Ace2p is visible during early telophase in both mother and daughter nuclei. The amount of Ace2p in mother and daughter nuclei is similar. During later stages of separation Ace2 accumulates exclusively in the daughter cell's nucleus. Ace2 is asymmetrically visible in the daughter nucleus during actomyosin ring contraction and remains in the daughter nucleus until shortly after mother/daughter separation is complete.

Doolin et al. (2001) summarized expression data of genes connected with regulation of the cell cycle (Cho et al. 1998; Spellman et al. 1998) and experimentally supported previously published data using northern blot analyses. Figure 20 shows predicted targets of the Ace2 and Swi5 transcription factors.

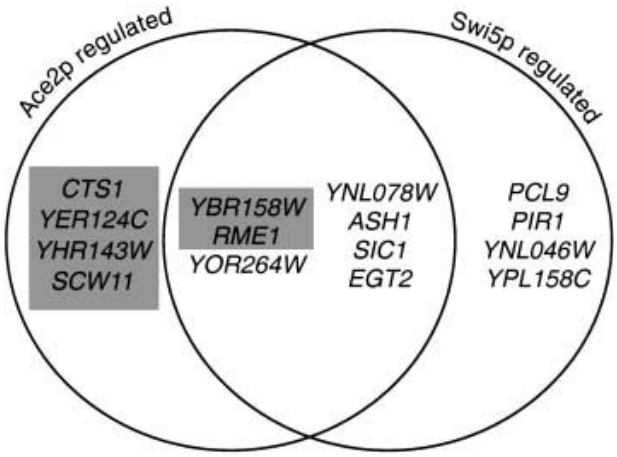


Figure 20. Summary of genes regulated by Ace2p and Swi5p. The shaded genes are expressed 10 min later than others. The central segment consists of genes regulated both by Ace2p and Swi5p.

Deletion of ACE2 and SWI5 genes results in a defect of cell separation with a clumpy phenotype (Figure 21).

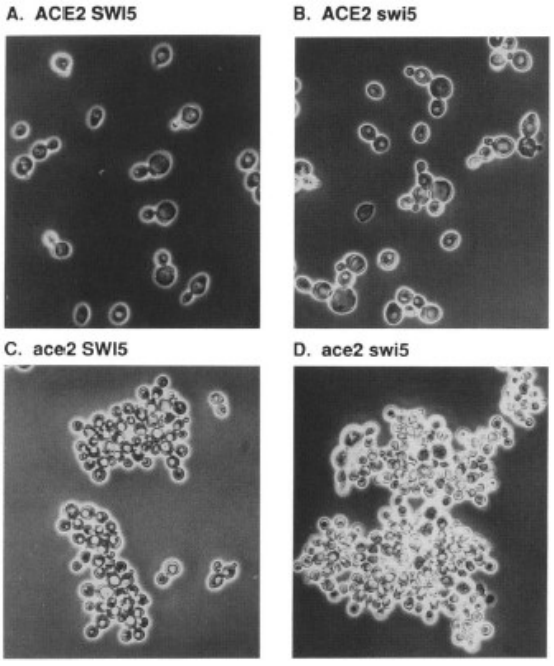


Figure 21. *Δace2* and *Δswi5* mutants have clumpy phenotype.

S. cerevisiae ACE2 and SWI5 have a homologue in Candida albicans – the CaACE2 gene. Deletion of CaACE2 leads to a similar clumped phenotype (Kelly et al. 2004).

Ca*Δace2* cells have a defect in cell separation, pseudohyphal growth and avirulent in a mouse model.

Ace2 also controls expression BUD9 and DSE1, which participate in new bud selection.(Bidlingmaier et al. 2001; Voth et al. 2005; Draper et al. 2009; Frydlova et al. 2009).

5. Materials and methods

5.1 Materials

5.1.1 List of used strains

Strain	Genotype	Source
BY4742 (wt)	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	EUROSCARF
BY-Uth1p-HA	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* UTH1-6HA-kanMX18	this study
BY-Dse2p-HA	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*, DSE2-6HA-natNT2	this study
BY-Sun4p-HA	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SUN4-6HA-kanMX18	this study
BY-Sim1p-HA	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SIM1-6HA-kanMX18	this study
BY-Dse4p-HA	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* DSE4-6HA-natNT2	this study
BY-Sun4p-myc	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SUN4-9myc-natNT2	this study
BY-Sun4p-HA, *ace2Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SUN4-6HA-kanMX18, ace2Δ-natNT2	this study
BY-Sun4p-HA, *swi5Δ*	MATα*, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SUN4-6HA-kanMX18, *swi5Δ*-natNT2	this study
BY-Sun4p-HA, *dse2Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SUN4-6HA- kanMX18, *dse2Δ*-natNT2	this study
BY-Sun4p-HA, *dse4Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SUN4-6HA- kanMX18, *dse4Δ*-kanMX18	this study
BY-Sun4p-HA, *egt2Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SUN4-6HA-kanMX18, *egt2Δ*-natNT2	this study
BY-Dse2p-HA, *sun4Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* DSE2-6HA-natNT2, *sun4Δ*-kanMX18	this study

BY-Dse2p-HA, *dse4Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* DSE2-6HA-natNT2, *dse4Δ*-kanMX18	this study\ EUROSCARF
BY-Dse2p-HA, *egt2Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* DSE2-6HA-natNT2, *egt2Δ*-kanMX18	this study\ EUROSCARF
BY-Sun4p-HA, *hof1Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SUN4-6HA-natNT2, *hof1Δ*-kanMX18	this study\ EUROSCARF
BY-Sun4p-HA, *aim44Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* SUN4-6HA-natNT2, *aim44Δ*-kanMX18	this study\ EUROSCARF
BY-Dse2p-HA, *hof1Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* DSE2-6HA-natNT2, *hof1Δ*-kanMX18	this study\ EUROSCARF
BY-Dse2p-HA, *aim44Δ*	MATα*, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* DSE2-6HA-natNT2, *aim44Δ*-kanMX18	this study\ EUROSCARF
BY-HA-Sun4p	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-SUN4	this study
BY-HA-Sun4p*, dse4Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-SUN4, *dse4Δ*-kanMX18	this study
BY-HA-Sun4p, *egt2Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-SUN4, *egt2Δ*-kanMX18	this study
BY-HA-Dse2p	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-DSE2	this study
BY-HA-Dse2p, *sun4Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-DSE2, *sun4Δ*-kanMX18	this study
BY-HA-Dse2p, *dse4Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*, 6HA-DSE2, *dse4Δ*-kanMX18	this study
BY-HA-Dse2p, *egt2Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-DSE2, *egt2Δ*-kanMX18	this study
BY-HA-Dse4p	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-DSE4	this study
BY-HA-Dse4p, *sun4Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-ΔDSE4, *sun4Δ*-kanMX18	this study
BY-HA-Dse4p, *cdc11Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-ΔDSE4, *cdc11Δ*-kanMX18	this study
BY-HA-Dse4p, *shs1Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-ΔDSE4, *shs1Δ*-kanMX18	this study

BY-HA-Sun4p, Dse2p-myc	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-SUN4, DSE2-9myc-natNT2	this study
BY-HA-Dse2p, Dse2p-myc	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-DSE2, DSE2-9myc-natNT2	this study
BY-HA-Dse4p, Dse2p-myc	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-DSE4, DSE2-9myc-natNT2	this study
BY-HA-Dse4p, Sun4p-myc	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-DSE4, SUN4-9myc-natNT2	this study
BY-HA-Dse4p, Dse4p-myc	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-DSE4, DSE4-9myc-natNT2	this study
BY-HA-Dse4p, myc-Dse2p	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-ΔSE4, 9myc-ΔSE2	this study
BY-HA-Sun4p, Dse4p-myc	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0,* 6HA-SUN4, DSE4-9myc-natNT2	this study
BY-*sun4Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, sun4Δ*-kanMX18	this study
BY-*uth1Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, uth1Δ*-kanMX4	EUROSCARF
BY-*nca3Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, nca3Δ*-kanMX4	EUROSCARF
BY-*sim1Δ*	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, sim1Δ*-kanMX4	EUROSCARF
BY-pTEF-UTH1	MATα, *his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0* pTEF-UTH1-kanMX18	this study

4.1.2 List of used plasmids

Plasmid name	Tag or marker gene	Selectable phenotype	Source
pAG25	PAgTEF1-**natMX**-TAgTEF1	clonNATR	EUROSCARF
pAG34	dr-PAgTEF1-**hphMX**-TAgTEF1-dr	Hygromycin B	EUROSCARF

pYM14	6HA	kanMX4	EUROSCARF
pYM15	6HA	HIS3MX6	EUROSCARF
pYM17	6HA	natNT2	EUROSCARF
pYM-N18	TEF Promoter	kanMX4	EUROSCARF
pYM21	9myc	natNT2	EUROSCARF
pOM12	6HA	KI-URA3	EUROSCARF
pOM22	9myc	KI-URA3	EUROSCARF

4.1.3 List of used primers

SUN4-HA-fw	GTT GTA CCG TTT CCG TTA CTG CTG GCA AAG CTA AGT TTG TTC TAT ACA ACC GTA CGC TGC AGG TCG AC	C-terminal fusion of *SUN4* with HA
SUN4-HA-rev	GAA TGG GGT AAT AAT ACA ATC AAC TTA CTC AAC TGT TGA TGC GCC TAA GTA TCG ATG AAT TCG AGC TCG	C-terminal fusion of *SUN4* with HA
NCA3-HA-fw	ATG GTT GCA CAG TTT CTG TTT TAT CTG GAT CTG CTG AAT TTG TTT TCT ATC GTA CGC TGC AGG TCG AC	C-terminal fusion of *NCA3* with HA
NCA3-HA-rev	ATT GAG GGT ATC AAA ATA CAA GAC ATT CTT TTA CCG AAA AGA AGA ATG ACA TCG ATG AAT TCG AGC TCG	C-terminal fusion of *NCA3* with HA
UTH1-HA-fw	GAC GGT TGT ACT GTT TCA GTT ACT TCT GGT TCT GCT AAC TTT GTC TTC TAC CGT ACG CTG CAG GTC GAC	C-terminal fusion of *UTH1* with HA
UTH1-HA-rev	AAA AAA GTA CTA GCA AAA GCT TAT TTG CAA TAT TCA AGG AAA AAA GGC CTA ATC GAT GAA TTC GAG CTC G	C-terminal fusion of *UTH1* with HA
pTEF-UTH1-fw	CCT TCC TTT AAA CAA AAA TTT ACC CTC CCT TAA TTT TTC AAG AAA TTC CAG TAT GCG TAC GCT GCA GGT CGA C	Insertion of pTEF in front of *UTH1*
pTEF-UTH1-rev	CTG GAG CGG CCA AGA CGG CGG TGG AGG CTG ATA AAG CTA ATA GAG CGG ATA ATT TCA TCG ATG AAT TCT CTG TCG	Insertion of pTEF in front of *UTH1*

SIM1-HA-fw	GTG TAC CGT CTC TGT TAC TTC CGG TAA AGC TCA TTT CGT CTT ATA CAA TCG TAC GCT GCA GGT CGA C	C-terminal fusion of *SIM1* with HA
SIM1-HA-rev	GAT GTG TTC GAA AAA AGA AAA AAA AAA GGA AAA GTA GTA GTC ACG TAG CAT CGA TGA ATT CGA GCT CG	C-terminal fusion of *SIM1* with HA
DSE2-HA-fw	ACC ACT ATA ACT AGT ACA AAC ACA ATT TAT GCT ACG GTT ACC ATT CGT ACG CTG CAG GTC GAC	C-terminal fusion of *DSE2* with HA
DSE2-HA-rev	GAA AAC AAA TAG AAA AGA ATG CAC CAC CAT TAT GCC TAA GCG CTA ATC GAT GAA TTC GAG CTC G	C-terminal fusion of *DSE2* with HA
DSE4-HA-fw	ACA TGG GCA TTA GCA TTT TCA GGG GGA CTG GCC AAC TCA ATT GCT CGT ACG CTG CAG GTC GAC	C-terminal fusion of *DSE4* with HA
DSE4-HA-rev	TAG TGG GAA AGA CTC GAC AGA CCT GCT TGC CAA GTT CT CTT TCT AAT CGA TGA ATT CGA GCT CG	C-terminal fusion of *DSE4* with HA
DSE2-myc-fw	ACC ACT ATA ACT AGT ACA AAC ACA ATT TAT GCT ACG GTT ACC ATT CGT ACG CTG CAG GTC GAC	C-terminal fusion of *DSE2* with myc
DSE2-myc-rev	GAA AAC AAA TAG AAA AGA ATG CAC CAC CAT TAT GCC TAA GCG CTA ATC GAT GAA TTC GAG CTC G	C-terminal fusion of *DSE2* with myc
DSE4-myc-fw	ACA TGG GCA TTA GCA TTT TCA GGG GGA CTG GCC AAC TCA ATT GCT CGT ACG CTG CAG GTC GAC	C-terminal fusion of *DSE4* with myc
DSE4-myc-rev	TAG TGG GAA AGA CTC GAC AGA CCT GCT TGC CAA GTT CT CTT TCT AAT CGA TGA ATT CGA GCT CG	C-terminal fusion of *DSE4* with myc
HA-SUN4-fw	CTT ACC GCG GCT TCA TTG ATC GGT TAT AGC ACA ATC GTT TCC GCT TGC AGG TCG ACA ACC CTT AAT	N-terminal fusion of *SUN4* with HA
HA-SUN4-rev	AGC AGT AGT AGT ACA TCC TGT GTC AAT ATC AGC CGC ATA CGG CAA GCG GCC GCA TAG GCC ACT	N-terminal fusion of *SUN4* with HA
HA-DSE2-fw	ACA ATT TTC AAT ATC TTA TTC TTT TTA TTC ACC TTA ATT GAA GCC TGC AGG TCG ACA ACC CTT AAT	N-terminal fusion of *DSE2* with HA
HA-DSE2-rev	AAT ACC ATC AGA AGT AAT AAG TTT GAC TGT TTC CCC ATT GCT GTT GCG GCC GCA TAG GCC ACT	N-terminal fusion of *DSE2* with HA
HA-DSE4-fw	CAA TTA TAT CTG ACA CTT CTT TTT CTA TTA AGT TTC GTC GAA TGT TGC AGG TCG ACA ACC CTT AAT	N-terminal fusion of *DSE4* with HA
HA-DSE4-rev	TTC CAA TAT TTC GTC TGC ATT ATT CGA TAT GAA ACT TAT ATA TGA GCG GCC GCA TAG GCC ACT	N-terminal fusion of *DSE4* with HA
Δace2-fw	AAG AAA TAA CTA AAG AAA TCT ATA GGA CCA AAA ACG GTG TTA ATA CAA TCC AGC TGA AGC TTC GTA CGC	Deletion of ACE2 gene

Δace2-rev	TAT TGT TAC TAT TAT TTA TTA TGT TAA TAT CAT GCA TAG ATA AAT GTT CGG CAT AGG CCA CTA GTG GAT CTG	Deletion of ACE2 gene
Δswi5-fw	ATT AAA GCG CAA TCA AAT AAA ATA TTA ACG TCA TTG TTT TTT CGA AAA AGC AGC TGA AGC TTC GTA CGC	Deletion of SWI5 gene
Δswi5-rev	TTT TTA TTA TTA AAT ATT AAA AAA AGT GTC CAT AAC ATC AAT GTT TTT TTG CAT AGG CCA CTA GTG GAT CTG	Deletion of SWI5 gene
Δsun4-fw	TTT ATT TAC TAA CCC ATC CCA AAT TTA TTT TAG TAA TTT GAT AAA CAG CTG AAG CTT CGT ACG C	Deletion of SUN4 gene
Δsun4-rev	GGG TAA TAA TAC AAT CAA CTT ACT CAA CTG TTG ATG CGC CTA AGT GCA TAG GCC ACT AGT GGA TCT G	Deletion of SUN4 gene
Δdse2-fw	CTT TCA CAG AGT AGA AAT AAA GCC ACT CCT TTA ACA AAT TAC AAA GAA CAG CTG AAG CTT CGT ACG C	Deletion of DSE2 gene
Δdse2-rev	GCC TGA GAA AAC AAA TAG AAA AGA ATG CAC CAC CAT TAT GCC TAA GCG GCA TAG GCC ACT AGT GGA TCT G	Deletion of DSE2 gene
Δcdc11-fw	TAG AAA GTC AAT CAT CAC AAG GCC TAA AGT TGC TAA CCA CCA GCC CAG CTG AAG CTT CGT ACG Cc	Deletion of CDC11 gene
Δcdc11-rev	TAT ATA TAG AGA AAG AAG AAA TAA GTG AGG AAG CCA AAA GCG GAC GCA TAG GCC ACT AGT GGA TCT G	Deletion of CDC11 gene
Δshs1-fw	CCC CAA AGA TCT GCT TAT AAT TGC TAG AAA AAT ATA TTA TTA ATC CAG CTG AAG CTT CGT ACG C	Deletion of SHS1 gene
Δshs1-rev	TTT ATT TAT TTA TTT GCT CAG CTT TGG ATT TTG TAC AGA TAC AAC GCA TAG GCC ACT AGT GGA TCT G	Deletion of SHS1 gene

4.1.4 Antibiotics

Ampicillin

G418

Hygromycin

Nourseothricin

4.1.5 Internet database and software

Yeast genome database - http://www.yeastgenome.org/

SPELL (Serial Pattern of Expression Levels Locator) - http://spell.yeastgenome.org/

Oligocalculator - http://mbcf.dfci.harvard.edu/docs/oligocalc.html

PubMed database - http://www.ncbi.nlm.nih.gov/pubmed/

O- and N-glycosylation sites - http://www.oppf.ox.ac.uk/opal/

4.1.6 Equipments

Analytic weight Meter AE200

Camera Jenoptic ProgRes CT3

Equipment for SDS PAGE and blotting BIO-RAD

Fluorescent microscope Leica DMR

Fluorescent microscope Zeiss

Incubation shaker N-BIOTEK NB 2015

Incubation shaker for Eppendorf tubes

Microcentrifuge and centrifuge Schoeller UNIVERSAL 320R

PCR thermocycler Minicycler

PCR thermocycler

pH-meter (Mettler Toledo)

Spectrophotometer NANODROP1000 (Thermo Scientific)

Spectrophotometer OPTIZE1412V

UV transilluminator Electronic Dual light

Votex Genie 2 (Scientific Industries)

Thermostat THRMOBOX 168B

4.1.7 Solutions and media

Note: Solutions and media were prepared with ultrapure water which was purified using an Ultrapur water purification system (Watrex).

4.1.7.1 Solid media

YEPDA	1 % yeast extract, 1 % peptone, 2 % agar, 2 % glucose (sterilise separately)

YEPDA + antibiotics	After sterilisation was added: G418 – 400 mg/L Hygromycin B – 400 mg/L Nourseothricin – 200 mg/L
YEPDA + ergosterol.	After sterilisation 20 ml of YEPG agar was supplemented with 1 ml of freshly prepared ergosterol solutionmlml: 2 mg of Ergosterol dissolved in 800µL Ethanol and 200µL Tween 80
GMA	1 % yeast extract, 0,05 % glucose, 150 µM uracil, 30 mM $CaCl_2$, 3 % glycerol, pH 5
GMA + boric acid	Before sterilisation GMA agar was supplemented with boric acid to a final concentration of 4 mg/L
GMA + Congo red	Before sterilisation GMA agar was supplemented with Congo red to a final concentration of 800 mg/L
GMA + Calcofluor white	After sterilisation GMA agar was supplemented with calcofluor white to a final concentration of 1 g/L
GMA + SDS	After sterilisation GMA agar was supplemented with SDS to a final concentration of 1,2 g/L
SDA	(2 % glucose, 100 mM KH_2PO_4, 15mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 0.15 % Wickerham's yeast nitrogen base supplemented with 150 µg/ml of uracil, leucine, histidine and lysine, 2 % agar (all components sterilised separately)
SDA+5-Fluoroorotic acid	Complete SDA supplemented with 0,1 % 5-Fluoroorotic acid

Nutrient agar + ampicillin	(4 % nutrient agar, ampicillin 100 mg/L)

4.1.4.2 Liquid media

SOC	(0,5 % yeast extract, 2 % bactopeptone, 10 mM NaCl, 2,5 mM KCl, 20 mM glucose, separately sterilised: 10 mM $MgCl_2 \cdot 6H_2O$ and 10 mM $MgSO_4 \cdot 7H_2O$)
Nutrient broth	Nutrient broth 2 %
YED	(1 % yeast extract, 2 % glucose),
GM	(1 % yeast extract, 3 % glycerol, pH 5)

4.1.5 Simple solutions

1M LiAc	10,2 g CH_3COOLi in 100 ml H_2O
1000x EtBr	10 mg ethidium bromide in 1 ml H_2O
1 % agarose gel	500 mg boiled in 50 ml 0,5 x TBE buffer
50 % PEG	10 g Polyethyleneglycol in 20 ml H_2O
10 mM MES	192 mg in 100 ml H_2O, (pH 6, HCl)
100 mM PMSF	17,4 mg Phenylmethylsulfonyl fluoride in 1 mlL isopropanol
1 mM AEBSF	24 mg 4-(2- aminoethyl) benzenesulfonyl fluoride in 1 ml deionised H_2O
PIC	1 pill of EDTA-free protease inhibitor mixture in 2 ml H_2O
5 % NaCl	50 g in 1 L H_2O

3 % NaCl	30 g in 1 L H_2O
1 % NaCl	30 g in 1 L H_2O
10 mM Tris-HCl	121 mg Tris in 100 ml H_2O
30 % Acrylamide	30 g Acrylamide in 100 ml H_2O
2 % BSA	66 µl 30 % Bovine serum albumin in 1 ml PEM buffer
3 M CH_3COONa	24,6 g CH_3COONa in 100 ml H_2O

4.1.6 Complex solutions

TBE 0,5x buffer	20 mM Tris, 1 mM EDTA, 45 mM Boric acid
RI	1 % glucose, 25 mM Tris-HCl pH7,5, 10 mM EDTA-NaOH
RII	0,2 M NaOH, 1 % SDS
RIII	29,44 g CH_3COOK, 11,5 ml ice-cold acetic acid, add dH_2O up to a final volume of 100 ml
Wickerham solution	0,2 mg biotin, 200 mg inositol, 20 mg riboflavin, 40 mg thiamine, 40 mg calcium pantothenate, 20 mg aminobenzoic acid, 40 mg nicotinic acid, 0,2 mg folic acid, 100 ml dH_2O
Electrode running buffer	3 g Tris, 14,4 g Glycine, 1g SDS per 1 L dH_2O

Separation buffer	18,15 g Tris, 400 mg SDS in 100 ml dH_2O, (pH 8,8, HCl)
Stacking buffer	6 g Tris, 400 mg SDS in 100 dH_2O, (pH 6,8 HCl)
Laemmli sample buffer	1,25 ml of 2,5 M Tris, pH 6,8, 1 g SDS, 2,5 ml dH_2O 5 ml glycerol 5 mg Bromphenol blue
Staining solution	100 ml Methanol, 25 ml Acetic acid, 125 ml dH_2O, 250 mg Coomassi blue R-250
Bleaching solution	400 ml Methanol, 100 ml Acetic acid, 500 ml dH_2O
TGM buffer	3,025g Tris, 14,42 g glycine, 150 ml methanol, 850 ml dH_2O
PBS buffer	9 g NaCl, 3,58 g $Na_2HPO_4 \cdot 12H_2O$, 50 µL Tween20 in 1 L dH_2O
20 mM potassium phosphate buffer	Mix 40 ml of 100 mM $K_2HPO_4 \cdot 3H_2O$ (2,28 g per 100 ml dH_2O) with 10 ml of KH_2PO_4 (1,36 g per 100 ml dH_2O), pH 7,4
Polyacrylamide gel	Separation gel 3 ml 30 % acrylamide, 2,5 mlL separation buffer, 4,5 ml deionised H_2O, 10 µl TEMED, 20 µl fresh prepared 10 % ammonium persulfate. Stacking gel 650 µl 30 % acrylamide, 1,25 ml stacking solution, 3,05 ml deionised H_2O, 10 µl TEMED, 25 µl freshly prepared 10 % ammonium persulfate.

PEM x4 buffer stock solution	0,4 M PIPES, 20 mM EGTA, 20 mM $MgCl_2$, pH 6,9 (KOH)
TE buffer	1 mM EDTA, 10 mM Tris, pH 8 (HCl)

4.1.7 Antibodies and fluorescent dye

0,2 % Calcofluor (Sigma)	2 µg Calcofluor in 1 ml dH_2O
Wheat Germ Agglutinin, Alexa Fluor 594 Conjugated (Life technology)	Stock solution: 5 mg WGA in 1 ml dH_2O Final volume for use 10 µg in 1 ml of cell suspension
HA-Tag (6E2) Mouse mAb (Alexa Fluor 488 Conjugate) Cell signaling	Dilution 1:50 in PEM 1x buffer
c-MYC (9E10) Mouse mAb SANTA CRUZ	Dilution 1:1000 in PBS buffer (pH7,4 H_3PO_4) + 0,02 % Sodium azide
HA-Tag (6E2) Mouse mAb, Cell Signaling	Dilution 1:1000 in PBS buffer (pH7,4 H_3PO_4) + 0,02 % Sodium azide + 0,05 % Tween20
Goat anti-mouse IgG-HRP, SANTA CRUZ	Dilution 1:5000 in PBS buffer (pH7,4 H_3PO_4) + 0,05 % Tween20

c-MYC (9E10) Mouse mAb (Alexa Fluor 647 Conjugate) SANTA CRUZ	Dilution 1:50 in PEM 1x buffer

4.2 Methods

4.2.1 Strain construction.

For amplification of the cassette, we used primers specific to the appropriate genes and an appropriate plasmid as the template. Strains were prepared using the plasmids shown in Chapter 4.1.2. The cassettes were amplified using specific primers (Chapter 4.1.3) according to (Guldener et al. 1996; Wach 1996; Janke et al. 2004; Gauss et al. 2005) and transformed into BY4742 cells.

PCR program for transformation cassette preparation:

95 ºC – 5 min
95 ºC – 30 sec
55 ºC – 30 sec
72 ºC – 2:30 min
} 30x
72 ºC – 15 min
4 ºC – ∞

Cassete reaction for amplification :

12,5 µl – PCR mix
11 µl – deionised H_2O
0,5 µl – forward primer (100 pmol/ µl)
0,5 µl – reverse primer (100 pmol/ µl)
0,5 µl – template DNA (plasmid)

For yeast transformation the LiAc/SS Carrier DNA/PEG method was used (Gietz and Woods 2002). The transformation mixture (36 µl 1M LiAc, 50 % PEG, 54 µl H2O, 10 µl ssDNA (boiled before adding), 20 µl cassette reaction) was mixed with 100 µl of yeast competent cells (2x109 cells/ml) and incubated at 42 ºC for 40 min.
As an additional step, the transformation mixture was centrifuged and the cell sediment resuspended in 2 ml YPD and incubated for 3-4 hours at 30 °C. Subsequently cells were collected by centrifugation, resuspended in 1 ml deionized water and plated in 330 µl aliquots on selective YEPDA plates supplemented with antibiotics (G418, Hygromycin B – 400 mg/L, Nourseothricin - 200 mg/L,) or on auxotrophic GMA selection plates, lacking specific amino acids. After 3 days growth cells from developing colonies were transferred to fresh selective medium. Positive strains were verified by PCR, sequenced and stored as glycerol stocks at – 75 ºC.
In the case of N-terminal c-myc or HA tagging, deletion of the selection marker was carried out. The transformation procedure used was similar to that shown above. Instead of a PCR reaction mix 2 µl of pSH62 plasmid, carrying the Cre recombinese gene and histidine selection marker was used. Cre-loxP recombination is used to rescue the URA3 selection marker. Verified strains containing the HA or c-myc tag and URA3 selection marker were transformed with plasmid pSH62 and plated on

selection plates without histidine. Cells from positive colonies were taken directly from selective plates and resuspended in liquid selective (-his) medium and incubated overnight. 10 µl of cell suspension was diluted in 1 ml of H2O and plated on GMA plates supplemented with 0,1 % 5-Fluoroorotic acid (5-FOA). Cells which contain a URA3 marker do not grow on the 5-FOA. Positive strains were verified by PCR, sequenced and stored as glycerol stocks – 75 ºC.

4.2.2. Extracellular and cellular protein extraction and separation using SDS–PAGE

4.2.2.1 Extracellular protein extraction from colonies.

The biomass of whole colonies was collected, weighed and washed with an equal volume of 10 mM MES buffer, pH 6 supplemented with protease inhibitors (Complete, EDTA-free protease inhibitor mixture (Roche Applied Science)), 100 mM PMSF (Phenylmethylsulfonyl fluoride, Sigma) in isopropanol and 1 mM AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride, Sigma)), i.e. for example 10 mg of biomass was washed with 10 µl of buffer. After centrifugation (5 min, 4 °C, 1180 g) supernatants and cell sediments were collected separately and stored at -75 °C. Supernatants were loaded into the wells of an SDS-PAGE gel in equal volumes, which were equivalent to the amount of washed cells.

4.2.2.2 Extracellular protein extraction from liquid cultures

Cell lysates and extracellular proteins from liquid cultures were prepared as follows: The same cell biomass was collected by the centrifugation (5 min, 4 °C, 1180 g) of a specific volume of cell culture (calculated according to the OD600 of the culture) grown in liquid GM/YED medium. The collected cells were used for the preparation of cell lysates. Proteins from the medium supernatant were precipitated with 100% Trichloroacetic acid, added in a 1:10 ratio and kept on ice for 2 hours for quantitative precipitation. Precipitated proteins were centrifuged (15 min, 4 °C, 12000 g), washed twice with acetone and resuspended in 200 µl of 10 mM MES buffer, pH6 with protease inhibitors. The amount of extracellular protein loaded into the well of an SDS-PAGE gel was proportional to the original number of washed cells and to the amount of cellular protein loaded.

4.2.2.3 Cellular protein extraction

The total-cell lysates were prepared from cell sediment after extracellular protein extraction. All steps were performed at 4 °C. Cells were washed with 1 ml of distilled water. After centrifugation (5 min, 4 °C, 1180 g) cells were broken with glass beads in 10 mM MES buffer, pH 6 with protease inhibitors in a FastPrep homogenizer (Qbiogene). Cell debris was removed by centrifugation at 1000 g for 3 min and subsequently 2000 g for 5 min. The protein concentration in the supernatant was determined using a protein detection kit (Bio-Rad) and the aliquots were stored at -75 °C.

4.2.3 Cell wall isolation

Cells were disrupted with glass beads in ice-cold 10 mM Tris-HCl, pH 7,4 buffer supplemented with protease inhibitors. Sediments obtained after 1000 g and 2000 g centrifugations were combined and cell walls were washed with ice-cold 10 mM Tris-HCl, pH 7,4, 1 mM PMSF (Phenylmethylsulfonyl fluoride, Sigma) and centrifuged at 1000 g, 4 °C for 10 min. Sediment was washed with 50 ml ice-cold wash solution A (1 mM PMSF), and subsequently with wash solution B (5 % NaCl, 1 mM PMSF), C (2 % NaCl, 1 mM PMSF) then D (1 % NaCl, 1 mM PMSF). Each wash was repeated four times and followed by centrifugation at 1000 g, 4 °C for 10 min. A final wash was carried out with ice-cold solution A. Isolated cell walls were suspended in 100-200 µl of 10 mM MES buffer, pH 6 supplemented with protease inhibitors.

Cell wall proteins were solubilized and denatured using Laemmli sample buffer and subjected to SDS-PAGE. The amounts of cell walls, loaded onto the SDS-PAGE gel, were equivalent to the amount of cellular and extracellular proteins loaded onto the gel.

4.2.4 Determination of amount of particular proteins

The proteins of cell lysates were denatured in Laemmli sample buffer and separated by SDS-PAGE using 9 % gels. 5 – 30 µg of protein was loaded onto the gel. After transfer to a PVDF membrane (Immobilon-P, Millipore), the amount of loaded proteins was verified by Coomassie blue staining of each membrane (loading control). The HA-tagged and c-MYC tagged proteins were detected using mouse anti-HA or anti-MYC antibodies (Cell Signaling Technology) (Santa Cruz

Biotechnology) in combination with goat anti-mouse IgG-HRP as the secondary antibody (Santa Cruz Biotechnology). The peroxidase signal was visualized with Super Signal West Pico (Pierce) on Super RX medical X-ray film. The level of the individual protein was evaluated by UltraQuant 6.0. To minimize the effects of band saturation, less exposed Western blots were used for the quantification. In cases where large differences in signal intensity occurred among quantified samples, the quantification of the more concentrated sample/s could be affected by signal saturation.

4.2.5 Spot assays

To determine the sensitivity of cells to cell wall toxic compounds (Calcofluor white, Congo red), an antifungal drug (Boric acid) or detergent (SDS) particular strains were grown overnight on YEPDA agar at 28 °C and cells resuspended in water at a concentration of 12 mg/ml. 10 µl aliquots were spotted onto GMA supplemented with toxic agents and incubated at 28 °C to produce 6 giant colonies per plate. The whole population of a colony was harvested.

For the spot tests an equal wet cell biomass of each strain was suspended in sterile distilled water and a series of 10-fold dilutions were prepared in water over a range of concentrations of 10^{-1} to 10^{-5} relative to the initial suspension. Aliquots of 5 µl from each dilution series were then spotted on the indicated media and cultivated at either 28 or 37 °C for 4 or 7 days, depending on the particular medium and treatment. All spot assays were repeated at least three times and a representative result is shown.

4.2.6 Cell wall sensitivity to zymolyase.

The cell biomass of whole colonies grown on GMA plates was collected and resuspended in 20 mM potassium phosphate buffer, pH7.4 to a cell concentration with an optical density at 600 nm (OD600) after 100-fold dilution. After 1 min of preheating, Zymolyase 100T (Zymo Research) was added (time zero) to a final concentration of 0.2 U/µl and incubated at 30 °C for 20 min. Cell wall resistance to Zymolyase was determined as the decrease in OD600 of a cell suspension; i.e. at 2 min intervals, 10 µl of treated cell suspension was diluted 100 times into 1 ml of distilled water and the OD600 was determined.

4.2.7 Immunofluorescence microscopy

Cells were grown in liquid GM medium overnight. After 1000 g, 10 min. centrifugation cells were washed with dH2O and resuspended at a concentration of approximately 10^7 cells in 200 ml PEM buffer (4X diluted stock solution 0,4 M PIPES, 20 mM EGTA, 20 mM MgCl2; pH 6,9 (KOH)). After 1000 g, 10 min. centrifugation the sediment was resuspended in 25 ml PEM buffer with 2 % Bovine serum albumin (BSA) and incubated for 30 min at room temperature. Alexa Fluor conjugated mouse IgG1 antibody, raised against the HA-tag (Cell Signaling Technology) or myc-tag (Santa Cruz Biotechnology) was added to a final dilution of 1:50 and incubated at room temperature for 1 hour. Cells were washed twice in dH2O, centrifuged 1000 g, 10 min. and used for microscopy. Fluorescence/DIC microscopy was performed using a Carl Zeiss AxioObserver.Z1 fluorescence microscope equipped with an Axiocam 506 camera and Plan-Apochromat 63x/1.40 Oil DIC M27 objective. Filter sets were used for Alexa Fluor 488 (excitation 450-490 nm; emission 500-550 nm) or for Alexa Fluor 647 (excitation 625-655 nm; emission 665-715 nm). For fluorochroming of the cell wall Calcofluor White (Sigma) was used at a concentration of 2 µg/ml and filter set for DAPI (excitation 335-383 nm; emission 420-470 nm). Alternatively Wheat Germ Agglutinin, Alexa Fluor 594 conjugate (Invitrogen) was used at a concentration of 10 µg/ml, together with a filter set for DsRed (excitation 538-562 nm; emission 570-640 nm).

4.2.8 Plasmid preparation

4.2.8.1 Electroporation

50 µl of competent E.coli cells were mixed with 1-2 µl (approximately 100-150 ng) of plasmid DNA solution and incubated on ice for 1 min. The suspension was transferred to a chilled electroporation cuvette, loaded into the sample chamber of the electroporator and a pulse applied (25 mFD, 200 Ω, and 2.5 kV, time constant = 4.6-4.8 msec). After pulse 1 ml SOS medium was immediately added to the cuvette. The mix was transferred (~1.05 ml total volume) into a 15 ml Falcon tube and incubated for 1 hour at 37 °C, with moderate shaking.

100 µl aliquots of cell culture were spread on selection plates (2 % Nutrient agar supplemented with ampicillin) and incubated overnight at 37 °C. Selected bacterial

colonies were resuspended in 50 ml of 2 % Nutrient broth with ampicillin and incubated overnight at 37 °C.

4.2.6.2 Plasmid isolation

Bacterial suspension was centrifuged (1000 g, 5 min) and washed once with distilled water. The bacterial pellet was resuspended in 2,5 ml of Solution I (50 mM glucose; 25 mM Tris-HCl, pH 8; 10 mM EDTA, pH 8), mixed with 5 ml of freshly prepared Solution II (0,2 N NaOH, 1 % SDS) in centrifuge tube and incubated at room temperature for 5-10 minutes. 3,5 ml of ice-cold Solution III (5 M potassium acetate 60 ml, glacial acetic acid 11,5 ml, H_2O 28,5 ml) was added and the mixture incubated on ice for 20 minutes. After centrifugation of the bacterial lysate (1000 g, 15 min, 4 °C) the supernatant was filtered through four layers of cheesecloth into a 50 ml centrifuge tube, mixed with 0,7 volumes of isopropanol and incubated for 10 min at room temperature. The nucleic acids were recovered by centrifugation at 2000 g for 20 minutes at room temperature. The pellet of nucleic acids and the walls of the tube were rinsed with 70 % ethanol at room temperature, dissolved in 600 µl TE buffer with RNAse (final volume 1:100) and incubated for 30 minutes at room temperature.

4.2.6.3 Phenol-chloroform extraction

The solution of DNA was mixed with an equal volume of phenol, vortexed and centrifuged at 10000 g for 10 minutes. The water phase was transferred to a fresh tube and mixed with 0,5 volumes of phenol and 0,5 volumes of chloroform. After centrifugation (10000 g, 10 min.) the water phase was transferred to a fresh tube and mixed with an equal volume of chloroform, vortexed and centrifuged (10000 g, 10 min.). The water phase was mixed with 2,5 volumes of ice cold ethanol and 1/10 volume of 3M CH_3COONa pH 5,4 and stored at -75 °C for 1 hour. Precipitated DNA was centrifuged at 16000 g for 30 minutes at 4 °C. The pellet was washed with 80 % ethanol and dissolved in deionized H_2O. For transformation cassette preparation the solution of DNA was diluted to a final plasmid concentration of 60 pmol/µl.

5. Results and discussion

5.1 SUN family proteins Sun4p, Uth1p and Sim1p are secreted from *Saccharomyces cerevisiae* and production is dependent on oxygen level

5.1.1 Extracellular localization of SUN family proteins

Aim:

The aim of first part of project was determination of extracellular localization of SUN proteins in extracellular space of the developing colonies and in the liquid incubation medium. Also, monitoring of production and release of SUN proteins from cells, incubated in respiratory and fermentative medium at different time-points.

5.1.1.1 Three of four SUN proteins are secreted from cells grown in liquid cultures

Using the yeast genome database and an application for predicting sites of glycosylation, known data about SUN family genes are summarized in Table 1. All SUN proteins have putative signaling secretion sequences on their N-termini and may be highly O-mannosylated. In addition, Sun4p and Nca3p may be N-glycosylated.

Table 1. Predicted properties of SUN family proteins

Properties of SUN proteins	Uth1p	Sun4p	Sim1p	Nca3p	
Predicted Mw (kDal)	36.955	43.442	48.19	35.412	http://www.yeastgenome.org/
Putative signal peptide for secretion	1-17 AA	1-22 AA	1-19AA	1-18AA	http://www.yeastgenome.org/
N-glycosylation sites	0	1 (395 AA)	0	1 (117 AA)	http://www.oppf.ox.ac.uk/opal/
O-mannosylation sites	28	49	78	9	http://www.oppf.ox.ac.uk/opal/

Kex2 cleavage sites (LysArg)	1 (32-33 AA)	1 (44-45 AA)	1 (34-35 AA)	1 (76-77 AA)	
Percent identity matrix of SUN proteins	**Uth1p**	**Sun4p**	**Sim1p**	**Nca3p**	http://www.ebi.ac.uk/Tools/msa/clustalo/
Uth1p	100	56.42	59.15	66.27	
Sun4p	56.42	100	72.20	54.93	
Sim1p	59.15	72.20	100	58.41	
Nca3p	66.27	54.93	58.41	100	

AA…. amino acid

The presence of signaling secretion sequences and indications of extracellular localization of some SUN proteins in previous publications suggest that all SUN proteins could be routed through the secretary pathway.

As evidence of extracellular localization and to monitor the production of SUN proteins *S. cerevisiae* BY4742 strains were prepared, containing SUN4, UTH1, SIM1 and NCA3 genes fused with a HA tag directly in the genome. Direct HA fusions within the genome guarantee the stability of the constructs and the natural regulation of SUN gene expression by own promoter. Cell and colony morphology analyses suggested that the HA-tag did not change the functionality of tagged proteins, as it did not affect the properties of the strains compared to the wild type.

Production of Sun4p-HA, Uth1p-HA, Sim1p-HA and Nca3p-HA in liquid respiratory GM medium with glycerol as a non-fermentative carbon source was monitored at different time-points. Biomass from medium was harvested at particular time-points and used for lysate preparation and protein analysis on Western blots. In parallel, extracellular proteins were precipitated from aliquots of cell-free cultivation medium. SDS-PAGE was performed on the amount of extracellular proteins precipitated from a culture volume equivalent to the amount of biomass used for the preparation of the lysate (see Material and Methods) (Figure 1 A).

Accumulation of SUN proteins in the constant aliquots of cultivation medium was analyzed at each time-point (Figure 1, B). The amount of the cellular variant of all SUN proteins (Uth1p-HA cell) increased during early growth phases. Uth1p-HA^{cell}

concentration increased and reached maximum level during early logarithmic growth phase (6-10 hrs of cultivation). Uth1p-HA^{cell} concentration started to decrease at the end of the logarithmic growth phase but Uth1p-HA^{cell} was still present in medium even at 72 h. The extracellular Uth1p-HA^{ex} level, i.e. the amount of Uth1p-HA^{ex} produced by an equal biomass volume, approximately matched the cellular level profile. Sun4p-HA^{cell} and Sim1p-HA^{cell} had similarly high levels from early logarithmic growth phase to the end of the exponential growth phase. After 48 hours of cultivation the amount of Sun4p-HA^{cell} decreased to about half that during exponential-phase; Sim1p-HA^{cell} almost disappeared from the cells.

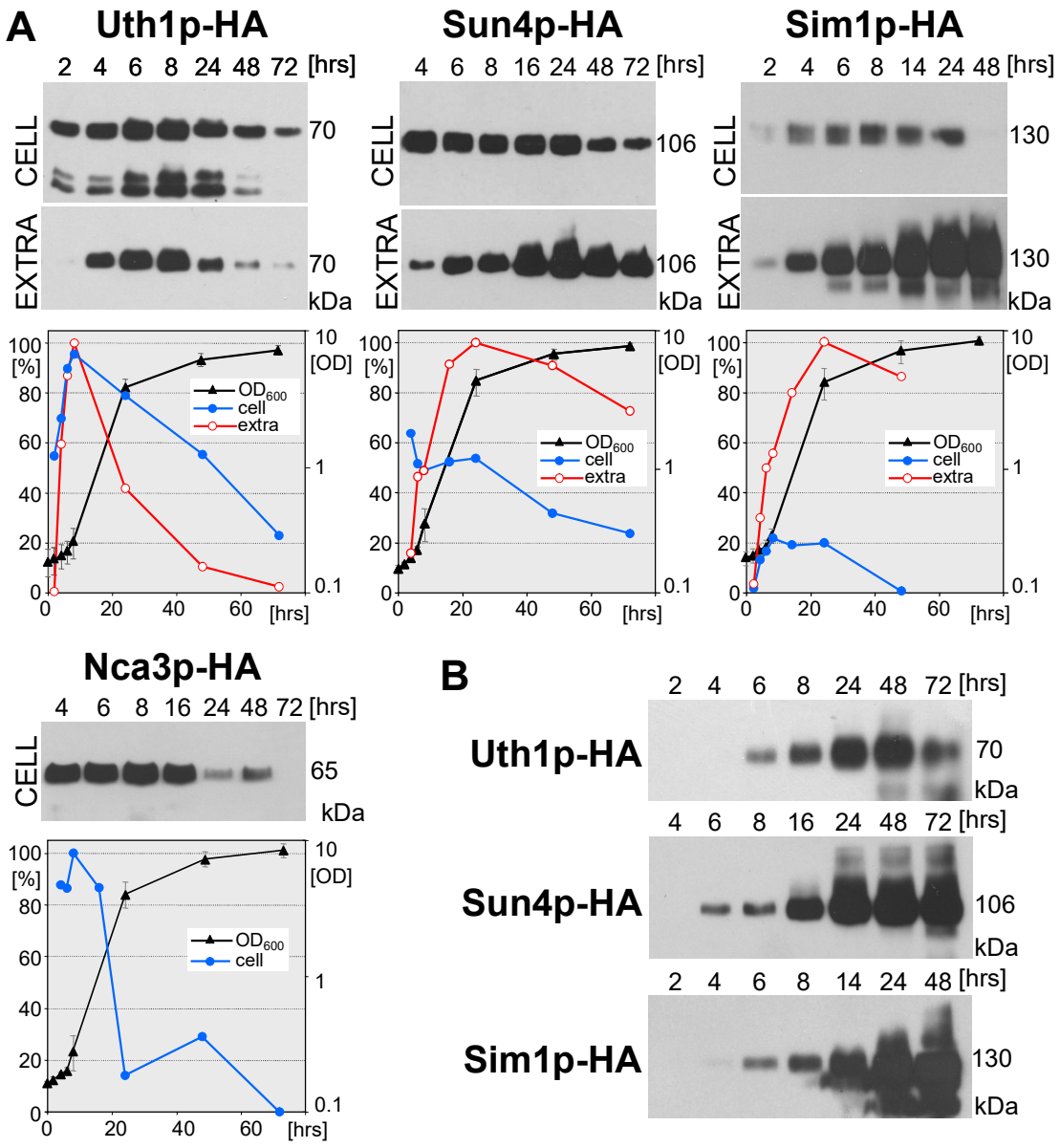


Figure 1. A, Cellular (Purcell et al.) and extracellular (EXTRA) level of SUN proteins in liquid GM medium. Amounts of the lysate-proteins corresponding to amounts of extracellular extracts were loaded onto the gel. The highest amount of the particular protein in each immunoblot quantified by densitometry was set as 100%. Growth curves of cultures of individual strains are shown (right axis). B, extracellular accumulation of SUN proteins. Proteins precipitated from a constant volume of the medium were loaded onto the gel. The value of Mw represents Mw of particular SUN protein linked to the HA tag.

The amount of both Sun4p-HA^{ex} and Sim1p-HA^{ex} begin to increase at the end of exponential growth phase. Nca3p-HA was not detected in medium during the cultivation. The amount of cellular Nca3p-HA^{cell} remained constant.

The absolute level (i.e. amount of protein in a constant volume of the medium) of Sun4p-HA^{ex} and Simp1p-HA^{ex} in the medium was still high even after 3 days of cultivation, while the extracellular accumulation of Uth1p-HA^{ex} in the medium decreased (Figure 1, B).

The level of cellular and extracellular SUN proteins was analyzed in a liquid cell culture grown in fermentative YED medium (Figure 2). The profile of production of Uth1p-HA, Sun4p-HA and Sim1p-HA in YED medium is similar to that observed in GM medium (Figure 1).

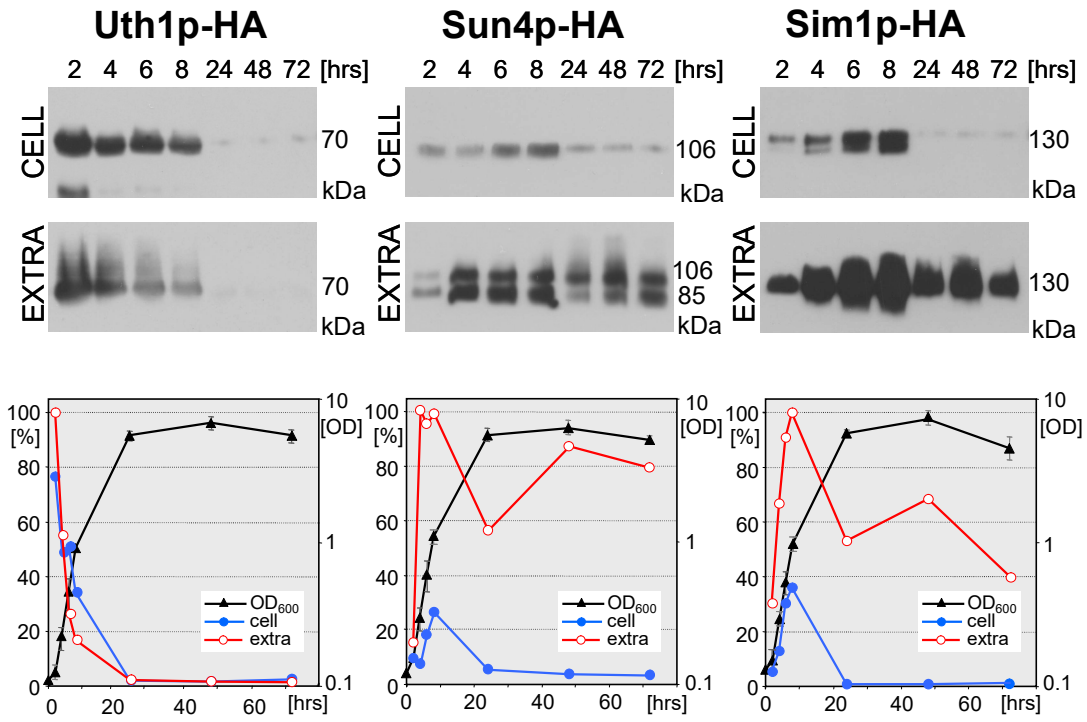


Figure 2. Cellular (Purcell et al.) and extracellular(EXTRA) level of SUN proteins during growth of liquid cell cultures in glucose YED medium. The standardized amounts of lysate-proteins or the corresponding amounts of extracellular extracts were loaded onto the gel. The highest amount of the particular protein in each immunoblot quantified by densitometry was set as 100%. Growth curves of individual strains are shown (right axis). The value of Mw represents Mw of particular SUN protein linked to the HA tag.

There is a difference in grow-rate of yeast in respiratory versus fermentative medium. Despite this, general profiles of production and release of SUN proteins are similar in both media.

5.1.1.2 Uth1p, Sun4p and Sim1p are present in extracellular space of developing colonies

The presence of SUN proteins in yeast shaken liquid cultures suggests that these proteins may be present in the extracellular matrix of multicellular yeast colonies that grow on solid nutrient agar.

Colonies were harvested at various developmental time-points. For Western blot analyses two protein samples were prepared; extracellular proteins washed from the cells and the cell lysate. The colony extracellular protein that was loaded during SDS-PAGE was produced from a similar cell biomass to that used to prepare the cell lysate loaded for detection of cellular SUN proteins. All SUN proteins other than Nca3p-HA have significant levels of both cellular and extracellular variants of proteins (Figure 3). The expression level of NCA3-HA was not sufficient to permit detection of Nca3p-HA by Western blot.

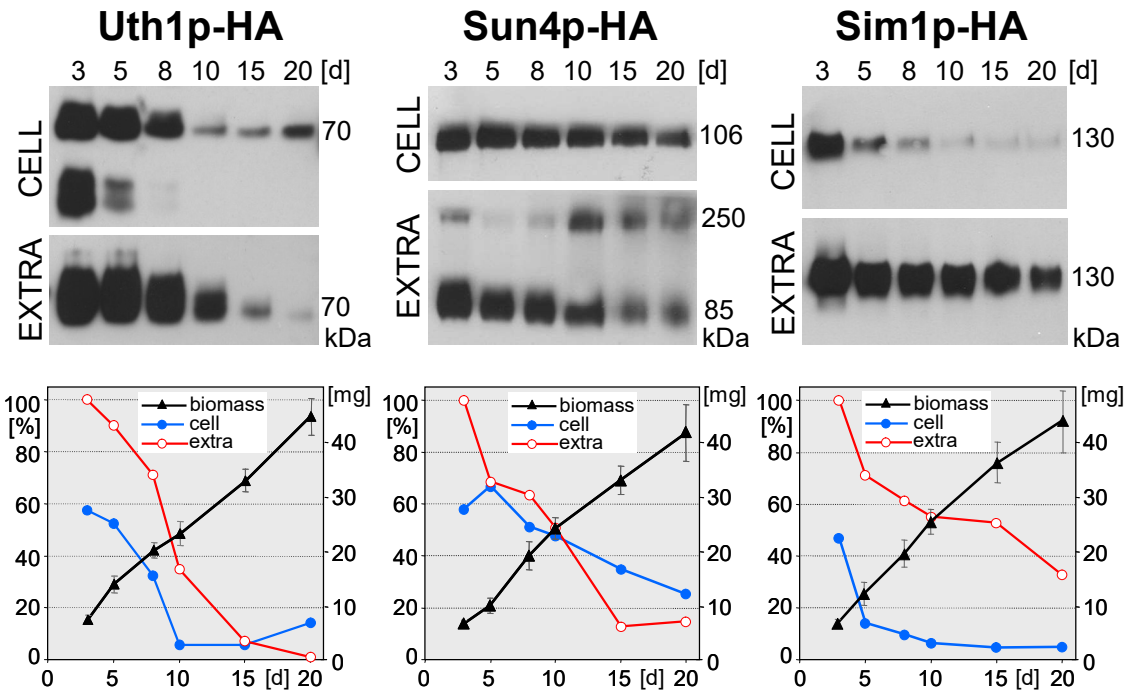


Figure 3. Cellular/extracellular level of individual SUN proteins during development of colonies growing on GMA plates. Standardized amounts of lysate-proteins were loaded onto the gel and corresponding amounts of extracellular extracts. The highest amount of the particular protein in each immunoblot quantified by densitometry was set as 100%. Growth curves of colonies formed by individual strains are shown as wet weight per one colony (right axis). The value of Mw represents Mw of particular SUN protein linked to the HA tag.

Uth1p-HA and Sun4p-HA were present in high levels in cells and the extracellular space of young colonies (3-8-days-old) from the onset of growth. The amount of BY-Uth1p-HA^{cell} decreased after 10 days of cultivation. Uth1p-HA^{ex} was absent from the extracellular space of the colony from about the 15th day. In contrast to both Uth1p-HA and Sim1p-HA, the cellular level of Sun4p-HA was relatively constant until late incubational phases (20-day-old colonies). Sim1p-HA^{cell} was presents in high amounts only in young colonies (3-days-old) and was almost absent from cells after the 5th day. The level of Sim1p-HA^{ex} remained constant at a relatively high concentration until the end of colony development (20-days-old colonies). The absence of any of the three SUN proteins did not affect significantly viability of cells within colonies (Figure 4)

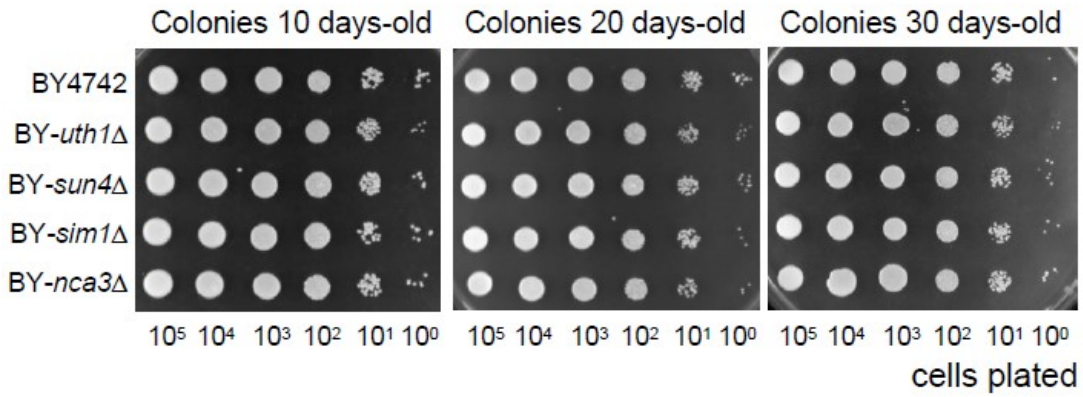


Figure 4. Viability of BY4742, BY-uth1Δ, BY-sun4Δ, BY-sim1Δ and BY-nca3Δ strains within colonies grown on GMA during 30 days of colony development.

Discussion I

It was found that Uth1p, Sun4p and Sim1p are released from cells to the extracellular space of colonies growing on solid media and in liquid cell cultures. Only Nca3p was not detected extracellular fraction and even the cellular level of this protein was very low in colonies.

Monitoring the amount of SUN proteins showed that these proteins are present in high amounts in the cellular fractions during exponential growth phase. Later, when the cultures enter the stationary phases their cellular concentrations decreased. Profiles of individual protein concentrations in cellular and extracellular fractions differed. The amounts of Sun4p and Sim1p gradually increase at the end of exponential growth phase and accumulate in the extracellular space. The amount of Uth1p released by a biomass unit correlates with its intracellular concentration, which indicates that a fraction of Uth1p is always released when this protein is produced. In contrast to Sun4p and Sim1p, extracellular Uth1p is partly degraded and the total concentration in the medium decreases as the cells stop producing it. The extracellular levels of Uth1p and Sun4p in liquid medium do not fully correspond to the cellular concentrations of these proteins, while in colonies extracellular and cellular profiles for these proteins are similar. Cell wall-attached Uth1p-HA and Sun4p-HA were present in much smaller amounts in the purified cell wall fraction of relatively young colonies (3-days old) (and almost no cell wall-attached Sim1p-HA was detected) relative to the amounts in the cellular and extracellular.

The extracellular Sun4p variant is about 20 kDa shorter than the intracellular one. During the following work it was found that the difference in molecular weight between extracellular and intracellular variants of Sun4p was attributable to different methods of sample preparation (See Results and Discussion). For all SUN proteins we observed molecular weight that differs from the molecular weight calculated from gene sequences (Table 1). All four proteins are predicted to contain multiple O-glycosylation sites and, in addition, Sun4p and Nca3p each contain one N-glycosylation site.

5.1.2 SUN genes are regulated differently according to the level of oxygen

Aim:
The aim of this part of work was to investigate whether SUN proteins production was dependent on the level of oxygen in environment.

In a previous publication (Velours et al. 2002, Kissova et al. 2006, Comaugrand et al. 2002, Deffieu et al. 2009, Comogaund et al. 2004, 2003, Kissova et al. 2007). was described connection between the functions of SUN proteins and the mitochondria. The expression of SIM1, a SUN homology gene in *Candida albicans* is upregulated under hypoxic conditions (Sosinska et al. 2008). The link between SUN protein function and mitochondrial function indicated a possible link between the expression of SUN genes and oxygen level. The production of Sun4p, Uth1p and Sim1p was analyzed under various oxygen levels, i.e. normoxic, hypoxic (1% O_2) and anoxic conditions. Colonies of the strains with HA-tagged versions of the SUN proteins were grown for 3 days in parallel under normoxic, hypoxic and anoxic conditions on YEPDA-Erg or on GMA plates. YEPDA-Erg was supplemented with glucose as the fermentative carbon source and ergosterol, a compound essential for yeast growth under anoxic conditions (Ishtar Snoek and Yde Steensma 2007). The total level (including cellular and extracellular protein) of both Uth1p-HA and Sim1p-HA decreased with decreased oxygen concentration. The total amount of Sun4p was relatively stable (Figure 5, A)

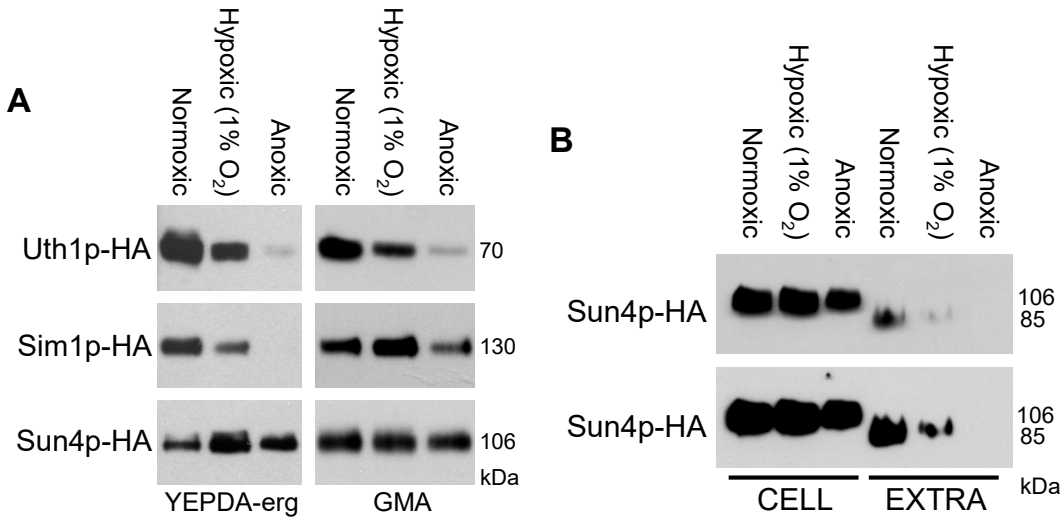


Figure 5. O2 concentration affects SUN protein production. A, Protein amounts in cell lysates of unwashed colonies of BY-Uth1p-HA, BY-Sun4p-HA and BY-Sim1p-HA strains. Strains were grown on either YEPDA-erg or GMA under normoxic, hypoxic or anoxic atmosphere. B, Sun4p-HA amounts in cell lysates (Purcell et al.) and in extracellular extracts (EXTRA) from colonies. BY-Sun4p-HA strain was grown on YEPDA-erg under normoxic, hypoxic or anoxic conditions for 3 days. The value of Mw at the right side of immunoblots represents Mw of particular SUN protein linked to the HA tag.

However, extracellular Sun4p-HA was not detected in anoxic conditions and the level of Sun4p-HAex was significantly decreased in hypoxic conditions (Figure 5B). This finding indicates that Sun4p-HAex undergoes degradation or that extracellular secretion of this protein decreases during anoxia/hypoxia.

A similar experiment was performed with incubation on respiratory GMA plates. Cells cannot divide and grow on medium with glycerol as carbon source under low oxygen conditions. After 3 days of incubation under normoxic condition the colonies were transferred to an anoxomat system and incubated for an additional 2 days under hypoxic conditions. Figure 5A shows that profiles of Uth1p and Sim1p concentration closely resemble those when grown on YEPDA-erg, while the level of Sun4p remained stable.

Discussion II

We suggest that SUN family proteins represent a group of proteins with similarities to a group of cell wall DAN/TIR mannoproteins. Members of this group are regulated by oxygen in various ways (Cohen et al. 2001). SUN proteins may be involved in cell wall remodeling at various oxygen concentrations. This suggestion is supported by the finding of significantly decreased sensitivity of the BY-*uth1*Δ strain to boric acid on YEPDA-erg plates under hypoxic/anoxic conditions (see Discussion IV). This finding is consistent with a decrease in the total amount of Uth1p under anoxic conditions and indicates a possible role for Uth1p in cell wall remodeling.

5.1.3 SUN protein-deficient yeast strains differ in sensitivity to cytostatic compounds.

Aim:
Analyze sensitivity of yeast strains, with specific SUN gene deletions, to different toxic compounds, particularly cell wall disrupting agents.

Previous publications described the cell wall localization of Uth1p and Sun4p and the possible effect on cell wall properties of these proteins (Velours et al. 2002; Ritch et al. 2010). We analyzed the sensitivity of SUN deficient strains to various yeast cytostatic agents, affecting the yeast cell wall or membranes (SDS, Congo red, Calcofluor, Boric acid) (Figure 6). Testing of yeast cell resistance to zymolyase show a significantly increased resistance of SUN-deficient strains to enzymatic treatment (Figure 6D). The resistance was measured using cells from 4-days-old colonies growing on GMA. Analysis of sensitivity to a low concentration of detergent SDS (0.012%) show that only the BY-*uth1*Δ and BY-*sim1*Δ strains were significantly more sensitive. On the other hand, overexpression of UTH1 under the control of the constitutive TEF promoter increased BY-p_{TEF}-UTH1 strain resistance tenfold when compared to the wild-type (Figures 6B).
Similar changes in sensitivity were observed to the cell wall toxic compounds, Congo red or Calcofluor white (CFW) dyes. The BY-*uth1*Δ strain was more sensitive to Congo red and Calcofluor (Figures 6A). This effect was suppressed in the BY-p_{TEF}-UTH1 strain with overexpression of the UTH1 gene.

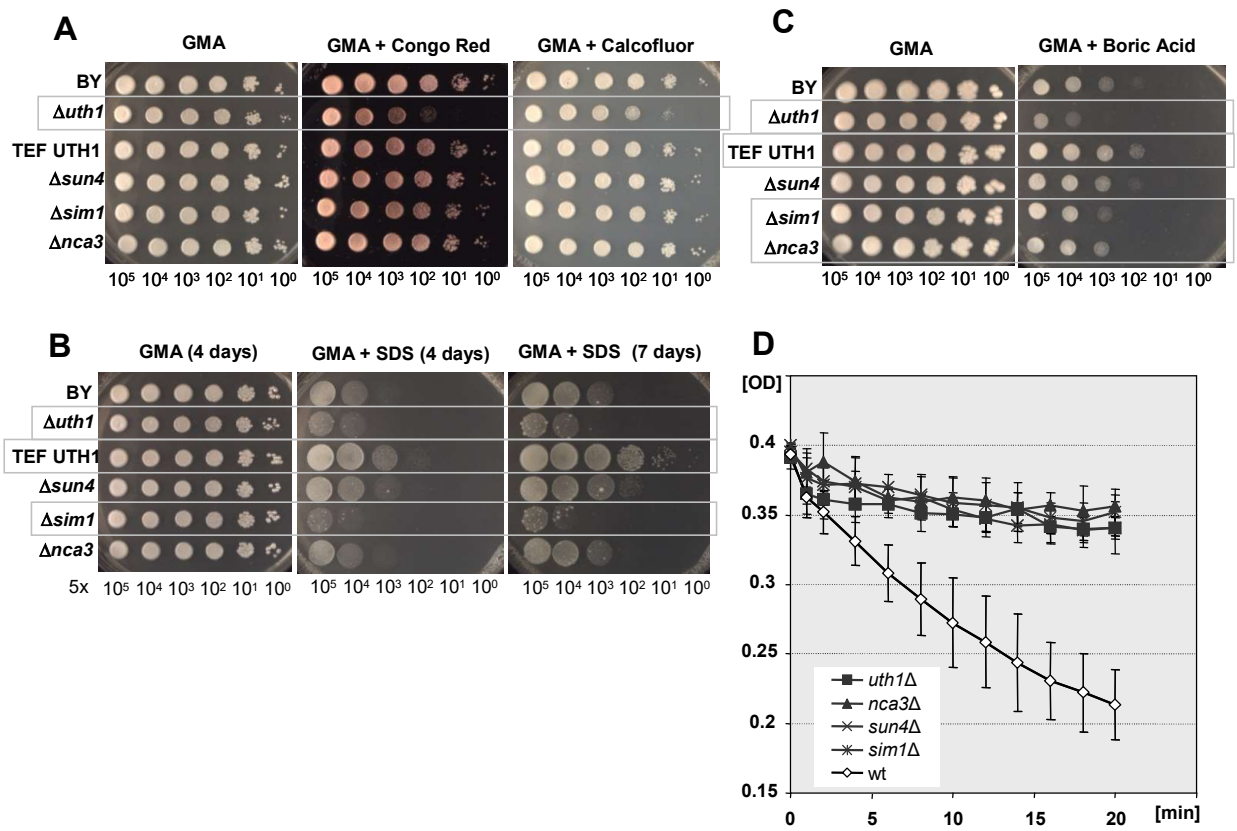


Figure 6. SUN deficient strains have different sensitivity to zymolyase and various drugs when grown on respiratory GMA agar. A, effect of Congo red (800 µg/ml) or Calcofluor white (1 mg/ml); cells were grown at 37º C for 4 days. B, effect of SDS (0.012%), cells were grown at 28º C for 4 or 7 days as indicated. C, effect of boric acid (0.4%), cells were grown at 28º C for 7 days. D, cell resistance to zymolyase (0.2 U/µl) presented as the decrease in density of cell suspensions.

The literature describes a disrupting effect of boric acid on the yeast cell wall (Schmidt et al. 2010). Boric acid is often used as an antifungal compound in the treatment of mycoses. The mechanism of boric acid's action is still unknown. We discovered that the BY-*uth1*Δ strain is approximately 500-fold more sensitive to 0.012% boric acid than the wild-type strain. On the other hand, overexpression of the UTH1 gene leads to a tenfold increase in resistance of the BY-p_{TEF}-UTH1 strain to boric acid treatment (Figures 6C).

Discussion III

An increased resistance to zymolyase treatment was described previously for strain BY-*uth1*Δ (Ritch et al. 2010). Resistance was explained by an increase in glucan level, especially β-1-6 glucan, in the yeast cell wall (Aguilar-Uscanga and Francois 2003). This fact and the finding that SUN deficient strains are more resistant to zymolyase treatment support the suggestion that these proteins participate in cell wall remodeling and that disrupting any of the SUN genes leads to a change in yeast cell wall composition. Analysis of sensitivity of cells from 20-days-old colonies (Figure 7), when SUN proteins are released from the cells, showed that cells still increased resistance to zymolyase treatment.

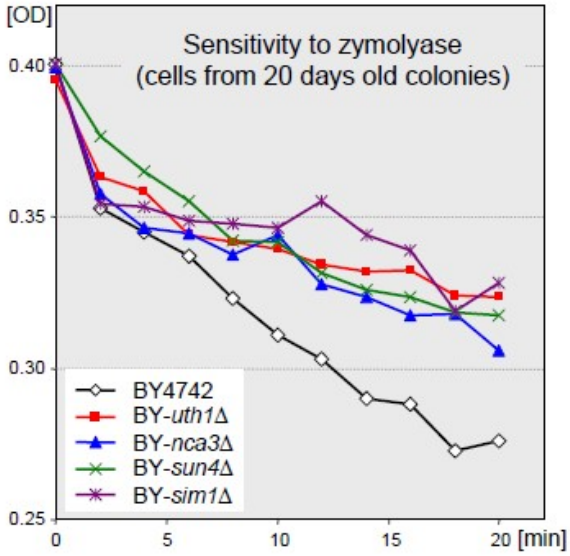


Figure 7. Resistance of cells from 20 days old colonies grown on GMA to zymolyase presented as decrease in density of cell suspension.

Decrease in SUN protein cellular concentration was observed and a decreased expression of UTH1 and SIM1 genes in aging colonies was described previously (Palkova et al. 2002; Vachova et al. 2009). Expression of SUN4, UTH1 and SIM1 genes is decreased during the entry of liquid culture cells to the stationary phase (Gasch et al. 2000). Both facts correspond to an observed decrease in SUN protein cellular concentration. SUN proteins, especially Sim1p, persist much longer outside the cells could play important roles, even in later phases of cell culture development. We analyzed the sensitivity of yeast SUN deletion strains to Calcofluor white, Congo red and boric acid, compounds that have previously been linked to increased cellular

chitin content in treated cells (Roncero and Duran 1985; Vannini et al. 1987; Schmidt et al. 2010). The growth of strain BY-*uth1*Δ on GMA plates was more sensitive to these agents than the parental BY4742 strain. UTH1 gene overexpression completely reversed all of the BY-*uth1*Δ sensitive phenotypes. This finding together with the previously described decrease in chitin content in the BY-*uth1*Δ strain (Ritch et al. 2010) suggests that Uth1p could be somehow involved in the synthesis or assembly or deposition or correct localization of cellular chitin. BY-*uth1*Δ and BY-*sim1*Δ strains were more sensitive to a low concentration of SDS, a detergent that affects cell wall integrity. Cell walls of these strains may be more fragile than cell walls of wild type cells. This suggestion however does not correlate with zymolaze treatment data.

5.1.4 Sensitivities of SUN-protein-deficient strains to toxic compounds differ when cells grow on different carbon sources, irrespective of SUN protein cellular concentration

Aim: Analyze sensitivity of yeast strains, with specific SUN gene deletions, to different toxic compounds in growth conditions with different carbon sources.

Rich et al. described an increase in the resistance of strain BY-*uth1*Δ, derived from the W303 background, to CFW when incubated on glucose plates. We therefore repeated the assays using YEPDA supplemented with CFW or Congo red. Surprisingly we did not observe effects of these cell wall damage agents on BY-*uth1*Δ growth. On the other hand, BY-p_{TEF}-UTH1 was significantly more sensitive to CFW and Congo red than the parental strain when grown on YEPDA (Figure 8).

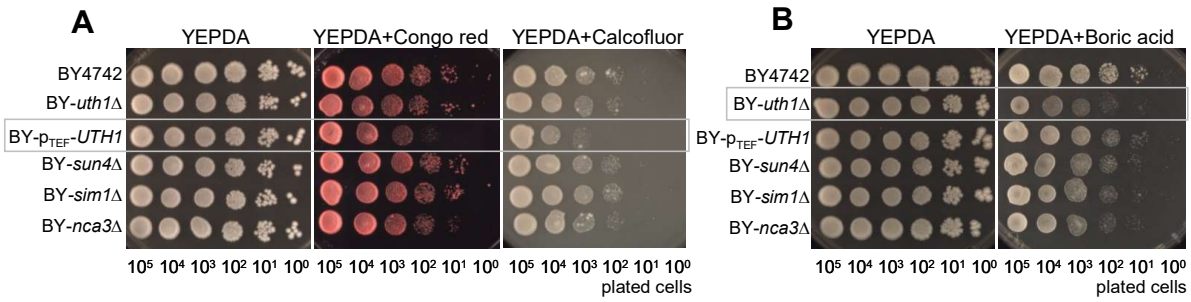


Figure 8. Sensitivity of strains deficient in SUN proteins to toxic compounds on fermentative YEPDA medium. A, B, Drop assays on YEPDA plates supplemented with Calcofluor, Congo red and boric acid.

Effect of boric acid treatment on BY-*uth1*Δ and BY-p_{TEF}-*UTH1* on YEPDA plates was similar, or slightly milder, to that observed on GMA plates. However, sensitivity of strain BY-*uth1*Δ to boric acid on YEPDA-erg plates under hypoxic/anoxic conditions was similar to that of the parental BY4742 strain (Figure 9A).

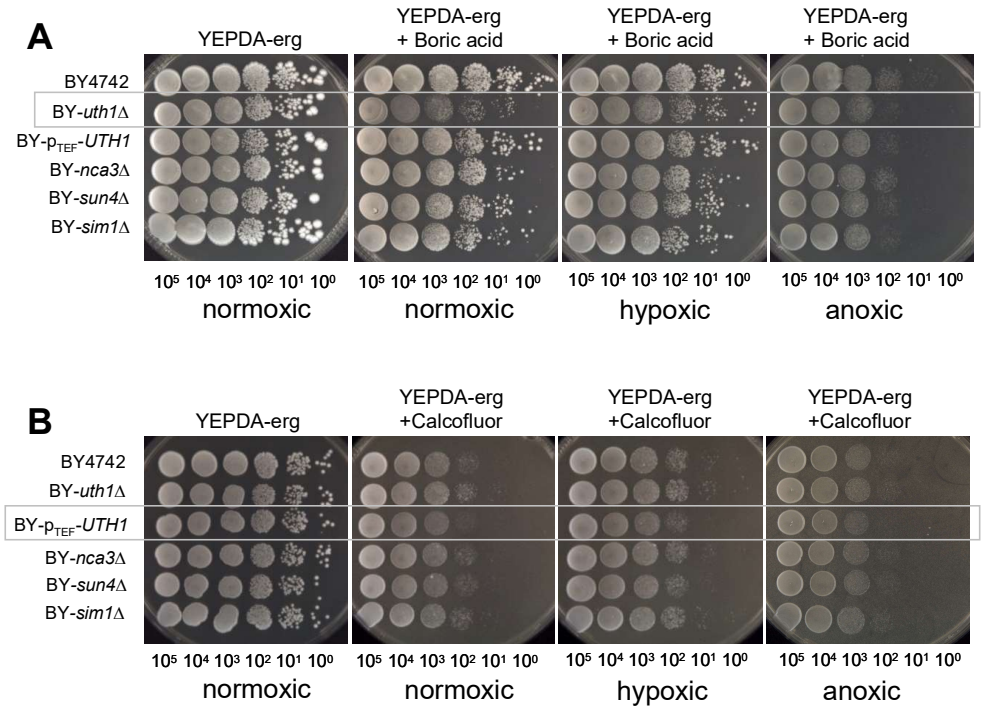


Figure 9. Sensitivity of strains deficient in SUN proteins to toxic compounds on fermentative medium under various levels of oxygen tension. Drop assays on YEPDA-erg plates supplemented with either boric acid or Calcofluor white and grown under normoxic, hypoxic (1% O_2) or anoxic conditions. A, boric acid (0.4 %), cells were grown at 28ºC for 6 days. B, Calcofluor white (2 mg/ml), cells were grown at 28º C for 3 days.

Changes in oxygen tension did not affect sensitivity of BY-p_{TEF}-UTH1 strain, in which the UTH1 gene is overexpressed, to Calcofluor and Congo Red (Figure 9B).

Discussion IV

We showed that the carbon source affects BY-*uth1*Δ strain sensitivity to CFW and Congo red. The BY-*uth1*Δ strain is more sensitive on respiratory GMA plates and of a similar sensitivity on fermentative YEPDA plates, when compared with the sensitivity of the BY4742 parental strain. On the other hand, BY-*uth1*Δ was more sensitive to boric acid than the parental strain on both media. We hypotheses that Uth1p could have a different effect on yeast cell walls under different environmental conditions. Previously it was shown that different carbon sources significantly affect the chitin and β-glucan composition of the cell wall (Aguilar-Uscanga and Francois

2003). Therefore we are led to conclude that Uth1p and (maybe also other SUN proteins) could be involved in such an environmental adaptation.

5.1.5 Summary discussion

What function could SUN proteins have in the extracellular space? Functions of similar cell wall attached proteins, released from cells, are mostly unknown. We suggest that SUN proteins may take part in cell wall remodeling during the exponential-to-diauxic/stationary phase transition and participate in affecting cell wall structures from the “outside”. This suggestion is supported by the finding that BY-Uth1p-HA and BY-Sun4p-HA were detected in much smaller amounts, and BY-Sim1p-HA was almost absent, in the purified cell wall fraction relative to levels in cellular and extracellular fractions.

A second possible reason for the release of SUN proteins from cells is that these proteins are removed from the cell wall after cell separation and that their presence in the extracellular medium is simply a result of this release. We prefer the first hypothesis. In summary, we found that SUN-family proteins are released from cells and regulated differently during particular phases of yeast culture development and under different environmental conditions, apparently being involved in remodeling of the cell wall and changes in its resistance to extracellular compounds. In addition, we found that cell sensitivity to boric acid is dependent on the presence (and level) of Uth1p. Boric acid is a fungistatic compound that is used in the medical treatment of recurrent and resistant yeast vaginitis. Consequently this protein is an interesting target for studies of boric acid's action, which are still unknown.

5.2 Cellular localization of Sun4p and its interaction with proteins or the yeast birth scar

5.2.1 Sun4p localizes to the birth scar of yeast cells and this localization is dependent on the Ace2p transcription factor.

Aim: Development and use of a novel method of visualization of cell wall proteins. Visualization of Sun4p to birth scar. Provide new data about regulation of Sun4p production and release.

In order to obtain more detailed information on Sun4p cell wall localization, we used yeast strain BY-Sun4p-HA with an HA tag on the C-terminus of Sun4p and we analyzed Sun4p-HA localization using a novel approach to the visualization of cell wall localized proteins. We developed a method of direct immunofluorescence in non-fixed and thus non-permeabilised living cells using anti-HA or anti-myc Alexa Fluor conjugated antibodies. To allow for the possibility that C-terminal tagging affects Sun4p localization we also prepared and analyzed a strain with N-terminally tagged Sun4p (BY-HA-Sun4p). Both Sun4p-HA and HA-Sun4p are visible at the same precisely defined position in each cell (Figure 1 A, F). For separate visualization of bud and birth scars we used co-staining with Calcofluor white, a dye that stains yeast bud scars with the exception of the birth scar and Alexa-588 labeled Wheat Germ Agglutinin (WGA-588) which stains all scars including the birth scar. A combination of co-staining and immunofluorescence showed that Sun4p localizes to the birth scar of yeast cells with both C and N termini exposed to the outside (Figure 1 A, F).

A comparison of the expression profile of *SUN4* in various genome wide expression experiments using SPELL (http://spell.yeastgenome.org/) revealed similarities in SUN4 expression with those of a group of other genes which localize to the cell wall (Figure S1). Microarray data from SPELL show that proteins coded by annotated genes may be involved in cytokinetic cell separation and be part of the fungal-type cell wall. Expression of some of these genes (EGT2, DSE1, DSE2, DSE4) is positively regulated by transcription factors Ace2p and Swi5p (Doolin et al. 2001). In addition, gene ontology data from SPELL show that genes from a list at Figure S1 may be involved in similar biological processes (cell division, cytokinesis) or be part of a similar cellular component (fungal-type cell wall, extracellular region).

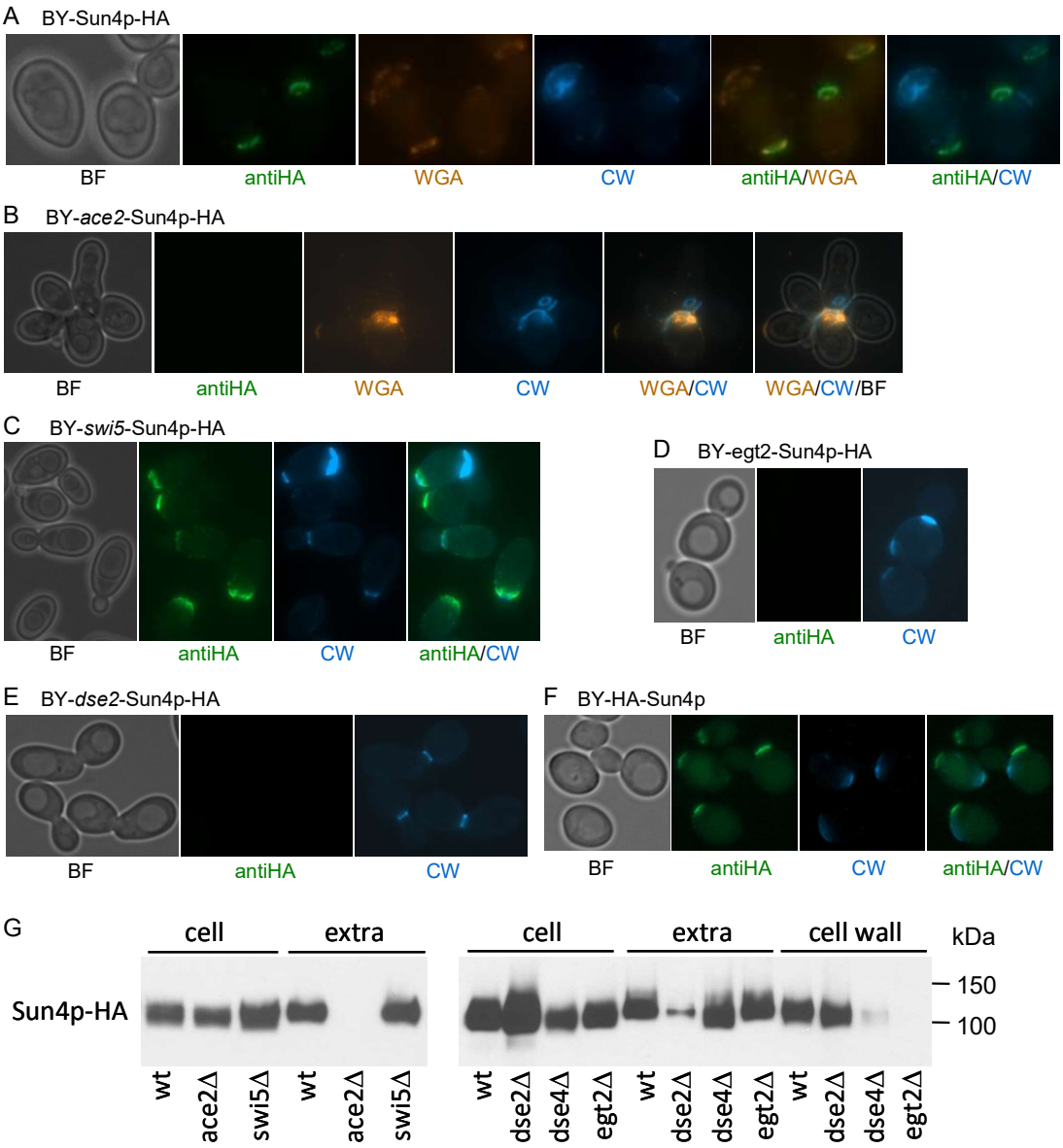
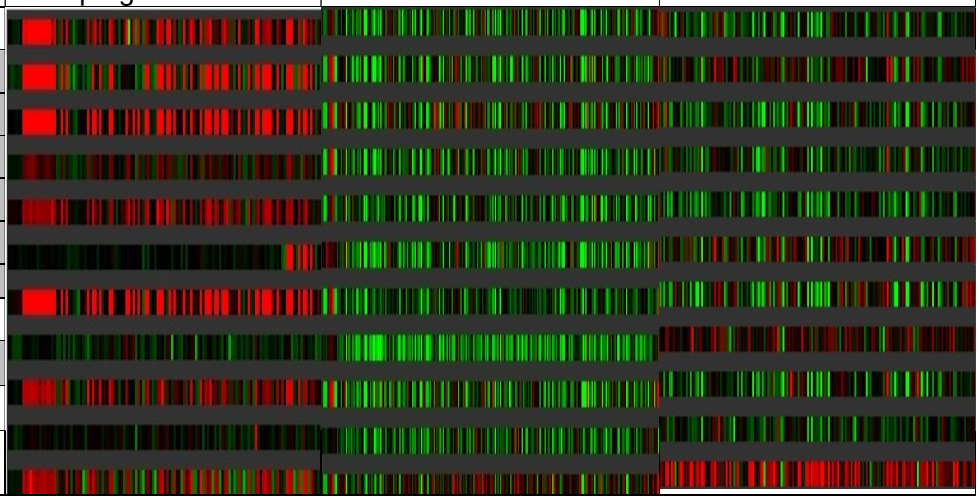


Figure 1. Sun4p localization in cells of wild type and knock-out strains.

Sun4p-HA and HA-Sun4p localization to the birth scar in the wt strain (A, F); absence of Sun4p-HA in the birth scar of *ace2*Δ (B), *egt2Δ* (D) and *dse2Δ* (E) strains; and diffuse Sun4-HA localization and budding-within-the-birth scar phenotype of *swi5Δ* strain (C) similar to phenotype of *aim44Δ* (not shown). Sun4p-HA cellular, extracellular and cell wall localization estimated in wild type and knock-out strains by Western Blot (G). Projections of Z-stacks are used to show Sun4p-HA localization to the birth scar ring, A, B and C. Transverse optical sections of cells are shown in D, E and F. BF, bright field; antiHA, antibody against HA-tag; WGA, WGA-588; CW, calcofluor white.

We therefore prepared strains derived from BY-Sun4p-HA in which SWI5 and *ACE2* had been deleted. Western blot analysis showed that Sun4p-HA is synthesized at comparable levels in wt and knockout strains but that this protein is not released extracellularly in the absence of Ace2p (Figure 1 G).

Immunofluorescence revealed that Sun4p-HA disappears from birth scars in the absence of Ace2p (Figure 1 B). The deletion of the SWI5 gene caused clear "budding-within-the-birth-scar phenotype" described previously for the *isw2*Δ strain (Frydlova et al. 2009) and more diffuse localization of Sun4p-HA in the region of the birth scar, when compared with the wild type (Figure 1 C).

Gene name	ASC[1)]	Genetic variation in gene expression among parents and progenies.[2)]	Genetic reconstruction of a functional transcriptional regulatory network. [3)]	Exploration of essential gene functions via titratable promoter alleles[4)]
SUN4	-			
DSE2	3.7			
SCW11	3.7			
EGT2	3.4			
AMN1	3.2			
CTS1	3.0			
DSE1	2.9			
TOS1	2.9			
DSE4	2.9			
EMP70	2.9			
PRY3	2.8			

1) ACS (Adjusted Correlation Score). This is a measure of weighted correlation for the gene with the query set across all datasets.
2) (Brem and Kruglyak 2005)
3) (Hu et al. 2007)
4) (Mnaimneh et al. 2004)

In grey marked genes used for screening

Figure S1. SPELL correlation of genes with the expression profile similar to that of SUN4. Data from the three published transcriptomics analyses are shown as an example. Full data can be found at http://spell.yeastgenome.org/search/show_results?search_string=YNL066W. The genes marked in grey were selected for screening of Sun4p-HA production and localization

These data revealed that some proteins encoded by genes, the expression of which is positively regulated by Ace2p, are indispensable for birth scar localization of Sun4p as well as for the release of this protein from cells. Thus, Ace2p effect on Sun4p localization is not direct but most probably mediated by some mediator/docking protein the expression of which is controlled by Ace2p. In addition, protein products of some genes regulated by Swi5p participate in proper selection of new budding site or in prevention of budding within the birth scar (Described in chapter 5.2.4).

Discussion:

During this work a novel approach was developed to the visualization of yeast cell wall proteins. This method helped to increase current knowledge of the composition of a relatively little studied yeast cell wall structure – the birth scar. First Sun4p was visualized and shown to be localized to the birth scar and it was determined that Sun4p localization was not directed by Ace2p at a transcriptional level. The amount of cellular Sun4p in the *ace2*Δ strain is similar to that in the wild type, but Sun4 protein disappeared from the birth scar and from the extracellular space in the ACE2-disrupted strain. We suspect that Sun4p forms a complex with other daughter cell-specific proteins that are under the regulation of the Ace2p transcription factor. In addition, visualization of the birth scar generates new data about new bud site selection. Disruption of the SWI5 transcription factor-encoding gene leads to altered Sun4p localization with a “budding-within-birth scar” phenotype where new buds are formed within birth scars, i.e., within the zone that is restricted for budding in the parental strain. This result shows that the Swi5 transcription factor controls the expression of protein/s which participates in new bud selection and/ or prevents budding within the birth scar area.

5.2.2 Dse2p, Dse4p and Egt2p proteins are involved in proper localization of Sun4p-HA to the birth scar and affect amount of Sun4p-HA that is released extracellularly.

Aim: Identifications of cell wall localized, ACE2-dependent proteins involved on Sun4p-HA localization to the birth scar.

First, a set of the strains was prepared, expressing SUN4-HA in EUROSCARF strain backgrounds, deleted for genes with similar expression patterns in SPELL (Figure S1) supplemented with a number of other genes, potentially localized to the birth scar. The Sun4p-HA-knockout strains were then screened for Sun4p-HA production and cellular localization by Western blot and immunofluorescence (data not shown). Four of the 15 analyzed strains (*dse2*Δ, *dse4*Δ, *egt2*Δ and *aim44*Δ) showed significantly changed patterns of Sun4p-HA production and/or localization. New knockout strains were then prepared, derived from isogenic parental BY-Sun4-HA and BY-HA-Sun4p strains. In the absence of Dse2p or Egt2p, Sun4p-HA disappeared completely from the birth scar similarly to the situation in *ace2*Δ (Figure 1 E, D) and its concentration (determined by Western blot) in the extracellular space (*dse2*Δ) or in the cell wall (*egt2*Δ) was significantly reduced (Figure 1G). In strain BY-*dse4*-Sun4p-HA, the amount of Sun4p-HA in the extracellular space and in the cell wall was reduced but the effect of DSE4 deletion was less prominent and Sun4p-HA was present in the birth scar normally as in the wild type (not shown). High Sun4p levels were identified by Western blot in cell wall extracts of strain BY-*dse2*-Sun4p-HA (the protein level being similar to that in the wild type). This, in parallel with the absence of Sun4p-HA in the birth scars (as observed by immunofluorescence), imply that Sun4p is present in the cell wall also with its C-and N-termini facing the interior of the cell, i.e., in the opposite alignment to that observed in the birth scar. Cell wall localization of Sun4p is also supported by the fact, that strain *sun4*Δ has significantly reduced zymolyase sensitivity (Kuznetsov et al. 2013) implicating Sun4p in cell wall remodeling. Dual localization of Sun4p may signify a dual function for Sun4p: one related to birth scar localization during the separation of daughters from mother cells (a role shared with other glucanases probably involved in secondary septum destruction, (Weiss 2012) and another (linked to its cell wall localization) in cell wall organization during bud growth (Mouassite et al. 2000; Weiss 2012)

Discussion:

The western blot and immunofluorescence data indicate that particularly Dse2p and Egt2p proteins are involved in proper localization of Sun4p-HA to the birth scar. Sun4p-HA disappears from the birth scar and extracellular space in the *egt2*Δ and *dse2*Δ strains. With regard to DSE2 destruction, the amount of Sun4p significantly decreased in the extracellular space of the developing colony. We propose that GPI-anchored Egt2p keeps complex Sun4p in the birth scar or helps in exposing part of these proteins to the external space. Both DSE2 and EGT2 genes are regulated by Ace2p, which is in agreement with the observed Sun4p-HA localizations.

5.2.3 Dse2p-HA localizes to the birth scar is dependent on Egt2p and independent of Sun4p

Aim: Determination of localization of Dse2p to the birth scar. Analysis of presence of Dse2p in birth scar and cell wall of strains with deleted for genes (SUN4, EGT2, DSE4) encoding proteins with predicted cell wall/birth scar localization.

Similar to Sun4p we prepared a series of strains with Dse2p labeled at its C terminus (Dse2p-HA) or N terminus (HA-Dse2p) in backgrounds lacking other cell wall/birth scar genes (*SUN4*, *EGT2* or *DSE4*) and we analyzed production and localization of Dse2p-HA and HA-Dse2p proteins. In the wild type strain, as described previously, Sun4p-HA and Dse2p-HA localized to the birth scar and were present in the cell lysate, cell wall and extracellular space of developing colonies. Co-staining with non-specific scar fluorescent dye WGA-588 indicated that similarly to Sun4p-HA, Dse2p-HA localizes to the rim of the birth scar (Figure 2 A). However, N-terminally tagged HA-Dse2p localized predominantly to the cell wall with a punctate pattern and fluorescence in the birth scar position was detectable only in a few cells. In addition, distribution of cell-wall-localized fluorescence was asymmetric in some cells, forming a gradient in the cell wall with higher HA-Dse2p concentration closer to the birth scar (Figure 2 B). The cell wall fluorescence was absent in the cell wall of small buds and present in daughter cells separated from mother cell or in large buds before separation. These data indicate either that N- and C-terminal tagging affect Dse2p localization in different ways, or that theDse2p termini are differently accessible to the antibody in different locations on the cell surface. The latter would suggest that Dse2p could be present in different complexes with other proteins and/or polysaccharides in the cell wall and in the birth scar. To determine which of these two possibilities is correct, we prepared ta strain containing double tagged Dse2p with an HA tag on the N-terminus and a Myc tag on the C-terminus. We than detected both tagged tails using anti HA-Alexa488 antibody in parallel with anti-MYC-Alexa647 antibody.

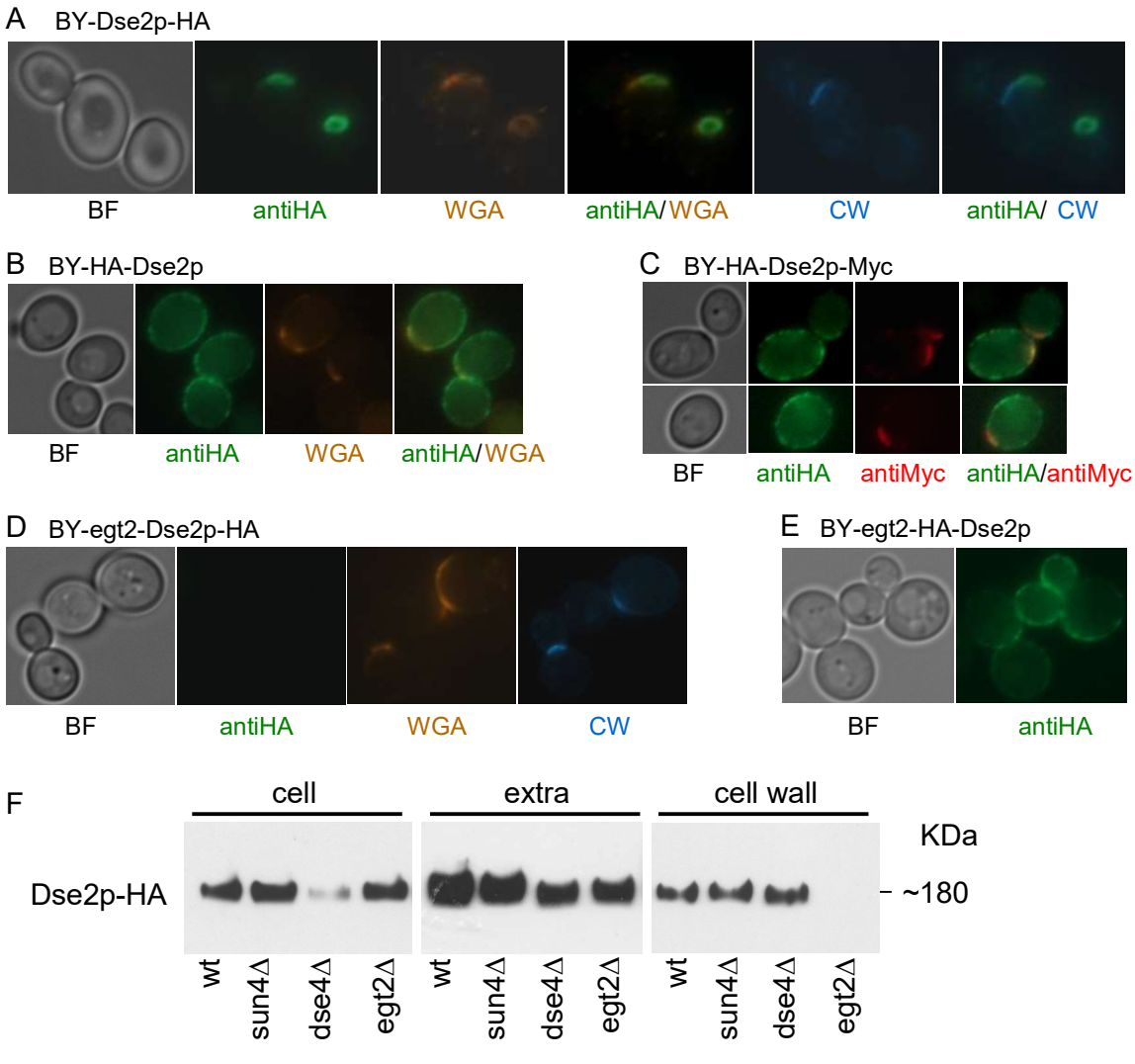


Figure 2. Dse2p localization in cells of wild type and knock-out strains.

Dse2p-HA localization to the birth scar (A) and HA-Dse2p localization to the cell wall (B) of the wt strain; absence of Dse2p-HA in the birth scar (D) and presence of HA-Dse2p in the cell wall (E) of the egt2Δ strain; and presence of HA-Dse2p-Myc both in the birth scar and cell wall (C). Dse2p-HA cellular, extracellular and cell wall localization estimated in wild type and knock-out strains by western blot (F). Projections of Z-stacks are used to show Dse2p-HA localization to the birth scar ring (A). Transverse optical sections of cells are shown in B, C, D and E. BF, bright field; anti-HA, antibody against HA-tag; anti-Myc, antibody against Myc tag; WGA, WGA-588; CW, calcofluor white.

The results (Figure 2 C) showed red fluorescence (C-terminus) exclusively in the birth scar together with green fluorescence (N-terminus) mostly distributed in the cell wall (and rarely in the birth scar) and thus proved that the N and C terminal tails of Dse2p are exposed differently in these two regions of the cell surface. Such dual localization of tagged Dse2p variants disproves the potential attachment of this protein to the cell surface by GPI anchor, which was predicted in a genome-wide approach (De Groot et al. 2003).

We then analyzed localization of Dse2p in different strains with deleted genes coding for proteins predicted to localize to the birth scar region (Figure 2 F). In the absence of Egt2p, Dse2p-HA was not visible in the birth scar (similar to Sun4p-HA in *egt2*Δ). Dse2p-HA levels in the cell wall decreased as shown by western blot but this protein was still present in cells and released to the extracellular space. In BY-*egt2*-HA-Dse2p, however, HA-Dse2p was still detectable by immunofluorescence in the cell wall, as in the wild type. These data indicate that Egt2p is important for proper Dse2p localization to the birth scar but that it is not important for Dse2p targeting to the cell wall. However, in the absence of Egt2p, Dse2p (similarly to Sun4p) disappeared from cell wall extracts as determined by western blot. This observation indicates that Egt2p plays a role also in the cell wall, possibly in stabilizing Dse2p and Sun4p attachment to the cell wall. In the absence of Egt2p, Dse2p and Sun4p are more easily released and thus can disappear from the cell wall during its purification. Unfortunately, we did not succeed tin analyzing the localization of Egt2p, because neither GFP nor HA tagging allowed us to visualize this protein in the cells (not shown). In the absence of Dse4p, Dse2p-HA localized to the birth scar and HA-Dse2p to the cell wall similarly to the situation in the wild type strain (not shown). Also in the absence of Sun4p both Dse2p-HA and HA-Dse2p localization was not affected (not shown), which indicates that Sun4p is not required for correct Dse2p localization, while Dse2p is indispensable for proper localization of Sun4p to the birth scar. Egt2p is crucial for birth scar localization of both Sun4p-HA and Dse2p-HA.

Discussion:

We showed that Dse2p-HA, similarly to Sun4p-HA, localized to the birth scar. In additiona, we used N-terminal tagging to demonstrate that HA-Dse2p was mostly distributed in the cell wall. This distribution is asymmetric and closer to the birth scar region. These results indicate that the N and C terminal tails of Dse2p are exposed differently in the yeast cell wall. Localization of Dse2p is dependent on GPI-

anchored Egt2p which maintains the interaction between the complex and Sun4p in the birth scar. Evidence of the involvement of Egt2p in complex organization includes the disappearance of Dse2p from the birth scar and cell wall in the *egt2*Δ strain. In contrast to Sun4p which depends on the presence of Dse2p, localization of Dse2p to the birth scar is independent of the presence of Sun4p.

5.2.4 Dse2p, Dse4p localizes to the birth scar and the cell wall.

Aim: Determination of localization of Dse4p to the birth scar and cell wall. Visualization of Dse4p, Dse2p and Sun4p co-localized within birth scar.

As with Sun4p and Dse2p to analyze the localization of Dse4p, we prepared strains with N- and C-terminally tagged Dse4p and analyzed Dse4p localization by direct immunofluorescence. In the wild type, Dse4p-HA was visible in the birth scars of some cells, although the fluorescence was very weak and often visible only in a small part of the birth scar (Figure 3H). The stronger fluorescence of HA-Dse4p present as a punctate pattern was detectable in the cell wall of the BY-HA-Dse4p strain. In contrast to the asymmetric cell wall localization of HA-Dse2p close to the birth scar, HA-Dse4p was almost evenly distributed in the cell wall (Figure 3G). These data showed that Dse2p and Dse4p exhibit similar dual localization in the birth scar and the cell wall with the same protein orientation.

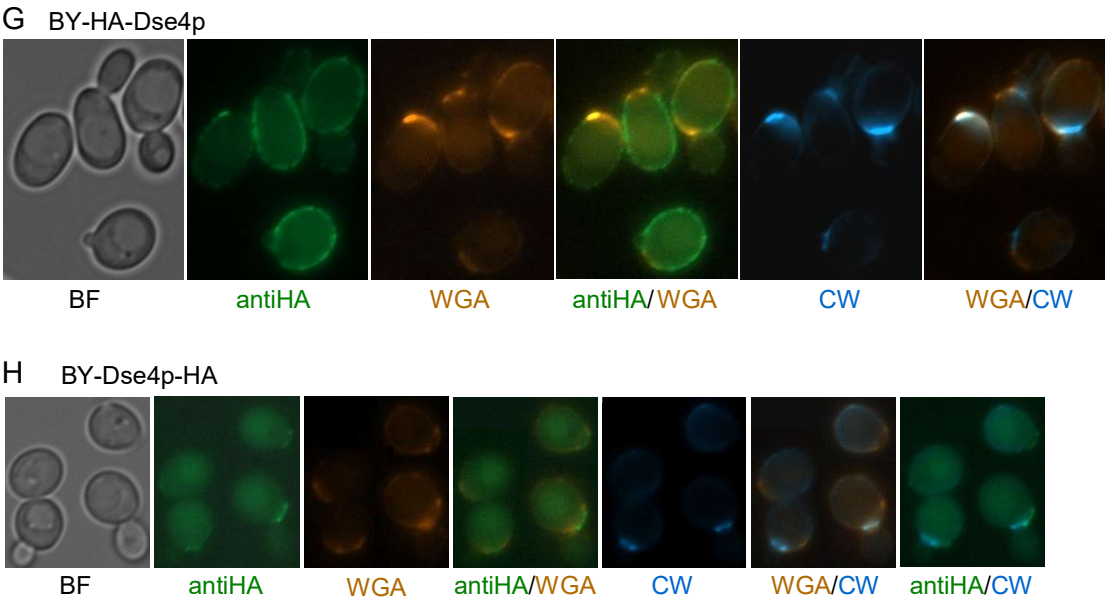
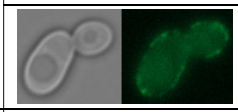
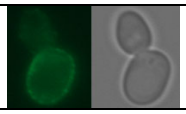
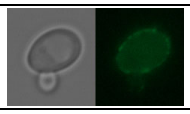
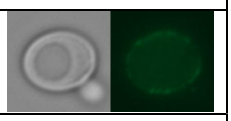


Figure 3. Dse4p localization in cells of wild type and knocked-out strains. Dse4p-HA localization to the birth scar (H) and HA-Dse2p localization to the cell wall (G) of the wild type strain. Transverse optical sections of cells are shown in G and H. BF, bright field; anti-HA, antibody against HA-tag; WGA, WGA-588; CW, calcofluor white.

In the absence of Sun4p, HA-Dse4p localized to the cell wall as in wild type. Unfortunately, construction of strains with HA-Dse4p derived from *sun4*Δ, *dse2*Δ and *egt2*Δ knock out strains failed, which may indicate that tagged Dse4p protein is not fully functional and its combination with a deletion of DSE2 or EGT2 is lethal to cells. The identification of Dse4p localization to the daughter site of the septum in fixed yeast cells and to the whole daughter cell after partial cell wall digestion (Baladron et al. 2002) supports our finding of dual Dse4p localization. However, in our experiment performed with living non-fixed cells, Dse4p was detected in the cell walls of both the daughter and mother cells. Interestingly, small buds have no signal or a lower fluorescent signal, using HA-Dse4p, in comparison with the mother's cell wall. We checked for the possible participation of septins Cdc11p and Shs1p, required components of the septin ring, in HA-Dse4 localization. We prepared statistical analyses of fluorescence level in the buds of strains BY-HA-Dse4, BY-cdc11-HA-Dse4 and BY-shs1-HA-Dse4 (Table S1).

Table S1

Strain	Numerous of analyzed cells	Middle bud		Small bud	
		Similar signal in mother cell and bud	Stronger signal in mother cell	Weak signal	Without signal
					
BY-HA-Dse4p	364	141	120	57	46
BY-*cdc1*-HA-Dse4p	291	80	123	61	27
BY-*shs1*-HA-Dse4p	266	104	88	49	25

Analysis did not reveal significant differences between deletion strains and the wild type (Figure 4). Most buds have weak fluorescent signal. Only 10% of all buds have no fluorescence.

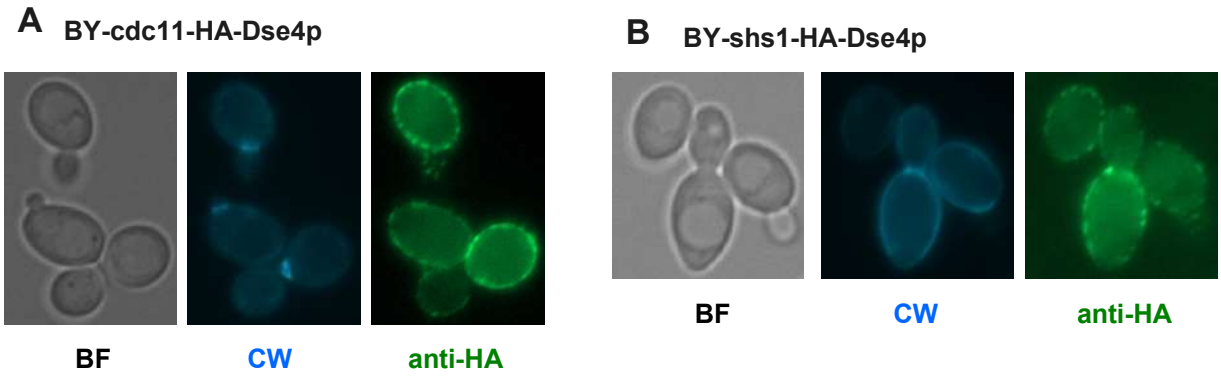


Figure 4. Dse4p localization in cells knocked-out strains.
Dse4p-HA localization to the cell wall in strain BY-cdc11-HA-Dse4p (A) and in strain BY-shs1-HA-Dse4p (B)

Discussion:

Dse4 is other novel identified protein in the birth scar. Similar to Dse2p, Dse4p showed dual localization on the yeast cell surface, being present within the birth scar and in the rest of the cell wall. This protein exposes different termini of their structure (Figure 6), indicating either different orientations of Dse4p or the presence of additional factors (proteins and/or carbohydrates) that cover specific domains of Sun4p and/or Dse2p

5.2.5 Sun4p co-localizes with Dse2p but not with Dse4p to the birth scar

Aim: Primary aim of this part of project was to show co-localization of Sun4p, Dse2p and Dse4p, newly determined and visualized in different parts of yeast cell surface.

The above data show a relationship among Sun4p, Dse2p and Dse4p localization. To examine the mutual localization of these proteins in more detail, we prepared strains with different combinations of tagged versions of these proteins. Strain BY-HA-Sun4p-Dse2p-Myc showed a clear co-localization of Dse2p and Sun4p to the birth scar (Figure 5A), indicating that these two proteins form a complex. As the birth scar localization of both proteins is dependent on the presence of functional Egt2p (Figures 1 and 2), and in addition, Dse2p is essential for Sun4p localization, we assume that Egt2p helps retain the Sun4p-Dse2p complex at the birth scar through interaction with Dse2p.

Analysis of potential co-localization of Sun4p with Dse4p to the birth scar using the BY-HA-Sun4p-Dse4p-Myc strain did not provide clear results (Figure 5B). In this strain, HA-Sun4p exhibited standard birth scar localization. Weak Dse4p-Myc fluorescence predominantly localized also to the birth scar but to other positions compared to HA-Sun4p. The analyses were complicated by rather weak Dse4p-Myc fluorescence but the results indicated that Dse4p does not form a complex with Sun4p (and thus probably not with Dse2p) in the birth scar. This conclusion is in agreement with the observation that the deletion of the DSE4 gene does not affect birth scar localization of Sun4p and Dse2p. In the BY-HA-Dse4p-Dse2p-Myc and BY-HA-Dse4p-Sun4p-Myc strains (Figures 6A and 5C), HA-Dse4p present in the cell wall did not co-localize with any of the other proteins present in the birth scar. As expected, complementary fluorescence of N-terminal-tagged Dse4p in the cell wall and of both Sun4p and C-terminal-tagged Dse2p in the birth scars was clearly visible. The question remained whether Dse4p co-localizes with Dse2p in the cell wall. Analysis of the BY-HA-Dse4p-Myc-Dse2p strain, however, showed mostly complementary fluorescence of HA-Dse4p and Myc-Dse2p in the cell wall (Figure 6B). Both proteins exhibited a typical punctuate pattern, which was generally more polarized for HA-Dse2p and more evenly distributed for HA-Dse4p. These data indicate that these proteins localize to different positions in the cell wall and therefore most likely do not interact.

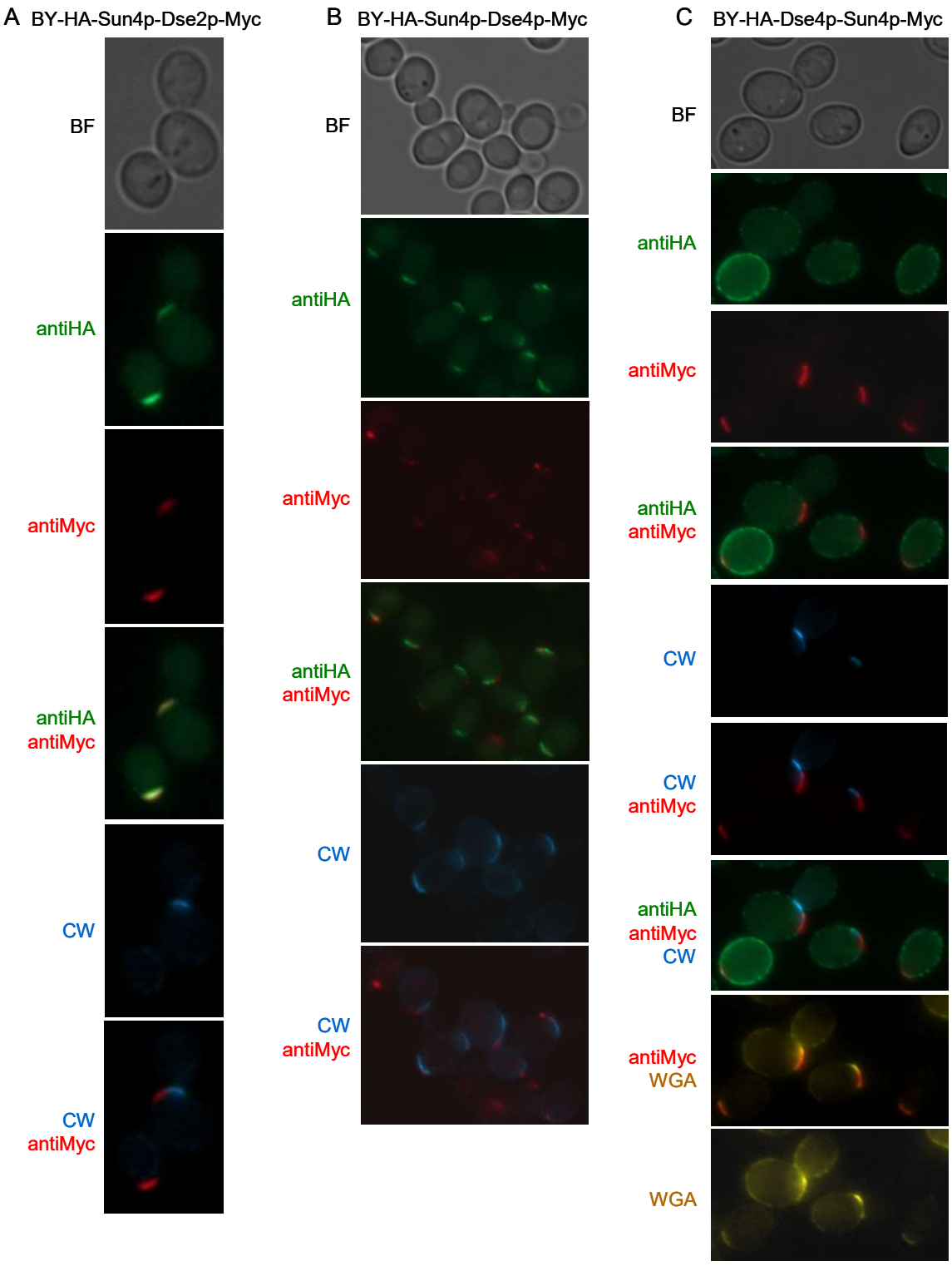


Figure 5. Co-localization of Sun4p with Dse2p and Dse4p in wild type cells. Transverse optical sections of cells (A, B, C). BF, bright field; anti-HA, antibody against HA-tag; anti-Myc, antibody against Myc tag; WGA, WGA-588; CW, Calcofluor white.

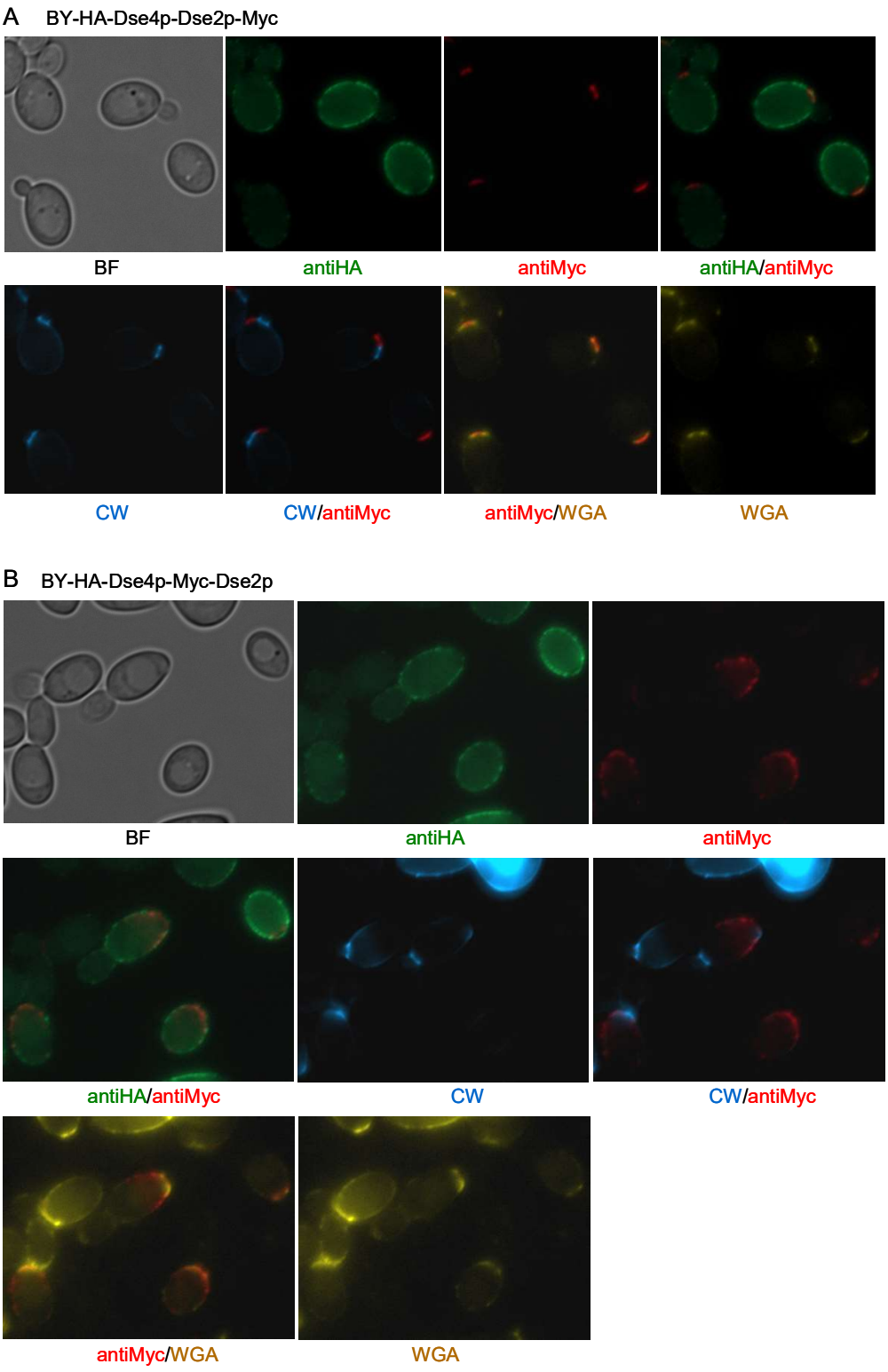


Figure 6. Co-localization of Dse2p and Dse4p in wild type cells.

Transverse optical sections of cells (A, B). BF, bright field; anti-HA, antibody against HA-tag; anti-Myc, antibody against Myc tag; WGA, WGA-588; CW, Calcofluor white.

Discussion:

The data presented clearly show the co-localization of HA-Sun4p and Dse2p-myc in the yeast birth scar. HA-Sun4p overlaps with Dse2-myc within the birth scar, indicating the possible formation of a complex by these proteins (Figure 5A). On the other hand, Dse4p was also present in the birth scar (described in chapter 5.2.4) but was not co-localized with HA-Sun4 and was present in other parts of the birth scar (Figure 5B). Similarly to HA-Dse2p, N-terminal-tagged Dse4p shows localization to the cell wall. The C- and N- terminal tagged HA-Dse4p-Myc-Dse2p strain showed mostly complementary fluorescence of HA-Dse4p and Myc-Dse2p in the cell wall with a punctuate pattern (Figure 6B). It is most likely that Dse4p-HA does not participate on the formation of a complex with Sun4p (and thus probably not with Dse2p), does not interact with these proteins and localizes to different positions in the cell wall.

5.2.6 The absence of Aim44p causes changes in the localization of birth scar proteins Sun4p and Dse2p and the "budding within the birth scar" phenotype

Aim: Experimentally show connection of Aim44p function with the formation of correctly assembled birth scars and consequently with the selection of new bud-sites outside of the birth scar.

We prepared strains with HA-tagged Sun4p and Dse2p in the *Δaim44* strain. Budding pattern and birth scar localization of Sun4p-HA and Dse2p-HA significantly changed in strains with a disrupted AIM44 gene. The BY-*aim44*-Sun4p-HA and BY-*aim44*-Dse2p-HA strains exhibited a "budding within the birth scar" phenotype, similar to the strain deleted in the SWI5 gene that codes for Swi5p, the transcriptional regulator of AIM44. Both Sun4p-HA and Dse2p-HA exhibited diffuse birth scar localization, similarly to Sun4p-HA localization in the *swi5*Δ strain (shown in Figure 1C). These findings indicate that Aim44p is involved in assembly of the birth scar Sun4p-Dse2p-Egt2p protein complex.

Discussion:

Meitenger et al. (2013) showed that the function of Swi5p-regulated gene Aim44p/Gps1 is closely connected with the bud-neck-co-localising G-proteins, Rho1 and Cdc42, which play critical roles in the maintenance of cell polarity and cell separation. This method of birth scar visualisation showed that Aim44p is necessary for correct new bud selection. Aim44p localizes to the birth scar region and prevents new bud site selection within this restricted for budding zone. Similarly the "budding within the birth scar" phenotype of strain *swi5*Δ is probably explained by changes in Swi5p-regulated AIM44 gene expression (Figure 1C). Only a portion of *swi5*Δ cells had the "budding within the birth scar" phenotype, whereas all cells of the *aim44*Δ strain had new buds within the birth scar. In addition, immunofluorescence showed diffused birth scar localization of both Sun4p-HA and Dse2p-HA, which indicates that Aim44p is involved in assembly of the birth scar Sun4p-Dse2p-Egt2p protein complex.

5.2.7 Summary discussion

We identified novel proteins in the yeast birth scar, a structure of largely unknown composition. Dse2p, Dse4p and most likely Sun4p showed dual localization on the yeast cell surface, being present within the birth scar and in the rest of the cell wall. Figure 7 shows a schematic model of localization of these proteins within the yeast cell surface. In both locations – birth scar and the rest of the cell wall- these proteins expose different termini of their structures, indicating either different orientations of these proteins or the presence of additional factors (proteins and/or carbohydrates) that cover specific domains of Sun4p and/or Dse2p. We suppose that GPI-anchored Egt2p keeps the Dse2p/Sun4p complex in the birth scar or helps to expose parts of these proteins to the external space. According to western blot, Egt2p also helps to stabilize Dse2p and Sun4p binding to the cell wall.

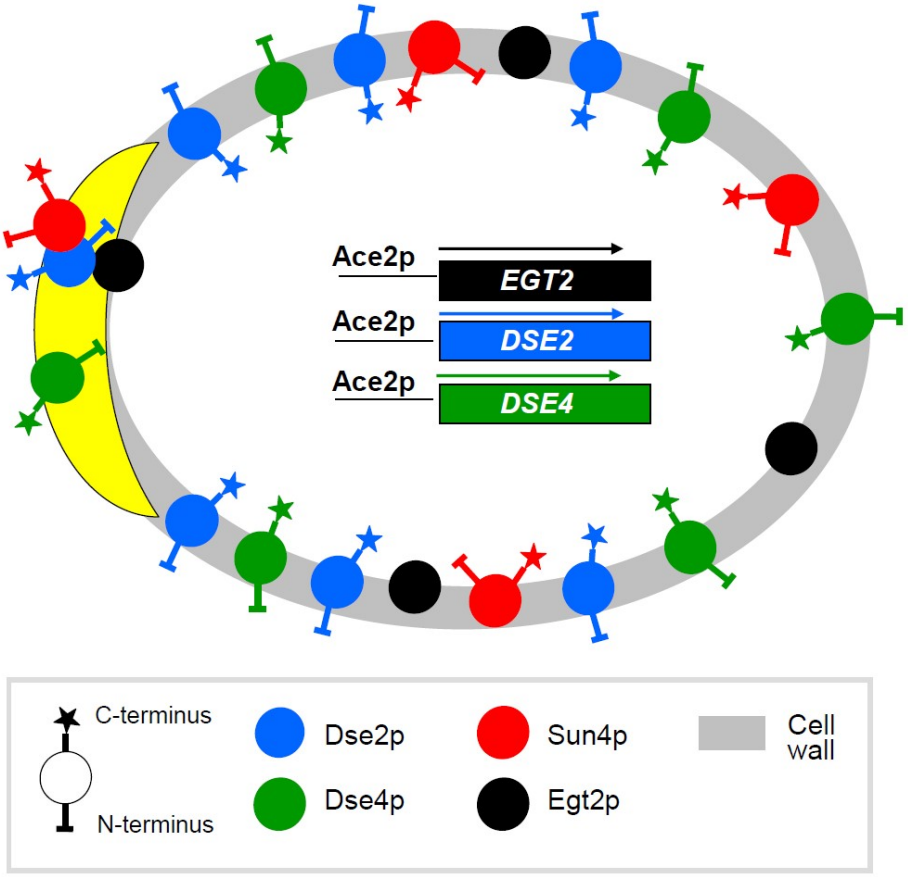


Figure 7. Schematic model of localization of Sun4p, Dse2p, Dse4p and Egt2p proteins.

The Ace2p transcriptional regulator is necessary for Sun4p localization to the cell wall because it induces the expression of Egt2p and Dse2p. *SUN4* expression is not affected by Ace2p.

Our results provide new data about birth scar composition and offer direct evidence of Egt2p-dependent Dse2p and Sun4p birth scar localization that can explain the decreased cell separation efficiency in strains with deletions of *DSE2*, *EGT2* or *SUN4* encoding proteins with predicted glucanase activity (Kovacech et al. 1996; Cappellaro et al. 1998; Colman-Lerner et al. 2001). We therefore hypothesize that these proteins form complexes that first localize to the daughter side of the bud neck during mitosis, similar to Dse4p (Baladron et al. 2002), and later to the birth scar of the daughter cell. In late mitosis, this complex presumably participates in mother-daughter cell separation. Sun4p exhibits asymmetric localization to the birth scar and is absent in bud scars, although it is not regulated by the daughter-cell-specific Ace2p transcription factor, which only activates genes in daughter cell nuclei (Roncero and Sanchez 2010). Sun4p asymmetric localization is likely ensured by forming a complex with daughter cell-specific Egt2p and Dse2p. In addition, fractions of Dse4p, Dse2p and Sun4p, which localize to the cell wall, may function in cell wall remodeling, which is important for proper bud growth (Weiss 2012).

The deletion of AIM44 and SWI5 lead to altered Sun4p and Dse2p localization, together with a “budding within the birth scar” phenotype, where new buds form within the birth scar, i.e., within the zone that is restricted for budding in the wild type strain. This phenotypic similarity of *aim44*Δ and *swi5*Δ can be explained by the fact that Swi5p is a transcriptional regulator of AIM44 (Meitinger et al. 2013). These findings implicate Aim44p in the formation of correctly assembled birth scars and consequently, in the selection of new bud-sites outside of the birth scar.

6. Conclusion

We found that Uth1p, Sun4p and Sim1p are released from cells to the extracellular space of colonies growing on solid media and in liquid cell cultures. SUN deficient strains are more resistant to zymolyase treatment that support the suggestion that these proteins participate in cell wall remodeling and that disrupting any of the SUN genes leads to a change in yeast cell wall composition. SUN family proteins represent a group of proteins which regulated differently by oxygen (similarly to a group of cell wall DAN/TIR mannoproteins). In additional, SUN-protein-deficient strains have different sensitivity to toxic compounds when cells grow on different carbon sources. We hypotheses that Uth1p could have a different effect on yeast cell walls under different environmental conditions and (maybe also other SUN proteins) could be involved in an environmental adaptation. This protein is an interesting target for studies of boric acid's action, which are still unknown.

This work increases current knowledge of the composition of a relatively little studied yeast cell wall structure – the birth scar. We found that Sun4p localizes to the birth scar of yeast cells and this localization is dependent on the Ace2p transcription factor. We determinated group of proteins (Dse2p, Dse4p and Egt2p) which are involved in proper localization of Sun4p-HA to the birth scar and affect amount of Sun4p-HA that is released extracellularly. We showed that Dse2p-HA, similarly to Sun4p-HA, localized to the birth scar. In addition, we used N-terminal tagging to demonstrate that HA-Dse2p was mostly distributed in the cell wall. The absence of Aim44p or of transcription regulator Swi5p causes changes in the localization of birth scar proteins Sun4p and Dse2p and the “budding within the birth scar” phenotype.

We proposed model of localization of Sun4p, Dse2p, Dse4p and Egt2p proteins within the yeast cell surface.

In summary, our results generate new knowledge about the localization of SUN family proteins and their roles in cell wall biogenesis, cell separation and birth scar composition.

7. Supplements

7.1 Publication 1

SUN Family Proteins Sun4p, Uth1p and Sim1p Are Secreted from Saccharomyces cerevisiae and Produced Dependently on Oxygen Level

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Aim:

To ascertain the localization of SUN family proteins and test the hypothesis that SUN proteins are involved in the remodeling of the yeast cell wall under different environment conditional.

Summary:

Results described in publication address questions about localization, oxygen-depended regulation and the possible involvement of SUN proteins in cell wall remodeling. We showed that three members of the SUN family of proteins (Uth1p, Sun4p, Sim1p) are released from cells and presents in extracellular space of colonies growing on solid media and in liquid cell cultures. In additional, we found that expression of UTH1, SUN4 and SIM1 genes regulated differently under different oxygen levels and during particular phases of yeast culture. We suppose that SUN proteins can be involved in remodeling of the cell wall and changes in its resistance to extracellular toxic compounds (Calcofluor White, Congo Red, SDS and Boric acid). Sensitivities of SUN-protein-deficient strains to toxic compounds differ when cells grow on different carbon sources (incubation on respiratory GMA plates or fermentative YEPDA plates). We indicated that Uth1 may be an interesting target for study of the mode of boric acid's action.

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SUN Family Proteins Sun4p, Uth1p and Sim1p Are Secreted from *Saccharomyces cerevisiae* and Produced Dependently on Oxygen Level

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Abstract

The SUN family is comprised of proteins that are conserved among various yeasts and fungi, but that are absent in mammals and plants. Although the function(s) of these proteins are mostly unknown, they have been linked to various, often unrelated cellular processes such as those connected to mitochondrial and cell wall functions. Here we show that three of the four *Saccharomyces cerevisiae* SUN family proteins, Uth1p, Sim1p and Sun4p, are efficiently secreted out of the cells in different growth phases and their production is affected by the level of oxygen. The Uth1p, Sim1p, Sun4p and Nca3p are mostly synthesized during the growth phase of both yeast liquid cultures and colonies. Culture transition to slow-growing or stationary phases is linked with a decreased cellular concentration of Sim1p and Sun4p and with their efficient release from the cells. In contrast, Uth1p is released mainly from growing cells. The synthesis of Uth1p and Sim1p, but not of Sun4p, is repressed by anoxia. All four proteins confer cell sensitivity to zymolyase. In addition, Uth1p affects cell sensitivity to compounds influencing cell wall composition and integrity (such as Calcofluor white and Congo red) differently when growing on fermentative versus respiratory carbon sources. In contrast, Uth1p is essential for cell resistance to boric acids irrespective of carbon source. In summary, our novel findings support the hypothesis that SUN family proteins are involved in the remodeling of the yeast cell wall during the various phases of yeast culture development and under various environmental conditions. The finding that Uth1p is involved in cell sensitivity to boric acid, i.e. to a compound that is commonly used as an important antifungal in mycoses, opens up new possibilities of investigating the mechanisms of boric acid's action.

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Introduction

The SUN family of 4 genes, (*SIM1*, *UTH1*, *NCA3* and *SUN4*) coding for homologous proteins similar to cell wall glucanases, is related to a variety of cellular processes. These proteins share a C-terminal amino acid domain (about 258 amino acid long) that is highly homologous among the members of the group (75–85% amino acid identity). This domain contains a putative Fe-binding domain with 4 cysteins (Cys-X5-Cys-X3-Cys-X24-Cys motif) [1]. The most studied member of the group *UTH1* was first described as a yeast-ageing gene, the deletion of which confers increased resistance to various stresses, including high temperature and oxidative stress, prolonged replicative life-span and increased mutant cell longevity [2–5]. Surprisingly, Uth1p was described to have a dual localization in the cell wall and in the outer mitochondrial membrane [6]. Uth1p was suggested to be important for mitochondrial autophagy (mitophagy) [7] and the presence of this protein was required for a proapoptotic effect of the mammalian BAX protein when expressed in yeast [4]. These findings, together with the observation that *uth1*Δ cells reduce the level of some mitochondrial proteins, such as cytochromes and citrate synthase, indicate that Uth1p may be a regulator of mitochondrial function. On the other hand, other findings suggest that Uth1p, like some other members of SUN family, may affect the function of the yeast cell wall. *uth1*Δ cells were described to have a cell wall that differs in its β-D-glucan and chitin composition from wild-type cells and that is more resistant to zymolyase treatment [8].

Little is known of the function of the three other members of SUN family, Nca3p, Sim1p and Sun4p. The function of Nca3p may be also related to mitochondria, as this protein was identified as a multicopy suppressor of a deficiency in the mitochondrial synthesis of some subunits of the ATP synthase [9]. Sim1p may be somehow involved in the regulation of cyclin-dependent kinase activity, since the *sim1*Δ strain with additional deletions of cyclines Clb1p and Clb4p exhibited an altered replication [10]. Sim1p when overproduced from multicopy plasmid, functioned also as a high copy extracellular suppressor of mutations in *PAG1* and *CBK1* genes involved in cellular morphogenesis [11]. Like Uth1p, Sun4p/Scw3p also exhibits a dual localization, being identified in mitochondria and in the cell wall, and this protein is supposed to be involved in cell septation [12,13]. All SUN family proteins

are homologous to the β-glucosidase of *Candida wickerhamii*, but no proof of their β-glucosidase activity has yet been found [14].

The SUN family protein Sun41p from *C. albicans* plays a role in cell attachment to a substrate and in biofilm formation. The *sun41*Δ strain forms aberrant hyphae and has decreased virulence [15]. A later study revealed that Sun41p is involved in cell separation and hyphal differentiation in *C. albicans*, and it exhibits synthetic lethality with Sun42p, another SUN family member of *C. albicans*. It has been therefore proposed that the SUN proteins of *Candida* sp. could be involved in cell wall remodeling linked to the maintenance of cell integrity during cell division [16].

Despite these mostly fragmentary data that relate the function of SUN proteins to either the cell wall or to mitochondria, the actual function of these proteins is currently unknown. Here we show that three members of the *S. cerevisiae* SUN family, Uth1p, Sim1p and Sun4p, are effectively released from cells growing either in liquid or on solid media. In addition, the production of Uth1p and Sim1p is controlled by oxygen concentration. The absence of each of the SUN proteins affects cell resistance to zymolyase and some other compounds affecting the cell wall. In addition, the absence of Uth1p markedly increases cell sensitivity to boric acid, a fungistatic agent widely used in the treatment of vaginal yeast infections. This effect is reversed by Uth1p overexpression. Our findings support the hypothesis of the function of SUN proteins being related to the function of the cell wall and indicate that Uth1p in particular could be involved in resistance to boric acid and thus could be a potential target in mycose treatment.

Results

Three of Four SUN Proteins are Secreted from Cells Grown in Liquid Cultures

SUN family genes code for proteins that are homologous, but involved in various unrelated processes and could act in different cellular compartments. Table 1 summarizes predicted features of these proteins, such as their mutual homology, Mw, presence of signaling secretion sequence and of glycosylation sites. Each of the SUN proteins contains potential signaling secretion sequence on their N-terminus and could be highly O-mannosylated. In addition, Sun4p and Nca3p could be N-glycosylated. These features suggest that all SUN proteins could be routed through the secretory pathway.

To monitor the production of SUN proteins, we prepared *S. cerevisiae* BY4742 strains containing *SUN4*, *UTH1*, *SIM1* and *NCA3* genes fused with a HA tag directly in the genome (Table 2). Genomic SUN-HA fusions guarantee the stability of the constructs as well as the natural regulation of SUN gene expression and of the amounts of proteins produced. The HA-tag did not change the functionality of the particular protein, as it did not affect the properties of the strains compared to the parental strain (data not shown).

Three of the four SUN proteins have been related to mitochondria either functionally or by their cellular localization [1,4,7,17–19]. Therefore, we monitored the level and timing of production of individual SUN proteins in respiratory GM medium with glycerol as a non-fermentative carbon source. We harvested cells for lysate preparation and protein analysis on Western blots at particular time-points throughout the growth of the shaken liquid cultures of each of the four strains. In parallel, we took aliquots of cell-free cultivation medium for an analysis of extracellular proteins. To be able to quantify the amount of SUN proteins released by cells to the cultivation medium and compare it with the amount of cellular SUN proteins, we performed an SDS-PAGE on the amount of extracellular proteins precipitated from a culture volume equivalent to the amount of biomass used for the preparation of the lysate (see Materials and Methods) (Figure 1A). In addition, we followed the time course of accumulation of SUN proteins in the cultivation medium as the amount of them present in constant aliquots taken at each time-point, as shown in Figure 1B.

Figure 1A shows that three of the four SUN proteins, Uth1p-HA, Sun4p-HA and Sim1p-HA are released to the cultivation medium with high efficiency. On the other hand, no Nca3p-HA was detected extracellularly during the cultivation (data not shown). Individual SUN proteins differed in the ratio of their cellular/extracellular protein concentration as well as in the timing of their production in the various growth phases. The cellular levels of all SUN proteins started to increase early during the cultivation, with Sim1p-HA being the latest. The cellular level of Uth1p-HA (Uth1p-HA^{cell}) gradually increased and reached its maximal values during the early logarithmic growth phase (after 6–10 hrs of cultivation). Later, at the end of the logarithmic growth phase, Uth1p-HA^{cell} started to decrease, however, some protein was still present even at 72 h. The extracellular Uth1p-HA

Table 1. Predicted properties of SUN family proteins.

Properties of SUN proteins	**Uth1p**	**Sun4p**	**Sim1p**	**Nca3p**	
Predicted Mw (kDal)	36.955	43.442	48.19	35.412	http://www.yeastgenome.org/
Putative signal peptide for secretion	1–17 AA	1–22 AA	1–19AA	1–18AA	http://www.yeastgenome.org/
N-glycosylation sites	0	1 (395 AA)	0	1 (117 AA)	http://www.oppf.ox.ac.uk/opal/
O-mannosylation sites	28	49	78	9	http://www.oppf.ox.ac.uk/opal/
Kex2 cleavage sites (LysArg)	1 (32–33 AA)	1 (44–45 AA)	1 (34–35 AA)	1 (76–77 AA)	
Percent identity matrix of SUN proteins	**Uth1p**	**Sun4p**	**Sim1p**	**Nca3p**	http://www.ebi.ac.uk/Tools/msa/clustalo/
Uth1p	100	56.42	59.15	66.27	
Sun4p	56.42	100	72.20	54.93	
Sim1p	59.15	72.20	100	58.41	
Nca3p	66.27	54.93	58.41	100	

AA.... amino acid.
doi:10.1371/journal.pone.0073882.t001

Table 2. Strains.

Strain	Genotype	source
BY4742 (wt)	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0*	EUROSCARF
BY-Uth1p-HA	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, UTH1-6HA-HIS3MX6*	this study
BY-Nca3p-HA	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, NCA3-6HA-HIS3MX6*	this study
BY-Sun4p-HA	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, SUN4-6HA-HIS3MX6*	this study
BY-Sim1p-HA	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, SIM1-6HA-HIS3MX6*	this study
BY-p_{TEF}-*UTH1*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0* p_{TEF}-*UTH1-kanMX18*	this study
BY-*uth1Δ*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, uth1Δ-kanMX4*	EUROSCARF
BY-*nca3Δ*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, nca3Δ-kanMX4*	EUROSCARF
BY-*sun4Δ*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, sun4Δ-kanMX4*	EUROSCARF
BY-*sim1Δ*	*MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0, sim1Δ-kanMX4*	EUROSCARF

doi:10.1371/journal.pone.0073882.t002

level (Uth1p-HA^{ex}), i.e. the amount of Uth1p-HA produced by a biomass unit that is released from the cells to the extracellular medium, approximately matched the cellular level profile. Cellular Sun4p-HA (Sun4p-HA^{cell}) and Sim1p-HA (Sim1p-HA^{cell}) remained at about the same high level from approximately the 4th to 24th h of cultivation, i.e. to the end of the exponential growth phase. After this point, Sun4p-HA^{cell} dropped to about half of its exponential-phase level; Sim1p-HA^{cell}, however, almost disappeared from the cells. The amount of both Sun4p-HA^{ex} and Sim1p-HA^{ex} released by the unit of biomass increased, this increase beginning at the end of the exponential growth phase. Cellular Nca3p-HA (Nca3p-HA^{cell}) was kept at a constant level, similarly to Sun4p-HA^{cell} and Sim1p-HA^{cell}; its level began to decrease from the 24th h onwards. The finding of different levels of extracellular SUN proteins released from the cells of post-exponential and stationary cultures is consistent with the observation that the extracellular accumulation (i.e. amount of protein in a constant volume of the medium) of Sun4p-HA^{ex} and Simp1p-HA^{ex} in the medium was still high even after 3 days of cultivation, while the absolute level of Uth1p-HA^{ex} in the medium decreased (Figure 1B).

To test whether the SUN protein production and secretion differs in respiratory and fermentative media, we analyzed the level of cellular and extracellular SUN proteins in a liquid cell culture grown in YED medium. As shown in Figure 2, Uth1p-HA, Sun4p-HA and Sim1p-HA are produced in YED medium during the exponential phase of population growth and are released out of the cells with profile similar to that observed in GM medium (Figure 1). Thus, in spite of differences related to different grow-rate of yeast in respiratory versus fermentative medium, general profiles of production and release of SUN proteins are similar in both media.

Uth1p, Sun4p and Sim1p are Present in Extracellular Space of Developing Colonies

Time-line analyses of SUN protein production and localization (especially the ability to release the proteins from the cells) in yeast shaken liquid cultures revealed significant differences between the individual SUN proteins. We therefore asked the question of when these proteins are produced and where they localize in multicellular yeast colonies that exhibit typical linear growth on solid nutrient agar [20,21]. We therefore harvested colonies at various developmental time-points and prepared the two protein samples; extracellular proteins washed out from the cells and the cell lysate. Both fractions were analyzed for the presence of SUN proteins using Western blots. As with the samples from liquid cultures, the amount of extracellular proteins from colonies that were loaded into the SDS-PAGE was produced by approximately the same amount of cell biomass that was used to prepare the cell lysate loaded for the detection of cellular SUN proteins.

While the amount of Nca3p-HA within developing colonies was below the detection limit of the method used, we detected a significant level of both cellular and extracellularly released Uth1p-HA, Sun4p-HA and Sim1p-HA (Figure 3). As in liquid cultures, the profile of the presence of these proteins within colonies differed significantly. Uth1p-HA was present in high levels in relatively young colonies (3-8-days-old) and was released into the extracellular space from the beginning. From day 10 of colony development, Uth1p-HA^{cell} significantly decreased and the protein also disappeared from the extracellular space of the colony from about the 15th day. Sun4p-HA was also found in colonies from the early phases of their growth, but in contrast to both Uth1p-HA and Sim1p-HA, its cellular level was constant until the late developmental phases (20-days-old colonies). Sun4p-HA^{ex} followed approximately the same profile, with a significant decrease in 15- and 20-days-old colonies. Only Sim1p-HA^{ex} persisted at a constant relatively high concentration until the end of the monitored period of colony development (20-days-old colonies). On the other hand, Sim1p-HA^{cell} dropped quickly, being only high in young colonies (3-days-old) and becoming almost undetectable from day 8 of colony development. The absence of any of the three SUN proteins did not affect significantly viability of cells within colonies (Figure S1).

SUN Genes are Regulated Differently according to the Level of Oxygen

The function of some of the SUN proteins was related to the function of mitochondria [1,4,7,17–19] and in addition, there are indications that the expression of the *SIM1* gene from *Candida albicans* is induced under hypoxic conditions [22]. We therefore analyzed the production of the three SUN proteins that were detected at relatively high levels within colonies (Uth1p-HA, Sim1p-HA and Sun4p-HA) under various levels of oxygen tension, i.e. normoxic, hypoxic (1% O_2) and anoxic conditions. Two experimental setups were designed. First, colonies of the strains with HA-tagged versions of the SUN proteins were grown for 3 days in parallel under normoxic and anoxic conditions and in an atmosphere with 1% O_2 on YEPDA-Erg with glucose as the

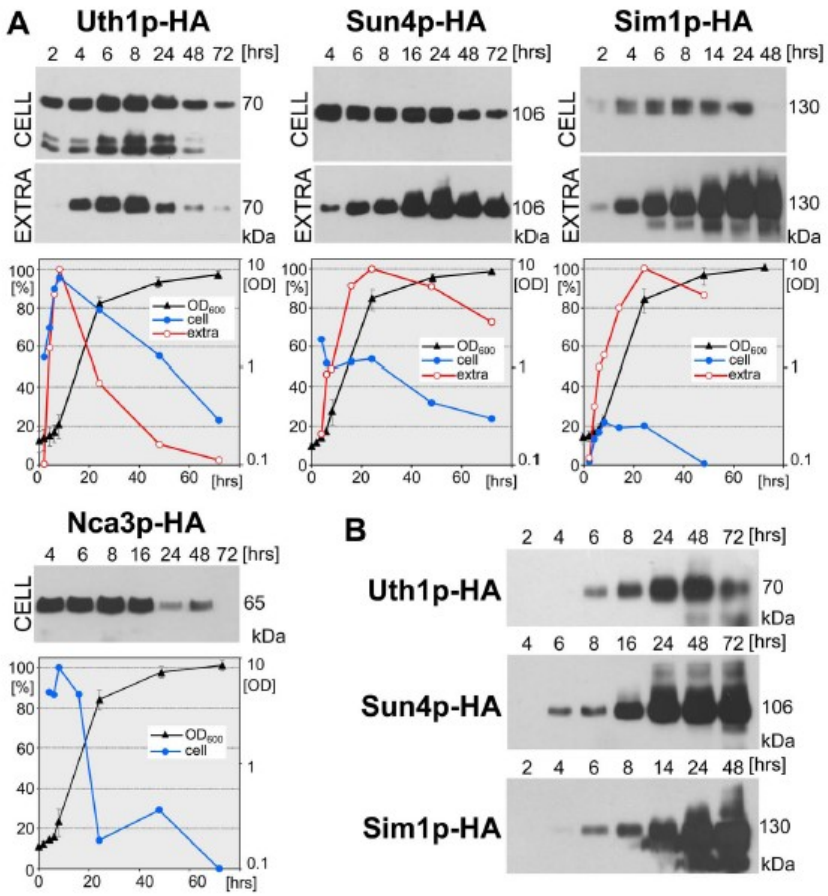

Figure 1. Cellular/extracellular level of individual SUN proteins during growth of liquid cell cultures in GM medium. A, Protein amounts in cell lysates (CELL) and in extracellular extracts (EXTRA), respectively, prepared from liquid cell cultures of BY-Uth1p-HA, BY-Sun4p-HA, BY-Sim1p-HA strains and the protein amounts in cell lysates from a liquid cell culture of BY-Nca3p-HA strain. The standardized amounts of lysate-proteins or the corresponding amounts of extracellular extracts were loaded onto the gel (loading controls, see Figure S3 A). A representative Western blot of 3 to 4 independent biological replicates is shown. The highest amount of the particular protein in each immunoblot quantified by densitometry was set as 100%. Growth curves of cultures of individual strains are shown (right axis). **B**, extracellular accumulation of Sun4p-HA, Uth1p-HA and Simp1p-HA proteins. Proteins precipitated from a constant volume of the medium were loaded onto the gel. A representative Western blot of 2 to 4 independent biological replicates is shown. The value of Mw at the right side of immunoblots represents Mw of particular SUN protein linked to the HA tag. S.D. values were calculated from 3–5 independent biological replicates.
doi:10.1371/journal.pone.0073882.g001

fermentative carbon source supplemented with ergosterol, a compound essential for yeast growth under anoxic conditions [23]. Figure 4A shows that the total level (comprised of both cellular and extracellular protein) of both Uth1p-HA and Sim1p-HA decreased with decreased oxygen concentration, being negligible under anoxic conditions. On the other hand, the total level of Sun4p was relatively stable, or even slightly higher under conditions of decreased oxygen tension. However, extracellular Sun4p-HA was not detected in anoxic conditions and the level of Sun4p-HA^{ex} in hypoxia was significantly lowered when compared to normoxic conditions (Figure 4B). This finding indicates that either extracellular secretion of this protein is decreased or Sun4p-HA^{ex} is more efficiently degraded in anoxia/hypoxia.

In a parallel experiment, colonies were grown for 3 days on respiratory GMA plates under normoxic conditions and then the colonies were transferred to the anoxomat device and incubated for an additional 2 days under low oxygen, i.e. under conditions where the cells cannot divide and grow. Figure 4A shows that the level of Uth1p and Sim1p also partially dropped under these non-growing conditions, while the level of Sun4p remained stable.

SUN-protein-deficient Yeast Strains Differ in Properties of their Cell Walls

Previous data also suggested that Uth1p and Sun4p proteins localize to the cell wall and may affect its properties [6,8]. We therefore analyzed the sensitivity of the strains individually deleted in each of the SUN genes to a spectrum of compounds that are known to be cytostatic for yeast, affecting their cell wall, plasma membrane or other cellular processes (Figure 5).

Figure 5D shows that a deletion of any of the SUN genes significantly increases yeast cell resistance to zymolyase treatment. The resistance was determined using cells from 4-days-old colonies

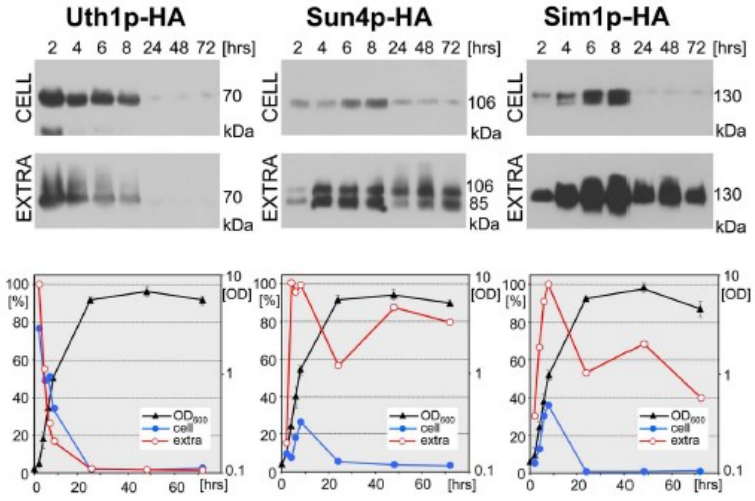

Figure 2. Cellular/extracellular level of SUN proteins during growth of liquid cell cultures in glucose YED medium. Protein amounts in cell lysates (CELL) and in extracellular extracts (EXTRA), respectively, prepared from liquid cell cultures of BY-Uth1p-HA, BY-Sun4p-HA and BY-Sim1p-HA strains. The standardized amounts of lysate-proteins or the corresponding amounts of extracellular extracts were loaded onto the gel (loading controls see Figure S3 B). A representative Western blot of the 2 independent biological replicates is shown. The highest amount of the particular protein in each immunoblot quantified by densitometry was set as 100%. Growth curves of individual strains are shown (right axis). The value of Mw at the right side of immunoblots represents Mw of particular SUN protein linked to the HA tag.
doi:10.1371/journal.pone.0073882.g002

growing on GMA. On the other hand, only the BY-*uth1*Δ strain was significantly more sensitive to Congo red or Calcofluor white (CFW) dyes when present in cultivation GMA agar (Figures 5A and S4A). The effect of these compounds that affect the cell wall was more prominent at a higher temperature (37°C) than at 28°C (not shown). This effect was suppressed in the BY-p_{TEF}-*UTH1* strain containing the *UTH1* gene under the control of the constitutive *TEF* promoter. BY-*uth1*Δ was also more sensitive to a

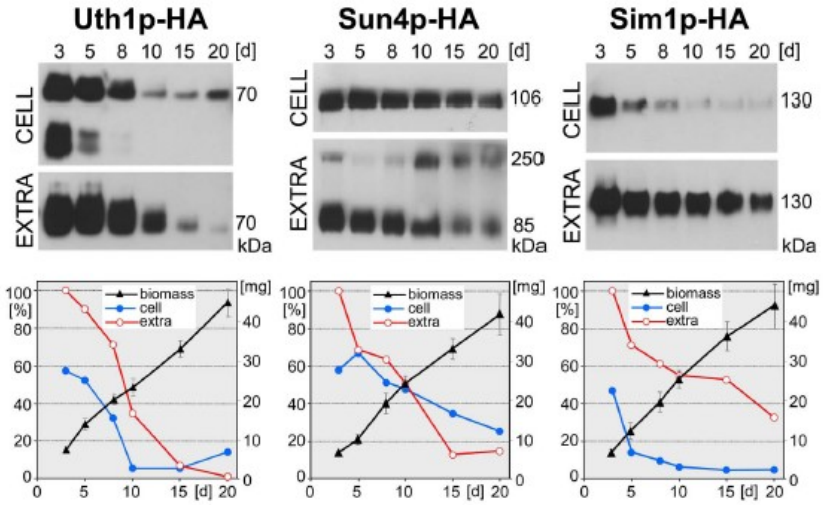

Figure 3. Cellular/extracellular level of individual SUN proteins during development of colonies growing on GMA plates. Protein amounts in cell lysates (CELL) and in extracellular extracts (EXTRA), respectively, prepared from colonies of BY-Uth1p-HA, BY-Sun4p-HA and BY-Sim1p-HA strains. The standardized amounts of lysate-proteins were loaded onto the gel (loading controls, see Figure S3 C) and corresponding amounts of extracellular extracts. The representative Western blot of 3–4 independent biological replicates is shown. The highest amount of the particular protein in each immunoblot quantified by densitometry was set as 100%. Growth curves of colonies formed by individual strains are shown as wet weight per one colony (right axis). The value of Mw at the right side of immunoblots represents Mw of particular SUN protein linked to the HA tag. S.D. values were calculated from 3–4 independent biological replicates.
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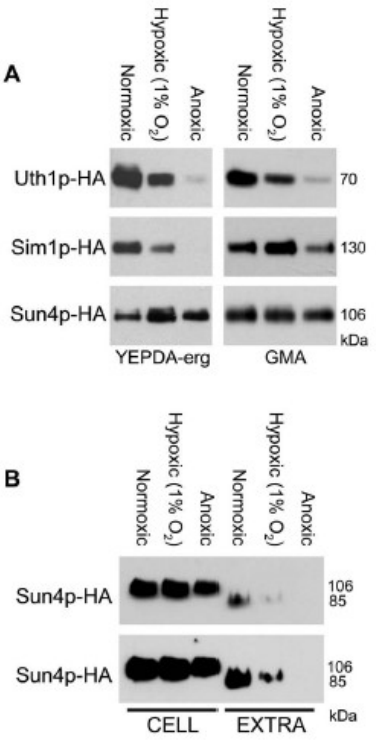

Figure 4. O_2 concentration affects SUN protein production. A, Protein amounts in cell lysates of unwashed colonies of BY-Uth1p-HA, BY-Sun4p-HA and BY-Sim1p-HA strains. Strains were either grown on YEPDA-erg or incubated on GMA under normoxic, hypoxic or anoxic atmosphere. The representative Western blot of the 2 independent biological replicates is shown. **B**, Sun4p-HA amounts in cell lysates (CELL) and in extracellular extracts (EXTRA) from colonies. BY-Sun4p-HA strain was grown on YEPDA-erg under normoxic, hypoxic or anoxic conditions for 3 days. Two film-expositions are shown to better visualize differences in protein concentrations in individual samples. The value of Mw at the right side of immunoblots represents Mw of particular SUN protein linked to the HA tag. The standardized amounts of lysate-proteins or the corresponding amounts of extracellular extracts were loaded onto the gel (loading controls, see Figure S3 D).
doi:10.1371/journal.pone.0073882.g004

low concentration of SDS (0.012%) than the wild type strain BY4742, while *UTH1* gene overexpression increased the BY-p_{TEF}-*UTH1* strain resistance tenfold compared to the wild-type strain (Figures 5B and S4A). In addition to BY-*uth1*Δ, the BY-*sim1*Δ strain was also significantly more sensitive to SDS than the wild-type strain. BY-*sun4*Δ and BY-*nca3*Δ strains exhibited the same resistance to SDS as the wild-type strain.

Boric acid, being an important antimicrobial, is often used as an antifungal compound in the treatment of vulvovaginal mycoses. Although current knowledge on the mechanisms of boric acid's action is still fragmentary, indications exist that among other things, this compound affects yeast morphogenesis. Figures 5C and S4A show that the BY-*uth1*Δ strain is about 500-fold more sensitive to 0.012% boric acid and the BY-p_{TEF}-*UTH1* strain is about 10 times more resistant to boric acid treatment than the wild-type strain. Of the other SUN knockout strains, only the BY-*sim1*Δ strain was slightly more sensitive to boric acid; BY-*sun4*Δ and BY-*nca3*Δ did not exhibit any differences.

Sensitivities of SUN-protein-deficient Strains to Toxic Compounds Differ when Cells Grow on Different Carbon Sources, Irrespective of SUN Protein Cellular Concentration

Data obtained on the sensitivity of BY-*uth1*Δ partially contradict the previous findings of [8] that showed an increased resistance of the *uth1*Δ strain derived from the W303 background to CFW when tested on glucose plates. We therefore repeated the assays using YEPDA supplemented with CFW or Congo red, i.e. with compounds that affect the yeast cell wall. Figure 6 shows that BY-p_{TEF}-*UTH1* was significantly more sensitive to CFW and Congo red than the parental strain when grown on YEPDA, while we did not observe any significant differences in the sensitivity of BY-*uth1*Δ. However, the resistance of BY-*uth1*Δ and BY-p_{TEF}-*UTH1* to boric acid on YEPDA plates was similar to that observed on GMA plates, only the effect of boric acid treatment was milder on YEPDA. Sensitivity of BY-*uth1*Δ strain to boric acid was significantly decreased on YEPDA-erg plates under hypoxic/anoxic conditions (Figure 7A) where the Uth1p-HA level is decreased (Figure 4A). As expected, changes in oxygen tension did not affect sensitivity of BY-p_{TEF}-*UTH1* strain (where native control of *UTH1* gene expression is abolished) to CFM and Congo Red (Figure 7B and not shown). As shown in Figures 1 and 2, the profiles of SUN protein production and secretion in relation to distinct growth phases of yeast population are similar. The observed differences in the cell sensitivity of SUN-gene-deleted strains on YEPDA and GM seem therefore not simply related to the level of SUN protein production on the two media.

Discussion

The SUN family is comprised of a group of fungus-specific proteins exhibiting high similarity, especially in their C-terminal domain (Table 1) [13,16]. SUN family members have been predicted to be involved in various unrelated cellular processes, such as mitochondrial biogenesis and autophagy (mitophagy), cytokinesis, cell wall structure and DNA replication [7,8,17]. In addition, the β-glycosidase activity of these proteins was predicted on the basis of the homology of SUN proteins to the β-glycosidase of *C. wickerhamii* [14]. Localization of the three SUN proteins of *S. cerevisiae* Sun4p, Uth1p and Sim1p to the cell wall was shown [6], together with a secondary localization of Uth1p and Sun4p to the mitochondria.

In this paper we show that three of the SUN family proteins, Uth1p, Sun4p and Sim1p, are released from cells to the extracellular space during cultivation in both liquid cell cultures and in colonies growing on solid media. As for the fourth member of the group, Nca3p, its extracellular localization was not detected and even the cellular level of this protein was below the detection limit in colonies. In exponentially growing liquid cultures, all SUN proteins are present in high amounts in the cellular fractions, while their cellular concentrations decrease later when the cultures enter the postdiauxic and stationary phase. Even exponentially growing cells are able to release a detectable amount of Sun4p and Sim1p; this ability however gradually increases at the end of the exponential growth phase, especially with Sim1p. This, together with an increasing amount of cell biomass, leads to an accumulation of high concentrations of both proteins in the extracellular fluid. In contrast, the extracellular amount of Uth1p released by a biomass unit correlates with its intracellular concentration, which indicates that a fraction of Uth1p is always released when this protein is produced. In addition, it seems that at least extracellular Uth1p is partially degraded, as its absolute amount in the medium decreases in the stationary phase when the

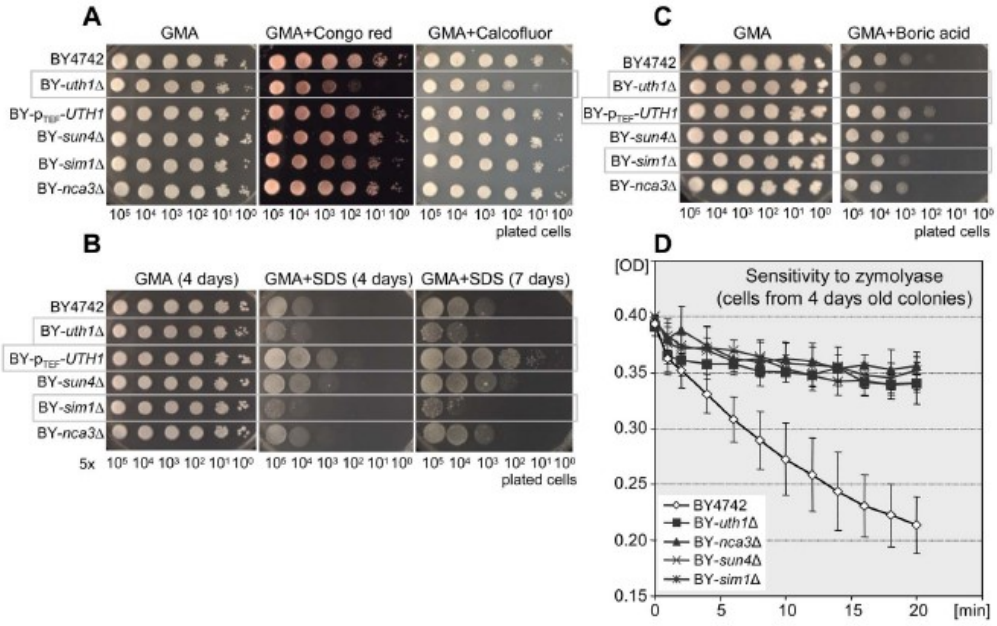

Figure 5. Strains deficient in SUN proteins have different sensitivity to zymolyase and various drugs when grown on respiratory GMA agar. A–C, drop assays of cells of BY4742 (wt), BY-*uth1*Δ, BY-p_{TEF}-*UTH1*, BY-*sun4*Δ, BY-*sim1*Δ and BY-*nca3*Δ strains on GMA plates supplemented with various drugs. Representative experiments of at least three biological replicates are presented. Significant drug effects on particular strains are marked by grey boxes; the quantification of the drug effect is shown in Figure S4A. **A**, effect of Congo red (800 μg/ml) or Calcofluor white (1 mg/ml); cells were grown at 37°C for 4 days. **B**, effect of SDS (0.012%), cells were grown at 28°C for 4 or 7 days as indicated. **C**, effect of boric acid (0.4%), cells were grown at 28°C for 7 days. **D**, cell resistance to zymolyase (0.2 U/μl) presented as decrease in density of cell suspension. S.D. values were calculated from four independent biological replicates for the mutant strains and 10 replicates for the wt; the significance of the difference between BY4742 and the other four strains was determined using two-way ANOVA with $p \leq 0.0001$.
doi:10.1371/journal.pone.0073882.g005

cells stop releasing it. Due to their continuous release from the cells, we cannot assess the extracellular stability of the other two proteins, Sun4p and Sim1p. In colonies, the profile of the extracellular level of Uth1p and Sun4p roughly corresponds to the cellular concentration of these proteins. As in liquid cultivations, the intracellular concentration of Sim1p drops rapidly even in relatively young 5-days-old colonies and the protein is released into the extracellular space over the next 15 days, which means that its concentration relative to a unit of biomass is maintained at a constant level. Interestingly, while the molecular weight (Mw) of extracellular Sim1p and Uth1p corresponded to that of the cellular proteins, the extracellular Sun4p variant is about 20 kDa shorter than the intracellular one, which indicates either Sun4p processing during its release or a different level of modification (possibly glycosylation). In addition, a smaller amount of Sun4p protein with an even higher Mw than the Mw of intracellular Sun4p was present in the extracellular space. This Mw difference of cellular versus extracellular Sun4p was not detectable in liquid cultivations in respiratory GM medium, while both forms (98 kDal and 77 kDal) were identified in fermentative YED medium. The Mw

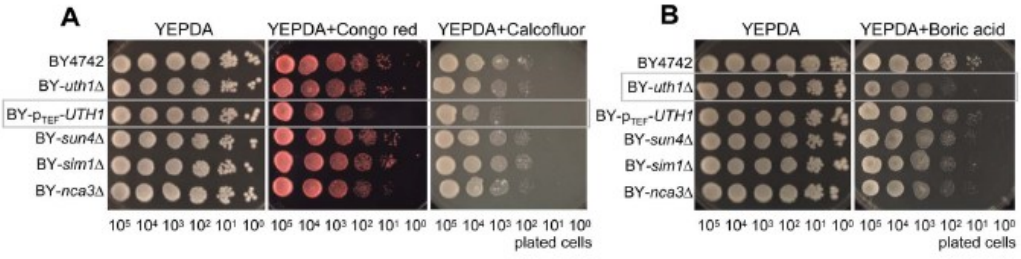

Figure 6. Sensitivity of strains deficient in SUN proteins to toxic compounds on fermentative YEPDA medium. A, B, Drop assays of cells of BY4742, BY-*uth1*Δ, BY-p_{TEF}-*UTH1*, BY-*sun4*Δ, BY-*sim1*Δ and BY-*nca3*Δ strains on YEPDA plates supplemented with various drugs. Representative experiments of at least three biological replicates are presented. Significant drug effects on particular strains are marked by grey boxes; the quantification of the drug effect is shown in Figure S4 B. **A**, Congo red (800 μg/ml) or Calcofluor white (600 μg/ml), cells were grown at 28°C for 4 days. **B**, boric acid (0.2%), cells were grown at 28°C for 5 days.
doi:10.1371/journal.pone.0073882.g006

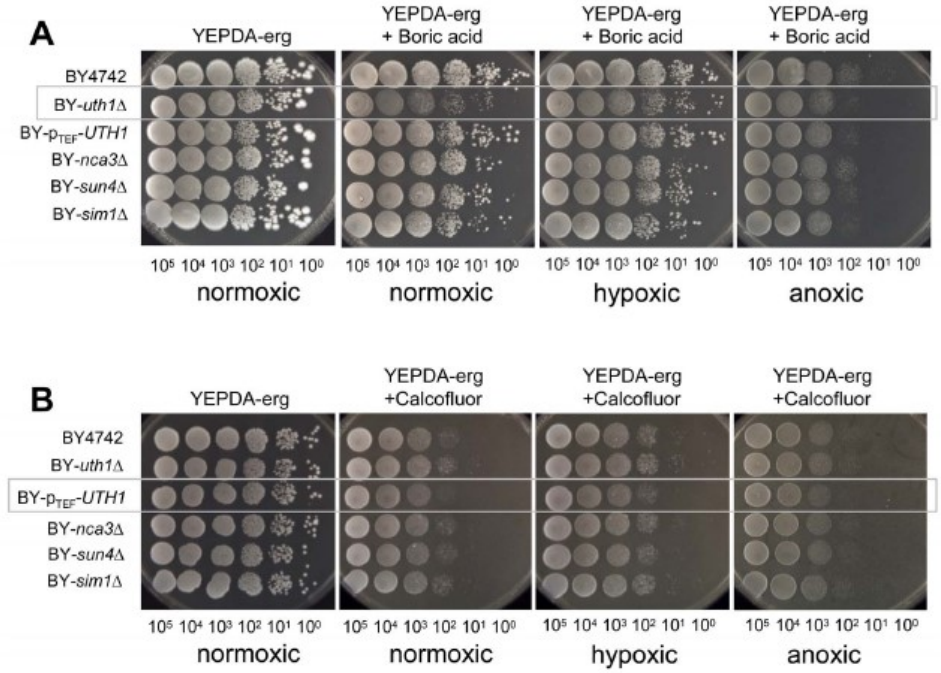

Figure 7. Sensitivity of strains deficient in SUN proteins to toxic compounds on fermentative medium under various levels of oxygen tension. Drop assays of cells of BY4742, BY-*uth1*Δ, BY-p_{TEF}-*UTH1*, BY-*sun4*Δ, BY-*sim1*Δ and BY-*nca3*Δ strains on YEPDA-erg plates supplemented with either boric acid or Calcofluor white and grown under normoxic, hypoxic (1% O_2) or anoxic conditions. Representative experiments of three biological replicates are presented. Significant drug effects on particular strains are marked by grey boxes. **A**, boric acid (0.4%), cells were grown at 28°C for 6 days. **B**, Calcofluor white (2 mg/ml), cells were grown at 28°C for 3 days.
doi:10.1371/journal.pone.0073882.g007

of intracellular Uth1p (62 kDa), Sun4p (98 kDa) and Sim1p (122 kDa) roughly corresponded to the Mws determined previously [6]. The differences from the Mws calculated from gene sequences (Table 1) were attributed to a high level of glycosylation of SUN proteins [6]. The Mw of Nca3p (57 kDa) that we observed was also higher than the Mw predicted from the sequence (35.4 kDa), suggesting that this protein is also modified. According to the prediction (Table 1), all four proteins contain multiple O-glycosylation sites and, in addition, Sun4p and Nca3p each contain one N-glycosylation site.

What could be the reason for the release of the three SUN proteins from cells? Several proteins attached to the cell wall have been described to be also present in free form in the extracellular space, including the surface adhesin Flo11p [24]. The functions of these released protein variants are mostly unknown. One can speculate that SUN proteins may participate in remodeling the cell wall during the exponential-to-diauxic/stationary phase transition and in stationary cells they could affect cell wall structures from the "outside". SUN proteins may affect cell morphogenesis, as was described for *C. albicans* Sun41p, which is involved in hyphae formation [15,16,25] and possibly secreted [25]. A second possibility that cannot be excluded is that these proteins are removed from the cell wall after accomplishing their task during cell division and septation, and that their presence in the extracellular medium is just a consequence of this release. However, comparison of the level of cellular, extracellular and cell-wall attached Uth1p-HA, Sun4p-HA and Sim1p-HA proteins from 3-days-old GMA grown colonies showed much smaller amount of Uth1p-HA and Sun4p-HA and almost no Sim1p-HA in purified cell wall fractions when compared to cellular and extracellular fractions (Figure 8). In addition, presence of these proteins also affects the sensitivity of aged colony cells to zymolyase (Figure S2) and in particular Sim1p accumulates in the extracellular space of ageing colonies as well as in liquid cultures and is therefore produced and directly released by stationary cells. All these findings favor the first possibility.

An increased resistance of the strain with a deleted *UTH1* gene to zymolyase treatment has been reported [8]. Here we show that the deletion of any of the four SUN genes confers cells grown on GMA plates with high resistance to this lytic enzyme. As the increased resistance to zymolyase was attributed to an increased level of β-1-6 glucan in the yeast cell wall [26], we can speculate that the deletion of any of the SUN genes leads to a remodeling of the yeast cell wall accompanied by an increasing in β-1-6 glucans. The finding that the level of β-glucans increased in the *uth1*Δ strain [8] supports this. Interestingly, an increased resistance of the SUN-gene-deleted strains to zymolyase was not only apparent in cells with a high cellular concentration of SUN proteins, but also later when SUN proteins are released from the cells. For example, in 20-days-old colonies, most of the Sim1p is released from the cells, but deletion of the *SIM1* gene still increases cell resistance to zymolyase (Figure S2). These data indicate that although expression of *UTH1* and *SIM1* decreases during colony ageing [27,28] and the expression of all three genes *SUN4*, *UTH1* and *SIM1* is decreased during the entry of liquid culture cells to the stationary phase [29], which both corresponds to an observed

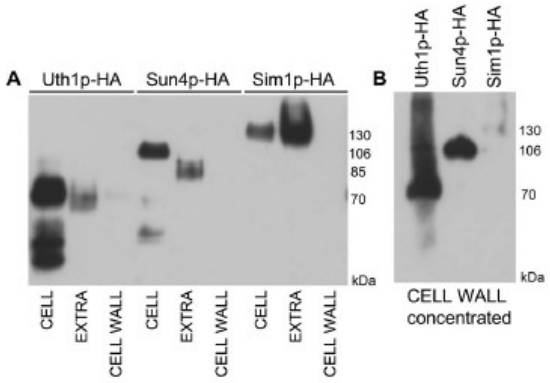

Figure 8. Localization of SUN proteins in cells from 3-days-old colonies grown on GMA plates. A, Protein amounts in cell lysates (CELL), extracellular extracts (EXTRA) and the purified cell walls (CELL WALL), respectively, prepared from colonies of BY-Uth1p-HA, BY-Sun4p-HA and BY-Sim1p-HA strains. The standardized amounts of lysate-proteins were loaded onto the gel and corresponding amounts of extracellular extracts and cell wall extracts. **B,** Proteins from 20-times concentrated cell wall extracts used in panel A.
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decrease in SUN protein cellular concentration, these proteins, and especially Sim1p, can persist much longer outside the cells and could play important roles, even in later phases of cell culture development.

In addition to the increased resistance to zymolyase observed in strains with any of their SUN genes knocked out, the BY-*uth1*Δ strain grown on GMA plates was more sensitive than the parental BY4742 strain to other compounds affecting the composition and/or structure of the yeast cell wall. Three of these compounds, Calcofluor white, Congo red and boric acid have been described to be linked to an increased cellular chitin content in treated cells [30–32]. The increased sensitivity of the BY-*uth1*Δ strain to these compounds together with the previously observed decrease in chitin content [8] suggests that Uth1p could be somehow involved in the synthesis or assembly or deposition or correct localization of cellular chitin. In addition, both the BY-*uth1*Δ and BY-*sim1*Δ strains were more sensitive to a low concentration of SDS detergent than the parental BY4742, BY-*sun4*Δ and BY-*nca3*Δ strains. The fact that SDS affects cell wall integrity [33] and observed sensitivity to this compound suggests that the cell walls of the BY-*uth1*Δ and BY-*sim1*Δ strains are more fragile than those of the cells of the other two strains, although all SUN gene delta strains exhibit an increased resistance to zymolyase treatment. SDS apparently affects different components of yeast cell surface structures than zymolyase does. The BY-*uth1*Δ sensitive phenotype to all four compounds can be completely reversed by *UTH1* gene overexpression controlled by the constitutive strong TEF promoter. The BY-p_{TEF}-*UTH1* strain even reaches a resistance to boric acid and SDS that surpasses the resistance of the parental strain. The finding that the presence of Uth1p is important for yeast resistance to CFW and Congo red contradicts the previous observation of Ritch et al [8] that a *uth1*Δ strain derived from a different background (W303) is more resistant to these compounds according to drop tests. As this difference could be due to either the different strain backgrounds or the different growth conditions used in the screen, we tested BY-*uth1*Δ strain sensitivity using YEPDA plates, as were used by Ritch et al [8]. The results clearly showed that BY-*uth1*Δ strain sensitivity to CFW and Congo red is affected by the carbon source, being higher on respiratory GMA plates and the same on fermentative YEPDA plates (where Uth1p overproduction leads to increased sensitivity to these compounds) as compared with the sensitivity of the BY4742 parental strain. On the other hand, BY-*uth1*Δ was more sensitive to boric acid than the parental strain on both media. Thus, Uth1p could have a different effect on yeast cell walls under different environmental conditions. It was shown that different carbon sources significantly affect the chitin and β-glucan composition of the cell wall [26]. One can therefore speculate that Uth1p (and maybe also other SUN proteins) could be involved in such an environmental adaptation.

SUN41 of *C. albicans* is one of the genes induced by hypoxia [34]. Our data showed that in *S. cerevisiae*, the production of Uth1p and Sim1p is decreased under conditions of decreased oxygen (anoxia or 1% oxygen), while the production of Sun4p was relatively stable, exhibiting no dependence on the oxygen level. These findings imply that in addition to group of cell wall DAN/TIR mannoproteins that is comprised of members regulated by oxygen in various ways [35], the SUN-family proteins represent another group of proteins that may be involved in cell wall remodeling at various oxygen concentrations. This prediction is supported by the finding that sensitivity of BY-*uth1*Δ strain to boric acid was diminished under conditions of decreased oxygen tension, i.e. under conditions where cells decrease Uth1p-HA protein production and thus probably do not need this protein.

In summary, our new data suggest that SUN-family proteins are regulated differently during particular phases of yeast culture development and under different environmental conditions, apparently being involved in remodeling of the cell wall and changes in its resistance to extracellular compounds. In addition, the finding that cell sensitivity to boric acid, i.e. to a fungistatic compound that is used in the medical treatment of recurrent and resistant yeast vaginitis, is dependent on the presence (and level) of Uth1p makes this protein an interesting target for studies of boric acid's action.

Materials and Methods

Yeast Strains and Media

The strains used in this study (Table 2) were derived from *Saccharomyces cerevisiae* BY4742 (MATα, *his3Δ1*, *leu2Δ0*, *lys2Δ0*, *ura3Δ0*) obtained from the EUROSCARF collection. Cells were grown at 28°C either in liquid YED medium (1% yeast extract, 2% glucose), or in liquid GM medium (1% yeast extract, 3% glycerol, pH 5) or on GMA agar (GM, 2% agar, 30 mM $CaCl_2$, pH 5). For spot assays, GMA agar was supplemented with 0.012% SDS, 0.4% boric acid, 800 µg/ml Congo red and 1 mg/ml Calcofluor white (CFW). YEPDA agar (YED, 1% pepton, 2% agar,) was supplemented with 0.2% boric acid, 800 µg/ml Congo red and 600 µg/ml Calcofluor white. YEPDA-erg, YEPDA supplemented with 0.5 ml/100 ml ergosterol solution (2 mg ergosterol, 800 µl ethanol, 200 µl Tween 80) was used for experiments under anoxic conditions. SDA agar (2% glucose, 100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 0.15% Wickerham's yeast nitrogen base supplemented with 150 µg/ml of uracil, leucine and lysine, 2% agar) and YEPDA-G418 agar (YEPDA with 400 µg/ml geneticin G418) were used for the selection of transformants.

Strain Construction

A hemagglutinin protein (HA) gene tag and artificial constitutive TEF promoter (p_{TEF}) were fused to the appropriate gene directly in the chromosome [36]. For amplification of the cassette, we used the primers specific for the *UTH1*, *NCA3*, *SUN4* and *SIM1*

genes and the appropriate plasmid as the template. Strains were prepared using the plasmids pYM15 (HA-tag) and pYM-N18 (p_{TEF}) [37]. The cassettes were amplified using specific primers (Table S1) and transformed according to [38] into BY4742 cells. Transformants were selected either on SDA agar with auxotrophic supplements or on YEPDA-G418. Correct genomic integration of the cassette was verified by PCR using specific primers and by sequencing.

Extracellular and Cellular Protein Extraction and Separation using SDS–PAGE

Cell lysates and extracellular material from colonies was collected as follows: The biomass of whole colonies was collected, weighed and washed with an equal volume of the 10 mM MES buffer, pH 6 supplemented with protease inhibitors (Complete, EDTA-free protease inhibitor mixture (Roche Applied Science), 100 mM PMSF (Phenylmethylsulfonyl fluoride, Sigma) in isopropanol and 1 mM AEBSF (4-(2- aminoethyl) benzenesulfonyl uoride, Sigma)), i.e. for example 10 µg of the biomass was washed with 10 µl of the buffer. After the centrifugation (5 min, 4°C, 1180 g) supernatants and sediments with cells were collected separately and stored at −75°C. Supernatants were loaded onto the slots of SDS-PAGE gel in equal volumes which were equivalent to the amount of washed cells.

Cell lysates and extracellular proteins from liquid cultures were prepared as follows: The same cell biomass was collected by the centrifugation (5 min, 4°C, 1180 g) of a specific volume of cell culture (calculated according to the OD_{600} of the culture) grown in liquid GM/YED medium. The collected cells were used for the preparation of cell lysates containing SUN^{cell} proteins. SUN^{ex} proteins from the medium supernatant were precipitated with 100% Trichloroacetic acid added in a 1:10 ratio and kept on ice for 2 hours for quantitative precipitation. Precipitated proteins were centrifuged (15 min, 4°C, 12000 g), washed twice with acetone and resuspended in 200 µl of 10 mM MES buffer, pH6 with protease inhibitors. The amounts of extracellular proteins were loaded onto the slots of SDS-PAGE to be equivalent to the amount of cells that produced those proteins as well as cellular proteins loaded onto the gel.

The total-cell lysates were prepared from cells and collected as indicated above. All steps were performed at 4°C. The cells were broken with glass beads in 10 mM MES buffer, pH 6 with protease inhibitors in a FastPrep (Qbiogene). Cell debris was removed by centrifugation at 1000 g for 3 min and subsequently 2000 g for 5 min. The protein concentration in the supernatant was determined using a protein detection kit (Bio-Rad) and the aliquots were stored at −75°C.

Cell Wall Isolation

Cells were disrupted as in the case of cell lysate preparation. Sediments obtained after 1000 g and 2000 g centrifugations were combined, cell walls isolated as described [39], washed with ice-cold 10 mM Tris-HCl, pH 7,4, 1 mM PMSF (Phenylmethylsulfonyl fluoride, Sigma) and centrifuged at 1000 g, 4°C for 10 min. Sediment was washed with 50 ml ice-cold wash solution A (1 mM PMSF), and subsequently with wash solution B (5% NaCl, 1 mM PMSF), C (2% NaCl, 1 mM PMSF) and D (1% NaCl, 1 mM PMSF); each of the washings was repeated four times. Isolated cell walls were suspended in 100 µl of 10 mM MES buffer, pH 6 supplemented with protease inhibitors. Cell wall proteins were solubilized and denatured by Laemmli sample buffer and subjected to SDS-PAGE.

Determination of Amount of Particular SUN Proteins

The proteins of cell lysates were denatured in Laemmli sample buffer and separated by SDS-PAGE using 9% gels. 20 µg of protein was loaded onto the slots with the exception of Nca3p-HA, where 30 µg was loaded. After transfer to a PVDF membrane (Immobilon-P, Millipore), the amount of loaded proteins was verified by Coomassie blue staining of each membrane (loading control). The HA-tagged SUN family proteins were detected by mouse anti-HA antibodies (Cell Signaling Technology) in combination with goat anti-mouse IgG-HRP as the secondary antibody (Santa Cruz Biotechnology). The peroxidase signal was visualized with Super Signal West Pico (Pierce) on Super RX medical X-ray film (Fuji). The level of the individual protein was evaluated by UltraQuant 6.0. To minimize an effect of band saturation, less exposed Western blots were usually used for the quantification. Only in those cases where large differences in signal intensity were present among quantified samples, the quantification of most concentrated sample/s could be partially affected by their saturation.

Cell Wall Sensitivity to Zymolyase

The cell biomass of whole colonies grown on GMA plates was collected and resuspended in 20 mM potassium phosphate buffer, pH7.4 to a cell concentration providing $OD_{600} = 0.4$ after 100-fold dilution. After 1 min of preheating, Zymolyase 100 T (Zymo Research) was added (time zero) to a final concentration of 0.2 U/µl and incubated at 30°C for 20 min. Cell wall resistance to Zymolyase was determined as the decrease in OD_{600} of a cell suspension; i.e. in 2 min intervals, 10 µl of treated cell suspension was diluted 100 times into 1 ml of distilled water and the OD_{600} was determined.

Spot Assays

To determine sensitivity of cells to toxic compounds, particular strains were grown overnight on YEPDA agar at 28°C. To compare the viability of colony population of different strains, the giant colonies were grown 6 per plate on GMA at 28°C [28] and the whole population of a colony was harvested.

For the spot tests an equal wet cell biomass of each strain was diluted in distilled water as follows: A series of 10-fold dilutions were prepared in water over a range of concentrations from 10^{-1} to 10^{-5} relative to the initial culture. Spots of 5 µl from each dilution series were then plated on the indicated media and cultivated at either 28 or 37°C for 4 or 7 days, depending on the particular medium and treatment. All spot assays were repeated at least three times and a representative experiment is shown.

Cell Incubation in Anoxomat Device

Cells were incubated under either hypoxic (1% O_2, 79.3% N_2, 10.3% CO_2, 9.5 H_2) or anoxic (0% O_2, 80% N_2, 9.9% CO_2, 9.9 H_2) conditions. For incubation, we used an Anoxomat™ device (Mart Microbiology b.v.).

Supporting Information

Figure S1 The viability of BY4742, BY-*uth1*Δ, BY-*sun4*Δ, BY-*sim1*Δ and BY-*nca3*Δ populations during 30 days of colony development. At specified time points the whole colony populations were harvested and viability of particular strains was compared by spot assays on YEPDA plates.
(PDF)

Figure S2 Resistance of cells from 20 days old colonies grown on GMA to zymolyase presented as decrease in density of cell suspension. Values represent averages from 4 independent biological replicates for the mutant strains and 6

replicates for the wt; the significance of the difference between BY4742 and the other four strains was determined using two-way ANOVA with $p<0.05$.
(PDF)

Figure S3 Loading controls for Western blots shown in Figures 1, 2, 3 and 4.
(PDF)

Figure S4 Sensitivity of strains deficient in SUN proteins to toxic compounds either on respiratory GMA (A) or on fermentative YEPDA (B) agar. Quantification of drop assays shown at Figures 5 and 6.
(PDF)

Table S1 List of the primers.
(PDF)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: EK LV ZP. Performed the experiments: EK HK. Analyzed the data: EK LV ZP. Wrote the paper: ZP LV.

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7.2 Publication 2

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Cellular localization of Sun4p and its interaction with proteins in the yeast birth scar (manuscript)

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Aim:

Determination of cellular localization of Sun4p and its interaction with cell wall proteins or the yeast birth scar.

Summary:

The results of publication focus on the role of Sun4p in birth scar composition. We implemented a novel method of visualization of cell wall proteins using antibodies conjugated with fluorescent dyes. We determined the localization of Sun4p, Dse2p and Dse4p to the birth scar. We showed that the specific localization of Sun4p depends on the presence of Dse2p and that the localization of both Sun4p and Dse2p to birth scars depends on GPI-anchored Egt2p. We hypothesize that these proteins are parts of the septum destruction complex which localizes to the daughter side of the bud neck and the birth scar and are required for mother–daughter cell separation at late mitosis. In addition, we showed that the presence of Sun4p within birth scars and the extracellular matrix depends indirectly on the Ace2p transcription factor

Using a novel immunofluorescence approach to yeast birth scar visualization we found that Aim44p is necessary for correct new bud selection. A strain with a disrupted AIM44 gene showed a so called “budding-within-birth scar” phenotype where new buds always appear within birth scars, the zone restricted for budding. A similar phenotype is seen in a strain deleted for gene SWI5, encoding a transcriptional regulator of AIM44. In summary, our results generate new knowledge about the localization of SUN family proteins and their roles in cell wall biogenesis, cell separation and birth scar composition.

Cellular localization of Sun4p and its interaction with proteins in the yeast birth scar

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Running Title: Proteins of yeast birth scar

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Keywords: yeast birth scar, cell wall, glucanases, SUN family of proteins.

Abbreviations:

IF, immunofluorescence; WB, Western blot; KO, knockout; BF, bright field; WGA-588, Alexa-588 labeled Wheat Germ Agglutinin.

Abstract

Yeast harbor several proteins with predicted glucanase activity that are potentially involved in cell wall remodeling during different processes, including mitosis. Here, we showed that two of these putative glucanases, Sun4p and Dse2p, co-localize to the yeast birth scar, dependently on presence of the third glucanase, Egt2p. The absence of these glucanases results in inefficient mother-daughter cell separation. The Sun4p, Dse2p and Egt2p localize to the daughter side of the bud neck, possibly forming a complex, and are involved in the separation of the virgin daughter from the mother cell during mitosis. The formation of properly assembled birth scars that delimitate cell wall area restricted in the next budding is dependent on the presence of Aim44p and its transcriptional regulator, Swi5p. *AIM44* or *SWI5* deletion caused the "budding within the birth scar" phenotype, together with altered localization of the birth scar proteins Sun4p and Dse2p, indicating the impairment of birth scar protein complexes.

Introduction

Sun4p protein is a member of the SUN family of proteins that are homologous to □-glucosidases and exhibit different localization and functions. Sun4p has been identified in the cell wall and possibly also in mitochondria [1]. This protein is also efficiently released from cells growing either in liquid medium or within yeast colonies [2]. Although little is known about the function of Sun4p and other SUN family proteins, Sun4p may be involved in cell separation, which is delayed in the *sun4*Δ strain; this strain often possesses more than one bud, and daughter cells often remain attached to the mother cell [3]. Sun4p homologue from *Candida albicans,* Sun41p, plays a role in virulence and biofilm formation, cell attachment to a substrate, and cell separation and hyphae formation, leading to the hypothesis that the SUN family proteins in *C. albicans* are involved in cell integrity maintenance during cell division, accompanied by cell wall remodeling [4]. The Sun4p homologue from *Aspergillus fumigatus* is also functionally related to the cell wall, being involved in hyphae growth [5].

The number of cell divisions of a particular yeast cell is often reflected by the number of bud scars (chitin-rich rings stainable by Calcofluor dye) remaining on the cell surface after daughter cell

separation. These bud scars are relatively small in diameter (1.8-2.4 μm) and are different in structure and composition from the so-called birth scar, i.e., the ring structure that remains on the surface of the daughter cell after its separation from the mother [6-8]. In contrast to bud scars, each cell has only one birth scar, which is not stainable by Calcofluor white, as it contains much less chitin than bud scars, but it binds wheat-germ agglutinin that can be used for birth scar visualization when labeled with fluorescent dye. The birth scar is a zone restricted for budding, it is larger than bud scars (3-3.7 μm), and its size increases with cell age [8]. Although birth scar composition and function are rather unknown, mutants (such as *isw2*Δ or cells with Dse1p overproduction) exhibiting the "budding within the birth scar" phenotype have been isolated [9]. Daughter cell separation includes a step in which the septum is degraded from the daughter cell side by different hydrolytic enzymes [10-13] encoded by genes regulated by the Ace2p transcription factor that accumulates in daughter cell nuclei during cell division [12].

Here, we identified Sun4p, Dse2p and Dse4p as new proteins that localize to the yeast birth scar. Dse2p birth scar localization depends on Egt2p, and Sun4p localization depends on both Dse2p and Egt2p. Co-localization studies indicate that Egt2p, Dse2p and Sun4p form a complex in the birth scar, while Dse4p is excluded from this complex. Ace2p does not regulate *SUN4* expression, but it is essential for proper Sun4p localization to the birth scar via expression of Sun4p docking proteins, Dse2p and Egt2p. Proper birth scar localization of Sun4p and Dse2p is disrupted in a strain with *AIM44* deletion, displaying a budding within the birth scar phenotype.

Results

Sun4p localizes to the birth scar of yeast cells in an Ace2p transcription factor-dependent manner

With the aim to obtain information on Sun4p cell wall localization, we used the yeast strain BY-Sun4p-HA with an HA tag on the C-terminus of Sun4p [2] and analyzed Sun4p-HA localization in living cells by direct immunofluorescence (IF) using anti-HA-Alexa Fluor 488 antibodies. To decrease the possibility that C-terminal tagging affects Sun4p localization, we also prepared the strain with N-terminally tagged Sun4p (BY-HA-Sun4p). Figures 1A and B show that both Sun4p-HA and HA-Sun4p are visible at the same precisely defined position in each cell. Co-staining with Calcofluor

white, the dye that stains yeast bud scars with the exception of the birth scar, together with staining with Alexa-588 labeled Wheat Germ Agglutinin (WGA-588), which stains all scars including the birth scar, showed that Sun4p localizes to the birth scar of yeast cells. Immunofluorescence was assayed in non-fixed and thus non-permeabilized cells; Sun4p thus localize to the birth scar with both C- and N-termini exposed on the outside of the cell.

A comparison of the expression profile of the *SUN4* gene in various genome wide expression experiments using SPELL (http://spell.yeastgenome.org/) revealed similarities in *SUN4* expression with a group of other genes that localize to the cell wall (Figure S1). The expression of some of these genes (e.g., *EGT2*, *DSE1*, *DSE2*, *DSE4*) is positively regulated by the transcription factors Ace2p and Swi5p [12, 13]. We therefore prepared strains derived from BY-Sun4p-HA (wt) with individual deletions of *SWI5* and *ACE2*. Western blot (WB) analysis showed that Sun4p-HA is synthesized at comparable levels in wt and knockout (KO) strains, but this protein is not released extracellularly in the absence of Ace2p (Figure 1 H). IF revealed that Sun4p-HA disappears from birth scars in the absence of Ace2p (Figure 1 C). The deletion of the *SWI5* gene caused a clear "budding within the birth scar phenotype", described previously for the *isw2*Δ strain [9], and a more diffuse localization of Sun4p-HA in the region of the birth scar compared to wt (Figures 1D and S2).

These data revealed that some proteins encoded by genes, the expression of which is positively regulated by Ace2p, are indispensable for Sun4p birth scar localization and its release from cells. Thus, Ace2p effect on Sun4p localization is not direct but most likely mediated by some mediator/docking protein whose expression is controlled by Ace2p. In addition, the protein products of some genes regulated by Swi5p are predicted to participate in the proper selection of a new budding site or in the prevention of budding within the birth scar.

Figure 1

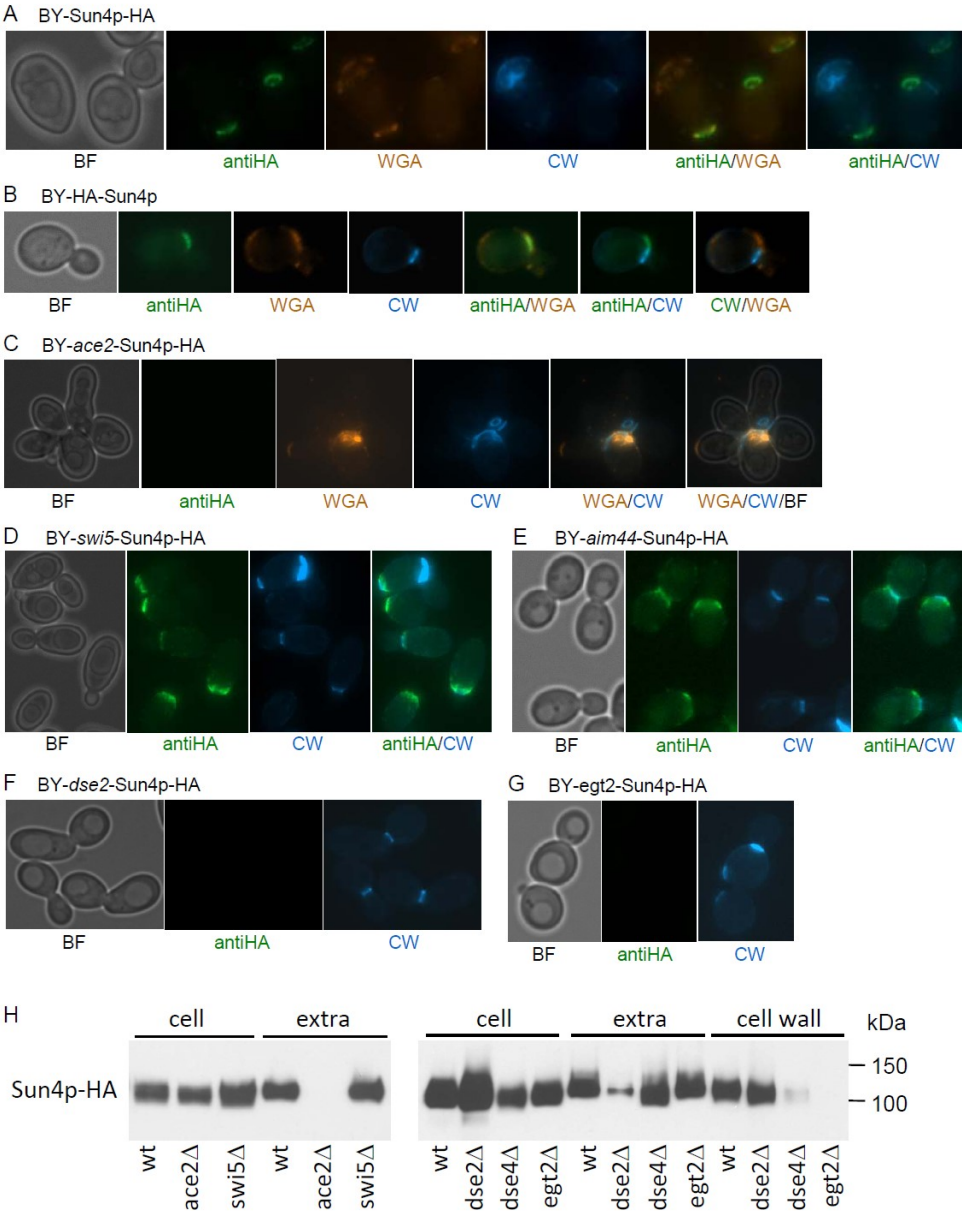

Figure 1. Sun4p localization in wt and KO cells. Sun4p-HA and HA-Sun4p localization to the birth scar in the wt strain (A, B); the absence of Sun4p-HA in the birth scar of *ace2*Δ (C), *egt2*Δ (G) and *dse2*Δ (F) strains; and diffuse Sun4-HA localization and budding within the birth scar phenotype of the *swi5*Δ strain (D), similar to the phenotype of *aim44*Δ (E). Sun4p-HA cellular, extracellular and cell wall localization estimated in wt and KO strains by WB (H). Loading controls for WB are in Figure S5 A. Projections of Z-stacks are used to show Sun4p-HA localization to the birth scar ring, A, C and D. Transversal optical sections of cells are shown in B, E, F and G. BF, bright field; anti-HA, antibody against HA-tag; WGA, WGA-588; CW, Calcofluor white. A-F, Representative cells from at least 10 fields (>1000 cells per strain) are shown; H, representative results of 4 independent biological experiments are shown.

Dse2p, Dse4p and Egt2p proteins are involved in proper localization of Sun4p-HA to the birth scar and affect the levels of Sun4p-HA that are released extracellularly

With the aim to identify the cell wall proteins involved in Sun4p-HA localization, we first prepared a set of the strains with *SUN4*-HA derived from EUROSCARF strains knocked out in genes with a similar expression pattern in SPELL (Figure S1) and in other genes potentially localized to the birth scar (Table S1). The Sun4p-HA-knockout strains were then screened for Sun4p-HA production and cellular localization by WB and IF (data not shown). Four of the 14 analyzed strains (*dse2*Δ, *dse4*Δ, *egt2*Δ and *aim44*Δ) showed significantly changed pattern of Sun4p-HA production and/or localization. New KO strains were then prepared, derived from parental BY-Sun4-HA and BY-HA-Sun4p strains (Table S1). In the absence of Dse2p or Egt2p, Sun4p-HA disappeared completely from the birth scar, similar to the situation in the *ace2*Δ strain (Figures 1 F, G and C), and its amount (determined by WB) in the extracellular space (*dse2*Δ) or in the cell wall (*egt*2Δ) was significantly reduced (Figure 1H). In the BY-*dse4*-Sun4p-HA strain, the amount of Sun4p-HA in the cell wall was reduced (Figure 1H), but the Sun4p-HA was present in the birth scar normally, as in the wt (Figure S2). High Sun4p levels identified by WB in cell wall extracts of the BY-*dse2*-Sun4p-HA strain (with protein levels similar to wt) in parallel with the absence of Sun4p-HA in the birth scars (as observed by IF) indicate that Sun4p is also present in the cell wall, with its C- and N-termini facing inside the cells, which is the direction opposite to that observed in the birth scar. Cell wall localization of Sun4p is also supported by the fact that the *sun4*Δ strain significantly decreases sensitivity to zymolyase [2], implicating Sun4p role in cell wall remodeling. The dual localization of Sun4p may signify a dual function, with one related to birth scar localization in the separation of daughter cells from mother cells (together with other glucanases probably involved in secondary septum destruction) [14] and the other function linked to the cell wall localization of proteins in cell wall organization during bud growth [3, 14]. Presented data indicate that Dse2p and Egt2p proteins are involved in the proper localization of Sun4p-HA to the birth scar. Both *DSE2* and *EGT2* genes are regulated by Ace2p, which is in agreement with the observed Sun4p-HA localization.

Dse2p-HA localizes to the birth scar dependently of Egt2p and independently of Sun4p

We prepared a series of strains with Dse2p labeled on its C-terminus (Dse2p-HA) or N-terminus (HA-Dse2p), together with other cell wall/birth scar genes (*SUN4*, *EGT2*, *DSE4*) individually deleted. In the wt strain, similar to Sun4p-HA, Dse2p-HA localized to the birth scar (Figure 2A) and was present in the cell lysate, cell wall and extracellular space (Figure 2F). Co-staining with WGA-588 indicated that similar to Sun4p-HA, Dse2p-HA localizes to the rim of the birth scar. However, N-terminally-labeled HA-Dse2p localized mostly to the cell wall in a punctuate pattern (Figure 2B), and fluorescence in the birth scar was detectable only in a few cells. In addition, the distribution of cell wall-localized fluorescence was asymmetric in some cells, forming a gradient in the cell wall with higher HA-Dse2p concentration closer to the birth scar. Cell wall fluorescence was not typically present in the cell wall of small buds, but it was visible in those that were already enlarged. These data indicated that either N- or C-terminal tagging affects Dse2p localization or both termini of Dse2p are differently accessible for the antibody in different locations on cell surfaces. The latter would suggest that Dse2p could be present in different complexes with other proteins and/or polysaccharides in the cell wall and the birth scar. To distinguish between these two possibilities, we prepared the strain containing double tagged Dse2p with a HA tag on the N-terminus and a Myc tag on the C-terminus. We then detected both tagged tails using anti HA-Alexa488 antibody in parallel with anti-Myc-Alexa647 antibody. The results (Figure 2C) showed red fluorescence (C-terminus) exclusively in the birth scar and green fluorescence (N-terminus) largely distributed in the cell wall (and rarely in the birth scar), thus proving that N- and C-terminal tails of Dse2p are differently exposed in these two regions of the cell surface. Such dual localization of tagged Dse2p variants disproved the potential attachment of this protein to the cell surface by a GPI anchor, which was predicted by a genome-wide approach [15].

We then analyzed Dse2p localization in different KO strains. In the absence of Egt2p, Dse2p-HA was not visible in the birth scar (similar to Sun4p-HA in *egt2*Δ) (Figure 2D). Dse2p-HA amounts in the cell wall disappeared as shown by WB, but this protein was still present in cells and released to the extracellular space (Figure 2F). In BY-*egt2*-HA-Dse2p, however, HA-Dse2p was still detectable by IF in the cell wall, as in the wt strain (Figure 2E). These data indicate that Egt2p is important for proper Dse2p localization to the birth scar, but it is not important for Dse2p targeting to the cell wall.

Figure 2

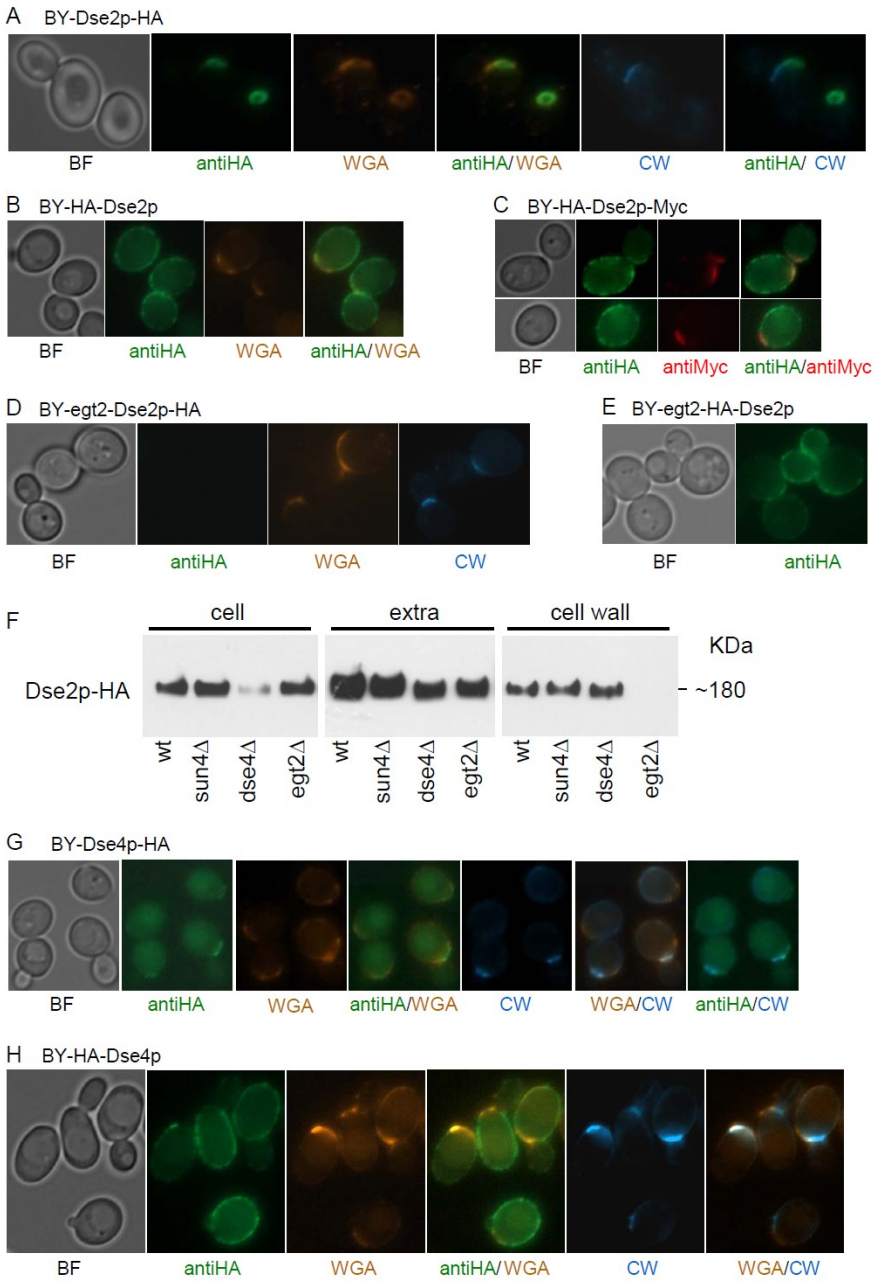

Figure 2. Dse2p and Dse4p localization in wt and KO cells. Dse2p-HA and Dse4p-HA localization to the birth scar (A, G) and HA-Dse2p and HA-Dse4p localization to the cell wall (B, H) of the wt strain; the absence of Dse2p-HA in the birth scar (D) and the presence of HA-Dse2p in the cell wall (E) of the *egt2*Δ strain; and the presence of HA-Dse2p-Myc both in the birth scar and cell wall (C). Dse2p-HA cellular, extracellular and cell wall localization estimated in wt and KO strains by WB (F). Projections of Z-stacks are used to show Dse2p-HA localization to the birth scar ring (A). Transversal optical sections of cells are shown in B, C, D, E, G and H. BF, bright field; anti-HA, antibody against HA-tag; anti-Myc, antibody against Myc tag; WGA, WGA-588; CW, Calcofluor white. A-E, G and H, Representative cells from at least 10 fields (>1000 cells per strain) are shown; F, representative result of 4 independent biological experiments is shown.

However, in the absence of Egt2p, Dse2p (similarly to Sun4p) disappeared from cell wall extracts as determined by WB. Loading controls for WB are in Figure S5 B. This observation indicates that Egt2p also plays a role in the cell wall, possibly in stabilizing Dse2p and Sun4p attachment to the cell wall. In the absence of Egt2p, Dse2p and Sun4p are more easily released and thus can disappear from the cell wall during its purification. Unfortunately, we did not succeed in analyzing the localization of Egt2p because neither GFP nor HA tagging allowed us to visualize this protein in the cells (not shown).

In the absence of Dse4p, Dse2p-HA localized to the birth scar and HA-Dse2p to the cell wall in a similar manner as in the wt strain (Figure S3 A). Additionally, in the absence of Sun4p, the localization of both Dse2p-HA and HA-Dse2p was also not affected (Figure S3 A), which indicates that Sun4p is not required for correct Dse2p localization, while Dse2p is indispensable for the proper localization of Sun4p to the birth scar. Egt2p is crucial for the birth scar localization of both Sun4p-HA and Dse2p-HA.

Similar to Dse2p, Dse4p localizes to the birth scar and the cell wall

To analyze the localization of Dse4p, we prepared strains with N- and C-terminally tagged Dse4p and analyzed Dse4p localization by direct IF. In wt, Dse4p-HA was visible in the birth scars of some cells, although the fluorescence was very weak and often visible only in small part of the birth scar (Figure 2G). The stronger fluorescence of HA-Dse4p presented as a punctate pattern was detectable in the cell wall of the BY-HA-Dse4p strain. These data showed that Dse2p and Dse4p exhibit similar dual localization in the birth scar and the cell wall with the same protein orientation. In contrast to the asymmetric cell wall localization of HA-Dse2p close to the birth scar, HA-Dse4p was almost evenly distributed in the cell wall of mother cells (Figure 2H). However, HA-Dse4p cell wall fluorescence was in some cases weaker or absent in the cell wall of the buds (Figure S4 B). Asymmetric HA-Dse4p localization was not affected by deletion of genes *CDC11* and *SHS1* coding for septins (Figure S4 B).

In absence of Sun4p, HA-Dse4p localized to the cell wall as in wt (Figure S4 A). Unfortunately, construction of strains with HA-Dse4p derived from *dse2*Δ and *egt2*Δ KO strains failed, which may indicate that tagged Dse4p protein is not fully functional and its combination with a deletion of *DSE2*

or *EGT2* is lethal to cells. Similarly, we were not able to prepare the strain with the two tags (C and N-terminal) of Dse4p.

Figure 3

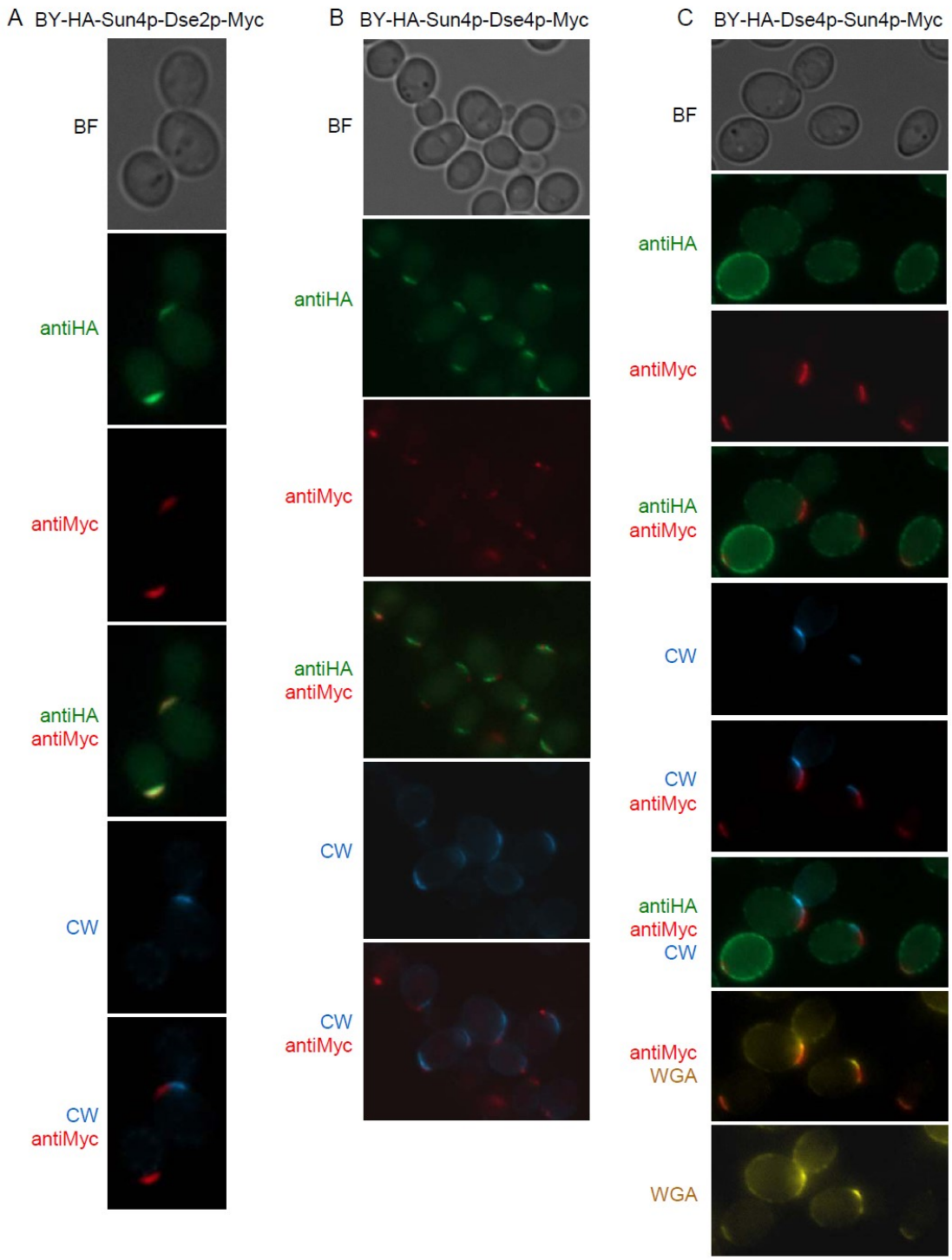

Figure 3. Co-localization of Sun4p with Dse2p and Dse4p in wt cells. Transversal optical sections of cells (A, B, C). BF, bright field; anti-HA, antibody against HA-tag; anti-Myc, antibody against Myc tag; WGA, WGA-588; CW, Calcofluor white. Representative cells from at least 10 fields (>1000 cells per strain) are shown.

The identification of Dse4p localization to the daughter site of the septum in fixed yeast cells and to the whole daughter cell after partial cell wall digestion [16] supports our finding of dual Dse4p localization. However, in our experiment performed with living non-fixed cells, Dse4p was detected in addition to cell walls of mother cells also in cell walls of some of the buds (Figure S4 B).

Sun4p co-localizes with Dse2p but not with Dse4p to the birth scar

The above data show a relationship among Sun4p, Dse2p and Dse4p localization. To examine the mutual localization of these proteins in more detail, we prepared strains with different combinations of tagged versions of these proteins. Strain BY-HA-Sun4p-Dse2p-Myc showed the clear co-localization of Dse2p and Sun4p to the birth scar (Figure 3A), indicating that these two proteins could form a complex. As the birth scar localization of both proteins is dependent on the presence of functional Egt2p (Figures 1 and 2), and in addition, Dse2p is essential for Sun4p localization, we assume that Egt2p keeps Sun4p and Dse2p at the birth scar through interaction with Dse2p.

Analysis of potential co-localization of Sun4p with Dse4p in the birth scar using the BY-HA-Sun4p-Dse4p-Myc strain indicates partially different localization of Sun4p and Dse4p in the birth scar (Figure 3B). In this strain, HA-Sun4p exhibited standard birth scar localization. Weak Dse4p-Myc fluorescence predominantly localized also to the birth scar, but to other positions compared to HA-Sun4p. The analyses were complicated by rather weak Dse4p-Myc fluorescence, but the results indicated that Dse4p does not form a complex with Sun4p (and thus probably not with Dse2p) in the birth scar. This conclusion is in agreement with the observation that the deletion of the *DSE4* gene does not affect the birth scar localization of Sun4p and Dse2p. In the BY-HA-Dse4p-Dse2p-Myc and BY-HA-Dse4p-Sun4p-Myc strains (Figures 4A and 3C), HA-Dse4p present in the cell wall did not co-localize with any of the other proteins present in the birth scar. As assumed, complementary fluorescence of N-terminal-tagged Dse4p in the cell wall and of both Sun4p and C-terminal-tagged Dse2p in the birth scars was clearly visible.

Figure 4

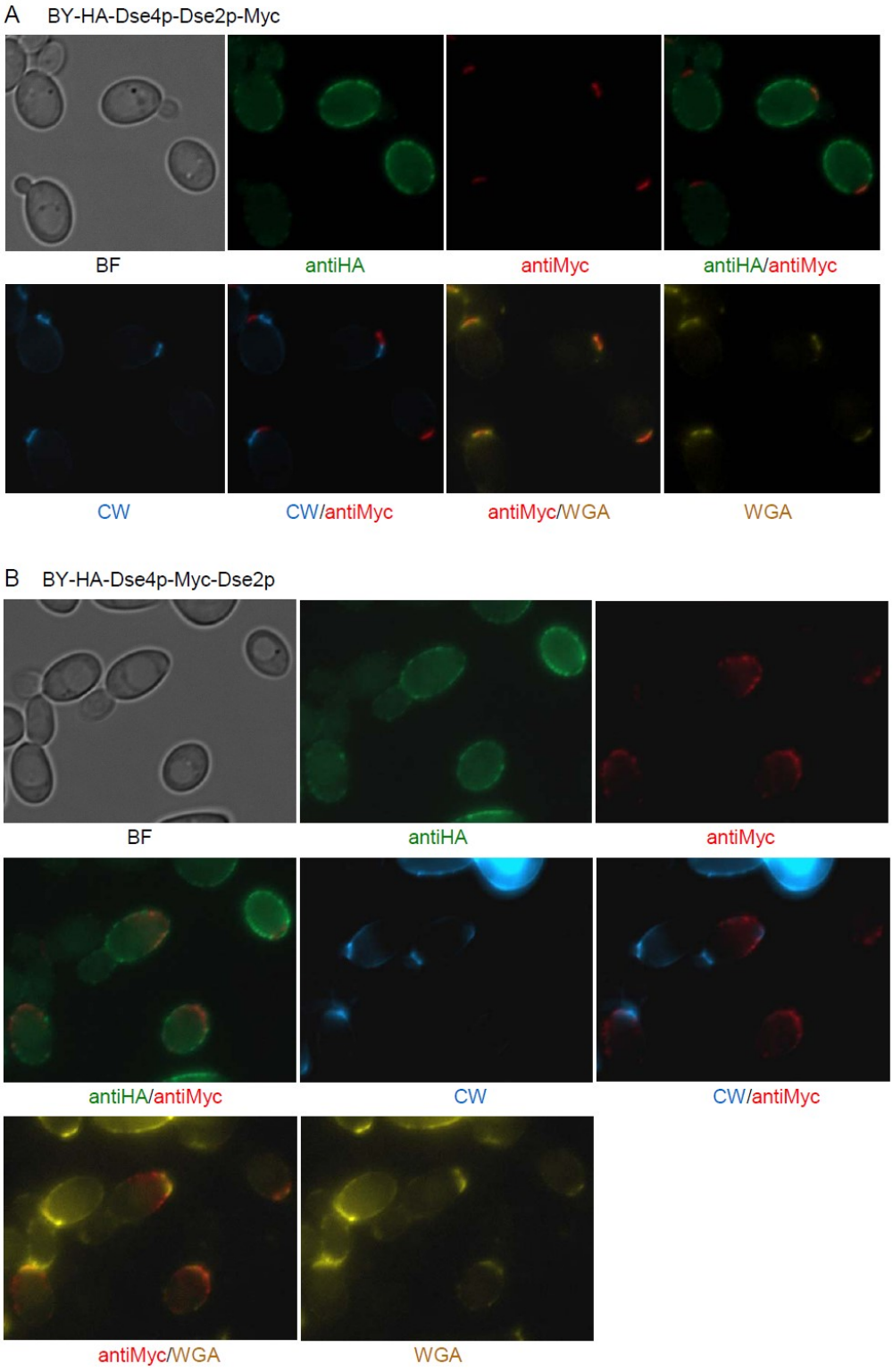

Figure 4. Co-localization of Dse2p and Dse4p in wt cells. Transversal optical sections of cells (A, B). BF, bright field; anti-HA, antibody against HA-tag; anti-Myc, antibody against Myc tag; WGA, WGA-588; CW, Calcofluor white. Representative cells from at least 10 fields (>1000 cells per strain) are shown.

The question remained whether Dse4p co-localizes with Dse2p in the cell wall. Analysis of the BY-HA-Dse4p-Myc-Dse2p strain, however, showed mostly complementary fluorescence of HA-Dse4p and Myc-Dse2p in the cell wall (Figure 4B). Both proteins exhibited a typical punctate pattern, which was generally more polarized for HA-Dse2p and more evenly distributed for HA-Dse4p. These data indicate that these proteins localize to different positions in the cell wall and therefore most likely do not interact.

The absence of Aim44p causes changes in the localization of birth scar proteins Sun4p and Dse2p and the "budding within the birth scar" phenotype

Deletion of another Swi5p-regulated gene, *AIM44,* in the BY-Sun4p-HA and BY-Dse2p-HA strains significantly changed the budding pattern and birth scar localization of Sun4p-HA and Dse2p-HA. The BY-*aim44*-Sun4p-HA and BY-*aim44*-Dse2p-HA strains exhibited a "budding within the birth scar" phenotype, similar to the strain deleted in the *SWI5* gene that codes for Swi5p, the transcriptional regulator of *AIM44*. Both Sun4p-HA and Dse2p-HA exhibited diffuse birth scar localization (Figures 1E and S3 B), similar to Sun4p-HA localization in the *swi5*ΔΔ strain (shown in Figures 1D). These findings indicate that Aim44p is involved in assembly of the birth scar proteins Sun4p, Dse2p and Egt2p.

Discussion

We identified novel proteins in the yeast birth scar, the structure of largely unknown composition. Dse2p, Dse4p and most likely Sun4p showed dual localization on the yeast cell surface, being present within the birth scar and in the rest of the cell wall. In both locations, these proteins expose different termini of their structures (Figure 5), indicating either different orientations of these proteins or the presence of additional factors (proteins and/or carbohydrates) that cover specific domains of Sun4p and/or Dse2p and/or Dse4p. According to our model-scheme, GPI-anchored Egt2p keeps the complex of Dse2p and Sun4p in the birth scar or helps to expose parts of these proteins to the external space. According to WB, Egt2p also helps to stabilize Dse2p and Sun4p binding to the

cell wall. The Ace2p transcriptional regulator is indispensable for Sun4p localization to the cell wall because it induces the expression of Egt2p and Dse2p. *SUN4* expression is not affected by Ace2p.

Our results provide direct evidence of Egt2p-dependent Dse2p and Sun4p birth scar localization that can explain the decreased cell separation efficiency in strains with deletions of *DSE2*, *EGT2* or *SUN4* encoding proteins with predicted glucanase activity [11, 12, 17]. Hydrolytic activity against β-(1,3)-glucan of Sun4p homologues, *Af*Sun1p of *Aspergillus fumigatus* and *Ca*Sun41p of *Candida albicans*, was confirmed [5]. We therefore hypothesize that Egt2p, Dse2p and Sun4p proteins form complexes that first localize to the daughter side of the bud neck during mitosis, similar to Dse4p [16], and later to the birth scar of the daughter cell. In late mitosis, this complex presumably participates in mother-daughter cell separation. Sun4p exhibits asymmetric localization to the birth scar (not to bud scars), although it is not regulated by the daughter-cell-specific Ace2p transcription factor, which only activates genes in daughter cell nuclei [12, 18]. Sun4p asymmetric localization is likely ensured by forming a complex with daughter-cell-specific Egt2p and Dse2p. In addition, fractions of Dse4p, Dse2p and Sun4p, which localize to the cell wall, may function in cell wall remodeling, which is important for proper bud growth [14].

Figure 5

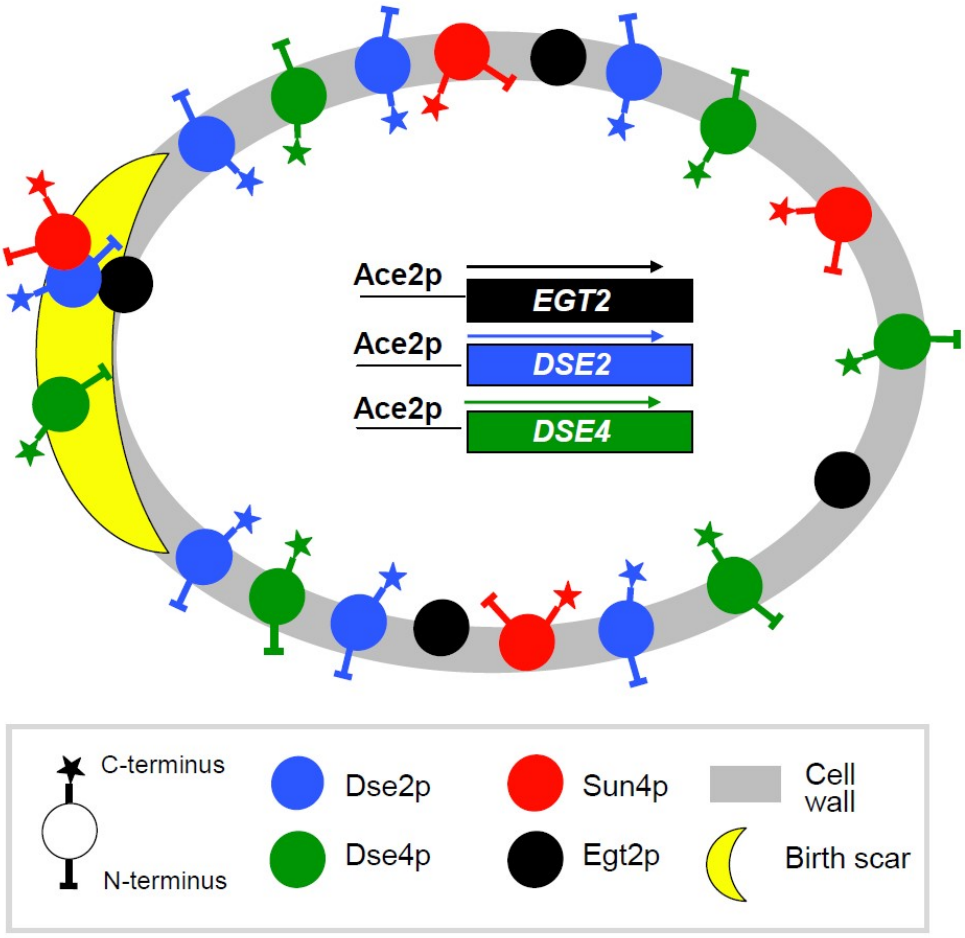

Figure 5. Schematic model of cell localization and interaction of Sun4p, Dse2p, Dse4p and Egt2p proteins.

The disruption of *AIM44* and *SWI5* lead to altered Sun4p and Dse2p localization, together with a "budding within the birth scar" phenotype, where new buds form within the birth scar, i.e., within the zone that is restricted for budding in the wt strain. This phenotypic similarity of *aim44*Δ and *swi5*Δ can be explained by the fact that Swi5p is a transcriptional regulator of *AIM44* [19]. These findings implicate Aim44p in the formation of correctly assembled birth scars and consequently, in the selection of new bud-sites outside of the birth scar.

Materials and methods

Yeast strains and media

The strains used in this study (Table S1) were all derived from *Saccharomyces cerevisiae* BY4742 (MATα, his3Δ1, leu2Δ0, lys2Δ0, ura3Δ0). The strains were either obtained from the EUROSCARF collection or prepared in this study. Yeast cells were grown at 28°C in liquid YD medium (1% yeast extract, 2% glucose) or GM medium (1% yeast extract, 3% glycerol, pH 5) or in giant colonies (six per plate) on GMA agar (GM, 2% agar, 30 mM $CaCl_2$). YPDA agar (YD, 1% peptone, 2% agar) with antibiotic supplements (G-418, 400 mg/l; nourseothricin, 200 mg/l or hygromycin B, 400 mg/l) or SDA agar (2% glucose, 100 mM KH_2PO_4, 15 mM $(NH_4)_2SO_4$, 0.8 mM $MgSO_4$, 0.15% Wickerham's yeast nitrogen base supplemented with 150 mg/ml of uracil, leucine and lysine, 2% agar) were used for strain construction; when needed, SDA plates were supplemented with 1 mg/ml 5-fluoroorotic acid (5-FOA).

Strain construction

Deletions and both C-and N-tagged (HA or Myc) strains were made by PCR amplification of marked cassettes using appropriate plasmids and by subsequent transformation [20] of the particular strain. For deletions, primers with homology overhangs flanking the open reading frame were used to amplify the cassette. For C-tagged strains, primers flanking the stop codon of the target genes were used. N-tagging was performed according to [21]. Cassette with N-terminal HA-tag was inserted after the sequence encoding signal peptide as predicted in SGD (http://www.yeastgenome.org/); Terminal amino acids of the signal peptides are Ala (GCC) for Dse2p, Ala (GCT) for Sun4p and Cys (TGT) for Dse4p. Correct genomic integration of cassettes was verified by PCR using specific primers and by sequencing.

Isolation of fractions of extracellular, cellular and cell wall proteins

Cell lysates and extracellular material from colonies were collected as follows. The biomass of 3-day old whole colonies was collected and washed with 10 mM MES buffer, pH 6 supplemented with protease inhibitors (Complete, EDTA-free protease inhibitor mixture (Roche Applied Science),

100 mM PMSF (phenylmethylsulfonyl fluoride, Sigma) and 1 mM AEBSF (4-(2- aminoethyl) benzenesulfonyl fluoride, Sigma) with a volume equal to the biomass. Wash buffer (supernatant) containing extracellular material and cells were collected separately and stored at -75°C. The cells were broken with glass beads in the same buffer as above using FastPrep FP120 (Thermo Savant, NY, USA). The supernatant obtained by centrifugation at 1000 g for 3 min and subsequently 2000 g for 5 min was used as the cell lysate; the sediments of both centrifugations were combined and used for cell wall isolation according to [22]. In brief, the sediments were washed with ice-cold 1 mM PMSF followed by solutions of 1 mM PMSF containing 5%, 2% and 1% of NaCl stepwise; each of the washings was repeated four times. All steps were performed at 4°C.

Determination of particular protein amounts

The proteins within the cell lysates (20 µg of proteins per slot, determined using a protein detection kit, Bio-Rad), purified cell walls, and extracellular proteins in amounts equivalent to the amount of cells that produced 20 µg of cell proteins were separated by SDS-PAGE using 9% gels, transferred to a PVDF membrane (Immobilon-P, Millipore) and stained with Coomassie blue (loading control). The HA-tagged and Myc-tagged proteins were detected using mouse monoclonal anti-HA (Cell Signaling Technology, #2367) and anti-Myc antibodies (Santa Cruz Biotechnology, sc-40) in combination with goat anti-mouse IgG-HRP as the secondary antibody (Santa Cruz Biotechnology, sc-2005). The peroxidase signal was visualized with Super Signal West Pico (Pierce) on Super RX medical X-ray film (Fuji).

Fluorescence microscopy

Cells grown in liquid GM medium overnight were washed in PEM buffer (0.1 M PIPES, 5 mM EGTA, 5 mM $MgCl_2$; pH 6.9 (KOH)) and treated with 2% Bovine serum albumin for 30 min at room temperature. Alexa Fluor 488-conjugated mouse IgG1 antibody against HA-tag (Cell Signaling Technology, #2350) or antibody against Myc-tag conjugated with Alexa Fluor 647 (Santa Cruz Biotechnology, sc-40 AF647) were added to final dilutions of 1:50 and incubated at room temperature for 1 hour. Cells were washed with dH_2O before Fluorescence/DIC/BF microscopy, which was performed using a Carl Zeiss AxioObserver.Z1 fluorescence microscope equipped with Axiocam 506

and a Plan-Apochromat 63x/1.40 Oil DIC M27 objective. Filter sets for Alexa Fluor 488 (excitation 450-490 nm; emission 500-550 nm) or for Alexa Fluor 647 (excitation 625-655 nm; emission 665-715 nm) were used. For visualization of bud/birth scars stained with Calcofluor White (2 μg/ml, Sigma) and/or Wheat Germ Agglutinin, Alexa Fluor 594 conjugate (10 μg/ml, Invitrogen), filter set for DAPI (excitation 335-383 nm; emission 420-470 nm) and for DsRed (excitation 538-562 nm; emission 570-640 nm) were used, respectively. Image acquisition was processed with ZEN 2012 (blue edition) software (Zeiss).

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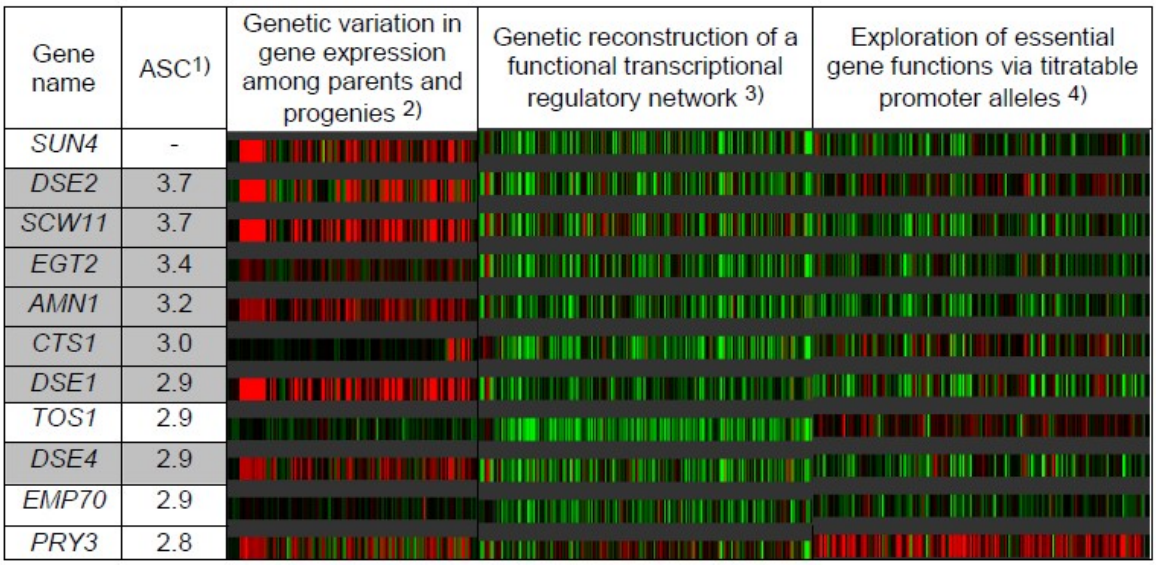
Supplementary

Figure S1



1) ACS (Adjusted Correlation Score) is a measure of weighted correlation for the gene with the queryset across all datasets.

2) Brem et al., PNAS 102: 1572-1577, 2005

3) Hu et al., Nat Genet 39: 683-687, 2007

4) Mnaimneh et al., Cell 118: 31-44, 2004

Figure S1. SPELL correlation of genes with the expression profile similar to that of SUN4.
Data from the three published transcriptomics analyses are shown as an example. Full data can be found at http://spell.yeastgenome.org/search/show_results?search_string=YNL066W. The genes marked in gray were selected for screening of Sun4p-HA production and localization.

Figure S2

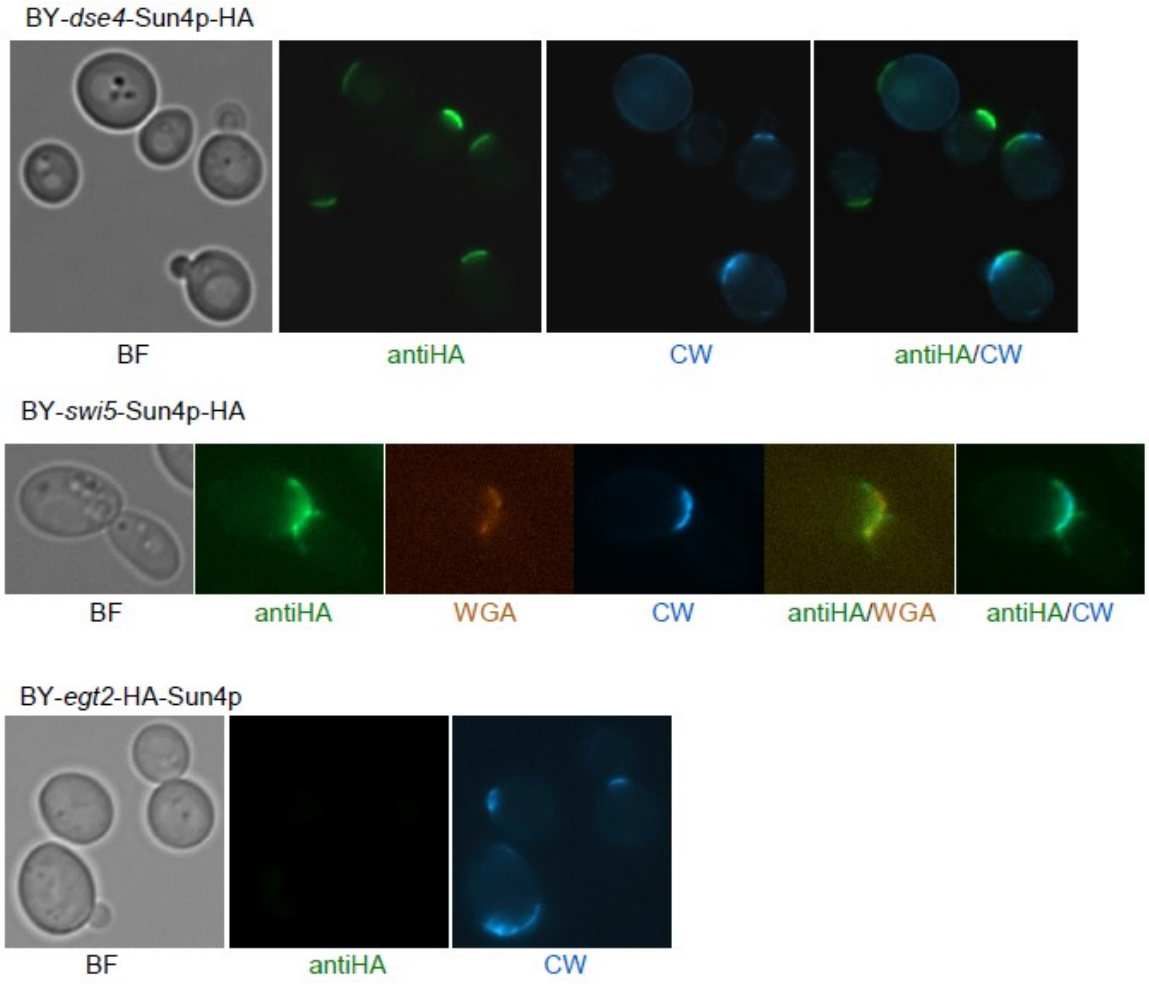


Figure S2. Sun4p-HA and HA-Sun4p localization in wt and KO cells.

Transversal optical sections of cells are shown. BF, bright field; anti-HA, antibody against HA-tag; CW, Calcofluor white, WGA, WGA-588.

Figure S3

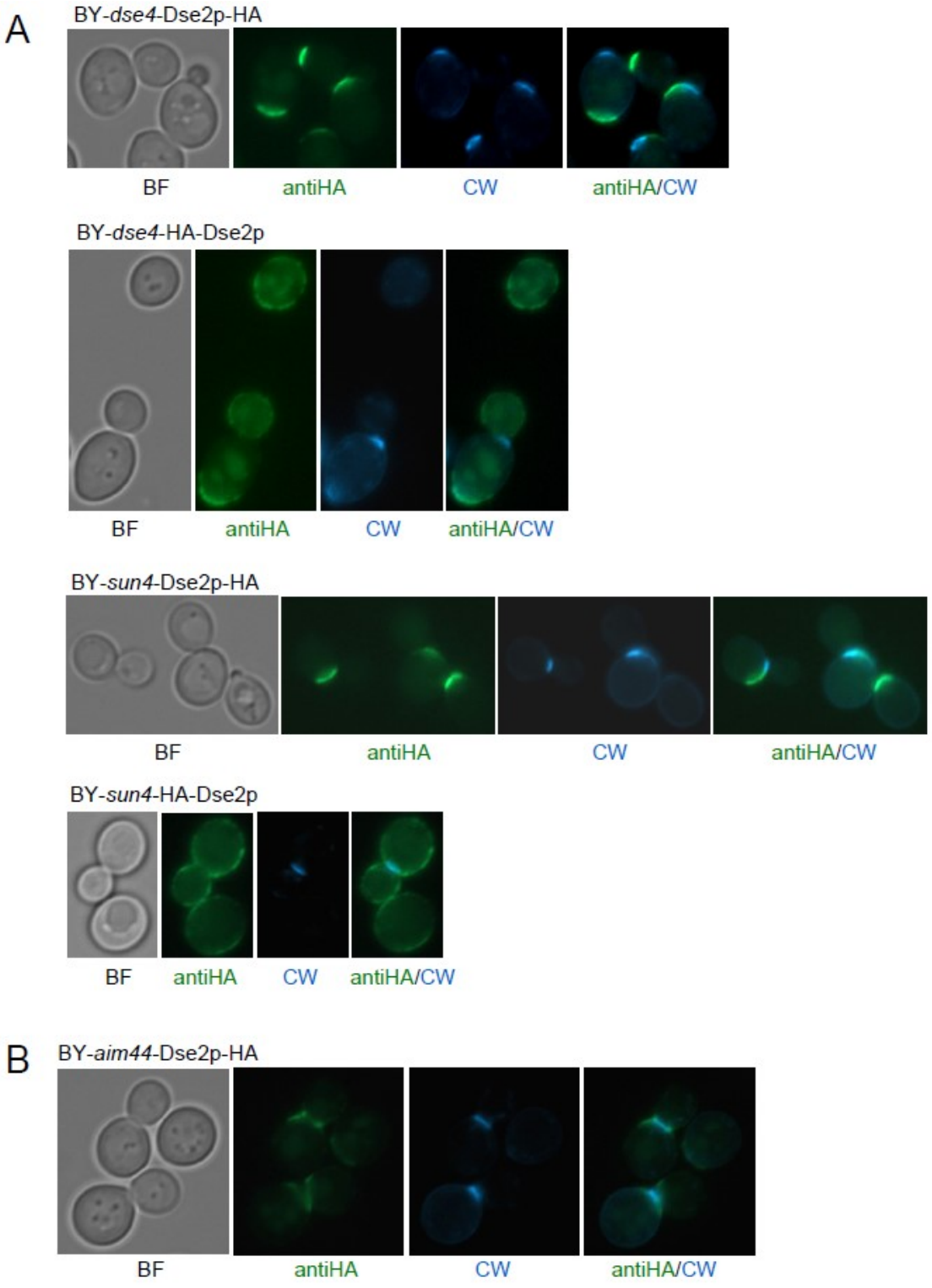
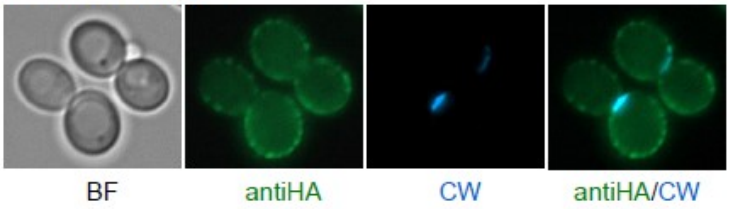


Figure S3. Dse2p-HA and HA-Dse2p localization in wt and KO cells.

Transversal optical sections of cells are shown. BF, bright field; anti-HA, antibody against HA-tag; CW, Calcofluor white.

Figure S4

A



B

Strain		Total	Large bud		Small bud	
			Mother cell and the bud exhibited similar cell wall fluorescence	Cell wall fluorescence of the bud was weaker	Cell wall fluorescence of the bud was weaker	The bud had no cell wall fluorescence
						
BY-HA-Dse4p	Counted cell number	364	141	120	57	46
	%	100%	~39%	~33%	~16%	~12%
BY-*cdc11*-HA-Dse4p	Counted cell number	291	80	123	61	27
	%	100%	~28%	~42%	~21%	~9%
BY-*shs1*-HA-Dse4p	Counted cell number	266	104	88	49	25
	%	100%	~39%	~33%	~18%	~10%

Figure S4. HA-Dse4p localization in wt and KO cells.

A, Transversal optical sections of cells are shown. BF, bright field; anti-HA, antibody against HA-tag; CW, Calcofluor white. B, Intensity of HA-Dse4p fluorescence in the cell walls of mother cells and the buds of the BY-HA-Dse4p strain and strains defective in septins.

Figure S5

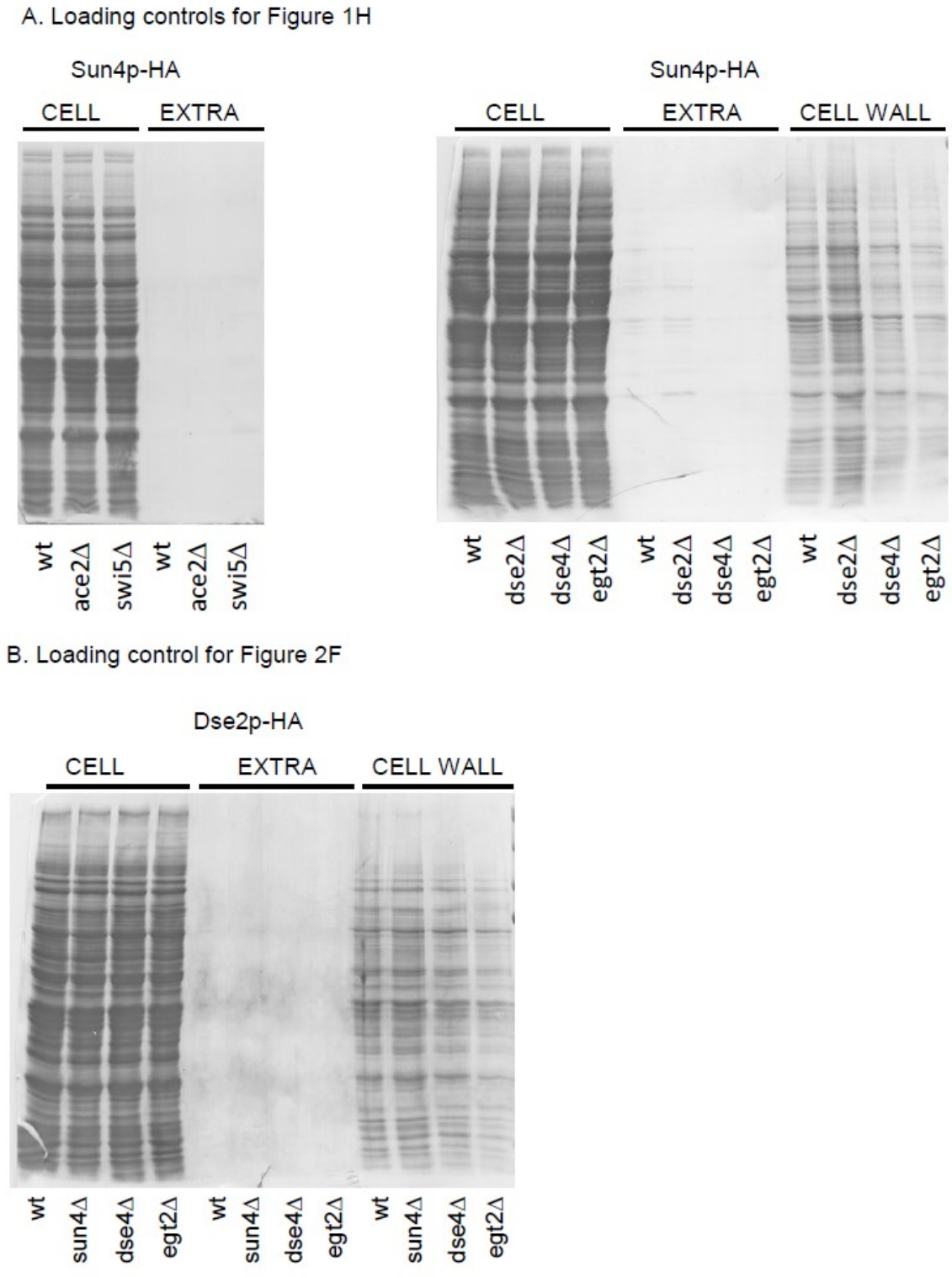


Figure S5. Loading controls for Western blots in Figures 1H and 2F

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